Essential Nutrients Handbook

Peyton Turner

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Preface

The main aim of this book is to educate learners and enhance their research focus by presenting diverse topics covering this vast field. This is an advanced book which compiles significant studies by distinguished experts in the area of analysis. This book addresses successive solutions to the challenges arising in the area of application, along with it; the book provides scope for future developments.

Nutrients are essential for the sustenance of life on Earth. They are fundamental to all biological processes performed by all species of organisms and for their structural growth. Nutrients such as fatty acids, vitamins, minerals, etc. are necessary and must be obtained on a regular basis through dietary supply. However excess consumption of nutrients can lead to nutrient toxicity. Nutrients are divided into two categories- micronutrients and macronutrients. Nutrition deficiency can result in various health conditions such as obesity, diabetes, metabolic syndrome, osteoporosis and many others. This book on nutrients provides in-depth knowledge of some of the key concepts and theories while also examining the modern advancements in this field of study. From theories to research to practical applications, case studies related to all contemporary topics of relevance to this field have been included herein. Constant effort has been made during the compilation of this book to enhance the understanding of the difficult concepts of nutrients, their structures and functions as easy and informative as possible, for the readers.

It was a great honour to edit this book, though there were challenges, as it involved a lot of communication and networking between me and the editorial team. However, the end result was this all-inclusive book covering diverse themes in the field.

Finally, it is important to acknowledge the efforts of the contributors for their excellent chapters, through which a wide variety of issues have been addressed. I would also like to thank my colleagues for their valuable feedback during the making of this book.

Editor

Effect of Human Milk Appetite Hormones, Macronutrients, and Infant Characteristics on Gastric Emptying and Breastfeeding Patterns of Term Fully Breastfed Infants

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Abstract: Human milk (HM) components influence infant feeding patterns and nutrient intake, yet it is unclear how they influence gastric emptying (GE), a key component of appetite regulation. This study analyzed GE of a single breastfeed, HM appetite hormones/macronutrients and demographics/anthropometrics/body composition of term fully breastfed infants (n = 41, 2 and/or 5 mo). Stomach volumes (SV) were calculated from pre-/post-feed ultrasound scans, then repeatedly until the next feed. Feed volume (FV) was measured by the test-weigh method. HM samples were analyzed for adiponectin, leptin, fat, lactose, total carbohydrate, lysozyme, and total/whey/casein protein. Linear regression/mixed effect models were used to determine associations between GE/feed variables and HM components/infant anthropometrics/adiposity. Higher FVs were associated with faster (−0.07 [−0.10, −0.03], $p < 0.001$) GE rate, higher post-feed SVs (0.82 [0.53, 1.12], $p < 0.001$), and longer GE times (0.24 [0.03, 0.46], $p = 0.033$). Higher whey protein concentration was associated with higher post-feed SVs (4.99 [0.84, 9.13], $p = 0.023$). Longer GE time was associated with higher adiponectin concentration (2.29 [0.92, 3.66], $p = 0.002$) and dose (0.02 [0.01, 0.03], $p = 0.005$), and lower casein:whey ratio (−65.89 [−107.13, −2.66], $p = 0.003$). FV and HM composition influence GE and breastfeeding patterns in term breastfed infants.

Keywords: human milk; term breastfed infants; gastric emptying; feeding frequency; ultrasound; stomach volumes; appetite hormones; macronutrients; feed volume; anthropometrics; body composition

1. Introduction

Breastfeeding and its longer duration are associated with reduced risks of developing obesity and other chronic non-communicable diseases later in life [1,2]. This unique protection could be the result of many mechanisms associated with both nutritive and non-nutritive components of human milk (HM) [3] as well as breastfeeding patterns and behaviour [4,5]. It has been shown that HM has the pleiotropic role, providing immune and anti-inflammatory protection [6,7] and endocrine, developmental, neural, and psychological benefits [2]. Non-nutritive HM components such as hormones, growth factors, neuropeptides, and anti-inflammatory and immune-modulating

agents influence the growth, development, and function of the gastrointestinal (GI) tract during early infancy [8], while some micronutrients act as nutritional antioxidants, improving GI functions [9]; however, there is much to be learned about the spectrum of HM programming agents, how their patterns change throughout lactation period, and their short-term effect on the gastric emptying (GE) rate of the breastfed infants.

GE is a process by which ingested food is mechanically and chemically partially broken down and delivered to the duodenum at a controlled rate for further digestion and absorption [10,11]. While well studied in the preterm population [12–14], in healthy term fully breastfed infants the GE rate and its relationship with breastfeeding patterns are not fully understood.

GE rate and patterns are known to depend on the nature and macronutrient composition of the ingested meal. HM or formula in the infant stomach separates into two phases, a liquid phase consisting of water, whey proteins, lactose, etc., and a semi-solid phase consisting of curd formed by casein and lipids. The semi-solid phase typically empties more slowly than the liquid phase. Different proportions of these phases in part explain the difference between GE patterns of formula-fed and breastfed infants—linear and curvilinear, respectively [12,15].

HM has a unique composition, including nutrients, growth factors, immune factors, and hormones. Despite numerous investigations into the different effects of HM and formula, few components, including major macronutrients, have been studied in connection with the GE of breasted term infants.

Fatty acids profiles are not associated with GE rate in preterm infants [16], while in term infants more rapid GE has been attributed to the fat and protein components of feeds with similar lactose concentration and osmolality [17].

Both osmolality and carbohydrate content are known to influence the rate of GE in adults [18], but in infants results are dependent on the type of carbohydrate [19,20].

Proteins from different HM fractions such as whey and casein are resistant to proteolysis in the infant stomach [21] and the protein content of a food has also been shown to influence appetite and its regulation [22]. Infant formula generally empties more slowly than HM in term infants; further, formulas with different casein:whey protein ratio exhibit different GE rates, with casein-predominant formulas emptying slower than whey-predominant formulas [23]. Thus the casein:whey ratio of HM could play an important role in controlling GE in the breastfed infant.

HM lysozyme, also present in whey in a relatively high concentration, catalyzes the hydrolysis of specific bonds in Gram-negative bacteria cell walls and plays multiple roles in digestive strategy, such as controlling the microbiome in the stomach and speeding up the digestion of microbial protein, which may affect gastric motility and GE rate [24,25].

The satiety hormone leptin and the appetite-stimulating hormone adiponectin are also present in HM. Although not transferred to the infant circulation in direct manner, levels of HM leptin and adiponectin from HM have been found to correlate with levels of these hormones in infant serum [26,27] and are known to affect both appetite control and infant body composition (BC) [28,29], but are yet to be investigated in relation to GE in the term infant. In animal models (rat, mouse), injection of leptin into the fourth ventricle has been shown to delay GE [30] and oral administration reduced food intake [31]. Leptin in HM is by far the most studied appetite hormone, but predominantly in skim milk [32]. Leptin measured in skim HM was not associated with time between feeds [33,34] or GE [34] in term breastfed infants, emphasizing the need for studies including whole milk leptin, where the levels of leptin are shown to be higher [32]. Adiponectin has the highest concentration of any appetite hormone in HM. It is present in a biologically active form that is resistant to digestion [35]. In the animal model adiponectin inhibits tension-sensitive gastric vagal afferent mechanosensitivity, modulating satiety signals in both lean and obese animals, while simultaneously increasing the mechanosensitivity of mucosal gastric vagal afferent in the obesity-induced model [36]. In humans, elevated serum levels of adiponectin are associated with more rapid GE in diabetic patients [37]. It is not known whether adiponectin levels impact GE in the infant and this warrants further investigation.

The volume of milk taken at a single feed varies greatly both within and between infants [38]. This may be affected by HM composition, with greater breastfeeding frequency associated with lower total 24-h protein intakes and higher lactose concentrations [39]. This suggests that the variations in HM components between mothers may potentially influence GE rate and time, and therefore feeding patterns.

This study investigated the effects of HM appetite hormones (whole milk adiponectin and leptin, skim milk leptin) and macronutrients (fat, total carbohydrates, lactose, oligosaccharides, total protein, casein and whey protein, lysozyme) on feeding frequency and GE. Further exploration of infant demographics, anthropometrics, and BC was carried out to determine relationships with infant feeding and GE.

2. Materials and Methods

2.1. Participants

Lactating mothers and their infants (n = 27) were recruited predominantly through the Australian Breastfeeding Association. Inclusion criteria were: healthy singletons, gestational age $\geq$ 37 weeks, fully breastfed on demand at the point of measurement. Exclusion criteria were: infant health issues requiring medication that could potentially influence GE rate (e.g., reflux), indications of low maternal milk production or infant growth issues. All mothers provided written informed consent to participate in the study, which was approved by the University of Western Australia, Human Research Ethics Committee (RA/1/4253) and registered with the Australian New Zealand Clinical Trials Registry (ACTRN12616000368437).

2.2. Study Design

Participants arrived at our laboratory at King Edward Memorial Hospital for Women (Subiaco, Perth, WA, Australia) in the morning (09:30–11:30 a.m.) to avoid circadian influence on the outcomes, and stayed for two consecutive breastfeeding sessions. Before the first feed (F1) infants were weighed and had ultrasound stomach volumes recorded (pre-feed residual, R1). Mothers expressed a pre-feed sample (fore-milk) of milk from the feeding breast/breasts and then breastfed their infants as usual. Immediately after F1, infant stomach volumes images and infant weights were taken, and mothers expressed a post-feed (hind-milk) milk sample. Subsequent scans of the stomach were scheduled at 15–20 min intervals (although attending infants' needs caused some variation) until the infant cued for the next feed (F2), when a final stomach volume immediately before F2 was measured (pre-feed residual, R2).

To assess infant BC bioimpedance spectroscopy measurements were taken pre-feed, unless impractical—then they were taken post-feed [40]. Ultrasound skinfold, length, and head circumference measurements were taken post-feed. This combination of two methods for measuring infant BC was used to ensure safe, non-invasive and accurate assessment and to avoid the inherent limitations of a singular technique [41]. Clothing was removed for the measurements except for a dry diaper and a singlet.

2.3. Feeding Frequency

Mothers were asked how frequently their infant feeds, and the self-reported typical time between the feeds (e.g., every three hours) during the week prior to the study session was taken as a proxy measure of feeding frequency.

2.4. Feed Volume Measurement

The volume of milk transferred from a breast/breasts by the infant was determined by weighing the infant immediately before and after the breastfeed using electronic scales ($\pm$2.0 g, Medela Electronic Baby Weigh Scales, Medela Inc., McHenry, IL, USA). Milk intake (g) was calculated by deducting the

initial weight from the final weight of the infant [42] and was converted to mL (feed volume; FV) using HM density of 1.03 g/mL [43].

2.5. Stomach Measurements with Ultrasound

The infant's stomach was scanned using the Aplio XG (Toshiba, Tokyo, Japan) machine, with a high-resolution PVT-674BT (6MHz) transducer and Parker ultrasonic gel (Fairfield, NJ, USA). Three to nine (median [IQR]: 5 [5; 6]) serial measurements of infant stomachs were taken 3 to 62 min apart (16 ± 10). Scans were performed with the infant in the semi-supine position according to the method validated in preterm infants [44]. Briefly, the sagittal and transverse planes of the stomach were used to measure the longitudinal (L), anterior-posterior (AP) and transverse (T) diameters directly from images on the ultrasound screen using electronic calipers (Figure 1). One experienced sonographer with good intra- and interrater reliability [44] performed all of the measurements. Gastric volume (mL) was calculated from the above measured diameters using following equation for an ellipsoidal body:

$$\textit{Stomach volume}\ (\text{mL}) = \text{L}\ (\text{mm}) \times \text{AP}\ (\text{mm}) \times \text{T}\ (\text{mm}) \times 0.52. \quad (1)$$

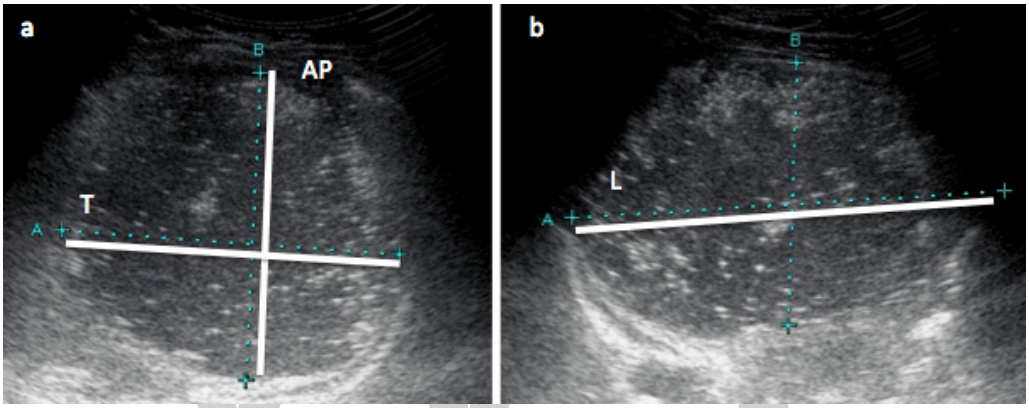

Figure 1. Measurements of infant's stomach with ultrasound. Ultrasound images of infant's stomach: (**a**) transverse view with anterior-posterior (AP) and transverse (T) diameter measurements; (**b**) longitudinal view with longitudinal (L) diameter (maximum length) measurement. Stomach volume (mL) = longitudinal diameter (mm) × anterior-posterior diameter (mm) × transverse diameter (mm) × 0.52.

2.6. Milk Sample Collection

Mothers hand-expressed or pumped small (1–2 mL) pre- and post-feed milk samples into separate 5-mL polypropylene plastic vials (Disposable Products, Adelaide, SA, Australia). Fat concentration was measured (below) and samples were frozen at −20 °C for further biochemical analysis.

2.7. Biochemical Analysis

2.7.1. Fat Content

Percentage fat was measured in pre- and post-feed samples immediately after sample collection with the creamatocrit method [45] using the Creamatocrit Plus device (Medela Inc., McHenry, IL, USA). Fat concentration of the pre- and post-feed milk samples (g/L) was calculated from the cream content of the milk samples, based on the equation [46]:

$$\textit{Fat}\ (\text{g/L}) = 3.56 + (5.917 \times \text{cream percentage}). \quad (2)$$

Fat concentration in the volume consumed by the infant was further calculated [47]:

$$Fat\ (g/L) = 0.53 \times \text{Fat}_{\text{pre-feed}} + 0.47 \times \text{Fat}_{\text{post-feed}}. \quad (3)$$

2.7.2. Sample Preparation

Prior to further analysis, all samples were thawed for two hours at room temperature (RT) and aliquoted into 1.5-mL tubes (Sarstedt, Numbrecht, Germany). Components' concentrations were determined in both pre- and post-feed samples in case of adiponectin, skim and whole milk leptin, fat, and lactose, and in pooled samples in case of total protein, casein, whey protein, total carbohydrates, and lysozyme. Concentrations of pre- and post-feed samples were averaged to arrive at the concentration used for statistical analyses. Whole milk was used for measuring whole milk adiponectin and leptin concentration. Milk samples were defatted (by centrifugation at RT in a Beckman Microfuge 11 (Aberdon Enterprise Inc., Elk Grove Village, IL, USA) at 10,000× *g* for 10 min and removing the fat layer by clipping it off with the top of the tube [48]) for analysis of skim milk leptin, total protein, lysozyme, lactose, and total carbohydrates concentrations. The standard assays were adapted for and carried out using a JANUS workstation (PerkinElmer, Inc., Waltham, MA, USA) and measured on EnSpire (PerkinElmer, Inc., Waltham, MA, USA).

2.7.3. Leptin

Leptin concentration in HM was measured using the R & D Systems Human Leptin enzyme linked immunosorbent assay (ELISA) DuoSet kit (Minneapolis, MN, USA) optimized to measure leptin in sonicated skim HM, as previously described by Cannon et al. [33] and further modified to measure leptin in skim and whole HM milk as described by Kugananthan et al. [32]. Recovery of leptin was 97.7% ± 9.7% (n = 10) with a detection limit of 0.05 ng/mL and an inter-assay CV of <7.2%.

2.7.4. Adiponectin

Adiponectin concentration in whole milk was measured using the Biovendor Human Adiponectin Sandwich ELISA kit (Life Technologies, Asheville, NC, USA). Adiponectin recovery was 96.2% ± 3.2% (n = 10) with a detection limit of 1 ng/mL and an inter-assay CV of <2.5%.

2.7.5. Protein

Casein and whey proteins were separated by the method fully described by Kunz and Lonnerdal [49], and Khan et al. [50]. Protein concentrations (total protein of skim HM, casein and whey proteins) were measured using the Bradford Protein Assay adapted from Mitoulas et al. [51]. Recovery of protein was 100.6% ± 5.2% (n = 5) with a detection limit of 0.031 g/L and an inter-assay CV of 7.8% (n = 18). Casein:whey ratio was calculated as follows:

$$\textit{Casein:whey ratio} = \text{casein concentration/whey protein concentration}. \quad (4)$$

2.7.6. Lysozyme

Lysozyme concentration was determined using a modified turbidimetric assay [52]. Hen egg white lysozyme (EC 3.2.1.17, Sigma, St. Louis, MA, USA) standards (range 0.00075–0.0125 g/L) and skim milk samples were diluted 10-fold with 0.1 M of Na_2HPO_4/1.1 mM of citric acid (pH 5.8) buffer. Twenty-five microliters of standards or diluted skim milk samples were placed into the wells of a plate (Greiner Bio-One, Frickenhausen, Germany), 175 µL of *Micrococcus lysodeiltikus* suspension (0.075% w/v, ATCC No. 4698, Sigma, St. Louis, MA, USA) was added into each well and plate was incubated at RT for 1 h. The absorbance was measured at 450 nm. Recovery of lysozyme was 97.0% ± 5.0% (n = 8) with a detection limit of 0.007 g/L and an inter-assay CV of 13.0% (n = 8).

2.7.7. Carbohydrates

Defatted milk was deproteinized with trichloroacetic acid [53] before dehydration by sulphuric acid [54]. This technique reliably estimates concentrations and carbon content for monosaccharides, disaccharides, and polysaccharides. Total carbohydrates were analyzed by UV-spectrophotometry. Recovery of total carbohydrates was 101.4% ± 2.1% (n = 7) with a detection limit of 0.007 g/L and an inter-assay CV of 3.3% (n = 7).

Lactose concentration was measured using the enzymatic spectrophotometric method of Kuhn and Lowenstein [55], adapted from Mitoulas et al. [51], with recovery of 98.2% ± 4.1% (n = 10), detection limit of 30 mM and inter-assay CV of 3.5%.

The human milk oligosaccharides (HMO) concentration (g) was calculated by deducting concentration of lactose (g) from concentration of total carbohydrates (g). The glucose and galactose were not measured or accounted for as their concentrations in HM are small and comparable or less than the assays errors [56].

2.8. Hormone and Macronutrient Dose

Doses were defined as the amount of hormone/macronutrient ingested during a breastfeed and calculated as average of the pre- and post-feed HM component concentration, multiplied by the corresponding FV. When an infant fed from both breasts at the breastfeeding session, hormone/macronutrient doses from these individual breastfeeds were calculated separately and added together.

2.9. Infants' Anthropometrics and Body Composition

2.9.1. Anthropometric Measurements

Infants' weight was determined by weighing before breastfeeding using Medela Electronic Baby Weigh Scales (±2.0 g; Medela Inc., McHenry, IL, USA). Clothing was removed except for a dry diaper and a singlet. Infant crown-heel length was measured once to the nearest 0.1 cm using non-stretch tape and headpiece and footpiece, both applied perpendicular to the hard surface. Infant head circumference was measured with non-stretch tape. Infant BMI was calculated according to the following formula:

$$BMI = \text{Body weight (kg)}/(\text{Height (m)})^2. \quad (5)$$

2.9.2. Body Composition with Bioelectrical Impedance Spectroscopy

Infants' whole body bioimpedance were measured using the Impedimed SFB7 bioelectrical impedance analyzer (ImpediMed, Brisbane, Queensland, Australia) applying an adult protocol (wrist to ankle) according to the manufacturer's instructions and analyzed with settings customized for each infant according to Lingwood et al. [57] and Gridneva et al. [41]. Values of resistance (ohm) at frequency of 50 kHz (R_{50}) were determined from the curve of best fit, averaged for analysis purposes and used in the Lingwood et al. age matched (3 and 4.5 mo infants) equations for fat-free mass (FFM) of 2 and 5 mo infants respectively [57]:

$$FFM\ 3\ \text{mo} = 1.458 + 0.498 \times W - 0.197 \times S + 0.067 \times L^2/R_{50} \quad (6)$$

$$FM\ 4.5\ \text{mo} = 2.203 + 0.334 \times W - 0.361 \times S + 0.185 \times L^2/R_{50}, \quad (7)$$

where L is body length (cm), R_{50} is resistance (Ω), S is sex (male = 1, female = 2) and W is infant weight (kg).

%FM was calculated as follows:

$$\%FM = 100(\text{Weight (kg)} - \text{FFM (kg)})/\text{Weight (kg)}. \quad (8)$$

2.9.3. Body Composition with Ultrasound Skinfold Measurements

Infant ultrasound skinfold measurements were carried out using the Aplio XG (Toshiba, Tokyo, Japan) ultrasound machine, PLT-1204BX 14-8 MHz transducer and sterile water-based Parker ultrasonic gel (Fairfield, NJ, USA). Single ultrasound scans of four anatomical sites (biceps, subscapular, suprailiac, and triceps) were performed on the left side of the body with minimal compression. Skinfold thickness (skin thickness and the skin–fat interface to fat–muscle interface distance) was measured directly from images on the screen using electronic calipers. One experienced sonographer (DG) with good intra- and interrater reliability [44] performed all of the measurements.

The doubled ultrasound skinfold thickness was used in Brook body density (d) age-matched (3–18 mo) equations [58] developed for skinfolds measured with calipers:

$$\textit{Male } d = 1.1690 - 0.0788 \times \log\left(\sum \text{SFT}\right) \tag{9}$$

$$\textit{Female } d = 1.2063 - 0.0999 \times \log\left(\sum \text{SFT}\right), \tag{10}$$

where d is infant body density (kg/L) and $\sum$SFT is a sum of four skinfolds (mm).

Predicted body density was converted to %FM using the Lohman equation [59]:

$$\%FM = 100 \times (5.28/d - 4.89), \tag{11}$$

where d is the infant body density (kg/L).

2.10. Statistical Analysis

Statistical analysis was performed in R 2.9.0 [60] for Mac OSX using additional packages nlme [61]; lattice [62], lattice extra [63], and car [64]; MASS [65], sfsmisc [66] and multcomp [67] for mixed effects modeling, data representation, robust regression, and multiple comparisons of means, respectively. Descriptive statistics are reported as mean ± standard deviation (SD) (range) or median (IQR) unless otherwise stated; model parameters are presented as estimate ± standard error (SE), and, where appropriate, an approximate 95% confidence interval (95% CI).

Measurements missing due to insufficient sample volume: skim milk leptin, whole milk leptin, adiponectin, total protein, whey and casein protein, lactose and total carbohydrate ($n = 3$); lysozyme ($n = 5$). Measurements of fat ($n = 14$) were missing as a result of either insufficient sample volumes or absence of separate feed volumes from breasts where both breast were offered during one feed. Also missing were feeding frequency as reported by mothers ($n = 6$), measurements of length, head circumference, infant BMI, %FM measured with bioelectrical spectroscopy ($n = 4$) and %FM measured with ultrasound skinfolds ($n = 5$).

GE time was determined as the time from the start of F1 to the start of F2 and included the time between two feeds and feed duration. Feed duration was included as up to 80% of HM consumed by term healthy breastfed infants in the first 4–5 min [68]. GE during breastfeeding was defined as the volume of milk to have left the stomach, calculated as the difference between the immediate post-feed stomach volumes and the sum of R1 and FV.

Due to the lack of term infant gastric-emptying studies focusing on stomach volume, no power calculation/sample size determination could be performed for this study. A goal of 20 infants at each two and five months was selected with the expectation that this would be sufficient to show overall patterns. When available, infants were included in both subsets to allow for investigation of longitudinal patterns. Linear mixed effects models allow us to treat the individual feeds as separate, without having to assume independence, when there may be correlations between feeds within infants.

Influences on GE rate were analyzed by first fitting a time curve to the sequential post-feed stomach volumes using linear mixed effects models; as curves differed significantly within and between infants ($p < 0.001$), random time curves were fitted to feeds within infants. Time terms (linear, square root) were selected as per the fractional polynomial method of [69]; this model also considered

possible confounding effects of FV (median-centred) and feed duration (median-centred). Interaction terms involving the time curve indicated changes in the GE rate; main effects indicated overall effects on post-feed stomach volumes but not the GE rate. The addition of one term to this base model was used to investigate associations with (a) concentrations/doses of hormones/macronutrients; (b) infant characteristics/anthropometrics/BC; (c) R1. Whether the overall effect of HM component concentrations differs by feed volume was investigated by including interactions between FV and concentration measures. Models using the selected technique did not converge for fat concentration, lysozyme concentration, or lysozyme dose. Omitting the random effect of feed within infant provided converging models, but no evidence of an association with fat or lysozyme was seen. Given the complexity of linear mixed effects models used to analyze GE rate, no further adjustments were performed and $p < 0.05$ was considered to be statistically significant.

Associations between pre-feed residual stomach volumes, FV, immediate post-feed stomach volumes, feed duration, feeding frequency and both hormone and macronutrient concentrations and doses, and infant anthropometrics/BC parameters were tested using robust linear regression. Mixed effects models were considered, but were not significantly better ($p > 0.1$) Robust linear regression (rlm) was chosen so as to address heteroscedasticity in the data and points with high leverage in the majority of the predictors; MM-estimation (M-estimation with Tukey's biweight, initialized by a specific S-estimator) accounting for appropriate covariates was used [65]. Approximate p-values were determined using the Wald test. Multivariate models accounting for FV were used for testing the relationship with FV-dependent predictor (fat dose and concentration).

Possible age differences in HM components, infant characteristics, and GE/breastfeeding parameters were analyzed with either linear mixed effects models or robust linear regression models; model type was determined using likelihood ratio tests. Linear mixed effects models were used to analyze relationships of GE during feed time with HM components and infant characteristics. R1, FV and feed duration were not associated with stomach volume reduction during the feed time, therefore univariate models were run. Multivariate linear mixed effects models accounting for R1, FV and feed duration were used in analysis of relationships of immediate post-feed stomach volumes with HM components and infant characteristics.

Owing to the large number of comparisons, a false discovery rate adjustment [70] was performed on associated subgroupings of results with one or more p-values < 0.05. p-values were considered to be significant at <0.011 for GE time, <0.031 for feeding frequency, <0.038 for R2, and <0.008 for associations between HM components' concentrations.

3. Results

3.1. Participants

Characteristics of the 27 participants (2 months (n = 20; longitudinal: 7 females, 7 males; cross-sectional: 2 females, 4 males); 5 months (n = 21; longitudinal: 7 females, 7 males; cross-sectional: 6 females); overall n = 41 feeds) are described in Table 1. At the study session, infants fed from one (n = 23) or both (n = 18) breasts.

3.2. Influence of Infant Age

Infant anthropometrics and %FM measured with bioimpedance spectroscopy significantly differed by infant age ($p < 0.001$), while breastfeeding and GE parameters did not change significantly ($p > 0.067$) (Table 1).

Lower whey protein concentration (5.51 $\pm$ 0.96 g/L 5 mo vs. 6.41 $\pm$ 1.39 g/L 2 mo, $p = 0.034$) and subsequently a higher casein:whey ratio (0.32 $\pm$ 0.14 5 mo vs. 0.22 $\pm$ 0.07 2 mo, $p = 0.035$) were observed at 5 months. All other measured appetite hormones and macronutrient concentrations did not differ significantly by infant age ($p > 0.053$).

3.3. Analyzed Human Milk Components

Appetite hormones and macronutrient concentrations and doses per feed are presented in Table 2. Higher skim milk leptin concentrations were associated with lower whole milk leptin concentrations (−0.25 [−0.34, −0.16], $p < 0.001$) and higher protein concentrations were associated with higher whey protein concentrations (0.68 [0.41, 0.95], $p < 0.001$). Higher HMO concentrations were associated with higher total carbohydrates concentrations ($p < 0.001$) and lower lactose concentrations ($p < 0.001$).

Table 1. Participant characteristics expressed as mean ± SD and range.

Characteristics	2 mo [a]		5 mo [b]		Total [c]	
	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Infant characteristics						
Infant age (weeks)	9 ± 1	6–10	22 ± 1	18–23	16 ± 7	6–23
Infant length (cm)	57 ± 2	53–61	65 ± 2 ***	62–69	61 ± 4	53–69
Infant weight (kg)	5.3 ± 0.8	4.2–6.3	7.2 ± 1.0 ***	5.8–9.5	6.3 ± 1.3	4.2–9.5
Infant BMI	15.9 ± 1.3	13.9–18.1	17.6 ± 1.7 ***	14.9–20.4	16.7 ± 1.7	13.9–20.4
HC (cm)	39 ± 1	37–42	43 ± 2 ***	40–46	41 ± 2	37–46
Fat Mass with BIS (%)	21.4 ± 3.6	11.1–27.1	28.9 ± 3.2 ***	21.7–35.8	25.3 ± 5.0	11.1–35.8
Fat Mass with US (%)	24.2 ± 3.6	17.5–30.5	26.6 ± 3.6	20.8–35.9	25.5 ± 3.8	17.5–35.9
BF/GE characteristics						
Feed volume (mL)	86 ± 34	35–140	85 ± 33	36–180	86 ± 33	35–180
SV after feed 1 (mL)	87 ± 36	32–141	93 ± 41	22–189	90 ± 38	22–189
Feed duration (min)	28 ± 14	11–72	20 ± 8	6–37	24 ± 12	6–72
SV reduction (mL) [d]	5 ± 21	(−42)–33	4 ± 26	(−57)–56	4 ± 24	(−57)–56
GE time (min) [e]	94 ± 29	44–153	88 ± 18	50–140	91 ± 24	44–140
Residual 1 (mL)	6 ± 12	0–50	11 ± 19	0–62	9 ± 16	0–62
Residual 2 (mL)	20 ± 20	0–81	15 ± 15	0–55	18 ± 18	0–81
Feeding frequency (h) [f]	2.3 ± 0.7	1.0–4.0	2.7 ± 0.8	1.5–4.0	2.5 ± 0.7	1.0–4.0

Data are mean ± SD and ranges. [a] $n = 20$; [b] $n = 21$. [c] $n = 41$ feeds. [d] Stomach volume reduction during feed time is calculated as the difference between the sum of residual 1 and feed volume and the immediate stomach volume after Feed 1 . [e] GE time is the time from the start of Feed 1 to the start of Feed 2 (time between feeds plus feed duration). [f] Feeding frequency self-reported by mothers as to how often infant feeds (e.g., every three hours). *** Indicates significant differences ($p < 0.001$) between two- and five-month-old infants. Abbreviations: BF—breastfeeding; BIS—bioimpedance spectroscopy; GE—gastric emptying; HC—head circumference; SV—stomach volume; US—ultrasound.

Table 2. Concentrations and doses of measured HM hormones and macronutrients.

Components	Concentration		Dose Per Feed	
	Mean ± SD	Range	Mean ± SD	Range
Adiponectin (ng/mL, ng)	10.02 ± 4.08	6.18–22.58	868.62 ± 491.32	238.60–2536.91
WM leptin (ng/mL, ng)	0.51 ± 0.18	0.23–1.10	44.80 ± 24.30	10.15–115.03
SM leptin (ng/mL, ng)	0.28 ± 0.12	0.20–0.84	24.8 ± 15.0	6.91–73.00
Total protein (g/L, g)	11.29 ± 2.56	7.60–24.16	0.99 ± 0.39	0.35–2.29
Casein (g/L, g)	1.54 ± 0.53	0.69–3.45	0.14 ± 0.07	0.04–0.29
Whey protein (g/L, g)	5.97 ± 1.26	3.82–9.08	0.52 ± 0.19	0.17–0.95
Casein:whey ratio	0.27 ± 0.11	0.10–0.73	n/a [a]	n/a [a]
Lysozyme (g/L, g)	0.14 ± 0.12	0.05–0.48	0.01 ± 0.01	0.003–0.030
TCH (g/L, g)	82.72 ± 7.89	67.08–97.49	7.28 ± 2.62	3.28–15.18
Lactose (g/L, g)	65.84 ± 5.14	53.49–77.94	5.86 ± 2.22	2.19–12.06
HMO (g/L, g)	16.88 ± 9.89	(−10.86) [b]–35.77	1.42 ± 0.94	(−1.09) [b]–3.78
Fat (g/L, g)	42.74 ± 12.10	17.42–66.79	3.57 ± 1.45	0.64–6.40

Data are mean ± SD and ranges, $n = 41$ feeds. [a] Casein:whey ratios for doses are the same as for concentrations. [b] Negative values are seen for human milk oligosaccharides (HMO) when lactose measurements are higher than total carbohydrates. Abbreviations: SM—skim milk; TCH—total carbohydrates; WM—whole milk.

3.4. Gastric Emptying Rate

The overall decreasing curvilinear pattern of GE (linear: 0.04 [−0.17, 0.24], $p = 0.72$; square root: −10.5 [−12.7, −8.2], $p < 0.001$) is shown in Figure 2. Higher FVs were associated with faster

(−0.07 [−0.10, −0.03], $p < 0.001$) GE rate (Figure 3) and higher overall post-feed stomach volumes (0.82 [0.53, 1.12], $p < 0.001$). No association was seen between feed duration and post-feed stomach volume (−0.25 [−0.68, 0.18], $p = 0.23$).

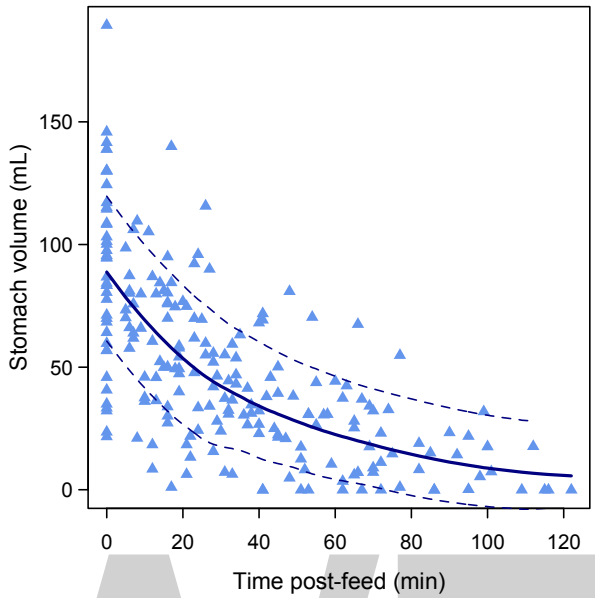

Figure 2. Overall curvilinear pattern of gastric emptying ($n = 41$ feeds). The lines represent the overall pattern of changes in stomach volume as measured by ultrasound imaging. Bold line represents local regression smoother (LOESS, span = 0.9). Dotted lines represent confidence interval.

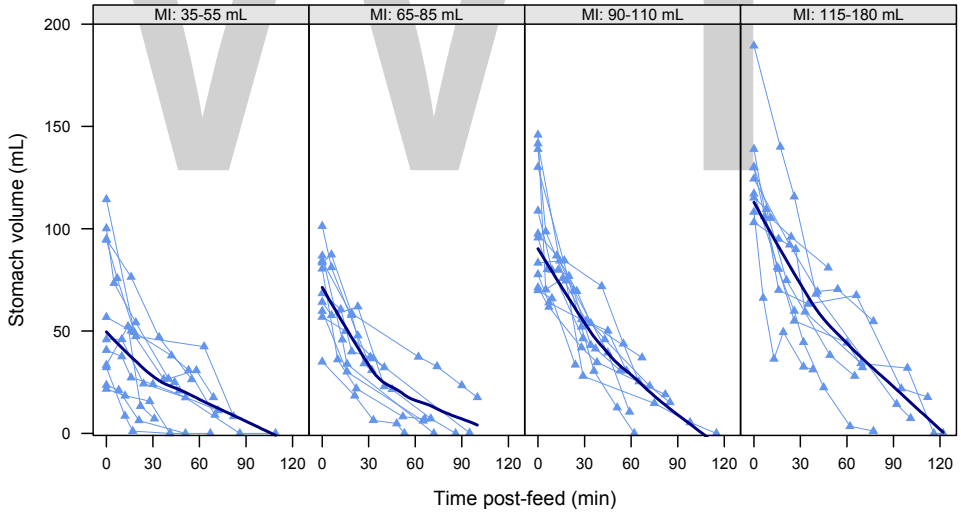

Figure 3. Gastric emptying of individual feeds in term breastfed infants ($n = 41$ feeds). Feeds are grouped by milk intake (MI) to illustrate the effect of the feed volumes; approximately equal numbers are included in each panel. Data points represent stomach volumes calculated from ultrasound images; connecting lines link measurements from the same feed. Bold line represents local regression smoother (LOESS, span = 0.9).

Immediate post-feed stomach volumes were not associated with R1 ($p = 0.91$).

After accounting for time post-feed, FV, and feed duration, as per the above model, larger R1 volumes (0.55 [0.24, 0.86], $p = 0.003$) and higher whey protein concentrations (4.99 [0.84, 9.13], $p = 0.023$) were associated with larger post-feed stomach volumes, while the casein:whey ratio (2.2 ± 0.88, $p = 0.030$) and lactose concentration (−0.04 ± 0.02, $p = 0.037$) modified the GE curve depending on

FV. Higher casein:whey ratios at lower FVs were associated with faster GE, and at higher FVs with slower GE, while higher lactose concentrations at lower FVs were associated with slower GE, and at higher FVs with faster GE. No other associations with post-feed stomach volumes or changes to the GE curves were found (Table 3).

Table 3. HM components and infant characteristics and their associations with feed variables and gastric emptying.

Predictors	Feed Volume [a]		Gastric Emptying Time [a]		Post-Feed Stomach Volumes [b]	
	Estimate ± SE (95% CI)	*p*-Value	Estimate ± SE (95% CI)	*p*-Value	Estimate ± SE (95% CI)	*p*-Value
Concentrations						
Adiponectin (ng/mL)	1 ± 1.3 (−36.6, 134.5)	0.44	2.3 ± 0.7 (0.9, 3.7)	**0.002** [c]	1.3 ± 0.7 (−0.2, 2.7)	0.081
Whole milk leptin (ng/mL)	9.9 ± 29.9 (−36.6, 134.5)	0.74	6.8 ± 15.8 (−24.2, 37.8)	0.67	−9.5 ± 13.3 (−35.8, 16.9)	0.48
Skim milk leptin (ng/mL)	49 ± 43.6 (−36.6, 134.5)	0.26	6.7 ± 24.3 (−41, 54.4)	0.78	39.8 ± 18.4 (−0.8, 80.3)	0.054
Total protein (g/L)	−2.1 ± 2.1 (−6.2, 1.9)	0.30	−0.9 ± 1.1 (−3.1, 1.3)	0.41	1.1 ± 1 (−1.2, 3.4)	0.30
Whey protein (g/L)	−5.5 ± 4.2 (−13.8, 2.7)	0.19	5.8 ± 2.2 (1.6, 10.1)	0.011	5 ± 1.9 (0.8, 9.1)	**0.023**
Casein (g/L)	2.6 ± 10.2 (−17.4, 22.5)	0.80	−12.4 ± 4.7 (−21.5, −3.2)	0.013	−2 ± 4.4 (−11.6, 7.6)	0.66
Casein:whey ratio	24.5 ± 46.1 (−65.9, 114.9)	0.59	−65.9 ± 21 (−107.1, −24.7)	**0.003**	−17.3 ± 20.1 (−61.4, 26.9)	0.41
Lysozyme (g/L)	−81.4 ± 46.2 (−172, 9.1)	0.079	−19.5 ± 28.3 (−75, 36)	0.49	23.3 ± 15.4 (−7.2, 53.8)	0.13
Total carbohydrates (g/L)	−1.1 ± 0.7 (−2.3, 0.2)	0.12	−0.6 ± 0.4 (−1.3, 0.1)	0.10	−0.5 ± 0.3 (−1.2, 0.1)	0.089
Lactose (g/L)	0.7 ± 1.1 (−1.4, 2.7)	0.51	0.2 ± 0.6 (−0.9, 1.3)	0.76	0.03 ± 0.49 (−1, 1.1)	0.96
HMO (g/L)	−0.8 ± 0.5 (−1.9, 0.2)	0.13	−0.4 ± 0.3 (−1, 0.2)	0.16	−0.4 ± 0.2 (−0.9, 0.1)	0.13
Fat (g/L)	−0.69 ± 0.6 (−1.8, 0.5)	0.26	−0.1 ± 0.3 (−0.6, 0.5)	0.79	−0.1 ± 0.3 (−0.9, 0.6)	0.71
Doses						
Adiponectin (ng)	n/a [d]	n/a [d]	0.02 ± 0.01 (0.01, 0.03)	**0.005**	0.01 ± 0.01 (−0.003, 0.03)	0.094
Whole milk leptin (ng)	n/a	n/a	−0.1 ± 0.2 (−0.4, 0.2)	0.44	−0.2 ± 0.2 (−0.5, 0.2)	0.28
Skim milk leptin (ng)	n/a	n/a	−0.2 ± 0.2 (−0.7, 0.3)	0.38	0.4 ± 0.2 (−0.1, 0.8)	0.086
Total protein (g)	n/a	n/a	−25.9 ± 12.4 (−50.2, −1.7)	0.040	15 ± 13.1 (−13.7, 43.7)	0.27
Whey protein (g)	n/a	n/a	47.6 ± 18.7 (10.8, 84.3)	0.015	50.6 ± 24.3 (−2.8, 104)	0.061
Casein (g)	n/a	n/a	−119 ± 53.3 (−223.4, −14.6)	0.030	0.4 ± 47.6 (−104.2, 105.1)	0.99
Lysozyme (g)	n/a	n/a	−276.2 ± 370.7 (−1002.9, 450.4)	0.46	395.5 ± 258.3 (−114.7, 905.6)	0.13
Total carbohydrates (g)	n/a	n/a	−4.1 ± 1.8 (−7.6, −0.5)	0.030	−4.6 ± 3 (−11.2, 2.1)	0.16
Lactose (g)	n/a	n/a	−5.8 ± 2.7 (−11.1, −0.6)	0.037	−3.1 ± 5.4 (−15.1, 8.9)	0.58
HMO (g)	n/a	n/a	−3.1 ± 3.1 (−9.2, 3)	0.32	−3.2 ± 2.7 (−9.1, 2.8)	0.27
Fat (g)	n/a	n/a	−2.8 ± 2.7 (−8.1, 2.4)	0.30	−4.9 ± 2.8 (−12.7, 2.8)	0.15

Table 3. *Cont.*

Predictors	Feed Volume [a]		Gastric Emptying Time [a]		Post-Feed Stomach Volumes [b]	
	Estimate ± SE (95% CI)	*p*-Value	Estimate ± SE (95% CI)	*p*-Value	Estimate ± SE (95% CI)	*p*-Value
Demographics						
Infant sex (Male)	−2.2 ± 10.7 (−23.1, 18.8)	0.84	−1.5 ± 7.5 (−16.3, 13.2)	0.84	−8.4 ± 4.6 (−17.8, 1.1)	0.081
Infant age (months)	−0.9 ± 3.6 (−7.9, 6)	0.80	−1.8 ± 2.5 (−6.6, 3)	0.47	−1.5 ± 1.4 (−4.5, 1.6)	0.32
Anthropometrics						
Infant length (cm)	−0.03 ± 1.3 (−2.6, 2.6)	0.98	−1.3 ± 0.9 (−3, 0.4)	0.15	−0.5 ± 0.6 (−1.8, 0.8)	0.44
Infant weight (kg)	0.7 ± 4.1 (−7.4, 8.8)	0.87	−2.3 ± 2.9 (−7.9, 3.4)	0.43	−2.3 ± 1.8 (−6.3, 1.7)	0.23
Head circumference (cm)	−2.5 ± 2.6 (−7.5, 2.5)	0.34	−1.4 ± 1.8 (−4.9, 2.1)	0.42	−1.8 ± 1.2 (−4.5, 0.8)	0.15
Infant BMI	−0.2 ± 3.2 (−6.5, 6)	0.94	−1.5 ± 2.2 (−5.8, 2.8)	0.48	−3.2 ± 1.5 (−6.6, 0.2)	0.062
Body composition						
Fat mass with US (%)	0.6 ± 1.4 (−2.2, 3.4)	0.67	−0.3 ± 0.9 (−2.2, 1.5)	0.71	−0.6 ± 0.7 (−2.1, 1.0)	0.42
Fat mass with BIS (%)	0.4 ± 1.1 (−1.8, 2.5)	0.74	−0.4 ± 0.7 (−1.9, 1)	0.56	−0.5 ± 0.5 (−1.5, 0.5)	0.35

Data are parameter estimate ± SE and 95% CI, n = 41 feeds. [a] Effects of predictors taken from univariate regression models; [b] Effects of predictors taken from linear mixed effects models that accounted for postprandial time, feed volume and feed duration. [c] After the false discovery rate adjustment the p-values were considered to be significant at <0.011 for GE time (bold font); [d] n/a—dosage is dependent on feed volume. Abbreviations: BIS—bioimpedance spectroscopy; HMO—human milk oligosaccharides; US—ultrasound skinfolds.

3.5. Feed Volume, Feed Duration, and Gastric Emptying during Breastfeeding

Higher FVs were associated with higher stomach volumes measured immediately post-feed (0.79 [0.51, 1.07], $p < 0.001$) and longer GE times (0.24 [0.03, 0.46], $p = 0.033$). FV was not associated with either concentrations of measured HM components or infant's characteristics/anthropometrics/BC (Table 3).

Feed duration was not associated with FV (0.06 [−0.03, 0.15], $p = 0.20$) or R1 volume (0.01 [−0.17, 0.19], $p = 0.91$).

After accounting for R1 (1.07 [0.47, 1.7], $p = 0.002$), FV (1.00 [0.71, 1.3], $p < 0.001$) and feed duration (−0.30 [−0.96, 0.36], $p = 0.34$), immediate post-feed stomach volumes were not associated with either measured HM components ($p > 0.068$) or infant's demographics/anthropometrics/BC ($p > 0.46$). Stomach volume reduction during breastfeeding was not associated with either measured HM components ($p > 0.11$); infant's demographics/anthropometrics/BC ($p > 0.48$); R1, FV or feed duration ($p > 0.34$).

3.6. Gastric Emptying Time

The GE time was not associated with feed duration (0.35 [−0.29, 0.98], $p = 0.28$), but was negatively associated with R2 (−0.63 [−1.05, −0.21], $p = 0.005$) after accounting for FV ($p < 0.001$). Longer GE times were associated with higher adiponectin concentration (2.3 [0.9, 3.7], $p = 0.002$) and dose (0.02 [0.01, 0.03], $p = 0.005$), and lower casein:whey ratio (−65.9 [−107.1, −24.7], $p = 0.003$). No associations with infant characteristics were seen (Table 3).

3.7. Pre-Feed Residuals

Infants cued for F1 and F2 with different residual volumes (R1 and R2) present in their stomachs (Table 1). Larger FVs were associated with smaller R1 volumes ($p = 0.002$), with each −0.92 [−1.47, −0.37] mL of R1 volume resulting in extra mL of FV. Larger R2 volumes were associated with larger FVs ($p = 0.006$), each additional mL of FV resulting in 0.21 [0.07, 0.35] mL greater R2.

There was no association between R2 and R1 in univariate model (0.11 [−0.19, 0.42], $p = 0.46$). After accounting for FV and GE time ($p < 0.001$ for both) larger R2 volumes were associated with larger R1 volumes (0.36 [0.11, 0.60], $p = 0.005$).

After accounting for FV, R2 was not associated with any concentration of HM components ($p \geq 0.038$ after adjusting for multiple comparisons).

3.8. Feeding Frequency

A longer time between the feeds was seen when infants were longer, heavier, and had higher %FM measured with BIS (Table 4) in univariate models. The associations for length and weight were not significant after accounting for the other ($p > 0.38$); the association for %FM measured with BIS was not significant after accounting for infant length ($p = 0.095$).

Table 4. Associations between infant feeding frequency and HM components and infant characteristics.

Predictors	Feeding Frequency (h) [a]	
	Estimate ± SE (95% CI) [b]	*p*-Value
Concentrations		
Adiponectin (ng/mL)	−0.001 ± 0.03 (−0.06, 0.06)	0.96
Whole milk leptin (ng/mL)	−1.1 ± 0.7 (−2.5, 0.3)	0.13
Skim milk leptin (ng/mL)	0.8 ± 1.6 (−2.3, 4)	0.60
Total protein (g/L)	−0.05 ± 0.05 (−0.15, 0.04)	0.28
Whey protein (g/L)	−0.1 ± 0.1 (−0.3, 0.1)	0.42
Casein (g/L)	0.04 ± 0.2 (−0.4, 0.5)	0.86
Casein:whey protein ratio	0.4 ± 1.1 (−1.7, 2.5)	0.68
Lysozyme (g/L)	−0.4 ± 1.1 (−2.5, 1.7)	0.71
Total carbohydrates (g/L)	0.01 ± 0.02 (−0.03, 0.04)	0.73
Lactose (g/L)	−0.05 ± 0.02 (−0.1, −0.01)	0.031
HMO (g/L)	0.01 ± 0.02 (−0.03, 0.04)	0.73
Fat (g/L)	−0.02 ± 0.01 (−0.04, 0.01)	0.19
Doses		
Adiponectin (ng/mL)	0.0002 ± 0.0003 (−0.0004, 0.0008)	0.50
Whole milk leptin (ng/mL)	−0.002 ± 0.01 (−0.01, 0.01)	0.80
Skim milk leptin (ng/mL)	0.01 ± 0.01 (−0.01, 0.03)	0.59
Total protein (g/L)	0.1 ± 0.3 (−0.5, 0.8)	0.67
Whey protein (g/L)	0.1 ± 0.7 (−1.3, 1.5)	0.89
Casein (g/L)	2.1 ± 2 (−1.7, 5.9)	0.27
Lysozyme (g/L)	−5.7 ± 17.2 (−39.4, 27.9)	0.73
Total carbohydrates (g/L)	0.1 ± 0.1 (0, 0.2)	0.22
Lactose (g/L)	0.04 ± 0.06 (−0.08, 0.17)	0.49
HMO (g/L)	0.3 ± 0.1 (0, 0.5)	0.051
Fat (g/L)	−0.2 ± 0.1 (−0.5, 0)	0.085
Demographics		
Infant sex (Male)	−0.2 ± 0.3 (−0.7, 0.4)	0.53
Infant age (months)	0.2 ± 0.1 (0, 0.3)	0.078
Anthropometrics		
Infant length (cm)	0.1 ± 0.03 (0.04, 0.15)	**0.004** [c]
Infant weight (kg)	0.2 ± 0.1 (0.1, 0.4)	**0.010**
Head circumference (cm)	0.1 ± 0.1 (0, 0.2)	0.23
Infant BMI	0.13 ± 0.1 (0, 0.3)	0.10
Body composition		
% fat mass with US	0.07 ± 0.03 (0, 0.13)	0.040
% fat mass with BIS	0.08 ± 0.02 (0.03, 0.12)	**0.002**

Data are parameter estimate ± SE and 95% CI, $n = 41$ feeds. [a] Feeding frequency self-reported by mothers as to how often infant feeds (e.g., every three hours). [b] Effects of predictors are results of univariate regression model. [c] After the false discovery rate adjustment the *p*-values were considered to be significant at <0.031 (highlighted). Abbreviations: BIS—bioimpedance spectroscopy; HMO—human milk oligosaccharides; US—ultrasound skinfolds.

4. Discussion

Our research shows that HM components, such as adiponectin, whey protein, casein:whey ratios, lactose, total carbohydrates, and oligosaccharides are associated with gastric emptying and breastfeeding patterns of breastfed infants. GE is a mechanism involved in satiety, therefore milk components influencing GE have the potential to affect infant milk intake and therefore growth and development in early life and subsequently health later in life.

Given the assumption that HM composition potentially influences GE [14,71], in term infants we expected the appetite hormones to be associated with infant GE rate such that high concentrations and/or doses of leptin would result in slower GE [30], whereas adiponectin might induce faster GE [37] consistent with both animal and human models. However, neither the concentrations nor doses of these hormones were related to GE rate. Previously skim milk leptin was not found to be associated with either GE rate or GE time [33,34], which we have confirmed with this larger study cohort. It was speculated that whole milk leptin, which is known to be of higher concentration, might be the reason for the negative finding [32]. While our measures of whole milk leptin were typically higher, there is an opinion that values of this magnitude are unlikely to contribute considerably to infant serum levels [72] so only the local pathways would be engaged in GE regulation. As such we were unable to find a relationship between whole milk leptin and GE. This is in contrast to animal studies showing reduced GE [30] or food intake [31] after injection or oral administration of leptin, respectively. However, it is possible that the long-term energy expenditure regulatory effect of leptin [73] may mask its short-term satiety effect on GE. Alternatively, if levels of leptin are contributing significantly to serum levels, there is a possibility that the number of receptors in the stomach of the young infant is low. Further, short-term satiety signaling through hypothalamic neurons is not fully mature, both of which would allow the infant to maintain a high physiological drive to feed to ensure adequate growth [28,73]. Gender differences in infant serum leptin levels associated with adiposity [74] have also been speculated to play a role in gastric response to HM leptin, although we did not find any relationships between infant sex/adiposity and both GE rate and GE time.

In contrast to leptin, we found that increased levels and doses of adiponectin were associated with longer GE times. This finding may partially explain the growth-regulating effect of adiponectin in infants in the first six months of life [29], when high HM adiponectin concentration is associated with lower infant weight and adiposity. Further adiponectin is 20-fold higher in concentration compared to leptin and is therefore likely to have greater biological significance [75]. The lack of association between adiponectin and GE rate is in agreement with studies of rats that showed that gastric epithelium and glands are populated with adiponectin receptors, which downregulate gastric motility [36,76]. Conversely, the findings are in contrast to studies of type 2 diabetic adults, in which elevated levels of adiponectin were associated with faster GE [37]. Further, other HM hormones such as ghrelin, cholecystokinin, and insulin may counteract or interact synergistically with leptin [77,78] and/or adiponectin.

Our study examined an extensive array of macronutrients beyond fat, total protein and lactose. Consistent with the findings of Cannon et al. [34] there were no associations with fat and total protein and either GE rate or GE time. Studies in dogs indicate that all three major macronutrients activate the ileal brake, resulting in reduction of GE; limited human studies support the findings for fat and carbohydrates while associations with protein are not so straightforward [79,80]. We were unable to find associations between the HM fat content and GE consistent with findings of Khan et al. [39] and Kent et al. [38] regarding the feeding frequency. This may be because lipids initiate the ileal brake when they reach the ileum via hydrolysis of triacylglycerol into fatty acids, thereby producing a delay in GE in humans [79]. Further analysis of HM fatty acids may shed more light on GE in breastfed infants.

However, we have found that higher whey protein concentrations are associated with larger post-feed stomach volumes, although we did not see any interaction with time, so no effect on GE rate was detected. This contradicts the results of studies of GE conducted on breastfed and formula-fed infants or studies of formula with different casein:whey ratio [15,81] in which a fast or slow GE rate

was explained by concentrations of whey protein or casein, respectively. Previous studies, however, could not adequately analyze the effect of the whey protein concentration in conjunction with volume, as they only reported gastric half-emptying time, restricted monitoring time, and/or controlled infants' volume intakes. The whey fraction of HM is highly soluble in the gastric juices and rapidly empties from the stomach compared to other proteins such as casein. Whey isolate, however, was associated with a lower gastric inhibitory polypeptide (GIP) response in adults, consistent with decreased rate of GE [82]. It may very well be that whey protein speeds up the initial stage of GE (probably during the breastfeeding time), but once it activates jejunal or ileal brakes the overall GE is reduced.

While lactose is related to GE rate, it is affected by FV; at the middle range FVs (71–108 mL) lactose has no relationship with GE, whereas at lower FVs higher lactose concentrations are associated with slower GE, and at higher FVs with faster GE. These results are consistent with Khan et al. [39], who reported an association of higher lactose concentration with increased feeding frequency. These findings could be an important addition to the evaluation of the digestive and metabolic effect of lower breastfeeding frequency and larger FVs, common in Western countries, contrary to the lactation practices in traditional societies [83].

In terms of casein:whey ratios the effect is opposite to that of lactose where at lower FVs higher casein:whey ratios are associated with faster GE, and at higher FVs with slower GE, which may explain the contradictory findings for casein associations with GE rate in previous studies [14,23]. Cows' milk casein was found to activate the ileal brake in adults, resulting in reduced food intake, although its effect on GE was not significant [84]. The finding of smaller volumes resulting in more rapid GE rate might be explained by the time casein spends in the acidic environment of the stomach. While soluble whey proteins rapidly enter the small intestine mostly intact, casein transit is delayed due to the curd formation. When it exits the stomach it is mainly in the form of degraded peptides [85]. If the FVs are small some casein may exit intact, thereby speeding up GE, while if the FVs are large, casein curdles and degrades to the opioid peptides that slow down GE [86]. However, this mechanism does not explain why higher casein:whey ratios of HM were associated with shorter GE time, which could be due to the smaller amounts of whey protein reaching the small intestine and having less effect on jejunal or ileal brakes [80]. Our finding that higher whey protein concentrations are associated with larger post-feed stomach volumes further supports this possible explanation.

Further, *k*-casein has been shown to inhibit the binding of *Helicobacter pylori* to human mucosa in vitro [87]. *Helicobacter pylori* are Gram-negative bacteria present in the stomach, and are known to downregulate levels of ghrelin and leptin in the stomach [88], which may significantly affect GE. The protective action of HM *k*-casein is reinforced by lysozyme, one of the major whey proteins. While we have not seen any significant associations between lysozyme and GE, lysozyme contributes to the control of the GI bacterial population [89], and could be upregulated to control the bacterial population in the GI tract [90] and increase digestion of microbial protein [24], all of which could potentially influence GE. In a clinical study of preterm infants, lysozyme added to donor HM or formula was associated with increased body weight, normalization of the stool, and improved feed tolerance [91]. While all of this suggests that lysozyme could potentially have an effect on GE in certain circumstances, given that we have studied a healthy population the magnitude of the effect could be insignificant.

GE during feed administration has been previously documented in preterm infants. In this study, an average of 20% of feed volume is emptied from the stomach during breastfeeding compared with 10% in preterm [92]. This is probably due to a more mature GI tract in term infants and the effect of both larger FVs and present pre-feed residuals, which were associated with faster GE rate, but not to the longer feed duration time in term infants or milk composition as no associations were found.

While we speculated that milk composition might regulate the milk intake of the infant and/or the residual volume in the stomach prior to cueing for the next feed, we were unable to show this. Rather, FV is more strongly associated with GE rate than variations in milk composition. Gastric mechanosensation is an important factor in the regulation of satiation during food intake. Indeed, gastric distention is an important determinant of GE [93], and volume-related suppression of GE rate

has been reported in animal models [94]. The observed volume-related acceleration of GE with larger FVs emptying more quickly in term breastfed infants is consistent with our previous findings [34]. The biggest effect of volume was seen after the feed and as the post-prandial period progressed the magnitude of this effect decreased (Figure 2). This may also explain the variability in the time between each feed for an infant over a 24-h period [33,38]. Feeding frequency decreases between one and three months of lactation, while milk intake during each breastfeeding session increases, with both parameters remaining constant up to six months [95]. This is attributed to the fact that as infants mature they become able to consume larger FVs [38], resulting in a longer time between feeds. Also, larger FVs are generally consumed at night or in the early morning when the frequency of feeding declines [33,38]. This decline in feeding frequency also coincides with higher nocturnal concentrations of leptin and fat, and lower concentrations of lactose in HM [33], although relationships between both feeding frequency and FV and these components' concentrations are yet to be evaluated.

The recommendations for breastfeeding are to feed on demand. Interestingly, we found that the majority of infants cued for a feed when milk was still present in the stomach, albeit in variable volumes (Table 1). This suggests that the reduction of gastric distension, which regulates hunger sensations, plays a greater role in signaling time to feed [96]. Further, it may be beneficial to the developing infant to have the gastric mucosa exposed to HM anti-inflammatory components such as lysozyme or immunomodulatory agents and growth factors, all of which contribute to the maturation of the GI tract [8]. Thus it may be detrimental to prescribe decreasing the frequency of feeding in breastfed infants or expect the infant stomach to be empty in order to feed again [97].

Furthermore, interesting associations were observed between infant milk intake and volumes remaining in the stomach prior to the first and second feed. Smaller residual volumes prior to the first feed were associated with greater milk intakes, and greater milk intakes were associated with larger volumes in the stomach prior to feeding again. This suggests that breastfed infants may appear to be consuming HM volumes in a variable pattern, but due to varying residuals may actually be feeding to a predetermined stomach volume, which is also supported by the positive relationship between both pre-feed residuals (R1, R2). In fasting adults ghrelin was found to increase and spontaneously decrease at the time points of the customary meals [98], supporting the involvement of the brain in GI tract regulation. Further studies monitoring two or more consecutive feeds or even 24-h GE measurements and analyses of ghrelin in HM would clarify this finding.

In healthy adults post-lag GE and colonic transfer is reported to be faster in men than in women [99]. In this study infant sex, age, anthropometrics, and BC were not associated with GE and breastfeeding parameters, with the exception of feeding frequency. Feeding frequency decreases in the first three months of lactation and then remains stable until six months [95,100]. The absence of a significant association between feeding frequency and age, together with associations with anthropometric and body composition parameters, illustrates that feeding frequency is dictated by the growth and development of an infant rather than the infant age. These findings further underline the need for breastfeeding on demand, with the frequency linked to individual infant growth rates rather than scheduled feeding, which could exert a detrimental effect on infant growth.

While the monitoring of a single feed limits the analysis possibilities, examination of multiple feeds requires the study to be carried out in the mother's home for long periods of time. The sample size is not a limitation of the study, as although no associations between milk composition and GE rate were detected, we were able to clearly show a relationship between FV and GE rate as well as associations between milk composition and other GE parameters.

5. Conclusions

Human milk appetite hormones and macronutrients and feed volume affect gastric emptying and feeding patterns in term breastfed infants. Adiponectin, whey protein, and casein:whey ratio are associated with GE, while the effects of casein:whey ratios and lactose concentrations on GE vary with

feed volume. Larger feed volumes result in a faster GE rate. Thus, milk composition and feed volume play an important role in appetite regulation via gastric function.

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Author Contributions: Zoya Gridneva conceived and designed of the study, collected the data, performed biochemical analysis, performed the statistical analyses, and wrote the manuscript; Sambavi Kugananthan adapted the methods for measuring adiponectin and leptin in whole milk, performed the hormone analysis, and critically reviewed the manuscript; Anna R. Hepworth was involved in research design, data analysis, and interpretation, and critically reviewed the manuscript; Wan J. Tie performed biochemical analysis and critically reviewed the manuscript; Ching T. Lai performed biochemical analysis, provided technical support, and critically reviewed the manuscript; Leigh C. Ward provided technical and educational support, contributed to the design of the study, and critically reviewed the manuscript; Peter E. Hartmann substantially contributed to the conception and design of the study, contributed reagents/materials/analysis tools for research, and critically reviewed the manuscript; Donna T. Geddes conceived and designed of the study, carried out the ultrasonography component of the study, provided the ultrasound images, and critically reviewed the manuscript. All authors have read and approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

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Food Insecurity, Poor Diet Quality, and Suboptimal Intakes of Folate and Iron Are Independently Associated with Perceived Mental Health in Canadian Adults

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Abstract: Background: To address nutrition-related population mental health data gaps, we examined relationships among food insecurity, diet quality, and perceived mental health. Methods: Stratified and logistic regression analyses of respondents aged 19–70 years from the Canadian Community Health Survey, Cycle 2.2 were conducted (n = 15,546). Measures included the Household Food Security Survey Module, diet quality (i.e., comparisons to the *Dietary Reference Intakes*, Healthy Eating Index), perceived mental health (poor versus good), sociodemographics, and smoking. Results: In this sample, 6.9% were food insecure and 4.5% reported poor mental health. Stratified analysis of food security and mental health status by age/gender found associations for poor diet quality, protein, fat, fibre, and several micronutrients (p-values < 0.05); those who were food insecure tended to have higher suboptimal intakes (p-values < 0.05). After adjustment for covariates, associations in relation to mental health emerged for food insecurity (OR = 1.60, 95% CI 1.45–1.71), poor diet quality (1.61, 95% CI 1.34–1.81), and suboptimal intakes of folate (OR = 1.58, 95% CI 1.17–1.90) and iron (OR = 1.45, 95% CI 1.23–1.88). Conclusions: Population approaches that improve food security and intakes of high quality diets may protect people from poor mental health.

Keywords: food insecurity; diet quality; nutrient intakes; mental health

1. Introduction

A growing body of evidence indicates relationships among food insecurity, diet quality, and mental health; however, their simultaneous effects are rarely studied. Food insecurity occurs when people are physically or economically unable to consume a sufficient quantity of food or have uncertainty in their ability to do so [1], and it has been associated with various indicators of mental ill health such as depression, mania, disordered eating, impaired cognition, higher internalizing and externalizing behaviours, and suicidal ideation [2–5]. For indicators such as depression, the links with food insecurity and nutrition may be bidirectional [6]. Diet quality, which encompasses adequacy or sufficiency, moderation, variety or diversity, and balance or equilibrium of nutrient and food intakes [7], is also critical to mental health. The two concepts of food insecurity and diet quality are interrelated: food insecurity includes the components of insufficient food quantity and quality, feelings of deprivation, and disrupted eating [8], and so it is inversely associated with higher levels of diet quality [9].

The understanding about the relationship between food insecurity and diet quality with respect to mental health is evolving. Food insecurity can contribute to over- and under-nutrition, nutrient excesses, disproportions, and deficiencies, as well as eating disturbances [10–12]. Manifestations of nutritional deficiencies include psychiatric symptoms, and single nutrients such as omega-3 fatty acids and folate have received attention in epidemiologic and intervention studies targeting mental health [13,14]. Intervention studies that have utilized multi-nutrient formulas with both minerals and vitamins have shown an even greater benefit for mental health [15], indicating the need for all essential nutrients for optimal mental function. Studies of adults with mood disorders, for example, have indicated that when compared to a general population, a larger proportion had suboptimal intakes of essential nutrients [16]. Furthermore, nutrient intakes have been found to be correlated with psychological functioning [17], and when food insecurity was also present, nutrient intakes and mania symptoms were worse [18]. Eating disturbances (e.g., fasting, binging), which can impact nutritional status, are also associated with both food insecurity and mental health [19–21]. Compromised diet quality caused by food insecurity may also contribute to alterations in the gut–microbiota–brain axis [22], creating metabolic, immune, and inflammatory responses [23] that contribute to worsening mental health. Conversely, intake of a good quality diet combined with food security can contribute to anti-inflammatory and protective effects of various nutrients and other bioactive components that optimize the brain's biochemistry and support cognitive health.

While both food security and diet quality are critical to mental health, there has been limited research examining their relationships. Specifically, there are knowledge gaps about how food insecurity may impact nutrient intakes in the context of poor and good levels of mental health and how these relationships may be influenced by different determinants of health such as income. Furthermore, despite burgeoning research in this area, studies have focused on specific mental health conditions and have narrowly defined a "good outcome" as a reduction in symptoms of mental illness and/or improved functional ability. In recent years, however, the concept of mental health has moved to a broader interest in individuals' appraisals about their experiences and the meaning that they attribute to these experiences [24], as this may impact health service use.

There has been increasing recognition that barriers to better nutrition must be understood at the population level among groups who are most likely to have poorer-quality diets and to be at risk for mental health conditions. Therefore, examining measures of mental health in a general population and their potential associations with food insecurity and diet quality can provide direction on population-level interventions and policies that foster mental health for individuals without diagnosed mental health conditions, potentially prevent or delay the development of a mental health condition, and optimize outcomes for those with mental health conditions that are symptomatic. The importance of this research is emphasized by the fact that food security status and diet quality are modifiable. To help address these knowledge gaps, we analysed data from a large well-designed cross-sectional study to examine: (1) differences in energy and nutrient intakes by food security and perceived mental health status; (2) differences in adequacy of macro- and micronutrient intakes by food security and perceived mental health status; and (3) the simultaneous effects of food insecurity, diet quality, and suboptimal micronutrient intakes in relation to perceived mental health. We hypothesized that: (1) diet quality is compromised by poor mental health and food insecurity; and (2) food insecurity and diet quality are independent predictors of poor mental health.

2. Materials and Methods

2.1. Sample

The sample was derived from cycle 2.2 (Nutrition) of the Canadian Community Health Survey (2004) conducted by Statistics Canada [25]. A complete description of the survey data is provided in documentation from Health Canada [26]. The survey sample consisted of 35,107 respondents (0 years+) living in private residences in Canada's 10 provinces. Initial interviews were conducted in-person

and collected information about the respondent's demographics, general health, dietary intake (24-h recall), and food security. A second 24-h recall was conducted by telephone with 10,786 respondents 3 to 10 days after the initial interview. For our analysis, we used data from adult respondents aged 19 to 70 (n = 20,498). Adults were selected, as associations between food insecurity and dietary quality is less consistently associated with low dietary quality in children. This is believed to be due to adults shielding children from compromised diets in the context of household food insecurity [9]. Thus, those less than 19 years of age (n = 15,190) were excluded. In addition, adults aged 70 years+ (n = 4371) were excluded, as there was limited data available on the second 24-h diet recalls in this age group.

2.2. Measures

2.2.1. Perceived Mental Health

The variable "perceived mental health" is a general indicator of individuals who are suffering from some form of mental disorder, mental or emotional problems, or distress, which is not necessarily reflected in the more global measure "self-perceived health" [27], and can affect service use [27,28]. This measure of overall mental health status is considered to align with the World Health Organization's definition of mental health where a person with or without a diagnosed mental health condition can experience well-being in which they realize their own abilities, can cope with the normal stresses of life, can work productively, and contribute to their community [29]. Research suggests perceived mental health is associated with mental morbidity measures such as non-specific psychological distress, depressive symptoms, activity limitations, and physical and emotional role functioning [30]. Strong positive associations between all mental morbidity measures and perceived mental health have been reported, with stronger associations between past month prevalence as compared to past 2- to 12-month prevalence and lifetime disorder [30].

Respondents rated perceived mental health based on answers to the question: "How would you say your mental health is? Excellent? Very good? Good? Fair? Poor?" The responses were dichotomized as poor/fair (poor mental health) and good/very good/excellent (good mental health). This treatment of the perceived mental health measure has been done in other studies [31,32], and is an established approach to modifying self-reported health measures [33].

2.2.2. Food Security

Food security was measured using the 18-item Household Food Security Survey Module (HFSSM) [34]. Food insecurity was determined based on affirmative responses to either of the "food sufficiency questions" which asked whether the household, in the past 12 months, sometimes did not have enough to eat or often did not have enough to eat. Food security status was classified as: (1) Food secure: access at all times in the previous year to enough food for an active, healthy life for all household members; (2) Food insecure: included categories of moderate or severe food insecurity where any household member had compromises in quality and/or quantity of food consumed which may have disrupted eating patterns.

2.2.3. Diet Quality

There are various diet quality indicators, and it is recommended that more than one be selected when testing associations with health outcomes [35]. For the current study, the indicators of diet quality included measures of energy and nutrient intakes and comparisons to North American dietary standards, as well as an index of diet quality. Nutrient intakes were compared to the Dietary Reference Intakes (DRIs) [36] by specified age/gender categories to review patterns of nutrient inadequacies. To assess intakes of the major nutrients, the Acceptable Macronutrient Distribution Ranges (AMDR) were used as the reference. The AMDR is a range of intakes for a particular energy source that is expressed as a percentage of total energy intakes, and is associated with reduced risk of chronic disease while providing adequate intakes of essential nutrients [37]. To estimate the prevalence of

potentially inadequate micronutrient nutrient intakes, the Estimated Average Requirement (EAR) cut point method of the *Dietary Reference Intakes* was used. The EAR is a nutrient intake value that is estimated to meet the requirements of half of the healthy individuals in a group. For nutrients with an EAR, the percentage below the EAR reflects the prevalence of potentially inadequate intakes for a given nutrient. For iron, "the full probability approach" [36] was used, which accounts for skewness in the requirement distributions of this nutrient.

The Canadian Healthy Eating Index (HEI) [35] was also used as an indicator of diet quality. The HEI includes nine scored components of the intakes of the four food groups of the Canadian Food Guide [38], total fat (percent of energy intake), saturated fat (percent of energy intake), total cholesterol intake, total sodium intake, and diet variety. The diet variety component is based on having at least one serving from each food group (i.e., vegetables and fruit, grain products, meat and alternatives, milk and alternatives). A final score of $\leq$50 indicates poor diet quality, 51 to 80 indicates a diet that needs improvements, and 81 to 100 indicates good diet quality.

2.2.4. Covariates

The covariates sex, age (categories of 19–30, 31–50, 51–70 years), income (five categories: lowest income, lower middle income, middle income, upper middle income, highest income), education (secondary school graduate or less vs. education above secondary school level), relationship status (married or common-law vs. single), and smoking status (current daily smoker vs. not a current daily smoker) were also included, as these factors can influence dietary intakes and food security. A five-level variable describes income adequacy according to total household income in the past 12 months and the number of people in the household. The highest level of education obtained by the respondent was classified as less than secondary school graduation, secondary school graduation, some postsecondary education, and postsecondary graduation. Relationship status included the categories of married, common-law, widowed/separated/divorced, and single/never married. A dichotomous variable differentiated those who were and were not current daily smokers (i.e., smoking daily or occasionally at the present time).

2.3. Analysis

The secured data was analysed in the Statistics Canada Research Data Centre at the University of British Columbia using SAS (version 9.1, 2003, SAS Institute, Cary, NC, USA) and SIDE-IML (Software for Intake Distribution Estimation in IML language, version 1.11, 2001, Iowa State University, Ames, IA, USA). Survey weights are incorporated into the calculations to provide national representation [25]. To account for survey design effects, the bootstrap resampling technique was used [39].

To assess for quality in dietary intake reporting, energy intake (EI) was examined in relation to estimated energy requirements (EER) [40,41] based on respondents' sex, age, self-reported physical activity level, as well as self-reported or measured height and weight to estimate requirements [42]. The physical activity coefficients used in the EER equation were based on three levels which account for the frequency and duration of activity: active, moderately active, or inactive [26].

To compare differences between food security status and nutrient intakes in relation to mental health, Mann–Whitney U tests, chi-square tests, ANOVAs, or their non-parametric equivalents (e.g., Kruskal–Wallis tests) were used where applicable. The nutritional adequacy of intakes by age/sex group and food security status was determined using data from the first (full sample) and second (subsample) dietary recalls [25]. The prevalence of potentially inadequate intakes were estimated using the EAR cut point approach for nutrients which have an EAR, accounting for age/sex categories and increased requirements of vitamin C in current smokers [43–47].

To examine relationships between the outcome variable mental health (poor vs. good) in relation to food security, diet quality, and suboptimal intakes of micronutrient intakes as defined by EAR cut-offs, logistic regression analysis was conducted which controlled for the covariates. Goodness-of-fit

chi-squared tests were applied to assess for model fit. The level of significance for all statistical tests was $p < 0.05$.

3. Results

3.1. Sample

The sample consisted of more females (55%) than males (45%); about two-thirds (68.4%) of the sample were less than 50 years. Almost 7% (6.9%) were food insecure, and 4.5% reported poor or fair mental health. Within the adult sample (19 to 70 years), about 8% (7.8%) were post-secondary graduates, 44.1% earned an income considered to be at the lower middle range or less, and 50.1% were in a relationship.

3.2. Bivariate Analyses

The medians of EI:EER tended to be lower among those who reported food insecurity, however no significant differences were found (Table S1). Poor quality diets as defined by the HEI were more prevalent among those with poor mental health (33.6%) compared to those with good mental health (26.9%; $p < 0.001$), and there were significant associations between food security status, diet quality, and mental health (all p-values < 0.05). For macronutrient and fibre intakes, there were significant associations between mental health and food insecurity for protein (grams) in males between 31 to 50 years and 51 to 70 years, carbohydrates (g) and fat (g) in females between 31 to 50 years and 51 to 70 years, and fibre (g) in males between 31 to 50 years and females 51 to 70 years (Table S1). For micronutrients, there was a relatively consistent trend observed for intakes by gender categories where those with good mental health and food secure status had higher intakes than those reporting poor mental health and food insecure status (Table S2). For females between 19 to 30 years, vitamin C intakes were significantly lower for those with poor mental health and food insecure status ($p < 0.05$). Thiamin and folate intakes among males and females 31 to 50 years were also significantly lower for those with poor mental health and food insecure status. For males in the same age group, significantly lower intakes of vitamins B_3, B_6, and B_9, as well as the minerals phosphorus, potassium, and zinc in those with poor mental health and food insecure status. For males and females 51 to 70 years, there was significant association for vitamin C when stratified by mental health and food security. For females in this age range, many significant associations were also found that included vitamins A, B_1, B_2, B_3, B_6, B_9, B_{12}, and D, as well as the minerals magnesium, phosphorus, and potassium. Interestingly, for many of the B vitamins, intakes were slightly higher among females with food insecure status who reported poor mental health.

When compared to the DRIs, a significantly higher proportion of respondents that were food insecure had protein intakes below the AMDRs, regardless of mental health status (Table 1). A significantly higher proportion of the sample with food insecure and poor mental health status had micronutrient intakes below the EARs for all vitamins and minerals analysed (exception: vitamin B_9).

3.3. Multivariate Analyses

After adjusting for the covariates, those with poor mental health status had an increased odds of being food insecure (OR = 1.60, 95% CI 1.45 to 1.71), having poor diet quality as measured by the HEI (1.61, 95% CI 1.34 to 1.81), protein intakes below the AMDR (OR = 1.01, 95% CI 1.00 to 1.02), and potentially inadequate intakes of folate (OR = 1.58, 95% CI 1.17 to 1.90) and iron (OR = 1.45, 95% CI 1.23 to 1.88) (Table 2).

Table 1. Prevalence estimates for categories of the acceptable macronutrient distribution ranges (AMDRs) and estimated average requirements (EARs) by mental health and food security [a].

Variable	Good Mental Health		Poor Mental Health	
	Food Secure	Food Insecure	Food Secure	Food Insecure
		AMDRs		
		Protein		
<20%	9.9	15.4 **	10.6	18.6 **
20% to 35%	52.7	51.7	55.4	53.1
>35%	37.4	32.9 **	34.0	28.3 **
		Fat		
<45%	20.3	16.3 **	19.0	21.3
45% to 65%	52.8	58.4 **	56.9	64.5 **
>65%	26.4	25.2	24.1	14.2 **
		EARs		
Vitamin A	52.3	62.8 **	54.4	67.0 **
Vitamin B_1 (Thiamin)	18.1	21.7 **	24.4	39.2 **
Vitamin B_2 (Riboflavin)	15.8	21.2 **	19.7	37.7 **
Vitamin B_3 (Niacin)	2.5	4.3 **	6.4	8.7 **
Vitamin B_6 (Pyridoxine)	26.7	33.0 **	39.6	42.5 **
Vitamin B_9 (Folate)	79.4	82.7 **	87.1	87.2
Vitamin C	35.9	46.0 **	43.2	55.7 **
Iron	7.3	11.3 **	12.5	18.9 **
Magnesium	49.8	59.5 **	58.5	61.2 **
Phosphorus	10.4	17.1 **	16.7	23.7 **
Zinc	33.5	43.9 **	37.9	45.6 **

[a] Carbohydrates and some micronutrients with EARs are not reported because stratified analysis created cell sizes <5; ** $p < 0.001$; test-statistics (z-statistic) range from 2.56 to 75.75; p-values 0.011 to <0.0001.

Table 2. Logistic regression estimates of food insecurity, healthy eating index (HEI), acceptable macronutrient distribution ranges (AMDRs), and estimated average requirements (EARs) in relation to poor mental health.

Variable	Odds Ratio (95% CI)	p-Value
Food insecurity	1.60 (1.45–1.71)	0.0048
	HEI	
Poor vs. good	1.61 (1.34–1.81)	0.0296
Needs improvement vs. good	1.06 (1.00–1.11)	0.2298
	AMDRs	
Fat	1.00 (0.97–1.03)	0.8252
Protein	1.01 (1.00–1.02)	0.0312
	EARs	
Vitamin A	0.88 (0.59–1.31)	0.5253
Vitamin B_1 (Thiamin)	1.45 (0.98–1.13)	0.0604
Vitamin B_2 (Riboflavin)	1.35 (0.66–2.76)	0.4056
Vitamin B_3 (Niacin)	0.53 (0.21–1.32)	0.1727
Vitamin B_6 (Pyridoxine)	0.79 (0.46–1.38)	0.4133
Vitamin B_9 (Folate)	1.58 (1.17–1.90)	0.0148
Vitamin C	1.37 (0.90–2.08)	0.1376
Iron	1.45 (1.23–1.88)	0.0192
Zinc	1.35 (0.90–2.01)	0.1479

Significant associations were also indicated for income, smoking status, and marital status ($p < 0.05$).

4. Discussion

This study provided evidence that dietary quality was lower for those who were food insecure and had poor mental health. Based on stratified analysis by age and gender, significant associations were found between mental health and food insecurity for protein, fat, carbohydrates, fibre, vitamins

A, B_1, B_2, B_3, B_6, B_9, B_{12}, C, and D, as well as the minerals magnesium, phosphorus, potassium, and zinc—particularly for older females. Furthermore, for those who were food insecure and had poor mental health, there was a higher prevalence of potentially inadequate intakes of most vitamins and minerals. When food insecurity, diet quality, and potentially inadequate intakes were simultaneously analysed with adjustment for covariates, food insecurity, poor diet quality, and suboptimal intakes of protein (marginal significance), folate, and iron all independently predicted poor mental health. Food insecurity and poor diet quality appeared to be the most significant predictors of poor mental health.

This study is the first to concurrently analyse food insecurity, diet quality, suboptimal nutrient intakes, and perceived mental health, and found results that are consistent with previous investigations that individually showed lower nutrient intakes, poor diet quality, and food insecurity are associated with poor perceived mental health [3,17,48,49]. In addition, our findings indicated that food insecurity is associated with dietary compromises that are of sufficient magnitude to impact nutritional and mental health, regardless of whether respondents did or did not have a diagnosed mental health condition.

Food insecurity tended to systematically drive increases in the proportion of individuals consuming a poor-quality diet and low intake levels of essential nutrients. Vitamins and minerals have important roles in various brain physiological processes, and have been linked with mental health [17]. Insufficient micronutrient levels can have harmful effects on the brain by reducing proteins such as brain-derived neurotropic factor (BDNF) and by up-regulating the stress response, immune, and oxidative systems [50]. Iron is involved in myelin production [51] and is a cofactor for neurotransmitter synthesis [52]. Zinc is involved in neuron migration, synaptogenesis, and neurogenesis [53]. Vitamins B_6, B_9, and B_{12} affect methylation in the central nervous system [54], and maintain the integrity of the myelin sheath [55]. Indeed, all dietary vitamins and minerals have critical roles in brain metabolism [15].

Multiple integrated mechanisms could explain the various findings reported here. For example, diet quality may represent the concerted action of various compounds within foods working synergistically [56] that support mental health. Individually, inadequate intakes of nutrients such as folate and iron independently predicted mental health, suggesting that there is a heightened need for some specific nutrients in relation to mental well-being. The results are particularly surprising for folate intakes, given that in 1998 the Government of Canada instituted mandatory folic acid fortification for all white flour and enriched pasta and cornmeal products [57], and the proportion of respondents with poor mental health consuming levels below the EAR did not differ significantly by food security status. While food insecurity encompasses components of dietary quality, many individual (e.g., stress and anxiety associated with attempts to access food), household (e.g., deprivation, parent's placement of a child's nutrition needs above theirs), and structural (e.g., poverty, stigma) drivers also explain independent links between food insecurity and mental health [58,59]. Clearly, future research is needed to help disentangle the complex relationships between nutritional and population mental health, which can then better direct food and health policies.

There are several limitations of this study. With the cross-sectional design, causality cannot be inferred. However, results from longitudinal studies suggest that bidirectional relationships occur between poor mental health (defined as depression), food insecurity, and diet quality [6,60]. The HFSSM focuses on income and does not consider factors such as diet self-efficacy (i.e., the belief in one's ability to manage diet even in the face of obstacles such as stress) and other important psychosocial variables that relate to dietary behaviours [61,62]. The HFSSM also does not account for intra-household variations [63], and the measures of food insecurity (previous year) and diet quality (previous 24 h) reflected incongruent time frames. The reporting of suboptimal macronutrient, vitamin, and mineral nutrient intakes were confined to those where reference levels (e.g., EARs) exist and sufficient data was available to analyse. As is common with dietary surveys, underreporting did occur and may have overestimated prevalences of suboptimal intakes. However, no significant differences were observed in EI:EER and food security status among the age/gender categories. It is difficult to

determine how these results may translate to other countries, as food insecurity and mental health measures vary. In national surveys, the US and Canada use versions of the HFSSM, and estimates of food insecurity in the US appear to be double that observed in Canada [64,65]. However, in Canada, food insecurity appears to have more of an impact on nutritional inadequacy [66]. None of the other industrialized countries use the same self-report mental health measure as the CCHS. The monitoring of self-reported mental health has only emerged in recent years, and this measure is increasingly recognized to be relevant as poor mental health has been associated with physical health, health service utilization, and psychiatric morbidities [67].

The results of this study suggest that alternatives for food and social policy need to be explored. In the Canadian context, there are limited publicly funded food programs that provide assistance to vulnerable households. In the US, for example, food subsidy programs such as the US Department of Agriculture's Supplemental Nutrition Assistance Program (SNAP) for Women, Infants, and Children (WIC) exist to improve food security for low-income individuals and households. Evidence suggests that participation in these programs contributes to small increases in the intake of targeted nutrients and foods, and that the nutrition education component of the programs (SNAP-Ed) improves skills in food resource management [68–70]. Thus, contextual factors such as food and nutrition assistance programs, food supply, food pricing, and other policies and programs need to be explored as means to ameliorate food insecurity, improve diet quality, and foster mental health within populations.

5. Conclusions

Overall, the results suggested that food insecurity, poor diet quality, and inadequate intakes of selected micronutrients were independently associated with poor mental health in a general national sample. These findings suggest that public policies that support both food security and high quality dietary intakes could promote mental health and well-being. Population-based interventions that have the potential to improve diet quality and food security—such as the July 2016 Canadian Universal Child Care Benefit for families below specified income levels—should be evaluated in relation to mental health.

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Validity of the Food Frequency Questionnaire Assessing the Folate Intake in Women of Reproductive Age Living in a Country without Food Fortification: Application of the Method of Triads

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Abstract: The study aimed to examine the external validity of the Folate Food Frequency Questionnaire (F-FFQ) designed for assessing the folate intake in Serbian women of reproductive age. The F-FFQ was tested against repeated 24 h dietary recalls and correspondent nutritional biomarkers (red blood cells (RBC) and serum folate concentrations) using the method of triads. In a cross sectional study, 503 women aged 18–49 years completed dietary questionnaires and representative validation subsample (n = 50) provided fasting blood samples for biomarker analyses. Correlation coefficients were calculated between each of the dietary methods and three pair-wise correlations were applied for the calculation of validity coefficients. Correlation coefficients observed between F-FFQ and three 24 h recalls were r = 0.56 ($p < 0.001$) and r = 0.57 ($p < 0.001$) for total sample and validation group, respectively. Bland–Altman plot and cross-classification analyses indicated good agreement between methods. High validity coefficients were determined between the true intake (I) and dietary assessment methods, F-FFQ (Q) and 24 h dietary recalls (R) (ρQI_{rbc} = 0.871 and ρQI_{ser} = 0.814; ρRI_{rbc} = 0.652 and ρRI_{ser} = 0.698), and moderate ones for biomarkers (B) (ρBI_{rbc} = 0.428 and ρBI_{ser} = 0.421). The F-FFQ is valid instrument for the assessment of dietary folate intake in women living in Serbia, a country without mandatory folic acid food fortification.

Keywords: folate; FFQ; validation; women; method of triads

1. Introduction

Nutritional imbalances during pregnancy can influence gene expression and cause abnormalities of fetal phenotype. Scientific progress in the comprehension of congenital anomalies has led to the conclusion that optimally balanced maternal diet with adequate intake of macro- and micronutrients can contribute to reducing the incidence of these disorders [1]. For the prevention of adverse pregnancy outcomes and normal fetal development folate, water-soluble B_9 vitamin, is recognized as a nutrient of particular importance [2].

The term folate refers to a group of related compounds including folates naturally present in foods and the synthetic, fully oxidized form, folic acid. The biological functions of folate as a co-enzyme are essentially based on single-carbon units transfer in the processes of purine and pyrimidine nucleotides biosynthesis and metabolism of amino acids methionine, serine, glycine and histidine [3]. Since the pteridine cycle, an element of the folate structure, cannot be synthesized de novo in mammalian body,

adequate amount of these compounds with a critical role in numerous biochemical processes must be provided through food and/or supplementation [4]. Unlike naturally occurring folate vitamers, which are labile and prone to losing biological activity during storage, food processing and preparation, folic acid retains stability and is therefore successfully used in the form of supplements and for food fortification [5,6].

There is substantial scientific evidence that maintaining adequate folate status before conception and during the first trimester of pregnancy significantly reduces the risk of occurrence and recurrence of neural tube defects (NTD) [2,7]. Given that the closure of the neural tube, fundamental for the proper formation of the nervous system, occurs during the third and fourth week (i.e., from Day 21 to Day 28) from conception, and that up to 41% of pregnancies are unplanned, it is clear that throughout this period the majority of women are still not, or have only become aware of the pregnancy [8,9]. For this reason, it is crucial to ensure optimal folate intake and status in all women of childbearing age.

Recommendation for optimal dietary intake of naturally-occurring mixed forms of folate for adults is 400 μg according to US Institute of Medicine (IOM) and World Health Organization/Food and Agricultural Organization of the United Nations (WHO/FAO) Expert Consultation group [10]. These organizations emphasize that, in addition to healthy diet, due to problematic stability and bioavailability of food folate, for optimal NTD prevention, it is necessary to provide 400 μg of folic acid daily by supplementation when planning pregnancy or throughout childbearing age [11]. Since it is difficult to attain general compliance with advice on nutrition and supplement usage, many countries worldwide have introduced controlled and strictly regulated folic acid food fortification. In Serbia, such policy has not been implemented and availability of voluntarily fortified food is limited. Furthermore, prior studies indicate that the intake of folate in this country is bellow values recommended by authorities [12], while only 3.9% of women report taking folic acid supplements during periconceptional period [13]. Concern regarding folate inadequacy and associated health issues is widespread across Europe. Majority of European countries lack fortification policy and current strategies, based primarily on dietary counseling and promotion of folic acid supplementation, have not had appreciable public health effect on reducing the prevalence of suboptimal folate intake and NTDs [14–17].

In facing the challenge of achieving folate adequacy, the first step is reliable and objective nutritional assessment performed with standardized and validated instruments. To address this concern, we developed the Food Frequency Questionnaire for folate intake assessment (F-FFQ). Food Frequency Questionnaires are used to assess usual diet during a defined period of time in a simple and cost-effective manner with relatively small burden imposed on researchers and respondents. Due to aforementioned advantages, this instrument is commonly used in nutritional epidemiological studies all over the world. However, it is important to highlight that a universal FFQ, which could be applied for all population groups and all research questions, does not exist. Demographic, socio-economic, geographical, climatic, cultural and medical status factors all have influence on the diet and FFQ must be created or adapted in accordance with the characteristics of a particular study population [18]. Furthermore, in order to ensure proper interpretation of data obtained by FFQ, it is important to determine the association between reported intakes from the FFQ and true dietary intake [19].

The aim of the present study is to determine whether the F-FFQ is valid tool for assessing the dietary intake of folate in Serbian women of reproductive age.

2. Materials and Methods

In this study, the relative ability of the F-FFQ to estimate folate intake was tested against reference method (repeated 24 h dietary recalls), and correspondent biomarkers of folate intake (concentration of folate in serum and red blood cells (RBC)) using the method of triads [20,21]. This triangular approach relies on the availability of quantitative intake information from three methods and uses the correlations between each of them to calculate the validity coefficient. The validity coefficient represents the correlation between the dietary intake reported by the F-FFQ and the unknown true dietary intake.

Given that FFQ and 24 h recalls commonly share errors related to misreporting, the main benefit of applying the triads method is the inclusion of biomarkers—objective and independent indicators of nutrient intake [20–22]. This approach enables broadening of validation parameters and more comprehensive analyses.

2.1. Study Participants

In a one-year period, from June 2014 to July 2015, a cross-sectional study was conducted as a part of a national integrated project with an objective to estimate dietary intake and biomarkers of folate status among women of reproductive age in Serbia. During the recruitment process, flyers with the invitation for participation in the study were available in selected primary health care facilities (community pharmacies and health centers) and educational institutions throughout country. Recruitment material contained detailed information regarding the purpose and objectives of the research, study protocol, as well as rights and expectations of the potential participants. The inclusion criteria for the study were: female sex, age between 18 and 49 years and a regular menstrual cycle. Exclusion criteria comprised: pregnancy, breast feeding, use of drugs that interact with folate metabolism, hormonal substitution and menopause.

Recruitment process and sample overview are presented in Figure 1.

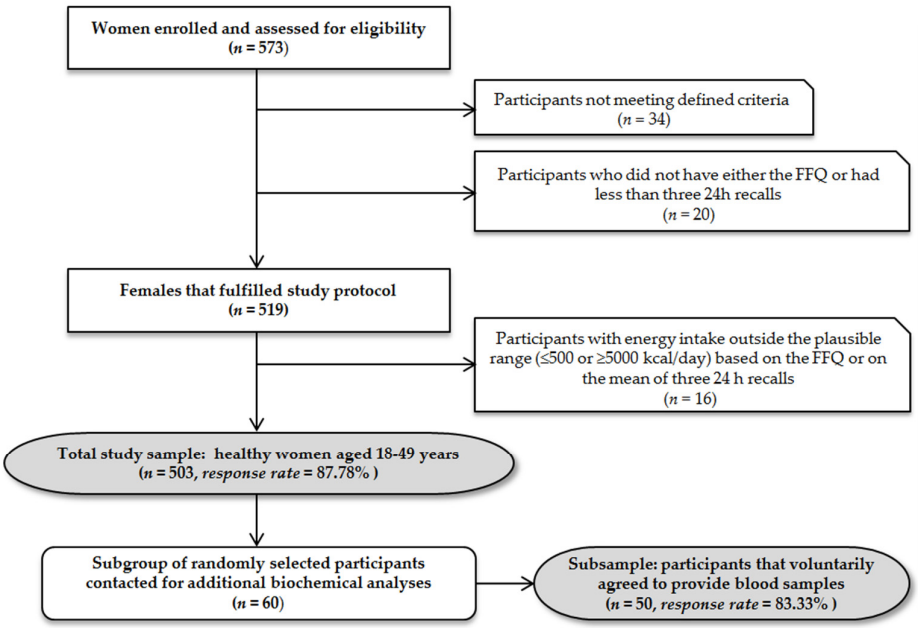


Figure 1. Recruitment process and sample overview (FFQ, Food Frequency Questionnaire).

2.2. Anthropometry

Anthropometry included height and weight measurement of participants dressed only in light clothing. Height was measured to the nearest 0.1 cm (Perspective Enterprises, Kalamazoo, MI, USA) and weight to the nearest 0.1 kg (TBF-300, Tanita Corp., Tokyo, Japan). Body Mass Index (BMI) was calculated as weight (kg)/height-squared (m^2) [23].

2.3. Food Frequency Questionnaire

The FFQ for folate (F-FFQ) was developed by combining a validated FFQ for folate in Croatia region [24] with the NCI/Block Health Habits and History Questionnaire [25]. Further adaptation of the F-FFQ included folate-rich traditional foods consumed in Serbia (such as baked beans "prebranac", stuffed dock leaves, ajvar/pindjur (traditional roasted red pepper spread), sun-dried peppers stuffed with beans, and dock/spinach pie) (Table S1). Face and content validity of the questionnaire were

assessed by an expert panel consisting of five researchers from the Center of Research Excellence in Nutrition and Metabolism in Serbia. The questionnaire was pilot tested for clarity and format improvement among 20 women of reproductive age who did not participate in the main study.

The F-FFQ was designed to capture habitual intake over the previous three months and was self-administered in the presence of trained dietitians. Photographs of food portion sizes (small, medium, and large) were included in the FFQ. In addition to pre-specified portion size options, there was a possibility for respondents to determine usual portion in an open-ended manner. The questionnaire included the following frequency of consumption options: "never", "once per month", "2–3 times per month", "once a week", "2–3 times per week", "4–6 times per week" and "every day". In the case respondents consumed certain food more than once per day they were instructed to specify portion that corresponds to daily intake. All the reported frequencies for ninety food items listed in F-FFQ were converted to frequencies per day with reference to a base value of 1.0 for the "every day" option. Estimates of amounts of food consumed per day were calculated by multiplying the daily equivalent frequency of consumption of food items by the chosen portion size.

In addition, to enhance interpretation of F-FFQ estimates, general questions related to age, education, previous medical conditions, detailed vitamin supplements and medication use and lifestyle habits regarding smoking, physical activity/exercise, alcohol as well as coffee and tea consumption were also incorporated in questionnaire.

2.4. Twenty-Four-Hour Dietary Recalls

Three 24 h dietary recalls per participant were performed by multiple pass during the last two weeks of the period covered by the F-FFQ. Interviews were conducted on nonconsecutive days, with two of the recalls being on weekdays and one on a weekend day. Survey calendar was defined so that adequate proportion of weekdays is captured on a group level. Within the structured interview participants reported the complete consumption of food and beverages in preceding 24 h. Data regarding the type of the food or dishes, time and place of consumption, cooking or processing method and the amounts consumed were recorded in suitable survey form in chronological order. All the interviews were done face to face and led by a trained professional according to standardized protocol. The estimated time of data collection was 15–30 min. In order to improve the accuracy of the portion estimate, the questionnaires were administered in conjunction with Food Atlas [26,27]. This amount estimation tool contains color photographs of various portions of foods and dishes whose selection was made on the basis of previously conducted national studies. For each item four to nine serving sizes, measured using calibrated digital scale, were available. All photographs were made in a standardized, uniform manner, with identical lighting, background and shooting distance, so that comparative factors (e.g., plate, cup and cutlery of defined dimensions) were clearly shown as an aid for selection.

2.5. Dietary Data Assessment

Quantitative food consumption data, obtained with both instruments (i.e., 24 h dietary recall and FFQ), were processed with DIET ASSESS & PLAN (DELTA Electronic Ltd., Subotica, Serbia), advanced dietary intake assessment and nutrition planning software tool, which has been applied previously in national, regional and international nutritional surveys and evaluated in European Food Safety Authority (EFSA) trial ring [26–29]. Nutrient intake calculation was performed using the Serbian Food Composition Database, harmonized with EuroFIR standards and embedded in EuroFIR Food Platform and Balkan food platform [30]. For dietary supplement users, content specified by manufacturer and reported information regarding dosage, consumption frequency and duration were taken into account. Total folate intake was estimated in μg dietary folate equivalents (DFE)/day using formula: μg of DFE = [μg of food folate + (1.7 × μg of synthetic folic acid)] and related to recommended values proposed by WHO—Estimated Average Requirements (EAR; 320 μg/day) and Recommended Nutrient Intake (RNI; 400 μg/day) for adults [10].

2.6. Biochemical Assessment

Within two days after completing the F-FFQ, blood samples of fifty randomly selected participants were collected by trained medical staff via venipuncture. Prior to specimen collection participants fasted overnight and analyses were performed immediately. Samples were collected in plastic Serum Separator Tube and in tripotassiumethylendiaminetetraacetic acid (K_3 EDTA) tube (BD Vacutainer; Becton, Dickenson and Company, Plymouth, United Kingdom) for serum and RBC folate concentration analyses, respectively. Biomarkers were determined using the ARCHITECT Folate kit (Abbott Laboratories, Abbott Park, IL, USA), based on Chemiluminescent Microparticle Immunoassay (CMIA) technology and traceable to the Folate WHO International Standard 03/178, on Architect i2000 analyzer (Abbott Laboratories, Abbott Park, IL, USA). Special attention was paid to keeping samples, calibrators and controls protected from light. Prior to RBC hemolysate preparation hematocrit of the EDTA specimen was determined according to the manufacturer's instructions [31]. The accuracy of the assay was verified with three level control materials (ARCHITECT Folate Controls, Abbott Laboratories, Abbott Park, IL, USA) and all were within manufacturer's specified range. All assays were done in duplicate. The intra-assay coefficients of variation for folate serum and RBC were 4.3% and 4.7%, while the inter-assay coefficients of variation were 6.1% and 6.3%, respectively.

Based on cut-off values proposed by WHO, folate serum concentrations <6.8 nmol/L were related to folate deficiency, concentrations between 6.8 nmol/L and 13.4 nmol/L to possible deficiency, and values >13.5 nmol/L to adequate status [32]. RBC folate concentrations below 340 nmol/L were considered indicative for folate deficiency and concentrations ≥906 nmol/L as optimal for achieving the greatest reduction of risk for NTD-affected pregnancy [33].

2.7. Statistical Analyses

The normality of the data distribution was analyzed using Shapiro–Wilk test for the total sample and the validation group separately. When data were not normally distributed, values were log transformed before analyses. For all parameters, differences between the total sample and validation group were tested using the Wilcoxon Rank Sum test.

Several statistical techniques were applied to evaluate validity of the F-FFQ. Pearson correlation coefficients were calculated to assess association between dietary folate intake estimated by F-FFQ and 24 h recalls on crude and energy-adjusted data for both total sample and validation subgroup [34]. Agreement between methods was further examined by classification of the variables into the quartiles (i.e., in four groups divided by 25th percentile, median and 75th percentile). We calculated the quartiles of folate intake assessed by F-FFQ for the validation group and cross-tabulated these with respective quartiles of 24 h-recall-derived estimates and biomarker levels. Rank of the variable that corresponds to the Pth percentile in the sample was calculated using formula $(P/100)(1 + n)$, where n is the number of observations. Since the number of participants in validation subsample in our study was 50 calculated ranks were not integer. Therefore, they were rounded to the nearest rank [35]. Discordance and agreement in quartile ranking was assessed as the percentage classification in the same, same or adjacent, opposite (first and third or second and fourth quartile) and absolutely opposite quartile (grossly misclassified—first and fourth quartile). Linear regression analyses were performed to test for significant linear trends between folate intake assessed by F-FFQ, 24 h recalls and folate biomarkers, adjusting for research settings.

Furthermore, to visualize agreement between folate intake results obtained from F-FFQ and 24 h recalls the Bland–Altman plot was constructed [36]. The arithmetic difference in folate intakes between the two methods for each individual was plotted against the average estimation of the two methods. The 95% limit of agreement was calculated as the mean difference ±1.96 standard deviation (SD).

The comprehensive triangular approach to validation, known as the method of triads, was applied as well. Validity coefficient (VC) for the FFQ was calculated using the correlations between the FFQ

and the biomarker (r_{QB}), 24 h recalls and the biomarker (r_{BR}) and the FFQ and 24 h recalls (r_{QR}) as shown in the following equation:

$$\rho_{QI} = \sqrt{(r_{QB} \times r_{QR}/r_{BR})}$$

This method has been described in detail by Ocke and Kaaks [20]. Correlation between the biomarker and the FFQ and calculated validity coefficient were used as lower and upper limits of validity coefficients. The 95% confidence intervals for the validity coefficients were estimated using bootstrap sampling where 1000 samples of equal size (n = 50) were obtained with replacement from the study subjects [20,37].

A p value < 0.05 was considered statistically significant. All statistical analyses were performed using R software package (R Foundation for Statistical Computing, Vienna, Austria) [38].

2.8. Ethical Approval

This study was conducted in accordance with the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institute for Medical Research Ethics Committee in Serbia (EO112/2015). Written informed consent for inclusion was obtained from all participants.

3. Results

The average age of participants was 34.09 (SD = 10.74) years. Distributed in age groups, 88, 179, 159 and 78 of the studied women were 18–25, 25–35, 35–45 and 45–49 years old, respectively. Total sample and validation group were not statistically different in weight (65.09 (SD = 8.15) kg vs. 62.24 (SD = 6.06) kg), height (168.89 (SD = 5.21) cm vs. 169.94 (SD = 4.42) cm), BMI (22.83 (SD = 2.64) kg/m^2 vs. 21.99 (SD = 1.93) kg/m^2) and waist to hip ratio (0.77 (SD = 0.06) vs. 0.76 (SD = 0.04)).

According to anthropometric measurements, 4.17% of the women were classified in the category of underweight (BMI < 18.5 kg/m^2), 20.08% in overweight (BMI = 25.0–29.9 kg/m^2) and 5.57% in obese (BMI $\geq$ 30.0 kg/m^2) while 70.18% had BMI within normal range (BMI = 18.5–24.9 kg/m^2) [23]. At the time of the survey, 2.58% of the participants had primary education, 58.65% secondary and 38.77% post-secondary education. Consumption of folic acid in the form of single or multivitamin/multivitamin–multimineral supplements at least once a week was reported by 4.77% of women. Smoking habit was reported by 29.22% of participants in total sample and 26.04% in validation group. Based on F-FFQ self-reports, among 503 studied women 41.75% consumed alcohol on a weekly basis, 89.66% consumed coffee and 63.61% tea at least a cup a day. Moderate physical activity between 30 min and 3.5 h weekly was stated by 76.34% of women in the main sample and 77.73% in validation subgroup.

Estimated daily energy, macronutrient and folate intake together with correlations between the estimates of the dietary intakes by the F-FFQ and 24 h recalls are presented in Table 1. Pearson correlation coefficients between folate intake assessed by F-FFQ and 24 h dietary recalls did not substantially change after adjustment for total energy intake based on residual model, neither for total sample (crude data: r = 0.56, p-value < 0.001 vs. energy-adjusted data: r = 0.53, p-value < 0.001) nor for validation subgroup (crude data: r = 0.57, p-value < 0.001 vs. energy-adjusted data r = 0.59, p-value < 0.001). There were no significant differences in intake estimates between the total sample and validation group (p > 0.05). Only 6.16% and 6.76% of all the participants reached folate EAR benchmark while 96.22% and 93.64% had folate intakes bellow recommended 400 μg DFE/day based on repeated 24 h recalls and FFQ, respectively.

Table 1. Daily energy and nutrient intake assessed by the F-FFQ and average of the three 24 h dietary recalls with correlations between the estimates of the dietary intake by applied questionnaires among Serbian women of reproductive age.

Nutrient	Total Sample (n = 503)				Pearson Correlation Coefficient	Validation Group (n = 50)				Pearson Correlation Coefficient
	F-FFQ		24 h Recalls			F-FFQ		24 h Recalls		
	Mean	SD	Mean	SD	r	Mean	SD	Mean	SD	r
Energy (kcal)	1724.62	330.02	1734.20	318.50	0.53 **	1727.29	200.36	1718.93	219.40	0.59 **
Carbohydrates (% TEI)	52.23	9.21	50.94	7.23		51.02	5.51	49.88	6.08	
Carbohydrates (g)	225.19	42.72	220.85	59.78	0.51 *	220.31	23.58	214.35	28.21	0.52 **
Fat (% TEI)	27.81	10.61	28.30	7.21		29.34	6.74	31.41	6.30	
Fat (g)	53.29	23.90	54.53	23.72	0.41 **	56.31	14.90	59.99	14.85	0.47 *
Protein (% TEI)	19.52	2.93	20.22	3.50		19.20	2.05	18.49	2.62	
Protein (g)	84.16	13.87	87.66	17.51	0.39 **	82.91	7.38	79.46	8.92	0.40 *
Folate (µg/1000 kcal)	118.98	35.44	121.66	42.73	0.53 **	123.95	32.27	119.90	35.76	0.53 **
Folate (µg)	205.20	61.30	211.00	81.06	0.56 **	214.10	60.78	206.10	65.31	0.57 **

[1] F-FFQ, Folate Food Frequency Questionnaire; %TEI, percentage of total energy intake; * p-value < 0.01, ** p-value < 0.001.

In Table 2 the median values and 5th and 95th percentiles of the daily intake levels of twelve food groups and the corresponding relative contributions of these food groups to the daily folate intake based on 24 h dietary recalls are presented. Major folate food sources, identified for total 503 women using both dietary methods, are presented in Table 3.

Table 2. Daily intake levels presented as the median values and 5th and 95th percentiles of twelve food groups and their corresponding contributions to daily folate intake based on repeated 24 h dietary recalls among Serbian women of reproductive age.

Food Groups	Intake of the Food Group (g/Day)			Contribution to Total Folate Intake	
	Median	P5	P95	%	Folate Intake (µg/Day)
Vegetables and vegetable products	175.07	63.95	318.20	37.35	79.66
Grains and grain products	195.91	90.68	338.82	23.39	49.89
Fruits and fruit products	130.75	9.31	378.17	11.52	24.57
Milk and milk products	241.69	15.68	481.12	10.48	22.35
Meat and meat products	95.62	12.31	200.87	5.77	12.31
Nuts, seeds and kernel products	6.00	0.00	66.00	3.01	6.42
Beverages (non-milk)	1.14	0.04	32.59	2.6	5.55
Miscellaneous	5.87	0.00	78.48	2.3	4.91
Eggs and egg products	8.88	0.00	64.10	1.45	3.09
Sea food and related products	0.00	0.00	107.45	1.11	2.37
Sugar and sugar products	17.55	0.75	64.97	0.98	2.09
Fat and oil	11.69	0.00	32.62	0.04	0.09

Table 3. Major folate food sources assessed by the F-FFQ and 24 h dietary recalls among Serbian women of reproductive age.

Food	24 h Dietary Recall (n = 503)		F-FFQ (n = 503)		
	µg DFE/Day (Total Sample)	µg DFE/Day (Consumers Only)	Number of Consumers	% of Consumers	Average Frequency of Consumption
Bread white	15.67	30.11	328	65.21	every day
Beans	10.08	218.38	434	86.28	2–3 times per month
Tomato, raw	9.45	18.75	482	95.83	4–6 times a week
Yoghurt 2.8% mf	7.65	16.05	419	83.30	once a week
Chicken, liver	7.14	251.19	456	90.66	once a week
Peas, green	6.11	93.57	422	83.90	2–3 times per month
Banana, raw	6.06	48.38	492	97.81	2–3 times per week
Egg, hen, whole	5.78	22.45	487	96.82	2–3 times per week
Pepper, red	5.03	51.07	321	63.82	2–3 times per month
Potato	4.98	12.91	225	44.73	2–3 times per month
Strawberries, raw	4.84	26.95	419	83.30	once a week
String beans	4.43	67.11	248	49.30	2–3 times per month
Lettuce, green leaf	4.29	17.98	354	70.38	2–3 times per month
Orange juice, fresh	3.17	62.16	232	46.12	once a week
Cabbage, white	2.71	12.06	393	78.13	2–3 times per month

DFE, dietary folate equivalents.

Mean folate serum level in the studied women was 12.29 (SD = 6.59) nmol/L and RBC folate 438.66 (SD = 144.63) nmol/L. Using a cut-off value of 6.8 nmol/L for serum folate and 340 nmol/L for RBC folate, the prevalence of blood folate levels indicative for folate deficiency was 30% (n = 15) and 24% (n = 12), respectively. Adequate folate serum concentrations were observed in 38% (n = 19) of studied participants. None of the women met or exceeded 906 nmol/L, the value of RBC folate associated with lowest risk of having NTD-affected pregnancy. A significant increase of serum and RBC folate concentrations were identified with an increase of folate intake ($p < 0.05$; Table 4).

The ability of F-FFQ to classify participants into the correct quartile of folate intake assessed by 24 h recalls and biochemical indices is summarized in Table 5. The F-FFQ classified more than 80% of subjects correctly or closely as 24 h recalls and folate biomarkers. Figure 2 illustrates correlations between the F-FFQ, 24 h dietary recalls and biomarkers of folate status (i.e., serum and RBC folate). Furthermore, Bland–Altman plots indicated good agreement between methods, as less than 5% (precisely 20 cases, i.e., 3.98%) of cases fell beyond the limits of agreement (Figure 3).

Table 4. Estimated serum and RBC folate concentrations by quartile of intake assessed by F-FFQ among Serbian women of reproductive age.

Quartiles	Folate Intake (μg/Day)	*n*	Serum Folate (nmol/L)			RBC Folate (nmol/L)		
			Mean	95% CI	*p* for Trend	Mean	95% CI	*p* for Trend
1st quartile	<168.43 (147.31)	13	9.54	5.73–13.35		378.29	304.73–451.84	
2nd quartile	168.43–194.12 (182,60)	13	11.48	7.51–15.45		399.05	306.61–491.48	
3rd quartile	194.12–245.62 (217.37)	12	12.18	8.78–15.58		447.27	358.51–536.02	
4th quartile	245.62–362.34 (306.81)	12	15.90	11.51–20.29	0.0139	526.05	444.62–607.48	0.0113

RBC, red blood cells.

Table 5. Cross-classification (%) of folate intake into quartiles by the F-FFQ and validation methods (the average of three 24 h recalls and biomarkers of folate status, i.e., serum and red blood cells (RBC folate concentrations) for 50 participants of validation subsample, Serbian women of reproductive age.

Folate Intake/Status Assessed by	Folate Intake Assessed by F-FFQ			
	Same Quartile	Same or Adjacent Quartile	Opposite Quartile	Grossly Misclassified
24 h recall	68	84	12	4
Folate RBC	50	82	14	4
Folate serum	48	82	12	6

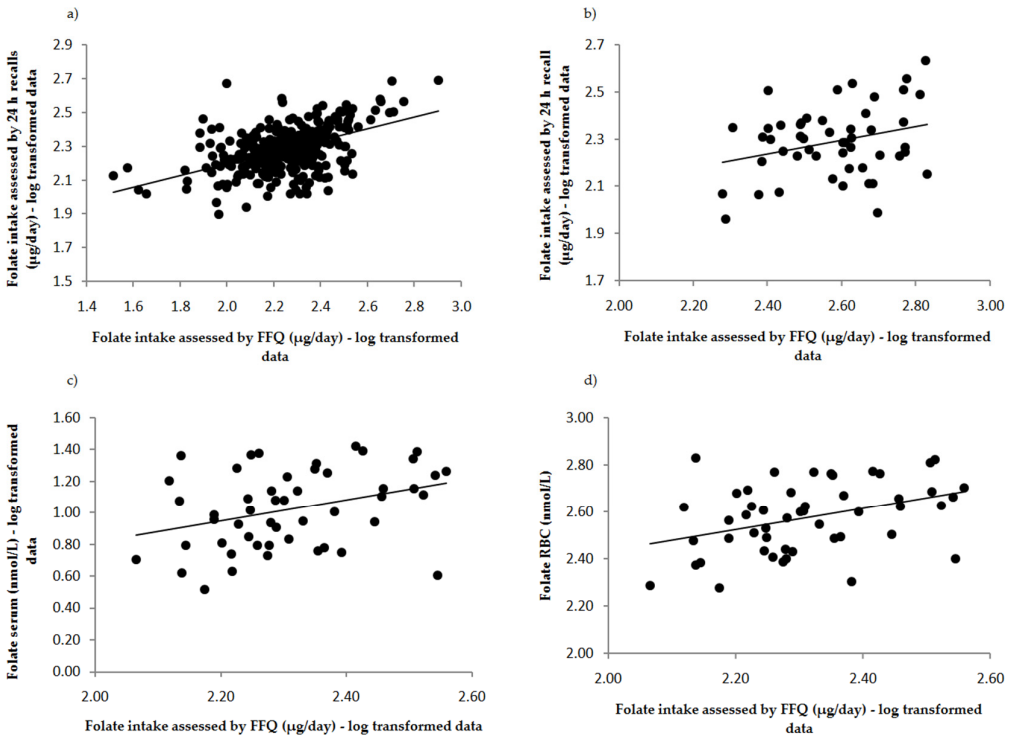


Figure 2. Correlations between folate intake assessed by the F-FFQ and the average of 24 h recalls in: (**a**) total sample (n = 503); and (**b**) validation subsample (n = 50); and folate intake assessed by the FFQ and: (**c**) folate serum concentration; and (**d**) red blood cells (RBC) folate concentration among Serbian women of reproductive age. All correlations were significant: (**a**) $r = 0.56$, $p < 0.001$; (**b**) $r = 0.57$, $p < 0.001$; (**c**) $r_{ser} = 0.28$, $p < 0.01$; and (**d**) $r_{rbc} = 0.37$, $p < 0.01$.

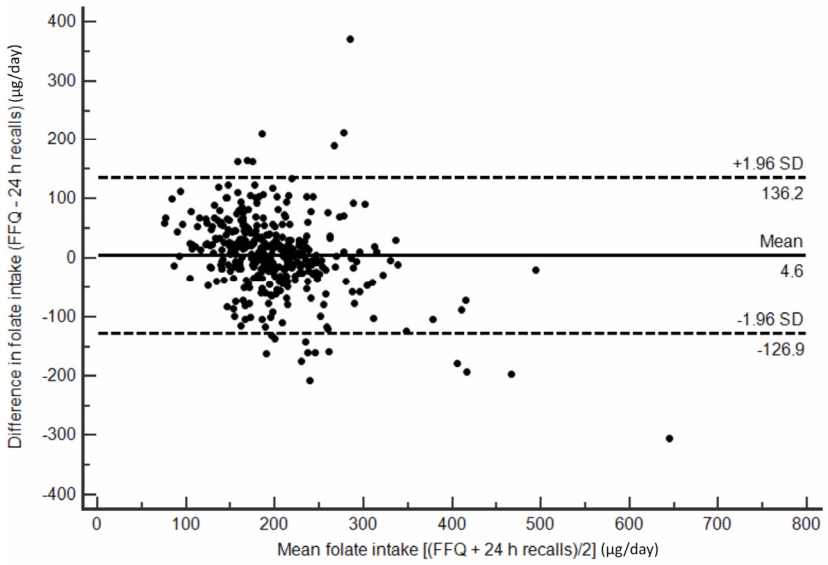
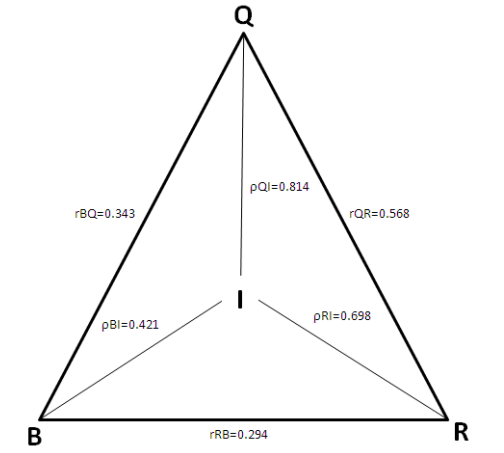


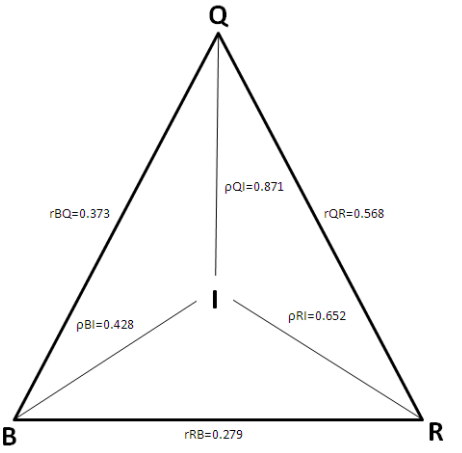
Figure 3. Bland–Altman plot assessing the agreement between the F-FFQ and the average of three 24 h dietary recalls for estimating folate intake, Serbian women of reproductive age. For each participan, the difference in folate intakes between the two methods was plotted against the mean folate intake by two methods: solid line, mean difference; and dotted line, 95% limits of agreement (LOA).

Application of the method of triads enabled triangular comparisons of the correlation coefficients between F-FFQ, reference method (repeated 24 h recalls) and biochemical measurements—serum folate (Figure 4a) and RBC folate level (Figure 4b). The validity coefficient of the F-FFQ was high for both serum and RBC folate, indicating a strong relationship between true folate dietary intake and F-FFQ estimates (Figure 4a,b).



Folate SERUM	Range for the VC*	95% CI for the VC
FFQ (ρ_{QI})	0.343-0.814	0.473-0.901
24 h recalls (ρ_{RI})	0.294-0.698	0.148-0.733
Folate serum ($\rho_{BI\ serum}$)	0.343-0.421	0.676-0.833

a)



Folate RBC	Range for the VC*	95% CI for the VC
FFQ (ρ_{QI})	0.373-0.871	0.693-0.881
24 h recalls (ρ_{RI})	0.279-0.652	0.087-0.693
Folate RBC ($\rho_{BI\ RBC}$)	0.373-0.428	0.462-0.852

b)

Figure 4. Triangular relationship of the correlation coefficients (r_{QB}, r_{RB}, r_{QR}) between folate intake estimated by the FFQ (Q), the average of the three 24 h dietary recalls (R) and the biomarkers (B) of folate status: (**a**) folate concentration in serum; and (**b**) red blood cells (RBC) folate concentration, and validity coefficients (VC: ρ_{QI}, ρ_{BI}, ρ_{RI}) between true intake (I) and estimated intakes, with 95% confidence intervals. * The lower limit of the true validity coefficient is correlation between the biomarker and the two dietary methods (F-FFQ and 24 h recalls), and the upper limit is calculated by the method of triads.

4. Discussion

Nutritional status assessment is nowadays recognized as an important part of medical care at individual and population level. In order to generate reliable information in the sphere of nutrition research, the use of valid, comprehensive instruments adapted to specific characteristics of the population of interest is essential. The aim of the present study was to validate the Food Frequency Questionnaire for the assessment of folate intake (F-FFQ) among women of reproductive age in Serbia, a country without mandatory folic acid food fortification. Therefore, we collected dietary intake data by applying two methods (24 h dietary recalls and FFQ), determined concentrations of two biochemical indicators of folate status and applied the method of triads [20]. Validity coefficients for F-FFQ were high regardless of biomarker used (ρQI_{rbc} = 0.871 and ρQI_{ser} = 0.814). Moreover, a high level of agreement between the F-FFQ and 24 h dietary recalls was determined with Bland–Altman plot and cross-classification analysis.

The estimated daily folate intakes by F-FFQ were 206.2 μg DFE for all 503 studied women and 214.1 μg DFE for the validation subsample. Results of this study are in agreement with previously published data for the population of Serbian women assessed by seven-day food record (228.0 μg/day) [12]. In addition, similar values have been reported in studies conducted in other European countries that assessed folate intake in women using an alternative FFQ (Sweden: 225.0 μg/day [39], The Netherlands: 177.0 μg/day [40], Italy: 222.4 μg/day [41], Norway: 209.0 μg/day [42]), 24 h dietary recalls (Poland: 211.0 μg/day [43], Finland: 205.0 μg/day [44], Greece: 227.2 μg/day [45], Austria: 212.7 μg/day [46]) and 48-h dietary recalls (Spain: 196.9 μg/day [47]). In our study, the main food groups contributing folate intake were identified as vegetables/vegetable products (37.35% of average daily intake), grains/grain products (23.39% of average daily intake) and fruits/fruit products (11.52% of average daily intake). These results are in accordance with the recent comparison of standardized dietary folate intake across ten countries participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) study [48]. A number of national dietary surveys indicate widespread prevalence of suboptimal folate intake among women of childbearing age in Europe highlighting the perspective of achieving folate status associated with lowest risk of folate-related disease (e.g., NTDs), rather than merely preventing evident folate deficiency (i.e., megaloblastic anemia) [14,49,50]. More than 90% of women in our study had folate intake bellow the recommended 400 μg DFE/day. To ensure adequate folate intake and status in general population and particularly in women of reproductive age mandatory folic acid food fortification policies have been established in many countries worldwide, which is not the case in Serbia. Availability of imported and voluntarily fortified foods is rather limited in Serbian market. In addition, results of our study confirm previous findings that folic acid in the form of dietary supplements is rarely consumed by Serbian women of reproductive age [13]. Potential consequences resulting from inadequate folate status on pregnancy outcomes and overall health should be perceived by Serbian public health authorities. It would be wise to consider implementation of educational programs to raise awareness about the significance of this nutrient, as well as nutritional interventions in the form of controlled fortification of selected foodstuffs or targeted supplementation.

It has been suggested that associations between dietary instruments (FFQ and 24 h recalls) estimated by correlation coefficients should be greater than or equal to 0.3, preferably over 0.4 and optimally in the range of 0.5–0.7 [18]. Correlation coefficient observed in the present study between F-FFQ and the average values of three 24 h dietary recalls were $r = 0.56$ ($p < 0.001$) for the total sample, and $r = 0.57$ ($p < 0.001$) for the validation group. The strength of these correlations compare favorably to findings reported in other FFQ validation studies. Pauwels et al. [51] developed FFQ to assess usual intake of methyl-group donors and validated it against seven-day food record among Flemish women of reproductive age. They reported a correlation coefficient between two dietary methods of $r = 0.58$. Similar correlation coefficient ($r = 0.56$) was found by Jackson et al. [52] during validation of the 120-item FFQ against repeated 24 h recalls in 70 men and women in Jamaica. Another study compared folate intake assessed by short FFQ and seven-day weighted food record

among 37 men and women and reported partial correlation coefficient (controlling for gender) of r = 0.53 [53]. In the Northern Sweden, a region without food fortification, FFQ was validated against 24 h recalls where Spearman correlation coefficient between estimates of folate intake based on two methods was r = 0.57 for female participants [39]. French et al. [54] developed FFQ to assess the folate intake of women of childbearing age in Canada and validated it against seven-day food record. The observed correlation coefficient between the two methods was r = 0.51. Recent study conducted among 67 British women of reproductive age explored the validity of purposefully designed FFQ for the assessment of usual dietary intake of micronutrient methyl donors (folate, choline and betaine) and selected antioxidants. The observed deattenuated correlation coefficient between the FFQ and estimates from three multiple-pass 24 h dietary recalls for folate was r = 0.47 for diet only and r = 0.80 when supplements were included [55].

In the present validation study serum and RBC folate concentrations were used to validate the dietary estimates of folate intake. Both biomarkers correlated significantly with the intake assessed by dietary methods. Correlation coefficients between F-FFQ and biomarkers were comparable with results of previously published studies for both folate concentration in erythrocytes (r_{rbc} = 0.37 versus 0.34 [24], 0.33 [52] and 0.35 [56]) and in the serum (r_{ser} = 0.28 versus 0.25 [40], 0.20 [52] and 0.26 [57]). Folate serum level is considered to reflect recent intake, while the concentration in red blood cells indicates long-term folate exposure and tissue stores. Taking into consideration that folate accumulates in erythrocytes only during erythropoiesis, RBC folate represents integrative measure of folate intake for the period which corresponds to average lifespan of erythrocytes (i.e., 120 days) [58]. Given that this time-frame is similar to the defined reference period of F-FFQ (i.e., three months), the higher value of correlation coefficient between folate intake assessed by the F-FFQ and the concentration in erythrocytes in relation to the serum is logical. Based on WHO recommendation RBC folate concentration in women of reproductive age should be at least 906 nmol/L so as to ensure optimal prevention of neural tube defects [33]. This value has not been recorded in none of the study participants.

Although correlation analysis is a popular technique which is relatively simple to interpret and compare with results of previously conducted studies, it measures the strength of association between variables, but not the agreement between them [36]. In accordance with recommendations that various statistical approaches should be applied when assessing validity of FFQ, we constructed Bland–Altman plots and performed classification in categories of consumption and status indicators [59]. Bland–Altman plot showed that that the estimates of folate intake obtained by the F-FFQ were comparable to those from repeated 24 h dietary recalls and unlikely to cause systematic bias. Our FFQ performed well in quartile assignment considering that 84% of respondents were correctly classified in the same or adjacent quartile as 24 h recalls and 82% as biochemical status indicators. Similar results of cross-classification analyses were reported in other FFQ validation studies. Percentage of subjects classified within one quartile of folate intake (FFQ versus 24 h recalls) was 84% and 83% for FFQs developed and validated by Johansson et al. [39] and Fayet et al. [56], respectively. In the present study F-FFQ grossly misclassified 4% of women, which is comparable with 4.3% reported by Jackson et al. [52].

Application of the method of triads enabled simultaneous comparison of the F-FFQ with 24 h dietary recalls and biomarkers. In our study, high validity coefficients were determined between the true intake (I) and dietary assessment methods (F-FFQ and repeated 24 h dietary recalls) (ρQI_{rbc} = 0.871 and ρQI_{ser} = 0.814; ρRI_{rbc} = 0.652 and ρRI_{ser} = 0.698) and moderate ones for biomarkers (ρBI_{rbc} = 0.428 and ρBI_{ser} = 0.421). Similar to other studies where the triangulation approach to validation of FFQ has been applied, the obtained validity coefficients for the F-FFQ and 24 h recalls were higher in relation to values for both biomarkers [40,53,60–62]. This could be explained by the fact that FFQ and 24 h recalls are methods designed to estimate dietary intake, while the biochemical markers are likely to be influenced by other factors in addition to diet. Our results for validity coefficients are comparable with other studies that used the triads model in validation of FFQ for assessment of folate intake (ρQI_{rbc} = 0.871 versus 0.75, 0.690_{man} and 0.410_{women}; ρQI_{ser} = 0.814 versus 0.940, 0.850_{man}, 0.690_{women}

and 0.720) [40,53,63], as well as other nutrients such as vitamin D (0.847) [60], α-carotene (0.850) [64], β-carotene (0.760) [37] and vitamin B_{12} (0.950) [65]. This triangulation analyses provided additional valuable insight into F-FFQ's performance and strengthened the evidence supporting its validity.

The F-FFQ is specific in respect to gender, age and geographic determiners, and, based on our knowledge, this is the first FFQ created and validated for the assessment of folate intake by women of reproductive age in Serbia. Advantages of this validation study include: method of data collection (i.e., interviewer-administered questionnaires according to standardized protocol), application of a detailed food list with incorporated photographs of various food portion sizes and comprehensive statistical validity confirmation with healthy women of reproductive age from different regions of Serbia. Additional advantage is consideration of dietary supplements consumption as well as analysis of folate biomarkers of recent intake and long-term status. Given that there were 503 participants in the total sample, among which 50 provided blood samples for biomarker validation analyses, the sample size in this study was harmonized with recommendations for validation studies [66]. Similar or smaller sample size has been reported for previously conducted studies using the triads method approach (n = 50 versus 53 [40], 36 [53], 20 [60] and 28 [64]). Furthermore, narrow 95% confidence intervals for the validity coefficients confirm adequate sample size in our study. It is noteworthy that in this study the so-called Heywood cases (values of validation coefficients greater than 1) have not occurred, which implies absence of random sampling fluctuation between methods or violation of basic model assumptions [20]. Additionally, in order to ensure questionnaire appropriateness regarding cultural and geographic determinants of diet, attention was dedicated to inclusion of locally available food items and traditional dishes that are consumed in Serbia [67]. Due to similar dietary patterns and cultural background, F-FFQ might be useful tool for the assessment of folate intake among other population groups in the Balkans' region. However, before F-FFQ is used in another population, additional studies are required to explore its validity.

A potential limitation of this study is that the blood sampling for biomarker analyses was conducted only once. Moreover, reproducibility of F-FFQ has not been assessed in this study. However, according to the Altman, method with poor repeatability will never agree well with another method [68]. Good results of F-FFQ and 24 h dietary recalls agreement evaluation suggest that weak reproducibility of this instrument is quite unlikely. Future studies should explore in detail seasonal variations in folate dietary intake by collecting dietary data for every participant in each season over the year, as this was outside the scope of this study due to organizational and financial constraints. However, is unlikely to be a significant limitation since the recent large-scale survey across Europe reported absence of systematic variations for folate intake according to the season of dietary intake collection [48]. Finally, as in most researches in the field of nutrition, general limitations of dietary assessment instruments should be considered. Both dietary methods depend on the memory of respondents and their perception of portion sizes. Nevertheless, potential restrictions were minimized by highly trained interviewers, use of comprehensive Food Atlas and relatively young age of participants.

5. Conclusions

Several approaches were used to examine the external validity of the F-FFQ and it performed consistently well. The application of the triads method resulted in high validity coefficients between the true intake and the F-FFQ regardless of biomarker used (ρQI_{rbc} = 0.871 and ρQI_{ser} = 0.814) with narrow 95% confidence intervals and the absence of Haywood cases. Furthermore, Bland–Altman and cross-classification analyses indicated good agreement between methods and satisfactory ranking potential of the F-FFQ. Therefore, considering presented results and similarities with other validation studies, we could conclude that F-FFQ is valid instrument for the assessment of dietary folate intake in women living in Serbia, a country without mandatory folic acid food fortification. In addition, the presented data strongly imply that the Serbian public health strategy should include nutrition initiatives targeting improvement of folate intake and status among women of reproductive age.

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Individual Diet Modeling Shows How to Balance the Diet of French Adults with or without Excessive Free Sugar Intakes

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Abstract: Dietary changes needed to achieve nutritional adequacy for 33 nutrients were determined for 1719 adults from a representative French national dietary survey. For each individual, an iso-energy nutritionally adequate diet was generated using diet modeling, staying as close as possible to the observed diet. The French food composition table was completed with free sugar (FS) content. Results were analyzed separately for individuals with FS intakes in their observed diets ≤10% or >10% of their energy intake (named below FS-ACCEPTABLE and FS-EXCESS, respectively). The FS-EXCESS group represented 41% of the total population (average energy intake of 14.2% from FS). Compared with FS-ACCEPTABLE individuals, FS-EXCESS individuals had diets of lower nutritional quality and consumed more energy (2192 vs. 2123 kcal/day), particularly during snacking occasions (258 vs. 131 kcal/day) (all p-values < 0.01). In order to meet nutritional targets, for both FS-ACCEPTABLE and FS-EXCESS individuals, the main dietary changes in optimized diets were significant increases in fresh fruits, starchy foods, water, hot beverages and plain yogurts; and significant decreases in mixed dishes/sandwiches, meat/eggs/fish and cheese. For FS-EXCESS individuals only, the optimization process significantly increased vegetables and significantly decreased sugar-sweetened beverages, sweet products and fruit juices. The diets of French adults with excessive intakes of FS are of lower nutritional quality, but can be optimized via specific dietary changes.

Keywords: sugars; linear programming; nutrient recommendations; dietary habits; snacking; France; INCA2

1. Introduction

In the current context of rising prevalence of non-communicable diseases, sugar intake is increasingly singled out as a public health issue because of its implication in dental caries [1] and weight gain [2], and potentially type 2 diabetes [3,4] and cardiovascular diseases [5–7]. Additionally, higher intakes of added sugars seem to be associated with poorer diet quality and lower micronutrient intakes [8]. Evidence ranges depending on health issues and sugar forms. However, the World Health Organization (WHO) recently focused on the prevention and control of unhealthy weight gain and dental caries, making recommendations for the intake of free sugars in adults and children [9].

For the WHO, the term "sugars" refers to all mono- and disaccharides, and "added sugars" include mono- and disaccharides added to food and beverages by the manufacturer, cook or consumer, and sugars naturally present in honey and syrups, while "free sugars" comprise added sugars plus sugars from fruit juices and concentrates [10].

The WHO recommends reducing the intake of free sugars to less than 10% of energy intake for both adults and children [9]. Today this is the most widely recognized recommendation, though the WHO concurrently makes a "conditional recommendation" of less than 5% of energy intake from free sugars, a threshold adopted by the Scientific Advisory Committee on Nutrition in the UK [11]. More recently, the 2015–2020 Dietary Guidelines for Americans (DGA) recommended limiting energy intakes from added sugars to a maximum of 10% [12]. In Europe, the European Food Safety Agency (EFSA) Panel on "Dietetic Products, Nutrition, and Allergies" declared in 2010 that "there are insufficient data to set an upper limit for (added) sugar intake" [13]. Similarly, in France, no recommendation has been set yet for free sugars.

Worldwide intakes of sugars vary widely by country [13–16] and subject characteristics, such as age [17] and eating patterns, including snacking habits [18]. Additionally, levels of information on sugar intakes (total, added, and free sugars) differ widely among food surveys, with little or no data on free sugars.

In this study, we were able for the first time to characterize the diet of French adults with excessive free sugar intakes, in comparison with those with acceptable free sugar intakes. We then determined the minimum dietary changes needed to achieve adequacy for all nutrients—including 10% maximum energy from free sugars—using diet modeling in individuals with and without excessive intakes of free sugars.

2. Materials and Methods

2.1. Dietary Survey and Population Sample

Data from the French national cross-sectional food consumption survey, named INCA2 (étude Individuelle Nationale des Consommations Alimentaires, 2006–2007) were used in this analysis. This cross-sectional survey, performed on nationally representative samples of children (3–17 years) and adults (18–79 years), using a multi-stage cluster sampling technique, has been described elsewhere [19,20]. To ensure national representativeness, each individual was assigned a weighting factor for unequal sampling probabilities and for differential non-responses. In terms of ethics of human subject participation, this survey was approved by the CNIL, the French authority of data protection (CNIL: "Commission Nationale Informatique et Libertés" No. 2003X727AU) and the CNIS, the French national council for statistical information (CNIS: "Conseil National de l'Information Statistique"). Verbal informed consent was obtained from all participants and formally recorded. The present study focuses on the adult population, aged between 20 and 75 years (n = 2486). Under-reporting individuals (i.e., those who have under-reported their food intake, voluntarily or not), were identified using the Goldberg method, based on the deviation between total energy reported and estimation of energy requirement (based on age, gender, weight, height, physical activity) [21] and excluded from the analysis (26.9% of the total adult sample). Additionally, only respondents who participated in the study for all seven days were retained, which left a final sample of 1726 individuals (Figure S1).

2.2. Demographic, Socio-Economic, Behavioral and Anthropometric Variables

Age, gender, socio-professional status, household type and income, current smoking status, sedentary behavior, frequency of snacking occasions and interest in diet were collected using self-reported and face-to-face questionnaires. Socio-professional status was classified as "active", "unemployed", "student", "retired" or "homemaker". The household type was described as: "in couple with at least one child", "in couple with no child", "single with at least one child" or "single with no child". Income per consumption unit (ICU) was calculated as self-reported household total net

income divided by the number of consumption units in the household, calculated using the scale from INSEE, the French national institute of statistics and economic studies (INSEE: "Institut National de la Statistique et des Etudes Economiques") [22]. Smoking status was divided into "smoker" and "non-smoker". Frequency of eating between the 3 main meals (breakfast, lunch, and dinner), as declared was divided in five frequencies ("more than four per day", "2–3 times per day", "one time per day", "less than one time per day", or "never"). Three levels of physical activity ("low", "moderate", or "high") were determined according to the short version of the International Physical Activity Questionnaire (IPAQ) [23]. A variable assessing time spent looking at a screen was used as a proxy for sedentariness. This variable was calculated as the sum of the time declared spent in front of the television and computer (including at work), during the week preceding the diet record (minutes (min) per day) [20].

Interest in diet was classified into "a lot", "little", "not really" and "not at all". Trained interviewers measured individual weight and height to calculate body mass index (BMI), divided into four classes (underweight, normal weight, overweight, obesity), according to the WHO definition [24].

2.3. Dietary Assessment

In a seven-day dietary diary, individuals recorded each food and each beverage consumed at home or outside home, split into six moments of consumption: three main meals (breakfast, lunch, and dinner) and three snacking occasions defined as food or beverage consumption between meals (morning, afternoon or evening). During the first face-to-face interview, the diary and a self-administered questionnaire were delivered at home by a trained and certified investigator, who explained to the subjects how to complete them. Just after the survey week, the investigator came back and checked the accuracy of the information reported in both documents [19]. Participants were told to complete the diary during the day in as close as real time as possible, in a pen and paper format. Portion sizes were estimated using a photographic booklet [25] or expressed by weight or household measures (spoon). All foods declared as consumed by the individual during the survey (n = 1314 foods and non-alcoholic beverages, including water) were placed in nine food categories and 30 sub-categories. In addition, to differentiate intrinsic sugar from free sugars, "fruits", "milk" and "yogurts" sub-categories were split into "fresh fruits" and "processed fruits"; "plain milk" and "sweet milk"; "plain yogurts" and "sweet yogurts". The "yogurts" sub-category included yogurts, fermented milks and associated French specialties ("fromage blanc" and "petit-suisses"). Alcoholic beverages were excluded from food analyses because they are not considered as food sources of essential nutrients in dietary recommendations, and therefore could not be optimized.

2.4. Food Composition Database and Free Sugars

The French food composition database [26] was used to estimate the energy and nutrient content of diets. We completed the national food composition table with an additional variable giving the free sugar content of foods. We used the WHO definition [10] which defines free sugars as all monosaccharides and disaccharides added to foods and beverages by the manufacturer, cook or consumer, and sugars naturally present in honey, syrups, fruit juices and fruit juice concentrates. Based on the systematic method to estimate added sugar content [27], the amount of equivalent sugars in all assimilated sugar ingredients was estimated using converting factors (e.g., equivalent sugars accounted for 100% in white sugar and only 80% in honey). Finally, the amount of free sugars for 100 g was estimated using the weight (in the recipe) of assimilated sugar ingredients and their corresponding amounts of sucrose.

In foods from the French food composition table [26], free sugars equal total sugars for 98 foods: honey, 2 syrups and 95 beverages including water. For 627 foods, the amount of free sugars was estimated using average recipes developed by ANSES, the French agency for food, environmental and occupational health and safety (ANSES: "Agence Nationale de SEcurité Sanitaire de l'alimentation, de l'environnement et du travail") and by nutritional expertise. For the remaining 589 foods considered

with no recipe (mainly mono-ingredient foods such as vegetables, non-processed fruits, meats, eggs, fish, etc.), the amount of free sugars was estimated by nutritional expertise, and was nil for 538 of them.

2.5. Diet Quality Indicators

Solid energy density (SED), food variety, mean adequacy ratio (MAR), mean excess ratio (MER), and a diet quality index based on the Probability of Adequate Nutrient intake (PANDiet) were used as indicators of diet quality, and were estimated for each individual observed diet. SED (kcal/100 g) was calculated based on items typically consumed as foods, including soups, but excluded drinking water and items typically consumed as beverages, such as milk, juices and soft drinks [28]. SED was calculated by dividing energy provided by solid foods by their weight. A high SED is associated with low diet quality [29]. Food variety was assessed by the number of different foods declared as consumed by each individual during the 7 days food record [30,31]. As originally proposed, the MAR was used as an indicator of good nutritional quality, and was calculated for each individual observed diet as mean percentages (capped at 100%) of recommended intakes [32] over a week for a list of nutrients. In the present study, it was calculated for 23 key nutrients [33]. The MER, an indicator of poor nutritional quality, was calculated as the mean percentages (minus 100%) of maximum recommended values over a week for sodium, saturated fatty acids and added sugars [33,34]. The updated version of the PANDiet index, integrating free sugars, was also used to estimate the overall nutritional quality of individual diets [35]. It summarizes in a single score the probability of having adequate intakes for 25 positive and negative nutrients. The score ranges from 0 to 100; the higher the score, the better the nutrient adequacy of the diet.

2.6. Diet Modeling

The present modeling approach was based on the previously described Individual Diet models (ID models) [36]. However, to improve its relevance, some changes were made to the original ID models and are described in the Supplementary Materials—Methods. Briefly, the present modeling approach was used to design, for each individual in the dietary survey, a diet at the same energy level which met a set of 33 nutritional recommendations (including 10% maximum energy from free sugars if the intake was greater than 10% or a "no increase" constraint when energy from free sugars was lower than or equal to 10%), while departing the least from the observed diet. To design a diet as similar as possible to the corresponding observed one, the model was parameterized to: (i) preferentially choose repertoire foods (i.e., foods declared as consumed by the individual); (ii) minimize the reduction of the repertoire foods; and (iii) control the introduction of non-repertoire foods (i.e., foods declared as consumed at least once in the survey, but not by this individual). The constraints to be met were a set of nutritional constraints based on dietary reference intakes, a set of acceptability constraints (maximum amounts of foods and food groups) and a set of other constraints, in particular total diet weight and total diet cost (Table S1). "Energy-free" drinks (i.e., drinks containing less than 4 kcal/100 mL) were excluded from the calculation of total diet weight to avoid competition between energy-free drinks and nutrient-dense foods with low energy content.

2.7. Identification of the Most Binding Nutrients

It is possible to identify the constraints the most difficult to fulfill by calculating, for each constraint, a factor named dual value. A null dual value indicates that the constraint is inactive: it has no impact on the optimized solution. In contrast, a non-null dual value means that the constraint is binding or active: it is influencing the result of the optimization process. To identify the most binding constraint, nutritional constraints were ranked in decreasing order according to their percentage of non-null dual values, estimated on the 1719 individuals.

2.8. Statistical Analyses

Of the 1726 adults, the diet optimization was unfeasible for 7 (i.e., no modeled diet able to simultaneously meet all the constraints could be mathematically designed with the list of food variables available for diet modeling). A final sample of 1719 adults was therefore taken for the statistical analysis. Two groups of individuals were defined, depending on the energy contribution from free sugars in their observed diets. Based on the WHO recommendation [9], individuals with a contribution greater than 10% were assigned to the "FS-EXCESS" group (excessive free sugar intakes), and those who had a contribution lower or equal to 10% were assigned to the "FS-ACCEPTABLE" group (acceptable free sugar intakes).

Individual characteristics were described and compared between FS-ACCEPTABLE and FS-EXCESS groups using a chi-squared test for categorical variables and general linear model (GLM) for continuous variables, with and without adjustment for gender and age.

Mean observed nutritional intakes, diet quality indicators, and intakes from food categories and sub-categories (as well as from fresh and processed fruits, plain and sweet milk and plain and sweet yogurts) were described for the whole sample and the two groups. Comparisons of observed food intake, nutritional intake and diet quality indicators between FS-EXCESS and FS-ACCEPTABLE individuals were made using GLM. Observed energy and free sugar intakes from main meals and from snacking occasions were also described and compared between the FS-ACCEPTABLE and FS-EXCESS groups with GLM.

GLM were used to compare the characteristics of observed and optimized diets in the two groups and to compare the variation in grams between optimized and observed diets among FS-EXCESS and FS-ACCEPTABLE individuals. To study changes in sugar balance after diet modeling, the variation in total, free and non-free sugars between observed and optimized diets, from main food categories and sub-categories were calculated and compared using GLM.

Observed energy intake, age and gender were used as a first set of adjustment variables. In a second set of adjustment variables, the current smoking status, BMI, socio-professional status were added to the first set, and, in a third set of adjustment variables, the composition of the family and sitting time were added to the second set. All values were survey-weighted and all analyses accounted for the complex INCA2 sampling frame design [19]. The Operational Research and the STAT packages of SAS version 9.4 (SAS Institute, Cary, NC, USA) were used to run linear programming models and perform statistical analysis, respectively. An alpha level of 1% was used for all statistical tests.

3. Results

3.1. Sample Characteristics

In this representative sample of French adults (n = 1693, weighted value), 41% of individuals (FS-EXCESS group, n = 690) had mean free sugar intakes above the 10% of energy intake level recommended by the WHO (mean intake 14.2% $\pm$ 4.2% of energy intake) and 59% (FS-ACCEPTABLE group, n = 1003) had acceptable intakes, i.e., below 10% of energy intake (mean intake 6.3% $\pm$ 2.5% of energy intake).

Demographic, anthropometric, socio-economic and behavioral characteristics are given in Table 1. Individuals were on average 10 years younger in the FS-EXCESS group than in the FS-ACCEPTABLE group. Individuals in the FS-EXCESS group had a lower BMI (23.6 vs. 25.2 kg/m^2), and the percentage of overweight or obese individuals among them was lower than in the FS-ACCEPTABLE group, even after adjustment for age and gender. In the FS-EXCESS group, the percentages of single individuals and couples with children were higher than in the FS-ACCEPTABLE group, while the percentage of couples without children was lower. In addition, the percentages of professionally active people and students were higher, while the percentage of retirees was lower in the FS-EXCESS group than in the FS-ACCEPTABLE group. There were proportionately more smokers in the FS-EXCESS group; this difference between groups was no longer significant after adjustment for age and gender.

Table 1. Demographic, anthropometric, socio-economic and behavioral characteristics of the total sample, FS-ACCEPTABLE and FS-EXCESS groups.

	ALL	FS-ACCEPTABLE	FS-EXCESS	p [1]	p [2]
Individuals, *n*	1693	1003	690		
Age, year [3]	47.0 ± 15.02	51.1 ± 14.0	41.1 ± 14.5	<0.001	-
Age, %					
20–34	25.4	15.1	40.3		
35–49	29.8	28.9	31.3		
50–64	29.0	35.0	20.3		
65–75	15.8	21.0	8.2		
Gender, %				0.087	-
Male	47.6	49.4	45.0		
Female	52.4	50.6	55.0		
BMI, kg/m^2 [3,4]	24.5 ± 4.3	25.2 ± 4.3	23.6 ± 4.0	<0.001	0.001
BMI, %				<0.001	0.001
<18.5 kg/m^2	4.2	3.0	6.0		
18.5 to <25 kg/m^2	55.7	49.2	65.1		
25 to <30 kg/m^2	30.6	35.4	23.7		
>30 kg/m^2	9.5	12.4	5.3		
Household composition, %					
Couple with at least one child	31.7	28.2	36.8	<0.001	
Couple with no child	42.3	49.3	32.0		
Single with at least one child	5.4	4.2	7.0		
Single with no child	20.6	18.3	23.9		
Missing information	0.1	.	0.2		
Socio-professional status, %				<0.001	
Active	56.2	52.6	61.5		
Unemployed	4.2	3.4	5.3		
Student	4.9	1.6	9.6		
Retired	25.9	34.3	13.8		
Homemaker	8.8	8.1	9.8		
ICU, euros/month [3]	1328 ± 837	1359 ± 830	1285 ± 847	0.069	
Current smoking status, %				<0.001	0.0216
Smoker	27.9	23.7	34.0		
Non-smoker	70.3	74.8	63.7		
Missing	1.8	1.5	2.3		
IPAQ, %				0.156	0.239
Low	22.6	20.9	25.1		
Moderate	30.5	31.2	29.6		
High	45.7	46.8	44.0		
Missing information	1.2	1.1	1.3		
Screen for leisure time, minutes/day [3,4]	205 ± 138	195 ± 128	221 ± 151	0.002	0.020
In front of computer	60 ± 97	52 ± 94	73 ± 101	<0.001	0.249
In front of TV	145 ± 98	143 ± 89	148 ± 110	0.442	0.040
Frequency of eating between meals, as declared %				<0.001	<0.001
≥4 times/day	2.4	1.3	4.0		
2 to 3 times/day	15.1	11.8	19.8		
1/day	31.5	28.1	36.3		
>0 and <1/day	25.3	26.6	23.4		
Never	23.4	29.6	14.4		
Missing/invalid answers	2.4	2.6	2.1		
Interest in diet, %				0.001	0.010
A lot	32.8	36.4	27.7		
Little	44.7	44.6	45.0		
Not really	16.3	13.3	20.7		
Not at all	4.9	4.5	5.3		
Missing/invalid answers	1.3	1.2	1.3		

Abbreviations: BMI, body mass index; ICU, income per consumption unit; IPAQ, International Physical Activity Questionnaire. [1] p value provided by chi-squared test for categorical variables and GLM for continuous variables; [2] Gender-age adjusted p values provided by logistic regression for categorical variables and GLM for continuous variable; [3] Results are Mean ± SD; [4] One missing information items for BMI and seven missing information items for screen for leisure time variable.

Physical activity (IPAQ) level did not significantly differ between groups. However, the FS-EXCESS individuals spent significantly more time sitting in front of computers or television (+25 min per day) than the FS-ACCEPTABLE individuals, but this difference between groups was no longer significant after adjustment for age and gender. The FS-EXCESS individuals declared that they ate more often

between meals, and they were less interested in their diet; these results remained significant after adjustment for age and gender.

3.2. Observed Nutritional Intakes and Diet Quality Indicators

Observed nutritional intakes and diet quality indicators are detailed in Table 2. Compared with the FS-ACCEPTABLE group, individuals in the FS-EXCESS group had higher daily energy intakes (2192 vs. 2123 kcal/day), with a higher energy contribution of carbohydrates (45.4% energy vs. 40.8%) and lower energy contributions from proteins and fats (respectively 15.3% and 37.1% vs. 17.3% and 39.4%) after adjustment for age, gender and energy intake (except for energy intake, only adjusted for age and gender) or further adjustment for other sociodemographic and lifestyle parameters (see footnote to Table 2 for details). With all adjustments, energy intakes at main meals did not significantly differ between the two groups, unlike energy intakes at snacking occasions, higher in FS-EXCESS vs. FS-ACCEPTABLE groups (258 kcal/day vs. 131 kcal/day). The quantity of free sugars at each moment of consumption (meals or snacking occasion) was higher in FS-EXCESS vs. FS-ACCEPTABLE individuals. For FS-ACCEPTABLE individuals, the quantity of free sugars consumed in main meals was 4.3 times greater than at snacking occasions, while this ratio was only 2.6 for the FS-EXCESS group (data not shown). Compared with FS-ACCEPTABLE individuals, those in the FS-EXCESS group ate a more energy-dense diet (185 versus 165 kcal/100 g), and had lower nutritional quality diets, as shown by a lower PANDiet score, a lower MAR and a higher MER. For 11 out of the 23 nutrients of the MAR, capped percentages of recommended intakes were lower for FS-EXCESS group compared with FS-ACCEPTABLE group. There were no significant differences for the 12 remaining nutrients. When looking at the MER, among the three nutrients, free sugars were driving the difference between the two FS groups (Table S2).

Table 2. Observed nutritional intakes and diet quality indicators for the total sample and for FS-ACCEPTABLE and FS-EXCESS groups (mean ± SD).

	ALL	FS-ACCEPTABLE	FS-EXCESS	p^{1}	p^{2}	p^{3}
Individuals, *n*	1693	1003	690			
		Mean ± SD				
Energy intake (kcal/day) [4]	2151 ± 536	2123 ± 539	2192 ± 529	0.016	0.007	0.008
from main meals (kcal/day)	1969 ± 501	1992 ± 509	1935 ± 487	0.158	0.316	0.359
from snacking occasions (kcal/day)	183 ± 194	131 ± 154	258 ± 220	<0.001	<0.001	<0.001
Proteins, % of energy	16.5 ± 2.7	17.3 ± 2.7	15.3 ± 2.2	<0.001	<0.001	<0.001
Fats, % of energy	38.5 ± 5.7	39.4 ± 6.0	37.1 ± 4.8	<0.001	<0.001	<0.001
Carbohydrates, % of energy	42.7 ± 6.1	40.8 ± 6.2	45.4 ± 5.0	<0.001	<0.001	<0.001
Free sugars, % of energy	9.5 ± 5.1	6.3 ± 2.5	14.2 ± 4.2	<0.001	<0.001	<0.001
Starch, g/day	141.1 ± 51.2	143.5 ± 55.7	137.7 ± 43.6	<0.001	<0.001	<0.001
Total sugars, g/day	90.2 ± 37.3	75.1 ± 29.2	112.1 ± 37.1	<0.001	<0.001	<0.001
Free sugars g/day	51.9 ± 33.1	33.5 ± 16.6	78.7 ± 33.1	<0.001	<0.001	<0.001
from main meals (g/day)	39.4 ± 24.5	27.2 ± 14.4	57.0 ± 25.5	<0.001	<0.001	<0.001
from snacking occasions (g/day)	12.6 ± 16.3	6.3 ± 7.2	21.7 ± 20.9	<0.001	<0.001	<0.001
Non-free sugars, g/day	38.3 ± 19.6	41.6 ± 21.1	33.4 ± 16.0	<0.001	<0.001	<0.001
Alcohol, g/day	0.22 ± 0.74	0.18 ± 0.63	0.27 ± 0.87	0.086	0.052	0.050
Solid energy density, kcal/100 g	173.4 ± 32.7	165.2 ± 14.9	185.3 ± 31.6	<0.001	<0.001	<0.001
Variety, number of foods/week	58.4 ± 14.9	57.4 ± 7.6	60.0 ± 14.7	0.017	0.006	0.014
PANDiet	62.7 ± 7.5	64.3 ± 12.8	60.4 ± 6.6	<0.001	<0.001	<0.001
MAR, %	83.8 ± 9.0	84.8 ± 23.9	82.4 ± 9.3	<0.001	<0.001	<0.001
MER, %	32.2 ± 30.0	25.1 ± 14.9	42.6 ± 34.6	<0.001	<0.001	<0.001

Abbreviations: PANDiet, probability of adequate nutrient intake; MAR, mean adequacy ratio; MER, mean excess ratio. [1] GLM with survey design adjusted for age, gender and energy intake (except for energy intake, adjusted for age and gender only); [2] GLM with survey design adjusted for age, gender, energy intake, smoking status, BMI and socio-professional status (except for energy intake, adjusted for age and gender only); [3] GLM with survey design adjusted for age, gender, energy intake, smoking status, BMI, socio-professional status, composition of the family and sitting time (except for energy intake, adjusted for age and gender only); [4] 1 kcal =4.184 kJ.

3.3. Food Amounts in Observed Diets

Food amounts in the observed diets of FS-ACCEPTABLE and FS-EXCESS groups are detailed in Table 3. The amounts of fruits, vegetables, starchy foods (except ready-to-eat cereals), meat/eggs/fish, cheese, water and added fats were higher in the FS-ACCEPTABLE than in the FS-EXCESS group. By contrast, the amounts of sweet products (all sub-categories), sugar-sweetened beverages, fruit juices and sweet yogurts were higher in the FS-EXCESS than in the FS-ACCEPTABLE group. All these differences were significant after adjustment for all the variables considered, except for water (significantly different between groups after adjustment for age, gender and energy intake only).

3.4. Food Amounts and Weight Variations after Optimization

Food amounts in optimized diets are given in Table 3, and food weight variations between observed and optimized diets (i.e., dietary changes induced by the optimization process) are shown in Figure 1.

At the food category level (Figure 1A), for both FS-ACCEPTABLE and FS-EXCESS individuals, the optimization process significantly increased the amount of fruits/vegetables/nuts and starchy foods, and significantly decreased the amount of meats/eggs/fish, mixed dishes/sandwiches and added fats (all p values < 0.001 except for added fats in FS-EXCESS, $p = 0.012$). The amount of dairy products and beverages was significantly increased for the FS-ACCEPTABLE individuals only, while sweet products were decreased for the FS-EXCESS individuals only (all p values < 0.001). The other changes at food category level were not significantly different from 0.

At the sub-category level, for both FS-ACCEPTABLE and FS-EXCESS individuals, fresh fruits (Figure 1B) and both refined and unrefined starchy foods (Figure 1C) were increased. The amount of vegetables was increased only for FS-EXCESS individuals (Figure 1B). Plain yogurts significantly increased and cheese decreased for both groups, whereas plain milk and sweet yogurts increased significantly only for FS-ACCEPTABLE individuals (Figure 1D). All sub-categories of sweet products were decreased for FS-EXCESS individuals (Figure 1E). For the beverage category (Figure 1F), water and hot beverage sub-categories were significantly increased for both FS-ACCEPTABLE and FS-EXCESS, whereas sugar-sweetened beverages and fruit juices were decreased only for FS-EXCESS individuals.

3.5. Identification of the Most Binding Nutrients

Based on dual values, the most binding constraints (in decreasing order) were those on total energy, the maximal amounts of sodium, free sugars and saturated fatty acids and the minimal amount of total carbohydrates. They presented non-null dual values for more than 75% of individuals in the total sample (data not shown).

3.6. Changes in Sugar Balance after Optimization

The amounts of total, free and non-free sugars in the observed and optimized diets (g/day), from main food category contributors are shown in Figure 2 for FS-ACCEPTABLE and FS-EXCESS individuals.

For FS-ACCEPTABLE individuals, total sugars were significantly increased after optimization (+17.5 g/day) resulting from an increase in non-free sugars (+18.7 g/day), mainly due to an increase in fresh fruits (+16 g/day) and dairy products (+1.6 g/day) and a small decrease in free sugars (−1.2 g/day) from sweet products and beverages.

For FS-EXCESS individuals, to reach the maximum 10% energy from free sugars allowed by the model, the optimization significantly reduced free sugars (−25.5 g/day) through a decrease in sweet products (−14.3 g/day), sugar-sweetened beverages (−7.8 g/day) and fruit juices (−2.6 g/day) (Figure 2 and Table 3). Non-free sugars were significantly increased (+22.1 g/day), mainly due to an increase in fresh fruits (+19.5 g/day), in the fruits/vegetables/nuts category. All these changes led to a slight but significant decrease in total sugars (−3.4 g/day).

Table 3. Amounts of food categories and sub-categories in observed and optimized diets for the total sample and for FS-ACCEPTABLE and FS-EXCESS groups (g/day).

	Observed Diets				Optimized Diets				FS-Acceptable	FS-Excess
	ALL	FS-ACCEPTABLE	FS-EXCESS	p [1]	ALL	FS-ACCEPTABLE	FS-EXCESS	p [1]	p [2]	p [2]
Individuals, n	1693	1003	690		1693	1003	690			
		Mean ± SD				**Mean ± SD**				
Fruits/vegetables/nuts	379.7 ± 236.7	438.0 ± 248.1	294.8 ± 189.8	a,b,c	562.3 ± 177.2	583.8 ± 184.3	531.1 ± 161.4	a,b,c	<0.001	<0.001
Fruits	163.1 ± 148.8	190.8 ± 162.9	122.9 ± 114.1	a,b,c	322.3 ± 138.9	336.1 ± 144.9	302.2 ± 127.1	a,b,c	<0.001	<0.001
Fresh	149.6 ± 144.4	178.5 ± 159.1	107.4 ± 106.6	a,b,c	307.0 ± 137.2	321.8 ± 143.5	285.4 ± 124.5	a,b,c	<0.001	<0.001
Processed	13.6 ± 31.7	12.3 ± 31.1	15.5 ± 32.4	a,b,c	15.3 ± 31.9	14.3 ± 32.8	16.8 ± 30.4		<0.001	0.063
Vegetables	214.5 ± 142.3	245.2 ± 149.6	169.8 ± 117.4	a,b,c	234.6 ± 99.4	242.3 ± 102.8	223.4 ± 93.2		0.593	<0.001
Nuts	2.1 ± 5.8	2.0 ± 5.7	2.1 ± 6.0		5.4 ± 9.2	5.4 ± 9.3	5.5 ± 9.1		<0.001	<0.001
Starchy foods	254.2 ± 118.9	270.0 ± 127.1	231.2 ± 101.6	a,b,c	366.7 ± 109.4	375.4 ± 112.2	354.1 ± 104.1	a,b,c	<0.001	<0.001
Refined	168.8 ± 96.4	181.2 ± 102.5	150.7 ± 83.5	a,b,c	222.9 ± 92.2	229.1 ± 94.2	214.0 ± 88.4	a,b,c	<0.001	<0.001
Unrefined	80.5 ± 59.4	85.1 ± 64.6	73.9 ± 50.1	a,b,c	139.3 ± 56.6	142.7 ± 58.1	134.3 ± 53.8	a,b,c	<0.001	<0.001
Ready-to-eat cereals	4.9 ± 16.3	3.6 ± 13.4	6.7 ± 19.6		4.5 ± 14.2	3.6 ± 13.1	5.9 ± 15.5		0.881	0.033
Meats/eggs/fish	166.9 ± 70.2	179.9 ± 71.7	147.9 ± 63.4	a,b,c	142.8 ± 43.2	144.5 ± 42.2	140.2 ± 44.7		<0.001	0.001
Mixed dishes and sandwiches	120.9 ± 91.4	117.4 ± 93.7	125.9 ± 87.9		70.4 ± 53.8	65.1 ± 51.9	78.1 ± 55.6		<0.001	<0.001
Dairy products	209.4 ± 173.7	201.5 ± 174.4	221.0 ± 172.1		222.7 ± 146.0	222.7 ± 142.1	222.7 ± 151.6		<0.001	0.754
Milk	94.4 ± 151.6	85.5 ± 153.1	107.5 ± 148.4		109.7 ± 138.0	106.7 ± 133.2	114.1 ± 144.7		<0.001	0.183
Plain milk	86.8 ± 145.8	80.9 ± 151.6	95.5 ± 136.5		104.0 ± 134.3	102.9 ± 132.9	105.7 ± 136.4		<0.001	0.034
Sweet milk	7.6 ± 38.2	4.6 ± 28.1	12.0 ± 49.0		5.7 ± 29.3	3.8 ± 23.7	8.3 ± 35.8		0.202	0.004
Yogurts	80.9 ± 81.3	78.9 ± 79.9	83.7 ± 83.1		94.7 ± 83.6	95.8 ± 83.3	93.2 ± 84.0		<0.001	0.000
Plain yogurts	39.5 ± 60.5	44.8 ± 67.0	31.7 ± 48.6		48.1 ± 66.1	54.7 ± 71.6	38.7 ± 55.9		<0.001	<0.001
Sweet yogurts	41.4 ± 59.5	34.1 ± 53.4	52.0 ± 66.0	a,b,c	46.6 ± 61.4	41.1 ± 58.4	54.5 ± 64.8		<0.001	0.227
Cheese	34.1 ± 28.8	37.1 ± 29.8	29.8 ± 26.9	a,b,c	18.3 ± 14.0	20.2 ± 14.5	15.5 ± 12.9		<0.001	<0.001
Sweet products	119.9 ± 76.0	91.1 ± 58.7	161.7 ± 79.2	a,b,c	103.4 ± 65.5	91.3 ± 62.5	121.2 ± 65.7	a,b,c	0.907	<0.001
Milk or eggs-containing desserts	18.4 ± 31.0	12.3 ± 21.7	27.3 ± 39.3	a,b,c	17.4 ± 29.4	13.1 ± 23.9	23.6 ± 35.2	a,b,c	0.082	<0.001
Cakes and pastries	48.3 ± 44.7	41.1 ± 38.5	58.9 ± 50.6	a,b,c	39.1 ± 37.1	37.7 ± 36.3	41.0 ± 38.1		0.006	<0.001
Biscuits	8.2 ± 18.4	4.6 ± 10.8	13.6 ± 24.9	a,b,c	7.9 ± 15.3	5.8 ± 12.5	10.8 ± 18.1	a,b,c	<0.001	0.001
Croissants	17.5 ± 26.0	14.2 ± 23.6	22.1 ± 28.5	a,b,c	15.7 ± 24.3	15.3 ± 24.5	16.4 ± 24.0		0.267	<0.001
Confectionery (incl. chocolate)	5.8 ± 14.1	3.1 ± 6.4	9.9 ± 20.0	a,b,c	4.4 ± 7.9	3.2 ± 6.2	6.0 ± 9.6	a,b,c	0.229	<0.001
Honey, marmalade and chocolate spread	13.8 ± 19.8	10.4 ± 15.8	18.6 ± 23.8	a,b,c	13.5 ± 17.5	11.3 ± 16.3	16.7 ± 18.7	a,b,c	0.009	0.003
Table sugar	7.8 ± 10.2	5.5 ± 7.6	11.3 ± 12.4	a,b,c	5.5 ± 6.9	4.8 ± 6.7	6.7 ± 7.0	a,b,c	<0.001	<0.001
Beverages including water	1322.6 ± 630.3	1288.5 ± 655.7	1372.1 ± 588.3		1398.4 ± 403.0	1398.1 ± 421.3	1398.8 ± 374.9		<0.001	0.259
Water	798.6 ± 569.3	821.1 ± 596.0	765.7 ± 526.7	a	898.4 ± 412.1	911.2 ± 426.8	879.8 ± 389.3		<0.001	<0.001
Hot beverages	389.4 ± 335.3	403.7 ± 332.1	368.4 ± 339.0		412.4 ± 334.3	428.4 ± 325.0	389.1 ± 346.2		<0.001	<0.001
Diet beverages	12.9 ± 62.2	10.2 ± 58.3	16.9 ± 67.3		17.6 ± 72.2	14.6 ± 69.2	21.9 ± 76.3		0.018	0.009
Sugar-sweetened beverages	62.4 ± 175.4	17.0 ± 46.4	128.3 ± 255.5	a,b,c	23.5 ± 60.8	10.1 ± 31.1	43.1 ± 83.9	a,b,c	<0.001	<0.001
Fruit juices 100%	59.4 ± 89.9	36.4 ± 62.2	92.8 ± 111.4	a,b,c	46.5 ± 67.2	33.7 ± 55.0	65.0 ± 78.4	a,b,c	0.012	<0.001
Added fats and sauces	45.5 ± 23.4	48.1 ± 23.3	41.8 ± 23.1	a,b,c	39.4 ± 18.4	39.2 ± 19.0	39.8 ± 17.6		<0.001	0.012
Foods based on soya	3.5 ± 25.4	4.0 ± 25.9	2.9 ± 24.7		3.8 ± 25.5	4.0 ± 25.4	3.5 ± 25.6		0.907	0.168

Abbreviations: FS, free sugars; incl. chocolate, including chocolate. [1] Letters indicate a significant ($p < 0.01$) difference between FS-ACCEPTABLE and FS-EXCESS based on GLM with survey design at three levels of adjustments: a, GLM adjusted for age, gender and energy intake; b, GLM adjusted for age, gender, energy intake, smoker status, BMI and socio-professional status; c, GLM adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time; [2] GLM to test differences in dietary intakes between observed and optimized diets, with survey design adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time.

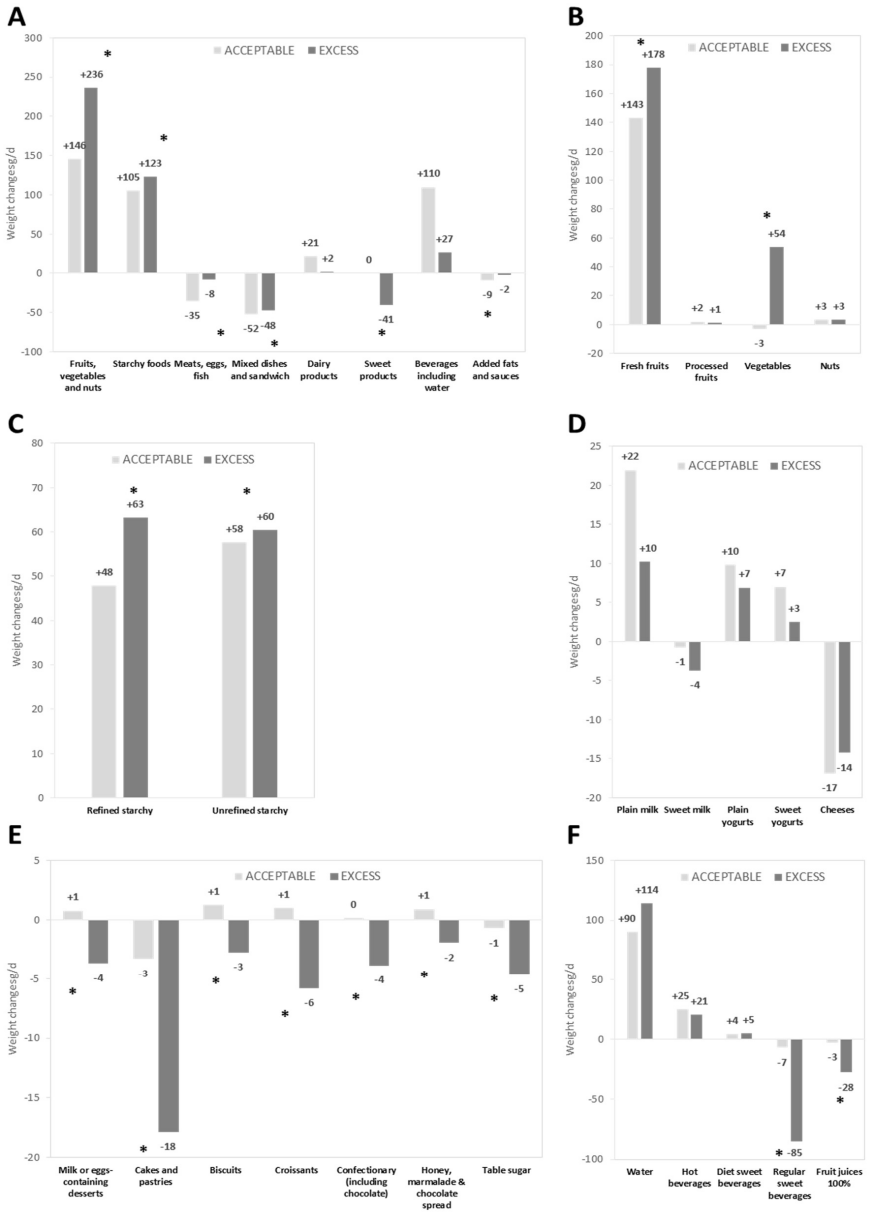

Figure 1. Weight changes [1] between observed and optimized diets (g/day) in food categories (**A**); and in food sub-categories for: fruits/vegetables/nuts (**B**); starchy foods (**C**); dairy products (**D**); sweet products (**E**); and beverages including water (**F**), in FS-ACCEPTABLE and FS-EXCESS individuals [2]. [1] Italic and bold values indicate a weight change significantly different from zero adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time; [2] the * symbol means that the weight changes were significantly different between FS-ACCEPTABLE and FS-EXCESS groups, adjusted for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time.

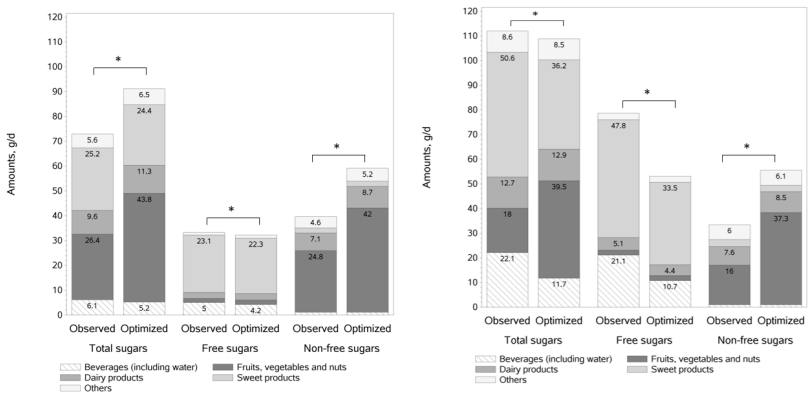

Figure 2. Amount of sugars (total, free, non-free) [1] in observed and optimized diets (g/d), from main food category contributors in: FS-ACCEPTABLE (**a**); and FS-EXCESS (**b**) individuals [2]. [1] Amounts of sugars lower than 4 g not labeled; [2] For both FS-ACCEPTABLE and FS-EXCESS, total sugars, free sugars and non-free sugars were significantly different between observed and optimized diets after adjustment for age, gender, energy intake, smoker status, BMI, socio-professional status, composition of the family and sitting time.

4. Discussion

In this representative sample of French adults, individuals with excessive intakes of free sugars represented 41% of the total population. Compared to the diets of individuals with acceptable free sugars intakes, their diets were found to be of lower nutritional quality, but could be optimized mostly via an increase in fresh fruits, vegetables and starchy foods, and a decrease in sweet products and sweet beverages including sugar-sweetened beverages and fruit juices.

Free sugar intakes represented 9.5% of energy intake in the French adult population. This is one of the lowest levels estimated by the WHO in European countries, and just below the WHO cut-off of 10%. Even so, individuals with free sugar intakes above this cut-off value (i.e., the FS-EXCESS group) represented 41% of the French adult population. Despite the existence of national and international recommendations on free sugars [9,11], it is currently difficult to estimate intakes of free sugars accurately, because information from nutrient composition tables is insufficient. Published studies on sugar intakes are mostly based on data on total and added sugars [13,15–17]. To our knowledge, only one other study recently conducted in the Dutch population [37] has also estimated free sugar intakes in a nationally representative sample. Compared with our French sample, a higher free sugar contribution (around 13.5% vs. 9.5% of energy intake) and with a lower adherence to the 10% WHO guidelines (around 30% vs. 59% respectively) was found in Dutch adults [37]. Interestingly, within the Dutch adult population, free sugar intakes decreased with age (from 16% to 11% of energy intake), in line with our findings, with FS-EXCESS individuals being 10 years younger than FS-ACCEPTABLE individuals.

Overall, in our sample, FS-EXCESS individuals had lower quality diets than FS-ACCEPTABLE individuals, as shown by a more energy-dense diet, lower MAR and PANDiet scores, and higher MER. These results are consistent with the conclusions of a recent review indicating that higher intake of added sugars is associated with poorer diet quality (in 20 out of 21 studies) and lower micronutrient intakes (in 21 out of 30 studies) [8]. Our results can be explained by food choices characterized by a lower consumption of foods of higher nutritional quality (e.g., fruits and vegetables) and a higher consumption of foods of lower nutritional quality (e.g., sweet products and sugar-sweetened beverages) [38] in FS-EXCESS individuals than in FS-ACCEPTABLE individuals.

FS-EXCESS individuals had a lower BMI than FS-ACCEPTABLE ones, despite higher energy intakes (+70 kcal/day) and greater sedentariness. A similar counterintuitive inverse relation between sugar intake and BMI was previously reviewed and discussed [39]. Selective underreporting of high sugar foods and drinks by overweight and obese people was listed as a potential explanatory factor.

The present survey being based on cross-sectional data, unhealthier eating patterns and lifestyles observed in FS-EXCESS individuals, may lead over time to weight gain, the extent of which could be estimated through the use of simplified dynamic energy balance models [40].

The top three food contributors to free sugar intakes were the same for both FS-ACCEPTABLE and FS-EXCESS groups, but with different contribution levels (measured in g/day of free sugars): sweet products (23.1 g/day and 47.8 g/day for FS-ACCEPTABLE and FS-EXCESS respectively) followed by beverages (5.0 g/day and 21.1 g/day) and dairy products (2.5 g/day and 5.1 g/day). Looking at food changes needed to achieve nutrient adequacy, the optimized diets showed similarities in the FS-ACCEPTABLE and FS-EXCESS groups (increase in fresh fruits, starchy foods, water, hot beverages and plain yogurts; decrease in mixed dishes/sandwiches, meat/eggs/fish and cheese). Additional food changes were found only in FS-EXCESS individuals, and consisted in a decrease in sweet products, sugar-sweetened beverages and fruit juices. Overall, the models were aimed not only at reducing free sugars, but also at ensuring a broad set of 33 nutritional recommendations were met without changing the energy level and thereby promoting nutrient density. This explains why food sub-categories containing free sugars were not necessarily decreased after optimization. For example, sweet yogurts were significantly increased in the FS-ACCEPTABLE individuals (+70 g/week). In other words, despite their free sugar content, the ID model selected sweet yogurts as a source of nutrients to favor.

Overall, the dietary changes needed to achieve nutrient adequacy were in line with the ongoing PNNS, the French national nutrition and health program (PNNS: "Programme National Nutrition Santé"), designed to improve health by helping people eat a healthier diet [41], where particular emphasis was placed on encouraging fruit and vegetable consumption, physical activity, and the consumption of whole grains, while reducing the consumption of foods with added sugars. In the US, to meet nutrient needs within calorie limits, advice has been recently given to choose a variety of nutrient-dense foods across and within all food groups, to limit calories from added sugars and saturated fats and to reduce sodium intake [12].

The strength of the present study lies in the ability to identify and quantify the dietary changes that may help any individual meet all 33 nutrient recommendations at the same time. Diet modeling with linear programming was early described as a unique tool to help develop food based dietary guidelines and public health messages [42]. Recently, the mean UK population diet was optimized to design the new Eatwell guide [43]. In the present study, individual diet modeling was used, rather than population diet modeling, in order to take into account the variability of individual food consumption. In addition, it is only with individual diet modeling that statistical analyses can be performed, therefore providing more robust conclusions. For FS-EXCESS individuals, dietary changes would mean halving sugar-sweetened beverages and fruit juices (from one glass/day to 1/2 glass/day) and table sugar (from 2 teaspoons/day to one teaspoon/day), reducing by about 1 portion /week for cakes and pastries, whereas fresh fruits would have to be greatly increased, with the addition of two portions of 80 g per day. If some of these changes could be difficult to integrate in daily life, our results do not seem to be drastically different from advice from a Register Dietician (RD). The RD would probably focus on the few major food changes able to rebalance the diet. The objective would not be to achieve adequacy for all nutrients at the same time but to correct major mistakes related to excesses or deficiencies. Overall, where a step-by-step approach would be taken by the RD, all the changes are considered at the same time with our mathematical model. Today, both approaches could be considered as complementary.

The present study has limitations. The choice was made to exclude alcoholic beverages from the present analyses because nutrient recommendations usually apply to non-alcoholic energy intakes. Similar choices were previously made in diet modeling with linear programming studies [43,44]. However, this limitation would appear negligible, since free sugars contained in alcoholic beverages contributed to only 0.16% of total energy intakes in our population (data not shown). In addition, allowing alcoholic beverages as variables in individual diet models was recently reported as difficult to manage, because they can contribute to energy requirements and some essential nutrients

(e.g., B vitamins and iron) for some individuals, without supplying any detrimental nutrients (e.g., sodium and saturated fatty acids) [44]. After optimization, the increase in total sugars seen in the FS-ACCEPTABLE group could also be considered as a limitation. However, we believe this increase would not have negative health effects since it is related to an increase in non-free sugars, mainly coming from fresh fruits. Indeed, in the absence of starch and total sugars recommendations, as fruits are more nutrient dense than starch, they are increased in large amounts by the optimization process to help meeting both micro-nutrient recommendations and the minimum carbohydrate energy contribution, leading to an increase in total sugars. Finally, the modeled diets could be questioned in terms of acceptability and feasibility. Despite its recognized interest in public health and nutrition [42], individual diet modeling with linear programming has only been used in the field of epidemiology. To improve realism, diets were optimized while staying as close as possible to current habits of each individual, such as preferentially using foods from his/her repertoire, which means that foods or drinks with lower nutritional profiles were not necessarily decreased or suppressed in the optimized diet [41]. For example, in the FS-ACCEPTABLE group, the amount of sweet products (91 g/day) did not change between observed and optimized diets. In terms of behavior change, adjusting food quantities rather than banning foods of low nutritional quality could be criticized, as some people may have difficulties keeping control over amounts consumed, highlighting the importance of considering individual psychological traits [45]. Future work could integrate complementary information on individual eating behavior characteristics, especially more refined acceptability parameters for a given individual. Generating results in portion sizes as well as integrating moments of consumption in the model would better target specific food changes in meals and snacking occasions. This would be particularly relevant for FS-EXCESS individuals studied here, as their dietary habits led to higher intakes of energy (in particular from sweet foods and drinks) specifically in snacking occasions compared with FS-ACCEPTABLE individuals. Once these model improvements have been made, these theoretical results will become more realistic, allowing some individual advice. Then, improvement of diet adequacy and metabolic parameters could be tested in a clinically relevant way.

5. Conclusions

In conclusion, the diet quality of French adults with excessive intakes of free sugars can be optimized by food changes that do not overly challenge their eating habits. To improve the estimation of free or added sugars and quality of food composition databases, initiatives such as the nutritional labeling of added sugars to be implemented on US food packages [46,47], are of interest to follow up. Finally, intervention studies are now needed to assess the feasibility, together with their short-term and long-term impact, of the changes in diet suggested by our study results.

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Author Contributions: A.L. contributed to the design of the study, interpreted the results, wrote the manuscript, and was responsible for the final content of the manuscript; M.M., R.G., F.V. conducted the study, performed the statistical analysis, interpreted the results and helped produce a final draft of the manuscript; F.D. contributed to the design of the study, interpreted the results, and helped produce a final draft of the manuscript; S.V. and N.D. contributed to the design of the study, interpreted the results, and wrote the manuscript; and all authors: read and approved the final version of the manuscript.

Conflicts of Interest: A.L., F.D. and S.V. are employees of Danone Nutricia Research. M.M., R.G., F.V. and N.D. declare no conflict of interest.

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Iron Intake and Dietary Sources in the Spanish Population: Findings from the ANIBES Study

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Abstract: Background: Iron deficiency is one of the most common nutritional problems in the world. It is frequent in both developed and developing countries and mainly affects women of childbearing age and children. Methods: Results were derived from the ANIBES cross-sectional study using a nationally-representative sample of the Spanish population (9–75 years, n = 2009). A three-day dietary record, collected by means of a tablet device, was used to obtain information about food and beverage consumption and leftovers. Results: Total median dietary iron intake was 9.8 mg/day for women and 11.3 mg/day for men. Highest intakes were observed among plausible adolescent reporters (13.3 mg/day), followed by adults (13.0 mg/day), elderly (12.7 mg/day), and children (12.2 mg/day). Prevalence of adequacy for iron intakes as assessed by EFSA criteria was higher than for the Spanish Recommended Iron Intake values in all age groups. Females had lower adequacy than males for both criteria, 27.3% and 17.0% vs. 77.2% and 57.0% respectively. Cereals or grains (26.7%–27.4%), meats and derivatives (19.8%–22.7%), and vegetables (10.3%–12.4%) were the major iron contributors. Conclusion: Higher iron intakes were observed in adolescents and were highest for non-heme iron. The prevalence of adequate iron intake according to EFSA criteria was higher than compared to national recommendations, and women had the lowest intakes. Therefore, there is a need to define standard dietary reference intake to determine inadequate iron intakes in the Spanish population.

Keywords: micronutrients; iron; ANIBES Study; dietary survey; food sources

1. Introduction

Iron is an essential nutrient of public health relevance required for many metabolic processes in the human body. It is part of haemoglobin and therefore crucial for the delivery of oxygen to the cells. It is also a structural component of many enzymes needed for a wide range of processes, such as phagocyte antimicrobial activity, neurotransmitter synthesis and function, and the production of DNA, collagen, and bile acids [1]. Iron deficiency is the most common and widespread nutritional disorder in the world [2,3]. As well as affecting a large number of children and women in non-industrialized countries, it is the only nutrient deficiency that is also significantly prevalent in virtually all industrialized nations. There are no current global figures for iron deficiency, but using iron deficiency anaemia as an indirect indicator, it can be estimated that most preschool children and pregnant women in non-industrialized countries, and at least 30%–40% in industrialized countries, are iron deficient [2]. Women, especially adolescents consuming low-energy diets, are at high risk of iron deficiency: anaemia is three times more frequent in adolescent girls who have tried to lose weight during the preceding 12 months compared with those who have not [3]. According to the World Health Organization (WHO), in the Spanish population anaemia prevalence ranges from 14% to 18% in children and in women of reproductive age, respectively [4]. Román-Viñas et al. observed a prevalence of iron intake inadequacy of 10%–21% of the Estimated Average Requirement (EAR) in a number of European populations when analyzing the European Nutrition and Health Report [5].

Considerable amounts of iron must be provided by the diet to replace the iron that is lost from the body (through blood loss and exfoliation of skin and gastrointestinal cells) and growth requirements [1]. Many dietary factors can hamper or promote absorption of this mineral, but the most important determinant of dietary iron absorption is systemic iron need: more is absorbed in a state of iron deficiency and less is absorbed when iron depots are replete [6]. In circumstances of marked iron requirements, however, the influence of dietary factors on iron absorption may become limiting. There are three main dietary factors related to iron status: quantity of iron, quality of iron, and the composition of diet [7].

Dietary iron is present in different forms and varying concentrations in a broad range of foods. There are two kinds of iron in the usual diet with respect to mechanisms of absorption: haem and non-haem iron. The richest sources of iron are cereals, vegetables, nuts, eggs, fish, and meat. Iron is also added to food as a fortificant in many countries [8,9] and is available as supplements [10]. Haem iron is 2–6 times more bioavailable from the diet than non-haem iron [11]. Daily Recommended Iron Intakes (DRI) for women of childbearing age is 18 mg/day and 8 mg/day for men and postmenopausal women [12]. Estimated Average Requirement (EAR) for iron is 8.1 mg/day for fertile women, 6 mg/day for men, and 5 mg/day for postmenopausal women [13].

Among the most remarkable limitations of dietary surveys in Spain are the use of non-harmonized methods for collecting intake data and the important occurrence of potential misreporting [9]. Assessing the validity of self-reporting among study participants might increase overall accuracy and representativeness of results. In addition, the use of new methods for collecting dietary information is necessary: completing food records is time-consuming hard work and can therefore be very inconvenient. Methods using information and communication technologies may also improve quality and accuracy [14].

There is a need for better and updated knowledge of micronutrient intakes in the Spanish population to prevent and/or delay adverse effects resulting from inadequate intakes at different stages of life. Dietary intake assessment of major iron sources is relevant because of the high anaemia prevalence amongst women of childbearing age. The ANIBES study is one of the few national dietary surveys in Spain to have collected comprehensive data with novel collection methods. Moreover, is the only representative one of the Spanish population using new technologies such as tablet devices to record food intakes and leftovers. The present study focuses on evaluating iron dietary intakes in the Spanish population according to age, gender and considering misreporting, but also to examine the main food sources that contribute to the mineral dietary intake.

2. Materials and Methods

The complete design, protocol and methodology of the ANIBES study have been described in detail elsewhere [15–17].

2.1. Sample

The ANIBES study is a cross-sectional study conducted using stratified multistage sampling. To guarantee better coverage and representativeness, the fieldwork was performed at 128 sampling points across Spain. The design of the ANIBES study aims to define a sample size that is representative of all individuals living in Spain, aged 9–75 years, and living in municipalities of at least 2000 inhabitants. The initial potential sample consisted of 2634 individuals, and the final sample comprised 2009 individuals (1013 men, 50.4%; 996 women, 49.6%). In addition, for the youngest age groups (9–12, 13–17, and 18–24 years), an "augment sample" was included to provide at least $n = 200$ per age group (error $\pm$ 6.9%) (Figure 1). The augment sample is the process of increasing the amount of interviews for a particular subgroup within the population in order to achieve an adequate number of interviews to allow analysis of population subgroups or segments that wouldn't normally yield a sufficient number of interviews in a main random survey, without the expense of increasing the sample size for the whole survey. Therefore, the random sample plus augment sample comprised 2285 participants. The sample quotas according to the following variables were age groups (9–12, 13–17, 18–64, and 65–75 years), gender (men/women), and geographical distribution (Northeast, East, Southwest, North–Central, Barcelona, Madrid, and Balearic and Canary Islands) and locality size: 2000 to 30,000 inhabitants (rural population); 30,000 to 200,000 inhabitants (semi-urban population), and over 200,000 inhabitants (urban population). The following exclusions were applied: people that lived in colleges, hospitals, and other institutions; those strictly following a diet for medical tests or to determined pathologies; those who had an acute disease (common cold, gastroenteritis, chickenpox, etc.); and those working in fields related to market research, advertising, or journalism. In addition, people not strictly following a medical diet, those following recommendations for diabetes, hypertension, hypercholesterolemia, hypertriglyceridemia or hyperuricemia, women who were pregnant or at lactation period, those with allergies or intolerances, and those with metabolic diseases such as hyperthyroidism or hypothyroidism were included. Finally, other factors for sample adjustment were considered: unemployment rate, percentage of foreigners (immigrant population), physical activity level assessed by The International Physical Activity Questionnaire (IPAQ) [18], tobacco use, and education or economic level. The fieldwork for the ANIBES study was conducted from mid-September 2013 to mid-November 2013, and two previous pilot studies were also performed. To equally represent all days of the week, study subjects participated during two weekdays and one weekend day. The final protocol was approved by the Ethical Committee for Clinical Research of the Region of Madrid, Spain [16].

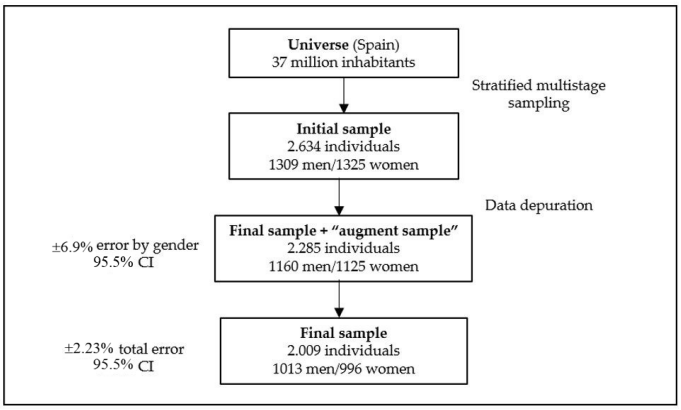

Figure 1. Sample collection design for the ANIBES study.

2.2. Food and Beverage Records

Study participants were provided with a tablet device (Samsung Galaxy Tab 2 7.0, Samsung Electronics, Suwon, Korea) and trained in recording information by taking photos of all food and drinks consumed during the three days of the study, both at home and outside. Photos had to be taken before beginning to eat and drink, and again after finishing, so as to record the actual intake. Additionally, a brief description of meals, recipes, brands, and other information was recorded using the tablet. Participants who declared or demonstrated that they were unable to use the tablet device were offered other options, such as using a digital camera and paper record and/or telephone interviews. A total 79% of the sample used a tablet, 12% a digital camera, and 9% opted for a telephone interview. As no differences in the percentage of misreporting were found according to the type of device used to assess dietary intake, we used the measurements of the three assessment methods in the analysis. In addition to details of what and how much was eaten, for each eating/drinking event, participants recorded where they were, who they were eating with, and whether they were watching television and/or sitting at a table. After each survey day, participants recorded if their intake was representative for that day (or the reason why if it was not), and details of any dietary supplements taken. The survey also contained a series of questions about participants customary eating habits (e.g., the type of milk usually consumed) to facilitate further coding. Food records were returned from the field in real time, to be coded by trained coders who were supervised by dieticians. An ad hoc central server software/database was developed for this purpose, to work in parallel with the codification and verification processes. Food, beverage, and energy and nutrient intakes were calculated from food consumption records using VD-FEN 2.1 software, a Dietary Evaluation Program from the Spanish Nutrition Foundation (FEN), Spain, which was newly developed for the ANIBES study by the FEN and is based mainly on Spanish food composition tables [19], with several expansions and updates. Data obtained from food manufacturers and nutritional information provided on food labels were included. A food photographic atlas was used to assist in assigning gram weights to portion sizes. The VD-FEN 2.1 software was developed to receive information from field tablets every two seconds, and the database was updated every 30 min. Energy distribution and objectives for the Spanish population were used to analyze the overall quality of the diet [16,20].

2.3. Evaluation of Misreporting

National diet and nutritional surveys are the most used tools to assess diet, nutrient intake, and the nutritional status of the population. Data collected in the surveys is based on subjects self-reporting. As this method is indirect and has a pseudo-quantitative nature, the surveys frequently report data that do not represent the habitual intake of the studied population and estimates energy intakes (EI) that are not plausible physiologically [21]. In this respect, EFSA has published a protocol that has a harmonized approach to identify misreporting based on a review of the methods used in representative samples of people aged 10–74 years in Europe [22]. EFSA suggests that the data should be reported for the whole population as well as divided into plausible and non-plausible reporters, and so this is included in the present article. EFSA recommendations were followed to calculate misreporting; in which the proposed protocol is based mainly on Goldberg [23] and Black [24]. This method evaluates the reported EI (EIrep) against the presumed energy requirements. EIrep is expressed as a multiple of the mean basal metabolic rate estimate (BMRest), and it is compared with the presumed energy expenditure of the studied population. Then, the ratio EIrep:BMRest is referred to as the physical activity levels (PAL) [22].

2.4. Statistical Analysis

Values are expressed as median (interquartile range) or as percentage. The non-parametric data were statistically analyzed by the Kruskal–Wallis test. When the Kruskal–Wallis test resulted in differences, multiple comparisons between medians were studied by Mann-Whitney's U test,

while significant differences among groups were allocated by post hoc Student-Newman-Keuls test. Differences were considered significant at $p < 0.05$. To establish if the samples were parametric or non-parametric, the Kolmogorov–Smirnov test was used. Data analysis was performed with SPSS 22.0 software package (IBM Corp., Armonk, NY, USA).

Values obtained for two diagnostic criteria on iron Recommended Dietary Intakes (RDI): iron Dietary Reference Values by EFSA [25] and iron daily Recommended Intakes for the Spanish population as reviewed by Moreiras et al. [12] were used (Table 1). Prevalence of adequacy for iron intakes (% population above 80% RDI) was calculated for each of the RDI and then compared using the McNemar test for paired proportions. The kappa coefficient (k) was used to assess the degree of agreement of the two classification criteria: EFSA and Moreiras et al. Agreement interpretation was based on established categorizations: "poor" (k < 0.000), "slight" (0.000–0.200), "fair" (0.210–0.400), "moderate" (0.410–0.600), "substantial" (0.61–0.80), and "almost perfect" (0.810–1.000).

Table 1. Recommended dietary intakes (RDI) by Moreiras and EFSA.

Age Group (Years)	Moreiras et al. [12]	EFSA [25]
	Fe (mg/Day)	Fe (mg/Day)
Men		
9	9	11
10	12	11
11	12	11
12	12	11
13–17	15	11
18–19	15	11
20–49	10	11
50–59	10	11
≥60	10	11
Women		
9	9	11
10	18	11
11	18	11
12	18	13
13–17	18	13
18–19	18	16
20–49	18	16 [a]
50–59	10	16 [a]
≥60	10	11

[a] For postmenopausal women, Daily Recommended Iron Intake (DRI) is the same as for women ≥60 years.

3. Results

A total of 2009 individuals, 996 women and 1013 men, participated in the study. The distribution by age and sex of the sample and the study population were not significantly different from the Spanish population for these age groups. A higher proportion of non-plausible reporters was identified amongst men (53.3%, Table 2). In addition, 75.9% of adults were acknowledged as non-plausible reporters (Table 3). Conversely, a lower proportion of non-plausible reporters was observed in children (5.8%) and adolescents (8.4%).

Table 2. Description of the ANIBES sample by gender and reporting.

Gender	Reporting	
	Plausible Reporters	Non-Plausible Reporters
Female	57.3% (n = 311)	46.7% (n = 685)
Male	42.7% (n = 232)	53.3% (n = 781)

Table 3. Description of the ANIBES sample by gender reporting and age group.

		Children	Adolescents	Adults	Elderly
Gender	Female	7.7% (n = 87)	6.6% (n = 74)	76.2% (n = 857)	9.5% (n = 107)
	Male	10.9% (n = 126)	11.8% (n = 137)	68.8% (n = 137)	8.5% (n = 137)
Reporting	Plausible reporters	17.8% (n = 120)	11.3% (n = 76)	64.2% (n = 433)	6.7% (n = 45)
	Non-plausible reporters	5.8% (n = 93)	8.4% (n = 135)	75.9% (n = 1222)	10.0% (n = 161)

Median daily iron intakes for the Spanish population aged 9–75 years are shown in Table 4. Males had higher iron intakes than females in the whole sample. Total median iron intakes amongst female were 9.8 mg/day while males were 11.3 mg/day. Significantly higher intake values ($p < 0.001$) were observed in the plausible reporters group in both female and male, with an iron intake of 12.0 mg/day and 14.7 mg/day, respectively.

Table 4. Iron intake (mg/day) and prevalence of adequate (% population above 80% RDI) in ANIBES sample by gender and reporting and agreement (k) between Spanish [12] and EFSA references [25].

Gender	Iron (mg/Day)	% Above 80% RDI Moreiras (Spain)	% Above 80% RDI EFSA	RDI Agreement (Kappa) Moreiras vs. EFSA
Women				
Total n = 996	9.8 (7.9–11.9)	17.0	27.3 ##	0.526
Plausible n = 331	12.0 *** (10.3–13.8)	24.8	50.5 ###	0.570
Non-plausible n = 685	8.8 (7.3–10.6)	13.4	19.8 ###	0.462
Men				
Total n = 1013	11.3 (9.0–14.0)	57.3	77.2	0.697
Plausible n = 232	14.7 *** (12.4–17.1)	84.0	100.0 ##	-
Non-plausible n = 781	10.3 (8.4–12.7)	49.3	70.4 ##	0.694

Values are median (interquartile range) per group. *** $p < 0.001$ difference plausible vs. non-plausible (Mann-Whitney'S U test). ## $p < 0.01$ differences between Moreiras et al. and EFSA references (McNemar test). ### $p < 0.001$ differences between Moreiras et al. and EFSA references (McNemar test).

Prevalence of adequacy for iron intakes (% population above 80% RDI) in the study population is presented by sex and reporting in Table 4 according to the different diagnostic criteria: national (Moreiras et al. [12]) and international (EFSA [25]). The proportion of adequacy for total iron intake in female was 17.0% and 27.3%, and for male it was 57.3% and 77.2% according to the Spanish [12] and EFSA [25] references, respectively (Table 4). The degree of agreement (k) between the two diagnostic criteria, observed in the present study, indicated that it was "moderate" for the prevalence of adequacy between Spanish [12] and EFSA [25] criteria in the case of women (k = 0.526) and "substantial" for men (k = 0.697).

Results of the analysis by age group and reporting are shown in Table 5. Adolescents (11.4 mg/day) and children (11.0 mg/day) had higher total iron intakes than adults (10.4 mg/day) and the elderly (10.2 mg/day). In all age groups, iron intake values in plausible reporters were significantly higher ($p < 0.001$) than in non-plausible reporters. Noteworthy, iron intakes were higher in the adolescent's plausible reporters group (13.3 mg/day). Conversely, children's plausible reporters group presented lower iron intake levels (12.2 mg/day). The degree of agreement (k) between the two diagnostic criteria was "fair" for the prevalence of adequacy between Spanish [12] and EFSA [25] criteria in all age groups (0.210–0.400).

Table 5. Iron intake (mg/day) and prevalence of adequate (% population above 80% RDI) in ANIBES sample by age group and reporting and agreement (k) between Spanish [12] and EFSA [25] references.

Age Group	Iron (mg/Day)	% Above 80% RDI Moreiras (Spain)	% Above 80% RDI EFSA	RDI Agreement (Kappa) Moreiras vs. EFSA
Children				
Total n = 213	11.0 (9.2–12.8)	40.9	77.9 ###	0.345
Plausible n = 120	12.2 *** (10.4–14.0)	54.2	94.2 ###	0.255
Non-plausible n = 93	9.2 (8.0–11.1)	23.7	57.0 ##	0.540
Adolescents				
Total n = 211	11.4 (9.1–13.4)	15.2	73.0 ###	0.243
Plausible n = 76	13.3 *** (11.6–15.4)	27.6	90.8 ###	0.212
Non-plausible n = 135	10.0 (8.1–11.8)	8.2	63.0 ###	0.277
Adults				
Total n = 1655	10.4 (8.4–12.9)	36.9	47.9 ###	0.233
Plausible n = 433	13.0 *** (11.0–15.6)	47.8	63.3 ##	0.730
Non-plausible n = 1222	9.6 (7.8–11.8)	33.0	42.5 ###	0.709
Elderly				
Total n = 206	10.2 (7.9–12.6)	52.9	68.0 ###	0.170
Plausible n = 45	12.7 *** (10.9–17.2)	88.9	100.0 ###	-
Non-plausible n = 161	9.5 (7.5–11.5)	42.9	59.0 ###	0.842

Values are median (interquartile range) per group. *** $p < 0.001$ difference No misreporting vs. Misreporting (Mann-Whitney's U test); ## $p < 0.01$ differences between Moreiras and EFSA references (McNemar test); ### $p < 0.001$ differences between Moreiras and EFSA references (McNemar test).

When iron intakes were evaluated by geographical distribution, overall, we observed that North Central region (11.4 mg/day) and Northeast (10.9 mg/day) presented higher daily iron intakes, while the Center of the peninsula (9.9 mg/day), Canary Islands (10.1 mg/day) and the South region (10.1 mg/day) had the lowest intakes (Table 6).

Table 6. Iron intake (mg/day) by geographical distribution.

Geographical Distribution (Nielsen Areas)	Iron (mg/Day)
Barcelona (Metropolitan Area)	10.8 (8.8–13.1)
Canary Islands	10.1 (7.8–13.3)
Center	9.9 (8.2–13.2)
East	10.6 (8.3–13.2)
Madrid (Metropolitan Area)	10.2 * (8.1–12.7)
Northeast	10.9 (8.7–13.4)
Northwest	10.6 (8.6–12.7)
North Central	11.4 (9.6–14.0)
South	10.1 ** (8.2–12.4)

Values are median (interquartile range per group); * $p < 0.05$ difference vs. North Central (Bonferroni test); ** $p < 0.01$ difference vs. North Central (Mann-Whitney's U test).

Contribution of Food and Beverage Groups to Iron Intake

The contribution (%) of food and beverage categories to the daily iron intake is shown, categorized by genre, in Figure 2. The food groups with the highest mean proportional contribution to total iron intake in both males and females were firstly cereal and grain products (26.7%–27.4%) and meat and meat products (19.8%–22.7%), of which intakes were significantly higher in males ($p < 0.001$). Thirdly, vegetables accounted for a 10.3%–12.4% of iron intakes, being significantly higher ($p < 0.001$) in females. Together, these three food groups contributed to ≥60% of iron intakes of the studied population.

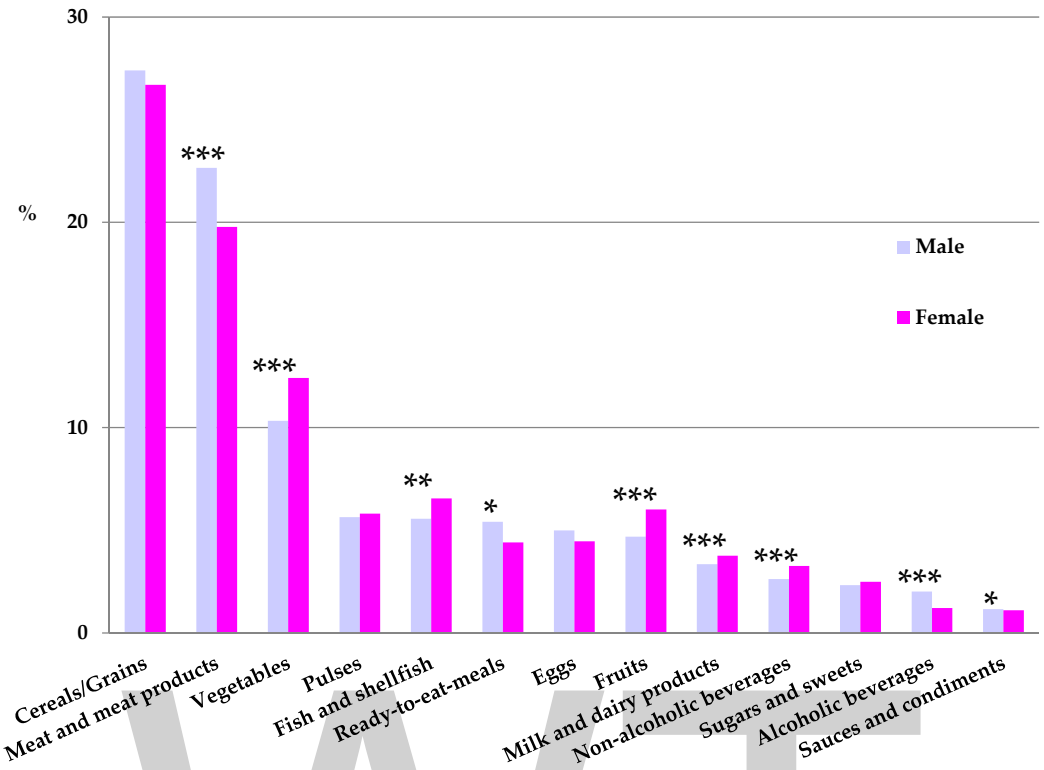

Figure 2. Contribution of food and beverages to iron intake by gender. * $p < 0.05$ difference vs. women (Mann-Whitney's U test); ** $p < 0.01$ difference vs. women (Mann-Whitney's U test); *** $p < 0.001$ difference vs. women (Mann-Whitney's U test).

When analyzing main food group sources for different age groups (Table 7), cereals and grain products were the main sources of iron for the entire sample, especially for adolescents (31.3%–33.1%) and children (30.7%–31.8%), where they were significantly higher than adults and the elderly ($p < 0.05$). Meat and meat products were the second largest contributors, being lowest for the elderly population (17.0%) and significantly highest for children, adolescents, and adult males (23.5%) (Table 7).

Table 7. Contribution of food and beverages to iron intake by gender and age group.

	Children		Adolescents		Adults		Elderly	
	Male	Female	Male	Female	Male	Female	Male	Female
Cereals/Grains (%)	30.7 ^{a}	31.8 ^{a}	33.1 ^{a}	31.3 ^{a}	26.9 ^{b}	26.9 ^{b}	24.0 ^{c}	24.6 ^{b,c}
Meat and meat products (%)	23.1 ^{d}	20.1 ^{d,e}	23.5 ^{d}	20.3 ^{d,e}	22.9 ^{d}	20.1 ^{e}	18.0 ^{e}	17.0 ^{e}
Vegetables (%)	7.1 ^{f}	7.9 ^{f}	6.7 ^{f}	7.7 ^{f}	10.8 ^{g}	12.4 ^{h}	13.0 ^{h,i}	14.7 ^{i}
Pulses (%)	4.5	6.4	4.9	5.7	5.6	5.8	7.6	6.0
Fish and shellfish (%)	3.8 ^{j}	4.0 ^{j}	3.7 ^{j}	4.3 ^{j}	5.7 ^{k}	6.5 ^{l}	6.9 ^{l,m}	7.9 ^{m}
Ready-to-eat-meals (%)	6.6 ^{n}	5.7 ^{n,o,p}	7.6 ^{n,o}	6.2 ^{n}	5.3 ^{o,p}	4.6 ^{p}	2.9 ^{p}	2.1 ^{q}
Eggs (%)	4.6	4.3	5.0	4.7	5.0	4.4	5.5	5.3
Fruits (%)	3.3 ^{r}	3.8 ^{r}	2.4 ^{s}	3.8 ^{r}	4.6 ^{r}	5.7 ^{t}	8.4 ^{u}	10.2 ^{w}
Milk and dairy products (%)	4.7 ^{x}	4.3 ^{y}	3.8 ^{y}	4.0	3.3 ^{y}	3.7 ^{z}	2.8 ^{z}	3.9 ^{y}
Non-alcoholic beverages (%)	2.5 $^{\alpha}$	2.6 $^{\alpha,\beta}$	2.0 $^{\alpha}$	2.6 $^{\alpha}$	2.8 $^{\beta}$	3.2 $^{\gamma}$	2.9 $^{\beta,\gamma}$	3.4 $^{\delta}$
Sugars and sweets (%)	6.1 $^{\varepsilon}$	5.8 $^{\varepsilon,\zeta}$	4.7 $^{\zeta}$	6.5 $^{\varepsilon,\zeta}$	2.0 $^{\eta}$	2.4 $^{\theta}$	1.2 $^{\iota}$	0.7 $^{\iota}$
Alcoholic beverages (%)	-	-	-	-	2.1 $^{\kappa}$	1.3 $^{\lambda}$	4.8 $^{\mu}$	2.0 $^{\lambda}$
Sauces and condiments (%)	1.0 $^{\nu}$	1.6 $^{\nu}$	1.1 $^{\nu}$	1.3 $^{\nu}$	1.2 $^{\nu}$	1.1 $^{\nu}$	0.7 $^{\xi}$	0.8 $^{\pi}$

Values are percentage. All differences are $p < 0.05$ (Student-Newman-Keuls test). Different superscript lowercase letters indicate statistical significance in each row.

Since meat and meat products are the food group with the highest sources of bioavailable heme iron, we decided to further explore the different subgroups and their contribution to iron intakes (Table 8). Total meat contribution was significantly higher ($p < 0.05$) for children (male) (12.9%), adolescent males (13.5%), and adult males (13.3%) vs. the rest of age groups. Red meat provided the same contribution for all age groups. Secondly, sausages and other meat products provided 5.5%–10.2% of total iron intakes, especially in children, adolescents and adult male which were significantly higher ($p < 0.05$) than in adult female and the elderly. On the other hand, the fish and shellfish group contribution was significantly higher ($p < 0.05$) in groups older than 18 years.

Table 8. Contribution of main meat and fish types to iron intake by gender and age group.

	Children		Adolescents		Adults		Elderly	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
Meat (total) (%)	12.9 ^{a,b}	9.8 ^{a}	13.5 ^{b}	10.7 ^{a}	13.3 ^{b}	12.1 ^{a}	10.6 ^{a}	10.9 ^{a}
Red meat (%)	5.3	5.1	5.4	2.8	5.5	4.8	5.1	4.8
White meat (%)	2.9 ^{c,d}	1.7 ^{e}	3.3 ^{c,d}	3.1 ^{c,d}	3.3 ^{d}	3.0 ^{c,d}	2.4 ^{c,d,e}	2.1 ^{c,e}
Poultry (%)	4.7 ^{f}	3.0 ^{g}	4.8 ^{f}	4.9 ^{f,g}	4.5 ^{f}	4.3 ^{f,g}	3.1 ^{f,g}	4.0 ^{f,g}
Viscera and offal (%)	0.0 ^{h,i}	0.3 ^{h,i}	0.0 ^{h}	0.1 ^{h,i}	0.6 ^{i}	0.4 ^{h,i}	1.0 ^{j}	0.6 ^{h,i}
Sausages and other meat products (%)	10.2 ^{k}	10.0 ^{k}	10.0 ^{k}	9.5 ^{k}	9.0 ^{k}	7.5 ^{l}	6.4 ^{l,m}	5.5 ^{m}
Fish and shellfish (total) (%)	3.8 ^{n}	4.0 ^{n}	3.7 ^{n}	4.3 ^{n}	5.7 ^{o}	6.5 ^{p}	6.9 ^{p,q}	7.9 ^{q}
White fish (%)	1.1 ^{r,s,t}	1.6 ^{s,t,u}	0.7 ^{r}	0.8 ^{r}	1.1 ^{r}	1.2 ^{r,s}	2.0 ^{t,u}	2.5 ^{u}
Blue Fish (%)	0.5 ^{v,w}	0.7 ^{v,w}	0.3 ^{v}	1.0 ^{v,w}	0.8 ^{w}	1.0 ^{w}	1.6 ^{y}	0.9 ^{w}
Shellfish (%)	1.6 $^{z,\alpha}$	1.1 ^{z}	1.7 ^{z}	1.5 ^{z}	2.2 $^{\alpha}$	2.6 $^{\alpha}$	2.2 $^{\alpha}$	2.7 $^{z,\alpha}$
Canned Fish (%)	0.6 $^{\beta}$	0.6 $^{\beta}$	1.0 $^{\beta}$	0.9 $^{\beta}$	1.7 $^{\gamma}$	1.7 $^{\gamma}$	1.0 $^{\beta,\gamma}$	1.8 $^{\gamma}$

Values are percentage. All differences are $p < 0.05$ (Student-Newman-Keuls test). Different superscript lowercase letters indicate statistical significance in each row.

4. Discussion

4.1. Iron Deficiency and Vulnerable Population Groups

In Europe, iron deficiency is considered to be one of the main nutritional deficiency disorders affecting large fractions of the population, particularly groups such as children and fertile or pregnant women. Moreover, adolescents consuming low energy diets, vegetarians, and vegans are at high risk of iron deficiency [26]. Children and adolescents are consistently considered a group at risk for nutritional deficiencies as their needs increase due to high growth requirements, in addition to some deleterious dietary habits (high consumption of empty calories, "meal skipping", etc.) [27]. Median total iron intake in children and adolescents from our study was 11 and 11.4 mg/day respectively. Even significantly higher values were observed for plausible reporters and adequacy prevalence (% higher than 80% RDI) was 40.9% and 15.2% for children and adolescents respectively, according to national recommendations. Results from the EnKID Study [28,29], back in 1998 observed that mean daily iron intakes were 14.4 ± 3.1 mg/day and 11.8 ± 2.1 mg/day in boys and girls, respectively. In this population-based, cross-sectional study, that evaluated the dietary habits and nutritional status of Spanish schoolchildren and adolescents aged 2–24 year ($n = 3534$), researchers used 24 h recalls and a food frequency questionnaire. Food patterns of this population group revealed moderate milk consumption, high consumption of dairy products and meat intake, and low consumption of fish, fruit, and vegetables. Noteworthy, in this study, under-reporters were excluded from the analysis [27].

Women of childbearing age are another vulnerable population for iron deficiency for the reasons already discussed. Overall, our results showed that women had a median total iron intake of 9.8 mg/day, with a significantly higher intake of 12.0 mg/day within plausible reporters. Adequacy prevalence as a percentage of population above 80% of national [12] and EFSA [25] recommendations was 17.0% and 27.3% respectively, while in the case of men adequacy increased to 57.0% and 77.2% respectively. The large difference between men and women regarding prevalence of adequate intake

calls for attention. Women are at high risk of iron deficiency and should be the ones to increase their iron intake. However, women's RDI for iron are more difficult to reach (18 mg/day compared to 8 mg/day for men and postmenopausal women). In view of these results, nutritional policies should be created for the female population, from different public administrations, to encourage the consumption of iron-rich foods, as well as to establish nutritional education programs to teach how to promote the ingestion of iron when ingested with foods with less bioavailable iron content. Quintas et al. studied a group of Spanish women (19–35 years, n = 130), showing a high incidence of iron deficiency at blood level (10.7%) and a low iron intake (11.1 $\pm$ 3.0 mg/day) [30]. Iron intakes and status were also assessed in the Spanish National Survey of Dietary Intake (Encuesta Nacional de Ingesta Dietética España, ENIDE) [9]. Their findings reveal that men's average daily iron intakes were higher than women's (15 mg/day vs. 12–14 mg/day) with a high percentage of fertile women that did not achieve recommended intakes. Median intake values were 14–15 mg/day in women and 16 mg/day in men, so that according to EAR, iron intakes were adequate.

4.2. Misreporting

The purpose of this pioneer study was to evaluate dietary intakes of iron in the Spanish population according to age, gender, and misreporting, and to examine the contribution from different foods to this mineral's total intake. To our knowledge, this is the first representative Spanish study considering plausible and non-plausible reporters for assessing dietary iron intake. Understanding the error in self-reported data may help both improving data collection and analyzing the relationships between dietary intake and health outcomes [31]. Maintaining or excluding subjects identified as under-reporters in the analyses is still a matter of debate. When the main issue is to describe eating patterns in a national representative sample, for example, excluding under-reporters is likely to induce a selection bias [32]. The latter may be of special importance when considering micronutrients intakes.

According to the literature, women are more likely to under-report than men, and under-reporting is more common among overweight and obese individuals [10,29]. Other associated characteristics, for which there is less consistent evidence, include age, smoking habits, level of education, social class, physical activity, and dietary restraint. [33]. Our results show that men represent the higher proportion of non-plausible reporters (53.3%). Also remarkably, adults were identified as the major age group who were non-plausible reporters (75.9%), while other studies find that children and adolescents represent higher percentages of misreporting [33].

Low-energy reporters are characterized by a relatively less favorable profile, in terms of education, weight status, sedentary behavior, and eating behaviors. People with low educational background and thus low knowledge on nutrition and health are likely to be both less interested and less compliant in recording food intake [34]. A number of authors have used the Goldberg cut-off to identify "low energy reporters" and to explore their characteristics or to examine the effects of low energy reporting on the data and the conclusions to be drawn from it [9].

Macdiarmid et al. [33] reviewed the main causes of misreporting and stated that the prevalence of under-reporting in large nutritional surveys could range from 18 to 54% of the whole sample, but can be as high as 70% in particular subgroups. Authors declare that the most consistent differences are between men and women and between groups differing in body mass index [33]. Although less likely to occur, the possibility of over-reporting cannot be overlooked. Foods which have a positive *health image* may be over-reported to describe a healthy diet [11].

4.3. Iron Food Sources

Detailed information on dietary iron sources is essential to better understand the strengths and weaknesses of the Spanish diet quality and to identify vulnerable population groups. Our results show that higher percentages of iron are provided by cereal and grain products (26.7%–27.4%) throughout all the studied population. This could indicate that the higher iron proportion comes from non-haem iron dietary sources, thus its bioavailability may be compromised. Meat and meat products are the second

contributing group (19.8%–22.7%). Results of the ANIBES study regarding main energy sources of the diet showed that the contribution of meat and meat products was categorized as "very high" amongst all age groups [16]. A number of recommendations by public health authorities designate that meat and processed meat products intake should be limited [35]. But, in this regard, as they represent the best haem iron dietary source, a compromise should be reached. According to Hercberg et al., haem iron in haemoglobin and myoglobin in meat, poultry, and fish usually constitutes only 10% or less of the total iron intake in European mixed diets, but the average absorption of haem iron is usually around 25% (but may vary from about 10% to 40%) [26]. The authors find that non-haem iron in cereals, vegetables, fruits, roots, pulses and beans constitutes the main part of dietary iron, although its bioavailability is low (1% ± 5%). Pulses and legumes are minor as iron sources amongst the studied population (4.5%–7.6%), being lower in children and higher in the elder group. Results obtained in the ENIDE dietary survey in Spain [36] showed that the higher percentage of iron contribution was from legumes and seeds (23%), with fish and shellfish and meat and meat products being second (19%) and third (16%), respectively. Noteworthy, cereal and grain products were the fourth iron source, accounting for 11% of total intakes [36]. It is important to acknowledge that there are a number of factors that affect iron absorption into the gastrointestinal system and thus iron bioavailability: calcium, phytates in cereals and legumes, and phenolic compounds found in tea, coffee, and other beverages bind iron and restrict its availability for absorption, while meat and vitamin C found in fruit and vegetables enhance the potential availability of iron for mucosal uptake [11]. The fact that between 6.7% and 14.7% of total dietary iron comes from the vegetable group indicates that vitamin C from this sources is potentially also consumed, although more information is required on iron absorption enhancers and inhibitors.

Mandatory iron fortification of wheat flour is implemented in a number of European countries such as the UK [26], where it accounts for 6%–10% of dietary intakes; but at present, only a voluntary food fortification scheme takes place in Spain, where food products such as fortified breakfast cereals and bars are available. It is noteworthy this type of products were not assessed in our survey.

The strengths of this study include the careful design, protocol, and methodology used in the ANIBES study, conducted among a random representative sample of the Spanish population aged 9–75 years. Food consumption assessment was performed using digital tablets and included a thorough quality control process. Main limitations are the cross-sectional design and the use of newly developed technologies for some age groups (i.e., the elderly).

The existence of potential iron insufficiency may be related to the rapid evolution of the dietary model and lifestyle modifications over the last few decades in our country. Indeed, in the last several generations, a reduction in total calorie intake was observed (due in part to a reduction in physical activity) [16] which has possibly led to a decrease in iron intake along with that of most other dietary micronutrients. Moreover, the increase in consumption of foods containing "empty calories," lacking or being low in trace elements or vitamins, has contributed to a decrease in the micronutrient density in diet.

5. Conclusions

In conclusion, the iron intakes from the ANIBES study population in Spain were studied and assessed, including plausible and non-plausible reporters. Total median daily iron intake levels observed were low for women and for men. Significantly higher iron intake values were observed among plausible reporters from both sexes and all age groups. The major proportion of dietary iron sources were cereal and grain products, which could indicate that the proportion of non-heme iron intake is higher in our study population.

Our results shows that the prevalence of adequacy for iron by the EFSA [25] criteria was higher than the one from the national standard (Moreiras et al. [12]). Therefore, there is a need to define standard dietary reference intake to determine inadequate mineral intakes in the Spanish population.

Finally, to optimize iron status, it is still desirable to encourage a varied diet with adequate attention to sources of haem iron, and more emphasis should be given to the enhancing or inhibitory factors influencing non-haem iron absorption by means of adequate recommendations regarding dietary habits.

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6

Body Composition, Nutritional Profile and Muscular Fitness Affect Bone Health in a Sample of Schoolchildren from Colombia: The Fuprecol Study

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Abstract: The objective of the present study is to investigate the relationships between body composition, nutritional profile, muscular fitness (MF) and bone health in a sample of children and adolescents from Colombia. Participants included 1118 children and adolescents (54.6% girls). Calcaneal broadband ultrasound attenuation (c-BUA) was obtained as a marker of bone health. Body composition (fat mass and lean mass) was assessed using bioelectrical impedance analysis. Furthermore height, weight, waist circumference and Tanner stage were measured and body mass index (BMI) was calculated. Standing long-jump (SLJ) and isometric handgrip dynamometry were used respectively as indicators of lower and upper body muscular fitness. A muscular index score was also computed by summing up the standardised values of both SLJ and handgrip strength. Dietary intake and degree of adherence to the Mediterranean diet were assessed by a 7-day recall questionnaire for food frequency and the Kidmed questionnaire. Poor bone health was considered using a z-score cut off of ≤ -1.5 standard deviation. Once the results were adjusted for age and Tanner stage, the predisposing factors of having a c-BUA z-score ≤ -1.5 standard deviation included being underweight or obese, having an unhealthy lean mass, having an unhealthy fat mass, SLJ performance, handgrip performance, and unhealthy muscular index score. In conclusion, body composition (fat mass and lean body mass) and MF both influenced bone health in a sample of children and adolescents from Colombia. Thus promoting strength adaptation and preservation in Colombian youth will help to improve bone health, an important protective factor against osteoporosis in later life.

Keywords: calcaneal ultrasound; bone health; body fat; muscular strength; calcium

1. Introduction

Quantitative ultrasound (QUS) measurements are widely used to assess bone (especially calcaneal) health because the measurements are non-invasive and both less expensive, and simpler than laboratory-based techniques (i.e., Dual-Energy X-ray Absorptiometry, DXA). However experience and standardization are required to achieve precise measurement [1]. Ultrasound has special appeal for use in youth because of its speed, low cost, and complete lack of ionizing radiation [1]. In recent years, c-BUA at the calcaneus site measured by QUS has been used to determine bone health status in adults [2,3], children [4] and adolescents [5]. Epidemiological evidence shows that peak bone health acquired through bone mineral accrual during childhood and adolescence may be a key determinant of bone health and future fracture risk during a person's lifespan [6]. It is well known that childhood and adolescence are crucial periods for the development of the skeleton [7]. However, bone quality is determined by a number of factors such as genetic-ethnic factors, body composition, and hormonal status as well as lifestyle behaviours, such as physical activity and diet, that influence bone gain during growth and bone loss later in life [8]. Changes in ultrasound measurements have been shown to be correlated with changes in bone mineral density (BMD) in children and adolescents, and can discriminate between a normal and low BMD [1–5]. A number of studies have also reported QUS parameters to be significantly associated with bone structure independently of BMD [1–3,9,10].

Among healthy children and adolescents, bone mass was positively influenced by certain measures of physical fitness as well as by age, weight, and pubertal stage [11]. Previous systematic reviews [12], cross-sectional studies [13,14], and experimental trials [15,16] have examined the influence of body composition, muscular fitness (MF), and nutritional profile, on bone health in children and adolescents, showing controversial findings. Body composition has been implicated in the accrual of bone mass and bone health in children and adolescents [17,18]. Scientific evidence has addressed the relationship between c-BUA with anthropometric and body composition variables such as weight, BMI, and lean mass, even in the long-term [19,20].

Regarding physical fitness, few previous studies have reported on the relationship between MF and c-BUA in youth. Studies based on DXA measurements reported controversial results. Torres-Costoso et al. [13] showed a positive association between upper-limb MF and bone health in schoolchildren, while Cole et al. [21] observed a negative association with volumetric bone density independent of lean mass. Similarly, Gracia-Marco et al. [16] found a positive relationship between MF, speed/agility and cardiorespiratory fitness, and accrual of total-body bone mineral content in 373 adolescents, while Vicente-Rodriguez found in a study of 278 Spanish adolescents that lean mass and MF are an independent predictor of bone health in youth populations [18,22].

On the other hand, healthy diet is an important modifiable factor in the development of bone mass during the critical periods of growth and maturation [23]. In this vein, Tylavsky et al. [24] reported relationships between high fruit and vegetable intakes and bone size/radius in the whole body in early-pubertal white girls after controlling for age, BMI, and physical activity. In addition, calcium intake is considered to be the most important nutrient for bone health throughout a person's lifespan [25], yet previous cross-sectional and longitudinal studies using QUS have yielded variable results, with a positive association found in some [17,26] but not in others [27]. For this reason, bone measurements are important, even at younger age, to detect a 'lagged behind' bone formation at an early stage or, at an older age, to detect a risk profile in time.

In the Latin-American region, most research on QUS has been conducted mainly on adults [28,29], while studies with children and adolescents are scarce [4,30]. At the local level, we have previously reported on body composition correlates of heel QUS among children and adolescents [4], but there is

currently no relevant information regarding body composition, MF, and nutritional profile bone gain during growth.

Therefore, the objective of the present study was to investigate the relationship between bone health, body composition, MF, and nutritional profile on a sample of children and adolescents from Colombia.

2. Methods

2.1. Study Desing and Sample Population

We performed cross-sectional analyses of baseline data from participants in The FUPRECOL study, which focused on the associations between fitness, health and non-communicable diseases. We have recently published a complete description of The FUPRECOL Study design, methods, and primary outcomes for our current cohort [4,31,32]. In this study, we included a sub-sample (n = 1118) of 9 to 17.9 years old healthy Colombian children and adolescents (boys n = 508 and girls n = 610). The participants were recruited between April 2015 and June 2015 [32]. Individuals with endocrine disorders, psychiatric disorders, pregnancy, cardiovascular disease, obesity, systemic infections, asthma, or other physical impairments that made them unable to participate in this study, as well as those using any prescribed drugs or actively using illegal or illicit drugs, were excluded from this investigation.

2.2. Data Collection

All data were collected at the same time in the morning, between 7:00 a.m. and 10:00 a.m. Body weight and height were measured following standard procedures and using an electronic scale (Tanita® BC544, Tokyo, Japan) and a mechanical stadiometer platform (Seca® 274, Hamburg, Germany), respectively. BMI was calculated as body weight in kilograms divided by the square of height in metres. BMI was classified as underweight, normal weight, overweight, or obese using the International Obesity Task Force (IOTF) criteria [33]. Waist circumference (WC) was measured at the midpoint between the last rib and the iliac crest using a tape measure (Ohaus® 8004-MA, Parsippany, NJ, USA). In all measures, we found almost excellent test-retest reliability [body weight (intraclass correlation, ICC = 0.983), height (ICC = 0.973), BMI (ICC 0.897), and WC (ICC = 0.967)]. To classify WC, we used criterion-referenced health-related cut-points derived from de Ferranti et al. [34] because of its large sample size, age-specificity, and relatively generalizable ethnicity. In addition, we also calculated the waist-to-height ratio (WHtR). Ashwel et al. [35] proposed a universal WHtR cutoff >0.5, that might identify early cardiovascular risk in children and adolescents. All cut-off values were based on data obtained from schoolchildren internationally [36–38]. Lean mass (kg) and body fat (percentage) were determined for bioelectrical impedance analysis (BIA) by Tanita BC-418®. A detailed description of BIA technique can be found elsewhere [39]. The corresponding intra-observer technical error (reliability) of the measurements was 0.95%. Ramírez-Vélez et al. [39] proposed a surrogate cut-off to unhealthy levels of body fatness (24% in boys and 26% in girls) to identify individuals at risk of excess adiposity. Abnormal body compositions such as high body fatness, low lean mass, or a combination of the two phenotypes are relevant indices, but data on their prevalence in youth populations are still limited. In our study, we divided participants into subgroups as proposed by Siervo et al. [37]: healthy lean mass, (upper 50th percentile) and healthy body fatness ($\leq$24% in boys and $\leq$26% in girls) and unhealthy lean mass (lower 50th percentile) and overfat (>24.1% in boys and >26.1% in girls).

The MF protocols used are appropriate for use in this age group and have shown acceptable validity and reliability [36,40]. We used standing long-jump (SLJ) and isometric handgrip dynamometry as indicators of lower and upper body MF, respectively. To assess lower body MF, subjects were instructed to jump as far as possible using a two footed take-off and landing technique. They were encouraged to flex then extend their knees, ankles, and hips and to swing their arms to maximise performance. SLJ performance was calculated as the distance between the toes at take-off to the heels at the landing point. The best score from two correctly performed jumps was used [40]. Handgrip

strength was assessed as an indicator of upper-body MF using an adjustable analogue handgrip dynamometer T-18 TKK SMEDLY III® (Takei Scientific Instruments Co., Ltd, Niigata, Japan). Students watched a brief demonstration of technique and were given verbal instructions on how to perform the test. The dynamometer was adjusted according to the child's hand size according to predetermined protocols [40]. Monthly, each dynamometer was tested using a standardized calibration procedure which showed that the device was within 1 kg of accuracy over the whole measuring range (from 0 to 100 kg), and with a 100 g sensitivity [41]. SLJ and handgrip measurements in a subsample (n = 229, similar in demographics and biological characteristics to the whole sample) were recorded to ensure reproducibility on the day of the study. The reproducibility of our data were r = 0.78 to SLJ and r = 0.96 to handgrip test. Rather than relying on performance-based normative values, we categorized MF data using cut-off points shown to have cardio-metabolic health predictive values [42]. A muscular index score was computed by summing up the standardized values of SLJ and handgrip strength. The score was calculated separately for boys and girls and by 1-year age groups [38]. To date, there are no established reference cut-off points for MF, assessed using either handgrip strength or SLJ. Previous studies have shown that participants with low MF (lowest quartile) have the poorest cardio-metabolic profile [43,44]; therefore, suggesting that participants falling below the threshold for this quartile could be considered "unhealthy". Taking this into account, we have used the 20th percentile as a threshold for unhealthy MF, as reported in adolescents from Europe [40].

Food consumption was assessed by the Kidmed questionnaire [45,46]. In this study, we divided participants into two groups: less or equal to 8 points (ideal healthy diet), and less or equal to 7 points (non-ideal healthy diet). In order to obtain information related to calcium-containing foods and soda, we used a seven-day recall. Participants were asked this specific question: "over the past 7 days, on average how many servings of food such as cheese, yogurt, milk, and calcium-fortified orange juice did you have per day?" (or per week if less frequent). Researchers helped participants to better estimate serving sizes (e.g., one cup of milk, one slice of cheese). Soda consumption and its type, in cups per day, was also collected. The 7-day recall test-retest reliability was 0.68. Calcium-containing foods and soda data were analysed with the Nutritionist-diet analysis software (ICBF, Bogota, Colombia), amended to include traditional Colombian recipes according to the Kidmed values validated by Flores Navarro-Pérez et al. [46]. Based on the NIH Consensus Development Program we used 1200 mg/day as optimal calcium intake in children and adolescents [47]. A registered nutritionist conducted the dietary assessment.

c-BUA is dependent on the commercial ultrasound densitometer used. Mathematical adjustments were made to the true c-BUA according to Jaworski et al, [48] and Ramírez-Vélez et al. [4] who used the Achilles ultrasound densitometer (Lunar Corporation, Madison, WI, USA) to measure the c-BUA values in young individuals [3]. A detailed description of c-BUA technique has been described elsewhere [4]. The coefficient of variation (CV) for within-day measurements has previously been reported as 1.8% for c-BUA. The CV for between-day measurements is 0.69 [49]. Poor bone health was considered using a z-score cut off of ≤ -1.5 standard deviation, as suggested in clinical practice [50].

Maturation status (self-reported) was assessed by the classification described by Tanner (five stages: I–V) as; pre-pubertal (I–II), pubertal (III), and post-pubertal (IV–V) [51]. Each participant entered into an isolated room where, using a set of images exemplifying the various stages of sexual maturation, they categorized the development of their own genitalia (for boys), breasts (for girls), armpits (for boys) and pubic hair (for both genders). The reproducibility of our data reached 85%.

2.3. Ethics Statement

The study protocol was explained verbally to the participants and their parents/guardians before they gave their written consent. Participation in the study was fully voluntary and anonymous, with no incentives provided to participants. The Review Committee for Research on Human Subjects at the University of Rosario (code No. CEI-ABN026-000262) approved all study procedures. The protocol

was in accordance with the latest revision of the Declaration of Helsinki and current Colombian laws governing clinical research on human subjects (Resolution 008430/1993 Ministry of Health).

2.4. Statistical Analysis

A power analysis showed that this sample size was sufficient to estimate c-BUA values with a precision of 10% and a power of 90%. The sample size was estimated at 30 participants per age-sex group. Body composition, nutritional profile, sexual maturation, and MF characteristics of the study sample are presented as means, SD or relative frequencies *n* (%). The normality of the variables was verified using histograms and Q-Q plots. Differences were analysed using two-way analysis of variance (ANOVA) or chi-square test (χ^2) in order to explore sex and age-group (children 9 to 11.9 years vs. adolescents 12 to 17.9 years) differences. Linear regression models and Pearson's correlation coefficients were used to examine the relationships between c-BUA values and body composition (lean mass and body fat percentage) outcomes by sex and age-group. To estimate the relationship between poor bone health and body composition and anthropometric variables (WC, BMI, WHtR, and age), nutrition profiles (Mediterranean diet quality (low, medium and high), calcium intake (compliance of calcium intake dietary recommendations), sugar-sweetened soft/drink intake (daily, weekly and never)), MF variables (SLJ, handgrip, handgrip/body mass, and muscular index score), and binary logistic regression models were used and adjusted for age and sexual maturation. *Odds ratios* were considered a confounder if they shifted the model in a constant direction with a proportional increase in the exposure level of at least 10%. We used SPSS V. 21.0 software for Windows (SPSS, Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Descriptive Characteristics

Table 1 shows the demographic descriptive statistics of the sample. The final sample had a mean age of 13.0 ± 2.3 (range 11–14) years and contained slightly more females (54.6%). Girls had lower levels of weight, z-score BMI, SLJ, handgrip, handgrip/body mass (kg) and muscular index scores than boys ($p < 0.05$). c-BUA average was different by age-group and sex; in girls (children 60.5 (14.8) vs. adolescents 80.8 (15.0) and in boys (children 58.5 (12.1) vs. adolescents 82.9 (18.0) The prevalence of overweight and obesity scores was 23.6% and 10.6% in girls (children 9 to 11.9 years), and 19.9% and 11.2% in boys ($p < 0.05$), according to the IOTF criteria. The average and prevalence of calcium intake foods (portions/day) and compliance with calcium intake daily were 1.5 and 8.7 in boys (adolescents 12 to 17.9 years), and 1.3 and 9.3% in girls, according to the dietary recommendations.

3.2. Effects of Anthropometric, Body Composition, Muscular Fitness and Calcium Intake Characteristics on Bone Health (c-BUA)

Correlations between the subjects' characteristics (anthropometric, body composition, muscular fitness and calcium intake) for both sexes and the c-BUA values were examined by Pearson correlations (Table 2). In children and adolescents, particularly boys aged 0 to 11.9 years, the c-BUA (dB/MHz) parameter correlated positively with age, weight, height, BMI, WC, fat mass, lean mass, handgrip, SLJ, muscular index score and calcium intake dietary and negatively with WHtR ($r = -0.141$, $p = 0.048$).

3.3. Characteristics Associated with Healthy and Poor Bone Health

The c-BUA ranged from 59.0 to 84.0 dB/MHz (mean 70.3 SD (18.8) dB/MHz). Participants falling within unhealthy lean mass, underweight status, unhealthy muscular index score and handgrip showed a higher poor bone health prevalence (29.6%, 20.7%, 17.9% and 15.5%, respectively). A total of 14.4% of children and adolescents had poor bone health (Table 3).

Table 1. Characteristics among a sample of children and adolescents from Bogota, Colombia (mean (SD) or frequencies).

Characteristics	Total (n = 1118)	Girls (n = 610)			Boys (n = 508)		
		Children 9–11.9 Years (n = 246)	Adolescents 12–17.9 Years (n = 364)	p-Value	Children 9–11.9 Years (n = 246)	Adolescents 12–17.9 Years (n = 364)	p-Value
Age (years)	13.0 (2.3)	10.6 (1.1)	14.6 (1.3)	<0.001	10.4 (1.2)	14.6 (1.2)	<0.001
Weight (kg)	46.6 (11.7)	37.0 (8.5)	51.0 (8.4)	<0.001	37.4 (9.2)	54.0 (10.4)	<0.001
Height (m)	152.0 (12.4)	141.5 (9.0)	155.2 (6.1)	<0.001	140.7 (10.0)	163.0 (9.6)	<0.001
Body mass index (kg/m^2)	19.9 (3.2)	18.5 (2.9)	21.2 (3.1)	<0.001	18.5 (2.8)	20.2 (3.0)	<0.001
Lean mass (kg)	34.4 (8.7)	60.4 (14.7)	80.0 (15.0)	<0.001	58.5 (12.0)	82.9 (18.0)	<0.001
Body mass index status (%) *							
Underweight	164 (14.7)	43 (17.5)	53 (14.6)	0.004	14 (7.1)	54 (17.3)	<0.001
Normal weight	658 (58.9)	119 (48.3)	207 (56.9)		121 (61.7)	211 (67.6)	
Overweight	221 (19.8)	58 (23.6)	89 (24.5)		39 (19.9)	35 (11.2)	
Obese	75 (6.7)	26 (10.6)	15 (4.1)		22 (11.2)	12 (3.8)	
Waist circumference (cm)	82.6 (9.3)	75.5 (7.5)	88.0 (7.1)	<0.001	75.5 (7.7)	85.6 (7.6)	<0.001
Waist-to-height ratio	0.426 (0.045)	0.427 (0.046)	0.422 (0.043)	0.189	0.447 (0.045)	0.418 (0.043)	<0.001
Fat mass (%)	21.7 (7.5)	60.4 (14.7)	80.8 (15.0)	<0.001	58.5 (12.0)	82.9 (18.0)	<0.001
Tanner stage * Prepuber/Puber/Pospuber (%)	31.5/35.9/32.6	54.1/33.3/12.6	15.4/40.1/44.5	<0.001	62.8/29.6/7.7	12.8/36.9/50.3	<0.001
Standing long-jump (cm)	130.8 (29.2)	107.4 (18.8)	127.5 (21.5)	<0.001	120.9 (21.5)	158.8 (26.2)	<0.001
Handgrip (kg)	22.5 (8.1)	16.0 (4.3)	23.0 (4.2)	<0.001	16.4 (4.5)	30.7 (8.4)	<0.001
Muscular index score	0.0 (0.8)	−0.1 (0.8)	0.0 (0.8)	0.046	−0.2 (0.9)	0.0 (0.8)	0.008
c-BUA (dB/MHz)	73.0 (18.9)	60.5 (14.8)	80.8 (15.0)	<0.001	58.5 (12.1)	82.9 (18.0)	<0.001
Mediterranean diet adherence * Low, Medium, High (%)	30.4/60.2/9.4	29.7/58.5/11.8	35.2/58.0/6.9	0.068	27.6/60.7/11.7	27.2/63.8/9.0	0.577
Sugar-sweetened soft drink intake * Daily/Weekly/Never	13.2/78.4/8.4	14.6/72.4/13.0	11.8/79.4/8.8	0.114	14.3/79.1/6.6	13.1/81.7/5.2	0.705
Sugar-sweetened drink intake * Daily/Weekly/Never	6.3/53.7/40.0	5.7/49.6/44.7	5.2/48.4/46.4	0.904	7.7/58.7/33.6	7.1/60.3/32.6	0.930
Calcium intake foods (portions/day)	1.3 (2.0)	1.1 (1.7)	1.3 (2.0)	0.152	1.0 (1.7)	1.5 (2.4)	0.002
Calcium intake dietary recommendations (portions/day)	3.7 (0.7)	3.3 (0.4)	3.5 (0.0)	<0.001	3.2 (0.5)	4.7 (0.6)	<0.001
Compliance with calcium intake dietary recommendations * (daily, yes)	8.7	8.1	9.3	0.606	8.2	8.7	0.847

Data are shown as mean (SD) or frequencies; Significant between-sex differences (ANOVA one way test or chi-square; * $p < 0.001$); c-BUA, calcaneus quantitative ultrasound parameter; Calcium intake and compliance was measured by 7-day recall test.

Table 2. Pearson correlation matrix for c-BUA and anthropometric, body composition, muscular fitness and calcium intake characteristics.

Characteristics	Total (n = 1118)		Girls (n = 610)				Boys (n = 508)			
			Children 9–11.9 Years (n = 246)		Adolescents 12–17.9 Years (n = 364)		Children 9–11.9 Years (n = 246)		Adolescents 12–17.9 Years (n = 364)	
	r	p-Value	r	p-Value	r	p-Value	r	p-Value	r	p-Value
Age (years)	0.663	<0.001	0.429	<0.001	0.283	<0.001	0.443	<0.001	0.492	<0.001
Weight (kg)	0.700	<0.001	0.550	<0.001	0.484	<0.001	0.630	<0.001	0.535	<0.001
Height (m)	0.649	<0.001	0.449	<0.001	0.310	<0.001	0.631	<0.001	0.443	<0.001
Body mass index (kg/m^2)	0.480	<0.001	0.416	<0.001	0.376	<0.001	0.417	<0.001	0.352	<0.001
Lean mass (kg)	0.689	<0.001	0.543	<0.001	0.460	<0.001	0.679	<0.001	0.575	<0.001
Waist circumference (cm)	0.475	<0.001	0.328	<0.001	0.404	<0.001	0.409	<0.001	0.347	<0.001
Waist-to-height ratio	0.015	0.624	0.021	0.995	0.085	0.103	−0.141	<0.001	0.018	0.747
Fat mass (%)	0.082	0.006	0.320	<0.001	0.325	<0.001	0.212	0.090	0.044	0.451
Standing long-jump (cm)	0.391	<0.001	0.229	<0.001	0.121	0.020	0.170	0.017	0.199	<0.001
Handgrip (kg)	0.644	<0.001	0.469	<0.001	0.323	<0.001	0.526	<0.001	0.580	<0.001
Muscular index score	0.259	<0.001	0.277	<0.001	0.151	<0.001	0.391	<0.001	0.278	<0.001
Calcium intake foods (portions/day)	0.069	0.021	0.028	0.661	0.016	0.760	0.015	0.829	0.041	0.469
CIDR (portions/day)	0.453	<0.001	0.236	<0.001	0.281	<0.001	0.320	<0.001	0.338	<0.001

r = correlation coefficient (Pearson's r); CIDR, calcium intake dietary recommendations.

Table 3. Prevalence and anthropometric, body composition, muscular fitness and nutritional profile characteristics associated with healthy and poor bone.

Characteristics	Adequate Bone Healthy (n = 956)				Poor Bone Healthy (n = 162)				p-Value
	n	%	CI 95%		n	%	CI 95%		
Girls	522	54.6	51.7	57.8	88	54.3	46.3	62.3	0.947
Boys	434	45.4	42.2	48.3	74	45.7	37.7	53.7	
Children (aged 9–11.9 years)	378	39.5	36.5	42.6	64	39.5	31.5	46.9	0.994
Adolescents (aged 12–17.9 years)	578	60.5	57.4	63.5	98	60.5	53.1	68.5	
Underweight (BMI)	119	12.4	10.6	14.4	45	27.8	20.4	34.6	<0.001
Normal (BMI)	565	59.1	56.0	62.1	93	57.4	49.4	64.8	
Overweight (BMI)	199	20.8	18.3	23.4	22	13.6	8.6	19.1	
Obese (BMI)	73	7.6	6.0	9.3	2	1.2	0.0	3.1	
Lean mass (unhealthy)	491	51.4	48.0	54.4	48	29.6	22.8	36.4	
Lean mass (healthy)	465	48.6	45.6	52.0	114	70.4	63.6	77.2	

Table 3. *Cont.*

Characteristics	Adequate Bone Healthy (n = 956)				Poor Bone Healthy (n = 162)				p-Value
	n	%	CI 95%		n	%	CI 95%		
Waist circumference (unhealthy)	44	4.6	3.3	6.0	5	3.1	0.6	6.2	0.387
Waist circumference (healthy)	912	95.4	94.0	96.7	157	96.9	93.8	99.4	
Waist to height-ratio (unhealthy)	74	7.7	6.2	9.5	6	3.7	1.2	6.8	0.072
Waist to height-ratio (healthy)	882	92.3	90.5	93.8	156	96.3	93.2	98.8	
Fat mass (unhealthy)	252	26.4	23.7	29.1	23	14.2	9.3	20.4	<0.001
Fat mass (healthy)	704	73.6	70.9	76.3	139	85.8	79.6	90.7	
Tanner stage (pre-pubertal)	302	31.6	28.6	34.4	50	30.9	24.1	37.7	0.945
Tanner stage (pubertal)	341	35.7	32.6	38.6	60	37.0	29.6	44.4	
Tanner stage (post-pubertal)	313	32.7	29.8	35.8	52	32.1	25.3	39.5	
Standing long-jump (unhealthy)	526	55.0	52.0	57.9	106	65.4	58.6	72.2	0.014
Standing long-jump (healthy)	430	45.0	42.1	48.0	56	34.6	27.8	41.4	
Handgrip (unhealthy)	637	66.6	63.5	69.7	143	88.3	82.7	93.2	<0.001
Handgrip (healthy)	319	33.4	30.3	36.5	19	11.7	6.8	17.3	
Muscular index score (unhealthy)	92	9.6	7.7	11.5	31	19.1	13.6	25.3	<0.001
Muscular index score (healthy)	864	90.4	88.5	92.3	131	80.9	74.7	86.4	
Mediterranean diet adherence (low)	289	30.2	27.4	33.3	51	31.5	24.7	38.9	0.908
Mediterranean diet adherence (medium)	578	60.5	57.5	63.6	95	58.6	50.6	66	
Mediterranean diet adherence (high)	89	9.3	7.5	11.2	16	9.9	5.6	14.8	
Sugar-sweetened soft drink intake (daily)	132	13.8	11.7	15.9	16	9.9	5.6	14.8	0.186
Sugar-sweetened soft drink intake (weekly)	749	78.3	75.7	81.0	128	79.0	72.2	85.2	
Sugar-sweetened soft drink intake (never)	75	7.8	6.2	9.6	18	11.1	6.8	16	
Sugar-sweetened drink intake (daily)	60	6.3	4.7	7.9	10	6.2	3.1	9.9	0.413
Sugar-sweetened drink intake (weekly)	520	54.4	51.0	57.6	81	50.0	42	57.4	
Sugar-sweetened drink intake (never)	376	39.3	36.3	42.7	71	43.8	36.4	51.8	
Compliance CIDR (No)	871	91.1	89.1	92.9	150	92.6	88.9	96.3	0.536
Compliance CIDR (Yes)	85	8.9	7.1	10.9	12	7.4	3.7	11.1	

BMI, body mass index; CIDR, calcium intake dietary recommendations.

3.4. Factors Associated with Poor Bone Health

Figure 1, shows results from the logistic regression analysis. Once the adjustment was performed (by age and Tanner stage), the predisposing factors of having a c-BUA *z*-score ≤ -1.5 standard deviation included; being underweight [OR 2.30 (95% CI 1.53 to 1.69)], being obese [OR 0.17 (95% CI 0.04 to 0.69)], having an unhealthy lean mass [OR 2.51 (95% CI 1.74 to 3.60)], unhealthy levels of fat mass [OR 0.46 (95% CI 0.29 to 0.74)], unhealthy SLJ performance [OR 1.55 (95% CI 1.09 to 2.19)], unhealthy handgrip performance [OR 3.77 (95% CI 2.29 to 6.20)], and unhealthy muscular index score [OR 2.22 (95% CI 1.42 to 3.47)].

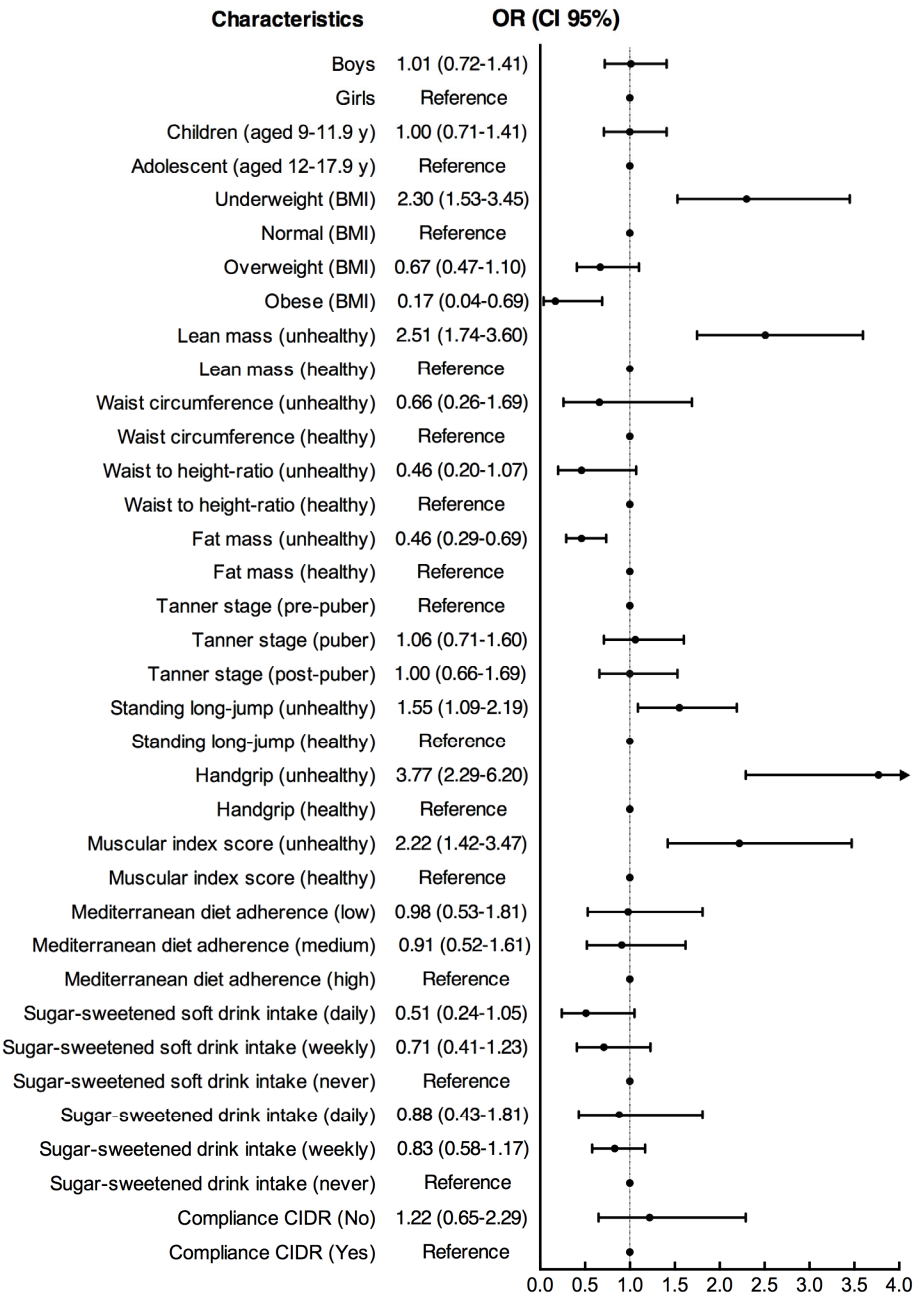

Figure 1. Factors associated with poor bone health.

4. Discussion

The results obtained in this study presented for the first time the relationship between bone health and body composition, nutritional profile, and MF in a sample of children and adolescents from Colombia. Our results show that poor bone health values were significantly related to BMI, lean body, fat mass, SLJ, handgrip, and muscular index score.

Regarding the c-BUA and body composition, in both genders and age-groups, the c-BUA (dB/MHz) parameter correlated positively with age, weight, height, waist circumference and BMI and negatively with WHtR. Our results are consistent with those from previous studies, demonstrating a strong association between anthropometric variables and body composition with c-BUA [17]. However, logistic regression analysis showed that the strongest positive associations were the weight status (overweight and obesity) or having a low lean mass, while the high fat mass was negatively associated. The associations observed in this study confirm previous findings, which are mainly based on studies in children and adolescents and on cross-sectional data. For example, a study of 245 Spanish children showed a positive correlation between c-BUA and weight, body mass index (BMI), and lean mass [14]. This is further supported by Eliakim et al. [52] and Correa-Rodriguez et al. [53], who showed reduced QUS measures in obese children compared to non-obese peers. In addition, fat mass may also have a pathophysiological effect on bone metabolism. This could suggest that schoolchildren from Bogotá have higher levels of adiposity compared to studies developed in other countries [54,55]. Body fat changes during childhood are ethnic-related, which has also been evident in pre-pubertal children and adolescents just from South America. The latter highlights the importance of our study, which is ethnic-specific [1,4]. As the growth spurt occurs during puberty, the natural process of pubertal development can explain these results as well as changes in body composition [56]. When children and adolescents grow, body fat (proportion and content) changes; this occurs more obviously in the pre-adolescent phases when girls continue to present an increase in fat mass [57,58]. Our results also show that obese and underweight youth were respectively associated with healthy or poor bone health, highlighting the role of body weight and lean mass in bone health. These findings could be due to the lower lean mass developed as consequence of their lower fat mass [7]. The hypothesis that the association between body size and QUS parameter may be parameter-specific has been supported by a study in Japanese adolescents [59].

Regarding the MF, the physiological basis for explaining the association between MF and bone mass remains uncertain. Our logistic regression analyses showed that unhealthy MF (SLJ, handgrip and muscular index score) predicts poor bone health. In this regard, a cross-sectional study of 693 school children found that physical fitness using the SLJ had a significant influence on the QUS parameters in children [60]. Also, a 20-year prospective study reported that childhood fitness levels, particularly in females and in the pre- or early pubertal years, were predictive of adult skeletal status as measured by QUS, explaining up to 8% of the variation in adult bone mass [18]. Therefore, our findings are also supported by studies demonstrating a significant association between MF and bone health using DXA measurements. Regarding the MF of upper-limbs, such as handgrip strength, Torres-Costoso et al. [7] findings showed that schoolchildren with good performance in handgrip test had more bone mineral content and BMD in a number of body regions. However, there are controversial results in relation to lower-limbs MF, showing positive [15], negative [8] or no association [61]. Discrepancies between results might be due to several factors, such as genetics, diet, physical activity or sport participation, skeletal age and hormone levels. In addition, one consistent finding between studies was of the role of lean mass to explain bone-related variables [7]. These results suggest the need to promote physical activity with an emphasis on fitness improvement through skeletal loading, which should constitute a key element in the physical education curriculum.

There is a solid body of evidence for the association of nutritional profile and calcium intake with bone mass, especially in adults, but there is a controversy on whether the influence of diet on bone health is mediated by body composition. Also, it is not known whether diet and body composition are independent predictors of poor bone health or whether the risk for poor bone health involved

with obesity is modified by MF. In our study, we did not detect significant effect of Mediterranean diet adherence, sugar-sweetened soft drink or calcium intake on c-BUA. This is also confirmed by the results of the multivariate model. Previously, it has been shown in paediatric population that excessive intake of sugar-sweetened beverages may have several adverse effects on human health such as low bone mineral density [62], hypocalcaemia [63] and bone turnover variables [64]. Some of the nutrients contained in sugar-sweetened beverages, like fructose, caffeine and phosphoric acid, have already been proposed to affect the link of such beverages with poor bone health [24,26–28]. However, a clear mechanism was not apparent on the basis of these observational data.

Despite calcium being considered the most important nutrient for bone health throughout a person's lifespan [48], previous studies using QUS have shown controversial results, with positive [12,27] and no associations [7,8,65]. However, recent reports have shown low calcium intake and dairy product consumption globally, especially in children and adolescents [66,67]. We found that inadequate calcium intake is highly prevalent in Colombian children and adolescents. Failure to observe an effect of calcium on c-BUA in the Colombian population compared to another studies might be explained to the criteria selected to define optimal calcium intake (i.e., 1200 mg/day). For example, in young [68] and adolescent females [23,48], it was reported that those subjects whose calcium intake was greater than 1000 mg/day had higher bone measurements (c-BUA) using calcaneus QUS. In contrast, an Iranian cross-sectional study on calcium intake factors in 9 to 12 years old schoolchildren found that dietary calcium intake was not significantly correlated with serum calcium and other selected biochemical indicators of bone health [69]. In Colombia, according to Velásquez et al. [70], calcium is the most limiting nutrient in the youth diet. On the other hand, previous research conducted in Piedecuesta, Colombian reported the prevalence of hypocalcaemia in approximately 42% of six to twelve years old schoolchildren [71]. The need to encourage children and adolescents to eat more calcium-rich products in order to meet their calcium needs should be emphasized.

This study has some limitations. Firstly the cross-sectional design cannot make cause–effect inferences. Secondly it included participants from only a single region in Colombia; therefore, inferences for all Colombian children and adolescents should be made cautiously. The FUPRECOL study was deployed in collaboration with the Bogotá District Education Department, which only has jurisdiction among public schools. Thirdly we have not considered the potential impact of recognized determinants such as socioeconomic status, metabolic biomarkers, physical activity patterns and ethnic factors that modulate growth and levels of adiposity. However, body fat has been considered as a determinant of bone mass and previous studies shown consistent evidence regarding the association of BMC with fat mass [3,19,72]. Furthermore, we have not adjusted our analyses for a wide range of confounders, including vitamin D intake and/or serum 25-hydroxy vitamin D concentration, social class and cardiorespiratory fitness [7,16,18,19,22,53]. A limitation of BMI as a marker of adiposity relates to the variability in fat mass and fat free mass that result in the same BMI in non-overweight children and adolescents. Because it is a weight-for-height measure, BMI does not distinguish between fat mass and lean body mass. Thus individuals with increased lean mass may also have increased BMI. Another limitation of BMI is that the characterization of adiposity may differ across ethnicity and gender and the cut-off point selection in percentiles at the overweight categories [73,74]. The relationship between BMI and c-BUA was reported for many youth populations [4,6,8,14]. Nevertheless, the role of obesity as a risk factor for unhealthy bone mass remains unclear. Finally, QUS data provide only quantitative information and do not allow assessing qualitative factors contributing to bone fragility. Also we have not analysed from QUS measurement the speed of sound and stiffness index. Despite these limitations, the study also has various strong points that should be highlighted. The results presented, for the first time, the relationship between bone health and body composition (fat mass and lean body), MF, and nutritional profile on a large sample of children and adolescents from Colombia.

5. Conclusions

Body composition and MF influence bone health on a large sample of children and adolescents from Colombia. Although poor bone health is a serious consequence of the future risk of osteoporosis, the deterioration of bone health requires increased attention among schoolchildren in Bogotá. Thus, promoting strength adaptation and preservation in Colombian youth will help to maximize bone health, an important protective factor against osteoporosis later in life.

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Abbreviations

The following abbreviations are used in this manuscript:

BIA	bioelectrical impedance analysis
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
c-BUA	calcaneus quantitative ultrasound parameter
DXA	dual-energy X-ray absorptiometry
MF	muscular fitness
SLJ	standing long-jump
QUS	quantitative ultrasonography
WHtR	waist-to-height ratio

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Lactational Stage of Pasteurized Human Donor Milk Contributes to Nutrient Limitations for Infants

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Abstract: Background. Mother's own milk is the first choice for feeding preterm infants, but when not available, pasteurized human donor milk (PDM) is often used. Infants fed PDM have difficulties maintaining appropriate growth velocities. To assess the most basic elements of nutrition, we tested the hypotheses that fatty acid and amino acid composition of PDM is highly variable and standard pooling practices attenuate variability; however, total nutrients may be limiting without supplementation due to late lactational stage of the milk. Methods. A prospective cross-sectional sampling of milk was obtained from five donor milk banks located in Ohio, Michigan, Colorado, Texas-Ft Worth, and California. Milk samples were collected after Institutional Review Board (#07-0035) approval and informed consent. Fatty acid and amino acid contents were measured in milk from individual donors and donor pools (pooled per Human Milk Banking Association of North America guidelines). Statistical comparisons were performed using Kruskal–Wallis, Spearman's, or Multivariate Regression analyses with center as the fixed factor and lactational stage as co-variate. Results. Ten of the fourteen fatty acids and seventeen of the nineteen amino acids analyzed differed across Banks in the individual milk samples. Pooling minimized these differences in amino acid and fatty acid contents. Concentrations of lysine and docosahexaenoic acid (DHA) were not different across Banks, but concentrations were low compared to recommended levels. Conclusions. Individual donor milk fatty acid and amino acid contents are highly variable. Standardized pooling practice reduces this variability. Lysine and DHA concentrations were consistently low across geographic regions in North America due to lactational stage of the milk, and thus not adequately addressed by pooling. Targeted supplementation is needed to optimize PDM, especially for the preterm or volume restricted infant.

Keywords: preterm infants; human milk; nutrition; donor milk; DHA; lysine

1. Introduction

The American Academy of Pediatrics (AAP) [1] and the World Health Organization (WHO) [2] recommend mother's own human milk feeding because of immunological benefits which lessen disease in the high-risk neonate [3,4]. Epidemiological investigations have determined that feeding mother's own milk decreases the likelihood of necrotizing enterocolitis and late-onset sepsis in preterm infants [4–6], resulting in shorter hospital stays and decreased cost of care [7,8]. When mother's

own milk is not available, pasteurized donor milk (PDM) is used as a reasonable alternative for the preterm infant [9–13]. To achieve this practice, mother's own milk is often augmented with pasteurized donor milk [14]. In fact, recent retrospective work demonstrated cost effectiveness in the intensive care unit that optimized human milk feeding [15]. However, a major gap in knowledge is the nutritional consequences of feeding infants with low volumes of PDM that is often provided by mothers feeding older term infants who consume much greater quantities of milk and receive food sources at 4–6 months [16]. Furthermore, whether from the mother or donor, specific human milk components can be influenced by maternal diet [17]. The reality is that many preterm infants fed even fortified human milk may demonstrate growth, meeting specified weight goals, but 43% are still documented as small for gestational age and are in the lower growth percentiles at discharge—unlike their term counterparts [18–20]. Most concerning is that growth failure—particularly linear velocity—is associated with neurodevelopmental morbidities [21–23]. In addition, a recent multi-site trial randomly assigning infants to donor milk or preterm formula did not find developmental advantages [24]. Consequently, understanding the nutrient characteristics of pooled human milk is essential to improving fortification strategies.

The composition of human milk changes throughout the course of lactation as the infant matures, and these changes are consistent with infants consuming increasing quantities of milk and ingesting other food sources [25,26]. Consequently, the nutrient composition of most PDM alone is not considered adequate to meet the needs of the preterm or volume-restricted infant due to the late lactational stage of the donor milk, which is nutritionally less concentrated than early milk [27,28], and there is consensus that PDM requires fortification [29–32]. Furthermore, a recent meta-analysis of term and preterm human milk composition confirmed that human milk is profoundly variable [33]. There are a multitude of factors that may contribute to this variability, including stage of lactation, diurnal cycles, exposure to environmental contaminants such as cigarette smoke, and the treatment of expressed milk (i.e., storage, containers, or tubing delivery) [33–35]. While many of these variables are normalized in mother's own milk fed consistently around the clock and for months, milk obtained for donation is subject to the circumstances at that time. Expert opinion suggests more research is needed in the area of donor milk and fortification [11]. As a starting point to address the most basic elements in donor milk composition, we chose to measure fatty acid and amino acid contents. The purpose of this study was to determine the variability in fatty acid and amino acid composition in individual and pooled PDM using current pooling practices in place at milk banks compliant with Human Milk Banking Association of North America (HMBANA) guidelines, specifically for protein (0.7–1.0 g/100 mL) and caloric contents (67–81 kcal/100 mL) [36].

2. Materials and Methods

2.1. Study Design/Participants

This is a prospective cross-sectional sampling of human milk from five HMBANA member milk banks: California, Colorado, Texas-Ft Worth, Michigan, and Ohio. Prospective enrollees were mothers donating to milk banks between December 2008 and June 2010. Participants were enrolled after Institutional Review Board approval and informed consent of the human milk donors. Milk samples from 15 to 16 individual mothers per Bank were collected from December 2008 through November of 2010. Collected samples were pasteurized, and 1 mL was removed and immediately stored at −80 °C for laboratory measurements. The remaining milk was pooled into five pools per bank to meet the required caloric content and protein concentrations following HMBANA guidelines [37]. The samples were shipped frozen to Nationwide Children's Hospital, Columbus, OH, USA and stored at −80 °C until analyzed.

2.2. Nutrient Analyses

Fatty acid concentrations were determined by gas chromatography and included capric (C10:0), lauric (C12:0), myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1ω7), steric (C18:0), oleic (C18:1ω9), vaccenic (C18:1ω7), linoleic (18:2ω6), α-linolenic (18:3ω3), γ-linolenic (C18:3ω3), arachidonic (20:4ω6), eicosapentaenoic (20:5ω3), and docosahexaenoic (22:6ω6) acids, as described previously [28]. Amino acid concentrations were measured by high pressure liquid chromatography and included phosphoserine, taurine, phosphoethanolamine, aspartic acid, threonine, serine, glutamic acid, alpha-aminoadipic, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, lysine, histidine, and arginine, as described previously [28]. Each individual donor milk sample and the pooled samples from each milk bank were analyzed independently.

2.3. Statistical Analysis

Statistical comparisons were performed using Kruskal–Wallis for the demographic data (Table 1). Fatty acid and amino acid contents were compared across Centers with multivariate analysis of variance using a linear regression model. Results of the multivariate tests are reported in the figure legends, and coefficient of variation (adjusted R^2) and the between-subject differences (p-values) are reported within the graph. All milk composition data were analyzed using lactational stage as a co-variate. Correlations between lactational stage and amino acids or fatty acids were performed by Spearman's correlation. Statistical significance was set at $p < 0.05$ for all analyses.

Table 1. Age and lactational stage of donors by milk bank.

Region	*n*	Age				Lactational Stage (months) *			
		Median	Range	Mean	Std. Dev.	Median	Range	Mean	Std. Dev.
California	16	33.5	24–41	32.9	5.3	4.0	1–13	4.8	3.4
Colorado	16	31.5	25–38	31.7	3.6	1.0 #	1–8	2.2	1.9
Michigan	15	32.0	26–41	33.5	4.9	3.0	1–12	4.4	3.8
Ohio	15	30.0	20–42	31.7	5.9	2.0 #	0–11	2.7	2.8
Texas-Ft Worth	16	32.0	25–37	31.2	4.4	5.5	2–11	6.1	2.7

* Data analyzed by Kruskal–Wallis, $p = 0.002$, with Dunn's Multiple Comparison test; # different than Texas. Std. Dev. = standard deviation.

3. Results

3.1. Participants

Women currently donating to HMBANA member banks were included in this study. Prior to donating, the participants received rigorous medical, dietary, and laboratory screening. No women refused participation. There were 16 women enrolled from California and Texas, and 15 women enrolled from each site in Colorado, Michigan, and Ohio. Participant median ages across Centers were 31.5–33.5 years (range 20–42 years) and median lactational stages were 1–5.5 months (range 0–13 months) (Table 1).

3.2. Nutrient Analyses

After caloric content and protein measurements were performed in the individual samples, the milk samples at each individual site were pooled to achieve an overall caloric content of 67–81 kcal per 100 mL and an overall total protein content of 0.7–1 g per 100 mL. Protein-to-calorie ratio with these combinations reaches 3.3 with standard fortification; however, baseline assumptions may change with mother's lactational stage.

Fatty acid analysis of the individual samples revealed substantial differences across Banks in all fatty acids ranging from C16:0 to C20:5ω3 (Figure 1 and Table S1). Fatty acid analysis of the pooled

samples indicated no differences between Centers (Table 2); however, the average docosahexaenoic acid (DHA) concentration in all samples was 7.1 mg/100 mL, which is substantially lower than the 65 mg/100 mL (0.8 mol %) required to match fetal accretion concentrations (based on intakes of 150 mL/kg/day) [38].

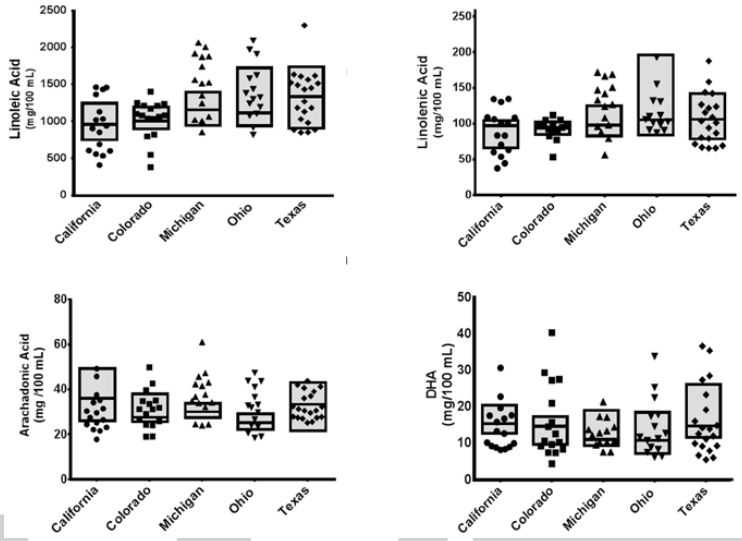

Figure 1. Fatty acid levels in pasteurized donor milk samples. The individual variability of linoleic acid, linolenic acid, arachidonic acid, and docosahexaenoic acid (DHA) were calculated as mg/kg/day, assuming 150 mL per day and a 1 kg infant. Individuals within each Bank are represented by the small symbols. The boxes represent the median and range for the pooled samples. Data are representative of 15–16 amino acids; analyses in individual milk samples revealed that all but two of the measured amino acids were different across Banks—these were taurine and tryptophan (Figure 2 and Table S2). Once samples were pooled, no statistical differences in amino acid contents across Banks were indicated (Table 3).

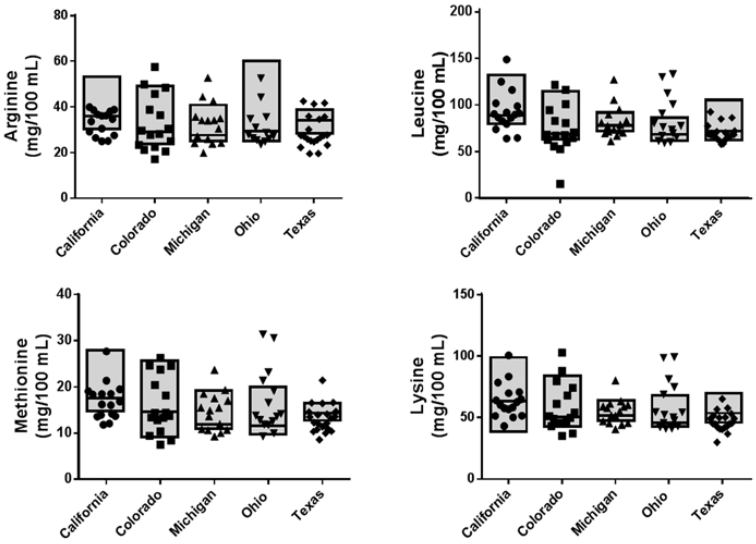

Figure 2. Amino acid levels in pasteurized donor milk samples. The individual variability of arginine, leucine, methionine, and lysine were calculated as mg/kg/day, assuming 150 mL per day and a 1 kg infant. Individuals within each Bank are represented by the small symbols. The boxes represent the median and range for the pooled samples. Data are representative of 15–16 individual samples and five pooled samples per Bank.

Table 2. Fatty acid levels in pooled samples obtained from Regional Milk Banks.

mg/100 mL	California		Colorado		Michigan		Ohio		Texas		r^2 (Adjusted)	p
	mean ± SD	med.	mean ± SD	med.	mean ± SD	med.	mean ± SD	med.	mean ± SD	med.		
C10:0	19.0 ± 8.5	17.6	26.1 ± 10.8	23.7	22.2 ± 7.3	21.1	15.3 ± 5.0	12.2	28.9 ± 13.6	31.6	0.055	0.314
C12:0	118 ± 31.0	117	130 ± 60.0	108	134 ± 28.5	125	123 ± 35.1	116	151 ± 45.8	134	0.151	0.864
C14:0	144 ± 32.5	142	156 ± 63.8	135	182 ± 33.6	184	162 ± 43.6	147	172 ± 37.8	153	0.140	0..835
C16:0	553 ± 86.8	580	614 ± 159	577	666 ± 1125	651	570 ± 79.6	569	677 ± 179	601	0.005	0.456
C16:1ω7	68.4 ± 14.4	71.3	68.9 ± 20.3	59.3	76.1 ± 13.8	75.1	54.1 ± 8.21	58.3	74.0 ± 26.0	68.1	0.047	0.331
C18:0	184 ± 34.1	191	193 ± 36.8	178	214 ± 55.4	195	193 ± 32.0	197	239 ± 67.7	202	0.051	0.322
C18:1ω9	935 ± 189	983	923 ± 157	880	1019 ± 173	1004	1008 ± 216	975	1134 ± 275	1033	0.019	0.396
C18:1ω7	69.5 ± 15.8	63.3	81.9 ± 18.0	72.8	85.6 ± 20.9	75.4	74.1 ± 16.9	79.2	89.7 ± 35.7	81.3	0.050	0.581
C18:2ω6	494 ± 90	483	510 ± 57	506	593 ± 82	584	661 ± 171	626	603 ± 156	562	0.127	0.184
C18:3ω6	6.64 ± 0.68	6.49	6.63 ± 0.62	6.58	7.16 ± 0.82	7.13	4.42 ± 1.04	4.55	6.49 ± 2.45	5.73	0.241	0.065
C18:3ω3	44.2 ± 7.47	46.8	44.9 ± 3.26	45.3	48.5 ± 7.46	47.3	52.1 ± 12.8	51.7	57.8 ± 21.4	51.1	0.044	0.338
C20:4ω6	18.1 ± 4.97	18.1	14.9 ± 2.50	13.9	15.4 ± 1.41	15.1	16.3 ± 4.23	15.8	12.7 ± 1.56	12.7	0.074	0.274
C20:5ω3	3.74 ± 0.47	3.55	3.80 ± 0.53	3.53	3.15 ± 0.82	2.94	2.20 ± 0.44	2.35	3.56 ± 2.51	2.36	0.016	0.403
C22:6ω3	7.81 ± 1.62	7.82	7.29 ± 1.58	7.46	6.17 ± 2.00	5.62	8.20 ± 3.02	6.89	6.09 ± 2.63	5.48	0.066	0.627

Data were analyzed by multivariate regression with Bank as the fixed variable and lactational stage as a co-variate. Multivariate tests (Pillai's Trace) revealed statistical differences between Banks (p = 0.000) across the model. Between-subject effects are indicated on the table. Data are representative of five pooled samples perBank; med = median. Data in gray indicates statistical significance at 0.05.

Table 3. Amino acid levels in pooled samples obtained from Regional Milk Banks.

mg/100 mL	California		Colorado		Michigan		Ohio		Texas		r^2 (Adjusted)	p
	mean ± SD	med.	mean ± SD	med.	mean ± SD	med.	mean ± SD	med.	mean ± SD	med.		
Phosphoserine	97.8 ± 41.4	80.4	85.5 ± 28.0	84.4	80.2 ± 13.4	73.3	82.2 ± 23.1	81.1	83.1 ± 17.2	74.9	0.050	0.333
Taurine	8.44 ± 3.63	8.17	7.00 ± 4.72	8.06	4.89 ± 3.42	2.59	5.5 ± 2.6	9.00	8.3 ± 4.9	6.57	0.051	0.332
Aspartic Acid	102 ± 24.4	94.4	88.2 ± 26.1	82.6	83.6 ± 13.0	79.9	83.4 ± 8.8	74.6	87.5 ± 22.8	85.9	0.097	0.240
Threonine	44.1 ± 13.0	39.8	33.4 ± 10.0	29.3	35.9 ± 3.25	34.8	35.0 ± 6.58	34.0	34.4 ± 7.96	32.5	0.130	0.185
Serine	56.9 ± 14.4	52.7	46.2 ± 14.1	44.4	44.8 ± 8.21	41.1	44.8 ± 5.02	38.4	47.3 ± 14.4	43.5	0.105	0.227
Glutamic Acid	206 ± 43.4	185	173.8 ± 37.0	162	171 ± 14.2	164	179 ± 23.46	151	164 ± 23.7	175	0.152	0.156
Proline	101 ± 25.8	83.5	78.9 ± 25.2	66.2	81.4 ± 5.71	79.7	79.3 ± 20.1	67.1	66.6 ± 6.81	70.3	0.205	0.096
Glycine	25.2 ± 5.9	23.0	22.3 ± 5.85	20.5	21.3 ± 2.26	21.0	20.5 ± 3.5	20.0	23.4 ± 7.17	21.0	0.027	0.385
Alanine	47.1 ± 11.4	44.7	39.5 ± 13.1	37.9	38.1 ± 7.51	35.0	37.4 ± 3.87	31.6	40.3 ± 13.5	36.4	0.066	0.299
Valine	41.1 ± 12.6	36.9	30.9 ± 9.45	30.7	32.2 ± 4.32	33.0	27.2 ± 9.96	23.4	25.9 ± 7.56	28.1	0.140	0.172
Methionine	19.7 ± 5.8	17.6	15.1 ± 6.48	14.6	14.2 ± 3.86	11.9	14.4 ± 1.38	11.5	13.8 ± 4.59	14.3	0.212	0.090
Isoleucine	37.4 ± 12.7	32.9	27.0 ± 8.69	25.1	28.8 ± 3.08	27.7	28.4 ± 6.71	23.6	23.8 ± 3.35	25.5	0.199	0.102
Leucine	99.1 ± 24.0	88.1	76.8 ± 21.2	68.6	79.4 ± 7.85	77.5	76.5 ± 16.8	67.8	72.6 ± 10.8	71.2	0.221	0.082
Tyrosine	55.6 ± 20.9	52.1	44.1 ± 19.5	44.6	40.4 ± 11.6	33.1	44.6 ± 6.6	32.2	42.1 ± 17.0	45.1	0.006	0.469
Phenylalanine	38.1 ± 11.4	34.4	29.7 ± 8.90	26.5	30.0 ± 3.65	29.3	29.8 ± 5.10	25.1	30.4 ± 9.03	29.8	0.098	0.238
Tryptophan	188.7 ± 39.6	176	172 ± 28.6	169	173 ± 9.39	173	173 ± 29.8	161	157 ± 16.4	158	0.064	0.624
Lysine	66.3 ± 23.2	62.9	54.8 ± 16.7	50.1	54.0 ± 7.41	51.1	54.5 ± 8.8	45.3	52.1 ± 11.4	53.2	0.022	0.399
Histidine	22.4 ± 6.3	18.7	18.1 ± 4.78	16.4	53.2 ± 78.2	18.6	19.0 ± 4.14	16.1	17.5 ± 3.31	17.5	0.042	0.564
Arginine	38.7 ± 9.8	36.0	31.7 ± 10.3	29.2	31.3 ± 7.6	27.6	33.8 ± 3.85	29.3	37.3 ± 15.5	34.1	0.038	0.360

Data were analyzed by multivariate regression with Banks as the fixed variable and lactational stage as a co-variate. Multivariate tests (Pillai's Trace) revealed no statistical differences between Banks or lactational stage across the model. Between-subject effects are indicated on the table. Data are representative of five pooled samples perBank; med = median. Data in gray indicates statistical significance at 0.05.

The composition of human milk is highly dependent upon lactational stage, which was highly significant in the multivariate regression analyses. Consequently, we analyzed both amino acids and fatty acids for correlation with lactational stage in individual and in pooled samples. In the individual samples, all amino acids were negatively correlated with lactational stage ($p \leq 0.006$), but these correlations were no longer significant after the samples were pooled ($p \geq 0.131$). Only the fatty acids eicosapentaenoic acid (EPA, 20:5ω3) ($p = 0.029$) and docosahexaenoic acid (DHA, 22:6ω3) ($p = 0.025$) were significantly correlated with lactational stage in the individual samples, and these correlations were no longer significant after the samples were pooled ($p = 0.819$ and $p = 0.690$, respectively).

4. Discussion

Mother's own human milk is recommended by the AAP as a unique biologic source of nutrition for both term and preterm infants [1]. When mother's milk is not available, PDM is a reasonable alternative for the preterm infant; however, PDM does not meet the nutritional needs of infants that are preterm or ingesting low volumes of milk if fed as the sole source of nutrition, and thus requires supplementation [9–11,39]. While women donating to human milk banks undergo extensive medical screening, there are many variables that are not controlled. The milk content of fatty acids of an individual woman may vary substantially depending upon her recent diet and/or other demographic factors. Human milk collection, storage, and fortification and biologic components are often described [40], but in light of the recent clinical practice of using donor milk, and publications on variability in human milk, the effectiveness of pooling to adequately address the nutritional differences in individual donor milk needs to be evaluated [33].

Our data indicate that donor milk protein-to-calorie ratio is within the range for nitrogen retention of 2.6–3.6 [41]; however, these studies were done with preterm formula, and other factors such as individual variation, heat treatment, absorption, or micronutrition may be limiting. We did find substantial differences between individuals and Banks in the nutrient contents of milk samples; these differences were largely overcome by pooling. These data demonstrate that infants fed DM that has not been pooled may experience dramatic differences from one day to the next in the composition of the milk they are fed—especially if the milk is from different individual donors. While the effects of nutrient variability are largely normalized in pooled PDM, there remain some deficiencies. This is especially important in the preterm population, because poor somatic growth correlates with low mental developmental scores and development of cerebral palsy [42].

Milk collected at milk banks in the United States is variable and is not a "single point in time", but likely to have been collected longitudinally over several months. Each milk bank is pasteurizing different volumes of milk with varying mothers in a pool. For example, the OhioHealth Mothers' Milk Bank typically pasteurizes 1500–2000 ounces per day from 4 to 10 different mothers, each having varying amounts of milk. The number of moms in a pool is dependent upon the available volume and calorie content of each. Current pooling practices utilizing HMBANA guidelines normalize the composition of PDM such that minimal differences in fatty acids or amino acids are observed among pooled samples and no correlations with lactational stage remain [36]. This observation may be explained by the fact that the sample size for pooled samples was one third that of the individual samples, and consequently there was less power to detect differences. Another explanation could be that the statistical differences between Banks Centers in the individual samples are largely driven by the outliers (up to five-fold different) and not actual differences from site to site. Despite the normalized contents in pooled samples, the absolute values vary two-to-three-fold and may still constitute a stressor on the infant. These findings would support customized supplementation for infants fed PDM to optimize nutrients.

DHA is a long chain poly-unsaturated omega-3 fatty acid that is a fundamental lipid in the human cortex and gray matter, and is correlated to developmental scores in preterm infants [43,44]. Furthermore, low concentrations of whole blood DHA have been correlated to late-onset sepsis and chronic lung disease, indicating the importance of DHA in overall infant health [45]. Throughout the

last trimester of pregnancy, DHA is accreted at a rate of 50–75 mg/kg/day [46]. Infants born before the last trimester miss this accretion period and current DHA concentrations typically fed in the neonatal intensive care unit (NICU) fall short of the fetal accretion levels. Depending on diet, human milk concentrations of DHA can range from 0.2 to 2 mol % [28,47]. In the current study, measured levels of DHA in the regional PDM samples ranged between 2.22 and 20.20 mg/100 mL (equivalent to 0.08 and 0.67 mol %). This demonstrates that DHA levels in PDM are insufficient to consistently provide intrauterine accretion levels for preterm infants. In a previous study, supplementation of donors with 1 g of DHA per day resulted in human milk DHA concentrations that reached intrauterine accretion levels for the infant (0.8 mol %) [48]. These data suggest that mothers donating milk—especially milk to be used for preterm infants—should be advised to increase their daily intake of DHA, or direct supplementation of the PDM milk may be required.

Typical clinical practice is to increase total protein intake to improve growth without consideration of the concentrations of specific amino acids. Most requirements for amino acids are established for parenteral nutrition, and few address the needs for the enterally-fed infant [49]. Lysine is an essential amino acid used in protein synthesis and along with methionine is a precursor for carnitine synthesis, which is essential for fatty acid metabolism. After total protein, lysine is the amino acid most rate-limiting for growth and is essential to the biological value of food sources [49,50]. Huang et al. has demonstrated that infants require 130 mg/kg/day of lysine for optimal growth [50]. We observed substantial variability across Banks among amino acid contents in individual milk samples, but this variability was no longer evident in pooled milk samples. Several of the individual amino acids were lower than that recommended for parental nutrition (even in the pooled samples), but the relevance for enteral feeding is unknown (tyrosine, 74 mg/k/day; methionine, 49 mg/k/day; threonine, 33 mg/kg/day) [49]. Most importantly, lysine concentrations ranged from 52.1 to 66.3 mg/150 mL (the amount consumed by a healthy preterm per day), which is lower than the recommended 130 mg/kg/day assuming that most preterm infants weigh between 0.5 and 1 kg. Since many essential amino acids have defined functions, supplementing PDM with a specific quality of protein to ensure these amino acids are available may be as important as increasing total protein content.

One limitation of our study is that donated milk was from a wide range of lactational stages and included newborn milk to 12 months post-birth. Our study captured a median of 1 to 5.5 months, which can vary widely in protein and lipid content. A targeted donor process could perhaps improve the amino acid and fatty acid profile. The strength of our study is that it included a cross-sectional prospective examination of many regions in the United States to mimic current clinical practice to evaluate baseline nutrition in the donor milk.

5. Conclusions

The occurrence of failure to thrive currently witnessed in many infants fed PDM is concerning [18], and requires a further examination as to the adequacy of PDM as a sole source of nutrition. Our data indicate that individual PDM is highly variable, and that pooling lessens this variability; however, infants fed pooled PDM may still experience nutritional differences that may affect their overall growth. Our data supports the observed insufficiency of DHA and now lysine contents in most human milk across the United States and the need for supplementation—specifically in the case of the preterm infant. These findings support further studies on personalized maternal and infant supplementation strategies to meet the needs of high-risk infants receiving PDM [51].

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Conflicts of Interest: The authors have no conflict of interest. Dr. Valentine is currently an Associate Professor at The University of Cincinnati, Cincinnati Ohio and Medical Director of Mead Johnson Nutrition, Evansville, IN, USA.

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A Village-Based Intervention: Promoting Folic Acid Use among Rural Chinese Women

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Abstract: Background: Folic acid supplementation is effective in reducing the risk of neural tube defects (NTDs). However, the use of folic acid is low among rural women in China. Nutrition education can provide information about folic acid and encourage its use. The primary objective of this study was to test the effectiveness of a village-based nutrition intervention on folic acid use among rural women. Methods: Sixty villages were randomly selected using multiple-stage sampling and were divided into control and intervention groups. The intervention included nutritional education at village clinics, written materials, and text messages (SMS). Folic acid use knowledge and behavior was assessed at baseline and after the intervention. Results: Self-reported compliance with folic acid supplement use increased from 17.0%–29.2% at baseline to 41.7%–59.2% one year post-intervention. During the same period, the folic acid knowledge score in the intervention group increased from 3.07 to 3.65, significantly higher than the control group (3.11 to 3.35). Multivariate binary logistic regression showed that the women who received folic acid education and SMS intervention were more likely to comply with folic acid supplement recommendations. Conclusions: The results indicated that an integrated village-based folic acid education intervention may be an effective way of promoting folic acid use for the prevention of NTDs in rural women.

Keywords: folic acid; neural tube defects; rural; women at birth age; village-based intervention; China

1. Introduction

Neural tube defects (NTDs) are the most common birth defects that contribute to infant mortality and serious disability [1]. NTDs occur in approximately the third or fourth week of pregnancy, before many women even know that they are pregnant. NTDs are highly prevalent in China. National birth surveillance showed that the NTD incidence is still high in rural areas of China, occurring in 12.9 of 10,000 births in 2007. The distribution of NTDs varies by region, with a higher prevalence in the north compared with the south and in rural compared to urban populations [2]. NTDs are preventable if women consume a daily supplement of 400 μg folic acid (FA) before and during conception. Therefore, many governments (including China's) recommend that all women take a daily folic acid supplement for 6 months, starting from 3 months before conception and continuing throughout the first trimester. Some research has indicated that blood folate deficiency is high among Chinese adults [3,4]. However, there are disparities in the consumption of folic acid in China, with 24.69%–41.0% of rural women reporting daily folic acid consumption compared with 57.1%–86.58% of urban women [5–8].

Since 1993, the Chinese government has developed strategies targeting women of child-bearing age to promote the use of folic acid for the prevention of NTDs. In some western regions with a high risk of NTDs, folic acid fortification of flour or rice has been reported as a potential health strategy to tackle folate deficiency [9,10]. However, small privately-owned flour milling factories account for a large proportion in China. Flour fortified with folic acid is not easy to implement broadly, because the specialized techniques require high quality control. Moreover, a shortage of related regulations and standards hinders the development of the folic acid-fortified food in China. For the time being, China still adopts folic acid supplements to prevent neural-tube defects. One strategy that provided free folic acid supplements in women's hospitals was interrupted with the abolition of mandatory premarital check-ups in 2003. In the meantime, the incidence of birth defects including NTDs in China has increased [11]. In June 2009, a health policy called "Supplementing Folic Acid to Prevent NTD" (SFAPN) was announced by the Chinese government. Under this policy, rural women of childbearing age can receive a daily supplement of 400 μg of folic acid supplied free at village health posts. The Chinese health authority aims for 60% of the targeted women to know about folic acid and for 60% of them to be taking folic acid by the end of 2009.

Although free folic acid supplements have been provided for rural women, lack of compliance has been a barrier to the program's success. The level of folic acid awareness and adequate intake remain relatively low in rural regions of China—below 30% in some villages [12]. Improving folic acid knowledge is an essential step in promoting the use of folic acid to prevent NTDs [13,14]. The major barrier for the effective delivery of folic acid knowledge is limited knowledge among village health workers. A study examining folic acid awareness among primary health workers showed that only 39.0%–48.1% of the respondents knew the benefits of folic acid supplementation [15]. No previous research has been conducted on how folic acid knowledge and folic acid supplementation can be increased in villages.

The aim of this article is to evaluate the results of a village-based folic acid education intervention to increase the knowledge and use of folic acid to prevent NTDs among rural women.

2. Methods

2.1. Study Design

This study was conducted in Hunan province in central south China. A recent study showed that alongside the decline of birth defects in northern regions with a high risk of NTDs, the incidence of birth defects gradually increased in some southern regions of China [16]. In Hunan province, the incidence of birth defects increased from 10.709/1000 in 1996–2004 to 13.836/1000 in 2005–2012 [16], and was even higher in rural regions (26/1000 in 2009, the SFAPN office of Hunan).

Multi-stage sampling based on NTD incidence was used to select townships that represented the same levels of SFAPN implementation in Changsha county in Hunan province. Ten townships were selected using simple random sampling. Then, six SFAPN villages were selected from each township using simple random sampling, and the sixty total selected villages were randomly assigned to the control group or the intervention group. The two groups were matched in terms of birth rate, NTD incidence, population size, economy level, and when SFAPN started in the village. For each SFAPN village, all the women of childbearing age who planned to have a baby were selected using systematic sampling.

Based on the pilot study results, we anticipated differences of 20% in compliance with folic acid use, ranging from 30% to 50%. The sample size for an average cluster size of 10 women was calculated for 80% power and 95% of the primary outcome, resulting in approximately 300 women in each intervention and control group. An intervention was implemented in all 30 intervention villages for one year. It consisted of folic acid education at village clinics and SMS messages regarding folic acid information for rural women. The unit of randomization was the village. Entry criteria for villages were as follows: (1) a minimum of 30 women planning pregnancy in the village; (2) the presence

of a village health post. Entry criteria for the women were (1) childbearing age and (2) planning a pregnancy.

2.2. Ethical Approval

This study was approved by the independent ethics committee of the Institute of Clinical Pharmacology, Central South University (project number CTXY-110013, December 2011). Written informed consent was obtained from all the subjects.

2.3. Intervention

All village doctors and family planning staff in the intervention villages received a two-day training program delivered by the Changsha county health department. The duration of the folic acid intervention for the women in the intervention group was one year, from September 2013 to September 2014. Figure 1 shows the pathway of the intervention: (1) Folic acid education: folic acid education and counseling sessions were provided monthly in village clinics by village doctors; (2) Monitoring of folic acid use: family planning staff in the intervention group informed every participant of the free folic acid supplements available at the village health posts and followed up their adherence to folic acid use monthly; (3) SMS intervention: the women who consented received short SMS text messages designed to address a range of common issues with folic acid supplements, including the benefits of folic acid, where and when to obtain free folic acid supplements, and the rules for folic acid supplementation (dose, frequency, and period). Five SMS text messages were delivered automatically twice per month via an open source integrated communication service called Fetion. It cost RMB 0.6 (USD 0.1) per month per woman to send these messages.

For the control group, the village doctors provided their usual services.

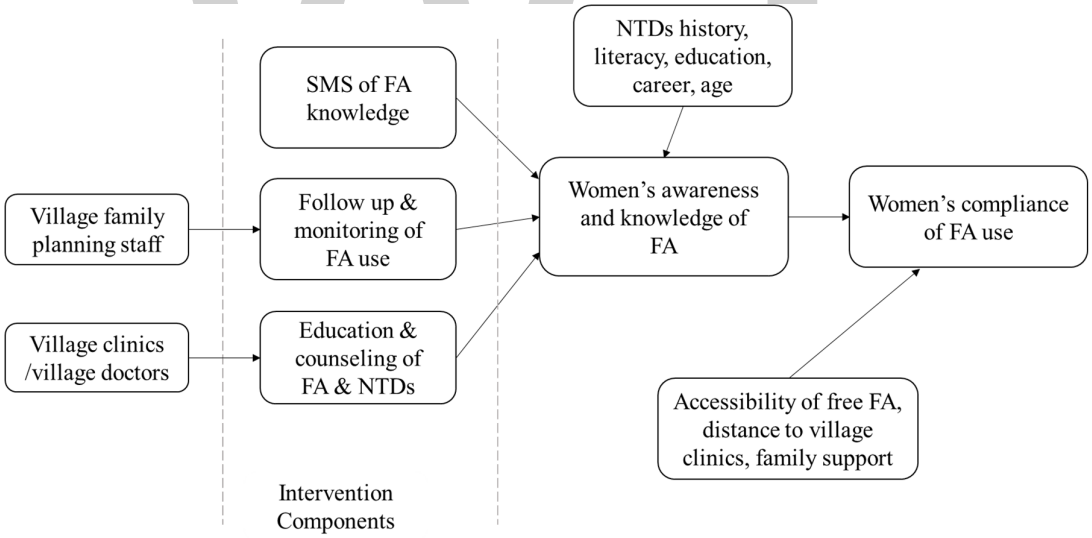

Figure 1. Pathway of the intervention and primary outcome. FA: folic acid; NTD: neural tube defect.

2.4. Outcome Measures

Face-to-face questionnaire interviews were used to investigate the knowledge and use of folic acid supplements in both the control and intervention groups before and after the intervention. Compliance with folic acid supplement uses during the three months before pregnancy and in the first trimester were investigated as the primary outcome. "Compliance with folic acid use" was defined as using folic acid for more than 3 weeks in one month. The secondary outcome was the folic acid knowledge score, which was based on five indicators assessing whether the participants knew (1) that folic acid prevents NTDs; (2) that folic acid from food sources was not sufficient; (3) the best timing to start using

folic acid; (4) the correct duration recommended by Chinese SFAPN for folic acid supplement use: 3 months before to 3 months after conception; and (5) the recommended daily dose of folic acid.

A score of 1 was assigned for correct answers and 0 for wrong answer or "don't know". The scores varied from 0–5 points and were classified into three levels: score 4 and above (cut-off 70%–100%); score 2–3 (cut-off 50%–70%); score 0–2 (less than 49%).

2.5. Statistical Analysis

Data cleaning and analysis were conducted using the SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA). The awareness of and compliance with folic acid use were analyzed using Pearson's chi-squared test and reported by the groups of women who were currently pregnant, gave birth in the last 12 months, or planned to have a baby. Logistic regression was used to examine the influence of various factors on whether the women continued to use folic acid.

3. Results

3.1. Sample Characteristics

A set of questionnaires was administered to the 759 participants of the target villages, and 757 completed questionnaires were returned for a response rate of 99.7%. Most of the women surveyed were of Han ethnicity (98.7%). The median age of the participants was 26 years, and the interquartile range was 24–30 years. Of the respondents, 43.7% were farmers, and 32.5% were employed. Approximately half of the women (45.6%) did not attain a high school diploma or post-high school degree. Approximately 19.4% of the participants reported a per capita annual net income below the Changsha county average level for 2012 (17,070 RMB). Among the 757 women planning pregnancies, 435 women (57.7%) had delivered their first child, 36.1% were planning their first pregnancy, and the others had had more than two previous live births. There were no significant differences in the women's demographic characteristics between the intervention and control group.

3.2. Knowledge Regarding Folic Acid at Baseline

The baseline investigation showed that 742 out of 757 women (98.0%) had heard about folic acid (Table 1). Nearly 80% were aware that folic acid supplements were recommended before and during early conception, but only 32.8% knew that folic acid specifically helps to decrease the risk of NTDs. No differences were found between the control group and intervention group. Among the 742 women who reported "have heard about folic acid", the main source of folic acid information was the village family planning staff (64.0%) or village doctors (52.0%).

Table 1. Awareness and knowledge of folic acid among the rural women at baseline.

Knowledge Questions	Control Group (n = 384)	Intervention Group (n = 373)	Total (n = 757)
Have heard about folic acid	377 (98.2%)	365 (97.9%)	742 (98.0%)
Know that "Using of folic acid can prevent NTDs"	122 (31.7%)	126 (33.8%)	248 (32.8%)
Know that "Food sources cannot provide enough folic acid"	168 (43.8%)	151 (40.5%)	319 (42.1%)
Know "Best timing to start using folic acid"	302 (78.6%)	300 (80.4%)	602 (79.5%)
Know "the recommended duration of folic acid use (3 months before to 3 months after conception "	303 (78.9%)	308 (82.6%)	611 (80.8%)
Know the recommended daily dose of folic acid	330 (85.9%)	316 (84.7%)	646 (85.4%)
Source of folic acid information *			
Village family planning staff	243 (64.6%)	232 (63.4%)	475 (64.0%)
Village doctors	195 (51.9%)	191 (52.2%)	386 (52.0%)
Family/Friends	58 (15.4%)	60 (16.4%)	118 (15.9%)
Other	47 (12.5%)	43 (11.7%)	90 (12.1%)

* women who reported "have heard about folic acid", n = 742 (376 in control group and 366 in intervention group).

3.3. Repeated Measures ANOVA of Knowledge Regarding Folic Acid after Intervention

At the final follow-up, 38 women in the control group and 70 women in the intervention group had dropped out. At baseline, there were no significant differences between the folic acid knowledge scores in the intervention and control groups ($t = 0.547$, $p = 0.585$). One year after intervention, knowledge scores increased in both intervention and control group. Mean knowledge scores increased by 18.9% (3.07 to 3.65) in the intervention group and by 7.7% (3.11 to 3.35) in the control group. As shown in Table 2, repeated measures ANOVA results indicated a significant interaction between intervention and time ($F = 12.232$, $p = 0.001$). Knowledge score increased faster in the intervention group than in the control group. This revealed that the intervention has an impact on increasing knowledge regarding folic acid over time.

Table 2. Repeated measures ANOVA for intervention and time for knowledge regarding folic acid.

Group	Before Intervention Mean ± SD	12-Months after Intervention Mean ± SD	Repeated Measures ANOVA		
			Between Subjects (Group)	Time Effect (Within Group Comparison)	Time × Intervention Effect (Between Group Comparison)
Control ($n = 347$)	3.11 ± 1.15	3.35 ± 0.99	$F = 3.293$	$F = 53.966$	$F = 12.232$
Intervention ($n = 302$)	3.07 ± 1.16	3.65 ± 1.00	$p = 0.070$	$p < 0.01$	$p = 0.001$

3.4. Use of Folic Acid after Intervention

Before the intervention, 239 out of 338 women (70.7%) in the intervention group and 260 out of 357 women (72.8%) in the control group reported having received folic acid supplements, with no difference between the two groups. Similar compliance with folic acid supplement use (less than 30%) was observed for both groups before intervention. Folic acid education, follow-up, and monitoring of folic acid use and SMS messages containing folic acid information were provided for the intervention group for one year. As presented in Table 3, the majority of the women in the intervention group (85.4%) reported having obtained folic acid supplements after intervention, significantly higher than the control group (68.6%). Compliance with folic acid use was also significantly higher in the intervention group (41.7%–59.2%) compared with the control group (17.0%–29.4%).

Table 3. Comparison of the intervention group with the control group regarding folic acid use among rural women after intervention.

Folic Acid Use	Control Group	Intervention Group	Chi-Square	*p*
	194 (68.6%)	239 (85.4%)	22.38	<0.01
Complied with folic acid supplement recommendations during the three months before pregnancy	57 (29.4%)	141 (59.2%)	38.39	<0.01
Complied with folic acid supplement recommendations during the first trimester	53 (27.7%)	133 (56.3%)	35.14	<0.01
Complied with folic acid supplement recommendations from 3 months before to 3 months after conception	32 (17.0%)	93 (41.7%)	29.36	<0.01

3.5. Effects of the Intervention on Use of Folic Acid

At the final follow-up, 164 women self-reported having complied with the folic acid supplement recommendations for 6 months—three months before to three months after conception. We applied multivariable binary logistic regression models to identify the effects of the intervention and other factors on compliance with folic acid supplement recommendations. A total of 10 variables with a

p-value ≤ 0.10 in the bivariate analysis were entered into the multivariable logistic regression analysis. As presented in Table 4, the women who participated in an intervention that integrated nutrition education, SMS information, and family planning staff visits were more likely to use folic acid supplements. We also found that women with family support and higher folic acid knowledge score were significantly more likely to use folic acid supplements.

Table 4. Factors associated with compliance with folic acid supplement use from 3 months before to 3 months after conception among rural women according to multivariate logistic regression.

Variable	Women Who Complied with Folic Acid Supplement Recommendations 3 Months before to 3 Months after Conception						
	B	S.E.	Wald	*df*	Sig	OR	95% CI
Constant	−3.885	0.337	132.915	1	<0.001	0.021	
Received FA education at village clinics	1.246	0.261	22.703	1	<0.001	3.475	2.082–5.802
Received SMS intervention	0.947	0.344	7.568	1	0.006	2.578	1.313–5.063
Family planning staff followed-up and monitored FA use	1.339	0.179	55.923	1	<0.001	3.813	2.685–5.416
Family support	0.812	0.266	9.319	1	0.002	2.252	1.337–3.792
FA score above 4	0.840	0.236	17.580	1	<0.001	2.692	1.694–4.277

S.E. = standard error.

4. Discussion

The present study found a wide deficiency in folic acid knowledge and the use of folic acid supplements. A large proportion of the sample was unaware of the need for folic acid supplementation. While most of the respondents indicated that they had heard of folic acid, only 32.8% knew that it could protect against NTDs. Similar results were found in other studies. In a survey of 2094 pregnant women in rural regions of western China, 56.0%–80.6% had heard about folic acid, but only 18.4%–38.9% indicated knowledge of the benefits of folic acid supplements [17]. In a study of 1907 rural women of childbearing age in Jiangsu province, approximately 99.6% of participants reported that they had heard of folic acid, but only 33.2%–43.8% knew that folic acid can prevent NTDs [18]. The use of folic acid supplements was also poor in this study; approximately 70% of the women had never taken folic acid supplements, and the compliance was less than 30%. Factors that contribute to this poor compliance may include inequalities in health resources, poor awareness and knowledge regarding folic acid, unplanned pregnancy, and other personal characteristics.

The best way to prevent NTDs is to ensure that rural women of childbearing age are taking appropriate amounts of folic acid daily. This requires them to be aware of when and where to obtain the folic acid supplements. Women in our study with higher folic acid knowledge scores were 2.7 times more likely to comply with folic acid recommendations, which was similar to findings of another study [19]. Strategies to increase the awareness of folic acid supplementation to prevent NTDs among rural women are important. In the SFAPN program, village doctors oversee nutrition education, providing folic acid supplements and following up on the use of folic acid. However, low education levels and insufficient professional training are barriers for delivering health information [20]. Some previous studies evaluated the effect of nutrition education on folic acid use among pregnant women, but only included pregnant women or doctors in town hospitals or county hospitals [21–23]. This study developed integrated interventions for both rural women and village health workers. Very few studies addressed folic acid training among Chinese village doctors. As we showed in another article, short-term training could effectively increase their knowledge regarding folic acid [24]. The training enhanced folic acid education in village clinics and promoted compliance with folic acid recommendations among rural women. SMS interventions have been shown to improve outcomes in rural health care settings [25]. Our study also indicated that SMS text messages encouraged rural women to use folic acid supplements (OR = 2.578, p = 0.006). In addition to providing reminders, the periodic SMS text messages sent information about folic acid to the rural women. Integrating SMS text messages into village-based health education may promote the delivery of folic acid information.

Village doctors are the gatekeepers of rural health care. However, gender imbalance is a barrier to women's health care in some rural regions of China. Influenced by custom, some rural women will not seek maternal and gynecological care from a male health worker. In our study, only 23.3% of village doctors were female, similar to the findings of another study [26]. We hypothesized that among family planning staff, a female with a high reputation in the village may play an important role in the intervention. The family planning staff's job includes regular monthly or bi-monthly follow-up visits to record the conditions of rural women's pregnancies and improve management. It is easy for family planning staff to follow up and monitor folic acid use in their day-to-day work. Our results showed that women who received visits from family planning staff were 3.8 times more likely to comply with folic acid recommendations. Therefore, family planning staff should be considered the main implementers of SFAPN and other female health care interventions.

Our study has several strengths. To our knowledge, it is one of few that has developed an integrated folic acid intervention for both health care providers and health care recipients. We prepared detailed intervention plans. We worked with local health departments and developed a set of folic acid education materials that is still being used to train primary health workers. The communication service for delivering SMS text messages was open source and low cost.

The study also had some limitations inherent in its design. We only chose one site to implement the intervention. It was not powered to identify the effect of the intervention on the compliance with folic acid recommendations among women with low literacy. In addition, because we did not require responses to the SMS messages or reports of having taken the folic acid supplements, it was difficult to determine the exact effect of the SMS intervention. Furthermore, a one-year follow-up was too short to observe the effect of the intervention on birth defects.

5. Conclusions

The main barriers for SFAPN implementation include a lack of professional training, a lack of village doctors, time conflicts and work responsibilities, and gender imbalance. Our integrated intervention increased folic knowledge and compliance with folic acid supplement use among rural women. Future studies should be conducted to evaluate which components of the interventions are most effective. Additionally, further studies need to assess the effectiveness of the intervention for reducing birth defects.

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Conflicts of Interest: The authors have no conflicts of interest.

Abbreviations

FA	Folic Acid
NTD	Neural Tube Defect
SFAPN	Supplementing Folic Acid to Prevent NTD

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Reference Values of 14 Serum Trace Elements for Pregnant Chinese Women: A Cross-Sectional Study in the China Nutrition and Health Survey 2010–2012

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Abstract: The development of reference values of trace elements is recognized as a fundamental prerequisite for the assessment of trace element nutritional status and health risks. In this study, a total of 1400 pregnant women aged 27.0 ± 4.5 years were randomly selected from the China Nutrition and Health Survey 2010–2012 (CNHS 2010–2012). The concentrations of 14 serum trace elements were determined by high-resolution inductively coupled plasma mass spectrometry. Reference values were calculated covering the central 95% reference intervals (P2.5–P97.5) after excluding outliers by Dixon's test. The overall reference values of serum trace elements were 131.5 (55.8-265.0 μg/dL for iron (Fe), 195.5 (107.0–362.4) μg/dL for copper (Cu), 74.0 (51.8–111.3) μg/dL for zinc (Zn), 22.3 (14.0–62.0) μg/dL for rubidium (Rb), 72.2 (39.9–111.6) μg/L for selenium (Se), 45.9 (23.8-104.3) μg/L for strontium (Sr), 1.8 (1.2–3.6) μg/L for molybdenum (Mo), 2.4 (1.2–8.4) μg/L for manganese (Mn), 1.9 (0.6–9.0) ng/L for lead (Pb), 1.1 (0.3-5.6) ng/L for arsenic (As), 835.6 (219.8–4287.7) ng/L for chromium (Cr), 337.9 (57.0–1130.0) ng/L for cobalt (Co), 193.2 (23.6–2323.1) ng/L for vanadium (V), and 133.7 (72.1–595.1) ng/L for cadmium (Cd). Furthermore, some significant differences in serum trace element reference values were observed between different groupings of age intervals, residences, anthropometric status, and duration of pregnancy. We found that serum Fe, Zn, and Se concentrations significantly decreased, whereas serum Cu, Sr, and Co concentrations elevated progressively compared with reference values of 14 serum trace elements in pregnant Chinese women. The reference values of serum trace elements established could play a key role in the following nutritional status and health risk assessment.

Keywords: reference values; trace elements; high-resolution inductively coupled mass spectrometry; China Nutrition and Health Survey 2010–2012

1. Introduction

Trace elements are inorganic constituents needed in minute quantities but considered nutrients essential for human health. Both a deficiency and an excessive accumulation can result in multiple physiological dysfunctions [1]. It has been shown that the status of trace elements can reflect total exposure of all possible sources. The determination of specimens in humans is valuable for characterizing the body burden of metals, toxics, and nutrients [2]. Moreover, compared to environmental analyses, it is a more direct way of estimating exposure, as it can distinctly depict where stronger environmental actions or medical decisions are required. Hence, the development of trace element reference values for specific populations has long been considered a prerequisite for assessing the exposure and absorption of trace elements, despite its complexity [3].

Pregnancy is a period of increased metabolic demands, mainly due to changes in the woman's physiology and the requirements of the growing fetus [4]. Oxygen consumption, central hemodynamic alterations, and oxidative stress are altered, contributing to a determination of the long-term health in pregnant women. Some trace element concentrations are also altered during this vulnerable period. Therein, it is worth noting that the deficiency or overexposure of certain trace elements could be detrimental to the health of both pregnant women and their fetuses [5]. Pregnant women are often exposed to inadequate macro- and micronutrition in developing countries or regions. Thus, the establishment of trace element reference values for pregnant women is essential for the assessment of trace element nutrition and potential health risks.

In general, the levels of trace element uptake are more easily affected by dietary habits, lifestyles, and environmental conditions. There is clearly a difference in trace elements in different countries and regions around the world. Germany [6], the United States [7], Canada [8], and other countries [9,10] have derived reference values of trace elements using their own data based on large-scale population-based studies. Regrettably, China has not paid much attention to trace element status. As a result, the extent of deficiency or overexposure can hardly be evaluated because no studies have been performed on a national level. Therefore, currently, a deeper health-risk evaluation of the health status of pregnant women in China in terms of trace elements cannot be performed. Fortunately, the China Nutrition and Health Survey 2010–2012 (CNHS 2010–2012) can provide trace elements profiles in all population groups in China, including pregnant women.

The purpose of this study was to obtain the concentrations of 14 serum trace elements from pregnant Chinese women and to determine their reference values according to the recommendations of the International Federation of Clinical Chemistry [11], so as to play a key role in protecting populations at greater risk.

2. Materials and Methods

2.1. Study Population

The CNHS 2010–2012 is a well-designed nationally representative cross-sectional study using a multistage stratified cluster sampling method. It covered all 31 provinces, autonomous regions, and municipalities directly under the central government throughout China. The whole county was divided into urban and rural regions according to their economic status and social development. The present study was performed based on the CNHS 2010–2012, and we had randomly chosen seemingly healthy pregnant women aged 18–44 years as the study subjects in this study from the 150 monitoring sites around China.

The CNHS 2010–2012 was conducted in accordance with the principles of the Declaration of Helsinki [12]. All detailed field procedures involving human subjects were approved by the Ethics Committee of the National Institute for Nutrition and Health, Chinese Center for Disease Control and Prevention. All subjects provided consent for their participation in the CNHS 2010–2012 after the nature of the survey was explained.

2.2. Sample Collection, Preparation, and Determination

Blood samples were drawn from the antecubital vein by venipuncture into a separation gel vacuum blood collection tubes after a fasting period of 10–12 h in the morning. All blood samples were centrifuged at 1500× g for 10 min within 0.5–1 h following collection. Serum samples were then extracted and aliquoted immediately into screw-top cryogenic vials, and stored at −70 °C and subjected to repeated freeze-thaw until analysis.

In this study, all serum specimens were chosen according to the identification number of the subjects' vials labeled, taking great care to avoid hemolysis samples. The concentrations of 14 serum trace elements, namely iron (Fe), copper (Cu), zinc (Zn), rubidium (Rb), selenium (Se), strontium (Sr), molybdenum (Mo), manganese (Mn), lead (Pb), arsenic (As), chromium (Cr), cobalt (Co), vanadium

(V), and cadmium (Cd), were determined by high-resolution inductively coupled plasma mass spectrometry (HR-ICP-MS) following 1:20 dilutions of 100 μL of serum with diluent containing 0.5% (v/v) HNO_3. The detailed analytical method has been reported in previous literature [13]. During the determination, accuracy and precision were checked by certified commercial serum reference materials (Clinchek Level-1, Level-2, Recipe, Germany) and samples of manual serum sample with spiked concentrations of certain elements.

2.3. Anthropometric Status

Height and weight were measured according to a standard protocol suggested by the World Health Organization (WHO). Height was measured to the nearest 0.1 cm without shoes, using a portable stadiometer and weight was measured in lightweight clothing to the nearest 0.1 kg with a calibrated beam scale. The anthropometric status for pregnant women was classified into four subgroups according to the cutoff values of body mass index (BMI): Underweight (BMI < 18.5), Normal (18.5 $\leq$ BMI < 25), Overweight (25 $\leq$ BMI < 30), and Obesity (30 $\leq$ BMI) [14].

2.4. Statistical Analysis

All calculations for determining reference values were in the light of the guidelines found in the Clinical and Laboratory Standards Institute [15]. We had employed the box plot that was used to identify possible outliers. Outliers were removed according to Dixon's test [15]. When the D/R ratio was over 1:3, the outliers were excluded from this study, where D is the absolute difference between the extreme value and the values nearest to it, and R is the range of all values. The reference values of 14 serum trace elements were expressed as the median anthinnessd the central 95% reference intervals (P2.5–P97.5). The differences in the concentrations of the serum trace elements between subgroups were further assessed using the Kruskal–Wallis test. To analyze the associations of age, residence, anthropometric status, and pregnancy with respect to serum trace elements, a general linear model factorial analysis was applied with Tukey's post hoc comparisons. All statistical analyses were performed using SAS 9.3 (SAS Institute, Inc., Cary, NC, USA). The statistical values were considered significantly different at $p < 0.05$.

3. Results

3.1. Population Characteristics

A total of 1400 pregnant women (average age: 27.0 $\pm$ 4.5 years) were randomly selected from the urban and rural regions in the CNHS 2010–2012 (Table 1). The study subjects were further divided into the different groupings by age interval (18–25 years, 26–30 years, and 31–44 years), residence (Urban and Rural regions), anthropometric status (Underweight, Normal, Overweight, and Obesity) and duration of pregnancy (Trimester 1: Gestation weeks < 12 weeks; Trimester 2, 12 $\leq$ Gestation weeks < 28 weeks; Trimester 3: Gestation weeks $\geq$ 28 weeks). Of these, the data of pregnant women for age intervals and anthropometric status (63, 4.5%) and for residences and duration of pregnancy (151, 10.8%) was removed for lacking of the relevant information and possible outliers.

Table 1. Characteristics of pregnant Chinese women selected from the China Nutrition and Health Survey 2010–2012 (CNHS 2010–2012).

Variables	Number	Percent (%)
Total population	1400	100
Age (year)		
18–25	528	39.5
26–30	505	37.8
31–44	304	22.7
Residences		
Urban	586	46.9
Rural	663	53.1

Table 1. *Cont.*

Variables	Number	Percent (%)
Anthropometric status		
Underweight	78	5.8
Normal	789	59.0
Overweight	388	29.0
Obesity	82	6.2
Pregnancy		
Trimester 1	291	21.8
Trimester 2	555	41.5
Trimester 3	491	36.7

3.2. Analytical Performances

Table 2 summarizes that the limits of method quantification (LOQs) calculated to the undiluted serum range from 0.02 μg/L for ^{208}Pb, ^{51}V, and ^{111}Cd to 2.2 μg/L for ^{77}Se. The comparison of target values with measured values shows sufficient agreement for all trace elements, without significant outliers for samples with lower concentrations. The coefficient variation (CV) % of intra-day precision was from 1.7 for ^{56}Fe to 7.5 for ^{208}Pb, whereas the CV % of inter-day precision ranged from 1.9 for ^{66}Zn to 10.6 for ^{208}Pb. In addition, the recoveries calculated by the measured concentrations compared with the certified concentrations of control materials were in the range 89.1% for ^{111}Cd and 112.5% for ^{208}Pb. In addition, the percentage of trace element values below the LOQs in this study were as follows: 14.8% for the ^{55}Mn results, 0.3% for ^{208}Pb, 24.3% for ^{75}As, 5.8% for ^{52}Cr, 5.2% for ^{59}Co, and 4.4% for ^{51}V.

Table 2. Selected isotope, limits of quantification (LOQs), measured and certified concentrations of reference materials, coefficient variation (CV) % of intra-day and inter-day and recoveries %.

Isotopes	LOQs (μg/L)	Concentration (μg/L)		CV %		Recoveries %
		Certified/Spiked	Measured	Intra-Day	Inter-Day	
^{56}Fe	0.4	407	420	1.7	2.4	103.2
^{63}Cu	0.2	1390 ± 210	1426 ± 216	2.9	3.1	102.6
^{66}Zn	1.6	1090 ± 160	987 ± 145	1.8	1.9	90.6
^{85}Rb	0.2	244	220	2.7	3.1	90.2
^{77}Se	2.2	158 ± 31	174 ± 34	6.3	8.4	110.1
^{88}Sr	0.03	46	45	3.8	3.4	97.8
^{95}Mo	0.15	4.18 ± 1.26	4.4 ± 1.3	4.6	6.2	105.3
^{55}Mn	1.5	2.4	2.5	2.4	2.4	104.2
$^{2\circ8}$Pb	0.02	0.4	0.5	7.5	10.6	112.5
^{75}As	0.7	19.3 ± 3.8	20.3 ± 4.0	5.5	6.8	105.2
^{52}Cr	0.25	6.31 ± 1.26	6.5 ± 1.3	3.6	4.0	103.0
^{59}Co	0.06	5.77 ± 1.15	6.3 ± 1.3	2.8	2.9	109.2
^{51}V	0.02	12.6 ± 2.6	13.5 ± 2.8	2.7	2.9	107.1
^{111}Cd	0.02	5.39 ± 1.08	4.8 ± 1.0	4.4	3.5	89.1

3.3. Reference Values of Trace Elements in Serum

The reference values of 14 serum trace elements for pregnant women from the CNHS 2010–2012 are listed in Tables 3 and 4. In addition to the results for the total study population, information on groupings classified according to age interval, residence, anthropometric status, and duration of pregnancy is provided. Overall, the median concentration of 14 serum trace elements ranges from 133.7 ng/L for Cd in the lower ng/L range up to 195.5 μg/dL for Cu or 131.5 μg/dL for Fe. Essential trace element concentrations are in a relative small range for Fe (55.8–265.0 μg/dL), Cu (107.0–362.4 μg/dL), Zn (51.8–111.3 μg/dL), and Se (39.9–111.6 μg/L). Other essential trace element concentration ranges are as follows: Mo (1.2–3.6 μg/L), Mn (1.2–8.4 μg/L), Co (57.0–1130.0 ng/L), and V (23.6–2323.1 ng/L). Potential essential trace element concentrations ranges are as follows: Rb (14.0–62.0 μg/dL) and Sr (23.8–104.3 μg/L). By contrast, toxic trace element concentrations are in a larger range for Pb (0.6–9.0 μg/L), As (0.3–5.6 μg/L), and Cd (72.1–595.1 ng/L).

Table 3. Reference values ranges of serum major trace elements for pregnant Chinese women from the CNHS 2010–2012.

Variables	Fe (μg/dL)		Cu (μg/dL)		Zn (μg/dL)		Rb (μg/dL)		Se (μg/L)		Sr (μg/L)		Mo (μg/L)	
	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5
Total	131.5	55.8–265.0	195.5	107.0–362.4	74.0	51.8–111.3	22.3	14.0–62.0	72.2	39.9–111.6	45.9	23.8–104.3	1.8	1.2–3.6
Age (year)														
18-25	127.0 ^{a}	55.6–247.0	196.0 ^{a}	102.5–356.0	72.8 ^{a}	51.3–107.4	22.4 ^{a}	14.0–71.2	68.3 ^{a}	37.3–107.5	47.8 ^{a}	24.1–108.6	1.8 ^{a}	1.2–3.5
26-30	138.0 ^{a}	57.0–271.0	195.9 ^{a}	109.0–360.5	75.2 ^{b}	54.5–114.0	22.4 ^{a}	14.4–61.0	75.0 ^{b}	42.0–112.0	43.5 ^{a}	24.0–100.5	1.8 ^{a}	1.2–3.7
30-44	132.0 ^{a}	56.0–273.0	194.0 ^{a}	113.0–373.7	73.4 ^{a,b}	51.6–110.0	21.6 ^{b}	13.6–48.8	73.0 ^{b}	41.0–118.0	45.0 ^{a}	23.0–100.5	1.7 ^{a}	1.1–3.5
Residences														
Urban	135.0 ^{a}	56.9–250.7	194.0 ^{a}	108.0–302.0	74.8 ^{a}	51.3–110.6	22.1 ^{a}	14.0–43.2	75.2 ^{a}	42.5–111.5	40.2 ^{a}	23.0–97.3	1.7 ^{a}	1.2–3.4
Rural	127.6 ^{a}	55.0–276.3	196.0 ^{a}	107.0–426.0	73.2 ^{a}	52.1–111.8	22.7 ^{a}	14.0–80.4	69.5 ^{b}	38.0–110.0	51.0 ^{b}	24.3–109.8	1.8 ^{b}	1.2–3.8
Anthropometric status														
Underweight	152.5 ^{a}	82.7–281.4	193.0 ^{a}	99.7–624.9	82.4 ^{a}	58.0–150.3	24.9 ^{a}	15.1–70.6	75.2 ^{a}	52.2–144.1	43.4 ^{a}	24.5–109.8	1.8 ^{a}	1.2–3.9
Normal	136.1 ^{b}	57.0–265.3	193.2 ^{a}	104.7–343.5	74.6 ^{b}	53.4–110.5	22.4 ^{a}	14.1–67.2	73.6 ^{a}	40.6–111.9	44.9 ^{a,b}	23.0–101.9	1.8 ^{a}	1.2–3.7
Overweight	118.8 ^{c}	53.9–242.6	199.7 ^{a}	117.0–378.4	72.7 ^{c}	50.4–106.8	21.8 ^{a}	13.9–60.9	68.2 ^{b}	38.6–105.3	48.9 ^{b,c}	24.9–109.9	1.8 ^{a}	1.1–3.4
Obesity	110.6 ^{d}	48.8–222.7	212.2 ^{b}	116.9–292.4	69.9 ^{d}	50.9–90.8	21.5 ^{a}	13.7–31.0	66.8 ^{b}	37.3–99.5	50.1 ^{c}	25.9–100.4	1.7 ^{a}	1.1–2.8
Pregnancy														
Trimester 1	150.0 ^{a}	70.9–285.6	157.5 ^{a}	89.7–366.0	83.2 ^{a}	57.7–129.5	22.9 ^{a}	13.7–80.9	77.6 ^{a}	44.0–112.0	43.2 ^{a}	22.6–102.0	1.8 ^{a}	1.2–3.6
Trimester 2	135.0 ^{b}	59.2–261.3	198.6 ^{b}	122.6–343.5	73.0 ^{b}	53.4–102.6	21.9 ^{a}	14.5–62.0	76.0 ^{a}	42.5–112.0	42.2 ^{a}	22.5–92.5	1.8 ^{a}	1.2–3.5
Trimester 3	110.3 ^{c}	52.2–260.7	208.2 ^{c}	121.0–375.0	71.0 ^{c}	49.4–110.0	22.4 ^{a}	13.9–52.2	65.0 ^{b}	38.0–105.0	52.8 ^{b}	26.0–119.5	1.8 ^{a}	1.1–3.7

M_0: Median Concentration. A general linear model was performed with Least Squares Means post hoc analysis to compare the effect of age. Values not sharing the same superscript letter (a–d) denote a significant difference between subgroups, $p < 0.05$.

Table 4. Reference values ranges of serum major trace elements for Chinese pregnant women in the CNHS 2010–2012 (continue).

Variables	Mn (μg/L)		Pb (μg/L)		As (μg/L)		Cr (ng/L)		Co (ng/L)		V (ng/L)		Cd (ng/L)	
	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5	M_0	P2.5–P97.5
Total	2.4	1.2–8.4	1.9	0.6–9.0	1.1	0.3–5.6	835.6	219.8–4287.7	337.9	57.0–1130.0	193.2	23.6–2323.1	133.7	72.1–595.1
Age (year)														
18–25	2.4	1.3–9.0	1.9	0.6–8.2	1.1 [a]	0.3–7.3	879.2	248.9–4295.3	353.2	68.5–1129.8	172.4	29.7–2130.5	126.2 [a]	71.9–614.3
26–30	2.4	1.2–7.7	2.0	0.6–8.9	1.1 [a]	0.4–4.5	798.0	205.0–3688.0	333.6	48.5–1085.5	208.3	17.5–2323.0	140.0 [b]	73.0–549.0
31–44	2.3	1.1–8.4	2.0	0.5–10.5	1.3 [b]	0.4–5.2	799.0	211.0–5546.0	333.0	89.0–1250.5	215.0	20.0–2486.0	137.5 [b]	71.0–598.5
Residences														
Urban	2.2 [a]	1.1–8.4	2.1 [a]	0.7–8.9	1.3 [a]	0.4–4.7	845.5	79.0–5395.0	338.0	52.0–1250.6	218.4 [a]	18.5–2625.5	139.3 [a]	72.4–624.8
Rural	2.5 [b]	1.2–7.8	1.8 [b]	0.5–9.2	1.0 [b]	0.3–7.5	827.4	297.9–3387.0	339.0	73.9–1041.6	174.9 [b]	31.0–1797.0	129.5 [b]	71.3–563.9
Anthropometric status														
Underweight	2.2 [a]	1.2–6.5	2.2 [a]	0.7–9.2	1.5 [a]	0.4–4.4	909.0 [a]	317.4–3135.6	234.0 [a]	21.6–917.0	125.0 [a]	13.6–2000.3	150.3 [a]	72.8–426.2
Normal	2.4 [a]	1.2–9.2	1.9 [a]	0.6–10.1	1.1 [a]	0.4–5.4	815.2 [a]	236.0–4587.5	317.9 [b]	52.9–1083.3	201.2 [b]	25.3–2485.7	137.8 [a]	72.6–660.7
Overweight	2.4 [a]	1.2–7.0	1.9 [a]	0.5–7.0	1.0 [a]	0.3–5.7	835.5 [a]	158.8–3913.2	391.0 [c]	93.6–1219.9	209.3 [b]	22.2–1965.6	125.5 [b]	68.2–488.0
Obesity	2.3 [a]	1.4–9.2	1.7 [a]	0.6–8.1	1.3 [a]	0.4–7.3	881.9 [a]	263.6–3320.8	394.3 [c]	112.4–1113.1	182.0 [b]	30.9–1885.6	115.3 [b]	71.9–290.7
Pregnancy														
Trimester 1	2.4	1.1–9.2	2.1 [a]	0.7–9.2	1.0 [a]	0.4–4.7	888.0 [a]	219.8–4287.7	217.6 [a]	26.0–1093.9	206.8 [a]	22.2–2401.3	136.5 [a]	71.9–618.3
Trimester 2	2.3	1.1–8.4	1.8 [b]	0.6–6.5	1.1 [a]	0.3–6.4	789.5 [a]	248.9–4295.3	313.0 [b]	45.2–1083.0	178.4 [a]	23.6–2564.2	133.2 [a]	71.9–456.3
Trimester 3	2.5	1.4–7.7	2.0 [a]	0.6–12.3	1.3 [a]	0.3–5.0	837.0 [a]	205.0–3688.0	432.0 [c]	136.2–1206.0	203.0 [a]	23.7–1965.6	134.0 [a]	74.0–757.0

M_0: Median Concentration. A general linear model was performed with Least Squares Means post hoc analysis to compare the effect of age. Values not sharing the same superscript letter (a–d) denote a significant difference betweenin subgroups, $p < 0.05$.

There are some remarkable changes in the 14 serum trace element concentrations in the different groupings. Among different age intervals, the concentrations of serum Zn, Se, As, and Cd significantly increased with increasing age ($p < 0.05$), whereas serum Rb concentration significantly decreased ($p < 0.05$). Among residences, significant differences were observed in serum Se, Sr, Mo, Mn, Pb, As, and Cd concentrations between the urban and rural regions ($p < 0.05$). Among different anthropometric statuses, we found that there was a distinctly difference in serum Fe, Cu, Zn, Se, Sr, Co, V, and Cd concentrations ($p < 0.05$). With respect to pregnancy, serum Fe, Zn, and Se concentrations significantly negatively decreased as the duration of pregnancy advanced, while serum Cu, Sr, and Co concentrations markedly positively increased ($p < 0.05$).

4. Discussions

The assessment of essential and toxic elements levels during pregnancy was complicated by the dynamic physiological changes that take place in pregnant women. At present, our study had established reference values of 14 serum trace elements for pregnant women selected from the CNHS 2010–2012. It had examined the remarkable differences of 14 serum trace element reference values in terms of age interval, residence, anthropometric status, and duration of pregnancy. Furthermore, it is worth noting that the 14 trace elements have inconsistent variation in the duration of pregnancy. We found that the concentrations of serum Fe, Zn, and Se significantly decreased, whereas serum Cu, Sr, and Co concentrations progressively elevated with advanced anthropometric status and duration of pregnancy.

In our study, the reference values of 14 serum trace elements are relatively accurate and reliable, reflecting the real status of serum trace elements in the human body. One possible reason could be that HR-ICP-MS was applied and "tailored" to the serum trace elements at ultra-trace analysis, ensuring low limits of detection for its excellent characteristics [16]. This makes us able to analyze the essential and toxic elements at trace and ultra-levels in a significantly high number of serum samples, producing reliable estimations. Another possible reason is that the representative samples of pregnant women were selected from the CNHS 2010–2012. The concentrations of serum trace elements are reported in the literature for the partial elements investigated, but there are some significant differences in content when compared to analogous studies [17–20]. In our study, the concentrations of serum Fe, Cu, Zn, Se, Sr, and Mn were 131.5 (55–265.0) μg/dL, 195.5 (107–362.4) μg/dL, 74.0 (51–111.0) μg/dL, 72.2 (39–111.6) μg/L, 45.9 (23–104.3) μg/L, and 2.4 (1.2–8.4) μg/L, respectively, which are in agreement with values reported in some of the previous literature [21,22], but were lower or higher when compared to those reported in other studies [23–26]. As regards the discrepancy, possible reasons include different environmental factors, different socioeconomic status, different dietary patterns, different lifestyles, and racial differences.

In addition, prior to deriving conclusions on comparison, several factors were required for consideration. As mentioned, age is the one of the first parameters that can significantly affect the body burden of trace elements [27]. In this study, we observed significant variations in serum trace elements between age intervals. Moreover, the concentrations of serum Se, Sr, Mo, Mn, V, As, Pb, and Cd have significant changes by residence. Therefore, economic factors might play an important role. Similarly, the levels of some serum trace elements are significantly different in different levels of anthropometric status. It was considered that the development and progression of obesity could be involved in the dysregulation of trace element metabolism via the increase in excretion and the decrease in bioavailability or redistribution among various pools [28]. With respect to the influence of pregnancy duration, the reasons might be multifactorial. Plasma volume expansion may explain why most trace elements show a decrease in concentration. However, regarding the increase in the concentration of serum Cu, Sr, and Co, we considered that metabolic changes might cause a certain amount of trace elements to be released into the blood. For example, the increase in serum Cu with the progression of pregnancy could be partly related to the synthesis of ceruloplasmin, a major Cu binding protein, as a result of elevated levels of maternal estrogen. Another potential reason is the

decreased biliary Cu excretion induced by the hormonal changes that are typical during pregnancy [29]. Apart from the above reasons, other specific reasons could also include natural background conditions, such as geographical location, climate, the composition of soil, and element concentrations in water and food.

To the best of our knowledge, there have been no national large-scale population studies of serum trace elements similar to this study. In the absence of such studies, our findings can be used to provide the baseline data on serum trace element concentrations for pregnant Chinese women. There are, however, some limitations that must be noted. Firstly, the sample size only involves 1400 pregnant women and this is relatively insufficient and cannot yield a reliably accurate estimation of pregnant women on a national level. Secondly, the concentrations of some trace elements such as Co, V, Cr, Pb, As, and Cd are at very low levels. Thus, the risk for external contamination has to be seriously considered during the process of blood collection, sample preparation, and determination. Thirdly, it is unclear as to what the status of health of the pregnant women in this study was. Thus, the influence of subclinical infections and inflammation on serum trace elements is difficult to further evaluate. Therefore, our future studies will be directed to overcome these limitations.

5. Conclusions

In summary, the results of this study obtained baseline data regarding 14 serum trace element concentrations for pregnant women in China based on the CNHS 2010–2012. This valuable data can help to establish the reference values of 14 serum trace elements and provide clear evidence that all of the selected elements are visibly altered when classified by age interval, residence, anthropometric status, and duration of pregnancy. Furthermore, these findings shall be useful for future research to assess the trace element nutritional status and health risks of environmental metal exposure, and to protect population in China at greater risk.

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Does the Valuation of Nutritional Claims Differ among Consumers? Insights from Spain

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Abstract: The presence in the market of food products with nutritional claims is increasing. The objective of this paper is to assess consumers' valuation of some nutritional claims ('high in fiber' and 'reduced saturated fat') in a European country and to test for differences among consumers. An artefactual non-hypothetical experiment was carried out in a realistic setting (mock/real brick-and-mortar supermarket) with a sample of 121 Spanish consumers stratified by gender, age, and body mass index. A latent class model was specified and estimated with the data from the experiment. Results indicate that consumers positively valued both nutritional claims, but the valuation was heterogeneous, and three consumer segments were detected. Two of them positively valued both nutritional claims (named 'nutritional claim seekers'), while the third segment's valuation was negative (named 'nutritional claim avoiders'). This last segment is characterized by being younger males with university studies who give the least importance to health, natural ingredients, and the calorie/sugar/fat content when shopping. They pay less attention to nutritional information, and they stated that they use this information to a lesser extent. These consumers showed the least interest in healthy eating, and they reported that they do not have health problems related to their diet.

Keywords: choice experiment; heterogeneous preferences; nutritional claims

1. Introduction

Within the European context, chronic diseases constitute the main cause of death, the most important ones being heart disease, cerebrovascular disease, cancer, and diabetes. The most significant preventable risk factors are unhealthy diet, physical inactivity, and tobacco use [1]. Moreover, it is widely accepted that current eating habits in general tend to be less than healthy. One reason for this phenomenon is the increase in the consumption of pre-prepared or quick-to-prepare foods and a subsequent reduction in consumers who cook food every day from fresh raw ingredients [2]. As a consequence, in many countries, dietary recommendations and actual food intake do not coincide at the general population level [3–5]. Thus, a change in dietary behavior is crucial to reduce the effect of unhealthy diets on consumers' health. One way to improve the quality of consumers' diets is to increase the consumption of fiber and decrease the consumption of fat, in particular saturated fat, to reduce the risk of chronic disease [4,6] by eating more fresh food products (fruits and vegetables), and cooking food at home from healthier ingredients (i.e., using non-saturated fats instead of saturated fats, less salt, etc.). However, nowadays this behavior is difficult to follow because consumers are searching for convenience and consumption of convenient food products is steady increasing [7]. In this context, another way in which consumers can improve the quality of their diet while satisfying their need for convenience is to choose processed food with claims of improved nutritional and health characteristics, for example 'fat free'. The presence of food products with nutritional and/or health

claims in the market has risen because food companies are increasingly offering healthier processed food alternatives to fulfill consumers' desires to follow a healthier diet. From the institutional point of view, the European Union has introduced certain regulations for the nutritional and health information on food products' labels to avoid misleading consumers [8]. This regulation applies to all nutrition and health claims on processed food, indicating that nutritional and health claims (NHCs) should not be misleading and should be substantiated by generally accepted scientific data.

Several papers have analyzed the prevalence of different nutritional and health claims in different European markets [9–12]. Except for the study by Hieke et al. (2016) [9], which includes several European countries (Germany, Slovenia, Spain, the Netherlands, and the United Kingdom), the papers focus only on Ireland, the UK, and Slovenia, respectively. Hieke et al. (2016) [9] found that 26% of foods carried at least one nutritional (21%) or health claim (11%). In addition, 64% of the claims were nutritional, with the highest proportion corresponding to Spain (74%). Foods carrying claims tended to carry multiple claims, with an average number of nutritional claims per product of 2 in the European countries and 2.1 in Spain. Apart from vitamins and minerals, the nutritional claims mainly referred to fat content (24%), followed by sugar (12%) and fiber (9%).

Thus, consumers have access to healthier food product alternatives that could help them to follow a healthier diet. However, making information available and ensuring that this information is correct is only the first step in inducing healthier diets. For consumers to choose healthier food products, it is necessary that they value positively the nutritional and/or health claims and are willing to buy and pay for them. Several empirical papers analyzed consumers' acceptance of food products with health-related claims by studying motivations, attitudes, and intention to purchase food products with these claims [13–15]. However, only a few papers were conducted to investigate consumers' preferences for them and to assess consumers' valuation for nutritional claims [16–19]. The latter papers estimated the extra price consumers are willing to pay for different nutritional and health claims for different products. All of them found that consumers would pay an extra price for a food product with nutritional or health claims. Accordingly, the objective of this paper is to investigate consumers' preferences for two relevant nutritional claims in Spain: in other words, to assess consumers' valuation for these claims. In addition, we are mainly interested in the possible differences in consumers' valuation of nutritional claims and in explaining these differences. This will allow us to profile consumers in terms of their willingness to pay for nutritional claims. This second objective is highly relevant because the analyzed nutritional claims exist in the market and the characterization of consumers would allow food companies and public authorities to tailor their marketing and information strategies and educational programs to help consumers to buy healthier food alternatives.

Spain was selected because of the higher number of nutritional claims in its market. The selection of the food product and the nutritional claims analyzed was made simultaneously based on the previous studies on the prevalence of nutritional and health claims in the market and on direct observations of the shelves in different supermarkets. As mentioned above, the most prevalent nutritional claims in the market are those related to fat content, sugar, and fiber. Hieke et al. (2016) [9] also indicated that, apart from baby and dietary foods, the cereal food category had the highest proportion of nutritional claims (30%). Therefore, a direct observation of different supermarkets was conducted to gather information on the different nutritional claims available in different cereal products (biscuits, toast bread, and breakfast cereals). Based on this information, we chose breakfast biscuits due to the high prevalence of nutritional claims and because they are a highly consumed product in Spain. Within breakfast biscuits, the most frequent nutritional claims were related to fiber. In addition, the evidence to date indicates that high-fiber diets are beneficial for weight control and protective of the development of diabetes and heart disease [6]. Accordingly, we chose the nutritional claim 'high in fiber'. Second, we were interested in the reduction of saturated fats (SFA) because it has been scientifically proven that reducing the intake of saturated fats and replacing them with polyunsaturated fatty acids (PUFA) reduces the risk of some health diseases related to diet, in particular cardiovascular diseases [20]. Thus, we chose the 'reduced saturated fat' claim as our second claim to

analyze, although this claim was only used in a few breakfast biscuits. This fact also supports our decision because consequently we analyze a claim with high prevalence in the market ('high in fiber') and another with low prevalence ('reduced saturated fat').

To assess consumers' valuation of the 'high in fiber' and 'reduced saturated fat' claims, we designed a non-hypothetical artefactual choice experiment and implemented it in a close-to-real environment (mock/real brick-and-mortar supermarket). The experiment was carried out in a Spanish town where this mock/real brick-and-mortar supermarket was located in June 2015 with a sample of consumers stratified by age, gender and body mass index.

2. Materials and Methods

We used an artefactual non-hypothetical experiment conducted in a realistic setting, specifically a mock/real brick-and-mortar supermarket, to increase the external validity of the study.

2.1. Study Design

First, we decided to use an artefactual experiment [21] to ensure that the recruited participants were representative food purchasers and had experience with the concerned good [22]. In addition, to ensure that the respondents had experience with the good, the target population consisted of participants who were responsible for the purchase of food products in their household and who consumed breakfast biscuits.

Second, we decided to conduct a choice experiment instead of other valuation methods because of its ability to value multiple attributes simultaneously, its consistency with the random utility theory, and the similarity of the choice task asked of the participants to their real purchase decisions [23]. In addition, we designed a non-hypothetical experiment instead of using a hypothetical choice experiment to avoid hypothetical bias. Several papers have analyzed the hypothetical bias in choice experiments and compared results from both hypothetical and non-hypothetical versions ([24–27], among others). They have all provided strong evidence of hypothetical bias, suggesting the use of non-hypothetical experiments. In addition, Chang et al. (2009) [24] found that non-hypothetical choices are a better approximation of true preferences than hypothetical ones, based on a comparison not only between hypothetical and non-hypothetical choice experiments but also with actual market shares. The interpretation of this finding is that the willingness to pay (WTP) values of non-hypothetical choice experiments (CEs) can be assumed to be the true values corresponding to actual payments in the marketplace [24].

Third, as the proposed research has an empirical orientation to provide stakeholders (private and public) with information on consumers' valuation of different nutritional claims, the external validity of the experiment is vital to allow for the generalization of the results. To increase the external validity of our experiment by increasing its ecological validity, the choice experiment was carried out in a close-to-real setting. In other words, we used a setting as similar to a real supermarket as possible. In particular, we used a mock/real brick-and-mortar supermarket. This mock/real supermarket is located in a logistic facility in the town where the experiment took place. This logistic space is available for companies to demonstrate how their product and service technology can help to create innovative solutions and improve productivity and competitiveness in the field of logistics. The logistic demonstration center is divided into several modules; Smart Store, Smart Point of Sale, Supply Chain Module, and Intelligent Transport Module. In this research, we used the Smart Point of Sale, which consists of a Smart Point of Sale Terminal and Smart Shelving for automatic inventory control to undertake the experiments in a close-to-real environment (Figure A1 in the Appendix A).

We designed a non-hypothetical choice experiment, introducing real economic incentives and real products. The participants received €10 because at the end of the experiment one choice set was randomly selected as binding and the respondents were required to purchase the food product chosen in the binding situation at the corresponding price.

We selected a box of half a kilo of breakfast biscuits with three different attributes: price, fiber, and fat content claims. The price levels were set, based on the market prices at the time of the experiment, at 0.5 €/box, 1.5 €/box, 2.5 €/box, and 3.5 €/box. The other two attributes had two options; the product carried the claim 'high in fiber' (FIBER) or the claim 'reduced saturated fat' (FAT) or did not carry a claim. The attributes selected and their levels are summarized in Table 1.

The choice set design was generated following the Street and Burgess (2007) [28] approach. For the main effects, three attributes were chosen with four, two, and two levels, respectively, as well as two options; we obtained eight pairs, and this design was 96.66% efficient compared with the optimal design. Thus, each respondent was asked to make four choices because we randomly split the choice sets into two blocks. Each choice set included three alternatives; two designed alternatives consisting of different products and a non-buy option.

Table 1. Breakfast biscuits attributes and levels.

Attributes	Levels
Price (Euro/liter)	0.5 €/box, 1.5 €/box, 2.5 €/box and 3.5 €/box
Fiber claim	None 'High in Fiber'
Fat claim	None 'Reduced Saturated Fat'

2.2. Participants and Recruitment

The experiment was conducted in June 2015 in a medium-sized town in Spain where the mock/real supermarket is located. This town is widely used by food marketers and market research consulting companies since its socio-demographics are representative of the Spanish Census of Population (Table A1 in the Appendix A). The target population was consumers who were responsible for the purchase of food products in their households and who consumed breakfast biscuits. The participants were recruited by an external company using our requirements: (i) food shoppers; (ii) breakfast biscuit consumers; and (iii) stratified by age, gender, and body mass index. The sample size for the experiment was chosen based on similar real experiments for agro-food products [29–33]. A total of 121 consumers stratified by gender, age, and body mass index participated in the experiment.

2.3. Implementation Procedure

The experiment was conducted by the research team as follows. On arrival, the consumers received information on the nature of the experiment and signed informed consent for participation. An ID number was assigned to each respondent to guarantee anonymity. The monitor provided a general overview of the working session and informed the participants that, at the end of the experiment, they would receive €10 to purchase a box of biscuits (the one that they chose in the binding choice set) at the corresponding price. The monitor insisted that it was in their best interest to choose only the product that they were really interested in purchasing because this product could be the selected type of biscuit in the binding choice set. In addition, the participants received all this information in clear written instructions together with the information on the biscuits and the attributes presented in the different choice tasks. Then, the respondents were asked to choose four times between two boxes of biscuits or the non-buy option in front of the supermarket shelves with the real biscuit boxes (Figure A1 in the Appendix A). Afterwards, they went to the cashier and another monitor asked them to select randomly one card out of four cards numbered from 1 to 4 (choice sets) to determine the binding choice set (see Figure A1 in the Appendix A). Then, the respondent received €10 to purchase the box of breakfast biscuits selected in this binding choice set at the corresponding price.

The respondents were also required to complete a brief questionnaire with the following structure: (i) food and breakfast product purchase and consumption; (ii) objective nutritional knowledge and use of nutritional information; (iii) interest in healthy eating; (iv) weight, height, and health status;

and (v) socio-demographic and economic characteristics (gender, family size and composition, age, educational level, and income range).

2.4. Measures

The measurement of consumers' choice were made by asking the respondents to choose four times between two boxes of biscuits or the non-buy option in front of the supermarket shelves, as mentioned above. In the questionnaire the participants were asked first whether they were responsible for food purchases. In addition, consumers were required to rate on a seven-point scale the importance that they attached to different attributes when shopping for food products. Finally, the respondents were asked about their frequency of consumption of cereals and breakfast biscuits; the options included never/once a month or less, 2–3 times a month, 1–2 times a week, 3–4 times a week, 5–6 times a week, once a day, and more than once a day.

The objective nutritional knowledge was measured following Grunert et al.'s (2010) [34] scale based on the knowledge of dietary recommendations. The participants were asked about their knowledge of health expert recommendations (should eat more, about the same, less, or try to avoid) regarding a series of nutrients or substances.

The use of nutritional information was assessed using four items on a seven-point Likert scale (e.g., 'I usually pay attention to nutrition information when I see it in an ad or elsewhere') based on Moorman (1998) [35]. The Cronbach's alpha for both four-item measures was 0.78, indicating good internal consistency reliability. Interest in healthy eating was measured on a seven-point Likert scale using the Roininen et al. (1999) [36] scale (e.g., 'The healthiness of food has little impact on my food choices') The Cronbach's alpha for both eight-item measures was 0.84, indicating very good internal consistency reliability.

Finally, the participants were required to report, apart from their socio-demographic and economic characteristics, their weight, height, and health problems. With this information, each participants' Body Mass Index (BMI) was calculated, and the participants were classified into different groups following Aranceta-Bartrino et al. (2016) [37]. Details on how each item is measured can be found in the results section.

2.5. Data Analysis: Discrete Choice Modeling

The data gathered in the choice experiment were used to estimate a utility function derived from the Lancastrian consumer theory of utility maximization [38]. Lancaster (1966) [38] proposed that the total utility associated with the provision of a good can be decomposed into separate utilities for their attributes. However, this utility is known to the individual but not to the researcher. The researcher observes some attributes of the alternatives, but some components of the individual utility are unobservable and treated as stochastic (random utility theory by McFadden, 1974) [39]. Thus, the utility is taken as a random variable for which the utility from the nth individual facing a choice among j alternatives within choice set J on each of t choice occasions is represented as follows:

$$U_{njt} = \beta X_{njt} + \varepsilon_{njt} \quad (1)$$

where β is a vector of parameters associated with the vector of explanatory variables X_{njt}, and ε_{njt} is an independent identically distributed (i.i.d.) error term over time, people, and alternatives. Traditionally, it was assumed that consumers were homogeneous in terms of taste, and conditional logit models were fitted [39]. However, numerous empirical papers using choice experiments have found that consumers' preferences for food products are heterogeneous. In this case the specification of the model should allow the parameters to vary in the population. Two alternatives have gained popularity when addressing this issue of heterogeneity, the random parameter logit model (RPL) and the latent class logit model (LC), both of which are versions of a mixed logit model [40]. In the RPL, each individual has a unique set of preferences and estimates of the utility function. Then,

heterogeneity is included by adding a vector of parameters that incorporates individual preference deviations with respect to the mean preference values; β in (1) is not constant but varies across individuals, β_n. However, if the preferences are assumed not to be 'unique' for each individual but rather distinct for a determined number of individual classes, the LC suits the modeling of choices better. In this model, consumers are assumed to belong to different segments or classes, each of them characterized by different class-specific utility parameters. In other words, within each segment, consumers' preferences are homogeneous, but preferences vary between segments, reflecting a 'lumpy' spread of preferences and allowing a more in-depth understanding of heterogeneity [40]. The latter modeling approach has gained popularity and has recently been used in several studies on consumers' valuation of food products [41–46].

In the LC model, the utility of individual 'n' choosing alternative j on the tth choice occasion is:

$$U_{njt|S} = \beta_S X_{njt} + \varepsilon_{njt|S} \quad (2)$$

where β_s is the parameter vector of class s associated with the vector of the explanatory variable, and X_{njt} and $\varepsilon_{njt\,|\,s}$ are error terms that follow a Type I (or Gumbel) distribution.

Thus, the probability that an individual will select alternative j, conditional on being in segment s, can be expressed as follows:

$$P_{nj} = \sum_{s=1}^{S} P_{ns} \prod_{t=1}^{T} P_{njt|s} \quad (3)$$

where P_{ns} is the allocation of individual n to the s class (probability of class s), and $P_{njt\,|\,s}$ is the choice probability that individual n, conditional on belonging to class s ($s = 1, \ldots, S$), chooses alternative j from a particular set J, comprising j alternatives, on a particular choice occasion t [47].

The parameters for the attributes are estimated by maximizing the likelihood function in the state of incomplete prior information on class membership or choice probabilities [46]. Then, the number of segments is endogenously determined jointly with the utility coefficients. The latent class model was estimated using NLOGIT 5.0 (Econometric Software Inc., Plainview, NY, USA).

In our empirical specification, the utility function includes the product attributes as explanatory variables, as well as an alternative-specific constant (α) representing the non-buy option. The utility function is specified as follows:

$$U_{njt/S} = \alpha + \beta_{S1} PRICE_{njt} + \beta_{S2} FIBER_{njt} + \beta_{S3} FAT_{njt} + \varepsilon_{njt/S} \quad (4)$$

The constant α represents the alternative-specific constant coded as a dummy variable that takes a value of 1 for the non-buying option and a value of 0 otherwise. It is expected that the constant α will receive a negative and significant value, indicating that consumers obtain a lower level of utility when they select the non-buying option than they do when selecting the other two alternatives (A and B). The price was defined by the price levels in the design. The other two variables (FIBER and FAT) were defined as dummies.

One of the key issues in latent class modeling is the selection of the number of segments to be considered. As Swait (1994) [48] stated, the optimal number of latent segments must be selected by looking at different multiple statistical criteria but also by assessing whether additional segments provide any further economic information, with the overall aim of attaining segment parsimony. To determine the best number of classes, we calculated four information criteria; the Akaike Information Criterion (AIC), the modified Akaike Information Criterion (AIC3), the Bayesian Information Criterion (BIC), and the ρ^2, called the Akaike Likelihood Ratio Index [49]. The preferred model should be the one with the lowest AIC, AIC3, and BIC and the highest $\overline{\rho}^2$.

Using the estimated parameters, the marginal WTP was calculated as the negative ratio of the partial derivative of the utility function with respect to the attribute of interest, divided by the derivative of the utility function with respect to the variable price.

$$WTP_{Attribute} = -\frac{\frac{\partial U_{njt}}{\partial\ Attribute}}{\frac{\partial u_{njt}}{\partial Price}} = -\frac{\beta_{Attibute}}{\beta_{Price}} \quad (5)$$

The marginal *WTP* was calculated for each of the obtained segments.

3. Results

The final sample was representative of the Spanish population (reference population) in terms of gender, age, and body mass index, as the results of tests of differences indicate (p-values of 0.64, 0.167 and 0.577, respectively, in Table 2). Slightly more than half of the respondents were female (52.0%), with an average age of 47 years. However, regarding the education level, the null hypothesis of equality was rejected (p-value = 0.001). In particular, we can see that people with secondary education were under-represented while people with higher education were over-represented. The higher proportion of people with university education occurs frequently in studies because more educated people are more prone to participate. The under- or over-representation of the sample is a feature common to many other surveys and empirical studies [50].

Table 2. Sociodemographic and economics characteristics and Body Mass Index.

	Sample	Population	Segment 1	Segment 2	Segment 3
Female (%) * (0.22 (0.641)) [1]	52.07	50.90 [2]	63.04	48.94	39.29
Age (average) **	46.98	42.68 [2]	50.00 [a]	48.11 [a]	40.14 [b]
Age (%) (21.31 (0.167)) [1]					
18–34 ***	24.79	24.12 [2]	15.22 [a]	17.02 [a]	53.57 [b]
35–44	19.01	20.62 [2]	23.91	19.15	10.71
45–54	25.62	18.56 [2]	21.74	31.91	21.43
55–64 **	10.74	14.32 [2]	17.39	10.64	0.00
≥65	19.83	22.38 [2]	21.74	21.28	14.29
Education level (%) (18.06 (0.001)) [1]					
Primary	17.36	17.00 [3]	15.22	17.02	21.43
Secondary	35.54	50.00 [3]	39.13	38.30	25.00
Higher	47.11	33.00 [3]	45.65	44.68	53.57
Income level (%) [4]					
≤1500 €/month *	42.71	N/A	47.83	25.53	35.71
1501–2500 €/month	33.01	N/A	23.91	31.91	28.57
>2500 €/month	24.28	N/A	17.39	25.53	17.86
Body mass index (%) (2.89 (0.577)) [1]					
Obese and over weight	52.07	60.90 [5]	58.07	53.19	39.29
Normal weight	46.28	37.80 [5]	39.13	44.68	60.71
Under weight	1.65	1.20 [5]	2.17	2.13	0.00

Note: ***, **, * denotes statistical significance at 1%, 5%, and 10%, respectively. [a,b] Superscript letters indicate that group means are different for continuous variables using the Bonferroni Test and that the percentages are different for discrete variables using χ^2-square Test. [1] The χ^2-square (p-value) Test between the sample and the population; [2] INE—Padrón continuo (1 January 2015); [3] Education at a glance: Organisation for Economic Co-operation and Development (OCDE) Indicators, OCDE (2014); [4] The 14.88% of the participants don't know or prefer not to say; [5] Aranceta-Bartrina et al., 2016 [37].

3.1. Statistical Results

To select the number of optimal clusters, we estimated the model using two, three, and four latent classes and calculated the different information criteria presented in Table 3. The calculated information criteria were constantly decreasing or increasing. However, the improvement from two to three

segments was greater than the change from three to four (Table 3). Moreover, we observed in the model for four classes that the value of the estimated parameters started to deteriorate, giving a larger standard error, which is considered as an indication to stop looking for more classes [51]. Thus, we chose the estimations of the model with three segments.

Table 4 shows the results of the LC model for three segments and of the one-segment model for comparison.

Table 3. Statistics to determine the optimal number of consumer segments.

Number of Segments	Number of Parameters (p)	Log Likelihood at Convergence (LL)	AIC [a]	AIC3 [b]	BIC [c]	$\bar{\rho}^2$ [d]
2	8	−434.69	885.37	893.37	446.30	0.05
3	12	−404.53	833.06	845.06	421.95	0.10
4	16	−395.36	822.72	838.72	418.59	0.11

Note: Log likelihood evaluated at zero is −464.20. [a] AIC (Akaike Information Criterion) is calculated using −2(LL − p); [b] AIC3 (Bozdogan Akaike Information Criterion) is calculated using (−2LL + 3p); [c] BIC (Bayesian Information Criterion) is calculated using (−LL + (p/2)xln(N)); [d] $\bar{\rho}^2$ is calculated using (1 − AIC/2LL(0)).

Table 4. Parameter values for biscuits latent class choice model.

			Latent Classes					
Variables	**One-Segment Model**		**Segment 1**		**Segment 2**		**Segment 3**	
	Coef.	*t*-ratio	Coef.	*t*-ratio	Coef.	*t*-ratio	Coef.	*t*-ratio
α	−0.28 **	−1.99	1.406 ***	3.11	−2.282 ***	−3.80	−1.302 ***	−2.93
PRICE	−0.539 ***	−9.18	−1.752 ***	−4.56	−0.353 ***	−415	−0.674 ***	−3.44
FIBER	0.647 ***	4.91	2.647 ***	3.52	0.807 ***	3.48	−1.294 ***	−2.74
FAT	0.632 ***	4.71	3.206 ***	5.04	0.647 ***	2.60	−1.440 ***	−2.79
Segment Size [a]			36.0 ***	6.40	38.2 ***	6.67	25.8 ***	5.30
WTPs			Coef.	*t*-ratio	Coef.	*t*-ratio	Coef.	*t*-ratio
FIBER	1.20		1.510 ***	5.24	2.285 ***	3.64	−1.921 ***	−2.03
FAT	1.17		1.829 ***	5.16	1.832 ***	3.16	−2.138 ***	−2.15

Note: ***, ** denotes statistical significance at 1% and 5%, respectively. [a] Estimated latent class probabilities (%).

In the one-segment model, as expected, the alternative-specific constant was negative and statistically significant; therefore, consumers attained a higher utility from choosing any alternative to the non-buy option. Moreover, as expected, the price variable (PRICE) was negative and statistically significant in accordance with the economic theory indicating that increments in the price decrease consumers' utility. The positive and statistically different from zero value of the parameter estimate for the two nutritional claims indicated that the utility for the breakfast biscuits with each of the claims was higher than that for the biscuits without claims. However, these results are not the best representation of consumers' behavior, as the LC model with three classes was superior in terms of statistical properties.

The estimated parameters for the three segments corroborated that heterogeneity across segments exists because the estimated values were substantially different between them, not only in magnitude but also in sign. The only estimated parameter that was consistently negative across segments was the price, although it varies considerably in absolute values. The results show that consumers in general, according to the economic theory, gain lower utility as the price of the product decreases. However, the consumers in the first segment were the most price-sensitive, while the consumers in segment 2 were the least price-sensitive. The estimated coefficients for the two nutritional claims were still positive for segment 1 and segment 2 but negative and statistically significant for segment 3. This result indicates that the utility of consumers in segment 3 for the breakfast biscuits with each of the claims was lower than that for the biscuits without claims. The contrary was still found for the consumers in segments 1 and 2. Therefore, we can conclude that the majority of consumers (36% of segment 1 plus 38% of segment 2) gain higher utility from breakfast biscuits with nutritional claims than from those

without these claims, with a small group of consumers (25.8% of segment 3) presenting a higher utility for the biscuits without claims.

3.2. Economic Valuation Results

In order to interpret the estimated parameters, the marginal willingness to pay (WTP) was calculated for each of the segments using equation (5). The WTP is the premium or extra-price that consumers are willing to pay for the food product with the claims in relation to the food product without the claim. For instance, the WTPs for the first segment were of 1.51 €/box for the 'high in fiber' claim and 1.83 €/box for the 'reduced saturated fat' claim. These values indicate that consumers in segment 1 were willing to pay an extra-price of €1.51 for a box of breakfast biscuits with the 'high in fiber' claim in relation to a box without this claim. In the same way, consumers in segment 1 were willing to pay an extra-price of €1.83 for a box of breakfast biscuits with the 'reduce saturated fat' claim in relation to the biscuits without the claim. Then, consumers were willing to pay more for the 'reduced saturated fat' claim than for the 'high in fiber' claim. Similar results were found for segment 2 (38% of consumers) because the consumers positively valued both nutritional claims, but the extra-price they were willing to pay for the 'high in fiber' claim (2.28 €/box) was higher than that for the 'reduced saturated fat' claim (1.83 €/box). Therefore, both segments can be named 'nutritional claim seekers,' but the first one can be considered 'reduced saturated fat' lovers and the second 'high in fiber' lovers. Finally, segment 3 (25.8% of consumers) differs from the previous ones because the consumers presented negative WTP for both nutritional claims. This result indicated that consumers would pay for a box of breakfast biscuits without claims more than for the box with each of the nutritional claims. Consequently, segment 3 can be named 'nutritional claim avoiders'.

3.3. Explaining Differences in Consumers' Valuation

To achieve our second objective, to explain the differences in consumers' valuation of nutritional claims, we characterized these consumer segments using the information on participants described in Section 2.3 and presented in Tables 2, 5 and 6. First, we conducted a series of bivariate analyses between the three segments and all of these participants' characteristics to test for differences among the segments. In particular, a chi-square or analysis of variance test was used depending on the nature of the characterization variables. Second, we profiled the three segments according to the characteristics found to be statistically different.

The first segment includes more women, older people, and people with a lower income level (Table 2). In addition, the proportion of consumers who are always the person responsible for the food purchases in the household is the highest (Table 5). The importance that they attach to the price when shopping is the highest, although they also give importance to health, natural ingredients, and the calorie/sugar/fat content. In addition, the proportion of consumers who eat breakfast biscuits once a day or more is smaller. The consumers in segment 1 are the least knowledgeable about saturated fat because a smaller proportion stated that experts recommend eating less saturated fat (although 80% knew). The consumers in this segment stated that they usually pay attention to and use nutritional information and read about nutrition in magazines and books (with almost 5 points on a 7-point scale). These consumers showed a high interest in healthy eating because they value healthy aspects to a greater extent and unhealthy ones to a lesser extent (Table 6). Finally, the proportion of consumers with osteoporosis or other bone problems is the highest.

Table 5. Food purchase, knowledge and use of nutritional information.

	Sample	Segment 1	Segment 2	Segment 3
Food purchase				
Who is doing the groceries in your household? Always me (%) *	37.19	47.83	36.17	21.43
Importance attached when buying food to these aspects (average)				
Convenience	4.64	4.72	4.80	4.25
Price **	5.36	5.67 [a]	5.11 [b]	5.29 [b]
Health **	5.63	5.74 [a]	5.81 [a]	5.14 [b]
Taste	5.79	5.85	5.74	5.75
Familiarity	4.83	4.72	5.02	4.71
Natural ingredients ***	5.29	5.37 [a]	5.62 [a]	4.61 [b]
Calorie/sugar/fat content ***	5.20	5.46 [a]	5.66 [a]	4.00 [b]
Consumption Frequency (once a day or more) (%)				
Breakfast cereals	17.36	23.92	17.03	10.71
Breakfast biscuits *	29.76	26.09 [a]	44.68 [b]	25.00 [a]
Objective nutritional knowledge (% right answers)				
Calcium	86.26	82.61	91.49	96.43
Salt	95.87	97.83	91.49	100.0
Fiber	95.87	95.65	97.87	92.86
Saturated fats *	87.60	80.43 [a]	95.74 [b]	85.71 [c]
Sugar	72.73	73.91	74.47	67.86
Calories	61.98	60.87	63.83	60.71
Fats	66.94	67.39	59.57	78.57
Use of nutritional information (%)				
I usually pay attention to nutrition information when I see it in an ad or elsewhere ***	4.88	4.96 [a]	5.51 [b]	3.68 [c]
I use nutrition information on the label when making most of my food selections **	4.85	4.96 [a]	5.19 [a]	4.11 [b]
I do not spend much time in the supermarket reading nutrition information *	3.62	3.28 [a]	3.57 [a]	4.25 [b]
I read about nutrition in magazines and books ***	4.03	4.43 [a]	4.43 [a]	2.71 [b]

Note: ***, **, * denotes statistical significance at 1%, 5%, and 10%, respectively. [a–c] Superscript letters indicate that group means are different for continuous variables using Bonferroni Test and that the percentages are different for discrete variables using χ^2-square Test.

Table 6. Interest in healthy eating and self-reported health problems.

	Sample	Segment 1	Segment 2	Segment 3
Interest in healthy eating (average)				
The healthiness of food has little impact on my food choices	2.86	2.85	2.66	3.21
I am very particular about the healthiness of food I eat ***	4.86	5.13 [a]	5.21 [a]	3.82 [b]
I eat what I like and I do not worry much about the healthiness of food	3.21	3.15	2.98	3.71
It is important for me that my diet is low in fat **	4.74	4.96 [a]	5.00 [a]	3.96 [b]
I always follow a healthy and balanced diet ***	4.70	4.74 [a]	5.13 [a]	3.93 [b]

Table 6. *Cont.*

	Sample	Segment 1	Segment 2	Segment 3
It is important for me that my daily diet contains a lot of vitamins and minerals **	5.02	5.24 [a]	5.15 [a]	4.43 [b]
The healthiness of snacks makes no difference to me ***	2.82	2.61 [a]	2.45 [a]	3.79 [b]
I do not avoid foods, even if they may raise my cholesterol *	2.92	2.76 [a]	2.68 [a]	3.57 [b]
Self-reported health problems (%)				
Overweight or obesity	28.93	30.43	29.79	25.00
Cardiovascular diseases (heart, . . .)	1.65	2.17	2.13	0.00
Hypertension (high blood pressure)	8.26	6.52	10.64	7.14
High levels of blood cholesterol	11.57	10.87	14.89	7.14
Diabetes	0.83	2.17	0.00	0.00
Osteoporosis or other bone problems *	15.70	23.91	12.77	7.14
None of the above *	58.68	56.52	51.06	75.00

Note: ***, **, * denotes statistical significance at 1%, 5%, and 10%, respectively. [a,b] Superscript letters indicate that group means are different for continuous variables using Bonferroni Test and that the percentage are different for discrete variables using χ^2-square Test.

The consumers in the second segment share some characteristics with the consumers in Segment 1 but differ in others. They are similar in terms of age; the importance that they give when shopping to price, health, natural ingredients, and the calorie/sugar/fat content; their attention to and use of nutritional information; and their interest in healthy eating. However, the proportion of women and lower-income households is smaller and the consumers are younger. In addition, the proportion of consumers who are always the person responsible for food purchases in the household is smaller than that in segment 1. The proportion of consumers who eat breakfast biscuits once a day or more is the largest among the segments. The consumers in Segment 2 are the most knowledgeable about saturated fat because a larger proportion stated that experts recommend eating less saturated fat. Finally, the proportion of consumers with osteoporosis or other bone problems is smaller than that in segment 1.

The third segment differs more from the previous ones. It consists of the smallest proportion of women, the youngest consumers, and the largest proportion of consumers with university studies. The proportion of consumers who are always the person responsible for the food purchases in the household is the smallest. They give the least importance when shopping to health, natural ingredients, and the calorie/sugar/fat content. In addition, the proportion of consumers who eat breakfast biscuits once a day or more is the smallest. Their knowledge about saturated fat is between the other two segments because the proportion of consumers who stated that experts recommend eating less saturated fat is average. The consumers in this segment stated to a lesser extent that they usually pay attention to and use nutritional information and read about nutrition in magazines and books than the consumers in the other two segments. These consumers showed the least interest in healthy eating because they value the healthy aspects the least and the unhealthy ones the most. Finally, the proportion of consumers without any health problems is the largest among the segments.

4. Discussion

This study aimed to investigate consumer preferences and WTP for two nutritional claims with different degrees of prevalence in the market and related to two different nutrients (one beneficial and the other harmful to health). Specifically, a highly prevalent claim for a beneficial nutrient ('high in fiber') and a less prevalent one for a harmful nutrient ('reduced saturated fat') were selected. The selected food carrier for the claims was breakfast biscuits. The results indicated that the general consumer positively values both nutritional claims with similar WTP. This finding is consistent with the previous results obtained by Øvrum et al. (2012) [17] and Van Wezemael et al. (2014) [18],

who found that a low saturated fat claim is positively valued for cheese and beef, respectively. However, heterogeneous preferences across consumers were found, and, using a latent class model, three segments of consumers based on the WTP for the two nutritional claims were detected. Two of the segments were considered 'nutritional claim seekers,' because their WTP for both nutritional claims was positive. The difference between these two segments is that the consumers in the first one (36% of consumers) present a higher valuation for 'reduced saturated fat' than for 'high in fiber.' This finding is similar to those obtained by Øvrum et al. (2012) [17] and Van Wezemael et al. (2014) [18], who found that the WTP for the low saturated fat claim was higher than the WPT for other nutritional claims (low fat in the first study and iron and protein claims in the second study). By contrast, the second segment (38% of the consumers) values the 'high in fiber' claim more than the 'reduced saturated fat' claim, although the WTP for 'reduced saturated fat' has almost the same magnitude. Thus, the second segment is named 'fiber lovers' and the first 'reduced saturated fat lovers'. On the other hand, the members of the third segment (25.8% of consumers) negatively value both nutritional claims and are considered to be 'nutritional claim avoiders'. This last segment is characterized by being younger males with university studies who are not responsible for the food purchases in the household. These consumers give the least importance to health, natural ingredients, and the calorie/sugar/fat content when shopping. They pay less attention to nutritional information, and they stated that they use this information to a lesser extent. These consumers showed the least interest in healthy eating, and they reported that they do not have health problems related to their diet. From these results we can derive some practical implications for food companies and public authorities. As most consumers positively value both nutritional claims (74%), we propose to food companies to offer breakfast biscuits with both claims to reach the market segment willing to buy and pay for these nutritional claims. As there is still a segment of consumers with a low level of interest in healthy eating, who are not willing to pay a positive premium for nutritional claims, we can make some suggestions to public authorities to change the preferences of this unwilling group. Our recommendation is to implement educational activities promoting the importance of following a healthy diet to people's health. These activities should focus on male and younger consumers, advising them that, although they do not yet have health problems related to their diet, healthy eating is the best way of preventing them from arising. This could induce changes in the preferences for the nutritional claims, reversing their negative valuation for them.

Another interesting result is that differences were not found among obese, overweight, and normal weight people but among consumers with different interests in healthy eating and with a different prevalence of health problems. This result indicates that the consumer's weight status itself has no effect on the willingness to pay for nutritional claims, while health problems and an interest in following a healthy diet do.

In summary, we found that most of the consumers are willing to pay for the two nutritional claims 'high in fiber' and 'reduced saturated fat', while the rest of the consumers (a quarter) value them negatively. This result is promising for both public and private stakeholders. For public authorities, it means that the use of nutritional claims can lead to healthier diets, which will reduce the prevalence of diet-related health problems for a big part of the population. For food companies, it provides information that they can use to tailor their marketing and advertising campaigns to reach the different segments.

Finally, this work poses some limitations that could undermine the generalization of the results. In particular, the study was only conducted in one European country (Spain) with a small sample. Therefore, to increase the external validity of our experiment, similar studies should be undertaken in other European countries. Moreover, the study of preferences for the two nutritional claims was applied only to one food product, breakfast biscuits. As preferences for nutritional claims may be product-specific, the study should be replicated for other food products.

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Appendix A

Figure A1. Smart Point of Sale for experimental settings.

Table A1. Population by sex and age in Spain and in the town (%).

		Sex		Age				
	Total	**Female**	**Male**	**18–34**	**35–44**	**45–54**	**55–64**	**More than 64**
Spain	46,624,382	50.90	49.10	24.12	20.62	18.56	14.32	22.38
Town	956,006	50.90	49.10	22.34	20.13	18.29	14.68	24.56

Source: Spanish Census of Population, 2015. Instituto Nacional de Estadística (www.ine.es), Spain.

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Validation of an Online Food Frequency Questionnaire against Doubly Labelled Water and 24 h Dietary Recalls in Pre-School Children

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Abstract: The development of easy-to-use and accurate methods to assess the intake of energy, foods and nutrients in pre-school children is needed. KidMeal-Q is an online food frequency questionnaire developed for the LifeGene prospective cohort study in Sweden. The aims of this study were to compare: (i) energy intake (EI) obtained using KidMeal-Q to total energy expenditure (TEE) measured via doubly labelled water and (ii) the intake of certain foods measured using KidMeal-Q to intakes acquired by means of 24 h dietary recalls in 38 children aged 5.5 years. The mean EI calculated using KidMeal-Q was statistically different ($p < 0.001$) from TEE (4670 $\pm$ 1430 kJ/24 h and 6070 $\pm$ 690 kJ/24 h, respectively). Significant correlations were observed for vegetables, fruit juice and candy between KidMeal-Q and 24 h dietary recalls. Only sweetened beverage consumption was significantly different in mean intake ($p < 0.001$), as measured by KidMeal-Q and 24 h dietary recalls. In conclusion, KidMeal-Q had a relatively short answering time and comparative validity to other food frequency questionnaires. However, its accuracy needs to be improved before it can be used in studies in pre-school children.

Keywords: doubly labelled water; food frequency questionnaire; 24 h dietary recalls; pre-school

1. Introduction

Diet is just as important in children as in adults, and possibly even more so because the habits developed in the early years often persist throughout the lifespan [1]. Being able to measure diet in pre-school children is important since childhood obesity often continues into adulthood [2]. An unhealthy diet is a contributing risk factor for some of the most common non-communicable diseases, such as cardiovascular disease, diabetes and cancer [3]. In order to investigate the relationships between diet and disease it is imperative to be able to easily and accurately measure energy and food intake, especially in large epidemiological settings.

Traditional methods to assess energy and food intake are 24 h dietary recalls, dietary history, diet records and food frequency questionnaires (FFQ). However, these are burdensome for both the participants and researchers and their accuracy is limited [4]. Thus, the development of easy-to-use and accurate methods to assess the intake of energy, foods and nutrients in pre-school children are

required. Over the past 10 years, there has been great interest in using telecommunications and computer technology to develop such methods [5]. The use of online questionnaires in epidemiology can be advantageous by allowing for faster response rates, reducing incomplete data via automated controls and increasing the ease of data processing through immediate storage of digital data [6,7].

The LifeGene prospective cohort study is an ongoing epidemiological study in Sweden, which will include approximately 300,000 people, aged 18 to 45. It aims to provide new information on various diseases and health issues affecting society such as cancer, cardiovascular disease, obesity and allergies [8]. LifeGene is currently assessing diet in adult participants using an online questionnaire, MiniMeal-Q. MiniMeal-Q has been validated against doubly labelled water (DLW) and has been shown to be valid for use in epidemiological studies [9]. As there are many differences in the types and amounts of food consumed, it is necessary to develop and validate online questionnaires for other age groups, such as pre-school children.

We included the FFQ KidMeal-Q in the population-based randomized controlled trial called Mobile-based intervention intended to stop obesity in pre-schoolers (MINISTOP). Overall, this trial aims to determine the effectiveness of a six-month mobile-phone-based intervention to improve body composition, dietary habits, physical activity, sedentary behaviour and physical fitness in healthy pre-school children [10]. The specific aims of this nested validation study are to compare: (i) energy intake (EI) obtained using KidMeal-Q to total energy expenditure (TEE) measured via DLW and (ii) the intake of certain foods measured using KidMeal-Q to intakes acquired by means of 24 h dietary recalls in 38 children aged 5.5 years.

2. Materials and Methods

2.1. Participants and Study Design

The MINISTOP trial was based in the county Östergötland in Sweden. A total of 40 parent couples and their children agreed to participate in a validation of dietary intake [11], body composition [12], and physical activity methods at the final follow-up assessment, which began in February 2015 when their children were 5.5 years of age. Details of the recruitment and population have been published previously [11,12]. The age, weight, height, body mass index (BMI), as well as the parental age, BMI and education were comparable between this sample and those in the whole MINISTOP trial (n = 315). Two children had missing information and in total 38 (18 from the intervention group and 20 from the control group) 5.5-year-olds participated in this validation.

This study was conducted according to the guidelines laid down by the Declaration of Helsinki, all procedures involving human subjects were approved by the Research and Ethics Committee in Stockholm, Sweden (2013/1607-31/5; 2013/2250-32), and informed consent was obtained from all parents. The MINISTOP trial is registered as a clinical trial (https://clinicaltrials.gov/ct2/show/NCT02021786).

2.2. Protocol

Parents of the children collected two urine samples at home and brought them to the measurement session at the Linköping University Hospital. The weight and height of the children were recorded when they were wearing minimal clothing and no shoes. Thereafter, the child received a dose of stable isotopes mixed with fruit juice to measure their TEE during the subsequent two-week period. The parents were instructed to collect urine samples on days 1, 5, 10 and 14 after dosing and to note the time of sampling. Within the same two-week period the intake of food and drink was assessed using 24 h dietary recalls. After the measurement at the hospital, all parents received an e-mail with a link to the online FFQ KidMeal-Q and were instructed to fill it in directly after the visit.

2.3. Energy Expenditure

TEE has been measured as previously described [11]. Briefly, each child was given an accurately weighed dose of stable isotopes, 0.14 g $^{2}H_2O$ and 0.35 g $H_2{}^{18}O$, per kg body weight. Five urine samples

were collected (on days 1, 5, 7, 10 and 14), stored and analysed for isotope enrichments as previously described [11]. CO_2 production was calculated according to Davies et al. [13], assuming that 27.1% of the total water losses was fractionated [13,14]. TEE was calculated by means of the Weir equation [15], assuming a food quotient of 0.85 [16]. The mean change in body weight from day one to 14 was 0.07 ± 0.32 kg.

2.4. KidMeal-Q

KidMeal-Q is an online meal-based FFQ designed for pre-school children aged three to six. This FFQ measures the child's dietary intake over the past couple of months and includes between 42 and 86 food items, drinks and dishes, depending on the number of follow-up questions. The following pre-defined frequency categories were used: for breakfast food items as well as fruit (1 time/day, 2 times/day or more, 1–2 times/week, 3–6 times/week, 1–3 times/month), for dishes, snacks as well as sweets (1–2 times/week, 3–6 times/week, 7 times/week or more, 1–3 times/month), and for vegetables (1 time/day, 2 times/day, or 3 times a day or more). See the Supplementary Materials for the questions provided in KidMeal-Q. For each of the following food groups, five photos of portion sizes were included: (1) rice, potatoes and pasta; (2) meat, chicken, fish and vegetarian substitutes; and (3) vegetables (raw or cooked).The photos were used to calculate portion sizes for cooked dishes and vegetables. For other food items, standard portions were used. EI was calculated from reported intakes of food items and dishes by linkage to the food composition database provided by the National Food Administration [17] by means of KidMealCalc (Epiqcenter, Stockholm, Sweden), a software developed and validated for this purpose. The grams of fruits, vegetables, fruit juice, sweetened beverages, candy, ice cream and bakery products were then summarized. These foods were selected as they represent healthy and unhealthy food habits relevant for childhood obesity [11].

2.5. 24 h Dietary Recalls

Four 24 h dietary recalls were performed over the phone in the two-week period following the measurement at the hospital, as published previously [11]. The days used in the 24 h dietary recalls were scheduled with the parents when they were at the hospital for measurements. Briefly, each parent was asked to recall the foods and beverages their child consumed. Information on the type of food products used in mixed dishes and the cooking method was recorded. The portion sizes were reported by the parents using household measurements (decilitres, tablespoons or teaspoons). Words such as slices or pieces were used for other foods such as bread, candy or potatoes. The reported intakes were converted into grams using a standardized weight table provided by the Swedish Food Agency [18] and the grams of fruits, vegetables, fruit juice, sweetened beverages, candy, ice cream and bakery products were then summarized. EI and nutrients were calculated from reported intakes of foods and beverages by linkage to the food composition database [17].

2.6. Statistics

Values are given as means and standard deviations (SD). Significant differences between mean values were identified using paired samples *t*-tests and the Wilcoxon Signed Rank test for parametric data (EI, TEE and selected nutrients) and non-parametric data (food groups). Pearson or Spearman correlations were used to evaluate relationships between variables. The Bland and Altman procedure [19] was used to compare EI using KidMeal-Q to TEE measured via DLW. Thus, the difference (y) between EI and TEE was plotted versus the average of the two estimates (x). The mean difference with $\pm$2SD (limits of agreement) were then calculated. To test for a relationship between x and y in the Bland and Altman plot, linear regression was used. Significance (two-sided) was accepted when $p < 0.05$. Analyses were performed using SPSS version 23 (IBM, Armonk, NY, USA).

The classification capacity of KidMeal-Q was assessed using TEE. This was done by ranking EI (KidMeal-Q) and TEE (DLW) in a sequence. Thus, the children with the lowest EI and TEE had the lowest number and the difference between this child and the second in the sequence was the

smallest possible. This principle of the smallest possible difference was maintained for all children, producing a sequence with gradually increasing values. The children were then divided into tertiles (low, medium and high) with increasing values. The classification capacity for KidMeal-Q was then evaluated as the number of children placed in the same (0), in the next higher (+1) or lower (−1) and in the second next higher (+2) or lower (−2) group.

3. Results

The descriptive characteristics and the energy expenditure for the 38 children (22 boys and 16 girls) are displayed in Table 1. There were no significant differences in anthropometric measures between boys and girls and therefore all analyses are presented for boys and girls combined. There was a wide range for weight and energy expenditure for the children. The parents were highly educated, with between 65%–75% of the parents having a university degree.

Table 1. Age, weight, height and energy expenditure of the study participants (n = 38).

Variables	Mean ± SD	Range
Age (years)	5.5 ± 0.1	5.2–5.7
Weight (kg)	20.6 ± 4.3	14.9–35.8
Height (cm)	114 ± 5	105–126
BMI (kg/m^2)	15.6 ± 2.3	13.3–25.6
TEE (kJ/24 h)	6070 ± 690	4910–7700
EI (kJ/24 h) [1,3]	4670 ± 1430	1810–7480
EI (kJ/24 h) [2]	6025 ± 665	4515–7530

SD, standard deviation; BMI, body mass index; TEE, total energy expenditure measured via doubly labelled water; BW, body weight; EI, energy intake. [1] EI assessed using KidMeal-Q; [2] EI assessed using 24 h dietary recalls; [3] Significantly lower than TEE as well as EI from 24 h dietary recalls (both $p < 0.001$).

On average it took the parents of the participating children 13.2 ± 6.2 min to complete KidMeal-Q. The mean EI calculated using KidMeal-Q was statistically different ($p < 0.001$) from TEE assessed via DLW. The mean EI was 4670 ± 1430 kJ/24 h and TEE was 6070 ± 690 kJ/24 h. Figure 1 displays the Bland and Altman Plot for EI assessed using KidMeal-Q to TEE measured using DLW. The limits of agreement were wide and a significant association was found for the average and difference (r = 0.711, $p < 0.001$). A significant trend was found, showing that lower EIs were underestimated to a greater extent. In comparison to TEE, KidMeal-Q underestimated EI in 84.2% (n = 32).

A significant correlation was found between EI (KidMeal-Q) and TEE (DLW), r = 0.320 (p = 0.05). When dividing the children into tertiles (low, medium and high) for EI and TEE 42.1% (n = 16) were classified correctly, 47.4% (n = 18) were classified plus or minus one group, and 10.5% (n = 4) were classified plus or minus two groups.

Table 2 shows the mean intakes and the correlations for the seven foods and drinks assessed using KidMeal-Q and 24 h dietary recalls. Only sweetened beverage consumption was significantly different in mean intake ($p < 0.001$) as measured by KidMeal-Q and 24 h dietary recalls. Significant correlations were observed for vegetables, fruit juice and candy between the two methodologies. Table 3 displays the mean intakes and correlations for selected nutrients estimated using KidMeal-Q and 24 h dietary recalls. For the percentage of energy obtained from the macronutrients no significant differences were observed, however a significant difference in percent energy from sucrose ($p < 0.001$) was found. Significant differences were also found for fibre and calcium (both $p < 0.001$). Significant correlations were found for the majority of the selected nutrients. EI from KidMeal-Q and the 24 h dietary recalls were correlated (r = 0.532, p = 0.001).

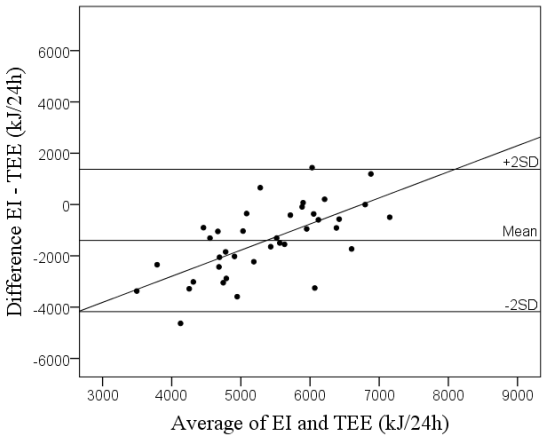

Figure 1. Bland and Altman Plot comparing energy intake using KidMeal-Q and total energy expenditure calculated using doubly labelled water in 38 children aged 5.5 years. The mean difference between methods was −1400 kJ/24 h with limits of agreement (2SD) of 2775 kJ/24 h.

Table 2. Mean intakes and correlations for the selected foods and beverages estimated by KidMeal-Q and 24 h dietary recalls (n = 38).

	KidMeal-Q (g/Day)	24 h Dietary Recall (g/Day) [1]	p-Value [2]	Rho [3]	p-Value [4]
Fruit	91 ± 44	111 ± 76	0.126	0.306	0.062
Vegetables	73 ± 63	67 ± 53	0.874	0.603	<0.001 *
Fruit juice	44 ± 72	45 ± 90	0.764	0.350	0.031 *
Sweetened beverages	7 ± 12	89 ± 94	<0.001 *	0.301	0.067
Candy	12 ± 11	15 ± 16	0.122	0.441	0.006 *
Ice cream	6 ± 8	11 ± 15	0.156	0.295	0.072
Bakery products	13 ± 20	17 ± 16	0.069	0.102	0.542

[1] Number of recorded days using 24 h dietary recalls: four days (n = 26, 68%), three days (n = 6, 16%), two days (n = 4, 11%), and one day (n = 2, 5%); [2] p-value is the difference between the intake between KidMeal-Q and 24 h dietary recalls calculated using the Wilcoxon Signed Ranks test; [3] Spearman rank order correlation; [4] p-value for the Spearman rank order correlation (rho). * Significant difference observed ($p < 0.05$).

Table 3. Mean intakes and correlations for selected nutrients estimated by KidMeal-Q and 24 h dietary recalls (n = 38).

	KidMeal-Q	24 h Dietary Recall	p-Value [1]	r [2]	p-Value [3]
Protein (E% [4])	16 ± 2	16 ± 2	0.693	0.537	0.001 *
Fat (E% [4])	30 ± 3	31 ± 4	0.398	0.384	0.017 *
Carbohydrates (E% [4])	52 ± 4	52 ± 5	0.707	0.325	0.047 *
Saturated fat E(% [4])	13 ± 2	13 ± 2	0.877	0.279	0.090
Monounsaturated fat (E% [4])	11 ± 1	11 ± 2	0.145	0.282	0.087
Polyunsaturated fat (E% [4])	3.9 ± 0.6	3.9 ± 1.1	0.726	0.104	0.534
Sucrose (E% [4])	7 ± 2	11 ± 4	<0.001 *	0.459	0.004 *
Fibre (g/MJ)	3.0 ± 0.8	2.2 ± 0.6	<0.001 *	0.603	<0.001 *
Vitamin A (ug/MJ)	97 ± 30	91 ± 63	0.562	0.467	0.003 *
Vitamin D (ug/MJ)	0.78 ± 0.21	0.67 ± 0.36	0.051	0.426	0.008 *
Vitamin E (ug/MJ)	1.00 ± 0.15	0.95 ± 0.29	0.272	0.430	0.007 *
Thiamin (mg/MJ)	0.18 ± 0.03	0.17 ± 0.05	0.065	0.460	0.004 *
Vitamin C (mg/MJ)	13 ± 6	11 ± 7	0.115	0.610	<0.001 *
Vitamin B12 (ug/MJ)	0.65 ± 0.19	0.61 ± 0.25	0.364	0.439	0.006 *
Calcium (mg/MJ)	143 ± 40	113 ± 28	<0.001 *	0.491	0.002 *
Iron (mg/MJ)	1.5 ± 0.4	1.3 ± 0.5	0.069	0.486	0.002 *

[1] p-value is the difference between the intake between KidMeal-Q and 24 h dietary recalls calculated using a paired samples t-test; [2] Pearson correlation coefficient; [3] p-value for the Pearson correlation coefficient (r); [4] Percent energy of total energy intake. * Significant difference observed ($p < 0.05$).

4. Discussion

KidMeal-Q is an interactive and user-friendly questionnaire with a relatively short answering time and has comparable validity to other corresponding epidemiological tools. KidMeal-Q underestimated

EI in the majority of children. However, in regards to the seven investigated food groups only one significant difference was found (sweetened beverages) for the mean intakes assessed using KidMeal-Q and 24 h dietary recalls.

KidMeal-Q is quick and simple for parents to respond to, which increases user-friendliness and the likelihood of completion [20]. On average a FFQ takes between 30 to 60 min [21], while KidMeal-Q took only a quarter to a half of that time. A systematic review and meta-analysis conducted by Edwards et al. [20] found that response rates are inversely related to the questionnaire length, and found an even stronger relationship in extremely short questionnaires. Time is not the only factor that affects user-friendliness; the wording and layout of the questions also plays a large role [22]. The interactive design of KidMeal-Q allows for easy navigation throughout the questionnaire through prompts, error messages and letting participants skip irrelevant questions, all of which increases completion rates [6,23]. The short answering time and interactive design of the questionnaire allows it to be used on a large number of people as well as in epidemiological studies, where dietary habits are not part of the main research question and only play a small part in the study.

As found with other FFQs [9,24–27] KidMeal-Q had wide limits of agreement, demonstrating that it is not a valid tool on an individual level. In comparison with TEE from DLW KidMeal-Q underestimated EI by 23%. One FFQ in pre-school children overestimated EI in comparison to TEE from DLW by 59% [28] and two others underestimated EI by 3% [26] and 5% [25]. Similar to KidMeal-Q, two of the FFQs [26,28] were conducted under unsupervised conditions, whereas that by Collins et al. [25] was conducted in a supervised setting, possibly allowing for the questionnaire to better predict EI through allowing parents to ask questions and clarify statements. The more questions in the Dutman et al. FFQ are more detailed [26], as demonstrated by its longer answering time of 25 min, may have led to a more accurate reporting of EI intake in comparison to KidMeal-Q. KidMeal-Q differed from the aforementioned FFQ in terms of how the parents reported their child's food intake, online versus pen and paper, which also may have led to the observed differences. The underestimation of EI could possibly be due to the questionnaire itself; for instance, the portion sizes provided were perhaps too small and thus led to the observed underestimation. It is important to note that KidMeal-Q underestimated sweetened beverages on average by 82 grams per day. This amount corresponds to approximately 1370 kJ, which is a considerable amount of energy. As FFQs have been shown to both under- and overestimate EI, more research needs to be conducted to improve the accuracy of these tools. Further work should focus on examining the provided portion sizes as well as gain additional understanding of parental reporting of dietary data. Specifically for KidMeal-Q, a revision of the questions regarding sweetened beverages is required.

Even though correlations are not optimal when evaluating methods, they are often used when comparing dietary assessment methods. In this study a significant moderate correlation was found between EI from KidMeal-Q and TEE measured with DLW, with similar results being found in other studies [9,24]. However, the correlation was lower than Kroke et al. [27] and Dutman et al. [26], but higher than Collins et al. [25] and Perks et al. [29]. Four of the six FFQs studied [9,24,27,29] were conducted in an adult or youth population and were traditional paper-based questionnaires, except for Christensen et al. [9]. A stronger correlation was found in the Dutman et al. [26] study; however, this may be attributed to the fact that they extensively reviewed their FFQ results and contacted parents about peculiar answers, which should provide more accurate estimates of EI. KidMeal-Q demonstrated a decent ranking ability compared to DLW, which is similar to other studies [9,26,27]. In regards to the correlations for the seven investigated food groups, they are also similar to those found in previous studies in pre-school children [30,31].

A strength of this validation study was the use of DLW as a reference method, which is considered the gold standard for assessing TEE and recommended for usage when validating EI [32]. Furthermore, the use of 24 h dietary recalls allowed us to assess KidMeal-Q's ability to evaluate certain food groups, which is of great importance in epidemiological studies. This study was limited by the fact that TEE was the average of 14 days, while KidMeal-Q assessed dietary habits over the past couple of

months; however, the day-to-day variation in TEE is low [33,34], thus we do not think this fact has largely influenced our results. We were unable to obtain four 24 h dietary recalls from all participants; however, when we re-ran the analyses including only children with four 24 h dietary recalls (n = 26), our conclusions remained the same. Furthermore, this nested validation study was conducted at the final follow-up within the MINISTOP trial and the parents in the intervention group were given advice on how to make their child's diet more healthy, which could have affected how they answered the FFQ. We do not believe this is an issue as there were no significant differences in EI as measured by KidMeal-Q or 24 h dietary recalls, TEE, or the food groups between the children in the intervention and control group. Additionally, the majority of the 24 h dietary recalls were weekend days as the MINISTOP trial targeted the home environment. However, as we have stated previously [11], we do not believe this is a major issue because the majority of Swedish parents with a child this age work and would have their child in daycare, so when they filled out the FFQ they would more than likely be filling it out with their child's food habits from the home environment. This study also had a relatively small sample size (n = 38) and only four 24 h dietary recalls were applied. Finally, the fact that the parents were on average more highly educated than the general Swedish population may limit the generalizability of the results.

In conclusion, the online FFQ, KidMeal-Q, has been demonstrated to be interactive and user-friendly. It has a relatively short answering time and has comparative validity to other FFQs. However, more work is needed to further improve the questionnaire's accuracy before it can be used in studies in pre-school children.

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Author Contributions: M.L. is the Principal Investigator for the MINISTOP trial. C.D.N. was responsible for data collection and data analysis, and drafted the manuscript. H.H. contributed to data collection. C.A. contributed to data analysis. K.B. developed the KidMeal-Q questionnaire. S.B. was responsible for data analysis for KidMeal-Q. A.B. contributed to the development of the KidMeal-Q questionnaire. The manuscript was then subsequently reviewed by H.H., C.A., A.B., S.B., K.B. and M.L. and all authors approved the final version.

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Abbreviations

The following abbreviations are used in this manuscript:

DLW	Doubly labelled water
EI	Energy intake
FFQ	Food frequency questionnaire
SD	Standard deviation
TEE	Total energy expenditure

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Dietary Fatty Acids and Changes in Blood Lipids during Adolescence: The Role of Substituting Nutrient Intakes

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Abstract: The relevance of dietary fatty acids (FA) for blood lipids should be assessed in the context of substituting nutrients. Such evidence is lacking for adolescents. This study describes prospective associations of dietary FA with changes in serum lipids during adolescence, and considers the theoretical isocaloric replacements of saturated FA (SFA) with other FA or carbohydrates (CHO). Children from the GINIplus and LISAplus birth cohorts, with data on FA intakes (at age 10 years) and serum lipids (at age 10 and 15 years), were included (n = 1398). Associations of SFA, monounsaturated FA (MUFA), n-3 polyunsaturated FA (n-3 PUFA) and n-6 PUFA, with changes in low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides (TAG), and total cholesterol to HDL ratio (TOTAL:HDL), were assessed by linear regression. Substitution models assessed isocaloric replacements of SFA with MUFA, n-3 PUFA, n-6 PUFA or CHO. Higher SFA intakes were associated with decreasing TAG. No associations were observed for fatty acid intakes with LDL, HDL or TOTAL:HDL. In females, replacing SFA with CHO was associated with increasing LDL, TAG and TOTAL:HDL. Our findings confirm observations in adults, although sex-specific determinants seem relevant in our adolescent population. Overlooking the nutrient context when limiting SFA intakes might have detrimental consequences appreciable as early as adolescence.

Keywords: fatty acids; lipids; isocaloric substitution; diet; carbohydrates; adolescence; epidemiology

1. Introduction

Since the first appearance of evidence suggesting a detrimental role of saturated fatty acids (SFA) in the development of coronary heart disease [1,2], the advice to reduce SFA consumption has become a major component of health-promoting strategies [3]. Nevertheless, inconsistent findings among emerging studies have led scientists to question the independent association of SFA with the development of cardiovascular disease (CVD) [4–6]. It has become clear that evidence supporting

a reduction of SFA intake must be interpreted in the context of the specific nutrients consumed in its place [7,8]. In 2008, the FAO and the WHO stated convincing evidence for an improved lipoprotein profile in adults when replacing SFA with polyunsaturated fatty acids (PUFA) and, to a lesser extent, with monounsaturated fatty acids (MUFA). On the other hand, replacing SFA with carbohydrates (CHO) was reported to reduce low-density lipoprotein (LDL) but also high-density lipoprotein (HDL) levels [9].

It is currently well established that CVD risk factors progress from childhood and adolescence into adulthood [10]. Results from numerous longitudinal cohort studies have indicated strong tracking of serum lipids from childhood to adulthood [11–13]. Considering the implications this can have for later disease development, improving our understanding of the role of dietary fatty acid intakes in children is of major importance for the early implementation of dietary advice. The period concerning pubertal development is of interest due to the rapid growth and development as well as behavioral changes occurring at this stage [14,15]. However, despite the growing evidence in adults [16–18], the amount of reliable and comparable data on dietary fatty acid intakes in children and adolescents is scarce [19]. Studies observing the associations of total [20–22] and saturated fat [23,24] with blood lipid concentrations have reported mixed results. In particular, longitudinal studies on the theoretical implication of different replacements of SFA on lipid profiles in children and adolescents are lacking. A 2002 study using repeated measures at ages 8 and 11 years, suggested associations with serum lipids similar to those observed in adults when replacing SFA with MUFA or PUFA [25]. Further studies are required to learn whether such associations persist during the period of pubertal development.

The current study therefore aims to describe the prospective associations of fatty acid intakes during childhood with changes in serum lipid concentrations during adolescence. Furthermore, we are interested in observing how associations with SFA may depend on the choice of substituting nutrient. We therefore consider changes in blood lipids following the theoretical reduction of SFA in the context of different isocaloric replacements with other fatty acids or with carbohydrates.

2. Materials and Methods

2.1. Participants

The present study used data from the 10- and 15-year follow-up assessments of the ongoing GINIplus (German Infant Nutritional Intervention plus environmental and genetic influences on allergy development) and LISAplus (Influence of Lifestyle-Related Factors on the Immune System and the Development of Allergies in Childhood plus the Influence of Traffic Emissions and Genetics) birth cohort studies. Healthy full-term newborns were recruited from obstetric clinics in four German cities. Information was collected using identical questionnaires and at physical examinations. The study designs, recruitment and exclusion criteria have been described previously [26,27]. For both studies, approval by the local ethics committees (Bavarian General Medical Council, University of Leipzig, Medical Council of North-Rhine-Westphalia) and written consent from participants' families were obtained.

2.2. Dietary Intake

Dietary intake data were collected at the 10-year follow-up assessment, using a self-administered food frequency questionnaire (FFQ) designed to assess food and nutrient intake over the past year in school-aged children, and validated to estimate energy, fatty acid and antioxidant intake [28]. In brief, subjects were asked to report estimated frequency and portion size of intakes of 80 food items. A quality control procedure was applied based on recommendations by Willett et al. for data cleaning in nutritional epidemiology [29,30]. Total daily energy intake and the intakes of SFA, MUFA, *n*-6 and *n*-3 PUFA, protein, carbohydrate and alcohol were calculated (in kcal/day) based on the German Food Code and Nutrient Database (BLS) version II.3.1 [31]. Each nutrient was expressed as its percentage contribution towards total daily energy intake (%EI), calculated as the ratio of energy from each nutrient to total daily energy intake, multiplied by 100.

2.3. Blood Lipids

Blood samples were obtained during the 10- and 15-year follow-up physical examinations. The concentrations (mmol/L) of total cholesterol, LDL, HDL, and triglycerides (TAG) were measured in serum using homogenous enzymatic colorimetric methods on a Modular Analytics System from Roche Diagnostics GmbH Mannheim according to the manufactures instructions. External controls were used in accordance with the guidelines of the German Society of Clinical Chemistry and Laboratory Medicine. The ratio of total to HDL cholesterol (TOTAL:HDL) was calculated by dividing total cholesterol by HDL.

2.4. Statistical Analyses

Participants with complete data on FA intakes at age 10 years, serum lipids at age 10 and 15 years, and all adjustment variables were included in the study (Figure 1). To test for differences due to attrition bias, we compared characteristics of participants lost to follow-up (data only available for exposure and outcome at 10 years) to those included in the present study analyses, who adhered at follow-up (data available for exposure at 10 years and outcome at 10 years and 15 years). Categorical variables, presented as percentages, were tested by Fisher's exact test (binary variables) or Pearson's Chi-squared test (variables with more than 2 levels). Continuous variables, presented as means (standard deviation), were tested by Student's *t*-test.

Statistical analyses were carried out in the total population and stratified by sex. Subject characteristics at ages 10 and 15 years were described by medians (25th percentile; 75th percentile) or counts (%). Differences from 10 to 15 years were tested using paired Wilcoxon signed rank test for continuous variables and McNemar's χ^2-test for categorical variables. Differences in characteristics between males and females at each assessment were tested using Wilcoxon signed rank test for continuous variables and χ^2-test for categorical variables (Fisher's exact test for binary variables). Changes (Δ) in lipid concentrations and in TOTAL:HDL ratio were calculated by subtracting each measurement at the 10-year follow-up from its respective measurement at the 15-year follow-up.

Using linear regression, two modelling approaches were applied. First, single nutrient models were fit to observe the changes in blood lipids when increasing habitual intakes of a single nutrient at a constant energy intake. Intakes of different fatty acids assessed at age 10 years were considered as the exposures of interest. Separate regression models were run for each exposure (SFA, MUFA, *n*-3 PUFA or *n*-6 PUFA) with the different blood lipid parameters (ΔLDL, ΔHDL, ΔTAG, ΔTOTAL:HDL). Through this prospective approach, we aim to avoid any misleading findings emerging from the possible bidirectional relationship between fatty acid intakes and blood lipids assessed at a single time-point only. Second, substitution models were fit to observe the effect of replacing SFA with other fatty acids (MUFA, *n*-6 PUFA and *n*-3 PUFA) or with CHO, on the different blood lipid parameters (ΔLDL, ΔHDL, ΔTAG, ΔTOTAL:HDL). These models included the exposure nutrient of interest as well as all other energy-bearing nutrients except SFA (the nutrient being "replaced"). In this way, energy intakes of protein, carbohydrate, alcohol and other fats are held constant; and by additionally including total energy intake in the model it is possible to interpret the resulting coefficients for each nutrient as its theoretical substitution for an equal amount of energy (%EI) from saturated fat, being the only energy-bearing nutrient not accounted for in the model. All models were adjusted for potential covariates in two steps. First, we adjusted for basic covariates (M_{BASIC}): study (GINI observation arm; GINI intervention arm; LISA), recruitment region (Munich; Wesel; Bad Honnef; Leipzig), sex (male; female)—not in sex-stratified models—exact age at 10-year blood sampling (years), fasting status at blood sampling (not fasted (46%); fasted at one assessment (45%); fasted at both assessments (9%)), BMI (kg/m^2) at age 10 years, screen-time (daily hours spent on activities in front of a screen: $\leq$2 h = low; >2 h = high) at age 10 years, total energy intake (kcal/day) at age 10 years, and lipid concentration (mmol/L) at age 10 years. In a second step, models were further adjusted for other potential confounders (M_{ADJ}): parental education level (highest level achieved by mother or father: $\leq$10th grade = low/medium; >10th grade = high) and pubertal onset at age

10 years (oestrogen $\geq$ 18.5 pmol/L or testosterone $\geq$ 0.1 nmol/L = yes; oestrogen < 18.5 pmol/L or testosterone < 0.1 mmol/L = no). Given the high intercorrelation typically present amongst dietary components [32], we calculated correlation coefficients between pairs of nutrient variables, using Pearson's product-moment correlation coefficient. A high negative correlation was observed between MUFA and CHO. By linearly regressing MUFA onto CHO and vice-versa, we computed residuals ($MUFA_{RESID}$ and CHO_{RESID}), which were uncorrelated with each other [33]. In order to avoid multicollinearity, these were included in the models as a stand-in for the original variable only when acting as a covariate (i.e., when assessing the effect of replacing SFA with CHO, CHO was included in its original form as the main predictor variable, and $MUFA_{RESID}$ was included in place of MUFA, along with all other covariates, and vice versa). Results from the linear regression analyses are presented as regression coefficients (β) per interquartile range (IQR) increase in the relevant exposure variable, along with their 95% confidence interval (95% CI). A two-sided α-level of 5% was considered significant for the total population analyses. For the sex-stratified analysis we corrected for multiple testing using Bonferroni correction: the α-level was divided by 2 (2.5%) as the dataset was analyzed by sub-groups of two levels (male/female). Statistical analyses were conducted using R (www.r-project.org), version 3.3.0 [34].

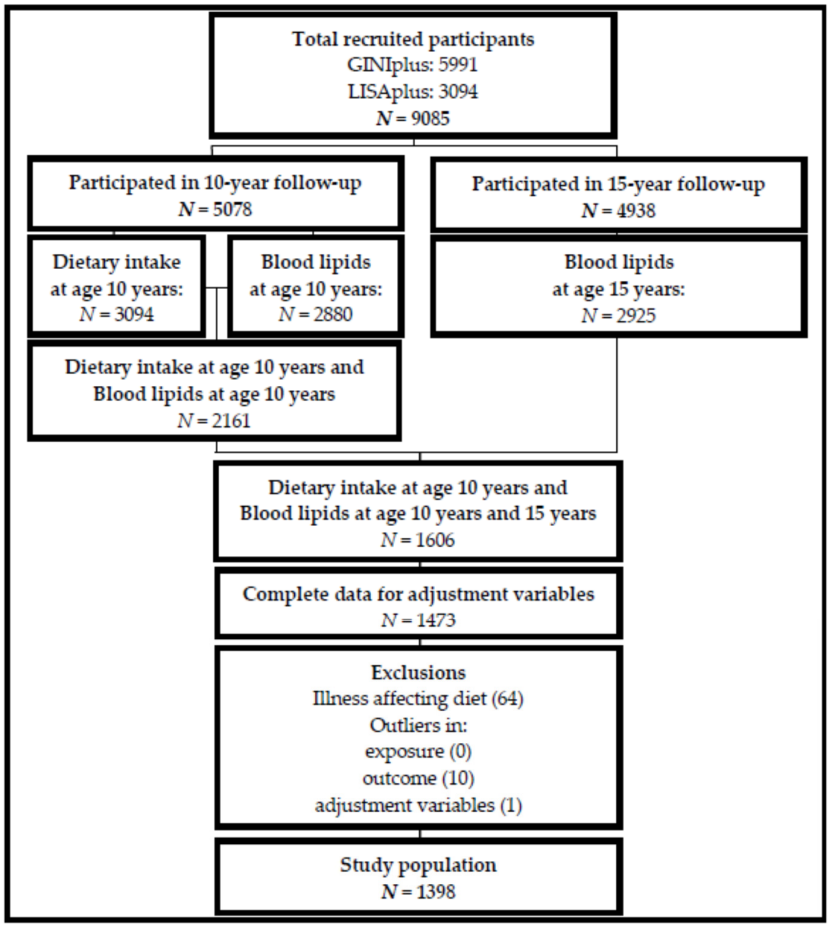

Figure 1. Study participants Dietary intake: intakes of fatty acids (saturated, monounsaturated, *n*-3 polyunsaturated, *n*-6 polyunsaturated), carbohydrate, protein, and alcohol obtained from FFQ; Blood lipids: low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides; adjustment variables: study, region, age, fasting status, BMI, screen-time, total energy intake, lipid concentration at age 10 years, parental education and pubertal onset; Illness affecting diet: e.g., diabetes, anorexia, coeliac disease, cancer, or medical dietary indications (e.g., gluten-free, lactose-free diets).

3. Results

3.1. Study Population

The present analyses comprised of 1398 participants (681 females and 717 males). The derivation of the study population is presented in Figure 1. Subjects providing complete dietary intake data at age 10 years, measures of serum lipids at both 10 and 15 years, as well as information on all adjustment variables, were included (n = 1473). Differences in descriptive characteristics between participants included in the analyses and participants lost to follow-up are presented in the Table S1). Participants were excluded if they reported an illness affecting diet (e.g., diabetes, anorexia, coeliac disease, cancer) or medical dietary indications, such as gluten-free or lactose-free diets (n = 64). Clear outliers in blood lipid concentrations (n = 10), or adjustment variables (n = 1) were visually identified using descriptive plots and excluded from the analyses. Basic characteristics of the study population at age 10 and 15 years are described in Table 1. Both sexes had higher levels of LDL and HDL and lower levels of TAG and TOTAL:HDL at age 15 years compared to 10 years. Significant differences over time were observed for BMI, fasting status at blood sampling, screen-time and total daily energy intake, with higher values at age 15 years in both sexes (except energy intake, which decreased in females). Males reported higher screen-time and daily energy intake than females at both time-points, as well as higher fat and protein intakes at age 15. On the other hand, females at age 15 years reported higher carbohydrate intakes. Overall, most participants were from Munich (57.7%) with a high parental education (71.3%). Notably, more females than males had reached pubertal onset at the age of 10 years (74.4% females vs. 24.1% males).

3.2. Single Nutrient Models

The prospective associations of dietary fatty acid intakes (in %EI) at age 10 years with changes in serum lipid concentrations from age 10 to age 15 years are described in Table 2. Values are presented for basic (M_{BASIC}) and fully adjusted models (M_{ADJ}). The resulting β-coefficients indicate the changes in blood lipid concentrations (mmol/L) per IQR increase in the %EI of a given fatty acid, while maintaining total energy intake constant. A significant inverse association was observed between the intake of SFA (IQR increase in %EI) at age 10 years and the change in TAG concentrations from age 10 to age 15 years (M_{ADJ}: β = −0.038 (95% CI = −0.075; −0.001), p-value = 0.042). A similar association was observed in females only, which was borderline statistically significant when corrected for multiple testing (M_{ADJ}: −0.053 (−0.100; −0.007), p-value = 0.025). No associations were observed for any of the fatty acid exposures with the other assessed blood lipid parameters.

3.3. Substitution Models

Table 3 shows the prospective associations of different dietary fatty acid intakes and CHO at age 10 years with changes in serum lipid concentrations from age 10 to age 15 years, when considering their theoretical substitution for SFA. Values are presented for basic (M_{BASIC}) and fully adjusted models (M_{ADJ}). Coefficients (β) obtained from these models represent an isocaloric substitution, i.e., the change in blood lipid concentrations when theoretically replacing the intake of SFA with another (specific) fatty acid or CHO, while maintaining total energy intake constant. A direct association was observed in the basic model for the substitution of CHO (IQR increase in %EI) for SFA with ΔLDL (M_{BASIC}: 0.063 (0.000; 0.127), p-value = 0.05). Sex-stratified analyses indicated significant associations in females only, after correction for multiple testing: direct associations were observed for the substitution of CHO (IQR increase in %EI) for SFA with ΔLDL (M_{ADJ}: 0.125 (0.021; 0.229), p-value = 0.019), ΔTAG (M_{ADJ}: 0.098 (0.020; 0.176), p-value = 0.014), and ΔTOTAL:HDL (M_{ADJ}: 0.115 (0.015; 0.215), p-value = 0.024).

Table 1. Basic characteristics of the study population.

Variables	Total (N = 1398)			Females (N = 681)			Males (N = 717)		
	10 Years	15 Years	p-Value [a]	10 Years	15 Years	p-Value [a]	10 Years	15 Years	p-Value [a]
Blood lipids									
LDL (mmol/L)	2.1 (1.7; 2.5)	2.3 (1.9; 2.7)	<0.01	2.1 (1.8; 2.5) [†]	2.4 (2.0; 2.9) [†]	<0.01	2.0 (1.7; 2.5)	2.2 (1.8; 2.6)	<0.01
HDL (mmol/L)	1.2 (1.1; 1.4)	1.5 (1.2; 1.7)	<0.01	1.2 (1.1; 1.4)	1.6 (1.4; 1.8) [†]	<0.01	1.3 (1.1; 1.5) [§]	1.4 (1.2; 1.6)	<0.01
TAG (mmol/L)	1.2 (0.9; 1.6)	1.0 (0.8; 1.4)	<0.01	1.2 (0.9; 1.6)	1.0 (0.8; 1.3)	<0.01	1.1 (0.8; 1.6)	1.0 (0.7; 1.4)	<0.01
TOTAL:HDL	3.8 (3.2; 4.5)	2.9 (2.5; 3.4)	<0.01	3.9 (3.4; 4.6) [†]	2.9 (2.5; 3.4)	<0.01	3.6 (3.2; 4.4)	3.0 (2.5; 3.5) [§]	<0.01
Fatty acids									
SFA (%EI)	12.6 (10.9; 14.7)	12.7 (10.8; 14.7)	0.621	12.5 (10.7; 14.7)	12.6 (10.6; 14.6)	0.190	12.8 (11.1; 14.8)	12.9 (10.9; 14.9)	0.512
MUFA (%EI)	10.7 (9.3; 12.3)	10.8 (9.4; 12.3)	0.133	10.7 (9.2; 12.1)	10.5 (9.1; 12.2)	0.608	10.7 (9.5; 12.4)	11.2 (9.6; 12.6) [§]	<0.01
n-3 PUFA (%EI)	0.54 (0.49; 0.62)	0.56 (0.49; 0.65)	<0.01	0.55 (0.49; 0.63)	0.57 (0.49; 0.64)	0.009	0.54 (0.48; 0.62)	0.56 (0.48; 0.65)	<0.01
n-6 PUFA (%EI)	3.7 (3.2; 4.3)	3.9 (3.3; 4.6)	<0.01	3.7 (3.2; 4.3)	3.9 (3.3; 4.7)	0.002	3.7 (3.2; 4.3)	3.9 (3.3; 4.6)	<0.01
Covariates									
Age (years)	10.2 (10.1; 10.3)	15.1 (15.0; 15.3)	<0.01	10.2 (10.1; 10.3)	15.1 (15; 15.3)	<0.01	10.2 (10.1; 10.3)	15.1 (15; 15.3)	<0.01
BMI (kg/m^2)	16.7 (15.6; 18.4)	20.2 (18.7; 22.2)	<0.01	16.8 (15.6; 18.5)	20.4 (18.9; 22.3)	<0.01	16.7 (15.7; 18.4)	20 (18.6; 22.1)	<0.01
Fasting (yes)	237 (17.0)	649 (46.4)	<0.01 [b]	121 (17.8)	309 (45.2)	<0.01 [b]	116 (16.2)	341 (47.6)	<0.01 [b]
Screen-time (high)	134 (9.6)	763 (55.3)	<0.01 [b]	48 (7.0)	322 (47.8)	<0.01 [b]	86 (12.0) [§]	442 (62.5) [§]	<0.01 [b]
Energy intake (kcal)	1933 (1591; 2292)	2011 (1584; 2532)	<0.01	1798 (1486; 2124)	1734 (1360; 2115)	0.016	2061 (1705; 2447) [§]	2361 (1884; 2866) [§]	<0.01
Fat (%EI)	30.1 (26.7; 34.2)	30.5 (27.1; 34.8)	0.128	29.9 (26.1; 33.9)	30.0 (26.5; 34.2)	0.982	30.2 (27.3; 34.4)	30.9 (27.6; 35.3) [§]	0.029
Carbohydrate (%EI)	54.1 (49.6; 58.0)	53.2 (48.6; 57.7)	0.004	54.3 (49.7; 58.4)	54.1 (49.1; 58.4) [†]	0.696	53.7 (49.6; 57.5)	52.4 (47.7; 56.7)	<0.01
Protein (%EI)	14.5 (12.9; 16.0)	14.8 (13.1; 16.6)	<0.01	14.4 (12.8; 16.1)	14.5 (12.7; 16.3)	0.452	14.5 (13.1; 16.0)	15.1 (13.4; 16.8) [§]	<0.01
Study									
GINI observation	452 (32.3)			221 (32.5)			231 (32.2)		
GINI intervention	437 (31.3)			224 (32.9)			213 (29.7)		
LISA	509 (36.4)			236 (34.7)			273 (38.1)		
Region									
Munich	807 (57.7)			389 (57.1)			418 (58.3)		
Leipzig	123 (8.8)			60 (8.8)			63 (8.8)		
Bad Honnef	65 (4.6)			29 (4.3)			36 (5.0)		
Wesel	403 (28.8)			203 (29.8)			200 (27.9)		
Parental education (High)	997 (71.3)			497 (73.0)			500 (69.7)		
Pubertal onset (Yes)	680 (48.6)			507 (74.4) [†]			173 (24.1)		

Values are medians (25th percentile; 75th percentile) or counts (%); LDL = low-density lipoprotein; HDL = high-density lipoprotein; TAG = triglycerides; SFA = saturated fatty acids; TOTAL:HDL = total cholesterol to HDL ratio; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; [a] tested by paired Wilcoxon signed rank test; [b] tested by McNemar's chi-squared test; [†] value is significantly greater in females than in males at the respective time-point (p-value < 0.05, tested by Wilcoxon signed rank test or Fisher's exact test); [§] value is significantly greater in males than in females at the respective time-point (p-value < 0.05, tested by Wilcoxon signed rank test or Fisher's exact test).

Table 2. Single nutrient model: prospective associations of dietary fatty acid intakes at age 10 years (per IQR increase in %EI) with changes in blood lipid concentrations (mmol/L) from age 10 to 15 years.

Fatty Acids		ΔLDL			ΔHDL			ΔTAG			ΔTOTAL:HDL		
		β	95% CI	*p*-Value	β	95% CI	*p*-Value	β	95% CI	*p*-Value	β	95% CI	*p*-Value
TOTAL													
SFA	M_{BASIC}	−0.038	−0.077; 0.001	0.057	0.005	−0.017; 0.027	0.653	**−0.038**	**−0.075; −0.001**	**0.042**	−0.030	−0.075; 0.015	0.196
	M_{ADJ}	−0.036	−0.075; 0.003	0.068	0.005	−0.017; 0.027	0.638	**−0.038**	**−0.075; −0.001**	**0.042**	−0.029	−0.074; 0.016	0.209
MUFA	M_{BASIC}	−0.012	−0.048; 0.024	0.519	0.011	−0.010; 0.031	0.306	−0.012	−0.046; 0.023	0.507	−0.017	−0.059; 0.025	0.428
	M_{ADJ}	−0.011	−0.048; 0.025	0.534	0.011	−0.009; 0.031	0.297	−0.012	−0.046; 0.023	0.503	−0.017	−0.059; 0.025	0.430
***n*-3 PUFA**	M_{BASIC}	−0.027	−0.058; 0.003	0.075	0.005	−0.012; 0.022	0.590	0.001	−0.028; 0.030	0.953	−0.017	−0.052; 0.018	0.342
	M_{ADJ}	−0.027	−0.057; 0.003	0.082	0.005	−0.012; 0.022	0.540	0.000	−0.028; 0.029	0.981	−0.017	−0.053; 0.018	0.332
***n*-6 PUFA**	M_{BASIC}	−0.003	−0.034; 0.028	0.866	0.015	−0.002; 0.032	0.089	0.009	−0.020; 0.039	0.525	−0.012	−0.048; 0.024	0.504
	M_{ADJ}	−0.003	−0.034; 0.028	0.849	0.016	−0.002; 0.033	0.074	0.009	−0.021; 0.038	0.559	−0.013	−0.049; 0.023	0.465
FEMALES													
SFA	M_{BASIC}	−0.048	−0.110; 0.014	0.131	0.012	−0.022; 0.045	0.491	−0.053	−0.100; −0.006	0.026	−0.057	−0.116; 0.002	0.060
	M_{ADJ}	−0.047	−0.110; 0.015	0.139	0.012	−0.022; 0.045	0.484	−0.053	−0.100; −0.007	0.025	−0.057	−0.116; 0.002	0.060
MUFA	M_{BASIC}	−0.004	−0.061; 0.053	0.888	0.020	−0.011; 0.050	0.211	−0.017	−0.060; 0.026	0.430	−0.026	−0.081; 0.028	0.342
	M_{ADJ}	−0.005	−0.062; 0.053	0.876	0.020	−0.011; 0.050	0.209	−0.018	−0.061; 0.025	0.420	−0.027	−0.081; 0.028	0.336
***n*-3 PUFA**	M_{BASIC}	−0.030	−0.078; 0.018	0.219	0.011	−0.015; 0.037	0.406	0.000	−0.036; 0.036	0.990	−0.032	−0.077; 0.014	0.173
	M_{ADJ}	−0.030	−0.079; 0.018	0.214	0.011	−0.015; 0.037	0.403	0.000	−0.036; 0.036	0.998	−0.032	−0.078; 0.014	0.169
***n*-6 PUFA**	M_{BASIC}	−0.020	−0.068; 0.028	0.409	0.019	−0.007; 0.044	0.148	0.016	−0.020; 0.052	0.390	−0.026	−0.071; 0.020	0.269
	M_{ADJ}	−0.021	−0.069; 0.027	0.397	0.020	−0.006; 0.046	0.124	0.014	−0.022; 0.050	0.446	−0.028	−0.074; 0.018	0.231
MALES													
SFA	M_{BASIC}	−0.029	−0.078; 0.019	0.237	−0.002	−0.031; 0.026	0.868	−0.016	−0.072; 0.04	0.577	0.002	−0.066; 0.070	0.962
	M_{ADJ}	−0.026	−0.075; 0.022	0.282	−0.003	−0.031; 0.026	0.863	−0.015	−0.072; 0.041	0.591	0.003	−0.065; 0.071	0.929
MUFA	M_{BASIC}	−0.021	−0.066; 0.025	0.372	0.000	−0.027; 0.027	0.975	−0.002	−0.055; 0.051	0.952	−0.001	−0.065; 0.063	0.972
	M_{ADJ}	−0.019	−0.065; 0.026	0.400	0.001	−0.026; 0.027	0.964	−0.002	−0.056; 0.051	0.927	−0.001	−0.065; 0.063	0.968
***n*-3 PUFA**	M_{BASIC}	−0.026	−0.064; 0.012	0.181	−0.003	−0.025; 0.02	0.818	0.005	−0.040; 0.049	0.826	0.001	−0.053; 0.055	0.971
	M_{ADJ}	−0.025	−0.063; 0.013	0.199	−0.001	−0.023; 0.022	0.952	0.003	−0.042; 0.048	0.891	0.000	−0.054; 0.054	0.992
***n*-6 PUFA**	M_{BASIC}	0.011	−0.029; 0.051	0.583	0.010	−0.013; 0.034	0.392	0.001	−0.045; 0.048	0.953	0.000	−0.056; 0.056	0.991
	M_{ADJ}	0.013	−0.027; 0.052	0.534	0.012	−0.011; 0.036	0.299	−0.001	−0.047; 0.046	0.975	−0.001	−0.057; 0.055	0.971

IQR = interquartile range; %EI = % of total energy intake; M_{BASIC} = single nutrient model adjusted for study, region, sex (not in sex-stratified models), exact age at blood sampling, BMI at 10 years, total daily energy intake at 10 years, screen-time at 10 years, fasting status at blood sampling, and lipid concentration at 10 years; M_{ADJ} = single nutrient model further adjusted for pubertal onset and parental education; Δ = change from age 10 to 15 years; Significant associations marked in bold (*p*-value < 0.05 for total population analyses, *p*-value < 0.025 for sex-stratified analyses—Bonferroni correction for multiple testing: 0.05/2).

Table 3. Substitution model: prospective associations of fatty acids and carbohydrates (CHO) (when replacing SFA) at age 10 years (per IQR increase in %EI), with changes in blood lipid concentrations (mmol/L) from age 10 to 15 years.

Substituting Nutrient		ΔLDL			ΔHDL			ΔTAG			ΔTOTAL:HDL		
		β	95% CI	*p*-Value	β	95% CI	*p*-Value	β	95% CI	*p*-Value	β	95% CI	*p*-Value
TOTAL													
MUFA	M_{BASIC}	−0.037	−0.085; 0.011	0.134	0.002	−0.025; 0.029	0.897	−0.041	−0.087; 0.005	0.077	−0.027	−0.083; 0.029	0.346
	M_{ADJ}	−0.034	−0.082; 0.014	0.163	0.002	−0.025; 0.029	0.894	−0.041	−0.086; 0.005	0.081	−0.025	−0.082; 0.031	0.378
***n*-3 PUFA**	M_{BASIC}	−0.027	−0.064; 0.011	0.164	−0.003	−0.024; 0.018	0.752	0.002	−0.033; 0.037	0.913	−0.008	−0.052; 0.036	0.715
	M_{ADJ}	−0.026	−0.063; 0.012	0.179	−0.003	−0.024; 0.018	0.775	0.002	−0.034; 0.037	0.916	−0.008	−0.052; 0.036	0.723
***n*-6 PUFA**	M_{BASIC}	0.018	−0.019; 0.056	0.341	0.016	−0.005; 0.037	0.143	0.019	−0.017; 0.055	0.303	−0.001	−0.045; 0.043	0.978
	M_{ADJ}	0.017	−0.021; 0.054	0.384	0.016	−0.005; 0.038	0.129	0.018	−0.018; 0.054	0.324	−0.002	−0.047; 0.042	0.916
CHO	M_{BASIC}	0.063	0.000; 0.127	0.050	−0.001	−0.036; 0.035	0.970	0.057	−0.003; 0.117	0.061	0.043	−0.031; 0.117	0.257
	M_{ADJ}	0.060	−0.004; 0.123	0.064	−0.001	−0.037; 0.034	0.947	0.057	−0.003; 0.117	0.063	0.041	−0.033; 0.115	0.278
FEMALES													
MUFA	M_{BASIC}	−0.053	−0.130; 0.024	0.175	0.005	−0.037; 0.046	0.825	−0.065	−0.122; −0.007	0.029	−0.054	−0.127; 0.020	0.153
	M_{ADJ}	−0.052	−0.129; 0.025	0.188	0.005	−0.037; 0.046	0.824	−0.064	−0.122; −0.007	0.029	−0.053	−0.127; 0.020	0.154
***n*-3 PUFA**	M_{BASIC}	−0.020	−0.080; 0.039	0.502	−0.004	−0.036; 0.028	0.822	0.002	−0.043; 0.047	0.928	−0.003	−0.060; 0.053	0.907
	M_{ADJ}	−0.020	−0.079; 0.039	0.510	−0.004	−0.036; 0.028	0.788	0.003	−0.042; 0.048	0.895	−0.002	−0.059; 0.055	0.942
***n*-6 PUFA**	M_{BASIC}	−0.004	−0.062; 0.054	0.896	0.015	−0.016; 0.046	0.341	0.034	−0.009; 0.078	0.125	−0.005	−0.060; 0.050	0.856
	M_{ADJ}	−0.005	−0.063; 0.053	0.868	0.017	−0.014; 0.048	0.292	0.032	−0.012; 0.075	0.155	−0.008	−0.064; 0.047	0.774
CHO	M_{BASIC}	**0.127**	**0.023; 0.231**	**0.017**	−0.003	−0.059; 0.053	0.916	**0.097**	**0.019; 0.175**	**0.015**	**0.114**	**0.014; 0.213**	**0.025**
	M_{ADJ}	**0.125**	**0.021; 0.229**	**0.019**	−0.004	−0.060; 0.052	0.891	**0.098**	**0.020; 0.176**	**0.014**	**0.115**	**0.015; 0.215**	**0.024**
MALES													
MUFA	M_{BASIC}	−0.027	−0.088; 0.033	0.373	−0.001	−0.036; 0.035	0.976	−0.015	−0.085; 0.055	0.668	−0.006	−0.091; 0.079	0.898
	M_{ADJ}	−0.024	−0.084; 0.036	0.435	−0.001	−0.037; 0.035	0.948	−0.015	−0.085; 0.056	0.683	−0.004	−0.089; 0.082	0.934
***n*-3 PUFA**	M_{BASIC}	−0.043	−0.091; 0.005	0.079	−0.008	−0.037; 0.020	0.570	0.006	−0.050; 0.062	0.833	−0.009	−0.076; 0.059	0.796
	M_{ADJ}	−0.043	−0.090; 0.005	0.081	−0.007	−0.035; 0.021	0.630	0.005	−0.050; 0.061	0.851	−0.009	−0.077; 0.058	0.788
***n*-6 PUFA**	M_{BASIC}	0.041	−0.009; 0.090	0.106	0.017	−0.012; 0.046	0.256	−0.004	−0.061; 0.054	0.898	0.001	−0.069; 0.071	0.985
	M_{ADJ}	0.041	−0.008; 0.090	0.103	0.018	−0.011; 0.048	0.224	−0.004	−0.062; 0.053	0.880	0.000	−0.070; 0.070	0.995
CHO	M_{BASIC}	0.017	−0.059; 0.093	0.658	−0.001	−0.046; 0.044	0.961	0.021	−0.068; 0.109	0.647	−0.008	−0.115; 0.099	0.884
	M_{ADJ}	0.012	−0.064; 0.088	0.755	0.000	−0.045; 0.045	0.991	0.020	−0.069; 0.108	0.663	−0.011	−0.118; 0.096	0.842

IQR = interquartile range; %EI = % of total energy intake; M_{BASIC} = substitution model adjusted for study, region, sex (not in sex-stratified models), exact age at blood sampling, BMI at 10 years, total daily energy intake at 10 years, screen-time at 10 years, fasting status at blood sampling, lipid concentration at 10 years, and all energy-bearing nutrients except SFA; M_{ADJ} = substitution model further adjusted for pubertal onset and parental education; Δ = change from age 10 to 15 years; Significant associations marked in bold (*p*-value < 0.05 for total population analyses, *p*-value < 0.025 for sex-stratified analyses—Bonferroni correction for multiple testing: 0.05/2).

4. Discussion

The present study used data from two large German birth cohorts to assess the prospective associations of fatty acid intakes with changes in blood lipid concentrations during adolescence. We found that higher intakes of SFA at age 10 years were associated with decreasing TAG between ages 10 and 15 years. Furthermore, we observed that the consumption of CHO at the expense of SFA in females was associated with increasing LDL, TAG and TOTAL:HDL.

4.1. Single Nutrient Model

Our findings regarding the prospective association of SFA intakes with reduced TAG concentrations are in line with existing literature in adults [35]. The observed relationship might be considered somewhat counterintuitive, given the suggestions for a possible detrimental role of SFA in the development of coronary heart disease [1,2]. Conflicting evidence has been reported in younger populations, although existing studies are scarce and heterogeneous in terms of design, statistical methods and outcome measurements. A study in children aged 6 to 12 years, reported a positive association between SFA and total cholesterol concentrations [36], whereas an inverse association was observed in a study in 15-year-olds [37]. Other studies have reported no association between dietary fatty acids and blood lipids in pre-pubertal and pubertal children [24,38]. The often-observed association of SFA consumption with increased LDL in adults [35] was not present in our adolescent population. In fact, we observed a negative relationship in our total study sample, although the association did not reach statistical significance (p-value = 0.068). Although the evidence for an association of SFA with LDL has been widely accepted, recent studies in adults have emerged, reporting no association between SFA and CVD risk [4]. Nonetheless, the present results should be interpreted in the context of possible correlations among different nutrients. In our dietary data, SFA was highly positively correlated with MUFA (r = 0.77), and also presented strong negative correlations with CHO (r = −0.81), which limits the ability to disentangle the individual effects of SFA [32]. In light of this and the inverse association observed between SFA and TAG levels in the current study, we speculate that increasing the intake of SFA might have led to decreasing TAG levels indirectly through a reduction in CHO intake. Indeed, CHO, in particular simple sugars, have been shown to have a detrimental impact on blood lipids through raising TAG levels [39]. This has been suggested to result mainly from increased hepatic secretion of very-low-density lipoprotein (VLDL) as well as impaired plasma TAG clearance, possibly induced by reduced insulin sensitivity [40].

4.2. Substitution Model

Results from our substitution analyses showed that replacing SFA with CHO was associated with increasing LDL, TAG and TOTAL:HDL in females. Our findings are in line with studies in adults which report a detrimental effect on blood lipids when substituting CHO for SFA [41]. However, the specific effects on blood lipid parameters differed from those observed in adults, who typically present lower LDL levels [9,42,43] occurring in parallel with decreasing HDL levels, and having no effect on the TOTAL:HDL ratio [44]. An increase in LDL is, however, plausible if we consider results from randomized controlled trials which have reported positive linear associations of CHO with small-dense LDL [45]. Other studies have shown positive relationships between dietary sugars with plasma LDL and TAG [46]. In agreement with this and other studies [8,9,41,44] females in our study population also presented positive associations between CHO and TAG. This relationship can be attributed to the increased secretion of VLDL and impaired plasma TAG clearance described above. The greater number of VLDL particles in the blood could also have led to the increase in the LDL production rate [40], which was further reflected by the lower TOTAL:HDL ratio observed amongst our findings. A previous study, including a subset of our study population, showed that the highest contribution towards total energy intake at age 10 years came from “refined grains” and “sugar-sweetened foods” [30], which might suggest that the CHO consumed by children in our study

population consisted largely of refined grains and sugars. Investigation into the effects of replacing SFA with different quality CHO is beyond the scope of the present study, but should be considered for further research in this age group.

Comparison of our results with existing evidence in adolescents is restricted due to the absence of studies carried out during this life period. One similar study observed theoretical effects of substituting MUFA or PUFA for SFA, and total fat for CHO from ages 8 to 11 years [25]. The study findings included slightly lowered total cholesterol when replacing SFA with MUFA or PUFA and higher HDL when replacing CHO with fat. Based on these findings, the authors suggested a similar effect of diet on serum lipids to that observed in adults [25]. Unfortunately, LDL, TAG and TOTAL:HDL were not included among the serum lipid measurements and so comparison with our study is limited. Nevertheless, our findings also seem to be to some extent comparable to observations in adult populations. Our results further suggest a sex-specific role of SFA (when replaced by CHO), acting mainly in female adolescents. The reasons for this gender discrepancy are unknown but we speculate that it could be related to possible sex differences in dietary patterns, hormones or pubertal stage. A greater proportion of girls in our study had entered pubertal onset at age 10 years (Table 1). It has been shown that physiological insulin resistance occurs during puberty [47], which may explain why females in the present study were more vulnerable than boys to the potentially adverse effects of carbohydrates. Additionally, girls in our study had slightly higher CHO intakes, which persisted at a high level, whilst they decreased in boys.

4.3. Strengths and Limitations

One of the main strengths of this study is its focus on the prospective role of dietary fatty acids on blood lipids during adolescence, a life period not often addressed and becoming increasingly relevant in terms of later disease development. Furthermore, we consider isocaloric replacements of SFA, which can contribute toward better understanding its independent role in the context of other nutrients. For our analyses, we benefited from a large homogenous population of females and males, providing data covering a five-year period from childhood to adolescence. The longitudinal nature of this study is a key aspect which allows us to add to the limited knowledge regarding fatty acid intake and prospective changes in markers of cardiometabolic risk during adolescence. Given the observational nature of the study, causality cannot be implied; nevertheless, the prospective analysis offers a temporal component which provides stronger grounds for a causal interpretation. Whether the observed effect sizes in this study can be considered clinically relevant might be a point for discussion. Furthermore, considering that children in the present study are not a high risk population and present normal blood lipid levels, the observation of associations at this stage provide only an indication of a possible early role of dietary nutrients in the long-term development of CVD risk factors. Nevertheless, given the increasing evidence for the progression of risk factors from childhood to adulthood, preventive measures might already consider this age group. Our findings provide a relevant indication of possible dietary targets which could support the development of recommendations for early disease prevention.

A main drawback in nutritional epidemiology is the high intercorrelation amongst different nutrients, which, if overlooked, can lead to incorrect conclusions. The use of substitution models can provide additional insight through the adjustment for other nutrients. However, the method can result in multicollinearity within statistical models, again generating misleading associations [32]. In our analyses, we tackle this problem by residualizing highly correlated variables, allowing the new variable to be included in the same model as the previously correlated nutrient, while avoiding multicollinearity [33]. A further limitation in the present study was non-random loss-to-follow-up, which meant that, for example, children of lower social classes might be underrepresented in our analyses. Therefore the generalizability of our findings is limited, as these cannot be considered representative of the study area (Table S1). Finally, we are aware of problems associated with misreporting of dietary intake with the use of FFQs. However, the FFQ was validated to estimate fatty acids and antioxidants in school-aged children. We observed plausible values in terms of energy intake

and believe that any misreporting was likely detected through extensive quality control, which was done at the expense of reducing the sample size, but with no substantial loss of power.

5. Conclusions

In conclusion, our findings suggest that higher SFA intakes might lead to reductions in TAG concentrations during adolescence. We highlight that observed associations in this context are not independent of other correlated nutrients. Furthermore, replacement of SFA with CHO in female children is associated with increasing levels of LDL, TAG and TOTAL:HDL during adolescence. Our findings confirm observations in adult populations, where detrimental aspects of increased consumption of CHO at the expense of SFA have been reported. Sex-specific determinants may however play a greater role during adolescence. It is important that recommendations to reduce SFA intakes do not overlook the possible effects of other nutrients consumed in their place.

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Relationship between Self-Reported Dietary Nutrient Intake and Self-Reported Sleep Duration among Japanese Adults

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Abstract: Several studies have reported that short sleep duration is a risk factor for obesity and metabolic disease. Moreover, both sleep duration and sleep timing might independently be associated with dietary nutrient intake. In this study, we investigated the associations between self-reported sleep duration and dietary nutrient intake, with and without adjustments for variations in sleep timing (i.e., the midpoint of sleep). We conducted a questionnaire survey, comprising a validated brief self-administered diet history questionnaire (BDHQ) and the Japanese version of the Pittsburgh Sleep Quality Index (PSQI) among 1902 healthy Japanese adults and found that the dietary intakes of several nutrients correlated with sleep duration among men regardless of adjustment for the midpoint of sleep. Particularly, (1) small but significant correlations were observed between sleep duration and the percentage of energy from protein, regardless of adjustment for the midpoint of sleep; (2) energy-adjusted intakes of sodium, vitamin D, and vitamin B12 also significantly correlated with sleep duration; and (3) intakes of bread, pulses, and fish and shellfish correlated with sleep duration. In contrast, no significant correlations were observed between sleep duration and dietary intakes among women. This study revealed that after controlling for the midpoint of sleep, sleep duration correlated significantly with the dietary intake of specific nutrients and foods in a population of Japanese men.

Keywords: sleep duration; midpoint of sleep; dietary nutrients; nutrition; food

1. Introduction

In recent years, laboratory and epidemiologic evidence has identified short sleep duration as a risk factor for the development of obesity and metabolic disease [1–4]. Indeed, in humans, sleep duration plays an important role in regulating the levels of leptin and ghrelin, which are among the key modulators of appetite and energy expenditure [1,5]. Several studies have shown associations of repetitive partial sleep deprivation and/or chronic short sleep duration with a significant decrease in

leptin levels and an increase in ghrelin levels [4,6,7]. This might be related to an increase in subjective hunger experienced by self-restricted individuals [1].

Several studies have investigated the association between sleep duration and dietary intake. In a NHANES (National Health and Nutrition Examination Survey)-based study, Grandner et al. found that relative to normal sleepers (7–8 h), short sleepers (5–6 h) reported higher intakes of absolute protein, carbohydrate, and total fat but a lower intake of dietary fiber, whereas very short sleepers (<5 h) reported lower intakes of protein, carbohydrates, dietary fiber, and total fats [8]. In an analysis of Chinese adults, individuals with a self-reported short sleep duration (<7 h) had a lower carbohydrate intake and higher fat intake, compared to normal-duration sleepers (7–9 h) [9]. Kant et al. also observed that both short and long sleepers reported receiving lower percentages of energy from protein, compared to normal duration sleepers in the NHANES [10].

Several previous studies have therefore emphasized the relationship between short sleep duration and poor dietary intake. A recent report suggested that in addition to sleep duration, sleep timing exhibited an important correlation with obesity [11]. Individuals with later sleep timing were 1.5 times more likely to be obese than were individuals with an early sleep timing, despite reasonably similar sleep durations [11]. Regarding nutrient intake, two recent studies showed the influence of sleep timing on the dietary intakes of certain nutrients [12,13]. A cross-sectional study of a large representative sample of the Finnish population also revealed that individuals with later sleep timing exhibited less healthy dietary habits, such as lower vitamin and higher fat consumption, compared to individuals with earlier sleep timing [12]. Another study of young Japanese women found that later sleep timing (i.e., later midpoint of sleep) was significantly associated with a lower percentage of energy intake from protein and carbohydrates; a lower energy-adjusted intake of cholesterol, potassium, calcium, magnesium, iron, zinc, vitamin A, vitamin D, thiamin, riboflavin, vitamin B6, and folate; and a higher percentage of energy intake from alcohol and fat [13].

Considering the results of the above-mentioned studies, we hypothesized that both sleep duration and sleep timing are associated with dietary nutrient intake. To test this hypothesis, we conducted a survey of sleep and dietary nutrient intake in a Japanese adult population. Our primary aim was to identify the existence of an association between the self-reported sleep duration and the intakes of specific dietary nutrients, after adjusting for variations in sleep timing.

2. Methods

2.1. Ethical Approval

This study was approved by the ethics committee of Tokyo Medical University (No. 2586), and written informed consent was obtained from all study participants.

2.2. Patient Selection

A total of 2007 healthy individuals, aged between 30 and 69 years, participated in this cross-sectional survey. Participants were recruited via study advertisements run by a Clinical Research Organization. Individuals who met the following criteria were excluded from the present study: previous or current diagnosis of psychiatric disorders, history of neurologic illness, use of medication with known effects on sleep or daytime alertness, or shift worker. We also excluded those with self-reported extremely low or high energy intake (<725 or >3235 kcal/day) (n = 92), and those whose midpoint of sleep was outside the range of midnight to noon (n = 15). After these exclusions, the final sample included in the subsequent analyses comprised 1902 adults.

2.3. Demographic Variables

The participants filled out self-administered questionnaires containing questions related to demographics and lifestyle. The demographic items included age, sex, height, weight, current

smoking habits (yes or no), current exercise routine (≥2 times/week, ≥30 min/session; yes or no), family structure (living alone or living with other family members).

2.4. Assessment of Dietary Intake

Dietary intake during the preceding one month was assessed with a validated, self-administered, brief diet history questionnaire (BDHQ) [14]. The BDHQ is a four-page structured questionnaire that enquires about the consumption frequency of a total of 56 foods and beverages that are commonly consumed in the general Japanese population. Dietary intakes, in terms of energy and selected nutrients, were estimated by applying an ad hoc computer algorithm to the 56 foods and beverages of the BDHQ and the Standard Tables of Food Composition in Japan [15].

2.5. Assessment of Sleep Duration and Sleep Timing

Sleep duration was assessed using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI) [16,17]. The PSQI is a self-rated questionnaire that measures sleep difficulty retrospectively for a one-month period, with a global score ranging from 0 to 21. Higher PSQI scores indicate a lower quality of sleep. In the PSQI, the subjects reported bedtimes, sleep onset latency, and rise times. Using these data, we calculated the sleep duration by subtracting the sleep onset time from the rise time and the midpoint of sleep as the halfway point between sleep onset time and rise time to determine sleep timing [13].

2.6. Statistical Analysis

The average values of dietary intake and anthropometric variables were calculated. For energy adjustment, we used the percentages of total energy intakes (% energy) from macronutrients and alcohol and the intakes per 1000 kcal (/1000 kcal) for other nutrients and food. In this study, we evaluated energy (kcal/day), alcohol (% energy), protein (% energy), total fat (% energy), carbohydrate (% energy), cholesterol (mg/1000 kcal), sodium (mg/1000 kcal), potassium (mg/1000 kcal), calcium (mg/1000 kcal), magnesium (mg/1000 kcal), iron (mg/1000 kcal), zinc (mg/1000 kcal), vitamin A (μg/1000 kcal), vitamin D (μg/1000 kcal), vitamin E (mg/1000 kcal), thiamin (mg/1000 kcal), riboflavin (mg/1000 kcal), vitamin B6 (mg/1000 kcal), vitamin B12 (μg/1000 kcal), folate (μg/1000 kcal), and vitamin C (mg/1000 kcal), as well as rice (g/1000 kcal), noodles (g/1000 kcal), bread (g/1000 kcal), confections (g/1000 kcal), potatoes (g/1000 kcal), fat and oil (g/1000 kcal), fruits (g/1000 kcal), vegetables (g/1000 kcal), pulses (g/1000 kcal), fish and shell fish (g/1000 kcal), meat (g/1000 kcal), eggs (g/1000 kcal), and milk and milk products (g/1000 kcal).

The Student's *t*-test was used to compare continuous variables, and the chi-square test was used to compare categorical variables between men and women. The multicolinearity effect was checked using VIF (Variance Inflation Factor) <10/tolerance tests >0.10. A Pearson's correlation analysis was performed to examine the relationships between sleep duration and dietary intakes. A partial correlation procedure was used to examine the linear relationships between these variables after controlling for the effects of other variables (age, sleep timing). All analyses were performed using the statistical software SPSS version 15.0 (SPSS Japan, Inc., Tokyo, Japan). p-values < 0.05 were considered statistically significant.

3. Results

The mean age of all the subjects was 48.0 (10.3) years (mean (standard deviation)), and 54.1% of the subjects were male. The mean body mass index (BMI) was 22.4 (3.3). The characteristics and dietary intakes, stratified by sex, are shown in Table 1. We observed significant differences in age ($t(1900) = 15.2$, $p = 0.001$), sleep latency ($t(1900) = 6.3$, $p = 0.001$), and rise time ($t(1900) = 0.66$, $p = 0.036$) between men and women. We further observed significant differences in the percentages of current smokers ($\chi^2(1) = 51.0$, $p = 0.001$), subjects with a current exercise routine ($\chi^2(1) = 47.1$, $p = 0.001$), subjects who lived alone ($\chi^2(1) = 43.0$, $p = 0.001$), and occupational statuses ($\chi^2(3) = 432.4$, $p = 0.001$). A residual analysis revealed that among men, the percentage of full-time workers was significantly

higher and the percentages of part-time workers and homemakers were significantly lower than among women.

Table 1. Participant characteristics and dietary intakes, stratified by sex (n = 1902).

	All (n = 1902)		Male (n = 1029)		Female (n = 873)		p-Value
	Mean	SD	Mean	SD	Mean	SD	
Age, years	48.0	10.3	51.1	10.2	44.3	9.2	0.001
Body mass index, kg/m^2	22.4	3.3	23.3	3.1	21.4	3.4	0.063
Bedtime, h:min	23:58	1:26	0:03	1:28	23:52	1:23	0.544
Sleep latency, min	19.4	19.0	16.9	15.0	22.4	22.6	0.001
Rise time, h:min	6:46	1:28	6:47	1:32	6:45	1:22	0.036
Midpoint of sleep, h:min	3:32	1:21	3:34	1:24	3:29	1:16	0.059
Sleep duration, h:min	6:26	1:05	6:23	1:04	6:30	1:07	0.311
Current smoking, %	22.0		28.3		14.7		0.001
Current exercise routine, %	27.7		34.2		20.1		0.001
Living alone, %	16.6		21.8		10.5		0.001
Occupation							
Full-time worker	61.8		77.9		42.6		0.001
Part-time worker	8.5		3.0		15.1		
Homemaker	11.6		0.0		25.3		
Unemployed	18.2		19.1		17.0		
Energy, kcal/day	1772.0	527.0	1922.5	521.6	1596.7	478.8	0.001
Alcohol, % energy	5.5	9.0	7.8	10.1	2.8	6.5	0.001
Nutrients							
Protein, % energy	14.5	2.9	13.9	2.7	15.3	3.0	0.001
Total fat, % energy	25.4	5.8	24.1	5.8	27.0	5.6	0.001
Carbohydrate, % energy	53.2	8.8	52.8	9.5	53.8	8.1	0.012
Cholesterol, mg/1000 kcal	196.0	75.0	184.3	75.5	210.4	73.1	0.001
Sodium, mg/1000 kcal	2313.0	491.0	2293.2	483.7	2337.1	499.8	0.052
Potassium, mg/1000 kcal	1336.0	404.0	1245.8	351.9	1442.8	435.0	0.001
Calcium, mg/1000 kcal	275.0	108.0	255.0	99.7	300.6	112.6	0.001
Magnesium, mg/1000 kcal	134.0	32.0	129.3	28.3	141.7	35.0	0.001
Iron, mg/1000 kcal	4.1	1.1	3.9	1.0	4.4	1.2	0.001
Zinc, mg/1000 kcal	4.2	0.7	4.1	0.7	4.4	0.7	0.001
Vitamin A, μg/1000 kcal	374.0	230.0	357.2	218.1	395.6	243.6	0.001
Vitamin D, μg/1000 kcal	6.5	4.3	6.1	3.9	6.9	4.6	0.001
Vitamin E, mg/1000 kcal	3.8	1.1	3.5	1.0	4.1	1.1	0.001
Thiamin, mg/1000 kcal	0.37	0.1	0.37	0.1	0.43	0.1	0.001
Riboflavin, mg/1000 kcal	0.69	0.2	0.66	0.2	0.73	0.2	0.001
Vitamin B6, mg/1000 kcal	0.65	0.2	0.62	0.2	0.69	0.2	0.001
Vitamin B12, μg/1000 kcal	4.6	2.4	4.5	2.3	4.8	2.6	0.006
Folate, μg/1000 kcal	176.0	68.0	164.0	58.7	190.9	75.6	0.001
Vitamin C, mg/1000 kcal	58.0	29.0	53.5	26.5	64.8	31.0	0.001
Food Group (g/1000 kcal)							
Rice	147.0	76.0	152.1	76.6	142.0	75.6	0.004
Noodles	44.0	30.0	49.2	33.1	38.2	26.3	0.001
Bread	49.0	28.0	43.5	25.8	55.6	30.9	0.001
Confections	27.0	21.0	22.5	17.9	33.5	24.1	0.001
Potatoes	18.0	17.0	19.2	18.4	25.7	21.8	0.001
Fat and oil	5.9	2.6	5.8	2.6	6.0	2.8	0.092
Fruits	62.0	58.0	61.4	59.2	63.9	57.2	0.362
Vegetables	134.0	82.0	116.3	66.2	155.0	95.1	0.001
Pulses	34.0	26.0	30.2	23.1	39.5	29.0	0.001
Fish and Shell fish	36.0	22.0	35.2	20.5	38.3	24.3	0.002
Meat	37.0	20.0	35.8	18.3	40.4	21.8	0.001
Eggs	20.0	14.0	19.8	14.7	21.8	14.7	0.003
Milk and milk products	68.0	61.0	63.5	61.4	73.4	61.2	0.001

SD: standard deviation.

Table 2. Correlation between sleep duration and dietary intakes among men (n = 1029).

	Mean	SD	[1] r	[1] β	[2] r	[2] β	[3] r	[3] β
Energy, kcal/day	1922.5	521.6	0.052	−0.05	0.134	−0.06	0.139	−0.06
Alcohol, % energy	7.8	10.1	0.024	0.02	0.150	0.02	0.156	0.02
Nutrients								
Protein, % energy	13.9	2.7	**0.076b**	0.08	**0.126b**	0.07	**0.126b**	0.07
Total fat, % energy	24.1	5.8	0.031	0.03	0.032	0.03	0.052	0.03
Carbohydrate, % energy	52.8	9.5	**0.068b**	−0.07	0.182	−0.06	0.183	−0.06
Cholesterol, mg/1000 kcal	184.3	75.5	0.058	0.06	0.113	0.05	0.114	0.05
Sodium, mg/1000 kcal	2293.2	483.7	**0.087a**	0.09	**0.093a**	0.09	**0.115a**	0.08
Potassium, mg/1000 kcal	1245.8	351.9	0.004	0.00	0.220	−0.02	0.224	−0.01
Calcium, mg/1000 kcal	255.0	99.7	0.011	0.01	0.212	0.00	0.218	0.00
Magnesium, mg/1000 kcal	129.3	28.3	0.030	0.03	0.226	0.02	0.230	0.02
Iron, mg/1000 kcal	3.9	1.0	0.045	0.05	0.180	0.04	0.182	0.04
Zinc, mg/1000 kcal	4.1	0.7	0.048	0.05	0.048	0.05	0.056	0.05
Vitamin A, μg/1000 kcal	357.2	218.1	0.014	0.01	0.074	0.01	0.096	0.02
Vitamin D, μg/1000 kcal	6.1	3.9	**0.088a**	0.09	**0.218a**	0.08	**0.218a**	0.08
Vitamin E, mg/1000 kcal	3.5	1.0	0.022	0.02	0.132	0.01	0.133	0.01
Thiamin, mg/1000 kcal	0.4	0.1	0.036	0.04	0.130	0.03	0.131	0.03
Riboflavin, mg/1000 kcal	0.7	0.2	0.026	0.03	0.187	0.02	0.193	0.02
Vitamin B6, mg/1000 kcal	0.6	0.2	0.058	0.06	0.208	0.05	0.213	0.05
Vitamin B12, μg/1000 kcal	4.5	2.3	**0.093a**	0.09	**0.192a**	0.08	**0.192a**	0.08
Folate, μg/1000 kcal	164.0	58.7	0.020	0.02	0.209	0.01	0.222	0.01
Vitamin C, mg/1000 kcal	53.5	26.5	0.007	0.01	0.242	−0.01	0.243	−0.01
Food Group (g/1000 kcal)								
Rice	152.1	76.6	0.025	−0.03	0.193	−0.01	0.197	−0.01
Noodles	49.2	33.1	0.038	0.04	0.114	0.04	0.143	0.04
Bread	43.5	25.8	0.056	−0.06	0.074	−0.06	**0.092b**	−0.06
Confections	22.5	17.9	0.054	−0.05	0.062	−0.06	0.083	−0.06
Potatoes	19.2	18.4	0.005	−0.01	0.044	−0.01	0.045	−0.01
Fat and oil	5.8	2.6	0.008	0.01	0.132	0.02	0.136	0.01
Fruits	61.4	59.2	0.005	−0.01	0.166	−0.02	0.167	−0.02
Vegetables	116.3	66.2	0.005	0.01	0.170	−0.01	0.178	0.00
Pulses	30.2	23.1	**0.086a**	0.09	**0.134a**	0.08	**0.141a**	0.08
Fish and Shell fish	35.2	20.5	**0.096a**	0.10	**0.183a**	0.09	**0.183a**	0.09
Meat	35.8	18.3	0.016	0.02	0.133	0.02	0.134	0.02
Eggs	19.8	14.7	0.046	0.05	0.075	0.04	0.081	0.05
Milk and milk products	63.5	61.4	0.031	−0.03	0.093	−0.04	0.099	−0.03

SD: standard deviation; r: Pearson's correlation coefficient r; β: standardized coefficient β; [1]: Univariate regression analysis; [2]: Multivariate regression analysis (sleep duration, age); [3]: Multivariate regression analysis (sleep duration, age, midpoint of sleep); a: $p < 0.01$, b: $p < 0.05$, number in bold showed significant variables.

Table 3. Correlation between sleep duration and dietary intakes among women (n = 873).

	Mean	SD	[1] r	[1] β	[2] r	[2] β	[3] r	[3] β
Energy, kcal/day	1596.7	478.8	0.002	0.00	0.097	0.01	0.109	0.01
Alcohol, % energy	2.8	6.5	0.018	−0.02	0.031	−0.02	0.068	−0.02
Nutrients								
Protein, % energy	15.3	3.0	0.019	0.02	0.147	0.03	0.160	0.03
Total fat, % energy	27.0	5.6	0.051	−0.05	0.064	−0.05	0.068	−0.05
Carbohydrate, % energy	53.8	8.1	0.035	0.04	0.056	0.03	0.057	0.03
Cholesterol, mg/1000 kcal	210.4	73.1	0.026	0.03	0.043	0.03	0.081	0.03
Sodium, mg/1000 kcal	2337.1	499.8	0.049	0.05	0.097	0.06	0.099	0.06
Potassium, mg/1000 kcal	1442.8	435.0	0.039	−0.04	0.217	−0.02	0.222	−0.02
Calcium, mg/1000 kcal	300.6	112.6	0.047	−0.05	0.204	−0.03	0.208	−0.03
Magnesium, mg/1000 kcal	141.7	35.0	0.014	−0.01	0.231	0.01	0.234	0.00
Iron, mg/1000 kcal	4.4	1.2	0.008	−0.01	0.157	0.01	0.161	0.00
Zinc, mg/1000 kcal	4.4	0.7	0.048	0.05	0.119	0.06	0.137	0.06
Vitamin A, μg/1000 kcal	395.6	243.6	0.008	−0.01	0.037	−0.01	0.038	−0.01
Vitamin D, μg/1000 kcal	6.9	4.6	0.017	0.02	0.140	0.03	0.145	0.03
Vitamin E, mg/1000 kcal	4.1	1.1	0.018	−0.02	0.133	−0.01	0.146	−0.01
Thiamin, mg/1000 kcal	0.4	0.1	0.013	−0.01	0.160	0.00	0.166	0.00
Riboflavin, mg/1000 kcal	0.7	0.2	0.050	−0.05	0.177	−0.04	0.178	−0.04
Vitamin B6, mg/1000 kcal	0.7	0.2	0.021	−0.02	0.159	−0.01	0.166	−0.01
Vitamin B12, μg/1000 kcal	4.8	2.6	0.013	−0.01	0.131	0.00	0.131	0.00
Folate, μg/1000 kcal	190.9	75.6	0.015	−0.02	0.166	0.00	0.170	0.00
Vitamin C, mg/1000 kcal	64.8	31.0	0.013	−0.01	0.186	0.00	0.187	0.00
Food group (g/1000 kcal)								
Rice	142.0	75.6	0.045	0.05	0.090	0.04	0.106	0.04
Noodles	38.2	26.3	0.014	0.01	0.089	0.01	0.137	0.01
Bread	55.6	30.9	0.043	−0.04	0.043	−0.04	0.044	−0.04
Confections	33.5	24.1	0.029	−0.03	0.069	−0.03	0.093	−0.03
Potatoes	25.7	21.8	0.038	−0.04	0.051	−0.04	0.068	−0.04
Fat and oil	6.0	2.8	0.024	−0.02	0.060	−0.03	0.060	−0.03
Fruits	63.9	57.2	0.008	0.01	0.135	0.02	0.137	0.02
Vegetables	155.0	95.1	0.034	−0.03	0.138	−0.02	0.141	−0.02
Pulses	39.5	29.0	0.006	0.01	0.121	0.02	0.122	0.02
Fish and Shell fish	38.3	24.3	0.018	0.02	0.129	0.03	0.129	0.03
Meat	40.4	21.8	0.002	0.00	0.039	−0.01	0.046	−0.01
Eggs	21.8	14.7	0.002	0.00	0.020	0.00	0.094	0.00
Milk and milk products	73.4	61.2	**0.066b**	−0.07	0.132	−0.06	0.132	−0.06

SD: standard deviation, r: Pearson's correlation coefficient r; β: standardized coefficient β; [1]: Univariate regression analysis; [2]: Multivariate regression analysis (sleep duration, age); [3]: Multivariate regression analysis (sleep duration, age, midpoint of sleep); a: $p < 0.01$, b: $p < 0.05$, number in bold showed significant variables.

Total energy and alcohol (% energy) intakes were significantly higher in male versus female subjects. On the other hand, male subjects had significantly lower intakes of protein (% energy), fat (% energy), and carbohydrates (% energy), as well as energy-adjusted (per 1000 kcal) nutrients other than sodium. Regarding food-group intakes (g/1000 kcal), those of bread, confections, potatoes, vegetables, pulses, fish and shell fish, meat, eggs, milk, and milk products were significantly higher among women than among men, whereas the reverse was true for rice and noodles.

Data regarding total energy; % energy from alcohol, protein, fat, and carbohydrates; and energy-adjusted (per 1000 kcal) nutrient and food-group intakes by sex are presented in Tables 2 and 3, respectively. Regarding macronutrients, small but significant correlations were observed between sleep duration and the percentage of energy derived from protein, both with and without adjustments for the midpoint of sleep among men (r = 0.126, $p < 0.05$). The energy-adjusted intakes of sodium, vitamin D, and vitamin B12 correlated significantly with sleep duration in men after adjusting for the midpoint of sleep (sodium: r = 0.115, $p < 0.01$; vitamin D: r = 0.218, $p < 0.01$; vitamin B12: r = 0.192, $p < 0.01$).

Regarding food-group intakes, we observed significant correlations between sleep duration and the intakes of bread, pulses, and fish and shellfish among men, both with and without adjustment for

the midpoint of sleep (bread: $r = 0.092$, $p < 0.05$; pulses: $r = 0.141$, $p < 0.01$; fish and shellfish: $r = 0.183$, $p < 0.01$).

In contrast, we observed no significant correlations between dietary intakes and sleep duration among women (Table 3).

4. Discussion

To the best of our knowledge, this is the first study to investigate the relationship between sleep duration and dietary intake of specific nutrients, while considering variations in sleep timing (i.e., the midpoint of sleep). In an earlier survey of a US population, energy intakes across sleep duration groups exhibited an inverse U-shaped distribution [8]. Another previous study also found an association of sleep deprivation with increased energy intake [7]. However, our results did not indicate a significant correlation between energy intakes and sleep duration. The reason for this discrepancy should be clarified in future studies.

We observed a significant sex-based difference in energy (kcal/day) intakes; specifically, male subjects had higher energy intakes. The observed sex-related differences in the intakes of several energy-adjusted nutrients, such as protein, calcium, and iron, are attributed to differences in total energy intake. After adjusting for the midpoint of sleep, we found that the intakes of specific dietary nutrients were correlated with sleep duration among men. The percentage of energy from protein and the energy-adjusted intakes of sodium, vitamin D, and vitamin B12 exhibited small but significant increases that correlated with sleep duration. In addition, the intakes of bread, pulses, and fish and shellfish were correlated with sleep duration, regardless of whether we adjusted for the midpoint of sleep. These results agree with those of a previous study that investigated the relationship between sleep duration and dietary intake in the NHANES; in that study, short sleepers reported lower intakes of protein, carbohydrates, dietary fiber, and total fats than did normal sleepers [8]. Another previous study of adolescents with short sleep durations observed decreased intakes of healthy foods such as vegetables, fruits, and fish, and increased intakes of unhealthy fast foods such as pizza, hamburgers, pasta dishes, and snack products [18]. Short sleep duration–induced changes in food preferences may be accompanied by changes in nutrient intakes, possibly consequent to changes in the secretion of appetite-related hormones such as leptin and ghrelin [5,6,19]. Previous studies also reported that total blood levels and circadian changes in cortisol, insulin, and thyroid-stimulating hormone levels were affected by a short sleep duration [20–22]. Additionally, a short sleep duration was found to enhance activity in brain reward and food-sensitive centers in response to unhealthy food stimuli [23]. A short sleep duration also led to extended hours of wakefulness, thus presenting additional opportunities for increased food intake [5]. Although these parameters were not evaluated objectively in the present study, they should be addressed in future studies. However, we observed no significant correlations between sleep duration and dietary intake among women in this study. We cannot clearly explain this sex-based difference. Previous studies either combined the data of men and women for analysis [8,12] or surveyed only women [13,24]. However, sex has been suggested as an important factor regarding food and nutrient intakes [25]. Our present study suggested a sex-based difference in the influence of sleep duration on dietary intake. Thus, the results of this study suggest a significant and independent association of sleep duration with dietary intakes of certain nutrients and foods in a Japanese adult male population after controlling for variations in the midpoint of sleep. Clock genes may influence the relationship between sleep timing and dietary intake. Circadian clocks, which are controlled by clock genes, regulate various biological rhythms, including sleep timing and the endocrine system [26]. In addition, mouse clock gene mutants exhibit increased alcohol intake [27]. Clock genes were also found to regulate metabolism [26]. In the meantime, periodic meal intake was found to be an important circadian clock entrainment signal in animals [28]. Furthermore, certain nutrients and food components, such as glucose, ethanol, caffeine, thiamine, and retinoic acid, can induce phase-shifts in circadian rhythms [29]. However, we could not identify if reverse causation

occurred because this epidemiological study was cross-sectional. Further studies are required to understand the relationships between dietary intakes and circadian clocks in humans.

We should note several limitations of our study. First, information about both dietary nutrient intake and sleep duration was based on the participants' self-reports. However, the participants might have overestimated their vegetable intake and/or underestimated their intakes of sweets and high-fat foods [14] during the preceding month. Still, the BDHQ was validated and used in several previous studies. Therefore, we used self-reporting methods to obtain data from our large sample. Further studies involving biomarkers and digital dietary records of nutrient intake are warranted. We also did not obtain information about the participants' usage of caffeine, antidepressants, or other medications that could influence appetite and/or sleep. Furthermore, the sleep duration and midpoint of sleep were derived from the same questionnaire, despite the lack of a significant correlation between these two variables. The subjective sleep duration might also have been misclassified because of reporting errors, which warrants the use of an objective sleep measurement such as actigraphy. Second, the results of this study might have been affected by sampling bias, as health-conscious people may have been more likely to participate in this type of health survey. However, the mean nutrient and food intake values in this population were almost the same as those reported by similarly aged adults in the National Nutrition and Health Survey in Japan [30]. Therefore, the subjects of the present study may be representative of the general Japanese population, at least regarding the study variables.

5. Conclusions

This study found that sleep duration was significantly and independently associated with the dietary intakes of certain nutrients and foods in a Japanese adult male population after controlling for variations in the midpoint of sleep.

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Impact of Early Nutrition on Body Composition in Children Aged 9.5 Years Born with Extremely Low Birth Weight

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Abstract: To evaluate body composition, metabolism and growth as well as their interaction with early nutrition in former extremely low birth weight infants (ELBW), we assessed qualitative and quantitative nutritional intake during initial hospitalization and infantile growth parameters in 61 former ELBW infants with a birth weight <1000 g. In two follow-up exams, physical and biochemical development were measured at 5.7 and at 9.5 years. At the second follow-up, in addition to biochemical reassessment, body composition was analyzed by dual-energy x-ray absorptiometry (DEXA). Protein intake between birth and discharge was associated with weight gain in the first six months of life ($r = 0.51$; $p < 0.01$). Weight catch-up preceded height catch-up. Protein intake in early infancy correlated highly significantly with abdominal fat mass ($r = 0.49$; $p < 0.05$), but not with lean body mass at 9.5 years ($r = 0.30$; not significant (n.s.). In contrast to nutrient intake, birth weight was associated with lean body mass ($r = 0.433$; $p < 0.001$). Early protein and carbohydrate intake were associated with high-density lipoprotein (HDL)-cholesterol, and early catch-up growth correlated with fasting insulin at follow-up. Stepwise linear regression demonstrated that protein intake predicted fat mass ($p < 0.05$), whereas only gender and birth weight standard deviation score (SDS) contributed significantly to lean body mass variation ($p < 0.05$). Our results suggest an important impact of early nutrient intake on body composition and metabolism in later childhood in ELBW children.

Keywords: preterm; early nutrition; body composition

1. Introduction

Decreased birth weight can have a persisting impact on metabolic health in later life [1]. In addition to metabolic dysfunction, subjects with low birth weight exhibit an increased risk of cardiovascular impairments in adulthood [2]. Catch-up growth in children with low birth weight has been associated with impaired metabolic parameters in later life [3]. On the other hand, catch-up of head circumference is important for neurological development [4].

Preliminary animal data suggest that the macronutrient content of late fetal or early postnatal nutrition might influence later body composition [5]. Therefore, early life macronutrient composition and total caloric intake currently receive much attention in humans, with special regard to the feeding

protocols of infants with low birth weight or born prematurely. Despite the continual improvement of dietary composition and a faster increase in quantitative food intake during initial in-patient stay, premature infants build up an energy deficit which leads to growth failure until discharge from the hospital [6,7].

In part, this might be due to the fact that energy supply in extremely low birth weight infants (ELBWs) often does not reach recommendations from guidelines in the first weeks after birth [8]. Missing these aims has been ascribed to the extent of prematurity. It depends on the clinical condition of the infants between birth and discharge, in particular on the number and severity of infections, the duration of the mechanical ventilation, and on medical treatment.

On the other hand, rapid catch-up growth following an initial growth restraint after preterm birth bears a higher risk of metabolic alterations later in life [9]. This is characterized by a higher abdominal fat mass, arterial hypertension, lower insulin sensitivity, and hyperlipidemia. The association of preterm birth and the occurrence of metabolic syndrome were mainly confirmed in studies in young adulthood [3]. Studies conducted in preterms at a younger age are sparse [10].

We hypothesized that in the special cohort of extremely low birth weight infants, the first signs of metabolic syndrome and altered body composition may be already present in early childhood and that these are related to early life events including the nutritional regimen.

To establish clear cause/effect relationships between early nutrition and subsequent morbidity, controlled human intervention trials in these groups at risk would be highly desirable. However, even today, feeding of critically ill preterm infants in the neonatal intensive care units (NICUs) remains a balancing act and makes controlled trials with strict adherence to nutritive protocols very difficult if not impossible. We thus performed a longitudinal single-center follow-up study in ELBW infants and focused on the development of height, weight, and metabolism. In addition, we assessed body composition at the last follow-up exam at a mean age of 9.5 years.

2. Materials and Methods

2.1. Patients

The initial study population consisted of 175 extremely low birth weight infants who were born at the Department of Neonatology at the University Hospital of Bonn in the years 1999–2002, per definition born with a birth weight below 1000 g. A total of 49 children died in the postnatal period, 27 had moved and were not accessible, and 38 refused to take part in the study.

First follow-up examination: A total of 61 children with a mean age of 5.7 $\pm$ 0.9 years participated in the first follow-up examination at the Children's Hospital in Bonn in 2006. The focus of the first follow-up examination was on auxological development. Subjects with chronic or syndromal disorders or with handicaps after severe intraventricular hemorrhages or necrotizing enterocolitis were excluded. For more details see [11].

Second follow-up examination: At the second follow-up in 2010, a total of 39 (22 female, 17 male) healthy children (mean age: 9.5 $\pm$ 1.0 years; range: 7.9–11.9) participated in the re-examination. Twenty-two families from the initial cohort could not be convinced to take part in the follow-up. The main reasons for not participating were having moved to more distant locations and unwillingness to travel, fear of stress to the children or refusal to participate in the dual energy X-ray absorptiometry (DEXA) analysis.

All but eight of the children (six male) were prepubertal. The children's parents and the children themselves were asked for written informed consent and agreed to the study. The protocol was approved by the Medical Ethics Committee of the Faculty of Medicine at the University of Bonn and the Federal Agency for Radiation Safety (Ethics approval code EK 115/06).

2.2. Measurement of Nutritional Intake

Nutritional intake from birth until discharge was collected retrospectively from patients' files. The following components were analyzed: total daily energy-, carbohydrate-, lipid- and protein-intake. For analysis, we calculated the average daily nutritional intake of each component over the whole in-patient stay, expressed in g/kg/day. Due to the fact that our cohort consisted of ELBW infants, strict adherence to a detailed nutritional protocol was not possible, in particular in critically ill infants. Several ELBW infants had to switch from oral to parenteral nutrition during clinical deterioration; therefore, the amino acid composition of the protein intake differed between patients and was not part of this analysis. Not prescribed, but actually taken amounts of nutrients were used for calculations.

2.3. Measurement of Auxological Parameters

Growth parameters (weight and height) at birth and during postnatal in-patient stay were gathered retrospectively from patients' files. Data concerning growth during infancy were collected from regular paediatric screening examinations. Children were seen at a mean age of one, two and four years as part of the recommended German prevention program. The first follow-up examination as part of the study protocol, was performed at a mean age of 5.7 years. Mean age at the second follow-up examination was 9.5 years. All children underwent standardized auxological measurements which were always conducted by the same person. All children were weighed with the same scales while wearing underclothes. Height was determined by the same fixed stadiometer in all cases. For analysis, age was corrected for preterm birth until two years of age. Weight and height standard deviation-scores were calculated using German reference data [12,13]. Body-mass-index in kg/m^2 (BMI) was standardized in agreement with national reference data [14]. To illustrate weight and height gain over the whole period, we calculated the change in weight/BMI, resp. height standard deviation score (SDS) between successive examinations.

In addition to the outlined auxological parameters, we measured triceps' skinfold thickness with a calibrated calliper according to standardized reference manuals. For analysis, reference data for standard deviation scores were derived from Gerver and coworkers 2001 [15].

2.4. Measurement of Body Composition

Measurement of body composition only took place at the second follow-up examination with a mean age of 9.5 years. Lean body mass, total and regional (abdominal, hip) fat mass were measured using dual-energy x-ray absorptiometry (Lunar DXA, GE Healthcare). The DXA scans were all performed with the same standard dose apiece (76 kV/0.15 mA/0.4 μGym^2). During the examination, every child wore shorts and lay in a standardized position on the back. We did not have to use sedatives. For analysis, reference data for standard deviation scores were provided from GE Lunar Body Composition Software (enCORE 2010; Version 13.31, GE healthcare, Madison, WI, USA).

2.5. Biochemical Analyses

Venous blood samples for laboratory analysis were collected from all children between 08:00 a.m. and 10:00 a.m. in a fasting state. Levels of insulin were measured with an Immulite 2000 analyzer (Pharmacia and Upjohn Diagnostics AB, Uppsala, Sweden) with the lowest detection limit at 2.0 μU/mL). Total cholesterol, HDL- and low-density lipoprotein (LDL)-cholesterol, triglycerides were measured with Vista 3000T (Siemens Healthcare, Newark, NJ, USA).

2.6. Definitions

We defined "catch-up growth" as an increase of weight or length of >1 SDS during an interval of six months.

2.7. Statistical Analysis

Statistical analysis was performed using the SPSS software (SPSS, Version 20, IBM Deutschland GmbH, 71137 Ehningen, Germany). Non-parametric tests were used whenever data were not normally distributed. Relationships between variables were examined by non-parametric Spearman's rank correlation, partial correlations or linear regression analysis. Linear regression analyses were calculated with lean body mass, fat mass, HDL-cholesterol and fasting insulin as dependent variables and sex, birth weight, early macronutrient intake, weight development and pubertal stage as independent variables. Sex was used as a categorical variable in these models. All variables not normally distributed were log-transformed in these models. Significance was defined as p value < 0.05; a statistical trend was assumed for p values between 0.05 and 0.1.

3. Results

3.1. Growth from Birth until 9.5 Years of Age

A comparison of weight and height development of our study cohort is shown in Table 1. From birth onwards, weight-SDS declined continuously to an averaged minimum of −2.2 SDS at discharge. The highest gain in weight took place during the first six months after discharge. Mean weight-SDS at 0.5 years of age almost reached the initial value at birth. Between 0.5 and 5.7 years, weight-SDS stabilized at around −1 SDS followed by a further improvement in weight gain until the second follow-up-examination.

Table 1. Weight, height, and body mass index (BMI) development of ELBW subjects during follow-up.

	n	Mean Weight SDS (Range)	Mean Height SDS (Range)	Mean BMI SDS (Range)
at birth (mean GA 27.2 wks)	61	−0.88 (−2.60 to 0.44)	−0.80 (−2.49 to 0.25)	
at discharge (mean GA 40.3 wks)	52	−2.16 (−3.88 to 0.05)	−3.00 (−5.19 to 1.22)	
0.5 yrs	61	**−0.95 *** (−3.15 to 1.25)	−6.56 (−13.9 to 2.45)	−1.49 (−5.31 to 1.64)
1 yr	61	−1.04 (−3.77 to 1.31)	**−2.28 *** (−5.9 to 0.03)	−1.85 (−4.83 to 1.01)
2 yrs	61	−1.03 (−4.03 to 0.78)	**−0.94 *** (−3.97 to 1.75)	−1.76 (−5.81 to 1.76)
4 yrs	61	−1.29 (−3.41 to 1.23)	−1.13 (−3.06 to 0.21)	−1.71 (−6.5 to 1.54)
5.7 yrs (4.5–7.7)	61	−1.29 (−4.45 to 1.04)	−0.97 (−3.71 to 0.9)	−1.36 (−4.55 to 1.29)
9.5 yrs (7.9–11.9)	39	−0.75 (−3.56 to 1.76)	−0.23 (−2.26 to 1.57)	−0.85 (−3.71 to 1.88)

A asterisk (*) represents a change of >1 SDS between the actual and the preceding time point. GA = gestational age; SDS = standard deviation score; wks = weeks; yr = year.

The time pattern of height development differed from weight development: Height-SDS also declined from birth onwards. In contrast to weight development, the lowest mean height occurred at 0.5 years of age. The highest height catch-up took place between 0.5 and 1 year of age (+4.28 SDS) followed by a slower catch-up growth in height up to 2 years of age. At 9.5 years of age, target height-SDS (mean: 0.1 SDS; range: −2.25 to +1.58) was reached by one third of the cohort.

BMI-SDS declined until 1 year of age and continually increased up to the time of the second follow-up examination.

3.2. Relationship between Early Postnatal Nutrition and Auxological Parameters

The higher the average protein intake between birth and discharge from hospital, the more pronounced was the subsequent weight gain. This correlation was particularly present in the time interval of catch-up growth from discharge to 0.5 years of age ($r = 0.505$; $p < 0.01$). Weight development during further follow-up is depicted in Table 1, exhibiting only minor changes in weight SDS between 0.5 and 5.7 years, followed by a moderate increase in weight between 5.7 and 9.5 years of age. Correlation coefficients between early protein intake and delta weight SDS between subsequent follow-up examinations were, between 0.5 and 1.0 year −0.146 (n.s.); between 1.0 and 2.0 years −0.198 (n.s.); between 2.0 and 4.0 years 0.005 (n.s.); between 4.0 and 5.7 years 0.045 (n.s.); and between 5.7 and 9.5 years 0.440 ($p < 0.05$). None of the other nutritional components (average energy, carbohydrate, lipid intake) correlated with catch-up growth in weight between discharge and 0.5 years of age. Catch-up growth in height between 0.5 and 1 year of age was not influenced by nutritional intake.

3.3. Association between Auxological Parameters and Body Composition at 9.5 Years of Age

As mentioned above, body composition was measured at the second follow-up of our cohort. There was a positive correlation between birth weight-SDS and lean body mass at 9.5 years of age (Figure 1 and Table 2). We found no correlation between birth weight-SDS and fat mass at the same age.

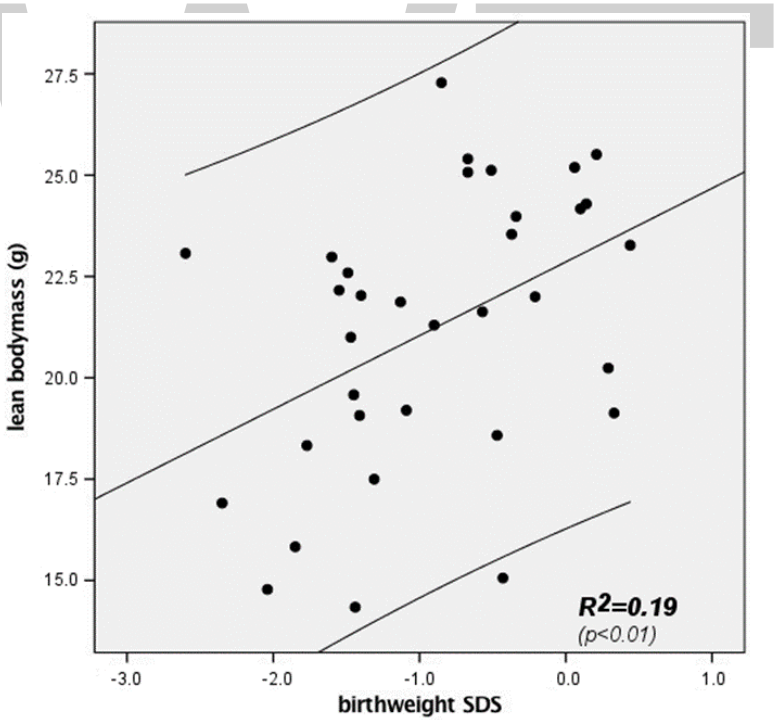

Figure 1. Correlation between birth weight and lean body mass in former ELBW subjects at 9.5 years of age. Lines represent the regression line and the upper and lower limit of the 95% confidence interval ($n = 35$).

As outlined in Table 1, we found no major changes in weight development between 0.5 and 5.7 years and a moderate weight gain between 5.7 and 9.5 years. Correspondingly, the positive correlation between early protein intake and weight gain between discharge and 0.5 years of age (reported in 3.2) remained significant for comparable calculations with subsequent timepoints. Correlation coefficients between early protein intake and delta weight SDS between subsequent follow-up examinations were: between discharge and 0.5 years 0.505 ($p < 0.01$); between discharge and 1.0 year 0.464 ($p < 0.05$); between discharge and 2.0 years 0.364 ($p = 0.052$); between discharge and

4.0 years 0.211 (n.s.); between discharge and 5.7 years 0.428 ($p < 0.05$); and between discharge and 9.5 years 0.542 ($p < 0.01$).

Table 2. Stepwise linear regression analyses of body composition and metabolic parameters in former ELBW infants at 9.5 years of age ($n = 30$).

	Regression Coefficients (ß)							
Independent Variable	***Sex*** [b]	***Birth Weight*** [c]	***Early Macronutrient Intake***			***Weight Dev*** [d]	***Pubertal Stage*** [e]	$R^2_{corr.}$
			Carb.	*Protein*	*Fat*			
Lean body mass (g)	−0.479 $p = 0.003$	0.476 $p = 0.004$	NS	NS	NS	NS	NS	0.44 $F = 11.11$ $p < 0.001$
Fat Mass (%)	NS	NS	NS	0.515 $p = 0.006$	NS	NS	NS	0.24 $F = 9.05$ $p < 0.01$
HDL-cholesterol (mg/dL)	NS	NS	−0.336 $p = 0.038$	−0.531 $p = 0.002$	NS	NS	NS	0.41 $F = 10.15$ $p = 0.001$
Fasting Insulin [f] (mU/L)	NS	NS	NS	NS	NS	0.559 $p = 0.008$	NS	0.28 $F = 8.64$ $p < 0.01$

Carb = carbohydrates; dev = development; DEXA = dual energy X-ray absorptiometry; NS = not significant. [b] Males had a significantly higher lean body mass than females ($p < 0.05$). [c] Birth weight was expressed as an SDS value. [d] weight development was calculated as the difference between birth weight SDS and weight SDS at the age of six months. [e] Pubertal stage was expressed in Tanner stages. [f] Fasting insulin concentrations were log-transformed before analysis.

Absolute height-SDS was positively associated with lean body mass at every measurement from birth onwards. Contrary to weight development, absolute height-SDS did not correlate with fat mass at any point of time. Stepwise linear regression demonstrated that birth weight-SDS and gender contributed significantly to lean body mass variation ($p < 0.05$).

Height development from birth to six months of age correlated significantly with variation in lean body mass ($r = 0.396$; $p = 0.020$), whereas weight from birth to 6 months of age correlated only with variation in fat mass ($r = 0.411$; $p = 0.022$), but not lean body mass ($r = 0.004$; $p = 0.984$). However, in the stepwise multiple regression analysis, neither catch-up in weight nor height contributed significantly to the variation in lean body mass or fat mass, respectively.

3.4. Influence of Early Postnatal Nutrition on Body Composition at 9.5 Years of Age

Average protein intake during early life correlated significantly with absolute fat as well as abdominal and hip fat mass (see Figure 2 and Table 3).

Table 3. Relationship between early macronutrient intake and parameters of body composition in former ELBW subjects at 9.5 years. of age as indicated by Spearman's correlation coefficients ($n = 30$).

	Total Fat Mass (%)	**Abdominal Fat Mass (%)**	**Hip Fat Mass (%)**	**Lean Body Mass (g)**	**Triceps Skinfold (SDS)**
Protein intake (g/kg/day)	**$r = 0.481$** **$p = 0.007$**	**$r = 0.488$** **$p = 0.006$**	**$r = 0.443$** **$p = 0.014$**	$r = 0.299$ $p = 0.108$	**$r = 0.381$** **$p = 0.05$**
Lipid intake (g/kg/day)	$r = 0.275$ $p = 0.141$	$r = 0.157$ $p = 0.406$	$r = 0.247$ $p = 0.189$	$r = -0.006$ $p = 0.975$	$r = 0.230$ $p = 0.248$
Carb. Intake (g/kg/day)	$r = -0.036$ $p = 0.849$	$r = 0.027$ $p = 0.888$	$r = -0.093$ $p = 0.624$	$r = -0.138$ $p = 0.468$	$r = 0.117$ $p = 0.562$
Energy intake (kcal/kg/day)	**$r = 0.371$** **$p = 0.043$**	$r = 0.332$ $p = 0.073$	$r = 0.319$ $p = 0.086$	$r = -0.004$ $p = 0.984$	$r = 0.342$ $p = 0.08$

Carb = carbohydrate; g = grams; SDS = standard deviation score. Average carbohydrate and average lipid intake were not associated to any parameter of body composition. In addition, stepwise linear regression demonstrated that protein intake predicted fat mass ($p < 0.05$), whereas macronutrient intake did not contribute to the observed variation of lean body mass (Table 2).

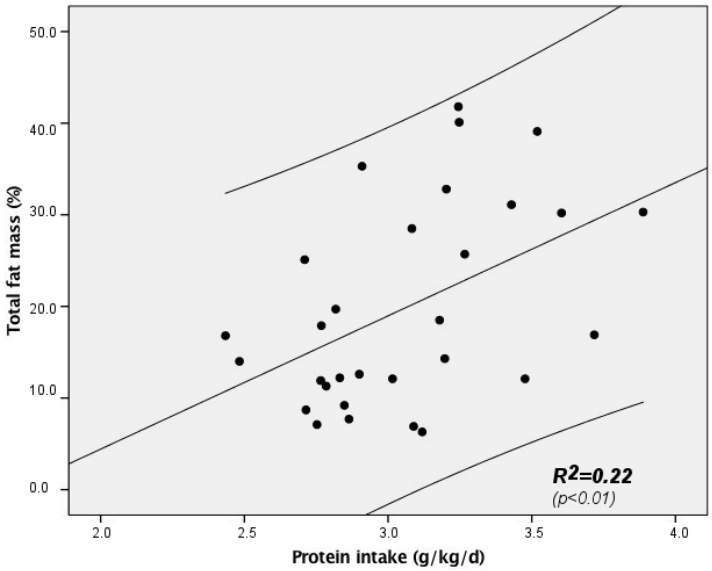

Figure 2. Correlation between early protein intake and fat mass in former ELBW subjects at 9.5 years of age. Lines represent the regression line and the upper and lower limit of the 95% confidence interval (n = 30).

In addition, triceps' skinfold thickness also correlated with average protein intake. Lean body mass did not correlate with the amount of protein intake. There was also a positive statistical trend between the average energy intake and absolute, abdominal and hip fat mass as well as to triceps' skinfold thickness at 9.5 years of age (Table 3).

Since the stepwise linear regression analysis revealed an interrelation between sex and lean body mass, we re-calculated the correlation coefficients presented in Tables 3 and 4 as partial correlations with sex as a control variable. Except for the correlation between triceps' skinfold SDS and protein intake which showed a borderline significant correlation when calculating Spearman's rank correlation coefficient and which no longer reaches statistical significance with sex as a control variable, correction for sex did not lead to relevant changes (see Supplementary Materials Tables S1 and S2).

3.5. Nutritional Intake and Metabolic Parameters at 9.5 Years of Age

Protein and carbohydrate intake during the interval between birth and discharge correlated negatively with HDL-cholesterol at the age of 9.5 years (Table 4). Lipid intake was not associated with any metabolic parameter. Fasting insulin levels and Homeostasis Model Assessment (HOMA) at the age of 9.5 years were highly significantly associated with protein intake, but with none of the other nutritional parameters (Table 4 and supplementary table S2).

Table 4. Association between early macronutrient and energy intake and metabolic markers in former ELBW subjects at 9.5 years of age as indicated by Spearman's correlation coefficients (n = 31).

	Protein Intake (g/kg/day)	Carbohydrate Intake (g/kg/day)	Lipid Intake (g/kg/day)	Energy Intake (kcal/kg/day)
HDL-cholesterol (mg/dL)	**−0.445** **p = 0.014**	**−0.417** **p = 0.022**	−0.188 p = 0.320	**−0.393** **p = 0.032**
LDL-cholesterol (mg/dL)	0.143 p = 0.450	−0.029 p = 0.879	−0.081 p = 0.670	−0.077 p = 0.722
Total cholesterol (mg/dL)	−0.056 p = 0.768	−0.336 p = 0.070	−0.169 p = 0.371	−0.327 p = 0.078
Fasting insulin (mU/L)	**0.581** **p = 0.003**	0.155 p = 0.469	0.077 p = 0.722	0.232 p = 0.274
HOMA index	**0.566** **p = 0.003**	0.152 p = 0.468	0.073 p = 0.728	0.230 p = 0.269

Stepwise linear regression demonstrated that protein and carbohydrate intake predicted HDL concentration at 9.5 years ($p = 0.001$), whereas regarding fasting insulin levels, only catch-up in weight between birth and six months of age contributed significantly ($p < 0.05$).

4. Discussion

To the best of our knowledge, this is the first study which demonstrates that quantity and quality of macronutrient intake during the first weeks of life seem to exert a long-term effect on body composition and metabolic health in former very premature ELBW children and that this is already measurable in childhood.

To achieve a sufficient weight and height catch-up, current guidelines focus on an amino-acid enriched formula nutrition [16]. This has been suggested in particular because the intake of protein-enriched formula has resulted in improved cognitive function [17–19]. In our study, we demonstrate that early nutrition with increased protein intake might not only be advantageous, but can be associated with an increased risk of metabolic disturbances. We found that early protein intake was associated with an increase in fat mass (absolute, abdominal and hip fat mass) which was already detectable at 9.5 years of age, whereas lean body mass at this age was not affected by protein intake (Table 3, Figure 2). Only birth weight SDS contributed to the observed variability in lean body mass, which is in accordance to previous reports [20,21].

In addition, protein intake explained a significant part of the observed variation of HDL-cholesterol with a decrease in HDL concentration correlating with increasing protein intake. Early protein and energy intake were significantly correlated to weight gain during the first six months of life. In the regression analysis, early weight gain was associated with an increase in fasting insulin level in later childhood. This is in congruence with already reported findings by others [3], although the studied patients in the cohort of Kerkhof et al. were all born at term with the majority being born small for gestational age (SGA) with a birth length < - 2SD.

The exact timing of catch-up growth has been a topic of discussion in the recent literature. In our cohort, weight-SDS decreased dramatically between birth and discharge. This phenomenon has often been described in previous clinical studies [6,7], particularly in extremely low birth weight infants [22]. Although most subjects were appropriate for gestational age at birth, the majority of subjects would have been considered small for gestational age at the expected date of delivery. In our study group, the main catch-up growth in weight took place after discharge until 0.5 years of corrected age.

An accelerated gain of weight within three months after term age caused an unfavourable body composition in young adulthood [3]. However, as stated above, the cohort reported by Kerkhof et al. differed from this cohort by a significantly higher gestational age and higher frequency of infants born SGA. Henriksen et al reported that severely ill ELBW infants exhibited a lower weight gain in the first months as compared to very low birth weight infants (VLBW) infants [23]. As our cohort consisted of very premature, frequently critically ill infants, we used a larger window for catch-up growth of six months.

With regards to mechanisms underlying metabolic changes, our data suggest that weight catch-up and the hereby associated metabolic risk might solely reflect the amount of protein intake during these critical first weeks of life. The risk of lifetime metabolic disturbances in ELBW children might be even more aggravated by the implementation of current guidelines, which recommend an even higher neonatal protein intake [16]. In addition, our analysis of body composition was performed in early childhood. Thus, we speculate that metabolic disturbances might even increase in the later life of these subjects

Height development differed from weight development in our cohort: After a constant decrease from birth up to 0.5 years of age, the main catch-up in height took place during the second half of the first year of life. At 9.5 years of age, target height was reached by only one-third of our cohort. Not unexpectedly, we found that the larger the change in weight-SDS from 0.5 years onwards, the higher

were both fat and lean body mass as measured by DEXA in our patients. Height development only correlated with lean body mass.

The interaction between protein intake after birth and the infants' auxological development as well as body composition later on turned out to be the focal point of our work. Protein-supplementation after birth has been discussed, in particular for extremely low birth weight infants. In our institution, protein intake for extremely low birth weight infants was usually increased from 0.5 to 1 g/kg/day on the first day of life up to a maximum of 2.5 g/kg/day (parenteral intake) and 4 g/kg/day (oral intake) in accordance to current recommendations [16,24].

It has been controversially debated whether higher protein intake during initial hospitalization directly causes uremia, acidosis and hyperammonemia [25]. Concerning long-term adverse health outcomes, protein intake may also entail a higher occurrence of long-term adverse health outcomes caused, for example, by bronchopulmonary disease (BPD) or necrotizing enterocolitis (NEC) [26]. In our cohort, we could neither verify short-term metabolic disturbances nor any occurrence of BPD or NEC which were directly affected by the amount of protein intake [8].

However, the advantages of an earlier and higher amino acid intake in form of a high protein intake, on auxological parameters were also shown by many international studies: Concerning short-term consequences, Poindexter and coworkers could demonstrate that a parenteral supplementation of amino acids of >3 g/kg/day during the first five days of life was associated with a better growth outcome at 36 postmenstrual weeks in extremely low birth weight infants compared to infants who received less amino acids [27]. At 18 months of age, however, in their study, the differences in growth parameters between groups had disappeared. A review by Premji et al. focused on studies which compared a low (<3 g/kg/day) versus a high (>3 g/kg/day but <4 g/kg/day) protein intake in low birth weight children postnatally [28]. On average, in these studies, higher protein intake resulted in an improved weight gain during the first months of life whereas gain in length was not influenced. Sufficient data about long-term auxological or cognitive outcome were missing in this review. In our cohort, average daily protein intake until discharge correlated highly significantly with main catch-up growth in weight between discharge from hospital and 0.5 years of age. However, it must be pointed out that we were not informed about the nutrition that our cohort received after discharge. In contrast to weight development, main catch-up growth in height between 0.5 and 1 years of age was not influenced by nutritional intake until discharge.

Considering long-term outcome parameters, most studies evaluated the effect of early protein administration on cognitive performance [17–19], whereas data on body composition of preterm infants as a result of early nutritional intake are sparse. For this purpose, dual-energy x-ray-absorptiometry is currently the most reliable method, since BMI does not accurately reflect the degree of central adiposity [29]. This is particularly important when considering that the mean BMI of our cohort at 9.5 years still remained below average. Garnett et al. revealed that mainly children with a lower birth weight and a subsequently high gain of weight afterwards tended to have a higher amount of abdominal fat in a prepubertal state [30]. Our patients were born with extremely low birth weight and frequently experienced a rapid catch-up in weight during the first six months of life. We thus hypothesize that a further increase of BMI during puberty might be associated with an even more pronounced, potentially unfavorable, fat distribution with its associated metabolic risks.

5. Limitations

Due to the high morbidity of our ELBW cohort and the fact that this was a single center study, our sample size was small. Furthermore, since our analysis was focused on quantity and not quality of protein intake, we were unable to analyze whether the amino acid composition of early infant nutrition might play an additional role on the long-term metabolic health of ELBW infants. On the other hand, the single center setting was advantageous with regards to the homogeneous nutritional regime and the early intensive care of our study patients. In addition, it has to be taken into account that besides

early postnatal nutrition, several other pre- and postnatal factors contribute to the outcome of preterm infants but were not part of our analysis.

6. Conclusions

Postnatal weight and height development in extremely low birth weight infants occur during different time periods. A high amount of protein intake seems to ensure a rapid catch-up growth during the first months of life. At 9.5 years of age, however, the higher early protein supply was associated with a potentially negative impact on both body composition profile and metabolic phenotype. The higher the postnatal daily protein supply, the higher the percentage of abdominal fat mass and fasting insulin, and the lower the level of HDL-cholesterol. If confirmed in further studies, this should lead to a critical reevaluation of the nutrient regimen in low birth weight infants.

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Conflicts of Interest: The authors declare no conflict of interest.

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Usefulness of the Waist Circumference-to-Height Ratio in Screening for Obesity and Metabolic Syndrome among Korean Children and Adolescents: Korea National Health and Nutrition Examination Survey, 2010–2014

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Abstract: The aims of this study were to assess the diagnostic value of the weight-to-height ratio (WHtR) for the detection of obesity and metabolic syndrome (MS) in Korean children and adolescents, and to determine the advantages of WHtR as a population-based screening tool in comparison with other obesity indicators, such as body mass index (BMI) and waist circumference (WC). We performed a cross-sectional analysis of data from 3057 children and adolescents (1625 boys, 1332 girls) aged 10–19 years who were included in the fifth Korean National Health and Nutrition Examination Survey (KNHANES, 2010–2012) up to the second year of the sixth KNHANES (2013–2014). Receiver operation characteristic (ROC) curves were generated to determine the optimal cutoff value and accuracy of WHtR for predicting individual obesity indicators or more than two non-WC components of MS. The area under the ROC curve (AUC) is a measure of the diagnostic power of a test. A perfect test will have an AUC of 1.0, and an AUC equal to 0.5 means that the test performs no better than chance. The optimal WHtR cutoff for the evaluation of general obesity and central obesity was 0.50 in boys and 0.47–0.48 in girls, and the AUC was 0.9. Regarding the assessment of each MS risk factor, the optimal WHtR cutoff was 0.43–0.50 in boys and 0.43–0.49 in girls, and these cutoffs were statistically significant only for the detection of high triglyceride and low High-density lipoprotein (HDL) cholesterol levels. When a pairwise comparison of the AUCs was conducted between WHtR and BMI/WC percentiles to quantify the differences in power for MS screening, the WHtR AUC values (boys, 0.691; girls, 0.684) were higher than those of other indices; however, these differences were not statistically significant (boys, $p = 0.467$; girls, $p = 0.51$). The WHtR cutoff value was 0.44 (sensitivity, 67.7%; specificity, 64.6%) for boys and 0.43 (sensitivity, 66.4%; specificity, 66.9%) for girls. There was no significant difference between the diagnostic power of WHtR and that of BMI/WC when screening for MS. Although the use of WHtR was not superior, WHtR is still useful as a screening tool for metabolic problems related to obesity because of its convenience.

Keywords: waist-to-height ratio; children; adolescents; overweight; obesity; metabolic syndrome; cutoff values; body mass index; waist circumference

1. Introduction

Childhood obesity and metabolic syndrome (MS) associated with obesity are considered major health problems worldwide [1]. The global prevalence of obesity has been increasing [2], and the prevalence of obesity in children and adolescents in Korea has also increased from 5.8% in 1997 to 9.7% in 2005, 10.95% in 2007, 10.8% in 2010, and 11.5% in 2014 [3]. Childhood obesity is more than 50% likely to lead to adult obesity [4,5], and it increases the risk of MS, a cluster of cardiovascular risk factors such as visceral obesity and lipid metabolism abnormalities [6]. In addition, since atherosclerosis can begin at an early age [7], obesity and MS in pediatric individuals can continue into adulthood [7,8]; thus, early detection and management of MS is very important for the prevention of cardiovascular disease in adulthood [4,5,9].

Detecting MS early requires a long timeframe and is costly, as each parameter of MS must be investigated [10]. In addition, although the prevalence of MS is increasing in pediatric individuals, its prevalence is still low. Therefore, it is not cost effective to conduct blood tests for early detection of metabolic abnormalities in all individuals at risk [11]. For this reason, anthropometric parameters, including body mass index (BMI), waist circumference (WC), and waist-to-height ratio (WHtR), have been suggested as screening tools for use in children and adolescents with cardiovascular risk factors [11].

A good screening test should be not only highly predictive but also easy to perform and interpret [12]. BMI and WC have been the most commonly used predictors of obesity and cardiovascular disease to date. Unlike adults, for whom a single obesity criterion is used, both BMI and WC percentiles by race, sex, and age are used to determine obesity in children and adolescents. Therefore, determining the obesity status for medical treatment is difficult using these methods [13,14] and it is not easy for the general population to understand these criteria. On the other hand, WHtR can be applied to a single reference point regardless of race, sex, or age. Consequently, it has been proposed as a population-based screening tool for cardiometabolic risk prediction in large-scale epidemiological studies and during medical examinations [12,15,16].

Several previous studies have shown that WHtR has a high sex match and is a useful index for evaluating abdominal, especially visceral, fat associated with cardiovascular risk factors [17]. In a recent meta-analysis investigating children and adolescents by Lo et al. [12], WHtR was shown to be a good predictor of cardiometabolic risk, and in some studies, its screening capacity was better than that of BMI and WC. However, the overall analysis concluded that the screening ability of WHtR was not significantly better than that of the other two indices. However, it has been suggested that using WHtR rather than BMI and WC may result in easier and more rapid identification of children and adolescents with cardiovascular risk factors [12]. In addition, the measurement and interpretation of WHtR are more convenient than those of the other two indicators [12]. Only one study has confirmed WHtR cutoff values for the assessment of cardiometabolic risk in Korean pediatric patients, performed in 314 obese adolescents visiting an obesity clinic [18]. Regardless of age and sex, a WHtR cutoff value of 0.5 is a significant indicator for predicting cardiovascular metabolic risk [16]. However, some studies have suggested other cutoff values with better sensitivity and specificity as indicators of obesity or metabolic abnormalities in children and adolescents [19–24]. Therefore, it is necessary to confirm an appropriate WHtR cutoff value in screening for cardiovascular metabolic disease risk in Korean children and adolescents [20].

In this study, the optimal WHtR cutoff value for screening for obesity and MS among children and adolescents was determined using recent data representative of Korean children and adolescents. Moreover, the usefulness of WHtR as a population-based screening tool for cardiovascular risk factors was compared with that of other obesity indicators including BMI and WC.

2. Materials and Methods

2.1. Study Population

In this study, data were analyzed from the fifth (2010–2012) and sixth (2013–2014) Korean National Health and Nutrition Examination Surveys (KNHANES) investigating children and adolescents aged

10–19 years conducted by the Korean Centers for Disease Control and Prevention (KCDC) [3,25,26]. The KNHANES is a nationwide health and nutrition survey performed annually to identify the health behaviors of the Korean population, the current status of chronic diseases, and data concerning food and nutrition consumption [26]. After selecting 20 households by applying a two-stage stratified sampling method using sampling units and households as first and second sampling units, respectively, health surveys, examinations, and nutrition surveys were conducted for household members aged 1 year or older. Of the 4885 children aged 10 to under 19, those who missed physical or blood pressure (BP) measurements or blood test results and those who did not fast for more than 8 h were excluded. Thus, the final study subjects numbered 3057 (1625 boys, 1432 girls). All participants provided informed consent before data collection, and the survey was approved by the KCDC Bioethics Committee (approval numbers: 2010-02CON-21-C, 2011-02CON-06-C, 2012-01EXP-01-2C, 2013-07CON-03-4C, and 2013-12EXP-03-5C in 2010, 2011 2012, 2013, and 2014, respectively). The study protocol was exempt from examination by the clinical examination committee of Seoul Paik Hospital of Inje University (Institutional Review Board no. IIT-2016-318).

2.2. Anthropometric Measurements

2.2.1. Anthropometric Measurements

All anthropometric measurements were performed by trained individuals using the same methods. The methods performed by the KNHANES are as follows. Height was measured with the subject on his/her side by contacting the subject's heels, hips, back, and back of the head with the vertical plate of the extensometer (SECA 225, SECA, Hamburg, Germany) while the subject maintained a horizontal gaze. The measuring plate was pressed to the vertex of the head with only enough pressure to press the hair down, and the measurement was recorded to the nearest 0.1 cm. Weight was measured to the nearest 0.1 kg while the subjects wore a disposable gown. Then, BMI was calculated according to the height and weight measurements as follows: BMI = body weight (kg)/height (m^2).

Using a tape measure (SECA 200, SECA), WC was measured to the nearest 0.1 cm while the subjects were breathing out. A mark was made using a water-based ink pen at the midpoint between the lower end of the last rib and the upper rim of the iliac crest at the central axillary line. If the degree of obesity was severe, the subject was asked to touch two points (the lower end of the last rib and the upper rim of the iliac crest) directly, or the subject was instructed to lean the upper body forward, and the lower part of the last rib was touched directly. If the two points could not be discerned, WC was measured at a point slightly above the navel. WHtR was obtained by dividing WC by height [27].

2.2.2. Blood Pressure

BP was measured using a mercury sphygmomanometer (Baumanometer Desk Model 0320; WA Baum, Co., Copiague, NY, USA). The subjects were asked to stop smoking at least 30 min prior to the BP measurement and to rest in a sitting position for 5 min before the test. The subjects sat in a chair with a backrest and armrests during the measurements. After palpating the brachial artery of the right arm such that the middle part of the cuff bladder was on top, the cuff was rapidly expanded to the maximum inflation pressure. The valves were regulated such that the pressure dropped constantly at a rate of 2 mmHg/s, and the systolic and diastolic BPs were monitored. The systolic BP was defined as the first Korotkoff sound and the diastolic BP as the fifth Korotkoff sound. BP was measured three times, and the mean of the second and third measurements was used for analysis.

2.2.3. Laboratory Tests

Blood tests were performed after the subjects had fasted for more than 8 h. Fasting plasma glucose (FPG), total cholesterol, triglyceride, and low-density lipoprotein (LDL)-cholesterol levels were measured using an automatic analyzer (Hitachi Automatic Analyzer 7600, Hitachi, Japan).

High-density lipoprotein (HDL) cholesterol was defined as the value calculated from the conversion formula for standardization [26].

2.3. Diagnostic Criteria

Obesity was defined using the 2007 Pediatric Adolescent Standard Growth Chart [28] from the Center for Disease Control and the Association of Korean Pediatrics. Weight classifications according to BMI diagnostic criteria were: normal weight, ≥the 5th percentile and <the 85th percentile by sex and age; overweight, ≥the 85th percentile and <the 95th percentile; and obesity, ≥the 95th percentile or BMI ≥25 kg/m^2 [29]. Moreover, obesity was defined as WC ≥the 90th percentile by sex and age [30,31]. The risk factors for MS were defined according to the modified National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP III) criteria [30]: (1) abdominal obesity, WC ≥ the 90th percentile by sex and age; (2) high triglyceride, fasting triglyceride level ≥110 mg/dL; (3) low HDL-cholesterol: HDL level <40 mg/dL (fasting); (4) high BP, systolic BP ≥130 mmHg or diastolic BP ≥85 mmHg for subjects >18 years of age, and systolic or diastolic BP ≥the 90th percentile by age, sex, and height for subjects <18 years of age; and (5) high FPG, FPG ≥110 mg/dL. MS was diagnosed when more than three of the above risk factors were detected. Non-WC components of MS was defined as having all of the above risk factors for MS except abdominal obesity.

2.4. Statistical Analysis

All analyses were performed for all participants and separately for boys and girls using the composite sample design and weighting of the KNHANES, except for receiver operating characteristic (ROC) curve analysis. The independent *t*-test and chi-square test were used to analyze differences according to sex. Continuous variables were expressed as means and standard errors and nominal variables as frequencies and percentages (standard error). ROC analysis was used to determine the optimal threshold and accuracy of WHtR in predicting the obesity indices (BMI and WC percentiles) and individual or more than two non-WC components of MS, including high FPG, high BP, high triglyceride, and low HDL cholesterol. The optimal threshold was determined using the Youden index (maximum value of (sensitivity + specificity − 1)), and all sensitivities, specificities, and J-values (sensitivity + specificity − 1) are presented [32]. The sensitivity, specificity, positive predictive value, negative predictive value, and J-values of 0.5 and 0.6 that were suggested as the optimal WHtR cutoff levels for predicting cardiovascular disease in previous studies [16,33], and this study's optimal cut-off values for predicting two or more non-WC components of MS are presented. To determine differences in the predictive values of abdominal obesity indices (WHtR, WC, and BMI) for more than one, more than two, or individual MS risk factors. WC and BMI were stratified to calculate percentiles, and ROC curve analysis was performed subsequently. The area under the curve (AUC) values were compared using non-parametric methods to confirm significance [34]. An AUC ≥0.5 is less accurate but remains useful for screening tests, an AUC >0.7 indicates an accurate value for screening, and an AUC >0.9 indicates a very accurate value [35,36]. Moreover, ROC analysis can be applied to measure differences in AUC values, thereby enabling identification of the most powerful variables [37]. ROC analysis was employed to avert assumptions of normal distribution and to analyze multiple predictors concurrently [37]. Continuous variables were adjusted into the binary classification (0 = normal, 1 = abnormal) according to the given threshold value [37]. In addition, calculations were corrected for multiple testing by computing 95% confidence intervals (CIs) [37]. Statistical analysis of the AUC difference between the three indexes (BMI percentile, WC percentile, WHtR) was performed using the STATA software ver. 12.0 (StataCorp LP, College Station, TX, USA). Other statistical analyses were performed using the SAS software ver. 9.4 (SAS Institute Inc., Cary, NC, USA). $p < 0.05$ was considered to indicate statistical significance.

3. Results

The general characteristics of the study subjects are shown in Table 1, according to sex. The mean age of the subjects was 14.26 ± 0.0 years, and the mean BMI was 20.96 ± 0.12 kg/m^2 for boys and 20.42 ± 0.11 kg/m^2 for girls. The WC and WHtR values were significantly larger in boys than girls (girls, 67.29 ± 0.2 cm and 0.427 ± 0.00, respectively; boys, 71.33 ± 0.33 cm and 0.432 ± 0.00, respectively). Overall, 12.0% of the subjects (14.1% of boys and 9.7% of girls) were obese, and 6.0% (4.4% of boys and 7.9% of girls) were overweight. The prevalence of obesity was higher in boys, and the prevalence of overweight was higher in girls. There were no significant differences in the prevalence of underweight or normal weight participants according to sex. Overall, a high triglyceride level was the most common risk factor, while a high fasting blood glucose level was the least common risk factor among the study subjects. Apart from central obesity, all other MS components were more prevalent among boys than girls, albeit without statistical significance; however, low HDL-cholesterol levels were significantly higher in boys than girls. In this sample, 45.6% of the subjects (boys, 48.4%; girls, 42.5%) had one non-WC components of MS, and 12.4% (boys, 14.4%; girls, 10.2%) had two non-WC components of MS, while 6.2% (boys, 7.0%; girls, 5.2%) were diagnosed with MS. There were no significant differences in MS prevalence between boys and girls.

Table 1. Baseline characteristics of study subjects.

	Total (n = 3057)	Boys (n = 1625)	Girls (n = 1432)	p-Value
Age (years)	14.26 ± 0.06	14.26 ± 0.07	14.26 ± 0.09	0.965
Height (cm)	161.69 ± 0.25	165.25 ± 0.38	157.61 ± 0.25	<0.001 **
Weight (kg)	54.80 ± 0.32	58.03 ± 0.49	51.10 ± 0.36	<0.001 **
BMI, (kg/m^2)	20.71 ± 0.08	20.96 ± 0.12	20.42 ± 0.11	0.001 **
WC (cm)	69.45 ± 0.22	71.33 ± 0.33	67.29 ± 0.29	<0.001 **
WHtR	0.430 ± 0.00	0.432 ± 0.00	0.427 ± 0.00	0.038 *
Systolic BP (mmHg)	107.22 ± 0.25	109.56 ± 0.34	104.54 ± 0.33	<0.001 **
Diastolic BP (mmHg)	66.33 ± 0.23	66.72 ± 0.32	65.88 ± 0.28	0.035 *
FPG (mg/dL)	89.15 ± 0.18	89.49 ± 0.22	88.76 ± 0.22	0.006 **
Total cholesterol (mg/dL)	157.75 ± 0.66	153.03 ± 0.85	163.16 ± 0.87	<0.001 **
Triglyceride (mg/dL)	83.15 ± 1.22	81.67 ± 1.58	84.82 ± 1.64	0.135
HDL cholesterol (mg/dL)	49.97 ± 0.25	48.53 ± 0.29	51.62 ± 0.34	<0.001 **
LDL cholesterol (mg/dL)	91.92 ± 0.55	88.98 ± 0.73	95.28 ± 0.75	<0.001 **
Obesity (BMI percentile ≥95 or BMI ≥25 kg/m^2) (%)	12.0 (0.7)	14.1 (1.1)	9.7 (1.1)	0.009 **
Overweight (BMI percentile 85–94) (%)	6.0 (0.5)	4.4 (0.5)	7.9 (0.8)	<0.001 **
Normal weight (BMI percentile 5–84) (%)	76.2 (0.9)	75.6 (1.3)	76.9 (1.3)	0.519
Underweight (BMI percentile <5) (%)	5.8 (0.5)	6.1 (0.8)	5.5 (0.7)	0.582
Central obesity (WC ≥the 90th percentile) (%)	8.9 (0.6)	7.6 (0.8)	10.5 (1.0)	0.026 *
High BP (%)	25.6 (1.0)	26.7 (1.4)	24.2 (1.3)	0.166
High FPG (%)	0.5 (0.1)	0.6 (0.2)	0.4 (0.2)	0.604
High triglyceride (%)	19.7 (1.0)	20.3 (1.3)	19.0 (1.3)	0.435
Low HDL cholesterol (%)	14.5 (0.8)	17.9 (1.2)	10.7 (1.1)	<0.001 **
At least one non-WC components of MS † (%)	45.6 (1.2)	48.4 (1.5)	42.5 (1.6)	0.005 **
Two or more non-WC components of MS † (%)	12. (0.8)	14.4 (1.2)	10.2 (1.0)	0.007 **
MS (%)	6.2 (0.5)	7.0 (0.8)	5.2 (0.8)	0.102

BMI: body mass index; WC: waist circumference; WHtR: waist circumference-height ratio; BP: blood pressure; FPG: fasting plasma glucose; HDL: high density lipoprotein; LDL: low density lipoprotein; MS: metabolic syndrome; Data expression as estimated mean ± standard error or estimated percent (standard error), as appropriate; * $p < 0.05$, ** $p < 0.01$ (p-value were analyzed by chi-square test or t-test.); † The non-WC components of MS were defined according to modified National Cholesterol Education Program, Adult Treatment Panel III (NCEP ATP III) criteria (1) triglycerides ≥110 mg/dL; (2) HDL cholesterol <40 mg/dL; (3) systolic or diastolic BP ≥90th percentile; (4) fasting plasma glucose level ≥110 mg/dL.

Table 2 shows the results of the ROC curve analysis performed to identify the optimal WHtR cutoff values for predicting general obesity, central obesity, and MS. The optimal WHtR cutoff value for predicting obesity according to BMI was 0.47 for both sexes. The AUC for predicting obesity according to BMI in boys and girls was 0.950 (sensitivity, 91.1%; specificity, 85.9%) and 0.965 (sensitivity and specificity, 90.2%), respectively.

Table 2. Results of ROC curve analysis to identify optimal WHtR to predict overweight, obesity, central obesity, and two or more non-waist circumference components of metabolic syndrome among children and adolescents.

	AUC	Cutoff Values ‡	Sensitivity (%)	Specificity (%)	J Value †	*p*-Value
Total (*N* = 3057)						
Overweight (BMI ≥ 85th percentiles)	0.846	0.44	93.1	70.5	0.636	<0.001 **
Obesity (BMI ≥ 95th percentiles or BMI ≥ 25 kg/m^2)	0.957	0.47	92.6	87.1	0.767	<0.001 **
WC (≥75th percentiles)	0.973	0.45	93.9	90.2	0.841	<0.001 **
WC (≥90th percentiles)	0.978	0.48	96.4	90.6	0.870	<0.001 **
High BP [a]	0.513	0.49	18.5	87.6	0.061	0.298
High FPG [b]	0.623	0.43	68.8	61.0	0.297	0.167
High triglyceride [c]	0.664	0.43	62.2	64.8	0.270	<0.001 **
Low HDL cholesterol [d]	0.658	0.44	56.0	71.4	0.273	<0.001 **
Two or more non-WC components of MS	0.690	0.43	69.8	62.2	0.320	<0.001 **
Boys (*n* = 1625)						
Overweight (BMI ≥ 85th percentiles)	0.861	0.45	95.8	70.2	0.660	<0.001 **
Obesity (BMI ≥ 95th percentiles or BMI ≥ 25 kg/m^2)	0.950	0.47	91.1	85.9	0.770	<0.001 **
WC (≥75th percentiles)	0.978	0.46	96.9	89.9	0.868	<0.001 **
WC (≥90th percentiles)	0.990	0.50	97.5	94.4	0.919	<0.001 **
High BP [a]	0.523	0.50	17.7	89.4	0.071	0.191
High FPG [b]	0.621	0.43	70.0	58.4	0.284	0.206
High triglyceride [c]	0.689	0.44	63.3	66.2	0.296	<0.001 **
Low HDL cholesterol [d]	0.631	0.44	56.3	67.8	0.240	<0.001 **
Two or more non-WC components of MS	0.691	0.44	67.7	64.6	0.323	<0.001 **
Girls (*n* = 1432)						
Overweight (BMI ≥ 85th percentiles)	0.856	0.44	90.2	75.5	0.657	<0.001 **
Obesity (BMI ≥ 95th percentiles or BMI ≥ 25 kg/m^2	0.965	0.47	92.7	90.2	0.829	<0.001 **
WC (≥75th percentiles)	0.980	0.44	94.7	89.9	0.846	<0.001 **
WC (≥90th percentiles)	0.985	0.48	94.6	94.6	0.892	<0.001 **
High BP [a]	0.501	0.49	14.7	90.9	0.056	0.9474
High FPG [b]	0.611	0.49	50.0	92.4	0.424	0.5554
High triglyceride [c]	0.638	0.43	58.0	67.8	0.258	<0.001 **
Low HDL cholesterol [d]	0.699	0.44	60.8	71.2	0.320	<0.001 **
Two or more non-WC components of MS	0.684	0.43	66.4	66.9	0.333	<0.001 **

ROC: receiver operating characteristic; AUC: area under curve; BMI: body mass index; WC: waist circumference; WHtR: Waist circumference-height ratio; BP: blood pressure; FPG: fasting plasma glucose; HDL: high density lipoprotein; MS: metabolic syndrome; * $p < 0.05$, ** $p < 0.01$ (Null hypothesis: area = 0.5); † J = sensitivity + specificity − 1; ‡ The optimal cut off point was obtained from Youden index as [maximum (J = sensitivity + specificity − 1)]; [a] High BP was diagnosed if systolic or diastolic blood pressure ≥90th percentile; [b] High FPG was diagnosed if FPG was ≥110 mg/dL; [c] High triglyceride was diagnosed if triglycerides was ≥110 mg/dL; [d] Low HDL cholesterol was diagnosed if HDL cholesterol was <40 mg/dL.

The optimal WHtR cutoff value for predicting obesity according to WC >the 90th percentile was 0.50 for boys, with an AUC of 0.990 (sensitivity, 97.5%; specificity, 94.4%), and 0.48 for girls,

with a AUC of 0.985 (sensitivity, 94.6%; specificity, 94.6%) (Table 2). The optimal WHtR cut-off values for predicting each MS risk factor ranged from 0.43 to 0.50 in boys and 0.43 to 0.49 in girls; these values were statistically significant only for hypertriglyceridemia and low HDL cholesterolemia (Table 2). The optimal WHtR cutoff value used to discriminate more than two non-WC components of MS was 0.44 in boys, with a J-value of 0.323 (sensitivity, 0.67; specificity, 64.6%) and an AUC of 0.691, and 0.43 in girls, with a J-value of 0.333 (sensitivity, 66.4%; specificity, 66.9%) and an AUC of 0.684 (Table 2).

When we compared an optimal WHtR cutoff value of 0.43 for the prediction of two or more non-WC components of MS with the values of 0.5 and 0.6, our values were lower. The sensitivity of our analysis was 69.8%, which was higher than that for other cutoff values; however, the specificity of 62.2% was lower than that for other cutoff values, and the positive predictive value of 19.0% was lower than other cutoff values. Similar results were found in both boys and girls (Table 3).

Table 3. Sensitivity and specificity of WHtR to detect two or more non-waist circumference components of metabolic syndrome.

		Cutoff Values †	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	J Value *
Total (*N* = 3057)							
WHtR	Optimal	0.43	69.8	62.2	19.0	94.2	0.320
		0.5	27.8	91.6	29.1	90.9	0.189
		0.6	2.6	99.7	52.9	89.0	0.023
Boys (*n* = 1625)							
WHtR	Optimal	0.44	67.7	0.646	21.5	93.3	0.323
		0.5	32.4	0.889	29.5	90.2	0.212
		0.6	4.4	0.996	60.0	87.9	0.040
Girls (*n* = 1432)							
WHtR	Optimal	0.43	66.4	66.9	17.9	94.8	0.333
		0.5	20.0	94.4	28.0	91.6	0.144
		0.6	0.7	99.9	33.3	90.3	0.006

WHtR: Waist circumference-height ratio; PPV: positive predictive value; NPV: negative predictive value; * J = sensitivity + specificity − 1; † The optimal cut off point was obtained from Youden index as [maximum (J = sensitivity + specificity − 1)].

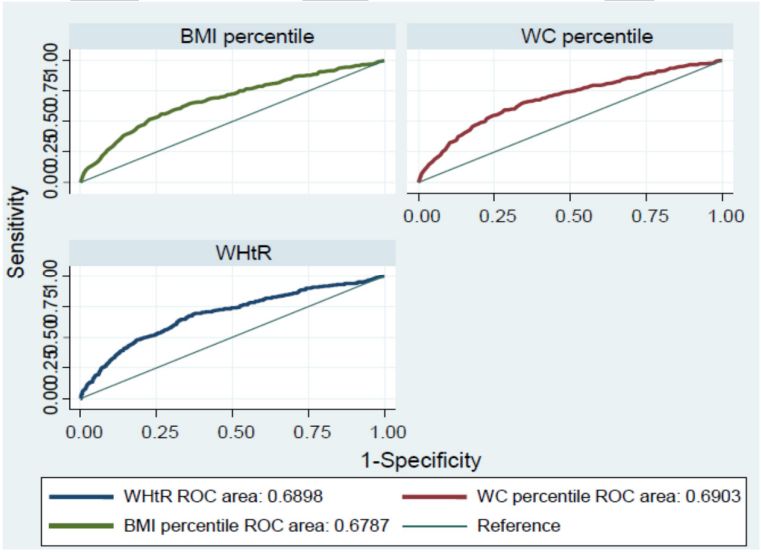
Table 4 presents a comparison of the usefulness of WHtR and other obesity indicators (BMI and WC percentile) for the prediction of two or more non-WC components of MS using AUC values. No indicators for predicting high FPG or high BP were statistically significant in either girls or boys. For high triglyceride, WHtR had the highest AUC value of 0.664, and overall the AUCs for WC percentile and WHtR were larger than those for BMI percentile (boys, 0.689; girls, 0.638); however, these differences were not statistically significant. For low HDL-cholesterol, the AUCs for WC percentile were 0.655 for boys and 0.715 for girls, respectively, which were superior to those of other indices, and statistically significant in boys. The AUC for WHtR for two or more non-WC components of MS was 0.690 for total, 0.691 for boys and 0.684 for girls, which were larger than the AUC values for the other two indices, albeit without statistical significance (total, $p = 0.216$; boys, $p = 0.469$; girls, $p = 0.512$) (Table 4, Figure 1).

Study subjects were divided into two groups based on the WHtR cutoff (0.43) and the MS index and other cardiovascular risk factors were compared (Table S1). In the group with a WHtR above the cutoff value, systolic BP, fasting blood glucose, total cholesterol, triglyceride, and LDL-cholesterol levels were significantly higher and HDL-cholesterol levels lower compared with those in the group with a WHtR below the cutoff value. The rate of central obesity, prevalence of MS risk factors, and rate of MS diagnosis were also significantly higher. Moreover, hemoglobin A1c levels, Glutamic oxaloacetic transaminase (GOT), Glutamic pyruvic transaminase (GPT), and Gamma glutamyl transpeptidase (GGTP) were also significantly higher.

Table 4. Area under the ROC curve of obesity indices to predict the presence of non-waist circumference components of metabolic syndrome according to sex.

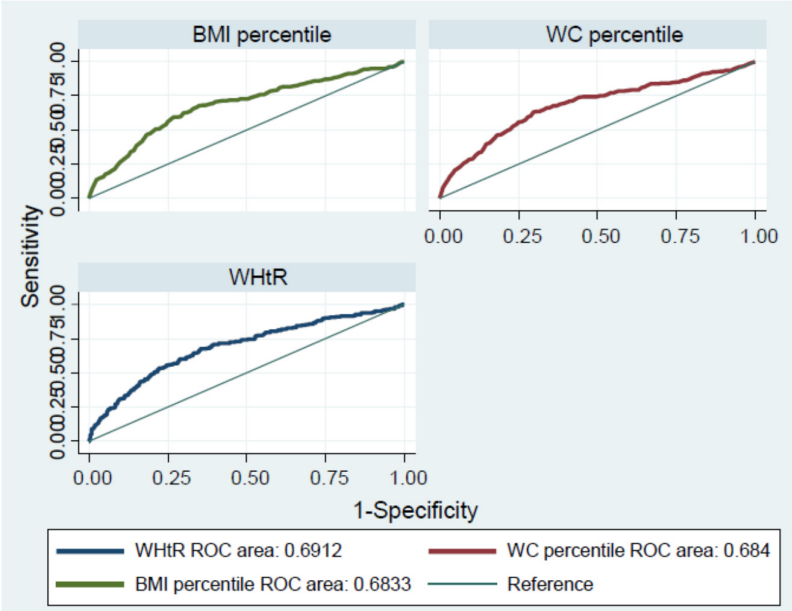
	BMI Percentile	WC Percentile	WHtR
	AUC (95% CI)	AUC (95% CI)	AUC (95% CI)
Total (*N* = 3057)			
High BP [#]	0.515 (0.491–0.540)	0.502 (0.477–0.527)	0.513 (0.488–0.538)
High FPG [†]	0.668 (0.504–0.832) *	0.653 (0.480–0.825)	0.623 (0.449–0.796)
High triglyceride [‡]	0.654 (0.629–0.679) **	0.659 (0.634–0.684) **	0.664 (0.639–0.689) **
Low HDL cholesterol [§]	0.668 (0.639–0.698) **	0.687 (0.658–0.715) **	0.658 (0.628–0.689) **
At least one non-WC components of MS	0.600 (0.580–0.620) **	0.592 (0.572–0.612) **	0.592 (0.572–0.613) **
Two or more non-WC components of MS	0.679 (0.647–0.710) **	0.690 (0.659–0.721) **	0.690 (0.658–0.721) **
Boys (*n* = 1625)			
High BP [#]	0.528 (0.494–0.562)	0.515 (0.481–0.549)	0.523 (0.489–0.557)
High FPG [†]	0.670 (0.482–0.857)	0.654 (0.457–0.851)	0.621 (0.434–0.808)
High triglyceride [‡]	0.678 (0.644–0.712) **	0.689 (0.655–0.723) **	0.689 (0.656–0.723) **
Low HDL cholesterol [§]	0.649 (0.612–0.686) **	0.655 (0.617–0.693) **	0.631 (0.592–0.669) **
At least one non-WC components of MS	0.612 (0.584–0.639) **	0.606 (0.578–0.634) **	0.599 (0.571–0.627) **
Two or more non-WC components of MS	0.683 (0.642–0.725) **	0.684 (0.641–0.727) **	0.691 (0.650–0.732) **
Girls (*n* = 1432)			
High BP [#]	0.501 (0.466–0.536)	0.522 (0.486–0.557)	0.501 (0.465–0.537)
High FPG [†]	0.646 (0.316–0.976)	0.612 (0.242–0.981)	0.611 (0.241–0.981)
High triglyceride [‡]	0.629 (0.591–0.660) **	0.638 (0.601–0.674) **	0.638 (0.600–0.675) **
Low HDL cholesterol [§]	0.690 (0.642–0.738) **	0.715 (0.670–0.761) **	0.699 (0.652–0.747) **
At least one non-WC components of MS	0.585 (0.555–0.615) **	0.573 (0.543–0.603) **	0.583 (0.553–0.613) **
Two or more non-WC components of MS	0.668 (0.620–0.717) **	0.682 (0.635–0.730) **	0.684 (0.636–0.733) **

ROC: receiver operating characteristic; AUC: area under curve; CI: confidence interval; BMI: body mass index; WC: waist circumference; WHtR: waist circumference-height ratio; BP: blood pressure; FPG: fasting plasma glucose; HDL: high density lipoprotein; MS: metabolic syndrome; * $p < 0.05$; ** $p < 0.01$ (Null hypothesis: area = 0.5); [#] High BP was diagnosed if systolic or diastolic blood pressure ≥90th percentile; [†] High FPG was diagnosed if FPG ≥110 mg/dL; [‡] High triglyceride was diagnosed if triglycerides was ≥110 mg/dL; [§] Low HDL cholesterol was diagnosed if HDL cholesterol was <40 mg/dL.

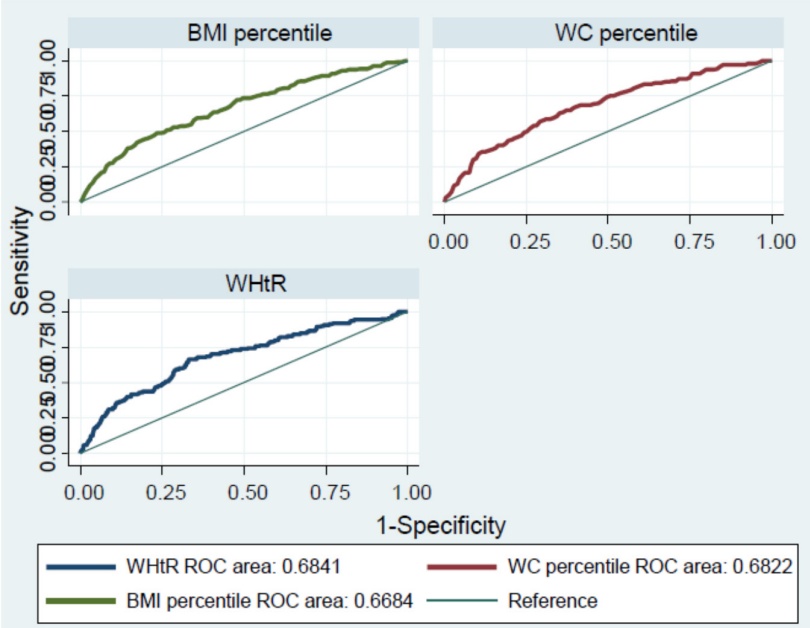


(**a**)

Figure 1. *Cont.*



(**b**)



(**c**)

Figure 1. The receiver operating characteristic (ROC) curves for body mass index (BMI) percentile, waist circumference (WC) percentile, and the waist-to-height ratio (WHtR) to predict two or more non-waist circumference components of metabolic syndrome according to sex. (**a**) Total; (**b**) Boys; (**c**) Girls.

4. Discussion

In this study, the optimal WHtR cutoff value for predicting obesity and MS was obtained using data representative of the Korean population. The usefulness of this cutoff value as a screening tool for the prediction of MS was compared with those for BMI and WC, which are conventional obesity indices. The WHtR cutoff value for MS diagnosis was less than the optimal WHtR cutoff value used for general and central obesity screening. WHtR was found to be a very accurate index for obesity screening; however, when comparing AUC values, its accuracy in detecting MS was statistically similar to those of BMI and WC percentiles.

To prevent adult-onset cardiovascular disease, it is important to determine those at high risk for obesity and metabolic abnormalities during childhood and adolescence [24]. BMI and WC are simple and inexpensive screening measurements for predicting obesity and MS, and they are commonly used in adults and children [38]. BMI is associated with body fat content but not always with abdominal obesity [13]. WC may reflect the extent of visceral fat accurately, but it can overestimate or underestimate the risk of cardiovascular disease, as individuals with similar WCs may vary in height [39]. In addition, the above two indicators are inconvenient for screening in children because of various differences in race, age, and sex-dependent aspect [40,41]. WHtR has the advantage of taking into account abdominal obesity as well as height associated with body fat accumulation or distribution [14,42]. Moreover, WHtR was found to be associated with variables relating to body fat, including trunk and body fat percentages, both of which are measured by dual energy X-ray absorptiometry, in both children and adults [43]. Ashwell et al. [44] suggested that WHtR is more sensitive than BMI for early prediction of obesity-related complications, is easy to measure by the general public, and is commonly used for all ages, sexes, and races. WHtR can be assessed in children and adolescents, whose height and WC change as they grow regardless of age [17], and used to evaluate the risk of cardiovascular disease accurately, compared with WC alone, since WHtR also takes into account height [12].

There has been controversy as to whether WHtR is independent of race and age [45]. Studies have also been performed to determine if a more accurate cutoff value than 0.5 exists for determining obesity or metabolic disorders in different races and sexes, especially in children and adolescents [23]. In this study, the optimal cutoff WHtR value for predicting MS risk was 0.44 in boys and 0.43 in girls. The optimal cutoff values for diagnosing general obesity and central obesity were 0.47 and 0.5 for boys and 0.47 and 0.48 for girls, respectively, which were higher than the cutoff values for diagnosing MS. Previous studies proposed optimal cutoff values for obesity screening in children and adolescents of 0.51 and 0.49 for Korean boys and girls, respectively [20], 0.47 and 0.45 for Chinese boys and girls, respectively [19], and 0.48 and 0.47 for Australian boys and girls, respectively [21]. Similar to this study, all AUC values were close to 1, which indicates a high predictive ability. The optimal WHtR cutoff value for predicting MS risk in children was 0.41 to 0.44 in Japan [22], 0.52 in the United States [24], and 0.465 and 0.455 for boys and girls in Africa, respectively [23]. In a prospective study of children and adolescents in Australia, the WHtR value used in children to predict cardiometabolic risk factors during adolescence was 0.47 for boys and 0.44 for girls [46]. In previous studies, the optimal cutoff value for MS screening was usually less than 0.5, except in studies conducted in obese children [18,47,48], which is generally consistent with the values presented in the literature [19,23,46,49,50]. There are several reasons why optimal cutoff values differ among studies. First, the diagnostic criteria used to evaluate MS and individual metabolic risk factors may differ [51]. Next, since body fat patterns show ethnic differences, visceral fat distribution associated with cardiometabolic risk may have a variety of effects depending on race [51]. It is also important to note that the prevalence of obesity and MS differ according to subject characteristics, including race, age, and geographic region [23]. Bauer et al. [24], who used an optimal cutoff value of 0.5 or greater, reported an obesity rate of 29.5% and an overall mean WHtR of 0.50, which was higher than the rate in our study. Lastly, different WC measurement methods have been used among studies. Gil et al. [20] reported an optimal WHtR cutoff value corresponding to a BMI $\geq$the 95th percentile in Korean children. In that study, unlike our method of measurement, the lowest WC value was used, and as a result, the optimal cutoff value was 0.48 for boys and 0.47 for girls. Therefore, for WHtR to be used as an indicator on its own, it is necessary to measure WC uniformly across studies; measuring weight and height in large epidemiological studies and clinical practice should always be encouraged [52,53].

Ashewell [54] suggested an optimal WHtR cutoff value of 0.5 for the prediction of obesity and metabolic abnormalities in children and adults and proposed that individuals with values $\geq$0.5 should be followed up, while immediate action should be taken in individuals with values $\geq$0.6. The sensitivity and specificity for MS diagnosis using WHtR cutoffs of 0.5 and 0.6 were analyzed. Sensitivity was

lower and specificity higher when using these values compared with our optimal cutoffs. When we applied a cutoff value of 0.6, which was proposed in obese children and adolescents, the specificity reached 99.7%, but the sensitivity was only 2.6%. In a prospective study of adolescents in Australia, it was reported that a WHtR cutoff value of ≥0.5 was sufficiently high to identify cardiometabolic risk co-occurrence, but the sensitivity was very low [46]. The lower the WHtR cut-off value, the higher the probability of an incorrect cardiovascular risk factor diagnosis, resulting in unnecessary resources and efforts to manage these risk factors [46]. On the other hand, increasing the cutoff value reduces the probability of misdiagnosing but increases the likelihood that a child with MS is missed [46]. Taking into consideration the critical role that the sensitivity plays in screening for childhood metabolic risk in population health studies involving an extensive number of subjects, it may be necessary to place emphasis on increasing the sensitivity rather than specificity. A cutoff value of 0.5, which is feasible clinically, can be used to predict obesity in Korean children. However, its estimated sensitivity for the detection of metabolic risk is 32% in boys and 20% in girls. For the purpose of screening for early detection and prevention of MS in Korea, it is appropriate to use a general value of 0.43 (boys, 0.44; girls, 0.43) as a cutoff, considering its high sensitivity and negative predictive value. In research of pre-indicators of cardiovascular disease, this study demonstrates perturbed children's metabolic profile since those with a WHtR above the cutoff value (0.43) are likely to exhibit adverse clinical factors beyond obesity and MS as shown in supplementary Table S1. In such context, screening children with a general cutoff value of 0.43 (boys, 0.44; girls, 0.43) is appropriate for early detection and prevention of MS in Korea, considering its high sensitivity and negative predictive value.

Previous studies have shown that WHtR is either a more appropriate [22,55–58], similar [59,60], or inferior [27,38] method than is BMI for screening for metabolic abnormalities. In this study, except for low HDL cholesterol values in girls, the AUCs of all indices were less than 0.7, indicating mediocre results. WHtR performed better than did BMI and WC percentiles as a screening tool for the prediction of two or more non-WC components of MS, but there was no statistically significant difference. The best predictors for high triglyceride were WC and WHtR, and the best predictor for low HDL cholesterol was WC in both boys and girls. These results suggest that WC and WHtR, which better reflect abdominal obesity, have a higher AUC than that for BMI, as triglycerides and HDL cholesterol are highly correlated with the amount of intra-abdominal fat in adolescents [61]. Lo et al. [12] conducted a meta-analysis of cross-sectional studies and found that the AUCs for high BP, high triglyceride, and low HDL cholesterol levels were <0.7, but the AUCs for the prediction of MS was ≥0.7 in adolescents. The majority of the outcomes had very high heterogeneity [12]. This suggests that the prevalence of MS is generally low in children and adolescents, and the prevalence of a high BP, high triglyceride or glucose level, low HDL cholesterol level, and MS varies according to the study subjects. However, a meta-analysis of adults showed that WHtR was superior to BMI and WC as an indicator of risk factors for cardiovascular metabolic disease, and the calculated AUC value was ≥0.7 [16]; similar results were obtained in a study investigating Korean adults [62]. In a meta-analysis of cohort studies, WHtR was demonstrated to be superior to BMI in detecting outcomes such as incidence of cardiovascular disease and cardiovascular or all-cause mortality, particularly in Asians [63]. The reason why the efficacy of WHtR is lower in children and adolescents compared with adults may be explained by variations in WHtR according to age [12,64]. As children become adolescents, increases in height are relatively greater than increases in WC, which suggests the potential for misclassification of children with excess abdominal fat as healthy [12]. It is also important to note that the prevalence of MS is lower in children than in adults [23]. Since childhood obesity is related to various metabolic factors, biochemical abnormalities usually do not appear until later in life, and the morbidity rate is lower in children than in adults [23,65]. In addition, it is also possible that high lipid profiles may be related to family history rather than weight in pediatric individuals [66]. Nevertheless, WHtR is an excellent tool to replace the existing obesity index because the AUC values of WHtR for detecting obesity defined by BMI and WC converge to 1. Use of WHtR also resulted in an accurate prediction of risk factors for cardiovascular metabolic disease, to the same extent as BMI and WC. Because WHtR is easy to

measure and apply clinically and for the general public to comprehend, it can be used in place of the existing obesity index to identify children and adolescents at risk of cardiometabolic disorders at an early age.

This study is not without limitations. First, it was a cross-sectional study; therefore, it did not provide enough evidence to determine the predictive value of WHtR for metabolic risk. However, it provided diagnostic value for obesity and MS. Future cohort studies using a prospective design are warranted to evaluate the validity of the obtained cutoff values. Next, the cutoff values obtained in this study cannot be applied to other ethnic groups. The prevalence of obesity and MS in children and adolescents were lower than those in other countries, which may have affected the results. Third, "complex sample analysis" could not be performed for ROC curve analysis because of limitations in the statistical program used; however, we performed ROC curve analysis for BMI and WC percentiles by stratifying according to age. Finally, the puberty status of the study population was not used because of the lack of collected date, although sexual maturity is related to obesity or metabolic diseases in youth [67].

To our knowledge, this is the first study to examine the utility of WHtR as a screening tool for MS in Korean children and adolescents. The strengths of our study include a comparison of various anthropometric indices and the identification of WHtR as a screening tool for obesity and MS among children and adolescents, using a sample representative of the Korean population. Unlike WHtR, which changes only slightly with age [59], BMI is usually lowest at 5–6 years of age and subsequently increases in proportion to weight and height until adolescence [68]. WC is characterized by a relatively constant increase [68]. Therefore, it is important to perform ROC curve analysis after stratifying by age for BMI and WC; the percentiles were calculated to compensate for errors associated with low percentiles in younger subjects with a low probability of metabolic abnormalities and relatively low WC and BMI values.

Since early detection of individuals at risk for MS is important for prevention and appropriate interventions [24], future prospective cohort studies should be performed to continue investigating the WHtR cutoff and its usefulness in predicting MS.

5. Conclusions

In this study, WHtR was shown to be a simple and effective measurement for screening for childhood obesity and MS in Korean children and adolescents. The optimal WHtR cutoff point for MS was 0.44 in boys and 0.43 in girls, which is slightly less than half of the height (0.5). Considering that today children and adolescents have a long life expectancy, early detection and management of metabolic risk are crucial. Therefore, the optimal WHtR cutoff value presented in this study is of value in the field of public health, as it is easy to use for screening for MS risk in large-scale epidemiological studies and in clinical practice.

Author Contributions: Dong-Hyun Choi contributed to the interpretation, the study results and wrote the paper; Yang-Im Hur contributed to the study concept and design, analysis and interpretation of the data, statistical analysis, and manuscript writing; Jae-Heon Kang contributed to the study concept and design, and gave professional advice on statistical analysis and interpretation of the data; Kyoungwoo Kim contributed to administrative support for the study and commented on manuscript.; Young Gyu Cho contributed to the study concept and design and gave comments on manuscript; Soo-Min Hong contributed to the study concept and design and the manuscript preparation, and commented on manuscript; Eun Byul Cho contributed to the study concept and design and manuscript preparation. All authors reviewed and approved the final manuscript.

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The contributors of this book come from diverse backgrounds, making this book a truly international effort. This book will bring forth new frontiers with its revolutionizing research information and detailed analysis of the nascent developments around the world.

We would like to thank all the contributing authors for lending their expertise to make the book truly unique. They have played a crucial role in the development of this book. Without their invaluable contributions this book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students.

This book was conceptualized with the vision of imparting up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers.

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The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for researchers, students, practitioners and scholars across the globe.

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