UNIVERSITY OF SÃO PAULO
FACULTY OF PHARMACEUTICAL SCIENCES
GRADUATE PROGRAM IN FOOD SCIENCES
AREA OF EXPERIMENTAL NUTRITION

LÍVIA NEDEL CHASSOT

Comparison between red wine and isolated *trans*-resveratrol in the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice

São Paulo
2018

LÍVIA NEDEL CHASSOT

Comparison between red wine and isolated *trans*-resveratrol in the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice

Original Version

Dissertation presented to the Faculty of Pharmaceutical Sciences, University of Sao Paulo, to obtain the degree of Master of Science.

Concentration area: Experimental Nutrition

Advisor: Prof. Dr. Inar Alves de Castro

São Paulo
2018

Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

C488c

Chassot, Lívia
Comparison between red wine and isolated trans-resveratrol in the prevention and regression of atherosclerosis in LDLr (-/-) mice / Lívia Chassot. - São Paulo, 2018.
72 p.

Dissertação (mestrado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Alimentos e Nutrição Experimental.
Orientador: Castro, Inar

1. vinho tinto. 2. resveratrol. 3. aterosclerose. 4. camondongo. 5. estresse oxidativo. I. T. II. Castro, Inar, orientador.

Name: CHASSOT, Lívia Nedel

Title: Comparison between red wine and isolated *trans*-resveratrol in the prevention and regression of atherosclerosis in LDLr $^{-/-}$ mice

Dissertation presented to the Faculty of Pharmaceutical Sciences, University of Sao Paulo to obtain the degree of Master of Science.

Commission of Thesis for the degree of Master of Science

Prof. Dr. _____

Institution: _____

Verdict: _____

Prof. Dr. _____

Institution: _____

Verdict: _____

Prof. Dr. _____

Institution: _____

Verdict: _____

São Paulo, _____, 2018.

I dedicate this work to my mom and dad, for guiding me in these 24 years with all the love of the universe.

AKNOWLEDGMENTS

Firstly, I would like to thank God, for lighting my path every day, and for all the blessings.

I would like to thank my family for everything. Mom and dad, thanks for the support in my (sometimes crazy) dreams, for believing in me, for all the conversations, for the patience, for being so present in my life. Julia, thanks for all the funny songs and dances on Friday, Saturday and Sunday. You make my life way more fun. Thanks for the advises as well. Who would say you would be so wise?

Inar, “profe”, thank you for the opportunity of being part of your team. Thanks for believing in me, and for making me believe in myself too. Your creativity is very inspiring! No words to express the privilege that has been, to me, developing this project. I am very thankful.

Prof. Dennys Cintra, thanks for giving such a great beauty to the Nutrition Science. Thank you for being so enthusiastic in every class in Unicamp, and for all the guidance for the Masters, as well. You are a great part of this achievement!

Well, LADAF. It would take lots of pages to express how thankful I am for each person of this amazing team. Marina, thank you for being SO helpful. Really! You are unique! Oráculo/poor little Japanese/Cérebro/Tico, thank you for everything. Mini, “voz da razão”, thank you for all your wisdom and attention. You are a role model to me! Every time that I feel I need to think hard, I will blink my eyes like, 400 times, very fast. Gabi, a Malucona, thank you for being so dedicated, I feel pleased for the opportunity of having such an authentic person as my partner, and friend! Leo, thank you for all the analyses, for the patience with all my emails, and for making your “visits” to the lab so fun. What would I do without the “microlas” and “sopa de bacon” talks? Thanks also, to all the IC’s, you make the difference! LADAF, we’re really the best, that’s the only explanation. Thank you!

I would like to thank Lívia (Lics) for becoming one of my closest friends, for having such a good energy, for being a great listener and advisor, for all the coffees and

moments. Your friendship is worth gold! Nat, Agatha, Gabi's, Camile, Lu, Mari, Vanessa, all prof. Thomas Ong team, thanks for sharing the laboratory, knowledge and also so many good and funny moments! I miss you all! "Amigas da Márcia", Mayara, Fla and Ju, thanks for all the lunches, the laughs, the non-sense conversations, the dances… Thank you so much for this great friendship! You made my master's days much lighter. Lucky me to meet you all!

I would like to thank prof. Silvia Cozzolino and her students Bruna and Grazi, for allowing me to use their laboratory and for all the assistance. Thanks to prof. Dulcineia Abdalla, Marcela and Walter from the "Departamento de Análises Clínicas e Toxicológicas", for all the instructions, support and materials for the histological analyses of the aortas. I would also like to thank prof. Silvya Stuchi and her students for receiving me in their laboratory and for being so kind.

Prof. Bruno Cogliati, thank you for all the help and learning, from liver to aortas, and for being so attentive!

Lurdinha, I would like to thank you for your friendship, for being a great listener and for making the most delicious tea ever!

Thanks to my great friends Valéria, Thais, Beibe and Keka, with whom I have shared not just the apartment, but memorable moments! This postgraduate experience would not have been the same without you. Rep de Boas is the one and only, and so are you!

For all my family, Nedel and Chassot, thank you so much for being great enthusiasts of me in Masters!

For those who are not here anymore, but who would definitely vibrate with this achievement, thanks to my grandparents, to my uncle Curt and my uncle of heart Bernd. You are part of who I am today!

This work would not have been possible without the financial support of CNPq (159523/2015-0), and without the commitment from all the employees from the Food and Experimental Nutrition Department towards all students. Thank you.

“If you are willing to walk the path of a dreamer, then anything is possible.”

Jared Leto

RESUMO

CHASSOT, Lívia Nedel. **Comparação entre o vinho tinto e o *trans*-resveratrol isolado na prevenção e regressão da aterosclerose em camundongos LDLr $^{(-/-)}$**. 2018. 72 f. Dissertação (Mestrado em Ciência dos Alimentos) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2018.

O consumo moderado de vinho tinto tem sido amplamente associado à redução do risco cardiovascular, principalmente devido à sua composição em compostos fenólicos com atividade antioxidante, como o *trans*-resveratrol. Nosso objetivo foi o de comparar o efeito do consumo de vinho tinto *vs trans*-resveratrol na prevenção e regressão da aterosclerose em camundongos LDLr $^{(-/-)}$. Este estudo consistiu em dois protocolos: "PREVENÇÃO" (PREV) e "REGRESSÃO" (REGR). Ambos os protocolos incluíram quatro grupos: vinho tinto (WINE), vinho tinto sem álcool (EXT), *trans*-resveratrol (RESV) e controle (CONT). No protocolo PREV, os animais receberam uma dieta normal durante 8 semanas e trocaram para uma dieta aterogênica durante as 8 semanas seguintes, enquanto o oposto foi realizado no protocolo REGR. Os animais que receberam dieta aterogênica após um período inicial em dieta normal (PREV) ganharam mais peso corporal (39.25 ± 2.30%) do que o oposto (29.27 ± 1.91%, *p=0.0013*), sugerindo uma interação entre idade e ganho de peso. O t*rans*-resveratrol mostrou efeito hipocolesterolêmico mais elevado no protocolo PREV, reduzindo colesterol total, LDL-C e VLDL-C, mas também o HDL-C. A suplementação com *trans*-resveratrol e vinho tinto sem álcool alterou o perfil de ácidos graxos do fígado em ambos os protocolos, levando a um aumento das concentrações de MDA e da atividade da SOD no protocolo PREV. Todas as três formas de suplementação alteraram biomarcadores do estresse oxidativo e lipidemia, mas não apresentaram efeito sobre a prevenção ou regressão de estrias gordurosas. Esses resultados sugerem que a proteção cardiovascular associada ao "Paradoxo francês" pode ser resultado de efeitos sinérgicos entre o vinho e a dieta mediterrânea.

Palavras-chave: vinho tinto, resveratrol, aterosclerose, camundongo, estresse oxidativo

ABSTRACT

CHASSOT, Lívia Nedel. **Comparison between red wine and isolated *trans*-resveratrol in the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice**. 2018. 72 p. Dissertation (Master in Food Science) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2018.

Moderate consumption of red wine has been widely associated with reduced cardiovascular risk, mainly due to its composition in phenolic compounds with antioxidant activity, such as *trans*-resveratrol. Our aim was to compare the effect of red wine *vs trans*-resveratrol consumption on the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice. This study consisted of two protocols: "PREVENTION" (PREV) and "REGRESSION" (REGR). Both protocols included four groups: red wine (WINE), dealcoholized red wine (EXT), *trans*-resveratrol (RESV), and control (CONT). In PREV protocol, animals received a normal diet for 8 weeks and then switched to an atherogenic diet for the following 8 weeks, while the opposite was performed during REGR protocol. Animals that received atherogenic diet after an initial period on a normal diet (PREV) gained more body weight (39.25 ± 2.30%) than the opposite (29.27 ± 1.91%, $p=0.0013$), suggesting an interaction between age and weight gain. *Trans*-resveratrol showed the highest hypocholesterolemic effect in PREV protocol, reducing total cholesterol, LDL-C and VLDL-C, but also HDL-C. The supplementation with *trans*-resveratrol and dealcoholized red wine changed the fatty acids profile in the liver in both protocols, leading to an increase of MDA concentrations and SOD activity in PREV protocol. All three forms of supplementation altered biomarkers of oxidative stress and lipidemia but presented no effect on the prevention or regression of fatty streaks. These results suggest that the cardiovascular protection associated with the "French Paradox" may be a result of synergistic effects between wine and the Mediterranean diet.

Key-words: red wine, resveratrol, atherosclerosis, mice, oxidative stress

LIST OF FIGURES

Introduction

Manuscript

LIST OF TABLES

Manuscript

ABBREVIATIONS

BHT, butylated hydroxytoluene;
CAT, catalase;
CONT, control group;
COX, cyclooxygenase;
eNOS, endothelial nitric oxide synthase;
EXT, dealcoholized wine group;
GPx, glutathione peroxidase;
GR, glutathione reductase;
GSH, reduced glutathione;
GSSG, oxidized glutathione;
HDL-C, high-density lipoprotein cholesterol;
HE, hematoxylin-eosin;
HPLC, high-performance liquid chromatography;
HX/XO, hypoxanthine/xanthine oxidase;
LDL-C, low-density lipoprotein cholesterol;
LXRs, liver X receptors;
MDA, malonaldehyde;
MPO, myeloperoxidase;
NADH, reduced nicotinamide adenine nucleotide;
NADPH, nicotinamide adenine dinucleotide phosphate;
NAFLD, non-alcoholic fatty liver disease;
NO, nitric oxide;
NAS, NAFLD Activity Score;
ORAC, oxygen radical absorbance capacity;
Pex, peroxidase;
PPARs, peroxisome proliferator-activated receptors family;
PREV, prevention protocol;
REGR, regression protocol;
RESV, *trans*-resveratrol group;
SMCs, smooth muscle cells;
SOD, superoxide dismutase;
SRs, scavenger receptors;
TEP, 1,1,3,3-tetraethoxypropane;
TG, triglycerides;
TH, transhydrogenase;
TNF-α, tumor necrosis factor-alpha;
UHPLC, ultra-high performance liquid chromatography;
VLDL-C, very low-density lipoprotein cholesterol;
WINE, wine group.

SUMMARY

1. INTRODUCTION

1.1. Atherosclerosis

Atherosclerosis is a multifactorial chronic-inflammatory disease of the arterial wall. This disease represents the underlying cause of vascular complications such as stroke and heart attack (Feig, 2014); besides, it constitutes one of the main causes for morbidity and mortality in the world (Hovland et al., 2015). There are two kinds of risk factors for the atherosclerosis development; the modifiable ones, which include hypertension, dyslipidemia, type 2 Diabetes, smoking and a sedentary lifestyle, and the non-modifiable ones like age, sex and heredity (Badimon; Vilahur; Padro, 2010). However, a diet composed by a high content of fat, generating an increase in the total cholesterol, triglycerides (TG) and low-density lipoproteins concentration and a reduction in the concentration of high-density lipoproteins, presents a prominent role in the atherosclerosis development (Houston et al., 2009).

The beginning of the atherosclerotic plaque development in the arteries is characterized by an endothelial dysfunction. Changes in the endothelium permeability and in its extracellular matrix composition, for example, may promote the entry and retention of low-density lipoproteins cholesterol (LDL-C) molecules that are circulating in excess in the endothelial lumen, into the arterial wall. Concomitantly, blood monocytes are recruited to the endothelial monolayer, migrate into the intima and maturate into macrophages, and chemokines are released. Once in the endothelium, LDL-C molecules may suffer chemical alterations, especially oxidation by reactive species (RS), originating an oxidized LDL-C (**Figure 1**). This may occur when these RS are being produced in excess or in a sustained way, exceeding the available antioxidant defense systems, leading to oxidative stress, which is a key process in the atherosclerosis development.

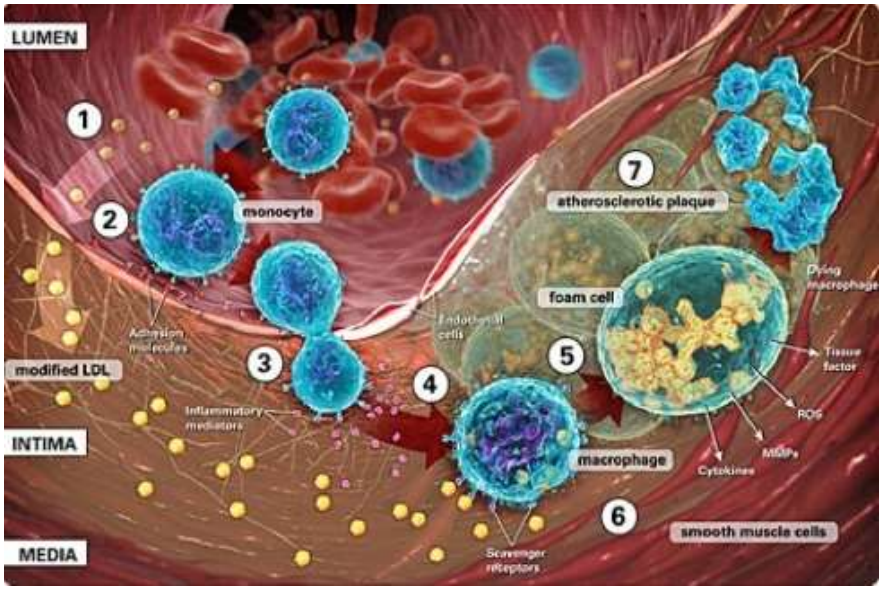

***Figure 1**: The beginning of the atherosclerotic lesion is characterized by an endothelial dysfunction, in which particles of LDL-C that are circulating in excess in the endothelial lumen (**1**) infiltrate between the endothelial cells junctions and suffer oxidation, originating the oxidized LDL-C. Then, it occurs the activation of adhesion molecules in the endothelial cell surface; these adhesion molecules recruit monocytes (**2**) that are internalized (**3**), with the liberation of inflammatory mediators and differentiation of the monocytes into macrophages (**4**). Next, the macrophages, through scavenger receptors, phagocyte the oxidized LDL-C forming foam cells (**5**), accompanied by a reactive species production and differentiation of smooth muscle cells (SMCs) (**6**) starting the atherosclerotic plaque development (**7**). Adapted from Glaudemans et al. (2010).*

Then, this oxidized LDL-C undergoes endocytosis by monocyte-derived macrophages through scavenger receptors, leading to the intracellular accumulation of cholesterol and constituting the foam cells, which are the initial lesions of atherosclerosis (**Figures 1 and 2b**).

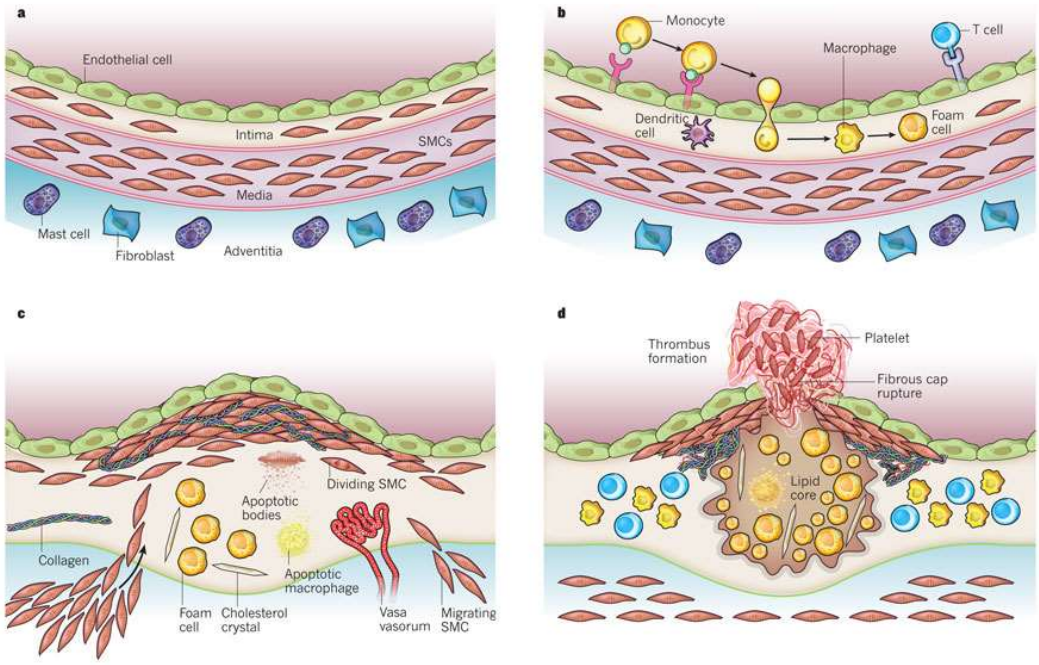

***Figure 2**: The stages of the atherosclerotic lesion are described. (**a**) The artery contains three layers; the tunica intima has an endothelial cells monolayer that is in contact with blood, and contains resident SMCs. The tunica media contains SMCs in a complex extracellular matrix. Lastly, the adventitia contains mast cells, nerve endings and microvessels. (**b**) The beginning of the lesion is characterized by the migration of blood monocytes to the intima through adhesion molecules, their differentiation to macrophages, and the uptake of oxidized LDL-C by these macrophages, yielding foam cells. (**c**) The progression of the lesion involves the migration of SMCs from the media to the intima, the proliferation of these cells, and the synthesis of collagen, proteoglycans and elastin. SMCs and macrophages can go through apoptosis, and the lipids contained in these dead cells can accumulate, forming a lipid core. It can also be seen cholesterol crystals and microvessels in this stage. (**d**) Finally, a thrombus development occurs, characterized by a disruption of the atherosclerotic plaque. Blood coagulation components are exposed to plaque's tissue factors, and the thrombus is released to the lumen, possibly impeding blood flow. Adapted from Libby, Ridker and Hansson (2011).*

As the lesions progresses, SMCs migrate from the media to the intima layer and proliferate, and the synthesis of collagen and proteoglycans, for instance, is intensified (**Figure 2c**). Plaque macrophages and SMCs can die as the lesion advances, and its derived extracellular lipid accumulates in the central region of the plaque, characterizing the lipid core (**Figure 2c**). The final stage of atherosclerosis is the plaque rupture, or thrombosis. In this process, the tissue factor in the plaque's interior is exposed to blood coagulation components, triggering the thrombus that extends into the vessel lumen, which can disturb the blood circulation (**Figure 2d**).

1.2. The oxidative stress in the genesis and progression of atherosclerosis

As elucidated below, the atherosclerosis development is characterized, among several processes, by an oxidative stress. The oxidative stress consists in the increase of radical species such as superoxide anion and hydroxyl radical, non-radical species like hydrogen peroxide, and in the reduction of the cellular antioxidant capacity, determined by the action of antioxidant enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and transhydrogenase (TH)) and non-enzymatic molecules like glutathione, resulting in direct or indirect damage to nucleic acids, proteins and lipids (Choi, 2016). These reactive species are the product of aerobic cellular metabolism for the reduction of one hydrogen molecule (**Figure 3**) (Nickening, Harrison, 2002; Ray, Shah, 2005), being generated extracellularly or in specific intracellular compartments (Griendling, Fitzgerald, 2003).

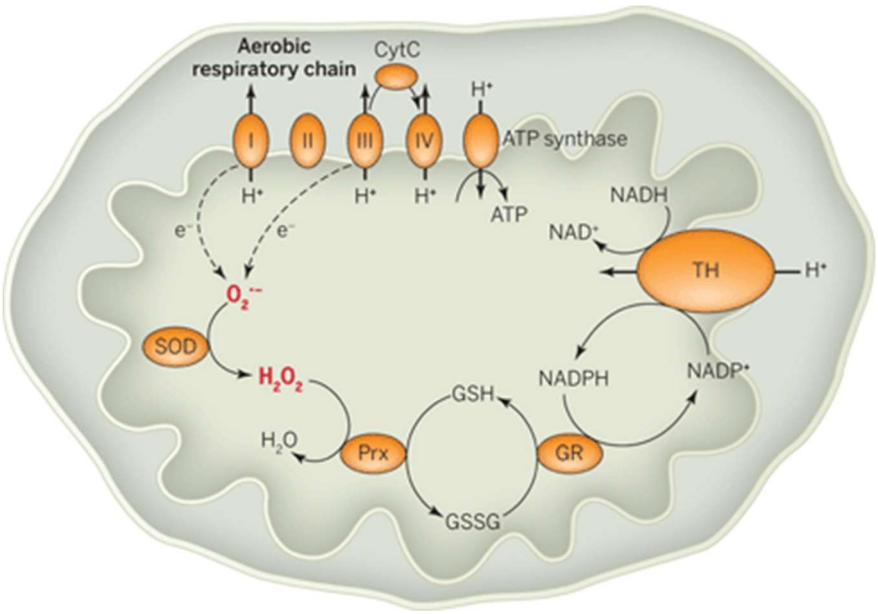

***Figure 3**: The TH contributes to the mitochondrial antioxidant defense producing nicotinamide adenine dinucleotide phosphate (NADPH). During respiration, electrons migrate to oxygen molecules, primarily by the complexes I and III, generating superoxide anion, which is converted in hydrogen peroxide by SOD, and finally in water by peroxidase (Pex), using reduced glutathione as hydrogen source. The levels of reduced glutathione are kept by glutathione reductase (GR), which uses the NADPH formed by TH to reduce oxidized glutathione. Adapted from Krengel and Tornroth-Horsefield (2015).*

Physiologically, the defense antioxidant mechanism adapts to changes in the oxidant levels keeping the homeostasis (Sies, 1997; Sies, 2007). However, in pathological conditions, the reactive species production may exceed the natural

antioxidant defense of the cells leading to dysfunction and apoptosis (Vaziri, 2008; Cai, Harrison, 2000; Sies, 2007; Majzunova et al., 2013).

The inflammatory process in atherosclerosis is associated to an increased production of reactive species and, as consequence, to oxidative stress (Libby; Ridker, Hansson, 2011). The main sources of reactive species in the vascular system are the enzymatic complexes: NADPH-oxidase, hypoxanthine/xanthine oxidase (HX/XO), mitochondrial respiratory chain, uncoupled endothelial nitric oxide (NO) synthase (Landmesser et al., 2007), cyclooxygenase (COX) (Muñoz et al., 2015) and myeloperoxidase (MPO) (Nicholls. Hazen, 2005). In **Figure 4** it can be observed the process of activation and action of the MPO enzyme in the development of atherosclerosis.

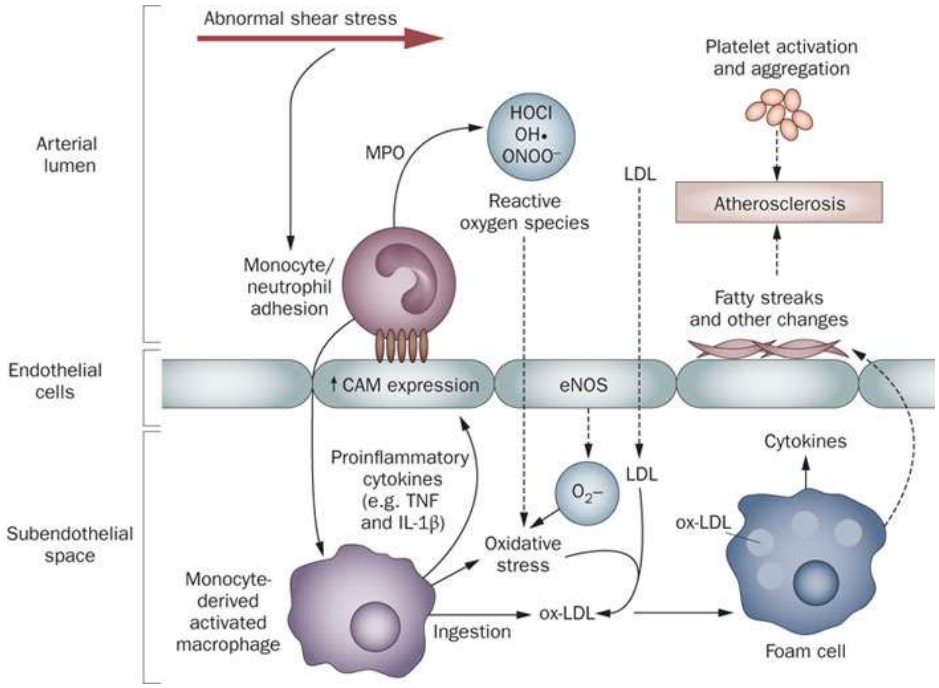

***Figure 4**: The endothelial dysfunction in the arteries causes an increase in the expression of adhesion molecules, which promotes the adhesion of monocytes. The infiltration of monocytes with their posterior activation to macrophages, leads to the release of MPO, which converts hydrogen peroxide in potent reactive species (hypochlorous acid, hydroxyl radical and peroxynitrite), leading to lipid nitrosylation and peroxidation of the tissue in the subendothelial space. The accumulation of oxidized LDL-C in the subendothelial space amplifies the inflammatory process. Macrophages that phagocyted the oxidized LDL-C become foam cells and appear as fatty streaks at the initial stage of atherosclerosis. The subsequent local inflammation, MPO deposition and fibrosis lead to the atherosclerotic plaque development. Adapted from Mok and Lau (2010).*

The oxidized LDL-C also reduces the activation of endothelial nitric oxide synthase (eNOS), which consequently inhibits the liberation of NO, generating vessel constriction (Ma et al., 2006). Besides, the superoxide anion, one of the main reactive species of biological interest, reacts with NO, also inhibiting its bioactivity and producing secondary reactive species, such as peroxynitrite, capable of oxidizing LDL-C (Griendling, Fitzgerald, 2003). The oxysterols, considered the most toxic constituents of oxidized LDL-C, induce apoptosis in the human endothelial cells through an important increase in the cytosolic calcium influx, followed by the activation of caspase 3, an enzyme that impedes DNA repair (Spyridopoulos et al., 2001). These events generate cytotoxicity and contribute to the atherosclerotic process.

1.3. The French Paradox as model of prevention and regression of the atherosclerotic plaque

Renaud and De Lorgeril (1992) observed that the mortality rate due to atherosclerosis presented by French and Swiss citizens was lower than that of individuals residing in other industrialized countries, such as United Stated and United Kingdom, although the intake of saturated fat was similarly elevated in them, besides the presence of other risk factors such as smoking. This paradox was attributed, in part, to the moderate and regular consumption of red wine (Renaud, De Lorgeril, 1992). Given this evidence, several studies with the proposal of elucidating the biochemical mechanisms responsible by the protective effects of red wine consumption have been published.

Red wine is rich in polyphenols, bioactive compounds of low molecular weight found in seeds, flowers and fruits. These compounds can be divided in two main classes, with base in their molecular structures: flavonoids and non-flavonoids (**Figure 5**).

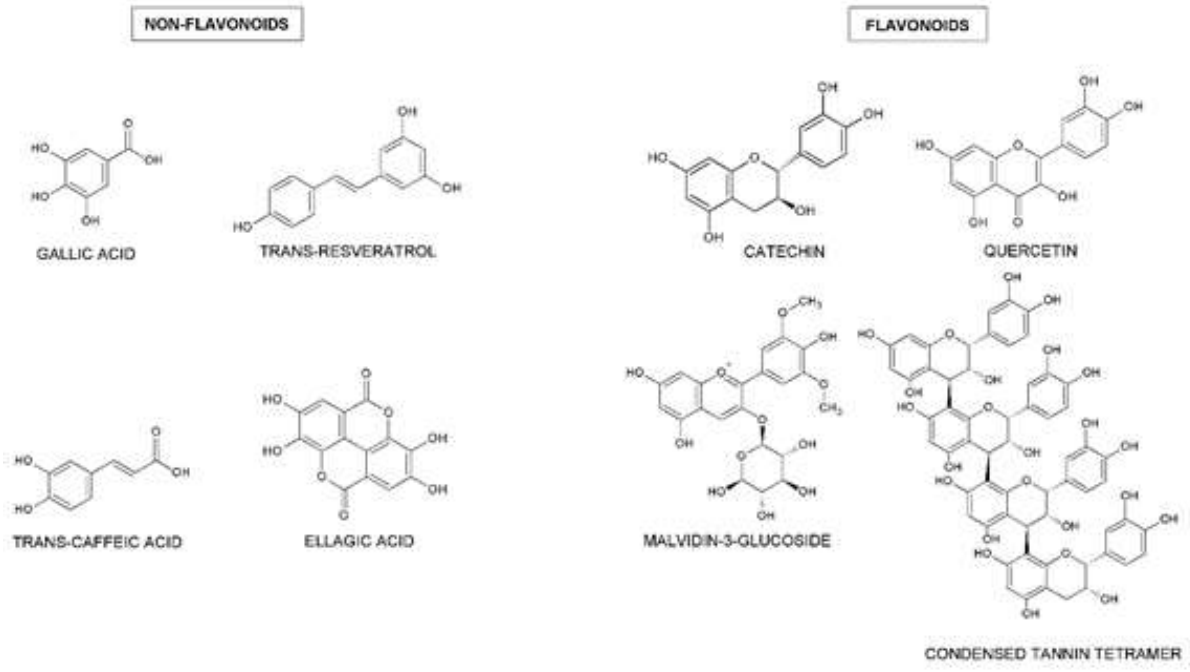

***Figure 5**: Examples of some phenolic compounds with high antioxidant activity found in the red wine (Adapted from Heim, Tagliaferro, Bobilya, 2002).*

The flavonoids include the anthocyanins (malvidin, delphinidin, petunidin, peonidin and cyanidin glucosides), flavonols (quercetin, rutin, myricetin and kaempferol), flavanols (catechin, epicatechin, epicatechin-3-gallate and gallocatechin), flavones (luteolin, apigenin) and flavonones (naringenin). The main non-flavonoid polyphenols include the cinnamic acids (caffeic, p-coumaric and ferulic), benzoic acids (gallic, vanilic and syringic), stilbens (resveratrol) (Cheynier, 2006), coumarins (Crozier, Jaganath, Clifford, 2009), lignins (Mkaouar et al., 2016) and lignans (Du et al., 2016).

The polyphenols exert antioxidant action reacting with reactive species (Mladenka et al., 2010; Obrenovich et al., 2010), or chelating transition metals involved in oxidative reactions (Rice-Evans, Miller, Paganga, 1995). The polyphenols present in red wine, like resveratrol and quercetin, have been shown to reduce oxidation of LDL-C and to reduce inflammation and endothelial dysfunction, contributing to the reduction of the atherosclerotic process (Serafini, Maimani, Ferro-Luzzi, 1998; Loke et al., 2010; Hayek et al., 1997). Beneficial effects of red wine in the initial events of atherosclerosis were demonstrated in several studies, and these could depend on some factors, like wine's phenolic composition and amount, and its antioxidant capacity (Macedo et al., 2013). Other conditions, like cultivar and price, for example,

may influence the wine functionality (Llobodanin, Barroso, Castro, 2014), and as a possible consequence, its antioxidant protective action.

In a crossover placebo-controlled study conducted by Apostolidou et al. (2015), 40 healthy individuals were divided in 2 age-adjusted groups according to their total cholesterol levels (hyper and normocholesterolemic) and consumed a specific variety of “tannat” (due to its content of phenolic compounds and high antioxidant activity) daily in their diet (125 mL per day for women and 250 mL per day for men) for one month. After a washout period of one month, they consumed a placebo drink daily for another month. Blood lipids, vitamin E and Total Antioxidant Capacity were measured before and after wine and placebo consumption. Total antioxidant activity increased after wine intervention in hypercholesterolemic ($p<0.001$) and normocholesterolemic ($p=0.002$) individuals. Vitamin E increased in hypercholesterolemic (13.1%) and in normocholesterolemic (5.41%) after red wine consumption. As asymptomatic hypercholesterolemic individuals are more likely to develop cardiovascular diseases, because of their high cholesterol levels and low serum vitamin E concentrations, this risk may decrease with an early intervention with a chronic consumption of red wine (Apostolidou et al., 2015).

In another crossover study, conducted by Chiva-Blanch and others (2013), the effects of red wine, dealcoholized red wine and gin were evaluated on glucose metabolism and lipid profile. Sixty-seven men at high cardiovascular risk received red wine (30 g alcohol/d and 798 mg polyphenols/d), dealcoholized red wine (1.14 g alcohol/d and 733 mg polyphenols/d) and gin (30 g alcohol/d) for 4 weeks, in a randomized order. Fasting plasma glucose and insulin, HOMA-IR, plasma lipoproteins, apolipoproteins and adipokines were determined at baseline and after each intervention. As observed, mean adjusted plasma insulin and HOMA-IR decreased after red wine and dealcoholized red wine intervention; LDL-C decreased (4.5% from baseline) after red wine intervention, and, after red wine and gin intervention, HDL-cholesterol and apolipoprotein A-2 increased (7% for both and 9% and 5%, respectively, from baseline). The study attributed these findings to the polyphenols naturally present in the red wine, which could lead to a greater protection on cardiovascular disease compared with other alcoholic beverages.

1.4. Resveratrol, oxidative stress and atherosclerosis

Resveratrol is a polyphenol that has been studied for decades in an ample spectrum of therapeutic research areas, occurring naturally in a variety of foods, like the blackberries, red grapes and peanuts. This polyphenol has been popularized due to the possibility of its use as a nutritional supplement (Kulkarni, 2015), being known as a potent antioxidant. Resveratrol reduces lipid peroxidation by scavenging reactive species (**Figure 5**) (Fremont, Belguendouz, Delpal, 1999; Belguendouz, Fremont, Linard, 1997).

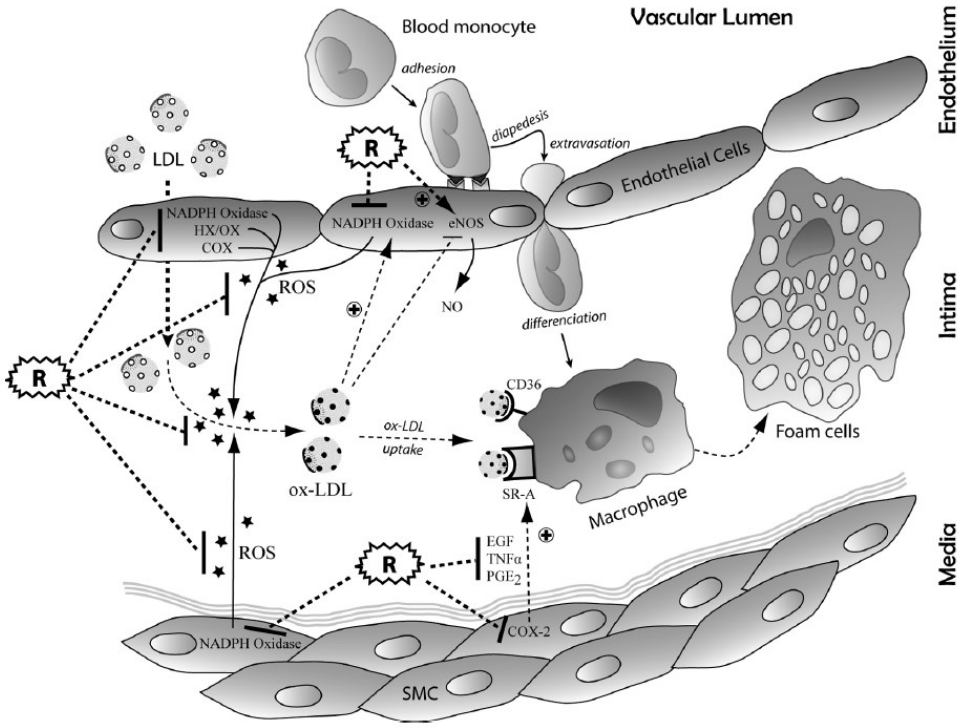

***Figure 6**: Effect of resveratrol in the initial events of atherosclerosis. Resveratrol (R), prevents the initial events by scavenging reactive oxygen species (ROS), inhibiting enzymatic systems that produce reactive species (NADPH oxidase, HX/XO, COX), regulating scavenger receptors class A (SR-A) and inducing eNOS, affecting vasodilatation. Adapted from Delmas, Jannin and Latruffe (2005).*

Besides, various enzymatic systems present in the endothelial cells or macrophages are implicated in LDL-C oxidation (**Figure 5**). Resveratrol can act in some of these systems, like in NADPH oxidase (Leiro et al., 2004) and HX/XO (Cao, Li, 2004) contributing to the reduction of the reactive species formation (Touyz et al., 2002). Resveratrol can also induce an increase in the activity of antioxidant enzymes,

like superoxide dismutase, catalase and glutathione peroxidase (Spanier et al., 2009; Lee et al., 2003).

So far, results observed in clinical studies with resveratrol supplementation and atherosclerosis are still controversial; factors like dosage, period of intervention and health condition of the volunteers can be decisive. In a randomized double-blind crossover study conducted by Timmers and colleagues (2011), 11 healthy obese men were treated with placebo and 150 mg/day of resveratrol for 30 days, with the objective of evaluating the metabolic effects of resveratrol. By the end of the 30 days, it was observed that resveratrol decreased intrahepatic lipid content ($p<0.05$), circulating glucose ($p=0.05$), TG ($p=0.03$), alanine-aminotransferase ($p=0.02$), and inflammatory markers, like tumor necrosis factor-alpha (TNF-α) ($p=0.04$) in comparison with placebo. Besides, in the postprandial state, resveratrol decreased fat oxidation ($p=0.007$), indicating positive consequences of a 30-day resveratrol supplementation in the metabolism of obese humans. Promising results were also observed in a similar study reported by Dash and others (2013), in which the production of apolipoproteins was analyzed in eight overweight or obese individuals with hypertriglyceridemia 4 to 6 weeks apart, after a treatment with resveratrol or placebo. They randomly received two weeks of placebo or resveratrol [1 g/day (500 mg twice per day) for one week, followed by 2 g resveratrol/day (1 g twice per day) for the second week]. Resveratrol reduced apoB-48 production rate by 22% ($p=0.007$) and apoB-100 production rate by 27% ($p=0.02$), indicating that 2 weeks of high-dose resveratrol can reduce lipoprotein produced in the enterocytes and hepatocytes.

Based on this brief literature review, although results are still controversial, it seems to be a tendency of consensus about the action attributed to a moderate consumption of red wine and a supplementation with resveratrol for cardiovascular protection. The discrepancy observed in results can be due to factors, such as different animal models used in the trials, antioxidant activity of the wine, evaluated biomarkers and administered dosages. Generally, most used animal models include genetically modified mice for the atherosclerosis development, such as Apolipoprotein E knockout mice and LDL receptor knockout mice, both under an atherogenic diet. The use of these animal models occurs due to the ethical difficulty to conduct clinical trials with humans, in which is preconized the alcohol consumption, even if moderate. Therefore, the direct supplementation with isolated resveratrol could be an alternative to those who look for the cardiovascular protection offered by a moderate consumption of red

wine, but do not want to or cannot adopt it because of the presence of alcohol. Thus, it is necessary to investigate if the substitution of a moderate consumption of red wine by the daily supplementation with isolated resveratrol could bring equivalent benefits in terms of reducing oxidative stress, and consequently bringing cardiovascular protection. Why isolated resveratrol, and not a mixture of the main red wine phenolic compounds? Because isolated resveratrol is a supplement already consumed in several countries by people who present various levels of cardiac risk, without any medical prescription. Despite of this reality, literature does not bring enough information about the effectiveness of this substitution in reducing oxidative stress, under a preventive and regressive approach of the atherosclerotic process.

1.5. REFERENCES

APOSTOLIDOU, C. et al. Cardiovascular risk and benefits from antioxidant dietary intervention with red wine in asymptomatic hypercholesterolemics. *Clin. Nutr. ESPEN*, v. 10, n. 6, e224-e233, 2015.

BADIMON, L.; VILAHUR, G.; PADRO, T. Nutraceuticals and atherosclerosis: human trials. *Cardiovasc. Ther.*, v. 28, n. 4, p. 202-215, 2010.

BELGUENDOUZ, L., FREMON, L., LINARD, A. Resveratrol inhibits metal ion-dependent and independent peroxidation of porcine low-density lipoproteins. *Biochem. Pharmacol.*, v. 53, n. 9, p. 1347-1355, 1997.

CAI, H.; HARRISON, D. G. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ. Res.*, v. 87, n. 10, p. 840-844, 2000.

CAO, Z.; LI, Y. Potent induction of cellular antioxidants and phase 2 enzymes by resveratrol in cardiomyocytes: protection against oxidative and electrophilic injury. *Eur. J. Pharmacol.*, v. 489, n. 1-2, p. 39-48, 2004.

CHIVA-BLANCH, G. et al. Effects of red wine polyphenols and alcohol on glucose metabolism and the lipid profile: A randomized clinical trial. *Clinical Nutrition*, v. 32, n. 2, p. 200-206, 2013.

CHEYNIER, V. Flavonoids in wine. In: ANDERSON, M.; MARKHAM, K. R. (Ed.(s)). Flavonoids: Chemistry, biochemistry and applications. New York: Boca Raton, p. 263-270, 2006.

CHOI, K. et al. Oxidative stress response in canine in vitro liver, kidney and intestinal models with seven potential dietary ingredients. *Toxicology Letters*, v. 241, p. 49-59, 2016.

CROZIER, A.; JAGANATH, I. B.; CLIFFORD, M. N. 2009. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.*, v. 8, p. 1001-1043, 2009.

DASH, S. et al. High-dose resveratrol treatment for 2 weeks inhibits intestinal and hepatic lipoprotein production in overweight/obese men. *Arterioscler. Thromb. Vasc. Biol.*, v. 33, n. 12, p. 2895-2901, 2013.

DELMAS, D.; JANNIN, B.; LATRUFFE, N. Resveratrol: preventing properties against vascular alterations and ageing. *Mol. Nutr. Food Res.*, v. 49, n. 5, p. 377-395, 2005.

DU, G. et al. Polyphenols: Potential source of drugs for the treatment of ischaemic heart disease. *Pharmacol. Ther.*, v.162, p. 23-34, 2016.

FEIG, J. E. Regression of atherosclerosis: insights from animal and clinical studies. *Ann. Glob. Health*, v. 80, n. 1, p. 13-23, 2014.

FREMONT, L., BELGUENDOUZ, L. DELPAL, S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life Sci.*, v. 64, n. 26, p. 2511-2521, 1999.

GLAUDEMANS, A. W. et al. Molecular imaging in atherosclerosis. *Eur. J. Nucl. Med. Mol. Imaging*, v. 37, n. 3, p. 2381-2397, 2010.

GRIENDLING, K. K.; FITZGERALD G. A. Oxidative stress and cardiovascular injury. Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation*, v. 108, n. 16, p. 1912-1916, 2003.

HEIM, K. E.; TAGLIAFERRO, A. R., BOBILYA, D. J. Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. *J. Nutr. Biochem.*, v. 13, n. 10, p. 572-584, 2002.

HAYEK, T. et al. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is

associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler. Thromb. Vasc. Biol*., v. 17, n. 11, p. 2744-2752, 1997.
HOUSTON, M. C. et al. Nonpharmacologic treatment of dyslipidemia. *Prog. Cardiovasc. Dis*., v. 52, n. 2, p. 61-94, 2009.
HOVLAND, A. et al. The complement system and toll-like receptors as integrated players in the pathophysiology of atherosclerosis. *Atherosclerosis*, v. 241, n. 2, p. 480-494, 2015.
KRENGEL, U.; TORNROTH-HORSEFIELD, S. Coping with oxidative stress. *Science*, v. 347, n. 6218, pp. 125-126, 2015.
KULKARNI, S. S.; CANTÓ, C. C. The molecular targets of resveratrol. *Biochim. Biophys. Acta*, v. 1852, n. 6, p. 1114-1123, 2015.
LANDMESSER, U.; DREXLER, H. Endothelial function and hypertension. *Curr. Opin. Cardiol.*, v. 22, n. 4, p. 316-320, 2007.
LEE, S. E. et al. Antioxidant activity of extracts from Alpinia katsumadai seed. *Phytother. Res.*, v. 17, n. 9, p. 1041-1047, 2003.
LEIRO, J. et al. Effects of *cis*-resveratrol on inflammatory murine macrophages: antioxidant activity and down-regulation of inflammatory genes. *J. Leukoc. Biol*., v. 75, n. 6, p. 1156-1165, 2004.
LIBBY, P.; RIDKER, P. M.; HANSSON, G. K. Progress and challenges in translating the biology of atherosclerosis. *Nature*, v. 473, n. 7347, p. 317-325, 2011.
LLOBODANIN, L.; BARROSO, L.; CASTRO, I. Sensory characterization of young South American red wines classified by varietal and origin. *Journal of Food Science*, v. 79, n. 8, p. 1595-1603, 2014.
LOKE, W. M. et al. Specific dietary polyphenols attenuate atherosclerosis in apolipoprotein E-knockout mice by alleviating inflammation and endothelial dysfunction. *Arterioscler. Thromb. Vasc. Biol*., v. 30, n. 4, p. 749-757, 2010.
MA, F. X. et al. Oxidized low-density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. *J. Lipid Res*., v. 47, n. 6, p. 1227-1237, 2006.
MACEDO, L. F. et al. Effect of red wines with different in vitro antioxidant activity on oxidative stress of high-fat diet rats. *Food Chem*., v. 137, n. 1-4, p. 122-129, 2013.
MAJZUNOVA, M. et al. Redox signaling in pathophysiology of hypertension. *J. Biomed. Sci*., v. 20, n. 1, p. 69, 2013.
MLADENKA, P. et al. Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity. *Free Radic. Biol. Med*., v. 49, n. 6, p. 963-975, 2010.
MKAOUAR, S. et al. Kinetic study of polyphenols extraction from olive (Olea europaea L.) leaves using instant controlled pressure drop texturing. *Separation and Purification Technology*, v. 161, p. 165-171, 2016.
MOK, M. Y.; LAU, C. S. The burden and measurement of cardiovascular disease in SSc. *Nature Reviews Rheumatology*, v. 6, p. 430-434, 2010.
MUÑOZ, M. et al. COX-2 is involved in vascular oxidative stress and endothelial dysfunction of renal interlobar arteries from obese Zucker rats. *Free Radic. Biol. Med*., v. 84, p. 77-90, 2015.
NICHOLLS, S. J.; HAZEN, S. L. Myeloperoxidase and cardiovascular disease. *Arterioscler. Thromb. Vasc. Biol*., v. 6, n. 1102-1111, 2005.
NICKENING, G.; HARRISON, D. G. The AT(1)-type angiotensin receptor in oxidative stress and atherogenesis: part I: oxidative stress and atherogenesis. *Circulation*, v. 105, n. 3, p. 393-396, 2002.
OBRENOVICH, M. E. et al. The role of polyphenolic antioxidants in health, disease and aging. *Rejuvenation Res*., v. 13, n. 6, p. 631-643, 2010.

RAY, R.; SHAH, A. M. NADPH oxidase and endothelial cell function. *Clin. Sci.* (Lond), v. 109, n. 3, p. 217-226, 2005.
RENAUD, S.; DE LORGERIL, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet*, v. 339, n. 8808, p. 1523-1526, 1992.
RICE-EVANS, C.A. et al. The relative anti-oxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Research*, v. 22, p. 375-388, 1995.
SERAFINI, M.; MAIMANI, G.; FERRO-LUZZI, A. Alcohol-free red wine enhances plasma antioxidant capacity in humans. *J. Nutr.*, v. 128, n. 6, p. 1003-1007, 1998.
SIES, H. Oxidative stress: oxidants and antioxidants. *Exp. Physiol.*, v. 82, n. 2, p. 291-295, 1997.
SIES, H. Total antioxidant capacity: appraisal of a concept. J Nutr, v. 137, n. 6, p. 14931495, 2007.
SPANIER, G. et al. Resveratrol reduces endothelial oxidative stress by modulating the gene expression of superoxide dismutase 1 (SOD1), glutathione peroxidase 1 (GPx1) and NADPH oxidase subunit (Nox4). *J. Physiol. Pharmacol.*, v. 60, Suppl. 4, p. 111-116, 2009.
SPYRIDOPOULOS I. et al. Alcohol enhances oxysterol-induced apoptosis in human endothelial cells by a calcium-dependent mechanism. *Arterioscler. Thromb. Vasc. Biol.*, v. 21, n. 3, p. 439-444, 2001.
TIMMERS, S. et al. Calorie restriction effects of 30 days of Resveratrol (resVida ™) supplementation on energy metabolism and metabolic profile in obese humans. *Cell Metab.*, v. 14, n. 5, 2011.
TOUYZ, R. M. et al. Effects of low dietary magnesium intake on development of hypertension in stroke-prone spontaneously hypertensive rats: role of reactive oxygen species. *J. Hypertens.*, v. 20, n. 11, p. 2221-2232, 2002.
VAZIRI, N. D. Mechanisms of lead-induced hypertension and cardiovascular disease. *Am. J. Physiol. Heart Circ. Physiol.*, v. 295, n. 2, H454-H465, 2008.

2. MANUSCRIPT

Comparison between red wine and isolated *trans*-resveratrol on the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice

Livia N. Chassot[1], Bianca Scolaro[1], Gabriela G. Rochel[1], Bruno Cogliati[2], Marcela F. Cavalcanti[3], Dulcineia S.P. Abdalla[3], Inar A. Castro[1]*

[1]LADAF, Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Av. Lineu Prestes, 580, B14, 05508-000, Sao Paulo, Brazil.

[2]Department of Pathology, School of Veterinary Medicine and Animal Sciences, University of Sao Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, 05508-270, Sao Paulo, Brazil.

[3]Department of Clinical Analysis (DSPA), Faculty of Pharmaceutical Sciences, University of Sao Paulo, Sao Paulo, Brazil.

**Corresponding author. Address: Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Av. Lineu Prestes, 580, B14, 05508-900, Sao Paulo, Brazil. Tel.: +55 (11) 3091-1152*

E-mail address: inar@usp.br; www.ladaf.com.br *(I. Castro).*

Acknowledgments

This study was financially supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Process 16/11664-5) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

2.1. ABSTRACT

Purpose Moderate consumption of red wine has been widely associated with reduced cardiovascular risk, mainly due to its composition in phenolic compounds with antioxidant activity, such as *trans*-resveratrol. Our aim was to compare the effect of red wine *vs trans*-resveratrol consumption on the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice.

Methods This study consisted of two protocols: "PREVENTION" (PREV) and "REGRESSION" (REGR). Both protocols included four groups: red wine (WINE), dealcoholized red wine (EXT), *trans*-resveratrol (RESV), and control (CONT). In PREV protocol, animals received a regular diet for 8 weeks and then switched to an atherogenic diet for the following 8 weeks, while the opposite was performed in REGR protocol.

Results Animals that received atherogenic diet after an initial period on a normal diet (PREV) gained more body weight (39.25 ± 2.30%) than the opposite (29.27 ± 1.91%, *p=0.0013*), suggesting an interaction between age and weight gain. *Trans*-resveratrol showed the highest hypocholesterolemic effect during PREV protocol, reducing total cholesterol, LDL-C and VLDL-C, but also HDL-C. The supplementation with *trans*-resveratrol and dealcoholized red wine changed the fatty acids profile in the liver in both protocols, leading to an increase of MDA concentrations and SOD activity during PREV protocol.

*Conclusions S*upplementation with *trans*-resveratrol, red wine and the same wine without alcohol altered biomarkers of oxidative stress and lipidemia but had no effect on the prevention or regression of fatty streaks. These data suggest that the cardiovascular protection associated with the "French Paradox" may be a result of synergistic effects between wine and the Mediterranean diet.

Key-words: red wine, resveratrol, atherosclerosis, mice, oxidative stress

2.2. INTRODUCTION

Since the first epidemiological study published by Renaud and de Lorgeril in 1992 [1], moderated daily consumption of red wine has been associated with the lower incidence of deaths caused by cardiovascular disease among individuals living in France and Switzerland, compared to individuals from other occidental countries. Although the negative association between alcohol consumption and total mortality has been well documented [2], some studies have strongly supported the cardioprotective effect of red wine's phenolic compounds, that are either naturally present in grapes skin and seeds, or formed during wine production and storage [3–5]. Among these compounds, *trans*-resveratrol, catechin, quercetin, malvidin, tannin, gallic acid, caffeic acid and ellagic acid (**Figure S1, Online Resource 1**) present a high antioxidant activity, as reported by a number of studies [6–13].

Therefore, phenolic compounds have been hypothesized to reduce cardiovascular risk upon regular intake, by acting as antioxidants *in vivo*. Despite rather low bioavailability, these compounds can reach the bloodstream and target tissues such as the endothelium, where they would supposedly act as free radical scavengers and could protect LDL apolipoprotein from oxidation [2, 14, 15]. Considering that oxidized LDL is an important trigger for fatty streaks formation and further development of atherosclerosis, the antioxidant activity promoted by phenolic compounds from red wine could contribute both to prevent atheroma plaques formation and to regress already established plaques.

Among the phenolic compounds present in red wine, *trans*-resveratrol, from the class of stilbenes, occurs naturally in fruits like grapes and berries [16] and has been extensively studied in several areas due to its association with lifespan [17] and cardiovascular benefits [18], for instance. Since the classic but also polemic studies reported by David Sinclair (i.e. Baur et al. [19]), the consumption of isolated *trans*-resveratrol supplements has increased [20]. The label of these supplements that are commercialized in drugstores brings a claim of potential antioxidant effect to the consumers. Therefore, individuals who wish to have beneficial effects of red wine consumption but have restrictions on the alcohol intake could

replace wine by capsules of *trans*-resveratrol. Although the consumption of *trans*-resveratrol capsules is already current, there is no study comparing the physiological consequence of this replacement until this moment. Hence, the objective of this study was to compare the effect of red wine *vs* isolated *trans*-resveratrol consumption on the prevention and regression of atherosclerosis using an LDLr $^{(-/-)}$ mice as a model.

2.3. MATERIALS AND METHODS

The red wine used in this study was selected from a previous research carried out in our group [21] in which 666 samples of South American red wines were analyzed according to their functionality, including antioxidant activity. Based on this analysis, eight bottles of Cuvée Alexandre, Carménère 2010 wine were purchased from a local importer (Adega Brasil, Pinhais, Brazil) and kept under refrigeration (4°C). Capsules of *trans*-resveratrol (Rite Aid, Camp Hill, United States) were purchased at a local drugstore. Fluorescein, epicatechin, caffeic acid, gallic acid, ferulic acid, rutin, *trans*-resveratrol, myricetin, quercetin, kaempferol, 1,1,3,3-tetraethoxypropane (TEP), reduced nicotinamide adenine dinucleotide (NADH), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione reductase (GR), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione peroxidase (GPx) and oxidized glutathione (GSSG) were purchased from Sigma-Aldrich (Sigma Chemical Co, St. Louis, United States). The anthocyanins cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside, and malvidin-3-glucoside were obtained from Polyphenols Laboratories AS (Polyphenols AS, Sandnes, Norway). To carry out the histological analyses, the following reagents were used: Oil Red-O, Harris Hematoxylin and Light Green SF Yellowish, obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, United States). All solvents were HPLC grade.

Animal Protocol

The experiment was carried out with male, homozygous, LDLr knockout mice (3-month-old), housed in plastic cages (five animals per cage) at constant room temperature (22±2°C) and relative humidity (55±10%), under a 12h light-12h dark cycle. Food and water were available *ad libitum*. This study was separated into two protocols, here designated as "PREVENTION" and "REGRESSION", which are detailed in **Figure 1**. Both protocols included four groups according to the following supplementation: wine (WINE), dealcoholized wine (EXT), *trans*-resveratrol (RESV), and a control group that did not receive any supplementation (CONT). Groups WINE and EXT received the exact same wine, except for the alcohol. The procedure applied to remove the alcohol from the wine is detailed in the **Online Resource 2**. To investigate the effects of the supplementation on fatty streaks formation, animals were fed an atherogenic diet according to Nogueira et al. [22], with some modifications. Normal and atherogenic diets composition are described in **Table S1 (Online Resource 3).** Wine, dealcoholized wine or *trans*-resveratrol were added to the water offered *ad libitum* to the animals. All bottles containing 120 mL of the solution were protected from the light and replaced every two days. The dose of *trans*-resveratrol offered to the animals was calculated based on Chen et al. [23], while the wine dose was based on human consumption (2 glasses/day), according to previous studies [24]. The supplementation applied in this study (wine, dealcoholized wine and *trans*-resveratrol) was characterized according to its antioxidant activity and phenolic composition (**Table S2, Online Resource 3**). Based on this profile, and on animal's hydric consumption, the supplementation dose was estimated in terms of *trans*-resveratrol, total phenolics, and alcohol (**Tables S3 and S4, Online Resource 3**). In the PREVENTION protocol, animals received the supplementation during 16 weeks, and the daily dosage corresponded to 0.69 mL of wine (18.35 µg of phenolic compounds), 0.81 mL of dealcoholized wine (20.45 µg of phenolic compounds) and 0.82 mg of *trans*-resveratrol. In the REGRESSION protocol, animals received the supplementation during 8 weeks, and the daily dosage was 0.64 mL of wine (16.42 mg of phenolic compounds), 0.73 mL of dealcoholized wine (18.49 µg of phenolic compounds) and 0.83 mg of *trans*-resveratrol. After 8 or 16 weeks,

depending on the protocol, mice were deprived of food for 8h and anesthetized with isoflurane. Blood samples were collected by heart puncture, immediately centrifuged (1,600 g for 15 minutes at 4°C), frozen in liquid N_2 and stored (-80°C) until analysis. Serum lipoprotein concentrations (total cholesterol – Liquiform Cholesterol, Cat.13; LDL-cholesterol (LDL-C) – LDL Liquiform, Cat. 111; HDL-cholesterol – HDL Liquiform, Cat. 98; TAG – Triglicerides Liquiform, Cat. 87) and glucose levels (Glucose Liquiform, Cat. 133) were quantified using commercial kits for enzymatic colorimetric tests (Labtest Diagnostica SA, Lagoa Santa, MG). The liver was excised, weighed, and small pieces of the larger lobe were frozen at -80°C for analyses. The heart and aorta were collected and fixed in 10% formol for 24h, and then stored in 70% ethanol for 48h. Subsequently, the samples were frozen in Tissue-Tek at -80°C, until histological analysis. The animal experimental protocol was maintained in accordance with "*National guidelines for the care and use of laboratory animals*" published by NIH [No. 85-23, revised 1996], and was approved (Protocol CEUA/FCF 511) by the Ethics Committee for Animal Studies of the Faculty of Pharmaceutical Sciences, University of Sao Paulo.

Supplements characterization

Antioxidant activity of wine, dealcoholized wine and *trans*-resveratrol was determined by applying the ORAC (Oxygen Radical Absorbance Capacity) methodology as reported by Huang et al. [25]. Phenolic compounds, including major anthocyanins, were identified and quantified by Ultra-High Performance Liquid Chromatography (UHPLC) Agilent 1290 coupled to a mass spectrometer 6460 triple quadrupole (Agilent Technologies, Palo Alto, United States), as reported by Long [26] and Jaitz et al. [27]. A detailed description of each method is described in the **Online Resource 2.**

Fatty acids composition

The oil present in the diets was extracted following the procedure described by Bligh & Dyer [28]. Samples were esterified according to a previously described procedure [29]. Oil (1.5 mg) and liver homogenate (50 µl) samples were transferred to tubes containing 50 µl of the Internal

Standard (C23:0), 50 µl of 0.5% butylated hydroxytoluene (BHT) and 1 ml of 0.5 M methanolic NaOH. Fatty acids quantification was carried out according to AOCS [30], using a gas chromatographer equipped with a G3243A mass spectrometer detector (Agilent 7890A GC System; Agilent Technologies, Palo Alto, United States). A fused silica capillary column (J&W DB-23 Agilent 122–236; 60 m × 250 mm inner diameter; Agilent Technologies, Palo Alto, United States) was used to inject 1 µl of the sample. High-purity He was used as the carrier gas at a flow rate of 1.3 mL/min with a split injection of 50:1. The fatty acids were identified by NIST and by comparing the retention time with those of four purified standard mixtures of fatty acid methyl esters (4-7801; 47085-U; 49453-U and 47885-U; Sigma Chemical Co., St. Louis, United States). Samples were analyzed in triplicate.

Liver oxidative stress biomarkers

Malonaldehyde (MDA) concentration was determined by reverse-phase high-performance liquid chromatography (HPLC), as previously described [31], with some modifications. Activity of the antioxidant enzymes SOD [32], GPx [33],GR [34], and CAT [35, 36], was determined according to the above mentioned published protocols. A detailed description of each method is described in the **Online Resource 2.**

Histological analyses

The preparation of histological sections and the measurement of atherosclerotic lesion area were performed as previously reported [37]. The ventricles were sectioned from the apex and base in a plane parallel to a line defined by the edges of the lateral atria. The hearts were then embedded in KilliK (Easy Path, Sao Paulo, Brazil) inside the cryostat-freezing chamber. Consecutive cuts with 5 µm thick were collected between the aortic sinus and the aortic onset with an average length of 250–300 microns [38]. The slices were stained with Oil Red-O and counter-stained with Harris Hematoxylin and Light Green SF Yellowish. The sections were analyzed by Nikon optical microscope coupled to a camera for image capture program performed by the NIS-Elements AR (tm) version 3.10 (Nikon, USA). To obtain the area of the

lesions and quantify atherosclerotic lesions close to the aortic valve in the aortic root, Axio Vision® and ImageJ were used for image acquisition, processing and analyses of all histological sections. All analyses were double-blind and were performed independently by two observers.

Liver tissue samples were fixed in 10% formalin for 24h and then embedded in paraffin wax following the standard procedures established by Kleiner et al. [39]. The samples were cut into 5μm sections and stained with hematoxylin-eosin (HE) to evaluate steatosis and inflammation. Steatosis, hepatocellular ballooning, and lobular inflammation were determined histopathologically and graded as described elsewhere [39]. The degree of steatosis was graded using the following four-point scale: grade 0, steatosis involving hepatocytes; grade 1, steatosis involving up to 33% of hepatocytes; grade 2, steatosis involving 33-66% of hepatocytes; and grade 3, steatosis involving >66% of hepatocytes. Lobular inflammation was also graded on a four-point scale: grade 0, no foci; grade 1, fewer than two foci per 20 × field; grade 2, four foci per 20 × field; and grade 3, more than four foci per 20 × field. Hepatocyte ballooning was graded on a three-point scale: 0, none; 1, a few balloon cells; and 2, any/prominent balloon cells. For non-alcoholic fatty liver disease (NAFLD) activity score, features of steatosis, lobular inflammation and hepatocyte ballooning were combined, and the range of values were from 0 to 8.

Statistical analysis

Results observed in the four groups at the end of the experiment or % of changes were compared using ANOVA followed by HSD Tukey test. Data were transformed or Kruskal Wallis analysis was applied when necessary. Data from histological analysis were evaluated by Chi-Square test. An alpha value of 0.05 was adopted to reject the null hypothesis. Data analysis and graphs were carried out using the Statistica v.9 software (Statsoft Inc., Tulsa, USA).

2.4. RESULTS

The body weight gain observed in the animals according to the protocol and type of intervention is showed in **Figure 2a**. These data showed that the animals that first received the normal diet for 8 weeks and then the atherogenic diet for 8 weeks more (PREVENTION) had a higher body weight gain (39.25 ± 2.30%) at the end of the study than the groups that were fed an atherogenic diet at the beginning of the protocol (REGRESSION) (29.27 ± 1.91%, *p=0.0013*). It can be suggested that the age of the mice interacts with the body weight gain driven by the atherogenic diet. The body weight gain/diet (**Figure 2b**) confirms the higher effect of the atherogenic diet on the weight gain compared to the normal diet and reflects the higher amount of fatty acids observed in the liver of the animals who ended the study under the atherogenic diet (**Figure 2c**). As expected, both total fatty acids and MDA fatty acids precursors (**Figure 2d**) were found in higher concentration in the groups that received the atherogenic diet for last (PREVENTION) than in the groups fed with the normal diet during the last 8 weeks (REGRESSION).

Regarding the PREVENTION protocol, no difference was observed in the body weight at the beginning (*p=0.0739*), after 8 weeks (*p=0.0773*) or at the end of the intervention time (*p=0.7868*) among the four experimental groups. **Figure 3** shows the biomarkers of lipidemia and oxidative stress measured in plasma and liver of the animals submitted to the PREVENTION protocol. Supplementation with *trans*-resveratrol (RESV) reduced total-cholesterol (*p<0.0001*), LDL-C (**Figure 3a**), VLDL-C (**Figure 3b**) and HDL-C (**Figure 3c**) compared to the WINE and CONT groups, with no difference to the EXT group. The treatments did not alter glucose (*p=0.1074*) and TAG (*p=0.1296*) concentrations in plasma. The amount of fatty acids precursors of MDA in the liver was higher (**Figure 3d**) in the animals supplemented with dealcoholized wine (EXT) and *trans*-resveratrol (RESV) than in the animals who received wine (WINE) and water (CONT). This result was similar to the MDA concentration showed in **Figure 3e**. Regarding the antioxidant enzymes, dealcoholized wine (EXT) and *trans*-resveratrol (RESV) increased the activity of SOD (**Figure 3f**) compared with the WINE

and CONT groups. No differences were observed in CAT (*p=0.1746*), GPx (*p=0.0663*) and GR activity (*p*=0.*5471*) among the four groups. Aorta histology is showed in **Figure 4**. No differences were observed among the groups for aorta inner area (1,017.7 ± 54.5 nm^2; *p=0.2788*), inner perimeter (5,167.6 ± 274.0 µm; *p=0.2093*), lesion area (186,804.8 ± 999.8 μm^2; *p=0.9402*) and lesion/inner area ratio (0.19 ± 0.01; *p=0.7265*). Supplementation also did not affect the biomarkers measured in the liver (**Figure S2, Online Resource 1)**. Ballooning (*p=0.3105*), Steatosis (*p=0.2010*), Lobular Inflammation (*p=0.0530*) and NAS (*p=0.3409*) did not differ among the groups.

In the REGRESSION protocol, a group fed with the Atherogenic diet from week 0 to 8 was adopted as "Baseline". Thus, in this protocol, most of the results taken after 16 weeks of intervention was compared with this "Baseline" group and expressed as % of change. At the beginning, the EXT group presented a higher body weight (27.35 ± 0.37g; *p=0.0027*) than the others (25.58 ± 0.48 g). After 8 weeks under atherogenic diet, the animals located in the EXT group (34.11 ± 0.64 g) did not show differences in the body weight compared with the other groups (33.50 ± 0.86 g), except for the Baseline group (30.28 ± 0.64 g; *p=0.0009*). After 16 weeks, differences in the body weight were no longer observed among the groups (30.73 ± 0.43 g; *p=0.7514*). Dealcoholized wine (EXT) and *trans*-resveratrol (RESV) groups showed greater HDL-cholesterol reduction than the WINE and CONT groups (**Figure 5a**). EXT group also showed a higher amount of MDA fatty acids precursors in the liver, followed by the RESV group (**Figure 5b**), and consequently, the highest concentration of MDA in the liver (**Figure 5c**) when compared with all other groups. In this protocol, all three supplementations reduced SOD activity (**Figure 5d**) with no changes in the GPx activity (*p=0.3565*). WINE group showed reduction of CAT activity (**Figure 5e**). The reduction observed in the GR activity in the WINE group was higher than the reduction found after *trans*-resveratrol and dealcoholized red wine supplementation (**Figure 5f**). From the histological analysis (**Figure 6**), no differences were observed among the groups for aorta inner area (-1.80% ± 4.77; *p=0.1116*), inner perimeter (-3.91% ± 2.59; *p=0.1386*), lesion area (-1.60% ± 13.84; *p=0.8810*) and lesion/inner area ratio (-5.65% ± 17.45; *p=0.7212*). Finally, liver histology of the supplemented animals was

compared with those of the baseline group (**Figure S3, Online Resource 1**). In this case, all groups showed an improvement when compared with baseline according to the following parameters: Ballooning (*p=0.0464*), Steatosis (*p=0.01517*), Lobular Inflammation (*p=0.0265)* and NAS (*p=0.0405*), but no differences were observed among the groups.

2.5. DISCUSSION

In this study, the supplementation with *trans*-resveratrol was compared with the consumption of wine (regular or dealcoholized) in terms of prevention and regression of fatty streaks in LDLr $^{(-/-)}$ mice. In the PREVENTION protocol, it was observed that *trans*-resveratrol and dealcoholized wine reduced total cholesterol and fractions, modulated the lipid metabolism in the liver and also increased SOD activity. When compared with control, the consumption of red wine did not change any plasmatic and hepatic biomarker, and it did not promote any damage to the hepatic tissue. In terms of endpoint, none of the treatments was able to prevent the fatty streaks formation caused by the atherogenic diet in this animal model. In the REGRESSION protocol, the treatments were compared with data observed in the “Baseline” group, and most of the results were expressed as % of change. *Trans*-resveratrol and dealcoholized wine reduced HDL cholesterol. Compared with control, consumption of red wine reduced the activity of the antioxidant enzymes in a more expressive way than *trans*-resveratrol and dealcoholized wine. As also observed in the PREVENTION protocol, both *trans*-resveratrol and dealcoholized red wine treatments modulated the lipid metabolism in the liver in a similar way, and no changes were observed in terms of damage to the hepatic tissue or fatty streaks formation.

To the best of our knowledge, this is the first study that evaluates and compares wine and resveratrol effects on plaque regression. Previous findings suggest that inhibition of atherosclerosis progression after intake of red wine or wine polyphenols occurs as a consequence of a hypocholesterolemic effect. Back in 2008, Do and colleagues showed that 20 weeks of resveratrol supplementation (0.02% diet/w) inhibited the progression of fatty

streak formation in ApoE $^{(-/-)}$ mice fed a normal diet, along with a reduction in total cholesterol, LDL-C and the hepatic activity of HMG-CoA reductase [40]. According to Berbée et al. [41], resveratrol (11 mg/kg/day) reduces atherosclerosis lesion and improves plaque stability in a similar extent as atorvastatin in an animal model for human-like lipoprotein metabolism (APOE*3-Leiden.CETP mice) mainly as a result of lowering plasma VLDL cholesterol levels [41]. Increased excretion of sterols and bile acids after intake of resveratrol and wine anthocyanidin have also been proposed as a mechanism for hypocholesterolemic effects observed in mice [23, 42] and rats [43]. Reduction of total cholesterol or LDL-C after the intake of *trans*-resveratrol (250 mg/day for 3 months) [44] and red wine (30 g alcohol/day for 4 weeks) [45] have also been observed in human clinical trials.

Interestingly, our results showed a reduction of HDL-C after consumption of *trans*-resveratrol and dealcoholized wine in both protocols. However, the relevance of HDL-C in the settings of cardiovascular disease is currently under question, whereas the concept of HDL particles functionality have been gaining strength [46]. In a study conducted by Bentzon et al. [47], consumption of red wine for 19 weeks (0.3 mL/day of ethanol) did not alter the amount or composition of advanced atherosclerosis in ApoE $^{(-/-)}$ mice, despite a 12% increase in HDL-cholesterol levels.

More than a decade ago, the dealcoholized red wine was shown to slow the progression of atherosclerotic lesion in ApoE $^{(-/-)}$ mice at 26 weeks of intervention, but not earlier on [48]. This protective activity was shown to be independent of any effects on lipid peroxidation, which was corroborated by others [49, 50]. In a previous study carried out by our group, we showed that the intake of a wine with high *in vitro* antioxidant activity decreased MDA and increased protein expression of SOD in the liver of rats fed a high-fat diet, while CAT and GPx activities were increased after the intake of a wine with low antioxidant activity [51]. More recently, red wine ethanolic extract was also shown to attenuated the levels of lipid peroxides induced by a high-fat diet in rats [52].

In the present study, *trans*-resveratrol and dealcoholized wine increased SOD activity when added to a high-fat diet (PREVENTION), while all three supplementations overall decreased

the activity of the antioxidant enzymes after 8 weeks of normal diet (REGRESSION). Similar results were reported by Rocha et al. [53] where *trans*-resveratrol supplementation was shown to increase SOD activity in rats fed a high-fat diet, but not in animals fed a standard diet. In healthy human subjects, a single dose of *trans*-resveratrol was shown to decrease SOD and CAT activity in erythrocytes [54]. These findings suggest that *trans*-resveratrol or wine polyphenols might induce the increase of SOD expression and activity under conditions of metabolic stress, downregulating its activity when further antioxidant mechanism is not required.

In both protocols it was observed that the increase of MDA was associated with the increased amount of available substrate for MDA production (polyunsaturated fatty acids containing more the two double bonds in their molecular structure [55]) (Figure **3d,e,** and **5b,c**) and supposedly not a consequence of an increase in oxidant species/oxidative stress. However, the mechanism by which *trans*-resveratrol or wine polyphenols could affect fatty acids metabolism in the liver is still not clear.

Alterations in the biomarkers that are not followed by an improvement of the disease endpoints are not rare in the literature. Actually, this is a controversial subject even in the pharmaceutical area, where drugs are able to improve classical biomarkers, but they do not reduce mortality in the target public. Regarding the studies involving food and supplements, the majority does not evaluate endpoints.

The lack of effect of the supplementation on fatty streaks progression and regression in our animal model could be attributed to a low dosage of wine and *trans*-resveratrol or to insufficient timeframe for fatty streaks to achieve a higher level of damage. However, both parameters (dose and time) were adapted, based on previous studies in which the authors found significant results. The period of diet-induced atherosclerosis applied in our protocol was based on information available in a clinical review by Whitman [56]. Thus, although other studies have used longer trials [57–59], fatty streaks were already observed in the endothelium of the animals fed with our atherogenic diet for 8 weeks.

Concerning the supplementation, similar doses have been reported by other studies that found improvement of some biomarkers associated with atherosclerosis using animal models [49]. Hayek and colleagues [60] conducted one of the first studies on red wine's effect on atheroma plaques and observed that the consumption of 0.5 mL red wine/day by apoE knockout mice for 6 weeks significantly reduced the atherosclerotic lesion area by 48% in comparison with the placebo group. The intake of wine (0.67 mL/day) and dealcoholized wine (0.77 mL/day) applied in our study corresponded to about 360 mL in a human model. In fact, the wine selected to compose this study presented the highest *in vitro* antioxidant activity among 666 samples of South American red wines [21]. Concerning to *trans*-resveratrol, the dose was 0.82 mg *trans*-resveratrol/animal/day, which corresponds to 2,30 g *trans*-resveratrol/day for a human of 70 Kg.

Although it was not the focus of this study, it was observed that older animals gained more weight when fed with the atherogenic diet (PREVENTION protocol) than the animals who received it at an early stage of life (REGRESSION protocol). This data suggests that there is an interaction between the age of the animals and their body weight gain. This result may be associated with differences in the metabolic rate of the animals according to the age. Liu and colleagues [61] observed that aging worsened liver injury, insulin resistance, dyslipidemia and fat storage in C57BL/6 mice fed a high-fat diet, leading to a reduced liver function, which can affect hepatic lipid metabolism. A reduction of β-oxidation with aging has been reported, which was related to the reduced hepatic expression of the nuclear receptors of the peroxisome proliferator-activated receptors family (PPARs), whereas the expression of liver X receptors (LXRs) was enhanced [62]. Aging is also associated with an increased amount of adipocytes in animal models fat depots [63].

2.6. CONCLUSION

The supplementation with *trans*-resveratrol, wine and the same wine without alcohol showed significant alterations in the biomarkers of oxidative stress and lipidemia but did not cause any

effect in terms of prevention or regression of fatty streaks in LDLr $^{(-/-)}$ mice submitted to an atherogenic diet. These data suggest that the cardiovascular protection associated with the “French Paradox” is, in fact, a consequence of the Mediterranean diet and it cannot be achieved out of this context.

2.7. CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

2.8. REFERENCES

1. Renaud S, De Longeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339:1523–1526
2. Haseeb S, Alexander B, Baranchuk A (2017) Wine and Cardiovascular Health: A Comprehensive Review. Circulation 136:1434–1448 . doi: 10.1161/CIRCULATIONAHA.117.030387
3. Medina-Remón A, Tresserra-Rimbau A, Pons A, et al (2015) Effects of total dietary polyphenols on plasma nitric oxide and blood pressure in a high cardiovascular risk cohort. The PREDIMED randomized trial. Nutr Metab Cardiovasc Dis 25:60–7 . doi: 10.1016/j.numecd.2014.09.001
4. Rockenbach II, Gonzaga LV, Rizelio VM, et al (2011) Phenolic compounds and antioxidant activity of seed and skin extracts of red grape (Vitis vinifera and Vitis labrusca) pomace from Brazilian winemaking. Food Res Int 44:897–901 . doi: 10.1016/j.foodres.2011.01.049
5. Fernández De Simón B, Martínez J, Sanz M, et al (2014) Volatile compounds and sensorial characterisation of red wine aged in cherry, chestnut, false acacia, ash and oak wood barrels. Food Chem 147:346–356 . doi: 10.1016/j.foodchem.2013.09.158
6. Mallebrera B, Maietti A, Tedeschi P, et al (2017) Antioxidant capacity of trans -

resveratrol dietary supplements alone or combined with the mycotoxin beauvericin. Food Chem Toxicol 105:315–318 . doi: 10.1016/j.fct.2017.04.027

7. Katalinić V, Milos M, Modun D, et al (2004) Antioxidant effectiveness of selected wines in comparison with (+)-catechin. Food Chem 86:593–600 . doi: 10.1016/j.foodchem.2003.10.007
8. An D, Zhang Q, Wu S, et al (2010) Changes of metabolic profiles in urine after oral administration of quercetin in rats. Food Chem Toxicol 48:1521–1527 . doi: 10.1016/j.fct.2010.03.019
9. Figueroa-Espinoza MC, Zafimahova A, Alvarado PGM, et al (2015) Grape seed and apple tannins: Emulsifying and antioxidant properties. Food Chem 178:38–44 . doi: 10.1016/j.foodchem.2015.01.056
10. Maurya H, Mangal V, Gandhi S, et al (2014) Prophylactic antioxidant potential of gallic acid in murine model of sepsis. Int J Inflam 2014:Article ID 580320 . doi: 10.1155/2014/580320
11. Foley S, Navaratnam S, McGarvey DJ, et al (1999) Singlet oxygen quenching and the redox properties of hydroxycinnamic acids. Free Radic Biol Med 26:1202–1208
12. Hayes JE, Allen P, Brunton N, et al (2011) Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (Olea europaea L.), lutein, sesamol and ellagic acid. Food Chem 126:948–955 . doi: 10.1016/j.foodchem.2010.11.092
13. Matsunaga N, Imai S, Inokuchi Y, et al (2009) Bilberry and its main constituents have neuroprotective effects against retinal neuronal damage in vitro and in vivo. Mol Nutr Food Res 53:869–877 . doi: 10.1002/mnfr.200800394
14. Motilva MJ, Macià A, Romero MP, et al (2016) Human bioavailability and metabolism of phenolic compounds from red wine enriched with free or nano-encapsulated phenolic extract. J Funct Foods 25:80–93 . doi: 10.1016/j.jff.2016.05.013
15. Del Pino-García R, Rivero-Pérez MD, González-SanJosé ML, et al (2016) Bioavailability of phenolic compounds and antioxidant effects of wine pomace seasoning after oral

administration in rats. J Funct Foods 25:486–496 . doi: 10.1016/j.jff.2016.06.030

16. Shen Y, Xu Z, Sheng Z (2017) Ability of resveratrol to inhibit advanced glycation end product formation and carbohydrate-hydrolyzing enzyme activity, and to conjugate methylglyoxal. Food Chem 216:153–160 . doi: 10.1016/j.foodchem.2016.08.034
17. Bhullar KS, Hubbard BP (2015) Lifespan and healthspan extension by resveratrol. Biochim Biophys Acta - Mol Basis Dis 1852:1209–1218 . doi: 10.1016/j.bbadis.2015.01.012
18. Zordoky BN, Robertson IM, Dyck JR (2015) Preclinical and clinical evidence for the role of resveratrol in the treatment of cardiovascular diseases. Biochim Biophys Acta 1852:1155–1177 . doi: 10.1016/j.bbadis.2014.10.016
19. Baur JA, Pearson KJ, Price NL, et al (2006) Resveratrol improves health and survival of mice on a high-calorie diet. Nature 444:337–342 . doi: 10.1038/nature05354
20. Smoliga JM, Vang O, Baur JA (2012) Challenges of translating basic research into therapeutics: Resveratrol as an example. Journals Gerontol - Ser A Biol Sci Med Sci 67 A:158–167 . doi: 10.1093/gerona/glr062
21. Llobodanin LG, Barroso LP, Castro IA (2014) Prediction of the functionality of young South American red wines based on chemical parameters. Aust J Grape Wine Res 20:15–24 . doi: 10.1111/ajgw.12057
22. Nogueira MS, Kessuane MC, Lobo Ladd AAB, et al (2016) Effect of long-term ingestion of weakly oxidised flaxseed oil on biomarkers of oxidative stress in LDL-receptor knockout mice. Br J Nutr 116:258–269 . doi: 10.1017/S0007114516001513
23. Chen Q, Wang E, Ma L, Zhai P (2012) Dietary resveratrol increases the expression of hepatic 7α-hydroxylase and ameliorates hypercholesterolemia in high-fat fed C57BL/6J mice. Lipids Health Dis 11:56 . doi: 10.1186/1476-511X-11-56
24. Fuhrmann B, Lavy A, Aviram M (1995) Consumption of red wine with meals reduces the susceptibility to human plasma and low density lipoprotein to lipid peroxydation. Am J Clin Nutr 61:549–554
25. Huang D, Ou B, Hampsch-Woodill M, et al (2002) High-throughput assay of oxygen

radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 50:4437–4444 . doi: 10.1021/jf0201529

26. Long WJ Analysis of Anthocyanins in Common Foods Using an Agilent Poroshell 120 SB-C18. Appl Note Agil Technol Inc
27. Jaitz L, Siegl K, Eder R, et al (2010) LC-MS/MS analysis of phenols for classification of red wine according to geographic origin, grape variety and vintage. Food Chem 122:366–372 . doi: 10.1016/j.foodchem.2010.02.053
28. Bligh EG, Dyer W (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917 . doi: 10.1080/24740527.2017.1337467
29. Shirai N, Suzuki H, Wada S (2005) Direct methylation from mouse plasma and from liver and brain homogenates. Anal Biochem 343:48–53 . doi: 10.1016/j.ab.2005.04.037
30. AOCS. Official Method Ce1e-91. Determination of Fatty Acids in Edible Oils and Fats by Capillary GLC. American Oil Chemists' Society. Official Methods and Recommended Practices. 5th ed. Champaignon: AOCS, 2004
31. Hong Y, Yeh S, Chang C, Hu M (2000) Total Plasma Malondialdehyde Levels in 16 Taiwanese College Students Determined by Various Thiobarbituric Acid Tests and an Improved High-Performance Liquid Chromatography-based Method. Clin Biochem 33:619–625 . doi: 10.1016/S0009-9120(00)00177-6
32. Ewing JF, Janero DR (1995) Microplate superoxide dismutase assay employing a nonenzymatic superoxide generator. Anal Biochem 232:243–248 . doi: 10.1006/abio.1995.0014
33. Flohé L, Günzler WA (1984) Assays of glutathione peroxidase. Methods Enzymol 105:114–120
34. Torres LL, Quaglio NB, de Souza GT, et al (2011) Peripheral oxidative stress biomarkers in mild cognitive impairment and Alzheimer's disease. J Alzheimer's Dis 26:59–68 . doi: 10.3233/JAD-2011-110284
35. Nabavi SF, Nabavi SM, Abolhasani F, et al (2012) Cytoprotective effects of curcumin

on sodium fluoride-induced intoxication in rat erythrocytes. Bull Environ Contam Toxicol 88:486–490 . doi: 10.1007/s00128-011-0495-5

36. Bonaventura J, Schroeder WA, Fang S (1972) Human erythrocyte catalase: an improved method of isolation and a reevaluation of reported properties. Arch Biochem Biophys 150:606–6017
37. Cavalcante MF, Kazuma SM, Bender EA, et al (2016) A nanoformulation containing a scFv reactive to electronegative LDL inhibits atherosclerosis in LDL receptor knockout mice. Eur J Pharm Biopharm 107:120–129 . doi: 10.1016/j.ejpb.2016.07.002
38. Paigen B, Morrow A, Holmes PA, et al (1987) Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis 68:231–240
39. Kleiner DE, Brunt EM, Van Natta M, et al (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41:1313–1321 . doi: 10.1002/hep.20701
40. Do GM, Kwon EY, Kim HJ, et al (2008) Long-term effects of resveratrol supplementation on suppression of atherogenic lesion formation and cholesterol synthesis in apo E-deficient mice. Biochem Biophys Res Commun 374:55–59 . doi: 10.1016/j.bbrc.2008.06.113
41. Berbée JFP, Wong MC, Wang Y, et al (2013) Resveratrol protects against atherosclerosis, but does not add to the antiatherogenic effect of atorvastatin, in APOE*3 - Leiden.CETP mice. J Nutr Biochem 24:1423–1430 . doi: 10.1016/j.jnutbio.2012.11.009
42. Wang D, Xia M, Gao S, et al (2012) Cyanidin-3-O-β-glucoside upregulates hepatic cholesterol 7α-hydroxylase expression and reduces hypercholesterolemia in mice. Mol Nutr Food Res 56:610–621 . doi: 10.1002/mnfr.201100659
43. Miura D, Miura Y, Yagasaki K (2003) Hypolipidemic action of dietary resveratrol, a phytoalexin in grapes and red wine, in hepatoma-bearing rats. Life Sci 73:1393–1400 . doi: 10.1016/S0024-3205(03)00469-7
44. Bhatt JK, Thomas S, Nanjan MJ (2012) Resveratrol supplementation improves glycemic

control in type 2 diabetes mellitus. Nutr Res 32:537–541 . doi: 10.1016/j.nutres.2012.06.003

45. Chiva-Blanch G, Arranz S, Lamuela-Raventos RM, Estruch R (2013) Effects of wine, alcohol and polyphenols on cardiovascular disease risk factors: Evidences from human studies. Alcohol Alcohol 48:270–277 . doi: 10.1093/alcalc/agt007
46. Kontush A (2015) HDL particle number and size as predictors of cardiovascular disease. Front Pharmacol 6:1–6 . doi: 10.3389/fphar.2015.00218
47. Bentzon JF, Skovenborg E, Hansen C, et al (2001) Red wine does not reduce mature atherosclerosis in apolipoprotein E-deficient mice. Circulation 103:1681–1687
48. Waddington E, Puddey IB, Croft KD (2004) Red wine polyphenolic compounds inhibit atherosclerosis in apolipoprotein E-deficient mice independently of effects on lipid peroxidation. Am J Clin Nutr 79:54–61
49. Stocker R, O'Halloran RA (2004) Dealcoholized red wine decreases atherosclerosis in apolipoprotein E gene-deficient mice independently of inhibition of lipid peroxidation in the artery wall. Am J Clin Nutr 79:123–130
50. Martínez N, Casós K, Simonetti P, et al (2013) De-alcoholised white and red wines decrease inflammatory markers and NF-κB in atheroma plaques in apoE-deficient mice. Eur J Nutr 52:737–747 . doi: 10.1007/s00394-012-0379-4
51. Macedo LFL, Rogero MM, Guimarães JP, et al (2012) Effect of red wines with different in vitro antioxidant activity on oxidative stress of high-fat diet rats. Food Chem 137:122–129 . doi: 10.1016/j.foodchem.2012.10.017
52. Auberval N, Dal S, Maillard E, et al (2017) Beneficial effects of a red wine polyphenol extract on high-fat diet-induced metabolic syndrome in rats. Eur J Nutr 56:1467–1475 . doi: 10.1007/s00394-016-1192-2
53. Rocha KKR, Souza GA, Ebaid GX, et al (2009) Resveratrol toxicity: Effects on risk factors for atherosclerosis and hepatic oxidative stress in standard and high-fat diets. Food Chem Toxicol 47:1362–1367 . doi: 10.1016/j.fct.2009.03.010
54. Pignitter M, Schueller K, Burkon A, et al (2015) Concentration dependent effects of

resveratrol and metabolites on the redox status of human erythrocytes in single dose studies. J Nutr Biochem 27:2–8 . doi: 10.1016/j.jnutbio.2015.08.032

55. Frankel EN, Regional N (1984) Lipid Oxidation: Mechanisms, Products and Biological Significance. 61:1908–1917

56. Whitman SC (2004) A practical approach to using mice in atherosclerosis research. Clin Biochem Rev 25:81–93

57. Chira EC, McMillen TS, Wang S, et al (2007) Tesaglitazar, a dual peroxisome proliferator-activated receptor alpha/gamma agonist, reduces atherosclerosis in female low density lipoprotein receptor deficient mice. Atherosclerosis 195:100–109 . doi: 10.1016/j.atherosclerosis.2006.12.012

58. Hartvigsen K, Binder CJ, Hansen LF, et al (2007) A diet-induced hypercholesterolemic murine model to study atherogenesis without obesity and metabolic syndrome. Arterioscler Thromb Vasc Biol 27:878–885 . doi: 10.1161/01.ATV.0000258790.35810.02

59. Graham TL, Mookherjee C, Suckling KE, et al (2005) The PPARδ agonist GW0742X reduces atherosclerosis in LDLR-/-mice. Atherosclerosis 181:29–37 . doi: 10.1016/j.atherosclerosis.2004.12.028

60. Hayek T, Fuhrman B, Vaya J, et al (1997) Reduced Progression of Atherosclerosis in Apolipoprotein E Deficient Mice Following Consumption of Red Wine, or Its Polyphenols Quercetin or Catechin, Is Associated With Reduced Susceptibility of LDL to Oxidation and Aggregation. Arterioscler Thromb Vasc Biol 17:2744–2752 . doi: 10.1161/01.ATV.17.11.2744

61. Liu C, Chang C, Lee H, et al (2016) Metabolic Damage Presents Differently in Young and Early-Aged C57BL/6 Mice Fed a High-Fat Diet. Int J Gerontol 10:105–111 . doi: 10.1016/j.ijge.2015.10.004

62. Cohen J, Horton J, Hobbs H (2011) Human fatty liver disease: old questions and new insights. Science (80-) 332:1519–1523 . doi: 10.1126/science.1204265.Human

63. Bertrand HA, Lynd FT, Masoro EJ, Yu BP (1980) Changes in adipose mass and

cellularity through the adult life of rats fed ad libitum or a life-prolonging restricted diet. Journals Gerontol 35:827–835 . doi: 10.1093/geronj/35.6.827

2.9. FIGURES

Figure 1: Animal protocols.

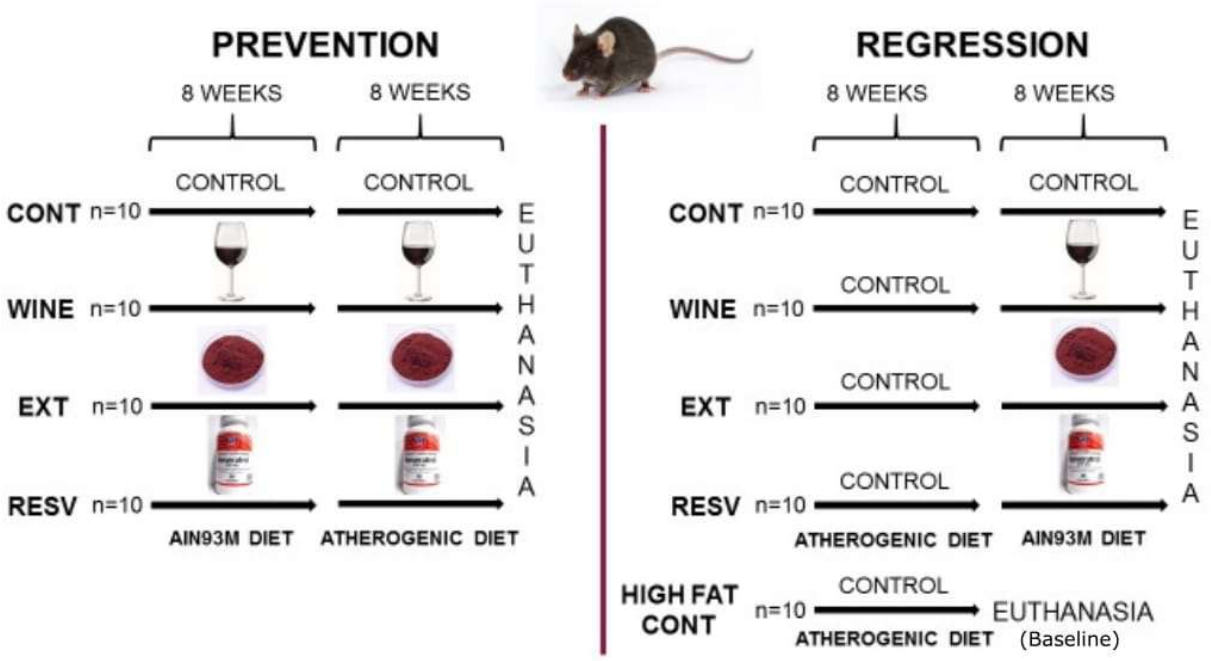

Fig. 1

Experiment time course and interventions on animals allocated in the PREVENTION and REGRESSION protocols. WINE: group supplemented with 0.69 mL of wine/day (PREVENTION) or 0.64 mL of wine/day (REGRESSION); EXT: group supplemented with 0.81 mL of dealcoholized wine/day (PREVENTION) or 0.73 mL of dealcoholized wine/day (REGRESSION); RESV: group supplemented with 0.82 mg of *trans*-resveratrol/day (PREVENTION) or 0.83 mg of *trans*-resveratrol/day (REGRESSION); and CONT: group not supplemented.

Figure 2: Comparison between the two protocols and respective treatment.

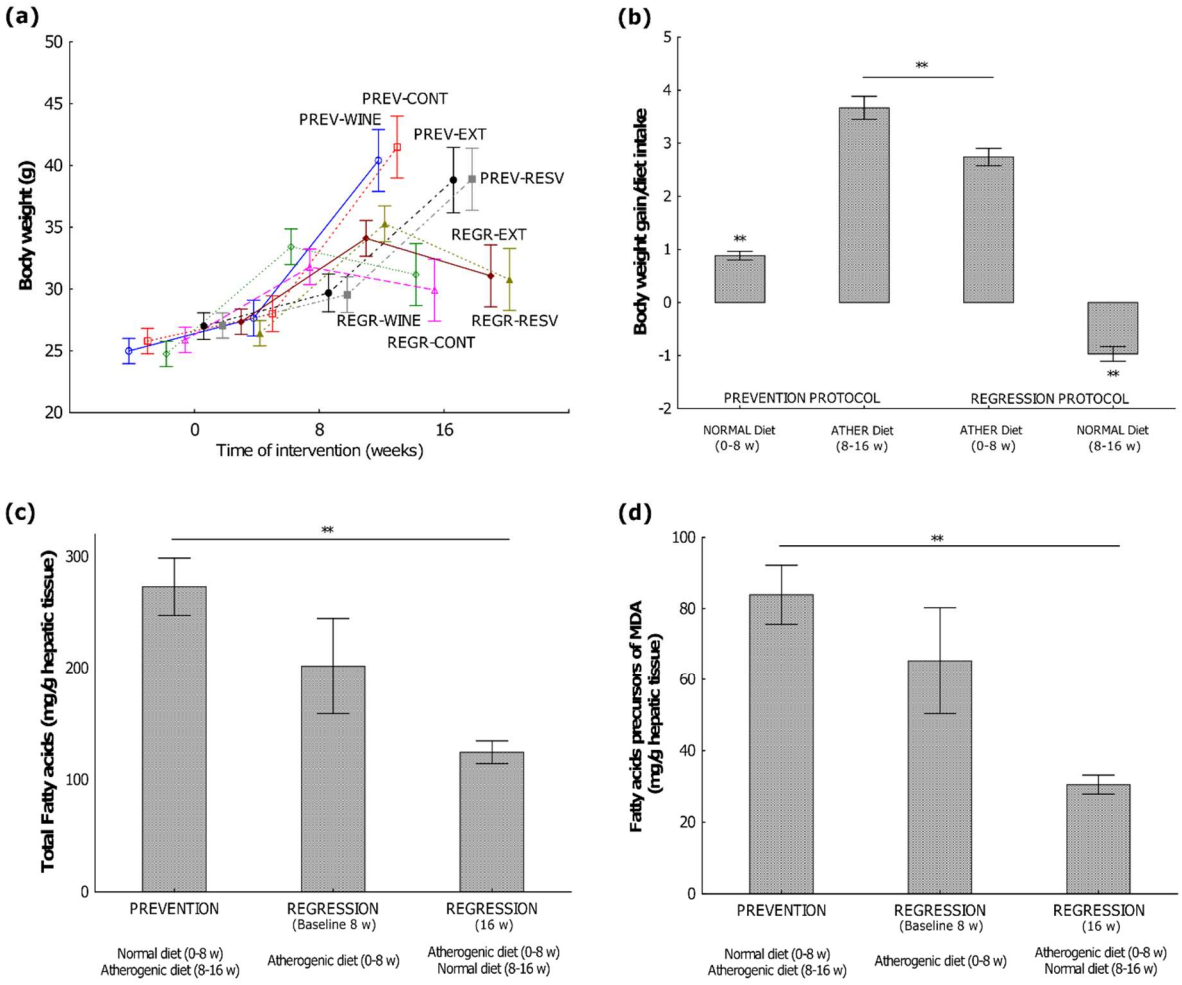

Fig. 2

Comparison between PREV (PREVENTION) and REGR (REGRESSION) protocols and respective treatment. (**a**) Body weight of the animals according to the protocol and respective treatment; (**b**) Body weight gain/diet consumption according to the diets: Normal diet at the beginning (0-8 weeks), Normal diet at the end (8-16 weeks), Atherogenic diet at the beginning (0-8 weeks) and Atherogenic diet at the end (8-16 weeks). (**c**) Fatty acids in the liver according to the protocols; (**d**) Fatty acids precursors of malonaldehyde according to the protocols. Data are presented as mean ± SD (n>40 for protocols and n>10 for treatments). Kruskal-Wallis ANOVA followed by Multiple Comparisons was applied to compare the body gain according to the diets; ***p<0.001*. One-way ANOVA and Tukey test were used to compare the PREVENTION and REGRESSION groups and the Baseline group of the REGRESSION protocol ***p<0.001*. WINE: group supplemented with 0.69 mL of wine/day (PREVENTION) or 0.64 mL of wine/day (REGRESSION); EXT: group supplemented with 0.81 mL of dealcoholized wine/day (PREVENTION) or 0.73 mL of dealcoholized wine/day (REGRESSION); RESV: group supplemented with 0.82 mg of *trans*-resveratrol/day (PREVENTION) or 0.83 mg of *trans*-resveratrol/day (REGRESSION); and CONT: group not supplemented.

Figure 3: Biomarkers of lipidemia and oxidative of the PREVENTION protocol.

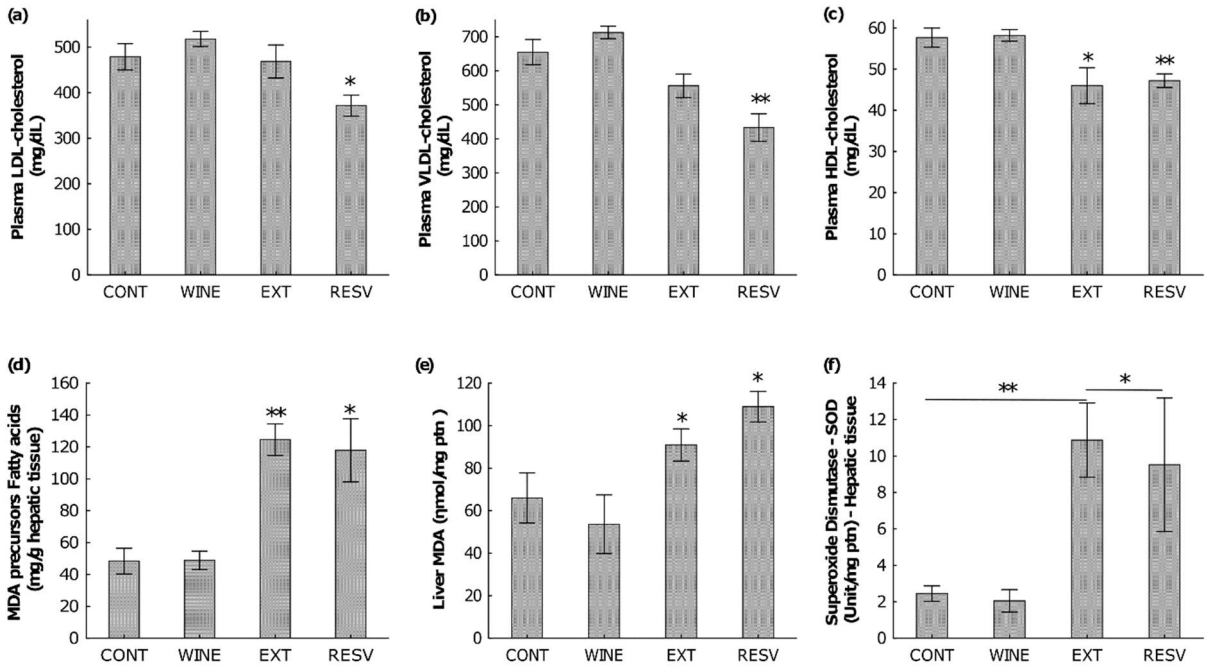

Fig. 3

Biomarkers of lipidemia and oxidative stress measured in the plasma and liver of the animals submitted to the PREVENTION protocol. (**a**) Plasma LDL-cholesterol; (**b**) Plasma VLDL-cholesterol; (**c**) Plasma HDL-cholesterol; (**d**) Fatty acids precursors of malonaldehyde in the liver; (**e**) Liver malonaldehyde; (**f**) Superoxide dismutase activity in liver. Data are presented as mean ± SD (n= 10/treatment). One-way ANOVA followed by Tukey test was applied to compare the four treatments; *$p<0.05$ and **$p<0.001$. WINE: group supplemented with 0.69 mL of wine/day; EXT: group supplemented with 0.81 mL of dealcoholized wine/day; RESV: group supplemented with 0.82 mg of *trans*-resveratrol/day; and CONT: group not supplemented.

Figure 4: Atherosclerotic lesion development of the PREVENTION protocol.

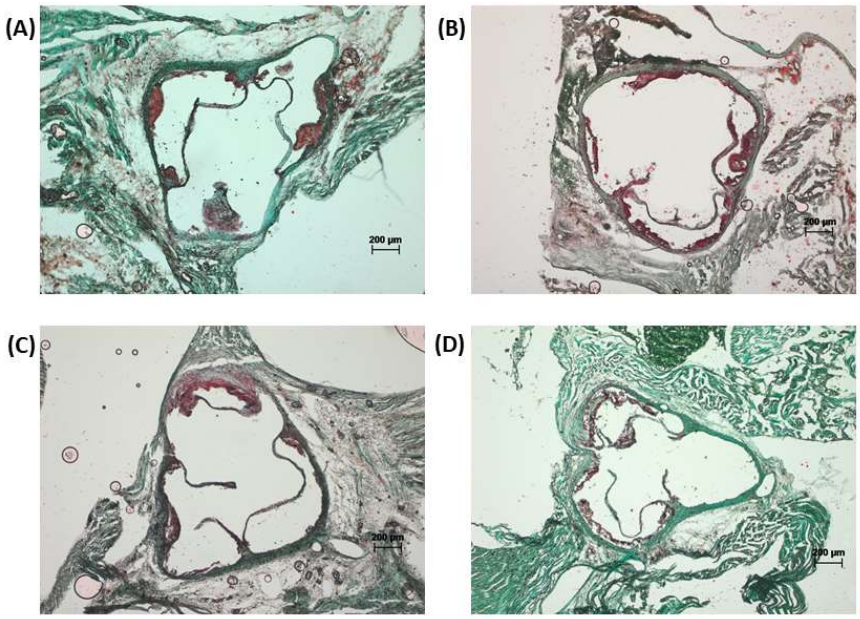

Fig. 4

Effect of the PREVENTION protocol on the atherosclerotic lesion development at the aortic sinus of LDLr $^{(-/-)}$ mice. Red color indicates lipid accumulation and green color indicates collagen fibrils. (**A**) WINE: group supplemented with 0.69 mL of wine/day; (**B**) EXT: group supplemented with 0.81 mL of dealcoholized wine/day. (**C**) RESV: group supplemented with 0.82 mg of *trans*-resveratrol/day. (**D**) CONT: group not supplemented. 10 x magnification images. Scale bar represents 200 µm.

Figure 5: Biomarkers of lipidemia and oxidative of the REGRESSION protocol.

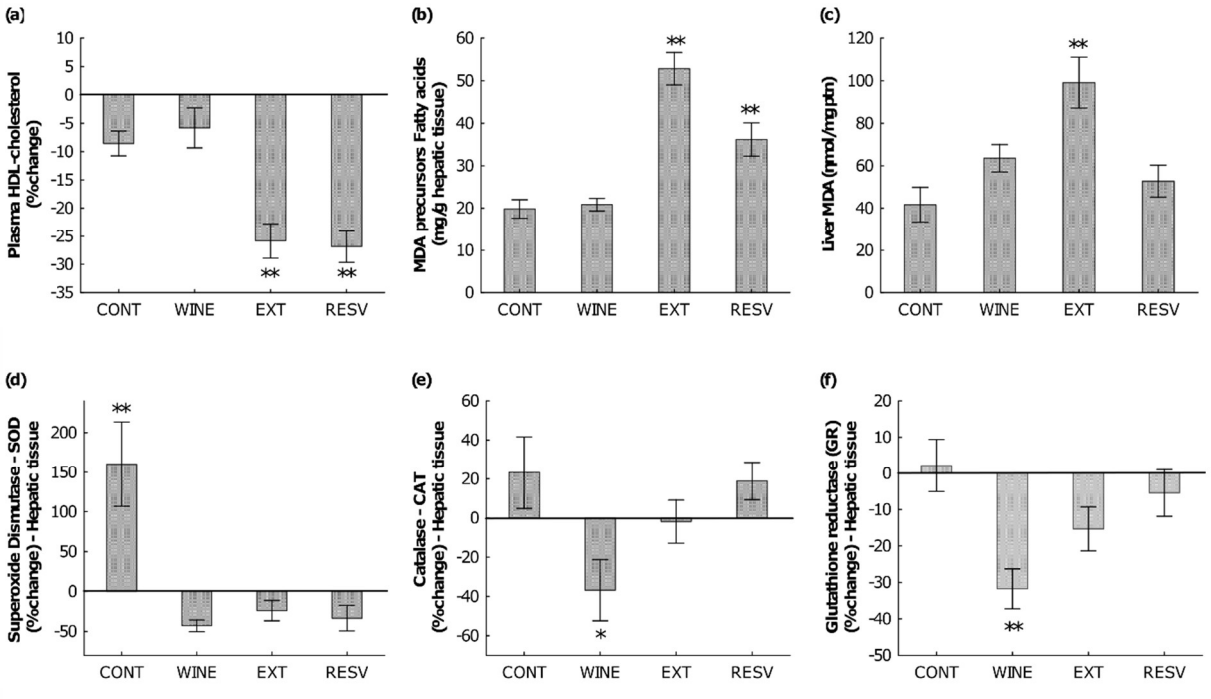

Fig. 5

Biomarkers of lipidemia and oxidative stress measured in the plasma and liver of the animals submitted to the REGRESSION protocol. Bars represent the % of change related to the Baseline group. (**a**) Plasma HDL-cholesterol; (**b**) Fatty acids precursors of malonaldehyde in the liver; (**c**) Liver malonaldehyde; (**d**) Superoxide dismutase activity in liver; (**e**) Catalase activity in liver; (**f**) Glutathione Reductase activity in liver. Data are presented as mean ± SD (n= 10/treatment). One-way ANOVA followed by Tukey test was applied to compare the four treatments; *$p<0.05$ and **$p<0.001$. WINE: group supplemented with 0.64 mL of wine/day; EXT: group supplemented with 0.73 mL of dealcoholized wine/day; RESV: group supplemented with 0.83 mg of *trans*-resveratrol/day; and CONT: group not supplemented.

Figure 6: Atherosclerotic lesion development of the REGRESSION protocol.

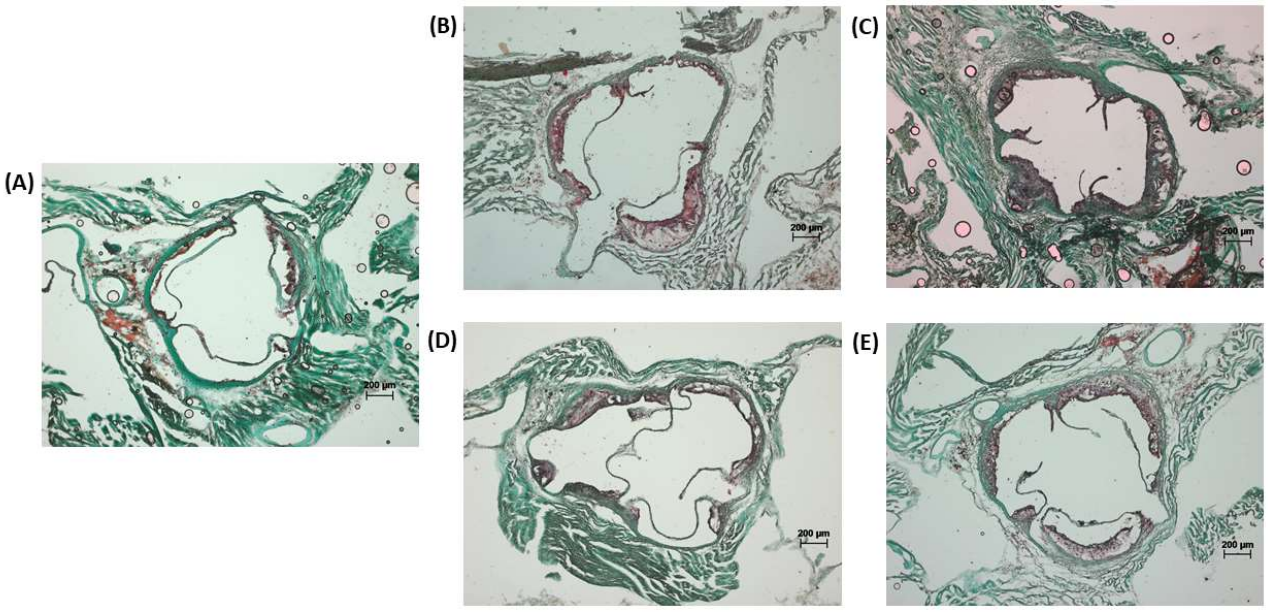

Fig. 6

Effect of the REGRESSION protocol on the atherosclerotic lesion development at the aortic sinus of LDLr $^{(-/-)}$ mice. Red color indicates lipid accumulation and green color indicates collagen fibrils. (**A**) WINE: group supplemented with 0.64 mL of wine/day. (**B**) EXT: group supplemented with 0.73 mL of dealcoholized wine/day. (**C**) RESV: group supplemented with 0.83 mg of *trans*-resveratrol/day. (**D**) CONT: group not supplemented. 10 x magnification images. Scale bar represents 200 µm.

European Journal of Nutrition

Comparison between red wine and isolated *trans*-resveratrol on the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice

Livia N. Chassot, Bianca Scolaro, Gabriela G. Rochel, Bruno Cogliati, Marcela F. Cavalcanti, Dulcineia S.P. Abdalla, Inar A. Castro*

**Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Av. Lineu Prestes, 580, B14, 05508-900, Sao Paulo, Brazil. Tel.: +55 (11) 3091-1152*
E-mail address: inar@usp.br; www.ladaf.com.br

2.10. SUPPLEMENTARY FIGURES

Figure S1: Phenolic compounds in red wine.

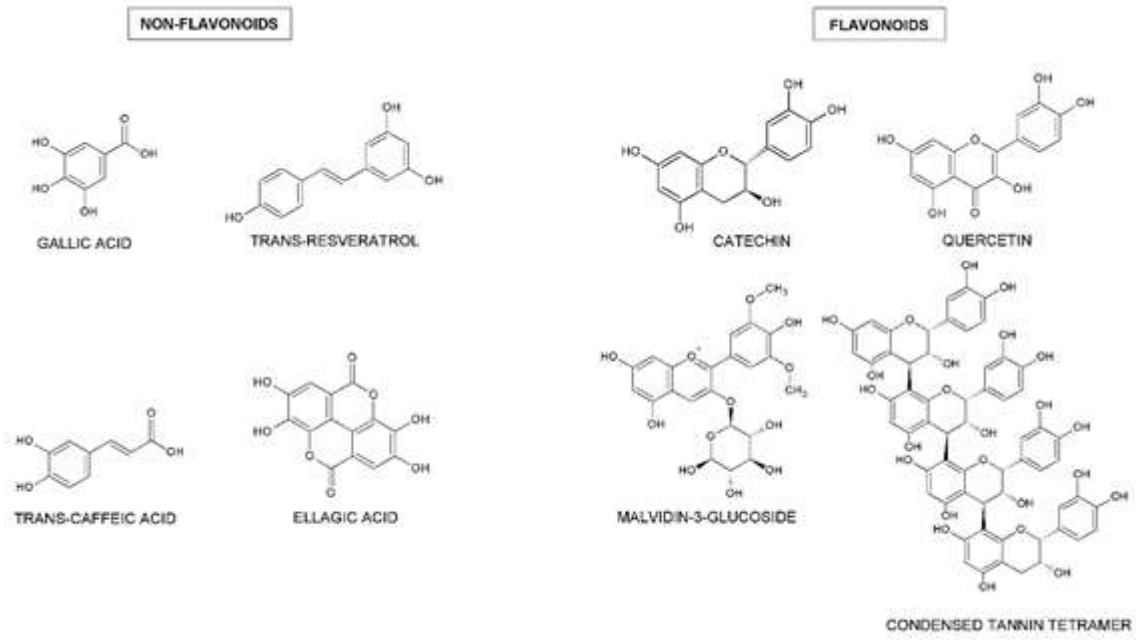

Figure S1: Major phenolic compounds with high antioxidant activity found in red wine (Adapted from Heim et al., 2002).

Heim KE, Tagliaferro AR, Bobilya DJ (2002) Flavonoid antioxidants: chemistry, metabolism and structure-activity relationships. J Nutr Biochem 13:572–584

Figure S2: Liver histology of the PREVENTION protocol.

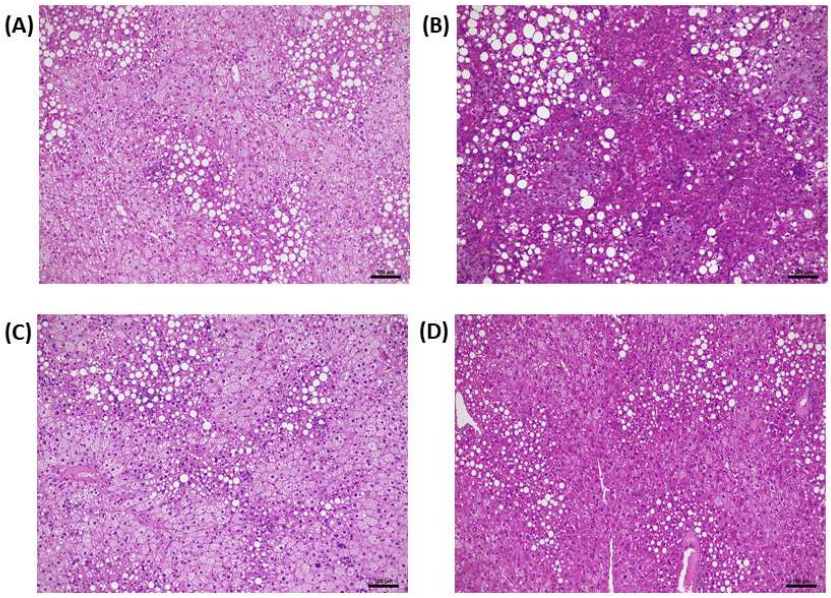

Figure S2: Effect of the PREVENTION protocol on the liver histology of LDLr $^{(-/-)}$ mice. Liver tissue stained with hematoxylin-eosin. (**A**) WINE: group supplemented with 0.69 mL of wine/day; (**B**) EXT: group supplemented with 0.81 mL of dealcoholized wine/day; (**C**) RESV: group supplemented with 0.82 mg of *trans*-resveratrol/day; (**D**) CONT: group not supplemented. 100 x magnification images. Scale bar represents 100 µm.

Figure S3: Liver histology of the REGRESSION protocol.

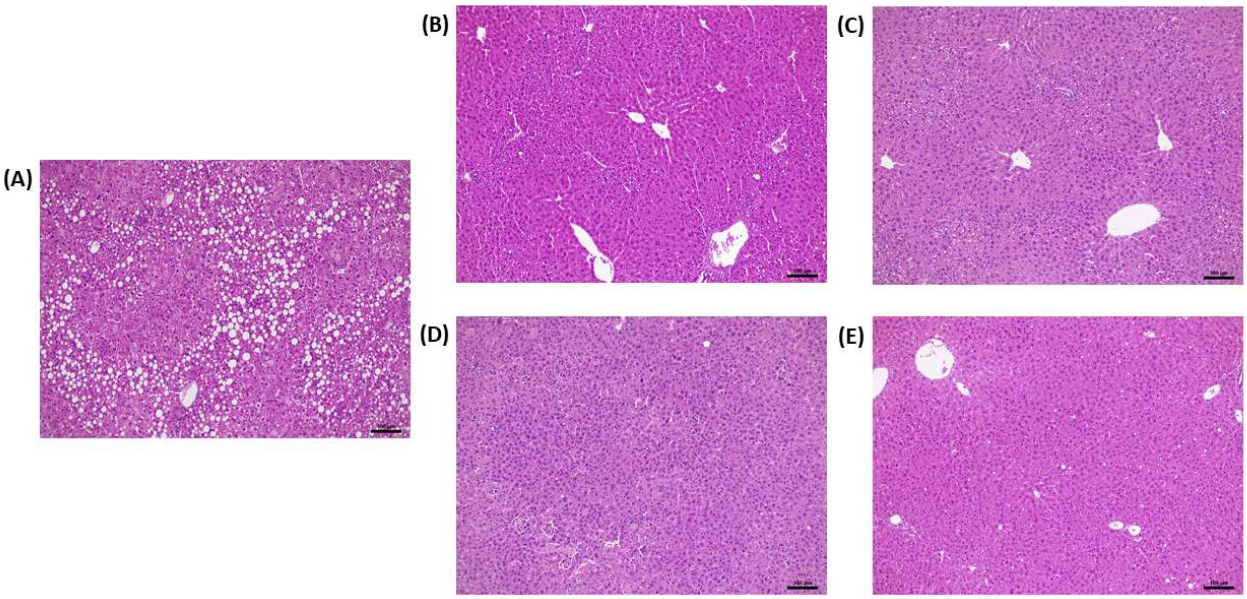

Figure S3: Effect of the REGRESSION protocol on the liver histology of LDLr $^{(-/-)}$ mice. Liver tissue stained with hematoxylin-eosin. (**A**) Baseline (8 weeks); (**B**) WINE: group supplemented with 0.64 mL of wine/day; (**C**) EXT: group supplemented with 0.73 mL of non-alcoholic wine/day; (**D**) RESV: group supplemented with 0.83 mg of *trans*-resveratrol/day; (**E**) CONT: group not supplemented. 100 x magnification images. Scale bar represents 100 µm.

European Journal of Nutrition

Comparison between red wine and isolated *trans*-resveratrol on the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice

Livia N. Chassot, Bianca Scolaro, Gabriela G. Rochel, Bruno Cogliati, Marcela F. Cavalcanti, Dulcineia S.P. Abdalla, Inar A. Castro*

**Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Av. Lineu Prestes, 580, B14, 05508-900, Sao Paulo, Brazil. Tel.: +55 (11) 3091-1152*
E-mail address: inar@usp.br; www.ladaf.com.br

2.11. SUPPLEMENTARY METHODS

Dealcoholized wine preparation.

The wine content of the four bottles was subjected to vacuum filtration for the removal of the water and ethanol at 40°C and 60 rpm using a rotavapor (Buchi Rotavapor R-215, Buchi Brasil Ltda., Valinhos, Brazil) as described by Soulat et al. (2006). The samples were evaluated for the phenolic composition before and after this procedure.

Antioxidant activity of the samples.

The ORAC method was performed according to Huang et al. (2002), with slight modifications. A volume of 25 µL of wine or dealcoholized wine (diluted 900 times in 75 mmol/L phosphate buffer, pH 7.1), or *trans*-resveratrol capsules content (1 µM in 75 mmol/L phosphate buffer, pH 7.1) was transferred to the microplate wells, filling the wells around the microplate with Milli-Q water. Then, 75 µL of a fluorescein solution (40 nmol/L in phosphate buffer) were added in each well. After an incubation at 37°C for 30 min, 25 µL of AAPH (153 nmol/L in phosphate buffer) were added in each well. Immediately, the fluorescence was measured every min for 1h in a microplate reader Sinergy HT (Bio Tek Instruments Inc., Winooski, VT,

USA). The “blank” was prepared with 25 µL of the phosphate buffer. The area under the curve was integrated using the software Gen5 (BioTek Instruments, Inc.). Each sample was analyzed in quadruplicate. The calibration curve was prepared with known Trolox concentrations (6.25-100 µmol/L). The results are expressed as milimolar of Trolox equivalents per litter of wine or wine extract, or milimolar of Trolox equivalents per gram of *trans*-resveratrol capsules content.

Phenolic compounds and anthocyanins profile of the supplementations.

The phenolic compounds analysis was carried out using a UHPLC Agilent 1290 coupled to a mass spectrometer 6460 triple quadrupole (Agilent Technologies, Palo Alto, United States). The method was based on Jaitz et al. (2010), using a Rapid Resolution HT 2.1 x 50 mm, 1.8 µm column (Agilent Technologies, Palo Alto, United States). The method used to determine individual anthocyanins was based on Long (2011), using a superficially porous column: Poroshell 120 StableBond SB-C18 75 mm, 2.7 µm (Agilent Technologies, Palo Alto, United States). Standards were used to quantify the phenolic compounds (catechin, caffeic acid, gallic acid, ferulic acid, rutin, *trans*-resveratrol, myricetin, quercetin and kaempferol) and anthocyanins (cyanidin-3-glucoside, delphinidin-3-glucoside, peonidin-3-glucoside, petunidin-3-glucoside and malvidin-3-glucoside), using a curve of each compound (0.05-10 mg/L of water:methanol 35:65 v/v). Wine and dealcoholized wine were filtered (Iso-Disc™, N-4-4, Nylon, 4 mm x 0.45 µm, Supelco, Bellefonte, PA, United States) and applied in the UHPLC. The capsules content was also analyzed for *trans*-resveratrol amount, being diluted (10 mg/L of water:methanol 35:65 v/v), filtered and injected in the UHPLC. For the data processing, the software Mass Hunter (version B.07.01) was used.

MDA concentration in liver.

The MDA concentration was determined by reverse-phase HPLC, following a previously described protocol (Hong, 2000), with modifications. Liver homogenate samples (0.05 ml) were mixed with 12.5 µl of 0.2% BHT and 6.25 µl of 10 N NaOH. About 20 µl of the derivative

conjugate were injected in the HPLC (Agilent Technologies 1200 Series) in a Phenomenex reverse-phase C18 analytical column (250 mm × 4.6 mm; 5 µm; Phenomenex) with an LC8-D8 pre-column (Phenomenex AJ0-1287) and MDA was fluorometrically quantified at an excitation of 515 nm and emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 60% potassium phosphate buffer (PBS) (10 mM, pH 7.4) + 40% methanol at a flow rate of 1 ml/min. A standard curve was prepared using TEP (0.5-15 µmol). Samples were analysed in triplicate and results are expressed as ηmol MDA/mg protein.

Antioxidant enzymes activity in liver.

SOD activity was determined according to a previously described procedure (Ewing, Janero, 1995). Liver homogenate samples containing 0.024 µg protein/µl (25 µl) were added to the wells of a microplate containing 200 µl of freshly prepared 0.1 mM EDTA, 62 µM Nitrotetrazolium blue chloride (NBT) and 98 µM NADH. The reaction was initiated with the addition of 25 µl of freshly prepared 33 µM phenazine methosulphate (PMS) (pH 7.4) containing 0.1 mM EDTA. Absorbance at 560 nm was monitored over 5 min as an index of NBT reduction. A standard curve was prepared using SOD (Sigma Chemical Co.) (1.5625-25 U/ml). GPx activity was determined according to a previously described procedure (Flohe, Gunzler, 1984; Wheeler, 1990), with modifications. In brief, 30 µl of liver homogenate (2 µg protein/µl) were incubated at 37°C for 5 min with 125 µl of 0.1 M PBS pH 7.4 with 1 mM EDTA, 5 µl of freshly prepared 0.08 M GSH and 5 µl of freshly prepared GR (9.6 U). Next, 30 µl of 1.2 mM NADPH and 5 µl of 0.46% tert-butylhydroquinone (TBHQ) were added in each well. Absorbance at 340 nm was monitored over 4 min at 37°C. A standard curve was prepared using GPx enzyme (Sigma Chemical Co.) (0.5-6 U/mg ptn). GR activity was determined as previously described (Torres, 2011), with modifications. Liver homogenate samples (10 µl) containing 2 µg protein/µl were added to the wells of a microplate with 190 µl of reaction medium containing 2.5 ml of 0.1 M PBS pH 7.4 with 1 mM EDTA, 1.5 ml of 0.005 M EDTA, 1 ml of milli-Q water, 10 mg of GSSG and 2 mg of NADPH. Absorbance at 340 nm and 37°C

was monitored for 6 min. A standard curve was prepared using GR enzyme (Sigma Chemical Co.) (0.003–0.25 U/mg ptn). CAT activity in the liver was determined as previously described (Nabavi, 2012; Bonaventura, Shroeder, Fang, 1972), with modifications. Liver homogenate samples (20 µl) at the concentration of 0.05 µg protein/µl were added to the wells of a UV microplate containing 140 µl of 0.1 M PBS pH 7.4 with 1 mM EDTA. Lastly, 40 µl of a 30 mM hydrogen peroxide solution were added in each well. The reading was monitored for 8 min, at 240 nm and 30°C. The calibration curve was prepared with known CAT concentrations (0.44375-0.44375 U/mg protein). All enzymatic assays were performed using a plate reader (Multi-Detection microplate reader; Synergy – BioTek) integrated with Gen 5 software. Samples were analysed in triplicate.

European Journal of Nutrition

Comparison between red wine and isolated *trans*-resveratrol on the prevention and regression of atherosclerosis in LDLr $^{(-/-)}$ mice

Livia N. Chassot, Bianca Scolaro, Gabriela G. Rochel, Bruno Cogliati, Marcela F. Cavalcanti, Dulcineia S.P. Abdalla, Inar A. Castro*

**Department of Food and Experimental Nutrition, Faculty of Pharmaceutical Sciences, University of Sao Paulo, Av. Lineu Prestes, 580, B14, 05508-900, Sao Paulo, Brazil. Tel.: +55 (11) 3091-1152*
E-mail address: inar@usp.br; www.ladaf.com.br

2.12. SUPPLEMENTARY TABLES

Table 1: Normal and atherogenic diets formulation and chemical composition.

Ingredient (g)	**CONTROL DIET[1]**	**ATHEROGENIC DIET[2]**
Cornstarch	620.692	295.500
Casein (>85% protein)	140.000	151.200
Sucrose	100.000	100.000
Lard	-	87.500
Soybean oil (no additives)	40.000	-
Corn oil[3]	-	200.000
Fiber[4]	50.000	100.000
Cholesterol	-	12.500
Mineral mix (AIN-93M-MX)[5]	35.000	37.800
Vitamin mix (AIN-93-VX)	10.000	10.800
L-Cystine	1.800	1.940
Choline bitartrate (41.1% choline)	2.500	2.700
TBHQ (Tert-butylhydroquinone)[6]	0.008	0.060
Total	**1000.000**	**1000.000**
Chemical Composition (g/100g)		
Moisture (g/100g)	12.32 ± 0.12	8.45 ± 0.10
Ashes	2.64 ± 0.03	3.16 ± 0.06
Protein (Nx6.14)	13.41 ± 0.35	15.49 ± 0.87
Lipids	10.07 ± 0.13	32.27 ± 0.52
Carbohydrate[7]	61.57 ± 0.37	40.64 ± 1.08
Energy (Kcal/100g)	390.55 ± 1.05	514.95 ± 2.76
Fatty acids (%)		
C16:0	17.45 ± 0.55	19.57 ± 0.10
C16:1 n7	0.07 ± 0.04	0.43 ± 0.01
C17:0	0.03 ± 0.03	0.13 ± 0.00
C18:0	10.78 ± 0.35	10.95 ± 0.10
C18:1 n9	19. 65 ± 0.43	31.23 ± 0.55
C18:2 n6	42.08 ± 0.09	28.53 ± 0.54
C18:3 n3	4.07 ± 0.12	0.42 ± 0.01
C20:0	0.20 ± 0.01	0.21 ± 0.01
C22:0	0.19 ± 0.01	0.15 ± 0.00

[1]Based on Reeves et al. (1993).
[2]Based on Nogueira et al. (2016). The diet compensation was made based on the reduction of consumption estimated in a pilot test. Considering a reduction of 8%, it was elevated the proportion of casein, mineral mix, vitamin mix, L-cystine and choline bitartrate in 8%. This way, despite the reduced ingestion of the mice with atherogenic diet, it was guaranteed the supply of the essential macro and micronutrients.
[3]Corn oil was kept at 60ºC for 17 days.
[4]The amount of fiber was doubled to make viable obtaining a powder diet with 30% of oil, being 70% of this oil liquid at room temperature.
[5]Changes were made in the mineral mix in order to promote the oxidative stress: the proportion of NaCl was doubled, the proportion of iron was triplicated, and the proportion of selenium was reduced by half.
[6]Calculated from 200 ppm on the fat content, regardless of the degree of unsaturation.
[7]Obtained by difference.

Table 2: Characterization of the supplements applied in this study.

	Wine	**Dealcoholized wine**	***trans*-resveratrol**
ORAC[1] (mMol/L)	45.40 ± 0.62	44.66 ± 0.15	9.08 ± 0.05
Phenolics (mg/L)			
Gallic acid	8.29 ± 0.34	11.55 ± 0.29	nd
Caffeic acid	1.30 ± 0.01	1.67 ± 0.00	nd
Catechin	1.98 ± 0.01	0.71 ± 0.00	nd
Epicatechin	0.71 ± 0.00	0.81 ± 0.00	nd
Ferulic acid	0.03 ± 0.00	0.04 ± 0.00	nd
Myricetin	3.85 ± 0.01	4.71 ± 0.02	nd
Trans-Resveratrol[2]	**0.11 ± 0.00**	**0.20 ± 0.05**	**5.23 ± 0.01**
Quercetin	4.26 ± 0.00	2.55 ± 0.01	nd
Kaempferol	1.46 ± 0.01	0.16 ± 0.00	nd
Delphinidin-3-glucoside	1.91 ± 0.20	1.80 ± 0.07	nd
Cyanidin-3-glucoside	0.01 ± 0.00	nd	nd
Petunidin-3-glucoside	0.15 ± 0.03	0.13 ± 0.02	nd
Peonidin-3-glucoside	0.10 ± 0.04	0.13 ± 0.02	nd
Malvidin-3-glucoside	1.54 ± 0.12	0.86 ± 0.13	nd

[1]ORAC values expressed as mMol/L for wine and dealcoholized *wine, and as mMol/g for trans-resveratrol capsules.*
[2]trans-Resveratrol expressed as mg/L for wine and dealcoholized *wine, and as mg/L of solution for trans-resveratrol capsules.*

Table 3: Dose of supplementation based on the hydric consumption of the animals from the **PREVENTION** protocol.

Consumption (mL/d)	**Wine**	**Dealcoholized wine**	***trans*-Resveratrol**
Hydric	3.94 ± 0.31	4.63 ± 0.30	3.94 ± 0.46
Wine/dealcoholized wine	0.69 ± 0.05	0.81 ± 0.04	-
Phenolics (µg/d)[1]			
Gallic acid	5.72	9.35	-
Caffeic acid	0.90	1.35	-
Catechin	1.37	0.57	-
Epicatechin	0.49	0.66	-
Ferulic acid	0.02	0.03	-
Myricetin	2.66	3.81	-
***trans*-Resveratrol**	**0.70**	**0.16**	**820.00**
Quercetin	2.94	2.06	-
Kaempferol	1.00	0.13	-
Delphinidin-3-glucoside	1.32	1.46	-
Cyanidin-3-glucoside	-	-	-
Petunidin-3-glucoside	0.10	0.10	-
Peonidin-3-glucoside	0.07	0.10	-
Malvidin-3-glucoside	1.06	0.67	-
Total Phenolics (µg)	18.35	20.45	820.00

[1]Mean of 16 weeks.

Table 4: Dose of supplementation based on the hydric consumption of the animals from the **REGRESSION** protocol.

Consumption (mL/d)	**Wine**	**Dealcoholized wine**	***trans*-Resveratrol**
Hydric	3.63 ± 0.42	4.15 ± 0.59	3.96 ± 0.37
Wine/dealcoholized wine	0.64 ± 0.06	0.73 ± 0.09	
Phenolics (µg/d)[1]			
Gallic acid	5.30	8.43	-
Caffeic acid	0.83	1.22	-
Catechin	1.27	0.52	-
Epicatechin	0.45	0.60	-
Ferulic acid	0.02	0.03	-
Myricetin	2.46	3.44	-
***trans*-Resveratrol**	**0.07**	**0.15**	**830.00**
Quercetin	2.73	1.86	-
Kaempferol	0.93	0.12	-
Delphinidin-3-glucoside	1.22	1.31	-
Cyanidin-3-glucoside	-	-	-
Petunidin-3-glucoside	0.10	0.09	-
Peonidin-3-glucoside	0.06	0.09	-
Malvidin-3-glucoside	0.98	0.63	-
Total Phenolics (µg)	16.42	18.49	830.00

[1]Mean of 8 weeks.

3. FINAL CONCLUSIONS

This study represents an important contribution in “Food Science” research, because it “breaks the myth” that red wine chronic and moderate consumption, solely, is enough for preventing atherosclerosis, or even acting as a co-therapy for a person that has already been through a cardiovascular event. Few health improvements will happen if there is not a complete change in health style, and for that, the Mediterranean diet concept has already proven its value. More than simply red wine, it includes lots of fresh vegetables, olive oil, fish, grains and herbs for instance.

Our study also shows the importance in conducting trials with an endpoint since, far beyond the improvement in the classical biomarkers of atherosclerosis, for example, the fatty streaks in the aortas define whether a stroke will happen in the future, or not. Therapeutics as red wine or isolated *trans*-resveratrol must be studied in their broader context.

4. ATTACHMENTS

4.1. CALCULATIONS

It was established the hydric volume of 120 mL for each drinking fountain (5 animals/drinking fountain), to be replaced every two or three days.

Wine volume
Human (70 Kg): hydric ingestion of 2 L (300 mL wine + 1700 mL water)
Mice (25 g): medium hydric ingestion of 4mL/animal/day (estimated value)

300 mL wine – 2000 mL hydric ingestion
x mL wine – 4 mL hydric ingestion
➔ **x** = 0.6 mL wine/animal/day

For a drinking fountain with a total volume proposed in 120 mL:
4 mL hydric ingestion – 0.6 mL wine
120 mL (drinking fountain) – **y** mL wine
➔ **y** = 18 mL wine/drinking fountain

Drinking fountain (120 mL): 18 mL wine + 102 mL water

Amount of trans-resveratrol
It was proposed the dosage of 200 mg *trans*-resveratrol/Kg diet/day (Chen et al., 2012).
Mice: medium food ingestion of 3 g/animal/day

200 mg *trans*-resveratrol – 1000 g diet
x mg *trans*-resveratrol – 3 g diet
➔ **x** = 0.6 mg *trans*-resveratrol/animal/day

0.6 mg *trans*-resveratrol – 4 mL hydric ingestion
y mg *trans*-resveratrol – 120 mL (drinking fountain)
➔ **y** = 18 mg *trans*-resveratrol/drinking fountain

Drinking fountain (120 mL): 18 mg *trans*-resveratrol + 120 mL water*

***Observation**: the animal protocol with resveratrol initiated admitting a hydric ingestion of 3 mL/animal/day (previously estimated value).

0.6 mg resveratrol – 3 mL hydric ingestion
y mg resveratrol – 120 mL (drinking fountain)
➔ **y** = 24 mg resveratrol/drinking fountain

And before we analyzed the resveratrol capsules content. This way, we oriented ourselves by the amount of resveratrol proposed in the product's label (Rite Aid, Camp Hill, PA/USA), of 250 mg resveratrol/capsule. As each capsule weigh around 500 mg, it was admitted a purity of 50%.
This way, 48 mg of the capsule content were put in the drinking fountain, to achieve the value of 24 mg resveratrol/drinking fountain.
After the capsules analysis, we identified a purity of 52.3%.
So, the effective supplementation was of 25.1 mg resveratrol/drinking fountain (described below).

48 mg capsule content – 100%
z mg resveratrol – 52.3%
➔ **z** = 25.1 mg resveratrol/drinking fountain

Non-alcoholic wine volume
Non-alcoholic wine obtained after vacuum filtration of a volume of 400 mL of wine: 17 g of non-alcoholic wine.

400 mL wine – 17 mL non-alcoholic wine
100 mL wine – **x** non-alcoholic wine
➔ **x** = 4.25% (yield)

400 mL wine – 17 mL non-alcoholic wine
18 mL wine – y mL non-alcoholic wine
➔ y = 0.765 mL non-alcoholic wine/drinking fountain

Drinking fountain (120 mL): 0.765 mL non-alcoholic wine + 119.2 mL water

4.2. ETHICS COMMITTEE APPROVAL

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF **95.2015-P511**

CERTIFICADO

A Comissão de Ética no Uso de Animais, da Faculdade de Ciências Farmacêuticas, da Universidade de São Paulo, **CERTIFICA** que o Projeto de Pesquisa "**Efeito do vinho tinto na prevenção e na reversão da aterosclerose em modelo animal**" (Protocolo CEUA/FCF/511), de responsabilidade do(a) pesquisador(a) **Livia Nedel Chassot**, sob orientação do(a) **Profa. Dra. Inar Alves de Castro**, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi APROVADO em reunião de **04 de dezembro de 2015**. Conforme a legislação vigente, deverá ser apresentado, no encerramento deste Projeto de Pesquisa, o respectivo **relatório final**.

São Paulo, 15 de abril de 2016.

Prof. Dr. Joilson de Oliveira Martins
Coordenador da CEUA/FCF/USP

Av. Prof. Lineu Prestes, 580, Bloco 13 A, Cidade Universitária, CEP 05508-900, São Paulo, SP
Telefone: (11) 3091 3622 - e-mail: ceuafcf@usp.br

4.3. BIOSECURITY COMMITTEE APPROVAL

UNVERSIDADE DE SÃO PAULO
FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão Interna de Biossegurança

Of.CIBio/0072016/FCF

São Paulo, 28 de março de 2016

Senhora Professora,

Conforme parecer favorável do relator, informo a Vossa Senhoria que em reunião da Comissão Interna de Biossegurança da Faculdade de Ciências Farmacêuticas realizada no dia 22 de março p.p., o Projeto **"Efeito do consumo de vinho tinto na prevenção e na reversão da aterosclerose em modelo animal"** foi aprovado.

Lembramos que, quando da elaboração do Relatório Anual a ser encaminhado à Comissão Interna de Biossegurança esta Comissão solicitará a V.Sa. comprovante de participação em treinamentos de Biossegurança de sua equipe para manutenção do credenciamento.

Atenciosamente,

Prof. Dr. MARIO HIROYUKI HIRATA
Presidente da CIBio

Ilma. Sra.
Profa. Dra. **INAR ALVES DE CASTRO**
Departamento de Alimentos e Nutrição Experimental da FCF-USP
NESTA

Av. Prof. Lineu Prestes, nº 580, Bloco 13 A - Cidade Universitária - CEP 05508-900 - São Paulo - SP
Fone/Fax: (011) 3091-3678 - e-mail: atadfcf.usp.br