

**UNIVERSITY OF DE SÃO PAULO**

Faculty of Pharmaceutical Sciences

Graduate Program in Food Science

Area of Experimental Nutrition

**Effect of *trans*-resveratrol on oxidative stress biomarkers associated with atherosclerosis**

Tamires Miranda Santana

São Paulo

2022

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Tamires Miranda Santana

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**Advisor:** Inar Castro Erger

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## RESUMO

Santana, T.M. **Efeito do *trans*-resveratrol em biomarcadores de estresse oxidativo associados à aterosclerose.** 2022. 73f. Dissertação (Mestrado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, São Paulo, 2022.

Evidências crescentes indicam que o estresse oxidativo desempenha um papel importante na fisiopatologia de muitas doenças cardiovasculares, incluindo a aterosclerose. Nesse contexto, o uso de compostos bioativos com ação antioxidante pode trazer benefícios à saúde, principalmente na prevenção e controle de eventos fisiopatológicos. Estudos sugerem que o polifenol *trans*-resveratrol pode reduzir o estresse oxidativo atuando na via do fator nuclear eritroide 2 relacionado ao fator 2 (Nrf2) e que esse efeito estaria associado a dosagem. Assim, o presente estudo teve como objetivo investigar o efeito de diferentes doses de *trans*-resveratrol sobre biomarcadores relacionados à aterosclerose e estresse oxidativo. Na primeira etapa, 27 ensaios clínicos randomizados, que avaliaram o efeito do *trans*-resveratrol em biomarcadores relacionados à aterosclerose, foram classificados de acordo com suas características de protocolo e perfil de cada resultado. Dados bioquímicos de 12 biomarcadores foram selecionados para calcular a variação líquida (%). Usando análise multivariada, os ensaios foram distribuídos em 3 *Clusters*. Os estudos que compuseram os *Clusters* II e III foram mais eficazes na melhora da pressão arterial e na redução da dislipidemia, respectivamente. Esses estudos foram caracterizados por um tempo de intervenção mais longo (> 2 meses) com doses de cerca de 200-500 mg/dia. Esses resultados mostraram que os efeitos do *trans*-resveratrol estão relacionados principalmente à dosagem e ao tempo de intervenção. Com base nesses resultados, duas doses foram selecionadas para aplicar em um protocolo experimental para investigar o efeito do *trans*-resveratrol em biomarcadores de estresse oxidativo hepático mediados pela via do Nrf2. Camundongos LDLr<sup>(-/-)</sup> foram alimentados por 8 semanas com dieta padrão, seguidos por mais de 24 semanas com *Western diet*, ambos contendo *trans*-resveratrol nas doses de 250 mg/kg de dieta/dia (baixa dose de resveratrol, LRD) ou 400 mg/kg de dieta/dia (alta dose de resveratrol, HRD). Um grupo controle (CONT) foi mantido sem suplementação. Em geral, ambas as doses de *trans*-resveratrol não afetaram o peso corporal e o perfil lipídico dos animais. Apenas o grupo LRD apresentou níveis reduzidos de dois importantes biomarcadores de estresse oxidativo no fígado (razão GSH/GSSG e malonaldeído), além da redução da expressão de fator nuclear kappa B (NF-κB). No entanto, ao contrário da nossa hipótese, ambas as doses reduziram a expressão de Nrf2 no fígado em comparação com o grupo CONT. Em relação às citocinas inflamatórias, não foram observadas alterações nos níveis de TNF-α e IL-10. Além disso, ambas as doses aumentaram o nível da citocina pró-inflamatória IL-6. Em conjunto, nossos resultados sugerem que a suplementação de *trans*-resveratrol em doses menores de 500 mg/dia podem contribuir para a redução de biomarcadores relacionados à aterosclerose e ao estresse oxidativo.

**Palavras-chave:** estresse oxidativo, biomarcadores, *trans*-resveratrol, Nrf2

## ABSTRACT

Santana, T.M. **Effect of *trans*-resveratrol on oxidative stress biomarkers associated with atherosclerosis. 2022.** 73f. Dissertation (Master) – Faculty of Pharmaceutical Sciences, University of São Paulo, São Paulo, 2022.

Growing evidence indicates that oxidative stress plays an important role in the pathophysiology of many cardiovascular diseases, including atherosclerosis. In this context, the use of bioactive compounds with antioxidant action can bring health benefits, especially in the prevention and control of pathophysiological events. Studies suggest that the polyphenol *trans*-resveratrol can reduce oxidative stress by acting on the nuclear factor erythroid-2-related factor 2 (Nrf2) and this effect would be associated with dosage. Thus, the present study aimed to investigate the effect of different doses of *trans*-resveratrol on biomarkers related to atherosclerosis and oxidative stress. In the first step, 27 randomized clinical trials, which evaluated the effect of *trans*-resveratrol on atherosclerosis-related biomarkers, were classified according to their protocol characteristics and profile of each outcome. Biochemical data from 12 biomarkers were selected to calculate the net change (%). Using multivariate analysis, the trials were distributed into 3 clusters. The studies that composed Clusters II and III were more effective in improving blood pressure and reducing dyslipidemia, respectively. These studies were characterized by a longer intervention time (> 2 months) with doses of around 200-500 mg/day. These results showed that the effects of *trans*-resveratrol are mainly related to dosage and intervention time. Based on these results, two doses were selected to apply in an experimental protocol to investigate the effect of *trans*-resveratrol on hepatic oxidative stress biomarkers mediated by Nrf2 pathway. LDLr<sup>(-/-)</sup> mice were fed for 8 weeks on a standard diet, followed by over 24 weeks on a Western diet, both containing *trans*-resveratrol at doses of 250 mg/kg diet/day (low dose resveratrol, LRD) or 400 mg/kg diet/day (high dose resveratrol, HRD). A control group (CONT) was maintained without supplementation. In general, both doses of *trans*-resveratrol did not affect the body weight and lipid profile of the animals. Only the LRD group showed reduced levels of two important biomarkers of oxidative stress in the liver (GSH/GSSG ratio and malonaldehyde), besides to reduced expression of factor nuclear kappa B (NF-κB). However, contrary to our hypothesis, both doses reduced Nrf2 expression in the liver compared to the CONT group. Regarding inflammatory cytokines, no changes were observed in the levels of TNF-α and IL-10. Furthermore, both doses increased the level of the pro-inflammatory cytokine IL-6. Taken together, our results suggest that *trans*-resveratrol supplementation at doses lower than 500 mg/day may contribute to the reduction of biomarkers related to atherosclerosis and oxidative stress.

**Keywords:** oxidative stress, biomarkers, *trans*-resveratrol, Nrf2, mice

## ABBREVIATIONS

•OH: hydroxyl radical	Maf: musculoaponeurotic fibrosarcoma
ARE: antioxidant-responsive element	MAPK: mitogen-activated protein kinase
bZIP: basic region leucine zipper	MCP-1: monocyte chemoattractant protein-1
CAT: catalase	MDA: malondialdehyde
CD36: receptor CD36	MHC: major histocompatibility complex
CNC: Cap-n-Collar	MPO: myeloperoxidase
CONT: control group	Neh: Nrf2-ECH homology
COX: cyclooxygenase	NF-κB: nuclear factor kappa B
Cul3: Cullin 3-based ubiquitin E3 ligase	NO: nitric oxide
CVD: cardiovascular disease	NOX: NADPH oxidase
CYP7A1: cholesterol 7 alpha-hydroxylase	NQO1: NAD(P)H-quinone-oxidoreductase
GPx: glutathione peroxidase	Nrf2: nuclear factor erythroid 2-related factor 2
GPx: glutathione peroxidase	O <sub>2</sub> <sup>•-</sup> : superoxide anion
GSH: glutathione	ONOO <sup>-</sup> : peroxide nitrite
H <sub>2</sub> O <sub>2</sub> : hydrogen peroxide	oxLDL: oxidized low-density lipoprotein
HDL: high density lipoprotein	ROS: reactive oxygen species
HMG CoA: hepatic 3-hydroxy 3-methylglutaryl coenzyme A	SOD: superoxide dismutase
HO-1: heme-oxygenase 1	Th: T helper cells
HRD: high resveratrol dose group	TLR4: toll-like receptor 4 (TLR4)
ICAM-1: intracellular adhesion molecule	Treg: T regulatory cells
IKK: inhibitor of nuclear factor-κB kinase	VCAM: vascular cell adhesion molecule-1
IL: interleukin	
IκB: inhibitor of nuclear factor-κB	
Keap1: Kelch-like ECH-associated protein 1	
LDL: low density lipoprotein	
LDLr(-/-): low density lipoprotein receptor knockout	
LOX-1: lectin-like oxLDL receptor-1	
LRD: low trans-resveratrol dose group	



## LIST OF TABLES

### Chapter 1

<b>Table 1.</b> General characteristics presented in the selected studies.....	33
<b>Table 2.</b> Change (%) and Net Change (%) of biomarkers selected to the multivariate analysis.....	34
<b>Table 3.</b> Factor coordinates of the variables (Biomakers), based on correlations until factor 6.....	34
<b>Table 4.</b> Protocol parameters and net changes of biomarkers according to the Clusters.....	36

## LIST OF FIGURES

### Introduction

<b>Figure 1.</b> Beginning of atherosclerosis and formation of fatty streaks.....	13
<b>Figure 2.</b> Features of advanced atherosclerotic plaque.....	13
<b>Figure 3.</b> Sources of free radicals and their primary targets.....	14
<b>Figure 4:</b> Redox signaling on NF- $\kappa$ B and Nrf2 pathway.....	16
<b>Figure 5.</b> Chemical structure of isoforms of resveratrol.....	17
<b>Figure 6.</b> Metabolism of <i>trans</i> -resveratrol.....	18
<b>Figure 7.</b> The nuclear Nrf2 signaling pathway.....	20

### Chapter 1

<b>Figure 1.</b> Net changes (%) observed in the assays.....	34
<b>Figure 2.</b> Projection of the assays on the factor-plane (1 x 2).....	35
<b>Figure 3.</b> Lipoproteins profile (A) and arterial pressure (B) net change values according to the groups.....	36

### Chapter 2

<b>Figure 1.</b> Effects of <i>trans</i> -resveratrol in food consumption and body weight during the experimental period.....	57
<b>Figure 2.</b> Effects of <i>trans</i> -resveratrol in lipid parameters.....	57
<b>Figure 3.</b> <i>Trans</i> -resveratrol effects in oxidative stress in the liver.....	58
<b>Figure 4.</b> Effect of <i>trans</i> -resveratrol on the mRNA expression levels in the liver.....	59
<b>Figure 5.</b> Effects of <i>trans</i> -resveratrol in antioxidant enzymes activity in the liver....	60
<b>Figure 6.</b> Effects of <i>trans</i> -resveratrol in NF- $\kappa$ B expression and pro-cytokines levels in the liver.....	61
<b>Figure 7.</b> Heatmap of the biomarkers according to the three experimental groups (CONT: darkblue, HDR: blue and LDR: lightblue), obtained by Cluster analysis of the standardized values.....	62

## SUMMARY

<b>Introduction</b> .....	11
<b>Review of literature</b> .....	12
Major physiological mechanisms involved in the atherosclerosis .....	12
ROS generation and oxidative stress.....	14
<i>Trans</i> -resveratrol: chemistry, absorption and metabolism .....	17
Activation of the Nfr2 pathway in oxidative stress .....	19
Resveratrol as a nutritional and nutraceutical strategy against oxidative stress and Nrf2 modulation .....	20
<b>Hypotesis</b> .....	24
<b>Objective</b> .....	24
<b>Description of chapters</b> .....	24
<b>References</b> .....	25
<b>Chapter I: Effect of resveratrol supplementation on biomarkers associated with atherosclerosis in humans</b> .....	31
Introduction .....	32
Methods .....	33
Results .....	33
Discussion .....	35
Conclusion .....	37
References .....	37
<b>Chapter II: Effect of two doses of <i>trans</i>-resveratrol on oxidative stress biomarkers associated with atherosclerosis in LDLr knockout mice</b> .....	39
Introduction .....	42
Material and Methods .....	44
Results .....	48
Discussion .....	49
Conclusion .....	53
References .....	54
Supplementary Material .....	63
<b>Final Considerations</b> .....	65
<b>Future perspectives</b> .....	65
<b>Attachments</b> .....	66

## 1. Introduction

Growing evidence indicates that oxidative stress plays an important role in the pathophysiology of many cardiovascular diseases (CVD) including atherosclerosis, hypertension and heart failure (Burtenshaw et al., 2019). Reactive oxygen species (ROS) are important for physiology as functional signaling entities, whereas excessive ROS concentration results in damage to macromolecules and contributes to the disease progression (Sies and Jones, 2020). Considering the role of oxidative stress in the pathogenesis of atherosclerosis, the use of bioactive compounds, as part of food matrices or taken as supplements, could contribute to preventing CVD through mechanisms associated with reduced levels of oxidative stress, lipid peroxidation and inflammation (Moss et al., 2018).

*Trans*-resveratrol (3,5,4'-trihydroxystylbene) is a polyphenol that has been associated with a decreased risk of CVD, mainly due to the theory of the “French Paradox” and to studies reported by the “Sinclair group”, that associated *trans*-resveratrol with longevity (Baur et al., 2006; Renaud and de Lorgeril, 1992). Currently, *trans*-resveratrol is sold as a nutritional supplement with a wide range of pharmacological effects. It has been consumed in several countries by people who present different levels of cardiac risk, without any medical prescription (Salehi et al., 2018). Numerous studies indicate that *trans*-resveratrol protects against oxidative stress not solely because of its direct antioxidant capacity, but also by up-regulating other endogenous antioxidant pathways, such as the nuclear factor erythroid 2-related factor 2 (Nrf2) (Xia et al., 2017).

Although is widely described the antioxidant effects of *trans*-resveratrol, some studies have reported side effects or shown controversial results and this fact can be due to several factors including dosage, intervention time and characteristics of the studied population (Dyck et al., 2019; Shaito et al., 2020). In this regard, different doses have already been evaluated to identify which would be the ideal dose to achieve some of the beneficial effects reported for *trans*-resveratrol, such as anti-obesity (Macarulla et al., 2009), chemoprevention (Stocco et al., 2012) and neurologic functions (Bakheet et al., 2017). However, little is known about the effects of different *trans*-resveratrol doses in the Nrf2 pathway and end-points associated with cardiovascular events

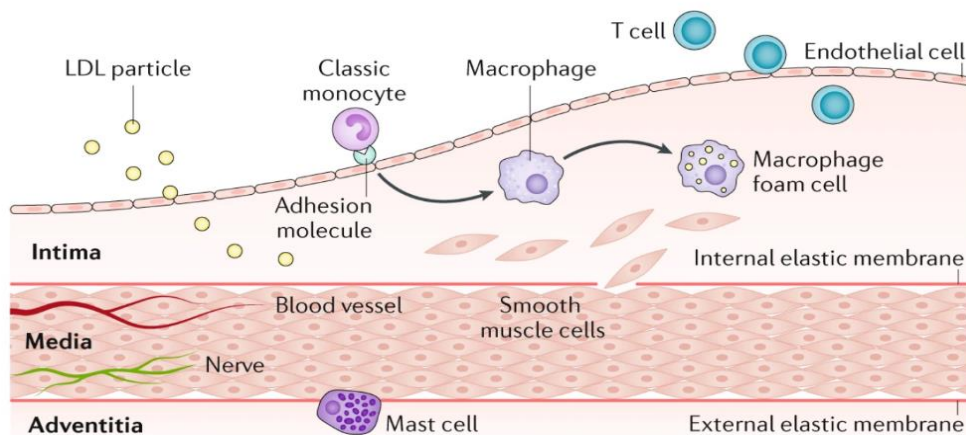
(Farkhondeh et al., 2020). Thus, it is important to understand the effects of different doses of *trans*-resveratrol, in order to optimize the *trans*-resveratrol efficiency towards biomarkers related to atherosclerosis.

## 2. Review of literature

### 2.1. Major physiological mechanisms involved in the atherosclerosis

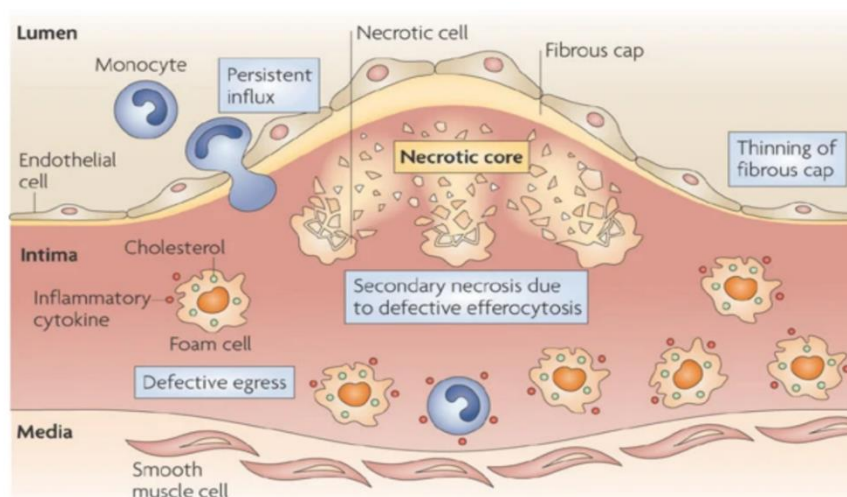
Atherosclerosis is a chronic inflammatory disease initiated by an endothelial dysfunction that occurs in sites where disturbed laminar flow is present (Soehnlein and Libby, 2021). Under normal conditions, the endothelium is primarily responsible for maintaining vascular homeostasis, mainly by the secretion of nitric oxide (NO) (Forstermann et al., 2017). The endothelial dysfunction increases the infiltration and retention of lipoproteins in the arterial intima, which is a key initiation event that triggers an inflammatory response that contributes to the progression and outcome of the disease (Ross, 1999; Soehnlein and Libby, 2021). Once in the subendothelial region, these lipoproteins may undergo oxidative modifications forming, for example, oxidized low-density lipoprotein (oxLDL) (Back et al., 2019; Witztum, 1994). The presence of oxLDL facilitates the recruitment and migration of immune cells via increased expression of adhesion molecules, such as vascular adhesion molecule (VCAM-1) and the intracellular adhesion molecule (ICAM-1) (Di Pietro et al., 2016; Libby et al., 2019). Innate and adapted immune cells promote an increase in proinflammatory cytokines and chemokines, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$  and IL-6, (Borén et al., 2020). Recruited monocytes are differentiated into macrophages, which through scavenger receptors, such as CD36, SR-A1, and lectin-like oxLDL receptor-1 (LOX-1) phagocyte oxLDL and form the foam cells, a hallmark of atherosclerosis (**Figure 1**) (Chistiakov et al., 2017; Libby, 2021; Marchio et al., 2019). Antigenic peptides from oxLDL presented bound to major histocompatibility complex (MHC) class II molecules of dendritic cells are recognized by Naive T cells (Saigusa et al., 2020). Naive T cells are activated and can differentiate into T helper (Th) or T regulatory (Treg) cell subtypes, which have a distinct role in atherosclerosis (Borén et al., 2020; Saigusa et al., 2020). In general, Th1 have been shown to promote atherosclerosis by secretion of TNF- $\alpha$  and IFN- $\gamma$ , while Treg cells promote inflammatory resolution of atherosclerosis progression via the production of IL-10 and TGF $\beta$  (Saigusa et al., 2020). The effect of others Th

subsets like Th2, Th9, and Th17 cells on the development of atherosclerosis remains controversial (Kong et al., 2022).



**Figure 1:** Beginning of atherosclerosis and formation of fatty streaks. Adapted from Libby

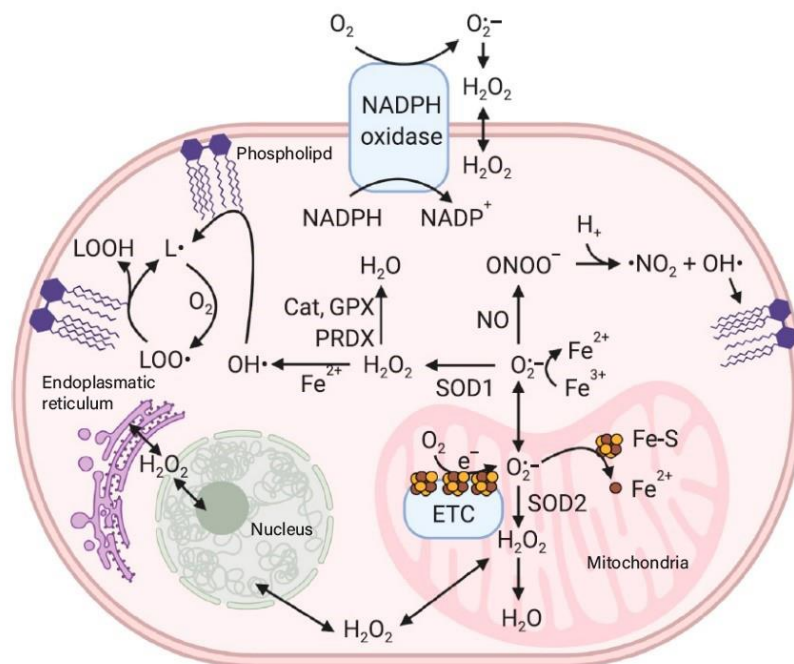
The progression of the atherosclerotic lesion and the chronic inflammation stimulates the migration of vascular smooth muscle cells (VSMC) in the tunica media to the intima (Tabas et al., 2015). Within the intima, VSMCs undergo metaplasia to become macrophage-like cells and take up oxLDL that contribute to produce foam cells in advanced lesions (Borén et al., 2020; Libby, 2021). At later stages, in the face of persistent inflammatory stimuli and other cytotoxic factors, these cells have their egress impaired and become apoptotic. The dead cells can become secondarily necrotic due the defective efferocytosis, which is a major driver of necrotic core formation (Yurdagul et al., 2018). The necrotic core is the primary feature of atherosclerotic plaque vulnerability, contributing to its rupture and luminal thrombosis, which underlies myocardial infarction and stroke (Back et al., 2019) (**Figure 2**).



**Figure 2:** Features of advanced atherosclerotic plaque. Adapted from Tabas (2009).

## 2.2. ROS generation and oxidative stress

Oxidative stress is defined as imbalance between production and accumulation of reactive species and antioxidant defenses that may result in damage to macromolecules (Sharifi-Rad et al., 2020; Sies, 1997). Reactive oxygen species (ROS) is an umbrella term to describes intermediary metabolites naturally produced in all cells and organisms (Sies and Jones, 2020). ROS include oxygen-free radicals, such as superoxide anion ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ), and nonradical oxygen derivatives, such as hydrogen peroxide ( $H_2O_2$ ) and peroxide nitrite ( $ONOO^-$ ) (Zhang et al., 2019). The main endogenous sources of ROS are NADPH oxidase (NOX), xanthine oxidase (XO), cyclooxygenase (COX), myeloperoxidase (MPO), enzymes of the mitochondrial electron transport chain and uncoupled endothelial NO synthase (Munzel et al., 2017). The generation of ROS and their redox targets are summarized in **Figure 3**.



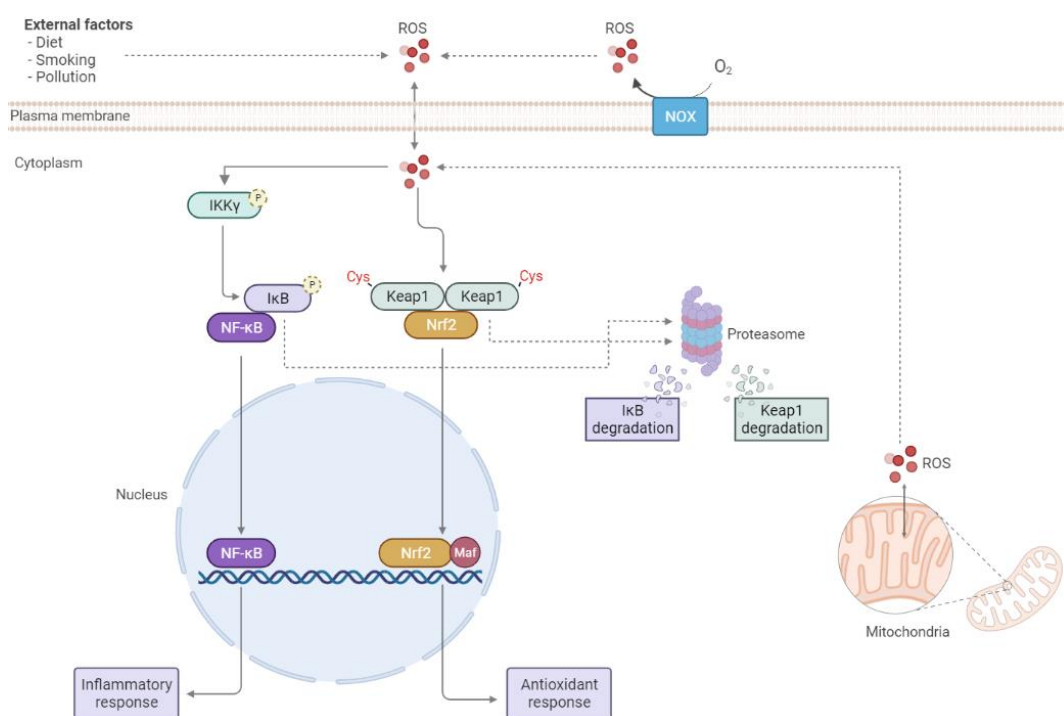
**Figure 3:** Sources of free radicals and their primary targets. Adapted from Harris and DeNicola (2020). Superoxide ( $O_2^{\cdot-}$ ) is primarily produced by the mitochondrial electron transport chain and NADPH oxidase enzymes, it targets iron-sulfur (Fe-S), which reduces ferric iron ( $Fe^{3+}$ ) to ferrous iron ( $Fe^{2+}$ ).  $O_2^{\cdot-}$  is moderately reactive and it is easily dismutation to hydrogen peroxide ( $H_2O_2$ ) by superoxide dismutases (SOD1 and SOD2).  $H_2O_2$  can diffuse across membranes and has effects distal to its site of production, targeting reactive Cys residues. It is detoxified to water by catalase (Cat), glutathione peroxidase (GPX) and peroxiredoxins (PRDX).  $O_2^{\cdot-}$  produces peroxynitrite ( $ONOO^-$ ) through a reaction with nitric oxide (NO) and  $ONOO^-$  decomposition generates the nitrogen dioxide radical ( $\cdot NO_2$ ). The hydroxyl radical ( $\cdot OH$ ) is formed when  $H_2O_2$  reacts with  $Fe^{2+}$  in the Fenton reaction and by decomposition of  $ONOO^-$ .  $\cdot OH$  reacts with lipids to form lipid radicals ( $L\cdot$ ), which react with oxygen to form lipid peroxide radicals ( $LOO\cdot$ ), which abstracts a hydrogen from another unsaturated lipid molecule to form lipid hydroperoxide (LOOH).

Beyond endogenous production, ROS can be formed as a consequence of external stimuli, like pollution, dietary nutrients, drugs and metabolic conditions (Sies and Jones, 2020). Diet influences these processes by supplying radical-producing substances (e.g., glucose; polyunsaturated fatty acids) (Matsuzawa-Nagata et al., 2008). The continued and increased delivery of these nutrients to mitochondria increases oxygen consumption and production of oxide anion as a consequence of an intensified reduction of the respiratory chain complexes (Matsuzawa-Nagata et al., 2008; Cardoso et al., 2013). Chronic consumption of a Western diet, typically described as high in calories and rich in sugars, trans and saturated fats, has been associated with hyperlipidemia and increased oxidative stress (Aleksandrova et al., 2021). At homeostatic levels, ROS act as signaling molecules, contributing to several cellular functions, such as regulation of vascular tone, cell growth, apoptosis and inflammatory responses (Chen et al., 2018; da Costa et al., 2019), while the non-pathological production of ROS has been associated as the primary cause of a wide range of pathologies, including atherosclerosis, diabetes and cancer (Forman and Zhang, 2021). Excessive levels of ROS lead to damage of cellular components including proteins (especially cysteine residues), lipids (lipid peroxidation), and nucleic acids (DNA damage) (Sies and Jones, 2020).

In this regard, ROS can mediate their biological effects through modification of target proteins, for example, kinases involved in direct or indirect activation of transcription factors (Sies and Jones, 2020). For example, the inhibitor of nuclear factor- $\kappa$ B kinase (IKK) is a serine/threonine kinase, primarily responsible for phosphorylate the inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B), which maintain and sequester factor nuclear kappa-B (NF- $\kappa$ B) in its latent form in the cytoplasm (Pantano et al., 2006). Redox modulation of IKK lead to the phosphorylation of the I $\kappa$ B, which results in its proteasomal degradation and translocation of NF- $\kappa$ B to the nucleus (Morgan and Liu, 2011; Sies and Jones, 2020). NF- $\kappa$ B is a central transcription factor in inflammation and immunity, besides, is also involved in regulating cell growth, differentiation, development and apoptosis (Morgan and Liu, 2011). On the other hand, ROS can modify the Kelch-like ECH-associated protein 1 (Keap-1) by oxidation of its cysteine residues, resulting in dissociation of the nuclear factor erythroid-2- related factor 2 (Nrf2), an essential transcription factor for protection



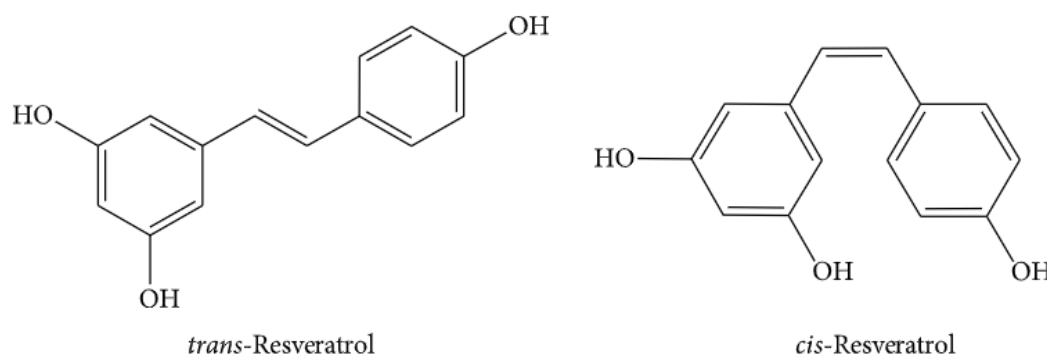
against oxidative stress response and also to represses inflammation (Kobayashi et al., 2016). Nrf2 is the major activator of the antioxidant-responsive element (ARE)-regulated genes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and genes of glutathione (GSH) metabolism (Tonelli et al., 2018; Zhang and Tsao, 2016). Interestingly, evidence suggests that Nrf2 signaling can be achieved also by natural products, such as sulforaphane, curcumin and *trans*-resveratrol, which contribute to increase the endogenous antioxidant defenses (Ooi et al., 2018). The redox signaling on NF- $\kappa$ B and Nrf2 pathway are summarized in the **Figure 4**.



**Figure 4:** Redox signaling on NF- $\kappa$ B and Nrf2 pathway. ROS from endogenous and exogenous sources induces the NF- $\kappa$ B pathway by activation of inhibitor of nuclear factor- $\kappa$ B kinase (IKK), which results in the phosphorylation of inhibitor of nuclear factor- $\kappa$ B (I $\kappa$ B) proteins and consequently their degradation by the proteasome, besides the subsequent liberation of NF- $\kappa$ B. Released NF- $\kappa$ B translocate to the nucleus and promote the transcription of genes involved in the inflammatory response. Redox and electrophile modification of Keap1 cysteine residues blocked it the negative regulation of Nrf2. Instead of being degraded, Nrf2 migrates to the nucleus, where it binds to Maf proteins and becomes transcriptionally active, promoting an antioxidant response. Abbreviation: ROS, reactive oxygen species; Cys, cysteines residues; IKK, inhibitor of nuclear factor- $\kappa$ B kinase; I $\kappa$ B, inhibitor of nuclear factor- $\kappa$ B; NOX, NADPH oxidase; NF- $\kappa$ B, factor nuclear kappa-B; Keap1, Kelch-like ECH-associated protein 1; Nrf2, nuclear factor erythroid-2- related factor 2. Based on Sies and Jones (2020).

### 2.3. *Trans*-resveratrol: chemistry, absorption and metabolism

*Trans*-resveratrol is a phytoalexin synthesized by several plants in response to microbial infections, ultraviolet radiation, or other stress stimuli. Thus, its concentration depends on environmental factors, such as temperature, soil and the presence of aggressive agents (Gambini et al., 2015; Pannu and Bhatnagar, 2019). *Trans*-resveratrol synthesis occurs by the phenylpropanoid pathway and its basic skeleton is composed of two phenolic rings linked by a double bond of styrene, which is responsible for the isomeric *cis* and *trans* forms available in nature (**Figure 5**). The *cis* form only occurs when the *trans* isomer is exposed to artificial light, UV radiation or pH > 11 (Gambini et al., 2015; Pannu and Bhatnagar, 2019). The *trans* isomer is the most stable and predominant form in food sources and supplements (Novelle et al., 2015).

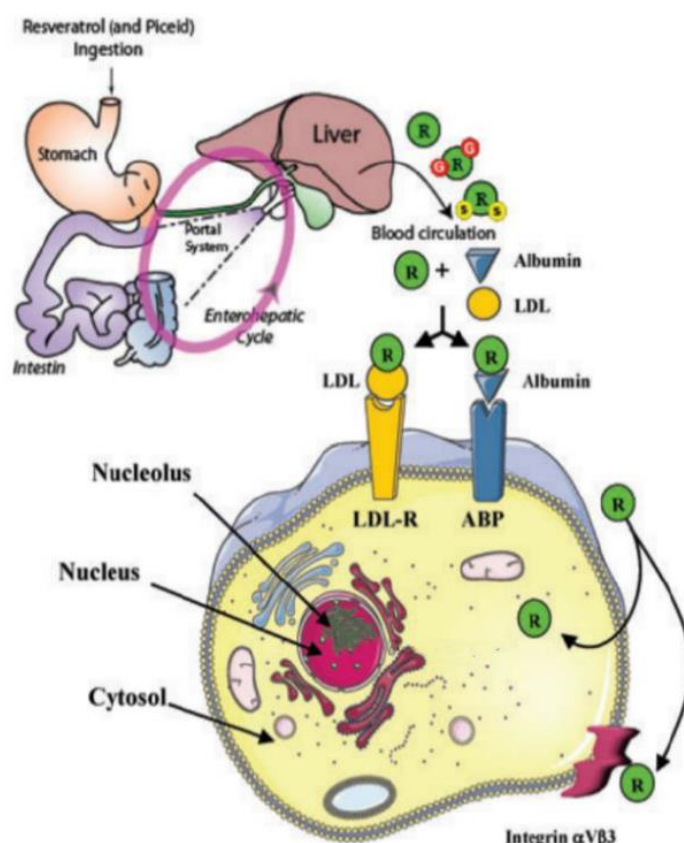


**Figure 5:** Chemical structure of isoforms of resveratrol. Adapted from Gambini (2015)

The major dietary sources of *trans*-resveratrol include grapes, wine, apples, peanuts and soy (Gambini et al., 2015). Due to the exogenous biological and physical stressors, the concentration of *trans*-resveratrol in all of these food products is highly variable, becoming difficult to accurately estimate the average daily intake (Weiskirchen and Weiskirchen, 2016). According to a study that included 40,685 subjects (aged 35–64 years), the estimated daily dose of *trans*-resveratrol intake and its glucoside *trans*-polydatin piceid is 100 and 933 µg/d, respectively, in Spanish adult population (Zamora-Ros et al., 2008). After ingestion, about 75% of *trans*-resveratrol is absorbed in the intestine by passive diffusion or forming complexes with intestinal membrane transporters, including integrins (Delmas et al., 2011; Lin et al., 2006; Springer and Moco, 2019). Once absorbed, *trans*-resveratrol reaches the liver via the hepatic portal system (Pannu and Bhatnagar, 2019). Like other xenobiotics, *trans*-

resveratrol undergoes phase II of drug metabolism, leading to the production of glucuronides and sulfate metabolites (Pannu and Bhatnagar, 2019; Springer and Moco, 2019). Conjugated resveratrol and metabolites undergo enterohepatic circulation, leaving the liver to be reabsorbed in the intestine after hydrolysis, or returning to the liver by the portal system for further metabolism Springer and Moco, 2019).

When *trans*-resveratrol and metabolites reach the bloodstream, they can be transported bound to albumin and lipoproteins, such as LDL (Delmas et al., 2011). This fact suggests that the transport of *trans*-resveratrol in lipoproteins could be a strategy to reduce LDL oxidation, as it would probably exert its antioxidant activity within the lipoprotein structure (Delmas et al., 2011). The metabolism of *trans*-resveratrol was summarized in **Figure 6**.



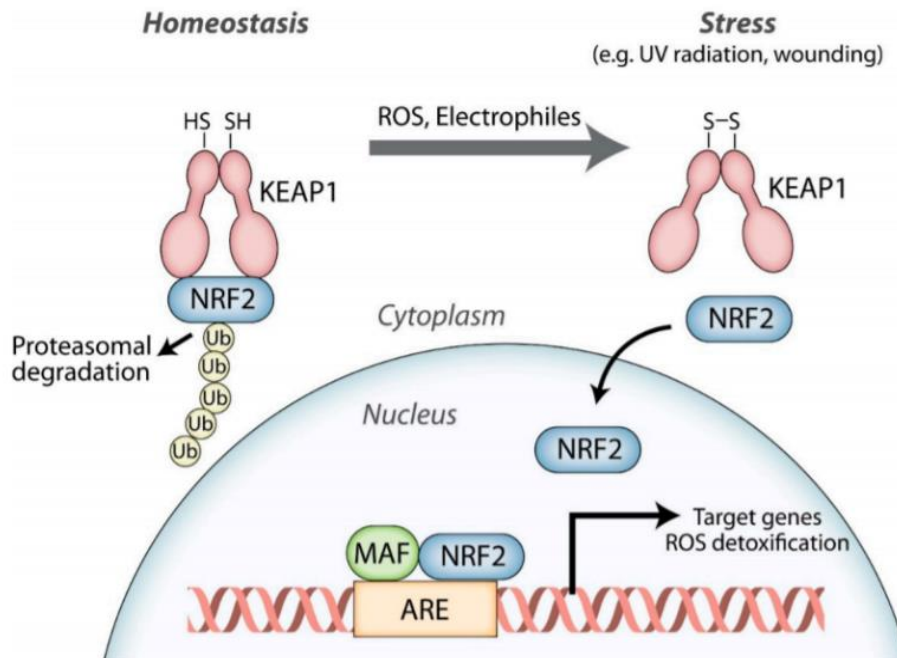
**Figure 6:** Metabolism of *trans*-resveratrol. Adapted from Delmas (2011). After ingestion, *trans*-resveratrol (R) is absorbed by enterocytes and, via the portal route, reaches the liver, where the metabolites sulfate and glucuronide are formed. These metabolites or free *trans*-resveratrol reach the blood system and can be conjugated with lipoproteins and albumin, reaching the cells through receptors for these proteins. Also, resveratrol metabolites can undergo further metabolism by enterohepatic circulation. Abbreviations: ABP, albumin-binding protein; LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor.

*Trans*-resveratrol has been shown to have a number of beneficial effects on cardiovascular health, including prevention of oxidative damage, promotion of vasodilatation and prevention of platelet aggregation. Besides, *trans*-resveratrol signaling could trigger the expression of antioxidant enzymes by the activation or repression of a wide range of transcription factors, such as Nrf2 (Bonnetfont-Rousselot, 2016).

#### **2.4. Activation of the Nrf2 pathway in oxidative stress**

Nrf2 is a member of the Cap-n-Collar (CNC) transcription factor family of the type bZIP (basic region leucine zipper), that regulates about 250 genes by binding to the ARE in the promoter of genes coding for antioxidant enzymes, such as SOD, GPx and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Cuadrado et al., 2019; Howden, 2013). Nrf2 is a protein containing 605 amino acids, presenting seven domains known as Neh (Nrf2-ECH homology) (Canning et al., 2015; Ooi et al., 2017).

Under unstressed conditions, the Nrf2 remains at basal levels due to constitutive ubiquitination mediated by Keap1 (Suzuki and Yamamoto, 2015). Keap1 is a cysteine-rich protein, which act as a highly redox-sensor and serves as an adaptor subunit of a Cullin 3 (Cul3)-based ubiquitin E3 ligase, which promotes ubiquitination and degradation of Nrf2 via the 26S proteasome (Canning et al., 2015). Upon oxidative and electrophilic stress, Keap1 cysteine residues are modified, causing dissociation of the Keap1:Nrf2 interaction (Levonen et al., 2004). This results in the stabilization of Nrf2, which escapes from ubiquitination, accumulate within the cell and translocate to the nucleus, where the Nrf2-Maf heterodimer induces the expression of its target genes (**Figure 7**) (Baird and Yamamoto, 2020; Suzuki and Yamamoto, 2015)



**Figure 7:** The nuclear Nrf2 signaling pathway. Adapted from Hiebert and Werner (2019). Under basal conditions, Nrf2 activity is suppressed by the Keap1 that leads Nrf2 to ubiquitination followed by proteasome degradation. Under oxidative stress, Nrf2 dissociates from Keap1 and then migrate to the nucleus and bind to the Maf protein, starting the ARE-genes transcription. Abbreviations: ARE, antioxidant response element; KEAP1, Kelch-like ECH-associated protein 1; Maf, musculoaponeurotic fibrosarcoma; Nrf2, nuclear factor erythroid 2-related factor 2; ROS, reactive oxygen species; Ub; ubiquitin.

The imbalance of Nrf2 levels increases oxidative stress and has been reported to be involved in multiple aspects of the genesis and progression of metabolic diseases. It is important to note that loss of Nrf2 reduces multiple antioxidant defense systems, increasing levels of multiple types of ROS that cause cell damage. Therefore, the activation of the Nrf2 pathway is an important mechanism to stimulate antioxidant enzyme synthesis in diverse diseases or pathological conditions (Sies and Jones, 2020).

## 2.5. *Trans*-resveratrol as a nutritional strategy against oxidative stress and Nrf2 modulation

The interest in *trans*-resveratrol began about thirty years ago, due to the association between red wine as part of the “French paradox” context (Renaud and de Lorgeril, 1992), and also because of the controversial studies reported by the “Sinclair group”, in which *trans*-resveratrol was associated with longevity (Baur et al., 2006). *Trans*-resveratrol has been shown to exert anti-platelet, anti-inflammatory, lipid-lowering, antioxidant and anti-thrombotic effects (Dyck and Schrauwen, 2015; Prasad, 2012; Zordoky et al., 2015). Frankel et al. (1993) demonstrated that *trans*-resveratrol

reduced the oxidation of human LDL by reducing *in vitro* copper-catalyzed oxidation. This effect was attributed to the chelation of copper, which is a pro-oxidant metal (Frémont, 2000).

The activation of Nrf2 mediated by bioactive compounds has attracted interest due to beneficial health effects, such as the prevention of oxidative stress. Evidence from *in vitro* and *in vivo* studies suggests that the use of *trans*-resveratrol and the activation of the Nrf2 pathway would play an important role in the antioxidant response. Ungvari et al. (2010) observed that *trans*-resveratrol in endothelial cells increased the expression of Nrf2 associated with the upregulation of Nrf2 target genes, such as NQO1 and heme-oxygenase 1 (HO-1), attenuating oxidative stress. In mice fed a high-fat diet, *trans*-resveratrol treatment attenuated oxidative stress and improved vasodilation, whereas in Nrf2 knockout mice that received the same diet, these effects were reduced (Ungvari et al., 2010).

In studies using animal models, *trans*-resveratrol supplementation has demonstrated improvement in the plasma lipid profile, increase in SOD and eNOS expression, reduction of oxidative stress, oxLDL and consequentially attenuated atherosclerotic lesions (Li et al., 2019; Rocha et al., 2009). In a previous trial conducted by our group, LDLr<sup>(-/-)</sup> mice previously supplemented with *trans*-resveratrol showed a reduction in total cholesterol and fractions and also increase SOD activity (Chassot et al., 2018). In another study, *trans*-resveratrol decreased expression of ICAM-1 via transcriptional regulation of Nrf2, blocking monocyte adhesion. This result supports a potential anti-inflammatory function of *trans*-resveratrol in the atherosclerotic process (Seo et al., 2019). The oxidative stress was also attenuated in diabetic mice that received *trans*-resveratrol for 30 days. The treatment normalized the renal expression of Nrf2 and increased the activity of antioxidant enzymes such as SOD, CAT and GPx, causing decline in hydroperoxides and protein carbonyl levels in the kidneys of diabetic mice (Palsamy and Subramanian, 2011). In another study, *trans*-resveratrol treatment in hypertensive rats for 9 weeks restored Nrf2 activity, reduced inflammation and oxidative stress and normalized antioxidant activity compared to the control group (Javkhedkar et al., 2015). In mice fed with high-fat diet, *trans*-resveratrol attenuated

oxidative stress and lipid accumulation through the reduced methylation status in the promoter regions of the Nrf2 genes in the liver tissue (Hosseini et al., 2020).

In humans, *trans*-resveratrol supplementation showed a reduction in intrahepatic lipid content, triglycerides, glycemia and markers of inflammation such as TNF- $\alpha$  and IL-6 (Timmers et al., 2011). Seyyedebrahimi et al. (2018) demonstrated increased expression of Nrf2 and SOD, as well as improved antioxidant capacity in subjects with type 2 diabetes that received 800 mg/day of *trans*-resveratrol for 2 months. A recent meta-analysis also correlated resveratrol supplementation with the downregulation of biomarkers of inflammation and oxidative stress, especially C-reactive protein and TNF- $\alpha$ , among patients with metabolic syndrome (Tabrizi et al., 2018). Taking these studies into account, it can be suggested that activation of Nrf2 by *trans*-resveratrol plays a critical role in reducing oxidative stress.

It is important to consider that the effect of *trans*-resveratrol appears to be dose-dependent. In general, *trans*-resveratrol doses in studies with animal models range from 0.125 to 500 mg/kg body weight/day mixed with diet; 2.5 to 800 mg/kg body weight/day by oral gavage or 0.4 to 50 mg/L in drinking water, during 2-16 weeks (Zordoky et al., 2015). In clinical trials, doses ranged from 8 to 3,000 mg/day with a duration between 28 and 360 days (Santana et al., 2022). Despite the beneficial effects of *trans*-resveratrol, some studies have demonstrated that a high dose may behave as a pro-oxidizing agent (Shaito et al., 2020). *In vitro* studies reported that *trans*-resveratrol concentrations ( $\geq 25\mu\text{M}$ ) can be cytotoxic (Radkar et al., 2007), increasing oxidative stress levels in endothelial cells (Posadino et al., 2015), impairing kidney function (Liu et al., 2019), and causing mitochondrial and DNA damage (de la Lastra and Villegas, 2007). In humans, high doses of *trans*-resveratrol were associated with gastrointestinal symptoms and also increased some cardiovascular biomarkers. Results of a pilot study reported by Mankowski et al. (2020) indicated that 1,000 mg/day of *trans*-resveratrol in overweight man elevated the levels of biomarkers related to cardiovascular risks, such as soluble vascular cell adhesion molecule and total plasminogen activator inhibitor, while individuals who received lower dose (300 mg/day) did not change these biomarkers. These unexpected results have been partly attributed to a failure to identify the optimal dose and key molecular targets to assess efficacy. In this regard, different doses have

already been evaluated to identify which would be ideal to achieve some of the beneficial effects reported for *trans*-resveratrol, such as anti-obesity (Macarulla et al., 2009), chemoprevention (Stocco et al., 2012) and neurologic functions (Bakheet et al., 2017).

Taking together, the effects of *trans*-resveratrol appear to be related to the dosage, which can play a deleterious effect at a high dose. Given that, it is important to understand which protocol characteristics and doses are effective in achieving some of the beneficial effects reported for this molecule.



### **3. Hypotesis**

According to the literature review, the following hypothesis were raised in this study:

(1) The beneficial effects of resveratrol on biomarkers related to atherosclerosis applied in clinical trials could be related to different protocol parameters, such as time of intervention and dosage.

(2) The antioxidant effect of *trans*-resveratrol was dose-dependent and could occur via Nfr2 pathway.

### **4. Objective**

The objective of this study was first to classify the clinical trials that applied *trans*-resveratrol to supplement humans according to the major atherosclerosis biomarkers, and identify which protocol characteristics could be associated with each result profile. Besides, the second objective was to evaluate the effects of two doses of *trans*-resveratrol on hepatic oxidative response mediated by Nrf2 in LDLr<sup>(-/-)</sup> mice in a prevention model.

### **5. Description of chapters**

This study was composed of two chapters. Chapter one brings a review published in the journal "Complementary Therapies in Clinical Practices" about studies that evaluated the effect of *trans*-resveratrol on biomarkers associated with atherosclerosis and how some protocols parameters were associated with each result. Chapter two describes an experimental study where two doses of *trans*-resveratrol were selected to supplement the LDLr<sup>(-/-)</sup> mice fed with a high-fat diet aiming to evaluate the effect on biomarkers of hepatic oxidative stress, using a prevention protocol.

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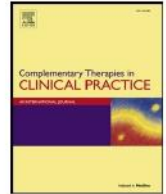
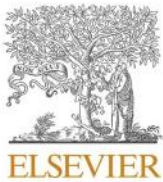
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CHAPTER 1: Effect of resveratrol supplementation on  
biomarkers associated with atherosclerosis in humans

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## Effect of resveratrol supplementation on biomarkers associated with atherosclerosis in humans

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### ABSTRACT

Previous studies have suggested the beneficial effects of resveratrol against cardiovascular disease (CVD). However, there are inconsistent results on cardiovascular-related biomarkers mainly because of variable dosage, intervention time and baseline characteristics of the population. Thus, the exact effect of resveratrol remains unclear. We conducted a review to classify the studies that applied resveratrol to supplement humans according to the major biomarkers and identify which protocol characteristics would be associated with each result profile. Randomized clinical trials that assessed resveratrol effect on biomarkers related to atherosclerosis were searched in databases. Biochemical data were collected from 27 studies on the baseline and post-intervention time. We selected 12 biomarkers to compose the matrix, based on their clinical relevance and higher variation level. A total of 32 assays were obtained from these 27 studies. The net change (%) was calculated for each biomarker. Applying multivariate analysis, the assays were grouped into 3 clusters. Studies that composed Cluster II were characterized by a mean dose of 454.14 mg/day for 74.21 days and showed higher reduction of triglyceride concentration and blood pressure, while those composing Cluster III applied doses around 273.75 mg/day for about 175.33 days and showed the highest HDL increase. Thus, interventions with resveratrol could be customized according to the patient condition, in terms of “dose/time of intervention”. This information can be applied to combine resveratrol with drugs to reduce blood pressure or improve lipid profile in further clinical studies.

### 1. Introduction

Cardiovascular disease (CVD) remains the main cause of morbidity and mortality globally [1]. Atherosclerosis, that underlies CVDs development, is an inflammatory disease characterized by endothelial dysfunction, accumulation of lipids in the arterial intima, in particular, low-density lipoprotein (LDL), immune system cell recruitment and inflammatory response [2].

Current therapeutic approaches in the treatment of atherosclerosis focus on reducing plasma cholesterol levels [3]. However, despite significant reductions in LDL many individuals remain at increased risk due to persistent elevations of inflammatory status [4]. This residual risk is linked to the role of inflammation in all stages of atherosclerosis [2,4].

Factors related to lifestyle, such as an unhealthy diet, favor the development of CVDs. The PREDIMED study (PREvención con Dieta MEDiterránea) demonstrated that the Mediterranean diet reduced cardiovascular events and inflammatory biomarkers related to atherosclerosis. This protective effect is attributed to foods rich in mono-unsaturated fatty acids, vitamins, minerals and polyphenols [5,6].

Among the polyphenols present in the Mediterranean diet, resveratrol stands out in grape and red wine [7]. The benefits attributed to resveratrol were raised since the postulation of “French Paradox” and were attributed to its antioxidant, antiplatelet and anti-inflammatory properties [8,9]. Resveratrol is a natural polyphenol found as *cis* and *trans* isomers, whose *trans*-isomer is the most well-known and stable natural form [7]. In this context, resveratrol has been popularized as a

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nutritional supplement [10]. Several studies conducted *in vitro* and *in vivo* have identified resveratrol as an important compound able to protect against diabetes, neurodegenerative diseases, cancer, aging, obesity and cardiovascular diseases [11]. However, nutritional interventions with resveratrol have shown controversial results and this fact can be due to several factors including dosage, intervention time and characteristics of the studied population [12]. Thus, our objective was to classify the studies that applied resveratrol to supplement humans according to the major atherosclerosis biomarkers, and identify which protocol characteristics could be associated to each result profile.

## 2. Methods

### 2.1. Search strategy

Randomized clinical trials that assessed resveratrol effect on biomarkers related to atherosclerosis were searched in Pubmed, Science Direct and Google Scholar databases from the last 10 years, using the following keywords: “resveratrol”, “cardiovascular disease”, “obesity”, “dyslipidemia”, “supplementation”, “clinical trial”, “biomarker”, “atherosclerosis” and “risk factors”.

### 2.2. Study selection

The manuscripts were selected first by title, then abstract, and lastly by an analysis of the full-text. To be included in our analysis, the studies had to meet the following criteria: (1) original articles with randomized double/triple blind controlled trial design; (2) studies conducted on humans; (3) use of resveratrol for intervention; and (4) be related to cardiovascular disease. From this analysis, only essays published in English and that met the inclusion criteria were selected.

### 2.3. Data extraction

Eligible studies were reviewed and the following data were inserted in the datasheets: study design, number of participants, age, sex, resveratrol dose and intervention duration. Also, the biochemical data were collected on baseline and after the intervention time. The net change of biomarkers from each study was calculated as the % difference between the values observed after and before (baseline) the intervention, discounted the placebo effect. In the studies in which more than one intervention was evaluated, the same placebo was applied to all interventions. About 81 biomarkers evaluated in at least one study were included (Supplementary Table 1), considering the initial and final values observed in the treated and control groups. However, the multivariate analysis and discussion were carried out with 12 biomarkers that presented higher physiological relevance to atherosclerotic process and higher variation level after the intervention.

### 2.4. Statistical analysis

Data were initially summarized and presented as mean  $\pm$  SD, median, mode and range (minimum and maximum values). After, from 81 biomarkers, 12 were selected to the multivariate analysis. The net change of these 12 biomarkers was applied as active variables (columns) and the 32 assays obtained from 27 studies were taken as cases (rows). First of all, the Principal Component Analysis (PCA) was used to plot the 32 assays according to the plane generated by the two first principal factors. In this analysis, mean substitution was applied when data was not present, and the analysis was based on correlation. After, Cluster Analysis was carried out on standardized data applying Ward’s method and Euclidean distance. Joining (Tree Clustering) analysis was used to visualize the association among the biomarkers and also for assays grouping. One-way ANOVA followed by Tukey test was applied to compare the protocol parameters and biomarkers net change among the groups. Calculations were performed using the Statistica v. 13.4 (TIBCO

Software Inc, Round Rode, Texas, USA) and graphs using the GraphPad Prism 8 (GraphPad Software, San Diego, California, USA).

## 3. Results

### 3.1. Included studies

From the studies published in the last 10 years found in the databases using the keywords, 27 [13–39] that followed the inclusion criteria were selected to our analysis. The major reasons to exclude the studies were the absence of Placebo group and open label or uniqueness of design. Some articles were decomposed in assays, excluding the part of the trial that evaluated a different compound than resveratrol.

### 3.2. Study characteristics

In general, studies showed variation in sample size (9–92) with an average of 26 individuals, median and mode of 25 individuals. The age of participants ranged from 32 to 67 years. The treatment duration ranged between 28 and 360 days, and the resveratrol dose ranged from 8 to 3000 mg/day, but most of trials used a dose of 500 mg/day. The detailed characteristics of the studies are presented in Table 1. The Supplementary Table 2 shows the general characteristics and routine drugs used by the patients according to the two groups.

### 3.3. Net change (%) of the major biomarkers

From 81 biomarkers, 12 were selected to the multivariate analysis based on the clinical relevance and the higher variation after the intervention. Table 2 shows the (%) change observed to these 12 selected biomarkers after the treatment, while Fig. 1 presents the respective net change (%).

### 3.4. Multivariate analysis

Table 3 presents the contribution of the net change of these 12 biomarkers to the PCA. Based on eigenvalues, 42.84% of the variation was explained by the two Principal Components (PCs). It was observed the positive contribution of the blood pressure (BP), total cholesterol (TC), triglyceride (TG), and LDL and negative contribution of the high-sensitivity C-reactive protein (hsCRP) and interleukin (IL)-6 to the Factor 1 of the PCA, while high density lipoprotein (HDL) contribute negatively and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and leptin positively to the Factor 2. Adiponectin contributed to Factor 3 and IL-10 to Factor 6. Fig. 2 shows the distribution of the assays according to the plane generated by the two first PCs, including the variables projection.

In general, except for the assays 21, 24, 10, 26, and 4c, all others showed similarity. The assays 21, 24 and 26 were separated in the horizontal axis (Factor 1). Assays 21 and 24 showed a reduction of BP in DM2 individuals supplemented with 800 mg/day of resveratrol for 8 weeks, however, no effect was observed in the lipoproteins or inflammatory biomarkers. In contrast, in the assay 26 it was observed an increase in LDL and a decrease of hsCRP and IL-6 in individuals with Non-alcoholic fatty liver disease (NAFLD) supplemented with 3000 mg/day for 8 weeks. The assays 10 and 4c were separated in the vertical axis (Factor 2) by the higher reduction in HDL and increase of TNF- $\alpha$  and

**Table 1**  
General characteristics presented in the selected studies.

	Mean $\pm$ SD	Median	Mode	Range
Individuals (n)	26 $\pm$ 17	25	25	9–92
Female/Male (%)	43/57	–	–	–
Age (y)	55 $\pm$ 9	58	60	32–67
Time (days)	105 $\pm$ 104	60	56	28–360
Dose (mg)	497.59 $\pm$ 658.36	225.00	500.00	8.00–3000.00

**Table 2**  
Change (%) and Net Change (%) of biomarkers selected to the multivariate analysis.

Biomarker <sup>a</sup>	Change after treatment <sup>b</sup>	Net change <sup>c</sup>	N (studies) <sup>d</sup>
SBP (%)	-3.34 ± 4.62	-2.76 ± 4.43	17
DBP (%)	2.00 ± 16.10	-1.84 ± 4.21	17
TC (%)	-4.61 ± 6.00	-2.69 ± 5.99	23
TG (%)	-2.85 ± 14.96	-1.33 ± 18.90	24
LDL (%)	-5.15 ± 8.68	-2.07 ± 10.25	22
HDL (%)	1.70 ± 7.15	0.92 ± 7.07	24
hsCRP (%)	12.34 ± 56.80	-4.23 ± 24.35	11
TNF-α (%)	1.76 ± 23.16	-2.58 ± 13.34	10
IL-6 (%)	1.05 ± 27.67	1.63 ± 32.05	11
IL-10 (%)	15.24 ± 22.31	14.82 ± 12.32	5
Leptin (%)	1.94 ± 15.59	2.46 ± 18.71	6
Adiponectin (%)	-16.61 ± 47.93	6.64 ± 9.85	9

Values are expressed as mean ± SD.

<sup>a</sup> SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; hsRCP, high sensitive C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10.

<sup>b</sup>  $((TF-T_0)/T_0)*100$  for Treated group.

<sup>c</sup>  $[(TF-T_0)/T_0]*100$  for Treated group] -  $[(TF-T_0)/T_0]*100$  for Control group].

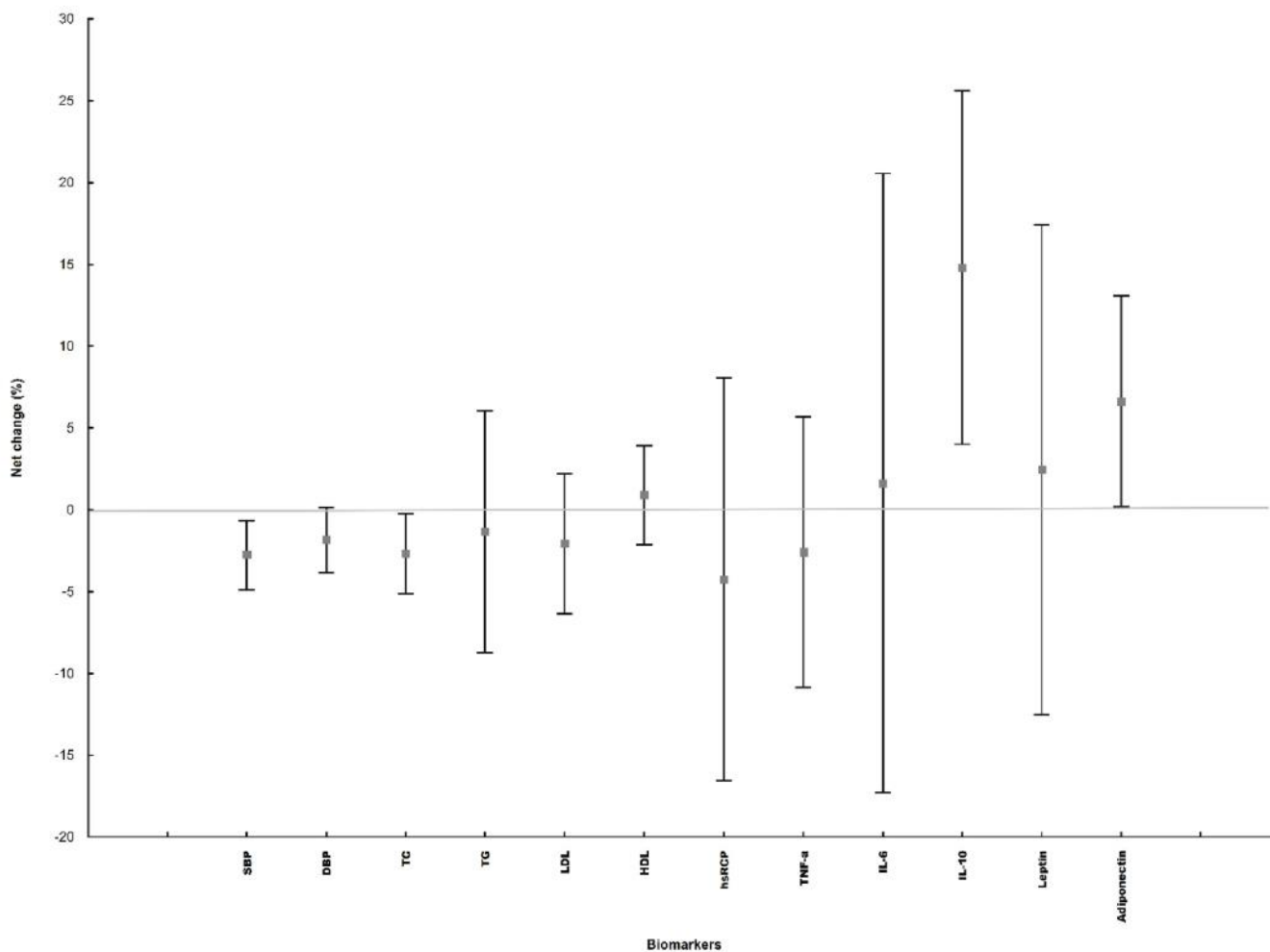
<sup>d</sup> From a total of 27 studies.

leptin observed in study 10 conducted with obese men supplemented with 1500 mg/day for 4 weeks and by the opposite results reported in the assay 4c, which decreased TNF-α and hsCRP. According to the dendrogram obtained from cluster analysis (Supplementary Figs. 1 and 2), the studies were divided into three clusters. Table 4 shows the

**Table 3**  
Factor coordinates of the variables (Biomakers), based on correlations until factor 6.

Biomarker <sup>a</sup>	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6
SBP	0.51	-0.30	-0.15	-0.42	0.11	-0.46
DBP	0.57	-0.30	-0.12	-0.39	-0.44	-0.27
TC	0.79	0.23	-0.31	0.17	-0.32	0.07
TG	0.55	0.05	-0.35	0.49	0.20	-0.29
LDL	0.64	0.26	0.03	0.41	-0.30	0.34
HDL	-0.03	-0.80	0.04	-0.01	-0.26	0.32
hsCRP	-0.63	0.16	-0.34	-0.10	-0.54	-0.11
TNF-α	-0.02	0.87	-0.21	-0.23	0.01	0.11
IL-6	-0.69	-0.03	-0.51	0.09	-0.22	-0.03
IL-10	0.36	-0.20	-0.20	-0.47	0.14	0.59
Leptin	0.14	0.65	0.41	-0.44	-0.13	-0.04
Adiponectin	0.02	-0.08	0.79	0.21	-0.33	-0.12

<sup>a</sup> SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; hsRCP, high sensitive C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10.



**Fig. 1.** Net changes (%) observed in the assays. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; hsRCP, high sensitive C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10. Values expressed as mean ± 1.96 SD.

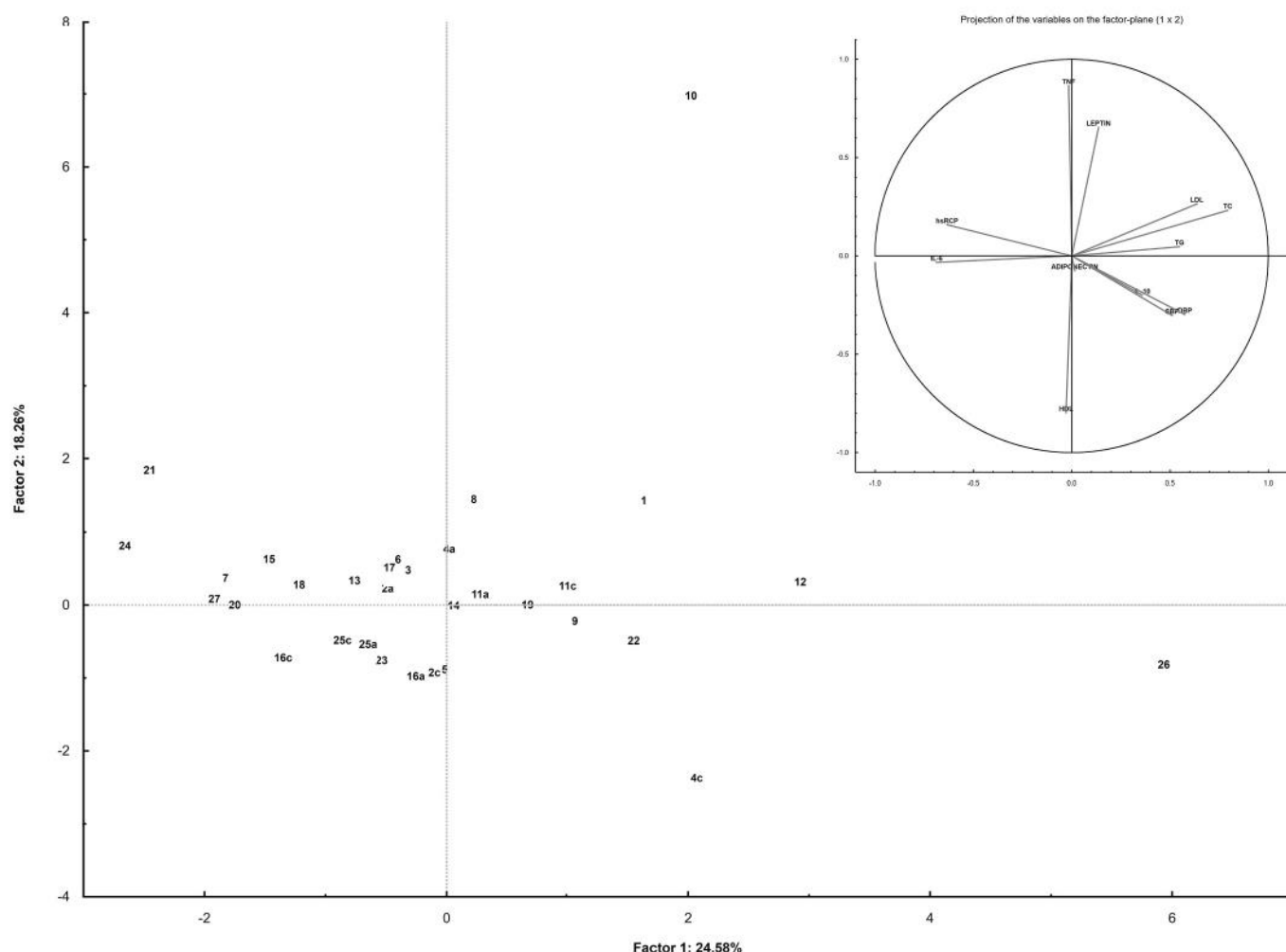


Fig. 2. Projection of the assays on the factor-plane (1 × 2).

characteristics of each Cluster. The clusters did not differ in terms of the number of participants, sex and age. Although the information about the baseline conditions of the patients and their prescription drugs was not shown in all studies, patients in the Cluster II and III presented comorbidities such as diabetes, dyslipidemia, hypertension, obesity and stable angina. Regarding to the protocol, it was observed a difference in the time of intervention ( $p = 0.007$ ) among the Clusters and a trend ( $p = 0.099$ ) of difference to the doses. The interventions that composed Cluster III presented the best results in terms of HDL change (Fig. 3a), while Cluster II showed the greater improvement of blood pressure (Fig. 3b). In addition, Cluster I assays showed a trend of reducing inflammatory biomarkers, such as IL-6 ( $p = 0.061$ ).

#### 4. Discussion

Resveratrol is widely commercialized as antioxidant and anti-inflammatory supplement to enhance cardiovascular protection without any prescription [40]. However, there is no consensus whether resveratrol really presents protective effects on cardiovascular health [41]. In this review we investigated the association between experimental conditions of clinical studies that evaluated the effects of resveratrol supplementation and biomarkers related to cardiovascular diseases, applying a multivariate statistical approach.

Atherosclerosis and other chronic diseases are inherent to aging and are usually evident in older people who are overweight or present dyslipidemia, diabetes or hypertension. For this reason, the mean age of the individual enrolled in the studies was 55 years old (Table 1), with some

of them under different drugs prescription (Table 4). Regarding safety, clinical trials have shown that resveratrol supplementation is safe and well-tolerated, but doses between 2.5 and 5.0 g/day can cause mild to moderate gastrointestinal symptoms [20,42]. For clinical practices, very low doses like 8.0 mg/day, use to be ineffective, while supra-physiological doses (>2.0 g/day) become very expensive and can also bring adverse effects. These aspects can justify the amount of 500 mg/day (Table 1) more often applied in the clinical assays. In general, the interventions last about 2 months, although protocols differed about this time, from 30 to 360 days.

In general, the resveratrol supplementation improved all biomarkers selected in our analysis (Fig. 1). This result was more effective to the patients of Clusters II and III (Table 4) and can be associated to the longer intervention time (>2 months) under lower doses (200–500 mg/day). Regarding to the lipid profile (Fig. 3 and Table 4), patients of Clusters II and III showed an expressive improvement, reducing dyslipidemia. The lipid-improving effect of resveratrol could be due to its downregulation of the hepatic enzyme 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase, responsible for cholesterol synthesis [43]. Moreover, resveratrol is able to increase the expression of LDL receptors and cholesterol 7 alpha-hydroxylase (CYP7A1), contributing to reduce LDL concentration in the circulation and increasing LDL excretion from the enterohepatic cycle, respectively [43,44]. In addition, according to Mansur et al. [26], resveratrol can increase of serum concentration of silent information regulator (Sirt-1) protein that has been associated with beneficial effects on inflammation, lipid metabolism and atherosclerosis.

**Table 4**  
Protocol parameters and net changes of biomarkers according to the Clusters.

Variables <sup>a</sup>	Cluster I (n = 6)	Cluster II (n = 14)	Cluster III (n = 12)	P value <sup>c</sup>
Individuals (n)	19.83 ± 5.41	21.86 ± 2.10	32.08 ± 5.30	0.107
Female (%)	15.74 ± 9.98	35.38 ± 7.96	45.47 ± 9.06	0.140
Age (y)	54.16 ± 3.30	53.63 ± 2.46	57.06 ± 1.93	0.541
Time (days)	38.50 ± 5.64 <sup>a</sup>	74.21 ± 10.38 <sup>a</sup>	175.33 ± 40.96 <sup>b</sup>	0.007
Dose (mg)	1066.67 ± 474.63	454.14 ± 129.00	263.75 ± 88.07	0.099
Diabetes (n) <sup>b</sup>	-	18.00 ± 7.77	15.33 ± 3.99	-
Dyslipidemia (n) <sup>b</sup>	-	-	43.50 ± 6.50	-
Obesity (n) <sup>b</sup>	-	-	26.00 ± 5.00	-
Hypertension (n) <sup>b</sup>	-	19.50 ± 0.5	31.43 ± 9.57	-
Stable Angina (n) <sup>b</sup>	-	2.00	2.00	-
Smokers (n) <sup>b</sup>	-	4.00 ± 0.84	8.25 ± 2.18	-
Aspirin (n) <sup>b</sup>	-	13.00	11.67 ± 0.67	-
Clopidogrel (n) <sup>b</sup>	-	1	1	-
Statins (n) <sup>b</sup>	-	18.00 ± 1.48	19.50 ± 5.50	-
β-Blockers (n) <sup>b</sup>	-	23.25 ± 2.06	14.00 ± 4.00	-
Calcium Channel Blockers (n) <sup>b</sup>	-	9.00 ± 1.00	3.00	-
ACE/ARB Inhibitors (n) <sup>b</sup>	-	21.50 ± 2.18	20.67 ± 1.68	-
SBP (%)	1.54 ± 1.23 <sup>a</sup>	-6.76 ± 1.06 <sup>b</sup>	-0.95 ± 1.19 <sup>a</sup>	p < 0.001
DBP (%)	2.17 ± 1.09 <sup>a</sup>	-4.51 ± 1.77 <sup>b</sup>	-1.39 ± 0.87 <sup>ab</sup>	0.026
TC (%)	4.95 ± 1.87 <sup>a</sup>	-6.04 ± 1.51 <sup>b</sup>	-3.28 ± 1.39 <sup>b</sup>	p < 0.001
TG (%)	23.26 ± 7.11 <sup>a</sup>	-10.13 ± 5.01 <sup>b</sup>	-3.51 ± 2.12 <sup>b</sup>	p < 0.001
LDL (%)	9.45 ± 2.07 <sup>a</sup>	-4.81 ± 3.40 <sup>b</sup>	-4.35 ± 2.38 <sup>ab</sup>	0.036
HDL (%)	-5.04 ± 5.37 <sup>a</sup>	-1.16 ± 1.42 <sup>a</sup>	6.23 ± 1.35 <sup>b</sup>	p < 0.001
hsCRP (%)	-18.00 ± 16.99	-2.92 ± 7.63	7.23 ± 9.55	0.361
TNF-α (%)	9.51 ± 20.08	-4.33 ± 3.14	-6.36 ± 4.26	0.395
IL-6 (%)	-45.52 ± 24.66	5.36 ± 10.83	14.82 ± 10.31	0.061
IL-10 (%)	22.05	-0.32	17.45 ± 7.13	-
Leptin (%)	29.73	-2.24 ± 18.00	-3.51 ± 5.87	-
Adiponectin (%)	2.41	1.82 ± 1.81	8.95 ± 4.74	-

Values are expressed as mean ± SD.

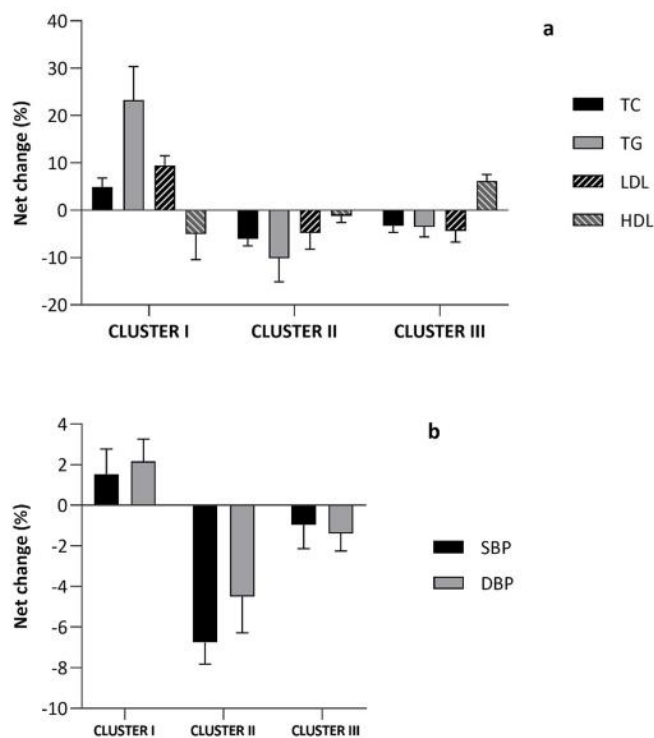
<sup>a</sup> SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; hsRCP, high sensitive C-reactive protein; TNF-α, tumor necrosis factor α; IL-6, interleukin 6; IL-10, interleukin 10.

<sup>b</sup> Mean considering only the studies in which this information was available.

<sup>c</sup> P value obtained by One-way ANOVA. Values followed by the same letter are not different.

The lesions observed in the arteries due to the increased variability of blood pressure, represent a link between blood pressure and atherosclerosis development [45]. In a meta-analysis, Liu, Ma, Zhang, He and Huang [46] reported that only supplementation with doses higher than 150 mg/day reduced systolic blood pressure. Fogacci, Banach and Cicero [47] also suggested that long-term (>3 months) resveratrol supplementation exerts a significant lowering effect on blood pressure in patients with non-alcoholic fatty liver disease (NAFLD), a known risk factor to cardiovascular diseases.

In our study, we observed that patients who took part of Cluster II were supplemented with about 450 mg/day and showed the best results in terms of both diastolic and systolic pressure reduction. Although Table 4 presents the drugs prescription only from the studies that showed this information, it seems that this positive result was not associated to drugs because they were present in both Cluster II and III. Based on a systematic review and meta-analysis including 17 clinical trials, it was observed that when the studies were categorized according



**Fig. 3.** Lipoproteins profile (a) and arterial pressure (b) net change values according to the clusters. Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein.

to the dose, there was a reduction of diastolic blood pressure between the two sub-sets (<300 and >300 mg/d), suggesting that resveratrol could promote cardiovascular health, mainly when used at higher dose in diabetic patients [48]. Thus, if the patient treatment is focused on blood pressure, the protocol applied in the studies of Cluster II could be suggested. The proposed pathway underlying the beneficial effect of resveratrol on SBP may involve mainly the increase of nitric oxide (NO) concentration via multiple mechanisms [49]. Resveratrol can stimulate endothelial NO synthase (eNOS) via activation of Sirt1, besides, resveratrol also enhances enzymatic activity of eNOS via phosphorylation at serine 1177 [50,51]. This pathway involves the activation of AMPK resulting in a subsequent increase of NO [49]. Lastly, the antioxidant activity of resveratrol attenuate vascular oxidative stress and prevents NO breakdown [50,51], mediating the vasodilatory effect of resveratrol.

Regarding to the anti-inflammatory action, it has been reported that resveratrol regulates key players in the inflammatory cascade, including molecular-targets such as nuclear factor kappa B (NF-κB), toll-like receptors (TLR) and activate nuclear factor erythroid 2-related factor 2 (Nrf2), suppressing pro-inflammatory cytokine production, such as IL-6, TNF-α and hsCRP [11,13,52]. Despite none of the three Clusters (Table 4) has showed difference among the inflammatory biomarkers, Cluster I assays showed a trend (p = 0.061) of higher reduction of IL-6, usually associated to episodes in which the immune system is activated [53]. In this context, the use of higher doses of resveratrol in a short time intervention may be more efficient. However, this hypothesis must be evaluated in further clinical trials. Obesity is a pro-inflammatory condition in which has a link between the adipose tissue and immune system that contributes to increase the level of cytokines and adipokines, which may present anti and proinflammatory effects [54]. These adipokines include mainly adiponectin and leptin. Changes in plasma levels of these adipokines result in metabolic disorders, such as insulin resistance and atherosclerosis [55]. In this regard, some experimental studies have evaluated the anti-inflammatory effects of resveratrol on

adipokines, nonetheless, the effect in humans is still inconsistent [56]. Results of a meta-analysis conducted by Mohammadi-Sartang et al. [57] showed that resveratrol supplementation changed serum adiponectin concentrations, but did not affect plasma leptin levels, independently of the supplementation dose. In our review, no difference of adiponectin and leptin was observed between the three groups (Table 4). It is important to highlight that the majority of individuals included in the studies were overweight (Supplementary Table 1). Additional studies are needed to assess the benefits of resveratrol on adipokines in humans, considering body weight as covariate in the statistical analysis.

Our review brought important practical information about interventions with resveratrol. It was clear that “dose/time of intervention” produces different results on biomarkers associated to atherosclerosis. The design applied in the Cluster II could be suggested as co-therapy to reduce blood pressure, while the design applied in the Cluster III studies would have better results as co-therapy to hypolipidemic drugs, specially to increase HDL. In a previous study reported by our group, Scolaro et al. [58] showed the importance of combining supplements with drugs to improve the patient’s treatment. In a recent review about the action of nutraceuticals in hypertensive disorders, resveratrol was suggested as a good alternative to be used together with traditional drugs to blood pressure control in pregnancy, reducing the exposition of the mother and fetus to additional risks [59]. It is also important to highlight that our analysis presents some limitations. In general, reviews and meta-analysis are mainly based on studies that successfully confirmed their hypothesis. In addition, many studies investigate the combination of several compounds, reducing the number of protocols that applies isolated resveratrol.

Another interesting aspect observed in our review is that although resveratrol has been commercialized as an antioxidant and its beneficial effect associated with the red wine consumption, as part of the Mediterranean diet [60], only 4 studies evaluated an oxidative stress biomarker before and after the intervention, probably due to the lack of reference values to these biomarkers.

## 5. Conclusion

Interventions with resveratrol should be customized according to the patient condition, mainly related to the “dose/time of intervention”. This information can be applied to combine resveratrol with drugs to reduce blood pressure or improve lipid profile in the future clinical studies.

## Author contribution

Conception and design by Inar A. Castro; development of methodology by Inar A. Castro, Tamires M. Santana and Lucas Y. Ogawa; literature search and data extraction by Tamires M. Santana and Lucas Y. Ogawa; statistical analysis by Inar A. Castro and Lucia P. Barroso; preparing the manuscript draft by Tamires M. Santana and Inar A. Castro; review and revision of the manuscript by Marcelo M. Rogero and Inar A. Castro. All authors read and approved the final version of the manuscript.

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## Declaration of competing interest

The authors declare no competing interests in any aspects.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ctcp.2021.101491>.

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CHAPTER 2: Effect of two doses of *trans*-resveratrol on hepatic oxidative stress biomarkers using a LDLr knockout mice model<sup>1</sup>

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**Effect of two doses of *trans*-resveratrol on hepatic oxidative stress  
biomarkers using a LDLr knockout mice model**

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**Short title:** *Trans*-resveratrol and oxidative stress in mice

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## Abstract

*Trans*-resveratrol is a stilbene largely used as supplement for humans aiming to improve the antioxidant defense. However, its potential antioxidant activity depends on dose and can occur by different metabolic pathways, including the activation of the nuclear factor erythroid 2-related factor 2 (Nrf2). Thus, the objective of this study was to investigate the effect of two doses of *trans*-resveratrol on hepatic oxidative stress biomarkers mediated by Nrf2 using a LRDr<sup>(-/-)</sup> mice model. Mice were fed for 8 weeks with a standard diet, followed for more 24 weeks with a Western diet, both containing *trans*-resveratrol in the doses of 250 mg/kg diet/day (low resveratrol dose, LRD) or 400 mg/kg diet/day (high resveratrol dose, HRD). A control group (CONT) was kept without supplementation. Both *trans*-resveratrol doses had no effect on body weight, lipid profile and antioxidant enzymes activity. However, LRD group showed a higher GSH/GSSG ratio, followed by a reduced MDA concentration, suggesting an antioxidant effect, and both doses reduced the expression of Nrf2 in the liver in comparison with CONT group. Furthermore, LRD group showed reduced nuclear factor- $\kappa$ B expression, while no other changes were observed in TNF- $\alpha$  and IL-10 levels. The low-dose of *trans*-resveratrol showed a more promising effect than the high dose in improving the hepatic oxidative status and this antioxidant effect was not associated with the expression of Nrf2 pathway. The relevance of this information must be considered in further clinical trials since *trans*-resveratrol has been consumed as an antioxidant without any dose control.

**Keywords:** *trans*-resveratrol, liver, oxidative stress, antioxidant, biomarkers

## Introduction

Unhealthy lifestyle practices and poor nutrition are risk factors that drastically contribute to increased oxidative stress and inflammation, and consequently, the progression of several chronic diseases, including cardiovascular diseases (CVD)<sup>(1)</sup>. Accumulating preclinical and clinical evidences indicate that diets rich in bioactive compounds (e.g., the Mediterranean diet) play a protective role against atherosclerosis through mechanisms associated with reduced levels of lipid peroxidation, oxidative stress and inflammatory response<sup>(2; 3)</sup>. The protective effect of these bioactive compounds has been attributed, in part, to their antioxidant activity<sup>(4)</sup>.

*Trans-resveratrol* (3,5,4'-trihydroxy-*trans*-stilbene) is a polyphenolic compound that belongs to the stilbene group and it is present in a large variety of plant foods, including grapes and red wine<sup>(5)</sup>. Indeed, since that moderate red wine consumption has been associated with a decreased risk of CVD, *trans-resveratrol* has attracted the most clinical interest<sup>(6)</sup>. Although the *cis* and *trans* isomers coexist in nature, the *trans* isomer is more stable and biologically active<sup>(7)</sup>. Numerous studies indicate that *trans-resveratrol* protects against oxidative stress not solely because of its direct antioxidant capacity, but also by up-regulating other endogenous antioxidant pathways<sup>(8)</sup>. A recent review showed that *trans-resveratrol* and other natural compounds can stimulate the nuclear factor erythroid 2-related factor 2 (Nrf2)<sup>(9; 10)</sup>. The Nrf2 is a transcription factor that responds to oxidative stress by binding to the antioxidant response element (ARE) in the promoter of genes encoding for antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and NAD(P)H:quinone oxidoreductase 1<sup>(11)</sup>.

Previous studies showed a preventive role of *trans-resveratrol* in chronic diseases such as type 2 diabetes and obesity<sup>(12)</sup>, kidney injury<sup>(13)</sup> and neurodegenerative

diseases<sup>(14)</sup> by stimulating the Nrf2 signaling pathway. A recent metabolomic analysis showed that *trans*-resveratrol metabolites could exert an atheroprotective effect, in part modulating metabolites in the liver<sup>(15)</sup>. It has also been reported that *trans*-resveratrol can activate Nrf2 pathway in different diseases or clinical conditions<sup>(16;17;18)</sup>.

Although is widely described the antioxidant effects of *trans*-resveratrol, some studies have reported side effects<sup>(19)</sup>. These effects include an increase in oxidative stress levels in endothelial cells<sup>(20)</sup>, impaired kidney function<sup>(21)</sup> and mitochondrial and DNA damage<sup>(22)</sup>. These unexpected results have been partly attributed to a failure to identify the optimal dose and key molecular targets to assess efficacy. In this regard, different doses have already been evaluated to identify which would be the ideal dose to achieve some of the beneficial effects reported for *trans*-resveratrol, such as anti-obesity<sup>(23)</sup>, chemoprevention<sup>(24)</sup> and neurologic functions<sup>(25)</sup>. However, little is known about the effects of different *trans*-resveratrol doses in the Nrf2 pathway in a diet-induced atherosclerosis mice model. Moreover, a recent review reported by our group showed that the effects of *trans*-resveratrol in clinical trials are conditioned to protocol parameters such as dose and time of intervention, which must be considered according to the objectives of the individual treatment. Interestingly, this review also showed that although *trans*-resveratrol supplements suggest an antioxidant activity on their labels, most of the studies evaluated in the review did not include the assessment of any oxidative stress biomarker<sup>(26)</sup>.

Taking into account the influence of the dose on *trans*-resveratrol interventions and the potential action on Nrf2 pathway, our objective was to evaluate two doses of *trans*-resveratrol on hepatic oxidative response mediated by Nrf2 in a LDLr<sup>(-/-)</sup> mice model for atherosclerosis.

## Materials and Methods

### Material

*Trans*-resveratrol was acquired from Eop Eireli Pharmacy (Santo André, SP, Brazil). 1,1,3,3-tetraethoxypropane (TEP), nicotinamide adenine dinucleotide (NADH), superoxide dismutase (SOD), nicotinamide adenine dinucleotide phosphate (NADPH), glutathione peroxidase (GPx) and reduced and oxidized glutathione (GSH and GSSG) were purchased from Sigma-Aldrich (Sigma Chemical Co, St. Louis, United States). *Trans*-resveratrol standard was also obtained from Sigma-Aldrich (Sigma Chemical Co., St. Louis, United States). All solvents were HPLC grade.

### Study design

Three-month-old male homozygous LDLr<sup>(-/-)</sup> mice in the C57BL/6 background were purchased from the Faculty of Pharmaceutical Sciences, University of São Paulo. The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmaceutical Sciences, University of São Paulo (CEUA/FCF 595). LDLr<sup>(-/-)</sup> mice have been applied as a model to evaluate oxidative stress and inflammation because these two conditions are present in atherosclerosis progression<sup>(27; 28)</sup>. Mice were housed in plastic cages (4 - 5 animals per cage) at constant room temperature (25 ± 2 °C) and relative humidity (55 ± 10 %), under a 12 h light–12 h dark cycle. Mice with an initial body weight of 25.49 ± 1.73 g were randomly allocated to three groups (n = 13/group) and fed *ad libitum* with a standard diet AIN-93M diet for 8 weeks without supplementation (CONT), or the same diet mixed with 250 mg of *trans*-resveratrol per kg diet/day (LRD) or 400 mg of *trans*-resveratrol per kg diet/day (HRD). After 8 weeks, the standard diet was replaced by a Western diet (D12492; Research Diets, New Brunswick, NJ) to promote the atherosclerotic process for 24 weeks,

following the same supplementation. The *trans*-resveratrol added to the diets showed a high degree of purity by comparing with the standard (Sigma - PHL89539) (**Supplementary Table 1 and Supplementary Figure 1**). The LRD dose was based on the concentration most frequent in the clinical assays<sup>(26)</sup> and also recommended in the commercial capsules label (500 mg/day), while the HRD dose (800 mg/day) was based on a clinical trial reported by Seyyedebrahimi *et al.* <sup>(29)</sup> that showed an increase of expression of genes involved in oxidative stress responses (Nrf2 and SOD), as well as increased plasma total antioxidant capacity and total thiol content in patients with type 2 diabetes. Diets formulation and chemical composition is shown in **Supplementary Table 2 and Table 3**. Individual body weight and food intake per cage were recorded two times weekly. After the experimental period, the mice were deprived of food for 8h, anesthetized with 3% isoflurane and euthanized. Blood was collected for plasma lipid profile analysis. The liver was excised and weighed and small pieces of the larger lobe were stored at -80 °C.

## **Methods**

### **Plasma lipids**

Plasma lipids (total cholesterol [TC]; triglyceride [TG]; high-density lipoprotein [HDL]; low-density lipoprotein [LDL]) concentration was quantified using Labtest Diagnóstica SA (Lagoa Santa, MG, Brazil) commercial kits for enzymatic colorimetric tests according to the manufacturer's instructions (n=11-12/group).

### **Antioxidant activity and biomarkers of oxidative stress**

Superoxide dismutase (SOD) activity was determined according to Ewing and Janero<sup>(30)</sup>; catalase activity was determined according to Bonaventura *et al.*<sup>(31)</sup> and

glutathione peroxidase (GPx) activity according to Flohé and Günzler<sup>(32)</sup>. GSH/GSSG ratio was quantified using a kit for oxidized and reduced glutathione from Sigma-Aldrich (38185). All of the enzymatic assays were performed in the liver homogenate using Synergy HTX Multi-Detection Microplate Reader (BioTekInstruments Inc., Winooski, VT, USA) and integrated with Gen 5 software. Hepatic malondialdehyde (MDA) concentration was determined by reverse phase HPLC (Hong et al., 2000), with minor modifications. Protein concentration was determined by Pierce BCA Protein Assay kit (Thermo Scientific) and expressed as  $\mu\text{g}/\text{mL}$ . Results were expressed as  $\mu\text{M}$  MDA/mg protein. Analysis was run in triplicate (n=10 - 13/group).

### **Cytokine content**

The cytokines interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- $\alpha$ ) and interleukin-10 (IL-10) were analyzed in the liver homogenate using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (MICYTOMAG-70K) (Millipore, St. Charles, MO, USA) (n=10/group).

### **Real-time PCR**

Total RNA from liver homogenate samples was extracted with TRIzol (Invitrogen, Carlsbad, CA), followed by incubation in DNase I RNase-free (Roche Applied Science, Indianapolis, IN) and then reverse transcription using 2  $\mu\text{g}$  of total RNA, SuperScript II Reverse Transcriptase (Invitrogen) and random primers p(dN)6 (Roche Applied Science). Real-time PCR was performed using the 7500TM Real-Time PCR System (Applied Biosystems, Warrington, UK), Power SYBR Green Gene Expression PCR Master Mix (Applied Biosystems) and specific primers for target genes: *Actb* (forward: gctccggcatgtgcaaag; reverse: catcacaccctggtgccta), *Gapdh* (forward: ctttggcggagggtgctagat; reverse: aggactcgtgcagccttacac), *Nfe2l2* (forward:

tgacatgagtcgcttgcc; reverse: cctgatgaggggcagtgaag), *Nfkb1* (forward: agcaacaaaacagaggggat; reverse: ctttgaggccccacatagt), *Ppia* (forward: ccgttcagctctgggatgac; reverse: gggcagcccagaacatcat), *Sod1* (forward: ggaacctccacttcgagca; reverse: cccatgctggccttcagtta) and *Sod2* (forward: gcctgctctaatacaggacc; reverse: tagtaagcgtgctccacac). The superoxide dismutase (SOD) family scavenge superoxide radicals ( $O_2^{\cdot-}$ ) by dismutation into hydrogen peroxide ( $H_2O_2$ ). SOD1 (Cu, ZnSOD) isoform has typically been considered a cytosolic enzyme, while SOD2 (MnSOD) is located inside the mitochondrial matrix and is considered the major protective barrier against the superoxide produced during mitochondrial respiration<sup>(33)</sup>. Data were normalized to the geometric average of *Actb*, *Gapdh* and *Ppia*. Relative quantification of mRNA was calculated by  $2^{-\Delta\Delta Ct}$  (n=8/group).

### **Statistical Analysis**

Data were evaluated by one-way ANOVA followed by Tukey test or non-parametric equivalent tests, depending on their distribution and homogeneity of variances evaluated by Shapiro-Wilk and Hartley test, respectively. Diets were compared by t-test for independent variables. The heatmap was based on Cluster Analysis, considering Wards method and Euclidean distance using the standardized columns and R Studio software. Values were expressed as mean  $\pm$  SD. A *p-value* of 0.05 was adopted to reject the null hypothesis. Calculations were performed using software Statistica v.13 (TIBCO Software Inc., Palo Alto, USA) and graphs using GraphPad Prisma v8 (GraphPad Software, CA, USA).



## Results

### Food intake, body weight and biomarkers of lipidemia

As shown in **Figure 1**, *trans*-resveratrol supplementation did not alter the food intake among the groups. After the first 8 weeks, the food intake was reduced by 15.7% ( $p < 0.001$ ) when the standard diet was replaced by the Western diet (**Figure 1A**). The groups started ( $25.49 \pm 1.73$  g;  $p = 0.971$ ) and ended the treatment with similar body weights ( $47.07 \pm 5.26$  g;  $p = 0.254$ ). After the dietary intervention with the Western diet, feeding was associated with a more pronounced and faster increase in body weight than standard feeding (**Figure 1B**). During treatment, the body weight gain ( $21.57 \pm 5.46$  g;  $p = 0.245$ ) showed no difference among the experimental groups (**Figure 1C**). Liver weight ( $2.05 \pm 0.46$  g;  $p = 0.134$ ) and relative liver weight ( $4.31 \pm 0.60$  %;  $p = 0.121$ ) did not change neither among the groups. Regarding to the lipid profile, **Figure 2** shows that supplementation with *trans*-resveratrol did not change plasma total cholesterol ( $p = 0.243$ ), triglycerides ( $p = 0.298$ ), HDL ( $p = 0.920$ ) and LDL concentrations ( $p = 0.331$ ).

### *Trans*-resveratrol supplementation effect on oxidative stress and inflammatory biomarkers

Concentrations of total glutathione and GSH were not different in any of the groups (**Figure 3A** and **3B**), but there was a decrease in GSSG in the LRD group ( $p < 0.001$ ) when compared with CONT ( $p < 0.001$ ) and HRD ( $p = 0.037$ ) groups. (**Figure 3C**). As consequence, the LRD group showed a higher GSH/GSSG ratio in comparison with CONT ( $p < 0.001$ ) and HRD ( $p < 0.001$ ) groups (**Figure 3D**). This result was corroborated by malondialdehyde (MDA) concentration evaluated in the liver homogenate. When compared with the control group, MDA concentration was 37% lower in the LRD group

( $p=0.022$ ) but did not change in comparison with HRD group ( $p=0.758$ ) (**Figure 3E**). We hypothesized that resveratrol mediates redox protection via antioxidant gene regulation by Nrf2. The mRNA expression of Nrf2 (**Figure 4A**) was reduced in both HRD and LRD groups. Thus, it was expected that SOD1 and SOD2 were also decreased. However, no difference in SOD1 expression was found among the groups (**Figure 4B**) while SOD2 gene expression (**Figure 4C**) was reduced only in HRD, but not in LRD group when compared with the control group. However, these results must be discussed with caution due to the variation among the groups when albumin expression was applied as control (Figure 4D). Treatment with *trans*-resveratrol does not affect the enzymatic activities of CAT (**Figure 5A**), SOD (**Figure 5B**) and GPx (**Figure 5C**) in comparison to the control group. Regarding the inflammatory biomarkers, there was a reduction of nuclear factor- $\kappa$ B (NF- $\kappa$ B) gene expression (**Figure 6A**) only in the LRD group in comparison to the CONT group. The pro-inflammatory cytokine IL-6 (**Figure 6B**) was increased by the supplementation in both doses, while no difference was found to TNF- $\alpha$  (**Figure 6C**) and IL-10 (**Figure 6D**). In addition, it was not observed difference in the TNF- $\alpha$ /IL-10 ratio (**Figure 6E**). **Figure 7** summarizes the biomarkers variation according to the three experimental groups.

## Discussion

*Trans*-resveratrol has shown considerable promising results due to multiple biological activities, such as vasorelaxant, neuroprotective, anti-inflammatory and antioxidant properties<sup>(34)</sup>. In a previous study carried in our group, it was observed that mice supplemented with about 800 mg/kg diet/day of *trans*-resveratrol, using a prevent protocol for 16 weeks, showed an increase in SOD activity in liver homogenate<sup>(35)</sup>. This result raised the hypothesis that the increase in SOD activity could have occurred via

Nrf2 activation. After, we showed that *trans*-resveratrol dose could be associated with different lipid profile in clinical trials<sup>(26)</sup>. Thus, in this study, it was investigated if the antioxidant effect of *trans*-resveratrol was dose-dependent and could occur via the Nrf2 pathway. To test this hypothesis, two doses of *trans*-resveratrol were selected to supplement the mice, using a prevention protocol.

It was observed that only the low dose (250 mg/kg diet/day) presented antioxidant effects, reducing MDA and increasing GSH/GSSG ratio in the liver, and that this effect was not a consequence of the Nrf2 pathway. In fact, mRNA expression of Nrf2 was reduced in both groups. In addition, the absence of changes in antioxidant enzymes activity (**Figure 5A-C**) and expression (**Figure 4B-C**) agrees with the Nrf2 result, since these enzymes have their expression modulated by Nrf2 pathway<sup>(36)</sup>. The reasons by which our results did not agree with those that identified Nrf2 as the mechanism responsible for the antioxidant action of *trans*-resveratrol can involve several factors. For example, *trans*-resveratrol can downregulate enzymes that are sources of ROS production, such as NADPH oxidase<sup>(8; 37)</sup>. Besides, the chemical formula of *trans*-resveratrol also influences Nrf2 activation. Hong *et al.* <sup>(38)</sup> observed that only *trans*-3,5,4'-trimethoxystilbene prevented the development of atherosclerotic lesions, increasing Nrf2 expression in THP-1 cell line.

Although the antioxidant activity found after the low dose supplementation has not been mediated by Nrf2 pathway, our hypothesis about the dose effect was confirmed, since no antioxidant response was observed to high dose of *trans*-resveratrol, except for the increase of SOD2 expression, that in turn, not reflected in MDA or GSH/GSSH ratio reduction. Some studies have demonstrated that a high dose may behave as a pro-oxidizing agent, leading to oxidative breakage of cellular DNA in

the presence of transition metal ions such as copper<sup>(22)</sup>. Under the chemical aspect, phenolic compounds in higher doses, including the stilbenes, instead of donating the hydrogen to the reactive specie promoting its neutralization, following then self-stabilizing by resonance of the double bond, they can directly react with the substrate causing its oxidation<sup>(39)</sup>. Take into accounting, it's possible that in our model the higher dose impaired the antioxidant response promoting an oxidative stress status.

In addition, it was also observed that the low dose (250 mg/Kg diet/day) of *trans*-resveratrol reduced NF-κB expression, without changing the other cytokines, except IL-6, which was increased by both doses. The NF-κB pathway is activated in the liver as a response to several stimuli, such as ligands of various cytokine receptors, pattern-recognition receptors (PRRs), TNF receptor superfamily members, or by oxidative stress<sup>(40)</sup>. According to Wang *et al.* <sup>(41)</sup>, *trans*-resveratrol suppresses the NF-κB and mitogen-activated protein kinase (MAPK) signaling cascades by inhibiting the toll-like receptor 4 (TLR4) signaling pathway. The TLR4 is a molecular link that is at the center of the events that connect the consumption of dietary fats with metabolic inflammation, and insulin resistance<sup>(42)</sup>. It is well known that prolonged and excessive consumption of the Western diet is associated with obesity-induced inflammation by continuously inducing the TLR4 signaling pathway <sup>(43; 44)</sup>. Besides, there is some debate as the Western diet might contribute to endotoxemia by causing changes in gastrointestinal barrier function which in turn leads to increased intestinal permeability and systemic exposure to bacterial lipopolysaccharide (LPS), a natural TLR4 ligand<sup>(45)</sup>.

In our model, NF-κB expression was stimulated by the high-fat diet associated with a lower capacity of clearing the LDL from the blood, due to the genetic alteration of the LDL receptors of the animals<sup>(46)</sup>. Thus, it can be suggested that the reduction of

NF- $\kappa$ B promoted by the low dose of *trans*-resveratrol has been a consequence of the reduced oxidative stress, since that ROS can modulate NF- $\kappa$ B activation induced by oxidative processes<sup>(47)</sup>. In the same way, IL-6 expression can be activated by other mechanisms than NF- $\kappa$ B, such as TLR, prostaglandins, adipokines, stress responses, and other cytokines<sup>(48)</sup>. IL-6 is known to be a pro-inflammatory cytokine, but also promotes liver regeneration and protects the liver against various forms of damage<sup>(49)</sup>. Previous studies have shown hepatic damage caused by the Western diet consumption for a long time, as it was applied in our model<sup>(50)</sup>. Thus, it can be suggested that *trans*-resveratrol could promote liver regeneration, but further studies are needed to clarify the role of *trans*-resveratrol supplementation in this clinical condition.

Only higher doses of *trans*-resveratrol may be able to improve the lipid profile, as has been observed in other studies, including our previous study<sup>(35)</sup>. Shao *et al.*<sup>(51)</sup> reported that *trans*-resveratrol can decrease plasma total cholesterol, triglycerides, and LDL concentrations, as well as increase HDL by regulating the level of the hepatic 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme or cholesterol 7 $\alpha$ -hydroxylase (CYP7A1). Nevertheless, results about lipid profile after *trans*-resveratrol supplementations are still controversial. Macarulla *et al.*<sup>(23)</sup> have indicated that low doses, up to 60 mg/kg body weight/day did not show any effect in lowering the lipid plasma levels. Also, there is clinical evidence suggesting no direct effect of *trans*-resveratrol on lipid-plasma levels<sup>(52)</sup>.

As shown in Figure 4D, albumin gene expression changed between the groups, with a high level in the CONT group, therefore, the gene expression results of this present study should be interpreted with caution. However, this unexpected result also suggests that the difference observed between the treated groups and control just

would be higher if the albumin expression was the same. It was not possible to identify the reasons by which *trans*-resveratrol increased albumin expression.

### **Conclusion**

Taking all results into account (**Figure 7**), it can be suggested that *trans*-resveratrol at a low dose showed a more promising effect than a high dose in improving oxidative status in the liver, and that this beneficial effect was not associated with an increase in the Nfr2 expression. The relevance of this information must be considered in further clinical trials, since *trans*-resveratrol has been consumed as an antioxidant without any dose control.

**Contributions:** T.M.S. and I.A.C designed the research. T.M.S., S.J.C. and G. C.G.C. performed animal experiments and laboratory analyses. J.D.Jr. and M.R.T. conducted RT-PCR analyses and data accurate. T.M.S. wrote original draft. M.M.R. and I.A.C. revised and edited the manuscript. All authors have read and approved this manuscript.

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**Conflict of Interest:** The authors declare no conflict of interest.

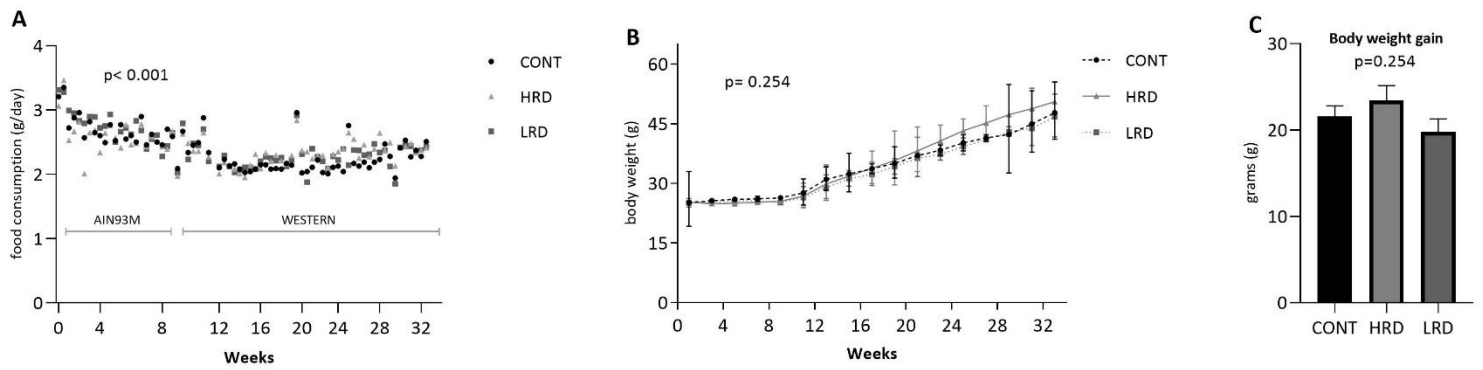
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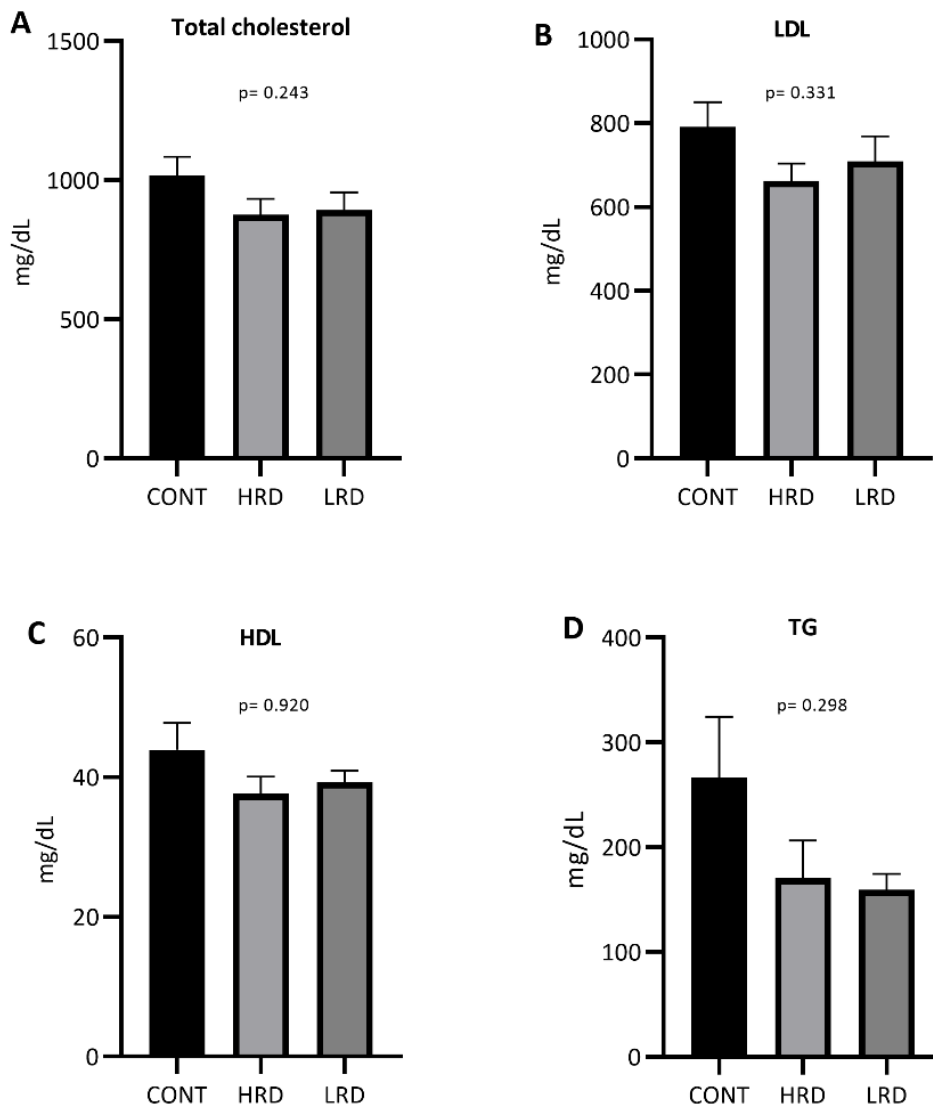
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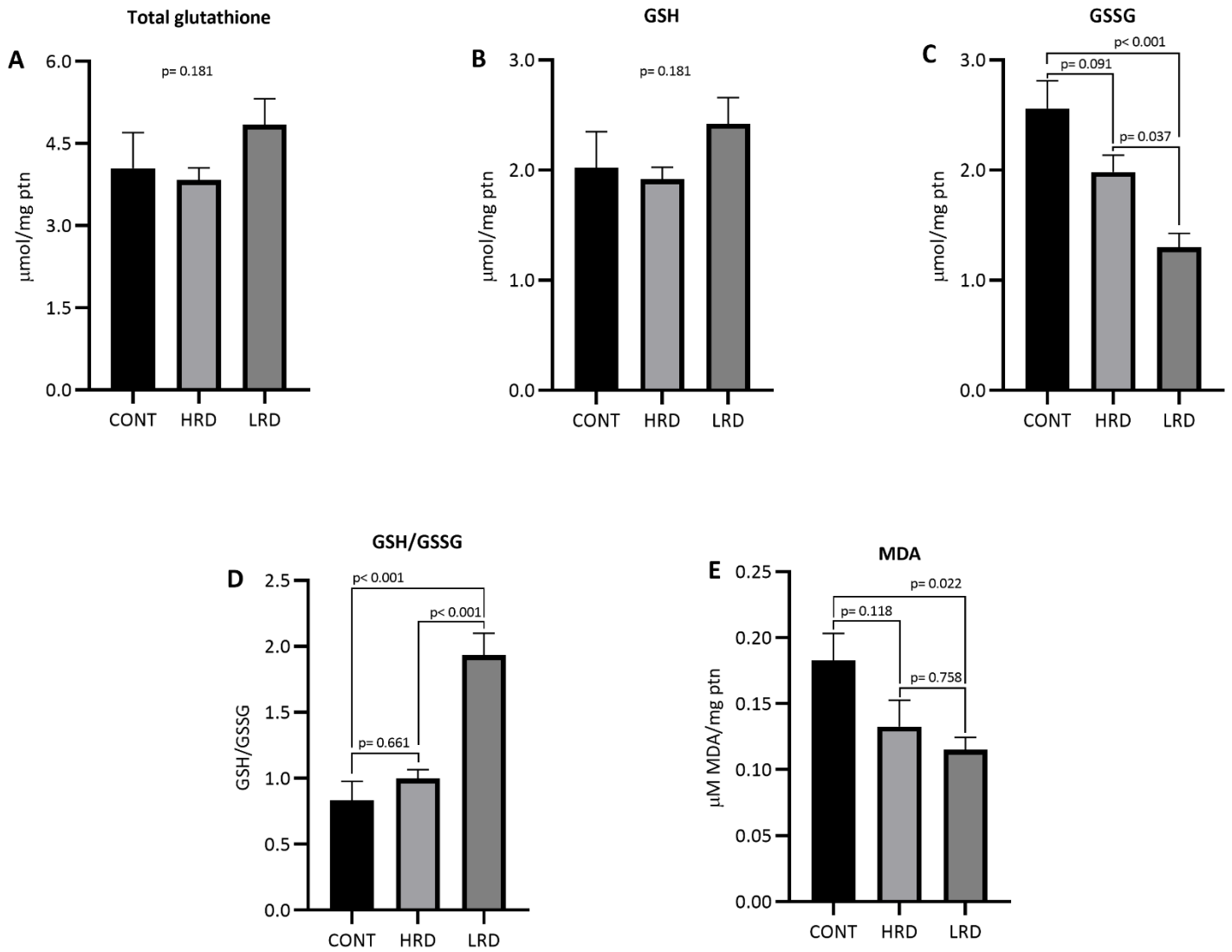
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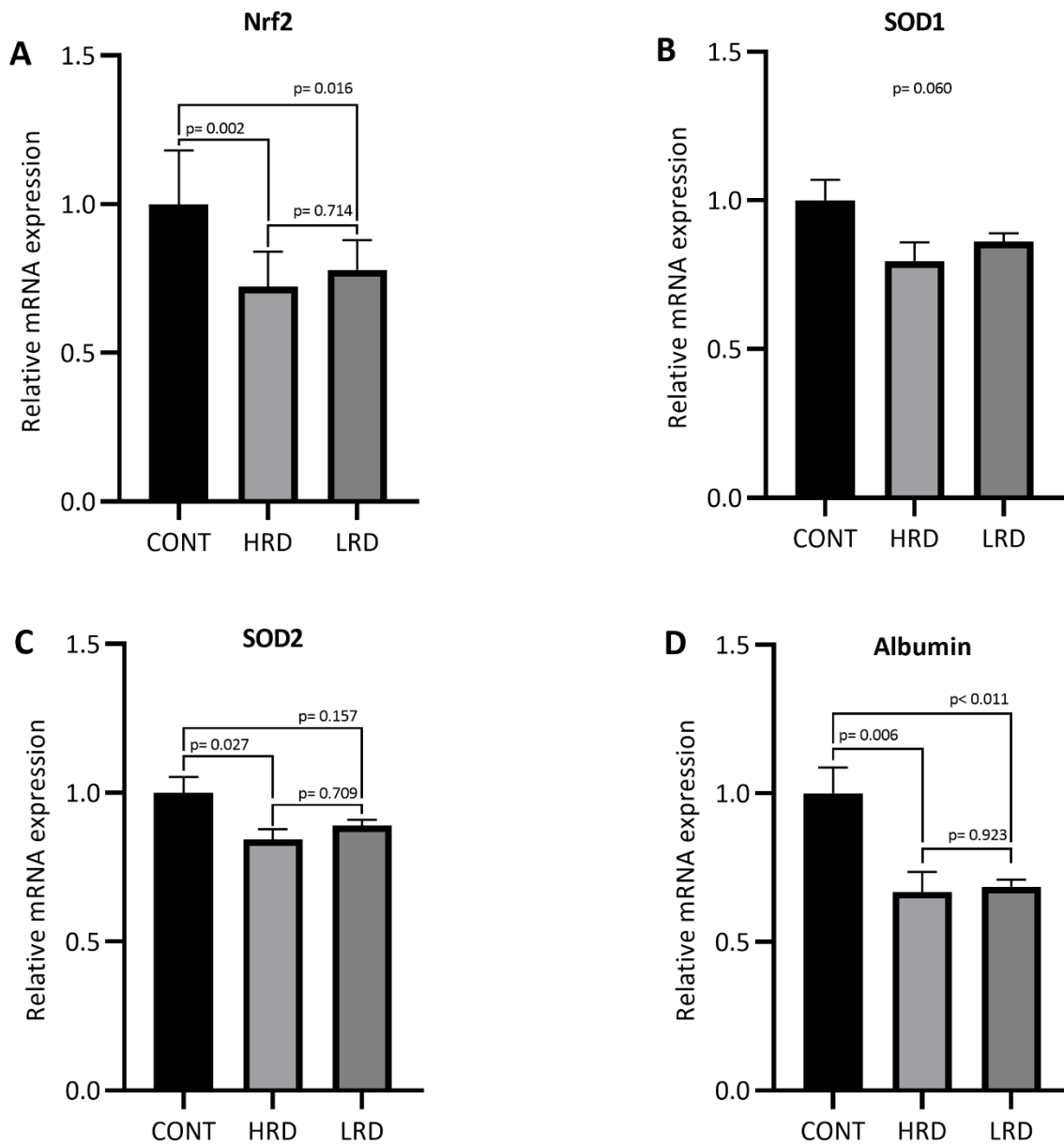
**Figure 1:** Effects of *trans*-resveratrol in food consumption and body weight during the experimental period. (A) food consumption, (B) body weight and (C) body weight gain. CONT: control group; HRD: high resveratrol dose group; LRD: low resveratrol dose group. Values are expressed as mean  $\pm$ SD.



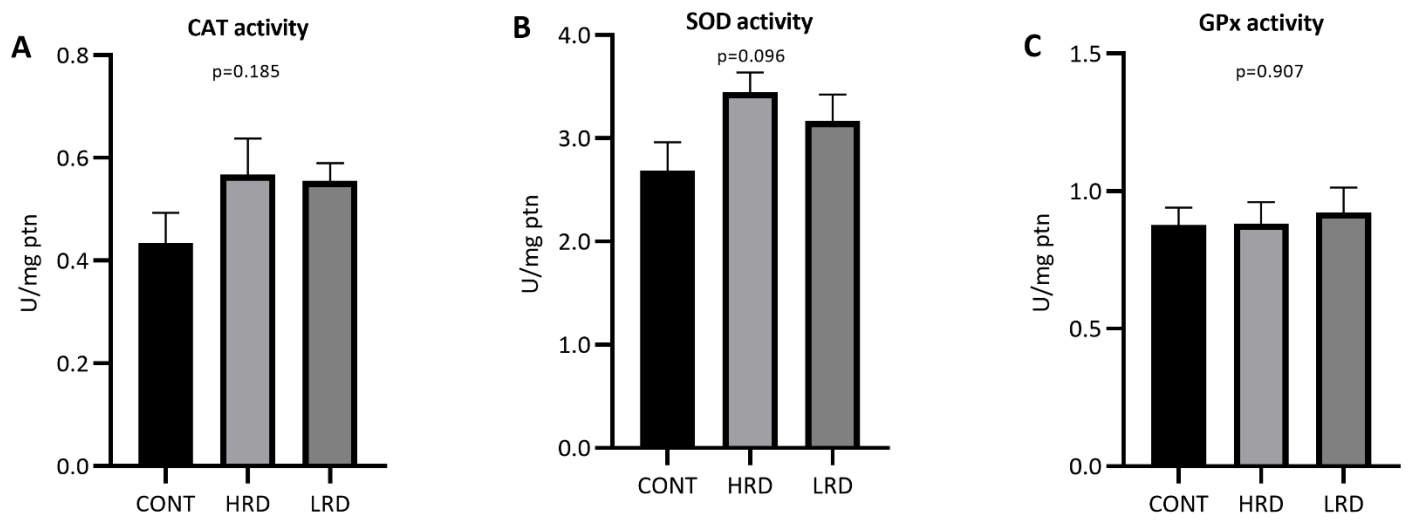
**Figure 2:** Effects of *trans*-resveratrol in lipid parameters. (A) Total cholesterol, (B) low-density lipoprotein, (C) high-density lipoprotein and (D) triglycerides. CONT, control group; HRD, high resveratrol dose group; LRD, low resveratrol dose group. Values are expressed as mean  $\pm$ SD.



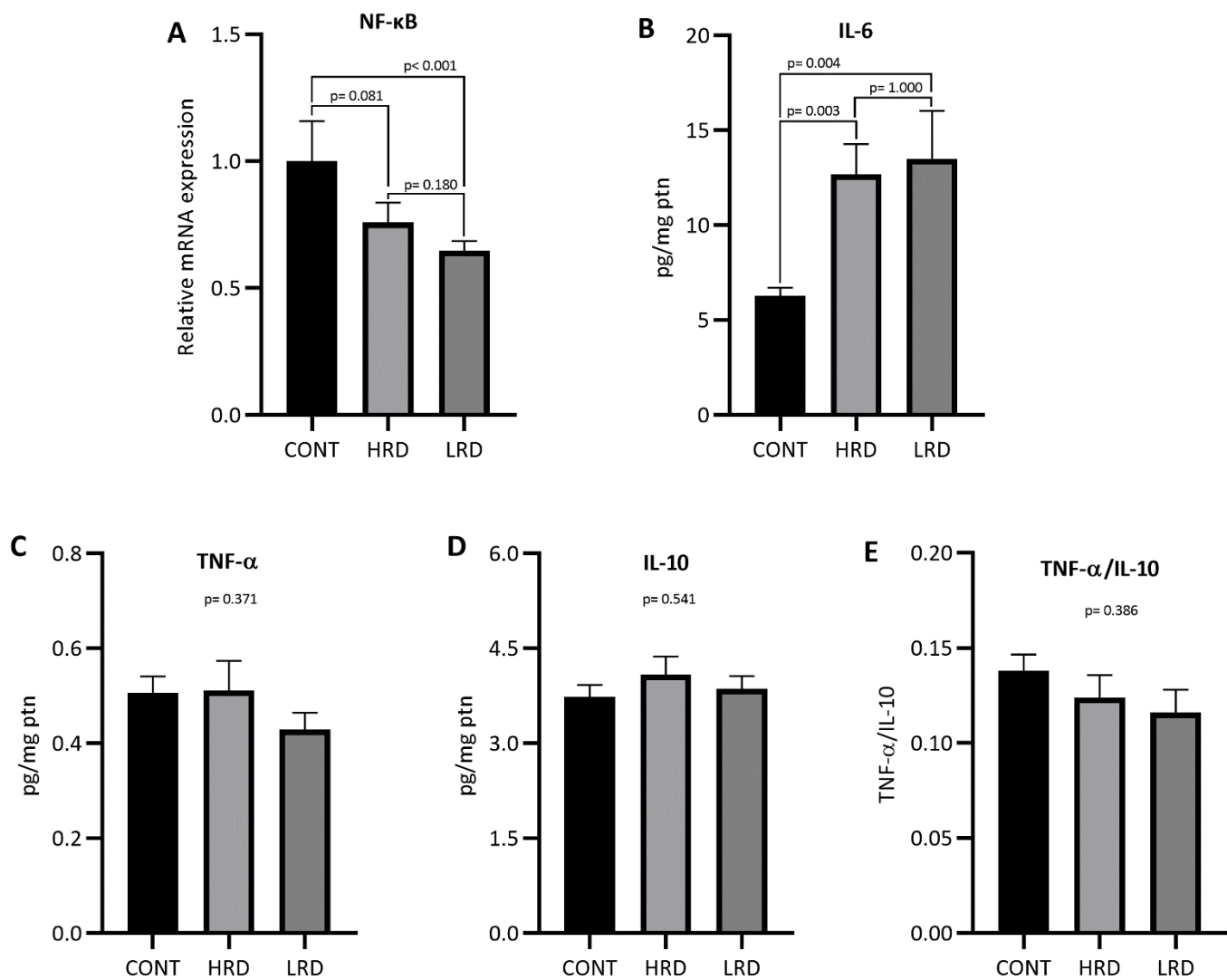
**Figure 3:** *Trans*-resveratrol effects in oxidative stress in the liver. (A) Total glutathione, (B) GSH, (C) GSSG, (D) GSH/GSSG and (E) MDA. CONT, control group; HRD, high resveratrol dose group; LRD, low resveratrol dose group. Values are expressed as mean  $\pm$ SD.



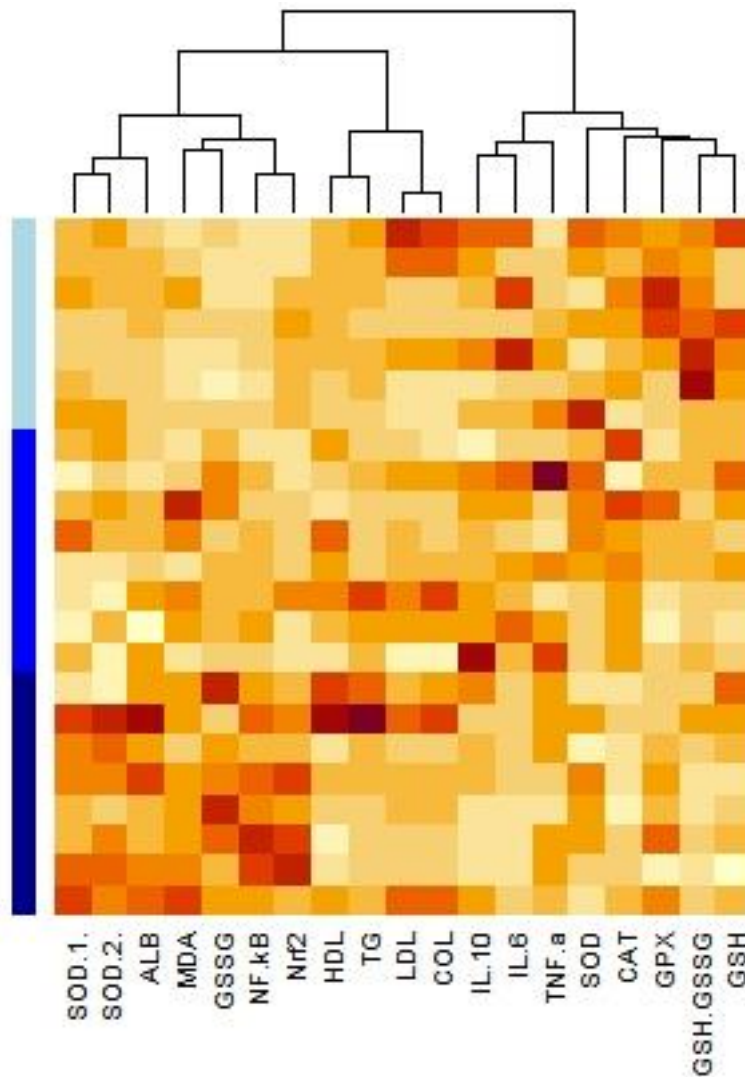
**Figure 4:** Effect of *trans*-resveratrol on the mRNA expression levels in the liver. (A) Nrf2, (B) SOD1, (C) SOD2 and (D) Albumin. CONT, control group; HRD, high resveratrol dose group; LRD, low resveratrol dose group. Values are expressed as mean  $\pm$ SD.



**Figure 5:** Effects of *trans*-resveratrol in antioxidant enzymes activity in the liver. (A) CAT, (B) SOD and (C) GPx. CONT, control group; HRD, high resveratrol dose group; LRD, low resveratrol dose group. Values are expressed as mean  $\pm$ SD.



**Figure 6:** Effects of *trans*-resveratrol in NF-κB expression and pro-cytokines levels in the liver. (A) NF-κB, (B) IL-6, (C) TNF-α, (D) IL-10 and (E) TNF-α /IL-10 ratio. CONT, control group; HRD, high resveratrol dose group; LRD, low resveratrol dose group. Values are expressed as mean ±SD.



**Figure 7:** Heatmap of the biomarkers according to the three experimental groups (CONT: darkblue, HRD: blue and LRD: lightblue), obtained by Cluster analysis of the standardized values. Abbreviations: SOD1, cytoplasmic superoxide dismutase mRNA expression; SOD2, mitochondrial superoxide dismutase mRNA expression; ALB, albumin; MDA, malondialdehyde; GSSG, glutathione disulfide; NF- $\kappa$ B, nuclear factor- $\kappa$ B; Nrf2, nuclear factor erythroid 2-related factor 2; HDL, high-density lipoprotein; TG, triglycerides; LDL, low-density lipoprotein; COL, total cholesterol; IL-10, interleukin-10; IL-6, interleukin-6; TNF- $\alpha$ , tumor necrosis factor alpha; SOD, superoxide dismutase activity; CAT, catalase activity; GPx, glutathione peroxidase; GSH, glutathione; GSG:GSSG ratio.

## Supplementary Data and Methods

### Supplementary Methods

#### *Trans*-resveratrol determination

The *trans*-resveratrol analysis was performed using high-performance liquid chromatography (HPLC; Agilent Technologies 1200 Series) following the method described by Piñeiro et al. (2006) with modifications. A solution of *trans*-resveratrol acquired to this study was evaluated in three concentrations (5.0, 25.0 and 60.0 mg/L) in methanol. Samples (30 µL) were injected in a reverse-phase C18 column (Agilent, Santa Clara, California, USA, 150 x 4.6 mm; 5µm) and quantified by fluorescence detection at an excitation wavelength of 310 nm and emission of 403 nm. The chromatographic analysis was performed using a mobile phase consisting of acetonitrile (A) and an aqueous formic acid solution 0.1% (B) with the following gradient: 8 – 100% of solvent A and 92 – 0% of solvent B in 0 – 11 min, at a flow rate of 1.0 mL/min. Column temperature was kept at 37°C. The identification of *trans*-resveratrol was carried out by comparing the retention time with the pure standard (Sigma - PHL89539), and the quantification was performed using a standard curve (5 – 60 mg/L;  $r=0.9961$ ). Analysis was run in triplicate.

### Supplementary Data

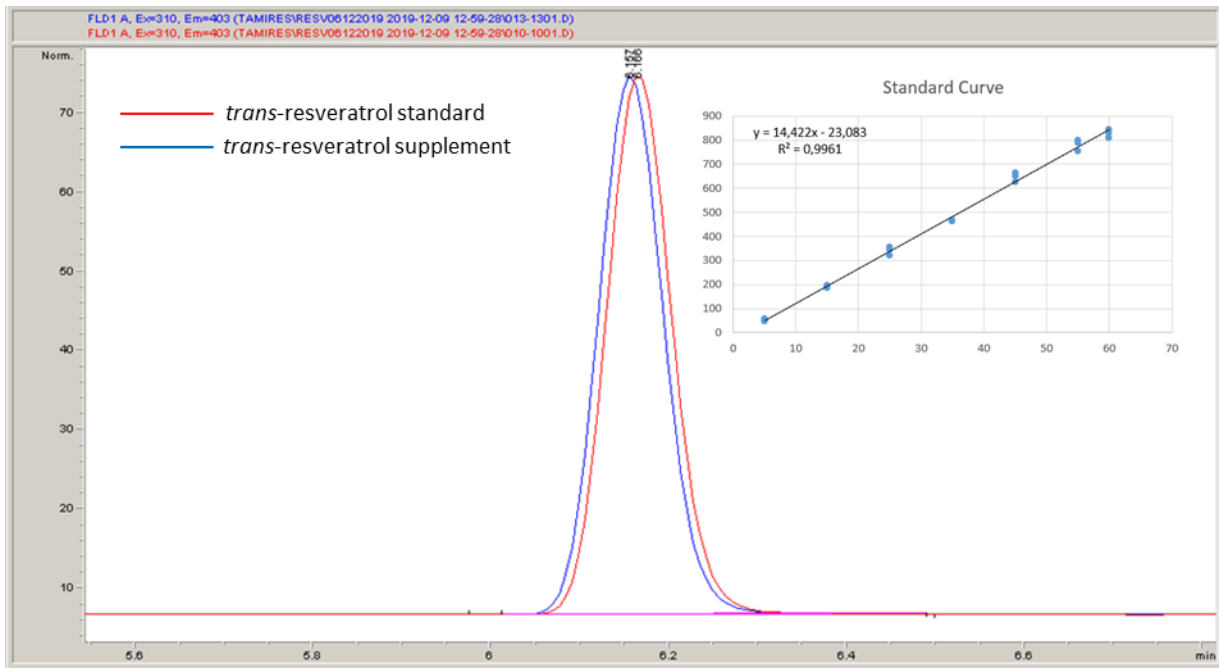
**Supplementary Table 1:** Expected and observed concentration of *trans*-resveratrol sample applied in the biological assay.

Expected concentration (mg/L)	Observed concentration <sup>1</sup> (mg/L)
5	5.46 ± 0.23
25	26.28 ± 0.56
60	61.72 ± 4.26

<sup>1</sup>Standard curve: 5 – 60 mg/L;  $R^2=0.9961$

Values expressed as mean ± SD





**Supplementary Figure 2:** Peak of *trans*-resveratrol sample applied in the biological assay and standard (SIGMA - PHL89539)

**Supplementary Table 2.** Composition of the diets

Ingredient (g)	AIN93M <sup>1</sup>	WESTERN <sup>2</sup>
Cornstarch	620.692	-
Maltodextrin	-	161.530
Casein (>85% protein)	140.000	258.448
Sucrose	100.000	88.900
Lard	-	316.600
Soybean oil (no additives)	40.000	32.300
Fiber	50.000	64.600
Cholesterol	-	6.250
Mineral mix (AIN93M – MX)	35.000	-
Mineral mix (S10026)	-	12.900
DiCalcium Phosphate	-	16.800
Calcium carbonate	-	7.100
Potassium citrate	-	21.300
Vitamin mix (AIN93M – VX)	10.000	-
Vitamin mix (V10001)	-	12.900
L-Cistine	1.800	3.870
Choline bitartrate (41,1% colina)	2.500	2.600
TBHQ (Terq-butyhydroquinone)	0.008	-
Total	1.000.000	1.000.000

<sup>1</sup>Based on Reeves et al. (1993)

<sup>2</sup>Western diet (D12492) according to Research Diets (2017)

**Supplementary Table 3.** Chemical composition of the diets.

Diet	Nutrients <sup>1</sup>					
	Moisture	Ash	Lipid	Protein	Carbohydrate <sup>2</sup>	Energy <sup>3</sup>
AIN93M – CONT	10.07 ± 0.12	3.05 ± 0.00	6.35 ± 0.52	16.17 ± 0.32	64.63 ± 0.43	380.75 ± 0.31
AIN93M – HRD	12.16 ± 0.12	2.97 ± 0.07	6.23 ± 0.52	13.75 ± 0.53	64.89 ± 0.93	369.44 ± 1.53
AIM93M – LRD	11.35 ± 0.03	3.05 ± 0.13	6.21 ± 0.54	13.62 ± 0.63	65.76 ± 1.09	371.74 ± 0.29
Western – CONT	3.80 ± 0.12	2.45 ± 0.11	36.46 ± 2.54	28.36 ± 0.99	28.94 ± 2.30	564.22 ± 5.97
Western - HRD	3.86 ± 0.03	3.13 ± 0.08	36.51 ± 0.32	32.06 ± 1.53	24.44 ± 1.24	554.54 ± 168
Western - LRD	3.82 ± 0.03	3.00 ± 0.06	37.30 ± 0.27	31.58 ± 0.94	24.29 ± 0.78	559.20 ± 1.68

<sup>1</sup> Values expressed in mean ± SD

<sup>2</sup> Obtained by difference

<sup>3</sup> Energy in Kcal/100g

## **6. Final considerations**

The potential of *trans*-resveratrol to modulate antioxidant pathways has garnered much attention by preventing the development of metabolic and cardiovascular diseases. Our study shows that the *trans*-resveratrol has different patterns of effects according to the dosage, which highlights the necessity to select the optimal dosage according to the target to be achieved. In general, we observed that *trans*-resveratrol in doses of about 500 mg/day represents an excellent approach to reducing biomarkers related to atherosclerosis and oxidative stress. However, we did not observe the expected results in the inflammation biomarkers and this effect appears to be mediated via a variety of intracellular signaling pathways. Therefore, further experimental studies and clinical trials are needed not only to investigate end-points, but also to elucidate the exact mechanism of activity, which is not yet fully understood.

## **7. Future perspectives**

Next studies could evaluate the effect of *trans*-resveratrol against oxidative stress and inflammation *in vitro* using macrophages and/or HUVECS challenged with a stressor, such as oxLDL, and apply molecular methodologies to describe its activity on signaling pathways. Based on safe and efficient dosage, clinical trials could be carried out aiming to combine *trans*-resveratrol with drugs applied to patients under primary or secondary prevention for CVD, improving their adherence to the treatment and general life quality.

## ATTACHMENTS

### Ethics commit approval



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



CEUA/FCF 083.2019-P595

### CERTIFICADO

Certificamos que a proposta intitulado **Efeito da suplementação de transresveratrol na expressão do fator de transcrição Nrf2 e seu impacto na prevenção da aterosclerose**, registrada com o nº 595, sob a responsabilidade do(a) pesquisador(a) **Tamires Miranda Santana**, sob orientação da **Profa. Dra. Inar Alves de Castro** – que envolve produção ou manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei Federal nº 11.794, de 8 de outubro de 2008, do Decreto Federal nº 6.899, de 15 de julho de 2009, e das normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovada pela Comissão de Ética no Uso de Animais (CEUA) da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (FCF/USP), em reunião de **01 de Novembro de 2019**.

Finalidade	Pesquisa Científica
Vigência da autorização	01/01/2020 a 31/12/2020
Espécie/linhagem/raça	Camundongo <i>Mus musculus</i> – C57BL/6 knockout LDLr
Número de animais	52
Sexo	Machos
Peso/Idade	3 meses – 25g
Origem	Biotério FCF-IQ-USP

Conforme a legislação vigente, deverá ser apresentado, no encerramento do projeto de pesquisa, o respectivo **relatório final**.

**Ressaltamos que após o período de término do projeto de pesquisa, nenhum ensaio poderá ser realizado.**

São Paulo, 04 de novembro de 2019.

**Profa. Dra. Cristina Stewart Bittencourt Bogsan**  
Vice Coordenadora 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



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



Of.CIBio/0272019/FCF

São Paulo, 19 de setembro de 2019.

Senhora Professora,

Informo a Vossa Senhoria que em reunião da Comissão Interna de Biossegurança da Faculdade de Ciências Farmacêuticas realizada nesta data., o Projeto *"Efeito da suplementação de trans-resveratrol na expressão do fator de transcrição Nrf2 e seu impacto na prevenção da aterosclerose"* foi **aprovado**, com os esclarecimentos apresentados em 29/07/2019, anexo ao presente.

Atenciosamente,

  
Prof. Dr. JOÃO CARLOS MONTEIRO DE CARVALHO  
Presidente da CIBio

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

Trans-resveratrol certificate analysis



Handwritten signature or initials.

**CERTIFICADO DE ANÁLISE**

Insumo:	Resveratrol (Trans.)	Data de Análise:	11-05-2019
Lote Interno:	19E01-B021-046784	Lote Fabricante:	20181224
Data de Fabricação:	24-12-2018	Data de Validade:	23-12-2020
Origem:	China	Procedência:	China
Condições de Armazenamento:	Temperatura até 25 °C	Ordem de Fracionamento:	046784

DCB:	11392	DCI:	-
CAS:	501-36-0	Peso Molecular:	228,246
Fórmula Molecular:	C14H12O3		
Observações:	Parte Utilizada:	Raiz	Nome Científico: Polygonum cuspidatum

Testes	Especificações	Resultados	Unidade	Referências
Descrição *	Pó quase branco, com odor característico.	Conforme		Fabricante
Sabor	Característico.	Conforme		Fabricante
Perda por dessecação *	<= 0,5 (2g/ 3 horas/ 105°C)	0,12 /	%	Fabricante
Ponto de fusão *	258 - 263	260,4	°C	Fabricante
Metais pesados *	<= 20	< 20	ppm	Fabricante
Solvente residual (CG) *	Etanol <= 5000	Conforme		Fabricante
Teor (HPLC) *	Resveratrol: >= 98,0	99,10	%	Fabricante
Testes microbiológicos				
Contagem total de bactérias *	<= 1000	< 10	UFC/g	Fabricante
Fungos e leveduras *	<= 100	< 10	UFC/g	Fabricante
Escherichia coli *	Negativo	Negativo		Fabricante
Salmonella *	Negativo	Negativo		Fabricante
Staphylococcus *	Negativo	Negativo		Fabricante
Testes adicionais				
Densidade aparente *	Informativo (Sem compactação)	0,33 /	g/mL	MGFB - V
Cinzas insolúveis em ácidos *	<= 1,5	0,13	%	MGFB - V
Matéria estranha microscópica *	Ácaros mortos <= 5,0	< 5,0		MGFB - V

\* Resultados obtidos em análises realizadas no Laboratório de Controle de Qualidade SM EMPREENDIMENTOS FARMACÉUTICOS LTDA. E os demais foram transcritos conforme certificado de análise do fabricante.

**Conclusão:**


Aprovado (X)  
Reprovado ( )



### CERTIFICADO DE ANÁLISE

Insumo:	Resveratrol (Trans.)	Data de Análise:	11-05-2019
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Farmacêutico Responsável  
Adriana M. Correia  
CRF-SP: Nº 72.989  
Organic Compounding  
SAC: (11) 3014-7100

Fim do Documento

AQUISIÇÃO MANIPULE CSGQ  
Nº IDENTIF. 010.765  
CHEGADA NF 24/05/19  
PESO CONFERIDO 100g  
DATA CONFERENC 24/05/19  
RESPONS ESTOQUE *[Signature]*

CQ MANIPULE CSGQ  
DATA ANÁLISE 04/07/19  
RESPONS CQ *[Signature]*  
FARMACÊUTICO *[Signature]*  
APROVADO

## Student record

12/05/2022 20:51

**Janus** - Sistema Administrativo da Pós-Graduação



### Universidade de São Paulo Faculdade de Ciências Farmacêuticas

#### FICHA DO ALUNO

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**9132 - 11302992/2 - Tamires Miranda Santana**

**Email:** tsantana@usp.br  
**Data de Nascimento:** 20/07/1993  
**Cédula de Identidade:** RG - 49.305.004-8 - SP  
**Local de Nascimento:** Estado de São Paulo  
**Nacionalidade:** Brasileira  
**Graduação:** Nutricionista - Universidade Anhembi Morumbi - São Paulo - Brasil - 2019

---

**Curso:** Mestrado  
**Programa:** Ciência dos Alimentos  
**Área:** Nutrição Experimental  
**Data de Matrícula:** 07/05/2019  
**Início da Contagem de Prazo:** 07/05/2019  
**Data Limite para o Depósito:** 03/11/2022  
**Orientador:** Prof(a). Dr(a). Inar Castro Erger - 07/05/2019 até o presente. Email: inarcastro@gmail.com  
**Proficiência em Línguas:** Inglês, 07/05/2019  
**Data de Aprovação no Exame de Qualificação:** Aprovado em 31/03/2021  
**Data do Depósito do Trabalho:**  
**Título do Trabalho:**  
**Data Máxima para Aprovação da Banca:**  
**Data de Aprovação da Banca:**  
**Data Máxima para Defesa:**  
**Data da Defesa:**  
**Resultado da Defesa:**  
**Histórico de Ocorrências:** Primeira Matrícula em 07/05/2019

---

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 6542 em vigor de 20/04/2013 até 28/03/2018).

**Última ocorrência:** Matrícula de Acompanhamento em 21/03/2022

**Impresso em:** 12/05/2022 20:50:51





**Universidade de São Paulo**  
**Faculdade de Ciências Farmacêuticas**  
**FICHA DO ALUNO**

9132 - 11302992/2 - Tamires Miranda Santana

Sigla	Nome da Disciplina	Início	Término	Carga Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBC5752-4/2	Danos em Biomoléculas e o seu Papel no Monitoramento da Exposição a Agentes Tóxicos	31/05/2019	05/07/2019	45	3	100	A	N	Concluída
BMF5864-3/2	Fisiopatologia das Espécies Reativas de Oxigênio (Instituto de Ciências Biomédicas - Universidade de São Paulo)	06/08/2019	07/10/2019	90	0	-	-	N	Turma cancelada
QBQ5893-3/3	Processos Redox em Bioquímica (Instituto de Química - Universidade de São Paulo)	06/08/2019	18/11/2019	60	4	93	A	N	Concluída
MCM5926-1/3	Células e Moléculas da Resposta Imune Inata (Faculdade de Medicina - Universidade de São Paulo)	23/09/2019	06/10/2019	60	4	100	A	N	Concluída
HNT5759-3/3	Genômica Nutricional no Contexto das Doenças Crônicas não Transmissíveis (Faculdade de Saúde Pública - Universidade de São Paulo)	05/11/2019	09/12/2019	60	4	100	A	N	Concluída
FBA5907-1/2	Lipídios – Estabilidade Oxidativa e Funcionalidade Cardiovascular	10/02/2020	23/02/2020	60	4	100	A	N	Concluída
MCM5865-3/3	Prática de Laboratório VI (Modelos Experimentais em Inflamação) (Faculdade de Medicina - Universidade de São Paulo)	02/03/2020	15/03/2020	60	4	100	A	N	Concluída
FBA5728-4/9	Aprimoramento Pedagógico	17/03/2020	13/04/2020	60	0	-	-	N	Turma cancelada
EDM5188-1/2	Preparação Pedagógica PAE: mediação pedagógica remota (Faculdade de Educação - Universidade de São Paulo)	03/09/2020	14/10/2020	60	4	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito da dissertação	
<b>Disciplinas:</b>	0	25	27
<b>Estágios:</b>			
<b>Total:</b>	0	25	27

Créditos Atribuídos à Dissertação: 71

**Conceito a partir de 02/01/1997:**

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

Última ocorrência: Matrícula de Acompanhamento em 21/03/2022

Impresso em: 12/05/2022 20:50:51

## **Complementary work**

**Poster presentation at EAS Congress 2021:** P609 / #1401, E-POSTERS TOPIC: 4. CLINICAL VASCULAR DISEASE / 4.07 NUTRITION, NUTRACEUTICALS. Santana, T. S., Ogawa, L. Y., Rogero, M. M., Barroso, L. P., & De Castro, I. A. (2021). Effect of supplementation with resveratrol on biomarkers associated with atherosclerosis in humans. *Atherosclerosis*, 331, e251.

**Video presentation at XXIV Semana Farmacêutica de Ciência e Tecnologia (FCF-USP):** Santana, T. S., Ogawa, L. Y., Rogero, M. M., Barroso, L. P., & De Castro, I. A. (2021). Effect of supplementation with resveratrol on biomarkers associated with atherosclerosis in humans

**Co-authorships:** Rogero, M. M., Leão, M. D. C., **Santana, T. M.**, de MB Pimentel, M. V., Carlini, G. C., da Silveira, T. F., ... & Castro, I. A. (2020). Potential benefits and risks of omega-3 fatty acids supplementation to patients with COVID-19. *Free Radical Biology and Medicine*.