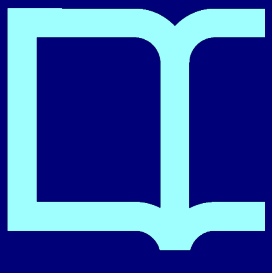

The non-alcoholic fraction of beer increases stromal cell derived factor 1 and the number of circulating endothelial progenitor cells in high-cardiovascular risk subjects: a randomized clinical trial

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ABSTRACT

Rationale: Moderate alcohol consumption is associated with a decrease in cardiovascular risk, but fermented beverages seem to confer greater cardiovascular protection due to their polyphenolic content. Circulating endothelial progenitor cells (EPC) are bone-marrow-derived stem cells with the ability to repair and maintain endothelial integrity and function and are considered as a surrogate marker of vascular function and cumulative cardiovascular risk. Nevertheless, no study has been carried out on the effects of moderate beer consumption on the number of circulating EPC in high cardiovascular risk patients.

Objective: To compare the effects of moderate consumption of beer, non-alcoholic beer and gin on the number of circulating EPC and EPC-mobilizing factors.

Methods: In this cross-over trial, 33 men at high cardiovascular risk were randomized to receive beer (30g alcohol/d), the equivalent amount of polyphenols in the form of non-alcoholic beer, or gin (30g alcohol/d) for 4 weeks. Diet and physical exercise were carefully monitored.

Results: The number of circulating EPC and EPC-mobilizing factors were determined at baseline and after each intervention. After the beer and non-alcoholic beer interventions, the number of circulating EPC significantly increased by 8 and 5 units respectively, while no significant differences were observed after the gin period. In correlation, stromal cell derived factor 1 increased significantly after the non-alcoholic and the beer interventions.

Conclusions: The non-alcoholic fraction of beer increases the number of circulating EPC in peripheral blood from high cardiovascular risk subjects.

Clinical Trial Registration: http://www.controlled-trials.com/ISRCTN95345245 ISRCTN95345245.

Keywords: beer, alcohol, polyphenols, endothelial progenitor cells, stromal cell derived factor 1.

INTRODUCTION

Endothelial progenitor cells (EPC) are bone-marrow-derived stem cells with the ability to differentiate into mature endothelial cells[1]. There is growing interest into circulating EPC as a mechanism to repair and maintain endothelial integrity and function by replacing denuded parts of the artery. Endothelial injury may play a role in the pathogenesis of atherosclerosis, arterial thrombosis and hypertension. Indeed, the balance between endothelial injury and recovery is extremely important to reduce cardiovascular events since mature endothelial cells possess limited regenerative capacity and, therefore, EPC are considered as one of the mechanisms to maintain endothelial function (reviewed in[2, 3]). Circulating EPC levels have been proposed as a surrogate marker for vascular function[4] and cumulative cardiovascular risk because of the strong correlation between the number of circulating EPC and flow-mediated brachial-artery reactivity and a decreased Framingham risk factor score[5].

On the other hand, moderate alcohol consumption is associated with a decreased risk of cardiovascular mortality independently of the type of beverage consumed[6]. Nevertheless, fermented alcoholic beverages (i.e. wine and beer) seem to confer greater cardioprotective effects than distilled beverages probably because of their higher polyphenolic content[7], although the mechanisms involved are not fully understood. *In vitro* or animal studies have observed that red wine polyphenols increase the number and functionality of EPC[8-12], suggesting a possible explanation for this greater cardioprotective effect. To our knowledge, few studies have evaluated the effects of red wine or beer polyphenols on circulating EPC[13, 14] in healthy individuals. An enhancement of circulating EPC was observed after red wine consumption[13, 14] but not after beer consumption[14]. Nevertheless, no studies have been carried out in high cardiovascular risk patients, who show greater endothelial damage than healthy individuals.

We embarked, therefore, on a study to evaluate the effects of moderate beer consumption and its fractions (alcoholic and non-alcoholic) on the number of circulating EPC and EPC-mobilizing factors in subjects at high risk of cardiovascular disease (CVD), in whom diet and exercise were carefully monitored.

SUBJECTS AND METHODS

Subjects

A total of 36 high risk male subjects aged between 55 and 75 years were recruited for the study in the outpatient clinic of the Internal Medicine Department at our institution. The subjects included in the trial were moderate alcohol consumers (1-3 drinks/day) and had diabetes mellitus or $\geq$3 of the following cardiovascular disease risk factors: tobacco smoking, hypertension, plasma LDL cholesterol $\geq$160 mg/dL, plasma HDL cholesterol $\leq$35 mg/dL, overweight or obesity (body mass index $\geq$25 kg/m^2), and/or family history of premature coronary heart disease (CHD). Exclusion criteria included documented CHD, stroke or peripheral vascular disease, human immunodeficiency virus infection, alcoholic liver disease, alcoholism or toxic abuse, malnutrition and neoplastic or acute infectious diseases. The Institutional Review Board of the hospital approved the study protocol and all participants gave written consent before participation in the study. The trial was registered in the Current Controlled Trials at London (http://www.controlled-trials.com/), International Standard Randomized Controlled Trial Number at http://www.isrctn.org/, as ISRCTN95345245.

Study design

The study was an open, randomized, crossover, controlled clinical trial, which included three 4-week intervention periods. Two weeks prior to the study the subjects were asked to maintain their usual diet and to refrain from consuming any alcoholic beverage. Baseline data were collected after this run-in period. Following this, participants were individually randomized in a crossover design among three treatment sequences lasting 4 weeks each, in which the test beverages were provided. Randomization was based on a computer-generated random number table, obtained by a secretary who did not participate in the recording and evaluation of the data, resulting in six possible diet sequences. Participants were assigned to the different interventions and received gin (100 mL – 30 g of ethanol/day), beer (660 mL – 30 g of ethanol/day), or the

same amount of polyphenols as beer in the form of non-alcoholic beer (990 mL). None of the participants consumed multivitamin or vitamin E supplements or anti-inflammatory drugs (steroids, NSAIDs or aspirin >100 mg/day). The beer and the non-alcoholic beer were of the lager type from the same Spanish commercial brand. The phenolic composition of the beers used in the study is detailed in **Supplemental Table 1**. The alcoholic degree of the beverages was 38, 5.4 and <0.1%, for gin, beer and non-alcoholic beer, respectively. The phenolic profile of beer and non-alcoholic beer was determined by the SPE-LC-ESI-MS/MS as described previously and adapted to beer samples[15]. The daily intake of polyphenols derived from non-alcoholic and beer interventions showed no significant differences (**Supplemental Table 1**), while the phenolic content of gin was negligible[16].

Diet and exercise monitoring

Subjects were asked to exclude alcoholic beverages as well as non-alcoholic beer 15-d before the first intervention (run-in period) and during the study. Subjects were also asked to maintain their lifestyle habits and not to change their dietary pattern during the study. Natural foods rich in antioxidants, especially fruit and vegetables, were especially monitored to ensure that individual diets had a similar antioxidant content throughout the study. After the run-in period and the day after each intervention period, the medical history was reviewed and the Minnesota Leisure Time Physical Activity Questionnaire was administered. In addition, the last week of the run-in period and the last week of each intervention period, the subjects were asked to fill in a validated 7-d food record questionnaire. The food records were used to assess nutrient intake and to monitor adherence to the study protocol. Compliance with the test drinks was also assessed by measures of urinary biomarkers of beer polyphenol intake. Foods were converted into nutrients using the Food Processor Nutrition and Fitness Software (esha Research, Salem, OR), adapted to local foods. At the end of each study intervention, a clinician assessed any adverse effects from the interventions by administering a checklist of symptoms, including

bloating, fullness or indigestion, altered bowel habit, dizziness and other symptoms possibly associated with the interventions.

Methods

Fasting blood samples and 24-h urine were collected at baseline (the last day of the run-in period) and the day after the last day of each intervention (beer, non-alcoholic beer and gin). Serum and urine samples were stored at –80°C until assayed. The clinical investigators and laboratory technicians were blinded to the interventions. To assess the compliance of interventions, isoxanthohumol (IX), a biomarker of beer intake, was determined in 24-h urine by SPE-LC-MS/MS as described previously[17].

EPC determination

A 10mL aliquot of whole blood after 12 hours fasting was used for EPC quantification. Samples were processed within one hour after collection and peripheral-blood mononuclear cells (PBMC) were isolated by Ficoll density-gradient centrifugation. Recovered cells were washed twice with phosphate buffered saline (PBS), and the pellet was resuspended at a concentration of 10^7 cells/mL of PBS/2% fetal bovine serum (FBS) after counting in a Neubauer chamber. A volume of 100 µL of the suspension (10^6 cells) was incubated for 1 hour in the dark at 4ºC with 10 µL of the monoclonal antibodies against human KDR (PE-labeled), CD34-FITC and CD133-APC (MAC's Milteny Biotech, all), known to be expressed in EPC[18]. At the end of the incubation 1 µL of 7-Aminoactinomycin D (7AAD) (Sigma-Aldrich) was added and cells were washed with PBS/2% FBS, and fixed in PBS/1% paraformaldehyde before analysis. Each analysis was carried out at a maximum of one hour after fixation and included 500,000 PBMC. The gating strategy to identify and quantify EPC is shown in **Figure 1**. Briefly, R1 was set according to EPC size and granularity and debris were excluded from R1 by the 7AAD staining (therefore $7AAD^-$ cells were selected, R2 region). $CD34^+$ cells (R3) were selected from R2 region; $CD133^+$ cells (R4) were selected from R3, and KDR^+ cells were identified from R4 as

EPC ($7AAD^-/CD34^+/CD133^+/KDR^+$). Results were expressed as number of EPC/500,000PBMC. Fluorescence was monitored with the Sphero™ Rainbow Calibration Particles (6 peaks) of 6.0-6.4μm (BD Biosciences). Cell counting (500,000 PBMC) and fluorescence analysis were performed in a FACSCalibur Flow Cytometer (Becton-Dickinson, San Jose, CA) using the CellQuestPro software.

Quantification of soluble of biomarkers of endothelial injury

As surrogate biomarkers of endothelial injury alpha-2-macroglobulin (A2M), interferon gamma induced protein 10 (IP10), interleukin-12 subunit p40 (IL12p40), matrix metalloproteinase-3 (MMP-3), matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinases 1 (TIMP-1), thrombopoietin, vascular endothelial growth factor (VEGF) and von Willebrand factor (vWF) were quantified by customized Human Multi Analyte Profiling (Human MAP) (Rules Based Medicine Inc., Austin, Texas, USA) following the manufacturer's instructions.

Quantification of soluble EPC-mobilizing and angiogenic factors

The following serum soluble cytokines and EPC-mobilizing and angiogenic factors were quantified by customized Human Multi Analyte Profiling (Human MAP) (Rules Based Medicine Inc., Austin, Texas, USA) following the manufacturer's instructions: alpha-fetoprotein (AF), beta-2-microglobulin (B2M), brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), epithelial-derived neutrophil-activating protein 78 (ENA78), erythropoietin (EPO), granulocyte colony-stimulating factor (GCSF), growth hormone (GH), myeloid progenitor inhibitory factor 1 (MPIF-1), stem cell factor (SCF), thrombopoietin and ferritin. Serum soluble stromal cell derived factor 1 (SDF1) and chemokine C-X-C-motif receptor 4 (CXCR4) were quantified with their respective ELISA kits from Cloud-Clone Corp., Houston, Texas, USA as per manufacturer's instructions and platelet-derived growth factor-BB (PDGF-BB) with the eBioscience ELISA kit (Bender MedSystems GmbH, Vienna, Austria).

Statistical analysis

Sample size was determined with the ENE 3.0 statistical program (GlaxoSmithKline, Brentford, United Kingdom) assuming a maximum loss of 10% participants. To detect the mean differences in the number of circulating EPC of 5 units with a conservative SD of 3, 24 subjects would be needed to complete the study (α risk = 0.05, power = 0.9). However, to obtain greater differences, the sample size was increased. The number of circulating EPC was considered the primary outcome and used to determine the sample size.

Statistical analysis was performed using the SAS Statistical Analysis Systems (version 9.2, SAS Institute Inc, Cary, North Carolina). Descriptive statistics [mean ± standard deviation (SD)] were used for the baseline characteristics of the participants. Values with a skewed distribution (AFP, ferritin and IP10) were transformed to their natural logarithm for analyses. To analyze the changes within each treatment a Student's t test for paired samples was performed between the data obtained before and after each intervention. One-factor analysis of variance (ANOVA) for repeated measures and the Bonferroni post-hoc test were used to compare the differences of the changes in outcome variables between the interventions. To exclude the presence of a carryover effect for the three periods, the interaction between treatment (beer, non-alcoholic beer and gin) and sequence of treatment was analyzed through repeated measures ANCOVA analyses with the value of the previous intervention (or the baseline value in the first intervention) as the covariate. Within- and between-group differences are expressed as means and 95% CIs. *P* was considered significant when <0.05.

RESULTS

Baseline characteristics, intervention compliance, diet, exercise monitoring, and side effects

Of the 36 subjects included, three withdrew before completing the three phases of the study because of journeys (n = 1) or incompatibility with the work schedule (n = 2). Therefore, 33 subjects were included in the study. **Table 1** shows the baseline characteristics of these

participants. Most were overweight or obese (85%), nearly three quarters had dyslipemia (70%), more than two thirds had hypertension (64%), and nearly one quarter had type-2 diabetes (21%) or were smokers (24%). Biochemical analytes (serum and intraerythrocytary folic acid, vitamin B_{12}, albumin, ASAT, ALAT and GGT) remained within the normal range throughout the study. None of the subjects reported adverse effects related to the interventions.

Protocol adherence was optimum in all subjects according to their self reports. As a measure of intervention compliance, IX -a biomarker of beer consumption- was determined in 24-h urine samples collected the last day of the run-in period and the last day of each intervention. After consumption of beer and non-alcoholic beer, 24-h urinary excretion of IX increased to 7.2±3.3 and 7.5±2.9 μg respectively (with no significant differences between them), whereas it was not detected at baseline and after the gin intervention ($P < 0.001$ between the two beer interventions and the gin and baseline interventions). According to these results, interventions compliance was excellent.

Data of dietary intake for the three intervention periods are shown in **Supplemental Table 2**. No significant differences were observed in nutrient and antioxidant intake at the beginning of the study and after each intervention or in the daily average energy expended in physical activity during the beer, non-alcoholic beer and gin intake periods. In addition, no changes were reported in drug intake in any of the subjects throughout the study. No carryover effect was observed for any of the variables studied.

Changes in circulating EPC

Changes in circulating EPC after the three interventions are shown in **Figure 2**. After the beer and non-alcoholic beer interventions, the number of circulating EPC significantly increased by 8 (95%CI 2 to 16) and 5 (CI 2 to 11) cells/500,000 PBMC ($P= 0.020$, both), while after the gin period, there was a trend to decrease the number of EPC, albeit not significantly, by 5 (CI -12 to 1) cells/500,000 PBMC ($P= 0.099$). Therefore, the effects of both beer interventions on the

number of circulating EPC were statistically different from those observed after the gin intervention (P= 0.008).

Changes in soluble biomarkers of endothelial injury

As can be observed in **Table 2**, serum concentrations of A2M, IP10, IL12p40, MMP-3, MMP-9, TIMP-1, thrombopoietin, VEGF and vWF remained constant throughout the study.

Changes in soluble EPC-mobilizing and angiogenic factors

Changes in soluble EPC-mobilizing and angiogenic factors are shown in **Table 3**. SCF decreased significantly after the non-alcoholic beer intervention (P= 0.020) while increasing significantly after the gin period (P= 0.043), but no changes were observed after the beer intervention. Therefore, the effects of gin and non-alcoholic beer on SCF serum concentrations were statistically different between them (P= 0.038). No correlation was observed between SCF and the number of circulating EPC (data not shown).

SDF1 increased significantly after the beer and non-alcoholic beer interventions (P=0.006 and 0.04, respectively), and no differences were observed after the gin period. Again, the effects of both beers achieved statistical significance compared to the gin intervention (P=0.029). On the other hand, a significant positive correlation was observed between the changes in SDF1 and circulating EPC after the beer and non-alcoholic beer interventions (P=0.031 R^2=0.310 and P=0.038 R^2=0.124, respectively).

No changes were observed after the three interventions in the serum concentrations of AF, B2M, BDNF, EGF, ENA78, EPO, ferritin, GCSF and GH.

DISCUSSION

The enhancement of circulating EPC and their functional capacity by changes in lifestyle (physical exercise[19]) or pharmacological treatments (statins[20]) may be relevant for patients who have had a cardiovascular event or subjects who are at high risk to suffer it. In this study, we

found that moderate alcoholic and non-alcoholic beer consumption enhanced the number of circulating EPC in high cardiovascular risk subjects. Since the effects of alcoholic and non-alcoholic beer consumption were similar, these results suggest that the non-alcoholic fraction of beer may be responsible for these effects. On the other hand, as gin did not change the number of circulating EPC, ethanol by itself does not seem to play a role in EPC. No synergistic effects were observed between the alcoholic and non-alcoholic fractions of beer. To our knowledge, this is the first time that circulating EPC and EPC-mobilizing factors have been evaluated after moderate beer consumption in a high cardiovascular risk population. Nevertheless, our findings were unexpected in view of those by Huang *et al.*[14], who observed a significant increase in the number of circulating EPC after moderate red wine but not beer consumption in healthy individuals. We observed that gin did not significantly modulate the number of circulating EPC, which is in accordance with Huang *et al.*[14], who observed no significant differences in the number of circulating EPC after a vodka intervention (both gin and vodka are distilled beverages without polyphenols). The differences observed after the beer intervention can partially be attributed to the different composition of the beers administered in both studies as well as the fact that populations with different cardiovascular risk burden respond to interventions in a different manner, as observed in previous studies by our group[16].

Increased mobilization or enhanced survival and/or differentiation have been proposed as mechanisms for the increase in circulating EPC [4]. According to our findings, the increase observed in circulating EPC is possibly due to their mobilization or enhanced survival through a SDF1-mediated mechanism, as the increase in the number of circulating EPC was correlated with the increase of SDF1 after both beer interventions, and the biomarkers of endothelial injury remained constant throughout the study. However, further studies are required to elucidate the compounds and intrinsic mechanisms by which the non-alcoholic fraction of beer increases the plasma concentration of SDF-1 in high cardiovascular risk subjects and other population subsets.

EPC have the ability to repair endothelial damage as well as contribute to the angiogenic process. Angiogenesis, the formation of new capillaries from the pre-existing vasculature by migration, proliferation, and structural rearrangement of endothelial cells, plays a fundamental role in both physiologic and pathologic situations[21]. Alcohol abuse is associated with increased VEGF-A serum levels[22], and *in vitro* studies have shown that even moderate amounts of ethanol are capable of inducing angiogenesis[23]. However, little is known about the effects of moderate alcohol consumption on the expression of angiogenic factors in humans. In addition, polyphenols from beer have shown antagonic angiogenic effects *in vitro*[24]. To our knowledge, this is the first time that soluble angiogenic factors have been analyzed after moderate alcohol consumption in a human population. We observed a decrease in SCF (known to be an EPC-mobilizing agent[25]) after the non-alcoholic beer intervention and an increase after the gin period, but its clinical significance seems negligible. Nevertheless, interestingly, the increase in circulating EPC was correlated with the increase of its mobilizing agent SDF1[26], which has been shown to reduce apoptosis, enhancing EPC survival *ex vivo*[27]. Therefore, the increased number of EPC after non-alcoholic beer fraction consumption should be attributed to an enhanced survival in the circulation. The fact that the population studied did not present any cardiovascular event during the intervention periods may explain why angiogenic factors remained constant. Overall, this increased circulating EPC could be translated into a re-endothelization more than a neovascularization process, thereby protecting the endothelium from possible chronic damage.

This study is not exempt of limitations. One of the limitations is that we did not include washout periods between the interventions. Washout periods would have extended the study 6 weeks more, decreasing subject compliance and increasing withdrawal. However, since no carryover effect was observed for any of the variables, the absence of a washout period would probably not have changed the results obtained. Another limitation is that the characteristics of the study design did not allow identifying the specific substances responsible for the effects observed.

Lastly, the results of this work can not be extrapolated to other populations and a 4-week intervention may not represent the potential effects of long-term consumption. Nonetheless, increased circulating EPC are associated with a reduced cardiovascular risk[28], giving our results potential clinical significance.

In conclusion, the non-alcoholic fraction of beer increases the number of circulating EPC in peripheral blood from high cardiovascular risk subjects, a mechanism that may explain the greater cardioprotective effects of beer compared to distilled alcoholic beverages in the high cardiovascular risk population.

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DISCLOSURES

Dr. Estruch reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN); serving on the boards of the Beer and Health

Foundation and the European Foundation for Alcohol Research (ERAB); receiving lecture fees from Cerveceros de España and Sanofi-Aventis; and receiving grant support through his institution from Novartis. Dr. Lamuela-Raventos reports serving on the board of and receiving lecture fees from FIVIN; receiving lecture fees from Cerveceros de España; and receiving lecture fees and travel support from PepsiCo. The other authors declare no conflict of interest.

REFERENCES

1. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964-967.
2. Urbich C, Dimmeler S. Endothelial progenitor cells: Characterization and role in vascular biology. *Circ Res*. 2004;95:343-353.
3. Shantsila E, Watson T, Lip GY. Endothelial progenitor cells in cardiovascular disorders. *J Am Coll Cardiol*. 2007;49:741-752.
4. Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M, Kamper U, Dimmeler S, Zeiher AM. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: Proof of concept for the clinical importance of endogenous vascular repair. *Circulation*. 2005;111:2981-2987.
5. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003;348:593-600.
6. Ronksley PE, Brien SE, Turner BJ, Mukamal KJ, Ghali WA. Association of alcohol consumption with selected cardiovascular disease outcomes: A systematic review and meta-analysis. *BMJ*. 2011;342:d671.
7. Costanzo S, Di Castelnuovo A, Donati MB, Iacoviello L, de Gaetano G. Wine, beer or spirit drinking in relation to fatal and non-fatal cardiovascular events: A meta-analysis. *Eur J Epidemiol*. 2011;26:833-850.

8. Balestrieri ML, Fiorito C, Crimi E, Felice F, Schiano C, Milone L, Casamassimi A, Giovane A, Grimaldi V, del Giudice V, Minucci PB, Mancini FP, Servillo L, D'Armiento FP, Farzati B, Napoli C. Effect of red wine antioxidants and minor polyphenolic constituents on endothelial progenitor cells after physical training in mice. *Int J Cardiol*. 2008;126:295-297.

9. Balestrieri ML, Schiano C, Felice F, Casamassimi A, Balestrieri A, Milone L, Servillo L, Napoli C. Effect of low doses of red wine and pure resveratrol on circulating endothelial progenitor cells. *J Biochem*. 2008;143:179-186.

10. Felice F, Zambito Y, Di Colo G, D'Onofrio C, Fausto C, Balbarini A, Di Stefano R. Red grape skin and seeds polyphenols: Evidence of their protective effects on endothelial progenitor cells and improvement of their intestinal absorption. *Eur J Pharm Biopharm*. 2012;80:176-184.

11. Huang PH, Tsai HY, Wang CH, Chen YH, Chen JS, Lin FY, Lin CP, Wu TC, Sata M, Chen JW, Lin SJ. Moderate intake of red wine improves ischemia-induced neovascularization in diabetic mice--roles of endothelial progenitor cells and nitric oxide. *Atherosclerosis*. 2010;212:426-435.

12. Lefevre J, Michaud SE, Haddad P, Dussault S, Menard C, Groleau J, Turgeon J, Rivard A. Moderate consumption of red wine (cabernet sauvignon) improves ischemia-induced neovascularization in apoe-deficient mice: Effect on endothelial progenitor cells and nitric oxide. *FASEB J*. 2007;21:3845-3852.

13. Hamed S, Alshiek J, Aharon A, Brenner B, Roguin A. Red wine consumption improves in vitro migration of endothelial progenitor cells in young, healthy individuals. *Am J Clin Nutr*. 2010;92:161-169.

14. Huang PH, Chen YH, Tsai HY, Chen JS, Wu TC, Lin FY, Sata M, Chen JW, Lin SJ. Intake of red wine increases the number and functional capacity of circulating endothelial progenitor cells by enhancing nitric oxide bioavailability. *Arterioscler Thromb Vasc Biol*. 2010;30:869-877.

15. Vallverdu-Queralt A, Jauregui O, Medina-Remon A, Lamuela-Raventos RM. Evaluation of a method to characterize the phenolic profile of organic and conventional tomatoes. *J Agric Food Chem*. 2012;60:3373-3380.

16. Chiva-Blanch G, Urpi-Sarda M, Llorach R, Rotches-Ribalta M, Guillen M, Casas R, Arranz S, Valderas-Martinez P, Portoles O, Corella D, Tinahones F, Lamuela-Raventos RM, Andres-Lacueva C, Estruch R. Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: A randomized clinical trial. *Am J Clin Nutr*. 2012;95:326-334.

17. Quifer Rada P, Martinez-Huelamo M, Jauregui O, Chiva-Blanch G, Estruch R, Lamuela-Raventos RM. Analytical condition setting is a crucial step in the quantification of unstable polyphenols in acidic conditions: Analysing prenylflavanoids in biological samples by lc-esi-ms/ms. *Anal Chem*. 2013; 85:5547-54.

18. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of vegfr-2 and ac133 by circulating human cd34(+) cells identifies a population of functional endothelial precursors. *Blood*. 2000;95:952-958.

19. Laufs U, Werner N, Link A, Endres M, Wassmann S, Jurgens K, Miche E, Bohm M, Nickenig G. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation*. 2004;109:220-226.

20. Liu Y, Wei J, Hu S, Hu L. Beneficial effects of statins on endothelial progenitor cells. *Am J Med Sci*. 2012;344:220-226.

21. Carmeliet P. Angiogenesis in life, disease and medicine. *Nature*. 2005;438:932-936.

22. Heberlein A, Muschler M, Lenz B, Frieling H, Buchl C, Groschl M, Riera R, Kornhuber J, Bleich S, Hillemacher T. Serum levels of vascular endothelial growth factor a increase during alcohol withdrawal. *Addict Biol*. 2010;15:362-364.

23. Gu JW, Elam J, Sartin A, Li W, Roach R, Adair TH. Moderate levels of ethanol induce expression of vascular endothelial growth factor and stimulate angiogenesis. *Am J Physiol Regul Integr Comp Physiol*. 2001;281:R365-372.

24. Negrao R, Costa R, Duarte D, Taveira Gomes T, Mendanha M, Moura L, Vasques L, Azevedo I, Soares R. Angiogenesis and inflammation signaling are targets of beer polyphenols on vascular cells. *J Cell Biochem*. 2010;111:1270-1279.

25. Jialal I, Fadini GP, Pollock K, Devaraj S. Circulating levels of endothelial progenitor cell mobilizing factors in the metabolic syndrome. *Am J Cardiol*. 2010;106:1606-1608.

26. Zheng H, Fu G, Dai T, Huang H. Migration of endothelial progenitor cells mediated by stromal cell-derived factor-1alpha/cxcr4 via pi3k/akt/enos signal transduction pathway. *Journal of cardiovascular pharmacology*. 2007;50:274-280.

27. Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, Bosch-Marce M, Masuda H, Losordo DW, Isner JM, Asahara T. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation*. 2003;107:1322-1328.

28. Hill JM, Finkel T, Quyyumi AA. Endothelial progenitor cells and endothelial dysfunction. *Vox Sang*. 2004;87 Suppl 2:31-37.

FIGURE CAPTIONS

Figure 1. Gating strategy to identify EPC in the FACS analyses.

R1 was set according to their size and granularity and debris were excluded from R1 by 7AAD staining (therefore 7AAD- cells were selected; R2). CD34+ cells were selected (R3) from this region (R2). CD133+ cells were selected (R4) from R3 and KDR+ cells were identified from R4 as EPC (7AAD-/CD34+/CD133+/KDR+).

Figure 2. Changes in the number of circulating EPC after the three interventions (expressed in terms of differences between before and after each intervention).

EPC: endothelial progenitor cells; PBMC: peripheral blood mononuclear cells.

P value of the differences between interventions (repeated measures ANOVA with the Bonferroni post-hoc test). *Significant differences between before and after the interventions (t-test).

TABLES

Table 1. Baseline characteristics of the 33 subjects included in the study.

	Mean ± SD*
Age (years)	61 ± 6
Hypertension [n (%)]	21 (64)
Dyslipemia [n (%)]	23 (70)
Type 2 Diabetes Mellitus [n (%)]	7 (21)
Current smokers [n (%)]	8 (24)
Sedentarism [n (%)]	6 (18)
Family history of premature CHD [n (%)]	2 (6)
Medications [n (%)]	
ACE Inhibitors	16 (48)
Diuretics	4 (12)
Statins	15 (45)
Fibrates	1 (3)
Oral hypoglycemic drugs	6 (18)
Aspirin or antiplatelet drugs	4 (12)
BMI (kg/m2)	28.8 ± 4.1
BMI≥ 25 kg/m2 [n (%)]	28 (85)
Abdominal perimeter (cm)	101 ± 10
WHR	0.95 ± 0.05
Systolic Blood Pressure (mmHg)	138 ± 16
Diastolic Blood Pressure (mmHg)	81 ± 8
Heart rate (beats/min)	68 ± 11
Glucose (mg/dL)	112 ± 27
Triglycerides (mg/dL)	99 ± 43
Total cholesterol (mg/dL)	185 ± 31
LDL cholesterol (mg/dL)	119 ± 26
HDL cholesterol (mg/dL)	44 ± 11
LDLc/HDLc ratio	3.03±0.88
Folic acid (serum) (ng/mL)	8.7 ± 3.7
Intraerythrocytary folic acid (ng/mL)	407 ± 94
Vitamin B12 (pg/mL)	432 ± 206
Albumin (mg/mL)	43 ± 2
ASAT (UI/L)	25 ± 12
ALAT (UI/L)	29 ± 16
GGT (UI/L)	29 ± 14

*Mean ± SD or *n* (%), when indicated (n=33).

CHD, Coronary Heart Disease; BMI, Body Mass Index; WHR, waist-to-hip ratio; ACE, Angiotensin-Converting Enzyme; LDL, Low Density Lipoprotein; HDL, High Density Lipoprotein; ASAT, Aspartate aminotransferase; ALAT, Alanine aminotransferase; GGT, Gamma glutamyl transpeptidase.

Table 2. Expression of serum soluble biomarkers of endothelial injury before and after each intervention in the 33 subjects included in the study.

	Beer intervention			Non-alcoholic beer intervention			Gin intervention			
	Mean±SD*		Mean differences	Mean± SD *		Mean differences	Mean± SD *		Mean differences	
	Before	After	(95% CI)†	Before	After	(95% CI)†	Before	After	(95% CI)†	*P*‡
A2M (mg/mL)	1.60 ± 0.47	1.62 ± 0.44	0.02 (-0.08, 0.12)	1.62 ± 0.40	1.62 ± 0.39	-0.01 (-0.10, 0.08)	1.68 ± 0.42	1.71 ± 0.58	0.02 (-0.13, 0.18)	0.888
IP10 (pg/ml)	240 ± 138	270 ± 201	29.11 (-7.42, 65.63)	235 ± 91	285 ± 214	50.10 (-21.74, 121.94)	259 ± 125	229 ± 64	-29.40 (-69.07, 10.26)	0.083
IL-12p40 (ng/mL)	0.16 ± 0.06	0.15 ± 0.05	-0.01 (-0.02, 0.01)	0.15 ± 0.05	0.15 ± 0.06	0.01 (-0.01, 0.02)	0.16 ± 0.04	0.16 ± 0.05	0.01 (-0.01, 0.02)	0.194
MMP-3 (ng/mL)	23.2 ± 8.2	23.5 ± 8.1	0.29 (-1.52, 2.11)	23.9 ± 10.1	22.7 ± 8.6	-0.74 (-2.46, 0.97)	23.4 ± 9.7	22.2 ± 7.0	-1.23 (-3.16, 0.70)	0.524
MMP-9 (ng/mL)	6.64 ± 3.06	7.28 ± 2.35	0.64 (-2.29, 1.01)	6.72 ± 2.40	7.45 ± 3.04	1.29 (-0.79, 3.37)	7.33 ± 2.95	6.65 ± 2.94	-1.944 (-3.90, 0.02)	0.085
TIMP- 1 (ng/mL)	138 ± 22	141 ± 24	3.37 (-2.20, 8.95)	138 ± 24	141 ± 22	2.68 (-5.01, 10.38)	141 ± 20	137 ± 20	-4.00 (-9.97, 1.97)	0.286
Thrombopoietin (ng/mL)	3.10 ± 0.84	3.01 ± 0.70	-0.09 (-0.25, 0.07)	3.00 ± 0.83	3.03 ± 0.79	0.01 (-0.15, 0.16)	2.90 ± 0.74	2.968 ± 0.85	0.05 (-0.13, 0.24)	0.550
VEGF (pg/mL)	1147 ± 407	1165 ± 369	8.53 (-47.77, 64.84)	1151 ± 364	1117 ± 356	-27.50 (-81.74, 26.74)	1173 ± 434	1205 ± 442	24.80 (-37.64, 87.24)	0.487
vWF (µg/mL)	48.1 ± 18.2	47.1 ± 19.5	-1.03 (-5.01, 2.95)	47.7 ± 19.6	48.788 ± 19.23	1.2 (-2.70, 5.10)	50.9 ± 18.3	45.8 ± 18.4	-6.53 (-13.54, 0.48)	0.156

Results are expressed as *mean ± SD (n=33) and †mean differences (95% CI) between before and after each intervention. Before each intervention is the value of the previous intervention or the baseline value in the first intervention. ‡P value of the repeated-measures ANOVA from the differences between interventions. §Significant differences (P<0.05) between before and after the intervention, measured by a Student's t test for paired samples. Rows with different superscript letters express differences between interventions (repeated-measures ANOVA and the Bonferroni post-hoc test).

Table 3. Expression of serum soluble EPC-mobilizing and angiogenic factors before and after each intervention in the 33 subjects included in the study.

	Beer intervention			Alcohol-free beer intervention			Gin intervention			
	Mean±SD*		Mean differences	Mean± SD *		Mean differences	Mean± SD *		Mean differences	
	Before	After	(95% CI)†	Before	After	(95% CI)†	Before	After	(95% CI)†	*P*‡
AF (ng/mL)	1.01 ± 1.26	1.12 ± 1.09	0.071 (-0.16, 0.30)	1.1 ± 1.13	1.11 ± 1.35	0.01 (-0.18, 0.20)	1.11 ± 1.04	1.10 ± 1.16	-0.01 (-0.11, 0.10)	0.714
B2M (µg/mL)	1.55 ± 0.49	1.63 ± 0.37	0.07 (-0.05, 0.19)	1.63 ± 0.33	1.65 ± 0.41	0.02 (-0.06, 0.11)	1.63 ± 0.37	1.59 ± 0.44	-0.09 (-0.21, 0.03)	0.204
BDNF (ng/mL)	17.9 ± 3.8	17.6 ± 4.8	-0.22 (-2.01, 1.57)	18.0 ± 5.0	17.1 ± 3.9	-0.90 (-2.22, 0.41)	16.6 ± 4.4	17.7 ± 4.6	0.99 (-0.73, 2.71)	0.355
CXCR4 (ng/mL)	0.94 ± 0.79	0.94 ± 0.49	0.01 (-0.17, 0.19)	0.92 ± 0.56	0.972 ± 0.72	0.06 (-0.07, 0.18)	1.06 ± 0.72	0.90 ± 0.59	-0.16 (-0.40, 0.07)	0.273
EGF (pg/mL)	163 ± 129	225 ± 178	49 (-17, 116)	197 ± 154	234 ± 196	42 (-23, 106)	166 ± 143	199 ± 165	33 (-30, 96)	0.948
ENA78 (ng/mL)	2.15 ± 1.19	2.34 ± 1.27	0.12 (-0.02, 0.26)	2.25 ± 1.24	2.21 ± 1.18	-0.05 (-0.19, 0.09)	2.25 ± 1.17	2.17 ± 1.12	-0.07 (-0.21,0.06)	0.140
EPO (pg/mL)	6.35 ± 3.90	6.84 ± 5.67	0.10 (-2.15, 2.34)	7.46 ± 5.92	6.49 ± 4.38	-0.95 (-3.21, 1.31)	6.32 ± 4.82	6.36 ± 4.24	0.16 (-1.53, 1.85)	0.752
Ferritin (ng/mL)	186 ± 149	176 ± 143	-9 (-21, 3)	230 ± 225	235 ± 203	4 (-5, 14)	178 ± 139	170 ± 1403	-7 (-8, 4)	0.185
GCSF (pg/mL)	6.55 ± 2.44	6.358 ± 2.343	-0.16 (-0.85, 0.53)	6.48 ± 2.11	6.91 ± 2.22	0.67 (-0.26, 1.60)	6.74 ± 2.68	6.30 ± 2.40	-0.43 (-1.39, 0.54)	0.277
GH (ng/mL)	0.79 ± 0.83	0.92 ± 0.94	0.13 (-0.17, 0.42)	0.80 ± 0.82	0.80 ± 0.97	0.002 (-0.34, 0.35)	0.72 ± 0.86	0.76 ± 0.78	0.04 (-0.21, 0.29)	0.842
MPIF-1 (ng/mL)	1.43 ± 0.46	1.41 ± 0.43	-0.06 (-0.17, 0.05)	1.43 ± 0.35	1.40 ± 0.39	-0.03 (-0.11, 0.04)	1.45 ± 0.42	1.45 ± 0.37	-0.01 (-0.11, 0.10)	0.818
PDGF-BB (pg/mL)	876 ± 390	864 ± 458	-13 (-133, 108)	860 ± 401	845 ± 361	-15 (-163, 133)	801 ± 408	832 ± 378	30 (-95, 156)	0.865
SCF (pg/mL)	358 ± 94	361 ± 116	3 (-28, 21)[a,b]	374 ± 117	347 ± 97	-24 (-47, -2)[a,§]	349 ± 112	366 ± 111	20 (1, 39)[b,§]	0.038
SDF1 (ng/mL)	1.45 ± 0.59	1.78 ± 0.79	0.34 (0.10, 0.58)[a,§]	1.56 ± 0.56	2.00 ± 1.23	0.43 (0.02, 0.87)[a,§]	1.67 ± 0.83	1.50 ± 0.54	-0.18 (-0.46, 0.10)[b]	0.029

Results are expressed as *mean ± SD (n=33) and †mean differences (95% CI) between before and after each intervention. Before each intervention is the value of the previous intervention or the baseline value in the first intervention. ‡P value of the repeated-measures ANOVA from the differences between interventions. §Significant differences (P<0.05) between before and after the intervention, measured by a Student's t test for paired samples. Rows with different superscript letters express differences between interventions (repeated-measures ANOVA and the Bonferroni post-hoc test).