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Effect of nut consumption on semen quality and functionality in healthy males consuming a western-style diet: a randomized controlled trial

--Manuscript Draft--

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Abstract:	Background: Human semen quality has declined in industrialized countries where pollution, smoking, and the consumption of a western-style diet are hypothesized as potential causes. Objectives: We evaluated the effect of chronic consumption of nuts on changes in conventional semen parameters and the potential mechanisms implicated. Design: The FERTINUTS study was a 14-week randomized, controlled, parallel trial. 119 healthy males aged 18-35 were allocated to two intervention groups: a) enriching the usual western-style diet with 60 g/d of a mixture of nuts (nut-group); b) following the usual western-style diet avoiding nuts (control-group). Semen and blood samples were collected at baseline and at the end of the intervention. Dietary information was recorded throughout the trial. Changes in conventional semen parameters (motility, volume, pH, sperm count and morphology) were determined as primary outcomes. The effect of nut consumption on spermatozoa (spz) DNA-fragmentation (SDF), reactive oxygen species (ROS) production, chromosome anomalies (X, Y, and 18), total DNA-methylation, and miRNAs expression were measured in sperm samples as potential causes of the changes in the seminogram.

	Results: Compared to the control group, an improvement in total sperm count (P-value=0.002), vitality (P-value=0.003), total motility (P-value=0.006), progressive motility (P-value=0.036), and morphology (P-value=0.008) was observed in the nut-group. Participants in the nut-group showed an increase in the consumption of total fat, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), magnesium, vitamin E, α-Linolenic acid (ALA), total omega 3 and omega-3/omega-6 ratio intake during the intervention. Participants in the nut-group showed a significant reduction in SDF (P-value<0.001), and in the expression of hsa-miR-34b-3p (P-value=0.036). No significant changes in ROS, sperm chromosome anomalies, or DNA-methylation were observed between groups. Conclusions: The inclusion of nuts in a western-style diet significantly improves the total sperm count, vitality, motility, and morphology. These findings could be partly explained by a reduction in the SDF.
Additional Information:	
Question	Response
REGISTRATION **A** - The NIH has very recently updated their position on which studies need to be registered in clinicaltrials.gov. They distinguish between a clinical study and a clinical trial. *The AJCN* will adhere to the NIH position. The NIH defines a clinical trial as a research study in which one or more human subjects are prospectively assigned to one or more interventions (which may include placebo or other control) to evaluate the effects of those interventions on health-related biomedical or behavioral outcomes. **Authors should use the following four questions to determine the difference between a clinical study and a clinical trial**: 1. Does the study involve human participants? 2. Are the participants prospectively assigned to an intervention? 3. Is the study designed to evaluate the effect of the intervention on the participants? 4. Is the effect being evaluated a health-related biomedical or behavioral outcome?	Trial registration number: ISRCTN12857940 URL of registration: http://www.isrctn.com/ISRCTN12857940

Note that if the answers to the 4 questions are yes, your study meets the NIH definition of a clinical trial and must be registered at clinicaltrials.gov or another trial registry, even if… • You are studying healthy participants • Your study does not have a comparison group (e.g., placebo or control) • Your study is only designed to assess the pharmacokinetics, safety, and/or maximum tolerated dose of an investigational drug • Your study is utilizing a behavioral intervention Studies intended solely to refine measures are not considered clinical trials. Studies that involve secondary research with biological specimens or health information are not clinical trials and are NOT required to be registered. You should consult the website https://grants.nih.gov/policy/clinical-trials/case-studies.htm and use the more than 30 examples to determine whether your research is a clinical trial. **B** ---For all studies, including those that don't require registration by the above rules, the authors must state explicitly in the Methods Section the pre-declared primary and secondary endpoints of their study and whether these changed during the course of the study or during post-hoc analyses. Also the paper must state explicitly that analyses not pre-specified	

are considered exploratory. To summarize, if you answer the 4 questions above with "yes" then you must register your trial before AJCN will consider it further. If you answer at least one of the 4 questions "no" you do not need to register your study. In either case you must revise your Methods section to conform to point 2 above. **Note that after 1 July 2018, AJCN will no longer allow retrospective registration**. All studies that fall under the NIH registration rules and recruited their first participant after 1 July must be registered prospectively. Appropriate public trial registries include ICMJE-approved public trials registries (http://www.clinicaltrials.gov, http://www.anzctr.org.au/, http://www.isrctn.org, http://www.umin.ac.jp, http://www.trialregister.nl). Please report the study ID number and the website where the clinical trial is registered on the title page of the paper.	
Please select a collection option from the list below:	Growth, Development, and Reproduction

MD. Dennis M. Bier
Editor-in-Chief of The American Journal of Clinical Nutrition

March 2018

Dear MD. Dennis M. Bier,

Please find enclosed the manuscript entitled “Effect of nut consumption on semen quality and functionality in healthy males consuming a western-style diet: a randomized controlled trial” by A. Salas-Huetos, R. Moraleda, S. Giardina, E. Anton, J. Blanco, J. Salas-Salvadó and M. Bulló for consideration for publication in The American Journal of Clinical Nutrition.

Human semen quality has declined in industrialized nations where pollution, smoking, and western-style diet eating habits are hypothesized to be potential causes. Recently, some studies suggest that healthy diets rich in omega-3, antioxidants (e.g. vitamin C and E, selenium and zinc), carnitines and folate might improve the quality of semen. Because nuts are rich in some of these nutrients, we hypothesize that adding nuts to a western-style diet could improve semen quality and functionality.

The FERTINUTS study was designed as a 14-week randomized, controlled, parallel two-group trial. Healthy males aged 18-35 were allocated to either a group that followed their usual western-style diet supplemented with 60 g/day of a mix of raw almonds, hazelnuts and walnuts (nut group), or to a group that followed their usual western-style diet and avoided eating nuts (control group).

In the present study we demonstrate that adding nuts to a western-style diet improved total sperm count, vitality, motility and morphology in a group of healthy reproductive-aged participants compared to an age-matched control group who avoided eating nuts. Of the mechanisms explored in order to understand these findings (i.e. sperm DNA fragmentation, reactive oxygen species production, chromosome anomalies -for X, Y, and 18-, total DNA methylation, and miRNAs expression), only a reduction in DNA fragmentation after nut consumption seems to be able to explain these beneficial effects observed for conventional sperm quality parameters.

These results support the potential benefits of certain nutrients contained in nuts for sperm quality.

In our opinion, the content of our randomized controlled trial fits the scope of The American Journal of Clinical Nutrition and will be of great interest to the journal’s readers.

No concurrent submission of this manuscript has been made to any other journal. The material contained in the manuscript is original and has not been published elsewhere.

We look forward to receiving your decision regarding the submitted article.

Yours sincerely,

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June 2018

Dr. Arne Astrup, and Dr. Dennis M Bier

Editors of The American Journal of Clinical Nutrition

Re: Manuscript Number: Manuscript **# ID AJCN-D-18-00232**

Dear Dr. Arne Astrup, and Dr. Dennis M Bier,

Thank you for your response to our manuscript.

Please find enclosed our manuscript entitled **"Effect of nut consumption on semen quality and functionality in healthy males consuming a western-style diet: a randomized controlled trial"**, which we would like to be accepted for publication in **THE AMERICAN JOURNAL OF CLINICAL NUTRITION.**

In submitting this revision, we have taken into account all of the reviewers' criticisms and the editors' suggestions. Our responses are itemised on the accompanying sheets and discuss the changes we have made to the revised text.

We hope that the new manuscript is now suitable for publication in **THE AMERICAN JOURNAL OF CLINICAL NUTRITION**.

I certify,

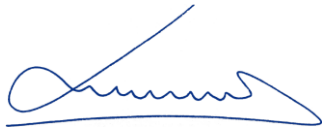
1. That the corresponding authors and all of the authors have read and approved the final submitted manuscript.

2. That no portion of the work has been or is currently under consideration for publication elsewhere.

3. That no portions of the manuscript other than the abstract has been published or posted on the Internet.

Looking forward to hearing from you,

Yours sincerely,



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Title: Effect of nut consumption on semen quality and functionality in healthy males consuming a western-style diet: a randomized controlled trial

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Abbreviations used: 3DDR, 3-day dietary records; ALA, α-Linolenic acid; BMI, body mass index; Ct, threshold cycle; FDR, false discovery rate; FISH, fluorescent in situ hybridization; FFQ, food frequency questionnaire; HDL, high-density lipoprotein; IM, immotility; ITT, intention-to-treat; IQR, interquartile rank; LDL, low-density lipoprotein; MUFA, monounsaturated fatty acids; NP, non-progressive motility; PP, per protocol; PR, progressive motility; PUFA, polyunsaturated fatty acids; qRT-PCR, quantitative PCR; RCT, randomized clinical trial; ROS, reactive oxygen species; RT-PCR, reverse transcription-polymerase chain reaction; SCLB, somatic cell lysis buffer; SDF, sperm DNA-fragmentation; SD, standard deviation; SDS, Sequence Detector Software; SE, standard error; spz., spermatozoa; TdT, terminal deoxynucleotidyl transferase; TLDAs, TaqMan® miRNAs Low-Density Arrays; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; VLDL, very-low-density lipoprotein; wk, week.

Trial registration: The trial was registered at ISRCTN (ISRCTN12857940).

ABSTRACT

Background:

Human semen quality has declined in industrialized countries where pollution, smoking, and the consumption of a western-style diet are hypothesized as potential causes.

Objectives:

We evaluated the effect of chronic consumption of nuts on changes in conventional semen parameters and the potential mechanisms implicated.

Design:

The FERTINUTS study was a 14-week randomized, controlled, parallel trial. 119 healthy males aged 18-35 were allocated to two intervention groups: a) enriching the usual western-style diet with 60 g/d of a mixture of nuts (nut-group); b) following the usual western-style diet avoiding nuts (control-group). Semen and blood samples were collected at baseline and at the end of the intervention. Dietary information was recorded throughout the trial. Changes in conventional semen parameters (motility, volume, pH, sperm count and morphology) were determined as primary outcomes. The effect of nut consumption on spermatozoa (spz) DNA-fragmentation (SDF), reactive oxygen species (ROS) production, chromosome anomalies (X, Y, and 18), total DNA-methylation, and miRNAs expression were measured in sperm samples as potential causes of the changes in the seminogram.

Results:

Compared to the control group, an improvement in total sperm count (P-value=0.002), vitality (P-value=0.003), total motility (P-value=0.006), progressive motility (P-value=0.036), and morphology (P-value=0.008) was observed in the nut-group. Participants in the nut-group showed an increase in the consumption of total fat, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), magnesium, vitamin E, α-Linolenic acid (ALA), total omega 3 and omega-3/omega-6 ratio intake

during the intervention. Participants in the nut-group showed a significant reduction in SDF (P-value<0.001), and in the expression of hsa-miR-34b-3p (P-value=0.036). No significant changes in ROS, sperm chromosome anomalies, or DNA-methylation were observed between groups.

Conclusions:

The inclusion of nuts in a western-style diet significantly improves the total sperm count, vitality, motility, and morphology. These findings could be partly explained by a reduction in the spermatozoa DNA-fragmentation.

Key words

Randomized clinical trial (RCT), western-style diet, nuts, semen quality, sperm DNA fragmentation

BACKGROUND

Infertility is the inability of a sexually active, non-contracepting couple to achieve pregnancy in one year or more (1). It is estimated that one in seven couples worldwide has problems conceiving (2,3), and male factors are responsible for 40-50% of these cases (4). Moreover, recent studies report that human semen quality has declined worldwide, pointing to changes in modifiable lifestyle factors as the main causes (5). Nowadays, pollution, stress, smoking, pesticides in food and trends towards a western-style diet are hypothesized as principal potential causes of this spermatozoa (spz) quality decline (6–9).

The western-style dietary pattern, which is high in saturated fats, red and processed meat, butter, high-fat dairy products, refined grains, white potatoes, refried beans, and rich in sugar drinks and fried foods, has been causally associated with the development of cardiovascular disease, diabetes, and some types of cancer, including breast and colorectal cancer (10). This type of diet has also been associated in some observational studies with low sperm quality in contrast to a healthy Mediterranean dietary pattern (11,12). More recently, some systematic reviews of observational studies and randomized controlled trials (RCTs) pointed out that male adherence to a healthy diet rich in omega-3, antioxidants (e.g. vitamin C and E, selenium and zinc), carnitines and folate may improve semen quality and fecundability (13,14).

Because nuts are nutrient-dense foods rich in some of the above-mentioned nutrients (15) and a key food in a healthy Mediterranean diet, we hypothesized that tree nut consumption added to an unhealthy diet (e.g. western-style) would beneficially affect semen quality and functionality. Therefore, the main objective of the present study was to evaluate the effect of chronic consumption of nuts on changes in conventional semen parameters related to male fertility in healthy individuals who eat a western-style diet. The study also aimed to identify potential mechanisms that modulate fertility status.

METHODS

Study design

The FERTINUTS (Effect of nut consumption on semen quality and functionality in healthy males) study was designed as a 14-week (wk), randomized, controlled, clinical trial with two parallel interventions. The trial was registered at ISRCTN (ISRCTN12857940).

The study participants were healthy males (18-35 years old) who reported routinely eating a western-style diet (according to the 15-item dietary screener modified from Martínez-González *et al.* (16); Supplemental Table 1). Participants were excluded if they had one of the following exclusion criteria: frequent consumption of nuts or a known history of allergy; use of plant sterol or fish oil supplements and multivitamins, vitamin E or other antioxidant supplements; history of reproductive disorders or vasectomy; current smokers; use of medications for chronic illness; or use of illegal drugs. This study was conducted according to the guidelines laid down in the Declaration of Helsinki, and the protocol was approved by the Institutional Review Board of the Hospital Universitari Sant Joan de Reus in October 2015. All the participants provided written informed consent.

The participants included in the study were randomized using a computerized random proportion model into one of two parallel groups: a) enriching the usual western-style diet with 60 g/d of a mixture of nuts (30 g of walnuts, 15 g of almonds and 15 g of hazelnuts) (nut group); b) following the usual western-style diet avoiding eating nuts (control group). Baseline visit was scheduled a maximum of 15 days after randomization. At baseline visit, nuts were provided free of charge to participants in pre-weighed packs. Detailed dietary instructions, including recipes (with or without nuts), were given to participants to increase adherence to the intervention.

General and anthropometric measurements

Weight, height and waist circumference were recorded using a high-quality electronic scale (TANITA TBF-300, Tanita, Amsterdam, The Netherlands). At baseline, general information on medical history, reproductive history (frequency of sexual relations, sexual relations with risk, hypospadias, hydrocele, varicocele, cryptorchidism, sexually transmitted diseases, and erectile dysfunction or sexual impotence, among others), and use of medication was collected by means of a general questionnaire. Blood pressure was measured at baseline and at the end of the intervention in duplicate with a 5-minute interval between each measurement by using a semiautomatic oscillometer in the non-dominant arm (Omron HEM-705CP, Netherlands).

Dietary measurements

At baseline and every month during the follow-up, dietary intake was estimated using the mean of 3-day dietary records (3DDR) including two workdays and a weekend day. The dietitian explained use of the record in an initial face-to-face interview and when collected, the dietitian checked for its completion with the participant. The participants were instructed to report all drinks and food consumed at any moment of the day. Energy and nutrient intake were calculated using Spanish food composition tables (17,18). Compliance with the intervention was assessed by counting the empty sachets of nuts returned by the participants.

During the follow-up, all participants completed a specific questionnaire reporting any adverse effects that were related or not to the intervention.

Blood sample analysis

Blood samples were collected at baseline and at the end of the intervention period in 12-hour fasting conditions. Fasting glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, very-low-density lipoprotein (VLDL) cholesterol, triglycerides, insulin, C-reactive protein and folate (a competitive test using folate-specific natural fixative proteins) were determined by

routine laboratory tests using standard enzymatic automated methods (COBAS; Roche Diagnostics Ltd, UK) (intra-assay coefficient of variation were: glucose=1.54%, total cholesterol=2.43%, HDL=2.58, insulin=6.18%, C-reactive protein=5.74 and folate=8.62%). LDL-cholesterol concentrations were estimated by using Friedewald's equation.

Semen sample analysis

Semen samples were collected by masturbation at baseline and at the end of the intervention period, after 3 days of sexual abstinence. Analyses of fresh samples were conducted at a maximum of 60 minutes after collection. Semen was diluted with 10% dimethyl sulfoxide, and frozen at -80ºC until further analysis. A slow freezing protocol (-1ºC/min) with a Nalgene Mr.Frosty™ (ThermoFisher Scientific) was used.

Conventional semen analysis

The conventional semen parameters including volume, pH, sperm motility, vitality and morphology were determined on fresh samples as primary outcomes. Total sperm count and concentration was determined with a 100-µm-deep haemocytometer chamber (Neubauer® chamber), using bright field optics at x400 magnification. Sperm motility was assessed under a light microscope at x400 magnification, classifying spermatozoa as follows: a) Progressive motility (PR), b) Non-progressive motility (NP); and c) Immotility (IM). Motility was then also expressed as a percentage of total motility (PR+NP). Sperm vitality was estimated by evaluating the sperm plasma membrane integrity using eosin-nigrosine at x1,000 magnification. Sperm morphology was assessed on semen smears for staining with Hemacolor® (Millipore, Billerica, MA, USA) at x1,000 magnification, identifying normal spermatozoa or defects in their head, midpiece, or principal piece (sole or combined). Morphology was expressed as the percentage of normal forms. These parameters were assessed in accordance with 2010 World Health Organization (WHO) parameters (1).

Sperm reactive oxygen species (ROS) production

The sperm ROS was measured in post-thawed semen and monitored by chemiluminescence using 5-Amino-2,3-dihydrophthalazine-1,4-dione (or luminol) (Sigma Aldrich, Karlsruhe, Germany). Briefly, 250 µl of sperm suspension (in a final quantity of $2.5x10^6$ spz) was placed in 96-well plates with 6.25 µl of luminol 5 mM. PBS was used both for blank and negative controls, whereas 31.25 µl of hydrogen peroxide was used for positive controls. All measurements were taken in duplicate and were calculated with a mean of 30 readings with a 96-plate reader (Victor III, Perkin Elmer, Waltham, MA, USA).

Sperm DNA fragmentation (SDF)

DNA fragmentation was measured by the Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling (TUNEL) method using the *In-Situ* Cell Death Detection® Kit (Roche Diagnostic Gmbh, Penzberg, Germany) according to the manufacturer's procedures. The SDF was evaluated by flow cytometer analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA). A total of 10,000 spermatozoa were analyzed (flow rate of 200-300 spz/s). A negative control (fixed and permeabilized cells with label solution-without TdT) and a positive control (fixed and permeabilized cells with rDNaseI and label solution-containing TdT) were used. Data were processed using the free Flowing Software v.2.5.1 (http://www.flowingsoftware.com/).

Sperm global DNA methylation

A somatic cell lysis buffer (SCLB), which contained both 0.1% SDS and 0.5% Triton X-100 (in Milli-Q® water), was used for sperm cell purification (19). Optical microscopic examination was used to verify the somatic cell elimination. Total sperm genomic DNA was isolated according to the QIAamp® DNA Mini (QIAGEN, Hilden, Germany) manufacturer protocol. Sperm cells were diluted with PBS, proteinase K, buffer AL and 10 µl of DTT 1M, and incubated for 20-30 min at 56ºC. DNA was finally eluted with 50 µl of elution solution (AE) and stored at -20ºC until analysis. DNA methylation profiles

were quantified with MethylFlash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek, Farmingdale, NY, USA) using 100-200 ng of genomic DNA according to the manufacturer's protocol. Samples were read at 450 nm absorbance (TECAN, Sunrise™, Grödig, Austria). A polynomial curve was used as the standard.

Sperm chromosome stability

Sperm chromosome stability was analyzed throughout the study of numerical anomalies for chromosomes X (DXZ1, Spectrum Green, Abbott Molecular Inc, Des Plaines, IL, USA), Y (DYZ3, Spectrum Orange), and 18 (D18Z1, Spectrum Aqua), using fluorescent in situ hybridization (FISH). Semen samples collected at baseline and at the final visit of 20 randomly selected participants (10 in the nut group and 10 in the control group) were processed for FISH evaluation as described (20). For each individual, 1,000 sperm nuclei were analyzed at x1,000 magnification using an Olympus BX-60 microscope (Olympus, Spain) equipped with specific filters: a triple-band filter for DAPI/Cy3/FITC, and single-band filters for FITC, Cy3 and Aqua. The analysis was performed using strict criteria concerning the intensity, the size and the signal distribution (21). Sperm nuclei were classified as disomic if there were two signals for a given chromosome and a single signal for the other chromosomes evaluated. Those sperm nuclei with no signal for a given chromosome and a single signal for the other chromosomes evaluated were classified as nullisomic. Nuclei with two signals for the sex chromosomes (XY, XX or YY) and two signals for chromosome 18 were recorded as diploid sperm.

Sperm miRNAs expression

Previous RNA isolation, sperm cell purification through SCLB, was used (see the section on sperm global DNA methylation). Total sperm RNA was isolated from these purified sperm cells using the *mir*Vana™ PARIS™ Kit (Invitrogen, ThermoFisher Scientific, Vilnius, Lithuania) according to the manufacturer's protocol with one modification: In the cell disruption step, sperm cells were diluted with cell disruption

buffer and freshly 10 µl of DTT 1M and incubated for 15 min at 56ºC. All RNA samples were treated with the DNA-free™ Kit (Ambion, ThermoFisher Scientific, Vilnius, Lithuania) and stored at -80ºC until further analysis.

RNA quality controls were applied in a sub-cohort of 10 randomly selected participants (5 in the nut group and 5 in the control group) at baseline and final visit. RNA concentration and purity were determined using the Nanodrop-2000 (Thermo Fisher Scientific, Wilmington, DE). Nano-RNA chips and Small-RNA chips (Agilent Technologies, Wilmington, DE, USA) were used to verify the proper elimination of somatic cells and to check the presence of small RNAs, respectively. Finally, a conventional reverse transcription-polymerase chain reaction (RT-PCR) and a conventional PCR amplification with exon-exon primers for the *PRM-1, GAPDH, c-KIT* and *CDH1* were performed to ensure the absence of DNA, and the absence of germinal and somatic RNA (Supplemental Table 2).

We employed a strategy consisting of initial screening by TaqMan® miRNAs Low-Density Arrays (TLDAs) for the 10-patient sub-cohort (previously mentioned) and a further validation in the whole population (n=98) with the selected TaqMan® miRNA Assays in a Custom Array. For miRNA screening, sperm RNA of the sub-cohort was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription kit and the Megaplex™ RT Primers, Human Pool A v2.1 and B v3.0 (Applied Biosystems Thermo Fisher Scientific, Pleasanton, CA, USA), and was pre-amplified using the TaqMan® PreAmp Master Mix and the Megaplex™ PreAmp Primers (Applied Biosystems Thermo Fisher Scientific, Pleasanton, CA, USA). Then, a quantitative PCR (qRT-PCR) was carried out using TaqMan® Universal PCR Master Mix, no AmpErase UNG (2x) (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) and the TLDAs cards A v2.1 and B v3.0 (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). The TLDA arrays were analyzed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Darmstadt, Germany). Data from qPCR

were obtained by Sequence Detector Software (SDS) v.2.2 and processed by RQ Manager v.1.2 software. The threshold cycle (Ct) values were normalized using the MCR method (22).

Then, we validated the TLDAs results in the whole population using a Custom Array Config-11 (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) that allows the analysis of up to 13 miRNAs (plus two endogenous controls). The aim was that these TLDAs would contain the differentially expressed miRNAs between groups. If needed, in order to complete the 13 available positions of these Custom Arrays, the other miRNAs added would include those with a P-value near the signification between groups, which has also been previously identified in human spermatozoa (23–26).

For the validation, after the cDNA amplification step (TaqMan® Advanced miRNA cDNA Synthesis Kit; Applied Biosystems, Thermo Fisher Scientific, Carlsbad, CA, USA), qRT-PCRs were performed in a 7900HT Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, Darmstadt, Germany) using TaqMan® Advanced MicroRNA Assays (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA) and TaqMan® Fast Advanced Master Mix (Applied Biosystems, Thermo Fisher Scientific, Austin, TX, USA) in a Custom Array (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA). The assays used to validate the results were showed in Supplemental Table 3. Hsa-miR-30a-5p (MIMAT0000087) and hsa-miR-100-5p (MIMAT0000098) were used as endogenous controls (27). All measurements were performed in triplicate and qPCR data were acquired using SDS v.2.2 and processed by RQ Manager v.1.2 software.

Statistical analysis

Sample size of 98 subjects were estimated based on the results of Robbins and collaborators (28) and using vitality (mean changes and standard deviation) as the primary outcome, with a 80% power and a level of confidence 0.05. Considering 20% of drop outs, we finally included a total of 119 participants. Normal distribution and

homogeneity of variances were evaluated using Kolmogorov-Smirnov and Levene's test, respectively. The data for the participants are shown as means±standard deviation (SD) for normal distributed variables and median (interquartile rank (IQR)) for non-normal continuous variables. A paired T-test (for normal distributed variables) or a paired Wilcoxon test (for non-normal distributed variables) was used to assess differences within intervention groups. ANCOVA models were applied to assess differences in changes between intervention groups after adjusting for baseline values. Spearman correlation coefficients were used to calculate pair-wise correlations. P-values in the sperm miRNAs analyses were adjusted by using the Benjamini-Hochberg false discovery rate (FDR). Statistical analysis for the primary outcomes (seminogram) and blood parameters were conducted using intention-to-treat (ITT) and per protocol (PP) (Supplemental Tables 5 and 6) approaches including all randomized participants at least fulfilling all baseline measurements. Secondary outcomes (ROS production, SDF, total DNA-methylation, chromosome stability, and miRNAs expression) were analyzed PP excluding participants who did not attend the last visit. Statistical analyses were carried out using the freely available R statistical computing environment v.2.14.2 (www.r-project.org) (29) and two additional packages, a high-throughput analysis of qPCR data v.1.13.1 (HTqPCR package at: http://www.bioconductor.org) (30) and Deducer for R (http://www.deducer.org/) (31), or SPSS 22.0 software (SPSS Inc, Chicago, IL). All the tests were two-sided, and significance was set at P-value<0.05.

RESULTS

Study participants

A total of 244 subjects were assessed for eligibility. Of these, 57 subjects declined to participate and 68 did not meet the inclusion criteria. Finally, 119 participants were randomly assigned to one of the two intervention groups. A total of 13 participants did not attend to the baseline visit. 8 participants (7 in the nut group and 1 in the control group) dropped out between the baseline and the last visit for personal reasons. The retention rate for study participants was 84.5% in the control group and 80.3% in the nut-supplemented group. Finally, 98 participants successfully completed the study (Figure 1).

The baseline general-anthropometric characteristics and blood biochemical parameters of the study participants are shown in Table 1 and Table 2, respectively.

Compliance with the intervention assessed by counting the empty sachets of nuts returned by the participants was high (>95% of empty sachets returned). Participants in the two groups reported similar adherence to the western-style diet at baseline (mean ± SD control group=8.63±2.34 and nut group=8.11±2.10; P-value=0.229) according to the 15-item dietary screener. No significant differences in baseline nut consumption were found between intervention groups. Moreover, there was an increase in the consumption of total fat, monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), magnesium, vitamin E, total omega 3 fatty acid, α-Linolenic acid (ALA), and omega-3/omega-6 ratio intake in the nut supplemented group (Supplemental Table 7).

Effect of nut consumption on conventional semen parameters (primary outcomes)

Table 3 shows baseline seminal parameters and changes that occurred during the intervention. No significant differences were observed in baseline values between the

two study groups. Significant differences in changes in total sperm count (P-value=0.002), vitality (P-value=0.003), total motility (P-value=0.006), progressive motility (P-value=0.036), and spermatozoa with normal forms (P-value=0.008) were observed between the two study groups. The improvement in these variables was greater in the nut group. Within the nuts group, total sperm motility and progressive motility significantly also improved.

Effect of nut consumption on ROS

No significant differences in changes in ROS were observed between the two study groups (P-value=0.359) (Supplemental Figure 1).

Negative significant correlations between total sperm motility and ROS (rho-value=-0.261; P-value=0.010), and total number of spermatozoa and ROS (rho-value=-0.281; P-value=0.005) were detected. However, no significant correlations between progressive motility and ROS (rho-value=-0.160; P-value=0.118) or vitality and ROS (rho-value=0.047; P-value=0.646) were detected.

Effect of nut consumption on SDF

Changes in the SDF index (reported as percentage of sperm DNA fragmentation) between study groups at the end of the study were significant (P-value<0.001) (Figure 2). A significant decrease in SDF between the baseline and the end of the intervention was also observed in the nut supplemented group (P-value=0.047).

Intragroup analyses did not show significant correlations between ROS and SDF changes (rho-value=0.045; P-value=0.657) or between the SDF index and progressive motility (rho-value=-0.108; P-value=0.294) or SDF-total motility (rho-value=-0.186; P-value=0.068). Negative significant correlations between vitality and SDF (rho=-0.225; P-value=0.027), between volume and SDF (rho=-0.274; P-value=0.006) and between total sperm count and SDF (rho=-0.317; P-value=0.002) were detected.

Effect of nut consumption on global human sperm methylation

No significant differences in changes in total DNA methylation were observed between the two study groups (P-value=0.827) (Supplemental Figure 2).

Effect of nut consumption on X, Y and 18 sperm chromosome anomalies

No significant differences in changes in sperm chromosome anomalies were observed across the two study groups (Supplemental Figure 3) in the 20 randomly selected samples (P-value X, Y and 18 disomic=0.193, P-value nullisomic=0.800, and P-value diploid=0.191).

Effect of nut consumption on miRNA expression

A significant reduction in the hsa-miR-34b-3p expression level (P-value=0.036; Δ value=-1.638) was observed in the group supplemented with nuts compared to the control group (Figure 3). No significant changes were observed in the rest of miRNAs.

DISCUSSION

In the present study, we demonstrated that adding 60 g/day of mixed nuts for 14-weeks to a western-style diet improved total sperm count, vitality, motility and morphology in a group of healthy reproductive-aged participants compared to an age-matched control group. Within the mechanisms explored only a reduction in the DNA fragmentation after nut consumption could explain these beneficial effects.

Only one previous study has analyzed the effect of nut consumption on conventional semen parameters (28). After 75 g/d of walnut administration for 12-weeks the authors reported an improvement in sperm vitality, motility and morphology, but not in the total sperm count. Therefore, our study agrees with some of these results related to conventional sperm parameters and extends the seminal improvements obtained from eating walnuts to other types of nuts. However, the aforementioned study did not explore the potential mechanisms responsible for these improvements. The differences in the effects found for some sperm parameters between the two studies could be explained mainly by the dissimilarities in the background dietary patterns, as well as the amount and type of nuts administered.

Nuts are energy-dense foods that contain about 50% fat, most of which in the form of MUFAs (except walnuts in which PUFAs predominate) and other key nutrients (32) for understanding the improvements in specific seminal parameters due to their potential effects on SDF and RNA expression.

Although the relationship between MUFAs and PUFAs, and sperm quality is largely unknown (33,34), there is more evidence about other nutrients contained in nuts (i.e. vitamin E, and omega-3 and 6 fatty acids) and their potential benefits for sperm quality and functionality. While some researchers have demonstrated that supplements of vitamin E significantly improved sperm motility (35), others found improved total sperm count and concentration, motility and morphology after omega-3 fatty acid supplementation (36). Not only omega-3 fatty acid but also the ratio omega-3/omega-6

410 in blood serum seems to be essential for sperm quality (37) and reproduction (38,39).
411 Similarly, a lower omega-3/omega-6 serum ratio was found in infertile patients
412 compared to fertile controls (37). In our study, participants in the nut group had a higher
413 intake of omega-3 and omega-6 fatty acids, but also had a higher omega-3/omega-6
414 ratio after nut consumption.

415 Some antioxidant nutrients and omega-3 fatty acids have been reported to have
416 beneficial effects on SDF, the only mechanistic effect shown in our study. For example,
417 an improvement in the SDF index after 2-months of vitamin C+E supplementation has
418 been previously reported in human sperm (40). Omega-3 docosahexaenoic acid plus
419 eicosapentanoic acid supplementation (990mg/d and 135mg/d, respectively) for 10-
420 weeks has also been demonstrated to improve SDF (41). In fact, sperm DNA
421 fragmentation has an important modulatory effect on sperm vitality and motility (42),
422 both parameters regulated by the consumption of nuts in our study.

423 We did not find a significant improvement in ROS related to nut consumption. Although
424 no correlation between SDF and ROS was shown in our study, high levels of ROS
425 have been strongly correlated with increased levels of sperm DNA damage and low
426 percentages of sperm motility in a previous large RCT conducted in healthy
427 participants (43). In fact, we found a significant correlation between total sperm motility
428 and ROS that partially confirmed this mechanistic relationship. Antioxidants contained
429 in nuts might decrease the levels of oxidative stress (OS) and consequently ROS,
430 potentially affecting sperm quality, mainly in terms of sperm count, motility, and vitality.
431 A recent study showed an improved sperm motility and morphology through a
432 decrease in sperm peroxidative damage after feeding the mice a walnut-enriched diet.
433 This suggests that oxidative stress may be involved in these sperm alterations (44).
434 Results from our study do not confirm this ROS pathway with potential effects on SDF.
435 Nevertheless, we cannot discard a possible lack of statistical power when changes in

436 ROS are measured, because the sample size of this trial was estimated using vitality
437 and motility as primary outcomes, not ROS.

438 Recent studies reported a close association between sperm DNA-methylation (mainly
439 in *H19, MEST* and *SNRPN* genes) and semen quality (45). For this reason, we
440 considered DNA-methylation as a potential mechanism inducing changes in the
441 seminogram. However, in our trial we did not observe significant changes in sperm
442 DNA-methylation after the consumption of nuts. This lack of effect on global sperm
443 DNA-methylation was also reported by Chan and collaborators (46) after the intake of
444 400µg/d of folic acid for 90 days. In our study, the amount of folate achieved by nut
445 supplementation was approximately 80µg/day, much less than the amount used by
446 Chan and collaborators and no differences in plasma folate were observed between
447 groups. Nevertheless, we cannot rule out completely the aforementioned mechanism
448 due to the global DNA-methylation measurement approach used in our study. Possibly
449 by employing more sensitive techniques (e.g. whole-genome BS-seq or microarrays)
450 for analyzing the methylation status of specific loci, we could identify some type of
451 effect. Moreover, although an association between hypomethylation and genomic
452 instability in cancer cell lines (47,48) or in sperm cells (49) has been reported, in our
453 study, no changes in the chromosome stability through FISH method occurred after the
454 intervention. Further RCTs analyzing these variables will help to confirm the influence
455 of nutrients or food on sperm DNA-methylation and chromosome numeric anomalies.

456 Many studies reflect that miRNAs play an essential role in spermatogenesis,
457 contributing to the mechanisms involved in human fertility (23–26,50). For this reason,
458 we considered miRNAs as a potential cause of the changes in the seminogram that
459 should be considered. Interestingly, in the present study we found that nut
460 supplementation reduced the sperm levels of hsa-miR-34b-3p. Previous studies have
461 observed that this miRNA is related to sperm motility (i.e. down-regulated in
462 ashtenozoospermic individuals (24)). Since in the present trial, a nut supplementation

improved sperm motility, our findings seem *a priori* to be a contradictory result. However, the validated target genes of this miRNA, through strong evidence methods (i.e. reporter assay, western blot and/or qRT-PCR), include *CDK6, MAP2K1, PAK1, SNCA*, and *VEGFA* (miRTarBase (51)). Interestingly, the functions associated with these genes include cell motility (GO:0048870) (*CDK6, MAP2K1* and *PAK1*), locomotion (GO:0040011) (*SNCA*) or cellular movement (GO:0006928) (*MAP2K1* and *VEGFA*), among others (www.genecards.org/). Therefore, it is apparent that miR-34b-3p is involved in the regulation of cell motility, but its target regulation might include antagonistic pathways. Unfortunately, the direct relationship between the miR-34b-3p and sperm motility has not been established yet and still deserves more exploration.

Several limitations related to our study need to be mentioned. First, this trial focuses on healthy and apparently fertile men. Therefore, the results cannot be extrapolated to the general population. Second, our study population followed a western diet in the context of a Mediterranean area that can be different from those usual in other western countries, making difficult the extrapolation of results to these populations. Third, the sample size of the present study (that was estimated using vitality as the primary outcome with 80% power and a level of confidence of 0.05) may not be sufficient to identify other possible mechanisms (as secondary outcomes) explaining our results at a molecular level, e.g. improvement of ROS. Fourth, it is important to mention that more than 10% of participants dropped out of the trial, therefore a specific risk of bias according to the COCHRANE recommendations must be acknowledged (52). Finally, the effect on semen parameters was observed with a supplement of 60g/d of nuts. Whether higher or lower amounts of nuts may have a differential effect on sperm parameters need to be determined.

The main strength of the present study is the design *per se,* because RCTs represent the cornerstone of evidence-based medicine, avoiding bias related to confounding factors (through a control group), and selection bias (through randomization). In

addition, the use of a mix of nuts allowed us to demonstrate the potential synergistic effects of their compounds (especially different types of polyphenols and other phytochemicals) on conventional semen parameters related to fertility. Also, the intervention period up to 14-weeks allowed us to cover all the cells, differentiating throughout one full spermatogenesis cycle. This made it possible to effectively explore the effects of food consumption on semen related parameters. We decided to use 60g of a mixture of those nuts that are more frequently consumed in our area in order to increase compliance with the intervention. Consuming between 60-90g of nuts has proved effective for down-lowering LDL-cholesterol (53), improving inflammation (54), glucose metabolism (54,55) and semen quality (28). Moreover, the administration of 30g of the same proportion of nuts we have used in the current study (2 parts of walnuts, 1 part of hazelnuts, and a part of almonds) was already demonstrated effective in the improvement of inflammation, endothelial and oxidative stress markers, blood pressure and insulin resistance, in the context of a Mediterranean Diet (56). Finally, motility and vitality parameters, both significantly improved after nut consumption, are those considered the most clinically relevant parameters of the seminogram by the WHO in 2010. Therefore, any variation in the aforementioned parameters could potentially has a clinical relevance.

In conclusion, in this parallel-randomized clinical trial, we observed that the supplementation of a western-style diet with walnuts, hazelnuts and almonds improves the main sperm quality parameters among healthy reproductive-aged people. These beneficial effects could possibly be explained by a reduction in the sperm DNA fragmentation. These results support the potential benefits of some nutrients contained in nuts for sperm quality.

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COMPETING INTERESTS

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AUTHORS' CONTRIBUTIONS

A.S-H. and J.S-S. initiated the idea of the trial. A.S-H., E.A., J.B., J.S-S., and M.B. were involved in study design. A.S-H. was involved in study conception and execution, acquisition and analysis of data, and wrote the manuscript; R.M. was involved in study execution and acquisition of data; S.G. was involved in acquisition of miRNAs data; E.A., J.B., J.S-S., and M.B. 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=56)**	**Control group (n=50)**
Age (years)	24 (4.6)	25 (4.7)
Weight (kg)	73.79 (9.74)	76.36 (11.52)
BMI (kg/m2)	23.55 (2.84)	24.09 (3.43)
Waist circumference (cm)	79.95 (7.37)	83.04 (8.57)
Systolic blood pressure (mmHg)	129.30 (11.56)	125.96 (11.46)
Diastolic blood pressure (mmHg)	73.04 (7.89)	71.63 (8.36)

Data are given as mean ± standard deviation (SD).

Abbreviations. BMI: Body-mass-index.

Table 2. Baseline values and changes after the intervention period in blood parameters.

Blood characteristics	Nut group (n=56)		Control group (n=50)		Treatment effect
	Baseline	Changes	Baseline	Changes	P-value
Fasting plasma glucose (mg/dl)	86.00 (82.75, 93.25)	0.00 (-5.00, 3.50)	86.00 (82.25, 91.00)	-1.00 (-6.00, 4.00)	0.292
Total cholesterol (mg/dl)	168.00 (150.00, 181.50)	-2.00 (-15.50, 5.00)	169.50 (150.00, 193.00)	-3.00 (-16.00, 8.00)	0.754
HDL-c (mg/dl)	56.00 (49.75, 65.25)	-1.00 (-4.50, 3.50)	55.50 (51.00, 63.75)	-1.00 (-7.00, 5.00)	0.420
LDL-c (mg/dl)	95.00 (74.75, 102.50)	-6.00 (-13.50, 5.50)	95.50 (78.50, 113.00)	-4.00 (-13.00, 8.00)	0.806
VLDL-c (mg/dl)	13.00 (12.00, 18.25)	0.00 (-2.00, 4.00)	13.00 (10.00, 17.75)	2.00 (-2.00, 5.00)	0.513
Triglycerides (mg/dl)	66.00 (59.00, 95.00)	2.00 (-8.00, 20.00)	65.50 (49.25, 87.50)	8.00 (-9.00, 24.00)	0.549
Fasting plasma insulin (mcUl/ml)	5.45 (2.88, 8.55)	0.00 (-1.60, 2.35)	5.48 (2.95, 6.80)	0.50 (-0.80, 1.80)	0.479
C-Reactive protein (mg/dl)	0.20 (0.20, 0.20)	0.00 (0.00, 0.00)	0.20 (0.14, 0.20)	0.00 (0.00, 0.03)	0.408
Folate (ng/ml)	6.30 (5.20, 7.78)	-0.40 (-1.18, 1.00)	6.30 (4.90, 7.80)	0.30 (-1.30, 1.00)	0.741

ANCOVA models were used to assess differences between intervention groups. Changes in biochemical variables were adjusted for baseline values of each biochemical variable. A paired Wilcoxon test was used to assess differences within intervention groups. Values are expressed as median and interquartile rank (IQR). 1 mg/dl plasma glucose=18.018 mmol/l, 1mg/dl total cholesterol= 38.610 mmol/l.

Abbreviations. HDL: high-density lipoprotein, LDL: low-density lipoprotein, VLDL: very-low-density lipoprotein.

Table 3. Baseline and changes after the intervention period in semen parameters.

Semen parameters	Nut group (n=56)		Control group (n=50)		Treatment effect
	Baseline	Changes	Baseline	Changes	P-value
pH	8.0 (8.0, 8.5)	0.0 (0.0, 0.0)	8.0 (8.0, 8.5)	0.0 (0.0, 0.0)	0.089
Volume (ml)	3.00 (1.98, 4.15)	0.00 (-0.83, 0.50)	3.15 (2.05, 4.99)	-0.65 (-1.38, 0.15)	0.112
Total spermatozoa (x10^6)	75.55 (29.95, 111.25)	4.45 (-15.60, 34.95)	69.75 (29.25, 123.25)	-15.15 (-42.05, 7.15)	**0.002**
Spermatozoa concentration (x10^6)	26.20 (14.85, 44.30)	0.10 (-2.11, 10.35)	21.20 (9.80, 37.60)	0.00 (-4.05, 4.14)	0.086
Vitality (%)	78.66 (71.03, 82.36)	3.42 (0.00, 7.44)	80.00 (73.93, 86.18)	-0.20 (-3.18, 3.59)	**0.003**
Motility:					
Total motility (progressive and non-progressive motility) (%)	64.23 (44.44, 70.45)	3.41 (-0.87, 13.14)*	67.96 (60.42, 77.93)	0.00 (-4.93, 6.30)	**0.006**
Progressive motility (%)	43.03 (27.19, 53.48)	3.78 (0.00, 15.14)*	49.72 (35.00, 61.22)	1.70 (-2.18, 5.93)	**0.036**
Non-progressive motility (%)	12.06 (9.13, 16.14)	-2.38 (-5.24, 0.06)	11.30 (7.36, 13.64)	-0.28 (-3.94, 2.66)	0.727
Immotile spermatozoa (%)	35.77 (29.05, 53.02)	-3.41 (-13.71, 0.88)*	32.04 (22.07, 39.59)	-0.92 (-5.73, 4.46)	**0.006**
Morphology:					
Normal forms (%)	6.55 (5.00, 8.08)	0.82 (-0.17, 2.12)	6.32 (5.47, 7.74)	-0.04 (-1.06, 0.65)	**0.008**
Abnormal head (%)	54.56 (45.10, 66.46)	0.00 (-2.32, 9.82)	52.43 (40.77, 66.19)	0.91 (-2.14, 8.53)	0.936
Abnormal midpiece (%)	10.79 (8.78, 15.03)	-0.21 (-2.86, 1.59)	11.83 (7.80, 14.44)	0.95 (-3.26, 2.93)	0.207
Abnormal principal piece (%)	11.27 (5.10, 27.43)	-0.46 (-3.90, 0.33)	14.49 (5.25, 30.85)	-1.15 (-4.22, 0.84)	0.829
Combined abnormality (%)	8.84 (6.46, 13.14)	0.14 (-3.39, 0.65)	7.59 (6.55, 13.46)	0.29 (-2.35, 2.20)	0.570

ANCOVA models were used to assess differences between intervention groups. Changes in seminogram variables were adjusted for baseline values of each seminogram variable. A paired Wilcoxon test was used to assess differences within intervention groups. Values are expressed as median and interquartile rank (IQR). *Significant difference ($P<0.05$) between baseline and the end of a particular intervention period.

FIGURE LEGENDS

Figure 1. Flow diagram of the FERTINUTS study.

Abbreviations. V0: baseline visit, V3: final visit.

Figure 2. Boxplot of the differences between the control group and nut group in the sperm DNA fragmentation (SDF) analysis.

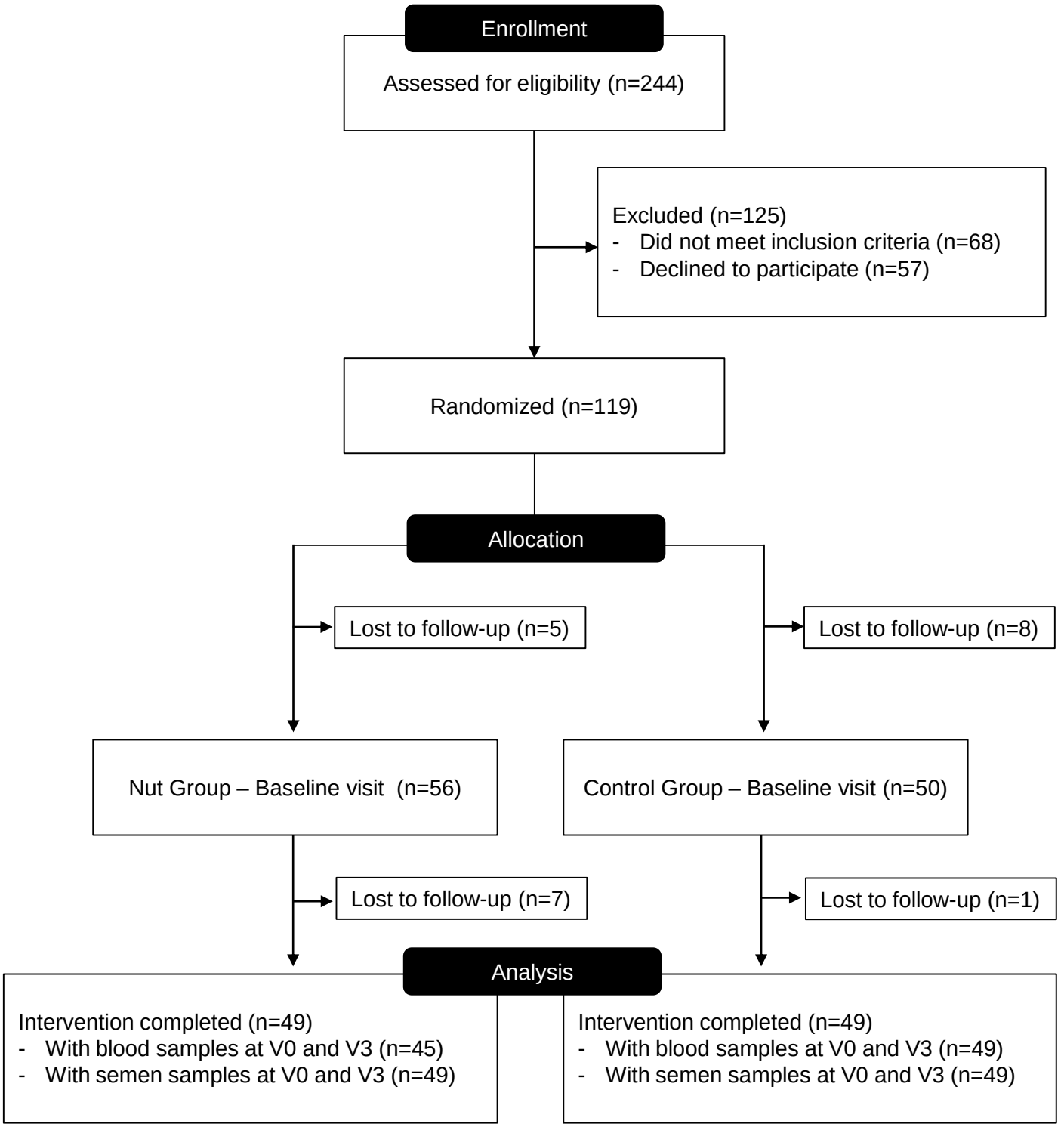
Legend: ANCOVA models were used to assess differences between intervention groups. Changes were adjusted for baseline values. A horizontal line in the boxplot illustrates the median value. The upper and lower bars indicate the 3rd and 1st percentiles, respectively.

Figure 3. Bar plot of the differences between the control group and nut group for the analyzed miRNAs.

Legend: We evaluated the differences with a Wilcoxon analysis for normality distribution reasons. The grey bars illustrate the mean value and the vertical lines the standard error. *Significant difference ($P<0.05$) between the nut supplemented group and the control group after the 14-wk intervention period.

Figure Click here to access/download;Figure;Figure 1_def.pptx

Figure 1. Flow diagram of the FERTINUTS study.



Figure

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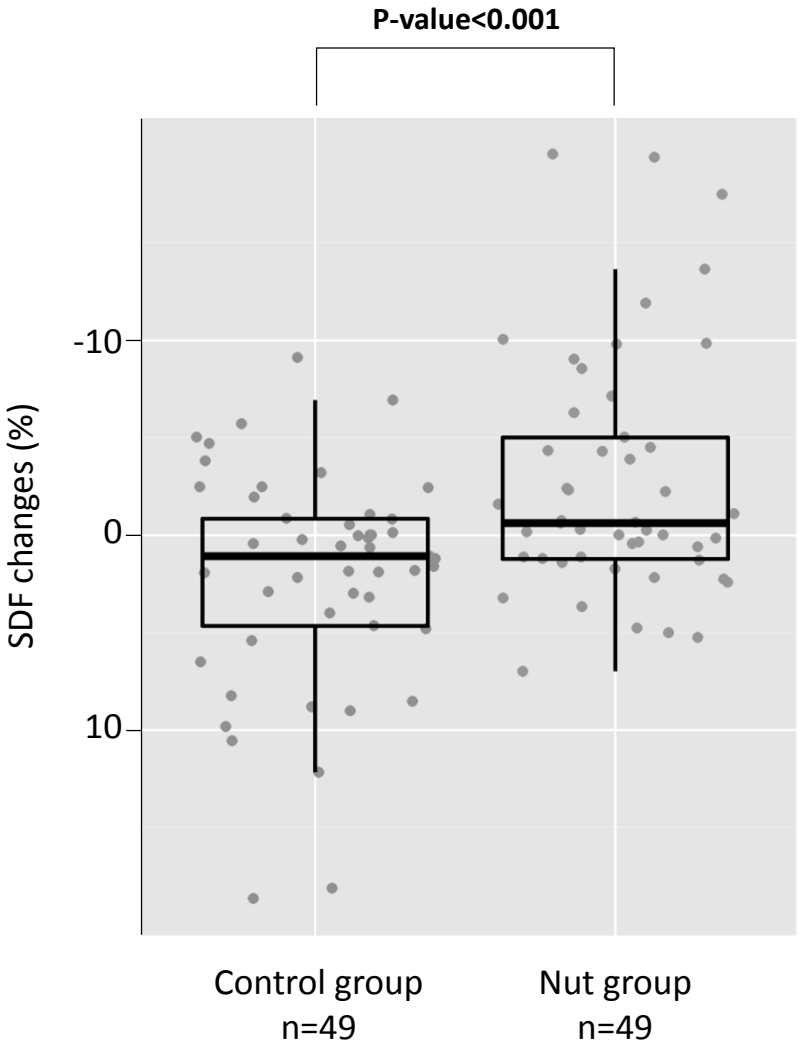
Figure 2. Boxplot of the differences between the control group and nut group in the sperm DNA fragmentation (SDF) analysis.



Figure

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Figure 3. Bar plot of the differences between the control group and nut group for the analyzed miRNAs.