FOOD & FUNCTION

A moderate red wine intake improves blood lipid parameters and erythrocytes membrane fluidity in post myocardial infarct patients

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While the cardioprotective effect of moderate and regular wine consumption in primary prevention has been well documented, the goal of the present investigation was to explore the effect of wine intake on blood parameters (lipid, anti-oxidant capacity, and erythrocyte membrane potential and fluidity) in post myocardial infarct patients to evaluate perspectives in secondary prevention. A clinical intervention trial has been undertaken on a group of selected post myocardial infarct patients who gave written informed consent for participation in this study prior to enrolment. This two-week study has been conducted on hospitalized patients during a cardiac readaptation period. During this period, patients were submitted to a ''Western prudent'' diet (inspired by the Mediterranean diet) and two groups have been compared on a drawn basis: patients receiving red wine (250 mL daily) to patients receiving water. Physical, clinical, and blood parameters were evaluated on Days 1 and 14. The data show a positive effect of low wine consumption on blood parameters (decrease in total cholesterol and LDL; increase in erythrocyte membrane fluidity and antioxidant status). The results show that a moderate consumption of red wine even for a short period associated with a ''Western prudent'' diet improves various blood parameters in lipid and anti-oxidative status in patients with previous coronary ischemic accidents.

Keywords:
Anti-oxidant / Blood / Cardiac alteration / Lipid / Wine

In 2004, the international multicenter case–control study INTERHEART in 52 countries from all continents was published [1]. The study revealed the existence of different cardiovascular risk factors and suggested three preventive factors (regular physical exercise, regular and moderate alcoholic beverage intake, and a fruit- and vegetable-based diet). The evidence of the cardioprotective effect of moderate and regular wine consumption in primary prevention has been confirmed either on the increase in plasma anti-oxidant capacity [2], the improvement of endothelial functions [3], the modulation of immunological functions of leukocytes [4], or recently, on the polyphenol dependant of red wine anti-oxidant effect [5]. The goal of this study was to explore the effects of red wine consumption in patients following a cardiovascular event. This trial has never been

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Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); **CRP,** C-reactive protein; **TEAC,** Trolox Equivalent Antioxidant Capacity Assay

undertaken yet. The originality of this intervention study conducted during hospitalization was its high level of standardization: the patients had the same lifestyle, the same level of exercise, and received the same diet during the study period. Overall, the results in that study showed that a moderate consumption of red wine associated with a ''Western prudent'' diet has some benefits on various blood parameters of the lipid and oxidative status after coronary ischemic accidents, i.e. decrease in total cholesterol and LDL; increase in erythrocyte membrane fluidity and antioxidant status. *Patients and diet protocol*: This was a clinical intervention trial on a group of patients conducted in the Cardiology Department of the Montbard Hospital (Montbard, France) over a three-month period (see Fig. 1 Supporting Information). The patients (39 subjects, 32 males and 7 females; mean age $\pm$ SD, 65 ± 7.9 years) were recruited by the department's cardiologists who selected patients with ischemic cardiopathies and previous myocardial infarct. Almost all of them were operated with coronarian bridge or stent. Following surgery, they received the classical medical prescriptions related to their cardiac pathologies: β-blockers, aspirin, anti-coagulant, conversion enzyme inhibitors, and/or statins. Importantly, it must be pointed out that at the start of the intervention trial study the selected patients received only aspirin as a medical prescription. They were welcomed for a three-wk cardiovascular rehabilitation programme. Patients with non-atheromatous cardiac pathologies were excluded as well as patients with alcohol addiction. The protocol of this clinical study was in agreement with the principles of the Helsinki declaration and was ethically approved by the local Committee on Human Research. All patients were included on a volunteer basis after description of the clinical project by the clinician. The information provided to the patients covered cardiovascular pathologies and the importance of a healthy diet; a brief history on the relationship between wine and health was also provided; the study and its objective were then described. All patients gave written informed consent for participation in this study prior to enrolment. The patients were divided into two drawn groups: a wine drinker group and a water drinker group. During the study, all patients were confined to a closed space: they were in the hospital for 3 wks with medical verification of the diet and physical activity. The cardiovascular rehabilitation program included a diet called the ''Western prudent'' diet, which is inspired by the Mediterranean diet principles of the Lyon study [6]. The diet (nutritional needs and the food composition of the meals) is shown in Table 1 (as Supporting Information) and corresponds to limited saturated fat (animal fat, oil), butter replaced by margarine, use of olive and coleseed oils, preference for fruits and vegetables, a limited supply of cheese (twice to three times a wk), water ad libitum for the patients of both groups. The ''red wine drinkers'' patients (15 patients; 11 males and 4 females; 66 ± 8 years) received two glasses of red wine per day, around 25 cl, distributed by the attending team: one glass at lunch and one glass at dinner. The wine was a red Burgundy, prepared from pinot noir grapes, vintage 1999, aged 7 years in oak barrel wine: Les Hautes-Côtes from the Château de Villars Fontaine, France, chosen for its high phenolic content ($3.81\ g/L \pm 0.17$ gallic acid equivalent). The detailed composition of polyphenol content is shown on table II (as Supporting Information). The ''water drinkers'' patients included 14 patients (11 males and 3 females; 64 ± 11 years). The patients (wine drinkers, water drinkers) were indexed in an individual file where their name was matched to a code number. The blood samples were then rendered anonymous by the cardiology service. The study was conducted over 15 days for each patient and blood samples were collected on Days 1 (D1) and 14 (D14). The blood was collected in the morning during the standard check-up included in the rehabilitation program. Blood samples collected on EDTA were sent to the hospital laboratory for the measurement of conventional parameters (total cholesterol, LDL, HDL, albumin) or to a specialized laboratory for the evaluation of the oxidative status (Lara-Spiral laboratory) and of erythrocyte membrane fluidity (INSERM 866). A summary of the protocol is shown in the chart (Fig. 1). Blood parameters measured at D1 and D14 for the two groups of patients were compared. *Blood samples*: Overnight fasting blood samples were collected in the morning in blood collection tubes (BD Biosciences, San Diego, CA, USA). The sera were immediately analyzed for their lipid profiles, albumin, C-reactive protein (CRP), and glucose. The whole blood was also immediately analyzed for its oxidative status with the KRL test (Kirial International/Spiral, Couternon, France), for erythrocyte transmembrane potential with $DiOC_6(3)$ and erythrocyte membrane fluidity with Merocyanine 540 (MC540) using flow cytometry. For further evaluation of pro-inflammatory cytokine levels (IL-1-β, IL-4, IL-6, IL-8, IFN-γ, TNF-α), the plasma samples were stored at $-80^{\circ}C$. *Estimation of clinical blood parameters*: Cholesterol, LDL, HDL, triglycerides, and CRP were measured on a Vista automate (Siemens, Munich, Germany) with specifically dedicated reagents. The reference values were the following: total cholesterol, 1.07 and 2.00 g/L; triglycerides, 0.35–1.35 g/L; HDL, 0.4–0.9 g/L; LDL, 1.0–1.6 g/L; CRP, < 0.3 mg/L. Measurement of free-radical-induced hemolysis with the KRL test: The antioxidant defenses were examined using a test based on in vitro free-radical-induced blood hemolysis based on [7, 8]. Blood samples were collected using EDTA as an anticoagulant. Hemolysis was started by adding 50 mM of 2,2′-azo-bis(2-amidinopropane) HCl (AAPH) and was assayed by monitoring absorbance at 620 nm (Kirial International/Spiral) of diluted patient blood. The results, given in triplicate, were expressed as 50% of maximal hemolysis time (HT_{50} in min) and referred to as the blood susceptibility to free radicals. Trolox equivalent antioxidant capacity assay (TEAC Assay) according to Re et al. [9]: The principle is the following: A ferryl myoglobin radical is formed from metmyoglobin and hydrogen peroxide. The ferryl myoglobin radical can oxidize

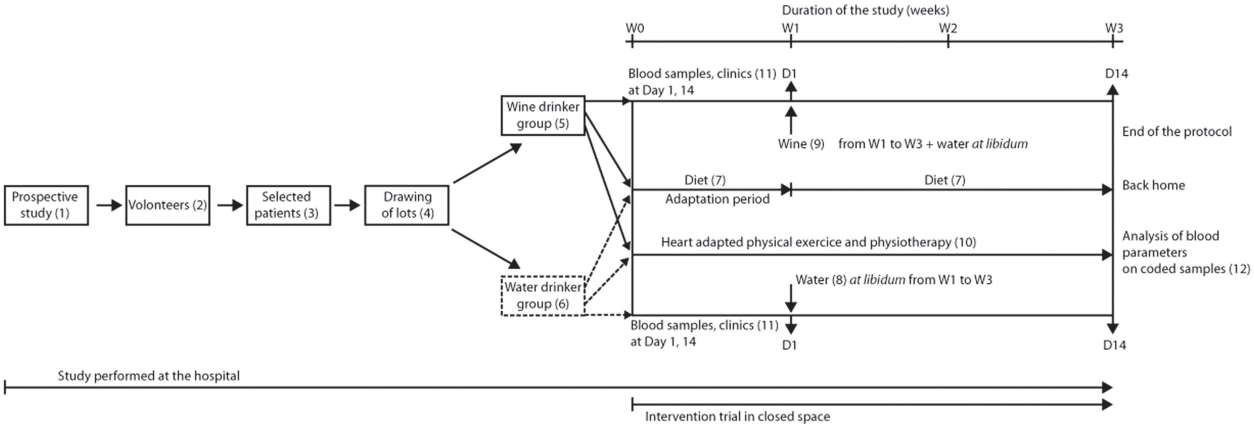

Figure 1. Chart of the intervention trial on post myocardial infarct patients. This time course summarizes the protocol described in ''Material and Method'' section ''Patient and diet protocol''. (1) Sixty patients suffering from ischemic cardiopathy and previous myocardial infarct (excluding non-atheromatous cardiopathies) were followed at the cardiology department of the hospital for a 3-month period of time. (2) Around 60 volunteers agree to participate to this study. Patients having a pre-existing alcoholism (revealed after a test validated by alcohologues physicians) were excluded. It must be pointed out that there were no patients suffering from infectious diseases. At this step, 39 subjects were included: 32 males and 7 females; mean of age $\pm$ SD, 65 $\pm$ 7.9 years. (3) The selected patients for the study have given written informed consents. Due to some personal reasons, treatments, and/or life style (i.e. treatment with insulin, thyroid hormone, anti-diuretic drugs or smokers), some patients have been excluded. So, 29 patients were finally selected. All accepted to possibly become ''wine drinker''. (4) For the intervention trial two groups ''wine drinker'' or ''water drinker'' were made by drawing of lots. (5) Composition of the ''wine drinker group'': 15 patients; 11 males and 4 females; 66 $\pm$ 8 years of age. (6) Composition of the ''water drinker group'': 14 patients; 11 males and 3 females; 64 $\pm$ 11 years of age. (7) The standard Western prudent diet type was provided over the three weeks of hospitalization (from W0 to W3). The first week (W0 to W1) was called adaptation period before the intervention trial (W1 to W3). (8) The ''water drinkers group'' received water ad libidum. (9) The ''red wine drinkers group'' received two glasses of red wine per day, around 2 $\times$ 12.5 cl, distributed by the attending team: one glass at lunch and one glass at dinner; and water ad libidum. (10) Cardiovascular readaptation programme for all patients; adapted daily physical exercise, physiotherapy over the three weeks of hospitalization (from W0 to W3). (11) Blood collection, clinical and physiological parameters measurements. (12) The samples of blood were taken in the morning. They were anonymous and coded for biochemical analysis.

ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) to generate a radical cation, $ABTS^{+}$, that is green in color and can be measured by absorbance at 405 nm. Antioxidants suppress this reaction by electron donation radical scavenging and inhibit the formation of the colored ABTS radical. The concentration of antioxidant in the test sample (15 μL of plasma) is inversely proportional to the ABTS radical formation. Flow cytometric methods used for the measurement of erythrocyte transmembrane potential with $DiOC_6(3)$ and erythrocyte membrane fluidity with Merocyanine 540 (MC540) [10, 11]. The incorporation of MC540 into the cytoplasmic membrane of erythrocytes mainly depends on its lipidic organization. Consequently, when biomembranes are dense and well organized (as in living cells), MC540 incorporation is lower than in disorganized membranes (as in damaged or dying cells) [11]. The accumulation of $DiOC_6(3)$ in the cytoplasmic membrane of erythrocytes provides information on the transmembrane potential of these cells and depends on the membrane lipid oxidation level leading to a decrease in membrane potential [11]. Staining with MC540 and $DiOC_6(3)$ was performed as follows. Blood samples were centrifuged (10 min at 900 $\times$ *g*) and plasma samples were stocked at 80°C, while erythrocytes were used for flow cytometric analyses. Oxidative compounds were added to the red blood cell suspension and the mix was incubated at 37°C. Every 30 min, 500 μL of the mix were stained with 500 μL of $DiOC_6(3)$ (3,3′-dihexyloxacarbocyanine iodide) (80 nM for 15 min at 37°C) or of MC 540 (5 μg/mL for 15 min at 37°C). Both dyes were purchased from Molecular Probes (Invitrogen, Cergy-Pontoise, France). For $DiOC_6(3)$, the green fluorescence was quantified on a Galaxy flow cytometer (Partec, Münster, Germany) at excitation and emission wavelengths of 488 and 515/10 nm, respectively. For MC540, the red fluorescence was quantified at excitation and emission wavelengths of 488 and 590/10 nm, respectively. Fluorescent signals were measured on a logarithmic scale. For each sample, 50 000 cells were acquired and the data were analyzed with the Flomax software (Partec). Kinetic experiments of membrane polarization were conducted every 30 min for 150 min. The data were expressed in arbitrary units as the area under curve obtained with the averaged fluorescence intensities measured every 30 min. *Statistical analysis*: Individual data were given as mean $\pm$ SEM (standard error of the mean). Statistical analyses were performed with the StatView software (Cary, StateNC, USA) using nonparametric tests including the Wilcoxon test and the Mann–Whitney test [12].

Table 1 summarizes the baseline characteristics and treatments received by both groups of participants. *Evolution of physiological parameters*: Table 3 (Supporting Information)

Table 1. Baseline characteristics and treatments received by both groups of participants

Characteristics and treatments	Wine drinker group		Water drinker group	
	Day 1	Day 14	Day 1	Day 14
Number of patients	19 (4 F, 15 M)	19	14 (3 F, 11M)	14
Average age of patients	66±8	66±8	64±11	64±11
Current therapeutic treatment (aspirin)[a)]	+	+	+	+
Hospital housed clinical trial	+	+	+	+
Diet (western prudent diet type)[b)]	+	+	+	+
Wine (2 × 125 mL/day)	+	+	–	–
Water (ad libidum)	+	+	+	+
Heart adapted physical exercise and physiotherapy	+	+	+	+

F, female; M, male. For further details, see Materials and Methods and chart of Supporting Information.

a) Were excluded of the study patients having insulin, thyroid hormone, and antidiuretic drug medical treatment; tobacco smokers and alcoholic addiction cases.

b) Daily consumption of fruits, vegetables, bread, margarine, from time to time olive and coleseed oils, fish and meat, low saturated animal fat. For details, see Table 2 Supporting Information.

reports the evolution of physical and clinical parameters over the two-week study (D1–D14). Part A does not show any significant changes in wine group versus water group for either glycemia, blood pressure (systolic and diastolic), body weight, and maximal oxygen consumption (VO_2). Part B reports the evolution from D1 to D14 in each group. Here, again no significant changes were seen, except for VO_2, which is improved both in the two groups after two weeks of the intervention study. This improvement can be explained by the adapted physical exercise and physiotherapy. Concerning the electrocardiograms, no changes were pointed out (not shown).

Evaluation of lipids, albumin, and CRP levels: Plasmatic levels of lipids (total cholesterol, triglycerides, lipoproteins (HDL, LDL)), albumin and CRP were estimated and reported in Table 2. As expected at the start of the study (Day 1) (Fig. 1A as Supporting Information), total cholesterol, triglycerides, lipoproteins (HDL, LDL), albumin, and CRP were not significantly different between the control group (water drinkers) and the study group (wine drinkers). Pro-inflammatory cytokines were not significantly changed (not reported). Interestingly, there were changes on Day 14, with a significant decrease (−16%) in total cholesterol in the study population as compared with the control group ($p = 0.042$) (Fig. 1B as Supporting Information). In addition, for the red wine drinkers (Fig. 1D as Supporting Information), there was a significant decrease in total cholesterol ($p = 0.047$) and LDL ($p = 0.012$) over the two-wk period of intervention. For the water drinkers, no significant differences were observed in the different parameters studied (total cholesterol, triglycerides, LDL, HDL, albumin, and CRP) (Fig. 1C as Supporting Information). Moreover, the ratio [Day 14/Day 1] for LDL was statistically lower (−18%) for red wine drinkers as compared with abstinent patients ($p = 0.0124$) (Fig. 1E as Supporting Information). *Evaluation of moderate red wine consumption on oxidative status and erythrocyte membrane properties*: Free radicals play a major role in atherosclerosis genesis even though organisms are naturally protected from ROS by detoxifying enzymes and antioxidant molecules. The antiradical defense system is complex since it involves several compounds (such as vitamins, antioxidants, pro-oxidants, and uric acid), enzymatic systems (glutathione peroxidase, catalase, superoxide dismutase, etc.). It is well accepted that the antioxidant capacity involved in the antiradical defense capital includes both negative (unbalanced nutrition, smoking, stressful lifestyle, etc.) and positive (vitamins, enzyme defense capital, etc.) factors. We attempted to evaluate in the whole blood the antioxidant capacity using the free-radical-induced hemolysis test, the so-called KRL test and to measure the erythrocyte transmembrane potential with $DiOC_6(4)$ and the erythrocyte membrane fluidity with Merocyanine 540 (MC540) (Fig. 2 as Supporting Information). No significant differences between the two groups at the start of the study (Day 1) were observed in these different parameters (Fig. 2A as Supporting Information). The comparison between wine drinkers and water drinker groups at Day 14 (Fig. 2A as Supporting Information) showed a significant difference (−35%) with the MC540 test ($p = 0.0032$). Concerning the changes from Day 1 to Day 14 for water drinkers and for red wine drinkers (Fig. 2B as Supporting Information), there was a statistically significant decrease (−37%) in erythrocyte staining with MC540 for red wine drinkers from Day 1 to 14 ($p = 0.047$) associated with a slight increase (+3%) in KRL values ($p = 0.024$). The KRL results of the blood anti-oxidant capacity of red wine drinkers were confirmed by the TEAC assay using the $ATBS^+$ discoloration method (Table 2). Thus, in agreement with data obtained in vitro, polyphenol supplementation associated with red wine consumption might be involved in better erythrocyte membrane fluidity [13] and in higher antioxidant defense potential. With MC540, which measures membrane fluidity, it is well

Table 2. Summarized effects of a moderate red wine intake on blood parameters and on physical/clinical parameters in post myocardial infarct patients

Parameters	Wine drinker group day 14 versus day 1	Wine drinker group versus water drinker group at day 14
Proteins		
Albumin	NS	NS
CRP	NS	NS
Lipids		
C	↘ −5%, $p = 0.047$	↘ −16%, $p = 0.042$
HDL	NS	NS
LDL	↘−5%, $p = 0.012$	↘−18%, $p = 0.043$
TG	NS	NS
Anti-oxidant capacity		
KRL	↗ +3%, $p = 0.024$	NS
TEAC		↗ +8%[a)]
Membrane erythrocyte properties		
Dioc6 (3)	NS	NS
Merocyanine 540	↘ −36%, $p = 0.047$	↘−35%, $p = 0.0032$
Glycemia		
Glucose	NS	NS
Physio-clinical		
Body weight	NS	NS
SBP	NS	NS
DBP	NS	NS
VO_2	↗ +26% $p = 0.02$	NS

C: total cholesterol; CRP: C-reactive protein; HDL: high-density lipoprotein; LDL: low density lipoprotein; TG: triglycerides; KRL: free radical-induced hemolysis test; TEAC assay: Trolox Equivalent Antioxidant Capacity Assay; Dioc6 (3): transmembrane potential probe; merocyanine 540: membrane fluidity probe; SBP: systolic blood pressure; DBP: diastolic blood pressure; VO_2: maximal oxygen consumption; NS: not statistically significant. For values and details, see Figs. 1, 2 and Table 3 in Supporting Information.

a) The increase in trolox concentration could not been statistically evaluated taking into account of limited blood sample remaining.

known that when membrane lipid density increases (disturbed lipid membrane organization), the MC540-associated fluorescence increases. In contrast, when membrane lipid density decreases (well-organized lipid membrane), the MC540-associated fluorescence decreases. Thus, in the case of red wine drinkers, the erythrocyte membrane appears better organized after 14 days of a wine-associated diet (positive effect of wine), while for water drinkers, the erythrocyte membrane appears less organized. On the other hand, a hyperpolarization measured by staining with $DiOC_6(3)$ has been reported in damaged erythrocytes in the preceding lysis stage [14]. Although not significant, we observed an increase in the water-drinking group compared with the red-wine-associated diet group (Fig. 2A as Supporting Information). This increase in hyperpolarization was associated in water drinking group with a slight decrease in the overall antioxidant status (KRL test). No significant differences were observed in the plasma concentration of total polyphenols on Days 1 and 14 for red wine drinkers and water drinkers (Fig. 3 as Supporting Information).

Of all alcoholic beverages, wine appears to be the richest in the diversity and in the content of polyphenols, which are classified into several families: flavonoids including tannins, anthocyanes, and stilbenes (resveratrol). The cardioprotective effect of wine is in part due to its polyphenol content, especially resveratrol [15]. The cardioprotective effects of moderate and regular wine consumption in primary prevention had been demonstrated before the present study [1, 16, 17]. Furthermore, increase in plasma anti-oxidant capacity [2], the improvement of endothelial functions [3, 18], the modulation of immunological functions [4], or the polyphenol-dependant red wine anti-oxidant effect [5] has been reported recently. While in 2007, a case–control study of post myocardial infarction related to dietary patterns was published showing a lower risk for wine drinkers [19], but no investigations studied on blood parameters. The goal of this study, although over a limited period (which unfortunately could not be extended due to the limitation of the established hospitalized cardiac readaptation protocols), was to explore and to test the possible beneficial effects of a low red wine consumption in patients with previous coronary ischemic accidents for a secondary prevention of a cardiovascular event. This short-term intervention trial indicates a beneficial effect on some blood parameters (total cholesterol (−16%), LDL (−18%), erythrocyte membrane fluidity (−36%)). It also shows that the expected antiradical effect provided by a ''Western prudent'' diet is present only when red wine is included in the overall diet. Of importance, in this study, both groups of patients were exposed to the same amount and equal quality of the diet antioxidants, the only difference between the groups was the supplementation with wine polyphenols in the presence of ethanol, with resveratrol (and piceid) fairly specific of the beverage [20]. In Supporting Information (Table 2), we show that the wine used in this study contains a high content of gallic acid, catechin, epicatechin, and caftaric acid. It must be pointed out that caffeic acid derivatives prevent the LDL oxidation [21]. Interestingly, our findings are in agreement with the meta-analysis of Covas et al. [22], who concluded that the postprandial oxidative stress after a meal is counteracted by the ingestion of red wine. Moreover, recently, Natella et al. reported a pilot study where the consumption of one wine intake with a cheese-rich meal could prevent the postprandial increase in plasma lipid hydroperoxides and oxysterols [23]. On the other hand, procyanidin, a polymerized wine tannin, has been reported to be a vasorelaxant [24]. Due to its high polyphenol content, red wine is therefore a real polyphenol concentrate since two glasses of wine contribute to nearly 1 g of polyphenols, which correspond to the daily polyphenol needs. In addition,

it is known that the effect of polyphenols is greater when ethanol is present [25], favoring better absorption since polyphenols are poorly hydrosoluble and are mainly accumulated in fruit skin, seeds, leaves, and other parts of comestible plants. This alcohol effect is similar to that observed during grape fermentation where polyphenols are extracted from grape skin by ethanol. Concerning the projection of biological and patho-physiological meaning, this two-week study already shows significant changes in blood parameters after a moderated red wine intake, especially a decrease in total cholesterol, and LDL. As it is well established that elevated cholesterol and LDL levels have major roles in the development of atherosclerosis, a decrease between 15 and 20% of the values associated with these parameters can represent an important statement in the secondary prevention of restenosis following coronarian surgery, especially after stent application. In addition, the strong increase in blood cell membrane fluidity can have a positive effect in cell signaling, the exchanges across the cytoplasmic membrane, and the plasticity of blood cells to facilitate blood cell circulation in the vessels and capillaries (prevention of thrombosis). It is, therefore, tempting to speculate that over a longer period we could obtain higher amplitude of changes in blood parameters. Nevertheless, this intervention trial on post myocardial infarct patients reveals that moderate and regular consumption of red wine may have some advantages in the secondary prevention of myocardial infarct when it is associated with a ''Western prudent'' diet and controlled physical exercise.

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