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The effect of resveratrol supplementation on the expression levels of factors associated with cellular senescence and sCD163/sTWEAK ratio in Patients with Type 2 Diabetes Mellitus: Study Protocol for a double blind Controlled Randomized Clinical Trial

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Complete List of Authors:	Abdollahi, Shima Salehi-Abargouei , Amin tabatabaie, Mahtab sheikhha, Mohammad Hasan Fallahzadeh, Hossein Rahmanian, Masoud Toupchian, Omid Karimi-Nazari, Elham mozaffari-khosravi, hassan; Shahid Sadoughi University of Medical Sciences and Health Services,
Keywords:	Type 2 diabetes mellitus, Cardiovascular Diseases, Resveratrol, Peroxisome Proliferator Activated Receptors, p53, p16

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Manuscripts

1 1 **The effect of resveratrol supplementation on the expression levels of**
2 2 **factors associated with cellular senescence and sCD163/sTWEAK ratio**
3 3 **in Patients with Type 2 Diabetes Mellitus: Study Protocol for a double**
4 4 **blind Controlled Randomized Clinical Trial**

5
6 6 Shima Abdollahi^{1,2}, Amin Salehi-Abargouei^{1,2}, Mahtab Tabatabaie^{1,2}, Mohammad
7 7 Hasan Sheikhha^{3,4}, Hossein Fallahzadeh⁵, Masoud Rahmanian⁶, Omid Toupchian⁷,
8 8 Elham Karimi-Nazari^{1,8}, Hassan Mozaffari-khosravi^{1,2*}

9
10 10 ¹ Nutrition and Food Security Research Center, Shahid Sadoughi University of Medical
11 11 Sciences, Yazd, Iran.

12 12 ² Department of Nutrition, School of Public Health, Shahid Sadoughi University of
13 13 Medical Sciences, Yazd, Iran.

14 14 ³ Department of Genetics, Faculty of Medicine, Yazd, Iran.

15 15 ⁴ Yazd Clinical and Research Center for Infertility, Yazd, Iran.

16 16 ⁵ Department of Biostatistics and Epidemiology, Research Center of Prevention and
17 17 Epidemiology of Non-Communicable Disease, School of Health, Shahid Sadoughi
18 18 University of Medical Sciences, Yazd, Iran.

19 19 ⁶ Department of Endocrinology and Metabolism, Shahid Sadoughi University of
20 20 Medical Sciences and Health Services, Yazd, Iran.

21 21 ⁷ School of Public Health, North Khorasan University of Medical Sciences, Bojnurd,
22 22 Iran.

23 23 ⁸ Biological Sciences and Technology Institute, Malek Ashtar University of
24 24 Technology, Tehran, Iran.

25
26 26 Address all correspondence and requests for reprints to: Hassan Mozaffari-khosravi,
27 27 Nutrition and Food Security Research Center, Shahid Sadoughi University of Medical

1
2 28 Sciences, Yazd, Iran and Department of Nutrition, School of Public Health, Shahid
3
4 29 Sadoughi University of Medical Sciences, Yazd, Iran.
5
6 30 E-mail: dr.mozaffarihasan@gmail.com
7
8 31 Tel: (9835)38209143
9
10 32 Email addresses:
11
12 33 Shima Abdollahi: sh.abd6864@yahoo.com
13
14 34 Amin Salehi-Abargouei: abargouei@gmail.com
15
16 35 Mohammad Hasan Sheikhha: sheikhha@yahoo.com
17
18 36 Hossein Fallahzadeh: fallahzadeh.ho@gmail.com
19
20 37 Masoud Rahmanian: drmasoudrahmanian@yahoo.com
21
22 38 Omid Toupchian: o.toupchian@gmail.com
23
24 39 Mahtab Tabatabaie: mahtab.tabatabaie.fb@gmail.com
25
26 40 elham karimi-Nazari: elham939k@gmail.com
27
28 41 Hassan Mozaffari-khosravi: dr.mozaffarihasan@gmail.com
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53 **Abstract**

54 **Introduction:** Type 2 Diabetes Mellitus (T2DM) is one of the challenges of the health
55 care system. Over the past decades, the numbers of people with diabetes has increased,
56 globally. One of the major complication in these patients is cardiovascular disease; it
57 seems that the cell cycle arrest can improve vascular function in these patients.
58 Resveratrol is a natural polyphenol and appears to improve the vascular function
59 through several cellular pathways. We will aim to evaluate the effects of resveratrol
60 supplementation on mRNA expression of peroxisome proliferator activated receptor
61 alpha (PPAR α), tumor suppressor protein p53, cyclin-dependent kinase inhibitor 1
62 (p21) and Cyclin-dependent kinase inhibitor 2A (p16) in patients with T2DM. We will
63 also measure serum levels of Cluster of Differentiation 163 (CD163) and TNF-like
64 weak inducer of apoptosis (TWEAK) as the indicators of cardiovascular status.

65 **Methods and analysis:** Seventy-two subjects suffering from T2DM will be participated
66 in this double blind randomized controlled clinical trial. Participants will be randomly
67 assigned to receive 1000 mg/day trans-resveratrol or placebo (methyl cellulose) for 8
68 weeks. The mRNA expression levels of PPAR α , p53, p21 and p16 genes will be
69 assessed using real-time polymerase chain reaction (PCR) and serum CD163 and
70 TWEAK levels will be measured using commercially available ELISA kits at the
71 baseline and the end of the study.

72 **Ethics and dissemination:** The study is performed in agreement with the Declaration
73 of Helsinki and is approved by the Ethics Committee of the Shahid Sadoughi University
74 of Medical Sciences (no: ir.ssu.sph.rec.1396.120). The results will be published in
75 scientific journals. We will also use conferences and social media to disseminate our
76 findings.

77 Trial registration number: IRCT20171118037528N1, Registered 29 December
78 2017)

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4 80 **Keywords:** *Type 2 diabetes mellitus, Cardiovascular Diseases, Resveratrol,*

5
6 81 *Peroxisome Proliferator Activated Receptors, p53, p16, p21, TWEAK, CD163*

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10 83 **‘Strengths and limitations of this study’**

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13 84 • To our knowledge, this is the first human study to investigate the effects of
14
15 85 resveratrol supplementation on the cellular factors associated with intima
16
17 86 hyperplasia through the cellular pathways.
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19 87 • This study is a first trial that uses resveratrol as a natural ligand for PPAR α .
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22 88 • The study is not designed to follow up the patients to determine the long-term
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24 89 effects of resveratrol supplementation.
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101 **Introduction**

102 With an increasing trend in its prevalence, type 2 diabetes mellitus (T2DM) has become
103 one of the important causes of mortality and morbidity worldwide (1-3). uncontrolled
104 T2DM might lead to a broad range of micro- and macro-vascular complications
105 including vascular dysfunction and cardiovascular disease (CVD) (4-6). Atherosclerosis
106 is one of the most important causes of CVD which leads to intima hyperplasia (IH) (4).
107 IH is associated with CVD and recently, it has been suggested to be added to the
108 Framingham risk factors (7). In addition, IH might lead to the restenosis after
109 percutaneous transluminal angioplasty or vascular graft and it is known as one of the
110 major complications during the treatment of CVD (8-10). Proliferation of Vascular
111 Smooth Muscle Cells (VSMCs) is increased during IH and is in accordance with
112 vascular stenosis and heart attack (11). Recent animal studies indicated that the
113 VSMCs' proliferation inhibition through cell cycle arrest can reduce the IH levels (12,
114 13).

115 Some cellular studies suggest that peroxisome proliferator activated receptors (PPARs)
116 also play a key role in VSMCs' proliferation (14, 15). PPARs are a group of nuclear
117 receptors with various isoforms, including α , β/δ and γ that are involved in transcription
118 regulation of a broad range of genes (16, 17). PPAR α is one of the members of this
119 family and has a critical role in the regulation of genes involved in fatty acid oxidation,
120 glucose metabolism, vascular function, obesity, cell proliferation, plaque stability and
121 inflammation (18, 19). Some body of evidences showed that PPAR α activation might
122 arrest the cell cycle progression in G₁/S phase through induction of the p16INK4a (6,
123 20). Cyclin-dependent kinase inhibitor 2A, which is also known as p16INK4a, is a
124 tumor suppressor that inhibits CDK4-mediated phosphorylation of retinoblastoma and
125 inhibits induction of E2F-dependent genes and therefore suppresses cell cycle
126 progression (21-23).

1
2 127 Resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is structurally known as stilbenoid
3
4 128 and phytoalexin, is a type of natural polyphenol found mostly in red grapes; it has been
5
6 129 introduced as a ligand of PPAR α and it seems to stimulate cellular senescence through
7
8 130 the above mentioned pathways (24-26). It also has been proposed that resveratrol has
9
10 131 the potential to activate p53, another important tumor suppressor, via phosphorylating
11
12 132 the serine residue in p53 protein through extracellular kinases (27, 28); phosphorylated
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14 133 p53 is proposed to be able to upregulate the cyclin-dependent kinase inhibitor 1 (p21)
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16 134 gene, which thereby inhibits CDK2 activity and induces the cell cycle arrest in S and
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18 135 G2 phase (29, 30). Animal studies indicated that p53 plays a key role in decreasing the
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20 136 intima thickness (31-34).

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22
23 137 Insulin resistance induces chronic inflammation via increased macrophage activity and
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25 138 overexpression of pro-inflammatory cytokines (35). TNF-related weak inducer of
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27 139 apoptosis (TWEAK) is a member of TNF superfamily which is mainly produced by
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29 140 macrophages and is released into the circulation in its soluble form (sTWEAK) (36).
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31 141 Studies have found that sTWEAK levels are reduced in T1DM, T2DM as well as in the
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33 142 presence of CVD risk factors (37-39). The main cause of reduced sTWEAK levels is its
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35 143 binding to fibroblast growth factor-inducible 14 (Fn14) receptor, which therefore can
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37 144 results in inflammatory responses (40).

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40 145 Intraplaque hemorrhage is a common feature of atherosclerotic plaques that is
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42 146 prevalent in patients with T2DM (41, 42); studies have shown that Intraplaque
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44 147 hemorrhage is most likely to occur in unstable plaques and is associated with ischemic
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46 148 stroke (43-45). On the other hand, cluster of differentiation 163 (CD163) is a
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48 149 macrophage scavenger receptor that is involved in the uptake of hemoglobin-
49
50 150 haptoglobin complexes and also known as a scavenger of sTWEAK (46, 47); sCD163 is
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52 151 the soluble form of this receptor and it has been proposed that sCD163/sTWEAK ratio
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54 152 can be used as an indicator of the severity and progression of vascular disease (37, 48,
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1
2 153 49). Resveratrol, as an antioxidant, can reduce inflammatory responses and
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4 154 macrophages activity (50) and thus, it seems that resveratrol might affect
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6 155 sCD163/sTWEAK ratio in patients with T2DM.
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8 156 We proposed to start a randomized clinical trial (RCT) with the following objectives:
9

10 157 i. To investigate the effect of resveratrol supplementation on the changes in
11
12 158 PPAR α , p16, p53 and p21 gene expression as well as serum level of sCD163
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14 159 and sTWEAK in patients with T2DM.
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16 160 ii. To compare the changes in serum levels of lipid profile, including
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18 161 triglyceride, total cholesterol, high density of lipoprotein cholesterol (HDL-
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20 162 C) and low density of lipoprotein cholesterol (LDL-C) as well as glycemic
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22 163 control indices including fasting blood sugar (FBS), fasting insulin,
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24 164 glycosylated hemoglobin (HBA1c) and pancreatic beta cell function and
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26 165 atherogenic index of plasma between the intervention and control groups.
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31 32 167 **Methods and analysis**

33 34 35 168 ***Study design***

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38 169 We designed a double-blind, randomized placebo controlled clinical trial among
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40 170 patients with T2DM; we randomly assigned patients to receive either 1000 mg
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42 171 resveratrol or placebo in a daily manner for two months. This monocentral study will be
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44 172 conducted in Diabetes Clinic Center in Yazd, Iran. The overall overview of the study is
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46 173 presented in Figure 1. Any methodological changes in the study design or sample size,
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48 174 which may potentially affect the patients' safety or study procedures, will be discussed
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50 175 in the committee of ethics before implementation.
51

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53 54 55 177 ***Randomization***

1
2 178 The present study will be an 8-week double-blind parallel RCT; patients will be
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4 179 randomized 1:1 according to the method of stratified block randomization based on sex
5
6 180 (male and female) and age (30-45 and 45-60 years).

7
8 181 Computer-generated random numbers will be used to randomly allocate eligible
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10 182 participants into the intervention group to receive two 500-mg capsules of resveratrol
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12 183 per day, or placebo group to receive two 500-mg capsules of methylcellulose per day;
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14 184 resveratrol supplements and placebo will be provided in the same shape, color and
15
16 185 appearance and will be packed in the same bottles and a person, who is not involved in
17
18 186 this project, will label the containers as A or B. Participants and administrators will be
19
20 187 unaware about the content of the bottles. Each bottle contains 60 capsules (providing
21
22 188 supplement for one month). All participants will be requested to bring back the first
23
24 189 bottle after first month and then will be given the second bottle. At the end of the study,
25
26 190 if the remained capsules of every patient exceed 10% of the total administered capsules
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28 191 (12 capsules), that patient will be categorized as non-adherent. There will be some
29
30 192 advices for enhancing the participant's compliance such as taking capsules with meals.
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32 193 Moreover, patients who complete the intervention will have an 8-hour nutrition
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34 194 education program for free. All randomized patients, including those who will complete
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36 195 the study or those who will not complete due to any reasons, will follow the same
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38 196 schedule.
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45 198 ***Eligibility criteria***

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47 199 30 to 60 year-old male and female, who have been diagnosed with established T2DM
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49 200 for at least three months prior to the intervention, will be invited to participate in the
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51 201 study; participants with the following criteria will be excluded: 1) diagnosis of any
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53 202 liver, kidney, cancer and Alzheimer's diseases or gastrointestinal ulcer; 2) pregnancy or
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55 203 lactation; 3) insulin therapy or the HbA1c levels at or above 8% at any point of study;
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2 204 4) consumption of supplements containing fish oil, vitamin E or C and red wine in the
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4 205 previous six months; 5) a history of allergic reaction to grapes; 6) consumption of
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6 206 anticoagulants, fibrates and anti-inflammatory agents; 7) a history of myocardial
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8 207 infraction or the presence of stent or battery in patients's heart.
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12 13 209 ***Sample size***

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15 210 Sample size is calculated based on a pervious human study regarding the PPAR α
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17 211 expression in peripheral blood nuclear cells (PBMCs) as the primary variable (51); the
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19 212 participant numbers needed in each group is calculated using a proposed formula for
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21 213 parallel clinical trials via considering $\alpha=0.05$ and a power of 80% (52). Assuming a
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23 214 20% of dropout rate, the final sample size is set to be 36 participants in each group.
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28 29 216 ***Intervention***

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31 217 Two trained researchers will introduce the study protocol to the participants. Written
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33 218 consent form will be obtained from all individuals who will decide to participate in the
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35 219 study. Patients will also receive information sheets. The related questionnaires will be
36
37 220 reviewed and approved by the ethical committee members.

38
39 221 Participants in the intervention group will take two capsules of resveratrol per day (one
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41 222 at breakfast and another at dinner) and individuals in the placebo group will take two
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43 223 capsules of 500 mg methylcellulose per day, at the same time for 8 weeks; each capsule
44
45 224 of resveratrol contains 500 mg of 99.71% micronized trans-resveratrol (particle size:
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47 225 <1.9 μm) which provides 495 mg trans-resveratrol without any inactive ingredients,
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49 226 fillers, additives or preservatives (Mega-Resveratrol, USA). Moreover, taking routine
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51 227 drugs prescribed by doctors, due to diabetes, will be permitted until their dosages
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53 228 remain unchanged during the study.
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2 229 Any possible adverse event will be reported to the medical ethics committee within a
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4 230 week and Shahid Sadoughi University of Medical Sciences will be responsible for any
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6 231 participation related problems. Some of the participants may withdraw from the study
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8 232 for any reason at any time, before or after signing the consent form; the investigator
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10 233 also may terminate an individual's participation in the study in order to keep the safety
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12 234 and protect the participant from excessive risks and/or to maintain the integrity of data
13
14 235 due to the improper follow-up of the procedures by participant.

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16
17 236 General information including age, diseases, medications and supplements will be
18
19 237 recorded through interviews at the beginning of the study. In order to obtain the
20
21 238 physical activity level, metabolic equivalents (METs) will be calculated through a
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23 239 questionnaire at the beginning and the end of study (53). For assessing the dietary
24
25 240 intakes, patients will be asked to complete a three-dietary record form (two weekdays
26
27 241 and one weekend day), once at the first week and another at the last week of the
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29 242 intervention; collected data will be analyzed using Nutritionist IV software (The Hearst
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31 243 Corporation, San Bruno, CA). All of the study related data will be stored confidentially.

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35 36 245 *Anthropometric measurements*

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39 246 Anthropometric parameters will be measured at the beginning and end of the
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41 247 intervention by the same person; body weight will be measured with light clothing with an
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43 248 accuracy level of 100 gram and height will be measured using Seca stadiometer with an
44
45 249 accuracy level of 0.5 cm; waist and hip circumferences will be measured to the nearest
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47 250 0.5 cm according to the standard methods, using a flexible tape (54). Body mass index
48
49 251 (BMI) will be calculated by dividing body weight (kg) by the height squared (m^2),
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51 252 waist-to hip-ratio (WHR) and waist-to-height ratio (WHtR) will be calculated via
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53 253 standard equations (6). Body composition analysis will be performed via InBody (USA)

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2 254 analyzer for estimating the measures of total body fat, abdominal fat, fat free mass and
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4 255 body liquids, before and after the intervention.
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8 257 ***Biochemical measurements***
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11 258 After 12 hours of fasting, 10 ml of venous blood will be collected. Biochemical
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13 259 analyses including fasting blood glucose, total cholesterol, triglycerides, HDL-C and
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15 260 LDL-C will be measured using automated enzymatic methods. Circulating insulin level
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17 261 and the percent of HbA1c will be assessed by enzyme-linked immunosorbent assay
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19 262 (ELISA) and high pressure liquid chromatography (HPLC), respectively. Commercially
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21 263 available ELISA kits will be used for estimating serum levels of sCD163 and
22
23 264 sTWEAK. Homeostatic model assessment of insulin resistance (HOMA-IR),
24
25 265 quantitative insulin sensitivity check index (QUICKI) as an insulin sensitivity index and
26
27 266 homeostasis model assessment of beta-cell function (HOMA-B) as well as atherogenic
28
29 267 index will be calculated using the suggested formulas (55, 56). All laboratory data will
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31 268 be identified by an ID number to maintain the confidentiality of every participant.
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37 270 ***Gene expression assay***
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40 271 After isolating PBMCs from whole blood by Ficoll-paque method (57), total mRNA
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42 272 will be extracted using Blood RNA kit (GeneAll Biotechnology, Korea); total extracted
43
44 273 mRNA will be reverse transcribed to cDNA by cDNA synthesis kit (GeneAll
45
46 274 Biotechnology, Korea). Real-time polymerase chain reaction (PCR) will be applied to
47
48 275 assess the mRNA expression levels of PPAR α , p53, p21 and p16. Glyceraldehyde
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50 276 phosphate dehydrogenase (GAPDH) will be the housekeeping gene in real-time PCR
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52 277 assessments.
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2 279 ***Statistical analysis***
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4 280 Principal researchers will have full access to the final data sets. Data entry and
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6 281 statistical analyses will be performed using SPSS for Windows (SPSS, Chicago, IL,
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8 282 USA), version 23.0; categorical data will be presented as number and percentages in
9
10 283 study groups. The intervention and the control arms will be compared with each other
11
12 284 for primary analysis. One-sample Kolmogorov-Smirnov test will be conducted to check
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14 285 normal distribution of continuous data; continuous variable will be expressed as means
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16 286 \pm SD or median and interquartile range. Independent sample t-test will be carried out
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18 287 for comparing parametric continuous data and Mann-Whitney U test will be used to test
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20 288 the differences in asymmetric variables between the two groups. Pearson's correlation
21
22 289 coefficient will be applied to show the correlation between biochemical and
23
24 290 anthropometric indices. General linear models will be used to assess the effects of
25
26 291 resveratrol relative to placebo after adjustment for baseline values. P-value ≤ 0.05 will
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28 292 be defined as statistically significance for all tests.
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32 293 Data analysis will be performed on two sets including the intention-to-treat (ITT) and
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34 294 the “per protocol” analysis; ITT analysis considers all patients in the intervention or
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36 295 control groups as originally allocated by randomization, independently of their actual
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38 296 adherence to the determined treatment and it ignores anything that happens after
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40 297 randomization including misallocation, noncompliance, withdrawal or protocol
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42 298 deviations; In the case that researchers observe a significant difference between those
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44 299 who will be allocated to receive the intervention and those who actually will adhere to
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46 300 the intervention, additional analysis will be performed considering the actual adherence
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48 301 to the treatment (per protocol analysis). The results of the two mentioned analyses will
49
50 302 be compared with each other.
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55
56 304 **Ethics and dissemination**
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1
2 305 Written consent form will be obtained from all patients before the study initiation. This
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4 306 protocol is approved by the Ethics Committee of the Shahid Sadoughi University of
5
6 307 Medical Sciences (no: ir.ssu.sph.rec.1396.120). This study is registered at the Iranian
7
8 308 Registry of Clinical Trials (IRCT20171118037528N1).
9

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13
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15
16

17 312 18 313 **Competing interests statement**

19
20
21 314 Part of the cost of this study will be borne by INSF. The funding body had no role in the
22
23 315 design of the study or in the writing of this manuscript or decision to submit the
24
25 316 manuscript for publication. They will also have no role in any aspect of the described
26
27 317 data management, analysis or the reporting of study results. The authors declare that
28
29 318 they have no competing interests.
30
31

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45
46

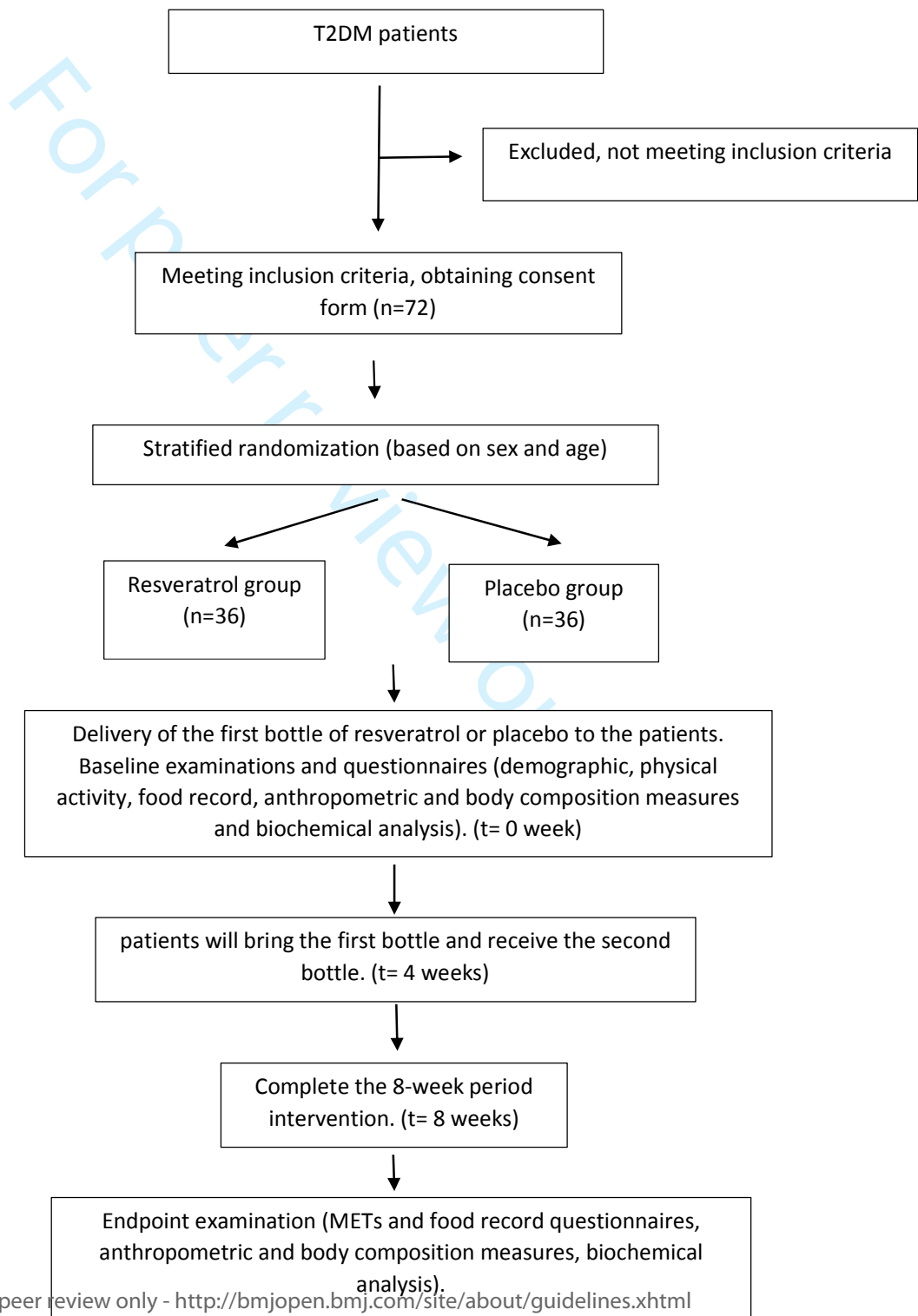
47 325 48 326 **Authors' contributions**

49
50
51 327 Ash: initial idea of this study, Study design, grant, major contributor in writing the
52
53 328 manuscript, reviewed all version of the paper. SA: Study design, grant, reviewed and
54
55 329 contributed to manuscript. MH: Study design, grant, reviewed and contributed to
56
57

1
2 330 manuscript, submission. FH: provided statistical expertise in clinical trial design,
3
4 331 sample size calculation. ShM and EK: Contributed to design of biochemical procedures,
5
6 332 reviewed and contributed to manuscript. RH: Contributed to study design, reviewed and
7
8 333 contributed to manuscript. TO: manuscript revisions and edit the final edition of the
9
10 334 manuscript. TM: contributed to manuscript and contributed to the edit of the
11
12 335 manuscript. All authors read and approved the final manuscript.
13
14
15 336

16 17 337 **List of abbreviations**

18
19 338 PPAR α : peroxisome proliferator activated receptor alpha; p21: cyclin-dependent kinase
20
21 339 inhibitor 1; p16: Cyclin-dependent kinase inhibitor 2A; CD163: Cluster of
22
23 340 Differentiation 163; TWEAK: TNF-like weak inducer of apoptosis; BMI: Body mass
24
25 341 index; GAPDH: Glyceraldehyde phosphate dehydrogenase; WHtR: waist-to-height
26
27 342 ratio; WHR: waist-to hip-ratio; ELISA: enzyme-linked immunosorbent assay; HPLC:
28
29 343 high pressure liquid chromatography; METs: metabolic equivalents; HDL: high density
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31 344 lipoprotein; LDL: low density lipoprotein; FBS: fasting blood sugar; HBA1c:
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33 345 glycosylated hemoglobin; T2DM: type 2 diabetes mellitus; CVD: cardiovascular
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35 346 disease; ITT: intention-to-treat.
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Figure 1: Overview of the Study

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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		
Protocol version	3	Date and version identifier	-
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	2 & 13
	5b	Name and contact information for the trial sponsor	-
	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	13
	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)	-
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	-
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)	7
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	7
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 & 9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8
	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)	-
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	7
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)	15
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	9
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	-
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	8
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	-
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	-
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors,	8

		data analysts), and how	
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	-
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	-
	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	8
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	10
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	11
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____
	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)	12
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	_____
	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	12
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	9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____

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)	7
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
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	10
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
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	11
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	9
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	_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
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	_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.

BMJ Open

The effect of resveratrol supplementation on the expression levels of factors associated with cellular senescence and sCD163/sTWEAK ratio in patients with type 2 diabetes mellitus: study protocol for a double-blind controlled randomized clinical trial

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1 **The effect of resveratrol supplementation on the expression levels of**
2 **factors associated with cellular senescence and sCD163/sTWEAK ratio**
3 **in patients with type 2 diabetes mellitus: study protocol for a double-**
4 **blind controlled randomized clinical trial**

6 Shima Abdollahi^{1,2,7}, Amin Salehi-Abargouei^{1,2}, Mahtab Tabatabaie^{1,2}, Mohammad
7 Hasan Sheikhha^{3,4}, Hossein Fallahzadeh⁵, Masoud Rahmanian⁶, Omid Toupchian⁷,
8 Elham Karimi-Nazari^{1,8}, Hassan Mozaffari-Khosravi^{1,2*}

10 ¹Nutrition and Food Security Research Center, Shahid Sadoughi University of Medical
11 Sciences, Yazd, Iran.

12 ²Department of Nutrition, School of Public Health, Shahid Sadoughi University of
13 Medical Sciences, Yazd, Iran.

14 ³Department of Genetics, Faculty of Medicine, Shahid Sadoughi University of Medical
15 Sciences, Yazd, Iran.

16 ⁴Yazd Clinical and Research Center for Infertility, Shahid Sadoughi University of
17 Medical Sciences, Yazd, Iran.

18 ⁵Department of Biostatistics and Epidemiology, Research Center of Prevention and
19 Epidemiology of Non-Communicable Disease, School of Health, Shahid Sadoughi
20 University of Medical Sciences, Yazd, Iran.

21 ⁶Yazd Diabetic Research Center, Shahid Sadoughi University of Medical Sciences,
22 Yazd, Iran.

23 ⁷ Department of Nutrition and Public Health, School of Public Health, North Khorasan
24 University of Medical Sciences, Bojnurd, Iran.

25 ⁸Biological Sciences and Technology Institute, Malek Ashtar University of Technology,
26 Tehran, Iran.

1
2 28 Address all correspondence and requests for reprints to: Hassan Mozaffari-Khosravi,
3
4 29 Nutrition and Food Security Research Center, Shahid Sadoughi University of Medical
5
6 30 Sciences, Yazd, Iran and Department of Nutrition, School of Public Health, Shahid
7
8 31 Sadoughi University of Medical Sciences, Yazd, Iran.

9
10
11 32 E-mail: dr.mozaffarihasan@gmail.com

12
13 33 Tel: (9835)38209143

14
15
16 34 Email addresses:

17
18 35 Shima Abdollahi: sh.abd6864@yahoo.com

19
20 36 Amin Salehi-Abargouei: abargouei@gmail.com

21
22 37 Mahtab Tabatabaie: mahtab.tabatabaie.fb@gmail.com

23
24 38 Mohammad Hasan Sheikhha: sheikhha@yahoo.com

25
26 39 Hossein Fallahzadeh: fallahzadeh.ho@gmail.com

27
28 40 Masoud Rahmanian: drmasoudrahmanian@yahoo.com

29
30 41 Omid Toupchian: o.toupchian@gmail.com

31
32 42 Elham Karimi-Nazari: elham939k@gmail.com

33
34 43 Hassan Mozaffari-Khosravi: dr.mozaffarihasan@gmail.com

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ABSTRACT

Introduction: Over the past decades, the number of people with type 2 diabetes (T2D) has increased, globally. One of the major complications in these patients is cardiovascular disease; it seems that the cell proliferation inhibition can improve vascular function in these patients. It is proposed that peroxisome proliferator activated receptor alpha (PPAR α), can induce cell cycle arrest via cyclin-dependent kinase inhibitor 2A (p16) activation. Also, it has been shown that phosphorylated tumor suppressor protein p53 is involved in cell senescence by cyclin-dependent kinase inhibitor 1 (p21) upregulation. Resveratrol is a natural polyphenol and appears to improve the vascular function through the mentioned pathways. We will aim to evaluate the effects of resveratrol supplementation on mRNA expression of PPAR α , p53, p21 and p16 in patients with T2D. We will also measure serum levels of cluster of differentiation 163 (CD163) and TNF-like weak inducer of apoptosis (TWEAK) as the indicators of cardiovascular status.

Methods and Analysis: Seventy-two subjects suffering from T2D will participate in this double blind randomized parallel placebo-controlled clinical trial. Participants will be randomly assigned to receive 1000 mg/day trans-resveratrol or placebo (methyl cellulose) for 8 weeks. The mRNA expression levels of PPAR α , p53, p21 and p16 genes will be assessed using real-time polymerase chain reaction (PCR) and serum CD163 and TWEAK levels will be measured using commercially available ELISA kits at baseline and the end of the study. Clinical outcomes parameters (glycemic and lipid profiles and body composition) will also be measured before and after study duration.

Ethics and dissemination: The study is performed in agreement with the Declaration of Helsinki and is approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (no: ir.ssu.sph.rec.1396.120). The results will be published in scientific journals.

Trial registration number: IRCT20171118037528N1, Registered 29 December 2017)

1
2 79 **Keywords:** *Type 2 diabetes mellitus, Cardiovascular Diseases, Resveratrol,*
3
4 80 *Peroxisome Proliferator Activated Receptors, p53, p16, p21, TWEAK, CD163*
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9 82 **‘Strengths and limitations of this study’**
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12 83 • To our knowledge, this is the first human study to investigate the effects of
13
14 84 resveratrol supplementation on the cellular factors associated with intimal
15
16 85 hyperplasia through the cellular pathways.
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18 86 • This study is a first trial that uses resveratrol as a natural ligand for PPAR α .
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20 87 • The study is not designed to follow up the patients to determine the long-term
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22 88 effects of resveratrol supplementation.
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100 INTRODUCTION

101 With an increasing trend in its prevalence, type 2 diabetes mellitus (T2DM) has become
102 one of the important causes of mortality and morbidity worldwide (1-3). Uncontrolled
103 T2DM might lead to a broad range of micro- and macro-vascular complications such as
104 vascular dysfunction and cardiovascular disease (CVD) (4-6). Atherosclerosis is one of
105 the most important causes of CVD which leads to intimal hyperplasia (IH) (4). IH is a
106 cardinal manifestation of atherosclerosis which is associated with CVD and recently, it
107 has been suggested to be added to the Framingham risk factors (7). In addition, IH might
108 lead to restenosis after percutaneous transluminal angioplasty or vascular graft and it is
109 known as one of the major complications during the treatment of CVD (8-10).
110 Proliferation of vascular smooth muscle cells (VSMCs) is increased during IH and is in
111 accordance with vascular stenosis and heart attack (11). Recent animal studies indicated
112 that the VSMCs' proliferation inhibition through cell cycle arrest can reduce the IH levels
113 (12, 13).

114 Some cellular studies suggest that peroxisome proliferator activated receptors (PPARs)
115 also play a key role in VSMCs' proliferation (14, 15). PPARs are a group of nuclear
116 receptors with various isoforms, including α , β/δ and γ that are involved in transcription
117 regulation of a broad range of genes (16, 17). PPAR α is one of the members of this family
118 and has a critical role in the regulation of genes involved in fatty acid oxidation, glucose
119 metabolism, vascular function, obesity, cell proliferation, plaque stability and
120 inflammation (18, 19). Some bodies of evidence showed that PPAR α activation might
121 arrest the cell cycle progression in G₁/S phase through induction of the p16INK4a (6, 20).
122 Cyclin-dependent kinase inhibitor 2A, which is also known as p16INK4a, is a tumor
123 suppressor that inhibits CDK4-mediated phosphorylation of retinoblastoma and inhibits
124 induction of E2F-dependent genes and therefore suppresses cell cycle progression (21-
125 23).

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2 126 Resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is structurally known as stilbenoid
3
4 127 and phytoalexin, is a type of natural polyphenol found mostly in red grapes; it has been
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6 128 introduced as a ligand of PPAR α and it seems to stimulate cellular senescence via the
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8
9 129 above mentioned pathways (24-26). It has also been proposed that resveratrol has the
10
11 130 potential to activate p53, another important tumor suppressor, by phosphorylating the
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13 131 serine residue in p53 protein through extracellular kinases (27, 28). Phosphorylated p53
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16 132 is proposed to be able to upregulate the cyclin-dependent kinase inhibitor 1 (p21) gene,
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18 133 thereby inhibiting CDK2 activity and induces the cell cycle arrest in S to G2 phase (29,
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20 134 30). Animal studies indicated that p53 plays a key role in decreasing the intimal thickness
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23 135 (31-34).

24
25 136 Insulin resistance induces chronic inflammation via increased macrophage activity and
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27 137 overexpression of pro-inflammatory cytokines (35). TNF-related weak inducer of
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29 138 apoptosis (TWEAK) is a member of TNF superfamily which is mainly produced by
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31 139 macrophages and is released into the circulation in its soluble form (sTWEAK) (36).
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34 140 Studies have shown that sTWEAK levels are reduced in T1DM, T2DM as well as in the
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36 141 presence of CVD risk factors (37-39). The main cause of reduced sTWEAK levels is its
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38 142 binding to fibroblast growth factor-inducible 14 (Fn14) receptor, which therefore can
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41 143 result in inflammatory responses (40).

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43 144 Intraplaque hemorrhage-common feature of atherosclerotic plaques- is prevalent in
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45 145 patients with T2DM (41, 42). Studies have shown that intraplaque hemorrhage is most
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47 146 likely to occur in unstable plaques and is associated with ischemic stroke (43-45). On the
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49 147 other hand, cluster of differentiation 163 (CD163) is a macrophage scavenger receptor
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51 148 that is involved in the uptake of hemoglobin-haptoglobin complexes and is also known
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53 149 as a scavenger of sTWEAK (46, 47); sCD163 is the soluble form of this receptor and it
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55 150 has been proposed that sCD163/sTWEAK ratio can be used as an indicator of the severity
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57 151 and progression of vascular diseases (37, 48, 49). Resveratrol, as an antioxidant, can

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2 152 reduce inflammatory responses and macrophages activity (50) and thus, it seems that
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4 153 resveratrol might affect sCD163/sTWEAK ratio in patients with T2DM. Given that no
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6 154 study has investigated this issue, this has prompted us to design a randomized clinical
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9 155 trial (RCT) with the following objectives:

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11 156 i. To investigate the effect of resveratrol supplementation on the changes in
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13 157 PPAR α , p16, p53 and p21 gene expression as well as serum levels of sCD163
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15 158 and sTWEAK in patients with T2DM.
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18 159 ii. To compare the changes in serum levels of lipid profile, including
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20 160 triglyceride, total cholesterol, high density lipoprotein cholesterol (HDL-C)
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22 161 and low density lipoprotein cholesterol (LDL-C) as well as glycemic control
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24 162 indices including fasting blood sugar (FBS), fasting insulin, glycosylated
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26 163 hemoglobin (HbA1c), pancreatic beta cell function and atherogenic index of
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28 164 plasma between the intervention and control groups.
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33 34 166 **METHODS AND ANALYSIS**

35 36 37 167 **Study design**

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40 168 We designed a double-blind, randomized parallel placebo-controlled clinical trial among
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42 169 patients with T2DM; we will randomly assign patients to receive either 1000 mg
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44 170 resveratrol or placebo in a daily manner for two months. This monocentral study will be
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46 171 conducted in Diabetes Clinic Center in Yazd, Iran. The overall overview of the study is
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48 172 presented in **Figure 1**. Any methodological changes in the study design or sample size,
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50 173 which may potentially affect the patients' safety or study procedures, will be discussed
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52 174 in the committee of ethics before implementation.
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58 59 176 **Randomization**

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2 177 The present study will be an 8-week double-blind parallel RCT; patients will be
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4 178 randomized 1:1 according to the method of stratified block randomization based on sex
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7 179 (male and female) and age (30-45 and 45-60 years).

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9 180 Computer-generated random numbers will be used to randomly allocate eligible
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11 181 participants into the one of the two trial groups by an independent statistician.
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13 182 Participants will be allocated to one of the two arms, using sealed envelope by a
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16 183 researcher who will not be involved in participant's enrollment (EKN) and assignment
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18 184 of intervention will be carried out by principal investigator (SA) who will be blinded to
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20 185 allocation. Resveratrol supplements and placebo will be provided in the same shape,
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22 186 color and appearance and will be packed in the same bottles and a person, who is not
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24 187 involved in this project, will label the containers as A or B. Participants and
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27 188 administrator will be unaware about the content of the bottles until data analyses.
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31 32 190 **Eligibility criteria**

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34 191 Thirty to sixty-year-old male and female, who have been diagnosed with established
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36 192 T2DM for at least three months prior to the intervention and are taking medication for
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38 193 diabetes, will be invited to participate in the study. Participants with the following
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40 194 criteria will be excluded: 1) diagnosis of any liver, kidney, cancer and Alzheimer's
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42 195 diseases or gastrointestinal ulcer; 2) pregnancy or lactation; 3) insulin therapy or the
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44 196 HbA1c levels at or above 8% at any point of study; 4) consumption of supplements
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46 197 containing fish oil, vitamin E or C in the previous six months; 5) a history of allergic
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48 198 reaction to grapes; 6) consumption of anticoagulants, fibrates and anti-inflammatory
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50 199 agents; 7) a history of myocardial infarction or the presence of stent or battery in
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52 200 patients's heart, and 8) consumption of red wine or supplements containing resveratrol
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54 201 in 6 months prior to intervention.
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203 **Sample size**

204 Sample size is calculated based on a previous human study regarding the PPAR α
205 expression in peripheral blood mononuclear cells (PBMCs) as the primary variable
206 (51). The participant numbers needed in each group is calculated using a proposed
207 formula for parallel clinical trials by considering $\alpha=0.05$ and a power of 80% (52).
208 Assuming a 20% of dropout rate, the final sample size is set to be 36 participants in
209 each group.

211 **Intervention**

212 Recruitment of participants will take place through installing announcements at Diabetes
213 Clinic Center in Yazd, Iran. Interested patients will be invited to a screening session and
214 two trained researchers (SA and MT) will introduce the study protocol to them and assess
215 eligibility criteria. Written consent form will be obtained from all eligible patients who
216 will decide to participate in the study. Participants will also receive information sheets.
217 Blood sample will be also obtained to assess HbA1c and eligible patients will be included
218 in the study. It should be mentioned that; ineligible patients will be excluded from the
219 study after receiving nutrition recommendations for diabetes.

220 General information including age, parity, education, medical information, duration of
221 the disease, etc. will be recorded through interviews at the beginning of the study. In
222 order to obtain the physical activity level, metabolic equivalents (METs) will be
223 calculated through a questionnaire at the beginning and the end of the study (53). To
224 assess the dietary intakes, participants will be asked to complete a three-dietary record
225 form (two weekdays and one weekend day), one at the first week and another at the last
226 week of the intervention; collected data will be analyzed using Nutritionist IV software
227 (The Hearst Corporation, San Bruno, CA). The questionnaires will be reviewed and

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2 228 approved by the ethical committee members. All the study related data will be stored
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4 229 confidentially.
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6 230 Participants in the intervention group will take two capsules of resveratrol per day (one
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8 231 at breakfast and another at dinner) and individuals in the placebo group will take two
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10 232 capsules of 500 mg methylcellulose per day, at the same time for 8 weeks; each capsule
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12 233 of resveratrol contains 500 mg of 99.71% micronized trans-resveratrol (particle size:
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14 234 <1.9 μm) which provides 495 mg trans-resveratrol without any inactive ingredients,
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16 235 fillers, additives or preservatives (Mega-Resveratrol, USA). Moreover, participants will
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18 236 continue taking diabetic medication prescribed by doctor during the study. Each bottle
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20 237 contains 60 capsules (providing supplement for one month). All participants will be
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22 238 requested to bring back the first bottle after first month and then they will be given the
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24 239 second bottle. At the end of the study, if the remaining capsules of every patient exceed
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26 240 10% of the total administered capsules (12 capsules), that patient will be categorized as
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28 241 non-adherent. There will be some advices for enhancing the participant's compliance
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30 242 such as taking capsules with meals. Moreover, patients who complete the intervention
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32 243 will have an 8-hour nutrition education program for free. All randomized patients,
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34 244 including those who will complete the study or those who will not complete due to any
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36 245 reasons, will follow the same schedule.
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38 246 Any possible adverse event will be reported to the medical ethics committee within a
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40 247 week and Shahid Sadoughi University of Medical Sciences will be responsible for any
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42 248 participation related problems. Some of the participants may withdraw from the study
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44 249 for any reason at any time, before or after signing the consent form; the investigator
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46 250 may also terminate an individual's participation in the study in order to keep the safety
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48 251 and protect the participant from excessive risks and/or to maintain the integrity of data
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50 252 due to the improper follow-up of the procedures by participant.
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2 254 **Data collection**

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4 255 *Anthropometric measurements*

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7 256 Anthropometric parameters will be taken at the beginning and end of the intervention by
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9 257 the same person. Height will be measured using a stadiometer (Seca, Hamburg, Germany)
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11 258 with an accuracy level of 0.5 cm; waist and hip circumferences will be measured to the
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13 259 nearest 0.5 cm according to the standard methods, using a flexible tape (54). Weight
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15 260 estimate and body composition analysis (% fat mass, % fat free mass and visceral fat)
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17 261 will be performed via InBody (USA) analyzer, with light clothing before and after the
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19 262 intervention. Body mass index (BMI) will be calculated by dividing body weight (kg) by
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21 263 the height squared (m^2), waist-to-hip-ratio (WHR) and waist-to-height ratio (WHtR) will
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23 264 be calculated via standard equations (6).
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30 266 *Biochemical measurements*

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33 267 After 12 hours of fasting, 10 ml of venous blood will be taken at baseline and week 8 of
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35 268 the study. A 6 ml of blood sample will be collected in the clot-activator tubes and
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37 269 centrifuged after 30 minutes clotting time (3000 g, 10 minutes at room temperature;
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39 270 Eppendorf AG, Hamburg, Germany) for serum isolation. Serum samples will be stored
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41 271 at $-70^{\circ}C$ until analyses. Remaining blood will be obtained in two EDTA-coated tubes for
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43 272 gene expression and HbA1c assessment, separately. Biochemical analyses including
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45 273 fasting blood glucose, total cholesterol, triglycerides, HDL-C and LDL-C will be
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47 274 measured using automated enzymatic methods and commercial kits (Pars Azmoon,
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49 275 Tehran, Iran). Laboratory kits will be used to assess circulating insulin levels and the
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51 276 percent of HbA1c will be assessed by enzyme-linked immunosorbent assay (ELISA,
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53 277 Monobind, USA) and high pressure liquid chromatography (HPLC, Pars Azmoon,
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55 278 Tehran, Iran), respectively. Commercially available ELISA kits will be used for
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1
2 279 estimating serum levels of sCD163 and sTWEAK. Homeostatic model assessment of
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4 280 insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI) as
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6 281 an insulin sensitivity index and homeostasis model assessment of beta-cell function
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8 282 (HOMA-B) as well as atherogenic index will be calculated using the suggested formulas
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10 283 (55, 56). All laboratory data will be identified by an identification number to maintain
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12 284 the confidentiality of participants.
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17 18 286 *Gene expression assay*

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21 287 Total RNA will be extracted directly from whole blood using GeneAll Hybrid-R
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23 288 purification kit protocol (GeneAll Biotechnology Co., Seoul, South Korea). The quality
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25 289 and purity (260/280 nm ratio between 1.8 to 2.2) of the RNA will be checked using
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27 290 spectrophotometer (NanoDrop, Thermo Scientific, USA). After normalization, high
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29 291 quality mRNA will be reverse transcribed to cDNA by cDNA synthesis kit (GeneAll
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31 292 Biotechnology Co., Seoul, South Korea) and according to the manufacturer's
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33 293 instruction. Three primer designing tools (Primer Blast, Oligocalc, and Gene runner
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35 294 5.0.99) are applied for sequencing the study primers (**Table 1**). Real-time polymerase
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37 295 chain reaction (PCR) and SYBR Green method (Takara Bio Inc., Japan) will be applied
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39 296 to assess the mRNA expression levels of PPAR α , p53, p21 and p16 in the StepOne
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41 297 system (Applied Biosystems, Foster City, California, USA). To this aim, 1 μ L of
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43 298 cDNA, 10 μ L of SYBR Green, 1 μ L of primers (revers and forward) and 0.4 μ L of
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45 299 Rox, will be mixed together. Final volume of solution will be reached to 20 μ L by
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47 300 adding ddH₂O (7/6 μ L). Real-time PCR will be adjusted for initial denaturation step at
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49 301 95 °C for 10 minutes, followed by 40 cycles of 90 °C for 15 seconds. The optimal
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51 302 annealing temperature for primers will be set at a range of 55-60 °C for 20 seconds. The
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53 303 final step will be set-up at 72 °C for 20 seconds, for primer extension. Glyceraldehyde
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55 304 phosphate dehydrogenase (GAPDH) will be the housekeeping gene in real-time PCR
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2 305 assessments. Real-time PCR efficacy and changes in expression levels will be tested
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4 306 using LinRegPCR software (57) and Pfaffl equation, respectively (58).
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10 308 **Statistical analysis**

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12 309 Principal researchers will have full access to the final data sets. Data entry and statistical
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14 310 analyses will be performed using SPSS for Windows (SPSS, Chicago, IL, USA), version
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16 311 23.0. The intervention and the control arms will be compared with each other for primary
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18 312 analysis. One-sample Kolmogorov-Smirnov test will be conducted to check normal
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20 313 distribution of data. Continuous variable will be expressed as means \pm SD or median and
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22 314 interquartile range and categorical data will be presented as number and percentages in
23
24 315 study groups. Independent sample t-test will be carried out for comparing parametric
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26 316 continuous data and Mann-Whitney U test will be used to test the differences in
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28 317 asymmetric variables between the two groups. Pearson's correlation coefficient will be
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30 318 applied to show the correlation between biochemical and anthropometric indices. General
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32 319 linear models will be used to assess the effects of resveratrol relative to placebo after
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34 320 adjustment for baseline values and participant's characteristics. P-value ≤ 0.05 will be
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36 321 defined as statistically significance for all tests.
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42 322 Data analysis will be performed on two sets including the intention-to-treat (ITT) and the
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44 323 "per protocol" analysis; ITT analysis considers all patients in the intervention or control
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46 324 groups as originally allocated by randomization, independently of their actual adherence
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48 325 to the determined treatment and it ignores anything that happens after randomization
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50 326 including misallocation, noncompliance, withdrawal or protocol deviations. In the case
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52 327 that researchers observe a significant difference between those who will be allocated to
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54 328 receive the intervention and those who will actually adhere to the intervention, additional
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2 329 analysis will be performed considering the actual adherence to the treatment (per protocol
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4 330 analysis). The results of the two mentioned analyses will be compared with each other.
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9 332 **Strengths and limitations**

11 333 This study has been designed as a double-blind randomized controlled clinical trial,
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13 334 which will investigate the effects of resveratrol supplementation on the cellular factors
14
15 335 associated with intimal hyperplasia for the first time. It will also be the first to use high-
16
17 336 bioavailable resveratrol supplement as a natural ligand for PPAR α in human. However,
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19 337 as a limitation, the labeling of the containers as A and B can result in unblinding entire
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21 338 group when unblinding is necessary; although, resveratrol has not shown serious
22
23 339 adverse events in previous studies. Another limitation in this study is the surrogate
24
25 340 markers that will be used for endothelial function assessment instead of gold-standard
26
27 341 methods such as flow-mediated dilation (FMD) or peripheral arterial tonometry (PAT).
28
29 342 Moreover, we will not perform oral glucose tolerance test (OGTT) or hyperinsulinemic
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31 343 clamp to evaluate glycemic control effects of resveratrol. Finally, this study is designed
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33 344 for short term assessment of resveratrol supplementation effects in patients with T2DM.
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41 346 **Patient and public involvement**

43 347 Patients or the public will not involve in the setting of the research question, outcome
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45 348 measures or study design and implementation. In this study, the intervention will
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47 349 involve taking daily supplement, and participants will not receive any lifestyle changes,
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49 350 so, participants will not be asked to assess the benefits and burdens of participating. The
50
51 351 summary results of the trial will be presented in a grouped form to scientific journals.
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53 352 Participants will be provided individual body composition report, as well as individual
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55 353 glycemic and lipid profile results, on request, when study is completed.
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1
2 355 **Ethical consideration**
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4 356 Written consent form will be obtained from all patients before the study initiation. This
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7 357 protocol is approved by the Ethics Committee of the Shahid Sadoughi University of
8
9 358 Medical Sciences (no: ir.ssu.sph.rec.1396.120). This study is registered at the Iranian
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11 359 Registry of Clinical Trials (IRCT20171118037528N1).
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15
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19
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21
22 364 (INSF).
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27
28 366 **Competing interests statement**
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30
31 367 Part of the cost of this study will be borne by INSF. The funding body had no role in the
32
33 368 design of the study or in the writing of this manuscript or decision to submit the
34
35 369 manuscript for publication. They will also have no role in any aspect of the described
36
37 370 data management, analysis or the reporting of study results. The authors declare that
38
39 371 they have no competing interests.
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45
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47

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52
53 376 scientific committee before the funding was approved (grant no: 96010660).
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58 378 **Authors' contributions**
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1
2 379 SA, ASA, OT, MHS, MR and HMK were involved in initial idea of this study and
3
4 380 designing the trial. SA, ASA and HMK were contributed in writing the manuscript and
5
6 381 getting grant. MT and EKN are co-investigator and will involve in collecting data,
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8
9 382 concealment procedure and counseling patients. HF provided statistical expertise in
10
11 383 clinical trial design, sample size calculation and blinding. MHS and EKN were
12
13 384 contributed to design of biochemical procedures. All authors read and approved the
14
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16 385 final manuscript.
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21 386

22 387 **List of abbreviations**

23 388 PPAR α , peroxisome proliferator activated receptor alpha; p21, cyclin-dependent kinase
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25 389 inhibitor 1; p16, cyclin-dependent kinase inhibitor 2A; CD163, cluster of differentiation
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27 390 163; TWEAK, TNF-like weak inducer of apoptosis; BMI, Body mass index; GAPDH,
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29 391 glyceraldehyde phosphate dehydrogenase; WHtR, waist-to-height ratio; WHR, waist-to
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31 392 hip-ratio; ELISA, enzyme-linked immunosorbent assay; HPLC, high pressure liquid
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33 393 chromatography; METs, metabolic equivalents; HDL, high density lipoprotein; LDL,
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35 394 low density lipoprotein; FBS, fasting blood sugar; HbA1c, glycosylated hemoglobin;
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37 395 T2DM, type 2 diabetes mellitus; CVD, cardiovascular disease; ITT, intention-to-treat.
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651 **Table 1:** Real-time PCR primer sequences

	Forward	Reverse
p53	GAGCTGAATGAGGCCTTGGA	CTGAGTCAGGCCCTTCTGTCTT
p21	TGGAGACTCTCAGGGTCGAAA	GGCGTTTGGAGTGGTAGAAATC
p16	CTTCCTGGACACGCTGGTG	GCATGGTTACTGCCTCTGGTG
PPAR α	CTATCATTTGCTGTGGAGATCG	AAGATATCGTCCGGGTGGTT
GAPDH (Reference gene)	TGGTATCGTGGAAGGACTCATG	GCTTCACCACCTTCTTGATGTC

652 GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase; PCR, Polymerase chain reaction; PPAR α ,

653 Peroxisome proliferator activated receptor alpha

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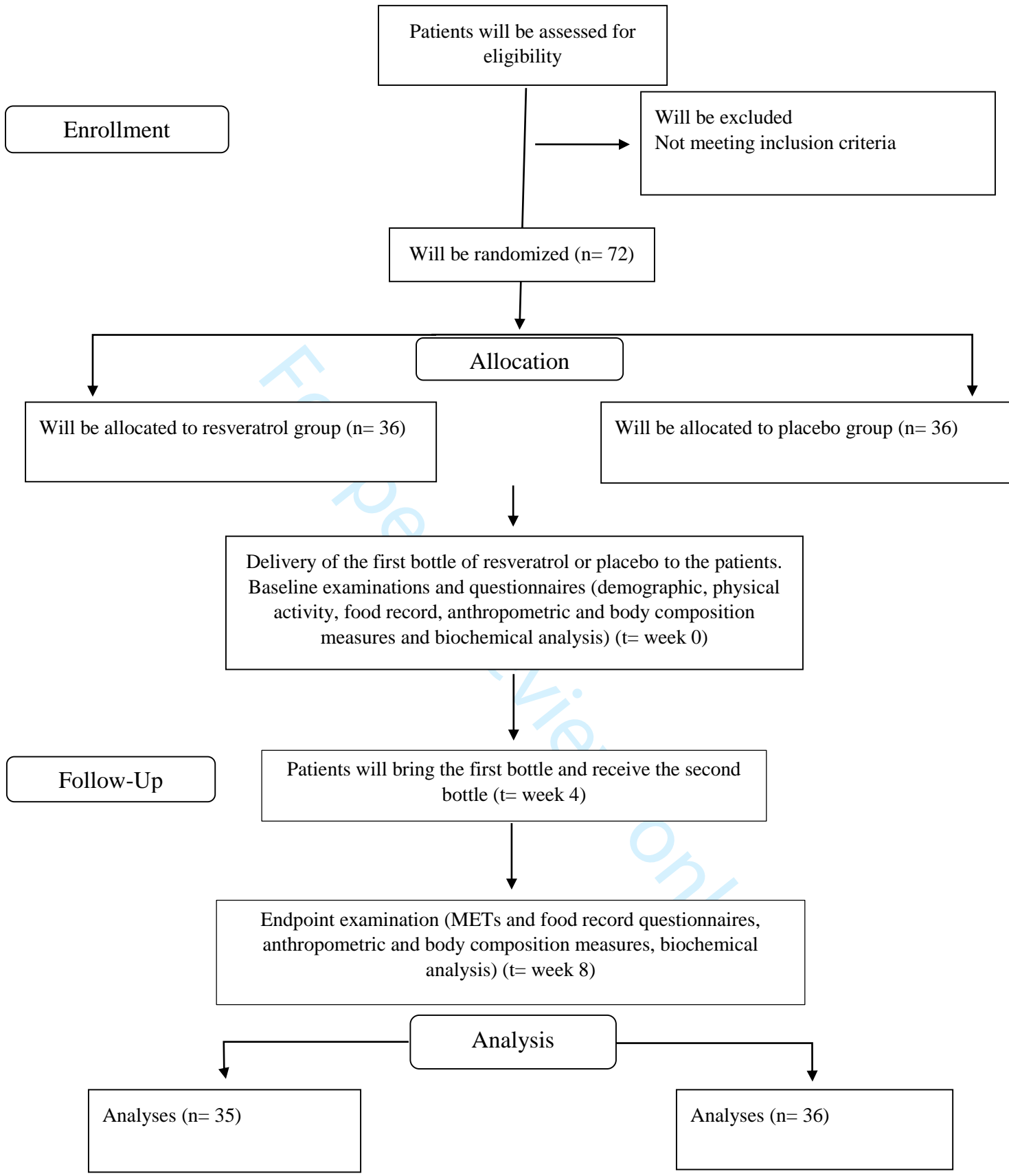


Figure 1: Overview of the study



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		
Protocol version	3	Date and version identifier	-
Funding	4	Sources and types of financial, material, and other support	13
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	2 & 13
	5b	Name and contact information for the trial sponsor	-
	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	13
	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)	-
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	-
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)	7
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	7
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 & 9
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	8
	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)	-
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	8
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	9
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	7
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)	15
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	9
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	-
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	8
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	-
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	-
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors,	8

		data analysts), and how	
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	-
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	-
	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	8
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	10
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	11
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	_____
	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)	12
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	_____
	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	12
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	9
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	_____
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	_____

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)	7
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	_____
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	_____
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	10
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	13
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	11
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	9
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	_____
	31b	Authorship eligibility guidelines and any intended use of professional writers	_____
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	_____
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	_____
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	_____

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "[Attribution-NonCommercial-NoDerivs 3.0 Unported](https://creativecommons.org/licenses/by-nc-nd/3.0/)" license.