DIPARTIMENTO SCIENZE DELLA VITA

**DOTTORATO DI RICERCA IN SCIENZE DELLA VITA**

CICLO **XXXIV**

COORDINATORE **Prof. Massimo Valoti**

# *IN VITRO* FUNCTIONAL INTERPLAY BETWEEN PERIVASCULAR ADIPOSE TISSUE AND FLAVONOIDS: CRITICAL ROLE OF BETA3 RECEPTOR AND SUPEROXIDE ANION

SETTORE SCIENTIFICO-DISCIPLINARE: BIO/14

Amer Ahmed Ghalb Faraa

Signed 11/05/2022

TUTOR: **Prof. Massimo Valoti** DOTTORANDO: **Faraa Amer Ahmed Ghalb**

CO-TUTOR: **Dr. Fabio Fusi**

A.A. 2021-2022

# Table of Contents

# List of Figures

# List of Tables

## List of Abbreviations

| | |
|---|---|
| ABC | ATP-binding cassette |
| ACE1/2 | Angiotensin converting enzyme 1/2 |
| ADRF | Adipocyte-derived relaxing factor |
| AT1R | Angiotensin II receptor type 1 |
| AT2R | Angiotensin II receptor type 2 |
| $BK_{Ca}$ | Large conductance $Ca^{2+}$-dependent $K^+$ channel |
| BMI | Body mass index |
| CBS | Cystathionine β-synthase |
| cGMP | Cyclic guanosine monophosphate |
| COMT | Catechol-O-methyltransferase |
| COX-1/2 | Cyclooxygenases 1/2 |
| CSE | Cystathionine γ-synthase |
| DAG | Diacylglycerol |
| eNOS | Endothelial NO synthase |
| GPCR | G-protein coupled receptors |
| HDL | High-density lipoprotein |
| IP3 | Inositol 3,4,5-triphosphate |
| $K_{ATP}$ | ATP-sensitive $K^+$ channel |
| $K_{ir}$ | Inward rectifier $K^+$ channel |
| KLF-2 | Krüpple like factor-2 |
| $K_V$ | Voltage-dependent $K^+$ channel |
| LDL | Low-density lipoprotein |
| L-NAME | N-ω-nitro-L-arginine methyl ester |
| LPH | Lactase phlorizin hydrolase |
| MLC | Myosin light chain |
| MLCK | Myosin light chain kinase |
| MLCP | Myosin light chain phosphatase |
| NF-kB | Nuclear factor kappa-light-chain enhancer of activated B cells |
| nNOS | Neuronal NO synthase |
| Nrf2 | Nuclear factor erythroid-derived 2-related factor 2 |
| PAME | Palmitic acid methyl ester |
| PDCF | PVAT-derived contracting factors |

| | |
|---|---|
| PDRF | PVAT-derived relaxing factors |
| PGE2 | Prostaglandin E2 |
| PGI2 | Prostacyclin |
| PKC | Protein kinase C |
| PLA2 | Phospholipase A2 |
| PPAR-γ | Peroxisome proliferator-activated receptor γ |
| PVAT | Perivascular adipose tissue |
| RAS | Renin-angiotensin system |
| ROCC | Receptor-operated $Ca^{2+}$ channels |
| ROCK | Rho kinase |
| ROS | Reactive oxygen species |
| sGC | Soluble guanylyl cyclase |
| SHR | Spontaneously hypertensive rat |
| SOCC | Store-operated $Ca^{2+}$ channels |
| SOD | Superoxide dismutase |
| TNF-α | Tumor necrosis factor-α |
| TXA2 | Thromboxane A2 |
| UCP-1 | Uncoupling protein-1 |
| VCAM-1 | Vascular cell adhesion protein 1 |
| VDCC | Voltage-dependent $Ca^{2+}$ channel |
| VLDL | Very low-density lipoprotein |
| VSMC | Vascular smooth muscle cells |

**Abstract (in English)**

Flavonoids, a class of natural polyphenols abundantly present in our diet, have been shown to exert *in vitro* vasorelaxant activity. This was ascribed to a direct effect on the smooth muscle or factors released by the endothelium. Nowadays, perivascular adipose tissue (PVAT) is emerging as a fine regulator of blood vessel contractility. Therefore, it is conceivable to hypothesize that flavonoids vasoactivity may occur also through or is influenced, either positively or negatively, by PVAT-released factors. This hypothesis was assessed *in vitro* on rat aorta rings. Several flavonoids proved to display both antispasmodic and spasmolytic activity towards noradrenaline-induced contraction of rings deprived of PVAT (-PVAT). However, when PVAT was present (+PVAT), both activities of some flavonoids were lost and/or much decreased. In rings-PVAT, the superoxide donor pyrogallol mimicked the effect of PVAT, whereas in rings+PVAT the antioxidant mito-tempol restored both activities of the two most powerful flavonoids, namely apigenin and chrysin. The Rho-kinase inhibitor fasudil, or apigenin and chrysin concentration-dependently relaxed the vessel active tone induced by the Rho-kinase activator NaF; the presence of PVAT counteracted apigenin spasmolytic activity though only in the absence of mito-tempol. Similar results were obtained in rings pre-contracted with phenylephrine. Finally, when $\beta_3$ receptors were blocked by SR59230A, the vasorelaxant activity of both flavonoids was no more affected by PVAT. These findings are coherent with the hypothesis that both noradrenaline and apigenin activated adipocyte $\beta_3$ receptors with the ensuing release of mitochondrial superoxide anion, which once diffused toward myocytes counteracted flavonoid vasorelaxant activity, thus underlining the control of adipocytes upon the vascular tone. This phenomenon might limit the beneficial health effects of this class of natural compounds in patients affected by either obesity and/or other pathological conditions characterized by sympathetic nerve overactivity.

**Abstract (In Italian)**

Numerosi studi dimostrano gli effetti benefici sul sistema cardiovascolare esercitati dai flavonoidi assunti con la dieta. Poiché il tessuto adiposo perivascolare (PVAT) ricopre un ruolo attivo nella regolazione del tono vasale, sia in condizioni fisiologiche sia patologiche, è lecito ipotizzare che l'attività vascolare dei flavonoidi possa manifestarsi anche attraverso il o sia influenzata, positivamente o negativamente, da fattori rilasciati dal PVAT. Quest ipotesi è stata valutata utilizzando come modello sperimentale anelli di aorta di ratto *in vitro*. Diversi flavonoidi tra quelli saggiati esercitavano un'azione sia antispasmodica sia spasmolitica nei confronti della contrazione indotta da noradrenalina in anelli privi di PVAT (-PVAT). Tuttavia, in presenza di PVAT (+PVAT), entrambe le attività venivano ridotte o addirittura perse. In anelli-PVAT, il donatore di anione superossido pirogallolo riproduceva gli effetti osservati in presenza di PVAT, mentre in anelli+PVAT l'antiossidante mitocondriale mito-tempol ripristinava entrambe le attività dei due flavonoidi più efficaci, apigenina e crisina. L'inibitore della Rho-chinasi fasudil, l'apigenina e la crisina rilassavano in maniera concentrazione-dipendente il tono attivo indotto dall'attivatore della Rho-chinasi NaF; la presenza di PVAT antagonizzava l'attività spasmolitica dell'apigenina, anche se solo in assenza di mito-tempol. Risultati simili venivano ottenuti in anelli pre-contratti con fenilefrina. Infine, quando i recettori $\beta_3$ venivano bloccati con SR59230A, l'attività vasorilassante di entrambi i flavonoidi non era più influenzata dalla presenza del PVAT. Questi risultati sono coerenti con l'ipotesi che sia la noradrenalina sia l'apigenina attivavano i recettori $\beta_3$ degli adipociti con il conseguente rilascio di anione superossido; questo, diffondendo nei sottostanti miociti, antagonizzava l'attività vasorilassante dei due flavonoidi, sottolineando così il controllo esercitato dagli adipociti sul tono vasale. Tale fenomeno potrebbe limitare gli effetti benefici sulla salute umana esercitati da questa classe di composti naturali in pazienti affetti da obesità o altre condizioni patologiche caratterizzate da una elevata attività simpatica.

# Acknowledgements

I am greatly delighted to place on record my profound gratitude and cordial thanks equally to my distinguished tutors **Prof. Massimo Valoti** and **Prof. Fabio Fusi**, for their excellent guidance and patience during my research, who extended all facilities and provided stimulating guidance for the victorious achievement of my research work. I believe it as my privilege to work in his competent leadership.

I would like also to thank Prof. Simona Saponara, Prof. Maria Frosini and Beatrice Gorelli for their great help, support, encouragement, and pleasant association extended throughout my research. I am also grateful to my friends and lab fellows: Dr. Alice Panti, Dr. Ahmed Shehata, Dr. Aasia Bibi, Dr. Adhban Omar, Gioele Mancini, Muhammad Jawad, Robel Alemu, Bilal Jamal, Alesio Mancini, Federica, Laura, Dumi, Anna, Elda, Sofia, Gemma, Rony, Giada, and Stefania for their cooperation, valuable suggestions, moral support, and help at every moment of my life and research work. My deepest gratitude goes to my beloved mother, brother and sisters for their endless love, prayers and encouragement.

Finally, I would also like to thanks the University of Siena for providing the PhD studentship that enable me to carry out the work presented in this thesis.

# CHAPTER 1

# INTRODUCTION

# 1. Introduction

Blood vessels are comprised of three layers. *Tunica intima* is the innermost layer consisting of a single sheet of endothelial cells lining and anchored to the lumen of vessels by a sub-endothelial layer/basal membrane (Milutinović et al., 2020). *Tunica media,* the middle layer, is primarily made of vascular smooth muscle cells (VSMCs) embedded in elastin and collagen fibres. *Tunica adventitia*, the outermost layer, contains fibroblast, *vasa vasorum*, nerves, and immune cells (Stenmark et al., 2013; Ahmed and Warren, 2018). Most blood vessels are surrounded by a fourth layer of adipose tissue called perivascular adipose tissue (PVAT), harbouring mainly adipocytes, resident immune cells (like macrophages, T-cells, and mast cells), perivascular nerves, and endothelial cells (Fig. 1.1). PVAT influences the vascular network in both health and disease states (Majesky, 2015). The following sections discuss these layers, focusing mainly on endothelium, VSMCs, and in more detail PVAT, and their relevance to vascular homeostasis.

## 1.1 Endothelium

The endothelium, a single layer of cells lining the lumen of the entire vascular system from the heart to the capillaries, contributes to the normal functioning of the vascular system. It forms a physical barrier separating the blood from underlying tissues, controls the movements of macromolecules, regulates vascular tone and blood flow, attenuates VSMCs proliferation and migration, reduces leukocyte adhesion and activation, minimizes platelet aggregation, and prevents thrombus formation (Rajendran et al., 2013; Li et al., 2018). Notably, endothelium exerts these functions by producing numerous factors such as NO, prostacyclins (PGI2), hydrogen peroxide ($H_2O_2$), and others, which act *via* autocrine, paracrine and/or endocrine modes (Triggle et al., 2012).

In particular, NO is a pivotal signalling molecule synthesized by vascular endothelial cells to coordinate their functions (Forstermann and Sessa, 2011). $Ca^{2+}$-dependent endothelial NO synthase (eNOS) synthesizes NO from L-arginine. Once synthesized, NO readily diffuses to neighbouring cells to elicit vasodilation of underlying VSMCs, attenuate their proliferation and migration, and or prevent platelet aggregation. In VSMC, NO binding to and the ensuing activation of soluble guanylyl cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP), initiates a signalling cascade leading to VSMC relaxation (Chen et al., 2008).

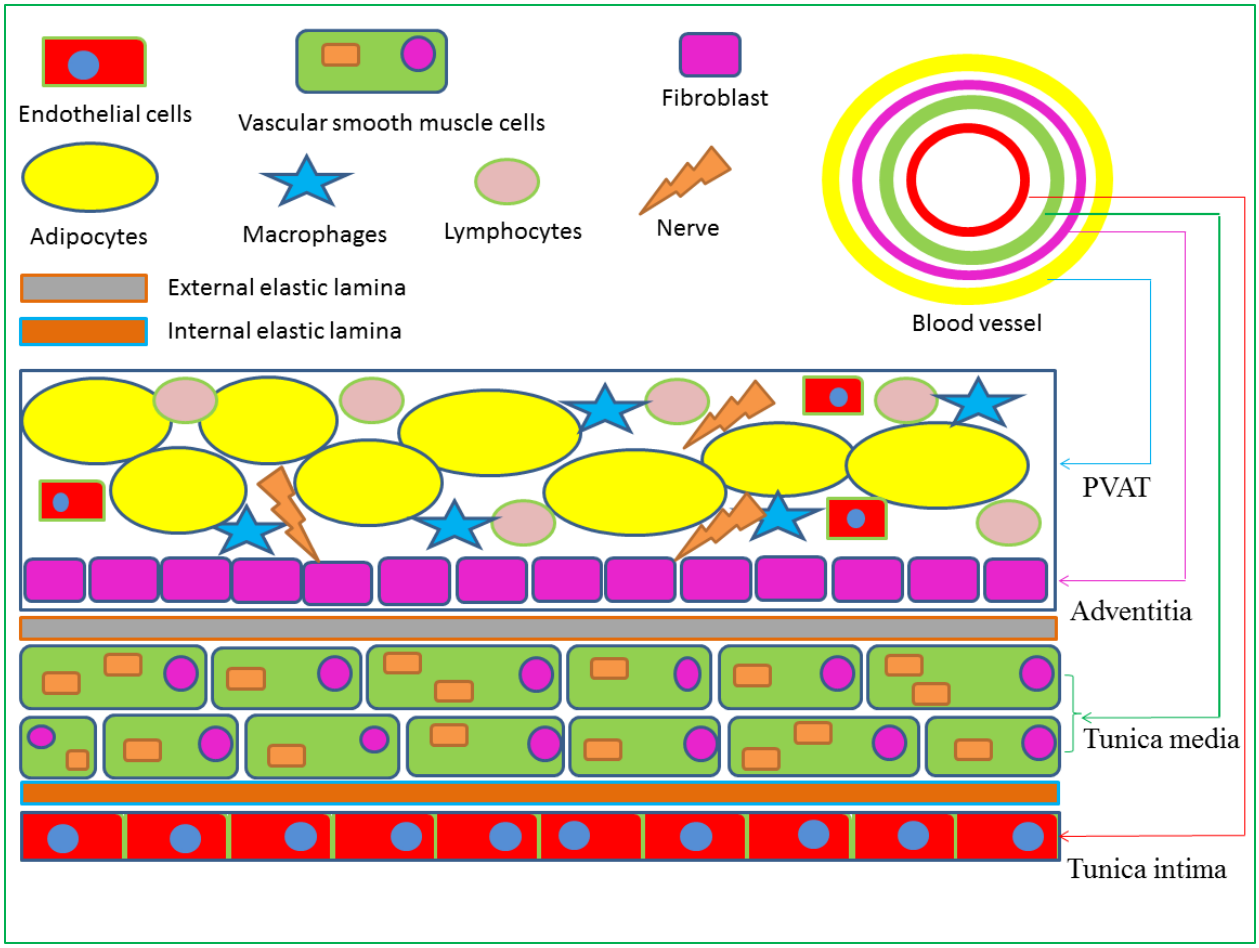



**Figure 1.1.** Schematic representation of blood vessel layers and cellular components of each layer.

Another key factor released by the endothelium is $PGI_2$, a potent vasodilator capable of attenuating VSMCs remodelling and inhibiting platelet activation (Mitchell et al., 2008). PGI2 is synthesized from membrane phospholipids by three enzymes, namely the $Ca^{2+}$-dependent phospholipase A2 (PLA2), cyclooxygenases (COX-1 and COX-2), and PGI2 synthase (Majed and Khalil, 2012). PGI2 synthase is highly expressed in endothelial cells compared to other prostaglandin synthase enzymes accounting for PGI2 as the predominant arachidonic acid metabolite produced by this tissue (Barac and Panza, 2009). Once synthesized, the labile and lipid-soluble molecule PGI2, diffuses to VSMCs and binds to its receptor, a member of G-protein coupled receptors (GPCR), leading to vasodilation (Tanaka et al., 2004).

In addition to NO and PGI2, the endothelium produces other mediators endowed with vasorelaxant and vasoconstricting, pro- and anti-inflammatory, pro- and anti-proliferative, and

pro- and anti-thrombotic activities (Galley and Webster, 2004). In a healthy endothelium, production of these factors is tightly shifted in favour of vasodilator, anti-inflammatory, anti-proliferative, and anti-thrombotic substances. However, disease states causing endothelial dysfunction are characterized by an over-production of pro-oxidants, vasoconstrictors, and pro-inflammatory, pro-proliferative, as well as pro-thrombotic factors (Park and Park, 2015; Jourde-Chiche et al., 2019).

### 1.2 Vascular smooth muscle cells

VSMCs are the structural and functional unit of blood vessels regulating, in healthy conditions, the degree of contraction and relaxation, and hence vascular tone and blood pressure (Shi et al., 2020). VSMCs are characterized by a quiescent, non-synthetic, contractile phenotype, and express several contractile proteins like α-actin and myosin heavy chains (Lacolley et al., 2017). Their function is controlled by the autonomic nervous system, hormones, and autocrine and paracrine agents produced by both endothelium and PVAT (Owens et al., 2004).

Contraction of VSMCs can be induced by electro-mechanical and/or pharmaco-mechanical coupling. The former involves depolarization of the cell membrane and $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (VDCCs). The latter is triggered by pharmacological agents acting on different GPCRs situated on the cell membrane. Receptor activation induces a conformational change resulting in the exchange of heterotrimeric Gq-protein GDP to Gq-GTP followed by dissociation of G-protein into α, and β-γ subunits. The α subunit activates a membrane-bound phospholipase C (PLC), which hydrolyzes membranous phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG). IP3 activates IP3 receptors located on the sarcoplasmic reticulum inducing $Ca^{2+}$ release, while DAG stimulates protein kinase C (PKC) activity that, in turn, phosphorylates receptor-operated, and store-operated $Ca^{2+}$ channels (ROCCs and SOCCs, respectively), which open allowing $Ca^{2+}$ influx (Amberg and Navedo, 2013; Brozovich et al., 2016; Ringvold and Khalil, 2017). Similarly, PKC phosphorylates and inhibits $K^+$ channels causing membrane depolarization, thus favouring VDCCs opening and augmenting $Ca^{2+}$ influx. The formation of the $Ca^{2+}$-calmodulin complex activates the myosin light chain (MLC) kinase (MLCK) that, in turn, phosphorylates MLC on Ser-19 and stimulates its ATPase activity, thus facilitating actin-myosin cross-linking and triggering muscle contraction (Hong et al., 2011). Simultaneously, also $Ca^{2+}$ sensitivity is

enhanced through, for instance, PKC phosphorylation of phosphorylation-dependent inhibitor of MLC phosphatase (CPI-17) that inhibits myosin light chain phosphatase (MLCP) and prevents removal of the phosphate group from MLC, thus maintaining the contractile status (Hamaguchi et al., 2000; Woodsome et al., 2001). PKC can also trigger muscle contraction by activating the MAPK kinase (MEK)/ERK1/2 pathway and/or Rho-Rho-kinase (ROCK) (Khalil, 2013; Liu and Khalil, 2018). Furthermore, some subtypes of GPCRs activate Rho-Rho-kinase (ROCK) which, in turn, phosphorylates and inhibits MLCP, thus enhancing $Ca^{2+}$ sensitivity and vascular tone (Wynne et al., 2009). A summary of these mechanisms is depicted in Fig. 1.2.

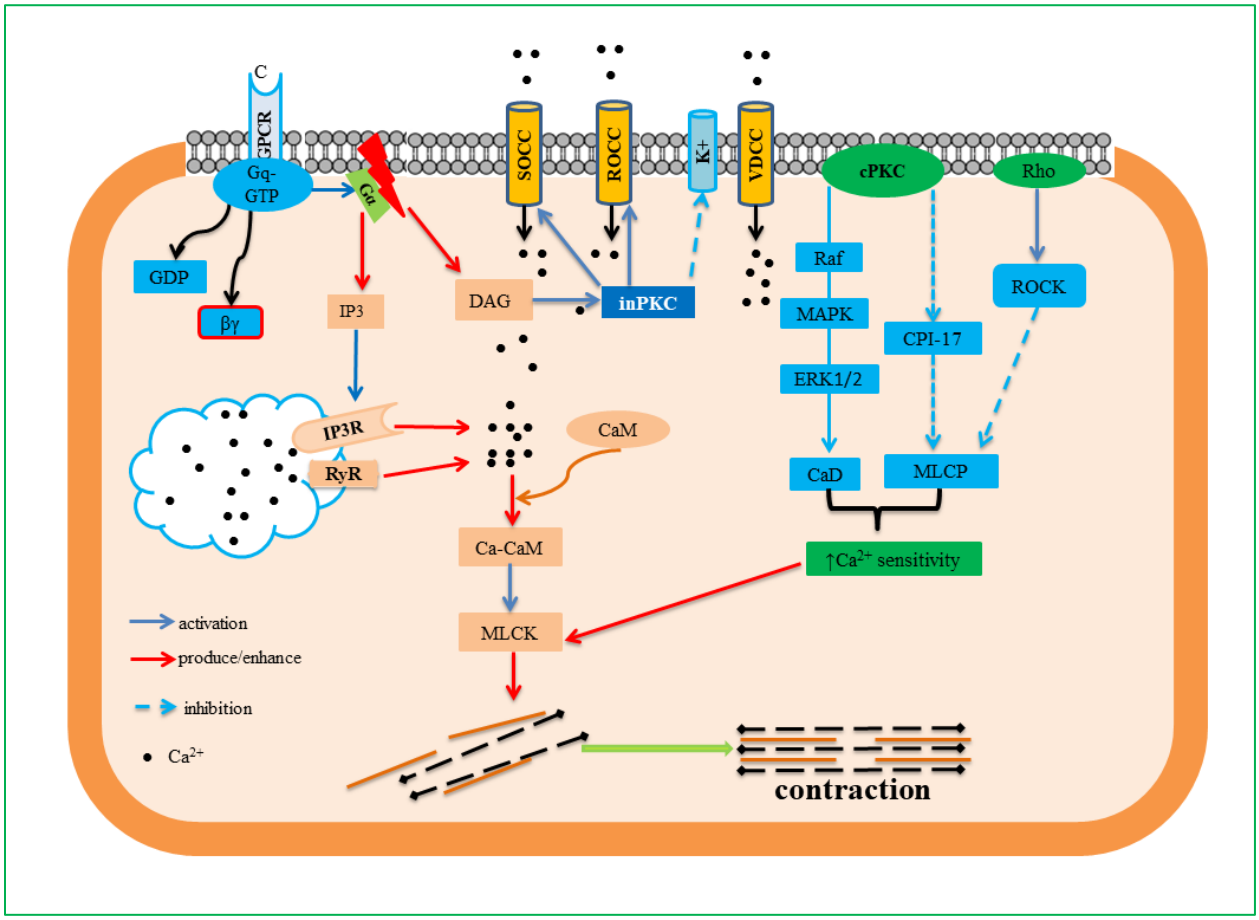



**Figure 1.2.** Mechanisms of VSMC contraction. Abbreviations: cPKC, active protein kinase C; CaD, caldesmon; DAG, diacylglycerol; C, contracting agent; GPCR, G-protein coupled receptor; inPKC, inactive protein kinase C; IP3R, IP3 receptor; $K^+$, $K^+$ channel; MLCP, myosin light chain phosphatase; ROCC, receptor-operated $Ca^{2+}$ channel; ROCK, rho-kinase; RyR, ryanodine receptor; SOCC, store-operated $Ca^{2+}$ channel; VDCC, voltage-dependent $Ca^{2+}$ channel.

VSMCs relaxation is induced mainly by decreasing $[Ca^{2+}]_i$ and/or $Ca^{2+}$ sensitivity. Several mechanisms contribute to this phenomenon including (1) decreasing $Ca^{2+}$ influx by blockade of VDCCs, ROCCs and/or SOCCs; (2) decreasing $Ca^{2+}$ release from intracellular store sites by inhibiting IP3 or ryanodine receptors; (3) increasing $Ca^{2+}$ extrusion/efflux from the cytoplasm by activating the plasmalemmal $Ca^{2+}$-ATPase and the $Na^{+}$-$Ca^{2+}$ exchanger; (4) enhancing $Ca^{2+}$ uptake into the sarcoplasmic reticulum by activating the corresponding $Ca^{2+}$-ATPase; (5) blocking stimulatory GPCRs like α-adrenergic receptors; (6) activating inhibitory GPCRs like β-adrenergic receptors; (7) opening $K^{+}$ channels including large-conductance $Ca^{2+}$-dependent ($BK_{Ca}$), voltage-dependent ($K_V$), ATP-dependent ($K_{ATP}$), and inward rectifier ($K_{ir}$) channels; (7) activating protein kinase A and protein kinase G; (8) inhibiting phosphodiesterases; and (9) activating sGC. Additionally, inhibition of PKC and ROCK or any of their downstream players like CPI-17, MLCP, etc., decreases the sensitivity of contracting machinery to $Ca^{2+}$, thus contributing to VSMC relaxation (Gurney, 1994; Liu and Khalil, 2018). A summary of these mechanisms is depicted in Fig. 1.3.

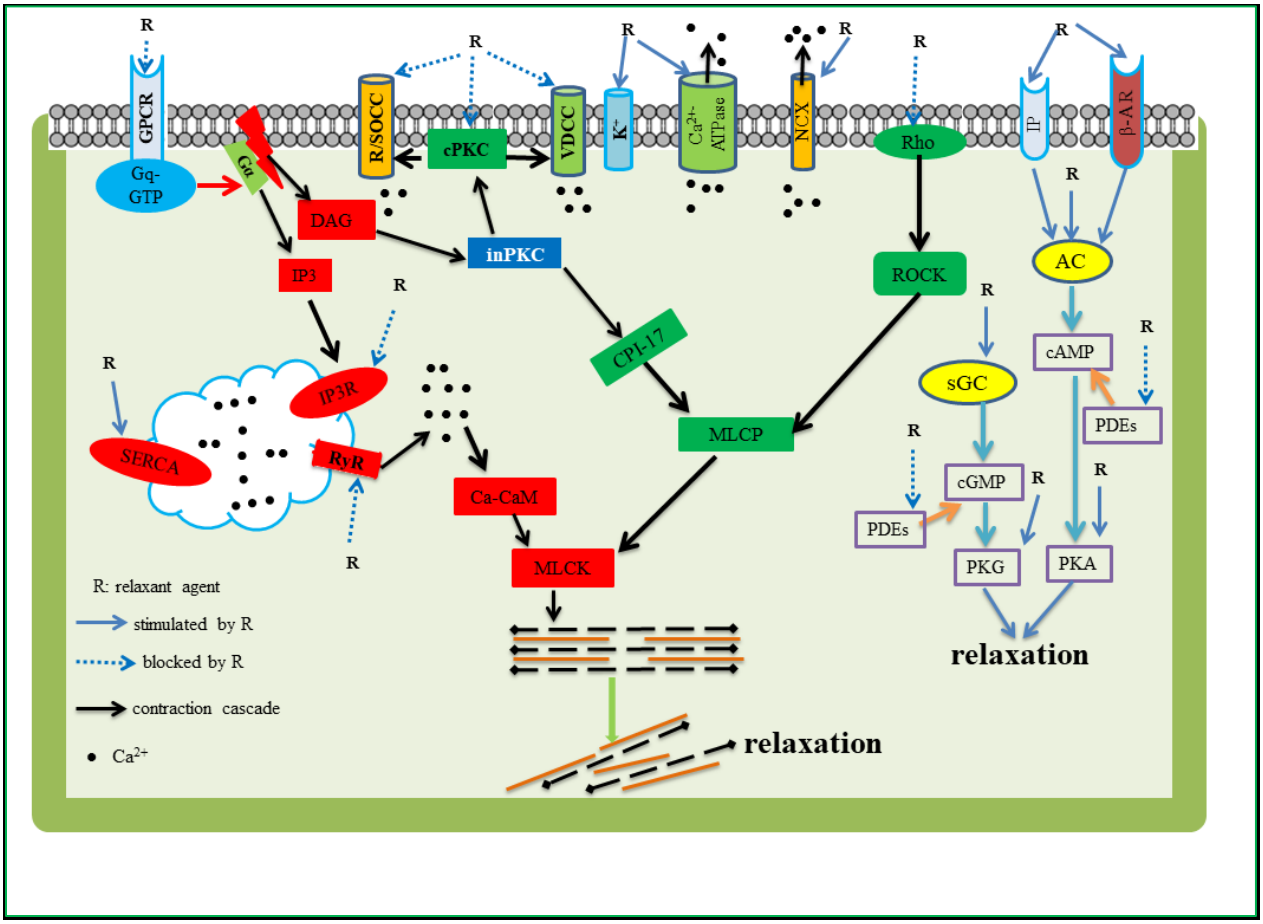


**Figure 1.3.** Mechanisms of VSMC relaxation. Abbreviations: AC, adenylyl cyclase; cPKC, active protein kinase C; β-AR, β-adrenergic receptor; $Ca^{2+}$-ATPase, plasmalemmal $Ca^{2+}$-ATPase; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; GPCR, G-protein coupled receptor; inPKC, inactive protein kinase C; IP3R; IP3 receptor; $K^+$, $K^+$ channel; MLCP, myosin light chain phosphatase; NCX, $Na^+$-$Ca^{2+}$ exchanger; PKA, protein kinase A; PKG, protein kinase G; PDEs, phosphodiesterases; R, relaxing agent; R/SOCC, receptor-/store-operated $Ca^{2+}$ channel; ROCK, Rho-kinase; RyR, ryanodine receptor; sCG, soluble guanylyl cyclase; SERCA, sarcoplasmic reticulum $Ca^{2+}$-ATPase; VDCC, voltage-dependent $Ca^{2+}$ channel.

### 1.3 Perivascular adipose tissue

PVAT is a type of adipose tissue that surrounds all blood vessels except cerebral and pulmonary arteries. Traditionally, PVAT was thought to act solely as a supporting matter, adhering blood vessels to other tissues; therefore, it is usually removed during *in vitro* studies. Three decades ago, Soltis and Cassis (1991) reported that the presence of PVAT attenuates noradrenaline-induced contraction; this effect was ascribed to catecholamine re-uptake into adrenergic nerves. The anti-contractile activity of PVAT was later observed with other vasoconstrictors that are not subjected to neuronal re-uptake (Löhn et al., 2002; Saxton et al., 2018b), thus promoting research to delineate the mechanisms underpinning this phenomenon and assess its role in cardiovascular diseases. Today, PVAT is recognized as an effective regulator of vascular tone and homeostasis *via* outside-in signalling (Nava and Llorens, 2019). Accumulating evidence indicates that PVAT, under certain pathological conditions like obesity, hypertension or diabetes, not only becomes dysfunctional but also exerts deleterious effects on the vasculature by secreting a plethora of pro-contractile and pro-inflammatory factors (Saxton et al., 2019).

#### 1.3.1 Characteristics of perivascular adipose tissue

PVAT displays several characteristics that distinguish it from other vascular bed layers or adipose tissues. First, it is not separated from the underlying blood vessel by a physical layer or an elastic lamina being rather in direct contact with the *tunica adventitia*. This allows factors produced by PVAT to directly affect blood vessels in a paracrine mode or *via* the *vasa vasorum* present within PVAT itself (Rajsheker et al., 2010). Second, unlike *tunica intima* and *tunica media* that are formed solely of endothelial cells and VSMCs, respectively, PVAT hosts pre- and mature adipocytes, endothelial cells derived from *vasa vasorum*, mesenchymal stem cells, and

immune cells including macrophages and lymphocytes (T and B cells), though adipocytes are the predominant cells. This cellular heterogeneity may be altered under certain pathological conditions such as inflammation, characterized by increased infiltration of macrophages (Fernández-Alfonso et al., 2017; Kumar et al., 2020). Third, PVAT adipocytes may originate from progenitors of the neighbouring cells such as VSMCs. Peroxisome proliferator-activated receptor gamma (PPAR-γ) is the master regulator of this adipogenic differentiation as its deletion in VSMCs results in a complete loss of PVAT in both thoracic and abdominal aorta, as well as in mesenteric arteries (Chang et al., 2012a). Additionally, PVAT situated in the aortic arch is believed to develop from neural crest cells, which can differentiate into brown and white adipocytes (Fu et al., 2019). Moreover, PVAT hosts multipotent mesenchymal stem cells that can differentiate into VSMCs or adipocytes, thus contributing to vascular remodelling (Pan et al., 2019). Fourth, PVAT displays phenotypic heterogeneity depending on the vascular bed, species, age, and/or disease status. For example, PVAT surrounding rat thoracic aorta exhibits a brown adipose tissue phenotype characterized by the presence of small lipid droplets, numerous mitochondria, and the expression of thermogenic genes such as uncoupling protein-1 (UCP-1). However, adult human aortic PVAT exhibits a white adipose tissue phenotype. Unlike thoracic PVAT, abdominal aortic PVAT is a mixture of white and brown adipose tissues, whereas that surrounding the mesenteric artery resembles white adipose tissue in both humans and rodents being characterized by the presence of large lipid droplets, few mitochondria, and a limited expression of UCP-1 (Gil-Ortega et al., 2015). Fifth, some pieces of evidence support the presence within PVAT of sensory and sympathetic innervation (Abu Baker et al., 2017; Saxton et al., 2018) essential for the release of leptin and adiponectin, respectively. However, these nerves might simply cross PVAT to reach the tunica *adventitia* or *media*. Further studies are required to demonstrate or rule out a direct innervation of PVAT.

Finally, PVAT contains functional catecholamines independent of sympathetic nerves but sensitive to tyramine (Ayala-Lopez et al., 2014). In this context, it can act as a buffer for noradrenaline released from sympathetic fibres and transported into PVAT *via* organic cation transporter 3, thereby preventing catecholamines from reaching blood vessels and causing contraction (Saxton et al., 2018a; Ahmad et al., 2019b).

### 1.3.2 Perivascular adipose tissue regulates vascular tone

A decade later to the seminal study by Soltis and Cassis (1991) demonstrating in rat thoracic aorta the anti-contractile effect of PVAT toward noradrenaline, Gollasch and colleagues observed that PVAT antagonizes the response to other vasoconstrictors, which are not subjected to neuronal reuptake (i.e., phenylephrine and angiotensin II) and to serotonin, which can be taken up by neurons. This antagonism was attenuated by the $K_{ATP}$ channel blocker glibenclamide and the tyrosine kinase inhibitor genistein, but was unaffected by other $K^+$ channel blockers (tetraethylammonium, 4-aminopyridine, and $Ba^{2+}$), by eNOS, COX-1/2, or cytochrome P450 inhibitors, or endothelium denudation. Noticeably, transferring solution from vessel rings with an intact PVAT (donor) to preparations deprived of PVAT (acceptor) caused a significant relaxation. The authors proposed that PVAT releases one or more factors (of unknown identity) termed adipocyte-derived relaxing factor (ADRF) (Löhn et al., 2002). ADRF release depends on extracellular $Ca^{2+}$ and is tuned by protein tyrosine kinase and protein kinase A without involving perivascular nerve endings or neuronal presynaptic $Na^+$ and N-type $Ca^{2+}$ channels, as well as vanilloid, cannabinoid, and calcitonin gene-related peptide receptors (Dubrovska et al., 2004).

Later, Gao et al. (2007) found that PVAT anti-contractile effect is mediated by two distinct pathways: endothelium-dependent and endothelium-independent. The former involves transferable factors, which stimulate eNOS that, in turn, activates $BK_{Ca}$ channels. The latter involves $H_2O_2$, which activates sGC. Noticeably, the anti-contractile effect of PVAT was also reported in other species including humans (Gao et al., 2005) and mice (Agabiti-Rosei et al., 2014).

In rat mesenteric arteries PVAT can also potentiate the response to perivascular neuronal activation elicited by electrical field stimulation in rat mesenteric arteries (Gao et al., 2006; Lu et al., 2010). Nowadays, it is well-established that PVAT influences vascular tone releasing numerous pro-contractile [known as PVAT-derived contracting factors (PDCFs)] and anti-contractile factors [PVAT-derived relaxing factors (PDRFs); Fig. 1.4]. Accordingly, the mechanisms of action triggered by these mediators have been explored to some extent, and PVAT therapeutic potential highlighted (Akoumianakis et al., 2017; Man et al., 2020).

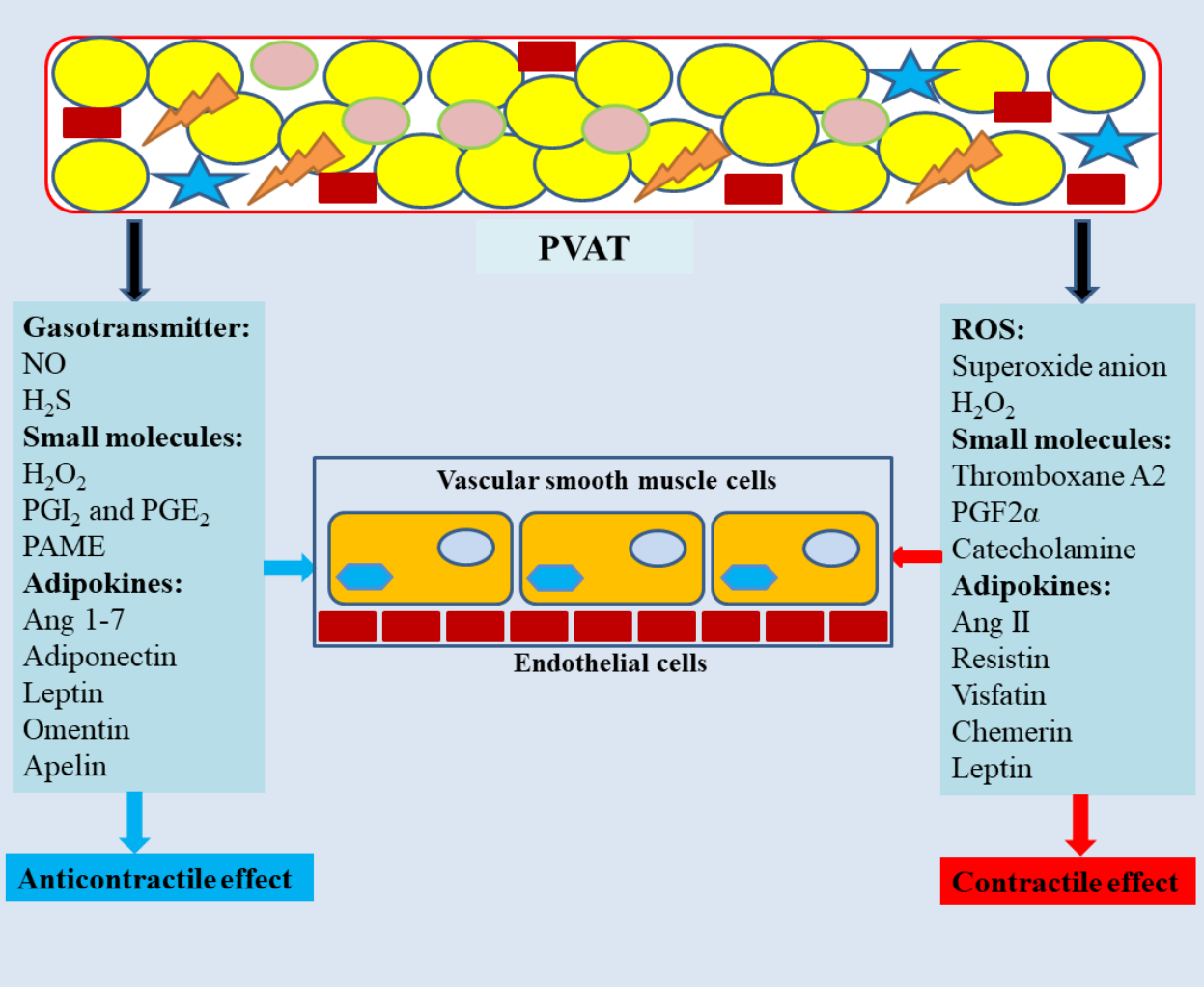



**Figure 1.4.** PVAT derived pro-contractile and anti-contractile factors. Abbreviations: Ang 1-7, angiotensin 1-7; Ang II, angiotensin II. PAME, palmitic acid methyl ester; $PGI_2$, prostacyclin; $PGE_2$, prostaglandin $E_2$; and PGF2α, prostaglandin 2α.

### 1.3.3 Anti-contractile factors released by perivascular adipose tissue

Similar to the endothelium, PVAT regulates vascular tone by synthesizing and secreting factors that act in a paracrine mode on the underlying VSMCs. Several PDRFs have been identified by different laboratories in various species and/or vascular beds: gasotransmitters (NO, $H_2S$), small molecules ($H_2O_2$, PGI2, palmitic acid methyl ester (PAME)), and adipocytokines (adiponectin, angiotensin 1-7, apelin, leptin, omentin) (Fig. 1.5). In this complex scenario, the search for selective drug modulating PVAT activity appears rather difficult.

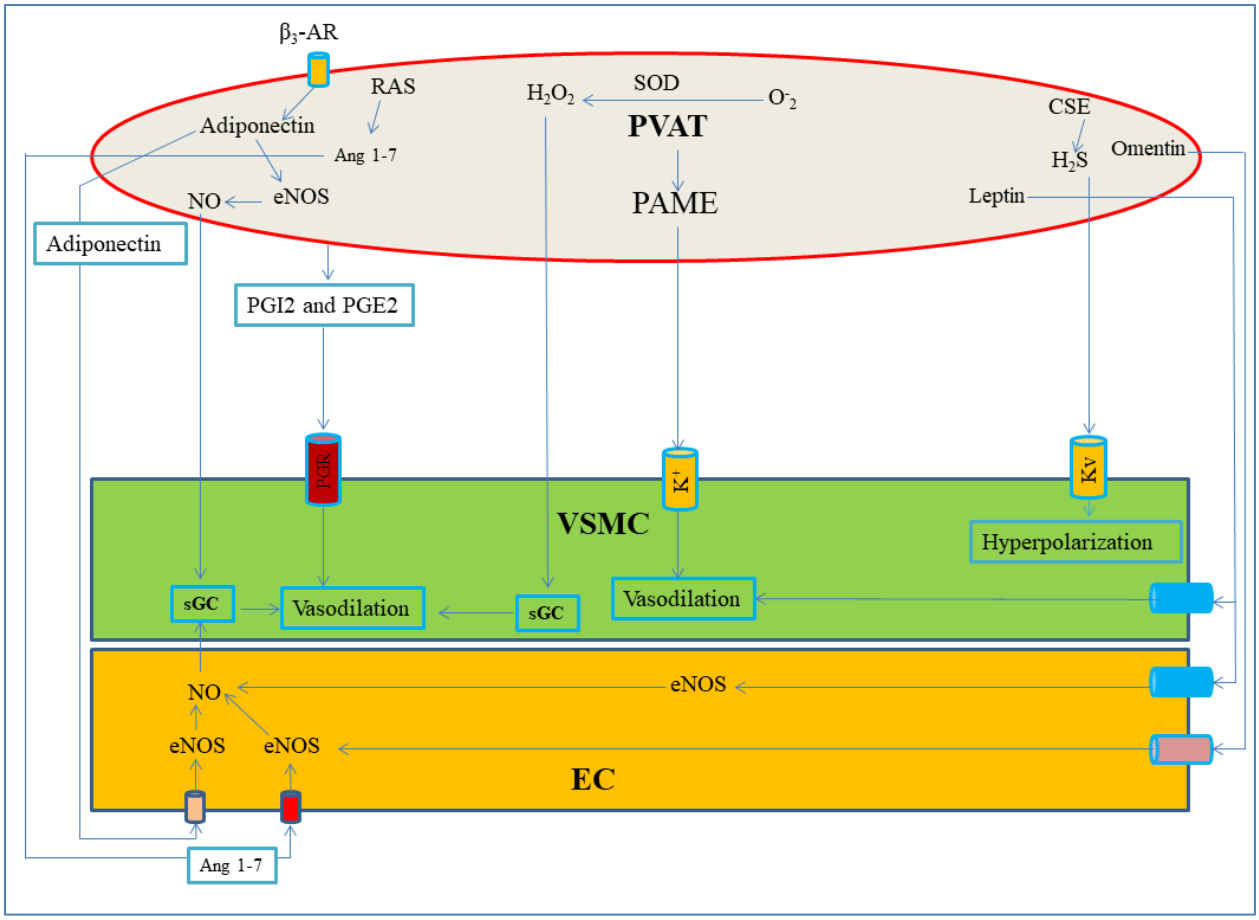



**Figure 1.5.** Mechanisms triggered by anti-contractile factors produced by PVAT. Abbreviation: Ang 1-7, angiotensin 1-7; $\beta_3$-AR, $\beta_3$-adrenergic receptor; CSE, cystathionine γ-lyase; ECs, endothelial cells; eNOS, endothelial nitric oxide synthase; PAME, palmitic acid methyl ester; $PGI_2$, prostacyclin; $PGE_2$, prostaglandin $E_2$; RAS, renin-angiotensin system; sGC, soluble guanylyl cyclase; SOD, superoxide dismutase; PVAT, perivascular adipose tissue; and VSMC, vascular smooth muscle cells.

*1.3.3.1 Nitric oxide*

NO is a gasotransmitter and potent vasodilator, produced mainly by endothelial cells capable of regulating vascular tone and blood homeostasis. Though Löhn and colleagues (2002) showed that the anti-contractile effect of PVAT is not dependent on NO synthesis, subsequently consistent studies revealed that NO is produced within PVAT and contributes to its anti-contractile effect (Dashwood et al., 2007; Gil-Ortega et al., 2010; Bussey et al., 2016; Victorio et al., 2016). Indeed, eNOS is expressed in PVAT adipocytes and endothelial cells of *vasa vasorum* within PVAT (Dashwood et al., 2007). Interestingly, NO derived from neuronal NOS (nNOS)

also contributes to the anti-contractile effect of PVAT (Nóbrega et al., 2019). Vasoconstriction triggered by the eNOS inhibitor N-ω-nitro-L-arginine methyl ester (L-NAME), in fact, is potentiated in the presence of PVAT suggesting that also PVAT-derived NO contributes to the overall basal NO production (Virdis et al., 2015). Similarly, L-NAME significantly enhances the response of endothelium-denuded PVAT-intact rat mesenteric preparations to noradrenaline, supporting the involvement of PVAT-derived NO in its anti-contractile effect (Aghamohammadzadeh et al., 2015). It is hypothesized that NO diffuses from PVAT to the neighbouring VSMCs and elicits vasodilation either *via* activation of the sGC-cGMP pathway and/or directly activating $BK_{Ca}$ channels, thus hyperpolarizing the membrane (Xia and Li, 2017). In conclusion, NO is produced by PVAT and contributes to the regulation of vascular tone supporting its role as a PDRF.

*1.3.3.2 Hydrogen sulfide*

$H_2S$ is a gasotransmitter synthesized endogenously from L-cysteine by the action of cystathionine β-synthase (CBS), and cystathionine γ-synthase (CSE). In the cardiovascular system, $H_2S$, produced in the endothelium and VSMCs by CSE, exerts vasodilation by activating either endothelial intermediate- and small-conductance $Ca^{2+}$-activated $K^+$ channels that hyperpolarize the underlying VSMCs or directly stimulating VSMC $K_{ATP}$ channel and inhibiting $Ca_V1.2$ channels (Streeter et al., 2012; Lv et al., 2020). PVAT expresses CSE and its anti-contractile effect is significantly decreased by CSE inhibition suggesting that $H_2S$ is a PDRF (Fang et al., 2009; Köhn et al., 2012). PVAT-derived $H_2S$ exerts its effect by activating KCNQ-type $K_V$ channels, but not $K_V7.1$ (Zavaritskaya et al., 2013; Tsvetkov et al., 2017; Gollasch et al., 2018). In summary, PVAT produces $H_2S$ that mediates its anti-contractile effect by hyperpolarizing the underlying VSMCs.

*1.3.3.3 Hydrogen peroxide*

$H_2O_2$, a small non-free radical reactive oxygen species (ROS), is recognized as the most pivotal mediator in oxidative signalling. $H_2O_2$, produced in both endothelial and VSMCs, plays a critical role in cardiovascular physiology and pathology (Bretón-Romero and Lamas, 2014; Byon et al., 2016). Gao and colleagues (2007) were the first to show the involvement of the $H_2O_2$/sGC/cGMP pathway in the endothelium-independent anti-contractile effect of PVAT.

Previous findings showed that $H_2O_2$ indeed induces vasodilation by sGC activation (Hayabuchi et al., 1998; Iesaki et al., 1999). Consistently, in rat thoracic aorta rings, the increase of propofol-induced relaxation caused by the presence of PVAT is significantly blocked by the $H_2O_2$ scavenger catalase and the sGC inhibitor ODQ (Kassam et al., 2011). Recent studies have confirmed the role of $H_2O_2$ in the PVAT anti-contractile effect (Costa et al., 2016; Emilova et al., 2016; Nóbrega et al., 2019). In fact, $H_2O_2$ is produced in PVAT mitochondria by Mn-Superoxide dismutase (SOD) (Costa et al., 2016), cytosolic and extracellular SOD (Gil-Ortega et al., 2014a), and nNOS (Nóbrega et al., 2019). Moreover, PVAT ameliorates endothelial dysfunction caused by an acute dose of ethanol, an effect ascribed to $H_2O_2$ (Gonzaga et al., 2018). In summary, PVAT-derived $H_2O_2$, produced by either mitochondrial or cytoplasmic pathways, diffuses down to VSMCs to mediate endothelium-independent PVAT anti-contractile effect.

*1.3.3.4 Prostacyclin*

PGI2 is a potent vasodilator produced mainly by the endothelium. Though Lohn et al. (2002) did not detect PGI2 among the PVAT factors mediating its anti-contractile effect, several studies have shown that it is produced by PVAT and contributes to its anti-contractile effect. In particular, this was observed in male Wistar-Kyoto rats (Zeng et al., 2009), in mice mesenteric arteries where the anti-contractile effect towards noradrenaline is inhibited by the cyclooxygenase inhibitor indomethacin (Lynch et al., 2013) and its level decrease with age or obesity (Chang et al., 2012b), in the human saphenous vein where the level of vasodilating prostaglandin E2 (PGE2) and PGI2 is markedly high (Ozen et al., 2013), and in male Wistar Hannover rats subjected to sepsis where PVAT anti-contractile effect decreases in the presence of indomethacin or the selective PGI2 receptor antagonist Ro1138452 while the level of superoxide anion and 6-keto-prostaglandin F1α (stable products of prostacyclin) increase (Awata et al., 2019). Finally, PVAT of mesenteric arteries from spontaneously hypertensive obese and Wistar-Kyoto rats shows enhanced COX-2 activity and releases PGE2, PGI2 and TXA2 (the latter is a pro-contractile factor) (Mendizábal et al., 2013). Taken together, these observations suggested that prostacyclin is synthesized by PVAT and contributes to its anti-contractile effect.

*1.3.3.5 Palmitic acid methyl ester*

PAME, a free fatty acid ester and a potent vasodilator released from the sympathetic ganglion (Lee et al., 2019), was first identified in aortic PVAT of Wistar Kyoto rats: its release is spontaneous and $Ca^{2+}$-dependent and causes vasodilation by opening 4-aminopyridine-sensitive $K^+$ channels. Both release and vasorelaxation are significantly reduced in spontaneously hypertensive rats (SHR) (Lee et al., 2011). Recently, in rat aorta and human mesenteric rings, PVAT exerts an anti-contractile effect on serotonin that is abolished by XE991, a $K_V7$ channel blocker (Wang et al., 2018). However, PAME induces vasodilation only in rat aorta, where it is also detected in PVAT (Wang et al., 2018). Interestingly, Liu et al. (2020) demonstrated that catechol-O-methyltransferase (COMT) is involved in PAME biosynthesis from palmitic acid and that both 3T3-L1 adipocytes and rat aortic PVAT express membrane-bound- and soluble-COMT proteins. Noticeably, the level of these enzymes is significantly reduced in SHR, suggesting a role for PAME in hypertension. These studies suggest that PAME is a PDRF that contributes to PVAT anti-contractile effect in various vascular beds.

*1.3.3.6 Angiotensin 1-7*

Angiotensin 1-7, a heptapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro) produced by the cleavage of angiotensin I or angiotensin II, counterbalances almost all physiological effects of angiotensin II (Ferrario et al., 2005). Though angiotensin 1-7 is produced mainly by endothelial cells and cardiomyocytes, PVAT contains complete renin-angiotensin system (RAS) components including renin, angiotensin I, angiotensin II, angiotensin 1-7, angiotensin-converting enzyme 1 (ACE1), ACE2, angiotensin II receptor type 1 (AT1R), and angiotensin II receptor type II (AT2R) (Averill et al., 2003; Galvez-Prieto et al., 2008). Angiotensin 1-7 was detected in aortic PVAT of Wistar rats where it exerts an endothelium-dependent anti-contractile effect. Of note, angiotensin 1-7 acts on the GPCR Mas (Santos et al., 2003), which is expressed in endothelial cells, VSMCs, and PVAT (Miao and Li, 2012). In fact, the anti-contractile effect is abolished by L-NAME, NO scavengers, the Mas receptor blocker A779, and the ACE2 inhibitor DX600 (Lee et al., 2009). In summary, PVAT produces angiotensin 1-7 that acts on endothelial cells *via* the Mas receptor thus contributing to its anti-contractile effect.

*1.3.3.7 Adiponectin*

Adiponectin is a hormone or adipokine, secreted mainly by adipose tissues, to regulate several physiological functions including metabolism and vascular tone (Achari and Jain, 2017). Fésüs et al. (2007) demonstrated that similarly to PVAT, it reduces serotonin-induced vasoconstriction by activating $K_V$ channels. However, the anti-contractile effect of PVAT in adiponectin gene-deficient mice is similar to that of wild-type animals, suggesting that it cannot be considered a PDRF candidate. This conclusion has been challenged by other studies showing that this hormone is a local vasodilator and a PDRF (Greenstein et al., 2009; Lynch et al., 2013; Withers et al., 2014). More recently, Withers et al. (2017) found that PVAT of eosinophil-deficient mice loses its anti-contractile activity which can be restored by eosinophil reconstitution. The authors demonstrated that adiponectin and NO are released from adipocytes upon activation of adipocyte $\beta_3$-adrenoceptors by catecholamines released from eosinophils. Furthermore, mice fed a high-fat diet exhibit reduced AMPK phosphorylation and adiponectin secretion, along with the loss of the anti-contractile effect of aortic PVAT. This phenomenon is reproduced in AMPK $\alpha_1$ knockout mice (Almabrouk et al., 2017; Almabrouk et al., 2018).

Adiponectin-induced vasodilation has been ascribed to numerous mechanisms including: stimulation of eNOS activity and enhanced biosynthesis of its substrate tetrahydrobiopterin (Chen et al., 2003; Xia and Li, 2017); direct activation of VSMC $BK_{Ca}$ or $K_V$ channels or indirect stimulation through NO release from adipocytes (Fésüs et al., 2007; Lynch et al., 2013; Withers et al., 2014); and VSMC NADPH oxidase inhibition *via* a PI3K/Akt-mediated block of Rac1 and down-regulation of p22phox gene expression (Antonopoulos et al., 2015). Altogether, adiponectin is a potent PVAT-derived vasodilator that contributes to its anti-contractile effect, at least in mouse species.

*1.3.3.8 Leptin*

Leptin is a hormone produced mainly by adipose tissue to regulate appetite and energy expenditure. Under physiological conditions, leptin stimulates the sympathetic nervous system (indirectly causing vasoconstriction) and directly induces vasodilation; both processes are in a balanced manner (Gu and Xu, 2013). Vasodilation may occur either through an endothelium-dependent eNOS activation (Vecchione et al., 2002) or endothelium-derived hyperpolarizing

factor (e.g., $H_2S$) (Jamroz-Wiśniewska et al., 2014) or through an endothelium-independent VSMC hyperpolarization (Momin et al., 2006). PVAT synthesizes and releases leptin in response to sensory nerve activation (Gálvez-Prieto et al., 2012; Fernández-Alfonso et al., 2013; Abu Bakar et al., 2017). However, its role as a possible PDRF remains controversial. Löhn et al. (2002), for instance, showed that leptin is not involved in the anti-contractile effect of PVAT, as Zucker obese rats lacking leptin receptors maintain PVAT activity. On the other hand, Gálvez-Prieto and colleagues (2012) demonstrated that leptin, likewise PVAT, antagonizes the response to angiotensin II. This antagonism is lost in SHR, where leptin mRNA and protein levels are significantly lower as compared to control animals. Therefore, the reduced synthesis of leptin and consequent impairment of eNOS stimulation may account for the loss of the PVAT anti-contractile effect. In this regard, it must be underlined that leptin can induce vasoconstriction of thoracic and pulmonary arteries of SHR increasing intracellular $Ca^{2+}$ release and $Ca^{2+}$ influx (Gomart et al., 2017). In Ossabaw swine with metabolic syndrome, PVAT-derived leptin worsens coronary endothelial dysfunction *via* PKC-β that, in turn, phosphorylates and inactivates eNOS (Payne et al., 2010). Notably, PVAT-derived leptin may also contribute to coronary pathogenesis through a Rho-kinase-mediated mechanism (Noblet, 2016) and may promote neointima formation and obesity-associated diseases (Schroeter et al., 2013). These findings demonstrate that leptin acts either as a PDRF or a PDCF: further studies are needed to clarify its role as a PVAT factor.

### 1.3.4 Pro-contractile factors released by perivascular adipose tissue

The effect of PVAT on vascular tone is still a matter of debate. Several studies have shown that, besides its anti-contractile effect, PVAT is also capable of potentiating vascular contraction under normal as well as pathological conditions. Owen et al. (2013) observed that the presence of PVAT from lean and obese Ossabaw swine augmented the contraction induced by high KCl in coronary arteries. Similarly, PVAT potentiates the contraction induced by electrical field stimulation in rat superior mesenteric arteries (Gao et al., 2006). Numerous studies have attempted to identify the factors released from PVAT that are directly or indirectly involved in this phenomenon. Some of them are detailed below and their mechanisms are depicted in Fig. 1.6.

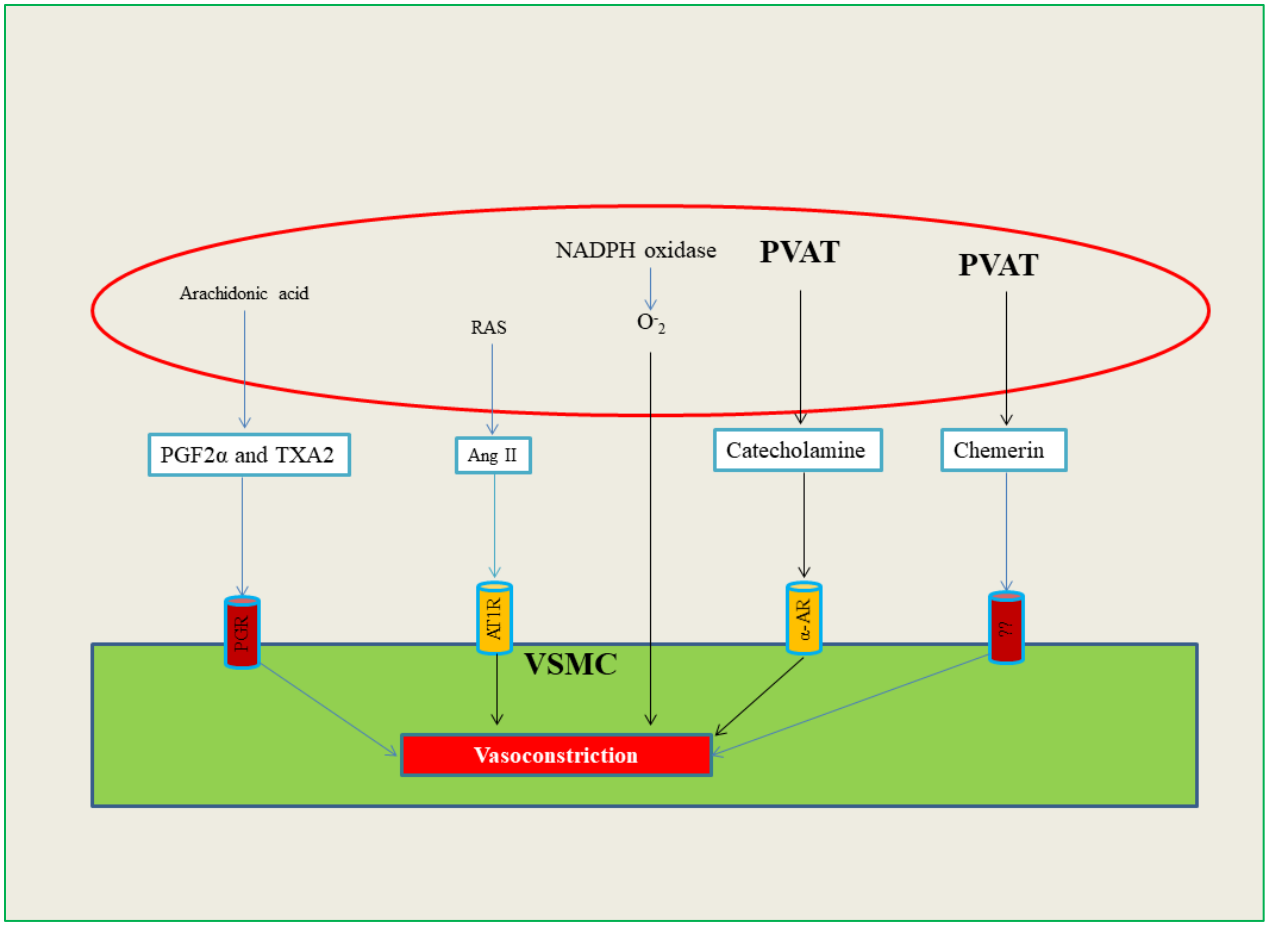



**Figure 1.6.** Mechanisms of pro-contractile factors produced by PVAT. Abbreviations: α-AR, α-adrenergic receptors; Ang II, angiotensin II; AT1R, angiotensin II receptor type 1; PGF2α, prostaglandin F2α; PGR, prostaglandin receptor; PVAT, perivascular adipose tissue; RAS, renin-angiotensin system; TXA2, thromboxane A2; and VSMC, vascular smooth muscle cells.

*1.3.4.1 Superoxide anion*

Superoxide anion, a free radical produced mainly by NADPH oxidase and mitochondrial electron transport chain complexes, is involved in several pathophysiological processes. In the vasculature, for example, it is produced in the endothelium, smooth muscle cells, and *adventitia* by the action of at least two enzymes: NADPH oxidase and xanthine oxidase (Staiculescu et al., 2014). In rat superior mesenteric arteries, Gao et al. (2006) showed that superoxide anion, produced by PVAT, increases the response to electrical field stimulation. Fluorescent labelling with dihydroethidium detected superoxide anion in PVAT-intact rings, isolated PVAT, and PVAT-derived adipocytes. Potentiation is mimicked by the exogenous superoxide donor pyrogallol, significantly reduced by SOD, NADPH oxidase inhibitors, and indomethacin, and is

not observed in PVAT-denuded rings. Tyrosine kinase and MAPK/ERK pathway inhibitors (i.e., tyrphostin A25 and U0126, respectively) suppress the response of electrical field stimulation in PVAT-intact but not in PVAT-denuded preparations, and attenuate the potentiation of the response operated by pyrogallol. Therefore, PVAT NADPH oxidase generates superoxide anions that potentiate the contraction of electrical field stimulation through a tyrosine kinase- and MAPK/ERK-dependent pathway.

Similarly, mesenteric arteries, isolated from C57BL/6J mice fed a high-fat diet for 32 weeks, lose the anti-contractile effect of PVAT, and exhibit high levels of superoxide anion and NADPH oxidase activity compared to vessels obtained from lean mice. This phenomenon was ascribed to a decreased expression of extracellular SOD and total SOD activity in PVAT, along with a two-fold increase in the ratio of oxidized to reduced glutathione (Gil-Ortega et al., 2014). High levels of superoxide anion and hydrogen peroxide as well as reduced endothelium-dependent vasodilation are observed in abdominal aortic PVAT of male C57Bl/6 mice fed a high-fat diet for 8 months. Finally, the anti-contractile effect of PVAT is abolished in obese mice as compared to lean controls and is partially restored by the NADPH oxidase inhibitor apocynin or the superoxide scavenger tiron (Ketonen et al., 2010). Taken together, these findings suggest that superoxide anions can be considered a PDCF limiting PVAT anti-contractile function under certain pathophysiological conditions.

*1.3.4.2 Angiotensin II*

Angiotensin II is an endogenous peptide hormone capable of eliciting vasoconstriction, thus increasing blood pressure. Similar to the adipose tissue, PVAT presents all the RAS components including angiotensinogen, angiotensin II, angiotensin 1-7, ACE1, ACE2, renin, AT1R, and AT2R. RAS components vary across the vascular tree: for example, angiotensin II levels are higher in mesenteric PVAT than in periaortic PVAT (Gálvez-Prieto et al., 2008; Cheng et al., 2018). Though its precise function in PVAT is not defined yet, angiotensin II can regulate vascular tone, VSMC growth and migration, as well as vascular remodelling (Miao and Li, 2012). Lu et al. (2010) demonstrated that PVAT-derived angiotensin II is the pro-contractile factor capable of potentiating the response of rat mesenteric arteries to electrical field stimulation. In fact, both inhibition of angiotensin II synthesis by the ACE inhibitor enalaprilat and blockade of AT1R by candesartan antagonize this stimulation in PVAT-intact but not in

PVAT-deprived tissues. Furthermore, likewise PVAT, exogenous angiotensin II augmented the response to electrical field stimulation. PVAT-derived angiotensin II is thought to act directly on AT1R, expressed on both adipocytes and vascular wall, to stimulate superoxide anion production by NADPH oxidase (Lu et al., 2008).

Noticeably, in rat mesenteric arteries, the anti-contractile effect of PVAT was abolished by *in vitro* hypoxic conditions and restored by pre-incubation with both the AT1R antagonist telmisartan and the ACE 2 inhibitor captopril, thus suggesting a role for angiotensin II in hypoxia-mediated loss of PVAT anti-contractile activity (Rosei et al., 2015). Recently, Fontes et al. (2020) showed that the loss of PVAT anti-contractile effect in a rat model of heart failure is caused by an overproduction of angiotensin II: in fact, the blockade of both AT1R and AT2R restored it. In summary, PVAT produces angiotensin II that, in turn, contributes to the loss of its anti-contractile activity under pathological conditions.

*1.3.4.3 Prostaglandins*

Prostaglandins are a group of arachidonic acid-derived metabolites endowed with diverse physiological activities such as vasodilation, vasoconstriction, as well as pro- and anti-inflammatory effects (Ricciotti and FitzGerald, 2011). Meyer et al. (2013) showed that aortic PVAT of monogenic G-protein coupled estrogen receptor-deficient obese or diet-induced obese C57Bl6 mice (but not lean mouse) significantly augments serotonin- and phenylephrine-induced vasoconstriction. This effect is blocked by COX inhibition or partially antagonized by inhibition of either COX-1 or COX-2. Furthermore, TXA2 is detected in the periaortic PVAT of these obese mice. PVAT-derived prostaglandins cause endothelial dysfunction in mesenteric arteries of spontaneously hypertensive obese rats as demonstrated by the reduced response of vessels to acetylcholine-induced vasodilation. This effect is reversed by the blockade of COX-2, TXA2 synthase, as well as PGI2 and TXA2 receptors (Mendizábal et al., 2013). Also, minced PVAT constricts thoracic aorta, carotid, and mesenteric arteries of C57BL/6J mice in an indomethacin- (a nonspecific COX inhibitor) dependent manner (Chang et al., 2012b). Indomethacin and flurbiprofen, another COX inhibitor, abolish PVAT-induced contraction of porcine coronary arteries (Ahmad et al., 2017). Interestingly, this study highlights sex differences in PVAT function because PGF2α receptor antagonist AL8810 attenuates PVAT-induced contraction in males while TXA2 receptor antagonist GR32191B in female's porcine coronary arteries,

respectively (Ahmad et al., 2017). Collectively, these studies point to PVAT-derived prostaglandins as PDCFs counteracting its anti-contractile effect and/or mediating its vasoconstriction.

*1.3.4.4 Catecholamines*

Catecholamines, produced mainly by the sympathetic nervous system and adrenal medulla, are monoamine neurotransmitters endowed with several functions such as regulation of metabolism and blood pressure. In addition to sympathetic nervous system innervation, PVAT contains its reservoir of catecholamines. A pioneering study by Ayala-Lopez and colleagues (2014), in fact, demonstrated that aortic and mesenteric PVAT of male Wistar rats contain detectable amounts of neurotransmitters; namely noradrenaline, dopamine, and serotonin. These catecholamines are sensitive to the indirect sympathomimetic tyramine, which elicits significantly higher contractions in arteries with intact PVAT compared to preparations devoid of PVAT. Tyramine-induced contraction is reduced by nisoxetine (a noradrenaline transporter inhibitor), tetrabenazine (a vesicular monoamine transporter inhibitor), and prazosin (an α adrenoreceptor antagonist), but not by either dopamine and serotonin transporters inhibition, or celiac ganglionectomy, indicating that noradrenaline release is independent of sympathetic neurons.

The origin of catecholamines in PVAT adipocytes is a matter of debate. First, PVAT adipocytes may synthesize these neurotransmitters *de novo* from their precursors, as they express the enzymes necessary for the synthesis (Stunes et al., 2011; Vargovic et al., 2011; Vargovic et al., 2013). Second, PVAT adipocytes may uptake catecholamines upon their release from the sympathetic nervous system. Indeed, in mesenteric PVAT accumulation of noradrenaline is reduced by nisoxetine, the serotonin transporter inhibitor citalopram, and the organic cation transporter 3 inhibitor corticosterone, as well as by the combinations of nisoxetine + corticosterone or citalopram + corticosterone (Ayala-Lopez et al., 2015). Saxton et al. (2018b) showed that PVAT of C57BL/6J mouse mesenteric arteries exhibits an anti-contractile effect to the electrical field stimulation-induced contraction that was ascribed to noradrenaline uptake into the adipocytes. Furthermore, PVAT adipocytes store noradrenaline *via* a vesicular monoamine transporter 1/2 (Ahmad et al., 2019). Finally, also other cells colonizing PVAT such as macrophages and lymphocytes may contribute to the pool, being capable of synthesizing catecholamines (Josefsson et al., 1996; Sá Gomes et al., 2019).

*1.3.4.6 Chemerin*

Chemerin, a small multifunctional protein produced mainly by adipose tissues and the liver, exerts a wide spectrum of functions by acting on chemokine-like receptor 1, now formally known as chemerin 1 (Kwiecien et al., 2020). Several reports have shown that PVAT, likewise endothelial cells and VSMC, can produce chemerin 1 (Watts et al., 2013; Darios et al., 2016; Flood and Watts, 2020). Chemerin and its active fragment chemerin-9 constrict various blood vessel preparations and this effect increases following endothelium removal or eNOS inhibition (Watts et al., 2013; Ferland et al., 2017; Omori et al., 2020). The mechanism underpinning chemerin-induced contraction seems to involve inhibitory G-protein and L-type $Ca^{2+}$ channel activation (Ferland et al., 2017). Additionally, chemerin potentiates the contraction induced by other agents such as endothelin-1 (Hanthazi et al., 2015), phenylephrine (Lobato et al., 2012), and electrical field stimulation (Flood and Watts, 2020). As its expression is enhanced in obesity and type 2 diabetes, chemerin may contribute to the loss of PVAT anti-contractile effect observed under these pathological conditions (Sell et al., 2009; Cao et al., 2017). Thus chemerin is a possible PDCF candidate; however, additional direct evidence demonstrating its involvement in PVAT-induced contraction or loss of its anti-contractile effect is needed.

## 1.4 Flavonoids

Flavonoids, the largest class of plant polyphenols, are synthesized from the amino acid phenylalanine through the shikimic acid pathway (Falcone et al., 2012). In plants, they protect against phytopathogens and ultraviolet radiations, contribute to flower pigmentation and pollinators' attraction, and play key roles in auxin transport, nodulation, as well as mediating plant-plant, plant-microbes, and plant-mammals interactions (Pandey and Rai, 2014; Nabavi et al., 2020). Hence, plants have evolved various metabolic pathways and enzymes involved in flavonoid biosynthesis: up to 9000 different flavonoids have been identified to date and this number is continuously growing (Wang et al., 2018b).

Human consumes a considerable amount of flavonoids from different plant-derived foods and beverages such as fruits, vegetables, tea, coffee, wine, and herbal medicines (Egert and Rimbach, 2011). These natural molecules are pleiotropic compounds endowed with excellent antioxidant, antimicrobial, anti-inflammatory, antidiabetic, antiatherosclerotic, antithrombotic, anticancer,

and neuro- and cardio-protective activities (Panche et al., 2016; Ayaz et al., 2019). In addition, clinical trials and epidemiological studies have found a significant inverse correlation between flavonoid intake and the incidence of diseases, among others, cardiovascular diseases and cancer (Peterson et al., 2012; Bondonno et al., 2019; Parmenter et al., 2020). The consequent increase in flavonoid market demand warrants production in large amounts; hence, principles of genetic engineering and synthetic biology are currently developed to increase their production either in plants or microorganisms (Zha et al., 2019). Furthermore, several flavonoid supplements and flavonoid-rich foods are present over-the-counter, e.g., lipo-flavonoids, ginkgo Biloba, milk thistle (silymarin), dark chocolate, and others.

### 1.4.1 Flavonoids: chemistry and classification

Flavonoids are characterized by a diphenylpropane skeleton consisting of 15 carbon atoms arranged into two 6-carbon rings (namely A and B) linked via an oxygen-containing, heterocyclic benzopyran C-ring (Panche et al., 2016). Based on oxidation and saturation of the C ring, or position of the B ring, flavonoids are classified into eight classes: flavone, flavonol, flavanone, flavanonol, isoflavone, flavan-3-ol, anthocyanidins, and chalcone (Fig. 1.7). Flavones have a double bond between C-2 and C-3 and the ketone group at position 4 of the C-ring. Similar to flavones, flavonols have a double bond between C-2 and C-3, a ketone at C-4, plus an OH group at C-3. Unlike flavones, isoflavones have the B ring attached to C-3 instead of C-2. Flavanones, also called dihydroflavones, have a saturated bond between C-2 and C-3 and a ketone group. Flavanonols, also called dihydroflavonols, have a saturated bond between C-2 and C-3 and an OH group at C-3 along with a ketone group. Flavan-3-ols, also called catechins, have an OH group at C-3 but lack both the ketone group and the double bond between C-2 and C-3. Chalcones, have the C-ring open. The last class, anthocyanidins, lacks the ketone group but has two double bonds between C-1 and C-2, and C-3 and C-4 (Rauter et al., 2018).

Flavonoids within the same class differ in their substitutions on A- and B-rings, hydroxylation being the most common, particularly at positions 5, 6, 7, 2`, 3`, 4`, and 5`. Flavonoids are also found in methylated, acylated, prenylated, phenyl-acylated and, most commonly, glycosylated forms. Besides contributing to flavonoid diversity, these modifications markedly influence their solubility, stability, and reactivity (Kumar and Pandey, 2013; Yang et al., 2015). Flavonoids can

also polymerize, either in plants or artificially, into larger molecules such as tannins or pro-anthocyanidins (Bridson et al., 2015; Santiago-Medina et al., 2017).

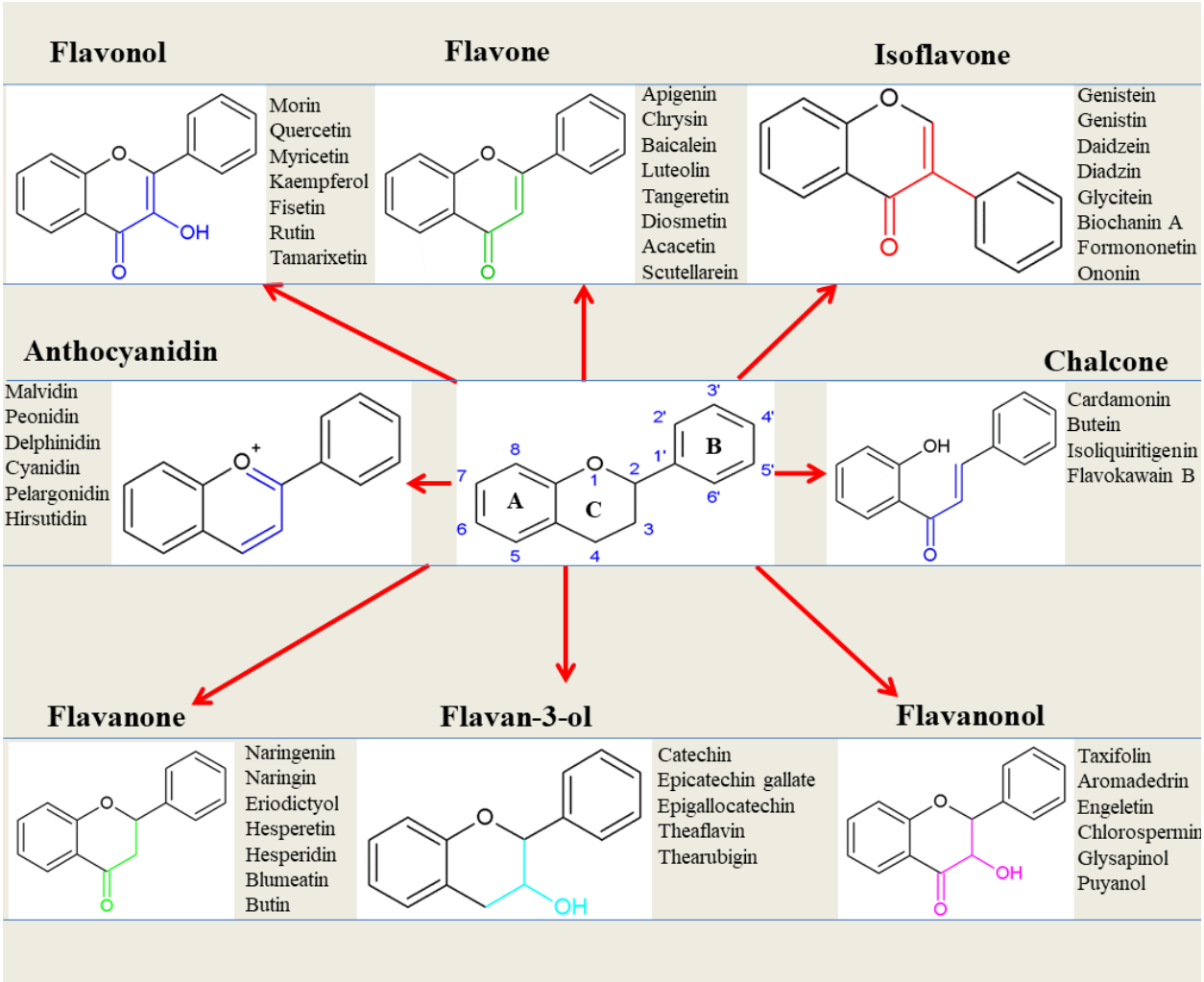



**Figure 1.7.** Structures and classification of the most common flavonoids

### 1.4.2 Flavonoids: occurrence and sources

Flavonoids are synthesized and widely distributed in all plant parts including roots, shoots, leaves, flowers, and fruits (Del Valle et al., 2015). Their content varies considerably depending on plant species, geographical area, genetic background, and developmental stage (Mouradov and Spangenberg, 2014). Since flavonoids play a crucial role as protecting agents, biotic and abiotic stressors such as pathogens, drought, temperature, radiation, salinity, and heavy metals also markedly influence their content and accumulation (Mierziak et al., 2014).

Dietary flavonoids can be found in fruits, vegetables, tea, coffee, wine, and herbal medicine (D'Archivio et al., 2007), different classes being characteristic of different plants. Celery, parsley, red peppers, chamomile, spinach, and ginkgo Biloba are rich in flavones (Hostetler et al., 2017). Flavonols are abundantly present in many vegetables, fruits, and beverages such as lettuce, onions, kale, tomatoes, berries, apples, grapes, tea, and red wine (Zhang et al., 2010). Flavanones are found in grapes and *Citrus* fruits such as oranges and lemons: they are responsible for the bitter taste of the juice and peel (Khan et al., 2013; Barreca et al., 2017). Isoflavones, also known as phytoestrogens, are abundant in soybeans and leguminous plants (Křížová et al., 2019). Tea, bananas, apples, blueberries, peaches, and pears are rich in flavan-3-ols or catechins (Auger et al., 2004). Flavanonols are limited in their distribution to *Citrus* fruits and *Glycosmis* species (Lukaseder et al., 2009). Finally, anthocyanidins are predominantly present in flowers and the outer layers of fruits such as cranberries, black currants, grapes, and berries: they confer beautiful colours that depend on pH and chemical modifications of A- and B-rings (Krga and Milenkovic, 2019). The most common flavonoids of each class and their sources are presented in Table 1.1.

### 1.4.3 Flavonoid intake

Daily flavonoid intake varies qualitatively and quantitatively depending on lifestyle, cultural dietary practices, dietary assessment methods, genetic variation of consumed food, food processing, and socioeconomic status of the individuals (Fanelli Kuczmarski et al., 2018; Ahn-Jarvis et al., 2019; Vieux et al., 2020). Recently, flavonoid intake has been estimated to range from 150 to 600 mg/day with considerable within- and between-individual variation (Escobar-Cévoli et al., 2017). The predominant sources of flavonoids in Western countries (Europe, the USA, and Australia) are tea, wine, citrus fruits, and vegetable juices. The major contributors to the total flavonoid intake in East Asia (China, Korea, and Japan) are soy foods and green tea whereas in the Middle East is black tea (Escobar-Cévoli et al., 2017). Globally, flavanols and pro-anthocyanidins are the most consumed flavonoids with daily intake ranging from 11 mg in Brazil to 629 mg in Australia, depending on tea and fruit consumption (Kent et al., 2015; Miranda et al., 2016). Flavanone intake varies from 5.0 mg in China to 130 mg in Spain, perfectly paralleling the consumption of citrus fruits and juices (Goetz et al., 2016). Flavonol intake seems similar globally with a daily range spanning from 15 to 60 mg/day, with

China, Korea, and Poland presenting the highest values (Chun et al., 2007). Similar to flavonols, the daily intake of anthocyanidins varies from 2.2 mg in Brazil to 60 mg in Northern Italy; this matches well with the ingestion of berries, grapes, and plums (Zamora-Ros et al., 2011). Flavone intake varies from 1 mg/day in the Asian population to 10 mg/day in Southern Europe, the latter arising from the Mediterranean diet (Escobar-Cévoli et al., 2017). Isoflavone intake is normally as low as 1-2 mg/day in Western countries where soy food is less consumed. Strikingly, in the Asian countries where soy foods are routinely consumed, the daily intake of isoflavones may reach 70 mg/day, for example, in China and Korea (He and Chen, 2013).

### 1.4.4 Flavonoids: absorption, transport, metabolism, and excretion

Except for catechins, flavonoids are generally glycosylated, thus being hydrophilic. Therefore, their absorbance through cellular membranes in the small intestine is enhanced by the enzymatic removal of sugar moieties (Hollman, 2004). Two main absorption pathways allow flavonoids uptake in the small intestine: a) hydrolysis of the glycoside by lactase phlorizin hydrolase (LPH) in the brush border of epithelial cells that readily absorbs the free aglycone (Németh et al., 2003) (LPH has a broad substrate specificity with more affinity toward glucosides, regardless of the aglycone (Day et al., 2000; Day et al., 2003); b) transport of polar glycosides by specific translocators, such as the $Na^+$-dependent glucose transporter, into the cytoplasm of epithelial cells, where they are hydrolyzed by a cytosolic β-glucosidase.

In epithelial cells, ATP-binding cassette (ABC) transporters (i.e., P-glycoprotein, multidrug resistance proteins, and breast cancer-resistance protein) pump flavonoids through the basolateral membrane into the bloodstream or back into the intestinal lumen, thus reducing their bioavailability (Williamson et al., 2018). Flavonoids that are not absorbed in the small intestine pass into the colon where they are hydrolyzed and metabolized by intestinal microbiota into phenolic and aromatic compounds and then absorbed into the bloodstream. Notably, the bioavailability of flavonoids is markedly influenced by the dietary source and food matrix in which they are consumed (Petersen et al., 2016; Kamiloglu et al., 2020). For instance, quercetin from onion is 4-fold more readily absorbed than that from apple and tea. As a general rule, flavonoids are absorbed according to the following order: isoflavones > flavonols > flavones > flavanones > anthocyanidins > pro-anthocyanins > flavanols (Viskupicova et al., 2008). Other factors like molecular weight, diet, age, sex, individual genetic variation, gut microbiome, and

concomitantly used medications may influence flavonoid absorption (Cassidy and Minihane, 2017; Eker et al., 2020).

Once absorbed from the small intestine into the systemic circulation, flavonoids are transported *via* the portal vein to the liver where they gain access to hepatocytes *via* organic anion transporters and ABC transporters (Wong et al., 2012). Here, they may undergo further phase I metabolism (oxidation or O-demethylation) by cytochrome P450 monooxygenases and/or phase II conjugation reactions by uridine-5-diphosphate glucuronosyltransferases, sulphotransferases, and catechol-O-methyltransferases catalyzing glucuronidation, sulfation, or methylation at different positions (Thilakarathna and Rupasinghe, 2013; Xiaoa and Högger, 2013). Sulfation and glucuronidation produce polar metabolites that are either released into the general circulation or excreted *via* bile acid. Of note, conjugation reactions are very efficient as evidenced by the fact that, in plasma and urine, flavonoids are found almost exclusively as conjugates, whereas aglycones are undetected or present at very low concentrations (Manach and Donovan, 2004). Finally, the ubiquitously expressed ABC transporters are involved either in flavonoid uptake from the circulation into the site of action, or excretion *via* the kidney (Sissung et al., 2014).

**Table 1.1.** Most common flavonoids and their main sources

| Parent class | Examples | Sources |
|---|---|---|
| Flavonol | Quercetin | Apple, honey, onion, red grape, cherries, citrus fruit, green leafy vegetable, nuts, broccoli, olive oil, green tea, red wine, dark cherries, and berries such as blueberries and cranberries |
| | Morin | Almond, guava, old fustic, onion, apple, tea, red wine, seaweeds, coffee and cereal grains |
| | Myricetin | Black plum, berries, oranges, nuts, tea, red wine, and vegetables e.g., tomato and fennel leave |
| | Kaempferol | Saffron, capers, spinach, kale, ginger, Chinese cabbage, broccoli, onion, and cherries |
| Flavone | Apigenin | Parsley, celery, onions, artichoke, oranges, tea, beer, wine, and herbs such as chamomile, thyme, oregano, and basil |
| | Chrysin | Propolis, honey, passion flowers like *Passiflora caerulea* and *P. incarnata*, and *Oroxylum indicum* |
| | Luteolin | Celery, broccoli, green pepper, parsley, chamomile, tea, carrots, olive oil, navel oranges, and oregano |
| | Diosmetin | Citron juice, bergamot juice, orange juice, rosemary, and oregano |
| Isoflavone | Genistein | Bean curd, fava beans, soybeans, kudzu (Japanese arrowroot), and lupin |
| | Daidzein | Soybeans (green, mature, cooked or boiled), black beans, currants and raisins |
| | Biochanin A | Clovers (zigzag, crimson, hares foot, red), alfalfa sprout, soybean, peanuts, chickpea, and cabbage |
| | Formononetin | Red clover, green beans, lima beans, and soybeans |
| Flavanone | Naringenin | Grapefruit, oranges, tomatoes, tart cherries, cocoa, bergamot, oregano, water mint, and beans |
| | Hesperetin | Oranges, lemons, balm, and mandarins |
| | Eriodictyol | *Eriodictyon californicum*, lemon balm, barley, rye, millet, and sorghum |
| Flavanol | Epicatechin | Apples, blackberries, raspberries, broad beans, chocolate, red wine, cherries, grapes, pears, apricots, green and black teas |
| | Gallocatechin | Green tea, bananas, persimmons, pomegranates, nectarine, peach, pecan nut, and chocolate |
| | Epigallocate-chin gallate | Teas (green, white, oolong, and black), cranberries, strawberries, blackberries, kiwis, cherries, pears, peaches, apples, avocados, and nuts (pecans, pistachios, and hazelnuts) |
| Flavanonol | Taxifolin | Non-glutinous rice, conifers like the Siberian larch, silymarin, wine, tea, cocoa, and cherry wood |
| | Aromadendrin | Siberian pine, loganberry, fenugreek, bog bilberry, and narrow-leaf cattail |
| Anthocyanidin | Malvidin | Bilberry, blueberry, bean, potatoes, black, blue or concord grapes, and grapes waste |
| | Pelargonidin | Ripe rasp-, straw-, blue-, black-, cran-, saskatoon-, and choke-berries, plums and pomegranates |
| | Cyanidin | Berries, cabbage, asparagus, plum, pomegranate, hawthorn, and cherry |
| | Delphinidin | Black-, rasp-, cran-, elderberries, blackcurrants, eggplant, red grapes, and soybean |

### 1.4.5 Flavonoids: biological and pharmacological activities

Flavonoids were first discovered in 1930 by Albert Szent-Gyorgyi, a Hungarian scientist, who termed them vitamin P. However, interest in the pharmacological and nutraceutical features of these natural compounds was boosted only during the 90s, after the publication of the French paradox, an epidemiological study highlighting the low incidence of coronary heart diseases in the French population despite its high daily intake of saturated fats. This paradox was ascribed to the consumption of red wine and polyphenol therein (Sun et al., 2002). This hypothesis stimulated researchers all over the world to investigate flavonoids beneficial effects at the cellular, animal, and human levels. Nowadays, flavonoids are recognized as pleiotropic compounds exhibiting a wide array of biological and pharmacological activities such as antioxidant, antitumor, antidiabetic, antimicrobial, cardioprotective, and neuroprotective activities (Fig. 1.8) (Busch et al., 2015; Jucá et al., 2020). More importantly, several epidemiological and meta-analysis studies have correlated flavonoid intake with a low incidence of cancer (Chang et al., 2018; Bondonno et al., 2019), cardiovascular diseases (Kim and Je, 2017; Parmenter et al., 2020), diabetes (Xu et al., 2018), obesity (Vernarelli and Lambert, 2017; Marranzano et al., 2018), Alzheimer's disease (Shishtar et al., 2020), and Parkinson disease (Gao et al., 2012).

The beneficial activity of this class of compounds has been ascribed to various mechanisms. First, a direct antioxidant activity ascribed to the presence of one or more phenolic groups that are effective ROS scavengers (Procházková et al., 2011; D'Amelia et al., 2018). Second, inhibition of enzymes like COXs, lipoxygenase, xanthine oxidase, aldehyde oxidase, SOD, ACE1/2, CYP3A4, and PKC, among others (Sadik et al., 2003; Pirouzpanah et al., 2009; Hou and Kumamoto, 2010; Ribeiro et al., 2015; Nile et al., 2018). Third, modulation of the expression of enzymes such as catalase, SOD, protein kinases, MAPK, RAF, etc. (Mansuri et al., 2014; Moskot et al., 2015). Fourth, the inhibition or activation of ion channels (Fusi et al., 2017; Fusi et al., 2020; Redford and Abbott, 2020). Fifth, modulation of receptors such as aryl hydrocarbon (Jin et al., 2018), liver X (Fouache et al., 2019), and γ-amino butyric acid-A receptors (Hanrahan et al., 2011). Sixth, modulation of transcription factors such as Nrf2, FoxO, and PPARγ, and signal transduction proteins (Pallauf et al., 2017; Mendonca and Soliman, 2020). Seventh, modulation of glucose (Strobel et al., 2005) and ABC transporters (Alvarez et

al., 2010; Miron et al., 2017). Eighth, interplay with cytoskeleton proteins (Böhl et al., 2007), cellular membranes (Oteiza et al., 2005; Hendrich, 2006), and blood proteins as well as red blood cells (Fiorani et al., 2003; Gecibesler and Aydin, 2020).

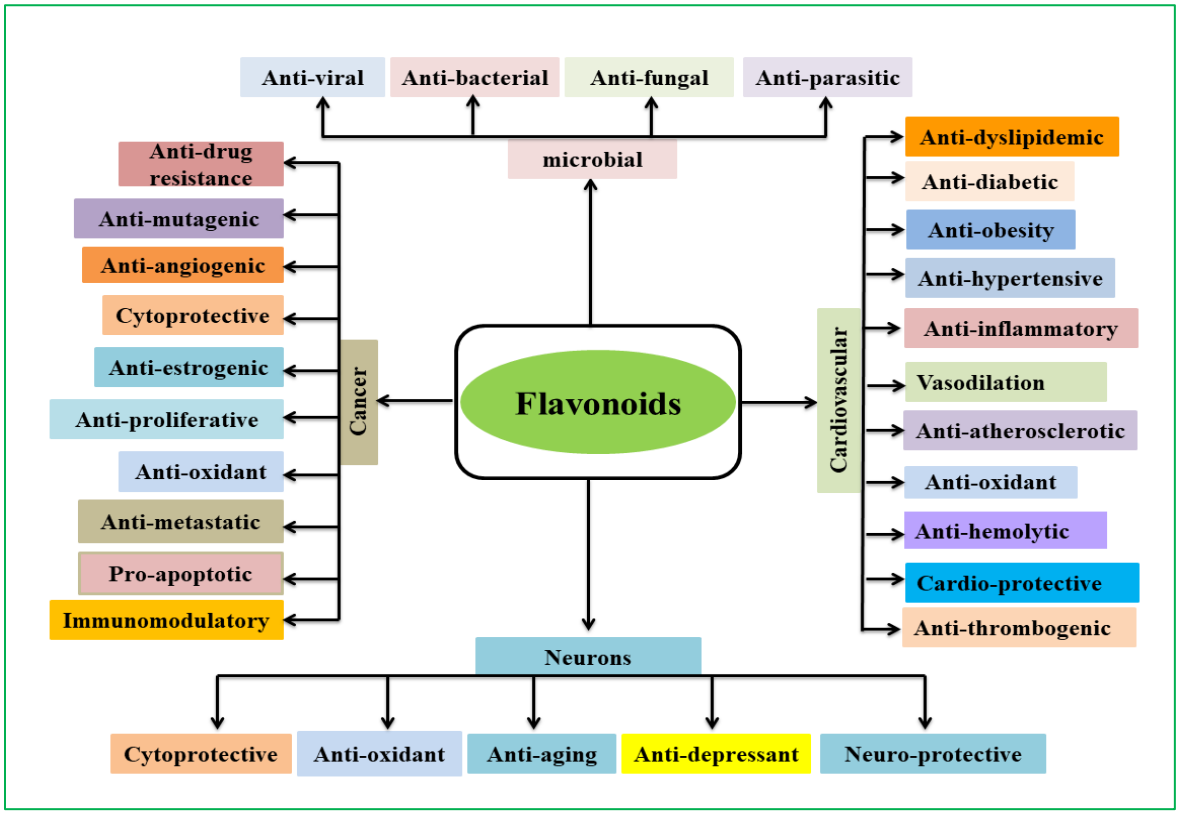



**Figure 1.8.** Biological and pharmacological activities of flavonoids.

### 1.4.6 Flavonoids and cardiovascular diseases

Coronary heart disease, myocardial infarction and stroke are cardiovascular diseases, a group of disorders that affect blood vessels and hearts: they represent the leading cause of death worldwide resulting in 17.8 million deaths in 2017 (Jagannathan et al., 2019). Cardiovascular diseases are generally preceded by many modifiable risk factors such as hypertension, hyperglycemia, obesity, oxidative stress, and others, which are not independent but often occur simultaneously. Additionally, these risk factors may be associated with genetic factors and/or bad lifestyles such as physical inactivity, smoking, inappropriate diet, mental stress, and ageing (Tangvarasittichai, 2015; Petrie et al., 2018). Accumulating evidence obtained from *in vitro*, *in vivo*, clinical and epidemiological studies point to flavonoids as beneficial agents capable of

improving cardiovascular health and ameliorating risk factors associated with cardiovascular diseases (Sánchez et al., 2019) (Fig. 1.9).

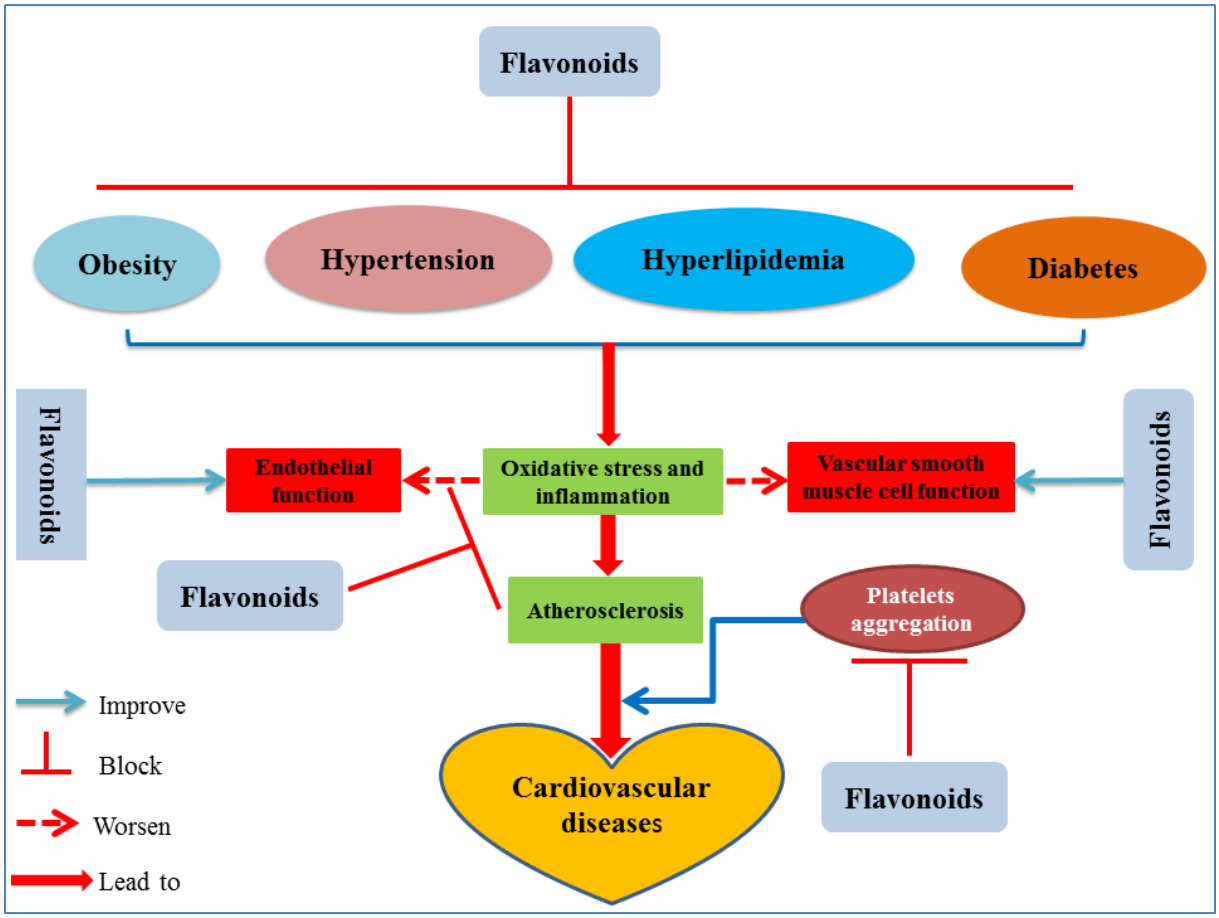



**Figure 1.9.** Flavonoids and cardiovascular diseases.

*1.4.6.1 Flavonoids improve endothelial function*

Endothelial dysfunction is an independent predictor and a risk factor for cardiovascular diseases (Versari et al., 2009; Bertoluci et al., 2015). Dietary flavonoids improve endothelial dysfunction by enhancing NO synthesis *via* the up-regulation of eNOS expression, and/or stimulation of its activity (Grassi et al., 2013; Duarte et al., 2014). Luteolin and cynaroside from artichoke (*Cynara scolymus* L.) increase eNOS expression in human umbilical vein cells (EA.hy 926 and HUVECs) and potentiate the relaxant response to acetylcholine in rat aortic rings (Li et al., 2004). Similarly, the red wine polyphenols cyanidin and the tea catechins epicatechin gallate and epigallocatechin gallate stimulate eNOS expression in EA.hy926 endothelial cells (Wallerath et al., 2005; Appeldoorn et al., 2009). Several flavonoids activate eNOS in a $Ca^{2+}$-independent

manner through the induction of Ser-1179 phosphorylation and/or Thr-495 dephosphorylation [e.g., epigallocatechin gallate (Lorenz et al., 2004), quercetin (Li et al., 2012), luteolin (Si et al., 2014), hesperetin (Rizza et al., 2011), hesperidin and naringin (Kim et al., 2015a)] or through a $Ca^{2+}$-dependent mechanism [quercetin (Khoo et al., 2010), and (-)-epicatechin (Ramirez-Sanchez et al., 2010)]. Furthermore, flavonoids down-regulate caveolin-1 (a negative regulator of eNOS) expression or uncouple it from eNOS (Sánchez et al., 2006; Ramirez-Sanchez et al., 2010), activate chaperonin protein HSP90 (involved in eNOS activation; Ramirez-Sanchez et al., 2012), reduce the level of asymmetrical dimethylarginine, an eNOS inhibitor (Li Volti et al., 2011; Wu et al., 2020; Yang et al., 2020), and inhibit arginase (Kim et al., 2013; Garate-Carrillo et al., 2020), thus stimulating eNOS activity and enhancing NO production. Moreover, quercetin, naringenin, and fisetin activate Sirtuin-1 that, in turn, deacetylates eNOS, thus improving endothelial function (Zhang et al., 2017; Iside et al., 2020). Finally, flavonoids up-regulate eNOS transcription factor Krüpple like factor-2 (KLF-2) to increase NO production (Tsai et al., 2014; Martínez-Fernández et al., 2015).

Flavonoids protect NO from inactivation and prevent eNOS uncoupling by: directly scavenging superoxide anion and preventing $ONOO^-$ formation (Grassi et al., 2010); inhibiting ROS-producing enzymes such as NADPH oxidase, 5-lipooxygenase, COXs, myeloperoxidase, xanthine oxidase; stimulating antioxidant enzymes such as SOD, catalase, and glutathione peroxidase (Izzi et al., 2012; Duarte et al., 2014); increasing the activity of erythroid-derived 2 related factor 2 (Nrf2) and up-regulating antioxidant enzymes such as NADPH:quinone oxidoreductase 1, heme oxygenase-1, glutathione-S-transferase, and γ-glutamylcysteine ligase (Zhang et al., 2013; Li et al., 2016; Yao et al., 2020).

A plethora of *in vitro* studies demonstrate the endothelium-dependency of flavonoid-induced relaxation of different vascular preparations (Loh et al., 2020). Furthermore, they protect endothelial function hampering the synthesis of prostaglandins (Kim et al., 2004), stimulating prostacyclin production (Polagruto et al., 2003; Yam et al., 2016), lowering the expression and release of endothelin-1 (Martínez-Fernández et al., 2015), and limiting the cellular damage caused by either angiotensin II (Wei et al., 2015; Jones et al., 2016) or glucose (Qian et al., 2017; Rezabakhsh et al., 2019). Altogether, these natural molecules improve endothelial function by

increasing NO production and inhibiting its degradation, modulating prostaglandins and prostacyclin synthesis, and by decreasing endothelial damage induced by pathological stimuli.

*1.4.6.2 Flavonoids improve vascular smooth muscle cell function*

Flavonoids relax a plethora of conduit and resistance arteries and veins (Loh et al., 2020). Mechanistically, this vasodilation can be mediated by: the blockade of $Ca^{2+}$ channels (Fu et al., 2005; Morello et al., 2006; Fusi et al., 2017); the opening of $K^{+}$ channels (Novakovic et al., 2015; Fusi et al., 2020); inhibition of $Ca^{2+}$ release (Huai et al., 2013; Tan and Yam, 2018) or stimulation of $Ca^{2+}$ uptake from and to intracellular stores, respectively (Marinko et al., 2018); inhibition of PKC (Duarte et al., 1993), ROCK (Baek et al., 2009; Je et al., 2014; Min et al., 2018), and phosphodiesterases (Orallo et al., 2005; Macêdo et al., 2014); stimulation of sGC (Mahobiya et al., 2018), protein kinase A (Mahobiya et al., 2018; Kumar et al., 2020), and protein kinase G (Iozzi et al., 2013); and/or stimulation of GPCRs like β adrenoreceptor (Revuelta et al., 1999). Notably, vasodilation is usually achieved through more than one of these pathways.

VSMCs are dynamic cells capable of changing their phenotype according to environmental cues. Under certain stimuli or pathological conditions such as hypertension, asthma, and atherosclerosis, VSMCs can transform from quiescent contractile into a synthetic non-contractile phenotype capable of proliferation, migration, and vascular remodelling (Lacolley et al., 2012). Luteolin inhibits the proliferation and migration of rat (A7r5) and human aortic vascular smooth muscle cells. This effect is mediated by inhibition of transforming growth factor-β (TGF-β) receptor 1 (TGFBR1) signalling because: 1) phosphorylation of TGFBR1 and its downstream player Smad 2/3 was decreased and 2) overexpression of TGFBR1 relieved the inhibitory effect of luteolin on VSMC proliferation. Furthermore, Molecular simulation demonstrated the direct binding of luteolin to TGFBR1 (Wu et al., 2018). A similar effect has been reported for quercetin (Alcocer et al., 2002), morin (Shin et al., 2018), cardamonin (Shen et al., 2014), xanthohumol (Liu et al., 2017a), baicalin (Liu et al., 2019), kaempferol (Kim et al., 2015b), naringenin (Chen et al., 2012; Xu et al., 2013), apigenin (Kim et al., 2002), and genistein (Tsai et al., 2017). Various mechanisms underpin this activity including a reduced activation of mitogen-activated protein kinase (Alcocer et al., 2002), modulation of transforming growth factor-β receptor 1 signalling (Wu et al., 2018), p27KIP1, Akt, and MMP-9 functions (Shin et al., 2018), down-

regulation of p38 MAPK, Akt, and extracellular regulated protein kinase phosphorylation (Shen et al., 2014), activation of maternally expressed gene 3 protein/p53 signalling pathway (Liu et al., 2019), modulation of microRNA expression (Kim et al., 2015b), and/or suppression of oxidative stress (Xu et al., 2013). In summary, flavonoids improve VSMC function by inducing vasodilation and/or inhibiting contraction and maintaining their non-synthetic, contractile phenotype by inhibiting proliferation, migration, and remodelling.

*1.4.6.3 Flavonoids inhibit platelet aggregation*

Platelet aggregation plays a pivotal role in the pathogenesis of acute and chronic cardiovascular diseases such as *angina pectoris*, stroke, and myocardial infarction (Gregg and Goldschmidt-Clermont, 2003). Platelet activation is a complex phenomenon, involving multiple players, that fundamentally depends on cytosolic $Ca^{2+}$ concentration. Several agents stimulate phospholipase C and $Ca^{2+}$ release from intracellular stores *via* IP3: $Ca^{2+}$ activates phospholipase A to release arachidonic acid that, in turn, is metabolized into TXA2 by COX-1, thus triggering and amplifying platelet aggregation (Li et al., 2010; Estevez and Du, 2017).

Flavonoids, either as flavonoid-rich food (tea, coffee, and wine) or as pure compounds, have shown an excellent anti-platelet aggregation activity in various experimental settings. Purple grape juice inhibits platelet aggregation *in vitro* and *in vivo*, simultaneously increasing platelet-derived NO production (Freedman et al., 2001). Ingestion of 200 ml of coffee reduces *ex vivo* platelet aggregation induced by collagen and arachidonic acid: this effect was caffeine-independent (Natella et al., 2008). The citrus flavonoids naringin and naringenin show anti-platelet activity, the former was more potent than the latter in peripheral blood platelets isolated from healthy controls (Zaragozá et al., 2016). Also, quercetin diminishes collagen-, thrombin- and ADP-dependent platelet aggregation in a concentration-dependent manner: this effect was mediated by the inhibition of ATP release, P-selectin expression, intracellular $Ca^{2+}$ mobilization, platelet integrin αIIbβ3 activation, and by an increase in the level of cyclic adenosine monophosphate (cAMP) and vasodilator-stimulated phosphoprotein (Oh et al., 2012). A similar effect has been reported for 3,6-dihydroxyflavone (Bojić et al., 2011), apigenin (Wright et al., 2013), chrysin (Liu et al., 2016), hesperetin (Jin et al., 2007), rutin (Sheu et al., 2004), kaempferol (Choi et al., 2015), isorhamnetin, and tamarixetin (Stainer et al., 2019). Ravishankar and coworkers (2018) found that hydroxyl groups in the A-ring are important structural

determinants for optimum platelet inhibitory activity, which decreases when OH is replaced with CHO; conversely, C=O substitution with C=S or B ring modification seems less critical. Flavonoids inhibition of platelet aggregation occurs preferentially through the blockade of signalling pathways initiated by activators such as ADP (Bojić et al., 2011; Iida et al., 2014), collagen (Liu et al., 2016), TXA2 (Guerrero et al., 2005; Muñoz et al., 2009), and thrombin (Choi et al., 2015). Furthermore, these natural products inhibit COX-1 (Jin et al., 2007) or phospholipase A/C activity (Sheu et al., 2004; Jin et al., 2007), reduce $Ca^{2+}$ mobilization from the sarcoplasmic reticulum (Hubbard et al., 2003), increase cyclic nucleotide levels through phosphodiesterase inhibition (Dell'Agli et al., 2008), inhibit protein disulfide isomerase involved in the activation of platelet integrin αIIbβ3 (Gaspar et al., 2020), stimulate NO production, ameliorate oxidative stress, and inhibit the expression of endothelial cell adhesion molecules such as VCAM-1, intercellular adhesive molecule (ICAM-1) and E-selectin (Stangl et al., 2005; Forte et al., 2016). Taken together, these observations indicate that flavonoids counteract platelet aggregation by exploiting several mechanisms including inhibition of the arachidonic acid pathway, and platelet degranulation (Fig. 1.10).

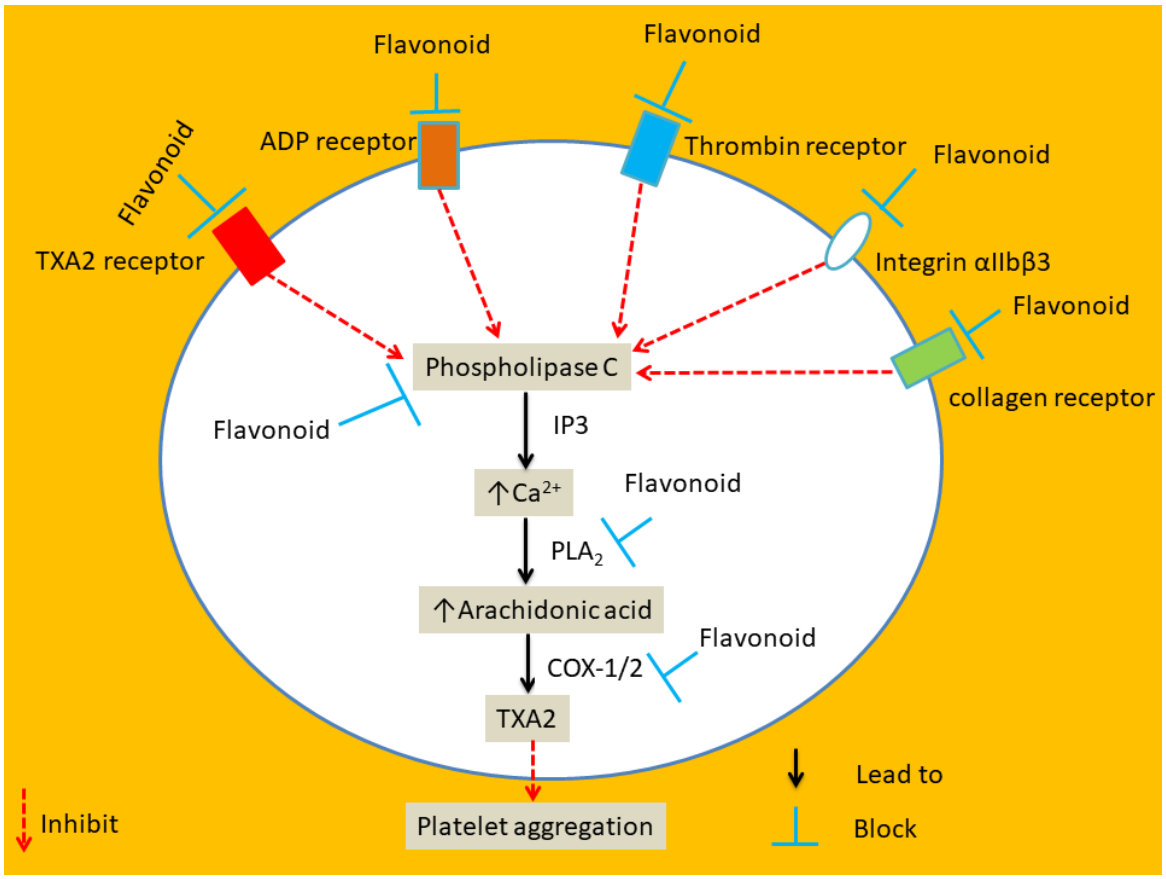


**Figure 1.10.** Mechanisms underpinning flavonoid-induced inhibition of platelet aggregation. Abbreviations: ADP, adenosine diphosphate, COX-1/2, cyclooxygenase-1/2; $IP_3$, inositol,3,4,5-triphosphate; PLA2, phospholipase A2; PLC, phospholipase C, TXA2, thromboxane A2.

*1.4.6.4 Flavonoids ameliorate oxidative stress and vascular inflammation*

Oxidative stress is caused by an imbalance between the production and scavenging of radicals and, more generally, oxidants. It plays a central role in the pathogenesis of several cardiovascular diseases such as vascular inflammation (Münzel et al., 2017), thus representing an important therapeutic target (Münzel et al., 2010).

Flavonoids can prevent oxidative stress and alleviate vascular inflammation through a plethora of mechanisms (Suen et al., 2016; Maiti et al., 2019). First, these polyphenols act as direct scavengers of oxidants such as superoxide anion, $H_2O_2$, hydroxyl radical, etc. The antioxidant activity of flavonoids is attributed to the presence of the double bond at position C2-C3, the OH group at position 3 on the C-ring, the polyhydroxylated B-ring, and the OH group at position 5 on the A-ring (Cao et al., 1997; Procházková et al., 2011). However, they cannot be regarded as direct scavengers of free radicals *in vivo* as the reported antioxidant activity is usually detected at micromolar concentrations, which are hardly attainable in the bloodstream due to their poor absorption, extensive metabolism, and rapid excretion. Hence, other mechanisms may account for oxidative stress amelioration, such as regulation of defence and repair systems along with the maintenance of a nucleophilic tone (i.e. protection against electrophiles, including radicals and oxidants (Forman et al., 2014). Second, flavonoids decrease the expression and/or directly inhibit ROS generating enzymes such as NADPH oxidase, xanthine oxidase, myeloperoxidase, lipoxygenase, COXs, inducible NOS, as well as limit ROS production at the mitochondrial electron transport chain (Shiba et al., 2008; Romero et al., 2009; Wang et al., 2015b; Kicinska and Jarmuszkiewicz, 2020). Third, some flavonoids modulate antioxidant enzymes like catalase, SOD, and glutathione peroxidase in favour of increased ROS elimination (Grassi et al., 2010; Al-Numair et al., 2015). Fourth, they activate the transcription factor nuclear factor erythroid-derived 2-related factor 2 (Nrf2), which regulates the expression of about 250 genes coding for antioxidant proteins, detoxifying enzymes, and cytoprotective proteins (Zheng et al., 2012; Yao et al., 2020). Under normal conditions, Nrf2 is located in the cytoplasm in association with the negative regulator Keap1, responsible for its ubiquitination and proteasomal degradation. Under oxidative stress conditions, Nrf2 disassociates from Keap1, translocates to the nucleus to form a

complex with several coactivators, and binds to the promoter region of antioxidant response elements, thus initiating the transcription of antioxidant enzymes (Smith et al., 2016; Satta et al., 2017). Fifth, flavonoids inhibit the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), thus alleviating the associated cardiovascular inflammation (Chekalina et al., 2018; Choy et al., 2019). NF-kB is a family of inducible transcription factors that play a pivotal role in the expression of pro-inflammatory mediators and cell cycle regulators. It is normally sequestered in the cytoplasm by proteins known as the IkB family, but once stimulated, enters the nucleus and binds to the NF-kB response site on DNA to initiate the transcription of a plethora of genes coding for inflammatory cytokines, chemokines, adhesion molecules, anti-apoptotic factors, and cell cycle regulators, which contribute to inflammation, endothelial dysfunction, angiogenesis, VSMC proliferation and migration, and platelet adhesion (Van der Heiden et al., 2010; Liu et al., 2017b). Sixth, they chelate transition metal ions such as iron and copper that may contribute to oxidative stress (Forman et al., 2014). Noticeably, flavonoids, like many antioxidants, show also pro-oxidant effects by direct production of hydroxyl radical *via* Fenton reaction, by oxidation of flavonoid phenoxyl radicals (end product of ROS scavenging reaction by flavonoids), inhibition of mitochondrial respiration, oxidation of low molecular antioxidants, and direct oxidation by peroxidases (Procházková et al., 2011). In summary, flavonoids protect the vascular system from oxidative stress due to their direct antioxidant activity and/or shifting the redox signalling balance towards a more antioxidant one.

*1.4.6.5 Flavonoids ameliorate dyslipidemia/hyperlipidemia*

Dyslipidemia, a persistent elevation of cholesterol, triglyceride, and low-density lipoprotein (LDL) levels along with a persistent decrease of high-density lipoprotein (HDL) levels, is the major risk factor for atherosclerosis and coronary heart diseases (Lacoste et al., 1995; Musunuru, 2010).

The lipid-lowering effect of flavonoids has been evaluated in different animal models (Koshy and Vijayalakshmi, 2001) and humans (Maron et al., 2003). Quercetin markedly reduces serum cholesterol and triglyceride in rabbits fed a high-fat diet for 12 weeks (Juźwiak et al., 2005). Similarly, administration of this flavonoid with a high-cholesterol diet to rats results in a significant reduction of liver triglycerides (-24%), liver and serum cholesterol (-22% and -20%,

respectively), and serum LDL (31%) while doubling serum HDL cholesterol as compared to control animals (Mariee et al., 2012).

Naringenin normalizes cholesterol, triglyceride, free fatty acid, very-low-density lipoprotein (VLDL), and LDL levels, increases plasma HDL, lipoprotein lipase, and lecithin cholesterol acyltransferase, and reduces the expression of β-hydroxy β-methylglutaryl-CoA reductase in rats fed 20% ethanol for two months (Jayachitra and Nalini, 2012). Similarly, naringenin also effectively decreases lipid (cholesterol and triglycerides) accumulation in the aorta and liver of mice lacking LDL receptors, fed a high-fat diet (Mulvihill et al., 2010).

Chrysin, orally administered to triton WR-1339-induced hyperlipidemic female C57BL/6 mice, significantly decreases total cholesterol, LDL cholesterol, and triglyceride levels (Zarzecki et al., 2014). Apigenin supplementation to C57BL/6J mice fed a high-fat diet for 16 weeks significantly decreases plasma levels of free fatty acids, total cholesterol, and apolipoprotein B by up-regulating the genes involved in fatty acid oxidation, Krebs's cycle, oxidative phosphorylation, and electron transport chain, down-regulating the expression of lipolytic and lipogenic genes, and inhibiting the activity of enzymes involved in triglycerides and cholesterol synthesis (Jung et al., 2016).

The black tea flavonoids theaflavin and thearubigins markedly reduce cholesterol, LDL, and triglyceride levels while increasing HDL levels in rats fed high cholesterol diet, theaflavin based drink being more effective than thearubigins alone or theaflavin and thearubigins combination (Imran et al., 2018). The lemon fruit flavonoid eriocitrin does not affect hepatic total cholesterol or triglycerides though significantly decreasing total serum cholesterol, VLDL, LDL, triglyceride, and phospholipid levels in rats fed a high-fat diet increasing their excretion in bile acid, clearance, or the expression of LDL receptor (Miyake et al., 2006). A similar effect is exerted by total flavonoid extracts from *Lomatogonium rotatum* (Bao et al., 2016), blueberry (Li et al., 2011), *Polygonum perfoliatum L*. (Wang et al., 2018a), and *Perilla frutescens* leaves (Feng et al., 2011) administrated to dyslipidemic rat models.

Studies concerning the anti-dyslipidemic effect of flavonoids in humans are scarce. For example, quercetin supplementation gives rise to contrasting results either decreasing triglycerides and LDL or being ineffective (Lee et al., 2011; Pfeuffer et al., 2013). A meta-analysis of five

randomized control trials performed on quercetin evidenced only a reduction of total triglycerides at doses >50 mg/day (Sahebkar, 2015). In conclusion, flavonoids seem to exert an excellent lipid-lowering effect in different dyslipidemic animal models by lowering the levels of total cholesterol, LDL, free fatty acids, and triglycerides as well as by increasing HDL levels.

*1.4.6.6 Flavonoids protect against hypertension*

Hypertension represents the main risk factor for various cardiovascular diseases including coronary heart disease, stroke, and cardiac arrhythmia (Oparil et al., 2018; Fuchs and Whelton, 2020). The antihypertensive effect of flavonoids has been claimed based on data obtained in *in vitro* blood vessel assays, animal models, and subsequently confirmed by human intervention and population studies (Clark et al., 2015). *In vitro* studies performed on blood vessels obtained from different animal species highlighted the excellent vasodilating activity of quercetin, naringenin, galangin, chrysin, apigenin, fisetin, tamarixetin, eupatorin, morin, kaempferol, genistein, and others (Loh et al., 2020).

In animal models, quercetin lowers the high blood pressure in spontaneously and salt-induced hypertensive rats (Duarte et al., 2001; Olaleye et al., 2014). Interestingly, the glucuronidated form of this flavonoid also decreases blood pressure of SHR after deconjugation at the target tissue by β-glucuronidase (Galindo et al., 2012). Similarly, chrysin shows an excellent antihypertensive effect in spontaneously and L-NAME induced hypertensive rats (Villar et al., 2002; Ramanathan and Rajagopal, 2016). This effect has also been reported for catechin (Jaffri et al., 2011), epigallocatechin gallate (Luo et al., 2020), hesperidin (Hashemzaei et al., 2020), genistein (Cho et al., 2007), luteolin (Lv et al., 2013), myricetin (Godse et al., 2010), naringin (Akintunde et al., 2020), and naringenin (Sánchez-Recillas et al., 2019; Oyagbemi et al., 2020) in various animal models of hypertension. Noticeably, these studies demonstrate that flavonoids are ineffective on normotensive rats.

Human data are limited and sometimes conflicting. Quercetin, for example, does not affect blood pressure in healthy men and women (Conquer et al., 1998; Bondonno et al., 2016) or in rheumatoid arthritis patients (Javadi et al., 2014), but reduces it in women with type 2 diabetes (Zahedi et al., 2013), in hypertensive patients (Edwards et al., 2007), in obese individuals with metabolic syndrome traits (Egert et al., 2009), in healthy male smokers (Lee et al., 2011), and in

healthy male carrying the apolipoprotein E genotype (Pfeuffer et al., 2013). A meta-analysis of seven clinical studies (enrolling 587 patients) shows that quercetin significantly reduces both systolic and diastolic blood pressure, though only at doses >500 mg/day (Serban et al., 2016).

In a cross-over clinical trial, a high-flavonoid sweetie juice (rich in naringin and narirutin) followed by a low-flavonoid sweetie juice (25% of naringin and 30% of narirutin as compared to the high-flavonoid sweetie juice), each for 5 weeks, significantly decrease both diastolic and systolic blood pressure in twelve stage-1 hypertensive patients. The former juice was particularly effective in reducing diastolic blood pressure (Reshef et al., 2005). Furthermore, the prolonged consumption or even a single dose administration of a hesperidin-enriched orange juice reduces systolic blood pressure in a randomized clinical trial involving pre- and stage-1 hypertensive individuals (Valls et al., 2020). Altogether, flavonoids exhibit an anti-hypertensive effect when administered to hypertensive models though only at high concentrations but do not affect the normotensive ones. This effect is mediated by their excellent endothelium-dependent and -independent vasorelaxant activity and alleviation of oxidative stress (Maaliki et al., 2019).

*1.4.6.7 Flavonoids exert anti-obesity effects*

Obesity, an accumulation of excessive fat (body mass index (BMI) ≥30), affects human health and reduces the quality of life. Its occurrence increases the incidence of hypertension and type-2 diabetes and is an independent risk factor for cardiovascular diseases such as stroke, coronary heart disease, and myocardial infarction (Poirier et al., 2006; Akil and Ahmad, 2011; Carbone et al., 2019).

Several cellular, animal models, and human intervention studies demonstrate the anti-obesity properties of flavonoids (Akhlaghi et al., 2018; Rufino et al., 2021). At the cellular level, the citrus flavonoid hesperetin reduces lipid accumulation by 63% at 25 µM and decreases ROS generation compared to control (Lee et al., 2017). Similarly, kaempferol inhibits adipogenesis in pre- and mature adipocytes. This effect is mediated by down-regulation of CCAAT enhancer binding protein alpha (Cebpa) mRNA (which encodes for a transcription factor involved in the modulation of proteins regulating cell cycle and body weight homeostasis) and up-regulation of *Pnpla2* and *Lipe* genes expression (both genes encode for enzymes involved in triglyceride hydrolysis) in 3T3-L1 cells (Torres-Villarreal et al., 2019). A similar effect has also been

described for quercetin (Seo et al., 2015a), chrysin (Choi and Yun, 2016), and apigenin (Gómez-Zorita et al., 2017), among others.

Galangin reduces body weight, energy intake, liver weight, and parametrial adipose tissue in obese female albino rats fed a cafeteria diet for 6 weeks. It also restores serum lipids; hence, counteracting lipid peroxidation and limiting the accumulation of hepatic triglycerides (Kumar and Alagawadi, 2013). Similarly, myricetin administered to obese C57BL/6 mice fed a high-fat diet significantly reduces body weight and restores several obesity-associated parameters such as serum glucose, triglyceride, and cholesterol levels. It also improves oxidative stress markers such as glutathione peroxidase activity, total antioxidant capacity, malondialdehyde level, and inflammatory markers (e.g., tumour necrosis factor-α (TNF-α)) (Su et al., 2016). Chrysin significantly reduces body weight, BMI, abdominal circumference/thoracic circumference ratio, and calorie intake while increasing locomotor activity and faecal cholesterol excretion in obese rats fed a high-fructose diet compared to control (Pai et al., 2020). More interestingly, apigenin administered to high fat-diet induced obese mice, reduces both body weight and visceral adipose tissues, by binding to non-phosphorylated STAT3 in visceral adipose tissues, thus reducing its transcriptional activity. This, in turn, decreases the expression of cluster of differentiation 36 (CD36) that, under physiological conditions, controls the level of peroxisome proliferator-activated receptor gamma (PPAR-γ), a critical nuclear factor for adipogenesis (Su et al., 2020). Similar effects are shown also by other flavonoids: quercetin (Seo et al., 2015a), fisetin (Liou et al., 2018), kaempferol (Wang et al., 2020), and morin (Madkhali, 2020).

So far, clinical trials assessing flavonoids in humans have produced conflicting results (Llaneza et al., 2012; Engelbert et al., 2016). Akhlaghi and coworkers (2018) meta-analyzed 58 studies published until 2018 concluding that flavanols have the potential for reducing the BMI and waist circumference in the overall population. Stratification analysis shows that flavanols decrease the BMI also in Asians, in subjects under 50 year-olds, or in those consuming higher doses >500 mg/day. Similarly, isoflavones reduce the BMI in non-Asian populations or in those consuming doses ≥75 mg/day. However, neither the BMI nor the waist circumference is modified by flavonols, flavanones, and anthocyanins.

Several mechanisms underlying the anti-obesity effect of flavonoids were hypothesized:

1. reduction of appetite and food intake *via* modulation of neuropeptide and leptin which control the hunger/satiety centre (Myoung et al., 2010);
2. reduction of carbohydrate and lipid digestion and/or absorption by decreasing activity of digestive enzymes such as pancreatic lipase (Rahim et al., 2015; Martinez-Gonzalez et al., 2017), α-amylase (Takahama and Hirota, 2018; Martinez-Gonzalez et al., 2019), and α-glucosidase (Proença et al., 2017; Hua et al., 2018);
3. modulation of lipid metabolism in favour of suppressed adipocyte differentiation and/or adipogenesis and enhanced lipolysis, β-oxidation, and apoptosis, through a complex hormonal and biochemical signalling involving CCAAT-enhancer binding protein (C/EBPα, β, δ), and PPARγ (Rufino et al., 2021);
4. induction of adipose tissue browning through the modulation of, for example, AMPK and or sirtuin-1 signalling pathways that, in turn, induces mitochondrial biogenesis and uncoupling protein-1 expression and, hence, energy expenditure, as demonstrated for rutin (Seo et al., 2015b), pentamethylquercetin (Han et al., 2017), quercetin (Choi et al., 2018), luteolin (Zhang et al., 2016), nobiletin (Lone et al., 2018), and other flavonoids (Zhang et al., 2019);
5. normalization of obesity-associated gut dysbiosis, as reported for quercetin, rutin, catechin, procyanidin, epigallocatechin gallate, and other flavonoids (Gil-Cardoso et al., 2016; Bai et al., 2019).

In summary, flavonoids exhibit a powerful anti-adipogenic effect at cellular and organism levels, particularly under obesity conditions. This effect involves a wide array of mechanisms: inhibition of lipid digestion and absorption, modulation of metabolism, and induction of adipose tissue browning.

#### *1.4.6.8 Flavonoids ameliorate hyperglycemia and diabetes*

Type-2 diabetes is not only a metabolic disorder characterized by persistent hyperglycemia but also an independent risk factor for cardiovascular diseases, the latter representing the most frequent cause of morbidity and mortality in diabetic patients (Leon and Maddox, 2015). Flavonoids seem to exert antidiabetic effects as demonstrated in several animal models and clinical trials (Fang et al., 2019; Russo et al., 2019). In particular, diosmin administered orally for 4 weeks to streptozotocin-induced diabetic rats significantly restores blood sugar levels,

improves lipid profile, reduces malondialdehyde levels, and increases glutathione levels and SOD activity (Jain et al., 2014). Similarly, apigenin improves diabetes-associated nephropathy in streptozotocin-induced diabetic rats as compared to control animals and inhibits renal dysfunction, oxidative stress, inflammation, collagen deposition, glomerulosclerosis, and fibrosis (Malik et al., 2017). A promising anti-diabetic effect characterizes also other flavonoids assessed in various diabetic animal models: fisetin (Prasath et al., 2014), quercetin (Shi et al., 2019), morin (Vanitha et al., 2014; Jiang et al., 2020a), hesperetin (Jayaraman et al., 2018), baicalein (Zhang et al., 2018), and kaempferol (Chandramohan et al., 2014).

Unlike studies on animal models, human clinical trials are limited to a few flavonoid-rich foods or flavonoid mixtures: furthermore, these studies show sometimes inconsistent results. For instance, flavonoids intake was not associated with a lower risk of type-2 diabetes in post-menopausal women (Nettleton et al., 2006) whereas, in a large cohort study involving 340,234 participants from 8 European countries, flavanols (monomer, dimer or pro-anthocyanidin with low degree of polymerization) and flavonols (in particular myricetin) were associated with a lower risk of type-2 diabetes (Zamora-Ros et al., 2014). A similar result was observed with quercetin in the Chinese population (Yao et al., 2019). However, the consumption of flavonols, flavones, quercetin, apigenin, myricetin, kaempferol and luteolin is not associated with type-2 diabetes incidence (Song et al., 2005).

In a cross-sectional study performed on 1997 women aged 18-76 years, a high intake of anthocyanins and flavones was associated with low peripheral insulin resistance and inflammation biomarkers; the other flavonoid subclasses assessed are ineffective (Jennings et al., 2014). Similarly, a follow-up study of 200,000 US women and men demonstrate that high anthocyanin consumption lowers the risk of type-2 diabetes; this beneficial effect seems to be specifically correlated to anthocyanins as no association was detected with the total flavonoid intake (Wedick et al., 2012). However, a subsequent meta-analysis considering four studies and 284,806 participants found that total intake of flavonoids is indeed associated with a lower risk of type-2 diabetes (Liu et al., 2014). The same conclusion was reached by an independent meta-analysis of nine prospective cohort studies involving 172,058 participants for flavonols, flavan-3-ols, and isoflavones (Guo et al., 2019). Xu et al. (2018) demonstrated in a meta-analysis of eight prospective studies involving 312,015 participants that, beyond total flavonoids,

anthocyanidins, flavan-3-ols, flavonols, and isoflavones are inversely correlated with type-2 diabetes risk.

In a meta-analysis of 18 studies involving 457,922 participants, a high intake of coffee, decaffeinated coffee, or tea was associated with a lower risk of type-2 diabetes (Huxley et al., 2009), in line with two studies that took into account tea consumption (Van Woudenbergh et al., 2012; Yang et al., 2014). In a previous study, however, tea consumption showed only a borderline effect on type-2 diabetes (Song et al., 2005).

Various mechanisms underpin the antidiabetic effect of these natural compounds (as depicted in Fig. 1.11): 1) modulation of digestive enzymes and, hence, lipid and carbohydrate absorption; 2) increased expression and translocation of glucose transporter-4; 3) modulation of hepatic enzymes involved in glucose metabolism, enhancing glucose uptake and storage; 4) protection of β-cells from apoptosis and enhancement of insulin secretion; 5) increased expression of and activation of PPAR-γ, thus improving glucose uptake and insulin sensitivity; 6) activation of the AMP-kinase pathways; 7) inhibition of tyrosine kinase activity; and 8) activation of the NF-κB pathway (Chen et al., 2015; Mahmoud et al., 2015; Al-Ishaq et al., 2019). Altogether, flavonoids seem to have a promising antidiabetic effect in particular in diabetic animal models. In humans, this effect appears to be conferred by flavonoid-rich foods rather than by individual flavonoids.

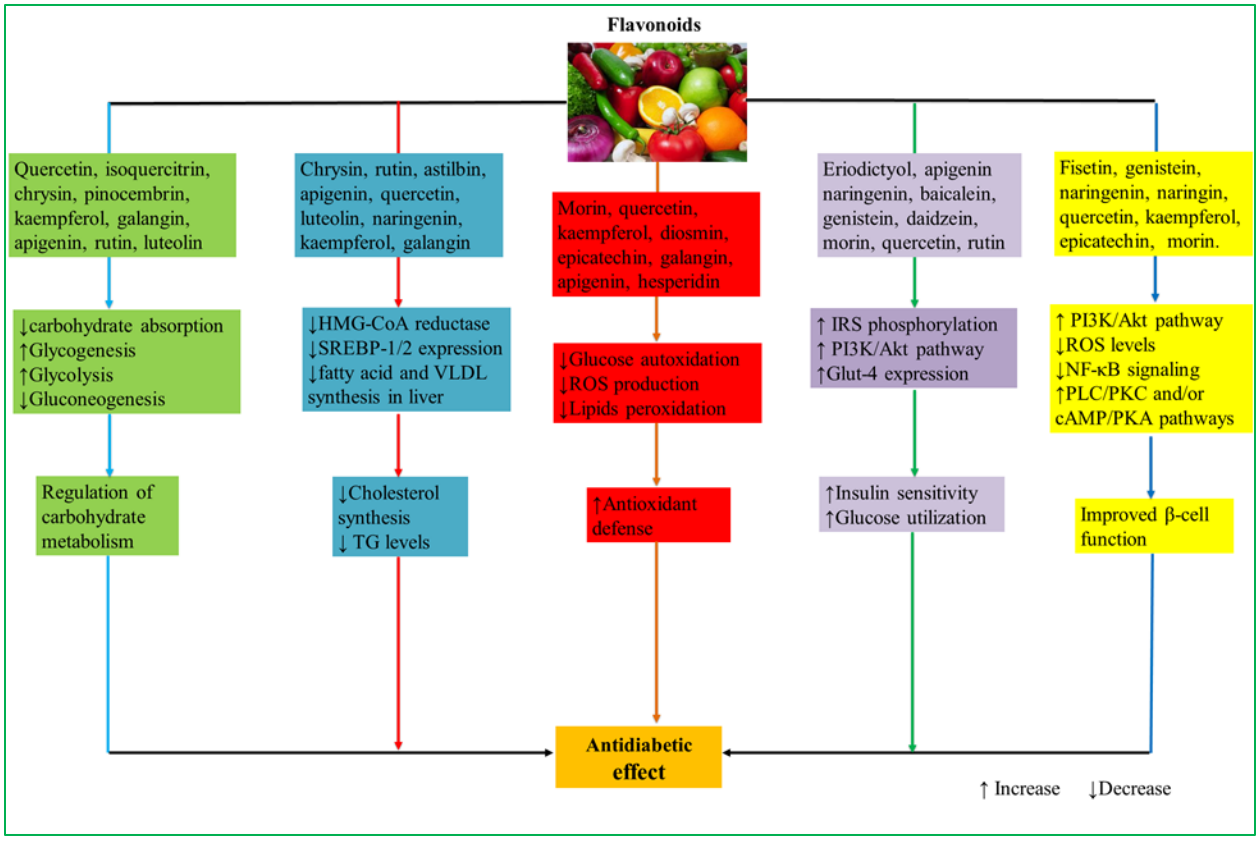



**Figure 1.11.** Mechanisms involved in flavonoid anti-diabetic effects. Abbreviations: cAMP, cyclic adenosine monophosphate; Glut-4, glucose transporter 4; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IRS; insulin receptor substrate; PI3K, phosphatidylinositol-3- 17 kinase; PKC, protein kinase C; PKA, protein kinase A; PLC, phospholipase C; ROS, reactive oxygen species; SREBP-1c, sterol regulatory element-binding protein; TG, triglycerides; VLDL, very-low-density lipoprotein.

*1.4.6.9 Flavonoids attenuate atherosclerosis development*

Atherosclerosis is the major direct cause of cardiovascular diseases such as myocardial infarction, stroke, and heart failure. It initiates with fat deposition on the vessel wall, which develops forming plaques that, in turn, either restrict blood flow or induce thrombosis (Frostegård, 2013).

Several flavonoids hamper atherosclerosis pathogenesis in various animal models (Salvamani et al., 2014). In particular, in apolipoprotein E knockout mice fed a high-fat diet, quercetin ameliorates atherosclerosis-associated alterations by enhancing autophagy, up-regulating P21 and P53 expression, and down-regulating TNFα, IL1β, IL18 and mTOR (Cao et al., 2019a).

Interestingly, apigenin shows an anti-atherosclerotic effect both in RAW264.7 macrophages and apolipoprotein E knockout mice by upregulation of ABCA1 expression *via* miR-33 repression that, in turn, increases cholesterol efflux and decreases foam cells formation, and the down-regulation of inflammation-associated markers like TLR-4, MyD88, p-IκB-α, and nuclear NF-κB p65 subunit (Ren et al., 2018). Also, fisetin shows a promising anti-atherosclerotic effect in apolipoprotein E knockout mice fed a high-fat diet: in fact, it reduces atherosclerotic plaque formation and lipid accumulation in the aortic sinus. This effect is mediated by the down-regulation of PCSK9, LOX-1, p53, p21, and p16 (Yan et al., 2021). Other flavonoids such as kaempferol (Kong et al., 2013), chrysin (Anandhi et al., 2014), and naringenin (Mulvihill et al., 2010) show similar effects in various atherosclerotic animal models.

The studies examining the effect of flavonoids on human atherosclerosis are limited and usually confined to flavonoid-rich food rather than individual flavonoids. For instance, tea but not coffee, caffeine or caffeinated coffee consumption, is inversely associated with coronary artery plaque progression (Reis et al., 2010). In the Danish population, a daily intake of flavonoids reduces by 14% the risk of atherosclerosis, particularly in smokers and alcohol consumers (Dalgaard et al., 2019).

The main mechanism responsible for the anti-atherosclerotic effect of flavonoids is inhibition of LDL oxidation and, hence, foam cell formation, which is considered the hallmark of atherosclerosis progression. This is achieved by, for example, quercetin enhancement of cholesterol efflux from foam THP-1 cells treated with oxidized LDL, an effect mediated by the up-regulation of PPARγ and ABCA1 expression (Sun et al., 2015). Quercetin also enhances the viability of RAW264.7 macrophages treated with oxidized LDL and decreases the intracellular accumulation of cholesterol and lipids. Up-regulation of ABCAl, ABCG1, and LXRα and down-regulation of PCSK9, p53, p21, and p16 seem~~s~~ involved in this activity (Li et al., 2018). Other flavonoids such as chrysin (Wang et al., 2015a), baicalin (He et al., 2016), kaempferol (Li et al., 2013), and fisetin (Jia et al., 2019) inhibit foam cell formation.

Other mechanisms participate in the anti-atherosclerotic effect of flavonoids: regulation of lipid metabolism in favour of enhanced cholesterol excretion (Deng et al., 2020); inhibition of endothelial cell senescence induced by oxidized LDL (Jiang et al., 2020b); enhanced autophagic activity of foam cells (Cao et al., 2019b); and reduced plaque rupture by metalloproteinase

inhibition (Song et al., 2001). In conclusion, flavonoids exhibit, at least in animal models, a promising anti-atherosclerotic effect that is mediated by reduced lipid oxidation, lipid accumulation, and foam cell formation.

### 1.4.7 Flavonoids and PVAT: what is known and what is missing?

Studies investigating the effect of flavonoids on PVAT are scarce. Hesperidin, for example, reverses PVAT advanced glycation end-products accumulation in old C57BL/6 mice to the level of young mice, thus improving the age-related PVAT-mediated arterial stiffening. This observation suggests a potential application of hesperidin in arterial de-stiffness and reduction of cardiovascular diseases *via* a PVAT mediated mechanism (Ouyang et al., 2017). Downregulation of the proinflammatory factor NF-κB p65 and the oxidative stress marker nitrotyrosine was hypothesized to underpin this beneficial effect of the flavonoid.

The standardized *Crataegus* extract WS® 1442, which contains considerable amounts of flavonoids, administered to high fat diet-induced obese male C57BL/6J mice, restored PVAT-mediated aorta response to acetylcholine without reducing the animal body weight. The mechanism underpinning this effect was hypothesized to be an increased phosphorylation and a decreased acetylation of PVAT eNOS (Xia et al., 2017; Halver et al., 2019).

In addition, several pathways are influenced and/or regulated by both PVAT and flavonoids. PVAT, for example, releases superoxide anions and $H_2O_2$, which are known to be scavenged by flavonoids (Yokomiza and Moriwaki, 2006; Khan et al., 2012). PVAT expresses different isoforms of NOS and releases NO that influences vascular tone. Flavonoids modulate NOS expression and activity by either increasing NO production or decreasing its degradation rate (Ahmed, 2021). PVAT secretes adiponectin (Withers et al., 2014); flavonoids improve its signalling in obese mice (Shabrova et al., 2011; Goto et al., 2012) and increase its serum level in diabetic patients (Yang et al., 2020). Finally, PVAT expresses a complete RAS system and secretes both angiotensin 1-7 and angiotensin II, while flavonoids inhibit ACE2 (Muchtaridi et al., 2020), thus protecting against angiotensin II-induced hypertension and endothelial dysfunction (Sarr et al., 2006; Wei et al., 2015).

Taken together, these observations suggest a potential overlapping control exerted by both PVAT and flavonoids on the vascular tone. Therefore, a possible functional interplay between

these two actors, occurring at the vascular level, was hypothesized. To vadilate this hypothesis, the choice of the best in-depth characterized model systems (in terms of animal species and vessel type) in the field of PVAT research is mandatory/crucial. A literature survey performed on the PubMed database identified the rat species and the aorta artery as the two best candidates (Fig. 1.12) which were, therefore, used in the present study. The fact that the cardiovascular physiology and pharmacology of the rat are well-known as are the mechanisms underpinning the contraction and relaxation of the rat aorta also contributed to this choice. Furthermore, the rat thoracic aorta is a large and long vessel: surgical and post-surgical procedures are easy to perform, and standard laboratory equipment allows for collecting a significant number of data in a short time from a few animals, in line with the 3Rs objectives. Finally, a plethora of studies have characterized the physiology of PVAT and the vascular effects of flavonoids in rat thoracic aorta, thus allowing a more rigorous assessment of their functional interaction.

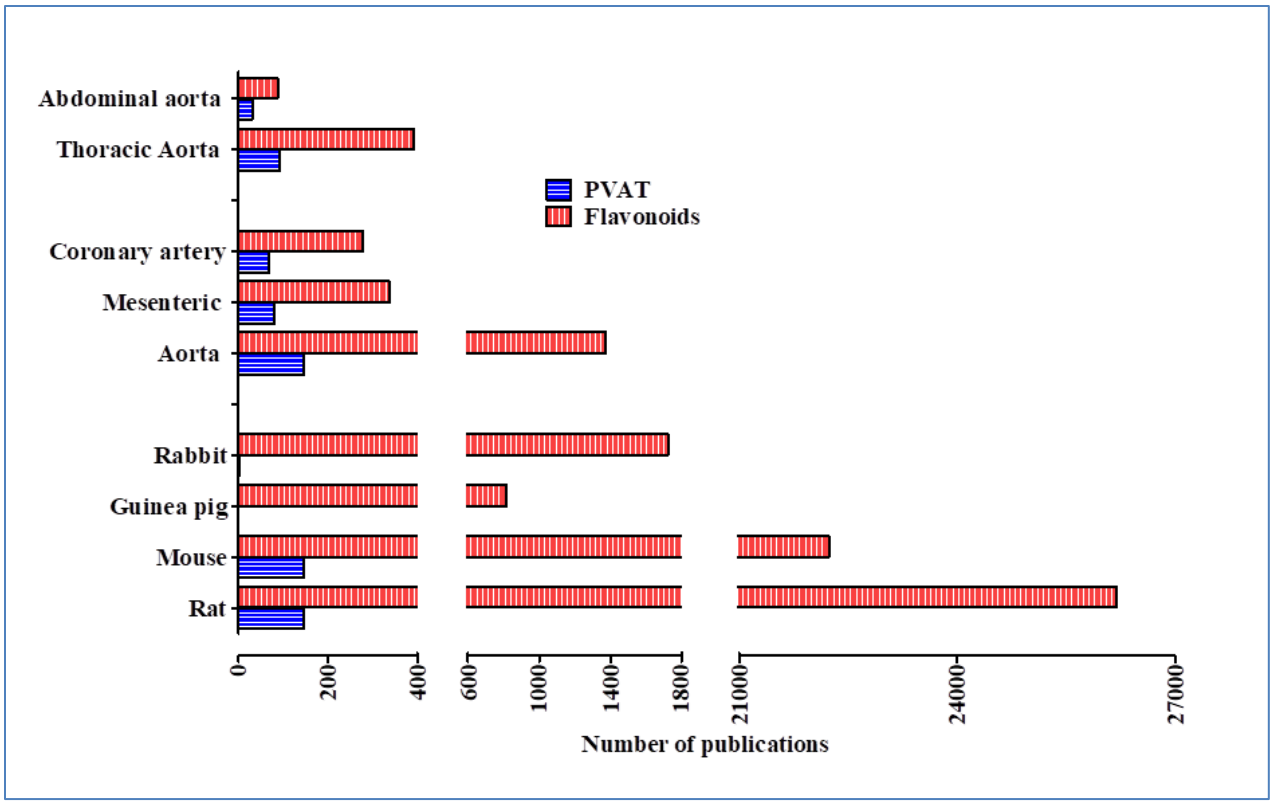



**Figure. 1.12.** Number of scientific publications provided by the PubMed database using the keywords “PVAT” or “flavonoids” in association with various animal models or vessels, as reported in the graph title.

# CHAPTER 2:

# MATERIALS AND METHODS

# 2. Materials and methods

## 2.1 Statement on Animal Care

All the study procedures were in strict accordance with the European Union Guidelines for the Care and the Use of Laboratory Animals (European Union Directive 2010/63/EU) and approved by the Animal Care and Ethics Committee of the University of Siena and the Italian Department of Health (7DF19.N.TBT). Male Wistar rats (weighing 347±42) were purchased from Charles River Italia (Calco, Italy) and maintained in an animal house facility at 25±1°C and 12:12 h dark-light cycle with access to standard chow diet and water *ad libitum*.

## 2.2 Preparation of aorta rings

Animals were anaesthetized with an isoflurane (4%) and $O_2$ gas mixture using Fluovac (Harvard Apparatus, Holliston, Massachusetts, USA), decapitated and exsanguinated. Following the opening of the abdominal and thoracic cavity, the thoracic aorta was gently removed along with adhered PVAT, washed from blood, and placed in a modified Krebs-Henseleit physiological solution (KHS) at 4°C containing (final concentrations expressed in mM): NaCl 118; KCl 4.75; $KH_2PO_4$ 1.19; $MgSO_4.7H_2O$ 1.19; $NaHCO_3$ 25; glucose 11.5; $CaCl_2.2H_2O$ 2.5; saturated with carbogen (95% $O_2$, 5% $CO_2$) to obtain a pH of 7.4. The thoracic aorta was cut into 10-12 rings of 3 mm long using a tissue slicer (2Biological Instruments, Besozzo, Italy). Two different types of aortic rings were prepared: rings with either intact (+PVAT) or deprived of (-PVAT). In some experiments, PVAT was detached from rings and left in the organ bath (Fig. 2.1). The endothelium was removed by gently rubbing the lumen of the ring with a forceps tip. Rings were mounted in organ baths between two parallel, L-shaped, stainless steel hooks, one fixed in place and the other connected to an isometric transducer, and subjected to 1 g passive tension (Carullo et al., 2021; Gao et al., 2007).

Preparations were allowed to equilibrate for 60 min to overcome the mechanical stress suffered during preparation. In this equilibration phase, KHS was renewed every 15 min and passive tension, if decreased by more than 25% within the first 15 min, was restored to the initial value. The mechanical activity of aorta rings was transformed into an electrical signal using the isometric transducer, amplified, and differentiated with an operational amplifier, and converted

from analogue to digital for analysis using a computerized acquisition system (LabChart 7.3.7; ADInstrument, Castle Hill, Australia).

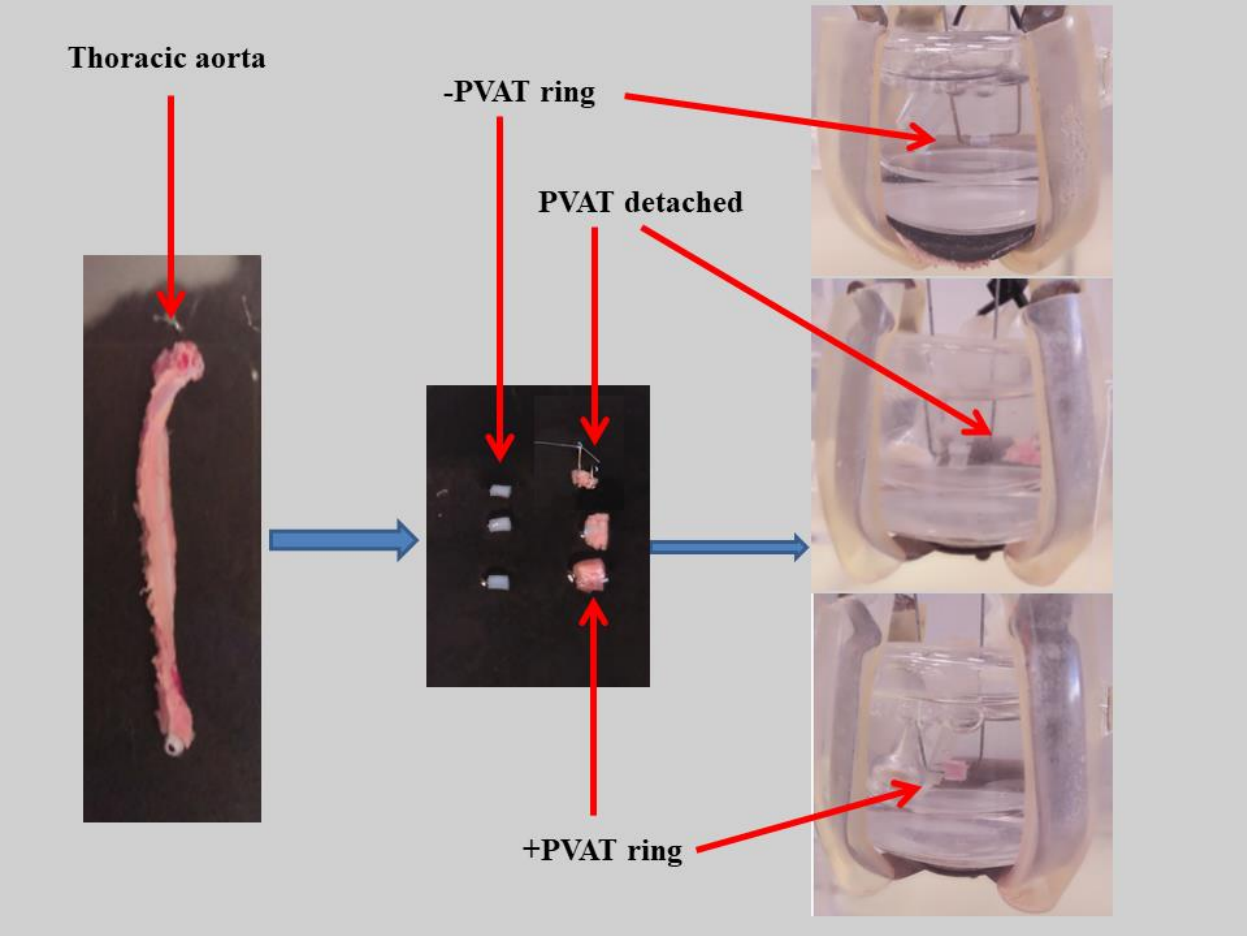



**Figure 2.1.** Schematic representation of the procedure followed to prepare aorta rings. On the right-hand column pictures of organ bath containing different preparations are shown.

## 2.3 Functional testing of the aorta preparations

### 2.3.1 Pharmaco-mechanical coupling and endothelium test

After 1 hour of initial equilibration, rings were pre-contracted with a sub-maximal concentration (0.3 µM) of the $\alpha_1$-adrenergic selective agonist phenylephrine. The contractile response induced by phenylephrine is mediated by both mobilization of $Ca^{2+}$ from intracellular stores and $Ca^{2+}$ influx through receptor-operated, store-operated, and voltage-gated $Ca^{2+}$ channels. Once the contraction reached a plateau, 10 µM acetylcholine was added: a relaxation less than 10% denoted the absence of a functional endothelium. Preparations were then washed for at least 60

min, once every 15 min before performing the electro-mechanical coupling test (Fig. 2.2).

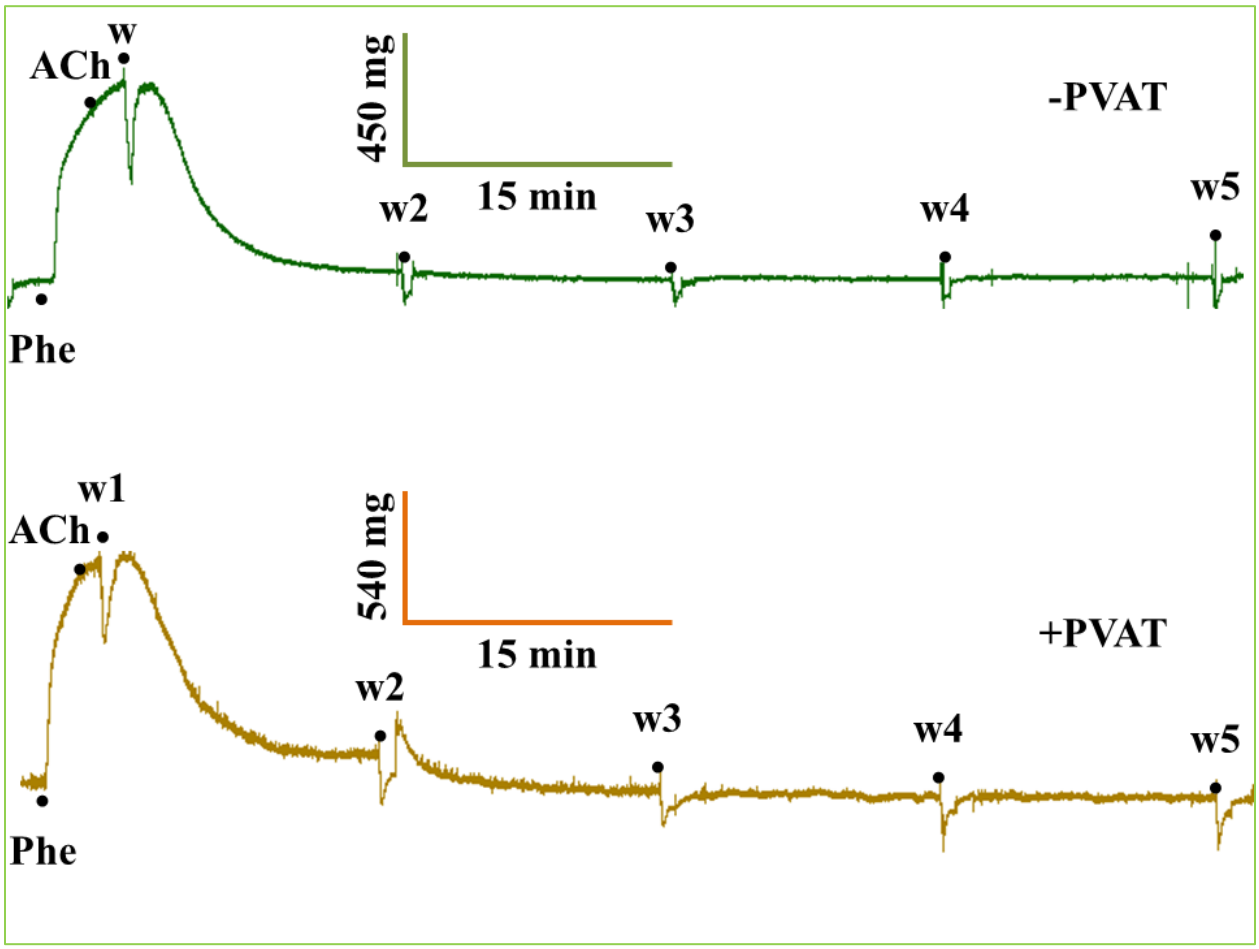



**Figure 2.2**. Pharmaco-mechanical coupling. Test of the viability of aorta rings and the functional integrity of the endothelium. Phe, phenylephrine; Ach, acetylcholine; w, wash.

### 2.3.2 Electro-mechanical coupling test

Preparations were challenged with 60 mM KCl (K60), which depolarizes the cell membrane, thus leading to the opening of voltage-gated $Ca^{2+}$ channels. The ensuing $Ca^{2+}$ influx triggers a tonic contraction. After 15 min, rings were washed with KHS at least for one hour once every 15 min to bring muscle tone back to the basal values before proceeding with the various experimental protocols (Fig. 2.3).

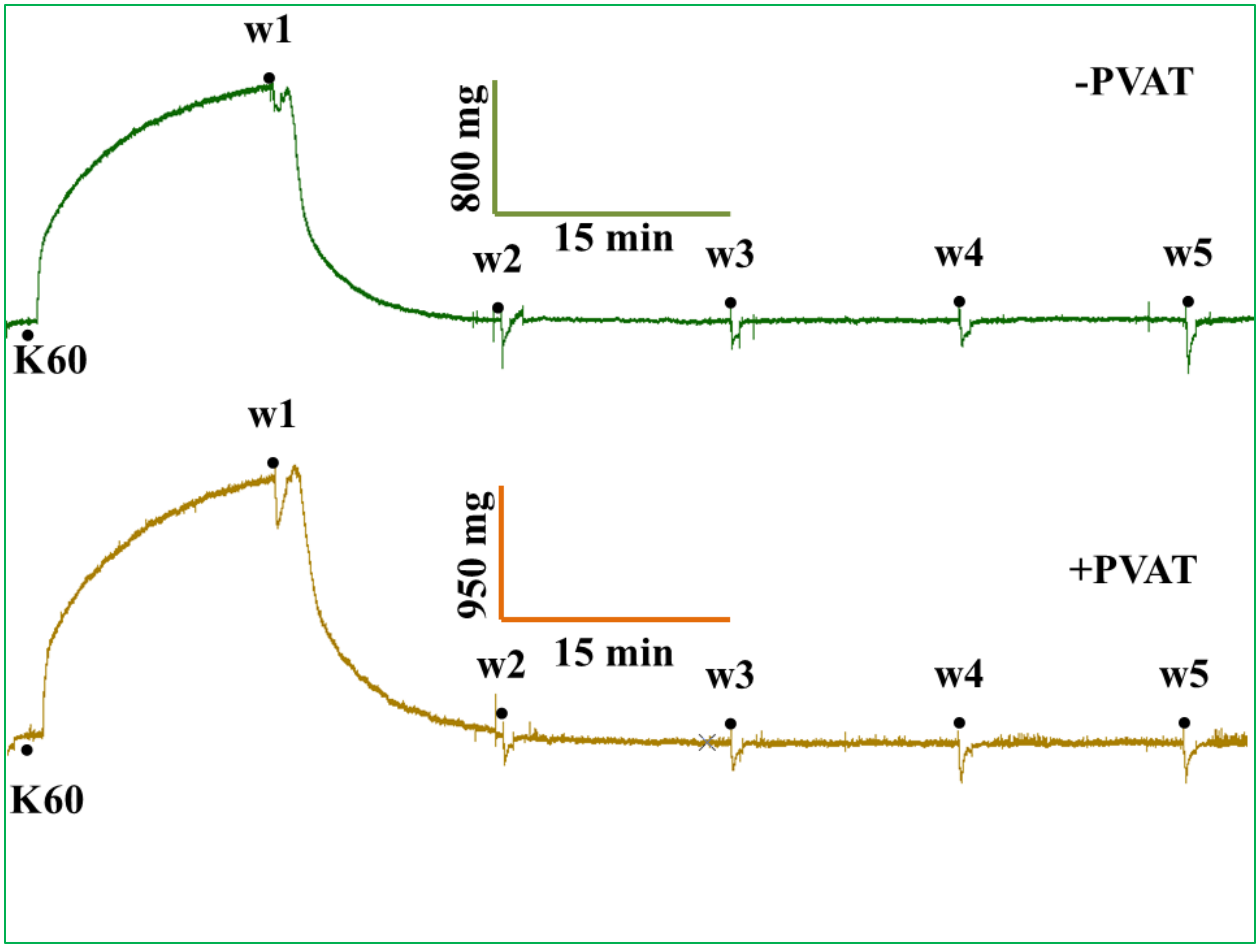



**Figure 2.3.** Electro-mechanical coupling and test of $Ca_V1.2$ channels functionality. K60, 60 mM KCl; w, wash.

## 2.4 Experimental protocols

### 2.4.1 Effect of PVAT on flavonoids antagonistic effect towards noradrenaline- or KCl-induced contraction

Both -PVAT and +PVAT preparations were pre-incubated with 10 µM flavonoid for 30 min; then, either noradrenaline or KCl was added cumulatively to construct a concentration-response curve. In a separate set of experiments, rings were pre-incubated with 10 µM mito-tempol for 30 min before the flavonoid addition. In the end, 1 µM nifedipine followed by 100 µM sodium nitroprusside (KCl-induced contraction) or 100 µM sodium nitroprusside alone (noradrenaline-induced contraction) were added to test the functional integrity of smooth muscle. In all these

experimental settings, the response was calculated as a percentage of the tone induced by 60 mM KCl in the ring functional assay (Table 2.1) (Kassam et al., 2012).

**Table 2.1:** Responses to KCl recorded in the ring functional assay.

| Flavonoids | KCl-induced contraction (mg) | | | |
|---|---|---|---|---|
| | DMSO-PVAT | DMSO+PVAT | Flavonoid-PVAT | Flavonoid+PVAT |
| Apigenin | | | | |
| NA | 1379±114 (9) | 1550±158 (9) | 1590±265 (9) | 1719±180 (9) |
| NA+Mito-tempol | 1352±161 (6) | 1547±150 (6) | 1533±107 (6) | 1490±72 (6) |
| NA+PVAT in bath | 1580±180 (5) | 1336±78 (5) | 1550±267 (5) | 1254±112 (5) |
| KCl | 1417±91 (10) | 1693±197 (10) | 1540±129 (10) | 1829±255 (10) |
| Chrysin | | | | |
| NA | 1279±208 (7) | 1393±164 (7) | 1340±181 (7) | 1941±177 (7) |
| NA+Mito-tempol | 1352±161 (6) | 1547±150 (6) | 1578±186 (6) | 1550±86 (6) |
| NA+PVAT in bath | 1522±158 (6) | 1343±63 (6) | 1393±229 (6) | 1657±112 (6) |
| KCl | 1441±98 (9) | 1817±171 (9) | 1567±203 (9) | 2000±280 (9) |
| 3,4′-Dihydroxyflavone | 1254±320 (5) | 1942±177 (5) | 1180±247 (5) | 1950±116 (5) |
| (-)-Epigallocatechin | 1413±247 (6) | 1972±115 (6) | 1840±189 (6) | 1567±74 (6) |
| (-)-Epigallocatechin gallate | 1138±241 (5) | 1768±46 (5) | 1448±259 (5) | 1772±133 (5) |
| Fisetin | 1097±281 (6) | 1263±159 (6) | 1247±202 (6) | 1478±113 (6) |
| Galangin | 1299±161 (8) | 1541±133 (8) | 1440±126 (8) | 1561±103 (8) |
| Genistein | 1203±144 (10) | 1358±137 (10) | 1382±171 (10) | 1725±178 (10) |
| Hesperetin | 996±162 (7) | 1680±146 (6) | 1061±173 (7) | 1687±133 (7) |
| Hesperidin | 1197±200 (7) | 1550±79 (7) | 1447±146 (7) | 1740±69 (7) |
| 5-Hydroxyflavone | 966±162 (7) | 1619±138 (7) | 966±187 (7) | 1511±145 (7) |
| Isorhamnetin | 1665±87 (4) | 1970±118 (4) | 1693±153 (4) | 2343±87 (4) |
| Kaempferol | 1153±206 (7) | 1503±81 (7) | 1400±146 (7) | 1671±157 (7) |
| Luteolin | 1878±110 (7) | 1730±111 (7) | 1495±197 (7) | 1723±121 (7) |
| Morin | 1379±275 (7) | 1769±203 (7) | 1583±267 (7) | 1951±189 (7) |
| (±)-Naringenin | 1836±104 (8) | 1715±97 (8) | 1584±111 (8) | 1677±88 (8) |
| Quercetin | 1701±151 (7) | 1877±144 (7) | 1420±142 (9) | 1845±155 (9) |
| Resokaempferol | 1254±320 (5) | 1942±177 (5) | 1428±296 (5) | 1826±197 (5) |
| Rutin | 1179±190 (7) | 1487±49 (7) | 1306±110 (8) | 1450±115 (8) |
| Scutellarein | 1390±211 (7) | 1797±200 (7) | 1687±122 (7) | 1723±164 (7) |
| Tamarixetin | 1256±167 (7) | 1884±83 (7) | 1411±241 (7) | 1889±175 (7) |
| (±)-Taxifolin | 1097±129 (6) | 1777±235 (6) | 1487±111 (6) | 1825±232 (6) |

Aorta rings were stimulated by 60 mM KCl and the ensuing contraction was measured in mg. The number of independent replicates, indicated in parenthesis, was obtained from at least three animals. The responses ranged between 1000 and 2000 mg demonstrating the strong viability of the preparations. NA: noradrenaline.

### 2.4.2 Effect of PVAT on flavonoid-induced vasodilation

-PVAT and +PVAT rings were pre-contracted with a submaximal concentration of noradrenaline or phenylephrine to a similar level. Once a plateau was achieved, flavonoids were added cumulatively to construct a concentration-response curve. In some experiments, preparations were pre-incubated with 10 µM mito-tempol for 30 min before stimulation by noradrenaline. In another series of experiments, -PVAT rings were pre-incubated with either 30 µM pyrogallol for 20 min before the addition of 1 µM noradrenaline. In the end, 100 µM sodium nitroprusside was added to test the functional integrity of smooth muscle. Responses were calculated as a percentage of the contraction induced by noradrenaline or phenylephrine (Table 2.2), taken as 100% (Kassam et al., 2011).

### 2.4.3 Effect of PVAT on apigenin- and chrysin-induced relaxation of NaF-evoked contraction

-PVAT and +PVAT rings were pre-contracted with 10 mM NaF. Once the plateau was achieved, apigenin or chrysin was added cumulatively to construct a concentration-response curve. In the end, 100 µM sodium nitroprusside was added to test the functional integrity of smooth muscle. Responses were calculated as a percentage of the contraction induced by NaF (Table 2.2), taken as 100%.

### 2.4.4 Effect of PVAT on fasudil-induced relaxation

-PVAT and +PVAT rings were pre-contracted with a submaximal concentration of noradrenaline or NaF to a similar level. Once a plateau was achieved, fasudil was added cumulatively to construct a concentration-response curve. In the end, 100 µM sodium nitroprusside was added to test the functional integrity of smooth muscle. Responses were calculated as a percentage of the contraction induced by noradrenaline or NaF (Table 2.2), taken as 100%.

### 2.4.5 Effect of SR59230A on the functional interaction between PVAT and flavonoids

-PVAT and +PVAT rings were pre-incubated with 0.3 µM SR59230A for 30 min and then contracted with noradrenaline to a similar level. Once a plateau was achieved, apigenin or chrysin was added cumulatively to construct a concentration-response curve. In the end, 100 µM sodium nitroprusside was added to test the functional integrity of smooth muscle. Responses

were calculated as a percentage of the contraction induced by noradrenaline (Table 2.2), taken as 100%.

**Table 2.2:** Responses to noradrenaline, phenylephrine or NaF recorded under various experimental conditions.

| Vasoconstrictor | Drug | Contraction (mg) | | P value |
|---|---|---|---|---|
| | | -PVAT | +PVAT | |
| NA | DMSO | 1674±120 (10) | 1670±118 (9) | 0.9814 |
| | Apigenin | 1970±145 (8) | 1971±171 (8) | 0.9956 |
| | Chrysin | 1658±156 (10) | 1909±107(9) | 0.1978 |
| | 3,4′-Dihydroxyflavone | 1704±115 (8) | 1832±111 (9) | 0.4355 |
| | Fisetin | 2100±168 (11) | 2009±123 (11) | 0.6664 |
| | Fasudil | 1433±174 (6) | 1284±157 (7) | 0.5365 |
| | Galangin | 2021±124 (7) | 2039±176 (7) | 0.9365 |
| | Genistein | 2194±101(8) | 2017±216 (9) | 0.4865 |
| | Hesperetin | 1919±232 (9) | 1684±142 (9) | 0.4016 |
| | 5-Hydroxyflavone | 1601±153 (11) | 1849±116 (12) | 0.2056 |
| | Kaempferol | 1829±171 (7) | 1883±80 (7) | 0.7785 |
| | Luteolin | 2180±387 (7) | 1583±170 (7) | 0.1831 |
| | Quercetin | 1907±195 (8) | 1542±1542 (9) | 0.1307 |
| | Resokaempferol | 1474±101 (8) | 1711±122 (8) | 0.1555 |
| | Tamarixetin | 1996±293 (4) | 2656±366 (5) | 0.1635 |
| | Apigenin (PVAT in bath) | 2040±165 (7) | 1973±136 (7) | 0.7591 |
| | Chrysin (PVAT in bath) | 2053±173 (8) | 1820±115 (8) | 0.2797 |
| NA+Mito-tempol | Apigenin | 1793±239 (7) | 1683±156 (7) | 0.7063 |
| | Chrysin | 1882±294 (6) | 1655±115 (6) | 0.3425 |
| NA+Pyrogallol | Apigenin NA<br>NA+pryo | 2368±174 (6)<br>2005±151 (6) | | 0.1454 |
| | Chrysin NA<br>NA+pryo | 1818±186 (10)<br>1914±154 (11) | | 0.6945 |
| NA+SR59230A | Apigenin NA<br>NA+SR | 2180±95 (5)<br>1713±143 (7) | 1844±152 (5)<br>1543±145 (7) | 0.0967<br>0.4202 |
| | Chrysin NA<br>NA+SR | 1747±229 (8)<br>1813±288 (7) | 1599±147 (9)<br>1655±153 (8) | 0.5842<br>0.6236 |
| Phenylephrine | DMSO | 1202±158 (6) | 1282±69 (6) | 0.6515 |
| | Apigenin | 2027±203 (6) | 1515±183 (6) | 0.0910 |
| | Chrysin | 1333±207 (7) | 1420±165 (7) | 0.7476 |
| NaF | DMSO | 2028±132 (5) | 1820±204 (5) | 0.4175 |
| | Apigenin | 2233±179 (8) | 2078±96 (9) | 0.4437 |
| | Chrysin | 2311±210 (7) | 2293±190 (8) | 0.9476 |
| | Fasudil | 1779±190 (11) | 1877±169 (11) | 0.6997 |
| NaF+Mito-tempol | Apigenin | 1050±173 (6) | 1503±101* (6) | 0.0471 |

Aorta rings were stimulated by noradrenaline (0.86 µM-0.93 µM), phenylephrine (0.92 µM-1.1 µM), or NaF (10 mM) and the ensuing contraction was measured in mg. The number of independent replicates is indicated in parenthesis. NA: noradrenaline; pyr: pyrogallol; SR: SR59230A.

### 2.5 Drugs and chemicals

The chemicals used were acetylcholine, apigenin, (-)-epigallocatechin, (-)-epigallocatechin gallate, fasudil, genistein, hesperetin, hesperidin, 5-hydroxyflavone, isorhamnetin, morin, (±)-naringenin, nifedipine, noradrenaline, phenylephrine, quercetin, rutin, scutellarein, SR59230A, (±)-taxifolin (Sigma Chimica, Milan, Italy), chrysin, galangin, kaempferol, resokaempferol, tamarixetin, 3,4′-dihydroxyflavone (Indofine, New Jersey, U.S.A), NaF, sodium nitroprusside (Riedel-De Haen AG, SeelzeHannover, Germany), pyrogallol (Merck, Darmstadt, Germany), fisetin, luteolin, and mito-tempol (Abcam, Milan, Italy). All other substances were of analytical grade and used without further purification. Phenylephrine was solubilized in 0.1 M HCl. Nifedipine was dissolved directly in ethanol, flavonoids in DMSO, pyrogallol, NaF, mito-tempol, and noradrenaline in distilled water.

### 2.6 Statistical analysis

Analysis of data was accomplished using LabChart 7.3.7 Pro (PowerLab; ADInstruments) and GraphPad Prism version 5.04 (GraphPad Software Inc.). Data are reported as mean±s.e.m; n is the number of rings analysed (indicated in parentheses), isolated from at least three animals. Statistical analyses and significance as measured by Student’s t test for unpaired samples (two-tailed) were obtained using GraphPad Prism version 5.04 (GraphPad Software Inc.). In all comparisons, $P<0.05$ was considered significant. The pharmacological response to drugs, described in terms of potency ($IC_{50}$ or $EC_{50}$ value, i.e. the drug concentration that caused a decrease or increase of muscle tone equal to 50% of the maximum response, respectively) and efficacy ($E_{max}$, i.e. the maximal response achieved with the highest concentration tested), was obtained by nonlinear regression analysis.

# CHAPTER 3

# RESULTS

## 3. Results

### 3.1 PVAT antagonism towards noradrenaline

As shown in Fig. 3.1, noradrenaline contracted rings -PVAT in a concentration-dependent manner ($EC_{50}$ 30.9±10.0 nM; $E_{max}$ 114.8±5.8%, n=8). The presence of PVAT, however, significantly reduced noradrenaline efficacy ($E_{max}$ 94.2±5.1%, n=7; P=0.021 vs -PVAT). Also, potency was reduced, though statistical significance was not reached ($EC_{50}$ 149.3±79.9 nM, n=7; P=0.14 vs -PVAT).

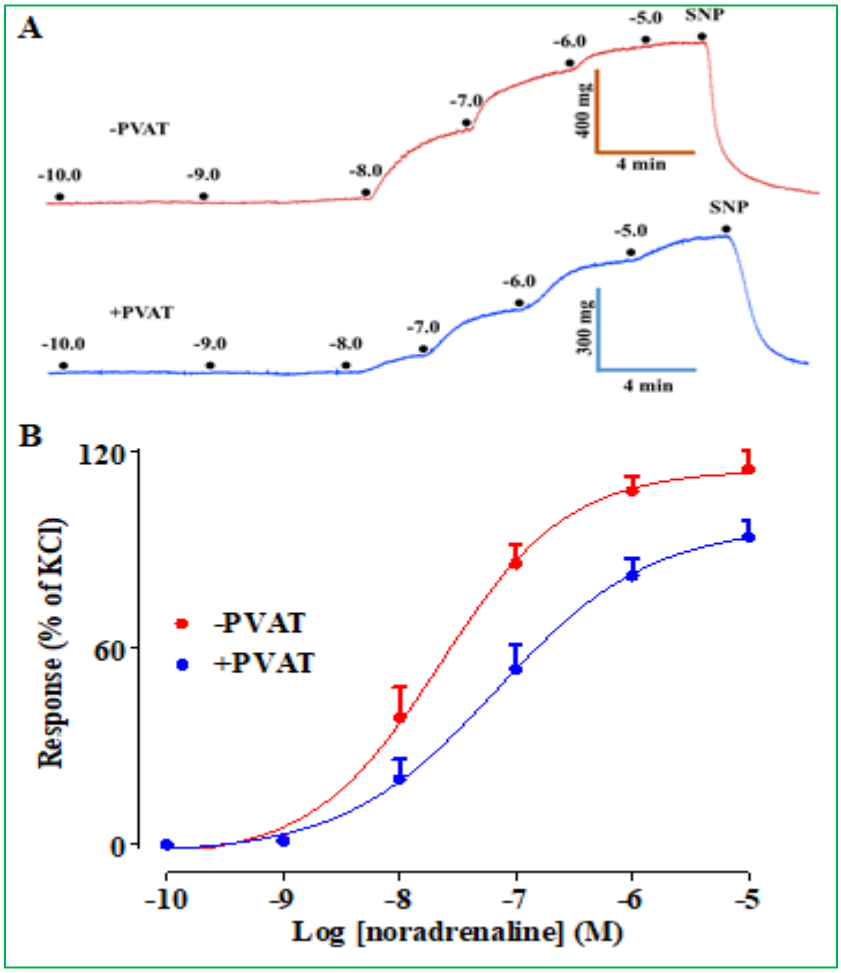



**Figure 3.1.** Effect of PVAT on noradrenaline response in endothelium-denuded rat aorta rings. (A) Traces (representative of 7-8 similar experiments) of the contraction induced by cumulative concentrations (in log units M) of noradrenaline in ring PVAT-deprived (-PVAT, upper panel), or PVAT-intact (+PVAT, lower panel). The effect of 100 μM sodium nitroprusside (SNP) is also shown. (B) Noradrenaline concentration-response curves constructed in endothelium-denuded rings -PVAT (red) or +PVAT (blue). In the ordinate scale, the response is reported as a

percentage of the contraction induced by 60 mM KCl in ring functional assay (-PVAT 1491±129 mg, n=8; +PVAT 1606±136 mg, n=7; P=0.553). Data points represent the mean±s.e.m.

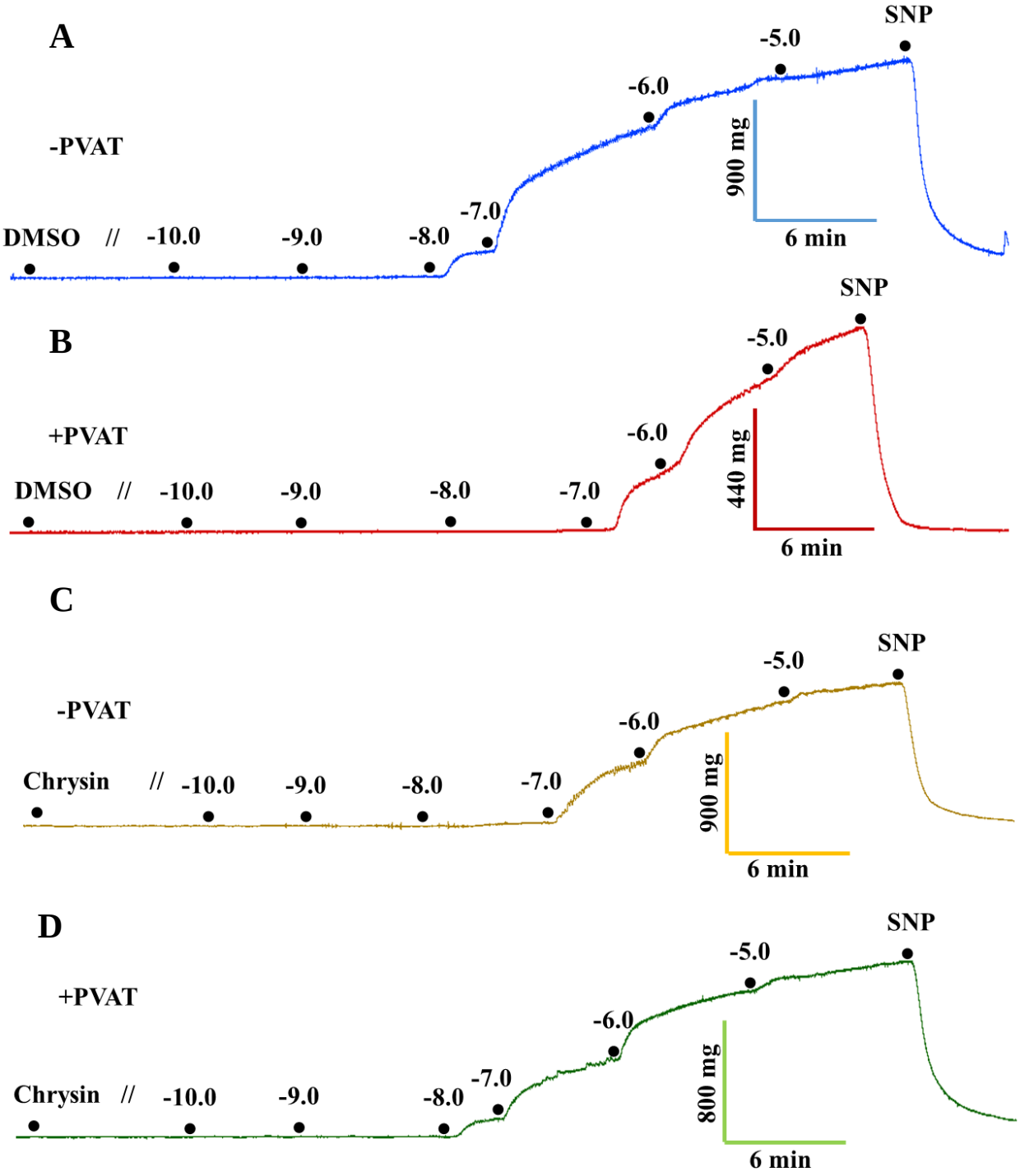
### 3.2 Effect of flavonoids on PVAT anti-contractile activity

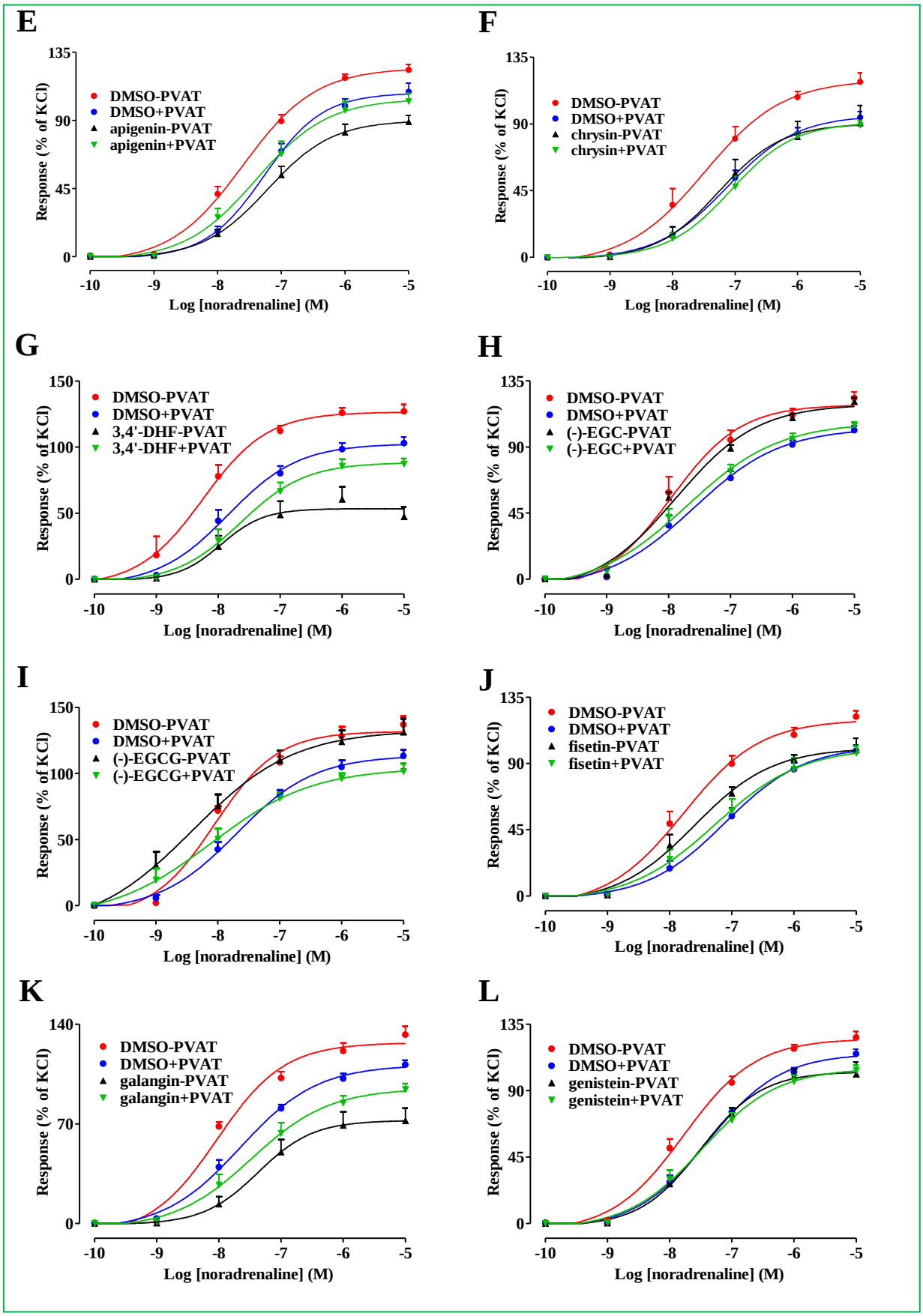
To assess the functional interaction between flavonoids and PVAT, a concentration-response curve to noradrenaline was constructed in both -PVAT and +PVAT rings in the presence of either various flavonoids (10 µM) or vehicle. The choice of this concentration was based on preliminary experiments performed with quercetin, chrysin, and genistein showing that 10 µM is the lowest concentration at which flavonoids significantly antagonized noradrenaline-induced response and relaxed aortic preparations pre-contracted by noradrenaline. Pre-incubation with flavonoids or vehicle, at the concentrations used in this study, did not affect the baseline tone (Fig. 3.2A-D). Noradrenaline was used to assess PVAT and flavonoid interplay under experimental conditions as physiological as possible. As shown in Table 3.1 and Fig. 3.2, in rings pre-incubated with vehicle PVAT reduced the efficacy of noradrenaline. Also, potency was decreased, though statistical significance was reached only in approximately half of the experiments. Among the flavonoids assessed, (-)-epigallocatechin (Fig. 3.2H), (-)-epigallocatechin gallate (Fig. 3.2I), hesperidin (Fig. 3.2N), isorhamnetin (Fig. 3.2P), morin (Fig. 3.2S), (±)-naringenin (Fig. 3.2T), rutin (Fig. 3.2W), scutellarein (Fig. 3.2X), and (±)-taxifolin (Fig. 3.2Z) neither modified the activity of noradrenaline in the absence of PVAT nor affected the anti-contractile effect of PVAT itself. All the remaining flavonoids significantly reduced noradrenaline efficacy in the order 3,4′-dihydroxyflavone (Fig. 3.2G) > galangin (Fig. 3.2K) > luteolin (Fig. 3.2R) > resokaempferol (Fig. 3.2V) > apigenin (Fig. 3.2E) ≅ tamarixetin (Fig. 3.2Y) ≅ chrysin (Fig. 3.2F) > genistein (Fig. 3.2L) > 5-hydroxyflavone (Fig. 3.2O) ≅ fisetin (Fig. 3.2J) ≅ kaempferol (Fig. 3.2Q) ≅ hesperetin (Fig. 3.2M) > quercetin (Fig. 3.2U). Surprisingly, in rings pre-treated with chrysin, fisetin, genistein, hesperetin, 5-hydroxyflavone, kaempferol, luteolin, quercetin, resokaempferol, and tamarixetin the efficacy of noradrenaline was independent of the presence of PVAT. Finally, PVAT counteracted the marked antagonism exerted by apigenin, galangin, and 3,4′-dihydroxyflavone towards noradrenaline-induced contraction.

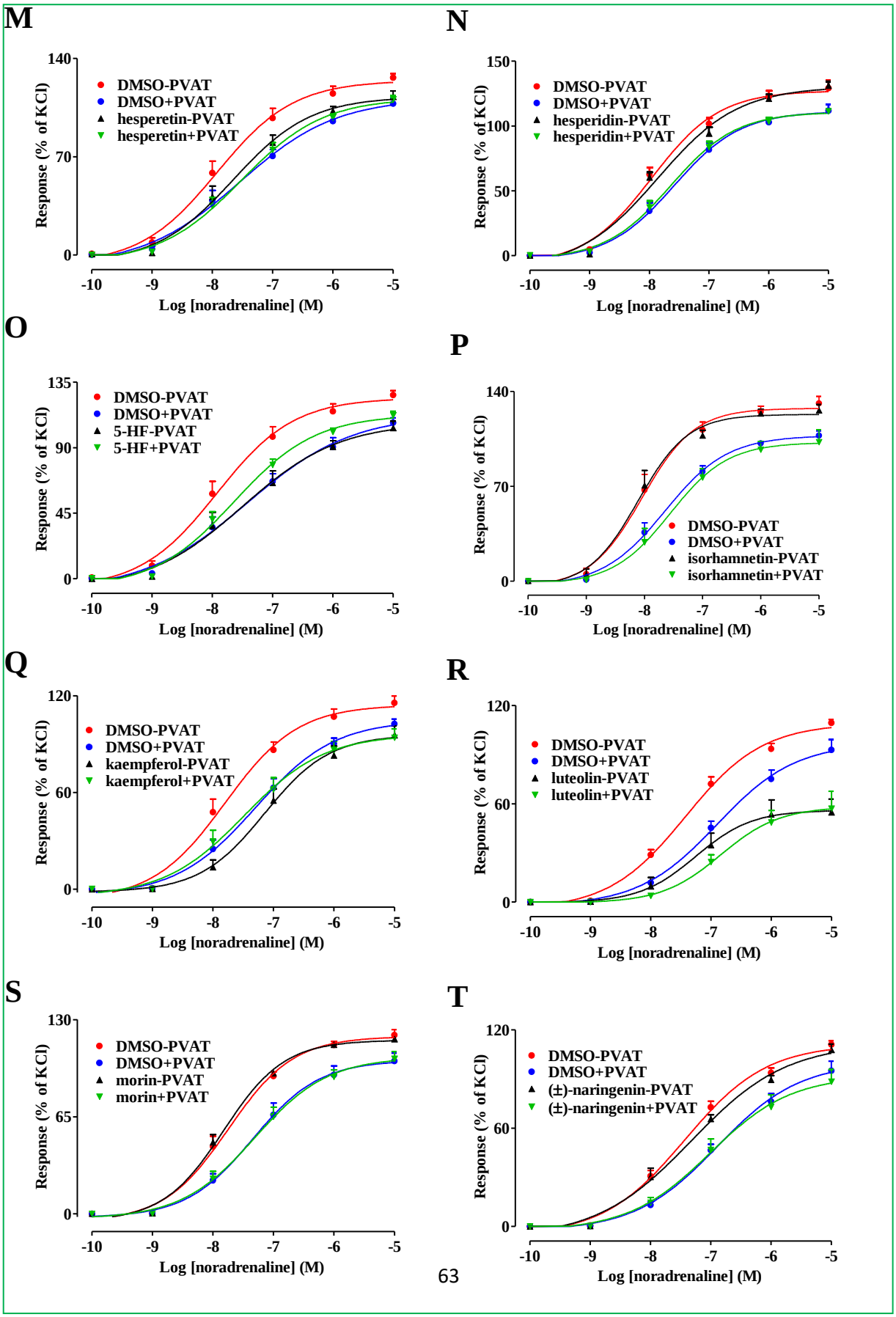
**Table 3.1. Effect of flavonoids on noradrenaline response and the anti-contractile activity of PVAT**

| Flavonoid | $EC_{50}$ (nM) | | | | $E_{max}$ (%) | | | | n |
|---|---|---|---|---|---|---|---|---|---|
| | DMSO-PVAT | DMSO+PVAT | Flavonoid-PVAT | Flavonoid+PVAT | DMSO-PVAT | DMSO+PVAT | Flavonoid-PVAT | Flavonoid+PVAT | |
| Apigenin | 29.9±6.3 | 60.3±8.0* | 81.0±18.8* | 56.6±16.4 | 123.3±3.6 | 108.9±5.7* | 89.1±4.1* | 102.5±5.4$^{\&}$ | 9 |
| Chrysin | 45.0±19.1 | 114.1±48.7 | 65.3±14.5 | 109.5±36.6 | 118.4±5.9 | 94.4±3.9* | 90.5±11.9* | 89.0±3.3 | 6-7 |
| 3,4′-Dihydroxyflavone | 6.6±1.6 | 17.7±4.4* | 42.1±30.5 | 30.4±11.4 | 127.0±5.4 | 102.9±4.6* | 47.2±7.5* | 87.3±4.1$^{\#,\&}$ | 5 |
| (-)-Epigallocatechin | 17.9±7.4 | 34.2±12.4 | 13.7±1.9 | 28.1±9.3 | 123.4±4.1 | 101.5±2.8* | 120.9±2.8 | 105.1±2.1$^{\&}$ | 6 |
| (-)-Epigallocatechin gallate | 9.0±0.5 | 22.1±6.4 | 7.5±4.9 | 13.5±5.3 | 137.0±6.2 | 113.2±4.6* | 131.2±10.2 | 101.5±5.9$^{\&}$ | 5 |
| Fisetin | 21.6±7.1 | 113.3±49.8* | 52.1±28.9 | 103.7±43.8 | 121.9±4.1 | 98.6±3.9* | 99.4±7.8* | 97.2±4.7 | 6 |
| Galangin | 9.6±0.6 | 24.3±4.9* | 120.3±70.4* | 89.8±58.8 | 132.8±5.9 | 111.6±3.1* | 72.3±8.8* | 94.3±4.1$^{\#,\&}$ | 8 |
| Genistein | 23.3±6.6 | 48.1±9.0* | 38.3±8.3 | 41.5±9.5 | 126.1±4.1 | 115.0±3.1* | 101.3±8.0* | 103.7±3.6$^{\#}$ | 9-10 |
| Hesperetin | 21.6±11.3 | 38.9±15.7 | 38.2±15.2 | 41.2±14.4 | 126.3±3.0 | 107.9±3.9* | 112.8±4.0* | 109.7±2.9 | 6-7 |
| Hesperidin | 12.5±1.9 | 33.6±10.2* | 15.9±3.3 | 27.0±6.9 | 129.1±5.9 | 111.8±4.9* | 131.1±3.7 | 111.4±2.1$^{\&}$ | 7 |
| 5-Hydroxyflavone | 21.6±11.3 | 74.2±37.7 | 102.3±70.9 | 28.1±5.9 | 126.3±2.9 | 107.2±3.4* | 103.6±4.6* | 112.4±2.7 | 7 |
| Isorhamnetin | 11.0±3.0 | 27.2±8.5 | 10.6±4.3 | 32.6±9.7 | 131.3±5.2 | 107.6±3.4* | 126.2±4.4 | 102.8±9.1$^{\&}$ | 4 |
| Kaempferol | 21.1±6.5 | 65.6±22.8* | 113.7±42.6* | 53.0±20.9 | 115.6±4.1 | 102.5±2.9* | 95.6±5.7* | 94.0±5.6 | 7 |
| Luteolin | 49.6±11.4 | 172.8±41.0* | 59.1±13.1 | 173.0±70.3 | 109.4±2.1 | 92.9±6.3* | 54.9±8.0* | 57.1±10.5$^{\#}$ | 7 |
| Morin | 20.8±4.8 | 73.3±31.1* | 16.3±2.8 | 63.5±20.3$^{\&}$ | 119.7±3.5 | 102.2±5.3* | 117.0±1.9 | 103.8±4.9$^{\&}$ | 7 |
| (±)-Naringenin | 47.5±10.1 | 170.0±35.6* | 62.1±14.0 | 122.2±28.0$^{\&}$ | 111.0±2.4 | 95.1±5.8* | 107.8±3.5 | 88.5±7.3$^{\&}$ | 8 |
| Quercetin | 23.0±4.5 | 123.6±29.7* | 9.5±2.4* | 80.7±21.8$^{\&}$ | 120.2±2.5 | 100.4±5.4* | 108.4±2.9* | 98.4±4.4 | 7-9 |
| Resokaempferol | 6.6±1.6 | 17.7±4.4* | 13.9±4.5 | 36.1±12.3 | 127.0±5.4 | 102.9±4.5* | 78.2±9.6* | 85.6±8.0 | 5 |
| Rutin | 14.7±2.0 | 43.5±19.3 | 16.4±2.0 | 30.0±8.9 | 129.9±5.9 | 108.7±4.3* | 125.9±4.1 | 108.3±4.7$^{\&}$ | 7-8 |
| Scutellarein | 16.1±6.6 | 35.2±10.6 | 13.4±4.9 | 51.4±23.6 | 116.8±4.4 | 103.5±3.2* | 113.6±3.2 | 104.5±2.0$^{\&}$ | 7 |
| Tamarixetin | 9.8±2.7 | 17.1±4.8 | 11.3±3.2 | 34.1±15.6 | 144.1±6.4 | 118.0±4.8* | 113.8±7.9* | 110.5±5.2 | 7 |
| (±)-Taxifolin | 13.3±2.7 | 21.4±5.9 | 10.8±2.6 | 15.8±6.5 | 138.4±6.3 | 119.0±5.7* | 137.1±5.0 | 115.8±3.2$^{\&}$ | 6 |

Endothelium-denuded thoracic rat aorta rings either -PVAT or +PVAT were pre-incubated with 10 µM flavonoids or vehicle for 30 min and then a concentration-response curve to noradrenaline was constructed. Potency ($EC_{50}$) and efficacy values ($E_{max}$, expressed as a percentage of 60 mM KCl-induced contraction) for noradrenaline are mean±s.e.m of n experiments performed. $^{*, \#, \&}$ $P<0.05$ vs DMSO-PVAT, DMSO+PVAT, and flavonoid-PVAT, respectively, Student's t test for unpaired samples.


A
-PVAT
DMSO
//
-10.0
-9.0
-8.0
-7.0
-6.0
-5.0
SNP
900 mg
6 min
B
+PVAT
DMSO
//
-10.0
-9.0
-8.0
-7.0
-6.0
-5.0
SNP
440 mg
6 min
C
-PVAT
Chrysin
//
-10.0
-9.0
-8.0
-7.0
-6.0
-5.0
SNP
900 mg
6 min
D
+PVAT
Chrysin
//
-10.0
-9.0
-8.0
-7.0
-6.0
-5.0
SNP
800 mg
6 min


E
DMSO-PVAT
DMSO+PVAT
apigenin-PVAT
apigenin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
F
DMSO-PVAT
DMSO+PVAT
chrysin-PVAT
chrysin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
G
DMSO-PVAT
DMSO+PVAT
3,4'-DHF-PVAT
3,4'-DHF+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
H
DMSO-PVAT
DMSO+PVAT
(-)-EGC-PVAT
(-)-EGC+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
I
DMSO-PVAT
DMSO+PVAT
(-)-EGCG-PVAT
(-)-EGCG+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
J
DMSO-PVAT
DMSO+PVAT
fisetin-PVAT
fisetin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
K
DMSO-PVAT
DMSO+PVAT
galangin-PVAT
galangin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
L
DMSO-PVAT
DMSO+PVAT
genistein-PVAT
genistein+PVAT
Response (% of KCl)
Log [noradrenaline] (M)


M
Response (% of KCl)
140
70
0
DMSO-PVAT
DMSO+PVAT
hesperetin-PVAT
hesperetin+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
N
Response (% of KCl)
150
100
50
0
DMSO-PVAT
DMSO+PVAT
hesperidin-PVAT
hesperidin+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
O
Response (% of KCl)
135
90
45
0
DMSO-PVAT
DMSO+PVAT
5-HF-PVAT
5-HF+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
P
Response (% of KCl)
140
70
0
DMSO-PVAT
DMSO+PVAT
isorhamnetin-PVAT
isorhamnetin+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
Q
Response (% of KCl)
120
60
0
DMSO-PVAT
DMSO+PVAT
kaempferol-PVAT
kaempferol+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
R
Response (% of KCl)
120
60
0
DMSO-PVAT
DMSO+PVAT
luteolin-PVAT
luteolin+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
S
Response (% of KCl)
130
65
0
DMSO-PVAT
DMSO+PVAT
morin-PVAT
morin+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)
T
Response (% of KCl)
120
60
0
DMSO-PVAT
DMSO+PVAT
(±)-naringenin-PVAT
(±)-naringenin+PVAT
-10
-9
-8
-7
-6
-5
Log [noradrenaline] (M)

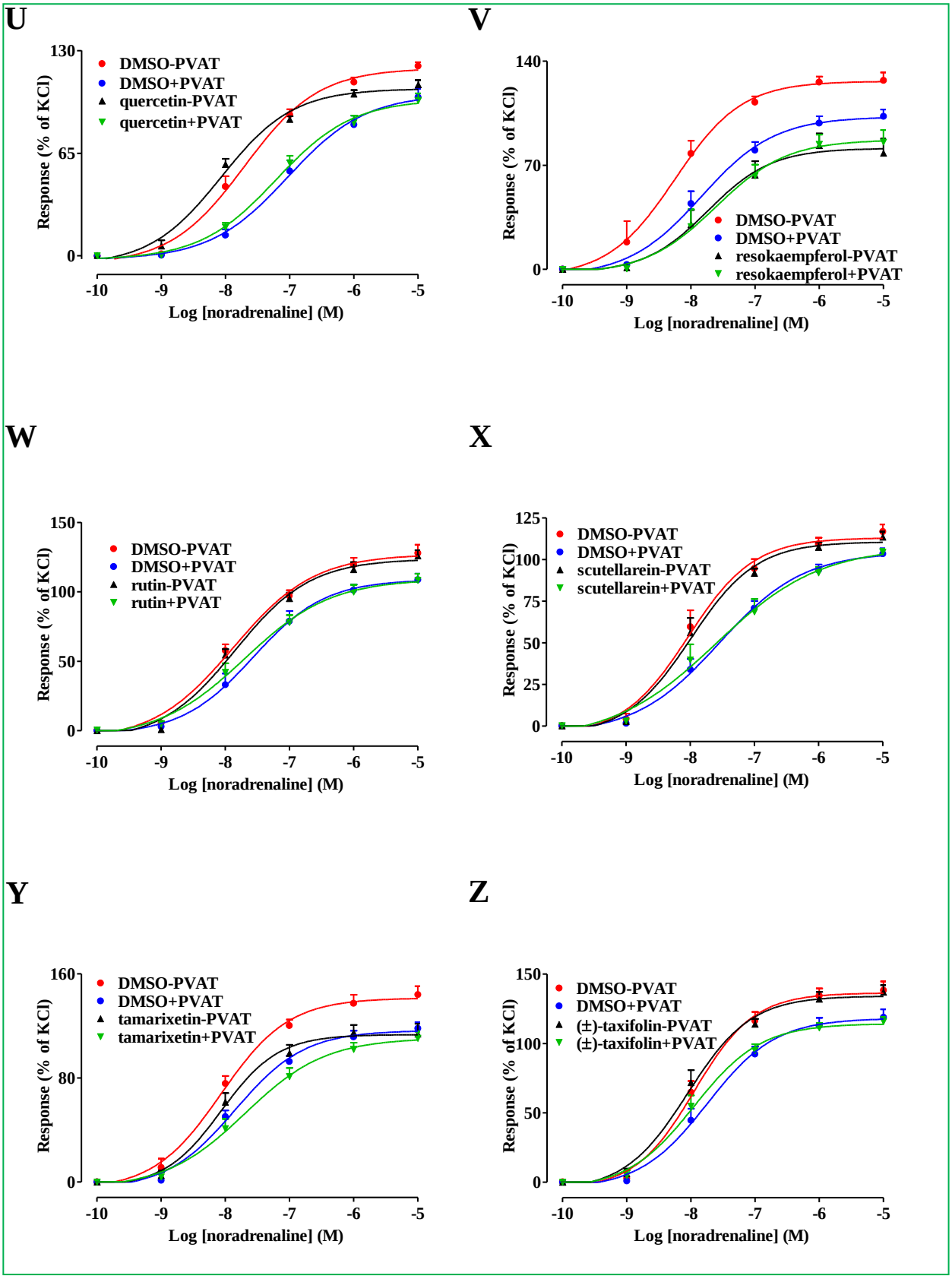

U
DMSO-PVAT
DMSO+PVAT
quercetin-PVAT
quercetin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
V
DMSO-PVAT
DMSO+PVAT
resokaempferol-PVAT
resokaempferol+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
W
DMSO-PVAT
DMSO+PVAT
rutin-PVAT
rutin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
X
DMSO-PVAT
DMSO+PVAT
scutellarein-PVAT
scutellarein+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
Y
DMSO-PVAT
DMSO+PVAT
tamarixetin-PVAT
tamarixetin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)
Z
DMSO-PVAT
DMSO+PVAT
(±)-taxifolin-PVAT
(±)-taxifolin+PVAT
Response (% of KCl)
Log [noradrenaline] (M)

**Figure 3.2.** Effect of flavonoids on noradrenaline-induced response in endothelium-denuded thoracic aorta rings either in the absence (-PVAT) or in presence of PVAT (+PVAT). (A-D) Aortic rings -PVAT (A,C) or +PVAT (B,D) were pre-incubated with (A,B) vehicle (DMSO) or (C,D) 10 µM chrysin and then cumulative concentrations of noradrenaline were added (in log units M); traces are representative of 6-7 similar experiments. (E-Z) Noradrenaline concentration-response curves constructed in preparation pre-incubated with vehicle (DMSO) or 10 µM of different flavonoids for 30 min. In the ordinate scale, the response is reported as a percentage of the tension induced by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m (n=4-10). (-)-EGC, (-)-epigallocatechin; (-)-EGCG, (-)-epigallocatechin gallate; 3,4′-DHF, 3,4′-dihydroxyflavone; 5-HF, 5-hydroxyflavone.

### 3.3 Effect of PVAT on the vasorelaxant activity of flavonoids

In the first series of experiments, the stability of vessel active tone induced by noradrenaline was assessed. As shown in Figure 3.3, in time control experiments, matching those performed with each flavonoid, noradrenaline-induced contraction in rings-PVAT showed a partial, significant, fading, though only after 60 min, corresponding to the highest concentration of flavonoids used. On the contrary, the residual tone measured in rings+PVAT was reduced by a mere 14%. Overlapping results were observed when assessing the effect of vehicle (i.e., DMSO).

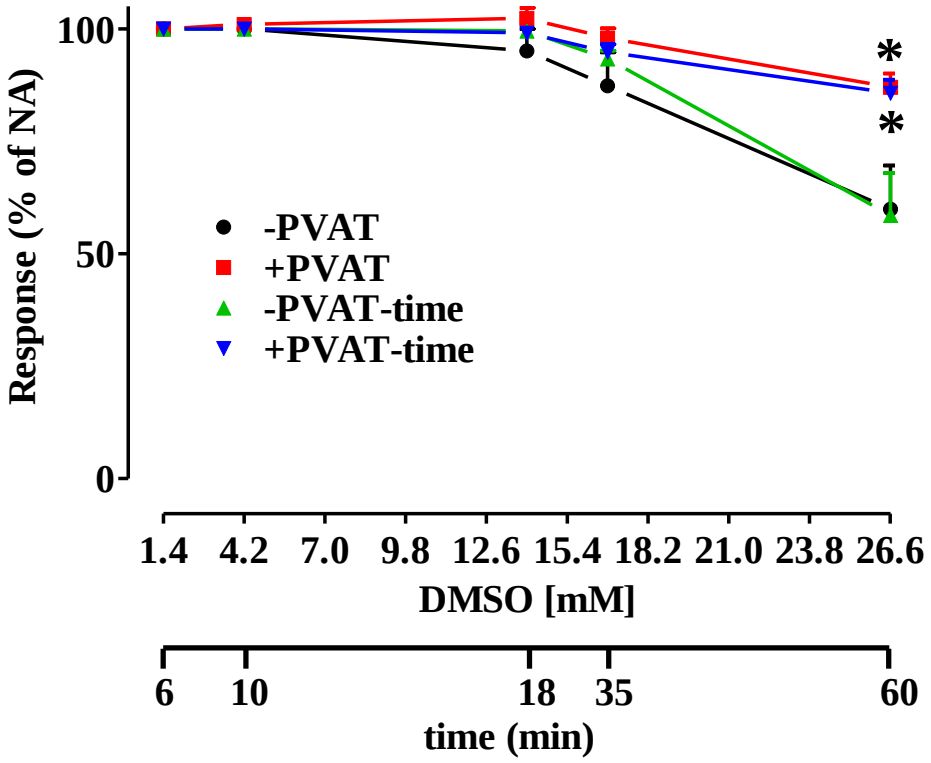


**Figure 3.3.** Effect of DMSO on and stability of the noradrenaline-induced tension in endothelium-denuded thoracic aorta rings. The steady-state contraction evoked by a submaximal concentration of noradrenaline in preparations without (-PVAT) or with PVAT (+PVAT) was monitored for 60 min as well as after the addition of cumulative concentrations of DMSO, matching those necessary to construct a concentration-response curve to each flavonoid (see below). In the ordinate scale, response is reported as a percentage of the initial tension induced by noradrenaline (NA). Data points represent the mean±s.e.m. (n=9-10). * P<0.05 vs. -PVAT, Student's t test for unpaired samples.

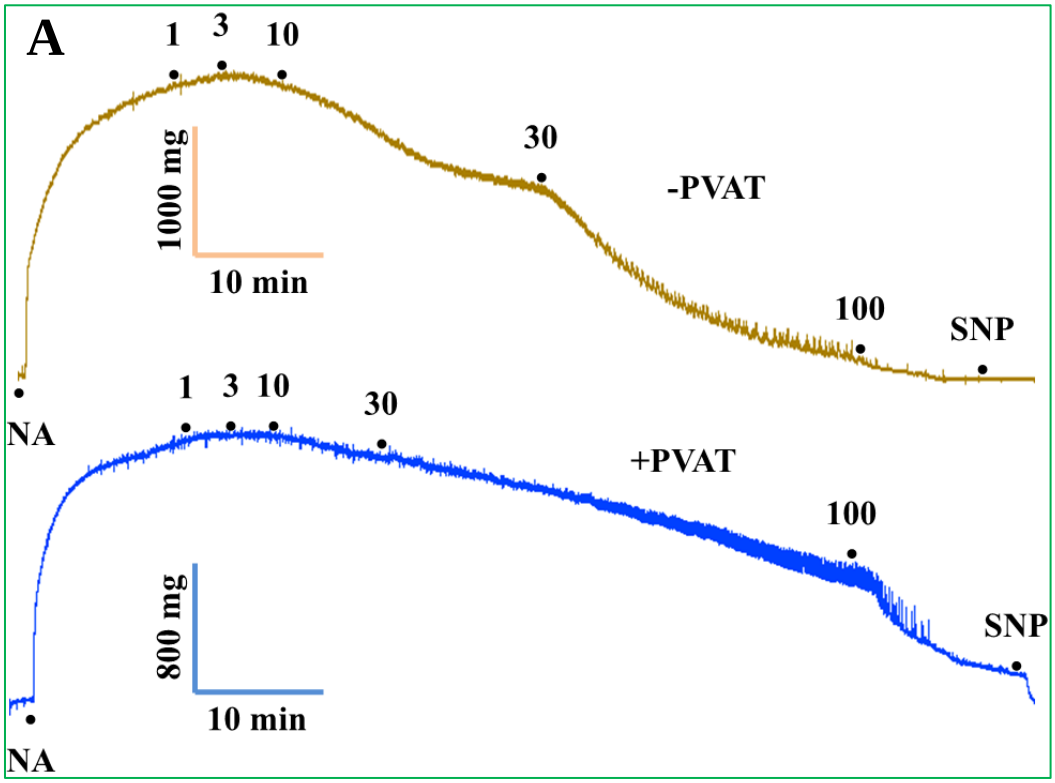
Another series of experiments were performed to assess PVAT modulation of flavonoid vasorelaxant activity towards noradrenaline-induced contraction (compounds that resulted ineffective in the previous experiments were not assessed further). As shown in Table 3.2 and Fig. 3.4, flavonoids caused ~~a~~ concentration-dependent relaxation of noradrenaline-induced contraction with $IC_{50}$ values in the range of 10-70 µM. The presence of PVAT significantly decreased the potency of all the compounds (except fisetin, kaempferol, luteolin, and quercetin) and the efficacy of chrysin, fisetin, genistein, hesperetin, 5-hydroxyflavone, and tamarixetin. The vasorelaxant activity of kaempferol, luteolin and quercetin was similar in both -PVAT and +PVAT preparations.

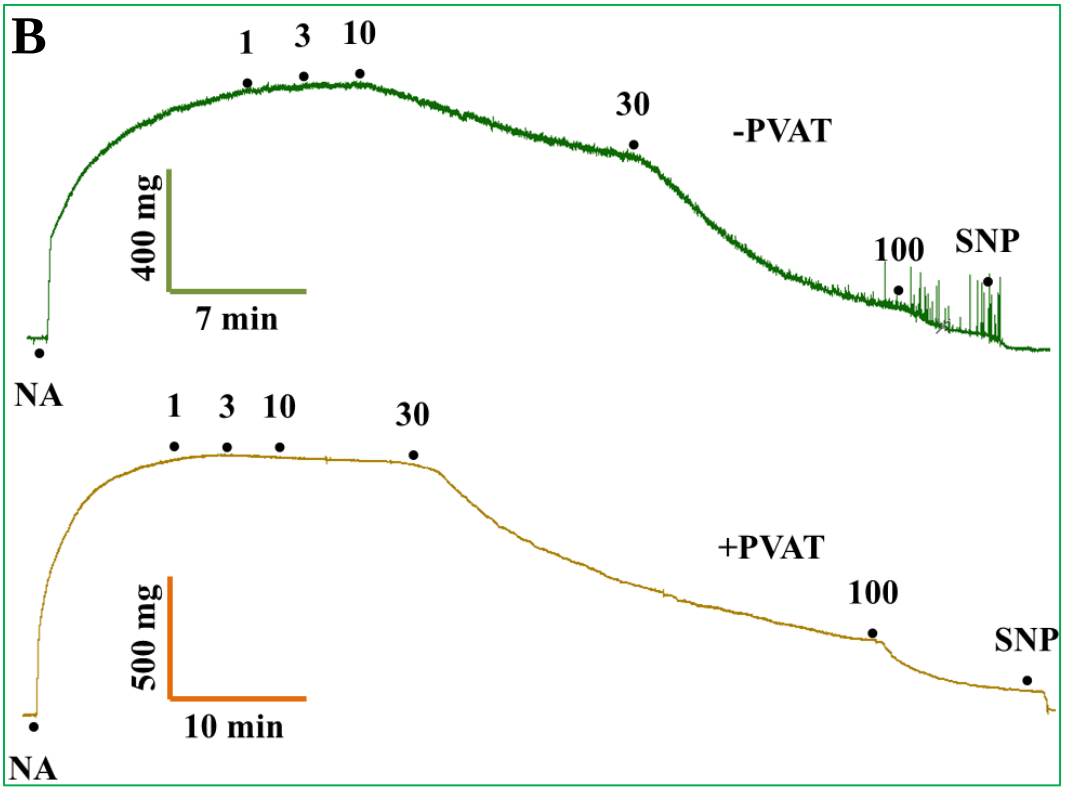
**Table 3.2. Effect of PVAT on flavonoid vasorelaxant activity**

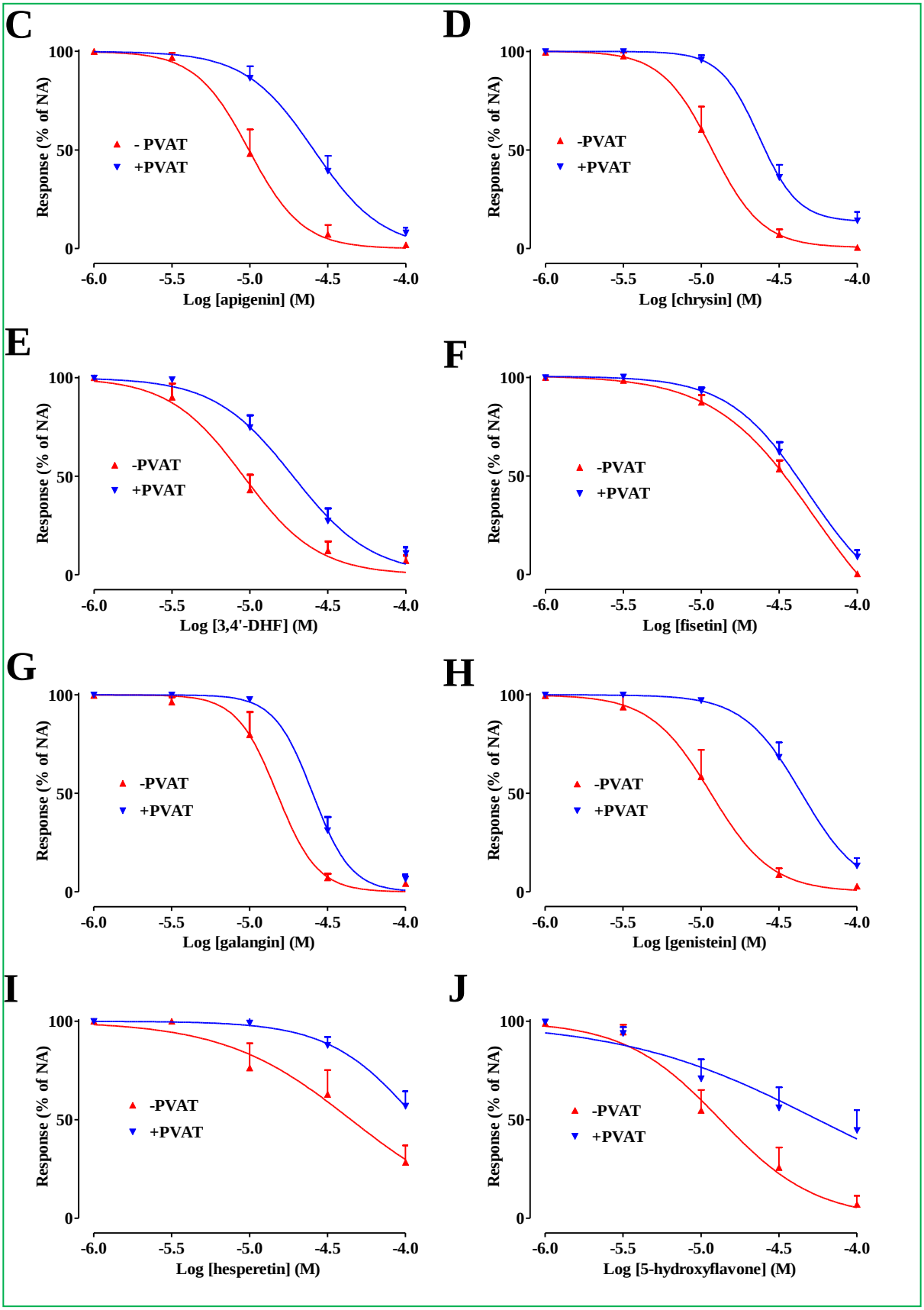
| Flavonoid | $IC_{50}$ (µM) | | $E_{max}$ (%) | |
|---|---|---|---|---|
| | -PVAT | +PVAT | -PVAT | +PVAT |
| DMSO | | | | |
| Apigenin | 11.4±2.2 (8) | 26.1±4.2* (8) | 98.0±1.3 (5) | 92.0±2.6 (7) |
| Chrysin | 13.4±1.9 (10) | 31.5±4.4* (9) | 99.4±0.4 (6) | 85.8±4.4* (9) |
| 3,4′-Dihydroxyflavone | 10.8±1.9 (8) | 21.8±3.9* (9) | 92.7±2.5 (7) | 89.1±3.1 (9) |
| Fisetin | 31.4±2.7 (11) | 40.5±4.9 (11) | 99.6±0.3 (11) | 91.0±3.4* (11) |
| Galangin | 16.4±2.3 (7) | 26.8±2.5* (7) | 94.9±1.5 (5) | 93.4±2.3 (7) |
| Genistein | 13.2±2.6 (8) | 46.7±6.9* (9) | 97.1±0.8 (5) | 87.8±3.9* (9) |
| Hesperetin | 74.6±24.2 (9) | >100 (9) | 71.6±8.5 (9) | 43.1±7.5* (9) |
| 5-Hydroxyflavone | 20.2±4.9 (11) | ~ 100 (12) | 93.0±4.3 (9) | 55.4±10.3* (12) |
| Kaempferol | 29.5±1.9 (7) | 27.1±1.8 (7) | 95.2±1.7 (7) | 96.3±1.2 (7) |
| Luteolin | 12.3±1.6 (7) | 13.4±1.3 (7) | 99.2±0.8 (7) | 98.1±0.8 (7) |
| Quercetin | 44.2±4.8 (8) | 33.1±5.3 (9) | 80.2±1.9 (8) | 85.9±2.7 (9) |
| Resokaempferol | 12.6±1.7 (8) | 24.3±3.1* (8) | 99.0±0.7 (6) | 96.2±0.6 (8) |
| Tamarixetin | 42.6±10.7 (4) | ~ 100 (5) | 78.7±6.6 (4) | 53.0±6.6* (5) |

Endothelium-denuded thoracic aorta rings were pre-contracted with noradrenaline to similar a level. When muscle tone reached a plateau, each flavonoid was added at cumulative concentrations. Potency ($IC_{50}$) and efficacy ($E_{max}$) were calculated by non-linear regression analysis. -PVAT $IC_{50}$ values for hesperetin and tamarixetin are estimated. Data represent the

mean±s.e.m; numbers in parenthesis indicate the experiments performed. *P<0.05 vs -PVAT, Student's t test for unpaired samples.




C
Response (% of NA)
100
50
0
- PVAT
+PVAT
-6.0
-5.5
-5.0
-4.5
-4.0
Log [apigenin] (M)
D
Response (% of NA)
-PVAT
+PVAT
Log [chrysin] (M)
E
Response (% of NA)
-PVAT
+PVAT
Log [3,4'-DHF] (M)
F
Response (% of NA)
-PVAT
+PVAT
Log [fisetin] (M)
G
Response (% of NA)
-PVAT
+PVAT
Log [galangin] (M)
H
Response (% of NA)
-PVAT
+PVAT
Log [genistein] (M)
I
Response (% of NA)
-PVAT
+PVAT
Log [hesperetin] (M)
J
Response (% of NA)
-PVAT
+PVAT
Log [5-hydroxyflavone] (M)

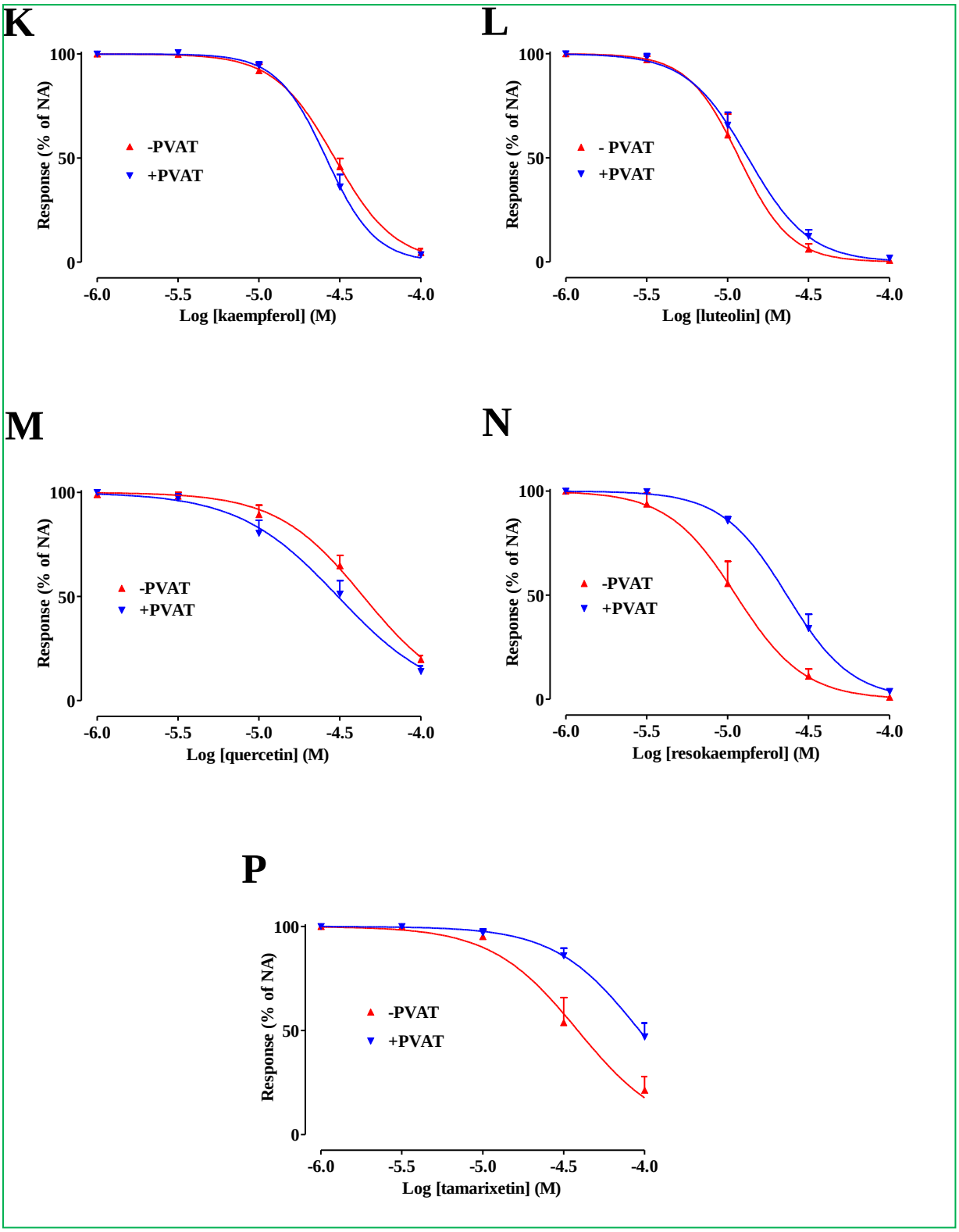

K
Response (% of NA)
100
50
0
-PVAT
+PVAT
-6.0
-5.5
-5.0
-4.5
-4.0
Log [kaempferol] (M)
L
Response (% of NA)
100
50
0
- PVAT
+PVAT
-6.0
-5.5
-5.0
-4.5
-4.0
Log [luteolin] (M)
M
Response (% of NA)
100
50
0
-PVAT
+PVAT
-6.0
-5.5
-5.0
-4.5
-4.0
Log [quercetin] (M)
N
Response (% of NA)
100
50
0
-PVAT
+PVAT
-6.0
-5.5
-5.0
-4.5
-4.0
Log [resokaempferol] (M)
P
Response (% of NA)
100
50
0
-PVAT
+PVAT
-6.0
-5.5
-5.0
-4.5
-4.0
Log [tamarixetin] (M)

**Figure 3.4.** Effect of PVAT on the vasorelaxant activity of different flavonoids in endothelium-denuded thoracic aorta rings. (A,B) The steady-state contraction was evoked by a submaximal concentration of noradrenaline (NA) to similar levels in preparations without (-PVAT) or with PVAT (+PVAT) and then (A) apigenin or (B) chrysin was added cumulatively. Traces are representative of 8-10 similar experiments. (C-P) Flavonoid concentration-response curves constructed in rings -PVAT and +PVAT. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by noradrenaline (NA). Data points represent the mean±s.e.m.

### 3.4 Effect of PVAT "in bath" on the vascular activity of apigenin and chrysin

Two reasons prompted us to select apigenin and chrysin to investigate the mechanism responsible for the functional interaction between flavonoids and PVAT. First, they relaxed aortic preparations with $IC_{50}$ values that markedly inhibited noradrenaline-induced response. Second, both their antispasmodic and spasmolytic activities were counteracted by the presence of PVAT.

As PVAT might function as a physical barrier limiting the diffusion of flavonoids towards vascular smooth muscle, in this series of experiments PVAT was detached from rings and left in the organ bath throughout the experiment. Then, the effect of the two flavonoids on noradrenaline response was re-assessed. As shown in Fig. 3.5A,B, under these experimental conditions, the anti-contractile effect of PVAT was lost; furthermore, the antagonistic effect of both chrysin and apigenin towards noradrenaline was not affected. In fact, the vasorelaxant activity of both apigenin and chrysin was not also modified by the presence of PVAT in the organ bath (Fig. 3.5C,D).

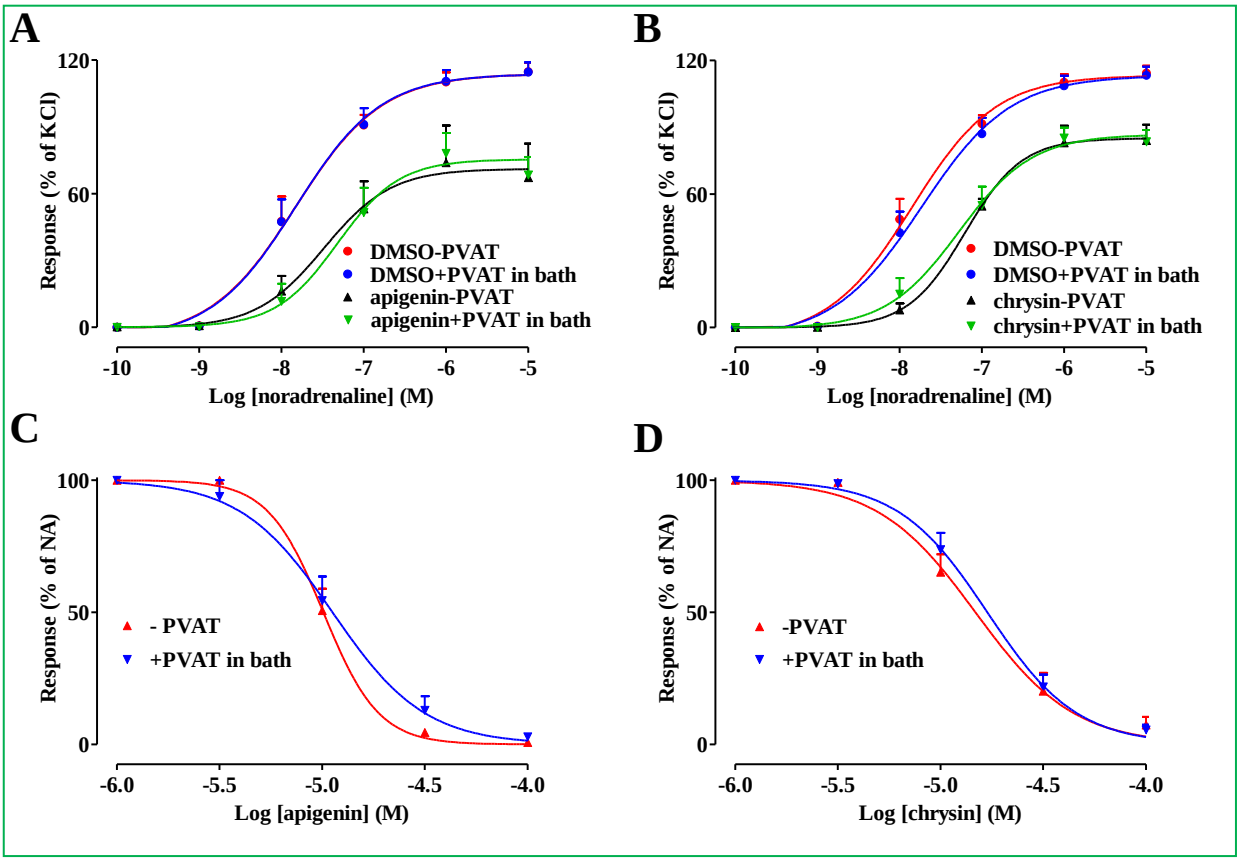



**Figure 3.5.** Effect of PVAT "in bath" either on noradrenaline-induced contraction or on flavonoid-induced relaxation. (A,B) Endothelium-denuded thoracic aorta rings pre-incubated with vehicle (DMSO), (A) 10 µM apigenin or (B) 10 µM chrysin for 30 min were challenged with cumulative concentrations of noradrenaline. PVAT was removed from the preparations (-PVAT) and, in some experiments, left in the organ bath (+PVAT in bath). (C,D) Concentration-response curves to (C) apigenin and (D) chrysin constructed in rings pre-contracted with 1 µM noradrenaline either in the absence (-PVAT) or presence of PVAT in the organ bath (+PVAT in bath). In the ordinate scale, the response is reported as a percentage of the tension induced either by (A,B) 60 mM KCl in ring functional assay or (C,D) by 1 µM noradrenaline (NA). Data points represent the mean±s.e.m.

### 3.5 Effect of PVAT on the anti-spasmodic activity of apigenin and chrysin

In this series of experiments, the anti-spasmodic activity of flavonoids was assessed in rings stimulated with cumulative concentrations of KCl in the absence or presence of PVAT. As previously reported (Soltis and Cassis 1991; Gao et al. 2005), PVAT did not affect the response to KCl. In fact, under control conditions, KCl contracted -PVAT and +PVAT preparations with

similar potency ($EC_{50}$ -PVAT 20.4±1.7 mM, +PVAT 23.1±1.3 mM, n=6-7; P=0.234) and efficacy ($E_{max}$ -PVAT 102.6±1.8%, +PVAT 105.7±1.6%, n=6-7; P=0.217). Similar results were obtained in rings pre-incubated with the vehicle (DMSO; Fig. 3.6). In rings -PVAT pre-incubated with either 10 µM apigenin ($EC_{50}$ DMSO 20.2±1.6 mM, apigenin 22.8±1.4 mM, n=10; P=0.242; $E_{max}$ DMSO 101.7±2.4%, apigenin 75.4±3.1%, n=10; P<0.0001) (Fig. 3.6A) or chrysin ($EC_{50}$ DMSO 19.6±1.7 mM, chrysin 25.4±1.9 mM, n=9; P=0.042; $E_{max}$ DMSO 102.8±2.4%, chrysin 70.4±4.1%, n=9; P<0.0001) (Fig. 3.6B), the response evoked by KCl was significantly reduced. As shown in Fig. 3.6, also in rings +PVAT the response to KCl was antagonized by both apigenin ($EC_{50}$ 25.1±1.9 mM; P=0.356 vs -PVAT; $E_{max}$ 75.1±5.5%, n=10; P=0.951) and chrysin ($EC_{50}$ 24.7±1.6 mM, P=0.779 vs -PVAT; $E_{max}$ 77.0±4.6%, n=9; P=0.299) similarly to what observed in rings -PVAT.

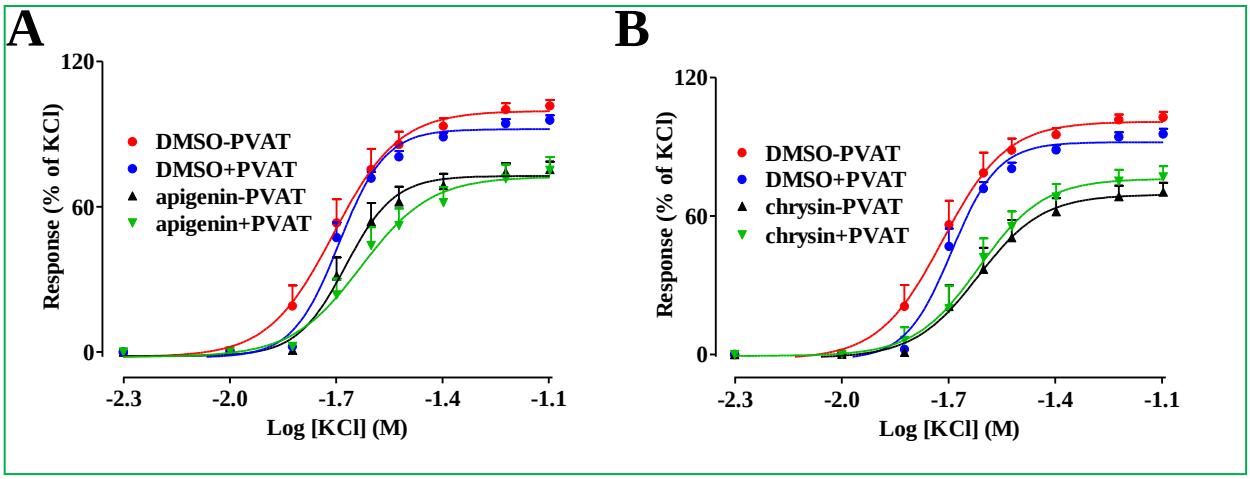



**Figure 3.6.** Effect of PVAT on the anti-spasmodic activity of apigenin and chrysin towards KCl. KCl concentration-response curves were constructed in endothelium-denuded thoracic aorta rings pre-incubated with vehicle (DMSO), (A) 10 µM apigenin or (B) 10 µM chrysin for 30 min. In the ordinate scale, the response is reported as a percentage of the contraction induced by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m.

### 3.6 Effect of mito-tempol on the functional interaction between flavonoids and PVAT

To test the hypothesis that the functional interaction between PVAT and apigenin or chrysin is mediated by the production of superoxide anion at PVAT mitochondrial level, -PVAT and +PVAT rings were pre-incubated with 10 µM mito-tempol for 30 min prior to construct a noradrenaline concentration-response curve. As shown in Fig. 3.7A,B, mito-tempol abolished the functional interaction between PVAT and both apigenin ($EC_{50}$ -PVAT 78.9±33.5 nM, +PVAT

99.5±66.9, n=6; P=0.790; $E_{max}$ -PVAT 65.6±2.9%, +PVAT 38.1±4.0%, n=6; P=0.0003) and chrysin ($EC_{50}$ -PVAT 33.2±3.4 nM, +PVAT 160.2±11.1 nM, n=6; P<0.0001; $E_{max}$ -PVAT 67.2±5.2%, +PVAT 49.5±2.3%, n=6; P=0.011) (see Fig. 3.2 E,F for a comparison).

In a second series of experiments, the vasorelaxant activity of apigenin and chrysin was assessed on noradrenaline pre-contracted rings in the presence of 10 µM mito-tempol. As shown in Fig. 3.7C,D, mito-tempol reverted the antagonism exerted by PVAT towards apigenin ($IC_{50}$ -PVAT 13.0±1.3 µM, +PVAT 14.2±2.5 µM, n=7; P=0.685; $E_{max}$ -PVAT 97.1±0.7%, +PVAT 93.7±4.4%, n=3-6; P=0.581) and chrysin ($IC_{50}$ -PVAT 11.9±2.3 µM, +PVAT 14.5±1.8 µM, n=6; P=0.374; $E_{max}$ -PVAT 90.5±3.6%, n=3; +PVAT 89.3±1.9%, n=4; P=0.768) (see Fig. 3.4C,D for a comparison).

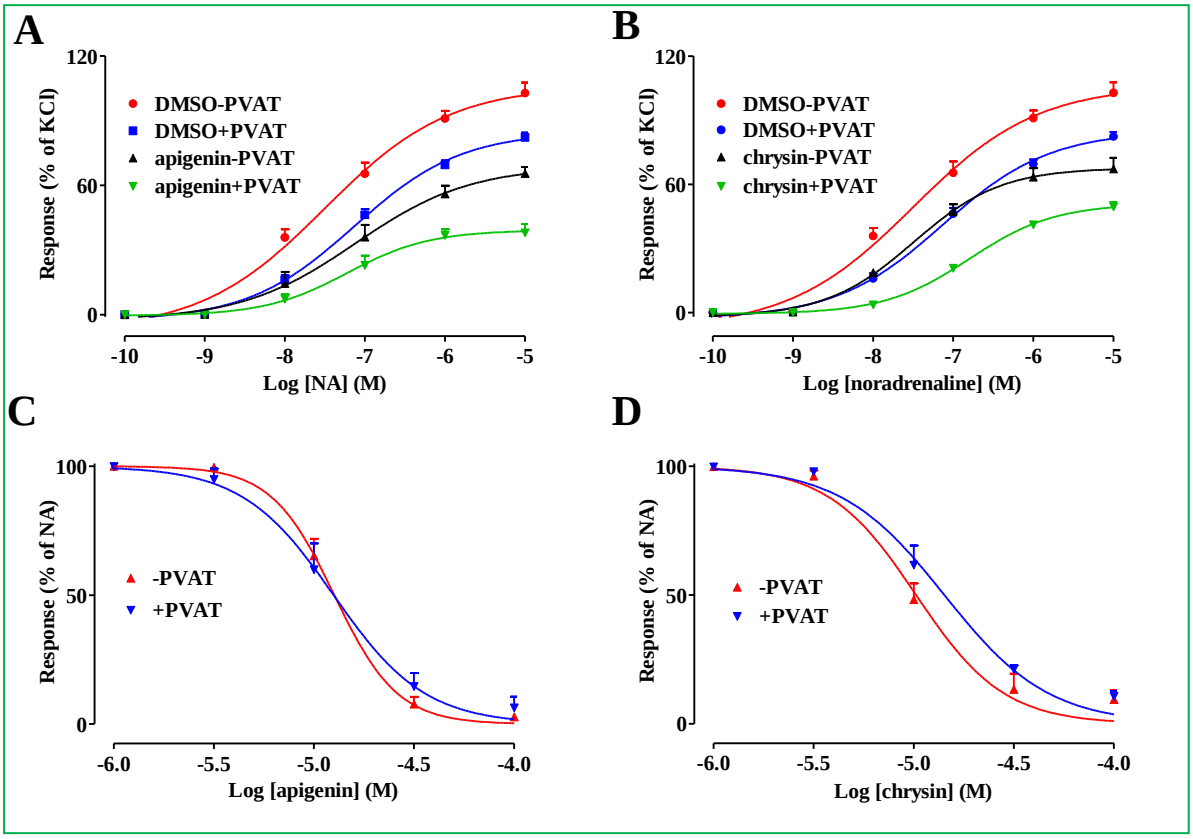



**Figure 3.7.** Effect of mito-tempol on the functional interaction between PVAT and flavonoids. (A,B) Endothelium-denuded thoracic aorta rings -PVAT or +PVAT were pre-incubated with 10 µM mito-tempol for 30 min and then treated with vehicle (DMSO), (A) 10 µM apigenin or (B) 10 µM chrysin for 30 min before constructing a noradrenaline concentration-response curve or pre-contracted by 1 µM noradrenaline before constructing a concentration-response curve to either (C) apigenin or (D) chrysin. In the ordinate scale, the response is reported as a percentage

of the tension induced by (A,B) 60 mM KCl in ring functional assay or (C,D) 1 μM noradrenaline (NA). Data points represent the mean±s.e.m.

### 3.7 Effect of pyrogallol on flavonoid vasodilatory activity

To strengthen the hypothesis of the superoxide radical involvement in the functional interaction between PVAT and apigenin or chrysin, rings -PVAT were pre-incubated with 30 μM pyrogallol, a superoxide donor (Gao et al. 2006), for 20 min, before the addition of 1 μM noradrenaline. Then, a cumulative concentration-response curve to either apigenin or chrysin was constructed. As shown in Fig. 3.8, under control conditions, both flavonoids relaxed noradrenaline-induced tone with $IC_{50}$ values of 14.3±1.7 μM (apigenin, n=6) and 15.3±4.3 μM (chrysin, n=10). In preparations pre-treated with pyrogallol, a significant rightward shift was observed, $IC_{50}$ values being 24.2±1.5 μM (n=6; P=0.001) and 29.1±4.3 μM (n=11; P=0.035) for apigenin and chrysin, respectively. Efficacy, however, remained unaffected.

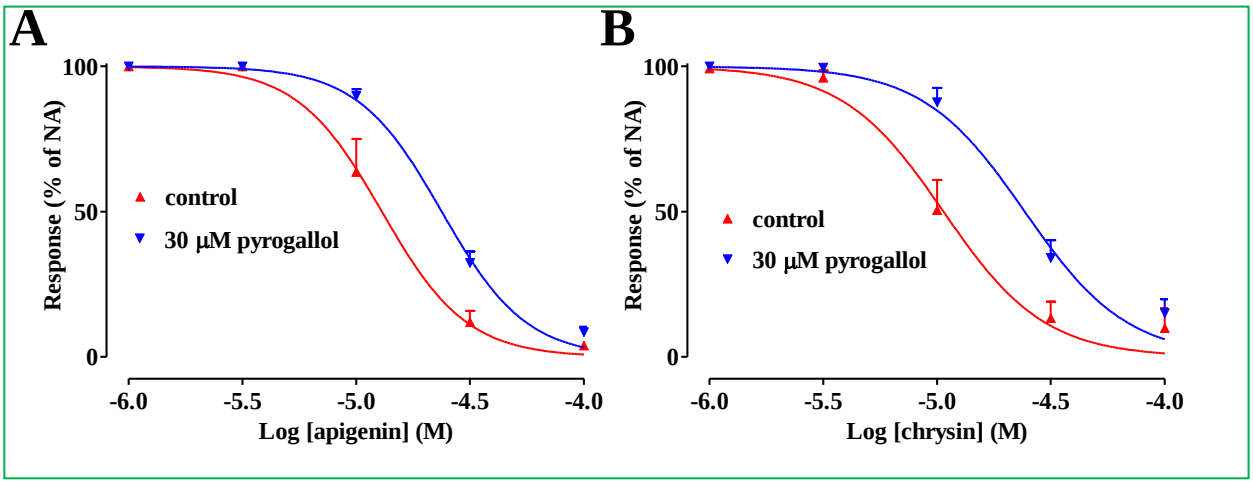



**Figure 3.8.** Effect of pyrogallol on apigenin and chrysin vasorelaxant activity. Endothelium-denuded thoracic aorta rings were pre-contracted with 1 μM noradrenaline and then either (A) apigenin or (B) chrysin was added cumulatively. In the ordinate scale, the response is reported as a percentage of the initial tension induced by noradrenaline (NA). Data points represent the mean±s.e.m.

### 3.8 Effect of flavonoids on NaF-induced contraction in the absence or presence of PVAT

In the first series of experiments, the effect of DMSO on vessel active tone induced by NaF was assessed. As shown in Figure 3.8, in rings both -PVAT and +PVAT, the addition of cumulative

concentrations of DMSO did not produce any significant effect, the residual tone measured at the maximal added concentration being reduced by a mere 10%.

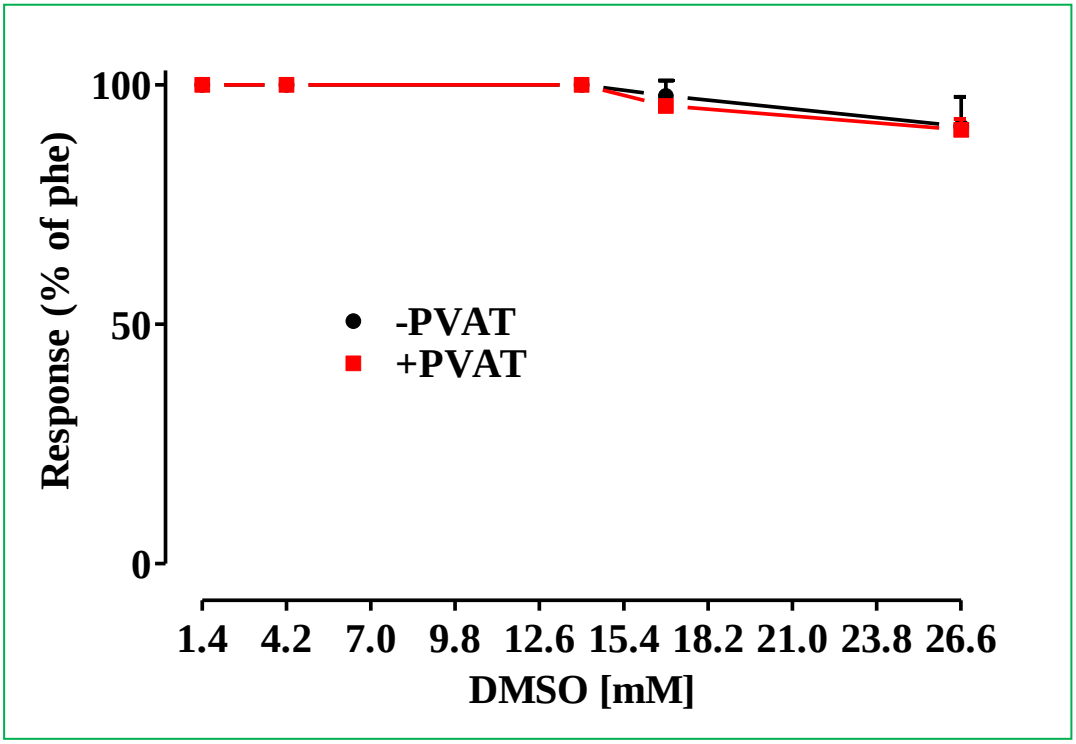



**Figure 3.9.** Effect of DMSO on NaF-induced tension in endothelium-denuded thoracic aorta rings. The steady-state contraction evoked by 10 mM NaF in preparations without (-PVAT) or with PVAT (+PVAT) was monitored after the addition of cumulative concentrations of DMSO, matching those necessary to construct a concentration-response curve to each flavonoid (see below). In the ordinate scale, response is reported as a percentage of the initial tension induced by NaF. Data points represent the mean±s.e.m. (n=5).

Another series of experiments were performed to assess a possible involvement of the Rho/Rho-kinase pathway in the functional interaction between PVAT and apigenin or chrysin. -PVAT and +PVAT rings were challenged by 10 mM NaF, a known activator of this enzyme (Yang et al. 2010), before constructing a concentration-response curve for both flavonoids. NaF evoked a slow and strong contraction (Fig. 3.10A) that was not affected by the presence of PVAT (see Table 2.2). In rings -PVAT (Fig. 3.10A,B), addition of apigenin induced a concentration-dependent relaxation of NaF-induced tone with a potency ($IC_{50}$ 17.2±2.5 µM, n=8; P=0.110) and an efficacy ($E_{max}$ 94.1±1.5%, n=6; P=0.083) not significantly different from those recorded in noradrenaline pre-contracted preparations (see Table 3.2). Chrysin, however, resulted less

effective under these experimental conditions (Fig. 3.10C), as the $IC_{50}$ value (24.9±5.7 µM, n=7; P=0.043) and $E_{max}$ value (90.3±1.6%, n=7; P=0.0003) were higher and lower, respectively, than those obtained in noradrenaline challenged rings. Vasorelaxation induced by chrysin was not significantly affected by the presence of PVAT ($IC_{50}$ 29.3±5.5 µM, n=8; P=0.583 vs -PVAT; $E_{max}$ 92.0±0.9%, n=8; P=0.349, Fig. 3.10C) whereas that of apigenin was significantly reduced ($IC_{50}$ 28.2±4.0 µM, n=9; P=0.038), though its efficacy remained unaltered ($E_{max}$ 93.1±0.9%, n=9; P=0.565; Fig. 3.10B). Noticeably, in rings pre-treated with 10 µM mito-tempol, PVAT antagonism towards apigenin was abolished ($IC_{50}$ -PVAT 6.5±0.6 µM, +PVAT 8.9±1.6 µM, n=6; P=0.200) (Fig. 3.10D), both concentration-response curves being significantly shifted to the left (-PVAT, P=0.004; +PVAT P=0.002).

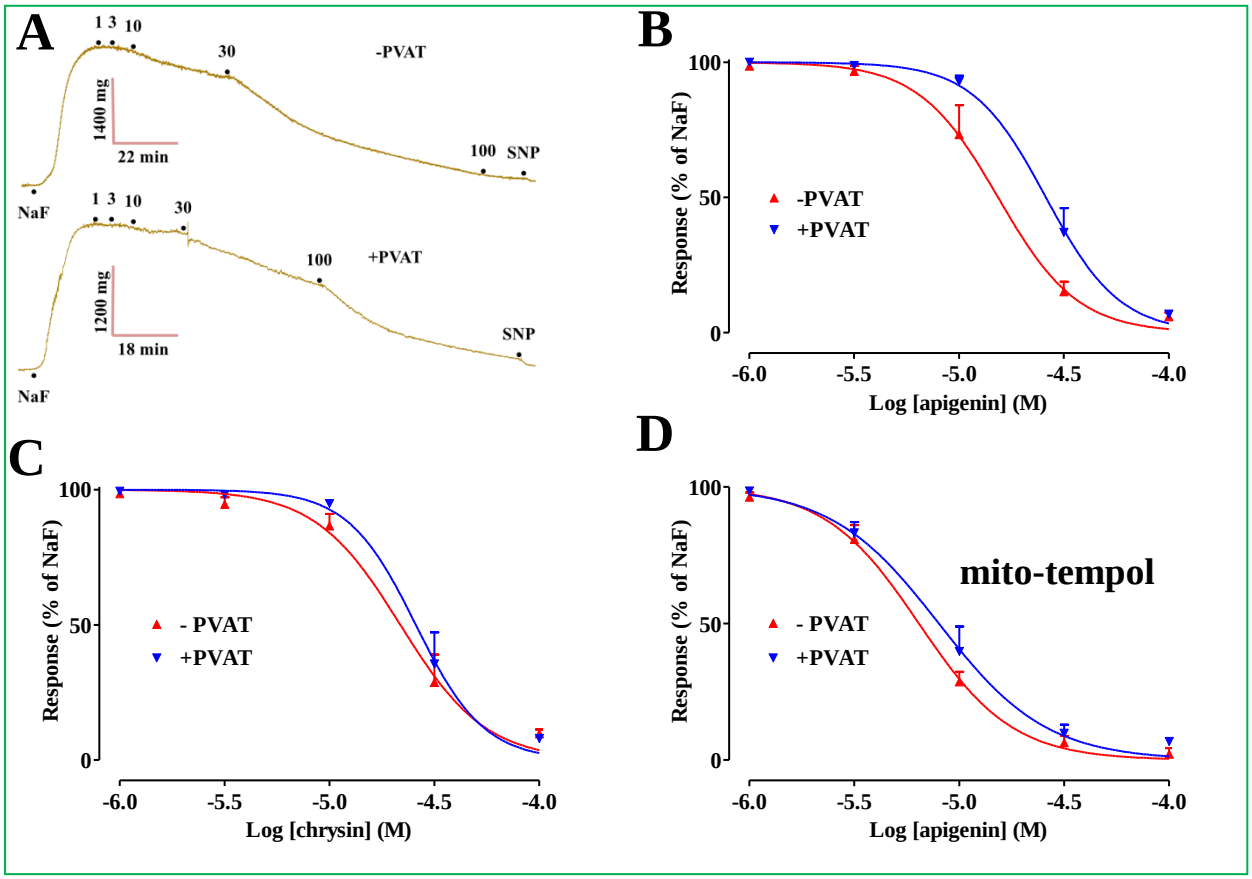



**Figure 3.10.** Effect of apigenin and chrysin on NaF-induced contraction. (A) The steady-state contraction was evoked in endothelium-denuded thoracic aorta rings -PVAT (upper panel) or +PVAT (lower panel) by 10 mM NaF and then apigenin (µM) was added cumulatively. Traces are representative of 8 similar experiments. (B-D) Concentration-response curves to (B,D) apigenin and (C) chrysin constructed in -PVAT and +PVAT rings pre-contracted by NaF (B,C)

under control conditions and (D) in the presence of 10 µM mito-tempol, pre-incubated for 30 min before NaF stimulation. In the ordinate scale, the response is reported as a percentage of the initial tension induced by NaF. Data points represent the mean±s.e.m.

### 3.9 Effect of PVAT on fasudil-induced vasorelaxation

To strengthen the hypothesis of Rho/Rho-kinase pathway involvement in the functional interaction between flavonoids and PVAT, the Rho-kinase inhibitor fasudil (Huang et al. 2018) was assessed in rings pre-contracted with either 1 µM noradrenaline or 10 mM NaF, both in absence or presence of PVAT. As shown in Fig. 3.11A, fasudil caused concentration-dependent relaxation of -PVAT preparations pre-contracted by noradrenaline, with an $IC_{50}$ value of 0.15±0.06 µM (n=6). The presence of PVAT significantly decreased the potency of fasudil ($IC_{50}$ 1.03±0.35 µM, n=7; P=0.041) without, however, affecting its efficacy ($E_{max}$ -PVAT 99.0±1.1%, +PVAT 98.4±1.2%, n=4; P=0.918). In rings pre-contracted by NaF (Fig. 3.11B), neither the potency ($IC_{50}$ -PVAT 0.83±0.32 µM, +PVAT 1.01±0.42 µM, n=11; P=0.731) nor the efficacy of fasudil ($E_{max}$ -PVAT 93.9±2.2%, +PVAT 93.7±1.9%, n=5; P=0.952) were modified by the presence of PVAT.

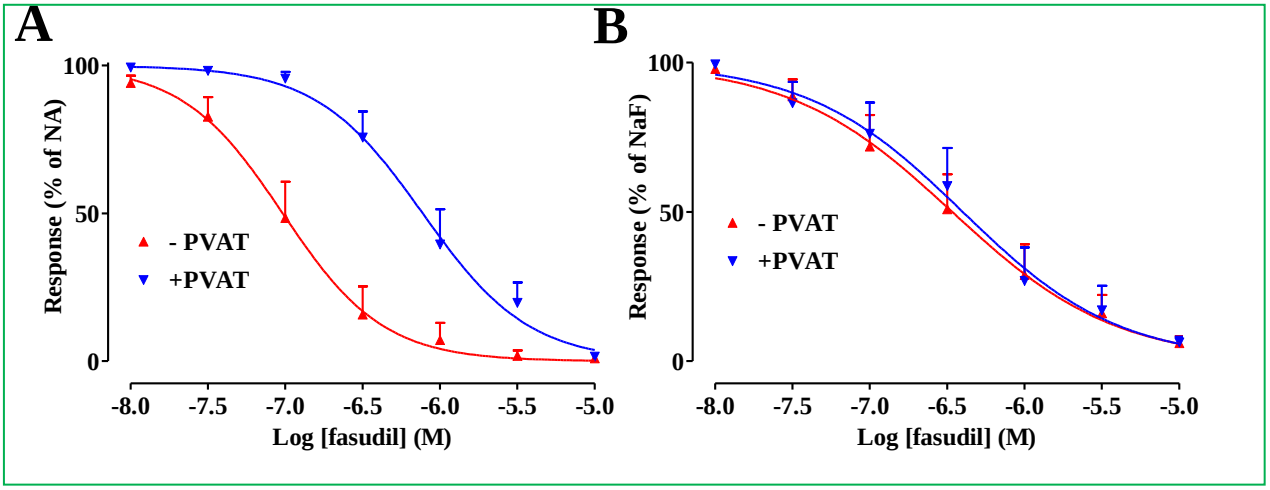



**Figure 3.11.** Effect of PVAT on fasudil vasorelaxant activity. Endothelium-denuded thoracic aorta rings were pre-contracted by either (A) 1 µM noradrenaline or (B) 10 mM NaF and then fasudil was added cumulatively. In the ordinate scale, the response is reported as a percentage of the initial tension induced by either noradrenaline (NA). Data points represent the mean±s.e.m.

### 3.10 Effect of PVAT on the vasorelaxant activity of apigenin and chrysin on phenylephrine-induced contraction

In the first series of experiments, the stability of vessel active tone induced by phenylephrine was assessed. As shown in Figure 3.12, in time control experiments, matching those performed with each flavonoid, phenylephrine-induced contraction in rings-PVAT displayed a partial fading accounting for less than 20% loss of tone in correspondence to the highest concentration of flavonoids used. This fading was not affected by the presence of PVAT. Overlapping results were observed when assessing the effect of vehicle (i.e., DMSO).

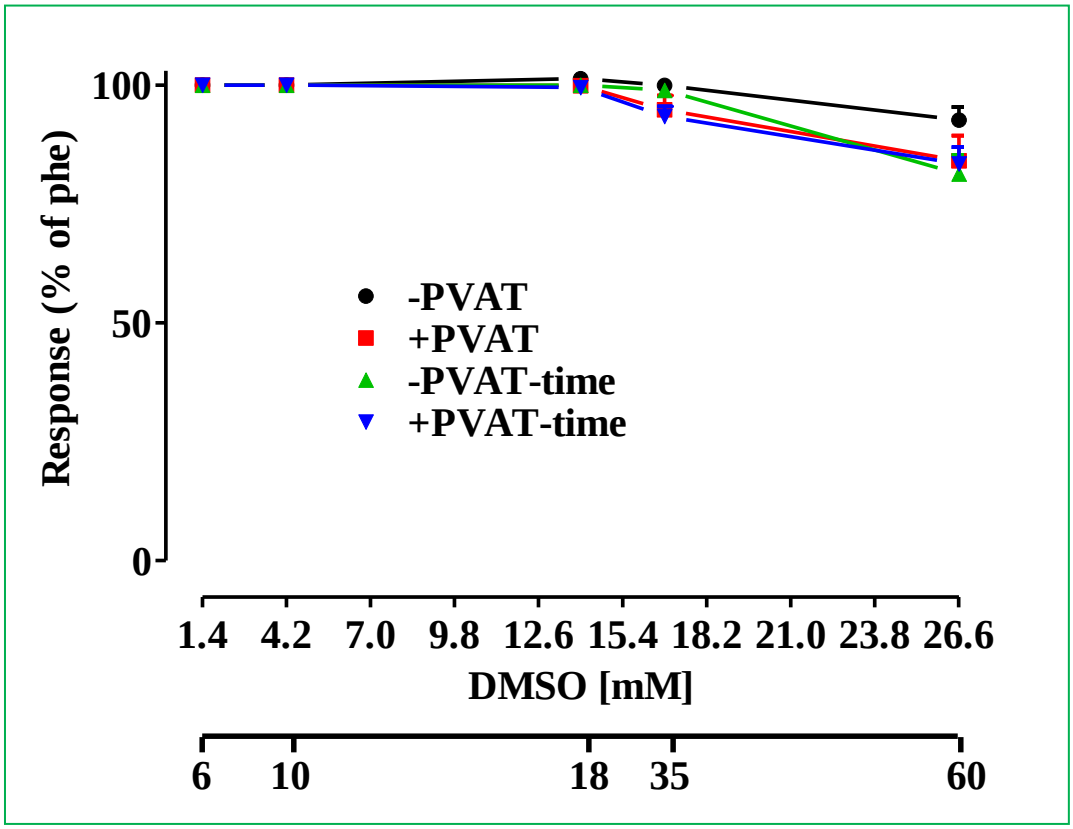



**Figure 3.12.** Effect of DMSO on and stability of the phenylephrine-induced tension in endothelium-denuded thoracic aorta rings. The steady-state contraction evoked by a submaximal concentration of phenylephrine in preparations without (-PVAT) or with PVAT (+PVAT) was monitored for 60 min as well as after the addition of cumulative concentrations of DMSO, matching those necessary to construct a concentration-response curve to each flavonoid (see below). In the ordinate scale, response is reported as a percentage of the initial tension induced by phenylephrine (phe). Data points represent the mean±s.e.m. (n=7-8).

Another series of experiments were performed to assess the possible involvement of $\beta_3$ receptors in mediating the functional interplay between PVAT and flavonoids. Both -PVAT and +PVAT

rings were pre-contracted with the $\alpha_1$ adrenergic receptor-selective agonist phenylephrine to similar levels and then apigenin or chrysin was added cumulatively. As shown in Fig. 13, in rings -PVAT both flavonoids relaxed phenylephrine-induced tone (apigenin $IC_{50}$ 11.4±1.9 µM, $E_{max}$ 99.2±0.3%, n=5-6; chrysin $IC_{50}$ 16.0±2.4 µM, $E_{max}$ 98.3±2.6%, n=6-7). In +PVAT preparations, both potency ($IC_{50}$ 25.1±5.1 µM, n=6; P=0.032 vs -PVAT) and efficacy of apigenin ($E_{max}$ 91.5±3.2%, n=5; P=0.044; Fig. 13A) of apigenin were significantly reduced. However, neither potency of chrysin (17.8±3.2 µM, n=7; P=0.659 vs -PVAT) nor efficacy ($E_{max}$ 94.4±2.5%, n=7; P=0.212; Fig. 13B) were affected by the presence of PVAT.

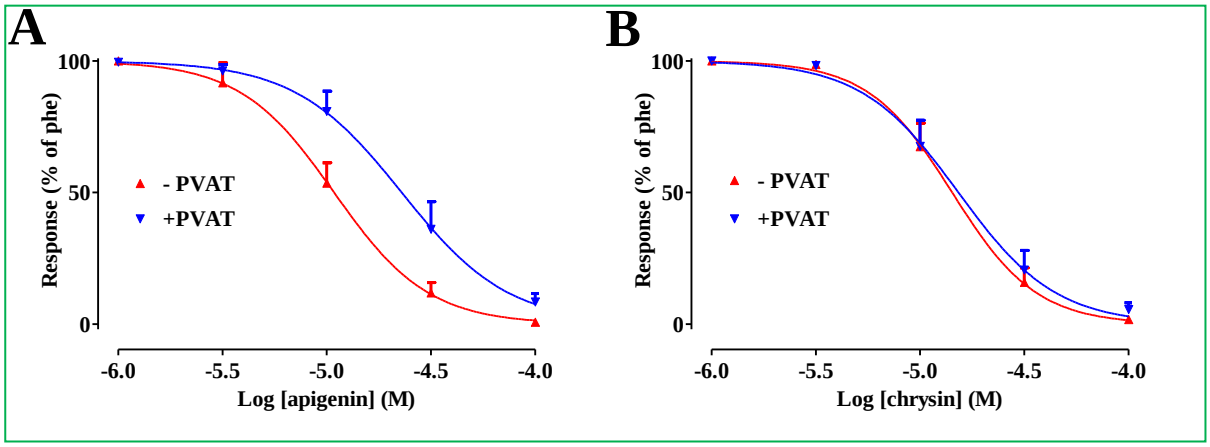



**Figure 3.13.** Effect of PVAT on apigenin and chrysin vasorelaxant activity towards phenylephrine-induced contraction. Both -PVAT and +PVAT endothelium-denuded thoracic aorta rings were pre-contracted by phenylephrine to similar levels and then either (A) apigenin or (B) chrysin was added cumulatively. In the ordinate scale, the response is reported as a percentage of the initial tension induced by phenylephrine (phe). Data points represent the mean±s.e.m.

### 3.11 Effect of SR59230A on the functional interplay between PVAT and flavonoids

To reinforce the hypothesis of superoxide anion release from PVAT as a result of $\beta_3$ receptor stimulation, both -PVAT and +PVAT rings were pre-incubated with 0.3 µM SR59230A (a selective $\beta_3$ receptor antagonist; (Gan et al. 2007) prior to stimulation by noradrenaline. As shown in Fig. 1.14, SR59230A abolished the antagonism exerted by PVAT towards apigenin ($IC_{50}$ -PVAT 3.5±0.9 µM, n=7, +PVAT 3.9±0.7 µM, n=8; P=0.746; $E_{max}$ -PVAT 98.9±1.1%, +PVAT 98.0±0.7%, n=4-8; P=0.475) and chrysin ($IC_{50}$ -PVAT 5.7±1.3 µM, n=7, +PVAT 5.9±1.2 µM, n=8; P=0.374; $E_{max}$ -PVAT 99.4±0.4%; +PVAT 99.1±0.9%, n=4; P=0.785). The

presence of SR59230A caused a significant leftward shift of the concentration response curves of both -PVAT and +PVAT rings (apigenin -PVAT+SR59230A, P=0.015; +PVAT+SR59230A, P=0.0008; chrysin -PVAT+SR59230A, P=0.040; +PVAT+SR59230A, P<0.0001).

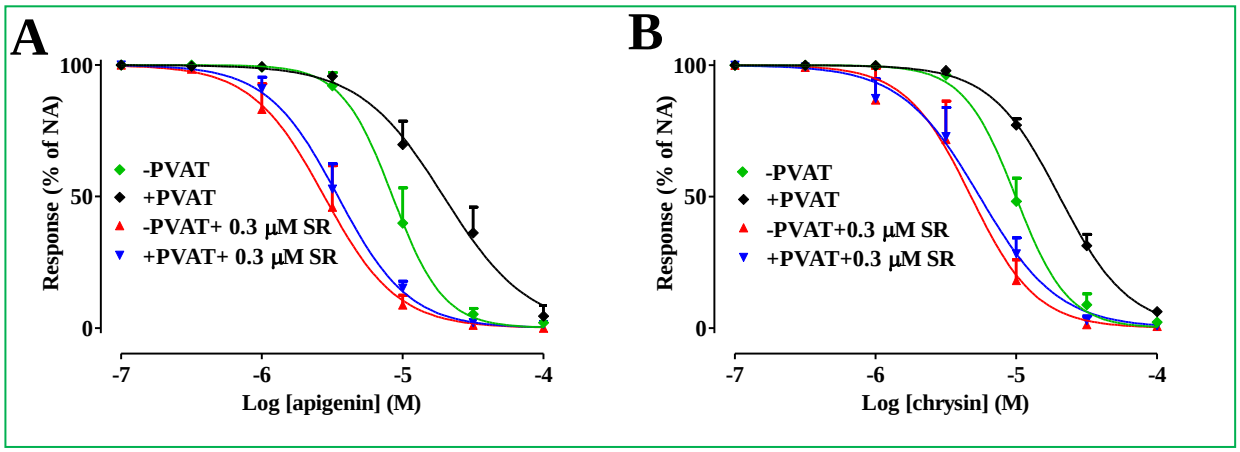



**Figure 3.14.** Effect of SR59230A on the functional interplay between PVAT and flavonoids. Both -PVAT and +PVAT endothelium-denuded thoracic aorta rings were pre-incubated with 0.3 µM SR59230A (SR) or none for 30 min and then contracted by noradrenaline to similar levels. On the plateau, either (A) apigenin or (B) chrysin was added cumulatively. In the ordinate scale, the response is reported as a percentage of the initial tension induced by noradrenaline (NA). Data points represent the mean±s.e.m.

# CHAPTER 4

# DISCUSSION

# 4. Discussion

The present study aimed to assess the possible functional interplay between PVAT and a series of dietary flavonoids. The main findings include: 1) flavonoids antagonized noradrenaline-induced response in a non-competitive and structure-dependent manner; 2) in the presence of PVAT, flavonoid antagonistic effect was either lost or reduced and the vasorelaxant activity either decreased or remained unaffected; 3) the vasorelaxant activity of apigenin and chrysin may be mediated, at least partially, by the Rho/Rho-kinase pathway; and 4) the stimulation of $\beta_3$ adrenergic receptors by noradrenaline and/or apigenin, likely resulted in the PVAT release of superoxide anions that, in turn, diffused down to VSMCs and activate the Rho/Rho-kinase pathway, thus inhibiting flavonoid vasorelaxant activity (Fig. 4.2).

## 4.1 Flavonoids antagonized noradrenaline response in a non-competitive and structure-dependent manner

Several flavonoids antagonized noradrenaline-induced response in a non-competitive manner. The structure-activity relationship revealed the structural requirements necessary for this antagonism (Fig. 4.1). The presence of one OH group in the flavonoid scaffold conferred a modest activity (5-hydroxyflavone), which markedly increased by adding another OH group, giving rise to the most potent compound (3,4′-dihydroxyflavone); the two OH groups, however, must be positioned on different rings (namely B and C rings) as the analogue chrysin was dramatically less effective. Flavonoids bearing three OH groups (apigenin, galangin, and resokaempferol) showed an inhibitory activity between 33% and 60%; in this case, however, the isoflavone structure (genistein) showed reduced activity. When the number of OH groups increased to four (one being methoxylated as in hesperetin), noradrenaline response was reduced by a mere 20% (fisetin and kaempferol), unless the groups were equally distributed on rings A and B only (luteolin) giving rise to an about 50% inhibition. In fact, unequal distribution occurring in scutellarein abolished the inhibitory activity. A further increase in the number of OH groups to five did not give rise to effective inhibition except for tamarixetin, bearing a methoxylated group in para position on the B ring (meta position in fact was ineffective as in isorhamnetin). Finally, the lack of the C2-C3 double bond, the presence of a bulky glycoside group(s), or catechins gave rise to structures devoid of activity. One limitation of this study, to our knowledge the first screening such a large number of flavonoids towards the adrenergic

receptor agonist noradrenaline, is that the molecular mechanism underpinning this antagonism was not investigated. However, it pointed to flavonols bearing one (3,4′-dihydroxyflavone) or two OH groups (galangin) as the most effective, non-competitive antagonists. Though the dietary flavonoids ingested, taken singularly, will never give rise to plasma concentrations comparable to those effective reported in this study, our diet is a rich mixture of these natural molecules that might act synergistically or additively to counteract endogenous $\alpha_1$ receptor agonists, reduce vascular smooth muscle tone and hence high blood pressure. These observations, therefore, might provide additional support to the substantial epidemiological evidence that people ingesting a high amount of food and/or beverages rich in flavonoids are more protected against hypertension development (Clark et al. 2015).

Noticeably, the non-competitive antagonism exerted by flavonoids towards noradrenaline indicates that the molecular target(s) underpinning this phenomenon is situated downstream to $\alpha_1$ receptors, where several stimulatory pathways occurred, thus supporting their effectiveness against hypertension inducers such as angiotensin II or endothelin. Indeed, flavonoids induce vasodilation of different vascular beds by blocking $Ca^{2+}$ channels, opening $K^+$ channels, inhibiting $Ca^{2+}$ release from or stimulating $Ca^{2+}$ uptake to intracellular stores, inhibiting enzymes such as protein kinase C, Rho-kinase, and phosphodiesterases, stimulating soluble guanylyl cyclase, protein kinase A, and protein kinase G, and stimulating of β receptor (Ahmed 2021). However, direct binding studies of flavonoids to these molecular targets, particularly adrenergic receptors, are lacking. Though, with regard to $Ca_V$ 1.2 channels, Fusi et al. (2003) demonstrated that myricetin did not significantly affect the binding of (+)-[$^3$H]PN-200-110 (a dihydropyridine capable to bind specifically to the α-subunit of $Ca_V$1.2 channels) to porcine aortic membranes, contrary to nicardipine and (S)-(-)-Bay K 8644, which displaced it in a concentration-dependent manner.

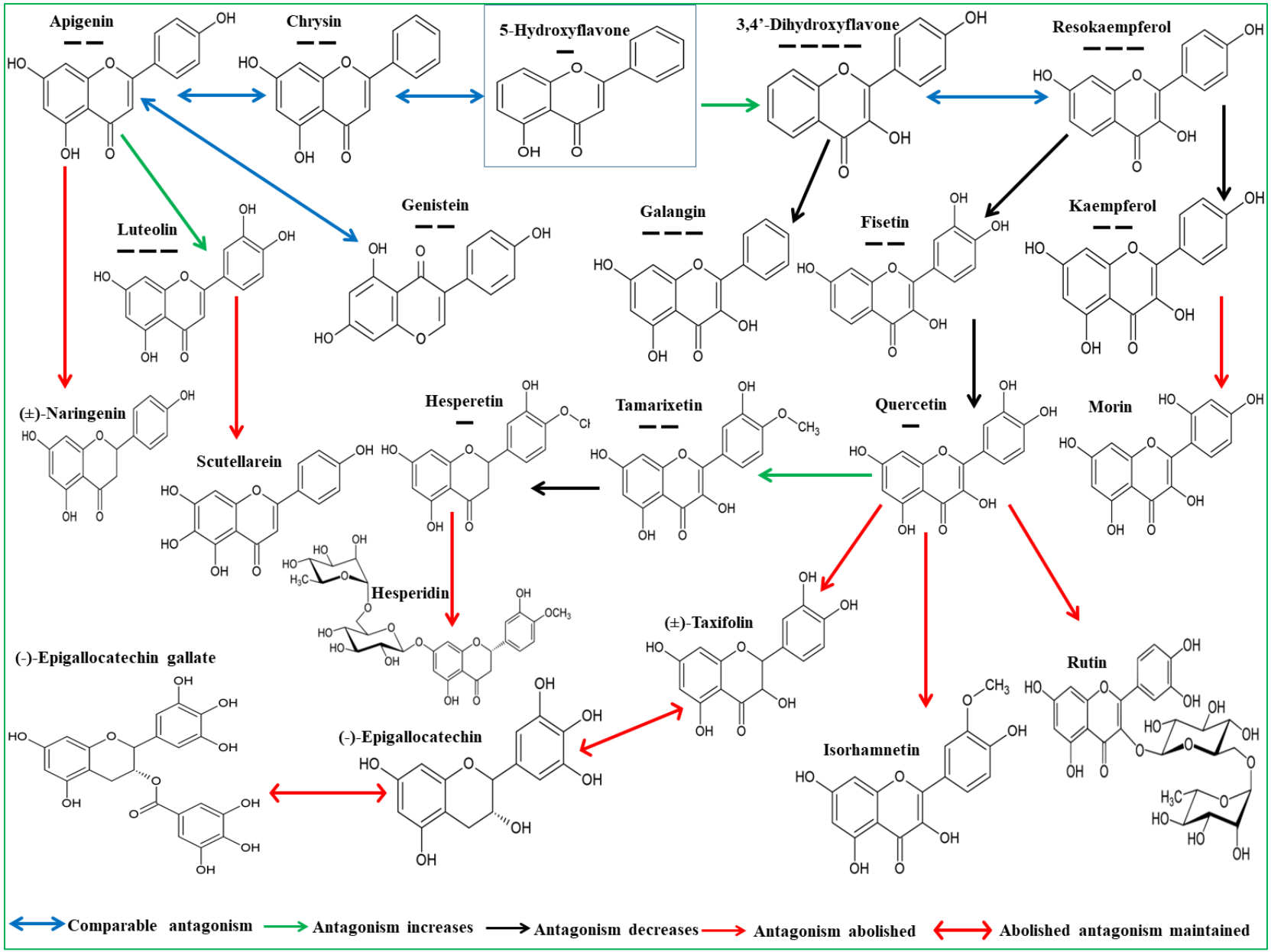

Apigenin
Chrysin
5-Hydroxyflavone
3,4'-Dihydroxyflavone
Resokaempferol
Luteolin
Genistein
Galangin
Fisetin
Kaempferol
(±)-Naringenin
Scutellarein
Hesperetin
Tamarixetin
Quercetin
Morin
Hesperidin
(±)-Taxifolin
(-)-Epigallocatechin gallate
(-)-Epigallocatechin
Isorhamnetin
Rutin
Comparable antagonism
Antagonism increases
Antagonism decreases
Antagonism abolished
Abolished antagonism maintained

**Figure 4.1** Structure-activity relationship for the flavonoids assessed for antagonism towards noradrenaline-induced contraction. Antagonism: ▬ very weak (<20%); ▬ ▬ weak (20-45%); ▬ ▬ ▬ moderate (45-75%); ▬ ▬ ▬ ▬ potent (>75%).

### 4.2 PVAT and flavonoids exhibited a multifaceted interaction

In the presence of PVAT, the compounds that did not antagonize the noradrenaline-induced response did not affect also the anti-contractile effect of PVAT. On the other hand, flavonoids antagonizing noradrenaline response either lost their inhibitory activity (their effect was not additive to the anti-contractile activity of PVAT; chrysin, fisetin, genistein, hesperetin, 5-hydroxyflavone, kaempferol, quercetin, resokaempferol, and tamarixetin) or significantly decreased (apigenin, 3,4′-dihydroxyflavone, and galangin). Consistently, the vasorelaxant activity of all these compounds was reduced except for kaempferol, luteolin and quercetin.

When considering the slow, time-dependent fading of noradrenaline-induced tone that was recorded either under control conditions or following the addition of DMSO, one could argue that this effect might have biased the direct vasorelaxant activity of flavonoids. However, several evidence suggest that this hypothesis can be ruled out. First, the vehicle did not exert any vasorelaxant acivity *per se*, because the loss of active tone caused by the highest concentrations of DMSO overlapped that observed in its absence. Second, most of the experiments here presented were performed using a flavonoid concentration (10 µM) at which noradrenaline-induced tone was still around 100%. Third, in most concentration-response curve for flavonoids, $IC_{50}$ values were below 30 µM, thus marginally affecting, if any, only $E_{max}$ values.

Altogether, this finding suggests that PVAT and flavonoids exhibit a multifaceted interplay: PVAT antagonizes the vascular activity of flavonoids or flavonoids inhibit the anti-contractile effect of PVAT.

Noticeably, kaempferol, luteolin, and quercetin, on one hand, exerted an antagonistic effect towards noradrenaline to variable levels; this effect was lost or independent of the presence of PVAT. On the other hand, their vasorelaxant activity was not affected by PVAT. This may imply that these flavonoids inhibit the anti-contractile effect of PVAT. However, this hypothesis or other plausible mechanisms underpinning this phenomenon were not investigated in this study and deserved further investigation in the future.

Of note, genistein inhibition of PVAT anti-contractile effect described here is in agreement with what was previously reported by Löhn and co-worker (Löhn et al. 2002) who pointed to tyrosine kinase inhibition as the mechanism underpinning this phenomenon. Furthermore, PVAT markedly inhibited genistein vasorelaxant activity in this work. A likely explanation for this observation is reported below.

### 4.3 PVAT does not act as a physical barrier to limit the diffusion of flavonoids

Apigenin and chrysin were selected as the most representative compounds, capable of antagonizing vascular response to noradrenaline and functionally interacting with PVAT, to elucidate the mechanism underpinning this interplay. First, the hypothesis that PVAT may act as a physical barrier limiting the diffusion of flavonoids to vascular smooth muscle cells was assessed. PVAT detached from rings, though left in the organ bath, lost its anti-contractile activity towards noradrenaline and did not counteract both antagonistic and vasorelaxant activities of flavonoids. However, apigenin and chrysin antagonized also KCl-induced contraction in both -PVAT and +PVAT rings with comparable potency and efficacy. This type of contraction induced by membrane depolarization is not affected by the presence of PVAT (Soltis and Cassis 1991; Löhn et al. 2002). These observations indicate that: PVAT does not limit the diffusion of flavonoids to the underlying vascular smooth muscle; factors released from PVAT, likely characterized by a brief half-life, are critical to the functional interplay between PVAT and flavonoids, observed only when PVAT was in contiguity to the smooth muscle. In this regard, Gao et al. (2007) demonstrated that aortic PVAT exerts its anti-contractile effects through either of two distinct mechanisms: one releasing a transferable relaxing factor that induces endothelium-dependent relaxation; the other involving an endothelium-independent factor. The latter hypothesis is in line with what is reported in the present work.

### 4.4 Superoxide anion mediates the functional interplay between flavonoids and PVAT

Superoxide anion, produced within PVAT, contributes to the pro-contractile effect observed in rat superior mesenteric artery in response to electrical field stimulation (Gao et al. 2006) and obese animal models (Gil-Ortega et al. 2014). As noradrenaline is capable to stimulate mitochondrial superoxide anion production from PVAT of rat aorta rings (Costa et al. 2016), it was hypothesized that this radical might be involved in the functional interplay between PVAT

and flavonoids. Two pieces of evidence supported this hypothesis. First, the mitochondria-targeted antioxidant, superoxide dismutase mimetic, mito-tempol abolished PVAT antagonism towards the spasmolytic and antispasmodic activities of apigenin and chrysin. Second, in rings - PVAT the superoxide donor pyrogallol decreased the vasorelaxant potency of both apigenin and chrysin, mimicking the effect of PVAT. ROS or superoxide anion produced from PVAT could be originated from mitochondria, NADPH oxidases, and endothelial nitric oxide synthase (eNOS) uncoupling. High vascular oxidative stress is an important pathologic and detrimental event in cardiometabolic complications of obesity, hypertension and type 2 diabetes mellitus (Zhou et al., 2021).

### 4.5 The Rho/Rho-kinase pathway mediates the vascular activity of apigenin and chrysin and the functional interplay between PVAT and flavonoids

Several studies have demonstrated that superoxide anion induces a contractile effect in different vascular beds through the modulation of the arachidonic acid pathway (Bharadwaj and Prasad 2002), and NO inactivation at the endothelial level (Wang et al. 1998), or activation of the smooth muscle Rho-kinase pathway (Jin et al. 2004). The former mechanism can be ruled out due to the absence of endothelium in the preparations used here. The latter mechanism was investigated by maximally stimulating the Rho/Rho-kinase pathway by NaF. First, both apigenin and chrysin concentration-dependently relaxed NaF-induced contraction suggesting that their vasorelaxant effect might occur, at least partially, through Rho/Rho-kinase inhibition. Second, under these experimental conditions, the presence of PVAT did not affect chrysin vasorelaxant activity but significantly reduced that of apigenin. The latter, however, was restored by pretreatment with mito-tempol suggesting that, besides noradrenaline, also apigenin can induce superoxide anion production from PVAT. Finally, to reinforce the role of Rho-kinase in mediating the functional interplay between PVAT and flavonoids, the Rho-kinase inhibitor fasudil was assessed in the absence or presence of PVAT. Indeed, PVAT significantly decreased the vasorelaxant potency of fasudil in noradrenaline but not in NaF pre-contracted rings. Taken together, these observations are consistent with the hypothesis that stimulation of $\beta_3$ receptor with noradrenaline or apigenin releases superoxide anions from PVAT mitochondria. These radicals likely diffuse into VSMCs and activates the Rho/Rho-kinase pathway, which counteracts flavonoids vasoactivity. In fact, increased generation of mitochondrial superoxide anion is

implicated in the loss of PVAT anti-contractile effect in a high-fat diet obese mice *via* Rho-kinase activation (da Costa et al. 2017).

Notably, Rho-kinase plays a fundamental role in vascular contraction by enhancing the sensitivity of the contractile machinery to $Ca^{2+}$. This effect is mediated by phosphorylation of myosin light chain phosphatase (MYLP)-regulatory subunit 1 (MYPT1) at T696 and T852 residues consequently inhibiting MLCP activity and maintaining myosin light chain in the phosphorylated form (Yang and Hori 2021). Additionally, Rho-kinase also phosphorylates the 17-kDa protein phosphatase-1 inhibitor protein (CPI-17) at the Thr38 residue that, in turn, inhibits the MLCP (Koyama et al. 2000; Liu and Khalil 2018).

### 4.6 Activation of $\beta_3$ receptor mediates the release of superoxide anions from PVAT

Adipose tissue $\beta_3$ receptor stimulation by noradrenaline increases cAMP levels and lipolysis (Schena and Caplan 2019); free fatty acids can then enter mitochondria where they are metabolized into energy and radicals as by-products (Chouchani et al. 2016). Therefore, the binding of noradrenaline to $\beta_3$ receptors might be the upstream event leading to superoxide anion production. Indeed, in rings pre-contracted by the selective $\alpha_1$ adrenergic receptor agonist phenylephrine that allowed the assessment of PVAT and flavonoid interplay in the absence of $\beta_3$ receptor stimulation, chrysin relaxation was not affected by the presence of PVAT, whereas both potency and efficacy of apigenin were reduced. However, pre-incubation of rings with the $\beta_3$ receptor antagonist SR59230A abolished the inhibitory activity exerted by PVAT towards both apigenin and chrysin vasorelaxation, suggesting that PVAT-flavonoids interplay is dependent on $\beta_3$ receptor stimulation by noradrenaline and/or apigenin. Interestingly, Pasha et al. (2019) hypothesized that apigenin can be a $\beta_3$ receptor agonist based on molecular docking studies and experiments performed on Ewing sarcoma A673 cells.

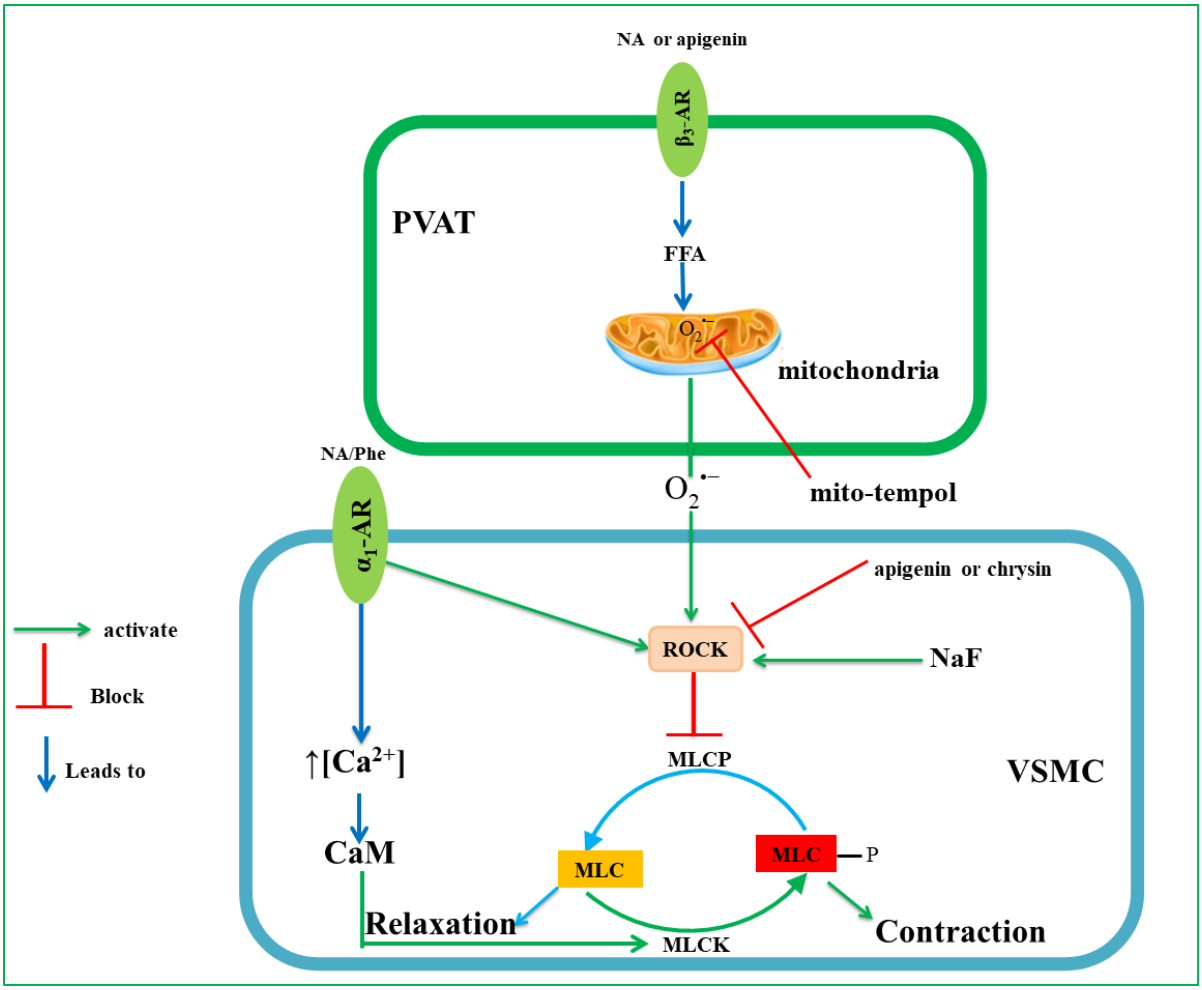



**Figure 4.2.** The proposed mechanism underpinning the interplay between flavonoids and PVAT. Abbreviations: $\alpha_1$-AR, $\alpha_1$-adrenergic receptor; $\beta_3$-AR, $\beta_3$-adrenergic receptor; CaM, $Ca^{2+}$ -calmodulin complex; FFA, free fatty acids; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NA, noradrenaline; Phe, phenylephrine; PVAT, perivascular adipose tissue; ROCK, Rho-kinase, VSMC, vascular smooth muscle cell, ↑, increase.

# CHAPTER 5

# CONCLUSION

## 5. Conclusion and limitations

The main actors of this PhD work are PVAT, an active regulator of vessel tone, and flavonoids, a class of dietary natural compounds endowed with excellent vasorelaxant activity. A multifaceted interaction between the two was pinpointed: PVAT hampering both the spasmolytic and anti-spasmodic activities of flavonoids, while flavonoids inhibiting the anti-contractile effect of PVAT. A mechanism underpinning this interplay was hypothesized as follows: noradrenaline and/or apigenin activate $\beta_3$ receptors within PVAT that, in turn, enhance lipolysis and free fatty acids production. These are metabolized by mitochondria into energy and ROS, in particular superoxide anions, which diffuse into VSMCs and activate the Rho/Rho-kinase pathway, the target of apigenin- and chrysin-induced vasorelaxation. Hence, in the presence of PVAT, the vascular activity of both flavonoids is hampered, a phenomenon that may limit the beneficial effects of these bioactive compounds in obesity-associated hypertension or diseases characterized by hyperactivity of the sympathetic nervous system.

This work suffered from a few limitations, which can be the focus of future investigation. First, though proper PVAT-denuded controls have been used, PVAT has not been studied separately; hence, a muscular origin of the effects observed cannot be ruled out. Second, among the 22 flavonoids analysed, only apigenin and chrysin were assessed in-depth for their interaction with PVAT; therefore, the hypothesized mechanism cannot be extrapolated to the whole class of natural compounds, particularly to those behaving differently toward PVAT.

Finally, the involvement of superoxide radicals and Rho-kinase in the functional interplay between flavonoids and PVAT has not been directly assessed by measuring their tissue levels and activity, respectively. Last, but not least, the cellular source of superoxide radicals has not been identified considering the heterogeneous cell types constituting PVAT.

# CHAPTER 6

# APPENDIX

## 6.1 Appendix 1

During his PhD, Amer Ahmed collaborated with other research groups and contributed to the following publications:

### 6.1.1 Vasoactivity of Mantonico and Pecorello grape pomaces on rat aorta rings: An insight into nutraceutical development

Journal of Functional Foods, 2019, 57, 328-334, (I.F. 3.451)

Gabriele Carullo[a], Miriam Durante[b], Fabio Sciubba[c], Donatella Restuccia[a], Umile Gianfranco Spizzirri[a], **Amer Ahmed[b]**, Maria Enrica Di Cocco[c], Simona Saponara[b,*], Francesca Aiello[a,*], Fabio Fusi[d]

[a]Department of Pharmacy, Health and Nutritional Sciences, Department of Excellence (2018–2022), University of Calabria, Ed. Polifunzionale, 87036 Arcavacata di Rende (CS), Italy
[b]Department of Life Sciences, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy
[c]Department of Chemistry, University of Rome La Sapienza, Piazzale Aldo Moro 5, 00185 Roma, Italy
[d]Department of Biotechnologies, Chemistry & Pharmacy, Department of Excellence (2018–2022), University of Siena, Via Aldo Moro 2, 53100 Siena, Italy

**Abstract**

The valorisation of agrochemical wastes has become a low cost and sustainable tendency for the development of functional food. In particular, these enriched products seem to be enjoyable tools to treat metabolic disorders. In this field, Calabrian autochthonous white grape pomaces (Mantonico and Pecorello cultivar) were firstly investigated for their vasoactive properties. Skins and seeds were extracted and characterized by NMR spectroscopy, revealing the presence of numerous vasoactive compounds. The effects of extracts were analysed in *in vitro* experiments on rat aorta rings (with and without endothelium), contracted by phenylephrine or KCl. Seeds extracts showed, differently from skins, an appreciable endothelium-dependent, eNOS-mediated

vasodilation in the range of 1–100 µg/ml. From a food market point of view, all the extracts were enclosed into a pectin polymer matrix. In the same experimental conditions, the polymers demonstrated the persistence of vasodilator activity only for Pecorello seed extract, which presented the richest chemical profile.

**Keywords:** Vasoactivity, White grape pomaces, Catechin, Pectin, Rat aorta rings

**His contributions consisted in:**

**6.1.1.1 Investigation of the mechanism of action underlying PSS-induced vasodilation**

This series of experiments was performed to explore the mechanism (s) involved in the vasoactivity of the most interesting pomace, i.e. PSS. First, we investigated a possible $K^+$ channel opening activity of the extract. Agents that open these channels enhance $K^+$ efflux from the cell and produce membrane hyperpolarization that, in turn, will cause $Ca_V1.2$ channels to close and muscle to relax. A characteristic property of these drugs is that they effectively inhibit vascular smooth muscle contraction caused by a moderate increase in the extracellular $K^+$ concentration, being ineffective when the $K^+$ concentration is raised to higher levels. Endothelium-denuded rings were, therefore, contracted with low extracellular $K^+$ concentrations (20–30 mM). Cumulative concentrations of PSS (up to 300 µg/ml) did not affect muscle active tone (Fig. 6.1A). Conversely, the subsequent addition of 100 µM pinacidil, a well-known $K_{ATP}$ channel opener, almost completely reverted $K^+$-induced contraction to 6.5±1.8% of control (n=6), thus demonstrating that PSS is not capable to open $K^+$ channels. In a second series of experiments, the endothelium-derived factor involved in PSS-induced relaxation was investigated. As shown in Fig. 6.1B, the cumulative addition of PSS relaxed endothelium-intact aorta rings pre-contracted with phenylephrine. This effect was suppressed by pre-incubation of tissues with 100 µM L-NAME, a well-known inhibitor of eNOS. The subsequent addition of sodium nitroprusside, a well-known NO-donor, completely reverted phenylephrine-induced contraction (0.0±0.0% of control, n=5), thus confirming that PSS relaxing effect is endothelium-dependent and mediated by eNOS. The production of an endothelium-dependent hyperpolarizing factor can be excluded because its relaxing effect is not modified by NO synthase inhibitors. Furthermore, as PSS-induced relaxation is abolished by the presence of L-NAME, also the production of prostanoids can be ruled out.

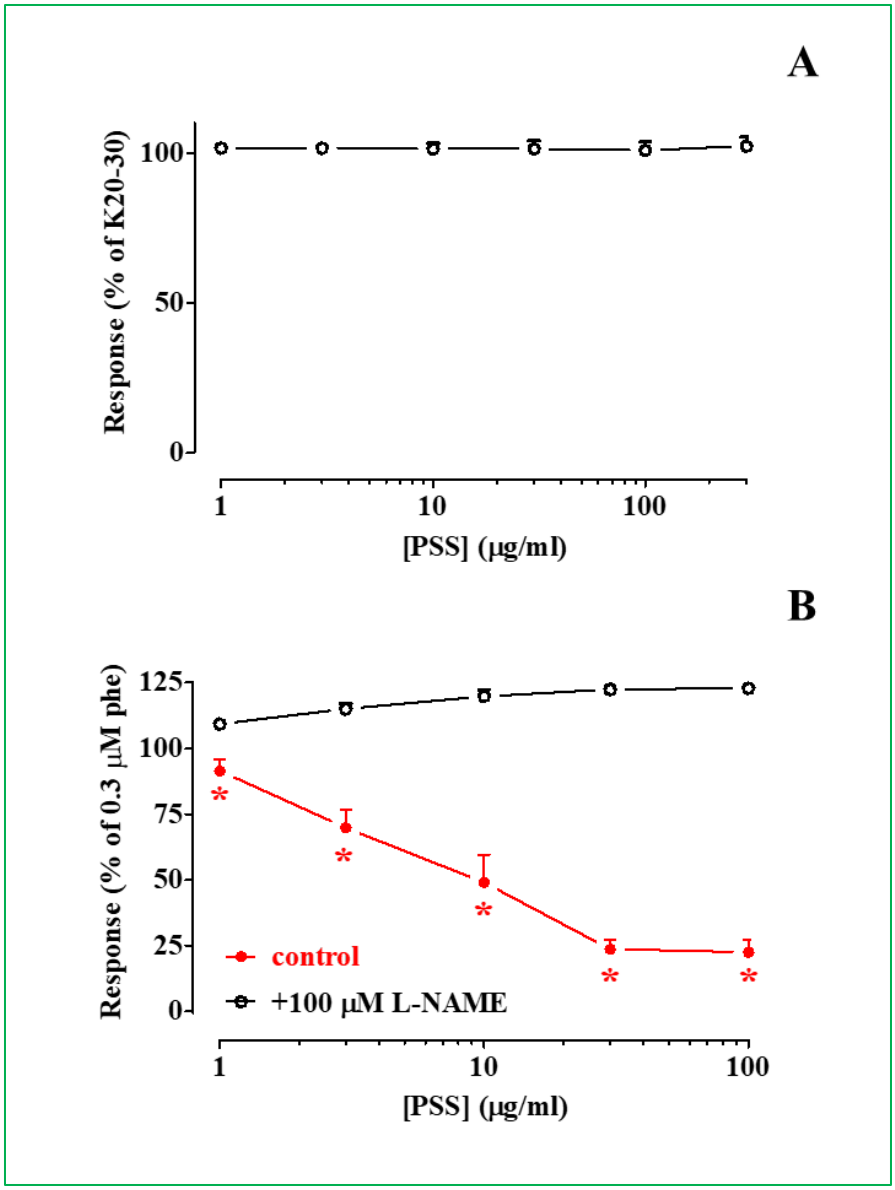



**Figure 6.1.** Effects of PSS on rat aorta rings. Concentration-response curves for PSS recorded either (A) in endothelium-denuded, 20–30 mM $K^+$ or (B) in endothelium-intact, 0.3 µM phenylephrine pre-contracted rings, the latter in the absence or presence of 100 µM L-NAME pre-incubated for 30 min. On the ordinate scale, relaxation is reported as a percentage of the initial tension induced by $K^+$ or phenylephrine (taken as 100%). Data points are means±s.e.m (n=5–8). *P<0.05, Student's t test for unpaired samples.

### 6.1.2. Vasorelaxing Activity of *R*-(−)-3′-Hydroxy-2,4,5-trimethoxy-dalbergiquinol from *Dalbergia tonkinensis*: Involvement of Smooth Muscle $Ca_V1.2$ Channels

Planta Medica, 2020, 86, 284–293 (I.F. 3.352)

Nguyen Manh Cuong[1,2], Ninh The Son[3], Ngu Truong Nhan[2, 4], Pham Ngoc Khanh[1], Tran Thu Huong[1], Nguyen Thi Thu Tram[5], Giampietro Sgaragli[6], **Amer Ahmed[6]**, Alfonso Trezza[7], Ottavia Spiga[7], Fabio Fusi [7]

[1]Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam
[2]Graduate University of Science and Technology, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam
[3]Institute of Chemistry, Vietnam Academy of Science and Technology (VAST), Hanoi, Vietnam
[4]Department of Science and Technology, Tay Nguyen University, Daklak, Vietnam
[5]Department of Chemistry, Faculty of Science, Can Tho University of Medicine and Pharmacy, Can Tho, Vietnam
[6]Dipartimento di Scienze della Vita, Università degli Studi di Siena, Siena, Italy
[7]Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Siena, Italy

**Abstract**

Dalbergia species heartwood, widely used in traditional medicine to treat various cardiovascular diseases, might represent a rich source of vasoactive agents. In Vietnam, *Dalbergia tonkinensis* is an endemic tree. Therefore, the aim of the present work was to investigate the vascular activity of *R*-(−)-3′-hydroxy-2,4,5-trimethoxydalbergiquinol isolated from the heartwood of *D. tonkinensis* and to provide circular dichroism features of its R absolute configuration. The vascular effects of *R*-(−)-3′-hydroxy-2,4,5-trimethoxydalbergiquinol were assessed on the *in vitro* mechanical activity of rat aorta rings, under isometric conditions, and on whole-cell $Ba^{2+}$ currents through $Ca_V1.2$ channels ($I_{Ba1.2}$) recorded in single, rat tail main artery myocytes by means of the patch-clamp technique. *R*-(−)-3′-Hydroxy-2,4,5-trimethoxydalbergiquinol showed concentration-dependent, vasorelaxant activity on both endothelium-deprived and endothelium

intact rings pre-contracted with the α1 receptor agonist phenylephrine. Neither the eNOS inhibitor L-NAME nor the cyclooxygenase inhibitor indomethacin affected its spasmolytic activity. *R*-(−)-3′-Hydroxy-2,4,5 trimethoxydalbergiquinol-induced vasorelaxation was antagonized by (S)-(−)-Bay K 8644 and unaffected by tetraethylammonium plus glibenclamide. In patch-clamp experiments, *R*-(−)-3′-hydroxy-2,4,5-trimethoxydalbergiquinol inhibited $I_{Ba1.2}$ in a concentration-dependent manner and significantly decreased the time constant of current inactivation. *R*-(−)-3′-Hydroxy-2,4,5- trimethoxydalbergiquinol likely stabilized the channel in its closed state, as suggested by molecular modelling and docking simulation to the $Ca_V1.2$ channel α1c subunit. In conclusion, *D. tonkinensis* species may represent a source of agents potentially useful for the development of novel antihypertensive drugs.

**Key words:** *Dalbergia tonkinensis*, Fabaceae, *R*-(−)-3′-hydroxy-2,4,5-trimethoxydalbergiquinol, rat aorta rings, docking simulation, $Ca_V1.2$ channel, open-chain neoflavonoid, patch-clamp

**His contribution consisted in:**

**6.1.2.1 Effect of DBQ on phenylephrine induced contraction**

The vasorelaxant activity of (*R*-(−)-3′-hydroxy-2,4,5-trimethoxydalbergiquinol) DBQ was first assessed on α1 adrenergic receptor-mediated smooth muscle contraction. As shown in Fig. 6.2, DBQ caused a concentration-dependent relaxation of endothelium-deprived rings contracted by 0.3 μM phenylephrine, almost fully reverting the active tone. The $IC_{50}$ value of DBQ was 31.4±8.6 μM (n=6). In a second series of experiments, DBQ was tested on rings with intact-endothelium, contracted by 0.3 μM phenylephrine. DBQ reverted phenylephrine-induced contractions with an $IC_{50}$ value that was not significantly different from that recorded in preparations devoid of endothelium (39.3±5.8 μM, n=7, P>0.05) (Fig. 6.2A,B). When tissues were pre-incubated with eNOS inhibitor L-NAME, DBQ potency (55.9±10.3 μM, n=7, P>0.05) marginally decreased, while efficacy remained unaffected. Finally, the concentration-response curve to DBQ recorded in endothelium-intact rings pre-incubated with indomethacin (32.3±4.6 μM, n=5, P>0.05) overlapped that obtained under control conditions.

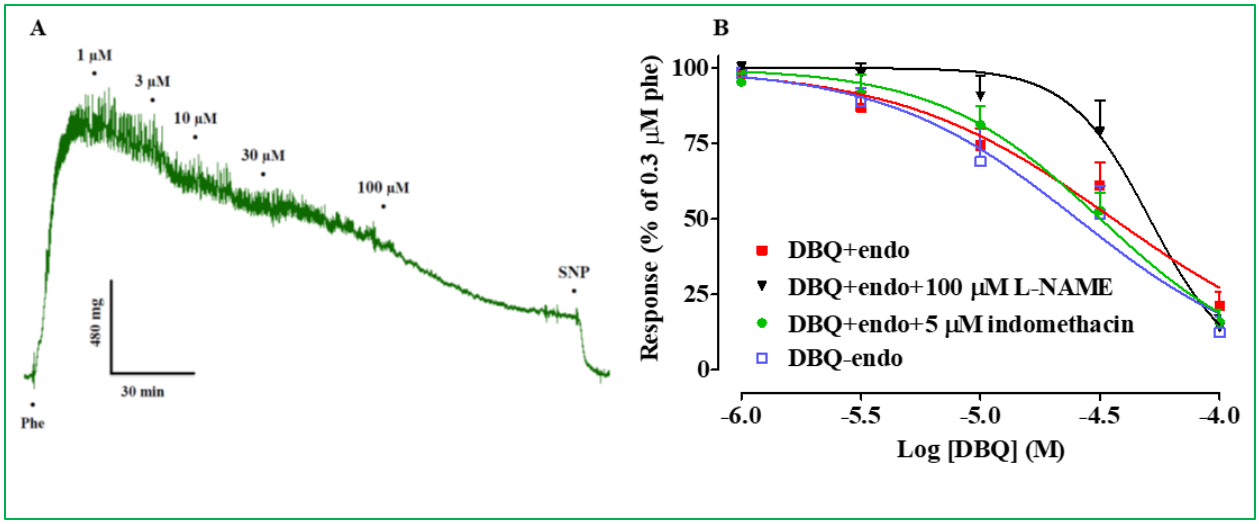



**Figure 6.2.** Effect of DBQ on phenylephrine-induced contraction of rat aorta rings. (A) Trace (representative of 7 similar experiments) of the relaxation developed in response to cumulative concentrations of DBQ, added at the plateau of a 0.3 µM phenylephrine (Phe)-induced contraction in the presence of an intact endothelium. The effect of 100 µM sodium nitroprusside (SNP) is also shown. (B) Concentration-response curves for DBQ recorded in endothelium-intact (+endo) and endothelium-denuded (-endo) rings in the absence or presence of either 100 µM L-NAME or 5 µM indomethacin. Relaxation is reported as a percentage of the initial tension induced by phenylephrine. Data points are the mean±s.e.m. (n=5–7).

#### 6.1.2.2 Effect of DBQ on high KCl induced contraction

To determine the involvement of $Ca_V1.2$ channels in the vascular activity of DBQ, its effect was evaluated on the contraction induced by both 25 mM and 60 mM KCl in endothelium-denuded aorta rings. As shown in Fig. 6.3, DBQ caused a concentration-dependent relaxation of the preparations pre-contracted by 60 mM KCl, with a relative $IC_{50}$ value of 15.5±3.3 µM (n=7), which was 1-fold lower than, but not significantly different from, that recorded in endothelium-deprived rings pre-contracted with phenylephrine (P=0.0941). Full relaxation was nearly attained, at least within the concentration range assessed, and efficacy ($E_{max}$) was 86.5±1.6%. When the rings were depolarized with 25 mM KCl, the concentration-response curve to DBQ was shifted leftward (Fig. 6.3B), though neither potency ($IC_{50}$ value of 7.0±2.1 µM, n=5, P=0.0802 vs. 60 mM KCl) nor efficacy ($E_{max}$ value of 86.6±6.0%, P=0.9793) were significantly different from those observed in the presence of 60 mM KCl. This leftward shift, however, may

suggest a potential activation of $K^+$ channels by DBQ, therefore, a new series of experiments was carried out to define the effect of the drug on those channels that play a fundamental role in the regulation of vascular smooth muscle tone, i.e., $K_{Ca}1.1$, $K_V$, and $K_{ATP}$ channels. As shown in Fig. 6.3B, pre-incubation of the rings with 10 mM tetraethylammonium plus 10 µM glibenclamide did not affect DBQ-induced relaxation ($IC_{50}$ value of 10.6±4.3 µM, n=5, P=0.4816; $E_{max}$ value of 86.2±8.9%, P=0.9683 vs. 25 mM KCl). To examine the role played by $Ca_V1.2$ channels in the vasorelaxant activity of DBQ, rings were contracted by 100 nM (S)-(−)- Bay K 8644 in the presence of 10–15 mM $K^+$. As shown in Fig. 6.3B, the concentration-response curve to DBQ significantly shifted to the right compared to that obtained in the 60 mM $K^+$-depolarized rings. The $IC_{50}$ value (23.5±4.5 µM, n=5, P=0.1729), however, was not significantly affected.

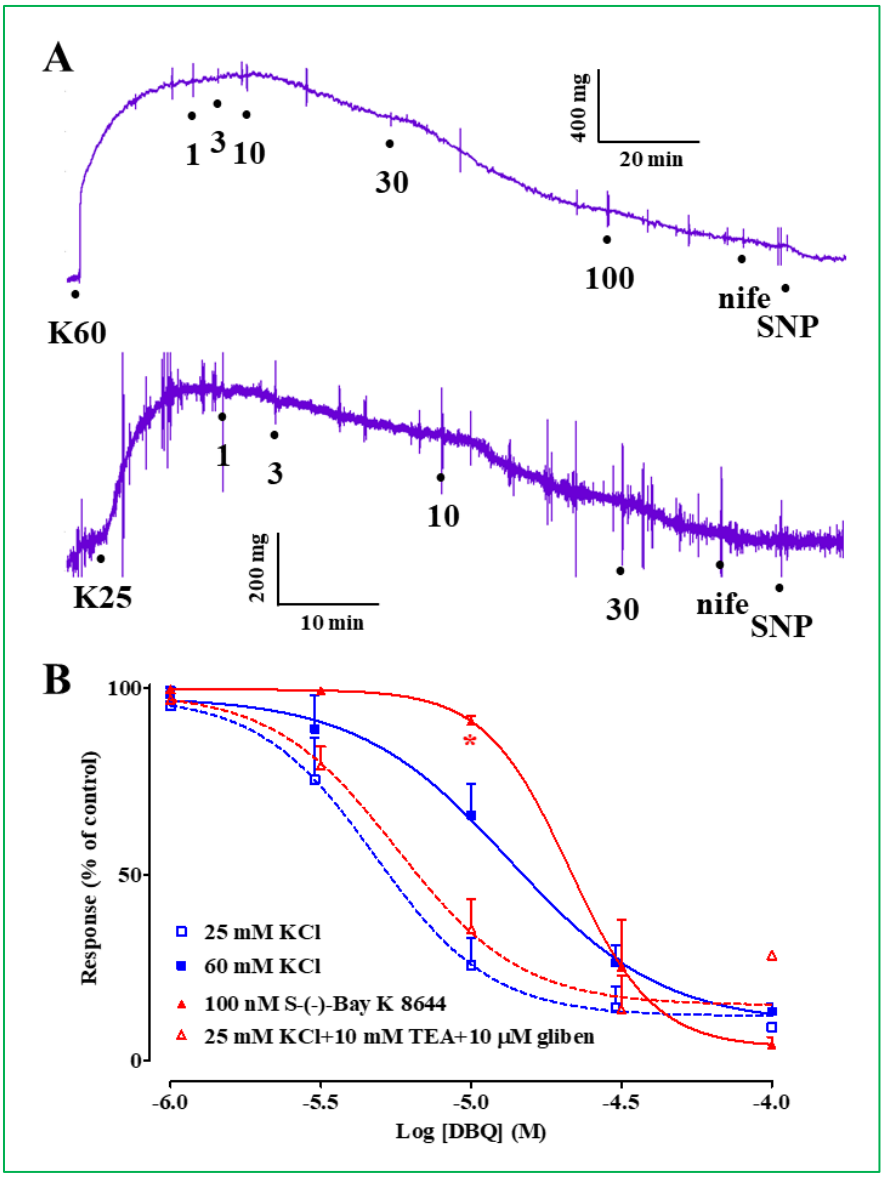


**Figure 6.3.** Effect of DBQ on high $K^+$-induced contraction in endothelium-denuded rat aorta rings. (A) Traces (representative of 5-7 similar experiments) of the relaxation developed in response to cumulative concentrations of DBQ (µM), added at the plateau of 60 mM (K60; upper trace) or 25 mM KCl (K25; lower trace). The effect of 1 µM nifedipine (nife) and 100 µM sodium nitroprusside (SNP) is also shown. (B) Concentration response curves for DBQ constructed in rings depolarized with 25 mM KCl, either in the absence or presence of 10 mM tetraethylammonium (TEA) plus 10 µM glibenclamide (gliben), 60 mM KCl, or stimulated with 100 nM (S)-(−)-Bay K 8644 in the presence of 10–15 mM KCl. Relaxation is reported as percentage of the initial tension induced by the stimulating agent. Data points are the mean±s.e.m. (n=5–7). *P=0.03 vs. 60 mM KCl, Student's t test for unpaired samples.

### 6.1.2.3 Effect of DBQ on $CaCl_2$ response curve

Furthermore, 20 µM DBQ caused a rightward, non-parallel shift of the concentration-response curve to $Ca^{2+}$ in preparations depolarized by 60 mM $K^+$ (Fig. 6.4).

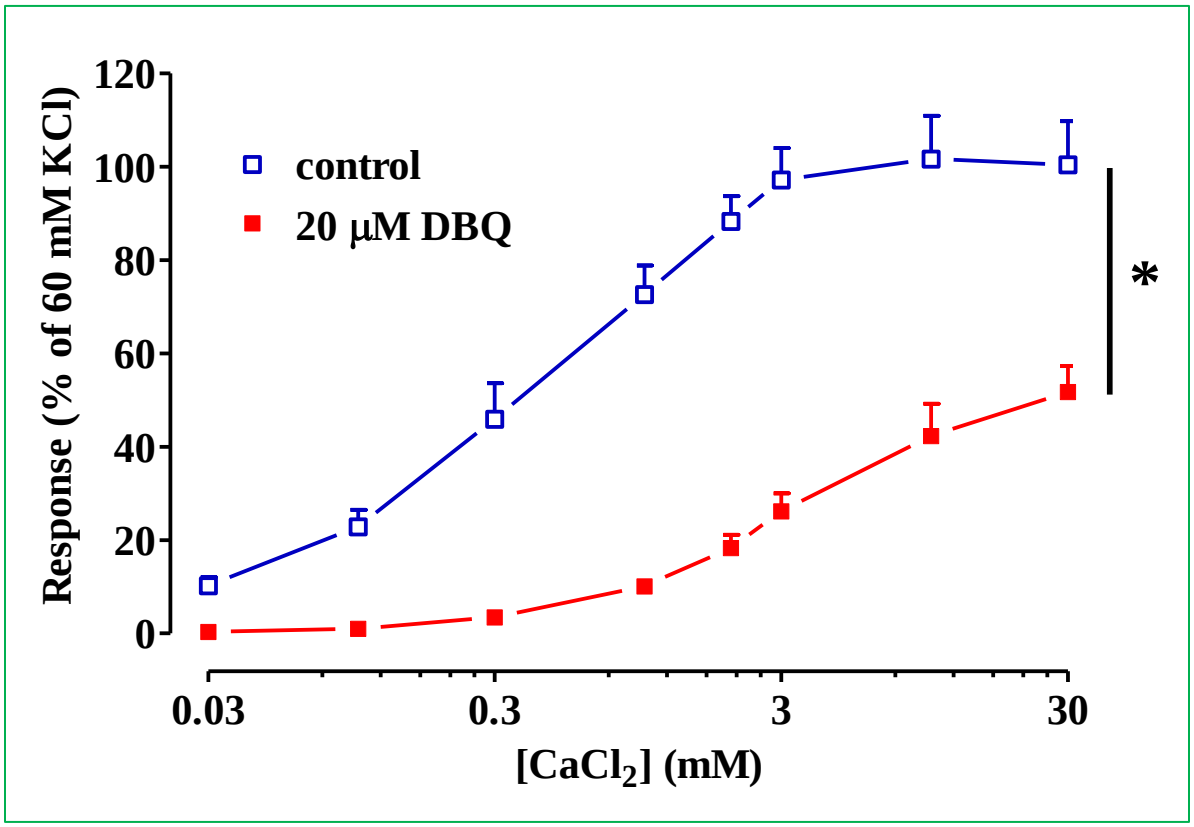


**Figure 6.4.** Effect of DBQ on the concentration-response curve to $Ca^{2+}$ in endothelium-denuded rat aorta rings. Concentration-response curves for $CaCl_2$ constructed in rings depolarized with a 60 mM KCl, $Ca^{2+}$-free PSS in the presence of either DMSO (control) or 20 µM DBQ. Contraction is reported as percentage of the tension induced by 60 mM KCl in the ring functional assay. Data points are the mean±s.e.m (n=3–5). *P<0.0001, two-way ANOVA.

### 6.1.3 Vasorelaxing Activity of Stilbenoid and Phenanthrene Derivatives from *Brasiliorchis porphyrostele*: Involvement of Smooth Muscle $Ca_V1.2$ Channel

Planta Medica, 2020, 86, 631–642, (I.F. 3.352)

Watcharee Waratchareeyakul[1, 2], Fabio Fusi[3], Miriam Durante[4], **Amer Ahmed[4]**, Walter Knirsch[5], Eduard Mas-Claret[1], Dulcie A. Mulholland[1, 6]

[1]Natural Products Research Group, Department of Chemistry, University of Surrey, Guildford, United Kingdom

[2]Department of Chemistry, Rambhai Barni Rajabhat University, Chanthaburi, Thailand

[3]Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, Siena, Italy

[4]Dipartimento di Scienze della Vita, Università degli Studi di Siena, Siena, Italy

[5]Institute of Plant Sciences, Karl-Franzens-University, Graz, Austria

[6]School of Chemistry and Physics, University of KwaZulu-Natal, Durban, South Africa

**ABSTRACT**

Five compounds, 3,4′-dihydroxy-3′,5,5′-trimethoxydihydro-stilbene, **1**; 3,4′-ihydroxy-3′,5′-dimethoxydihydrostilbene, **2**; 3,4′-dihydroxy-5,5′-dimethoxydihydrostilbene, **3**; 9,10-dihydro-2,7-dihydroxy-4,6-dimethoxyphenanthrene, **4**; and the previously unreported 1,2,6,7-tetrahydroxy-4-methoxyphenanthrene, **5** were isolated from the South American orchid, *Brasiliorchis porphyrostele*. An in-depth analysis of their vascular effects was performed on *in vitro* rat aorta rings and tail main artery myocytes. Compounds **1**–**4** were shown to possess vasorelaxant activity on rings pre-contracted by the α1 receptor agonist phenylephrine, the $Ca_V1.2$ stimulator (S)-(−)-Bay K 8644, or depolarized with high $K^+$ concentrations. However, compound **5** was active solely on rings stimulated by 25 mM but not 60 mM $K^+$. The spasmolytic activity of compounds **1** and **4** was significantly affected by the presence of an intact endothelium. The $K_{ATP}$ channel blocker glibenclamide and the $K_V$ channel blocker 4-aminopyridine significantly antagonized the vasorelaxant activity of compounds **4** and **1**, respectively. In patch-clamp experiments, compounds **1**–**4** inhibited $Ba^{2+}$ current through $Ca_V1.2$ channels in a concentration-dependent manner, whereas neither compound **4** nor compound **1** affected $K^+$ currents through $K_{ATP}$ and $K_V$ channels, respectively. The present *in vitro*,

comprehensive study demonstrates that *Brasiliorchis porphyrostele* may represent a source of vasoactive agents potentially useful for the development of novel antihypertensive agents that have now to be validated *in vivo* in animal models of hypertension.

**Key words:** *Brasiliorchis porphyrostele*, phenanthrenes, stilbenoids, vasorelaxing, vascular $Ca_V1.2$ channel, vascular endothelium, Orchidaceae

**His contribution consisted in:**

**6.1.3.1 Effect of compounds 1-5 on phenylephrine-induced contraction**

As shown in Fig. 6.5A, compounds **1**–**4** caused a concentration-dependent relaxation of endothelium-denuded rings contracted by 0.3 µM phenylephrine. Compound **4** showed the greatest efficacy, almost fully reverting phenylephrine-induced contraction. $pIC_{50}$ values of the 4 compounds ranged between 4.30 and 4.58 (50 µM and 26 µM, respectively). However, compound **5** was ineffective. In a second series of experiments, compounds **1**–**5** were tested on rings with intact endothelium contracted by 0.3 µM phenylephrine. Compounds **2** and **3** reverted phenylephrine-induced contractions with $pIC_{50}$ values not significantly different from those recorded in preparations devoid of endothelium. As the endothelium was not involved in their vasorelaxant activity, compounds **2** and **3** were not further investigated in this particular experimental setting. However, compound **1** was found to be less potent and compound **4** more potent, with an intact endothelium. Even in the presence of an intact endothelium, compound **5** was still ineffective. To elucidate the role played by the endothelium in the vasorelaxation caused by compounds **1** and **4**, the concentration-response curve was repeated in rings pre-incubated with either 5 µM indomethacin (to investigate the conceivable involvement of cyclooxygenase-derived vasoconstrictors) or 100 µM L-NAME (to investigate the conceivable involvement of endothelium-derived vasodilators such as NO), respectively. As shown in Fig. 6.5B, compound **1**-induced spasmolysis was markedly augmented, though not significantly, in the presence of indomethacin. However, pre-treatment with L-NAME significantly antagonized the relaxation caused by compound **4**. These results obtained on aorta ring preparations stimulated with phenylephrine (pharmaco-mechanical contraction) provided important information on the mechanism of action of compounds **1**–**4**. In particular, endothelium-derived vasodilators (e.g., NO) or vasoconstrictors (e.g., cyclooxygenase-derived) seem to be involved

only in compounds **4** and **1** vasoactivity, respectively, while compounds **2** and **3** relaxed both endothelium-intact and endothelium-denuded rings with similar potency.

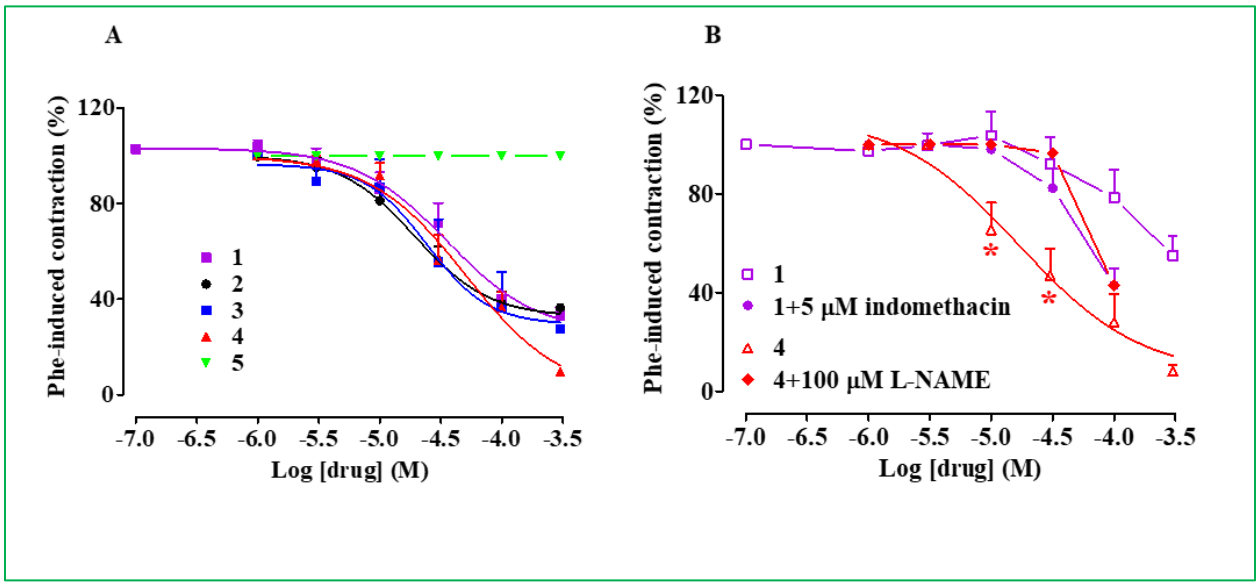



**Figure 6.5.** Effects of compounds **1**–**5** on phenylephrine-induced contraction in rat aorta rings. (A) Concentration-response curves for compounds **1**–**5** in endothelium-denuded rings pre-contracted by 0.3 µM phenylephrine. Steady tension was evoked by phenylephrine, and then each compound was added cumulatively. In the ordinate scale, response is reported as percentage of the initial tension induced by 0.3 µM phenylephrine (phe). (B) Concentration-response curves for compounds **1** and **4** in endothelium-intact rings pre-contracted by 0.3 µM phenylephrine, in the absence or presence of either 5 µM indomethacin or 100 µM L-NAME, pre-incubated for 20 min and 30 min, respectively, and left in contact with the preparation throughout the experiments. Data points are mean±s.e.m (n=3–16). *P<0.05 vs. L-NAME, Student's t-test for unpaired samples.

### 6.1.4 Vasorelaxant Effects Induced by Red Wine and Pomace Extracts of Magliocco Dolce *cv*.

Pharmaceuticals, 2020, 13, 87, (IF=5.856)

Gabriele Carullo[1], **Amer Ahmed[2]**, Fabio Fusi[3], Fabio Sciubba[4], Maria Enrica Di Cocco[4], Donatella Restuccia[1], Umile Gianfranco Spizzirri[1], Simona Saponara[2,] *****, Francesca Aiello[1,] *****

[1]Department of Pharmacy, Health and Nutritional Sciences, Department of Excellence 2018-2022, University of Calabria, Ed. Polifunzionale, 87036 Rende (CS), Italy
[2]Department of Life Sciences, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy
[3]Department of Biotechnology, Chemistry and Pharmacy, Department of Excellence 2018-2022, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy
[4]Department of Chemistry, University of Rome La Sapienza, Piazzale Aldo Moro 5, 00185 Roma, Italy

**Abstract**

Several epidemiological studies demonstrate that moderate (red) wine consumption may afford protection against cardiovascular diseases. Protection is ascribed to the biological activity of wine components, many of which, however, are discarded during winemaking. *In vitro* rat thoracic aorta rings contracted with phenylephrine or KCl were used to assess the vasorelaxant activity of extracts from wine pomaces (seeds and skins) of the Calabrian autochthonous grape variety Magliocco dolce (Arvino). NMR spectroscopy was used to ascertain their chemical composition. Data demonstrate that seed and skin, but not must, extracts are capable of relaxing vascular preparations in an endothelium-dependent manner, similarly to the red wine extract, due to the presence of considerable amounts of bioactive constituents. In rings pre-contracted with 20–30 mM KCl, only seed extracts showed a moderate relaxation. The most efficacious vasodilating extract (wine) showed a good antioxidant profile in both [(2,2-diphenyl-1-picrylhydrazyl)acid] radical (DPPH) and [2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] radical (ABTS) assays. In conclusion, winemaking from Magliocco dolce grape can provide potentially health-promoting by-products useful in cardiovascular disease management.

**His contribution consisted in:**

**6.1.4.1 Effect of LDC extracts on phenylephrine-induced contraction**

In rings with an intact endothelium, all LDC extracts except LDC4 caused a concentration dependent relaxation of phenylephrine-induced contraction. $E_{max}$ values were 58.1±12.1% (1 mg/mL LDC1, n=5), 65.8±5.8% (0.3 mg/mL LDC2, n=7), 80.6±5.3% (0.1 mg/mL LDC3, n=6), 79.8±3.6% (10 µg/mL LDC5, n=6), and 76.6 ± 5.1% (10 µg/mL LDC6, n=7) (Fig. 6.6A–F). Skin extracts LDC1 and LDC2 showed a biphasic relaxing behaviour, with a primary activity plateauing, and a secondary effect starting at concentrations of 300 µg/mL and 100 µg/mL, respectively (Fig. 6.6A,B). Seed extracts LDC5 and LDC6 were the most potent of the series ($IC_{50}$ values of 1.7±0.3 µg/mL, n=6, and 1.3±0.3 µg/mL, n=7, respectively; Fig. 6.6) but, at concentrations >10 µg/mL, they showed a hormetic behaviour giving rise to vessel contraction. Finally, even the lyophilized red wine LDC3 markedly relaxed aortic rings with an $IC_{50}$ value of 39.1±13.9 µg/mL (n=6). In endothelium-deprived preparations, LDC extracts did not cause vasorelaxation; only LDC5 and LDC6, at the maximal concentration assessed (1 mg/mL), significantly reverted phenylephrine-induced active tone ($E_{max}$ of 83.1±4.1% and 83.5±5.3%, respectively, n=5; Fig. 6.6E,F). The three most interesting extracts of the series (i.e., effective and devoid of a hormetic behaviour), namely LDC1-LDC3, were further investigated in order to shed light on the mechanism underpinning their endothelium-dependent antispasmodic activity. Pre-exposure of endothelium-intact rings to L-NAME antagonized the vasorelaxant activity of LDC1, LDC2, and LDC3 (Fig. 6.6A–D). Finally, vasorelaxation induced by the most effective mixture, i.e., LDC3, was assessed in the presence of a TRPV1 channel blocker: capsazepine did not affect LDC3-induced spasmolysis in endothelium-intact preparations (Fig. 6.7).

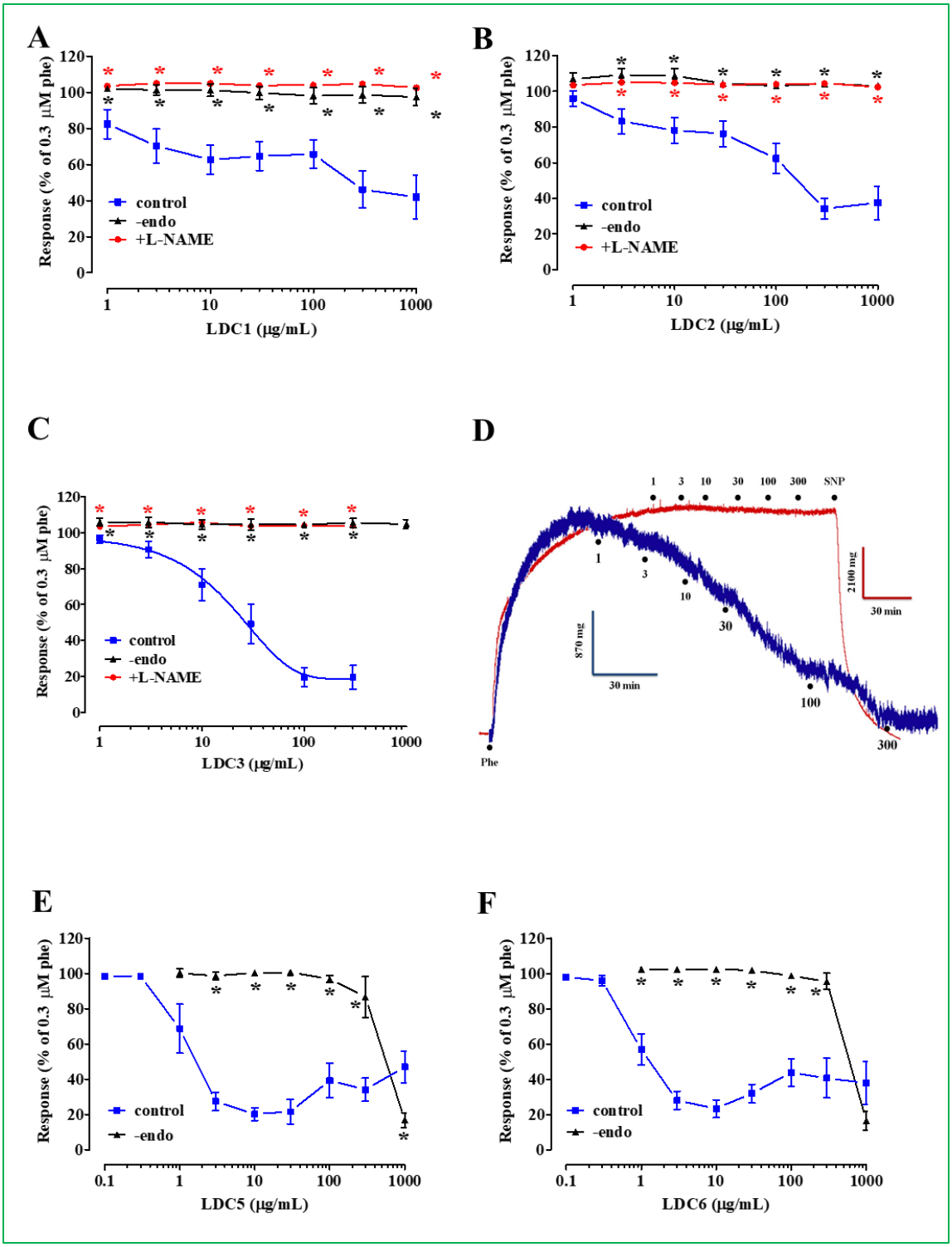



**Figure 6.6.** Effects of LDC extracts on phenylephrine-induced contraction in either endothelium-intact or deprived rat aorta rings. Endothelium-intact (control) or -denuded (-endo) rings, pre-contracted by 0.3 µM phenylephrine, were challenged with cumulative concentrations of (A) LDC1, (B) LDC2, (C) LDC3, (E) LDC5, and (F) LDC6. Some

experiments were performed in endothelium-intact rings pre-incubated with 100 µM L-NAME. (D) Traces representative of 5–6 similar experiments, showing the effects of LDC3 on endothelium-intact rings in the absence (blue trace) or presence of 100 µM L-NAME (red trace). In the ordinate scale, relaxation is reported as percentage of the initial tension induced by phenylephrine (Phe). In the abscissa, the concentration of each drug (as specified in the legend to symbols) is reported in µg/ml. Data points represent mean±s.e.m. (n=4–8). *P<0.05 vs control, one-way ANOVA or Student's t test for unpaired samples.

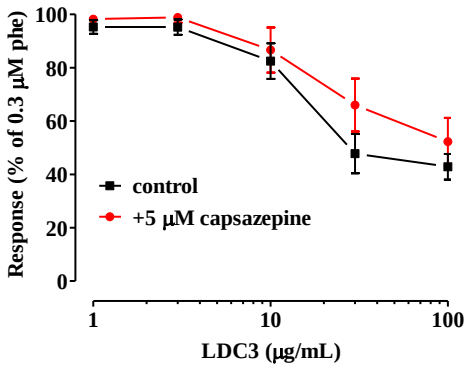



**Figure 6.7.** Effects of capsazepine on LDC3-induced relaxation. Endothelium-intact rings, pre-contracted by 0.3 µM phenylephrine, were challenged with cumulative concentrations of LDC3, in the absence (control) or presence of 5 µM capsazepine. In the ordinate scale, relaxation is reported as percentage of the initial tension induced by phenylephrine (Phe). In the abscissa, the concentration of drug (as specified in the legend to symbols) is reported in µg/ml. Data points represent mean±s.e.m. (*n* = 6).

### 6.1.4.2 Effects of LDC extracts on high KCl-induced contraction

The cumulative addition of the seed extracts LDC5 and LDC6 to endothelium-denuded rings depolarized with 20–30 mM KCl caused a modest decrease of the active tension ($E_{max}$ values of 42.1±18.2% and 47.3±15.5%, respectively; n=6; Fig. 6.8). The remaining extracts did not affect this contraction up to the maximal concentration assessed in the present work (Fig. 6.8). Contrary to LDC extracts showing no relaxing activity, the addition of 100 µM pinacidil completely reverted vessel tone to basal level (Fig. 6.8B).

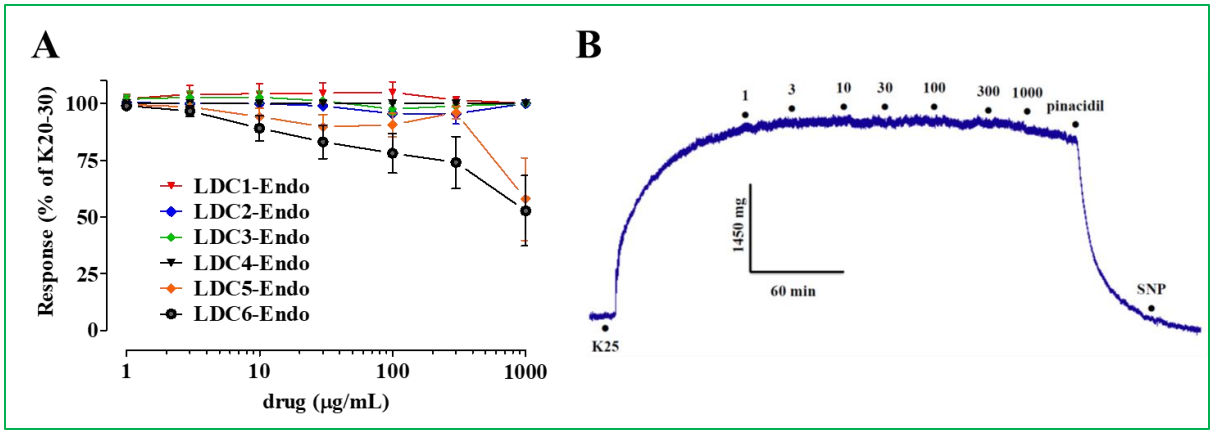



**Figure 6.8.** Effect of LDC extracts on high KCl-induced contraction of rat aorta rings. (A) Concentration-response curves for LDC extracts were constructed on endothelium-denuded preparations pre-contracted by 20–30 mM KCl. In the ordinate scale, relaxation is reported as percentage of the initial tension induced by KCl, taken as 100%. In the abscissa, the concentration of each drug (as specified in the legend to symbols) is reported in µg/mL. Data point represent mean±s.e.m. (n=4–6). (B) Trace representative of 4 similar experiments, showing the effects of LDC1 (µg/mL) on an endothelium-denuded ring pre-contracted by 25 mM KCl (K25). The effect of 100 µM pinacidil and 100 µM sodium nitroprusside (SNP) is also shown.

### 6.1.5 Design, synthesis and pharmacological evaluation of ester-based quercetin derivatives as selective vascular $K_{Ca}1.1$ channel stimulators

Bioorganic Chemistry, 105, 2020, 104404 (I.F. 5.275)

Gabriele Carullo[a,b], **Amer Ahmed**[c], Alfonso Trezza[b], Ottavia Spiga[b], Antonella Brizzi[b], Simona Saponara[c,*], Fabio Fusi[b,*] , Francesca Aiello[a]

[a]Department of Pharmacy, Health and Nutritional Sciences, DoE 2018-2022, University of Calabria, Edificio Polifunzionale, 87036 Rende (CS), Italy
[b]Department of Biotechnology, Chemistry and Pharmacy, DoE 2018-2022, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy
[c]Department of Life Sciences, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy

**Abstract**

Quercetin represents one of the most studied dietary flavonoids; it exerts a panel of pharmacological activities particularly on the cardiovascular system. Stimulation of vascular $K_{Ca}1.1$ channels contributes to its vasorelaxant activity, which is, however, counteracted in part by its concomitant stimulation of $Ca_V1.2$ channels. Therefore, several quercetin hybrid derivatives were designed and synthesized to produce a more selective $K_{Ca}1.1$ channel stimulator, then assessed both *in silico* and *in vitro*. All the derivatives interacted with the $K_{Ca}1.1$ channel with similar binding energy values. Among the selected derivatives, **1E** was a weak vasodilator, though displaying an interesting $Ca_V1.2$ channel blocking activity. The lipoyl derivatives **1F** and **3F**, though showing pharmacological and electrophysiological features similar to those of quercetin, seemed to be more effective as $K_{Ca}1.1$ channel stimulators as compared to the parent compound. The strategy pursued demonstrated how different chemical substituents on the quercetin core can change/invert its effect on $Ca_V1.2$ channels or enhance its $K_{Ca}1.1$ channel stimulatory activity, thus opening new avenues for the synthesis of efficacious vasorelaxant quercetin hybrids.

**Keywords:** $Ca_V1.2$ channel, $K_{Ca}1.1$ channel, Molecular docking, Quercetin, Vasoactivity, Lipoic acid, Hypertension, Ester-based derivatives

**His contribution consisted in:**

**6.1.5.1 Pharmacological evaluation of quercetin and its derivatives on phenylephrine- and high KCl-induced contraction**

In a first series of experiments, **1E**, **1F**, and **3F** were tested on rings pre-contracted with high KCl, which depolarizes the membrane, thus causing $Ca_V1.2$ channels to open. Quercetin was also assessed as positive control. In rings depolarized with 25 mM KCl, **1F**, **3F**, and quercetin caused a concentration dependent relaxation of equal potencies and efficacy (Fig. 6.9A). However, **1E** was only partially effective, showing a relaxant effect of 27.5±6.3% (n=4). In preparations depolarized with 60 mM KCl, **1F** and quercetin showed a vasorelaxant activity superimposable to that recorded on 25 mM KCl-stimulated rings (Fig. 6.9B). The spasmolytic activity of **3F** was significantly greater than that of the parent compound quercetin. Furthermore, **1E** efficacy was further reduced. High KCl-induced contraction is essentially due to the opening of $Ca_V1.2$ channels and the ensuing $Ca^{2+}$ influx from the extracellular space. In particular, the two KCl concentrations employed here (namely 25 mM and 60 mM) represented standardized experimental settings to detect $K^+$ channel openers and $Ca^{2+}$ antagonists, respectively. In fact, the former should be more efficacious on 25 mM KCl-induced contraction whereas the latter on that induced by 60 mM KCl. As no significant differences were observed between the two experimental settings, it can be hypothesized that the vasorelaxant activity of quercetin and its derivatives cannot be ascribed only to a $Ca_V1.2$ channel blockade or to a $K_{Ca}1.1$ channel stimulation. Of note two findings: the weak myorelaxant activity displayed by **1E** and the higher efficacy of **3F** as compared to quercetin in rings depolarized with 60 mM KCl. The effects of **1E**, **1F**, and **3F** were further investigated on rings precontracted with the α-adrenergic receptor agonist phenylephrine. Fig. 6.9C shows that **3F** and quercetin relaxed aorta ring preparations in a concentration-dependent manner, with a similar pattern of potency and efficacy. **1F** was less active, whereas **1E** spasmolytic activity accounted for a mere 10% relaxation at the maximal concentration assessed. Phenylephrine-induced contraction is the result of $Ca^{2+}$ release from the sarcoplasmic reticulum triggered by inositol trisphosphate ($IP_3$) and $Ca^{2+}$ influx from the extracellular space through receptor-, store-operated, and $Ca_V1.2$ channels.

Therefore, quercetin and **3F**, **1F** to a lesser extent, but not **1E**, likely affect one or more of these pathways leading to vasorelaxation. Taken together, aorta ring findings suggest that the introduction of a lipoyl moiety in C-7 position leaves unaltered or even improves quercetin vasorelaxant activity, whereas that in C-4′ position does not change or slightly reduces it. The latter observation is further supported by the marked reduction of vasorelaxant activity characterizing **1E** that bears a 3,4-methyelenedioxycynnamoyl moiety in C-4′ position.

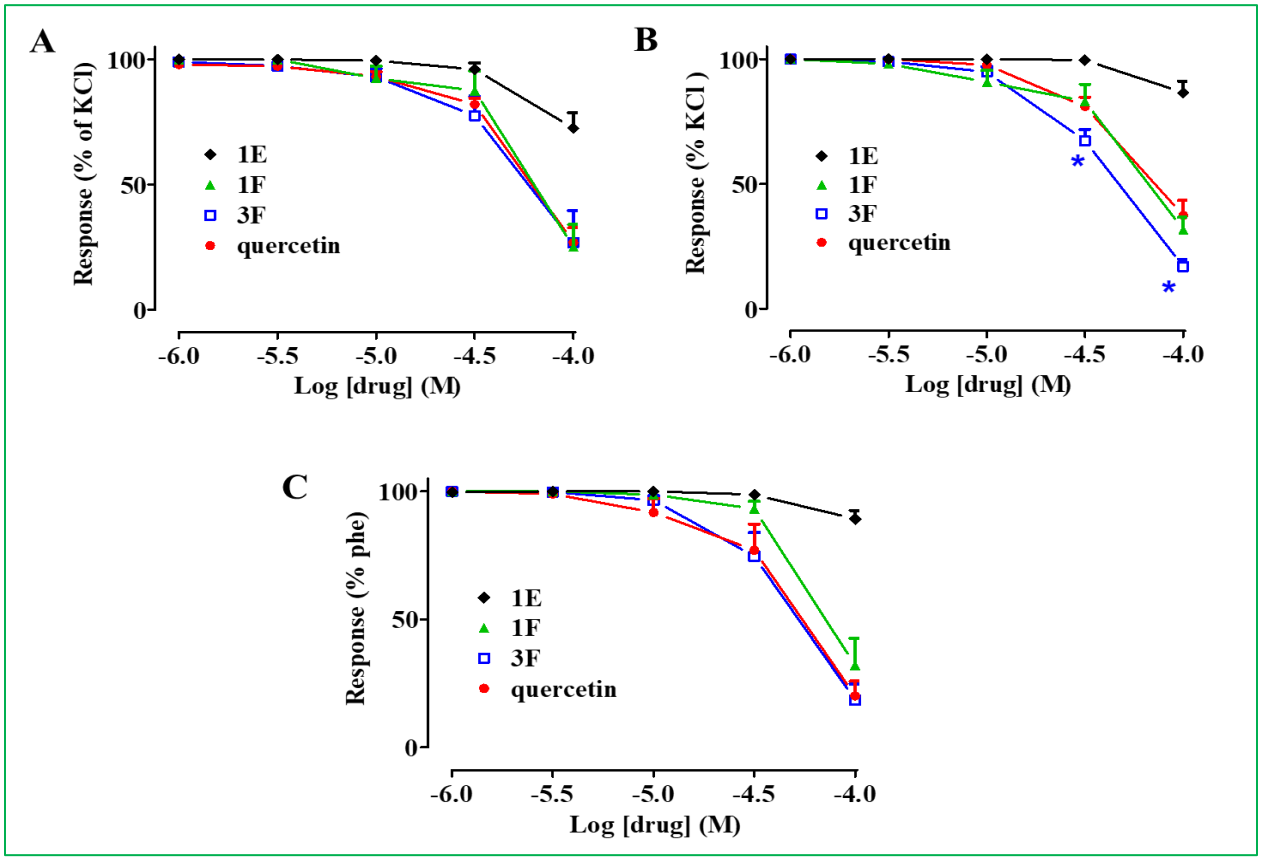



**Figure 6.9.** Effects of quercetin and its derivatives on rat aorta rings. Endothelium-deprived rings were pre-contracted with (A) 25 mM KCl (K25), (B) 60 mM KCl (K60), or (C) 0.3 µM phenylephrine (phe). On the plateau of the contraction, cumulative concentrations of quercetin, **1E**, **1F** or **3F** were added. On the ordinate axis, muscle tension is reported as percentage of the initial tone evoked by either KCl or phenylephrine. Data points represent mean±s.e.m. (n=4–6). *P<0.05 vs. quercetin, Student's t test for unpaired samples.

### 6.1.6 A multitarget semi-synthetic derivative of the flavonoid morin with improved *in vitro* vasorelaxant activity: Role of $Ca_V1.2$ and $K_{Ca}1.1$ channels

Biochemical Pharmacology, 2020, 185, 114429 (I.F. 5.858)

Gabriele Carullo[a,b], **Amer Ahmed**[c], Alfonso Trezza[a], Ottavia Spiga[a], Antonella Brizzi[a], Simona Saponara[c],*, Fabio Fusi[a],*, Francesca Aiello[b]

[a]Department of Biotechnology, Chemistry and Pharmacy, DoE 2018-2022, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy

[b]Department of Pharmacy, Health and Nutritional Sciences, DoE 2018-2022, University of Calabria, Edificio Polifunzionale, 87036 Arcavacata di Rende, CS, Italy

[c]Department of Life Sciences, University of Siena, Via Aldo Moro 2, 53100 Siena, Italy

**Abstract**

$Ca_V1.2$ channels play a fundamental role in the regulation of vascular smooth muscle tone. The aim of the present study was to synthesize morin derivatives bearing the nitrophenyl moiety of dihydropyridine $Ca^{2+}$ antagonists to increase the flavonoid vasorelaxant activity. The effects of morin and its derivatives were assessed on $Ca_V1.2$ and $K_{Ca}1.1$ channels, both *in vitro* and *in silico*, as well as on the contractile responses of rat aorta rings. All compounds were effective $Ca_V1.2$ channel blockers, positioning in the $\alpha_{1C}$ subunit region where standard blockers bind. Among the four newly synthesized morin derivatives, the penta-acetylated morin-1 was the most efficacious $Ca^{2+}$ antagonist, presenting a vasorelaxant profile superior to that of the parent compound and, contrary to morin, antagonized also the release of $Ca^{2+}$ from the sarcoplasmic reticulum; surprisingly, it also stimulated $K_{Ca}1.1$ channel current. Computational analysis demonstrated that morin-1 bound close to the $K_{Ca}1.1$ channel S6 segment. In conclusion, these findings open a new avenue for the synthesis of valuable multi-functional, vasorelaxant morin derivatives capable to target several pathways underpinning the pathogenesis of hypertension.

**His contribution consisted in:**

#### 6.1.6.1 Effects of morin and its derivatives on high $K^+$-induced contraction

In this series of experiments, the vasorelaxant activity of morin and its derivatives was assessed on rings pre-contracted by high KCl concentrations, which cause membrane depolarization and

$Ca_V1.2$ channel opening. In aorta preparations depolarized by 60 mM KCl, morin-1 and morin-2 caused a concentration-dependent relaxation (Table 1; Fig. 6.10A), whilst the parent compound morin had only weak inhibitory effects.

**Table 6.1.** Effects of morin and its derivatives on high $K^+$- or phenylephrine-induced contractions in rat aorta rings

| Drug | $K^+$-induced contraction | | | | | | Phenylephrine-induced contraction | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 25 mM KCl (-endothelium) | | | 60 mM KCl (-endothelium) | | | +endothelium | | | -endothelium | | |
| | $IC_{50}$ (μM) | $E_{max}$ | n | $IC_{50}$ (μM) | $E_{max}$ | n | $IC_{50}$ (μM) | $E_{max}$ | n | $IC_{50}$ (μM) | $E_{max}$ | n |
| **Morin** | N.D. | -28.2±6.6% | 5 | N.D. | -35.7±6.6% | 4 | N.D. | -34.4±4.7% | 5 | N.D. | -46.6±8.5% | 5 |
| **Morin-1** | 7.1±2.1* | -99.6±0.2% | 6 | 28.3±5.7 | -94.2±2.7% | 5 | 20.9±4.6 | -95.5±1.4% | 5 | 17.8±3.4 | -94.8±1.4% | 5 |
| **Morin-2** | 18.2±6.0* | -98.8±0.4% | 6 | 47.3±4.9 | -83.6±4.6% | 5 | 32.4±7.8 | -84.4±6.4% | 5 | 27.5±7.5 | -85.3±5.2% | 6 |
| **Morin-4** | N.D. | -2.2±2.2% | 3 | N.D. | -0.4±3.8% | 3 | N.D. | +55.3±16.0% | 3 | N.D. | +53.9±36.5% | 3 |

Rings, endothelium-denuded (-endothelium) or -intact (+endothelium), were contracted by 25 mM, 60 mM KCl or 0.3 μM phenylephrine. Potency ($IC_{50}$) and efficacy ($E_{max}$) are reported as mean±s.e.m and n represent the number of independent replicates. N.D.: Not detected. *P<0.05 vs 60 mM KCl, Student's *t* test for unpaired samples.

Additionally, morin-1 and morin-2 fully relaxed rings stimulated by 25 mM KCl, the former being apparently more potent than the latter compound (Table 6.10; Fig. 6.10B). Noticeably, both derivatives were significantly more potent on 25 mM KCl- than on 60 mM KCl-induced contraction; the parent compound morin weakly antagonized the former contraction, while morin-4 was almost devoid of effects on both KCl-induced contractions. These functional experiments indicated that morin-1 behaves either as a $Ca^{2+}$ antagonist or as a $K^+$ channel opener (being more active on 25 mM KCl- as compared to 60 mM KCl-induced contraction). Hence, its activity was assessed on rings incubated simultaneously with the $K^+$ channel blocker TEA (10 mM, a concentration known to block almost all the $K^+$ channels) and the $Ca_V1.2$ channel stimulator Bay K 8644. Under this experimental condition, both the efficacy (-60.6±7.9%, n=8; P<0.05 vs 60 mM KCl, see Table 6.1) and potency (83.1±11.7 μM, n=7; P<0.05) of morin-1 were significantly reduced.

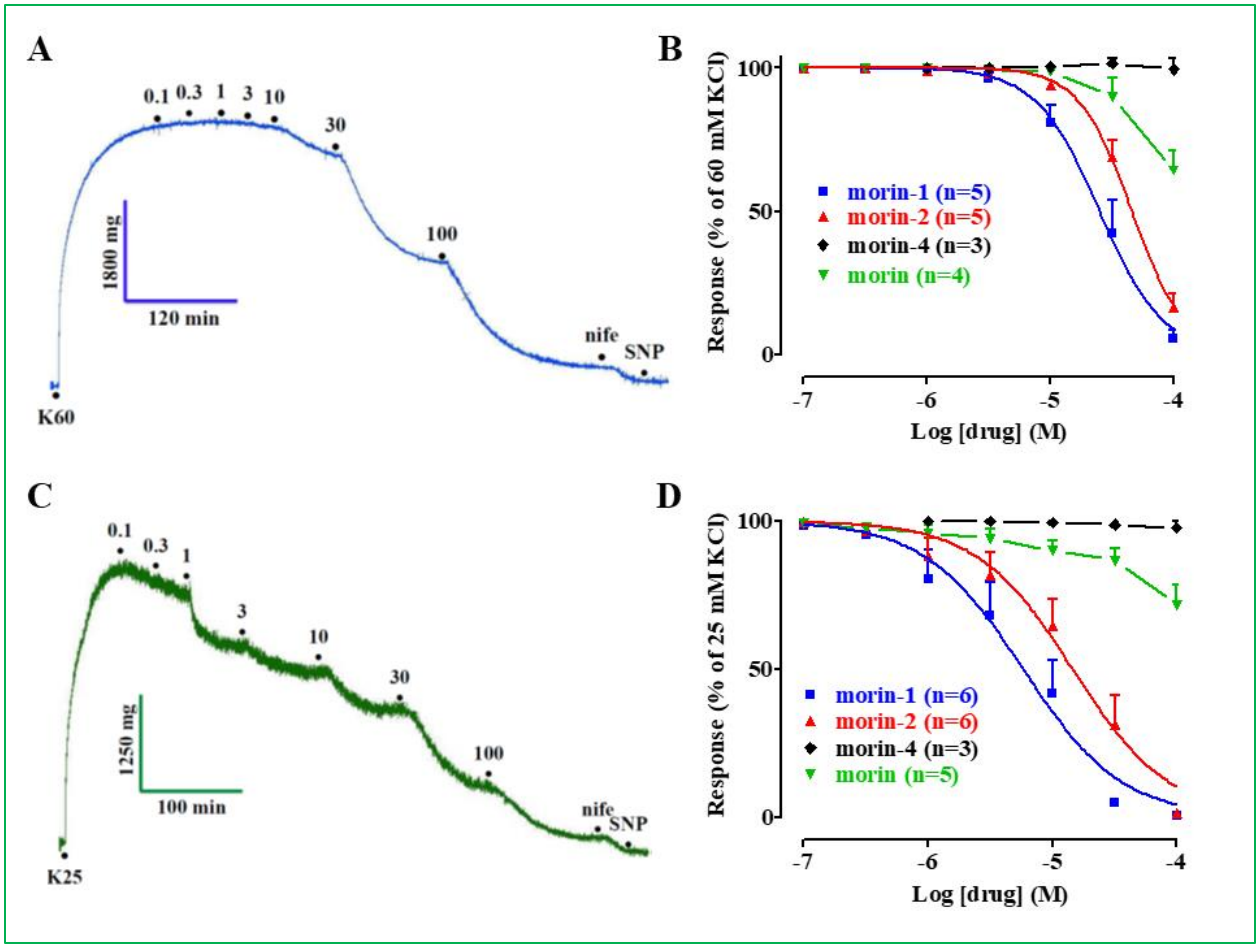



**Figure 6.10.** Effects of morin and its derivatives on high KCl-induced contraction of rat aorta rings. (A, C) Traces (representative of 5-6 similar experiments) of the relaxation developed in response to cumulative concentrations of morin-1 (μM), added at the plateau of (A) 60 mM (K60) or (C) 25 mM KCl (K25). The effect of 10 μM nifedipine (nife) and 100 μM sodium nitroprusside (SNP) is also shown. (B, D) Concentration-response curves for morin-1, morin-2, morin-4, and morin in endothelium-denuded rings pre-contracted with either (B) 60 mM KCl or (D) 25 mM KCl. In the ordinate scale, relaxation is reported as percentage of the initial tension induced by high $K^+$ (60 mM KCl 1895±137 mg, n=17; 25 mM KCl 1500±112 mg, n=20). Data points represent the mean±s.e.m.

### 6.1.6.2 Effects of morin and its derivatives on phenylephrine-induced contraction

This series of experiments was performed to investigate the effect of drugs on pharmaco-mechanical coupling. In endothelium-intact rings, both morin-1 and morin-2 reverted the $\alpha_1$ adrenergic receptor agonist phenylephrine-induced contraction in a concentration-dependent

manner (Table 6.1; Fig. 6.11A, B). Endothelium removal did not significantly affect either potency or efficacy (Table 6.1; Fig. 6.11). The parent compound morin weakly relaxed aorta preparations either in the presence or absence of a functional endothelium (Table 6.1; Fig. 6.11D). Surprisingly, morin-4 caused a marked concentration-dependent increase of phenylephrine-induced tone, which was not affected by endothelium removal (Table 6.1; Fig. 6.11C).

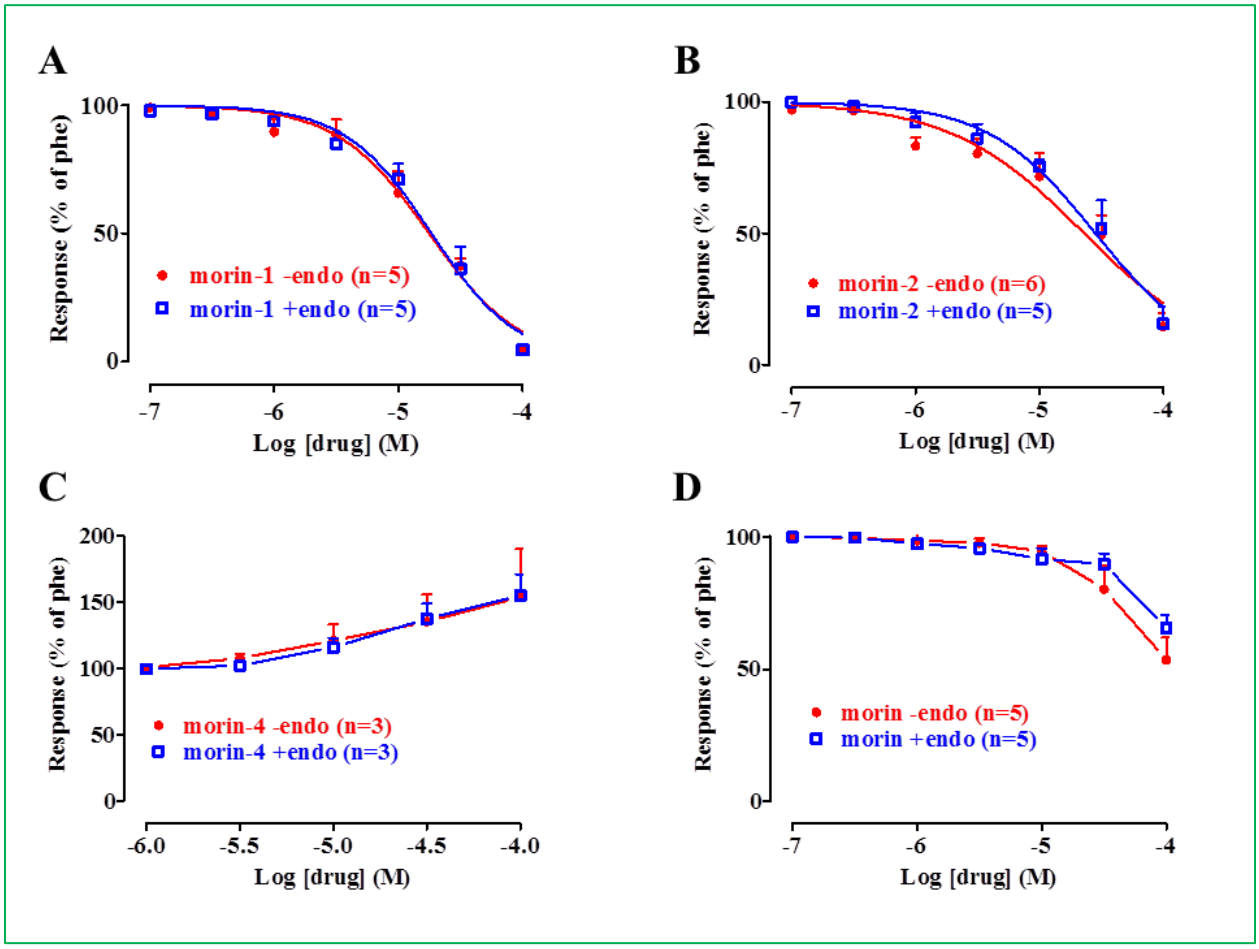



**Figure 6.11.** Effects of morin and its derivatives on phenylephrine-induced contraction of rat aorta rings. Concentration-response curves for (A) morin-1, (B) morin-2, (C) morin-4, and (D) morin in endothelium-intact (+endo) or denuded (-endo) rings pre-contracted by 0.3 µM phenylephrine (phe). Steady-state contraction was evoked by phenylephrine and then each drug was added cumulatively. In the ordinate scale, relaxation is reported as a percentage of the initial tension induced by phenylephrine (-endo 1149±112 mg, n=19; +endo 1156±127 mg, n=18). Data points represent the mean±s.e.m.

### 6.1.6.3 Effects of morin and morin-1 on $Ca^{2+}$ release from intracellular stores and extracellular $Ca^{2+}$ influx triggered by phenylephrine

The most effective compound of the series morin-1 as well as morin were assessed for their effects on phenylephrine-induced contraction elicited either in the absence or in presence of extracellular $Ca^{2+}$. As shown in Fig. 6.12A, pre-treatment with 30 µM morin-1 significantly reduced the contraction elicited by 10 µM phenylephrine in $Ca^{2+}$-free KHS. When the normal external $Ca^{2+}$ concentration was restored, still in presence of phenylephrine, morin-1 significantly reduced also the ensuing tonic contraction. On the contrary, morin did not affect the contraction elicited by phenylephrine both in the presence and in absence of extracellular $Ca^{2+}$ (Fig. 6.12B).

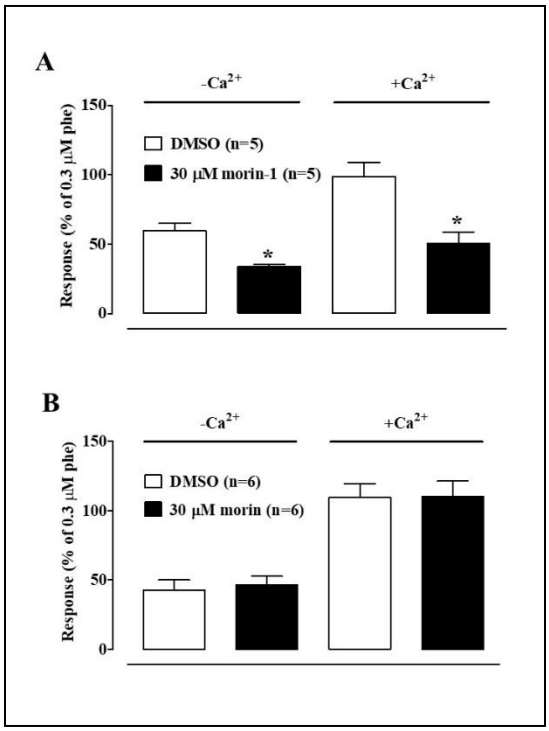



**Figure 6.12.** Effects of morin and morin-1 on $Ca^{2+}$ release from intracellular stores and on extracellular $Ca^{2+}$ influx induced by phenylephrine in endothelium-denuded rings. Effect of (A) morin-1 or vehicle only (DMSO) and (B) morin or vehicle only (DMSO) on 10 µM phenylephrine-induced contraction in the presence (+$Ca^{2+}$) and absence of extracellular $Ca^{2+}$ (-$Ca^{2+}$). Contractions were measured independently, the response to phenylephrine in the absence of extracellular $Ca^{2+}$ representing the baseline for that obtained after the addition of extracellular $Ca^{2+}$. Columns are the mean±s.e.m and represent the percentage of the response to 0.3 µM

phenylephrine (phe) in KHS (989±102 mg, n=20, morin-1; 701±52 mg, n=24, morin), taken as 100%. *P<0.05 vs. DMSO, Student's *t* test for unpaired samples.

**6.1.7 Flavonoids and hERG channels: Friends or foes?** (Review Article)

European Journal of Pharmacology, 2021, 899, 174030 (I.F. 4.432)

Simona Saponara[a], Fabio Fusi [b,*], Daniele Iovinelli[b], **Amer Ahmed[a]**, Alfonso Trezza[b], Ottavia Spiga[b], Giampietro Sgaragli [a,c], Massimo Valoti [a]

[a]Dipartimento di Scienze della Vita, Università degli Studi di Siena, via A. Moro 2, 53100, Siena, Italy

[b]Dipartimento di Biotecnologie, Chimica e Farmacia, Università degli Studi di Siena, via A. Moro 2, 53100, Siena, Italy

[c]Accademia Italiana della Vite e del Vino, via Logge degli Uffizi Corti 1, 50122, Florence, Italy

**Abstract**

The cardiac action potential is regulated by several ion channels. Drugs capable to block these channels, in particular the human ether-a-go-go-related gene (hERG) channel, also known as $K_V11.1$ channel, may lead to a potentially lethal ventricular tachyarrhythmia called *"Torsades de Pointes"*. Thus, evaluation of the hERG channel off-target activity of novel chemical entities is nowadays required to safeguard patients as well as to avoid attrition in drug development. Flavonoids, a large class of natural compounds abundantly presents in food, beverages, herbal medicines, and dietary food supplements, generally escape this assessment, though consumed in consistent amounts. Continuously growing evidence indicates that these compounds may interact with the hERG channel and block it. The present review, by examining numerous studies, summarizes the state-of-the-art in this field, describing the most significant examples of direct and indirect inhibition of the hERG channel current operated by flavonoids. A description of the molecular interactions between a few of these natural molecules and the *Rattus norvegicus* channel protein, achieved by an *in silico* approach, is also presented.

**Keywords:**

Docking simulation, Flavonoid, Patch-clamp, hERG channel, Long QT syndrome, *Torsades de pointes*

**His contribution consisted in:** drafting, and revising the manuscripts.

**6.1.8 Flavonoids and cardiovascular risk factors: a review** (review article)

Pharmadvances, 2021, 3(3):521-547.doi: 10.36118/pharmadvances.2021.11

**Amer Ahmed**

Dipartimento di Scienze della Vita, Università degli Studi di Siena, via A. Moro 2, 53100, Siena, Italy

**Abstract:**

Several modifiable and non-modifiable risk factors are associated with the incidence of cardiovascular diseases, the leading cause of death worldwide. A healthy diet, such as the Mediterranean diet, lowers the incidence of cardiovascular diseases. Intake of flavonoids, a class of plant-derived polyphenols widely distributed in fruits, vegetables, tea, coffee, and wine, is the hallmark of the Mediterranean diet that has been associated with reduced cardiovascular risk factors. Several mechanisms underpin this beneficial activity: a direct vasodilatory effect, prevention of endothelial dysfunction, inhibition of platelet aggregation, and smooth muscle cell proliferation along with an anti-oxidant, anti-inflammatory, anti-obesity, anti-diabetic, and anti-atherosclerotic effect. This review provides an updated overview of the mechanisms by which flavonoids ameliorate cardiovascular risk factors, thus retarding cardiovascular disease progression.

**Keywords:** cardiovascular diseases, flavonoids, endothelial cells, hypertension, obesity.

## 6.2 Appendix-2

During his PhD, Amer Ahmed has investigated on abdominal rat aorta rings the vascular activity of mitochondrial fission and dynamin protein inhibitors, namely mdivi-1, dynasore, and dyngo-4a.

### 6.2.1 Effect of mdivi-1 on high KCl induced contraction

The vasorelaxant activity of mdivi-1 was assessed on rings pre-contracted by high KCl concentrations, which cause membrane depolarization and $Ca_V1.2$ channel opening. As shown in Fig. 6.13, mdivi-1 caused a concentration-dependent relaxation of aorta preparations depolarized by 60 mM KCl, ($IC_{50}$ 4.8±0.5 µM, n= 5). This relaxation was not significantly affected by the presence of the dynamin inhibitors dynasore ($IC_{50}$ 4.4±0.7 µM, n=5; P=0.823 vs control) and dyngo-4a ($IC_{50}$ 5.2±0.2 µM, n=5; P=0.823 vs control; Fig. 6.13A) or the dynamin activator ryngo 1-23 ($IC_{50}$ 5.2±0.7 µM, n=5; P=0.823 vs control)

Mdivi-1 fully reverted also the contraction evoked by 25/30 mM KCl, though with a potency that was significantly greater than that recorded in rings pre-contracted by 60 mM KCl ($IC_{50}$ 1.1±0.3 µM, n=6; P=0.0001; Fig. 6.13B). This data suggest that mdivi-1 behaved both as a $Ca_V1.2$ channel blocker and as a $K^+$ channel opener: this hypothesis was supported by the shift to the right of mdivi-1 concentration-response curve observed in rings pre-contracted by the $K^+$ channel blocker tetraethylammonium (10 mM) and the $Ca_V1.2$ channel stimulator Bay K 8644 (100 nM). Under this experimental condition, an $IC_{50}$ value of 10.2±3.1 µM (n=6) and an efficacy of 86.4±9.4 were recorded.

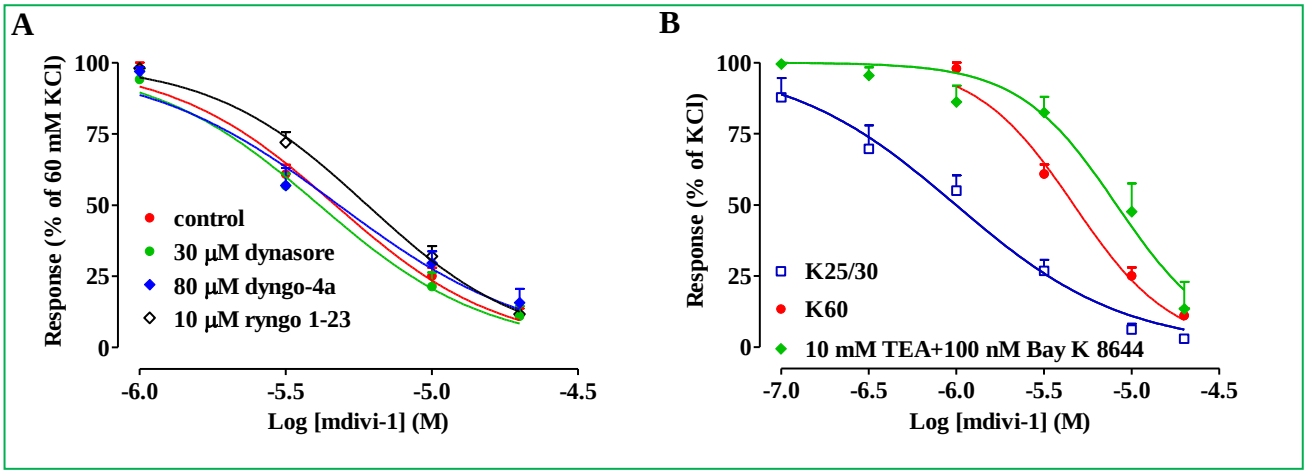


**Figure 6.13.** Effect of mdivi-1 on high KCl-induced contraction. (A) Mdivi-1 concentration response curves constructed on endothelium-denuded abdominal rat aorta rings pre-contracted by 60 mM KCl in the absence (control) or presence of 30 µM dynasore, 80 µM dyngo-4a, or 10 µM ryngo 1-23. (B) Mdivi-1 concentration-response curves constructed in rings pre-contracted by 60 mM KCl (K60; red), 25/30 mM KCl (K25/30; blue), or 10 mM tetraethylammonium (TEA) plus 100 nM Bay K 8644 (green). In the ordinate scale, response is reported as percentage of the initial tension induced (A) by 60 mM KCl (control 2152±332 mg, n=5; 30 µM dynasore 1548±85 mg; 80 µM dyngo-4a 1602±198 mg, n=5; ryngo 1-23 1972±151 mg, n=5) or (B) by (60 mM KCl 2152±332 mg, n=5; 25/30 mM KCl 2163±176 mg, n=6; TEA+Bay K 8644 1587±121 mg). Data points represent the mean±s.e.m.

### 6.2.2 Effect of mdivi-1 on phenylephrine- and NaF-induced contractions

The effects of mdivi-1 were assessed in endothelium-denuded rings were pre-contracted by either 0.3 µM phenylephrine (a selective $\alpha_1$ receptor agonist) or 10 mM NaF (a known activator of Rho-kinase). As shown in Fig. 6.14, mdivi-1 reverted both contractions with similar potency ($IC_{50}$ phenylephrine 4.0±0.8 µM, n=11; NaF 3.5±0.9 µM, n=6; P=0.706) and efficacy ($E_{max}$ phenylephrine 91.0±2.2%, n=9; NaF 86.8±5.6%, n=5; P=0.421). Interestingly, in aortic rings pre-incubated with the mitochondria-targeted antioxidant mito-tempol, both potency ($IC_{50}$ 10.2±1.4 µM, n=7; P=0.006 vs control) and efficacy of mdivi-1 towards phenylephrine ($E_{max}$ 77.8±5.7%, n=7; 0.034; Fig. 6.14A) were reduced.

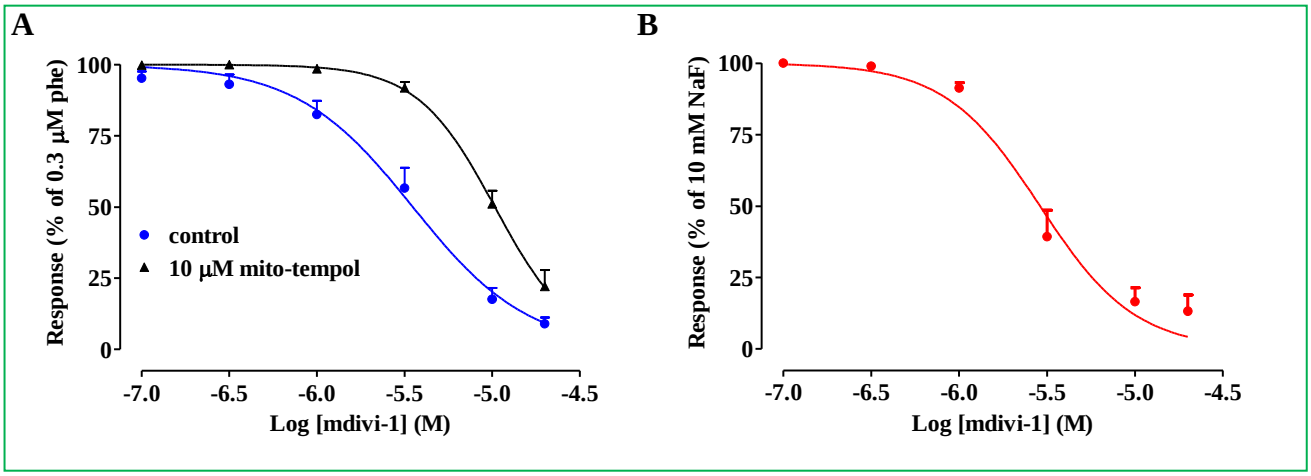


**Figure 6.14.** Effects of mdivi-1 on phenylephrine- and NaF-induced contractions. Mdivi-1 concentration-response curves were constructed in endothelium-denuded abdominal rat aorta rings pre-contracted either by (A) 0.3 µM phenylephrine (phe), in the absence (control) or presence of 10 µM mito-tempol, or by (B) 10 mM NaF. Steady-state contraction was evoked by phenylephrine or NaF and then mdivi-1 was added cumulatively. In the ordinate scale, response is reported as percentage of the initial tension induced by either (A) phenylephrine (control 1308±128 mg, n=11; mito-tempol 1908±138 mg, n=7) or (B) NaF (1443.1±199, n=6). Data points represent the mean±s.e.m.

### 6.2.3 Effect of mdivi-1 on intracellular $Ca^{2+}$ homeostasis

The effect of mdivi-1 on $Ca^{2+}$ release from intracellular store sites and $Ca^{2+}$ influx from extracellular environment triggered by phenylephrine was assessed. As shown in Fig. 6.15, pre-treatment with mdivi-1 did not affect $Ca^{2+}$ release from intracellular store sites (measured as muscle tension in the absence of extracellular $Ca^{2+}$). However, at the highest concentration tested (10 µM), $Ca^{2+}$ influx triggered by phenylephrine was significantly reduced (Fig. 6.15B).

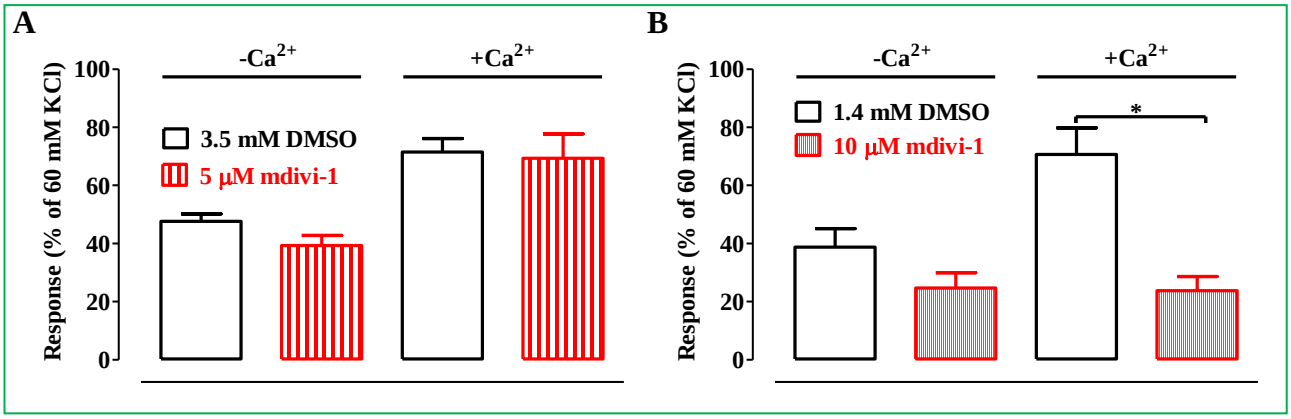



**Figure 6.15.** Effect of mdivi-1 on $Ca^{2+}$ release from intracellular store sites and on extracellular $Ca^{2+}$ influx induced by phenylephrine. Effects of vehicle only (DMSO), (A) 5 µM, and (B) 10 µM mdivi-1 recorded in endothelium-denuded abdominal rat aorta rings pre-contracted by 10 µM phenylephrine in the absence (-$Ca^{2+}$) and presence of extracellular $Ca^{2+}$ (+$Ca^{2+}$). Contractions were measured independently, the response to 10 µM phenylephrine in the absence of extracellular $Ca^{2+}$ representing the baseline for that obtained after the addition of extracellular $Ca^{2+}$. In the ordinate scale, response is reported as percentage of tension induced by 60 mM KCl

in ring functional assay (5 µM mdivi-1 1727±179 mg, n=18; 10 µM mdivi-1 2150±125 mg, n=13). Data points represent the mean±s.e.m. *P<0.05 vs DMSO, Student's t test for unpaired samples.

As mdivi-1 reduced extracellular $Ca^{2+}$ influx and relaxed 60 mM KCl-induced contraction, its effect on $Ca^{2+}$ influx through $Ca_V1.2$ channel was assessed. As shown in Fig. 6.16, pre-incubation with 5 µM mdivi-1 caused a rightward, non-parallel shift of the $Ca^{2+}$ concentration-response curve in preparations depolarized by 60 mM KCl, decreasing the area under curve from 895±124 (DMSO, n=5) to 420±72 (mdivi-1, n=6; P=0.007). Notably, addition of the $Ca_V1.2$ channel stimulator Bay K 8644 (100 nM) significantly increased muscle tension of both DMSO and mdivi-1 treated rings.

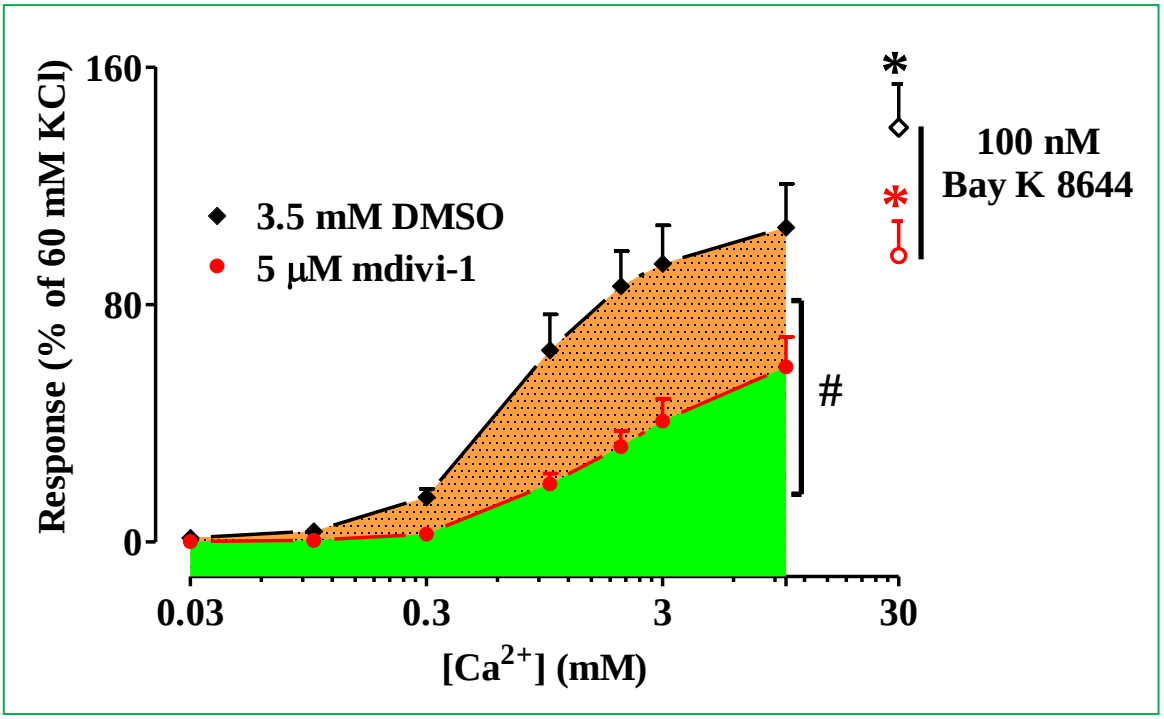



**Figure 6.16.** Effect of mdivi-1 on $Ca^{2+}$ influx through $Ca_V1.2$ channels. Concentration-response curves to $CaCl_2$ constructed in endothelium-denuded abdominal rat aorta rings depolarized with 60 mM KCl $Ca^{2+}$-free KHS in the presence of either vehicle (DMSO) or 5 µM mdivi-1. In the ordinate scale, response is reported as percentage of the tension induced by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m. (n=5-6). $^{*,\#}$ P<0.05 vs 10 mM $Ca^{2+}$ (paired samples) or vs DMSO (AUC; unpaired samples), Student's t test.

### 6.2.4 Effect of dynasore and dyngo-4a on high KCl-induced contraction

The vascular effects of dynamin inhibitors dynasore and its analogue dyngo-4a were assessed. As shown in Fig. 6.17, dynasore caused a concentration-dependent relaxation of 25/30 mM KCl- ($IC_{50}$ 4.3±1.5 µM, n=7; $E_{max}$ 90.1±3.4%; n=4) and 60 mM KCl-induced contraction (estimated $IC_{50}$ 56.8±18.5 µM; $E_{max}$ 66.0±6.7%; n=5). Surprisingly, its analogue dyngo-4a potentiated 60 mM KCl- induced response by about 50%.

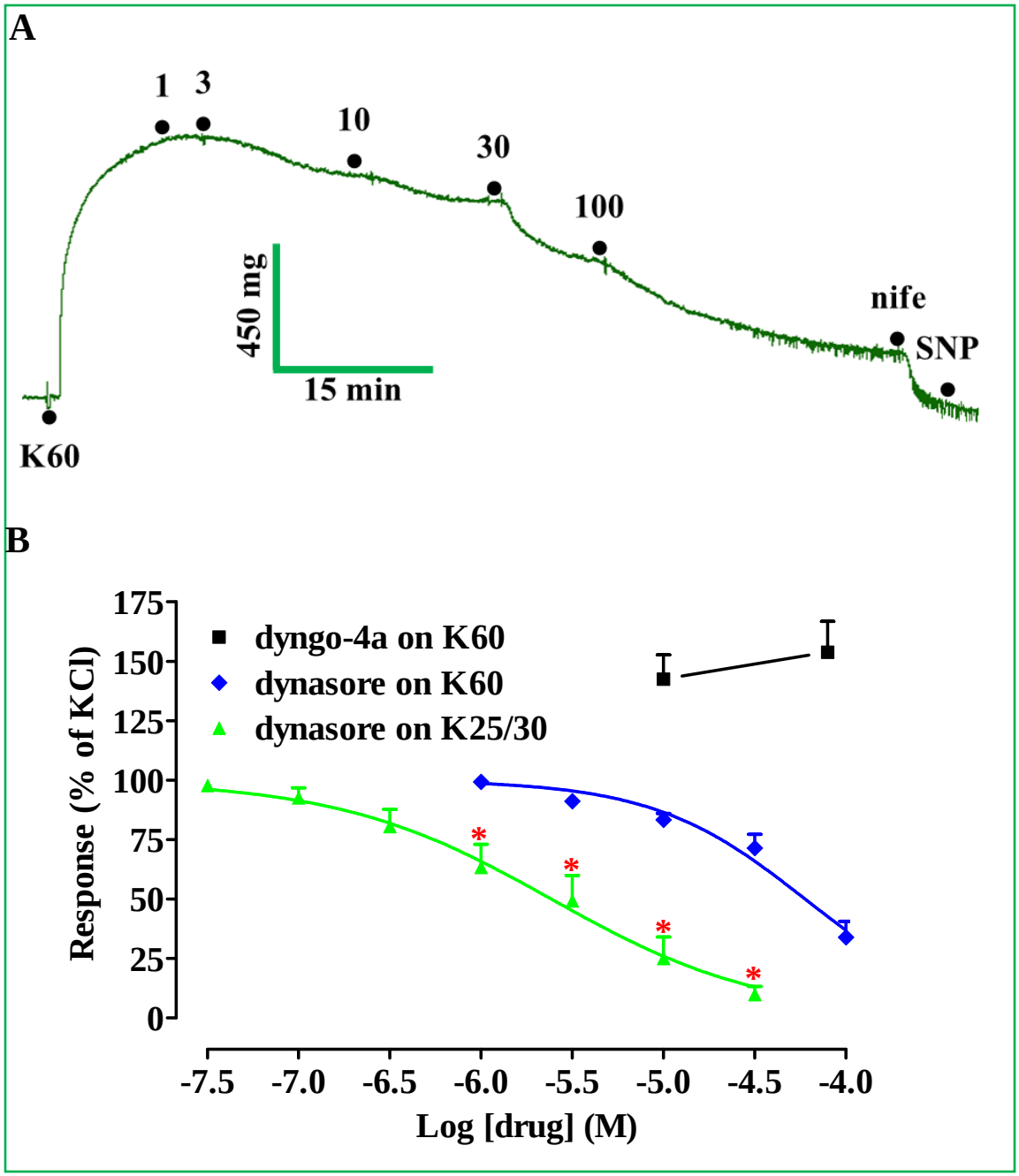


**Figure 6.17.** Effect of dynasore and dyngo-4a on high KCl-induced contraction. (A) Endothelium-denuded abdominal aorta rings were depolarized by 60 mM KCl (K60). Once a plateau was achieved, dynasore (μM) was added cumulatively. The effect of 1 μM nifedipine (nife) followed by 100 μM sodium nitroprusside (SNP) is also shown. The trace is representative of 5 similar experiments. (B) Concentration-response curves for dynasore and dyngo-4a in rings pre-contracted by 25/30 mM (green) or 60 mM KCl (blue and black). In the ordinate scale, response is reported as percentage of the initial tension evoked by KCl. Data points represent the mean±s.e.m. (n=4-7). *P<0.05 vs K60, Student's t test for unpaired samples.

As shown in Fig. 6.18, dyngo-4a induced a concentration-dependent contraction in rings stimulated by 25 mM KCl ($EC_{50}$ 10.6±4.7 μM, n=9; $E_{max}$ 90.6±11.5%, n=8). This effect was not affected by the presence of the $\alpha_1$ receptor antagonist prazosin (100 nM) ($EC_{50}$ 5.2±0.9 μM, n=8; P=0.322 vs control; $E_{max}$ 90.8±4.7%, n=5; P=0.987).

.

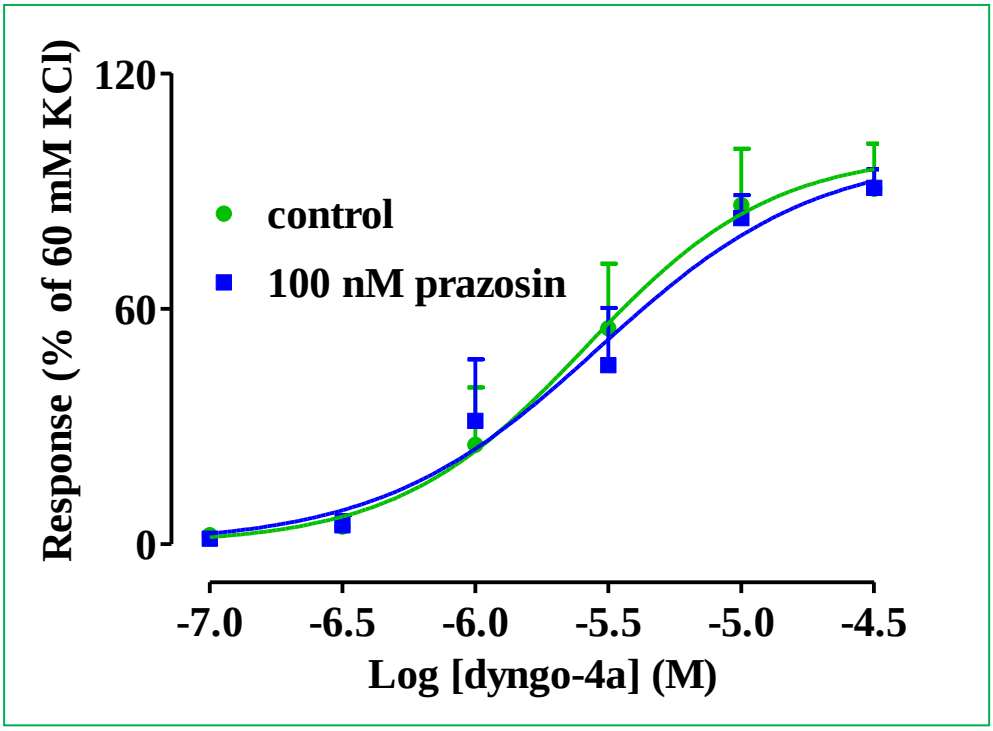



**Figure 6.18.** Effect of dyngo-4a on 25 mM KCl-induced contraction. Dyngo-4a concentration-response curves were constructed in endothelium-denuded abdominal rat aorta rings pre-contracted by 25 mM KCl in absence (control) or presence of 100 nM prazosin. In the ordinate

scale, response is reported as a percentage of the initial tension evoked by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m. (n=8-9).

### 6.2.5 Effect of dynasore on phenylephrine-induced contraction

The effect of dynasore on pharmaco-mechanical coupling was assessed in rings pre-contracted by 0.3 µM phenylephrine. As shown in Fig. 6.19, dynasore relaxed phenylephrine-induced tone in a concentration-dependent manner ($IC_{50}$ 10.8±2.9 µM; $E_{max}$ 88.3±5.7%, n=6). In rings pre-incubated with the mitochondria-targeted antioxidant mito-tempol, efficacy was reduced ($E_{max}$ 63.8±7.6%, n=6; P=0.028 vs control); also estimated potency was reduced, though statistical significance was not reached ($IC_{50}$ 23.1±7.7 µM, n=6; P=0.167).

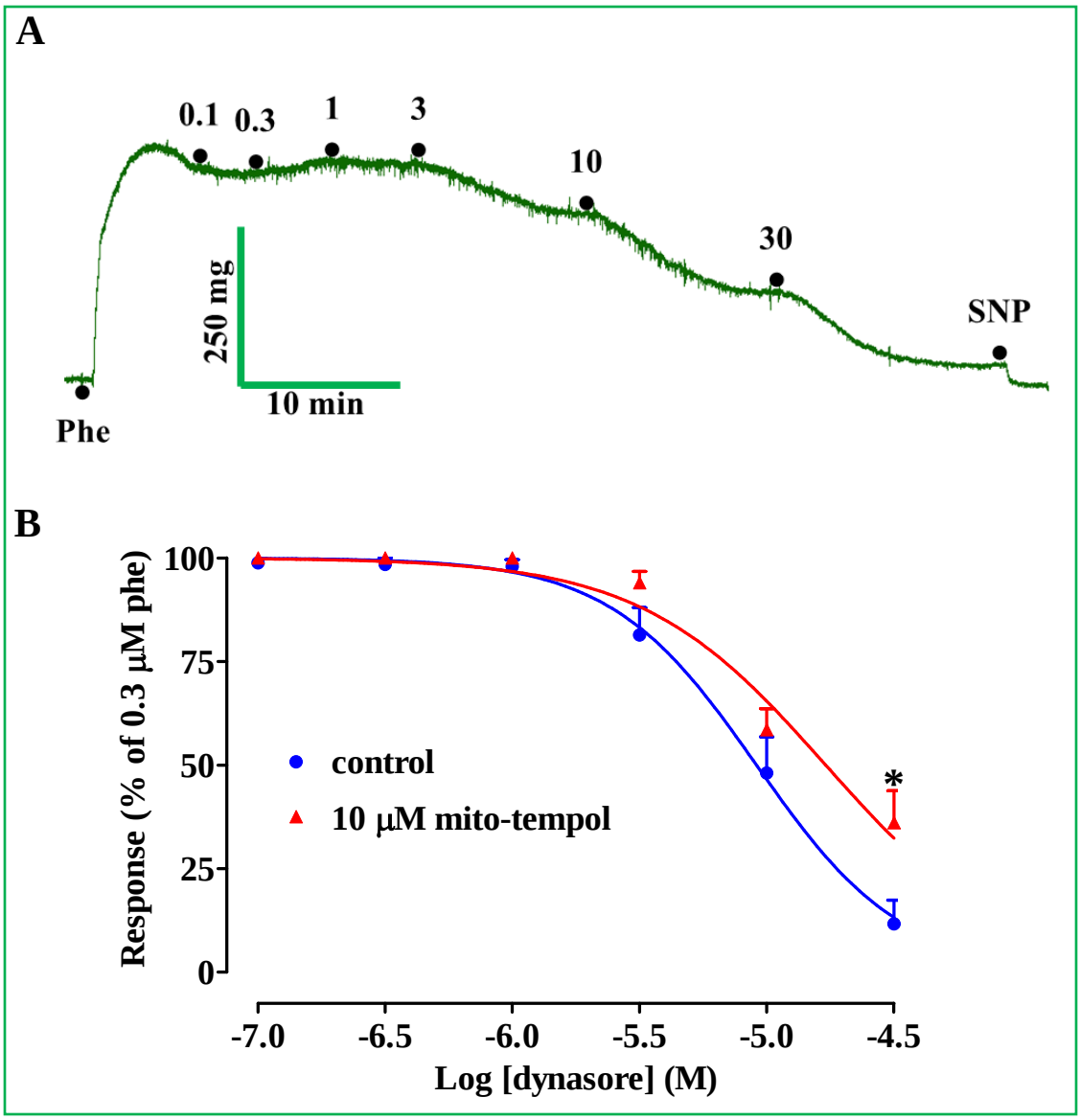


**Figure 6.19.** Effect of dynasore on pharmaco-mechanical coupling. (A) Steady-state contraction was evoked by 0.3 μM phenylephrine (phe) in endothelium-denuded abdominal rat aorta rings and then dynasore (μM) was added cumulatively; the effect of sodium nitroprusside (SNP) is also shown. Trace is representative of 6 similar experiments. (B) Dynasore concentration-response curves constructed in rings pre-contracted by phenylephrine in absence (control) or presence of mito-tempol. In the ordinate scale, response is reported as percentage of the initial tension induced by phenylephrine (control 1370±221 mg; mito-tempol 1360±74 mg; n=6). Data points represent the mean±s.e.m.

### 6.2.6 Effect of dyngo-4a on KCl concentration-response curve

The effect of dyngo-4a on KCl concentration-response curve was assessed in endothelium-denuded abdominal rat aorta rings. As shown in Fig. 6.20, dyngo-4a caused a concentration-dependent leftward shift of the curve ($EC_{50}$ 1.4 mM DMSO 28.4±1.8 mM; 10 μM dyngo-4a 21.2±1.2 mM, n=5; P=0.007; $EC_{50}$ 11.2 mM DMSO 26.6±1.9 mM; 80 μM dyngo-4a 15.7±2.1 mM, n=5; P=0.0001) ($E_{max}$ DMSO 94.9±5.2%; 10 μM dyngo-4a 113.3±1.8%; P=0.046; DMSO 96.9±2.1%; 80 μM dyngo-4a 106.0±3.5%; P=0.163). Noticeably, dyngo-4a evoked a concentration-dependent, weak contraction of basal tone during the incubation period (Fig. 6.20B).

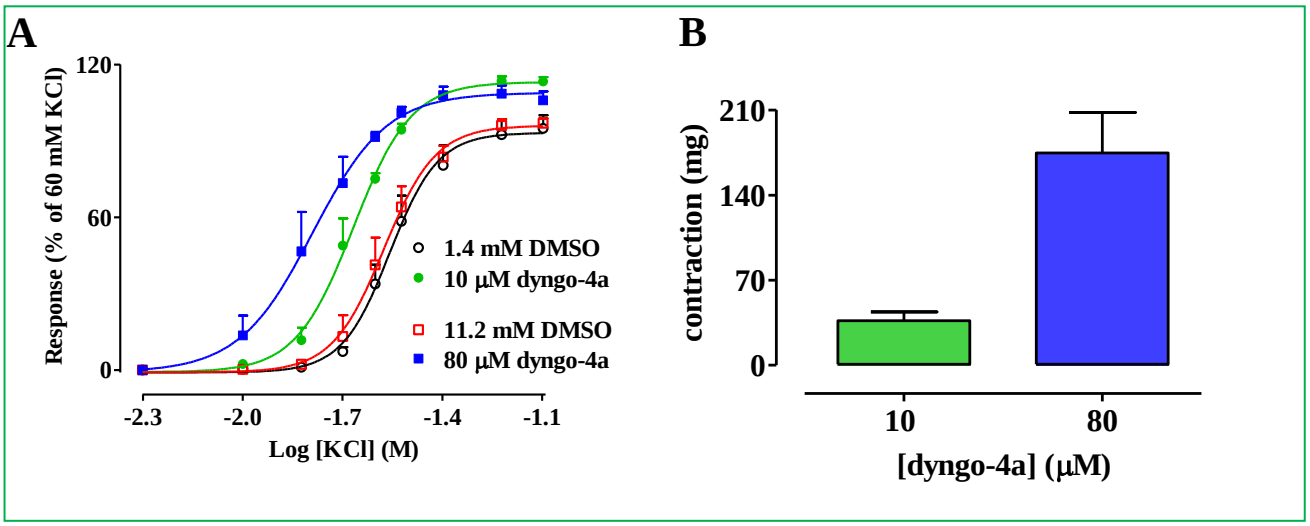



**Figure 6.20.** Effect of dyngo-4a on KCl concentration-response curve. (A) Abdominal rat aorta rings were pre-incubated with DMSO prior to construct KCl concentration-response curve. After

washout, a second concentration-response curve was constructed in the presence of 10 µM or 80 µM dyngo-4a. In the ordinate scale, response is reported as percentage of the tension induced by 60 mM KCl in ring functional assay. (B) Tension recorded in preparations incubated with dyngo-4a. Data points and columns represent the mean±s.e.m. (n=5).

### 6.2.7 Effect of dyngo-4a on phenylephrine concentration-response curve

The effect of dyngo-4a on the contractile response to the $\alpha_1$ receptor agonist phenylephrine was assessed. Two consecutive phenylephrine concentration-response curves were constructed; the first under control conditions, the second in the absence or presence of 10 µM, 80 µM dyngo-4a, or vehicle (DMSO) pre-incubated for 15 min. As shown in Fig. 6.21, the two consecutive phenylephrine concentration-response curves were superimposable ($EC_{50}$ 268±92 nM and 157±16 nM, n=5; P=0.2605; $E_{max}$ 135.5±9.7% and 147.4±12.4%; P=0.150). Similar results were obtained in rings pre-incubated with vehicle (DMSO) when compared to both the first curve ($EC_{50}$ 275±87 nM and 318±110 nM, n=5; P=0.185; $E_{max}$ 134.5±11.9% and 141.3±3.6%, n=5; P=0.651) and the second curve ($EC_{50}$ 157±16 nM, n=5; P=0.492; $E_{max}$ 147.4±12.4%, n=5; P=0.651; Fig.6.21B).

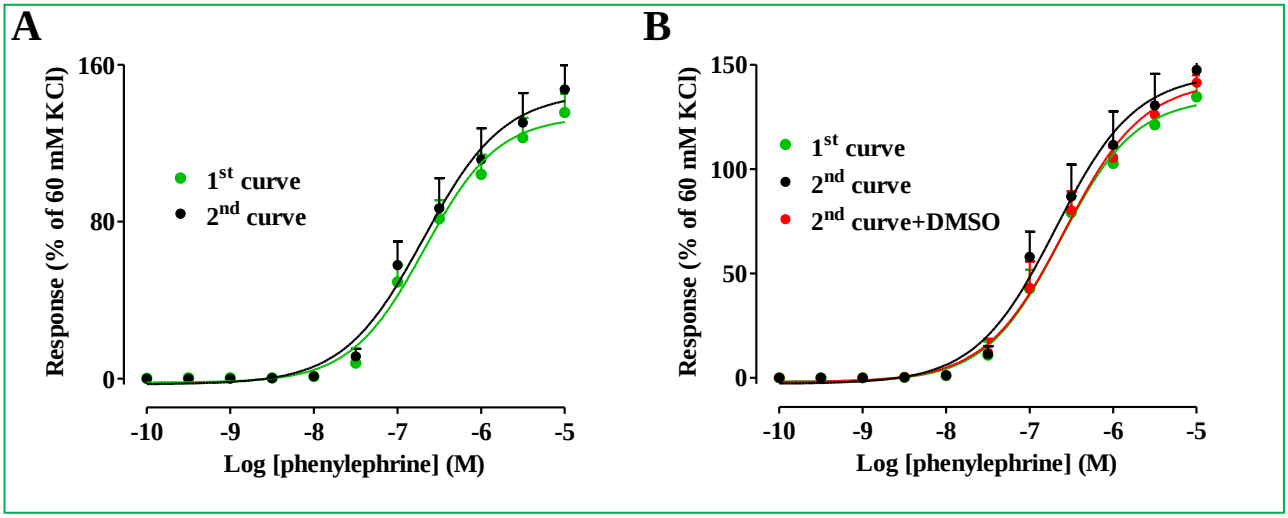



**Figure 6.21.** Reproducibility of the response to phenylephrine. Phenylephrine concentration-response curves were constructed in endothelium-denuded abdominal rat aorta rings. (A) Two consecutive curves measured in the same ring. (B) Effect of DMSO on the second phenylephrine concentration-response curve. In the ordinate scale, response is reported as percentage of the

contraction induced by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m. (n=5).

As shown in Fig. 6.22, pre-incubation with 10 μM dyngo-4a caused a significant leftward shift of phenylephrine concentration-response curve ($EC_{50}$ 63±18 nM; P=0.021 vs DMSO; $E_{max}$ 178.3±9.3%; n=7; P=0.009). Similarly, pre-incubation with 80 μM dyngo-4a significantly increased the potency of phenylephrine ($EC_{50}$ 55.0±17.2 nM, n=6; P=0.029); the increase in efficacy however, did not reach a statistical significance ($E_{max}$ 150.7±5.7%, n=6; P=0.218).

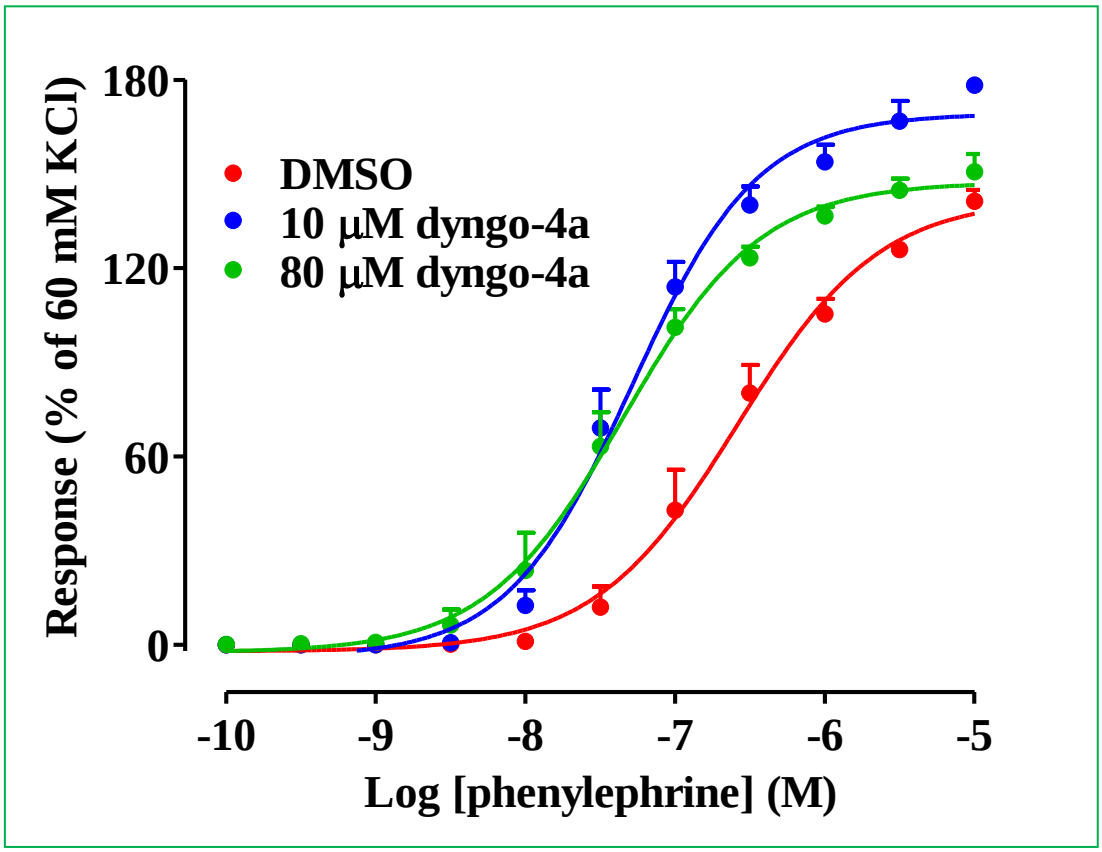



**Figure 6.22.** Effect of dyngo-4a on phenylephrine-induced contraction. Endothelium-denuded abdominal rat aorta rings were pre-incubated with DMSO, 10 μM, or 80 μM dyngo-4a for 15 min and then phenylephrine was added cumulatively. In the ordinate scale, response is reported as percentage of tension induced by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m. (n=5-7).

### 6.2.8 Effect of mito-tempol on dyngo-4a potentiation of phenylephrine-induced contraction

The involvement of superoxide anion in dyngo-4a potentiation of phenylephrine-induced contraction was assessed. As shown in Fig. 6.23, in the presence of vehicle phenylephrine evoked a concentration-dependent contraction of the preparations ($EC_{50}$ DMSO 408±133 nM; $E_{max}$ 97.2±6.1%, n=7). Dyngo-4a induced potentiation of this response ($EC_{50}$ 66±9 nM; P=0.014 vs DMSO; $E_{max}$ 131.1±7.0%; P=0.002) was counteracted by the mitochondria-targeted antioxidant mito-tempol ($EC_{50}$ mito-tempol+dyngo-4a 96±11 nM, n=7; P=0.060 vs dyngo-4a; $E_{max}$ 104.4±10.5%, n=7; P=0.0564; Fig. 6.23A). Notably, mito-tempol alone did not affect the response to phenylephrine (Fig. 6.23B).

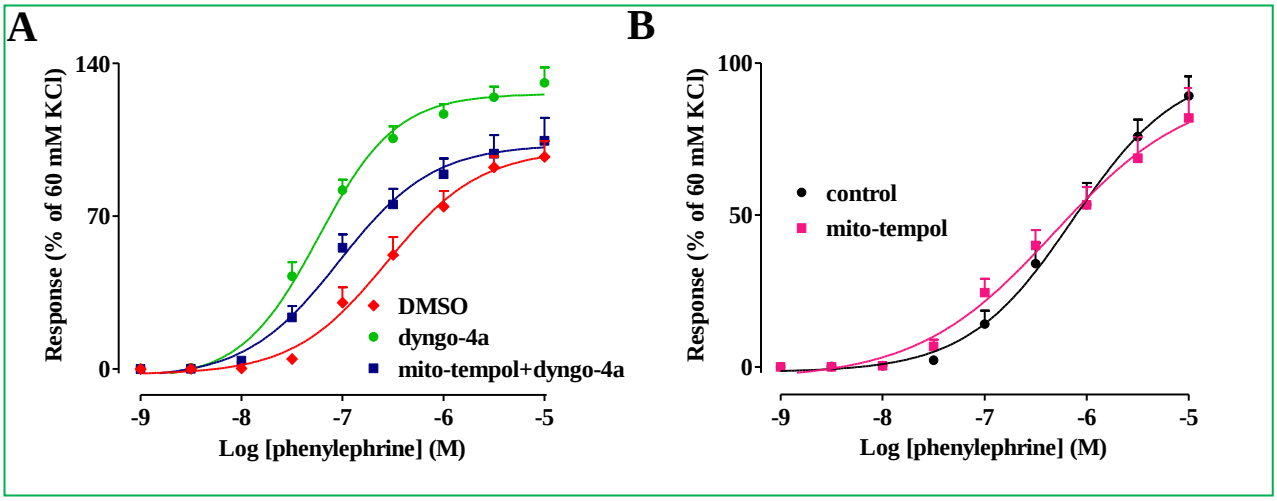



**Figure 6.23.** Effect of mito-tempol on dyngo-4a potentiation of phenylephrine-induced contraction. (A) Endothelium-denuded abdominal rat aorta rings, pre-incubated with DMSO, 10 µM dyngo-4a alone, or 10 µM dyngo-4a plus 10 µM mito-tempol, were stimulated with cumulative concentrations of phenylephrine. (B) Effect of 10 µM mito-tempol on phenylephrine-induced contraction. In the ordinate scale, response is reported as percentage of tension induced by 60 mM KCl in ring functional assay. Data points represent the mean±s.e.m. (n=7).

### 6.2.9 Effect of tyramine on dyngo-4a-induced contraction

The mechanism underpinning dyngo-4a-induced increase of basal tone (see Fig. 6.20B) was assessed. As shown in Fig. 6.24, 80 µM dyngo-4a alone evoked a modest contraction, which was lower than that caused by the indirect sympathomimetic agent tyramine. When dyngo-4a was

added on the plateau of tyramine-induced contraction, a further increase of tone was recorded (P=0.0002). Finally, the subsequent cumulative addition of prazosin (0.1-10 μM) fully reverted the rings vessel tone to basal level.

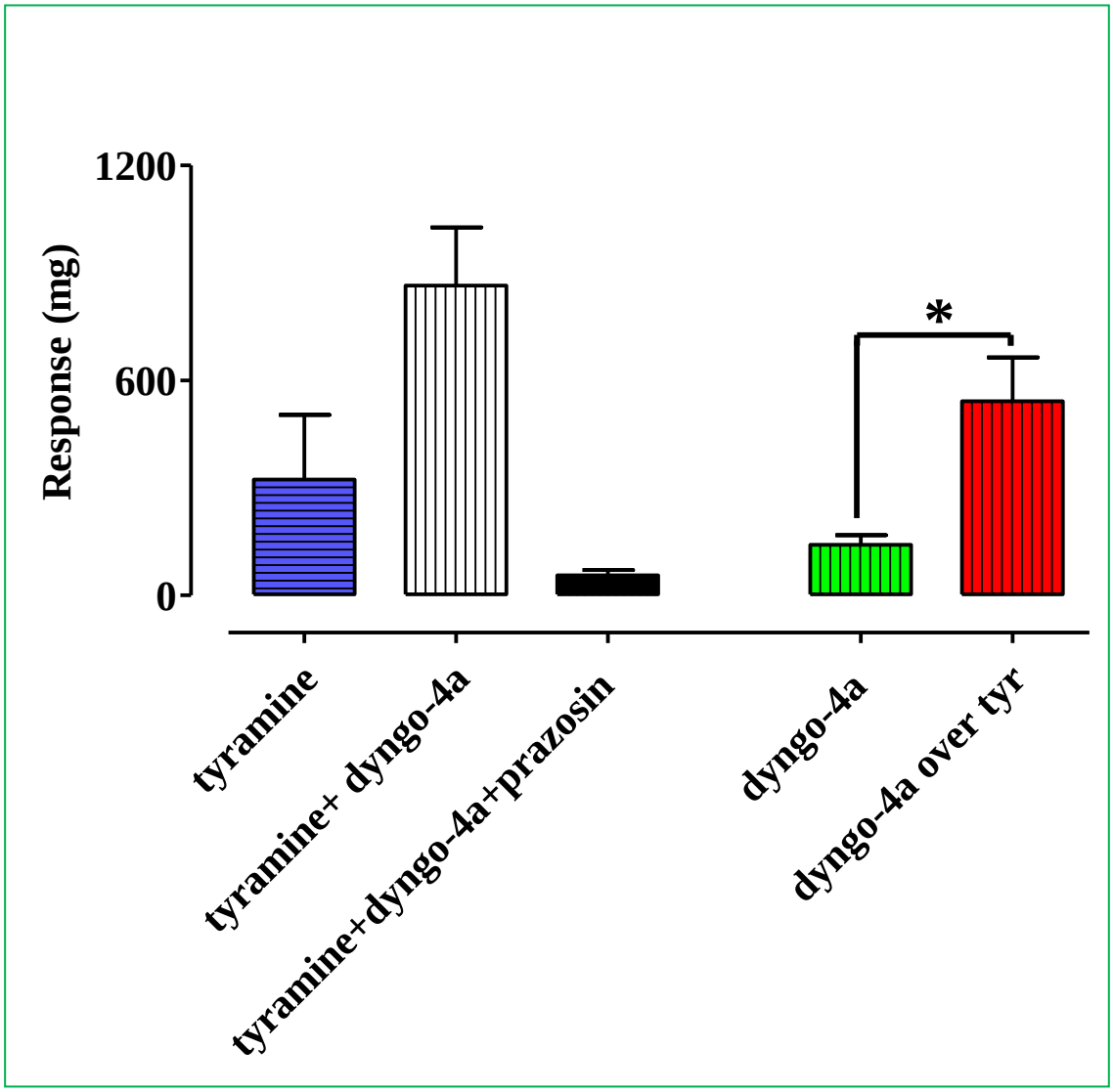



**Figure 6.24.** Effect of tyramine and prazosin on dyngo-4a-induced contraction. (Left) Endothelium-denuded abdominal rat aorta rings were pre-contracted by 100 μM tyramine and then, at the plateau of tyramine-induced contraction, 80 μM dyngo-4a was added followed by prazosin (10 μM). (Right) Effect of 80 μM dyngo-4a on the spontaneous tone in the absence or presence of 100 μM tyramine. In the ordinate scale, the response is reported in mg. Columns represent the mean±s.e.m. (n=5-11). *P<0.05, Student's t test for unpaired samples.

# CHAPTER 7:

# BIBLIOGRAPHY

## 7. References


Abu Bakar, H., Robert Dunn, W., Daly, C., Ralevic, V., 2017. Sensory innervation of perivascular adipose tissue: a crucial role in artery vasodilatation and leptin release. Cardiovasc Res 113, 962-972.

Achari, A.E., Jain, S.K., 2017. Adiponectin, a Therapeutic Target for Obesity, Diabetes, and Endothelial Dysfunction. International Journal of Molecular Sciences 18, 1321.

Agabiti-Rosei, C., De Ciuceis, C., Rossini, C., Porteri, E., Rodella, L.F., Withers, S.B., Heagerty, A.M., Favero, G., Agabiti-Rosei, E., Rizzoni, D., Rezzani, R., 2014. Anticontractile activity of perivascular fat in obese mice and the effect of long-term treatment with melatonin. J Hypertens 32, 1264-1274.

Aghamohammadzadeh, R., Unwin, R.D., Greenstein, A.S., Heagerty, A.M., 2015. Effects of Obesity on Perivascular Adipose Tissue Vasorelaxant Function: Nitric Oxide, Inflammation and Elevated Systemic Blood Pressure. J Vasc Res 52, 299-305.

Ahmad, A.A., Randall, M.D., Roberts, R.E., 2017. Sex differences in the role of phospholipase A(2) -dependent arachidonic acid pathway in the perivascular adipose tissue function in pigs. J Physiol 595, 6623-6634.

Ahmad, M.F., Ferland, D., Ayala-Lopez, N., Contreras, G.A., Darios, E., Thompson, J., Ismail, A., Thelen, K., Moeser, A.J., Burnett, R., Anantharam, A., Watts, S.W., 2019. Perivascular Adipocytes Store Norepinephrine by Vesicular Transport. Arteriosclerosis, Thrombosis, and Vascular Biology 39, 188-199.

Ahmed, A., 2021. Flavonoids and cardiovascular risk factors: a review. Pharmadvances 3(3):521-547

Ahmed, S., Warren, D.T., 2018. Vascular smooth muscle cell contractile function and mechanotransduction. Vessel Plus 2, 36.

Ahn-Jarvis, J.H., Parihar, A., Doseff, A.I., 2019. Dietary Flavonoids for Immunoregulation and Cancer: Food Design for Targeting Disease. Antioxidants (Basel) 8, 202.

Akhlaghi, M., Ghobadi, S., Mohammad Hosseini, M., Gholami, Z., Mohammadian, F., 2018. Flavanols are potential anti-obesity agents, a systematic review and meta-analysis of controlled clinical trials. Nutr Metab Cardiovasc Dis 28, 675-690.

Akil, L., Ahmad, H.A., 2011. Relationships between obesity and cardiovascular diseases in four southern states and Colorado. J Health Care Poor Underserved 22, 61-72.

Akintunde, J.K., Akintola, T.E., Hammed, M.O., Amoo, C.O., Adegoke, A.M., Ajisafe, L.O., 2020. Naringin protects against Bisphenol-A induced oculopathy as implication of cataract in hypertensive rat model. Biomedicine & Pharmacotherapy 126, 110043.

Akoumianakis, I., Tarun, A., Antoniades, C., 2017. Perivascular adipose tissue as a regulator of vascular disease pathogenesis: identifying novel therapeutic targets. Br J Pharmacol 174, 3411-3424.

Alcocer, F., Whitley, D., Salazar-Gonzalez, J.F., Jordan, W.D., Sellers, M.T., Eckhoff, D.E., Suzuki, K., Macrae, C., Bland, K.I., 2002. Quercetin inhibits human vascular smooth muscle cell proliferation and migration. Surgery 131, 198-204.

Al-Ishaq, R.K., Abotaleb, M., Kubatka, P., Kajo, K., Büsselberg, D., 2019. Flavonoids and Their Anti-Diabetic Effects: Cellular Mechanisms and Effects to Improve Blood Sugar Levels. Biomolecules 9, 430.

Almabrouk, T.A.M., Ugusman, A.B., Katwan, O.J., Salt, I.P., Kennedy, S., 2017. Deletion of AMPKα1 attenuates the anticontractile effect of perivascular adipose tissue (PVAT) and reduces adiponectin release. Br J Pharmacol 174, 3398-3410.

Almabrouk, T.A.M., White, A.D., Ugusman, A.B., Skiba, D.S., Katwan, O.J., Alganga, H., Guzik, T.J., Touyz, R.M., Salt, I.P., Kennedy, S., 2018. High Fat Diet Attenuates the Anticontractile Activity of Aortic PVAT via a Mechanism Involving AMPK and Reduced Adiponectin Secretion. Front Physiol 9.

Al-Numair, K.S., Chandramohan, G., Veeramani, C., Alsaif, M.A., 2015. Ameliorative effect of kaempferol, a flavonoid, on oxidative stress in streptozotocin-induced diabetic rats. Redox Rep 20, 198-209.

Alvarez, A.I., Real, R., Pérez, M., Mendoza, G., Prieto, J.G., Merino, G., 2010. Modulation of the activity of ABC transporters (P-glycoprotein, MRP2, BCRP) by flavonoids and drug response. Journal of Pharmaceutical Sciences 99, 598-617.

Amberg, G.C., Navedo, M.F., 2013. Calcium dynamics in vascular smooth muscle. Microcirculation 20, 281-289.

Anandhi, R., Thomas, P.A., Geraldine, P., 2014. Evaluation of the anti-atherogenic potential of chrysin in Wistar rats. Mol Cell Biochem 385, 103-113.

Antonopoulos, A.S., Margaritis, M., Coutinho, P., Shirodaria, C., Psarros, C., Herdman, L., Sanna, F., De Silva, R., Petrou, M., Sayeed, R., Krasopoulos, G., Lee, R., Digby, J., Reilly, S., Bakogiannis, C., Tousoulis, D., Kessler, B., Casadei, B., Channon, K.M., Antoniades, C., 2015. Adiponectin as a link between type 2 diabetes and vascular NADPH oxidase activity in the human arterial wall: the regulatory role of perivascular adipose tissue. Diabetes 64, 2207-2219.

Appeldoorn, M.M., Venema, D.P., Peters, T.H., Koenen, M.E., Arts, I.C., Vincken, J.P., Gruppen, H., Keijer, J., Hollman, P.C., 2009. Some phenolic compounds increase the nitric oxide level in endothelial cells in vitro. J Agric Food Chem 57, 7693-7699.

Auger, C., Al-Awwadi, N., Bornet, A., Rouanet, J.-M., Gasc, F., Cros, G., Teissedre, P.-L., 2004. Catechin and procyanidins in Mediterranean diet. Food Research International 37, 233-245.

Averill, D.B., Ishiyama, Y., Chappell, M.C., Ferrario, C.M., 2003. Cardiac angiotensin-(1-7) in ischemic cardiomyopathy. Circulation 108, 2141-2146.

Awata, W.M.C., Gonzaga, N.A., Borges, V.F., Silva, C.B.P., Tanus-Santos, J.E., Cunha, F.Q., Tirapelli, C.R., 2019. Perivascular adipose tissue contributes to lethal sepsis-induced vasoplegia in rats. Eur J Pharmacol 863, 27.
Ayala-Lopez N, Watts SW (2017) New actions of an old friend: perivascular adipose tissue's adrenergic mechanisms. Br J Pharmacol 174(20):3454-3465

Ayala-Lopez, N., Jackson, W.F., Burnett, R., Wilson, J.N., Thompson, J.M., Watts, S.W., 2015. Organic cation transporter 3 contributes to norepinephrine uptake into perivascular adipose tissue. Am J Physiol Heart Circ Physiol 309, 2.

Ayala-Lopez, N., Martini, M., Jackson, W.F., Darios, E., Burnett, R., Seitz, B., Fink, G.D., Watts, S.W., 2014. Perivascular adipose tissue contains functional catecholamines. Pharmacol Res Perspect 2, 41.

Ayaz, M., Sadiq, A., Junaid, M., Ullah, F., Ovais, M., Ullah, I., Ahmed, J., Shahid, M., 2019. Flavonoids as Prospective Neuroprotectants and Their Therapeutic Propensity in Aging Associated Neurological Disorders. Frontiers in Aging Neuroscience 11.

Baek, I., Jeon, S.B., Song, M.-J., Yang, E., Sohn, U.D., Kim, I.K., 2009. Flavone Attenuates Vascular Contractions by Inhibiting RhoA/Rho Kinase Pathway. The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology 13, 201-207.

Bai, Y.-f., Wang, S.-w., Wang, X.-x., Weng, Y.-y., Fan, X.-y., Sheng, H., Zhu, X.-t., Lou, L.-j., Zhang, F., 2019. The flavonoid-rich Quzhou Fructus Aurantii extract modulates gut microbiota and prevents obesity in high-fat diet-fed mice. Nutrition & Diabetes 9, 30.

Bao, L., Hu, L., Zhang, Y., Wang, Y., 2016. Hypolipidemic effects of flavonoids extracted from Lomatogonium rotatum. Exp Ther Med 11, 1417-1424.

Barac, A., Panza, J.A., 2009. Mechanisms of Decreased Vascular Function With Aging. Hypertension 53, 900-902.

Barreca, D., Gattuso, G., Bellocco, E., Calderaro, A., Trombetta, D., Smeriglio, A., Laganà, G., Daglia, M., Meneghini, S., Nabavi, S.M., 2017. Flavanones: Citrus phytochemical with health-promoting properties. Biofactors 43, 495-506.

Bertoluci, M.C., Cé, G.V., da Silva, A.M., Wainstein, M.V., Boff, W., Puñales, M., 2015. Endothelial dysfunction as a predictor of cardiovascular disease in type 1 diabetes. World J Diabetes 6, 679-692.

Bharadwaj LA, Prasad K (2002) Mechanism of superoxide anion-induced modulation of vascular tone. International Journal of Angiology 11(1):23-29 doi:10.1007/s00547-001-0049-5.

Böhl, M., Tietze, S., Sokoll, A., Madathil, S., Pfennig, F., Apostolakis, J., Fahmy, K., Gutzeit, H.O., 2007. Flavonoids affect actin functions in cytoplasm and nucleus. Biophysical journal 93, 2767-2780.

Bojić, M., Debeljak, Ž., Tomičić, M., Medić-Šarić, M., Tomić, S., 2011. Evaluation of antiaggregatory activity of flavonoid aglycone series. Nutr J 10, 73.

Bondonno, N.P., Bondonno, C.P., Rich, L., Mas, E., Shinde, S., Ward, N.C., Hodgson, J.M., Croft, K.D., 2016. Acute effects of quercetin-3-O-glucoside on endothelial function and blood pressure: a randomized dose-response study. The American Journal of Clinical Nutrition 104, 97-103.

Bondonno, N.P., Dalgaard, F., Kyrø, C., Murray, K., Bondonno, C.P., Lewis, J.R., Croft, K.D., Gislason, G., Scalbert, A., Cassidy, A., Tjønneland, A., Overvad, K., Hodgson, J.M., 2019. Flavonoid intake is associated with lower mortality in the Danish Diet Cancer and Health Cohort. Nature Communications 10, 3651.

Bretón-Romero, R., Lamas, S., 2014. Hydrogen peroxide signaling in vascular endothelial cells. Redox Biology 2, 529-534.

Bridson, J.H., Kaur, J., Zhang, Z., Donaldson, L., Fernyhough, A., 2015. Polymeric flavonoids processed with co-polymers as UV and thermal stabilisers for polyethylene films. Polymer Degradation and Stability 122, 18-24.

Brozovich, F.V., Nicholson, C.J., Degen, C.V., Gao, Y.Z., Aggarwal, M., Morgan, K.G., 2016. Mechanisms of Vascular Smooth Muscle Contraction and the Basis for Pharmacologic Treatment of Smooth Muscle Disorders. Pharmacol Rev 68, 476-532.

Busch, C., Burkard, M., Leischner, C., Lauer, U.M., Frank, J., Venturelli, S., 2015. Epigenetic activities of flavonoids in the prevention and treatment of cancer. Clinical Epigenetics 7, 64.

Bussey, C.E., Withers, S.B., Aldous, R.G., Edwards, G., Heagerty, A.M., 2016. Obesity-Related Perivascular Adipose Tissue Damage Is Reversed by Sustained Weight Loss in the Rat. Arterioscler Thromb Vasc Biol 36, 1377-1385.

Byon, C.H., Heath, J.M., Chen, Y., 2016. Redox signaling in cardiovascular pathophysiology: A focus on hydrogen peroxide and vascular smooth muscle cells. Redox Biology 9, 244-253.

Cao, G., Sofic, E., Prior, R.L., 1997. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. Free Radic Biol Med 22, 749-760.

Cao, H., Jia, Q., Shen, D., Yan, L., Chen, C., Xing, S., 2019a. Quercetin has a protective effect on atherosclerosis via enhancement of autophagy in ApoE(-/-) mice. Experimental and therapeutic medicine 18, 2451-2458.

Cao, H., Jia, Q., Yan, L., Chen, C., Xing, S., Shen, D., 2019b. Quercetin Suppresses the Progression of Atherosclerosis by Regulating MST1-Mediated Autophagy in ox-LDL-Induced RAW264.7 Macrophage Foam Cells. International Journal of Molecular Sciences 20, 6093.

Cao, Z.F.H., Stoffel, E., Cohen, P., 2017. Role of Perivascular Adipose Tissue in Vascular Physiology and Pathology. Hypertension 69, 770-777.

Carbone, S., Canada, J.M., Billingsley, H.E., Siddiqui, M.S., Elagizi, A., Lavie, C.J., 2019. Obesity paradox in cardiovascular disease: where do we stand? Vasc Health Risk Manag 15, 89-100.

Carullo, G., Ahmed, A., Trezza, A., Spiga, O., Brizzi, A., Saponara, S., Fusi, F., Aiello, F., 2021. A multitarget semi-synthetic derivative of the flavonoid morin with improved in vitro vasorelaxant activity: Role of CaV1.2 and KCa1.1 channels. Biochemical Pharmacology 185, 114429.

Case AJ, Roessner CT, Tian J, Zimmerman MC (2016) Mitochondrial Superoxide Signaling Contributes to Norepinephrine-Mediated T-Lymphocyte Cytokine Profiles. PLOS ONE 11(10):e0164609-e0164609 doi:10.1371/journal.pone.0164609

Cassidy, A., Minihane, A.-M., 2017. The role of metabolism (and the microbiome) in defining the clinical efficacy of dietary flavonoids. The American Journal of Clinical Nutrition 105, 10-22.

Chandramohan, G., Al-Numair, K.S., Alsaif, M.A., Veeramani, C., 2014. Antidiabetic effect of kaempferol a flavonoid compound, on streptozotocin-induced diabetic rats with special reference to glycoprotein components. Progress in Nutrition 17, 50-57.

Chang, H., Lei, L., Zhou, Y., Ye, F., Zhao, G., 2018. Dietary Flavonoids and the Risk of Colorectal Cancer: An Updated Meta-Analysis of Epidemiological Studies. Nutrients 10, 950.

Chang, L., Villacorta, L., Li, R., Hamblin, M., Xu, W., Dou, C., Zhang, J., Wu, J., Zeng, R., Chen, Y.E., 2012a. Loss of perivascular adipose tissue on peroxisome proliferator-activated receptor-γ deletion in smooth muscle cells impairs intravascular thermoregulation and enhances atherosclerosis. Circulation 126, 1067-1078.

Chang, L., Villacota, L., Dou, C., Chen, E., Zhang, J., 2012b. Abstract 662: Perivascular Adipose Tissue-derived Prostaglandins Constrict Vessel. Hypertension 60, A662-A662.

Chekalina, N., Burmak, Y., Petrov, Y., Borisova, Z., Manusha, Y., Kazakov, Y., Kaidashev, I., 2018. Quercetin reduces the transcriptional activity of NF-kB in stable coronary artery disease. Indian Heart J 70, 593-597.

Chen, H., Montagnani, M., Funahashi, T., Shimomura, I., Quon, M.J., 2003. Adiponectin stimulates production of nitric oxide in vascular endothelial cells. J Biol Chem 278, 45021-45026.

Chen, J., Mangelinckx, S., Adams, A., Wang, Z.-t., Li, W.-l., De Kimpe, N., 2015. Natural flavonoids as potential herbal medication for the treatment of diabetes mellitus and its complications. Natural product communications 10, 1934578X1501000140.

Chen, K., Pittman, R.N., Popel, A.S., 2008. Nitric oxide in the vasculature: where does it come from and where does it go? A quantitative perspective. Antioxidants & Redox Signaling 10, 1185-1198.

Chen, S., Ding, Y., Tao, W., Zhang, W., Liang, T., Liu, C., 2012. Naringenin inhibits TNF-α induced VSMC proliferation and migration via induction of HO-1. Food and Chemical Toxicology 50, 3025-3031.

Cheng, C.K., Bakar, H.A., Gollasch, M., Huang, Y., 2018. Perivascular Adipose Tissue: the Sixth Man of the Cardiovascular System. Cardiovascular Drugs and Therapy 32, 481-502.

Cho, T.M., Peng, N., Clark, J.T., Novak, L., Roysommuti, S., Prasain, J., Wyss, J.M., 2007. Genistein Attenuates the Hypertensive Effects of Dietary NaCl in Hypertensive Male Rats. Endocrinology 148, 5396-5402.

Choi, H., Kim, C.-S., Yu, R., 2018. Quercetin Upregulates Uncoupling Protein 1 in White/Brown Adipose Tissues through Sympathetic Stimulation. JOMES 27, 102-109.

Choi, J.-H., Park, S.-E., Kim, S.-J., Kim, S., 2015. Kaempferol inhibits thrombosis and platelet activation. Biochimie 115.

Choi, J.H., Yun, J.W., 2016. Chrysin induces brown fat-like phenotype and enhances lipid metabolism in 3T3-L1 adipocytes. Nutrition 32, 1002-1010.

Chouchani, E. T., L. Kazak, M. P. Jedrychowski, G. Z. Lu, B. K. Erickson, J. Szpyt, K. A. Pierce, D. Laznik-Bogoslavski, R. Vetrivelan, C. B. Clish, A. J. Robinson, S. P. Gygi, and B. M. Spiegelman. 2016. Mitochondrial ROS regulate thermogenic energy expenditure and sulfenylation of UCP1. Nature 532 (7597):112-116.

Choy, K.W., Murugan, D., Leong, X.-F., Abas, R., Alias, A., Mustafa, M.R., 2019. Flavonoids as Natural Anti-Inflammatory Agents Targeting Nuclear Factor-Kappa B (NFκB) Signaling in Cardiovascular Diseases: A Mini Review. Frontiers in Pharmacology 10, 1295-1295.

Chun, O.K., Chung, S.J., Song, W.O., 2007. Estimated dietary flavonoid intake and major food sources of U.S. adults. J Nutr 137, 1244-1252.

Clark, J.L., Zahradka, P., Taylor, C.G., 2015. Efficacy of flavonoids in the management of high blood pressure. Nutr Rev 73, 799-822.

Conquer, J.A., Maiani, G., Azzini, E., Raguzzini, A., Holub, B.J., 1998. Supplementation with quercetin markedly increases plasma quercetin concentration without effect on selected risk factors for heart disease in healthy subjects. J Nutr 128, 593-597.

Costa, R.M., Filgueira, F.P., Tostes, R.C., Carvalho, M.H., Akamine, E.H., Lobato, N.S., 2016. H2O2 generated from mitochondrial electron transport chain in thoracic perivascular adipose tissue is crucial for modulation of vascular smooth muscle contraction. Vascul Pharmacol 84, 28-37.

D'Amelia, V., Aversano, R., Chiaiese, P., Carputo, D., 2018. The antioxidant properties of plant flavonoids: their exploitation by molecular plant breeding. Phytochemistry Reviews 17, 611-625.

Da Costa RM, Fais RS, Dechandt CRP, et al. (2017) Increased mitochondrial ROS generation mediates the loss of the anti-contractile effects of perivascular adipose tissue in high-fat diet obese mice. Br J Pharmacol 174(20):3527-3541 doi:10.1111/bph.13687

Dalgaard, F., Bondonno, N.P., Murray, K., Bondonno, C.P., Lewis, J.R., Croft, K.D., Kyrø, C., Gislason, G., Scalbert, A., Cassidy, A., Tjønneland, A., Overvad, K., Hodgson, J.M., 2019. Associations between habitual flavonoid intake and hospital admissions for atherosclerotic cardiovascular disease: a prospective cohort study. The Lancet Planetary Health 3, e450-e459.

D'Archivio, M., Filesi, C., Di Benedetto, R., Gargiulo, R., Giovannini, C., Masella, R., 2007. Polyphenols, dietary sources and bioavailability. Ann Ist Super Sanita 43, 348-361.

Darios, E.S., Winner, B.M., Charvat, T., Krasinksi, A., Punna, S., Watts, S.W., 2016. The adipokine chemerin amplifies electrical field-stimulated contraction in the isolated rat superior mesenteric artery. American Journal of Physiology-Heart and Circulatory Physiology 311, H498-H507.

Dashwood, M.R., Dooley, A., Shi-Wen, X., Abraham, D.J., Souza, D.S., 2007. Does periadventitial fat-derived nitric oxide play a role in improved saphenous vein graft patency in patients undergoing coronary artery bypass surgery? J Vasc Res 44, 175-181.

Day, A.J., Cañada, F.J., Díaz, J.C., Kroon, P.A., McLauchlan, R., Faulds, C.B., Plumb, G.W., Morgan, M.R.A., Williamson, G., 2000. Dietary flavonoid and isoflavone glycosides are hydrolysed by the lactase site of lactase phlorizin hydrolase. FEBS Letters 468, 166-170.

Day, A.J., Gee, J.M., DuPont, M.S., Johnson, I.T., Williamson, G., 2003. Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. Biochem Pharmacol 65, 1199-1206.

Del Valle, J.C., Buide, M.L., Casimiro-Soriguer, I., Whittall, J.B., Narbona, E., 2015. On flavonoid accumulation in different plant parts: variation patterns among individuals and populations in the shore campion (Silene littorea). Frontiers in Plant Science 6, 939-939.

Dell'Agli, M., Maschi, O., Galli, G., Fagnani, R., Cero, E., Caruso, D., Bosisio, E., 2008. Inhibition of platelet aggregation by olive oil phenols via cAMP-phosphodiesterase. The British journal of nutrition 99, 945-951.

Deng, Q., Li, X.X., Fang, Y., Chen, X., Xue, J., 2020. Therapeutic Potential of Quercetin as an Antiatherosclerotic Agent in Atherosclerotic Cardiovascular Disease: A Review. Evidence-Based Complementary and Alternative Medicine 2020, 5926381.

Duarte, J., Francisco, V., Perez-Vizcaino, F., 2014. Modulation of nitric oxide by flavonoids. Food Funct 5, 1653-1668.

Duarte, J., Pérez-Palencia, R., Vargas, F., Ocete, M.A., Pérez-Vizcaino, F., Zarzuelo, A., Tamargo, J., 2001. Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats. British Journal of Pharmacology 133, 117-124.

Duarte, J., Pérez-Vizcaíno, F., Zarzuelo, A., Jiménez, J., Tamargo, J., 1993. Vasodilator effects of quercetin in isolated rat vascular smooth muscle. European Journal of Pharmacology 239, 1-7.

Dubrovska, G., Verlohren, S., Luft, F.C., Gollasch, M., 2004. Mechanisms of ADRF release from rat aortic adventitial adipose tissue. American Journal of Physiology-Heart and Circulatory Physiology 286, H1107-H1113.

Edwards, R.L., Lyon, T., Litwin, S.E., Rabovsky, A., Symons, J.D., Jalili, T., 2007. Quercetin reduces blood pressure in hypertensive subjects. J Nutr 137, 2405-2411.

Egert, S., Bosy-Westphal, A., Seiberl, J., Kürbitz, C., Settler, U., Plachta-Danielzik, S., Wagner, A.E., Frank, J., Schrezenmeir, J., Rimbach, G., Wolffram, S., Müller, M.J., 2009. Quercetin reduces systolic blood pressure and plasma oxidised low-density lipoprotein concentrations in overweight subjects with a high-cardiovascular disease risk phenotype: a double-blinded, placebo-controlled cross-over study. Br J Nutr 102, 1065-1074.

Egert, S., Rimbach, G., 2011. Which sources of flavonoids: complex diets or dietary supplements? Adv Nutr 2, 8-14.

Eker, M.E., Aaby, K., Budic-Leto, I., Rimac Brnčić, S., El, S.N., Karakaya, S., Simsek, S., Manach, C., Wiczkowski, W., de Pascual-Teresa, S., 2020. A Review of Factors Affecting Anthocyanin Bioavailability: Possible Implications for the Inter-Individual Variability. Foods 9, 2.

Emilova, R., Dimitrova, D.Z., Mladenov, M., Hadzi-Petrushev, N., Daneva, T., Padeshki, P., Schubert, R., Chichova, M., Lubomirov, L., Simeonovska-Nikolova, D., Gagov, H., 2016. Diabetes converts arterial regulation by perivascular adipose tissue from relaxation into H(2)O(2)-mediated contraction. Physiol Res 65, 799-807.

Engelbert, A.K., Soukup, S.T., Roth, A., Hoffmann, N., Graf, D., Watzl, B., Kulling, S.E., Bub, A., 2016. Isoflavone supplementation in postmenopausal women does not affect leukocyte LDL receptor and scavenger receptor CD36 expression: A double-blind, randomized, placebo-controlled trial. Molecular Nutrition & Food Research 60, 2008-2019.

Escobar-Cévoli, R., Castro-Espín, C., Béraud, V., Buckland, G., Zamora-Ros, R., Béraud, G., 2017. An overview of global flavonoid intake and its food sources. Flavonoids-From Biosynthesis to Human Health.

Estevez, B., Du, X., 2017. New Concepts and Mechanisms of Platelet Activation Signaling. Physiology 32, 162-177.

Falcone, F., Maria, L., Rius, S., Casati, P., 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Frontiers in Plant Science 3.

Fanelli Kuczmarski, M., Sebastian, R.S., Goldman, J.D., Murayi, T., Steinfeldt, L.C., Eosso, J.R., Moshfegh, A.J., Zonderman, A.B., Evans, M.K., 2018. Dietary Flavonoid Intakes Are Associated with Race but Not Income in an Urban Population. Nutrients 10, 1749.

Fang, J.Y., Lin, C.H., Huang, T.H., Chuang, S.Y., 2019. In Vivo Rodent Models of Type 2 Diabetes and Their Usefulness for Evaluating Flavonoid Bioactivity. Nutrients 11.

Fang, L., Zhao, J., Chen, Y., Ma, T., Xu, G., Tang, C., Liu, X., Geng, B., 2009. Hydrogen sulfide derived from periadventitial adipose tissue is a vasodilator. J Hypertens 27, 2174-2185.

Feng, L.-J., huan-yu, C., Ying, K.-J., Hua, J., Dai, X.-Y., 2011. Hypolipidemic and antioxidant effects of total flavonoids of Perilla Frutescens leaves in hyperlipidemia rats induced by high-fat diet. Food Research International - FOOD RES INT 44, 404-409.

Ferland, D.J., Darios, E.S., Neubig, R.R., Sjögren, B., Truong, N., Torres, R., Dexheimer, T.S., Thompson, J.M., Watts, S.W., 2017. Chemerin-induced arterial contraction is G(i)- and calcium-dependent. Vascul Pharmacol 88, 30-41.

Fernández-Alfonso, M.S., Gil-Ortega, M., García-Prieto, C.F., Aranguez, I., Ruiz-Gayo, M., Somoza, B., 2013. Mechanisms of Perivascular Adipose Tissue Dysfunction in Obesity. International Journal of Endocrinology 2013, 402053.

Fernández-Alfonso, M.S., Somoza, B., Tsvetkov, D., Kuczmanski, A., Dashwood, M., Gil-Ortega, M., 2017. Role of Perivascular Adipose Tissue in Health and Disease. Compr Physiol 8, 23-59.

Ferrario, C.M., Trask, A.J., Jessup, J.A., 2005. Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. Am J Physiol Heart Circ Physiol 289, 29.

Fésüs, G., Dubrovska, G., Gorzelniak, K., Kluge, R., Huang, Y., Luft, F.C., Gollasch, M., 2007. Adiponectin is a novel humoral vasodilator. Cardiovasc Res 75, 719-727.

Fiorani, M., Accorsi, A., Cantoni, O., 2003. Human red blood cells as a natural flavonoid reservoir. Free Radic Res 37, 1331-1338.

Flood, E.D., Watts, S.W., 2020. Endogenous Chemerin from PVAT Amplifies Electrical Field-Stimulated Arterial Contraction: Use of the Chemerin Knockout Rat. Int J Mol Sci 21.

Fontes, Milene T., Paula, Suliana M., Lino, Caroline A., Senger, N., Couto, Gisele K., Barreto-Chaves, M.L.de M., Mill, José G., Rossoni, Luciana V., 2020. Renin–angiotensin system overactivation in perivascular adipose tissue contributes to vascular dysfunction in heart failure. Clinical Science 134, 3195-3211.

Forman, H.J., Davies, K.J., Ursini, F., 2014. How do nutritional antioxidants really work: nucleophilic tone and para-hormesis versus free radical scavenging in vivo. Free Radic. Biol. Med. 66, 24–35.

Forstermann, U., Sessa, W., 2011. Nitric Oxide Synthases: Regulation and Function. European Heart Journal 33, 829-837, 837a.

Forte, M., Conti, V., Damato, A., Ambrosio, M., Puca, A.A., Sciarretta, S., Frati, G., Vecchione, C., Carrizzo, A., 2016. Targeting Nitric Oxide with Natural Derived Compounds as a Therapeutic Strategy in Vascular Diseases. Oxid Med Cell Longev 2016, 7364138.

Fouache, A., Zabaiou, N., De Joussineau, C., Morel, L., Silvente-Poirot, S., Namsi, A., Lizard, G., Poirot, M., Makishima, M., Baron, S., Lobaccaro, J.-M.A., Trousson, A., 2019. Flavonoids differentially modulate liver X receptors activity—Structure-function relationship analysis. The Journal of Steroid Biochemistry and Molecular Biology 190, 173-182.

Freedman, J.E., Parker, C., Li, L., Perlman, J.A., Frei, B., Ivanov, V., Deak, L.R., Iafrati, M.D., Folts, J.D., 2001. Select Flavonoids and Whole Juice From Purple Grapes Inhibit Platelet Function and Enhance Nitric Oxide Release. Circulation 103, 2792-2798.

Frostegård, J., 2013. Immunity, atherosclerosis and cardiovascular disease. BMC Medicine 11, 117.

Fu, M., Xu, L., Chen, X., Han, W., Ruan, C., Li, J., Cai, C., Ye, M., Gao, P., 2019. Neural Crest Cells Differentiate Into Brown Adipocytes and Contribute to Periaortic Arch Adipose Tissue Formation. Arteriosclerosis, Thrombosis, and Vascular Biology 39, 1629-1644.

Fu, X.C., Wang, M.W., Li, S.P., Zhang, Y., Wang, H.L., 2005. Vasodilatation produced by orientin and its mechanism study. Biol Pharm Bull 28, 37-41.

Fuchs, F.D., Whelton, P.K., 2020. High Blood Pressure and Cardiovascular Disease. Hypertension 75, 285-292.

Fusi F, Saponara S, Frosini M, Gorelli B, Sgaragli G. 2003. L-type Ca2+ channels activation and contraction elicited by myricetin on vascular smooth muscles. *Naunyn-Schmiedeberg's Archives of Pharmacology* 368: 470-8.

Fusi, F., Spiga, O., Trezza, A., Sgaragli, G., Saponara, S., 2017. The surge of flavonoids as novel, fine regulators of cardiovascular Ca(v) channels. Eur J Pharmacol 796, 158-174.

Fusi, F., Trezza, A., Tramaglino, M., Sgaragli, G., Saponara, S., Spiga, O., 2020. The beneficial health effects of flavonoids on the cardiovascular system: Focus on $K^+$ channels. Pharmacological Research 152, 104625.

Galindo, P., Rodriguez-Gómez, I., González-Manzano, S., Dueñas, M., Jiménez, R., Menéndez, C., Vargas, F., Tamargo, J., Santos-Buelga, C., Pérez-Vizcaíno, F., Duarte, J., 2012. Glucuronidated Quercetin Lowers Blood Pressure in Spontaneously Hypertensive Rats via Deconjugation. PLOS ONE 7, e32673.

Galley, H.F., Webster, N.R., 2004. Physiology of the endothelium. British Journal of Anaesthesia 93, 105-113.

Galvez-Prieto, B., Bolbrinker, J., Stucchi, P., de Las Heras, A.I., Merino, B., Arribas, S., Ruiz-Gayo, M., Huber, M., Wehland, M., Kreutz, R., Fernandez-Alfonso, M.S., 2008. Comparative expression analysis of the renin-angiotensin system components between white and brown perivascular adipose tissue. J Endocrinol 197, 55-64.

Gálvez-Prieto, B., Somoza, B., Gil-Ortega, M., F García-Prieto, C., de las Heras, A., González, M., Arribas, S., Aranguez, I., Bolbrinker, J., Kreutz, R., Ruiz-Gayo, M., Fernández-Alfonso, M., 2012. Anticontractile Effect of Perivascular Adipose Tissue and Leptin are Reduced in Hypertension. Frontiers in Pharmacology 3.

Gan, R. T., W. M. Li, C. H. Xiu, J. X. Shen, X. Wang, S. Wu, and Y. H. Kong. 2007. Chronic blocking of beta 3-adrenoceptor ameliorates cardiac function in rat model of heart failure. *Chin Med J* 120 (24):2250-2255.

Gao, X., Cassidy, A., Schwarzschild, M.A., Rimm, E.B., Ascherio, A., 2012. Habitual intake of dietary flavonoids and risk of Parkinson disease. Neurology 78, 1138-1145.

Gao, Y.J., Lu, C., Su, L.Y., Sharma, A.M., Lee, R.M., 2007. Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. Br J Pharmacol 151, 323-331.

Gao, Y.J., Takemori, K., Su, L.Y., An, W.S., Lu, C., Sharma, A.M., Lee, R.M., 2006. Perivascular adipose tissue promotes vasoconstriction: the role of superoxide anion. Cardiovasc Res 71, 363-373.

Gao, Y.-J., Zeng, Z.-h., Teoh, K., Sharma, A.M., Abouzahr, L., Cybulsky, I., Lamy, A., Semelhago, L., Lee, R.M.K.W., 2005. Perivascular adipose tissue modulates vascular function in the human internal thoracic artery. The Journal of Thoracic and Cardiovascular Surgery 130, 1130-1136.

Garate-Carrillo, A., Navarrete-Yañez, V., Ortiz-Vilchis, P., Guevara, G., Castillo, C., Mendoza-Lorenzo, P., Ceballos, G., Ortiz-Flores, M., Najera, N., Bustamante-Pozo, M.M., Rubio-Gayosso, I., Villarreal, F., Ramirez-Sanchez, I., 2020. Arginase inhibition by (-)-Epicatechin reverses endothelial cell aging. European Journal of Pharmacology 885, 173442-173442.

Gaspar, R.S., da Silva, S.A., Stapleton, J., Fontelles, J.L.d.L., Sousa, H.R., Chagas, V.T., Alsufyani, S., Trostchansky, A., Gibbins, J.M., Paes, A.M.d.A., 2020. Myricetin, the Main Flavonoid in Syzygium cumini Leaf, Is a Novel Inhibitor of Platelet Thiol Isomerases PDI and ERp5. Frontiers in Pharmacology 10.

Gecibesler, I.H., Aydin, M., 2020. Plasma Protein Binding of Herbal-Flavonoids to Human Serum Albumin and Their Anti-proliferative Activities. Anais da Academia Brasileira de Ciências 92.

Gil-Cardoso, K., Ginés, I., Pinent, M., Ardévol, A., Blay, M., Terra, X., 2016. Effects of flavonoids on intestinal inflammation, barrier integrity and changes in gut microbiota during diet-induced obesity. Nutr Res Rev 29, 234-248.

Gil-Ortega, M., Condezo-Hoyos, L., García-Prieto, C.F., Arribas, S.M., González, M.C., Aranguez, I., Ruiz-Gayo, M., Somoza, B., Fernández-Alfonso, M.S., 2014. Imbalance between pro and anti-oxidant mechanisms in perivascular adipose tissue aggravates long-term high-fat diet-derived endothelial dysfunction. PLOS ONE 9, e95312-e95312.

Gil-Ortega, M., Somoza, B., Huang, Y., Gollasch, M., Fernández-Alfonso, M., 2015. Regional differences in perivascular adipose tissue impacting vascular homeostasis. Trends in Endocrinology & Metabolism 26.

Gil-Ortega, M., Stucchi, P., Guzmán-Ruiz, R., Cano, V., Arribas, S., González, M.C., Ruiz-Gayo, M., Fernández-Alfonso, M.S., Somoza, B., 2010. Adaptative nitric oxide overproduction in perivascular adipose tissue during early diet-induced obesity. Endocrinology 151, 3299-3306.

Godse, S., Mohan, M., Kasture, V., Kasture, S., 2010. Effect of myricetin on blood pressure and metabolic alterations in fructose hypertensive rats. Pharmaceutical Biology 48, 494-498.

Goetz, M.E., Judd, S.E., Hartman, T.J., McClellan, W., Anderson, A., Vaccarino, V., 2016. Flavanone Intake Is Inversely Associated with Risk of Incident Ischemic Stroke in the REasons for Geographic and Racial Differences in Stroke (REGARDS) Study. The Journal of Nutrition 146, 2233-2243.

Gollasch, M., Welsh, D.G., Schubert, R., 2018. Perivascular adipose tissue and the dynamic regulation of K(v) 7 and K(ir) channels: Implications for resistant hypertension. Microcirculation 25, 12434.

Gomart, S., Gaudreau-Ménard, C., Jespers, P., Dilek, O.G., Hupkens, E., Hanthazi, A., Naeije, R., Melot, C., Labranche, N., Dewachter, L., Mc Entee, K., 2017. Leptin-Induced Endothelium-Independent Vasoconstriction in Thoracic Aorta and Pulmonary Artery of Spontaneously Hypertensive Rats: Role of Calcium Channels and Stores. PLOS ONE 12, e0169205-e0169205.

Gómez-Zorita, S., Lasa, A., Abendaño, N., Fernández-Quintela, A., Mosqueda-Solís, A., Garcia-Sobreviela, M.P., Arbonés-Mainar, J.M., Portillo, M.P., 2017. Phenolic compounds apigenin, hesperidin and kaempferol reduce in vitro lipid accumulation in human adipocytes. Journal of Translational Medicine 15, 237.

Gonzaga, N.A., Awata, W.M.C., do Vale, G.T., Marchi, K.C., Muniz, J.J., Tanus-Santos, J.E., Tirapelli, C.R., 2018. Perivascular adipose tissue protects against the vascular dysfunction induced by acute ethanol intake: Role of hydrogen peroxide. Vascul Pharmacol 111, 44-53.

Goto T, Teraminami A, Lee J-Y, Ohyama K, Funakoshi K, Kim Y-I, Hirai S, Uemura T, Yu R, Takahashi N, Kawada T. 2012. Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese–diabetic mice. *The Journal of Nutritional Biochemistry* 23: 768-76

Grassi, D., Desideri, G., Di Giosia, P., De Feo, M., Fellini, E., Cheli, P., Ferri, L., Ferri, C., 2013. Tea, flavonoids, and cardiovascular health: endothelial protection. Am J Clin Nutr 98, 30.

Grassi, D., Desideri, G., Ferri, C., 2010. Flavonoids: Antioxidants Against Atherosclerosis. Nutrients 2, 889-902.

Greenstein, A.S., Khavandi, K., Withers, S.B., Sonoyama, K., Clancy, O., Jeziorska, M., Laing, I., Yates, A.P., Pemberton, P.W., Malik, R.A., Heagerty, A.M., 2009. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. Circulation 119, 1661-1670.

Gregg, D., Goldschmidt-Clermont, P.J., 2003. Platelets and Cardiovascular Disease. Circulation 108, e88-e90.

Gu, P., Xu, A., 2013. Interplay between adipose tissue and blood vessels in obesity and vascular dysfunction. Reviews in Endocrine and Metabolic Disorders 14, 49-58.

Guerrero, J.A., Lozano, M.L., Castillo, J., Benavente-García, O., Vicente, V., Rivera, J., 2005. Flavonoids inhibit platelet function through binding to the thromboxane A2 receptor. J Thromb Haemost 3, 369-376.

Guerrero, L., Castillo, J., Quiñones, M., Garcia-Vallvé, S., Arola, L., Pujadas, G., & Muguerza, B. (2012). Inhibition of angiotensin-converting enzyme activity by flavonoids: structure-activity relationship studies. *PloS one*, *7*(11), e49493.

Guo, X.F., Ruan, Y., Li, Z.H., Li, D., 2019. Flavonoid subclasses and type 2 diabetes mellitus risk: a meta-analysis of prospective cohort studies. Crit Rev Food Sci Nutr 59, 2850-2862.

Gurney, A.M., 1994. Mechanisms of drug-induced vasodilation. J Pharm Pharmacol 46, 242-251.

Halver J, Wenzel K, Sendker J, Carrillo García C, Erdelmeier CAJ, Willems E, Mercola M, Symma N, Könemann S, Koch E, Hensel A, Schade D. 2019. Crataegus Extract WS®1442 Stimulates Cardiomyogenesis and Angiogenesis From Stem Cells: A Possible New Pharmacology for Hawthorn? *Frontiers in Pharmacology* 10.

Hamaguchi, T., Ito, M., Feng, J., Seko, T., Koyama, M., Machida, H., Takase, K., Amano, M., Kaibuchi, K., Hartshorne, D.J., Nakano, T., 2000. Phosphorylation of CPI-17, an Inhibitor of Myosin Phosphatase, by Protein Kinase N. Biochemical and biophysical research communications 274, 825-830.

Han, Y., Wu, J.-Z., Shen, J.-z., Chen, L., He, T., Jin, M.-w., Liu, H., 2017. Pentamethylquercetin induces adipose browning and exerts beneficial effects in 3T3-L1 adipocytes and high-fat diet-fed mice. Scientific Reports 7, 1123.

Hanrahan, J.R., Chebib, M., Johnston, G.A.R., 2011. Flavonoid modulation of GABA(A) receptors. British Journal of Pharmacology 163, 234-245.

Hanthazi, A., Gomart, S., Gaudreau Ménard, C., Jespers, P., Springael, J.-Y., Naeije, R., Dewachter, L., Mc Entee, K., 2015. Chemerin potentiates pulmonary artery reactivity to endothelin-1. European Respiratory Journal 46, PA2440.

Hashemzaei, M., Rezaee, R., Nabatzehi, M., Tsarouhas, K., Konstantinos Nikolouzakis, T., Lazopoulos, G., A Spandidos, D., Tsatsakis, A., Shahraki, J., 2020. Anti-hypertensive effect of crocin and hesperidin combination in high-fat diet treated rats. Experimental and therapeutic medicine 19, 3840-3844.

Hayabuchi, Y., Nakaya, Y., Matsuoka, S., Kuroda, Y., 1998. Hydrogen peroxide-induced vascular relaxation in porcine coronary arteries is mediated by $Ca^{2+}$-activated $K^{+}$ channels. Heart Vessels 13, 9-17.

He, F.-J., Chen, J.-Q., 2013. Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between Chinese women and women in Western countries and possible mechanisms. Food Science and Human Wellness 2, 146-161.

He, X.W., Yu, D., Li, W.L., Zheng, Z., Lv, C.L., Li, C., Liu, P., Xu, C.Q., Hu, X.F., Jin, X.P., 2016. Anti-atherosclerotic potential of baicalin mediated by promoting cholesterol efflux from macrophages via the PPARγ-LXRα-ABCA1/ABCG1 pathway. Biomed Pharmacother 83, 257-264.

Hendrich, A.B., 2006. Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. Acta Pharmacol Sin 27, 27-40.

Hollman, P.C.H., 2004. Absorption, Bioavailability, and Metabolism of Flavonoids. Pharmaceutical Biology 42, 74-83.

Hong, F., Haldeman, B.D., Jackson, D., Carter, M., Baker, J.E., Cremo, C.R., 2011. Biochemistry of smooth muscle myosin light chain kinase. Archives of Biochemistry and Biophysics 510, 135-146.

Hostetler, G.L., Ralston, R.A., Schwartz, S.J., 2017. Flavones: Food Sources, Bioavailability, Metabolism, and Bioactivity. Advances in Nutrition 8, 423-435.

Hou, D.-X., Kumamoto, T., 2010. Flavonoids as Protein Kinase Inhibitors for Cancer Chemoprevention: Direct Binding and Molecular Modeling. Antioxidants & Redox Signaling 13, 691-719.

Hua, F., Zhou, P., Wu, H.-Y., Chu, G.-X., Xie, Z.-W., Bao, G.-H., 2018. Inhibition of α-glucosidase and α-amylase by flavonoid glycosides from Lu'an GuaPian tea: molecular docking and interaction mechanism. Food & Function 9, 4173-4183.

Huai, R., Han, X., Wang, B., Li, C., Niu, Y., Li, R., Qu, Z., 2013. Vasorelaxing and Antihypertensive Effects of 7,8-Dihydroxyflavone. Am J Hypertens 27, 750-760.

Huang, Y.-y., J.-m. Wu, T. Su, S.-y. Zhang, and X.-j. Lin. 2018. Fasudil, a Rho-Kinase Inhibitor, Exerts Cardioprotective Function in Animal Models of Myocardial Ischemia/Reperfusion Injury: A Meta-Analysis and Review of Preclinical Evidence and Possible Mechanisms. *Frontiers in Pharmacology* 9 (1083).

Hubbard, G., Wolffram, S., Lovegrove, J., Gibbins, J., 2003. The role of polyphenolic compounds in the diet as inhibitors of platelet function. The Proceedings of the Nutrition Society 62, 469-478.

Huxley, R., Lee, C.M., Barzi, F., Timmermeister, L., Czernichow, S., Perkovic, V., Grobbee, D.E., Batty, D., Woodward, M., 2009. Coffee, decaffeinated coffee, and tea consumption in relation to incident type 2 diabetes mellitus: a systematic review with meta-analysis. Arch Intern Med 169, 2053-2063.

Iesaki, T., Gupte, S.A., Kaminski, P.M., Wolin, M.S., 1999. Inhibition of guanylate cyclase stimulation by NO and bovine arterial relaxation to peroxynitrite and H2O2. Am J Physiol 277.

Iida, Y., Doi, T., Matsushima-Nishiwaki, R., Tokuda, H., Ogura, S., Kozawa, O., Iida, H., 2014. (-)-Epigallocatechin gallate selectively inhibits adenosine diphosphate stimulated human platelet activation: Suppression of heat shock protein 27 phosphorylation via p38 mitogen activated protein kinase. Mol Med Rep 10, 1383-1388.

Imran, A., Butt, M.S., Arshad, M.S., Arshad, M.U., Saeed, F., Sohaib, M., Munir, R., 2018. Exploring the potential of black tea based flavonoids against hyperlipidemia related disorders. Lipids in Health and Disease 17, 57-57.

Iozzi, D., Schubert, R., Kalenchuk, V.U., Neri, A., Sgaragli, G., Fusi, F., Saponara, S., 2013. Quercetin relaxes rat tail main artery partly via a PKG-mediated stimulation of KCa1.1 channels. Acta Physiologica 208, 329-339.

Iside, C., Scafuro, M., Nebbioso, A., Altucci, L., 2020. SIRT1 Activation by Natural Phytochemicals: An Overview. Frontiers in Pharmacology 11.

Izzi, V., Masuelli, L., Tresoldi, I., Sacchetti, P., Modesti, A., Galvano, F., Bei, R., 2012. The effects of dietary flavonoids on the regulation of redox inflammatory networks. Frontiers in bioscience : a journal and virtual library 17, 2396-2418.

Jaffri, J.M., Mohamed, S., Rohimi, N., Ahmad, I.N., Noordin, M.M., Manap, Y.A., 2011. Antihypertensive and cardiovascular effects of catechin-rich oil palm (Elaeis guineensis) leaf extract in nitric oxide-deficient rats. J Med Food 14, 775-783.

Jagannathan, R., Patel, S., Ali, M., Narayan, K.M.V., 2019. Global Updates on Cardiovascular Disease Mortality Trends and Attribution of Traditional Risk Factors. Current Diabetes Reports 19.

Jain, D., Bansal, M.K., Dalvi, R., Upganlawar, A., Somani, R., 2014. Protective effect of diosmin against diabetic neuropathy in experimental rats. J Integr Med 12, 35-41.

Jamroz-Wiśniewska, A., Gertler, A., Solomon, G., Wood, M.E., Whiteman, M., Bełtowski, J., 2014. Leptin-induced endothelium-dependent vasorelaxation of peripheral arteries in lean and obese rats: role of nitric oxide and hydrogen sulfide. PLOS ONE 9, e86744-e86744.

Javadi, F., Eghtesadi, S., Ahmadzadeh, A., Aryaeian, N., Zabihiyeganeh, M., Foroushani, A.R., Jazayeri, S., 2014. The effect of quercetin on plasma oxidative status, C-reactive protein and blood pressure in women with rheumatoid arthritis. Int J Prev Med 5, 293-301.

Jayachitra, J., Nalini, N., 2012. EFFECT OF NARINGENIN (CITRUS FLAVANONE) ON LIPID PROFILE IN ETHANOL-INDUCED TOXICITY IN RATS. Journal of Food Biochemistry 36, 502-511.

Jayaraman, R., Subramani, S., Sheik Abdullah, S.H., Udaiyar, M., 2018. Antihyperglycemic effect of hesperetin, a citrus flavonoid, extenuates hyperglycemia and exploring the potential role in antioxidant and antihyperlipidemic in streptozotocin-induced diabetic rats. Biomedicine & Pharmacotherapy 97, 98-106.

Je, H.D., Kim, H.-D., La, H.-O., 2014. The Inhibitory Effect of Apigenin on the Agonist-Induced Regulation of Vascular Contractility via Calcium Desensitization-Related Pathways. Biomol Ther (Seoul) 22, 100-105.

Jennings, A., Welch, A.A., Spector, T., Macgregor, A., Cassidy, A., 2014. Intakes of anthocyanins and flavones are associated with biomarkers of insulin resistance and inflammation in women. J Nutr 144, 202-208.

Jia, Q., Cao, H., Shen, D., Yan, L., Chen, C., Xing, S., 2019. Fisetin, via CKIP-1/REGγ, limits oxidized LDL-induced lipid accumulation and senescence in RAW264.7 macrophage-derived foam cells. Eur J Pharmacol 865, 23.

Jiang, B., Geng, Q., Li, T., Mohammad Firdous, S., Zhou, X., 2020a. Morin attenuates STZ-induced diabetic retinopathy in experimental animals. Saudi J Biol Sci 27, 2139-2142.

Jiang, Y.-H., Jiang, L.-Y., Wang, Y.-C., Ma, D.-F., Li, X., 2020b. Quercetin Attenuates Atherosclerosis via Modulating Oxidized LDL-Induced Endothelial Cellular Senescence. Frontiers in Pharmacology 11.

Jin L, Ying Z, Webb RC (2004) Activation of Rho/Rho kinase signaling pathway by reactive oxygen species in rat aorta. Am J Physiol Heart Circ Physiol 287(4)

Jin, U.-H., Park, H., Li, X., Davidson, L.A., Allred, C., Patil, B., Jayaprakasha, G., Orr, A.A., Mao, L., Chapkin, R.S., Jayaraman, A., Tamamis, P., Safe, S., 2018. Structure-Dependent Modulation of Aryl Hydrocarbon Receptor-Mediated Activities by Flavonoids. Toxicological Sciences 164, 205-217.

Jin, Y.R., Han, X.H., Zhang, Y.H., Lee, J.J., Lim, Y., Chung, J.H., Yun, Y.P., 2007. Antiplatelet activity of hesperetin, a bioflavonoid, is mainly mediated by inhibition of PLC-gamma2 phosphorylation and cyclooxygenase-1 activity. Atherosclerosis 194, 144-152.

Jones, H.S., Gordon, A., Magwenzi, S.G., Naseem, K., Atkin, S.L., Courts, F.L., 2016. The dietary flavonol quercetin ameliorates angiotensin II-induced redox signaling imbalance in a human umbilical vein endothelial cell model of endothelial dysfunction via ablation of p47phox expression. Mol Nutr Food Res 60, 787-797.

Josefsson, E., Bergquist, J., Ekman, R., Tarkowski, A., 1996. Catecholamines are synthesized by mouse lymphocytes and regulate function of these cells by induction of apoptosis. Immunology 88, 140-146.

Jourde-Chiche, N., Fakhouri, F., Dou, L., Bellien, J., Burtey, S., Frimat, M., Jarrot, P.-A., Kaplanski, G., Le Quintrec, M., Pernin, V., Rigothier, C., Sallée, M., Fremeaux-Bacchi, V., Guerrot, D., Roumenina, L.T., 2019. Endothelium structure and function in kidney health and disease. Nature Reviews Nephrology 15, 87-108.

Jucá, M.M., Cysne Filho, F.M.S., de Almeida, J.C., Mesquita, D.d.S., Barriga, J.R.d.M., Dias, K.C.F., Barbosa, T.M., Vasconcelos, L.C., Leal, L.K.A.M., Ribeiro, J.E., Vasconcelos, S.M.M., 2020. Flavonoids: biological activities and therapeutic potential. Natural Product Research 34, 692-705.

Jung, U.J., Cho, Y.Y., Choi, M.S., 2016. Apigenin Ameliorates Dyslipidemia, Hepatic Steatosis and Insulin Resistance by Modulating Metabolic and Transcriptional Profiles in the Liver of High-Fat Diet-Induced Obese Mice. Nutrients 8.

Juźwiak, S., Wójcicki, J., Mokrzycki, K., Marchlewicz, M., Białecka, M., Wenda-Rózewicka, L., Gawrońska-Szklarz, B., Droździk, M., 2005. Effect of quercetin on experimental hyperlipidemia and atherosclerosis in rabbits. Pharmacol Rep 57, 604-609.

Kamiloglu, S., Tomas, M., Ozdal, T., Capanoglu, E., 2020. Effect of food matrix on the content and bioavailability of flavonoids. Trends in Food Science & Technology.

Kassam, S.I., Lu, C., Buckley, N., Gao, Y.J., Lee, R.M., 2012. Modulation of thiopental-induced vascular relaxation and contraction by perivascular adipose tissue and endothelium. Br J Anaesth 109, 177-184.

Kassam, S.I., Lu, C., Buckley, N., Lee, R.M., 2011. The mechanisms of propofol-induced vascular relaxation and modulation by perivascular adipose tissue and endothelium. Anesth Analg 112, 1339-1345.

Kent, K., Charlton, K.E., Russell, J., Mitchell, P., Flood, V.M., 2015. Estimation of Flavonoid Intake in Older Australians: Secondary Data Analysis of the Blue Mountains Eye Study. J Nutr Gerontol Geriatr 34, 388-398.

Ketonen, J., Shi, J., Martonen, E., Mervaala, E., 2010. Periadventitial adipose tissue promotes endothelial dysfunction via oxidative stress in diet-induced obese C57Bl/6 mice. Circ J 74, 1479-1487.

Khalil, R.A., 2013. Protein Kinase C Inhibitors as Modulators of Vascular Function and Their Application in Vascular Disease. Pharmaceuticals 6, 407-439.

Khan, M., Zill, E.H., Dangles, O., 2013. A comprehensive review on flavanones, the major citrus polyphenols. Journal of Food Composition and Analysis 33.

Khoo, N.K.H., White, C.R., Pozzo-Miller, L., Zhou, F., Constance, C., Inoue, T., Patel, R.P., Parks, D.A., 2010. Dietary flavonoid quercetin stimulates vasorelaxation in aortic vessels. Free Radic Biol Med 49, 339-347.

Kicinska, A., Jarmuszkiewicz, W., 2020. Flavonoids and Mitochondria: Activation of Cytoprotective Pathways? Molecules (Basel, Switzerland) 25, 3060.

Kim, C.E., Han, S., Kim, M.H., Kim, S.-W., 2015a. Flavonoids activate endothelial nitric oxide synthase by altering their phosphorylation via mitogen-activated protein kinase pathways in glucose-induced endothelial cells. Journal of Functional Foods 17, 676-684.

Kim, H.P., Son, K.H., Chang, H.W., Kang, S.S., 2004. Anti-inflammatory Plant Flavonoids and Cellular Action Mechanisms. Journal of Pharmacological Sciences 96, 229-245.

Kim, K., Kim, S., Moh, S.H., Kang, H., 2015b. Kaempferol inhibits vascular smooth muscle cell migration by modulating BMP-mediated miR-21 expression. Mol Cell Biochem 407, 143-149.

Kim, S.W., Cuong, T.D., Hung, T.M., Ryoo, S., Lee, J.H., Min, B.S., 2013. Arginase II inhibitory activity of flavonoid compounds from Scutellaria indica. Archives of Pharmacal Research 36, 922-926.

Kim, T.-J., Zhang, Y.-H., Kim, Y., Lee, C.-K., Lee, M.-K., Hong, J.-T., Yun, Y.-P., 2002. Effects of apigenin on the serum- and platelet derived growth factor-BB-induced proliferation of rat aortic vascular smooth muscle cells. Planta medica 68, 605-609.

Kim, Y., Je, Y., 2017. Flavonoid intake and mortality from cardiovascular disease and all causes: A meta-analysis of prospective cohort studies. Clinical Nutrition ESPEN 20, 68-77.

Köhn, C., Schleifenbaum, J., Szijártó, I.A., Markó, L., Dubrovska, G., Huang, Y., Gollasch, M., 2012. Differential effects of cystathionine-γ-lyase-dependent vasodilatory H2S in periadventitial vasoregulation of rat and mouse aortas. PLOS ONE 7, 3.

Kong, L., Luo, C., Li, X., Zhou, Y., He, H., 2013. The anti-inflammatory effect of kaempferol on early atherosclerosis in high cholesterol fed rabbits. Lipids Health Dis 12, 12-115.

Koshy, A., Vijayalakshmi, N., 2001. Impact of certain flavonoids on lipid profiles - Potential action of Garcinia cambogia flavonoids. Phytotherapy Research 15, 395-400.

Koyama M, Ito M, Feng J, et al. (2000) Phosphorylation of CPI-17, an inhibitory phosphoprotein of smooth muscle myosin phosphatase, by Rho-kinase. FEBS Lett 475(3):197-200.

Krga, I., Milenkovic, D., 2019. Anthocyanins: From Sources and Bioavailability to Cardiovascular-Health Benefits and Molecular Mechanisms of Action. Journal of Agricultural and Food Chemistry 67, 1771-1783.

Křížová, L., Dadáková, K., Kašparovská, J., Kašparovský, T., 2019. Isoflavones. Molecules (Basel, Switzerland) 24, 1076.

Kumar, R.K., Jin, Y., Watts, S.W., Rockwell, C.E., 2020. Naïve, Regulatory, Activated, and Memory Immune Cells Co-exist in PVATs That Are Comparable in Density to Non-PVAT Fats in Health. Frontiers in Physiology 11.

Kumar, S., Alagawadi, K.R., 2013. Anti-obesity effects of galangin, a pancreatic lipase inhibitor in cafeteria diet fed female rats. Pharmaceutical Biology 51, 607-613.

Kumar, S., Pandey, A.K., 2013. Chemistry and Biological Activities of Flavonoids: An Overview. The Scientific World Journal 2013, 162750.

Kumar, T., Sharma, M., Rana, A., Lingaraju, M.C., Parida, S., Kumar, D., Singh, T.U., 2020. Biochanin-A elicits relaxation in coronary artery of goat through different mechanisms. Research in Veterinary Science 131, 206-214.

Kwiecien, K., Brzoza, P., Bak, M., Majewski, P., Skulimowska, I., Bednarczyk, K., Cichy, J., Kwitniewski, M., 2020. The methylation status of the chemerin promoter region located from −252 to +258 bp regulates constitutive but not acute-phase cytokine-inducible chemerin expression levels. Scientific Reports 10, 13702.

Lacolley, P., Regnault, V., Nicoletti, A., Li, Z., Michel, J.-B., 2012. The vascular smooth muscle cell in arterial pathology: a cell that can take on multiple roles. Cardiovascular Research 95, 194-204.

Lacolley, P., Regnault, V., Segers, P., Laurent, S., 2017. Vascular Smooth Muscle Cells and Arterial Stiffening: Relevance in Development, Aging, and Disease. Physiological Reviews 97, 1555-1617.

Lacoste, L., Lam, J.Y.T., Hung, J., Letchacovski, G., Solymoss, C.B., Waters, D., 1995. Hyperlipidemia and Coronary Disease. Circulation 92, 3172-3177.

Lee, K.-H., Park, E., Lee, H.-J., Kim, M.-O., Cha, Y.-J., Kim, J.-M., Lee, H., Shin, M.-J., 2011. Effects of daily quercetin-rich supplementation on cardiometabolic risks in male smokers. Nutr Res Pract 5, 28-33.

Lee, R.H.-C., Couto E Silva, A., Possoit, H.E., Lerner, F.M., Chen, P.-Y., Azizbayeva, R., Citadin, C.T., Wu, C.Y.-C., Neumann, J.T., Lin, H.W., 2019. Palmitic acid methyl ester is a novel neuroprotective agent against cardiac arrest. Prostaglandins Leukot Essent Fatty Acids 147, 6-14.

Lee, R.M., Lu, C., Su, L.Y., Gao, Y.J., 2009. Endothelium-dependent relaxation factor released by perivascular adipose tissue. J Hypertens 27, 782-790.

Lee, Y.C., Chang, H.H., Chiang, C.L., Liu, C.H., Yeh, J.I., Chen, M.F., Chen, P.Y., Kuo, J.S., Lee, T.J., 2011. Role of perivascular adipose tissue-derived methyl palmitate in vascular tone regulation and pathogenesis of hypertension. Circulation 124, 1160-1171.

Lee, Y.-J., Seo, M.-J., Lee, O.-H., Kim, K.-J., Lee, B.-Y., 2017. Hesperetin inhibits lipid accumulation and ROS production during adipocyte differentiation in 3T3-L1 cells. Journal of Food Biochemistry 41, e12348.

Leon, B.M., Maddox, T.M., 2015. Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research. World J Diabetes 6, 1246-1258.

Li Volti, G., Salomone, S., Sorrenti, V., Mangiameli, A., Urso, V., Siarkos, I., Galvano, F., Salamone, F., 2011. Effect of silibinin on endothelial dysfunction and ADMA levels in obese diabetic mice. Cardiovascular Diabetology 10, 62.

Li, C., Zhang, W.-J., Frei, B., 2016. Quercetin inhibits LPS-induced adhesion molecule expression and oxidant production in human aortic endothelial cells by p38-mediated Nrf2 activation and antioxidant enzyme induction. Redox Biology 9, 104-113.

Li, H., Xia, N., Brausch, I., Yao, Y., Förstermann, U., 2004. Flavonoids from artichoke (Cynara scolymus L.) up-regulate endothelial-type nitric-oxide synthase gene expression in human endothelial cells. J Pharmacol Exp Ther 310, 926-932.

Li, M., Qian, M., Kyler, K., Xu, J., 2018. Endothelial–Vascular Smooth Muscle Cells Interactions in Atherosclerosis. Frontiers in Cardiovascular Medicine 5.

Li, P.G., Sun, L., Han, X., Ling, S., Gan, W.T., Xu, J.W., 2012. Quercetin induces rapid eNOS phosphorylation and vasodilation by an Akt-independent and PKA-dependent mechanism. Pharmacology 89, 220-228.

Li, S., Cao, H., Shen, D., Jia, Q., Chen, C., Xing, S.L., 2018. Quercetin protects against ox LDL induced injury via regulation of ABCAl, LXR α and PCSK9 in RAW264.7 macrophages. Mol Med Rep 18, 799-806.

Li, X.-Y., Kong, L.-X., Li, J., He, H.-X., Zhou, Y.-D., 2013. Kaempferol suppresses lipid accumulation in macrophages through the downregulation of cluster of differentiation 36 and the upregulation of scavenger receptor class B type I and ATP-binding cassette transporters A1 and G1. Int J Mol Med 31, 331-338.

Li, Y.C., Li, B.X., Geng, L.J., 2011. Hypolipidemic and antioxidant effects of total flavonoids from blueberry leaves. Eur Food Res Technol 233, 897-903.

Li, Z., Delaney, M.K., O'Brien, K.A., Du, X., 2010. Signaling During Platelet Adhesion and Activation. Arteriosclerosis, Thrombosis, and Vascular Biology 30, 2341-2349.

Liou, C.J., Wei, C.H., Chen, Y.L., Cheng, C.Y., Wang, C.L., Huang, W.C., 2018. Fisetin Protects Against Hepatic Steatosis Through Regulation of the Sirt1/AMPK and Fatty Acid β-Oxidation Signaling Pathway in High-Fat Diet-Induced Obese Mice. Cell Physiol Biochem 49, 1870-1884.

Liu, C.-H., Hsu, H.-J., Tseng, T.-L., Lin, T.-J., Weng, W.-H., Chen, M.-F., Lee, T.J.-F., 2020. COMT-catalyzed palmitic acid methyl ester biosynthesis in perivascular adipose tissue and its potential role against hypertension. Journal of Pharmacology and Experimental Therapeutics, jpet.119.263517.

Liu, G., Xie, W., He, A.D., Da, X.W., Liang, M.L., Yao, G.Q., Xiang, J.Z., Gao, C.J., Ming, Z.Y., 2016. Antiplatelet activity of chrysin via inhibiting platelet αIIbβ3-mediated signaling pathway. Mol Nutr Food Res 60, 1984-1993.

Liu, R., Heiss, E.H., Schachner, D., Jiang, B., Liu, W., Breuss, J.M., Dirsch, V.M., Atanasov, A.G., 2017a. Xanthohumol Blocks Proliferation and Migration of Vascular Smooth Muscle Cells in Vitro and Reduces Neointima Formation in Vivo. Journal of Natural Products 80, 2146-2150.

Liu, T., Zhang, L., Joo, D., Sun, S.-C., 2017b. NF-κB signaling in inflammation. Signal Transduction and Targeted Therapy 2, 17023.

Liu, Y., Jia, L., Min, D., Xu, Y., Zhu, J., Sun, Z., 2019. Baicalin inhibits proliferation and promotes apoptosis of vascular smooth muscle cells by regulating the MEG3/p53 pathway following treatment with ox LDL. Int J Mol Med 43, 901-913.

Liu, Y.-J., Zhan, J., Liu, X.-L., Wang, Y., Ji, J., He, Q.-Q., 2014. Dietary flavonoids intake and risk of type 2 diabetes: A meta-analysis of prospective cohort studies. Clinical Nutrition 33, 59-63.

Liu, Z., Khalil, R.A., 2018. Evolving mechanisms of vascular smooth muscle contraction highlight key targets in vascular disease. Biochemical Pharmacology 153, 91-122.

Llaneza, P., González, C., Fernández-Iñarrea, J., Alonso, A., Díaz, F., Pérez-López, F.R., 2012. Soy isoflavones improve insulin sensitivity without changing serum leptin among postmenopausal women. Climacteric 15, 611-620.

Lobato, N.S., Neves, K.B., Filgueira, F.P., Fortes, Z.B., Carvalho, M.H., Webb, R.C., Oliveira, A.M., Tostes, R.C., 2012. The adipokine chemerin augments vascular reactivity to contractile stimuli via activation of the MEK-ERK1/2 pathway. Life Sci 91, 600-606.

Loh, Y.C., Chan, S.Y., Tew, W.Y., Oo, C.W., Yam, M.F., 2020. New flavonoid-based compound synthesis strategy for antihypertensive drug development. Life Sciences 249, 117512.

Löhn, M., Dubrovska, G., Lauterbach, B., Luft, F.C., Gollasch, M., Sharma, A.M., 2002. Periadventitial fat releases a vascular relaxing factor. Faseb J 16, 1057-1063.

Lone, J., Parray, H.A., Yun, J.W., 2018. Nobiletin induces brown adipocyte-like phenotype and ameliorates stress in 3T3-L1 adipocytes. Biochimie 146, 97-104.

Lorenz, M., Wessler, S., Follmann, E., Michaelis, W., Düsterhöft, T., Baumann, G., Stangl, K., Stangl, V., 2004. A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-,

and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. J Biol Chem 279, 6190-6195.

Lu, C., Su, L.-Y., Lee, R., Gao, Y.-J., 2008. Superoxide anion mediates angiotensin II-induced potentiation of contractile response to sympathetic stimulation. European Journal of Pharmacology 589, 188-193.

Lu, C., Su, L.-Y., Lee, R.M.K.W., Gao, Y.-J., 2010. Mechanisms for perivascular adipose tissue-mediated potentiation of vascular contraction to perivascular neuronal stimulation: The role of adipocyte-derived angiotensin II. European Journal of Pharmacology 634, 107-112.

Lukaseder, B., Vajrodaya, S., Hehenberger, T., Seger, C., Nagl, M., Lutz-Kutschera, G., Robien, W., Greger, H., Hofer, O., 2009. Prenylated flavanones and flavanonols as chemical markers in Glycosmis species (Rutaceae). Phytochemistry 70, 1030-1037.

Luo, D., Xu, J., Chen, X., Zhu, X., Liu, S., Li, J., Xu, X., Ma, X., Zhao, J., Ji, X., 2020. (−)-Epigallocatechin-3-gallate (EGCG) attenuates salt-induced hypertension and renal injury in Dahl salt-sensitive rats. Scientific Reports 10, 4783.

Lv, B., Chen, S., Tang, C., Jin, H., Du, J., Huang, Y., 2020. Hydrogen sulfide and vascular regulation - An update. J Adv Res 27, 85-97.

Lv, G.Y., Zhang, Y.P., Gao, J.L., Yu, J.J., Lei, J., Zhang, Z.R., Li, B., Zhan, R.J., Chen, S.H., 2013. Combined antihypertensive effect of luteolin and buddleoside enriched extracts in spontaneously hypertensive rats. J Ethnopharmacol 150, 507-513.

Lynch, F.M., Withers, S.B., Yao, Z., Werner, M.E., Edwards, G., Weston, A.H., Heagerty, A.M., 2013. Perivascular adipose tissue-derived adiponectin activates BK(Ca) channels to induce anticontractile responses. Am J Physiol Heart Circ Physiol 304, 4.

Maaliki, D., Shaito, A.A., Pintus, G., El-Yazbi, A., Eid, A.H., 2019. Flavonoids in hypertension: a brief review of the underlying mechanisms. Current Opinion in Pharmacology 45, 57-65.

Macêdo, C.L., Vasconcelos, L.H.C., de Correia, A.C.C., Martins, I.R.R., de Lira, D.P., de O Santos, B.V., de A Cavalcante, F., Silva, B.A.d., 2014. Mechanisms underlying vasorelaxation induced in rat aorta by galetin 3,6-dimethyl ether, a flavonoid from Piptadenia stipulacea (Benth.) Ducke. Molecules (Basel, Switzerland) 19, 19678-19695.

Madkhali, H.A., 2020. Morin attenuates high-fat diet induced-obesity related vascular endothelial dysfunction in Wistar albino rats. Saudi Pharm J 28, 300-307.

Mahmoud, A., Ahmed, O., Ashour, M., Abdel Moneim, A., 2015. In vivo and in vitro antidiabetic effects of citrus flavonoids; A study on the mechanism of action. International Journal of Diabetes in Developing Countries 35, 250-263.

Mahobiya, A., Singh, T.U., Rungsung, S., Kumar, T., Chandrasekaran, G., Parida, S., Kumar, D., 2018. Kaempferol-induces vasorelaxation via endothelium-independent pathways in rat isolated pulmonary artery. Pharmacological Reports 70, 863-874.

Maiti, S., Nazmeen, A., Medda, N., Patra, R., Ghosh, T.K., 2019. Flavonoids green tea against oxidant stress and inflammation with related human diseases. Clinical Nutrition Experimental 24, 1-14.

Majed, B.H., Khalil, R.A., 2012. Molecular mechanisms regulating the vascular prostacyclin pathways and their adaptation during pregnancy and in the newborn. Pharmacol Rev 64, 540-582.

Majesky, M.W., 2015. Adventitia and perivascular cells. Arteriosclerosis, Thrombosis, and Vascular Biology 35, e31-e35.

Malik, S., Suchal, K., Khan, S.I., Bhatia, J., Kishore, K., Dinda, A.K., Arya, D.S., 2017. Apigenin ameliorates streptozotocin-induced diabetic nephropathy in rats via MAPK-NF-κB-TNF-α and TGF-β1-MAPK-fibronectin pathways. Am J Physiol Renal Physiol 313, F414-F422.

Man, A.W.C., Zhou, Y., Xia, N., Li, H., 2020. Perivascular Adipose Tissue as a Target for Antioxidant Therapy for Cardiovascular Complications. Antioxidants 9.

Manach, C., Donovan, J., 2004. Pharmacokinetics and metabolism of dietary flavonoids in humans. Free radical research 38, 771-785.

Mansuri, M.L., Parihar, P., Solanki, I., Parihar, M.S., 2014. Flavonoids in modulation of cell survival signalling pathways. Genes Nutr 9, 400-400.

Mariee, A.D., Abd-Allah, G.M., El-Beshbishy, H.A., 2012. Protective effect of dietary flavonoid quercetin against lipemic-oxidative hepatic injury in hypercholesterolemic rats. Pharm Biol 50, 1019-1025.

Marinko, M., Jankovic, G., Nenezic, D., Milojevic, P., Stojanovic, I., Kanjuh, V., Novakovic, A., 2018. (−)-Epicatechin-induced relaxation of isolated human saphenous vein: Roles of $K^+$ and $Ca^{2+}$ channels. Phytotherapy Research 32, 267-275.

Maron, D.J., Lu, G.P., Cai, N.S., Wu, Z.G., Li, Y.H., Chen, H., Zhu, J.Q., Jin, X.J., Wouters, B.C., Zhao, J., 2003. Cholesterol-Lowering Effect of a Theaflavin-Enriched Green Tea Extract: A Randomized Controlled Trial. Archives of Internal Medicine 163, 1448-1453.

Marranzano, M., Ray, S., Godos, J., Galvano, F., 2018. Association between dietary flavonoids intake and obesity in a cohort of adults living in the Mediterranean area. Int J Food Sci Nutr 69, 1020-1029.

Martínez-Fernández, L., Pons, Z., Margalef, M., Arola-Arnal, A., Muguerza, B., 2015. Regulation of vascular endothelial genes by dietary flavonoids: structure-expression relationship studies and the role of the transcription factor KLF-2. J Nutr Biochem 26, 277-284.

Martinez-Gonzalez, A.I., Alvarez-Parrilla, E., Díaz-Sánchez, Á.G., de la Rosa, L.A., Núñez-Gastélum, J.A., Vazquez-Flores, A.A., Gonzalez-Aguilar, G.A., 2017. In vitro Inhibition of Pancreatic Lipase by Polyphenols: A Kinetic, Fluorescence Spectroscopy and Molecular Docking Study. Food Technol Biotechnol 55, 519-530.

Martinez-Gonzalez, A.I., Díaz-Sánchez Á, G., de la Rosa, L.A., Bustos-Jaimes, I., Alvarez-Parrilla, E., 2019. Inhibition of α-amylase by flavonoids: Structure activity relationship (SAR). Spectrochim Acta A Mol Biomol Spectrosc 206, 437-447.

Mendizábal, Y., Llorens, S., Nava, E., 2013. Vasoactive effects of prostaglandins from the perivascular fat of mesenteric resistance arteries in WKY and SHROB rats. Life Sci 93, 1023-1032.

Mendonca, P., Soliman, K.F.A., 2020. Flavonoids Activation of the Transcription Factor Nrf2 as a Hypothesis Approach for the Prevention and Modulation of SARS-CoV-2 Infection Severity. Antioxidants 9.

Meyer, M.R., Fredette, N.C., Barton, M., Prossnitz, E.R., 2013. Regulation of Vascular Smooth Muscle Tone by Adipose-Derived Contracting Factor. PLOS ONE 8, e79245.

Miao, C.-Y., Li, Z.-Y., 2012. The role of perivascular adipose tissue in vascular smooth muscle cell growth. British Journal of Pharmacology 165, 643-658.

Mierziak, J., Kostyn, K., Kulma, A., 2014. Flavonoids as Important Molecules of Plant Interactions with the Environment. Molecules 19, 16240-16265.

Milutinović, A., Šuput, D., Zorc-Pleskovič, R., 2020. Pathogenesis of atherosclerosis in the tunica intima, media, and adventitia of coronary arteries: An updated review. Bosn J Basic Med Sci 20, 21-30.

Min, Y.S., Yoon, H.-J., Je, H.D., Lee, J.H., Yoo, S.S., Shim, H.S., Lee, H.Y., La, H.-O., Sohn, U.D., 2018. Endothelium Independent Effect of Pelargonidin on Vasoconstriction in Rat Aorta. Biomolecules & therapeutics 26, 374-379.

Miranda, A.M., Steluti, J., Fisberg, R.M., Marchioni, D.M., 2016. Dietary intake and food contributors of polyphenols in adults and elderly adults of Sao Paulo: a population-based study. British Journal of Nutrition 115, 1061-1070.

Miron, A., Aprotosoaie, A.C., Trifan, A., Xiao, J., 2017. Flavonoids as modulators of metabolic enzymes and drug transporters. Annals of the New York Academy of Sciences 1398, 152-167.

Mitchell, J.A., Ali, F., Bailey, L., Moreno, L., Harrington, L.S., 2008. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. Experimental Physiology 93, 141-147.

Miyake, Y., Suzuki, E., Ohya, S., Fukumoto, S., Hiramitsu, M., Sakaida, K., Osawa, T., Furuichi, Y., 2006. Lipid-Lowering Effect of Eriocitrin, the Main Flavonoid in Lemon Fruit, in Rats on a High-Fat and High-Cholesterol Diet. Journal of Food Science 71, S633-S637.

Momin, A.U., Melikian, N., Shah, A.M., Grieve, D.J., Wheatcroft, S.B., John, L., El Gamel, A., Desai, J.B., Nelson, T., Driver, C., Sherwood, R.A., Kearney, M.T., 2006. Leptin is an endothelial-independent vasodilator in humans with coronary artery disease: evidence for tissue specificity of leptin resistance. European Heart Journal 27, 2294-2299.

Morello, S., Vellecco, V., Alfieri, A., Mascolo, N., Cicala, C., 2006. Vasorelaxant effect of the flavonoid galangin on isolated rat thoracic aorta. Life Sciences 78, 825-830.

Moskot, M., Jakóbkiewicz-Banecka, J., Kloska, A., Smolińska, E., Mozolewski, P., Malinowska, M., Rychłowski, M., Banecki, B., Węgrzyn, G., Gabig-Cimińska, M., 2015. Modulation of expression of genes involved in glycosaminoglycan metabolism and lysosome biogenesis by flavonoids. Scientific Reports 5, 9378.

Mouradov, A., Spangenberg, G., 2014. Flavonoids: a metabolic network mediating plants adaptation to their real estate. Frontiers in Plant Science 5.

Muchtaridi M, Fauzi M, Khairul Ikram NK, Mohd Gazzali A, Wahab HA. Natural Flavonoids as Potential Angiotensin-Converting Enzyme 2 Inhibitors for Anti-SARS-CoV-2. Molecules. 2020;25(17):3980.

Mulvihill, E.E., Assini, J.M., Sutherland, B.G., DiMattia, A.S., Khami, M., Koppes, J.B., Sawyez, C.G., Whitman, S.C., Huff, M.W., 2010. Naringenin decreases progression of atherosclerosis by improving dyslipidemia in high-fat-fed low-density lipoprotein receptor-null mice. Arterioscler Thromb Vasc Biol 30, 742-748.

Muñoz, Y., Garrido, A., Valladares, L., 2009. Equol is more active than soy isoflavone itself to compete for binding to thromboxane A2 receptor in human platelets. Thrombosis Research 123, 740-744.

Münzel, T., Camici, G.G., Maack, C., Bonetti, N.R., Fuster, V., Kovacic, J.C., 2017. Impact of Oxidative Stress on the Heart and Vasculature: Part 2 of a 3-Part Series. Journal of the American College of Cardiology 70, 212-229.

Münzel, T., Gori, T., Bruno, R.M., Taddei, S., 2010. Is oxidative stress a therapeutic target in cardiovascular disease? European Heart Journal 31, 2741-2748.

Musunuru, K., 2010. Atherogenic Dyslipidemia: Cardiovascular Risk and Dietary Intervention. Lipids 45, 907-914.

Myoung, H.J., Kim, G., Nam, K.W., 2010. Apigenin isolated from the seeds of Perilla frutescens britton var crispa (Benth.) inhibits food intake in C57BL/6J mice. Arch Pharm Res 33, 1741-1746.

Nabavi, S.M., Šamec, D., Tomczyk, M., Milella, L., Russo, D., Habtemariam, S., Suntar, I., Rastrelli, L., Daglia, M., Xiao, J., Giampieri, F., Battino, M., Sobarzo-Sanchez, E., Nabavi, S.F., Yousefi, B., Jeandet, P., Xu, S., Shirooie, S., 2020. Flavonoid biosynthetic pathways in plants: Versatile targets for metabolic engineering. Biotechnology Advances 38, 107316.

Natella, F., Nardini, M., Belelli, F., Pignatelli, P., Di Santo, S., Ghiselli, A., Violi, F., Scaccini, C., 2008. Effect of coffee drinking on platelets: inhibition of aggregation and phenols incorporation. Br J Nutr 100, 1276-1282.

Nava, E., Llorens, S., 2019. The Local Regulation of Vascular Function: From an Inside-Outside to an Outside-Inside Model. Frontiers in Physiology 10.

Németh, K., Plumb, G.W., Berrin, J.G., Juge, N., Jacob, R., Naim, H.Y., Williamson, G., Swallow, D.M., Kroon, P.A., 2003. Deglycosylation by small intestinal epithelial cell beta-glucosidases is a critical step in the absorption and metabolism of dietary flavonoid glycosides in humans. Eur J Nutr 42, 29-42.

Nettleton, J.A., Harnack, L.J., Scrafford, C.G., Mink, P.J., Barraj, L.M., Jacobs, D.R., Jr., 2006. Dietary flavonoids and flavonoid-rich foods are not associated with risk of type 2 diabetes in postmenopausal women. J Nutr 136, 3039-3045.

Nile, S.H., Keum, Y.S., Nile, A.S., Jalde, S.S., Patel, R.V., 2018. Antioxidant, anti-inflammatory, and enzyme inhibitory activity of natural plant flavonoids and their synthesized derivatives. Journal of Biochemical and Molecular Toxicology 32, e22002.

Noblet, J.N., 2016. Coronary perivascular adipose tissue and vascular smooth muscle function: influence of obesity.

Nóbrega, N., Araújo, N.F., Reis, D., Facine, L.M., Miranda, C.A.S., Mota, G.C., Aires, R.D., Capettini, L., Cruz, J.D.S., Bonaventura, D., 2019. Hydrogen peroxide and nitric oxide induce anticontractile effect of perivascular adipose tissue via renin angiotensin system activation. Nitric Oxide 84, 50-59.

Novakovic, A., Marinko, M., Vranic, A., Jankovic, G., Milojevic, P., Stojanovic, I., Nenezic, D., Ugresic, N., Kanjuh, V., Yang, Q., He, G.W., 2015. Mechanisms underlying the vasorelaxation of human internal mammary artery induced by (-)-epicatechin. Eur J Pharmacol 762, 306-312.

Oh, W.J., Endale, M., Park, S.C., Cho, J.Y., Rhee, M.H., 2012. Dual Roles of Quercetin in Platelets: Phosphoinositide-3-Kinase and MAP Kinases Inhibition, and cAMP-Dependent Vasodilator-Stimulated Phosphoprotein Stimulation. Evid Based Complement Alternat Med 485262, 17.

Olaleye, M., Crown, O., Akinmoladun, A., Akindahunsi, A., 2014. Rutin and quercetin show greater efficacy than nifedipin in ameliorating hemodynamic, redox, and metabolite imbalances in sodium chloride-induced hypertensive rats. Human & Experimental Toxicology 33, 602-608.

Omori, A., Goshima, M., Kakuda, C., Kodama, T., Otani, K., Okada, M., Yamawaki, H., 2020. Chemerin-9-induced contraction was enhanced through the upregulation of smooth muscle chemokine-like receptor 1 in isolated pulmonary artery of pulmonary arterial hypertensive rats. Pflugers Arch 472, 335-342.

Oparil, S., Acelajado, M.C., Bakris, G.L., Berlowitz, D.R., Cífková, R., Dominiczak, A.F., Grassi, G., Jordan, J., Poulter, N.R., Rodgers, A., Whelton, P.K., 2018. Hypertension. Nat Rev Dis Primers 4, 14.

Orallo, F., Camiña, M., Álvarez, E., Basaran, H., Lugnier, C., 2005. Implication of Cyclic Nucleotide Phosphodiesterase Inhibition in the Vasorelaxant Activity of the Citrus-Fruits Flavonoid (±)-Naringenin. Planta Med 71, 99-107.

Oteiza, P., Erlejman, A., Verstraeten, S., Keen, C., Fraga, C., 2005. Flavonoid-membrane Interactions: A Protective Role of Flavonoids at the Membrane Surface? Clinical & developmental immunology 12, 19-25.

Ouyang A, Garner TB, Fleenor BS. 2017. Hesperidin reverses perivascular adipose-mediated aortic stiffness with aging. Experimental Gerontology 97: 68-72

Owen, M.K., Witzmann, F.A., McKenney, M.L., Lai, X., Berwick, Z.C., Moberly, S.P., Alloosh, M., Sturek, M., Tune, J.D., 2013. Perivascular Adipose Tissue Potentiates Contraction of Coronary Vascular Smooth Muscle. Circulation 128, 9-18.

Owens, G.K., Kumar, M.S., Wamhoff, B.R., 2004. Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease. Physiological Reviews 84, 767-801.

Oyagbemi, A.A., Omobowale, T.O., Adejumobi, O.A., Owolabi, A.M., Ogunpolu, B.S., Falayi, O.O., Hassan, F.O., Ogunmiluyi, I.O., Asenuga, E.R., Ola-Davies, O.E., Soetan, K.O., Saba, A.B., Adedapo, A.A., Nkadimeng, S.M., McGaw, L.J., Oguntibeju, O.O., Yakubu, M.A., 2020. Antihypertensive power of Naringenin is mediated via attenuation of mineralocorticoid receptor (MCR)/ angiotensin converting enzyme (ACE)/ kidney injury molecule (Kim-1) signaling pathway. European Journal of Pharmacology 880, 173142.

Ozen, G., Topal, G., Gomez, I., Ghorreshi, A., Boukais, K., Benyahia, C., Kanyinda, L., Longrois, D., Teskin, O., Uydes-Dogan, B.S., Norel, X., 2013. Control of human vascular tone by prostanoids derived from perivascular adipose tissue. Prostaglandins Other Lipid Mediat 107, 13-17.

Pai, S.A., Martis, E.A., Munshi, R.P., Gursahani, M.S., Mestry, S.N., Juvekar, A.R., 2020. Chrysin mitigated obesity by regulating energy intake and expenditure in rats. Journal of Traditional and Complementary Medicine 10, 577-585.

Pallauf, K., Duckstein, N., Hasler, M., Klotz, L.-O., Rimbach, G., 2017. Flavonoids as Putative Inducers of the Transcription Factors Nrf2, FoxO, and PPAR-γ. Oxid Med Cell Longev 2017, 4397340.

Pan, X.X., Ruan, C.C., Liu, X.Y., Kong, L.R., Ma, Y., Wu, Q.H., Li, H.Q., Sun, Y.J., Chen, A.Q., Zhao, Q., Wu, F., Wang, X.J., Wang, J.G., Zhu, D.L., Gao, P.J., 2019. Perivascular adipose tissue-derived stromal cells contribute to vascular remodeling during aging. Aging Cell 18, 14.

Panche, A.N., Diwan, A.D., Chandra, S.R., 2016. Flavonoids: an overview. J Nutr Sci 5, e47-e47.

Pandey, N., Rai, S.P., 2014. Biochemical Activity and Therapeutic Role of Antioxidants in Plants and Humans, pp. 191-224.

Park, K.H., Park, W.J., 2015. Endothelial Dysfunction: Clinical Implications in Cardiovascular Disease and Therapeutic Approaches. J Korean Med Sci 30, 1213-1225.

Parmenter, B.H., Croft, K.D., Hodgson, J.M., Dalgaard, F., Bondonno, C.P., Lewis, J.R., Cassidy, A., Scalbert, A., Bondonno, N.P., 2020. An overview and update on the epidemiology of flavonoid intake and cardiovascular disease risk. Food & Function 11, 6777-6806.

Pasha, A., M. Vignoli, A. Subbiani, A. Nocentini, S. Selleri, P. Gratteri, A. Dabraio, T. Casini, L. Filippi, I. Fotzi, C. Favre, and M. Calvani. 2019. β3-Adrenoreceptor Activity Limits Apigenin Efficacy in Ewing Sarcoma Cells: A Dual Approach to Prevent Cell Survival. International journal of molecular sciences 20 (9):2149.

Payne, G.A., Borbouse, L., Kumar, S., Neeb, Z., Alloosh, M., Sturek, M., Tune, J.D., 2010. Epicardial perivascular adipose-derived leptin exacerbates coronary endothelial dysfunction in metabolic syndrome via a protein kinase C-beta pathway. Arteriosclerosis, Thrombosis, and Vascular Biology 30, 1711-1717.

Petersen, B., Egert, S., Bosy-Westphal, A., Müller, M., Wolffram, S., Hubbermann, E., Rimbach, G., Schwarz, K., 2016. Bioavailability of quercetin in humans and the influence of food matrix comparing quercetin capsules and different apple sources. Food Research International 88.

Peterson, J.J., Dwyer, J.T., Jacques, P.F., McCullough, M.L., 2012. Associations between flavonoids and cardiovascular disease incidence or mortality in European and Us populations. Nutrition Reviews 70, 491-508.

Petrie, J.R., Guzik, T.J., Touyz, R.M., 2018. Diabetes, Hypertension, and Cardiovascular Disease: Clinical Insights and Vascular Mechanisms. Can J Cardiol 34, 575-584.

Pfeuffer, M., Auinger, A., Bley, U., Kraus-Stojanowic, I., Laue, C., Winkler, P., Rüfer, C.E., Frank, J., Bösch-Saadatmandi, C., Rimbach, G., Schrezenmeir, J., 2013. Effect of quercetin on traits of the metabolic syndrome, endothelial function and inflammation in men with different APOE isoforms. Nutr Metab Cardiovasc Dis 23, 403-409.

Pirouzpanah, S., Hanaee, J., Razavieh, S.-V., Rashidi, M.-R., 2009. Inhibitory effects of flavonoids on aldehyde oxidase activity. Journal of Enzyme Inhibition and Medicinal Chemistry 24, 14-21.

Poirier, P., Giles, T.D., Bray, G.A., Hong, Y., Stern, J.S., Pi-Sunyer, F.X., Eckel, R.H., 2006. Obesity and Cardiovascular Disease: Pathophysiology, Evaluation, and Effect of Weight Loss. Circulation 113, 898-918.

Polagruto, J., Schramm, D., Wang-Polagruto, J., Lee, L., Keen, C., 2003. Effects of Flavonoid-Rich Beverages on Prostacyclin Synthesis in Humans and Human Aortic Endothelial Cells: Association with Ex Vivo Platelet Function. Journal of Medicinal Food 6, 301-308.

Prasath, G.S., Pillai, S.I., Subramanian, S.P., 2014. Fisetin improves glucose homeostasis through the inhibition of gluconeogenic enzymes in hepatic tissues of streptozotocin induced diabetic rats. Eur J Pharmacol 740, 248-254.

Procházková, D., Boušová, I., Wilhelmová, N., 2011. Antioxidant and prooxidant properties of flavonoids. Fitoterapia 82, 513-523.

Proença, C., Freitas, M., Ribeiro, D., Oliveira, E.F.T., Sousa, J.L.C., Tomé, S.M., Ramos, M.J., Silva, A.M.S., Fernandes, P.A., Fernandes, E., 2017. α-Glucosidase inhibition by flavonoids: an in vitro and in silico structure-activity relationship study. Journal of Enzyme Inhibition and Medicinal Chemistry 32, 1216-1228.

Qian, Y., Babu, P.V.A., Symons, J.D., Jalili, T., 2017. Metabolites of flavonoid compounds preserve indices of endothelial cell nitric oxide bioavailability under glucotoxic conditions. Nutrition & Diabetes 7, e286-e286.

Rahim, A., Takahashi, Y., Yamaki, K., 2015. Mode of pancreatic lipase inhibition activity in vitro by some flavonoids and non-flavonoid polyphenols. Food Res Int 75, 289-294.

Rajendran, P., Rengarajan, T., Thangavel, J., Nishigaki, Y., Sakthisekaran, D., Sethi, G., Nishigaki, I., 2013. The Vascular Endothelium and Human Diseases. International Journal of Biological Sciences 9, 1057-1069.

Rajsheker, S., Manka, D., Blomkalns, A.L., Chatterjee, T.K., Stoll, L.L., Weintraub, N.L., 2010. Crosstalk between perivascular adipose tissue and blood vessels. Curr Opin Pharmacol 10, 191-196.

Ramanathan, V., Rajagopal, S., 2016. Chrysin Ameliorates the Lipid Profiles in Nω-nitro-l-arginine-methylester-induced Hypertensive Rats. American Journal of Biochemistry and Molecular Biology 6, 60-66.

Ramirez-Sanchez, I., Aguilar, H., Ceballos, G., Villarreal, F., 2012. (-)-Epicatechin-induced calcium independent eNOS activation: roles of HSP90 and AKT. Molecular and Cellular Biochemistry 370, 141-150.

Ramirez-Sanchez, I., Maya, L., Ceballos, G., Villarreal, F., 2010. (−)-Epicatechin Activation of Endothelial Cell Endothelial Nitric Oxide Synthase, Nitric Oxide, and Related Signaling Pathways. Hypertension 55, 1398-1405.

Rauter, A.P., Ennis, M., Hellwich, K.-H., Herold, B.J., Horton, D., Moss, G.P., Schomburg, I., 2018. Nomenclature of flavonoids (IUPAC Recommendations 2017). Pure and Applied Chemistry 90, 1429.

Ravishankar, D., Salamah, M., Akimbaev, A., Williams, H.F., Albadawi, D.A.I., Vaiyapuri, R., Greco, F., Osborn, H.M.I., Vaiyapuri, S., 2018. Impact of specific functional groups in flavonoids on the modulation of platelet activation. Scientific Reports 8, 9528.

Redford, K.E., Abbott, G.W., 2020. The ubiquitous flavonoid quercetin is an atypical KCNQ potassium channel activator. Communications Biology 3, 356.

Reis, J.P., Loria, C.M., Steffen, L.M., Zhou, X., Horn, L.v., Siscovick, D.S., Jacobs, D.R., Carr, J.J., 2010. Coffee, Decaffeinated Coffee, Caffeine, and Tea Consumption in Young Adulthood and Atherosclerosis Later in Life. Arteriosclerosis, Thrombosis, and Vascular Biology 30, 2059-2066.

Ren, K., Jiang, T., Zhou, H.F., Liang, Y., Zhao, G.J., 2018. Apigenin Retards Atherogenesis by Promoting ABCA1-Mediated Cholesterol Efflux and Suppressing Inflammation. Cellular Physiology and Biochemistry 47, 2170-2184.

Reshef, N., Hayari, Y., Goren, C., Boaz, M., Madar, Z., Knobler, H., 2005. Antihypertensive effect of sweetie fruit in patients with stage I hypertension. Am J Hypertens 18, 1360-1363.

Revuelta, M.P., Hidalgo, A., Cantabrana, B., 1999. Involvement of cAMP and beta-adrenoceptors in the relaxing effect elicited by flavonoids on rat uterine smooth muscle. J Auton Pharmacol 19, 353-358.

Rezabakhsh, A., Rahbarghazi, R., Malekinejad, H., Fathi, F., Montaseri, A., Garjani, A., 2019. Quercetin alleviates high glucose-induced damage on human umbilical vein endothelial cells by promoting autophagy. Phytomedicine 56, 183-193.

Ribeiro, D., Freitas, M., Tomé, S.M., Silva, A.M., Laufer, S., Lima, J.L., Fernandes, E., 2015. Flavonoids inhibit COX-1 and COX-2 enzymes and cytokine/chemokine production in human whole blood. Inflammation 38, 858-870.

Ricciotti, E., FitzGerald, G.A., 2011. Prostaglandins and inflammation. Arteriosclerosis, Thrombosis, and Vascular Biology 31, 986-1000.

Ringvold, H.C., Khalil, R.A., 2017. Chapter Six - Protein Kinase C as Regulator of Vascular Smooth Muscle Function and Potential Target in Vascular Disorders, in: Khalil, R.A. (Ed.), Advances in Pharmacology. Academic Press, pp. 203-301.

Rizza, S., Muniyappa, R., Iantorno, M., Kim, J.-a., Chen, H., Pullikotil, P., Senese, N., Tesauro, M., Lauro, D., Cardillo, C., Quon, M.J., 2011. Citrus polyphenol hesperidin stimulates production of nitric oxide in endothelial cells while improving endothelial function and reducing inflammatory markers in patients with metabolic syndrome. J Clin Endocrinol Metab 96, E782-E792.

Romero, M., Jiménez, R., Sánchez, M., López-Sepúlveda, R., Zarzuelo, M.J., O'Valle, F., Zarzuelo, A., Pérez-Vizcaíno, F., Duarte, J., 2009. Quercetin inhibits vascular superoxide production induced by endothelin-1: Role of NADPH oxidase, uncoupled eNOS and PKC. Atherosclerosis 202, 58-67.

Rosei, C.A., Withers, S.B., Belcaid, L., De Ciuceis, C., Rizzoni, D., Heagerty, A.M., 2015. Blockade of the reninâ–“angiotensin system in small arteries and anticontractile function of perivascular adipose tissue. Journal of hypertension 33, 1039-1045.

Rufino, A.T., Costa, V.M., Carvalho, F., Fernandes, E., 2021. Flavonoids as antiobesity agents: A review. Medicinal Research Reviews 41, 556-585.

Russo, B., Picconi, F., Malandrucco, I., Frontoni, S., 2019. Flavonoids and Insulin-Resistance: From Molecular Evidences to Clinical Trials. International Journal of Molecular Sciences 20, 2061.

Sá Gomes, A., Leite, F., Ribeiro, L., 2019. Adipocyte-macrophage crosstalk upon the expression of catecholamine synthesis pathway genes. Molecular biology of the cell 30, 3075.

Sadik, C.D., Sies, H., Schewe, T., 2003. Inhibition of 15-lipoxygenases by flavonoids: structure-activity relations and mode of action. Biochem Pharmacol 65, 773-781.

Sahebkar, A., 2015. Effects of Quercetin Supplementation on Lipid Profile: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. Critical reviews in food science and nutrition 57.

Salvamani, S., Gunasekaran, B., Shaharuddin, N.A., Ahmad, S.A., Shukor, M.Y., 2014. Antiartherosclerotic Effects of Plant Flavonoids. Biomed Res Int 2014, 480258.

Sánchez, M., Galisteo, M., Vera, R., Villar, I.C., Zarzuelo, A., Tamargo, J., Pérez-Vizcaíno, F., Duarte, J., 2006. Quercetin downregulates NADPH oxidase, increases eNOS activity and prevents endothelial dysfunction in spontaneously hypertensive rats. J Hypertens 24, 75-84.

Sánchez, M., Romero, M., Gómez-Guzmán, M., Tamargo, J., Pérez-Vizcaino, F., Duarte, J., 2019. Cardiovascular Effects of Flavonoids. Curr Med Chem 26, 6991-7034.

Sánchez-Recillas, A., Gonzalez, N., Barrera, V., Ibarra-Barajas, M., Estrada-Soto, S., Ortiz-Andrade, R., 2019. Vasorelaxant and Antihypertensive Activities of Citroflavonoids (Hesperidin/Naringenin Mixture): Potential Prophylactic of Cardiovascular Endothelial Dysfunction. Pharmacognosy Magazine 15, S84-91.

Santiago-Medina, F.-J., Pizzi, A., Basso, M.C., Delmotte, L., Celzard, A., 2017. Polycondensation Resins by Flavonoid Tannins Reaction with Amines. Polymers 9, 37.

Santos, R.A., Simoes e Silva, A.C., Maric, C., Silva, D.M., Machado, R.P., de Buhr, I., Heringer-Walther, S., Pinheiro, S.V., Lopes, M.T., Bader, M., Mendes, E.P., Lemos, V.S., Campagnole-Santos, M.J., Schultheiss, H.P., Speth, R., Walther, T., 2003. Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci U S A 100, 8258-8263.

Sarr M, Chataigneau M, Martins S, Schott C, El Bedoui J, Oak MH, Muller B, Chataigneau T, Schini-Kerth VB. Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase. Cardiovasc Res. 2006 Sep 1;71(4):794-802.

Satta, S., Mahmoud, A.M., Wilkinson, F.L., Yvonne Alexander, M., White, S.J., 2017. The Role of Nrf2 in Cardiovascular Function and Disease. Oxid Med Cell Longev 2017, 9237263.

Saxton, S., Ryding, K., Aldous, R., Withers, S., Ohanian, J., Heagerty, A., 2018a. Role of Sympathetic Nerves and Adipocyte Catecholamine Uptake in the Vasorelaxant Function of Perivascular Adipose Tissue. Arteriosclerosis, Thrombosis, and Vascular Biology 38, ATVBAHA.118.310777.

Saxton, S.N., Clark, B.J., Withers, S.B., Eringa, E.C., Heagerty, A.M., 2019. Mechanistic Links Between Obesity, Diabetes, and Blood Pressure: Role of Perivascular Adipose Tissue. Physiological Reviews 99, 1701-1763.

Saxton, S.N., Ryding, K.E., Aldous, R.G., Withers, S.B., Ohanian, J., Heagerty, A.M., 2018b. Role of Sympathetic Nerves and Adipocyte Catecholamine Uptake in the Vasorelaxant Function of Perivascular Adipose Tissue. Arterioscler Thromb Vasc Biol 38, 880-891.

Schena, G., and M. J. Caplan. 2019. Everything You Always Wanted to Know about β(3)-AR* (* But Were Afraid to Ask). Cells 8 (4):357.
Schroeter, M.R., Eschholz, N., Herzberg, S., Jerchel, I., Leifheit-Nestler, M., Czepluch, F.S., Chalikias, G., Konstantinides, S., Schäfer, K., 2013. Leptin-dependent and leptin-independent paracrine effects of perivascular adipose tissue on neointima formation. Arterioscler Thromb Vasc Biol 33, 980-987.
Sell, H., Laurencikiene, J., Taube, A., Eckardt, K., Cramer, A., Horrighs, A., Arner, P., Eckel, J., 2009. Chemerin Is a Novel Adipocyte-Derived Factor Inducing Insulin Resistance in Primary Human Skeletal Muscle Cells. Diabetes 58, 2731-2740.
Seo, M.-J., Lee, Y.-J., Hwang, J.-H., Kim, K.-J., Lee, B.-Y., 2015a. The inhibitory effects of quercetin on obesity and obesity-induced inflammation by regulation of MAPK signaling. The Journal of nutritional biochemistry 26, 1308-1316.

Seo, S., Lee, M.-S., Chang, E., Shin, Y., Oh, S., Kim, I.-H., Kim, Y., 2015b. Rutin Increases Muscle Mitochondrial Biogenesis with AMPK Activation in High-Fat Diet-Induced Obese Rats. Nutrients 7, 8152-8169.

Serban, M.C., Sahebkar, A., Zanchetti, A., Mikhailidis, D.P., Howard, G., Antal, D., Andrica, F., Ahmed, A., Aronow, W.S., Muntner, P., Lip, G.Y., Graham, I., Wong, N., Rysz, J., Banach, M., 2016. Effects of Quercetin on Blood Pressure: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. J Am Heart Assoc 5, 002713.

Shabrova EV, Tarnopolsky O, Singh AP, Plutzky J, Vorsa N, Quadro L. 2011. Insights into the Molecular Mechanisms of the Anti-Atherogenic Actions of Flavonoids in Normal and Obese Mice. *PLOS ONE* 6: e24634),

Shen, Y.-J., Zhu, X.-X., Yang, X., Jin, B., Lu, J.-J., Ding, B., Ding, Z.-S., Chen, S.-H., 2014. Cardamonin inhibits angiotensin II-induced vascular smooth muscle cell proliferation and migration by downregulating p38 MAPK, Akt, and ERK phosphorylation. Journal of Natural Medicines 68, 623-629.

Sheu, J.R., Hsiao, G., Chou, P.H., Shen, M.Y., Chou, D.S., 2004. Mechanisms involved in the antiplatelet activity of rutin, a glycoside of the flavonol quercetin, in human platelets. J Agric Food Chem 52, 4414-4418.

Shi, G.-J., Li, Y., Cao, Q.-H., Wu, H.-X., Tang, X.-Y., Gao, X.-H., Yu, J.-Q., Chen, Z., Yang, Y., 2019. In vitro and in vivo evidence that quercetin protects against diabetes and its complications: A systematic review of the literature. Biomedicine & Pharmacotherapy 109, 1085-1099.

Shi, J., Yang, Y., Cheng, A., Xu, G., He, F., 2020. Metabolism of vascular smooth muscle cells in vascular diseases. Am J Physiol Heart Circ Physiol 319, H613-H631.
Shiba, Y., Kinoshita, T., Chuman, H., Taketani, Y., Takeda, E., Kato, Y., Naito, M., Kawabata, K., Ishisaka, A., Terao, J., Kawai, Y., 2008. Flavonoids as substrates and inhibitors of myeloperoxidase: molecular actions of aglycone and metabolites. Chem Res Toxicol 21, 1600-1609.

Shin, S.S., Ko, M.C., Noh, D.H., Hwang, B., Park, Y., Park, S.L., Kim, W.J., Moon, S.K., 2018. Morin inhibits PDGF-induced proliferation, migration, and invasion of vascular smooth muscle cells via modulating p27KIP1, AKT, and MMP-9 activities. Gen Physiol Biophys 37, 633-645.

Shishtar, E., Rogers, G.T., Blumberg, J.B., Au, R., Jacques, P.F., 2020. Long-term dietary flavonoid intake and risk of Alzheimer disease and related dementias in the Framingham Offspring Cohort. The American Journal of Clinical Nutrition 112, 343-353.

Si, H., Wyeth, R.P., Liu, D., 2014. The flavonoid luteolin induces nitric oxide production and arterial relaxation. European Journal of Nutrition 53, 269-275.

Sissung, T.M., Goey, A.K.L., Ley, A.M., Strope, J.D., Figg, W.D., 2014. Pharmacogenetics of membrane transporters: a review of current approaches. Methods Mol Biol 1175, 91-120.

Smith, R.E., Tran, K., Smith, C.C., McDonald, M., Shejwalkar, P., Hara, K., 2016. The Role of the Nrf2/ARE Antioxidant System in Preventing Cardiovascular Diseases. Diseases (Basel, Switzerland) 4, 34.

Soltis, E.E., Cassis, L.A., 1991. Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. Clin Exp Hypertens A 13, 277-296.

Song, L., Xu, M., Lopes-Virella, M.F., Huang, Y., 2001. Quercetin Inhibits Matrix Metalloproteinase-1 Expression in Human Vascular Endothelial Cells through Extracellular Signal-Regulated Kinase. Archives of Biochemistry and Biophysics 391, 72-78.

Song, Y., Manson, J.E., Buring, J.E., Sesso, H.D., Liu, S., 2005. Associations of dietary flavonoids with risk of type 2 diabetes, and markers of insulin resistance and systemic inflammation in women: a prospective study and cross-sectional analysis. J Am Coll Nutr 24, 376-384.

Staiculescu, M.C., Foote, C., Meininger, G.A., Martinez-Lemus, L.A., 2014. The role of reactive oxygen species in microvascular remodeling. International Journal of Molecular Sciences 15, 23792-23835.

Stainer, A.R., Sasikumar, P., Bye, A.P., Unsworth, A.J., Holbrook, L.M., Tindall, M., Lovegrove, J.A., Gibbins, J.M., 2019. The Metabolites of the Dietary Flavonoid Quercetin Possess Potent Antithrombotic Activity, and Interact with Aspirin to Enhance Antiplatelet Effects. TH Open 3, e244-e258.

Stangl, V., Lorenz, M., Ludwig, A., Grimbo, N., Guether, C., Sanad, W., Ziemer, S., Martus, P., Baumann, G., Stangl, K., 2005. The flavonoid phloretin suppresses stimulated expression of endothelial adhesion molecules and reduces activation of human platelets. J Nutr 135, 172-178.

Stenmark, K.R., Yeager, M.E., El Kasmi, K.C., Nozik-Grayck, E., Gerasimovskaya, E.V., Li, M., Riddle, S.R., Frid, M.G., 2013. The adventitia: essential regulator of vascular wall structure and function. Annu Rev Physiol 75, 23-47.

Streeter, E., Hart, J., Badoer, E., 2012. An investigation of the mechanisms of hydrogen sulfide-induced vasorelaxation in rat middle cerebral arteries. Naunyn Schmiedebergs Arch Pharmacol 385, 991-1002.

Strobel, P., Allard, C., Perez-Acle, T., Calderon, R., Aldunate, R., Leighton, F., 2005. Myricetin, quercetin and catechin-gallate inhibit glucose uptake in isolated rat adipocytes. The Biochemical journal 386, 471-478.

Stunes, A.K., Reseland, J.E., Hauso, O., Kidd, M., Tømmerås, K., Waldum, H.L., Syversen, U., Gustafsson, B.I., 2011. Adipocytes express a functional system for serotonin synthesis, reuptake and receptor activation. Diabetes Obes Metab 13, 551-558.

Su, H., Feng, L.-n., Zheng, X.-d., Chen, W., 2016. Myricetin protects against diet-induced obesity and ameliorates oxidative stress in C57BL/6 mice. Journal of Zhejiang University SCIENCE B 17, 437-446.

Su, T., Huang, C., Yang, C., Jiang, T., Su, J., Chen, M., Fatima, S., Gong, R., Hu, X., Bian, Z., Liu, Z., Kwan, H.Y., 2020. Apigenin inhibits STAT3/CD36 signaling axis and reduces visceral obesity. Pharmacological Research 152, 104586.

Suen, J., Thomas, J., Kranz, A., Vun, S., Miller, M., 2016. Effect of Flavonoids on Oxidative Stress and Inflammation in Adults at Risk of Cardiovascular Disease: A Systematic Review. Healthcare 4.

Sun, A.Y., Simonyi, A., Sun, G.Y., 2002. The "French Paradox" and beyond: neuroprotective effects of polyphenols. Free Radic Biol Med 32, 314-318.

Sun, L., Li, E., Wang, F., Wang, T., Qin, Z., Niu, S., Qiu, C., 2015. Quercetin increases macrophage cholesterol efflux to inhibit foam cell formation through activating PPARγ-ABCA1 pathway. Int J Clin Exp Pathol 8, 10854-10860.

Takahama, U., Hirota, S., 2018. Interactions of flavonoids with α-amylase and starch slowing down its digestion. Food Funct 9, 677-687.

Tan, C.S., Yam, M.F., 2018. Mechanism of vasorelaxation induced by 3′-hydroxy-5,6,7,4′-tetramethoxyflavone in the rats aortic ring assay. Naunyn-Schmiedeberg's Archives of Pharmacology 391, 561-569.

Tanaka, Y., Yamaki, F., Koike, K., Toro, L., 2004. New insights into the intracellular mechanisms by which PGI2 analogues elicit vascular relaxation: cyclic AMP-independent, Gs-protein mediated-activation of MaxiK channel. Curr Med Chem Cardiovasc Hematol Agents 2, 257-265.

Tangvarasittichai, S., 2015. Oxidative stress, insulin resistance, dyslipidemia and type 2 diabetes mellitus. World J Diabetes 6, 456-480.

Thilakarathna, S.H., Rupasinghe, H.P.V., 2013. Flavonoid bioavailability and attempts for bioavailability enhancement. Nutrients 5, 3367-3387.

Torres-Villarreal, D., Camacho, A., Castro, H., Ortiz-Lopez, R., de la Garza, A.L., 2019. Anti-obesity effects of kaempferol by inhibiting adipogenesis and increasing lipolysis in 3T3-L1 cells. J Physiol Biochem 75, 83-88.

Triggle, C.R., Samuel, S.M., Ravishankar, S., Marei, I., Arunachalam, G., Ding, H., 2012. The endothelium: influencing vascular smooth muscle in many ways. Can J Physiol Pharmacol 90, 713-738.
Tsai, T.-N., Lin, W.-S., Wu, C.-H., Lin, W.-Y., Chu, K.-M., Cheng, C.-C., Hsu, C.-H., Tsai, W.-C., Cheng, S.-M., Yang, S.-P., 2014. Activation of Krüppel-Like Factor 2 with Ginkgo Biloba Extract Induces eNOS Expression and Increases NO Production in Cultured Human Umbilical Endothelial Cells. Acta Cardiol Sin 30, 215-222.

Tsai, Y.C., Leu, S.Y., Peng, Y.J., Lee, Y.M., Hsu, C.H., Chou, S.C., Yen, M.H., Cheng, P.Y., 2017. Genistein suppresses leptin-induced proliferation and migration of vascular smooth muscle cells and neointima formation. Journal of Cellular and Molecular Medicine 21, 422-431.

Tsvetkov, D., Kaßmann, M., Tano, J.-Y., Chen, L., Schleifenbaum, J., Voelkl, J., Lang, F., Huang, Y., Gollasch, M., 2017. Do K(V) 7.1 channels contribute to control of arterial vascular tone? British Journal of Pharmacology 174, 150-162.

Valls, R.M., Pedret, A., Calderón-Pérez, L., Llauradó, E., Pla-Pagà, L., Companys, J., Moragas, A., Martín-Luján, F., Ortega, Y., Giralt, M., Romeu, M., Rubió, L., Mayneris-Perxachs, J., Canela, N., Puiggrós, F., Caimari, A., Del Bas, J.M., Arola, L., Solà, R., 2020. Effects of hesperidin in orange juice on blood and pulse pressures in mildly hypertensive individuals: a randomized controlled trial (Citrus study). European Journal of Nutrition.

Van der Heiden, K., Cuhlmann, S., Luong le, A., Zakkar, M., Evans, P.C., 2010. Role of nuclear factor kappaB in cardiovascular health and disease. Clin Sci 118, 593-605.

Van Woudenbergh, G.J., Kuijsten, A., Drogan, D., van der, A.D., Romaguera, D., Ardanaz, E., Amiano, P., Barricarte, A., Beulens, J.W., Boeing, H., Bueno-de-Mesquita, H.B., Dahm, C.C., Chirlaque, M.D., Clavel, F., Crowe, F.L., Eomois, P.P., Fagherazzi, G., Franks, P.W., Halkjaer, J., Khaw, K.T., Masala, G., Mattiello, A., Nilsson, P., Overvad, K., Ramón Quirós, J., Rolandsson, O., Romieu, I., Sacerdote, C., Sánchez, M.J., Schulze, M.B., Slimani, N., Sluijs, I., Spijkerman, A.M., Tagliabue, G., Tjønneland, A., Tumino, R., Forouhi, N.G., Sharp, S., Langenberg, C., Feskens, E.J., Riboli, E., Wareham, N.J., 2012. Tea consumption and incidence of type 2 diabetes in Europe: the EPIC-InterAct case-cohort study. PLOS ONE 7, 30.

Vargovic, P., Ukropec, J., Laukova, M., Cleary, S., Manz, B., Pacak, K., Kvetnansky, R., 2011. Adipocytes as a new source of catecholamine production. FEBS Lett 585, 2279-2284.

Vargovic, P., Ukropec, J., Laukova, M., Kurdiova, T., Balaz, M., Manz, B., Ukropcova, B., Kvetnansky, R., 2013. Repeated immobilization stress induces catecholamine production in rat mesenteric adipocytes. Stress 16, 340-352.

Vecchione, C., Maffei, A., Colella, S., Aretini, A., Poulet, R., Frati, G., Gentile, M.T., Fratta, L., Trimarco, V., Trimarco, B., Lembo, G., 2002. Leptin Effect on Endothelial Nitric Oxide Is Mediated Through Akt–Endothelial Nitric Oxide Synthase Phosphorylation Pathway. Diabetes 51, 168-173.

Vernarelli, J.A., Lambert, J.D., 2017. Flavonoid intake is inversely associated with obesity and C-reactive protein, a marker for inflammation, in US adults. Nutrition & Diabetes 7, e276-e276.

Versari, D., Daghini, E., Virdis, A., Ghiadoni, L., Taddei, S., 2009. Endothelial Dysfunction as a Target for Prevention of Cardiovascular Disease. Diabetes Care 32, S314-S321.

Victorio, J.A., Fontes, M.T., Rossoni, L.V., Davel, A.P., 2016. Different Anti-Contractile Function and Nitric Oxide Production of Thoracic and Abdominal Perivascular Adipose Tissues. Front Physiol 7.

Vieux, F., Maillot, M., Rehm, C.D., Drewnowski, A., 2020. Flavonoid Intakes in the US Diet Are Linked to Higher Socioeconomic Status and to Tea Consumption: Analyses of NHANES 2011-16 Data. J Nutr 150, 2147-2155.

Villar, I., Jiménez, R., Galisteo, M., Garcia-Saura, M., Zarzuelo, A., Duarte, J., 2002. Effects of Chronic Chrysin Treatment in Spontaneously Hypertensive Rats. Planta medica 68, 847-850.

Vinayagam, R., Xu, B. (2015). Antidiabetic properties of dietary flavonoids: a cellular mechanism review. Nutrition & Metabolism, 12.

Virdis, A., Duranti, E., Rossi, C., Dell'Agnello, U., Santini, E., Anselmino, M., Chiarugi, M., Taddei, S., Solini, A., 2015. Tumour necrosis factor-alpha participates on the endothelin-1/nitric oxide imbalance in small arteries from obese patients: role of perivascular adipose tissue. Eur Heart J 36, 784-794.

Viskupicova, J., Ondrejovič, M., Sturdik, E., 2008. Bioavailability and metabolism of flavonoids. Journal of food and nutrition research 47, 151-162.

Wallerath, T., Li, H., Gödtel-Ambrust, U., Schwarz, P.M., Förstermann, U., 2005. A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 12, 97-104.

Wang HD, Pagano PJ, Du Y, et al. (1998). Superoxide Anion From the Adventitia of the Rat Thoracic Aorta Inactivates Nitric Oxide. Circulation Research 82(7):810-818 doi:doi:10.1161/01.RES.82.7.810

Wang, J., Wang, Y., Xu, C., Wang, B., Yu, J., Kang, X., Liu, X., Zhou, L., Qin, Y., Liao, L., Zhang, Y., 2018a. Effects of total flavonoids extracted from Polygonum perfoliatum L . on hypolipidemic and antioxidant in hyperlipidemia rats induced by high-fat diet.

Wang, N., Kuczmanski, A., Dubrovska, G., Gollasch, M., 2018. Palmitic Acid Methyl Ester and Its Relation to Control of Tone of Human Visceral Arteries and Rat Aortas by Perivascular Adipose Tissue. Frontiers in Physiology 9.

Wang, S., Zhang, X., Liu, M., Luan, H., Ji, Y., Guo, P., Wu, C., 2015a. Chrysin inhibits foam cell formation through promoting cholesterol efflux from RAW264.7 macrophages. Pharm Biol 53, 1481-1487.

Wang, T., Wu, Q., Zhao, T., 2020. Preventive Effects of Kaempferol on High-Fat Diet-Induced Obesity Complications in C57BL/6 Mice. Biomed Res Int 2020, 4532482.

Wang, T.-y., Li, Q., Bi, K.-s., 2018. Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. Asian Journal of Pharmaceutical Sciences 13, 12-23.

Wang, Y., Zhang, G., Pan, J., Gong, D., 2015b. Novel insights into the inhibitory mechanism of kaempferol on xanthine oxidase. J Agric Food Chem 63, 526-534.

Watts, S.W., Dorrance, A.M., Penfold, M.E., Rourke, J.L., Sinal, C.J., Seitz, B., Sullivan, T.J., Charvat, T.T., Thompson, J.M., Burnett, R., Fink, G.D., 2013. Chemerin connects fat to arterial contraction. Arteriosclerosis, Thrombosis, and Vascular Biology 33, 1320-1328.

Wedick, N.M., Pan, A., Cassidy, A., Rimm, E.B., Sampson, L., Rosner, B., Willett, W., Hu, F.B., Sun, Q., van Dam, R.M., 2012. Dietary flavonoid intakes and risk of type 2 diabetes in US men and women. Am J Clin Nutr 95, 925-933.

Wei X, Zhu X, Hu N, Zhang X, Sun T, Xu J, Bian X. Baicalin attenuates angiotensin II-induced endothelial dysfunction. Biochem Biophys Res Commun. 2015 Sep 11;465(1):101-7.

Wei, X., Zhu, X., Hu, N., Zhang, X., Sun, T., Xu, J., Bian, X., 2015. Baicalin attenuates angiotensin II-induced endothelial dysfunction. Biochem Biophys Res Commun 465, 101-107.

Williamson, G., Kay, C.D., Crozier, A., 2018. The Bioavailability, Transport, and Bioactivity of Dietary Flavonoids: A Review from a Historical Perspective. Comprehensive reviews in food science and food safety 17, 1054-1112.

Withers, S.B., Bussey, C.E., Saxton, S.N., Melrose, H.M., Watkins, A.E., Heagerty, A.M., 2014. Mechanisms of adiponectin-associated perivascular function in vascular disease. Arteriosclerosis, Thrombosis, and Vascular Biology 34, 1637-1642.

Withers, S.B., Forman, R., Meza-Perez, S., Sorobetea, D., Sitnik, K., Hopwood, T., Lawrence, C.B., Agace, W.W., Else, K.J., Heagerty, A.M., Svensson-Frej, M., Cruickshank, S.M., 2017. Eosinophils are key regulators of perivascular adipose tissue and vascular functionality. Sci Rep 7.

Wong, C., Akiyama, Y., Abe, T., Lippiat, J., Orfila, C., Williamson, G., 2012. Carrier-mediated transport of quercetin conjugates: Involvement of organic anion transporters and organic anion transporting polypeptides. Biochemical Pharmacology 84, 564-570.

Woodsome, T.P., Eto, M., Everett, A., Brautigan, D.L., Kitazawa, T., 2001. Expression of CPI-17 and myosin phosphatase correlates with Ca(2+) sensitivity of protein kinase C-induced contraction in rabbit smooth muscle. The Journal of Physiology 535, 553-564.

Wright, B., Spencer, J.P., Lovegrove, J.A., Gibbins, J.M., 2013. Flavonoid inhibitory pharmacodynamics on platelet function in physiological environments. Food Funct 4, 1803-1810.

Wu, W., Yang, B., Qiao, Y., Zhou, Q., He, H., He, M., 2020. Kaempferol protects mitochondria and alleviates damages against endotheliotoxicity induced by doxorubicin. Biomedicine & Pharmacotherapy 126, 110040.

Wu, Y.-T., Chen, L., Tan, Z.-B., Fan, H.-J., Xie, L.-P., Zhang, W.-T., Chen, H.-M., Li, J., Liu, B., Zhou, Y.-C., 2018. Luteolin Inhibits Vascular Smooth Muscle Cell Proliferation and Migration by Inhibiting TGFBR1 Signaling. Frontiers in Pharmacology 9.

Wynne, B.M., Chiao, C.-W., Webb, R.C., 2009. Vascular Smooth Muscle Cell Signaling Mechanisms for Contraction to Angiotensin II and Endothelin-1. J Am Soc Hypertens 3, 84-95.

Xia N, Weisenburger S, Koch E, Burkart M, Reifenberg G, Förstermann U, Li H. Restoration of perivascular adipose tissue function in diet-induced obese mice without changing bodyweight. Br J Pharmacol. 2017 Oct;174(20):3443-3453.

Xia, N., Li, H., 2017. The role of perivascular adipose tissue in obesity-induced vascular dysfunction. Br J Pharmacol 174, 3425-3442.

Xiaoa, J., Högger, P., 2013. Metabolism of Dietary Flavonoids in Liver Microsomes. Current drug metabolism 14.

Xu, C., Chen, J., Zhang, J., Hu, X., Zhou, X., Lu, Z., Jiang, H., 2013. Naringenin inhibits angiotensin II-induced vascular smooth muscle cells proliferation and migration and decreases neointimal hyperplasia in balloon injured rat carotid arteries through suppressing oxidative stress. Biol Pharm Bull 36, 1549-1555.

Xu, H., Luo, J., Huang, J., Wen, Q., 2018. Flavonoids intake and risk of type 2 diabetes mellitus: A meta-analysis of prospective cohort studies. Medicine 97, e0686.

Yam, M.F., Tan, C.S., Ahmad, M., Shibao, R., 2016. Mechanism of vasorelaxation induced by eupatorin in the rats aortic ring. European Journal of Pharmacology 789, 27-36.

Yan, L., Jia, Q., Cao, H., Chen, C., Xing, S., Huang, Y., Shen, D., 2021. Fisetin ameliorates atherosclerosis by regulating PCSK9 and LOX 1 in apoE<sup> / </sup> mice. Exp Ther Med 21, 25.

Yang Q, Hori M (2021) Characterization of Contractile Machinery of Vascular Smooth Muscles in Hypertension. Life 11(7):702

Yang, D., Yang, Z., Chen, L., Kuang, D., Zou, Y., Li, J., Deng, X., Luo, S., Luo, J., He, J., Yan, M., He, G., Deng, Y., Li, R., Yuan, Q., Zhou, Y., Jiang, P., Tan, S., 2020. Dihydromyricetin increases endothelial nitric oxide production and inhibits atherosclerosis through microRNA-21 in apolipoprotein E-deficient mice. Journal of Cellular and Molecular Medicine 24, 5911-5925.

Yang, E., S. B. Jeon, I. Baek, M. J. Song, Y. R. Yoon, and I. K. Kim. 2010. Fluoride induces vascular contraction through activation of RhoA/Rho kinase pathway in isolated rat aortas. Environ Toxicol Pharmacol 29 (3):290-296.

Yang, L., Ling, W., Qiu, Y. et al. Anthocyanins increase serum adiponectin in newly diagnosed diabetes but not in prediabetes: a randomized controlled trial. *Nutr Metab* (Lond) 17, 78 (2020).,

Yang, W.S., Wang, W.Y., Fan, W.Y., Deng, Q., Wang, X., 2014. Tea consumption and risk of type 2 diabetes: a dose-response meta-analysis of cohort studies. Br J Nutr 111, 1329-1339.

Yang, X., Jiang, Y., Yang, J., He, J., Sun, J., Chen, F., Zhang, M., Yang, B., 2015. Prenylated flavonoids, promising nutraceuticals with impressive biological activities. Trends in Food Science & Technology 44, 93-104.

Yao, H., Sun, J., Wei, J., Zhang, X., Chen, B., Lin, Y., 2020. Kaempferol Protects Blood Vessels From Damage Induced by Oxidative Stress and Inflammation in Association With the Nrf2/HO-1 Signaling Pathway. Frontiers in Pharmacology 11, 1118-1118.

Yao, Z., Gu, Y., Zhang, Q., Liu, L., Meng, G., Wu, H., Xia, Y., Bao, X., Shi, H., Sun, S., Wang, X., Zhou, M., Jia, Q., Wu, Y., Song, K., Gao, W., Guo, C., Niu, K., 2019. Estimated daily quercetin intake and association with the prevalence of type 2 diabetes mellitus in Chinese adults. Eur J Nutr 58, 819-830.

Yokomizo A, Moriwaki M. Effects of uptake of flavonoids on oxidative stress induced by hydrogen peroxide in human intestinal Caco-2 cells. Biosci Biotechnol Biochem. 2006 Jun;70(6):1317-24.

Zahedi, M., Ghiasvand, R., Feizi, A., Asgari, G., Darvish, L., 2013. Does Quercetin Improve Cardiovascular Risk factors and Inflammatory Biomarkers in Women with Type 2 Diabetes: A Double-blind Randomized Controlled Clinical Trial. International journal of preventive medicine 4, 777-785.

Zamora-Ros, R., Forouhi, N.G., Sharp, S.J., González, C.A., Buijsse, B., Guevara, M., van der Schouw, Y.T., Amiano, P., Boeing, H., Bredsdorff, L., Fagherazzi, G., Feskens, E.J., Franks, P.W., Grioni, S., Katzke, V., Key, T.J., Khaw, K.T., Kühn, T., Masala, G., Mattiello, A., Molina-Montes, E., Nilsson, P.M., Overvad, K., Perquier, F., Redondo, M.L., Ricceri, F., Rolandsson, O., Romieu, I., Roswall, N., Scalbert, A., Schulze, M., Slimani, N., Spijkerman, A.M., Tjonneland, A., Tormo, M.J., Touillaud, M., Tumino, R., van der, A.D., van Woudenbergh, G.J., Langenberg, C., Riboli, E., Wareham, N.J., 2014. Dietary intakes of individual flavanols and flavonols are inversely associated with incident type 2 diabetes in European populations. J Nutr 144, 335-343.

Zamora-Ros, R., Knaze, V., Lujan-Barroso, L., Slimani, N., Romieu, I., Touillaud, M., Kaaks, R., Teucher, B., Mattiello, A., Grioni, S., Crowe, F., Boeing, H., Foerster, J., Quirós, J., Molina-Montes, E., Huerta, J., Engeset, D., Skeie, G., Trichopoulou, A., González, C., 2011. Estimation of the intake of anthocyanidins and their food sources in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. The British journal of nutrition 106, 1090-1099.

Zaragozá, C., Monserrat, J., Mantecón, C., Villaescusa, L., Zaragozá, F., Álvarez-Mon, M., 2016. Antiplatelet activity of flavonoid and coumarin drugs. Vascul Pharmacol 87, 139-149.

Zarzecki, M.S., Araujo, S.M., Bortolotto, V.C., de Paula, M.T., Jesse, C.R., Prigol, M., 2014. Hypolipidemic action of chrysin on Triton WR-1339-induced hyperlipidemia in female C57BL/6 mice. Toxicol Rep 1, 200-208.

Zavaritskaya, O., Zhuravleva, N., Schleifenbaum, J., Gloe, T., Devermann, L., Kluge, R., Mladenov, M., Frey, M., Gagov, H., Fésüs, G., Gollasch, M., Schubert, R., 2013. Role of KCNQ

channels in skeletal muscle arteries and periadventitial vascular dysfunction. Hypertension 61, 151-159.

Zeng, Z.H., Zhang, Z.H., Luo, B.H., He, W.K., Liang, L.Y., He, C.C., Su, C.J., 2009. The functional changes of the perivascular adipose tissue in spontaneously hypertensive rats and the effects of atorvastatin therapy. Clin Exp Hypertens 31, 355-363.

Zha, J., Wu, X., Gong, G., Koffas, M.A.G., 2019. Pathway enzyme engineering for flavonoid production in recombinant microbes. Metabolic Engineering Communications 9, e00104.

Zhang, B., Sun, W., Yu, N., Sun, J., Yu, X., Li, X., Xing, Y., Yan, D., Ding, Q., Xiu, Z., Ma, B., Yu, L., Dong, Y., 2018. Anti-diabetic effect of baicalein is associated with the modulation of gut microbiota in streptozotocin and high-fat-diet induced diabetic rats. Journal of Functional Foods 46, 256-267.

Zhang, T., Wang, F., Xu, H.X., Yi, L., Qin, Y., Chang, H., Mi, M.T., Zhang, Q.Y., 2013. Activation of nuclear factor erythroid 2-related factor 2 and PPARγ plays a role in the genistein-mediated attenuation of oxidative stress-induced endothelial cell injury. Br J Nutr 109, 223-235.

Zhang, W., Huang, Q., Zeng, Z., Wu, J., Zhang, Y., Chen, Z., 2017. Sirt1 Inhibits Oxidative Stress in Vascular Endothelial Cells. Oxid Med Cell Longev 2017, 7543973.

Zhang, X., Li, X., Fang, H., Guo, F., Li, F., Chen, A., Huang, S., 2019. Flavonoids as inducers of white adipose tissue browning and thermogenesis: signalling pathways and molecular triggers. Nutrition & Metabolism 16, 47.

Zhang, X., Zhang, Q.X., Wang, X., Zhang, L., Qu, W., Bao, B., Liu, C.A., Liu, J., 2016. Dietary luteolin activates browning and thermogenesis in mice through an AMPK/PGC1α pathway-mediated mechanism. Int J Obes 40, 1841-1849.

Zhang, Y., Li, Y., Cao, C., Cao, J., Chen, W., Zhang, Y., Wang, C., Wang, J., Zhang, X., Zhao, X., 2010. Dietary Flavonol and Flavone Intakes and Their Major Food Sources in Chinese Adults. Nutrition and Cancer 62, 1120-1127.

Zheng, Y., Morris, A., Sunkara, M., Layne, J., Toborek, M., Hennig, B., 2012. Epigallocatechin-gallate stimulates NF-E2-related factor and heme oxygenase-1 via caveolin-1 displacement. The Journal of nutritional biochemistry 23, 163-168.

## 8. List of Publications

Pozzetti L., Ferrara F., Marotta L., Gemma S., Butini S., Benedusi M., Fusi F., **Ahmed, A**., Pomponi S., Ferrari S., Perini M., Ramunno A., Pepe G., Campiglia P., Valacchi G., Carullo G., Campiani G. 2022. Extra Virgin Olive Oil Extracts of Indigenous Southern Tuscany Cultivar Act as Anti-Inflammatory and Vasorelaxant Nutraceuticals. Antioxidants 11: 437

**Ahmed, A**. 2021. Flavonoids and cardiovascular risk factors: a review. Pharmadvances, 3(3):521-547

Saponara, S., Fusi, F., Iovinelli, D., **Ahmed, A**., Trezza, A., Spiga, O., Sgaragli, G., Valoti, M., 2021. Flavonoids and hERG channels: Friends or foes? European Journal of Pharmacology, 899, 174030. IF= 4.432

Carullo, G., **Ahmed, A**., Trezza, A., Spiga, O., Brizzi, A., Saponara, S., Fusi, F., Aiello, F., 2021. A multitarget semi-synthetic derivative of the flavonoid morin with improved in vitro vasorelaxant activity: Role of CaV1.2 and KCa1.1 channels. Biochemical Pharmacology 185, 114429. IF= 5.858

Carullo, G., **Ahmed, A**., Trezza, A., Spiga, O., Brizzi, A., Saponara, S., Fusi, F., Aiello, F., 2020. Design, synthesis and pharmacological evaluation of ester-based quercetin derivatives as selective vascular KCa1.1 channel stimulators. Bioorganic Chemistry 105, 104404. IF=5.275

Carullo, G., **Ahmed, A**., Fusi, F., Sciubba, F., Di Cocco, M.E., Restuccia, D., Spizzirri, U.G., Saponara, S., Aiello, F., 2020a. Vasorelaxant Effects Induced by Red Wine and Pomace Extracts of Magliocco Dolce cv. Pharmaceuticals 13, 87. IF=5.863

Waratchareeyakul, W., Fusi, F., Durante, M., **Ahmed, A**., Knirsch, W., Mas-Claret, E., Mulholland, D.A., 2020. Vasorelaxing Activity of Stilbenoid and Phenanthrene Derivatives from Brasiliorchis porphyrostele: Involvement of Smooth Muscle CaV1.2 Channels. Planta Med 86, 631-642. IF=3.352

Cuong, N.M., Son, N.T., Nhan, N.T., Khanh, P.N., Huong, T.T., Tram, N.T.T., Sgaragli, G., **Ahmed, A**., Trezza, A., Spiga, O., Fusi, F., 2020. Vasorelaxing Activity of R-(−)-3′-Hydroxy-2,4,5-trimethoxydalbergiquinol from Dalbergia tonkinensis: Involvement of Smooth Muscle CaV1.2 Channels. Planta Med 86, 284-293. IF=3.352

Carullo, G., Durante, M., Sciubba, F., Restuccia, D., Spizzirri, U.G., **Ahmed, A**., Di Cocco, M.E., Saponara, S., Aiello, F., Fusi, F., 2019. Vasoactivity of Mantonico and Pecorello grape pomaces on rat aorta rings: An insight into nutraceutical development. Journal of Functional Foods 57, 328-334. IF=4.451.