EFFECT OF A DAILY SNACK CONTAINING GREEN LEAFY VEGETABLES ON WOMEN'S FATTY ACID STATUS– A RANDOMIZED CONTROLLED TRIAL IN MUMBAI, INDIA

Running title: Green leafy vegetables and fatty acid status

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ABSTRACT

Background and objectives: There are few data on the fatty acid status of non-pregnant Indian women. Our objective was to investigate the effect of a snack containing green leafy vegetables (GLVs) on women's erythrocyte long chain polyunsaturated fatty acid status (LCPUFA).

Methods and Study Design: Non-pregnant women (n=222) aged 14-35 years from Mumbai slums were randomized to consume a snack containing GLVs, fruit and milk (treatment) or a control snack containing foods of low micronutrient content such as potato and onion, daily under observation. One treatment snack contained a mean (SD) of 54.1 (33.7) mg alpha-linolenic acid (ALA) and one control snack contained 4.1 (3.4) mg ALA. Blood was collected at baseline (0 weeks) and after 12 weeks of supplementation. Erythrocyte fatty acids were analyzed using gas chromatography and expressed as g/100g fatty acids. Plasma malondialdehyde, homocysteine, and erythrocyte superoxide dismutase and glutathione peroxidase were measured. The effect of the treatment on 12 week LCPUFA was assessed using ANCOVA models.

Results: Median (IQR) erythrocyte DHA in the treatment group increased from 1.50 (1.11, 2.03) at baseline to 1.86g/100g (1.50, 2.43) (**p<0.001**) at 12 weeks, and fell in controls from 1.78 (1.37, 2.32) to 1.60 (1.32, 2.04) (**p<0.001**). The total n-3 fatty acids increased in the treatment group. There was no effect on malondialdehyde and antioxidant enzyme levels. Plasma Hcy at 0 and 12 weeks was inversely associated with erythrocyte DHA at 12 weeks.

Conclusion: Daily consumption of a snack containing GLV improved women's erythrocyte DHA levels without increasing oxidative stress.

Keywords: Alpha-linolenic acid; erythrocyte; docosahexaenoic acid; green leafy vegetables; intervention study

Introduction

Alpha linolenic acid (ALA; 18:3*n*-3) is obtained from flaxseed oil, nuts, pulses and green leafy vegetables (GLV) [1] and is the 'parent' essential n-3 polyunsaturated fatty acid which is metabolized to EPA (20:5*n*-3) and DHA (22:6*n*-3). DHA is a structural and functional component of the eye and central nervous system cell membranes.[2, 3] The DHA status of the mother during pregnancy is positively associated with length of gestation, birth weight, and visual and cognitive development of the child.[4-6] DHA status in pregnancy also reflects metabolic adaptations to DHA synthesis.[7] The natural dietary source of DHA is oily fish.[1] Vegetarians are reliant on conversion of ALA in plant-based foods to DHA. There is debate about the extent to which this conversion can meet DHA requirements.[8, 9] In India, many people are vegetarian or have low fish intakes for cultural, availability and/or economic reasons. There have been no randomized studies examining the effect of a dietary supplement containing GLV on DHA status. Although increasing the dietary intake of n-3 long chain polyunsaturated fatty acid (n-3 LCPUFA) is considered an effective preventive strategy, there are concerns that long chain polyunsaturated fatty acid (LCPUFA) rich diet may lead to an increase in lipid peroxidation.[10] Several studies have shown a ''paradoxical'' role of DHA, demonstrating both pro-oxidant and antioxidant activities.[11-13] Studies also demonstrate that n-3 fatty acids enhance the activity and expression of antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase.[14, 15] Further, there is evidence that DHA is linked with the 1-Carbon cycle and a deficiency of micronutrients (folic acid, vitamin B_{12}) can influence LCPUFA status.[16] Studies in humans have reported an inverse association between plasma and serum DHA status and homocysteine (Hcy) concentrations.[17, 18] Human studies have also linked higher Hcy levels to an increase in oxidative stress.[19]

In our recent trial (The Mumbai Maternal Nutrition Project (MMNP)) women in slums were randomized to consume a daily snack containing GLV, fruit and milk or a control snack with foods of low micronutrient content before conception and throughout pregnancy.[20] The primary outcome of the trial was birth weight and there was a positive effect of the intervention among women with a normal to high BMI. The treatment snack was made using GLV, fruit and milk along with flour and mixed spices. The ALA content of GLV is approximately 250mg/100g food. Women consumed the snack six times in a week; therefore it is possible that the increased birth weight observed was related to improved maternal DHA status due to conversion of ALA to DHA. It was not feasible to measure pre-and post-supplementation blood nutrient levels in the MMNP as this may have discouraged women from participating.

The primary objective of the current study was to assess the effect of the intervention snack on erythrocyte LCPUFAon non-pregnant women residing in a nearby slum area. Secondary objectives were to: 1) report baseline fatty acid status in this population; and 2) assess whether the snacks affected MDA concentrations and antioxidant enzyme activity and 3) study associations between Hcy and erythrocyte fatty acid DHA

Materials and methods

The present study was conducted from October 2009 to March 2010 and was an extension to the larger trial, the MMNP (ISRCTN No 62811278) which ran from January 2006 to May 2012. The extension study was conducted in a separate group of non-pregnant women living in a similar slum community (Shivaji Nagar) approximately 15 km from the MMNP study area. The extension study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the Institutional ethics committee of Grant Medical College and Sir JJ Group of Hospitals, Mumbai.

Subjects and setting: Women were eligible for the study if they were 14-35 years of age, non-pregnant and not exclusively breast-feeding at the time of enrolment (based on self-report). Migration rates are high in this community and women were asked to enrol only if they intended to remain in the study area for at least 3 months. Women were invited by health workers to attend community meetings where the study was explained by the research team. Informed written consent was obtained from all eligible women who wished to participate (n=222) and patient anonymity was preserved

Height at baseline was measured to the nearest mm using a portable stadiometer (Microtoise, UK) and weight to the nearest 100g using electronic scales (Salter, UK). Demographic and lifestyle data (age, religion, education level, occupation, tobacco use, and alcohol consumption) were collected by questionnaire. Socio-economic status was assessed using the Standard of Living Index (SLI), based on housing type, utilities and household possessions; a higher score indicates higher socio-economic status.[21]

Dietary assessment: A 238-item FFQ developed for this population,[22] with a reference period of the previous 7 days, was administered by a trained interviewer and intake frequency scores were calculated. For example, an item consumed once a week had a score of 1 while items consumed once daily had a score of 7. FFQ data were collected to estimate habitual frequency of intake of foods including: GLV; fruit; milk; meat; fish; eggs; pulses and legumes; fried foods; nuts, oils & oilseeds. In order to determine whether there were differences in ALA intake between groups pre- and post-intervention we created a crude score in which we summed the frequencies of consumption of ALA-rich foods defined as containing ≥0.15g/100g ALA. These foods included GLV, pulses, legumes, nuts, oils & oilseeds.[23-25] We compared the change in this crude score over time between the two groups.

Sample collection and processing: A non-fasted10ml venous blood sample was collected in EDTA tube and centrifuged within 30 minutes of collection at 2000 rpm for 35 min to separate the plasma and erythrocytes. Non-fasted blood samples were collected since reports indicate that there is no difference in the fatty acids between the fasting and non-fasting state.[26, 27] After separating the plasma, the erythrocyte fraction was washed thrice with normal saline and the erythrocytes were transferred into vials, which were kept on dry ice for up to 8 hours before storage in a -80^{o}C freezer.

The women were randomized, stratified by age (3 groups) and BMI (3 groups), into the treatment or control group, using a block-randomization program in STATA (StataCorp, Texas). Height and weight measurements, FFQ and blood sampling were repeated after 12 weeks of supplementation.

Intervention: The intervention was a cooked snack made from locally available food ingredients.[22] The treatment snacks contained approximately 25g fresh GLV (e.g. spinach, colocasia, coriander, fenugreek leaves), 4g dried fruit (e.g. figs, dates, raisins) and 12g whole milk powder. The control snacks contained foods of lower micronutrient content such as potato and onion. All snacks were prepared with binding ingredients such as wheat, chickpea and maize flour and added spices. They were fried in sunflower oil except for one recipe in each group which was not cooked. The active ingredients in the intervention snacks were GLV, fruit and milk. The mean total weight of one treatment snack was 65g whereas the mean total weight of the control snack was 36g. Because of the differences in ingredients, despite our best efforts, it was not possible to produce treatment and control snacks that were identical in terms of weight and energy. The differences in the ingredients meant that treatment snacks (160 kcal) had on average a higher energy content than control snacks (90 kcal). Several varieties of the treatment (n=18) and control snacks (n=19) were used, to prevent monotony for the women (Supplementary Table 1). The snacks were prepared and cooked fresh each day in the MMNP study kitchen, and transported to the study area, where they were provided to the women at centres close to their homes between 3:00pm and 6:00pm, Monday to Saturday. Snack consumption was observed and recorded (1= full; 0.5= $\geq$half; 0= <half). Compliance was defined as the proportion of snacks consumed out of the 72 provided over the 12 wk period. Blinding was not feasible in this study as the treatment and control snacks obviously contained different foods. However, in order to provide some degree of allocation concealment, each arm of the intervention was divided into 2 groups to give a total of four sub-groups each with its own set of recipes. Women were issued with color-coded identity cards corresponding to color-coded packaging for the snacks. The two treatment groups and the two control groups were merged for data analysis. Data and blood

were collected in study centres within the slum community for the convenience of the women. The project staff who collected data were not aware of the snack composition.

Biochemical investigations: All biochemical analyses took place in a laboratory away from the subject recruitment site and technicians were blinded to treatment group.

Erythrocyte fatty acids analysis: Analyses took place within 6-8 months of blood collection. The procedure was modified from Manku et al.[28] Briefly 200µl of the stored erythrocytes were directly pipetted into a glass esterification tube and trans-esterification of the total fatty acids was performed using hydrochloric acid-methanol. Methyl esters were separated using a Perkin Elmer gas chromatograph (SP 2330, 30m capillary Supelco column, Perkin Elmer, Shelton, CT, USA). Helium was used as the carrier gas at 1mL/min. The oven temperature was held at 150ºC for 10 min, programmed to rise from 150 to 220ºC at 10ºC/min, and then held at 220ºC for 10 min. The detector temperature was 275ºC and the injector temperature was 240ºC. Integration of peak areas was done manually and the peaks were identified by comparison with relative retention time of standard fatty acid methyl esters (Sigma-Aldrich). Fatty acids were expressed as g per 100g fatty acid, i.e. as percentages of total fatty acids measured.

Laboratory analysis of fatty acids in the snacks: We measured fatty acids in the treatment and control snacks. Samples of the snacks were homogenized, moisture was removed by drying in an oven at 100°C to a constant weight, and finally the samples were sealed in polythene bags. Lipids were extracted using the Folch method, [29] after which the analytic procedure was the same as for erythrocyte fatty acids.

Absolute ALA content in the snacks: Samples of the snacks were homogenized, moisture was removed by drying in an oven at 100°C to a constant weight, and the samples were sealed in polythene bags. A known concentration of internal standard was added to 100mg snack, and lipids were extracted using the Folch method[29] using chloroform/methanol (2:1, v/v) mix solution. Due to presence of polar compounds, fatty acids dissolved better in chloroform compared to hexane; hence chloroform was used. The extract was washed with NaCl solution (0.58%), dried under argon, and then the extract was taken up to 300µl chloroform/methanol. For methyl ester synthesis 100 µl of the extract from 300µl chloroform/methanol was taken in an esterification tube and 1.5 ml methanolic HCl (3 N) was added. The tubes were closed, shaken for 30 seconds, and heated to 85° C for 45 min. Samples were cooled to room temperature. 1 ml hexane was added for FAME extraction. After centrifugation at 900 * g for 5 min the upper hexane phase was transferred into a further glass tube. The extracts were taken to dryness under argon gas at room temperature, and taken up in 50 µl chloroform. Methyl esters were separated using a Perkin Elmer gas chromatograph (SP 2330, 30m capillary Supelco column, Perkin Elmer, Shelton, CT, USA). Helium was used as the carrier gas at 1mL/min. The oven temperature was held at 150ºC for 10 min, programmed to rise from 150 to 220ºC at 10ºC/min, and then held at 220ºC for 10 min. The detector temperature was 275ºC and the injector temperature was 240ºC. Peak area of ALA and peak area of internal standard in the snacks, were recorded.

The absolute ALA concentration [30] was calculated by dividing the area of the internal standard by the amount of internal standard added, and then dividing the area of each fatty acid by this result to obtain the absolute concentration of each fatty acid within the amount used.

Analysis of plasma malondialdehyde (MDA): Plasma MDA concentrations were estimated using the Oxis kit (MDA-586, Oxis International, Foster City, CA, USA). Briefly, thiobarbituric acid reacts with MDA to form a pink solution and the absorbance was measured at 586 nm. Tetramethoxypropane was used as a standard.MDA concentration was expressed as μmol/ml.

Analysis of Plasma Hcy: Total plasma Hcy was measured by fluorescence polarization immunoassay (FPIA) using Abbott homocysteine kits on an Abbott Axsym system (Abbott Laboratories, Abbott Park, IL 60064 USA).[31] The Axsym Hcy assay is based on the FPIA technology. Bound Hcy (oxidized form) is reduced to free Hcy, which is enzymatically converted to S-adenosyl-L-homocysteine (SAH). SAH and labelled fluorescein tracer compete for the sites on the monoclonal antibody molecule. The intensity of the polarized fluorescent light was measured by the FPIA optical assembly.

Analysis of antioxidant enzymes: Antioxidant enzyme (SOD and GPx) activity was estimated using the erythrocyte lysates. The erythrocyte pellet was washed and re-suspended in 4 packed cell volumes of ice cold deionised water and the tubes were kept on ice for 10 minutes. Further, these lysates were diluted with sample diluent and assayed within one hour. The SOD assay was performed using the Cayman's kit (Catalog Number 706002). It utilizes a tetrazolium salt for the detection of superoxide radicals which are generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme required to exhibit 50% dismutation of the superoxide radical. The reaction was initiated by adding a radical detector and the colour change was measured between 440 - 460nm spectrophotometrically using an ELISA plate reader. SOD activity was expressed as U/ml.

The GPx assay is an indirect measure of the activity of cellular-GPx. The method uses a colorimetric assay (GPx-340™: Oxis Research International; Bioxytech®). Oxidized glutathione (GSSG) produced upon reduction of organic peroxide by cellular-GPx was recycled to its reduced state by the enzyme glutathione reductase. The oxidation of nicotinamide adenine dinucleotide phosphate was accompanied by a decrease in absorbance at 340nm, (A340) providing a spectrophotometric means for monitoring GPx enzyme activity. The enzyme reaction was initiated by adding the substrate, tertbutyl hydroperoxide and the absorbance was recorded at 340nm. The rate of decrease in the A340 was directly proportional to the GPx activity in the sample. GPx activity was expressed as mU/ml.

Statistical analysis: Statistical analysis was performed using SPSS software (version 21, Chicago, IL). Data are presented as mean and standard deviation (SD) values for normally distributed variables and median and inter quartile range (IQR) for skewed variables. Before making between-group comparisons, skewed variables were normalized by log transformation (age, weight, BMI, baseline fatty acids). The level of statistical significance was considered to be $p<0.05$. Differences in subject characteristics between the treatment and control groups were determined using t tests for continuous variables and chi-squared tests for categorical variables. The mean (95% CI) change in concentrations from baseline to 12 wk (δ) was calculated (12 wk value – baseline value) within the treatment group (δ^t) and within the control group (δ^c). We used ANCOVA models to assess the effect of group allocation on 12 wk fatty acid status, MDA, SOD and GPx. Initially we added only baseline (0 wk) concentration or activity as a covariate. We then ran a further model adding age, BMI, standard of living index, compliance with supplementation and 12 wk frequency of intake of GLV and fish as covariates. In the MMNP there was a greater effect of the intervention in women of higher BMI. We therefore tested for an interaction between allocation group and

BMI as a continuous variable. The associations between plasma Hcy concentration at 0 wk and 12 wk with DHA at 0 wk and 12 wk were assessed using linear regression.

Power calculations: When designing the study we were unable to find any previous studies from which to specifically estimate an effect size for fatty acids from our intervention. Along with assessing the effect of the treatment snacks on changes in fatty acid status, we also studied the effects on several micronutrients (please see Kehoe S et al; British Journal of Nutrition 2015, 113, 813–821). Retinol was the only nutrient for which we had good quality data on prevalence of deficiency in India at the time of designing the study, so we based our power calculations on this particular micronutrient. On that basis 82 subjects were required in each group to detect a difference in the proportion of vitamin A deficiency (defined as serum retinol < 0.7 μmol/l) of the order of 35% in the control group *v.* 10% in the treatment group at 90% power and at the 0.05 significance level.[32] We conducted a post hoc calculation for fatty acids and found that our study had 80% power to detect a difference of 0.16 units of DHA (g/100g fatty acid) between the 0-12 week changes in control and treatment groups at the 0.05 significance level.

Results

Analysis sample: A total of 222 women were eligible and agreed to participate in the study. Of these, 114 were randomized to the control and 108 to the treatment group (Figure 1). The analysis sample comprised 167 women (control: 85; treatment: 82) for whom blood samples were available at both baseline and after 12 wk supplementation. There were no significant differences in baseline characteristics between the women who were included in the final analysis sample (n=167) and those who were not (n=55), except for age (Table 1). The women who dropped out were older than those who remained (22 years vs. 20 years, respectively; p=0.033).

Subject characteristics: Over half of the women were underweight (BMI<18.5kg/m^2). Three quarters of them had completed secondary education and two thirds were not doing any paid work. About 17% of the women consumed tobacco by chewing, either in the form of 'meisheri' (tooth powder) or recreationally along with betel leaves. Approximately 15% of the women lived in housing made from plastic sheeting, sticks, and textiles. The remainder had houses with solid walls and floors made from cement, with corrugated metal roofs. Three quarters of houses consisted of only one room. Most (>80%) women used a public pit toilet. There were no significant differences between the treatment and control groups with regard to socio-demographic characteristics (Supplementary Table 2). Compliance was 95.1% in the treatment group and 96.5% among controls.

Dietary intakes: These data represent the women's habitual diet and do not include the intervention. The frequency of dietary intakes in the week prior to registration did not differ between groups (Table 2). Frequencies of intake of GLV, fruit, milk, meat, egg, and fish were low compared with Indian recommendations.[23] Almost half the women ate no fish. The

291 median (IQR) frequency of intakes of green leafy vegetables, pulses and legumes increased in
292 both the treatment and control groups across the 12 wk study; there were no significant
293 differences between groups in the change of intakes of these or total ALA-rich foods.

294 *Fatty acid content of the snacks:* ALA was the only n-3 fatty acid, and LA the only n-6 fatty
295 acid, present in the snacks (Table 3). The relative amounts of ALA were higher in the
296 treatment than in the control snacks, while LA was higher in the control snacks. The relative
297 amounts of all saturated fatty acids except SA were higher in the treatment than in the control
298 snacks. There were no significant differences in relative amounts of monounsaturated fatty
299 acids between the control and treatment snacks except for MOA, which was higher in the
300 treatment snacks.
301
302 *Absolute ALA content of the snacks:* The mean (SD) absolute amount of ALA in the
303 treatment group was 54.1 (33.7) mg per snack and only 4.1 (3.4) mg per control snack.
304
305 *Erythrocyte fatty acids:* The ANCOVA statistics presented in Table 4 show that there was no
306 effect of group allocation on 12 wk relative amounts of ALA or EPA when controlled for
307 baseline amounts. DHA increased over the study period in the treatment group and decreased
308 in the control group. There was a significant effect of the treatment snacks on 12 wk DHA
309 and total n-3 fatty acids (Table 4). DHA increased in the treatment group relative to the
310 control group by 0.45mg/100g which was approximately 25% of the median baseline values.
311 This effect remained significant in the fully adjusted model (Table 5). There was no
312 significant interaction between group allocation and women's BMI.

313 LA increased in both the treatment and control groups between baseline and 12 wk, and there
314 was a borderline significant effect of group allocation, with a greater effect in the treatment

group, on 12 wk values when controlled for baseline values (Table 4). The treatment snacks did not have an effect on the proportions of other n-6 fatty acids measured. The treatment snack was associated with a decreased n-6 to n-3 fatty acid ratio at 12 wk. There were no changes in the other saturated or monounsaturated fatty acids or their totals in either group, nor any differences between groups.

Plasma malondialdehyde: MDA concentrations decreased in both groups without significant differences between the groups (Table 4).

Erythrocyte antioxidant enzymes: SOD activity decreased, whereas GPx activity increased, in both groups, without significant differences between the groups for either enzyme (Table 4).

Hcy concentrations and association between Hcy and DHA: Baseline and 12 wk median (IQR) plasma Hcy level were 11.47 (9.06, 14.19) and 10.55 (8.73, 13.11) (μmol/L) respectively in the total group. In the treatment and control groups, these values were 11.34 (8.78, 14.56) and 11.57 (9.38, 14.11) respectively at baseline and 10.55 (8.62, 13.22) 10.62 (8.85, 13.27) respectively after 12 wk. There was no effect of supplementation on plasma Hcy levels.

Among all subjects there was a borderline significant inverse association between baseline Hcy (μmol/L) and 12 wk DHA (mg/100g) (B= -0.22; 95% CI -0.47, 0.03; p=0.078). We observed a significant negative association between 12 wk plasma Hcy (μmol/L) and 12 wk erythrocyte DHA (mg/100g) (B= -0.26; 95% CI -0.51,-0.02; p= 0.037).

Discussion

This is the first study to report an increase in total n-3 fatty acids due to a food-based intervention among women of reproductive age. The intervention aimed to increase intakes of green leafy vegetables, fruit and milk rather than test the effect of a specific nutrient or fatty acid.

The baseline proportions of erythrocyte n-3 fatty acids, n-6 fatty acids, total polyunsaturated and monounsaturated fatty acids in our population were lower than in women of reproductive age in the USA [33] and the UK, [34] while the proportion of saturated fatty acids was higher.

Dietary intakes of n-3 source foods in the Indian population: Dietary intakes of n-3 foods are low among Indian women [35] compared with recommendations [36] and with the UK population.[37] Oily fish is a rich source of DHA. The DHA content of the fish commonly consumed by Indians is between 6.3% and 28.5% of total fatty acids.[38, 39] Indian women may therefore be more reliant on the conversion of ALA in plant-based foods to DHA. GLV are widely available, relatively inexpensive [40] and acceptable to the majority, compared with other sources such as flaxseed and walnuts. Nevertheless, national surveys and research studies have shown that reported intakes of GLV are low [41, 42] and more so among slum dwellers than the general population.[43, 44] In another slum community in Mumbai, we showed that a quarter of women ate GLV less than once every alternate day, and three quarters ate them less than once a day.[42]

Interventions to increase DHA status: A number of intervention studies in humans have examined the effect of ALA in plant-based preparations (seeds, seed oil or margarine) on DHA status and reports are inconsistent. Some studies report no increase in blood levels of

DHA,[45-48] while two studies found an increase in DHA, of the order of 2-14% among adults [49, 50] and another study reported a 38% increase in infants.[51] Hypercholesterolemic patients in a Japanese study consumed 320g per day of green vegetable juice made from radish leaves, spinach, and lettuce for 3 weeks which resulted in an increase in serum DHA from 2.1% to 4.4%.[49] Substitution of soyabean oil with perilla oil, providing a dose of 3g of ALA per day, for 10 months increased serum DHA levels by 21% of baseline values from 5.3mg/100g to 6.4mg/100g.[50] Fish oils have been shown to increase DHA levels by over 60% [52, 53] as the amount of EPA or DHA from fish oils ranged from 600mg to 6g/day.

The varying results from other plant-based intervention studies, and the generally small changes in DHA that they achieved, have led to controversy over whether conversion of ALA from plant sources in humans can meet DHA requirements.[54, 55] It is important to note that most of this evidence is from studies in omnivorous populations. In populations with poor fatty acid status due to low intakes of animal-source foods, it is possible that plant sources of ALA are more readily converted to DHA.[49, 50, 56]

An observational study in Europe [56] collected dietary intake data and measured plasma phospholipid n-3 fatty acids among 14,422 men and women aged 39–78 y. Based on these data, conversion between dietary ALA and circulating long-chain n-3 PUFAs (EPA, DPA and DHA) was estimated and compared between fish-eaters and non-fish-eaters. The total intake of n-3 fatty acids was 57-80% lower among non-fish eaters but differences in circulating long chain n-3 PUFAs were considerably smaller. The authors speculated that this was because the product: precursor ratio, i.e. (EPA+DPA+DHA)] / ALA, was greater in non-fish-eaters indicating more efficient conversion of ALA to long chain n-3 PUFAs in this group.

The higher post-intervention erythrocyte DHA concentration in our study may also be attributed to other ingredients present in the snack. Turmeric is a common staple spice in all the curries and vegetables cooked in India. A recent study in animals found that curcumin, found in turmeric, improved the conversion of ALA to DHA.[57]

Contradictory results have been reported regarding dietary flavonoids (anthocyanins).[58, 59] An animal study [58] reported an increase in DHA after consumption of dietary flavonoids by increasing the activation of EPA and DHA biosynthesis from ALA; whereas a recent study in rodent and human cells indicated that anthocyanin intake has little effect on omega 3 fatty acid status in mammals.[59]

In the present study we observed a small increase in the proportion of erythrocyte DHA of approximately 25% of baseline values in the treatment group compared with the control group, without increasing oxidative stress. Since the ALA content of the treatment snacks was much lower than that expected to increase DHA, and since intermediate fatty acids such as EPA did not change, it may be that the increased DHA in the treatment group was not a reflection of increased conversion, but rather reduced degradation. Higher DHA levels were associated with lower plasma homocysteine concentrations. Although homocysteine concentrations did not differ between allocation groups, we speculate that low homocysteine may be protective of DHA itself and/or homocysteine status may be a marker for another nutrient or metabolite that is protective of DHA. There is evidence that Hcy is associated with poor DHA preservation, and many human studies have reported inverse associations between DHA and Hcy.[17, 18]

There were no effects of our supplement on the levels of MDA or on the activity of anti-oxidant enzymes SOD and GPx. Being a food based intervention, the lack of effect on MDA

410 or anti-oxidant enzymes may be attributed to micronutrients present in the treatment snack
411 which might have prevented lipid peroxidation. We have previously reported that the snack
412 increased plasma beta-carotene levels, [32] which may have prevented oxidative stress.
413
414 *Strengths and limitations:* Strengths of the present study were its randomized design, a
415 sustained intervention over three months with high rates of compliance, and measurement of
416 a large range of fatty acids, Hcy as well as markers of oxidative stress and antioxidant
417 enzyme activity. The subjects started and completed the intervention at the same time, thus
418 creating an internal control for seasonal changes in food intakes. A food based intervention
419 was used because: a) people eat food and not tablets/ capsules so it was not a major change in
420 their usual routine/lifestyle, b) it was thought that compliance would be better in a food based
421 intervention than with tablets, and c) the snacks comprised locally sourced foods which are
422 inexpensive, available, and culturally acceptable, so the intervention has potential for public
423 health scalability/sustainability and provides income and employment to local people. The
424 nature of food based interventions is that we cannot know which components are beneficial,
425 and blinding is not possible. The lack of blinding is unlikely to have affected the laboratory
426 measurements, but it may have influenced the women's dietary behaviour in ways that
427 affected their fatty acid status. However, GLV intakes increased to a similar degree in both
428 groups over the study period. We speculate that this was due to seasonal changes in
429 availability. We have previously reported that in a separate group of women living in
430 Mumbai, intakes of fruit and GLV vary by season. [42] GLV tend to become cheaper and more
431 abundant during the months towards the end of our study.

432 The control snacks contained fewer calories and less protein than the treatment snacks. This
433 was because the intervention was an increase in daily intakes of GLV, fruit and milk. The
434 control snacks represented the calories provided in the binding ingredients of the treatment

435 snacks. However, this small difference in energy intake may have caused the observed
436 differences in fatty acids. .

437 The precise amount of ALA in the women's diet would have been desirable in order to ensure
438 that there was no difference between groups at baseline. Our crude score did not suggest a
439 difference between groups in the change in ALA content of the habitual diet over the study
440 period.

441 **Conclusion**

442 There was an increase in erythrocyte DHA levels as a result of consuming the treatment
443 snack. However, this increase was small and may not be explained only by the ALA content
444 of the snack. It is likely that other constituents in the treatment snack may have also
445 influenced DHA levels. Therefore we suggest a need to conduct further research to better
446 understand this result. The enzymes involved in the fatty acid biosynthesis pathway could be
447 examined in the future to understand the biosynthesis of long chain PUFA, especially DHA.

448

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452

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454

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458

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Table 1: Baseline characteristics of study subjects

	Completed (n=167) **Median (IQR)**	**Withdrawn(n=55)** **Median (IQR)**	**p***
Age (years)	20 (17, 26)	22 (20, 29)	0.033
Weight (kg)	40.7 (36.4, 46.6)	40.5 (36.1, 47.1)	0.346
Height†(cm)	149.4 (5.8)	150.1 (5.0)	0.389
BMI (kg/m^2)	18.42 (16.05, 20.38)	18.69 (16.76, 20.72)	0.455
Monthly Income (Rs)	4000 (3000, 5000)	4000 (3000, 5000)	0.478
Systolic Blood Pressure (mmHg)†	107.5 (11.2)	104.6 (13.5)	0.114
Diastolic Blood Pressure (mmHg)†	68.6 (8.4)	66.5 (8.2)	0.109
Standard of Living Index	23 (18, 27)	21 (16, 26)	0.122
	n (%)	**n (%)**	
Parity‡			0.085
0	95 (56.9)	21 (38.2)	
1	11 (6.6)	7 (12.7)	
2	19 (11.4)	7 (12.7)	
≥3	42 (25.1)	20 (36.4)	
Education‡			0.209
Graduate	5 (3.0)	2 (3.6)	
Higher Secondary	9 (5.4)	2 (3.6)	
Secondary	124 (74.3)	34 (61.8)	
Primary	12 (7.2)	4 (7.3)	
Any Other	4 (2.4)	2 (3.6)	
Illiterate	13 (7.8)	11 (20.0)	
Occupation‡			0.386
Professional	4 (2.4)	1 (1.8)	
Graduate- semi professional	0 (0.0)	1 (1.8)	
Self employed	5 (3.0)	1 (1.8)	
Skilled worker	10 (6.0)	3 (5.5)	
Semi-skilled	7 (4.2)	1 (1.8)	
Unskilled worker	29 (17.4)	15 (27.3)	
Not working	112 (67.1)	33 (60.0)	
Tobacco Consumption‡			0.188
Yes	29 (17.4)	14 (25.5)	
No	138 (82.6)	41 (74.5)	

† Mean (SD) or ‡ N (%) presented instead of median (IQR) for normally distributed or categorical variables respectively. *T test and Chi-square tests for differences between women who completed the study and those who withdrew.

Table 2: Median (IQR) frequency of habitual intake of foods per week excluding the intervention snacks

Foods	Control (N=85)		Treatment (N=82)		
ALA-rich foods	0 wk Median (IQR)	12 wk Median (IQR)	0 wk Median (IQR)	12 wk Median (IQR)	p*
Green leafy vegetables	1 (0, 2)	3 (1, 4)	1(0, 2)	2 (0, 3)	0.084
Pulses & Legumes	1 (1, 3)	2 (0,4)	1 (1,3)	1.5 (0, 3)	0.547
Nuts, Oils & Oilseeds	1 (0,14)	1 (0,5)	1 (0,7)	0 (0, 1)	0.807
Total intake of ALA-rich foods	5 (2, 15)	7 (4, 12)	4 (2,11)	4 (2,10)	0.703
Other foods					
Fruits	1 (0, 2)	2 (1,6)	1 (0,2)	3 (1,6)	0.596
Milk & Milk Products	1 (0, 2)	1 (0,3)	1 (0,2)	1 (0,3)	0.307
Meat	2 (1, 4)	2 (0,3)	2 (1,3)	2 (1,3)	0.597
Fish	0 (0, 1)	0 (0,1)	1 (0,1)	0 (0,1)	0.263
Egg	1 (0, 1)	1 (0,2)	1 (0,2)	1 (0,2)	0.977

*P values are from t tests comparing the change in intakes from 0 wk to 12 wk (12 wk frequency – 0 wk frequency) between allocation groups.

Table 3: Fatty acid content (g/100g total fatty acids measured) of the control and treatment snacks

Fatty acids	Control	Treatment	
	g/100g total fatty acids measured		
	Median (IQR)	Median (IQR)	p*
n-3 PUFA			
ALA	0.52 (0.44, 0.72)	2.12 (1.42, 3.42)	p<0.001
EPA	0	0	-
DHA	0	0	-
n-6 PUFA			
LA	62.6 (59.1, 63.7)	52.1 (49.4, 54.8)	0.016
DGLA	0	0	-
AA	0	0	-
DPA	0	0	-
GLA	0	0	-
Saturated Fatty Acids			
MA	0.22 (0.19,0.26)	1.47 (0.87, 2.03)	0.047
PA	7.7 (7.6, 8.2)	11.7 (10.4, 13.5)	0.001
SA	3.7 (3.6, 4.1)	4.6 (4.2, 5.1)	0.613
Total Saturated	11.7 (11.5, 13.2)	17.6 (15.2, 20.6)	0.007
MUFA			
MOA	0.01(0.01, 0.02)	0.13 (0.07, 0.21)	p<0.01
POA	0.10 (0.09, 0.12)	0.30 (0.22, 0.39)	0.922
OA	24.5(23.7, 26.3)	26.2 (24.9, 27.5)	0.642
NA	0	0	-
Total Monounsaturated	24.6 (23.8, 26.4)	26.7 (25.3, 28.0)	0.619
Total	99.8 (99.5, 99.8)	99.5 (99.2, 99.8)	0.910

*p relates to the difference between the control and treatment snacks assessed using t tests.

ALA=alpha linolenic acid (18:3n-3); EPA=eicosapentaenoic acid (20:5n-3); DHA=docosahexaenoic acid (22:6n-3); LA=linoleic acid (18:2n-6); GLA=gamma linolenic acid (18:3n-6); DGLA=dihomo-gamma linolenic acid (20:3n-6); DPA=docosapentaenoic acid (22:5n-6); AA=arachidonic acid (20:4n-6); MA=myristic acid (14:0); PA=palmitic acid (16:0); SA=stearic acid (18:0); MUFA; mono-unsaturated fatty acid; MOA=myristoleic acid

(14:1n-5); POA=palmitoleic acid (16:1n-7); OA=oleic acid (18:1n-9); NA=nervonic acid (24:1n-9).

Table 4 Median (IQR) erythrocyte fatty acids, antioxidant enzymes and plasma MDA at baseline and at 12 week of supplementation and the difference between groups in the change over the study period controlled for baseline status

Fatty acids (g/100g fatty acid)	**Control**		**Treatment**		**Confidence**		
	0 week Median(IQR)	12 week Median (IQR)	0 week Median (IQR)	12 week Median (IQR)	$\delta^c - \delta^t$ Mean	lower bound, upper bound	p*
n-3 PUFA							
ALA	0.05 (0.04, 0.08)	0.05 (0.03, 0.09)	0.06 (0.04, 0.08)	0.06 (0.04, 0.09)	-0.01	-0.02, 0.01	0.270
EPA	0.25 (0.17, 0.41)	0.22 (0.16, 0.40)	0.24 (0.14, 0.32)	0.22 (0.16, 0.45)	-0.06	-0.18, 0.06	0.295
DHA	1.78 (1.37, 2.32)	1.60 (1.32, 2.04)	1.50 (1.11, 2.03)	1.86 (1.50, 2.43)	**-0.45**	**-0.56, -0.34**	**<0.0001**
Total n-3	2.3 (1.7, 2.8)	1.9 (1.6, 2.6)	1.8 (1.4, 2.6)	2.2 (1.8, 2.9)	**-0.45**	**-0.62,-0.27**	**<0.0001**
n-6 PUFA							
LA	9.4 (8.4, 10.3)	9.7 (8.8, 10.4)	9.8 (9.0, 11.2)	10.4 (9.5,11.4)	-0.4	-0.7, 0.0	0.050
DGLA	1.16 (0.93, 1.47)	1.10 (0.98, 1.51)	1.19 (0.97, 1.51)	1.12 (1.00, 1.35)	-0.01	-0.84, 0.11	0.799
AA	12.9 (11.7,14.1)	13.4 (12.0, 14.3)	12.9 (11.7, 14.0)	13.0 (11.7, 13.9)	0.27	-0.22, 0.75	0.283
DPA	0.55 (0.45, 0.76)	0.54 (0.40, 0.82)	0.55 (0.41, 0.76)	0.56 (0.41, 0.67)	-0.03	-0.05, 0.10	0.483
GLA	0.06 (0.04, 0.08)	0.06 (0.04, 0.08)	0.07 (0.04, 0.09)	0.06 (0.03, 0.08)	0.01	-0.01, 0.02	0.268
Total n-6	24.2 (22.3, 25.5)	24.9 (23.2, 26.2)	24.6 (23.5, 26.2)	24.8 (23.2, 27.0)	-0.2	-0.9, 0.5	0.558
n-6:n-3	10.9 (8.7, 14.6)	12.9 (9.4, 15.4)	13.4 (9.9, 17.9)	10.8 (8.7, 14.3)	**3.2**	**2.3, 4.0**	**<0.0001**
Saturated Fatty Acids							
MA	0.36 (0.24, 0.47)	0.35 (0.28, 0.44)	0.34 (0.24, 0.44)	0.33 (0.27, 0.41)	0.0	-0.05, 0.05	0.989
PA	28.8 (24.7, 31.9)	29.8 (25.7, 31.2)	28.5 (24.8, 31.6)	29.3 (27.0, 31.8)	-0.2	-1.2, 0.9	0.749
SA	16.4 (15.6, 17.4)	16.9 (15.7, 17.4)	16.2 (15.4, 17.1)	16.5 (15.7, 17.4)	0.1	-0.3, 0.5	0.567
Total saturated	46.1 (41.6, 49.0)	46.5 (43.1, 48.7)	45.7 (41.4, 48.8)	47.1 (43.6, 49.3)	-0.1	-1.3, 1.1	0.901
MUFA							
MOA	0.03 (0.01, 0.07)	0.03 (0.01, 0.07)	0.03 (0.02, 0.07)	0.02 (0.018, 0.06)	0.00	-0.01, 0.02	0.592
POA	0.42 (0.33, 0.56)	0.40 (0.35, 0.56)	0.42 (0.33, 0.59)	0.40 (0.31, 0.53)	0.01	-0.06, 0.09	0.723
OA	11.1 (10.6, 11.9)	10.8 (10.1, 11.7)	11.2 (10.5, 12.0)	10.3 (10.0, 11.1)	0.3	-0.2, 0.8	0.237
NA	2.2 (1.0, 3.0)	2.2 (0.9, 3.0)	2.2 (0.8, 2.9)	2.5 (0.7, 3.0)	-0.01	-0.34, 0.32	0.942
Total MUFA	14.0 (12.5, 15.1)	13.4 (12.7, 14.4)	13.9 (12.5, 15.0)	13.3 (11.7, 14.1)	0.30	-0.25, 0.86	0.286

Indicator of Oxidative Stress							
MDA (μmol/ml)	2.0 (1.2, 6.2)	1.3 (1.0, 1.9)	1.7 (1.2, 3.6)	1.3 (1.0, 1.9)	-0.40	-0.89, 0.09	0.106
SOD (U/ml)	2599 (1544, 3640)	1852 (1165, 3332)	2353 (1472, 3633)	1981 (1161, 2825)	-5	-576, 565	0.986
GPx (mU/ml)	137 (105, 173)	151 (123, 181)	127 (112, 150)	141 (116, 161)	4.9	-6.8, 16.0	0.429

δ^c, change in relative amounts in control group (12 week – baseline), δ^t change in relative amounts in treatment group (12 week – baseline). 95% confidence interval. *p relates to ANCOVA test assessing effect of treatment group on 12week concentrations with 0 week concentrations as a covariate. PUFA=polyunsaturated fatty acids; ALA=alpha linolenic acid (18:3n-3); EPA=eicosapentaenoic acid (20:5n-3); DHA=docosahexaenoic acid (22:6n-3); LA=linoleic acid (18:2n-6); GLA=gamma linolenic acid (18:3n-6); DGLA=dihomo-gamma linolenic acid (20:3n-6); DPA=docosapentaenoic acid (22:5n-6); AA=arachidonic acid (20:4n-6); MA=myristic acid (14:0); PA=palmitic acid (16:0); SA=stearic acid (18:0); MUFA=monounsaturated fatty acids; MOA=myristoleic acid (14:1n-5); POA=palmitoleic acid (16:1n-7); OA=oleic acid (18:1n-9); NA=nervonic acid (24:1n-9). The n-6:n-3 fatty acid ratio=(LA, GLA, DGLA, DPA and AA)/(ALA, EPA and DHA). MDA=malondialdehyde; SOD=superoxide dismutase; GPx=glutathione peroxidase. Values in bold type indicate statistically significant differences.

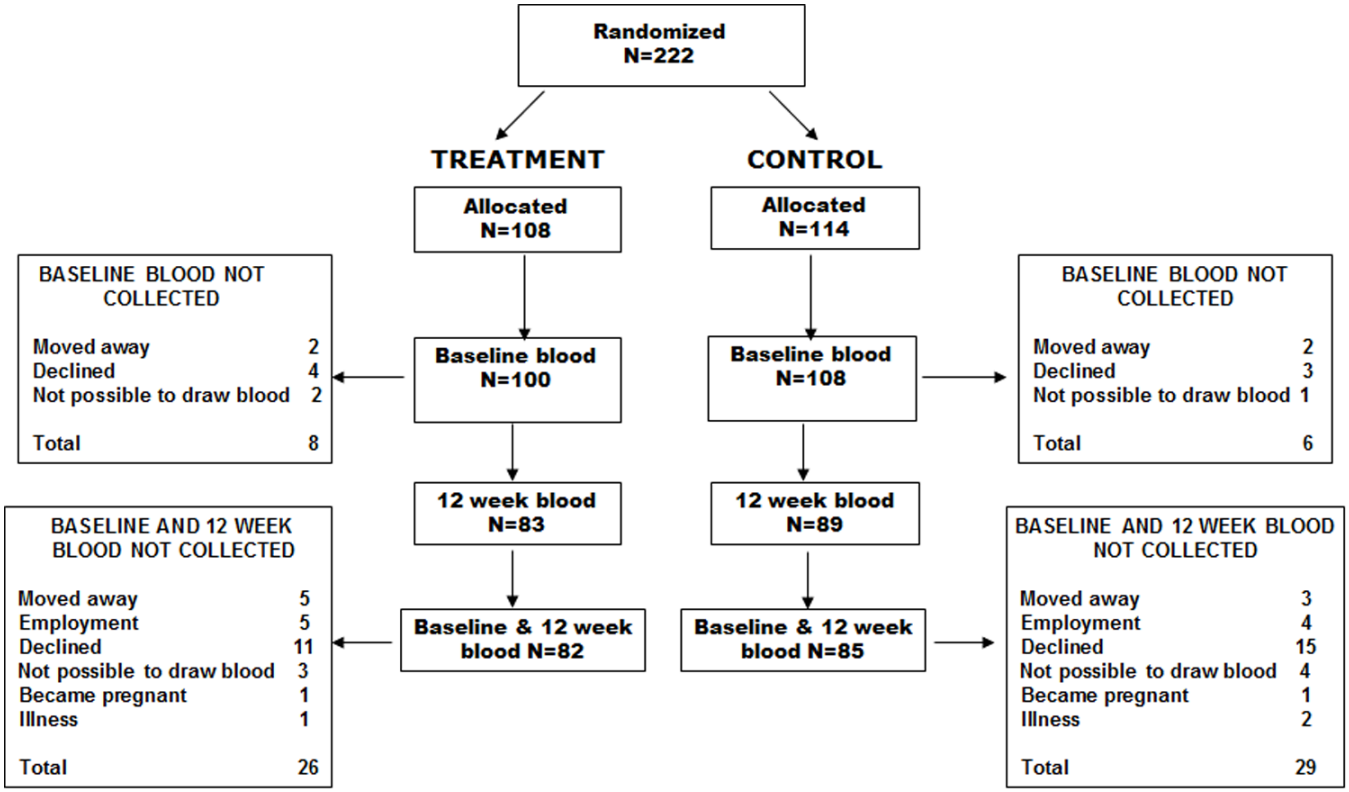
Table 5: Summary of ANCOVA model with 12 week DHA (%) as the outcome

			Confidence interval	
Predictor Variable	P	B	lower Bound	upper Bound
Treatment Group[†]	**< 0.001**	0.447	0.333	0.562
Baseline erythrocyte DHA (g/100g)	**< 0.001**	0.764	0.678	0.849
Age (years)	0.664	0.001	-0.011	0.011
BMI (kg/m^2)	0.977	0.003	-0.014	0.021
SLI score	0.805	0.001	-0.008	0.010
Compliance[†]	0.912	0.004	-0.066	0.074
Habitual GLV intake (12 week)	0.831	-0.003	-0.032	0.042
Fish Intake(12 weeks)	0.558	0.019	-0.046	0.084

[†] control Group =0, Treatment Group =1, BMI, Body Mass Index; SLI, Standard of Living Index; GLV, green leafy vegetables. [†]Proportion of snacks consumed of those provided.

Figure 1: Participant flow during the study

Original to this manuscript


Randomized
N=222
TREATMENT
CONTROL
Allocated
N=108
Allocated
N=114
BASELINE BLOOD NOT COLLECTED
Moved away 2
Declined 4
Not possible to draw blood 2
Total 8
Baseline blood
N=100
Baseline blood
N=108
BASELINE BLOOD NOT COLLECTED
Moved away 2
Declined 3
Not possible to draw blood 1
Total 6
12 week blood
N=83
12 week blood
N=89
BASELINE AND 12 WEEK BLOOD NOT COLLECTED
Moved away 5
Employment 5
Declined 11
Not possible to draw blood 3
Became pregnant 1
Illness 1
Total 26
Baseline & 12 week blood N=82
Baseline & 12 week blood N=85
BASELINE AND 12 WEEK BLOOD NOT COLLECTED
Moved away 3
Employment 4
Declined 15
Not possible to draw blood 4
Became pregnant 1
Illness 2
Total 29