# Blackcurrant anthocyanin induces activation of NO pathway : role of sodium glucose cotransporter 1 and 2

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# THÈSE

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## Activation de la voie du monoxyde d'azote dans les cellules endothéliales par les anthocyanes du cassis : Caractérisation des molécules actives et rôle des co-transporteurs sodium-glucose 1 et 2

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*When we live, everyone has to endure a hard time. Sometimes, we need to be patient to give back the love. All of us, we are still on my way.*

## Father and mother,

Your efforts, sacrifice, motivation, encouragement and unconditional love
navigate me to arrive great moment,
This thesis is your glory, your success and your happiness.

## My wife Boyoung and my daughter Bareum,

Always appreciate your supporting to achieve my goal,
Sorry for cannot be with you in such a holy moment,
I would like to say "I love you" on this occasion
To my sweet baby,
All the world is waiting your birth and blessing you,
Be happy and healthy.

## My sister and her husband,

Your comfort erasing the sorrows and encouraged me,
Praying your health and prosperous of your family.

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To only my Korean friend Sinhee and Italian Friend Dr. Belcastro

Thank you for having fun and motivate each other. It was really contributing to endure the hard moments.

# ***RESUME EN FRANÇAIS***

**Activation de la voie du monoxyde d'azote dans les cellules endothéliales par les anthocyanes du cassis : Caractérisation des molécules actives et rôle des co-transporteurs sodium-glucose 1 et 2**

Les anthocyanes sont des polyphénols appartenant au groupe des flavonoïdes et constituent des pigments naturels apparaissant dans le bleu, le violet et le rouge. Ils sont présents en grande quantité dans le vin rouge, les légumes et dans de nombreuses variétés de baies.

Le cassis (*Ribes nigrum*. L), issu d'un arbuste ligneux, est une baie largement répandue et cultivée de l'Europe de l'Ouest à l'Asie Centrale. De plus, il a été montré que la cassis est riche en polyphénols notamment issus des anthocyanes tels que la cyanidin-3-*O*-glucoside (C3G), le rutinoside (C3R) et la delphinidin-3-*O*-glucoside (D3G), -rutinoside (D3R). Les anthocyanes extraits du cassis possèdent des propriétés antioxydantes et ont montré des effets protecteurs sur le système cardiovasculaire en promouvant la formation de monoxyde d'azote endothélial (NO, facteur vasoprotecteur), en régulant le tonus vasculaire, en inhibant la formation des plaques d'athérosclérose et en protégeant les cellules endothéliales de l'oxydation des lipoprotéines de faible densité (Ox-LDL). De plus, plusieurs études cliniques ont suggéré que les anthocyanes améliorent la santé générale malgré le fait que leur concentration plasmatique est inférieure au µM et que ces composés sont rapidement éliminés par l'organisme. Cependant, peu de données décrivent le mécanisme de transport des anthocyanes ainsi que les mécanismes de signalisation intracellulaire responsables des effets biologiques. Une publication récente a montré que la D3G, l'anthocyane la plus abondante dans le cassis, a prévenu la dysfonction mitochondriale induite par l'oxydation des LDL dans les cellules endothéliales par transport de la D3G par le cotransporteur sodium glucose de type 1

(SGLT1). Récemment, l'expression des cotransporteurs sodium glucose de type 1 et 2 a été mise en évidence au sein de cellules endothéliales en culture. De ce fait, nous émettons l'hypothèse que les anthocyanes entrent dans les cellules endothéliales *via* les transporteurs SGLT1 et SGLT2 afin d'induire les réponses biologiques.

Le but de l'étude est de caractériser les anthocyanes actives issues du cassis, d'identifier les voies de signalisation intracellulaire promouvant la formation de monoxyde d'azote endothélial ainsi que de clarifier le rôle des transporteurs SGLT1 et 2 dans le transport des anthocyanes.

Un extrait de cassis riche en anthocyanes (BCE) a été préparé par chromatographie en utilisant une colonne Sephadex LH-20. La fraction riche en anthocyanes a été analysée par chromatographie liquide à ultra-haute performance (UPLC) afin d'identifier les anthocyanes présent dans l'extrait de cassis. L'effet du BCE sur la relaxation dépendante de l'endothélium due au NO a été mesuré par des expériences de réactivité vasculaire et les voies de signalisation ont été étudiées par la technique de Western Blot à partir de cellules endothéliales en culture. Le transport des anthocyanes dans les cellules endothéliales en culture a été étudié de manière indirecte par mesure de la fluorescence du réactif A de Naturstoff par microscopie confocale et cytométrie en flux.
Les cellules endothéliales ont été isolées à partir d'artères coronaires de porc et incubées 15 min avec de la collagénase. Des cellules endothéliales, jeunes, de passage P1 ont été utilisées afin d'évaluer la voie de signalisation Akt-eNOS après traitement avec le BCE. Des cellules P3, sénescentes, pathologiques, ont été obtenues à partir des cellules P1 par repiquages successifs après 4 à 5 jours de culture jusqu'à atteindre le troisième passage.

Le BCE entraine de puissantes relaxations due au NO d'anneaux d'artères coronaires pourvus d'endothélium, significativement réduites par le LX4211 (inhibiteur mixte des SGLT 1 et 2), par la canagliflozine (inhibiteur faiblement sélectif de SGLT2) mais pas par la dapagliflozine (inhibiteur hautement sélectif de SGLT2). En revanche, les relaxations induites par l'épigallocatéchine gallate (EGCG), un flavonoïde glucoconjugué, ne sont pas affectées par les inhibiteurs de SGLT1 et 2. Ces résultats indiquent que le BCE induit la relaxation due au NO dans les anneaux d'artères coronaires de porc, vraisemblablement grâce au transporteur SGLT1 et dans une moindre mesure, *via* le transporteur SGLT2. Nous émettons l'hypothèse que la structure glucosidique dans la structure des anthocyanes joue un rôle important dans la stimulation de la formation de NO endothélial via SGLT1 et 2.

L'activation de l'eNOS et d'Akt induite par le BCE est fortement augmentée après 2, 5 et 15 min comme le montre les analyses par la technique de Western Blot. La phosphorylation de la eNOS est significativement prévenue par le LX-4211, la dapagliflozine et la canagliflozine. Afin d'identifier les anthocyanes actives contenues dans le BCE, l'induction de la phosphorylation de la eNOS a été réalisée à partir des 4 types majeurs d'anthocyanes purifiés. La C3G, l'anthocyane majoritairement présente dans le BCE, augmente significativement la phosphorylation de la eNOS comparée au contrôle qui est inhibée par le LX-4211. Le rutinoside phosphoryle faiblement la eNOS. De plus, la D3G, une anthocyane de type glucosidique, affecte dans une faible mesure la phosphorylation de la eNOS mais sans effets significatifs. Ces résultats indiquent que les anthocyanes extraites du BCE que l'activation de la voie du NO est fortement dépendante de la structure chimique, et plus précisément de la présence d'une structure glucosidique au sein des anthocyanes.

Le réactif A de Natustoff génère de la fluorescence par chélation des anthocyanes. On observe une augmentation du niveau de fluorescence par cytométrie en flux d'un facteur

2 dans les cellules endothéliales exposées à la C3G, d'un facteur 1,5 pour les cellules endothéliales exposées à la D3G. Le traitement des cellules au LX-4211 inhibe significativement l'augmentation du signal de fluorescence. De la même façon, les expériences de microscopie confocale montrent une forte intensité de fluorescence, visualisée par un signal lumineux rouge, dans les cellules endothéliales exposées soit à la D3G soit à la C3G, alors qu'aucun effet n'a été observé pour les anthocyanes de nature non glucosidique. Cet effet a également été inhibé par le LX-4211. Ces observations indiquent que seuls les anthocyanes conjuguées au glucose (C3G et D3G) entrent dans les cellules endothéliales *via* SGLT1 et 2. En outre, le transport des anthocyanes a été inhibé de manière compétitive par le D-glucose de manière dépendante de la dose, mais le transport n'a pas été affecté par le mannitol (contrôle osmotique) ce qui suggère que l'influx d'anthocyanes se fait *via* les transporteurs SGLT1 et 2. L'ensemble de ces observations suggère le potentiel de protection du système cardiovasculaire des anthocyanes issues du cassis par une inhibition compétitive de l'influx de glucose.

Des données récentes du laboratoire d'accueil, ont montré une augmentation de l'expression des transporteurs SGLT1 et 2 dans des cellules endothéliales en culture soumises à des conditions pathologiques telles que une haute concentration de glucose ou un stress oxydant mais aussi dans des cellules en sénescence réplicative. C'est pourquoi, il est possible que la surexpression des transporteurs SGLT1 et 2 conduise à un transport accru des anthocyanes aboutissant à un meilleur effet vasoprotecteur. Par la suite, l'expression des transporteurs SGLT1 et 2 a été comparée entre les cellules endothéliales jeunes (P1) et les cellules endothéliales sénescentes (P3) par expérience de Western Blot. Les transporteurs SGLT1 et 2 ont été significativement surexprimés dans les cellules endothéliales sénescentes de passage P3 comparé aux cellules endothéliales jeunes de passage P1. De plus, la phosphorylation de la eNOS induite par

le BCE, a été comparée entre les cellules endothéliales P1 et P3. Un plus haut niveau de phosphorylation de la eNOS a été observé dans les cellules P3 comparativement aux cellules P1. Le LX-4211 a inhibé de manière plus importante la phosphorylation de la eNOS dans les cellules P3 que dans les cellules P1. De même, le transport des anthocyanes a été significativement augmenté dans les cellules P3 comparées aux cellules contrôles P1 ce qui valide une expression différentielle des transporteurs SGLTs et explique l'effet inhibiteur du LX-4211. Il est donc possible d'émettre l'hypothèse que l'effet protecteur des anthocyanes est plus prononcé dans les artères pathologiques plutôt que dans les vaisseaux sains.

Ainsi, on peut conclure que les anthocyanes conjugués au glucose (C3G et D3G) induisent la relaxation dépendante de l'endothélium par leur transport *via* SGLT1 et dans une moindre mesure, par SGLT2 dans les artères saines mais également et de manière plus marquée dans les artères pathologiques. La contribution des transporteurs SGLT1 et 2 dans le transport des anthocyanes promeut la protection du système cardiovasculaire et permettra de mieux comprendre la biodisponibilité des anthocyanes au sein de l'organisme.

# *Abbreviations*

## List of abbreviation

**12-HETE**: 12-hydroxyeicosetetetraenoic acid

**15-HETE**: 15-hydroxyeicosetetetraenoic acid

**4CL**: 4-coumaroyl CoA ligase

**ADP**: Adenosine diphosphate

**AGE**: Advanced glycation end-product

**ANS**: Anthocyanidin synthase

**C3G**: Cyanidin-3-*O*-glucoside

**C3R**: Cyanidin-3-*O*-rutinoside

**CAM**: Calmodulin

**CH4**: Cinnamate 4-hydroxylase

**CHD**: Coronary heart disease

**CHI**: Chalcone isomerase

**CHS**: Chalcone synthase

**COX-1**: Cyclooxygenase-1

**COX-2**: Cyclooxygenase-2

**CRP**: C-reactive protein

**CVD**: Cardiovascular disease

**CYP 450**: Cytochrome P450

**D3G**: Delphinidin-3-*O*-glucoside

**D3R**: Delphinidin-3-*O*-rutinoside

**DNA**: Deoxyribonucleic acid

**EDH**: Endothelium-derived hyperpolarization

**ER**: Endoplasmic reticulum

**ET-1**: Endothelin-1

**FAK**: Focal adhesion kinase

**FDA**: U.S food and drug administration

**FGF**: Fibroblast growth factor

**GLUT**: Glucose transporter

**GM-CSF**: Granulocyte-macrophage colony stimulating factor

**HUVEC**: Human umbilical vein endothelial cell

**ICAM-1**: Intracellular adhesion molecule-1

**IHD**: Ischemic heart disease

**$IK_{Ca}$**: Intermediate $Ca^{2+}$-dependent $K^+$ channels

**IL-1β**: Interleukin-1β

**IP**: $PGI_2$ receptor

**$K_{Ca}$**: $Ca^{2+}$-dependent $K^+$ channels

**LDL**: Low density lipoprotein

**LDOX**: Leucoanthocyanidin dioxygenase

**MAPK**: Mitogen-activated protein kinase

**MCP-1**: Monocyte chemoattractant protein-1

**MMP-2**: Matrix metalloproteinase-2

**MMP-9**: Matrix metalloproteinase-9

**NADPH**: Nicotinamide adenine dinucleotide phosphate

**NF- ΚB**: nuclear factor kappa B

**NO**: Nitric oxide

**$O_2^-$**: Superoxide anion

**$ONOO^-$**: Peroxynitrite anion

**PAF**: Platelet activating factor

**PB**: Phenobarbital

**PDGF**: Platelet-derived growth factor

**$PGI_2$**: Prostacyclin

**PI3K**: Phosphoinositide-3-kinase

**PKA**: Protein kinase A

**PKG**: Protein kinase G

**RNA**: Ribonucleic acid

**ROS**: Reactive oxygen species

**SGLT1**: Sodium-glucose cotransporter 1

**SGLT2**: Sodium-glucose cotransporter 2

**$SK_{Ca}$**: Small conductance $Ca^{2+}$-dependent $K^+$ channels

**SMC**: Smooth muscle cell

**SNP**: Sodium nitroprusside

**SRA**: Scavenger receptor A

**TF**: Tissue factor

**TLR4**: Toll-like receptor 4

**TNF**: Tumor necrosis factor

**$TXA_2$**: Thromboxane

**VCAM-1**: Vascular cell adhesion molecule-1

**VEGF**: Vascular endothelial growth factor

**WHO**: World health organization

**cAMP**: Cyclic adenosine-3',5'-monophosphate

**eNOS**: Endothelial nitric oxide synthase

**hs-CRP**: High sensitive C-reactive protein

**nNOS**: Neuronal nitric oxide synthase

**sGC**: guanylyl cyclase

**t-PA**: Tissue type plasminogen activator

**$vCa^{2+}$**: Voltage-sensitive $Ca^{2+}$ Channel

**vWF**: Von Willebrand factor

## List of Figures and tables

# *Chapter One*

# Cardiovascular diseases

## 1. Prevalence of cardiovascular diseases

Cardiovascular diseases (CVDs) have been reported as the most pivotal causes of global deaths among non-communicable diseases during the last 16 years (Hausenloy and Yellon, 2013). In 2016, World Health Organization (WHO) announced that ischemic heart disease (IHD) and stroke are world's biggest killers accounting for a combined 15.2 millions of deaths (WHO, 2018). IHD is the leading cause of myocardial infarction and ischemic heart failure characterized by reduction of blood flow due to the occlusive coronary artery subsequent to atherothrombosis (Kerrigan and Stotland, 1993). For the last few decades, incidence and mortality of IHD have dropped in high-income countries more likely due to an increased level of medical healthcare (Ng et al., 2014).

However, the prevalence of IHD is still increasing in low-income counties such as Eastern Europe and Central Asia due to the insufficient medical coverage (Moran et al., 2014). Accumulated data indicated that the onset of CVDs appears to be dependent on many factors such as gender, geographical localization, economic scale of country, acquired factors, and life style. For example: i) The number of women dying and/or surviving with CVDs and stroke are exceeding the number of men in the United States. whereas more men are dying with IHD (Mosca et al., 2011), ii) Age related prevalence of IHD is estimated as 10.88 million people in the world at the age between 50 to 54, 3-fold higher than those at the age of 40 to 44.

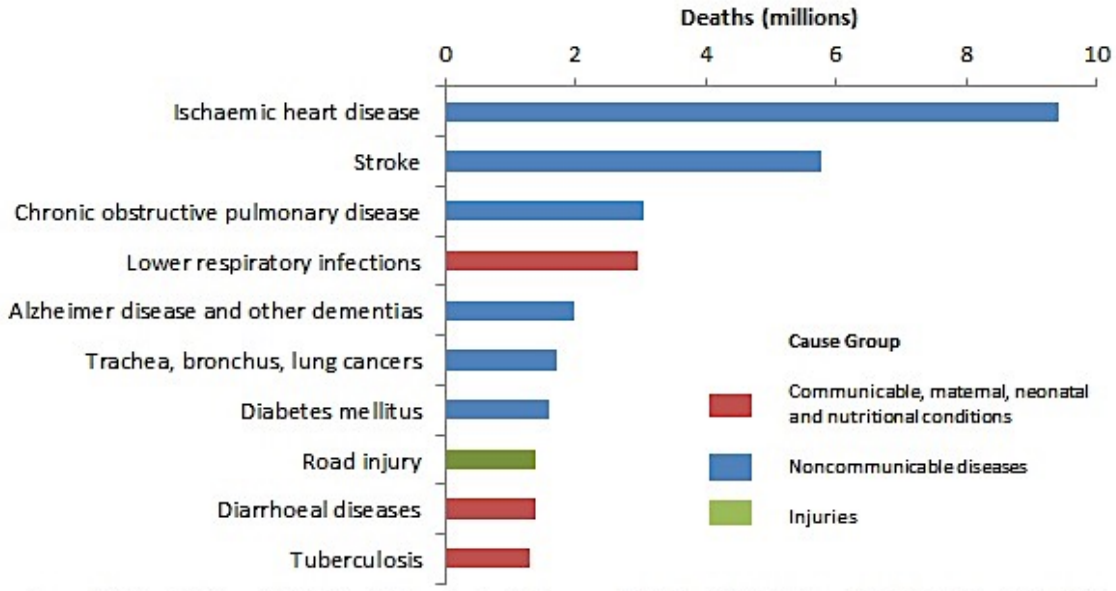



**Figure 1. Top 10 global causes of death.** Of the 56. 9 million deaths worldwide in 2016, more than half were includ in the top 10 causes. WHO announced that in 2018 17.9 million people die each year by CVDs (WHO, 2018).

## 2. Main causes of death in cardiovascular diseases

CVD is a term including diseases related to heart and blood vessels including IHD, which may lead to the development of myocardial ischemia, stroke, congenital heart defects and peripheral artery diseases (Kannel et al., 1976). Atherosclerosis is the pathological process predominantly in coronary arteries, cerebral arteries, iliac and femoral arteries, and carotid artery that is responsible for CHD, stroke, and peripheral arterial diseases. It is initiated in the intima of the arteries with deposits of lipids, principally cholesterol and its esters, in macrophages and smooth muscle cells, which contribute to the formation of an atherosclerotic plaque development (Council, 1989). Therefore, understanding the cellular and molecular mechanisms and the genetic contribution in the development of atherosclerosis are key to better understand the pathology. At atherosclerotic susceptible sites, the complex of cells, connective-tissue

elements, lipid crystals and blood born inflammatory immune cells may lead to intimal thickening and narrowing of the lumen in the coronary artery (Hansson, 2005). IHD, also known to CHD (coronary heart disease), is occurring due to the presence of atherosclerotic plaques leading ultimately to plaque erosion on major branches of coronary arteries (Crossman and Morton, 2007). Thus, the progression of atherosclerotic plaques leads to progressive narrowing of the lumen of the affected arteries, flow through the vessel will diminish, blood pressure distal to the stenosis will decrease (Crossman and Morton, 2007). As discussed in the previous section, stroke is the second most common cause of death in the world population (WHO, 2018). Stroke is characterized by ischemia due to the interruption or reduction of blood supply to part of brain tissue. It is an acute medical condition due to the damage of distal tissues by deficiency or poor supply of oxygen in distal tissues causing an extension of brain cell death. Stroke has been classified into two different types: ischemic stroke due to the low blood flow, and hemorrhagic stroke with bleeding subsequent to the rupture of brain capillaries (NIH, 2014).

## 3. Risk factors of CVDs

Several lines of evidences are pointing out that many risk factors are contributing to the development of CVDs. As a key hallmark of CVDs, it has been well-established that reactive oxygen species (ROS) play a critical role in the initiation and development of CVDs.

ROS are known as highly reactive molecules containing oxygen with uncoupled electrons, which may lead to alterations of biological molecules through oxidation of carbohydrates, lipids, enzymes, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Although oxygen by itself is not a strong oxidant, reduced oxygen with unpaired electrons

requires additional three electrons until it is transformed to $H_2O$ (Turrens, 2003).

In the cardiovascular system, a variety of enzymes such as cytochrome P450, cyclooxygenase-2 (COX-2), xanthine oxidase, lipoxygenase, NADPH oxidase and uncoupled eNOS contribute to the cytosolic formation of ROS (Ghosh et al., 2015). Previous studies have shown that smoking represents the most preventable risk factor of CVDs which is contributing to the development of atherosclerosis. In part, due to the fact that cigarette smoke contains approximately 4000 different chemicals. These chemicals often contribute to the generation of ROS in the body after involving the endoplasmic reticulum (ER) stress induced protein miss-folding (Lin et al., 2012). C57/BL6 mice have shown that chronic cigarette smoking can cause oxidative stress and impair endothelium-dependent relaxations by reducing nitric oxide (NO) bioavailability (Guo et al., 2006).

Another modifiable risk factor of CVDs is physical activity or exercise. Moderate frequency and intensity of physical exercise contributes to maintain cardiovascular health and protects the cardiovascular system through various of mechanisms: i) Physical activity influence-fibrinolysis activity by increasing the tissue-type plasminogen activator (t-PA) function (DeSouza et al., 1998), ii) Physical training significantly reduced neointima formation and has been shown to increases NO bioavailability in spleen-derived endothelial progenitor cells (Laufs et al., 2004), iii) A clinical study with obese children showed decreased systolic blood pressure (exercise: 6.9±13.5 mmHg vs. control: 3.8±7.9 mmHg) by the daily anaerobic exercise for 30 min (Farpour-Lambert et al., 2009). In addition, numerous studies suggest that well-balanced diets reduce the risk of type 2 diabetes and may lower the risk of obesity (Davis et al., 2004).

Especially, consumption of fruit, vegetables and fish, containing high quantity of antioxidants, was associated with an inversed relationship relative to risk factors (Joshipura et al., 1999).

# *Chapter Two*

# Endothelium and

# Cardiovascular diseases

# 1. Vascular physiology

## 1.1. Role and structure of blood vessels

The circulatory system in our body consists of two essential parts: i) the cardiovascular system including the heart and blood vessels, and ii) the lymphatic system (Rizzo, 2015). The major function of blood vessels is transportation of blood to peripheral organs for supplying oxygen and nutrients to every tissue in the body, and immunological properties such as recruitment of immune cells by the release of chemoattractants. In order to fulfill these functions, blood vessels have specific structures (Murray, 1926).

The histological structure of blood vessels is divided into three main layers such as tunica intima, tunica media and tunica adventitia (Fig. 2). Tunica intima, known as endothelium, is the innermost part of blood vessels composed of a single layer of endothelial cells, which directly faces blood flow due to their exposure to the lumen (Alberts et al., 2002).

Endothelium is supported by elastic lamina, that bears much of the wall tension in blood vessels (Wong and Langille, 1996). Elastic lamina represents a flexible barrier between tunica intima and tunica media and known to have an important role in atherogenesis via its modulation of diffusion processes across the artery wall (Sandow et al., 2009).

Tunica media is the central part of blood vessels made up of numerous layers of smooth muscle cells, fibrous and non-fibrous matrix proteins such as laminin, collagen, elastin (Spina et al., 1983).

The outermost part of blood vessels is called tunica adventitia or tunica externa, which consists of connective tissue collagen fibre bundles, elastic fibre nets and also

vasa vasorum and nervi vasorum. Its most obvious function is the integration of the artery into surrounding tissues and innervation of the vessel wall. Nervi vasorum, one component in adventitia, plays a key role to regulate vascular tone by neurotransmission. Blood is circulating through large blood vessels relatively quickly, therefore, there is insufficient opportunity for blood in the lumen of the vessel to provide nutrition to or remove waste from the surrounding cells. Furthermore, the large blood vessel walls are too thick for nutrients to diffuse towards the outermost part of blood vessels. Therefore, the structure of larger arteries contains small blood vessels within their walls known as the vasa vasorum (Witter et al., 2017).

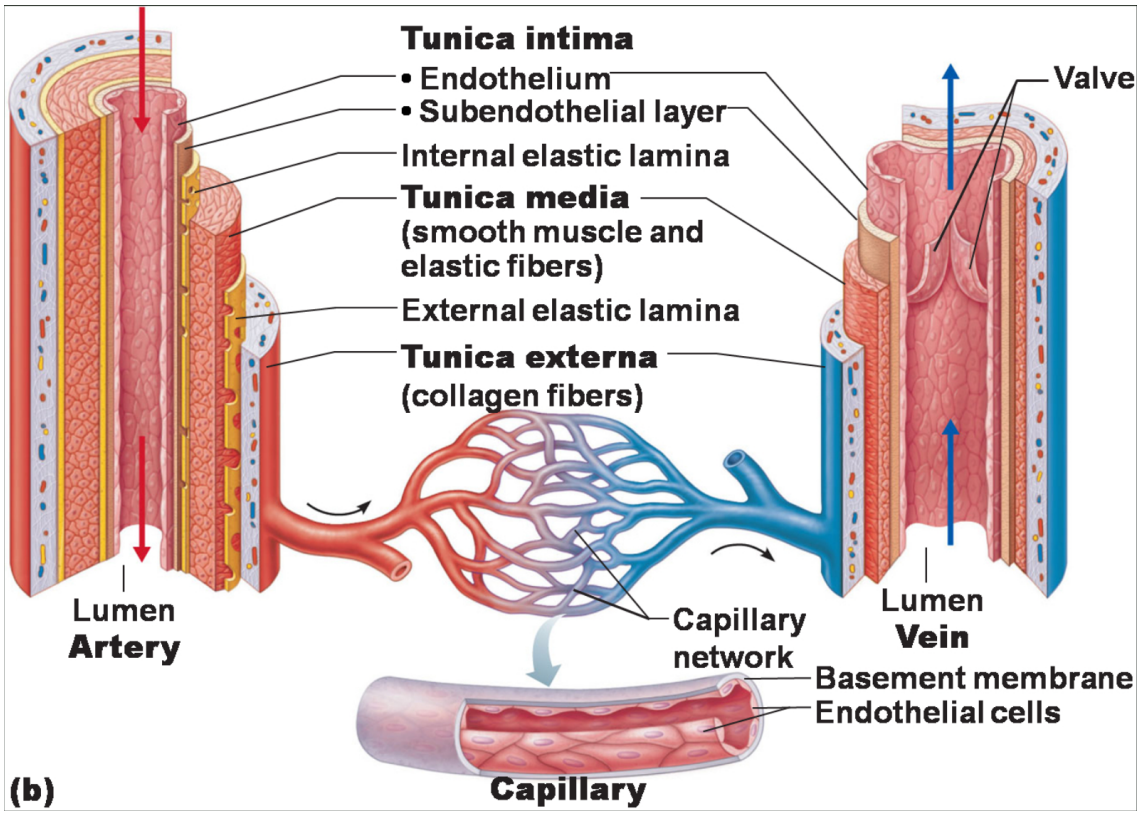



**Fig. 2 General structure of blood vessels.** Arteries and veins are composed of three tissue layers including tunica intima, tunica media and tunica adventitia (Adopted from Wayne W. La Morte, MD, PhD, MPH, Boston University School of Public Health).

## 1.2 The endothelium

### 1.2.1 Role of the endothelium in blood vessels

The endothelium is a permeable thin layer consisting of an endothelial cell monolayer covering the luminal surface of all blood vessels (Alberts et al., 2002). The endothelium plays a key role (Fig. 3) in the maintenance of vascular health through the regulation of blood fluidity and blood flow, the control of vessel wall permeability and the communication with circulating blood and immune cells (Pober and Sessa, 2007). Due to its barrier function, the endothelium controls to selective transfer of various molecules and acts as a semi-permeable layer.

Endothelial cells have many paracrine and endocrine functions regulating the response of the vascular smooth muscle and also circulating immune cells such as lymphocytes and platelets (Sumpio et al., 2002). They release a variety of vasoactive mediators such as prostacyclin and NO in response to shear stress and chemical stimulators, such as thrombin, bradykinin or adenosine diphosphate (ADP) to inhibit platelet aggregation and regulate vascular tone (Woulfe et al., 2001).

They have two distinct transport mechanisms defined as paracellular and transcellular pathways (Mann et al., 2003). The paracellular pathway, comprising tight junctions between neighboring endothelial cells, is restrictive the macromolecular transport and increasing in response to inflammatory mediators such as thrombin, bradykinin, vascular endothelial growth factor (VEGF), platelet activating factor (PAF) and histamine to promote the dilation of the intercellular space. The transcellular pathway is the primary mechanism of the transport of macromolecules including albumin, steroid hormones, lipids, vitamins and other substances that bind to albumin across the restrictive endothelium barrier by the caveolae-mediated endocytosis (Minshall and

Malik, 2006). Previous research has indicated that GLUT1 and GLUT4 are expressed in endothelial cells and that they contribute to glucose transportation into various tissues like umbilical veins, adrenal capillaries, aorta, retina, heart, placenta and testis (Kahn et al., 1991).

In addition, the endothelium plays a most important role to maintain vascular tone through the release of vasodilatory factors such as, nitric oxide (NO), prostacyclin ($PGI_2$) and endothelium-derived hyperpolarization (EDH) and/or vasocontractile factors such as thromboxane ($TXA_2$) and endothelin-1 (ET-1) (Sandoo et al., 2010). Often, inflammatory processes can be observed in blood vessels where endothelial cells are activated by release of proinflammatory cytokines and growth factors (Danese et al., 2007).

In addition, endothelial cells control several lineage of immune cells trafficking and also act directly as an immune cell by the release of endothelium-derived growth factors (i.e. VEGF, FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony stimulating factor) and recruiting factors such as monocyte chemoattractant protein-1 (MCP-1), expression of toll-like receptor 4 (TLR4) to recognize antigens like lipopolysaccharide (LPS), and to release tumor necrosis factor (TNF) and interleukin-1β (IL-1β). Endothelial cells not only recruit immune cells but also directly and/or indirectly participate in immunological processes in the body (Bell, 2009).

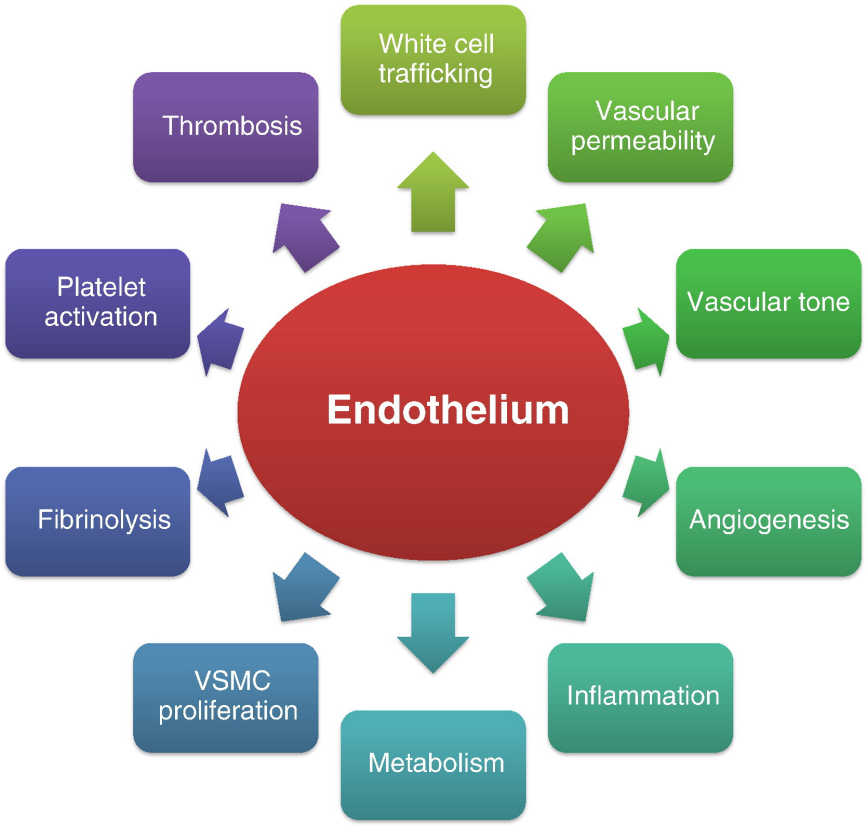



**Fig 3. Physiological functions of endothelium.** Endothelium have critical role in physiology **(Sena et al., 2013).**

### 1.2.2. Regulation of vascular tone

#### 1.2.2.1. Nitric oxide

Several vasoactive substances are released from the endothelium that regulate basal arterial tone by controlling the relaxation/contraction status of the vascular smooth muscle. In our body, three different enzymes have been reported to produce NO. The neuronal 'nNOS' (nNOS, NOS I or bNOS) and endothelial 'eNOS' (eNOS, NOS III) isoforms are generally regulated by $Ca^{2+}$/calmodulin (CAM) and by phosphorylation. The

third one is the inducible nitric oxide synthase, which expression is initiated by proinflammatory cytokines and lipopolysaccharide (Fleming, 2010).

NO is one of best known endothelium-dependent vascular relaxing factor. It is a soluble gas continuously synthesized by the conversion of L-arginine to L-citrulline by the enzyme called eNOS (Maron and Michel, 2012). A reduced bioavailability of NO is a one of the major markers of CVDs (Naseem, 2005).

The signaling cascade leading to the activation of eNOS is targeted by several kinds of upstream pathways. Early studies demonstrated that VEGF-induced formation of NO in endothelial cells is inhibited by a phosphoinositide-3-kinase (PI3K) inhibitor, which suggested that VEGF stimulates the PI3K/Akt-mediated activation of eNOS by the phosphorylation of Ser1177, an activator site on eNOS (Bian et al., 2008). Numerous publications suggested that NO is not only a vasodilatory mediator but it can also protect the cardiovascular system from oxidative stress and prevent the onset of CVDs.

NO at vascular sites plays an important role to control both pro- and anti-atherosclerotic responses. A reduced formation and/or bioavailability of NO is a key mechanism for the development of atherosclerosis prolong as LDL oxidation, leukocyte adhesion, smooth muscle cell (SMC) migration and proliferation and platelet aggregation (Naseem, 2005).

In CVD patients, it has been frequently observed as increased leukocyte invasion to the intima and an increased LDL (low density lipoprotein) oxidation due to the changes of endothelium permeability and antioxidant properties, respectively. Furthermore, a reduced formation of NO will also promote SMC migration and proliferation and excessive extracellular thrombus formation common features of atherosclerotic plaques, associated with neointimal thickening (Jeremy et al., 1999). Hence, an optimal NO bioavailability is key importance to prevent and protect the arterial wall.

#### 1.2.2.2. Endothelium-derived hyperpolarization (EDH)

Since the discovery of NO and $PGI_2$, it has been observed that those two mediators cannot fully account for relaxation induced by agonists in several types of blood vessels such as in the coronary circulation, which suggested the involvement of an additional mechanism defined as endothelium-dependent non-NO and non-$PGI_2$ mediated relaxation (Komori et al., 1988).

This non-NO, non-$PGI_2$ endothelium-dependent relaxation involves SMC hyperpolarization and is abolished by potassium channel ($K^+$-channel) blockers, and has been termed endothelium-dependent hyperpolarization (EDH) (Félétou and Vanhoutte, 2007).

The endothelium-dependent relaxation involving EDH gets more important compared to NO as the size of the artery decreases. Moreover, there is a great variability of EDH resource between species and tissues. Also, EDH mediated endothelium-dependent relaxation is more prominent after eNOS inhibition, suggesting that EDH-mediated endothelium-dependent relaxation is limited in the presence of sufficient levels of NO (Shimokawa et al., 1996).

The mechanism of EDH-mediated relaxation involves two stages depending on the place where the event takes place i) Once endothelial cells are activated by the agonist such as bradykinin, an increase in the intracellular calcium level ($[Ca^{2+}]_i$) and the entry of extracellular $Ca^{2+}$ are observed. The calcium activator signal activates $Ca^{2+}$-dependent $K^+$-channels ($K_{Ca}$) of intermediate ($IK_{Ca}$) and small conductance ($SK_{Ca}$) and induces $K^+$ efflux. ii) EDH is transmitted to SMC, activates $K_{Ca}{}^{2+}$ channels and induces SMC hyperpolarization accompanied by a reduced opening of voltage-sensitive $Ca^{2+}$ ($vCa^{2+}$) channels that leads to SMC relaxation (Luksha et al., 2009). The hyperpolarization is transmitted from endothelial cells to the vascular smooth muscle cell

via myoendothelial gap junction. An alternatively in some type of blood vessels, EDH has been suggested to involve cytochrome P450 (CYP 450) products (Fleming, 2014), high level of extracellular $K^+$ released from endothelial cells, $H_2O_2$ and C-type natriuretic peptide (Luksha et al., 2009).

### 1.2.2.3. Prostacyclin ($PGI_2$)

Prostacyclin is also called prostaglandin $I_2$ or $PGI_2$, which is a member of the eicosanoid family. It is a lipid molecule synthesized from arachidonic acid by the activation of the cyclooxygenase pathway (COX) (Marcus et al., 1980). The expression of COX-2, an inducible type of COX, is controlled by numerous signal transductions such as mitogen-activated protein kinases (MAPKs) and inflammatory mediators activating NF-ΚB (nuclear factor kappa B) signaling pathway, while COX-1 gene expression is predominantly constitutive. In endothelial cells, COX-1 may also be inducible in response to shear stress, VEGF and thrombin (Morita, 2002). Like NO, prostacyclin is a cardio-protective mediator in healthy endothelial cells.

Also, it is known to prevent and/or inhibit platelet aggregation and to induce vasodilatory responses (Mitchell et al., 2008). The action of prostacyclin to induce vascular relaxation is determined by specific $PGI_2$ receptors (IP) of the G-protein coupled receptor family on vascular smooth muscle cells. $PGI_2$/IP interaction in the cell membrane stimulates G-protein complex such as Gs, which stimulates cyclic adenosine-3',5'-monophosphate (cAMP)/protein kinase A (PKA) leading to $Ca^{2+}$ extrusion from cytosol and sarcoplasmic reticulum $Ca^{2+}$ pump (Dusting et al., 1977). In addition, PKA activates different $K^+$ channels including ATP-sensitive $K^+$ channels and MaxIK channels promoting the smooth muscle cell hyperpolarization and relaxation (Majed and Khalil, 2012).

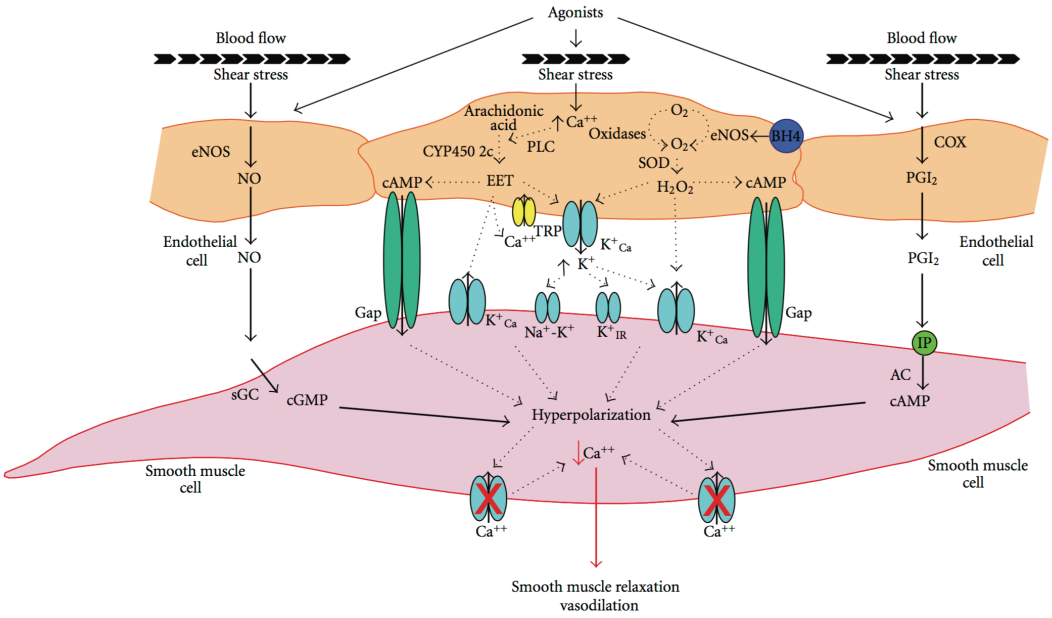



**Fig 4. Mechanism of endothelium-dependent relaxation (Ozkor and Quyyumi, 2011).** NO synthase: eNOS; cyclooxygenase: COX; prostacyclin: $PGI_2$; phospholipase C: PLC; CYP450 2c: cytochrome P4502C; eicosatrienoic acids: EETs; calcium-dependent potassium: $K_{Ca}^{+}$; gap junctions: Gap; transient receptor potential: TRP; adenylyl cyclase: AC; cyclic adenosine monophosphate: cAMP; cyclic guanosine monophosphate: cGMP; soluble guanylyl cyclase: sGC; prostacyclin receptor.

## 2. Endothelial dysfunction and cardiovascular diseases

### 2.1. Pathophysiology of cardiovascular diseases

#### 2.1.1. Atherosclerosis

The common causes of CVDs are related to numerous disorders such as hypercholesterolemia, hypertension, hyperglycemia and atherosclerosis. Atherosclerosis plaque erosion and/or rupture is a major event underlying IHD, stroke

and myocardial infarction. These pathologies are often severe acute ischemic conditions requiring hospital care, and can induce severe chronic disabilities.

The pathogenesis of atherosclerosis involves an inflammatory response and major structure changes of the arterial wall (Scott, 2004). LDL gets oxidized to become oxidized low density lipoprotein (LDL) by the oxidative stress in the arterial wall, leading to the recruitment of circulatory leukocytes and the release of pro-inflammatory cytokines like MCP-1, TNF-α, IL-1β and granulocyte-macrophage colony stimulating factor (GM-CSF) (Chen and Khismatullin, 2015).

Cytokines and growth factors released under stress conditions, in turn, leading to activate SMC migration to the intimal layer and circulatory monocyte adhesion through the expression of cell adhesion molecules such as VCAM-1, ICAM-1 in the early phase of atherosclerosis (Xing et al., 2012). Once resident in the arterial intima, monocytes will become macrophages that engulf oxidized LDL through the increased expression of the scavenger receptor A (SRA) or CD36 and, then they are undergoing a series of changes that lead to foam cell formation (Libby et al., 2002).

A number of clinical studies have observed that atherosclerotic plaques from patients contain lipid deposition including cholesterol crystals. After the early formation of fatty streaks, the progression of atheroma involves the participation of SMCs and the disruption of internal elastic lamina located between the intimal and the medial layer of blood vessels. The secretion of active matrix metalloproteinases (MMP)-2 and MMP-9 from both endothelial cells and SMCs contribute to remodeling of the lipid-rich atherosclerotic plaque via digestion of collagen and elastin, promoting of SMC migration and proliferation (Johnson and Galis, 2004). Integrins are heterodimeric transmembrane proteins, composed of an α and a β subunit, that link the extracellular matrix to the cytoskeleton of SMCs. Integrin ligation promotes for SMC adhesion to matrix, and it triggers pro-migratory intracellular signaling cascades involving the phosphorylation

focal adhesion kinase (FAK) and cytoskeletal remodeling through the expression of lamellipodia and filopodia (Gerthoffer, 2007).

### 2.1.2 Diabetes mellitus

Numerous lines of evidences indicate that diabetes mellitus is not only an endocrine disorder but also that promotes CVDs. A key feature in diabetes mellitus is chronic hyperglycemia. Different molecular mechanisms involving in hyperglycemia have been observed such as increased glucose influx leading to activation of the polyol pathway, formation of advanced glycation end-products (AGEs) and activation of the 12/15 lipoxygenase pathway. All these mechanisms finally increase the level of oxidative stress in blood vessels. The polyol pathway is a two-step pathway converting glucose to the sorbitol and to the fructose. It is activated when large amount of glucose is present such as in hyperglycemic conditions. Sorbitol cannot cross the cell membrane and it promotes osmotic stress and oxidative stress to blood vessels due to the consumption of NADPH in the polyol pathway (Brownlee, 2001). High levels of blood glucose in hyperglycemia leads to over-production of reactive dicarbonyls, which may react with amino acids of the proteins and to form AGE. Glycated proteins and lipids, AGEs, may alter the specific function of the proteins and they directly bind to AGE receptors (RAGE), which promote endothelial dysfunction through the increase expression of adhesion molecules such as ICAM-1 and VCAM-1 and also the expression of pro-inflammatory cytokines such as TNF-α, VEGF and tissue factor (TF) (Goldin et al., 2006). 12/15 lipoxygenases are enzymes promote the insert of oxygen molecules to the 12 or 15 carbon position in arachidonic acids. The expression of 12/15 lipoxygenases have been observed in endothelial cells, smooth muscle cells and macrophages at a different metabolic pathophysiologies including hyperglycemia. In a previous study, 12-

hydroxyeicosetetetraenoic acid (12-HETE) and 15-hydroxyeicosetetetraenoic acid (15-HETE), final metabolites of arachidonic acid, by activation of 12/15 lipoxygenases have been shown to accelerate atherogenesis in LDL receptor deficiency mice through the modification of endothelial cell permeability and induction of LDL oxidation (Parthasarathy et al., 1989).

Type 1 diabetes results from deficiency of insulin secretion subsequent to loss of beta cells in pancreas, and is also termed insulin-dependent diabetes mellitus. In contrast, type 2 diabetes is non-insulin dependent and associated with insulin resistance when cells fail to respond properly to insulin. But both type 1 and type 2 are characterized by chronic hyperglycemia promoting CVDs (Kitabchi et al., 2009).

Hyperglycemia is well-known to activate NF-KB, which induces the expression of a great number of pro-atherothrombotic genes in endothelial cells, monocyte-derived macrophages and SMCs. In addition, AGE-RAGE engagement leads to the formation of ROS subsequent an increased NADPH oxidase (Fukami et al., 2014) that promote LDL oxidation and autoxidation of glucose in the blood thereby promoting tissue damage in the artery wall (Pennathur and Heinecke, 2007). Previous studies have indicated that hyperglycemia enhances monocyte adhesion to cultured endothelial cells via the activation of NF-KB, and also stimulates the expression of several pro-inflammatory genes related to atherogenesis (Piga et al., 2007).

The reduction of NO generation is induced by high glucose and AGEs and is associated with impaired endothelium-dependent relaxations to acetylcholine in the streptozotocin-induced diabetic mice model. Furthermore, it is associated with an increased levels of peroxynitrite, nitro-tyrosine expression and lipid peroxidation involving eNOS uncoupling (Camici et al., 2007).

### 2.1.3. Hypertension

Hypertension is designed as a high level of blood pressure that can lead to coronary artery disease, stroke, heart failure, atrial fibrillation and peripheral vascular diseases (Lackland and Weber, 2015). For adults, hypertensive patients show blood pressure greater than 140/90 mmHg as systolic/resting blood pressure (Whelton et al., 2018). The hypertension is classified with two different states defined as primary (essential) and secondary high blood pressure. More than 95% of patients are categorized in primary hypertension, and the onset of 5% secondary hypertensive patients generally has no family history but it is caused by renal and endocrine disorders or an iatrogenic trigger, such as use of oral contraceptives (Poulter et al., 2015).

## 2.2 Endothelial dysfunction

Endothelium is semi-permeable monolayer in inner surface of blood vessel, which is particularly maintaining vascular tone and regulating oxidative stress by the formation of NO and controlling the local angiotensin II activity (Sitia et al., 2010). Endothelial dysfunction is termed as a pathophysiological state characterized by loss of endothelium dependent relaxation activity, impaired fibrinolytic ability, hemodynamic dysregulation, increase of growth factors and inflammatory cytokines, increase expression of adhesion molecules, excessive generation of ROS, enhanced endothelial permeability (Abraham and Distler, 2007). The mechanism involving development of endothelial dysfunction has been linked to several causes, such as hypertension, inflammation, dyslipidemia and hyperglycemia resulting oxidative stress (Sitia et al., 2010).

Healthy endothelium maintains the homeostasis of vascular tone by NO production, decrease of oxidative stress, inhibition of platelet aggregation and inflammation (Table

1).

Common feature of endothelial dysfunction is characterized by decrease NO bioavailability due to the NO trapping by free radicals and also decrease of eNOS activity by the uncouple eNOS. NO reacts with superoxide anion ($O_2^-$) to form peroxynitrite anion ($ONOO^-$). In 24 months old C57 BL/6J mice, the level of eNOS was non significantly different compared to 2 month old mice. However, the aged mice (24 months old mice) had significantly increase ratio of eNOS monomer to dimers and also the formation of peroxynitrite. It is suggesting that peroxynitrite is an extremely potent oxidant that can initiate lipid peroxidation of LDL. In addition, peroxynitrite promotes the recruitment of immune cells by the increase level of adhesion molecules such as VCAM-1 and ICAM-1 and also release of MCP-1 in endothelial cells (Hansson and Hermansson, 2011).

| Healthy Endothelium | Dysfunctional Endothelium |
|---|---|
| • Vasodilatory (↑ NO, $PGI_2$)<br>• ↓ Oxidative stress , low uric acid<br>• Anti-coagulant (↓ PAI-1, vWF, P-selectin)<br>• Anti-inflammatory (↓ sICAM, sVCAM, E-selectin, CRP, TNF-α, IL-6, MCP-1)<br>• ↑ Repair (EPCs), ↓ Damage (CECs, MPs) | • Impaired vasodilation (↓ NO, $PGI_2$)<br>• ↑ Oxidative stress , uric acid<br>• Pro-coagulant (↑ PAI-1, vWF, P-selectin)<br>• Pro-inflammatory (↑ sICAM, sVCAM, E-selectin, CRP, TNF-α, IL-6, MCP-1)<br>• ↓ Repair (EPCs), ↑ Damage (CECs, MPs) |

**Table 1. Differences of healthy and dysfunctional endothelium (Sena et al., 2013).**

# *Chapter Three*

# Role of anthocyanin rich products in cardiovascular health

# 1. Introduction of anthocyanins

## 1.1 Anthocyanin rich foods and intake

Anthocyanins are water-soluble natural pigments that belong to the flavonoid group of polyphenolic compounds. Due to their frequent presence in fruit, vegetables and red wine, they are one of the major nutrient of the human diet. Anthocyanins occur naturally in plants with the form of glucoside conjugation in which intact anthocyanidins coupled to a sugar moiety (Tsuda, 2012). There are about 17 different anthocyanins found in nature, only 6 major anthocyanins are widely distributed such as, delphinidin, cyanidin, petunidin, peonidin, pelargonidin and malvidin (Prior and Wu, 2006). These compounds are well contained in the human diet, especially, in berry species (Table 2).

| Source | Antho.[a] | Concentration mg/100 g FW |
|---|---|---|
| Black grapes (*Vitis vinifera*) | mv-3-glc | 39.23 |
| Red wine | mv-3-glc | 9.97[d] |
| Bilberries (*Vaccinium myrtillus*) | cy-3-glc | 405.00 |
| | cy-3-gal | 370.00 |
| Blueberries (*Vaccinium corymbosum*) | peo-3-glc | 365.00 |
| Blackberries (*Rubus sp.*) | cy-3-glc | 138.72 |
| | cy-3-rut | 8.86 |
| Blackcurrants (*Ribes nigrum*) | dp-3-rut | 304.91 |
| | cy-3-rut | 160.78 |
| | dp-3-glc | 86.68 |
| | cy-3-glc | 25.07 |
| Chokeberries (*Aronia melanocarpa*) | cy-3-gal | 557.67 |
| | cy-3-ara | 252.76 |
| Strawberries (*Fragaria x ananassa*) | pl-3-glc | 47.14 |
| Elderberries (*Sambucus nigra*) | cy-3-sam | 462.96 |
| | cy 3-O-glc | 794.13 |

**Table 2. Dietary source of anthocyanins (Pojer et al., 2013).**

Since the past few decades, there are a lot of studies suggesting that anthocyanins consumption induced a wide range of biological activities such as an antioxidant effect, anti-inflammatory effects, anticarcinogenic effects, and protection against CVDs (Zafra-

Stone et al., 2007). A 8-week randomized clinical trial of blackberry juice ingestion by 72 dyslipidemia patients showed significantly increased HDL and apo A-I, apo B, whereas high sensitive C-reactive protein (hs-CRP) was significantly decreased (Aghababaee et al., 2015). It is suggesting that anthocyanin-rich food have a beneficial effect on the level of serum apolipoproteins, a marker of dyslipidemia. Furthermore, the findings of Lobro *et al*., indicated that cyanidin-3-*O*-glucoside induced protective effects on global ischemia reperfusion injury in isolated heart, and antioxidant properties in endothelial cells (Ziberna et al., 2012). A high intake of anthocyanins for long period was associated with a reduction of CVDs risk factors with a significantly lower blood pressure and arterial stiffness in 1898 individual women aged 18-75 in cross-sectional study (Jennings et al., 2012).

Thus, anthocyanin-rich food, based on accumulated evidences, appear to have a preventive effect on various diseases with different mechanisms.

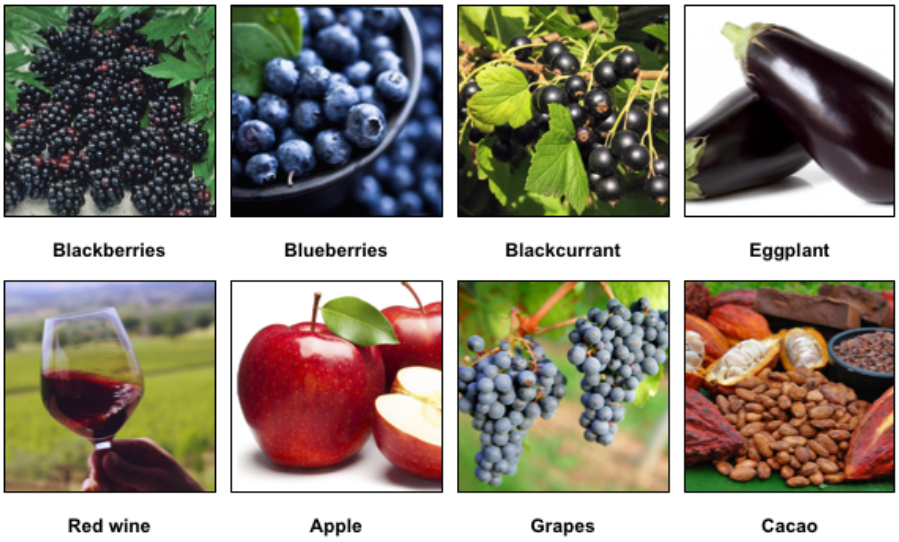



**Figure 5. Anthocyanin rich foods**

### 1.2 Classification and characteristic of anthocyanins

Anthocyanins are a class of flavonoid synthesized by the phenylpropanoid pathway. Phenypropanoid pathway is initiated from the conversion of amino acid phenylalanine to the cinnamate by the action of enzyme phenylalanine ammonialyase. Cinnamate is transformed to 4-coumarate by the cinnamate 4-hydroxylase (CH4) and from 4-coumarate to the 4-coumaroyl-CoA by the 4-coumaroyl CoA ligase (4CL) (Whelton et al.). Then, 4-coumaroyl-CoA is combined with three malonyl-CoA to make up for the flavonoid backbone called chalcones. The metabolic pathway continues by serial modification with enzymes to yield flavone, dihydroflavonol and anthocyanins, respectively (Fig. 6), (Winkel-Shirley, 2001).

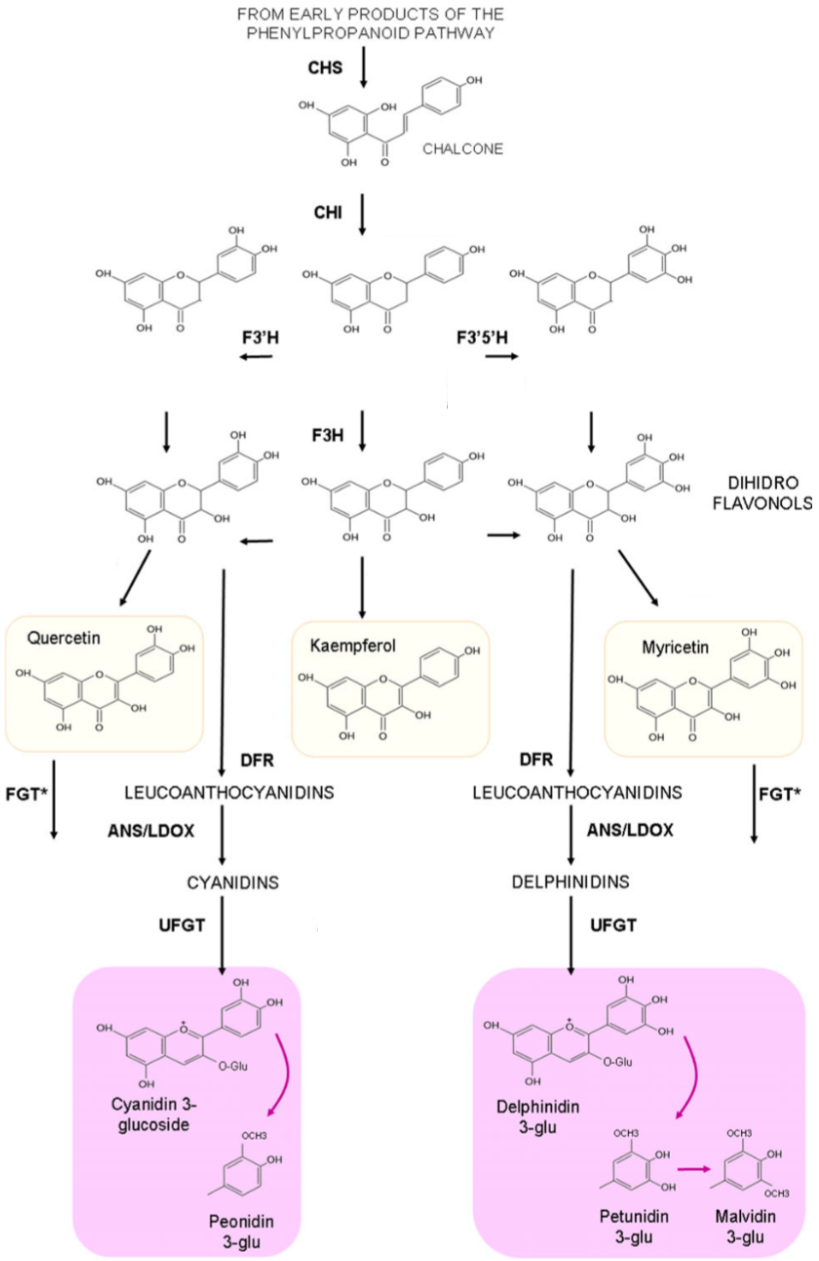
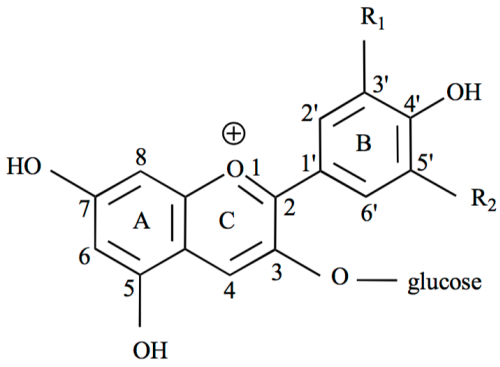



**Figure 6. Schematic pathway of anthocyanin biosynthesis.** CHS, chalcone synthase; CHI, chalcone isomerase; F3H/F3'H/F3'5'H, flavonoid hydroxylases; DFR, dihydroflavonol-4-reductase; ANS/LDOX, anthocyanidin synthase/leucoanthocyanidin dioxygenase; UFGT, UDP glucose:flavonoid-3-*O*-glucosyltransferase; FLS, flavonol synthase (Matus et al., 2009).

Anthocyanins are derived from anthocyanidins by the addition of different types of sugars and hydroxyl group. In detail, anthocyanidins are sugar free chemical structure of anthocyanins based on flavylium ion. The distribution of six major anthocyanidins in the plants is cyanidin (50%), peonidin (12%), pelargonidin (12%), petunidin (7%), delphinidin (12%) and malvidin (7%). There are four main classes of anthocyanins: 3-monosides, 3-biosides, 3, 5-diglycosides and 3,7-diglycosides (Kong et al., 2003).




Pelargonidin-3-glucoside: $R_1$=H, $R_2$=H

Cyanidin-3-glucoside: $R_1$=OH, $R_2$=H

Delphinidin-3-glucoside: $R_1$=OH, $R_2$=OH

Peonidin-3-glucoside: $R_1$=$OCH_3$, $R_2$=H

Petunidin-3-glucoside: $R_1$=$OCH_3$, $R_2$=OH

Malvidin-3-rutinoside: $R_1$=$OCH_3$, $R_2$=$OCH_3$

**Figure 7. Structure of six common anthocyanidin with glycosides.**

Generally, anthocyanins are stable in low pH condition and they are affected by various of environmental conditions, such as light, temperature, oxygen, metal ion and intramolecular association with other compounds (Shipp and Abdel-Aal, 2010). Anthocyanins can be found in different structures depending of the pH of the solution containing anthocyanins. At pH 1, the fravylium cation is stable and characterized by a puple or blue color (Fig 7. A). Between pH 2 and 4 fravylium cation is transformed to blue quinoidal species (Fig 7. B-D). Colorless species are detected at the pH 5 and 6 (Fig 7

(E-F)), carbinol pseudobase and chalcone, respectively. Then, anthocyanins start to degrade when pH is increased more than 7 (Castaneda-Ovando et al., 2009).

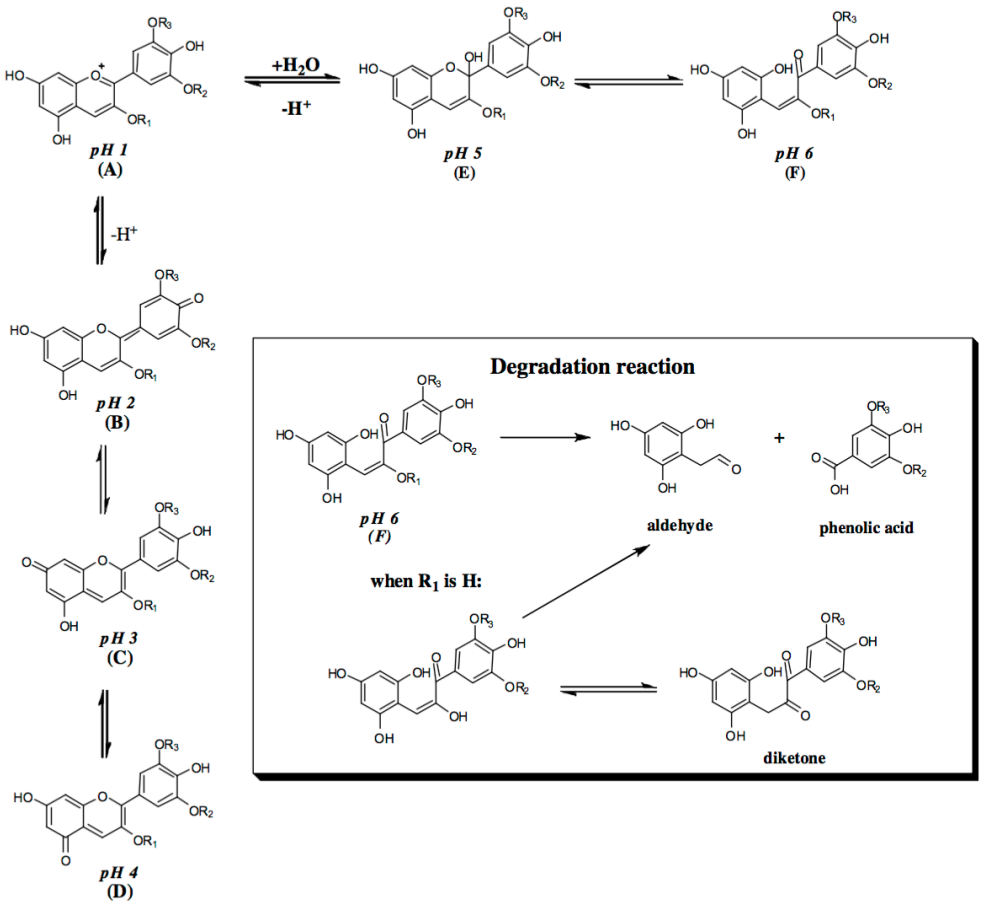



**Figure 8. Changes of anthocyanin structure in different pH condition (Castaneda-Ovando et al., 2009).**

## 2. Effect of anthocyanins in cardiovascular health

### 2.1. Bioavailability of anthocyanins

Based on previous studies, it is known that anthocyanins can be found as an aglycone, intact and acylated structures in urine (Kurilich et al., 2005). It is suggesting

that various structures of anthocyanins can be absorbed after metabolism and also, their original structure without structural modification. A previous study has shown that the rate and quantity of absorption of anthocyanins is dependent on their chemical structures such as the aglycone and the glucose moiety (Tian et al., 2006). The maximum concentration of anthocyanins in plasma is detected 30 min to 2 h after consumption of anthocyanins rich-fruit. The systemic bioavailability of anthocyanins is estimated less than 2% in *in vivo* studies (Borges et al., 2013). The relatively low plasma level of anthocyanins suggested that they undergo rapid and extensive metabolism after absorption and/or that they rapidly enter into target cells.

In a human study, anthocyanins are detected in blood within minutes after consumption, suggesting that anthocyanins are very quickly absorbed from stomach (Mallery et al., 2011). Previously, Passamonti *et al*., have shown that the stomach is a major site for anthocyanins absorption via bilitranslocase. Bilitranslocase is a plasma membrane organic anion carrier expressed in the liver and gastric epithelium including stomach (Tsuda et al., 1999). Several investigators reported that anthocyanins are transported to intestinal epithelium in the body exclusively as an intact glycoside (Miyazawa et al., 1999). Therefore, a recent publication has shown that delphinidin-3-*O*-glucoside, the most abundant anthocyanin in blackcurrant, prevents oxidized LDL-induced mitochondrial dysfunction in endothelial cells following delphinidin-3-*O*-glucoside uptake via sodium-glucose cotransporter 1 (SGLT 1), (Jin et al., 2013).

A great number of anthocyanin metabolites are detected with different structures in the urine and blood. The consumption of an anthocyanin with four different glucosides (cyanidin-3-glactoside, cyanidin-3-arabinoside, cyanidin-3-glucoside, cyanidin-3-xyloside) resulted in the appearance of at least ten different metabolites subsequent to the involvement of different metabolism pathway such as methylation, sulfonation, glucuronidation and hydrolysis (Kay et al., 2004).

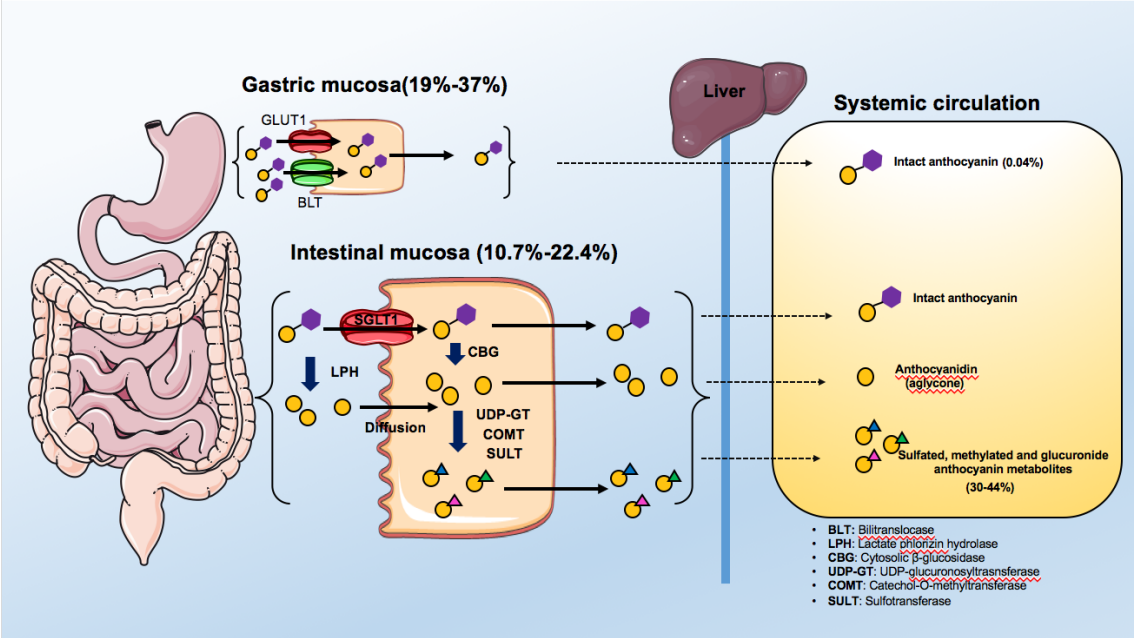



**Figure 9. The metabolism of anthocyanin in the body.**

## 2.2 Role of anthocyanin in cardiovascular health

### 2.2.1 *In vitro* study

Anthocyanins are the most abundant flavonoid constituents in fruits and vegetables. So, daily intake of anthocyanins in people of United States is estimated between 180 to 215 mg/day, which is 9-fold higher than the other dietary flavonoids. Many *in vitro* data are suggesting a beneficial effect of anthocyanins in human health through a variety of mechanisms. The phenolic structure of anthocyanins is responsible for antioxidant activities, which has the ability to trap ROS molecules such as superoxide ($O_2^{\cdot -}$), singlet oxide anion ($O_2$), peroxide ($ROO^-$), hydrogen peroxide ($H_2O_2$) and the hydroxyl radical (OH.) (Wang and Jiao, 2000). The antioxidant properties of anthocyanins have been observed in different *in vitro* systems such as endothelial cell (Bagchi et al., 2004), liver

(Meyers et al., 2003), colon cancer (Renis et al., 2008), breast cancer (Singletary et al., 2007), and leukemia cell lines (Feng et al., 2007). Previous results have shown that antioxidant property of anthocyanins are based on their structural characteristics of hydroxyl residues in anthocyanin molecules, and also observed directly by interaction with amino acids of proteins C=O, and C=N groups (Jaldappagari et al., 2008, Kong et al., 2003).

Minnie et al., suggested that anthocyanin-rich extract from *Aronia meloncapa* E. induces cell cycle arrest in G1 phase targeting colon cancer cell line but not normal colonic epithelial cell. This effect was determined within 24 h by the increase expression of cyclin-dependent kinase p21 and p27 (Malik et al., 2003). In addition, anthocyanin-rich berry extract inhibited total and facilitated glucose transportation to Caco-2 cells by a decreased expression of GLUT1 transporter, interestingly, the expression of SGLT 1 was not affected. It is suggesting that the mechanism induced inhibition of glucose uptake differ depending on the type of glucose transporters (Alzaid et al., 2013).

Due to the instability of anthocyanins in physiological pH, often experiments have also investigated precursors and metabolites, such as cyanidin and protocatechuic acid. In contrast to cyanidin-3-*O*-glucopyranoside, cyanidin (aglycone of cyanidin-3-*O*-glucopyranoside) and protocatechuic acid protect neuronal cells against $H_2O_2$ -induced apoptosis through an increased antioxidant activity at the cytosol level.

### 2.2.2. *In vivo* study

Although previous results indicated low bioavailability of anthocyanins, there are a lot of *in vivo* studies showing that anthocyanins are associated with numerous biological mechanisms.

Anthocyanin-rich blackcurrant extract exhibits anti-carcinogenetic properties in

diethylnitrosamine (DENA)-induced hepatocellular tumorigenesis on Sprague-dawley rats. The oral administration of anthocyanins-rich blackcurrant extract dose-dependently inhibited the early hepatic preneoplastic events, in a two-stage model of hepatocarcinogenesis initiated with DENA and promoted by phenobarbital (PB). This result demonstrats that dietary anthocyanins contained in blackcurrant extract clearly possessed beneficial effects on chemically-induced rat liver tumorigenesis (Bishayee et al., 2011). Moreover, several studies indicated that dietary anthocyanins inhibit metabolic disorders through the regulation of leptin, insulin and blood glucose resistance. For example, cyanidin-3-*O*-glucoside suppress hypertrophy of adipocytes in a high fat diet fed C57BL/6J mice developing obesity. In this research, mRNA levels of enzymes involved in fatty acid, triglycerol synthesis and sterol regulatory elements binding protein-1 were suppressed by oral administration of cyanidin-3-*O*-glucoside (Tsuda et al., 2003). Previous results showed that anthocyanins have phytoestrogenic activities, such as genistein, kamperol and quercetin (Zava and Duwe, 1997). It also has been reported that anthocyanin-rich blackcurrant extract and its four major anthocyanins possessed phytoestrogenic activities in ovariectomized rats and normal human female skin fibroblast cell line by the up regulation of estrogen-related genes (Nanashima et al., 2018). The data suggested that anthocyanins might be contribute to hormonal control by the maintenance of estrogen-related genes.

### 2.2.3. Anthocyanins in clinical trials

As discussed in the previous section, a number of clinical aspects have estimated that anthocyanins have the beneficial effects in human health, despite the great range of variation on the daily consumption of anthocyanins as such 5 mg to 225 mg. In dyslipidemia patients, anthocyanin supplementation improved serum LDL and HDL

cholesterol concentration associated with inhibition of cholesterol ester transfer protein in dyslipidemia patients (Qin et al., 2009). Moreover, previous epidemiological study implicated that anthocyanin consumption may reduce oxidative stress induced DNA damage by increase of glutathione levels in healthy subjects (Weisel et al., 2006). Iris et al., in their previous case study, suggested daily intake of berries (blackcurrant, lingonberry, bilberry, chokeberry mixed with raspberry) reduced blood pressure, increase HDL cholesterol level and CVDs risk factors, such as vWF (Von Willebrand factor), fibrinogen, ICAM-1 (intracellular adhesion molecule-1) (Erlund et al., 2008).

## 3. Blackcurrant anthocyanin and cardiovascular health

### 3.1. Blackcurrant anthocyanins and stability

Blackcurrant (*Ribes nigrum*), a fruit of woody shrub in the family of Grossulariaceae, is one of the most popular berries and is widely cultivated from Eastern Europe to middle Asia. In previous report, blackcurrant juices were subjected to high performance liquid chromatography with UV and mass spectroscopy detectors. As shown in Fig. 9, there are four major anthocyanins that are most abundant in the blackcurrant juice such as, delphinidin-3-O-rutinoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside and cyanidin-3-O-glucoside, respectively. Then, evaluation of stability to compare between

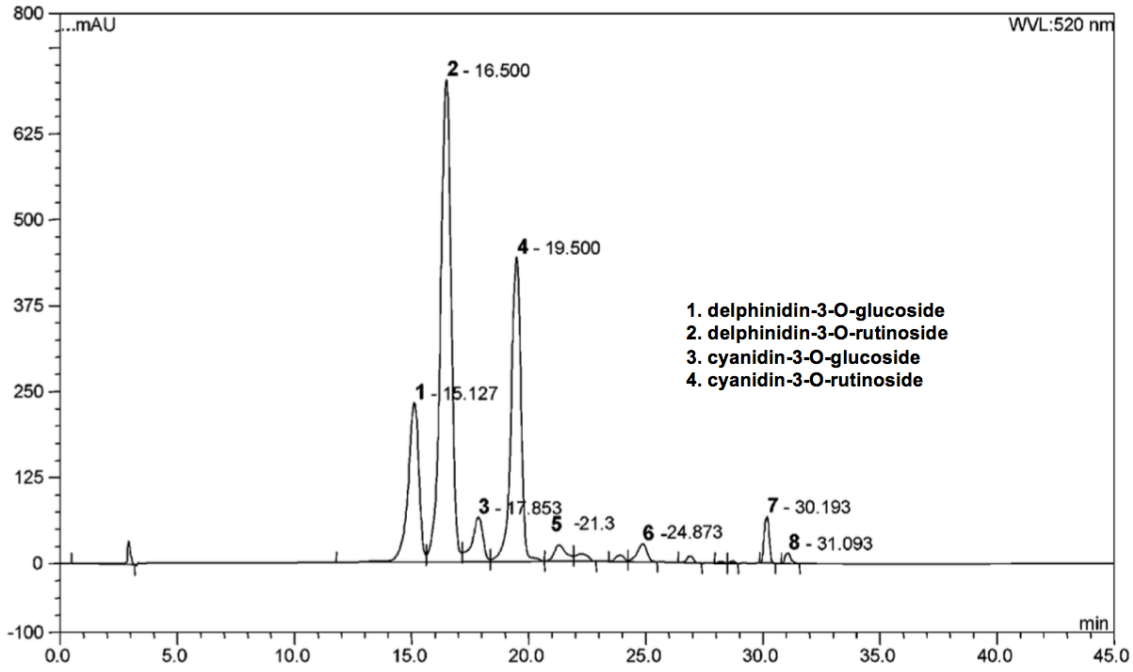



**Figure. 10. Anthocyanin composition in blackcurrant (Kapasakalidis et al., 2006).**

four major anthocyanins under the irradiation of UV and high temperature condition was analyzed. The total content of anthocyanins after 4 h of irradiation of UV decreased on average by 44-55%. Furthermore, cyanidin-3-*O*-rutinoside was the most thermally stable anthocyanin in the blackcurrant (Kapasakalidis et al., 2006).

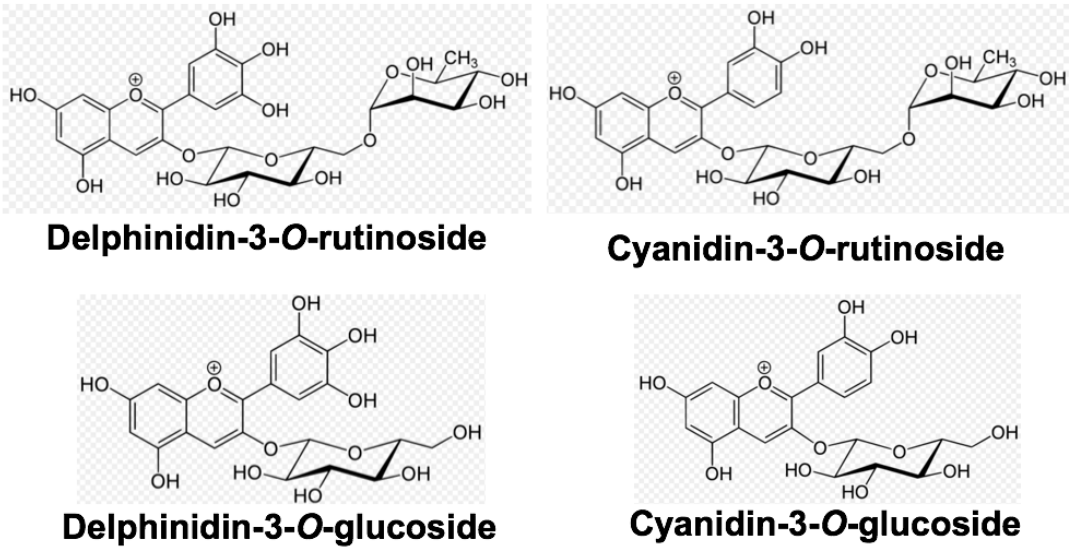



**Figure 11. Four major anthocyanins in blackcurrant.**

### 3.2. Blackcurrant and cardiovascular diseases

Anthocyanins are major flavonoid contained in diverse dietary sources such as, vegetables, berries, red wine and colored crops. In past decades, numerous studies suggested anthocyanins decrease the risk of CVDs mortality through the regulation of various risk factors (Hertog et al., 1995).

Clinical trials in which 250 ml of blackcurrant juice was given to 48 peripheral arterial disease patients, it was observed that the patients group treated by blackcurrant juice had a 11% decrease of peripheral arterial disease level and C-reactive protein (Dalgård et al., 2008). Keiko *et al*., proposed that the mechanism of blackcurrant on decreasing hind-limb perfusion pressure through involved an increase NO formation in endothelium (Iwasaki-Kurashige et al., 2006). In addition, blackcurrant anthocyanins activate Akt-eNOS signaling pathways in human umbilical endothelial cell (HUVEC) and it is abolished in the presence of wortmannin a PI3K inhibitor (Edirisinghe et al., 2011). Taken

together, blackcurrant anthocyanins stimulate the activation of PI3K-Akt-eNOS signaling pathway triggering NO formation in the blood vessel. Cyanidin-3-*O*-glucoside, typical anthocyanin in blackcurrant, shows free radical scavenging activity, suppresses inflammation, prevents endothelial dysfunction, vascular failure, and myocardium damage and seems to help prevent cardiovascular disease (Amorini et al., 2003, Serraino et al., 2003). Moreover, delphnidin-3-*O*-glucoside neutralized oxidized LDL induced oxidative stress and apoptosis in cultured endothelial cells by the reduction of mitochondria respiratory chain complex and intracellular superoxide anion formation (Xie et al., 2012).

In addition, Oak et al., observed that delphinidin and cyanidin inhibit platelet-derived growth factor (PDGF) induced VEGF release in conditioned medium of SMC by a preventing the activation of MAPK signaling pathway (Oak et al., 2006). The evaluation of lipid lowering capacity of blackcurrant anthocyanins has indicated that a polyphenol-rich blackcurrant pomace extract down-regulates intestinal and serum lipid formation in the high fat diet fed rabbit. In this study, the blackcurrant pomace extract ameliorated hyperlipidemia by decreasing triglyceride, total cholesterol, non-HDL cholesterol and free fatty acid levels in blood and increased the antioxidant activities (Jurgoński et al., 2014).

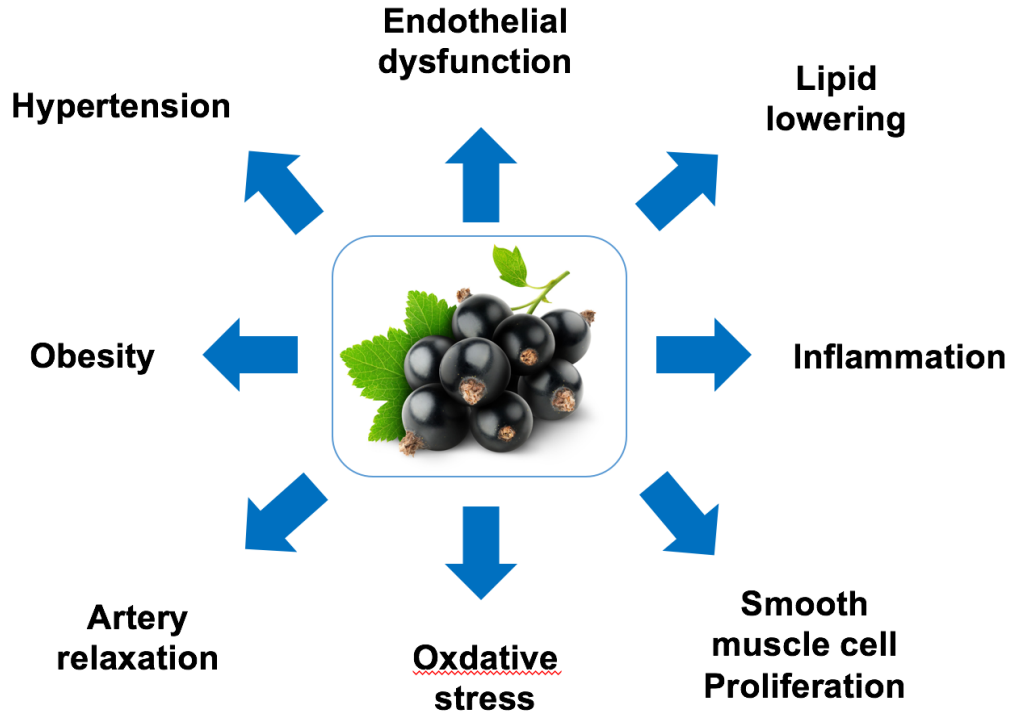



**Figure 12. The effect of blackcurrant on CVDs risk factors.**

# *Chapter Four*

# Sodium-glucose cotransporters

## 1. Physiology of sodium-glucose cotransporters

### 1.1 Structure and types

Sodium-glucose cotransporters (SGLTs) are a family of glucose transporters expressed in the intestinal mucosa of small intestine and the proximal tubule of the kidney. There are two most well-known SGLT family members, SGLT1 and SGLT2. SGLT 1 and 2 are responsible for the reabsorption of glucose to the blood in the kidney. SGLT 1, encoded by the gene *SLC5A1*, is mainly expressed in the intestinal lumen that contributes to transport glucose and galactose, which is about a 74-kDa glycoprotein of 14 postulated membrane spans localized in the brush border of the intestinal epithelium (Turk et al., 1994). As shown in Fig. 12, all transmembrane spans are an alpha helix, the N-terminal is located extracellular and C-terminal is inside the cytoplasm. The functional core is located on MS6 to 11 containing a galactose binding site and sodium binding site is placed on MS11 to 12 (Turk and Wright, 1997).

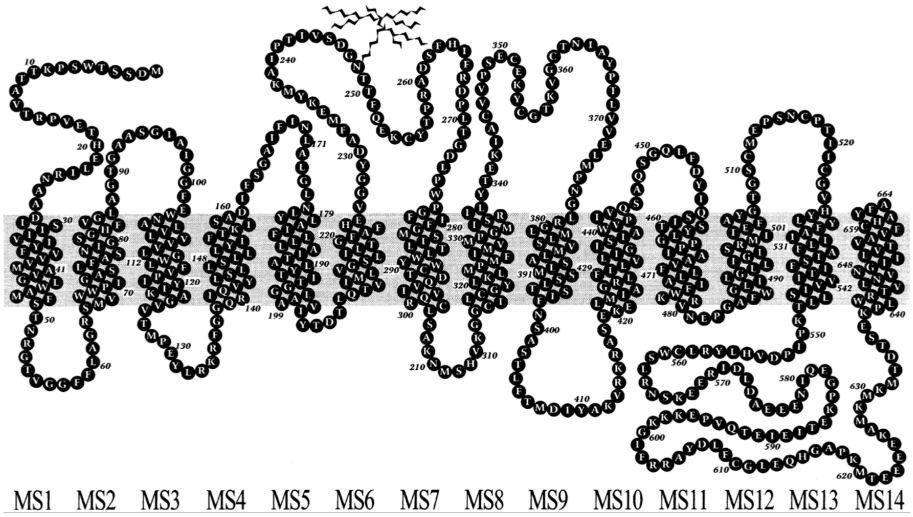



**Figure 13. Structure of SGLT1**

Figure 13 shows the human homologue model of SGLT2 based on the inward facing. In the sugar occluded state, glucose is coordinated by glucose binding site, and is excluded from contact with the external solution by hydrophobic residues (Ghezzi et al., 2018).

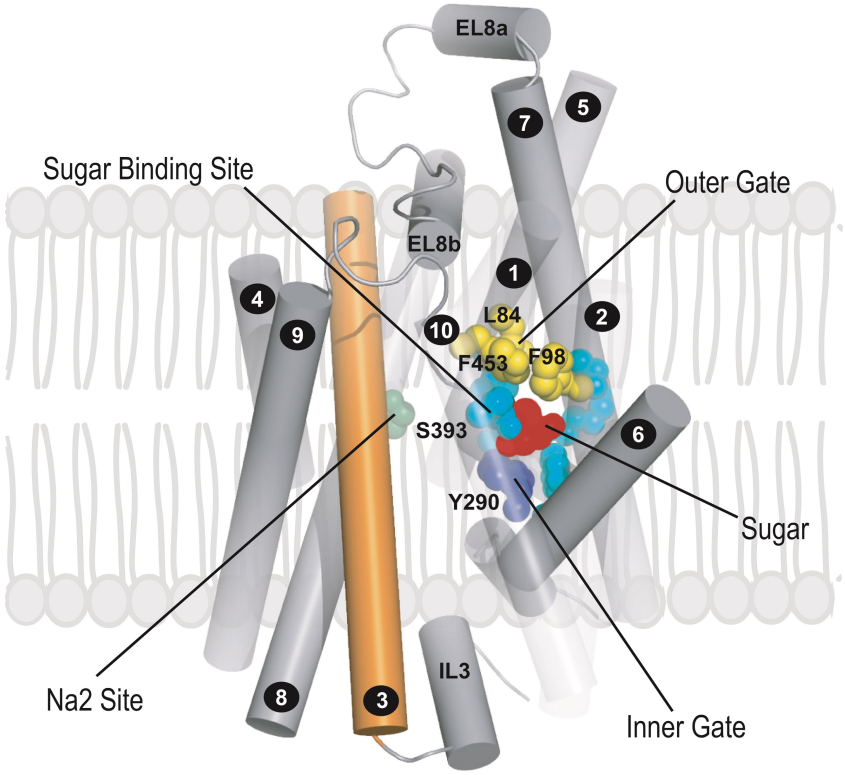



**Figure 14. The structure of human SGLT2 homologue (Ghezzi et al., 2018).**

### 1.2. Role of Sodium-glucose cotransporters

Glucose is an essential energy source of cells in the human body. Thus, glucose transporter plays key role for the energy production in the different organs. Glucose transporters are classified into two families: facilitative glucose transporters (GLUTs) and sodium-dependent glucose cotransporters (SGLTs). Facilitative glucose transporters

have been identified six different isoforms, Glut1 to Glut6. The primary function of the facilitative glucose carrier is to mediate glucose exchange between the blood and cytoplasm. Facilitative transportation systems are often termed passive diffusion, and that is energy independent system, which only can transport their substrate on a concentration gradient-manner. The Gluts are widely expressed in the human body, and distinct distribution of whole body glucose might be regulated by the tissue specific expression with several isoforms (Mueckler, 1994).

SGLTs have a different way of glucose transportation, which accept glucose and $Na^+$ by and electrochemical gradient across the membrane. SGLT1 is mainly expressed in the intestine and kidney responsible for galactose/glucose transportation. In contrast, SGLT2 is highly observed in the proximal tubule of kidney contributing ~90% of renal glucose reabsorption.

In the kidney, SLGT1 is expressed in S2 and S3 segments of renal tubules, and it reabsorbs one glucose molecule coupled with two sodium ions. SGLT2 has 60% of genetic similarity with SGLT1 and located on the S1 segment of proximal tubules. SGLT2 has low glucose affinity and transport one glucose molecule with one sodium ion, but high capacity. It is considering that SGLT1 and SGLT2 are contributing 10% and 90% of filtered glucose reabsorption, respectively (Harada and Inagaki, 2012).

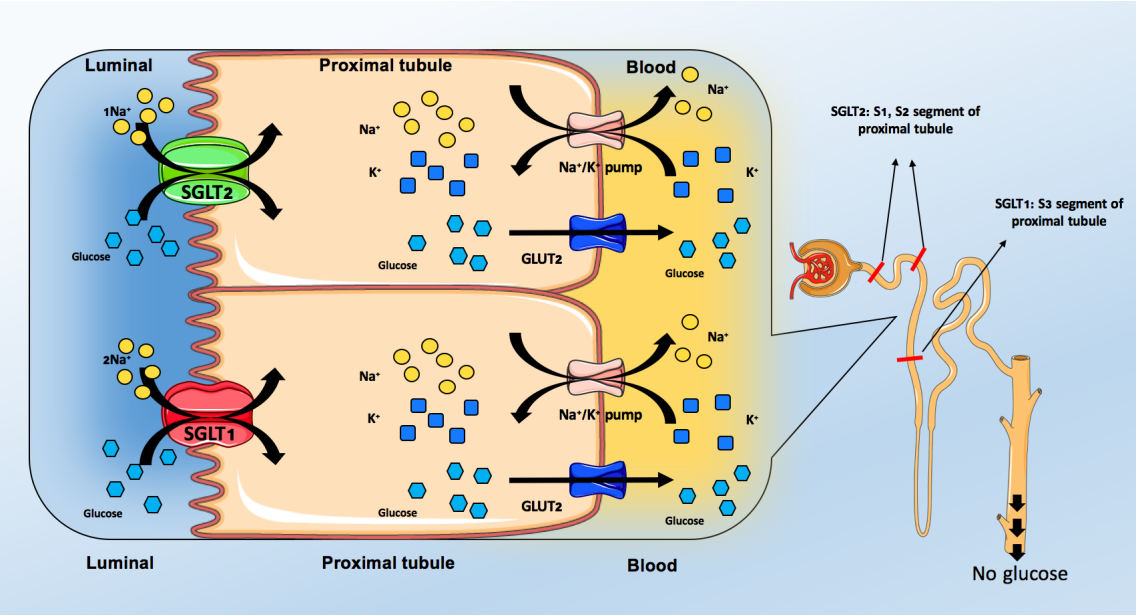



**Figure 15. The role of SGLT1 and SGLT2 in the proximal tubule of kidney.**

## 1. 3. Classification of SGLT inhibitors and application

SGLT inhibitors, approved by U.S food & drug administration (FDA), are a class of prescription medicine used with diet and exercise for treatment of type 2 diabetes. Most of SGLT inhibitors are targeting SGLT2 classified as canagliflozin, dapagliflozin and empagliflozin. Selectivity of SGLT inhibitors for SGLT2 over SLGT1 is important, due to

| Compound | SGLT-2 (AMG) | SGLT-1 (AMG) | SGLT-4 (AMG) | SGLT-5 (mannose) | SGLT-6 (myo-inositol) |
|---|---|---|---|---|---|
| C-glucosides | | | $IC_{50}$<br>$pIC_{50} \pm SEM$ | | |
| Empagliflozin | 3.1 | 8300 | 11000 | 1100 | 2000 |
| (BI 10773) | 8.50 ± 0.02 | 5.08 ± 0.03 | 4.94 ± 0.09 | 5.98 ± 0.15 | 5.70 ± 0.08 |
| Dapagliflozin | 1.2 | 1400 | 9100 | 820 | 1300 |
| (BMS-512148) | 8.94 ± 0.06 | 5.86 ± 0.07 | 5.04 ± 0.12 | 6.09 ± 0.22 | 5.88 ± 0.09 |
| Canagliflozin | 2.7 | 710 | 7900 | 1700 | 240 |
| (JNJ-28431754;TA-7284) | 8.56 ± 0.02 | 6.15 ± 0.06 | 5.10 ± 0.02 | 5.77 ± 0.12 | 6.61 ± 0.09 |

**Table 3. Potency of selectivity of SGLT 2 inhibitors.**

inhibition of SGLT1 in the intestine can lead to glucose-galactose malabsorption, a disease characterised by severe dehydration and diarrhoea observed in individuals with mutations in the SGLT-1 gene (Meeuwisse, 1970). Grempler et al., in their previous publication, studied that empagliflozin has a high degree of selectivity over SGLT1, 4, 5 and 6 compared with other SGLT inhibitors (Table. 3), (Grempler et al., 2012).

SGLT1 and SGLT2 inhibition by LX4211 (sotagliflozin), targeting type 1 diabetes mellitus, delays postprandial glucose absorption through the renal glucose reabsorption and local SGLT1 inhibition in the intestine. Furthermore, unique benefit of SLGT inhibitors is able to lower the requirement of bolus insulin interaction associated with glycemic variability and hypoglycemia.

# *Aim of the study*

## Aim of the study

Anthocyanins are secondary metabolites of plants, known to protect the part from natural enemies in the nature. Anthocyanin molecules are responsible for red, blue and purple colors used as food, pharmaceutical ingredients, and having potential health benefits. Most anthocyanins are being conjugated with one or two sugar molecules. In the last few decades, it has been suggested that anthocyanins have a potential health-promoting effect, although their structure is instable and is affected by pH, light, temperature, enzyme and oxygen. The potential effect of anthocyanins in cardiovascular health has been related to various mechanisms including an antioxidant effect, modulation of vascular tone and blood pressure, serum lipid lowering.

The bioavailability of anthocyanins is supposed to be very low. Clinical studies of anthocyanins bioavailability showed total anthocyanin metabolites including intact anthocyanins excreted in urine are about 1.8% from the ingested amount. The peak of anthocyanin metabolites in urine is detected 2 h after the ingestion, and among the metabolites, glucuronide anthocyanins were the most represented (Felgines et al., 2003). However, appearance of blackberry-derived C3G in serum in a 15-day blackberry fed rat was about 41.7% from the total ingested amount, suggesting that stomach is an essential organ in anthocyanin absorption (Talavéra et al., 2005).

Regarding endothelial cells, dietary anthocyanins (cyanidin-3-*O*-glucoside, delphinidin-3-*O*-glucoside and pelargonidin-3-*O*-glucoside) protect against peroxynitrite-induced mitochondrial dysfunction by inhibiting the activation of caspase-9 and -3 in cultured bovine aortic endothelial cells (Paixão et al., 2011). Auger *et al*., observed that high amounts of anthocyanins containing fruit juices and purees induce endothelium-dependent relaxation of porcine coronary artery rings. The most active ones were predominantly berries including aronia, blackcurrant, lingoberry, blueberry and cranberry, berries containing high levels of anthocyanins (Auger et al., 2011). Furthermore, C3G

and its 11 different metabolites altered the expression of inflammatory mediators IL-6 and VCAM-1 expression induced by oxidized LDL and CD40L (Amin et al., 2015).

Sodium-glucose cotransporters are a family of glucose transporters, contributing the renal glucose reabsorption. SGLT2 is located S1 and S2 segment of proximal tubule and has a high capacity but a low affinity for glucose. On the contrary, SGLT1 is expressed in S3 segment of proximal tubule with a low capacity but with a high affinity. By lowering the renal threshold for glucose excretion, SGLT inhibitors suppress renal glucose reabsorption leading to an improve hyperglycemia. In addition, the selective empagliflozin normalized glucose levels, and also improved endothelium-dependent relaxation, decreased expression of RAGE, COX 2, iNOS in Zucker diabetic fatty rats (Steven et al., 2017).

The aim of this study was to determine the contribution of SGLT1 and 2 in blackcurrant anthocyanins entry into endothelial cells and activation of vasoprotective endothelial NO pathway.

In detail, the aims were

- To identify the mechanisms underlying blackcurrant anthocyanin-induced endothelium-dependent NO-mediated relaxation in porcine coronary artery rings,
- Determination of active anthocyanins in the blackcurrant extract using cultured ECs,
- Study the contribution of SGLT1/2 in anthocyanins uptake into ECs,
- Evaluate the potential of blackcurrant anthocyanins to restore the protective endothelial function in senescent ECs, and entry via role of SGLT1 and SGLT2.
-

# ***Results***

# Article I

# Glucose-conjugated blackcurrant anthocyanins activate the endothelial NO synthase pathway following uptake via sodium-glucose cotransporter 1 and 2

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**Words count: 6532**

**Number of figures and tables: 9**

**Key words:** anthocyanin, blackcurrant, endothelial cell, nitric oxide, sodium-glucose cotransporters

## Abstract

**Introduction:** Blackcurrant (BC) anthocyanins, including predominantly glucoside- and rutinoside-conjugated cyanidin and delphinidin, have been shown to protect the vascular system, in part, by stimulating the endothelial formation of nitric oxide (NO). This study evaluated the possibility that sodium-glucose cotransporters (SGLT) 1 and 2 contribute to BC sugar-conjugated anthocyanins entry into endothelial cells (ECs) and the subsequent activation of endothelial NO synthase (eNOS) using isolated blood vessels and cultured ECs.

**Methods:** BC extract (BCE) was prepared from a BC juice using Sephadex LH-20 column. Vascular reactivity was assessed using organ chambers. Cultured ECs were used at P1 and P3 (senescent). The expression level of proteins was assessed by Western blot analysis, and the uptake of anthocyanins by Naturstoff's reagent A and confocal microscopy and flow cytometry.

**Results**: BCE caused concentration-dependent NO-mediated relaxations of coronary artery rings with endothelium, and phosphorylation of the eNOS activator site Ser1177 in ECs at P1, which were significantly inhibited by the dual SGLT1/2 inhibitor LX4211 and the SGLT2 inhibitors dapagliflozin and canagliflozin but not by the highly selective SGLT2 inhibitor empagliflozin. Cyanidin-3-*O*-glucoside (C3G) also increased eNOS phosphorylation, an effect prevented by LX4211. Uptake of BCE, C3G and delphinidin-3-O-glucoside (D3G) but not their rutinoside-conjugated forms was observed in ECs at P1, which was prevented by LX4211 but not empagliflozin. Both SGLT1 and 2 protein expression levels were observed in ECs at P1 and these signals were significantly higher in P3. An increased uptake of BCE and BCE-induced phosphorylation of eNOS was observed in ECs at P3 compared to P1, both of these effects were markedly inhibited by LX4211 and also, to some extent, by empagliflozin.

**Conclusion:** The present findings indicate that uptake of BC glucoside-conjugated

anthocyanins involves SGLT1 in young ECs, and both SGLT1 and SGLT2 in senescent ECs leading to stimulation of the endothelial NO pathway. The enhanced uptake of BC anthocyanins into senescent ECs might be an interesting strategy to protect senescence-prone atherosusceptible arterial sites.

## Introduction

Cardiovascular diseases (CVDs) are the critical cause of death worldwide in 2016 [1]. Several epidemiological studies have indicated that regular consumption of polyphenol-rich fruits (i.e., red and dark berries), vegetables (i.e., purple potato and red onion) and beverages (i.e., red wine, berry juices) is associated with a reduced risk of CV mortality [2-5]. The beneficial effect has been attributable, at least in part, to their high content of flavonoids and, in particular, anthocyanins. Amongst anthocyanin-rich products, the fruit of blackcurrant (BC, *Ribes nigrum* L.) is an interesting source with up to 250 mg of anthocyanins/100 g of fresh fruit [6]. Analysis of BC extracts by high-performance liquid chromatography has indicated the presence of several types of anthocyanins including cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin conjugated with either 3-*O*-rutinoside or 3-*O*-glucoside. Moreover, 3-*O*-rutinoside and 3-*O*-glucoside of delphinidin and cyanidin appear to be the major BC anthocyanins accounting for more than 97% of the total anthocyanin content [7].

The protective effect of BC anthocyanins on the CV system is likely to involve their ability to enhance the endothelial formation of the most important vasoprotective factor, nitric oxide (NO). Indeed, the comparison of 13 different fruit juices and purees has indicated that BC juice and puree are amongst the most active products to induce endothelium-dependent NO-mediated relaxations in porcine coronary artery rings [8, 9]. Moreover, cyanidin-3-*O*-glucoside (C3G) induced the phosphorylation of Akt in a time-dependent manner leading to the subsequent phosphorylation of endothelial nitric oxide synthase (eNOS) at the activator site Ser1179 to stimulate the NO/cyclic GMP pathway in cultured bovine aortic endothelial cells [10]. Alternatively, it may also involve their ability to decrease the level of oxidative stress promoting endothelial dysfunction and inflammatory responses [11]. Indeed, C3G prevented peroxynitrite-induced arterial dysfunction in rat aortic rings, and DNA damage in human umbilical vein ECs [12].

Anthocyanins are highly instable and very susceptible to degradation depending on several factors such as pH, temperature, light and their chemical structures [13]. The peak concentration of C3G in blood is detected 15 min after oral administration of a red fruit anthocyanin extract in rats, and 30 min in humans, which indicates that anthocyanins are absorbed by the gastro-intestinal track [14]. Moreover, bilitranslocase expressed in the gastric epithelium and also in the liver, has been suggested to contribute to the gastric absorption of anthocyanins in rats [15]. Interestingly, cyanidin-3-*O*-galactoside and -glucoside but not the aglycone competitively inhibited uptake of sulfobromophthalein, a bilitranslocase substrate, in rat liver plasma membranes suggesting that the sugar moiety of anthocyanins is important [16]. Recently, D3G has been shown to protect ECs against oxidized LDL-induced mitochondrial dysfunction subsequent to its entry via sodium-glucose cotransporter1 (SGLT1) [17]. Indeed, the protective effect of D3G was abolished by the non-selective SGLT inhibitor phlorizin, D-glucose and it was also not observed following SGLT1 knock out using SGLT1 siRNA [17]. A role for SGLTs is also supported by the fact that D3G significantly inhibited the uptake of radioactive 3-*O*-methyl-glucose (3-OMG) in the mouse intestine, an effect that was inhibited by phlorizin [18].

Therefore, the major aim of the present study was to evaluate the possibility that SGLT1 and 2, the two major SGLTs, contribute to BC extract and its major glucoside- and rutinoside-conjugated anthocyanins uptake into endothelial cells (ECs) promoting the subsequent activation of eNOS using isolated blood vessels and cultured ECs.

## Materials and methods

### Preparation and phytochemical analysis of BCE

To prepare BCE, concentrate blackcurrant juice was loaded into sephadex LH-20 column (Sigma) eluted with 1L of acetified methanol (v/v%, 0.01% acetic acid) at 12

ml/min. The anthocyanin rich fraction was concentrated by rotary evaporator at temperature not exceeding 40 °C. The anthocyanin-rich BCE compounds are yielded total 160 mg.

High Performance Liquid Chromatography coupled with Diode Array Detector (HPLC-DAD) analysis was performed in a liquid Elite Lachrom chromatograph (Merck Hitachi) equipped with an L2450 photodiode array detector. Separation of anthocyanins was carried out according to Tabart *et al*., [19]. Absorbance was recorded at 518nm. Standards of cyanidin-3-O-glucoside, cyanidin-3-O-rutinoside, delphinidin-3-O-glucoside, and delphinidin-3-O-rutinoside were purchased from Extrasynthese (Genay Cedex, France).

**Cell culture**

Porcine hearts were purchased from the local slaughterhouse (SOCOPA, Holtzheim, France). The left anterior coronary arteries were carefully collected, cleaned and flushed with warm PBS. To carry out primary cultures of porcine coronary artery ECs, the arteries were incubated with collagenase (type I, Worthington, 1 mg/ml) for 15 min at 37 °C. ECs were cultured in T75 flasks containing MCDB131 (Invitrogen) culture medium supplemented with 15% fetal bovine serum, fungizone (250 µg/ml), penicillin (100 UI/ml), streptomycin (100 UI/ml), L-glutamine (2 mM, all from Lonza, Levallois-Perret, France), and grown for 48–72 h until they reach 80-90 % of confluence (passage 0, P0). Thereafter, ECs were detached with trypsin (trypsin-EDTA; Life Technologies SAS) and further passaged at a ratio of 1:3 at regular intervals to induce replicative senescence (passage 3; P3).

**Vascular reactivity**

Left anterior coronary arteries were isolated, carefully cleaned to remove connective

tissue and fat, and cut into rings of 3-4 mm length. Thereafter, rings were suspended organ chambers containing Kreb's bicarbonate solution (mM: NaCl 119, KCl 4.7, $KH_2PO_4$ 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and D-glucose 11, pH 7.4, 37 °C) and oxygenated with a mixture of 95% $O_2$ and 5% $CO_2$. The resting tension was set at 5 g before the determination of changes of isometric tension. Coronary artery rings were contracted with U46619 before a concentration-relaxation curve to BCE was constructed. In some experiments, rings were incubated with an inhibitor: N*w*-nitro-L-arginine (L-NA, 30 μM, inhibitor of endothelial NO synthase), indomethacin (10 μM, COX inhibitor), inhibitors of endothelium-dependent hyperpolarization Tram-34, (1 μM, small conductance $Ca^{2+}$-activated $K^+$ channel blocker) plus UCL1684 (1 μM, intermediate conductance $Ca^{2+}$-activated $K^+$ channel blocker) or the combination of L-NA, indomethacin, Tram-34 and UCL-1684 for 30 min before the addition of U46619 and the subsequent relaxation to BCE. To determine the role of SGLT1 and/or 2 in BCE-induced NO-mediated relaxation, rings were incubated with either LX4211 ($10^{-7}$ M), empagliflozin ($10^{-7}$ M), dapagliflozin ($10^{-7}$ M) or canagliflozin ($10^{-7}$ M) for 5 min before addition of BCE. In some rings, the endothelium was removed mechanically by gently rubbing the lumen of the rings with a pair of forceps.

**Western blot analysis**

Following treatment ECs were washed twice with cold-PBS then lysed in protein extraction buffer (composition in mM: NaCl 150; $Na_3VO_4$ 1; sodium pyrophosphate 10; NaF 20; okadaic acid 0.01, Sigma; Tris/HCl 20, pH 7.5, QBiogene; a tablet of protease inhibitor, Roche and 1% Triton X-100, QBiogen). Equal amounts of proteins were loaded to 10-12% of denaturing SDS (10-12%) polyacrylamide gel and separated by gel-electrophoresis. Separated proteins were transferred to nitrocellulose membrane (GE Healthcare Life Sciences) at 4 °C and blocked with 5% BSA containing TBST (Tris-

buffered saline with 1% tween 20, Sigma) buffer. To detect proteins, membranes were incubated with a primary anti-body for overnight: rabbit polyclonal anti-SGLT1 (diluted 1:1.000; Alomone labs; cat. nº AGT-031), a rabbit polyclonal anti SGLT2 (diluted 1:1.000; Alomone labs; cat. nº AGT-032), a rabbit polyclonal anti-peNOS (diluted 1:1000; Cell signaling; cat. nº #9571), a rabbit monoclonal anti-pAkt (diluted 1:3000; Cell signaling; cat. nº #4060S) or mouse monoclonal anti-eNOS (diluted 1:1500; BD transduction; cat. nº 610297), a mouse monoclonal anti-β-tubulin (diluted 1:10.000; Sigma-Aldrich; cat. nº T7816) overnight at 4 °C. Membranes were washed with TBST three times at ten min intervals. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti-rabbit or anti-mouse IgG, dilution of 1:5000; Cell Signaling Technology; cat. nº #7074, #7076, respectively) at room temperature for 60 min. The expression level of proteins was detected by chemiluminescence reaction (ECL; Amersham, Les Ulis, France) followed by densitometric analysis using Image J software.

**Determination of anthocyanin uptake by flow cytometry**

ECs were seeded in a 6-well plate for 24 h. After 12 h of starvation, BCE (100 µg/ml) or an anthocyanin (100 µM; cyanidin-3-*O*-glucoside, C3G; cyanidin-3-*O*-rutinoside, C3R; delphinidin-3-*O*-glucoside, D3G; delphinidin-3-*O*-rutinoside, D3R) was added to ECs for 15 min in the absence or presence of the dual SGLT1/2 inhibitor LX4211 or the highly selective SGLT2 inhibitor empagliflozin. Following the incubation time, ECs were washed twice with PBS and incubated with 0.2% Naturstoff's reagent A (2-Aminoethyl diphenyl borate; Sigma) in PBS for 5 min. Subsequently, cells were washed with PBS and scraped in 500 µl of freshly added PBS. Fluorescence intensity was detected in the FL4-channel using a FACS Calibur (Becton-Dickinson, San Jose, CA, USA).

**Determination of anthocyanin uptake by confocal microscopy**

ECs were seeded into Lab-Tek® chamber slide for 24 h. Following treatment of ECs, they were washed and mounted in DAKO medium (fluorescence editing medium, DAKO) and examined under confocal microscope (Leica TCS SPE) at an emission wavelength of 620–705nm (excitation=488nm). Images were analyzed using Image J software.

### Statistical analysis

Data are presented as mean±SEM or as otherwise stated of n different experiments. Mean values were compared using two-way or one-way ANOVA followed by Dunnett's multiple comparisons test to identify significant differences between treatments using GraphPad Prism (Version 7).

## Results

### BCE composition

A shown in Table 1, the HPLC-DAD analysis of the BCE indicated the presence of four major sugar-conjugated anthocyanins, namely delphinidin-3-*O*-rutinoside (76.25 ± 1.82 µg/ml), cyanidin-3-*O*-rutinoside (38.81 ± 0.20 µg/ml), delphinidin-3-*O*-glucoside (17.49 ± 0.69 µg/ml), cyanidin-3-*O*-glucoside (6.11 ± 0.04 µg/ml), peonidin (7.22 ± 0.11 µg/ml) and several other minor compounds, including malvidin, delphinidin, peonidin-3-*O*-glucoside and peonidin-3-*O*-rutinoside (Table 1).

### BCE-induced NO-mediated relaxation in coronary artery rings involves SGLTs

BCE caused concentration-dependent relaxations starting at 3 µg/ml reaching near maximal relaxation at 30 µg/ml in rings with endothelium whereas no such effect was observed in rings without endothelium (Fig.1A). BCE-induced endothelium-dependent relaxation was abolished by the eNOS inhibitor, $N^G$-nitro-L-arginine (L-NA), and not affected by inhibitors of endothelium-dependent hyperpolarization, Tram-34 plus UCL-

1684, and also by the non-selective inhibitor of cyclooxygenase, indomethacin indicating the exclusive involvement of NO (Fig. 1B). To evaluate the role of SGLT1 and 2 in the BCE-induced NO-mediated relaxation, rings with endothelium were incubated with the dual SGLT1/2 inhibitor LX4211 or a selective SGLT2 inhibitor (canagliflozin, dapagliflozin, empagliflozin) before the addition of BCE (Fig. 1C-F). Both LX4211 and canagliflozin significantly inhibited BCE-induced relaxation whereas the more selective SGLT2 inhibitors dapagliflozin and, in particular, empagliflozin were without effect (Fig. 1C-F), suggesting the involvement, at least to some extent, of SGLT1.

In addition, none of the SGLT inhibitors affected the endothelium-dependent relaxation to bradykinin and the natural non-sugar conjugated epigallocatechin gallate, and to the NO donor sodium nitroprusside (supplementary Fig. 1).

**Expression level of SGLT1 and 2 proteins in young ECs**

Previous studies have indicated that SGLT1 is expressed predominantly in the kidney and intestine, and SGLT2 mainly in the kidney [20] [21] [22]. Although native and cultured ECs have been shown to express SGLT1 mRNA, SGLT2 mRNA levels were undetectable [23]. The present findings using immunofluorescence staining of ECs at P1 revealed both SGLT1 and for SGLT2 fluorescence signals as assessed by confocal microscopy (Fig. 2A). To obtain further evidence, the protein expression level of SGLT1 and 2 was assessed in ECs lysates using Western blot analysis. A protein of about 65 kDa was detected by SGLT1 labeling in lysates of ECs and also in the positive control protein lysates of porcine kidney and intestine (Fig. 2B). Similarly, a protein of about 70 kDa was detected by SGLT2 labeling in lysates of ECs and kidney and, to a lower level, in intestine (Fig. 2B).

**Role of SGLTs in the BCE- and C3G-induced activation of the eNOS signaling**

**pathway in young ECs**

Next, the role of SGLTs in the BCE-induced activation of the eNOS pathway was characterized in cultured ECs at P1. BCE (100 µg/ml) caused a time-dependent phosphorylation of the Akt activator site Ser473 and the eNOS activator site Ser1177 starting within 2 min and reaching a plateau level at 5 min and, thereafter, the signal remained elevated for at least up to 30 min (Fig. 3A,B). To determine the role of SGLTs, ECs were incubated with an SGLT inhibitor prior to the addition of BCE for 15 min and the subsequent assessment of the phosphorylation level of eNOS at Ser1177. The dual SGLT1/2 inhibitor LX4211 and the selective SGLT2 inhibitors canagliflozin and dapagliflozin abolished the stimulatory effect of BCE whereas the highly selective SGLT2 inhibitor empagliflozin was without effect, suggesting a major role of SGLT1 (Fig. 4). Thereafter, experiments have been performed to determine BC anthocyanins capable of stimulating the endothelial eNOS. Exposure of ECs at P1 to the four major BC anthocyanins has indicated that the glucose-conjugated anthocyanin C3G increased the eNOS phosphorylation level at Ser1177 whereas C3R, D3G and D3R were inactive (Fig. 5). The stimulatory effect of C3G tested at 100 µM was significantly lower than that induced by the BCE (100 µg/ml) on eNOS phosphorylation (Fig. 5A). In addition, LX4211 markedly reduced the stimulatory effect of C3G and that of the BCE on the eNOS activator site Ser1177 (Fig. 5). Thus, these findings indicate that glucose-conjugated cyanidin is a major active anthocyanin contributing to the BCE-induced activation of eNOS most likely subsequent to its uptake via SGLT1.

**LX4211 but not empagliflozin prevents uptake of BCE and glucose-conjugated BC anthocyanins into young ECs**

Next, experiments were performed to evaluate the role of SGLT1 and 2 in the uptake of BC anthocyanins into ECs using Naturstoff reagent A, a common fluorescent dye used

to detect flavonoids [24]. Exposure of ECs at P1 to BCE was associated with a pronounced increase in the Naturstoff reagent A fluorescence signal that was inhibited in a concentration-dependent manner by D-glucose and not by mannitol (an osmotic control) demonstrating the involvement of a glucose-dependent mechanism (Fig. 6A). LX4211 significantly inhibited the BCE uptake by about 20% whereas empagliflozin was without effect (Fig. 6B). The study evaluating the uptake of the four major BC anthocyanins has indicated that a significant increased fluorescence signal in ECs in response to C3G and also, to some extent, D3G, but not to the rutinoside-conjugated anthocyanins C3R and D3R (Fig. 6C-D). Moreover, the uptake of both C3G and D3G was abolished by LX4211 (Fig. 6D). Altogether, these findings suggest the involvement predominantly of SGLT1 in the uptake of BC glucose-conjugated anthocyanin into young healthy ECs.

**LX4211 and empagliflozin inhibit BCE anthocyanin uptake and activation of eNOS in senescent ECs**

Previous studies have shown that high glucose-induced oxidative stress up-regulates the expression level of SGLT1 and 2 in proximal tubule cells [25]. Since oxidative stress is a key inducer of endothelial senescence promoting endothelial dysfunction, experiments have determined the expression level and the role of SGLT1 and SGLT2 in the uptake of anthocyanins into senescent ECs and the subsequent activation of eNOS. Immunofluorescence labeling indicated an increased SGLT1 and SGLT2 fluorescence signal in senescent ECs at P3 compared to that in ECs at P1 (Fig. 7A). Similarly, Western blot analysis indicated an increased protein expression level of both SGLT1 and SGLT2 in ECs at P3 compared to ECs at P1 (Fig. 7B,C). Exposure of ECs at P3 was associated with a significantly enhanced uptake of BCE anthocyanins compared to that observed in ECs at P1 (Fig. 8A,B). The BCE uptake was prevented by

LX4211 to a greater in ECs at P3 than P1 (inhibitory effect was 20% in ECs at P1 and 86% in ECs at P3, Fig. 8A). Although the highly selective SGLT2 inhibitor empagliflozin did not affect BC anthocyanins uptake in ECs at P1, is significantly inhibited that in ECs at P3 by 30% (Fig. 8B). Consistent with an increased uptake, BCE induced an increased phosphorylation level of eNOS at Ser1177 in ECs at P3 than in those at P1, and this effect was inhibited by LX4211 (Fig. 8C).

## Discussion

The major findings of the present study indicate that BC anthocyanins are potent activators of the endothelial NO pathway in both native and cultured ECs. Moreover, they show that the BC stimulatory effect involves predominantly C3G, and that it is induced following BC anthocyanin uptake via SGLT1 in young healthy ECs, and both SGLT1 and SGLT2 in senescent ECs. Thus, the enhanced entry of BC anthocyanins in senescent ECs characterized by an endothelial dysfunction with a reduced formation of NO and an up-regulation of pro-atherothrombotic responses, suggests that BC anthocyanins might be of interest to target senescence-prone atherosusceptible sites to restore vascular protection.

Vascular reactivity studies using porcine coronary artery rings have indicated that BC anthocyanins are natural products with strong vasorelaxing properties. The characterization of the BCE-induced relaxation indicated that it is entirely dependent on the presence of a functional endothelium and exclusively mediated by the endothelial formation of NO since the eNOS inhibitor $N^G$-nitro-L-arginine abolished the relaxation whereas inhibitors of the endothelium-dependent hyperpolarization pathway and of the cyclooxygenase pathway were inactive. Besides BC anthocyanins, endothelium-dependent NO-mediated relaxations have also been observed in artery rings in response to several other rich sources of anthocyanins including blueberry, aronia and grape

products [26-28]. The stimulatory effect of anthocyanins on ECs is mediated via activation of the PI3-kinase/Akt pathway triggering the phosphorylation of the eNOS activator sites at Ser1177 to enhance the endothelial formation of NO [26]. These natural products are well-known to contain high levels of anthocyanins conjugated to different sugars such as glycoside, rutinoside and arabinoside [7]. Indeed, the phytochemical analysis of the BC extract has indicated the presence of 4 major anthocyanins including glycosides and rutinosides of cyanidin and delphinidin with C3G and D3G accounting for more than 15%. Despite the fact that anthocyanins are potent activators of the endothelial formation of NO, the underlying mechanisms remain largely unknown such as the role of the anthocyanin structure, the type of sugar, as well as the mechanisms contributing to cellular uptake and the intracellular targets.

Recent findings by Jin *et al*,. have indicated that D3G is able to protect human umbilical vein ECs against oxidized LDL-induced mitochondrial dysfunction and that this effect is associated with an intracellular accumulation of the anthocyanin [17]. Moreover, the characterization of the D3G uptake has shown that it is dependent on the presence of extracellular $Na^+$, inhibited by increasing concentrations of glucose and the non-selective SGLT inhibitor phlorizin, and also following knocked-down of SGLT1, suggesting that SGLT1 mediates D3G entry [17]. Therefore, we have performed investigations to determine the role of SGLT1 and 2, the two major SGLTs, in the BC anthocyanins-induced activation of the endothelial NO pathway using isolated coronary artery rings with endothelium and cultured ECs.

The findings indicate that the dual SGLT1/2 inhibitor LX4211 and the selective SGLT2 inhibitor canagliflozin but not dapagliflozin and empagliflozin, inhibited, to some extent, BCE-induced NO-mediated relaxation. The fact that none of the SGLTs inhibitors affected endothelium-dependent NO-mediated relaxations to bradykinin and to the non-sugar conjugated natural product epigallocatechin gallate [29, 30], and to the NO donor

sodium nitroprusside, rules out an action of the SGLT inhibitors on the signal transduction pathway leading to eNOS activation and also on the soluble guanylyl cyclase/cyclic GMP relaxing pathway in the vascular smooth muscle. The ability of canagliflozin to inhibit BCE-induced relaxation is most likely explained by its reduced selectivity for SGLT2 compared to SGLT1. Indeed, amongst the three SGLT2 inhibitors, empagliflozin is known to have the highest selectivity for SGLT2 compared to SGLT1 (>2500 fold), followed by dapagliflozin (>1200 fold) and canagliflozin (>250 fold) [31]. Altogether, these findings suggest a role for SGLT1 in the stimulatory effect of BCE on the endothelial NO pathway in native ECs.

In order to better determine the mechanisms involved in the anthocyanin-induced activation of the eNOS pathway, investigations were performed with cultured young coronary artery ECs at P1. These studies have indicated that the BCE-induced phosphorylation of eNOS at Ser1177 is abolished by LX4211, canagliflozin and dapagliflozin but not by the highly selective SGLT2 inhibitor empagliflozin suggesting a preferential role of SGLT1. Moreover, the evaluation of the four major BC anthocyanins (C3G, C3R, D3G, D3R) has revealed that only C3G was able to cause eNOS phosphorylation at Ser1177 showing the importance of the anthocyanin structure and also of the type of sugar moiety (glucoside vs rutinoside). Since LX4211 abolished the stimulatory effect of C3G on eNOS phosphorylation at Ser1177, SGLT1 most likely contributes to its entry into ECs. Moreover, Western blot analysis and immunofluorescence staining revealed the presence of SGLT1 and SGLT2 in ECs at P1. The fact that a high concentration of C3G (100 µM) was required to detect the activation of the eNOS pathway in ECs is best explained by the poor stability of anthocyanins in cell culture medium. Indeed, only 0.4% of cyanidin was recovered after a 2-h incubation period of human Caco-2 cell enterocytes [32].

Next, the uptake of BC anthocyanins into ECs was followed using Naturstoff reagent

A, a common fluorescent dye used to detect flavonoids. Indeed, depending on the substitution with hydroxyl groups on the B and C ring, flavonoids and Naturstoff reagent A form a chelate with characteristical fluorescence [33-35]. The uptake of BCE anthocyanins, and of glucosides of cyanidin and delphindin was observed but not that of their rutinosides derivatives in ECs at P1. The BCE uptake was concentration-dependently inhibited by D-glucose, (a SGLTs substrate), and also, to some extent, by LX4211 but not empagliflozin. Moreover, LX4211 abolished the uptake of C3G and D3G. Altogether, these findings provide evidence that SGLT1 predominantly mediates the entry of glucoside-conjugated cyanidin and delphinidin whereas additional mechanisms contribute to that of the mixture of BC anthocyanins in young healthy EC at P1.

Both experimental and clinical studies have established that endothelial dysfunction associated with oxidative stress is a hallmark of major cardiovascular diseases including hypertension, hypercholesterolemia and diabetes [36]. Oxidative stress is thought to trigger premature endothelial aging characterized major changes in cell phenotype including the expression of regulators of the cell cycle such as the p53/p21 and p16 pathways, the down-regulation of the NO pathway, and the up-regulation of pro-atherothrombotic responses including the expression of adhesion molecules and tissue factor [37-39]. Moreover, signs of endothelial senescence appear initially at arterial branches and curvatures that are exposed to disturbed or turbulent flow and prone to atherogenesis [40, 41]. Since oxidative stress has been shown to upregulate the expression of SGLT1 and SGLT2 in proximal tubule cells [25], experiments have compared the protective effect of BC anthocyanins in young healthy ECs at P1 and replicative senescent ECs at P3 as shown previously [37-39]. These investigations have indicated that the uptake of BC anthocyanins is increased in senescent compared to healthy ECs, and that this effect leads to an increased activation of the endothelial NO pathway. Since LX4211 inhibited BC anthocyanins uptake to a greater extent in

senescent compared to young ECs, and that despite not affecting BC anthocyanins uptake in young ECs, empagliflozin significantly reduced the uptake in senescent ECs, SGLT1 appears to mediate predominantly anthocyanins uptake in healthy ECs whereas both SGLT1 and 2 contribute in senescent ECs. Moreover, an increased expression level of SGLT1 and 2 has been observed in senescent ECs by immunofluorescence staining and Western blot analysis, most likely accounting for the increased uptake of BC anthocyanins in senescent ECs.

Altogether, the present findings indicate that BC anthocyanins are potent activator of the endothelial NO pathway in native and cultured endothelial cells. Amongst BC anthocyanins, the glucoside derivatives of cyanidin and delphinidin appear to be the most active natural compounds. They further indicate that EC senescence is associated with an increased uptake of BC anthocyanins involving both SGLT1 and SGLT2 most likely subsequent to the up-regulation of their expression level, leading to an improved endothelial NO pathway. Thus, anthocyanins appear as interesting natural compounds to target an early event in the development of atherogenesis at atherosusceptible sites by preferentially accumulating in senescent ECs to restore the protective endothelial function.

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**References**

[1] WHO, 2018.

[2] Han, K.-H., Sekikawa, M., Shimada, K.-i., Hashimoto, M.*, et al.*, Anthocyanin-rich purple potato flake extract has antioxidant capacity and improves antioxidant potential in rats. *British journal of nutrition* 2006, *96*, 1125-1134.

[3] Mink, P. J., Scrafford, C. G., Barraj, L. M., Harnack, L.*, et al.*, Flavonoid intake and cardiovascular disease mortality: a prospective study in postmenopausal women–. *The American journal of clinical nutrition* 2007, *85*, 895-909.

[4] Renaud, S. d., de Lorgeril, M., Wine, alcohol, platelets, and the French paradox for coronary heart disease. *The Lancet* 1992, *339*, 1523-1526.

[5] Santhakumar, A. B., Kundur, A. R., Fanning, K., Netzel, M.*, et al.*, Consumption of anthocyanin-rich Queen Garnet plum juice reduces platelet activation related thrombogenesis in healthy volunteers. *Journal of Functional Foods* 2015, *12*, 11-22.

[6] Koeppen, B. H., Herrmann, K., Flavonoid glycosides and hydroxycinnamic acid esters of blackcurrants (Ribes nigrum). *Zeitschrift für Lebensmittel-Untersuchung und Forschung* 1977, *164*, 263-268.

[7] Slimestad, R., Solheim, H., Anthocyanins from black currants (Ribes nigrum L.). *Journal of Agricultural and Food Chemistry* 2002, *50*, 3228-3231.

[8] Auger, C., Kim, J.-H., Trinh, S., Chataigneau, T.*, et al.*, Fruit juice-induced endothelium-dependent relaxations in isolated porcine coronary arteries: evaluation of different fruit juices and purees and optimization of a red fruit juice blend. *Food & function* 2011, *2*, 245-250.

[9] Auger, C., Pollet, B., Arnold, C., Marx, C., Schini-Kerth, V. B., Great heterogeneity of commercial fruit juices to induce endothelium-dependent relaxations in isolated porcine coronary arteries: role of the phenolic content and composition. *J Med Food* 2015, *18*, 128-136.

[10] Xu, J.-W., Ikeda, K., Yamori, Y., Cyanidin-3-glucoside regulates phosphorylation of endothelial nitric oxide synthase. *FEBS letters* 2004, *574*, 176-180.

[11] Rashid, S., Idris-Khodja, N., Auger, C., Kevers, C.*, et al.*, Polyphenol-Rich Blackcurrant Juice Prevents Endothelial Dysfunction in the Mesenteric Artery of Cirrhotic Rats with Portal Hypertension: Role of Oxidative Stress and the Angiotensin System. *Journal of medicinal food* 2018, *21*, 390-399.

[12] Serraino, I., Dugo, L., Dugo, P., Mondello, L.*, et al.*, Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-induced endothelial

dysfunction and vascular failure. *Life sciences* 2003, *73*, 1097-1114.
[13] Castaneda-Ovando, A., de Lourdes Pacheco-Hernández, M., Páez-Hernández, M. E., Rodríguez, J. A., Galán-Vidal, C. A., Chemical studies of anthocyanins: A review. *Food chemistry* 2009, *113*, 859-871.
[14] Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K., Someya, K., Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3, 5-diglucoside, into rats and humans. *Journal of agricultural and food chemistry* 1999, *47*, 1083-1091.
[15] Talavera, S., Felgines, C., Texier, O., Besson, C.*, et al.*, Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *The Journal of nutrition* 2003, *133*, 4178-4182.
[16] Passamonti, S., Vrhovsek, U., Mattivi, F., The interaction of anthocyanins with bilitranslocase. *Biochemical and biophysical research communications* 2002, *296*, 631-636.
[17] Jin, X., Yi, L., Chen, M.-l., Chen, C.-y.*, et al.*, Delphinidin-3-glucoside protects against oxidized low-density lipoprotein-induced mitochondrial dysfunction in vascular endothelial cells via the sodium-dependent glucose transporter SGLT1. *PloS one* 2013, *8*, e68617.
[18] Hidalgo, J., Teuber, S., Morera, F. J., Ojeda, C.*, et al.*, Delphinidin Reduces Glucose Uptake in Mice Jejunal Tissue and Human Intestinal Cells Lines through FFA1/GPR40. *International journal of molecular sciences* 2017, *18*, 750.
[19] Tabart, J., Kevers, C., Pincemail, J., Defraigne, J.-O., Dommes, J., Comparative antioxidant capacities of phenolic compounds measured by various tests. *Food Chemistry* 2009, *113*, 1226-1233.
[20] Kashiwagi, Y., Nagoshi, T., Yoshino, T., Tanaka, T. D.*, et al.*, Expression of SGLT1 in human hearts and impairment of cardiac glucose uptake by phlorizin during ischemia-reperfusion injury in mice. *PLoS One* 2015, *10*, e0130605.
[21] Zambrowicz, B., Freiman, J., Brown, P., Frazier, K.*, et al.*, LX4211, a dual SGLT1/SGLT2 inhibitor, improved glycemic control in patients with type 2 diabetes in a randomized, placebo-controlled trial. *Clinical Pharmacology & Therapeutics* 2012, *92*, 158-169.
[22] Gorboulev, V., Schürmann, A., Vallon, V., Kipp, H.*, et al.*, Na+-D-glucose cotransporter SGLT1 is pivotal for intestinal glucose absorption and glucose-dependent incretin secretion. *Diabetes* 2012, *61*, 187-196.
[23] Han, Y., Cho, Y.-E., Ayon, R., Guo, R.*, et al.*, SGLT inhibitors attenuate NO-

dependent vascular relaxation in the pulmonary artery but not in the coronary artery. *American Journal of Physiology-Lung Cellular and Molecular Physiology* 2015, *309*, L1027-L1036.

[24] Tattini, M., Gravano, E., Pinelli, P., Mulinacci, N., Romani, A., Flavonoids accumulate in leaves and glandular trichomes of Phillyrea latifolia exposed to excess solar radiation. *The New Phytologist* 2000, *148*, 69-77.

[25] Han, H. J., Lee, Y. J., Park, S. H., Lee, J. H., Taub, M., High glucose-induced oxidative stress inhibits Na+/glucose cotransporter activity in renal proximal tubule cells. *American Journal of Physiology-Renal Physiology* 2005, *288*, F988-F996.

[26] Anselm, E., Chataigneau, M., Ndiaye, M., Chataigneau, T., Schini-Kerth, V. B., Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src-and Akt-dependent activation of eNOS. *Cardiovascular research* 2007, *73*, 404-413.

[27] Kalea, A. Z., Clark, K., Schuschke, D. A., Kristo, A. S., Klimis-Zacas, D. J., Dietary enrichment with wild blueberries (Vaccinium angustifolium) affects the vascular reactivity in the aorta of young spontaneously hypertensive rats. *The Journal of nutritional biochemistry* 2010, *21*, 14-22.

[28] Kim, J. H., Auger, C., Kurita, I., Anselm, E.*, et al.*, Aronia melanocarpa juice, a rich source of polyphenols, induces endothelium-dependent relaxations in porcine coronary arteries via the redox-sensitive activation of endothelial nitric oxide synthase. *Nitric Oxide* 2013, *35*, 54-64.

[29] Auger, C., Kim, J.-H., Chabert, P., Chaabi, M.*, et al.*, The EGCg-induced redox-sensitive activation of endothelial nitric oxide synthase and relaxation are critically dependent on hydroxyl moieties. *Biochemical and biophysical research communications* 2010, *393*, 162-167.

[30] Schini-Kerth, V. B., Auger, C., Kim, J.-H., Étienne-Selloum, N., Chataigneau, T., Nutritional improvement of the endothelial control of vascular tone by polyphenols: role of NO and EDHF. *Pflügers Archiv-European Journal of Physiology* 2010, *459*, 853-862.

[31] Grempler, R., Thomas, L., Eckhardt, M., Himmelsbach, F.*, et al.*, Empagliflozin, a novel selective sodium glucose cotransporter-2 (SGLT-2) inhibitor: characterisation and comparison with other SGLT-2 inhibitors. *Diabetes, Obesity and Metabolism* 2012, *14*, 83-90.

[32] Kay, C. D., Kroon, P. A., Cassidy, A., The bioactivity of dietary anthocyanins is likely to be mediated by their degradation products. *Molecular nutrition & food research* 2009, *53*, S92-S101.

[33] Brasseur, T., Angento, L., Reagents for densitomeric determination of flavonoids. *J Chromatogr* 1986, *351*, 351-355.
[34] Ernst, I., Wagner, A., Lipinski, S., Skrbek, S*., et al.*, Cellular uptake, stability, visualization by 'Naturstoff reagent A', and multidrug resistance protein 1 gene-regulatory activity of cyanidin in human keratinocytes. *Pharmacological research* 2010, *61*, 253-258.
[35] Neu, R., A new reagent for differentiating and determining flavones on paper chromatograms. *Naturwissenschaften* 1956, *43*, 10.1007.
[36] Thuesen, A. D., Andersen, K., Lyngsø, K. S., Burton, M*., et al.*, Deletion of T-type calcium channels Ca v 3.1 or Ca v 3.2 attenuates endothelial dysfunction in aging mice. *Pflügers Archiv-European Journal of Physiology* 2018, *470*, 355-365.
[37] Abbas, M., Jesel, L., Auger, C., Amoura, L*., et al.*, Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging and ThrombogenicityClinical Perspective: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways. *Circulation* 2017, *135*, 280-296.
[38] Khemais-Benkhiat, S., Idris-Khodja, N., Ribeiro, T. P., Silva, G. C*., et al.*, The redox-sensitive induction of the local angiotensin system promotes both premature and replicative endothelial senescence: preventive effect of a standardized Crataegus extract. *Journals of Gerontology Series A: Biomedical Sciences and Medical Sciences* 2015, *71*, 1581-1590.
[39] Silva, G. C., Abbas, M., Khemais-Benkhiat, S., Burban, M*., et al.*, Replicative senescence promotes prothrombotic responses in endothelial cells: Role of NADPH oxidase-and cyclooxygenase-derived oxidative stress. *Experimental gerontology* 2017, *93*, 7-15.
[40] Suo, R., Zhao, Z.-Z., Tang, Z.-H., Ren, Z*., et al.*, Hydrogen sulfide prevents H2O2-induced senescence in human umbilical vein endothelial cells through SIRT1 activation. *Molecular medicine reports* 2013, *7*, 1865-1870.
[41] Warboys, C. M., de Luca, A., Amini, N., Luong, L*., et al.*, Disturbed flow promotes endothelial senescence via a p53-dependent pathway. *Arteriosclerosis, thrombosis, and vascular biology* 2014, *34*, 985-995.

**Figure legends**

**Figure 1.** Effect of SGLT1 and 2 inhibitors on BCE-induced NO-mediated relaxation in porcine coronary artery rings. Porcine coronary artery rings with or without endothelium were contracted with U46619 before the addition of increasing concentrations of BCE (A to F). B) Rings with endothelium were incubated with either $N^G$-nitro-L-arginine (L-NA, 30 μM, inhibitor of endothelial NO synthase), indomethacin (10 μM, COX inhibitor), Tram-34 (1 μM, small conductance $Ca^{2+}$-activated $K^+$ channel blocker) plus UCL-1684 (1 μM, intermediate conductance $Ca^{2+}$-activated $K^+$ channel blocker) or the combination of L-NA, indomethacin, Tram-34 and UCL-1684 for 30 min before contraction with U46619 and subsequent relaxation with BCE. (C to F) Rings with endothelium were incubated with an SGLT inhibitor after contraction with U46619 and the subsequent relaxation to BCE was induced. Results are expressed as mean±SEM of 7 to 9 different experiments. *$P$<0.05 vs respect control.

**Figure 2.** Expression of SGLT1 and SGLT2 in porcine coronary artery endothelial cells at passage 1. SGLT1 and 2 immunofluorescence staining in ECs was observed by confocal microscopy (A), and Western blot analysis (B). A) Representative photo showing immunofluorescence staining using either a SGLT1 or a 2 antibody in ECs at passage 1. DAPI staining (Blue) was used for the detection of nuclei in ECs. B) Representative Western blot showing SGLT1 and 2 in ECs. Proteins from porcine kidney and intestine were used as positive controls for SGLT1 and 2, respectively. Similar findings were observed in 2 additional experiments.

**Figure 3.** BCE induces activation of the Akt-eNOS signaling pathway in a time-dependent manner. Porcine coronary artery ECs were incubated with BCE (100 μg/ml) for increasing times before analysis of the level of pAkt (Ser473) and peNOS (Ser1177)

by Western blot analysis. Results are expressed as mean±SEM values of 3-4 different experiments. *P**<0.05 vs BCE.

**Figure 4.** Effect of SGLT1/2 inhibitors on the BCE-induced phophorylation of eNOS at Ser1177 in ECs. ECs were incubated with either a dual SGLT1/2 inhibitor LX4211 ($10^{-7}$ M) or a selective SGLT2 inhibitor (EMPA; empagliflozin, DAPA; dapagliflozin, CANA; canagliflozin, $10^{-7}$ M) for 5 min before addition of BCE (100 µg/ml) for 15 min. The phosphorylation level of the eNOS activation site at Ser1177 (peNOS) was assessed by Western blot analysis. Results are expressed as mean±SEM of 3-4 different experiments. *$P$<0.05 vs BCE.

**Figure 5.** LX4211 prevents activation of eNOS induced by the glucose-conjugated anthocyanins, cyanidin-3-*O*-glucoside. ECs were incubated with the dual SGLT1/2 inhibitor, for 5 min, before the addition of a BCE anthocyanin A) cyanidin-3-*O*-glucoside, B) cyanidin-3-*O*-rutinoside, C) delphinidin-3-*O*-glucoside, and D) delphinidin-3-*O*-rutinoside for 100 µM, or BCE (100 µg/ml) for 15 min. Thereafter, the phosphorylation level of eNOS at Ser1177 was determined by Western blot analysis. Results are expressed as mean±SEM by 3 to 4 different experiments. *$P$<0.05 vs BCE-treated cells, $^{\#}P$<0.05 vs BCE or C3G, $^{\$}P$<0.05 vs non-treated cells.

**Figure 6.** LX4211 prevents BCE, C3G and D3G uptake in coronary artery ECs at passage 1. Anthocyanin uptake into ECs was determined using Naturstoff's reagent A and assessed by flow cytometry (A, B and D) and confocal microscopy (C). ECs were incubated either with D-glucose, mannitol or a SGLT inhibitor for 5 min before the addition of BCE (100 µg/ml) or a BC anthocyanin (100 µM) for 15 min. Original FACS flow chart of BCE uptake in ECs in the absence (Blue arrow) or presence (Red arrow) of LX4211

as assessed by flow cytometry (right panel). Results are expressed as mean±SEM by 3 to 4 different experiments. *$P$<0.05 vs control, $^{\#}P$<0.05 vs C3G or D3G.

**Figure 7.** Up-regulation of SGLT1 and 2 expressions in senescent coronary artery ECs. A) Representative photo showing immunofluorescence staining using either a SGLT1 or 2 antibody in ECs at passage 1 and 3. Similar observations were made in 2 additional experiments. B, C) Expression level of SGLT1 and 2 in ECs at P1 and P3 as assessed by Western blot analysis. Results are expressed as mean±SEM by 3-4 different experiments. *$P$<0.05 vs ECs at P1.

**Figure 8.** Up-regulation of BCE anthocyanins uptake and activation of eNOS in senescent ECs is inhibited by LX4211 and also by empagliflozin. ECs at P1 and at P3 were exposed to LX4211 (A, C) and empagliflozin (B, C) for 5 min before the addition of BCE for 15 min. Thereafter, the uptake of anthocyanins was assessed using Naturstoff's reagent A and protein expression by Western blot analysis. Results were expressed as mean±SEM of 3 different experiments. *$P$<0.05 vs BCE at P1, $^{\#}P$<0.05 vs respective BCE-treated cells.

**Supplementary file**

**Figure 1.** Relaxations induced by either sodium nitroprusside, bradykinin or a non-glucose conjugated flavonoid epigallocatechin gallate are not affected by LX4211 and selective SGLT2 inhibitors in coronary artery rings with endothelium. Porcine coronary artery rings were contracted with U46619 before the addition of increasing concentrations of a relaxing agent. (A) sodium nitroprusside (a NO donnor), (B) bradykinin (an endothelium-dependent vasodilator), and (C) epigallocatechin gallate (a non-glucose conjugated flavonoid inducing NO-mediated relaxation). Results are

expressed as mean±SEM of 7-9 different experiments.

**Table 1**. Analysis of the anthocyanins content of the blackcurrant extract.

**Figure 1.**

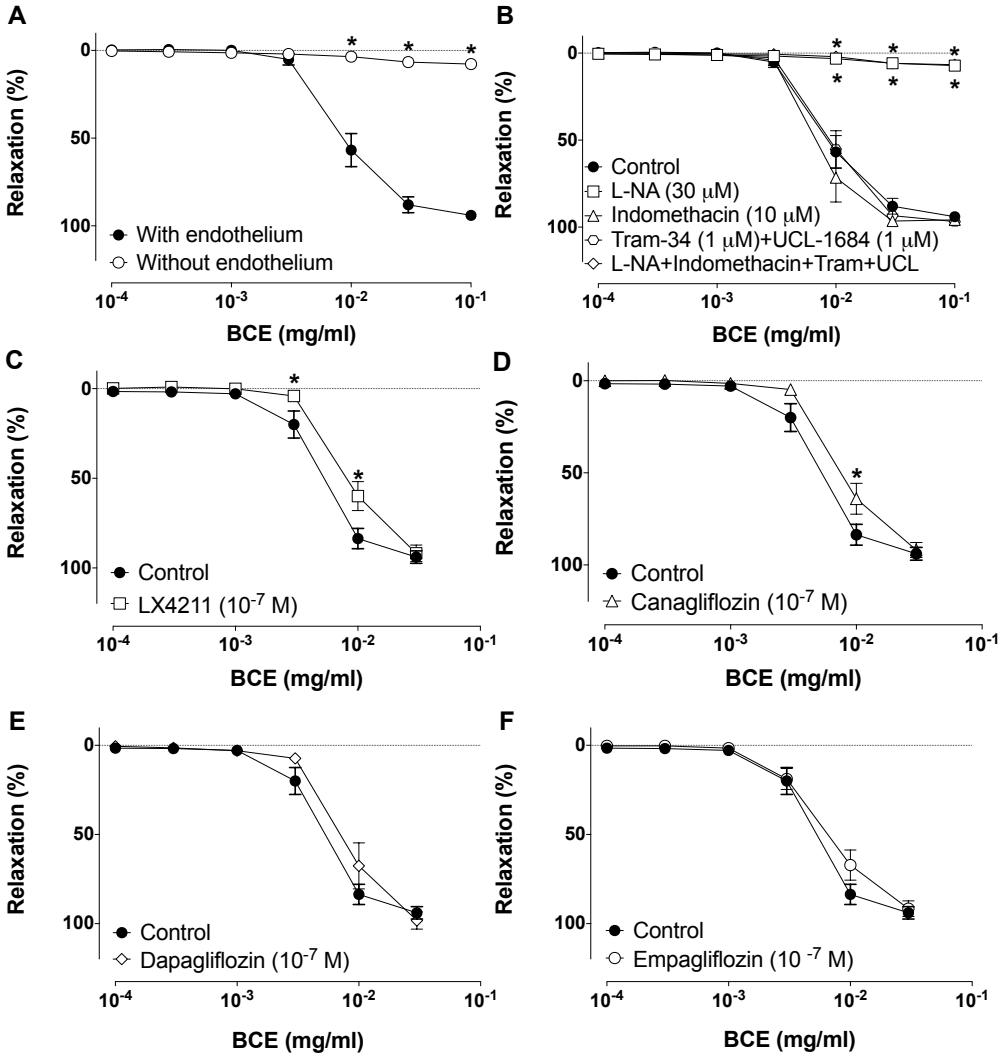

A
Relaxation (%)
0
50
100
With endothelium
Without endothelium
10-4
10-3
10-2
10-1
BCE (mg/ml)
B
Relaxation (%)
Control
L-NA (30 μM)
Indomethacin (10 μM)
Tram-34 (1 μM)+UCL-1684 (1 μM)
L-NA+Indomethacin+Tram+UCL
BCE (mg/ml)
C
Relaxation (%)
Control
LX4211 (10-7 M)
BCE (mg/ml)
D
Relaxation (%)
Control
Canagliflozin (10-7 M)
BCE (mg/ml)
E
Relaxation (%)
Control
Dapagliflozin (10-7 M)
BCE (mg/ml)
F
Relaxation (%)
Control
Empagliflozin (10 -7 M)
BCE (mg/ml)

**Figure 2.**

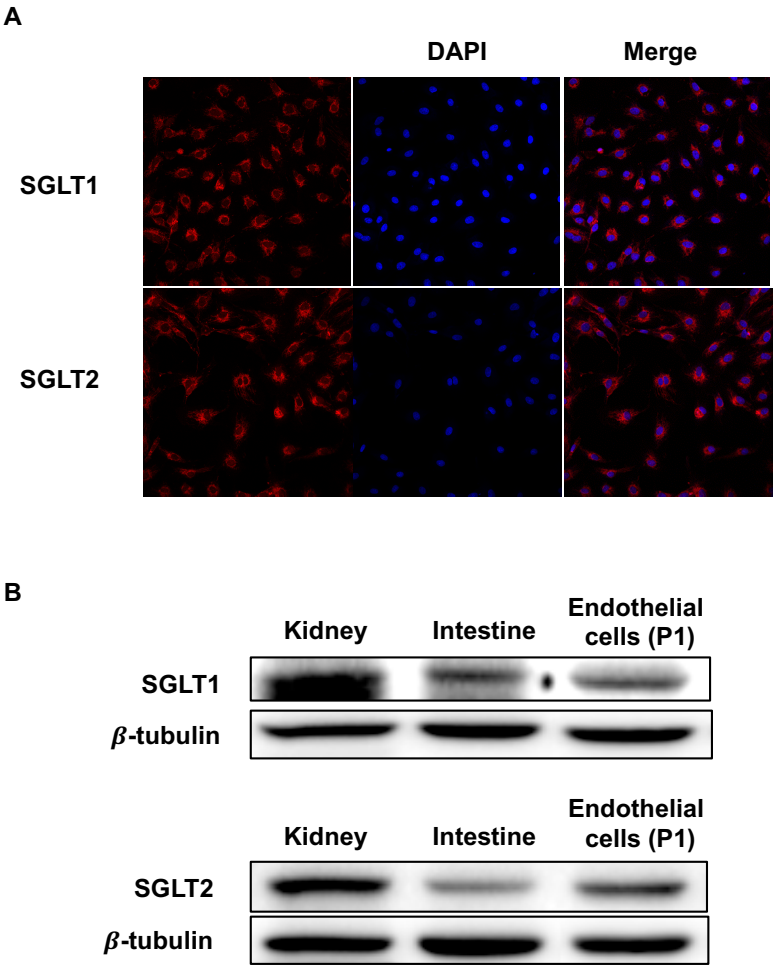

A
DAPI
Merge
SGLT1
SGLT2
B
Kidney
Intestine
Endothelial cells (P1)
SGLT1
β-tubulin
Kidney
Intestine
Endothelial cells (P1)
SGLT2
β-tubulin

**Figure 3.**

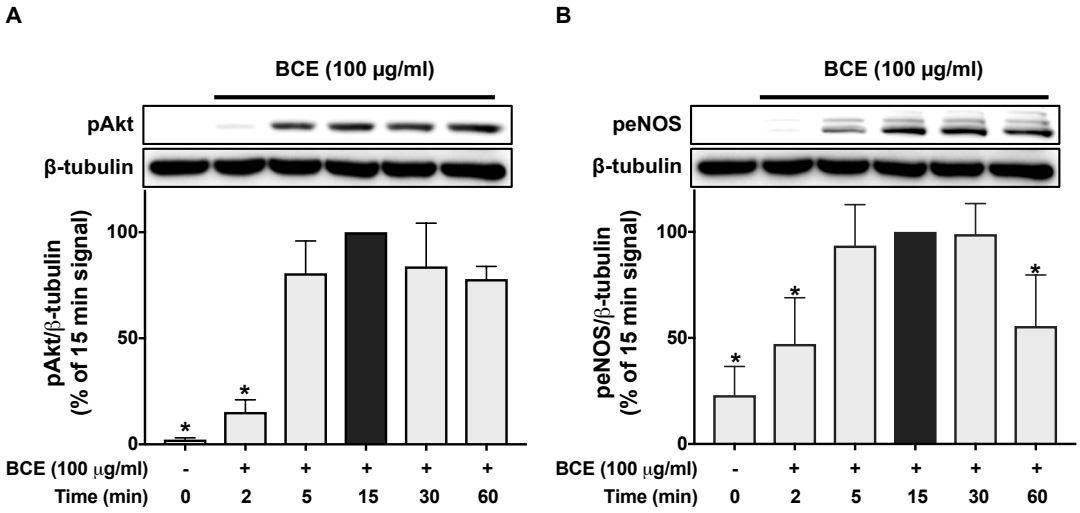

A
BCE (100 μg/ml)
pAkt
β-tubulin
pAkt/β-tubulin (% of 15 min signal)
100
50
0
*
*
BCE (100 μg/ml) - + + + + +
Time (min) 0 2 5 15 30 60
B
BCE (100 μg/ml)
peNOS
β-tubulin
peNOS/β-tubulin (% of 15 min signal)
100
50
0
*
*
*
BCE (100 μg/ml) - + + + + +
Time (min) 0 2 5 15 30 60

**Figure 4.**

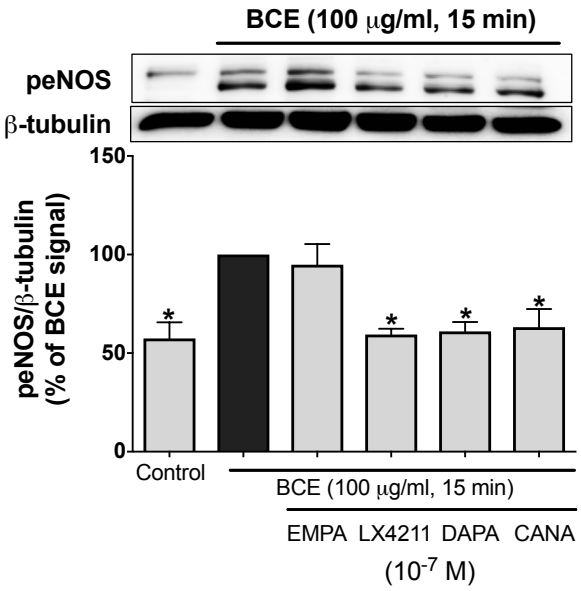

BCE (100 μg/ml, 15 min)
peNOS
β-tubulin
peNOS/β-tubulin (% of BCE signal)
150
100
50
0
*
*
*
*
Control
BCE (100 μg/ml, 15 min)
EMPA LX4211 DAPA CANA
(10-7 M)

**Figure 5.**

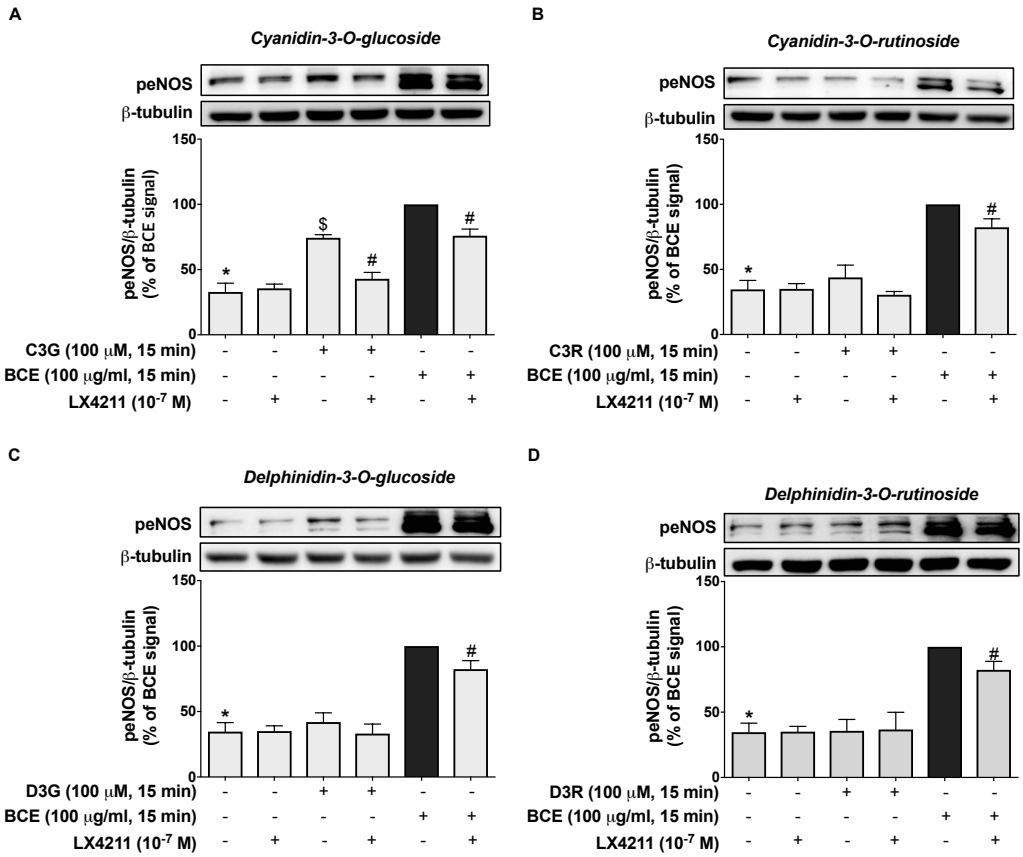

A
Cyanidin-3-O-glucoside
peNOS
β-tubulin
peNOS/β-tubulin (% of BCE signal)
150
100
50
0
*
$
#
#
C3G (100 μM, 15 min) - - + + - -
BCE (100 μg/ml, 15 min) - - - - + +
LX4211 (10-7 M) - + - + - +
B
Cyanidin-3-O-rutinoside
peNOS
β-tubulin
peNOS/β-tubulin (% of BCE signal)
150
100
50
0
*
#
C3R (100 μM, 15 min) - - + + - -
BCE (100 μg/ml, 15 min) - - - - + +
LX4211 (10-7 M) - + - + - +
C
Delphinidin-3-O-glucoside
peNOS
β-tubulin
peNOS/β-tubulin (% of BCE signal)
150
100
50
0
*
#
D3G (100 μM, 15 min) - - + + - -
BCE (100 μg/ml, 15 min) - - - - + +
LX4211 (10-7 M) - + - + - +
D
Delphinidin-3-O-rutinoside
peNOS
β-tubulin
peNOS/β-tubulin (% of BCE signal)
150
100
50
0
*
#
D3R (100 μM, 15 min) - - + + - -
BCE (100 μg/ml, 15 min) - - - - + +
LX4211 (10-7 M) - + - + - +

**Figure 6.**

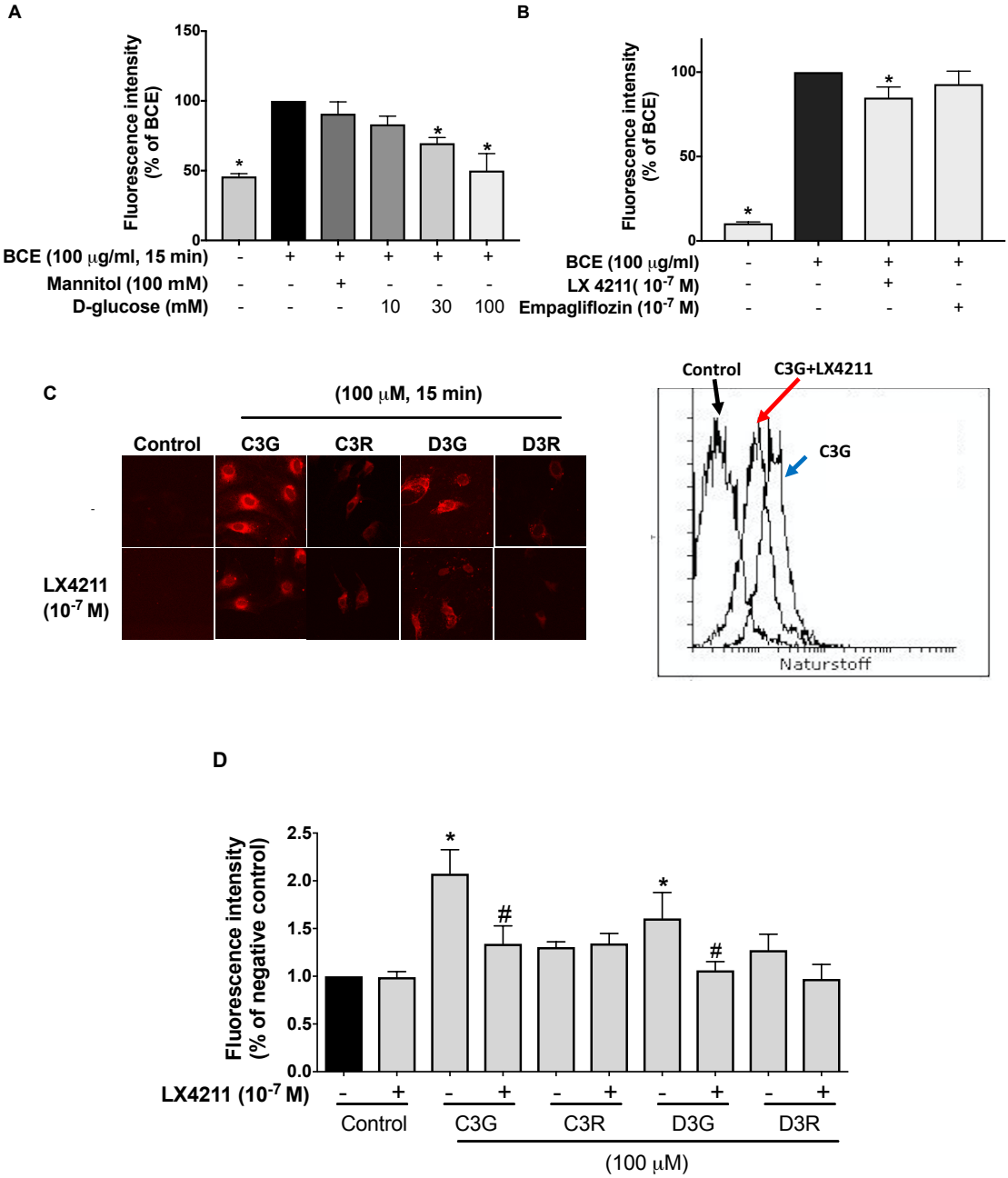

A
Fluorescence intensity (% of BCE)
150
100
50
0
BCE (100 μg/ml, 15 min) - + + + + +
Mannitol (100 mM) - - + - - -
D-glucose (mM) - - - 10 30 100
B
Fluorescence intensity (% of BCE)
100
50
0
BCE (100 μg/ml) - + + +
LX 4211( 10-7 M) - - + -
Empagliflozin (10-7 M) - - - +
C
(100 μM, 15 min)
Control
C3G
C3R
D3G
D3R
LX4211 (10-7 M)
Control
C3G+LX4211
C3G
Naturstoff
D
Fluorescence intensity (% of negative control)
2.5
2.0
1.5
1.0
0.5
0.0
LX4211 (10-7 M) - + - + - + - + - +
Control
C3G
C3R
D3G
D3R
(100 μM)

Figure 7.

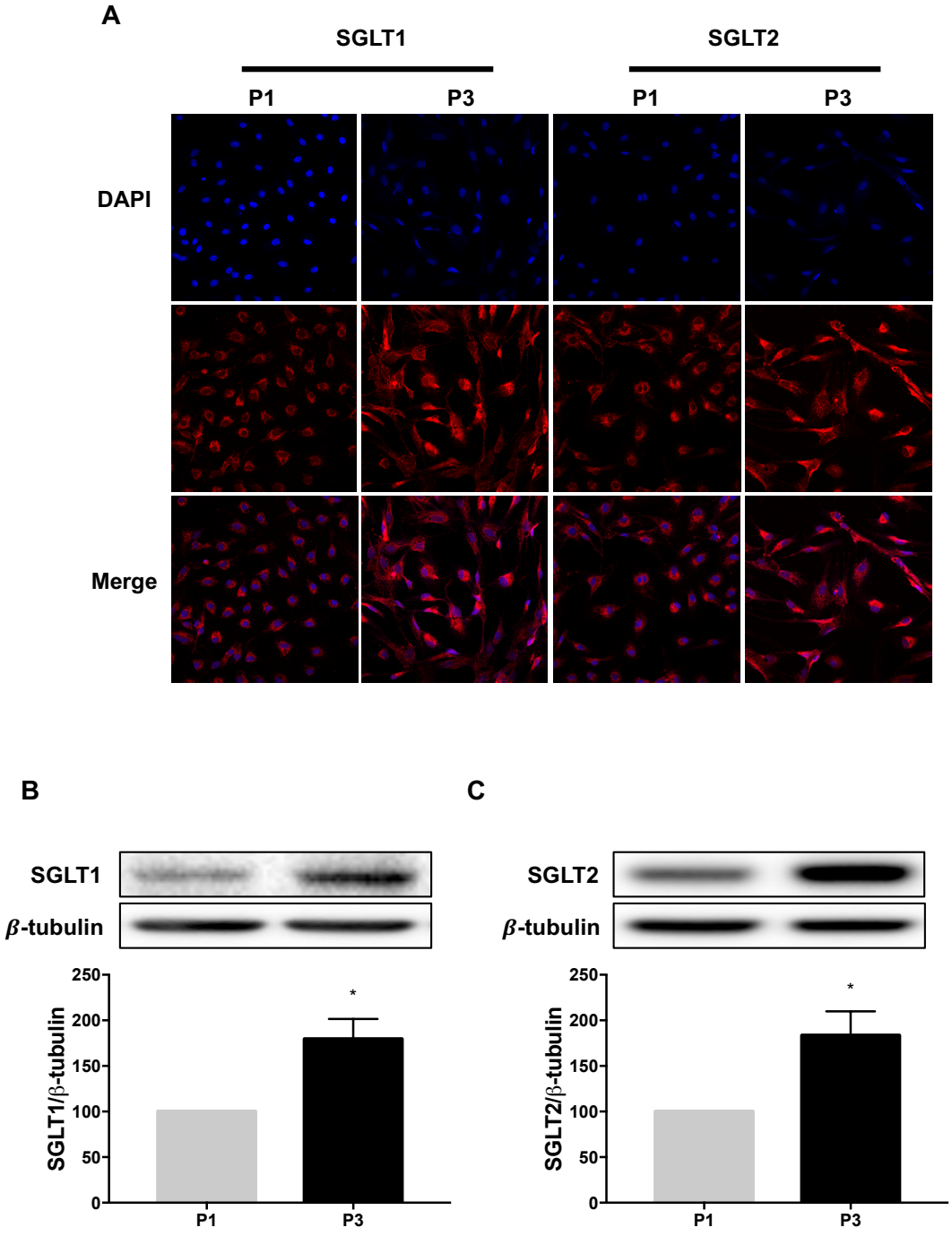

A
SGLT1
SGLT2
P1
P3
P1
P3
DAPI
Merge
B
SGLT1
β-tubulin
SGLT1/β-tubulin
250
200
150
100
50
0
*
P1
P3
C
SGLT2
β-tubulin
SGLT2/β-tubulin
250
200
150
100
50
0
*
P1
P3

**Figure 8.**

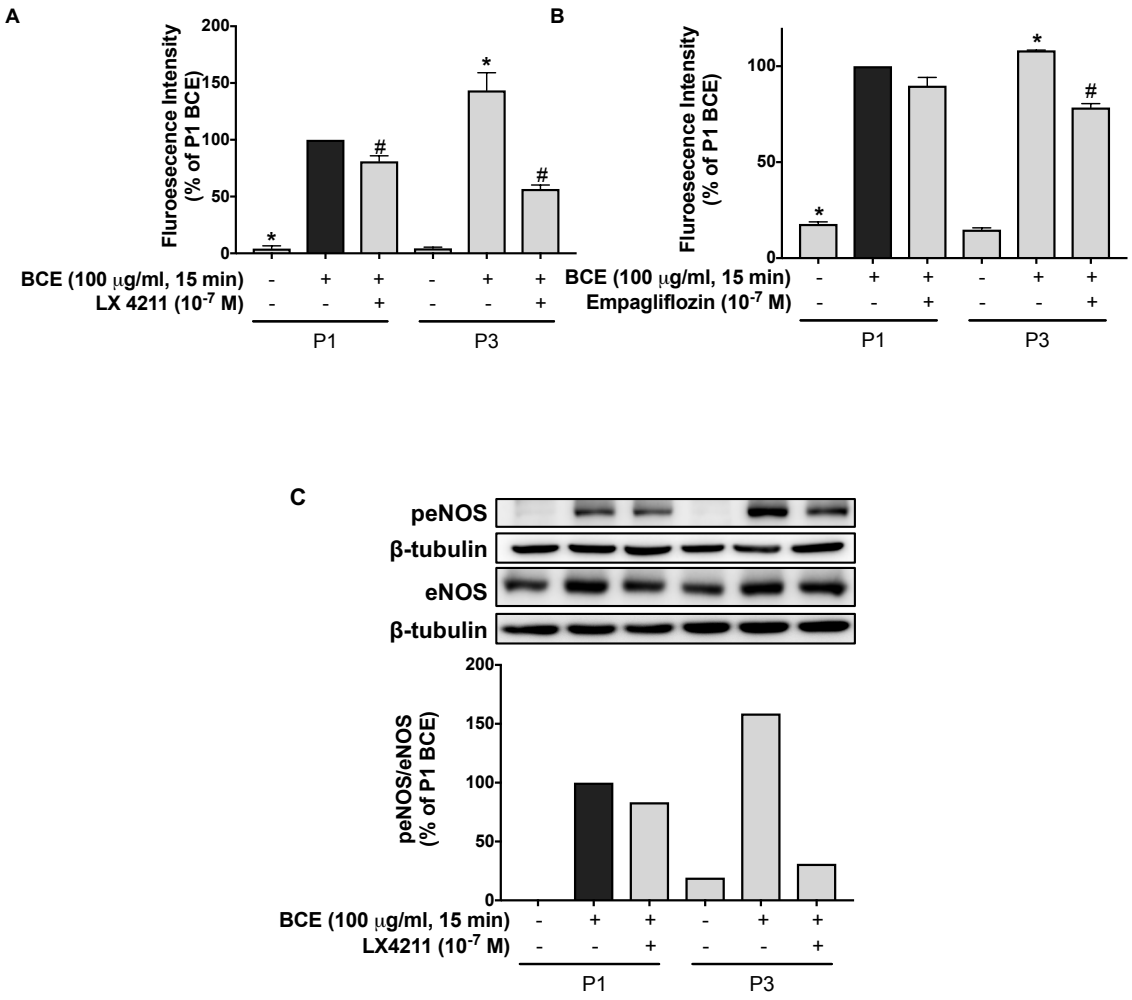
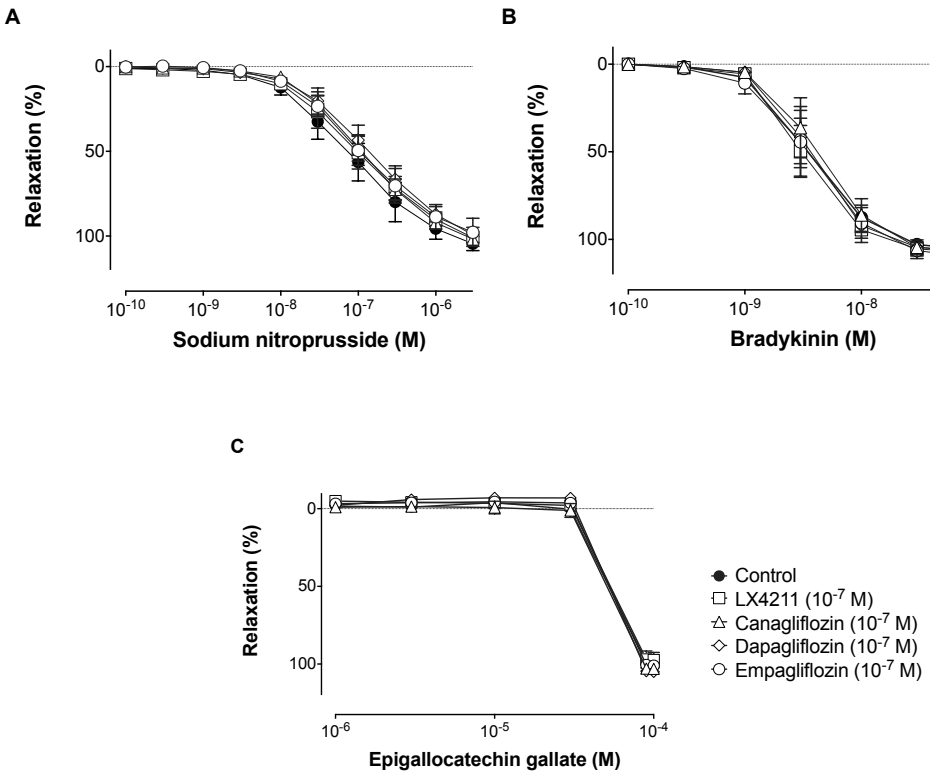

A
Fluroesecence Intensity (% of P1 BCE)
200
150
100
50
0
*
#
*
#
BCE (100 μg/ml, 15 min) - + + - + +
LX 4211 ($10^{-7}$ M) - - + - - +
P1
P3
B
Fluroesecence Intensity (% of P1 BCE)
100
50
0
*
*
#
BCE (100 μg/ml, 15 min) - + + - + +
Empagliflozin ($10^{-7}$ M) - - + - - +
P1
P3
C
peNOS
β-tubulin
eNOS
β-tubulin
peNOS/eNOS (% of P1 BCE)
200
150
100
50
0
BCE (100 μg/ml, 15 min) - + + - + +
LX4211 ($10^{-7}$ M) - - + - - +
P1
P3

**Table 1.**

| | Anthocyanin | (µg/ml) with S.D |
|---|---|---|
| 1 | Delphinidin-3-O-glucoside | 17.49 ± 0.69 |
| 2 | Delphinidin-3-O-rutinoside | 76.25 ± 1.82 |
| 3 | Cyanidin-3-O-glucoside | 6.11 ± 0.04 |
| 4 | Cyanidin-3-O-rutinoside | 38.81 ± 0.20 |
| 5 | Delphinidin | 0.10 ± 0.01 |
| 6 | Peonidin-3-O-glucoside | 0.05 ± 0.00 |
| 7 | Peonidin-3-O-rutinoside | 0.34 ± 0.01 |
| 8 | Peonidin | 7.22 ± 0.11 |
| 9 | Malvidin | 0.90 ± 0.01 |

**Supplementary**



# Article II

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# Potential mechanisms underlying cardiovascular protection by polyphenols: Role of the endothelium

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ABSTRACT

Epidemiological studies have indicated that regular intake of polyphenol-rich diets such as red wine and tea, are associated with a reduced risk of cardiovascular diseases. The beneficial effect of polyphenol-rich products has been attributable, at least in part, to their direct action on the endothelial function. Indeed, polyphenols from tea, grapes, cacao, berries, and plants have been shown to activate endothelial cells to increase the formation of potent vasoprotective factors including nitric oxide (NO) and to delay endothelial ageing. Moreover, intake of such polyphenol-rich products has been associated with the prevention and/or the improvement of an established endothelial dysfunction in several experimental models of cardiovascular diseases and in Humans with cardiovascular diseases. This review will discuss both experimental and clinical evidences indicating that polyphenols are able to promote endothelial and vascular health, as well as the underlying mechanisms.

## 1. Introduction

Polyphenols are an abundant and diverse group of secondary plant metabolites, which are present in a wide variety of dietary foods and traditional plant medicines [1]. Numerous epidemiological studies have indicated that diets rich in fruit and vegetables, and beverages such as red wine and tea, are associated with a reduced risk of cardiovascular diseases [2–8]. The study of the association between dietary factors and mortality from cardiovascular diseases has indicated that the diet-related cardiometabolic deaths are predominantly observed in a population characterized by a low intake of vegetables and fruits [9]. Furthermore in the US, a 26.5% reduction of cardiometabolic deaths per year has been observed between 2002 and 2012 and this effect has been related to an improvement of the intake of polyphenol-rich products such as nuts/seeds, whole grains, and fruits regardless of sex, age and race [9]. The cardiovascular protective effect of polyphenols has been attributable to their antioxidant activities and also to several additional effects such as an anti-inflammatory effect, prevention of the oxidation of low-density lipoproteins, inhibition of platelet aggregation and adhesion, and of smooth muscle cell migration and proliferation [10–13]. Alternatively, vascular protection may also be due to the direct action of polyphenols on the endothelial function. The aim of the present review is to summarize the current experimental and clinical evidence of the vascular protective effects of polyphenols on both healthy and pathological blood vessels, and to discuss the underlying molecular mechanisms.

## 2. Dietary polyphenols

Polyphenols naturally exist in plants and plant products, including fruits, vegetables, nuts, herbs, cocoa, and tea. There are currently over 8000 phenolic structures known, of which more than 4000 belong to the flavonoid class, and several hundred are present in edible vegetables. However, it is believed that the total content of polyphenols in plants is underestimated because many compounds present in fruit, vegetables and derived products have not yet been identified, and the polyphenol composition of most fruits and some grain varieties is not yet known. The structure of polyphenols is characterized by at least a

*Abbreviations:* Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; CDK, cyclin-dependent kinases; COX, cyclooxygenase; EDH, endothelium-derived hyperpolarization; EGCg, epigallocatechin gallate; eNOS, endothelial NO synthase; ET-1, endothelin-1; FMD, flow-mediated dilatation; ICAM-1, intercellular adhesion molecule-1; NO, nitric oxide; $PGI_2$, prostacyclin; ROS, reactive oxygen species; SOD, superoxide dismutase; $TXA_2$, thromboxane $A_2$; VCAM-1, vascular cell adhesion molecule-1

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simple phenol core bearing at least one hydroxyl group. More than 8000 polyphenolic structures are described and they are classified according to the arrangement of the carbon atoms and their substituents in two main classes: flavonoids and non-flavonoids.

The largest and best-studied polyphenols are flavonoids, which include several thousand compounds. Flavonoids are composed of a 15-carbon skeleton (C6-C3-C6) with two aromatic rings linked by a 3-carbon bridge forming an oxygenated heterocycle. They are subdivided into several subclasses including flavonols, flavanones, flavan-3-ols, flavones, anthocyanins, and isoflavones that can be further substituted by hydroxyl groups, sugars, organic acids, methyl groups or isopentyl units [7]. Flavonols such as quercetin and kaempferol and theirs derivatives are found in most kind of plants, except in algae and fungi. They are mainly present in form of *O*-glycosides in commonly consumed fruits and vegetables [14]. Flavanones are found as *O*-glycosides mainly in citrus fruits along with polymethoxylated flavones. Flavan-3-ols, also sometimes referred to as catechins, are the most structurally complex subclasses of flavonoids. Indeed, the simple (+)-catechin and its isomer (-)-epicatechin can undergo hydroxylation into gallocatechin, esterification with gallic acid, and/or polymerization into complex proanthocyanidins (condensed tannins). Moreover, they can undergo further transformation during food processing such as fermentation and drying of tea leaves during the production of black tea, leading to formation of theaflavins and thearubigins. They are abundant in tea, wine and grapes, cocoa and chocolates, beer and cider. Flavones occur predominantly in some herbs such as parsley or celery, whereas isoflavones are found in vegetables such as soybean and derived products including soy milk, miso and tofu [15]. Anthocyanidins are the pigments responsible for the pink/red to purple/blue colors of fruits, vegetables and flowers and are prominent in foods such as berries and red wine [16]. In plants they are always found conjugated to sugars and named anthocyanins, and they can be further conjugated to hydroxycinnamic acid and/or organic acids.

The major non-flavonoids include mainly phenolic acids, hydroxycinnamates, hydrolysable tannins, and stilbenes. The most abundant phenolic acid, gallic acid, can form large complexes such as non-sugar galloyl esters and ellagitannins that can be found in strawberries, raspberries, blackberries, mango, persimmon, grapes and wine, tea as well as walnuts and hazelnuts [7]. Hydroxycinnamates are C6-C3 structures including caffeic acid, ferulic acid, *p*-coumaric acid and sinapic acid. They are found mainly as ester of tartric acid or quinic acid under the term of chlorogenic acids in coffee. The stilbenes are primarily defined by *trans*-resveratrol, a 1,2-diarylethene structure found in red wine [17]. In addition, additional polyphenolic structures are present in certain types of foods and are combined into a separate class, termed “Other Polyphenols” [18]. These additional polyphenols include such compounds as tyrosol and curcuminoids. Polyphenols of the tyrosol subclass are present in olive oil and are thought to contribute to the health benefits seen with olive oil consumption.

While high amounts of specific polyphenols are observed in certain kinds of food, no food contains only a single class of polyphenols, and it is likely that the complementary and/or synergistic nature of these natural compounds in food contribute to their biological activity.

Dietary intake of polyphenols is highly variable. Due to the poor standardization of assays used for polyphenols separation and quantification and the great variability of polyphenols content within food [19], an accurate information regarding the polyphenol composition and their amount in food still remains challenging. Moreover, polyphenols are absorbed to various extent in the digestive tracts and can be highly metabolized depending on the chemical nature of the molecules [20]. Thus, the molecular mechanism reported with isolated compounds using *in vitro* or *ex vivo* models need to be evaluated very cautiously since metabolites/catabolites of the parent compound most likely reach the target tissue at a low micromolar concentration. In addition, the circulating concentration may not reflect the actual concentration in target tissues since an accumulation of polyphenols has been observed in specific cells [21,22].

Nevertheless, there is nowadays more and more evidence provided by *in vitro/ex vivo* studies, *in vivo* studies, and clinical trials that polyphenols can have a positive impact on health [20]. In particular during the last decade, there has been much interest in the potential health benefit of dietary plant polyphenols on the cardiovascular system, in part mediated by targeting blood vessels and, more specifically, the endothelium [2,12,23–25].

## 3. Role of endothelium on vascular function

Blood vessels are made of three layers including the intima consisting of a single cobblestone-like layer of endothelial cells, the media containing predominantly smooth muscle cells and elastic fibers, and the adventitia composed of fibroblasts, collagen fibers, and perivascular nerves. The endothelium is nowadays well-defined as a metabolically active organ that has a key role in the control of vascular structure and function mostly *via* the generation of several vasoactive factors that will determine a balance between vasodilator and vasoconstrictor responses depending on the local environment and needs [26]. Endothelium-dependent vasodilator responses involve predominantly factors such as NO, endothelium-derived hyperpolarization (EDH) and prostacyclin ($PGI_2$), while endothelium-dependent vasocontractile responses involve mostly endothelin-1 (ET-1), angiotensin II (Ang II), reactive oxygen species (ROS) and thromboxane $A_2$ ($TXA_2$) [26]. Amongst endothelium-derived factors, NO generated by endothelial NO synthase (eNOS) from L-arginine is considered as the most important vasoprotective factor in the body due to its ability to regulate a great variety of responses contributing to vascular homeostasis [26]. In particular, NO is able to prevent the adhesion of monocytes to the endothelial surface and inhibit the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) [27]. NO is also a potent inhibitor of platelet adhesion and aggregation. Moreover, NO prevents the proliferation of vascular smooth muscle cells and the expression of extracellular matrix molecules thereby contributing to limit vascular remodeling and the formation of vascular lesions. Altogether, endothelium-derived NO is an important vasodilator and anti-atherothrombotic factor that helps to prevent and/or to delay the initiation and development of cardiovascular diseases [28,29]. Despite a minor role in large arteries, EDH contributes to inhibit vascular tone in the coronary artery and coronary microcirculation and also in arterioles following the transmission of the hyperpolarization from the endothelium to the underlying vascular smooth muscle and, as a consequence, a reduced opening of voltage-operated calcium channels and, hence, calcium entry. In addition to NO and EDH, other endothelium-derived factors contribute to regulate vascular responses such as the potent vasoconstrictor and mitogen ET-1, the vasodilator $PGI_2$ generated by the arachidonic acid cascade via cyclooxygenase (COX) that inhibits vascular tone in some arteries and acts in synergy with NO to prevent platelet activation, fibrinolytic factors (tissue plasminogen activator and plasminogen activator inhibitor-1), factors that affect coagulation (tissue factor, heparin, and von Willebrand factor), and pro-inflammatory factors (*e.g.* adhesion molecules and pro-inflammatory cytokines) [30]. Thus, the endothelium has a key central role in the regulation of the balance between a vasodilating and anti-proliferative state and a vasoconstrictor pro-atherothrombotic state to maintain vascular homeostasis. Any disturbance to this subtle and precious balance will lead to the appearance of an endothelial dysfunction characterized by blunted endothelium-dependent vasodilatation often associated with oxidative stress, which will promote the development of cardiovascular diseases. Since endothelial dysfunction is observed before changes in the structure of the arterial wall it is thought to contribute to the initiation and development of cardiovascular diseases. Endothelial dysfunction is observed with increasing age, and also prematurely in the presence of smoking and major cardiovascular risk factors including hypertension,

hypercholesterolemia, diabetes, metabolic syndrome, obesity and heart failure.

Recent findings suggest that endothelial senescence characterized by cell cycle arrest and morphological and biochemical changes, contributes to promote endothelial dysfunction [31]. Endothelial senescence is a state characterized by the appearance of senescence-associated beta-galactosidase activity, a reduced expression of cyclin and cyclin-dependent kinases (CDK), and an increased expression of CDK inhibitors including the p53/p21 and the p16 pathways [32]. Since adenovirus-mediated transfection of p53 in the endothelium of aortic rings from healthy young rats leads to endothelial dysfunction and reduced bioavailability of NO, endothelial senescence appears to be an early event promoting endothelial dysfunction [33]. Premature endothelial senescence can be induced by several noxious atherothrombotic stimuli such as oxidative stress, Ang II, high glucose, and also by low levels of shear stress [34]. The induction of senescence can severely affect the ability of endothelial cells to contribute to the regulation of vascular homeostasis as indicated by the fact that senescent endothelial cells have a reduced eNOS-derived NO formation, oxidative stress and the induction of pro-atherothrombotic factors including VCAM-1, tissue factor and pro-inflammatory factors [35–37]. Moreover, endothelial senescence is observed prematurely *in vivo* at atheroprone arterial sites such as bifurcations and curvatures characterized by disturbed blood flow, reduced eNOS-derived NO formation and oxidative stress in young healthy rats, and also in the aorta of old and diabetic rats, and in the endothelium overlying atherosclerotic human plaques [38–40]. Thus, an attractive novel target to protect the vascular system is to prevent and/or delay the induction of endothelial senescence, thereby favoring a sustained endothelial eNOS-derived NO formation to protect the arterial wall and to promote healthy vascular ageing.

## 4. Protective effect of polyphenols on the endothelium

There is now increasing evidence that certain polyphenolic-rich products and authentic polyphenols are able to increase the protective effect of endothelial cells on the vascular function (Fig. 1).

Indeed, several polyphenol-rich plants and fruit extracts derived from grape, tea, berries and traditional medicinal plants have been shown to cause pronounced relaxations of pre-contracted arterial rings with an intact endothelium, whereas only small relaxations are observed in those without endothelium [41]. Since an inhibitor of either eNOS or soluble guanylyl cyclase markedly blunted the polyphenol-induced endothelium-dependent relaxation, the involvement of eNOS-derived NO has been suggested. Thereafter, investigations using electron paramagnetic resonance spectroscopy have provided direct proof that red wine polyphenols increased the formation of NO in the rat aorta and also in cultured endothelial cells [42,43]. Since the vasorelaxant effect of grape-derived polyphenols such as purple grape juice and red wine in porcine coronary artery rings is only partially inhibited by an eNOS inhibitor and that a further inhibition is obtained by the addition of inhibitors of the EDH pathway, it implies that, besides NO, EDH contributes also to the vasorelaxant effect of polyphenols. Although it is widely recognized that polyphenols have anti-oxidant effects, several lines of evidence indicate that the polyphenols-induced endothelium-dependent relaxations and formation of NO is rather dependent on their intracellular pro-oxidant response in healthy endothelial cells. Indeed, membrane permeant analogues of superoxide dismutase (SOD) and catalase markedly reduced the vasorelaxant effect of polyphenols whereas non-permeant anti-oxidants such as native SOD and catalase were inactive [44,45]. Moreover, grape-derived polyphenols increased in a time- and concentration-dependent manner the intracellular formation of ROS in cultured coronary artery endothelial cells and also in the endothelium but not in the underlying vascular smooth muscle of healthy coronary arteries [46,47]. The pro-oxidant response to polyphenols has been shown to cause the Src-mediated phosphatidylinositol-3-kinase (PI-3-kinase)-dependent phosphorylation of Akt, which, in turn, induces the activation of eNOS by phosphorylation of the activator site Ser1177 to increase the formation of NO in endothelial cells (Fig. 1) [43–45,48,49]. The polyphenol-induced intracellular oxidative stress in endothelial cells does not involve major enzymatic sources of ROS such as NADPH oxidase and cyclooxygenases, and the mitochondrial respiratory chain, and is dependent on hydroxyl moieties at key positions at the polyphenol structure, suggesting that auto-oxidation of the polyphenol structure might be of importance [43,50,51].

When compared to the short-lasting (within minutes) endothelial formation of NO by physiological stimulators such as bradykinin, that induced by polyphenols is characterized by a relatively sustained eNOS activation since the phosphorylation of eNOS at Ser1177 persists for several hours, resulting in a long-lasting formation of NO, and, hence, appears to be optimal for a sustained vascular protection [52].

Because polyphenols represent an extraordinary diversity of chemical structures, the identification of the active compounds of polyphenols-rich products acting on endothelial cells and the characterization of their underlying mechanism remain challenging. Nevertheless, several representative active polyphenols such as *trans*-resveratrol of the stilbene subclass, quercetin of the flavonol subclass, and delphinidin of the anthocyanin subclass have been shown to cause endothelium-dependent relaxations *via* the Src-PI-3-kinase-Akt-eNOS pathway in endothelial cells. The study of the structure-activity relationship of anthocyanins revealed that the activation of eNOS is dependent on the molecular structure of the compound, with a key role of both the B ring hydroxylation and the substitution at C3 [43,50,51]. Indeed petunidin-3-*O*-coumaroylglucoside induced eNOS phosphorylation at Ser1177 whereas closely related compounds such as malvidin-3-*O*-coumaroylglucoside, petunidin-3-*O*-glucoside, and petunidin were poorly active [43]. Besides the Src/PI-3-kinase/Akt pathway, certain polyphenols have also been shown to stimulate the endothelial formation of NO through a transient increase in the calcium signal [53], activation of estrogen receptors [54–56], AMPK [57,58], and of Sirt1/KLF2 [58] pathways in certain vascular beds and/or species.

Recent studies have reported that premature endothelial senescence is involved in various pathological conditions, such as endothelial dysfunction and atherosclerosis [31,59]. Premature endothelial senescence is triggered by the progressive induction of a pro-oxidant state and can be initiated by different stimuli such as Ang II, the inhibition of eNOS-derived NO formation, the upregulation of pro-oxidant enzymes such as NADPH oxidase and COXs, and the activation of the local angiotensin system that all promote the induction of the cell cycle inhibitory pathways including the p53/p21 and the p16 pathways [10,36,60–64]. It is suggested that the endothelial formation of NO contributes to protect endothelial cells against excessive oxidative stress, and, thus, that compounds stimulating the eNOS-derived formation of NO may help to delay endothelial senescence [62,65,66]. Recent investigations have indicated that polyphenols are able to prevent and/or retard endothelial senescence induced by different stimuli (Fig. 2). Indeed, a polyphenol-rich *Crataegus* extract was able to effectively delay replicative endothelial senescence by preventing the down-regulation of eNOS-derived formation of NO associated with a reduced expression level of the cell cycle regulatory proteins (p53, p21, and p16), the pro-oxidant enzymes NADPH oxidase, COX-1 and COX-2, and the local angiotensin system [36]. Several isolated polyphenols have also been reported to have anti-endothelial ageing properties. Curcumin attenuated the $H_2O_2$-induced premature senescence on endothelial cells *via* activation of the Sirt1/eNOS pathway [67]. Paeonol protected endothelial cells against oxidative stress-induced premature senescence and selaginellin against homocysteine-induced senescence, in part, *via* their antioxidant properties and an increased expression level of Sirt1 [68,69]. Resveratrol prevented high glucose-induced endothelial senescence by reducing the intracellular generation of ROS and *via* the AMPK/Sirt1 and p300/p53/p21 signaling pathway [70]. Besides

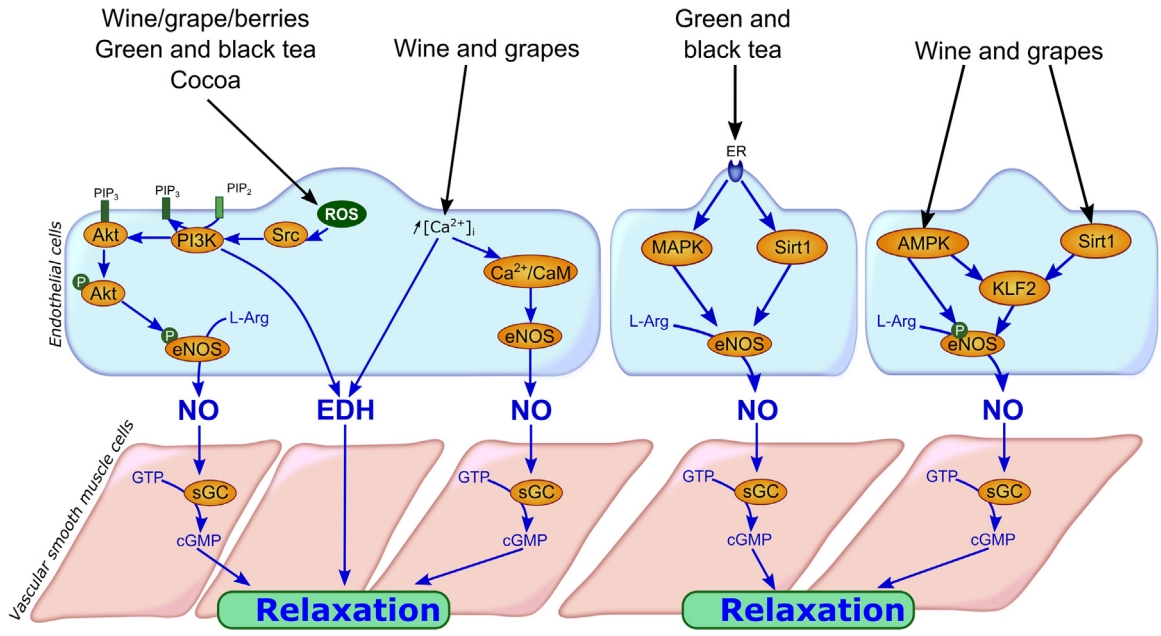


**Fig. 1.** Schematic representation summarizing potential mechanisms contributing to induce endothelium-dependent vasorelaxation subsequent to an increased formation of nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) in response to several polyphenols and polyphenol-rich products. Endothelial NO synthase-derived NO is a potent vasodilator *via* the activation of soluble guanylyl cyclase and the subsequent activation of the cyclic GMP relaxing pathway in the underlying vascular smooth muscle, and EDH by hyperpolarizing the vascular smooth muscle leading to a reduced opening of voltage-operated calcium channels, and, hence, a reduced intracellular activator calcium signal. The different pathways mediating the NO formation in response to certain polyphenols include the redox-sensitive activation of the Src/PI-3-kinase/Akt pathway, the $Ca^{2+}$/CaM signaling pathway, the activation of estrogen receptors, and the AMPK and Sirt1/KLF2 pathways. Abbreviations: GTP, guanosine-5'-triphosphate; cGMP, cyclic 3',5'-guanosine monophosphate; sGC, soluble guanylyl cyclase; SMCs, smooth muscle cells; $Ca^{2+}$/CaM, calcium/calmodulin complex; $[Ca^{2+}]i$, intracellular free calcium concentration; ROS, reactive oxygen species; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase, Src, proto-oncogene tyrosine-protein kinase; Akt, protein kinase B; ER, estrogen receptor; ERK 1/2, extracellular signal-regulated kinases 1/2; MAPKs, mitogen-activated protein kinases; Sirt1, sirtuin 1; AMPK, 5' adenosine monophosphate-activated protein kinase; KLF2, Krüppel-like Factor 2.

endothelial cells, polyphenol-rich blackberry, raspberry, and black raspberry extracts attenuated also Ang II-induced senescence in vascular smooth muscle cells through NADPH oxidase-dependent and -independent mechanisms [71].

Taken together, these data indicate that polyphenols can exert a beneficial effect on the vascular and endothelial functions through several mechanisms including an increased and sustained formation of NO, a normalization of the local angiotensin system, and an inhibition of oxidative stress, in part, by preventing the expression of pro-oxidant enzymes such as NADPH oxidase and COXs, all of them contributing to improve the endothelial function and to prevent vascular ageing.

To clarify the intracellular molecular mechanism of polyphenols, it is important to elucidate how polyphenols interact with target cells. Because diffusion of flavonoids across lipid membranes is very slow or does not occur, specific membrane receptors and/or transport systems facilitating the entry of polyphenols into endothelial cells most likely contribute to trigger intracellular responses. Previous studies have suggested the existence of specific receptors of the green tea polyphenol EGCg [72] and of a polyphenol transport system in endothelial cells [73]. In addition, a recent study suggested that the uptake of delphinidin-3-*O*-glucoside by endothelial cells depends on the sodium-glucose cotransporter 1 [74]. Further studies are required to better understand

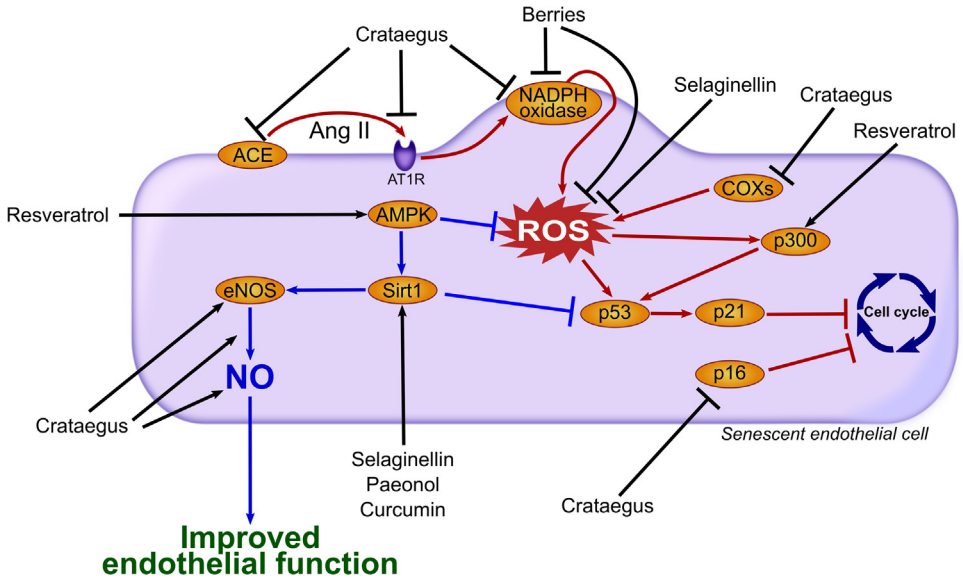


**Fig. 2.** Schematic representation summarizing potential mechanisms contributing to the beneficial effect of several polyphenols and polyphenol-rich products by protecting endothelial cells against oxidative-stress-induced premature senescence. Polyphenols can prevent endothelial senescence due to their antioxidant properties, by decreasing the formation of ROS by targeting NADPH oxidases, by preventing the down-regulation of the eNOS-derived formation of NO leading to a reduced expression level of the cell cycle regulatory proteins (p53, p21, and p16), by activating the Sirt1/eNOS and the AMPK/Sirt1 and p300/p53 signaling pathways. Blue lines indicate endothelial protective effects, red lines indicate pro-senescent pathways. Arrows indicate stimulatory effects and T-bar lines inhibitory effects. Abbreviations: ROS, reactive oxygen species; ACE, angiotensin-converting enzyme; Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; AMPK, 5' adenosine monophosphate-activated protein kinase; NO, nitric oxide; eNOS, endothelial nitric oxide synthases; Sirt1, sirtuin 1; COXs, cyclooxygenases.

how polyphenols interact with both extracellular and intracellular targets to trigger signal transduction pathways leading to biological responses.

## 5. Effect of polyphenols on the endothelial and vascular function in experimental models of cardiovascular diseases

Although *in vitro* and *ex vivo* studies are helpful to determine the direct vasoprotective effect of natural products on the endothelial and vascular function, and to characterize the underlying mechanism, they need to be associated with *in vivo* investigations to evaluate the potential of natural products to protect the cardiovascular system. In particular, the *in vivo* experimentation will take into account the bioavailability of natural products following oral absorption, and, hence, the fact that they are often highly metabolized to generate a great variety of secondary circulating metabolites or catabolites that contribute to mediate the biological response. In addition, *in vivo* experimentation will also evaluate the biological activity of natural products in an integrated system taking into account the influence of mechanical forces exerted by blood flow, the interaction of blood and blood cells with the arterial wall and also of neurovascular regulatory mechanisms [75].

Hypertension, a major cardiovascular risk factor, is characterized by an endothelial dysfunction associated with vascular oxidative stress in several experimental models of hypertension [76]. Studies evaluating the antihypertensive potential of natural products have indicated that the intake of red wine or red wine polyphenols reduced systolic blood pressure in the spontaneously hypertensive rats [77–79]. Similarly, an antihypertensive effect has also been observed in response to the consumption of a grape seed extract [80], green and black tea extracts [81], blueberry [82], and an azuki bean extract [83] in the spontaneously hypertensive rat. Of interest, intake of a polyphenol-rich cocoa powder up to 300 mg/kg body weight was able to reduce blood pressure to a similar level as 50 mg/kg of Captopril, an angiotensin-converting enzyme inhibitor, in the spontaneously hypertensive rat [84]. While the doses used in this study seem high, they are equivalent to 3.40 g of cocoa polyphenols and 568 mg of Captopril for a 70 kg Human, respectively, when using metabolic conversion factors [85]. Although the dose of Captopril exceeds the recommended maximal prescription dose of 450 mg/day, the dose of 3.40 g of cocoa polyphenols are within the range of Human consumption. Similar reductions in blood pressure after intake of grape-derived products have also been reported in other experimental models of hypertension including the $N^G$-nitro L-arginine-induced hypertension [86–88], the DOCA-salt-induced hypertension [89,90], and the Ang II-induced hypertension in rats [61]. Moreover, the antihypertensive effect of red wine and red wine extracts is associated with an improved endothelial function in the DOCA-salt rat [89,90], the spontaneously hypertensive rat [77,79,91], and the Ang II-induced hypertensive rat [61]. Intake of a maritime pine bark extract also improved blood pressure and endothelial function in the DOCA-salt rat [92].

Interestingly in a model of ageing-related endothelial dysfunction, the chronic intake of a red wine polyphenolic extract was able not only to delay the onset of the endothelial dysfunction [93], but also to improve an established ageing-related endothelial dysfunction in middle-aged rats [94,95]. Intake of a hawthorn extract prevented also ageing-related endothelial dysfunction in rats [96], and pomegranate juice and extract in female obese Zucker rats [97].

Besides polyphenol-rich products and extracts, an antihypertensive effect has also been observed following the chronic intake of isolated polyphenols such as chlorogenic acid [98], EGCg [99], genistein [100], quercetin [101–105], and *trans*-resveratrol [106] in several models of hypertension. Moreover, the antihypertensive effect was associated with an improved endothelial dysfunction in response to (-)-epicatechin [107,108], EGCg [99], genistein [100], flavone, and quercetin [109]. The chronic intake of catechin by normotensive rats [110], and baicalein by hypertensive rats [109] were also associated with an improved endothelial function without reduction of blood pressure. Both an improved NO component and indomethacin-sensitive relaxation explained the ability of EGCg to restore the endothelial function in the streptozotocin-induced diabetic rats [111].

In experimental models of cardiovascular diseases and ageing, the endothelial dysfunction is associated with an increased vascular level of oxidative stress predominantly superoxide anion, known to chemically inactivate NO, throughout the arterial wall, and is due, at least in part, to an increased expression and activity of NADPH oxidase [61,95,112]. Other pro-oxidant sources that have been involved include COXs, xanthine oxidase, cytochrome P450, the mitochondrial respiratory chain and uncoupled eNOS [94,95]. Red wine and hawthorn extracts have been shown to reduce the level of oxidative stress in the arterial wall by reducing the expression level of NADPH oxidase sub-units in old rats and Ang II-induced hypertensive rats [61,93–96]. Alternatively, polyphenols have also been suggested to decrease the level of vascular oxidative stress by inducing the expression of antioxidant enzymes such as catalase [113,114].

The local angiotensin system has been identified to play a determinant role in the induction of endothelial dysfunction and oxidative stress in several models of cardiovascular diseases [60]. Indeed, an increased expression of angiotensin-converting enzyme and angiotensin type 1 receptors (AT1R) is observed in the arterial wall of experimental models of hypertension, diabetes, atherosclerosis and ageing [115–119]. The fact that both angiotensin-converting inhibitors and AT1R antagonists are able to improve the endothelial dysfunction in old, in hypertensive, and also in diabetic rats, implies a determinant role of the local angiotensin system [120–124]. The fact that chronic intake of a red wine extract improved ageing- and hypertension-related endothelial dysfunction associated with the normalization of the expression of both angiotensin-converting enzyme and AT1R in the arterial wall, indicates that the local angiotensin system is an important target of polyphenols [94,95].

Altogether, these data indicate that polyphenols can exert a beneficial effect on the endothelial and vascular function through several mechanisms including an increased formation of both NO and EDH, a normalization of the local angiotensin system and of NADPH oxidase-derived oxidative stress to sustain the protective effect of endothelial cells on the vascular system.

## 6. Effect of polyphenols on the endothelial and vascular function in Humans

In agreement with epidemiological studies and experimental investigations, several clinical studies support the concept that polyphenol-rich products are able to improve the vascular function in Humans. The endothelial function can be assessed in Humans by flow-mediated dilatation (FMD), which uses non-invasive ultrasound to measure the percentage of dilatation of the brachial artery in response to blood flow and is due to an increased endothelial formation of NO in response to shear stress. A blunted FMD has been observed in Humans presenting major cardiovascular risk factors, and is known to be an independent predictor of the cardiovascular risk [125].

In healthy subjects, polyphenol-rich products have been shown to increase FMD by relatively low doses such as the consumption of two glasses of red wine with or without alcohol, or of a flavonoid-rich dark chocolate (46 g) for 2 weeks [126–130]. The beneficial effect of dark chocolate appears to be related to its content in (-)-epicatechin and to activation of the endothelial NO formation [131]. Indeed, the increased FMD after intake of the flavanol-rich cocoa drink was associated with a peak in plasma concentration of flavanols and (-)-epicatechin metabolites, as well as of plasma nitroso species indicating an increased formation of NO. Moreover, the inhibition of NO synthase abolished the cocoa-induced increase in FMD [131]. Polyphenol-rich blueberries, particularly rich in anthocyanins, improved also FMD in a time- and

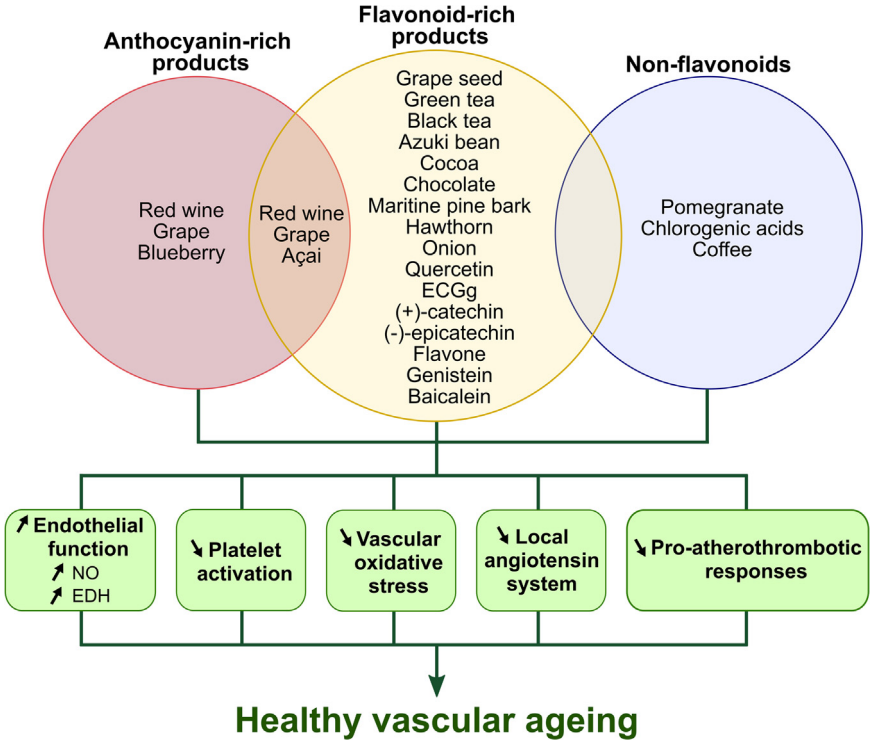


**Fig. 3.** Several polyphenol-rich natural products and polyphenols have been shown to improve vascular ageing by reducing endothelial dysfunction, platelet activation, vascular oxidative stress, over-activation of the local angiotensin system and increased pro-thrombotic responses associated with cardiovascular risk factors in preclinical and clinical studies.

dose-dependent manner (up to a concentration of 766 mg total polyphenol) in healthy volunteers and this effect was associated with an increased level of circulating metabolites [132]. Similar effects were also observed after consumption of a pine bark extract (180 mg/day for 2 weeks) [133], a grape seed extract (2 g/day) [129], purified (-)-epicatechin (1 or 2 mg/kg body weight) [131], coffee (two cups of 200 ml extracted from 18 g of coffee) [134], and a flavonoid-rich açai meal (694 mg polyphenols) [135].

Several clinical studies indicate also that polyphenol-rich products might contribute to improve hypertension and the related endothelial dysfunction. Indeed, daily ingestion of 100 g of dark chocolate for two weeks reduced blood pressure in mildly hypertensive patients [136], in diabetes and hypertensive patients [137] and in elderly subjects [138]. Consumption of pomegranate juice for two weeks by hypertensive patients reduced systolic blood pressure by 5% following intake of 50 ml [139], and by about 6 mmHg by 150 ml [140]. Intake of purple grape juice, equivalent to about two glasses, for 8 weeks improved blood pressure in hypertensive patients in one study [141] but not in another [142]. In addition, chronic consumption of red wine or dealcoholized red wine for 4 weeks (272 ml/day) reduced blood pressure by about 22.3 and 25.8 mmHg, respectively in subjects with a high normal blood pressure or grade 1 hypertension [143]. However, the daily consumption of a glass of red wine (200 ml/day) for up to 20 weeks did not affect blood pressure in normotensive patients with carotid atherosclerosis [144]. Chronic intake of a beverage containing 300 mg of grape seed extract for six weeks significantly reduced systolic blood pressure by about 5.6% and diastolic blood pressure by 4.7% in subjects with mild hypertension, and a much greater effect was observed in subjects with the highest initial blood pressure level [145]. In addition, the consumption of a low-fat meal with 80 mg of soybean isoflavones for 2 weeks or dark chocolate for 1 week increased FMD in postmenopausal women, a population with an increased risk of cardiovascular diseases [146,147]. Acute and/or chronic ingestion of polyphenol-rich products including grapes, cocoa, onions, red wine [148–151] and also the polyphenolic compound resveratrol [152] improved metabolic disease-related endothelial dysfunction. Moreover in patients with coronary artery disease, an improved FMD was observed in response to the acute ingestion of red wine (250 ml or 4 ml/kg) [153,154], EGCg (300 mg) [155], and red grape polyphenols (600 mg) [156], and also to the chronic intake of black tea for 4 weeks (900 ml/day) [157] and cranberry juice for 4 weeks (480 ml/day) [158].

## 7. Concluding remarks

There is now increasing evidence that several polyphenol-rich natural products are able to improve the endothelial function in ageing and in both experimental models of cardiovascular diseases and in patients with major cardiovascular risk factors mostly by re-adjusting the balance between the endothelial formation of NO and the vascular level of oxidative stress (Fig. 3). These findings further suggest that despite the relatively limited information regarding the active ingredients of the food products and their bioavailability, their structure-activity relationship, and their interaction with cell membranes and activation of intracellular signal transduction pathways, regular intake of polyphenol-rich natural products appears to be an attractive approach for promoting healthy ageing.

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## References

[1] V. Neveu, J. Perez-Jimenez, F. Vos, V. Crespy, L. du Chaffaut, L. Mennen, C. Knox, R. Eisner, J. Cruz, D. Wishart, A. Scalbert, Phenol-Explorer: an online comprehensive database on polyphenol contents in foods, Database (2010), http://dx.doi.org/10.1093/database/bap024.
[2] S. Renaud, M. de Lorgeril, Wine, alcohol, platelets, and the French paradox for coronary heart disease, Lancet 339 (1992) 1523–1526.
[3] F. Sofi, C. Macchi, R. Abbate, G.F. Gensini, A. Casini, Mediterranean diet and health status: an updated meta-analysis and a proposal for a literature-based adherence score, Public Health Nutr. 17 (2014) 2769–2782.
[4] L. Dauchet, P. Amouyel, S. Hercberg, J. Dallongeville, Fruit and vegetable

consumption and risk of coronary heart disease: a meta-analysis of cohort studies, J. Nutr. 136 (2006) 2588–2593.
[5] F.J. He, C.A. Nowson, G.A. MacGregor, Fruit and vegetable consumption and stroke: meta-analysis of cohort studies, Lancet 367 (2006) 320–326.
[6] L.M. Oude Griep, W.M. Verschuren, D. Kromhout, M.C. Ocke, J.M. Geleijnse, Variety in fruit and vegetable consumption and 10-year incidence of CHD and stroke, Public Health Nutr. 15 (2012) 2280–2286.
[7] A. Crozier, I.B. Jaganath, M.N. Clifford, Dietary phenolics: chemistry, bioavailability and effects on health, Nat. Prod. Rep. 26 (2009) 1001–1043.
[8] J. Rienks, J. Barbaresko, U. Nothlings, Association of polyphenol biomarkers with cardiovascular disease and mortality risk: a systematic review and meta-analysis of observational studies, Nutrients 9 (2017).
[9] R. Micha, J.L. Penalvo, F. Cudhea, F. Imamura, C.D. Rehm, D. Mozaffarian, Association between dietary factors and mortality from heart disease, stroke, and type 2 diabetes in the United States, JAMA 317 (2017) 912–924.
[10] A. Scalbert, C. Manach, C. Morand, C. Remesy, L. Jimenez, Dietary polyphenols and the prevention of diseases, Crit. Rev. Food Sci. Nutr. 45 (2005) 287–306.
[11] J.G. Gormaz, N. Valls, C. Sotomayor, T. Turner, R. Rodrigo, Potential role of polyphenols in the prevention of cardiovascular diseases: molecular bases, Curr. Med. Chem. 23 (2016) 115–128.
[12] V.B. Schini-Kerth, N. Etienne-Selloum, T. Chataigneau, C. Auger, Vascular protection by natural product-derived polyphenols: in vitro and in vivo evidence, Planta Med. 77 (2011) 1161–1167.
[13] L. Marzocchella, M. Fantini, M. Benvenuto, L. Masuelli, I. Tresoldi, A. Modesti, R. Bei, Dietary flavonoids: molecular mechanisms of action as anti- inflammatory agents, Recent Pat. Inflamm. Allergy Drug Discov. 5 (2011) 200–220.
[14] M.G.L. Hertog, P.C.H. Hollman, M.B. Katan, Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands, J. Agric. Food Chem. 40 (1992) 2379–2383.
[15] K. Zaheer, M. Humayoun Akhtar, An updated review of dietary isoflavones: nutrition, processing, bioavailability and impacts on human health, Crit. Rev. Food Sci. Nutr. 57 (2017) 1280–1293.
[16] J. Fang, Classification of fruits based on anthocyanin types and relevance to their health effects, Nutrition 31 (2015) 1301–1306.
[17] R. Vidavalur, H. Otani, P.K. Singal, N. Maulik, Significance of wine and resveratrol in cardiovascular disease: French paradox revisited, Exp. Clin. Cardiol. 11 (2006) 217–225.
[18] R. Garcia-Villalba, A. Carrasco-Pancorbo, G. Zurek, M. Behrens, C. Bassmann, A. Segura-Carretero, A. Fernandez-Gutierrez, Nano and rapid resolution liquid chromatography-electrospray ionization-time of flight mass spectrometry to identify and quantify phenolic compounds in olive oil, J. Sep. Sci. 33 (2010) 2069–2078.
[19] C. Manach, A. Scalbert, C. Morand, C. Remesy, L. Jimenez, Polyphenols: food sources and bioavailability, Am. J. Clin. Nutr. 79 (2004) 727–747.
[20] D. Del Rio, A. Rodriguez-Mateos, J.P. Spencer, M. Tognolini, G. Borges, A. Crozier, Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases, Antioxid. Redox Signal. 18 (2013) 1818–1892.
[21] Y. Kawai, T. Nishikawa, Y. Shiba, S. Saito, K. Murota, N. Shibata, M. Kobayashi, M. Kanayama, K. Uchida, J. Terao, Macrophage as a target of quercetin glucuronides in human atherosclerotic arteries: implication in the anti-atherosclerotic mechanism of dietary flavonoids, J. Biol. Chem. 283 (2008) 9424–9434.
[22] Y. Kawai, H. Tanaka, K. Murota, M. Naito, J. Terao, (-)-Epicatechin gallate accumulates in foamy macrophages in human atherosclerotic aorta: implication in the anti-atherosclerotic actions of tea catechins, Biochem. Biophys. Res. Commun. 374 (2008) 527–532.
[23] C. Auger, A. Said, P.N. Nguyen, P. Chabert, N. Idris-Khodja, V.B. Schini-Kerth, Potential of food and natural products to promote endothelial and vascular health, J. Cardiovasc. Pharmacol. 68 (2016) 11–18.
[24] R. Andriantsitohaina, C. Auger, T. Chataigneau, N. Etienne-Selloum, H. Li, M.C. Martinez, V.B. Schini-Kerth, I. Laher, Molecular mechanisms of the cardiovascular protective effects of polyphenols, Br. J. Nutr. 108 (2012) 1532–1549.
[25] J.H. Kim, C. Auger, V.B. Schini-Kerth, Activation of eNOS by polyphenol-rich products and polyphenolic compounds, Curr. Pharm. Des. 20 (2014) 3521–3529.
[26] P.M. Vanhoutte, H. Shimokawa, M. Feletou, E.H. Tang, Endothelial dysfunction and vascular disease – a 30th anniversary update, Acta Physiol. 219 (2017) 22–96.
[27] S. Moncada, R.M. Palmer, E.A. Higgs, Nitric oxide: physiology, pathophysiology, and pharmacology, Pharmacol. Rev. 43 (1991) 109–142.
[28] J.A. Vita, J.F. Keaney Jr., Endothelial function: a barometer for cardiovascular risk? Circulation 106 (2002) 640–642.
[29] A.G. Herman, S. Moncada, Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis, Eur. Heart J. 26 (2005) 1945–1955.
[30] M.E. Widlansky, N. Gokce, J.F. Keaney Jr., J.A. Vita, The clinical implications of endothelial dysfunction, J. Am. Coll. Cardiol. 42 (2003) 1149–1160.
[31] T. Minamino, H. Miyauchi, T. Yoshida, Y. Ishida, H. Yoshida, I. Komuro, Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction, Circulation 105 (2002) 1541–1544.
[32] J. Chen, X. Huang, D. Halicka, S. Brodsky, A. Avram, J. Eskander, N.A. Bloomgarden, Z. Darzynkiewicz, M.S. Goligorsky, Contribution of p16INK4a and p21CIP1 pathways to induction of premature senescence of human endothelial cells: permissive role of p53, Am. J. Physiol. Heart Circ. Physiol. 290 (2006) H1575–H1586.
[33] C.S. Kim, S.B. Jung, A. Naqvi, T.A. Hoffman, J. DeRicco, T. Yamamori, M.P. Cole, B.H. Jeon, K. Irani, p53 Impairs endothelium-dependent vasomotor function through transcriptional upregulation of p66shc, Circ. Res. 103 (2008) 1441–1450.
[34] M.S. Goligorsky, J. Chen, S. Patschan, Stress-induced premature senescence of endothelial cells: a perilous state between recovery and point of no return, Curr. Opin. Hematol. 16 (2009) 215–219.
[35] M. Abbas, L. Jesel, C. Auger, L. Amoura, N. Messas, G. Manin, C. Rumig, A.J. Leon-Gonzalez, T.P. Ribeiro, G.C. Silva, R. Abou-Merhi, E. Hamade, M. Hecker, Y. Georg, N. Chakfe, P. Ohlmann, V.B. Schini-Kerth, F. Toti, O. Morel, Endothelial microparticles from acute coronary syndrome patients induce premature coronary artery endothelial cell aging and thrombogenicity: role of the Ang II/AT1 receptor/NADPH oxidase-mediated activation of MAPKs and PI3-kinase pathways, Circulation 135 (2017) 280–296.
[36] S. Khemais-Benkhiat, N. Idris-Khodja, T.P. Ribeiro, G.C. Silva, M. Abbas, M. Kheloufi, J.O. Lee, F. Toti, C. Auger, V.B. Schini-Kerth, The redox-sensitive induction of the local angiotensin system promotes both premature and replicative endothelial senescence: preventive effect of a standardized crataegus extract, J. Gerontol. 71 (2016) 1581–1590.
[37] G.C. Silva, M. Abbas, S. Khemais-Benkhiat, M. Burban, T.P. Ribeiro, F. Toti, N. Idris-Khodja, S.F. Cortes, V.B. Schini-Kerth, Replicative senescence promotes prothrombotic responses in endothelial cells: role of NADPH oxidase- and cyclooxygenase-derived oxidative stress, Exp. Gerontol. 93 (2017) 7–15.
[38] C.M. Warboys, A. de Luca, N. Amini, L. Luong, H. Duckles, S. Hsiao, A. White, S. Biswas, R. Khamis, C.K. Chong, W.M. Cheung, S.J. Sherwin, M.R. Bennett, J. Gil, J.C. Mason, D.O. Haskard, P.C. Evans, Disturbed flow promotes endothelial senescence via a p53-dependent pathway, Arterioscler. Thromb. Vasc. Biol. 34 (2014) 985–995.
[39] T. Minamino, H. Miyauchi, T. Yoshida, K. Tateno, T. Kunieda, I. Komuro, Vascular cell senescence and vascular aging, J. Mol. Cell. Cardiol. 36 (2004) 175–183.
[40] H. Matsui-Hirai, T. Hayashi, S. Yamamoto, K. Ina, M. Maeda, H. Kotani, A. Iguchi, L.J. Ignarro, Y. Hattori, Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence in vitro and in vivo: a relationship between telomeres and nitric oxide, J. Pharmacol. Exp. Ther. 337 (2011) 591–599.
[41] D.F. Fitzpatrick, S.L. Hirschfield, T. Ricci, P. Jantzen, R.G. Coffey, Endothelium-dependent vasorelaxation caused by various plant extracts, J. Cardiovasc. Pharmacol. 26 (1995) 90–95.
[42] E. Andriambeloson, A.L. Kleschyov, B. Muller, A. Beretz, J.C. Stoclet, R. Andriantsitohaina, Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta, Br. J. Pharmacol. 120 (1997) 1053–1058.
[43] C. Auger, M. Chaabi, E. Anselm, A. Lobstein, V.B. Schini-Kerth, The red wine extract-induced activation of endothelial nitric oxide synthase is mediated by a great variety of polyphenolic compounds, Mol. Nutr. Food Res. 54 (Suppl. 2) (2010) S171–S183.
[44] E. Anselm, M. Chataigneau, M. Ndiaye, T. Chataigneau, V.B. Schini-Kerth, Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS, Cardiovasc. Res. 73 (2007) 404–413.
[45] M. Ndiaye, M. Chataigneau, I. Lobysheva, T. Chataigneau, V.B. Schini-Kerth, Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery, FASEB J. 19 (2005) 455–457.
[46] M. Ndiaye, T. Chataigneau, R. Andriantsitohaina, J.C. Stoclet, V.B. Schini-Kerth, Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism, Biochem. Biophys. Res. Commun. 310 (2003) 371–377.
[47] S.V. Madeira, C. Auger, E. Anselm, M. Chataigneau, T. Chataigneau, R. Soares de Moura, V.B. Schini-Kerth, eNOS activation induced by a polyphenol-rich grape skin extract in porcine coronary arteries, J. Vasc. Res. 46 (2009) 406–416.
[48] J.A. Kim, G. Formoso, Y. Li, M.A. Potenza, F.L. Marasciulo, M. Montagnani, M.J. Quon, Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn, J. Biol. Chem. 282 (2007) 13736–13745.
[49] J.H. Kim, C. Auger, I. Kurita, E. Anselm, L.O. Rivoarilala, H.J. Lee, K.W. Lee, V.B. Schini-Kerth, Aronia melanocarpa juice, a rich source of polyphenols, induces endothelium-dependent relaxations in porcine coronary arteries via the redox-sensitive activation of endothelial nitric oxide synthase, Nitric Oxide: Biol. Chem./Off. J. Nitric Oxide Soc. 35 (2013) 54–64.
[50] C. Auger, J.H. Kim, P. Chabert, M. Chaabi, E. Anselm, X. Lanciaux, A. Lobstein, V.B. Schini-Kerth, The EGCg-induced redox-sensitive activation of endothelial nitric oxide synthase and relaxation are critically dependent on hydroxyl moieties, Biochem. Biophys. Res. Commun. 393 (2010) 162–167.
[51] I. Kurita, J.H. Kim, C. Auger, Y. Kinoshita, T. Miyase, T. Ito, V.B. Schini-Kerth, Hydroxylation of (-)-epigallocatechin-3-O-gallate at 3", but not 4", is essential for the PI3-kinase/Akt-dependent phosphorylation of endothelial NO synthase in endothelial cells and relaxation of coronary artery rings, Food Funct. 4 (2013) 249–257.
[52] M. Alhosin, E. Anselm, S. Rashid, J.H. Kim, S.V. Frota Madeira, C. Bronner, V.B. Schini-Kerth, Redox-sensitive Up-regulation of eNOS by purple grape juice in endothelial cells: role of PI3-kinase/Akt, p38 MAPK, JNK, FoxO1 and FoxO3a, PLoS One 8 (2013) e57883.
[53] S. Martin, E. Andriambeloson, K. Takeda, R. Andriantsitohaina, Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signalling pathways leading to nitric oxide production, Br. J. Pharmacol. 135 (2002) 1579–1587.
[54] M.O. Kane, E. Anselm, Y.D. Rattmann, C. Auger, V.B. Schini-Kerth, Role of gender and estrogen receptors in the rat aorta endothelium-dependent relaxation to red wine polyphenols, Vasc. Pharmacol. 51 (2009) 140–146.
[55] M. Chalopin, A. Tesse, M.C. Martinez, D. Rognan, J.F. Arnal, R. Andriantsitohaina, Estrogen receptor alpha as a key target of red wine polyphenols action on the endothelium, PLoS One 5 (2010) e8554.

[56] D. Leonetti, R. Soleti, N. Clere, L. Vergori, C. Jacques, L. Duluc, C. Dourguia, M.C. Martinez, R. Andriantsitohaina, Estrogen receptor alpha participates to the beneficial effect of red wine polyphenols in a mouse model of obesity-related disorders, Front. Pharmacol. 7 (2016) 529.
[57] M.O. Kane, M. Sene, E. Anselm, S. Dal, V.B. Schini-Kerth, C. Augier, Role of amp-activated protein kinase in NO- and EDHF-mediated endothelium-dependent relaxations to red wine polyphenols, Indian J. Physiol. Pharmacol. 59 (2015) 369–379.
[58] X. Cui, X. Liu, H. Feng, S. Zhao, H. Gao, Grape seed proanthocyanidin extracts enhance endothelial nitric oxide synthase expression through 5'-AMP activated protein kinase/Surtuin 1-Krupple like factor 2 pathway and modulate blood pressure in ouabain induced hypertensive rats, Biol. Pharm. Bull. 35 (2012) 2192–2197.
[59] K.F. Burrig, The endothelium of advanced arteriosclerotic plaques in humans, Arterioscler. Thromb. 11 (1991) 1678–1689.
[60] D.G. Harrison, H. Cai, U. Landmesser, K.K. Griendling, Interactions of angiotensin II with NAD(P)H oxidase, oxidant stress and cardiovascular disease, J. Renin-Angiotensin-Aldosterone Syst.: JRAAS 4 (2003) 51–61.
[61] M. Sarr, M. Chataigneau, S. Martins, C. Schott, J. El Bedoui, M.H. Oak, B. Muller, T. Chataigneau, V.B. Schini-Kerth, Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase, Cardiovasc. Res. 71 (2006) 794–802.
[62] H. Ota, M. Eto, S. Ogawa, K. Iijima, M. Akishita, Y. Ouchi, SIRT1/eNOS axis as a potential target against vascular senescence, dysfunction and atherosclerosis, J. Atheroscler. Thromb. 17 (2010) 431–435.
[63] H. Shan, D. Guo, X. Li, X. Zhao, W. Li, X. Bai, From autophagy to senescence and apoptosis in Angiotensin II-treated vascular endothelial cells, APMIS 122 (2014) 985–992.
[64] Y. Zu, L. Liu, M.Y. Lee, C. Xu, Y. Liang, R.Y. Man, P.M. Vanhoutte, Y. Wang, SIRT1 promotes proliferation and prevents senescence through targeting LKB1 in primary porcine aortic endothelial cells, Circ. Res. 106 (2010) 1384–1393.
[65] T. Hayashi, H. Matsui-Hirai, A. Miyazaki-Akita, A. Fukatsu, J. Funami, Q.F. Ding, S. Kamalanathan, Y. Hattori, L.J. Ignarro, A. Iguchi, Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes, Proc. Natl. Acad. Sci. USA 103 (2006) 17018–17023.
[66] M. Vasa, K. Breitschopf, A.M. Zeiher, S. Dimmeler, Nitric oxide activates telomerase and delays endothelial cell senescence, Circ. Res. 87 (2000) 540–542.
[67] Y. Sun, X. Hu, G. Hu, C. Xu, H. Jiang, Curcumin attenuates hydrogen peroxide-induced premature senescence via the activation of SIRT1 in human umbilical vein endothelial cells, Biol. Pharm. Bull. 38 (2015) 1134–1141.
[68] J. Jamal, M.R. Mustafa, P.F. Wong, Paeonol protects against premature senescence in endothelial cells by modulating Sirtuin 1 pathway, J. Ethnopharmacol. 154 (2014) 428–436.
[69] C.J. Wang, C.P. Hu, K.P. Xu, G.S. Tan, Y.J. Li, Effects of selaginellin on homocysteine-induced senescence in human umbilical vein endothelial cells, J. Cardiovasc. Pharmacol. 55 (2010) 560–566.
[70] E. Zhang, Q. Guo, H. Gao, R. Xu, S. Teng, Y. Wu, Metformin and resveratrol inhibited high glucose-induced metabolic memory of endothelial senescence through SIRT1/p300/p53/p21 pathway, PLoS One 10 (2015) e0143814.
[71] R.G. Feresin, J. Huang, D.S. Klarich, Y. Zhao, S. Pourafshar, B.H. Arjmandi, G. Salazar, Blackberry, raspberry and black raspberry polyphenol extracts attenuate angiotensin II-induced senescence in vascular smooth muscle cells, Food Funct. 7 (2016) 4175–4187.
[72] H. Tachibana, K. Koga, Y. Fujimura, K. Yamada, A receptor for green tea polyphenol EGCG, Nat. Struct. Mol. Biol. 11 (2004) 380–381.
[73] D.D. Schramm, H.E. Collins, J.B. German, Flavonoid transport by mammalian endothelial cells, J. Nutr. Biochem. 10 (1999) 193–197.
[74] X. Jin, L. Yi, M.L. Chen, C.Y. Chen, H. Chang, T. Zhang, L. Wang, J.D. Zhu, Q.Y. Zhang, M.T. Mi, Delphinidin-3-glucoside protects against oxidized low-density lipoprotein-induced mitochondrial dysfunction in vascular endothelial cells via the sodium-dependent glucose transporter SGLT1, PLoS One 8 (2013) e68617.
[75] R. Busse, I. Fleming, Regulation of endothelium-derived vasoactive autacoid production by hemodynamic forces, Trends Pharmacol. Sci. 24 (2003) 24–29.
[76] C. Bleakley, P.K. Hamilton, R. Pumb, M. Harbinson, G.E. McVeigh, Endothelial function in hypertension: victim or culprit? J. Clin. Hypertens. 17 (2015) 651–654.
[77] R. Lopez-Sepulveda, R. Jimenez, M. Romero, M.J. Zarzuelo, M. Sanchez, M. Gomez-Guzman, F. Vargas, F. O'Valle, A. Zarzuelo, F. Perez-Vizcaino, J. Duarte, Wine polyphenols improve endothelial function in large vessels of female spontaneously hypertensive rats, Hypertension 51 (2008) 1088–1095.
[78] K. Mizutani, K. Ikeda, Y. Kawai, Y. Yamori, Extract of wine phenolics improves aortic biomechanical properties in stroke-prone spontaneously hypertensive rats (SHRSP), J. Nutr. Sci. Vitaminol. 45 (1999) 95–106.
[79] S.-L. Chan, C. Capdeville-Atkinson, J. Atkinson, Red wine polyphenols improve endothelium-dependent dilation in rat cerebral arterioles, J. Cardiovasc. Pharmacol. 51 (2008) 553–558.
[80] N. Peng, J.T. Clark, J. Prasain, H. Kim, C.R. White, J.M. Wyss, Antihypertensive and cognitive effects of grape polyphenols in estrogen-depleted, female, spontaneously hypertensive rats, Am. J. Physiol. Regul. Integr. Comp. Physiol. 289 (2005) R771–R775.
[81] H. Negishi, J.W. Xu, K. Ikeda, M. Njelekela, Y. Nara, Y. Yamori, Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats, J. Nutr. 134 (2004) 38–42.
[82] K.S. Shaughnessy, I.A. Boswall, A.P. Scanlan, K.T. Gottschall-Pass, M.I. Sweeney, Diets containing blueberry extract lower blood pressure in spontaneously hypertensive stroke-prone rats, Nutr. Res. 29 (2009) 130–138.
[83] Y. Mukai, S. Sato, Polyphenol-containing azuki bean (Vigna angularis) extract attenuates blood pressure elevation and modulates nitric oxide synthase and caveolin-1 expressions in rats with hypertension, Nutr. Metab. Cardiovasc. Dis. 19 (2009) 491–497.
[84] E. Cienfuegos-Jovellanos, M. Quinones Mdel, B. Muguerza, L. Moulay, M. Miguel, A. Aleixandre, Antihypertensive effect of a polyphenol-rich cocoa powder industrially processed to preserve the original flavonoids of the cocoa beans, J. Agric. Food Chem. 57 (2009) 6156–6162.
[85] S. Reagan-Shaw, M. Nihal, N. Ahmad, Dose translation from animal to human studies revisited, FASEB J. 22 (2008) 659–661.
[86] R.S. de Moura, D.Z. Miranda, A.C. Pinto, R.F. Sicca, M.A. Souza, L.M. Rubenich, L.C. Carvalho, B.M. Rangel, T. Tano, S.V. Madeira, A.C. Resende, Mechanism of the endothelium-dependent vasodilation and the antihypertensive effect of Brazilian red wine, J. Cardiovasc. Pharmacol. 44 (2004) 302–309.
[87] I. Bernatova, O. Pechanova, P. Babal, S. Kysela, S. Stvrtina, R. Andriantsitohaina, Wine polyphenols improve cardiovascular remodeling and vascular function in NO-deficient hypertension, Am. J. Physiol. Heart Circ. Physiol. 282 (2002) H942–H948.
[88] O. Pechanova, I. Bernatova, P. Babal, M.C. Martinez, S. Kysela, S. Stvrtina, R. Andriantsitohaina, Red wine polyphenols prevent cardiovascular alterations in L-name-induced hypertension, J. Hypertens. 22 (2004) 1551–1559.
[89] R. Jimenez, R. Lopez-Sepulveda, M. Kadmiri, M. Romero, R. Vera, M. Sanchez, F. Vargas, F. O'Valle, A. Zarzuelo, M. Duenas, C. Santos-Buelga, J. Duarte, Polyphenols restore endothelial function in DOCA-salt hypertension: role of endothelin-1 and NADPH oxidase, Free Radic. Biol. Med. 43 (2007) 462–473.
[90] N. Lopez Andres, A. Tesse, V. Regnault, H. Louis, V. Cattan, S.N. Thornton, C. Labat, A. Kakou, S. Tual-Chalot, S. Faure, P. Challande, M. Osborne-Pellegrin, M.C. Martinez, P. Lacolley, R. Andriantsitohaina, Increased microparticle production and impaired microvascular endothelial function in aldosterone-salt-treated rats: protective effects of polyphenols, PLoS One 7 (2012) e39235.
[91] S.L. Chan, A. Tabellion, D. Bagrel, C. Perrin-Sarrado, C. Capdeville-Atkinson, J. Atkinson, Impact of chronic treatment with red wine polyphenols (RWP) on cerebral arterioles in the spontaneous hypertensive rat, J. Cardiovasc. Pharmacol. 51 (2008) 304–310.
[92] C.J. Kwak, E. Kubo, K. Fujii, Y. Nishimura, S. Kobuchi, M. Ohkita, M. Yoshimura, Y. Kiso, Y. Matsumura, Antihypertensive effect of French maritime pine bark extract (Flavangenol): possible involvement of endothelial nitric oxide-dependent vasorelaxation, J. Hypertens. 27 (2009) 92–101.
[93] S. Dal-Ros, J. Zoll, A.L. Lang, C. Auger, N. Keller, C. Bronner, B. Geny, V.B. Schini-Kerth, Chronic intake of red wine polyphenols by young rats prevents aging-induced endothelial dysfunction and decline in physical performance: role of NADPH oxidase, Biochem. Biophys. Res. Commun. 404 (2011) 743–749.
[94] S. Dal-Ros, C. Bronner, C. Auger, V.B. Schini-Kerth, Red wine polyphenols improve an established aging-related endothelial dysfunction in the mesenteric artery of middle-aged rats: role of oxidative stress, Biochem. Biophys. Res. Commun. 419 (2012) 381–387.
[95] N. Idris Khodja, T. Chataigneau, C. Auger, V.B. Schini-Kerth, Grape-derived polyphenols improve aging-related endothelial dysfunction in rat mesenteric artery: role of oxidative stress and the angiotensin system, PLoS One 7 (2012) e32039.
[96] N. Idris-Khodja, C. Auger, E. Koch, V.B. Schini-Kerth, Crataegus special extract WS ((R))1442 prevents aging-related endothelial dysfunction, Phytomedicine 19 (2012) 699–706.
[97] F. de Nigris, M.L. Balestrieri, S. Williams-Ignarro, F.P. D'Armiento, C. Fiorito, L.J. Ignarro, C. Napoli, The influence of pomegranate fruit extract in comparison to regular pomegranate juice and seed oil on nitric oxide and arterial function in obese Zucker rats, Nitric Oxide: Biol. Chem./Off. J. Nitric Oxide Soc. 17 (2007) 50–54.
[98] A. Suzuki, N. Yamamoto, H. Jokura, M. Yamamoto, A. Fujii, I. Tokimitsu, I. Saito, Chlorogenic acid attenuates hypertension and improves endothelial function in spontaneously hypertensive rats, J. Hypertens. 24 (2006) 1065–1073.
[99] M.A. Potenza, F.L. Marasciulo, M. Tarquinio, E. Tiravanti, G. Colantuono, A. Federici, J.A. Kim, M.J. Quon, M. Montagnani, EGCG, a green tea polyphenol, improves endothelial function and insulin sensitivity, reduces blood pressure, and protects against myocardial I/R injury in SHR, Am. J. Physiol. Endocrinol. Metab. 292 (2007) E1378–E1387.
[100] R. Vera, M. Sanchez, M. Galisteo, I.C. Villar, R. Jimenez, A. Zarzuelo, F. Perez-Vizcaino, J. Duarte, Chronic administration of genistein improves endothelial dysfunction in spontaneously hypertensive rats: involvement of eNOS, caveolin and calmodulin expression and NADPH oxidase activity, Clin. Sci. 112 (2007) 183–191.
[101] M. Sanchez, M. Galisteo, R. Vera, I.C. Villar, A. Zarzuelo, J. Tamargo, F. Perez-Vizcaino, J. Duarte, Quercetin downregulates NADPH oxidase, increases eNOS activity and prevents endothelial dysfunction in spontaneously hypertensive rats, J. Hypertens. 24 (2006) 75–84.
[102] M.F. Garcia-Saura, M. Galisteo, I.C. Villar, A. Bermejo, A. Zarzuelo, F. Vargas, J. Duarte, Effects of chronic quercetin treatment in experimental renovascular hypertension, Mol. Cell. Biochem. 270 (2005) 147–155.
[103] M. Galisteo, M.F. Garcia-Saura, R. Jimenez, I.C. Villar, R. Wangensteen, A. Zarzuelo, F. Vargas, J. Duarte, Effects of quercetin treatment on vascular function in deoxycorticosterone acetate-salt hypertensive rats. Comparative study with verapamil, Planta Med. 70 (2004) 334–341.
[104] J. Duarte, R. Jimenez, F. O'Valle, M. Galisteo, R. Perez-Palencia, F. Vargas, F. Perez-Vizcaino, A. Zarzuelo, J. Tamargo, Protective effects of the flavonoid quercetin in chronic nitric oxide deficient rats, J. Hypertens. 20 (2002) 1843–1854.
[105] J. Duarte, R. Perez-Palencia, F. Vargas, M.A. Ocete, F. Perez-Vizcaino, A. Zarzuelo,

J. Tamargo, Antihypertensive effects of the flavonoid quercetin in spontaneously hypertensive rats, Br. J. Pharmacol. 133 (2001) 117–124.
[106] L. Rivera, R. Moron, A. Zarzuelo, M. Galisteo, Long-term resveratrol administration reduces metabolic disturbances and lowers blood pressure in obese Zucker rats, Biochem. Pharmacol. 77 (2009) 1053–1063.
[107] M. Galleano, I. Bernatova, A. Puzserova, P. Balis, N. Sestakova, O. Pechanova, C.G. Fraga, (-)-Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanism, IUBMB Life 65 (2013) 710–715.
[108] M. Gomez-Guzman, R. Jimenez, M. Sanchez, M.J. Zarzuelo, P. Galindo, A.M. Quintela, R. Lopez-Sepulveda, M. Romero, J. Tamargo, F. Vargas, F. Perez-Vizcaino, J. Duarte, Epicatechin lowers blood pressure, restores endothelial function, and decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension, Free Radic. Biol. Med. 52 (2012) 70–79.
[109] A. Machha, M.R. Mustafa, Chronic treatment with flavonoids prevents endothelial dysfunction in spontaneously hypertensive rat aorta, J. Cardiovasc. Pharmacol. 46 (2005) 36–40.
[110] S. Benito, D. Lopez, M.P. Saiz, S. Buxaderas, J. Sanchez, P. Puig-Parellada, M.T. Mitjavila, A flavonoid-rich diet increases nitric oxide production in rat aorta, Br. J. Pharmacol. 135 (2002) 910–916.
[111] M. Roghani, T. Baluchnejadmojarad, Chronic epigallocatechin-gallate improves aortic reactivity of diabetic rats: underlying mechanisms, Vasc. Pharmacol. 51 (2009) 84–89.
[112] M.O. Kane, N. Etienne-Selloum, S.V. Madeira, M. Sarr, A. Walter, S. Dal-Ros, C. Schott, T. Chataigneau, V.B. Schini-Kerth, Endothelium-derived contracting factors mediate the Ang II-induced endothelial dysfunction in the rat aorta: preventive effect of red wine polyphenols, Pflug. Arch. 459 (2010) 671–679.
[113] P. Mladenka, L. Zatloukalova, T. Filipsky, R. Hrdina, Cardiovascular effects of flavonoids are not caused only by direct antioxidant activity, Free Radic. Biol. Med. 49 (2010) 963–975.
[114] H. Negishi, J.W. Xu, K. Ikeda, M. Njelekela, Y. Nara, Y. Yamori, Black and green tea polyphenols attenuate blood pressure increases in stroke-prone spontaneously hypertensive rats, J. Nutr. 134 (2004) 38–42.
[115] E. Ferrannini, W.C. Cushman, Diabetes and hypertension: the bad companions, Lancet 380 (2012) 601–610.
[116] A. Pende, F. Dallegri, Renin-angiotensin antagonists: therapeutic effects beyond blood pressure control? Curr. Pharm. Des. 18 (2012) 1011–1020.
[117] A. Durante, G. Peretto, A. Laricchia, F. Ancona, M. Spartera, A. Mangieri, D. Cianflone, Role of the renin-angiotensin-aldosterone system in the pathogenesis of atherosclerosis, Curr. Pharm. Des. 18 (2012) 981–1004.
[118] E.S. Jones, M.J. Black, R.E. Widdop, Influence of Angiotensin II subtype 2 receptor (AT(2)R) antagonist, PD123319, on cardiovascular remodelling of aged spontaneously hypertensive rats during chronic Angiotensin II subtype 1 receptor (AT(1)R) blockade, Int. J. Hypertens. 2012 (2012) 543062.
[119] S. Conti, P. Cassis, A. Benigni, Aging and the renin-angiotensin system, Hypertension 60 (2012) 878–883.
[120] Y. Kansui, K. Fujii, K. Goto, I. Abe, M. Iida, Angiotensin II receptor antagonist improves age-related endothelial dysfunction, J. Hypertens. 20 (2002) 439–446.
[121] K. Goto, K. Fujii, U. Onaka, I. Abe, M. Fujishima, Angiotensin-converting enzyme inhibitor prevents age-related endothelial dysfunction, Hypertension 36 (2000) 581–587.
[122] K. Goto, K. Fujii, U. Onaka, I. Abe, M. Fujishima, Renin-angiotensin system blockade improves endothelial dysfunction in hypertension, Hypertension 36 (2000) 575–580.
[123] S. Nemoto, T. Kobayashi, K. Taguchi, T. Matsumoto, K. Kamata, Losartan improves aortic endothelium-dependent relaxation via proline-rich tyrosine kinase 2/Src/Akt pathway in type 2 diabetic Goto-Kakizaki rats, Am. J. Physiol. Heart Circ. Physiol. 301 (2011) H2383–H2394.
[124] S. Matsumoto, M. Shimabukuro, D. Fukuda, T. Soeki, K. Yamakawa, H. Masuzaki, M. Sata, Azilsartan, an angiotensin II type 1 receptor blocker, restores endothelial function by reducing vascular inflammation and by increasing the phosphorylation ratio Ser(1177)/Thr(497) of endothelial nitric oxide synthase in diabetic mice, Cardiovasc. Diabetol. 13 (2014) 30.
[125] J. Yeboah, J.R. Crouse, F.C. Hsu, G.L. Burke, D.M. Herrington, Brachial flow-mediated dilation predicts incident cardiovascular events in older adults: the Cardiovascular Health Study, Circulation 115 (2007) 2390–2397.
[126] M. Boban, D. Modun, I. Music, J. Vukovic, I. Brizic, I. Salamunic, A. Obad, I. Palada, Z. Dujic, Red wine induced modulation of vascular function: separating the role of polyphenols, ethanol, and urates, J. Cardiovasc. Pharmacol. 47 (2006) 695–701.
[127] S. Agewall, S. Wright, R.N. Doughty, G.A. Whalley, M. Duxbury, N. Sharpe, Does a glass of red wine improve endothelial function? Eur. Heart J. 21 (2000) 74–78.
[128] M. Hashimoto, S. Kim, M. Eto, K. Iijima, J. Ako, M. Yoshizumi, M. Akishita, K. Kondo, H. Itakura, K. Hosoda, K. Toba, Y. Ouchi, Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery, Am. J. Cardiol. 88 (2001) 1457–1460 (A1459).
[129] S.M. Hampton, C. Isherwood, V.J. Kirkpatrick, A.C. Lynne-Smith, B.A. Griffin, The influence of alcohol consumed with a meal on endothelial function in healthy individuals, J. Hum. Nutr. Diet. 23 (2010) 120–125.
[130] M.B. Engler, M.M. Engler, C.Y. Chen, M.J. Malloy, A. Browne, E.Y. Chiu, H.K. Kwak, P. Milbury, S.M. Paul, J. Blumberg, M.L. Mietus-Snyder, Flavonoid-rich dark chocolate improves endothelial function and increases plasma epicatechin concentrations in healthy adults, J. Am. Coll. Nutr. 23 (2004) 197–204.
[131] H. Schroeter, C. Heiss, J. Balzer, P. Kleinbongard, C.L. Keen, N.K. Hollenberg, H. Sies, C. Kwik-Uribe, H.H. Schmitz, M. Kelm, (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans, Proc. Natl. Acad. Sci. USA 103 (2006) 1024–1029.
[132] A. Rodriguez-Mateos, C. Rendeiro, T. Bergillos-Meca, S. Tabatabaee, T.W. George, C. Heiss, J.P. Spencer, Intake and time dependence of blueberry flavonoid-induced improvements in vascular function: a randomized, controlled, double-blind, crossover intervention study with mechanistic insights into biological activity, Am. J. Clin. Nutr. 98 (2013) 1179–1191.
[133] K. Nishioka, T. Hidaka, S. Nakamura, T. Umemura, D. Jitsuiki, J. Soga, C. Goto, K. Chayama, M. Yoshizumi, Y. Higashi, Pycnogenol, French maritime pine bark extract, augments endothelium-dependent vasodilation in humans, Hypertens. Res. 30 (2007) 775–780.
[134] E.A.J. Boon, K.D. Croft, S. Shinde, J.M. Hodgson, N.C. Ward, The acute effect of coffee on endothelial function and glucose metabolism following a glucose load in healthy human volunteers, Food Funct. (2017).
[135] R.M. Alqurashi, L.A. Galante, I.R. Rowland, J.P. Spencer, D.M. Commane, Consumption of a flavonoid-rich acai meal is associated with acute improvements in vascular function and a reduction in total oxidative status in healthy overweight men, Am. J. Clin. Nutr. 104 (2016) 1227–1235.
[136] D. Taubert, R. Berkels, R. Roesen, W. Klaus, Chocolate and blood pressure in elderly individuals with isolated systolic hypertension, JAMA 290 (2003) 1029–1030.
[137] A. Rostami, M. Khalili, N. Haghighat, S. Eghtesadi, F. Shidfar, I. Heidari, S. Ebrahimpour-Koujan, M. Eghtesadi, High-cocoa polyphenol-rich chocolate improves blood pressure in patients with diabetes and hypertension, ARYA Atheroscler. 11 (2015) 21–29.
[138] D. Mastroiacovo, C. Kwik-Uribe, D. Grassi, S. Necozione, A. Raffaele, L. Pistacchio, R. Righetti, R. Bocale, M.C. Lechiara, C. Marini, C. Ferri, G. Desideri, Cocoa flavanol consumption improves cognitive function, blood pressure control, and metabolic profile in elderly subjects: the Cocoa, Cognition, and Aging (CoCoA) study – a randomized controlled trial, Am. J. Clin. Nutr. 101 (2015) 538–548.
[139] M. Aviram, L. Dornfeld, Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure, Atherosclerosis 158 (2001) 195–198.
[140] S. Asgary, A. Sahebkar, M.R. Afshani, M. Keshvari, S. Haghjooyjavanmard, M. Rafieian-Kopaei, Clinical evaluation of blood pressure lowering, endothelial function improving, hypolipidemic and anti-inflammatory effects of pomegranate juice in hypertensive subjects, Phytother. Res. 28 (2014) 193–199.
[141] Y.K. Park, J.S. Kim, M.H. Kang, Concord grape juice supplementation reduces blood pressure in Korean hypertensive men: double-blind, placebo controlled intervention trial, Biofactors 22 (2004) 145–147.
[142] M.M. Dohadwala, N.M. Hamburg, M. Holbrook, B.H. Kim, M.A. Duess, A. Levit, M. Titas, W.B. Chung, F.B. Vincent, T.L. Caiano, A.A. Frame, J.F. Keaney Jr., J.A. Vita, Effects of concord grape juice on ambulatory blood pressure in pre-hypertension and stage 1 hypertension, Am. J. Clin. Nutr. 92 (2010) 1052–1059.
[143] G. Chiva-Blanch, M. Urpi-Sarda, E. Ros, S. Arranz, P. Valderas-Martinez, R. Casas, E. Sacanella, R. Llorach, R.M. Lamuela-Raventos, C. Andres-Lacueva, R. Estruch, Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide: short communication, Circ. Res. 111 (2012) 1065–1068.
[144] D.W. Droste, C. Iliescu, M. Vaillant, M. Gantenbein, N. De Bremaeker, C. Lieunard, T. Velez, M. Meyer, T. Guth, A. Kuemmerle, A. Chioti, A daily glass of red wine and lifestyle changes do not affect arterial blood pressure and heart rate in patients with carotid arteriosclerosis after 4 and 20 weeks, Cerebrovasc. Dis. Extra 3 (2013) 121–129.
[145] E. Park, I. Edirisinghe, Y.Y. Choy, A. Waterhouse, B. Burton-Freeman, Effects of grape seed extract beverage on blood pressure and metabolic indices in individuals with pre-hypertension: a randomised, double-blinded, two-arm, parallel, placebo-controlled trial, Br. J. Nutr. 115 (2016) 226–238.
[146] W.L. Hall, N.L. Formanuik, D. Harnpanich, M. Cheung, D. Talbot, P.J. Chowienczyk, T.A. Sanders, A meal enriched with soy isoflavones increases nitric oxide-mediated vasodilation in healthy postmenopausal women, J. Nutr. 138 (2008) 1288–1292.
[147] C.E. Marsh, H.H. Carter, K.J. Guelfi, K.J. Smith, K.E. Pike, L.H. Naylor, D.J. Green, Brachial and cerebrovascular functions are enhanced in postmenopausal women after ingestion of chocolate with a high concentration of cocoa, J. Nutr. 147 (2017) 1686–1692.
[148] J. Barona, J.C. Aristizabal, C.N. Blesso, J.S. Volek, M.L. Fernandez, Grape polyphenols reduce blood pressure and increase flow-mediated vasodilation in men with metabolic syndrome, J. Nutr. 142 (2012) 1626–1632.
[149] J. Balzer, T. Rassaf, C. Heiss, P. Kleinbongard, T. Lauer, M. Merx, N. Heussen, H.B. Gross, C.L. Keen, H. Schroeter, M. Kelm, Sustained benefits in vascular function through flavanol-containing cocoa in medicated diabetic patients a double-masked, randomized, controlled trial, J. Am. Coll. Cardiol. 51 (2008) 2141–2149.
[150] E.Y. Choi, H. Lee, J.S. Woo, H.H. Jang, S.J. Hwang, H.S. Kim, W.S. Kim, Y.S. Kim, R. Choue, Y.J. Cha, J.E. Yim, W. Kim, Effect of onion peel extract on endothelial function and endothelial progenitor cells in overweight and obese individuals, Nutrition 31 (2015) 1131–1135.
[151] S.R. Coimbra, S.H. Lage, L. Brandizzi, V. Yoshida, P.L. da Luz, The action of red wine and purple grape juice on vascular reactivity is independent of plasma lipids in hypercholesterolemic patients, Braz. J. Med. Biol. Res. 38 (2005) 1339–1347.
[152] R.H. Wong, P.R. Howe, J.D. Buckley, A.M. Coates, I. Kunz, N.M. Berry, Acute resveratrol supplementation improves flow-mediated dilatation in overweight/obese individuals with mildly elevated blood pressure, Nutr. Metab. Cardiovasc. Dis.: NMCD 21 (2011) 851–856.
[153] A.P. Whelan, W.H. Sutherland, M.P. McCormick, D.J. Yeoman, S.A. de Jong, M.J. Williams, Effects of white and red wine on endothelial function in subjects

with coronary artery disease, Intern. Med. J. 34 (2004) 224–228.
[154] K. Karatzi, C. Papamichael, K. Aznaouridis, E. Karatzis, J. Lekakis, C. Matsouka, G. Boskou, A. Chiou, M. Sitara, G. Feliou, D. Kontoyiannis, A. Zampelas, M. Mavrikakis, Constituents of red wine other than alcohol improve endothelial function in patients with coronary artery disease, Coron. Artery Dis. 15 (2004) 485–490.
[155] M.E. Widlansky, N.M. Hamburg, E. Anter, M. Holbrook, D.F. Kahn, J.G. Elliott, J.F. Keaney Jr., J.A. Vita, Acute EGCG supplementation reverses endothelial dysfunction in patients with coronary artery disease, J. Am. Coll. Nutr. 26 (2007) 95–102.
[156] J. Lekakis, L.S. Rallidis, I. Andreadou, G. Vamvakou, G. Kazantzoglou, P. Magiatis, A.L. Skaltsounis, D.T. Kremastinos, Polyphenolic compounds from red grapes acutely improve endothelial function in patients with coronary heart disease, Eur. J. Cardiovasc. Prev. Rehabil. 12 (2005) 596–600 (Working Groups on Epidemiology & Prevention and Cardiac Rehabilitation and Exercise Physiology).
[157] S.J. Duffy, J.F. Keaney Jr, M. Holbrook, N. Gokce, P.L. Swerdloff, B. Frei, J.A. Vita, Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease, Circulation 104 (2001) 151–156.
[158] M.M. Dohadwala, M. Holbrook, N.M. Hamburg, S.M. Shenouda, W.B. Chung, M. Titas, M.A. Kluge, N. Wang, J. Palmisano, P.E. Milbury, J.B. Blumberg, J.A. Vita, Effects of cranberry juice consumption on vascular function in patients with coronary artery disease, Am. J. Clin. Nutr. 93 (2011) 934–940.

# General discussion

NO, a major endothelium-dependent vasodilator, is a soluble gas continuously synthesized by vascular endothelial cells (Tousoulis et al., 2012). A released NO from endothelial cells stimulates soluble guanylyl cyclase (sGC) in vascular smooth muscle cells to produce cGMP, subsequent cGMP activates the protein kinase G (PKG) promoting the uptake of intracellular calcium into the sarcoplasmic reticulum and opening the calcium-dependent potassium channel. The decrease level of intracellular calcium induces vascular smooth muscle cell relaxation (Zhao et al., 2015). Another mechanism is endothelium-dependent relaxation mediated by EDH. The opening of $SK_{ca}$ and $IK_{ca}$ channel expressed in endothelial cells will generate a hyperpolarization, which will be transmitted to the underlying vascular smooth muscle to induce relaxation. $SK_{ca}$ and $IK_{ca}$ can be activated by increase concentration of intracellular calcium, which leads to $K^+$ efflux. A moderate increase of $K^+$ can promote smooth muscle cell hyperpolarization by activating the intracellular regulation of $K^+$ conductance and $Na^+/K^+$ pump (Félétou and Vanhoutte, 2003). The endothelium-derived hyperpolarizing factors such as $K^+$, CYP450, $PGI_2$ and $H_2O_2$ will act at the vascular smooth muscle to induce relaxation by the myo-endothelial gap junction. The $PGI_2$ is synthesized from membrane lipids by the action of $PLA_2$ and COX. $PGI_2$ release form endothelial cells induces smooth muscle cell hyperpolarization by the binding of $PGI_2$ to the receptor of $PGI_2$/IP. $PGI_2$/IP interaction in the cell membrane G-protein, which stimulates cAMP/PKA leading to $Ca^{2+}$ efflux from cytosol and endoplasmic reticulum via $Ca^{2+}$ pumps (Mitchell et al., 2008).

Endothelial dysfunction is characterized by reduced bioavailability of vasodilators, represents an early step of atherosclerosis promoting the evolution of atherosclerosis plaque accompanied with pro-inflammatory and pro-thrombotic responses. The imbalance between the release of endothelium-dependent relaxing and contracting

factor in blood vessels is a major feature of endothelial dysfunction (Hadi et al., 2005). All traditional risk factor of CVDs such as smoking, aging, hyperglycemia, hypertension, premature atherosclerosis are associate with endothelial dysfunction (Widlansky et al., 2003).

Recent study has shown that daily consumption of fresh fruit decreased systolic blood pressure by 4.0 mmHg and blood glucose by 9.0 mg/dl as compare to non-consumption of fresh fruit. Moreover, the decrease CVD event, such as cardiovascular death, major coronary events, ischemia, is inversely related to daily consumption of fresh fruit (Du et al., 2016).

Anthocyanins are plant derived natural pigments abundant in fruit, red wine and vegetables. An average of anthocyanin intake in the United States is estimated from 180 to 215 mg/day. Anthocyanins are not essential nutrients, and there is no disorder associated with lack of anthocyanin intake. However, several clinical studies showed high level of anthocyanins consumption is related to decrease of CVD prevalence. For instance, habitual intake of anthocyanin- and flavone-rich food in men showed that high intake of anthocyanins is lowering a 14% non-fatal myocardial infarction and high intake of flavanone is associated with a 22% lowering of ischemic stroke (Cassidy et al., 2016). One-month anthocyanin-rich strawberry supplements is associated with reduction of total cholesterol, triglyceride and LDL by about 8.78%, 13.72% and 20.80%, respectively (Alvarez-Suarez et al., 2014). Intake of C3G in normal diet in type 2 diabetic *db*/*db* mice for 8 weeks showed that and improvement of endothelium-dependent relaxation in the aorta associated with an increase eNOS phosphorylation at Ser 1177 and of the level of cGMP, and an increased expression of adiponectin (Liu et al., 2014). In addition, several studies are showing an upregulation of basal eNOS expression by C3G (Xu et al., 2004), an anthocyanin-rich purple potatoes extract inhibited D-galactose-induced endothelial senescence by the inhibition of intracellular AGE formation (Sun et al., 2015).

Regarding anthocyanins, they are characterized by a poor stability, which is affected by many environmental factors such as light, pH, temperature and also by their structure. Despite the low absorption and instability of anthocyanins, many studies showed beneficial effect of anthocyanins on cardiovascular health. However, the mechanism of anthocyanin regarding the absorption and subsequent mechanism in endothelial cells are still unclear.

SGLT include two isoforms SGLT1 and SGLT2 which facilitate the renal glucose reabsorption. SGLT2 has a low affinity and a high capacity glucose transporter located in S1 segment of proximal tubule of kidney responsible for ~90% of renal glucose reabsorption, SGLT1 is contributing ~10% of renal glucose reabsorption. SGLT inhibitors prevent glucose reabsorption in the kidney and subsequently lower blood glucose and promotes the elimination of glucose by the urine. Recently, D3G has been shown to protect ECs against oxidized LDL-induced mitochondrial dysfunction subsequent to its entry via SGLT1, suggesting that SGLT1 is, at least in part, an active transporter of D3G (Jin et al., 2013).

Thus, the aim of our study is to identify the effect of blackcurrant anthocyanins and their underlying mechanism in endothelial cells, and in particular, the vascular reactivity studies have indicated that contribution of SGLTs to blackcurrant anthocyanin absorption.

BCE is a potent activator of NO-mediated relaxation in porcine coronary artery rings with endothelium. LX4211 and canagliflozin significantly prevented the NO-mediated relaxation induced by BCE. These finding suggested that blackcurrant anthocyanins induce NO-mediated coronary artery relaxation is mediated by most likely by SGLT1. However, SGLT inhibitors did not affect endothelium dependent and independent relaxation induced by bradykinin and sodium nitroprusside (SNP), respectively. In addition, typical non-glucose conjugated flavonoid epigallocatechin gallate induced NO-mediated relaxation was not affected by SGLT inhibitors. These findings indicated that

the glucose moiety in the anthocyanin structure might be of importance to stimulate the eNOS possibly via SGLT1. In a previous study, blackberry anthocyanins absorption in rat stomach indicated that high levels of C3G and galactoside-conjugated cyanidin was identified, whereas cyanidin-rutinoside was lower than monoglycoside conjugated cyanidin (Talavera et al., 2003). Total anthocyanins contents of BCE showed four major anthocyanins including cyanidin and delphinidin conjugated with glucoside or rutinoside moiety. Indica *et al*., showed blackcurrant anthocyanins induced eNOS phosphorylation through the redox-sensitive activation of PI3K/Akt signaling pathway in human umbilical vein endothelial cell. In addition, each major anthocyanin, C3G: 2.3 μg, C3R: 20 μg, D3G: 5.8 μg and D3R: 29.6 μg, are able to induce eNOS phosphorylation. However, an efficacy of anthocyanins in activation of eNOS are not comparable due to different doses were treated (Edirisinghe et al., 2011). The kinetic study of the activation of Akt and eNOS by BCE indicated a peak level of pAkt and peNOS at 15 min. The stimulatory effect of BCE on eNOS significantly prevented by SGLT inhibitors except empagliflozin. These finding suggest a reduced in BCE uptake into endothelial cells by inhibition of most likely to SGLT1 resulting in the prevention of the subsequent activation of eNOS.

According to the previous research, mRNA expression of SGLT2 is high in the kidney and also to some extent in other tissues such as ileum, brain, trachea, thyroid gland, testis, prostate, skeletal muscle and lung. The SGLT1 mRNA is distinct from SGLT2 mRNA and is highly observed in the ileum, skeletal muscle, coronary artery and kidney (Chen et al., 2010). However, we successfully identified the expression of SGLT1 and 2 proteins in porcine coronary artery endothelial cells using confocal microscopy and Western blot analysis. Moreover, an increased level of expression of both SGLT1 and 2 is observed during sequential subculture of ECs. Present findings suggest, at least in part, the possibility that BCE anthocyanins target the pathological increased expression of SGLT1 and 2 resulting in an increased influx into endothelial cells. The glucoside

conjugated anthocyanin, C3G, showed significant activation of eNOS, that was prevented by inhibition of SGLT1/2, and D3G showed increased extent to eNOS activation but not significantly. It is showing that anthocyanins with sugar moiety is more selectively transported by SGLT1 into endothelial cells. Similarly, anthocyanin uptake is of BCE, C3G and D3G is observed in P1 endothelial cells using flow cytometry and confocal microscopy, and the anthocyanins uptake was prevented by LX4211. However, SGLT2 inhibition by high selective empagliflozin did not prevent BCE anthocyanin uptake. SGLT2 is responsible for about the ~90% of renal glucose reabsorption by the low affinity and high capacity transportation in the kidney, whereas SGLT1 has high affinity and low capacity, may suggest that SGLT2 is more specialized for the transport of high amounts of substrate. In addition, an increase expression of SGLT1 and 2 in P3 endothelial cells showed the prevention of BCE anthocyanin uptake in the presence of LX4211 and empagliflozin, and subsequent preventive effect on the activation of eNOS, including the SGLT1 and SGLT2 contribute the anthocyanin uptake in senescent ECs.

Many lines of evidences are supporting that anthocyanins possess various biological activities including prevention of CVDs. However, various arguments are raised regarding the low bioavailability of anthocyanins. Anthocyanins have strong antioxidant propeties able to protect blood vessels by chelating ROS and also inhibiting ROS formation (Dai et al., 2012, Singletary et al., 2007). In addition, anthocyanins inhibit the expression of adhesion molecules preventing monocyte infiltration to the intimal layer observed in early stages of atherosclerosis (Amin et al., 2015). Moreover, the present findings indicate that BCE anthocyanins promote the activation of eNOS resulting in NO-mediated relaxation in coronary artery rings. Additionally, it can also be suggested that anthocyanins competitively reduce the glucose uptake into the endothelial cells by the sharing the SGLT1/2 leading to a behind effect.

In conclusion, the present finding suggest SGLT1 is contributing to anthocyanin

uptake in healthy endothelial cells, and both SGLT1 and 2 in pathologic endothelial cells, which is promoting the activation of eNOS resulting NO-mediated protective effect.

# Communication and publication

## Communication and publication

### Publication

**Hyun-Ho LEE**, Sin-Hee Park, Eugenia Belcastro, Cyril Auger, MinHo Oak, Claire Kevers, Joel Pincemail, Valérie B. SCHINI-KERTH, Glucose-conjugated blackcurrant anthocyanins activate the endothelial NO synthase pathway following uptake via sodium-glucose cotransporter 1 and 2**,** (Molecular nutrition & Food research, In preparation).

**Hyun-Ho LEE***, Kushal* Sharma, Hira hasan, Dal-seong Kong, Min-Ho Oak, Particulate matter 10 induces endothelial senescence by the activation of redox sensitive local angiotensin system (Environmental health perspectives, In preparation), * equal contribution.

Hira hasan, Eugenia belcastro, **Hyun-Ho LEE**, Malak abbas, Abdul wahid qureshi, Muhammad akmal farooq, Patrick ohlmann, Florence toti, Cyril auger, Valérie. B. SCHINI-KERTH, Olivier morel, Laurence jesel morel, Thrombin promotes premature atrial endothelial cell senescence leading to the induction of pro-infiltrative and pro-fibrotic responses: Role of the local angiotensin II/AT 1 receptor system (In preparation).

Min-ho Oak, Cyril Auger, Eugenia Belcastro, Sin-Hee Park, **Hyun-Ho LEE**, Valérie. B. SCHINI-KERTH, Potential mechanisms underlying cardiovascular protection by polyphenols: Role of the endothelium (*Free Radical Biology and Medicine*, In press, 2018).

Mbaye Sene, Modou Oumy Kane, **Hyun-Ho LEE**, Cyril Auger, Philippe Chabert, Cathérine Vonthron-Sénécheau, Aminata Sall Diallo, Valérie. B. SCHINI-KERTH. Endothelium-dependent relaxation by a hydroethanolic extract of *Adansonia digitata* leaves in porcine coronary artery rings and rat thoracic aorta, mesenteric, carotid artery rings: Role of NO and EDH (In preparation).

### Oral presentation

**Hyunho LEE**, An anthocyanin-rich blackcurrant extract induced NO-mediated relaxation in coronary artery rings

and eNOS phosphorylation in cultured endothelial cells: Role of sodium-glucose cotransporters 1 and 2, (ICMAN-IUPHAR Natural Products, Aberdeen, Scotland, 2017).

**Hyunho LEE,** An anthocyanin-rich blackcurrant extract induced NO-mediated relaxation in coronary artery rings and eNOS phosphorylation in cultured endothelial cells: Role of sodium-glucose cotransporters 1 and 2, (Selected presenter, Doctoral school day conference 2018, Strasbourg, FRANCE)

**Poster presentation**

**Hyunho LEE**, Sonia Khemais-Benkhiat, Philippe Chabert, Cyril Auger, Claire Kevers, Joel Pincemail, Valérie. B. SCHINI-KERTH. An anthocyanin-rich black currant extract prevents high glucose-induced senescence and dysfunction in cultured coronary artery endothelial cells, (Achieves of cardiovascular diseases, Nantes and JCI, Illkirch, France, 2017).

**Hyun-Ho LEE**, Sonia Khemais-Benkhiat, Philippe Chabert, Cyril Auger, Sin-Hee Park, Claire Kevers, Joel Pincemail, Min-Ho Oak, Valérie. B. SCHINI-KERTH. An anthocyanin-rich blackcurrant extract induced NO-mediated relaxation in coronary artery rings and eNOS phosphorylation in cultured endothelial cells: Role of sodium-glucose cotransporters 1 and 2, (ICMAN-IUPHAR Natural Products, Aberdeen, Scotland, 2017).

Mbaye Sene, Modou Oumy Kane, **Hyun-Ho LEE**, Cyril Auger, Philippe Chabert, Cathérine Vonthron-Sénécheau, Aminata Sall Diallo, Valérie. B. SCHINI-KERTH. Endothelium-dependent relaxation by a hydroethanolic extract of *Adansonia digitata* leaves in porcine coronary artery rings and rat thoracic aorta, mesenteric, carotid artery rings: Role of NO and EDH (ICMAN-IUPHAR Natural Products, Aberdeen, Scotland, 2017).

# *References*

## References


ABRAHAM, D. & DISTLER, O. 2007. How does endothelial cell injury start? The role of endothelin in systemic sclerosis. *Arthritis research & therapy,* 9**,** S2.

AGHABABAEE, S. K., VAFA, M., SHIDFAR, F., TAHAVORGAR, A., GOHARI, M., KATEBI, D. & MOHAMMADI, V. 2015. Effects of blackberry (Morus nigra L.) consumption on serum concentration of lipoproteins, apo AI, apo B, and high-sensitivity-C-reactive protein and blood pressure in dyslipidemic patients. *Journal of research in medical sciences: the official journal of Isfahan University of Medical Sciences,* 20**,** 684.

ALBERTS, B., JOHNSON, A., LEWIS, J., RAFF, M., ROBERTS, K. & WALTER, P. 2002. Blood vessels and endothelial cells.

ALVAREZ-SUAREZ, J. M., GIAMPIERI, F., TULIPANI, S., CASOLI, T., DI STEFANO, G., GONZÁLEZ-PARAMÁS, A. M., SANTOS-BUELGA, C., BUSCO, F., QUILES, J. L. & CORDERO, M. D. 2014. One-month strawberry-rich anthocyanin supplementation ameliorates cardiovascular risk, oxidative stress markers and platelet activation in humans. *The Journal of nutritional biochemistry,* 25**,** 289-294.

ALZAID, F., CHEUNG, H.-M., PREEDY, V. R. & SHARP, P. A. 2013. Regulation of glucose transporter expression in human intestinal Caco-2 cells following exposure to an anthocyanin-rich berry extract. *PLoS One,* 8**,** e78932.

AMIN, H. P., CZANK, C., RAHEEM, S., ZHANG, Q., BOTTING, N. P., CASSIDY, A. & KAY, C. D. 2015. Anthocyanins and their physiologically relevant metabolites alter the expression of IL-6 and VCAM-1 in CD40L and oxidized LDL challenged vascular endothelial cells. *Molecular nutrition & food research,* 59**,** 1095-1106.

AMORINI, A. M., LAZZARINO, G., GALVANO, F., FAZZINA, G., TAVAZZI, B. & GALVANO, G. 2003. Cyanidin-3-O-β-glucopyranoside protects myocardium and erythrocytes from oxygen radical-mediated damages. *Free radical research,* 37**,** 453-460.

AUGER, C., KIM, J.-H., TRINH, S., CHATAIGNEAU, T., POPKEN, A. M. & SCHINI-KERTH, V. B. 2011. Fruit juice-induced endothelium-dependent relaxations in isolated porcine coronary arteries: evaluation of different fruit juices and purees and optimization of a red fruit juice blend. *Food & function,* 2**,** 245-250.

BAGCHI, D., SEN, C., BAGCHI, M. & ATALAY, M. 2004. Anti-angiogenic, antioxidant, and anti-carcinogenic properties of a novel anthocyanin-rich berry extract formula. *Biochemistry (Moscow),* 69**,** 75-80.

BELL, E. 2009. Innate immunity: Endothelial cells as sentinels. *Nature reviews immunology,* 9**,** 532.

BIAN, K., DOURSOUT, M. F. & MURAD, F. 2008. Vascular system: role of nitric oxide in cardiovascular diseases. *The journal of clinical hypertension,* 10**,** 304-310.

BISHAYEE, A., MBIMBA, T., THOPPIL, R. J., HÁZNAGY-RADNAI, E., SIPOS, P., DARVESH, A. S., FOLKESSON, H. G. & HOHMANN, J. 2011. Anthocyanin-rich black currant (Ribes nigrum L.) extract affords chemoprevention against diethylnitrosamine-induced hepatocellular carcinogenesis in rats. *The Journal of nutritional biochemistry,* 22**,** 1035-1046.

BORGES, G., LEAN, M. E., ROBERTS, S. A. & CROZIER, A. 2013. Bioavailability of dietary (poly) phenols: a study with ileostomists to discriminate between absorption in small and large intestine. *Food & function,* 4**,** 754-762.

BROWNLEE, M. 2001. Biochemistry and molecular cell biology of diabetic complications. *Nature,* 414**,** 813.

CAMICI, G. G., SCHIAVONI, M., FRANCIA, P., BACHSCHMID, M., MARTIN-PADURA, I., HERSBERGER, M., TANNER, F. C., PELICCI, P., VOLPE, M. & ANVERSA, P. 2007. Genetic deletion of p66Shc adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. *Proceedings of the National Academy of Sciences,* 104**,** 5217-5222.

CASSIDY, A., BERTOIA, M., CHIUVE, S., FLINT, A., FORMAN, J. & RIMM, E. B. 2016. Habitual intake of anthocyanins and flavanones and risk of cardiovascular disease in men, 2. *The American journal of clinical nutrition,* 104**,** 587-594.

CASTANEDA-OVANDO, A., DE LOURDES PACHECO-HERNÁNDEZ, M., PÁEZ-HERNÁNDEZ, M. E., RODRÍGUEZ, J. A. & GALÁN-VIDAL, C. A. 2009. Chemical studies of anthocyanins: A review. *Food chemistry,* 113**,** 859-871.

CHEN, C. & KHISMATULLIN, D. B. 2015. Oxidized low-density lipoprotein contributes to atherogenesis via co-activation of macrophages and mast cells. *PloS one,* 10**,** e0123088.

CHEN, J., WILLIAMS, S., HO, S., LORAINE, H., HAGAN, D., WHALEY, J. M. & FEDER, J. N. 2010. Quantitative PCR tissue expression profiling of the human SGLT2 gene and related family members. *Diabetes Therapy,* 1**,** 57-92.

COUNCIL, N. R. 1989. *Diet and health: implications for reducing chronic disease risk*, National Academies Press.

CROSSMAN, D. C. & MORTON, A. C. 2007. Pathophysiology of Ischemic Heart Disease. *Heart Disease and the Surgical Patient.* CRC Press.

DAI, L.-P., DONG, X.-J. & MA, H.-H. 2012. Antioxidative and chelating properties of anthocyanins in Azolla imbricata induced by cadmium. *Polish Journal of Environmental Studies,* 21.

DALGÅRD, C., NIELSEN, F., MORROW, J. D., ENGHUSEN-POULSEN, H., JONUNG, T., HØRDER, M. & DE

MAAT, M. P. 2008. Supplementation with orange and blackcurrant juice, but not vitamin E, improves inflammatory markers in patients with peripheral arterial disease. *British journal of nutrition,* 101**,** 263-269.

DANESE, S., DEJANA, E. & FIOCCHI, C. 2007. Immune regulation by microvascular endothelial cells: directing innate and adaptive immunity, coagulation, and inflammation. *The Journal of Immunology,* 178**,** 6017-6022.

DAVIS, M. S., MILLER, C. K. & MITCHELL, D. C. 2004. More favorable dietary patterns are associated with lower glycemic load in older adults. *Journal of the American Dietetic Association,* 104**,** 1828-1835.

DESOUZA, C. A., JONES, P. P. & SEALS, D. R. 1998. Physical activity status and adverse age-related differences in coagulation and fibrinolytic factors in women. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 18**,** 362-368.

DU, H., LI, L., BENNETT, D., GUO, Y., KEY, T. J., BIAN, Z., SHERLIKER, P., GAO, H., CHEN, Y. & YANG, L. 2016. Fresh fruit consumption and major cardiovascular disease in China. *New England Journal of Medicine,* 374**,** 1332-1343.

DUSTING, G. J., MONCADA, S. & VANE, J. R. 1977. Prostacyclin (PGX) is the endogeneous metabolite responsible for relaxation of coronary arteries induced by arachidonic acid. *Prostaglandins,* 13**,** 3-15.

EDIRISINGHE, I., BANASZEWSKI, K., CAPPOZZO, J., MCCARTHY, D. & BURTON-FREEMAN, B. M. 2011. Effect of black currant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) in vitro in human endothelial cells. *Journal of agricultural and food chemistry,* 59**,** 8616-8624.

ERLUND, I., KOLI, R., ALFTHAN, G., MARNIEMI, J., PUUKKA, P., MUSTONEN, P., MATTILA, P. & JULA, A. 2008. Favorable effects of berry consumption on platelet function, blood pressure, and HDL cholesterol–. *The American journal of clinical nutrition,* 87**,** 323-331.

FARPOUR-LAMBERT, N. J., AGGOUN, Y., MARCHAND, L. M., MARTIN, X. E., HERRMANN, F. R. & BEGHETTI, M. 2009. Physical activity reduces systemic blood pressure and improves early markers of atherosclerosis in pre-pubertal obese children. *Journal of the American College of Cardiology,* 54**,** 2396-2406.

FÉLÉTOU, M. & VANHOUTTE, P. M. 2003. Third pathway: Endothelium-dependent hyperpolarization. *Drug development research,* 58**,** 18-22.

FÉLÉTOU, M. & VANHOUTTE, P. M. 2007. Endothelium-dependent hyperpolarizations: past beliefs and present facts. *Annals of medicine,* 39**,** 495-516.

FELGINES, C., TALAVÉRA, S. V., GONTHIER, M.-P., TEXIER, O., SCALBERT, A., LAMAISON, J.-L. & RÉMÉSY, C. 2003. Strawberry anthocyanins are recovered in urine as glucuro-and sulfoconjugates in humans. *The Journal of nutrition,* 133**,** 1296-1301.

FENG, R., NI, H.-M., WANG, S. Y., TOURKOVA, I. L., SHURIN, M. R., HARADA, H. & YIN, X.-M. 2007. Cyanidin-3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemic cells by induction of oxidative stress. *Journal of Biological Chemistry,* 282**,** 13468-13476.

FLEMING, I. 2010. Molecular mechanisms underlying the activation of eNOS. *Pflügers Archiv-European Journal of Physiology,* 459**,** 793-806.

FLEMING, I. 2014. The pharmacology of the cytochrome P450 epoxygenase/soluble epoxide hydrolase axis in the vasculature and cardiovascular disease. *Pharmacological reviews,* 66**,** 1106-1140.

FUKAMI, K., YAMAGISHI, S.-I. & OKUDA, S. 2014. Role of AGEs-RAGE system in cardiovascular disease. *Current pharmaceutical design,* 20**,** 2395-2402.

GERTHOFFER, W. T. 2007. Mechanisms of vascular smooth muscle cell migration. *Circulation research,* 100**,** 607-621.

GHEZZI, C., LOO, D. D. & WRIGHT, E. M. 2018. Physiology of renal glucose handling via SGLT1, SGLT2 and GLUT2. *Diabetologia***,** 1-11.

GHOSH, R., ALAJBEGOVIC, A. & GOMES, A. V. 2015. NSAIDs and cardiovascular diseases: role of reactive oxygen species. *Oxidative medicine and cellular longevity,* 2015.

GOLDIN, A., BECKMAN, J. A., SCHMIDT, A. M. & CREAGER, M. A. 2006. Advanced glycation end products: sparking the development of diabetic vascular injury. *Circulation,* 114**,** 597-605.

GREMPLER, R., THOMAS, L., ECKHARDT, M., HIMMELSBACH, F., SAUER, A., SHARP, D., BAKKER, R., MARK, M., KLEIN, T. & EICKELMANN, P. 2012. Empagliflozin, a novel selective sodium glucose cotransporter-2 (SGLT-2) inhibitor: characterisation and comparison with other SGLT-2 inhibitors. *Diabetes, Obesity and Metabolism,* 14**,** 83-90.

GUO, X., OLDHAM, M., KLEINMAN, M., PHALEN, R. & KASSAB, G. 2006. Effect of cigarette smoking on nitric oxide, structural, and mechanical properties of mouse arteries. *American Journal of Physiology-Heart and Circulatory Physiology,* 291**,** H2354-H2361.

HADI, H. A., CARR, C. S. & AL SUWAIDI, J. 2005. Endothelial dysfunction: cardiovascular risk factors, therapy, and outcome. *Vascular health and risk management,* 1**,** 183.

HANSSON, G. K. 2005. Inflammation, atherosclerosis, and coronary artery disease. *New England Journal of Medicine,* 352**,** 1685-1695.

HANSSON, G. K. & HERMANSSON, A. 2011. The immune system in atherosclerosis. *Nature immunology,* 12**,** 204.

HARADA, N. & INAGAKI, N. 2012. Role of sodium-glucose transporters in glucose uptake of the intestine and

kidney. *Journal of diabetes investigation,* 3**,** 352-353.

HAUSENLOY, D. J. & YELLON, D. M. 2013. Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *The Journal of clinical investigation,* 123**,** 92-100.

HERTOG, M. G., KROMHOUT, D., ARAVANIS, C., BLACKBURN, H., BUZINA, R., FIDANZA, F., GIAMPAOLI, S., JANSEN, A., MENOTTI, A. & NEDELJKOVIC, S. 1995. Flavonoid intake and long-term risk of coronary heart disease and cancer in the seven countries study. *Archives of internal medicine,* 155**,** 381-386.

IWASAKI-KURASHIGE, K., LOYAGA-RENDON, R. Y., MATSUMOTO, H., TOKUNAGA, T. & AZUMA, H. 2006. Possible mediators involved in decreasing peripheral vascular resistance with blackcurrant concentrate (BC) in hind-limb perfusion model of the rat. *Vascular pharmacology,* 44**,** 215-223.

JALDAPPAGARI, S., MOTOHASHI, N., GANGEENAHALLI, M. P. & NAISMITH, J. H. 2008. Bioactive mechanism of interaction between anthocyanins and macromolecules like DNA and proteins. *Bioactive Heterocycles VI.* Springer.

JENNINGS, A., WELCH, A. A., FAIRWEATHER-TAIT, S. J., KAY, C., MINIHANE, A.-M., CHOWIENCZYK, P., JIANG, B., CECELJA, M., SPECTOR, T. & MACGREGOR, A. 2012. Higher anthocyanin intake is associated with lower arterial stiffness and central blood pressure in women–. *The American journal of clinical nutrition,* 96**,** 781-788.

JEREMY, J. Y., ROWE, D., EMSLEY, A. M. & NEWBY, A. C. 1999. Nitric oxide and the proliferation of vascular smooth muscle cells. *Cardiovascular research,* 43**,** 580-594.

JIN, X., YI, L., CHEN, M.-L., CHEN, C.-Y., CHANG, H., ZHANG, T., WANG, L., ZHU, J.-D., ZHANG, Q.-Y. & MI, M.-T. 2013. Delphinidin-3-glucoside protects against oxidized low-density lipoprotein-induced mitochondrial dysfunction in vascular endothelial cells via the sodium-dependent glucose transporter SGLT1. *PloS one,* 8**,** e68617.

JOHNSON, C. & GALIS, Z. S. 2004. Matrix metalloproteinase-2 and− 9 differentially regulate smooth muscle cell migration and cell-mediated collagen organization. *Arteriosclerosis, thrombosis, and vascular biology,* 24**,** 54-60.

JOSHIPURA, K. J., ASCHERIO, A., MANSON, J. E., STAMPFER, M. J., RIMM, E. B., SPEIZER, F. E., HENNEKENS, C. H., SPIEGELMAN, D. & WILLETT, W. C. 1999. Fruit and vegetable intake in relation to risk of ischemic stroke. *Jama,* 282**,** 1233-1239.

JURGOŃSKI, A., JUŚKIEWICZ, J., ZDUŃCZYK, Z., MATUSEVICIUS, P. & KOŁODZIEJCZYK, K. 2014. Polyphenol-rich extract from blackcurrant pomace attenuates the intestinal tract and serum lipid changes induced by a high-fat diet in rabbits. *European journal of nutrition,* 53**,** 1603-1613.

KAHN, B. B., ROSSETTI, L., LODISH, H. F. & CHARRON, M. J. 1991. Decreased in vivo glucose uptake but

normal expression of GLUT1 and GLUT4 in skeletal muscle of diabetic rats. *The Journal of clinical investigation,* 87**,** 2197-2206.

KANNEL, W. B., MCGEE, D. & GORDON, T. 1976. A general cardiovascular risk profile: the Framingham Study. *The American journal of cardiology,* 38**,** 46-51.

KAPASAKALIDIS, P. G., RASTALL, R. A. & GORDON, M. H. 2006. Extraction of polyphenols from processed black currant (Ribes nigrum L.) residues. *Journal of agricultural and food chemistry,* 54**,** 4016-4021.

KAY, C. D., MAZZA, G., HOLUB, B. J. & WANG, J. 2004. Anthocyanin metabolites in human urine and serum. *British Journal of Nutrition,* 91**,** 933-942.

KERRIGAN, C. L. & STOTLAND, M. A. 1993. Ischemia reperfusion injury: a review. *Microsurgery,* 14**,** 165-175.

KITABCHI, A. E., UMPIERREZ, G. E., MILES, J. M. & FISHER, J. N. 2009. Hyperglycemic crises in adult patients with diabetes. *Diabetes care,* 32**,** 1335-1343.

KOMORI, K., LORENZ, R. R. & VANHOUTTE, P. M. 1988. Nitric oxide, ACh, and electrical and mechanical properties of canine arterial smooth muscle. *American Journal of Physiology-Heart and Circulatory Physiology,* 255**,** H207-H212.

KONG, J.-M., CHIA, L.-S., GOH, N.-K., CHIA, T.-F. & BROUILLARD, R. 2003. Analysis and biological activities of anthocyanins. *Phytochemistry,* 64**,** 923-933.

KURILICH, A. C., CLEVIDENCE, B. A., BRITZ, S. J., SIMON, P. W. & NOVOTNY, J. A. 2005. Plasma and urine responses are lower for acylated vs nonacylated anthocyanins from raw and cooked purple carrots. *Journal of Agricultural and Food Chemistry,* 53**,** 6537-6542.

LACKLAND, D. T. & WEBER, M. A. 2015. Global burden of cardiovascular disease and stroke: hypertension at the core. *Canadian Journal of Cardiology,* 31**,** 569-571.

LAUFS, U., WERNER, N., LINK, A., ENDRES, M., WASSMANN, S., JÜRGENS, K., MICHE, E., BÖHM, M. & NICKENIG, G. 2004. Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis. *Circulation,* 109**,** 220-226.

LIBBY, P., RIDKER, P. M. & MASERI, A. 2002. Inflammation and atherosclerosis. *Circulation,* 105**,** 1135-1143.

LIN, Y., HOFFMAN, N., AKSOY, M., MUNISWAMY, M. & KELSEN, S. 2012. Cigarette smoke-induced reactive oxygen species (ROS) production in human airway epithelial cells is calcium and NADPH-oxidase (NOX) dependent. *C53. COPD pathogenesis: in vitro and in vivo studies.* American Thoracic Society.

LIU, Y., LI, D., ZHANG, Y., SUN, R. & XIA, M. 2014. Anthocyanin increases adiponectin secretion and protects against diabetes-related endothelial dysfunction. *American Journal of Physiology-Endocrinology and Metabolism,* 306**,** E975-E988.

LUKSHA, L., AGEWALL, S. & KUBLICKIENE, K. 2009. Endothelium-derived hyperpolarizing factor in vascular physiology and cardiovascular disease. *Atherosclerosis,* 202**,** 330-344.

MAJED, B. H. & KHALIL, R. A. 2012. Molecular mechanisms regulating the vascular prostacyclin pathways and their adaptation during pregnancy and in the newborn. *Pharmacological reviews***,** pr. 111.004770.

MALIK, M., ZHAO, C., SCHOENE, N., GUISTI, M. M., MOYER, M. P. & MAGNUSON, B. A. 2003. Anthocyanin-rich extract from Aronia meloncarpa E. induces a cell cycle block in colon cancer but not normal colonic cells. *Nutrition and cancer,* 46**,** 186-196.

MALLERY, S. R., BUDENDORF, D. E., LARSEN, M. P., PEI, P., TONG, M., HOLPUCH, A. S., LARSEN, P. E., STONER, G. D., FIELDS, H. W. & CHAN, K. K. 2011. Effects of human oral mucosal tissue, saliva and oral microflora on intraoral metabolism and bioactivation of black raspberry anthocyanins. *Cancer Prevention Research***,** canprevres. 0040.2011.

MANN, G. E., YUDILEVICH, D. L. & SOBREVIA, L. 2003. Regulation of amino acid and glucose transporters in endothelial and smooth muscle cells. *Physiological reviews,* 83**,** 183-252.

MARCUS, A. J., WEKSLER, B. B., JAFFE, E. A. & BROEKMAN, M. J. 1980. Synthesis of prostacyclin from platelet-derived endoperoxides by cultured human endothelial cells. *The Journal of clinical investigation,* 66**,** 979-986.

MARON, B. A. & MICHEL, T. 2012. Subcellular localization of oxidants and redox modulation of endothelial nitric oxide synthase. *Circulation Journal,* 76**,** 2497-2512.

MATUS, J. T., LOYOLA, R., VEGA, A., PEÑA-NEIRA, A., BORDEU, E., ARCE-JOHNSON, P. & ALCALDE, J. A. 2009. Post-veraison sunlight exposure induces MYB-mediated transcriptional regulation of anthocyanin and flavonol synthesis in berry skins of Vitis vinifera. *Journal of experimental botany,* 60**,** 853-867.

MEEUWISSE, G. 1970. Glucose-galactose malabsorption. Studies on renal glucosuria. *Helvetica paediatrica acta,* 25**,** 13-24.

MEYERS, K. J., WATKINS, C. B., PRITTS, M. P. & LIU, R. H. 2003. Antioxidant and antiproliferative activities of strawberries. *Journal of agricultural and food chemistry,* 51**,** 6887-6892.

MINSHALL, R. & MALIK, A. 2006. Transport across the endothelium: regulation of endothelial permeability. *The Vascular Endothelium I.* Springer.

MITCHELL, J. A., ALI, F., BAILEY, L., MORENO, L. & HARRINGTON, L. S. 2008. Role of nitric oxide and prostacyclin as vasoactive hormones released by the endothelium. *Experimental physiology,* 93**,** 141-147.

MIYAZAWA, T., NAKAGAWA, K., KUDO, M., MURAISHI, K. & SOMEYA, K. 1999. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3, 5-diglucoside, into rats and humans. *Journal*

*of agricultural and food chemistry,* 47**,** 1083-1091.

MORAN, A. E., FOROUZANFAR, M. H., ROTH, G., MENSAH, G. A., EZZATI, M., FLAXMAN, A., MURRAY, C. J. & NAGHAVI, M. 2014. The global burden of ischemic heart disease in 1990 and 2010: the Global Burden of Disease 2010 study. *Circulation***,** CIRCULATIONAHA. 113.004046.

MORITA, I. 2002. Distinct functions of COX-1 and COX-2. *Prostaglandins & other lipid mediators,* 68**,** 165-175.

MOSCA, L., BARRETT-CONNOR, E. & WENGER, N. K. 2011. Sex/gender differences in cardiovascular disease prevention: what a difference a decade makes. *Circulation,* 124**,** 2145-54.

MUECKLER, M. 1994. Facilitative glucose transporters. *European journal of biochemistry,* 219**,** 713-725.

MURRAY, C. D. 1926. The physiological principle of minimum work: I. The vascular system and the cost of blood volume. *Proceedings of the National Academy of Sciences,* 12**,** 207-214.

NANASHIMA, N., HORIE, K., MAEDA, H., TOMISAWA, T., KITAJIMA, M. & NAKAMURA, T. 2018. Blackcurrant Anthocyanins Increase the Levels of Collagen, Elastin, and Hyaluronic Acid in Human Skin Fibroblasts and Ovariectomized Rats. *Nutrients,* 10**,** 495.

NASEEM, K. M. 2005. The role of nitric oxide in cardiovascular diseases. *Molecular aspects of medicine,* 26**,** 33-65.

NG, M., FLEMING, T., ROBINSON, M., THOMSON, B., GRAETZ, N., MARGONO, C., MULLANY, E. C., BIRYUKOV, S., ABBAFATI, C. & ABERA, S. F. 2014. Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The lancet,* 384**,** 766-781.

NIH. 2014. *What is stroke?* [Online]. Available: https://www.nhlbi.nih.gov/health-topics/stroke [Accessed 2, 8 2015].

OAK, M. H., BEDOUI, J., MADEIRA, S. F., CHALUPSKY, K. & SCHINI-KERTH, V. 2006. Delphinidin and cyanidin inhibit PDGFAB-induced VEGF release in vascular smooth muscle cells by preventing activation of p38 MAPK and JNK. *British journal of pharmacology,* 149**,** 283-290.

OZKOR, M. A. & QUYYUMI, A. A. 2011. Endothelium-derived hyperpolarizing factor and vascular function. *Cardiology research and practice,* 2011.

PAIXÃO, J., DINIS, T. C. & ALMEIDA, L. M. 2011. Dietary anthocyanins protect endothelial cells against peroxynitrite-induced mitochondrial apoptosis pathway and Bax nuclear translocation: an in vitro approach. *Apoptosis,* 16**,** 976.

PARTHASARATHY, S., WIELAND, E. & STEINBERG, D. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proceedings of the National Academy of Sciences,* 86**,**

1046-1050.

PENNATHUR, S. & HEINECKE, J. W. 2007. Mechanisms for oxidative stress in diabetic cardiovascular disease. *Antioxidants & redox signaling,* 9**,** 955-969.

PIGA, R., NAITO, Y., KOKURA, S., HANDA, O. & YOSHIKAWA, T. 2007. Short-term high glucose exposure induces monocyte-endothelial cells adhesion and transmigration by increasing VCAM-1 and MCP-1 expression in human aortic endothelial cells. *Atherosclerosis,* 193**,** 328-334.

POBER, J. S. & SESSA, W. C. 2007. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology,* 7**,** 803.

POJER, E., MATTIVI, F., JOHNSON, D. & STOCKLEY, C. S. 2013. The case for anthocyanin consumption to promote human health: a review. *Comprehensive Reviews in Food Science and Food Safety,* 12**,** 483-508.

POULTER, N. R., PRABHAKARAN, D. & CAULFIELD, M. 2015. Hypertension. *Lancet,* 386**,** 801-12.

PRIOR, R. L. & WU, X. 2006. Anthocyanins: structural characteristics that result in unique metabolic patterns and biological activities. *Free radical research,* 40**,** 1014-1028.

QIN, Y., XIA, M., MA, J., HAO, Y., LIU, J., MOU, H., CAO, L. & LING, W. 2009. Anthocyanin supplementation improves serum LDL-and HDL-cholesterol concentrations associated with the inhibition of cholesteryl ester transfer protein in dyslipidemic subjects–. *The American journal of clinical nutrition,* 90**,** 485-492.

RENIS, M., CALANDRA, L., SCIFO, C., TOMASELLO, B., CARDILE, V., VANELLA, L., BEI, R., LA FAUCI, L. & GALVANO, F. 2008. Response of cell cycle/stress-related protein expression and DNA damage upon treatment of CaCo2 cells with anthocyanins. *British journal of nutrition,* 100**,** 27-35.

RIZZO, D. C. 2015. *Fundamentals of anatomy and physiology*, Cengage Learning.

SANDOO, A., VAN ZANTEN, J. J. V., METSIOS, G. S., CARROLL, D. & KITAS, G. D. 2010. The endothelium and its role in regulating vascular tone. *The open cardiovascular medicine journal,* 4**,** 302.

SANDOW, S. L., GZIK, D. J. & LEE, R. M. 2009. Arterial internal elastic lamina holes: relationship to function? *Journal of anatomy,* 214**,** 258-266.

SCOTT, J. 2004. Pathophysiology and biochemistry of cardiovascular disease. *Current opinion in genetics & development,* 14**,** 271-279.

SENA, C. M., PEREIRA, A. M. & SEIÇA, R. 2013. Endothelial dysfunction—a major mediator of diabetic vascular disease. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease,* 1832**,** 2216-2231.

SERRAINO, I., DUGO, L., DUGO, P., MONDELLO, L., MAZZON, E., DUGO, G., CAPUTI, A. P. & CUZZOCREA, S. 2003. Protective effects of cyanidin-3-O-glucoside from blackberry extract against peroxynitrite-

induced endothelial dysfunction and vascular failure. *Life sciences,* 73**,** 1097-1114.

SHIMOKAWA, H., YASUTAKE, H., FUJII, K., OWADA, M. K., NAKAIKE, R., FUKUMOTO, Y., TAKAYANAGI, T., NAGAO, T., EGASHIRA, K. & FUJISHIMA, M. 1996. The importance of the hyperpolarizing mechanism increases as the vessel size decreases in endothelium-dependent relaxations in rat mesenteric circulation. *Journal of cardiovascular pharmacology,* 28**,** 703-711.

SHIPP, J. & ABDEL-AAL, E.-S. M. 2010. Food applications and physiological effects of anthocyanins as functional food ingredients. *The Open Food Science Journal,* 4.

SINGLETARY, K. W., JUNG, K.-J. & GIUSTI, M. 2007. Anthocyanin-rich grape extract blocks breast cell DNA damage. *Journal of medicinal food,* 10**,** 244-251.

SITIA, S., TOMASONI, L., ATZENI, F., AMBROSIO, G., CORDIANO, C., CATAPANO, A., TRAMONTANA, S., PERTICONE, F., NACCARATO, P. & CAMICI, P. 2010. From endothelial dysfunction to atherosclerosis. *Autoimmunity reviews,* 9**,** 830-834.

SPINA, M., GARBISA, S., HINNIE, J., HUNTER, J. C. & SERAFINI-FRACASSINI, A. 1983. Age-related changes in composition and mechanical properties of the tunica media of the upper thoracic human aorta. *Arteriosclerosis: An Official Journal of the American Heart Association, Inc.,* 3**,** 64-76.

STEVEN, S., OELZE, M., HANF, A., KRÖLLER-SCHÖN, S., KASHANI, F., ROOHANI, S., WELSCHOF, P., KOPP, M., GÖDTEL-ARMBRUST, U. & XIA, N. 2017. The SGLT2 inhibitor empagliflozin improves the primary diabetic complications in ZDF rats. *Redox biology,* 13**,** 370-385.

SUMPIO, B. E., RILEY, J. T. & DARDIK, A. 2002. Cells in focus: endothelial cell. *The international journal of biochemistry & cell biology,* 34**,** 1508-1512.

SUN, C., FAN, S., WANG, X., LU, J., ZHANG, Z., WU, D., SHAN, Q. & ZHENG, Y. 2015. Purple sweet potato color inhibits endothelial premature senescence by blocking the NLRP3 inflammasome. *The Journal of nutritional biochemistry,* 26**,** 1029-1040.

TALAVÉRA, S., FELGINES, C., TEXIER, O., BESSON, C., GIL-IZQUIERDO, A., LAMAISON, J.-L. & RÉMÉSY, C. 2005. Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain. *Journal of agricultural and food chemistry,* 53**,** 3902-3908.

TALAVERA, S., FELGINES, C., TEXIER, O., BESSON, C., LAMAISON, J.-L. & RÉMÉSY, C. 2003. Anthocyanins are efficiently absorbed from the stomach in anesthetized rats. *The Journal of nutrition,* 133**,** 4178-4182.

TIAN, Q., GIUSTI, M. M., STONER, G. D. & SCHWARTZ, S. J. 2006. Urinary excretion of black raspberry (Rubus occidentalis) anthocyanins and their metabolites. *Journal of agricultural and food chemistry,* 54**,** 1467-1472.

TOUSOULIS, D., KAMPOLI, A.-M., TENTOLOURIS NIKOLAOS PAPAGEORGIOU, C. & STEFANADIS, C. 2012.

The role of nitric oxide on endothelial function. *Current vascular pharmacology,* 10**,** 4-18.

TSUDA, T. 2012. Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. *Molecular nutrition & food research,* 56**,** 159-170.

TSUDA, T., HORIO, F. & OSAWA, T. 1999. Absorption and metabolism of cyanidin 3-O-β-D-glucoside in rats. *FEBS letters,* 449**,** 179-182.

TSUDA, T., HORIO, F., UCHIDA, K., AOKI, H. & OSAWA, T. 2003. Dietary cyanidin 3-O-β-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice. *The Journal of nutrition,* 133**,** 2125-2130.

TURK, E., MARTÍN, M. G. & WRIGHT, E. M. 1994. Structure of the human Na+/glucose cotransporter gene SGLT1. *Journal of Biological Chemistry,* 269**,** 15204-15209.

TURK, E. & WRIGHT, E. 1997. Membrane topology motifs in the SGLT cotransporter family. *The Journal of membrane biology,* 159**,** 1-20.

TURRENS, J. F. 2003. Mitochondrial formation of reactive oxygen species. *The Journal of physiology,* 552**,** 335-344.

WANG, S. Y. & JIAO, H. 2000. Scavenging capacity of berry crops on superoxide radicals, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. *Journal of Agricultural and Food Chemistry,* 48**,** 5677-5684.

WHELTON, P. K., CAREY, R. M., ARONOW, W. S., CASEY, D. E., COLLINS, K. J., HIMMELFARB, C. D., DEPALMA, S. M., GIDDING, S., JAMERSON, K. A. & JONES, D. W. 2018. 2017 ACC/AHA/AAPA/ABC/ACPM/AGS/APhA/ASH/ASPC/NMA/PCNA guideline for the prevention, detection, evaluation, and management of high blood pressure in adults: a report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Journal of the American College of Cardiology,* 71**,** e127-e248.

WHO. 2018. *The top 10 global causes of deaths* [Online]. Available: http://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death [Accessed 25, 5 2018].

WIDLANSKY, M. E., GOKCE, N., KEANEY, J. F. & VITA, J. A. 2003. The clinical implications of endothelial dysfunction. *Journal of the American College of Cardiology,* 42**,** 1149-1160.

WINKEL-SHIRLEY, B. 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. *Plant physiology,* 126**,** 485-493.

WITTER, K., TONAR, Z. & SCHÖPPER, H. 2017. How many layers has the adventitia?–Structure of the arterial tunica externa revisited. *Anatomia, histologia, embryologia,* 46**,** 110-120.

WONG, L. C. & LANGILLE, B. L. 1996. Developmental remodeling of the internal elastic lamina of rabbit arteries: effect of blood flow. *Circulation research,* 78**,** 799-805.

WOULFE, D., YANG, J. & BRASS, L. 2001. ADP and platelets: the end of the beginning. *The Journal of clinical investigation,* 107**,** 1503-1505.

XIE, X., ZHAO, R. & SHEN, G. X. 2012. Influence of delphinidin-3-glucoside on oxidized low-density lipoprotein-induced oxidative stress and apoptosis in cultured endothelial cells. *Journal of agricultural and food chemistry,* 60**,** 1850-1856.

XING, K., MURTHY, S., LILES, W. C. & SINGH, J. M. 2012. Clinical utility of biomarkers of endothelial activation in sepsis-a systematic review. *Critical Care,* 16**,** R7.

XU, J.-W., IKEDA, K. & YAMORI, Y. 2004. Upregulation of endothelial nitric oxide synthase by cyanidin-3-glucoside, a typical anthocyanin pigment. *Hypertension,* 44**,** 217-222.

ZAFRA-STONE, S., YASMIN, T., BAGCHI, M., CHATTERJEE, A., VINSON, J. A. & BAGCHI, D. 2007. Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular nutrition & food research,* 51**,** 675-683.

ZAVA, D. T. & DUWE, G. 1997. Estrogenic and antiproliferative properties of genistein and other flavonoids in human breast cancer cells in vitro.

ZHAO, Y., VANHOUTTE, P. M. & LEUNG, S. W. 2015. Vascular nitric oxide: Beyond eNOS. *Journal of pharmacological sciences,* 129**,** 83-94.

ZIBERNA, L., TRAMER, F., MOZE, S., VRHOVSEK, U., MATTIVI, F. & PASSAMONTI, S. 2012. Transport and bioactivity of cyanidin 3-glucoside into the vascular endothelium. *Free radical biology and medicine,* 52**,** 1750-1759.

Hyunho LEE

# Activation de la voie du monoxyde d'azote dans les cellules endothéliales par les anthocyanes du cassis : Caractérisation des molécules actives et rôle des co-transporteurs sodium-glucose 1 et 2


## Résumé

Depuis quelques décennies, de nombreuses données suggèrent que l'effet protecteur cardiovasculaire des anthocyanes implique vraisemblablement une amélioration de la fonction endothéliale par une augmentation de la formation de monoxyde d'azote (NO). Cependant, les mécanismes protecteurs du transport intracellulaire des anthocyanes dans la cellule endothéliale demeurent mal compris. L'objectif de cette thèse est d'évaluer la contribution de SGLT1 et SGLT2, les co-transporteurs majeurs du sodium et du glucose, dans l'entrée des anthocyanes issues du cassis et de ses dérivés glucoside et rutinoside dans les cellules endothéliales. Cette entrée promeut l'activation de la voie de la monoxyde d'azote synthase endothéliale (eNOS) qui est ici étudiée par l'utilisation de vaisseaux isolés et de cellules endothéliales en culture. Un extrait de cassis riche en anthocyanes (BCE) induit la relaxation dépendante de l'endothélium par la voie du NO sur des anneaux d'artère coronaire de porc et active la voie de signalisation Akt-eNOS au sein des cellules endothéliales en culture. De plus, des expériences additionnelles suggèrent que l'effet protecteur des anthocyanes dépend à la fois du type de glucoside présent dans la structure des anthocyanes mais aussi de la contribution des transporteurs SGLTs dans l'influx cellulaire des anthocyanes. La capacité des anthocyanes à lutter contre la dysfonction endothéliale est hautement potentialisée dans un modèle cellulaire de sénescence réplicative par l'augmentation de l'influx des anthocyanes due à une forte expression des SGLTs. L'ensemble de ces données indique que les anthocyanes extraits du cassis sont de puissants activateurs de la voie du NO endothélial dans les cellules natives et en culture. Parmi les anthocyanes contenus dans le cassis, les dérivés glycosidiques comme la cyanidine et la delphinidine-3-*O*-glucoside, sont les anthocyanes les plus puissantes afin d'activer la voie du NO. En conclusion, les anthocyanes peuvent être particulièrement intéressantes afin de cibler précocement les sites à risque d'athérosclérose par leur effet de stimulation de l'expression des transporteurs SGLT1 et 2.


Mots-clés : Cassis, sénescence endothéliale, co-transporteur sodium glucose, monoxyde d'azote.


## Abstract

Since last few decades, considerable data have been suggested that the protective effect of anthocyanin on cardiovascular system is likely to involve an improvement of endothelial function by increase nitric oxide (NO) formation. However, comprehensive studies on the subsequent mechanisms of protective effect by anthocyanin intracellular transportation in vascular endothelial cell is poorly understood. The aim of this thesis is to evaluate the possibility that SGLT1 and 2, the two major sodium-glucose cotransporters (SGLT), contribute to blackcurrant anthocyanins and its major glucoside- and rutinoside-conjugated anthocyanins uptake into endothelial cells that promoting the subsequent activation of endothelial nitric oxide synthase (eNOS) pathway using isolated blood vessels and cultured endothelial cells. An anthocyanin rich blackcurrant extract (BCE) induced NO-mediated endothelium dependent relaxation in porcine coronary artery rings and activated Akt-eNOS signaling pathway in cultured endothelial cell. Furthermore, additional experiments suggested that such a protective effect of anthocyanin is based on the type of glucoside in anthocyanin structure and contribution of SGLTs for the intracellular transportation of anthocyanins. An ability of anthocyanin against endothelial dysfunction is highly potentiated in the endothelial cell replicative senescence model by the increase anthocyanin efflux according to the high expression of SGLTs. Altogether, the present findings indicate that blackcurrant anthocyanins are potent activator of the endothelial NO pathway in native and cultured endothelial cells. Among blackcurrant anthocyanins, glucose derivatives such as cyanidin and delphinidin -3-*O*-glucoside are the most potent anthocyanins for activation of NO pathway. In conclusion, anthocyanin can be more prominent by preferentially targeting an early stage of atherosclerotic site by their increase expression of SGLT1 and 2.


Key word: Blackcurrant, endothelial senescence, sodium-glucose cotransporter, nitric oxide