

# 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 randomised clinical trial

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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 $\alpha$ ) can induce cell cycle arrest via cyclin-dependent kinase inhibitor 2A (p16) activation. Also, it has been shown that phosphorylated tumour 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 $\alpha$ , p53, p21 and p16 in patients with T2D. We will also measure serum levels of cluster of differentiation 163 (CD163) and tumour necrosis factor (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 randomised 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 $\alpha$ , p53, p21 and p16 genes will be assessed using real-time PCR and serum CD163 and TWEAK levels will be measured using commercially available ELISA kits at baseline and the end of the study. Clinical outcome parameters (glycaemic 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.

## Strengths and limitations of this study

- To our knowledge, this is the first human study to investigate the effects of resveratrol supplementation on the cellular factors associated with intimal hyperplasia through the cellular pathways.
- This study is the first trial that uses resveratrol as a natural ligand for PPAR $\alpha$ .
- This study is not designed to follow-up the patients to determine the long-term effects of resveratrol supplementation.

**Trial registration number** IRCT20171118037528N1; Pre-results.

## INTRODUCTION

With an increasing trend in its prevalence, type 2 diabetes mellitus (T2DM) has become one of the important causes of mortality and morbidity worldwide.<sup>1–3</sup> Uncontrolled T2DM might lead to a broad range of micro- and macrovascular complications such as vascular dysfunction and cardiovascular disease (CVD).<sup>4–6</sup> Atherosclerosis is one of the most important causes of CVD which leads to intimal hyperplasia (IH).<sup>4</sup> IH is a cardinal manifestation of atherosclerosis which is associated with CVD, and recently it has been suggested to be added to the Framingham risk factors.<sup>7</sup> In addition, IH might lead to restenosis after percutaneous transluminal angioplasty or vascular graft and it is known as one of the major complications during the treatment of CVD.<sup>8–10</sup> Proliferation of vascular

smooth muscle cells (VSMCs) is increased during IH and is in accordance with vascular stenosis and heart attack.<sup>11</sup> Recent animal studies indicated that the VSMCs' proliferation inhibition through cell cycle arrest can reduce the IH levels.<sup>12 13</sup>

Some cellular studies suggest that peroxisome proliferator-activated receptors (PPARs) also play a key role in VSMCs' proliferation.<sup>14 15</sup> PPARs are a group of nuclear receptors with various isoforms, including  $\alpha$ ,  $\beta/\delta$  and  $\gamma$  that are involved in transcription regulation of a broad range of genes.<sup>16 17</sup> PPAR $\alpha$  is one of the members of this family and has a critical role in the regulation of genes involved in fatty acid oxidation, glucose metabolism, vascular function, obesity, cell proliferation, plaque stability and inflammation.<sup>18 19</sup> Some bodies of evidence showed that PPAR $\alpha$  activation might arrest the cell cycle progression in G<sub>1</sub>/S phase through induction of the p16INK4a.<sup>6 15</sup> Cyclin-dependent kinase inhibitor 2A, which is also known as p16INK4a, is a tumour suppressor that inhibits CDK4-mediated phosphorylation of retinoblastoma and inhibits induction of E2F-dependent genes and therefore suppresses cell cycle progression.<sup>20–22</sup>

Resveratrol (3,5,4'-trihydroxy-trans-stilbene), which is structurally known as stilbenoid and phytoalexin, is a type of natural polyphenol found mostly in red grapes; it has been introduced as a ligand of PPAR $\alpha$  and it seems to stimulate cellular senescence via the above mentioned pathways.<sup>23–25</sup> It has also been proposed that resveratrol has the potential to activate p53, another important tumour suppressor, by phosphorylating the serine residue in p53 protein through extracellular kinases.<sup>26 27</sup> Phosphorylated p53 is proposed to be able to upregulate the cyclin-dependent kinase inhibitor 1 (p21) gene, thereby inhibiting CDK2 activity and induces the cell cycle arrest in S to G<sub>2</sub> phase.<sup>28 29</sup> Animal studies indicated that p53 plays a key role in decreasing the intimal thickness.<sup>30–33</sup>

Insulin resistance induces chronic inflammation via increased macrophage activity and overexpression of pro-inflammatory cytokines.<sup>34</sup> Tumour necrosis factor (TNF)-related weak inducer of apoptosis (TWEAK) is a member of TNF superfamily which is mainly produced by macrophages and is released into the circulation in its soluble form (sTWEAK).<sup>35</sup> Studies have shown that sTWEAK levels are reduced in T1DM, T2DM as well as in the presence of CVD risk factors.<sup>36–38</sup> The main cause of reduced sTWEAK levels is its binding to fibroblast growth factor-inducible 14 (Fn14) receptor, which therefore can result in inflammatory responses.<sup>39</sup>

Intraplaque haemorrhage—common feature of atherosclerotic plaques—is prevalent in patients with T2DM.<sup>40 41</sup> Studies have shown that intraplaque haemorrhage is most likely to occur in unstable plaques and is associated with ischaemic stroke.<sup>42–44</sup> On the other hand, cluster of differentiation 163 (CD163) is a macrophage scavenger receptor that is involved in the uptake of haemoglobin-haptoglobin complexes and is also known as a scavenger of sTWEAK<sup>45 46</sup>; sCD163 is the soluble form of this receptor and it has been proposed that sCD163/sTWEAK ratio can

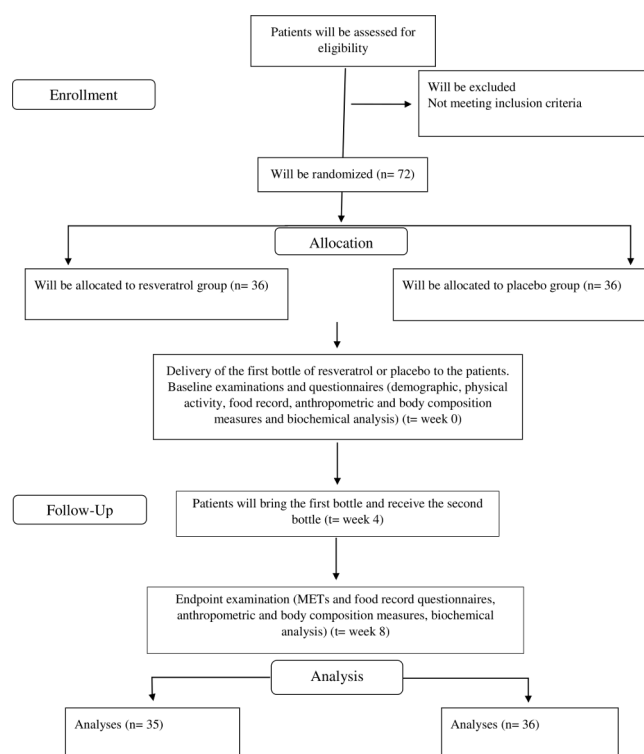
be used as an indicator of the severity and progression of vascular diseases.<sup>36 47 48</sup> Resveratrol, as an antioxidant, can reduce inflammatory responses and macrophages activity<sup>49</sup> and thus, it seems that resveratrol might affect sCD163/sTWEAK ratio in patients with T2DM. Given that no study has investigated this issue, this has prompted us to design a randomised clinical trial (RCT) with the following objectives:

- To investigate the effect of resveratrol supplementation on the changes in PPAR $\alpha$ , p16, p53 and p21 gene expression as well as serum levels of sCD163 and sTWEAK in patients with T2DM.
- To compare the changes in serum levels of lipid profile, including triglyceride, total cholesterol, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) as well as glycaemic control indices including fasting blood sugar, fasting insulin, glycosylated haemoglobin (HbA1c), pancreatic beta cell function and atherogenic index of plasma between the intervention and control groups.

## METHODS AND ANALYSIS

### Study design

We designed a double-blind, randomised parallel placebo-controlled clinical trial among patients with T2DM; we will randomly assign patients to receive either 1000 mg resveratrol or placebo in a daily manner for 2 months. This monocentral study will be conducted in Diabetes Clinic Center in Yazd, Iran. The overall overview of the study is presented in [figure 1](#). Any methodological changes in the study design or sample size, which may potentially affect



**Figure 1** Overview of the study.

the patients' safety or study procedures, will be discussed in the committee of ethics before implementation.

### Randomisation

The present study will be an 8-week double-blind parallel RCT; patients will be randomised 1:1 according to the method of stratified block randomisation based on sex (male and female) and age (30–45 and 45–60 years).

Computer-generated random numbers will be used to randomly allocate eligible participants into the one of the two trial groups by an independent statistician. Participants will be allocated to one of the two arms, using sealed envelope by a researcher who will not be involved in participant's enrolment (EKN) and assignment of intervention will be carried out by principal investigator (SA) who will be blinded to allocation. Resveratrol supplements and placebo will be provided in the same shape, colour and appearance and will be packed in the same bottles and a person, who is not involved in this project, will label the containers as A or B. Participants and administrator will be unaware about the content of the bottles until data analyses.

### Eligibility criteria

Thirty to sixty-year-old men and women, who have been diagnosed with established T2DM for at least 3 months prior to the intervention and are taking medication for diabetes, will be invited to participate in the study. Participants with the following criteria will be excluded: (1) diagnosis of any liver, kidney, cancer and Alzheimer's diseases or gastrointestinal ulcer; (2) pregnancy or lactation; (3) insulin therapy or the HbA1c levels at or above 8% at any point of study; (4) consumption of supplements containing fish oil, vitamin E or C in the previous 6 months; (5) a history of allergic reaction to grapes; (6) consumption of anticoagulants, fibrates and anti-inflammatory agents; (7) a history of myocardial infarction or the presence of stent or battery in patients's heart and (8) consumption of red wine or supplements containing resveratrol in 6 months prior to intervention.

### Sample size

Sample size is calculated based on a previous human study regarding the PPAR $\alpha$  expression in peripheral blood mononuclear cells (PBMCs) as the primary variable.<sup>50</sup> The participant numbers needed in each group is calculated using a proposed formula for parallel clinical trials by considering  $\alpha=0.05$  and a power of 80%.<sup>51</sup> Assuming a 20% of dropout rate, the final sample size is set to be 36 participants in each group.

### Intervention

Recruitment of participants will take place through installing announcements at Diabetes Clinic Center in Yazd, Iran. Interested patients will be invited to a screening session and two trained researchers (SA and MT) will introduce the study protocol to them and assess eligibility criteria. Written consent form will be obtained from all eligible patients who will decide to participate in

the study. Participants will also receive information sheets. Blood sample will be also obtained to assess HbA1c and eligible patients will be included in the study. It should be mentioned that ineligible patients will be excluded from the study after receiving nutrition recommendations for diabetes.

General information including age, parity, education, medical information, duration of the disease, etc. will be recorded through interviews at the beginning of the study. In order to obtain the physical activity level, metabolic equivalents will be calculated through a questionnaire at the beginning and the end of the study.<sup>52</sup> To assess the dietary intakes, participants will be asked to complete a three-day dietary record form (2 weekdays and 1 weekend day), one at the first week and another at the last week of the intervention; collected data will be analysed using Nutritionist IV software (The Hearst Corporation, San Bruno, California, USA). The questionnaires will be reviewed and approved by the ethical committee members. All the study related data will be stored confidentially.

Participants in the intervention group will take two capsules of resveratrol per day (one at breakfast and another at dinner) and individuals in the placebo group will take two capsules of 500 mg methylcellulose per day at the same time for 8 weeks; each capsule of resveratrol contains 500 mg of 99.71% micronized trans-resveratrol (particle size:  $<1.9\mu\text{m}$ ) which provides 495 mg trans-resveratrol without any inactive ingredients, fillers, additives or preservatives (Mega-Resveratrol, USA). Moreover, participants will continue taking diabetic medication prescribed by doctor during the study. Each bottle contains 60 capsules (providing supplement for 1 month). All participants will be requested to bring back the first bottle after first month and then they will be given the second bottle. At the end of the study, if the remaining capsules of every patient exceed 10% of the total administered capsules (12 capsules), that patient will be categorised as non-adherent. There will be some advices for enhancing the participant's compliance such as taking capsules with meals. Moreover, patients who complete the intervention will have an 8-hour nutrition education programme for free. All randomised patients, including those who will complete the study or those who will not complete due to any reason, will follow the same schedule.

Any possible adverse event will be reported to the medical ethics committee within a week and Shahid Sadoughi University of Medical Sciences will be responsible for any participation-related problems. Some of the participants may withdraw from the study for any reason at any time before or after signing the consent form; the investigator may also terminate an individual's participation in the study in order to keep the safety and protect the participant from excessive risks and/or to maintain the integrity of data due to the improper follow-up of the procedures by the participant.

## Data collection

### Anthropometric measurements

Anthropometric parameters will be taken at the beginning and end of the intervention by the same person. Height will be measured using a stadiometer (Seca, Hamburg, Germany) with an accuracy level of 0.5 cm; waist and hip circumferences will be measured to the nearest 0.5 cm according to the standard methods using a flexible tape.<sup>53</sup> Weight estimate and body composition analysis (% fat mass, % fat free mass and visceral fat) will be performed via InBody (USA) analyser, with light clothing before and after the intervention. Body mass index will be calculated by dividing body weight (kg) by the height squared (m<sup>2</sup>); waist-to-hip ratio and waist-to-height ratio will be calculated via standard equations (6).

### Biochemical measurements

After 12 hours of fasting, 10 mL of venous blood will be taken at baseline at week 8 of the study. A 6 mL of blood sample will be collected in the colt-activator tubes and centrifuged after 30 min clotting time (3000g, 10 min at room temperature; Eppendorf AG, Hamburg, Germany) for serum isolation. Serum samples will be stored at -70°C until analyses. Remaining blood will be obtained in two ethylenediamine tetraacetic acid (EDTA)-coated tubes for gene expression and HbA1c assessment separately. Biochemical analyses including fasting blood glucose, total cholesterol, triglycerides, HDL-C and LDL-C will be measured using automated enzymatic methods and commercial kits (Pars Azmoon, Tehran, Iran). Laboratory kits will be used to assess circulating insulin levels and the percent of HbA1c will be assessed by ELISA (Monobind, California, United State) and high pressure liquid chromatography (Pars Azmoon, Tehran, Iran), respectively. Commercially available ELISA kits will be used for estimating serum levels of sCD163 and sTWEAK. Homeostatic model assessment of insulin resistance (HOMA-IR), quantitative insulin sensitivity check index (QUICKI) as an insulin sensitivity index and homeostasis model assessment of beta-cell function (HOMA-B) as well as atherogenic index will be calculated using the suggested formulas.<sup>54 55</sup> All laboratory data will be identified by an identification number to maintain the confidentiality of participants.

### Gene expression assay

Total RNA will be extracted directly from whole blood using GeneAll Hybrid-R purification kit protocol (GeneAll Biotechnology Co., Seoul, South Korea). The quality and purity (260/280 nm ratio between 1.8 and 2.2) of the RNA will be checked using spectrophotometer (NanoDrop, Thermo Fisher Scientific, Waltham, Massachusetts, United States). After normalisation, high-quality mRNA will be reverse transcribed to cDNA by cDNA synthesis kit (GeneAll Biotechnology Co.) and according to the manufacturer's instruction. Three primer designing tools (Primer Blast, Oligocalc, and Gene runner 5.0.99) are applied for sequencing the study primers (table 1). Real-time PCR and SYBR Green method (Takara Bio Inc., Japan) will be applied to assess the mRNA expression levels of PPAR $\alpha$ , p53, p21 and p16 in the StepOne system (Applied Biosystems, Foster City, California, USA). To this aim, 1  $\mu$ L of cDNA, 10  $\mu$ L of SYBR Green, 1  $\mu$ L of primers (reverse and forward) and 0.4  $\mu$ L of Rox will be mixed together. Final volume of solution will be reached to 20  $\mu$ L by adding ddH<sub>2</sub>O (7/6  $\mu$ L). Real-time PCR will be adjusted for initial denaturation step at 95°C for 10 min, followed by 40 cycles of 90°C for 15 s. The optimal annealing temperature for primers will be set at a range of 55°C–60°C for 20 s. The final step will be set up at 72°C for 20 s for primer extension. Glyceraldehyde phosphate dehydrogenase (GAPDH) will be the housekeeping gene in real-time PCR assessments. Real-time PCR efficacy and changes in expression levels will be tested using LinRegPCR software<sup>56</sup> and Pfaffl equation, respectively.<sup>57</sup>

### Statistical analysis

Principal researchers will have full access to the final data sets. Data entry and statistical analyses will be performed using SPSS for Windows V.23.0 (SPSS). The intervention and the control arms will be compared with each other for primary analysis. One-sample Kolmogorov-Smirnov test will be conducted to check normal distribution of data. Continuous variable will be expressed as means $\pm$ SD or median and IQR and categorical data will be presented as number and percentages in study groups. Independent sample t-test will be carried out for comparing parametric continuous data and Mann-Whitney U test will be used to test the differences in asymmetric variables between the two groups. Pearson's correlation coefficient will be

**Table 1** Real-time PCR primer sequences

	Forward	Reverse
p53	GAGCTGAATGAGGCCTTGGGA	CTGAGTCAGGCCCTTCTGTCTT
p21	TGGAGACTCTCAGGGTTCGAAA	GGCGTTTGGAGTGGTAGAAATC
p16	CTTCCTGGACACGCTGGTG	GCATGGTTACTGCCTCTGGTG
PPAR $\alpha$	CTATCATTTGCTGTGGAGATCG	AAGATATCGTCCGGGTGGTT
GAPDH (Reference gene)	TGGTATCGTGAAGGACTCATG	GCTTCACCACCTTCTTGATGTC

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PPAR $\alpha$ , peroxisome proliferator-activated receptor alpha.

applied to show the correlation between biochemical and anthropometric indices. General linear models will be used to assess the effects of resveratrol relative to placebo after adjustment for baseline values and participant's characteristics. P value  $\leq 0.05$  will be defined as statistically significant for all tests.

Data analysis will be performed on two sets including the intention-to-treat (ITT) and the 'per protocol' analysis; ITT analysis considers all patients in the intervention or control groups as originally allocated by randomisation, independently of their actual adherence to the determined treatment and it ignores anything that happens after randomisation including misallocation, noncompliance, withdrawal or protocol deviations. In the case that researchers observe a significant difference between those individuals who will be allocated to receive the intervention and those individuals who will actually adhere to the intervention, additional analysis will be performed considering the actual adherence to the treatment (per protocol analysis). The results of the two mentioned analyses will be compared with each other.

### Strengths and limitations

This study has been designed as a double-blind randomised controlled clinical trial, which will investigate the effects of resveratrol supplementation on the cellular factors associated with IH for the first time. It will also be the first to use high-bioavailable resveratrol supplement as a natural ligand for PPAR $\alpha$  in human. However, as a limitation, the labelling of the containers as A and B can result in unblinding entire group when unblinding is necessary; although, resveratrol has not shown serious adverse events in previous studies. Another limitation in this study is the surrogate markers that will be used for endothelial function assessment instead of gold-standard methods such as flow-mediated dilation or peripheral arterial tonometry. Moreover, we will not perform oral glucose tolerance test or hyperinsulinaemic clamp to evaluate glycaemic control effects of resveratrol. Finally, this study is designed for short-term assessment of resveratrol supplementation effects in patients with T2DM.

### Patient and public involvement

Patients or the public will not involve in the setting of the research question, outcome measures or study design and implementation. In this study, the intervention will involve taking daily supplement, and participants will not receive any lifestyle changes. So participants will not be asked to assess the benefits and burdens of participating. The summary results of the trial will be presented in a grouped form to scientific journals. Participants will be provided individual body composition report, as well as individual glycaemic and lipid profile results, on request, when study is completed.

### Ethical consideration

Written consent form will be obtained from all patients before the study initiation. This study is registered at the Iranian Registry of Clinical Trials.

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**Competing interests** The authors declare that they have no competing interests.

**Patient consent for publication** Obtained.

**Ethics approval** This protocol is approved by the Ethics Committee of the Shahid Sadoughi University of Medical Sciences (no: ir.ssu.sph.rec.1396.120).

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