Nutrition and Dietetics

Ashley Martin

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Edited by **Ashley Martin**

Published by White Word Publications,
5 Penn Plaza,
19th Floor,
New York, NY 10001, USA

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International Standard Book Number: 978-1-9789-2730-8

Contents

Preface

Dietetics is a scientific discipline that focuses on human nutrition, regulation of diet and improving human health. This book on nutrition and dietetics encompasses various clinical trials, experiments and case studies that analyze and evaluate current knowledge on nutrition sources and treatment techniques for nutritional deficiency. The aim of this book is to present researches that have transformed this discipline and aided its advancement. With state-of-the-art inputs by acclaimed experts of this field, this book targets students and professionals.

The information contained in this book is the result of intensive hard work done by researchers in this field. All due efforts have been made to make this book serve as a complete guiding source for students and researchers. The topics in this book have been comprehensively explained to help readers understand the growing trends in the field.

I would like to thank the entire group of writers who made sincere efforts in this book and my family who supported me in my efforts of working on this book. I take this opportunity to thank all those who have been a guiding force throughout my life.

Editor

Higher iron pearl millet (*Pennisetum glaucum* L.) provides more absorbable iron that is limited by increased polyphenolic content

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Abstract

Background: Our objective was to compare the capacity of iron (Fe) biofortified and standard pearl millet (*Pennisetum glaucum* L.) to deliver Fe for hemoglobin (Hb)-synthesis. Pearl millet (PM) is common in West-Africa and India, and is well adapted to growing areas characterized by drought, low-soil fertility, and high-temperature. Because of its tolerance to difficult growing conditions, it can be grown in areas where other cereal crops, such as maize, would not survive. It accounts for approximately 50% of the total world-production of millet. Given the widespread use of PM in areas of the world affected by Fe-deficiency, it is important to establish whether biofortified-PM can improve Fe-nutriture.

Methods: Two isolines of PM, a low-Fe-control ("DG-9444", *Low-Fe*) and biofortified ("ICTP-8203 Fe",*High-Fe*) in Fe (26 μg and 85 μg-Fe/g, respectively) were used. PM-based diets were formulated to meet the nutrient requirements for the broiler (*Gallus-gallus*) except for Fe (Fe concentrations were 22.1±0.52 and 78.6±0.51 μg-Fe/g for the *Low-Fe* and *High-Fe* diets, respectively). For 6-weeks, Hb, feed-consumption and body-weight were measured (n = 12).

Results: Improved Fe-status was observed in the *High-Fe* group, as suggested by total-Hb-Fe values (15.5±0.8 and 26.7±1.4 mg, *Low-Fe* and *High-Fe* respectively, $P<0.05$). DMT-1, DcytB, and ferroportin mRNA-expression was higher ($P<0.05$) and liver-ferritin was lower ($P>0.05$) in the *Low-Fe* group versus *High-Fe* group. *In-vitro* comparisons indicated that the High-Fe PM should provide more absorbable-Fe; however, the cell-ferritin values of the *in-vitro* bioassay were very low. Such low *in-vitro* values, and as previously demonstrated, indicate the presence of high-levels of polyphenolic-compounds or/and phytic-acid that inhibit Fe-absorption. LC/MS-analysis yielded 15 unique parent aglycone polyphenolic-compounds elevated in the *High-Fe* line, corresponding to $m/z = 431.09$.

Conclusions: The *High-Fe* diet appeared to deliver more absorbable-Fe as evidenced by the increased Hb and Hb-Fe status. Results suggest that some PM varieties with higher Fe contents also contain elevated polyphenolic concentrations, which inhibit Fe-bioavailability. Our observations are important as these polyphenols-compounds represent potential targets which can perhaps be manipulated during the breeding process to yield improved dietary Fe-bioavailability. Therefore, the polyphenolic and phytate profiles of PM must be carefully evaluated in order to further improve the nutritional benefit of this crop.

Keywords: Pearl millet, Biofortification, Iron bioavailability, Polyphenols, In vitro digestion/Caco- 2 cell model, Broiler chicken

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Introduction

The World Health Organization estimates that approximately one-third of worldwide infant deaths and one half in developing countries can be attributed to malnutrition. More specifically, iron (Fe) deficiency is the most common nutritional deficiency worldwide [1]. Fe deficiency is particularly widespread in low-income countries because of a general lack of consumption of animal products (which can promote non-heme iron absorption and contain highly bioavailable heme Fe) coupled with a high consumption of cereal grains and legumes replete with antinutrients (e.g., polyphenolic compounds and phytic acid) that are inhibitors of Fe bioavailability [2,3].

Poor dietary quality is more often characterized by micronutrient deficiencies or reduced mineral bioavailability, than by insufficient energy intake [3,4]. Diets with chronically poor Fe bioavailability which result in high prevalence of iron deficiency and anemia increase the risk of all-cause child mortalities and also may lead to many pathophysiological consequences including stunted growth, low birth weight, delayed mental development and motor functioning, and others [5-7]. Thus, a crucial step in alleviating Fe deficiency anemia is through understanding how specific dietary practices and components contribute to the Fe status in a particular region where Fe deficiency is prevalent.

Pearl millet (PM) is a resilient cereal crop, grown mostly in marginal environments in the arid and semi-arid tropical regions of Asia and Africa [8-10]. It is a major dietary constituent for peoples living in Western India and the Sahel region of the African continent, and is often served as a complementary food for infants and young children [11,12]. For example, among the rural poor in India, PM intake can reach nearly 60% of all cereal grain consumption [8]. A major non-nutritional advantage to PM consumption is that it can be grown in areas with very limited rainfall, where crops such as maize or sorghum are very likely to fail during most growing seasons [8,13]. As a well-adapted crop to growing areas characterized by drought, low soil fertility, and high temperature, it performs well in soils with high salinity or low pH [13,14].

With regard to nutritional quality, PM is at least equivalent to maize and generally superior to sorghum in protein content/quality and metabolizable energy levels [10], as well as digestibility [15]. Furthermore, PM does not usually contain significant amounts of condensed polyphenols, such as the tannins commonly found in other staple crops such as sorghum, which can decrease digestibility [16]. PM grain is also rich in important micronutrients such as Fe and Zn, and has a more complete amino acid profile than maize or sorghum [15]. Taken in totality, these qualities make PM a major contributor of dietary protein, Fe, and Zn intake in a variety of rural populations in India and sub-Saharan Africa [10,11].

Recently, conventional plant breeding at ICRISAT (International Crops Research Institute For the Semi-Arid Tropics, Andhra Oradesh, India) has developed biofortified PM containing up to 90 μg Fe/g PM, a substantial increase over standard PM containing 36-50 μg Fe/g PM [17]. A previous study that assessed biofortified PM line to deliver more absorbable Fe to young women indicated that consumption of the Fe biofortified PM increased the amount of absorbable Fe [18]. Another study in young children assessed the absorption of Fe and Zn from biofortified PM, and found that the concentrations of both Fe and Zn absorbed were more than adequate to meet the physiological requirements for these micronutrients [11].

However, an increase in Fe concentration in PM may not necessarily translate into a proportional increase in absorbed Fe since genotypes with high Fe concentrations may also have increased (or decreased) concentrations of Fe absorption inhibitors or enhancers [19]. Therefore, it is necessary to measure both the amount of bioavailable Fe and the concentration of Fe in these new iron-enhanced crops, as well as potential inhibitors (e.g., polyphenols) of Fe bioavailability [19,20].

The *Gallus gallus* model has been used extensively for nutritional research and has shown to be an excellent animal to model Fe bioavailability [21], as chicks respond quickly to Fe malnutrition, and their micronutrient deficient phenotypes include poor Fe status, growth stunting, and organ hypertrophy. Further, this model agrees well with *in vitro Caco-2* cell results [19,20,22-26]. Hence, the objective of the current study was to compare the capacities of two pearl millet varieties to deliver Fe for Hb synthesis and to improve the Fe status of Fe deficient broiler chickens.

Materials and methods

Diets, animals and study design

The two pearl millet isolines used in the study were developed from a low Fe commercial variety for India (DG-9444, *"Low-Fe"*) and an introgressed, open pollinated variety line (ICTP 8203 Fe, *"High-Fe"*). Seed was multiplied in Andrha Pradesh, India under phosphorus fertilized, standard agronomic conditions and shipped to Ithaca, New York in sealed containers imported as grain.

Forty eight Cornish cross fertile broiler eggs were obtained from a commercial hatchery (Moyer's chicks, Quakertown, PA). The eggs were incubated under optimal conditions at the Cornell University Animal Science poultry farm incubator. Upon hatching (hatchability rate was 93%), chicks were allocated into 2 treatment groups on the basis of body weight, gender and blood Hb concentration (aimed to ensure equal distribution between groups, n = 12): 1. *High-Fe*: 75% pearl millet diet (78 μg/g Fe); 2. *Low-Fe*: 75% pearl millet diet (22 μg/g Fe). Experimental diets had no supplemental Fe. Diets compositions are shown in Table 1.

Table 1 Composition of the experimental diets[1–3]

Ingredient	*High-Fe* (Biofortified)	*Low-Fe* (Standard)
	g/kg (by formulation)	
High-Fe Pearl millet (84.9 μg/g Fe)	750	–
Low-Fe Pearl Millet (25.9 μg/g Fe)	–	750
Skim milk, dry	100	100
DL- Methionine	2.5	2.5
Corn starch	47.5	47.5
Corn oil	30	30
Choline chloride	0.75	0.75
Vitamin/mineral premix (no Fe)	70	70
Total (g)	1000	1000
Selected components	**mean ± SEM, n = 5 (by analysis)**	
Dietary Fe concentration (μg/g)	78.6 ± 0.51[a]	22.1 ± 0.52[b]
Phytic Acid (μg/g)	9940 ± 1380[a]	10500 ± 230[a]
Phytate:Fe molar ratio[3]	10.7 ± 0.55[b]	40.2 ± 0.35[a]

[1]Vitamin and mineral premix provided/kg diet (330002 Chick vitamin mixture; 235001 Salt mix for chick diet; *Dyets* Inc. Bethlehem, PA).
[2]Iron concentrations in the diets were determined by an inductively-coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co. Franklin, MA) following wet ashing.
[3]Method for determining phytate is described in the materials and methods section.
[a,b]Within a row, means without a common letter are significantly different ($P < 0.05$).

Chicks were housed in a total-confinement building (1 chick per 0.5 m^2 metal cage). Birds were under indoor controlled temperatures and were provided 16 h of light. Each cage was equipped with an automatic nipple drinker and manual self-feeder. All birds were given *ad libitum* access to water (Fe content was 0.379 ± 0.012 mg/L). Feed intakes were measured daily (as from day 1). Dietary Fe intake was calculated from feed intake and Fe concentration in the diets.

Blood analysis and Hemoglobin (Hb) measurements

Blood samples were collected weekly from the wing vein (n = 12, ~100 μL) using micro-hematocrit heparinized capillary tubes (Fisher, Pittsburgh, PA). Samples were collected in the morning (starting at 08:00) following an 8 h overnight fast. The samples were analyzed for Hb concentration. Body weights and Hb concentrations were measured weekly.

Fe bioavailability was calculated as hemoglobin maintenance efficiency (HME) [21]:

$$\mathrm{HME} = \frac{Hb\ Fe,\ mg\ (final) - Hb\ Fe,\ mg\ (initial)}{Total\ Fe\ Intake,\ mg} \times 100$$

Where Hb-Fe (index of Fe absorption) = total body Hb-Fe. Hb-Fe was calculated from Hb concentrations and estimates of blood volume based on body weight (a blood volume of 85 mL per kg body weight is assumed) [21]:

$$\text{Hb- Fe (mg)} = \text{B.W. (kg)} \times 0.085\ \text{L blood/kg} \times \text{Hb (g/L)} \times 3.35\text{mg Fe/g Hb.}$$

At the end of the experiment (day 42), birds were euthanized by CO_2 exposure. The digestive tracts and livers were quickly removed (within 1 min post death) from the carcass and separated into various sections for tissue (duodenum and liver ~ 1-2 cm; ~2-3 g, respectively). The samples were immediately frozen in liquid nitrogen, and then stored in a −80°C freezer until analysis. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Blood Hb concentrations were determined spectrophotometrically using the cyanmethemoglobin method (H7506-STD, Pointe Scientific Inc. Canton, MI) following the kit manufacturer's instructions.

Isolation of total RNA

Total RNA was extracted from 30 mg of duodenal (proximal duodenum, n = 12) and liver tissues (n = 12) using Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. All steps were carried out under RNase free conditions. RNA was quantified by absorbance at 260–280 nm. Integrity of the 28S and 18S rRNA was verified by 1.5% agarose gel electrophoresis followed by ethidium bromide staining [19-26].

DMT1, DcytB and ferroportin gene expression analysis

As previously described [19-26], PCR was carried out with primers chosen from the fragments of chicken (*Gallus gallus*) duodenal and hepatic tissues [Divalent Metal Transporter-1, DMT1 gene (GeneBank database; GI 206597489) (forward: 5'-AGC CGT TCA CCA CTT ATT TCG-3'; reverse: 5'-GGT CCA AAT AGG CGA TGC TC-3'), Duodenal Cytochrome B, DcytB gene (GI 20380692) (forward: 5'-GGC CGT GTT TGA GAA CCA CAA TGT T-3'; reverse: 5'-CGT TTG CAA TCA CGT TTC CAA AGA T-3') and Ferroportin gene (GI 61098365) (forward: 5'-GAT GCA TTC TGA ACA ACC AAG GA'; reverse: 5'-GGA GAC TGG GTG GAC AAG AAC TC-3')].Tissue-specific 18S rRNA was used to normalize the results [(GI 7262899) (forward: 5'- CGA TGC TCT TAA CTG AGT-3'; reverse: 5'-CAG CTT TGC AAC CAT ACT C-3')]. All PCR products were separated by electrophoresis on 2% agarose gel, stained with ethidium bromide, and quantified using the Quantity One 1-D analysis software (Bio-Rad, Hercules, CA).

In-vitro Fe bioavailability assessment

An *in vitro* digestion/*Caco-2* cell culture model [19] was used to assess *in vitro* Fe bioavailability. In this method, the cooked pearl millet samples and the formulated diets were subjected to simulated gastric and intestinal digestion. Exactly 0.5 g of the freeze-dried cooked pearl millet and diets samples were utilized for each replication of the *in vitro* digestion process.

Harvesting of *Caco-2* cells for ferritin analysis

The protocols used in the ferritin and total protein contents analyses of *Caco-2* cells were similar to those previously described [19]. *Caco-2* cells synthesize ferritin in response to increases in intracellular Fe concentration. Therefore, we used the ratio of ferritin/total protein (expressed as ng ferritin/mg protein) as an index of the cellular Fe uptake. All glassware used in the sample preparation and analyses were acid washed.

Ferritin and Fe in the liver, electrophoresis, staining and measurement of gels

Liver ferritin and liver Fe quantification were conducted as previously described [21]. The gels were scanned with Bio-Rad densitometer. Measurements of the bands were conducted using the Quantity-One 1-D analysis program (Bio-Rad, Hercules, CA). All samples (n = 6) were analyzed in duplicates (n = 6).

Polyphenolic relative amounts in diets

A list of reported compounds was obtained by generation of high accuracy mass-to-charge (*m/z*) data derived from analysis of the PM samples using a UPLC/MS system and related software [19,27]. From this *m/z* data, the METLIN database (METLIN, Scripps Center, La Jolla, CA) was used to identify a further list of potential flavonoid aglycones present in greater concentration in the *High-Fe* PM, and compiled in Table 2.

Table 2 Aglycone of polyphenolic compounds corresponding to an *m/z* = 431.09 highly-enriched in the *High-Fe* PM

Class	Compound	Putative *in vitro* effect on Fe absorption/ bioavailability	Citation
Flavones	Apigenin	↓	[28,29]
	Baicalein	↓	[30,31]
	Luteolin	↓	[28]
	Norwogonin	*	
	Scutellarein	*	
	5,7,2'-Trihydroxyflavone	*	
	7,3',4'-Trihydroxyflavone	*	
	7,3',4',5'-Tetrahydroxyflavone	*	
Flavonol	Galangin	↓	[32]
	Kaempferol	↓	[28]
Isoflavones	Dihydrodaidzein	↓	[28]
	Genistein	↓	[29,33]
	Trihydroxyisoflavone	*	
	6,7,4'-trihydroxyisoflavone	*	
Anthocyanins	Pelargonidin	↓	[34]

*As of the writing of this paper, no data on the putative effects of these compounds relating to Fe absorption/ bioavailability exist.
↓ Decrease of Fe bioavailability/absorption *in vitro*.

Polyphenol extraction

As previously described [19,27], to one gram of ground PM material, 5 mL of methanol:water (50:50) was added. The slurry was vortexed for one minute, placed in a sonication water bath for 10 minutes, vortexed again for one minute, and centrifuged at 4000 × g for 15 min. The supernatant was filtered with a 0.45 μm syringe filter and stored for later use in a −20°C freezer.

LC/MS analysis

As previously described [19,27], Extracts were analyzed by LC-MS with an Acquity UPLC coupled to a Xevo G2 QTof spectrometer (Waters Corp. Milford, MA). For LC analysis, 5 μL samples of extract were injected and passed through a HSS C18 1.8 μm 2.1 × 100 mm column (Waters) at 0.4 mL/min. The mobile phase consisted of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). Polyphenols were eluted using linear gradients of 2.4 to 20% B in 2.5 min, 20 to 40% B in 0.5 min, 40 to 52% B in 2 min, 52 to 95% B in 0.5 min, 95 to 2.4% B in 1 min, and a 0.5 min hold at 2.4% B. ESI mass spectrometry was performed in positive ionization mode with a scan speed of 5/s in the mass range from 50 to 1200 Da. Lock-mass correction was used, with leucine-enkephalin as the external lock-mass standard. Instrumentation and data acquisition were controlled by MassLynx (Waters Corp., Milford, MA) software. Eluted compounds were marked by mass (m/z) and relative abundance using MarkerLynx (Waters Corp., Milford, MA) software. Potential polyphenol identities of individual masses were obtained by reference to METLIN database (Scripps Center for Metabolomics).

Determination of phytic acid concentration in the diet samples

Dietary phytic acid (phytate)/total phosphorus was measured as phosphorus released by phytase and alkaline phosphatase, following the kit manufacturer's instructions (n = 5) (K-PHYT 12/12, Megazyme International, Ireland).

Statistical analysis

Results were analyzed by ANOVA using the general linear models procedure of SAS software (SAS Institute

Inc. Cary, NC). Differences between treatments were compared by Tukey's test and values were considered statistically different at $P < 0.05$ (values in the text are means ± SEM).

Results

Growth rates, Hb, Hb-Fe and HME

There were no significant differences in feed intakes at any time throughout the study. However, Fe intakes were consistently higher in the *High-Fe* group versus *Low-Fe* group. As from day 14 of the study, body weights were higher ($P < 0.05$) in the *High-Fe* group versus *Low-Fe* group. Also, as from day 35 of the study, Hb concentrations were higher ($P < 0.05$) in the *High-Fe* group versus *Low-Fe* group (Figure 1). The increase in total body Hb-Fe from day 14 until study conclusion was significantly greater in the *High-Fe* group versus *Low-Fe* group (25.6 ± 1.4 mg and 14.4 ± 0.8 mg, respectively, $P < 0.05$, Figure 1). HME was significantly different between groups at all-time points, with a higher percent obtained in the bird group receiving the standard PM diet (*Low-Fe*, $n = 6$, $P < 0.05$).

Gene expression of iron transporters (DMT-1, Ferroportin) and DcytB in the duodenum

Gene expression analysis of duodenal DMT-1, Ferroportin and DcytB, with results reported relative to 18S rRNA, revealed increased mRNA expression of DMT-1, Ferroportin, and DcytB in the *Low-Fe* group compared to the *High-Fe* group (Figure 2) ($n = 6$, $P < 0.05$).

Caco-2 cell ferritin protein formation

Ferritin concentrations were significantly higher in cells exposed to the *High-Fe* diet versus the *Low-Fe* diet, as well as higher in cells exposed to the *High-Fe* PM versus *Low-Fe* PM only ($P < 0.05$, $n = 6$, Table 3).

Ferritin and Fe in the liver

The avian ferritins corresponded to a weight of approximately 470 to 500 kDa [21]. No significant differences in liver Fe or liver ferritin concentrations (with liver specimens collected on day 42) were measured between the treatment groups ($n = 6$, $P > 0.05$, Table 4).

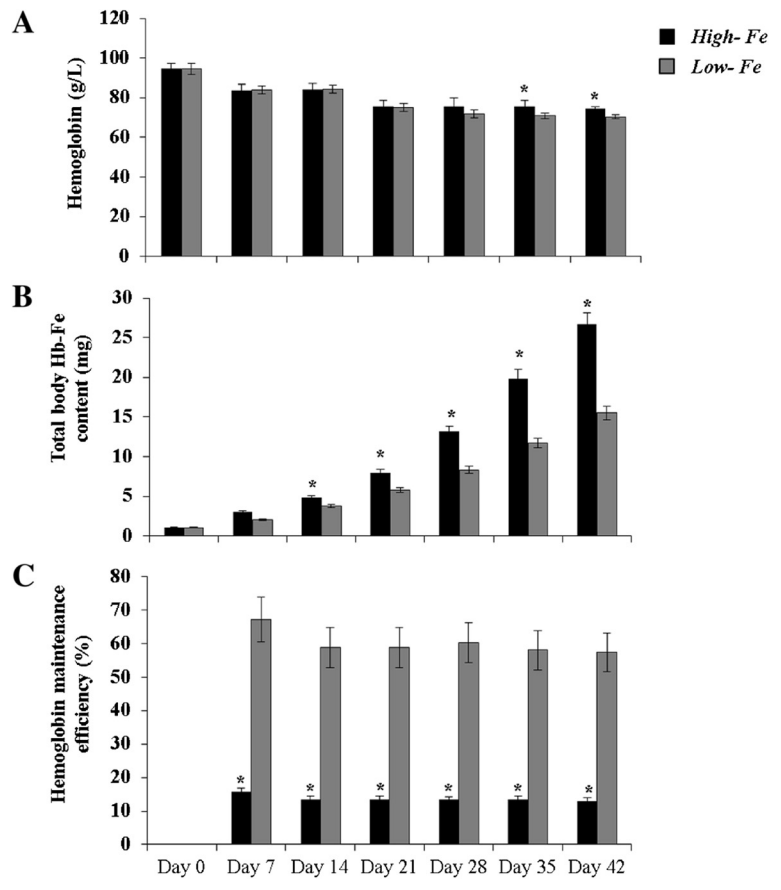

Figure 1 Iron status parameters of chicken fed the tested diets from days 0- 42[1]. (A) Hb (g/L), **(B)** Total body Hb-Fe content (mg), and **(C)** % HME. [1]Values are mean daily feed intakes for the 7 days preceding the day designated in the column heading (n=12).

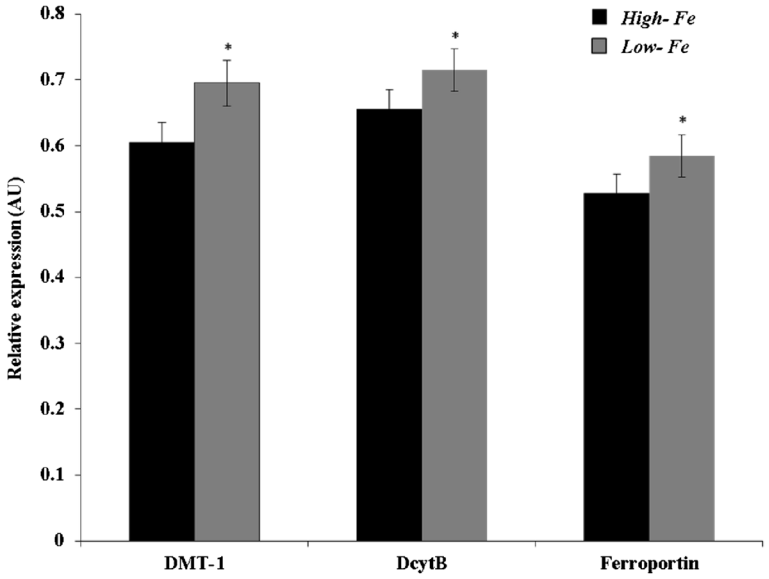

Figure 2 Duodenal mRNA expression of DMT-1, DcytB, and ferroportin on day 42. [1]Changes in mRNA expression are shown relative to expression of 18S rRNA in arbitrary units (AU, n = 12, P < 0.05).

Polyphenolic relative amounts in the diets samples

Phenolic analysis [19,27] of the PM samples detected three specific mass-to-charge ratios (*m/z*), one of which significantly higher in the *High-Fe* (biofortified) PM variety (AU, P < 0.05). The elevated mass (*m/z* = 431.09) corresponds to 15 possible candidate glycosylated phenolic compounds. The aglucones of these compounds, as well as their purported effect on Fe absorption and bioavailability [28-34], can be found in Table 2.

Phytate concentration and Phytate:Fe molar ratios in the diet samples

No significant differences in phytate concentration were measured between *High-Fe* and *Low-Fe* diets (n = 5, P > 0.05). Dietary phytate concentrations (as inositol hexaphosphate, IP6) are shown in Table 1. The concentrations of phytic acid ($IP_{1\rightarrow6}$) and Fe in the diets were used to calculate the phytate to Fe molar ratios. However, as expected, the ratios of phytate:Fe significantly differ between diets (40.2 ± 0.35 and 10.7 ± 0.55 for the *Low-Fe* and *High-Fe* PM diets, respectively, n = 5, P > 0.05, Table 1).

Table 3 Ferritin concentrations in *Caco-2* cells exposed to samples of PM only and PM-based diets

Tested sample[1]	Ferritin/cell protein (ng/mg)
Cell Baseline[2]	$1.54d^{e} \pm 0.12$
$FeCl_3$	$58.69^{b} \pm 2.29$
$FeCl_3$ + Ascorbic Acid	$364.95^{a} \pm 19.55$
Low-Fe PM only	$1.22^{e} \pm 0.05$
High-Fe PM only	$2.61^{c} \pm 0.36$
Low-Fe PM-based diet	$1.47^{de} \pm 0.27$
High-Fe PM- based diet	$2.46^{c} \pm 0.13$

[1]*Caco-2* bioassay procedures and preparation of the digested samples are described in the materials and methods sections (mean ± SEM).
[2]Cells were exposed to only MEM (minimal essential media) without added food digests and Fe (n = 6).
[a-e] Within a column, means without a common letter are significantly different (P < 0.05).

Discussion

PM is a pervasive and nutritious grain harvested in many parts of the world; it is common primarily in West Africa and the Indian subcontinent, where micronutrient deficiencies are rampant [8]. It is an unusually hardy food crop, and consequently there is a progressive increase in the use of these grains as a major food staple, especially among subsistence farmers and the rural poor in large areas of India and sub-Saharan Africa [8,35]. In terms of biofortification, target levels for PM Fe concentration have been set at nearly 77 μg/g or higher, which should likely represent a 30–40 μg/g differential from the more typical PM Fe levels [17]. In the present study, the differential in Fe content between the two PM lines was 56 μg/g, thus confidence was high going into the

Table 4 Ferritin protein and the iron concentration in the liver[1]

Treatment diet	Ferritin (μg/g wet weight)	Iron (μg/g wet weight)	Iron/Ferritin (μmol)
High-Fe	285 ± 8.5^{a}	25.2 ± 3.9^{a}	34.5 ± 3.5^{a}
Low-Fe	277 ± 7.1^{a}	19.3 ± 2.7^{a}	29.7 ± 5.3^{a}

[1]Atomic mass for iron used for calculations defined as 55.8 g/mol.
[a]Within a column, means with a common letter are not significantly different (n = 12, P > 0.05, mean ± SEM).

study that a nutritional benefit would be observed. In addition, it was recently demonstrated that Fe biofortified PM improved Fe status in Indian school children, and the authors concluded that dietary supplementation with Fe biofortified PM for six months significantly resolved Fe deficiency [36]. Hence, the objectives of the current study were to assess the capacity of a Fe-biofortified PM line to provide bioavailable Fe for Hb synthesis, as well as to establish a polyphenolic profile of the PM variety.

The *in vitro* results showing increased ferritin concentrations in *Caco-2* cells exemplify that the *High-Fe* (biofortified) PM does in fact provide additional, absorbable Fe. However, the assay also suggests that the bioavailability is relatively low compared to other foods, as the ferritin values are only slightly higher than the "baseline" conditions. As was previously demonstrated, such low values are typical of an inhibitory effect by polyphenols [19,20,22,27]. Although hepatic ferritin and Fe concentrations were not significantly different between groups, increases in Hb (on days 35 through 42 in the *High-Fe*) and total body Hb-Fe (higher as from day 14 in the *High-Fe*) indicate birds receiving the *High-Fe* diet had moderately higher Fe available for Hb synthesis. Further, % HME was significantly elevated at all time points in the *Low-Fe* indicating an adaptive response (e.g., a relative up-regulation of absorption) to less absorbable dietary Fe. In addition, significant differences in duodenal mRNA abundance of DMT-1, DcytB and ferroportin were obtained between groups, with a higher relative mRNA expression of all three genes in the *Low-Fe* group. Similar to previous observations [19,20,24,25], these results suggest, again, a compensatory, or adaptative, mechanism in the *Low-Fe* group due to a relative deficiency of absorbable Fe in the diet. In totality, however, these results suggest that the *High-Fe* PM diet provided more absorbable Fe to the birds, and thus yielded an improved Fe status throughout the duration of the study.

The interference of Fe uptake, relative to control diets high in bioavailable Fe, reflected in the *Caco-2* cell results (Table 3) is indicative of the strong inhibitory effect that so-called anti-nutrients (e.g., polyphenolic compounds) have on Fe bioavailability [37]. Although, for example, differences in ferritin concentration in *Caco-2* cells exposed to the *High-Fe* PM diet versus the *Low-Fe* PM diet were obtained, however, this relatively higher amount of ferritin (in the *High-Fe* PM) is not proportional to the significantly increased Fe content in the biofortified *High-Fe* PM. Although the *High-Fe* PM contained a greater Fe concentration than did the *Low-Fe* PM, concentration of polyphenolic compounds, known inhibitors of Fe bioavailability [38], also increased. Therefore, as part of the breeding process, it is incumbent upon researchers to assess the polyphenolic profile of the biofortified crop in question, since these chemicals have significant effects on Fe absorption and bioavailability in a variety of cell culture, animal, and human models [37-39].

From our LC/MS analysis, we determined a *m/z* ratio of 431.09 corresponding to 15 unique parent polyphenolic aglycones, significantly elevated in the *High-Fe* PM compared to the *Low-Fe* PM. The plant metabolites identified belong to chemical families including flavones, flavonols, isoflavones, and anthocyanins, many of which have been shown to inhibit Fe absorption [28-34], [Table 2] either by direct mineral chelation and Fe efflux or, in the case of the phytoestrogen isoflavones, by modulating membrane Fe receptor expression and thus affecting Fe homeostasis [33]. For example, [31] elucidated antioxidant effects of baicalein through Fe-binding in a physiologically-relevant *in vitro* model. It was determined that baicalein bound Fe^{2+} more strongly than ferrozine, a well-known Fe^{2+} chelator. Our results are consistent with others [40-42] who have found a variety of phenolic and polyphenolic compounds, namely kaempferol, luteolin, and apigenin, in different varieties of millet (mainly *E. coracana*, a utricles millet). For a detailed review of relevant phenolic compounds found in millet, please see [43] and [44].

Indeed the purpose of the current study was to assess Fe bioavailability in biofortified PM, however, future research is certainly needed to elucidate what, if any, affects these other compounds mentioned in Table 2 have on mineral absorption and bioavailability. In light of the significant biological effects these polyphenols have in modulating many aspects of health and chronic disease [45], a goal of future research should be to identify and modulate concentration of specific families, and perhaps individual compounds, which display Fe inhibitory properties. Using this tailored, individualistic approach, the health-promoting properties of these compounds may remain largely intact in PM and other crops, while the effects of Fe inhibition suppressed. In India alone, about 50 million people rely upon PM as a major source of their dietary energy. Its tolerance to drought, heat and soil salinity and its high water use efficiency makes it a climate-smart crop. In addition, given its high protein and mineral content (especially Fe), and high dietary fiber, the area under PM cultivation is expected to increase, including its adoption in non-traditional growing environments [2,8-13]. Hence, we suggest continued research using the *in vitro*/ *Caco-2* cell and *Gallus gallus* models as guiding tools to further investigate these effects.

Conclusion

This study provides evidence that increasing Fe concentration in biofortified PM by nearly 60 μg/g provides modest, yet noticeable, increases in bioavailable Fe *in vitro* and

improved Fe status *in vivo*. Concurrent increases in polyphenolic compounds, inhibitors of Fe utilization, in the biofortified PM suggest that these compounds must be considered when using high- Fe PM lines to improve the Fe status of at-risk populations. Future feeding trials must continue to characterize the polyphenolic and phytate profiles of PM, and evaluate the effects such compounds have on Fe absorption and bioavailability. Modification of the PM polyphenol profile may be a means to improve Fe bioavailability in PM. We conclude that PM is a promising vehicle for increasing intakes of bioavailable Fe.

Abbreviations

PM: Pearl millet; Fe: Iron; Zn: Zinc; Hb: Hemoglobin; Hb- Fe: Hemoglobin iron; HME: Hemoglobin maintenance efficiency; BW: Body weight; ICRISAT: International Crops Research Institute for the Semi-Arid tropics; LC/MS: Liquid Chromatography/ Mass Spectrometry; ESI: Electron Mass Spectrometry; UPLC: Ultra Performance Liquid Chromatography; PCR: Polymerase chain reaction; DMT-1: Divalent metal transporter 1; DcytB: Duodenal cytochrome B; Da: Dalton; MEM: Minimum essential media; AU: Arbitrary Units.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ET was a co-principal investigator in developing the study protocol and design, performing the data collection, statistical analyses, in vivo and in vitro analyses, and writing and editing of the manuscript. SR assisted with the *in vivo* data collection, data analysis and writing and editing of the manuscript. JB assisted with the *in vivo* data collection. JH performed the polyphenolic bean analysis. RG was a co-principal investigator who participated in the study design, and editing of the manuscript. All authors approved the final manuscript.

Acknowledgements

We thank Dr. Binu Cherian (HarvestPlus) and Dr. Kedar N Rai (ICRISAT) for providing the pearl millet seeds that were used in the study. We also wish to thank Mrs. Pei-pei Chang and Mrs. Mary Bodis for their excellent technical support in the USDA Nutrition laboratory.

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Foods advertised in US weekly supermarket sales circulars over one year: a content analysis

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Abstract

Background: The nutritional content of Americans' shopping carts is suboptimal despite federal dietary guidance, in this case, the MyPlate consumer icon which displays desired proportions of vegetables, fruits, dairy, grains and protein foods for consumption. Consumers mention print advertising—such as weekly sales circulars—frequently as influencing their grocery shopping decisions.

Methods: To examine and describe the relative proportions of advertised foods aggregated into the MyPlate food grouping system, a content analysis of 9 209 foods advertised in 52 weekly supermarket newspaper sales inserts in 2009 from a local grocery chain was conducted in a Midwestern community.

Results: Overall, the protein foods group was most often represented in sales circulars (25% of total items), followed by grains (18%); dairy (10%); vegetables (8%) and fruits (7%). Less than 3% of sales advertisements were for dark green and red & orange vegetables. Over twice as much whole fruit versus 100% fruit juice was advertised (70% vs. 30%, respectively; $P < 0.001$). Significantly fewer protein foods and more grains than expected were advertised in the fall, and slightly more dark green vegetables were advertised in winter and spring than in summer and fall ($P = 0.05$).

Conclusions: The average American diet, including underconsumption of fruits and vegetables but overconsumption of protein foods, was reflected in the relative frequency of food groups advertised in weekly sales circulars. Modifying sales circulars to represent healthier food groups may preserve retail profits (considering these groups' higher profit margin) while promoting adherence to federal dietary guidance.

Keywords: Dietary guidelines, Advertising, Supermarkets, Grocery stores, Promotion

Background

Americans reported spending an average of ~ $400.00 USD each month at supermarkets in 2012, and spending has increased slightly since 2006 [1]. While some of the monthly expenditure can be explained by consumer demand for specific food items (e.g., organic products), more can be explained by supermarket strategies to promote specific products that maximize store profitability [2,3]. One very effective way supermarkets promote specific products is through weekly sales circulars, both in print and online.

Weekly sales circulars provide information to consumers about not only price discounts, but also what foods to consider purchasing. Price discount information is important considering economic downturns and rising food prices [1,4-8]. Indeed, 88% of consumers say that price is somewhat or very important when buying food [9]. Information regarding what foods to purchase is important considering consumers have to contend with multiple possible grocery stores in which to shop and an average of 39 000 items from which to choose within each store [10]. Weekly grocery store sales circulars help facilitate food purchasing decisions [11].

Over 70% percent of US adults read newspaper circulars [11,12]. Half of shoppers report using technology when grocery shopping and 23% of these shoppers report that they check prices at multiple stores before shopping

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[1]. Circulars have been shown to increase targeted versus untargeted item purchasing by 100% [13]. In fact, supermarket sales circulars are so effective in stimulating demand [13-16] that it is difficult to find a supermarket that does not use them.

Considering weekly sales circulars' effectiveness on influencing consumer purchases, it is not surprising that seasonal changes in promotion would affect consumer purchase types and amounts. For example, there is evidence that food pricing and advertising vary seasonally [17], as do purchases of vegetables and fruits [18]. Furthermore, seasonality may be associated with food intake [19-21], suggesting that advertising different foods more heavily at certain times of the year, such as vegetables and fruits in the summer or meats over holidays, may influence demand. Despite being an important new area of research given the influence of advertising on purchasing and its potential to positively impact dietary habits, the healthfulness of food items advertised in sales circulars is understudied. To our knowledge, there have been only two short-term studies published to date [22,23] that have described the contents of weekly grocery store sales circulars. Examination of supermarket sales circular content is critical to diet quality research to help understand commercial promotion of food types, which—inadvertently or advertently—are used as consumption recommendations [24]. Given that American's food purchases [25] and dietary intake [26] fall short of the Dietary Guidelines for Americans (DGA) [27] recommendations, and that supermarket sales circulars are widely used by consumers to guide their purchases, circulars have the potential to have a positive influence on an individual's diet quality [24].

Accordingly, the goal of this study was to examine the content of a year's worth of sales circulars to determine whether food groups advertised in sales circulars varied from recommended food group intake guidance overall and by season. We hypothesized that circulars would reflect seasonal changes and advertise more vegetables and fruits in summer and fall compared to the winter and spring seasons.

Methods

Fifty-two weekly supermarket sales circulars dating from January 1 to December 31, 2009 were collected from a local Midwestern supermarket chain. The chain consists of eight stores located in a city of 69 000 predominantly Non-Hispanic white individuals (86.7%) [28], and is considered small compared to other chain supermarkets [29]. As all stores belonged to the same chain, there was no variation in the circulars by individual store. Each food item in the weekly circulars was dual-coded by trained research personnel to assure data entry accuracy; all discrepancies were resolved by a supervisory research dietitian. A total of 9 209 food items were coded. The coding scheme for the advertised items was as follows: all items advertised in the circulars were classified as food or nonfood items. Food items were further classified into food groups and subgroups of food groups. Guidelines for classifying were based upon the following: The initial groupings were based on the Food Group Description File from the USDA National Nutrient Database for Standard Reference, Release 22 [30] and subgroups from USDA Handbook 8 (AH8) [31] with minor modifications to include products not originally designated in AH8. Food items were then grouped by major food groups (vegetables, fruits, grains, protein foods, and dairy) and subgroups of MyPlate, the consumer icon implementing the DGA [32] (n = 6 366; 69%) using the MyPyramid Equivalents Database [33]. The DGA makes recommendations to limit or reduce intake of the food components termed empty calories (solid fats and added sugars), therefore this group was also included. The solid fats group contains fats that are solid at room temperature, such as margarine spreads and added sugars refers to sugar that is not naturally-occurring and is added to foods during processing. The added sugars group in this study includes foods that are comprised predominantly of added sugar, such as sugar-sweetened beverages and hard candy [32]. Advertised items that did not have enough detail to determine the food group were categorized as "other" (n = 2 843). The "other" group included mixed dishes that could not be categorized into food groups. Wherever possible, the major food groups were disaggregated into subgroups. For instance, the fruit group was divided into whole fruit and 100% fruit juice, and dairy, protein, and vegetable groups were broken into individual components. It was not possible to identify whole versus non-whole grain products from the advertisements, however, we subjectively categorized grain products as "with" or "without" added sugars. For instance, cakes, cookies, and sweet rolls were categorized as "with added sugar" while bread, pasta, and crackers were classified "without added sugar". Seasons were categorized using the meteorological definition as follows: winter (December, January, February), spring (March, April, May), summer (June, July, August), and fall (September, October, November).

To determine whether observed frequencies of the five recommended food groups in the advertisements (n = 6 366) varied significantly from what was expected across seasons, a contingency table analysis was conducted using PASW Statistics 18, Release Version 18.0 [34]. Significant discrepancies between observed versus expected frequencies were determined by computing adjusted chi-square residuals for each contingency table cell. As adjusted chi-square residuals are normally distributed, any residual value greater than or equal to ±1.96 is interpreted as a significant departure from what was expected [35].

A generalized linear model was used to test for seasonal differences in the relative proportions of advertised items for each food group and subgroup using Proc GENMOD in SAS Version 9.2 [36]. If the overall model was significant, Tukey's contrasts were used to do pairwise comparisons between all seasons.

Results

The proportions of all food and subgroups advertised over the year are presented in Figures 1 and 2. Over the year, the groups frequently advertised of the recommended food groups was the protein foods group, which comprised one-fourth of all items described. Grains were the next most often advertised group (18%), followed by dairy (10%), vegetables (8%), and fruits (7%). Sixteen percent of foods were categorized as empty calories while another 14% were not able to be categorized. Meat, poultry and cheese were the most often advertised items in the protein and dairy groups, respectively.

Sixty-nine percent of all food items were categorized into the five major food groups. The frequency of items advertised approached significance as a function of season (overall chi-square test of independence, χ^2 (12, $N = 6$, 316) =20.1, $P = 0.066$). The adjusted chi-square residuals are plotted in Figure 3. Advertised frequencies of grains were significantly higher in fall ($z = 2.97$, $P = 0.003$) and lower than expected in the spring ($z = 1.95$, $P = 0.051$). The frequency of protein foods advertised was lower than expected in fall ($z = 2.62$, $P < 0.009$). There were no significant differences in the observed frequencies in the vegetable, fruit, or dairy groups by season.

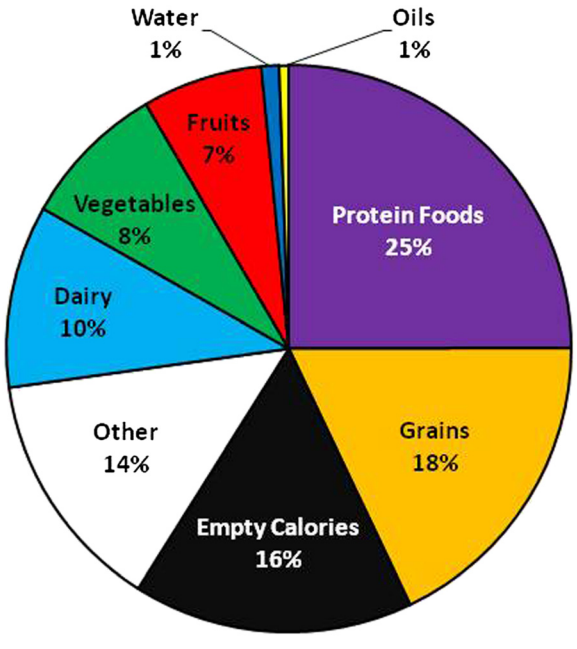

Figure 1 Proportions of food groups advertised in 52 weekly US supermarket sales circulars in 2009.

Next, we examined differences in groups and subgroups by season using generalized linear regression (Table 1). Items in the grain group were advertised significantly more often in fall compared to spring or summer ($P < 0.001$) and grains without added sugar was lowest in the spring months ($P < 0.001$). There were no seasonal differences in frequency of advertisments of grains with added sugar. Overall, there were no significant differences in the dairy group ($P = 0.35$), but fluid milk advertisements varied seasonally ($P = 0.02$). Advertised dark green vegetables also varied by season ($P = 0.05$); as did seafood, which was more heavily advertised in the winter months ($P < 0.001$).

Discussion

In this study we described the seasonal content of one year's worth of Sunday supermarket circulars. There was little variation between the frequencies of food groups advertised by season. In particular, advertisements for the major fruit and vegetable groups did not vary significantly during the summer or fall seasons, as one would expect, since one might assume that they are more easily available to supermarkets during the harvest months of the year. Only the promotion of dark green vegetables varied by season, with the highest numbers in spring compared to summer, although the absolute number of items advertised was neligible. However, advertisements for grains were greater in the fall and lower in spring whilst the frequency of protein foods was lower in the fall season than expected. The increased advertising of grain products in the fall was not due to changes in the grains with added sugar category, as might be anticipated with the demand for holiday baked goods, such as cookies and other sweetened desserts, but due to changes in the grains without added sugar category.

Overall, the frequency of advertisements for most food groups by this supermarket chain did not reflect current recommendations. Although nearly 50% of the recommended foods by the DGA are fruits and vegetables, we found that only a small proportion of advertisements were for items from those two food groups (15%). Encouragingly, one-fifth of items advertised were grain products. Although we were unable to distinguish whole grain items from refined items, we roughly categorized grains with added sugar and grains without added sugar, and found that over half of all grain products advertised were in the without added sugar category. As many food manufacturers are reformulating their grain products to contain more whole grains, the relatively high frequency of advertisements for grain products without added sugar has the potential to increase whole grain purchases and consumption [37].

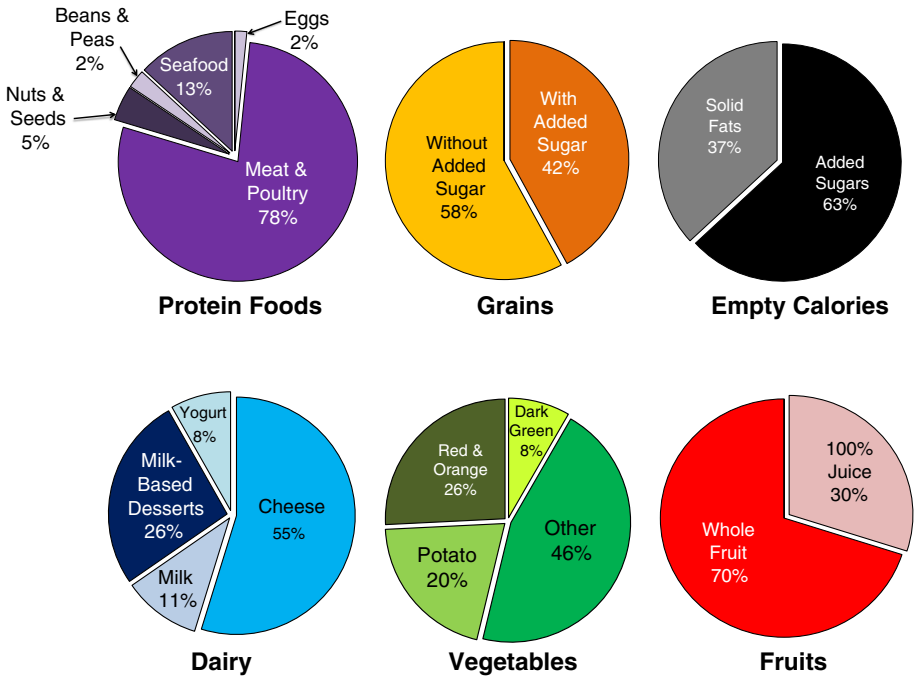

Figure 2 Proportions of food subgroups advertised in 52 weekly US supermarket sales circulars in 2009.

Our findings are similar to those of Martin-Biggers et al. [22], who to our knowledge were the first investigators to compare the content of supermarket flyers to dietary recommendations and obesity prevalence in a national sample of sales circulars. Their approach compared the physical space devoted to the major food groups recommended by the DGA on the front page of single weekly supermarket sales circulars nationwide to the space allocated on the MyPlate icon. Despite the fact that their approach differed significantly from ours and they did not examine advertisements over time, they also found that while protein foods were over-represented, dairy, fruits, and vegetables were underrepresented; only the grains group was represented in approximately the same proportions as those shown in the MyPlate icon. It is of interest that the same pattern was found both when

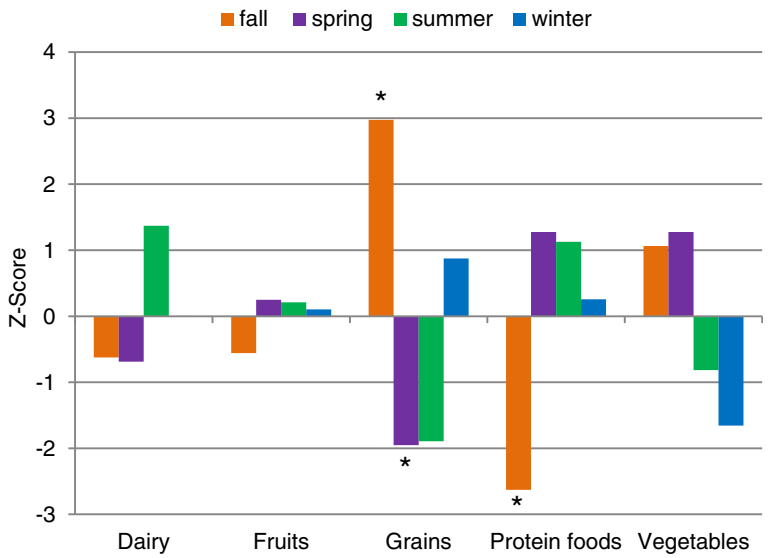

Figure 3 Expected compared to observed frequency of food groups advertised seasonally. Footnotes: Adjusted chi-square residuals were computed for each contingency table cell. Any residual value greater than or equal to ±1.96 is interpreted as a significant departure from what was expected in each season and marked with an *.

Table 1 Differences in the proportions of food groups advertised in weekly supermarket sales circulars by season[1]

MyPlate major food groups and subgroups	Season								p-value
	Winter		Spring		Summer		Fall		
	n = 2 006		n = 2 622		n = 2 154		n = 2 427		
	n	%	n	%	n	%	n	%	
Grains	388	19.3[ac]	427	16.3[b]	360	16.7[ab]	481	19.8[c]	< 0.001
Without added sugar	224	11.2[ac]	233	8.9[b]	205	9.5[ab]	298	12.2[c]	<0.001
With added sugar	164	8.2	194	7.4	155	7.2	183	7.5	0.67
Vegetables	156	7.8	227	8.7	171	7.9	214	8.8	0.50
Other vegetables	65	3.2	114	4.4	85	4.0	85	3.5	0.20
Red & orange	40	2.0	47	1.8	48	2.2	63	2.6	0.25
Potatoes	34	1.7	40	1.5	29	1.4	54	2.2	0.12
Dark green	17	0.9	26	1.0	9	0.4	12	0.5	0.05
Fruits	144	7.2	177	6.8	150	7.0	160	6.6	0.88
Fruit	94	4.7	126	4.8	115	5.3	107	4.4	0.53
100% fruit juice	50	2.5	51	2.0	35	1.6	53	2.2	0.23
Oils	9	0.5	13	0.5	7	0.3	21	0.9	0.08
Dairy	218	10.9	257	9.8	242	11.2	245	10.1	0.35
Cheese	125	6.2	143	5.5	134	6.2	125	5.2	0.29
Milk-based desserts	44	2.2	76	2.9	72	3.3	62	2.6	0.13
Fluid milk	31	1.6	21	0.8	16	0.7	34	1.4	0.02
Yogurt	18	0.9	17	0.7	20	0.9	24	1.0	0.55
Protein Foods	525	26.2	657	25.1	557	25.9	560	23.1	0.66
Meat & poultry	391	19.5[ab]	513	19.6[ab]	457	21.2[a]	433	17.8[b]	0.04
Seafood	97	4.8	85	3.2[a]	50	2.3[a]	70	2.9[a]	< 0.001
Nuts and seeds	20	1.0	30	1.1	23	1.1	36	1.5	0.45
Beans & peas	10	0.5	18	0.7	16	0.7	13	0.5	0.69
Eggs	7	0.4	11	0.4	11	0.5	8	0.3	0.78
Water	21	1.0	30	1.1	20	0.9	23	1.0	0.87
Empty calories	300	15.0	408	15.6	376	17.5	388	16.0	0.15
Added sugars	182	9.1	253	9.7	242	11.2	252	10.4	0.10
Solid fats	118	5.9	155	5.9	134	6.2	136	5.6	0.85
Other[2]	245	12.2[a]	426	16.3[b]	271	12.6[a]	335	13.8[ab]	< 0.001

[1]Values with the same superscript letters are not significantly different from each other.
[2]Includes combination foods; not enough detail available to separate out into groups.

examining either the front page only or the entire circular, and either in a national sample at a single time point or in a regional sample over a year. Ethan et al. reported the nutrition content of items on the first page of online sales circulars in the Bronx area [23]. They found that, at least on the front page, approximately 16% of advertisements were for vegetables or fruits, including 100% juice, compared to our findings of 15% and also reported that grain products accounted for 15% of ads compared to our results of 18%. Again, using different methods and time periods, results are consistent, indicating that perhaps overall, the front page of circulars is representative of the content of the full advertisement. Regardless of the methodology, this important initial research consistently points to a lack of concordance between dietary guidance and food items advertised in sales circulars.

Compared to recommendations, purchases for fruits and vegetables are suboptimal while products high in solid fats and added sugars are excessive [25]. While there is little research linking food purchases to food consumption [38], a growing body of literature suggests that price reductions or provision of coupons and food vouchers can improve both purchases and dietary intake of healthy food [39]. Online coupons are becoming popular with retailers; however, current research indicates that online coupons are overwhelmingly for processed snack

foods, not for healthier foods [40]. Many intervention efforts to promote the consumption of nutrient dense foods, especially fruits and vegetables, have turned to retail outlets. Economic incentives are often coupled with behavioral change strategies such as price manipulation, targeted coupons, in-store education, printed promotional materials, changes in product placement, and newspaper messages [41]. Other novel approaches include influencing purchasing by manipulating social meaning during shopping [42] and providing direct guidance for fruit and vegetable recommendations built into shopping carts [43]. Yet, to our knowledge, none have partnered with stores to target weekly circulars as a method of promoting price discounts of healthy, nutrient dense, foods. Relatively minor and inexpensive changes in content and placement of advertised items in circulars hold great potential to influence purchasing behavior and dietary intake to more closely align with recommendations.

The sales circulars were from a local grocery chain in the Midwest and may not reflect advertised products in other parts of the country. Other locations may also have a more pronounced seasonal variation in items advertised, especially vegetables and fruits, however, as our results were not dissimilar to other studies [22,23], they may be relevant to other parts of the country. Although coders worked in teams, coding was subjective and not all advertisements could be accurately classified, such as promotions where a variety of food items by one company were advertised together. We could not discriminate refined grains from whole grains as generally advertisements for ready-to-eat cereals and breads contained a variety of both whole- and non-whole grains. Because we counted each advertised item as it was displayed, we did not account for "buy one get one free" multiples. Although we did not distinguish between high- and low-fat meats or fried vs fresh fish, food items were categorized by subcategories of the major MyPlate food groups. Federal guidance recommends decreasing intake of solid fats and added sugars, but this is not reflected in the MyPlate icon. Strengths of the study include the use of foods in the entire circular, the use of a full years' worth of advertisements, and the comparison to federal dietary guidance. This novel approach adds to the sparse literature on grocery store sales circulars.

Stores use sales circulars to increase purchases by existing customers and to attract customers away from their usual grocery store by offering price discounts. However, if the sales circulars are not only interpreted as communicating purchasing deals but also function as intake guidance, then the relationship between sales circulars, purchasing behavior, and food consumption patterns needs to be investigated further.

Using a calendar year of supermarket sales circulars to ascertain information on the types of foods advertised helps us to understand consumer choices and can inform policy changes to promote healthier diets. Consumers indicate that weekly sales circulars are main factors in food purchasing decisions; thus, modification of the items advertised has the potential to significantly support people's efforts to eat healthier.

Reconfiguring sales circulars to promote healthier items may result in at least four possible outcomes: 1. No effect (i.e., new circulars are ignored); 2. A decrease in total sales and sales of healthier foods (i.e., consumer reactance to new circulars); 3. A decrease in total sales, but increase in healthier food sales (i.e., switching from less healthy food to more healthy food because of new circulars); 4. An increase in total sales and sales of healthier foods (i.e., keeping current purchasing patterns plus new healthy circular foods). Given what is known about price discounts and in-store marketing [24,43], we would expect outcomes #3 or #4. Currently, however, there is no empirical evidence examining the impact of systematic circular changes on purchases of healthier foods. Consequently, we expect this to be a fruitful area of future research regarding attempts to encourage healthier purchases. Increased focus on promoting fruits and vegetables in sales circulars may result in increased retail profits, through promotion of sales and simultaneous reduction in waste due to spoilage [44,45] —a beneficial effect for the stores that would likely keep these foods consistently in weekly circulars.

Conclusions

The results of this study demonstrated that the food items advertised in weekly grocery circulars in this particular geographic location did not correspond to the current DGA recommendations. Nevertheless, health interventions via supermarket advertising are largely underexplored to date. If changes in advertisements were partnered with interventions sensitive to the needs of retail stakeholders, the goal to increase the proportion of Americans meeting national dietary guidance, thereby decreasing the risk of chronic disease, could be paralleled by increased profits for the participating retailers.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LJ conceived the study. LJ, SK, CRP, and LDW developed the research questions and study design, interpreted data and drafted the manuscript. LKJ: performed statistical analysis, interpreted data and provided critical revisions of the manuscript: BSH and AJS: oversaw the study, collected data, performed analysis, interpreted data, and critically revised the manuscript. All authors approved the final version of this paper for publication.

Acknowledgements

This study was funded by the United States Department of Agricultural/ Agricultural Research Service, USDA 5450-51000-049-00D. The contents of this publication do not necessarily reflect the views or policies of the USDA or the Agricultural Research Service, nor does mention of trade names, commercial products, or organizations imply endorsement from the U.S. government.

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Effect of calcium supplementation on bone resorption in pregnancy and the early postpartum: a randomized controlled trial in Mexican Women

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Abstract

Background: Calcium needs are physiologically upregulated during pregnancy and lactation to meet demands of the developing fetus and breastfeeding infant. Maternal calcium homeostasis is maintained by hormonal adaptive mechanisms, thus, the role of dietary calcium supplementation in altering maternal responses to fetal-infant demand for calcium is thought to be limited. However, increased calcium absorption is directly related to maternal calcium intake and dietary supplementation has been suggested to prevent transient bone loss associated with childbearing.

Methods: In a double-blind, randomized placebo-controlled trial, we randomly assigned 670 women in their first trimester of pregnancy to 1,200 mg/day calcium (N = 334) or placebo (N = 336). Subjects were followed through 1-month postpartum and the effect on urinary cross-linked N-telopeptides (NTx) of type I collagen, a specific marker of bone resorption, was evaluated using an intent-to-treat analysis. Women with a baseline and at least one follow-up measurement (N = 563; 84%) were included. Subsequent analyses were conducted stratifying subjects by compliance assessed using pill counts. In random subsets of participants, bone-specific alkaline phosphatase (BAP) (N = 100) and quantitative ultrasound (QUS) (N = 290) were also measured.

Results: Calcium was associated with an overall reduction of 15.8% in urinary NTx relative to placebo ($p < 0.001$). Among those who consumed ≥50%, ≥67%, and ≥75% of pills, respectively, the effect was associated with 17.3%, 21.3%, and 22.1% reductions in bone resorption (all $p < 0.001$). There was no significant effect of calcium on bone formation measured by BAP. However, by 1-month postpartum, those in the calcium group had significantly lower NTx/BAP ratios than those in the placebo group ($p = 0.04$) indicating a net reduction in bone loss in the supplement group by the end of follow-up. Among subjects who consumed ≥50% and ≥75% of pills, respectively, calcium was also associated with an increase of 26.3 m/s ($p = 0.03$) and 59.0 m/s ($p = 0.009$) in radial SOS relative to placebo by 1-month postpartum.

Conclusions: Calcium administered during pregnancy and the early postpartum period, to women with intakes around adequacy, was associated with reduced bone resorption and, thus, may constitute a practical intervention to prevent transient skeletal loss associated with childbearing.

Trial registration: ClinicalTrials.gov Identifier NCT00558623

Keywords: Bone-specific alkaline phosphatase, Calcium, Clinical trials, Lactation, Pregnancy, Quantitative ultrasound bone speed of sound, Urinary N-telopeptide of type I collagen

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Background

Calcium needs are physiologically-upregulated during pregnancy and lactation to meet the demands of the developing fetus and breastfeeding infant for skeletal mineralization and growth [1,2]. Maternal calcium homeostasis is maintained by hormonal adaptive mechanisms that control intestinal calcium absorption, renal calcium excretion, and mobilization of skeletal mineral stores [3,4]. The role of dietary calcium supplementation in altering maternal responses to fetal-infant demand for calcium is thought to be limited; however, increased calcium absorption is directly related to maternal calcium intake [5,6].

Pregnancy- and lactation-associated bone loss has also been demonstrated through decreases in bone mineral density (BMD). An estimated five percent or more of total maternal bone mass may be mobilized [7,8], although, this bone loss is reversible with levels rebounding to pre-pregnancy levels after cessation of lactation [9]. There is clear histological and biochemical evidence that the maternal skeleton undergoes increased bone resorption during pregnancy [10,11].

Biochemical markers of bone resorption (osteoclast activity) and bone formation (osteoblast activity) have been found change drastically during pregnancy suggesting a physiological state of high bone turnover [12]. These markers of bone turnover may identify changes in bone remodeling and microarchitecture within a relatively short time interval (several days to months) before changes in BMD can be detected [13] and, thus, may provide insights into mechanisms of bone loss [14]. The long-term effects of these transient changes in maternal bone on child bone health are not fully understood [15], but new data indicate that maternal dietary deficiency during pregnancy may be associated with lower peak bone mass in offspring [16,17].

It is recommended that U.S. pregnant and breastfeeding women over the age of 18 years consume at least 1,000 mg calcium per day [18], but these recommendations are based largely on studies in non-pregnant adults [2]. High dietary calcium intake has been shown to decrease bone mobilization during pregnancy [19,20] suggesting that dietary calcium supplementation may be an effective means to prevent maternal bone loss. A number of studies have demonstrated an association with calcium supplementation and changes in bone turnover in non-pregnant adults [21], but data on the effects among pregnant women are scarce and there have been relatively few controlled supplementation trials that have studied the relationships directly [22]. The previously published trials of calcium supplementation and bone turnover in pregnant women [23-25] have been limited by their sample sizes and varying study designs making inferences from their results difficult. In addition, the trials in Gambia and China studied populations with low habitual dietary calcium intakes which limit their generalizability to populations with intakes approaching adequacy (such as the general U.S. population).

The objective of the present study was to evaluate the effect of dietary calcium supplementation on bone turnover during pregnancy and the early postpartum period using a double-blind, randomized placebo-controlled trial design. The hypothesis was that a daily supplement of 1,200 mg calcium carbonate would decrease bone resorption over the course of pregnancy among a relatively large sample of women with near adequate dietary calcium intakes.

Methods

Study population and design

First trimester pregnant women were enrolled from January 1, 2001 to April 26, 2004 at Mexican Social Security Institute prenatal clinics which serve a low-to-moderate income population in Mexico City. In brief, a total of 3,836 women were assessed for eligibility, of whom 1,981 did not meet study eligibility criteria (pregnancy of no more than 14 weeks gestation; not a high-risk pregnancy; plans to reside in Mexico City for study period; and no other reasons for exclusion) or were not able to be reached for contact (N = 2). When pregnant women were screened for initial recruitment, they were excluded if they exhibited any of the following conditions: any factor that could interfere with maternal calcium metabolism, intention not to breastfeed, preeclampsia, kidney or cardiac diseases, gestational diabetes, history of urinary infections, family or personal history of kidney stone formation, seizure disorder requiring daily medications, or ingestion of corticosteroids. Of the remaining 1,853 eligible women, 670 (36%) agreed to participate, signed informed consent, and were randomly assigned to receive a daily supplement of 1,200 mg calcium carbonate (two-600 mg tablets (Lederle, Inc.); N = 334) or placebo (N = 336) (Figure 1). Neither participants nor study personnel were aware of treatment group assignments and placebo tablets were formulated to be indistinguishable from the active treatment tablets.

Calcium carbonate is ~40% elemental calcium by weight [26]; therefore, for 1,200 mg calcium carbonate, the elemental calcium equivalent is: 480 mg. All treatment and control subjects were provided with a daily supplement of 30 mg iron (Fe) sulfate from study entry through 12 months postpartum since prenatal vitamins were not included in the standard of care. Women were instructed to consume Fe supplements at the mid-day "comida" (main meal) to decrease side effects that may accompany Fe ingestion [27]. Supplement levels were selected to meet two criteria: ensured adequacy and safety of total dietary intake. Doses consistent with the AI for calcium [28] and the Recommended Dietary Allowance (RDA) for Fe [29] to ensure normal physiologic requirements for pregnancy and lactation [30] would be met among women in the

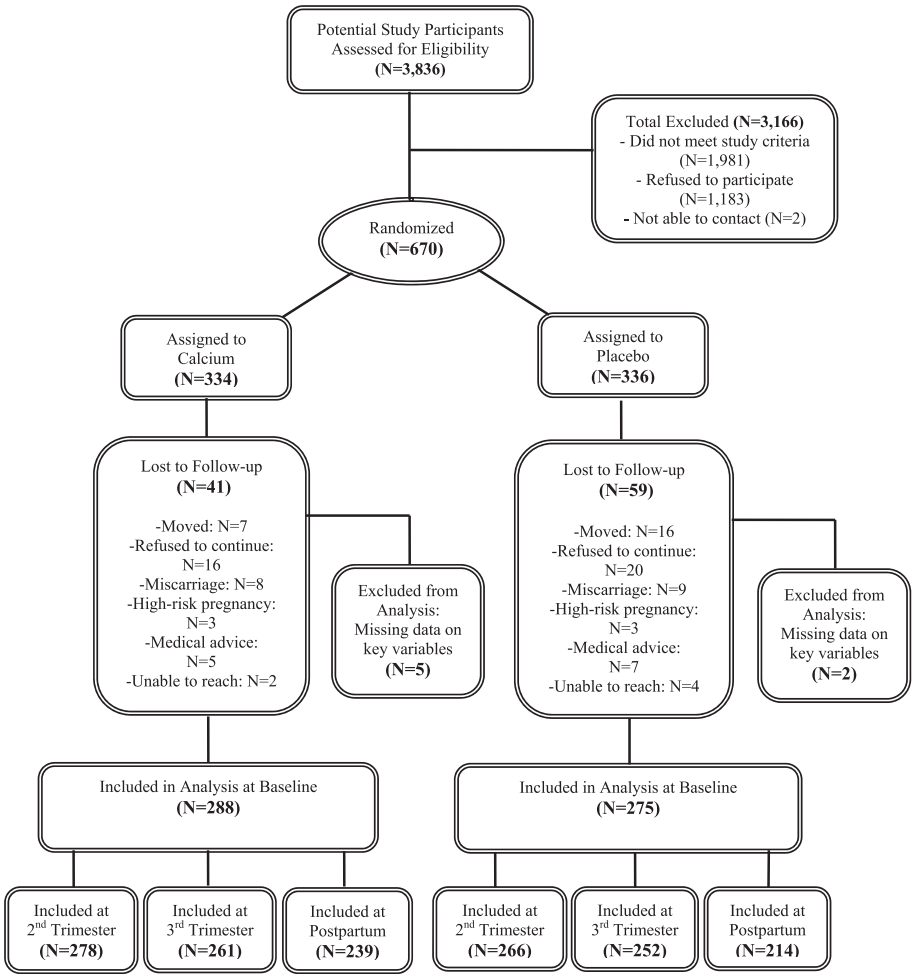

Figure 1 Study sample profile.

lower quartile of intake in our study population. Calcium was suggested to be consumed at bedtime, rather than in the morning, due to recent evidence (at the time of study planning) of potentially greater effects on bone turnover which was shown to be greater during the night than daytime hours [31]. Given potential problems with compliance, a split-dose regimen is not usually suggested for long-term supplementation trials as simplified drug-dosing regimens have been shown to improve adherence to therapy [32].

Participants were assessed at four time points: baseline (1st trimester) prior to initiation of treatment, and after having consumed calcium or placebo at 6 (2nd trimester) and 8 (3rd trimester) months of gestation and 1-month postpartum. Immediately following the baseline assessment, women were instructed to consume tablets daily at bedtime and compliance was assessed by pill count at each follow-up visit. Women who had a baseline and at least one follow-up measurement (calcium, N = 288; placebo, N = 275) were defined as having completed follow-up and included in this analysis (N = 563; 84%). The reasons for loss-to-follow up and the final numbers of women included at each stage of the analysis are detailed in Figure 1.

The research protocol was approved by the Human Subjects Committees of the Mexican National Institute of Public Health, the Mexican Social Security Institute, and participating institutions and has complied with all federal guidelines governing the use of human subjects. All participants received a detailed explanation of the study intent and procedures prior to signing the informed consent.

Markers of bone turnover

Urinary excretion of cross-linked N-telopeptides (NTx) of type I collagen was measured in urine from second-morning void collected by participants prior to each visit. NTx is a specific marker of osteoclast activity (bone resorption) that has been shown to be stable and resistant to degradation in stored samples [33]. Samples were analyzed with a commercially available competitive-inhibition

enzyme-linked immunosorbent assay (Osteomark; Ostex International, Seattle, Washington). NTx concentrations were controlled for urine dilution using creatinine concentration and expressed as nanomoles of bone collagen equivalents (BCE) per millimole of creatinine (nM BCE/mM creatinine). The intra-assay CV was 8.9% (at 406 nM BCE) and 8.7% (at 1563 nM BCE); the inter-assay CV was 8.6% (at 427 nM BCE) and 5.6% (at 1513 nM BCE).

Bone-specific alkaline phosphatase (BAP) was measured in plasma stored at −70°C from a subset of participants (N = 100) using the Ostase® BAP immunoenzymetric assay (Immunodiagnostic Systems Inc., Fountain Hills, AZ). BAP levels reflect the metabolic status of osteoblasts and, thus, serve as an indicator of bone formation [34,35].

Bone ultrasound measurement

Bone speed of sound (SOS, in meters per second) was measured at the distal radius using quantitative ultrasound (QUS) (Sunlight Omnisense 7000, Zicon Ltd. Petah-Tikva, Israel) in a random subset of participants (N = 290). Dual-energy x-ray absorptiometry (DXA) is the gold standard for measuring BMD [36], however, due to the potential for ionizing radiation exposure to the fetus, its use during pregnancy is inadvisable and specifically prohibited by Mexican law. QUS allows for an inexpensive, convenient, and radiation-free method by which to assess bone quality during pregnancy and several previous epidemiologic studies have used quantitative ultrasound to assess bone changes over the course of pregnancy [37-39].

Dietary intake

Daily intakes of calcium and total energy were assessed at each visit using a semi-quantitative food frequency questionnaire designed to estimate usual dietary intake over the prior month. The questionnaire was modified and validated among women living in Mexico City [40] and included questions specific to pregnancy such as any additional use of dietary supplements.

Statistical analysis

To assess whether randomization was successful in achieving comparability, baseline characteristics were compared between the calcium and placebo groups using the Wilcoxon rank-sum (Mann–Whitney U) two-sample test of equality. A similar comparison was performed between those who were included in the analyses and those who were lost to follow-up in order to assess whether selective attrition occurred. All tests of statistical significance were two-sided.

The effect of the calcium supplement on bone resorption was evaluated using an intent-to-treat strategy. A first approach was to conduct a comparison of the log-transformed NTx concentrations between treatment groups at each follow-up stage, both unadjusted (t-test) and adjusting for covariates (linear regression). A second approach was fitting a by mixed-effects regression model with a random intercept for each subject in order to adjust for imbalances at baseline and to gain precision in treatment effect estimates by including covariates. Mixed-effects models take into account the correlation between repeated measures on subjects over time. In addition, as mixed models are flexible with respect to incomplete data, all subjects with at least one follow-up measurement were included to increase the study's power. The outcome variable was natural log-transformed NTx in the 2^{nd} and 3^{rd} trimesters and 1-month postpartum. Models included the following baseline variables: treatment assignment (calcium vs. placebo), age (years), primigravidity (yes/no), NTx (nM BCE/mM creatinine), daily calcium (g/day) and energy intake (kcal/day), and time. We fitted a model including time*-treatment interactions to test for heterogeneity of treatment effects at different timepoints. To assess if breastfeeding at 1-month postpartum modified the effect of the supplement, a cross-sectional model with an interaction term between lactation (0,1 variable that defines whether the woman was lactating at the time of the 1 month postpartum visit) and supplement group was also fitted.

A secondary strategy was to estimate the efficacy of the supplement by performing a dose–response analysis to further assess the effect of the supplement by estimated compliance. Compliance was analyzed as the proportion of the expected number of pills taken by subjects between consecutive visits and then categorized into three groups: ≥50% of pills consumed, ≥67% of pills consumed, and ≥75% of pills consumed.

We also fitted a model with the NTx/BAP ratio as the outcome variable, in the subset with both measures available (N = 100 subjects, 270 observations), to observe if the relative levels of bone resorption-to-bone formation changed over the course of the pregnancy and to evaluate if this change was different between treatment groups. All statistical analyses were performed using STATA for Windows, version 12.0 (StataCorp LP, College Station, Texas).

Results

A total of 670 eligible women were randomized to receive calcium supplementation (N = 334) or placebo (N = 336) (Figure 1). Baseline characteristics were similar for the calcium and placebo groups with the exception of maternal age which was one year higher on average in controls (26.9 years) than in the supplement group (25.9 years; p = 0.02) (Table 1). Approximately 35.4% of women were primigravid and there were no significant differences by treatment. Dietary calcium intake, also not significantly different between treatment groups, was about 1,100 milligrams per day on average. Geometric mean (and geometric standard deviation (GSD)) pre-treatment NTx levels

Table 1 Baseline characteristics of subjects by treatment assignment and follow-up status

	Treatment assignment		Follow-up status	
	Calcium (N = 334)	Placebo (N = 336)	Included[a] (N = 563)	Not included (N = 107)
Variable	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Age (years)	26.9 (5.6)	25.9 (5.3)[b]	26.5 (5.5)	26.2 (5.3)
Education (years)	10.8 (2.9)	11.0 (3.2)	10.9 (3.1)	10.6 (2.9)
Number of pregnancies	2.0 (1.0)	2.1 (1.1)	2.1 (1.0)	2.0 (0.9)
Number of children	0.8 (0.8)	0.8 (0.9)	0.8 (0.9)	0.7 (0.7)
Number of months previous breastfeeding (cumulative lifetime)	5.6 (8.9)	6.8 (9.0)	6.4 (9.2)	5.1 (7.2)
BMI (kg/m^2)	25.9 (4.1)	25.8 (3.7)	25.9 (3.9)	25.9 (3.9)
Energy intake (kcal/day)	1888 (592)	1862 (637)	1860 (613)	1951 (619)
Calcium intake (mg/day)	1108 (492)	1083 (532)	1096 (515)	1091 (497)
Hematocrit (%)	39.1 (3.3)	39.1 (3.0)	39.1 (3.2)	39.1 (2.7)
NTx (nM BCE/mM creatinine)[c]	62.3 (1.7)	62.9 (1.7)	62.9 (1.7)	52.2 (1.7)

[a]Defined as having at least one visit completed after baseline and included in final model.
[b]$p < 0.05$ Wilcoxon rank-sum (Mann–Whitney U) two sample test of equality of distributions.
[c]Geometric mean and GSD; n = 291 treated, n = 285 with placebo.

were 62.3 (1.7) and 62.9 (1.7) nM BCE/mM creatinine for the calcium and placebo groups, respectively (p = 0.73).

A total of 563 women (84%) had at least one follow-up assessment and were included in the analyses. Comparing those included in the analysis (placebo N = 275; calcium N = 288) to those who were not included (placebo N = 61; calcium N = 46) revealed no significant differences by treatment assignment suggesting that those women who remained in the study were not systematically different than those who did not complete follow-up. Overall, the proportion of lactating women at 1-month postpartum was 89.6% and there were no differences by treatment group (calcium, 89.9% vs. placebo, 89.3%; p = 0.8).

In the unadjusted intent-to-treat analysis, calcium was associated with average reductions of 15.1, 16.4, and 20.2% in NTx concentrations in the 2nd and 3rd trimesters, and 1 month post-partum respectively (all $p \leq 0.001$). The corresponding visit-specific covariate-adjusted reduction estimates were 13.8, 15.6 and 19.2% (all $p \leq 0.001$) (Table 2). The overall covariate-adjusted average reduction in NTx concentrations relative to placebo was 15.8% ($p < 0.001$).

Results of the mixed effects regression model with treatment-by-time interactions showed a significantly different effect of the calcium supplementation on bone resorption at each study assessment when compared to baseline difference between treatment groups. The reduction was more evident at 1-month postpartum than in the 2nd and 3rd trimesters, but these reductions were significant for each of the three assessments: 2nd trimester (–13.7% reduction, p = 0.002); 3rd trimester (–15.6% reduction, p = 0.001); and 1-month postpartum: (–18.6% reduction, $p < 0.001$) (Figure 2).

Since response to treatment could depend on baseline dietary calcium intake, we tested a dietary calcium-by-treatment group interaction. There was no significant interaction between dietary calcium intake (either as a continuous variable or as quartiles) and supplement group at baseline. However, when examining lactation status, there was no effect of supplement in the non-lactating women (p = 0.57) compared to a 23% reduction in lactating women ($p < 0.0001$), indicating that lactation is an effect modifier for the effect of calcium supplementation on bone resorption.

When the effect of calcium supplementation was assessed for women "as treated" (N = 563) using models stratified by compliance (Table 3), we saw a dose–response effect of calcium on NTx concentration. Among those women who consumed ≥50% of pills, calcium was associated, on average, with a 17.3% reduction in NTx in comparison to placebo ($p < 0.001$). This increased to 21.3% ($p < 0.001$) and 22.1% ($p < 0.001$) for those who consumed ≥67% of pills and ≥75% of pills.

Table 2 Effect of calcium supplementation on NTX (Log-transformed) (N = 563)

	Unadjusted			Adjusted[a]		
	N	%Δ[b]	p-value	N	%Δ[b]	p-value
Study visit						
2nd trimester	548	15.1	0.001	544	13.8	0.001
3rd trimester	517	16.4	<0.001	513	15.6	<0.001
1-month postpartum	456	20.2	<0.001	453	19.2	<0.001
Average	567	16.8	<0.001	563	15.8	<0.001

[a]Adjusted for baseline: age, primigravidity, NTx, and dietary calcium and total energy intakes.
[b]Percent reduction: 1- e^{β}.

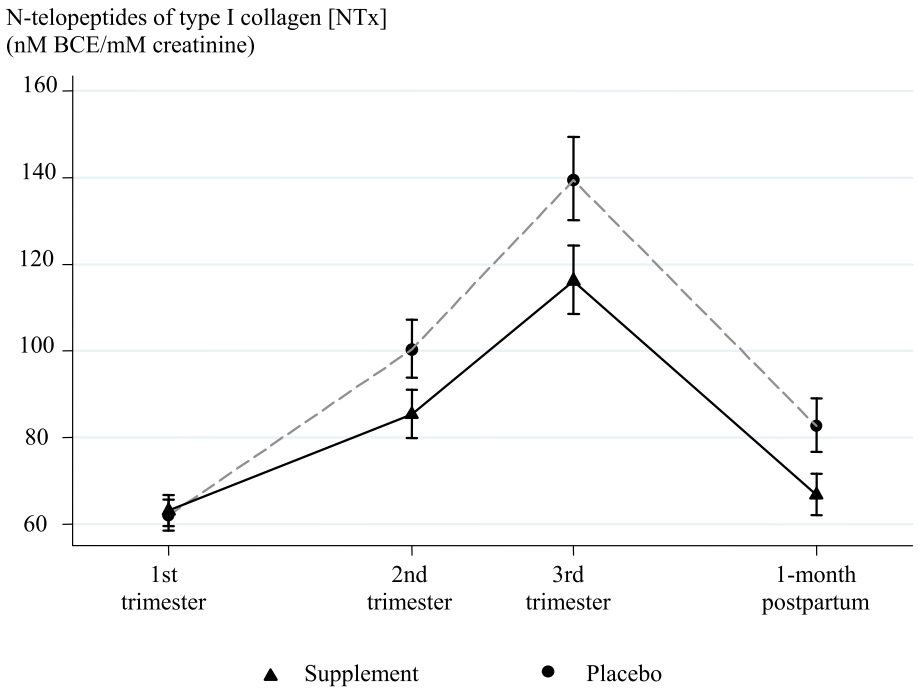

Figure 2 Effect of calcium supplementation on urinary N-telopeptides of type I collagen [NTx] (nM BCE/mM creatinine) at each trimester during pregnancy and at 1-month postpartum (Intent-to-Treat Analysis, N = 563); adjusting for baseline variables: NTx, age, primigravidity, dietary calcium and daily energy intake.

The subset of women with serum BAP measurements (N = 100) were not significantly different than those who did not have the measurements available (N = 463) except for years in school (those with BAP had 0.7 more years, on average, p = 0.04) and hematocrit (those with BAP 0.7 percentage points higher, on average, p = 0.04). There was no significant effect of calcium on BAP alone at any stage (p-values: 0.61, 0.20, 0.32 for 2nd trimester, 3rd trimester, and 1-month postpartum, respectively) (data not shown). Adjusting for age, primigravidity, baseline dietary calcium and total energy intakes, and baseline NTx/BAP ratio, the calcium group had lower, though not statistically significant, NTx/BAP ratio estimates at the 2nd (−10.1%, p = 0.32) and 3rd trimester (−13.4%, p = 0.20) visits. By 1-month postpartum, those in the calcium group had statistically significant lower NTx/BAP ratios than those in the placebo group (−21.5%, p = 0.04) indicating a greater net reduction in bone loss in the supplement group by the end of follow-up.

The subset of women with SOS available (N = 290) were not significantly different than those who did not have the measurements available except for years in school (those with SOS had 0.6 more years, p = 0.01) and total energy intake (women with SOS consumed ~190 kcal less, on average, p < 0.001). While radial SOS decreased over the course of pregnancy in both groups, declines in the supplement group were relatively attenuated and, by 1-month postpartum, those in the supplement group had higher, though not significantly, radial SOS than those in the placebo group (p = 0.13) (data not shown). Calcium was

Table 3 Effect of calcium supplementation[a] on NTx by treatment compliance[b]

		Average (Overall)		2nd trimester		3rd trimester		1-month postpartum	
Compliance	N (Obs)	%Δ[c]	p-value	%Δ[c]	p-value	%Δ[c]	p-value	%Δ[c]	p-value
ALL	563 (1510)	15.8	<0.001	13.7	0.002	15.6	0.001	18.6	<0.001
<50%	161[d] (270)	11.2	0.110	10.9	0.256	10.7	0.383	12.6	0.361
≥50%	505[d] (1240)	17.3	<0.001	14.9	0.003	15.6	0.002	19.2	<0.001
≥67%	378 (790)	21.3	<0.001	19.0	0.005	19.2	0.001	23.0	<0.001
≥75%	267 (423)	22.1	<0.001	25.0	0.171	19.0	0.006	21.9	0.002

[a]Adjusted for baseline: age, primigravidity, NTx, and dietary calcium and total energy intakes.
[b]Compliance assessed by pill count at each visit and analyzed as proportion of expected pills used between consecutive visits.
[c]Percent reduction: $1-e^{\beta}$.
[d]Numbers of subjects do not add to 563 because subjects may appear in more than one stratum due to time-varying nature of compliance.

associated with an overall average increase of 9.05 m/s in radial SOS relative to placebo though this difference was not significant (p = 0.216). However, among those subjects who consumed 50% or more of pills (N = 251), calcium was associated with an increase of 26.3 m/s in radial SOS relative to placebo by 1-month postpartum (p = 0.03). Among those subjects who consumed at least 75% of pills, calcium supplementation was associated with an increase of 59.0 m/s in radial SOS relative to placebo by 1-month postpartum (p = 0.009).

Discussion

In this randomized controlled trial, a 1,200 mg daily calcium carbonate supplement administered during pregnancy and the early postpartum period was associated with reductions in NTx, compared to placebo, both during pregnancy and at one month postpartum, indicating that dietary calcium supplementation may help to suppress maternal bone mobilization. These effects were stronger with increasing treatment compliance, suggesting a dose–response effect, with a greater than 22% average overall reduction observed among the most compliant women. These results are consistent with a previous randomized crossover trial in a small group of women which showed that dietary calcium supplementation reduced NTx levels by an average of 14% when administered in the third trimester of pregnancy [23]. To place the magnitude and direction of these changes into context, this is consistent with a 28% reduction in urinary NTx observed after 1-month of hormone replacement therapy among women randomized to receive 0.625 mg conjugated equine estrogen (Premarin, Wyeth Ayerst, Philadelphia, PA) [41].

The results of this study are also consistent with the findings of a study among 36 pregnant Chinese women with low habitual dietary calcium intake that found calcium supplementation was associated with significant decreases in markers on bone resorption; although in contrast to our findings, they also reported increases in bone formation [25]. Unlike our study, calcium was provided by supplementing the "usual diet" with 45 g milk powder (350 mg calcium) or milk powder plus 600 mg calcium supplement (950 mg calcium). In that study, dietary calcium supplementation during pregnancy was associated, in a dose-dependent manner, with greater BMD measured by DXA at 6 weeks postpartum at the spine and whole body ($p < 0.05$), but not at the hip site.

In the present study, calcium was associated with significantly higher radial SOS, a marker of bone density, by 1-month postpartum among the most compliant subjects. While the overall effect, including all subjects regardless of compliance, was not statistically significant, the direction of the effect is consistent with our hypothesis and radial SOS measurements were available in only about half of the subjects, thus, the study was underpowered to detect an effect of calcium on SOS. In addition, calcium's impact on bone density may differ depending on the type of bone. We measured SOS in the distal radius, a site with a predominance of cortical bone, and calcium may be acting on bone sites where trabecular bone dominates.

In a study of 125 Gambian women, supplementation with 1,500 mg/day calcium was associated with lower BMD measured by DXA in a subset of participants at the distal and midshaft of the radius, but with increases in measures of BMD in the lumbar spine and whole body [24]. Like the Chinese study, the Gambian study also measured the effect of calcium supplementation among women with low dietary calcium intake. However, unlike our study and the one by Liu et al. [25], the Gambian study did not continue supplementation into the postpartum period which may be partially responsible for their findings of rebound demineralization following cessation of lactation [42]. We found that the ratio of bone resorption-to-bone formation was significantly lower in the calcium group by 1-month postpartum suggesting that calcium is effective in reducing net bone loss measured after pregnancy. The observed effects at 1-month postpartum were being driven by lactating women in our study which suggests that the need for continuation of calcium supplementation may extend into the postpartum period.

A limitation of our study is that we used QUS, and not DXA, to assess bone quality in pregnant women and this measurement was available in only about half of the women. QUS has been demonstrated to predict fracture risk [43] and has been widely used in epidemiologic studies to measure bone density particularly where DXA is not available [44] or not advisable, such as during pregnancy [37-39] due to the potential for radiation exposure to the fetus. QUS has been found to be well-correlated with DXA at all sites measured over 7 years of follow-up [45] and provides our study with the advantage that we were able to include repeated measures of bone density, in addition to biochemical markers of bone turnover, over the entire course of pregnancy and the early postpartum period.

Pregnancy and lactation may impact a woman's peak bone mass which is an important determinant of subsequent osteoporosis risk [46]. In addition, calcium may also have potential benefits for child bone health [16,17,47]. The possibility that intrauterine programming of fetal bone growth may be an important determinant of osteoporosis and the risk of other chronic diseases in later life is now being considered [48]. New evidence indicates that maternal dietary deficiencies during pregnancy may be associated with lower peak bone mass in offspring later in life [16,17].

Average baseline dietary calcium intake for women in our trial was within the current recommended dietary guidelines of 1,000-1,300 mg/day for pregnant and lactating

women [18]. It is possible that high amounts of calcium are needed to counterbalance the nutritional needs of the developing fetus [49]; thus, previous trials among women with low habitual dietary calcium intakes may have been unable to detect an effect. Bone mineralization does not depend solely on the availability of calcium: protein, energy, and other nutrients are also important to bone formation and mineralization. Vitamin D is essential for calcium homeostatis and now recognized as an important nutrient for bone health including modest support for maternal vitamin D status and increased offspring bone mass among [50]. However, this study was planned and carried out on the basis of the 1997 IOM guidelines [28]. Vitamin D was not specifically recommended with calcium supplementation as is currently common practice. Nonetheless, other prior studies of calcium supplementation in adult pregnancy [23-25], to which we compare our results, also did not measure or administer Vitamin D. One small randomized study of pregnant Brazilian adolescents with habitually low calcium intake [51] found that 600 mg calcium carbonate plus vitamin D_3 (200 IU) resulted in higher lumbar spine bone mass and a reduced rate of femoral neck bone loss during lactation which is consistent with our results. The maternal response to fetal calcium demand may also be highly individualized and other genetic, hormonal, or lifestyle factors may be involved [52].

Conclusion

In summary, dietary calcium intake likely plays a modest, but important role in suppressing maternal bone mobilization during pregnancy and the early postpartum. Calcium supplementation during pregnancy may also reduce the risk of hypertensive disorders of pregnancy [53,54], pre-eclampsia [55,56], and lead exposure [57] which themselves pose risks to the mother and fetus. The risks posed by calcium supplementation at levels approximating the upper limit of recommended daily intake are relatively minor [2,18] and U.S. guidelines for calcium in pregnancy and lactation are based on studies in non-pregnant adults [2]. The World Health Organization now recognizes the importance of calcium supplementation in pregnancy [58]. Thus, dietary supplementation of calcium intake among pregnant and lactating women should be considered particularly in populations where dietary calcium intake is low.

Abbreviations

BCE: Bone collagen equivalents; BAP: Bone-specific alkaline phosphatase; BMD: Bone mineral density; DXA: Dual-energy x-ray absorptiometry; QUS: Quantitative ultrasound; SOS: Speed of sound; NTx: Urinary cross-linked N-telopeptides of type I collagen.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Study design: HH, MHA, and KEP. Study conduct: ASE, HLF, AMG, KK, RJW, KEP, HH, MHA, MMT. Data collection: AMG, RJW. Data analysis and interpretation: ASE, HLF, MMT. Drafting and revising manuscript content: ASE. Approving final version of manuscript: ASE, HLF, AMG, KK, RJW, KEP, HH, MHA, MMT. ASE, HLF, and MMT take responsibility for the integrity of the data analysis. All authors read and approved the final manuscript.

Acknowledgements

The authors gratefully acknowledge the contribution of the American British Cowdray Hospital in Mexico City for use of its research facilities. This work was supported by the U.S. National Institutes of Health (NIH) [grant numbers: P42-ES05947, R01-ES07821, R01-ES021446, P30-ES00002, and K01-ES014907] and by México Consejo Nacional de Ciencia y Tecnología (CONACYT) (The National Council of Science and Technology) [grant number 4150M9405]. The contents of this article are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

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Human milk and neurodevelopment in children with very low birth weight: a systematic review

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Abstract

Human milk (HM) contains critical nutrients and possibly other neurotrophic factors that could benefit the less developed brain of preterm infants, particularly those with very low birth weight (VLBW). This study aims to systematically review the original studies to determine whether there is a reproducible independent effect of HM feeding on neurodevelopment outcome in preterm VLBW infants. Search of seven databases (PubMed, Cochrane, CINAHL, Embase, Proquest Research Library, Google Scholar, and Web of Science) identified 24 original studies. Each study was evaluated by two authors independently for 8 non-nutritive (study design, target population, a priori power calculation, adjustment for baseline growth status, postnatal complication, other confounders, observer blinding to feeding status, effect size) and 5 nutritive (definition and duration of HM intake, use of HM fortifier, source of HM data, infant formula used) methodology parameters, and consistency and directness of outcome measures. Thirteen reports of preterm infants with wide ranges of birth weights were excluded as none provided sufficient data to delineate the effects of HM feeding on developmental outcome of subjects with VLBW. Eleven reports included only VLBW children and 7 studies were reviewed after elimination of preliminary data from same cohort or lack of appropriate standardized testing or control group. These 7 studies (n = 18 to 704, median 219) were performed at <3 years (3 studies) and at 5 to 11 years (4 studies). Six studies were secondary analysis of data from other studies. Each study met or only partially met 4 to 10 methodological parameters. VLBW children with no neurological impairment fed HM achieved normal or low normal range of test scores. Formula feeding using older formulations was associated with a lower subtest score in 4 studies. There is no randomized clinical trial comparing the neurodevelopment outcome of HM versus formula or minimal HM feeding that included only children with VLBW. The role of HM in the neurodevelopment and cognitive function of VLBW children needs reassessment with high quality studies in the context of current formulations of HM fortifier and preterm formula.

Keywords: Preterm, Very low birth weight, Milk, Human, Breast milk, Donor milk, Neurodevelopment or cognitive function

Introduction

Preterm very low birth weight (VLBW, <1,500 g) infants are at high risk for growth failure and co-morbidities that result in delayed neurodevelopment and academic achievement [1-3]. Early nutrition support is recognized as critical to growth and development and exclusive breastfeeding is universally recommended as beneficial to the health and well-being for all infants [4-6]. However, human milk (HM) alone does not support optimal growth for VLBW infants, so multinutrient fortification, focusing on protein, minerals, vitamins and other nutrients is recommended [4-6]. Preterm VLBW infants are born at a period of significant phase of in utero organ development and are at risk for deficiency of essential nutrients and trophic factors critical to the growth and function of the nervous system. The less developed brain of preterm infants, particularly those with VLBW, theoretically could benefit from feeding maternal milk since it contains critical nutrients such as long chain polyunsaturated fatty acids (LCPUFA) and possibly other neurotrophic factors. This is supported by a review of earlier studies which indicated that HM has greater neurodevelopment benefits than formula for feeding preterm infants [7].

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Significant methodological issues arise in the determination of the effect of feeding HM on neurodevelopment outcome. Since it is neither feasible nor ethical to assign breastfeeding randomly, determining the effect of breastfeeding is invariably based on observation. This has challenges particularly in controlling for factors to minimize the risk of bias [8-10]. One quasi-randomized trial in preterm infants of varying birth weights and gestational ages showed significant neurodevelopment benefit for infants fed HM [11]. This and other reports [7], did not adequately control for perinatal and postnatal complications, social and environmental factors that can affect neurodevelopment, and had limited data on the role of HM feeding on neurodevelopment of the group at greatest risk for neurodevelopment impairment, namely children with VLBW. The aim of this study is to systematically review the original studies to determine whether there is a reproducible independent effect of HM feeding on neurodevelopment outcome in preterm VLBW infants.

Methods

Identification of articles

A systematic search of the literature was conducted for studies published in English that examined the effect on neurodevelopment and cognitive outcomes from breast milk feeding to VLBW infants. Literature searches of the databases Medline (via PubMed) from 1988, Cochrane Library from 1982, Cumulative Index to Nursing and Allied Health Literature (CINAHL) from 1992, Embase from 1988, Proquest Research Library from1990, Google Scholar from 1994, and Web of Science from 1992 were performed on several occasions with a final search performed on August 11, 2014.

The PubMed search strategy employed a 5 step process using medical subject headings (mh) and related subject/keyword/text word (tw) terms. The first 4 searches were performed independently followed by the 5th search which combined the results from first 4 searches within each database to obtain the articles to be screened for relevance and subsequent review. The first search include infant, premature (mh), OR infant, very low birth weight (mh) OR Very low birth weight (tw) OR extremely low birth weight (tw) OR preterm infants (tw). The second search include breast feeding (mh) OR milk, human (mh) OR breast milk (tw) OR donor milk (tw) OR donor human milk (tw) OR maternal milk (tw). The third search include child development (mh) OR cognition (mh) OR intelligence (mh) OR neurodevelopment (tw) OR cognitive development (tw) OR brain development (tw) OR cognitive outcomes (tw) OR development cognitive (tw). The fourth search include outcome (all fields) OR effect (all fields). The fifth search combined the results from first 4 searches. This procedure was followed for all databases except for some variation in the search terms specific to a database.

For the purpose of this review, "human milk" was defined as breast milk from the mother (own mother's milk, OMM) or one or more donors (donor milk, DM), whether it was delivered by gavage or a nipple from the bottle or breast. "Neurodevelopment outcome" was defined as the attainment of age-appropriate developmental milestones or specific testing of intelligence or educational achievement. Study outcome limited to behavior/temperament tests or motor ability alone were not considered since their value as the sole predictor of long term neurodevelopment or cognitive function is not well established.

Titles and available abstracts of all studies compiled from the final electronic database search were screened by the investigators to determine eligible studies. Peer reviewed original studies independently assessed the relationship between HM and neurodevelopment outcome were identified. Reports of VLBW children studied as part of a larger cohort of preterm children with greater range of birth weights were included if the data clearly delineated to allow assessment of neurodevelopment effect on VLBW children from HM feeding. Bibliographies from these articles were also searched for additional applicable studies. For each cohort with multiple publications, only the publication with the longest duration of neurodevelopment follow-up was included in this review.

Evaluation of articles

We evaluated each article following the principles of systematic review [12] and similar to previous reports [8-10,13] but with modifications pertinent to the VLBW situation. To minimize bias of this systematic review, each study was evaluated independently by two authors (WK and ST) according to a list generated a priori, and the final result was a consensus reached by both authors.

To minimize bias within each study and across studies, each study was reviewed according to a list of non-nutritive and nutritive parameters. The non-nutritive parameters included 1) study design and whether the study's primary goal was the determination of the effect of breast milk on neurodevelopment or a secondary analysis in a non-breast milk related project, 2) target population, whether VLBW infants were included as part of the preterm population with higher birth weights or were the sole target, 3) predetermined sample size for different feeding groups, 4) whether adjustments were made for baseline differences in other variables such as the presence of intrauterine growth retardation (IUGR), 5) documentation of the extent of postnatal complications that could compromise the neurodevelopment outcome including the extent of intracranial hemorrhage, chronic lung disease, necrotizing enterocolitis, retinopathy of prematurity, severe neurosensory impairment, documented sepsis; how this information was managed

and whether infants with serious complications were excluded from the data analysis, 6) control for bias in neurodevelopment and cognitive outcome, namely whether studies were controlled for socioeconomic status, maternal intelligence, and child rearing environment using Child Life Experience [14], Home Observation for Measurement of the Environment [15] or similar assessment tool, 7) whether observers of the outcome were blind to feeding status, 8) whether the study reported an effect size or some other strategy to interpret the clinical impact of the results. Nutritive parameters on the availability of feeding data included 1) definition and 2) duration of HM intake, 3) the type and amount of fortification, 4) source of HM feeding data, and the 5) type of non-HM feeding support. To accommodate the varied ages and circumstances of included children, all data from standardized tests of neurodevelopment or general intelligence were included. All quantitative and statistical data presented were based on each publication without any assumption or modification.

We also assessed the quality of each study [16] according to study design, whether the methodological criteria were met, and consistency and directness of outcome measures.

Results

Figure 1 indicates the number of articles screened and the final number of studies reviewed. A total of 24 reports of original studies that included HM feeding and neurodevelopment outcome in VLBW children were identified. Thirteen reports were excluded as none of these publications provided sufficient data to delineate the effects of HM feeding on developmental outcome of subjects with VLBW [11,17-28]. Of these 13 publications, six [11,22-26] were reports of selected subsets (n = 50 to 438) from the same original study populations of 926 subjects. The average birth weights were ~1400 g and 26 to 38% of the subjects were small for gestation (SGA). Two population-based cohorts [17,28] from the same country included >1400 preterm infants in each cohort. The mean (SD) birth weights of HM fed groups were 1430 (SD 280) g and 1460 (SD 400) g respectively. The birth weights were significantly higher (average 100 g) and as was the mean gestation (average ~0.5 week) than non HM groups. Five other publications [18-21,27] reported neurodevelopment of subjects with birth weights up to 2000 g (n = 39 to 388). Two of these publications [19,21] reported on the same cohort.

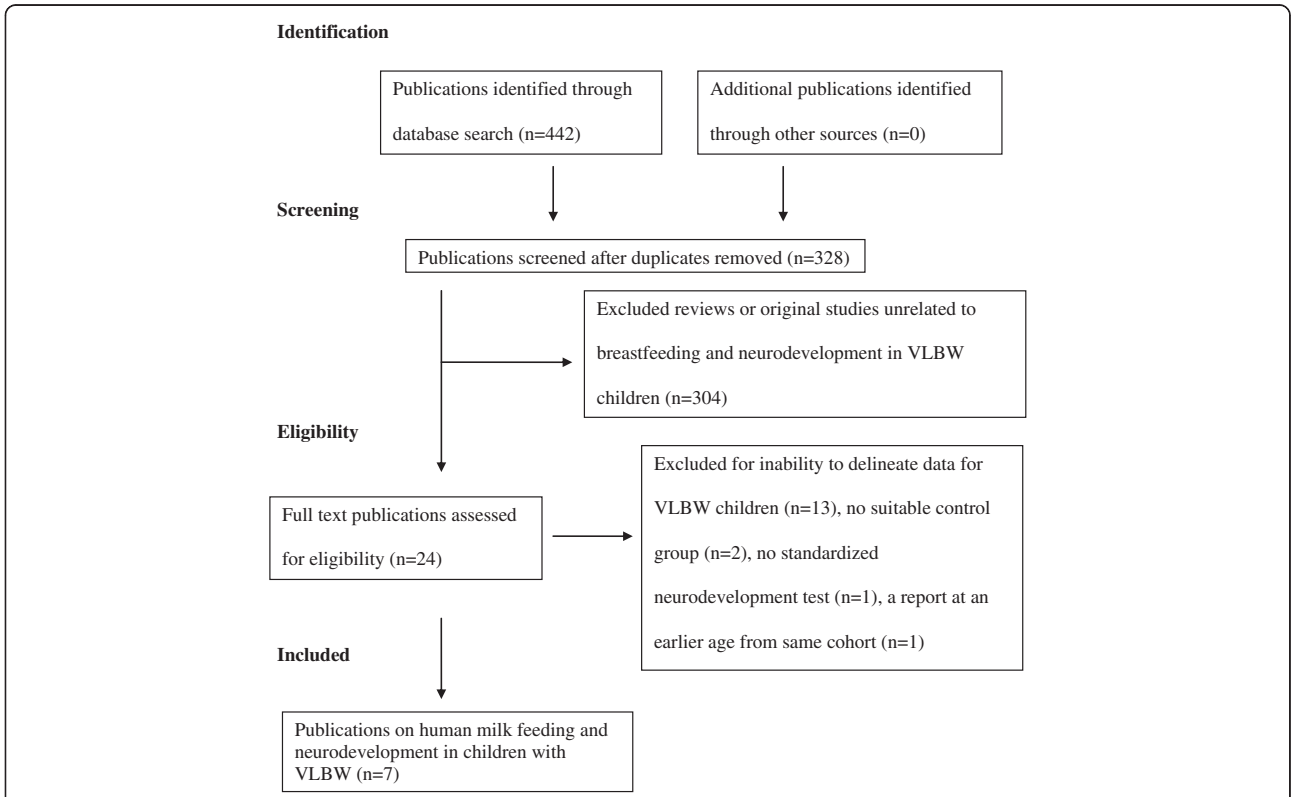

Figure 1 Search strategy to obtain original studies assessing the effect of feeding human milk on neurodevelopment of children with very low birth weight.

Eleven reports included only VLBW children [29-39]. Four of these publications were excluded because there was another publication of the same cohort at a younger age [36], no formal standardized neurodevelopment tests were performed [37] and no suitable control group was defined as the study involved supplementation of docosahexaenoic acid and arachidonic acid of HM fed infants [38,39]. Of the remaining 7 studies of VLBW children, 2 included only children with birth weights <1000 g [34,35].

Table 1 shows clinical characteristics of 7 studies of developmental outcome associated with HM feeding in VLBW infants. Three studies [30-32] reported the number of SGA infants with birth weight of <10th percentile as surrogate for IUGR. SGA was reported to be as high as 62% in one study [31]. The numbers of children from multiple births also were not well documented.

Table 2 shows details of non-nutritive methodological parameters. All were observational studies dependent on maternal choice whether to provide breast milk with varying amount of OMM provided to their VLBW infant. In 6 of 7 studies, the effects of HM feeding on neurodevelopment were extracted via secondary analysis of data from other projects. The design of the primary studies was epidemiologic observational with 2 interventional studies: one on structured counseling to promote breastfeeding [29] and the other on glutamine supplementation in parenteral nutrition [35]. Three studies [31,33,35] included only subsets of the study population from primary study. Exclusion criteria were generally clearly defined although varied among different studies. Three studies [29,30,35] included children assessed at younger than 3 years and 4 studies included children from 5 to 11 years [31-34]. Sample sizes varied from 18 to 704 (median 219) children and none stated a priori power calculation to measure the effect of breast milk. The attrition rate of the subjects assessed tends to increase with increased duration of follow-up. Blinding of the observers to the feeding status of the subjects was reported in 3 studies [29,30,34].

In all studies, VLBW children without neurological impairment fed HM achieved normal or low normal scores on standardized tests of neurodevelopment or cognitive function. Thus any advantage of HM feeding is due to the lower scores of formula-fed infants. Two studies [29,31] used dichotomous grouping with 80% of the intake of HM as a cutoff point. For the group that received the most HM, one of these two studies reported a higher raw score in one subtest of neurodevelopment at 5 years of age but no adjustments were made to account for confounders [31]. The other study showed no significant difference in adjusted scores [29]. One study at 30 months corrected age showed HM feeding during the hospitalization resulted in mean Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) scores on the Bayley Scales of Infant Development (BSID) in the low normal range; those fed infant formula have significantly lower scores [35]. Two of the 3 studies with neurodevelopment assessment at 6 to 11 years showed that HM feeding at highest volume was associated with intelligence test scores in the normal range. The formula fed group showed a significantly lower score in verbal intelligence quotient (IQ) in one study [32] and in visual motor skill subtest in another study [33]. The third study [34] reported that children born extremely preterm without major neurological impairment showed significantly poorer academic attainment on reading and mathematics than their term peers; HM feeding positively affected reading, but not mathematics attainment, at 11 years. In the same study, breast milk consumption together with perinatal and neonatal complications and socio-economic status accounted for 29% of the variance in reading attainment at 11 years.

Table 3 shows every study obtained some data on perinatal, postnatal, social and environmental factors. However, the extent of the details varied greatly. The statistical handling of the numerous variables also varied among studies and only one study adjusted for the SGA status.

Table 4 shows the nutritive parameters assessed in each study. Details on the extent and duration of HM feeding varied. The source of the feeding data was referenced in 4 studies. Those with short-term follow-up generally had sufficient details to allow categorization of the amount of HM intake. Long-term follow-up studies generally relied on maternal recall, and the amount of HM intake was not quantified [31-34]. Only one study reported use of health clinic record [32] as an additional measure to minimize recall bias. No study reported the use of donor milk. Use of HM fortifier was reported in 2 studies. Preterm infant formula usage was reported in 3 studies but the type of infant formula used was not documented in the other 4 studies. In 6 of 7 studies, the control group used as a comparison with HM-fed group was from the same cohort of VLBW infants who were fed exclusively infant formula or whose daily feedings consisted of up to 80% HM. Only one study included children born at term and matched for sex and ethnicity and attending the same mainstream school or special education facility.

All but one study were secondary analyses of data from primary studies that may have an independent effect on neurodevelopment. The effect of HM on neurodevelopment was based on observation of selected cohorts. The quality and the risk of bias, as determined by the extent to which the methodological parameters are met, were varied among different studies (Table 5). Of the 8 non-nutritive parameters, each study has at least 4 parameters that were either not met or only partially met with the use of surrogate markers. Of the 5 nutritive parameters, one study did not meet one parameter while the other studies did not

Table 1 Human milk (HM) and developmental outcome in very low birth weight (VLBW) infants: clinical characteristics

Reference	Pinelli et al. [29]	Furman et al. [30]	Tanaka et al. [31]	Horwood et al. [32]	Smith et al. [33]	Johnson et al. [34]	Vohr et al. [35]
Birth weight g	<1500	600-1499	<1500	<1500	<1500	<1000	<1000
Gestation: mean (weeks, SD if available)	HM (29, 3) vs. Control (29, 3).	All subjects (27, 2).	HM (28.7, 3.2); Control (30.7, 1.6).	*	HM (28.7, 2.4) vs. Control (27.8, 2.5).	HM <26 vs. Control (term birth).	HM 26.7 vs. Control 26.2.
Range of HM intake	>80% vs. <80%	>50 to 0 mL/kg/d	>80% vs. <80%		Breastfeeding at discharge vs. no HM at any stage	VLBW infants: any breast milk vs. none during hospitalization. Control: regardless of HM intake	>80% vs. 0
% small for gestation	NS	8	HM 40%; "Control" 62%	*	NS	NS	NS
% singleton	100	100	NS	*	61.5	NS	NS
Year/s of birth	NS	Jan 1997-Feb 1999	1999-2000	1986	1991-1993	Mar-Dec 1995	Oct 99-Jun 2001

NS – not stated or specified.
*Small for gestation and multiple births entered into analysis but no raw data presented.

Table 2 Human milk (HM) and developmental outcome in very low birth weight (VLBW) infants: non-nutritive methodological parameters

Reference	Pinelli et al. [29]	Furman et al. [30]	Tanaka et al. [31]	Horwood et al. [32]	Smith et al. [33]	Johnson et al. [34]	Vohr et al. [35]
Primary study for the subjects	Structured breast feeding counseling to sustain lactation	Self selected provision of breast milk vs. preterm formula	RBC DHA in breastfed VLBW infants at 4 weeks	Retinopathy of prematurity	Epidemiology of brain injuries in VLBW infants and Epidemiologic study of multiple births	Early predictors of education attainment at 11 y	Parenteral glutamine trial
Subject source	One center, Canada	One center, USA	One center, Japan	All VLBW births, New Zealand	5 centers, USA	All extremely preterm births in United Kingdom and Ireland	12 of 15 NICHD NRN sites, USA
Exclusions	Multiple birth, severe congenital, surgical, chromosome abnormality, non-English speaking parents	Positive drug screen, major congenital anomalies, intrauterine infection, overwhelming maternal social concerns	Cerebral palsy, no RBC DHA data, severe chronic lung disease, minor anomalies, hearing problem	Sensorineural deficit and no breast milk data	NS	None	Unable to test including those with sensorineural deficit
Assessment age	6 and 12 m corrected	20 m corrected	5y	91 m	6 to 8y	Median 10y 11 m	30 m corrected
Sample size (assessed/eligible)*	138/148	98/119	18/26	298/338	439/770	219/307; and 153 term "Control"	704/939
Observers blinded to feeding protocol	Yes	Yes	NS	NS	NS	Yes	NS
Neurodevelopment tests	BSID II: MDI and PDI	BSID II: MDI and PDI	KABC	WISC-R: verbal and performance IQ	CELF; CCVL; KABC; PPVT; WRAVMA	KABC; TAAS; WIAT-II	BSID-II
Effect size for human milk†	See below	See below	See below	See below	See below	See below	See below

CI – confidence interval; NICHD NRN – National Institute of Child Health and Human Development Neonatal Research Network; NS – not stated or specified or not significant; RBC DHS red blood cell - Docosahexaenoic acid; SD – standard deviation.

Neurodevelopment tests: BSID II Bayley Scales of Infant Development (2nd edition), MDI Mental Developmental Index, PDI Psychomotor Developmental Index; CCVL California Children's Verbal Learning Test; CELF Clinical Evaluation of Language Fundamentals 3rd ed; KABC - Kaufman Assessment Battery for Children; PPVT - Peabody picture vocabulary test 3rd ed; TAAS Teachers Academic Attainment Scale; WIAT-II Wechsler Individual Achievement Test – II; WISC-R - Revised Wechsler intelligence scale for children; WRAVMA - Wide Range Assessment of Visual Motor Abilities.

*None had stated a priori power calculation to measure the neurodevelopment effect of HM. For Smith et al. [33], total sample size included 4 gestation matched VLBW controls for each VLBW subject with abnormal cranial ultrasound. For Johnson et al. [34], control subjects included one subject selected randomly from 3 classmates born at term with same sex and ethnicity.

†Effect size for human milk (maximum amount or as specified) vs none or limited human milk. Mean (SD or CI if available) scores after adjustment for covariates and confounders:

Pinelli et al. [29]. Dichotomous groups based on 80% HM intake as cut point: No significant difference in MDI 92 (15) vs. 91 (12) or PDI 78 (15, SD) vs. 77 (14).

Furman et al. [30]. No significant difference in MDI 85 +/−21 vs. 80 +/−16 or PDI 76 +/−16 vs. 80 +/−16.

Tanaka et al. [31]. Dichotomous groups based on 80% HM intake as cut point: higher raw score for sequential 106.7 (14.5) vs. 94.7 (11.6) but not simultaneous or composite mental processing. No adjusted scores.

Horwood et al. [32]. Higher mean verbal IQ (102.1 vs 96.1 $p < 0.05$) and performance IQ (103.3 vs. 99.6, $p > 0.15$) after >8 m HM.

Smith et al. [33]. Significantly different in visual motor skills only: WRAVMA drawing 97.7 (14.6) vs. 90.6 (13.5), 95% CI = 1.0-9.2; and K-ABC triangle completion 10.6 (3.0) vs. 9.1 (2.5), 95% CI = 0.1-1.7. No HM effect from increased HM duration (>4 m, in infants with abnormal cranial ultrasound or <28 weeks gestation).

Johnson et al. [34]. Regardless of HM intake, significantly lower composite scores in extremely preterm children without serious neurosensory or cognitive impairment for reading 91 (13.4) vs. 98.7 (11.5); for mathematics 84.0 (15.6) vs. 98.8 (14.8). Data analyzed for children with and without serious functional or cognitive impairment while attending mainstream or special schools. Multivariate model show breast milk is one of the independent predictors of reading scores but not mathematic scores at 11 years. Other independent predictors included BSID-II MDI, and head circumference at 30 m, and perinatal and social factors. All independent predictors accounted for 31% of the variance for reading scores at 11 years.

Vohr et al. [35]. MDI and PDI in the highest 3 quintiles of human milk groups were higher ($p < 0.05$) than no human milk group. Mean values for highest vs. no human milk for MDI 89.7 vs. 76.5, and for PDI 90.2 vs 78.4. HM intake as a continuous measure show that each 10 mL/kg/d increase in human milk ingestion, the estimated increase of 0.59 points in MDI and 0.56 points in PDI.

Table 3 Human milk (HM) and developmental outcome in very low birth weight (VLBW) infants: perinatal, postnatal, social and environmental data*

Reference	Perinatal/postnatal factors	Social and environmental factors
Pinelli et al. [29]	Type of delivery	Maternal and paternal age, education and occupation, 1 or 2 parent home, social classes I-V (Hollingshead index)
Furman et al. [30]	Delivery at perinatal center, antenatal steroid and cesarean section. Apnea, sepsis, jaundice, necrotizing enterocolitis, chronic lung disease, cranial ultrasound abnormalities.	Maternal education and ethnicity, and marital status
Tanaka et al. [31]	Chronic lung disease, cranial ultrasound, necrotizing entercolitis. Intrauterine growth retardation	Maternal age and education
Horwood et al. [32]	Sex, multiple births, birth weight, gestational age, intrauterine growth retardation, 5 min Apgar score	Maternal age, education and smoking, 1 or 2 parents, family income, child ethnicity, birth order
Smith et al. [33]	Length of hospital stay	Maternal age, verbal ability, education, cigarette smoking and marital status, Home observation for measurement of the environment inventory – short version, annual household income, gender, parity
Johnson et al. [34]	Birth weight, gestation, antenatal steroid, premature rupture of membranes, vaginal breech delivery, chorioamnionitis, admission temperature <35°C, CRIB score, abnormal last cranial ultrasound, necrotizing entercolitis, postnatal steroid, duration of NICU admission. Neurodevelopmental assessment results at 30 m and 6 y	Socioeconomic (UK National Statistics Socio-Economic classification), maternal age, race and highest education.
Vohr et al. [35]	Gestation, gender, sepsis, intraventricular hemorrhage grade 3 to 4, periventricular leukomalacia, oxygen need at 36 weeks, necrotizing enterocolitis, and weight <10th percentile at 18 months.	Maternal age and education, marital and health insurance status, race, and income.

*The variables entered into the final model to determine the independent effect of HM feeding were varied and not always fully described. Some investigators [30] used composite scores to minimize the number of variables entered into data analysis and no specific modeling was performed by other investigators [31].

meet 2 or 3 parameters (Table 5). Lack of documentation for a methodological parameter or the use of surrogate markers negatively affected the quality of many studies. Consistency of the effect is variable with the advantage of feeding HM in adjusted neurodevelopment or educational attainment test scores in 4 of the 7 reports [32-35]. Three of these studies [32-34] of VLBW children at 6 to 11 years of age showed an advantage from HM with selected subtests rather than overall test scores. Dose effect of HM intake was reported with 2 studies [32,35]. Directness of the outcome was supported with the use of age-appropriate standardized tests but suffered from secondary analysis of other studies, incomplete sampling, poor selection of control group, and use of surrogate markers.

Discussion

For infants born at term, the benefits of HM on neurodevelopment and cognitive function may be limited according to reports on studies that adequately controlled for maternal intelligence and other social and environmental factors [8-10]. HM may provide greater benefit for the preterm infants when there is an added need for specific nutrients and trophic effects. Meta-analysis of earlier studies with larger preterm infants supported this assumption [7]. However, the meta-analysis did not attempt to evaluate each study's methods or interpret results on the basis of the quality of the investigation. As a result, the pooled effect estimates obtained reflect the average of a heterogeneous group of studies.

Our systematic review on the independent effect of HM feeding on neurodevelopment outcome, taking into account the additional confounders unique to VLBW children, provided a better understanding of the strengths and limitations of each study. It appears that significant limitations exist with each study. These limitations may involve study design or the quality of the study in the fulfillment of non-nutritional and nutritional methodological criteria, which can affect the applicability of the outcome data. The inconsistent effect on neurodevelopment test scores and variable advantage in different subtest scores when assessed at school ages also contributed to the difficulty in interpreting the HM effect on neurodevelopment outcome.

Table 4 Human milk (HM) and developmental outcome in very low birth weight (VLBW) infants: nutritive parameters

Reference	Pinelli et al. [29]	Furman et al. [30]	Tanaka et al. [31]	Horwood et al. [32]	Smith et al. [33]	Johnson et al. [34]	Vohr et al. [35]
HM feeding definition. None specified whether donor milk was used.	Maternal milk intake as % of total fluid intake and by duration	Maternal milk at 0, 1–24, 25–49, >50 mL/kg/d	Maternal milk	Any maternal milk from birth	Expressed maternal milk without or with progression to direct breastfeeding	Any breast milk	Maternal milk intake by quintiles
HM feeding duration	Continuous measures till 12 m corrected	Up to 4w	HM group 72 +/− 45.2 (SD)d, Formula group received HM for 59 +/−32.1d	None, <4 m, 4-7 m, 8 + m	<1w, 1-4w, 1-3 m, 4–6 m, >6 m	Neonatal period	Up to 120d.
HM fortification	Milk based powder if intake <180-200 mL/kg/d (21% of infants)	Milk based powder or concentrated PTF	NS	NS	NS	NS	NS
HM feeding data source	Maternal questionnaires, 24 h expressed milk volume, test weighing one feeding each 3 m	*	*	Maternal recall and child health record	Maternal recall	*	Database from hospital records
Infant formula data	20 exclusively PTF	PTF	NS	NS, n = 76	NS	NS	PTF, 180 (23%) exclusively FF

PTF - Preterm formula in hospital, FF - formula fed, NS - not stated or specified.
*Not stated and presumably were obtained from review of hospital record.

Table 5 Studies of human milk (HM) feeding and developmental outcome in very low birth weight (VLBW) infants: meeting criteria for methodological quality

Reference	Pinelli et al. [29]	Furman et al. [30]	Tanaka et al. [31]	Horwood et al. [32]	Smith et al. [33]	Johnson et al. [34]	Vohr et al. [35]
As primary outcome of original study*	-	+	-	-	-	-	-
VLBW only	+	+	+	+	+	+†	+†
A priori power calculation	-	-	-	-	-	-	-
Baseline adjustment for SGA	-	-	-	+	-	-	-
Postnatal complication	-	+	-	+	-	+	+
Maternal intelligence	+/–	+/–	+/–	+/–	+/–	+/–	+/–
Social class or Socioeconomic status	+	-	-	+/–	+/–	+	+/–
Child rearing environment	+/–	+/–	-	+/–	+	-	+/–
Observers blinded to feeding protocol	+	+	-	-	-	+	-
Effect size after adjustment	+	+	NA	+‡	+‡	+‡	+‡
Human milk definition	+	+	+	+	+	+	+
Human milk duration	+	+	+	+	+	+	+
Human milk fortification	+	+	-	-	-	-	-
Human milk feeding data source	+	-	-	+	+	-	+
Formula type	+	+	-	-	-	-	+

SGA = Small for gestational age, NA = not available.
+Met methodological criterion.
-Did not meet methodological criterion; or not stated or not specified in the publication.
+/–Use surrogates such as income for socioeconomic status, maternal education for maternal intelligence, marital status or one or two parent family for child rearing environment.
*All studies were observational and most were secondary analysis of study cohort from other studies.
†Only children with birth weights <1000 g.
‡Limited consistency of neurodevelopment outcome. Human milk showed variable advantage in some test scores [32-35].

Majority of publications determining the effect of breast milk feeding on neurodevelopment in VLBW children is based on observational data from other studies. The source of VLBW population for example those with postnatal complications [32,33]; and intervention performed in the original studies, such as counseling to improve breastfeeding [29] or glutamine supplementation in parenteral nutrition [35] potentially may influence the outcome measures by indirect means. Furthermore, secondary analysis of data generates more questions for hypothesis testing rather than providing a definitive cause and effect of HM feeding.

Quality of the studies, as indicated by adherence to methodological criteria that minimizes risks for bias, is generally low. Numerous factors other than nutritional intake have been identified as confounding variables in relation to child development [40] and may have origin even before birth. Many preterm infants experienced variable adverse growth in utero but not all studies reported the rate of IUGR or SGA. In preterm infants, SGA is an independent predictor of severe cognitive deficit [17]. For extremely preterm infants with VLBW, SGA as an indicator of IUGR has an odds ratio of 3.91 for increased risk of death or neurodevelopment impairment [41]. Some studies of children born preterm have reported IUGR or SGA rates from 34% [26] to as high as 60% [31], and not all studies reported whether or how the data analysis accounted for IUGR or SGA. Extra uterine growth retardation also occurs frequently in VLBW infants and may be another marker for neurodevelopment delay [42]. A small head circumference at 8 months corrected age is an independent marker of neurodevelopment and cognitive impairment, independent of the type of feeding [43,44]. Multiple births are at risk for preterm delivery and discrepant in utero growth resulting in VLBW and IUGR, and discordant neurodevelopment outcome has been reported for VLBW twins [45]. Not all studies have accounted for multiple births and some studies have restricted the study population to singletons [29,30]. In addition, the effect on breast milk production in mothers with both twins admitted to a neonatal intensive care unit is not well-defined.

For extremely preterm infants, a difference in 100 g in birth weight or one week of gestation can have major impact on perinatal and postnatal complications [46] that can directly or indirectly impact neurodevelopment outcome and confound the effects of HM. Thus it is important not to generalize the neurodevelopment effect of HM from preterm infants with higher birth weight and gestation, since they have a relatively longer period of development in utero and less serious postnatal complications. The earlier reports included large numbers of preterm children with higher birth weights than VLBW which could reflect better intrauterine growth at the same gestation or had IUGR at a more advanced gestation [11,17-28]. Interaction between gender and diet has been reported in some studies, with males showing more benefit from nutritional intervention [11,26,34]. One study of children with birth weight <1000 g reported that being male has a small negative predictive effect on reading but not mathematics attainment at 11 years [34].

The age at follow-up varies, although few studies [32-34] assessed cognitive scores at school ages which are considered as much better predictors of adult scores. One report of significant positive effect of HM on neurodevelopment at 30 months corrected age was generated from secondary analysis of a subpopulation from another project [35]. In the same cohort, there was a significant increase in MDI scores by 2.7 points in the HM group and a trend to lower PDI scores by 2.3 points in the non-HM group since an earlier assessment at 18 months [36]. This drift in test scores could bias the outcome that showed HM group has higher MDI and PDI scores at 30 months [35]. The advantage associated with HM feeding appears to diminish with older children, as the improvement in test scores is limited to selected and different subtests [32-34]. It is possible that the effect of HM may be less important as genetic and environmental factors play bigger roles at school age. In some cohorts with follow up at older ages, the validity of mother's milk effect on IQ at adolescence is questionable since the data is based on <10% of the subjects from the original cohort [22].

Age-appropriate developmental or cognitive tests standardized to normal age matched children allow the use of a control group from the same VLBW cohort [29-33,35]. Only one study used selected classmates in the same educational setting and born at term with same sex and ethnicity and tested during the same period to minimize any secular drift in test scores over time [34]. Selection of a control group based on the volume of human milk ingested heavily influences the outcome. Any HM effect on neurodevelopment may be difficult to detect when the data analysis is dichotomized using a large volume of HM consumption as a cutoff point [29,31]. Not all reports indicated whether the testers were blind to feeding status thus contributing to the risk for bias.

The extensive numbers of potential confounders of neurodevelopment and variable exclusion criteria based on the type and extent of postnatal complications support the need for appropriate statistical modeling and large sample size to provide meaningful interpretation of the neurodevelopment effect of feeding HM. One study employed a composite neonatal risk score and a composite socioeconomic score to minimize the number of independent variables and to avoid multi-colinearity of variables in statistical modeling [30]. However, there is

no uniform approach to statistical modeling and none of the studies had stated a priori power calculation to measure the neurodevelopment effect of HM.

Both breastfeeding and neurodevelopment outcome are confounded by maternal intelligence, social and socioeconomic status, and child rearing environment, and possibly from intangible psychobiology of maternal behavior and mother-infant relationship [10,47,48]. Mothers elected to provide HM and breastfeeding are often highly motivated and possibly more health conscious and more likely to stimulate their infants thus contributing to self-selection bias. None of the studies reviewed have formal assessment of maternal intelligence and few studies specifically assessed the other confounders. The use of surrogate for these critical independent determinants of neurodevelopment outcome limits the validity of HM effect.

It is important to recognize that VLBW children with no neurological impairment and fed HM achieved normal or low normal scores on standardized tests of neurodevelopment or cognitive function. The VLBW children fed a lower amount of HM or fed infant formula have lower test scores. One should consider feeding HM as protective rather than providing an added advantage to neurodevelopment.

Nutritive factors are important in the evaluation of the role of HM in neurodevelopment and cognitive function of VLBW children. In a cohort of children born preterm and including those with VLBW enrolled in a quasi-randomized study of supplementing OMM with DM or infant formulas, preliminary data at 7.5 to 8 years from the first 300 children out of 926 subjects showed that the children who received OMM had IQ scores in the normal range. However, those who exclusively received formula have overall test scores that were 8.3 points lower [11]. In a subsequent report of 377 subjects from several subsets in the same cohort of infants, those who received infant formula (nutrient enriched versus regular formula), and either exclusively or as a supplement to OMM, experienced a beneficial effect to neurodevelopment that appeared primarily to be related to the use of the nutrient enriched formula [26]. Unfortunately, no quantifiable data specific to VLBW children was presented in either report.

In the studies reviewed, the contents of nutrients in infant formulas and HM fortifiers are in much lower quantity and lack additional nutrients such as LCPUFA when compared to the current formulations. If better nutrient profile is critical to neurodevelopment, then it is possible that all VLBW infants could benefit with the use of newer and better fortified formulas and HM fortifiers.

The timing, volume and duration of HM consumed could be important for neurodevelopment. Significant advantage in neurodevelopment effect appears to occur even after a brief period of consumption of OMM during initial hospitalization as >88% of infants in one study [11] and 77% of infants in another study [35] received no HM by the time of hospital discharge. Unfortunately, these details are extremely limited in long-term follow-up studies.

A dose effect of OMM also may be present. Preterm infants whose mothers intended to breastfeed but could not provide any breast milk, performed at the level of exclusively formula fed children on cognitive testing at 7.5 to 8 years [11], and a dose effect was also demonstrated when the analysis was performed with HM intake as a continuum [35]. One long-term VLBW population cohort at 7 to 8 years of age showed a significant benefit of prolonged breastfeeding and reached a mean of 6 points advantage in verbal IQ after receiving OMM for 8 months or more [32]. However, no additional benefit beyond 4 months of breastfeeding was reported in another study of VLBW children at 6 years [33]. A modest independent beneficial effect of feeding OMM during the neonatal period on higher reading but not mathematics attainment at 11 years was also reported [34].

It is important to determine whether OMM or DM was used in the assessment of HM's effect on neurodevelopment. Fresh OMM contains many components that may provide trophic actions which can directly or indirectly influence the growth and development of the nervous system but are inactivated or destroyed during processing of the DM [6,49]. The use of DM alone or as supplement to OMM resulted in poorer growth and neurodevelopment [23]. It appears that VLBW children in all studies reviewed were provided with OMM; none reported that any DM was used. The use of HM fortifiers was reported in two studies [29,30]. In another study, the use of human milk fortifier was not specified but was likely provided since the cohorts were born in the era when HM fortification was the standard of care [35].

It is possible that space limitation imposed by the journals may have precluded detail description by the investigators, although it is unlikely to eliminate all significant limitations that exist with each study. Limitations to our study included at least the following: we evaluated original peer reviewed studies only in English and did not pursue details from published abstracts or the authors. However, abstracts have not undergone the same rigor in review process as the full publication and are unlikely to have sufficient data to allow meaningful systematic review of the data. Information from the authors is unlikely to resolve the many methodological concerns in the studies reviewed, and additional information would be unlikely to alter the overall conclusions. Our study also excluded 13 reports [11,17-28] because of insufficient data to delineate the effect of HM feeding on

neurodevelopment outcome in children with VLBW. The data from these reports included many children with higher birth weights and thus at lower risk of neurodevelopment deficit than VLBW children. Furthermore, reports based on subsets of original cohort make it difficult to interpret the significance of the finding in the context of the whole population and should be considered as hypothesis generating rather than definitive data on the neurodevelopment benefit of HM.

In addition, the two population observational studies [17,28] were confounded by the HM fed cohorts having significantly greater birth weights and gestations compared to non HM group. In any case, the neurodevelopment scores in those fed predominantly OMM [11,17-28] are generally within the normal ranges and consistent with reports that include only children with VLBW [29-35].

Conclusions

There is no randomized clinical trial comparing the neurodevelopment outcome of HM versus formula or minimal HM feeding that included only children with VLBW. Studies to date have significant methodological limitations although limited data suggest a possible protective effect on neurodevelopment from feeding OMM for a short period after birth and a possible dose effect on the volume and duration of feeding OMM. If better overall nutrient profile is more important for optimal neurodevelopment, it is theoretically possible that the use of current formulations of HM fortifier and preterm infant formulas could improve the neurodevelopment outcome of all VLBW children. Thus, the role of maternal milk in neurodevelopment and cognitive function of VLBW infants needs to be reassessed with high quality studies in the context of current formulations of HM fortifier and preterm formula. With increasing use of DM to the exclusion of preterm formula in NICU, a separate assessment of the role of DM in neurodevelopment is needed in view of the numerous differences between OMM and DM.

Abbreviations

DM: Donor milk; HM: Human milk; IQ: Intelligence quotient; IUGR: Intrauterine growth retardation; LCPUFA: Long chain polyunsaturated fatty acids; OMM: Own mother's milk; SGA: Small for gestation; VLBW: Very low birth weight.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

WK conceptualized and designed the study, contributed to data acquisition and interpretation, and drafted the initial manuscript. ST and SM contributed to study design, data acquisition and interpretation, and review of manuscript. SM was also responsible for the literature search. RS contributed to study design, data interpretation, and review of manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors thank Alicia LaDuke, BS, MLIS, for assistance in the initial literature search; and Lawrence Ku, MD and Monica Salari BS, for assistance in the preliminary screening and review of the publications.

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Folate, Vitamin B12 and Homocysteine status in the post-folic acid fortification era in different subgroups of the Brazilian population attended to at a public health care center

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Abstract

Background: Folate and vitamin B12 are essential nutrients, whose deficiencies are considerable public health problems worldwide, affecting all age groups. Low levels of these vitamins have been associated with high concentrations of homocysteine (Hcy) and can lead to health complications. Several genetic polymorphisms affect the metabolism of these vitamins. The aims of this study were to assess folate, vitamin B12 and homocysteine status in distinct Brazilian individuals after the initiation of folic acid fortification by Brazilian authorities and to investigate the effects of RFC1 A80G, GCPII C1561T and MTHFR C677T polymorphisms on folate, vitamin B12 and Hcy levels in these populations.

Methods: A total of 719 individuals including the elderly, children, as well as pregnant and lactating women were recruited from our health care center. Folate, vitamin B12 and Hcy levels were measured by conventional methods. Genotype analyses of RFC1 A80G, GCPII C1561T and MTHFR C677T polymorphisms were performed by PCR-RFLP.

Results: The overall prevalence of folate and vitamin B12 deficiencies were 0.3% and 4.9%, respectively. Folate deficiency was observed only in the elderly (0.4%) and pregnant women (0.3%), whereas vitamin B12 deficiency was observed mainly in pregnant women (7.9%) and the elderly (4.2%). Plasma Hcy concentrations were significantly higher in the elderly (33.6%). Pregnant women carrying the MTHFR 677TT genotype showed lower serum folate levels ($p = 0.042$) and higher Hcy levels ($p = 0.003$). RFC1 A80G and GCPII C1561T polymorphisms did not affect folate and Hcy levels in the study group. After a multivariate analysis, Hcy levels were predicted by variables such as folate, vitamin B12, gender, age and RFC1 A80G polymorphism, according to the groups studied.

Conclusion: Our results suggest that folate deficiency is practically nonexistent in the post-folic acid fortification era in the subgroups evaluated. However, screening for vitamin B12 deficiency may be particularly relevant in our population, especially in the elderly.

Keywords: Folate, Vitamin B12, Homocysteine, Folic acid fortification, Reduced folate carrier, Glutamate carboxypeptidase II, Methylenetetrahydrofolate reductase

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Background

Folate and vitamin B12 are essential nutrients, and their deficiencies represent public health problems worldwide, affecting all age groups and leading to complications such as anemia, birth defects and neurological disorders [1,2]. Low concentrations of folate and vitamin B12 are also associated with high homocysteine (Hcy) levels, considered a risk factor for cardiovascular disease, cognitive decline and adverse pregnancy outcomes [2-4].

Folate deficiency can be caused by inadequate dietary intake, medications (methotrexate and anticonvulsants), alcoholism and conditions associated with increased cell turnover. In addition, a few genetic polymorphisms have been shown to influence folate levels. On the other hand, vitamin B12 deficiency results mainly from gastrointestinal conditions leading to vitamin B12 malabsorption, and less frequently from intestinal parasitosis and genetic polymorphisms [5].

Genetic polymorphisms such as reduced folate carrier (RFC1) A80G and glutamate carboxypeptidase II (GCPII) C1561T have also been shown to impair folate transport and absorption, respectively, thus affecting the bioavailability of the dietary folate [6,7]. The methylenetetrahydrofolate reductase (MTHFR) C677T polymorphism is associated with elevated Hcy levels and reduced folate and vitamin B12 levels [8,9].

Fortification of white flour with folic acid is mandatory in several countries and has proved to be a successful public health intervention. Although the major purpose of folic acid fortification is to reduce the occurrence of neural tube defects (NTD) during pregnancy, an additional benefit is a potential protection against chronic diseases, through its association with lower Hcy levels [10,11]. In Brazil, this strategy has been applied since 2004 by the National Health Surveillance Agency, ANVISA [12]. Fortification was implemented to provide an adequate intake of folate mainly for vulnerable populations, such as pregnant women, lactating women, children and the elderly [13,14].

In this context, the aims of this study were: (1) to assess folate and vitamin B12 status, as well as the frequency of elevated Hcy levels among the elderly, children, pregnant women and lactating women consecutively assisted at a public health care center after folic acid fortification; and (2) to investigate the effects of RFC1 A80G, GCPII C1561T and MTHFR C677 polymorphisms on folate, vitamin B12 and Hcy levels in the same study groups.

Methods

Study population

Seven hundred and nineteen participants were enrolled from April 2006 to May 2007, and divided into the following subgroups: 262 elderly individuals (≥60 year old), 106 children (≤6 year old), 291 pregnant women and 60 lactating women. The study group was chosen based on their vulnerability to vitamin deficiencies. We excluded individuals who had hypothyroidism, renal and hepatic disease, and those using agents that affect vitamin B metabolism such as methotrexate and anticonvulsants. In addition, individuals with acute illnesses were also excluded. Information regarding age, gender, height, weight, medical history, smoking, use of multivitamin supplements and gestational stage were collected.

The study groups were composed of individuals consecutively attended to at a public health care center in the northern area of the city of Campinas, Brazil, during visits to primary care physicians. Our recruitment goal was 250 individuals from each group, but due to the exclusion criteria and rate of refusal to participate this number could not be reached. Thus, all eligible participants, who were in accordance with the study protocol and who agreed to participate in the study, provided a written informed consent. For children, the written informed consent was obtained from mothers or guardians. The study protocol was approved by the Ethics Committee of the University of Campinas.

Blood collection

Fasting blood samples were obtained from all participants in tubes without anticoagulant, for measurement of folate and vitamin B12, and in tubes containing EDTA to measure Hcy levels and for the genetic analyses (RFC1 A80G, GCPII C1561T and MTHFR C677T). Serum and plasma samples were processed within 3 hours of collection and stored below −80°C until analyses.

Laboratory assays

Serum levels of folate and vitamin B12 were determined using chemiluminescence immunoassays (Elecsys/Roche Diagnostics, Mannheim, Germany). Hcy in plasma was assessed by high-performance liquid chromatography (HPLC) with fluorescence detection [15]. Genomic DNA was isolated from whole blood by standard methods. The analyses of RFC1 A80G, GCPII C1561T and MTHFR C677T polymorphisms were accomplished by PCR-RFLP. The amplified fragments were digested by restriction enzymes (*HinfI*, *HhaI* and *AccI*, respectively), as previously described [6,16,17].

We separated folate, vitamin B12 and Hcy levels into categories, based on the reference values used in our service. For folate levels, categories were: <4 ng/mL, ≥4 ng/mL; for vitamin B12: <200 pg/mL, 200 – 300 pg/mL, >300 pg/mL; and for Hcy levels, these categories were: <15 μmol/L, ≥15 μmol/L. Individuals with levels of folate below 4 ng/mL and vitamin B12 below 200 pg/mL were categorized as having deficiency. Vitamin B12 levels between 200 – 300 pg/mL were considered as marginal status [5,14].

Levels of Hcy ≥15 μmol/L were considered elevated [18]. It is noteworthy that the reference values used to describe folate and vitamin B12 deficiencies are similar to those cited by WHO [19].

Statistical analysis

Data are expressed as median, percentiles 25 and 75, absolute number and percentages. Medians were compared using the Mann–Whitney test for 2-group comparisons and the Kruskal-Wallis test for 3-group comparisons. The Hardy-Weinberg equilibrium test was performed for all genotypes using the chi-square test. Multiple linear regression analysis with stepwise criteria was performed for Hcy as the dependent variable and folate, vitamin B12, polymorphisms, age, gender, body mass index, hypertension, diabetes and smoking as the independent variables. This analysis was adjusted for gestational stage in pregnant women. Statistical tests were performed using SPSS software for Windows version 17.0 (SPSS Inc, Chicago, IL, USA) and SAS System for Windows version 9.2 (SAS Institute Inc, Cary, NC, USA) with the level of significance set at $p < 0.05$.

Results

During the enrollment period, all individuals who assisted at a public health care center were invited to participate in the study. A total of 719 individuals were included in our study. Among them, 40.5% were pregnant women, 36.5% were elderly, 14.7% were children and 8.3% were lactating women. The demographic characteristics of the studied population are summarized in Table 1.

Folate, vitamin B12 and homocysteine levels

Among the distinct study groups, children had higher vitamin B12 levels, the elderly had higher Hcy levels and lactating women had lower folate levels (Table 2).

The overall frequency of folate and vitamin B12 deficiencies were estimated to be 0.3% and 4.9% respectively (Table 3). Interestingly, we observed no folate or vitamin B12 deficiencies in the children included in our study, and only 1% presented marginal status of vitamin B12. Folate deficiency could be identified in 0.4% of elderly and 0.3% of pregnant women. None of the lactating women showed folate deficiency. The pregnant women group was the one which presented a higher frequency of vitamin B12 deficiency (7.9%), followed by the elderly (4.2%) and lactating women (1.9%). Marginal status of vitamin B12 was observed in 14.5% of the elderly, 33.7% of pregnant women and 7.4% of lactating women. The frequency of elevated Hcy levels was observed mainly in the elderly (33.6%). Among pregnant and lactating women, the frequencies of elevated Hcy levels were 0.7 and 5.0%, respectively.

Effects of polymorphisms RFC1 A80G, GCPII C1561T and MTHFR C677T in the studied population

In order to assess the effects of polymorphisms on folate, vitamin B12 and Hcy levels we compared the median of these parameters in individuals with different genotypes (Table 4). Folate, vitamin B12 and Hcy concentrations were not affected by the RFC1 A80G, GCPII C1561T and MTHFR C677T polymorphisms in the elderly, children and lactating women. Pregnant women carrying the MTHFR 677TT genotype showed lower

Table 1 Demographic characteristics of the study population

	Elderly (N = 262)	Children (N = 106)	Pregnant women (N = 291)	Lactating women (N = 60)
Age (y)	67 (60 – 91)	3 (0.5 - 6)	26 (14–43)	26.5 (14–40)
Gender (male)	114	54	-	-
BMI (mean ± SD)	26.7 (23.9, 29.9)	15.9 (14.8, 17.9)	26.4 (23.4, 30.7)	23.4 (21.5, 26.4)
Supplementation[1] [n (%)]	43 (16.4)	22 (16.6)	138 (47.4)	10 (16.6)
Smoking [n (%)]	20 (7.6)	-	27 (9.3)	8 (13.3)
Hypertension[2] [n (%)]	158 (60.3)	-	42 (14.4)	5 (8.3)
Diabetes [n (%)]	65 (24.8)	3 (2.8)	12 (4.1)	1 (1.6)
Dyslipidemia [n (%)]	65 (24.8)	-	2 (0.7)	-
Gestational stage[3] n (%)]				
First trimester	-	-	73 (27.8)	-
Second trimester	-	-	125 (47.5)	-
Third trimester	-	-	65 (24.7)	-

Age is expressed as median and range in parentheses. Body Mass Index (BMI) is expressed as median and percentiles (25 and 75) in parentheses. Gender is expressed as number of individuals.
[1]Users of multivitamins or folic acid supplements.
[2]Hypertension definition: blood pressure exceeding 140 over 90 mmHg.
[3]Gestational stage was not available for 28 pregnant women.

Table 2 Folate, vitamin B12, and Hcy levels in different study groups

Groups	Folate (ng/mL)	Vitamin B12 (pg/mL)	Hcy[1] (μmol/L)
Elderly	11.2(8.7, 13.6)	443(333, 620.2)	13.5(11.1, 17.1)
	N = 262	N = 262	N = 262
Children	12.4(9.4, 14.6)	853.0(611, 1188)	6.2(5.2, 7.3)
	N = 103	N = 103	N = 105
Pregnant women	10.7(8.3, 14.1)	325.0(257, 424)	6.4(5.3, 7.5)
	N = 291	N = 291	N = 291
Lactating women	9.8(7.6, 12.2)	523.0(415.7, 641.5)	9.2(7.6, 10.8)
	N = 54	N = 54	N = 60
***P*[2]**	0.003	p < 0.001	p < 0.001

All values are expressed as median and percentiles (25 and 75).
[1]Hcy, homocysteine.
[2]Kruskal-Wallis test.

serum folate levels (p = 0.042) and higher Hcy levels (p = 0.003).

Homozygosity for the GCPII C1561T polymorphism was not found in our population.

Genotype distributions of RFC1 A80G, GCPII C1561T and MTHFR C677T polymorphisms were in Hardy-Weinberg equilibrium. Table 5 summarizes the frequencies of genotypes for each polymorphism studied.

Impact of clinical and laboratory parameters on Hcy levels

We next evaluated the impact of clinical and laboratorial parameters on Hcy levels (Table 6). Using a multiple linear regression analysis with stepwise criteria, the variables independently associated with Hcy levels were: folate, vitamin B12, gender, age and RFC1 A80G polymorphism (genotype AA) in the elderly; vitamin B12 in children; and folate in pregnant women. None of the variables evaluated showed any impact on Hcy levels in lactating women.

Discussion

The present study suggests that fortification of flour with folic acid has been effective, as folate deficiency was practically nonexistent (0.3%), whereas vitamin B12 deficiency was present in 4.9% of the studied group. A previous study performed with Brazilian adults reported that folate deficiency was not detected and vitamin B12 deficiency was over 6% [20]. Similarly, folate deficiency is practically nonexistent and vitamin B12 deficiency is approximately 5% in the Canadian population [11]. In developed countries, folate deficiency is uncommon; however this deficiency can still be observed in some developing countries [21]. In addition, the prevalence of folate and vitamin B12 deficiencies vary between different studies due to differences in the assays employed [13].

We believe that our results represent the profile of these vitamins in the investigated group, as the measurements were performed in 719 individuals of various ages under several clinical conditions using laboratory methods considered suitable for analysis. Furthermore, the subgroup-specific evaluation is also important, enabling the identification of individuals at an increased risk of developing vitamin deficiencies. These individuals should be considered for specific prophylactic measures, as a problem in clinical practice is that sometimes the deficiencies are identified only when complications, such as anemia, NTD, and neurological disorders, have already occurred. Thus, prevention of folate and vitamin B12 deficiencies becomes a major challenge for health worldwide.

As described previously, folate deficiency may occur at any age, mostly in individuals ingesting a poor diet or suffering from intestinal malabsorption [22]. Moreover, vitamin B12 levels frequently decrease with age due to malabsorption of vitamins from food, which is more common in the elderly [11]. Approximately 10% of the elderly are estimated to present reduced levels of vitamin B12, with this prevalence increasing approximately 5% at the age of 65 years and to 20% at the age of

Table 3 Folate, vitamin B12 and Hcy status according to cut-off values

	Cut-off values	All	Elderly	Children	Pregnant women	Lactating women
		N, %	N, %	N, %	N, %	N, %
Folate						
	<4 ng/mL	2, 0.3	1, 0.4	-	1, 0.3	-
	≥4 ng/mL	708, 99.7	261, 99.6	103, 100	290, 99.7	54, 100
Vitamin B12						
	<200 pg/mL	35, 4.9	11, 4.2	-	23, 7.9	1, 1.9
	200 - 300 pg/mL	141, 19.9	38, 14.5	1, 1.0	98, 33.7	4, 7.4
	>300 pg/mL	534, 75.2	213, 81.3	102, 99.0	170, 58.4	49, 90.7
Homocysteine						
	<15 μmol/L	625, 87.0	174, 66.4	105, 100	289, 99.3	57, 95.0
	≥15 μmol/L	93, 13.0	88, 33.6	-	2, 0.7	3, 5.0

Table 4 Folate, vitamin B12 and Hcy levels according to different polymorphisms in the study groups

	GROUPS	RFC1 A80G[3]			P^1	GCPII C1651T[4]		P^2	MTHFR C677T[5]			P^1
		AA	AG	GG		CC	CT		CC	CT	TT	
Folate (ng/mL)												
	Elderly	11.2 (8.4,13.2)	11.2 (8.8, 13.9)	11 (8.6, 13.2)	0.840	11 (8.5, 13.7)	12.2 (11.1, 13)	0.252	11.2 (8.6, 13.3)	11 (8.8, 14.2)	10.2 (7.3, 12.4)	0.158
		N = 49	N = 125	N = 86		N = 241	N = 14		N = 103	N = 118	N = 36	
	Children	12.5 (8.2,16.1)	12.5 (9.4, 15.1)	12.5 (10.2, 14.1)	0.863	12 (9.4, 15.1)	11.5 (8.2, 18.3)	0.932	12.0 (9.3, 14.4)	12.5 (9.2, 14.9)	13.8 (9.6, 15.6)	0.441
		N = 20	N = 60	N = 20		N = 79	N = 6		N = 51	N = 32	N = 11	
	Pregnant women	9.8 (8.0,13.6)	11 (8.4, 15)	10.9 (8.5, 14.2)	0.346	10.8 (8.2, 14.3)	11.5 (8.5, 13.3)	0.927	11.2 (9, 15)	10.8 (7.7, 14)	8.8 (7.3, 11.6)	0.042
		N = 74	N = 129	N = 83		N = 250	N = 21		N = 139	N = 113	N = 26	
	Lactating women	10.6 (9.4,12.7)	9.5 (7, 12.1)	9.5 (7.3, 11.7)	0.300	9.8 (7.7, 12)	12.6 (7.5, 14.3)	0.429	10 (9.1, 12.5)	9.3 (7.1, 13.1)	9.7 (6.8, 10.8)	0.723
		N = 12	N = 28	N = 14		N = 49	N = 3		N = 27	N = 21	N = 4	
Vitamin B12 (pg/mL)												
	Elderly	418 (310, 687.5)	446 (341, 623)	442 (337.5, 584.7)	0.650	444 (333, 616.5)	373.5 (288.2, 647.7)	0.317	463 (345, 594)	431 (330.5, 627.5)	391.5 (276.2, 577.5)	0.443
		N = 49	N = 125	N = 86		N = 241	N = 14		N = 103	N = 118	N = 36	
	Children	827 (638.5, 1047.5)	874 (650.2, 1200)	770.5 (513, 1207.2)	0.840	901 (611, 1204)	772.5 (518.7, 1000.5)	0.359	790 (572, 1109)	918.5 (660.2, 1206)	756 (553, 1049)	0.333
		N = 20	N = 60	N = 20		N = 79	N = 6		N = 51	N = 32	N = 11	
	Pregnant women	341.5 (267.7, 462)	324 (253, 419)	319 (240, 412)	0.218	322 (253.7, 424)	324 (263.5, 399.5)	0.738	321 (249, 420)	334 (257.5, 425)	308.5 (236.2, 441.7)	0.703
		N = 74	N = 129	N = 83		N = 250	N = 21		N = 139	N = 113	N = 26	
	Lactating women	563 (470, 648)	467 (400.2, 540.7)	624 (408.7, 682)	0.114	488 (414, 622.5)	627 (576, 699)	0.094	488 (437, 627)	485 (377, 551.5)	617 (520, 712)	0.137
		N = 12	N = 28	N = 14		N = 49	N = 3		N = 27	N = 21	N = 4	
Homocysteine (μmol/L)												
	Elderly	14.1 (11.7, 17.9)	12.9 (10.7, 16.4)	13.8 (11.4, 17.2)	0.170	13.5 (11.2, 17.1)	13.3 (10.7, 18.4)	0.896	13.2 (10.9, 15.7)	13.3 (10.8, 17.2)	14.8 (12.1, 18.4)	0.087
		N = 49	N = 125	N = 86		N = 241	N = 14		N = 103	N = 118	N = 36	
	Children	6.0 (5.3, 6.7)	6.3 (5.1, 7.4)	6.7 (5.2, 7.4)	0.582	6.2 (5.2, 7.1)	5.9 (5.2, 8.4)	0.876	6.2 (5.4, 7.2)	5.8 (4.9, 7.5)	6.8 (6.5, 8)	0.099
		N = 20	N = 60	N = 20		N = 78	N = 6		N = 51	N = 32	N = 11	
	Pregnant women	5.9 (5.1, 7.6)	6.4 (5.4, 7.6)	6.3 (5.5, 7.5)	0.151	6.4 (5.4, 7.5)	6.2 (5.1, 7.8)	0.710	6.2 (5.4, 7.4)	6.3 (5.2, 7.3)	7.8 (6.3, 9.2)	0.003
		N = 74	N = 129	N = 83		N = 250	N = 21		N = 139	N = 113	N = 26	
	Lactating women	9.4 (8.2, 10.4)	9.7 (7.4, 11.5)	8.7 (7.8, 10.9)	0.929	9.4 (7.5, 10.8)	12.6 (8.9, 12.6)	0.193	9.4 (7.6, 10.7)	9.9 (8, 11.9)	7.8 (6.8, 11.4)	0.529
		N = 12	N = 28	N = 14		N = 49	N = 3		N = 27	N = 21	N = 4	

Values are expressed as median and percentiles (25 and 75).
[1]Kruskal-Wallis test.
[2]Mann-Whitney test.
[3]AA, wild type; AG, heterozygous; and GG homozygous mutant for the RFC1 A80G polymorphism.
[4]CC, wild type; and CT, heterozygous for the GCPII C1561T polymorphism.
[5]CC, wild type; CT, heterozygous; and TT, homozygous mutant for the MTHFR C677T polymorphism.

Table 5 Overall genotype frequencies of RFC1 A80G, GCPII C1561T and MTHFR C677T polymorphisms

Polymorphisms	Genotypes	Study groups	
		N	%
RFC1 A80G[1]			
	AA	158	22.3
	AG	343	48.4
	GG	207	29.3
GPCII C1561T[2]			
	CC	619	93.4
	CT	44	6.6
MTHFR C677T[3]			
	CC	326	47.3
	CT	286	41.5
	TT	77	11.2

[1]AA, wild type; GA, heterozygous; and GG homozygous mutant for the RFC1 A80G polymorphism.
[2]CC, wild type; and CT, heterozygous for the GCPII C1561T polymorphism.
[3]CC, wild type; CT, heterozygous; and TT, homozygous mutant for the MTHFR C677T polymorphism.

85 years [13,18]. In our study, we identified 0.4% and 4.2% of the elderly with folate and vitamin B12 deficiencies respectively, whereas 14.5% showed marginal levels of vitamin B12. Coussirat *et al.* evaluated 545 Brazilian elderly individuals, and detected 0.5% with folate deficiency, 5.5% with vitamin B12 deficiency, while 23.3% had marginal levels of vitamin B12. Xavier *et al.* showed that 7.2% of the elderly had a vitamin B12 deficiency [20,23].

Our results show a situation that can often go unnoticed in the elderly, which is a vitamin B12 deficiency.

Table 6 Predictors of Hcy levels in the elderly, children and pregnant women

Groups	Independent variables	β	R^2	*P*
Elderly				
	Folate	−4.594	0.0441	p < 0.001
	Vitamin B12	−0.044	0.0324	p < 0.001
	Gender (male)	52.785	0.1622	p < 0.001
	Age	3.219	0.0716	p < 0.001
	RFC1 (AG x AA)	−26.995	0.0192	0.011
	(GG x AA)	−11.736		
Children				
	Vitamin B12	−0.023	0.0782	0.011
Pregnant women				
	Folate	−4.556	0.0750	p < 0.001

Multiple linear regression analysis with stepwise criteria. Independent variables: age, gender (male versus female), smoking, hypertension, diabetes, dyslipidemia, BMI, polymorphisms (RFC1 A80G, GCPII C1561T e MTHFR C677T), folate and vitamin B12. In pregnant women, the analysis was adjusted for gestational stage.

Atrophy of the gastric mucosa, the presence of autoantibodies against intrinsic factors (often undiagnosed), or the presence of *H. pylori* may play a role as an etiological factor of vitamin B12 deficiency in this age group, because it results in malabsorption of vitamin B12.

Although almost all of these individuals in our study showed no anemia (data not shown), it should be emphasized that symptoms such as depression, dementia and impaired cognitive function, which have been associated with vitamin B12 deficiency, may be misinterpreted as aging-related co-morbidities rather than vitamin B12 dependent co-morbidities [24-26]. In this sense, our findings have great relevance in clinical practice, suggesting that the measurement of this vitamin should be part of routine diagnosis in patients over 60 years, even in the absence of hematological symptoms. The high frequency of elderly individuals with marginal levels of vitamin B12 is another important fact, although the clinical significance of these levels on their health are not clear [27].

Children aged from 0.5 – 6 years, included in our study, did not exhibit folate and vitamin B12 deficiencies. Several studies conducted in developing countries, such as Colombia, have described a very low prevalence of deficiency of these vitamins [28]. In Brazil, a study that included 1111 Amazonian children reported a folate and vitamin B12 deficiency of 2.5 and 3.7%, respectively [29]. Another study of 164 Brazilian children showed that deficiency of folate and vitamin B12 were present in 2.2 and 11.7%, respectively. It is important to note that this study included children under 2 years of age, who continued to receive cow's milk and porridge or did not consume vegetables, fruits, and animal products until over the age of one; a fact that might have contributed to the high prevalence of vitamin B12 deficiency [30]. Shakur *et al.* showed that since food fortification with folic acid, folate deficiency has been reduced to practically 0% in children aged 1 – 14 years in Canada [31]. These results suggest that there is apparently no additional advantage of supplementation with folic acid in countries where food products are folic acid-fortified [14].

Our results suggest that fortification was adequate to prevent folate deficiency in these children, and that diet was capable of meeting vitamin B12 requirements. Vitamin B12 deficiency in children is exceptional as daily requirements are very small, and children normally consume food that contains this nutrient.

During pregnancy, folate and vitamin B12 are essential for normal fetal development. Furthermore, pregnant women have an increased physiological need for these nutrients, and their inadequate intake increases the risk of developmental abnormalities, including NTD [32,33]. The results of the present study showed that the median of folate (10.7 ng/mL) and vitamin B12 (325.0 pg/mL) in pregnant women were higher than those reported by

Guerra-Shinohara *et al.* (5.6 ng/mL and 181.1 pg/mL, respectively) before folic acid fortification in Brazil [34].

We highlight that folate deficiency during pregnancy was virtually nonexistent in our study. According to some studies, the mandatory fortification of flour with folic acid resulted not only in an improvement in input and levels of folate in the blood, but also in a reduction in the occurrence of NTD [35-37]. Despite the lack of data on the outcome of these pregnancies, our results corroborate the effectiveness of this program, since normal folate levels were present in all pregnant women, irrespective of folate supplementations. However, we cannot exclude that supplementation may have contributed to achieving adequate folate levels in some of these pregnant women.

Vitamin B12 deficiency (7.9%) or marginal levels (33.7%) were frequent in the group of pregnant women. A high prevalence of this deficiency in this group has also been described in other populations [38]. Ray *et al.* suggested that 1 in 20 Canadian women may be vitamin B12 deficient during the critical period of closing the embryonic neural tube [39]. Increased risk of NTD has also been associated with vitamin B12 deficiency, especially after the fortification of flour with folic acid [40,41].

In the current study, practically all pregnant women with vitamin B12 deficiency were in their second and third trimester of pregnancy. It is known that vitamin B12 decreases through gestation due to an increase in fetal requirements [34,42,43], and therefore, the deficiency in this group should be interpreted with caution. In addition, we cannot rule out the effect of hemodilution upon the levels of vitamin B12 in pregnant women. Moreover, the cut-off used to identify the vitamin B12 deficiency in the general population may not apply during pregnancy.

In lactating women, a higher folate intake is also required. Folate concentration in human milk is strongly regulated and not affected by maternal folate status, except in clinically folate-deficient mothers [14]. Maternal vitamin B12 levels have also been correlated with milk vitamin B12, and infant urinary methylmalonic acid levels, inversely related to milk vitamin B12 levels. However, in breast-fed infants the deficiency may become clear due to the low milk concentration of vitamin B12 [44,45]. In this study, we did not observe folate deficiency in lactating women; however 1.9% showed vitamin B12 deficiency.

Although the main purpose of fortifying flour with folic acid is the reduction of NTD, the potential benefit of reducing the risk of cardiovascular disease by reducing the levels of Hcy is also relevant [10]. Hyperhomocysteinemia has been considered a risk factor for vascular diseases and its increase is related to folate and vitamin B12 deficiencies, and genetic polymorphisms.

Some population-based studies suggest that a decline in mortality related to strokes coincided with the introduction of folic acid fortification in the United States and Canada [46]. However, some meta-analysis studies that evaluated the risk of cardiovascular disease or death in patients with or without previous disease, failed to show any beneficial effect of this strategy. Apparently, for some subgroups of patients, such as those with kidney disease, supplementation may have a beneficial effect [47,48].

Our results showed a higher frequency of hyperhomocysteinemia, mainly in the elderly. Hyperhomocysteinemia may be a consequence of ageing, oxidative stress, hypertension, diabetes and dyslipidemia. These factors were common in this group and could justify the high frequency of this condition. Moreover, our results corroborate with studies that consider the reduction of folate and vitamin B12 and age as factors related to hyperhomocysteinemia [49,50].

On the other hand, hyperhomocysteinemia was nonexistent in all the children included, and multivariate analysis demonstrated the effect of vitamin B12 levels. A study performed in 207 children from the region of Campinas demonstrated that acquired factors, vitamin B12 and folate, were the most important factor in defining the levels of plasma Hcy [51].

We have also observed that Hcy levels were normal in most of the pregnant women (99.3%). Hcy levels are known to be lower in pregnant women than in non-pregnant women [52,53]. However, studies have reported that elevated Hcy levels (>15.33 μmol/L) were observed in mothers of infants with NTD [54,55]. Furthermore, several studies have also associated high levels of Hcy to a variety of adverse effects during pregnancy [56].

In lactating women, hyperhomocysteinemia was present in 5%. Ramlau-Hansen *et al.* demonstrated that breastfeeding mothers who did not take folic acid supplements had a higher prevalence of elevated Hcy, compared to breastfeeding mothers taking folic acid supplements, and to a control population [57]. Despite this relationship between folate and hyperhomocysteinemia in lactating women, the multivariate analysis showed no factor that might interfere with Hcy levels, and this fact can be attributed to the number of participants included.

Among the polymorphisms investigated, only MTHFR C677T was significantly associated with folate and Hcy levels in pregnant women. Folate status is well-known to play a crucial role in the development of hyperhomocysteinemia in individuals with this polymorphism [58,59]. Furthermore, Hcy levels tend to be independent of the genotype in populations having high T allele frequency (≥20%) [60,61].

No interactions of the RFC1 A80G and GCPII C1561T polymorphisms were found on folate and Hcy levels,

corroborating with previous studies. The effects of polymorphisms in folate-metabolizing genes on Hcy levels may be masked by the interaction with other polymorphisms or with environmental factors that influence folate status [62].

The frequencies of MTHFR C677T and RCF1 A80G genotypes were similar to those reported in others populations [17,62], whereas genotype frequencies of the GCPII C1561T polymorphism were different from those described in other studies [6,7].

Finally, we were able to demonstrate that Hcy levels are influenced mainly by folate and vitamin B12. Plasma Hcy may serve as an indicator of status and perhaps of the intake of vitamins such as folate and vitamin B12 [49]. These results also corroborate with previous findings where acquired factors contributed more to hyperhomocysteinemia than genetic factors [51].

In Brazil, a few studies have evaluated vitamin deficiencies after folic acid fortification, mainly in the population investigated and, therefore, we considered this analysis of an impressive number of more than 700 participants as the strength of the current study. However some limitations should be acknowledged, such as: the fact that our population is not representative of the entire country's population which presents great diversity; the small number of children and lactating women included; plasma Hcy samples were not kept on ice until separation, and the length of time between the blood collection and plasma preparation could have a significant impact on plasma total Hcy; the lack of data from the pre-fortification era to compare with the post-fortification era; and also the lack of data regarding food-intake to correlate with folate and vitamin B12 levels. Also, we did not perform the measurements of red blood cell folate and methylmalonic acid. We could not exclude that the real impact of fortification was compromised in lactating women because this group comprised of only 60 individuals. These limitations should be considered when analyzing our data.

Conclusion

Folate deficiency is practically nonexistent in the post-folic acid fortification era in the studied population. However, our study suggests that screening for vitamin B12 deficiency may be particularly relevant, especially in the elderly, and the impact of the relatively high frequency of this deficiency on the overall health of our population deserves additional studies. Regarding the influence of genetic polymorphisms, we observed no evidence that RFC1 A80G and GCPII C1561T polymorphisms-influenced folate, vitamin B12 and Hcy. Finally, we confirmed that folate and vitamin B12 are important determinants of Hcy levels.

Abbreviations

ANVISA: National Health Surveillance Agency; GCPII: Glutamate carboxypeptidase II; Hcy: Homocysteine; HPLC: High-performance liquid chromatography; MTHFR: Methylenetetrahydrofolate reductase; NTD: Neural tube defect; RFC1: Reduced folate carrier.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The authors' contributions were as follows – AB: responsible for laboratory analysis, statistical analysis, interpretation of results and writing the manuscript; ACA: responsible for study design and conducting research; LFB: contributed to subject recruitment and collection data; BMM: responsible for polymorphisms analysis; AMBZ: responsible for study supervision; EVP, NFH, JMAB: responsible for interpretation of results and critically reviewed the manuscript; and all authors read and approved the final manuscript.

Acknowledgements

This study was supported by a grant from the National Counsel of Technological and Scientific Development (CNPq), project n° 402257/2005-7. The funder had no role in the design, analysis and writing of this article.

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A randomized, controlled, crossover trial to assess the acute appetitive and metabolic effects of sausage and egg-based convenience breakfast meals in overweight premenopausal women

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Abstract

Background: Dietary protein at breakfast has been shown to enhance satiety and reduce subsequent energy intake more so than carbohydrate or fat. However, relatively few studies have assessed substitution of protein for carbohydrate on indicators of appetite and glucose homeostasis simultaneously.

Methods: The acute appetitive and metabolic effects of commercially-prepared sausage and egg-based breakfast meals at two different protein levels (30 g and 39 g/serving), vs. a low-protein pancake breakfast (3 g protein) and no breakfast (water), were examined in premenopausal women (N = 35; age 32.5 ± 1.6 yr; BMI 24.8 ± 0.5 kg/m^2). Test products provided ~280 kcal/serving and similar fat (12–14 g) and fiber contents (0–1 g). Visual Analog Scale ratings for appetite (hunger, fullness, prospective consumption, desire to eat) and repeated blood sampling for plasma glucose and insulin concentrations were assessed throughout each test day. Energy intake was recorded at an *ad libitum* lunch meal at 240 min.

Results: Results showed increased satiety ratings for both the 30 and 39 g protein meals vs. the low-protein and no breakfast conditions ($p < 0.001$ for all). Postprandial glucose and insulin excursions were lower following the 30 g and 39 g protein conditions vs. the low-protein condition, with smaller responses following the 39 g vs. 30 g protein condition ($p < 0.05$ for all). Energy intake at lunch was significantly less ($p < 0.001$) following the 39 g protein meal (692 kcal) vs. the low-protein and no breakfast conditions (789 and 810 kcal, respectively). Total energy intake from the test condition + lunch was higher ($p < 0.01$) for the 30 and 39 g meals (982 and 983 kcal, respectively) vs. no breakfast (810 kcal), and less than the low protein breakfast (1064 kcal; $p < 0.01$ vs. 39 g condition only).

Conclusions: Results suggest that convenience meals providing 30 or 39 g protein/serving produce greater appetite control, lower postprandial glycemia and insulinemia, and reduced subsequent intake at lunch relative to a low-protein control, or no breakfast.

Trial registration: NCT01713114

Keywords: Hunger, Fullness, Appetite, Protein, Glycemic control

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Background

Protein is generally regarded to be more satiating than an equivalent amount of digestible carbohydrate or fat [1-4]. This may be particularly true when protein is consumed at breakfast versus later in the day, as studies that have fed protein at lunch or dinner have shown more variable results [5]. Acute intervention studies have shown that protein-rich breakfast meals reduce appetite and increase satiety throughout the morning relative to moderate or low-protein breakfast meals [6-8]. Protein-rich breakfast meals have also been shown to reduce energy intake at a subsequent *ad libitum* lunch meal [6,7,9-11]. Such effects have been demonstrated at protein intakes ≥20 g, and most consistently at intakes ≥30 g of protein per meal [6,7,9-12].

The effects of a protein-rich breakfast may extend beyond the immediate postprandial period. Several investigators have reported reduced energy intakes over the 24 hour period following egg-based breakfast meals rich in protein [7,10], although not all studies have produced similar results [13]. Leidy et al. [12] found that higher protein intake at breakfast (35 vs. 13 g) was associated not only with greater satiety and less hunger throughout the morning, but also with reduced energy intake from snacks in the evening hours, particularly high-fat snacks.

Limited data exist regarding postprandial glucose and insulin excursions and their relationships to appetitive responses following high vs. lower protein meals. In addition to the satiety properties of dietary protein, the consumption of higher protein meals has been proposed to improve glucose homeostasis. Several meta-analyses from long-term, higher protein, weight loss and/or weight maintenance diets report reductions in glycated hemoglobin and/or fasting insulin concentrations with higher vs. normal protein diets [14,15]. Since larger postprandial glucose elevations have been shown to be associated with an increased risk for the development of type 2 diabetes mellitus [16] and cardiovascular disease [17], it is of interest to identify dietary strategies, such as higher protein intake at breakfast, that might improve glucose homeostasis through the reductions in these responses.

Average dietary protein intake in the US is adequate based on current recommendations [18]. However, data from the National Health and Nutrition Examination Survey (NHANES) survey suggest that the majority of dietary protein is consumed at dinner, with protein intakes at breakfast averaging ~10 g in women and 15 g in men, well below levels shown to favorably affect appetite and metabolism [19]. In addition, approximately 20% of US men and women do not consume breakfast [20]. In an analysis of NHANES data, our group found that higher protein intake at breakfast was inversely associated with energy intake at lunch, and higher non-protein intake at breakfast was positively associated with energy intake at lunch [19].

Given ready-to-eat cereals and other foods requiring little preparation are frequently consumed at breakfast, convenient breakfast options high in protein would be potentially beneficial for individuals interested in reducing morning hunger and energy intake later in the day as well as glycemic excursions. The present study was undertaken to evaluate the effects of consuming two higher-protein sausage and egg-based frozen convenience breakfast meals, providing 30 or 39 g of protein, compared with a lower-protein, higher carbohydrate frozen convenience breakfast meal (pancakes and syrup, 3 g protein), and breakfast skipping, on appetite ratings, postprandial glycemic and insulinemic responses, and *ad libitum* energy intake at a lunch meal in normal weight to overweight, premenopausal women.

Methods

Design

This was a randomized, controlled, crossover study conducted at Biofortis Clinical Research (Addison, IL) according to Good Clinical Practice Guidelines, the Declaration of Helsinki (2000), and the United States 21 Code of Federal Regulations. An institutional review board (Quorum Review IRB, Seattle, WA) approved the protocol before initiation of the study and subjects provided written informed consent before any study procedures were performed. The study included 1 screening visit and 4 test visits, each separated by at least 5 days.

Participants

Healthy premenopausal women aged 18 to 55 y, each with a body mass index (BMI) 18.5 to 29.9 kg/m^2 and who were regular consumers of breakfast and lunch (≥5 days/week), were recruited to participate. Subjects were excluded if they were self-defined smokers; reported a recent weight change of ±2.7 kg; had history of surgical intervention for the treatment of obesity; used weight loss medications, supplements, programs, or meal replacement products; used medications or dietary supplements likely to markedly affect taste, smell, or appetite; or scored >11 on a dietary restraint scale [21]. Subjects with a history of cardiac, renal, hepatic, endocrine, pulmonary, biliary, pancreatic, gastrointestinal or neurologic disorders, or cancer (in the last 2 years); known sensitivity, allergy, or taste aversion to any of the ingredients in the study products; a history of eating disorders or alcohol abuse; and use of medications known to influence carbohydrate metabolism were also excluded from the study. Subjects who developed symptoms of active infection during the study period (e.g., upper respiratory infection) or reported antibiotic use were allowed to continue only after symptoms had been resolved and antibiotic use had been discontinued at least 5 days prior to testing.

In total, 34 women participated in the study (Table 1). Test visits were scheduled during the follicular phase of each woman's menstrual cycle, defined as days 1 to 14, where day 1 is the first day of menses. Participants were instructed to contact the clinic once menses began to schedule their test visits. Additional study instructions included avoidance of vigorous physical activity, consumption of alcoholic beverages, and maintenance of habitual caffeine intake within 24 hours of each test visit. Subjects were also dispensed a food record prior to their first test visit, which was copied and dispensed to each participant with instructions to replicate the same food and beverage intakes to the best of their ability after 1400 hour the day prior to subsequent test visits.

Procedures

On test days, subjects reported to the clinic (~0800 hour) following an overnight fast (10–12 hour). An intravenous (IV) catheter was inserted for collection of venous blood. At approximately t = −60 min, subjects were offered 6 oz of water, a caffeinated, non-caloric cola, caffeinated coffee, or tea with non-caloric sweetener. The beverage of their choice was replicated at subsequent test visits. At t = −12 min, subjects were administered one of three study products with 175 g of water or water only, and instructed to consume approximately one-third of the study product and water every 4 min (Figure 1). Participants were instructed to consume the study product and water in their entirety. Participants were provided with 500 g of water and allowed to drink *ad libitum* throughout the remainder of the visit. Actual water intake was recorded.

Validated Visual Analog Scale (VAS) ratings [22] for appetite sensations (hunger, fullness, desire to eat, and prospective food consumption) were completed prior to study product intake (t = −25 and −15 min) and at 30 min intervals until 240 min. VAS ratings were recorded on a 100-mm horizontal line anchored by "not at all" to "extremely" in response to questions worded as "How strong is your feeling of". Palatability was assessed by a 9-point scale with the anchors "dislike extremely" and "like extremely" immediately following the last bite of study product.

Table 1 Subject characteristics

Characteristic	Efficacy evaluable population
	N (%)
Female	34 (100)
Race/Ethnicity	
Non-Hispanic white	20 (58.8)
African American	7 (20.6)
Other	7 (20.6)
	Mean (SEM)
Age (y)	32.2 (1.6)
Weight (kg)	66.9 (1.5)
Body mass index (kg/m^2)	24.9 (0.5)
Restraint score (Arbitrary units)	6.5 (0.6)

Blood draws were performed at specific times (Figure 1). Seven blood samples (4 ml/sample; 28 ml/testing day) were collected throughout each testing day. The samples were collected in test tubes containing ethylenediaminetetraacetic acid. Within 10 min of collection, the samples were centrifuged at −4 °C for 10 min. The plasma was separated and stored in microcentrifuge tubes at −80 °C for future analysis. Plasma glucose was measured through an in-house glucose oxidase assay (Thermo Fisher Scientific, USA). Plasma insulin was measured using the Milliplex MAP magnetic bead-based multi-analyte, metabolic panel (Millipore, St. Charles, MO) and Magpix Luminex technologies (Luminex Corporation, Austin, TX).

An *ad libitum* lunch consisting of tortellini and sauce was provided to participants following the last blood collection. Subjects were allowed 25 min for lunch and instructed to eat until "comfortably full". Food was weighed to the nearest gram prior to and following consumption. Subjects were provided with a standard amount of water during lunch and the quantity of water consumed was recorded. A final VAS rating was recorded following lunch at t = 270 min.

Test products

Test products included two commercially-prepared, frozen breakfast meals consisting of egg white, poultry sausage, potatoes, and cheese at two different protein levels [30 g and 39 g/serving; Hillshire Brands); a low protein (LP) meal consisting of mini-pancakes (Eggo®, Kellogg's), syrup (Aunt Jemima®, The Quaker Oats Company), butter (Land O'Lakes); and no breakfast (NB; water only) (Table 2). All test products were prepared according to manufacturer instructions. Each meal was similar in energy, total fat, and fiber contents (except water only condition), but differed in total weight (77 g, 189 g, and 178 g for the LP, 30 g and 39 g bowls, respectively).

Statistical analyses – VAS responses and subsequent food intake

Statistical analyses were conducted using SAS version 9.1.3 (SAS Institute, Cary, NC). An evaluable sample size of 33 subjects was expected to provide 85% power to detect a difference of 2400 mm * min in the net incremental area under the curve (niAUC) for postprandial VAS scores between treatment conditions, assuming a standard deviation of 3816 mm * min (based on prior work by our group), for an effect size of 62%. A nominal alpha of 0.017 was used for sample size calculations to account for three

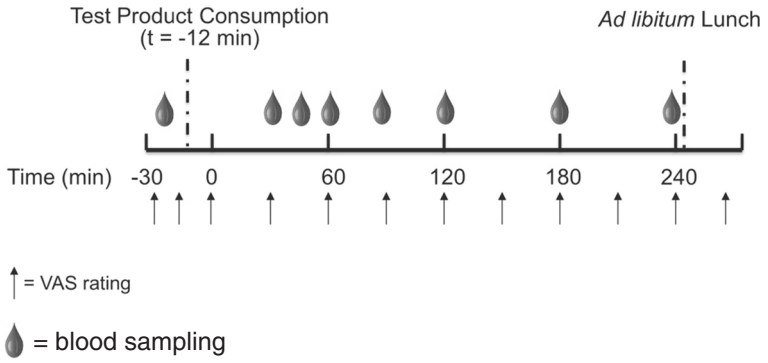

Figure 1 Test visit flow diagram.

primary comparisons between the three energy-containing meal conditions [23].

VAS rating niAUCs were calculated by applying the trapezoidal rule for both positive and negative increments from pre-meal (average of timepoints, nominal time 0 min was calculated as the average of the values at −25 and −15 min) to 240 min, whereas total AUC was calculated for the plasma glucose and insulin concentrations throughout the 240 min [24]. Repeated measures analysis of variance (ANOVA) was used to assess differences between test conditions for continuous outcome variables. Initial models contained terms for treatment condition, sequence, period, age and baseline BMI, with subject included as a random effect. Models were reduced in a stepwise manner until only significant terms or treatment condition remained in the model. The Shapiro-Wilk test was used to assess normality of the distribution of residuals in the analysis models. The normality assumption was rejected for palatability, energy intake at lunch, and preload plus energy intake, therefore rank transformations were employed and the models were rerun using the transformed data. When treatment condition main effects were detected, pairwise comparisons between conditions were conducted using Tukey's adjustment for multiple comparisons. Sensitivity analyses were conducted using the overall liking palatability score as a covariate in models for appetite ratings and energy intake at lunch with the no breakfast condition excluded.

For the appetite and food intake outcomes, the primary efficacy analysis was completed on an efficacy evaluable (EE) sample that included all subjects who consumed the 39 g protein breakfast and at least one other condition. A secondary analysis was completed on a per protocol (PP) sample, a subset of the EE population excluding those subjects who had significant protocol deviations (e.g., use of an excluded medication, illness that caused rescheduling of visits) during the treatment period. Subjects in the PP population completed all test visits. All decisions were made prior to breaking the treatment code or locking the database by people unaware of the order of treatments. Results were similar for the EE and PP analyses; therefore, outcomes are presented only for the EE sample. However, for the glucose and insulin measures, only those completing all test conditions were included and thus a PP analysis was performed.

Table 2 Test meal characteristics[1]

	NB	LP	30 g Pro	39 g Pro
Energy (kcal)	0	288	280	294
Protein (g)	0	3	30	39
Carbohydrate (g)	0	44	13	3
Total fat (g)	0	11	12	14
Fiber (g)	0	1	0	0
Palatability (au)[2]	–	7 (7, 8)	7 (6, 8)	6 (4, 7)

[1]NB, no breakfast; LP, low protein; Pro, protein.
[2]Palatability was assessed by a 9-point scale with 1 = "dislike extremely" and 9 = "like extremely." Median values (interquartile limits) are presented.

Results

Palatability

Ratings for appearance, texture, flavor, and overall liking were higher (more palatable) for the LP and 30 g protein conditions versus the 39 g protein conditions ($p < 0.02$ for all). There were no differences between the 30 g and LP conditions. Despite the fact that there were differences, there were no statistically significant associations between overall liking and any of the appetitive ratings or energy intake at lunch. Similarly, when palatability rating was included as a covariate in a subset analysis that excluded the no breakfast condition, palatability rating was did not significantly reduce the unexplained variance.

Appetite sensation ratings

Results for hunger, fullness, desire to eat, and prospective food consumption are depicted in the line graphs

(individual timepoints) and bar graphs (niAUC) in Figures 2 and 3. For each appetite response, there was a main effect of treatment condition ($p < 0.001$). Pairwise comparisons showed that both the 30 g and 39 g protein conditions led to greater appetite control and satiety based on the niAUC values ($p < 0.001$ for all) compared to the NB and LP breakfast. The LP condition also produced greater ratings for satiety and reduced hunger relative to the no breakfast condition ($p < 0.01$ for all). There were no differences between the 30 and 39 g conditions for any appetite sensation rating. An exploratory analysis was conducted to compare treatment conditions for appetite sensation ratings at the 240 min timepoint immediately prior to the lunch meal. Results are summarized in Table 3. All treatments except the 30 g and 39 g protein conditions differed significantly for ratings of hunger, fullness and desire to eat ($p < 0.05$), with the 30 g and 39 g protein conditions producing the lowest ratings for hunger and desire to eat and the highest ratings for fullness. Prospective food consumption ratings did not differ significantly between the LP and 30 g protein conditions ($p = 0.075$), as well as between the 30 g and 39 g protein conditions ($p = 0.587$), but each of these conditions produced significantly lower ratings compared to NB (all $p < 0.05$).

Metabolic responses

Results for plasma glucose and insulin are depicted in the line graphs (individual timepoints) and bar graphs (AUC) in Figure 4. No main effect of treatment condition was detected for the glucose AUC response. However, a main effect of treatment condition was detected for glucose peak ($p < 0.001$) and the postprandial change in glucose ($p < 0.001$). Pairwise comparisons showed that both the NB and the 39 g protein conditions led to a lower glucose peak (both, 96 ± 1 mg/dL; $p < 0.05$ for both) compared to the LP (112 ± 3 mg/dL) and the 30 g protein (101 ± 2 mg/dl) conditions, while the 30 g protein condition led to a lower glucose peak ($p < 0.001$) compared to the LP condition. Additionally, pairwise comparisons showed that both the NB and the 39 g protein condition led to a smaller postprandial glucose change from pre-breakfast (−14 ± 1 mg/dL and −16 ± 1 mg/dL, respectively; $p < 0.001$ for both) compared to the LP (−41 ± 2 mg/dL) and the 30 g protein condition (−23 ± 2 mg/dL), while the 30 g protein condition led to a smaller postprandial glucose change ($p < 0.001$) vs. the LP condition. Lastly, the 39 g protein condition led to a smaller postprandial change in glucose ($p < 0.05$) compared to the NB condition.

Main effects of treatment condition were detected for the insulin AUC response, peak insulin, and postprandial changes ($p < 0.001$ for all). Pairwise comparisons showed that the NB condition led to lower insulin AUC ($p < 0.001$ for all) compared to all other breakfast conditions. The 30 g and 39 g protein conditions led to lower insulin AUC ($p < 0.05$ for both) vs. the LP breakfast with no differences between the 30 g and 39 g protein conditions. The NB

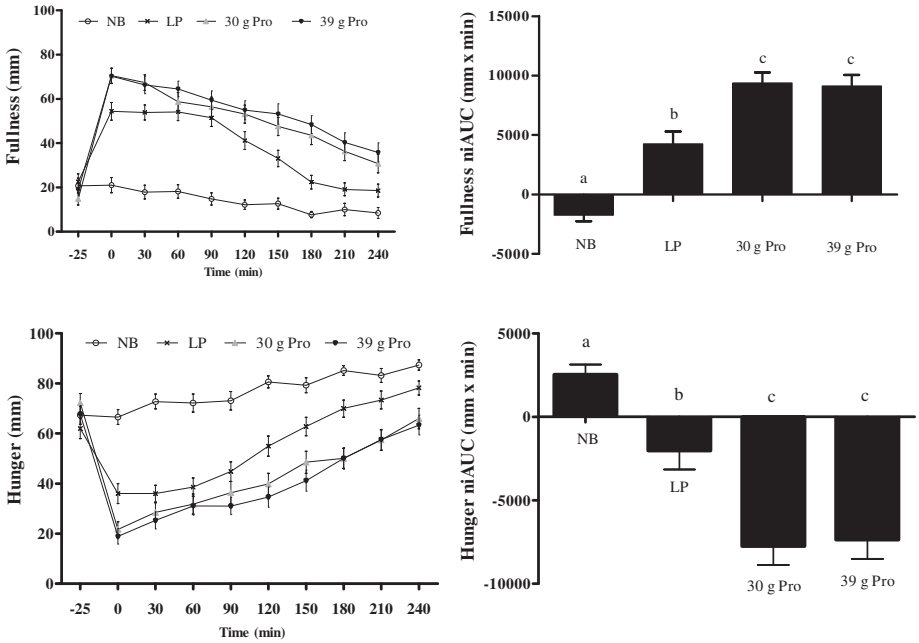

Figure 2 Appetite VAS ratings (hunger and fullness) at each timepoint and niAUC values for each condition. Data are presented as mean ± SEM. Different letters indicate differences between conditions ($p < 0.0001$). Pairwise comparisons between conditions were conducted using Tukey's adjustment for multiple comparisons.

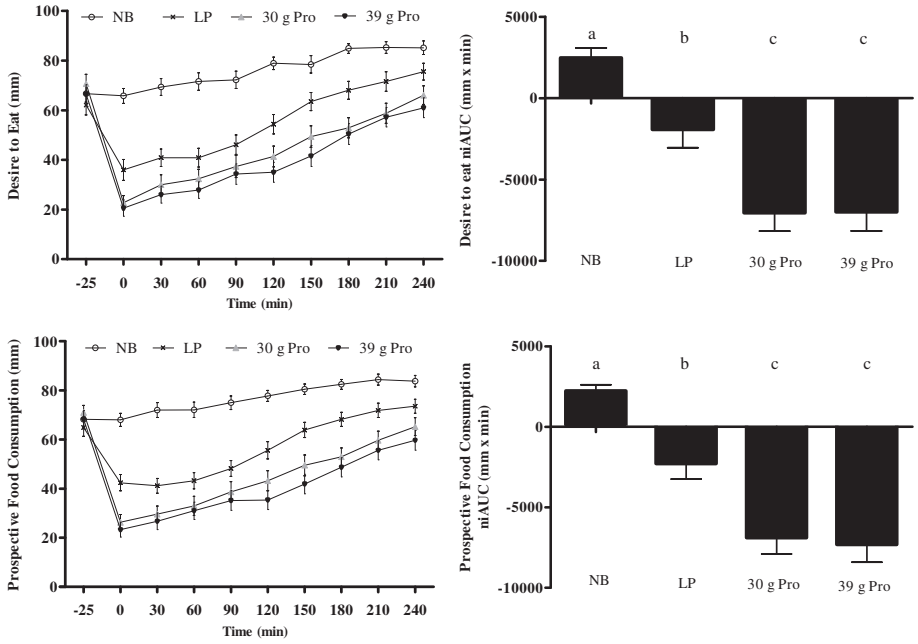

Figure 3 Appetite VAS ratings at each timepoint (left) and niAUC values (right) for each condition. Data are for desire to eat and prospective food consumption. Data are presented as mean ± SEM. Different letters indicate differences between conditions (p < 0.0001). Pairwise comparisons between conditions were conducted using Tukey's adjustment for multiple comparisons.

condition led to a lower insulin peak (7 ± 1 pg/mL; $p < 0.001$ for all) compared to all breakfast conditions (i.e., low-protein (pancakes): 37 ± 3 pg/ml; 30 g protein: 20 ± 2 pg/mL; and 39 g protein: 16 ± 2 pg/mL). The 39 g protein condition led to a lower insulin peak ($p < 0.05$ for both) compared to the LP and the 30 g protein conditions. The 30 g protein condition led to a lower insulin peak ($p < 0.001$) vs. LP condition. Lastly, the NB condition led to the smallest postprandial change from pre-breakfast in insulin (−4 ± 1 pg/mL; $p < 0.001$ for all) compared to all breakfast meals (i.e. LP: −34 ± 3 pg/ml; 30 g protein: −17 ± 2 pg/mL; and 39 g protein: −11 ± 2 pg/mL. The 39 g protein condition led to a smaller postprandial change in insulin ($p < 0.01$ for both) compared to the LP and the 30 g protein conditions. The 30 g protein condition led to a smaller postprandial change in insulin ($p < 0.001$) vs. the LP condition.

Lunch energy intake

Median energy intake at the lunch meal was reduced by approximately 15% following the 30 g and 39 g protein conditions versus the NB condition ($p \leq 0.03$; Figure 5). Compared to the LP breakfast, energy intake was lower following the 39 g protein condition ($p < 0.001$), and was lower with the 30 g protein breakfast, but this result was only marginally statistically significant (p = 0.053). There was no significant difference in energy intake between the LP condition and NB condition. Median total energy intake from the test condition + lunch meal was higher for all meals versus the no breakfast condition ($p < 0.001$). Compared to the LP meal, total energy intake was lower after the 39 g protein meal ($p < 0.01$). There were no differences between the 30 and 39 g conditions for energy intake at lunch or preload + energy intake at lunch.

Table 3 Appetite sensation ratings at 240 min by breakfast meal condition[1]

	NB	LP	30 g Pro	39 g Pro	p-value
	Median (interquartile limits)				
Hunger (mm)	91 (79, 96)[a]	81 (70, 91)[b]	71 (50, 84)[c]	69 (49, 80)[c]	<0.0001
Fullness (mm)	3 (1, 12)[a]	15 (9, 24)[b]	26 (10, 54)[c]	33 (14, 59)[c]	<0.0001
Desire to eat (mm)	89 (81, 95)[a]	76 (69, 92)[b]	69 (47, 83)[c]	69 (39, 80)[c]	<0.0001
Prospective food consumption (mm)	85 (77, 93)[a]	77 (64, 84)[b]	67 (50, 82)[b,c]	69 (40, 79)[c]	<0.0001

[1]NB, no breakfast; LP, low protein; Pro, protein.
[a,b,c]Different superscripted letters denote statistically significant differences (p < 0.05).

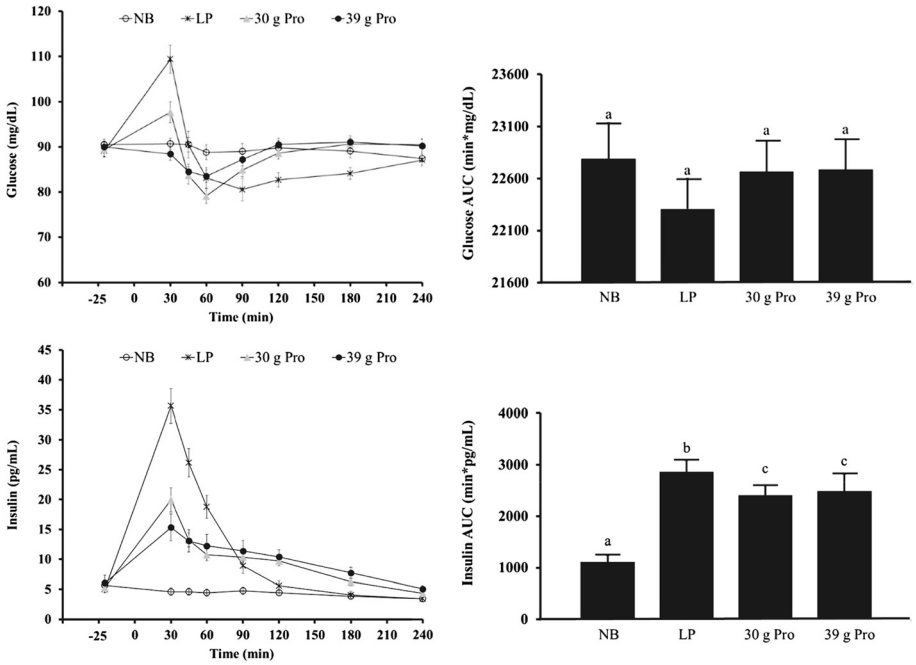

Figure 4 Plasma glucose and insulin at each timepoint (left) and AUC values (right) for each condition. Data are presented as mean ± SEM. Different letters indicate differences between conditions ($p < 0.0001$). Pairwise comparisons between conditions were conducted using Tukey's adjustment for multiple comparisons.

Discussion

The results of this study showed that consumption of protein-rich convenience breakfast meals led to reductions in perceived hunger, increased satiety, and reductions in postprandial glucose and insulin excursions compared to a low-protein meal in normal to overweight, premenopausal women. The protein-rich breakfast meals also resulted in reduced energy intake at the *ad libitum* lunch meal, although results only reached significance for the 39 g protein meal. Thus, consumption of a high protein, sausage and egg-based, ready-to-heat meal may be an option for facilitating satiety throughout the morning, reducing postprandial glycemic and insulinemic excursions, and moderating energy intake at lunch.

The satiety and food intake findings are similar to results reported by other investigators in acute intervention trials using breakfasts based on solid food sources of protein. Eggs and meat as sources of protein, in particular, have been associated with greater perceived satiety and/or improved glycemic control in several studies [1,6,7,10,12,25,26]. For example, Ratliff et al. [10] compared the effects of an egg-based breakfast to a bagel-based breakfast in a group of healthy men. Similar to the results of the present study, perceived hunger, glucose, insulin, and energy intake at the lunch meal were all reduced following the higher protein breakfast. However, to the best of our knowledge, very few prior studies have evaluated the appetitive effects of protein-based, frozen convenience

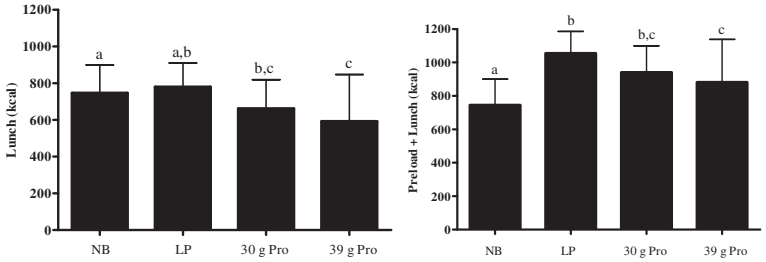

Figure 5 Lunch (left) and total (preload + lunch; right) energy intake following each condition (t = 240 min). Data are presented as median (75th percentile). Different letters indicate differences between conditions ($p < 0.05$, note - energy intake at lunch for the 30 g Pro breakfast vs. LP breakfast $p = 0.053$). Pairwise comparisons between conditions were conducted using Tukey's adjustment for multiple comparisons.

meals. Such meals serve as an easy to prepare option, compared to other high-protein foods traditionally consumed at breakfast in the U.S. (e.g., fresh breakfast meats, fresh eggs), which require a greater degree of preparation. This is further supported by data indicating that a primary barrier associated with breakfast is the lack of availability and convenience [27].

The present work included two levels of protein served as part of a commonly consumed breakfast meal. There were minimal differences in responses between the protein-containing meals, suggesting that both protein levels were sufficient to elicit a greater satiety response and to reduce postprandial glycemic and insulinemic excursions compared with the low-protein meal. The differences in glucose and insulin responses elicited by the test meals were expected because a substitution of protein for carbohydrate was employed, thus reducing the available carbohydrate load. Nevertheless, chronic reduction of dietary carbohydrate has been demonstrated to produce notable metabolic effects, including lowering the circulating concentration of triglycerides, reducing blood pressure, and lessening the demand for insulin production by the pancreatic beta-cells [28]. Moreover, ingestion of protein at a meal tends to increase insulin secretion without significantly increasing the plasma glucose concentration (compared to water ingestion) in the postprandial period [29]. Lastly, elevations in plasma insulin and glucose have been shown to reduce appetitive sensations [30,31]. Thus, it is notable that the appetitive effects observed with higher protein and lower carbohydrate were present despite lower insulin and glucose concentrations.

Other investigations utilizing beverages or semi-liquid applications (e.g., yogurt or custard) to evaluate the satiating properties of protein have shown similar effects to those observed in the present study [32-36]. However, such vehicles are not among the top breakfast choices in the U.S. [27]. Further, the present work included overweight and normal weight women, a group more likely to engage in strategies to reduce body weight than their male counterparts [37]. Results from several long-term intervention trials have provided evidence that higher protein, reduced carbohydrate diets may help to enhance weight loss and/or maintain lean body mass during weight loss [14,15,38-40]. Increased satiation, and therefore better adherence to caloric restriction, is one potential mechanism by which high protein diets may facilitate weight loss. High protein, easy to prepare breakfast options with greater satiating potential would likely facilitate the consumption of calorically-restricted, protein-rich diets.

The mechanisms whereby dietary protein promotes satiety are not completely understood. It has been hypothesized that a high-protein meal may modulate the post-absorptive release of hormones and neurochemicals in the gastrointestinal tract that down-regulate appetite [41]. In particular, consumption of high-protein meals has been shown to decrease levels of the hunger-stimulating hormone ghrelin and/or promote the increase in the satiety-stimulating hormones peptide YY (PYY) and glucagon-like peptide-1 (GLP-1), resulting in increased perceptions of satiety [8,9,26,42,43]. Unfortunately, data for ghrelin, PYY, and GLP-1 for the present study are not available. A majority of studies that have assessed the relationship between postprandial insulin levels and appetite sensations have suggested that insulin has an acute effect to suppress appetite [30,44], although conflicting results have been reported [45,46]. Intracerebroventricular or systemic injections of insulin suppress food intake in a dose-dependent manner in animal models, suggesting a direct involvement in satiety [47]. It is therefore notable that appetite ratings were reduced with the high protein conditions compared to the low protein (higher carbohydrate) breakfast, despite lower insulin (and glucose) responses. Some investigators have suggested that increased thermogenesis following consumption of high-protein meals, as well as changes in substrate oxidation may influence appetitive signals that affect food intake [48,49]. Additional studies will be needed to more fully define the mechanisms responsible for the effects of substituting protein for carbohydrate observed in the present study. It is also suggested that additional research is needed to assess the substitution of protein for dietary fat. Future research of interest would also include studies in which protein is substituted for fat, since the current study cannot separate the effects of increasing the protein content of the study breakfast meals from those of reducing the carbohydrate content.

Both buffet lunch and uniform food models, such as the tortellini and sauce lunch used in the present study, have been used extensively in appetite research [50]. A uniform food model was used in the present investigation because the main objective was related to energy intake rather than food selection. Thus, a limitation of the study is that possible differences in food or macronutrient preferences at lunch could not be assessed.

Another limitation is the short-term nature of the measurement period. It is possible that appetitive sensations or energy intake would increase later in the day to compensate, or even overcompensate, for reductions in energy intake observed at the lunch meal. However, results from Leidy et al. [12] suggest that this may not be the case, as total daily energy intake was reduced when a high-protein breakfast was consumed, compared with a lower protein breakfast meal. Notably, in that trial, a 7-day period of acclimation was employed for both breakfast conditions, suggesting that the effects of higher protein breakfasts on appetite do not dissipate, at least after several days of consumption.

The possibility cannot be ruled out that expectations surrounding each breakfast condition and differences in

the sensory characteristics influenced the results. The present trial evaluated commercially-available breakfast options, therefore matching the sensory properties of the test conditions was not possible. However, all products were considered to be relatively well-liked and there were only measurable differences in the palatability of the highest protein condition relative to the low protein and 30 g protein conditions. Inclusion of the overall palatability rating as a covariate in sensitivity analyses for appetite ratings and energy intake at lunch did not significantly reduce the unexplained variance, suggesting that differences in palatability were unlikely to have materially influenced the results.

Differences in the physical characteristics of the study products may have also influenced orogastric transit time. In an effort to control this to the degree possible, subjects were instructed to eat a portion of each condition at specific intervals over a 12-minute period (approximately one-third during each of three 4-min periods). However, we cannot rule out the possibility that differences in consumption patterns (e.g., chewing) or orogastric transit time influenced our findings. Further, the weight and volume of the products differed. Such characteristics have been previously shown to affect perceived satiety, however our objective was to evaluate conventional breakfast options at an equivalent caloric level, and as such, differences in weight and volume were unavoidable due to the nature of the breakfast meals [51].

Conclusion

In conclusion, the results of the present investigation suggest that sausage/egg convenience meals providing 30 g or 39 g protein per serving produce greater appetite control, reduce postprandial glycemic and insulinemic responses, and lower energy intake at lunch relative to a lower-protein, higher carbohydrate, breakfast meal.

Competing interests

TR, HL, KM, KS, and AL received research funding from Hillshire Brands.

Authors' contributions

TR, HL, and KM conceived the study concept and design and analyzed/interpreted the data. Additionally, TR, HL, and KM drafted and revised the manuscript for important intellectual content. AL, KS, and KM participated in study supervision. All authors read and approved the final manuscript.

Acknowledgements

The authors thank the study participants for their dedication and compliance during the testing days. This trial was funded by Hillshire Brands, Chicago, IL. Financial supporters had no role in the design, implementation, analysis or interpretation of data or preparation of the manuscript.

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Association between quality of the diet and cardiometabolic risk factors in postmenopausal women

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Abstract

Background: Climateric is a phase of women's life marked by the transition from the reproductive to the non-reproductive period. In addition to overall weight gain, the menopause is also associated with the increase of abdominal fat. We used The Healthy Eating Index as a summary measure to evaluate the major components and the quality of women's diet after the onset of the menopause. This study aims at examining the association between the quality of the diet and cardiometabolic risk factors in postmenopausal women.

Methods: Cross-sectional study including 215 postmenopausal women attending a public outpatient clinic. The 24-hour dietary recall method was used to assess the food intake and to establish the Healthy Eating Index. Diets were then classified as appropriate diet (>80 points), diet "requiring improvement" (80–51 points), and poor diet (<51 points). Cardiometabolic risk factors included abdominal obesity, dyslipidemia, diabetes mellitus, and hypertension. The Fisher's exact test was utilized for the Statistical analysis.

Results: The analysis of the food intake showed that the average daily intake of lipids (36.7%) and sodium (2829.9 mg) were above the recommended. Only 8.8% of the women performed moderate or intense physical exercises on a regular basis. The diet was considered poor in 16.3%, "requiring improvement" in 82.8%, and appropriate for only 0.9% of the women. The study detected increased waist circumference in 92.1% of the participants. The mean concentration of triglycerides was of 183.3 mg/dl, and 130.7 mg/dl for cholesterol (Low Density Lipoprotein).

Conclusion: Women consume a low quality diet, possibly due to the low intake of vegetables and fruits and excessive consumption of sodium. These inappropriate eating habits are associated with and, have a negative impact on the cardiometabolic risk factors such as abdominal obesity.

Keywords: Aging, Menopause, Eating habits

Background

The World Health Organization (WHO) defines climateric as a biological phase and not a pathological process, a significant period of female aging, characterized by the establishment of a physiological progressive state of hypoestrogenism, which ends with the permanent cessation of the menstrual cycles. Menopause is a mark of this phase and is acknowledged only one year after the occurrence of the last menstrual cycle [1]. In addition to an overall increase in body weight, menopause has been associated with a greater accumulation of abdominal fat [2]. This unfavorable change in the body fat distribution contributes to explain the increased cardiovascular risk during this stage of life [3].

The association among nutrients, food, and non-communicable diseases (NCD) can be studied with the same tools used to assess individual food consumption, such as the 24-hour recall, which reflects the previous day of the individual food intake. The Healthy Eating Index (HEI) developed using data from the 24-hour

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dietary recall, is a summary measure of the main components of an individual's diet. It facilitates the assessment of a diet's quality, of either populations or groups of individuals. Without reducing the assessment of a single component alone, it takes into consideration the complexity of a given diet, and allows for an indirect assessment of nutrients [4].

Climateric is a topic of concern in women's health, not only for the uncomfortable symptoms it engenders, but also for its potential impact on public health. The latter includes the high prevalence of non-communicable diseases due to a new food pattern of the Brazilian population, the increased consumption of processed foods with high levels of saturated fat, sugar, and salt, in parallel with a growing elderly population [5].

Within this context, the primary purpose of this study is to verify the association between diet's quality and cardiometabolic risk factors in postmenopausal women as well as to contribute to the validation of findings in different living conditions and geographic settings.

Methods

A cross-sectional observational study consisting of a convenience sample of 234 postmenopausal women undergoing treatment at the gynecology outpatient clinic of Instituto Fernandes Figueira (IFF), within the endocrinology and urogynecology subspecialties, from October 2011 to October 2012. The inclusion criteria were: women aged ≥ 45 years and without a menstrual period for 12 consecutive months or more. Exclusion criteria were: dietary counseling with physician or nutritionist, uncontrolled thyroid disease, special diet or vegetarian and extremely low dietary intake (<500 kcal/day) or extremely high (>4000 kcal/day). Two hundred and fifteen women met the criteria and underwent all the required exams. The Research Ethics Committee of IFF approved this study and each participant signed a Statement of Informed Consent.

Nutritional status was estimated by The Body Mass Index (BMI = weight/height^2). Weight and height were measured with a standard beam balance scales (Filizola®, Brazil) and with a stadiometer (Wiso®, Brazil), respectively [6,7]. The nutritional status was interpreted according to WHO recommendations. Women (<65 years) were classified as normal weight (18.5–24.9Kg/m^2), as overweight (25–29.9Kg/m^2), or obese (≥30Kg/m^2). Elderly women (≥65 years) as lean (<22Kg/m^2), normal weight (22–27Kg/m^2), or overweight (>27Kg/m^2) [8].

Waist circumference (WC) was measured at the midpoint between the iliac crest and the lower border of the last floating rib at the end of a normal expiration, using inelastic tape. Each measurement was duplicated and the mean value recorded. Abdominal fat was estimated indirectly by the measurement of the waist circumference, and rated high when waist >80 cm [8].

We used the cutoff point of the Brazilian Society of Cardiology for adults, with isolated systolic hypertension ≥ 140 × 90 mmHg to classify blood pressure level [9].

Blood was collected from each subject after 12-hour fasting. These were used to determine Triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLC) and glucose levels. The low-density lipoprotein cholesterol (LDLC) was calculated using Friedewald et al.'s formula: LDLC = Total cholesterol - (triglycerides/5 + HDLC) [10]. Normal values were: TC <200 mg/dl, HDLC > 50 mg/dl, LDLC <100 mg/dl, TG <150 mg/dl and glucose levels <100 mg/dl [11].

Regarding lifestyle, women were classified as active (self-evaluation) or sedentary. Furthermore, this study adopted the recommendation of the Centers for Disease Control and Prevention (2008) that defines as active women those who undergo moderate or intense aerobic activity for 30 minutes at least five times a week or muscle-strengthening activities two or more days per week [12].

Regarding food intake we used the acceptable macronutrient Distribution Ranges proposed in 2005 by the National Academy of Sciences being 45%–65% for carbohydrates, 10%–35% for proteins, and 20–35% for lipids of a standard total daily intake of 2000Kcal [13]. According to the Feeding Guidelines for the Brazilian Population, the daily intake of saturated fat, cholesterol, and sodium should be less than, 10%, 300 mg and 2400 mg respectively. In addition, the Ministry of Health recommends in the Feeding Guidelines for the Brazilian Population, a minimum daily intake of 3 servings of fruits (each serving of 70 kcal) and 4 servings of greeneries and vegetables (each serving of 15 kcal) [14].

Dietary intake data were obtained through dietary recall of each participant, with reported information on food intake over a 24-hour period preceding the interview or more often the previous day [15]. The nutritional values of food products, consumed and registered in the 24-hour dietary recall, were analysed using the Nutrition Support Programme (Nutwin, [16]). Food products that were not in the database were introduced, using the Brazilian Food Composition Tables (TACO, [17]) and Pinheiro et al. [18]. The data on food intake were used to calculate the HEI scores. The HEI comprises ten components being five food groups (cereal, breads, roots and tubers; fruits and vegetables; milk and dairy products; meat, eggs and beans), four nutrients (saturated fat, total fat, cholesterol, and sodium), plus a measure of dietary variety. Each of ten components contributes with 10 points to the maximum possible score of 100 [19].

The servings of the food follow the daily recommendation of the Feeding Guide for the Brazilian Population as per 1000 Kcal. Based on the total energy calculated by adding up all items of a given food group, we calculated the number of consumed portions of this group, based

on the energy content of a defined portion [20]. Each component of the HEI had a score from zero to ten, and intermediate values were recorded as a proportion of the consumption by group. Thus, ten points, would be scored if the consumption in the group of cereals, breads, tubers and roots was of 450/1000 kcal. Likewise for the group of greenery and vegetables, 22.5/1000 kcal and the fruits group 105/1000 kcal. The same for the consumption of milk and dairy products 180/1000 kcal and the meat, eggs and beans 122.5/1000 kcal. The calculation of all other components followed the same rational being total fat <30%; saturated fat <10%; cholesterol < 300 mg/day, sodium ≤ 2,4 mg/day, and the variety in the diet characterized by the consumption of 8 or more different types of food daily [21]. According to the final score, the consumption was classified as "poor" diet (HEI <51 points), diet "requiring improvement" (HEI between 51 and 80 points), and healthy diet (HEI > 80 points) [19].

The data was stored with double entry on access database and processed by a software developed in R language and the statistical analysis performed with the Statistical Package for Social Sciences software – SPSS, version 13. The continuous variables depicted by measures of central tendency (mean or median), scatter plot, and of minimum and maximum values. For the comparison of categorical variables, the Fisher's exact test or they Chi-squared test were used with $\alpha = 0.05$ for statistical significance and 95% confidence interval.

Results and discussion

Table 1 shows the main characteristics of the study group. Median age was 58.9 years and median age at menopause 49.0 years. Regarding the pattern of food consumption, the results of this study are analogous to those reported by Tardivo et al. [21] on the association between diet quality and metabolic risk indicators in postmenopausal women in the state of São Paulo. The median HEI scores was 60.0 and the total daily calorie intake averaged 1607.8 Kcal showed by these authors were close to our findings, respectively 60.7 for HEI scores and 1619.4 Kcal (Table 1). The participants of this study consumed a slightly higher percentage of protein (median 17.3 versus 15.4%), and carbohydrates (49.9 versus 46.0%), but a lower consumption of lipids (35.6 versus 38.3%). Most women surveyed consumed either a diet "requiring improvement" (82.8%) or poor (16.3%), and only 0.9% ate an adequate diet. Tardivo et al. also showed a similar pattern regarding poor diet and requiring improvement (48.5%), with an adequate diet recorded for 3% of women [21].

The vast majority (94.9%) reported a protein intake within the recommended limits and it did not account significantly to the quality of the diet (Table 2) as opposed to Tardivo's study where all macronutrients contributed significantly to determining the quality of the diet [21]. Regarding women who consumed a poor diet our results are comparable to their findings as to higher consumption of calories, lipids, saturated fat, cholesterol and sodium, as well as carbohydrates below the recommended intake (Table 2).

When evaluating the mean quality scores separately for each component of the HEI, the worst records relate to the fruits and greeneries and, the vegetables groups (Table 3). The group of meat, eggs and beans stood out as the best score, possibly linked to the daily consumption of beans, a

Table 1 Clinical characteristics, laboratory values, dietary intakes and Healthy Eating Index score

Characteristics	Average	Median (P25; P75)*	Range**
Sociodemographic			
Age (years)	59.3	58.9 (53.4; 64.6)	44.5–90.1
Education (years)	5.85	8 (4.0; 8.0)	0–8
N° of people living in the house	2.88	3 (2.0; 4.0)	1–8
Per capita income (R$)	1678.5	1240 (850; 2000)	300–12000
Outpatient clinics			
Age at Menopause (years)	47.2	49 (44; 51)	30–60
Length of menopause (years)	12.0	10.7 (5.5;17.2)	0.8–34.3
BMI (Kg/m^2)	28.3	28.0 (24.6;31.6)	18.1–42.5
WC (cm)	95.4	94.7 (87.3; 103)	65–134
Diastolic blood pressure (mmHg)	81.5	80 (70; 90)	60–140
Systolic blood pressure (mmHg)	131.2	130 (120; 140)	90–260
Lifestyle			
Exercises leisure (minutes/week)	256.1	225 (120; 300)	20–900
Inflammatory markers			
Glucose levels (mg/dL)	111.9	103 (94; 117)	66–469
TC (mg/dl)	222.3	218 (196; 250)	91–336
LDLC (mg/dl)	130.7	128 (106; 154)	63–246
HDLC (mg/dl)	55.7	55 (46; 64)	29–100
TG (mg/dl)	183.3	165 (120; 220)	60–558
Food Intake			
Total energy value (kcal/d)	1737.8	1619.4 (1286.1; 2041.2)	698.8–3955.3
Protein (%)	18.2	17.3 (14.2; 21.6)	8.1–44.5
Carbohydrate (%)	48.8	49.9 (41.5; 56.0)	14.6–73.6
Lipids (%)	36.7	35.6 (30.2; 41.7)	16.6–68.2
Saturated Fat (%)	10.3	9.8 (7.8; 12.7)	3.1–25.2
Cholesterol (mg)	230.5	178.2 (122.8; 282.3)	14.7–989.8
Sodium (mg)	2829.9	2521.5 (1843.5; 3603,8)	530.5–10596.2
HEI (score)	60.3	60.7 (54.2; 66.9)	33.0–84.2

*Data are expressed as median with 25th and 75th percentiles in parentheses.
**Values minimum–maximum.

Table 2 Association between Healthy Eating Index scores and clinical characteristics, inflammatory markers and dietary intake

Characteristics	N Total = 215	p-value[a]	Poor diet[b] n = 35 (16.3%)	Diet needs improvement[c] n = 178 (82.8%)	Good diet[d] n = 2 (0.9%)
Outpatient clinics					
Hormone therapy		0.57			
Yes	30 (14.0%)		3 (10.0%)	27 (90.0%)	0 (0%)
No	185 (86.0%)		32 (17.3%)	151 (81.6%)	2 (1.1%)
BMI <65 years old		0.45			
Eutrophic	48 (28.7%)		5 (10.4%)	43 (89.6%)	0 (0%)
Overweight	63 (37.7%)		13 (20.6%)	49 (77.8%)	1 (1.6%)
Obese	56 (33.6%)		11 (19.6%)	44 (78.6%)	1 (1.8%)
BMI ≥ 65 years old		0.15			
Lean	5 (10.4%)		0 (0%)	5 (100.0%)	0 (0%)
Eutrophic	14 (29.2%)		0 (0%)	14 (100.0%)	0 (0%)
Overweight	29 (60.4%)		6 (20.7%)	23 (79.3%)	0 (0%)
WC (cm)		0.78			
<80	17 (7.9%)		2 (11.8%)	15 (88.2%)	0 (0%)
≥80	198 (92.1%)		33 (16.7%)	163 (82.3%)	2 (1.0%)
High blood pressure		0.38			
Yes	68 (31.6%)		14 (20.6%)	54 (79.4%)	0 (0%)
No	147 (68.4%)		21 (14.3%)	124 (84.4%)	2 (1.4%)
Self-reported lifestyle		0.09			
Active	71 (33.0%)		9 (12.7%)	60 (84.5%)	2 (2.8%)
Sedentary	144 (67.0%)		26 (18.1%)	118 (81.9%)	0 (0%)
Aerobic exercises		0.81			
Yes	14 (6.5%)		2 (14.3%)	11 (78.6%)	1 (7.1%)
No	25 (11.6%)		2 (8.0%)	22 (88.0%)	1 (4.0%)
Strengthening exercises		0.58			
Yes	5 (2.3%)		1 (20.0%)	4 (80.0%)	0 (0%)
No	34 (15.8%)		3 (8.8%)	29 (85.3%)	2 (5.9%)
Inflammatory markers					
Glucose levels (mg/dL)		0.73			
<100	80 (37.2%)		14 (17.5%)	66 (82.5%)	0 (0%)
≥100	135 (62.8%)		21 (15.6%)	112 (83.0%)	2 (1.5%)
TC (mg/dl)		0.27			
<200	60 (27.9%)		13 (21.7%)	47 (78.3%)	0 (0%)
≥200	155 (72.1%)		22 (14.2%)	131 (84.5%)	2 (1.3%)
LDLC (mg/dl)		0.75			
<100	40 (18.6%)		5 (12.5%)	35 (87.5%)	0 (0%)
≥100	175 (81.4%)		30 (17.1%)	143 (81.7%)	2 (1.1%)
HDLC (mg/dl)		0.37			
<50	75 (34.9%)		15 (20.0%)	60 (80.0%)	0 (0%)
≥50	140 (65.1%)		20 (14.3%)	118 (84.3%)	2 (1.4%)
TG (mg/dl)		0.58			
<150	88 (40.9%)		13 (14.8%)	75 (85.2%)	0 (0%)
≥150	127 (59.1%)		22 (17.3%)	103 (81.1%)	2 (1.6%)

Table 2 Association between Healthy Eating Index scores and clinical characteristics, inflammatory markers and dietary intake *(Continued)*

Food intake					
Total energy intake (kcal/day)		**<0.001**			
<2000	160 (74.4%)		10 (6.3%)	148 (92.5%)	2 (1.3%)
≥2000	55 (25.6%)		25 (45.5%)	30 (54.5%)	0 (0.0%)
Protein (%)		0.21			
<10	7 (3.3%)		0 (0.0%)	7 (100.0%)	0 (0.0%)
10–35	204 (94.9%)		33 (16.2%)	169 (82.8%)	2 (1.0%)
>35	4 (1.9%)		2 (50.0%)	2 (50.0%)	0 (0.0%)
Carbohydrate (%)		**0.001**			
<45	77 (35.8%)		20 (26.0%)	57 (74.0%)	0 (0.0%)
45–65	129 (60.0%)		15 (11.6%)	112 (86.8%)	2 (1.6%)
>65	9 (4.2%)		0 (0.0%)	9 (100.0%)	0 (0.0%)
Lipids %		**0.001**			
<20	7 (3.3%)		0 (0.0%)	7 (100.0%)	0 (0.0%)
15–30	94 (43.7%)		4 (4.3%)	88 (93.6%)	2 (2.1%)
>30	114 (53.0%)		31 (27.2%)	83 (72.8%)	0 (0.0%)
Saturated fat (%)		**<0.001**			
<10	111 (51.6%)		9 (8.1%)	100 (90.1%)	2 (1.8%)
≥10	104 (48.4%)		26 (25.0%)	78 (75.0%)	0 (0.0%)
Cholesterol (mg)		**<0.001**			
≤300	168 (78.1%)		14 (8.3%)	152 (90.5%)	2 (1.2%)
>300	47 (21.9%)		21 (44.7%)	26 (55.3%)	0 (0.0%)
Sodium (mg)		**<0.001**			
≤2400	96 (44.7%)		4 (4.2%)	91 (94.8%)	1 (1.0%)
>2400	119 (55.3%)		31 (26.1%)	87 (73.1%)	1 (0.8%)
Vegetables and greenery intake		0.1			
≥4 servings	23 (10.7%)		5 (21.7%)	17 (73.9%)	1 (4.3%)
<4 servings	192 (89.3%)		30 (15.6%)	161 (83.9%)	1 (0.5%)
Fruit Intake		1			
≥3 servings	2 (0.9%)		0 (0.0%)	2 (100.0%)	0 (0.0%)
<3 servings	213 (99.1%)		35 (16.4%)	176 (82.6%)	2 (0.9%)

Data are expressed in numbers and percentage between parentheses.
[a]statistical difference between groups p <0.05 (Fisher's Exact Test).
[b]HEI < 51 points.
[c]HEI between 51 and 80 points.
[d]HEI > 80 points.

typical and vital component of the Brazilian diet. These findings are in agreement with the results of the Household Budget Survey (POF 2008–2009), which showed a profile of food consumption that combines traditional Brazilian diet of rice and beans with foods that are high in calories with low content of nutrients and, well below the recommended intake for fruits, greeneries and vegetables [22].

Levy Costa et al. reported an evolving change of food availability, distribution and food intake patterns at the household and population levels since the 70's, leading to the current profile. Their findings are consistent with the rising prevalence of overweight and obesity and the increasing rate of NCDs and their contribution to the morbidity and mortality profiles of the population. This diet configuration, called "western" or "westernized" includes a high intake of salt and sugar, a reduced consumption of fruits, fiber and vegetables and an increased level of total and saturated fats [23].

Pereira et al. compared the food consumption of women aged 35 or more in two population based cross sectional

Table 3 Mean, median, minimum and maximum score of the Healthy Eating Index components

HEI components	Mean	Median	Score Min	Score Max
Cereal, bread, tubers, roots	3.96	3.77	0.52 (0.5)	9.8 (0.5)
Vegetables and greenery	2.13	0.00	0 (67.4)	10 (10.7)
Fruits	1.83	1.39	0 (1.9)	10 (0.9)
Milk and dairy	4.87	4.52	0 (9.8)	10 (17.2)
Meat, eggs and legumes	10.0	10.0	9.0 (0.5)	10 (99.5)
Total fat	5.57	6.20	0 (19.5)	10 (24.7)
Saturated fat	7.29	10.0	0 (10.2)	10 (51.6)
Cholesterol	8.47	10.0	0 (11.6)	10 (78.1)
Sodium	7.33	9.49	0 (7.9)	10 (44.7)
Variety of diet	8.90	10	2.0 (1.9)	10 (65.6)
Diet quality index	60.3	60.7	33.0 (0.5)	84.2 (0.5)

Data are expressed as number and percentage in parentheses.

studies undertook in Rio de Janeiro, Brasil, in 1995–1996 (n = 1.014) e 2004–2005 (n = 1.001). The prevalence of obesity (BMI ≥ 30 kg/m2) raised from 16.6% para 24% in 10 years. Pereira et al. also show that changes in dietary intake of adult women in the Metropolitan Region of Rio de Janeiro are in disagreement with the recommendations for healthy eating. There was an increase in the consumption of processed foods with high-energy and there was a decrease in the consumption of fruits, milk, beans, roots, tubers and meats. In this population group, the shift from the traditional Brazilian food habits is detrimental to the overall quality of diets and possibly contributes to the occurrence of overweight and obesity. There is also an increased risk of developing metabolic disorders and other non-communicable chronic diseases [24]. Most women of this study had an inadequate consumption of fruits (99.1%) and of greeneries and vegetables (89, 3%) (Table 2). There was a significant association between high concentrations of LDL-cholesterol and lower fruit intake (Table 4). Hung et al. assessed the association between fruit intake and cardiovascular disease, fruits being protective when consumption was of ≥ 3 fruit servings per day [25].

Another study found that low intake of fruits represented both, overall risk for obesity and for abdominal fat Perozzo et al. [26] studied the association between food patterns and obesity in a population based cross sectional study, with a representative sample of 1.026 women (20–60 years) in São Leopoldo, Rio Grande do Sul, Brazil. Overall and abdominal obesity were present in 18% and 23.3% women respectively. After controlling for confounding factors, low fruit intake was positively associated with BMI [26]. The present study did not find such an association.

Identifying the type of body fat distribution is crucial because the accumulation of fat in the abdominal region is directly linked to metabolic changes that can lead to the development of cardiovascular diseases and diabetes mellitus. During the menopause, the decrease in estrogen and the overall increase of body weight are concurrent with the augmentation of visceral fat (abdominal). This characterizes an android profile associated with higher cardiovascular risks in postmenopausal women [27]. Toth et al. reported a 49% increase in abdominal fat and 22% of subcutaneous fat in postmenopausal women compared to women between the first and last natural menstruation [2].

In this study, the prevalence of high WC as a cardiometabolic risk factor was high (92.1%), as well as glucose levels (62.8%) and triglycerides (59.1%) (Table 2). Cardiometabolic risk factors and diet quality are not significantly associated in this study (Table 2), corroborating the findings reported by Tardivo et al. [21]. These authors also found an association between diet quality and total body fat estimated by skinfold, a measurement not collected measurement in the present study. Nonetheless, this study showed that women under 65 and overweight have a higher prevalence of hypertension and hyperglycemia and that the excessive intra-abdominal fat relates to glucose intolerance and insulin resistance (Table 5).

The high WC as surrogate measure for the accumulation of intra-abdominal fat, is directly associated with the prevalence of diabetes, increasing the risk of cardiovascular disease. Before menopause, women have lower levels of blood pressure than men of the same age group do. After menopause, the blood pressure levels of women exceed those of men in the same age range. Hypoestrogenism during the postmenopause causes a tendency to increase blood pressure thus increasing the risk of cardiovascular diseases [1].

The mean daily sodium intake was of 2829.9 mg (Table 1), is above the maximum recommended intake for adults (2400 mg) [14] and less than that reported by the Research Project on Household Budgets (POF) 2008–2009 (12 g of salt or 4700 mg of sodium) [22]. Women (7%) also reported adding salt to prepared meals. Being aware that this is quite a popular practice, we may consider this figure underrated. Martinazzo et al. reported a prevalence of 16.7% of excessive sodium intake, which is roughly 2.4 fold higher than the level found in this study [28]. However, this level of excessive sodium intake was associated with higher serum concentrations of total cholesterol and LDLC, increased lipids and dietary cholesterol, and even lack of hormone replacement therapy (Table 4). The profile of inadequate dietary intake of sodium and lipids is associated with lower levels of estrogens and favors atherogenesis. Along the aging process, women experience variations in their metabolic profile leading to changes in the composition and distribution of adipose

Table 4 Association between inadequate intake with clinical characteristics, inflammatory markers and food intake

Characteristics	N (Total = 215)	Inadequate intake		
		Fruits (< 3 servings/day)	Vegetables and greenery (< 4 servings/day)	Sodium (> 2400 mg/day)
Outpatient clinics				
Hormone therapy				
p-value*		0.26	0.34	**0.02**
Yes	30 (14.0%)	29 (96.7%)	28 (93.3%)	11 (36.7%)
No	185 (86.0%)	184 (99.5%)	164 (88.6%)	108 (58.4%)
Inflammatory markers				
Glucose levels (mg/dL)				
p-value		0.39	0.07	0.41
<100	80 (37.2%)	80 (100.0%)	75 (93.8%)	43 (53.8%)
≥100	135 (62.8%)	133 (98.5%)	117 (86.7%)	76 (56.3%)
TC (mg/dl)				
p-value		0.07	0.47	**<0.001**
<200	60 (27.9%)	58 (96.7%)	53 (88.3%)	46 (76.7%)
≥200	155 (72.1%)	155 (100.0%)	139 (89.7%)	73 (47.1%)
LDLC (mg/dl)				
p-value		**0.03**	0.56	**0.02**
<100	40 (18.6%)	38 (95.0%)	36 (90.0%)	28 (70.0%)
≥100	175 (81.4%)	175 (100.0%)	156 (89.1%)	91 (52.0%)
HDLC (mg/dl)				
p-value		0.42	0.59	0.5
<50	75 (34.9%)	75 (100.0%)	67 (89.3%)	33 (44.0%)
≥50	140 (65.1%)	138 (98.6%)	125 (89.3%)	63 (45.0%)
TG (mg/dl)				
p-value		0.65	0.52	0.09
<150	88 (40.9%)	87 (98.9%)	79 (89.8%)	54 (61.4%)
≥150	127 (59.1%)	126 (99.2%)	113 (89.0%)	65 (51.2%)
Food intake				
Total energy intake (kcal/day)				
p-value		0.55	0.09	**<0.001**
<2000	160 (74.4%)	158 (98.8%)	146 (91.3%)	68 (42.5%)
≥2000	55 (25.6%)	55 (100.0%)	46 (83.6%)	51 (92.7%)
Protein (%)				
p-value		1	0.72	0.06
<10	7 (3.3%)	7 (100.0%)	6 (85.7%)	1 (14.3%)
10–35	204 (94.9%)	202 (99.0%)	182 (89.2%)	115 (56.4%)
>35	4 (1.9%)	4 (100.0%)	4 (100.0%)	3 (75.0%)
Carbohydrate (%)				
p-value		**0.02**	0.18	0.05
<45	77 (35.8%)	75 (97.4%)	72 (93.5%)	48 (62.3%)
45–65	129 (60.0%)	129 (100.0%)	111 (86.0%)	69 (53.5%)
>65	9 (4.2%)	9 (100.0%)	9 (100.0%)	2 (22.2%)

Table 4 Association between inadequate intake with clinical characteristics, inflammatory markers and food intake *(Continued)*

Lipids (%)				
p-value		0.53	0.29	**0.01**
<20	7 (3.3%)	7 (100.0%)	7 (100.0%)	1 (14.3%)
20–30	94 (43.7%)	94 (100.0%)	87 (92.6%)	47 (50.0%)
>30	114 (53.0%)	112 (98.2%)	98 (86.0%)	71 (62.3%)
Saturated Fat (%)				
p-value		0.73	0.06	0.08
<10	111 (51.6%)	110 (99.1%)	103 (92.8%)	56 (50.5%)
≥10	104 (48.4%)	103 (99.0%)	89 (85.6%)	63 (60.6%)
Cholesterol (mg)				
p-value		0.06	**<0.001**	**<0.001**
≥300	168 (78.1%)	166 (98.8%)	161 (95.8%)	83 (49.4%)
>300	47 (21.9%)	47 (100.0%)	31 (66.0%)	36 (76.6%)

Data are expressed in numbers and percentage between parentheses.
*statistical difference between groups p <0.05 (Fisher's Exact Test).

Table 5 Association between cardiometabolic risk factors and clinical characteristics

Characteristics	N (Total = 215)	Increased WC (≥80 cm)	Low HDL (<50 mg/dl)	Increased TG (≥150 mg/dl)	High blood pressure (≥130 × 85 mmHg)	Increased glucose levels (≥100 mg/dl)
Outpatient clinics						
BMI <65 years old						
p-value*		**<0.001**	0.25	0.42	**0.03**	**0.01**
Eutrophic	48 (28.7%)	36 (75.0%)	19 (39.6%)	25 (52.1%)	9 (18.8%)	22 (45.8%)
Overweight	63 (37.7%)	62 (98.4%)	19 (30.2%)	39 (61.9%)	21 (33.3%)	44 (69.8%)
Obese	56 (33.6%)	56 (100.0%)	25 (44.6%)	36 (64.3%)	24 (42.9%)	39 (69.6%)
BMI ≥ 65 years old						
p-value*		**<0.001**	0.12	0.47	0.71	0.24
Lean	5 (10.4%)	3 (60.0%)	1 (20.0%)	3 (60.0%)	1 (20.0%)	4 (80.0%)
Eutrophic	14 (29.2%)	12 (85.7%)	1 (7.1%)	6 (42.9%)	3 (21.4%)	6 (42.9%)
Overweight	29 (60.4%)	29 (100.0%)	10 (34.5%)	18 (62.1%)	10 (34.5%)	20 (69.0%)
Lifestyle						
Self-reported lifestyle						
p-value*		0.51	0.24	0.09	0.49	0.39
Active	71 (33.0%)	65 (91.5%)	22 (31.0%)	37 (52.1%)	23 (32.4%)	46 (64.8%)
Sedentary	144 (67.0%)	133 (92.4%)	53 (36.8%)	90 (62.5%)	45 (31.3%)	89 (61.8%)
Aerobic exercises						
p-value*		0.64	0.2	0.48	0.06	0.52
Yes	14 (6.5%)	11 (78.6%)	2 (14.3%)	7 (50.0%)	2 (14.3%)	10 (71.4%)
No	25 (11.6%)	22 (88.0%)	8 (32.0%)	14 (56.0%)	11 (44.0%)	19 (76.0%)
Strengthening exercises						
p-value*		0.16	0.2	0.12	0.45	0.38
Yes	5 (2.3%)	3 (60.0%)	0 (0.0%)	1 (20.0%)	1 (20.0%)	3 (60.0%)
No	34 (15.8%)	30 (88.2%)	10 (29.4%)	20 (58.8%)	12 (35.3%)	26 (76.5%)

Data are expressed in numbers and percentage between parentheses.
*statistical difference between groups p <0.05 (Fisher's Exact Test).

tissue, favoring not only weight gain, but also the progression of atherosclerotic processes. Estrogen deficiency is not the only predisposing factor for weight gain after menopause. It is often parallel to lower basal metabolic rate and a tendency to adopt a more sedentary lifestyle, subsequent to the aging process [29].

The risk of developing coronary artery disease related to sedentary life is 1.5 to 2.4 times higher when compared to hypertension, dyslipidemia and smoking. Aerobic physical activity of moderate intensity when performed on a regular basis (at least 30 minutes, three times a week), can have an impact in reducing the risk of cardiovascular events in the range 30–40% [11]. In this study, although 33% of women are self-reported as active (Table 2) and 74.4% consumed a normal caloric diet (less than 2000 kcal/day), there is a high prevalence of overweight and obesity as well as high WC. Only 8.8% actually performed aerobic or strengthening exercises sufficiently enough to have an impact on the prevention of cardiovascular diseases [12]. Women who do not perform physical activities were more likely to have high blood pressure (44%) when compared to those who were active (14.3%) (Table 5). Regular physical activities is an important therapeutic, non-pharmacological preventive method against cardiovascular events.

The following study limitations are important when interpreting the findings reported herein. First, the sample size was relatively small due to the type of the study design (cross-sectional study, based on a convenience sample). Thus, the study results do not reflect either the nutritional status, or the health patterns of the population of Rio de Janeiro. Second, the variation of food consumption exists between individuals (inter-individual variability) and in the same individual, in relation to daily intakes (intra-individual variability), and these are inherent to studies of this type. Moreover, estimated food consumption methods are marked by variations along the evaluation process, from obtaining individuals reported information to the compilation of data. These could lead to a misleading use of data related to food consumption patterns and their association with health outcomes. Third, although the 24-hour recall method is widely used for dietary assessments, the intake of a single day does not represent the daily intake of an individual. However, it has been carefully considered to be the most appropriate and feasible tool regarding both, the purpose of this study and the study population. According to Willett, applying a single 24-hour recall may be suitable for estimating mean intakes in groups, given a sample size that suits this purpose [30].

Conclusions

This study concludes that the women consumed a low quality diet attributed to the low intake of fruits, vegetables and greeneries and excessive sodium. These inappropriate eating practices have a negative impact on cardiometabolic risk factors of postmenopausal women who also showed a high prevalence of abdominal obesity. Furthermore, women presented increased lipids, fasting glucose as well as higher blood pressure levels that are recognized markers of increased cardiovascular risks.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
DAV, VMF, EGR, LPFM contributed to the conceptualization and design, interpretation and writing of the article. DAV and VMF developed the research protocol and conducted the data collection. EGR were responsible for data analyses. RAGS, CRMMC and MVMP have been involved in drafting the manuscript or revising it critically for important intellectual content. All authors critically reviewed the manuscript, approved the final version submitted for publication.

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Acknowledgement
Study conducted at the outpatient clinic of Instituto Fernandes Figueira, Oswaldo Cruz Foundation (IFF/FIOCRUZ). The research field data used here is part of the Academic Master's thesis of Danyelle de Almeida Ventura, defended and approved at IFF/FIOCRUZ, in March 2013.

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Sodium intake in US ethnic subgroups and potential impact of a new sodium reduction technology: NHANES Dietary Modeling

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Abstract

Background: Because excessive dietary sodium intake is a major contributor to hypertension, a reduction in dietary sodium has been recommended for the US population. Using the National Health and Nutrition Examination Survey (NHANES) 2007–2010 data, we estimated current sodium intake in US population ethnic subgroups and modeled the potential impact of a new sodium reduction technology on sodium intake.

Methods: NHANES 2007–2010 data were analyzed using The National Cancer Institute method to estimate usual intake in population subgroups. Potential impact of SODA-LO® Salt Microspheres sodium reduction technology on sodium intake was modeled using suggested sodium reductions of 20-30% in 953 foods and assuming various market penetrations. SAS 9.2, SUDAAN 11, and NHANES survey weights were used in all calculations with assessment across age, gender and ethnic groups.

Results: Current sodium intake across all population subgroups exceeds the Dietary Guidelines 2010 recommendations and has not changed during the last decade. However, sodium intake measured as a function of food intake has decreased significantly during the last decade for all ethnicities. "Grain Products" and "Meat, Poultry, Fish, & Mixtures" contribute about 2/3rd of total sodium intake. Sodium reduction, using SODA-LO® Salt Microspheres sodium reduction technology (with 100% market penetration) was estimated to be 185–323 mg/day or 6.3-8.4% of intake depending upon age, gender and ethnic group.

Conclusions: Current sodium intake in US ethnic subgroups exceeds the recommendations and sodium reduction technologies could potentially help reduce dietary sodium intake among those groups.

Keywords: Sodium intake, Ethnic subgroups, Sodium reduction modeling, NHANES, Sodium reduction technology

Background

The prevalence of hypertension in America has increased over the past 20 years in men, women, Blacks, and Whites [1]. Based on 2007 to 2010 data, 33% (about 78 million) of US adults have hypertension and African American adults have among the highest prevalence of hypertension (44%) in the world [2]. In 2010, high blood pressure was estimated to be responsible for $156 billion in direct and indirect health care cost [3]. Because excessive dietary sodium intake is a significant contributor to hypertension [3-9], limiting sodium intake has been recommended for the US population by US public health agencies and other expert scientific organizations, such as the American Heart Association [5,7,10,11]. The Dietary Guidelines for Americans 2010 [11] recommend a maximum dietary sodium intake of 2,300 mg/day for the general population and 1500 mg/day for at-risk groups, including African Americans, older adults (age 51 years and above), and persons of any age with hypertension, diabetes, or chronic kidney disease (about half of the US population). The World Health Organization (WHO) [12] recommends adults consume less than 2,000 mg of sodium, or 5 grams of salt. Regardless of these recommendations, dietary sodium intake in the US is well above that needed for physiological function and is greater than recommended.

Sodium is primarily consumed as sodium chloride and the majority of sodium in the diet comes from sodium

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added during food processing as an ingredient for flavor, processing aid, and for food safety purposes [11,13]. Processed foods contribute more than 75% of dietary sodium intake in the US diet; about 10% of dietary sodium occurs naturally in foods and another 5-10% is discretionary salt [4].

In this study, we used the most recent (2007–2010) data from the National Health and Nutrition Examination Survey (NHANES) to estimate the current sodium intake in population subgroups and modeled the potential impact of SODA-LO® Salt Microspheres sodium reduction technology on sodium intake. SODA-LO® is a sodium-reduction ingredient that can reduce sodium in certain applications through its technology that turns standard salt crystals into free-flowing, hollow salt microspheres, which efficiently delivers salt taste and functionality by maximizing surface area.

Methods

Study population

NHANES, a large dietary survey of a nationally representative sample of the non-institutionalized US population, was used to assess sodium intake and its sources in the diet of ethnic subgroups in the US population [14]. The NHANES data are collected and released by the National Center for Health Statistics (NCHS) of the Center for Disease Control and Prevention (CDC), every two years. All participants or proxies provided written informed consent and the Research Ethics Review Board at the NCHS approved the survey protocol. Dietary intake data with reliable 24-hour recall dietary interviews (day one via in-person interview at the Mobile Examination Center and day two via telephone interview) using USDA's automated multiple-pass method (AMPM) were used. The data from NHANES 2007–2008 and 2009–2010 were combined for the analyses [15]. The combined sample included 3,626 Mexican American; 5,559 other Hispanic; 7,369 non-Hispanic White and 3,568 non-Hispanic Black participants ages 2 years and older. Children under age 2 years and pregnant and/or lactating females were excluded from the analyses.

Estimation of sodium intake

The USDA Nutrient Database for Standard Reference (SR) Releases 22 & 24 were used in conjunction with the Food & Nutrient Database for Dietary Studies (FNDDS) versions 4.1 & 5.0, to determine the sodium derived from foods consumed by NHANES 2007–2008 and NHANES 2009–2010 participants respectively [16-19]. Unadjusted sodium values were used in all analyses. The mean usual intakes (long-run average daily intakes) of sodium from all foods were determined using the National Cancer Institute (NCI) method [20] for a single dietary component, because sodium is consumed at some level on most days. All analyses were adjusted for the complex survey design of NHANES using the appropriate sample weight. Covariates in the usual intake models included age and gender groups, day of the week of dietary recall (weekend/weekday), and interview sequence of the dietary recall (in person versus via telephone).

Estimation of food sources of sodium

Food groups for NHANES 2007–2008 and NHANES 2009–2010 dietary intake data were defined using the USDA FNDDS 4.1 & FNDDS 5.0 databases, respectively [16,18]. Data for over 7000 foods were collapsed into 9 broad categories of FNDDS food groups. Sodium consumption (mg/day) and amount of sodium as percent of total dietary intake (mg/kcal and mg/g food) were computed for all FNDDS food groups.

Sodium intake modeling analysis

SODA-LO® Salt Microspheres is a sodium-reduction ingredient which can reduce sodium in certain applications through its technology that turns standard salt crystals into free-flowing, hollow salt microspheres which efficiently delivers salt taste and functionality by maximizing surface area. A 20% to 30% reduction in sodium content in 953 foods (17 foods in "Milk & Milk Products" for 20% reduction, 304 foods in "Meat, Poultry, Fish & Mixtures" for 20 to 25% reduction, 20 foods in "Egg" for 25% reduction, 30 foods in "Dry Beans, Peas, Other Legumes, Nuts & Seeds" for 25% reduction, 511 foods in "Grain Products" for 25% reduction, 35 foods in "Vegetable" for 20 to 30% reduction, and 36 foods in "Fats, Oils & Salad Dressings" for 25% reduction) was modeled. Various scenarios for potential reduction in usual intake of sodium were then computed by using a 0-100% reduction factor and 10-100% market penetration. The individual reductions were computed for foods using the reduction factor and market penetration factor, and were used to model usual intake after sodium reduction.

Statistical methods

SAS 9.2 (SASs Institute, Inc.; Cary, NC) and SUDAAN 11 (Research Triangle Institute, Research Triangle Park, NC, USA,) were used for all calculations. NHANES survey weights, strata and primary sampling units were used in all calculations to adjust for oversampling of certain groups, non-response by some selected sample persons, and to adjust for the complex sample design of NHANES to ensure nationally representative results. Data are presented as means ± standard errors (SE). $P < 0.01$ was considered statistically significant.

Results

Usual intakes of sodium across age, gender and ethnic groups are shown in Figure 1. Intake of sodium was

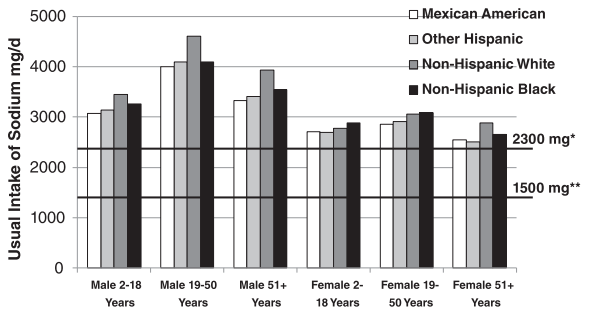

Figure 1 Usual intake of sodium by age and gender groups in population subgroups. Data from NHANES 2007–2010. Usual intakes from foods were estimated by using the National Cancer Institute method. *Dietary Guidelines for Americans 2010 recommended level for general population; **Dietary Guidelines for Americans 2010 recommended level for at risk group.

dependent on age, gender and ethnicity. The usual intake of adults (age 19–50 years) of any gender and ethnicity was higher compared to children (age 2–18 years) and older adults (age 51 years and above) of the same gender and ethnicity. The age related differences in usual intakes were much more pronounced in males than in females of any ethnicity. Males of any age and ethnic group consumed more sodium than females of the corresponding age and ethnic group. Non-Hispanic White (especially males) consumed more sodium than other ethnic groups (Figure 1). Usual intakes of all age, gender and ethnic groups were higher than 2300 mg/day. Intakes of sodium were below 1500 mg/day for less than 5% population of any age, gender and ethnicity (except for older adult Other Hispanic females).

Trends in sodium intake over the last 10 years (5 NHANES cycles) among adults (ages 19–50 years) and older adults (age 51 years and above) of different ethnicity are shown in Figure 2. Average sodium intake in each NHANES cycle was higher than 1500 mg/day as well as 2300 mg/day for all adults and older adults irrespective of gender and ethnicity. Moreover, in each NHANES cycle, male adults (ages 19–50 years) and male older adults (age 51 years and above) consistently consumed more sodium than female adults and female older adults respectively in all ethnic groups. Average intake of sodium (mg/day) during the past 5 NHANES cycles did not change significantly ($P > 0.01$) for adults and older adults for any gender or ethnic group (Figure 2). Similarly, the average intake of sodium among children (ages 2–18 years) over the past 5 NHANES cycles was always higher than 2300 mg/day and did not change significantly ($P > 0.01$) for any gender and ethnic group (data not presented).

Sodium intake was also measured as a function of energy intake (mg/kcal) and as a function of total food intake (mg/g food) in addition to absolute intake (mg/day) for all NHANES cycles in males and females of all ethnicities. Table 1 shows trends in sodium intake by different measures over the last 10 years (5 NHANES cycles). While there was no change in sodium intake measured as mg/day (absolute amount) and as mg/kcal (function of energy intake) for male or female adults or older adults of any ethnic subgroup, sodium intake measured as a function of food intake (mg/g food) decreased significantly ($P < 0.01$) among all adult and older adult males and females of all ethnicity (except for older adult male other Hispanic). The sodium intake values in adults (age 19 years and older) of all ethnicity were 3586 mg or 1.68 mg/kcal or 1.56 mg/g food in 2001–2002 and 3607 mg or 1.72 mg/kcal or 1.12 mg/g food in 2009–2010.

The contribution of various food groups (FNDDS defined 9 food groups) to the sodium in the diets of US adults and older adults by population subgroups is shown in Figure 3. No major overall age, gender or ethnicity related differences were noted. “Grain Products” were the top most contributors of dietary sodium, followed by “Meat, Poultry, Fish & Mixtures”. These two food groups contributed 60-70% of total sodium intake in adults and older adults. “Milk & Milk Products”, and “Vegetables”, were the next two major sodium contributors, providing more than 15% of total sodium. These four food groups (“Grain Products”, “Meat, Poultry, Fish & Mixtures”, “Milk & Milk Products”, and “Vegetables”) combined were responsible for more than 85% of total dietary sodium for all ethnic subgroups. The remaining five food groups (“Eggs”, “Dry Beans, Peas, Other Legumes, Nuts & Seeds”, “Fruits”, “Fats, Oils & Salad Dressings”, and “Sugars, Sweets & Beverages”) contributed less than 15% of the sodium in the diet (Figure 3).

Table 2 shows the maximum achievable reduction (using the maximum reduction factor and 100% market penetration) in sodium intake across all food categories to be 185–323 mg (6.3-8.4%). A lower reduction factor and/or lower market penetration would provide lower reductions. A somewhat higher reduction is expected for non-Hispanic Whites and non-Hispanic Blacks compared to Mexican Americans and Other Hispanics (Table 2). “Grain Products” and “Meat, Poultry, Fish & Mixtures” were the main contributors of sodium reduction, contributing to more than 80% of total sodium reduction. Sodium reduction in “Grain Products” contributed to 60-70% of total sodium reduction for Mexican Americans, other Hispanics and non-Hispanic Whites, and 50-60% of total sodium reduction for non-Hispanic Black adults and older adults. Sodium reduction in “Meat, Poultry, Fish & Mixtures” contributed about 16-24% of total sodium reduction for Mexican Americans, other Hispanics and non-Hispanic Whites, and about 25-30% of total sodium reduction for non-Hispanic Blacks adults and older adults (Table 3).

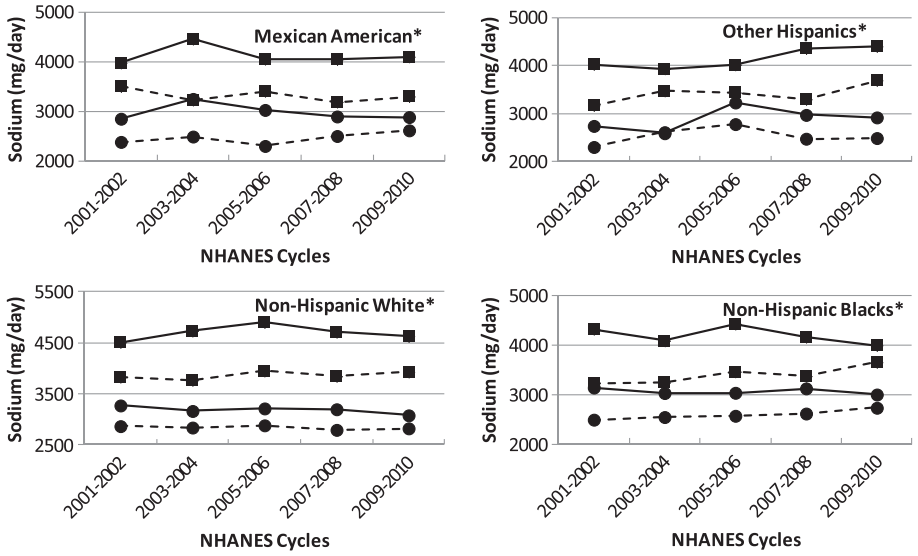

Figure 2 Sodium intake trends by age and gender groups over 5 NHANES cycles in population subgroups. Data from NHANES 2001–2010. Square: male, Circles: females, solid line: 19–50 years, dotted line: 51 + years. Usual intakes from foods were estimated by using the National Cancer Institute method. *No change over 5 NHANES cycles, P > 0.01.

Discussion

Average sodium intake across all age, gender and ethnic subgroups in 2007–2010 significantly exceeded most dietary recommendations, including those of the Dietary Guidelines for Americans 2010, American Heart Association 2010, World Health Organization 2012, Institute of Medicine's 2005 defined Adequate Intake (AI) or Tolerable Upper Intake Level (UL) and other scientific and public health organizations [4,11,12,21,22]. The Dietary Guidelines for Americans 2010 recommend that at risk population groups, such as older adults (age 51+ years) and African Americans of any age, should limit sodium

Table 1 Sodium intake trends by age and gender groups in population subgroups over 5 NHANES cycles

Ethnicity	Age (Years)	Gender	Sodium intake trend					
			mg/day		mg/g food		mg/kcal	
			beta*	P**	beta*	P**	beta*	P**
Mexican American	19-50	Male	−19.33	0.7652	−0.10	<0.0001	0.03	0.0342
		Female	−34.38	0.2673	−0.14	<0.0001	0.02	0.0544
	51+	Male	−39.13	0.4792	−0.09	<0.0001	−0.01	0.5585
		Female	53.75	0.1116	−0.13	<0.0001	0.02	0.1729
Other Hispanic	19-50	Male	110.83	0.0913	−0.10	<0.0001	0.04	0.0327
		Female	63.23	0.1697	−0.12	0.0004	0.04	0.0635
	51+	Male	91.07	0.4067	−0.11	0.0508	0.03	0.4286
		Female	26.09	0.6285	−0.14	<0.0001	0.03	0.1452
Non-Hispanic White	19-50	Male	23.42	0.5281	−0.12	<0.0001	0.03	0.0012
		Female	−34.81	0.1798	−0.15	<0.0001	0.01	0.2341
	51+	Male	29.05	0.3438	−0.11	<0.0001	0.00	0.6550
		Female	−13.25	0.4582	−0.13	<0.0001	−0.01	0.1134
Non-Hispanic Black	19-50	Male	−59.10	0.2367	−0.14	<0.0001	0.02	0.1816
		Female	−18.59	0.6458	−0.19	<0.0001	0.02	0.0936
	51+	Male	100.06	0.0619	−0.12	<0.0001	−0.02	0.3977
		Female	56.50	0.1167	−0.14	<0.0001	0.03	0.0242

Data from NHANES 2001–2010. Sodium intake was measured as absolute intake (mg/day) as a function of energy intake (mg/kcal) and as a function of food intake (mg/g food).
*beta – regression coefficient; **P < 0.01 significant.

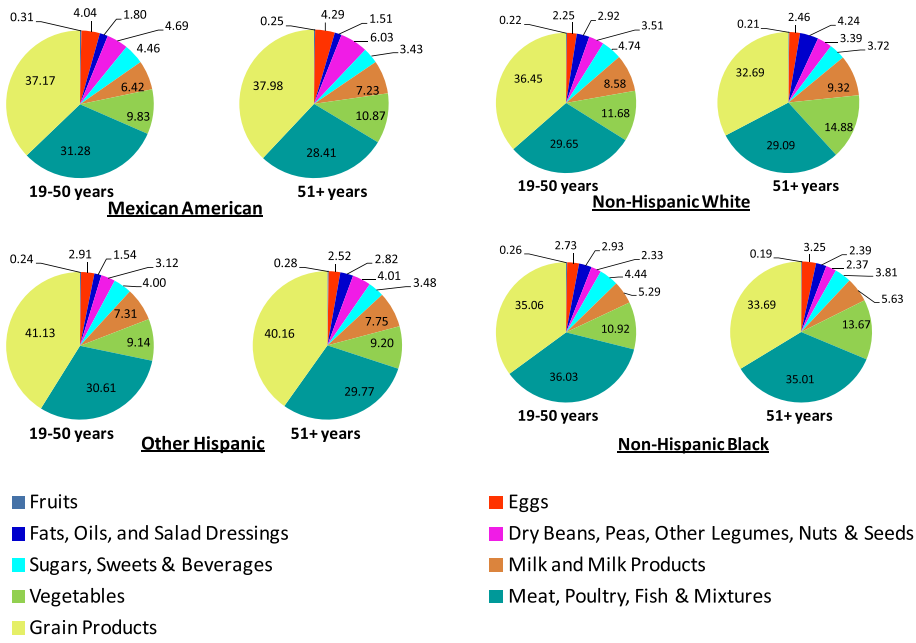

Figure 3 Dietary sodium contribution from nine FNDDS food groups by age groups in population subgroups. Data from NHANES 2007–2010. Data is presented as % of total dietary sodium.

intake to 1500 mg/day [11]. However, the average intake of non-Hispanic Blacks of any age/gender (except for female children and older adults) and male older adults of any ethnicity were more than twice the US Dietary Guideline recommendations and only a small proportion of the population (less than 5%) met these recommendations. Sodium intake above recommendations is a global issue as demonstrated by high average sodium intakes in other countries [23]. Current evidence suggests that excessive sodium intake is a risk factor for hypertension and consequent health outcomes, including coronary heart disease (CHD), stroke, and mortality [5-9].

The average intake of sodium was age and gender dependent, with males consuming more sodium than females, and adults (ages 19–50 years) consuming more sodium than children (ages 2–18 years) and older adults (age 51 years and above). This observation is most likely due to higher food and caloric intake among males and adults ages 19–50 years. In addition to age and gender dependence, the average intake of sodium was also dependent on ethnicity. The average intake of sodium was highest for Non-Hispanic Whites followed by non-Hispanic Blacks, and other Hispanics and Mexican Americans in every age and gender group (except for non-Hispanic Black female children and female adults). Earlier studies comparing ethnic subgroups also reported a higher mean intake of sodium among non-Hispanic Whites compared to non-Hispanic Blacks and Mexican Americans [13,24-26]. These

Table 2 Potential sodium intake reduction with SODA-LO® Salt Microspheres (Sodium Replacement Technology) in population subgroups

Ethnicity	Age	Potential reduction* (mg)	Current intake (mg)	Potential intake after reduction (mg)	% Reduction*
Mexican American	19-50 years	250 ± 14	3558 ± 68	3309 ± 59	6.8 ± 0.3
	51+ years	196 ± 12	2898 ± 86	2702 ± 78	6.8 ± 0.4
Other Hispanic	19-50 years	252 ± 11	3599 ± 45	3347 ± 39	6.9 ± 0.3
	51+ years	185 ± 11	2916 ± 68	2731 ± 63	6.3 ± 0.3
Non-Hispanic White	19-50 years	323 ± 8	3903 ± 55	3581 ± 53	8.3 ± 0.2
	51+ years	242 ± 5	3320 ± 49	3078 ± 47	7.3 ± 0.2
Non-Hispanic Black	19-50 years	296 ± 10	3544 ± 66	3248 ± 60	8.4 ± 0.2
	51+ years	222 ± 9	3046 ± 74	2824 ± 67	7.2 ± 0.2

Data from NHANES 2007–2010. Potential reduction was modeled using 20% to 30% targeted maximum reduction in sodium content in 953 foods with 100% market penetration.
*Average of reductions in individuals.

Table 3 Potential sodium intake reduction with SODA-LO® salt microspheres in population subgroups by FNDDS food groups

	Potential sodium intake reduction, mg & (%)			
	Mean ± SE			
	Mexican American	Other Hispanic	Non-Hispanic white	Non-Hispanic black
All foods (953 foods; 20-30% targeted reduction in sodium content)				
Male, 19–50 Years	294 ± 20 (6.9 ± 0.4)	297 ± 15 (7.0 ± 0.3)	398 ± 13 (8.7 ± 0.2)	338 ± 13 (8.3 ± 0.3)
Male, 51+ Years	236 ± 19 (7.3 ± 0.5)	222 ± 18 (6.7 ± 0.5)	290 ± 7 (7.4 ± 0.2)	258 ± 14 (7.2 ± 0.2)
Female, 19–50 Years	193 ± 10 (6.7 ± 0.3)	198 ± 9 (6.8 ± 0.3)	248 ± 7 (8.0 ± 0.2)	259 ± 14 (8.4 ± 0.3)
Female 51+ Years	161 ± 7 (6.3 ± 0.3)	152 ± 7 (6.0 ± 0.3)	200 ± 6 (7.2 ± 0.2)	195 ± 12 (7.2 ± 0.3)
Milk & milk products (17 foods; 20% targeted reduction in sodium content)				
Male, 19–50 Years	2 ± 1 (0.2 ± 0.1)	2 ± 1 (0.2 ± 0.1)	4 ± 1 (0.3 ± 0.1)	0.3 ± 0.2 (0.1 ± 0.0)
Male, 51+ Years	0.2 ± 0.1 (0.02 ± 0.01)	0.3 ± 0.2 (0.1 ± 0.0)	5 ± 1 (0.5 ± 0.1)	1 ± 0 (0.2 ± 0.2)
Female, 19–50 Years	2 ± 1 (0.4 ± 0.2)	2 ± 1 (0.3 ± 0.1)	5 ± 1 (0.5 ± 0.1)	2 ± 1 (0.4 ± 0.1)
Female 51+ Years	5 ± 2 (0.6 ± 0.2)	3 ± 1 (0.4 ± 0.1)	6 ± 1 (0.9 ± 0.1)	2 ± 1 (0.4 ± 0.2)
Meat, poultry, fish & mixtures (304 foods; 20-25% targeted reduction in sodium content)				
Male, 19–50 Years	67 ± 9 (4.7 ± 0.5)	72 ± 9 (4.8 ± 0.4)	87 ± 5 (5.5 ± 0.3)	117 ± 7 (7.4 ± 0.3)
Male, 51+ Years	44 ± 6 (4.4 ± 0.6)	39 ± 6 (3.9 ± 0.5)	59 ± 4 (4.5 ± 0.2)	79 ± 5 (5.5 ± 0.3)
Female, 19–50 Years	44 ± 7 (5.1 ± 0.6)	43 ± 5 (4.9 ± 0.5)	53 ± 4 (5.8 ± 0.4)	92 ± 7 (8.2 ± 0.5)
Female 51+ Years	37 ± 6 (4.8 ± 0.5)	31 ± 5 (3.9 ± 0.5)	31 ± 3 (3.9 ± 0.3)	49 ± 5 (5.0 ± 0.5)
Eggs (20 foods; 25% targeted reduction in sodium content)				
Male, 19–50 Years	4 ± 2 (1.2 ± 0.5)	3 ± 1 (1.0 ± 0.4)	4 ± 1 (1.6 ± 0.4)	4 ± 2 (1.2 ± 0.5)
Male, 51+ Years	2 ± 1 (0.4 ± 0.3)	1 ± 1 (0.3 ± 0.3)	5 ± 1 (2.0 ± 0.5)	4 ± 1 (1.0 ± 0.4)
Female, 19–50 Years	3 ± 1 (1.3 ± 0.5)	3 ± 1 (1.3 ± 0.4)	3 ± 1 (2.1 ± 0.7)	4 ± 2 (2.0 ± 0.7)
Female 51+ Years	2 ± 1 (0.6 ± 0.3)	1 ± 1 (0.4 ± 0.2)	2 ± 1 (0.7 ± 0.3)	3 ± 2 (0.7 ± 0.4)
Dry beans, peas, other legumes, nuts & seeds (30 foods; 25% targeted reduction in sodium content)				
Male, 19–50 Years	5 ± 1 (3.3 ± 0.6)	4 ± 1 (3.0 ± 0.5)	7 ± 1 (9.6 ± 0.8)	3 ± 0.7 (8.4 ± 0.9)
Male, 51+ Years	4 ± 1 (4.3 ± 0.9)	3 ± 1 (4.1 ± 0.8)	8 ± 1 (10.7 ± 0.8)	4 ± 1 (6.6 ± 1.0)
Female, 19–50 Years	2 ± 1 (4.0 ± 0.7)	2 ± 0.4 (4.6 ± 0.6)	5 ± 1 (8.0 ± 0.7)	2 ± 0.5 (7.4 ± 1.3)
Female 51+ Years	2 ± 1 (4.1 ± 0.9)	3 ± 1 (5.1 ± 0.8)	5 ± 0.4 (11.1 ± 0.8)	2 ± 0.4 (7.3 ± 1.0)
Grain products (511 foods; 25% targeted reduction in sodium content)				
Male, 19–50 Years	195 ± 16 (13.4 ± 0.8)	195 ± 13 (12.6 ± 0.6)	253 ± 11 (15.1 ± 0.4)	179 ± 10 (13.0 ± 0.4)
Male, 51+ Years	166 ± 13 (14.7 ± 0.6)	158 ± 13 (13.4 ± 0.6)	176 ± 6 (14.4 ± 0.4)	145 ± 12 (13.4 ± 0.5)
Female, 19–50 Years	125 ± 5 (11.2 ± 0.3)	132 ± 6 (11.3 ± 0.4)	156 ± 5 (14.0 ± 0.2)	126 ± 8 (11.5 ± 0.5)
Female 51+ Years	99 ± 6 (11.6 ± 0.6)	100 ± 4 (11.4 ± 0.5)	132 ± 5 (14.1 ± 0.3)	117 ± 7 (13.3 ± 0.5)
Vegetables (35 foods; 20-30% targeted reduction in sodium content)				
Male, 19–50 Years	18 ± 2 (5.0 ± 0.4)	17 ± 2 (5.0 ± 0.4)	37 ± 3 (7.1 ± 0.4)	29 ± 3 (8.5 ± 0.7)
Male, 51+ Years	15 ± 2 (4.7 ± 0.6)	14 ± 2 (4.6 ± 0.4)	25 ± 2 (4.7 ± 0.3)	18 ± 2 (4.3 ± 0.4)
Female, 19–50 Years	15 ± 1 (4.4 ± 0.4)	14 ± 1 (5.0 ± 0.4)	20 ± 1 (5.8 ± 0.4)	28 ± 3 (7.8 ± 0.6)
Female 51+ Years	12 ± 2 (3.6 ± 0.7)	10 ± 2 (3.3 ± 0.5)	16 ± 1 (3.9 ± 0.3)	17 ± 2 (5.3 ± 0.6)
Fats, oils & salad dressings (36 foods; 25% targeted reduction in sodium content)				
Male, 19–50 Years	4 ± 1 (16.1 ± 1.3)	4 ± 0 (16.4 ± 1.0)	7 ± 1 (14.4 ± 0.9)	5 ± 1 (14.4 ± 0.9)
Male, 51+ Years	4 ± 1 (6.0 ± 1.6)	6 ± 1 (15.8 ± 1.3)	12 ± 1 (15.5 ± 0.6)	8 ± 1 (18.6 ± 0.9)
Female, 19–50 Years	2 ± 0 (11.5 ± 1.0)	3 ± 0 (12.0 ± 0.8)	6 ± 1 (13.5 ± 0.7)	4 ± 1 (13.4 ± 1.2)
Female 51+ Years	4 ± 1 (16.1 ± 0.8)	4 ± 1 (15.6 ± 0.8)	9 ± 1 (13.6 ± 0.6)	6 ± 1(16.4 ± 0.8)

Data from NHANES 2007–2010. Potential reduction was modeled using 20% to 30% targeted maximum reduction in sodium content in 953 foods with 100% market penetration.

observed differences between ethnic population subgroups may be related to differences in dietary patterns. While non-Hispanic Blacks consume slightly less sodium than non-Hispanic Whites, it is recommended that they limit sodium intake to 1500 mg/day due to their likely sensitivity to sodium with a greater response to blood pressure-raising effects of sodium [27,11].

Our data on ethnic subgroups also showed that sodium intake has not changed in the past decade and has consistently remained higher than the Dietary Guidelines recommendations. There is very limited data available on sodium intake trends among ethnic subgroups in the US population. An analysis of 38 studies from 1957–2003 did not find any significant temporal trend in 24 h sodium excretion among males or females, or Blacks or Whites participants [28]. Another recent analysis of NHANES data reported a slight decline in mean sodium intake but no change in sodium density during 2003 to 2010 and suggested this to be related to declines in calorie consumption [29]. In the present study, sodium intake was also measured as a function of total food intake and total calorie intake, in addition to absolute intake. While there was no change in the absolute amount of sodium intake, we noted a significant decrease in sodium intake as a function of food intake in all ethnic subgroups over the last decade. This suggests that a reduction in the sodium content of foods occurred, but with a consequent increase in food intake during 2001–2010, potentially offsetting the decrease in sodium and resulting in no change in total sodium intake (mg/day) of the population. This finding emphasizes the need for innovative food technologies to help further reduce the sodium content of foods.

Different foods contain different amounts of sodium, which is either added (for flavor, food processing, and/or food safety) or occurs naturally [4,11,13]. However, both sodium content of foods and the frequency of food consumption contribute to sodium intake. Often the problem of excess sodium intake is due to frequently consumed foods which might be moderate in their sodium content [4]. Studies from CDC and NCI [30,31] analyzing NHANES 2005–2006 data, reported that grain and meat products were the top contributors of sodium in the US diet. The present study, using the most recent NHANES dataset (2007–2010) for various ethnic subgroups, shows that two food groups "Grain Products" and "Meat, Poultry, Fish & Mixtures" jointly provide 2/3 of dietary sodium, and four food groups "Grain Products", "Meat, Poultry, Fish & Mixtures", "Milk & Milk Products", and "Vegetables", jointly provide 4/5 of the dietary sodium for all age, gender and ethnic subgroups. These data suggest that sodium reduction strategies aiming at "Grain Products" and "Meat, Poultry, Fish & Mixtures", the two major food group sources of sodium in the diet, could have a bigger impact on dietary sodium reduction compared to other food groups.

Continued high sodium intake across the population, despite consistent recommendations to limit sodium and the accumulating evidence linking excessive sodium intake to hypertension, has led to calls for population wide interventions to reduce sodium in the US diet [13,32]. However, current sodium intake status (2007–2010 NHANES) and the sodium intake trend (2001–2010 NHANES) data presented above for population subgroups indicate that there has been no significant progress in the reduction of sodium intake. Our present dietary sodium modeling data using SODA-LO® Salt Microspheres, a sodium-reduction ingredient at its potential usage level in 953 foods shows a 185–323 mg/day reduction in sodium intake which translates to about 6.3-8.4% reduction of current sodium intake in the US population by ethnic subgroups. Utilizing this technology could add to the stepwise reduction in sodium content of foods that the food industry has been implementing.

Dietary sodium reduction is an important target for public health improvement, as reduced sodium intake has been demonstrated to reduce blood pressure and is also associated with a reduced risk of stroke and fatal coronary heart disease in adults [5-9,33,34]. Dietary sodium reduction is estimated (using statistical modeling) to be cost effective, may potentially improve overall health and provide substantial healthcare cost benefits [35-41]. Using the CHD Policy Model (a computer simulation of heart disease in US adults) to quantify the benefits of sodium reduction, Bibbins-Domingo et al. recently estimated that reducing dietary salt by 3 g (1200 mg sodium) per day would reduce CHD, stroke and myocardial infarction, prevent deaths, and save $10-24 billion in health care costs annually [35]. Interpolation of these data [35] suggest a potential for 0.45 to 0.88 mm Hg reduction in systolic blood pressure and $3.0 to 5.3 billion in reductions in health care cost with a 300 mg/day decrease in sodium intake. The present study demonstrated a possible 185–323 mg/day reduction in current sodium intake using SODA-LO®, which could lead to the 300 mg/day decrease in sodium that is attributed to blood pressure and health care cost reductions. Substantially higher potential health benefits due to sodium reduction are expected in at-risk groups, including African Americans, older adults (age 51 years and above), and persons of any age with hypertension, diabetes, or chronic kidney disease, as they may be more responsive to blood pressure-raising effects of sodium [11].

SODA-LO® Salt Microspheres is a sodium-reduction ingredient that converts standard salt crystals into free-flowing, hollow salt microspheres which efficiently delivers salt taste and functionality by maximizing surface area. As with many other sodium reduction approaches,

such a technology could modestly increase the cost of some foods, however the potential health benefits, health improvement and reduced healthcare costs from dietary sodium reduction is expected to vastly out-weigh the cost of the technology. Additionally, use of sodium reduction technologies which do not alter flavor may potentially delay the consumer's palate adaptation for less salt. However, changing consumer behavior is difficult and some attempts to lower dietary salt intake on an individual basis have largely proved to be ineffective [42]. Moreover, changing the palate may require a significant amount of time; in the interim technologies like SODA-LO® Salt Microspheres can provide an immediate solution for sodium intake reduction. Further research is needed to evaluate the effects of sodium reduction in the marketplace as the sodium reduction technologies, such as SODA-LO®, are introduced.

A major strength of our study is the use of a large nationally representative population sample to assess the total usual intake of sodium with the NCI method. One of the limitations of our study was the cross-sectional nature of NHANES data, which prevents definitive conclusions or causality.

Conclusions

In conclusion, this study reports that current sodium intake in the US in all ethnic subgroups exceeds public health recommendations. Sodium reduction using the technology of SODA-LO® Salt Microspheres could potentially reduce dietary sodium intake by 6-8% in these subgroups.

Abbreviations

AI: Adequate intake; AMPM: Automated multiple-pass method; CDC: Center for disease control and prevention; CHD: Coronary heart disease; FNDDS: Food & nutrient database for dietary studies; NCHS: National center for health statistics; NCI: National Cancer Institute; NHANES: National Health and Nutrition Examination Survey; SE: Standard error; SR: Standard release; UL: Tolerable upper intake level; WHO: World Health Organization.

Competing interests

VLF and SA are nutrition consultants and provide services to food industry. At the time of the research LS and PS were both full time employees of Tate & Lyle Ingredients Americas LLC. PS has since left Tate & Lyle. The work was funded by Tate & Lyle Ingredients Americas LLC.

Authors' contributions

VLF – participated in the design, NHANES dietary data analysis, dietary modeling, interpretation of the data, revising the manuscript and the approval of final version; SA – participated in interpretation of the data, drafting the manuscript, revising the manuscript and the approval of final version; LS – participated in the design, NHANES dietary data analysis, dietary modeling, interpretation of the data, revising the manuscript and the approval of final version; PS – conceptualized the research, participated in the design, NHANES dietary data analysis, dietary modeling, interpretation of the data, revising the manuscript and the approval of final version.

Acknowledgement

The present study was funded by Tate & Lyle Ingredients Americas LLC. The research and all publications arising out of or referable to it are considered proprietary data to which Tate & Lyle Ingredients Americas LLC claims exclusive right of reference in accordance with Regulation (EC) no. 1924/2006 of the European Parliament and of the Council on Nutrition and Health Claims Made on Foods.

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Survival and digestibility of orally-administered immunoglobulin preparations containing IgG through the gastrointestinal tract in humans

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Abstract

Oral immunoglobulin (Ig) preparations are prime examples of medicinal nutrition from natural sources. Plasma products containing Ig have been used for decades in animal feed for intestinal disorders to mitigate the damaging effects of early weaning. These preparations reduce overall mortality and increase feed utilization in various animal species leading to improved growth. Oral administration of Ig preparations from human serum as well as bovine colostrum and serum have been tested and proven to be safe as well as effective in human clinical trials for a variety of enteric microbial infections and other conditions which cause diarrhea. In infants, children, and adults, the amount of intact IgG recovered in stool ranges from trace amounts up to 25% of the original amount ingested. It is generally understood that IgG can only bind to antigens within the GI tract if the Fab structure is intact and has not been completely denatured through acidic pH or digestive proteolytic enzymes. This is a comprehensive review of human studies regarding the survivability of orally-administered Ig preparations, with a focus on IgG. This review also highlights various biochemical studies on IgG which potentially explain which structural elements are responsible for increased stability against digestion.

Keywords: Serum-derived bovine immunoglobulin, Bovine colostrum, Serum-derived human immunoglobulin, Immunoglobulin digestion, Oral immunoglobulin

Introduction

The biological role of immunoglobulins (Ig) in the protection of the gastrointestinal (GI) tract is well-established, particularly the role of Ig in colostrum and breast milk [1,2]. Hence, colostrum and breast milk are categorized as medicinal nutrition. Due to the need for pasteurization, however, Ig preparations from these sources will have inconsistent amounts of IgG since the amount of IgG denaturation depends upon both the quality of the colostrum and exact method of pasteurization [3]. Efficacy of these formulations in various enteropathies in humans also depends upon the Ig surviving past the stomach into the small and large bowels.

It is generally well-recognized that secretory IgA (sIgA), the primary class of Ig in human colostrum and breast milk as well as being found in the intestinal tract, are stable against enzymatic degradation. Other Igs, such as IgG and IgM, are also less susceptible than typical dietary proteins to digestion, yet this knowledge remains underappreciated despite numerous clinical studies in humans which illustrate recovery of intact and immunologically active IgG from the ileum and feces [4-7]. The purpose of this review is to summarize human clinical studies which assess digestibility of IgG purified from either colostrum or serum in ileal aspirates and stool. This review also summarizes *in vitro* biochemical studies that have assessed structural features of IgG which contribute to their overall stability and discusses for the first time the proposed structural basis for resistance to digestion in the GI tract. With the introduction to the market of the first nutritional therapy in the form of a physician supervised medical food containing high levels of IgG from bovine sera, it is important to understand the pharmacokinetics of these molecules. This understanding is necessary for the usefulness of any Ig-containing formulation

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as a natural therapeutic for the management of intestinal disorders [8].

Review

Immunoglobulin survival through the gastrointestinal tract: *clinical data*

Numerous studies have been performed demonstrating that Ig preparations derived from human sera as well as bovine colostrum and sera survive past the stomach, throughout the GI tract, and are present in fecal matter. These studies are summarized in Table 1. IgG was the predominant Ig in these preparations, but often either IgA and/or IgM was present in smaller amounts.

Recovery in infants

Many of the first published clinical studies on the digestibility of orally-administered IgG were conducted in infant populations. Zinkernagel et al. [9] fed 10 healthy infants at less than three weeks of age a lyophilized bovine colostrum preparation containing 70% IgG administered at 2 g/kg/d. An average 13%-20% of the Ig survived undigested in the stool as demonstrated by an agglutination assay against *E. coli* antigens [9]. In addition, recovered titer correlated with the amount of undigested IgG. In another study, 6 healthy immature, formula-fed infants ingested a 10% human immune serum globulin (HISG), predominantly IgG, in divided doses of 1 to 8 ml/kg/day over 5 consecutive days [10]. The survival of IgG in stool over a 24 hour period ranged between 4-12% of the original IgG ingested. Variability per subject was observed in the survival of active IgG in feces; however, increasing doses were associated with higher amounts of IgG excreted per day. There was no evidence of systemic absorption or adverse events.

In a larger randomized, controlled clinical trial, low-birth weight infants unable to breast feed were administered 600 mg daily of serum-derived human IgA (73%) and IgG (26%). The test group (n = 91) ingested the serum-derived IgA-IgG mixed into either infant formula or infant formula combined with pooled, pasteurized human milk. The control group (n = 88) ingested the same formula/milk preparation, less the serum-derived IgA-IgG [11]. The infants receiving oral IgA-IgG had fewer cases of necrotizing colitis (0 cases) compared to the controls (6 cases) and had "substantial amounts" of intact IgA and IgG recovered in stool compared to the controls. As in the previous study, there was no evidence of systemic absorption.

Bovine milk Ig concentrate purified from hyperimmunized cows against four human rotavirus serotypes has also been studied in a group (n = 164) of low birth weight infants [12]. The infants were dosed at 2 g Ig concentrate/kg/day for five days. Of the infants receiving Ig, stool samples from 47% had detectable bovine IgG and 43% maintained rotavirus-neutralization activity against bovine rotavirus V1005, human rotavirus Wa (serotype 1) and simian rotavirus SA-11 in cell culture. Furthermore, the infants with high amounts of neutralizing activity still present in feces demonstrated clinical benefit [12].

Recovery in children

One small (N = 3) and another larger study (N = 105) have been performed to assess recovery of active IgG against rotavirus from feces of children [13,14]. The smaller study was comprised of three pediatric patients ages 16 months and 4 yr, both with severe combined immunodeficiency disease, and 18-yr-old with common variable immunodeficiency disease [13]. All three had a history of intermittent positive excretion of rotavirus serotype 1 with chronic diarrhea, decreased weight gain and fat malabsorption. The children ingested a single dose of 150 mg/kg human sera Ig (IgG at 50 mg/ml) labeled with ^{125}I. Approximately 50% of the recovered radioactivity was excreted in the stools over a 3 d period. Half of the excreted radioactively labeled IgG, or 25% of the originally ingested IgG, retained immunological activity, as determined by the recovery of ^{125}I-labeled Ig bound to rotavirus [13].

In the second study, children drank 100 ml of whole cow's milk supplemented with hyperimmunized bovine colostrum against rotavirus, 3 times daily for a period of 6 days [14]. There were five groups based on the rotavirus-antibody titer of the Ig formulation: control (no rotavirus antibody titer), 1:2,500, 1:5,100, 1:8,000, and 1:8,200. After pooling results from the four experimental groups, approximately 5% of IgG was recovered while the level of antibody activity varied considerably. Approximately 88% of the experimental patients had detectable neutralization signals which correlated (r = 0.81) with percent reduction of rotavirus from stools and the titer of ingested milk/colostrum.

Recovery in adults

Two studies in adults measured the ileal recovery of orally-administered Igs [4,6]. Healthy, fasted subjects (n = 7) drank 400 ml of a ^{15}N-labeled bovine colostrum-derived Ig fraction, containing approximately ~5.2% IgG, 0.86% IgM, and 0.1% IgA (~2 g IgG, 0.34 g IgM, and 0.04 g IgA) and ileal effluents were collected every 20 min for 8 hrs [4]. Approximately 19% of IgG and IgM was still immunologically active from ileal effluent, although the titer was not reported. The authors also reported that 59% of IgG collected from 2 subjects in the jejunum remained active. Further purification of the ileal chyme samples using protein chromatography demonstrated that the fractions with the most immunological activity had a molecular weight of ~100 kDa

Table 1 Clinical studies assessing survival of orally-administered immunoglobulin preparations through the digestive tract in humans

Population (n), health status	Donor species	Preparation	Amount of Ig ingested daily	Material*	Recovery	Recovered immunological activity	Reference
Infant (10), healthy	Bovine	Powder	2 g/kg	IgG (70%), C	13%	Yes (titer correlated with % IgG recovered)	Zinkernagel, et al. (1972) [9]
Infant (6), healthy	Human	Liquid	1 – 8 ml/kg (152 – 1120 mg of IgG)	IgG (~99%), S	4-12%	Yes (titer, 1:2)	Blum, et al. (1981) [10]
Infant (179), low birth weight	Human	Liquid	600 mg	IgA (73%) –IgG (26%), S	1-10 mg/g of dry feces	NR	Eibl et al. (1988) [11]
Infant (164), rotaviral gastroenteritis	Bovine (hyperimmunized)	Liquid	2 g of concentrate/kg	IgG (% NR), M	10%	Yes (titer, 1:48)	Hilpert, et al. (1987) [12]
Children (3), immune deficiency with chronic diarrhea/rotavirus	Human	Liquid	150 mg/kg	IgG (% NR), S	~25%	Yes (titer NR, recovery as intact immune complex, Ig + rotavirus)	Losonsky, et al. (1985) [13]
Children (105), healthy	Bovine (hyperimmunized)	Liquid (one Powder Group)	NR as gram of Ig	Ig (% NR), C	5%	Yes (titer correlated with initial dose)	Pacyna, et al. (2001) [14]
Adult (65), cholera	Bovine (hyperimmunized)	Powder	4 g Ig or 16 g Ig	IgG (~94%), C	10-20%	Yes (titer NR)	McClead, et al. (1988) [17]
Adult (7), healthy	Bovine	Powder	24.4 g Ig	IgG (84%) – IgM (14%), C	~19%†	Yes (titer NR)	Roos, et al. (1995) [4]
Adult (6), healthy	Bovine (hyperimmunized)	Powder	2.1 g IgG	IgG (% NR), C	49%†	Yes (titer correlated with % IgG recovered)	Warny, et al. (1999) [6]
Adult (6), healthy	Bovine (hyperimmunized)	Powder	14.2 g IgG or 3.4 g IgG	IgG (% NR), C	1.6-32.7%	Yes (titer NR)	Kelly, et al. (1997) [5]
Adult (50), healthy & challenged with *S. flexneri*	Bovine (hyperimmunized)	Liquid	NR as gram of Ig	IgG (% NR), C	‡	Yes (titer, ≥ 1:8)	Tacket, et al. (1992) [16]
Adult (72), bone marrow transplant patients	Human	Liquid	50 mg/kg body weight	IgG (% NR), S	1-80 mg/dL of feces	NR	Copelan, et al. (1994) [18]
Adult (12), healthy	Bovine	Powder	NR as gram of Ig	IgG (% NR), S	‡	NR	Hanning, et al. (1994) – Unpublished, Data on File.
Adult (4), healthy	Bovine	Powder	0.5 g, 2.5 g or 10 g IgG	IgG (% NR), C	<0.01%	NR	Bogstedt, et al. (1997) [19]
Adult (8), healthy	Bovine	Liquid	7.65 g IgG	IgG, C	<0.1%	No	Lissner, et al. (1998) [20]

†Amount collected from the ileum.
‡ Indicates the presence of immunoglobulin in stool.
*S = serum; C = colostrum; M = milk concentrate.
NR = Not Reported.

(kilo-Dalton) which correlates well with *in vitro* pepsin and trypsin digestion experiments of the intact IgG (~160 kDa) into $Fab_{1,2}$ dimers (~100 kDa) and monomers (~50 kDa) [15]. Thus, in addition to demonstrating IgG survivability through the gastric environment into the ileum, these data establish that *in vivo* IgGs are digested in a step-wise manner into active, intact Fab fragment that still bind target antigen (s).

In the second ileal recovery study, 6 healthy volunteers with an end ileostomy ingested 5 g of bovine-Ig concentrate from hyperimmune bovine colostrum against *C. difficile* which contained 2.1 g of IgG alone, with an antacid, during treatment with omeprazole (a proton-pump inhibitor which decreases the acidity of the stomach), or within enteric-coated capsules in four separate experiments [6]. The difference in IgG recovered was not statistically significant: alone (49%), antacid (30%) and omeprazole (50%) ($p = 0.13$). Counterintuitively, enteric encapsulation resulted in statistically less IgG reaching the ileum (4%) during the time course, compared with both the alone and omeprazole group. Some capsules were recovered intact or partially digested, suggesting that enteric encapsulation inhibited release of IgG in the small intestine. Percent recovery of IgG correlated with neutralizing activity of toxin A. These two studies illustrate that a high percentage of orally-administered IgG (between ~19% and ~50%) can be recovered intact and active from the distal ileum in adult humans before entering the large intestine.

Fecal recovery of orally-administered Igs has also been demonstrated in healthy adults. In one study, healthy adult volunteers ($n = 6$) took a single oral dose of bovine Ig concentrate (BIC, 45 g with 14.2 g of IgG) from the colostrum of hyperimmunized cows against *C. difficile* [5]. Each subject had a 14-day wash-out period between crossing-over into one of six testing groups: fasting (45 g BIC), fed (45 g BIC), fed (8 g BIC), co-administered with antacid (45 g BIC), with omeprazole (45 g BIC), and enteric-coated capsules (8 g BIC) designed to release the product at $pH > 6$ in the intestinal tract. Total bovine IgG and specific anti-*C. difficile* IgG activity were measured in feces. The bovine IgG concentration of the fasting group compared to the ingested dose of 45 g BIC after 72 hr in feces was 3.8%, for fed 1.6%, and for antacid 2.7%. Omeprazole increased fecal bovine IgG levels to 8.8%, although not statistically significant over the other groups. In this study, the stool samples from enteric-coated capsules had 32.7% of the IgG in the original dose, 8 g BIC. For the fed group ingesting 8 g BIC, the recovery was 0.6% the original dose. *C. difficile* neutralizing activity correlated with the percentage of IgG recovered. This study showed that enteric encapsulation of oral Ig correlated with higher amounts of IgG recovered in stool and therefore delivered more intact IgG to the colon in healthy adults. Further studies are required to determine whether enteric encapsulation is necessary to protect orally delivered Ig for efficacy in specific intestinal disorders.

Tacket et al. [16] administered two specific preparations of bovine colostrum against *Shigella flexneri* 2a strain 5427 T liposaccharide, anti-LPS IgG which varied by titer of anti-LPS (1:2,560 and 1:40,960, respectively) and addition of chocolate dairy protein powder (1:40,960 titer only) to healthy adults ($n = 50$). The healthy adults were challenged with 10^3 *c.f.u.* of *S. flexneri* 2a strain 2457 T after three days of ingesting the Ig concentrates above. Bovine IgG was detected in feces at 91% (1:2,560) and 60% (1:40,960) of the original amounts. In both groups, the recovered titer of bovine anti-LPS was ≥ 1:8. This study illustrated a dose–response effect since the group with the higher titer was better protected from *S. flexneri* challenge. In another report of two trials, 2 g of bovine Ig concentrate from the colostrum of cows hyperimmunized against cholera toxin was administered to patients with active cholera diarrhea under different protocols: two doses (4 g bovine Ig concentrate, $n = 45$) or a single dose for every two hours for a total of eight doses (16 g of Ig concentrate, $n = 20$). From the 65 patients, a total of 35 individual stool samples were analyzed for bovine IgG and IgA. Low levels of active Ig, either as whole IgG or Fab fragments, was found in stool for roughly 60% of the patients ingesting bovine Ig. An average of about 10-20% cholera toxin neutralizing activity was still present in the recovered stools, although the titer was not reported [17]. In another randomized, controlled trial, patients undergoing bone marrow transplantation ($N = 72$) who received either 50 mg/kg human gammaglobulin or placebo daily in four divided doses for 28 days after the procedure (500 mg weekly), there was 1 to 80 mg/dL of IgG present in stool. Antibody reactivity was not reported [18].

Other trials reporting only trace amounts of IgG in feces have also been performed. In an unpublished study of 12 healthy adults, when 10 g of serum-derived bovine immunoglobulin/protein isolate (SBI) was given on two consecutive days followed by 2.5 g for 14 days, there were only low levels of detectable IgG in feces [Hanning, R. and M. Drew, *Bovine Immunoglobulin Feeding Trial.* Data on File, 1994]. There was no detectable bovine Ig in the serum of any subjects again supporting the notion of no intact systemic circulation of bovine-derived Ig. In another study, when 0.5, 2.5, or 10 g of bovine IgG was administered to 4 healthy adults, only 0.01% of the ingested IgG was detected in feces. Neutralizing activity of this recovered fraction was not assessed [19]. Similarly, when a 15 g colostrum preparation from non-immunized cows with an IgG concentration of 51% was given to healthy volunteers ($n = 8$), there was no

evidence of systemic absorption of bovine IgG (blood or urine) and in 3 of the 8 patients there were only trace amounts of bovine IgG detected in feces, but without antibody reactivity to *Yersinia enterocolitica* and *Campylobacter jejuni* antigens [20]. These data suggest that the study population may be an important determinant in recovery Ig and neutralizing activity. In healthy individuals with normal transit times, there appears to be less recovery of orally administered Ig compared to patients with accelerated intestinal transit due to infection or disease.

Recovered IgG values varied from study to study. This is not surprising since each study had different patient populations and various immunoglobulin preparations. Yet, these data summarized above strongly suggest that IgG are more resistant to complete digestion throughout the human GI tract than other dietary proteins since only three of the fifteen reports illustrate trace amounts of IgG recovered. It should be mentioned that with respect to nutrition literature, the term "digestibility" is most recently defined as the net absorption of an amino acid [21]. In this review, we are using the term "digestibility" to discuss the integrity of the quaternary and tertiary structure of IgG as it passes through the GI tract in humans, as it is generally understood that IgG can only bind to antigens within the GI tract if the antigen binding domain, the Fab, is intact. This would mean the Fab domain must resist complete denaturation through acidic pH and complete digestion by proteolytic enzymes. Most of the human studies summarized above for IgG assessed crude protein digestibility because they reported a percentage of intact IgG recovered, either from the ileum or from feces. As aforementioned, the many nutrition experiments which assess the digestibility of other dietary proteins are reported as percentages as a function of the net absorption of an amino acid. Therefore, it is difficult to compare the digestibility of IgG as reported in this review (crude protein) to the digestibility of other dietary proteins reported in literature (ileal digestibility as absorbed amino acids). In order to provide some sort of comparable measure, the true ileal digestibility - as defined as absorbed amino acids - of other dietary proteins in humans are: 95% for milk proteins, 94.1% for casein, 90% for pea protein, 91.5% for wheat protein [22]. There is one study reviewed in this manuscript which could conservatively be compared to the above numbers. Roos et al. [4], reported a prececal nitrogen absorption from the Ig preparation to be at 79%, below the absorption percentages mentioned above for other dietary proteins. In addition, recovered active fragments corresponding to ~100 kDa or two Fab domains were purified from the recovered chyme. This study therefore directly corroborates the step-wise process by which IgG are digested by enzymes *in vitro* also occurs in humans *in vivo* and that Ig preparations are digested more slowly, corresponding to less amino acid absorption *via* nitrogen monitoring. There are biochemical *in vitro* experiments which have assessed if various structural features of IgG contribute to overall stability. It is useful to discuss such experiments insofar that the majority of clinical studies reviewed in this manuscript illustrate a percentage of IgG and the Fab domains retained neutralizing activity albeit at a reduced level and more importantly, that a percentage of IgG and Fab domains retain their structural integrity, remaining intact.

Immunoglobulin structure

The base structure of Ig can be represented as a two-dimensional "Y" (Figure 1). The IgG molecule can be divided into two independent active domains: the variable antigen binding domain or fragment and the constant region. The antigen binding fragment, or Fab, is responsible for interacting with the target antigen or epitope *via* binding by the paratope or complementary determining region (CDR) (Figure 1B). IgGs are able to bind to various antigens with high specificity due to the variability of amino acids in the CDR, since the general folded Y-shape (tertiary structure) of Igs is conserved. The fragment crystallizable region (Fc) is an effector to initiate an immune response, and has no antigen binding ability (Figure 1B).

There are two post-translational structural features of Ig molecules which contribute to the overall stability of the molecule: intra- and interchain covalent disulfide bonds between cysteine residues and glycosylation (Figure 1C). Disulfides bonds are hallmark features of Ig stability and are also known to increase the stability of other proteins [23-28]. Other studies link IgG glycosylation, a variable feature for the Fab domain, but a conserved feature on the Fc domain, with stabilizing effects [29-32]. The region most susceptible to enzymatic degradation is the hinge region, which connects the Fab and Fc domains. The hinge region is the most flexible region of the IgG since it is the only single polypeptide region of IgG. Studies on the susceptibility to degradation of IgG to acidic conditions (the stomach) and digestion by enzymes (stomach and small intestine) are reviewed below.

Temperature and pH – in vitro

The protein melting temperature (T_M) is the point at which a protein unfolds, or denatures, and lacks the structure necessary for activity. T_M is a biochemical measurement to probe protein structure in different situations, such as pH. Thermal stability of an IgG correlates with pH: lower pH values, 2.8-3.4, corresponded to lower average global T_M values, 43.7°C-53.6°C, and higher pH, 5.0-7.5, corresponded to higher average global T_M values, 67.5°C-68°C [33]. Even in the acidic pH

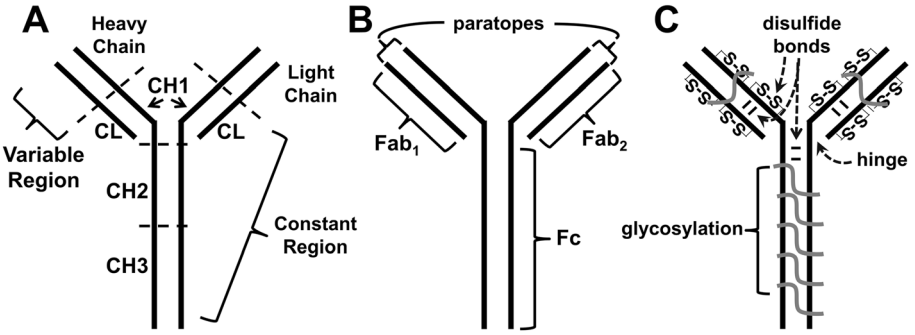

Figure 1 Simplified two-dimensional schematics of immunoglobulin G (IgG). A. Schematic showing the variable and constant regions of the IgG molecule for the heavy (CHx) and light chains (CL); **B**. Schematic of the antigen binding fragments ($Fab_{1,2}$) and the fragment crystallizable region (Fc) of the IgG molecule; **C**. Schematic portraying intra- and interchain disulfide bonds as well as glycosylation of the Fab, Fc regions, and paratope antigen binding regions of the IgG molecule.

range of 2.8-3.4, the T_M value does not fall below body temperature, 37°C, accounting for Ig structural integrity in the digestive tract. The addition of sugars, trehalose [34] and sucrose [35] to experimental buffers increased T_M values for all IgGs with pH values ranging from 4.0-8.0 suggesting that other environmental factors within the GI tract, like dietary molecules, could increase Ig stability. Salt content does not appear to affect IgG stability [35]. There have been isolated reports where the CH2 domain, the most thermally stable domain of the IgG, exhibits increased thermal stability after exposure to pH of 2.0 [36]. This increased stability is correlated with alternate conformations that various IgGs exhibit at this low pH [37-39]. Some IgG purification procedures, either from recombinant expression methods used to produce commercially-available biologic drugs or from naturally occurring sources, such as serum, require acidic or basic exposure steps [40,41]. IgG molecules purified utilizing pH shifts do not lose their capacity to bind antigens. In fact, certain intravenous immunoglobulin (IVIg) preparations purified with a low-pH step have enhanced *in vitro* and *in vivo* binding to antigens that correlates with better performance in mouse models of sepsis [41-43]. These studies underscore that IgG remain stable in acidic conditions at body temperature. It is also possible that transient exposure to acidic conditions in the stomach may increase stability for survival down the GI tract and to proteolytic enzymes.

Resistance to proteolytic digestion

Characterization of IgG structure using *in vitro* digestion with proteolytic enzymes dates to the 1960s [44-47]. It is well-established that enzymatic digestion of IgG at the hinge region with papain produces two active domains, $Fab_{1,2}$ and Fc (Figure 1BC). Furthermore, modulating reaction time, pH and temperature during enzymatic digestion will result in a variety of active domain fragments [48]. The primary digestive enzymes in humans for proteins are pepsin, in the stomach, followed by trypsin and chymotrypsin, in the small intestine [49] which digest IgG to Fab dimers (~100 kDa) and monomer Fab fragments (~50 kDa) [15], but this enzymatic susceptibility may vary by IgG subtype and species. Bovine IgG_1, for example, is more readily proteolyzed by pepsin than bovine IgG_2 [50]. Bovine IgG is more stable to proteolytic digestion compared to rabbit or human IgG [29]. Even after proteolytic digestion of IgG, Fab dimers and monomer fragments retain binding and antigen-neutralizing activity as long as they are not denatured. Proteolytic characterization of bovine derived IgG_1 *in vitro* demonstrates intact and reactive Fab domains [29,50-53]. This correlates with the human *in vivo* study by Roos et al. [4] demonstrating that immunologically active bovine Fabs were recovered in ileal aspirates.

Conclusion

Twelve of the 15 human studies in infants, children and adults that clearly demonstrate that orally-administered Ig (particularly IgGs), from human and bovine serum as well as from bovine colostrum and milk, survive gastric exposure and resist proteolytic digestion in the stomach and intestinal tract (Table 1) [4-6,9-14,16-18]. The stability of IgG is based on the structural properties of the molecules with contributions from intra- and interchain disulfide bonds, post-transcriptionally added sugars, and the three dimensional folded domains. Even when partially digested by proteolytic enzymes, Fab fragments retain not only binding but neutralizing activity through the digestive tract. In addition, there is no evidence of intact absorption of the protein, making oral IgG administration a safe, potentially effective therapy in a number of GI conditions and diseases. These physical properties and the ability of digested IgG fragments, Fab monomers and $Fab_{1,2}$ dimers to retain active binding activity make

them attractive natural therapeutic options for GI conditions [54] and mitigation of damage caused by bacterial enterotoxins, endotoxins and secreted exotoxins [55].

Studies in animals have illustrated that ingestion of serum-derived Ig preparations, containing primarily IgG, increase anti-inflammatory cytokines and decrease pro-inflammatory cytokines in mucosal jejunum [56,57]. Additionally, administration of serum-derived IgG has been shown to prevent increased intestinal permeability induced by enterotoxin challenge [58]. Improved nutrient utilization in animal models and human clinical studies after ingestion of serum-derived immunoglobulin preparations have also been observed [59,60]. More recent studies have also shown efficacy of oral serum-derived bovine immunoglobulin preparations, primarily containing IgG, in such conditions as irritable bowel syndrome with diarrhea (IBS-D) and HIV-associated enteropathy [59,61]. Other patients with chronic conditions, such as inflammatory bowel disease (IBD) or common variable immunodeficiency (CVID) might also benefit from orally administered Ig preparations, containing IgG, due to antigen-neutralizing activity as well anti-inflammatory properties of these preparations. In short, there is sufficient evidence illustrating that IgGs are less susceptible to digestion throughout the GI tract and therefore may provide for a distinctive nutritional requirement unique to patients with intestinal disorders and diseases which other dietary proteins cannot provide.

Abbreviations

Ig: Immunoglobulin(s); IgG: Immunoglobulin G; GI: Gastrointestinal; sIgA: Secretory IgA; HISG: Human immune serum globulin; kDa: Kilo-Dalton; BIC: Bovine Ig concentrate; *S. flexneri*: *Shigella flexneri*; SBI: Serum-derived bovine immunoglobulin/protein isolate; Fab: Antigen binding fragment; CDR: Complementary determining region; Fc: Fragment crystallizable region; T_M: Protein melting temperature; IBS-D: Irritable bowel syndrome, with diarrhea; IBD: Inflammatory bowel disease; CVID: Common variable immunodeficiency.

Competing interests

Victoria S Jasion and Bruce P Burnett are salaried employees of Entera Health, Cary, NC which markets an oral immunoglobulin preparation.

Author's contributions

BPB researched all literature for human clinical data and wrote the first draft of this section, VSJ reviewed the literature and edited this section. VSJ researched all articles on biochemical testing for the stability of IgG and wrote the first draft of this section, BPB reviewed that literature and edited this section. VSJ edited and responded to reviewer's comments. BPB created the first draft of Table 1, VSJ inserted more information and reformatted Table 1. BPB created Figure 1. All authors read and approved the final manuscript

Acknowledgements

We would like to thank Steven Polyak, MD (University of Iowa), Eamonn Quigley, MD (Houston Methodist) and Bryon Petschow, PhD (Entera Health) for their careful review of this manuscript and subsequent suggestions. Since VSJ and BPB are salaried employees of Entera Health and both VSJ and BPB researched, wrote and submitted this review, Entera Health, Inc. funded this review.

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A review of the systematic review process and its applicability for use in evaluating evidence for health claims on probiotic foods in the European Union

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Abstract

This paper addresses the use of systematic review and meta-analysis to evaluate the strength of evidence for health benefits of probiotic foods, especially relating to health claim substantiation in the European Union. A systematic review is a protocol-driven, transparent and replicable approach, widely accepted in a number of scientific fields, and used by many policy-setting organizations to evaluate the strength of evidence to answer a focused research question. Many systematic reviews have been published on the broad category of probiotics for many different outcomes. Some of these reviews have been criticized for including poor quality studies, pooling heterogeneous study results, and not considering publication bias. Well-designed and -conducted systematic reviews should address such issues. Systematic reviews of probiotics have an additional challenge – rarely addressed in published reviews - in that there must be a scientifically sound basis for combining evidence on different strains, species or genera. The European Food Safety Authority (EFSA) is increasingly adopting the systematic review methodology. It remains to be seen how health claims supported by systematic reviews are evaluated within the EFSA approval process. The EFSA Panel on Dietetic Products, Nutrition and Allergies deems randomized trials to be the best approach to generating evidence about the effects of foods on health outcomes. They also acknowledge that systematic reviews (with or without meta-analyses) are the best approach to assess the totality of the evidence. It is reasonable to use these well-established methods to assess objectively the strength of evidence for a probiotic health claim. Use of the methods to combine results on more than a single strain or defined blend of strains will require a rationale that the different probiotics are substantively similar, either in identity or in their mode of action.

Keywords: Systematic reviews, Meta-analysis, Probiotics, EFSA, Regulatory, Health claims

Introduction

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1]. Although probiotics can be administered in different regulatory categories of products, this paper focuses on probiotics used in foods. Probiotic foods include yogurt, cheese, juices, and cereal bars among others, the most common being yogurt. European Union (EU) consumers have used probiotic foods for decades, but with the implementation of EU legislation on health claims starting in 2009, no specific health claims for probiotic foods have been approved by the agency responsible for reviewing health claim substantiation in the EU, the European Food Safety Authority (EFSA). As a result, at this point in time (September 2014), probiotic food labels cannot communicate any health benefits to consumers in the EU. Determining what level of evidence is deemed sufficient to support health claims for probiotics has been much debated in recent years [2-8]. In addition to the lack of approved health claims for probiotics in the EU, the European Commission has indicated that the term "probiotic" in itself is an implied health claim, and subsequently the term "probiotic" should not be used on products in the absence of an approved health

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claim [9]. Also descriptors such as "live active cultures" or "active bacteria" have been banned as descriptors for foods by some member states: Ireland [10] and Sweden [11]. Therefore, no health claims can be made on probiotic foods in the EU, even though evidence for health benefits of probiotics mounts in the scientific literature [12-16]. One reason for this seeming disparity is that studies to substantiate health claims for foods must (1) be conducted on subjects reflecting the general population, and (2) target functional or reduction of disease risk endpoints rather than therapeutic ones, [17] rendering some probiotic research ineligible to substantiate food claims. The medical community may be less inclined to make such a distinction. For example, the European Society for Primary Care Gastroenterology [18], the World Gastroenterology Organisation [19] and the European Society for Paediatric Gastroenterology, Hepatology and Nutrition [20] have all published guidelines for probiotic use.

One tool that can provide the most objective assessment of evidence on a given endpoint is a systematic review (SR), with or without a meta-analysis (MA) (the statistical combination of the results of the included studies to provide an estimate of the size of an effect or association). By systematically and rigorously identifying and critiquing as much of the available evidence for a pre-specified intervention, comparator and endpoint as possible, an assessment of the relevant body of evidence can be made. Based on this assessment, it may be possible to draw conclusions about the strength of the evidence for a specific intervention versus a specific comparator. This is a well-established approach that has been used in many fields including health and social care [21] and has also been used to explore the benefits of probiotics. Here, we review what is required for a well-conducted systematic review to set the stage for discussion of applying this method to assess evidence to substantiate a health claim for a probiotic food.

Our focus is not to build a case for any specific probiotic health claim, but to discuss the scientific basis for appropriate application of the systematic review and meta-analysis approach to assessing the totality within the field of probiotics. Although the literature is replete with meta-analyses where data on different strains and probiotic preparations have been pooled, critics hold that such techniques should be reserved for data on the same probiotic strain or strain blend. We propose that in certain well-considered situations, it is possible to pool results on different probiotics. Developing a scientific rationale based on substantial identity or common modes of action may justify such an approach. Although some probiotic functions are certainly strain-specific, we suggest conversely that not all probiotics function differently. When it is possible to link different probiotic strains by a common mechanism of action, pooling data on these strains may be appropriate.

What are systematic review and meta-analysis?

A SR is a review that follows a pre-specified protocol to identify, select, critique, synthesize and summarize evidence to answer a focused research question [22-24]. Generally, there are seven steps to a SR [22,24-30]:

Step 1: Framing the question
Step 2: Identifying potentially relevant studies
Step 3: Study selection
Step 4: Data extraction
Step 5: Quality assessment
Step 6: Synthesizing the evidence
Step 7: Interpreting the findings

In brief, the first step involves defining and refining a research question [31] followed by the development of a protocol that sets out in detail how the SR steps will be conducted. The protocol presents the research question in terms of the **P**opulation of interest, **I**ntervention(s) received by the population, **C**omparator interventions, **O**utcome(s) (or endpoints), and **S**tudy types of interest. This type of conceptual breakdown of the research question is known as 'PICOS'. It is generally recommended that the protocol be peer-reviewed by external reviewers and then registered (e.g., through the International Prospective Register of SRs PROSPERO [32]. This is to encourage adherence to the protocol and to maximize transparency.

After the protocol is finalized, an extensive literature search is undertaken to identify relevant studies, including a search for both unpublished and published data, preferably regardless of language. Next, study titles and abstracts, and then full papers or unpublished reports are screened for inclusion in the SR with inclusion decisions made based on the defined 'PICOS'. The data required to answer the review question are then extracted from the included studies and the methodological quality of the studies is assessed. It is recommended that study selection and data extraction are conducted by at least two reviewers working independently in order to minimize human error in making decisions about study relevance and to maximize accurate reporting of the data. To maximize consistency and reduce opportunities for error, data extraction should be piloted within spreadsheet or review software, by both reviewers, and the agreed template used for all studies. Additional data may be collected from the original researchers who conducted the included studies [33].

Quality assessment of the included studies is a key component of the SR process as it provides a view on the reliability of the evidence reported in each of the

studies. There are a number of quality assessment and risk of bias tools available [22,24,34-38] and they are usually specific to the study type (i.e., a different quality assessment tool asking different questions would be used for randomized trials than for non-randomized observational studies).

Depending on the nature of the outcome data, the results of SRs may be presented in a narrative and/or quantitative synthesis. When it makes sense to combine data from similar studies (i.e., when they have similar participants, interventions and outcomes) it may be possible to quantitatively synthesize the outcomes using statistical techniques such as meta-analysis (MA). MA pools results from different studies to obtain an average estimate of effect across studies. In simple terms, studies may be weighted so that larger studies have a greater impact on the pooled result than smaller studies. This method increases the statistical power to detect a difference in effect that may not be detected in individual studies, and increases the precision of the estimate of the effect of the intervention [22,24]. Inevitably, there will be some variation in the estimated effects between studies included in a MA. If the variation is significant (statistically heterogeneous) subgroup analyses may be conducted. Ideally these should be specified *a priori* in the case of study characteristics that would be expected to influence treatment effects. Subgroup analyses are also used to explore inconsistencies between study results that are unlikely to have arisen by chance alone [22]. Sensitivity analyses may also be conducted (for example, omitting studies with lower quality from the analysis) to give an indication of the 'robustness' of the results [39]. Meta-regression is an extension to subgroup analysis that evaluates continuous and categorical variables [24].

The last step of the SR process includes interpretation of the findings in the context of the quality of the body of evidence [24,27]. If the SR includes a MA, the degree of consistency across studies should be considered to increase confidence in the pooled estimate of effect [35]. Without knowing the consistency of the results between studies (i.e., the degree of heterogeneity), it is impossible to determine the generalizability of the estimate for the average effects [35]. At this stage, the main results are also discussed within the context of other (systematic) review evidence and any gaps in the evidence.

When reporting a SR, transparent reporting of the review methods and assumptions should be the main objective. SRs should conform to reporting guidance such as the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) framework [28]. Ideally, systematic reviews should also be subject to peer review.

Use of systematic review and meta-analysis methods in assessing the effects of probiotics

The SR process is used in several arenas to assess the evidence base in support of regulatory and policy recommendations. Despite the robustness of SR methodology for assessing a body of evidence, we identify four issues relating to the use of meta-analysis in the field of probiotics:

- Was the intervention defined appropriately?
- Was the search extensive and was publication bias assessed? SRs which have not searched widely for studies and which have not assessed publication bias may produce biased results.
- Were results combined appropriately?
- Were analyses conducted to assess study quality? SRs and MAs that do not explore the effect of including lower quality studies run the risk of providing unreliable results.

Was the intervention defined appropriately?

Defining the intervention can be a particular challenge for SRs of probiotics, which comprise a broad range of different genera, species and strains of live microbes. An intervention, or the class of substance (such as a probiotic) that is the subject of the review, does not have to be a single substance. But in the case of grouping multiple substances into one class, there must be a sound rationale that the members of the class as defined are expected to function in a similar manner (Table 1). A probiotic intervention may comprise a single strain or a mixture of strains selected from a broad range of live microorganisms, including prokaryotic and eukaryotic microbes of different genera, species and strains. Common bacterial genera used as probiotics include *Lactobacillus*, *Bifidobacterium*, *Propionibacterium*, *Streptococcus* and *Bacillus*. Strains of the yeast, *Saccharomyces cerevisiae* (biovariant *boulardii*), are also used.

Observations by early researchers on probiotic functionality, especially with regard to outcomes from *in vitro* or animal studies, clearly indicate that different strains of the same species may behave differently. Van Hemert et al. [40] illustrate this point. They tested 42 *Lactobacillus plantarum* strains and found a 14-fold difference in the strains' ability to induce peripheral blood mononuclear cells to secrete interleukin-10. Similar strain-specific responses are seen in many other *in vitro* and animal studies that assess biological properties of different probiotics. The body of literature in human studies reveals few head-to-head comparisons of probiotics. A few examples are available in the literature where different probiotic products or different genera or species are compared. Canani et al. [41] compared 5 different probiotic preparations with a placebo

Table 1 Criteria that may be acceptable* for combining different probiotic strains into the same 'class of intervention' for a specific outcome

Rationale	Probiotic formulation defining the intervention class	Example	Comment
Common identity	Single strain of one specific genus and specie	*L. acidophilus* strain A	All studies include the same strain; may include studies conducted in different food matrices
Common identity	Single defined blend of multiple strains (strains A + B + C)	*L. acidophilus* strain A and *L. casei* strain B and *B. lactis* strain C	Strains must be maintained in equivalent relative doses in all studies
Common taxonomy	Studies used different strains of same species or subspecies?	*L. acidophilus* strain A, *L. acidophilus* strain B, or *L. acidophilus* strain C	Different studies using different strains included
Common structural or secreted product (e.g., beta-glycosidases, exopolysaccharides)	Different strains from a defined taxonomic group that may include different species or genera	All strains of *L. bulgaricus* and *S. thermophilus*	Different studies using different strains included
Common mechanism of action known to be necessary and sufficient for the effect (e.g., production of a specific bacteriocin or range of bacteriocins known to be active against a specific pathogen, or induction of immune mechanisms needed for the effect)	Different strains from a defined taxonomic group that may include different species or genera	*L. salivarius* strain A *L. salivarius* strain B	Different studies using different strains included
Common physiological effect previously proven by at least one human study of quality	Different strains from a defined taxonomic group that may include different species or genera	All strains	Different studies using different strains included

*To the extent that commonalities cannot be defined for different probiotic strains, studies on them should not be pooled into a meta-analysis. For example, probiotics such as *Saccharomyces boulardii*, *Bacillus coagulans* and *Bifidobacterium* species likely are too different biologically and mechanistically from each other to form one class of intervention. Additionally, even if taxonomically similar, if different strains have different mechanisms responsible for an effect, it may be inappropriate to pool studies. For example, a meta-analysis on anti-infective actions of probiotics likely should not pool data from a *Lactobacillus rhamnosus* strain where effects are known to be due exclusively through immune enhancing activity in the small intestine and a *Lactobacillus salivarius* strain where effects are known to be due primarily through bacteriocin production in the colon.

and found that two, but not the other three, were effective in treating children with acute diarrhoea. O'Mahony et al. [42] compared a *Lactobacillus salivarius* strain and a *Bifidobacterium infantis* strain in subjects with irritable bowel syndrome and found that only the *B. infantis* strain significantly alleviated symptoms. Such studies show that in some cases, different clinical outcomes result from using different strains. However, in other conditions, different strains of probiotics have a similar impact on a particular clinical endpoint. This is the case with necrotizing enterocolitis [43], antibiotic-associated diarrhoea [14] and upper respiratory tract infections [12], where SRs combining results of multiple probiotic preparations resulted in convincing evidence of efficacy for a broad range of probiotics. In these examples, not all tested probiotics seemed equivalently effective, but different probiotics were effective. In fact, many SRs published on probiotics have tended to combine all probiotic preparations, although criticism of this approach is building [19]. Some SRs that have included a wide range of probiotic strains conclude that not all strains are equally effective [44]. A few SRs have focused on single strains, such as *L. rhamnosus* GG [45,46], *S. boulardii* [47] or *L. reuteri* [48].

A scientific justification may exist for grouping multiple strains, species or even genera of probiotics into one class of intervention for the purpose of conducting a SR and MA. It may be appropriate to group multiple strains of a narrow taxonomic cluster, such as *Bifidobacterium animalis* subsp. *lactis* where evidence shows little diversity in the members of this subspecies. Lee and O'Sullivan state that four strains of *B. animalis* subsp. *lactis* exhibited >99% sequence identity over their entire genomes, indicating a very closely related group [49]. Masco et al. [50] have also shown that based on pulsed-field gel electrophoresis, all commercial *B. lactis* strains are indistinguishable. Barrangou et al. [51] also found a high degree of genome conservation indicative of a monomorphic subspecies. Collado et al. [52] showed that 7 of 8 *B. lactis* strains had essentially the same strain-specific pattern as indicated by a random amplified polymorphic DNA technique. Such taxonomic grouping may, however, be criticized in the absence of information on the mechanism of action for the benefit, since it is possible that the part of the genome that is not shared encodes the functional activity.

It may also be appropriate to propose that several probiotics might be legitimately grouped based on the production of common "structures", such as peptidoglycan, flagellae or exopolysaccharides, known to evoke specific physiological responses in the host or could be grouped based on a common mechanism of action. This latter

example is the case with the yogurt cultures, where aiding lactose digestion is linked to the microbial production of beta-galactosidase. EFSA approved the health claim that "yogurt cultures" (which encompass any strains from the species *Lactobacillus bulgaricus* and *Streptococcus thermophilus*) could improve lactose digestion [53]. Note that this claim does not extend to the *Lactobacillus* genus, as mixed results have been reported among other tested *Lactobacillus* species. In another example, it is conceivable that a class could be defined as members of a specific taxonomic cluster that also produce certain levels of a certain metabolite *in vivo*, such as specific short chain fatty acids.

When clustering different probiotics into one intervention group, the conclusions of the SR could only apply to the intervention class as defined. Therefore, if the intervention was defined as the subspecies, *B. animalis* subsp. *lactis*, rather than *B. animalis* subsp. *lactis* strain Bb-12, for example, then the conclusions of the SR would apply to all strains of *B. animalis* subsp. *lactis*, whether tested in a human study or not. This concept has already been accepted by EFSA in the case of yogurt cultures. The presumption that any benefits apply only to the individual strains used in the included studies reflects a lack of conviction by those defining the class as the subject of the SR that the class is logically and appropriately defined. Where strains express unique characteristics (not common to their genera or species) that enable a specific health benefit, they should not be grouped in a MA since results from one strain cannot inform (or predict) effects of another strain of the same genus or species. However, if there is a scientifically sound basis for defining a class that encompasses different strains, information on all members of that class can be used to provide a more comprehensive set of supporting data. If a study does not show an effect for one of the strains in the class, this may simply represent a study with null results, some of which will be expected in any body of research. If a strain is not represented in the collection of reviewed studies, it also does not mean that it should be excluded from the general conclusions on the benefit or otherwise of that class. Alternatively, subgroup analysis that shows efficacy for specific members of the defined class may enable researchers to refine the class more narrowly. Of note to this discussion is that in theory, EFSA accepts pooling data from studies of a well-defined food constituent that is responsible for a claimed effect when given at the appropriate dose [54]. However, if pooling data on different probiotic products (comprising different strains), we reiterate that it will be necessary to provide a scientific justification for considering this group as a defined class.

Challenges emerge if one intends to use pooled data from studies on different strains clustered into one intervention group as part of a dossier to substantiate an EU health claim. One pillar of such a dossier is characterization of the substance under consideration. This section clearly defines what food or food ingredient is the subject of the health claim. EFSA requires that characterization is very specific. A strategy for definition of the substance under review must be developed if data from different strains, potentially delivered in different matrices, are to be combined in a MA. It is possible that a SR for a defined set of probiotics can be used as evidence for a dossier for one specific member of that class. Then the characterization information should be specific for that one member.

Was the search extensive and was publication bias assessed?

Although the SR might be useful to bring the studies together, the MA should not be undertaken when there is likely to be serious publication bias, since this is likely to provide an unreliable estimate of the true effect size [24]. Publication bias occurs when publication is influenced by the results. An extensive search for both published and unpublished data and the use of a range of different search techniques mitigates this issue, but relevant studies may still remain inaccessible to specific reviews. This is why investigations of publication bias, where possible, are helpful in revealing the possible extent of missed studies and the potential for risk of publication bias should be considered within the conclusions of a SR [55]. Prospective trial registries such as ClinicalTrials.gov improve awareness of research that has been undertaken and already includes numerous probiotic trials.

Were studies combined appropriately?

When deciding whether to conduct a MA, input from a topic expert is often helpful to ascertain whether it is appropriate to combine the results of the identified studies [24]. Current guidance recommends that data should not be pooled when there is a mix of different comparisons of different treatments, or when the outcomes are too diverse (i.e., when they measure different variables) [24]. In the case of probiotics, this guidance would not preclude combining studies on different probiotic products as long as there is a solid scientific rationale for grouping the different probiotics into one class. As an extension to this, it may be misleading to combine results in a MA if there are substantial differences between study estimates of effect, particularly if they are in opposing directions [22]. If this occurs with probiotics studies, the studies could be grouped into logical subsets, for example by probiotic strain, species, or genera (defined *a priori).*

Were analyses conducted to assess study quality?

MAs of poor quality studies may result in an erroneous interpretation [24]. Different methods have been suggested to deal with this potential problem, such as only including high quality studies in a review, or conducting sub-group analyses, or sensitivity analysis by quality criteria, to allow a focus on the higher quality studies. Quality assessment may reflect concerns about study methods, but may also take into account other aspects of the study such as concerns about bias caused by the funding of the research or inadequate information about the study as a result of the publication format (in the case of conference abstracts). The inclusion of *only* poor quality studies in MA (or where a high proportion of the weight of the evidence is from poor quality studies) should be avoided, as they may cast doubt on any subsequent conclusions, or even hinder any correct interpretation.

Use of the systematic review/meta-analysis process by the European Food Safety Authority

In 2010, EFSA published its guidance on the conduct of SRs of relevance to food and feed safety [23]. EFSA noted that, although rarely used in these areas, SR methods had potential for application in the fields of food and feed safety. In 2012, EFSA advertised a series of framework agreements (Scientific Services to Support EFSA Systematic Reviews) [56] with suppliers to provide training in SR methods, to offer expert support at various stages of the SR process and to provide SRs to EFSA. Suppliers were requested to perform SRs in line with the methods described in the EFSA framework agreements [56].

The EFSA guidance [23] clearly stresses the importance of precisely defined, closed-framed questions (exposure or intervention, diagnostic accuracy, descriptive questions of populations or systems), which are captured *a priori* in clear eligibility criteria for studies to be included in the review.

In cases where there is a large quantity of evidence, the SR method can formally and systematically summarize that evidence (with MA where data permit) and provide more precise estimates of effects than an individual study can provide. The value of the SR approach also lies in its presentation of all of the available evidence with an assessment of the quality of that evidence: the strengths and limitations of the evidence base can thus be seen in a clearer way. The more controversial the topic, the more important it is for the SR process to be described in detail, so that the methods can be understood and alternative approaches discussed. The EFSA guidance provides a clear outline of a suitable process for conducting a SR and some recommendations on the reporting of a SR, without providing a formal reporting structure such as the PRISMA guidance described earlier. In the recent framework tender documents [56], suppliers are asked to consider documenting and reporting the method and results of the SR using the PRISMA statement and, where that is not applicable, to document and explain any discrepancies from the PRISMA statement.

The use of MA within SRs is guided by the suitability of the data for pooling; the considerations informing a decision to use MA are described in Appendix E of the EFSA guidance [23]. Although this is not a formal recommendation of the EFSA guidance, the presentation of the issues around MA in the guidance suggests that for SRs to be as transparent as possible, the rationale and justification for MA should be presented in the SR report. The discussion and conclusions of the SR should be grounded in the *a priori* objectives for the SR and the results identified. Again, adhering to this approach and documenting the rationale in full will contribute to the rigour and objectivity of a particular SR.

Use of the systematic review and meta-analysis for evaluation of evidence to substantiate health claims on foods in the European Union

In Europe, Article 6 of EC Regulation 1924/2006 states that health claims for food labeling must be based on and substantiated by 'generally accepted scientific evidence' [57]. In order to ensure that health claims made are truthful and can be understood by consumers, health claims undergo a specific procedure of assessment and authorization involving consultation to the EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA Panel) [57]. For the assessment of all types of health claim applications (article 13.1, 13.5 or 14), the NDA Panel considers whether the beneficial effect of the food is substantiated by generally accepted scientific evidence [57] using an assessment process of the highest possible standard. As described by the NDA Panel, this involves taking into account the totality of the available scientific data, and weighing the evidence obtained from individual studies [57].

The EFSA 'General guidance for Article 13.1, 13.5 and 14 health claims evaluation' [57] indicates that data from intervention and observational studies in humans should be presented with the most reliable data being assessed according to a hierarchy of study designs. Trials with full randomization and adequate allocation concealment (method of randomization reported) are deemed to be at the top of the hierarchy of human intervention studies for assessing a cause-effect relationship between the consumption of the food or food constituent and the claimed health effect [58]. Cohort studies are considered to be at the top of the hierarchy for human observational studies [58].

While the NDA panel did not propose a formula for how many or what type of studies are needed to substantiate a claim, the reproducibility of the effect of the food as indicated by consistency between studies is an important consideration for their final assessment. Thus, a comprehensive review of the data from human studies addressing the specific relationship between the food and the claimed effect is required from the applicant.

The NDA panel guidelines make it clear that in evaluating and weighing the evidence to substantiate health claims on foods, SR and MA can play an important role [58]. EFSA ask applicants to provide a comprehensive review in order to evaluate the totality of the evidence proposed in the dossier. This step must be included as a specific section of a health claim dossier. The NDA panel's final opinion will be based on a SR of the totality of the available data, but does not require that all individual randomized trials or other type of studies show statistical significance. For example, when considering the evidence for periconceptional folate supplementation for preventing birth defects, 5 randomized trials were considered [59]. Since the incidence of birth defects was quite low, the MA process allowed pooling of available data to reach a convincing conclusion about the effectiveness of folate. The MA reached a level of evidence that was not reached by the individual randomized trials.

The NDA panel does require that the SR provides convincing evidence on the consistency and reproducibility of the effect when analyzing the totality of the data. EFSA considers randomized trials as the best tools to generate evidence on the effects of interventions and considers SR and MA to be the best tools to evaluate the totality of the evidence and inform a final opinion. MA and SR may be useful to address the following scenarios that we envision could emerge in submitted dossiers:

- Studies are underpowered due to a smaller effect size than predicted, so none of the studies reaches statistical significance. But, when data are analyzed together as part of a MA, a statistically significant average result is obtained.
- One pivotal study has been conducted which is in favor of the intervention, and other studies with null results have been published. This reflects a genuine evidence base and the review of all contributing studies should allow exploration of heterogeneity and provide information on the true direction and size of the health benefit. All studies, with all results, contribute to an overall picture of the effect.
- Studies are underpowered due a heterogeneous population within each study, comprising responders and non-responders, which cancel each other out in the analysis of the individual study. The best approach to this situation is to better identify the target population *a priori* and perform randomized trials with the appropriate population subset, or to unpick the studies into the relevant subgroups. On the other hand, acceptance of the health claim has the potential to benefit a subset of the population consuming the product, which is of benefit to those consumers. Subgroup analyses defined *a priori* may enable identification of the responding subgroup and convincing evidence in support of a claim for the subgroup may be revealed.
- The incidence of the health endpoint is low (such as incidence of flu during the winter season) and very large sample sizes among the study population are required. Such sample sizes are better achieved by pooling the results of several randomized trials, providing that these studies collectively provide sufficient power.
- Many statistically significantly positive randomized trials exist, but no two trials investigate the same strain. If there is a scientifically valid basis to expect that a group of different strains would function similarly on an endpoint, then they can be reasonably grouped into one intervention for a meta-analysis, and the resulting meta-analysis would be sufficient evidence to support a health claim.

We recognize that SRs are only one input to a decision-making process regarding formulation of policy derived from evidence. The health technology assessment process, for example, also considers information on the economic and legal aspects of competing interventions.

Conclusions

SR and MA are transparent and rigorous approaches to synthesizing evidence. It is widely accepted in many scientific disciplines and by many policy-setting organizations as the best way to evaluate the strength of evidence available to answer a focused question. EFSA guidance acknowledges the validity of well-conducted SRs for closed-framed questions, which seems appropriate for assessing evidence for probiotics: *In population P, does intervention I affect outcome O when assessed using comparator C?*

EFSA endorses the SR process to inform their judgment when deciding health claims, as observed with the decision on maternal folate intake and reduced risk of neural tube defects. This suggests the method is also suitable for probiotic health claims.

Applying the SR approach to probiotics to obtain evidence on a single strain (e.g., Strain A) or on a single, defined multi-strain probiotic (e.g., Strain A + B + C) is scientifically justifiable. Applying the SR approach to studies conducted on a broader range of probiotics (i.e.,

studies of different strains of the same species or subspecies, different species or different genera, such that study 1 is conducted on strain A, study 2 is conducted on strain B, study 3 is conducted on strain C + D) requires a scientifically valid justification for definition of the class. However, EFSA precedent has allowed such 'clustering' of all yogurt cultures into a category of food that can improve lactose digestion, even though the possibility exists that a particular strain may not deliver adequate lactase to improve lactose digestion. EFSA seems to recognize that available evidence provides a reasonable certainty that yogurt cultures will provide this benefit. However, 100% certainty is not possible and should not be required.

The majority of SRs published on probiotics to date has been conducted on a broad group of live microorganisms without stated justification for clustering into one class. In many cases, this includes studies using *Lactobacillus*, *Bifidobacterium*, *E. coli*, *Saccharomyces* and others. If the overall effect is positive, the conclusion from such reviews has generally been that 'probiotics' are effective for the particular indication. However, they have not concluded that every possible probiotic strain will necessarily be effective, and they have generally acknowledged that effects may be due to only specific, tested strains. A strict application of the SR approach should enable application of the conclusions of the review to every member of the class as defined.

Procedures within EFSA, including their approach to definition and characterization of the substance under review, presents a challenge for use of the SR and MA approaches on a defined class of different strains of probiotics for primary health claim substantiation.

In conclusion, SR and MA represent a well-developed and widely applied means to evaluate the strength of research evidence and should be acceptable by EFSA for substantiating probiotic claims. Use of SR to combine studies on multiple probiotic strains, however, requires a valid scientific rationale that combining evidence from different strains is biologically and physiologically justified.

Abbreviations

EFSA: European food safety authority; EU: European Union; MA: Meta-analysis; NDA Panel: Panel on Dietetic Products, Nutrition and Allergies; PRISMA: Preferred reporting items for systematic reviews and meta-analyses; SR: Systematic review.

Competing interests

MES served as a paid consultant to the Global Alliance for Probiotics. FG, CH, JG and SK received honoraria from the Global Alliance for Probiotics for the preparation of this manuscript. JG and SK have received unrestricted educational grants from the Global Alliance of Probiotics.

Authors' contributions

JG, MES, SK, FG and CH: Contributed to the original plan for the paper, wrote sections of the paper and contributed to draft revisions. All authors read and approved the final manuscript.

Acknowledgement

The Global Alliance for Probiotics provided an unrestricted educational grant to fund the preparation of this paper. They commented on both the paper outline and the final paper, but had no control over the final version of this article. Authors gratefully acknowledge review of this paper by Professor Michael Clarke and Dr Heather McIntosh.

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Increased dietary α-linolenic acid has sex-specific effects upon eicosapentaenoic acid status in humans: re-examination of data from a randomised, placebo-controlled, parallel study

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Abstract

Background: There is a metabolic pathway by which mammals can convert the omega-3 (n-3) essential fatty acid α-linolenic acid (ALA) into longer-chain n-3 polyunsaturated fatty acids (LC n-3 PUFA) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). As far as we know there are currently no studies that have specifically examined sex differences in the LC n-3 PUFA response to increased dietary ALA intake in humans, although acute studies with isotope-labelled ALA identified that women have a significantly greater capacity to synthesise EPA and DHA from ALA compared to men.

Findings: Available data from a placebo-controlled, randomised study were re-examined to identify whether there are sex differences in the LC n-3 PUFA response to increased dietary ALA intake in humans. There was a significant difference between sexes in the response to increased dietary ALA, with women having a significantly greater increase in the EPA content of plasma phospholipids (mean +2.0% of total fatty acids) after six months of an ALA-rich diet compared to men (mean +0.7%, P = 0.039). Age and BMI were identified as predictors of response to dietary ALA among women.

Conclusions: Women show a greater increase in circulating EPA than men during increased dietary ALA consumption. Further understanding of individual variation in the response to dietary ALA could inform nutrition advice, with recommendations being specifically tailored according to habitual diet, sex, age and BMI.

Keywords: α-linolenic acid, Sex, Eicosapentaenoic acid

Findings

Background

There is a metabolic pathway by which mammals can convert the omega-3 (n-3) essential fatty acid α-linolenic acid (ALA; 18:3n-3) into longer-chain (LC) more unsaturated n-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) via a series of desaturase and elongase catalysed reactions [1]. Acute studies with stable isotope-labelled ALA have identified that females have a significantly greater capacity than males to synthesise EPA and DHA from ALA, with estimated net conversion rates of ALA to EPA of 21% vs. 8% and of ALA to DHA of 9% vs. 0% [2,3]. Correspondingly, females have been found to have significantly higher concentrations of DHA in plasma lipids and erythrocytes compared to males, regardless of their dietary intake of n-3 fatty acids [4]. Sex differences in EPA and DPA content have also been observed, with females having higher erythrocyte phospholipid EPA, lower adipose tissue EPA and lower plasma DPA content [4]. Studies of women using the contraceptive pill [5,6] or hormone-replacement therapy [7,8] and of trans-sexual subjects [6] suggest that

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sex hormones play a role in mediating these sex differences in LC n-3 PUFA status.

As far as we know there are currently no studies that have specifically examined sex differences in the fatty acid response to a chronic increase in dietary ALA intake in humans, with available data from male or mixed sex cohorts indicating modest but significant increases in circulating EPA and but not DHA, following increased ALA intake [9-11]. If there are significant sex differences in the response to increased ALA intake in humans, this must be considered during trials and has implications for dietary advice and recommendations. Here we re-examine data from a human dietary study [12] to test our hypothesis that sex can significantly influence the increment in LC n-3 PUFA status following ALA supplementation.

Methods

Data selected for secondary analysis were plasma phospholipid fatty acids from a study of healthy male (M; n = 87) and female (F; n = 63) participants aged 25–72 yr who had replaced their normal margarine or butter with specially formulated margarines for a period of 6 months [12]. The original study involved randomisation of participants to one of five groups, including a control group who consumed a standard high linoleic acid, low ALA margarine, two groups who consumed margarines enriched with ALA, and two groups who consumed margarines enriched with EPA + DHA [12]. Here data from the control and the "high-ALA" groups are used. The high-ALA margarine provided contained 41 g ALA/100 g compared with 1 g ALA/100 g in the control margarine. The study was approved by the University of Reading Ethics and Research Committee and the West Berkshire Health Authority Ethics Committee, and each participant gave written informed consent before participating. Participants in the control group (n = 30; 18 M, 12 F) consumed an average of 1.5 g ALA/day (from background diet and the control margarine), and those receiving the high-ALA margarine (n = 29; 17 M, 12 F) consumed an average of 9.5 g ALA/day (from background diet and the high-ALA margarine) over the 6 month period [12]. Available data for which paired baseline and 6 month values were available were included in this analysis (control group n = 21; high-ALA group n = 23). The data set supporting the results of this article is available in the University of Southampton Institutional Research Repository [ePrints Soton: http://eprints.soton.ac.uk/id/eprint/370208].

Lipid was extracted from plasma with chloroform:methanol (2:1, by vol) and phospholipids were isolated by thin-layer chromatography by using a mixture of hexane:diethyl ether:acetic acid (90:30:1, by vol) as the elution phase. Fatty acid methyl esters were prepared by incubation with 14% boron trifluoride at 80°C for 60 min. Fatty acid methyl esters were separated in a gas chromatograph and identified by comparison with standards.

Data were analysed using IBS SPSS Statistics 20 (IBM, Portsmouth, UK). Data are expressed as change from baseline following the six month dietary period and were assessed for the effect of diet, sex and diet*sex interactions by two-way ANOVA. Where significant effects were found, differences between dietary groups and the sexes were explored by independent T tests. Linear regression was conducted to investigate the influence of diet, age, weight and BMI upon the change in EPA content.

Results

Males weighed significantly more than females within both dietary groups, resulting in a significantly higher intake of ALA (expressed as g per kg body weight) among females in both dietary groups (Table 1). No sex differences were identified for any n-3 PUFA at baseline (Table 1). Participants randomised to the high-ALA diet had a significantly lower baseline DHA status than those in the control group (Table 1).

The diet provided significantly altered plasma phospholipid n-3 fatty acid content, with those receiving the high-ALA diet having significantly higher ALA and EPA contents (Table 2). Significant sex*diet interactions were observed for plasma phospholipid 16:0, 20:1n-9 and EPA content. Females receiving the high-ALA diet had a significantly greater increase in plasma phospholipid EPA than males consuming the same diet (Table 2). Women showed a mean 2.0% of total fatty acids increase in the EPA content of plasma phospholipids after the high-ALA diet compared with a mean 0.7% increase in men ($P = 0.039$). No significant sex*diet interaction was identified for any other n-3 PUFA, including DHA, or for any n-6 PUFA (Table 2). Inclusion of participant age, BMI or weight in the model as covariates did not alter the pattern of results observed for plasma phospholipid EPA content (data not presented).

Linear regression was conducted within each sex to determine the contribution of diet, age, weight and BMI to the change in plasma phospholipid EPA status. Among females, diet, age and BMI were significant predictors of the change in plasma phospholipid EPA content (Table 3). Age was inversely related to change in EPA status among females, while diet and BMI were significantly positively related with change in plasma phospholipid EPA status. None of these variables was a significant predictor of the change in plasma phospholipid EPA in men (Table 3). Data were examined for correlations between estimated ALA intake expressed as mg/d/kg bodyweight and change in EPA content in order to assess whether a higher relative dose was responsible for the sex differences observed. No significant correlations were observed (data not presented).

Table 1 Characteristics of participants in the study according to diet

	1.5 g/d ALA		9.5 g/d ALA		P value		
	Male (n = 11)	Female (n = 10)	Male (n = 13)	Female (n = 10)	Sex	Diet	Sex*diet
Age (y)	52.9 ± 13.7	53.0 ± 11.2	50.5 ± 12.7	53.5 ± 12.0	0.68	0.80	0.70
Weight (kg)	82.3 ± 8.8	62.9 ± 7.4*	84.7 ± 14.3	69.9 ± 9.2*	<0.001	0.15	0.47
BMI (kg/m^2)	26.4 ± 2.7	24.2 ± 3.1	27.3 ± 3.9	25.6 ± 2.6	0.053	0.24	0.82
ALA dose per kg body weight (mg/d)	18.4 ± 1.9	24.2 ± 2.6*	115.5 ± 22.0†	137.8 ± 16.8†*	0.003	<0.001	0.067
Baseline plasma phospholipid n-3 fatty acid status (% total fatty acids)							
ALA (18:3n-3)	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.63	0.20	0.75
EPA (20:5n-3)	0.9 ± 0.8	1.1 ± 0.9	0.8 ± 0.6	0.8 ± 0.5	0.55	0.48	0.73
DPA (22:5n-3)	2.1 ± 1.5	1.6 ± 0.7	1.9 ± 0.7	1.8 ± 0.9	0.34	0.98	0.63
DHA (22:6n-3)	4.4 ± 1.3	4.2 ± 1.3	3.5 ± 1.0	3.2 ± 0.9	0.63	0.008	0.86

Data are mean ± SD.
*Significantly different from males in same dietary group ($p < 0.05$, independent mean t-test). †Significant effect of diet within same sex ($p < 0.05$, independent mean t-test).

Discussion and conclusions

These data demonstrate that there are sex differences in the response to dietary ALA in humans, with females having a significantly greater increase in EPA content of plasma phospholipids compared to males over the same period on the same diet. This confirms that the sex differences observed in the synthesis of EPA from ALA in acute studies using stable isotopes [2,3] are also reflected in the response to chronic dietary regimes involving increased dietary supply of ALA. There was no sex specific enhancement in DPA or DHA content of plasma phospholipids after chronically increased ALA intake, nor any significant sex differences in DPA or DHA at baseline.

Data were not available on the menopausal status of women in the study, and insufficient data were available to perform a subset analysis of those participants under 45 years old (Females < 45 yr: 1.5g/d ALA n = 1, 9.5g/d ALA n = 2). However, an inverse relationship between the age of female participants and the change in EPA content after supplementation was observed, supportive of a role for female sex hormones in regulating the endogenous synthesis of LC n-3 PUFA. Larger studies will

Table 2 Plasma phospholipid fatty acid composition after control or ALA-rich diet for 6 months

	1.5 g/d ALA		9.5 g/d ALA		P value[1]		
	Male (n = 11)	Female (n = 10)	Male (n = 13)	Female (n = 10)	Sex	Diet	Sex*diet
	(% total fatty acids)						
16:0	26.7 ± 1.5	27.5 ± 1.4	28.0 ± 2.2	26.8 ± 1.3	0.82	0.44	0.073
18:0	13.2 ± 1.2	13.4 ± 1.5	12.5 ± 3.8	13.6 ± 1.6	0.82	0.84	0.20
22:0	1.4 ± 0.9	1.0 ± 0.5	1.3 ± 1.0	1.4 ± 1.1	0.11	0.50	0.69
18:1n-9	10.8 ± 3.0	10.9 ± 2.3	11.4 ± 2.9	11.2 ± 1.9	0.78	0.53	0.64
20:1n-9	0.4 ± 0.3	0.2 ± 0.1*	0.4 ± 0.2	0.3 ± 0.2	0.18	0.39	0.041
18:2n-6	22.2 ± 1.8	22.3 ± 2.4	22.1 ± 2.5	21.6 ± 2.6	0.55	0.58	0.61
18:3n-6	0.2 ± 0.2	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.60	0.14	0.60
20:3n-6	3.6 ± 1.0	4.2 ± 1.3	3.5 ± 1.4	3.0 ± 1.6	0.97	0.38	0.12
20:4n-6	10.3 ± 2.4	10.0 ± 2.0	9.1 ± 2.7	8.8 ± 1.3	0.74	0.26	0.32
18:3n-3	0.3 ± 0.1	0.3 ± 0.2	0.6 ± 0.3†	0.9 ± 0.3†	0.19	<0.001	0.11
20:5n-3	1.5 ± 1.5	1.2 ± 1.0	1.5 ± 1.3	2.8 ± 1.7†*	0.35	0.023	0.027
22:5n-3	2.0 ± 0.5	2.3 ± 0.7	2.5 ± 0.7	2.6 ± 0.7	0.11	0.22	0.39
22:6n-3	4.7 ± 1.2	3.9 ± 0.6	3.9 ± 1.3	3.7 ± 1.1	0.34	0.20	0.36

Data are mean ± SD.
[1]Statistics were conducted using change from baseline data. *Significantly different from males in same dietary group ($p < 0.05$, independent mean t-test). †Significant effect of diet within same sex ($p < 0.05$, independent mean t-test).

Table 3 Linear regression analysis of change in plasma phospholipid EPA status[1]

	Males (n = 24)	P	Females (n = 20)	P
Model R	0.28	0.80	0.79	0.004
Standardised coefficients (Beta)				
BMI	−0.06	0.88	1.06	0.008
Diet (1.5 g/d ALA vs. 9.5 g/d ALA)	0.02	0.94	0.63	0.003
Age	−0.15	0.56	−0.47	0.029
Weight	−0.15	0.68	−0.70	0.055

[1]Data used are from participants in both the control group (1.5 g ALA/day) and the high ALA group (9.5 g ALA/day).

be required to fully investigate the role that menopausal status has upon the sex differences observed in this analysis. Data from this analysis indicates that a diet rich in ALA does not contribute to circulating DHA status, and the consequences of this should be considered in circumstances where there may be specific needs for DHA, such as during pregnancy.

The observation that BMI was positively correlated to change in plasma phospholipid EPA content may have two explanations. Firstly, increasing BMI is typically indicative of increasing body fatness and so of a proportional decrease in lean mass. This may result in less use of ALA for oxidation with increasing fatness, sparing a greater proportion of the ALA consumed to be used for conversion to LC n-3 PUFA. Secondly adipose tissue may have a role in the synthesis of LC PUFA, either as a primary source of endogenous synthesis, or indirectly via the higher circulating estradiol status associated with increasing adiposity. For these same reasons, relative adiposity may also contribute to sex differences in fatty acid status, as female fat mass is significantly higher than that of males with the same BMI. Thus, young women may have a greater capacity to respond to an increased intake of ALA than males, and may therefore have a lower need for preformed dietary EPA. Further studies incorporating additional measures indicative of body composition and adiposity, such as waist circumference will be required to further investigate these hypotheses.

Further understanding of the sex-linked variation in the response to dietary ALA could inform future dietary recommendations, with advice specifically tailored to match that individual's sex. Studies which investigate other factors known to influence PUFA metabolism such as age, sex hormone status, adiposity and genotype may further identify those people who are most likely to benefit from ALA supplementation and inform personalised nutrition recommendations. The current recommended intake of EPA + DHA for adults in the UK is a minimum of 450 mg/day, yet it is estimated that over 70% of UK adults do not habitually consume any oily fish [13]. If a diet rich in ALA can contribute to EPA status in women, this could both inform dietary advice to women, especially those unable or unwilling to consume oily fish, and provide evidence for an alternative and sustainable dietary source of n-3 fatty acids.

Abbreviations

ALA: α-linolenic acid; DHA: Docosahexaenoic acid; DPA: Docosapentaenoic acid; EPA: Eicosapentaenoic acid; F: Female; LC: Longer-chain; M: Male; PUFA: Polyunsaturated fatty acids.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YEF, AMM, and ECL-F conducted the human study under the supervision of CMW; SK analysed the fatty acids from the human study under the supervision of PCC; CEC conducted the secondary analysis of the data; CEC and PCC wrote the paper. CEC had primary responsibility for final content. All authors read and approved the final manuscript.

Acknowledgements

The human study was funded by the Department for Environment, Food & Rural Affairs, the Biotechnology and Biological Sciences Research Council, Roche Vitamins Ltd. and by Unilever Research Vlaardingen, under the Agri-Food LINK programme (grant AFQ111). The authors would like to acknowledge the contributions of Gert W Meijer (Unilever Research) and Reto Muggli (Roche Vitamins) to the human study. None of the authors has a conflict of interest.

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A survey of diet self-efficacy and food intake in students with high and low perceived stress

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Abstract

Objective: Given the rise in obesity and obesity-related disorders, understanding the relationship between stress, self-efficacy and food choice in young adulthood may have implications for preventing negative health outcomes later in life that stem from poor eating habits. The current study examined whether stress levels and diet self-efficacy may be associated with unhealthy eating habits in young adults.

Methods: Male and female undergraduate students (N = 136) completed questionnaires that tap into diet self-efficacy (DSE), perceived stress (PS), sodium, and fat intake. Sex differences in choice of food were predicted, and low levels of perceived stress and high diet self-efficacy were expected to be associated with lower fat and sodium intake.

Results: Findings indicate an interaction between perceived stress and diet self-efficacy on fat intake and a main effect for diet self-efficacy on sodium intake in this population. As expected, low levels of perceived stress and high diet self-efficacy were associated with the lowest levels of fat and sodium intake in students. Findings were driven by females.

Conclusions: This study provides preliminary evidence that diet self-efficacy and perceived stress levels relate to nutrient intake in young adult females, and that increasing diet self-efficacy and reducing perceived stress in young adult females may lead to reductions in fat and sodium intake, leading to healthier eating habits.

Keywords: Food intake, Fat, Sodium, Stress, Diet self-efficacy

Introduction

The young adult North American population is found to ingest more fat and sodium than is considered healthy. According to Health Canada [1], 25% of males and 23% of females 19 years of age and older report fat intake above the recommended amount (25-35% of total energy intake). In addition, 99% of males and 73% of females aged 19-30 years old reportedly ingest more than the tolerable upper intake level of 2300 mg of sodium per day [1]. The question that arises from these statistics is: what is causing young adults to over-consume fat and sodium?

Over the past few years, reports of increased stress among college and university students have surfaced [2,3]. In a related vein, studies have shown that *stress* is a significant instigator of poor eating behaviors, especially in the young adult population [4]. Animal research shows that stress exposure increases both fat and sodium intake.

The majority of human studies have focused on fat intake in relation to stress. Studies show that females [4-8] and males [9-11] ingest more fat following exposure to a psychosocial stressor. Interestingly, Epel and colleagues [5] found that increased secretion of the stress hormone cortisol following a psychosocial stressor as well as stress induced by completion of visuospatial puzzles and serial subtraction tasks was related to fatty but not sodium-rich foods in female participants.

Fewer studies have examined the relationship between stress and sodium intake. One study by Miller et al. [12] found that males who scored high on hostility and who were more stress-reactive to a psychosocial stressor, reported greater sodium consumption on a food frequency questionnaire and consumed more sodium in the laboratory [12]. The cyclical nature of the stress response and food intake in these subjects demonstrates that sodium intake resulting from stress may only worsen subsequent physiological feelings of stress in individuals by increasing blood pressure [13,14].

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Although a number of studies demonstrate an increase in fat intake among highly stressed individuals, a handful of studies have reported decreased intake under stress [15,16]. Epel and colleagues [5] measured dietary restraint (those who are attempting to actively diet and place restraint on their food intake) and serum cortisol resulting from stressors such as visuospatial puzzles, serial subtraction tasks and deliverance of a speech in young adult females. The authors found high cortisol release to be related to intake of fatty but not salty foods, and that overall, psychophysiological responses to stress may induce uhealthy eating [5]. On the contrary, Stone and Brownell (in [15]) examined daily records of stress and eating habits and found that male and females students report eating less when faced with more severe stressors. Mixed findings within the literature are likely related to individual differences; not everyone responds to stress in the same way, and thus not everyone overeats, or eats high-fat or high-sodium foods when stressed. A potential mediating factor to consider in the relationship between stress and food intake is self-efficacy.

Research suggests that self-efficacy affects the cognitive appraisal of a stressor and thus the stress response that ensues [17,18]. General self-efficacy is loosely defined as one's confidence in his or her ability to manage a demand in the presence of obstacles [19]. Indeed, a number of studies have shown that greater level of general self-efficacy is related to lower reports of stress [18,20-22]. Ebstrup and colleagues [21] examined the role of general self-efficacy in the stress appraisals of male and female participants aged 18-69 years and found that general self-efficacy acts as a buffer to stress by increasing one's beliefs that he or she is able to overcome the external events or obstacles that are perceived as stressful. Given that self-efficacy decreases perceived stress, it may be suggested that this attribute may moderate the association between stress and nutrient intake. In other words, although stress levels may be heightened in a given situation, greater self-efficacy may reduce one's tendency to use unhealthy food intake as a way of reducing feelings of stress.

Findings regarding the relationship between self-efficacy and nutrient intake are mixed. Armitage and Connor [23] conducted a study with undergraduate males and females, and found that although self-efficacy did not directly correlate with eating behavior, it was a predictor of intention to reduce fat intake; a potential explanation for this is that their measure of self-efficacy consisted of a non-validated 2-item scale. O'Connor et al. [7] investigated general self-efficacy as a mediator for perceived stress, and also examined the relationship between self-efficacy and health behaviours. O'Connor and colleagues [7] found that stress leads to increased fat consumption; however, greater self-efficacy was associated with lower fat consumption in high stressed men and women. Similar results were found in a study conducted by Royal and Kurtz [24], although their sample consisted only of female undergraduates. In a study by Barrington et al. [25], it was found that high levels of perceived stress are associated with greater fast-food intake, especially among individuals with low eating awareness. However, no associations between self-efficacy and stress or food intake were found [25]. One potential explanation for this negative finding is that fast food intake and self-efficacy were measured using a non-validated 1- and 2-item scale respectively. Fewer studies have assessed the relationship between self-efficacy and sodium intake. A study conducted by Cornelio and colleagues [14] examined behavioral determinants of sodium consumption in individuals with hypertension; the researchers measured self-efficacy using a 3-item scale at baseline and found that higher self-efficacy predicted intention to avoid the use of sodium in cooking and to avoid consumption of foods with high sodium content over a two month period. Women with lower self-efficacy were found to add sodium to foods while cooking, although self-efficacy was not related to actual avoidance of high-sodium foods in either gender [14]; the authors did suggest that interventions to decrease sodium consumption should incorporate changes in self-efficacy.

Mixed findings pertaining to the relationship between stress, self-efficacy and nutrient intake likely result from the use of general self-efficacy scales, which are not specific to food intake behavior. Bandura [17] was one of the most prominent theorists to state that self-efficacy is primarily task-specific, and thus to measure potential behavior outcomes, measures of self-efficacy should be specific to that behavior. Importantly, displaying a high level of general self-efficacy does not indicate that an individual is efficacious in all self-efficacy components [26]. Another possible explanation is that general self-efficacy does not directly map onto eating behaviors and thus diet self-efficacy would be a more appropriate measure in assessing the moderating role of self-efficacy in the relationship between stress and food intake. Diet self-efficacy is one component of self-efficacy that depicts one's belief in his or her ability to manage a diet even in the face of obstacles such as stress or exposure to unhealthy foods; thus, diet self-efficacy may act as a moderator between perceived stress and food intake behavior.

To date, only one study has assessed the role of diet self-efficacy in the relationship between stress and food intake. Foreyt et al. [27] used a series of questionnaires and found that women reported lower diet self-efficacy and greater levels of stress compared to men. Further, obese participants reported significantly lower diet self-efficacy compared with that of average-weight individuals [27]. Although this study demonstrates positive

associations between diet self-efficacy, stress and weight, Foreyt and colleagues did not examine specific intake of nutrients, such as fat and salt, which may influence individuals' physical health status.

The goal of the current study was to investigate the relationship between perceived stress and fat and salt intake, and to evaluate the moderating role of self-efficacy in young adults. In order to address previous mixed findings on the role of self-efficacy, we measured both general self-efficacy and diet self-efficacy. It was hypothesized that increases in perceived stress would associate with increases in fat and salt intake. It was also hypothesized that diet self-efficacy would moderate the relationship between stress and food intake, in that high stressed individuals with high diet self-efficacy would report lower fat and salt intake compared with high stressed-low diet self-efficacy individuals. Overall, the highest sodium and fat intake was expected in individuals reporting high stress and low diet self-efficacy and the lowest sodium and fat intake was expected in students reporting low stress and high diet self-efficacy; low stress-low diet self-efficacy and high stress-high diet self-efficacy groups were not expected to differ in nutrient intake. Finally, in line with Bandura's theory, general self-efficacy was not expected to moderate the relationship between perceived stress and nutrient intake.

Methods

Ethical considerations

All research conducted within the current study was approved and overlooked by Ryerson University's Research Ethics Board. Necessary documents, including an ethics proposal, consent and debriefing forms, as well as all questionnaires were submitted and approved prior to conducting the study.

Measures

In order to test the predicted hypotheses, participants completed six questionnaires, including those that measure levels of perceived stress, self-efficacy, and nutrient intake.

Demographics questionnaire

A 15-item demographics questionnaire developed by the primary researcher was used to gain general demographic information from the participants, including age, sex, race, smoking status, program of study, work status, number of exams in the past month, hypertension diagnosis, medications being taken, height, and weight. All items on the demographics questionnaire were self-reported, with the exception of participants' height and weight, which was measured by researcher to calculate Body Mass Index (BMI).

Eating habits confidence scale

A 20-item self-report diet self-efficacy questionnaire designed to evaluate an individual's belief in his or her ability to successfully avoid eating certain unhealthy foods, namely high-fat and high-sodium foods. Participants' final scores on this scale may range from 0-100. An example of an item in the EHCS is "How sure are you that you can stick to your low fat, low salt foods when there is high fat, high salt food readily available at a party?". This questionnaire has been validated in the target population for the current study [28], and deemed a reliable measure of diet self-efficacy (alpha = 0.9) [29].

General perceived self-efficacy scale

A 10-item self-report measure used to assess general self-efficacy. The questionnaire is designed to measure one's general sense of perceived self-efficacy, with potential scores ranging from 0-40. An example of an item in the GPSES is "It is easy for me to stick to my aims and accomplish my goals". This questionnaire has been validated in the target population for this study [30], and has been deemed a reliable measure, with Cronbach's alpha ranging from 0.8-0.9 [31,32].

Cohen's perceived stress scale

This scale is a 14-item self-report questionnaire with a maximum potential score of 56. This scale is commonly used to assess an individual's perception of stress over a 1-month period. An example of an item in this questionnaire is "In the past month, how often have you felt nervous or stressed?". This scale has been deemed reliable and has been validated in the target population for this study [33]. This scale has a measured reliability of Cronbach's alpha of 0.9 [34,35].

Block fat screener

A 17-item self-report questionnaire used to evaluate an individual's fat intake over a 1-month period. This screener evaluates how often an individual has eaten a certain food in the past month. An example of an item found on this screener is "How often have you eaten doughnuts, pastries, cake, cookies (not low-fat) within the past month?". This screener has been validated in the target population for this study [36], and has been deemed reliable, with a Cronbach's alpha between 0.7 and 0.9 [37].

Block sodium screener

A 28-item self-report questionnaire used to evaluate an individual's sodium intake over a 1-month period. This screener evaluates how often an individual has eaten specific types of food in the past month, and how often they have done so within the average day. An example of an item found on this screener is "How often have you

eaten salad dressing in the past month, and within a day?". The Block food screeners have been deemed validated in the target population for studies similar to the current, although a reliability study has yet to be conducted [36].

Participant characteristics

Undergraduate students (n = 136; 23 male, 113 female; M_{age} = 20.62, SD = 3.41) were recruited from Ryerson University in Toronto, Ontario, Canada. Participants were eligible on the condition that they were enrolled in the Introductory Psychology courses at Ryerson University. Thus, students who were completing any degree which would allow them to take the Introductory Psychology courses were eligible to participate in the study. Those who were eligible were recruited through Sona, the university's online participant pool, which was then made up of hundreds of students. Fifty-five percent of the sample population identified themselves as Caucasian and the average body mass index (BMI) within the sample was 21.93 (SD = 4.97).

Procedure

Upon arrival at the Stress and Healthy Aging Research Lab at Ryerson University, participants were asked to read and sign an informed consent form. Following consent, participants completed the Eating Habits Confidence Scale, the General Perceived Self-Efficacy Scale, the Perceived Stress Scale, the Block Fat Screener, the Block Sodium Screener, and the demographics questionnaire. Following completion of the questionnaire battery, the primary researcher weighed the participant and measured the individual's height in order to calculate BMI. Upon completion of the study, participants were debriefed.

Research design and statistical analyses

Using a survey-based cross-sectional design, all participants completed the questionnaires in the same order in order to reduce contingency biases in responses to subsequent questionnaires.

Variables of perceived stress (PS), diet self-efficacy (DSE), general self-efficacy, and fat and sodium intake were assessed for normality and outliers. All variables were normally distributed and 6 outliers were removed from the data due to age and health conditions. One male and two female participants were removed from the dataset based on their age and program; they were all significantly older than the mean age (38, 40 and 58 years of age) in the continuing education program and they reported health concerns that may impact nutrient intake (hypertension and prescribed lowered sodium consumption). Two males were removed from the data set due to their outlying age (41 and 48 years of age) and health conditions (high cholesterol and Brown Adipose Tissue). One male was removed due to his stress score being significantly above the mean (more than three standard deviations). Following removal of these 6 participants, a total of 130 participants (19 male, 111 female) were included in subsequent analyses.

To assess the association between PS, DSE and fat and sodium intake, separate linear regression analyses were performed. In each model, PS, DSE and their interaction (PS × DSE) were entered in step one, followed by the covariates age, race, and sex in step two. This model was conducted for both fat and salt intake as the outcome variable of interest. Further, the same analyses were conducted using general self-efficacy (GSE) to confirm that diet self-efficacy is a more relevant measure than general self-efficacy in assessing the moderating role between stress and fat and sodium intake.

In order to assess and visualize the interaction between stress and self-efficacy on fat and sodium intake, a median split was created for perceived stress (HighPS-LowPS) and diet self-efficacy (HighDSE-LowDSE), which then enabled the creation of four groups: highPS-highDSE (n = 31), lowPS-lowDSE (n = 30), highPS-lowDSE (n = 36), and lowPS-highDSE (n = 33). Analyses of variances followed by post-hoc group comparisons were performed to assess group differences in the combined effects of PS and DSE on sodium and fat intake. Given that the proposed hypotheses were based on group (e.g. high PS and low DSE is associated with greater sodium intake), ANOVAs were conducted irrespective of the aforementioned regression analyses.

Finally, subgroup analyses were conducted to explore the relationship between stress and self-efficacy on fat and sodium intake in female and male participants. All analyses were performed using SPSS (Statistical Package for the Social Sciences) and results were considered significant at the 5% alpha level.

Results

For the purpose of the current analyses, *d* is meant to indicate Cohen's Effect Size. A larger effect size indicates that the difference between means is significant [38]. In addition, for the purpose of these analyses, the term 'intake' is meant to indicate fat and sodium scores based on self-report food intake questionnaires. All additional calculations with respect to fat and sodium intake (e.g. mg/day) are based on intake scores.

Sample characteristics

Participants were on average 20.62 years of age (SD = 3.41), with 85% of the sample being female. Fifty-five percent of the sample identified themselves as Caucasian, and the average BMI of the sample was 21.93 (SD = 4.97). See Table 1.

Table 1 Sample characteristics

Variable	Mean (SD) or %
Demographics	
Age (Min = 18.0, Max = 38.0)	20.62 (3.41)
Sex (% female)	85
Race (% Caucasian/White)	55
BMI (Min = 14.87, Max = 35.2)	21.93 (4.97)
Smoking status (% yes)	6.9
Program	
Psychology	19
Social work	17
Biology	12
Nursing	10
Other	42
Work	
No (%)	44
Part time (%)	55
Full time (%)	1
Exams	
0 (%)	6
1 to 2 (%)	12
3 to 4 (%)	25
5 to 6 (%)	33
7+ (%)	24
Hypertension (%yes)	1
Medication (%yes)	18
Stress and self efficacy	
PS (Min = 7.0, Max = 40.0)	26.72 (7.43)
GSE (Min = 20.0, Max = 40.0)	30.31 (4.0)
DSE (Min = 25.0, Max = 100)	75.88 (12.73)
Dietary intake	
Sodium score (Min = 8.0, Max = 64.00)	30.21 (10.85)
Sodium (mg/day) (Min = 194.6, Max = 6512.5)	2983.7 (1184.16)
Fat score (Min = 4.0, Max = 48.0)	20.66 (7.96)
Percent fat (%) (Min = 24.0, Max = 50.9)	34.16 (4.82)
Saturated fat (g) (Min = 9.4, Max = 48.1)	24.57 (7.16)
Total fat (g) (Min = 49.5, Max = 159.1)	91.94 (19.31)
Cholesterol (mg) (Min = 96.55, Max = 476.35)	254.06 (69.99)

Average GSE, DSE and PS scores were also calculated. The average GSE rating was 30.31 (SD = 3.96). The average DSE rating was 75.88 (SD = 12.73) and the average PS rating was 26.72 (SD = 7.43). See Table 1 for additional calculated means.

Sample characteristics by sex

Males and female participants differed in their report of GSE, DSE, and PS. Males reported significantly higher levels of GSE ($F(1,128) = 5.91$, $p = 0.02$; $d = .67$) than females, and females reported higher levels of PS ($F(1,128) = 7.06$, $p = 0.01$; $d = .77$) than males. There were no significant differences between males and females in terms of reported DSE ($F(1,128) = 0.06$, $p = 0.81$; $d = .07$).

Males and females differed in their reports of both fat and sodium intake. Saturated fat ($F(1,128) = 6.96$, $p = 0.01$; $d = .66$), cholesterol ($F(1,128) = 15.69$, $p < 0.001$; $d = .98$), and sodium intake in mg per day ($F(1,128) = 6.89$, $p = 0.01$; $d = .68$) was found to be significantly greater in males compared with female intake scores.

Effects of stress and diet self-efficacy on reported fat intake

Unadjusted regression analyses showed an interaction effect between PS and DSE (ß = -1.07, $p = 0.04$) on fat score. These findings did not change when controlling for age, sex, and race.

Mirroring the aforementioned regression analyses, ANOVA revealed a significant PSxDSE interaction effect on reported fat intake ($F(3,125) = 5.83$, $p = 0.001$; $r = .35$). Controlling for age, sex and race, ANCOVA revealed the same result, suggesting a significant PS×DSE effect on reported fat intake ($F(3,123) = 5.36$, $p = 0.002$; *partial* $\eta^2 = .12$). Subsequent post-hoc Tukey tests revealed that reported fat intake of highPS-lowDSE participants significantly differed from highPS-highDSE participants ($p = 0.001$) and from lowPS-highDSE participants ($p = 0.01$), but not from lowPS-lowDSE participants ($p = 0.23$) (see Table 2 for adjusted means). More specifically, highPS-lowDSE participants demonstrated significantly greater levels of reported fat intake than highPS-lowDSE participants and lowPS-highDSE participants. See Figure 1.

Effects of stress and diet self-efficacy on reported sodium intake

Unadjusted regression analyses revealed no interaction effect between DSE and PS. A main effect was revealed for DSE (ß = -0.33, $p < 0.001$) with trending effects for PS (ß = 0.15, $p = 0.07$) on sodium score. Specifically, participants who reported higher levels of DSE also reported lower levels of sodium intake, independent of perceived stress. These findings did not change when controlling for age, sex, and race.

Table 2 Adjusted means from DSExPS interactions for fat score

Group	Adjusted mean	Standard error
LowDSExLowPS	20.89	1.41
LowDSExHighPS	24.78	1.30
HighDSExLowPS	18.80	1.33
HighDSExHighPS	17.88	1.37

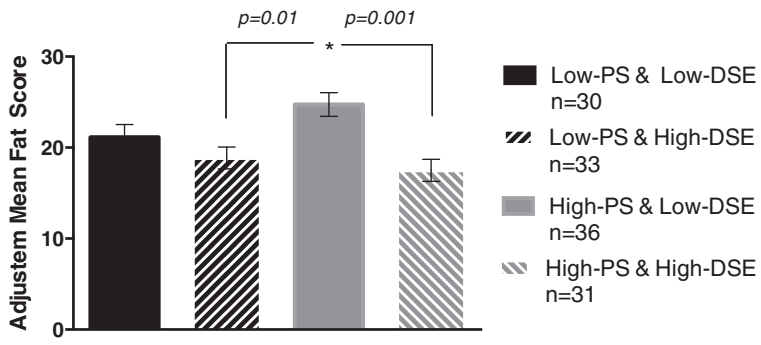

Figure 1 Adjusted mean fat intake score (and standard errors) based on high and low PS and DSE.

ANOVA revealed a significant PSxDSE interaction effect on sodium intake at the trend level ($F(3,126) = 2.47$, $p = 0.07$; $r = .23$). A similar interaction effect was found after controlling for age, sex, and race ($F(3, 123) = 2.31$, $p = 0.09$; *partial* $\eta^2 = .05$). Although the interaction was not statistically significant, subsequent post-hoc comparisons were conducted (see Table 3 for adjusted means) which revealed that reported sodium intake was significantly higher in highPS-lowDSE participants compared with lowPS-highDSE participants ($p = 0.04$); although not statistically significant, sodium intake in highPS-lowDSE participants was also greater compared with highPS-highDSE participants ($p = 0.07$) and lowPS-lowDSE participants ($p = 0.36$). See Figure 2.

General self-efficacy as a moderator for fat and sodium intake

Regression analyses revealed no significant associations between GSE and fat intake (ß = 0.12, $p = 0.25$), or interaction effects of PS and GSE on fat intake (ß = -0.62, $p = 0.26$). Regression analyses also revealed no significant associations between GSE and sodium intake (ß = -0.31, $p = 0.31$), or interactions between PS and GSE on sodium intake (ß = -0.44, $p = 0.42$).

Additional variables considered

Additional variables such as BMI, work and school commitments, age, race, and medications being taken were measured. None of these variables were found to correlate with nutrient intake, PS or DSE. Regression analyses and ANOVAs revealed no significant effects of an association between BMI, and PS or DSE.

Table 3 Adjusted means from DSExPS interactions for sodium score

Group	Adjusted mean	Standard error
LowDSExLowPS	29.49	2.00
LowDSExHighPS	34.29	1.84
HighDSExLowPS	28.13	1.89
HighDSExHighPS	28.55	1.94

Discussion

A robust association exists between stress and increased fat and sodium intake. In light of the growing obesity epidemic and an increase in the amount of stress being reported among college and university students, the current study evaluated perceived stress, self-efficacy and sodium and fat intake in a group of undergraduate students.

Overall, reported perceived stress within this sample was slightly above the standardized norm [39]. Current findings also coincide with previous research suggesting that unhealthy food intake is a common coping mechanism implemented in response to stress in undergraduate students [4].

Previous studies have demonstrated an increase in food intake as a result of increases in reported stress [5,40], but have failed to focus on the specific nutrients that high-stressed individuals tend to gear towards, such as foods that are high in sodium and fat. The current study has refined this association by examining fat and sodium intake instead of using more general measures of food intake. However, based on the current results, stress alone does not contribute to nutrient intake; rather, the effects of stress on sodium and fat intake are dependent on an individual's level of diet-self efficacy. The combination of high stress and low diet self-efficacy appears to be associated with the greatest amount of reported fat and sodium intake, and the combination of low levels of stress paired with high diet self-efficacy seems to be associated with the lowest reported intake of these nutrients. However, it should be noted that not all post-hoc comparisons were statistically significant; thus these findings must be interpreted with caution.

Research suggests that stress-induced eating of fatty and high-sodium foods may be a contributing factor to

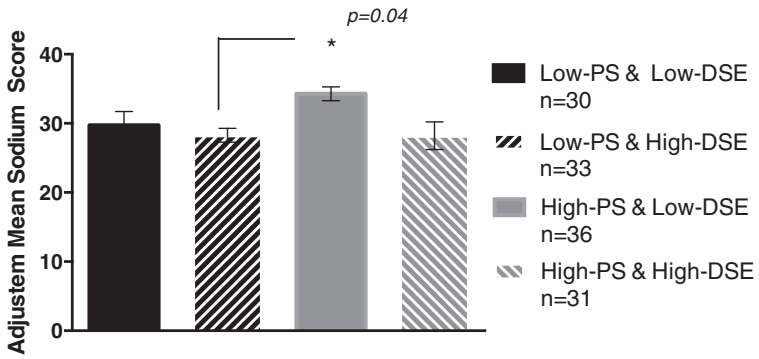

Figure 2 Adjusted mean sodium intake score (and standard errors) based on high and low PS and DSE.

the development of obesity - resulting from energy intake exceeding energy output over a long period of time. These diet patterns are also shown to be associated with a number of health problems [41-47]).

Previous studies have either examined general self-efficacy or general food intake (e.g. caloric intake), which have led to mixed findings. The current study shows the importance of matching self-efficacy to the type of behavior under investigation. Indeed, according to Bandura's [26] theory, it is important to focus on domain-specific self-efficacy, as general self-efficacy does not appear to consistently play a role in specific health behaviors. The present findings support this premise as general self-efficacy was not associated with nutrient intake, nor did it moderate the relationship between stress and nutrient intake. Specifically, this study suggests that the interaction between perceived stress and diet self-efficacy plays an integral role in determining sodium and fat intake. The distinctive moderating effect of diet self-efficacy compared to general self-efficacy reported in this study may explain the mixed and inconclusive findings within the literature. This distinction should also be considered in future research that examines other outcomes, such as assessing the moderating effect of exercise self-efficacy on physical activity behavior.

Interestingly, body mass index was not associated with fat and sodium intake. This may be due to other lifestyle behaviors that were not investigated (such as exercise), which may moderate the relationship between BMI, fat and sodium. Additionally, stress-inducing situations (e.g. number of exams and assignments one was recently required to complete) were not associated with perceived stress. This lack of association may be related to the importance of "perception" – as previously mentioned, one's stress level is highly dependent on perception of the stressors that are present in one's life [48]. The perception of stress is relative and involves other external factors such as interpersonal stressors or health stressors that may contribute to overall stress scores; five exams for one student may be considered as stressful as one exam for another student.

Although the present findings are important and significantly contribute to the existing literature, this study was not without limitations. First, the small number of male participants did not provide enough power to assess sex differences in the relationship between stress, diet self-efficacy and nutrient intake. However, based on observation of the means, male undergraduate students seem to be demonstrating the same pattern outcomes as females (data not shown). Second, the sample was relatively homogeneous, consisting of young adults (Age range = 18-38 years) living primarily in the Greater Toronto Area, which may undermine generalizability of the findings. Finally, the use of self-report questionnaires may be subject to recall bias and self-report bias that may have overestimated or underestimated the results. Future research should examine fat and sodium intake using a larger sample of male participants in order to perform subgroup analyses and investigate sex differences. Sugar intake should also be considered, as it is well-known that high sugar intake can put individuals at risk for negative health outcomes such as diabetes.

As young adults continue to ingest large amounts of sodium and fat, their risk for health conditions such as obesity, hypertension, cardiovascular disease and cognitive impairment also rise. The findings of the current study have important implications for the prevention of the aforementioned detrimental health conditions. Finally, these findings provide insight into the theoretical notion that improvements in diet self-efficacy (but not general self-efficacy), and reductions in perceived stress levels may reduce fat and sodium intake, thus reducing young adults' risk of developing poor health conditions.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
RN conducted the devised the objective for the current study, as well as the methods. RN also selected the measures, and was responsible for participant recruitment and data collection. Further, RN conducted the statistical analyses and wrote the manuscript. AJ supervised the entire research process, aiding in all aspects including selecting measures, submitting the ethics proposal, running statistical analyses, and revising the manuscript. All authors read and approved the final manuscript.

Acknowledgements
We thank Sasha Mallya for her contribution to the study, including feedback regarding access to measures, and appropriate statistical analyses to conduct. We also thank Nurit Postelnik for aiding in data collection.

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Sex differences in the impact of the Mediterranean diet on systemic inflammation

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Abstract

Background: Some intervention trials have reported a reduction in systemic inflammation with the Mediterranean diet (MedDiet) while others have observed no effect. Despite the fact that sex differences have been highlighted in the inflammatory regulation, it is still not known whether MedDiet exerts similar effects on systemic inflammation in men and women. The aim of this study was therefore to investigate sex differences in the effects of the MedDiet on high-sensitivity C-reactive protein (hs-CRP).

Findings: Participants were 35 men and 27 premenopausal women (24–53 years) presenting a slightly deteriorated lipid profile. All foods were provided to participants during a 4-week isocaloric MedDiet. At baseline, women had higher hs-CRP concentrations than men ($P = 0.03$). No sex difference was observed in hs-CRP response to the MedDiet (P for sex-by-time interaction = 0.36), with both men and women experiencing no change (respectively $P = 0.62$ and $P > 0.99$). When subgroups were formed according to hs-CRP concentration before the MedDiet phase, men with elevated baseline values (≥2 mg/l) experienced a reduction in hs-CRP over time with the MedDiet (−26.5 %) while an increase was observed in men with lower baseline values (+96.6 %; P for group-by-time interaction = 0.02). This pattern of change was not observed in women.

Conclusions: Results from this controlled feeding study suggest that men and women have similar effects from the MedDiet on systemic inflammation. The individual's overall inflammatory status seems to influence these effects, but only in men.

Trial registration: This clinical trial was registered at www.clinicaltrials.gov as NCT01293344.

Keywords: Sex, Mediterranean diet, C-reactive protein, Men, Women

Findings

Introduction

The implication of low-grade, chronic inflammation in the formation, progression and rupture of atherosclerotic plaques is now well-recognized [1]. Accordingly, elevated C-reactive protein (CRP), a marker reflecting the individual's systemic inflammatory status, has been consistently associated with increased risk of coronary heart disease events [2, 3] and type 2 diabetes [4]. There is growing evidence that adopting the traditional Mediterranean diet (MedDiet) reduces systemic inflammation [5]. The MedDiet is characterized by an abundance of plant-based foods, such as fruits, vegetables, whole grain cereals, nuts and legumes; olive oil as the main source of fat; moderate amounts of fish, poultry, dairy products and eggs; relatively low amounts of red meat and sweets and moderate amounts of red wine with meals [6]. However, even if most of the intervention trials have reported that this food pattern reduces CRP concentrations [7–11], some have observed no effects [12–15]. Accordingly, the investigation of factors that may influence the anti-inflammatory effects of this healthy food pattern is of great interest. Sex has been highlighted as a determinant of the inflammatory regulation. In fact women are characterized by a higher inflammatory overall burden than men [16]. Also, effects of sex hormones on inflammatory status have been documented, estrogens being now recognized for their anti-inflammatory properties in women [17]. However it is still not known whether MedDiet exerts the

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same effect on inflammation in men and women. The aim of this study was therefore to investigate sex differences in the effects of the MedDiet on high-sensitivity C-reactive protein (hs-CRP) concentrations. As estrogens have anti-inflammatory properties in premenopausal women [17], and that the MedDiet has been previously shown to reduce estrogen concentrations in women [18], we hypothesized that premenopausal women benefit less from the anti-inflammatory effects of the MedDiet than men.

Methods

Participants

Thirty-eight men and 32 premenopausal women (24–53 years) took part of this study. The main inclusion criteria were to have a slightly elevated low-density lipoprotein cholesterol (LDL-C) concentrations (between 3.4 and 4.9 mmol/l) or total cholesterol to high-density lipoprotein cholesterol (HDL-C) ratio ≥5.0, and at least one of the four following cardiovascular disease (CVD) risk factors: waist circumference >94 cm in men and >80 cm in women; triacylglycerol (TAG) concentration ≥1.7 mmol/l; fasting glycemia between 6.1 and 6.9 mmol/l and/or blood pressure levels ≥130/85 mm Hg. More details about inclusion and exclusion criteria have been reported elsewhere [19]. Women using systemic hormonal contraceptives were excluded. All subjects signed an informed consent form before their inclusion in the study, which has been approved by the Laval University Research Ethics Committee. Power analysis indicated that a total sample size of $n = 62$ is sufficient to detect significant changes in hs-CRP concentrations (repeated measures, within-between interaction) with a small effect-size estimate (Cohen's d of 0.20), and with an $\alpha = 0.05$ and a power (1-β error probability) of 0.95 (G*Power Version 3.0.10, Franz Faul, Universität Kiel, Germany).

Study design

The study protocol consisted in a 4-week run-in period, immediately followed by a 4-week fully-controlled MedDiet phase. Firstly, during the 4-week run-in period, participants had to comply with the recommendations of the Canada's Food Guide [20] as prescribed by a registered dietician. The purpose of this run-in period was to ensure similar dietary habits between men and women prior the controlled MedDiet phase, a goal that has been reached as previously reported [19]. Briefly, Canada's Food Guide is an educational tool which promotes healthy eating for Canadians in order to reduce the risk of many chronic diseases and to achieve overall health and vitality. It indicates the recommended number of food guide servings per day for each of the four food groups (vegetables and fruits, grain products, milk and alternatives, and meat and alternatives) according to the age and sex of individuals.

Thereafter, during a 4-week fully-controlled feeding phase, subjects consumed an experimental MedDiet formulated to be concordant with the characteristics of the traditional MedDiet [6]. Details about the composition of the MedDiet are given in Table 1 and Table 2, as previously reported in other publications [19, 21]. Subjects were instructed to consume only the foods and beverages provided to them, which corresponded to 100 % of their estimated energy needs. More precisely, energy needs were estimated by averaging the energy requirements estimated by a validated FFQ [22] administrated at the beginning of the run-in period and energy needs as determined by the Harris–Benedict formula. Body weight was measured on weekdays just before lunch and in case of body weight variation, energy intake was modified. The amount of each food/drink provided to each participant during the MedDiet was proportional to his/her estimated energy needs. In order to evaluate compliance, participants were asked to note on a checklist foods consumed and, if needed, the amount of foods not consumed for each day of the controlled MedDiet phase. The overall compliance calculated from the food checklist in men and women was respectively 97.9 ± 3.6 % and 97.6 ± 3.2 %. Since sex hormones may influence the inflammatory status [17], women's feeding was shortened or prolonged if needed in order to be able to carry out all tests in the early follicular phase of their

Table 1 Servings of key foods of the Mediterranean pyramid consumed daily during the experimental Mediterranean diet phase for a 10 460 kJ/d (2500 kcal/d) menu

Key foods[a]	MedDiet (servings/d)
Olive oil (ml)	43.3
Whole grains products	5.7
Fruits and Vegetables	16.1
Legumes	0.5
Nuts	0.9
Cheese and yogurt	2.0
Fish	1.3
Poultry	0.9
Eggs	0.3
Sweets	0.3
Red meat	0.2
Red wine	1.3

MedDiet Mediterranean diet

[a]Extra virgin and virgin olive oils were used. Serving size for whole grains products = 125 ml (rice, pasta, bulgur, couscous), one bread piece or 30 g cereal; Serving size for fruits and vegetables = 125 ml; Serving size for legumes = 175 ml and for nuts = 30 g; Serving size for fish, poultry and red meat = 75 g; Serving size for egg = 100 g; Serving size for dairy products (mostly low fat cheese and yogurt) = 50 g cheese, 175 g yogurt and 250 ml milk; Serving size for red wine = 150 ml

This table has been previously published in other publications [19, 21]

Table 2 Daily nutritional composition of the experimental Mediterranean diet for a 10 460 kJ/d (2500 kcal/d) menu

	MedDiet For 10 460 kJ/d (2500 kcal/d)
Energy (kJ)	10 460
Carbohydrate (% of total energy)	46.0
Fiber (g)	42.3
Protein (% of total energy)	17.0
Fat (% of total energy)	32.0
SFA (% of total energy)	6.7
MUFA (% of total energy)	18.1
PUFA (% of total energy)	4.7
Cholesterol (mg)	289.7
Alcohol (% of total energy)	5.0
MUFA to SFA ratio	2.7
Sodium (mg)	3039

MedDiet Mediterranean diet
This table has been previously published in other publications [19, 21]

menstrual cycle (mean duration of the feeding period in women 28.8 ± 4.3 days).

CRP measurements

Fasting blood samples were collected after the run-in period (*i.e.* just before the controlled MedDiet phase, referred as baseline values) and immediately after the MedDiet. Serum concentrations of hs-CRP were measured using a high-sensitivity enzyme immunoassay test kit (BioCheck Inc., Foster City, CA; coefficients of variation: intra-assay ≤ 7.5 %, inter-assay ≤ 4.1 %).

Statistical analysis

Statistical analyses were performed with the SAS statistical package version 9.4 (SAS Institute Inc., Cary, NC, USA). Time and sex-by-time interaction effects on hs-CRP concentrations were assessed by using MIXED procedures for repeated measurements followed by Tukey-Kramer tests. Participants with hs-CRP concentrations greater than 10 mg/l (indicative of an acute inflammation process [23]) before or after the MedDiet phase were excluded from our analyses (three men and five women). A $P \leq 0.05$ was considered as statistically significant.

Results

At baseline, men and women had similar mean age and body mass index (BMI) (Table 3). However, men were characterized by higher body weight and waist circumference, and displayed higher values for TAG, total cholesterol/HDL-C ratio, systolic and diastolic blood pressures and fasting glucose, and a lower value for HDL-cholesterol than women (Table 3). The degree of concordance of the diet with the traditional MedDiet, as assessed by the Mediterranean score after the run-in phase based on the Canada's Food Guide [24], was similar in men and women (Table 3).

At baseline, women had higher hs-CRP concentrations than men (1.53 ± 1.49 mg/l for men and 2.32 ± 1.63 mg/l for women; P for sex difference = 0.03). No change was observed for hs-CRP concentrations over time with the MedDiet in both men and women (respectively P = 0.62 and P > 0.99, P for sex-by-time interaction = 0.36; Fig. 1).

When subgroups were formed according to hs-CRP concentrations before the MedDiet phase, men with

Table 3 Characteristics of men and women at baseline[a]

	Men (n = 35)		Women (n = 27)		Sex difference[b]
	Mean	SD	Mean	SD	*P*-value
Age (years)	43.0	7.2	41.4	7.3	0.3928
Body weight (kg)[c]	92.1	14.1	74.9	9.7	<0.0001
BMI (kg/m^2)[c]	29.2	3.2	28.4	3.2	0.2881
Waist circumference (cm)[c]	102.7	11.0	94.7	8.1	0.0018
TAG (mmol/l)[c]	1.86	1.19	1.34	0.65	0.0273
LDL-cholesterol (mmol/l)	3.65	0.72	3.56	0.51	0.5896
HDL-cholesterol (mmol/l)[c]	1.12	0.30	1.33	0.25	0.0020
Total cholesterol/HDL-C	5.24	1.03	4.25	0.77	<0.0001
Systolic blood pressure (mm Hg)	117.3	12.9	107.2	10.2	0.0015
Diastolic blood pressure (mm Hg)	80.4	9.2	72.1	8.0	0.0005
Fasting glucose (mmol/l)	5.87	0.37	5.54	0.44	0.0019
Mediterranean score (arbitrary units)[d]	25.1	6.0	24.5	4.8	0.6541

SD standard deviation, *BMI* body mass index, *TAG* triacylglycerol, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein
[a]These characteristics were measured after the run-in period, *i.e.* immediately before the controlled MedDiet phase
[b]Sex differences were determined using the Student's *t*-test for unpaired data, except for age for which Wilcoxon-Mann–Whitney test was used
[c]Analysis was performed on transformed values
[d]From 0 to 44 points, a score of 44 implies a food pattern which is perfectly concordant with the traditional MedDiet

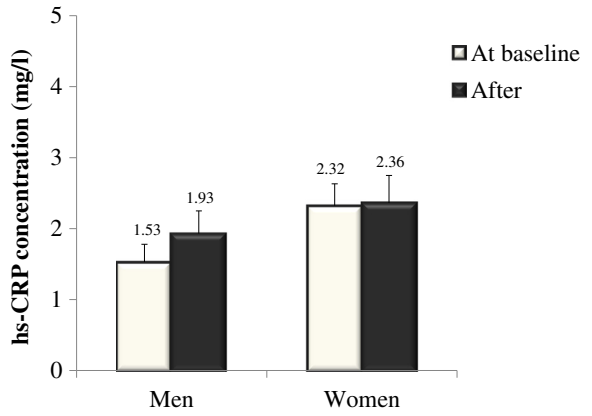

Fig. 1 hs-CRP concentrations observed in men (left, n = 35) and women (right, n = 27) at baseline and after the 4-week Mediterranean diet. MIXED procedures for repeated measurements followed by Tukey-Kramer tests were used. Data are means ± SEM

elevated baseline values (≥2 mg/l, as defined in the Canadian guidelines for the diagnosis and treatment of dyslipidemia and prevention of CVD in the adult [25, 26]) experienced a reduction in hs-CRP over time with the MedDiet (−26.5 %) while an increase was observed in men with lower baseline values (<2 mg/l, +96.6 %; P for group-by-time interaction among men = 0.02; Fig. 2a). This pattern of change was not observed in women (P for group-by-time interaction among women = 0.11; Fig. 2b).

Adjustments for the small but significant weight loss during the MedDiet phase (−1.2 kg or 1.3 % of initial body weight in men, P < 0.0001 and −0.6 kg or 0.7 % in women, P = 0.04; P for sex-by-time interaction = 0.09) did not influence results obtained (not shown). Changes in body weight during the controlled MedDiet were not associated with changes in hs-CRP concentrations (r = −0.02, P = 0.89 in men and r = −0.13, P = 0.52 in women). No change was observed for waist circumference in both sexes.

Discussion

Results from this fully-controlled feeding study suggest that men and women have similar effects from the MedDiet on systemic inflammation. In fact, in this sample of individuals characterized by moderately elevated CRP concentrations, no beneficial effects from the MedDiet were observed, irrespective of the sex. However, results suggest that the variability in the anti-inflammatory effects of the MedDiet might be attributed in part to the

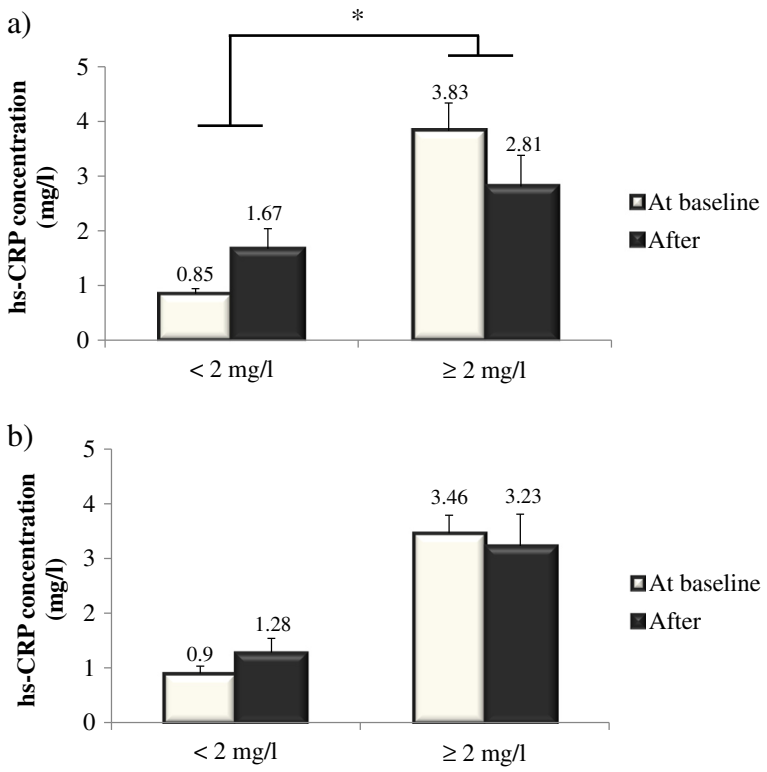

Fig. 2 hs-CRP concentrations observed in men (**a**) and women (**b**) at baseline and after the 4-week Mediterranean diet according to their hs-CRP baseline value. MIXED procedures for repeated measurements followed by Tukey-Kramer tests were used. *A group-by-time interaction was found in men (P = 0.02) but not in women (P = 0.11). Men with hs-CRP < 2 mg/l, n = 27; men with hs-CRP ≥ 2 mg/l, n = 8; women with hs-CRP < 2 mg/l, n = 12; women with hs-CRP ≥ 2 mg/l, n = 15. Data are means ± SEM

individual's overall inflammatory status but this observation seems to be more specific to men.

Despite the fact that men and women differ substantially with respect to inflammatory regulation, very limited data exist on sex differences in the impact of diet on inflammatory status. In the case of the MedDiet, an observational study has reported reduced CRP concentrations in men, but not in women, who consumed a diet more closely in accordance with the MedDiet [27] while another study observed this association irrespective of the sex [28]. For interventional trials, only the Prevención con Dieta Mediterránea (PREDIMED) study has previously documented the effects of the MedDiet on inflammatory status taking into account the sex [11]. Their study, consisting of a nutritional intervention among 772 high-risk individuals, indicates that the adherence to an energy-unrestricted MedDiet supplemented with extra-virgin olive oil reduces CRP concentrations compared with a low-fat diet, with subgroup analyses showing no difference between sexes. Results from our feeding study are therefore partly in line with those from the PREDIMED study, suggesting that the MedDiet has similar effect on inflammation in men and women.

However, our results are in disagreement with a meta-analysis of randomized controlled trials published in 2014 [5] which, as the PREDIMED study, reported a reduction of CRP concentrations with the MedDiet. However, further investigations of trials included in the meta-analysis highlight conflicting results between studies, some reporting a reduction of systemic inflammation with the MedDiet while almost half of the studies observed no significant effects. In an effort to improve our understanding of factors responsible for this divergence between studies, additional analyses from the present study suggest that the variability in the inflammatory response to the MedDiet might be attributed in part to the individual's systemic inflammatory status, *i.e.* those with elevated CRP concentrations having anti-inflammatory effects from the MedDiet while those with low baseline concentrations experiencing a clinically non-significant increase in response to this food pattern. However this pattern of change was more specific to men, suggesting that sex may modulate to a certain extent the impact of the MedDiet on the inflammatory status.

It is important to consider that participants included in the present study were characterized by healthy dietary habits at baseline. In fact, participants had to comply with the recommendations of the Canada's Food Guide during the four weeks preceding the controlled MedDiet phase [19]. It is therefore possible that changes in hs-CRP have started during this run-in phase, limiting subsequent changes during the controlled MedDiet phase. Therefore, results from the present study should not be over-interpreted and they suggest that, compared with the Canada's Food Guide recommendations, the MedDiet has no further impact on hs-CRP concentrations.

The small body weight loss observed during the MedDiet phase may be view as a limitation. However, several studies have demonstrated that body weight loss is the best nonpharmacologic modality to reduce inflammation [29], which is in contrast with the nonsingnificant increase in hs-CRP concentrations observed in the present study. In addition, some studies have highlighted that a weight loss of at least 10 % is needed to have a significant effect on the inflammatory markers in overweight and obese individuals [30]. Moreover, additional analyses showed that the adjustment for body weight changes did not influence any of the results obtained. Therefore, these observations suggest that body weight loss observed in the present study was not a major limitation.

Conclusions

Results from this feeding study suggest that the MedDiet exerts similar effects on inflammation in men and women. In addition, these results suggest that the variability in the anti-inflammatory effects of the MedDiet might be attributed in part to the individual's overall inflammatory status; however this observation seems to be more specific to men. Additional clinical studies including several inflammatory markers and a larger sample size are of importance to further document the impact of sex on the inflammatory response to the MedDiet.

Abbreviations

CRP: C-reactive protein; MedDiet: Mediterranean diet; hs-CRP: High-sensitivity C-reactive protein; LDL-C: Low-density lipoprotein cholesterol; HDL-C: High-density lipoprotein cholesterol; TAG: Triacylglycerol; FFQ: Food frequency questionnaire; BMI: Body mass index; SD: Standard deviation.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

SL designed the research; AB and LC conducted the research; SD supervised the medical condition of participants; AB analyzed the data and wrote the first draft of this paper; all authors participated to the interpretation of data and revised critically and approved the final version of the paper.

Acknowledgements

The authors would like to thank Johanne Marin for her contribution in the laboratory. This research was supported by the Canadian Institutes of Health Research (grant number: MOP 84568) and the Heart and Stroke Foundation of Quebec (grant number: 2007–180). AB is a recipient of a studentship from Canadian Institutes of Health Research (CIHR). BL is Chair of Nutrition at Laval University.

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Tomato juice intake increases resting energy expenditure and improves hypertriglyceridemia in middle-aged women: an open-label, single-arm study

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Abstract

Background: Tomato-based food products have health-promoting and disease-preventing effects. Some tomato juice ingredients may have health benefits for middle-aged women, including women with menopausal symptoms and cardiovascular diseases. We investigated the net effect of tomato juice intake on several health parameters in women in this age group.

Methods: An open-label, single-arm study was conducted, involving 95 women (40-60-years-old) who had at least one menopausal symptom. The participants refrained from foods and drinks rich in tomato and tomato-based products for 2 weeks prior to the study and during the 8 weeks of tomato juice consumption. After the run-in period, the women were asked to consume 200 mL of unsalted tomato juice, twice daily for 8 weeks. Their menopausal symptoms were evaluated using the Menopausal Symptom Scale (MSS), Hospital Anxiety and Depression Scale (HADS), and Athens Insomnia Scale (AIS) before the study, and at 4 and 8 weeks after study commencement. At the same times, body composition; blood pressure; heart rate; resting energy expenditures (REEs); and serum levels of triglyceride (TG), cholesterol, glucose, and hemoglobin A1c were measured.

Results: Ninety-three women (98%) completed the study. The following parameters showed significant changes, compared with baseline, at study weeks 4 and 8 (mean ± standard deviation at baseline, week 4, and week 8): (1) the MSS score improved (9.9 ± 5.2, 8.5 ± 5.0, 8.3 ± 5.0; $P < 0.0001$, repeated measures analysis of variance(ANOVA)), (2) the HADS-anxiety subscale score improved (5.3 ± 2.7, 4.8 ± 2.4, 4.9 ± 2.9; $P = 0.041$, Friedman test), (3) heart rate increased (62.6 ± 9.4 bpm, 64.4 ± 8.6 bpm, 63.8 ± 8.2 bpm; $P = 0.028$, Friedman test), (4) REE increased (1980 ± 368 kcal/day, 2108 ± 440 kcal/day, 2149 ± 470 kcal/day; $P = 0.0030$, repeated measures ANOVA), (5) serum TG level decreased in the subgroup of women ($n = 22$) who had high TG (150 mg/dL or higher) at baseline (237.8 ± 88.9 mg/dL, 166.7 ± 86.1 mg/dL, 170.9 ± 109.7 mg/dL; $P = 0.0002$, Friedman test).

Conclusions: Tomato juice intake alleviated menopausal symptoms, including anxiety, increased REEs and heart rate, and lowered high baseline serum TG levels in middle-aged women.

Trial registration: UMIN-CTR UMIN000011877.

Keywords: Menopausal symptoms, Anxiety, Basal metabolism, Dyslipidemia

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Background

Middle-aged women are not only bothered by the physical and psychological symptoms of menopause, but are also at increased risk for cardiovascular diseases (CVDs), such as central obesity, hypertension, dyslipidemia, and diabetes [1-4], which are partly induced as a result of diminished estrogen production [5]. CVD is the number one cause of death worldwide, accounting for 17.3 million (30%) deaths, globally, in 2008 [6]. Most CVDs could be theoretically prevented through an elimination of risk factors, such as tobacco use, unhealthy diets, obesity, high blood pressure, impaired glucose tolerance, and raised lipids [7]; however, modification of one's own lifestyle is often difficult.

Tomato juice contains a variety of bioactive ingredients, such as gamma-aminobutyric acid (GABA), lycopene, 13-oxo-9,11-octadecadienoic acid (13-oxo-ODA), and esculeoside A, which may provide physical and psychological health benefits for middle-aged women. For example, GABA reduces psychological stress [8] and lowers blood pressure [9]. Lycopene has been reported to have anti-cancer effects and anti-oxidative effects, and to be effective at mitigating cardiovascular diseases, osteoporosis, and mental disorders [10-14]. Recently, 13-oxo-ODA was shown to lower plasma and hepatic triglyceride (TG) levels in an animal model of obesity [15]. Esculeoside A, a tomato saponin, was also shown to reduce serum levels of TG and cholesterol, and to ameliorate atherosclerotic lesions in ApoE-deficient mice [16]. Compared to fresh tomatoes, tomato juice has several advantages, including an increased level of extractable lycopene [17], and elevated antioxidant capacity (as a result of activation through the canning (heating) process) [18], and additionally, 13-oxo-ODA is found only in tomato juice [15]. Moreover, a recent research showed that tomato vinegar beverage improved glucose tolerance in high-fat diet-induced obese mice [19].

In the present study, we investigated the net effect of tomato juice intake on a variety of health parameters in middle-aged women, based on the hypothesis that anti-oxidative effect of lycopene and relaxing effect of GABA may alleviate physical and psychological symptoms of menopause; 13-oxo-ODA and Esculeoside A may increase resting energy expenditure (REE) and lower the serum level of triglyceride.

Methods

We conducted an open-label, single-arm study at the Menopause Clinic of the Tokyo Medical and Dental University. The study protocol was reviewed and approved by the Tokyo Medical and Dental University Review Board, and written informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki [20].

Ninety-five Japanese women participated in this study. The inclusion criteria were as follows: ages between 40 and 60; having at least one menopausal symptom on the Menopausal symptom scale (MSS) (score >1). The exclusion criteria were as follows: medication for hypertension, dyslipidemia, diabetes, or other cardiovascular diseases; intake of vitamins, lycopene, GABA or other supplements that could affect the parameters to be evaluated; allergy to tomato or tomato products. The participants were recruited through advertisements posted in our hospital and in the patients' social network. The participants were classified as follows: premenopausal (regular menstrual cycles in the past 3 months), perimenopausal (a menstrual period within the past 12 months but a missed period or irregular cycles in the past 3 months), postmenopausal (no menstrual period in the past 12 months), or had surgically or medically induced menopause (hysterectomy or chemotherapy for breast cancer).

From 2 weeks before the start until study termination, the participants refrained from foods and drinks rich in tomatoes and tomato products. After the run-in period, each participant was asked to consume 200 mL of unsalted tomato juice (Nippon Del Monte, Gunma, Japan) twice daily, just before breakfast and dinner, for 8 weeks. The nutritional composition of the tomato juice used in the current study is shown in Table 1. The product was manufactured in compliance with the Food Safety System Certification (FSSC) 22000 adopted by the Global Food Safety Initiative (GFSI). Adherence to the study protocol was confirmed by checking the participants' diaries including the records of tomato juice consumption.

The participants' menopausal symptoms were evaluated using MSS, Hospital Anxiety and Depression Scale

Table 1 The nutritional composition of the tomato juice used in the current study

Nutrient	Value per 400 ml
Energy	82 kilocalories
Protein	4.4 g
Fat	0 g
Sugars	14.4 g
Dietary fiber	3.6 g
Sodium	32 mg
Calcium	46 mg
Potassium	1260 mg
Vitamin A	92 μg
Lycopene	44 mg
13-oxo-ODA	78.4 μg
GABA	198 mg
Esculeoside A	unknown

13-oxo-ODA, 13-oxo-9,11-octadecadienoic acid.
GABA, gamma-aminobutyric acid.

(HADS), and Athens Insomnia Scale (AIS) before, and after 4 and 8 weeks of study participation. At each time point, body composition, blood pressure, heart rate, and REE, as well as serum levels of TG, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and plasma levels of glucose and hemoglobin A1c (HbA1c) were measured.

The MSS was validated and used in previous studies for participants to rate the severity of ten menopausal symptoms [21]. We evaluated vasomotor symptoms (hot flashes, perspiration, and chilliness); somatic symptoms (irregular heartbeat, headache/dizziness, tiredness, and aching joints/muscles); and psychological symptoms (insomnia, irritability, and depressed mood) using a 4-point Likert scale, depending on how often each symptom affected their daily life: none (never, 0 points); mild (rarely, 1 point); moderate (sometimes, 2 points); severe (very often, 3 points). MSS scores were calculated as the total score for the 10 aforementioned symptoms.

Developed by Zigmond and Snaith as a questionnaire [22], the HADS is a reliable instrument for screening clinically significant anxiety and depression in women; the questionnaire was translated into Japanese by Kitamura et al. [23]. The AIS was developed as a brief, easy-to-administer self-assessment questionnaire for determining insomnia severity according to the International Classification of Disease, Tenth Revision. The internal consistency and test-retest reliability of the AIS was previously confirmed [24]. The current study followed the protocol reported earlier [25], who measured the effect of supplementation with grape seed proanthocyanidin extracts on determining insomnia severity using AIS [24] and screening of clinically significant anxiety and depression using the Japanese version of HADS questionnaire [23].

The body composition of the participants, including height, weight, body mass index, fat mass, and muscle mass, was assessed using a body composition analyzer (MC190-EM; Tanita, Tokyo, Japan). Participants' systolic and diastolic blood pressure and heart rate were also measured using a vascular screening system (VS-1000; Fukuda Denshi, Tokyo, Japan). Additionally, REE was measured using a portable, indirect calorimeter (Metavine-N VMB-005 N; Vine, Tokyo, Japan).

Blood samples were collected by antecubital venipuncture and were centrifuged for the collection of serum. The serum and plasma samples were sent within 3 days of sampling to SRL, Inc. (Tokyo, Japan). The levels of serum lipids, plasma glucose, and HbA1c were assayed according to standard techniques.

Statistical analyses were performed using GraphPad Prism version 5.02 (GraphPad Software, San Diego, CA, USA) and IBM SPSS Statistics version 20 (IBM Corporation, Armonk, NY, USA). After testing the normality of each valuable using Shapiro-Wilk test, the variables with Gaussian distribution were evaluated with parametric tests (One-way repeated measures ANOVA and paired *t*-test) and those without were with non-parametric tests (Friedman and Wilcoxon signed-rank tests). One-way ANOVA and Kruskal-Wallis test were used for the evaluations of baseline characteristics among four different menopausal status groups and postprandial time for blood sampling. P-values < 0.05 were considered statistically significant.

Results

Table 2 presents the characteristics at baseline and at 4 and 8 weeks after study commencement of the ninety-three women (98%), who completed the 10-week study. 41 women (44%) were premenopausal, whereas 17 (18%) were perimenopausal, 28 (30%) were postmenopausal, and 7 (8%) had surgically or medically induced menopause. Comparing the baseline data among the four different menopausal status groups, we observed no statistically significant differences except for serum levels of total cholesterol (premenopausal, 197.8 ± 31.2 mg/dL; perimenopausal, 231.9 ± 25.3 mg/dL; postmenopausal, 223.3 ± 31.1 mg/dL; had surgically or medically induced menopause, 216.3 ± 40.3 mg/dL; P = 0.0005, One-way ANOVA). The mean interval between the blood sampling time and the previous meal at the 0-, 4-, and 8-week measurements were: 3.8 ± 2.2 hours, 4.1 ± 2.6 hours, and 4.2 ± 2.5 hours, respectively ($P = 0.62$, Kruskal-Wallis test).

We evaluated the changes in menopausal symptom severity using 3 scales. The mean MSS score (9.9 ± 5.2, at baseline; mean ± standard deviation) was significantly improved after 4 (8.5 ± 5.0, $P = 0.0012$, paired *t*-test) and 8 (8.3 ± 5.0, $P < 0.0001$) weeks of tomato juice consumption. The mean HADS-anxiety subscale score also improved, relative to baseline (5.3 ± 2.7) after 4 (4.8 ± 2.4, $P = 0.0170$, Wilcoxon signed rank test) and 8 (4.9 ± 2.9, $P = 0.0337$) weeks. There were no significant changes in the mean HADS-depression subscale and AIS scores after 4 and 8 weeks of the intervention.

We also evaluated the changes in participant body compositions, blood pressure, heart rate, and REEs. The participant body composition parameters and blood pressures did not change significantly after 4 and 8 weeks of intervention. However, the mean heart rate was significantly increased, compared to baseline (62.6 ± 9.4 bpm), after 4 (64.4 ± 8.6 bpm, $P = 0.0046$, Wilcoxon signed rank test) and 8 (63.8 ± 8.2 bpm, P = 0.0020) weeks of the intervention. In addition, the mean REE increased after 4 (2108 ± 440 kcal/day, $P = 0.0082$, paired *t*-test) and 8 (2149 ± 470 kcal/day, $P = 0.0018$) weeks of tomato juice consumption, relative to baseline (1980 ± 368 kcal/day).

Table 2 Participant characteristics at baseline and at 4 and 8 weeks after study commencement

	Baseline	4 weeks	8 weeks	P-value
Menopausal symptom score				
MSS score	9.9 (5.2)	8.5 (5.0)**	8.3 (5.0)***	<0.0001[a]
HADS-anxiety subscale score	5.3 (2.7)	4.8 (2.4)[#]	4.9 (2.9)[#]	0.04[b]
HADS-depression subscale score	4.7 (3.2)	4.6 (3.4)	4.4 (3.3)	0.32[b]
Athens Insomnia Scale score	3.7 (2.3)	3.4 (2.4)	3.8 (2.8)	0.16[b]
Body composition				
Height, cm	157.5 (4.9)	157.5 (4.9)	157.5 (4.9)	0.50[b]
Weight, kg	55.0 (8.5)	54.9 (8.6)	55.0 (8.6)	0.13[a]
Body mass index, kg/m^2	22.2 (3.2)	22.1 (3.2)	22.2 (3.2)	0.34[a]
Fat mass, kg	15.7 (6.1)	15.8 (6.2)	15.7 (6.2)	0.58[b]
Muscle mass, kg	37.0 (3.1)	36.9 (3.0)	37.1 (3.0)	0.046[a]
Blood pressure and heart rate				
Systolic blood pressure, mmHg	116.9 (14.1)	117.8 (14.3)	117.0 (13.8)	0.87[b]
Diastolic blood pressure, mmHg	75.4 (10.4)	75.2 (9.7)	74.5 (9.3)	0.23[b]
Heart rate, min^{-1}	62.6 (9.4)	64.4 (8.6)[##]	63.8 (8.2)[##]	0.03[b]
Resting Energy Expenditure	1980 (368)	2108 (440)**	2149 (470)**	0.003[a]
Blood examination				
Triglycerides, mg/dL	115.9 (84.0)	116.3 (70.2)	107.4 (75.3)	0.25[b]
Total cholesterol, mg/dL	213.1 (33.4)	213.9 (36.1)	212.2 (36.8)	0.76[a]
LDL cholesterol, mg/dL	120.3 (28.8)	121.1 (31.2)	120.2 (32.2)	0.87[a]
HDL cholesterol, mg/dL	73.2 (16.3)	75.3 (14.9)	74.6 (15.0)	0.31[a]
Glucose, mg/dL	98.9 (16.6)	98.8 (11.6)	99.2 (19.0)	0.51[b]
HbA1c, %	5.3 (0.4)	5.3 (0.4)	5.4 (0.4)	0.36[b]

Data are shown as means (standard deviations).
[a]One-way repeated measures analysis of variance.
[b]Friedman test.
**P < 0.01, ***P < 0.001 versus baseline, Paired *t*-test.
[#]P < 0.05, [##]P < 0.01 versus baseline, Wilcoxon signed-rank test.

Finally, we evaluated the changes in serum levels of lipids and glucose. Although the serum TG level among all participants did not change significantly, in the subgroup of women (n = 22) with high TG levels at baseline (150 mg/dL or higher; actual, 237.8 ± 88.9 mg/dL), the level decreased significantly after 4 (166.7 ± 86.1 mg/dL, $P = 0.0002$, Wilcoxon signed rank test) and 8 (170.9 ± 109.7 mg/dL, $P = 0.0025$) weeks of intervention (Figure 1). The serum levels of total cholesterol, LDL cholesterol, HDL cholesterol, glucose, and HbA1c did not change significantly.

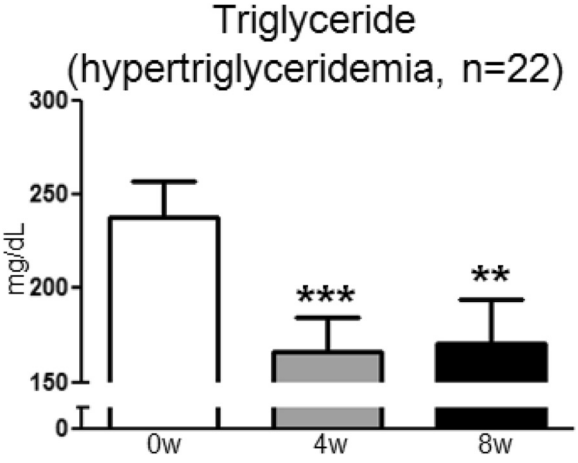

Figure 1 Change in serum TG level in the subgroup of women who had hypertriglyceridemia at baseline (≥150 mg/dL). Data are presented as means and standard errors. **$P < 0.01$, ***$P < 0.001$ versus baseline, Wilcoxon signed-rank test.

Discussion

This open-label, single-arm study investigated the net effect of tomato juice intake on a variety of health parameters in middle-aged women. The results indicated that tomato juice intake alleviated some menopausal symptoms, including anxiety; increased REE and heart rate; and lowered serum TG levels (in women with elevated baseline levels).

Tomato juice contains a variety of ingredients, and the outcomes of this study should be summed effects of them. Most of the clinical studies on the effects of tomatoes, tomato juice, and tomato products have focused on lycopene, a bioactive ingredient that is abundant in tomatoes. Dietary intake of fresh tomatoes or tomato juice is known to increase the serum lycopene level [26]. Lycopene is expected to reduce cardiovascular diseases, osteoporosis, and mental disorders through its anti-oxidative effects [10-14]. In 2012, Kim et al. isolated 13-oxo-ODA from tomato juice. They showed that this juice component acted as a potent peroxisome proliferator-activated receptor-alpha (PPARα) activator to lower plasma and hepatic TG levels in an animal model of obesity [15]. Further, in 2010, Nohara et al. demonstrated that esculeoside A, a tomato saponin, reduced serum levels of TG and cholesterol, and ameliorated atherosclerotic lesions in ApoE-deficient mice [16]. Human studies have not been published, to our knowledge. We believe that the current study is the first to investigate the net effects of tomatoes or tomato products on the health parameters, including menopausal symptoms, body composition, blood pressure, heart rate, and serum levels of lipids and glucose, of middle-aged women.

In our study, menopausal symptoms improved following regular consumption of tomato juice over a prolonged (8-week) period. Although the mechanisms causing the vasomotor symptoms that affect middle-aged women are not yet fully understood, oxidative stress could be one of the responsible factors [27]. Psychological symptoms are likewise prevalent around menopause, and may be associated with oxidative stress [28]. The improvements in menopausal symptoms observed in our study participants could partly be explained by the anti-oxidative effect of lycopene. A meta-analysis of 13 clinical trials [12] showed that the least effective amount of lycopene to reduce oxidative stress was 10 mg/day, much lower than the estimated dosage in the current study, 44 mg/day. Furthermore, GABA, much more abundant in tomatoes than in other vegetables and fruits [29], could be another candidate as its analog, gabapentin, has been proven to relieve hot flush [30].

Our study also revealed that tomato juice intake increased REE and heart rate, and, in women with elevated baseline TG levels, lowered serum TG levels. 13-oxo-ODA is a newly identified PPARα activator, first reported in 2012 [15]. In their study, Kim et al. showed that mouse rectal temperatures were significantly higher in 13-oxo-ODA-treated animals than in the controls, implying that 13-oxo-ODA increased energy metabolism. Their study also showed that plasma, liver, and skeletal muscle TG levels were lower in the mice treated with 47.6 mg/kg of 13-oxo-ODA than controls. On the other hand, Nohara et al. focused on a tomato saponin, esculeoside A [16]. They showed that administration of 100 mg/kg/day of esculeoside A inhibited the accumulation of cholesterol esters in macrophages, and reduced serum levels of TG and cholesterol in ApoE-deficient mice. The increase in energy metabolism and the decrease in serum TG level shown in our study could be attributable to these bioactive ingredients, although caution should be taken as the estimated dosage of 13-oxo-ODA in the current study, ~1.4 μg/kg, is far smaller than that in the animal study, and we do not know the exact amount of esculeoside A contained in the tomato juice used. Further human studies investigating the effects of 13-oxo-ODA and esculeoside A on energy metabolism are warranted. The increase in heart rate observed in the current study may be associated with raised REE, as it is well known that heart rate is linearly related to basal energy expenditure [31].

The present study has some limitations. First, it is designed as a single-arm study because an adequate placebo or active control for tomato juice is difficult to prepare. Second, blood samples should rather have been collected in fasting status, which were unfortunately not acceptable for many participants in our study. However, the fact that the average postprandial intervals were not significantly different among the groups would justify the comparison between them. Third, some women may have changed their eating and exercise habits during the course of the 10-week study, although we requested them to adhere to their current lifestyles. Finally, we should rather have evaluated insulin resistance by measuring plasma insulin concentration instead of HbA1c in our relatively healthy participants.

Conclusions

Tomato juice intake alleviated menopausal symptoms, including anxiety, increased REEs and heart rate, and lowered high baseline serum TG levels in middle-aged women.

Abbreviations

MSS: Menopausal symptom scale; HADS: Hospital anxiety and depression scale; AIS: Athens insomnia scale; REE: Resting energy expenditure; TG: Triglyceride; ANOVA: Analysis of variance; CVD: Cardiovascular disease; GABA: Gamma-aminobutyric acid; 13-oxo-ODA: 13-oxo-9,11-octadecadienoic acid; FSSC: Food Safety System Certification; GFSI: Global Food Safety Initiative; LDL: Low-density lipoprotein; HDL: High-density lipoprotein; PPARα: Peroxisome proliferation-activated receptor-alpha.

Competing interests

MT received a research grant from Kikkoman Corporation.

Authors' contributions

MT, AH, and TK were responsible for project development, data collection, and data analysis. All authors contributed to reviewing, editing, and approving the final manuscript.

Acknowledgments

This research was supported by Kikkoman Corporation.

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Predicting body composition using foot-to-foot bioelectrical impedance analysis in healthy Asian individuals

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Abstract

Background: The objectives of this study were to develop a regression model for predicting fat-free mass (FFM) in a population of healthy Taiwanese individuals using standing foot-to-foot bioelectrical impedance analysis (BIA) and to test the model's performance in predicting FFM with different body fat percentages (BF%).

Methods: We used dual-energy X-ray absorptiometry (DXA) to measure the FFM of 554 healthy Asian subjects (age, 16–75 y; body mass index, 15.8–43.1 kg/m^2). We also evaluated the validity of the developed multivariate model using a double cross-validation technique and assessed the accuracy of the model in an all-subjects sample and subgroup samples with different body fat levels.

Results: Predictors in the all-subjects multivariate model included height^2/impedance, weight, year, and sex (FFM = 13.055 + 0.204 weight + 0.394 height^2/Impedance − 0.136 age + 8.125 sex (sex: Female = 0, Male = 1), r^2 = 0.92, standard error of the estimate = 3.17 kg). The correlation coefficients between predictive FFM by BIA (FFM_{BIA}) and DXA-measured FFM (FFM_{DXA}) in female subjects with a total-subjects $BF\%_{DXA}$ of <20 %, 20 %–30 %, 30 %–40 % and >40 % were r = 0.87, 0.90, 0.91, 0.89, and 0.94, respectively, with bias ± 2SD of 0.0 ± 3.0 kg, −2.6 ± 1.7 kg, −1.5 ± 2.8 kg, 0.5 ± 2.7 kg, and 2.0 ± 2.9 kg, respectively. The correlation coefficients between FFM_{BIA} and FFM_{DXA} in male subjects with a total-subjects $BF\%_{DXA}$ of <10 %, 10 %–20 %, 20 %–30 %, and >30 % were r = 0.89, 0.89, 0.90, 0.93, and 0.91, respectively, with bias ± 2SD of 0.0 ± 3.2 kg, −2.3 ± 2.5 kg, −0.5 ± 3.2 kg, 0.4 ± 3.1 kg, and 2.1 ± 3.2 kg, respectively.

Conclusions: The standing foot-to-foot BIA method developed in this study can accurately predict FFM in healthy Asian individuals with different levels of body fat.

Keywords: Dual-energy X-ray absorptiometry, Foot-to-foot, Cross-validation, Fat-free mass

Background

In recent years, bioelectrical impedance analysis (BIA) has undergone major changes. The traditional electrodes that are pasted on with gel have been replaced with reusable stainless steel contact electrodes [1, 2], and the measuring position has been changed from supine to standing. In standing foot-to-foot BIA, impedance is measured through the electronic pathway of the lower extremities [3]. This technique is widely used to assess the whole-body composition [4–6].

Although many fat-free mass (FFM) predictive models based on BIA performed in the supine position have been validated, most of these models have been developed for Caucasian, African-American, Hispanic, and Native American populations [7–9]. Insufficient research has been performed to develop FFM predictive models based on BIA in Asian populations. Because different ethnic groups may exhibit different body composition characteristics, predictive models should be developed for specific

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populations to prevent ethnic bias and facilitate accurate estimates of FFM [10, 11].

Standing foot-to-foot BIA may be a convenient and safe method for assessing body composition. In the existing research, however, there is inconsistent support of the validity of BIA in estimating FFM in the general population [4, 6, 8, 12–17], BIA cannot accurately estimate FFM in obese populations [18–20], BIA tends to underestimate the body fat percentage (BF%) and overestimate the FFM, and body composition parameters are affected by the BF% in tested subjects.

Studies to date have only explored the regression mode for tested subjects because manufacturers retain the prediction equations of the measuring system as confidential information; therefore, knowledge of BIA devices is limited [13]. Additionally, studies to date have had relatively small sample sizes that do not represent the entire population, thereby minimizing the generalizability and external validity of the results.

Methods

Subjects

In total, 554 Taiwanese subjects were recruited by advertisement and volunteered to participate in this study. The subjects were asked to complete a health questionnaire that included personal background information, physical characteristics, and health status. The subjects were tested following 48 h without alcohol, 7 days without diuretic agents, and 24 h without strenuous physical activity. No urination was allowed within 30 min prior to the BIA and DXA measurements. Female participants were not tested during their menses. No subjects reported a history of endocrine, nutritional, or growth disorders; chronic illnesses such as high blood pressure, diabetes, cancer, kidney dysfunction, liver disease, or asthma; or electronic device insertion. Each subject underwent foot-to-foot BIA and DXA at the Department of Radiology of Dah Li County Jen-Ai Hospital in Taiwan. This study was approved by the institutional review board of Jen-Ai Hospital.

Anthropometry

The subjects were weighed to the nearest 0.1 kg on a Weight-Tronix scale, (Scale Electronics Development, NY, USA). Height was measured without shoes to the nearest 0.5 cm on a stadiometer (Holtain Ltd., Crosswell, Wales, UK). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2).

Measurements of body composition

The subjects were asked to wear a cotton gown and remove all metallic objects from the body. Body composition was measured using a DXA system (Lunar Prodigy; GE Healthcare, Madison, WI, USA). The estimation of body composition included measurement of total body fat, fat-free soft tissue, and bone mineral content. The BF% was calculated as 100 × fat mass/(fat mass + FFM), where FFM was the summation of the fat-free soft tissue and mineral content. The DXA measurements were taken at 2:00 PM using enCORE 2003 Version 7.0 software (GE Healthcare), and the foot-to-foot impedance measurements were conducted following the DXA measurements. With the subjects in a standing position, the measurements were repeated until they were stable to within 5 ohm (usually up to three times within an interval of 30 s), and the average value of three repeated measurements was used in the calculations.

Impedance analysis

The BIA measurements were taken using the impedance measurement device. We used an imbedded stainless steel contact plate with four electrodes, which was connected to a QuadScan 4000 (Bodystat, Douglas, UK). The QuadScan 4000 was in turn connected to stainless steel electrode plates. A body composition sensing platform (HBF-361; Omron Healthcare, Kyoto, Japan) was used as the impedance measuring base and included a stainless steel polar plate and cable. The subjects were asked to stand on the contact plate without shoes and with their feet slightly apart. A current of only 50 kHz was used to measure the impedance in the left-foot-to-right-foot pathway, expressed as $Z_{F\text{-}F}$.

The coefficient of variation (CV) (standard deviation [SD]/mean), expressed as a percentage, of the within-day and between-day estimates of impedance in all subjects was calculated to evaluate the repeatability of the impedance measurements. After referring to the amount of time spent during the DXA measurement, the subjects lay in the supine position for 20 min and then underwent the foot-to-foot standing position impedance measurement. The within-day CV was calculated by measuring the impedance of ten subjects (five males and five females) ten times within 1 h, and the between-day CV was calculated by measuring the impedance of 10 subjects on five consecutive days.

All measurements were conducted in a well-ventilated room with controlled temperature and humidity. The impedance measurements were performed three times within 3 min in each subject immediately after the DXA measurements.

Statistical analysis

The BF% derived by DXA (expressed as $BF\%_{DXA}$) was grouped by 10 % difference intervals for percent body fat [21]. The variables, which included age, height, weight, BMI, and impedance, are expressed as mean ± SD, and the numbers in parentheses are the minimum and maximum

values. The paired-samples *t*-test was used to evaluate the differences between the two measurement methods.

The estimates of FFM derived by DXA were used as reference values (expressed as FFM_{DXA}) to develop a stepwise multiple regression model by setting height squared/impedance ($h^2/Z_{F\text{-}F}$), weight (w), age (y), and sex (s, female: 0, male: 1) as predictive variables. The stepwise procedure included the variable with the highest correlation coefficient and the minimum standard error of estimation in the model first. The FFM model predicted by BIA was developed using a double cross-validation technique. The subjects were split into two groups, G_1 and G_2, based on BF%. Thus, the two groups were evenly matched for BF%. An FFM predictive model was then constructed for each group and cross-validated with each model. If the FFM predictive models were proved to be similar to each other by comparing the correlation and standard error of the estimate (SEE) [11], the two samples were combined to develop a pooled FFM predictive model [22, 23].

Furthermore, the root mean square error (RMSE) and pure error (PE) were used to test the accuracy of the FFM predictive model by BIA:

$$\mathrm{RMSE} = \sqrt{\sum \frac{(y_i' - y_i)^2}{n} - p - 1},$$

where y' the predicted FFM, y is the observed FFM, n is the number of subjects, and p is the number of predictor variables, and

$$\mathrm{PE} = \sqrt{\sum \frac{(y_i' - y_i)^2}{n}},$$

where the error of measurement estimates the magnitude of the error for a given measurement and is defined as the difference between measurements for the individual (i) (i = 1..., *n*, where n is the number of individuals).

A Bland–Altman plot [24] was performed to assess the agreement between the results from the FFM predictive model and the DXA measurements. Passing–Bablok regression describes a linear regression procedure with no special assumptions regarding the distribution of the samples and the measurement errors [25] and was used to evaluate the interchangeability of the two methods. All analyses were conducted using SPSS for Windows (Version 17.0; SPSS Inc., Chicago, IL, USA) and MedCalc (Version 9.0; MedCalc Inc., Mariakerke, Belgium). Statistical significance was set at $p < 0.05$ for all tests.

Results

The physical characteristics of the subjects grouped by sex and BF% are shown in Table 1. A total of 554 subjects met the criteria for this study: 257 females and 297 males. The subjects ranged in age from 16 to 75 years with a mean of 32.8 ± 14.8 years. Their weight varied from 39.0 to 125.5 kg, and their BMI ranged from 15.9 to 43.1 kg/m^2 with a mean of 24.0 ± 4.1 kg/m^2. The BMIs for G_1 and G_2 were 23.9 ± 4.2 (range, 16.0–43.1) and 24.0 ± 4.1 (range, 16.2–42.9) kg/m^2, respectively. The subjects were also divided into five different subgroups by 10 % body fat intervals (Table 1).

The CV of the within-day estimates of impedance in ten subjects was 0.3 % to 0.8 %, and the CV of the between-day estimates of impedance in 10 subjects was 0.9 % to 1.7 %.

Table 2 summarizes the cross-validation results in which the r^2, SE, PE, and RMSE show very similar values between the two groups. The regression lines of FFM_{BIA} against FFM_{DXA} developed using G_1 and G_2 data demonstrate similar trends deviated from the identical line (slope = 0.92 for G_1 and 0.93 for G_2).

The results of the FFM predictive model by multiple regression analysis for all 554 subjects are shown in Table 3. Figure 1a shows the correlation for all subjects measured by FFM_{DXA} and the predictive values of FFM_{BIA}. The Passing–Bablok regression analysis indicated a foot-to-foot BIA and DXA equation as follows: $FFM_{BIA} = 0.911\ FFM_{DXA} + 4.27$ with a 95 % confidence interval (CI) of 0.80 to 1.02 for the slope and a 95 % CI of −0.74 to 9.28 for the intercept of the regression model, indicating that the foot-to-foot BIA and DXA FFM estimate methods are interchangeable ($p > 0.10$). Figure 1b shows a Bland–Altman plot of the differences between the all-subjects FFM_{DXA} and the predictive values of FFM_{BIA}. For FFM, the −2SD to +2SD was −6.40 to 6.40 kg. The correlation between $FFM_{BIA} - FFM_{DXA}$ and FFM_{DXA} can be expressed as the regression line y = −0.089 × + 4.428 (r = 0.31, $p < 0.001$).

In the developed FFM predictive model, the cumulative SEE and r^2 by individual predictors are shown in Table 4. The determination coefficient between the predictive FFM by the single predictor $h^2/Z_{F\text{-}F}$ and FFM_{DXA} was $r^2 = 0.837$. As the predictors sex, weight, and age were added into the model, the determination coefficients changed to $r^2 = 0.881$, 0.893, and 0.922, respectively. The standard β coefficients of the predictors' height squared/impedance, sex, weight, and age were 0.43, 0.36, 0.25, and −0.18, respectively, and the variance inflation factor (VIF) values were 4.27, 2.25, 2.49, and 1.16, respectively.

Table 5 shows a comparison of the results for the mean and bias. The regression line and correlation coefficients between FFM_{DXA} and FFM_{BIA} predicted by the model for sex and BF% are shown in Table 5. The correlation coefficients of different $BF\%_{DXA}$ subgroups ranged from 0.89 to 0.94 (r = 0.89–0.94). The subgroups with the largest bias ± SD of FFM_{DXA} and FFM_{BIA} were the $BF\%_{DXA} > 40$ % (female subgroup) and the $BF\%_{DXA} > 30$ %

Table 1 Characteristics and impedances of subjects grouped by sex and %BF[a]

Female ($n = 257$)					
$BF\%_{DXA}$	<20 % ($n = 22$)	20 % - 30 % ($n = 80$)	30 %-40 % ($n = 94$)	>40 % ($n = 61$)	Total ($n = 257$)
Age(year)	21.6 ± 3.8	27.9 ± 12.5	37.5 ± 13.4	43.3 ± 15.4	34.7 ± 14.8
	(17, 59)	(16, 61)	(16, 67)	(18, 75)	(16,75)
Height(cm)	162.8 ± 6.8	162.1 ± 6.3	158.9 ± 6.4	158.9 ± 7.6	160.2 ± 6.9
	(151, 174)	(148, 174)	(143, 181)	(144, 178)	(143, 181)
Weight(kg)	49.3 ± 4.0	54.8 ± 6.3	58.3 ± 8.0	74.8 ± 14.5	60.2 ± 12.2
	(44, 58)	(39, 69)	(42, 84)	(53, 108)	(38, 108)
BMI (kg/m^2)	18.9 ± 1.3	20.8 ± 2.0	23.1 ± 2.9	29.1 ± 4.5	23.5 ± 4.5
	(16, 22)	(16, 25)	(16, 31)	(22, 43)	(16, 43)
Z_{F-F} (ohm)	560.5 ± 76.0	529.2 ± 60.2	443.9 ± 58.2	488.9 ± 68.0	521.5 ± 66.1
	(446, 733)	(391, 704)	(326,595)	(362, 706)	(326, 733)
$h^2/Z_{F-F}(cm^2/ohm)$	48.1 ± 7.0	50.4 ± 7.1	48.4 ± 6.3	52.6 ± 8.1	50.0 ± 7.2
	(33, 59)	(36, 69)	(33, 65)	(33, 79)	(33, 79)
Male ($n = 297$)					
$BF\%_{DXA}$	<10 % ($n = 46$)	10 % - 20 % ($n = 96$)	20 % - 30 % ($n = 104$)	>30 % ($n = 51$)	Total ($n = 297$)
Age(year)	20.3 ± 2.3	24.7 ± 10.7	35.4 ± 14.8	44.7 ± 14.6	31.2 ± 14.8
	(16, 29)	(16, 65)	(16, 71)	(21, 71)	(16,71)
Height(cm)	173.0 ± 5.7	173.5 ± 8.1	173.2 ± 6.7	172.3 ± 8.0	173.3 ± 7.3
	(160, 182)	(157, 196)	(156, 193)	(155, 200)	(155, 200)
Weight(kg)	65.0 ± 5.4	67.3 ± 8.1	76.4 ± 10.4	86.5 ± 14.5	73.4 ± 12.4
	(52, 77)	(42, 90)	(59, 122)	(66, 125)	(42, 125)
BMI (kg/m^2)	21.7 ± 1.6	22.3 ± 1.9	25.4 ± 2.7	28.8 ± 4.1	24.4 ± 3.7
	(18, 26)	(17, 27)	(19.7, 37)	(23, 42)	(17, 42)
Z_{F-F} (ohm)	441.3 ± 38.3	451.5 ± 46.8	449.8 ± 53.9	439.9 ± 49.9	447.3 ± 48.8
	(380, 530)	(365, 685)	(334, 625)	(345, 572)	(335, 685)
$h^2/Z_{F-F}(cm^2/ohm)$	68.4 ± 7.3	67.4 ± 8.4	67.8 ± 10.1	69.2 ± 10.0	68.0 ± 9.1
	(56, 84)	(36, 96)	(46, 97)	(49, 96)	(36, 97)

[a]All values are mean ± SD; minimum and maximum in parentheses, [2]BI, bioelectrical index; h^2/Z_{F-F}, $Height^2/Impedance$

(male subgroup), with bias ± SD of −2.0 ± 2.9 kg and 2.1 ± 3.2 kg, respectively. The subgroups with the smallest bias ± SD of FFM_{DXA} and FFM_{BIA} were the female subgroup and the male subgroup, with bias ± SD of 0.0 ± 3.0 kg and 0.0 ± 3.2 kg, respectively. Although the Bland–Altman plot indicates that there was a systematic error with the BIA method, this error was small. For example, the BIA method would underestimate FFM by an average of 2.2 % in an individual with an FFM of 40 kg and overestimate FFM by −1.5 % in an individual with an FFM of 60 kg.

Discussion

The present study measured FFM in 544 healthy Asian individuals using DXA (297 male, 257 female; age range, 16–75 years). We used BIA to measure the impedance at 50 kHz of the lower extremities in a standing position to develop a multivariable model for predicting FFM using DXA measurements. We also evaluated the validity of the developed multivariable model with a double cross-validation technique and assessed the accuracy of the model in an all-subjects sample and in different BF% subgroup samples. The results of the study indicated that the FFM predictive model based on BIA estimates is a valid method for assessing FFM in healthy subjects with different BF% values. The force of gravity has an effect on the fluid distribution in our body. Depending on the body position, gravity may also cause differences in blood pressures. As a result, regulation of blood volume may become challenging: standing still leads to rapid and persistent plasma volume loss of up to 7 % for a 30-min period [26]. Nunez *et al.* [1] performed foot-to-foot standing upright and supine position impedance measurements and obtained the following results. There was a high correlation between upright and supine position impedance measurements of the lower extremities. The

Table 2 Prediction equation for G_1 and G_2 subjects

G_1 subjects ($n = 277$)	
Measured FFM_{DXA}	50.54 ± 11.58 kg
Prediction FFM_{BIA}	12.518 + 0.215 w + 0.397 h^2/ Z_{F-F} − 0.143 y + 7.843 S (Female = 0, Male = 1), ($r^2 = 0.92$, SE = 3.23 kg, CV = 6.3 %) (1.a)
Prediction FFM	
Cross-validation using G_2 subjects FFM	50.48 ± 10.97 kg, r = 0.96, bias ± SD = −0.06 ± 3.22 kg, PE = 3.22 kg, RMSE = 2.31 kg, LOA = −6.46 to 6.38 kg
G_2 subjects ($n = 277$)	
Measured FFM_{DXA}	49.81 ± 10.91 kg
Prediction FFM_{BIA}	13.639 + 0.192 w + 0.392 h^2/ Z_{F-F} − 0.129 y + 8.355 S (Female = 0, Male = 1), ($r^2 = 0.92$, SEE = 3.13 kg, CV = 6.1 %) (1.b)
Prediction FFM	
Cross-validation using G_1 subjects FFM	49.86 ± 10.60 kg, r = 0.96, bias ± SD = 0.05 ± 3.13 kg, PE = 3.12 kg, RMSE = 2.18 kg, LOA = −6.21 to 6.31 kg

FFM, fat free mass; Regression coefficient estimate ± SE; FFM_{DXA}, DXA measurement of FFM; FFM_{BIA}, BIA prediction of FFM; h^2/Z_{F-F}, height ^{2}/impedance; SEE, standard error of estimate; LOA, limits of agreement

RMSE, Root mean square error= $\sqrt{\sum \frac{(y_i' - y_i)^2}{n} - p - 1}$, where y' the predicted FFM, y is the observed; n is the number of subjects, and p is the number of predictor variables; PE, Pure errors= $\sqrt{\sum \frac{(y_i' - y_i)^2}{n}}$; r, correlation coefficient between FFM_{BIA} and FFM_{DXA}

difference in impedance measured by the two methods versus the mean impedance for the two methods was evaluated as a Bland–Altman plot (r = 0.44, $p = 0.23$, NS). The plot showed a small but systematic difference between the two methods. In a study by Rush *et al.* [27], the foot-to-hand impedance decreased by up to 9 ohm (mean, 5 ohm; 1.0 %) over 10 min of standing and increased by up to 7 ohm (mean, 3 ohm; 0.7 %) in the lying position. Based on the results of both studies, the difference in the impedance measures was caused by the changes in the effects of gravity on the different positions and body fluids. Oshima [28] reported that the average foot-to-foot impedance value would decrease by 6.8 % after 6 h of continuous measurements. Kushner *et al.* [29] also reported a −3 % to 1 % change during a 5-min standing upright position and 10-min supine position in hand-to-foot impedance measurements. In the present study, regardless of standing or lying down, only a 1 % decrease in the foot-to-foot impedance measurement occurred over a 3-min period (data not shown).

The standing foot-to-foot BIA method described herein produced inconclusive results. The present study had the following characteristics: (a) We used the same instruments in the same setting to measure the impedance by foot-to-foot BIA in the standing position and FFM by DXA in a large, single-institution Asian sample; such patients have been insufficiently studied in BIA research to date. (b) Instead of evaluating the validity of existing commercial instruments, this study aimed to develop an FFM predictive model using standing foot-to-foot BIA. (c) This study tested not only the accuracy and suitability of BIA for assessing body composition in a general population, but also its performance in subjects with different BF% values.

The present study used h^2/Z_{F-F} and other anthropometric variables, such as weight, sex, and age, as predictive variables to develop the prediction model. We used h^2/Z_{F-F} instead of h^2/reactance (X_C) or resistance (R), as adopted by other studies [23, 30], as a predictor in the regression model for the following reasons: The QuadScan 4000 produces a 50-kHz frequency and provides measured results for resistance, impedance, and reactance. Nunez *et al.* [1] proposed a standing foot-to-foot bioimpedance analysis FFM estimate model in which they used Z (impedance) as

Table 3 Prediction equation for FFM using all subjects

Development group (n =554)	
Measured FFM_{DXA}	50.17 ± 11.25 kg
FFM prediction equation (FFM_{BIA})	13.055 + 0.204 w + 0.394 h^2/ Z_{F-F} − 0.136 y + 8.125 S (Female = 0, Male = 1), ($r^2 = 0.92$, SEE = 3.17 kg, CV = 6.3 %) (2)
Prediction FFM	50.17 ± 10.69 kg, PE = 3.20 kg, RMSE = 2.29 kg

FFM, fat free mass; SE, Standard error of estimate; FFM_{DXA}, DXA measurement of FFM; FFM_{BIA}, BIA prediction of FFM; h^2/Z_{F-F}, height^2/impedance; SEE, standard error of estimate;

Root mean square error (RMSE) = $\sqrt{\sum \frac{(y_i' - y_i)^2}{n} - p - 1}$

Pure errors (PE) = $\sqrt{\sum \frac{(y_i' - y_i)^2}{n}}$; where y' is the predicted FFM, y is the observed, n is the number of subjects and p is the number of predictor variables

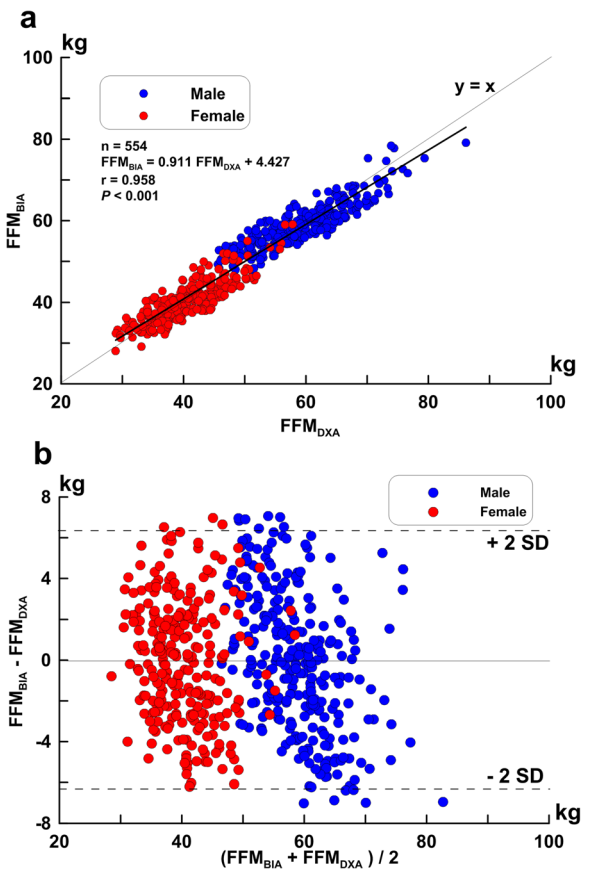

Fig. 1 Correlations (**a**) and difference (**b**) of FFM in all subjects estimated by FFM_{DXA} and FFM_{BIA}. The difference (calculated as FFM_{BIA} - FFM_{DXA} per Bland-Altman) is plotted against the mean of the measurements of FFM_{DXA} and FFM_{BIA}

the estimate variable; this variable has also been widely used in other studies of BIA standing-position body composition estimation [31]. Furthermore, in similar research, Kotler [32] and Lukaski *et al.* [33] indicated that when using resistance or impedance estimating FFM, TBW (total body water) and TBK (total body potassium) have no significant difference. For these reasons, we used impedance as the estimated variable. When developing a body composition predictive model, the predictors should be easy to measure, accurate, reproducible, and physiologically related to the dependent variable [34, 35]; the predictors in our model meet these requirements [36, 37].

When developing a regression model, the following issues should be taken into consideration to avoid violating assumptions and to ensure that the regression analyses have sufficient power: collinearity, sample size, number of predictors, cross-validation, and SEE [38]. When additional variables were added into the FFM predictive equation using BIA and other anthropometric variables, collinearity was present if the predictive variables were highly correlated. This might affect the estimation of the regression coefficient in a predictive model, which may lead to incorrect identification of the predictive variables. The VIF analysis was conducted to identify potential problems related to collinearity. When the VIF of the predictors had exceeded ten, the collinearity was considered to be severe. In this study, the VIF was smaller than five; thus, collinearity did not exist.

To ensure sufficient power, the minimum sample size was set to 91 when the effect size was medium, the number of predictors was five, the power was 0.8, and the alpha value was 0.05 [39]. When the effect size was small, the minimum sample size was 686. Although the number of subjects in the present study was less than 686, the sample was large enough to minimize variable inflation and improve reliability. Additionally, a cross-validated technique was used to validate the prediction model. In this study, height^2/impedance, sex, weight, and age were the predictors in the regression model. In the model, the correlation coefficient of height^2/impedance and FFM_{DXA} was 0.92, and the standardized coefficient β was 0.43; these values explain approximately 43 % of the variance of FFM. The prediction model developed using the G_1 and G_2 data was double cross-validated with an RMSE of 2.31 and 2.18 kg, respectively and a bias ± SD of −0.01 ± 3.22 and 0.05 ± 3.12 kg, respectively. The similar results derived by the G_1 and G_2 models indicate that the predictive models can accurately predict FFM. The all-subjects predictive model also showed results similar to the G_1 and G_2 models in terms of the correlation coefficient, SEE, and CV, thus validating the accuracy of the predictive model. We also randomly assigned subjects into two groups to double cross-validate the regression models.

Table 4 Multiple stepwise regression analysis results for $h^2/Z_{F\text{-}F}$ measured with foot-to-foot BIA as an independent variable and FFM_{DXA} as a dependent variable

Dependent variables used in model (All subjects, $n = 554$)								Dependent variable		
$h^2/Z_{F\text{-}F}$	Sex	Weight	Age	Intercept	SEE(kg)	r^2	VIF	β	r	SEE (kg)
0.84 ± 0.02**	-	-	-	−0.10 ± 0.96	4.54	0.84	4.27	0.43	0.92**	4.54
0.63 ± 0.02**	6.99 ± 0.49**	-	-	8.68 ± 1.03**	3.88	0.89	2.25	0.36	0.82**	6.52
0.52 ± 0.03**	7.56 ± 0.47**	0.14 ± 0.02**	-	7.09 ± 0.99**	3.68	0.90	2.49	0.25	0.73**	7.74
0.39 ± 0.02**	8.12 ± 0.41**	0.20 ± 0.02**	−0.14 ± 0.01**	13.05 ± 0.96**	3.16	0.92	1.16	−0.18	−0.29**	10.76

Regression coefficient estimate ± SE; r, variance; r^2, determination coefficient; $^{*}p < 0.05$, $^{**}p < 0.001$; β: Standardized coefficients; VIF, variance inflation factor

Table 5 Comparison of the results of FFM_{DXA} and FFM_{BIA} for different %BF subgroups[a]

Female: $BF\%_{DXA}$	<20 % ($n = 20$)	20 % - 30 % ($n = 80$)	30 %-40 % ($n = 94$)	>40 % ($n = 61$)	Total ($n = 257$)
FFM_{DXA} (kg)	41.8 ± 3.8	41.8 ± 5.2	38.4 ± 4.7	40.9 ± 6.9	40.3 ± 5.6
	(35, 48)	(29, 52)	(29, 51)	(30, 58)	(29, 58)
FFM_{BIA} (kg)	39.2 ± 3.6	40.3 ± 4.7	38.9 ± 4.4	42.9 ± 6.7	40.3 ± 5.2
	(32, 45)	(29, 51)	(28, 51)	(31, 60)	(28, 59)
Bias ± SD[b] (kg)	−2.6 ± 1.7	−1.5 ± 2.8	0.5 ± 2.7	2.0 ± 2.9	0.0 ± 3.0
r[c]	0.90	0.91	0.89	0.94	0.87
P[d]	0.04*	0.06	0.43	0.11	0.92
Slope[e]	0.86	0.83	0.79	0.89	0.79
Intercept[e]	3.17	5.34	9.77	6.70	8.41
Male: $BF\%_{DXA}$	<10 % ($n = 46$)	10 % - 20 % ($n = 96$)	20 % - 30 % ($n = 104$)	>30 % ($n = 51$)	Total ($n = 297$)
FFM_{DXA}(kg)	60.9 ± 5.2	58.6 ± 7.0	58.2 ± 7.9	57.9 ± 7.7	50.1 ± 11.4
	(47, 71)	(33, 76)	(46, 86)	(46, 77)	(33, 86)
FFM_{BIA}(kg)	58.6 ± 3.8	58.1 ± 5.3	58.6 ± 6.7	60.0 ± 7.4	50.0 ± 10.9
	(52, 67)	(39, 74)	(48, 79)	(47, 79)	(40, 79.2)
Bias ± SD[b](kg)	−2.3 ± 2.5	−0.5 ± 3.2	0.4 ± 3.1	2.1 ± 3.2	0.0 ± 3.2
r[c]	0.89	0.90	0.93	0.91	0.89
P[d]	0.02*	0.59	0.68	0.16	0.97
Slope[e]	0.65	0.68	0.79	0.87	0.70
Intercept[e]	19.13	18.52	12.71	9.60	17.41

[a]All values are mean ± SD; minimum and maximum in parentheses
[b]The biases and standard deviations between FFM_{DXA} and FFM_{BIA} indifferent subgroups
[c]The correlation coefficients of FFM_{DXA} and FFM_{BIA} in different subgroups
[d]The results of paired *t*-tests between FFM_{DXA} and FFM_{BIA} indifferent subgroups
[e]The slopes and intercepts of the regression model of FFM_{DXA} and FFM_{BIA} indifferent subgroups
*Denotes significantly different at the .05 level

The results were similar to the BF%-matched samples; the regression lines of FFM_{BIA} against FFM_{DXA} developed using randomly assigned data sets demonstrated a similar trend that deviated from an identical line (slope = 0.93 for G_1 and 0.92 for G_2; data not shown).

When comparing the results of our predictive model with those of previously published studies on supine-position hand-to-foot BIA measurements, the correlation coefficient and SEE were similar to those of Kotler *et al.* [32] ($r^2 = 0.83$, $n = 256$, SEE = approximately 3.0 kg), Sun *et al.* [8] ($r^2 = 0.92$, $n = 1095$, RMSE = 2.9 kg), Heitmann *et al.* [40] ($r^2 = 0.90$, $n = 139$, SEE = 3.6 kg), and Sun *et al.* [8] ($r^2 = 90$, n = 734, RMSE = 3.9 kg); however, they were lower than those of Kyle *et al.* [23] ($r^2 = 0.96$, $n = 343$, SEE = 1.8 kg) and Deurenberg *et al.* [41] ($r^2 = 0.92$, $n = 661$, SEE = 2.6 kg). These results may have been because the correlation coefficient of the predictive value and the measured FFM value tended to be higher in the hand-to-foot model than in the foot-to-foot model [42, 43]; this may be a shortcoming of the foot-to-foot model in assessing FFM.

The Geneva BIA equation published by Kyle *et al.* [23] provides ideal results of a high r^2 and low SEE ($r^2 = 0.96$, LOA = −3.4 to 3.5 kg, and SEE = 1.72 kg); however, their subjects' BMI range was narrower (17.0–33.8 kg/m^2) than that of the subjects in our study (15.9–43.1 kg/m^2). Sun *et al.* [20] and Deurenberg [44] indicated that the estimated results were affected by the level of adiposity. The developed predictive equations in our study overestimated FFM in subgroups with a higher BF% (male, $BF\%_{DXA} > 30$ %; female, $BF\%_{DXA} > 40$ %). When these subjects were excluded and used to develop another model, then the results ($n = 442$; BMI, 15.8–36.9 kg/m^2; $r^2 = 0.94$; SEE = 2.80 kg, LOA = not reported) were comparable with those reported by Kyle *et al.* [23]. Although these results may be appealing, they have limited application. We included subjects with a high BF% to broaden the application range. The average FFM_{DXA} of the subjects in our study was 50.17 ± 11.25 kg, while that in the Geneva BIA equation was 54.0 ± 10.5 kg. Their sample had a smaller SD for FFM_{DXA}, indicating that their data tended to be closer to the mean, resulting in a smaller SEE. The standing foot-to-foot impedance measurement may be convenient, but has a significantly smaller FFM correlation than the hand-to-foot impedance measurement [31, 42]. Based on the estimate equation suggested in our study, the LOA may be large; however, we consider it acceptable. This is one of the limitations of the present study. When estimating FFM using

our predictive equation in subjects with a high level of adiposity (female, $BF\%_{DXA} > 40$ %; male, $BF\%_{DXA} > 30$ %), the bias ± SD in female and male subjects was 2.0 ± 2.9 and 2.1 ± 3.2 kg, respectively. Although the bias and SD were higher than those in the other leaner subgroups in our study, the results show that our predictive equations performed better for estimating FFM in subjects with a high BF% than did the equation developed by Jakicic *et al.* [45] ($r^2 = 0.66$, SEE = 8.8 kg, $n = 123$, and LOA = not reported).

Age has not been included as a predictor in every model in other published studies [23, 36, 41]. Some models excluded age because it only explained limited variance in FFM [23]. However, in our predictive model, age explained approximately 18.0 % of the variance in FFM and was therefore included in the predictive model. Several studies have indicated that the concentration of potassium in fat-free tissue decreases systematically with age [46, 47]. There are important age-related changes in the composition of FFM. The main molecular components of the FFM are water, protein, osseous and nonosseous mineral, and glycogen. The proportion of water, protein, and osseous mineral in the FFM vary systematically with age. Kyle *et al.* [47] examined the accuracy of a predictive model with different age groups. Many studies have reported that the accuracy of BIA estimation is affected by the level of obesity [18–20]. Therefore, this study examined the accuracy of a model for predicting FFM in individuals with different percentages of body fat. The predictive value of FFM using our model was not significantly different from FFM_{DXA} among the subgroups of different BF% values and sexes, and the correlation coefficients were 0.87 ($p = 0.92$) in females and 0.89 ($p = 0.97$) in males. These results indicate that BIA is an accurate method for assessing FFM in individuals with a BF% in the range evaluated in our study. Moreover, standing foot-to-foot BIA can be used as a convenient method to assess the different BF% values in male and female adults. Clinical use of BIA in patients with abnormal hydration cannot be recommended until further validation has proven that a BIA algorithm is accurate in such conditions. The present study focused on the different foot-to-foot BIA BF% values; we did not discuss differences in foot-to-foot BIA FFM estimate measurements based on either regional composition or different body types; these are topics requiring further discussion. Moreover, in patients with body shape abnormalities, very small or large body heights, or relative sitting heights, the use of prediction equations in subjects with an abnormal body build (*e.g.*, acromegaly or amputation) should be interpreted with caution [48]. Many published studies on BIA estimate equations have used impedance as an estimate variable, but the present study applied the impedance variable in the standing foot-to-foot model and found satisfactory results for estimating FFM in a healthy Taiwanese population (BMI = 16–43 kg/m^2).

Conclusions

Our FFM predictive model based on standing foot-to-foot BIA can conveniently predict FFM in both male and female healthy Asian subjects with different BF% values.

Consent

Written informed consent was obtained from the patient for the publication of this report and any accompanying images.

Abbreviations

BF%: Body fat percentage; H: Height; Z_{F-F}: Foot-to-foot impedance; w: Weight; y: Age; s: Sex; Xc: Reactance; R: Resistance; LOA: Limits of agreement.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ACH, CLC, CLL, and LMC interpreted the results and critically reviewed the manuscript; CSW designed the study and conducted the research; CLC, and KCH contributed to subject recruitment and data collection; GBD, LMC, KCH, and YLH performed the laboratory analysis and statistical analysis, interpreted the results, and wrote the manuscript; YYC supervised the study; and all authors read and approved the final manuscript.

Acknowledgements

This study was supported by a grant (NSC 95-2413-H-028-003) from the National Science Council of the Republic of China. The authors thank Charder Electronic Co., Ltd. for participating in this study.

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"Fixing a heart": the game of electrolytes in anorexia nervosa

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Abstract

Case: A 25-year-old woman with chronic anorexia nervosa and depression presented with sudden weakness and fatigue. Psychosocial history was notable for binge-starve cycles over the past year and a decline in overall well-being. Vitals on presentation were notable for hypothermia, hypotension, and bradycardia. Initial exam was significant for emaciation, lethargy, and lower extremity edema. Laboratory work-up revealed markedly elevated LFTs, hypoglycemia, thrombocytopenia and elevated INR and lipase. ECG showed sinus bradycardia with prolonged QTc. Ultrasound revealed normal liver and biliary tree. Serum acetaminophen, alcohol level, and urinary toxicology were unremarkable. Work up for infectious, autoimmune, and genetic causes of hepatitis was negative. Echocardiogram revealed left ventricular hypokinesis and EF 10-15%. Nutritional support was begun slowly, however electrolyte derangements began to manifest on hospital day 2, with hypophosphatemia, hypokalemia, hypocalcemia, and hypomagnesemia. Multiple medical and psychiatric disciplines were consulted, and aggressive electrolyte monitoring and repletion were done. The patient's overall clinical status improved slowly during her hospital course. Her liver enzymes trended down, and her QTc interval eventually returned toward the normal range. Repeat echocardiogram following treatment revealed improvement of her EF to 40%.

Discussion: Anorexia nervosa is an eating disorder characterized by extremely low body weight, fear of gaining weight or distorted perception of body image, and amenorrhea. Anorexia can lead to life threatening medical complications, and thus constitutes a major challenge to manage. Central to the pathogenesis of the refeeding syndrome is a weakened cardiopulmonary system, electrolytes abnormalities, hepatic dysfunction, liver hypoperfusion and failure.

Conclusion: Given the clinical presentation, this patient likely presented on the brink of developing frank refeeding syndrome, with cardiac dysfunction and hypovolemia, leading to hepatic hypoperfusion and ischemic hepatitis. Subsequently, she developed electrolyte disturbances characteristic of refeeding syndrome, which were managed without major complication. Her hospital course is encouraging not only for her recovery, but for the collaboration of the different teams involved in her care, and it highlights the importance of a multidisciplinary approach to caring for patients with the potential dire complications of a complex psychiatric illness.

Case

A 25 year-old woman with a past medical history significant for anorexia nervosa, for which she had multiple psychiatric admissions, depression and Attention deficit hyperactivity disorder on fluoxetine and methylphenidate who presented with weakness for 1 day. She was in her usual state of health until the night prior to presentation, when she began to feel weak after having dinner. The next morning she had difficulty getting out of bed, felt dehydrated and was lethargic. The patient reports that her "normal" weight is between 90 and 100 pounds. Recently, she moved out of her parents' home, withdrew socially, resumed binging, and started to feel depressed. Since then, her eating habits have been characterized by cycles of binging and starving, during which she will consume an entire box of pasta or 20 apples, feel sick, and then starve until feeling hungry again. She denies fever, nausea and vomiting, diarrhea, urinary symptoms, and recent increase in exercise volume or frequency. She also denies current suicidal and homicidal ideation.

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In the emergency room, vital signs were significant for rectal temperature 93 F, heart rate 56BPM, blood pressure 80/55, and respiratory rate 14. Physical exam was remarkable for lethargy, emaciation and pitting lower extremity edema. Electrocardiogram showed sinus bradycardia, with a prolonged corrected QT interval of 0.54 and U waves, attributed initially to magnesium deficiency. She was given 2 grams of magnesium intravenously in the emergency department. Laboratory studies (Table 1) were notable for BUN 37 and Cr 0.6, glucose 31, Ca 7.9, Mg 1.9, phos 3.5, AST 1,386, ALT 1,208, alkaline phosphatase 378 and prolonged prothrombin time. She received 10% dextrose in water solution and was admitted to Medicine for further management of EKG abnormalities and elevated liver transaminases.

On the medical ward, patient was hypophosphatemic, hypokalemic, hypocalcemic, and hypomagnesemic, requiring IV fluids and frequent electrolytes repletion. She was closely monitored with multiple daily blood draws. Working closely with the Nutrition and Endocrine services, Her diet was slowly advanced with increase in calorie allowance. Throughout her nutritional rehabilitation, she had a vigorous appetite and had no problems eating or voiding.

Ultrasound of the right upper quadrant revealed a normal size liver, with no structural lesions, and a gallbladder polyp, with no evidence of cholestasis or cholelithiasis. Blood acetaminophen and alcohol levels and urinary toxicology were unremarkable. Work up for infectious, autoimmune, and genetic causes of hepatitis was unremarkable. During this time, the patient's AST and ALT peaked in the high 4,000 s and slowly trended downward. She refused a liver biopsy. Ultimately, the patient's liver failure was attributed to chronic malnutrition and possible liver hypoperfusion in the setting of poor oral intake.

Table 1 Important laboratory results from admission to discharge

	On Admission	Day 2	On discharge (day 10)
Sodium (137–145 mmol/l)	133	130	132
Potassium (3.5-5.1 mmol/l)	2.7	3.4	3.6
Phosphorus (2.5-4.5 mg/dl)	3.5	2.1	3.4
Urea Nitrogen (7–17 mg/dl)	37	13	15
Creatinine (0.52-1.04 mg/dl)	0.6	0.3	0.3
Calcium (8.4-10.3 mg/dl)	7.9	6.7	7.8
Glucose (74–106 mg/dl)	31	73	65
AST (15–46 u/l)	1386	2833	537
ALT (13–69 u/l)	1208	2114	1050
Alkaline Phosphatase (38–126 u/l)	378	471	47
PT (11.8-14.5 sec)	22	23.1	15.3
INR (0/9-1.1)	1.9	2.1	1.2
PTT (25–36.6 sec)	36.1	26.6	34.4
Magnesium (1.6-2.3 mg/dl)	1.9	1.6	1.8
Hemoglobin (12–16 g/dl)	13.5	7.7	7.4
WBC count (4.5-10.8 K/ul)	3.1	1	1.9
Platelets (150–450 K/ul)	83	21	67

The patient remained in sinus rhythm on telemetry, with heart rate ranging from 40 and 100 beats per minute and persistent QTc prolongation. Trans-thoracic echocardiogram (Figure 1) revealed a hypokinetic left ventricle, left ventricular wall atrophy, and an ejection fraction of 10-15%. Following carddiology recommendations, the patient's phosphorus, magnesium, and potassium were aggressively maintained at >3.5, >2.0, and >4.0, respectively, to reduce the risk of torsades de pointes. The corrected QT interval eventually downtrended toward the normal range.

The psychiatrist continued to meet and support the patient throughout her stay in the hospital and was present on rounds to discuss the patient's discharge disposition and plans for follow up. Her hospital course was otherwise notable for steadily decreasing blood counts. During her hospitalization, she was found to be pancytopenic with an absolute neutrophil count as low as 700 and platelet count of 21,000. She was placed briefly on reverse contact isolation for infection prophylaxis until her white count and ANC increased.

The patient improved dramatically over the first few days and by day 4 of hospitalization, she was able to ambulate without assistance and use the bathroom independently she was discharged by day 10.

Discussion

Anorexia nervosa is an eating disorder characterized by extremely low body weight, fear of gaining weight or distorted perception of body image, and amenorrhea [1]. Although described mostly in the context of psychiatric illness, anorexia can lead to devastating, and at times life threatening, medical complications and thus constitutes a challenging condition to manage [2]. Serious complications include electrolyte disturbances, often in the context of the refeeding syndrome, hypothermia, endocrine dysfunction, and multi-organ failure. Anorexia nervosa is most likely responsible for our patient's triad of weakness, liver dysfunction, and prolonged QT interval.

Refeeding malnourished patients with anorexia nervosa can be associated with hypophosphatemia, cardiac arrhythmia and delirium. Phosphorus repletion should be started early with and serum levels maintained above 3 mg/dL. Patients need close monitoring since cardiac and neurologic events associated are most likely to occur within the first weeks [3]. In chronically malnourished anorexia nervosa patients, a slow and a gradual increase in nutrition with nutritional counseling, psychotherapy and careful monitoring of body weight, heart rate and

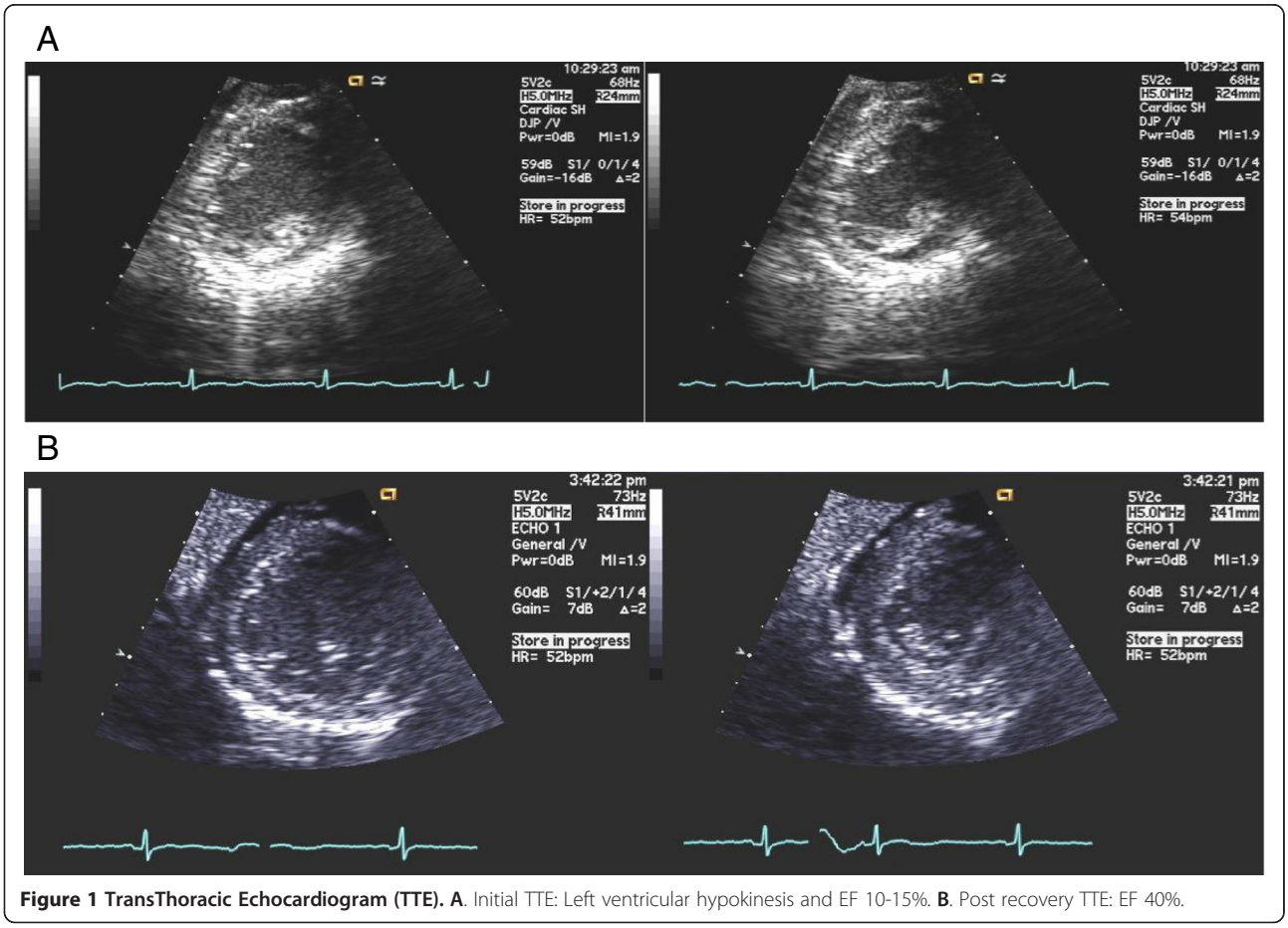

Figure 1 TransThoracic Echocardiogram (TTE). A. Initial TTE: Left ventricular hypokinesis and EF 10-15%. **B**. Post recovery TTE: EF 40%.

rhythm and serum electrolytes is recommended to deliver a safe and effective nutritional rehabilitation and to avoid rapid electrolyte shifts and fluid overload [4-6].

The patient's weakness and fatigue, in the context of a recent history of starting a regular diet while in a state of chronic malnutrition, are concerning for refeeding syndrome, which typically occurs 2 to 5 days after beginning nutritional repletion [7]. Depleted phosphate stores due to prolonged starvation, hypocalcemia, and hypokalemia can lead to impaired muscle contractility and subsequently weakness, myalgia, and tetany. Hypoglycemia and anemia or pancytopenia from chronic malnutrition may have also contributed to the patient's weakness.

Liver injury with an elevation of liver enzymes is a frequent complication, and steatosis of the liver is thought to be the major underlying pathology. The treatment is hydration, correction of electrolytes and fluid imbalance, and gradual nutritional support to prevent refeeding syndrome [8]. The patient exhibits elevated serum transaminases, elevated alkaline phosphatase, and prolonged prothrombin time, which collectively are suggestive of acute liver failure. Liver dysfunction likely also contributed to the patient's hypoglycemia by compromising hepatic gluconeogenesis. Liver hypoperfusion due to anorectic hypovolemia, phosphate and thiamine depletion secondary to refeeding syndrome may have synergistically caused rapid and profound injury to the hepatocytes, resulting in the leak of alanine and aspartate aminotransferases into the serum.

Bradycardia is a common finding in patients with anorexia nervosa secondary to hypothermia and perhaps as a compensatory mechanism to conserve energy in states of starvation. Hypokalemia, hypomagnesemia, and hypocalcemia are typical findings in the refeeding syndrome and may be contributing to the patient's EKG abnormalities showing prolonged QTc [9].

Electrolyte abnormalities occur in anorexia nervosa most often in the context of the refeeding syndrome, defined in 1990 by Solomon and Kirby as "the metabolic and physiologic consequences of the depletion, repletion, compartmental shifts and interrelationships of phosphorus, potassium, magnesium, glucose metabolism, vitamin deficiency and fluid resuscitation" [10]. Central to the pathogenesis of the refeeding syndrome is a weakened cardiopulmonary system, which is incapable of accommodating the fluid and sodium load presented to

the body during nutritional repletion. The resultant volume expansion and fluid retention can progress even to heart failure. Typical electrolyte abnormalities include hypophosphatemia, hypomagnesemia, hypokalemia, and hypocalcemia, with deficiencies in thiamine and other B complex vitamins, as seen during this patient's hospital course.

Hepatic dysfunction is a common medical complication of anorexia nervosa and its treatment. Its exact presentation and laboratory profile, however, remain widely variable, perhaps reflecting the lack of knowledge regarding the etiology and pathophysiology of this condition. One retrospective study of 126 patients with anorexia nervosa—with no history of prior liver disease, hepatotoxic drug exposure, or alcohol consumption—who were malnourished and subsequently hospitalized for parenteral nutrition found that 43% had elevated serum transaminases on admission [11]. The authors identified 4 risk factors associated with these laboratory findings: young age, low BMI, restrictive subtype of anorexia nervosa, and male gender. They also noted resolution of the transaminitis in most cases following nutritional repletion over a period of 4 weeks. Notably, the highest values observed for AST and ALT were 2,120 and 2,614, respectively, well below this patient's peak values in the 4,000 s.

A case report from Japan in 1999 describes a 20 year-old woman with anorexia nervosa (BMI 12.1) who presented to the hospital with lethargy and lightheadedness and was found to have prolonged PT, thrombocytopenia to 64,000, and AST and ALT of 5,000 and 3,980, respectively [12]. She was treated with plasmapheresis for liver dysfunction, but subsequently developed pulmonary edema, acute renal failure, gastrointestinal bleeding, and disseminated intravascular coagulation. Extensive work up for infectious and drug-induced hepatitis was unremarkable, as was investigation for fatty liver changes and antioxidant deficiency. The authors stopped short of undertaking a liver biopsy, as the patient eventually recovered, but they concluded, by exclusion, that malnutrition itself might have led to hepatic failure.

More recently, a case report from the UK of a patient with anorexia nervosa and a BMI of 9 admitted for seizure also described elevation of AST to 5,403 and coagulopathy with an INR of 2.0 [13]. There was no history of alcohol or hepatotoxic drug use, and work up for viral and autoimmune hepatitis and paracetamol toxicity was unremarkable. The liver enzymes normalized spontaneously as the patient was nutritionally rehabilitated, and her acute liver failure was attributed to an episode of hypotension (BP 80/50). Hypotension was due to poor nutritional intake and secondary to cardiac dysfunction from chronic malnutrition led to liver hypoperfusion and, ultimately, ischemic hepatitis.

Further investigation of the patient's cardiac abnormalities revealed left ventricular hypokinesis and EF 10-15%. Takotsubo cardiomyopathy, also known as stress-induced cardiomyopathy, has been described as a rare complication in young women with anorexia nervosa and usually presents in a manner similar to acute myocardial infarction [14]. The condition is characterized by transient hypokinesis, akinesis, or dyskinesis of the left ventricle with or without apical involvement; regional wall motion abnormalities extending beyond a single vascular distribution; presence of a stressful trigger in most cases; absence of coronary artery disease or evidence of plaque rupture; new EKG abnormalities or cardiac enzyme leak; and absence of pheochromocytoma and myocarditis. The pathophysiology of takotsubo cardiomyopathy remains to be elucidated but is thought to involve catecholamine excess leading to myocardial stunning.

Given the findings on history and physical exam and a review of relevant literature, the patient likely presented on the brink of developing frank refeeding syndrome with cardiac dysfunction and hypovolemia leading to hepatic hypoperfusion and ischemic hepatitis. Subsequently, she developed electrolyte disturbances characteristic of refeeding syndrome, which were managed without major complication. Her hospital course is encouraging not only for her recovery, but for the collaboration of the different teams involved in her care, and it highlights the importance of a multidisciplinary approach to caring for patients with the potential dire complications of a complex psychiatric illness.

Consent

An oral over the phone consent was obtained from the patient for publication of this case report and any accompanying images.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

JA, HJ, MK: writers. EA: acquisition of data, references, figure and table. RG, HA: reviewers. All authors read and approved the final manuscript.

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Cassia cinnamon does not change the insulin sensitivity or the liver enzymes in subjects with impaired glucose tolerance

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Abstract

Background: Published studies have reported conflicting results regarding the effects of cinnamon on glucose, lipids and insulin. To gain further insight into the metabolic effects of *Cinnamomum cassia* we performed randomized, double-blinded placebo-controlled study using euglycaemic-hyperinsulinaemic clamp.

Methods: Twenty-one subjects with impaired glucose tolerance (IGT) were included in the study (10 or 11 subjects in each group). The study groups were matched for age, gender and body mass index (BMI). Waist-to-hip ratio, BMI, blood pressure, fasting blood glucose, insulin, triglycerides, total cholesterol, low-density lipoprotein, high-density lipoprotein , HbA1c, ASAT, ALAT, bilirubin, ALP, GT and PK were measured before and after the intake of capsules equivalent to 6 g cinnamon twice a day for 12 weeks. The changes in insulin resistance were measured by euglycaemic-hyperinsulinaemic clamp. The Wilcoxon signed rank sum test, the Mann-Whitney U test and Pearson's chi-squared test were used to analyse the data. Values of $p < 0.05$ were considered to indicate statistically significant differences.

Results: At enrolment, the groups were similar in terms of age, gender and BMI. Of the 21 randomized patients with IGT, 17 completed the study (8 controls vs. 9 treated). The ingestion of 6 g cinnamon twice a day for 12 weeks had no significant effect on insulin sensitivity, HbA1c, fasting glucose or BMI. No significant changes were seen in lipids or liver enzymes.

Conclusions: This study showed that ingestion of 6 g *C. cassia* twice a day for 12 weeks did not change the insulin sensitivity or liver enzymes in subjects with IGT.

Background

Chronic diseases such as cardiovascular disease, cancer, chronic respiratory disease and diabetes mellitus are currently the major cause of death in almost every country worldwide, and not only in the developed countries. According to the World Health Organization chronic diseases cause almost 35 million deaths each year worldwide, which is 60% of the total mortality. Cardiovascular diseases account for almost 50% of deaths due to chronic diseases [1]. The aging population and changes in lifestyle, such as increased energy intake and decreased physical activity, are becoming a growing problem, resulting in an increase in the incidence of obesity and diabetes. Over the past three decades, the prevalence of diabetes mellitus has more than doubled. It has been estimated that the number of people with diabetes worldwide will rise from 6.4% in 2010 (285 million people) to 7.7% (439 million people) by 2030 [2], and the number of obese individuals is projected to rise from 33% (1.3 billion) in 2005 to 57.8% (3.3 billion) in 2030 [3]. Diabetes and obesity are two major risk factors for the development of cardiovascular disease, and the above predictions indicate that cardiovascular disease will soon reach epidemic proportions. It is still not completely understood why diabetes is one of the major risk factors for coronary heart disease, but it has been hypothesized that hyperglycaemia triggers an inflammatory response in the vessels and that inflammation leads to atherosclerosis. Seventy-five to eighty per cent of deaths in adult diabetics are related to cardiovascular disease [4].

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Impaired glucose tolerance (IGT) is the term used to describe the intermediate stage between normal glucose metabolism and type 2 diabetes mellitus. Subjects with IGT have an increased risk of developing type 2 diabetes mellitus and cardiovascular disease compared with normoglycaemic subjects, and early interventions are, therefore, important. The causes of type 2 diabetes mellitus are multifactorial, but diet plays an important role in the incidence, severity and management of the condition [5]. The relationship between dietary factors and coronary heart disease has been studied previously, and it has been found that dietary interventions that reduce the level of circulating blood sugar can reduce the risk of developing diseases such as type 2 diabetes mellitus and cardiovascular disease, which are the two major risk factors for early death [5].

Various types of natural remedies have been used historically for the treatment of ailments, among them *Cinnamomum cassia. C. cassia* is obtained from the bark of the outer skin of a tall evergreen tree, and contains active components including cinnamic aldehyde, cinnamyl aldehyde, tannin, mucus and carbohydrates. These components have been found to have anti-oxidant, anti-microbial, anti-inflammation, anti-diabetic and anti-tumour activity [6-8]. *C. cassia* and *C. zeylanicum* extracts have been shown to be among the most effective natural substances in the regulation of blood glucose [9]. However, some studies have shown that coumarin, found in *C. cassia,* may have detrimental effects on the liver, and the Federal Institute for Risk Assessment in Europe has, therefore, suggested that it be replaced by *C. zeylanicum* [10]. We have previously shown that the ingestion of 6 g *C. cassia* powder reduces the postprandial blood glucose concentration [11]. However, in a later study, we found that the ingestion of *C. zeylanicum* did not affect postprandial blood glucose or insulin levels in humans [12].

Other studies have shown conflicting results regarding the effect of cinnamon on insulin sensitivity, when measured indirectly [13-16]. In the present study, we investigated whether *C. cassia* increased insulin sensitivity in subjects with IGT, using the euglycemic-hyperinsulinaemic clamp, which provides a direct measure of insulin resistance. To the best of our knowledge, the direct measurement of the effect of cinnamon on insulin resistance been not been studied previously in humans. We also investigated whether *C. Cassia* supplementation had any effect on liver enzymes.

Subjects and methods

Twenty-one subjects with IGT, who met the study criteria, were included in this randomized, double-blinded placebo-controlled study. The inclusion criterion was diagnosis of IGT, by a standard 75 g OGTT, for less than 12 months before enrolment. Glucose tolerance status and fasting blood glucose levels were evaluated using the criteria established by the WHO [5]. The exclusion criteria were: thyroid disorders, or insulin, oral hypoglycaemics, or insulin-sensitizing drugs within 60 days before enrolment. The subjects were recruited from the population in southern Sweden and all examinations were done in the same institution. After diagnosis of IGT, all participants lifestyle advice but no medications. Four subjects dropped out of the study, one for personal reasons (treated group), one had difficulty swallowing the capsules (control group), and two had gastrointestinal problems (control group). These four subjects did not differ from the 17 participants at baseline. Patients were allocated to the treatment or control group using stratified randomized selection for age, sex and body mass index (BMI) (Table 1). They were randomly selected to either the A or B group by sealed envelopes.

All the capsules were prepared in advance by Scandinavian Nutrients AB (Strängnäs, Sweden), and contained either 700 mg cellulose or 500 mg *C. cassia* and 200 mg cellulose. Participants received both individual verbal and written information and signed a consent form. The

Table 1 Clinical and demographic characteristics of the subjects with IGT at baseline

Variables	Controls	Treated
Subjects	8	9
Female/Women	5 (63)	5 (56)
Male/Men	3 (37)	4 (44)
Age (years)	72 ± 2	73 ± 2
BMI (kg/m^2)	28.6 ± 1.9	25.7 ± 1.3
Waist circumference (cm)	0.9 ± 0.1	0.9 ± 0.09
Smoking		
No	8 (100)	6 (67)
Previously	0 (0)	3 (33)
Present	0 (0)	0 (0)
Blood pressure		
Systolic (mmHg)	122 ± 16.7	140.5 ± 4,7
Diastolic (mmHg)	80.0 ± 2.6	82.0 ± 2.8
Fasting-glucose (mmol/l)	6.7 ± 0.4	6.0 ± 0.3
Fasting-insulin (mIE/l)	11.1 ± 2.0	9.8 ± 2.1
HbA1c (mmol/mol)	40.1 ± 2.4	39.6 ± 1.3
Cholesterol (mmol/l)	4.5 ± 0.2	4.9 ± 0.4
LDL Cholesterol (mmol/l)	2.7 ± 0.2	3.2 ± 0.4
HDL Cholesterol (mmol/l)	1.5 ± 0.1	1.4 ± 0.1
Triglycerides (mmol/l)	1.1 ± 0.1	1.1 ± 0.1

Gender and smoking-related values are given as percentages, all other values are reported as the mean ± SD.
The groups did not differ for any variable, $p<0.05$.

participants were instructed to ingest one capsule each morning at breakfast time and one each evening at dinner time for 12 weeks. During this period, the subjects were examined three times, at baseline, after sex weeks and finally, after 12 weeks. Insulin sensitivity was measured at baseline and after 12 weeks, in the morning after a 12-hour fast. Smoking and snuff-taking were prohibited 8 hours prior to and during the test.

Insulin sensitivity was determined with the euglycaemic-hyperinsulinaemic clamp, according to DeFronzo et al. [17]. Intravenous catheters were inserted into antecubital veins in both arms. One arm was used for the infusion of glucose and insulin, and the contralateral arm was used for blood intermittent sampling. The catheter was kept patent by a slow infusion of 0.9% saline, to which 2 ml of the subject's blood per 100 ml infusate had been added to prevent the absorption of insulin to glassware and plastic surfaces. Baseline samples of glucose and insulin were taken. A primed constant infusion of insulin (Actrapid 100 IU/ml; NovoNordisk, Bagsvaerd, Denmark), was started at a constant infusion rate of 0.28 nmol/m^2 body surface area/min. After 4 min, glucose infusion (200 mg/ml) was started; the infusion rate being adjusted manually throughout the procedure to maintain the blood glucose level at 5.0 mmol/l. Blood glucose was determined at the bedside every 5 minutes. Blood samples were taken after 60 and 120 min for the analysis of the insulin concentration. Blood glucose concentrations were measured with the HemoCue Glucose system (HemoCue AB, Ängelholm, Sweden). The precision of the HemoCue Glucose system was better than 0.3 SD in the range 0 mmol/l to 22.2 mmol/l.

All analyses of plasma and whole blood were performed on samples obtained after overnight fasting. Analyses of fasting plasma triglycerides, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), HbA1c, ASAT, ALAT, bilirubin, ALP, GT, PK, whole-blood glucose (fasting blood glucose), and insulin were carried out at the time of baseline examination, at the Department of Clinical Chemistry, Skane University Hospital in Malmö, which is affiliated to a national standardization and quality control system. Insulin concentrations were measured using an immunoassay with an alkaline phosphatase conjugate (Access Ultrasensitive Insulin, Beckman-Coulter AB, Bromma, Sweden). The sensitivity of the insulin immunoassay was 0.03 mU/l, and the intra-assay coefficient of variation was less than 10% in the range 0.03 mU/l to 300 mU/l. Total cholesterol and HDL were measured by enzyme assay kits, using an automated analyser (Aeroset™, Abbott Labs, USA). LDL was calculated using the Friedewald equation [18]. All samples from each subject were analysed in the same run.

The study was approved by the Ethics Committee of Lund University and was performed according to the Helsinki Declaration. The study started in 2011 and was completed in 2013.

All statistical calculations were performed using SPSS for Windows software (version 22, 2013). The Wilcoxon signed rank sum test was used to compare quantitative variables within the group, and the Mann-Whitney U test was used to compare quantitative variables between groups. Pearson's chi-squared test for categorized variables was used to test for statistical significances between the groups. Values of $p < 0.05$ were considered to indicate statistically significant differences.

Results

No significant changes were seen in clinical or demographic characteristics such as BMI, waist:hip ratio, systolic or diastolic blood pressure between the two groups at baseline (Table 1). No significant differences were found in HbA1c, fasting blood glucose or insulin levels at baseline, but the placebo group had a significantly lower fasting insulin level after 12 weeks (Table 2). No significant differences were seen in total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides at baseline, and there were no significant changes during the study (Table 2). Neither were any significant changes seen in ALAT, ALP, GT or bilirubin (Table 2). No significant increase in insulin sensitivity was seen in the treated group after 60 or 120 min (Table 3). In the placebo group, a significant increase in insulin sensitivity was seen at 60 min, but not at 120 min.

Discussion

In this study, it was found that 12 weeks of *C. cassia* supplementation (12 g/d) did not improve fasting plasma glucose, insulin, blood lipids or insulin sensitivity in patients with IGT. Neither were any of the liver enzymes affected in this study. Although the effects of cinnamon on glucose, insulin resistance and lipid profile have been investigated in several studies, showing conflicting results, to the best of our knowledge, this is the first study to use a direct measurement of insulin resistance.

Cinnamon has demonstrated qualities that enhance glucose uptake by activating insulin receptor kinase activity, autophosphorylation of the insulin receptor, and glycogen synthase activity, *in vitro* and *in vivo* [19-22]. In a study on patients with type 1 diabetes who were given cinnamon (unknown species) no differences were found in HbA1c, total daily insulin intake, or number of hypoglycaemic episodes, compared to the placebo group [23]. This may be explained by the fact that cinnamon decreases insulin resistance, which is not the cause of type 1 diabetes. Insulin resistance plays a key role in the development of diabetes, and cinnamon may have qualities that decrease insulin resistance. However, this has not been verified in humans using direct measurements,

Table 2 Glycemic outcomes, lipids and liver enzymes in patients with IGT, at baseline and after 12 weeks' treatment or placebo

Variables	Baseline			12 wk.			
	Control	Treated	p-value	Control	p-value	Treated	p-value
Fasting-glucose (mmol/l)	6.7 ± 0.4	6.0 ± 0.3	0.370	6.1 ± 0.4	0.205	6.1 ± 0.4	0.735
Fasting-insulin (mIE/l)	11.1 ± 2.0	9.8 ± 2.1	0.481	8.5 ± 1.6	0.034*	9.0 ± 2.5	0.284
HbA1c (mmol/mol)	40.1 ± 2.4	39.6 ± 1.3	0.815	40.1 ± 1.8	0.786	40.2 ± 1.6	0.320
Cholesterol (mmol/l)	4.5 ± 0.2	4.9 ± 0.4	0.673	4.4 ± 0.2	0.670	4.6 ± 0.3	0.482
LDL Cholesterol (mmol/l)	2.7 ± 0.2	3.2 ± 0.4	0.606	2.6 ± 0.3	0.528	2.9 ± 0.2	0.553
HDL Cholesterol (mmol/l)	1.5 ± 0.1	1.4 ± 0.1	0.888	1.5 ± 0.1	0.774	1.4 ± 0.1	0.414
Triglycerides (mmol/l)	1.1 ± 0.1	1.1 ± 0.1	0.888	1.0 ± 0.2	0.686	1.0 ± 0.1	0.101
ASAT (μkat/l)	0.38 ± 0.04	0.40 ± 0.05	0.888	0.38 ± 0.04	0.752	0.41 ± 0.05	0.445
ALAT (μkat/l)	0.39 ± 0.07	0.50 ± 0.09	0.481	0.38 ± 0.08	0.528	0.49 ± 0.09	0.799
ALP (μkat/l)	0.89 ± 0.05	1.04 ± 0.13	0.541	0.86 ± 0.06	0.161	1.10 ± 1.28	0.128
GT (μkat/l)	0.37 ± 0.07	0.58 ± 0.15	0.370	0.36 ± 0.06	0.674	0.71 ± 0.25	0.313
Bilirubin (μmol/l)	9.2 ± 1.1	9.9 ± 1.6	0.963	8.5 ± 1.1	0.197	11.6 ± 2.0	0.087
Pk (INR)	1.04 ± 0.02	1.0 ± 0.02	0.888	1.01 ± 0.02	0.157	1.0 ± 0.02	0.317

Significant differences between the control and treated group in glycemic outcomes, lipids and liver enzymes were evaluated at baseline with the Mann-Whitney U test.
Significant differences within the groups in glycemic outcomes, lipids and liver enzymes were evaluated at 12 wk. with the Wilcoxons signed rank test, *$p < 0.05$.
Values are reported as means ± SD.

i.e. the euglycaemic-hyperinsulinaemic clamp. A reduction in insulin resistance was seen in six nondiabetic women after the intake of *C. burmannii*, 1 g per day, in capsules, for 8 weeks [13]. A reduction in insulin resistance was also reported in a recently published study in which 23 patients with nonalcoholic fatty liver disease were given 1.5 g cinnamon (unknown species) per day for 12 weeks [14]. Solomon et al. found improved insulin sensitivity in eight healthy men after a 14-day intervention with *C. cassia* (pills, 3 g per day) [15]. However, Vanschoonbeek et al. reported that insulin sensitivity was not improved in twelve women with type 2 diabetes mellitus after taking 1.5 g *C. cassia* per day for 6 weeks [16]. In the studies mentioned above, insulin resistance was measured using a homeostatic model assessment (Homa-IR), the quantitative insulin sensitivity check index (Quick) or the insulin sensitivity index (ISI) [24-26]. However, these provide indirect measures of insulin resistance. Both Homa-IR and Quick provide quantitative measurements based on fasting insulin and fasting glucose levels, while ISI is based on the oral glucose tolerance test. It has been recognized that Homa-IR gives relatively low values in patients with insulin resistance undergoing elective surgery [27] or who have advanced type 2 diabetes mellitus [28]. On the other hand, it has been reported in other studies that Homa-IR shows good correlation with the ISI assessed with the euglycaemic-hyperinsulinaemic clamp [29]. Methods of assessing insulin sensitivity using an oral glucose tolerance test have been proposed in a few studies [30,31]. Although this indirect method of measuring insulin resistance is simple and plays an important role in large-scale studies, the golden standard in smaller studies is the euglycaemic-hyperinsulinaemic clamp.

In 2003, Khan et al. reported remarkable results following the ingestion of 1, 3 and 6 g *C. cassia* powder per day for 40 days. Not only were the levels of fasting glucose reduced in subjects with type 2 diabetes mellitus, but positive effects were also seen on triglyceride, LDL and total cholesterol levels [31]. Following this study, several other studies on subjects with type 2 diabetes

Table 3 Insulin sensitivity measured by euglycaemic-hyperinsulinaemic clamp in patients with IGT, at baseline and after 12 weeks' treatment or placebo

	Baseline	12 weeks	p-value	Baseline	12 weeks	p-value
	Placebo (n = 8)			*C. cassia* (n = 9)		
60 min	6.3 ± 1.2	8.8 ± 1.9	0.012*	7.3 ± 1.3	7.8 ± 1.4	0.477
120 min	5.3 ± 1.8	7.0 ± 1.2	0.068	6.0 ± 1.1	6.3 ± 0.9	0.373

Significant differences in insulin sensitivity within the groups were evaluated at 60 min resp. 120 min at baseline and 12 wk. with the Wilcoxons signed rank test.
* = Significant difference within the groups between 60 min resp. 120 min at baseline and at 12wk., *$p < 0.05$.
Values are reported as means ± SD.

mellitus who had been given cinnamon were published, with varying results. The study by Mang et al. [32] revealed that 3 g *C. cassia* supplement per day for 4 weeks reduced fasting glucose in type 2 diabetes mellitus patients. In contrast, Vanschoonbeek et al. and Blevins et al. found no difference in fasting glucose in type 2 diabetes mellitus patients after taking 1.5 g *C. cassia* per day for 6 weeks, and 1 g *C. cassia* per day for 12 weeks, respectively [16,33]. No difference was observed in HbA1c, total cholesterol, LDL, HDL or triglyceride concentrations in any of the above studies [16,32,33]. A meta-analysis performed in 2008 revealed no effect of ingested cinnamon on glucose or lipid parameters in subjects with type 1 or type 2 diabetes mellitus [34].

However, in a recent study, HbA1c was found to be decreased in patients with type 2 diabetes mellitus (1 g *C. cassia* per day for 12 weeks) [35]. Similar results were obtained by Akilen (2 g *C. cassia* per day for 12 weeks) and Lu (120 mg per day or 360 mg *C. aromaticum* per day for 12 weeks) [36,37]. In these studies, the subjects had HbA1c levels above 8% and high fasting glucose levels, and were already using concomitant hypoglycaemic medication. The subjects in this present study had IGT, had normal HbA1c levels and were not receiving any medication for diabetes. These results suggest that individuals with poorly controlled diabetes may benefit more from cinnamon intake than those receiving adequate treatment. A new meta-analysis in 2013 of type 2 diabetes mellitus patients revealed a decrease in fasting plasma glucose, HbA1c, total cholesterol, LDL and triacylglycerol levels, and an increase in HDL, but no change in HbA1c, after the ingestion of cinnamon [38]. These conflicting results might be due to differences in prescribed anti-diabetic medication, the type and dose of cinnamon administered, the duration of the study, and/or the population studied.

Coumarin is present in cinnamon, especially *C. cassia*. Debate on the toxicity of coumarins started after experimental studies on hepatotoxicity in dogs. The Federal Institute for Risk Assessment issued a warning on the consumption of cinnamon and established a limit of 0.1 mg coumarin per kg body weight/day. In a study on 114 participants who ingested 30 mg coumarin combined with a vasoactive component, Schmeck-Lindenau et al. found that nine of the patients showed elevated levels of transaminases in serum [39]. But they concluded that the risk of elevated transaminases was limited after risk factors, as hepatitis in the history and other diseases in the liver were considered. In a biokinetic study, Abraham et al. showed that coumarin is well absorbed from ingested *C. cassia* [40]. According to the Federal Institute for Risk Assessment the average coumarin content in *C. cassia* is 3000 mg/kg. In our study, where the subjects ingested 12 g *C. cassia* per day for 12 weeks, i.e., about 36 mg coumarin, no significant changes were seen in transaminases. Askari et al. found a reduction in the level of transaminases in patients with nonalcoholic fatty liver disease, who were given 1.5 g cinnamon/day for 12 weeks [14]. Larger and long-term studies are needed to elucidate the effect of coumarin in hepatotoxicity.

The strengths of the present study were the double-blind placebo-controlled design with few drop-outs, and the direct measurement of insulin sensitivity. However, this study also had some limitations. Firstly, the sample size was small and all the participants were living in southern Sweden. Secondly, the control group had a significantly lower insulin response than the treated group after 12 weeks, which indicates that the placebo capsules had an active effect. The placebo capsules contained cellulose, which is a type of fibre, and the amount of fibre was probably sufficient to cause an effect on the gastrointestinal tract, leading to an indirect effect on insulin response. The relation between high fibre diets and a low insulin response is already known [41]. Thirdly, we had no means of precisely determining the compliance, but this was assessed by counting the remaining capsules and repeated follow-ups. Finally, although the cinnamon and placebo capsules appeared identical, it is possible that some of the participants could discern a difference between the two types of capsules because of the smell.

In conclusion, the findings of this study were that the ingestion of 6 g *C. cassia* twice a day for 12 weeks did not change the insulin sensitivity or liver enzymes in subjects with IGT.

Competing interest

None of the authors have any personal or financial competing interest.

Authors' contributions

The authors' contributions were as follows: JH, JN and JW contributed to the design of the study; JW was responsible for recruiting the subjects and carried out the practical aspects of the study. JW performed the statistical calculations and wrote the first draft of the manuscript. JH, SL, JN critically revised the manuscript. All authors have read and approved the final manuscript.

Acknowledgments

This study was supported by Dr Per Håkansson's Foundation and Erhold Lundström's Foundation for Medical Research. The authors are grateful to Ida Kapusta and Margaretha Persson for expert technical assistance.

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Serum concentration of eicosapentaenoic acid is associated with cognitive function in patients with coronary artery disease

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Abstract

Background: Recent studies have shown that intake of n-3 polyunsaturated fatty acids (PUFAs) is associated with reduced risk of cognitive impairment and coronary artery disease (CAD); however, it is currently unknown whether reduced serum n-3 PUFA is associated with cognitive impairment in patients with CAD.

Methods: We retrospectively evaluated cognitive function with the mini-mental state examination (MMSE), serum levels of PUFAs (including eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA], dihomogammalinolenic acid [DGLA], and arachidonic acid [AA]), cardiovascular risk factors (hypertension, dyslipidemia, diabetes mellitus, cerebrovascular disease, and history of current/previous smoking), and parameters of cardiac function (left ventricular ejection fraction and brain natriuretic peptide levels) in 146 Japanese CAD patients. The associations between the MMSE scores and the other parameters were evaluated.

Results: Pearson correlation analysis showed that EPA ($R = 0.25$, $P < 0.01$), EPA/AA ratio ($R = 0.22$, $P = 0.01$), and left ventricular ejection fraction ($R = 0.15$, $P = 0.04$) were positively associated with MMSE score, and that age ($R = -0.20$, $P < 0.01$) and brain natriuretic peptide levels ($R = -0.28$, $P < 0.01$) were inversely associated with MMSE score. Multiple regression analysis showed that age ($P < 0.05$) was negatively associated with MMSE score, while EPA ($P < 0.01$) and EPA/AA ratio ($P < 0.05$) were positively associated with MMSE score; however, sex; body mass index; left ventricular ejection fraction; levels of DHA, AA, and DGLA; DHA/AA ratio; brain natriuretic peptide; and presence of hypertension, dyslipidemia, diabetes mellitus, cerebrovascular disease, and history of current/previous smoking were statistically excluded.

Conclusions: Serum EPA concentration is associated with cognitive function in patients with CAD, suggesting that a low serum EPA level is a risk factor for cognitive impairment independent of cardiac function, including left ventricular ejection fraction. This correlation potentially lends further support to a role of dietary n-3 PUFAs in preventing the cognitive decline in CAD patients.

Keywords: Eicosapentaenoic acid, n-3 polyunsaturated fatty acids, Cognitive function, Mini-mental state examinations, Coronary artery disease

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Background

Cardiovascular disease has recently been implicated as a major factor in the development of dementia, as these diseases may be linked by shared common risks and pathogenic elements [1,2]. Accumulation of cardiovascular risk factors therefore leads to cognitive impairment. In addition, hypoxia/ischemia resulting from reduced cerebral blood flow due to cardiac dysfunction may be associated with dementia. Conversely, dementia itself could be an independent cardiovascular risk factor; patients with dementia have lifestyle-related problems, such as inappropriate food or alcohol intake, sedentary activity, and psychosocial stress, including depression [3]. Therefore, the modification of these lifestyle-related problems could be strategies for coronary artery disease (CAD) and dementia prevention. While several pharmacological agents, including cholinesterase inhibitors [4], have been developed for dementia, sufficiently effective and curative treatments have not yet been established. Therefore, the identification of residual risk factors is important for dementia prevention.

The Japan Eicosapentaenoic Acid Lipid Intervention Study showed that long-term use of eicosapentaenoic acid (EPA) is effective for prevention of major coronary events in Japanese hypercholesterolemic patients [5]. In addition, recent studies demonstrated that consumption of fish and n-3 polyunsaturated fatty acids (PUFAs) reduced the incidence of cognitive impairment [6]. These studies indicate that a reduced serum level of n-3 PUFAs may be a risk factor for both CAD and cognitive impairment.

However, it is currently unknown whether reduced serum levels of n-3 PUFAs are associated with cognitive impairment, and more specifically, which components of PUFAs are associated with cognitive function in CAD patients. Therefore, the aim of this study was to investigate the association between cognitive function and n-3 PUFA levels (including eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA], dihomogammalinolenic acid [DGLA], and arachidonic acid [AA]) in CAD patients, and to identify which components of PUFAs are associated with cognitive function in these patients.

We hypothesized that decreased level of EPA would be associated with cognitive impairment in patients with CAD.

Material and methods

Patients and study design

In patients with CAD, serum PUFA levels were measured for identification of residual risk factors for CAD. In addition, patients underwent mini-mental state examinations (MMSE) to screen cognitive function [1]. We retrospectively reviewed 146 consecutive Japanese patients diagnosed with CAD in the Department of Cardiology at Tokushima University Hospital between April 2013 and March 2014.

Patients with CAD were defined as patients with a history of myocardial infarction, angiographic evidence of at least 50% stenosis by area in at least 1 coronary artery, evidence of exercise-induced ischemia, or history of coronary revascularization. The exclusion criteria were as follows: use of fish oil supplements or n-3 fatty acid-containing drugs or a history of myocardial infarction

Table 1 Clinical characteristics of subjects

Variables	
Number of patients	146
Mini-mental state examination score	27.8 ± 3.1
Male, n (%)	111 (76.0%)
Age, y	70.9 ± 8.6
Body mass index, kg/m^2	23.5 ± 3.4
Systolic blood pressure, mmHg	127 ± 16.3
Diastolic blood pressure, mmHg	71 ± 11.6
Low-density lipoprotein cholesterol, mg/dL	96.0 ± 34.4
Triglycerides, mg/dL	124.5 ± 70.3
High-density lipoprotein cholesterol, mg/dL	55.0 ± 14.7
HbA1c, %	6.7 ± 5.5
Fasting plasma blood glucose, mg/dL	130.0 ± 58.7
Brain natriuretic peptide, pg/mL	151.6 ± 274.2
Left ventricular ejection fraction, %	58.6 ± 11.2
Fatty acids	
Docosahexaenoic acid, μg/mL	132.5 ± 52.2
Eicosapentaenoic acid, μg/mL	68.9 ± 40.4
Arachidonic acid, μg/mL	173.0 ± 43.4
Dihomogammalinolenic acid, μg/mL	34.5 ± 11.4
Complications	
Dyslipidemia, n (%)	126 (86.3%)
Hypertension, n (%)	131 (89.7%)
Diabetes mellitus, n (%)	80 (54.8%)
Cerebrovascular disease, n (%)	25 (17.1%)
Smoking, n (%)	108 (74.0%)
Drugs	
ACEI/ARB, n (%)	91 (62.3%)
β-blockers, n (%)	53 (36.3%)
Calcium channel blockers, n (%)	62 (42.5%)
Statins, n (%)	123 (84.2%)
Ezetimibe, n (%)	8 (5.5%)
Diuretics loop, n (%)	27 (18.5%)
Mineralocorticoid antagonists, n (%)	7 (4.8%)

Unless indicated otherwise, data are presented as the mean ± standard deviation.
ACEI, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers.

within 1 month. In addition, patients with symptomatic active malignant diseases or liver dysfunction (aspartate aminotransferase levels >100 IU/L, alanine aminotransferase levels >100 IU/L) were also excluded.

Hypertensive patients were defined as those with a systolic blood pressure of ≥140 mmHg and/or diastolic blood pressure of ≥90 mmHg and/or individuals receiving antihypertensive medications. Dyslipidemic patients were defined as those with a low-density lipoprotein cholesterol level (LDL-C) of ≥140 mg/dL, a triglyceride level of ≥150 mg/dL, a high-density lipoprotein cholesterol level (HDL-C) of <40 mg/dL, or individuals receiving lipid-lowering medications. Diabetic patients were defined as individuals receiving insulin or oral hypoglycemic agents or those with an HbA1c level of ≥6.5%, fasting plasma glucose level of 126 mg/dL, or non-fasting plasma glucose level of ≥200 mg/dL.

Serum fatty acid composition, including levels of EPA, DHA, DGLA, and AA, was measured by gas–liquid chromatography at a commercially available laboratory (SRL, Tokyo, Japan).

Since n-6 PUFAs, including AA, are often considered to be pro-inflammatory fatty acids and the EPA/AA ratio is associated with a low incidence of cardiac events [7], EPA/AA and DHA/AA ratios were also calculated.

In addition, other biochemical parameters, including LDL-C, HDL-C, triglycerides, fasting plasma glucose, and HbA1c, were also measured.

Cardiac function was evaluated by measuring brain natriuretic peptide (BNP), a biomarker which represents left ventricular endodiastolic pressure, and left ventricular ejection fraction (LVEF) evaluated by echocardiography. The LVEF was calculated using the modified Simpson's method with the apical 4-chamber and 2-chamber views.

Cognitive function was evaluated by MMSE, which is widely used as a screening tool for assessment of cognitive function [8].

This study protocol was approved by the Tokushima University Hospital Ethics Committee.

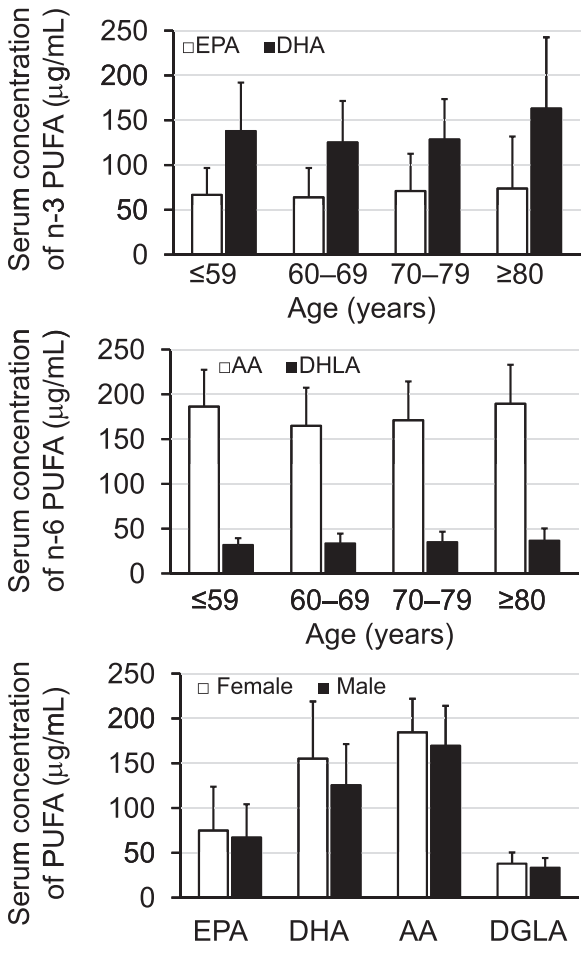

Figure 1 Distribution of levels of n-3 and n-6 polyunsaturated fatty acids (PUFAs) stratified by age and sex. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; DGLA, dihomogammalinolenic acid.

Statistical analysis

Continuous variables were averaged and expressed as the mean ± standard deviation, and categorical parameters were expressed as a percentage. MMSE scores and BNP levels were natural log transformed for statistical analysis because of non-normal distributions. Associations between CAD risk factors and MMSE scores were

Table 2 Pearson correlation analysis between MMSE scores and cardiovascular risk factors

Variables	R	P-value
Age	−0.20	< 0.01
Sex	-	0.30
Body mass index	0.09	0.23
DHA	0.12	0.06
EPA	0.25	<0.01
AA	0.1	0.99
DGLA	−0.001	0.20
EPA/AA	0.22	0.01
DHA/AA	0.10	0.20
Left ventricular ejection fraction	0.15	0.04
Brain natriuretic peptide	−0.28	< 0.01
Hypertension	-	0.96
Dyslipidemia	-	0.89
Diabetes mellitus	-	0.37
Cerebrovascular disease	-	0.79
Current/previous smoker	-	0.12

MMSE, mini-mental state examination; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; AA, arachidonic acid; DGLA, dihomogammalinolenic acid.

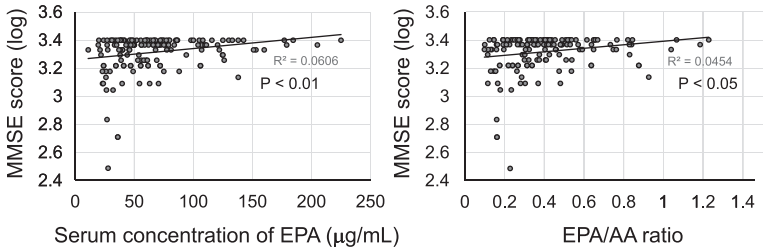

Figure 2 Association between serum concentration of eicosapentaenoic acid (EPA) and EPA/arachidonic acid (AA) ratio and mini-mental state examination (MMSE) score.

determined by the Student's t-test or the Pearson's correlation analysis. Multiple regression analysis (standard least squares method) was used to assess the degrees of association between the CAD risk factor variables and the MMSE scores. Residuals and residual vs. fit plots were examined to ensure homoscedasticity.

All statistical analyses were performed using JMP 10 software. Statistical significance was defined as $P < 0.05$.

Results

Clinical characteristics of subjects

Patient characteristics are shown in Table 1. The mean MMSE score was 27.8 ± 3.1, indicating that subjects generally had mild cognitive impairment. The serum concentration of n-3 PUFAs, including EPA and DHA, and n-6 PUFAs, including AA and DGLA, are shown in Figure 1 by patient age and sex. EPA and DHA concentrations were the highest among patients aged ≥80 years, and levels of EPA, DHA, DGLA, and AA were higher in women than in men.

Correlation between n-3 PUFAs and MMSE score

The Pearson correlation analysis showed that levels of EPA, EPA/AA ratio, and LVEF were positively associated with MMSE score, and that age and BNP was inversely associated with MMSE score (Table 2, Figure 2). There were no relationships between MMSE score and body mass index or levels of DHA, AA, DGLA, and DHA/AA ratio (Figure 3). In addition, there were no differences in MMSE scores according to patient sex and risk factors, including hypertension, dyslipidemia, diabetes mellitus, cerebrovascular disease, and history of current/previous smoking. Interestingly, both LVEF and BNP (parameters of cardiac function) were associated with serum levels of DHA and DHA/AA but not EPA or EPA/AA (Additional file 1: Tables S1 and S2).

Multiple regression analysis was then performed to elucidate independent determinants of MMSE score; age ($P < 0.05$) was negatively associated with MMSE score, while EPA levels ($P < 0.01$) and EPA/AA ratio ($P < 0.05$) were positively associated with MMSE score. However, sex; body mass index; levels of DHA, AA, DGLA, and DHA/AA ratio; LVEF; levels of BNP; presence of hypertension, dyslipidemia, diabetes mellitus, and cerebrovascular disease; and history of current/previous smoking were statistically excluded (Table 3).

Discussion

The present study demonstrated that decreased serum levels of EPA and a reduced EPA/AA ratio are associated with cognitive impairment in patients with CAD, indicating that decreased EPA is a risk factor for development of cognitive impairment this patient population. AA levels showed no association with MMSE scores, while low EPA levels were independently correlated with MMSE scores. Therefore, EPA/AA did not increase the predictive power of EPA alone. In addition, the Pearson correlation analysis exhibited an association between MMSE scores and LVEF or BNP; however, multiple regression in an age-adjusted model showed no association.

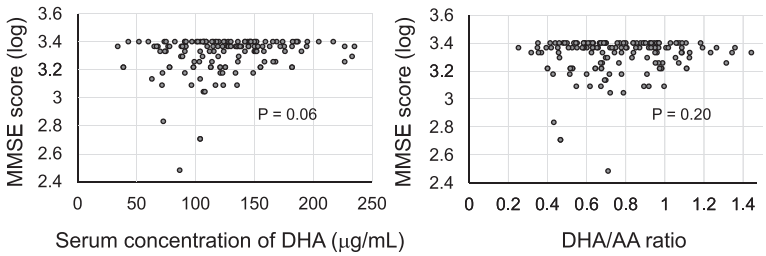

Figure 3 There were no associations between serum concentration of docosahexaenoic acid (DHA) and DHA/AA ratio and mini-mental state examination (MMSE) score.

Table 3 Multiple regression analysis for determinants of degree of MMSE score in patients with CAD

Variables	Coefficient	95% confidence interval	P-value
Model 1			
Age, years	−0.004	−0.007 to −0.001	0.02
Male sex	0.005	−0.03 to 0.04	0.76
Body mass index, kg/m^2	−0.0008	−0.001 to 0.007	0.85
Hypertension	0.02	−0.01 to 0.06	0.24
Dyslipidemia	−0.001	−0.04 to 0.03	0.91
Diabetes mellitus	0.001	−0.02 to 0.02	0.98
Cerebrovascular disease	−0.01	−0.04 to 0.02	0.50
Current/previous smoker	0.001	−0.03 to 0.04	0.80
EPA, μg/mL	0.001	0.0003 to 0.002	< 0.01
DHA, μg/mL	−0.0004	−0.001 to 0.0003	0.22
AA, μg/mL	0.0001	−0.0001 to 0.001	0.83
DGLA, μg/mL	0.001	−0.002 to 0.004	0.45
LVEF, %	0.001	−0.001 to 0.004	0.20
BNP, pg/mL	−0.015	−0.035 to 0.005	0.15
Model 2			
Age, years	−0.003	−0.006 to −0.0002	0.04
Male sex	0.002	−0.03 to 0.04	0.89
Body mass index, kg/m^2	0.0008	−0.006 to 0.008	0.83
Hypertension	0.02	−0.02 to 0.05	0.37
Dyslipidemia	0.003	−0.03 to 0.04	0.84
Diabetes mellitus	0.002	−0.03 to 0.04	0.85
Cerebrovascular disease	−0.01	−0.03 to 0.02	0.58
Current/previous smoker	0.003	−0.03 to 0.04	0.85
EPA/AA	0.15	0.03 to 0.28	0.01
DHA/AA	−0.04	−0.15 to 0.07	0.42
LVEF, %	0.001	−0.001 to 0.003	0.29
BNP, pg/mL	−0.018	−0.04 to 0.001	0.06

Model 1 $R^2 = 0.20$; $P < 0.05$.
Model 2 $R^2 = 0.17$; $P < 0.05$.
EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; DGLA, dihomogammalinolenic acid; LVEF, left ventricular ejection fraction; BNP, brain natriuretic peptide.

Although there is a possibility of confounding between cardiac function and age, our results indicate that MMSE scores were associated with EPA levels independent of cardiac function.

It has been reported that fish intake is associated with cognitive function [9,10], and n-3 PUFA-containing food improves cognitive function [11]. The exact mechanisms of EPA on neuronal function are unknown; however, EPA is involved in endothelial-dependent vascular function and plays important roles in the prevention of microcirculatory insufficiency. Therefore, one possible mechanism is that decreased EPA results in microcirculation insufficiency in the brain, thus leading to ischemia-induced brain damage. It has been reported that EPA enhances endothelial expression of nitric oxide synthase, and activation of endothelial nitric oxide synthase leads to vasodilation and endothelial protection [12,13]. Reduced nitric oxide synthase-induced microcirculation insufficiency may be involved in the development of cognitive impairment. Brain microvascular insufficiency, including cerebral white matter disease, is an early marker of cognitive impairment [14,15]. Thus, in patients with very mild cognitive impairment, such as those who were included in this study, microcirculation insufficiency rather than amyloid β deposition, the cause of Alzheimer's disease, may be the predominant contributor to vascular dementia.

It has been reported that vascular inflammation is associated with cognitive function [16]. EPA is known to have the ability to attenuate tumor necrosis factor-α-induced upregulation of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and monocyte chemoattractant protein-1 [17]. The anti-inflammatory effects of EPA might contribute to the prevention of cognitive impairment.

A previous meta-analysis demonstrated that subjects with predementia syndrome had significantly lower levels of EPA but not DHA or total n-3 PUFA [18]; thus, serum EPA may be a more sensitive biomarker for prediction of cognitive impairment compared to serum DHA. It has also been reported that administration of n-3 PUFA to patients with very mild Alzheimer's disease (MMSE >27 points) delayed the rate of cognitive dysfunction, indicating the importance of n-3 PUFA on brain function in early stages of cognitive impairment [19].

In addition, EPA is converted into DHA in the liver and at the blood-brain barrier and has been shown to cross the blood-brain barrier after dietary supplementation, thereby suggesting that EPA plays important roles in cognitive function after conversion into DHA [20,21]. EPA could be a rich source of DHA and may compensate for reduced levels of DHA in the brain; decreased serum EPA may thus be a more profound indicator of cognitive function than decreased serum DHA.

The present study had several limitations. In particular, this was a retrospective study with a small sample size in a single center. Larger clinical cohort studies are needed to clarify the effects of EPA on cognitive function.

Conclusions

Serum EPA concentration rather than serum DHA concentration is associated with cognitive function in patients with CAD, suggesting that a low serum EPA level is a risk factor for cognitive impairment independent of cardiac function. Our data support a role of dietary n-3 PUFAs in preventing the cognitive decline in CAD patients.

Additional file

Additional file 1: Table S1. Pearson correlation analysis between left ventricular ejection fraction and serum levels of polyunsaturated fatty acids. **Table S2.** Pearson correlation analysis between brain natriuretic peptide levels and serum levels of polyunsaturated fatty acids.

Abbreviations

PUFAs: Polyunsaturated fatty acids; EPA: Eicosapentaenoic acid; DHA: Docosahexaenoic acid; DGLA: Dihomogammalinolenic acid; AA: Arachidonic acid; CAD: Coronary artery disease; MMSE: Mini-mental state examinations; LDL-C: Low-density lipoprotein cholesterol level; HDL-C: High-density lipoprotein cholesterol level.

Competing interests

The authors declare that they have no conflicts of interest to disclose.

Authors' contributions

SY, TH, RU, DF, AT, JH, T Ise, KY, TT, T Iwase, HY, TS, TW, M Shimabukuro, MA, M Sata collected data and SY analyzed the data and wrote the manuscript. KA, MA, M Shimabukuro, and M Sata provided the suggestion for this study. All authors read and approved the final manuscript.

Acknowledgements

We thank the staff of the Hospital Information Center of Tokushima University Hospital for abstracting patients' data from medical records. This work was supported in part by JSPS KAKENHI (Grant Number 25461131 and 25860605) and Actelion Academia Prize 2013.

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Improved oxidative status in major abdominal surgery patients after N-acetyl cystein supplementation

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Abstract

Background: Increased levels of reactive oxygen species during and after surgery may affect inflammatory response, post-operative adhesion molecule formation, and hemodynamic stability. The glutathione redox cycle is an important regulator in oxidative stress and its reduced forms scavenge free radicals. N-acetyl cysteine, a precursor of reduced glutathione, is considered as a potentially therapeutic wide spectrum agent in clinical practice. We therefore examined whether N-acetyl cysteine improves some biochemical parameters in cancer patients undergoing major abdominal surgery.

Methods: Thirty-three patients diagnosed with pancreas, stomach, rectum, colon malignancies, and undergoing major abdominal surgery at Ankara Numune Training and Research Hospital were randomly divided into two groups; control (CON) and N-acetyl cysteine (NAC). The NAC group had 1,200 mg N-acetyl cysteine starting two days before the operation day, in addition to isonitrogenous and isocaloric total parenteral nutrition of 1.2 g/kg protein, 25 kcal/kg, and 60:40 carbohydrate/fat ratio. Blood and urine samples were drawn two days before the operation, on operation day, and on the first, third, and fifth days post-operation.

Results: Plasma malondialdehyde was significantly lower in the NAC group ($P < 0.001$). N-acetyl cysteine treatment did not affect plasma levels of vitamin A, C or E. The NAC group exhibited a higher ratio of reduced glutathione to oxidised glutathione ($P = 0.019$). Urinary nitrate level was also significantly lower in the NAC group ($P = 0.016$).

Conclusion: The study demonstrated the clinical importance of N-acetyl cysteine supplementation on antioxidant parameters in abdominal surgery patients. In these patients N-acetyl cysteine and vitamin administration can be considered as an effective method for improvement of oxidative status.

Keywords: Major abdominal surgery, N-acetyl cystein, Plasma amino acids, Oxidant parameters

Introduction

Free radicals are important compounds that may affect progress in trauma, tissue damage, and chronic degenerative diseases [1]. Under normal physiological conditions these reactive compounds are removed by an organism's antioxidant mechanisms [2]. However, lack of balance between oxidant and antioxidant molecules may trigger harmful effects of free radicals, especially under sub-optimal conditions such as cancer [3]. Therefore, reducing oxidative stress and supporting the antioxidant system are considered as substantial approaches in clinical practice [4,5].

N-acetyl cysteine (NAC) is the N-acetylated form of amino acid L-cysteine and is used in the first step of glutathione (GSH) synthesis, by extracting cysteine from the N-acetylated derivative [6]. Since GSH is the fundamental thiol antioxidant of the human body and NAC provides a rate-limiting cysteine needed for glutathione synthesis, much work to date has investigated the role of NAC as an oxidative stress suppressor in various diseases [7-11]. It was shown that NAC exerts these antioxidant effects through promoting glutathione synthesis [12]. The direct free radical chelating property of NAC has also been examined, although results relating to the reactivity

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of NAC with superoxide anion and hydrogen peroxide have been controversial [13]. In addition to these mechanisms, reduction in leukocyte-endothelium interaction, oxidative burst of neutrophils, anti-inflammatory, and mucolytic actions were all associated with NAC [12-14].

Most of the studies that assess the effectiveness of NAC have focused on cardiac, liver, and abdominal surgery, due to its protective effects against oxidant stress contributed by surgical operations. These studies have demonstrated some beneficial effects of NAC on postoperative atrial fibrillation, postoperative adhesion formation, ischemia/reperfusion injury, and peritoneal fibrinolytic activity [14-17]. However, results have been inconsistent with those of other trials in which the influence of NAC was examined [18,19]. Certainly, there is strong evidence to support the direct effect of NAC on antioxidant capacity of glutathione redox cycle *in vitro* [12]. Hence, it is of interest to investigate the effect of NAC treatment on related biomarkers. For this perspective, the objective of this study was to examine the effect of NAC treatment on oxidant, antioxidant, and plasma amino acid levels in major abdominal surgery patients.

Material and methods

Thirty-three non-smoker oncology patients (18 male and 15 female) undergoing major abdominal surgery at Ankara Numune Training and Research Hospital were included in the study. Patients were diagnosed with pancreas cancer (CON, n = 3; NAC, n = 3), stomach cancer (CON, n = 10; NAC, n = 7), rectum cancer (CON, n = 3; NAC, n = 4), and colon cancer (CON, n = 1; NAC, n = 2). The exclusion criteria were as follows: emergency operation; pregnancy; breast feeding; impaired renal function; preoperative IV feeding; inability to maintain hemodynamic conditions that allowed optimal conventional resuscitation; mean arterial pressure persistently under 70 mm Hg despite inotropic support; hemotocrit values below 30% or receiving blood transfusions; unable to keep a PaO_2 of 80 to 140 mm Hg and CO_2 of 35 to 50 mm Hg or requiring a fractional inspired oxygen concentration (FiO_2) of over 50; severe heart disease; or taking calcium channel antagonists, angiotensin converting enzyme inhibitors, corticosteroids, NAC, or other drugs with antioxidant activity. Patients were randomly divided into two groups: control (CON) and experimental (NAC) group. All patients received isonitrogenous and isocaloric total parenteral nutrition of 1.2 g/kg protein, 25 kcal/kg, and 60:40 carbohydrate/fat ratio. The NAC group was given 1,200 mg of NAC (300 mg in every 6 h) through total parenteral nutrition, starting from 2 days before the operation and lasting until the fifth post-operative day. This dose of NAC supplementation was considered to be clinically relevant in order to avoid pro-oxidant effects. Blood and 24 h urine samples were collected 2 days before operation (baseline), on operation day, and the first, third and fifth post-operation days. The study protocol was approved by the Minister of Health Research Ethics Committee (B100İEG0110011).

Anthropometric measurements

Body weight and body composition of patients were measured by a Bodystat 1500 (Bodystat Douglas Isle of Man, UK). In addition to body composition, mid-upper arm circumference (MUAC) and triceps skinfold thickness (TSFT) were measured. Nutritional Risk Index (NRI) was derived as follows: NRI = (1.519 × serum albumin, g/L) + (41.7 × present/usual body weight). A NRI score of >100 indicates no risk for the patient; 97.5 to 100, mild risk; 83.5 to 97.5, moderate risk; and < 83.5, severe risk.

Blood and urine samples

Pre-operative baseline blood samples were taken after 12 h of starvation. Following two days of NAC supplementation or control treatment, patients were operated. Operation day blood samples were taken 2 h after surgery. Post-operation day 1, 3 and 5 blood samples were taken. Twenty-four hour urine samples were collected on the same days in sterile containers and stored at -20°C. Blood samples were centrifuged and stored at -80°C. Plasma amino acid analyses were performed using a GC amino acid kit (EZ:faast).

Blood indicators of oxidative stress and antioxidants

Reduced (GSH) and oxidized glutathione (GSSG) were determined from whole blood samples and malondialdehyde (MDA) was analyzed from plasma samples using high pressure liquid chromatography [20,21]. Similarly, vitamin C, A, and E were assayed using high pressure liquid chromatography of plasma samples as previously described [22,23].

Nitrate and nitrite analysis

Urine nitrate and nitrite was determined using a spectrophotometer (UNICAM 1500) and read at an absorbance of 538 nm. Glycine–(NaOH) buffer and copper ($CuSO_4$) saturated cadmium were used to reduce nitrate to nitrite [24].

Statistical analysis

All data were analysed using the Statistical Package for Social Sciences (version 16; SPSS, Inc., Chicago, IL, USA). The effect of NAC supplementation on biochemical and antioxidant parameters was examined using ANOVA with factors of treatment and time (i.e., the day that the samples were taken [pre-operation, operation day, and the first, third, fifth days post-operation]). Tukey's post hoc test was performed when the main time effect was significant. Values are expressed as mean values with their

standard errors unless otherwise stated. $P < 0.05$ was considered statistically significant.

Results

Participants

All 33 subjects completed the study successfully. Table 1 shows the characteristics of the two study groups. Age and anthropometric measurements were similar between groups, yet BMI and body composition were different between male and female participants. NRI data indicated that all of the participants were at risk of malnutrition (Table 1). This was a mild risk for female participants of the NAC group, whereas female participants of the control group and all other male participants exhibited a moderate risk of malnutrition. Total and post-operative lengths of stay at hospital were similar between groups (Table 1).

Plasma amino acid levels

NAC treatment (1,200 mg) resulted in significantly increased plasma cystine concentration throughout the study (5.30 ± 2.05 μmol/L, CON versus 14.71 ± 2.08 μmol/L, NAC, $P = 0.002$) (Figure 1A). Although there was a trend towards a rise in plasma cystine concentration during post-operational days in the NAC group, the effect of day was not significantly important in either group. Plasma essential amino acid (EAA) levels was unaffected by NAC treatment at any stage of the study (398.28 ± 42.87 μmol/L, CON versus 433.05 ± 43.38 μmol/L, NAC, $P > 0.05$) (Figure 1B). However, time exhibited a significant effect on plasma EAA levels since there was a significant difference in plasma EAA concentration between the operation day and post-operational fifth day in both groups (286.49 ± 68.13 μmol/L, operation day versus 590.33 ± 69.26 μmol/L, post-operational fifth day, $P = 0.029$). Similarly, branched chain amino acid (BCAA) concentration was not influenced by NAC treatment, although it exhibited a significant difference between the operation day and post-operational fifth day (286.49 ± 68.13 μmol/L, operation day versus 590.33 ± 69.26 μmol/L, post-operational fifth day, $P = 0.046$) (Figure 1C). Plasma aromatic amino acid (AAA) concentration was found to be affected by both treatment ($P < 0.001$) and day ($P = 0.017$) (Figure 1D). While plasma AAA was significantly higher in the NAC group than the control group (87.37 ± 6.04 μmol/L, CON versus 129.77 ± 6.11 μmol/L, NAC, $P < 0.001$); and was also significantly lower on operation day when compared to pre-operational values in both groups (136.71 ± 9.45 μmol/L, pre-operation versus 93.88 ± 9.59 μmol/L, operation day, $P = 0.017$).

Oxidant and antioxidant parameters

Oxidant and antioxidant parameters of the two study groups are shown in Table 2. Plasma MDA exhibited significantly lower levels in the NAC group when compared to controls (0.44 ± 0.01 μmol/L, CON versus 0.36 ± 0.01 μmol/L, NAC, $P < 0.001$). This effect of NAC on plasma MDA was independent of the time of sampling, which suggests that plasma MDA concentration was not influenced by pre-, post-, or day of operation. A similar pattern was observed for urinary nitrate levels, which showed that the NAC group had significantly lower nitrate concentrations (350.73 ± 31.15 μmol/L, CON versus 242.17 ± 31.91 μmol/L, NAC, $P = 0.016$), irrespective of the day. The exception to this was the urinary nitrite level, which was similar between groups (10.83 ± 1.07 μmol/L, CON versus 9.30 ± 1.09 μmol/L, NAC, $P > 0.05$) and was not affected by sample day (Table 2).

NAC supplementation also had remarkable influence on the variables associated with glutathione redox cycle (Figure 2). Although there was a marked decrease in whole blood GSH levels during post-operation, when

Table 1 Characteristics of the two study groups

	Control group		NAC group	
	Male (n = 9)	Female (n = 8)	Male (n = 9)	Female (n = 7)
Age (y)	60.44 ± 4.35	57.38 ± 3.58	56.56 ± 4.05	65.29 ± 5.49
BMI (kg/m^2)[a]	20.05 ± 1.03	25.80 ± 1.45	20.99 ± 1.18	25.11 ± 0.79
Body fat (%)[a]	32.07 ± 2.65	44.44 ± 3.38	27.60 ± 4.05	42.81 ± 3.2
Body water (%)[a]	55.00 ± 3.98	46.90 ± 2.45	59.30 ± 3.47	48.20 ± 2.45
Fat-free mass (kg)[a]	38.93 ± 2.30	33.24 ± 2.82	43.44 ± 3.46	35.96 ± 3.87
Triceps skin fold thickness[a]	6.93 ± 0.86	18.05 ± 1.45	8.27 ± 1.49	18.96 ± 2.07
Mid upper arm circumference (cm)	22.76 ± 0.84	22.80 ± 0.76	23.43 ± 1.22	23.79 ± 1.08
Nutritional risk index	92.70 ± 3.30	95.65 ± 3.68	92.41 ± 5.06	98.97 ± 2.55
Total length of stay (d)	23.11 ± 3.85	27.89 ± 3.85	30.88 ± 4.09	23.57 ± 4.37
Post-operation length of stay (d)	15.89 ± 6.15	19.44 ± 4.11	21.50 ± 5.19	11.29 ± 1.59

Data presented as mean ± SEM.
[a]Significantly different between male and female participants in all groups ($P < 0.001$).

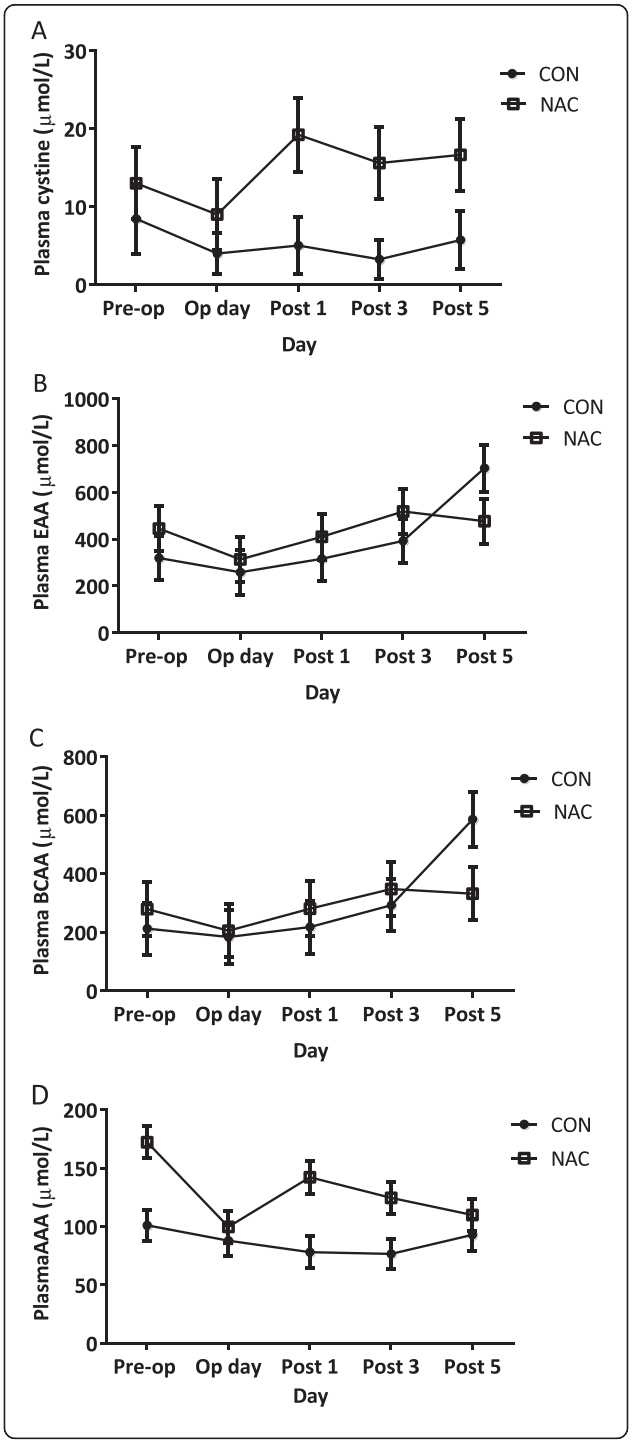

Figure 1 Data presented as mean ± SEM. CON n = 17, NAC n = 16. **A**: Plasma cystine levels. NAC group had significantly higher plasma cystine levels compared to the control group (P = 0.002). No significant effect of day was found on plasma cystine level. **B**: Plasma EAA levels. Tukey's post-hoc test showed a significant difference between operation day and post-operational fifth day on plasma EAA levels in both study groups (P = 0.029). No significant effect of treatment was found on plasma EAA. **C**: Plasma BCAA levels. Tukey's post-hoc test showed a significant difference between operation day and post-operational fifth day on plasma EAA levels in both study groups (P = 0.046). No significant effect of treatment was found on plasma BCAA. **D**: NAC group had significantly higher plasma AAA when compared to control group (P < 0.001). Tukey's post-hoc test showed a significant difference between pre-operation day and operation day on plasma AAA levels in both study groups (P = 0.017).

compared to operation day in both groups (690.78 ± 30.99 μmol/L, operation day versus 563.38 ± 30.53 μmol/L, post-operational third day, P = 0.016), NAC treatment resulted in significantly increased GSH levels when compared to controls (589.58 ± 24.80 μmol/L, CON versus 671.76 ± 25.30 μmol/L, NAC, P = 0.023). Whilst whole blood GSH levels increased in the NAC group, GSSG levels decreased significantly (268.74 ± 17.43 μmol/L, CON versus 206.07 ± 17.78 μmol/L, NAC, P = 0.014) and independently of the day (Figure 2). These effects of NAC treatment on GSH and GSSG levels were reflected in the GSH:GSSG ratio, which was higher in patients receiving NAC treatment (2.92 ± 0.88 μmol/L, CON versus 5.92 ± 0.90 μmol/L, NAC, P = 0.019) (Figure 2).

One of the aims of the present was to assess whether NAC treatment had any impact upon antioxidant vitamins. In this context, plasma vitamin A, C, and E levels were analyzed. Although oxidant parameters were influenced by NAC treatment from the early phase of the study, antioxidant vitamins did not exhibit the same trend (Table 2). Plasma vitamin A levels were significantly higher during the pre-operational period in both groups (1.32 ± 0.07 μmol/L, pre-operational day versus 0.82 ± 0.07 μmol/L, operation day, 0.58 ± 0.07 μmol/L, post-operational first day, 0.64 ± 0.07 μmol/L, post-operational third day, 0.83 ± 0.07 μmol/L, post-operational fifth day, P < 0.001). Interestingly, vitamin C level was found to be significantly lower in the NAC group, irrespective of the study days (2.59 ± 0.10 mg/L, CON versus 2.11 ± 0.10 mg/L, NAC, P = 0.001) and vitamin E levels were similar between all groups (Table 2).

Discussion

This study aimed to examine the influence of NAC supplementation on plasma amino acid levels, as well as antioxidant and oxidant parameters, in cancer patients undergoing major abdominal surgery. The results demonstrated that NAC treatment, starting from 2 days before the operation day and lasting until the post-operational

Table 2 Oxidant and antioxidant parameters

	Group	Day				
		Pre-op	Op day	Post-op 1	Post-op 3	Post-op 5
MDA (μmol/L)*	CON	0.45 ± 0.03	0.45 ± 0.03	0.45 ± 0.03	0.44 ± 0.03	0.43 ± 0.03
	NAC	0.35 ± 0.03	0.35 ± 0.03	0.37 ± 0.03	0.36 ± 0.03	0.36 ± 0.03
Nitrate (μmol/L)†	CON	293.58 ± 69.22	287.16 ± 69.22	262.63 ± 69.22	381.18 ± 69.22	529.09 ± 71.35
	NAC	197.44 ± 71.35	211.11 ± 71.35	285.81 ± 71.3	242.49 ± 71.35	274.01 ± 71.35
Nitrite (μmol/L)	CON	8.61 ± 2.38	11.28 ± 2.37	9.85 ± 2.37	14.96 ± 2.37	9.45 ± 2.45
	NAC	9.39 ± 2.45	8.13 ± 2.45	10.91 ± 2.45	9.83 ± 2.45	8.24 ± 2.45
Vitamin A (μmol/L)	CON	1.22 ± 0.09[a]	0.76 ± 0.09[b]	0.51 ± 0.09[b]	0.64 ± 0.09[b]	0.88 ± 0.09[b]
	NAC	1.43 ± 0.09[a]	0.89 ± 0.10[b]	0.64 ± 0.09[b]	0.64 ± 0.09[b]	0.77 ± 0.10[b]
Vitamin C (mg/L)‡	CON	2.66 ± 0.22	2.68 ± 0.23	2.65 ± 0.22	2.48 ± 0.22	2.49 ± 0.24
	NAC	2.29 ± 0.23	2.26 ± 0.23	2.07 ± 0.23	1.99 ± 0.23	1.96 ± 0.23
Vitamin E (μmol/L)	CON	29.24 ± 1.65	20.54 ± 1.65	16.04 ± 1.65	18.97 ± 1.65	21.22 ± 1.76
	NAC	34.86 ± 1.70	21.03 ± 1.70	16.30 ± 1.70	18.20 ± 1.70	20.09 ± 1.70

Data presented as mean ± SEM.
*Plasma malondialdehyde level was influenced by NAC treatment ($P < 0.001$).
†Urinary nitrate level was influenced by NAC treatment ($P = 0.016$).
‡Plasma vitamin C level was influenced by NAC treatment ($P = 0.001$).
[a,b]Mean values with unlike superscript letters in the same row were significantly different ($P < 0 \cdot 05$).

fifth day, influence some important markers of oxidative stress in these patients. MDA, which is a crucial indicator of lipid peroxidation, was 18.18% lower in the NAC treated group when compared to controls. Similarly, nitrate levels, which can be considered as an oxidative stress biomarker in urine, decreased 30.95% in the NAC group. Given that intracellular GSSG accumulates and the GSH: GSSG ratio decreases under increased levels of oxidative stress, increased GSH:GSSG ratio implies an improved cellular oxidative capacity [6]. Here we found that the GSH:GSSG ratio was 50.68% greater in the NAC group than controls.

Surgery initiates a wide spectrum of suboptimal alterations in body homeostasis and is closely associated with several complications of surgical stress, of which exposure to increased oxidative stress is considered as an important component [25]. From this perspective, improved antioxidant mechanisms may help to combat oxidative stress. Although the current study demonstrated diminished levels of oxidant stress factors, antioxidant factors such as vitamin A, C, and E did not exhibit improvement. In fact, vitamin C level was found to be lower in the NAC group. To date, only a few studies have examined the effects of NAC and antioxidant vitamins on disease and reported beneficial effects of these combined therapies [26,27], however, the principal reason behind the lower or unaltered levels of these vitamins in the current study remains unknown. A recent study has shown that NAC administration during high-intensity exercise did not change blood glutathione levels but diminished lipid peroxidation [28], and there is also evidence regarding the altered pre-operative and post-operative total oxidative stress and total antioxidant capacities following NAC treatment [29]. Therefore total oxidative stress and total antioxidant capacities should be measured in future studies to elucidate the antioxidant mechanisms that are associated with NAC action. In addition, cancer patients tend to have lower circulating concentrations of antioxidant vitamins, either due to altered nutritional status, increased catabolic processes, or inflammatory response [30]. Indeed, the current study revealed that all plasma antioxidant vitamin levels were lower in the NAC group than in healthy individuals. To further investigate the clinical relevance of this finding, future studies should focus on antioxidant vitamin supplementation together with NAC, and seek the oxidant status of these patients.

NRI is a useful tool for the early identification of nutritional depletion in hospitalised patients [31]. It was considered that determining the nutritional status of patients before surgery was needed to address the increased risk of malnutrition related disorders, and that NRI data reflected a mild risk of malnutrition in the current study. This situation emphasizes the importance of pre-operative care strategies in order to combat malnutrition and post-operational morbidity and mortality, since these factors were shown to be related [32]. Since hospital malnutrition is one of the most important challenges in clinical practice, novel strategies should be developed to avoid the progress of malnutrition in cancer patients undergoing surgery. A study which used NAC infusion at a rate of 0.3 mg/kg/min intravenously during surgery and 0.2 mg/kg/min for 24 h during post-operation showed a significantly shorter period in the intensive

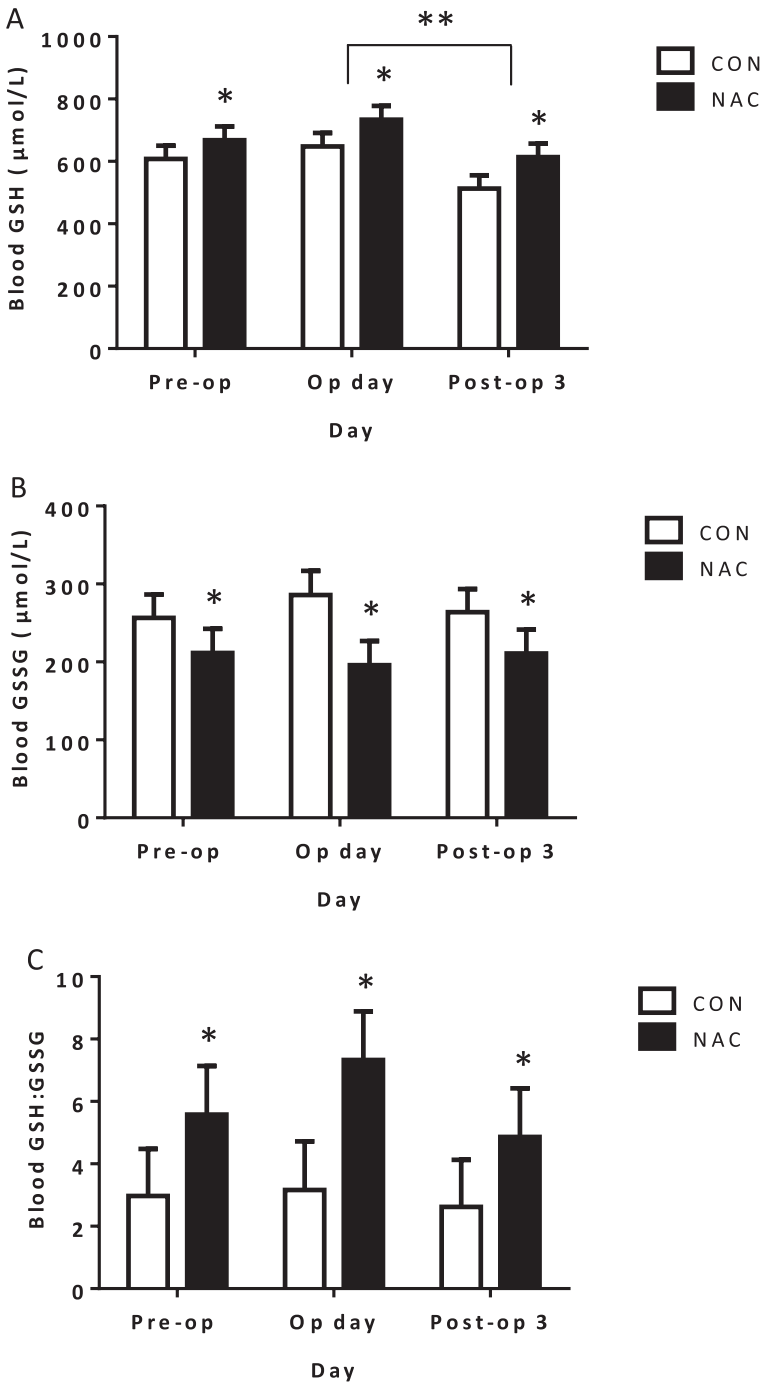

Figure 2 Data presented as mean ± SEM. CON n = 17, NAC n = 16. **A**: Blood reduced glutathione (GSH) levels. *Indicates the significant effect of NAC treatment when compared to control group (P = 0.023). **Indicates the significant effect of day. Tukey's post-hoc test showed a significant difference between operation day and post-operational third day on blood GSH levels in both study groups (P = 0.016). **B**: Blood oxidised glutathione (GSSG) levels. *Indicates the significant effect of NAC treatment when compared to control group (P = 0.014). No significant effect of day was found on GSSG levels. **C**: *Indicates the significant effect of NAC treatment when compared to control group (P = 0.019). No significant effect of day was found on GSH/GSSG ratio.

care unit length of stay [33]. Whereas a prophylactic high dose oral of NAC was reported to be ineffective on post-operative hospital stay after heart surgery [18]. The current study also did not exhibit a significant effect on length of stay in hospital. In fact, despite a non-significant outcome, post- or total length of stay

data at hospital appeared to be increased in males. The reason of this outcome can be attributable to specific gender differences rather than an experimental effect, since some studies indicated that gender may play a deterministic role in duration of length of stay in hospital [34,35]. Future studies should examine whether these differences can be attributable to specific responses to the treatment, preventable complications, or bias. In addition, the effect of NAC supplementation on hospital care should be considered in future studies, since NAC may exert a protective effect through shortening the length of stay, and lowering malnutrition rate and morbidity in the post-operative period.

The therapeutic feature of antioxidants relies on their capacity to cross the cell membranes [12]. When NAC is administered through oral or intravenous routes, it undergoes N-deacetylation [12]. There is some conflicting data about the mechanisms of the antioxidant action of NAC as it has not yet been clearly shown whether the effectiveness of NAC should be attributed to its direct involvement in synthesis of intracellular GSH after N-deacetylation, its ability to reduce extracellular cystine to cysteine, or its direct chelating property to free radicals [13]. The current study demonstrated a profound increase in GSH levels in the NAC treated group, as in other studies [13,36]. These data clearly indicate the impact of NAC supplementation on GSH synthesis. However, the effect of NAC supplementation on cystine to cysteine reduction is not clear since the cystine data introduced in this study did not distinguish the reduced cysteine and cystine. It is known that more than 90% of the total soluble cysteine in plasma is in the oxidized cystine form [37]. In another study, in which the kinetics of uptake and deacetylation of NAC in erythrocytes was investigated, NAC was found to replace a cysteine with a cystine molecule and enhance plasma free cysteine levels [38]. The current study exhibited a significant increase in cystine levels following NAC supplementation, especially after the operation day. Since the cysteine levels were undetected, the interpretation of this outcome becomes complicated. However, when the oxidative milieu of blood and short half-life of NAC is taken into account, rapid oxidation of cysteine to cystine should also be considered [39]. However, it should also be considered that the amount of available intracellular cysteine is the limiting factor in GSH synthesis. Therefore, our finding of increased GSH in the NAC treated group may suggest that the amount of NAC taken was sufficient to induce GSH synthesis in these patients. Moreover, continuous infusion of NAC through total parenteral nutrition may help to reduce the risk of oxidative injury. Further research should focus on the interactions between NAC and related biomarkers to reinforce current knowledge about the mechanisms of NAC.

In disease and trauma, adequacy of specific amino acids becomes an important issue. It is well-known that requirements of the amino acids may change under traumatic conditions [40]. Beyond their common functions, specific amino acid supplementation in total parenteral nutrition was shown to induce beneficial effects in operative cancer patients [41,42]. Therefore, one of the aims of the current was to determine plasma amino acid levels along with NAC supplementation. Despite the clear effect of NAC on plasma cysteine, as discussed above, no major effect of NAC was found on other plasma amino acid levels. In fact, operation day appeared to have a greater impact on plasma amino acid levels. A recent study that investigated the changes in plasma BCAA and glutamine concentrations in operative gastrointestinal cancer patients indicated that the fall in BCAA levels were partially prevented by total parenteral nutrition [43]. Our results did not exhibit such an effect on BCAA, whereas AAA appeared to remain at higher levels during the treatment. Further studies investigating the influence of total parenteral nutrition on plasma protein and amino acid levels in surgery patients should be performed.

N-acetyl cysteine supplementation was associated with pro-oxidant effects in experimental models, which caused concerns about its application in clinical practice when there is a lack of significant oxidative stress factor [12]. Certainly, the amount of NAC used is the determinative factor for the pro-oxidant effects. In the current study, NAC was infused at a rate of 300 mg in every 6 h (i.e., 1,200 mg/day). This amount is a low to intermediate level when compared to other studies in the literature [13,18]. In another study, even a low dose of NAC supplementation, which was 2 to 4 mmol/l in cardioplegia solution, significantly reduced the MDA levels [44]. In a study in which the pro-oxidant and deleterious effects of NAC were shown in healthy individuals, NAC was used up to 2,400 mg over longer periods [45]. Therefore, using 1,200 mg of NAC/day provided a safe and clinically relevant dose which could be considered in further studies and applications.

This study examined the hypotheses that NAC may improve oxidant and antioxidant parameters in cancer patients undergoing major abdominal surgery, and the data generated supports the assertion that NAC may provide beneficial effects in these patients. Despite these improved parameters, it should be taken into account that the present study has several limitations. One of the most important limitations is the lack of follow-up of patients. Monitoring the prognosis of patients and other physical outcomes would provide a complete understanding of the clinical consequences. Therefore, future studies should acknowledge the long-term and solid effects of NAC supplementation on prognosis, recovery, and survival rates of the patients in addition to the length of

hospitalization. Secondly, large-scale population studies are needed to generalise the beneficial effects of NAC on surgery patients. A recent systematic review that aimed to analyse the effectiveness of pharmacological modulation of oxidative stress in surgery related interventions of animal models, reported that NAC was effective at reducing oxidative stress markers [46]. Although the exact mechanisms of this action remains to be understood completely, these results indicate the clinical relevance of translating this approach to human studies. Consequently, supporting oxidative defence mechanisms in surgery patients should be evaluated within the limits of ordinary clinical practice, yet these practices may help to improve normal body homeostasis in these patients.

Competing interest
The authors declare that they have no competing interests to this study.

Authors' contribution
AK conducted the study, carried out laboratory analysis and collected data. AA examined data, performed statistical analysis and drafted manuscript. ZB carried out laboratory analysis and examined data. MMO provided clinical supervision and involved in assessment of patient inclusion. HTB designed the research, interpreted data, and wrote the manuscript. All authors read and approved the final manuscript.

Acknowledgement
The study was funded by Hacettepe University, Project number: 0102402004.

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Tea consumption is inversely related to 5-year blood pressure change among adults in Jiangsu, China: a cross-sectional study

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Abstract

Background: Data relating to the association between tea consumption and blood pressure change are inconsistent. The aim of this analysis was to investigate the association between tea consumption and the change in blood pressure (BP) in Chinese adults over a 5-year period.

Methods: Data from 1109 Chinese men (N= 472) and women (N= 637) who participated in the Jiangsu Nutrition Study (JIN) were analysed. BP was measured in 2002 and 2007. Tea (green, black and total tea) consumption was quantitatively assessed at the follow-up survey in 2007.

Results: Total tea and green tea consumption were inversely associated with 5-year diastolic BP (DBP) but not systolic BP (SBP) change. In the multivariable analysis, compared with no consumption of tea, those with daily total tea/green tea consumption of at least10 g had 2.41 mmHg and 3.68 mmHg smaller increase of DBP respectively. There was a significant interaction between smoking and total tea/green tea consumption and DBP change. The inverse association between total tea/green tea consumption and DBP change was significant only in non-smokers. Green tea consumption was inversely associated with SBP change only in non-smokers and those without central obesity.

Conclusion: The consumption of green tea is inversely associated with 5-year BP change among Chinese adults, an effect abrogated by smoking.

Keywords: Blood pressure change, Tea consumption, Epidemiology, Nutrition, Population study

Background

On average, worldwide, approximately 40% of adults aged 25 and above have hypertension [1]. In China the prevalence of hypertension in adults increased from 27.2% in 2002 to 33.5% in 2010 [2], and was comparable in urban and rural areas (34.7% vs 32.9%) [3]. Despite the well-established associations of hypertension with cardiovascular and renal disease [4-7], only about 19% of those with hypertension had adequate treatment [2]. Lifestyle factors including smoking, high salt intake, energy dense, low fibre, low fruit and vegetable diets are known risk factors of hypertension [8-11].

Tea is one of the most commonly consumed beverages worldwide and has a long history of use that originated about 5000 years ago in China. Tea contains a variety of antioxidants and other chemicals (e.g. flavonoids, caffeine, theanine, theaflavins, theophylline, phenolic acids and polyphenols) that have anti-mutagenic, anti-diabetic and anti-inflammatory effects [12-17]. An inverse association between tea consumption and blood pressure (BP) has been reported in cross-sectional epidemiological studies [13,18,19]. Experimental interventions in animal and humans suggest beneficial effects of tea on BP [14-17,19-23]. Conversely, some short-term trials in humans have shown a positive association between tea and BP [24-26]. Others have shown no effects [26-28]. A systematic review on five randomized clinical trials concluded that there was no effect of tea consumption on BP [12]. There is no longitudinal study on the association between regular tea consumption and

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BP, and the interactions between tea consumption and other lifestyle factors have not been assessed.

The objective of the study was to assess the association between tea consumption and 5-year BP changes, and the interaction between tea consumption and lifestyle factors in relation to BP changes among Chinese adults aged 20 years and above, based on a large population study in China: The Jiangsu Nutrition Study (JIN).

Methods

Study population

The JIN cohort study comprises men and women aged 20 years or older and the methods of sampling have been described previously [29-31]. In 2002, BP was measured in, and dietary information obtained from, 2849 participants living in two cities and six rural areas. In 2007, 1682 of the original participants were identified through household visits: of these 1492 agreed to a follow-up interview at home, with 1282 (76.2%) participants attending follow-up clinics. For the current analysis, we excluded those participants who had extreme values of weight change of more than 20 kg and those who had known diabetes, stroke or cancer at baseline (n= 40). In addition, 133 participants did not have information on tea consumption in 2007. The final sample size in this study consisted of 472 men and 637 women (total n= 1109) (Figure 1). Compared with the retained participants (n= 1682), those lost to follow-up (n= 1167) were generally younger, with a higher BMI, waist circumference and lower systolic BP (SBP), but there were no differences in energy intake, Diastolic BP (DBP) or gender (Additional file 1: Table S1). The study was conducted according to the guidelines laid down in the Declaration of Helsinki and the Jiangsu Provincial Centre for Disease Control and Prevention approved all procedures. Written informed consent was obtained from all participants.

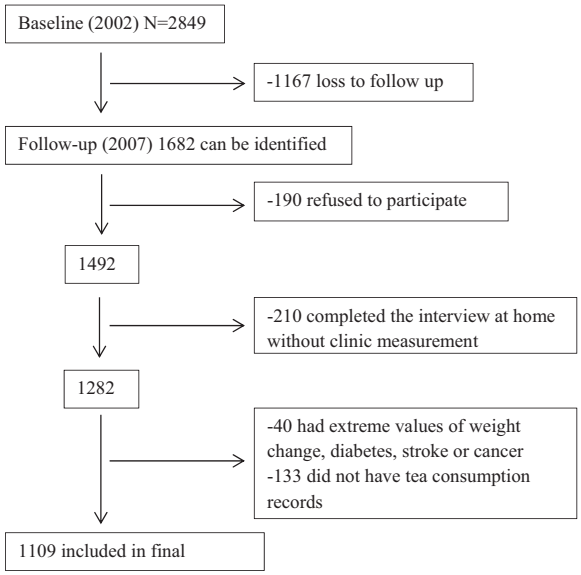

Figure 1 Sample description.

Data collection and measurements

Participants were interviewed at their homes by trained health workers using a standard questionnaire [29].

Exposure variables-tea consumption in 2007

The usual weekly green tea and black tea consumption was assessed by the question "How much tea do you drink each week? 1) green tea__*liang* 2) black tea__*liang*". "*Lang*" is a Chinese unit corresponding to 50 g. Total tea consumption was the sum of green and black tea in grams per day.

Outcome variables-change in BP between 2007 and 2002

After 5 minutes seated, BP was measured twice by mercury sphygmomanometer on the right upper arm at both baseline and follow-up. The mean of the two measurements was used in the analyses. The cuff size was selected on the basis of the upper arm circumference to ensure that the cuff did not overlap [2]. Hypertension was defined as SBP above 140 mmHg and/or DBP above 90 mmHg, or use of antihypertensive medications.

Dietary intake

In 2002, dietary intake patterns during the previous year were determined by a series of detailed questions about the usual frequency and quantity of intake of 33 food groups and beverages. The food frequency questionnaire (FFQ) has been validated [32,33] and reported to be a useful method for the collection of individual food consumption information in face-to-face interviews, but not in self-administered surveys due to the current level of education of the majority of the Chinese population. We assessed the intake of specific nutrients using a 3-day weighed food diary, which recorded all foods consumed by each individual on three consecutive days including the weekend. We did not consider under- and over-reporting of energy intake to be an issue because upon reviewing the food diaries with the participants the health workers would clarify any intake value for particular foods that fell below or above the usual value reportedly consumed by the population within the region. Food consumption data were analysed using the Chinese Food Composition Table [34].

Other lifestyle factors

These were assessed in both 2002 and 2007 by questionnaire which asked about cigarette smoking current, past smoking and passive; eating out frequently (coded as yes or no); the frequency and amount of alcohol consumed.

Table 1 Sample characteristic (in 2002) according to tea consumption (in 2007) among Chinese adults[a] (N= 1109)

	Total tea				Green tea				Black tea			
	0 g/day (N= 846)	<10 g/day (7.1 g/day[b], N= 148)	>10 g/day (14.3 g/day, N= 115)	P	0 g/day (N= 900)	<10 g/day (7.1 g/day, N= 139)	>10 g/day (14.3 g/day, N= 70)	P	0 g/day (N= 1018)	<10 g/day (7.1 g/day, N= 51)	>10 g/day (14.3 g/day, N= 40)	P
Age	48.9	50.2	50.0	0.37	48.9	50.8	49.7	0.11	49.1	50.4	49.8	0.74
(years)	(0.5)	(1.1)	(1.1)		(0.4)	(1.1)	(1.4)		(0.4)	(1.8)	(2.1)	
Men (%)	32.86	69.59	79.13	<0.01	35.2	71.2	80.0	<0.01	39.8	68.6	80.0	<0.01
Urban (%)	11.8	13.5	17.4	0.23	11.3	18.7	17.1	0.03	12.77	15.69	5.00	0.28
Low education (%)	55.2	50.7	43.5	0.01	55.0	48.9	41.4	<0.01	53.9	49.0	45.0	0.16
Manual job (%)	55.6	45.9	40.0	<0.01	54.8	43.2	44.3	0.01	53.6	47.1	35.0	0.05
No active commuting (%)	39.5	43.9	45.2	0.06	40.2	43.9	40.0	0.06	40.1	43.1	52.5	0.58
No leisure time physical activity (%)	92.8	87.8	85.2	0.03	92.7	85.6	85.7	0.03	91.9	84.3	87.5	0.03
Sleeping < 7 h/day	11.8	12.4	15.9	<0.01	12.2	11.8	14.7	0.02	12.0	13.7	17.5	0.09
Sedentary activity < 1 h/day	18.8	6.8	5.2	<0.01	18.2	6.5	2.9	<0.01	16.5	3.9	12.5	<0.01
Smoker (%)	19.9	51.4	58.3	<0.01	22.1	48.9	62.9	<0.01	25.7	51.0	57.5	<0.01
Alcohol drinker (%)	20.9	40.8	40.9	<0.01	22.7	36.2	42.9	<0.01	24.4	37.3	42.5	<0.01
Weight	59.6	60.6	60.7	0.37	59.5	61.4	61.5 (1.2)	0.04	59.97	58.53	58.48	0.38
(kg)[c]	(0.3)	(0.9)	(0.9)		(0.3)	(0.8)			(0.3)	(1.3)	(1.5)	
Waist circumference	78.5	79.5	79.8	0.26	78.4	80.3	80.3	0.05	78.86	77.59	78.24	0.60
(cm)[c]	(0.3)	(0.8)	(0.9)		(0.3)	(0.8)	(1.1)		(0.3)	(1.3)	(1.5)	
Central obesity (%)[e]	31.7	27.7	22.6	0.10	30.6	30.2	25.7	0.69	31.5	17.7	15.0	0.01
Obesity (%) (BMI ≥ 28 kg/m^2)	10.3	8.1	7.8	0.43	10.0	7.9	10.0	0.21	10.3	2.0	5.0	0.21
BMI	23.3	23.7	23.8	0.30	23.3	24.0	24.0	0.04	23.4	22.8	23.4	0.36
(kg/m^2)[c]	(0.1)	(0.3)	(0.3)		(0.1)	(0.3)	(0.4)		(0.1)	(0.5)	(0.5)	
Hypertension (%)	29.7	37.8	33.9	0.11	29.7	37.4	38.6	0.07	31.3	37.3	20.0	0.20
SBP	126.7	126.5	126.2	0.97	126.5	127.0	127.2	0.93	126.8	125.6	122.0	0.28
(mmHg)[c]	(0.7)	(1.6)	(1.8)		(0.6)	(1.6)	(2.3)		(0.6)	(2.7)	(3.0)	
DBP	79.6	80.3	81.0	0.47	79.5	80.9	82.5	0.06	80.0	81.2	75.6	0.04
(mmHg)[c]	(0.4)	(0.9)	(1.1)		(0.4)	(1.0)	(1.4)		(0.4)	(1.6)	(1.8)	
Energy	2376.5	2260.8	2288.2	0.07	2363.5	2286.3	2333.5	0.40	2361.6	2222.9	2272.3	0.21
(Kcal/day)[c]	(21.4)	(51.6)	(58.8)		(20.6)	(53.3)	(74.6)		(19.2)	(86.2)	(97.7)	
Fat	80.1	87.5	87.4	<0.01	80.8	86.5	86.2	0.02	81.0	95.1	84.6	<0.01
(g/day)[d]	(0.9)	(2.1)	(2.4)		(0.8)	(2.2)	(3.1)		(0.8)	(3.5)	(4.0)	

Table 1 Sample characteristic (in 2002) according to tea consumption (in 2007) among Chinese adults[a] (N= 1109) *(Continued)*

Protein	72.0	72.4	76.9	<0.01	72.1	74.0	75.6	0.09	72.3	71.8	79.9	<0.01
(g/day)[d]	(0.5)	(1.2)	(1.4)		(0.5)	(1.2)	(1.7)		(0.4)	(2.0)	(2.3)	
Carbohydrate	324.6	307.2	306.0	<0.01	321.8	314.8	312.3	0.27	322.9 (1.9)	283.4	302.4	<0.01
(g/day)[d]	(2.1)	(5.2)	(5.9)		(2.1)	(5.4)	(7.5)			(8.6)	(9.7)	
Fibre	12.6	9.6	9.5	<0.01	12.5	9.8	8.7 (1.1)	<0.01	12.0	10.5	10.5	0.30
(g/day)[d]	(0.3)	(0.7)	(0.8)		(0.3)	(0.8)			(0.3)	(1.3)	(1.4)	
Sodium	6.7	6.9	6.8	0.89	6.8	6.7	6.8	0.99	6.7	7.3	6.8	0.66
(g/day)[d]	(0.1)	(0.4)	(0.4)		(0.1)	(0.4)	(0.5)		(0.1)	(0.6)	(0.7)	

[a]Mean(SE), nutrients and alcohol intake were calculated from weighted food records, other food intakes were calculated from food frequency questionnaire.
[b]Median tea consumption. [c]Adjusted for age and sex. [d]Adjusted for age and sex and energy intake. [e]Based on IDF definition for Chinese population.

Questions on daily commuting were grouped into three categories: (1) motorized transportation, or 0 min of walking or cycling; (2) walking or cycling for 1–29 min; (3) walking or cycling for ≥30 min. Daily leisure time physical activity was grouped into three categories: 0, 1–29 and ≥30 min. Daily sleeping was grouped into three categories: <7, 7–8 and ≥9 hours. Daily time spent on sedentary activities (viewing television, operating a computer, playing video games and reading during leisure time) was classified into four categories: <1, 1–1.9, 2–2.9 and ≥3 hours. Education was recoded into 'Low' (illiteracy, primary school), 'Medium' (junior middle school) or 'High' (high middle school or higher), based on six categories of education levels in the questionnaire. Occupation was recoded into 'Manual' or 'Non-manual' based on a question with twelve occupational categories.

Anthropometric measurements

In both 2002 and 2007, anthropometric measurements were obtained using standard protocols and techniques. Body weight was measured in light indoor clothing without shoes to the nearest 100 g. Height was measured without shoes to the nearest millimetre using a stadiometer. Waist circumference was measured to the nearest millimetre midway between the inferior margin of the last rib and the crest of the ilium, in the mid-axillary line in a horizontal plane. Family history of hypertension was defined as the presence of known family members

Table 2 Linear regression β coefficients (95% confidence interval) for categories of total tea, green tea and black tea consumption predicting 5-year change in blood pressure in 1109 adults participating in the Jiangsu Nutrition Study

	<10 g/day[a] (7.1 g/day)[b], β(95% CI)	>10 g/day[a] (14.3 g/day), β(95% CI)	P for trend
Total tea			
SBP			
Model 1	-1.10(-4.56 to 2.36)	-2.41(-6.30 to 1.48)	0.20
Model 2	-0.29(-3.78 to 3.20)	-2.03(-5.97 to 1.91)	0.35
Model 3	0.31(-3.45 to 3.51)	-1.76(-5.70 to 2.19)	0.45
DBP			
Model 1	-1.92(-3.93 to 0.09)	-2.79(-5.05to -0.53)	<0.01
Model 2	-1.44(-3.47 to 0.60)	-2.64(-4.94 to -0.34)	0.02
Model 3	-1.30(-3.33 to 0.73)	-2.41(-4.71 to -0.11)	0.028
Green tea			
SBP			
Model 1	-1.17(-4.69 to 2.35)	-3.96(-8.73 to 0.81)	0.10
Model 2	-0.45(-4.01 to 3.11)	-3.28(-8.08 to 1.52)	0.23
Model 3	-0.14(-3.40 to 3.77)	-2.71(-7.50 to 2.08)	0.35
DBP			
Model 1	-2.37(-4.41 to -0.33)	-4.25(-7.02 to -1.49)	<0.01
Model 2	-1.87(-3.94 to 0.21)	-3.96(-6.76 to -1.16)	<0.01
Model 3	-1.67(-3.74 to 0.40)	-3.68(-6.47 to -0.89)	<0.01
Black tea			
SBP			
Model 1	-2.99(-8.41 to 2.44)	2.41(-3.70 to 8.51)	0.85
Model 2	-2.34(-7.79 to 3.10)	1.82(-4.26 to 7.90)	0.89
Model 3	-2.46(-7.90 to 2.98)	1.40(-4.66 to 7.46)	0.99
DBP			
Model 1	-3.42(-6.56 to -0.27)	3.69(0.15 to 7.24)	0.43
Model 2	-2.82(-5.99 to 0.35)	3.18(-0.37 to 6.72)	0.46
Model 3	-2.71(-5.88 to 0.46)	2.99(-0.54 to 6.52)	0.49

Values are β, 95% confidence interval (CI) from multilevel mixed-effects linear regression model adjusting for household clusters. Model 1 adjusted for age and sex. Model 2: model 1 + smoking (0, 1-19, ≥ 20 cigarettes/day), alcohol drinking (g/day), active commuting (no, 1-29 min/day, ≥30 min/day), leisure time physical activity (no, 1-29 min/day, ≥30 min/day), sleeping (<7 h/day, 7-8 h/day, ≥9 h/day), sedentary activity (<1 h/day,1-1.9 h/day, 2-2.9 h/day, ≥3 h/day), education (low, medium, high) and occupation (manual/non-manual), overweight (BMI > 24 kg/m^2, yes/no), BMI change (continuous), central obesity (defined as waist circumference: men ≥ 90 cm, women ≥ 80 cm), eating out, passive smoking, family history of hypertension. Model 3: model 2 + energy and sodium, fibre, potassium intake, fruit, vegetable, high blood pressure medication (baseline, follow-up), salt and fat intake. CI, confidence interval. [a]Referent category is non-tea drinkers. [b]Median tea consumption.

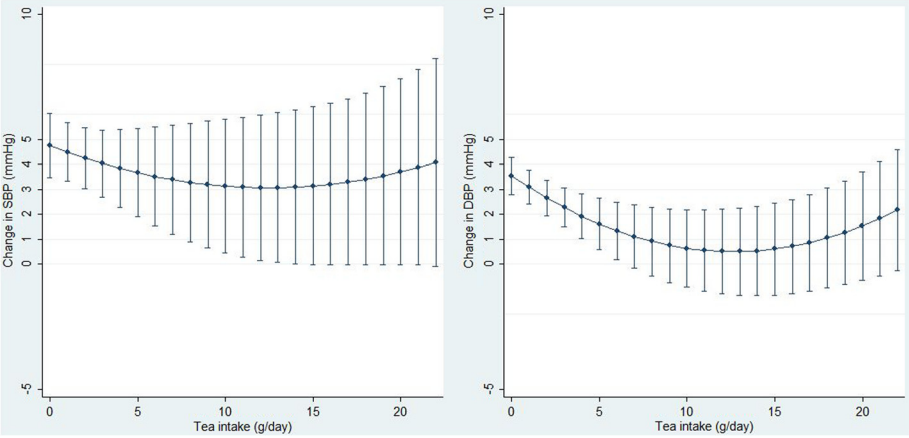

Figure 2 Predicted association between tea consumption and blood pressure changes over 5 years among Chinese adults[a]. Command *marginsplot* was used to generate the graph. Tea intake was treated as continuous variables. 11 participants with tea consumption more than 22 g/day were excluded. [a]Models adjusted for variables in model 3 of Table 2.

with hypertension in any of three generations (siblings, parents, or grandparents).

Statistics

Total tea, green tea and black tea consumption were recoded into three categories: 0, ≤10, >10 g/day. The χ^2-test was used to compare differences between categorical variables and ANOVA was used to compare differences in continuous variables between groups. Mixed-effects linear regression was used to determine the association between different types of tea consumption and BP change. In the full model we adjusted for age, sex, education, occupation, active commuting, leisure time physical activity, sedentary activity, smoking, passive smoking, alcohol drinking, overweight (yes/no) at the baseline, change in BMI, central obesity (yes/no), eating out, family history of hypertension, hypertension medication, energy, sodium, fibre, potassium, fat, fruit, vegetable and salt intake. These multivariable models were adjusted for household cluster, incorporated as random effects in these models. We tested for linear trends across the categories of tea consumed by assigning each participant the median value of the category and modelling this value as a continuous variable. After adjusting for the covariates described in the full model above, we graphically examined the relationship between tea consumption (continuous, g/day) and BP change. Both linear and quadratic terms of tea consumption were put in the model to allow for non-linear associations. All the analyses were performed using STATA 12 (Stata Corporation, College Station, Texas, USA). A two-sided P value less than 0.05 was considered to be statistically significant.

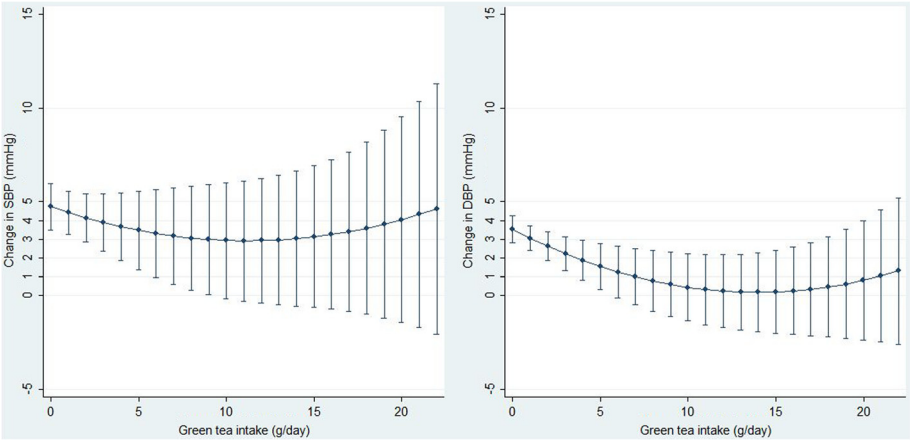

Figure 3 Predicted association between green tea consumption and blood pressure changes over 5 years among Chinese adults[a]. Command *marginsplot* was used to generate the graph. Green tea intake was treated as continuous variables. 11 participants with tea consumption more than 22 g/day were excluded. [a]Models adjusted for variables in model 3 of Table 2.

Table 3 Stratified regression coefficients (95% confidence interval) for SBP change according to the total tea, green tea and black tea consumption categories (β coefficients and 95% confidence intervals) among Chinese adults (n= 1109)[a]

		Categories of tea consumption			
	N	<10 g/day[b] (7.1 g/day)[c]	>10 g/day[b] (14.3 g/day)	P for trend	P for interaction
Total tea					
Central obesity					
Yes	334	0.20(-7.17 to 7.57)	0.68(-8.51 to 9.87)	0.89	0.20
No	768	-0.35(-4.18 to 3.47)	-2.81(-7.04 to 1.43)	0.23	
BMI					
>24 kg/m^2	443	0.56(-5.41 to 6.52)	0.84(-5.93 to 7.61)	0.78	0.21
<24 kg/m^2	659	0.19(-3.92 to 4.29)	-2.97(-7.74 to 1.79)	0.30	
Sex					
Male	469	-0.24(-4.55 to 4.06)	-1.29(-5.82 to 3.24)	0.60	0.79
Female	633	1.22(-4.67 to 7.1)	0.32(-7.7 to 8.34)	0.79	
Smoking					
Yes	310	0.54(-4.41 to 5.48)	0.97(-4.14 to 6.07)	0.71	0.31
No	792	0.66(-4.11 to 5.43)	-5.26(-11.12 to 0.6)	0.17	
Drinking					
Yes	281	0.91(-4.81 to 6.63)	-0.73(-7.05 to 5.59)	0.91	0.54
No	805	1.19(-3.41 to 5.78)	-3.51(-8.66 to 1.64)	0.33	
Green tea					
Central obesity					
Yes	334	0.69(-6.47 to 7.85)	1.39(-9.59 to 12.37)	0.77	0.11
No	768	-0.53(-4.51 to 3.46)	-5.57(-10.75 to -0.39)	0.07	
BMI					
>24 kg/m^2	443	-0.03(-5.95 to 5.89)	0.37(-7.75 to 8.50)	0.95	0.52
<24 kg/m^2	659	1.03(-3.28 to 5.34)	-4.13(-9.97 to 1.70)	0.37	
Sex					
Male	469	-0.36(-4.63 to 3.91)	-2.83(-8.11 to 2.45)	0.34	0.90
Female	633	0.78(-5.44 to 6.99)	-1.84(-12.37 to 8.68)	0.92	
Smoking					
Yes	310	1.19(-3.76 to 6.13)	0.43(-5.33 to 6.2)	0.78	0.052
No	792	-0.5(-5.35 to 4.35)	-10.06(-17.75 to -2.36)	0.038	
Drinking					
Yes	281	-1.06(-7.07 to 4.95)	-3.06(-10.23 to 4.12)	0.40	0.67
No	805	1.09(-3.47 to 5.66)	-4.7(-11.1 to 1.7)	0.36	
Black tea					
Central obesity					
Yes	334	-11.39(-25.64 to 2.87)	6.25(-11.07 to 23.58)	0.93	0.76
No	768	-0.23(-5.85 to 5.39)	1.06(-5.14 to 7.26)	0.80	
BMI					
>24 kg/m^2	443	-0.93(-11.69 to 9.83)	1.75(-9.06 to 12.56)	0.83	0.33
<24 kg/m^2	659	-2.04(-7.95 to 3.87)	1.55(-5.51 to 8.6)	0.96	

Table 3 Stratified regression coefficients (95% confidence interval) for SBP change according to the total tea, green tea and black tea consumption categories (β coefficients and 95% confidence intervals) among Chinese adults (n= 1109)[a] *(Continued)*

Sex					
Male	469	-2.92(-9.22 to 3.39)	1.63(-5.01 to 8.27)	0.95	0.15
Female	633	2.72(-7.23 to 12.67)	8.01(-5.32 to 21.34)	0.21	
Smoking					
Yes	310	-3.88(-10.87 to 3.11)	2.49(-4.9 to 9.88)	0.88	0.39
No	792	0.42(-7.48 to 8.32)	2.25(-7.17 to 11.66)	0.65	
Drinking					
Yes	281	3.76(-4.98 to 12.49)	3.43(-5.98 to 12.85)	0.33	0.67
No	805	-3.93(-11.24 to 3.39)	0.43(-7.5 to 8.36)	0.72	

CI, confidence interval. [a]Models adjusted for variables in model3 of Table 2. Stratifying variables are not adjusted for in corresponding models. [b]Referent category is non-tea drinkers. [c]Median tea consumption.

Results

The mean total tea, green tea and black tea consumption in the sample were 2.80 g/day, 1.88 g/day and 0.92 g/day. Of the 1109 participants, 846 reported no tea drinking. Table 1 shows the association between tea consumption and intake of nutrients and specific food items or food groups. Tea consumption was positively associated with fat and protein but inversely associated with carbohydrate and fibre intake. There were no significant differences in energy, sodium, potassium or salt intake across the tea consumption categories. Rice and vegetable intake was higher among individuals with high tea consumption as compared with those who did not drink tea. In contrast, wheat flour intake was significantly lower among those with the higher levels of tea consumption than those with no tea consumption. The prevalence of smoking and alcohol consumption increased with the increase of tea consumption (all $p< 0.05$). There was a positive association between tea consumption and socio-economic status (i.e. education, occupation), and physical activity. There was a negative association between tea consumption and sleep. There were no significant differences in SBP and DBP across tea consumption categories. There were no significant differences in cigarette smoking and alcohol consumption between the baseline and five-year follow-up time points (Additional file 1: Table S2). Seventy participants (6.3%) reported taking hypertension medication (at both baseline and follow-up). On average SBP increased by 4.5 mmHg (SD 19.1) and DBP increased by 3.0 mmHg (SD 11.2) over 5 years. The prevalence of hypertension at follow-up across total tea consumption categories of none, <10 g/day and ≥10 g/day was 41.8%, 43.9% and 41.7% respectively.

Table 2 shows the association between tea consumption and BP changes in multivariable regression analyses. There was an inverse association between total tea/green tea consumption and changes in DBP. In the fully adjusted model (model 3), including dietary and non-dietary covariates, the β values and 95% confidence intervals for DBP changes were 0, −1.30 (−3.33 to 0.73) and −2.41(−4.71 to −0.11) (p for trend= 0.028) for total tea consumption of 0, 1–10 g/day, and >10 g/day; 0, −1.67 (−3.74 to 0.40), −3.68(−6.47 to −0.89) ($p< 0.01$) for green tea consumption of 0, 1–10 g/day, and >10 g/day.

Figures 2 and 3 show the association between total tea/green tea consumption (as continuous variables) and BP changes with adjustment for all covariates. There was a dose–response relationship between total tea/green tea consumption and DBP change but not SBP change. The confidence intervals were wider at the right end due to the small number of participants with high tea consumption.

There were no significant interactions for tea consumption with central obesity, BMI, sex, smoking and drinking in relation to SBP change. However, high consumption of green tea was significantly inversely associated with SBP change among those who were non-obese and non-smokers (Table 3).

An inverse association between total /green tea consumption and DBP change was observed only among non-smokers. However, no association was found between black tea consumption and DBP change in any subgroup (Table 4).

Discussion

In this population study, we found an inverse association between green but not black tea consumption and 5-year change in both SBP and DBP. The beneficial effect of high green tea consumption on both SBP and DBP occurred only in non-smokers and in the case of SBP only in those without abdominal obesity. There was a clear dose–response relationship between green tea consumption and DBP change.

The inverse association between tea consumption and BP change in our study was limited to green tea consumption. A recent randomized trial which included 95

Table 4 Stratified regression coefficients (95% confidence interval) for DBP change according to the total tea, green tea and black tea consumption categories (β coefficients and 95% confidence intervals) among Chinese adults (n= 1109)[a]

		Categories of tea consumption			
	N	<10 g/day[b] (7.1 g/day)[c]	>10 g/day[b] (14.3 g/day)	P for trend	P for interaction
Total tea					
Central obesity					
Yes	334	0.55(-3.55 to 4.65)	0.03(-5.08 to 5.13)	0.91	0.32
No	768	-1.72(-4.03 to 0.60)	-2.94(-5.50 to -0.37)	0.02	
BMI					
>24 kg/m^2	443	-1.24(-4.57 to 2.08)	-2.29(-6.06 to 1.48)	0.20	0.64
<24 kg/m^2	659	-0.93(-3.43 to 1.57)	-2.78(-5.68 to 0.12)	0.06	
Sex					
Male	469	-1.92(-4.58 to 0.74)	-2.88(-5.69 to -0.08)	0.03	0.56
Female	633	0.06(-3.28 to 3.4)	-0.49(-5.04 to 4.07)	0.88	
Smoking					
Yes	310	0.76(-2.43 to 3.94)	-0.57(-3.86 to 2.71)	0.81	0.03
No	792	-2.43(-5.15 to 0.29)	-4.65(-7.98 to -1.32)	<0.01	
Drinking					
Yes	281	0.61(-2.88 to 4.1)	-0.9(-4.77 to 2.96)	0.76	0.52
No	805	-1.25(-3.86 to 1.35)	-2.84(-5.77 to 0.08)	0.047	
Green tea					
Central obesity					
Yes	334	-0.2(-4.18 to 3.77)	2.05(-4.04 to 8.15)	0.65	0.09
No	768	-1.88(-4.29 to 0.52)	-5.41(-8.54 to -2.28)	<0.01	
BMI					
>24 kg/m^2	443	-1.64(-4.94 to 1.66)	-1.73(-6.25 to 2.79)	0.289	0.64
<24 kg/m^2	659	-0.9(-3.52 to 1.72)	-5.13(-8.67 to -1.58)	0.01	
Sex					
Male	469	-1.81(-4.44 to 0.82)	-4.13(-7.39 to -0.87)	<0.01	0.85
Female	633	-1.09(-4.62 to 2.43)	-1.69(-7.66 to 4.27)	0.43	
Smoking					
Yes	310	0.82(-2.36 to 4)	-1.46(-5.17 to 2.25)	0.61	<0.01
No	792	-3.43(-6.19 to -0.68)	-7.6(-11.96 to -3.24)	<0.01	
Drinking					
Yes	281	-1.03(-4.7 to 2.64)	-3.52(-7.87 to 0.83)	0.12	0.91
No	805	-1.18(-3.77 to 1.4)	-4.13(-7.76 to -0.5)	0.03	
Black tea					
Central obesity					
Yes	334	-8.16(-15.99 to -0.32)	7.35(-2.22 to 16.93)	0.78	0.77
No	768	-1.55(-4.96 to 1.85)	2.44(-1.32 to 6.2)	0.48	
BMI					
>24 kg/m^2	443	-2.45(-8.44 to 3.55)	-1.73(-7.76 to 4.3)	0.41	0.70
<24 kg/m^2	659	-2.56(-6.15 to 1.02)	5.01(0.73 to 9.29)	0.20	

Table 4 Stratified regression coefficients (95% confidence interval) for DBP change according to the total tea, green tea and black tea consumption categories (β coefficients and 95% confidence intervals) among Chinese adults (n= 1109)[a] *(Continued)*

Sex					
Male	469	-2.68(-6.58 to 1.22)	2.45(-1.66 to 6.56)	0.62	0.32
Female	633	-1.02(-6.66 to 4.61)	7.25(-0.3 to 14.81)	0.17	
Smoking					
Yes	310	-2.61(-7.1 to 1.89)	3.31(-1.44 to 8.06)	0.46	0.95
No	792	-1.85(-6.36 to 2.65)	2.49(-2.88 to 7.86)	0.70	
Drinking					
Yes	281	1.75(-3.56 to 7.05)	5.01(-0.85 to 10.87)	0.09	0.41
No	805	-3.57(-7.72 to 0.57)	2.52(-1.98 to 7.02)	0.83	

CI, confidence interval. [a]Models adjusted for variables in model3 of Table 2. Stratifying variables are not adjusted for in corresponding models. [b]Referent category is non-tea drinkers. [c]Median tea consumption.

participants showed that consumption of black tea lowers BP in individuals with normal to high-normal range BPs [23]. We did not find any association between black tea and BP change in our study, possibly because relatively few participants consumed black tea.

There are relatively few epidemiological studies that examine the relationship between tea consumption and blood pressure [13,18,19,35]. The majority of these showed a protective effect of tea on BP [13,18,19]. In a cross-sectional study in Taiwan, Yang et al. found that habitual tea consumption, defined as daily consumption of moderate strength green tea or oolong tea of 120 mL/day or more for 1 year significantly lowers the risk of hypertension [18]. Another cross-sectional study in Western Australia undertaken by Hodgson et al. showed that green tea and black tea intake were associated with significantly lower SBP and DBP in older women: consuming 1 cup (250 ml) green/black tea per day was associated with a 2.2 mm Hg lower SBP and a 0.9 mm Hg lower in DBP [19]. In Norway, Stensvold et al. found that SBP decreased with increasing black tea consumption: comparing those who drank five or more cups/day of tea with those who drank less than one cup/day, the regression coefficients for SBP were −3.1 and −4.0 mm Hg in SBP in men and women, respectively [13]. There is only one study which found that tea consumption was positively associated with BP [35]. In Algiers, it was found that DBP was higher among tea drinkers than non-drinkers (78.1 ± 9.9 mm Hg vs 75.2 ± 9.1 mm Hg). However, the study has a very small sample size of 124 tea drinkers with no adjustment for other dietary factors, and the type of tea consumed was not assessed.

There are a number of potential mechanisms by which tea might lower BP. Tea flavinoids inhibit the activity of angiotensin converting enzyme activity, augment nitric oxide and reduces endothelin-1 concentrations, thereby improving endothelial function and lowering BP [36,37]. Epigallocatechin gallate (EGCG), a tea polyphenol has been shown to improve endothelial function and insulin sensitivity and lower BP in animals [16]. In another animal study, ɣ -Aminobutyric acid (GABA) in tea can block nicotine-induced contraction of isolated ileum and prevent the BP elevation caused by vagal or splanchnic nerve stimulation [21]. Moreover, green tea may induce vascular relaxation in the isolated aortic strips via the blockade of adrenergic α_1-receptors in rats [38]. Green tea has an anti-inflammatory effect [39]. It is known that inflammation is a risk factor for hypertension [40]. Antioxidants in tea may reduce the vascular sclerosis that occurs with ageing [41]. In addition, one recent meta-analysis has shown moderate consumption of tea substantially enhances endothelial-dependent vasodilation [42].

Dietary patterns that are high in fruit and vegetables and low in sodium are associated with lower blood pressure [43,44]. In a group of chimpanzees consuming an optimal vegetable diet, progressive addition of up to 15.0 g/d of salt caused large rises in blood pressure, which reversed when the added salt was removed [45]. In the current study, salt intake was not significantly different across the categories of tea consumption, and tea drinking was positively associated with rice and vegetable intake. However, even after adjusting for dietary factors including salt intake, the association between tea consumption and BP change persists. Tea consumption in China increased from 573 million kilogram in 2005 to about 864 million kilogram in 2007, and about 34% of Chinese drink tea, with 58% of those consuming green tea [46,47]. In addition, salt consumption in China has been decreasing over the past few decades [48]. The increasing prevalence of hypertension therefore prompts questions about the importance of either tea or salt consumption as mitigating factors. Other lifestyles factors, for example obesity, smoking and excess alcohol consumption contribute to hypertension and have been increasing in prevalence in China. A recent study shows that more than 50% of Chinese men were smokers [49].

We observed a beneficial effect of high tea consumption on limiting an increase in DBP only in non-smokers. In addition, an inverse association between tea consumption and DBP change was also found among non-obese and non-alcohol drinkers. In other words, there seems to be no beneficial effects of tea drinking on BP among those with unhealthy lifestyle factors.

The strengths of the study include a large population based sample, and a long time to follow-up (5 years). The data collection and management were undertaken by intensively trained health workers to reduce information bias. We were able to adjust for a range of dietary and non-dietary factors.

The main limitations of the study are that the baseline for tea consumption in 2002 was not collected, and the inability to account for the change in tea consumption during the 5-year follow-up period may affect the BP change. As other lifestyle factors (smoking, alcohol drinking) seemed to be quite stable in the study, we would assume that tea drinking habits were relatively stable also over the five year period. Secondly, there was a relatively high attrition rate of loss to follow-up in the study; this can be attributed to the large number of job migrations from rural areas to urban areas in China [50]. However, there were no differences in energy intake, DBP or gender between those lost to follow-up and those retained, thus limiting bias. Sample power limits the subgroup analyses (e.g. few women drank tea). Finally, although we have adjusted for a few potential covariates, residual confounding may still be present.

Conclusion

We found that the consumption of total/green tea is inversely associated with 5-year BP change among Chinese adults, an effect abrogated by smoking and obesity.

Additional file

Additional file 1: Table S1. Sample characteristics between those retained and those lost to follow up. **Table S2.** Changes in variables [a]between baseline and follow-up (n=1109).

Abbreviations

BP: Blood pressure; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; JIN: Jiangsu nutrition study; FFQ: Food frequency questionnaire; EGCG: Epigallocatechin gallate; GABA: γ-Aminobutyric acid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

XT analyzed and interpreted the data, and wrote the paper. LG and ZS contributed to assist the analysis. ZS conducted the study. AT, GW and ZS contributed to the writing and editing of the paper. All authors contributed to the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors thank the participating regional Centers for Disease Control and Prevention in Jiangsu province, including the Nanjing, Xuzhou, Jiangyin, Taicang, Suining, Jurong, Sihong, and Haimen centers for their support for data collection.

Funding sources

The study is supported by Jiangsu Provincial Natural Science Foundation (BK2008464, PI ZS) and the Jiangsu Provincial Health Bureau, China.

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Dietary intake of patients with moderate to severe COPD in relation to fat-free mass index: a cross-sectional study

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Abstract

Background: Fat-free mass (FFM) depletion has been shown to be a better predictor of mortality than BMI in chronic obstructive pulmonary disease (COPD) patients. The specific aim of the current study was to assess the nutritional status of stable COPD patients in relation to fat free mass index profiles.

Methods: We investigated 65 male moderate-to-severe stable COPD patients. A self-reported questionnaire was applied about general characteristics and smoking history. Nutritional intake was assessed by using a 54–item quantitative food frequency questionnaire. Weight, height, mid-upper arm circumference (MUAC), waist circumference (WC), handgrip strength and body composition measurements were taken by a trained dietitian. The data were analyzed with SPSS 15.0 software.

Results: The mean age of the patients was 62.1 ± 8.9 years. Among all of the patients 13.8% was underweight (BMI < 21 kg/m^2) and 18.5% had a low fat-free mass index (FFMI < 16 kg/m^2). The percentages of the patients who did not meet the daily recommended intakes (RNI) were highest for magnesium (93.8%) and calcium (92.3%). Mean daily consumptions of milk-yogurt, red meat and fruits were significantly low in the low FFMI group compared to normal FFMI group (for all; $p < 0.05$). Patients with normal FFMI had significantly higher weight, height, WC, MUAC, handgrip strength, fat and fat-free mass than the patients with low FFMI (for all; $p < 0.05$).

Conclusions: Dieticians should be aware of COPD patients with low FFMI in order to evaluate the nutritional intake and therefore plan nutritional strategies to improve prognosis of the disease.

Keywords: COPD, Nutritional status, Dietary intake, Body composition, Fat-free mass

Background

Chronic Obstructive Pulmonary Disease (COPD) is a preventable disease characterized by persistent airflow limitation that is usually progressive. It is a leading cause of morbidity and mortality worldwide and results in an economic and social burden [1]. COPD should not be considered as a localized pulmonary disorder but as a systemic disease. Well-characterized systemic features are muscle atrophy and weakness and osteoporosis [2].

The nutritional status of patients with COPD has been considered an important factor that influences the prognosis of the disease [3]. Approximately 20–40% of COPD outpatients have been reported as underweight or malnourished [4]. Body composition is reported to be one of the main determinants of functional disability of COPD patients independent of respiratory functions [5]. Weight loss and depletion of fat-free mass (FFM) may be observed in stable COPD patients, irrespective of the degree of airflow limitation and they are reported to contribute to morbidity, disability, and handicap [6]. Increased muscle protein break-down is a key feature in muscle wasting. This process of cachexia can be considered the result of interplay of systemic factors, including systemic inflammation, oxidative stress, and growth factors that may synergize with local factors leading to protein imbalance [7]. It is important to recognize that muscle mass may be reduced in COPD patients despite a normal BMI [8]. Fat-free mass index (FFMI) has been reported to provide information

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beyond that provided by body mass index (BMI) [9,10]. It has been shown that fat-free mass (FFM) depletion is a better predictor of mortality than BMI alone in COPD patients [9]. A recent study on 564 moderate to severe COPD patients in the Netherlands showed that disturbances in body composition were associated with dramatic differences in macro- and micronutrient intake [11].

Therefore, the specific aims of the current study were to examine the nutritional status of stable moderate to severe male COPD patients and to evaluate energy, macro- and micronutrient intakes in relation to fat free mass index profiles.

Methods

Design and participants

A cross-sectional study was conducted to determine the nutritional status of male COPD patients. The study population was recruited from COPD patients who visited the outpatient respiratory clinics of Ankara Ataturk Chest Diseases and Thoracic Surgery Training and Research Hospital, Turkey. Patients who are over 45 years of age, without any signs of infection and cognitively intact to answer the questions were invited to the study. The diagnosis and staging of COPD was made by pulmonologists according to American Thoracic Society/European Respiratory Society (ATS/ERS) and Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [1,6]. To be included in the study, patients had to be in a stable condition and not have reported acute symptoms or therapy modifications in the 30 days before enrollment. Patients with chronic kidney failure, diabetes, malignancy, congestive heart failure, myocardial infarction in the last 2 years were excluded from the study. In total, 65 male COPD patients (33 moderate, 32 severe) were included in the final data analysis of this study. The study protocol complied with the principles laid down in the Declaration of Helsinki and was approved by the Hacettepe University Senate Ethics Committee (B.30.2. HAC.0.70.00.01/431-1855 2010). Written informed consent was obtained from all subjects.

Measurements

Characteristics of participants

A self-reported questionnaire was applied to the patients by face-to-face interview method to collect information about age, gender, educational level, total monthly family income, duration since diagnosed with COPD and smoking history.

Nutritional intake and physical activity

Nutritional intake and habitual food consumption was assessed by an 54–item quantitative food frequency questionnaire asking for dietary habits in the last 6 months by a trained dietitian. In order to assist respondents in identifying the actual quantity of the foods, a Turkish food photograph catalogue was used. Reported information was converted into a daily intake frequency of each item, which was in turn converted into the daily intake in grams per day for each food. The dietary data was analysed using BeBIS-6.1 (Nutrition Information Systems Software) and total intake of energy, carbohydrate, protein, fat, fiber, vitamin A, vitamin E, vitamin C, vitamin B12, calcium, iron, zinc and magnesium were calculated. Dietary intake was individually compared with gender- and age group- specific Turkish recommendations given in the Dietary Guidelines for Turkey (DGT) [12]. The exact RNI values were used as strict cutoff values to categorize dietary intake (e.g., <RNI and ≥ RNI). The physical activity levels (PAL) were assessed by a 24-h physical activity recall by a trained dietitian.

Anthropometric measurements and body composition

Anthropometric assessment consisted of determination of weight (kg), height (m), mid-upper arm circumference (MUAC) (cm) and waist circumference (cm). Height was measured with a clinical stadiometer while the patient was standing barefoot. Body weight was measured using a calibrated scale, with the patient wearing light clothes and no shoes. BMI (kg/m^2) was calculated as weight/height^2 and was classified according to European Respiratory Society (ERS) and American Thoracic Society's (ATS) recommendation [6]. Mid-upper arm circumference was measured on the non-dominant arm using a non-stretch tape measure at the mid point between acromion and olecranon. Waist circumference (WC) measurement was made around patient's bare midriff, at the midpoint between the lowest rib and the iliac crest, at the end of gentle expiration while standing without shoes with a non-stretch tape.

Handgrip strength was assessed as a measure of peripheral skeletal muscle strength. A digital handgrip-dynamometer (Takei TKK-5401) was used to determine the isometric grasp in each hand by measuring the maximally developed strength of the flexors of the fingers. Two measurements were made in each hand with the arm unsupported. The mean value of left and right hand strength was used for statistical analysis.

Body composition was assessed using bioelectrical impedance analysis (BIA) (Bodystat 1500; Bodystat Ltd, Douglas, UK) with subjects lying supine, with four surface electrodes placed on the right wrist and ankle. Measurements were obtained in the morning after a fast of at least 3 hours and urination 30 minutes prior to the procedure. The fat-free mass index (FFMI) (kg/m^2) was calculated as the ratio of FFM to height in meters squared. Fat free mass index was classified as low FFMI (<16 kg/m^2) and normal FFMI (≥16 kg/m^2) [8]. All of the anthropometric measurements were performed by a trained dietitian.

Spirometry and disease severity

Spirometric evaluation of the patients was performed using a computerized spirometer (Spirolab III SFT) by spirometry

technicians. Spiromeric values of the forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), and FEV_1/FVC were recorded as percent predicted values. The FEV_1% predicted was measured with the highest value from at least three technically acceptable spirometric manoevres being used. Classification of disease stage was based on the forced expiratory volume in the first second (FEV_1) value as per GOLD guideline i.e. stage I FEV1 ≥ 80%predicted, stage II 50% ≤ FEV1 < 80%predicted, stage III 30% ≤ FEV1 < 50%predicted and stage IV < 30% of predicted [1].

Blood parameters

Serum sample from each subject was obtained by a trained nurse at a fasting state of ≥8 hours and they were analyzed for visceral protein stores represented by total protein and serum albumin. The measurements were performed by the clinical chemistry laboratory of Ankara Ataturk Chest Diseases and Thoracic Surgery Training and Research Hospital with the Mannheim/Hitachi 747 analyzer using the bromcresol green method (Roche, Oxford, CT).

Data analysis

The data were analyzed with SPSS (Statistical Package for the Social Sciences) WIN 15.0. Descriptive statistics reported frequencies, percentages, mean (±SD), minimum and maximum values where appropriate as well as chi-square or Fisher differences. The normality of the data distribution was tested using visual (histogram and probability graphics) and analytical methods (Kolmogrov-Smirnov/Shapiro-Wilk tests). Student's *t* test (under parametric conditions) or Mann–Whitney U test (under non-parametric conditions) for unpaired data was used for comparison of general characteristics, nutritional and anthropometric data between the low FFMI and normal FFMI groups. Spearman correlation test examined the relationship between FFMI (kg/m^2) and age, spirometry, handgript strength, total energy expenditure (TEH), physical activity level (PAL), serum albumin and total protein levels.

Results

General characteristics

The mean age of the patients (n = 65) was 62.1 ± 8.9 years and most were retired (81.5%) and married (90.8%). The mean FEV_1% predicted was 50.2 ± 13.6%. Duration since diagnosed with COPD was significantly lower in moderate patients than severe patients (5.6 ± 8.7 and 8.2 ± 9.6 years respectively; p = 0.043). Mean age for starting to smoke was 14.9 ± 4.4 years and the cumulative smoking was 53.5 ± 28.8 pack.years (Table 1). All participants had health insurance and 60% of the patients reported a monthly family income of less than 700 Turkish Liras (≈US$314). Distributions of BMI classification of the patients according to the classification of European Respiratory Society and American Thoracic Society (ERS/ATS) and FFMI are given in Figure 1. Among all of the patients 13.8% was underweight, 32.3% was overweight and 15.4% was obese. On the other hand, low FFMI was assessed in the 18.5% of the patients. While 11% of the patients had both low BMI and FFMI, 8% of the patients had a low FFMI despite a normal BMI (Figure 2). Moderate and severe COPD patients did not show significant difference in context of BMI and FFMI distributions when evaluated separately (data not shown).

Table 1 General characteristics of COPD patients (n = 65)

Characteristics	
Age (y)	62.1 ± 8.9
45-64	38 (58.5)
≥65	27 (41.5)
FEV_1 (% of predicted)	50.2 ± 13.6
FVC (%)	64.5 ± 16.6
FEV_1/FVC	61.4 ± 7.7
Smoking	
Initiation age (y)	14.9 ± 4.4
Pack.years	53.5 ± 28.8
Duration of COPD (y)	6.9 ± 9.2
BMI (kg/m^2)	25.3 ± 4.3
FFMI (kg/m^2)	18.9 ± 2.6

Nutritional intake and habitual food consumption

Table 2 lists the participants' nutritional intake status assessed by quantitative food frequency questionnaire. The mean total energy intake was 1906.3 ± 567.4 kcal and did not show significant difference between low FFMI and normal FFMI groups (p = 0.478). Seventy five percent of the patients' energy intakes were not meeting the recommended daily intake levels. Although the difference was not statistically significant, the frequency of patients whose dietary energy intake did not meet the RNI levels was higher in the low FFMI group (91.7%).

Daily intakes of carbohydrate, protein, fat and fiber were 273.2 ± 85.7 g, 72.7 ± 25.0 g, 54.1 ± 20.4 g and 20.7 ± 6.5 g respectively. Mean fiber intake was 20.7 ± 6.5 g and 90.8% of the patients could not meet RNI for fiber. Neither vitamin nor mineral intakes were different between the two FFMI groups. The mean percentages of meeting the daily recommended intakes (RNI) were lowest for magnesium (57.0 ± 21.3%) and calcium (62.2 ± 29.1%). None of the patients with low FFMI could meet the RNI levels for fiber, magnesium, calcium and zinc.

Habitual daily food consumption of COPD patients assessed by quantitative food frequency questionnaire is given in Table 3. Mean daily consumption of dairy products was 220.1 ± 170.4 g and 95.4% of the patients' dairy consumption was below the recommended levels. Mean

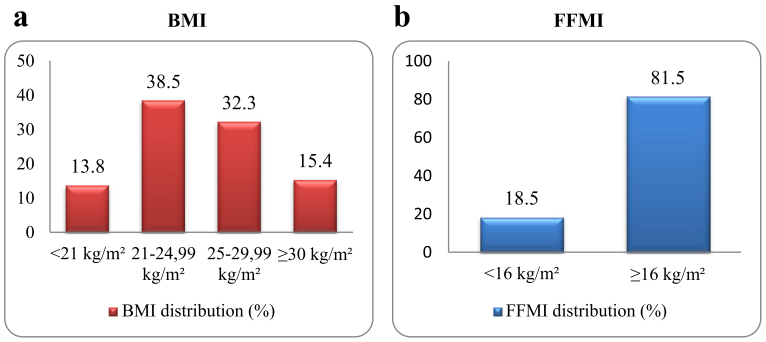

Figure 1 BMI and FFMI distributions of COPD patients (n = 65). a. Values indicating the patients with BMI values <21 kg/m^2 (underweight), between 21-24.99 kg/m^2 (normal weight), between 25-29.99 kg/m^2 (overweight) and ≥30 kg/m^2 (obese) are shown as percentages (%). **b**. Values indicating the patients with FFMI values <16 kg/m^2 (low FFMI) and ≥16 kg/m^2 (normal FFMI) are shown as percentages (%). Abbreviations: BMI: Body mass index; FFMI: Fat-free mass index.

consumption of legumes among COPD patients was 8.9 ± 5.6 g/day and nearly 97% was not meeting the RNI for legumes. Mean daily fruit and vegetable consumption was 389.9 ± 200.1 g and 92.3% of the patients' consumption was below the RNI for fruits and vegetables. Mean daily consumptions of milk-yogurt, red meat and fruits were found to be significantly low in the low FFMI group compared to normal FFMI group (for all; $p < 0.05$).

Anthropometric measurements, body composition, physical activity level and blood parameters

Anthropometric measurements, handgrip strength, total energy expenditure (TEE), physical activity level (PAL) and some blood parameters of COPD patients are given in Table 4. Patients with normal FFMI had significantly higher weight, height, WC, MUAC, fat and fat-free mass than the patients with low FFMI. Mean handgript strength of the low FFMI group was 26.1 ± 4.7 kg while it was 34.6 ± 7.9 kg in the normal FFMI group ($p = 0.001$). Total energy expenditure (TEE) assessed by 24-h recall was also significantly low in the low FFMI group, but the physical activity levels (PAL) were similar. There was a positive weak correlation between FFMI and handgript strength ($r = 0.331$, $p = 0.007$) and a positive moderate correlation between FFMI and TEE ($r = 0.548$, $p = 0.000$) (Table 5). Hemoglobin and hemotoctrit levels were significantly lower in the low FFMI group (for all; $p < 0.05$) (Table 4). A positive weak correlation was found between FFMI and serum albumin ($r = 0.288$, $p = 0.022$) (Table 5).

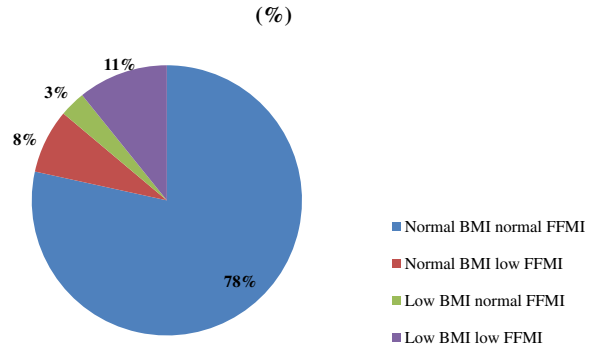

Figure 2 The prevalence of different depletion categories. Blue: normal BMI and FFMI; red: normal BMI and FFM depletion; green: low BMI and normal FFM; purple: low BMI and FFMI.

Discussion

General characteristics

The present study which included male COPD patients with moderate to severe airway obstruction showed that dietary intake and habitual food consumption differ in relation to fat-free mass index. The link between aging and the pathogenesis of COPD is strongly supported [10] and longer duration of the disease since diagnose in severe COPD patients in our study can be explained by the progressive nature of the disease [1]. Cigarette smoking is the most important environmental risk factor for the development of COPD [13]. In our study the mean age for starting to smoke corresponded to adolescence, similar to the findings of a recent study by Kim et al. [14], who found the mean initiation age of smoking of COPD patients as 16.4 ± 4.8 years.

In the current study 18.5% of patients were characterized by a low FFMI. Similarly, a study among 72 COPD outpatients suffering from a moderate degree of airflow obstruction showed that prevalance of fat-free mass depletion was 18% in male patients [15]. In another study, among moderate to severe COPD patients the prevalance of fat free mass depletion was 20.3% in male patients [16]. A recent study by Van de Bool et al. [11] showed that almost 25% of moderate to severe COPD patients who were eligible for pulmonary rehabilitation were characterized by a low FFMI. These little discrepancies may be explained by different inclusion criteria of the studies. On the other hand, in a cross-sectional study among male COPD patients, depletion of fat-free mass

Table 2 Energy and nutrient intakes of COPD patients assessed by food frequency questionnaire

	Low FFMI (n = 12)	Normal FFMI (n = 53)		Total (n = 65)
	X ± SD	X ± SD	p	X ± SD
Energy (kcal)	1770.0 ± 338.2	1937.2 ± 605.7	0.478	1906.3 ± 567.4
RNI (%)	81.3 ± 15.1	86.1 ± 24.4	0.519	85.2 ± 22.9
<RNI	11 (91.7)	38 (71.7)	0.266	49 (75.4)
Carbohydrate (g)	258.4 ± 46.9	276.6 ± 92.3	0.488	273.2 ± 85.7
Protein (g)	68.4 ± 15.4	73.7 ± 26.7	0.946	72.7 ± 25.0
Fat (g)	48.1 ± 13.6	55.5 ± 21.5	0.398	54.1 ± 20.4
Ratio of C:P:F	60:16:24	58:16:26	-	58:16:26
Fiber (g)	19.3 ± 3.8	21.0 ± 6.9	0.499	20.7 ± 6.5
RNI (%)	66.4 ± 13.2	72.3 ± 23.9	0.254	71.2 ± 22.3
<RNI	12 (100.0)	47 (88.7)	0.583	59 (90.8)
Vitamin A (mcg)	1029.2 ± 1365.4	782.2 ± 621.6	0.710	827.8 ± 802.3
RNI (%)	114.4 ± 151.2	86.9 ± 69.1	0.340	92.0 ± 89.1
<RNI	8 (66.7)	41 (77.4)	0.470	49 (75.4)
Vitamin E (mg)	16.3 ± 5.1	17.3 ± 7.0	0.710	17.1 ± 6.6
RNI (%)	108.5 ± 33.7	115.6 ± 46.5	0.618	114.3 ± 44.3
<RNI	4 (33.3)	20 (37.7)	1.000	24 (36.9)
Vitamin B12 (mcg)	4.9 ± 4.8	3.8 ± 3.0	0.442	4.0 ± 3.4
RNI (%)	202.1 ± 201.1	160.0 ± 123.3	0.350	167.7 ± 139.9
<RNI	3 (25.0)	21 (39.6)	0.511	24 (39.6)
Vitamin C (mg)	115.2 ± 34.7	115.6 ± 60.3	0.389	115.5 ± 56.2
RNI (%)	128.0 ± 38.6	128.5 ± 67.0	0.979	128.4 ± 62.4
<RNI	1 (8.3)	20 (37.7)	0.084	21 (32.3)
Calcium (mg)	654.0 ± 141.7	759.8 ± 334.8	0.302	740.2 ± 310.2
RNI (%)	54.5 ± 11.8	65.2 ± 31.4	0.057	63.2 ± 29.1
<RNI	12 (100.0)	48 (90.6)	0.575	60 (92.3)
Iron (mg)	9.1 ± 1.9	9.9 ± 3.3	0.594	9.8 ± 3.1
RNI (%)	91.2 ± 19.2	99.2 ± 33.1	0.423	97.7 ± 31.0
<RNI	7 (58.3)	30 (56.6)	0.913	37 (56.9)
Zinc (mg)	8.1 ± 1.7	9.0 ± 3.2	0.660	8.8 ± 3.0
RNI (%)	74.0 ± 15.8	81.8 ± 29.2	0.375	80.3 ± 27.3
<RNI	12 (100.0)	42 (79.2)	0.109	54 (83.1)
Magnesium (mg)	205.3 ± 53.8	247.0 ± 94.2	0.217	239.3 ± 89.3
RNI (%)	48.9 ± 12.8	58.8 ± 22.4	0.145	57.0 ± 21.3
<RNI	12 (100.0)	49 (92.5)	1.000	61 (93.8)

Energy and nutrient intakes of COPD patients assessed by food frequency questionnaire. Values for actual nutrient intakes and percentage of meeting the RNI levels are shown as mean ± SD. Values indicating the number of patients with nutrient intakes below RNI (<RNI) are shown as number and percentage (%) in parenthesis. None of the differences between the low FFMI group and the normal FFMI group were significant. RNI: recommended nutrient intake.

based on fat-free mass index was about 42% [17]. This discrepancy can be due to more severe COPD being present in the study as they mentioned that the prevalance of low FFMI was high among severe to very severe (stage IV) COPD patients.

Increased BMI does not protect against fat-free mass depletion in COPD, since there is a preferential loss of muscle tissue in this disease [18]. In our study 8% of the patients had low FFMI despite a BMI ≥21 kg/m^2. A study by Pirabbasi et al. [17] showed that among 271 male COPD patients 11.1% had a low FFMI (<16 kg/m^2) despite a normal BMI (≥21 kg/m^2). In the study by Vermeeren et al. [16], the prevalence of being underweight was 17% whereas prevalence of FFM depletion was 42%. It should be noted however that in their study they used <18.5 kg/m^2 as the BMI cutoff for being underweight.

Table 3 Daily food consumption of COPD patients assessed by quantitative food frequency questionnaire (n = 65)

	Low FFMI (n = 12)	Normal FFMI (n = 53)		Total (n = 65)
Food groups (g/day)	**X ± SD**	**X ± SD**	**p**	**X ± SD**
Dairy products	117.9 ± 86.6	243.3 ± 176.7	0.007*	220.1 ± 170.4
<RNI	12 (100.0)	50 (94.3)	1.000	62 (95.4)
Milk-yogurt	85.4 ± 86.4	208.1 ± 174.0	0.004*	185.5 ± 167.9
<RNI	12 (100.0)	51 (96.2)	1.000	63 (96.9)
Cheese	32.5 ± 19.9	35.2 ± 22.9	0.986	34.7 ± 22.2
<RNI	3 (25.0)	17 (85.0)	0.741	20 (30.8)
Meats, eggs, legumes, nuts	110.7 ± 43.8	123.2 ± 62.1	0.800	120.9 ± 59.0
<RNI	9 (75.0)	36 (67.9)	0.673	55 (84.6)
Red meat	4.8 ± 6.5	12.7 ± 14.3	0.044*	11.2 ± 13.5
Chicken, turkey	33.6 ± 31.6	44.7 ± 34.2	0.260	42.7 ± 33.8
Fish	23.0 ± 26.9	28.9 ± 27.1	0.450	27.8 ± 26.9
Eggs	27.1 ± 14.2	20.7 ± 17.6	0.116	21.9 ± 17.1
<RNI	0 (0.0)	8 (15.1)	0.333	8 (12.3)
Legumes	8.2 ± 4.8	9.1 ± 5.8	0.428	8.9 ± 5.6
<RNI	12 (100.0)	51 (96.2)	1.000	63 (96.9)
Nuts	14.1 ± 15.5	7.2 ± 11.1	0.531	8.5 ± 12.2
Bread-grains	310.9 ± 112.3	343.4 ± 140.1	0.434	337.3 ± 135.1
<RNI	10 (83.3)	40 (75.5)	0.717	38 (58.5)
Fruits	124.0 ± 109.3	222.3 ± 121.8	0.013*	204.1 ± 124.8
Vegetables	167.5 ± 104.1	190.0 ± 129.0	0.576	185.8 ± 124.3
Fruits and vegetables	150.3 ± 43.4	412.2 ± 204.4	0.058	389.9 ± 200.1
<RNI	12 (100.0)	48 (90.6)	0.575	60 (92.3)
Oils-fats	30.3 ± 20.2	26.8 ± 11.2	0.906	27.4 ± 13.2
<RNI	8 (66.7)	42 (79.2)	0.449	50 (76.9)
Sugar	41.6 ± 30.5	42.5 ± 36.5	0.946	42.3 ± 35.3
<RNI	10 (83.3)	36 (67.9)	0.484	50 (76.9)

Daily food consumption of COPD patients assessed by quantitative food frequency questionnaire (n = 65). Values for actual intakes are shown as mean ± SD. Values indicating the number of patients with food consumption below RNI (<RNI) are shown as number and percentage (%) in parenthesis. Asterisks indicate a significant difference between groups: *P < 0.05. Daily dairy, milk-yogurt, red meat and fruit consumptions of patients with low FFMI were significantly lower. RNI: recommended nutrient intake.

Nutritional intake and habitual food consumption

In the current study, mean daily energy intakes of COPD patients was 1906 kcal similarly to a group of 275 moderate to severe COPD patients (93% male) in Spain, whose mean daily energy intake was 2033 kcal [19]. According to Turkey Health and Nutrition Survey-2010, mean energy intake of Turkish individuals was 1918 kcal in the 51–64 years age group and 1706 kcal in the 65–74 year age group [20]. A study in Malaysia with 149 COPD patients showed that dietary energy intake of patients (assessed by one day or two days record) was below Malaysian RNI [18]. Compared to the Malaysian sample, in our study prevalance of patients with energy intake below RNI is lower yet clinically significant (93% vs 75%, respectively). A recent study by Van de Bool et al., which is the only study to evaluate dietary intake of COPD patients in relation to body composition until now, showed that COPD patients with low FFMI reported higher energy intake than patients with normal FFMI [11]. On the contrary, in the current study mean energy intake of patients in the low FFMI group was lower than normal FFMI group (1770 ± 338 and 1937 ± 606 kcal, respectively).

Selective wasting of fat-free mass is suggesting a disturbed protein balance in COPD patients [21]; hence, protein intake of depleted COPD patients is recommended to exceed 1.5 g/kg/day [22]. Increased protein intake and physical activity, in the form of resistance training, stimulate muscle protein synthesis in the elderly [23]. In the study of Van de Bool et al. [11], COPD pateints with low FFMI reported significantly higher protein intake per kg body weight. In the current study, protein intake of patients did not differ between low and normal FFMI groups. However, daily consumptions of dairy products and red meat

Table 4 Body composition, handgrip strength, physical activity level (PAL) and some blood parameters of COPD patients

	Low FFMI (n = 12)	Normal FFMI (n = 53)		Total (n = 65)
Characteristics	**X ± SD**	**X ± SD**	**p**	**X ± SD**
Weight (kg)	55.1 ± 6.4	75.4 ± 10.9	0.000**	71.6 ± 12.9
Height (cm)	164.1 ± 5.9	168.8 ± 5.9	0.015*	167.9 ± 6.1
WC (cm)	83.9 ± 7.6	101.0 ± 11.4	0.000**	97.8 ± 12.6
MUAC (cm)	25.1 ± 1.7	30.2 ± 3.0	0.000**	29.3 ± 3.4
FM (g)	14.8 ± 3.4	18.8 ± 6.4	0.042*	18.1 ± 6.1
FM (%)	26.7 ± 3.6	24.5 ± 5.5	0.155	24.9 ± 5.2
FFM (g)	40.3 ± 4.0	56.3 ± 6.3	0.000**	53.4 ± 8.6
FFM (%)	73.3 ± 3.6	75.5 ± 5.5	0.146	75.1 ± 5.2
Handgript strength (kg)	26.1 ± 4.7	34.6 ± 7.9	0.001*	33.1 ± 8.1
TEE (kkal/d)	2031.9 ± 344.3	2463.5 ± 426.6	0.002*	2382.8 ± 443.5
PAL	1.5 ± 0.2	1.6 ± 0.2	0.156	1.5 ± 0.2
WBC	8.3 ± 2.2	8.4 ± 1.8	0.882	8.4 ± 1.9
HG (g/dL)	14.5 ± 1.5	15.4 ± 1.3	0.038*	15.3 ± 1.4
HCT (%)	43.4 ± 4.3	46.3 ± 3.8	0.024*	45.7 ± 4.0
Serum albumin (g/dL)	4.1 ± 0.4	4.3 ± 0.3	0.051	4.2 ± 0.3
Serum total protein (g/dL)	6.8 ± 0.4	7.0 ± 0.5	0.289	7.0 ± 0.5

Body composition, handgrip strength, physical activity level (PAL) and some blood parameters of COPD patients. Values are shown as mean ± SD. Asterisks indicate a significant difference between groups: *P < 0.05; **P < 0.001. Patients with low FFMI had significantly lower weight, height, WC, MUAC, FM, FFM, handgrip strength, TEE, HG and HCT values. *Abbreviations: WC* waist circumference, *MUAC* mid-upper arm circumference, *FM* fat mass, *FFM* fat-free mass, *TEE* total energy expenditure, *PAL* physical activity level, *WBC* white blood cells, *HG* hemglobin, *HCT* hematoctrit.

were significantly low in the low FFMI group. This finding is considerable since high-quality protein sources such as whey protein, milk, and beef have been shown to improve protein synthetic response in the elderly [24].

In the current study, majority of patients' daily milk and yogurt consumption was below RNI and this finding was more marked in the patients with low FFMI. Milk proteins (casein and whey) are known for their high branched-chain amino acid (BCAA) content, which include leucine (LEU), isoleucine (ILE) and valine (VAL) [25]. Since skeletal muscle is a major site of (BCAA) catabolism in disease state, they are used for maintenance of protein quality and repair process of tissues [26]. Plasma levels of BCAAs, particularly leucine, are reduced in patients with COPD [27] and a significant association was found between low levels of BCAAs and depletion of FFM [17]. Engelen et al. showed an elevated anabolic response to sip feeding of a casein protein meal in patients with COPD [28]. All of these findings make milk proteins an important preventive approach to conserve muscle mass in COPD.

Table 5 Spearman's rank correlation between FFMI (kg/m^2) and some parameters

	FFMI (kg/m^2)	
	r	**p**
Age	−0.059	0.641
FEV_1 (%)	0.092	0.465
FEV_1/FVC (%)	0.217	0.082
Handgript strength (kg)	0.331	0.007*
TEE (kkal/d)	0.548	0.000**
PAL	0.216	0.084
Serum albumin (g/dL)	0.288	0.022*
Serum total protein (g/dL)	0.159	0.213

Spearman's rank correlation between FFMI (kg/m^2) and some parameters. Asterisks indicate a significant correlation between groups: *P < 0.05; **P < 0.001.

In this study, a vast majority (92%) of the COPD patients could not meet RNI for fruits and vegetables with mean daily consumption of fruits being significantly lower in the low FFMI group compared to normal FFMI group. This finding is concerning since cross-sectional studies have showed a significant positive association between fruit and vegetable (FV) intake and forced expiratory volume in 1 s (FEV_1), with stronger evidence for fruit consumption [29,30]. Data from the MORGEN study showed that higher intakes of antioxidants such as vitamin C, beta-carotene and flavonoids are associated with higher FEV_1 values, compared with low intakes [31,32]. Moreover, Walda et al. [33] demonstrated an inverse association between fruit intake and 20 yr COPD mortality.

The major deficiencies were assessed in magnesium and calcium intakes in the current study. Mean magnesium intake of COPD patients was 239.3 ± 89.3 mg in

our study. According to National Turkish Health and Nutrition Survey (NTHNS) 2010, mean magnesium intake was 290.8 mg in the 51–64 year old group, 271.3 mg in the 65–74 year old group and 241.7 mg in 75 years and older [20]. Mean magnesium intake of COPD patients in our study was lower than all of the age groups in the national survey. Low consumption of dark leafy vegetables, nuts and seeds due to chewing problems [34], or legumes due to gastrointestinal disturbances [35] might be the reason of low magnesium intake in our group of COPD patients.

Mean calcium intake in the current study was 740.2 ± 310.2 mg and 92% of the patients' intake could not meet RNI. Calcium intake in the NTHNS-2010 was 712.7 mg in the 51–64 year olds, 677.2 mg in the 65–74 year olds and 592.6 mg in 75 years and older [20]. In the study by Van de Bool et al. which evaluated the dietary intake of COPD patients assessed by using a cross-check dietary history in in Netherlands, calcium intake was reported as "too low" since 72% of the patients' dietary intake could not meet the RNI [11]. In the COPD patients in this study, the reported percentage of patients with calcium intake below recommendations was remarkably higher. In a Spanish group of 275 moderate to severe COPD patients, prevalence of complience with recommendations was 31% for magnesium and 49% for calcium and similar to our study magnesium was the major mineral deficieny [19].

We did not find any significant differences in daily macro- and micronutrient intakes between patients with low FFMI and normal FFMI. Unlikely to our study, Van de Bool et al. recently showed that intakes of calcium and vitamin A in COPD patients with low FFMI were significantly higher [11]. The consumption of legumes, dairy products, fruits and vegetables were lowest as majority of the patients' intakes did not comply with the recommendations in our sample. In a Spanish group of 275 moderate to severe COPD patients daily legume consumption was 31 ± 21 g while in our study it is 8.9 ± 5.6 g. This difference may be attributed to cultural differences between the countries.

Anthropometric measurements, body composition and physical activity level

In the present study mean weight, height, waist circumference (WC), mid-upper arm circumference (MUAC), fat mass and fat-free mass were significantly lower in patients with low FFMI. Mid-upper arm circumference correlate with total muscle mass and is therefore used to predict changes in the protein nutritional status [36]. Accordingly, in the present study mean MUAC of patients with low FFMI was significantly low.

Patients with COPD have a significantly reduced duration, intensity, and counts of daily physical activity when compared to healthy control subjects [37]. Low fat-free mass has been shown to impair exercise performance in COPD patients [38]. A recent study by Andersson et al. [39] showed that COPD patients who were more physically active were characterized not only by better pulmonary function but also higher BMI and FFMI. In the current study, mean total daily energy expenditure of patients with low FFMI was significantly low. Additionally, mean physical activity levels was lower in the low FFMI group, but the difference was not statistically significant.

Serum albumin is synthesized in the liver and is a marker of nutritional status. Data suggest that low serum albumin is associated with low appendicular skeletal muscle mass in elderly women and men. Reduced protein metabolism with aging may occur concurrently in the liver and muscle causing similar decrements in both serum albumin and muscle mass [40]. Although in our study mean serum albumin levels were not markedly different between low and normal FFMI groups, there was a positive weak correlation between FFMI and serum albumin.

Cesari et al. showed in their study that hemoglobin levels were associated with muscle and fat mass changes, and that decreased muscular strength occured in the presence of anemia in individuals who were 65 years and older [41]. Similarly to these findings, in our study patients with low FFMI had significantly low hemoglobin and hematocrit levels.

Limitations

Some shortcomings of the current study need to be considered. First, no healthy control group could be included in the present analyses in order to compare the nutritional intake between COPD patients and healthy subjects. Nevertheless, results were compared with general findings in general older Turkish adults from the Natonal Turkish Nutrition and Health Survey-2010 [20]. Second, loss of FFM seems to be more frequent in patients with emphysema-type COPD than in patients with chronic bronchitis. Unfortunately, we were unable to differentiate COPD subtypes in our study. Third, the assessment of dietary intake of fat might be underestimated in food frequency questionnaire. The food frequency method is generally applied in order to assess the quality of dietary intake because it is able to provide data about particular food groups. While there is concern that food frequency questionnaires can be prone to measurement error [42], they have been shown to identify similar patterns of diet as other dietary methods [43].

Fourth, in the current study physical activity was assessed by a 24-h recall questionnaire. It has been reported that COPD patients overestimate the time spent walking and underestimate time spent standing. Therefore, using a multisensor armband or an accelerometer to assess physical activity would be more reliable [37].

Conclusions

Health proffesionals, especially dietitians should be aware of COPD patients with low FFMI in order to evaluate the nutritional intake and therefore plan nutritional strategies to improve prognosis of the disease. Dietary strategies to prevent fat-free mass loss in COPD patient should be further investigated.

Abbreviations

ATS: American Thoracic Society; BCAA: Branched-chain amino acids; BIA: Bioelectrical impedance analysis; BMI: Body mass index; COPD: Chronic obstructive pulmonary disease; DGT: Dietary Guidelines for Turkey; ERS: European Respiratory Society; FEV_1: Forced expiratory volume in the first second; FFM: Fat-free mass; FFMI: Fat-free mass index; FVC: Forced vital capacity; GOLD: Global Initiative for Chronic Obstructive Lung Disease; HCT: Hematoctrit; HG: Hemoglobin; ILE: Isoleucine; LEU: Leucine; MUAC: Mid-upper arm circumference; NTHNS: National Turkish Health and Nutrition Survey; PAL: Physical activity level; RNI: Recommended nutrient intakes; TEH: Total energy expenditure; VAL: Valine; WBC: White blood cells; WC: Waist circumference.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Statement of authorship: HTB and DY were responsible for the original ideas and methodology of the study. DY with the undersupervision of HTB carried out the studies and data analyses and drafted the manuscript. NC and SC contributed to patient recruitment. HTB provided significant advice and consultation. All authors contributed significantly, read and approved the final manuscript.

Acknowledgement

We are very grateful to all participants included in the present study, and the outpatient respiratory clinics of Ankara Ataturk Chest Diseases and Thoracic Surgery Training and Research Hospital for their kind assistance in collecting the data.

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Relationship of vitamin D status and bone mass according to vitamin D-binding protein genotypes

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Abstract

Background: Vitamin D-binding protein (DBP) may alter the biological activity of total 25-hydroxyvitamin D [25(OH)D]; this could influence on the effects of vitamin D in relation to bone mineral density (BMD) and fractures. Emerging data suggest that fetuin-A may be involved in bone metabolism. We aimed to investigate the influence of *DBP* gene polymorphism on the relationship of vitamin D status and fetuin-A levels to BMD and bone markers.

Methods: This cross-sectional study was part of a health survey of employees of the Electricity Generating Authority of Thailand (1,734 healthy subjects, 72% male). Fasting blood samples were assayed for 25(OH)D, fetuin-A, N-terminal propeptides of type 1 procollagen (P1NP), C-terminal cross-linking telopeptides of type I collagen (CTx-I), and *DBP* rs2282679 genotypes. L1–L4 lumbar spine and femoral BMD were measured using dual-energy X-ray absorptiometry.

Results: The *DBP* rs2282679 genotype distribution conformed to the Hardy–Weinberg equilibrium. There were no correlations between 25(OH)D levels and BMD and bone markers. But a trend of positive correlation was observed for the *DBP* genotypes with total hip BMD, and for the interaction between 25(OH)D and *DBP* genotypes with BMD at all femoral sites. We further analyzed data according to *DBP* genotypes. Only in subjects with the AA (common) genotype, 25(OH)D levels were positively related to BMD and bone markers, while fetuin-A was negatively related to total hip BMD, independently of age, gender and BMI.

Conclusions: The interaction between vitamin D status, as measured by circulating 25(OH)D and *DBP* rs2282679 genotypes, modified the association between 25(OH)D and BMD and bone markers. Differences in *DBP* genotypes additionally influenced the correlation of fetuin-A levels with femoral BMD.

Keywords: BMD, Bone turnover markers, Fetuin-A, 25(OH)D, *DBP* rs2282679 genotypes

Background

Vitamin D plays important roles in bone and calcium metabolism. It enhances intestinal calcium absorption and suppresses bone resorption through its negative regulatory influence on parathyroid hormone secretion [1]. Moreover, vitamin D affects osteoblast by inhibiting proliferation but promoting mineralization and maturation [2,3]. Osteomalacia is a clinical feature of severe vitamin D deficiency due to impaired bone mineralization [4]. The influence of vitamin D on bone mass and the propensity to osteoporosis is less clear. Despite its biological effects related to bone mass, results from clinical studies investigating the effects of vitamin D on osteoporosis or osteoporotic fractures have been inconsistent [5,6]. Observational studies regarding the effect of vitamin D are usually performed using circulating 25-hydroxyvitamin D [25(OH)D], which is mostly bound to vitamin D-binding protein (DBP). It has been shown that genetic polymorphisms of *DBP*, for example three major polymorphic forms of *DBP*: GC1F, GC1S and GC2 are highly associated with 25(OH)D levels[7,8]. Recently, large genome-wide

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association studies in European [9,10] and two studies in Chinese [11,12] reported another *DBP* polymorphism, rs2282679, had an association with vitamin D deficiency. Nonetheless, data of the relationship between *DBP* rs2282679 genotypes and BMD and bone markers is scanty. It is unclear if there is an interaction of DBP or *DBP* genetic polymorphism and circulating 25(OH)D that affects bone mass; this may underlie the inconsistent results of some studies.

Fetuin-A is a multifunctional protein of hepatic origin. Besides glucose and energy homeostasis [13], fetuin-A may be involved in bone metabolism, as suggested by recent findings in elderly men and women [14,15]. With regard to the influence of vitamin D, it has been shown that vitamin D administration increase circulating fetuin-A in both experimental animals [16] and humans [17]. However, the relative influence of fetuin-A versus vitamin D and their possible interaction on bone mass is unknown at present. Therefore, the purpose of the present study was to investigate the influence of the interrelationship of vitamin D status, *DBP* gene polymorphism and fetuin-A levels on bone mineral density (BMD).

Methods

This study was part of a health survey of 1,734 employees of the Electricity Generating Authority of Thailand (EGAT). Prior to commencement, the study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine, Ramathibodi Hospital, Mahidol University; all subjects gave written informed consent. As described in detail elsewhere [18], survey data was collected through self-administered questionnaires, physical examinations, electrocardiography, chest radiography, and blood analysis. Anthropometric variables, including weight, height and waist circumference (WC), were measured using standard techniques. Body mass index (BMI) was derived by weight (kg)/height $(m)^2$. Fasting blood samples were obtained and assayed for 25(OH)D, fetuin-A, N-terminal propeptides of type 1 procollagen (P1NP), C-terminal cross-linking telopeptides of type I collagen (CTx-I), and *DBP* rs2282679 genotypes.

BMD

The measurement method was described in an earlier report [19]. Each subject changed into light clothing before undergoing BMD assessment by dual-energy X-ray absorptiometry (DXA) at the lumbar spine (L1–L4 vertebrae) and total hip. All procedures were performed according to the recommendations of the International Society for Clinical Densitometry (ISCD) [20] by ISCD-certified technologists using a Hologic QDR-4500 DXA scanner (Bedford MA, USA). Quality assurance procedures using a spine phantom were performed daily. The precision error was less than 1.5%. The BMD coefficients of variation were 0.82% and 1.51% for the lumbar spine and total hip, respectively.

DBP rs2282679 genotypes

Genomic DNA was isolated from peripheral blood leukocytes using a standard phenol–chloroform extraction method. The *DBP* rs2282679 polymorphism on chromosome 4q12-q13 was genotyped using a TaqMan® assay with allele-specific probes on an ABI Prism® 7500 Real-Time PCR System (Applied Biosystems, Foster City CA, USA). These polymorphisms were chosen because of recently large genome-wide association studies in populations of European descent reported that this genotype had the strongest association with vitamin D deficiency [9,10] and a similar result was reported in two studies of Chinese people [11,12].

Fetuin-A level

Serum fetuin-A level was measured by sandwich enzyme immunoassay (R&D Systems, Minneapolis MN, USA). Intra- and inter-assay precisions were 4.9% and 7.3%, respectively.

Serum 25(OH)D measurement

Serum $25(OH)D_2$ and $25(OH)D_3$ were analyzed by LC-MS/MS with an Agilent 1200 Infinity liquid chromatograph (Agilent Technologies, Waldbronn, Germany) coupled to a QTRAP® 5500 tandem mass spectrometer (AB SCIEX, Framingham MA, USA) using a MassChrom® 25-OH-Vitamin D_3/D_2 diagnostics kit (ChromSystems, Gräfelfing, Germany). The summation of serum $25(OH)D_2$ and $25(OH)D_3$ [total 25(OH)D] was used to reflect vitamin D status. Vitamin D deficiency was defined as having 25(OH)D levels of less than 50 nmol/L [20 ng/mL] [21]. The inter-assay and intra-assay coefficients of variation of total serum 25(OH)D level were 6.3% and 5.0%, respectively.

Serum P1NP and CTx-I levels

Serum P1NP and CTx-I levels were determined by electrochemiluminescence immunoassay on a Cobas e 411 analyzer (Roche Diagnostics, Mannheim, Germany). The assays had intra-assay precision of 5.4% and 3.8%, respectively.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). All data were normally distributed. Differences between males and females were assessed by Student's *t*-test. Multiple linear regression analysis was performed to identify the association between lumbar spine L1–L4 BMD, femoral neck BMD, total hip BMD, serum P1NP and serum CTx-I (the dependent variables) and age,

BMI, gender, 25(OH)D, *DBP* rs2282679 genotypes, the interaction between 25(OH)D and *DBP* genotypes [25 (OH)D × *DBP* rs2282679 genotypes] and fetuin-A. Subjects were then classified into three groups according to *DBP* rs2282679 genotype, and the association between all dependent variables and age, BMI, gender, 25(OH)D and fetuin-A were reassessed by multiple linear regression analysis. The polymorphism of *DBP* rs2282679 genotype were assigned as the following; AA: homozygous referent genotype, CA: heterozygous genotype and CC: homozygous variant genotype. A p value less than 0.05 was considered statistically significant. All analyses were performed using the SPSS statistical software package, version 17.0 (SPSS, Chicago IL, USA).

Results

The mean age of the subjects was 39.9 ± 6.6 years, and most of the subjects were males (72%) due to the demographics of the EGAT workforce. A data comparison between males and females (Table 1) revealed that males were slightly older, and had significantly higher BMI, WC, femoral neck and total hip BMD, serum P1NP and serum CTx-I. With regard to vitamin D status, mean total 25(OH)D concentrations were significantly higher in males than in females (65.20 ± 14.74 vs. 53.64 ± 12.90 nmol/L, $p < 0.001$; Table 1). As expected, females had a higher prevalence of vitamin D deficiency: 41.4% of females had 25(OH)D less than 50 nmol/L, whereas 13.6% of males were classified as vitamin D deficient. The distribution of 25(OH)D levels in total subject, male and female was shown in Table 2.

The *DBP* rs2282679 genotype distribution conformed to the Hardy–Weinberg equilibrium: i.e. AA (999; 57.6%), CA (637; 36.7%), and CC (98; 5.7%). Other than 25(OH)D levels, there were no differences in clinical characteristics between subjects in each *DBP* genotype of the entire cohort (combine males and females; Table 2) or in subgroup of males or females (data not showed). For vitamin D status, subjects with the AA genotype had the highest 25(OH)D levels (64.6 ± 15.5 nmol/L) compared with those in the CA and CC groups (59.2 ± 14.2 and 53.0 ± 10.6 nmol/L, respectively; p <0.001, Table 1). For each *DBP* genotype, 25(OH)D levels in males were higher than in females, as presented in Figure 1. 25(OH)D levels in each *DBP* genotype were all significantly different in males (Figure 2). In female, the difference in 25 (OH)D levels were found only between those in CC vs. AA and CA vs. AA genotype (Figure 2).

Table 3 shows the associations between BMD, serum P1NP and serum CTx-I, and demographic parameters, 25(OH)D and fetuin-A. Age, gender and BMI were independently associated with BMD at all skeletal sites, as well as serum P1NP and serum CTx-I. 25(OH)D levels were not correlated with any dependent variables, whereas *DBP* genotype tended to be associated with total hip BMD and serum P1NP ($p = 0.07$ and $p = 0.09$, respectively). A weak correlation between fetuin-A and total hip BMD was found ($\beta = -0.05$, $p = 0.02$). Since circulating 25(OH)D binds to DBP, it is likely that there is an interaction between 25(OH)D and *DBP* genotypes. The femoral BMD tended to correlate to the interaction between 25(OH)D and *DBP* genotypes ($p = 0.09$). On the

Table 1 The clinical characteristics of the study population

	Total (n = 1,734)	Male (n = 1,246)	Female (n = 488)	p value (male vs. female)
Age (years)	39.87 ± 6.65	40.07 ± 6.80	39.35 ± 6.23	<0.01
BW (kg)	66.42 ± 12.93	70.68 ± 11.38	55.55 ± 9.92	<0.001
BMI (kg/m^2)	23.94 ± 3.76	24.59 ± 3.54	22.27 ± 3.80	<0.001
Waist circumference (cm)	86.11 ± 10.46	89.09 ± 9.42	78.51 ± 9.08	<0.001
Waist–hip ratio	0.88 ± 0.06	0.90 ± 0.05	0.82 ± 0.06	<0.001
Lumbar spine (L1–L4) BMD (g/m^2)	0.98 ± 0.12	0.98 ± 0.12	0.98 ± 0.10	NS
Femoral neck BMD (g/m^2)	0.80 ± 0.12	0.82 ± 0.12	0.75 ± 0.10	<0.001
Total hip BMD (g/m^2)	0.92 ± 0.15	0.95 ± 0.14	0.86 ± 0.15	<0.001
Serum P1NP (ng/mL)	46.28 ± 18.02	48.55 ± 18.48	40.48 ± 15.35	<0.001
Serum CTx-I (ng/mL)	0.35 ± 0.16	0.39 ± 0.16	0.25 ± 0.12	<0.001
Total 25(OH)D (nmol/L)	61.95 ± 15.17	65.20 ± 14.74	53.64 ± 12.90	<0.001
Subgroup stratified by 25(OH)D levels				
<50 nmol/L	42.93 ± 5.82 (n = 371)	44.14 ± 4.40 (n = 169)	41.92 ± 6.63 (n = 202)	<0.001
50 to < 75 nmol/L	61.68 ± 6.67 (n = 1,051)	62.28 ± 6.61 (n = 790)	59.87 ± 6.55 (n = 261)	<0.001
≥75 nmol/L	85.45 ± 10.25 (n = 312)	85.64 ± 10.46 (n = 287)	83.26 ± 7.23 (n = 25)	NS
Fetuin-A (μg/mL)	559.16 ± 110.64	560.87 ± 110.29	554.79 ± 111.53	NS

Data is expressed as mean ± SD.

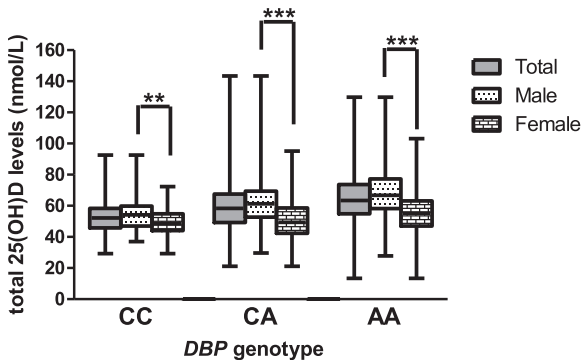

Figure 1 25(OH)D levels in total subjects, male and female subjects according to *DBP* rs2282679 genotype. The square box represented mean ± SD. The upper and lower bar represented the upper and lower values. $^{**}P < 0.01$ for comparison of 25(OH)D levels between male and female in CC group. $^{***}P < 0.001$ for comparison of 25(OH)D levels between male and female in CA and AA group.

other hand, this interaction did not significantly correlate to L1–L4 BMD and bone turnover markers. Subgroup analysis in subject with 25(OH)D < 50 nmol/L (n = 371) did not demonstrate the association between 25(OH)D levels and BMD at all skeletal sites and bone markers (Additional file 1: Table S1).

Because of the potential interactions, data were analyzed according to *DBP* genotype. 25(OH)D levels were significantly related to BMD and bone markers, independently of age, gender and BMI – but only in subjects with the AA genotype (Table 4C). Interestingly, fetuin-A was significantly correlated with total hip BMD (β = –0.09, p <0.01), and tended toward a negative correlation with femoral neck BMD (β = –0.05, $p = 0.08$) in subjects with the AA genotype (Table 3C). The correlation effect of both 25(OH)D and fetuin-A was comparable, but in an opposite direction, for total hip BMD, as suggested by the standardized regression coefficients of 0.08 and –0.09, respectively (Table 4C). The result of the correlation

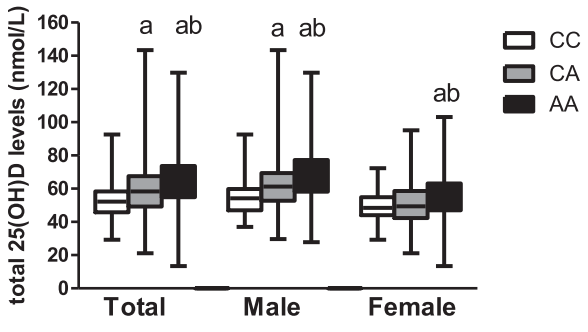

Figure 2 25(OH)D levels of each *DBP* rs2282679 genotype in total subject, male and female. The square box represented mean ± SD. The upper and lower bar represented the upper and lower values. **a**: p < 0.05, different from CC group. **b**: p < 0.05, different from CA group.

between 25(OH)D and BMD and bone markers in subject with vitamin D deficiency was slightly different from the result of the entire cohort. Multiple regression analysis demonstrated the association between 25(OH)D levels and serum P1NP (β = –0.18, $p = 0.02$) in subjects with the CA genotype, independently of age, gender and BMI (Additional file 1: Table S2). In subjects with the AA genotype, 25(OH)D levels were significantly related to L1–L4 BMD, femoral neck BMD and serum CTx (β = 0.30, $p <$ 0.001; β = 0.15, $p = 0.035$ and β = -0.15, $p = 0.03$, respectively). On the other hand, the correlation of 25(OH)D levels and total hip BMD and serum P1NP were no longer existed (Additional file 1: Table S2).

Discussion

The present findings demonstrated a correlation between femoral BMD and the interaction between vitamin D status, as measured by circulating 25(OH)D, and *DBP* genotypes. It should be noted that statistical interaction does not necessarily reflect true biological interaction. However, a number of biological bases exist which may underlie the observed statistical interaction.

Vitamin D metabolites are mainly transported in the circulation by vitamin D-binding protein (DBP); that is, about 85–90% of 25(OH)D and $1,25(OH)_2D$ are bound to DBP. Ten to 15% of vitamin D metabolites circulate weakly bound to albumin, while less than 1% of circulating vitamin D is in free form [22]. Vitamin D bound to DBP is transported within the organism, facilitating access of vitamin D to various tissues and cell types as well as regulating the total amount of vitamin D available for the organism [23]. For example, at the renal proximal tubules, megalin (a cell surface receptor for DBP) internalizes DBP-bound 25(OH)D through endocytosis, and thus free 25(OH)D is further metabolized by renal 1α-hydroxylase [7,24]. In addition, cubilin (another receptor for DBP) and megalin were both detected in osteoblast-like cell lines and human primary osteoblasts cell culture, suggesting that osteoblast are able to internalize DBP-25(OH)D complex *in vivo* [2]. DBP not only acts as a high-affinity serum transporter, but can function as a macrophage-activating factor and actin binder. The direct effect of DBP on bone, independently of its ligands, is not clearly understood. However, most extra-renal tissues do not appear to express megalin or its associated co-receptors, suggesting that these tissues are more likely to acquire 25(OH)D in free form or as bio-available 25 (OH)D – i.e. the sum of the free and the albumin-bound fraction of 25(OH)D – and not DBP-bound 25(OH)D [7]. In other words, the free and/or bio-available fraction of 25 (OH)D may be more strongly linked to biological effects than the total form. Since only total 25(OH)D is generally assessed in observational and interventional studies, this may explain inconsistencies in the association of total

Table 2 The clinical characteristics of the study population stratified by *DBP* rs2282679 genotype

	DBP genotype			*P* value
	CC (n = 98)	CA (n = 637)	AA (n = 999)	
M/F (n,%)	69/29 (70.4/29.6)	465/172 (73/27)	712/287 (71.3/28.7)	NS
Age (years)	40.08 ± 6.97	39.96 ± 6.60	39.79 ± 6.66	NS
BW (kg)	66.51 ± 12.09	66.60 ± 13.19	66.30 ± 12.84	NS
BMI (kg/m^2)	23.67 ± 3.40	23.93 ± 3.82	23.97 ± 3.76	NS
WC (cm.)	86.41 ± 9.90	86.28 ± 10.80	85.98 ± 10.31	NS
WHR	0.88 ± 0.06	0.88 ± 0.07	0.88 ± 0.06	NS
Lumbar spine L1-4 BMD (g/m^2)	0.98 ± 0.11	0.98 ± 0.12	0.98 ± 0.12	NS
Femoral neck BMD (g/m^2)	0.80 ± 0.11	0.80 ± 0.12	0.80 ± 0.12	NS
Total hip BMD (g/m^2)	0.92 ± 0.11	0.92 ± 0.13	0.92 ± 0.16	NS
Serum P1NP (ng/mL)	46.35 ± 15.20	46.22 ± 18.55	46.31 ± 17.95	NS
Serum CTx (ng/mL)	0.36 ± 0.15	0.35 ± 0.16	0.35 ± 0.16	NS
Total 25(OH)D (nmol/L)	53.0 ± 10.6	59.2 ± 14.2	64.6 ± 15.5	<0.001
Fetuin-A (μg/mL)	563.71 ± 112.35	563.01 ± 110.24	556.25 ± 110.75	NS

Data is expressed as mean ± SD.

25(OH)D and health outcomes, including BMD. Supporting this free-hormone hypothesis, some studies have suggested that free or bio-available 25(OH)D, as opposed to total 25(OH)D, is more strongly correlated with BMD [25,26].

Accumulated evidence suggests the influence of *DBP* polymorphism on circulating 25(OH)D. Most studies have investigated three major polymorphic forms of *DBP*: GC1F, GC1S and GC2 (rs7041 and rs4588) [27]. These *DBP* variants exhibit differences in affinity to 25(OH)D and 1,25$(OH)_2$D, with the hierarchy of affinity binding GC1F > GC1S > GC2 [8]. Thus, subjects with GC1F alleles had the highest total 25(OH)D levels, while subjects with GC2 had the lowest [28]. Recently, two large genome-wide association studies in populations of European descent reported that rs2282679, another *DBP* polymorphism, had the strongest association with vitamin D deficiency [9,10]. A similar result was reported in two studies of Chinese people [11,12]. That is consistent with the present findings, where total 25(OH)D levels in subjects who had the minor genotype (CC) were about 11 nmol/L lower than in those who had the major genotype (AA). We noticed that the prevalence of *DBP* rs2282679 genotypes in Thais was slightly different than those reported in Chinese [11]: i.e. AA genotype, 57% vs. 45–48%; CA genotype, 37% vs. 42–48%; and CC genotype, 6% vs. 10–11% (in Thais and Chinese, respectively).

Findings from in vitro studies, such as in monocytes [29], dendritic cells [30] and keratinocytes [31], suggest that the biological effects of vitamin D are dependent on both the serum concentration of free 25(OH)D and the *DBP* genotype. For example, monocytes exposed to 25(OH)D showed less induction of antimicrobial cathelicidin in the presence of DBP, while there was much more potent induction of cathelicidin in human cell cultures containing lower-affinity forms of DBP [29]. Similarly, the ability of 25(OH)D to induce dendritic cells to become tolerogenic regulatory T cells was found to be enhanced by either a lower concentration of DBP or by the presence of lower-affinity genetic variants of *DBP* [30].

Table 3 The association between BMD, serum P1NP, serum CTX and age, BMI, gender, 25(OH)D, *DBP* rs2282679 genotype, 25(OH)D χ *DBP* rs2282679 genotype and fetuin-A by multiple regression analysis

	Lumbar spine L1-L4 BMD		Femoral neck BMD		Total hip BMD		Serum P1NP		Serum CTx-I	
	β	p	β	p	β	p	β	p	β	p
Age	-0.09	<0.001	-0.20	<0.001	-0.12	<0.001	-0.19	<0.001	-0.21	<0.001
BMI	0.22	<0.001	0.39	<0.001	0.37	<0.001	-0.05	0.05	-0.08	<0.001
Male gender	-0.06	0.03	0.13	<0.001	0.17	<0.001	0.25	<0.001	0.44	<0.001
25(OH)D	-0.02	NS	-0.10	NS	-0.11	NS	0.10	NS	0.08	NS
DBP rs2282679 genotype	-0.09	NS	-0.14	NS	-0.17	0.07	0.17	0.09	0.13	NS
25(OH)D χ *DBP* rs2282679 genotype	0.11	NS	0.25	0.09	0.26	0.09	-0.25	NS	-0.21	NS
Fetuin-A	-0.02	NS	-0.02	NS	-0.05	0.02	0.04	NS	-0.01	NS

Based on biological plausibility and statistical interaction between 25(OH)D and *DBP* genotypes, we classified subjects according to *DBP* genotype. Our method differed from previous studies [25,26] in that we did not assess DBP levels directly and did not calculate the amounts of free and bio-available 25(OH)D. Nevertheless, it was found that 25(OH)D was significantly related to BMD and bone markers, independently of age, gender and BMI – but only in subjects with the AA genotype. The analysis in subjects with vitamin D deficiency was mostly corresponded with the result of the entire cohort. Even more the stronger correlation between 25(OH)D levels to L1–L4 BMD, femoral neck BMD and serum CTx were found (Additional file 1: Table S2). We propose that the difference in affinity of vitamin D ligands for DBP and the difference in amount of free and bio-availability forms of 25(OH)D in each *DBP* genotype could underlie our finding. Our study demonstrated that 25(OH)D levels was highest in subjects in AA genotype. Thus, the affinity of vitamin D ligands for DBP is possibly highest in the AA genotype and lowest in the CC genotype. It would be the case that DBP-bound 25 (OH)D of subject in AA genotype more reuptake at the proximal tubule, provide more 25(OH)D for renal synthesis of $1,25(OH)_2D$ to facilitate circulating levels of this hormone and support endocrine function [27], including bone health. About free hormone hypothesis, a study of Johnsen et al. which explored the effects of rs7041 and rs4588 polymorphisms on BMD, reported that the correlation of the free and bio-available forms of 25(OH)D with bone density were stronger after adjusting for these common polymorphisms [25]. Otherwise, to date there has been little published information concerning the influence of *DBP* rs2282679 polymorphism on BMD. Measurement of DBP levels and additional calculation for DBP-bound/free 25(OH)D in the recent cohort are further warranted to prove this hypothesis. With regard to gender, 25(OH)D of subjects with AA genotype was independently associated with L1-L4 BMD only in females and associated with femoral neck and total hip BMD only in males (data not showed).

Fetuin-A was also demonstrated in the present study to be related to BMD at femoral sites. Fetuin-A is a multifunctional protein mainly of hepatic origin, and plays key roles in calcium and bone metabolism as well as in glucose and energy homeostasis [32]. Fetuin-A is a natural inhibitor of metastatic calcification [32]. However, it has been demonstrated in a number of studies that fetuin-A is positively correlated with bone mass [14,15]. This seemingly contradictory observation has been reconciled by a recent study taking into account the size-selective permeability of collagen fibrils [33]. Although the present study revealed an association between fetuin-A and BMD, the direction of the association was the opposite of previous findings among the elderly

Table 4 The association between BMD, serum P1NP, serum CTX and age, BMI, gender, 25(OH)D and fetuin-A by multiple regression analysis in subjects stratified by the *DBP* rs2282679 genotype

	Lumbar spine L1-L4 BMD		Femoral neck BMD		Total hip BMD		Serum P1NP		Serum CTx	
	β	p	β	p	β	p	β	p	β	p
A: *DBP* genotype = CC (n = 98)										
Age	-0.13	NS	-0.14	NS	-0.15	NS	-0.25	0.02	-0.20	0.04
BMI	0.23	0.04	0.42	<0.001	0.51	<0.001	0.01	NS	-0.07	NS
Male gender	0.05	NS	0.25	0.01	0.23	0.02	0.08	NS	0.41	<0.001
25(OH)D	0.02	NS	0.01	NS	-0.05	NS	0.05	NS	0.05	NS
Fetuin-A	-0.07	NS	-0.10	NS	-0.08	NS	0.19	0.07	0.16	0.08
B: *DBP* genotype = CA (n = 637)										
Age	-0.09	0.03	-0.21	<0.001	-0.15	<0.001	-0.15	<0.001	-0.19	<0.001
BMI	0.21	<0.001	0.38	<0.001	0.36	<0.001	-0.04	NS	-0.07	0.06
Male gender	-0.03	NS	0.13	<0.01	0.20	<0.01	0.22	<0.001	0.40	<0.001
25(OH)D	0.01	NS	0.02	NS	0.02	NS	-0.01	NS	-0.01	NS
Fetuin-A	0.00	NS	0.04	NS	0.03	NS	0.05	NS	-0.03	NS
C: *DBP* genotype = AA (n = 999)										
Age	-0.08	0.01	-0.19	<0.001	-0.10	<0.01	-0.21	<0.001	-0.23	<0.001
BMI	0.22	<0.001	0.39	<0.001	0.37	<0.001	-0.06	0.07	-0.09	0.003
Male gender	-0.09	0.01	0.13	<0.001	0.15	<0.001	0.28	<0.001	0.46	<0.001
25(OH)D	0.08	0.01	0.10	<0.01	0.08	<0.01	-0.11	0.001	-0.10	0.002
Fetuin-A	-0.04	NS	-0.05	0.08	-0.09	<0.01	0.02	NS	-0.01	NS

[14,15], which demonstrated a positive correlation. One of the differences in the present study which may account for this anomaly is the relatively young age of the study population. Fetuin-A apparently possesses a biphasic response, the underlying basis of which is not entirely clear [34]. The present study also demonstrated that the association of fetuin-A with bone mass varied according to *DBP* genotype, and that this effect was independent of vitamin D status. This observation requires further confirmation, however, and should be taken into account in future studies investigating the effect of fetuin-A on bone.

A number of limitations are present in our study. As mentioned above, our study population was relatively young, with an age range of 25–54 years. Generalizability, if applicable, of our results is therefore limited to this particular age group. The circulating levels of vitamin D-binding protein were not measured, and so we were not able to determine if the interaction between the vitamin D-binding protein gene and vitamin D status was also applicable and could be explained by variations in circulating vitamin D-binding protein. Finally, calcium intake data was not available in the present study.

In conclusion, in young healthy Thai adults, interaction between vitamin D status, as measured by circulating 25(OH)D and *DBP* rs2282679 genotypes, modified the association between total 25(OH)D and bone density and bone turnover markers. Differences in *DBP* genotypes additionally influenced the correlation of fetuin-A levels with femoral sites BMD.

Additional file

Additional file 1: Table S1. The association between BMD, serum P1NP, serum CTX and age, BMI, gender, 25(OH)D, *DBP* rs2282679 genotype, 25(OH)D χ *DBP* rs2282679 genotype and fetuin-A by multiple regression analysis in subjects with 25(OH)D < 50 nmol/L (n = 371). **Table S2.** The association between BMD, serum P1NP, serum CTX and age, BMI, gender, 25(OH)D and fetuin-A by multiple regression analysis in subjects with 25(OH)D < 50 nmol/L and stratified by the *DBP* rs2282679 genotype.

Abbreviations

DBP: Vitamin D-binding protein; 25(OH)D: 25-hydroxyvitamin D; BMD: Bone mineral density; P1NP: N-terminal propeptides of type 1 procollagen; CTx-I: C-terminal cross-linking telopeptides of type I collagen; EGAT: Electricity Generating Authority of Thailand; WC: Waist circumference; BMI: Body mass index; DXA: Dual-energy X-ray absorptiometry; ISCD: The International Society for Clinical Densitometry; SD: Standard deviation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

HN, WR, PS and BO conceived of the study, participated in its design and coordination, performed the statistical analysis and helped to draft the manuscript. CS carried out the BMD measurement. LC carried out the vitamin D metabolites measurement (LC-MS/MS). SC carried out the genotyping of rs2282679 in the *DBP* gene. All authors read and approved the final manuscript.

Acknowledgements

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This study was supported by the Thailand Research Fund and Mahidol University.

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Dietary animal and plant protein intakes and their associations with obesity and cardio-metabolic indicators in European adolescents: the HELENA cross-sectional study

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Abstract

Background: Previous studies suggest that dietary protein might play a beneficial role in combating obesity and its related chronic diseases. Total, animal and plant protein intakes and their associations with anthropometry and serum biomarkers in European adolescents using one standardised methodology across European countries are not well documented.

Objectives: To evaluate total, animal and plant protein intakes in European adolescents stratified by gender and age, and to investigate their associations with cardio-metabolic indicators (anthropometry and biomarkers).

Methods: The current analysis included 1804 randomly selected adolescents participating in the HELENA study (conducted in 2006–2007) aged 12.5-17.5 y (47% males) who completed two non-consecutive computerised 24-h dietary recalls. Associations between animal and plant protein intakes, and anthropometry and serum biomarkers were examined with General linear Model multivariate analysis.

Results: Average total protein intake exceeded the recommendations of World Health Organization and European Food Safety Authority. Mean total protein intake was 96 g/d (59% derived from animal protein). Total, animal and plant protein intakes (g/d) were significantly lower in females than in males and total and plant protein intakes were lower in younger participants (12.5-14.9 y). Protein intake was significantly lower in underweight subjects and higher in obese ones; the direction of the relationship was reversed after adjustments for body weight (g/(kg.d)). The inverse association of plant protein intakes was stronger with BMI z-score and body fat percentage (BF%) compared to animal protein intakes. Additionally, BMI and BF% were positively associated with energy percentage of animal protein.

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Conclusions: This sample of European adolescents appeared to have adequate total protein intake. Our findings suggest that plant protein intakes may play a role in preventing obesity among European adolescents. Further longitudinal studies are needed to investigate the potential beneficial effects observed in this study in the prevention of obesity and related chronic diseases.

Keywords: Protein intake, Adolescence, Body composition, Biomarkers, HELENA study

Introduction

The prevalence of overweight (OW) and obesity (OB) in adolescents, defined on the basis of body mass [1], has increased rapidly worldwide. In 2010, the estimated prevalence of OW and OB in European children and adolescents was approximately 38%, including 10% OB [2]. As a consequence of OB-related co-morbidities, over 20000 children suffer from type 2 diabetes and more than 400000 have impaired glucose levels [2]. Childhood OW and OB both influence long-term health and evidence suggest an association with coronary events and mortality later in life [3,4].

Nutrition during the early years of life is a critical factor of OB in adolescence further impacting on adulthood OW and OB, and the consequences of chronic diseases [5,6]. High protein intakes were reported to improve cardiovascular risk factors including abdominal OB, dyslipidemia, glucose intolerance, and hypertension in European children (5–18 y) [7]. Previous randomised trials [8,9] suggest that a high-protein diet defined as ≥20% of total energy lowers the risk of OW and promotes weight maintenance among adolescents [10]. The association between dietary protein intake and adolescent OW and OB has mainly been investigated in relation to its increased thermic effect and satiety when compared to fats and carbohydrates [9,11]. Others, however, have reported that higher protein content in the diet did not confer any benefit in the treatment of OB among children 9–18 y old [12].

The debate on protein sources is still ongoing, addressing the nutritional quality of dietary proteins based on their amino acids composition. The protein quality or biological value of proteins from animal sources is high, whereas most plant proteins lack one or more essential amino acids and are therefore considered as incomplete proteins. What some seem to be concerned with is that the majority of high-protein foods are significant sources of fat and/or sugar as well (such as meat and meat products, cheese, and dairy desserts), and should therefore be carefully selected. Hermanussen *et al.* reported a positive correlation between the energy contribution of animal proteins to the diet and the body mass index (BMI) in adolescents [13]. On the other hand, Bradlee *et al.* found no association between OB and meat consumption among adolescents [14], while, plant-based diets were inversely associated with normal BMI in children in Hermanussen's study [13]. A Western dietary pattern high in animal sources is associated with an increased risk of metabolic syndrome (MetS) [15,16], whereas diets high in fruits, vegetables and whole grains are associated with a decreased risk [17]. Evidence showed that plant protein, soy in particular, can bind phytoestrogen compounds to stimulate lipid metabolism resulting in a better blood profile, by lowering total cholesterol (TC), triglyceride (TG), low-density lipoprotein-cholesterol (LDL-C) and reducing insulin resistance [18,19].

The aim of the current study was to evaluate total, animal and plant protein intakes in European adolescents and to investigate their association with cardio-metabolic indicators (anthropometry: BMI z-score and body fat percentage (BF%); and biomarkers: TC, TG, LDL-C, very LDL-C (VLDL-C), high-density lipoprotein cholesterol (HDL-C), C-reactive protein (CRP), glucose, insulin and leptin).

Methods

Survey population

The Healthy Lifestyle in Europe by Nutrition in Adolescence-Cross Sectional Study (HELENA-CSS) is a European Commission funded project on lifestyle and nutrition among adolescents from 10 cities of European countries: Stockholm, Athens, Heraklion, Rome, Zaragoza, Ghent, Lille, Dortmund,Vienna, and Pecs that ran between October 2006 and December 2007. Due to logistical reasons, adolescents from Heraklion and Pecs were excluded for the dietary intake assessments. A multi-stage random cluster sampling procedure was used to select 3528 adolescents, stratified by geographical location, age and socioeconomic status (SES). Schools were randomly selected after stratification to guarantee diversity of the sample in culture and SES.

Male and female adolescents, aged 12.5-17.5 y, not participating simultaneously in a clinical trial, free of any acute infection lasting less than 1 week before inclusion year, and who provided two 24-h recall interviews with valid information and complete anthropometric measurements, were included in the final analysis of the current study. Details on sampling procedures, study design and non-respondents have been reported elsewhere [20,21].

The study was approved by the Research Ethics Committees of each city involved. Written informed consent

was obtained from the adolescents' parents and the adolescents themselves [22].

Dietary intake assessment

Two non-consecutive computerised 24-h dietary recalls (HELENA-DIAT), instructed by dieticians/researchers, were used to collect food consumption data. During interviews, adolescents were allowed to ask questions and following completion the recall was checked for completeness. Each participant was asked to complete the recall twice in a time-span of 2 weeks during the school time.

HELENA-DIAT is a self-administered computer program based on the Young Adolescents' Nutrition Assessment on Computer (YANA-C) [23], consisting of a single computerised 24-h recall with a structured program based on six meal occasions. The validated YANA-C [23], was designed to obtain a detailed description and quantification of foods consumed, and eventually included about 800 food items hierarchically organized in 25 food groups, and about 300 colored photograph sets of foods in different portions [24,25].

Dietary intakes were linked to the German Food Code and Nutrient DataBase (BLS (Bundeslebensmittelschlüssel), version II.3.1, 2011) [26]. However, the estimated percentage of animal and plant protein intakes were calculated by linking the 24-h recall food consumption data to the Belgian NUBEL [27], the Dutch NEVO [28] and the USDA [29] food composition databases which used the Kjeldahl method for analysing protein [30], because no differentiation was made between plant and animal proteins in the BLS database. Protein intakes were calculated in absolute terms (g/d) and relative terms (energy percentages (E%); per kg body weight).

Under-reporters, excluded in the current study, were considered as individuals with a ratio of energy intake over estimated basal metabolic rate lower than 0.96 [31].

Anthropometric measurements

Weight (kg) and height (m) were measured in underwear and barefoot to the nearest 0.1 kg and 0.1 cm, respectively, by trained researchers. BMI was calculated as weight (kg)/height (m^2). Participants were classified into four BMI categories according to the International Obesity Task Force (IOTF) cut-offs for adolescents [1]: equivalent to underweight (UW) (<18.5 kg/m^2), normal weight (NW) (18.5-24.9 kg/m^2), OW (25.0-29.9 kg/m^2), and OB (≥30.0 kg/m^2). Standard deviation score of BMI (BMI z-score) was calculated using the lmsGrowth method [32]. The cut-off of BMI z-score [33]: UW (<−2), NW (−2 -1), OW (>1) and OB (>2). Skinfold thickness was measured to the nearest 0.2 mm in triplicate [34]. The same trained investigators made all measurements (inter-rater reliability >95 %). BF% was calculated using Slaughter's equations [35]. More details about the anthropometric measurements are given in a previous manuscript [34]. Physical maturations were examined by a physician during a medical examination to determine the pubertal status based on Tanner stages [36]. The final physical maturations were classified into three categories: pre-pubertal (stage 1); pubertal (stage 2 to 4) and post-pubertal (stage 5).

Blood samples

Blood samples were collected in a randomly selected subsample of the total HELENA-CSS. Adolescents who agreed to be involved in the blood sampling were asked to fast after 8 pm on the previous day. Fasting blood samples, information of adolescents' medical history and recent acute diseases were collected by venipuncture between 8–10 a.m. at schools or hospitals by a medical doctor, A blood sampling questionnaire was completed by the participants for the purposes of assessing fasting status, acute infection, allergies, smoking, vitamin and mineral supplements, and medication. A specific handling, transport and traceability system for biological samples was developed for the HELENA study. All samples were analyzed centrally. The blood sampling procedure has been described elsewhere [37].

Physical activity

Physical activity (PA) was assessed for 7 days by an uniaxial accelerometer (Actigraph GT1M), described previously [38]. At least 3 days of recording with a minimum of 8 hours' registration per day was set as an inclusion criterion. PA, used in the current study, was categorized in the following categories: at least 1 hour of PA per day, no PA or less than 1 hour of PA per day.

Statistical analysis

Descriptive data is presented as means with standard deviation or frequency distributions. Energy and total, animal and plant protein intakes were corrected for within-person variation using the Multiple Source Method (MSM), which is suitable for estimating population's usual intakes [39]. Statistical differences for total energy and total, animal and plant protein intakes between subgroups (gender and age) were assessed using the Student *T*-test and ANOVA.

GLM multivariate analysis was used to investigate the associations of indicators (dependent variables) with animal and plant protein intakes, and animal (E%) and plant (E%) energy percentages (independent variables) through three models (stepwise approach): (1) model 1 = unadjusted model; (2) model 2 = model 1 + adjusted for fat intake; (3) model 3 = model 2 + further adjusted for PA, confounding factors and interactions, and controlling for the country clustering effect. Potential confounding factors including age (younger group (12.5-14.9 y) and

older group (15.0-17.5 y)), gender, tanner stage (pre-puberty, puberty and post-puberty) and two-way interactions between potential confounding factors and independent variables were included in the model 3. Anthropometry and serum biomarkers were investigated separately. In addition, animal and plant protein intakes, and the energy percentage (E%) from animal and plant protein were examined in a separate model due to colinearity.

All statistical analysis were performed using the statistical software SPSS for Windows version 18 (SPSS Inc, Chicago, IL, USA). Results were considered statistically significant at α two-tailed level of 0.05.

Results

A total of 1804 out of 3528 adolescents (47% males) from 8 centres with valid and complete dietary data and measurements of weight and height were included in the analysis (Table 1). 74% participants were classified in tanner stage 2–4, including 7% in tanner 2, 24% in tanner 3 and 41% in tanner 4. In total 279 adolescents were classified as OW and OB. Mean BMI z-score for both genders was in the NW range. Females had higher BF %, but lower BMI z-score compared to males. Furthermore, higher serum lipid profiles and leptin levels were found in females.

Total energy and total, animal and plant protein intakes

Median total protein contributing to energy intake was 15.5%. Average total protein intakes exceeded the World Health Organization (WHO) recommendations (10.0 – 15.0% of the total energy intake) [40] and the estimated average requirements (EAR) and population reference intake (PRI) of the European Food Safety Authority (EFSA) (EAR: 0.66 g/(kg.d) for both genders; PRI: males, 0.70-0.74 (g/(kg.d), and females, 0.67-0.72 g/(kg.d)) [41] (Table 2). All but one adolescent met the EAR, while, fourteen and two adolescents did not reach the WHO recommendations for protein intakes and the PRI, respectively.

Mean total protein intake (384 kcal/d) contributed 15.8% to total energy intake. Mean animal protein intakes were the main contributor (59%) to total protein intakes, as opposed to mean plant protein (Table 3). Total and plant protein intakes were significantly lower in females and the younger group. Body weight adjusted total protein intakes and E% from total protein were significantly lower in the older group. Total energy, total and animal protein intakes and total protein (E%) were higher in obese adolescents than non-obese ones. More specifically, body weight adjusted total protein intake (g/(kg.d)) was significantly lower in OB, and higher in UW peers.

Associations between total, animal and plant protein intakes and cardio-metabolic indicators

Figure 1 shows a significant decline in BF% across the total protein tertiles ($P < 0.001$) by age. But no significance was observed in males and females. The results of the GLM multivariate analysis showed that crude BF% was inversely associated with absolute animal and plant protein in model 1, but crude BMI z-score and BF% were positively associated with animal protein (E%) (Table 4). Absolute animal protein intake was inversely associated with crude serum biomarkers including TC, TG, VLDL-C and leptin, but positively with serum fasting glucose. While absolute plant protein intake was inversely associated with crude TC, HDL-C, and leptin, but positively with serum fasting glucose. After adjustments for fat intake (Model 2), BMI z-score became positively associated with absolute animal protein intake, but several significant associations found in model 1 disappeared. Leptin kept to be inversely associated with absolute animal protein intake in model 2, and BF%, TC and HDL-C with absolute plant protein intake. Only serum HDL-C became positively associated with absolute animal protein intake, after further adjusting for confounding factors, PA and interaction factors (Model 3). Inverse associations were observed between BMI z-scores and BF%, and absolute plant protein intake. Whereas both BMI z-scores and BF% were positively associated with animal protein (E%). No biomarker was associated with percentage of energy intake derived from animal and plant protein (data not shown).

Discussion

The HELENA study is the first large-scale European adolescent population-based dietary survey of 8 European countries providing data on the nutritional intake, status, main determinants of food choices and preferences among European adolescents. The current study is the first to provide information on intakes of total, animal and plant proteins and their associations with OB and cardio-metabolic indicators.

Total energy and total, animal and plant protein intakes

The contribution of protein to energy intake in our study was similar to that reported in Greek and Italian adolescents, lower than that of Spanish peers (male: 17.2%, female: 17.8%) [42], but higher than adolescents in review studies of Western, Central and Eastern European countries [43-45]. In addition, total protein intake was reported to be slightly lower in Italian peers (male: 99 g/d, female: 82 g/d) [46], Spanish males (male: 105 g/d, female: 86 g/d) [42], and Western European adolescents [43,45]. The adolescents in this study had much higher animal and plant protein intakes than those of Belgian peers (male: 52 g/d, female: 37 g/d;

Table 1 Anthropometric characteristics and levels of obesity-related biomarkers in adolescents participating in the HELENA-CSS

	Total	Males	Females
Total participants (n)	1804	855	949
Age (y) (mean (range))	14.7 (12.5-17.4)	14.8 (12.5-17.4)	14.7 (12.5-17.4)
12.5-14.9 y (n)	1032	481	551
15.0-17.5 y (n)	772	374	398
Tanner Stage (n = 1752)		n (%)	
Tanner 1	9 (0.514)	9 (1.1)	0 (0.0)
Tanner 2-4	1294 (73.9)	614 (74.2)	680 (73.5)
Tanner 5	449 (25.6)	204 (24.7)	245 (26.5)
Weight status (n = 1804)[μ]			
Underweight	142 (7.9)	58 (6.8)	84 (8.9)
Normal weight	1383 (76.7)	649 (75.9)	734 (77.3)
Overweight	222 (12.3)	114 (13.3)	108 (11.4)
Obesity	57 (3.2)	34 (4.0)	23 (2.4)
		Mean (SD)	
Anthropometry			
BMI z-score (n = 1804)	0.270 (1.1)	0.358 (1.1)	0.190 (1.0)
BF% (n = 1764)	22.0 (8.6)	18.4 (9.1)	25.1 (6.8)
Biomarkers			
TC (mg/dL) (n = 552)	159.1 (27)	151.9 (24.9)	165.8 (27.1)
TG (mg/dL) (n = 552)	67.6 (31.1)	64.5 (31.5)	70.5 (30.5)
LDL-C (mg/dL) (n = 552)	92.6 (24.2)	89.0 (23.2)	96.0 (24.7)
VLDL-C (mg/dL) (n = 552)	13.5 (6.2)	12.9 (6.3)	14.1 (6.1)
HDL-C (mg/dL) (n = 552)	55.6 (10.3)	53.3 (9.3)	57.8 (10.7)
CRP (mg/L) (n = 524)	1.2 (4.0)	1.5 (5.5)	0.841 (1.3)
Glucose (mg/dL) (n = 552)	90.1 (7.0)	91.9 (7.2)	88.5 (6.4)
Insulin (μIU/mL) (n = 545)	9.5 (6.0)	9.0 (6.6)	10.0 (6.6)
Leptin (ng/mL) (n = 518)	18.5 (21.9)	8.1 (12.9)	27.5 (24.1)

SD, standard deviation; BMI, body mass index; BF%, body fat percentage; TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein- cholesterol; VLDL-C, very low-density lipoprotein- cholesterol; HDL-C, high-density lipoprotein- cholesterol; CRP, c-reactive protein.
[μ]BMI categories is classified based on the International Obesity Task Force cut-offs, underweight: <18.5 kg/m^2, normal weight: 18.5-24.9 kg/m^2, overweight: 25.0-29.9 kg/m^2, obesity: ≥30.0 kg/m^2.

Table 2 Percentile of total protein intakes and the number of the subjects below the recommendations of European food safety authority in the European adolescents

Characteristics	N	Total protein (g/d)			Total protein (g/(kg.d))			The number of subjects below the recommendations	
		25%	50%	75%	25%	50%	75%	EAR[μ]	PRI[μ]
Total	1804	76	91	109	1.3	1.6	2.0	1	2
Gender									
Males	855	90	106	127	1.5	1.8	2.3	0	0
Females	949	68	80	94	1.2	1.5	1.8	1	2
Age									
12.5-14.9 y	1032	74	90	108	1.4	1.7	2.1	1	1
15.0-17.5 y	772	77	94	112	1.3	1.6	1.9	0	1

EAR: estimated average requirement; PRI: population reference intake.
[μ]EAR: 0.66 g/(kg.d) for both genders; PRI : males, 0.70-0.74 g/(kg.d) and females, 0.67-0.72 g/(kg.d).

Table 3 Estimated means of energy, total, animal and plant protein intakes, and energy percentage of protein intakes of adolescents participating in the in HELENA-CSS stratified by gender, age, tanner and BMI category

Characteristics	N	Energy (kcal/d)	Total protein (g/d)	Total protein (g/(kg.d))	Animal protein (g/d)	Plant protein (g/d)	Total protein	Plant protein
							% energy contributing to total energy intake	
				Mean intake (SD)				
Total	1804	2450 (637)	96 (28)	1.7 (0.6)	58 (23)	38 (13)	15.8 (2.8)	6.2 (1.3)
Gender								
Males	855	2792 (655)	110 (29)	1.9 (0.6)	66 (24)	43 (13)	15.9 (3.0)	6.2 (1.3)
Females	949	2141 (428)*	83 (20)*	1.6 (0.5)*	50 (18)*	33 (10)*	15.6 (2.7)	6.3 (1.3)
Age								
12.5-14.9 y	1032	2358 (637)	94 (28)	1.8 (0.6)	57 (22)	37 (12)	16.1 (2.9)	6.2 (1.4)
15.0-17.5 y	772	2752 (713)**	98 (29)**	1.6 (0.5)**	58 (23)	39 (12)**	15.4 (2.8)**	6.2 (1.2)
Weight status								
Underweight	142	2443 (631)	94 (28)	2.2 (0.7)	56 (21)	39 (12)	15.5 (2.7)	6.3 (1.2)
Normal weight	1383	2458 (635)	96 (28)[a]	1.8 (0.6)[a]	58 (22)	38 (13)	15.7 (2.8)	6.2 (1.3)
Overweight	222	2397 (636)	96 (29)[ab]	1.4 (0.4)[ab]	59 (24)	37 (11)	16.2 (3.0)[b]	6.2 (1.3)
Obesity	57	2476 (701)	102 (33)[abc]	1.2 (0.4)[ab]	63 (27)	38 (12)	16.5 (3.1)[b]	6.2 (1.2)

SD, standard deviation.
*Mean value was significantly different between males and females by Student *T*- test ($P < 0.05$).
**Mean value was significantly different from the young group (12.5-14.9 y) by Student *T*- test ($P < 0.05$).
[a]Mean value was significantly different from underweight by ANOVA, ($P < 0.05$, Bonferroni correction.
[b]Mean value was significantly different from normal weight by ANOVA, ($P < 0.05$, Bonferroni correction).
[c]Mean value was significantly different from overweight by ANOVA, ($P < 0.05$, Bonferroni correction).

male: 30 g/d, female: 24 g/d, respectively) [43] and higher plant protein intake (male: 30 g/d, female: 25 g/d), but lower animal protein intake than Spanish peers (male: 74 g/d, female: 60 g/d).

Associations between total, animal and plant protein intakes and cardio-metabolic indicators

Obese HELENA participants consumed more total protein than non-obese participants. Evidence from other European studies indicate higher contribution of animal sources [44,47] to total protein and lower from plant protein consumptions [45], which might point to a relationship between increasing prevalence of OB in European adolescents. Our results suggest that increasing total protein intakes may be inversely associated with adolescents' BF%, which can be explained by plant protein intakes being significantly inversely associated with BMI z-score and BF%, after adjustment for fat intake, PA and confounding factors. Consistent with our findings, observed benefits of increasing total and plant protein intakes on body composition [14,48] could be attributed to the protein effect on increasing stimulated fat oxidation and building of lean body mass [49]. Conversely, the results of a previous randomized trial on obese adolescents (11–16 y) demonstrated that increasing protein consumption conferred no benefit on weight loss and body composition in the treatment of adolescent OB [12]. The different study design and target population might partly explain differences observed. Remarkably, the level of serum leptin was found to be extremely low among males in our study. High levels of leptin can easily be observed in female adolescents, because leptin was reported to play a critical role in the regulation of puberty, especially in females [50]. Serum leptin is proven to be related to BF% [51], and this might partly explain our finding on why females kept high BF% when increasing total protein intake, whereas BF% in males decreased gradually.

Evidence shows that plant protein from vegetables, fruits, and legumes not only improves body composition, but also results in lower body weight compared to animal protein [13,52]. In our study, although animal protein intake was found to be weakly inversely associated with BF%, animal protein (E%) was observed to be positively associated with BF%. Previous studies concluded that total and animal protein intakes might be responsible for increasing body weight and BMI in adolescents [12,13]. Mirkopoulou *et al.* suggested that extremely high protein intakes, animal protein in particular, might increase the risk of adolescents' OB due to higher energy consumption [53]. Furthermore, the results of a longitudinal study suggested that a high animal protein intake in mid-childhood might be associated with an earlier pubertal growth and spurt peak height velocity, whereas a higher plant protein intake could delay puberty [54]. On the contrary, some studies disagreed the above hypothesis of increased intake of total and animal protein

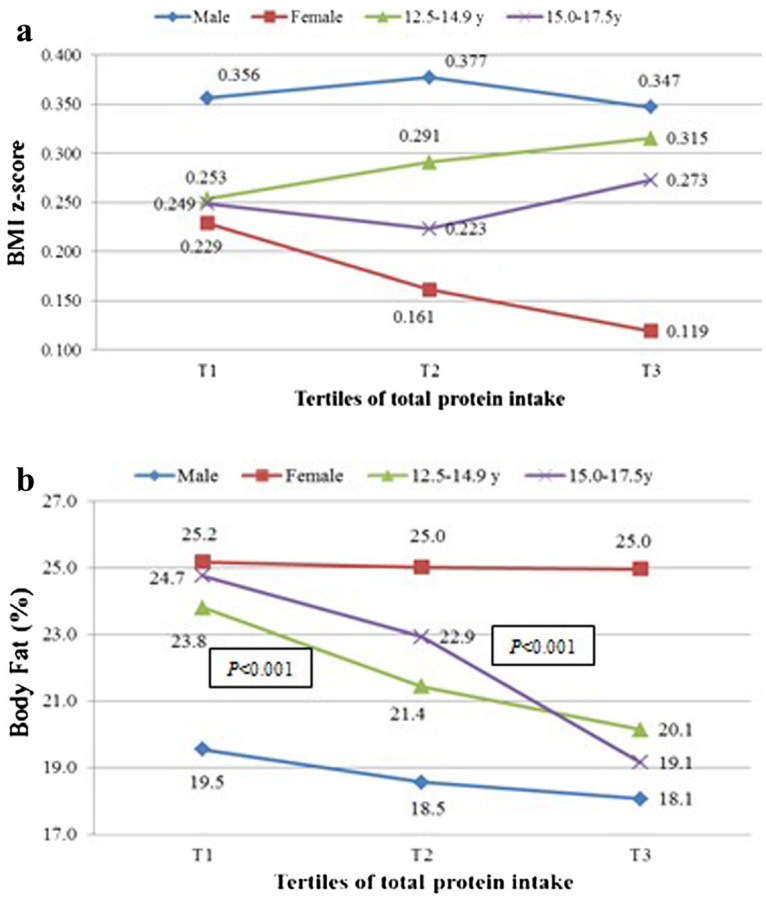

Figure 1 Tertiles [μ] of total protein intake (g/d) and anthropometric indicators in adolescents participating in HELENA-CSS (n = 1804).
[μ]Tertile 1 (T1): <81 g/d; tertile 2 (T2): 81 g/d to 103 g/d; tertile 3 (T3): ≥103 g/d.

resulting in decreasing the risk of OW and OB [55,56] by affecting the appetite. A randomized 8-weeks parallel intervention trial suggested that seafood protein sources from cod and salmon were efficient to treat OB because of caloric restriction and lower saturated fatty acids intake [55]. Therefore, the amount of total, animal and plant proteins in the diet may be a critical factor on prevention against OW and OB.

Evidence also shows that increasing protein intake results in improvement of serum lipids [57]. Plant protein based diets in childhood could be responsible for lowering the risk of MetS and its consequence in the adulthood [58]. In the current study, only serum HDL-C was found to be weakly positively associated with animal protein intake. The increases in HDL-C might possibly be explained by the inverse association of animal protein intake with BF%. Mirkopoulou *et al.* reported that no association with blood lipid profile was observed in Greek adolescents [53], supporting most of our results, as similarities in the study design and target population might explain similarities in observations. Some cross-sectional studies showed that plant based diets were associated with more favourable lipid levels in adolescents by lowering TC and LDL-C, but increasing the HDL-C levels [17,59], whereas high intakes derived from animal sources were associated with an increased risk of MetS [15]. However, it has to be considered that adolescence is a critical period with inevitable increases in energy and nutrient intakes to regulate hormone balances resulting in physical, behaviour and social development. Leptin is a protein hormone that has a key role in regulating energy intake and energy expenditure, including appetite in the longer term [60,61]. In the current study, no significance of serum leptin was found in model 3, but it was negatively associated with animal and plant protein intakes in model 1 and model 2, respectively. The status of statistical significance between serum leptin and plant protein intake changed in the model 2 compared to model 1 due to fat intake. In addition, fat intake can be a critical factor for the serum lipid profile and plant protein intake. No study has provided evidence on clear mechanisms, though it is possible that plant protein intake might stimulate serum leptin via homeostasis impacting on body weight and BF%. In addition, female, OW and obese adolescents in particular,

Table 4 Associations between dietary animal and plant protein intakes (g/d and E%) and body composition of adolescents participating in the HELENA-CSS (n = 1804)

Dependent variables[µ]	Animal protein (g/d)				Plant protein (g/d)			
	β	SE	95% CI	*P*	β	SE	95% CI	*P*
BMI z-score								
Model 1	0.001	0.001	−0.001, 0.004	0.206	−0.002	0.002	−0.006, 0.002	0.414
Model 2	0.003	0.001	0.000, 0.006	0.023	0.000	0.002	−0.005, 0.004	0.847
Model 3	−0.000002	0.000001	−0.000007, 0.000	0.421	−0.012	0.005	−0.023, −0.001	0.027
Body fat (%)								
Model 1	−0.054	0.009	−0.071, −0.036	<0.001	−0.162	0.016	−0.194, −0.131	<0.001
Model 2	−0.009	0.010	−0.030, 0.011	0.386	−0.106	0.019	−0.144, −0.069	<0.001
Model 3	−0.000052	0.000018	−0.000087, − 0.000016	0.004	−0.139	0.040	−0.217, −0.060	0.001
	Animal protein (E%)				Plant protein (E%)			
BMI z-score								
Model 1	0.021	0.008	0.005, 0.038	0.011	0.012	0.019	−0.026, 0.050	0.533
Model 2	0.021	0.008	0.005, 0.037	0.011	0.008	0.020	−0.031, 0.046	0.692
Model 3	0.024	0.009	0.006, 0.043	0.010	−0.027	0.021	−0.067, 0.013	0.188
Body fat (%)								
Model 1	0.209	0.067	0.077, 0.341	0.002	0.124	0.156	−0.181, 0.429	0.426
Model 2	0.196	0.065	0.068, 0.325	0.003	−0.179	0.154	−0.482, 0.123	0.245
Model 3	0.168	0.070	0.030, 0.305	0.017	−0.229	0.151	−0.526, 0.068	0.130

SE, standard error of coefficient β; CI, confidence interval.
[µ]Model 1, unadjusted; model 2, adjusted for fat intake; model 3, model 2 further adjusted for age, sex, tanner stage, physical activity, country cluster, and interactions between potential confounding factors and animal / plant protein (separate model).

during puberty might most likely underestimate energy and dietary intakes, which may bias the associations. Confounding factors, such as gender, age, Tanner stage and region, may account for some unexpected findings, serum biomarkers in particular.

Strengths and limitations

This European nutrition survey is the first large-scale study among European adolescents that used a standardized approach accross 8 participating centers. Additionally, it is the first study evaluating total, animal and plant protein intakes in European adolescents stratified by gender and age, and investigating associations with anthropometry and serum biomarkers as studies with the same standardised methodology across European countries are limited.

The current study has also some limitations including the dietary assessment method used to assess diet that only included dietary information of two non-consecutive days. The 24-h dietary recall method does not allow quantifying proportions of non-consumers for particular food items, especially for those less frequently consumed. In order to decrease the influence of such limitation, nutrient intakes were corrected for within- person variability by applying the MSM method. Moreover, accuracy of collected data relies on the individual's ability to remember foods and beverages consumed in the past 24 hours, and might, therefore, be biased towards misreporting. In this respect, the 24-h dietary recalls were performed through computer-assisted HELENA-DIAT software to standardize the recall procedures as much as possible. Food pictures, showing daily foods consumed by European adolescents, were used in order to facilitate the participants to recall the potion size of the foods consumed in the previous days, which assisted participants and interviewers in accurately assessing the consumed amounts. The same food composition table for conversion of food intake data to estimated nutrient intakes was used for all survey centres. In this way, differences in definitions, analytical methods, units and modes of expression were overcome. However, missing foods of protein contents in the BLS table were calculated via recipes or taken from local food composition tables. In addition, the small sample size of serum biomarkers may also be a potential influencing factor leading to weak linear relationship between animal and plant protein intakes and serum biomarkers. Furthermore, the cross-sectional study design of this study cannot assess causality between health outcomes and dietary intakes.

Recommendations

Protein is critical for the development of bone and muscle mass, and health in adolescents. An increased protein intake is one of the most common approaches to the dietary management of OB and related chronic diseases.

However, extra high protein intake can result in side-effects due to imbalance in energy intake and food consumption. The findings of current study indicate that plant protein had more protective effect against OB compared to animal protein, although HDL-C was found to be weakly positively associated with absolute animal protein intake. We noticed that participants exceeded protein intake based on WHO requirement, and almost 2/3 sources were from animal origin rather than from plants, which may influence body weight and body composition. The findings of our study highlight that future public health policies and school policies need to be developed and implemented to help establishing healthy food preferences, and adjusting food concepts and dietary behaviors in adolescents. Possible prevention strategies could include the development of multicomponent school-based interventions combining education and environmental changes towards increased intakes of plant proteins from legumes and vegetables.

Conclusion

The total protein intake of European adolescents exceeded the recommendations and animal proteins contribute most to the energy intake derived from total protein intake. Total and animal protein intake and E% derived from protein intake were higher in obese subjects. A negative association of total protein intake was found with BF%. GLM multivariate analysis indicates inverse associations, on one hand, between BMI z-score and plant protein intake, and on the other hand between BF% and animal and plant protein intakes. Both BMI z-score and BF% were positively associated with animal protein (E%). In conclusion our findings suggest that plant protein intakes may play a role in preventing OB among European adolescents. Further longitudinal studies should be conducted to investigate these potential beneficial effects of plant protein intakes in the prevention of OB and related chronic diseases.

Abbreviations

BF%: Body fat percentage; BMI: Body mass index; BMI z-score: Standard deviation score of BMI; BLS: Bundeslebensmittelschlüssel, the German food code and nutrient database; CRP: C-reactive protein; EAR: Estimated average requirement; EFSA: European food safety authority; HDL-C: High-density lipoprotein cholesterol; HELENA-CSS: The healthy lifestyle in Europe by nutrition in adolescence-cross sectional study; IOTF: The international obesity task force; LDL-C: Low-density lipoprotein cholesterol; MetS: Metabolic syndrome; MSM: The multiple source method; NEVO: Dutch food composition table; NUBEL: Belgium food composition table; NW: Normal weight; OB: Obesity; OW: Overweight; PA: Physical activity; PRI: Population reference intake; SES: Socioeconomic status; TC: Total cholesterol; TG: Triglyceride; VLDL-C: Very low-density lipoprotein cholesterol; USDA: The USDA national nutrient database; UW: Underweight; WHO: World health organization; YANA-C: The young adolescents' nutrition assessment on computer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

YL was responsible for the data analyses and the drafting of the manuscript. All authors contributed to conception and the interpretation of the results. All authors read and approved the final manuscript.

Acknowledgements

The HELENA-study took place with the financial support of the European Community Sixth RTD Framework Programme. This work was also partially supported by the European Union in the framework of the Public Health Programme, the Swedish Council for Working Life and Social Research (FAS), the Spanish Ministry of Education, and the Spanish Ministry of Health, Maternal, Child Health and Development Network and the Spanish Ministry of Science and Innovation AGL2007-29784-E. The content of this paper reflect only the authors' views and the rest of HELENA-study members and the European Community is not liable for any use that may be made of the information contained therein. Augusto César F. de Moraes is in receipt of a *PhD scholarship* from the São Paulo State Research Foundation (FAPESP: proc. 2011/11137-1 and 2011/20662-2) and *Postdoctoral Research Fellow* scholarship from National Counsel of Technological and Scientific Development (CNPq: proc. 313772/2014-2); Luis A. Moreno was given scholarship of *Visiting Professor* from Brazilian government by Science without Borders Program by CNPq and CAPES (Coordination of Improvement of Higher Education Personnel) (proc. 007/2012). The GENUD Research Group co-financed by the European Regional Development Fund (MICINN-FEDER). Many thanks to Petra Pickert, Anke Carstensen and Rosa Maria Torres for their contribution to laboratory work.

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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.

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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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