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DR ALBERT SALAS-HUETOS (Orcid ID : 0000-0001-5914-6862)

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Title: Sperm DNA methylation changes after short-term nut supplementation in healthy males consuming a Western-style diet

Authors

Albert Salas-Huetos, M.Sc., Ph.D. [a,b,c,d]; Emma R. James, B.Sc. [d,e]; Jordi Salas-Salvadó, Ph.D., M.D. [a,b,c,f]; Mònica Bulló, Ph.D. [a,b,c]; Kenneth I. Aston, Ph.D. [d]; Douglas T. Carrell, Ph.D. [d,e,*]; and Timothy G. Jenkins, Ph.D. [d,g,*]

Affiliations

[a] Human Nutrition Unit, Biochemistry and Biotechnology Department, Faculty of Medicine and Health Sciences, Universitat Rovira i Virgili (URV), 43201 Reus, Spain.

[b] Institut d'Investigació Sanitària Pere i Virgili (IISPV), 43204 Reus, Spain.

[c] Consorcio CIBER, M.P., Physiopathology of Obesity and Nutrition (CIBERobn), Instituto de Salud Carlos III (ISCIII), 28029 Madrid, Spain.

[d] Andrology and IVF Laboratory, Division of Urology, Department of Surgery, University of Utah School of Medicine, 84108 Salt Lake City, UT, USA.

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[e.] Department of Human Genetics, University of Utah School of Medicine, 84108 Salt Lake City, UT, USA.

[f.] Hospital Universitari Sant Joan de Reus (HUSJR), 43204 Reus, Spain.

[g.] Department of Physiology and Developmental Biology, Brigham Young University, 84062 Provo, UT, USA.

*** Corresponding authors**

Douglas T. Carrell

Andrology and IVF Laboratory, Division of Urology, Department of Surgery, University of Utah School of Medicine, 84108 Salt Lake City, UT, USA. Tel: (+1) 801-581-3740, Email: douglas.carrell@hsc.utah.edu

Timothy G. Jenkins

Department of Physiology and Developmental Biology, Brigham Young University, 84062 Provo, UT, USA. Tel: (+1) 801-422-6797, Email: tim_jenkins@byu.edu

Availability of the data: The array methylation data have been deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) (GSE140004).

Key words: Diet, epigenetics, healthy individuals, nuts, sperm DNA methylation.

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ABSTRACT

BACKGROUND: Many environmental and lifestyle factors have been implicated in the decline of sperm quality, with diet being one of the most plausible factors identified in recent years. Moreover, several studies have reported a close association between the alteration of specific sperm DNA methylation signatures and semen quality.

OBJECTIVES: To evaluate the effect of tree nuts consumption on sperm DNA methylation patterns in healthy individuals reporting eating a Western-style diet.

MATERIAL AND METHODS: This is a post-hoc analysis conducted in a subset of participants (healthy, non-smoking, and young) from the FERTINUTS 14-wk randomized-controlled, parallel-trial, recruited between December 2015 and February 2017. The participants included in the current study (n=72) were randomly selected in a proportion 2:1 from the original FERTINUTS trial between the 98 participants that completed the entire dietary intervention (nut group, n=48; control group, n=24). Sperm DNA methylation patterns were examined at baseline and after 14 weeks in 48 individuals consuming 60 g/d of mixed nuts (nuts group) and in 24 individuals following the usual Western-style diet avoiding consumption of nuts (control group).

RESULTS: Over the course of the trial, no significant changes in global methylation were observed between groups. However, in the nuts group, we identified 36 genomic regions that were significantly differentially methylated between the baseline and the end of the trial and 97.2% of the regions displayed hypermethylation. We identified no such change in the control group over the same period of time. We also utilized the recently developed germ line age calculator to determine if nut consumption resulted in alterations to the epigenetic age of cells and no significant differences were found.

DISCUSSION AND CONCLUSION: Adding nuts to a regular Western-style diet subtly impacts sperm DNA methylation in specific regions, demonstrating that there are some sperm epigenome regions that could respond to diet.

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INTRODUCTION

Male infertility is a complex disorder of the reproductive system affecting nearly 1 in 10 men aged 16–74 years.[1] This highly prevalent condition has worsened during the last 45 years with a 50–60% decline in total sperm count and concentration in North America, Europe and Australia.[2] Many physiological, genetic, environmental and lifestyle factors, have been implicated in this decline in sperm quality, with diet being one of the most plausible factors identified in recent years.[3–10]

Moreover, recent studies have reported a close association between the alteration of specific sperm DNA methylation signatures (e.g. *H19, MEST*, and *SNRPN*) and semen quality[11–13] that could be associated with assisted reproductive techniques (ART) outcomes.[14] Therefore, DNA methylation could be a potential mechanism whereby diet can induce changes to basic semen parameters. In fact, this mechanism was suggested six years ago in an animal study. Two different groups of mice were administered diets supplemented with 2 mg folic acid per kg/d (folic sufficient diet) or 0.3 mg folic acid per kg/d (folic deficient diet) over 2 weeks. The results suggested that a father's diet can influence not only their sperm quality and sperm DNA damage, but also offspring health, including the incidence of birth defects (craniofacial defects, limb hyperextension, spine malformation and dorsal malformations, among others). The study pointed out that adequate paternal dietary folate is essential to maintain normal sperm epigenetic patterns necessary for offspring health.[15] However, main results in humans to date have shown a highly stable human sperm methylome after a short-term (90 days) folic acid supplementation.[16]

Until now, no randomized clinical trials (RCT) testing foods have been published in the literature with the objective to evaluate the effects in sperm DNA methylation. Within the framework of the FERTINUTS trial, the objective of this analysis is to evaluate the effect of a short-term consumption of a mixture of tree nuts on sperm DNA methylation patterns in healthy individuals reporting eating a Western-style diet.

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MATERIALS AND METHODS

Study design

The present study was conducted in a sub-cohort of participants that were submitted to a dietary intervention within the FERTINUTS trial between December 2015 and February 2017. The FERTINUTS study was a parallel 14 weeks, randomized, controlled clinical trial aimed to assess the effects of nut supplementation on sperm quality parameters, and the design and methods of the study was published elsewhere.[6] Trial registration: ISRCTN12857940. The protocol was approved by the Institutional Review Board of the *Hospital Universitari Sant Joan de Reus* in October 2015. All participants provided a written informed consent, and the study was done according to the Declaration of Helsinki for Medical Research involving Human Subjects.

The study participants were healthy, non-smoking and young men (18–35 years old) who reported routinely eating a Western-style diet (according to a 15-item dietary screener modified from Martínez-González *et al.* 2012 [17]) without any of these exclusion criteria: severe chronic illness, alcohol or drug abuse, frequent consumption or allergy of nuts, use of supplements (i.e. plant sterol, fish oil supplements, multivitamins, vitamin E or other antioxidant supplements), history of reproductive disorders or vasectomy, current smokers, or use of drugs for chronic diseases.

The participants included in the FERTINUTS trial were randomly assigned in a 1:1 manner using a computerized random model into 1 of 2 parallel groups: one group consumed the usual Western-style diet enriched with 60 g of a mixture of nuts/d (30 g of walnuts, 15 g of almonds, and 15 g of hazelnuts; nuts group), and the other consumed the usual Western-style diet while avoiding nuts (control group).

In the main trial a total of 244 subjects were assessed for eligibility. Excluding the subjects who declined to participate or did not meet the inclusion criteria, a total of 119 participants were randomly assigned to 1 of the 2 intervention groups, and finally, 98 participants successfully completed the study (49 per group).

Population selected

The participants included in the current study (n=72) were randomly selected from the original FERTINUTS trial [6] (in a 2:1 treatment versus control manner using a computerized random model) between the 98 participants that completed the entire dietary intervention: nut group; n=48, and control group; n=24 **(Supplemental Figure 1)**. The results were analyzed pre- and post-treatment,

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using the pre-treatment as a self-control for every subject taking into account the values of both groups (control and nut group) to establish causal effects benefiting from the clinical trial design and to detect possible sources of bias.

Participants characteristics

General anthropometric characteristics, general information on medical history, reproductive history and use of medication was collected by means of a questionnaire at baseline by trained staff. Weight, height and waist circumference were recorded using a high-quality electronic scale (TANITA TBF-300, Tanita), and blood pressure was recorded using a semiautomatic oscillometer (Omron HEM-705CP, Netherlands).

During the follow-up (in four different visits), all the participants completed a specific questionnaire reporting any adverse effects related or not related to the intervention, and a 3-day dietary record (3DDR) including 2 workdays and a weekend day in face-to-face interviews with an expert nutritionist-dietitian. Only at baseline the participants completed a 143-item, semi-quantitative, validated food frequency questionnaire (FFQ) over the past year.[18] Energy and nutrient intake were calculated using Spanish food composition tables.[19,20] Compliance with the intervention was assessed by counting the empty sachets of nuts returned by the participants and by the 3DDR questionnaire.

At baseline and at the end of the intervention, blood samples in 12 h fasting conditions and semen samples after 3 days of sexual abstinence were collected. Fasting glucose, total cholesterol, HDL cholesterol, LDL cholesterol, VLDL cholesterol, triglycerides, insulin, C-reactive protein (CRP) and folate were determined in blood by routine laboratory tests using standard enzymatic automated methods (COBAS; Roche Diagnostics Ltd, UK). Seminogram parameters (pH, volume, vitality, total sperm count and concentration, motility and morphology) were assessed in fresh semen samples with a maximum of 60 minutes after collection (detailed information can be found in [6,10]) according the 2010 World Health Organization's report [21] and following the Björndahl checklist.[22] After the seminogram analysis, semen samples were frozen at -80ºC with a slow freezing protocol (-1ºC/min) following dilution in 10% dimethyl sulfoxide (DMSO) until DNA isolation.

Sperm purification and DNA isolation

Prior to DNA isolation, a somatic cell lysis methodology was performed by incubation sperm samples with somatic cell lysis buffer, which contained both 0.1% SDS and 0.5% Triton X-100 (in Milli-Q®

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water), on ice followed by two high volume wash steps.[23] Optical microscopic examination was used to verify somatic cell elimination before proceeding. To establish the efficacy of this lysis protocol, a post-hoc analysis of somatic cell contamination was conducted using the *DLK1* locus. That region contains fourteen CpGs that are differentially methylated between sperm cells (low methylation levels) and somatic round cells (high methylation levels). In sperm we have established a threshold of the average methylation across those CpGs=0.20 (or 20% methylation).[12]

Total sperm DNA was isolated according to the QIAamp® DNA Mini (QIAGEN, Hilden, Germany) manufacturer protocol with the addition of a sperm-specific lysis solution (that contains proteinase K and dithiothreitol) and stored at -20ºC until analysis. DNA concentration and purity were determined using a Nanodrop-1000 spectrophotometer (Thermo Fisher Scientific).

Bisulfite conversion, microarray analysis and data processing

A total of 500ng of extracted DNA was bisulfite converted with the EZ-96 DNA Methylation kit (Zymo Research, Irvine, CA, USA) according to manufacturer's recommendations specifically for use with Illumina array platforms. The converted DNA was then hybridized to Infinium® MethylationEPIC BeadChip microarrays (Illumina) and analyzed according to Illumina protocols at the University of Utah Genomics Core Facility. The EPIC microarray targets more than 850k methylated CpGs in promoter, gene body and enhancer regions at single-nucleotide resolution.

To generate beta-values (β-values), data from microarrays were processed using the *minfi* Bioconductor package (*minfi*; package at http://www.bioconductor.org) [24,25] an additional package for the freely available R statistical computing environment v.3.5.0 (www.r-project.org).[26] First, the array data for all samples were evaluated for standard data quality indicators (QC and density plots; data not shown). Beta-values were then generated by analyzing the intensities for methylation or no methylation at each CpG using the following equation: β-value = methylated/(methylated + unmethylated). The resultant β-value ranges from 0 to 1 and indicates the relative levels of methylation at each CpG; a value of 1 represents a completely methylated CpG and 0 represents a completely unmethylated CpG. These intensity values were subjected to a removal of poorly performing probes from the downstream analysis (probes with a QC $p<0.05$) and SWAN normalization.[27] Normalized β-values were then logit transformed to generate M-values for further analyses. P-values of <0.05 were considered significant after Benjamini-Hochberg (BH) False Discovery Rate (FDR) correction to control for multiple comparisons.[28]

Differential methylation analysis

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Global methylation analysis was conducted by averaging β-values across all probes on the array for pre- and post-treatment individuals in both groups. β-values were also averaged considering CpG island context and gene association. The average values were compared by a paired t-test.

A single CpG analysis was also applied. This analysis was performed using a paired t-test between pre- and post-treatment individuals, and the P-values were subjected to a Benjamini-Hochberg FDR correction.

Moreover, an analysis of methylation alteration involving genomic regions (approximately 100–2,000 bps in size) was performed. Such analyses can identify more subtle alterations that occur over multiple neighboring CpGs. This analysis was performed using the Useq bioinformatics software package (https://sourceforge.net/projects/useq/). Briefly, paired data from each subject (at baseline and at 14 weeks of intervention in both intervention groups) was subjected to a 1,000 base pair sliding window analysis. To diminish the influence of outliers in the data set, methylation for a specific window was reported as a pseudo-median and differences between the samples were reported as log 2 ratios. Two thresholds were applied to identify windows with significant differential methylation: 1) a Benjamini-Hochberg FDR corrected Wilcoxon Signed Rank Test of ≤0.0001 (≥ transformed FDR of 40), and 2) an absolute log2 ratio ≥0.2. Raw FDR values were transformed with the following formula: ($-10 \log_{10}$ (q-value FDR)), such that a 13=0.05, 20=0.01, 25=0.003, 30=0.001, and 40=0.0001, etc. This approach has been successfully implemented multiple times in our laboratory.[12,29,30] From this regional data, the single CpG contained within the significantly differentially methylated regions were subsequently extracted for further interrogation. A complementary analysis of the primary results was applied to prevent batch effects and other possible confounding factors (e.g. 14 weeks of aging).

In order to predict the functions of the significant regions, the Genomic Regions Enrichment of Annotations Tool (GREAT; http://great.stanford.edu/) from the Bejerano Lab at Stanford University was used.[31]

Large scale data information

The GRCh37 (UCSC GCRh37/hg19, Feb/2009) *Homo sapiens* assembly was used for all the aforementioned analysis. The array methylation data have been deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) (GSE140004).

Germ line aging analysis

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We additionally performed a germ line aging analysis on the samples to determine if the epigenetic age signatures in sperm were altered as a result of nut consumption. Based in 51 regions of the genome identified previously by our group to accurately predict chronological age, we conducted an age calculation prediction for all samples [32]. In this study, the authors suggested that the rate of germ line aging can be affected by environmental exposures or lifestyles (smoking, obesity, etc.). With this hypothesis in mind we tested whether nut supplementation could affect the germ line age, and investigated whether 14 weeks of intervention are sufficient to detect differences in the methylation-based germ line age calculation.

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RESULTS

A total of 72 subjects were included in the present analysis (48 in the nuts supplemented group and 24 in the control group) at two time points (at baseline and at the end of the dietary intervention). No differences in the main baseline general characteristics of the participants between originally randomized and those included in the present study were shown. The baseline general anthropometric characteristics of the individuals selected for the present study are shown in **Table 1**. No significant differences were observed between baseline values in any of the analyzed parameters validating the sequence of randomization used in the initial RCT. Moreover, no significant differences were observed in baseline blood biochemical and semen parameters, however significant differences in total sperm count, and concentration, total motility, progressive motility, and normal morphology were observed between the two study groups, being greater in the nut group **(Supplemental Table 1)** as reported in the main paper.[6]

Baseline and 14-wk changes in nutrients and food consumption after the intervention are shown in **Supplemental Table 2**. As a result of consuming nuts, treatment subjects showed significantly higher intake of total proteins, total fat, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), fiber, magnesium, total omega-3, α-linolenic acid (ALA) and omega-6.

The *DLK1* locus quality control data confirmed that the samples assessed in our study are clear of round cell contamination (mean ± standard deviation; SD of the 14 CpGs in all the individuals analyzed=0.169±0.055) **(Supplemental Figure 2)**.

We analyzed intraindividual DNA methylation data for differences in global methylation (methylation across the entire genome considered as a single average) over the course of the trial. Changes in global methylation (reported as absolute average difference of methylation β-values) between study groups at the end of the study were non-significant (mean ± SD; in control group=0.0027±0.0054 and nut group=0.0033±0.0039) **(Figure 1-A)**. Additionally, we evaluated average difference of methylation across different genomic features (CpG islands, gene bodies, or non-CpG loci interrogated) **(Figure 1-B, C and D)**, and again no significant differences were observed across the two study groups.

Moreover, we evaluated methylation alterations at specific loci. Site-specific DNA methylation analysis revealed no significantly differentially CpGs associated with nut intervention following Benjamini-Hochberg FDR correction for multiple comparisons.

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In addition to the global analysis and single CpG evaluation, we performed a high-resolution analysis of region-specific methylation differences. After different complementary analyses to exclude batch effects and other possible confounding factors (e.g. 14 weeks of aging), we identified 36 genomic regions that were significantly differentially methylated between the baseline and the end of the trial in the nuts group **(Table 2)**. A paired comparison of the individuals in the control group did not show regional statistical differences. 97.2% of differentially methylated regions displayed increased methylation at the end of the trial in the nut consuming group (only one region showed a decrease of methylation). The differentially methylated regions included 904 individual CpGs (718 increased and 186 decreased methylation). CpG islands (54.98% vs. 18.62%) were significantly over-represented and imprinted genes (0% vs. 0.81%) were significantly under-represented compared to the background of the entire array (866,836 CpGs totally analyzed) **(Figure 2)**.

All of the differentially methylated regions are associated with a gene and the majority (84.9%) are proximal (<50kb) to transcription start sites (TSS) **(Figure 3)**. The genes encompassed in the differentially methylated regions were subjected to gene ontology (GO) analysis, but no one GO term or Pathway was significantly enriched.

We additionally assessed methylation patterns in these samples using the recently developed age prediction algorithm for sperm which enables the identification of “germ line age” to determine whether nut supplementation impacted DNA methylation-based germ line age **(Figure 4)**. However, the assessment of this specific metric yielded no significant findings.

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DISCUSSION

In the present study, we demonstrated that adding 60 g of mixed nuts/d to a Western-style diet for 14 weeks (short-term nutritional intervention) has a subtle impact on the sperm DNA methylome in a group of healthy, reproductive-aged, non-smoking participants. Regarding the impact of the consumption of nuts on the sperm methylation profiles, no significant changes in global methylation were observed between both groups. However, 36 regions were significantly differentially methylated (35/36 hypermethylated) between the baseline and the end of the trial only in the nuts group. Interestingly, this bias toward regions with an increase in methylation is in contrast to changes observed as a result of increasing age,[29] smoking,[30] or elevated BMI,[33] where most differentially methylated regions display reduced DNA methylation, but our direct analysis of germ line age showed no significant decrease in the aging signal.

This is the first study analyzing the relation of mixed nuts (e.g. walnuts, hazelnuts and almonds) and sperm DNA methylation patterns at specific loci and regions, as a follow-up to a previous study where the authors examined total sperm DNA methylation in the same cohort of healthy men. This was made possible because of the unique nature of the FERTINUTS trial. In the main study we showed that the supplementation of a Western-style diet with walnuts, hazelnuts, and almonds improves sperm quality parameters among healthy reproductive-aged men, and these beneficial effects could possibly be explained by a reduction in sperm DNA fragmentation.[6] However, we pointed out that sperm DNA methylation could be another potential mechanism involved in these changes. Even though the main study did not observe significant changes in global sperm DNA methylation after the consumption of nuts, the technique used had a very low sensitivity (ELISA-based), and higher resolution analyses were necessary to confirm this finding. In fact, the present analysis did identify changes to the methylome, suggesting additional effects of nut consumption on sperm physiology.

As has been observed in previous studies, the sperm methylome in this cohort was highly homogeneous (no interindividual differences at baseline).[34] Despite this, we were able to identify some subtle changes that occurred over the course of the study in the nut consuming group. However, we did not find that the nut consumers developed a change of methylation globally compared to the control group. It is not surprising that we did not identify widespread and overarching changes to the epigenome due to the short duration of the study and the likelihood for variability. In fact, studies assessing supplementation of various antioxidants (such as those found in

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nuts) are often plagued by similar issues of variability in the groups of interest.[35] Interestingly though, some recent experiments have found a rapid response to a dietary intervention on other epigenetic system (e.g. tsRNA) in human sperm.[36] This could indicate that sperm DNA methylation is more attenuated or requires longer to manipulate.

Moreover, when analyzing specific regions with a high predictive aging value (germ line age calculation), we could not identify evidence of young or aging-like signals in the sperm methylome of these individuals.[32] This finding may be due to the fact that the changes are too subtle over such a short period of time to be detected with this methodology.[37]

However, when regional results are compared with other studies, the results become more interesting. A recent case-control study performed by our group analyzed the impact of cigarette smoking on sperm DNA methylation patterns in 78 men who smoke and 78 never-smokers. The authors found 141 significantly altered CpGs, with 74% of those loci displaying a loss of methylation.[30] In general, these types of studies showed that unhealthy lifestyle factors appear to result in hypomethylation effects. In contrast, we found 36 differentially methylated regions in the current study, with 97.2% of regions (35/36) displaying an increase of methylation. As we know, dietary nut supplementations are a healthy habit conferring beneficial effects not only in the prevention of cardiovascular diseases,[38] but also in the improvement of sperm quality parameters [6] and sexual function.[9]

Nuts contain significant amounts of folic acid and other nutrients with potential ability to change DNA methylation. According to the US Department of Agriculture Nutrient Database (available from: https://ndb.nal.usda.gov/ndb/search/list) this mixture of nuts offers approximately 53µg of folate/day of supplementation among other minerals and vitamins (**Supplemental Table 3**). While folic acid taken independently seems not to be related with DNA methylation changes in humans [16] it is possible that when consumed in a native form with other important nutrients it could play a role in methylation regulation the effect is observed. Beyond folic acid content, nuts are a nutrient-dense food with a special nutrient content, and some of the bioactive compounds that are present in hazelnuts, almonds and walnuts (e.g. genistein, epigallocatechin-3-gallate) have been previously shown to affect DNA methyltransferase (DNMT) activity.[39] For example, epigallocatechin-3-gallate treatment lowered DNMT activity in esophageal cancer cells and resulted in the reversal of the hypermethylated state of tumor suppressor genes including *p16, RARB, MLH1* and *MGMT*.[40] Similarly, genistein, the most studied isoflavone in humans, has demonstrated estrogen-like

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properties, andtreatment of esophageal carcinoma cells with Genistein resulted in a partial reversal of DNA hypermethylation and reactivated *p16, RARB* and *MGMT*.[41] The direct relation between these nutrients and the sperm DNA methylation changes should be investigated in future studies.

Several limitations related to our study should be mentioned. First, this is a post-hoc analysis conducted in a subset of participants of a previous clinical trial and this could weaken our findings, though sample size is reasonable for a study of intra-individual changes. Second, the trial focuses on healthy and apparently fertile men, therefore, the results cannot necessarily be extrapolated to other populations. Third, no validation of any of the differentially methylated regions using a different method was done. Fourth, we acknowledge that the period of intervention is probably too short to represent epigenetic changes which can affect sperm DNA over an entire lifetime. Lastly, the scope of the findings is limited to impacts on the sperm epigenome and it is still unknown whether these modifications could be relevant for the embryo development or offspring health. Considering these limitations, this study should be considered as an extremely worthy hypothesis-generating study.

However, these concerns are outweighed by the study strengths. This is a powerful research design sufficiently powered to explore differences in sperm quality parameters as well as epigenetic modifications. Moreover, the study has an adequate phenotypic evaluation of the men included and the study population was composed of relatively homogenous settings (healthy, non-smoking and young men).

In conclusion, our findings provide the first evidence that adding nuts to a regular Western-style diet subtly impacts sperm DNA methylation in specific regions, demonstrating that there are some environment sensitive regions of the sperm epigenome that could respond to diet. The potential health benefits of our findings deserve further investigation. Additionally, it is important to further explore these findings in other patient populations, as it is possible that alternate populations, such as aged men may respond more drastically, or differently, than a young, healthy population of men.

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DECLARATIONS

Ethics approval and consent to participate

Trial registration: ISRCTN12857940. The protocol was approved by the Institutional Review Board of the *Hospital Universitari Sant Joan de Reus* in October 2015. All participants provided a written informed consent, and the study was done according to the Declaration of Helsinki for Medical Research involving Human Subjects.

Consent for publication

Not applicable.

Availability of data

The array methylation data have been deposited in the National Center for Biotechnology Information's (NCBI) Gene Expression Omnibus (GEO) (GSE140004).

Competing interests

J.S.-S. reports serving on the board of and receiving grant support through his institution from International Nut and Dried Fruit Council; receiving consulting personal fees from Danone; and receiving grant support through his institution from Nut and Dried Fruit Foundation. K.I.A., D.T.C. and T.G.J. are equity holders in Inherent Biosciences Inc. None of the other authors reported a conflict of interest related to the study.

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Authors' contributions

A.S-H. and J.S-S. initiated the idea of the trial. A.S-H., E.R.J., M.B., J.S-S., and T.G.J. were involved in study design. A.S-H. was involved in study conception and execution, acquisition and analysis of

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data, and wrote the manuscript; E.R.J. and T.G.J. were involved in acquisition and analysis of the data; E.R.J., M.B., J.S-S., K.I.A., D.T.C., and T.G.J. supervised the analysis and critically revised the manuscript. All authors provided substantial intellectual contributions and approved the final version of the manuscript.

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TABLES

Table 1. Baseline characteristics of the study population.

Baseline characteristics	**Nut group (n=48)**	**Control group (n=24)**	**P-value**
Age (years)	24.21 (4.81)	25.63 (4.86)	0.472
Weight (kg)	74.32 (10.00)	76.38 (13.77)	0.582
BMI (kg/m²)	23.71 (2.94)	24.10 (4.10)	0.645
Waist circumference (cm)	80.38 (7.62)	82.78 (9.87)	0.259
Systolic blood pressure (mmHg)	129.90 (11.51)	127.33 (12.07)	0.384
Diastolic blood pressure (mmHg)	73.08 (8.23)	72.05 (8.76)	0.623

Data are given as mean ± standard deviation (SD). Differences between groups were calculated by t-tests assuming equal variances (subjected to Levene's homogeneity of variance analysis).

Abbreviations. BMI: Body-mass-index.

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Table 2. Genomic regions of alteration in the nuts group after 14-wk of nuts consumption (n=36). Represented in this table are the windows of significance that were identified in our study as well as their transformed Wilcoxon FDR value, the gene(s) identified in the region and the distance to TSS (ordered by Chromosome/Start-Stop). Genome Version: Homo sapiens (UCSC GCRh37/hg19, Feb/2009).

Chromosome	Start	Stop	Genomic Region size (b)	Wilcoxon FDR Value	Gene(s) in the region (distance to TSS)
chr1	26798177	26798965	788	40.299	*HMGN2* (-439)
chr1	35658023	35659183	1160	62.960	*SFPQ* (+146)
chr1	167905730	167906690	960	45.376	*MPC2* (-932), *DCAF6* (+154)
chr1	230882603	230883553	950	47.706	*CAPN9* (-52)
chr2	26395155	26396788	1633	58.735	*GAREML* (+12)
chr4	146018582	146020237	1655	65.668	*ANAPC10* (-57), *ABCE1* (+326)
chr5	176513311	176514433	1122	57.218	*FGFR4* (-15)
chr6	28863544	28864833	1289	52.486	*SCAND3* (-309,077), *TRIM27* (+27,577)
chr6	30522985	30525528	2543	54.591	*PRR3* (-406), *GNL1* (+694)
chr6	30653799	30655746	1947	56.047	*PPP1R18* (+899)
chr6	31619513	31621766	2253	64.126	*APOM* (-2,608), *BAG6* (-163)
chr6	31829793	31831891	2098	65.668	*NEU1* (-159)
chr6	31864682	31870991	6309	65.668	*EHMT2* (-2,376)
chr6	32133929	32135804	1875	65.668	*EGFL8* (+2,485), *AGPAT1* (+9,990)
chr6	32764865	32765403	538	65.668	*HLA-DQB2* (-33,867), *HLA-DOB* (+19,691)
chr6	33167488	33169607	2119	58.762	*HSD17B8* (-3,871), *RXRB* (-157), *SLC39A7* (-102)

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chr6	33215649	33218095	2446	61.494	*VPS52* (+22,799), *RING1* (+40,600)
chr6	33265133	33268072	2939	51.989	*RGL2* (+498)
chr6	43027125	43028005	880	51.626	*KLC4* (-30), *MRPL2* (-21)
chr6	168709593	168710204	611	47.578	*FRMD1* (-230,060), *DACT2* (+10,503)
chr7	140097579	140098580	1001	55.167	*SLC37A3* (+269)
chr7	150754731	150756774	2043	51.637	*CDK5* (-136), *SLC4A2* (+454)
chr9	125666989	125667945	956	47.985	*RC3H2* (-7,078), *ZBTB6* (+8,142)
chr10	43047784	43048735	951	49.732	*ZNF33B* (+85,732)
chr10	48416391	48417339	948	47.953	*GDF2* (-12)
chr10	88728073	88728948	875	40.457	*ADIRF* (+562)
chr11	27721088	27723790	2702	62.498	*BDNF* (+161)
chr11	118662123	118662892	769	48.435	*DDX6* (-663)
chr12	5620611	5621457	846	51.009	*NTF3* (+79,755), *ANO2* (+434,364)
chr14	90722653	90723463	810	44.545	*PSMC1* (+219)
chr15	23085583	23087240	1657	46.756	*NIPA1* (+24)
chr17	76100510	76101392	882	41.099	*TMC6* (+23,827), *TNRC6C* (+99,814)
chr18	77335412	77336268	856	44.841	*CTDP1* (-103,961), *NFATC1* (+175,504)
chr19	46525566	46526864	1298	44.447	*PGLYRP1* (+108)
chr21	34863117	34864412	1295	54.268	*DNAJC28* (+15)
chrX	68047520	68049086	1566	65.668	*EFNB1* (-537)

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Abbreviations. b: bases; TSS: Transcription start site.

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FIGURES CAPTIONS

Figure 1. Box plot of the differences between the control group and the nut group based on genomic context (entire array, A; CpG islands, B; gene bodies, C; and non-CpGs loci, D). No differences in average methylation were observed.

Figure 2. Number and percentage of CpGs islands, imprinted and enhancers regions found in the hypermethylated significative regions compared to the background of the entire array.

Figure 3. (A) Bar plot showing the number of associated genes per region. (B) Histogram displaying the enrichment of regions with methylation alteration associated with nut intervention in the context of the distance of regions from transcription start sites (TSS).

Figure 4. (A). Boxplot depicting the age prediction analysis. (B). Density plot shows the accuracy of age prediction in baseline and final intervention period in both analyzed groups.

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SUPPORTING INFORMATION

Supplemental Tables

Supplemental Table 1. Baseline values and changes after the intervention period in blood and sperm parameters.

Supplemental Table 2. Nutrient intake at baseline and changes by intervention group.

Supplemental Table 3. Average minerals and vitamins composition of studied nuts (per 100 g).

Supplemental Figures

Supplemental Figure 1. Flow diagram.

Supplemental Figure 2. Scatter plot of DNA methylation levels at the DLK1 locus in the analyzed samples. In green the 20% methylation threshold (round sperm cells) and in blue the methylation mean of all the samples.

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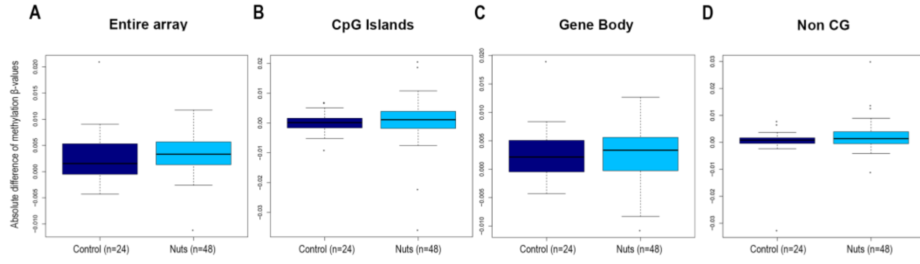

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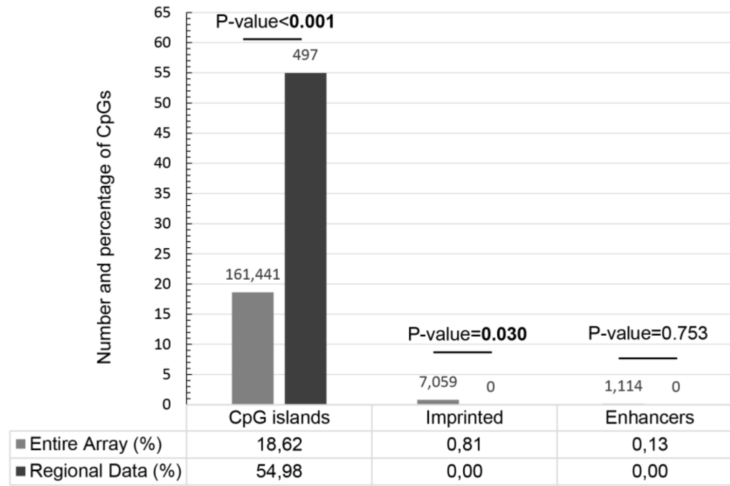

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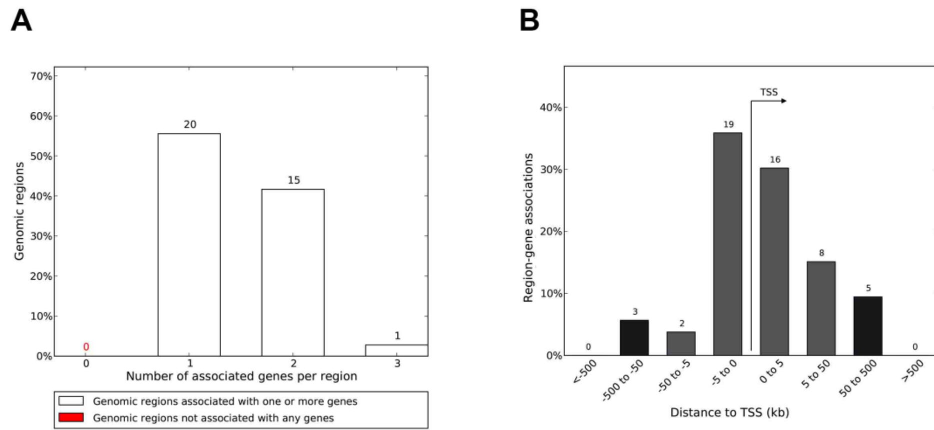

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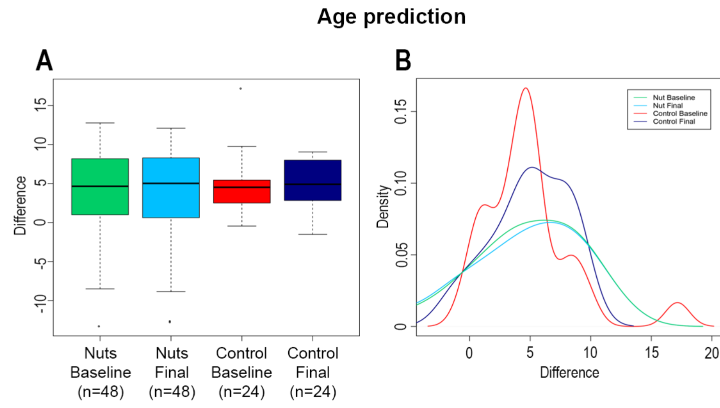

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