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The effect of adding chocolate with a high percentage of cocoa and polyphenols to a normal diet on blood pressure, vascular function, body composition, quality of life and cognitive performance in postmenopausal women. Randomized clinical trial. ECCAMP Study.

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

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1 **TITLE: The effect of adding chocolate with a high percentage of cocoa and**
2 **polyphenols to a normal diet on blood pressure, vascular function, body**
3 **composition, quality of life and cognitive performance in postmenopausal**
4 **women. Randomized clinical trial. ECCAMP Study.**

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For peer review only

1
2
3 31 **ABSTRACT**

4 32 **Introduction:** The intake of polyphenols has shown certain health benefits. The aim of
5
6 33 this study is to assess the effect of adding a daily amount of chocolate high in cocoa
7
8 34 content and polyphenols to the normal diet on blood pressure, vascular function,
9
10 35 cognitive performance, quality of life and body composition in postmenopausal women.

11
12 36 **Methods and analysis:** Randomized clinical trial with two parallel groups involving a
13
14 37 total of 140 women between 50 and 64 years of age in the postmenopausal period,
15
16 38 defined by amenorrhea of at least 12 consecutive months. The main variable will be the
17
18 39 change in blood pressure. Secondary variables will be changes in vascular function,
19
20 40 quality of life, cognitive performance and body composition. The intervention group will
21
22 41 be given chocolate containing 99% cocoa, with instructions to add 10 g daily to their
23
24 42 normal diet for 6 months. The daily nutritional contribution of this amount of chocolate
25
26 43 is 59 Kcal and 65.4 mg of polyphenols. There will be no intervention in the control
27
28 44 group. All variables will be measured at the baseline visit and at 3 and 6 months after
29
30 45 randomization, except cognitive performance and quality of life, to be assessed only at
31
32 46 baseline and at 6 months. Recruitment is scheduled to begin on June 1, 2018, and the
33
34 47 study will continue until May 31, 2019.

35
36 48 **Ethics and dissemination:** This study was approved by the Clinical Research Ethics
37
38 49 Committee of the Health Area of Salamanca, Spain ("CREC of Health Area of
39
40 50 Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The
41
42 51 clinical trial has been registered at ClinicalTrials.gov provided by the US National
43
44 52 Library of Medicine, number NCT03492983.

45
46 53 **Keywords:** Chocolate, postmenopause, arterial pressure, vascular stiffness, body
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48 54 composition, quality of life.
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3 57 **Strengths and limitations of this study:**

- 4
5 58 - The effect of commercially available chocolate high in cocoa and polyphenols on
6
7 59 the health of postmenopausal women is not known since most of the available
8
9 60 studies have used laboratory compounds prepared with high composition of these
10
11 61 substances.
12
13 62 - Blood pressure and vascular function will be measured objectively using a
14
15 63 sphygmomanometer and a Vasera VS-2000 device (Fukuda Denshi), with body
16
17 64 composition measured by impedance analysis, while quality of life and cognitive
18
19 65 performance will be assessed using validated instruments.
20
21 66 - Due to the nature of the intervention, the participants cannot be blinded, although
22
23 67 the researchers who perform the measurements and the statistical analysis will be
24
25 68 blinded.
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69 INTRODUCTION

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

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

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

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

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

1
2
3 97 associated with a dose-dependent improvement of vascular function, in particular of
4 98 arterial stiffness measured by pulse wave speed^{9 10 15}. However, this relationship is not
5
6 99 evident in people with mild hypertension when cardio-ankle vascular index (CAVI) is
7
8
9 100 used as a measure of arterial stiffness¹⁶.

10
11 101 There is also evidence of the influence of these polyphenols in reducing the
12
13 102 augmentation index (AIx). The study by West et al¹⁷, involving subjects with excess
14
15 103 weight and moderate obesity, concludes that the treatment with dark chocolate
16
17 104 decreases AIx in women, although it seems that this association may affect more the
18
19 105 elasticity of the large arteries, especially in subjects with obesity and diabetes mellitus
20
21 106 type 2¹⁸.

22
23 107 **Cocoa polyphenols and cognitive performance:** There is evidence to suggest that
24
25 108 chocolate rich in polyphenols may be beneficial for cognitive performance and state
26
27 109 since it improves mental processing speed and attenuates the increase of mental
28
29 110 fatigue among healthy young adults^{19 20}. An improvement in cognitive performance
30
31 111 among older age groups after eating chocolate has also been observed²¹. Several
32
33 112 studies also show that polyphenol-rich chocolate causes an improvement in executive
34
35 113 function and categorical fluency²², in working memory^{23 24}, and a slowing of mental
36
37 114 fatigue²⁵. Furthermore, a positive influence of cocoa polyphenols on physiological
38
39 115 processes has been reported, with a neuroprotective effect²⁶ and improved cognitive
40
41 116 performance²⁷.

42
43 117 **Cocoa polyphenols and quality of life:** The quality of life linked to health is
44
45 118 represented by the individual's perception of well-being in various aspects of life,
46
47 119 including physical and mental aspects. The effect of chocolate and polyphenols on the
48
49 120 quality of life has scarcely been studied, with not a great deal of evidence available and
50
51 121 even less of a conclusive nature. In a study conducted among healthy people, where
52
53 122 regular consumption of chocolate was recorded over a year, no evidence was found of
54
55 123 a clear association between chocolate intake and the physical or mental components of

1
2
3 124 quality of life ²⁸. Nevertheless, it has been observed that the consumption of dark
4
5 125 chocolate may be beneficial for the quality of life of women with fibromyalgia ²⁹.

6
7 126 **Cocoa polyphenols and body composition:** The menopause period leads to various
8
9 127 changes in the body composition of women ³⁰. Regarding the connection between
10
11 128 cocoa polyphenols and body composition, results diverge. Some clinical trials involving
12
13 129 healthy people and overweight or obese patients have not reported significant
14
15 130 differences that link chocolate consumption to anthropometric measures ^{12 16 17 31}. Other
16
17 131 studies indicate that chocolate consumption may have positive effects on body
18
19 132 composition in adolescents ³², patients with diabetes ³³ or women with obesity ³⁴. Two
20
21 133 recent systematic reviews also indicate that eating chocolate is associated with
22
23 134 reduced body mass index (BMI) and waist circumference ^{35 36}, and one of them also
24
25 135 concludes that the amount and the length of time during which it is eaten play a key
26
27 136 role in these beneficial effects ³⁶. Conversely, other studies such as that carried out
28
29 137 with the cohort of the Atherosclerosis Risk in Communities (ARIC) study have observed
30
31 138 a dose-dependent increase in weight after habitual chocolate consumption ³⁷.

32
33 139 In sum, the polyphenols present in chocolate seem to have a positive effect on BP,
34
35 140 vascular function, cognitive performance and quality of life, especially in populations
36
37 141 with increased cardiovascular risk such as postmenopausal women ³⁸. However, the
38
39 142 conflicting results obtained in different studies suggest that the real contribution of
40
41 143 these compounds to health and the underlying mechanisms remain unclear. Moreover,
42
43 144 most of these studies have used preparations with high concentrations of polyphenols
44
45 145 that are usually not present in the normal diet.

46
47 146 This study aims to evaluate the effect of adding a daily amount of 10 g of chocolate
48
49 147 high in cocoa content (99%) and polyphenols to the normal diet on blood pressure,
50
51 148 vascular function, cognitive performance, quality of life and body composition in
52
53 149 postmenopausal women.

54 150 **METHODS AND ANALYSIS**

55 56 151 **Design and setting:**

1
2
3 152 This controlled and randomized clinical trial involves two parallel groups. The study will
4
5 153 be carried out in the Research Unit of the La Alamedilla Health Centre in Salamanca
6
7 154 (Spain), which is part of the Biomedical Research Institute of Salamanca (IBSAL) and
8
9 155 the Primary Care Prevention and Health Promotion Research Network (REDIAPP).
10
11 156 The recruitment schedule is set to start on June 1, 2018, and the study will run until
12
13 157 May 31, 2019. There will be a baseline assessment and two follow-ups, at 3 and 6
14
15 158 months.

16
17 159 **Study population:**

18
19 160 Those subjects who meet the selection criteria and sign the informed consent after
20
21 161 receiving information about the objectives and implementation of the study will take
22
23 162 part.

24
25 163 Inclusion criteria: women between 50 and 64 years of age in postmenopause, defined
26
27 164 by and checked against amenorrhea during at least 12 consecutive months.

28
29 165 Exclusion criteria: personal history of cardiovascular disease; personal history of
30
31 166 diabetes mellitus, arterial hypertension or dyslipidemia under pharmacological
32
33 167 treatment; hypocaloric diets; clinically demonstrable neurological and/or
34
35 168 neuropsychological disease; treatment with hormone replacement therapy; intolerance
36
37 169 and/or allergy to cocoa or any of the components of the supplement.

38
39 170 Participants will be selected using a consecutive sample of women who meet the
40
41 171 selection criteria in the GP surgeries of four urban primary care centres in Salamanca,
42
43 172 from June 1, 2018.

44
45 173 **Sample size:**

46
47 174 The size of the sample has been estimated based on the potential modification of the
48
49 175 main variable, systolic blood pressure (SBP). Given alpha and beta risks of 0.05 and
50
51 176 0.20 respectively in bilateral contrast and a standard deviation (SD) of 5.8 mmHg, 140
52
53 177 participants (70 per group) will be necessary to detect a minimum difference of 2.9
54
55 178 mmHg in the SBP between the two groups. A predicted drop-out rate of 10% during
56
57 179 follow-up has been taken into account. This estimate has considered the results

1
2
3 180 obtained in a similar study in which a decrease in SBP of 6.5 was observed \pm 5.8
4
5 181 mmHg¹⁰.

6
7 182 **Randomization:**

8
9 183 Participants will be assigned to the intervention group (IG) or control group (CG) at
10
11 184 random. The allocation sequence will be generated by an independent researcher
12
13 185 using the Epidat 4.2 program³⁹ and will remain hidden until the participants are
14
15 186 assigned to each group.

16
17 187 **Intervention:**

18
19 188 No type of intervention will be carried out with the CG participants.

20
21 189 IG participants will be given chocolate with 99% cocoa content and asked to eat 10 g
22
23 190 daily for a period of 6 months. Participants will also be given instructions on eating and
24
25 191 keeping the product, with the recommendation, for example, that the daily chocolate
26
27 192 intake be eaten at the same time. In addition, they will be given a calendar on which to
28
29 193 record the time it was eaten each day. This calendar will be returned to the researchers
30
31 194 at each replenishment visit.

32
33 195 This amount of chocolate provides the following daily nutritional contribution: 59 Kcal,
34
35 196 0.8 g of carbohydrates, 1.5 g of protein, 5.1 g of fat, of which 3.1 g are saturated fats.
36
37 197 The proportion of polyphenols per 10 g is 65.4 mg. The polyphenolic profile of this
38
39 198 compound can be seen in table 1. On each visit, IG participants will receive the amount
40
41 199 of chocolate they need until the next replenishment visit. In addition to the baseline
42
43 200 visit, there will be 5 replenishment visits in months 1, 2, 3 (coinciding with the
44
45 201 evaluation visit), 4 and 5. The sole purpose of the replenishment visits will be to supply
46
47 202 the amount of chocolate needed until the next visit, without any other intervention being
48
49 203 carried out.

50
51 204 Participants in both groups will be instructed to continue with the dietary pattern they
52
53 205 usually follow, without changing their eating habits during the study period.

54
55 206 **Procedures:**

1
2
3 207 For each participant a baseline visit and two follow-up visits at 3 and 6 months after the
4
5 208 initial one are scheduled (Figure 1). The IG will also make 5 replenishment visits, in
6
7 209 months 1, 2, 3 (coinciding with the first follow-up visit), 4 and 5. In the replenishment
8
9 210 visits they will be given the amount of chocolate needed until the next visit and will
10
11 211 hand in the calendar with the record of the chocolate eaten.

12 **Primary and secondary endpoints:**

13
14 213 The primary variable will be the decrease in clinical BP, measured with a digital
15
16 214 sphygmomanometer. Secondary variables will include vascular function, quality of life,
17
18 215 cognitive performance and body composition.

19
20 216 All variables will be measured at 3 and 6 months after randomization, except for
21
22 217 cognitive performance and quality of life, to be assessed only after 6 months.

23
24 **Blood pressure:**

25
26 219 Clinical systolic and diastolic blood pressure will be measured with a validated Omron
27
28 220 M10-IT sphygmomanometer (Omron Healthcare, Kyoto, Japan). Three measurements
29
30 221 will be taken in the dominant arm of the subject in a sitting position after at least 5
31
32 222 minutes of rest with an appropriately sized cuff, following the recommendations of the
33
34 223 European Society of Hypertension ⁴⁰. The average of the last two measurements will
35
36 224 be recorded.

37
38 **Vascular function:**

39
40 226 The Vasera VS-2000 device (Fukuda Denshi) will be used to measure the CAVI and
41
42 227 the brachial-ankle pulse wave velocity (ba-PWV) at rest. CAVI is a good indicator of
43
44 228 arterial stiffness, providing an accurate estimate of the degree of atherosclerosis
45
46 229 without depending on blood pressure ⁴¹. $CAVI \geq 9$ and $ba-PWV \geq 18.3$ will be
47
48 230 considered pathological ⁴². Pathological CAVI is representative of subclinical
49
50 231 atherosclerosis ⁴³.

51
52 **Cognitive performance:**

53
54 233 Attention and executive functions: Trail Making Test A will be used to measure
55
56 234 attention and Trail Making Test B for processing speed and executive functions ⁴⁴.

1
2
3 235 Immediate verbal memory will be assessed with the Rey Auditory Verbal Learning
4 236 Test. The immediate recall of a list of 15 words is measured in three attempts, followed
5
6 237 by delayed verbal memory through the free recall of the words learned in the first part
7
8 238 of the test after 10 minutes ⁴⁵.

9
10 239 Working memory will be assessed with the WAIS Digit Span Backward test ⁴⁶.

11
12 240 Phonological fluency will be explored by naming as many words as possible starting
13
14 241 with different letters of the FAS Questionnaire in the space of one minute ⁴⁷.

15
16 242 Categorical fluency measures verbal semantic fluency and will be assessed by naming
17
18 243 as many animals as possible in one minute ⁴⁸.

19
20 244 **Quality of life:**

21
22 245 The quality of life linked to health will be assessed through the EuroQol 5-D
23
24 246 questionnaire (EQ-5D). We will use the adapted Spanish version of this questionnaire,
25
26 247 which has been validated in the Spanish population ⁴⁹. This questionnaire consists of
27
28 248 three elements: the assessment by the individuals of their state of health in level of
29
30 249 severity by dimension (mobility, personal care, daily activities, pain/discomfort and
31
32 250 anxiety/depression), the assessment of their state of health on an analogue visual
33
34 251 scale, and finally an index of social values obtained for each state of health generated
35
36 252 by the instrument.

37
38 253 The quality of life will also be studied using the Cervantes Scale ⁵⁰. This questionnaire
39
40 254 is specifically designed for menopause and postmenopause and has been validated for
41
42 255 Spanish women. Its 31 structured items cover the 4 dimensions of menopause:
43
44 256 menopause and health, sexuality, psychic domain and relationships.

45
46 257 **Body composition:**

47
48 258 Body composition will be measured with the Inbody 230 Monitor ⁵¹. This analyzer
49
50 259 provides data on skeletal muscle mass, fat mass, total body water, fat-free mass,
51
52 260 percentage of body fat, waist-hip ratio, basal metabolism, and also a segmental
53
54 261 analysis.

1
2
3 262 Body weight will be measured twice with an electronic scale (Scale 7830, Soehnle
4
5 263 Professional GmbH & Co, Backnang, Germany) after proper calibration (accuracy ± 0.1
6
7 264 kg). Height will be measured by recording the average of two readings rounded to the
8
9 265 nearest centimetre using a stadiometer (Seca 222, Medical scale and measurement
10
11 266 system, Birmingham, UK). Both measurements will be made with the subject barefoot
12
13 267 and wearing light clothing. Body mass index will be calculated by dividing weight (kg)
14
15 268 by height squared (m^2). Waist circumference will be assessed in accordance with the
16
17 269 recommendations of the Spanish Society for the Study of Obesity (SEEDO)⁵² and will
18
19 270 be measured in duplicate before and after inhalation, using a flexible tape parallel to
20
21 271 the floor, at the level of the mid-point between the lowest rib and the iliac crest, with the
22
23 272 subject standing up and without clothes.

273 **Other variables**

274 **Clinical and sociodemographic variables**

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

285 **Evaluation of chocolate consumption and habitual diet**

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

1
2
3 289 Nutritional habits will be assessed by a 24-hour log on 3 non-consecutive days prior to
4
5 290 each visit.

6 291 **Evaluation of other lifestyles**

7
8 292 The use of tobacco will be assessed with a questionnaire on the personal history and
9
10 293 pattern of smoking.

11
12 294 Alcohol use will be recorded with a questionnaire covering the previous 7 days which
13
14 295 will include specific beverages and the amount by volume drunk of each.

15
16 296 Physical activity will be measured using the International Physical Activity
17
18 297 Questionnaire (IPAQ) in its short version and validated in Spanish⁵³. This
19
20 298 questionnaire measures activity over the previous 7 days, classifying the subjects
21
22 299 according to three activity levels (low, moderate and high) with respect to three types of
23
24 300 activities: walking, moderate-intensity activities and vigorous-intensity activities. The
25
26 301 amount of physical exercise will be estimated in METs-minute/week.

27 302 **Evaluation of laboratory variables**

28
29 303 At baseline and follow-up visits at 6 months, we will measure plasma fasting glucose
30
31 304 values (mg/dL), glycated haemoglobin (%), total cholesterol (mg/dL), total triglycerides
32
33 305 (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), creatinine (mg/L),
34
35 306 insulinemia (mg/dL). Creatinine in urine (mg/dL) and microalbuminuria (mg/dL) will also
36
37 307 be measured.

38
39 308 Insulin resistance will be determined using the HOMA index (Homeostasis Model
40
41 309 Assessment Insulin Resistance) estimated using the following equation: Fasting
42
43 310 glucose (mmol/l) X insulin (mU/ml)/22.5.

44 311 **Blinding strategy:**

45
46 312 Due to the nature of the intervention itself, the participants and the person responsible
47
48 313 for delivering the chocolate to IG participants, cannot be blinded. However, the person
49
50 314 responsible for carrying out the study measurements at each visit and for the statistical
51
52 315 analysis will be blind to the intervention.

1
2
3 316 **Statistical analysis:**

4
5 317 **General analysis**

6
7 318 Results for the quantitative variables will be expressed by mean \pm standard deviation or
8
9 319 by frequency distribution in the case of qualitative ones. The normality of the variables
10
11 320 will be assessed using the Kolmogorov-Smirnov test. In cases where a normal
12
13 321 distribution cannot be assumed, the corresponding nonparametric tests will be applied.
14
15 322 The association between independent qualitative variables will be analyzed by means
16
17 323 of the chi-square test or Fisher's exact test. The means between the two groups will be
18
19 324 compared using the Student's t test or the Mann-Whitney U test, and the Pearson or
20
21 325 Spearman correlation coefficients will be calculated to analyze the relationship between
22
23 326 quantitative variables.

24
25 327 The analysis of the results for the main variable and the secondary variables will be
26
27 328 carried out by intention to treat. In addition, a secondary analysis will be run taking into
28
29 329 account chocolate intake adherence (< 50% days and > 50% days) and other relevant
30
31 330 subgroups in relation to their physical activity or previous chocolate consumption.

32
33 331 All analyses will be performed with the SPSS version 23.0 (IBM Corporation, Armonk,
34
35 332 NY, USA) and an alpha risk of 0.05 will be set as the limit of statistical significance.

36
37 333 **Analysis of the intervention's effect on primary and secondary outcomes.**

38
39 334 To analyze the changes at 3 and 6 months from baseline in the primary outcome
40
41 335 (blood pressure) and in the secondary outcomes within the same group, the Student's t
42
43 336 test for paired data or the Wilcoxon test will be used. The McNemar test will be applied
44
45 337 with quantitative or dichotomous variables.

46
47 338 Effects of the intervention will be analyzed in a comparison of the changes in blood
48
49 339 pressure and the secondary variables between the IG and the CG, using ANCOVA and
50
51 340 adjusting for possible confounders. Effects of the intervention during follow-up will be
52
53 341 studied with an analysis of the variance of repeated measures.

54
55 342 **Analysis by subgroups.**

1
2
3 343 The effect of the intervention could be influenced by age, sociocultural level and
4 344 adherence to the study's chocolate intake. The same analyses described above will be
5
6 345 performed for each of the aforementioned subgroups.
7

8
9 346 **Secondary analyses.**

10 347 A multivariate multiple regression analysis will be performed to identify the variables
11
12 348 with the greatest influence on blood pressure changes and the secondary variables
13
14 349 analyzed.
15

16
17 350 **Methodological limitations:**

18 351 Due to the nature of the intervention, the participating subjects cannot be blinded.
19
20 352 However, the researcher who analyses the data and the person who makes the
21
22 353 measurements during follow-up visits will be blinded with respect to the group to which
23
24 354 the participants belong.
25

26 355 Assessment of the quality of life and lifestyles will be carried out through self-reported
27
28 356 data; however, previously validated instruments will be used to obtain these. To make
29
30 357 compliance with the intervention in the IG easier, IG participants will be provided with
31
32 358 instructions on eating the chocolate and a calendar to record each intake.
33

34 359 **ETHICS AND DISSEMINATION**

35
36 360 **Ethical considerations:**

37
38 361 The study was approved by the Clinical Research Ethics Committee of the Salamanca
39
40 362 Health Area ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT
41
42 363 checklist is available for this protocol. The clinical trial has been registered at
43
44 364 ClinicalTrials.gov with the identifier NCT03492983.
45

46 365 Participants must sign informed consent in accordance with the Declaration of Helsinki.
47
48 366 Subjects will be informed of the objectives of the project and the risks and benefits of
49
50 367 the explorations to be carried out, including sample collection. None of the tests will
51
52 368 pose risks that could endanger the lives of participants. Confidentiality of participant
53
54 369 data will be guaranteed at all times in accordance with the provisions of the Organic
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1
2
3 370 Law on the Protection of Personal Data (15/1999 of December 13, LOPD), and under
4
5 371 the conditions established by Law 14/2007 of biomedical research.

6
7 372 **Dissemination plan:**

8
9 373 The research group plans to achieve rapid and widespread dissemination of results to
10
11 374 ensure maximum visibility of this study. To this end, the results of the study will be
12
13 375 published in scientific journals with peer review. At least one publication of the main
14
15 376 results and others with the secondary results are planned. This will be complemented
16
17 377 by presentation of the results of the study at relevant scientific conferences and
18
19 378 seminars of national and international scope. In addition, a doctoral thesis based on
20
21 379 this project will be prepared. Appropriate dissemination will likewise be carried out
22
23 380 through social networks and other media. Moreover, given the involvement of a
24
25 381 commercial product, the transfer to clinical practice is expected to be very fast if the
26
27 382 results are as expected.

28
29 383 **DISCUSSION**

30
31 384 In recent years, there has been an increase in attention to polyphenols and their
32
33 385 beneficial effects on health, with numerous studies being carried out to assess this ^{15 17}.
34
35 386 Similarly, the therapeutic use of these compounds has been suggested for certain
36
37 387 diseases or population groups ^{29 54}. The menopause increases the risk of developing
38
39 388 cardiovascular disease compared to the previous period ³⁸. However, we have not
40
41 389 found any study that assesses the effect of adding commercially available chocolate
42
43 390 high in cocoa content to the usual diet in this population at special risk. Similarly, no
44
45 391 studies have been found that evaluate the effects on cognitive performance, quality of
46
47 392 life and body composition of adding commercial chocolate with high cocoa content to
48
49 393 the usual diet in postmenopausal women.

50
51 394 The results of this work will provide new evidence in this regard for the development of
52
53 395 strategies in nutritional education of particularly vulnerable populations, given their high
54
55 396 risk of developing cardiovascular disease, including non-pharmacological therapies and
56
57 397 strategies that employ lifestyle modification. This intervention may also have

1
2
3 398 implications for the preparation of recommendations in clinical practice guidelines and
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5 399 quality improvement programs aimed at the care of postmenopausal women.
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For peer review only

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31 577 **AUTHORS' CONTRIBUTIONS:**

32
33 578 JIR, JAM, LGO and IGY contributed to the conception and design of the study. IGY,
34
35 579 JIR and JAM prepared the manuscript of the study protocol. JIR, JAM, LGO, RAD,
36
37 580 SMS, JGS, SMG, ERS, MGM and IGY contributed to the development of the study
38
39 581 protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided
40
41 582 assistance with statistical methodology and knowledge. JIR, JAM, LGO, RAD, SMS,
42
43 583 JGS, SMG, ERS, MGM and IGY provided a critical review of the manuscript.
44
45 584 All authors have read and accepted the final version of the protocol.
46

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48
49 586 This study was supported in part by grants funded by la Gerencia Regional de Castilla
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51 587 y León (GRS 1583/B/17).
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3 588 “Lindt & Sprüngli will provide the necessary chocolate for the implementation of the
4
5 589 study. This company will not play any role in the design of the study, data analysis,
6
7 590 reporting of results, or the decision to present the manuscript for publication”.

8
9 591 **COMPETING INTERESTS STATEMENT:**

10 592 The authors declare that they have no conflicts of interest.

11
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21
22 598 Lugones-Sánchez, Benigna Sánchez-Salgado, Carmen Castaño-Sánchez, Emiliano
23
24 599 Rodríguez-Sánchez, Susana González-Manzano, Olaya Tamayo-Morales, Susana
25
26 600 González-Sánchez.

27
28 601 **FIGURE LEGEND:**

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30 602 Figure 1. Study flow chart

31
32 603 Table 1. Polyphenol composition of 99% cocoa chocolate

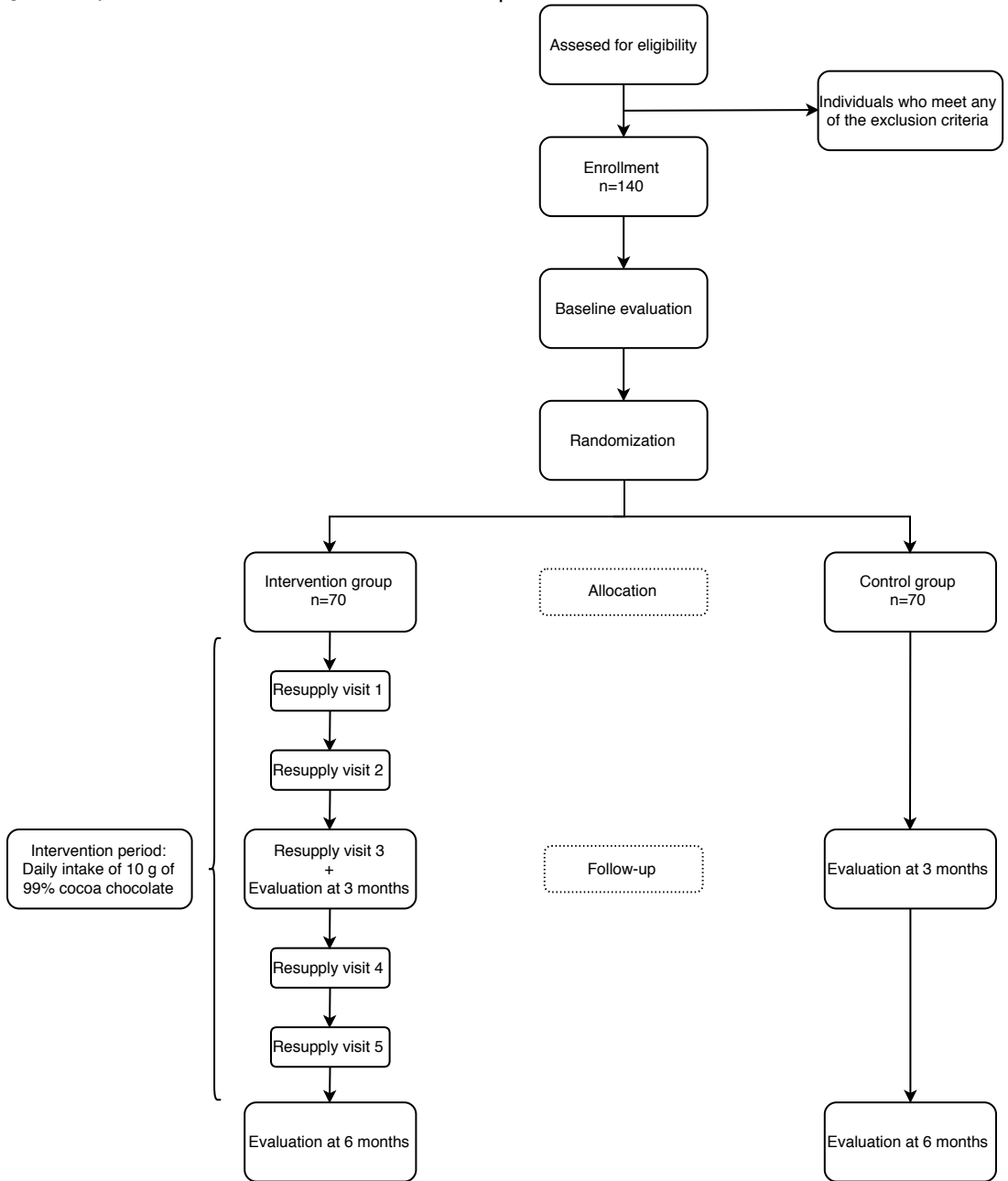
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605 **Table 1.** Polyphenols composition of 99% cocoa chocolate.

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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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

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47**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

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2				
3	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
4				
5	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	8
6				
7				

8 **Methods: Assignment of interventions (for controlled trials)**

9 Allocation:

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11				
12	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
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17	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
18				
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21	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	9
22				
23				
24	Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	13
25				
26				
27		17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	13
28				
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31 **Methods: Data collection, management, and analysis**

32				
33	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
34				
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38		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
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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



1				
2				
3	Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	15
4				
5				
6		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
7				
8	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
9				
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11	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	22
12				
13				
14	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
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16				
17	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
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20	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
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25		31b	Authorship eligibility guidelines and any intended use of professional writers	16
26				
27		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	16
28				
29	Appendices			
30				
31	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	NA
32				
33				
34	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
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36				

37 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.
 38 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons
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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

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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.

AUTHORS:

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3 29 **ABSTRACT**

4 30 **Introduction:** The intake of polyphenols has shown certain health benefits. The aim of
5
6 31 this study is to assess the effect of adding a daily amount of chocolate high in cocoa
7
8 32 content and polyphenols to the normal diet on blood pressure, vascular function,
9
10 33 cognitive performance, quality of life and body composition in postmenopausal women.

11
12 34 **Methods and analysis:** Randomized clinical trial with two parallel groups involving a
13
14 35 total of 140 women between 50 and 64 years of age in the postmenopausal period,
15
16 36 defined by amenorrhea of at least 12 consecutive months. The main variable will be the
17
18 37 change in blood pressure. Secondary variables will be changes in vascular function,
19
20 38 quality of life, cognitive performance and body composition. The intervention group will
21
22 39 be given chocolate containing 99% cocoa, with instructions to add 10 g daily to their
23
24 40 normal diet for 6 months. The daily nutritional contribution of this amount of chocolate
25
26 41 is 59 Kcal and 65.4 mg of polyphenols. There will be no intervention in the control
27
28 42 group. All variables will be measured at the baseline visit and at 3 and 6 months after
29
30 43 randomization, except cognitive performance and quality of life, to be assessed only at
31
32 44 baseline and at 6 months. Recruitment is scheduled to begin on June 1, 2018, and the
33
34 45 study will continue until May 31, 2019.

35
36 46 **Ethics and dissemination:** This study was approved by the Clinical Research Ethics
37
38 47 Committee of the Health Area of Salamanca, Spain ("CREC of Health Area of
39
40 48 Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The
41
42 49 clinical trial has been registered at ClinicalTrials.gov provided by the US National
43
44 50 Library of Medicine, number NCT03492983. The results will be disseminated through
45
46 51 open access peer-reviewed journals, conference presentations, broadcast media as
47
48 52 well as presentation to stakeholders.

49
50 53 **Keywords:** Chocolate, postmenopause, arterial pressure, vascular stiffness, body
51
52 54 composition, quality of life.

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3 57 **Strengths and limitations of this study:**

- 4
5 58 - This study used a commercially available chocolate with high content of cocoa and
6
7 59 polyphenols during the intervention.
8
9 60 - Blood pressure and vascular function will be measured objectively using a
10
11 61 sphygmomanometer and a Vasera VS-2000 device (Fukuda Denshi), with body
12
13 62 composition measured by impedance analysis, while quality of life and cognitive
14
15 63 performance will be assessed using validated instruments.
16
17 64 - Due to the nature of the intervention, the participants cannot be blinded, although
18
19 65 the researchers who perform the measurements and the statistical analysis will be
20
21 66 blinded.

22 67 **INTRODUCTION**

23
24 68 Polyphenols are bioactive compounds found in many plants, fruits and vegetables. The
25
26 69 beneficial effects on human health associated with the consumption of a diet rich in
27
28 70 polyphenols has generated great scientific interest in these substances.[1-3] The action
29
30 71 of polyphenols is based on their antioxidant capacity through the uptake of free
31
32 72 radicals, the chelation of metals with redox properties and the modulation and inhibition
33
34 73 of enzymatic activity.[4]

35
36
37 74 The most abundant polyphenols in cocoa are flavonoids, which have been linked to a
38
39 75 protective effect against cardiovascular disease, decreasing the risk of cardiovascular
40
41 76 morbidity and mortality and favouring the prevention of other chronic diseases such as
42
43 77 diabetes mellitus type 2.[1-3, 5-7] The ability to reduce cardiovascular risk could be due
44
45 78 to an improvement in the elements that define metabolic syndrome, the improvement of
46
47 79 vascular endothelial dysfunction, insulin resistance and the inhibition of platelet
48
49 80 activation and aggregation.[8, 9] However, although current evidence suggests that
50
51 81 polyphenols produce an improvement in cardiovascular health, it is not enough to
52
53 82 determine the minimum amount of intake necessary to achieve health benefits.[10]

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2
3 83 **Cocoa polyphenols and blood pressure:** The effect of consuming polyphenols
4
5 84 present in chocolate on the blood pressure (BP) statistics of healthy individuals is not
6
7 85 clear. Cocoa consumption has been associated with an improvement in endothelial
8
9 86 function and a decrease in blood pressure in both healthy subjects and those with risk
10
11 87 factors and cardiovascular diseases.[11, 12] Some studies have observed a dose-
12
13 88 dependent relationship between cocoa intake and clinical BP, with higher consumption
14
15 89 equated to lower blood pressure and better vascular function.[13, 14] Conversely, other
16
17 90 research has not obtained significant changes in these parameters related to the
18
19 91 supplementation of cocoa or pure polyphenols such as epicatechin or quercetin.[15,
20
21 92 16]

22
23 93 Endothelial dysfunction in postmenopausal women causes changes that favour the
24
25 94 development of cardiovascular risk factors and atherosclerosis, which lead to the
26
27 95 appearance and maintenance of hypertension.[17, 18] A decrease in BP has been
28
29 96 observed in this group after daily consumption of cocoa with a flavonol content of 40.12
30
31 97 mg. Below this level, however, no changes have been observed.[19]

32
33
34 98 **Cocoa polyphenols and vascular function:** Among healthy individuals as well as
35
36 99 postmenopausal women, the consumption of polyphenols present in cocoa has been
37
38 100 associated with a dose-dependent improvement of vascular function, in particular of
39
40 101 arterial stiffness measured by pulse wave speed.[13, 14, 19] One of these studies also
41
42 102 suggests that the reduction in arterial stiffness observed in postmenopausal women
43
44 103 after consumption of cocoa is independent of the frequency of the intake.[19] However,
45
46 104 this relationship is not evident in people with mild hypertension when cardio-ankle
47
48 105 vascular index (CAVI) is used as a measure of arterial stiffness.[20]

49
50 106 There is also evidence of the influence of these polyphenols in reducing the
51
52 107 augmentation index (AIx). The study by West et al,[21] involving subjects with excess
53
54 108 weight and moderate obesity, concludes that the treatment with dark chocolate
55
56 109 decreases AIx in women, although it seems that this association may affect more the

1
2
3 110 elasticity of the large arteries, especially in subjects with obesity and diabetes mellitus
4
5 111 type 2.[22]

6
7 112 **Cocoa polyphenols and cognitive performance:** There is evidence to suggest that
8
9 113 chocolate rich in polyphenols may be beneficial for cognitive performance and state
10
11 114 since it improves mental processing speed and attenuates the increase of mental
12
13 115 fatigue among healthy young adults.[23, 24] An improvement in cognitive performance
14
15 116 among older age groups after eating chocolate has also been observed[25] and
16
17 117 especially in subjects with higher risk of cardiovascular disease.[26] Several studies
18
19 118 also show that polyphenol-rich chocolate causes an improvement in executive function
20
21 119 and categorical fluency,[27] in working memory,[28, 29] and a slowing of mental
22
23 120 fatigue[30] and also that a higher frequency of chocolate consumption has been
24
25 121 associated with better cognitive function.[29] Furthermore, a positive influence of cocoa
26
27 122 polyphenols on physiological processes has been reported, with a neuroprotective
28
29 123 effect[31] and improved cognitive performance.[32] In this regard, it has been
30
31 124 suggested that the brain-derived neurotrophic factor (BDNF) may play a role in the
32
33 125 cognitive enhancement induced by the flavonoides.[33] Favorable effects on
34
35 126 cerebrovascular function have also been observed in postmenopausal women after
36
37 127 consumption of chocolate with high concentration of cocoa.[34]

38
39 128 **Cocoa polyphenols and quality of life:** The quality of life linked to health is
40
41 129 represented by the individual's perception of well-being in various aspects of life,
42
43 130 including physical and mental aspects. The effect of chocolate and polyphenols on the
44
45 131 quality of life has scarcely been studied, with not a great deal of evidence available and
46
47 132 even less of a conclusive nature. In a study conducted among healthy people, where
48
49 133 regular consumption of chocolate was recorded over a year, no evidence was found of
50
51 134 a clear association between chocolate intake and the physical or mental components of
52
53 135 quality of life.[35] Nevertheless, it has been observed that the consumption of dark
54
55 136 chocolate may be beneficial for the quality of life of women with fibromyalgia.[36]

1
2
3 137 **Cocoa polyphenols and body composition:** The menopause period leads to various
4
5 138 changes in the body composition of women.[37] Regarding the connection between
6
7 139 cocoa polyphenols and body composition, results diverge. Some clinical trials involving
8
9 140 healthy people and overweight or obese patients have not reported significant
10
11 141 differences that link chocolate consumption to anthropometric measures.[16, 20, 21,
12
13 142 38] Other studies indicate that chocolate consumption may have positive effects on
14
15 143 body composition in adolescents,[39] patients with diabetes[40] or women with
16
17 144 obesity.[41] Two recent systematic reviews also indicate that eating chocolate is
18
19 145 associated with reduced body mass index (BMI) and waist circumference,[42, 43] and
20
21 146 one of them also concludes that the amount and the length of time during which it is
22
23 147 eaten play a key role in these beneficial effects.[43] Conversely, other studies such as
24
25 148 that carried out with the cohort of the Atherosclerosis Risk in Communities (ARIC)
26
27 149 study have observed a dose-dependent increase in weight after habitual chocolate
28
29 150 consumption.[44]

30
31 151 In sum, the polyphenols present in chocolate seem to have a positive effect on BP,
32
33 152 vascular function, cognitive performance and quality of life, especially in populations
34
35 153 with increased cardiovascular risk such as postmenopausal women.[45] However, the
36
37 154 conflicting results obtained in different studies suggest that the real contribution of
38
39 155 these compounds to health and the underlying mechanisms remain unclear. Moreover,
40
41 156 most of these studies have used preparations with high concentrations of polyphenols
42
43 157 that are usually not present in the normal diet.

44
45 158 This study aims to evaluate the effect of adding a daily amount of 10 g of chocolate
46
47 159 high in cocoa content (99%) and polyphenols to the normal diet on blood pressure,
48
49 160 vascular function, cognitive performance, quality of life and body composition in
50
51 161 postmenopausal women.

52 53 54 162 **METHODS AND ANALYSIS**

55 56 163 **Design and setting:**

1
2
3 164 This controlled and randomized clinical trial involves two parallel groups. The study will
4
5 165 be carried out in the Research Unit of the La Alamedilla Health Centre in Salamanca
6
7 166 (Spain), which is part of the Biomedical Research Institute of Salamanca (IBSAL) and
8
9 167 the Primary Care Prevention and Health Promotion Research Network (REDIAPP).
10
11 168 The recruitment schedule is set to start on June 1, 2018, and the study will run until
12
13 169 May 31, 2019. There will be a baseline assessment and two follow-ups, at 3 and 6
14
15 170 months.

171 **Study population:**

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

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

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

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

185 **Patient and public involvement**

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

189 **Sample size:**

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

1
2
3 192 0.20 respectively in bilateral contrast and a standard deviation (SD) of 5.8 mmHg, 140
4
5 193 participants (70 per group) will be necessary to detect a minimum difference of 2.9
6
7 194 mmHg in the SBP between the two groups. A predicted drop-out rate of 10% during
8
9 195 follow-up has been taken into account. This estimate has considered the results
10
11 196 obtained in a similar study in which a decrease in SBP of 6.5 was observed \pm 5.8
12
13 197 mmHg.[14]

14
15 198 **Randomization:**

16
17 199 Participants will be assigned to the intervention group (IG) or control group (CG) at
18
19 200 random. The allocation sequence will be generated by an independent researcher
20
21 201 using the Epidat 4.2 program[46] and will remain hidden until the participants are
22
23 202 assigned to each group.

24
25 203 **Intervention:**

26
27 204 No type of intervention will be carried out with the CG participants.

28
29 205 IG participants will be given chocolate with 99% cocoa content and asked to eat 10 g
30
31 206 daily for a period of 6 months. According to the EFSA (European Food Safety
32
33 207 Authority), 10 g of high-flavanol dark chocolate consumed in the context of a balanced
34
35 208 diet could help maintain endothelium-dependent vasodilation.[47] Participants will also
36
37 209 be given instructions on eating and keeping the product, with the recommendations, for
38
39 210 example, that the chocolate can be consumed in small pieces leaving them unmated in
40
41 211 the mouth, without chewing them. In addition, a series of recommendations will be
42
43 212 given remembering the organoleptic characteristics of the product, as well as the
44
45 213 recommendations of trying to consume the product at the same time or refrain from
46
47 214 ingesting it dissolved in milk. In addition, they will be given a calendar on which to
48
49 215 record the time it was eaten each day. This calendar will be returned to the researchers
50
51 216 at each replenishment visit.

52
53 217 This amount of chocolate provides the following daily nutritional contribution: 59 Kcal,
54
55 218 0.8 g of carbohydrates, 1.5 g of protein, 5.1 g of fat, of which 3.1 g are saturated fats.

56
57 219 The proportion of polyphenols per 10 g is 65.4 mg. The polyphenolic profile of this

1
2
3 220 compound can be seen in table 1. On each visit, IG participants will receive the amount
4
5 221 of chocolate they need until the next replenishment visit. In addition to the baseline
6
7 222 visit, there will be 5 replenishment visits in months 1, 2, 3 (coinciding with the
8
9 223 evaluation visit), 4 and 5. The sole purpose of the replenishment visits will be to supply
10
11 224 the amount of chocolate needed until the next visit, without any other intervention being
12
13 225 carried out.

14
15 226 Participants in both groups will be instructed to continue with the dietary pattern they
16
17 227 usually follow, without changing their eating habits during the study period.

18
19 228 **Procedures:**

20
21 229 For each participant a baseline visit and two follow-up visits at 3 and 6 months after the
22
23 230 initial one are scheduled (Figure 1). The IG will also make 5 replenishment visits, in
24
25 231 months 1, 2, 3 (coinciding with the first follow-up visit), 4 and 5. In the replenishment
26
27 232 visits they will be given the amount of chocolate needed until the next visit and will
28
29 233 hand in the calendar with the record of the chocolate eaten.

30
31 234 **Primary and secondary endpoints:**

32
33 235 The primary variable will be the decrease in clinical BP, measured with a digital
34
35 236 sphygmomanometer. Secondary variables will include vascular function, quality of life,
36
37 237 cognitive performance and body composition.

38
39 238 All variables will be measured at 3 and 6 months after randomization, except for
40
41 239 cognitive performance and quality of life, to be assessed only after 6 months.

42
43 240 **Blood pressure:**

44
45 241 Clinical systolic and diastolic blood pressure will be measured with a validated Omron
46
47 242 M10-IT sphygmomanometer (Omron Healthcare, Kyoto, Japan). Three measurements
48
49 243 will be taken in the dominant arm of the subject in a sitting position after at least 5
50
51 244 minutes of rest with an appropriately sized cuff, following the recommendations of the
52
53 245 European Society of Hypertension.[48] The average of the last two measurements will
54
55 246 be recorded.

56
57 247 **Vascular function:**

1
2
3 248 The Vasera VS-2000 device (Fukuda Denshi) will be used to measure the CAVI and
4
5 249 the brachial-ankle pulse wave velocity (ba-PWV) at rest. CAVI is a good indicator of
6
7 250 arterial stiffness, providing an accurate estimate of the degree of atherosclerosis
8
9 251 without depending on blood pressure.[49] CAVI ≥ 9 and ba-PWV ≥ 18.3 will be
10
11 252 considered pathological.[50] Pathological CAVI is representative of subclinical
12
13 253 atherosclerosis.[51]

14
15 254 **Cognitive performance:**

16
17 255 The instructions are presented visually at the start of the baseline measurement to
18
19 256 ensure limiting a learning effect over the subsequent testing periods. Attention and
20
21 257 executive functions: Trail Making Test A will be used to measure attention and Trail
22
23 258 Making Test B for processing speed and executive functions.[52]

24
25 259 Immediate verbal memory will be assessed with the Rey Auditory Verbal Learning
26
27 260 Test. The immediate recall of a list of 15 words is measured in three attempts, followed
28
29 261 by delayed verbal memory through the free recall of the words learned in the first part
30
31 262 of the test after 10 minutes.[53]

32
33 263 Working memory will be assessed with the WAIS Digit Span Backward test.[54]

34
35 264 Phonological fluency will be explored by naming as many words as possible starting
36
37 265 with different letters of the FAS Questionnaire in the space of one minute.[55]

38
39 266 Categorical fluency measures verbal semantic fluency and will be assessed by naming
40
41 267 as many animals as possible in one minute.[56]

42
43 268 **Quality of life:**

44
45 269 The quality of life linked to health will be assessed through the EuroQol 5-D
46
47 270 questionnaire (EQ-5D). We will use the adapted Spanish version of this questionnaire,
48
49 271 which has been validated in the Spanish population.[57] This questionnaire consists of
50
51 272 three elements: the assessment by the individuals of their state of health in level of
52
53 273 severity by dimension (mobility, personal care, daily activities, pain/discomfort and
54
55 274 anxiety/depression), the assessment of their state of health on an analogue visual

1
2
3 275 scale, and finally an index of social values obtained for each state of health generated
4
5 276 by the instrument.

6
7 277 The quality of life will also be studied using the Cervantes Scale.[58] This questionnaire
8
9 278 is specifically designed for menopause and postmenopause and has been validated for
10
11 279 Spanish women. Its 31 structured items cover the 4 dimensions of menopause:
12
13 280 menopause and health, sexuality, psychic domain and relationships.

14 281 **Body composition:**

15
16 282 Body composition will be measured with the Inbody 230 Monitor.[59] This analyzer
17
18 283 provides data on fat mass and body fat percentage as principal outcomes and also
19
20 284 skeletal muscle mass, total body water, fat-free mass, waist-hip ratio, basal
21
22 285 metabolism, and a segmental analysis.

23
24
25 286 Body weight will be measured twice with an electronic scale (Scale 7830, Soehnle
26
27 287 Professional GmbH & Co, Backnang, Germany) after proper calibration (accuracy ± 0.1
28
29 288 kg). Height will be measured by recording the average of two readings rounded to the
30
31 289 nearest centimetre using a stadiometer (Seca 222, Medical scale and measurement
32
33 290 system, Birmingham, UK). Both measurements will be made with the subject barefoot
34
35 291 and wearing light clothing. Body mass index will be calculated by dividing weight (kg)
36
37 292 by height squared (m^2). Waist circumference will be assessed in accordance with the
38
39 293 recommendations of the Spanish Society for the Study of Obesity (SEEDO)[60] and will
40
41 294 be measured in duplicate before and after inhalation, using a flexible tape parallel to
42
43 295 the floor, at the level of the mid-point between the lowest rib and the iliac crest, with the
44
45 296 subject standing up and without clothes.

46 47 297 **Other variables**

48 49 298 **Clinical and sociodemographic variables**

50
51 299 At the baseline visit, information on clinical and sociodemographic variables will also be
52
53 300 collected via questions about age, marital status, educational level and occupation.
54
55 301 Family history of cardiovascular disease and personal history of anxiety and/or
56
57
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1
2
3 302 depression, gestational diabetes, hypertension, dyslipidemia and the prescribed
4
5 303 pharmacological treatment (antiaggregants, anticoagulants, thyroid hormone treatment,
6
7 304 anxiolytics) will also be recorded, as well as the taking of NSAIDs in the last two weeks.
8
9 305 In subsequent visits, personal histories of cardiovascular disease, diabetes mellitus,
10
11 306 arterial hypertension or dyslipidemia in treatment, as well as the prescribed
12
13 307 pharmacological treatment (hypolipidemic, antihypertensive, antidiabetic) will also be
14
15 308 noted.

309 **Evaluation of chocolate consumption and habitual diet**

18 310 Chocolate consumption will be assessed at each evaluation visit by a series of
19
20 311 questions about the amount, type and frequency of consumption in the period between
21
22 312 visits.

24 313 Nutritional habits will be assessed by a 24-hour log on 3 non-consecutive days prior to
25
26 314 each visit.

315 **Evaluation of other lifestyles**

30 316 The use of tobacco will be assessed with a questionnaire on the personal history and
31
32 317 pattern of smoking.

34 318 Alcohol use will be recorded with a questionnaire covering the previous 7 days which
35
36 319 will include specific beverages and the amount by volume drunk of each.

38 320 Physical activity will be measured using the International Physical Activity
39
40 321 Questionnaire (IPAQ) in its short version and validated in Spanish.[61] This
41
42 322 questionnaire measures activity over the previous 7 days, classifying the subjects
43
44 323 according to three activity levels (low, moderate and high) with respect to three types of
45
46 324 activities: walking, moderate-intensity activities and vigorous-intensity activities. The
47
48 325 amount of physical exercise will be estimated in METs-minute/week.

326 **Evaluation of laboratory variables**

52 327 At baseline and follow-up visits at 6 months, we will measure plasma fasting glucose
53
54 328 values (mg/dL), glycated haemoglobin (%), total cholesterol (mg/dL), total triglycerides
55
56 329 (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), creatinine (mg/L),

1
2
3 330 insulinemia (mg/dL). Creatinine in urine (mg/dL) and microalbuminuria (mg/dL) will also
4
5 331 be measured. Insulin resistance will be determined using the HOMA index
6
7 332 (Homeostasis Model Assessment Insulin Resistance) estimated using the following
8
9 333 equation: Fasting glucose (mmol/l) X insulin (mU/ml)/22.5.

10 334 The evaluation visits will be made in the morning between 8:00 and 10:00 a.m. Each
11
12 335 participant will be informed prior to the visit to go fasting for at least 12 hours, having
13
14 336 avoided the 24 hours prior to visiting the consumption of polyphenol-rich foods,
15
16 337 including cocoa, chocolate, apples, and red wine as well as alcoholic drinks or the
17
18 338 performance of programmed physical activity. All evaluation visits, including blood
19
20 339 pressure measurements and evaluations of vascular function, will be carried out in a
21
22 340 room with conditions of lighting and temperature standardized, recommending that
23
24 341 patients attend the appointment with a prior rest of at least 8- 10 hours.

25 26 27 342 **Data collection procedure, data management and monitoring**

28
29 343 Data collection of the baseline and follow-up evaluation visits at 3 and 6 months will be
30
31 344 carried out by a nurse specifically trained to do so. The intervention visit after the
32
33 345 baseline evaluation will be carried out by another nurse, different from the one who
34
35 346 does the data collection. Each participant will have a unique identification code within
36
37 347 the study. All measurements will be compiled in a data collection notebook and kept in
38
39 348 a secure place that will remain closed within the health center. A database will be
40
41 349 created in SPSS to which only the members of the research team and the people
42
43 350 related to the statistical analyzes will have access. The principal investigator or a
44
45 351 person designated for this purpose will perform a weekly process of monitoring the
46
47 352 study, taking into account the inclusion of patients, cleaning and debugging of
48
49 353 databases and adaptation of the procedures to the protocol.

50 51 354 **Blinding strategy:**

52
53 355 Due to the nature of the intervention itself, the participants and the person responsible
54
55 356 for delivering the chocolate to IG participants, cannot be blinded. However, the person

1
2
3 357 responsible for carrying out the study measurements at each visit and for the statistical
4
5 358 analysis will be blind to the intervention.

6
7 359 **Statistical analysis:**

8
9 360 **General analysis**

10 361 Results for the quantitative variables will be expressed by mean \pm standard deviation or
11
12 362 by frequency distribution in the case of qualitative ones. The normality of the variables
13
14 363 will be assessed using the Kolmogorov-Smirnov test. In cases where a normal
15
16 364 distribution cannot be assumed, the corresponding nonparametric tests will be applied.
17
18 365 The association between independent qualitative variables will be analyzed by means
19
20 366 of the chi-square test or Fisher's exact test. The means between the two groups will be
21
22 367 compared using the Student's t test or the Mann-Whitney U test, and the Pearson or
23
24 368 Spearman correlation coefficients will be calculated to analyze the relationship between
25
26 369 quantitative variables.

27
28 370 The analysis of the results for the main variable and the secondary variables will be
29
30 371 carried out by intention to treat. In addition, a secondary analysis will be run taking into
31
32 372 account chocolate intake adherence (< 50% days and > 50% days) and other relevant
33
34 373 subgroups in relation to their physical activity or previous chocolate consumption.

35
36 374 All analyses will be performed with the SPSS version 23.0 (IBM Corporation, Armonk,
37
38 375 NY, USA) and an alpha risk of 0.05 will be set as the limit of statistical significance.

39
40 376 **Analysis of the intervention's effect on primary and secondary outcomes.**

41
42 377 To analyze the changes at 3 and 6 months from baseline in the primary outcome
43
44 378 (blood pressure) and in the secondary outcomes within the same group, the Student's t
45
46 379 test for paired data or the Wilcoxon test will be used. The McNemar test will be applied
47
48 380 with quantitative or dichotomous variables.

49
50 381 Effects of the intervention will be analyzed in a comparison of the changes in blood
51
52 382 pressure and the secondary variables between the IG and the CG, using ANCOVA and
53
54 383 adjusting for possible confounders as the smoking status. Effects of the intervention
55
56 384 during follow-up will be studied with an analysis of the variance of repeated measures.

1
2
3 385 **Analysis by subgroups.**

4 386 The effect of the intervention could be influenced by age, sociocultural level and
5
6 387 adherence to the study's chocolate intake. The same analyses described above will be
7
8 388 performed for each of the aforementioned subgroups.
9

10 389 **Secondary analyses.**

11
12 390 A multivariate multiple regression analysis will be performed to identify the variables
13
14 391 with the greatest influence on blood pressure changes and the secondary variables
15
16 392 analyzed.
17

18 393 **Methodological limitations:**

19
20 394 Due to the nature of the intervention, the participating subjects cannot be blinded.
21
22 395 However, the researcher who analyses the data and the person who makes the
23
24 396 measurements during follow-up visits will be blinded with respect to the group to which
25
26 397 the participants belong. The smoking status in the 12 months prior to the time of
27
28 398 inclusion could influence the outcome measures related to vascular function and blood
29
30 399 pressure so, although participants will not be excluded for this reason, this aspect will
31
32 400 be controlled in statistical analysis. Assessment of the quality of life and lifestyles will
33
34 401 be carried out through self-reported data; however, previously validated instruments
35
36 402 will be used to obtain these. To make compliance with the intervention in the IG easier,
37
38 403 IG participants will be provided with instructions on eating the chocolate and a calendar
39
40 404 to record each intake.
41
42

43 405 **ETHICS AND DISSEMINATION**

44
45 406 **Ethical considerations:**

46
47 407 The study was approved by the Clinical Research Ethics Committee of the Salamanca
48
49 408 Health Area ("CREC of Health Area of Salamanca") in February 2018. A SPIRIT
50
51 409 checklist is available for this protocol. The clinical trial has been registered at
52
53 410 ClinicalTrials.gov with the identifier NCT03492983.
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2
3 411 Participants must sign informed consent in accordance with the Declaration of Helsinki.
4
5 412 Subjects will be informed of the objectives of the project and the risks and benefits of
6
7 413 the explorations to be carried out, including sample collection. None of the tests will
8
9 414 pose risks that could endanger the lives of participants. Confidentiality of participant
10
11 415 data will be guaranteed at all times in accordance with the provisions of the Organic
12
13 416 Law on the Protection of Personal Data (15/1999 of December 13, LOPD), and under
14
15 417 the conditions established by Law 14/2007 of biomedical research.

16
17 418 **Dissemination plan:**

18
19 419 The research group plans to achieve rapid and widespread dissemination of results to
20
21 420 ensure maximum visibility of this study. To this end, the results of the study will be
22
23 421 published in open-access scientific journals with peer review. At least one publication
24
25 422 of the main results and others with the secondary results are planned. This will be
26
27 423 complemented by presentation of the results of the study at relevant scientific
28
29 424 conferences and seminars of national and international scope. In addition, a doctoral
30
31 425 thesis based on this project will be prepared. Appropriate dissemination will likewise be
32
33 426 carried out through social networks and other media. Moreover, given the involvement
34
35 427 of a commercial product, the transfer to clinical practice is expected to be very fast if
36
37 428 the results are as expected.

38
39 429 **DISCUSSION**

40
41 430 In recent years, there has been an increase in attention to polyphenols and their
42
43 431 beneficial effects on health, with numerous studies being carried out to assess this.[19,
44
45 432 21] Similarly, the therapeutic use of these compounds has been suggested for certain
46
47 433 diseases or population groups.[36, 62] The menopause increases the risk of
48
49 434 developing cardiovascular disease compared to the previous period.[45] However, we
50
51 435 have not found any study that assesses the effect of adding commercially available
52
53 436 chocolate high in cocoa content to the usual diet in this population at special risk.
54
55 437 Similarly, no studies have been found that evaluate the effects on cognitive

1
2
3 438 performance, quality of life and body composition of adding commercial chocolate with
4
5 439 high cocoa content to the usual diet in postmenopausal women.
6
7 440 The results of this work will provide new evidence in this regard for the development of
8
9 441 strategies in nutritional education of particularly vulnerable populations, given their high
10
11 442 risk of developing cardiovascular disease, including non-pharmacological therapies and
12
13 443 strategies that employ lifestyle modification. This intervention may also have
14
15 444 implications for the preparation of recommendations in clinical practice guidelines and
16
17 445 quality improvement programs aimed at the care of postmenopausal women.
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5
6 651 JIR, JAM, LGO and IGY contributed to the conception and design of the study. IGY,
7
8 652 JIR and JAM prepared the manuscript of the study protocol. JIR, JAM, LGO, RAD,
9
10 653 SMS, JGS, SMG, ERS, MGM and IGY contributed to the development of the study
11
12 654 protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided
13
14 655 assistance with statistical methodology and knowledge. JIR, JAM, LGO, RAD, SMS,
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16 656 JGS, SMG, ERS, MGM and IGY provided a critical review of the manuscript.
17
18 657 All authors have read and accepted the final version of the protocol.

19
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23
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25
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27
28 662 study. This company will not play any role in the design of the study, data analysis,
29
30 663 reporting of results, or the decision to present the manuscript for publication".

31
32 664 **COMPETING INTERESTS STATEMENT:**

33
34 665 The authors declare that they have no conflicts of interest.

35
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39
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49
50 673 González-Sánchez.

51
52 674 **FIGURE LEGEND:**

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54 675 Figure 1. Study flow chart

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56 676 Table 1. Polyphenol composition of 99% cocoa chocolate

677

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

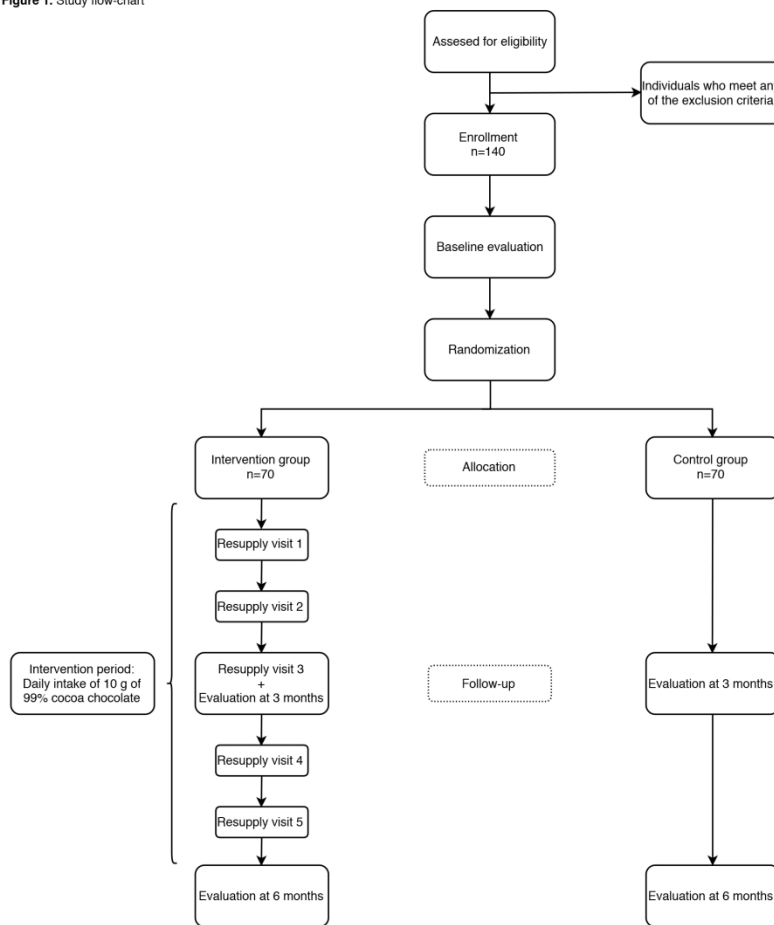


Figure 1

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

1
2
3 Sample size 14 Estimated number of participants needed to achieve study objectives and how it was determined, including Pag 7, line 190
4 clinical and statistical assumptions supporting any sample size calculations

5
6 Recruitment 15 Strategies for achieving adequate participant enrolment to reach target sample size Pag 7, line 182
7

8 **Methods: Assignment of interventions (for controlled trials)**
9

10 Allocation:

11
12 Sequence generation 16a Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any Pag 8, line 199
13 factors for stratification. To reduce predictability of a random sequence, details of any planned restriction
14 (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants
15 or assign interventions
16

17 Allocation concealment mechanism 16b Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, Pag 8, line 199
18 opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned
19
20

21 Implementation 16c Who will generate the allocation sequence, who will enrol participants, and who will assign participants to Pag 8, line 200
22 interventions
23

24 Blinding (masking) 17a Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome Pag 13, line 355
25 assessors, data analysts), and how
26

27 17b If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's Pag 13, line 355
28 allocated intervention during the trial
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31 **Methods: Data collection, management, and analysis**
32

33 Data collection methods 18a Plans for assessment and collection of outcome, baseline, and other trial data, including any related Pag 9, line 238
34 processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of
35 study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known.
36 Reference to where data collection forms can be found, if not in the protocol
37

38 18b Plans to promote participant retention and complete follow-up, including list of any outcome data to be NA
39 collected for participants who discontinue or deviate from intervention protocols
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3	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
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7	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
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10		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Pag 15, line 385
11				
12		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
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15 **Methods: Monitoring**

16				
17	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
18				
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22		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
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24				
25	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
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28	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
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32 **Ethics and dissemination**

33				
34	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Pag 15, line 407
35				
36				
37	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
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3	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
4				
5				
6		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
7				
8	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
9				
10				
11	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Pag 22, line 665
12				
13				
14	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
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17	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
18				
19				
20	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
21				
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25		31b	Authorship eligibility guidelines and any intended use of professional writers	Pag 16, line 419
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27		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Pag 16, line 426
28				
29	Appendices			
30				
31	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	NA
32				
33				
34	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
35				
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37 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.
 38 Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons
 39 "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.
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BMJ Open

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

Journal:	<i>BMJ Open</i>
Manuscript ID	bmjopen-2018-024095.R2
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Primary Subject	Nutrition and metabolism

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



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

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1
2
3 27 **ABSTRACT**

4 28 **Introduction:** The intake of polyphenols has certain health benefits. This study will aim
5
6 29 to assess the effect of adding a daily amount of chocolate high in cocoa content and
7
8 30 polyphenols to the normal diet on blood pressure, vascular function, cognitive
9
10 31 performance, quality of life and body composition in postmenopausal women.

11
12 32 **Methods and analysis:** Here we plan a randomized clinical trial with two parallel
13
14 33 groups involving a total of 140 women between 50- and 64-years-old in the
15
16 34 postmenopausal period, defined by amenorrhea of at least 12 consecutive months. The
17
18 35 main variable will be the change in blood pressure. Secondary variables will be
19
20 36 changes in vascular function, quality of life, cognitive performance, and body
21
22 37 composition. The intervention group will be given chocolate containing 99% cocoa, with
23
24 38 instructions to add 10 g daily to their normal diet for 6 months. The daily nutritional
25
26 39 contribution of this amount of chocolate is 59 Kcal and 65.4 mg of polyphenols. There
27
28 40 will be no intervention in the control group. All variables will be measured at the
29
30 41 baseline visit and 3 and 6 months after randomization, except cognitive performance
31
32 42 and quality of life, which will only be assessed at baseline and at 6 months.
33
34 43 Recruitment is scheduled to begin on June 1, 2018, and the study will continue until
35
36 44 May 31, 2019.

37
38 45 **Ethics and dissemination:** This study was approved by the Clinical Research Ethics
39
40 46 Committee of the Health Area of Salamanca, Spain ("CREC of Health Area of
41
42 47 Salamanca") in February 2018. A SPIRIT checklist is available for this protocol. The
43
44 48 clinical trial has been registered at ClinicalTrials.gov provided by the US National
45
46 49 Library of Medicine, number NCT03492983. The results will be disseminated through
47
48 50 open access peer-reviewed journals, conference presentations, broadcast media, and
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50 51 a presentation to stakeholders.

51
52 52 **Keywords:** Chocolate, postmenopause, arterial pressure, vascular stiffness, body
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54 53 composition, quality of life.

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For peer review only

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3 56 **Strengths and limitations of this study:**

- 4
5 57 - This study will use commercially available chocolate with a high content of cocoa
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7 58 and polyphenols during the intervention.
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9 59 - Blood pressure and vascular function will be measured objectively using a
10
11 60 sphygmomanometer and a Vasera VS-2000 device (Fukuda Denshi), with body
12
13 61 composition measured by impedance analysis, while the quality of life and cognitive
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15 62 performance will be assessed using validated instruments.
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17 63 - Due to the nature of the intervention, the participants cannot be blinded, although
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19 64 the researchers who perform the measurements and the statistical analysis will be
20
21 65 blinded.
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25 67 **INTRODUCTION**

26
27 68 Polyphenols are bioactive compounds found in many plants, fruits and vegetables. The
28
29 69 beneficial effects on human health associated with the consumption of a diet rich in
30
31 70 polyphenols has generated great scientific interest in these substances.[1-3] The action
32
33 71 of polyphenols is based on their antioxidant capacity through the uptake of free
34
35 72 radicals, the chelation of metals with redox properties and the modulation and inhibition
36
37 73 of enzymatic activities.[4]
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41 75 The most abundant polyphenols in cocoa are flavonoids, which have been linked to a
42
43 76 protective effect against cardiovascular disease, decreasing the risk of cardiovascular
44
45 77 morbidity and mortality and favouring the prevention of other chronic diseases, such as
46
47 78 diabetes mellitus type 2.[1-3, 5-7] The ability to reduce cardiovascular risk could be due
48
49 79 to an improvement in the elements that define metabolic syndrome, the improvement of
50
51 80 vascular endothelial dysfunction, insulin resistance and the inhibition of platelet
52
53 81 activation and aggregation.[8, 9] However, although current evidence suggests that
54
55 82 polyphenols produce an improvement in cardiovascular health, this is insufficient to
56
57 83 determine the minimum amount of intake necessary to achieve health benefits.[10]

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3 844
5 **85 Cocoa polyphenols and blood pressure:**

6
7 86 The effect of consuming polyphenols present in chocolate on the blood pressure
8
9 87 statistics of healthy individuals is unclear. Cocoa consumption has been associated
10
11 88 with an improvement in endothelial function and a decrease in blood pressure in both
12
13 89 healthy subjects and those with risk factors and cardiovascular diseases.[11, 12] Some
14
15 90 studies have observed a dose-dependent relationship between cocoa intake and
16
17 91 clinical BP, with higher consumption equated to lower blood pressure and better
18
19 92 vascular function.[13, 14] Conversely, other research has not obtained significant
20
21 93 changes in these parameters related to the supplementation of cocoa or pure
22
23 94 polyphenols, such as epicatechin or quercetin.[15, 16]

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27 96 Endothelial dysfunction in postmenopausal women causes changes that favour the
28
29 97 development of cardiovascular risk factors and atherosclerosis, which lead to the
30
31 98 appearance and maintenance of hypertension.[17, 18] A decrease in blood pressure
32
33 99 has been observed in this group after daily consumption of cocoa with a flavanol
34
35 100 content of 40.12 mg. Below this level, however, no changes have been observed.[19]

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37 10138
39 102 **Cocoa polyphenols and vascular function:**

40
41 103 Among healthy individuals, as well as postmenopausal women, the consumption of
42
43 104 polyphenols present in cocoa has been associated with a dose-dependent
44
45 105 improvement of vascular function, in particular of arterial stiffness measured by pulse
46
47 106 wave speed.[13, 14, 19] One of these studies also suggests that the reduction in
48
49 107 arterial stiffness observed in postmenopausal women after consumption of cocoa is
50
51 108 independent of the frequency of the intake.[19] However, this relationship is not evident
52
53 109 in people with mild hypertension when cardio-ankle vascular index (CAVI) is used as a
54
55 110 measure of arterial stiffness.[20]

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3 112 There is also evidence of the influence of these polyphenols in reducing the
4
5 113 augmentation index (Aix). The study by West et al.,[21] involving subjects with excess
6
7 114 weight and moderate obesity, concludes that treatment with dark chocolate decreases
8
9 115 Aix in women, although it seems that this association might have a greater effect on
10
11 116 the elasticity of the large arteries, especially in subjects with obesity and diabetes
12
13 117 mellitus type 2.[22]

14
15 118

119 **Cocoa polyphenols and cognitive performance:**

18
19 120 There is evidence to suggest that chocolate rich in polyphenols is beneficial for
20
21 121 cognitive performance and state since it improves mental processing speed and
22
23 122 attenuates the increase of mental fatigue among healthy young adults.[23, 24] An
24
25 123 improvement in cognitive performance among older age groups after eating chocolate
26
27 124 has also been observed,[25] especially in subjects with higher risk of cardiovascular
28
29 125 disease.[26] Several studies also show that polyphenol-rich chocolate causes an
30
31 126 improvement in executive function, categorical fluency,[27] and working memory,[28,
32
33 127 29] and a slowing of mental fatigue.[30] Also, a higher frequency of chocolate
34
35 128 consumption has been associated with improved cognitive function.[29] Furthermore, a
36
37 129 positive influence of cocoa polyphenols on physiological processes has been reported,
38
39 130 with a neuroprotective effect[31] and improved cognitive performance.[32] In this
40
41 131 regard, it has been suggested that the brain-derived neurotrophic factor (BDNF) plays
42
43 132 a role in the cognitive enhancement induced by the flavonoides.[33] Favourable effects
44
45 133 on cerebrovascular function have also been observed in postmenopausal women after
46
47 134 consumption of chocolate with a high concentration of cocoa.[34]

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49 135

136 **Cocoa polyphenols and quality of life:**

52
53 137 The quality of life linked to health is represented by the individual's perception of well-
54
55 138 being in various aspects of life, including physical and mental aspects. The effect of
56
57 139 chocolate and polyphenols on the quality of life has scarcely been studied, with little

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2
3 140 available evidence and even less of a conclusive nature. In a study conducted among
4
5 141 healthy people, where regular consumption of chocolate was recorded over 1 year, no
6
7 142 evidence was found of a clear association between chocolate intake and the physical
8
9 143 or mental components of quality of life.[35] Nevertheless, it has been observed that the
10
11 144 consumption of dark chocolate might be beneficial for the quality of life of women with
12
13 145 fibromyalgia.[36]

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15 146

147 **Cocoa polyphenols and body composition:**

148 The menopause period leads to various changes in the body composition of
19
20 149 women.[37] Regarding the connection between cocoa polyphenols and body
21
22 150 composition, results diverge. Some clinical trials involving healthy people and
23
24 151 overweight or obese patients have not reported significant differences that link
25
26 152 chocolate consumption to anthropometric measures.[16, 20, 21, 38] Other studies
27
28 153 indicate that chocolate consumption might have positive effects on body composition in
29
30 154 adolescents,[39] patients with diabetes[40] or women with obesity.[41] Two recent
31
32 155 systematic reviews also indicate that eating chocolate is associated with reduced body
33
34 156 mass index (BMI) and waist circumference,[42, 43] and one of them also concludes
35
36 157 that the amount and the length of time during which it is eaten play a key role in these
37
38 158 beneficial effects.[43] Conversely, other studies such as that carried out with the cohort
39
40 159 of the Atherosclerosis Risk in Communities (ARIC) study have observed a dose-
41
42 160 dependent increase in weight after habitual chocolate consumption.[44]

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45 161

46 162 In sum, the polyphenols present in chocolate seem to have a positive effect on BP,
47
48 163 vascular function, cognitive performance, and quality of life, especially in populations
49
50 164 with increased cardiovascular risk, such as postmenopausal women.[45] However, the
51
52 165 conflicting results obtained in different studies suggest that the real contribution of
53
54 166 these compounds to health and the underlying mechanisms remain unclear. Moreover,

1
2
3 167 most of these studies have used preparations with high concentrations of polyphenols
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5 168 that are usually not present in the normal diet.

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8
9 170 This study aims to evaluate the effect of adding a daily amount of 10 g of chocolate
10
11 171 high in cocoa content (99%) and polyphenols to the normal diet on blood pressure,
12
13 172 vascular function, cognitive performance, quality of life, and body composition in
14
15 173 postmenopausal women.

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18 175 **METHODS AND ANALYSIS**

19 176 **Design and setting:**

20
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22 177 This controlled and randomized clinical trial involves two parallel groups. The study will
23
24 178 be carried out in the Research Unit of the La Alamedilla Health Centre in Salamanca
25
26 179 (Spain), which is part of the Biomedical Research Institute of Salamanca (IBSAL) and
27
28 180 the Primary Care Prevention and Health Promotion Research Network (REDIAPP).
29
30 181 The recruitment schedule is set to start on June 1, 2018, and the study will run until
31
32 182 May 31, 2019. There will be a baseline assessment and two follow-ups, at 3 and 6
33
34 183 months.

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36 184

37 185 **Study population:**

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40 186 Those subjects who meet the selection criteria and sign the informed consent after
41
42 187 receiving information about the objectives and implementation of the study will take
43
44 188 part.

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48 190 Inclusion criteria: women between 50- and 64-years-old in postmenopause, defined by
49
50 191 and checked against amenorrhea during at least 12 consecutive months.

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52 192 Exclusion criteria: a personal history of cardiovascular disease; personal history of
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54 193 diabetes mellitus, arterial hypertension or dyslipidemia under pharmacological
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56 194 treatment; hypocaloric diets; clinically demonstrable neurological and/or

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3 195 neuropsychological disease; treatment with hormone replacement therapy; intolerance
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5 196 and/or allergy to cocoa or any of the components of the supplement.
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9 198 Participants will be selected using a consecutive sample of women who meet the
10
11 199 selection criteria in the GP surgeries of four urban primary care centres in Salamanca,
12
13 200 from June 1, 2018.
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15 201

16 202 **Patient and public involvement:**

17
18 203 Patients and the public were not involved in the design of this study or outcome
19
20 204 measures. We hope that the results of the study will be disseminated through press
21
22 205 releases and information-sharing meetings with the study participants.
23
24 206

25 207 **Sample size:**

26
27
28 208 The size of the sample has been estimated based on the potential modification of the
29
30 209 main variable, systolic blood pressure (SBP). Given alpha and beta risks of 0.05 and
31
32 210 0.20 respectively in bilateral contrast and a standard deviation (SD) of 5.8 mmHg, 140
33
34 211 participants (70 per group) will be necessary to detect a minimum difference of 2.9
35
36 212 mmHg in the SBP between the two groups. A predicted drop-out rate of 10% during
37
38 213 follow-up has been taken into account. This estimate has considered the results
39
40 214 obtained in a similar study in which a decrease in SBP of 6.5 was observed \pm 5.8
41
42 215 mmHg.[14]
43
44 216

45 217 **Randomization:**

46
47
48 218 Participants will be assigned to the intervention group (IG) or control group (CG) at
49
50 219 random. The allocation sequence will be generated by an independent researcher
51
52 220 using the Epidat 4.2 program [46] before the inclusion of the first participant, using
53
54 221 masked block randomisation. Patients will receive their randomisation number based
55
56 222 on the order of their baseline evaluation visit and will remain hidden until the
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3 223 participants are assigned to each group. To ensure that the blinding is maintained,
4 224 patients will be given clear instructions not to disclose which treatment they have been
5
6 225 randomised to while being interviewed by the blind assessors. Information on treatment
7
8 226 allocation will be stored in a secure locker in case of emergency unblinding.
9

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13 228 **Intervention:**

14 229 No type of intervention will be carried out with the CG participants.
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18 231 IG participants will be given chocolate with 99% cocoa content and asked to eat 10 g
19
20 232 daily for 6 months. According to the EFSA (European Food Safety Authority), 10 g of
21
22 233 high-flavanol dark chocolate consumed in the context of a balanced diet could help
23
24 234 maintain endothelium-dependent vasodilation.[47] Participants will also be given
25
26 235 instructions on eating and keeping the product, with the recommendations, for
27
28 236 example, that the chocolate can be consumed in small pieces leaving them unmated in
29
30 237 the mouth, without chewing them. Also, a series of recommendations will be given
31
32 238 addressing the organoleptic characteristics of the product, as well as the
33
34 239 recommendations of trying to consume the product at the same time or refrain from
35
36 240 ingesting it dissolved in milk. Also, participants will be given a calendar on which to
37
38 241 record the time it was eaten each day. This calendar will be returned to the researchers
39
40 242 at each replenishment visit.
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43
44 244 This amount of chocolate provides the following daily nutritional contribution: 59 Kcal,
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46 245 0.8 g of carbohydrates, 1.5 g of protein, 5.1 g of fat, of which 3.1 g are saturated fats.
47
48 246 The proportion of polyphenols per 10 g is 65.4 mg. The polyphenolic profile of this
49
50 247 compound can be seen in Table 1. On each visit, IG participants will receive the
51
52 248 amount of chocolate they need until the next replenishment visit. In addition to the
53
54 249 baseline visit, there will be five replenishment visits in months 1, 2, 3 (coinciding with
55
56 250 the evaluation visit), 4 and 5. The sole purpose of the replenishment visits will be to

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3 251 supply the amount of chocolate needed until the next visit, without any other
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5 252 intervention being carried out.

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9 254 Participants in both groups will be instructed to continue with the dietary pattern they
10
11 255 usually follow, without changing their eating habits during the study period.

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15 257 **Procedures:**

16
17 258 For each participant a baseline visit and two follow-up visits at 3 and 6 months after the
18
19 259 initial one are scheduled (Figure 1). The IG will also make five replenishment visits, in
20
21 260 months 1, 2, 3 (coinciding with the first follow-up visit), 4 and 5. In the replenishment
22
23 261 visits, participants will be given the amount of chocolate needed until the next visit and
24
25 262 will hand in the calendar with the record of the chocolate eaten.

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29 264 **Primary and secondary endpoints:**

30
31 265 The primary variable will be the decrease in clinical BP, measured with a digital
32
33 266 sphygmomanometer. Secondary variables will include vascular function, quality of life,
34
35 267 cognitive performance and body composition.

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39 269 All variables will be measured at 3 and 6 months after randomization, except for
40
41 270 cognitive performance and quality of life, which will be assessed only after 6 months.

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45 272 **Blood pressure:**

46
47 273 Clinical systolic and diastolic blood pressure will be measured with a validated Omron
48
49 274 M10-IT sphygmomanometer (Omron Healthcare, Kyoto, Japan). Three measurements
50
51 275 will be taken in the dominant arm of the subject in a sitting position after at least 5 min
52
53 276 of rest with an appropriately sized cuff, following the recommendations of the European
54
55 277 Society of Hypertension.[48] The average of the last two measurements will be
56
57 278 recorded.

279

280 Vascular function:

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

287

288 Cognitive performance:

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

293

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

298

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

300 Phonological fluency will be explored by naming as many words as possible starting
301 with different letters of the FAS Questionnaire in the space of 1 min.[55]

302

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

305

306 Quality of life:

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2
3 307 The quality of life linked to health will be assessed through the EuroQol 5-D
4
5 308 questionnaire (EQ-5D). We will use the adapted Spanish version of this questionnaire,
6
7 309 which has been validated in the Spanish population.[57] This questionnaire consists of
8
9 310 three elements: the assessment by the individuals of their state of health in level of
10
11 311 severity by dimension (mobility, personal care, daily activities, pain/discomfort and
12
13 312 anxiety/depression), the assessment of their state of health on an analogue visual
14
15 313 scale, and finally an index of social values obtained for each state of health generated
16
17 314 by the instrument.

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19 315

20 316 The quality of life will also be studied using the Cervantes Scale.[58] This questionnaire
21
22 317 is specifically designed for menopause and postmenopause and has been validated for
23
24 318 Spanish women. Its 31 structured items cover the four dimensions of menopause:
25
26 319 menopause and health, sexuality, psychic domain and relationships.

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30 321 **Body composition:**

31
32 322 Body composition will be measured with the Inbody 230 Monitor.[59] This analyzer
33
34 323 provides data on fat mass and body fat percentage as principal outcomes and also
35
36 324 skeletal muscle mass, total body water, fat-free mass, waist-hip ratio, basal
37
38 325 metabolism, and a segmental analysis.

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41 326

42 327 Body weight will be measured twice with an electronic scale (Scale 7830, Soehnle
43
44 328 Professional GmbH & Co, Backnang, Germany) after proper calibration (accuracy ± 0.1
45
46 329 kg). Height will be measured by recording the average of two readings rounded to the
47
48 330 nearest centimetre using a stadiometer (Seca 222, Medical scale and measurement
49
50 331 system, Birmingham, UK). Both measurements will be made with the subject barefoot
51
52 332 and wearing light clothing. Body mass index will be calculated by dividing weight (kg)
53
54 333 by height squared (m^2). Waist circumference will be assessed in accordance with the
55
56 334 recommendations of the Spanish Society for the Study of Obesity (SEEDO)[60] and will

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3 335 be measured in duplicate before and after inhalation, using a flexible tape parallel to
4
5 336 the floor, at the level of the mid-point between the lowest rib and the iliac crest, with the
6
7 337 subject standing up and without clothes.
8

9 338

10 339 **Other variables**

11
12 340 Clinical and sociodemographic variables

13
14 341 At the baseline visit, information on clinical and sociodemographic variables will also be
15
16 342 collected via questions about age, marital status, educational level and occupation. The
17
18 343 family history of cardiovascular disease and personal history of anxiety and depression,
19
20 344 gestational diabetes, hypertension, dyslipidemia and the prescribed pharmacological
21
22 345 treatment (antiaggregants, anticoagulants, thyroid hormone treatment, anxiolytics) will
23
24 346 also be recorded, as well as the taking of NSAIDs in the last 2 weeks.
25

26 347

27
28 348 In subsequent visits, personal histories of cardiovascular disease, diabetes mellitus,
29
30 349 arterial hypertension or dyslipidemia in treatment, as well as the prescribed
31
32 350 pharmacological treatment (hypolipidemic, antihypertensive, antidiabetic) will also be
33
34 351 noted.
35

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37
38 353 Evaluation of chocolate consumption and habitual diet

39
40 354 Chocolate consumption will be assessed at each evaluation visit by a series of
41
42 355 questions about the amount, type and frequency of consumption in the period between
43
44 356 visits.
45

46 357

47
48 358 Nutritional habits will be assessed by a 24-h log on three non-consecutive days prior to
49
50 359 each visit.
51

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54 361 Evaluation of other lifestyles
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3 362 The use of tobacco will be assessed with a questionnaire on the personal history and
4
5 363 pattern of smoking.

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9 365 Alcohol use will be recorded with a questionnaire covering the previous 7 days, which
10
11 366 will include specific beverages and the amount by volume drunk of each.

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14
15 368 Physical activity will be measured using the International Physical Activity
16
17 369 Questionnaire (IPAQ) in its short version and validated in Spanish.[61] This
18
19 370 questionnaire measures activity over the previous 7 days, classifying the subjects
20
21 371 according to three activity levels (low, moderate and high) with respect to three types of
22
23 372 activities: walking, moderate-intensity activities and vigorous-intensity activities. The
24
25 373 amount of physical exercise will be estimated in METs-minute/week.

26
27 374
28
29 375 Evaluation of laboratory variables

30
31 376 At baseline and follow-up visits at 6 months, we will measure plasma fasting glucose
32
33 377 values (mg/dL), glycated haemoglobin (%), total cholesterol (mg/dL), total triglycerides
34
35 378 (mg/dL), HDL cholesterol (mg/dL), LDL cholesterol (mg/dL), creatinine (mg/L), and
36
37 379 insulinemia (mg/dL). Creatinine in urine (mg/dL) and microalbuminuria (mg/dL) will also
38
39 380 be measured. Insulin resistance will be determined using the HOMA index
40
41 381 (Homeostasis Model Assessment Insulin Resistance) estimated using the following
42
43 382 equation: Fasting glucose (mmol/l) × insulin (mU/ml)/22.5.

44
45 383
46
47 384 The evaluation visits will be made in the morning between 8:00 and 10:00 a.m. Each
48
49 385 participant will be informed prior to the visit to fast for at least 12 h, having avoided
50
51 386 during the 24 h prior to visiting the consumption of polyphenol-rich foods, including
52
53 387 cocoa, chocolate, apples, and red wine as well as alcoholic drinks or the performance
54
55 388 of the programmed physical activity. All evaluation visits, including blood pressure
56
57 389 measurements and evaluations of vascular function, will be carried out in a room with

1
2
3 390 standardized lighting and temperature, recommending that patients attend the
4
5 391 appointment with a prior rest of at least 8–10 h.

6
7 392

8
9 393 Data collection procedure, data management and monitoring

10
11 394 Data collection of the baseline and follow-up evaluation visits at 3 and 6 months will be
12
13 395 carried out by a nurse specifically trained to do so. The intervention visit after the
14
15 396 baseline evaluation will be carried out by another nurse, different from the one who
16
17 397 performs the data collection. Each participant will have a unique identification code
18
19 398 within the study. All measurements will be compiled in a data collection notebook and
20
21 399 kept in a secure place that will remain closed within the health centre. A database will
22
23 400 be created in SPSS to which only the members of the research team and the people
24
25 401 related to the statistical analyses will have access. The principal investigator or a
26
27 402 person designated for this purpose will perform a weekly process of monitoring the
28
29 403 study, taking into account the inclusion of patients, cleaning and debugging of
30
31 404 databases, and adaptation of the procedures to the protocol.

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35 406 **Blinding strategy:**

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37 407 Due to the nature of the intervention itself, the participants and the person responsible
38
39 408 for delivering the chocolate to IG participants cannot be blinded. However, the person
40
41 409 responsible for carrying out the study measurements at each visit and for the statistical
42
43 410 analysis will be blind to the intervention.

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47 412 **Statistical analysis:**

48
49 413 General analysis

50
51 414 Results for the quantitative variables will be expressed by mean \pm standard deviation or
52
53 415 by frequency distribution in the case of qualitative variables. The normality of the
54
55 416 variables will be assessed using the Kolmogorov-Smirnov test. In cases where a
56
57 417 normal distribution cannot be assumed, the corresponding nonparametric tests will be

1
2
3 418 applied. The association between independent qualitative variables will be analyzed
4
5 419 using the Chi-square test or Fisher's exact test. The means between the two groups
6
7 420 will be compared using the Student's t-test or the Mann-Whitney U test, and the
8
9 421 Pearson or Spearman correlation coefficients will be calculated to analyze the
10
11 422 relationship between quantitative variables.

12
13 423

14 424 The analysis of the results for the main variable and the secondary variables will be
15
16 425 carried out by intention to treat. Also, a secondary analysis will be made, taking into
17
18 426 account chocolate intake adherence (< 50% days and > 50% days) and other relevant
19
20 427 subgroups in relation to their physical activity or previous chocolate consumption.

21
22 428 All analyses will be performed using SPSS version 23.0 (IBM Corporation, Armonk,
23
24 429 NY, USA) and an alpha risk of 0.05 will be set as the limit of statistical significance.

25
26 430

27
28 431 Analysis of the intervention's effect on primary and secondary outcomes

29
30 432 To analyze the changes at 3 and 6 months from baseline in the primary outcome
31
32 433 (blood pressure) and in the secondary outcomes within the same group, the Student's
33
34 434 t-test for paired data or the Wilcoxon test will be used. The McNemar test will be
35
36 435 applied with quantitative or dichotomous variables.

37
38 436

39
40 437 Effects of the intervention will be analyzed in a comparison of the changes in blood
41
42 438 pressure and the secondary variables between the IG and the CG, using ANCOVA and
43
44 439 adjusting for possible confounders e.g. smoking status. Effects of the intervention
45
46 440 during follow-up will be studied with an analysis of the variance of repeated measures.

47
48 441

49
50 442 Analysis by subgroups

51
52 443 The effect of the intervention could be influenced by age, sociocultural level and
53
54 444 adherence to the study's chocolate intake. The same analyses described above will be
55
56 445 performed for each of the subgroups above.

446

447 Secondary analyses

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

451

452 Methodological limitations:

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

464

465 ETHICS AND DISSEMINATION**466 Ethical considerations:**

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

471

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

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2
3 474 benefits of the explorations to be carried out, including sample collection. None of the
4
5 475 tests will pose risks that could endanger the lives of participants. Confidentiality of
6
7 476 participant data will be guaranteed at all times in accordance with the provisions of the
8
9 477 Organic Law on the Protection of Personal Data (15/1999 of December 13, LOPD), and
10
11 478 under the conditions established by Law 14/2007 of biomedical research.

12
13 479

14 480 **Dissemination plan:**

15
16 481 The research group plans to achieve rapid and widespread dissemination of results to
17
18 482 ensure maximum visibility of this study. To this end, the results of the study will be
19
20 483 published in open-access scientific journals with peer review. At least one publication
21
22 484 of the main results and others with the secondary results are planned. This will be
23
24 485 complemented by the presentation of the results of the study at relevant scientific
25
26 486 conferences and seminars of national and international scope. Also, a doctoral thesis
27
28 487 based on this project will be prepared. Appropriate dissemination will likewise be
29
30 488 carried out through social networks and other media. Moreover, given the involvement
31
32 489 of a commercial product, the transfer to clinical practice is expected to be rapid if the
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34 490 results are as expected.

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37 38 492 **DISCUSSION**

39
40 493 In recent years, there has been an increase in attention to polyphenols and their
41
42 494 beneficial effects on health, with numerous studies being carried out to assess this.[19,
43
44 495 21] Similarly, the therapeutic use of these compounds has been suggested for certain
45
46 496 diseases or population groups.[36, 62] The menopause increases the risk of
47
48 497 developing cardiovascular disease compared to the previous period.[45] However, we
49
50 498 have not found any study that assesses the effect of adding commercially available
51
52 499 chocolate high in cocoa content to the usual diet in this population. Similarly, no studies
53
54 500 have been found that evaluate the effects on cognitive performance, quality of life and

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3 501 body composition of adding commercial chocolate with high cocoa content to the usual
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5 502 diet in postmenopausal women.

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8
9 504 This work will provide novel data helpful for the development of strategies in the
10
11 505 nutritional education of particularly vulnerable populations, given their high risk of
12
13 506 developing cardiovascular disease, including non-pharmacological therapies and
14
15 507 strategies that employ lifestyle modification. This intervention might also have
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17 508 implications for the preparation of recommendations in clinical practice guidelines and
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19 509 quality improvement programs aimed at the care of postmenopausal women.
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3 713 **AUTHORS' CONTRIBUTIONS:**

4 714 JIR, JAM, LGO and IGY contributed to the conception and design of the study. IGY,
5
6 715 JIR and JAM prepared the manuscript of the study protocol. JIR, JAM, LGO, RAD,
7
8 716 SMS, JGS, SMG, ERS, MGM and IGY contributed to the development of the study
9
10 717 protocol. JIR, JAM, LGO, RAD, SMS, JGS, SMG, ERS, MGM and IGY provided
11
12 718 assistance with statistical methodology and knowledge. JIR, JAM, LGO, RAD, SMS,
13
14 719 JGS, SMG, ERS, MGM and IGY provided a critical review of the manuscript.
15
16 720 All authors have read and accepted the final version of the protocol.
17

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19 721

20 722 **FUNDING STATEMENT:**

21
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23
24 724 y León (GRS 1583/B/17). "Lindt & Sprüngli will provide the necessary chocolate for the
25
26 725 implementation of the study. This company will not play any role in the design of the
27
28 726 study, data analysis, reporting of results or the decision to present the manuscript for
29
30 727 publication".
31

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34 729 **COMPETING INTERESTS STATEMENT:**

35
36 730 The authors declare that they have no conflicts of interest.
37

38
39 731

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53
54 739 González-Sánchez.
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741 **FIGURE LEGEND:**

742 Figure 1. Study flow chart.

743 Table 1. Polyphenol composition of 99% cocoa chocolate

744

745 **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

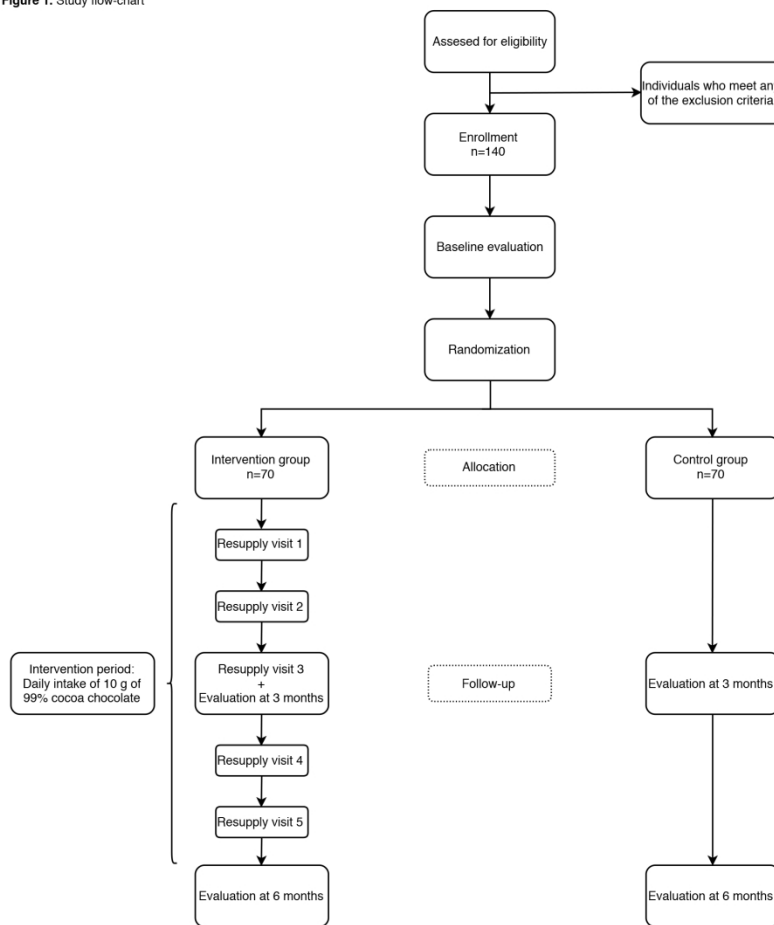


Figure 1

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 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

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3	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
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5	Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	Pag 9, line 198
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8	Methods: Assignment of interventions (for controlled trials)			
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10	Allocation:			
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12	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
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17	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
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21	Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	Pag 9, line 219
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24	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
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27		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
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31	Methods: Data collection, management, and analysis			
32				
33	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
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38		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
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3	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
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7	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
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10		20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	Pag 18, line 443
11				
12		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
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15 **Methods: Monitoring**

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17	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
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22		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
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25	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
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28	Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	NA
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32 **Ethics and dissemination**

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34	Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	Pag 18, line 467
35				
36				
37	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
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3	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
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6		26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	NA
7				
8	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
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11	Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	Pag 25, line 730
12				
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14	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
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17	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
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20	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
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25		31b	Authorship eligibility guidelines and any intended use of professional writers	Pag 19, line 481
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27		31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	Pag 19, line 481
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29	Appendices			
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31	Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	NA
32				
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34	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
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37 *It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items.
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