Moderate-to-high-intensity training and a hypocaloric Mediterranean diet enhance endothelial progenitor cells and fitness in subjects with the metabolic syndrome

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ABSTRACT

A reduction in EPC (endothelial progenitor cell) number could explain the development and progression of atherosclerosis in the MetS (metabolic syndrome). Although much research in recent years has focused on the Mediterranean dietary pattern and the MetS, the effect of this diet with/without moderate-to-high-intensity endurance training on EPCs levels and CrF (cardiorespiratory fitness) remains unclear. In the present study, the objective was to assess the effect of a Mediterranean diet hypocaloric model with and without moderate-to-high-intensity endurance training on EPC number and CrF of MetS patients. Thus 45 MetS patients (50–66 years) were randomized to a 12-week intervention with the hypocaloric MeD (Mediterranean diet) or the MeDE (MeD plus moderate-to-high-intensity endurance training). Training included two weekly supervised sessions [80 % MaxHR (maximum heart rate); leg and arm pedalling] and one at-home session (65–75 % MaxHR; walking controlled by heart rate monitors). Changes in: (i) EPC number [$CD34^+KDR^+$ (kinase insert domain-containing receptor)], (ii) CrF variables and (iii) MetS components and IRH (ischaemic reactive hyperaemia) were determined at the end of the study. A total of 40 subjects completed all 12 weeks of the study, with 20 in each group. The MeDE led to a greater increase in EPC numbers and CrF than did the MeD intervention ($P \leqslant 0.001$). In addition, a positive correlation was observed between the increase in EPCs and fitness in the MeDE group ($r = 0.72$; $r^2 = 0.52$; $P \leqslant 0.001$). Body weight loss, insulin sensitivity, TAGs (triacylglycerols) and blood pressure showed a greater decrease in the MeDE than MeD groups. Furthermore, IRH was only improved after the MeDE intervention. In conclusion, compliance with moderate-to-high-intensity endurance training enhances the positive effects of a model of MeD on the regenerative capacity of endothelium and on the fitness of MetS patients.

Key words: cardiorespiratory fitness, cardiovascular risk, endothelial progenitor cell, Mediterranean diet, microvascular reactivity, moderate-to-high-intensity training.

Abbreviations: AUC, area under the curve; BMI, body mass index; BP, blood pressure; CrF, cardiorespiratory fitness; EPC, endothelial progenitor cell; HDL-c, high-density lipoprotein cholesterol; HOMA, homoeostasis model assessment; HR, heart rate; IRH, ischaemic reactive hyperaemia; LDL-c, low-density lipoprotein cholesterol; KDR, kinase insert domain-containing receptor; maxHR, maximum HR; MeD, Mediterranean diet; MetS, metabolic syndrome; MeDE, MeD plus moderate-to-high-intensity endurance training; PORH, post-occlusive reactive hyperaemia; 6MWT, six-min walk test; StST, sit-to-stand test; TAG, triacylglycerol; VEGF, vascular endothelial growth factor; $\dot{V}o_{2\ max}$, maximal oxygen consumption.

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INTRODUCTION

The MetS (metabolic syndrome) increases the risk of cardiovascular morbidity and mortality [1]. Recently, this phenomenon has been linked to EPCs (endothelial progenitors cells). The EPC is a bone-marrow-derived cell subtype that modulates different and complex processes of endothelial and myocardial regeneration, such as angiogenesis, myogenesis and apoptosis of cardiomyocytes [2]. A reduction in $CD34^+KDR^+$ (kinase insert domain-containing receptor) cells (a putative EPCs phenotype) has been observed in MetS patients [3]; moreover, this decrease has also been observed along with an impaired functionality of such cells as expressed by a decrease in repair function of the damaged endothelium or neovascularization [4,5]. Such a phenomenon would favour the onset of atherosclerotic disease or its long-term progression [6].

In the primary prevention of the MetS and its individual components, the MeD (Mediterranean diet) has proven not only effective, but it can also be easily adopted by everybody, regardless of their biological and cultural characteristics [7–9]. The MeD has a positive cardiovascular effect, reducing BP (blood pressure) and improving endothelial dysfunction [10,11]. Moreover, it has been observed that a quantitative modification in the fatty and/or caloric intake of the MeD may induce weight loss and lipidic control in obese subjects [12]. However, there are no objective data on the possible effects of a therapeutic model of hypocaloric MeD on the factors involved in the endothelial regeneration processes in MetS patients.

On the other hand, exercise training is now considered as a key component for the therapeutic approach on obesity and the MetS [13]. Regular endurance exercise favours weight loss and improves insulin resistance [14]; it also improves postprandial lipidic control [15] and reduces oxidative stress and systemic inflammatory response [16]. However, most exercise protocols designed for obese or MetS patients use regular steady-state exercise, such as walking and jogging. In such programmes, the low-intensity effort that may be undertaken by MetS patients who were previously sedentary could result in insufficiency to induce significant cardiometabolic adaptations [17]. For this reason, another alternative could be including moderate-to-high-intensity efforts from the onset of the training programme. One strategy for such a purpose is to periodize the training, increasing the duration of high-intensity effort progressively during the programme, until the subject can maintain high-intensity effort in a prolonged endurance exercise. Although there is no data about the effects of such programmes on the endothelium regenerative capacity; recent studies have shown that high-intensity exercise training leads to greater improvements in CrF (cardiorespiratory fitness), fat mass and glucose tolerance, compared with low-intensity exercise training [18,19].

On the basis of the evidence discussed above, we aimed at investigating the effect of a model of hypocaloric MeD, by itself or combined with periodized moderate-to-high-intensity endurance training, on the endothelium regenerative capacity (i.e. EPC numbers), CrF and cardiovascular risk factors in previously sedentary MetS patients.

MATERIALS AND METHODS

Subjects

A total of 45 subjects (67 % postmenopausal women, $n = 30$; and 33 % men, $n = 15$), meeting three or more of the MetS criteria as proposed by the Third Report of the National Cholesterol Education Program ATP-III (NCEP ATPIII 2002), participated in the present study (40 completed). These subjects (age, 57.92 ± 5.00 years; range, 50–66 years) were selected from 150 voluntees, both men and women, undergoing nutritional/medical screening at the Lipids and Atherosclerosis Unit at Reina Sofia University Hospital (Cordoba, Spain) between September 2009 and July 2010. The exclusion criteria were statin therapy, Type 1 or 2 diabetes, antecedent of unstable angina, heart failure or stroke and medical therapy able to modify heart frequency such as β-blockers. Other exclusion criteria were age >70 years, smoking, significant chronic pulmonary illness, renal or hepatic insufficiency, exercise testing limited by angina or leg claudication and neurological or orthopaedic limitations. In addition, during the 6 months prior to the study, none of the subjects should have participated in any weight-loss programme, nor have practiced any regular physical exercise or intense physical activity (>5 h of weekly vigorous activity). During the first appointment with the physician, the subjects underwent detailed clinical evaluation and a 12-lead resting ECG as a final filter to accept or reject participation in the study.

All subjects gave written informed consent to participate and the study had been previously approved by The Human Investigation Review Committee at Reina Sofia University Hospital.

Study design

Upon enrollment, the participants were randomly assigned to a 12-week experimental intervention with either a model of hypocaloric normoproteic MeDE (MeD plus moderate-to-high-intensity endurance training) or to the same diet without exercise (MeD) (Figure 1). Both groups were closely supervised by a dietitian and a physician who made weekly phone calls and attended monthly appointments with participants in order to promote adherence and proper implementation of programmes. A 3-day food diary, a 132-food frequency

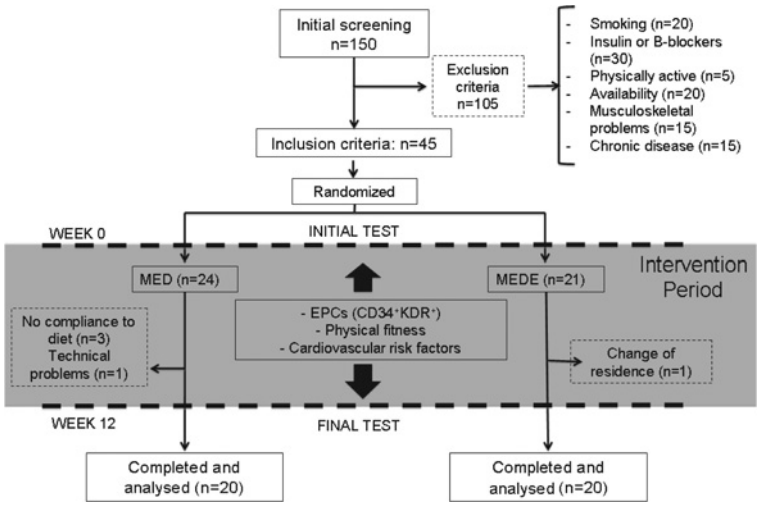

Figure 1 Study flow diagram, dropouts and reasons of dropouts

questionnaire and a validated Spanish version of the physical activity questionnaire used in the Nurses' Health Study and the Health Professionals' Follow-up Study [20] were used to measure dietary intake and physical habits at both the beginning and the end of the study. During these sessions, fasting blood samples were collected to determine circulating EPCs and measure glucose, insulin and lipidic parameters. Vascular and physical fitness tests were measured in all participants at the beginning and at the end of the study. BP, body weight, height and waist circumference were also measured and BMI (body mass index) were calculated.

Dietary intake and MeD prescription

At baseline and at the end of the intervention period, the volunteers were asked to keep two 3-day weighted food diaries and a qualitative/quantitative questionnaire of the frequency of food intake was administered by a registered dietitian. The weighted food intake over 2 week days and 1 weekend day was obtained by using scales provided by the investigators. The analysis of the food diaries and questionnaires of the frequency of food intake were carried out with the use of a dietary analysis software program (Dietsource version 2.0; Novartis).

At the beginning of the intervention period, all of the volunteers (in MeD and MeDE) were provided with information about the MeD. They were counselled on food groups and specific items on this type of eating pattern and they were asked to follow a specific daily and weekly food plan. The individual requirement of this plan was based on the restriction of 40 % of habitual energy intake (~500 kcal/day; where 1 kcal = 4.168 kJ) in order to promote weight loss. This restriction was progressive: 20 % during the first 4 weeks and 10 % in each of the following 4 weeks until completion of the 12 weeks of intervention. High-quality replica foods and photographs were used to explain the written prescription on size portions and frequency of each food group to be consumed daily in order to fulfil the individual energy requirements. The dietary targets for the percentage of macronutrients in the hypocaloric MeD model were: 50 % carbohydrates, 30 % lipids [20 % MUFAs (mono-unsaturated fatty acids), provided by virgin olive oil, 5 % SFAs (saturated fatty acids) and 5 % PUFAs (polyunsaturated fatty acids)] and approximately 20–22 % protein. In this line, the participants were instructed to consume 0.8 g protein/kg of body weight per day during the whole intervention period in order to maintain a normal protein intake.

Qualitatively, all food plans included daily consumption of extra virgin olive oil, red wine, whole-wheat grains and products, low-fat dairy products, salads, mixed fruits and whole raw almonds. The extra virgin olive oil as the main source of fat was supplied to the subject. Suggestions given to all subjects included the elimination or severe restriction of deep-fried foods, fatty and processed meats, confectionery, sweetened soft drinks, pies and sugars. The compliance throughout the study was assessed through a 14-item questionnaire of adherence to the traditional MeD [21], which was completed every 4 weeks. The objective of these 14-item questionnaires was also to reinforce the key principles of the MeD, including increased consumption of vegetables, fruits, red wine, nuts and the use of olive oil as main culinary fat.

Exercise intervention

The 12-week endurance training regime consisted of three weekly sessions of moderate-to-high-intensity training. Of these, two were supervised training sessions at the laboratory, whereas the other session was performed at home. Weekly supervised training was performed using

Table 1 Exercise prescription to hypocaloric MeDE group

*Exercise intensity was 80 % of maximum heart rate [23] for the moderate-to-high-intensity series; †active recovery intensity was 50 % of maximum HR.

	Supervised exercise (two per week)			At-home exercise (one per week)	
	[Cycle ergometer (10 min)–arm ergometer (10 min)–cycle ergometer (10 min)]			Continue walking exercise	
Week	Set*	Active recovery†	Session duration	Intensity	Session duration
1	6×1 min	4 min	30 min	65%	25 min
2	6×1.5 min	3.5 min			30 min
3	6×2 min	3 min			35 min
4	6×2.5 min	2.5 min			40 min
5	6×3 min	2 min		70%	35 min
6	6×3.5 min	1.5 min			40 min
7	6×4 min	2 min			45 min
8	6×4.5 min	0.5 min			50 min
9	3×7 min	3 min		75%	45 min
10	3×8 min	2 min			50 min
11	3×9 min	1 min			55 min
12	1×30 min	0 min			60 min

two different ergometers; a cycle ergometer to exercise lower limbs (Technogym Recline 500™) and an arm ergometer for the upper limbs (Technogym Ecxite™ 700). Meanwhile, home-based training consisted of outdoor continuous walking. In all sessions, the subjects used a heart rate monitor (FS1™; Polar Electro) to obtain the assigned exercise intensity.

Supervised training sessions

A periodized training programme including series at 80 % of maxHR (maximum heart rate) and active recovery periods of decreasing duration were designed, since evidence shows that intensity above 70 % maxHR produces important cardiometabolic benefits in obese and overweight patients [17]. Such periodization had as a goal to reach by the end of the intervention period a 30-min continuous exercise session at 80 % of maxHR. Different exercises (leg and arm pedalling) were used to avoid interrupting the session as a result of local fatigue. Details on the supervised exercise protocol are shown in Table 1.

At-home sessions

An at-home training programme was prescribed, including an increase in both exercise volume and intensity, as shown in Table 1. The patients were instructed to immediately stop home-based training if they had chest pain or any other distressing symptoms. In addition, all subjects recorded the sustained HR (heart rate) of each training session as well as the corresponding perceived exertion according to a 6–20 Borg scale. Subsequently, this information was analysed in the laboratory to check that exercise intensity and volume corresponded to what was prescribed for each session.

Anthropometric and BP measurements

Height and weight were recorded without shoes and wearing light clothing and waist circumference was measured at the level of the umbilicus. BP, measured after a 15-min rest, was recorded as the mean of three supine measurements using an oscillometric BP monitor (Omron M3™).

Analytic methods

Biochemical assays

Fasting venous blood was collected into tubes containing 1 g of EDTA and immediately centrifuged at 4 °C (15 min for 176.4 *g*). Serum and plasma were stored at −85 °C for later determinations of levels of glucose, insulin, total cholesterol, HDL-c (high-density lipoprotein cholesterol) and TAGs. Glucose and plasma TAG concentrations were measured by spectrophotometry using a modular analyser (ISE-4-DDPPEEPP; Hoffman La Roche). Plasma insulin levels were measured by chemiluminescent microparticle immunoassay using an analyser (Architect i-4000; Abbott).

Circulating EPC number

Much research has shown, in recent years, that EPCs represent approximately 0.01 % of total peripheral blood cells; their numbers vary depending on different factors such as antibody affinity, presence of cardiovascular risk factors, coronary artery disease or chronic heart failure [22]. For flow cytometric quantification of EPCs, 100 μl of whole blood was incubated during 20 min at 4 °C with 10 μl of TC (Tri colour)-conjugated anti-(human-CD34) antibody (Invitrogen) and FITC-conjugated anti-(human KDR) antibody (R&D Systems).

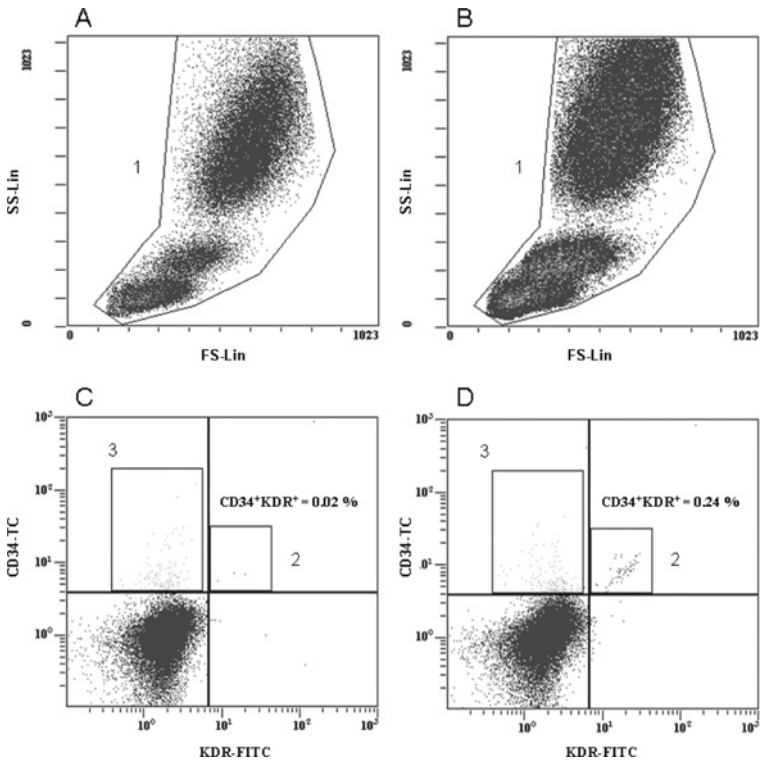

Figure 2 Representative example for enumeration of EPCs before (A and C) and after (B and D) the intervention

The general distribution of all cell populations is seen on area 1 in (A) and (B). The combination of $CD34^+KDR^+$ on area 2 in (C) and (D) was quantified as EPCs. $CD34^+$ cells were enumerated within the area 3 in (C) and (D). TC, Tri-colour.

Afterwards, erythocytes and platelets were lysed with Versalyse (Beckman Coulter) and the remaining cells were analysed by flow cytometry (Cytometer FC500; Beckman Coulter). $CD34^+KDR^+$ cells were quantified and defined as EPCs. Each analysis included 150 000 events passing the gate. The EPC level was considered as the number of $CD34^+KDR^+$ cells/$\times 10^6$ peripheral blood cells (Figure 2). In addition, data from $CD34^+$ only cells were also extracted. All data were analysed using commercially available software (CXP analysis software 2.2; Beckman Coulter).

Exercise tests

Submaximal exercise test in cycle-ergometer

The protocol used for graded submaximal exercise test was the one proposed by ACSM (American College of Sports Medicine) and YMCA (Young Mens' Christian Association) [23]; this protocol is a multistage protocol involving a progressive increase in workload based on the subject's HR response during exercise. In brief, 3-min stages on a cycle ergometer were used (Technogym Recline 500^{TM}). Before starting, the length of the saddle was adjusted according to the subject's leg length and was registered for later exercise sessions. HR was constantly monitored with a cardiotachometer (Polar Electro^{TM}). Moreover, at the end of each stage, BP was measured by auscultation, by a physician with accredited experience. The subjects were asked to keep a pedalling cadence of 50–60 rev./min.

Before they were tested, the participants were familiarized with the cycle ergometer exercise test and all tests were performed under identical environmental conditions (21–24 °C; 45–55 % relative humidity). The initial stage used for all testing was set at a load of 25 W, considered as a warm-up. The HR reached at the end of the first stage determined the load of the following stage: 50 W (HR, 100 beats/min), 75 W (HR, 90–100 beats/min), 100 W (HR, 80–89 beats/min) or 125 W (HR, <80 beats/min). Subsequently every 3-min stage was incremented by 25 W until one of the criteria established for interruption was met: (i) signs and symptoms of intolerance to exercise (dizziness, dyspnoea, disorientation, etc.) or for fatigue or precocious exhaustion; (b) HR over 100 % of the maxHR as estimated by Tanaka's formula [24]; however, it was sought that all subjects reached at least 85 % maxHR; (iii) an abnormal BP response (increase of systolic BP over 250 mmHg or diastolic BP over 120 mmHg); and (iv) impossibility of keeping a pedalling cadence of 50 rev./min. The loads used in the first test (baseline) following the above-mentioned protocol

were registered to be repeated in the second test after the intervention period (week 12). $\dot{V}o_{2\,max}$ (maximum oxygen consumption; in ml/kg of body weight per min) estimation was performed plotting the workloads and HR for the final two stages of the protocol into the multistage $\dot{V}o_{2\,max}$ prediction equation [25].

6MWT (6-min walk test)

The 6MWT is a simple test used in obesity and in a wide clinical spectrum of disease; it provides a valid assessment of functional exercise level for daily physical activities. The 6MWT was performed in a 45.72-m corridor according to the standards of the American Thoracic Society [26]. The purpose is to cover as much distance as possible in 6 min, although stopping and resting were allowed during the test. For this, the patients were asked to walk at a maximum tolerated speed and were encouraged verbally every minute. A timer with a countdown function was used to time the 6 min. Investigators calculate the distance covered during the 6MWT test after the study is completed [laps walked×distance per lap (in metres) + partial lap].

StST (sit to stand test)

The StST was performed using a steel-moulded chair (0.40 m height and 0.36 m depth). The subjects were asked to stand up from a sitting position and then to sit down as many times as quickly as possible during 30 s. The subjects were instructed to stand up fully and to place their buttocks on the chair in a sitting position between repetitions. The test started and stopped when the examiner said 'go' or 'stop' respectively. Prior to the measurements, practice trials with submaximal effort were performed for positioning and learning of the task. The StST was performed twice with an interval of 2 min between trials and the highest number of repetitions was adopted for the individual data.

IRH (ischaemic reactive hyperaemia)

A Periflux 5000 laser-Doppler monitor (Perimed) was used to measure PORH (post-occlusive reactive hyperaemia). The PORH was used because it provided a simple, swift method for measuring changes in acute microvascular endothelial reactivity in basal conditions [27]. In brief, with the patient lying in a supine position in a room with stable temperature (20–22 °C), the BP cuff (HG Erkameter 300) was placed 5 cm above the elbow, whereas the laser probe was attached to the palmar surface of the second finger of the same dominant hand. After a 20-min resting period, basal capillary flow was measured for 1 min (t_0). Thereafter, a 4-min distal ischaemia was induced by inflating the cuff to suprasystolic pressure (200–220 mmHg). Subsequently, the cuff was deflated and the flow was recorded for 1 min (t_d). The data were recorded and stored using PeriSoft for Windows. The values of the AUC (area under the curve) of the t_0 and t_d times were analysed. These data were used to calculate the increase in post-ischaemic flow by means of the equation: $iAUC = [(AUCt_d - AUCt_0) \times 100/AUCt_0]$. In addition, PORHmax was defined as PORHpeak (the maximum value achieved after 4 min of flow occlusion) referred to the baseline skin value and was calculated as follows: PORHmax= [(PORHpeak) – baseline skin flux]

Table 2 Characteristics of participants in MeD and MeDE groups at baseline

No significnat differences were observed between the groups.

Variable	MeD group ($n = 20$)	MeDE group ($n = 20$)
Age (years)	57.2 ± 4.29	59.05 ± 5.47
Sex (*n*)		
Female	14 (70 %)	13 (65 %)
Male	6 (30 %)	7 (35 %)
Routine medication (*n*)	3.23 ± 2.09	3.73 ± 2.52
Risk factors (*n*)		
Abdominal obesity	20 (100.00 %)	20 (100.00 %)
Hypertension	16 (80.00 %)	18 (90.00 %)
Dyslipidaemia	12 (60.00 %)	13 (65.00 %)
Impaired fasting glucose	13 (65.00 %)	12(60.00 %)

Statistics

The results are presented as means and S.E.M. Sample normality was calculated using the Shapiro–Wilk test. The effect of the different interventions (MeD and MeDE; independent variables) on EPCs, $CD34^+$, $\dot{V}o_{2\,max}$, 6MWT, StST, cardiometabolic risk factors and IRH (dependent variables) was subjected to ANOVA with repeated measurement of two factors: group and time: [2 (group)×2 (time)]. A Tukey correction was used to adjust the P value in relation to the number of contrasts performed. Statistical significance was set at $P \leqslant 0.05$; for all the statistical tests, the SPSS 15.0 package for Windows was used.

RESULTS

A total of 40 subjects completed all 12 weeks of the study, 20 in each group. The 'no-compliance to diet' was the most frequent complaint of dropouts in the MeD group, whereas 'change of residence' was the reason given in the MeDE group. There were no significant differences between the MeD and MeDE groups for age and sex (Table 2).

Dietary intake and physical activity

The self-reported physical activity (including endurance exercise programme) and composition of the study diet consumed by MeD and MeDE during the 12-week study period are presented in Table 3. There were no differences in total energy, carbohydrates, protein,

Table 3 Nutrient intake and physical activity at baseline and after 12 weeks in the MeD and MeDE groups

Values are means ± S.E.M. *Significant difference within group (baseline against week 12); †significant difference between groups (MeDE against MeD). MET, metabolic equivalent of task; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

	MeD group ($n = 20$)		MeDE group ($n = 20$)	
Variable	Baseline	Week 12	Baseline	Week 12
Energy (kcal/day)	2211.20 ± 155.97	1304.28 ± 93.77*	2126.37 ± 177.13	1206,18 ± 116.47*
Carbohydrate (% of energy)	46.85 ± 1.61	47.03 ± 1.91	47.19 ± 1.13	48.11 ± 1.31
Protein (% of energy)	17.31 ± 0.93	22,39 ± 0.74*	18,25 ± 0.59	22.31 ± 0.61*
Fat (% of energy)	35.85 ± 1.47	30,58 ± 1.33*	34.56 ± 1.51	29,42 ± 1.00*
SFA (% of energy)	13.74 ± 0.52	8.89 ± 1.28*	13.08 ± 0.45	7.54 ± 1.03*
MUFA (% of energy)	14.09 ± 1.10	20.33 ± 0.82*	14.02 ± 0.91	20.01 ± 0.78*
PUFA (% of energy)	3.41 ± 0.26	4.70 ± 0.74	3.49 ± 0.23	4.01 ± 0.61
Vitamin C (mg/day)	102.71 ± 57.96	220.06 ± 49.90*	99.59 ± 47.07	210.75 ± 60.39*
Activity				
Total physical activity (MET/W)	70.77 ± 9.65	75,00 ± 7.62	75.59 ± 9.02	97.59 ± 6.05*†
Sedentary activities (h/W)	89.60 ± 6.24	86,25 ± 7.34	90.37 ± 3.84	86,61 ± 4.20
Total energy expenditure (MET/W)	173.84 ± 8.44	176.26 ± 9.91	179.37 ± 10.79	201,25 ± 7.95*†

Table 4 MeD diet score and changes in the consumption of key foods rich in antioxidant compounds according to 14-item questionnaire of adherence to the traditional MeD at baseline and after 12 weeks in the MeD and MeDE groups

Values are means ± S.E.M. for the quantitative score and the percentage of compliance for specific items of 14-item questionnaire. Each item from the 14-item questionnaire was assigned values of 0 or 1 and summed for the total score. *Significant difference within group (baseline against week 12).

	MeD group ($n = 20$)		MeDE group ($n = 20$)	
Variable	Baseline	Week 12	Baseline	Week 12
Total MeD score (points)	6.10 ± 0.39	10.50 ± 0.70*	6.45 ± 0.39	11.18 ± 0.58*
Vegetables consumption ⩾ two servings/day (at least one of them in salad or raw) (% of compliance)	15 ($n = 3$)	85 ($n = 17$)*	10 ($n = 2$)	80 ($n = 16$)*
Fruits consumption ⩾ three pieces/day (including fresh juice) (% of compliance)	10 ($n = 2$)	90 ($n = 18$)*	10 ($n = 2$)	95 ($n = 19$)*
Red wine consumption ⩾ seven glasses/week (% of compliance)	15 ($n = 3$)	50 ($n = 10$)*	10 ($n = 2$)	45 ($n = 9$)*
Nuts consumption ⩾ three times/week (% of compliance)	15 ($n = 3$)	90 ($n = 18$)*	15 ($n = 3$)	95 ($n = 19$)*

total, monounsaturated, saturated and polyunsaturated fat intakes between the groups. In addition, compliance with Mediterranean eating pattern was good in both exercising and non-exercising groups as shown in the MED score in Table 4.

Weight and cardiometabolic risk factors

Both study groups exhibited significant weight loss, improvements in BMI and reductions in waist circumference between baseline and post-study measures but, as expected, weight and BMI showed greater changes in the MeDE group (Table 5). In addition, fasting glucose, total cholesterol, LDL-c (low-density lipoprotein cholesterol) and systolic BP were improved by a similar amount in both groups. However, plasma insulin concentration, TAGs, HOMA (homoeostasis model assessment) index and diastolic BP decreased in MeD and MeDE following the intervention, with a greater reduction observed in the exercising group. Analysis of HDL-c did not reveal any effect of time or trial. Finally, we found a statistically significant increase in PORHmax and iAUC after the 12-week treatment only with the MeDE (~50 and 90% respectively) (Table 5).

Endothelial progenitor cells and cardiorespiratory fitness

The MeDE and MeD groups exhibited a significant increase in EPC numbers between baseline and 12 weeks measure but, adherence to the MeDE treatment resulted in greater concentration of circulating EPCs than after the MeD ($P \leqslant 0.001$) (Figure 3). In order to measure whether improvements in CrF were achieved with and without exercise protocol, we estimated $\dot{V}o_{2\,max}$ using a submaximal cycle-ergometer test and a 6MWT and StST. There were no differences between the groups at baseline (Figure 4), but the MeDE resulted in a greater increase in $\dot{V}o_{2\,max}$, in the distance covered in 6 min

Table 5 Anthropometric, biochemical and clinical characteristics at baseline and after 12 weeks in MeD and MeDE groups

Values are means ± S.E.M. *Significant difference within group (baseline against week 12); †significant difference between groups (MeDE against MeD). iAUC, increase in post-ischaemic flow estimated using values from area under the curve of laser-Doppler test; LDL-c, low-density lipoprotein cholesterol; PORHmax, maximum value achieved after 4 min of flow occlusion — baseline skin value.

	MeD group ($n = 20$)		MeDE group ($n = 20$)	
Variable	Baseline	Week 12	Baseline	Week 12
Body weight (kg)	96.04 ± 3.54	90.66 ± 3.37*	97.21 ± 2.46	88.83 ± 2.22*†
BMI (kg/m^2)	38.44 ± 1.46	36.35 ± 1.37*	37.05 ± 0.72	33.79 ± 0.72*†
Waist circumference (cm)	114.41 ± 2.56	110.69 ± 2.71*	109.20 ± 2.00	105.02 ± 2.09*
Fasting glucose (mmol/l)	5.95 ± 0.13	5.65 ± 0.15*	5.69 ± 0.20	5.38 ± 0.25*
Fasting insulin (pmol/l)	92.02 ± 6.66	77.92 ± 9.30	86.60 ± 7.57	44.58 ± 4.93*†
HOMA index	3.53 ± 0.29	2.91 ± 0.39*	3.13 ± 0.27	1.56 ± 0.18*†
Total cholesterol (mmol/l)	5.27 ± 0.14	4.95 ± 0.16*	5.06 ± 0.23	4.62 ± 0.28*
LDL-c (mmol/l)	3.36 ± 0.12	3.11 ± 0.14*	3.27 ± 0.16	2.98 ± 0.20*
HDL-c (mmol/l)	1.23 ± 0.06	1.20 ± 0.06	1.20 ± 0.07	1.17 ± 0.05
TAGs (mmol/l)	3.44 ± 0.24	3.21 ± 0.27*	3.55 ± 0.26	2.63 ± 0.24*†
Systolic BP (mmHg)	140.55 ± 3.67	126.07 ± 3.51*	142.23 ± 2.76	124.42 ± 3.45*
Diastolic BP (mmHg)	83.29 ± 1.71	80.00 ± 2.19*	84.70 ± 1.71	73.31 ± 2.30*†
iAUC (AUC)	226.90 ± 45.92	227.03 ± 20.44	217.68 ± 25.10	327.77 ± 35.72*†
PORHmax	166.02 ± 15.61	169.11 ± 25.52	132.13 ± 18.89	176.36 ± 18.49*†

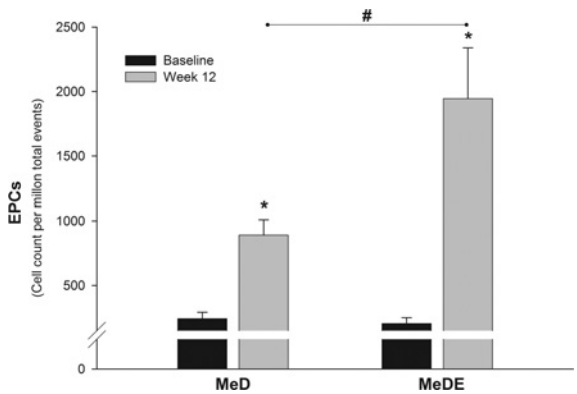

Figure 3 Levels of circulating EPCs defined as CD34$^+$KDR$^+$ at baseline and after 12 weeks in the MeD and MeDE groups

$n = 20$ patients in each intervention period. Black bars indicate baseline; grey bars, 12 weeks of intervention. Values are means ± S.E.M. *Significant difference within group (baseline against week 12); #significant difference between groups (MeD against MeDE).

and in the number of repetitions in StST at the post-study measures ($P \leqslant 0.001$). In addition, we found in the MeDE group, but not in the MeD group, a significant positive correlation between increase in EPCs and CRF, as expressed through 6MWT. Pearson's rank correlation is shown in Figure 5.

Moreover, the number of CD34$^+$ cells increased both in the MeD and MeDE groups after 12 weeks of treatment without observing significant differences between the two groups (570.46 ± 153.8 to 2246.77 ± 301.5 and 517.16 ± 167.9 to 2369.45 ± 488.9 respectively). The observed increase in CD34$^+$ cells in both groups was positively correlated with reductions in HOMA index ($r = 0.41$, $P < 0.05$; and $r = 0.55$, $P < 0.05$ respectively).

We found no significant correlation between EPCs or CD34$^+$ cells and the other studied variables.

DISCUSSION

There are two main findings derived from the present study in non-diabetic MetS patients without manifested cardiovascular disease who were previously sedentary. On the one hand, it was observed that 12 weeks of a hypocaloric MeD model improved the endothelium regenerative capacity as a result of the increase in EPCs, improving, at the same time, cardiovascular risk factors. On the other hand, the application of the same dietetic model plus periodized moderate-to-high-intensity endurance training (MeDE) resulted in a greater increase in EPCs. In addition, as expected after the exercise intervention, cardiorespiratory fitness showed a greater improvement than the diet model alone. The observed improved fitness was positively correlated with the circulating levels of EPCs. Finally, insulin sensitivity, plasma TAG concentrations and BP showed greater decreases in the MeDE intervention; microvascular endothelial reactivity, however, only improved in the combined intervention.

Recent reports by our group have shown that, at least in healthy adults, the endothelium regenerative capacity

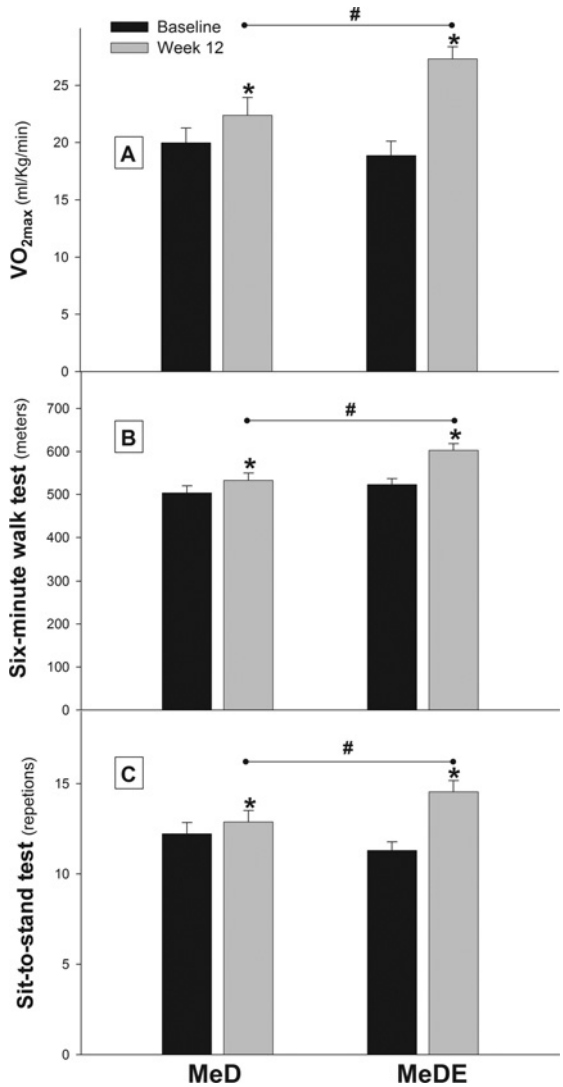

Figure 4 Maximal oxygen consumption (A), 6MWT (B) and StST (C) in the MeDE and MeD groups at baseline and after 12 weeks

Black bars indicate baseline; grey bars, 12 weeks of intervention. $n = 20$ patients in each intervention period. Values are means $\pm$ S.E.M. *Significant difference within group (baseline against week 12); #significant difference between groups (MeD against MeDE).

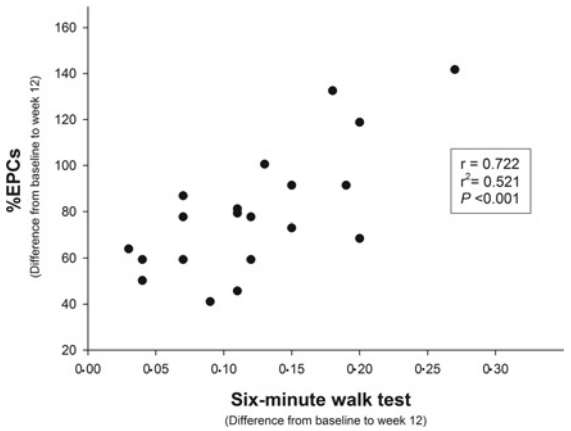

Figure 5 Pearson's correlation (r) between amount of circulating EPCs and distance covered in 6MWT, both expressed as differences from baseline to week 12

$n = 20$ patients.

(circulating EPCs) and endothelial damage (release of endothelial microparticles), are significantly improved after an 8-week intervention with a close adherence to a Mediterranean-style diet [28]. In the present study, it has also been shown that, in MetS patients, the number of EPCs significantly increases using the same dietary pattern yet modified by means of caloric restriction. With regard to the possible mechanism implicated in this phenomenon, in other populations without the MetS, a reduction in oxidative stress and the up-regulation in NO bioavailability, have been proposed as the link between regular intake of a typical MeD and the increase in EPCs [28,29]. The abundant content in phenolic acids, polyphenols and flavonoids of a typical MeD seems to confer a potent beneficial effect on the oxidative balance [30,31]. In this regard, a close adherence to the MeD pattern (including consumption of vegetable fats, that is, extra virgin olive oil and the reduction of animal fat intake) was shown through an increase in total quantitative score of adherence to the MeD at the end of the present study in both the MeD and MeDE groups (Table 4). Furthermore, a significant increase in weekly and daily consumption of antioxidant-rich foods (vegetables, fruits, red wine and nuts) was achieved by the subjects in the present investigation as derived from the analysis of a 14-item questionnaire of the traditional MeD. These findings support the hypothesis that an improvement in oxidative state could be one of the mechanisms involved in the increase of EPCs in the MeD group.

On the other hand, a few studies, mainly assessing chronic heart failure patients, have studied the effect of physical exercise on EPC number [32]. In such patients, Gatta et al. [33] found that a short-training period with endurance exercise (3 weeks) increases the number of $CD34^+KDR^+$ cells and the proliferation of colony-forming units-endothelial cells after training. In healthy subjects and chronic heart failure patients, both VEGF (vascular endothelial growth factor), as well as the increase in eNOS (endothelial NO synthase) activity, seem to acutely respond to the shear stress produced by an intense endurance exercise session [34,35]. Both VEGF and NO have been proposed as determining factors of the activation of stem cell mobilization processes from the bone marrow to the vascular area [36–38];

this would partly explain the time-dependent increase in $CD34^+KDR^+$ cells subsequently to an acute endurance exercise [39]. Specifically, a pivotal role for NO in EPC mobilization was demonstrated in humans when inhibiting the acute NO production [using non-selective NO synthase inhibitor L-NMMA (N^G-monomethyl-L-arginine) a complete blockade of EPC mobilization in response to endurance exercise of moderate intensity was observed [40].

To the best of our knowledge, this is the first study using an MeD model showing that the combination with a 12-week moderate-to-high-intensity endurance training increases the $CD34^+KDR^+$ cells level in MetS patients. One possible explanation for this phenomenon is that an endothelial adaptation (i.e. increase in EPC mobilizing factors such as NO) may have resulted in greater EPC level in the MeDE group. In addition, this intervention with moderate-to-high-intensity exercise did not only include intermittent effort sessions (allowing the MetS and previously sedentary subjects to reach greater effort intensity since the beginning of training) but it also was a periodized programme, that is, it allowed for the distribution of acute stimuli of increasing intensity over the 12-week period. In terms of vascular physiology, a periodized programme as the one used in the present study could mean a constant endothelial adaptation against new weekly stimuli (i.e. blood flow changes and ischaemic phenomena). In line with this, an increase in NO availability of early EPCs, along with an improved *in vivo* endothelial repair capacity and better endothelium-dependent flow-mediated vasodilatation, were observed in mice with defined carotid endothelial injury after EPCs transplantation from MetS subjects after 8 weeks with exercise training [41]. Accordingly, the MeDE group was the only one showing an increase in IRH as measured by laser Doppler (PORHmax and iAUC). Although IRH, as measured in the present study, had no demonstratable relationship with coronary endothelial function, previous studies have shown that an improvement in microvascular reactivity as determined by using laser Doppler is significantly related to an increase in NO bioavailability [28,42]. For such reason, we hypothesize that a greater bioavailability in NO could have occurred after the MeDE intervention.

Another phenomenon that could potentially explain the greater EPCs increase in the MeDE would be a specifically exercise-induced shedding of endothelial cells that may have been misleadingly counted as EPCs in this group. Yet, we have found that both the MeDE and MeD had a similar increase in $CD34^+$ cell population when it should be expected to observe a greater number of these cells in the MeDE group, especially if exercise had triggered the increased release of endothelial cells. Therefore we deem that a shift in circulating $CD34^+$ cells towards $CD34^+KDR^+$ cells could have occurred such as observed in previous studies after a single bout of exercise in healthy subjects [43]. Nevertheless, the observed increase in circulating $CD34^+KDR^+$ cells in MeDE should be interpreted with caution since this phenomenon could be merely part of a greater one. In other words, the potential benefit of a diet plus moderate-to-high-intensity endurance training on the endothelial regenerative capacity could be due not solely to an increase in $CD34^+KDR^+$ cells. In line with this, it can be observed, in a review by Urbich and Dimmeler [44], that cell populations, other than haemopoietic stem cells, can also give rise to endothelial cells, increasing tissue neovascularization after ischaemia and re-endothelialization after endothelial injury.

On the other hand, in the present study, it has been observed that subjects in the MeDE group increased their $\dot{V}o_{2\,max}$, and, in a specific way, they improved their local muscular performance and their physical work capacity with regard to an endurance exercise as was shown by StST and 6MWT respectively. Moreover, it has been observed that the increase in CrF was related to the increase in EPCs only in the MeDE group. This positive relation among variables such as EPCs and fitness highlights the importance, for the improvement of EPCs levels, of a training programme with mechanical and metabolic stimuli potent enough so as to reach significant cardiovascular and physical adaptation. Once more, the periodized moderate-to-high-intensity training used in the present study could have been a key factor to simultaneously induce the increase in EPCs and the improvement in CrF. In line with this, other researchers have found evidence to assume that the increased amount of EPCs observed in reference groups, or after exercise intervention, means a long-term adaptation to increased muscle work, new blood vessel formation and changes in blood flow [45,46]. Conversely, even though subjects in the MeD group also improved their physical fitness (6MWT and StST), this improvement, unlike what happened in the MeDE group, would be due to body weight loss and to biomechanical factors, but not to cardiovascular and metabolic adaptations. This mechanism would, therefore, explain the lack of relation between fitness and increased EPC levels observed in the MeD group. In other words, our findings would suggest that the cardiometabolic component of fitness is important for the mechanisms that explain the increased availability of EPCs. Conversely, the physical functioning (other component of fitness), which can be discreetly improved in obese patients with the MetS losing weight through diet, might not be important in this phenomenon.

Finally, the conventional risk factors of the MetS have also been proposed as variables related to EPCs reduction in this type of patient [5]. In the present study, it has been observed that the MeD reduced the fasting glycaemia and plasma concentration of TAG in the subjects analysed. Basically, the MeDE was more effective than the MeD for weight loss and for improving the cardiometabolic

risk factors, such as triglyceridaemia, BP and insulin sensitivity. Nevertheless, only the increase in $CD34^+$ cells, but not in $CD34^+KDR^+$ cells, was positively and discretely correlated with a reduction in the HOMA index after the 12-week treatment in both groups (MeD and MeDE). This finding is partially in accordance with observational studies that have shown that $CD34^+$ cells are the phenotype which relates best to cardiovascular risk factors in MetS patients [4,47]. The present study was not designed; however, to elucidate which were the mechanisms by which exercise could generate an increase in EPCs levels or to understand risk modulation by means of these interventions. Conversely, it was designed to know the effects on the EPC number and CrF after two treatments that had not been studied with regard to endothelium regenerative capacity and the subjects' fitness (i.e. MeD alone and plus periodical moderate-to-high-intensity endurance training).

A potential limitation of the present study is that we determined the number of EPCs, but not their functionality. However, like most studies published to date, this study aims to determine the change in one of the factors related to endothelial regenerative capacity (i.e. EPC number). Future designs must analyse whether the EPCs increase after treatment with MeD, with or without exercise, is synergistically related, or not, to their functionality. Only one of the several putative EPCs phenotypes ($CD34^+KDR^+$) was assessed since, to date, the $CD34^+KDR^+$ combination (a prototypical stem cell antigen plus a marker of the endothelial lineage) remains the most accepted EPC phenotype. In essence, this combination is the only putative EPCs phenotype that has been repeatedly and convincingly demonstrated to be an independent predictor of cardiovascular outcomes [48]. The present study lacks an arm without the MeD (i.e. with exercise alone). Unlike other designs that examine the independent effect of exercise and diet, our aim was to determine the cumulative effect of moderate-to-high-intensity training along with a variant of the MeD, on circulating EPCs. A strength of the present study is the careful selection of patients included in the study (without co-existent diseases and morbidity such as diabetes and peripheral vascular disease or concomitant medications) as well as the rigorous control of adherence to interventions (diet or diet plus exercise).

In conclusion, a therapeutic model of hypocaloric MeD improves endothelium regenerative capacity and cardiometabolic risk factors in MetS patients without manifested cardiovascular disease. Likewise, the present findings show, for the first time, that a 12-week moderate-to-high-intensity endurance training combined with this diet model produces a greater increase in $CD34^+KDR^+$ cell levels; this fact coincides with a significant improvement in cardiorespiratory fitness and with a beneficial influence on IRH and cardiovascular risk.

AUTHOR CONTRIBUTION

Francisco Fuentes-Jiménez conceived and designed the study, analysed and interpreted the data, drafted the paper, obtained funding, and had full access to and takes responsibility for the integrity of the data in the study; Juan Marcelo Fernández conceived and designed the study, acquired, analysed and interpreted the data, and drafted the paper; Marzo Edir Da Silva Grigoletto conceived and designed the study, provided statistical expertise and drafted the paper; Francisco Pérez-Jiménez conceived and designed the study, analysed and interpreted the data, supervised the study and provided critical revision of the paper for important intellectual content; Daniel Rosado-Álvarez acquired, analysed and interpreted the data, provided technical and logistical support, and drafted the paper; Oriol Alberto Rangel-Zúñiga acquired the data; Leslie Lorena Landaeta-Díaz acquired the data, and provided technical and logistic support; Javier Caballero-Villarraso provided technical and logistic support; José López-Miranda provided statistical expertise, supervised the study and made critical revisions of the paper for important intellectual content. All decisions regarding the design, conduct, collection, analysis or interpretation of the data and the decision to submit the paper for publication were made independently by the authors.

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