CHARLES UNIVERSITY

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

DOCTORAL THESIS

Title: Study of the mechanisms of action of phenolic compounds on the vascular smooth muscle

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Consultant: assoc. prof. Jana Pourová, Pharm.D., Ph.D.

Hradec Králové, 2023

DECLARATION

I hereby declare that I am the sole author of this doctoral thesis and that this thesis is my original work generated independently under the orientation of my supervisor. All the sources of information and literature I have used for the redaction of this doctoral thesis are cited in the text and properly listed in the "References" section of this thesis. This work has not been used to obtain another or the same degree.

I agree to make my thesis available through the information system of Charles University.

September 2023 M.Sc. Patrícia Dias

Columbus, OH, USA

ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové

Training Workplace	Department of Pharmacology and Toxicology
Doctoral Degree Program	Pharmacology and Toxicology
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Title of Doctoral Thesis	Study of the mechanisms of action of phenolic compounds on the vascular smooth muscle

Cardiovascular diseases including hypertension, coronary artery disease, peripheral artery disease, and cerebrovascular disease remain the leading cause of death worldwide. In addition, discouraging estimations have suggested a future increase in the number of cardiovascular patients. Thus, novel treatment modalities are clearly needed to prevent or reverse these epidemic trends.

Phenolic compounds contain one or more hydroxyl groups bound to a benzene ring. This class of chemicals includes: a) natural compounds (e.g., dietary polyphenols and small phenolic metabolites) referred to as nutraceuticals due to their claimed health-promoting effects and b) synthetic compounds (e.g., bisphenols) which, on the contrary, have been suggested to negatively affect human health.

Even if there are claims that polyphenol-rich diet is associated with cardioprotective effects, important questions remain to be elucidated. In particular, the parent compounds have mostly low bioavailability, so the bioactivities have been ascribed to their colonic metabolites. 3-hydroxyphenylacetic acid (3-HPAA) is a small phenolic metabolite that has previously shown vasorelaxant effects *ex vivo*. Hence, the first part of this dissertation aimed to investigate whether 3-HPAA exerts haemodynamic effects *in vivo* as well and to elucidate the mechanism of action. The intravenous administration of 3-HPAA as both a bolus and infusion decreased systolic and diastolic arterial blood pressure in spontaneously hypertensive rats. The blood pressure-decreasing effect was dose-dependent and was not accompanied by significant changes in heart rate. Additional *ex vivo* isometric tension recordings revealed that 3-HPAA relaxes the isolated porcine coronary artery, which was selected as a model for determination of the mechanism of action at the molecular level. The vasorelaxant effect was partially dependent on the integrity of the endothelial layer and was significantly decreased after endothelial nitric oxide synthase (eNOS) inhibition. On the contrary, the inhibition of small and intermediate conductance calcium-activated potassium channels (SKCa and IKCa), cyclooxygenases, or L-type calcium channels as well as antagonism at muscarinic receptors had no impact on 3-HPAA-induced vasorelaxation. In summary, the findings suggested that 3-HPAA decreased blood pressure *in vivo* through peripheral vasorelaxation *via* a mechanism likely involving nitric oxide release by the endothelial layer.

Bisphenols are endocrine-disrupting chemicals widely used by the industry in the production of polycarbonate plastic and epoxy resins in food packaging materials, beverage cans, thermal receipts, electronic devices, etc. Since the lead compound, bisphenol A (BPA), has been inculpated with many harmful effects on human health, it has been replaced by novel, so-called next-generation (NextGen) bisphenols. These alternative compounds are, however, much less studied and are now pervasive throughout the environment. Therefore, in the second part of this dissertation, we aimed firstly to systematically review the literature available on bisphenols and their potential impact on the cardiovascular system. Due to being present in many daily use products, humans are inevitably exposed to bisphenols. Indeed, bisphenols have been detected in different human biological samples. Reported total serum levels of BPA (i.e., including the conjugated metabolite) were up to ~ 430 nM, whereas those of free BPA were up to 80 nM. Although reports on the levels of NextGen bisphenols are scarcer, the number of studies has been increasing. For example, the maximum serum levels of bisphenol S reported were ~ 680 nM. I*n vitro* studies showed that bisphenols interact with ion channels, thyroid, oestrogenic and androgenic receptors. In the case of BPA, vasodilatory effects were shown *ex vivo*, while an unexpected increase in arterial blood pressure occurred *in vivo* and in observatory cross-sectional human studies. Additional negative effects have been described on hepatic lipid and glucose metabolism and coronary artery disease. However, due to inconsistencies and even contradictory findings, there is a need for novel studies, particularly focusing on the newly introduced NextGen bisphenols. For this reason, the next stage of this PhD project centred on testing 14 bisphenols (bisphenols A, AF, AP, B, BP, C, E, F, G, M, P, PH, S and Z) and comparing their effects *in vitro* (human and rat cell lines), *ex vivo* (isolated rat aorta) and *in vivo* (Wistar Han rats, acutely or chronically exposed to either low environmental or high toxic doses). Eight of the tested bisphenols relaxed the rat aorta with different potencies. Bisphenol AF (BPAF) was the most potent vasodilator, with an EC_{50} of 57 μM, and the mechanism of action seemed to be based on the blockade of L-type calcium channels. The cytotoxicity of bisphenols towards 4 human and rat cell lines (H9c2, A-10, MCF7/S0.5 and MCF7/182R-6) showed variable potencies ranging from micromolar units to millimolar concentrations. Hence, changes in arterial blood pressure and cardiotoxicity could occur. However, the *in vivo* acute effects of three doses (0.005, 0.05 and 2.5 mg/kg) of BPAF and 3 other analogues (bisphenols A, S and F) on the cardiovascular system were rather biologically negligible. BPAF was also administered chronically at a dose of 2.5 mg/kg daily for 4 weeks to normotensive Wistar Han rats, but there were no changes in arterial blood pressure. In summary, although bisphenols can relax vascular smooth muscles, the effective concentrations are too high to produce clear cardiovascular effects in relation to common biological exposure.

ABSTRAKT

Univerzita Karlova, Farmaceutická fakulta v Hradci Králové

Školicí pracoviště:	Katedra farmakologie a toxikologie
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Název disertační práce:	Studium mechanismů účinků fenolických látek na hladký cévní sval

Kardiovaskulární choroby zahrnující mj. vysoký krevní tlak, ischemickou chorobu srdeční, ischemickou chorobu dolních končetin a cerebrovaskulární choroby zůstávají celosvětově nejčastější příčinou úmrtí. Kromě toho existují predikce, které naznačují další vzestup počtu kardiovaskulárních pacientů v budoucnosti. Z těchto důvodů jsou jednoznačně potřebné nové léčebné postupy s cílem zpomalit nebo zvrátit tento epidemiologický trend.

Fenolické sloučeniny obsahují jednu nebo více hydroxylových skupin na benzenovém jádře. Tyto látky zahrnují a) přírodní sloučeniny jako například potravní polyfenoly a malé fenolické metabolity, u kterých se očekávají příznivé účinky na lidské zdraví, a b) syntetické látky jako např. bisfenoly, u kterých se naopak obáváme možného negativního působení.

I když je k dispozici řada studií ukazujících, že potrava bohatá na polyfenoly je spojena s kardioprotektivními účinky, stále zde zůstává řada nezodpovězených otázek. Především parentní sloučeniny mají nízkou biodostupnost a jejich biologické účinky jsou tak připisovány metabolitům tvořeným lidskou mikroflórou. Kyselina 3-hydroxyfenyloctová (3HFO) je malým fenolickým metabolitem, u kterého byl již dříve *ex vivo* nalezen vasodilatační potenciál. Cílem první část této dizertace je ověřit, zda 3HFO dokáže navodit hemodynamické účinky *in vivo* a jaký je mechanismus tohoto účinku. Intravenózní podání 3HFO ve formě jak bolusů, tak infuzí navodilo snížení systolického i diastolického krevního tlaku u spontánně hypertenzních potkanů. Tento účinek byl dávkově závislý a nebyl doprovázen vlivem na srdeční frekvenci. Následovaly *ex vivo* pokusy, kde jako model pro zjištění mechanismu účinku na molekulární úrovni byly vybrány isolované prasečí věnčité tepny s monitorováním isometrického napětí. Pokusy prokázaly, že 3HFO tyto cévy relaxuje. Vasodilatační účinek byl částečně závislý na celistvosti endoteliální vrstvy a významně poklesl při inhibici endotelové syntasy oxidu dusnatého (eNOS). Naopak inhibice malých nebo středních vápníkem-aktivovaných draslíkových kanálů, cyklooxygenas ani L-typu vápníkových kanálů stejně jako antagonismus na muskarinových receptorech vasodilatační účinek 3HFO neovlivnily. Z toho usuzujeme, že pokles krevního tlaku po podání 3HFO zřejmě nastává periferní vasodilatací přes mechanismus, který zahrnuje uvolňování oxidu dusnatého endotelem.

Bisfenoly jsou endokrinními disruptory, které se široce používají v průmyslu na produkci polykarbonátových plastů a epoxidových pryskyřic v obalových potravinářských materiálech, nápojových plechovkách, speciálním účtenkovém papíru, elektrických přístrojích apod. Jelikož hlavní zástupce, bisfenol A (BPA), je podezřelý z řady škodlivých účinků na lidské zdraví, byl nahrazen novějšími látkami, nazývanými další generací bisfenolů. Tyto alternativní látky jsou sice ještě relativně málo prozkoumány, ale v současné době se již běžně vyskytují v životním prostředí. Druhá část této dizertační práce je věnována nejprve systematickému shrnutí dostupné literatury na téma bisfenoly a jejich potenciálnímu vlivu na kardiovaskulární systém. Díky přítomnosti v řadě předmětů denního užívání, jsou lidé těmto látkám nevyhnutelně exponováni. V souladu s tím byly nalezeny v řadě lidských biologických vzorků. Celková sérová hladina BPA (tj. včetně konjugovaných metabolitů) byla naměřena až ~ 430 nM, zatímco u volného BPA to bylo do 80 nM. I když o hladinách látek z další generace bisfenolů existuje méně dostupných informací, počet studií v této oblasti se zvyšuje. Například pro bisfenol S byla zjištěna maximální sérová hladina ~ 680 nM. *In vitro* studie dále prokázaly, že bisfenoly interagují s iontovými kanály a také s tyroidními, estrogenními a androgenními receptory. V případě BPA byly popsány vasodilatační účinky *ex vivo*, zatímco v *in vivo* a observačních prevalenčních studiích byl překvapivě zjištěn nárůst arteriálního krevního tlaku. Další popsané nepříznivé účinky byly ve vztahu k jaternímu metabolismu lipidů i metabolismu glukosy a k ischemické chorobě srdeční. V dostupných výsledcích jsou nesrovnalosti nebo dokonce vyznívají protichůdně. Z tohoto důvodu jsou potřeba další studie zaměřené především na novou generaci bisfenolů. Další část tohoto doktorandského projektu se soustředí na 14 bisfenolů (bisfenoly A, AF, AP, B, BP, C, E, F, G, M, P, PH, S a Z) a porovnává jejich účinky *in vitro* (lidské a potkaní buněčné linie), *ex vivo* (isolovaná potkaní aorta) a *in vivo* (Wistar Han potkani akutně nebo chronicky exponovaní buď nízkým /environmentálním/ dávkám nebo vysokým toxickým dávkám). Osm z testovaných bisfenolů relaxovalo potkaní aortu s různou potencí. Bisfenol AF (BPAF) byl nejúčinnější. Jeho EC_{50} byla 57 μM, a mechanismus účinku závisel na blokádě L-typu vápníkových kanálů. Cytotoxicita bisfenolů na čtyři lidské a potkaní buněčné linie (H9c2, A-10, MCF7/S0.5 a MCF7/182R-6) byla variabilní a pozorována v koncentracích od mikromolárních až po milimolární. Proto jsme očekávali, že některé z těchto látek by mohly mít vliv na arteriální krevní tlak a navodit poškození srdce. Avšak *in vivo* akutní účinky tří dávek (0.005, 0.05 a 2.5 mg/kg) nejúčinnější látky BPAF a jeho tří dalších analogů (bisfenoly A, S a F) na kardiovaskulární systém byly biologicky spíše zanedbatelné. BPAF jsme kromě toho podávali i chronicky v dávce 2.5 mg/kg denně po dobu 4 týdnů normotenzním potkanům Wistar Han, ale ani v tomto experimentu nebyl pozorován významný vliv na arteriální krevní tlak. Závěrem lze tedy konstatovat, že ačkoliv bisfenoly relaxují hladké cévní svaly, účinné koncentrace, které by navodily jasné kardiovaskulární účinky, jsou výrazně vyšší, než běžná biologická expozice.

Anything we envision and aspire to achieve in our lives comes to fruition through a positive ambition, a high motivation combined with self-discipline, work, devotion, and a secret ingredient called love.

Patrícia Dias

To my parents Paula and Alberto,
for their unconditional love and for being
the biggest of my blessings.

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ABBREVIATIONS

3-HPAA	3-hydroxyphenylacetic acid
AC	Adenylate cyclase
BKCa	Large conductance calcium-activated K^+ channels
CaM	Calmodulin
CaMKII	Calcium-calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CO	Cardiac output
COX	Cyclooxygenases
CVD	Cardiovascular disease
DAG	1,2-diacylglycerol
DBP	Diastolic blood pressure
EDH	Endothelium-derived hyperpolarization
EDRF	Endothelium-derived relaxing factor
E_m	Membrane potential
eNOS	Endothelial nitric oxide synthase
EPAC	Exchange factor directly activated by cAMP
GPCRs	G protein-coupled receptors
GSH	Glutathione
HDL	High-density lipoprotein
HR	Heart rate
IKCa	Intermediate conductance calcium-activated K^+ channels
IK_S	Slow delayed rectifier potassium current
IP_3	Inositol 1,4,5-trisphosphate
IP_3Rs	Inositol 1,4,5-trisphosphate receptors
IRAG	Inositol 1,4,5-trisphosphate receptor-associated cGMP-kinase substrate
K_{ATP}	ATP-sensitive K^+ channels
K_{ir}	Inwardly-rectifying K^+ channels
K_V	Voltage-gated K^+ channels
LDL	Low-density lipoprotein
MAP	Mean arterial pressure
MAPK	Mitogen-activated protein kinases
MEJ	Myoendothelial projection
MLC	Myosin light chain
MLCK	Myosin light chain kinase

MLCP	Myosin light chain phosphatase
MYPT-1	Myosin phosphatase target subunit-1
NCX	Sodium-calcium exchanger
NextGen	Next generation
NHANES	National Health and Nutrition Examination Survey
NHE	Sodium-proton exchanger
NO	Nitric oxide
PG	Prostaglandin
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLB	Phospholamban
PLC	Phospholipase C
PMCA	Plasmalemmal Ca^{2+}-ATPase
ROCK	Rho-kinase
RyRs	Ryanodine receptors
SBP	Systolic blood pressure
SERCA	Sarcoplasmic reticulum Ca^{2+}-ATPase
sGC	Soluble guanylate cyclase
SHRs	Spontaneously hypertensive rats
SKCa	Small conductance calcium-activated K^+ channels
SR	Sarcoplasmic reticulum
SV	Stroke volume
SVR	Systemic vascular resistance
TG	Triglycerides
TPR	Total peripheral resistance
TRP	Transient receptor potential
TxA2	Thromboxane A2
VEGF	Vascular endothelial growth factor
VGCCs	Voltage-gated Ca^{2+} channels
VLDL	Very-low-density lipoprotein
VSM	Vascular smooth muscle

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[1] *Schematic pictures of endothelial and vascular smooth muscle cells are original and were created by the author using Microsoft PowerPoint software and ChemDraw (PerkinElmer).*

1 INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of mortality and morbidity worldwide, taking the lives of approximately 18 million people every year [1]. Vasculature is a major player involved in the protection and development of CVDs. Hallmarks of vascular disease include alterations in arteries that frequently appear more constricted, with thicker walls, and functional impairment [2]. Among histopathological findings are dysfunctional endothelium and phenomena of ion channel remodelling in vascular smooth muscle (VSM) cells [3-5]. Increased total peripheral resistance (TPR) typically observed ultimately leads to hypertension, one of the major risk factors for CVDs. Even though numerous antihypertensive drugs are available, hurdles such as unawareness, late diagnosis, nonadherence to prescriptions, and alarming epidemic incidence impose a tremendous socioeconomic burden on societies and healthcare systems.

Phenolic compounds are structurally characterized as one or more hydroxyl groups (-OH) bound to a benzene ring. These include natural phenolic compounds (e.g., dietary polyphenols and small phenolic metabolites) and synthetic compounds (e.g., bisphenols).

Dietary polyphenols have been suggested to correlate with protective effects on blood vessels [6-8]. The parent forms, however, have low bioavailability, while the gut microbiota-derived metabolites, mostly small phenolics, have been detected at much higher levels in human plasma. In recent years, our research team uncovered that a plethora of small phenolic metabolites exert vasorelaxant effects *ex vivo* [9]. Despite the promising findings unveiling their potential to modify CVDs, the mechanisms of action of these compounds are rather diverse or remain enigmatic.

In contrast, there are novel vascular risk factors in the physical environment that, albeit modifiable, cannot be pertained as individual responsibility, specifically concerning exposure to environmental pollutants, such as bisphenols. Multiple epidemiological studies have revealed that bisphenol A (BPA) exerts harmful effects on the cardiovascular system, including blood vessels [10, 11]. The replacement of BPA by surrogates, such as bisphenol S (BPS), bisphenol AF (BPAF) and bisphenol F (BPF), has raised new concerns as safety studies are scant. Considering the harsh lessons from the ‘’old culprit’’ BPA, independent researchers and regulatory agencies should pursue the investigation of these new alternatives.

Therefore, this doctoral project aimed to explore the effects and mechanisms of action of phenolic compounds on the cardiovascular system, given their apparent dichotomous role in vascular pathology.

2 THEORETICAL BACKGROUND

2.1 THE CARDIOVASCULAR SYSTEM

Since the discoveries of the English physician William Harvey (1578-1657), published in "*Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus",* the understanding of the cardiovascular system has advanced substantially [12, 13]. This chapter presents an overview of general concepts concerning the heart and vasculature.

2.1.1 The vasculature

In humans, the cardiovascular system is formed by the heart, blood and blood vessels, which work in perfect synchronicity to ensure adequate blood flow throughout the body [14]. The cardiovascular system is the first organ system to fully develop and become functional [15]. During the embryonic stage of gestation, vasculogenesis takes place with mesodermal precursors yielding to hemangioblasts and, subsequently, to primitive endothelium and endothelial tubes [16]. Cell-cell interactions, cell-matrix interactions, growth factors, e.g., vascular endothelial growth factor (VEGF), and morphogens modulate vasculogenesis [17]. Then, the endothelial tubes recruit smooth muscle cell progenitors that undergo differentiation [18]. Factors such as biophysical forces and biochemical signalling play a major role in vessel wall development [19]. Thereafter, angiogenesis, which consists of the sprouting of new vessels from pre-existing vasculatures, occurs [20].

2.1.1.1 Classification of blood vessels

The thickness and composition of the blood vessels vary according to the tissues perfused and the haemodynamic forces exerted upon their walls. As a result, blood vessels are divided into: arteries, arterioles, capillaries, venules and veins. Arteries assure the supply of blood and nutrients to organs and tissues, and despite the fact that only 10 to 15% of the total blood volume is present in arterial beds, these are high pressure vessels. Then, arteries give origin to arterioles, vessels of smaller lumen size, particularly rich in smooth muscle, and responsible for the regulation of the organ blood perfusion and arterial blood pressure (see section 2.1.3). Capillaries are formed by a single layer of endothelial cells, enabling the exchange of nutrients and metabolites through diffusion into tissues. Aside from the arterial system, there is the venous system which includes venules and veins. While venules take up and conduct the blood from capillaries into veins, veins are much larger vessels containing one-way valves that assure blood flow toward the heart. Contrarily to the arterial system, the venous system has low pressure but accommodates approximately 75 % of the blood volume in circulation [16].

2.1.1.2 Architecture of the vessel wall

The typical structure of a blood vessel consists of three layers: the tunica intima, the tunica media and the tunica adventitia (**Figure 1**) [21]. Capillaries are exceptions. The tunica intima is the layer formed by endothelial cells that lines the vessel lumen and, hence, is in contact with the contents of the blood. In the case of arteries, the so-called internal elastic lamina is also present and separates the tunica intima and the tunica media. In close vicinity, the tunica media (middle layer) ensures changes in the diameter of the vessels being composed of smooth muscle cells (mentioned VSM cells) and elastic fibres in variable proportions. There are two main types of arteries: elastic, also called conduit arteries (e.g., aorta), rich in elastic tissue (**Figure 1B**) and muscular arteries (e.g., mesenteric arteries), which have a high content of smooth muscle (**Figure 1C**) [21]. Lastly, the outer layer, tunica externa, is composed of fibrous connective tissue (collagen) and maintains the integrity of the vessels [22].

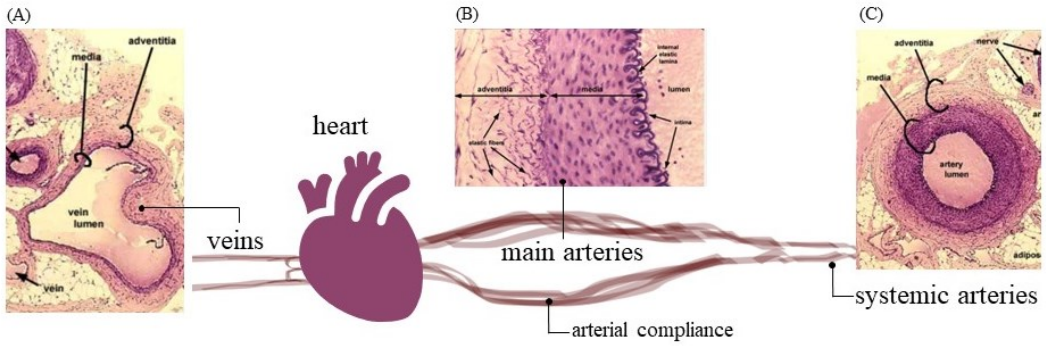

Figure 1. **Histological specimen of a vein (A), elastic artery (B) and muscular artery (C) and respective tunica layers: the adventitia (outer layer), the media (middle layer) and the intima (inner layer – not written).** Adopted and modified from [21].

2.1.2 The heart

The heart is a muscular organ whose primary function is to pump blood throughout the body, providing nutrients and oxygen to cells and removing waste products [14]. Human hearts are formed by four chambers: two thin-walled atria and two thick-walled ventricles**.** The blood circulation (**Figure 2A**) is divided into two main circuits: a) systemic circulation, which delivers oxygenated blood from the left ventricle *via* the aorta to the rest of the body and returns deoxygenated blood back to the right atrium *via venae cavae* and b) pulmonary circulation, which comprehends the transport of deoxygenated blood from the right ventricle through pulmonary arteries to the lungs and its return already oxygenated back to the left atrium *via* pulmonary veins [14, 23]. The unidirectional blood flow is proportioned by two atrioventricular valves and two semilunar valves that close and open depending on the pressure gradient [24]. The cardiac cycle comprises the repeating events between one heartbeat and the next. It includes a period of relaxation known as diastole and a period of contraction known as systole.

2.1.2.1 Coronary arterial circulation

The heart ensures its own blood supply through large epicardial coronary arteries whose distribution on the surface of the heart resembles a crown. In fact, the term coronary stems from the Latin *coronarius,* which means ‘’of a crown’’ [25]. **Figure 2B** illustrates the main coronary arteries. In short, the left main coronary artery emerges from the first main branch of the aorta and gives rise to the left anterior descending (LAD) and left circumflex artery. In the right heart, the right coronary artery also emerges from the aorta and feeds the posterior descending coronary artery (PDA) [26]. Figuratively speaking, the heart can be considered ‘’an altruistic organ’’ insofar as it is perfused only after it has perfused all other organs, i.e., during diastole. Therefore, an adequate diastolic filing period is essential to maintain sufficient coronary flow. Collectively, many factors contribute to the regulation of coronary vascular resistance and guarantee adequate perfusion and oxygenation of the heart.

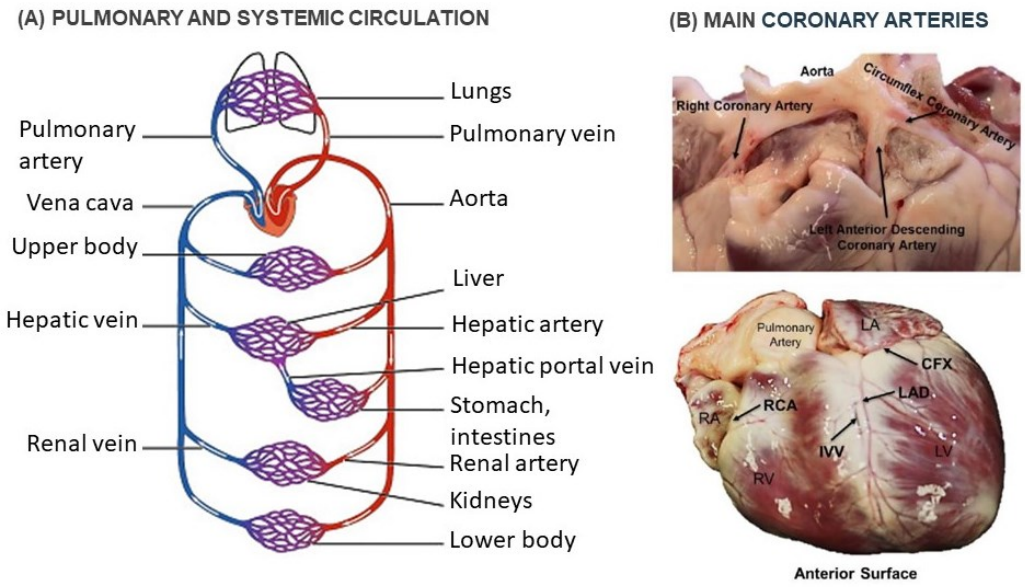

Figure 2. **(A) Schematic diagram of the circulation of the blood (pulmonary and systemic circuits). (B) Pictures of the main coronary arteries in human hearts.** RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle; RCA, right coronary artery; IVV, interventricular vein; LAD, left anterior descending artery; CFX, circumflex coronary artery. Adopted and modified from [26, 27].

2.1.3 Basic concepts of haemodynamics

Haemodynamics describes interactions between physiological parameters that govern the behaviour of the cardiovascular system. At rest, the heart pumps approximately 5 L of blood per minute, which constitutes the normal cardiac output (CO); however, the CO can increase up to 35 L/min at exercise. The volume of blood ejected with each contraction is called stroke volume (SV), and for a young, healthy man, it is around 70 mL, while for women is about 10 to 20% lower [28]. The CO is calculated by the product of SV and heart rate (HR) (Equation 1).

$$CO = SV \times HR \ (Equation\ 1)$$

Of note, the typical HR in adults ranges between 60 to 100 beats per minute, and it is under the control of the autonomic nervous system. SV is primarily influenced by the following factors: a) preload or the volume of blood inside the ventricle just before systole, i.e., the end-diastolic volume, which is mostly determined by the venous return, b) afterload or the opposing force to the outflow of the blood from the heart; an increased afterload is a consequence of increased systemic vascular resistance, and c) contractility or the force of contraction of the heart muscle [29].

Systemic vascular resistance (SVR) is the resistance or force exerted by blood vessels on circulating blood that contributes to the regulation of blood pressure, blood flow and heart function [30]. The arterial system is the major determinant of SVR. Specifically, resistance arteries and arterioles rule SVR because, compared with veins, these vessels have a much thicker media layer (**Figure 1**). Three factors influence SVR: (1) the length of the vessel, *L*; (2) the radius of the vessel, *r*; and (3) the viscosity of the blood, μ. By recalling the Hagen-Poiseuille equation, one can describe the flow mechanics in blood vessels as: $R = \frac{8L\mu}{(\pi.r^4)}$ [31]. SVR is dramatically affected by changes in arterial and arteriolar tone (i.e., vasoconstriction or vasodilation), as this implies a change in the radius of the vessel. VSM cells are responsible for the active and fine-tuned regulation of blood pressure. Blood pressure, in turn, by stretching VSM cells constitutes an important factor for the regulation of the myogenic tone and thus SVR [32]. The measurement of SVR is a useful and predictive approach in: (1) pathological conditions (e.g., acute congestive heart failure and cardiogenic shock [33, 34]); (2) during pregnancy (high SVR in the first weeks of gestation is an early marker of cardiovascular risk such as preeclampsia [35]), and (3) in healthy subjects (irrespective of the blood pressure, an elevation in SVR constitutes a valuable CVD risk marker).

Mean arterial pressure (MAP) is a haemodynamic factor that can be calculated by the product of CO and SVR (Equation 2).

$$MAP = CO \times SVR \ (Equation\ 2)$$

Most importantly, MAP is an important indicator of organ perfusion. Typically, MAP ranges between 70 and 100 mmHg, and a value of at least 60 mmHg is required to provide adequate blood flow [36]. MAP can also be estimated using the following formula: $MAP = DBP + \frac{1}{3}(SBP - DBP)$ where SBP

and DBP stand for systolic blood pressure and diastolic blood pressure, respectively. Systolic blood pressure reflects the pressure exerted by the blood on the arterial walls upon ventricular contraction (i.e., during systole), and, hence it mainly depends on CO and is inversely proportional to arterial compliance (**Figure 1**). On the other hand, diastolic blood pressure mirrors the peripheral resistance and for this reason was formerly considered a marker of vascular damage. Nowadays, it is well assumed that both systolic and diastolic blood pressure are important prognostic indicators, and according to the International Society of Hypertension (ISH), the recommended (normal) values for the systolic blood pressure are less than 130 mmHg and for the diastolic blood pressure less than 85 mmHg [37].

Figure 3 shows the fluctuation of systolic, diastolic, mean arterial pressure (MAP), and pulse pressure (i.e., SBP-DBP) in distinct vascular beds, which is in line with the above-mentioned regarding pressure gradients in the arterial and venous systems (see section 2.1.1.1).

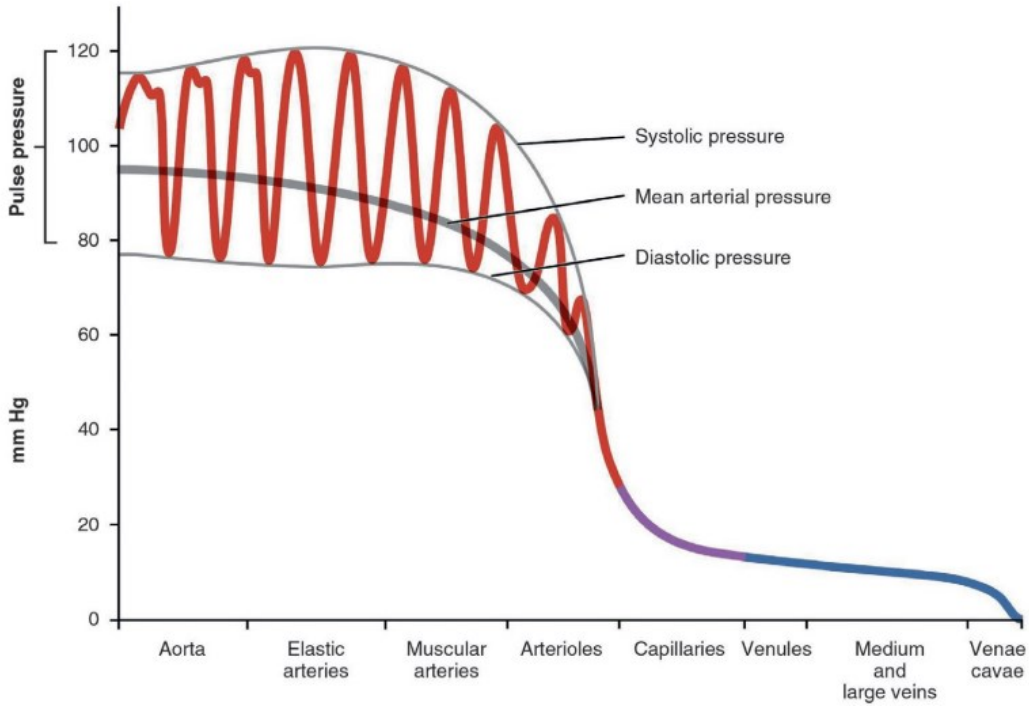

Figure 3. **Systolic, diastolic, mean arterial, and pulse pressures according to different types of blood vessels.** Adopted from [30].

2.2 REGULATION OF THE VASCULAR TONE

Under physiological conditions, numerous vasoactive stimuli permanently influence the vascular tone. Notably, an integrative control is executed centrally and locally. This chapter includes a description of the main molecular mechanisms of vasomotor control and selected targets pertinent to this study.

2.2.1 Mechanisms of VSM contraction

An adequate understanding of the mechanisms that lead to vasoconstriction furnishes a foundation for identifying aetiologies of vascular diseases and therapeutic targets. Succinctly, VSM contraction can occur through three canonical pathways involving the following players: calcium (Ca^{2+}), protein kinase C (PKC), and Rho-kinase (ROCK) (**Figure 4**).

2.2.1.1 Calcium (Ca^{2+})

Ca^{2+} is a major regulator of VSM contraction. Under resting conditions, free Ca^{2+} concentration in the cytosol of VSM cells, i.e., $[Ca^{2+}]_c$, is ~ 100 nM, whereas in the extracellular milieu is ~ 2 mM. The concentration gradient is maintained in these cells through a permanent Ca^{2+} extrusion executed by the plasmalemmal Ca^{2+}-ATPase (PMCA) and the sodium-calcium exchanger (NCX). Moreover, Ca^{2+} is stored in the sarcoplasmic reticulum (SR), an intracellular organelle that occupies nearly 5% of VSM cell volume and where reported free Ca^{2+} levels reach hundreds of μM [38]. Total Ca^{2+} levels in SR, however, are higher due to being bound to calsequestrin, a Ca^{2+}-binding protein [39]. Ca^{2+} is actively pumped from the cytosol back to the SR by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) [40]. However, due to limitations in the storage capacity of the SR, Ca^{2+} is also mobilized to the mitochondria through the mitochondrial Ca^{2+} uniporter (MCU) [41, 42]. A rise in $[Ca^{2+}]_c$ to μM levels triggers a myogenic response [43, 44] as a result of Ca^{2+} influx from the extracellular space and/or Ca^{2+} release from SR. The high electrochemical gradient generates a driving force that promotes a continuous Ca^{2+} entry (i.e., Ca^{2+} leak) into VSM cells that, despite not causing contraction (due to the mechanisms mentioned above of Ca^{2+} extrusion and Ca^{2+} uptake), is primordial for the maintenance of tonic tension [reviewed in [45]]. Additionally, Ca^{2+} influx occurs through voltage-dependent and -independent plasmalemmal Ca^{2+} channels. On the other hand, Ca^{2+} release from the SR occurs through ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3Rs). Furthermore, when cytosolic Ca^{2+} concentrations approach μM units near the SR, Ca^{2+}-induced Ca^{2+} release occurs. After elevation of $[Ca^{2+}]_c$, Ca^{2+} binds calmodulin (CaM), a highly conserved protein with four Ca^{2+}-binding sites and three isoforms (CaM1, CaM2, CaM3) known to interact with more than 300 different targets [46]. Subsequently, the Ca^{2+}-CaM complex activates myosin light chain kinase (MLCK), which phosphorylates the 20-kDa myosin light chain (MLC) and increases the activity of actin-activated magnesium (Mg^{2+})-ATPase allowing the interaction between actin and myosin (i.e., actomyosin cross-

bridge) and VSM contraction [47]. Later, other mechanisms leading to VSM contraction were discovered due to inconsistent relationships between $[Ca^{2+}]_c$, MLC phosphorylation and force, and that involve PKC and ROCK. Interestingly, VSM cell contraction of individual cells is synchronized and conducted along rat mesenteric arteries due to the existence of smooth muscle gap-junctions rich in connexins (e.g., Cx37, Cx43) that allow the spread of intercellular Ca^{2+} waves [48, 49].

Oppositely, VSM relaxation occurs through a decrease in $[Ca^{2+}]_c$, which is mediated by Ca^{2+} uptake by SR (through SERCA) and Ca^{2+} efflux through PMCA and NCX, with consequent dissociation of Ca^{2+}-CaM complex and dephosphorylation of MLC by myosin light chain phosphatase (MLCP) [47].

2.2.1.1.1 Voltage-gated Ca^{2+} channels (VGCCs)

Voltage-gated Ca^{2+} channels (VGCCs) are categorized according to their electrophysiological and pharmacological properties. L-type Ca^{2+} channels ("L" stands for long or lasting, in VSM $Ca_V1.2$) are high voltage-activated channels which are activated frequently at potentials positive to -10 mV [50], followed by a relatively slow inactivation which occurs in a Ca^{2+}-dependent manner. In VSM cells, Ca^{2+} influx occurs primarily *via* L-type Ca^{2+} channels, which can be blocked by dihydropyridines (nifedipine), phenylalkylamines (verapamil) and benzothiazepines (diltiazem). Intriguingly, other dihydropyridines, such as Bay K8644, may exert agonistic or antagonistic effects depending on concentration and precise chemical structure. It is noteworthy that also T-type Ca^{2+} channels ("T" stands for transient; Ca_V3) contribute to vascular tone, especially at low luminal pressures. Unlike their L-type counterparts, T-type Ca^{2+} channels are low voltage-activated as they activate at potentials positive to - 70 mV and inactivate rapidly at potentials greater than -40 mV [50]. $Ca_V3.1$ and $Ca_V3.2$ are expressed in rat, mouse, and human VSM cells [51]. Recent findings with knockout models shed more light on their functional roles. In VSM cells, $Ca_V3.1$ mediate vasoconstriction, whereas in endothelial cells, vasorelaxation occurs due to the activation of endothelial nitric oxide synthase (eNOS). $Ca_V3.2$ are involved in the negative feedback of pressure-induced vascular tone *via* activation of RyRs and a channel named BKCa (see section 2.2.2.2.3) and subsequent hyperpolarization and vasorelaxation [51].

2.2.1.1.2 Other channels

In addition to VGCCs, there are the so-called transient receptor potential (TRP) channels which are cationic channels that participate in the regulation of VSM membrane potential and contraction. TRP channels are grouped into six major subfamilies, namely: TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPA (ankyrin) and TRPML (mucolipin). TRPCs are receptor-operated calcium-permeable nonselective cation channels whose activation induces VSM cell depolarization by the entry of Na^+ and/or Ca^{2+} [52]. TRPCs were formerly suggested as potentially relevant channels for store-operated Ca^{2+} entry [52, 53]. However, shortly after the identification of STIM1 and Orai1 proteins, this hypothesis lost part of its plausibility. STIM1 is located on the SR membrane and acts as an intraluminal Ca^{2+}sensor, while Orai1 is present at the plasma membrane and allows Ca^{2+} entry. Thus, the STIM1-Orai1 pathway is crucial for detecting Ca^{2+} depletion in the SR and

ensuring Ca^{2+} homeostasis [54]. There are several TRPCs isoforms widely expressed in various cell types. TRPC1 and TRPC6 are highly abundant in VSM cells of the rat aorta and mesenteric arteries [52]. TRPC6 are activated by shear stress, allowing Ca^{2+} influx into VSM cells with subsequent vasoconstriction. Moreover, mechanical stimuli, such as intraluminal pressure and vessel wall stretching, also play a role in the maintenance of VSM tone. Counter-intuitively, an increase in the intraluminal blood pressure induces myogenic vasoconstriction, a phenomenon known as the ‘‘Bayliss effect’’ [55].

2.2.1.2 Protein kinase C (PKC)

Agonists at $G_{\alpha q}$ protein-coupled receptors (GPCRs) (e.g., norepinephrine, phenylephrine, angiotensin II (AngII), endothelin-1(ET-1), and thromboxane A2 (TxA2)) cause an increase in $[Ca^{2+}]_c$ by first inducing Ca^{2+} release from SR followed by maintained Ca^{2+} entry from the extracellular space. Specifically, these agonists activate phospholipase C (PLC), which, in turn, cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) [47]. Since IP_3 is water-soluble, it diffuses through the cytosol and binds to the IP_3Rs, resulting in Ca^{2+} release from the SR. Oppositely, because of its lipophilicity, DAG remains in the plasma membrane, where it activates protein kinase C (PKC). PKC is ubiquitously present in endothelial cells, VSM cells, and fibroblasts, with more than 10 isoforms identified (classic, novel and atypical) [56]. PKCα, β, δ and ε are expressed in human VSM cells [57]. PKCα, β, δ and ζ are expressed in the rat aorta [58, 59]; PKCα, γ, δ, ε and ζ in the rat mesenteric artery [60] and PKCα and ε in the porcine coronary artery [61]. Upon activation, PKC translocates from cytosol to the plasma membrane and stimulates VSM contraction through a multitude of ways [62]. PKC inhibits plasmalemmal potassium (K^+) channels, with consequent depolarization and increase in $[Ca^{2+}]_c$ [63]. PKC phosphorylates BKCa channels (see section 2.2.2.2.3) and decreases the chances of their activation by cGMP-dependent protein kinase (see section 2.2.2.1.1) [63-66]. Other actions of PKC include inhibition of Na^+/K^+-ATPase and activation of Na^+/H^+ antiport exchanger leading to VSM depolarization and alkalinization of the cytosol [67, 68]. In addition, PKC promotes VSM contraction through Ca^{2+} sensitizing pathways that lead to the inhibition of MLCP: a) *via* phosphorylation at the myosin phosphatase target subunit-1 (MYPT-1) and b) *via* phosphorylation of the protein kinase C-dependent phosphatase inhibitor, CPI-17 [69]. Other pathways by which PKC promotes VSM contraction include cascades of protein kinases and phosphorylation of downstream effectors, e.g., mitogen-activated protein kinases (MAPK) and actin-binding proteins (e.g., caldesmon) that can inhibit myosin binding to actin. The phosphorylation of caldesmon results in the release of its inhibition of Mg^{2+}-ATPase activity, increase in the actin-myosin interaction and VSM contraction [70].

Interestingly, despite PKC being mostly known for its vasoconstrictive effects, it has also been associated with vasorelaxation through an enhancement of C-natriuretic peptide (CNP) expression in

VSM cells [71, 72], whereas, in endothelial cells, it has been linked to modulation of eNOS activity [73].

2.2.1.3 Rho-kinase (ROCK)

Lastly, agonists at $G_{\alpha 12/13}$ PCRs also lead to VSM contraction by activating the small cytoplasmic GTPase RhoA. In the following step, RhoA binds to and activates ROCK which in turn phosphorylates the MYPT-1 leading to MLCP inhibition, and Ca^{2+} sensitization. There are two ROCK isoforms (ROCK-1 and ROCK-2), with ROCK-2 being the prominent isoform in VSM cells, binding directly to MLCP and promoting contraction [74]. Particularly, this process occurs without the need for a surge in $[Ca^{2+}]_c$. Other substrates of ROCK include CPI-17, 20-kDa MLC and calponin [47].

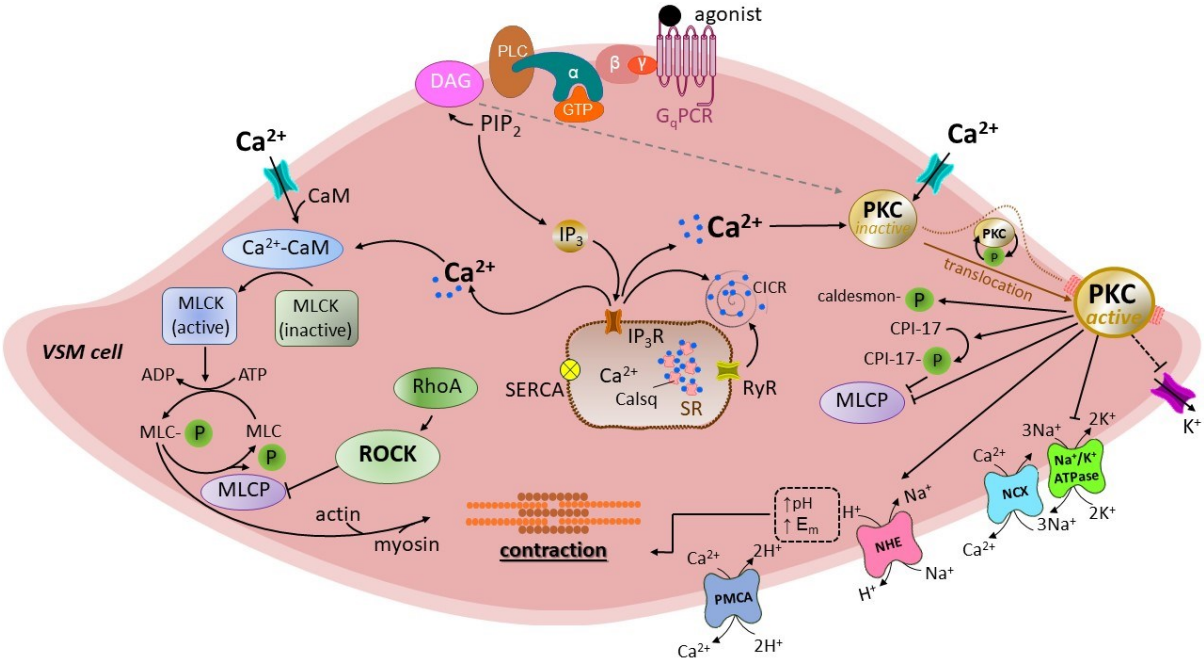

Figure 4. Main mechanisms leading to vascular smooth muscle (VSM) cell contraction. Calcium (Ca^{2+}) is stored in the sarcoplasmic reticulum (SR) bound to calsequestrin (Calsq). Ca^{2+} uptake to SR occurs *via* sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), whereas Ca^{2+} release occurs *via* ryanodine receptors (RyRs) and inositol 1,4,5-trisphosphate receptors (IP_3Rs). An increase in Ca^{2+} concentration in close vicinity to SR triggers calcium-induced calcium release (CICR). Agonists at Gq protein-coupled receptors (GqPCRs) activate phospholipase C (PLC), which, in turn, cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). IP_3 diffuses through the cytosol and binds to the IP_3Rs. Ca^{2+} release from the SR, along with Ca^{2+} entry cause an increase in cytosolic Ca^{2+} concentration. Subsequently, Ca^{2+} binds calmodulin (CaM), and the complex Ca^{2+}-CaM activates myosin light chain kinase (MLCK), which phosphorylates myosin light chain (MLC) and allows the formation of actomyosin cross-bridge and VSM cell contraction. DAG remains in the plasma membrane and activates protein kinase C (PKC), which then translocates to the plasma membrane. Active PKC promotes VSM contraction in many ways, including phosphorylation of caldesmon and the protein kinase C-dependent phosphatase inhibitor (CPI-17), inhibiting myosin light chain phosphatase (MLCP). Besides, PKC directly phosphorylates MLCP leading to its inhibition. Other actions of active PKC include inhibition of various potassium (K^+) channels, inhibition of sodium-potassium ATPase (Na^+/K^+-ATPase) and activation of sodium-proton exchanger (NHE). The alkalinisation of cytosol (↑pH) and cell membrane depolarization (↑E_m) together with Rho-kinase (ROCK) activation by RhoA which results in inhibition of MLCP contribute to VSM contraction. *Other abbreviations*: GTP, guanosine triphosphate; NCX, sodium-calcium exchanger; PMCA, plasmalemmal Ca^{2+}-ATPase; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

2.2.2 Mechanisms of VSM relaxation

Succinctly, VSM relaxation is mediated by a decrease in $[Ca^{2+}]_c$ or a decay in myofilament sensitivity to $[Ca^{2+}]_c$. A decrease in $[Ca^{2+}]_c$ may occur through a) a decrease in Ca^{2+} entry from the extracellular space, b) an increase in Ca^{2+} efflux, or c) Ca^{2+} uptake to the SR. Ca^{2+} desensitization occurs through the phosphorylation of MLCK or the activation of MLCP.

2.2.2.1 Cyclic nucleotide pathways

2.2.2.1.1 Cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG)

The activation of the cytosolic soluble guanylate cyclase (sGC) by nitric oxide (NO) or the membrane-bound particulate guanylate cyclase (pGC) by natriuretic peptides (e.g., CNP) leads to the hydrolysis of GTP to cGMP. cGMP subsequently activates protein kinase G (PKG), a kinase that has three isoforms, Iα, Iβ, and II [75]. PKGIα and Iβ are highly abundant in VSM cells [76]. In its active conformation, PKGIα activates MLCP directly, and indirectly through the inhibition of RhoA. Besides, PKGIα attenuates $G_{\alpha q}$-evoked vasoconstriction [77] (see section 2.2.1.2). PKGIβ, on the other hand, by activating inositol 1,4,5-trisphosphate receptor-associated cGMP-kinase substrate (IRAG), inhibits IP_3R-mediated Ca^{2+} release from the SR [78]. In addition, PKG: a) hyperpolarizes VSM cells through K^+ efflux *via* BKCa, K_V and K_{ATP} channels [79]; b) reduces open probability of L-type calcium channels and TRPC6 channels [80] c) phosphorylates phospholamban thus decreasing its inhibitory effect on SERCA [81], and d) has been associated to an enhancement of PMCA affinity for Ca^{2+} [82]. Last but not least, cGMP levels are modulated by phosphodiesterases (PDEs), mainly by the PDE_5 isoform (**Figure 5**). Interestingly, PKG phosphorylates PDE_5 and increases its activity as a mechanism of negative feedback [83]. Detailed information about the cGMP-PKG pathway is referred in the reference No. [84].

2.2.2.1.2 Cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)

Agonists that bind to $G_{\alpha s}$PCRs, e.g., prostacyclin (PGI_2) binding to IP receptors, lead to the formation of cAMP from ATP following the activation of adenylate cyclase (AC). Subsequently, cAMP activates protein kinase A (PKA), a kinase that has two subtypes PKAI and PKAII. Moreover, cAMP-mediated effects also occur through the activation of the ‘’novel cAMP effector’’ EPAC (exchange factor directly activated by cAMP). The prediction of cAMP-mediated effects on vascular tone is not straightforward. Indeed, opposite EPAC-mediated effects were reported in VSM cells isolated from vessels of a different calibre, i.e., relaxation was observed in large vessels, while contraction in small vessels [reviewed in [85]]. In rat aortic VSM cells, EPAC, through a Rap1-dependent Ca^{2+} desensitization, leads to the inhibition of RhoA and thus disinhibition of MLCP [86]. Intriguingly, cAMP through the activation of PKA leads to the phosphorylation of K_{ATP} and vasorelaxation, whereas, in the absence of PKA, an increase in $[Ca^{2+}]_c$ induced by EPAC inhibits K_{ATP} thus promoting vasoconstriction [87]. Regardless, cAMP signalling-induced vasorelaxation occurs in multiple ways,

including: a) increase in SR Ca^{2+} uptake through phosphorylation of phospholamban, thereby releasing its inhibitory effect on SERCA [88]; b) hyperpolarization of VSM cells by increasing the open probability of K^+ channels (K_{ATP}, K_{ir}, and BKCa) [79] and consequent reduction of the open probability of voltage-gated calcium channels; c) phosphorylation of MLCK, thereby decreasing myofilament Ca^{2+} sensitivity [89]; d) modulatory role on RhoA [90]; e) promotion of MLCP activity [91] and f) reduction of the inhibitory phosphorylation of MYPT-1 [92] (**Figure 6**).

cAMP levels are regulated through the activity of phosphodiesterases, mostly PDE_3 isoform.

Last but not least, agonists at $G_{\alpha i}$PCRs (e.g., EP3 prostanoid receptor) exert inhibitory effects on the activity of AC, being therefore vasoconstrictive.

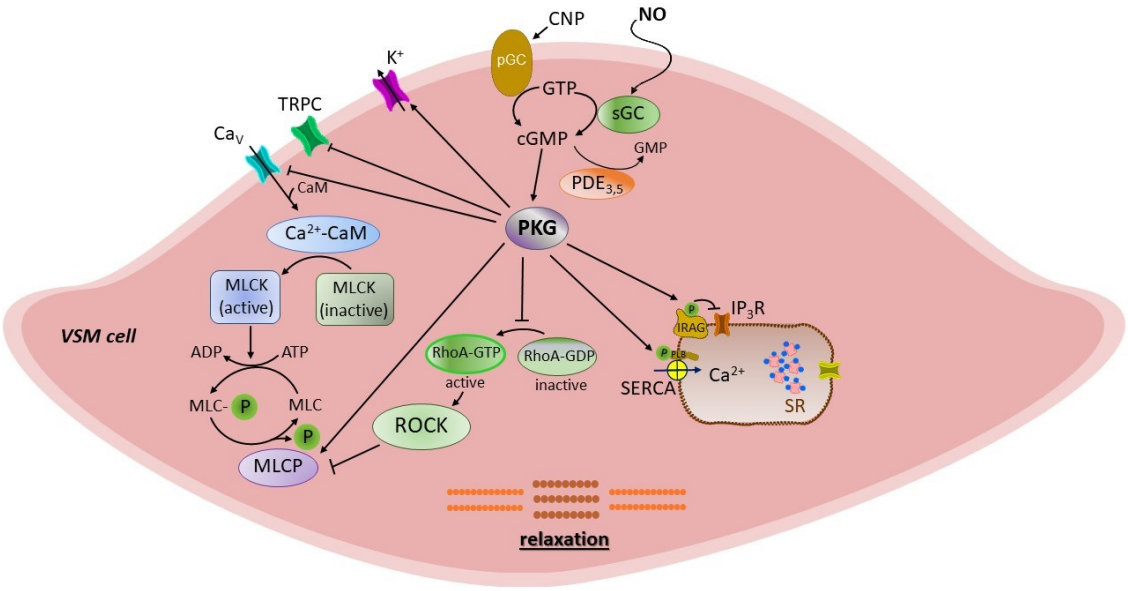

Figure 5. **Main cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG)-mediated pathways involved in vascular smooth muscle (VSM) cell relaxation.** Following the activation of the soluble guanylate cyclase (sCG) by nitric oxide (NO) or the membrane-bound guanylate cyclase (pCG) by natriuretic peptides (e.g., CNP), the hydrolysis of guanosine triphosphate (GTP) to cGMP occurs. cGMP activates protein kinase G (PKG), whose main effects include: a) activation of K^+ channels leading to K^+ efflux and VSM cell hyperpolarization; b) reduction of the open probability of L-type calcium channels and transient receptor potential (TRPC) channels; c) activation of myosin light chain phosphatase (MLCP); d) inhibition of RhoA; e) activation of inositol 1,4,5-trisphosphate receptor-associated cGMP-kinase substrate (IRAG), leading to the inhibition of inositol 1,4,5-trisphosphate receptor (IP_3R)-mediated Ca^{2+} release from the sarcoplasmic reticulum (SR) and phosphorylation of phospholamban (PLB) with consequent release of its inhibitory effect on sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). *Other abbreviations*: PDE, phosphodiesterase; CaM, calmodulin; Ca_V, L-type calcium channels; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ROCK, Rho-kinase.

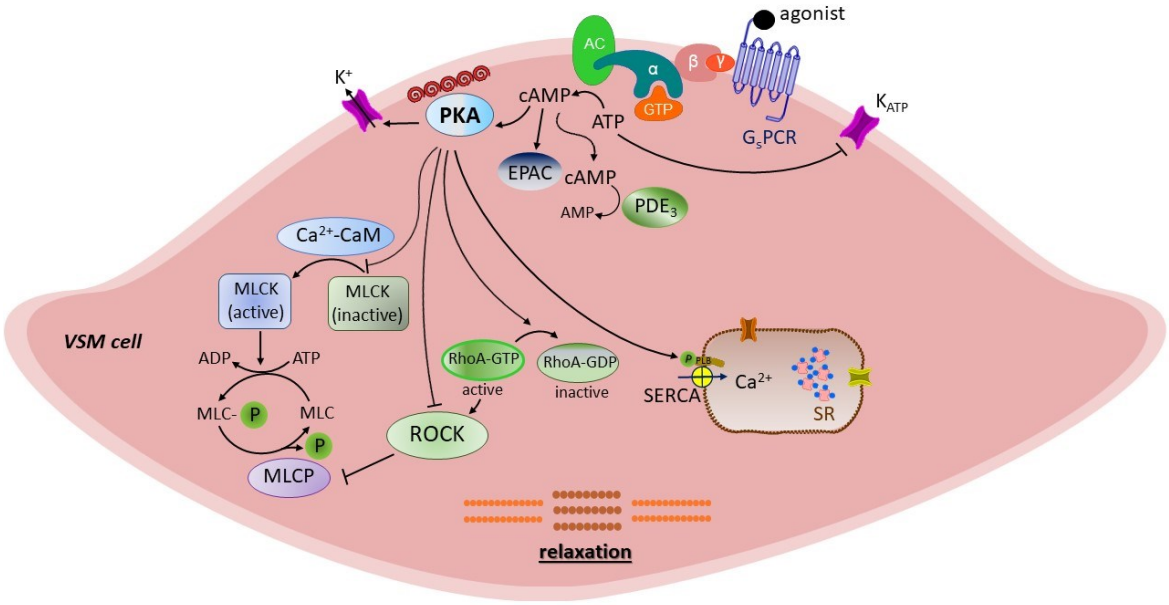

Figure 6. **Main cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)-mediated pathways involved in vascular smooth muscle (VSM) cell relaxation.** The binding of an agonist to Gs protein-coupled receptors (GsPCR) activates adenylate cyclase (AC). Next, AC leads to the hydrolysis of adenosine triphosphate (ATP) into cAMP. cAMP activates PKA, whose main effects include: a) increase in the open probability of K^+ channels and VSM cell hyperpolarization; b) phosphorylation of myosin light chain kinase (MLCK); c) promotion of myosin light chain phosphatase (MLCP) activity *via* modulatory role on RhoA and Rho-kinase (ROCK); and d) phosphorylation of phospholamban releasing its inhibitory effect on sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA). cAMP-signalling also occurs through exchange factor directly activated by cAMP (EPAC). *Other abbreviations*: GTP, guanosine triphosphate; PDE, phosphodiesterase; CaM, calmodulin; ADP, adenosine diphosphate; SR, sarcoplasmic reticulum; K_{ATP}, ATP-sensitive K^+ channels.

2.2.2.2 Membrane potential (E_m) and hyperpolarization

The resting membrane potential (E_m) of VSM cells ranges approximately between -55 mV and -40 mV. VSM cells from small resistance vessels are even more depolarized with an E_m ranging between -45 and -30 mV. K^+ conductance plays a crucial role in the maintenance of resting E_m and, thus, vascular tone. As already described, membrane depolarization leads to the activation of voltage-gated calcium channels, Ca^{2+} influx and provokes a contractile response. Opening of K^+ channels leads to K^+ efflux and consequent decrease in E_m, causing VSM cell hyperpolarization, hence counterbalancing membrane depolarization and smooth muscle contraction [reviewed in [93, 94]]. The diversity of K^+ channels is considerably large, and four major types have been identified in VSM: inwardly-rectifying (K_{ir}), ATP-sensitive (K_{ATP}), large conductance calcium-activated (BKCa), and voltage-gated (K_V) K^+ channels [2, 95].

2.2.2.2.1 Inwardly-rectifying K^+ (K_{ir}) channels

K_{ir} channels are activated by extracellular K^+, and their primary function is to continuously perform an inward rectification at E_m potentials negative to the K^+ equilibrium potential (E_K, ~-83 to -90 mV when extracellular K^+ levels are 5 mM). Contrarily, at more positive E_m (up to -30 mV), K_{ir} conduct a K^+ outward current. There are claims indicating that K_{ir} channels contribute to the maintenance of resting E_m, mostly in small resistance vessels [96]. Seven isoforms (IUPHAR K_{ir}1-7) have been identified, with K_{ir}2.1 being present in VSM cells of rat coronary and mesenteric arteries [97]. K_{ir} channels are inhibited by barium (Ba^{2+}).

2.2.2.2.2 ATP-sensitive K^+ (K_{ATP}) channels

ATP-sensitive K^+ channels are part of the inwardly rectifying channel family. Since K_{ATP} channels are sensitive to intracellular ATP (μM concentrations), they have been purported to act as metabolic sensors [2, 98]. A decrease in intracellular ATP levels triggers K_{ATP} activation. Structurally, they are formed by an inward rectifier K^+ channel pore (K_{ir}6.1 or K_{ir}6.2) and an ATP-binding regulatory subunit (sulfonylurea subunits, SUR_1/SUR_2A/SUR_2B) [99]. Reports on the occurrence of spontaneous coronary vasospasm in K_{ir}6.1 knockout mice highlight their importance in coronary vasculature [100]. The antidiabetic drug of the sulfonylurea class, glibenclamide, inhibits K_{ATP} channels in various vascular beds, including the rabbit mesenteric artery [101].

2.2.2.2.3 Calcium-activated K^+ (KCa) channels

Three subtypes, namely, large conductance (BKCa), intermediate conductance (IKCa), and small conductance (SKCa) calcium-activated K^+ channels, have been described.

BKCa (KCa1.1) are Ca^{2+} and voltage-sensitive channels highly abundant in VSM cells. BKCa opens upon increases in E_m (depolarization), increases in intracellular Ca^{2+} neighbouring BKCa due to Ca^{2+} release from RyRs, or elevation in $[Ca^{2+}]_c$ through L-type calcium channels. Consequently, negative feedback is ensured *via* K^+ efflux and VSM cell hyperpolarization, restricting further $[Ca^{2+}]_c$

elevations. While at resting E_m BKCa channels are practically quiescent, at a depolarized state, such as in resistance vessels, due to Ca^{2+}-induced Ca^{2+} release, the activation of these channels has been reported. In addition, increased activity of BKCa was observed in spontaneously hypertensive rats (SHRs), contributing to decreased aorta contractility [102].

SKCa (KCa2.1-2.3) and IKCa (KCa3.1) constitutively express calmodulin and hence are Ca^{2+}-sensitive. Under physiological conditions, IKCa are mostly present in endothelial cells instead, where they mediate endothelial-derived hyperpolarization of VSM (see section 2.2.3.1.2). However, in proliferating porcine coronary VSM cells, there are reports of both expression and enhanced activity of IKCa [103]. Particularly, IKCa have been associated with phenotypic modulation of coronary VSM cells, atherosclerosis and plaque formation [93, 104].

2.2.2.2.4 Voltage-gated K^+ (K_V) channels

K_V channels are postulated to be the main players responsible for setting the resting E_m in VSM cells. The resting E_m of VSM cells typically ranges between ~-40 mV and -55 mV, i.e., close to the activation threshold for K_V currents [105]. K^+ conductance, however, is not solely responsible for the resting E_m. K_V channels comprise 12 subfamilies (K_V1-12), and in VSM cells, at least six members, namely, K_V1.x, K_V2.1, K_V9, K_V3.1, K_V4.x and K_V7.x were identified [79]. K_V2.2 was described in the aorta, mesenteric and coronary arteries [2]. Furthermore, different isoforms are found in distinct vascular beds and species. For instance, K_V1.2, K_V1.5, K_V2.1, K_V7.1, K_V7.4, and K_V7.5 have been identified in blood vessels from rodents, rabbits, dogs and humans [reviewed in [3]]. Concerning K_V7, it is noteworthy that they are major players in the regulation of VSM contractility. K_V7 subfamily comprises five members (K_V7.1-K_V7.5). K_V7.1 are abundant in cardiomyocytes where together with the ancillary protein KCNE1 give origin to the slow delayed rectifier potassium current (IK_S) involved in ventricular repolarization [106]. On the other hand, K_V7.2, 7.3 and 7.5 allow the slow voltage-gated ‘’M-current’’ that underpins neuronal excitability and transmitter release [106], whereas K_V7.4 and K_V7.5 are highly abundant in VSM cells where, apparently, they play an important role in vasomotor control and total peripheral resistance [107]. Last, both cAMP and cGMP vasodilatory pathways exert modulatory roles on K_V7 channels. In particular, cAMP-signalling *via* PKA and EPAC is expected to enhance K_V7 activity, and K_V7 was found to participate in cGMP-mediated vasodilation [108-110].

2.2.3 Endothelium & vascular tone

2.2.3.1 Endothelium-derived relaxing factors

2.2.3.1.1 Nitric oxide (NO)

Initially denominated endothelium-derived relaxing factor (EDRF) by Furchgott and Zawadzki, the radical nitric oxide (NO) became a matter of intense research shortly after its discovery [111]. NO-synthases (NOS) are the enzymes responsible for NO synthesis by converting L-arginine to L-citrulline, with the endothelial isoform (eNOS) being predominant in the cardiovascular system. Besides its well-known vasodilatory properties, NO exerts a wide variety of other effects, such as inhibition of platelet aggregation, leucocyte and platelet adhesion, inhibition of smooth muscle proliferation and antioxidant activity [reviewed in [112]]. NO production is induced by the binding of agonists to endothelial $G_{\alpha q}$-PCRs (e.g., binding of acetylcholine to muscarinic receptors). Recalling the above-described (see section 2.2.1.2), agonists at $G_{\alpha q}$-PCRs lead to the production of IP_3 and DAG. Further binding of IP_3 to IP_3R leads to the release of Ca^{2+} from the SR and causes an elevation in $[Ca^{2+}]_c$ followed by the activation of CaM. In endothelial cells, however, differently from what occurs in VSM, CaM as a co-factor of eNOS and, by activating it, enhances NO production. Once formed, NO rapidly diffuses to VSM cells, targets sGC and induces vasorelaxation (see section 2.2.2.1.1). Additionally, eNOS can be activated without an increase in $[Ca^{2+}]_c$. This calcium-independent activation occurs through the phosphorylation of eNOS. For instance, VEGF phosphorylates eNOS mainly via the Ser/Thr kinase Akt (protein kinase B), while shear stress induces phosphorylation mainly *via* activation of PKA [113]. Interestingly, other actions of NO include post-translational modifications of proteins, such as: a) reversible S-glutathionylation of SERCA enhancing its activity as part of the Ach-induced relaxation of rabbit carotid arteries [114] and b) S-nitrosylation of sGC lessening its activity as negative feedback [115].

2.2.3.1.2 Endothelium-derived hyperpolarization (EDH)

In addition to the release of EDRFs, endothelial cells are able to induce vasorelaxation by generating an endothelium-derived hyperpolarization (EDH). The hyperpolarization of endothelial cells involves an increase in $[Ca^{2+}]_c$ through the activation of TRP channels, followed by the opening of SKCa and IKCa (see section 2.2.2.2.3). While SKCa are mostly located in close vicinity to endothelial gap junctions, IKCa are mostly found near myoendothelial projections (MEJs) that grant electrotonic propagation of hyperpolarization from endothelial to VSM cells. Moreover, the opening of endothelial SKCa and IKCa channels allows an outward current of K^+ ions that subsequently bind to and activate K_{ir} channels and Na^+/K^+-ATPase on the plasmalemma of VSM cells [reviewed in [5]]. MEJs allow the diffusion of molecules (e.g., Ca^{2+} and IP_3) between endothelial and VSM cells and the bidirectional conduction of hyperpolarization and depolarization [116, 117]. Notably, the contribution of EDH to vasorelaxation is more relevant in vessels of smaller calibre, where MEJs are highly abundant [118,

119]. In contrast, NO-mediated vasorelaxation is predominantly observed in vessels of larger calibre [120].

2.2.3.1.3 Prostacyclin (PGI_2)

Early work by Moncada, Vane and colleagues led to the discovery of prostacyclin (PGI_2) [121]. PGI_2 is a prostaglandin that arises from the metabolism of arachidonic acid by cyclooxygenases (COXs, also referred to as prostaglandin G/H synthases) and isomerization of PGH_2 *via* PGI synthase (CYP8A1). While COX-1 is ubiquitous and constitutively expressed, COX-2 is induced in inflammatory and oxidative stress states. Furthermore, in small vessels in the brain and heart, another isoform, COX-3, was reported [122]. PGI_2 is produced in both endothelial and VSM cells, and data from COX knockout mice revealed that in the endothelium of systemic blood vessels, COX-1 is the isoform responsible for the release of PGI_2 [123]. PGI_2, through the binding to IP receptors ($G_{\alpha s}$PCRs) on VSM cells, induces vasorelaxation by the increase in cAMP (see section 2.2.2.1.2 and **Figure 7**). The reported vasodilation has also been linked to the binding of PGI_2 to PPAR receptors. Besides vasodilation, PGI_2 has been associated with inhibition of platelet aggregation, atheroprotective effects, and angiogenesis [124].

2.2.3.2 Endothelium-derived contracting factors

Endothelin-1 (ET-1) is a potent endothelium-derived vasoconstrictor peptide. To date, two ET-1 receptor subtypes, ET_A and ET_B, have been identified [125]. By binding to the ET_A receptor, ET-1 induces a vasocontractile response, promotes cell proliferation and mediates thromboxane A_2-induced contractions [126]. Oppositely, ET-1 binding to ET_B receptors induce vasorelaxation. Nonetheless, vasoconstriction is by far the most prominent effect of ET-1 and ET_A is the preponderant receptor in the cardiovascular system.

Endothelial COXs also generate vasoconstrictive mediators. Such molecules include oxygen-derived free radicals, thromboxane A_2, and prostaglandin $F_{2\alpha}$ (**Figure 7**). In some cases, the vasoconstrictive effects are elicited by binding to thromboxane (TP) receptors located on VSM cells. Indeed, despite of thromboxane A_2 being the natural ligand for TP receptors, other mediators, namely, PGH_2, isoprostanes and hydroxyeicosatetraenoic acids (HETEs) can also activate these receptors. In addition to TP receptors, vasoconstriction might occur through the activation of PGE_2 type 3 receptor (EP3) which is a $G_{\alpha i}$PCR and thus inhibits AC (see section 2.2.2.1.2) [127]. Moreover, vasoconstriction can also result from the activation of PGE_2 type 1 receptor (EP1), a $G_{\alpha q}$PCR (see section 2.2.1.2) [127].

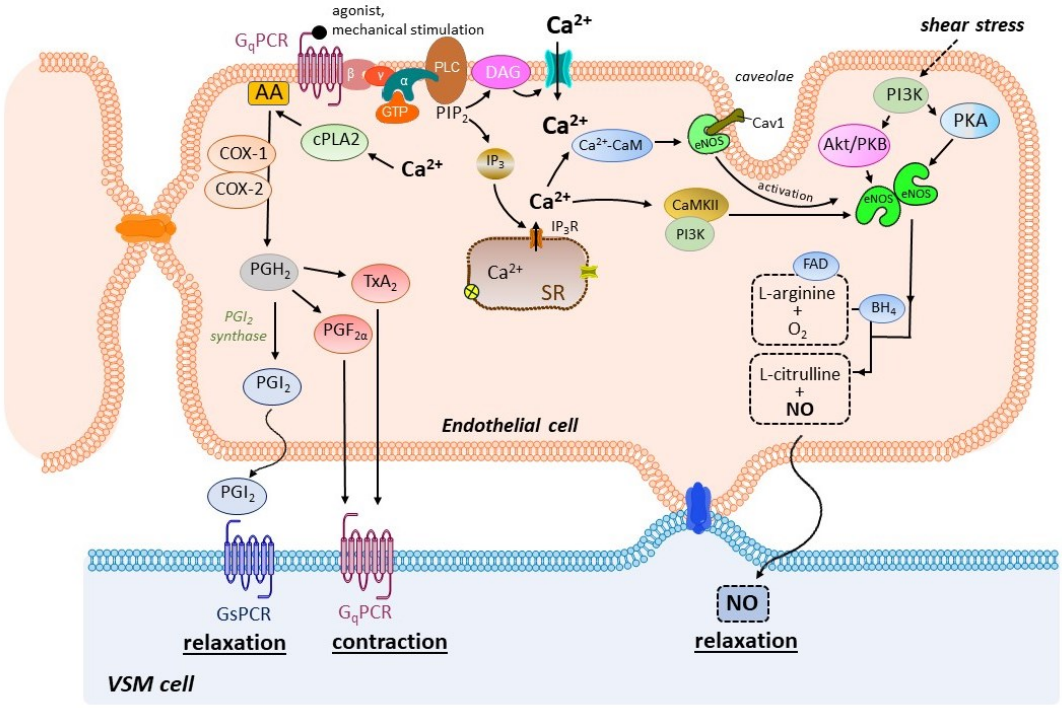

Figure 7. **The role of endothelium in the regulation of vascular tone.** Endothelial nitric oxide synthase (eNOS) is highly abundant in *caveolae* (plasmalemmal invaginations) bound to the protein caveolin-1 (Cav1). Agonists at Gq protein-coupled receptors (GqPCRs) lead to the activation of phospholipase C (PLC) and formation of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3) from phosphatidylinositol 4,5-bisphosphate (PIP_2). IP_3 elicits calcium release from the sarcoplasmic reticulum (SR) by binding to IP_3 receptors. The activation of eNOS occurs after the calcium-calmodulin (Ca^{2+}-CaM) complex displaces Cav1. Tetrahydrobiopterin (BH_4) and flavin adenine dinucleotide (FAD) are cofactors required for the production of nitric oxide (NO). BH_4 is important for the dimerization of eNOS with consequent production of NO and L-citrulline from L-arginine. Then, NO diffuses to vascular smooth muscle (VSM) cells leading to vasorelaxation (section 2.2.2.1.1). In addition, activation of eNOS might occur *via* phosphorylation by calcium-calmodulin-dependent protein kinase II (CaMKII) and phosphoinositide 3-kinase (PI3K), protein kinase A (PKA) and protein kinase B (Akt/PKB). Shear stress induces phosphorylation of eNOS mainly *via* activation of PKA. On the left upper corner: an increase in cytosolic calcium in endothelial cells, activates the cytosolic phospholipase A2 (cPLA2) which then hydrolyses arachidonic acid (AA) from the plasma membrane. AA is further metabolized by cyclooxygenases (COX-1 and COX-2) to several prostaglandins (PGs). Prostacyclin (PGI_2) is produced by PGI_2 synthase and then diffuses into VSM cells where it induces relaxation by binding to GsPCRs (section 2.2.3.1.3). Thromboxane A2 (TxA2) and $PGF_{2\alpha}$ induce vasoconstriction by binding to GqPCRs. *Other abbreviations*: GTP, guanosine triphosphate.

2.3 PHENOLIC COMPOUNDS

2.3.1 Dietary (poly)phenols and small phenolic metabolites

Plant (poly)phenols are secondary metabolites with diverse functions ranging from defence against herbivores and pathogens to the formation of hues (vibrant colours) that confer protection against ultraviolet radiation and attract pollinators and seed dispersing animals [128]. In the current meaning, polyphenols consist of at least one benzene ring with one or more hydroxyl groups attached. To date, more than 8000 structures have been identified and they are classified into: simple phenolics (e.g., catechol and pyrogallol), phenolic acids (e.g., caffeic and vanillic acids), lignans (e.g., podophyllotoxin), stilbenes (e.g., resveratrol), coumarins and flavonoids [128]. Flavonoids, in turn, are divided into the following subgroups: flavonols (e.g., quercetin), flavones (e.g., apigenin), isoflavones (e.g., daidzein and genistein), flavanones (e.g., hesperetin), anthocyanidins and flavan-3-ols (e.g., epicatechin).

2.3.1.1 Food sources and estimated daily intake

Flavonoid-rich foods and beverages include fruits (purple, blue, and red berries, citrus, and apples), vegetables (red onions), cocoa, nuts, tea, coffee, beer and red wine [129]. Additional information for more than 500 (poly)phenols present in plant products can be found in the Phenol-Explorer Database [130]. A large cohort population study (n = 36037) with 10 European countries revealed an estimated mean daily intake of total polyphenols of ~1170–1200 mg/day, with flavonoids accounting for 50%, although a large variability was observed between countries [131]. Another study with Finnish adults (n = 2007) reported a mean total intake of polyphenols of ~ 860 mg/day, with coffee and cereals being major food sources [132], and in the United States (US) population (n = 9773) a similar value was found, i.e., ~ 885 mg/day mostly obtained from coffee, beans and tea [133].

2.3.1.2 Pharmacokinetics

In diet, flavonoids occur mainly in glycosidic forms, i.e., with one or more sugar moieties bound to phenolic groups or to the -OH at C3 position. Due to being extensively metabolized, their bioavailability is influenced by the sugar moiety, food matrix, and interindividual variations concerning microbiome and proteins involved in biotransformation and pharmacokinetics [134, 135].

Prior to absorption, deglycosylation must occur. It can be mediated by the activity of hydrolases located in the brush border in the small intestine, namely lactase-phlorizin hydrolase (LPH) and cytosolic β-glucosidase (CBG) present in enterocytes [136]. Next, the free aglycones undergo phase II metabolism in the enterocyte performed by uridine-5'-diphosphate-glucuronosyltransferases (UGT), sulfotransferases (SULT) and catechol-*O*-methyltransferases (COMT) [137]. Phase I metabolism also occurs, albeit to a much smaller extent. In the following step, flavonoids are transported to the liver, *via* the portal vein, and undergo further conjugation. Subsequently, the hepatic conjugates can be excreted

into the intestine through the bile, and be reabsorbed after deconjugation by the gut microflora. The enterohepatic circulation results in the prolongation of flavonoids half-life ($t_{1/2}$) in human plasma (**Figure 8**).

It is worthy to note that only a small portion (~10 %) of flavonoid glycosides is absorbed in the small intestine while the remaining amount reaches the colon. For instance, rutin (quercetin-3-*O*-rutinoside) reaches the colon in its non-metabolized form. In the subsequent step, deglycosylation occurs by the colonic microbiota and quercetin, i.e., the aglycone form, is released. Further, reduction of double bond in 2,3-position takes place and C-ring fission occurs leading to the production of simple phenolic metabolites (**Figure 9**) [138]. Only flavonoids with 5 and 4' free -OH groups undergo C-ring cleavage [139]. The research interest in identifying flavonoid metabolites is not recent insofar as there are studies from the 1950s that identified metabolites of quercetin and rutin in the urine of rabbits [140]. Later, studies performed in germ-free rats [141] and ileostomists [142] showed that these metabolites result from colonic bacterial transformation. Compared with the parent forms, these phenolic metabolites are more readily absorbed and have a higher bioavailability. In human plasma, they were detected at concentrations up to tens of μM after ingestion of a cranberry juice containing 787 mg of polyphenols [143]. Furthermore, these gut microbiota-derived phenolics also undergo phase II hepatic metabolism, namely, *O*-methylation, glucuronidation and sulphation [136] and are excreted in the urine (**Figure 8**).

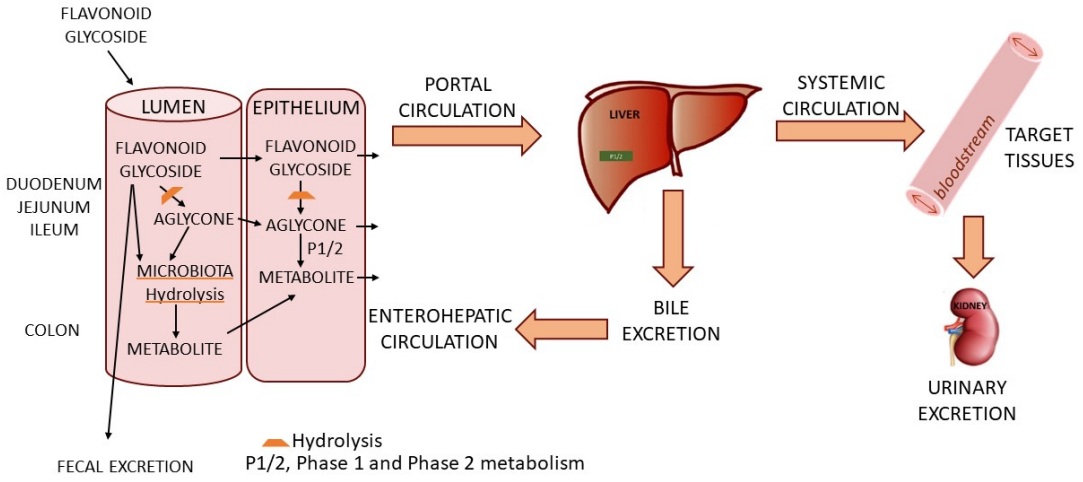

Figure 8. Schematic representation of absorption, metabolism and elimination of flavonoids. Adopted and modified from [135].

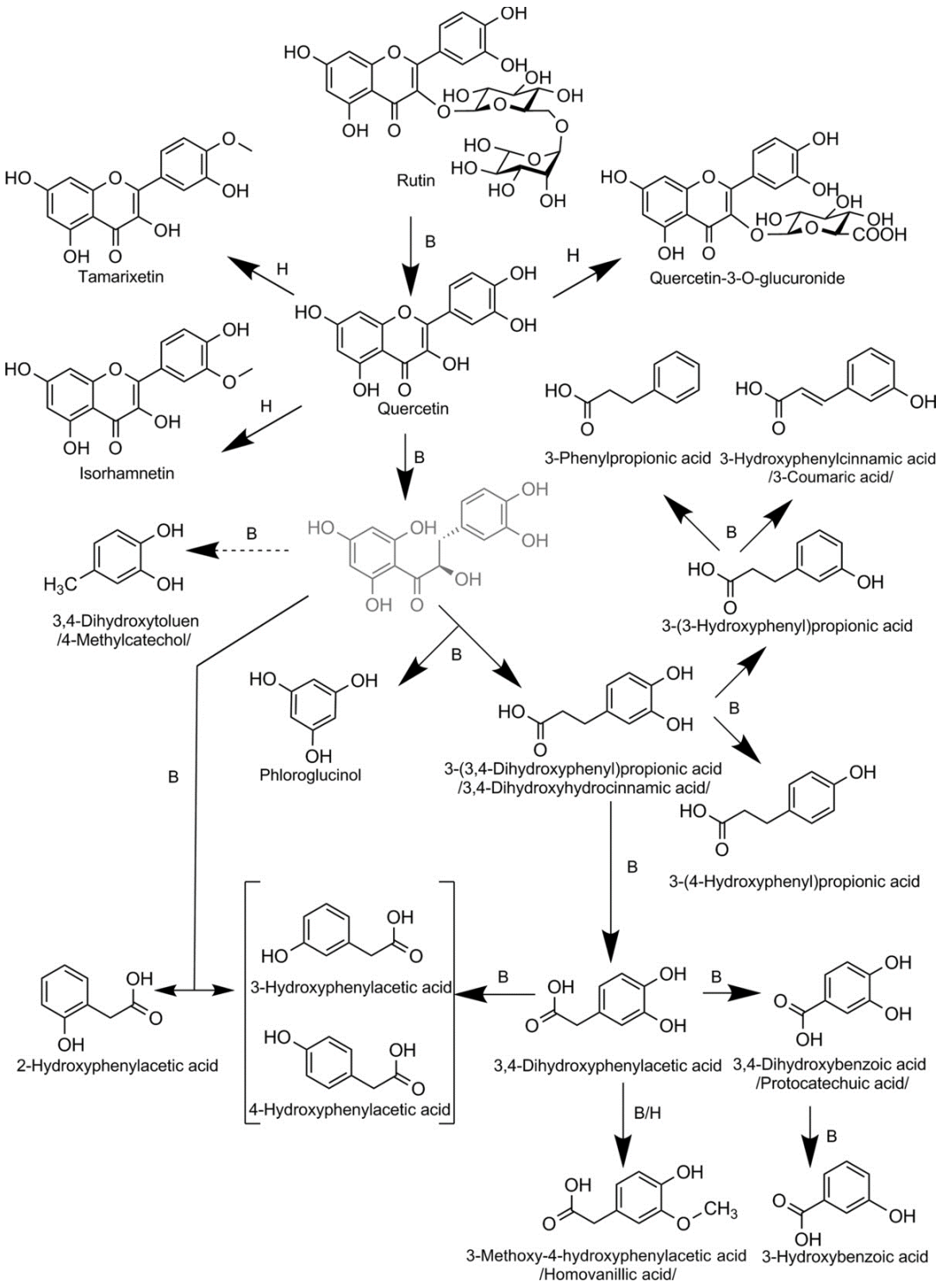

Figure 9. Scheme of the metabolism of quercetin. The intermediate represented in grey is hypothesised. B stands for bacterial enzymes (microflora), whereas H human enzymes. Adopted from [9].

2.3.1.3 Plasma levels of small phenolic metabolites

Overall, higher plasma concentrations are reported for small flavonoid metabolites than their parent forms. After ingestion of a cranberry juice with 787 mg of (poly)phenols, 60 different phenolic metabolites (e.g., hippuric acids, catechols, benzoic acids, phenylacetic acids, and cinnamic acid derivatives) were detected in human plasma. Among metabolites, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, 4-methylcatechol-*O*-sulfate and hippuric acid reached maximal concentrations of 476 nM, 615 nM, 3.5 µM, and 42.9 µM, respectively, 7 to 10 h after consumption [143]. Given the many factors influencing the nature of formed metabolites and their levels, the existence of considerable interindividual variability is anticipated. Nevertheless, commonly detected levels in plasma range between low nanomolar to low micromolar [143]. Additional information and comprehensive data on polyphenol metabolism can be found in the Phenol-Explorer database at http://www.phenol-explorer.eu.

2.3.1.4 Bioactivities of small phenolic metabolites and their effects on the vascular system

The bioactivities of small phenolic metabolites include antioxidant, anti-inflammatory, antidiabetic, anticarcinogenic, anti-osteoporotic, and neuroprotective activities [136, 138].

In regards to direct effects on blood vessels, prior studies conducted by my colleagues showed vasorelaxant properties in the rat aorta for several metabolites, such as 3-(3-hydroxyphenyl)propionic acid, 3,4-dihydroxyphenylacetic acid, and 3-hydroxyphenylacetic acid (**Figure 9**) [9, 144]. However, different vasodilatory potencies and efficacies were found. Further mechanistic investigations have shown that metabolites also differ in their mechanisms of action. In addition, these metabolites might have multiple cardiovascular effects. For instance, 4-methylcatechol (**Figure 9**) (2.5 mg/kg, intravenously) caused a decrease in mean arterial blood pressure by 10% in spontaneously hypertensive rats [144] but also, antiplatelet activity was reported for this compound [145].

Finally, it must be underlined that, despite the multiple beneficial health effects reported in the literature, evidence from human intervention trials remains scarce [146].

2.3.2 Bisphenols

Bisphenols are synthetic compounds widely used in the manufacture of polycarbonate plastic and epoxy resins lining food and beverage cans. They are also used as flame retardants and components of dental sealants, thermal paper, cigarette filters, and electronic devices [147, 148]. Due to depolymerisation, bisphenols leach into the environment, food, beverages, and even infant formula [149], and thus, humans are unavoidably exposed, as well as other animals in the ecosystem.

The first reports on the potential endocrine-disruptor activity of the lead compound, bisphenol A (BPA), date back to the early 1930s [150], but data on its harmful effects resulted in significant legislative changes only in the last two decades. BPA was prohibited from infant feeding bottles in Europe since 2011 [151], and in 2017, the European Chemicals Agency (ECHA) classified BPA as a substance of very high concern (SVHC) based on its endocrine-disrupting effects [152].

Due to such restrictions, next-generation (NextGen) bisphenols were readily introduced as alternatives to BPA. More than two dozen bisphenols exist with similar structures to BPA, albeit with different substituents. The most commonly used NextGen analogues are bisphenols S (BPS), F (BPF), and AF (BPAF) (**Figure 10**). Similar to BPA, reports show that most of these alternatives may also have endocrine-disrupting activities [153]. Fortunately, many European Union (EU) countries have proposed investigating and regulating these BPA alternatives [154].

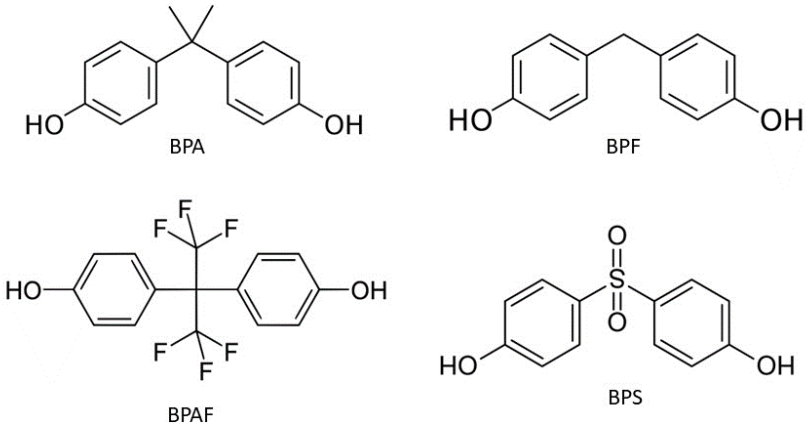

Figure 10. **Chemical structures of BPA, BPF, BPAF and BPS**.

At first sight, the introduction of BPA alternatives appeared to be a quite effective measure. However, new concerns emerged shortly after because, unlike BPA, safety data on these alternatives is scant [155, 156]. Moreover, because the industry increasingly uses these new analogues, they are emergent environmental pollutants.

2.3.2.1 Sources of human exposure

BPA, BPS and BPF were detected in surface water and seawater samples from Asian countries at units of μg/L [157]. Reported BPAF levels in surface waters in the Czech Republic were around 200 ng/L [158]. In a Chinese freshwater lake, BPA, BPF, BPAF, BPS and other bisphenols (AP, Z, B) were detected at levels ranging from units to hundreds of ng/L [159]. Bisphenols also occur in sediments, sewage and sludge [160], food [161], bottled water [162], personal daily care products [163], thermal receipts [164], and indoor dust [165].

The estimated daily BPA exposure of the population in general ranges between 0.5 and 1.5 μg/kg, and the exposure routes include oral, sublingual, transdermal, and inhalatory [11].

2.3.2.2 Toxicokinetics

Notably, toxicokinetic studies of BPA in human subjects showed that, after ingestion, BPA is rapidly absorbed and metabolised by conjugation giving origin to its glucuronide (the main metabolite) and sulphate. Maximum free (unconjugated) and conjugated BPA serum concentrations are attained after ~ 1h. It is noteworthy that the free BPA form accounts only for ~0.6% of the total BPA. Urinary excretion is the main route of elimination of BPA in humans, with the recovery of the administered dose exceeding 90%. The terminal $t_{1/2}$ of free BPA and its glucuronide is ~5.5h [166, 167]. In addition, toxicokinetic of BPS was also explored in humans. Free BPS is rapidly absorbed (t_{max}< 1h), and it is also conjugated. However, differently from BPA, BPS apparently undergoes enterohepatic circulation. Similar to BPA, BPS is predominantly excreted through urine [168, 169]. Concerning toxicokinetic studies of BPA in animals (e.g., rats, mice, piglets and non-human primates), interspecies differences must always be taken into account. For instance, in rats, biliary excretion of BPA and enterohepatic circulation of BPA-glucuronide occurs [170]. In the gut, BPA-glucuronide is hydrolysed by β- glucuronidases and free BPA is released and subsequently reabsorbed into blood [170]. In this respect, non-human primates are closest to humans since the elimination of BPA occurs through urinary excretion [171].

When assessing the potential negative effects of BPA on systems, one must consider the route of administration. Indeed, rat studies showed that BPA administered by gavage results in much lower exposure to free BPA (i.e., the biologically active form) compared with the same dose administered intravenously [11]. Besides the route of administration, age-dependent differences have been reported concerning free BPA levels attained [11].

2.3.2.3 Bisphenol levels in human biological fluids

The information contained in **Table 1** concerning bisphenol levels detected in human biological fluids is representative of a number of published articles.

Table 1. A brief outline of bisphenol levels detected in human biological samples [11, 172].

Biofluid	[Bisphenol] levels (nM)	Population
Serum	[free BPA] 1.4–2.6; [BPF] nd–31	General population
	[BPA] 2.4–140; [BPS] nd–0.4; [BPAF] nd–0.13;	Residents in an industrial area
	[BPA] nd–430	Workers in epoxy resin factories
	[BPA] nd–300; [BPS] nd–560; [BPAF] nd–1.2	Pregnant women
Umbilical cord blood	[BPA] nd–39	Foetuses
Amniotic fluid	[BPA] nd–25	Pregnant women
Breast milk	[BPA] nd–66; [BPS] nd–5; [BPAF] nd–0.274	Lactating women
Urine	[BPA] 0.44–152; [BPS] 0.08–84; [BPF] 0.69–5.5	General population
	[BPA] 3–16; [BPS] 0.7–4; [BPF] 1.5–6.4	Pregnant women
	[BPA] 20–8500	Industrial workers

Data are presented as min–max values. [free BPA]: unconjugated BPA levels; [bisphenol]: total or non-specified levels; nd: not detected.

2.3.2.4 Effects on the cardiovascular system

There has been intense research concerning potential BPA hazards, and although far less explored, there are also some data on NextGen bisphenols. The main effects of bisphenols on the cardiovascular system *in vitro*, *ex vivo* and *in vivo* are compiled in **Table 2**.

Of particular interest are human population studies addressing potential cardiovascular risks arising from exposure to bisphenols. A prospective cohort study with US participants from the National Health and Nutrition Examination Survey (NHANES) demonstrated an increased risk of cardiovascular mortality in subjects with higher urinary BPA levels [173]. Concerning the hallmarks of vascular disease, a cross-sectional study showed an association between serum BPA concentrations and the development of atherosclerotic plaques in elder Caucasian subjects [174]. In Asian adolescents and young adults, a positive association was found between BPA exposure and the thickness of the intima and media layers in the carotid artery [175]. In children, exposure to BPA was linked to an increase in oxidative stress biomarkers, and for BPS, independent of the lack of clarity, a possible correlation between higher BPS levels and endothelial dysfunction cannot be ruled out [176]. There are also reports of an association between higher urinary BPA concentrations and severe coronary artery disease, peripheral artery disease, and hypertension [10, 177]. In urine, also higher BPS levels were associated with a greater propensity to develop hypertension [178], while elevated BPA was associated with increased risk of preeclampsia [179].

Table 2. Effects of bisphenols on relevant targets for the cardiovascular system.

Ion channels & ionic currents	**Ref**
[BPA] activation of BKCa (KCa1.1) (human and canine coronary SMCs)	[180]
[BPA, BPF, BPS] blockade of rapid delayed rectifier K^+ current (IKr)	[181]
[BPA] blockade of voltage-gated Na^+ channel hNav1.5 (cardiomyocytes)	[182]
[BPA, BPF, BPS] blockade of peak and late Na^+ currents (I_{Na})	[181]
[BPA] blockade of L-type Ca^{2+} channel (W-CLTCC) currents (A7r5 cells)	[183]
[BPA] blockade recombinant human T-type Ca^{2+} channels Cav3.1, Cav3.2, Cav3.3 (HEK293 cells)	[184]
Heart	**Ref**
[BPA] ↑ oxidative stress, ↓ NO, ↓ acetylcholinesterase activity (heart, BPA-treated ♂ rats)	[185]
[BPA] ↑ malondialdehyde, ↓ GSH, ↑ CK-MB, myocardial injury (♂ rats)	[186]
[BPA] lengthening of the RR, prolongation of QT and PQ intervals (♂ rats)	[186]
[BPA] ↓ rate and force of atrial contractions (rat hearts)	[187]
[BPA] ↓ HR, heart block, ↓ left ventricular pressure, alterations in Ca^{2+} handling (rat hearts, cardiomyocytes)	[188]
[BPA, BPS] ↓ ventricular systolic pressure (mouse hearts)	[189]
[BPA, BPF, BPS] ↓ HR (rat hearts)	[181]
Blood vessels	**Ref**
[BPA] vasorelaxation (rat aortic rings)	[183]
[BPA] alterations of embryonic and maternal vessels in placenta (pregnant CD-1 mice)	[190]
[BPA] impairment of Ach-induced relaxation; ↑ Ang II; ↑eNOS; ↑ superoxide and peroxynitrite (carotid artery isolated from BPA-treated mice)	[191]
[BPA] oxidative stress and endothelial dysfunction (aorta isolated from BPA-treated rats)	[192]
Arterial blood pressure	**Ref**
[BPA] ↑ SBP ↑ DBP (CD1 mice)	[191]
[BPA] ↑ SBP ↑ DBP (♂ rats)	[186]
Angiogenesis	**Ref**
[BPA] ↑ VEGF production (swine aortic endothelial cell line)	[193]
[BPA] ↓ uterine VEGF expression (♀ rats neonatally exposed)	[194]
[BPA] ↑ VEGF expression in the uterus, vagina and pituitary (adult ovariectomized rats)	[195]
Atherosclerosis & lipid profile	**Ref**
[BPA] ↑ atherosclerotic lesions in the aorta and aortic sinus; ↑ non-HDL cholesterol ($ApoE^{-/-}$ mice)	[196]
[BPA] ↑ atherosclerotic lesions in the aortic arch; SMCs proliferation; inflammation (WHHL rabbits)	[197]
[BPS] ↑ serum cholesterol, ↑ TG, ↑ LDL, ↑ VLDL, ↓ HDL (♂ rats)	[198]
Endocrine system	
[BPA, BPAF, BPF, BPB] estrogenic activity (MCF-7 cells)	[199]
[BPA, BPF, BPB, BPS] anti-androgenic activity (DHT assay in NIH3T3 cells)	[199]
[BPA] agonist and antagonist at thyroid hormone (TH) receptors (GH3 cell line)	[200]
[BPA, BPF, BPS, BPAF] interference with genes related with TH synthesis (GH3 cell line, FRTL-5 cells)	[201]
[BPA, BPAF, BPB, BPF, BPS, BPZ] thyroid hormone-like activity (GH3 cell line)	[202]

3 AIMS OF THE DOCTORAL THESIS

This doctoral project aimed to investigate the cardiovascular effects and mechanisms of action of two major categories of phenolic compounds: dietary phenolic metabolites and possible pollutant bisphenols.

Considering that **small phenolic metabolites** derived from the gut microbiota have a higher bioavailability than parent compounds and, therefore, may be primarily responsible for the biological activities, the following objectives were stipulated:

a) To investigate whether the small metabolite, 3-hydroxyphenylacetic acid (3-HPAA), exerts beneficial cardiovascular effects *in vivo* and decipher the mechanism of action;

Given the structural resemblance of the **newly introduced NextGen bisphenols** to BPA and many still unresolved concerns due to the lack of safety studies, the following aims were set:

a) To elaborate a systematic literature review about the potential impact of bisphenols on the cardiovascular system;
b) To investigate whether a series of NextGen bisphenols exert harmful effects on the cardiovascular system *in vitro*, *ex vivo* and *in vivo.*

4 PUBLICATIONS INCLUDED IN THE DOCTORAL THESIS

The framework of the present manuscript-based doctoral thesis relies on an annotated collection of three articles published in peer-reviewed journals with impact factor. In particular, two original research articles (one with shared first-authorship) and one literature review.

P1. Dias P, Pourová J, Vopršalová M, Nejmanová I, Mladěnka P. **3-Hydroxyphenylacetic Acid: a blood pressure-reducing flavonoid metabolite.** Nutrients. 2022; 14(2):328 [IF_{2020}=5.7] ($Q2_{2020/AIS}$ in Nutrition & Dietetics)

The publication is available either as a printed dissertation or online:
https://doi.org/10.3390/nu14020328

P2. Dias P, Tvdrý V, Jirkovský E, Solner Dolenc M, Peterlin Mašič L & Mladěnka P. **The effects of bisphenols on the cardiovascular system.** Crit Rev Toxic. 2022;52(1): 66-87 [IF_{2020}=5.6] (1^{th} $decile_{2020/AIS}$ in Toxicology)

The publication is available either as a printed dissertation or online:
https://doi.org/10.1080/10408444.2022.2046690

P3. Tvrdý V[#], **Dias P**[#], Nejmanová I, Carazo A, Jirkovský E, Pourová J, Fadraersada J, Moravcová M, Peterlin Mašič L, Sollner Dolenc M & Mladěnka P. **The effects of bisphenols on the cardiovascular system *ex vivo* and *in vivo***. Chemosphere 2023;313: 137565 [IF_{2021} 8.9], ($Q1_{2021/IF}$, $Q2_{2021/AIS}$ in Environmental Sciences) [#]designates shared co-first authorship

The publication is available either as a printed dissertation or online:
https://doi.org/10.1016/j.chemosphere.2022.137565

5 COMMENTARY ON PUBLISHED WORKS

Part I. Gut microbiota-derived small phenolic metabolites

P1. 3-Hydroxyphenylacetic Acid: a blood pressure-reducing flavonoid metabolite.

Dias P, Pourová J, Vopršalová M, Nejmanová I, Mladěnka P.

Nutrients. 2022; 14(2):328 [IF_{2020}=5.7] ($Q2_{2020/AIS}$ in Nutrition & Dietetics)

A short summary of the paper:

Preliminary findings by my colleagues have shown that the flavonoid metabolite 3-hydroxyphenylacetic acid (3-HPAA) causes vasorelaxation of the rat aorta *ex vivo* [9]. In this article, we extended this study and investigated whether 3-HPAA-induced vasorelaxation is reflected *in vivo* by a reduction of arterial blood pressure. Besides, we performed a mechanistic study *ex vivo*.

In order to confirm the effect of 3-HPAA *in vivo*, this metabolite was administered to SHRs through intravenous bolus or infusion. Indeed, 3-HPAA caused a dose-dependent and significant decrease in both systolic and diastolic arterial blood pressure. Interestingly, a significant decreasing effect in diastolic blood pressure was noticed even after a dose of 10 $\mu g.kg^{-1}$. Since there were no alterations in heart rate, the pressure-decreasing effect was likely caused by peripheral relaxation. Furthermore, this was not accompanied by baroreceptor reflex. To simulate the slow absorption of the metabolite from the gastrointestinal tract, 5 min-lasting infusions of 3-HPAA (1 or 5 $mg.kg^{-1}.min^{-1}$) were also administered. Also, in these experiments, 3-HPAA reduced significantly both diastolic and systolic blood pressure.

In the next step, the *in vivo* study was complemented with a series of mechanistic experiments performed *ex vivo* using the left circumflex porcine coronary artery. In particular, the results showed that 3-HPAA acts in an endothelium-dependent manner *via* an enhancement of the activity (or activation) of endothelial nitric oxide synthase (eNOS).

Part II. Bisphenols

P2. The effects of bisphenols on the cardiovascular system.

Dias P, Tvrdý V, Jirkovský E, Solner Dolenc M, Peterlin Mašič L & Mladěnka P.

Crit Rev Toxic. 2022;52(1): 66-87 [IF_{2020}=5.6] (1^{th} $decile_{2020/AIS}$ in Toxicology)

A short summary of the paper:

This systematic review focused on the well-known endocrine disruptor bisphenol A (BPA), its novel congeners and their potentially harmful effects on the cardiovascular system. In the last decade, revolutionary legislative changes removed BPA from daily use products and led to its replacement by substitutes. However, these alternatives are structural analogues to BPA, and contrarily to the ‘’old culprit’’, studies on the latter are scant. Unsurprisingly, questions about their safety have been raised.

Considering the reported but inconclusive correlation between elevated BPA levels in human urine samples and a higher prevalence of CVDs, we intended to summarise and critically review the literature on the topic. The most commonly used alternatives, bisphenol S (BPS), bisphenol F (BPF) and bisphenol AF (BPAF), are emergent pollutants due to their ubiquitous presence in the environment. These new compounds have also been detected in human biological samples.

The main findings from *in vitro*, *ex vivo* and *in vivo* (animal models) studies regarding the effects of BPA on the cardiovascular system suggested relaxation of vascular smooth muscles and modulatory effects on numerous ion channels relevant to the regulation of vessel tone and heart contractility. Endocrine-disrupting activities through the interaction with sex and thyroid hormone receptors have also been extensively reported. Despite the countless number of published papers on BPA, the reported findings are often divergent and, in some cases, even controversial. Most intriguingly, due to its vasodilatory effects *ex vivo*, we anticipated that BPA would decrease arterial blood pressure, but unexpectedly, BPA exposure was associated with increased blood pressure. Nevertheless, we also identified several limitations inherent to these hypothesis-driven studies that preclude drawing clear conclusions. On the other hand, most human population studies indicate an association between exposure to bisphenols and harmful effects on the cardiovascular system.

P3. The effects of bisphenols on the cardiovascular system *ex vivo* and *in vivo*.

Tvrdý V[#], **Dias P[#],** Nejmanová I, Carazo A, Jirkovský E, Pourová J, Fadraersada J, Moravcová M, Peterlin Mašič L, Sollner Dolenc M & Mladěnka P.

Chemosphere 2023;313: 137565 [IF_{2021} 8.9], ($Q1_{2021/IF}$, $Q2_{2021/AIS}$ in Environmental Sciences) [#]designates shared co-first authorship

A short summary of the paper:

In this article, we focused on experimental confirmation of the cardiovascular effects of bisphenols based on caveats and inconsistencies summarized in our review paper.

Therefore, we have decided to assess a total of 14 bisphenols: A, AP, B, BP, C, E, G, M, P, PH, Z, including the most commonly used alternatives bisphenols S, F, and AF.

In the first step, the isolated rat aorta, a standard *ex vivo* model for testing vasoactive properties, was used. Although no vasoconstrictive effects were observed, eight bisphenols showed significant vasodilatory effects (with EC_{50} values ranging from 57 μM to hundreds of μM). BPAF, the most potent compound, inhibited L-type calcium channels according to our mechanistic experiments. A similar qualitative effect was observed for BPA, BPS and BPF. Although this is in harmony with the findings of Feiteiro et al. [183], who showed vasodilatory effects *ex vivo* for BPA, as far as we know, this is the first study demonstrating that next-generation bisphenols also have such effects. However, as bisphenols levels in human plasma are much lower (often hundreds of nM), we surmise a low or negligible clinical relevance of these findings.

Subsequent cellular toxicity studies in H9c2 cells (heart tissue model) showed high toxicity for BPAF (IC_{50} ~ 3 μM), while BPS and BPF had no cytotoxic effects (even at 100 μM). Notably, our cytotoxicity studies with four cell lines enabled us to systematically assess and directly compare the toxicity of all tested bisphenols, which has not been performed previously as far as we know.

In order to confirm or reject biologically important effects of bisphenols, these compounds were further given i.v. in different doses. However, low doses (mimicking real exposures) of BPA, BPF, BPAF, or BPS administered to normotensive Wistar Han rats did not significantly change arterial blood pressure or cardiac parameters. Additionally, BPAF given to Wistar Han rats by oral gavage at a high intoxication dose (2.5 $mg.kg^{-1}$, 28 days) had no significant impact on arterial blood pressure. No cardiotoxicity measured with sensitive marker cardiac troponin T was observed in contrast to the above-mentioned *in vitro* data with rat H9c2 cardiomyoblasts.

In conclusion, bisphenols in biologically achievable concentrations are unlikely to significantly affect blood pressure *in vivo*. In addition, cytotoxic effects were mainly observed at higher concentrations than typical exposure levels. Overall, our findings cast doubt on the alleged profound effects of bisphenols on the cardiovascular system.

6 DISCUSSION

6.1 GUT MICROBIOTA-DERIVED SMALL PHENOLIC METABOLITES

The studies demonstrating the cardiovascular protective effects of polyphenol-rich foods are manifold. For instance, ingestion of cranberry juice or cocoa products has been associated with a decrease in blood pressure [203, 204]. In contrast, research on pure flavonoids has many shortages due to challenges such as the highly complex pharmacokinetics. In recent years, the understanding of these biotransformations has led to a paradigm shift, with increasing attention being given to the small phenolic metabolites formed in the gastrointestinal tract, for which, as mentioned earlier, higher bioavailability and multiple bioactivities were reported (see sections 2.3.1.3 and 2.3.1.4).

Prior to this dissertation, my colleagues from the Research group of Cardiovascular pharmacology and toxicology showed that several gut microbiota-derived metabolites are endowed with biologically relevant vasodilatory properties and blood pressure-decreasing effects [9, 144, 205]. One of these small metabolites, 3-hydroxyphenylacetic acid (3-HPAA), was not able to induce maximal vasorelaxation of rat aortic rings *ex vivo* but was otherwise active [9]. As, in the meantime, my colleagues reported that the effect of some small phenolic compounds on arterial blood pressure might be at least additive [205], I started to concentrate in the ambit of this doctoral project on this compound. 3-HPAA is a metabolite of different classes of flavonoids, namely, flavonols (e.g., quercetin (**Figure 9**), isoflavones, and flavanols (oligomeric procyanidins and their monomers, catechin and epicatechin) [143, 206-209]. Thus, it can be inferred that 3-HPAA might be formed after the ingestion of a wide variety of foods such as onions, broccoli, apples, tea, red wine, and cocoa-based products [129].

On this basis, the following research questions were raised: a) whether 3-HPAA could produce beneficial haemodynamic effects also *in vivo* and b) which mechanism at the molecular level is responsible for the vasodilatory activity.

In vivo effects of 3-HPAA

The *in vivo* findings of this study (see section 5., P1) demonstrated that 3-HPAA, administered intravenously both as bolus or infusions, significantly and dose-dependently decreased systolic and diastolic arterial blood pressure in SHRs. Moreover, there were no changes in heart rate. SHRs are the most commonly used animal model of essential hypertension, as progressive changes in arterial blood pressure resemble those occurring clinically [210]. Surprisingly, the intravenous bolus doses from which significant decreasing effects on diastolic and systolic arterial blood pressure occurred were very low, i.e., 10 and 100 $\mu g.kg^{-1}$, respectively. Analogously, the intravenous infusion of 3-HPAA ($1\ mg.kg^{-1}.min^{-1}$) significantly decreased both systolic and diastolic blood pressure. Considering that absorption of colonic metabolites in the gastrointestinal tract occurs slowly and continuously, infusions are closer to real situations. Relevant data can also be compared with pharmacokinetic studies. In gerbils

fed by calafat berries extract through gavage (containing ~ 2.6 mg of phenolics), the maximal plasma concentrations of 3-HPAA were ~ 300 nM after 4h [211]. In rats, an intravenous bolus dose of 3-HPAA of 2 and 4 $mg.kg^{-1}$ resulted in maximal plasma concentrations of ~ 6 $mg.L^{-1}$ (~40 µM) and ~ 16 $mg.L^{-1}$ (100 µM), respectively [212]. Therefore, it is predictable that after a low dose of 10 $\mu g.kg^{-1}$, biologically relevant 3-HPAA serum levels could be attained, i.e., 100–200 nM [212]. Of note, the intravenous infusion of 3-HPAA (1 $mg.kg^{-1}.min^{-1}$) could yield serum levels up to tens of µM, a roughly 10-fold higher concentration than produced by diet [143], and the dose of 5 $mg.kg^{-1}.min^{-1}$ would generate plasma concentrations much superior to common attainable levels. Although the translatability of animal pharmacokinetic data to humans constitutes a real issue, the efficient concentration from our study is achievable in humans after consumption of a diet rich in flavonoids (see section 2.3.1.3). It is worth re-emphasising that as 3-HPAA is a common metabolite of several flavonoids present in different foods. Hence, higher serum concentrations could be achieved than when a single parent flavonoid is administered, again emphasizing that the observed blood pressure-decreasing effects might have a real impact. However, to verify this hypothesis, more studies are necessary to detect and quantify 3-HPAA in human plasma.

Importantly, the fact that 3-HPAA did not produce significant changes in heart rate at any of the administered doses suggested that the blood pressure-decreasing effect could be mediated by peripheral relaxation. Besides, the absence of a baroreceptor reflex is advantageous as it could otherwise lead to adverse effects, as reported with the calcium channel blocker nifedipine [213].

Elucidation of the mechanism of action ex vivo

Taking into account the hypothesis that the blood pressure-decreasing effect of 3-HPAA relies on a decrease in peripheral vascular resistance, a series of mechanistic experiments were performed *ex vivo*. 3-HPAA produced a dose-dependent relaxation of the left circumflex porcine coronary artery *via* endothelium-derived NO.

Vascular myography was employed in this experimental set. This technique has been used in pharmacological experiments for over a century, including those conducted by Furchgott and colleagues (see section 2.2.3.1.1) [111]. The use of the porcine coronary artery model enabled the reduction of the number of sacrificed animals in line with the 3Rs principle of Russel and Burch [214]. Although the concentration required to observe vasorelaxation was much higher than in rat aorta [9], the porcine coronary artery has many traits that made it suitable for this study. Regarding translatability, the porcine model is the most adequate for cardiovascular research due to its high resemblance to the human vasculature [215]. In addition, muscarinic receptors, for instance, of subtype M_3 expressed in porcine coronary vessels, exhibit a high homology (> 90%) with the human amino acid sequence [216]. During this study, several potential targets and pathways (described in section 2.2) of 3-HPAA-induced

vasorelaxation were investigated. In particular, through the use of selective pharmacological probes, muscarinic receptors, IKCa, SKCa, prostanoids and L-type Ca^{2+} channels were excluded.

Oppositely, the disruption of the endothelial layer and the inhibition of eNOS decreased significantly the extent of maximal relaxation achieved. Indeed, the effect was partially dependent on the endothelium, and these findings raised the question of whether 3-HPAA could activate eNOS and induce NO synthesis. NO would then diffuse to VSM cells, where it would activate sGC and cGMP-PKG pathway, resulting in vasorelaxation (see sections 2.2.3.1.1 and 2.2.2.1.1). Given that the pKa of 3-HPAA is ~ 4, a transporter for the penetration of this compound inside the cells could be involved, but no data are available yet. The mechanism leading to eNOS activation remains also unknown, though there was no evidence of an increase in $[Ca^{2+}]_c$. Besides vasorelaxation, NO exerts other beneficial effects such as prevention of VSM cell proliferation and migration, inhibition of expression of numerous pro-inflammatory and pro-atherothrombotic mediators, prevention of platelet adhesion and aggregation and monocyte adhesion [217].

In addition, studies showed that polyphenol-rich plants and fruit extracts (e.g., grape skin extracts and purple grape juice) relax porcine coronary arteries through an endothelium-dependent NO-mediated mechanism [218]. However, caveats include that most studies lack compositional analysis of the extracts, so information on the bioactive components is missing. Others have reported vasodilatory activities for the parent forms such as quercetin [219], but this is not very probable as, in plasma, aglycones are absent or circulate only in trace amounts, which contrasts with the predominant conjugates (i.e., glucuronides, sulphates). Galindo and colleagues [220] referred that flavonoid glucuronides, albeit inactive, could serve as reservoirs and plasma transport metabolites that, *via* deconjugation *in situ*, would allow the release of the aglycone form. Clear proofs are, however, missing. Furthermore, and most importantly, Galindo et al. in another study [221] reported that quercetin had greater antihypertensive effects when orally administered than injected intraperitoneally. These findings could signify that the antihypertensive effects are exerted by the small phenolic metabolites formed by the colonic microbiota, which is in concert with the hypothesis and aims of this dissertation.

3-HPAA and vascular diseases

Augmented arterial stiffness, increased vasoconstriction, and endothelial dysfunction are known abnormalities observed in hypertension. Although quite debatable, some authors consider endothelial dysfunction a risk factor and a consequence of hypertension [222]. Endothelial dysfunction has been correlated with impaired NO bioavailability [222]. Predisposing factors include a decrease in endogenous NO production, an increase in NO degradation, abnormally increased levels of vasoconstrictors, and hyperproduction of reactive oxygen and nitrogen species [223].

In patients with essential hypertension, there are reports of abnormalities in endothelium-derived NO [224]. However, the role of NO and endothelial dysfunction in hypertension in spontaneously hypertensive animals is not obvious [223] because the available studies are quite divergent. There can be more factors, such as different types of arteries studied, various experimental approaches and last but not least, miscellaneous ages of the animals. Regardless, SHR is a standard model of NO-deficient hypertension [225]. Concerning eNOS, a reduction in its activity and expression was reported in the aortae of SHRs [226]. In contrast, others described similar eNOS activities in the aortae of SHRs and normotensive Wistar-Kyoto rats, while an increased eNOS expression was observed in the aortas of SHRs [227]. Studies with mesenteric arteries of SHRs showed a reduction in eNOS activity [227] and a lower endothelium-dependent reactivity [228]. The small resistance arteries and arterioles generate resistance in the circulation, which is, however, mostly physiological due to their essential roles in the control of systemic blood pressure. One can speculate that the diastolic pressure-decreasing effect of a very low dose of 3-HPAA could have been mediated by the promotion of endothelial NO production in these resistance vessels. However, as mentioned previously, the relaxation of vessels of smaller calibre occurs primarily through a NO-independent manner (section 2.2.3.1.2). Intriguingly, in hypertensive states, compensatory mechanisms have been described that consist of a "shift" in the role of NO, which assumes greater importance in the relaxation of small vessels [229]. Of note, 3-HPAA could act through different mechanisms in different vascular beds. Indeed, the mechanism of action of a determined compound might differ according to the type of vessel. Moreover, there are also challenges associated with interspecies differences. The future use of human *in vitro* models in vascular myography, i.e., bioengineered blood vessels, could help solve these hurdles and reduce the number of sacrificed animals [230].

In the healthy coronary vessels, the vasorelaxant activity of 3-HPAA was not pronounced. Notwithstanding, it remains unknown whether, in pathological states such as coronary artery disease (CAD), in which endothelial dysfunction is present, 3-HPAA could be protective.

Limitations of the study

One of the limitations of this study consists of the fact that the ingestion of several foods throughout the day leads to the formation of a mixture of phenolic metabolites in the colon (e.g., **Figure 9**), which are thereafter mostly absorbed in the systemic circulation. Even more interesting, each metabolite has a unique mechanism of vasodilatory activity. For instance, 3-(3-hydroxyphenyl)propionic acid induced relaxation of the rat aorta *via* a NO-dependent mechanism [9], whereas 4-methylcatechol apparently acted in an endothelium-independent manner [144]. As already mentioned, the effects of different metabolites on arterial blood pressure might be at least additive [205]. On the contrary, we cannot deny, at the moment, that if some metabolites act at the same target, the final blood pressure-reducing effect might be contrarily lower. To further investigate this issue, studies reporting available plasma levels of different colonic metabolites from common food need to be published. Current literature is largely insufficient concerning this aspect.

6.2 BISPHENOLS

Due to extensive production and synthesis of bisphenols, these chemicals are ubiquitous in the environment and with alleged harmful health effects. Therefore, research on these compounds has been gathering increasing interest. More precisely, many papers have been published over the last two decades about the lead compound BPA. Ultimately, the confirmation of widespread human exposure and its potential detrimental effects have led to legislative changes and its replacement by NextGen bisphenols. However, the newly introduced, albeit far less studied, NextGen bisphenols are now emerging environmental pollutants and have been detected in human biofluids too (**Table 1**). Whether these substitutes are safer compared to BPA has captured the attention of many toxicologists and other experts. In this context, the second part of my PhD project focused on this question, particularly on the investigation of the potential impact of BPA and its structural variants on the cardiovascular system.

Systematic review

Our strategy relied on first screening and summarizing the literature on the topic, which enabled us to get in-depth insights into the current findings in this area (see section 5., **P2**). Although published papers on the topic are extensive, many reported data are inconsistent or contradictory. For instance, *ex vivo*, BPA exhibited vasodilatory properties, whereas, *in vivo*, a contrasting blood pressure-increasing effect was described. Another example is the disparate results regarding the effects of BPA on thyroid receptors since different studies reported that it acts as an agonist, antagonist or is devoid of an activity (**Table 2**).

As the apparent discrepancy among results could stem from differences in the experimental procedures (e.g., use of conventional or genetically modified animal models), design (e.g., tested doses, treatment duration) and data elaboration, the studies should be interpreted cautiously. Actually, the Consortium Linking Academic and Regulatory Insights on BPA Toxicity (CLARITY-BPA) program was created to combine the findings of a traditional regulatory toxicological study (core study) and academic studies (conducted by independent researchers). However, the conclusions regarding the potential harmful effects of BPA on the cardiovascular health of rodents were not clear. In the CLARITY-BPA study conducted by Gear and colleagues [231], NCTR-Sprague-Dawley rats were gavaged daily with BPA (2.5, 25, 250, 2500, or 25 000 µg/kg from gestational day 6 to birth followed by oral gavage of the offspring with the same doses for 1–2 years or until postnatal day 21). The main results included a significant decrease in left ventricle wall thickness at postnatal day 90 in females (2.5 µg/kg), a significant decrease in heart weight at 6 months in females (2.5 µg/kg), a decrease in collagen content in female hearts at postnatal day 90 (25 mg/kg) and progressive cardiomyopathy lesions [231]. However, major drawbacks included: a) a background level of cardiomyopathy also in non-treated animals, b) BPA was also detected in control animals, c) cardiac parameters and blood

pressure were not assessed, and d) non-neoplastic lesions in the heart were associated with the ageing of the rat strain used [232].

In addition, the issue of the translatability of animal data to humans logically arises and justifies the need for additional human population studies, especially prospective cohort studies, which are lacking. Additionally, there is a need for mechanistic studies in order to comprehend the deleterious effects of BPA on organs and systems. Nevertheless, numerous electrophysiological studies showed that BPA inhibits ion channels in cardiomyocytes and vascular smooth muscle cells. Moreover, in some cases, alterations in ion currents were registered at BPA concentrations reported in humans [184].

Beyond this, new concerns have emerged upon the restricted usage of BPA and its substitution by NextGen bisphenols, compounds to which humans are now unavoidably exposed (see section 2.3.2). Even though little is known about their toxicological profiles, various studies suggested that these alternatives could adversely affect the reproductive, endocrine, nervous and cardiovascular systems [233, 234]. *In vivo* exposure to bisphenol A, AF, F and S caused a disruption in vascular development in zebrafish embryos [235]. Furthermore, the same study reported an inhibition of angiogenesis *in vitro* [235]. Most importantly, an epidemiological study from China (n = 1437) reported an association between exposure to BPS and an increase in arterial blood pressure and hypertension [178].

Original research

Given the inconsistencies and unclear conclusions about the cardiovascular effects of bisphenols, especially NextGen bisphenols, we conducted a series of experiments *in vitro, ex vivo* and *in vivo* with 14 bisphenols.

Ex vivo vasodilation and elucidation of the mechanism

As mentioned previously, others have reported vasodilatory properties for BPA [183], hence, at the beginning, we aimed to investigate the hypothesis of whether BPA analogues could affect vessel tone as well. With that aim in mind, we tested 14 bisphenols (bisphenols A, AF, AP, B, BP, C, E, F, G, M, P, PH, S and Z) on the isolated rat aorta. None of the compounds caused vasoconstriction, and the results showed that most are vasodilators, albeit with different potencies and efficacies. Among the currently most common BPA alternatives (i.e., bisphenols S, F, and AF), BPAF was the most potent vasodilator with an EC_{50} ~57 μM. The EC_{50} values for the vasodilatory activity ranged in general up to hundreds of μM, thereby indicating that the vasodilatory effects are produced at much higher concentrations than are commonly detected levels in plasma/serum (**Table 1**). Therefore, we concluded that it is not very likely that bisphenols produce vasodilatory effects at biologically achievable concentrations. Regardless, and after taking into account electrophysiological reports showing that BPA inhibits L-type Ca^{2+} channels in aortic thoracic smooth muscle cells (A7r5 cells), we formulated the hypothesis as to whether BPAF could also inhibit these channels. On that basis, we performed a series

of mechanistic experiments on the isolated rat aorta. Our results showed that not only BPAF but also BPA, BPF and BPS are capable of inhibiting L-type Ca^{2+} channels, with potencies that align with their vasodilatory activities. Meaning that the potencies were in descending order: BPAF > BPA > BPF > BPS. Interestingly, our review (P2) included a study by Deutschmann et al. [236] in which BPA was shown to inhibit different VGCCs. Moreover, analysis of the structure-activity relationship revealed that effective inhibition was associated with the presence of: (1) a double-alkylated or double-trifluoromethylated sp^3-hybridized carbon atom between the two aromatic rings and (2) two aromatic moieties in angulated orientation [236]. In addition, Prudencio et al. [181] reported inhibitory effects of bisphenols on L-type Ca^{2+} channel currents, with potencies in descending order: BPA > BPF > BPS that align with our findings.

Cytotoxicity

Concomitantly, we considered particularly relevant to test the cytotoxicity of these bisphenols. Briefly, BPAF was the most toxic bisphenol with an IC_{50} ~3 μM, while BPF and BPS had no cytotoxic effects in H9c2 cells (a rat heart tissue model). These three bisphenols showed a lower toxicity (BPAF) or were quite inert (BPF and BPS) in A-10 rat smooth muscle cells. In breast cancer cell lines, BPA, BPF, BPS were quite non-toxic as well, while BPAF showed mild toxicity, albeit much lower than in H9c2 cells. However, one of the limitations of our study is that we did not investigate potential mechanisms for the observed toxicity. Others reported that BPAF increases the levels of reactive oxygen species in human cardiac myocytes [237]. Notably, our study showed that cytotoxicity generally occurs at greater concentrations than common human exposure concentrations and allowed, as far as we know, to compare directly the cytotoxicity of 14 bisphenols for the first time.

Acute haemodynamic changes in vivo

Next, we sought to explore whether the bisphenol-induced vasodilatory effects *ex vivo* would be reflected in acute haemodynamic changes *in vivo*. To answer this question, we administered four representative bisphenols with different *ex vivo* vasodilatory potencies (BPA, BPS, BPF or BPAF) to Wistar rats of both sexes intravenously. Three different doses were tested: two low doses (0.005 and 0.05 mg/kg) mirroring potential intake (environmental exposure) doses (see section 2.3.2.1) and a high dose of 2.5 mg/kg (an intoxication dose). The results showed that no significant changes in arterial blood pressure were detected at the two lowest doses. Differently, at the highest dose 2.5 mg/kg: a) BPF caused a significant decrease in diastolic blood pressure in females, and systolic blood pressure in males, and b) BPAF caused a significant reduction in diastolic blood pressure solely in males. No alterations in important cardiac parameters were detected. However, the experimental caveats include the fact that the vehicle used to prepare the highest dose had some mild and quite variable effect, hence making the interpretation of such findings rather difficult. Also, intravenous administration does not mimic the

main route of exposure to bisphenols. However, relatively recent studies have called attention to the risks of exposure to bisphenols through medical devices (e.g., catheters), especially during neonatal intensive care [238]. Furthermore, the levels of the biologically active form, i.e., free BPA, are expected to be higher when a given dose is administered intravenously compared with oral administration, as this route circumvents the first-pass metabolism. Notwithstanding, our findings suggest that, at common exposure doses, it is very improbable that, acutely, BPA, BPS, BPF and BPAF exert a measurable impact on arterial blood pressure. However, this does not necessarily mean that harmful effects could not occur in long-term exposure.

Chronic study in vivo

For this latter reason, we performed a chronic study (28 days) during which Wistar Han rats (males) were gavaged daily with the most potent vasodilator from the bisphenol class tested BPAF (2.5 mg/kg). BPAF was chosen mainly as it has the highest cytotoxic and vasodilatory potential of the tested compounds. It is also known to be able to interact with various endocrine receptors, which could also be the reason for changes in blood pressure. Although in 2008, the U.S. National Institute of Environmental Health Sciences nominated BPAF for toxicological evaluation [239], as far as we know, it had not been previously tested for its cardiovascular effects *in vivo*. Our main results showed that BPAF had no significant effects on arterial blood pressure. This was confirmed both through daily non-invasive continuous monitoring and invasive measurement through left ventricular catheterization at the end of the study. These findings contrast with reports about BPA showing that it causes an increase in arterial blood pressure (**Table 2**). Interestingly, no alterations in cardiac parameters were detected with the exception of an increase in ejection fraction. Moreover, since cardiac contractility was not elevated, we hypothesized that this could be derived from a decrease in peripheral resistance. In fact, BPAF also revealed a tendency to decrease the peripheral resistance index, a result that could support this hypothesis. Another important aspect is that we did not observe any signs of cardiotoxicity *in vivo*, as serum levels of cardiac troponin T (TnT) were not altered.

Summing up, the chronic administration of BPAF (2.5 mg/kg) did not exert a significant impact on the cardiovascular endpoints analysed.

Limitations of the study

Of note, the limitations in our chronic study include the fact that only male rats were included. Hence, a potential sex-dependent impact of BPAF was not investigated. For instance, in a mice study, a decrease in systolic blood pressure in females was observed after a 1000-times-higher dose of BPA compared with males [240]. Thus, the hypothesis that BPAF could also produce sex-dependent effects should not be overlooked. In addition, administration through gavage also has disadvantages as it causes stress to the animals. Finally, in real situations, humans are exposed to different bisphenols

simultaneously in addition to multiple other xenobiotics. Assessing potential hazards resulting from those combined exposures is extremely experimentally challenging.

7 CONCLUSION AND FUTURE DIRECTION IN RESEARCH

In summary, the small phenolic metabolite, 3-HPAA, exerted antihypertensive effects in SHRs. Notably, the effect occurred from a very low dose for which 3-HPAA plasma concentrations could be achievable in humans through diet. Furthermore, the findings suggested that 3-HPAA-induced vasorelaxation of coronary arteries occurs *via* endothelium dependent NO-mediated mechanism. However, it is not yet known whether 3-HPAA also relaxes small resistance vessels, essential for the regulation of blood pressure, by the same mechanism. Further studies should investigate the site of action, e.g., by employing different vascular beds like human mesenteric arteries.

Endothelial dysfunction and decreased NO bioavailability are characteristic histopathological findings in hypertension. Unfortunately, global epidemiological data show that the prevalence of hypertension is rising due to ageing and exposure to lifestyle risk factors. Multiple studies have shown that extracts of (poly)phenol-rich plants and fruits relax blood vessels *via* NO production. However, it remains to be clarified which substances are responsible for the reported bioactivities, particularly regarding the potential role played by colonic metabolites. Thus, additional pharmacokinetic and pharmacodynamic studies are needed. Given that endothelial dysfunction is an early predictor of cardiovascular outcomes, future research is warranted to elucidate whether not only 3-HPAA but also other metabolites might exert endothelial protective effects. Another interesting issue would be to see whether 3-HPAA and/or other metabolites can positively modulate the effect of conventional antihypertensives (e.g., angiotensin-converting enzyme inhibitors). It is well known that combinations of antihypertensive drugs with different mechanisms of action are more suitable than high doses given in monotherapy in both efficacy and side effect issues [241]. Also, for non-antihypertensive drugs such as statins, beneficial effects on vasculature have been reported [242]. Such studies can bring novel interesting findings with clinical overlap.

In the second part of this doctoral project, most of the NextGen bisphenols showed vasodilatory effects, albeit at much higher concentrations than their typical exposure levels. BPAF was the most potent vasodilator acting via inhibition of L-type Ca^{2+} channels on VSM. The same mechanism was observed for BPA, BPS and BPF. The intravenous administration of BPA or its most commonly used alternatives, BPS, BPF or BPAF to normotensive Wistar Han rats (at doses mimicking environmental exposure) did not produce significant changes in the arterial blood pressure nor cardiac parameters. In addition, the chronic administration of BPAF by gavage (2.5 $mg.kg^{-1}$, 28 days) to Wistar Han rats did not alter arterial blood pressure. There were no indicators of cardiotoxicity even if the cytotoxicity *in vitro* was observed in quite low concentrations ($IC_{50} \sim 3$ μM in rat embryonic cell line). Regardless, these relatively low concentrations are not expected to occur in blood, and hence, the toxicity might likely occur at higher concentrations than common exposure levels. Overall, these findings have raised questions about the claimed significant effects of bisphenols on the cardiovascular system. Specifically,

and within the constraints of our experimental setup, the profound effects of bisphenols on the cardiovascular system after exposure to common doses are improbable.

However, it is noteworthy that toxicological investigation of potential long-term harmful effects is extremely challenging. Therefore, at this juncture, making definitive conclusions concerning the hazardous nature of NextGen bisphenols is premature.

Indeed, our systematic literature review highlighted numerous needs in current bisphenol research. Since some studies used supraphysiological concentrations of bisphenols and specific genetic animal models, the translatability of those findings to the human population is debatable. Also, mechanistic studies are needed, especially about NextGen bisphenols. Furthermore, aspects related to the animal model (e.g., species, age, sex) and study design (e.g., bisphenol kinetics, doses, regimens and duration of exposure) should be stipulated taking in mind their predictive value for the human population. In sum, future studies are of utmost relevance and should be conducted cautiously.

Overall, the aims and objectives of this project were addressed and accomplished, although logically, it raised future questions. Most importantly, the studies included in this dissertation could serve as a tool for future pharmacological and therapeutic interventions as well as for the study of novel risk factors as ways of reversing the epidemic of cardiovascular diseases.

8 CONTRIBUTION OF THE CANDIDATE TO THE PUBLISHED WORKS IN THE DISSERTATION

P1. Dias P, Pourová J, Vopršalová M, Nejmanová I, Mladěnka P. **3-Hydroxyphenylacetic Acid: a blood pressure-reducing flavonoid metabolite.** Nutrients. 2022; 14(2):328 [IF_{2020}=5.7] ($Q2_{2020/AIS}$ in Nutrition & Dietetics)

- The first author, performed most of the research, validation, mathematical and statistical data analysis, writing – original draft preparation, and writing – review and editing.

P2. Dias P, Tvdrý V, Jirkovský E, Solner Dolenc M, Peterlin Mašič L & Mladěnka P. **The effects of bisphenols on the cardiovascular system.** Crit Rev Toxic. 2022;52(1): 66-87 [IF_{2020}=5.6] (1^{th} $decile_{2020/AIS}$ in Toxicology)

- The first author, responsible for the preparation, analysis, interpretation and writing of the manuscript.

P3. Tvrdý V[#], **Dias P[#],** Nejmanová I, Carazo A, Jirkovský E, Pourová J, Fadraersada J, Moravcová M, Peterlin Mašič L, Sollner Dolenc M & Mladěnka P. **The effects of bisphenols on the cardiovascular system *ex vivo* and *in vivo***. Chemosphere 2023;313: 137565 [IF_{2021} 8.9], ($Q1_{2021/IF}$, $Q2_{2021/AIS}$ in Environmental Sciences) [#]designates shared co-first authorship

- Equally contributed as a co-first author.
- Performed research: planned and executed the *ex vivo* experiments and also major contributions were given to the *in vivo* chronic study, particularly with the daily monitoring of blood pressure non-invasively (tail-cuff method), as well as in the end of the study.
- A significant contribution to the writing and review of the manuscript, in particular at phase of revision required by reviewers in the journal.

9 OVERVIEW OF THE SCIENTIFIC OUTPUTS OF THE CANDIDATE

9.1 LIST OF ALL PUBLICATIONS

- Sirakanyan SN, Hrubša M, Spinelli D, **Dias P**, Kartsev V, Carazo A, Hovakimyan AA, Pourová J, Hakobyan EK, Karlíčková J, Parvin S, Fadraersada J, Macáková K, Geronikaki A & Mladěnka P. **Synthesis of 3,3-dimethyl-6-oxopyrano[3,4-c]pyridines and their antiplatelet and vasodilatory activity.** J Pharm Pharmacol 2022;74(6): 887–895 [IF_{2019}= 2.6] ($Q3_{2019}$ in Pharmacology & Pharmacy)

- **Dias P**, Pourová J, Vopršalová M, Nejmanová I, Mladěnka P. **3-Hydroxyphenylacetic Acid: a blood pressure-reducing flavonoid metabolite.** Nutrients. 2022; 14(2):328 [IF_{2020}=5.7] ($Q2_{2020/AIS}$ in Nutrition & Dietetics)

- **Dias P,** Tvdrý V, Jirkovský E, Solner Dolenc M, Peterlin Mašič L & Mladěnka P. **The effects of bisphenols on the cardiovascular system.** Crit Rev Toxic. 2022;52(1): 66-87 [IF_{2020}=5.6] (1^{th} $decile_{2020/AIS}$ in Toxicology)

- Tvrdý V[#], **Dias P[#],** Nejmanová I, Carazo A, Jirkovský E, Pourová J, Fadraersada J, Moravcová M, Peterlin Mašič L, Sollner Dolenc M & Mladěnka P. **The effects of bisphenols on the cardiovascular system *ex vivo* and *in vivo***. Chemosphere 2023;313: 137565 [IF_{2021} 8.9], ($Q1_{2021/IF}$, $Q2_{2021/AIS}$ in Environmental Sciences) [#]designates shared co-first authorship

- Pourová J **Dias P,** Pour M, Fialová S, Czigle S; Nagy M, Tóth J, Balázs V, Horváth A, Csikós E, Farkas A, Horváth G & Mladěnka P. **Suggested mechanisms of action of herbal drugs and their biologically active constituents in the cough treatment: overview.** Peer J, 2023

9.2 PRESENTATIONS AT NATIONAL AND INTERNATIONAL CONFERENCES

- **Dias P,** Pourová J, Vopršálová M, Mladěnka P. *Voltage-gated potassium (K_V) channels contribute to 3-methoxycathecol-induced vasorelaxation on rat aorta ex vivo*. 13th Postgraduate and Postdoc Conference (2023) – Hradec Králové, CZ
- **Dias P**, Pourová J, Vopršálová M, Nejmanová I, Mladěnka P. *The flavonoid metabolite 3-hydroxyphenylacetic acid decreases arterial blood pressure in rats*. XXIV World Congress International Society for Heart Research (2022) – Berlin, Germany
- **Dias P**, Pourová J, Vopršálová M, Mladěnka P. *Flavonoid metabolite 3-hydroxyphenylacetic acid relaxes porcine coronary artery ex vivo: a mechanistic study*. 12th Postgraduate and Postdoc Conference (2022) – Hradec Králové, CZ
- **Dias P**, Tvrdý V, Carazo A, Jirkovský E, Pourová J, Mladěnka P. *Effects of the environmental pollutants bisphenols on isolated rat aorta*. TOXCON (2021) – Stara Lesná, Slovakia
- **Dias P**, Pourová J, Vopršálová M, Mladěnka P. *Vasodilatory effects of bisphenols in isolated rat aorta*. 11th Postgraduate and Postdoc Conference (2021) – Hradec Králové, CZ

9.3 SCIENTIFIC EXPERIENCE ABROAD

- 8-month laboratory training at the Dorothy M. Davis Heart & Lung Research Institute (Ohio State University, USA) under supervision of Assoc. Prof. Przemysław Radwański (from March to October, 2023)

9.4 GRANTS AND FELLOWSHIPS

Principal investigator

- Rector's Mobility Fund; 2022; Grant number: FM/c/2022-2-009
- Grant Agency of Charles University; 2020-2022; Grant number: 136120; Title of the project: *Arterial blood pressure decreasing effects of small phenolic metabolites of flavonoids: mechanistic ex vivo and in vivo study*

Team member

- Grant Agency of Charles University; 2020-2022; Grant number: 1322120; Title of the project: *Testing of potentially novel antiplatelet compounds using metabolites of natural phenolic compounds as templates*

9.5 OTHER ACADEMIC ACTIVITIES

- Laboratory training of the following diploma students: Nela Kuchařová, Kristýna Šmídová, Kateřina Kuzdřalová, Monika Štefancová, and Erasmus student Maurizio Contreras.

- Preparation of the studying materials for the European OEMONOM project (the **O**pen access **E**ducational **M**aterials on **N**aturally **O**ccurring **M**olecules – sources, biological activity and use, https://portal.faf.cuni.cz/OEMONOM/EN/Home/) on vitamin H.

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