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Keywords:	chocolate, postmenopause, arterial pressure, vascular stiffness, body composition, quality of life

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ABSTRACT

Introduction: The intake of polyphenols has shown certain health benefits. The aim of this study is to assess the effect of adding a daily amount of chocolate high in cocoa content and polyphenols to the normal diet on blood pressure, vascular function, cognitive performance, quality of life and body composition in postmenopausal women.

Methods and analysis: Randomized clinical trial with two parallel groups involving a total of 140 women between 50 and 64 years of age in the postmenopausal period, defined by amenorrhea of at least 12 consecutive months. The main variable will be the change in blood pressure. Secondary variables will be changes in vascular function, quality of life, cognitive performance and body composition. The intervention group will be given chocolate containing 99% cocoa, with instructions to add 10 g daily to their normal diet for 6 months. The daily nutritional contribution of this amount of chocolate is 59 Kcal and 65.4 mg of polyphenols. There will be no intervention in the control group. All variables will be measured at the baseline visit and at 3 and 6 months after randomization, except cognitive performance and quality of life, to be assessed only at baseline and at 6 months. Recruitment is scheduled to begin on June 1, 2018, and the study will continue until May 31, 2019.

Ethics and dissemination: This study was approved by the Clinical Research Ethics Committee of the Health Area of Salamanca, Spain ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov provided by the US National Library of Medicine, number NCT03492983.

Keywords: Chocolate, postmenopause, arterial pressure, vascular stiffness, body composition, quality of life.

Strengths and limitations of this study:

- The effect of commercially available chocolate high in cocoa and polyphenols on the health of postmenopausal women is not known since most of the available studies have used laboratory compounds prepared with high composition of these substances.
- Blood pressure and vascular function will be measured objectively using a sphygmomanometer and a Vasera VS-2000 device (Fukuda Denshi), with body composition measured by impedance analysis, while quality of life and cognitive performance will be assessed using validated instruments.
- Due to the nature of the intervention, the participants cannot be blinded, although the researchers who perform the measurements and the statistical analysis will be blinded.

INTRODUCTION

Polyphenols are bioactive compounds found in many plants, fruits and vegetables. The beneficial effects on human health associated with the consumption of a diet rich in polyphenols has generated great scientific interest in these substances [1-3]. The action of polyphenols is based on their antioxidant capacity through the uptake of free radicals, the chelation of metals with redox properties and the modulation and inhibition of enzymatic activity [4].

The most abundant polyphenols in cocoa are flavonoids, which have been linked to a protective effect against cardiovascular disease, decreasing the risk of cardiovascular morbidity and mortality, and favouring the prevention of other chronic diseases such as diabetes mellitus type 2 [1-3 5 6]. The ability to reduce cardiovascular risk could be due to an improvement in the elements that define metabolic syndrome, the improvement of vascular endothelial dysfunction, insulin resistance and the inhibition of platelet activation and aggregation [7 8].

Cocoa polyphenols and blood pressure: The effect of consuming polyphenols present in chocolate on the blood pressure (BP) statistics of healthy individuals is not clear. Some studies have observed a dose-dependent relationship between cocoa intake and clinical BP, with higher consumption equated to lower blood pressure and better vascular function [9 10]. Conversely, other research has not obtained significant changes in these parameters related to the supplementation of cocoa or pure polyphenols such as epicatechin or quercetin [11 12].

Endothelial dysfunction in postmenopausal women causes changes that favour the development of cardiovascular risk factors and atherosclerosis, which lead to the appearance and maintenance of hypertension [13 14]. A decrease in BP has been observed in this group after daily consumption of cocoa with a flavonol content of 40.12 mg. Below this level, however, no changes have been observed [15].

Cocoa polyphenols and vascular function: Among healthy individuals as well as postmenopausal women, the consumption of polyphenols present in cocoa has been

associated with a dose-dependent improvement of vascular function, in particular of arterial stiffness measured by pulse wave speed [9 10 15]. However, this relationship is not evident in people with mild hypertension when cardio-ankle vascular index (CAVI) is used as a measure of arterial stiffness [16].

There is also evidence of the influence of these polyphenols in reducing the augmentation index (AIx). The study by West et al [17], involving subjects with excess weight and moderate obesity, concludes that the treatment with dark chocolate decreases AIx in women, although it seems that this association may affect more the elasticity of the large arteries, especially in subjects with obesity and diabetes mellitus type 2 [18].

Cocoa polyphenols and cognitive performance: There is evidence to suggest that chocolate rich in polyphenols may be beneficial for cognitive performance and state since it improves mental processing speed and attenuates the increase of mental fatigue among healthy young adults [19 20]. An improvement in cognitive performance among older age groups after eating chocolate has also been observed [21]. Several studies also show that polyphenol-rich chocolate causes an improvement in executive function and categorical fluency [22], in working memory [23 24], and a slowing of mental fatigue [25]. Furthermore, a positive influence of cocoa polyphenols on physiological processes has been reported, with a neuroprotective effect [26] and improved cognitive performance [27].

Cocoa polyphenols and quality of life: The quality of life linked to health is represented by the individual's perception of well-being in various aspects of life, including physical and mental aspects. The effect of chocolate and polyphenols on the quality of life has scarcely been studied, with not a great deal of evidence available and even less of a conclusive nature. In a study conducted among healthy people, where regular consumption of chocolate was recorded over a year, no evidence was found of a clear association between chocolate intake and the physical or mental components of

quality of life [28]. Nevertheless, it has been observed that the consumption of dark chocolate may be beneficial for the quality of life of women with fibromyalgia [29].

Cocoa polyphenols and body composition: The menopause period leads to various changes in the body composition of women [30]. Regarding the connection between cocoa polyphenols and body composition, results diverge. Some clinical trials involving healthy people and overweight or obese patients have not reported significant differences that link chocolate consumption to anthropometric measures [12 16 17 31]. Other studies indicate that chocolate consumption may have positive effects on body composition in adolescents [32], patients with diabetes [33] or women with obesity [34]. Two recent systematic reviews also indicate that eating chocolate is associated with reduced body mass index (BMI) and waist circumference [35 36], and one of them also concludes that the amount and the length of time during which it is eaten play a key role in these beneficial effects [36]. Conversely, other studies such as that carried out with the cohort of the Atherosclerosis Risk in Communities (ARIC) study have observed a dose-dependent increase in weight after habitual chocolate consumption [37].

In sum, the polyphenols present in chocolate seem to have a positive effect on BP, vascular function, cognitive performance and quality of life, especially in populations with increased cardiovascular risk such as postmenopausal women [38]. However, the conflicting results obtained in different studies suggest that the real contribution of these compounds to health and the underlying mechanisms remain unclear. Moreover, most of these studies have used preparations with high concentrations of polyphenols that are usually not present in the normal diet.

This study aims to evaluate the effect of adding a daily amount of 10 g of chocolate high in cocoa content (99%) and polyphenols to the normal diet on blood pressure, vascular function, cognitive performance, quality of life and body composition in postmenopausal women.

METHODS AND ANALYSIS

Design and setting:

This controlled and randomized clinical trial involves two parallel groups. The study will be carried out in the Research Unit of the La Alamedilla Health Centre in Salamanca (Spain), which is part of the Biomedical Research Institute of Salamanca (IBSAL) and the Primary Care Prevention and Health Promotion Research Network (REDIAPP). The recruitment schedule is set to start on June 1, 2018, and the study will run until May 31, 2019. There will be a baseline assessment and two follow-ups, at 3 and 6 months.

Study population:

Those subjects who meet the selection criteria and sign the informed consent after receiving information about the objectives and implementation of the study will take part.

Inclusion criteria: women between 50 and 64 years of age in postmenopause, defined by and checked against amenorrhea during at least 12 consecutive months.

Exclusion criteria: personal history of cardiovascular disease; personal history of diabetes mellitus, arterial hypertension or dyslipidemia under pharmacological treatment; hypocaloric diets; clinically demonstrable neurological and/or neuropsychological disease; treatment with hormone replacement therapy; intolerance and/or allergy to cocoa or any of the components of the supplement.

Participants will be selected using a consecutive sample of women who meet the selection criteria in the GP surgeries of four urban primary care centres in Salamanca, from June 1, 2018.

Sample size:

The size of the sample has been estimated based on the potential modification of the main variable, systolic blood pressure (SBP). Given alpha and beta risks of 0.05 and 0.20 respectively in bilateral contrast and a standard deviation (SD) of 5.8 mmHg, 140 participants (70 per group) will be necessary to detect a minimum difference of 2.9 mmHg in the SBP between the two groups. A predicted drop-out rate of 10% during follow-up has been taken into account. This estimate has considered the results

obtained in a similar study in which a decrease in SBP of 6.5 was observed ± 5.8 mmHg [10].

Randomization:

Participants will be assigned to the intervention group (IG) or control group (CG) at random. The allocation sequence will be generated by an independent researcher using the Epidat 4.2 program [39] and will remain hidden until the participants are assigned to each group.

Intervention:

No type of intervention will be carried out with the CG participants.

IG participants will be given chocolate with 99% cocoa content and asked to eat 10 g daily for a period of 6 months. Participants will also be given instructions on eating and keeping the product, with the recommendation, for example, that the daily chocolate intake be eaten at the same time. In addition, they will be given a calendar on which to record the time it was eaten each day. This calendar will be returned to the researchers at each replenishment visit.

This amount of chocolate provides the following daily nutritional contribution: 59 Kcal, 0.8 g of carbohydrates, 1.5 g of protein, 5.1 g of fat, of which 3.1 g are saturated fats. The proportion of polyphenols per 10 g is 65.4 mg. The polyphenolic profile of this compound can be seen in table 1. On each visit, IG participants will receive the amount of chocolate they need until the next replenishment visit. In addition to the baseline visit, there will be 5 replenishment visits in months 1, 2, 3 (coinciding with the evaluation visit), 4 and 5. The sole purpose of the replenishment visits will be to supply the amount of chocolate needed until the next visit, without any other intervention being carried out.

Participants in both groups will be instructed to continue with the dietary pattern they usually follow, without changing their eating habits during the study period.

Procedures:

For each participant a baseline visit and two follow-up visits at 3 and 6 months after the initial one are scheduled (Figure 1). The IG will also make 5 replenishment visits, in months 1, 2, 3 (coinciding with the first follow-up visit), 4 and 5. In the replenishment visits they will be given the amount of chocolate needed until the next visit and will hand in the calendar with the record of the chocolate eaten.

Primary and secondary endpoints:

The primary variable will be the decrease in clinical BP, measured with a digital sphygmomanometer. Secondary variables will include vascular function, quality of life, cognitive performance and body composition.

All variables will be measured at 3 and 6 months after randomization, except for cognitive performance and quality of life, to be assessed only after 6 months.

Blood pressure:

Clinical systolic and diastolic blood pressure will be measured with a validated Omron M10-IT sphygmomanometer (Omron Healthcare, Kyoto, Japan). Three measurements will be taken in the dominant arm of the subject in a sitting position after at least 5 minutes of rest with an appropriately sized cuff, following the recommendations of the European Society of Hypertension [40]. The average of the last two measurements will be recorded.

Vascular function:

The Vasera VS-2000 device (Fukuda Denshi) will be used to measure the CAVI and the brachial-ankle pulse wave velocity (ba-PWV) at rest. CAVI is a good indicator of arterial stiffness, providing an accurate estimate of the degree of atherosclerosis without depending on blood pressure [41]. CAVI ≥ 9 and ba-PWV ≥ 18.3 will be considered pathological [42]. Pathological CAVI is representative of subclinical atherosclerosis [43].

Cognitive performance:

Attention and executive functions: Trail Making Test A will be used to measure attention and Trail Making Test B for processing speed and executive functions [44].

Immediate verbal memory will be assessed with the Rey Auditory Verbal Learning Test. The immediate recall of a list of 15 words is measured in three attempts, followed by delayed verbal memory through the free recall of the words learned in the first part of the test after 10 minutes [45].

Working memory will be assessed with the WAIS Digit Span Backward test [46].

Phonological fluency will be explored by naming as many words as possible starting with different letters of the FAS Questionnaire in the space of one minute [47].

Categorical fluency measures verbal semantic fluency and will be assessed by naming as many animals as possible in one minute [48].

Quality of life:

The quality of life linked to health will be assessed through the EuroQol 5-D questionnaire (EQ-5D). We will use the adapted Spanish version of this questionnaire, which has been validated in the Spanish population [49]. This questionnaire consists of three elements: the assessment by the individuals of their state of health in level of severity by dimension (mobility, personal care, daily activities, pain/discomfort and anxiety/depression), the assessment of their state of health on an analogue visual scale, and finally an index of social values obtained for each state of health generated by the instrument.

The quality of life will also be studied using the Cervantes Scale [50]. This questionnaire is specifically designed for menopause and postmenopause and has been validated for Spanish women. Its 31 structured items cover the 4 dimensions of menopause: menopause and health, sexuality, psychic domain and relationships.

Body composition:

Body composition will be measured with the Inbody 230 Monitor [51]. This analyzer provides data on skeletal muscle mass, fat mass, total body water, fat-free mass, percentage of body fat, waist-hip ratio, basal metabolism, and also a segmental analysis.

Body weight will be measured twice with an electronic scale (Scale 7830, Soehnle Professional GmbH & Co, Backnang, Germany) after proper calibration (accuracy ± 0.1 kg). Height will be measured by recording the average of two readings rounded to the nearest centimetre using a stadiometer (Seca 222, Medical scale and measurement system, Birmingham, UK). Both measurements will be made with the subject barefoot and wearing light clothing. Body mass index will be calculated by dividing weight (kg) by height squared (m^2). Waist circumference will be assessed in accordance with the recommendations of the Spanish Society for the Study of Obesity (SEEDO) [52] and will be measured in duplicate before and after inhalation, using a flexible tape parallel to the floor, at the level of the mid-point between the lowest rib and the iliac crest, with the subject standing up and without clothes.

Other variables

Clinical and sociodemographic variables

At the baseline visit, information on clinical and sociodemographic variables will also be collected via questions about age, marital status, educational level and occupation. Family history of cardiovascular disease and personal history of anxiety and/or depression, gestational diabetes, hypertension, dyslipidemia and the prescribed pharmacological treatment (antiaggregants, anticoagulants, thyroid hormone treatment, anxiolytics) will also be recorded, as well as the taking of NSAIDs in the last two weeks. In subsequent visits, personal histories of cardiovascular disease, diabetes mellitus, arterial hypertension or dyslipidemia in treatment, as well as the prescribed pharmacological treatment (hypolipidemic, antihypertensive, antidiabetic) will also be noted.

Evaluation of chocolate consumption and habitual diet

Chocolate consumption will be assessed at each evaluation visit by a series of questions about the amount, type and frequency of consumption in the period between visits.

Nutritional habits will be assessed by a 24-hour log on 3 non-consecutive days prior to each visit.

Evaluation of other lifestyles

The use of tobacco will be assessed with a questionnaire on the personal history and pattern of smoking.

Alcohol use will be recorded with a questionnaire covering the previous 7 days which will include specific beverages and the amount by volume drunk of each.

Physical activity will be measured using the International Physical Activity Questionnaire (IPAQ) in its short version and validated in Spanish [53]. This questionnaire measures activity over the previous 7 days, classifying the subjects according to three activity levels (low, moderate and high) with respect to three types of activities: walking, moderate-intensity activities and vigorous-intensity activities. The amount of physical exercise will be estimated in METs-minute/week.

Evaluation of laboratory variables

At baseline and follow-up visits at 6 months, we will measure plasma fasting glucose values (mg/dL), glycated haemoglobin (%), total cholesterol (mg/dL), total triglycerides (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), creatinine (mg/L), insulinemia (mg/dL). Creatinine in urine (mg/dL) and microalbuminuria (mg/dL) will also be measured.

Insulin resistance will be determined using the HOMA index (Homeostasis Model Assessment Insulin Resistance) estimated using the following equation: Fasting glucose (mmol/l) X insulin (mU/ml)/22.5.

Blinding strategy:

Due to the nature of the intervention itself, the participants and the person responsible for delivering the chocolate to IG participants, cannot be blinded. However, the person responsible for carrying out the study measurements at each visit and for the statistical analysis will be blind to the intervention.

Statistical analysis:

General analysis

Results for the quantitative variables will be expressed by mean ± standard deviation or by frequency distribution in the case of qualitative ones. The normality of the variables will be assessed using the Kolmogorov-Smirnov test. In cases where a normal distribution cannot be assumed, the corresponding nonparametric tests will be applied. The association between independent qualitative variables will be analyzed by means of the chi-square test or Fisher's exact test. The means between the two groups will be compared using the Student's t test or the Mann-Whitney U test, and the Pearson or Spearman correlation coefficients will be calculated to analyze the relationship between quantitative variables.

The analysis of the results for the main variable and the secondary variables will be carried out by intention to treat. In addition, a secondary analysis will be run taking into account chocolate intake adherence (< 50% days and > 50% days) and other relevant subgroups in relation to their physical activity or previous chocolate consumption.

All analyses will be performed with the SPSS version 23.0 (IBM Corporation, Armonk, NY, USA) and an alpha risk of 0.05 will be set as the limit of statistical significance.

Analysis of the intervention's effect on primary and secondary outcomes.

To analyze the changes at 3 and 6 months from baseline in the primary outcome (blood pressure) and in the secondary outcomes within the same group, the Student's t test for paired data or the Wilcoxon test will be used. The McNemar test will be applied with quantitative or dichotomous variables.

Effects of the intervention will be analyzed in a comparison of the changes in blood pressure and the secondary variables between the IG and the CG, using ANCOVA and adjusting for possible confounders. Effects of the intervention during follow-up will be studied with an analysis of the variance of repeated measures.

Analysis by subgroups.

The effect of the intervention could be influenced by age, sociocultural level and adherence to the study's chocolate intake. The same analyses described above will be performed for each of the aforementioned subgroups.

Secondary analyses.

A multivariate multiple regression analysis will be performed to identify the variables with the greatest influence on blood pressure changes and the secondary variables analyzed.

Methodological limitations:

Due to the nature of the intervention, the participating subjects cannot be blinded. However, the researcher who analyses the data and the person who makes the measurements during follow-up visits will be blinded with respect to the group to which the participants belong.

Assessment of the quality of life and lifestyles will be carried out through self-reported data; however, previously validated instruments will be used to obtain these. To make compliance with the intervention in the IG easier, IG participants will be provided with instructions on eating the chocolate and a calendar to record each intake.

ETHICS AND DISSEMINATION

Ethical considerations:

The study was approved by the Clinical Research Ethics Committee of the Salamanca Health Area ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov with the identifier NCT03492983.

Participants must sign informed consent in accordance with the Declaration of Helsinki. Subjects will be informed of the objectives of the project and the risks and benefits of the explorations to be carried out, including sample collection. None of the tests will pose risks that could endanger the lives of participants. Confidentiality of participant data will be guaranteed at all times in accordance with the provisions of the Organic

Law on the Protection of Personal Data (15/1999 of December 13, LOPD), and under the conditions established by Law 14/2007 of biomedical research.

Dissemination plan:

The research group plans to achieve rapid and widespread dissemination of results to ensure maximum visibility of this study. To this end, the results of the study will be published in scientific journals with peer review. At least one publication of the main results and others with the secondary results are planned. This will be complemented by presentation of the results of the study at relevant scientific conferences and seminars of national and international scope. In addition, a doctoral thesis based on this project will be prepared. Appropriate dissemination will likewise be carried out through social networks and other media. Moreover, given the involvement of a commercial product, the transfer to clinical practice is expected to be very fast if the results are as expected.

DISCUSSION

In recent years, there has been an increase in attention to polyphenols and their beneficial effects on health, with numerous studies being carried out to assess this [15 17]. Similarly, the therapeutic use of these compounds has been suggested for certain diseases or population groups [29 54]. The menopause increases the risk of developing cardiovascular disease compared to the previous period [38]. However, we have not found any study that assesses the effect of adding commercially available chocolate high in cocoa content to the usual diet in this population at special risk. Similarly, no studies have been found that evaluate the effects on cognitive performance, quality of life and body composition of adding commercial chocolate with high cocoa content to the usual diet in postmenopausal women.

The results of this work will provide new evidence in this regard for the development of strategies in nutritional education of particularly vulnerable populations, given their high risk of developing cardiovascular disease, including non-pharmacological therapies and strategies that employ lifestyle modification. This intervention may also have

implications for the preparation of recommendations in clinical practice guidelines and quality improvement programs aimed at the care of postmenopausal women.

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AUTHORS' CONTRIBUTIONS:

JIR, JAM, LGO and IGY contributed to the conception and design of the study. IGY, JIR and JAM prepared the manuscript of the study protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY contributed to the development of the study protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided assistance with statistical methodology and knowledge. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided a critical review of the manuscript.

All authors have read and accepted the final version of the protocol.

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"Lindt & Sprüngli will provide the necessary chocolate for the implementation of the study. This company will not play any role in the design of the study, data analysis, reporting of results, or the decision to present the manuscript for publication".

COMPETING INTERESTS STATEMENT:

The authors declare that they have no conflicts of interest.

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FIGURE LEGEND:

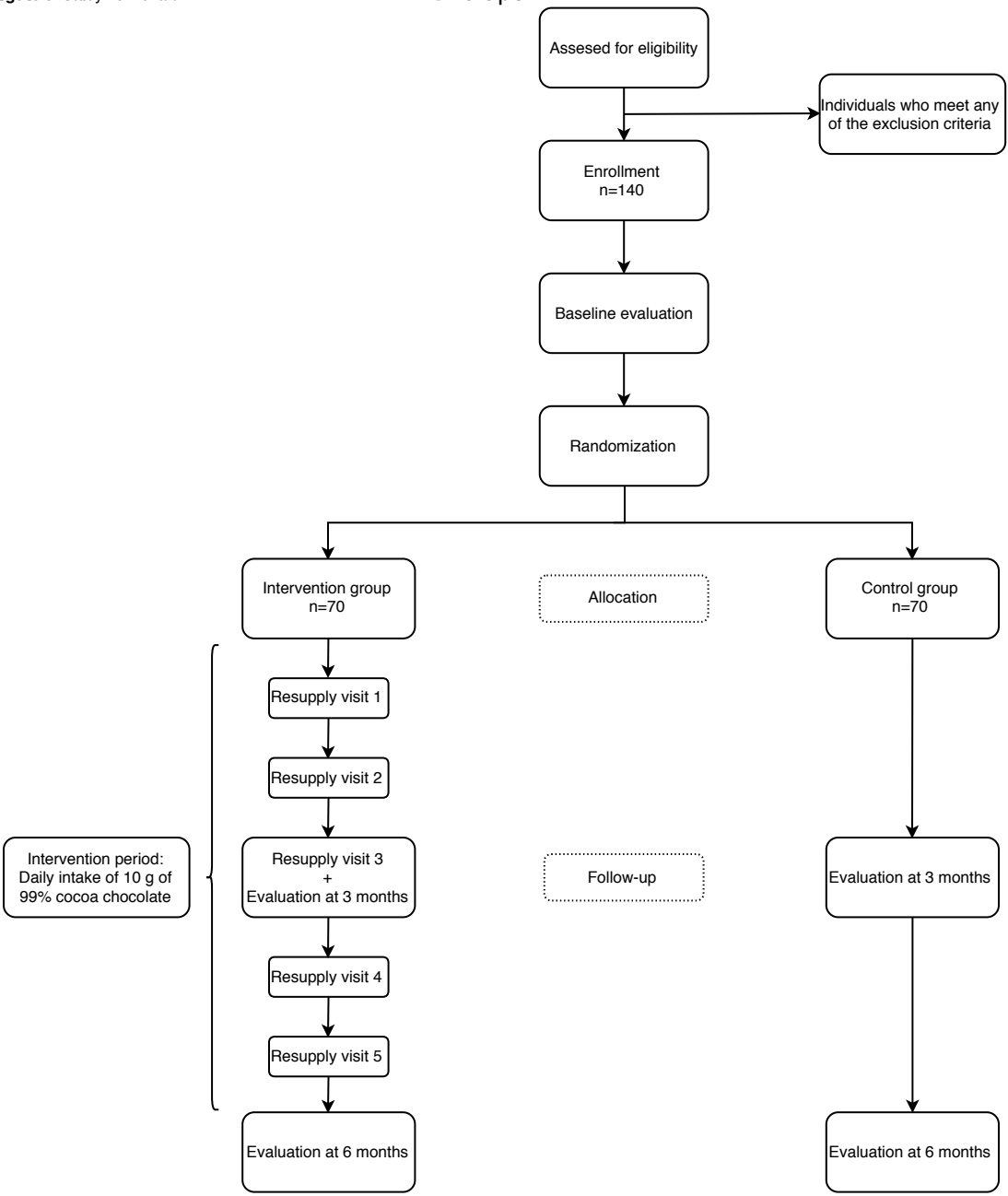
Figure 1. Study flow chart

Table 1. Polyphenol composition of 99% cocoa chocolate

Table 1. Polyphenols composition of 99% cocoa chocolate.

Compounds	Quantity
Protocatechuic acid (mg/g)	0.058 ± 0.008
Procyanidin dimer (B3) (mg/g)	0.176 ± 0.013
Catechin (mg/g)	1.035 ± 0.105
Procyanidin dimer (B2) (mg/g)	1.440 ± 0.055
Epicatechin (mg/g)	2.610 ± 0.075
Procyanidin trimer (C1) (mg/g)	0.853 ± 0.024
Procyanidin A hexoside (mg/g)	0.354 ± 0.007
Quercetin glucoside (mg/g)	0.002 ± 0.000
Quercetin arabinoside (mg/g)	0.003 ± 0.001

Figure 1. Study flow-chart



SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	3
	2b	All items from the World Health Organization Trial Registration Data Set	3
Protocol version	3	Date and version identifier	NA
Funding	4	Sources and types of financial, material, and other support	21-22
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	21
	5b	Name and contact information for the trial sponsor	21-22
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	22
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	21

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	5-7
	6b	Explanation for choice of comparators	5-7
Objectives	7	Specific objectives or hypotheses	7
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	8
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	8
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	8
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	9
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	NA
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	10
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	12
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	10-12
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Figure 1

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	8
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	8
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	9
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	9
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	9
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	13
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	13
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	10-12
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	NA
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	14
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	14-15
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	14-15
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	NA
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	NA
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	15
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	15

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	15
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	15
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	22
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	NA
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	16
	31b	Authorship eligibility guidelines and any intended use of professional writers	16
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	16
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	NA
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	NA

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated.

BMJ Open

Vascular and cognitive effects of chocolate with a high concentration of cocoa in postmenopausal women: a study protocol for a randomized clinical trial

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Primary Subject	Nutrition and metabolism

Heading:	
Secondary Subject Heading:	Cardiovascular medicine
Keywords:	chocolate, postmenopause, arterial pressure, vascular stiffness, body composition, quality of life

Note from the Editors: Instructions for reviewers of study protocols

Since launching in 2011, BMJ Open has published study protocols for planned or ongoing research studies. If data collection is complete, we will not consider the manuscript.

Publishing study protocols enables researchers and funding bodies to stay up to date in their fields by providing exposure to research activity that may not otherwise be widely publicised. This can help prevent unnecessary duplication of work and will hopefully enable collaboration. Publishing protocols in full also makes available more information than is currently required by trial registries and increases transparency, making it easier for others (editors, reviewers and readers) to see and understand any deviations from the protocol that occur during the conduct of the study.

The scientific integrity and the credibility of the study data depend substantially on the study design and methodology, which is why the study protocol requires a thorough peer-review.

BMJ Open will consider for publication protocols for any study design, including observational studies and systematic reviews.

Some things to keep in mind when reviewing the study protocol:

- Protocol papers should report planned or ongoing studies. The dates of the study should be included in the manuscript.
- Unfortunately we are unable to customize the reviewer report form for study protocols. As such, some of the items (i.e., those pertaining to results) on the form should be scored as Not Applicable (N/A).
- While some baseline data can be presented, there should be no results or conclusions present in the study protocol.
- For studies that are ongoing, it is generally the case that very few changes can be made to the methodology. As such, requests for revisions are generally clarifications for the rationale or details relating to the methods. If there is a major flaw in the study that would prevent a sound interpretation of the data, we would expect the study protocol to be rejected.

TITLE: Vascular and cognitive effects of chocolate with a high concentration of cocoa in postmenopausal women: a study protocol for a randomized clinical trial.

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ABSTRACT

Introduction: The intake of polyphenols has shown certain health benefits. The aim of this study is to assess the effect of adding a daily amount of chocolate high in cocoa content and polyphenols to the normal diet on blood pressure, vascular function, cognitive performance, quality of life and body composition in postmenopausal women.

Methods and analysis: Randomized clinical trial with two parallel groups involving a total of 140 women between 50 and 64 years of age in the postmenopausal period, defined by amenorrhea of at least 12 consecutive months. The main variable will be the change in blood pressure. Secondary variables will be changes in vascular function, quality of life, cognitive performance and body composition. The intervention group will be given chocolate containing 99% cocoa, with instructions to add 10 g daily to their normal diet for 6 months. The daily nutritional contribution of this amount of chocolate is 59 Kcal and 65.4 mg of polyphenols. There will be no intervention in the control group. All variables will be measured at the baseline visit and at 3 and 6 months after randomization, except cognitive performance and quality of life, to be assessed only at baseline and at 6 months. Recruitment is scheduled to begin on June 1, 2018, and the study will continue until May 31, 2019.

Ethics and dissemination: This study was approved by the Clinical Research Ethics Committee of the Health Area of Salamanca, Spain ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov provided by the US National Library of Medicine, number NCT03492983. The results will be disseminated through open access peer-reviewed journals, conference presentations, broadcast media as well as presentation to stakeholders.

Keywords: Chocolate, postmenopause, arterial pressure, vascular stiffness, body composition, quality of life.

Strengths and limitations of this study:

- This study used a commercially available chocolate with high content of cocoa and polyphenols during the intervention.
- Blood pressure and vascular function will be measured objectively using a sphygmomanometer and a Vasera VS-2000 device (Fukuda Denshi), with body composition measured by impedance analysis, while quality of life and cognitive performance will be assessed using validated instruments.
- Due to the nature of the intervention, the participants cannot be blinded, although the researchers who perform the measurements and the statistical analysis will be blinded.

INTRODUCTION

Polyphenols are bioactive compounds found in many plants, fruits and vegetables. The beneficial effects on human health associated with the consumption of a diet rich in polyphenols has generated great scientific interest in these substances.[1-3] The action of polyphenols is based on their antioxidant capacity through the uptake of free radicals, the chelation of metals with redox properties and the modulation and inhibition of enzymatic activity.[4]

The most abundant polyphenols in cocoa are flavonoids, which have been linked to a protective effect against cardiovascular disease, decreasing the risk of cardiovascular morbidity and mortality and favouring the prevention of other chronic diseases such as diabetes mellitus type 2.[1-3, 5-7] The ability to reduce cardiovascular risk could be due to an improvement in the elements that define metabolic syndrome, the improvement of vascular endothelial dysfunction, insulin resistance and the inhibition of platelet activation and aggregation.[8, 9] However, although current evidence suggests that polyphenols produce an improvement in cardiovascular health, it is not enough to determine the minimum amount of intake necessary to achieve health benefits.[10]

Cocoa polyphenols and blood pressure: The effect of consuming polyphenols present in chocolate on the blood pressure (BP) statistics of healthy individuals is not clear. Cocoa consumption has been associated with an improvement in endothelial function and a decrease in blood pressure in both healthy subjects and those with risk factors and cardiovascular diseases.[11, 12] Some studies have observed a dose-dependent relationship between cocoa intake and clinical BP, with higher consumption equated to lower blood pressure and better vascular function.[13, 14] Conversely, other research has not obtained significant changes in these parameters related to the supplementation of cocoa or pure polyphenols such as epicatechin or quercetin.[15, 16]

Endothelial dysfunction in postmenopausal women causes changes that favour the development of cardiovascular risk factors and atherosclerosis, which lead to the appearance and maintenance of hypertension.[17, 18] A decrease in BP has been observed in this group after daily consumption of cocoa with a flavonol content of 40.12 mg. Below this level, however, no changes have been observed.[19]

Cocoa polyphenols and vascular function: Among healthy individuals as well as postmenopausal women, the consumption of polyphenols present in cocoa has been associated with a dose-dependent improvement of vascular function, in particular of arterial stiffness measured by pulse wave speed.[13, 14, 19] One of these studies also suggests that the reduction in arterial stiffness observed in postmenopausal women after consumption of cocoa is independent of the frequency of the intake.[19] However, this relationship is not evident in people with mild hypertension when cardio-ankle vascular index (CAVI) is used as a measure of arterial stiffness.[20]

There is also evidence of the influence of these polyphenols in reducing the augmentation index (AIx). The study by West et al,[21] involving subjects with excess weight and moderate obesity, concludes that the treatment with dark chocolate decreases AIx in women, although it seems that this association may affect more the

elasticity of the large arteries, especially in subjects with obesity and diabetes mellitus type 2.[22]

Cocoa polyphenols and cognitive performance: There is evidence to suggest that chocolate rich in polyphenols may be beneficial for cognitive performance and state since it improves mental processing speed and attenuates the increase of mental fatigue among healthy young adults.[23, 24] An improvement in cognitive performance among older age groups after eating chocolate has also been observed[25] and especially in subjects with higher risk of cardiovascular disease.[26] Several studies also show that polyphenol-rich chocolate causes an improvement in executive function and categorical fluency,[27] in working memory,[28, 29] and a slowing of mental fatigue[30] and also that a higher frequency of chocolate consumption has been associated with better cognitive function.[29] Furthermore, a positive influence of cocoa polyphenols on physiological processes has been reported, with a neuroprotective effect[31] and improved cognitive performance.[32] In this regard, it has been suggested that the brain-derived neurotrophic factor (BDNF) may play a role in the cognitive enhancement induced by the flavonoides.[33] Favorable effects on cerebrovascular function have also been observed in postmenopausal women after consumption of chocolate with high concentration of cocoa.[34]

Cocoa polyphenols and quality of life: The quality of life linked to health is represented by the individual's perception of well-being in various aspects of life, including physical and mental aspects. The effect of chocolate and polyphenols on the quality of life has scarcely been studied, with not a great deal of evidence available and even less of a conclusive nature. In a study conducted among healthy people, where regular consumption of chocolate was recorded over a year, no evidence was found of a clear association between chocolate intake and the physical or mental components of quality of life.[35] Nevertheless, it has been observed that the consumption of dark chocolate may be beneficial for the quality of life of women with fibromyalgia.[36]

Cocoa polyphenols and body composition: The menopause period leads to various changes in the body composition of women.[37] Regarding the connection between cocoa polyphenols and body composition, results diverge. Some clinical trials involving healthy people and overweight or obese patients have not reported significant differences that link chocolate consumption to anthropometric measures.[16, 20, 21, 38] Other studies indicate that chocolate consumption may have positive effects on body composition in adolescents,[39] patients with diabetes[40] or women with obesity.[41] Two recent systematic reviews also indicate that eating chocolate is associated with reduced body mass index (BMI) and waist circumference,[42, 43] and one of them also concludes that the amount and the length of time during which it is eaten play a key role in these beneficial effects.[43] Conversely, other studies such as that carried out with the cohort of the Atherosclerosis Risk in Communities (ARIC) study have observed a dose-dependent increase in weight after habitual chocolate consumption.[44]

In sum, the polyphenols present in chocolate seem to have a positive effect on BP, vascular function, cognitive performance and quality of life, especially in populations with increased cardiovascular risk such as postmenopausal women.[45] However, the conflicting results obtained in different studies suggest that the real contribution of these compounds to health and the underlying mechanisms remain unclear. Moreover, most of these studies have used preparations with high concentrations of polyphenols that are usually not present in the normal diet.

This study aims to evaluate the effect of adding a daily amount of 10 g of chocolate high in cocoa content (99%) and polyphenols to the normal diet on blood pressure, vascular function, cognitive performance, quality of life and body composition in postmenopausal women.

METHODS AND ANALYSIS

Design and setting:

This controlled and randomized clinical trial involves two parallel groups. The study will be carried out in the Research Unit of the La Alamedilla Health Centre in Salamanca (Spain), which is part of the Biomedical Research Institute of Salamanca (IBSAL) and the Primary Care Prevention and Health Promotion Research Network (REDIAPP). The recruitment schedule is set to start on June 1, 2018, and the study will run until May 31, 2019. There will be a baseline assessment and two follow-ups, at 3 and 6 months.

Study population:

Those subjects who meet the selection criteria and sign the informed consent after receiving information about the objectives and implementation of the study will take part.

Inclusion criteria: women between 50 and 64 years of age in postmenopause, defined by and checked against amenorrhea during at least 12 consecutive months.

Exclusion criteria: personal history of cardiovascular disease; personal history of diabetes mellitus, arterial hypertension or dyslipidemia under pharmacological treatment; hypocaloric diets; clinically demonstrable neurological and/or neuropsychological disease; treatment with hormone replacement therapy; intolerance and/or allergy to cocoa or any of the components of the supplement.

Participants will be selected using a consecutive sample of women who meet the selection criteria in the GP surgeries of four urban primary care centres in Salamanca, from June 1, 2018.

Patient and public involvement

Patients and the public were not involved in the design of this study or outcome measures. We hope that the results of the study will be disseminated through press releases and information meetings with the study participants.

Sample size:

The size of the sample has been estimated based on the potential modification of the main variable, systolic blood pressure (SBP). Given alpha and beta risks of 0.05 and

0.20 respectively in bilateral contrast and a standard deviation (SD) of 5.8 mmHg, 140 participants (70 per group) will be necessary to detect a minimum difference of 2.9 mmHg in the SBP between the two groups. A predicted drop-out rate of 10% during follow-up has been taken into account. This estimate has considered the results obtained in a similar study in which a decrease in SBP of 6.5 was observed ± 5.8 mmHg.[14]

Randomization:

Participants will be assigned to the intervention group (IG) or control group (CG) at random. The allocation sequence will be generated by an independent researcher using the Epidat 4.2 program[46] and will remain hidden until the participants are assigned to each group.

Intervention:

No type of intervention will be carried out with the CG participants.

IG participants will be given chocolate with 99% cocoa content and asked to eat 10 g daily for a period of 6 months. According to the EFSA (European Food Safety Authority), 10 g of high-flavanol dark chocolate consumed in the context of a balanced diet could help maintain endothelium-dependent vasodilation.[47] Participants will also be given instructions on eating and keeping the product, with the recommendations, for example, that the chocolate can be consumed in small pieces leaving them unmated in the mouth, without chewing them. In addition, a series of recommendations will be given remembering the organoleptic characteristics of the product, as well as the recommendations of trying to consume the product at the same time or refrain from ingesting it dissolved in milk. In addition, they will be given a calendar on which to record the time it was eaten each day. This calendar will be returned to the researchers at each replenishment visit.

This amount of chocolate provides the following daily nutritional contribution: 59 Kcal, 0.8 g of carbohydrates, 1.5 g of protein, 5.1 g of fat, of which 3.1 g are saturated fats. The proportion of polyphenols per 10 g is 65.4 mg. The polyphenolic profile of this

compound can be seen in table 1. On each visit, IG participants will receive the amount of chocolate they need until the next replenishment visit. In addition to the baseline visit, there will be 5 replenishment visits in months 1, 2, 3 (coinciding with the evaluation visit), 4 and 5. The sole purpose of the replenishment visits will be to supply the amount of chocolate needed until the next visit, without any other intervention being carried out.

Participants in both groups will be instructed to continue with the dietary pattern they usually follow, without changing their eating habits during the study period.

Procedures:

For each participant a baseline visit and two follow-up visits at 3 and 6 months after the initial one are scheduled (Figure 1). The IG will also make 5 replenishment visits, in months 1, 2, 3 (coinciding with the first follow-up visit), 4 and 5. In the replenishment visits they will be given the amount of chocolate needed until the next visit and will hand in the calendar with the record of the chocolate eaten.

Primary and secondary endpoints:

The primary variable will be the decrease in clinical BP, measured with a digital sphygmomanometer. Secondary variables will include vascular function, quality of life, cognitive performance and body composition.

All variables will be measured at 3 and 6 months after randomization, except for cognitive performance and quality of life, to be assessed only after 6 months.

Blood pressure:

Clinical systolic and diastolic blood pressure will be measured with a validated Omron M10-IT sphygmomanometer (Omron Healthcare, Kyoto, Japan). Three measurements will be taken in the dominant arm of the subject in a sitting position after at least 5 minutes of rest with an appropriately sized cuff, following the recommendations of the European Society of Hypertension.[48] The average of the last two measurements will be recorded.

Vascular function:

The Vasera VS-2000 device (Fukuda Denshi) will be used to measure the CAVI and the brachial-ankle pulse wave velocity (ba-PWV) at rest. CAVI is a good indicator of arterial stiffness, providing an accurate estimate of the degree of atherosclerosis without depending on blood pressure.[49] CAVI ≥ 9 and ba-PWV ≥ 18.3 will be considered pathological.[50] Pathological CAVI is representative of subclinical atherosclerosis.[51]

Cognitive performance:

The instructions are presented visually at the start of the baseline measurement to ensure limiting a learning effect over the subsequent testing periods. Attention and executive functions: Trail Making Test A will be used to measure attention and Trail Making Test B for processing speed and executive functions.[52]

Immediate verbal memory will be assessed with the Rey Auditory Verbal Learning Test. The immediate recall of a list of 15 words is measured in three attempts, followed by delayed verbal memory through the free recall of the words learned in the first part of the test after 10 minutes.[53]

Working memory will be assessed with the WAIS Digit Span Backward test.[54]

Phonological fluency will be explored by naming as many words as possible starting with different letters of the FAS Questionnaire in the space of one minute.[55]

Categorical fluency measures verbal semantic fluency and will be assessed by naming as many animals as possible in one minute.[56]

Quality of life:

The quality of life linked to health will be assessed through the EuroQol 5-D questionnaire (EQ-5D). We will use the adapted Spanish version of this questionnaire, which has been validated in the Spanish population.[57] This questionnaire consists of three elements: the assessment by the individuals of their state of health in level of severity by dimension (mobility, personal care, daily activities, pain/discomfort and anxiety/depression), the assessment of their state of health on an analogue visual

scale, and finally an index of social values obtained for each state of health generated by the instrument.

The quality of life will also be studied using the Cervantes Scale.[58] This questionnaire is specifically designed for menopause and postmenopause and has been validated for Spanish women. Its 31 structured items cover the 4 dimensions of menopause: menopause and health, sexuality, psychic domain and relationships.

Body composition:

Body composition will be measured with the Inbody 230 Monitor.[59] This analyzer provides data on fat mass and body fat percentage as principal outcomes and also skeletal muscle mass, total body water, fat-free mass, waist-hip ratio, basal metabolism, and a segmental analysis.

Body weight will be measured twice with an electronic scale (Scale 7830, Soehnle Professional GmbH & Co, Backnang, Germany) after proper calibration (accuracy ± 0.1 kg). Height will be measured by recording the average of two readings rounded to the nearest centimetre using a stadiometer (Seca 222, Medical scale and measurement system, Birmingham, UK). Both measurements will be made with the subject barefoot and wearing light clothing. Body mass index will be calculated by dividing weight (kg) by height squared (m^2). Waist circumference will be assessed in accordance with the recommendations of the Spanish Society for the Study of Obesity (SEEDO)[60] and will be measured in duplicate before and after inhalation, using a flexible tape parallel to the floor, at the level of the mid-point between the lowest rib and the iliac crest, with the subject standing up and without clothes.

Other variables

Clinical and sociodemographic variables

At the baseline visit, information on clinical and sociodemographic variables will also be collected via questions about age, marital status, educational level and occupation. Family history of cardiovascular disease and personal history of anxiety and/or

depression, gestational diabetes, hypertension, dyslipidemia and the prescribed pharmacological treatment (antiaggregants, anticoagulants, thyroid hormone treatment, anxiolytics) will also be recorded, as well as the taking of NSAIDs in the last two weeks. In subsequent visits, personal histories of cardiovascular disease, diabetes mellitus, arterial hypertension or dyslipidemia in treatment, as well as the prescribed pharmacological treatment (hypolipidemic, antihypertensive, antidiabetic) will also be noted.

Evaluation of chocolate consumption and habitual diet

Chocolate consumption will be assessed at each evaluation visit by a series of questions about the amount, type and frequency of consumption in the period between visits.

Nutritional habits will be assessed by a 24-hour log on 3 non-consecutive days prior to each visit.

Evaluation of other lifestyles

The use of tobacco will be assessed with a questionnaire on the personal history and pattern of smoking.

Alcohol use will be recorded with a questionnaire covering the previous 7 days which will include specific beverages and the amount by volume drunk of each.

Physical activity will be measured using the International Physical Activity Questionnaire (IPAQ) in its short version and validated in Spanish.[61] This questionnaire measures activity over the previous 7 days, classifying the subjects according to three activity levels (low, moderate and high) with respect to three types of activities: walking, moderate-intensity activities and vigorous-intensity activities. The amount of physical exercise will be estimated in METs-minute/week.

Evaluation of laboratory variables

At baseline and follow-up visits at 6 months, we will measure plasma fasting glucose values (mg/dL), glycated haemoglobin (%), total cholesterol (mg/dL), total triglycerides (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), creatinine (mg/L),

insulinemia (mg/dL). Creatinine in urine (mg/dL) and microalbuminuria (mg/dL) will also be measured. Insulin resistance will be determined using the HOMA index (Homeostasis Model Assessment Insulin Resistance) estimated using the following equation: Fasting glucose (mmol/l) X insulin (mU/ml)/22.5.

The evaluation visits will be made in the morning between 8:00 and 10:00 a.m. Each participant will be informed prior to the visit to go fasting for at least 12 hours, having avoided the 24 hours prior to visiting the consumption of polyphenol-rich foods, including cocoa, chocolate, apples, and red wine as well as alcoholic drinks or the performance of programmed physical activity. All evaluation visits, including blood pressure measurements and evaluations of vascular function, will be carried out in a room with conditions of lighting and temperature standardized, recommending that patients attend the appointment with a prior rest of at least 8- 10 hours.

Data collection procedure, data management and monitoring

Data collection of the baseline and follow-up evaluation visits at 3 and 6 months will be carried out by a nurse specifically trained to do so. The intervention visit after the baseline evaluation will be carried out by another nurse, different from the one who does the data collection. Each participant will have a unique identification code within the study. All measurements will be compiled in a data collection notebook and kept in a secure place that will remain closed within the health center. A database will be created in SPSS to which only the members of the research team and the people related to the statistical analyzes will have access. The principal investigator or a person designated for this purpose will perform a weekly process of monitoring the study, taking into account the inclusion of patients, cleaning and debugging of databases and adaptation of the procedures to the protocol.

Blinding strategy:

Due to the nature of the intervention itself, the participants and the person responsible for delivering the chocolate to IG participants, cannot be blinded. However, the person

responsible for carrying out the study measurements at each visit and for the statistical analysis will be blind to the intervention.

Statistical analysis:

General analysis

Results for the quantitative variables will be expressed by mean ± standard deviation or by frequency distribution in the case of qualitative ones. The normality of the variables will be assessed using the Kolmogorov-Smirnov test. In cases where a normal distribution cannot be assumed, the corresponding nonparametric tests will be applied. The association between independent qualitative variables will be analyzed by means of the chi-square test or Fisher's exact test. The means between the two groups will be compared using the Student's t test or the Mann-Whitney U test, and the Pearson or Spearman correlation coefficients will be calculated to analyze the relationship between quantitative variables.

The analysis of the results for the main variable and the secondary variables will be carried out by intention to treat. In addition, a secondary analysis will be run taking into account chocolate intake adherence (< 50% days and > 50% days) and other relevant subgroups in relation to their physical activity or previous chocolate consumption.

All analyses will be performed with the SPSS version 23.0 (IBM Corporation, Armonk, NY, USA) and an alpha risk of 0.05 will be set as the limit of statistical significance.

Analysis of the intervention's effect on primary and secondary outcomes.

To analyze the changes at 3 and 6 months from baseline in the primary outcome (blood pressure) and in the secondary outcomes within the same group, the Student's t test for paired data or the Wilcoxon test will be used. The McNemar test will be applied with quantitative or dichotomous variables.

Effects of the intervention will be analyzed in a comparison of the changes in blood pressure and the secondary variables between the IG and the CG, using ANCOVA and adjusting for possible confounders as the smoking status. Effects of the intervention during follow-up will be studied with an analysis of the variance of repeated measures.

Analysis by subgroups.

The effect of the intervention could be influenced by age, sociocultural level and adherence to the study's chocolate intake. The same analyses described above will be performed for each of the aforementioned subgroups.

Secondary analyses.

A multivariate multiple regression analysis will be performed to identify the variables with the greatest influence on blood pressure changes and the secondary variables analyzed.

Methodological limitations:

Due to the nature of the intervention, the participating subjects cannot be blinded. However, the researcher who analyses the data and the person who makes the measurements during follow-up visits will be blinded with respect to the group to which the participants belong. The smoking status in the 12 months prior to the time of inclusion could influence the outcome measures related to vascular function and blood pressure so, although participants will not be excluded for this reason, this aspect will be controlled in statistical analysis. Assessment of the quality of life and lifestyles will be carried out through self-reported data; however, previously validated instruments will be used to obtain these. To make compliance with the intervention in the IG easier, IG participants will be provided with instructions on eating the chocolate and a calendar to record each intake.

ETHICS AND DISSEMINATION

Ethical considerations:

The study was approved by the Clinical Research Ethics Committee of the Salamanca Health Area ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov with the identifier NCT03492983.

Participants must sign informed consent in accordance with the Declaration of Helsinki. Subjects will be informed of the objectives of the project and the risks and benefits of the explorations to be carried out, including sample collection. None of the tests will pose risks that could endanger the lives of participants. Confidentiality of participant data will be guaranteed at all times in accordance with the provisions of the Organic Law on the Protection of Personal Data (15/1999 of December 13, LOPD), and under the conditions established by Law 14/2007 of biomedical research.

Dissemination plan:

The research group plans to achieve rapid and widespread dissemination of results to ensure maximum visibility of this study. To this end, the results of the study will be published in open-access scientific journals with peer review. At least one publication of the main results and others with the secondary results are planned. This will be complemented by presentation of the results of the study at relevant scientific conferences and seminars of national and international scope. In addition, a doctoral thesis based on this project will be prepared. Appropriate dissemination will likewise be carried out through social networks and other media. Moreover, given the involvement of a commercial product, the transfer to clinical practice is expected to be very fast if the results are as expected.

DISCUSSION

In recent years, there has been an increase in attention to polyphenols and their beneficial effects on health, with numerous studies being carried out to assess this.[19, 21] Similarly, the therapeutic use of these compounds has been suggested for certain diseases or population groups.[36, 62] The menopause increases the risk of developing cardiovascular disease compared to the previous period.[45] However, we have not found any study that assesses the effect of adding commercially available chocolate high in cocoa content to the usual diet in this population at special risk. Similarly, no studies have been found that evaluate the effects on cognitive

performance, quality of life and body composition of adding commercial chocolate with high cocoa content to the usual diet in postmenopausal women.

The results of this work will provide new evidence in this regard for the development of strategies in nutritional education of particularly vulnerable populations, given their high risk of developing cardiovascular disease, including non-pharmacological therapies and strategies that employ lifestyle modification. This intervention may also have implications for the preparation of recommendations in clinical practice guidelines and quality improvement programs aimed at the care of postmenopausal women.

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AUTHORS' CONTRIBUTIONS:

JIR, JAM, LGO and IGY contributed to the conception and design of the study. IGY, JIR and JAM prepared the manuscript of the study protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY contributed to the development of the study protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided assistance with statistical methodology and knowledge. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided a critical review of the manuscript.

All authors have read and accepted the final version of the protocol.

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"Lindt & Sprüngli will provide the necessary chocolate for the implementation of the study. This company will not play any role in the design of the study, data analysis, reporting of results, or the decision to present the manuscript for publication".

COMPETING INTERESTS STATEMENT:

The authors declare that they have no conflicts of interest.

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FIGURE LEGEND:

Figure 1. Study flow chart

Table 1. Polyphenol composition of 99% cocoa chocolate

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678 **Table 1.** Polyphenols composition of 99% cocoa chocolate.

Compounds	Quantity
Protocatechuic acid (mg/g)	0.058 ± 0.008
Procyanidin dimer (B3) (mg/g)	0.176 ± 0.013
Catechin (mg/g)	1.035 ± 0.105
Procyanidin dimer (B2) (mg/g)	1.440 ± 0.055
Epicatechin (mg/g)	2.610 ± 0.075
Procyanidin trimer (C1) (mg/g)	0.853 ± 0.024
Procyanidin A hexoside (mg/g)	0.354 ± 0.007
Quercetin glucoside (mg/g)	0.002 ± 0.000
Quercetin arabinoside (mg/g)	0.003 ± 0.001

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Figure 1. Study flow-chart

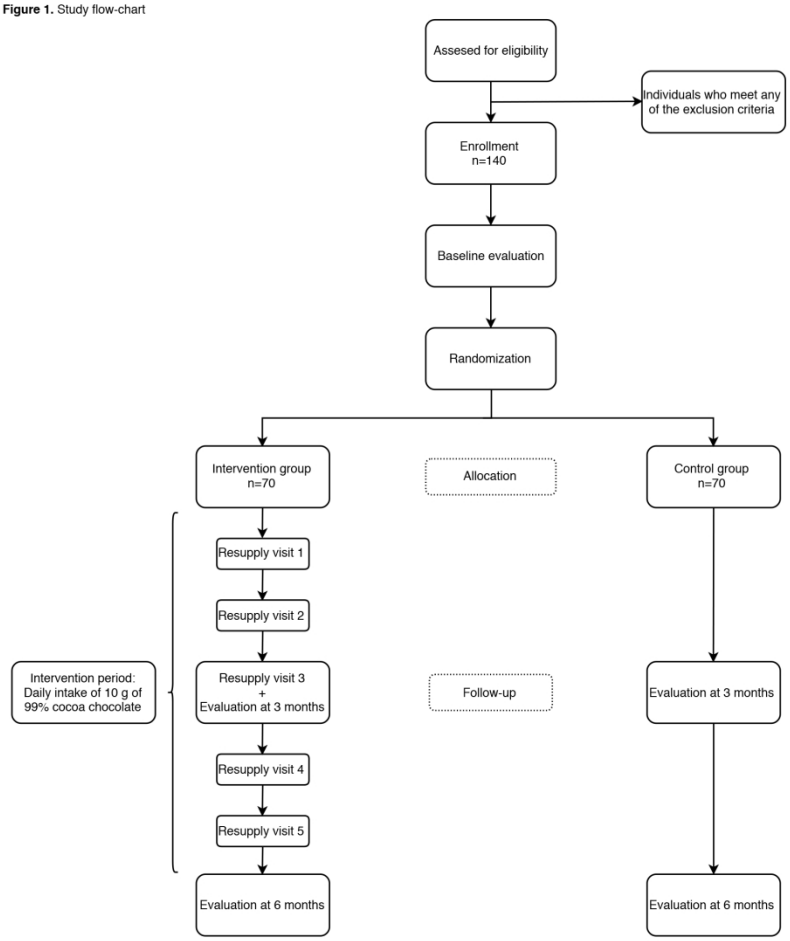

Figure 1

157x222mm (300 x 300 DPI)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Pag 1, line 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Pag 2, line 50
	2b	All items from the World Health Organization Trial Registration Data Set	NA
Protocol version	3	Date and version identifier	NA
Funding	4	Sources and types of financial, material, and other support	Pag 22, line 659
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Pag 22, line 651
	5b	Name and contact information for the trial sponsor	Pag 22, line 659
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	Pag 22, line 662
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Pag 22, line 651

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Pag 6, line 151
	6b	Explanation for choice of comparators	Pag 6, line 151
Objectives	7	Specific objectives or hypotheses	Pag 6, line 158
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Pag 7, line 164
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Pag 7, line 164
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Pag 7, line 175
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Pag 8, line 204
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	NA
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Pag 8, line 214
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Pag 8, line 208
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Pag 9, line 235
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Figure 1

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Pag 7, line 190
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Pag 7, line 182
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Pag 8, line 199
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Pag 8, line 199
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Pag 8, line 200
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Pag 13, line 355
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Pag 13, line 355
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Pag 9, line 238
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Pag 13, line 343
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Pag 14, line 377
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Pag 15, line 385
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Pag 15, line 387
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	NA
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	NA
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Pag 15, line 407
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	NA

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Pag 16, line 411
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Pag 16, line 414
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Pag 22, line 665
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Pag 13, line 349
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Pag 16, line 419
	31b	Authorship eligibility guidelines and any intended use of professional writers	Pag 16, line 419
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Pag 16, line 426
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	NA
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	NA

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.

BMJ Open

Vascular and cognitive effects of cocoa-rich chocolate in postmenopausal women: A study protocol for a randomized clinical trial

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Primary Subject	Nutrition and metabolism

Heading:	
Secondary Subject Heading:	Cardiovascular medicine
Keywords:	chocolate, postmenopause, arterial pressure, vascular stiffness, body composition, quality of life

TITLE: Vascular and cognitive effects of cocoa-rich chocolate in postmenopausal women: A study protocol for a randomized clinical trial.

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ABSTRACT

Introduction: The intake of polyphenols has certain health benefits. This study will aim to assess the effect of adding a daily amount of chocolate high in cocoa content and polyphenols to the normal diet on blood pressure, vascular function, cognitive performance, quality of life and body composition in postmenopausal women.

Methods and analysis: Here we plan a randomized clinical trial with two parallel groups involving a total of 140 women between 50- and 64-years-old in the postmenopausal period, defined by amenorrhea of at least 12 consecutive months. The main variable will be the change in blood pressure. Secondary variables will be changes in vascular function, quality of life, cognitive performance, and body composition. The intervention group will be given chocolate containing 99% cocoa, with instructions to add 10 g daily to their normal diet for 6 months. The daily nutritional contribution of this amount of chocolate is 59 Kcal and 65.4 mg of polyphenols. There will be no intervention in the control group. All variables will be measured at the baseline visit and 3 and 6 months after randomization, except cognitive performance and quality of life, which will only be assessed at baseline and at 6 months. Recruitment is scheduled to begin on June 1, 2018, and the study will continue until May 31, 2019.

Ethics and dissemination: This study was approved by the Clinical Research Ethics Committee of the Health Area of Salamanca, Spain ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov provided by the US National Library of Medicine, number NCT03492983. The results will be disseminated through open access peer-reviewed journals, conference presentations, broadcast media, and a presentation to stakeholders.

Keywords: Chocolate, postmenopause, arterial pressure, vascular stiffness, body composition, quality of life.

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Strengths and limitations of this study:

- This study will use commercially available chocolate with a high content of cocoa and polyphenols during the intervention.
- Blood pressure and vascular function will be measured objectively using a sphygmomanometer and a Vasera VS-2000 device (Fukuda Denshi), with body composition measured by impedance analysis, while the quality of life and cognitive performance will be assessed using validated instruments.
- Due to the nature of the intervention, the participants cannot be blinded, although the researchers who perform the measurements and the statistical analysis will be blinded.

INTRODUCTION

Polyphenols are bioactive compounds found in many plants, fruits and vegetables. The beneficial effects on human health associated with the consumption of a diet rich in polyphenols has generated great scientific interest in these substances.[1-3] The action of polyphenols is based on their antioxidant capacity through the uptake of free radicals, the chelation of metals with redox properties and the modulation and inhibition of enzymatic activities.[4]

The most abundant polyphenols in cocoa are flavonoids, which have been linked to a protective effect against cardiovascular disease, decreasing the risk of cardiovascular morbidity and mortality and favouring the prevention of other chronic diseases, such as diabetes mellitus type 2.[1-3, 5-7] The ability to reduce cardiovascular risk could be due to an improvement in the elements that define metabolic syndrome, the improvement of vascular endothelial dysfunction, insulin resistance and the inhibition of platelet activation and aggregation.[8, 9] However, although current evidence suggests that polyphenols produce an improvement in cardiovascular health, this is insufficient to determine the minimum amount of intake necessary to achieve health benefits.[10]

Cocoa polyphenols and blood pressure:

The effect of consuming polyphenols present in chocolate on the blood pressure statistics of healthy individuals is unclear. Cocoa consumption has been associated with an improvement in endothelial function and a decrease in blood pressure in both healthy subjects and those with risk factors and cardiovascular diseases.[11, 12] Some studies have observed a dose-dependent relationship between cocoa intake and clinical BP, with higher consumption equated to lower blood pressure and better vascular function.[13, 14] Conversely, other research has not obtained significant changes in these parameters related to the supplementation of cocoa or pure polyphenols, such as epicatechin or quercetin.[15, 16]

Endothelial dysfunction in postmenopausal women causes changes that favour the development of cardiovascular risk factors and atherosclerosis, which lead to the appearance and maintenance of hypertension.[17, 18] A decrease in blood pressure has been observed in this group after daily consumption of cocoa with a flavonol content of 40.12 mg. Below this level, however, no changes have been observed.[19]

Cocoa polyphenols and vascular function:

Among healthy individuals, as well as postmenopausal women, the consumption of polyphenols present in cocoa has been associated with a dose-dependent improvement of vascular function, in particular of arterial stiffness measured by pulse wave speed.[13, 14, 19] One of these studies also suggests that the reduction in arterial stiffness observed in postmenopausal women after consumption of cocoa is independent of the frequency of the intake.[19] However, this relationship is not evident in people with mild hypertension when cardio-ankle vascular index (CAVI) is used as a measure of arterial stiffness.[20]

There is also evidence of the influence of these polyphenols in reducing the augmentation index (AIx). The study by West et al.,[21] involving subjects with excess weight and moderate obesity, concludes that treatment with dark chocolate decreases AIx in women, although it seems that this association might have a greater effect on the elasticity of the large arteries, especially in subjects with obesity and diabetes mellitus type 2.[22]

Cocoa polyphenols and cognitive performance:

There is evidence to suggest that chocolate rich in polyphenols is beneficial for cognitive performance and state since it improves mental processing speed and attenuates the increase of mental fatigue among healthy young adults.[23, 24] An improvement in cognitive performance among older age groups after eating chocolate has also been observed,[25] especially in subjects with higher risk of cardiovascular disease.[26] Several studies also show that polyphenol-rich chocolate causes an improvement in executive function, categorical fluency,[27] and working memory,[28, 29] and a slowing of mental fatigue.[30] Also, a higher frequency of chocolate consumption has been associated with improved cognitive function.[29] Furthermore, a positive influence of cocoa polyphenols on physiological processes has been reported, with a neuroprotective effect[31] and improved cognitive performance.[32] In this regard, it has been suggested that the brain-derived neurotrophic factor (BDNF) plays a role in the cognitive enhancement induced by the flavonoides.[33] Favourable effects on cerebrovascular function have also been observed in postmenopausal women after consumption of chocolate with a high concentration of cocoa.[34]

Cocoa polyphenols and quality of life:

The quality of life linked to health is represented by the individual's perception of well-being in various aspects of life, including physical and mental aspects. The effect of chocolate and polyphenols on the quality of life has scarcely been studied, with little

available evidence and even less of a conclusive nature. In a study conducted among healthy people, where regular consumption of chocolate was recorded over 1 year, no evidence was found of a clear association between chocolate intake and the physical or mental components of quality of life.[35] Nevertheless, it has been observed that the consumption of dark chocolate might be beneficial for the quality of life of women with fibromyalgia.[36]

Cocoa polyphenols and body composition:

The menopause period leads to various changes in the body composition of women.[37] Regarding the connection between cocoa polyphenols and body composition, results diverge. Some clinical trials involving healthy people and overweight or obese patients have not reported significant differences that link chocolate consumption to anthropometric measures.[16, 20, 21, 38] Other studies indicate that chocolate consumption might have positive effects on body composition in adolescents,[39] patients with diabetes[40] or women with obesity.[41] Two recent systematic reviews also indicate that eating chocolate is associated with reduced body mass index (BMI) and waist circumference,[42, 43] and one of them also concludes that the amount and the length of time during which it is eaten play a key role in these beneficial effects.[43] Conversely, other studies such as that carried out with the cohort of the Atherosclerosis Risk in Communities (ARIC) study have observed a dose-dependent increase in weight after habitual chocolate consumption.[44]

In sum, the polyphenols present in chocolate seem to have a positive effect on BP, vascular function, cognitive performance, and quality of life, especially in populations with increased cardiovascular risk, such as postmenopausal women.[45] However, the conflicting results obtained in different studies suggest that the real contribution of these compounds to health and the underlying mechanisms remain unclear. Moreover,

most of these studies have used preparations with high concentrations of polyphenols that are usually not present in the normal diet.

This study aims to evaluate the effect of adding a daily amount of 10 g of chocolate high in cocoa content (99%) and polyphenols to the normal diet on blood pressure, vascular function, cognitive performance, quality of life, and body composition in postmenopausal women.

METHODS AND ANALYSIS

Design and setting:

This controlled and randomized clinical trial involves two parallel groups. The study will be carried out in the Research Unit of the La Alamedilla Health Centre in Salamanca (Spain), which is part of the Biomedical Research Institute of Salamanca (IBSAL) and the Primary Care Prevention and Health Promotion Research Network (REDIAPP). The recruitment schedule is set to start on June 1, 2018, and the study will run until May 31, 2019. There will be a baseline assessment and two follow-ups, at 3 and 6 months.

Study population:

Those subjects who meet the selection criteria and sign the informed consent after receiving information about the objectives and implementation of the study will take part.

Inclusion criteria: women between 50- and 64-years-old in postmenopause, defined by and checked against amenorrhea during at least 12 consecutive months.

Exclusion criteria: a personal history of cardiovascular disease; personal history of diabetes mellitus, arterial hypertension or dyslipidemia under pharmacological treatment; hypocaloric diets; clinically demonstrable neurological and/or

neuropsychological disease; treatment with hormone replacement therapy; intolerance and/or allergy to cocoa or any of the components of the supplement.

Participants will be selected using a consecutive sample of women who meet the selection criteria in the GP surgeries of four urban primary care centres in Salamanca, from June 1, 2018.

Patient and public involvement:

Patients and the public were not involved in the design of this study or outcome measures. We hope that the results of the study will be disseminated through press releases and information-sharing meetings with the study participants.

Sample size:

The size of the sample has been estimated based on the potential modification of the main variable, systolic blood pressure (SBP). Given alpha and beta risks of 0.05 and 0.20 respectively in bilateral contrast and a standard deviation (SD) of 5.8 mmHg, 140 participants (70 per group) will be necessary to detect a minimum difference of 2.9 mmHg in the SBP between the two groups. A predicted drop-out rate of 10% during follow-up has been taken into account. This estimate has considered the results obtained in a similar study in which a decrease in SBP of 6.5 was observed ± 5.8 mmHg.[14]

Randomization:

Participants will be assigned to the intervention group (IG) or control group (CG) at random. The allocation sequence will be generated by an independent researcher using the Epidat 4.2 program [46] before the inclusion of the first participant, using masked block randomisation. Patients will receive their randomisation number based on the order of their baseline evaluation visit and will remain hidden until the

participants are assigned to each group. To ensure that the blinding is maintained, patients will be given clear instructions not to disclose which treatment they have been randomised to while being interviewed by the blind assessors. Information on treatment allocation will be stored in a secure locker in case of emergency unblinding.

Intervention:

No type of intervention will be carried out with the CG participants.

IG participants will be given chocolate with 99% cocoa content and asked to eat 10 g daily for 6 months. According to the EFSA (European Food Safety Authority), 10 g of high-flavanol dark chocolate consumed in the context of a balanced diet could help maintain endothelium-dependent vasodilation.[47] Participants will also be given instructions on eating and keeping the product, with the recommendations, for example, that the chocolate can be consumed in small pieces leaving them unmated in the mouth, without chewing them. Also, a series of recommendations will be given addressing the organoleptic characteristics of the product, as well as the recommendations of trying to consume the product at the same time or refrain from ingesting it dissolved in milk. Also, participants will be given a calendar on which to record the time it was eaten each day. This calendar will be returned to the researchers at each replenishment visit.

This amount of chocolate provides the following daily nutritional contribution: 59 Kcal, 0.8 g of carbohydrates, 1.5 g of protein, 5.1 g of fat, of which 3.1 g are saturated fats. The proportion of polyphenols per 10 g is 65.4 mg. The polyphenolic profile of this compound can be seen in Table 1. On each visit, IG participants will receive the amount of chocolate they need until the next replenishment visit. In addition to the baseline visit, there will be five replenishment visits in months 1, 2, 3 (coinciding with the evaluation visit), 4 and 5. The sole purpose of the replenishment visits will be to

supply the amount of chocolate needed until the next visit, without any other intervention being carried out.

Participants in both groups will be instructed to continue with the dietary pattern they usually follow, without changing their eating habits during the study period.

Procedures:

For each participant a baseline visit and two follow-up visits at 3 and 6 months after the initial one are scheduled (Figure 1). The IG will also make five replenishment visits, in months 1, 2, 3 (coinciding with the first follow-up visit), 4 and 5. In the replenishment visits, participants will be given the amount of chocolate needed until the next visit and will hand in the calendar with the record of the chocolate eaten.

Primary and secondary endpoints:

The primary variable will be the decrease in clinical BP, measured with a digital sphygmomanometer. Secondary variables will include vascular function, quality of life, cognitive performance and body composition.

All variables will be measured at 3 and 6 months after randomization, except for cognitive performance and quality of life, which will be assessed only after 6 months.

Blood pressure:

Clinical systolic and diastolic blood pressure will be measured with a validated Omron M10-IT sphygmomanometer (Omron Healthcare, Kyoto, Japan). Three measurements will be taken in the dominant arm of the subject in a sitting position after at least 5 min of rest with an appropriately sized cuff, following the recommendations of the European Society of Hypertension.[48] The average of the last two measurements will be recorded.

Vascular function:

The Vasera VS-2000 device (Fukuda Denshi) will be used to measure the CAVI and the brachial-ankle pulse wave velocity (ba-PWV) at rest. CAVI is a good indicator of arterial stiffness, providing an accurate estimate of the degree of atherosclerosis without depending on blood pressure.[49] CAVI ≥ 9 and ba-PWV ≥ 18.3 will be considered pathological.[50] Pathological CAVI is representative of subclinical atherosclerosis.[51]

Cognitive performance:

The instructions are presented visually at the start of the baseline measurement to ensure limiting a learning effect over the subsequent testing periods. Attention and executive functions: Trail Making Test A will be used to measure attention and Trail Making Test B for processing speed and executive functions.[52]

Immediate verbal memory will be assessed with the Rey Auditory Verbal Learning Test. The immediate recall of a list of 15 words is measured in three attempts, followed by delayed verbal memory through the free recall of the words learned in the first part of the test after 10 min.[53]

Working memory will be assessed with the WAIS Digit Span Backward test.[54]
Phonological fluency will be explored by naming as many words as possible starting with different letters of the FAS Questionnaire in the space of 1 min.[55]

Categorical fluency measures verbal semantic fluency and will be assessed by naming as many animals as possible in 1 min.[56]

Quality of life:

The quality of life linked to health will be assessed through the EuroQol 5-D questionnaire (EQ-5D). We will use the adapted Spanish version of this questionnaire, which has been validated in the Spanish population.[57] This questionnaire consists of three elements: the assessment by the individuals of their state of health in level of severity by dimension (mobility, personal care, daily activities, pain/discomfort and anxiety/depression), the assessment of their state of health on an analogue visual scale, and finally an index of social values obtained for each state of health generated by the instrument.

The quality of life will also be studied using the Cervantes Scale.[58] This questionnaire is specifically designed for menopause and postmenopause and has been validated for Spanish women. Its 31 structured items cover the four dimensions of menopause: menopause and health, sexuality, psychic domain and relationships.

Body composition:

Body composition will be measured with the Inbody 230 Monitor.[59] This analyzer provides data on fat mass and body fat percentage as principal outcomes and also skeletal muscle mass, total body water, fat-free mass, waist-hip ratio, basal metabolism, and a segmental analysis.

Body weight will be measured twice with an electronic scale (Scale 7830, Soehnle Professional GmbH & Co, Backnang, Germany) after proper calibration (accuracy ± 0.1 kg). Height will be measured by recording the average of two readings rounded to the nearest centimetre using a stadiometer (Seca 222, Medical scale and measurement system, Birmingham, UK). Both measurements will be made with the subject barefoot and wearing light clothing. Body mass index will be calculated by dividing weight (kg) by height squared (m^2). Waist circumference will be assessed in accordance with the recommendations of the Spanish Society for the Study of Obesity (SEEDO)[60] and will

be measured in duplicate before and after inhalation, using a flexible tape parallel to the floor, at the level of the mid-point between the lowest rib and the iliac crest, with the subject standing up and without clothes.

Other variables

Clinical and sociodemographic variables

At the baseline visit, information on clinical and sociodemographic variables will also be collected via questions about age, marital status, educational level and occupation. The family history of cardiovascular disease and personal history of anxiety and depression, gestational diabetes, hypertension, dyslipidemia and the prescribed pharmacological treatment (antiaggregants, anticoagulants, thyroid hormone treatment, anxiolytics) will also be recorded, as well as the taking of NSAIDs in the last 2 weeks.

In subsequent visits, personal histories of cardiovascular disease, diabetes mellitus, arterial hypertension or dyslipidemia in treatment, as well as the prescribed pharmacological treatment (hypolipidemic, antihypertensive, antidiabetic) will also be noted.

Evaluation of chocolate consumption and habitual diet

Chocolate consumption will be assessed at each evaluation visit by a series of questions about the amount, type and frequency of consumption in the period between visits.

Nutritional habits will be assessed by a 24-h log on three non-consecutive days prior to each visit.

Evaluation of other lifestyles

The use of tobacco will be assessed with a questionnaire on the personal history and pattern of smoking.

Alcohol use will be recorded with a questionnaire covering the previous 7 days, which will include specific beverages and the amount by volume drunk of each.

Physical activity will be measured using the International Physical Activity Questionnaire (IPAQ) in its short version and validated in Spanish.[61] This questionnaire measures activity over the previous 7 days, classifying the subjects according to three activity levels (low, moderate and high) with respect to three types of activities: walking, moderate-intensity activities and vigorous-intensity activities. The amount of physical exercise will be estimated in METs-minute/week.

Evaluation of laboratory variables

At baseline and follow-up visits at 6 months, we will measure plasma fasting glucose values (mg/dL), glycated haemoglobin (%), total cholesterol (mg/dL), total triglycerides (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), creatinine (mg/L), and insulinemia (mg/dL). Creatinine in urine (mg/dL) and microalbuminuria (mg/dL) will also be measured. Insulin resistance will be determined using the HOMA index (Homeostasis Model Assessment Insulin Resistance) estimated using the following equation: Fasting glucose (mmol/l) × insulin (mU/ml)/22.5.

The evaluation visits will be made in the morning between 8:00 and 10:00 a.m. Each participant will be informed prior to the visit to fast for at least 12 h, having avoided during the 24 h prior to visiting the consumption of polyphenol-rich foods, including cocoa, chocolate, apples, and red wine as well as alcoholic drinks or the performance of the programmed physical activity. All evaluation visits, including blood pressure measurements and evaluations of vascular function, will be carried out in a room with

standardized lighting and temperature, recommending that patients attend the appointment with a prior rest of at least 8–10 h.

Data collection procedure, data management and monitoring

Data collection of the baseline and follow-up evaluation visits at 3 and 6 months will be carried out by a nurse specifically trained to do so. The intervention visit after the baseline evaluation will be carried out by another nurse, different from the one who performs the data collection. Each participant will have a unique identification code within the study. All measurements will be compiled in a data collection notebook and kept in a secure place that will remain closed within the health centre. A database will be created in SPSS to which only the members of the research team and the people related to the statistical analyses will have access. The principal investigator or a person designated for this purpose will perform a weekly process of monitoring the study, taking into account the inclusion of patients, cleaning and debugging of databases, and adaptation of the procedures to the protocol.

Blinding strategy:

Due to the nature of the intervention itself, the participants and the person responsible for delivering the chocolate to IG participants cannot be blinded. However, the person responsible for carrying out the study measurements at each visit and for the statistical analysis will be blind to the intervention.

Statistical analysis:

General analysis

Results for the quantitative variables will be expressed by mean ± standard deviation or by frequency distribution in the case of qualitative variables. The normality of the variables will be assessed using the Kolmogorov-Smirnov test. In cases where a normal distribution cannot be assumed, the corresponding nonparametric tests will be

applied. The association between independent qualitative variables will be analyzed using the Chi-square test or Fisher's exact test. The means between the two groups will be compared using the Student's t-test or the Mann-Whitney U test, and the Pearson or Spearman correlation coefficients will be calculated to analyze the relationship between quantitative variables.

The analysis of the results for the main variable and the secondary variables will be carried out by intention to treat. Also, a secondary analysis will be made, taking into account chocolate intake adherence (< 50% days and > 50% days) and other relevant subgroups in relation to their physical activity or previous chocolate consumption.
All analyses will be performed using SPSS version 23.0 (IBM Corporation, Armonk, NY, USA) and an alpha risk of 0.05 will be set as the limit of statistical significance.

Analysis of the intervention's effect on primary and secondary outcomes
To analyze the changes at 3 and 6 months from baseline in the primary outcome (blood pressure) and in the secondary outcomes within the same group, the Student's t-test for paired data or the Wilcoxon test will be used. The McNemar test will be applied with quantitative or dichotomous variables.

Effects of the intervention will be analyzed in a comparison of the changes in blood pressure and the secondary variables between the IG and the CG, using ANCOVA and adjusting for possible confounders e.g. smoking status. Effects of the intervention during follow-up will be studied with an analysis of the variance of repeated measures.

Analysis by subgroups
The effect of the intervention could be influenced by age, sociocultural level and adherence to the study's chocolate intake. The same analyses described above will be performed for each of the subgroups above.

Secondary analyses

A multivariate multiple regression analysis will be performed to identify the variables with the greatest influence on blood pressure changes and the secondary variables analyzed.

Methodological limitations:

Due to the nature of the intervention, the participating subjects cannot be blinded. However, the researcher who analyses the data and the person who makes the measurements during follow-up visits will be blinded with respect to the group to which the participants belong. The smoking status in the 12 months prior to the time of inclusion could influence the outcome measures related to vascular function and blood pressure; therefore, although these participants will not be excluded, this aspect will be controlled in the statistical analysis. Assessment of the quality of life and lifestyles will be carried out through self-reported data; however, previously validated instruments will be used to obtain these. To make compliance with the intervention in the IG easier, IG participants will be provided with instructions on eating the chocolate and a calendar to record each intake.

ETHICS AND DISSEMINATION

Ethical considerations:

The study was approved by the Clinical Research Ethics Committee of the Salamanca Health Area ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The clinical trial has been registered at ClinicalTrials.gov with the identifier NCT03492983.

Participants must provide informed consent in accordance with the Declaration of Helsinki. Subjects will be informed of the objectives of the project and the risks and

benefits of the explorations to be carried out, including sample collection. None of the tests will pose risks that could endanger the lives of participants. Confidentiality of participant data will be guaranteed at all times in accordance with the provisions of the Organic Law on the Protection of Personal Data (15/1999 of December 13, LOPD), and under the conditions established by Law 14/2007 of biomedical research.

Dissemination plan:

The research group plans to achieve rapid and widespread dissemination of results to ensure maximum visibility of this study. To this end, the results of the study will be published in open-access scientific journals with peer review. At least one publication of the main results and others with the secondary results are planned. This will be complemented by the presentation of the results of the study at relevant scientific conferences and seminars of national and international scope. Also, a doctoral thesis based on this project will be prepared. Appropriate dissemination will likewise be carried out through social networks and other media. Moreover, given the involvement of a commercial product, the transfer to clinical practice is expected to be rapid if the results are as expected.

DISCUSSION

In recent years, there has been an increase in attention to polyphenols and their beneficial effects on health, with numerous studies being carried out to assess this.[19, 21] Similarly, the therapeutic use of these compounds has been suggested for certain diseases or population groups.[36, 62] The menopause increases the risk of developing cardiovascular disease compared to the previous period.[45] However, we have not found any study that assesses the effect of adding commercially available chocolate high in cocoa content to the usual diet in this population. Similarly, no studies have been found that evaluate the effects on cognitive performance, quality of life and

body composition of adding commercial chocolate with high cocoa content to the usual diet in postmenopausal women.

This work will provide novel data helpful for the development of strategies in the nutritional education of particularly vulnerable populations, given their high risk of developing cardiovascular disease, including non-pharmacological therapies and strategies that employ lifestyle modification. This intervention might also have implications for the preparation of recommendations in clinical practice guidelines and quality improvement programs aimed at the care of postmenopausal women.

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AUTHORS' CONTRIBUTIONS:

JIR, JAM, LGO and IGY contributed to the conception and design of the study. IGY, JIR and JAM prepared the manuscript of the study protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY contributed to the development of the study protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided assistance with statistical methodology and knowledge. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided a critical review of the manuscript.

All authors have read and accepted the final version of the protocol.

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COMPETING INTERESTS STATEMENT:

The authors declare that they have no conflicts of interest.

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FIGURE LEGEND:

Figure 1. Study flow chart.

Table 1. Polyphenol composition of 99% cocoa chocolate

Table 1. Polyphenols composition of 99% cocoa chocolate.

Compounds	Quantity
Protocatechuic acid (mg/g)	0.058 ± 0.008
Procyanidin dimer (B3) (mg/g)	0.176 ± 0.013
Catechin (mg/g)	1.035 ± 0.105
Procyanidin dimer (B2) (mg/g)	1.440 ± 0.055
Epicatechin (mg/g)	2.610 ± 0.075
Procyanidin trimer (C1) (mg/g)	0.853 ± 0.024
Procyanidin A hexoside (mg/g)	0.354 ± 0.007
Quercetin glucoside (mg/g)	0.002 ± 0.000
Quercetin arabinoside (mg/g)	0.003 ± 0.001

Figure 1. Study flow-chart

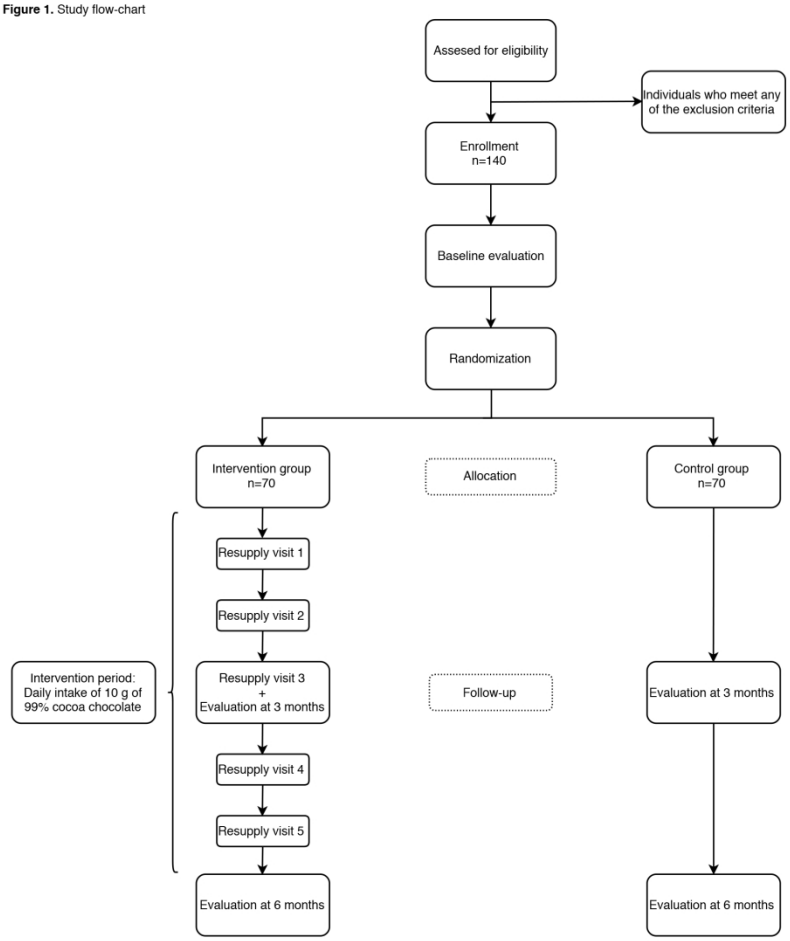

Figure 1

157x222mm (300 x 300 DPI)

SPIRIT
STANDARD PROTOCOL ITEMS: RECOMMENDATIONS FOR INTERVENTIONAL TRIALS

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	Pag 1, line 1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	Pag 2, line 48
	2b	All items from the World Health Organization Trial Registration Data Set	NA
Protocol version	3	Date and version identifier	NA
Funding	4	Sources and types of financial, material, and other support	Pag 25, line 723
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	Pag 25, line 714
	5b	Name and contact information for the trial sponsor	Pag 25, line 723
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	Pag 25, line 725
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	Pag 25, line 714

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	Pag 7, line 162
	6b	Explanation for choice of comparators	Pag 7, line 162
Objectives	7	Specific objectives or hypotheses	Pag 8, line 170
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	Pag 8, line 177
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	Pag 9, line 199
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Pag 8, line 190
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	Pag 10, line 2229
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	NA
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	Pag 10, line 240
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Pag 10, line 234
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	Pag 11, line 265
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Figure 1

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	Pag 9, line 208
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Pag 9, line 198
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	Pag 9, line 219
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	Pag 10, line 225
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Pag 9, line 219
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	Pag 16, line 407
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	Pag 16, line 407
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Pag 11, line 273
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	NA

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	Pag 16, line 401
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	Pag 17, line 424
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Pag 18, line 443
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	Pag 17, line 426
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	NA
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	NA
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	NA
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Pag 18, line 467
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	NA

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	Pag 18, line 472
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	Pag 19, line 475
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Pag 25, line 730
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	Pag 16, line 400
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	NA
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	Pag 19, line 481
	31b	Authorship eligibility guidelines and any intended use of professional writers	Pag 19, line 481
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Pag 19, line 481
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	NA
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	NA

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.