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THÈSE présentée par :

Ahmed Bey CHAKER

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Potential of anthocyanins to prevent age-related endothelial senescence and dysfunction

THÈSE dirigée par :

Mr AUGER cyril	Chargé de Recherche, Université de Strasbourg
Mme BENKIRANE-JESSEL Nadia	Directrice de Recherche, Université de Strasbourg

RAPPORTEURS :

Mr BARON Denis	Professeur des Université, Nestlé Institute of Health Sciences, Suisse
Mr PINCEMAIL Jöel	Professeur des Université, Université de Liège, Belgique

AUTRES MEMBRES DU JURY :

Mme BOURGUIGNON Marie-Pierre	Docteur, Institut de Recherche Servier
Mr HEINTZ Dimitri	Ingénieur de Recherche, Université de Strasbourg

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INVITÉS : (le cas échéant)
Mme SCHINI-KERTH Valérie

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Abbreviations

ACE: Angiotensin converting enzyme

ADMA: Asymmetric Dimethyl-L-Arginine

ADP: Adenosine diphosphate

AGEs: Advanced glycation end products

ARB: Anthocyanins-rich blackcurrant juice

Ang I: angiotensin I

AT1R: Angiotensin II Type 1 receptor

Ang II: Angiotensin II

AT2R: Angiotensin II Type 2 receptor

BH_4: Tetrahydrobiopterin

BMI: Body mass index

BP: Blood pressure

C3G: Cyanidin-3-*O*-glucoside

C3R: Cyanidin-3-*O*-rutinoside

Ca^{2+}: calcium

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

cGMP: Cyclic guanosine monophosphate

CHD: Coronary heart disease

COX: Cyclooxygenase

CRP: C-reactive protein

CTGF: Connective tissue growth factor

CVD: cardiovascular disease

CYP450: Cytochromes P450

D3G: Delphinidin-3-*O*-glucoside

D3R: Delphinidin-3-*O*-rutinoside

DNA: Deoxyribonucleic acid

E1A: Adenoviral protein

ECE: Endothelin converting enzyme

ECM: Extracellular matrix

EDH: Endothelium derived hyperpolarization

eNOS: Endothelial nitric oxide synthase

ET-1: Endothelin-1

FDA: U.S food and drug administration

FFA: Free fatty acids

FHS: Framingham Heart Study

GLUT: Glucose transporter

GSH: Glutathione

HDL: High density lipoproteins

HF: Heart failure

HO-1: Heme oxygenase 1

HUVEC: Human umbilical vein endothelial cell

IHD: Ischemic heart disease

IK_{Ca}: intermediate conductance Calcium-activated potassium channels

IL-1β: Interleukin-1β

IP : Prostacyclin receptor

LAS: Large artery stiffness

LDL: Low density lipoprotein

MAPK: Mitogen-activated protein kinase

MCP-1 : Monocyte chemoattractant protein-1

MMP-2: Matrix metalloproteinase- 2

MMP-9: Matrix metalloproteinase- 9

MnSOD: Manganese-dependent superoxide dismutase

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-ĸB: nuclear factor kappa B

nNOS: Neuronal nitric oxide synthase

NO: Nitric oxide

NOHA: N-hydroxy-L-arginine

NOS: Nitric oxide synthase

NOX: NADPH oxidases subunit

NQO1: Quinone oxidoreductase 1

$O2^{\bullet -}$: Superoxide anion

$ONOO^{\bullet -}$-: Peroxynitrite anion

PE / PHE: Phenylephrine

PG: Prostaglandin

PGH_2: Prostaglandin H2

PGI_2: Prostaglandin I2

PI3K: Phosphoinositide 3-kinase

PKB: Protein kinase B

PKC: Protein kinase C

PLA2: Phospholipase A2

PLC: Phospholipase C

PLD: Phospholipase D

PPET1: Preproendothelin-1

RAS: Renin-angiotensin system

RNA: Ribonucleic acid

ROS: Reactive oxygen species

SASP: Senescence-associated secretory phenotype

SA-β-gal: Senescence-associated β-galactosidase (SA-β-gal)

sGC: soluble guanylyl cyclase

SGLT: Sodium glucose co-transporter

SHR: Spontaneously hypertensive rats

SHRSP Spontaneously hypertensive stroke-prone rats

SIPS: stress-induced premature senescence

SK_{Ca}: Small conductance calcium-activated potassium channels

Smad2: Mothers against decapentaplegic homolog 2

SMC: Smooth muscle cell

SNP: Sodium nitroprusside

SOC: Store-operated Ca^{2+} channels

SOD: Superoxide dismutase

TF: Tissue factor

TGF: Transforming growth factors

TNF: Tumor necrosis factor

TXA_2: Thromboxane A2

TXAS: Thromboxane A synthase

VCAM-1: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

WHO: world health organization

Abstract / Résumé

Abstract

Cardiovascular diseases are actually the leading cause for mortality around the world. Indeed, in 2008 around 17.3 million deaths were due to cardiovascular diseases, that being 30 % of the total world mortality (OMS 2011). Amongst these death, 7.3 million were attributable to coronary heart diseases and 6.2 to ischemic stroke. Moreover, future projections indicate that cardiovascular diseases should remain the first cause of death with an estimated 23.3 million deaths by 2030. Cardiovascular diseases have several risk factors including elevated blood pressure, diabetes, dyslipidemias as well as physiological aging.

Several experimental studies and clinical trials have demonstrated that the development of cardiovascular diseases and their associated risk factors, including the physiological aging, is early associated with the appearance of an endothelial dysfunction characterized by a decreased formation of vasoprotective factors and an increased formation of vasoconstricting factors, resulting in an imbalance leading towards the accelerated development of vascular pathologies. Indeed, it has been shown that physiological ageing is associated with a progressive decrease in endothelium-dependent vasodilatation. Thus the age-related endothelial dysfunction could be an early event promoting the development of cardiovascular diseases, as endothelial dysfunction is involved in the mechanisms underlying the development of atherosclerotic lesions, including up-regulation of adhesion molecules, increased chemokine secretion and leukocyte adherence, increased cell permeability, enhanced low-density lipoprotein oxidation, platelet activation, cytokine elaboration, and vascular smooth muscle cell proliferation and migration.

In addition, several studies have demonstrated that endothelial dysfunction and cardiovascular diseases development are also associated with an increased vascular oxidative stress and an up-regulation of the local angiotensin system that also contributes to the development of

cardiovascular diseases through the induction of oxidative stress by up-regulating NADPH oxidase, the main producer of reactive oxygen species (ROS) in the vascular wall. Moreover, recent studies have suggested that the premature induction of senescence in endothelial cells could be an early event in the development of the endothelial dysfunction, especially in arterial zone at risk exposed to a turbulent blood flow and low shear stress such as bifurcations and curvatures. Studies from our laboratory have shown that in cultured endothelial cells, the induction of senescence (both replicative and premature) is associated with an increased oxidative stress, an overexpression and activation of the local angiotensin system, and an upregulation of the sodium-glucose co-transporters SGLT1 and SGLT2. Moreover, data indicate that both SGLTs and the local angiotensin system interact in a feed-forward self-amplification loop *via* a redox-sensitive mechanism.

Dietary factors also play a pivotal role in the modulation of the endothelial function. Indeed, the effects of dietary habits and compounds on the vascular health (vascular structure and function) have be the subject of numerous epidemiological and interventional studies. These studies reported that the endothelial function could be protected and/or improved by specific dietary patterns such as the Mediterranean diet, and an increased dietary intake of several items including fruits, vegetables, fatty fish, tea, red wine and micronutrients (polyphenolic compounds, omega-3 fatty acids, carotenoids and vitamins C, D and E).

Moreover, several epidemiological studies have demonstrated an inverse correlation between the cardiovascular morbimortality and dietary intake of products rich in polyphenols such as vegetables, fruits, teas, wine or coca. The beneficial effect of polyphenols on cardiovascular health has been attributed, at least in part, to their direct effect on blood vessels and in particular on the endothelium. Indeed, experimental studies and clinical trials have shown that polyphenols can increase the endothelial formation and release of vasoprotective factors such

as nitric oxide (NO), a potent vasodilatator and inhibitor of pro-inflammatory and pro-thrombotic responses and can improve the endothelial dysfunction and vascular oxidative stress that contribute to the development of cardiovascular diseases such as hypertension and atherosclerosis.

Thus, previous studies from our team have demonstrated that red fruits, particularly blackcurrant, polyphenol-rich extracts and several isolated compounds are able to potently induce the endothelial function *via* the redox-sensitive activation of the Src/PI3-kinase/Akt pathway leading to the activating phosphorylation of eNOS, the enzyme responsible for the endothelial formation of NO. In addition, a polyphenol-rich extract derived from red wine was able not only to prevent the appearance of an age-related endothelial dysfunction, but also to restore, at least in part, a normal endothelial function in animals with an established age-related endothelial dysfunction. Moreover, studies showed that products rich in anthocyanins, a specific type of polyphenols, are potent inducers of the endothelial function in both *in vitro* and *in vivo* models. Furthermore, recently published studies reported that some anthocyanins could enter in endothelial cells through SGLT1 and SGLT2.

The aim of the present study is thus to assess the potential of anthocyanin-rich products to improve age-related endothelial senescence and dysfunction, a major risk factor for the development of cardiovascular diseases. To that aim, we selected a blackcurrant juice rich in anthocyanin whose phytochemical analysis shown that it contains 4 major anthocyanins, namely cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside and delphinidin-3-*O*-rutinoside

To assess the effect on age-related endothelial dysfunction, old males Wistar rats (22-months-old) received daily in the drinking water either 60 or 120 mg/kg/day (in gallic acid equivalent) of the anthocyanin-rich blackcurrant juice for 2 weeks. After 2 weeks of treatment, rats were killed and organs collected. Systolic blood pressure (SBP) was assessed by tail-cuff sphygmomanometry, vascular reactivity using organ chambers, protein expression by immunofluorescence, oxidative stress using dihydroethidium, and anthocyanin uptake using Neu reagent.

The main results from the study indicate that, compared to young control rats, physiological aging was associated with an increase in systolic blood pressure. The intake of blackcurrant juice for 2 weeks was associated with a dose-dependent reduction of systolic blood pressure of 4 and 7 mmHg for the groups receiving 60 and 120 mg/kg/day, respectively. In the main mesenteric artery, aging was associated with an age-related endothelial dysfunction. Indeed, in mesenteric artery rings from old rats, the relaxation in response to acetylcholine was not affected by either indomethacin or the combination of Tram-34 plus UCL1684, but was abolished in presence of N^Gnitro L-arginine, indicating that the EDH component was abolished in aged rats. The intake of blackcurrant at 120 mg/kg/day restored the EDH-mediated relaxation, improved the response to levcromakalim, a potassium channel opener, and diminished the age-related increase in contractile responses to phenylephrine.

Most interestingly, chronic intake of the blackcurrant juice for 2 weeks was associated with a dose-dependent accumulation of anthocyanins in the vascular wall of the aorta, both in the thoracic aorta, a low-risk area, and at the level of aortic arch bifurcations, a high-risk area. In addition, the *ex vivo* incubation of aortic tissues with an anthocyanin-rich phenolic extract obtained from the blackcurrant juice demonstrated a rapid uptake of anthocyanin primarily in

the endothelium, which was more pronounced in old rats and in the area at risk. The cellular uptake of anthocyanins is associated with an overexpression of SGLT1 in the endothelium of old rats compared to young rats. Moreover, the intake of blackcurrant juice is associated with a further increased expression of SGLT1. The cellular uptake of anthocyanins in the endothelium was significantly inhibited in presence of either a dual SGLT1/2 inhibitor (sotagliflozin) or a selective SGLT2 inhibitor (empagliflozin), indicating the role of both SGLTs in the endothelial uptake of anthocyanins.

In addition, the physiological aging was associated with an increase vascular oxidative stress throughout the aortic wall. This vascular oxidative stress was normalized by the chronic intake of the anthocyanin-rich blackcurrant juice. Moreover, the vascular oxidative stress in the thoracic aorta of the old rats was significantly decreased by both inhibitors of SGLT, indicate that both SGLT1 and SGLT2 play a role in the age-related vascular oxidative stress.

Finally, the physiological aging is associated with an endothelial overexpression of eNOS, local angiotensin system components (ACE and AT1R), and markers of fibrosis (TGFβ, Collagen I), inflammation (MCP-1) and senescence (p53). Chronic intake of the blackcurrant juice is associated with a significant reduction in the endothelial expression of all markers. Anthocyanins could thus represent a new therapeutic approach to improve established vascular and endothelial dysfunction with a natural targeting of high-risk areas, and could be integrated in health policies from a healthy aging.

Résumé

Les maladies cardiovasculaires sont la première cause de mortalité dans le monde. Ainsi, en 2008, près de 17,3 millions de décès étaient dus aux maladies cardiovasculaires soit 30 % de la mortalité mondiale totale (OMS 2011). Parmi ces décès, 7,3 millions seraient dus à une coronaropathie et 6,2 millions à un accident vasculaire cérébral (AVC). De plus, les projections indiquent que les maladies cardiovasculaires devraient rester les premières causes de décès, avec près de 23,3 millions de personnes mourant d'une maladie cardiovasculaire d'ici 2030. Les maladies cardiovasculaires présentent de nombreux facteurs de risques dont l'hypertension vasculaire, les diabètes, les dyslipémies ou encore le vieillissement physiologique.

De nombreuses études expérimentale et cliniques ont montré que le développement des maladies cardiovasculaires et de leurs facteurs de risque, dont le vieillissement physiologique, étaient associés de façon précoce avec l'apparition d'une dysfonction endothéliale caractérisée par une diminution de la formation des facteurs protecteurs et une augmentation de la formation des facteurs vasoconstricteurs, le tout engendrant un déséquilibre menant au développement accéléré des pathologies vasculaires. En effet, il a été démontré que le vieillissement physiologique était associé à une diminution progressive de la vasodilatation dépendante de l'endothélium. Ainsi, la dysfonction endothéliale liée à l'âge pourrait être un évènement précoce favorisant le développement des maladies cardiovasculaires du fait que la dysfonction endothéliale est impliquée dans la formation de lésions athéromateuses en favorisant les mécanismes sous-jacents au développement de l'athérosclérose, notamment l'augmentation de l'expression des molécules d'adhésion, de l'adhésion des leucocytes, de l'oxydation des LDL, de l'activation plaquettaires, et de la prolifération et de la migration des cellules musculaires lisses vasculaires.

De plus, plusieurs études ont montré que la dysfonction endothéliale et le développement des maladies cardiovasculaires sont associés à une augmentation du stress oxydant vasculaire et à une surexpression du système angiotensine local qui contribue également au développement des maladies cardiovasculaires de par l'augmentation du stress oxydant vasculaire induit par la surexpression de la NAPDH oxydase, la principale source des espèces réactives de l'oxygène dans la paroi vasculaire. Par ailleurs, des études récentes ont suggéré que l'induction d'une senescence prématurée des cellules endothéliales constitue un évènement précoce dans le développement de la dysfonction endothéliale, notamment au niveau des zones à haut risque caractérisées par la présence d'un flux turbulent et à faible force de cisaillement comme les bifurcations et les courbures. Les études du laboratoire d'accueil ont ainsi montré que dans les cellules endothéliales en culture, l'induction d'une sénescence (réplicative ou prématurée) était associée à une augmentation du stress oxydant, une activation et une surexpression du système angiotensine local et une surexpression des cotransporteurs sodium-glucose SGLT1 et SGLT2. De plus, les résultats obtenus indiquent que le système angiotensine local et les SGLTs agissent comme une boucle d'auto-amplification *via* le stress oxydant cellulaire.

Les facteurs nutritionnels joueraient un rôle important dans la modulation de la fonction endothéliale. Ainsi, l'influence de l'alimentation sur la santé vasculaire (structure et fonction vasculaires) a fait l'objet de nombreuses études épidémiologiques et interventionnelles. Ces études ont rapporté une amélioration de la fonction endothéliale associée à la consommation de plusieurs aliments dont les fruits, les légumes, les poissons gras, les thés et le vin rouge, et de plusieurs micronutriments incluant les polyphénols, les acides gras n-3 (omega-3), les vitamines C, D et E, et les caroténoïdes.

De nombreuses études épidémiologiques ont montré qu'il existait une association inverse entre la morbi-mortalité cardiovasculaire et la consommation de produits riches en polyphénols tels

que les fruits, les légumes, le vin rouge, le cacao et le thé. L'effet bénéfique des polyphénols sur la santé cardiovasculaire a été attribué en partie à leur effet direct sur les vaisseaux sanguins, et plus particulièrement sur l'endothélium. En effet, des études expérimentales et cliniques ont révélé que les polyphénols sont capables d'augmenter la formation endothéliale de facteurs vasoprotecteurs comme le NO, un puissant vasodilatateur et un inhibiteur de réponses pro-inflammatoires et pro-thrombotiques, et d'améliorer la dysfonction endothéliale et le stress oxydant vasculaire qui contribuent au développement des pathologies cardiovasculaires majeures comme l'hypertension artérielle et l'atherosclérose.

Ainsi, les travaux précédents du laboratoire ont montré que les jus de fruits rouges, notamment le cassis, les extraits riches en polyphénols et certaines molécules isolées sont capables d'induire l'activation de la fonction endothéliale *via* une activation redox-sensible de la voie de signalisation Src/PI3-kinase/Akt aboutissant à l'activation par phosphorylation de la eNOS, l'enzyme responsable de la formation endothéliale de NO. De plus, un extrait riche en polyphénol issu du vin rouge a montré qu'il pouvait non seulement prévenir l'apparition de la dysfonction endothéliale liée à l'âge, mais aussi restaurer, au moins partiellement, une fonction endothéliale normale chez des animaux présentant une dysfonction endothéliale déjà installée. Des travaux ont indiqué que les produits riches en anthocyanes, une classe spécifique de polyphénols, sont de puissants activateurs de la fonction endothéliales *in vitro* et *in vivo*. De plus, des travaux récents ont indiqué que certaines anthocyanes pouvait pénétrer dans les cellules endothéliales *via* les cotransporteurs sodium-glucose SGLT1 et SGLT2.

Le but des travaux de recherche de cette thèse de doctorats est d'évaluer le potentiel de produits riches en anthocyanes à améliorer la dysfonction et la sénescence endothéliales liées à l'âge, un facteur de risque cardiovasculaire majeur. Pour ce faire, nous avons choisi d'utiliser un jus de

cassis riche en anthocyanes dont l'analyse phytochimique a montré qu'il contenait majoritairement 4 anthocyanines, à savoir la cyanidine-3-*O*-glucoside, la cyanidine-3-*O*-rutinoside, la delphinidine-3-*O*-glucoside et la delphinidine-3-*O*-rutinoside.

Pour cette étude, des rats Wistar males de 22 mois ont reçu quotidiennement pendant 2 semaines dans l'eau de boisson soit 60, soit 120 mg GAE/kg/j (équivalent d'acide gallique) de jus de cassis riche en anthocyanes, ou de l'eau (groupe contrôle). Après deux semaines de traitement, les rats ont été euthanasiés et les organes sont prélevés. L'artère mésentérique a été utilisée pour l'étude de la réactivité vasculaire, et l'artère mésentérique principale et l'aorte pour des études en immunofluorescence et histochimie fluorescente sur coupes congelées.

Les principaux résultats de notre étude indiquent que, par rapport à des rats jeunes de 12 semaines, le vieillissement physiologique était associé à une augmentation de la pression artérielle systolique. La consommation de jus de cassis pendant 2 semaines est associée à une diminution de la pression artérielle systolique de 4 et 7 mmHg pour le groupe 60 et 120 mg/kg, respectivement. Dans l'artère mésentérique principale, le vieillissement est associé à une dysfonction endothéliale. En effet, dans les anneaux d'artère mésentérique des rats âgés contrôles, la relaxation en réponse à l'acétylcholine n'était pas affectée par l'indométacine ou la combinaison Tram-34 plus UCL1684, mais abolie en présence de N^{G}nitro L-arginine, montrant une forte diminution de la composante EDH. Le traitement à la dose de 120 mg/kg/jour est associé à une amélioration de la composante EDH de la relaxation à l'acétylcholine, une amélioration de la relaxation en réponse à la levcromakalim, un ouvreur des canaux potassiques, et à une diminution des réponses contractiles à la phényléphrine.

Plus intéressant, le traitement chronique avec le jus de cassis riche en anthocyanes pendant 2 semaines est associé à une accumulation d'anthocyanes dépendante de la dose dans la paroi vasculaire de l'aorte, aussi bien au niveau de la zone thoracique à faible risque qu'au niveau des bifurcations de la crosse aortique, zone à haut risque. De plus, l'exposition *ex vivo* des tissus aortiques à un extrait phénolique issu du jus de cassis a montré une absorption rapide des anthocyanes préférentiellement dans l'endothélium vasculaire et de façon plus importante chez les animaux âgés ainsi que dans les zones à risques. Cette entrée cellulaire d'anthocyanes est associée à une surexpression de SGLT1 dans l'endothélium de l'aorte des animaux âgés par rapport aux rats jeunes. Le traitement chronique avec le jus de cassis est aussi associé à une augmentation de l'expression du SGLT1 par rapport aux animaux âgés non traités. L'entrée des anthocyanes dans l'endothélium de l'aorte thoracique a été significativement inhibée dans tous les groupes par une pré-incubation à la fois par un inhibiteur mixte des SGLT1 et SGLT2 (sotagliflozine), mais aussi par un inhibiteur spécifique du SGLT2 (empagliflozine), démontrant un rôle des deux co-transporteurs dans l'entrée cellulaire des anthocyanes.

Le vieillissement physiologique est également associé à une augmentation du stress oxydant vasculaire dans l'ensemble la paroi vasculaire. Le stress oxydant vasculaire est normalisé par le traitement chronique avec le jus de cassis riche en anthocyanes. De plus, le stress oxydant vasculaire dans l'aorte thoracique des animaux âgés est significativement diminué par l'application *ex vivo* des inhibiteurs des co-transporteurs, indiquant un rôle des SGLT1 et SGLT2 dans l'augmentation du stress oxydant vasculaire liée à l'âge.

Finalement, le vieillissement physiologique est associé à une surexpression de la eNOS, des composants du système angiotensine local (ACE et AT_1R), de marqueurs de fibrose (TGFβ, Collagène I), de marqueurs d'inflammation (MCP-1) et de sénescence (p53). L'ensemble de ces surexpressions ont été significativement réduites par le traitement chronique avec le jus de cassis riche en anthocyanes.

L’ensemble des résultats obtenus indique que la prise chronique de jus de cassis riche en anthocyanes pendant 2 semaines améliore significativement l’augmentation de la pression artérielle systolique et la dysfonction endothéliale liées à l’âge chez le rat. Les effets bénéfiques impliquent une amélioration de la composantes EDH de la relaxation dépendante de l’endothélium et une diminution des marqueurs de fibrose et d’inflammation, dues, au moins en partie à une diminution du stress oxydant vasculaire lié à l’activation du système angiotensine local. Cet effet bénéfique est associé à une accumulation des anthocyanes dans les tissus vasculaires via les co-transporteurs sodium-glucose surexprimés chez les rats âgés et dans les zones à haut risques. Les anthocyanes représentent donc une nouvelle approche thérapeutique permettant de reverser une dysfonction endothéliale et vasculaire établies en ciblant spécifiquement les zones à risques, et pourraient être préconisées dans les politiques de santé visant au vieillissement en bonne santé.

Production scientifique

Publications:

Anthocyanin-rich blackcurrant intake by old rats improves blood pressure, vascular oxidative stress and endothelial dysfunction associated with SGLT1- and 2-mediated vascular uptake of anthocyanin. **Chaker AB**, Algara-Suarez P, Remila L, Bruckert C, Park S-H, Houngue U, Belcastro E, Qureshi AW, El Itawi H, Toti F, Schini-Kerth VB, Auger C. En préparation pour soumission en aout 2020.

Empagliflozin treatment does not affect the hypertensive response to Ang II administration to rats but decreases oxidative stress in the arterial wall, and endothelial and cardiac dysfunction. Bruckert C, Remila L, Matsushita K, Auger C, Houngue U, **Chaker AB**, Park S, Algara-Suarez P, Belcastro E, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB. En préparation pour soumission en aout 2020.

Nanoencapsulation of EPA:DHA 6:1 potentiates the endothelium-dependent relaxation of coronary artery rings compared to the native form: role of NO, endothelium-dependent hyperpolarization end prostanoids. Remila L, Belcastro E, Guenday-Tuereli N, **Chaker AB**, Vandamme T, Tureli E, Kerth P, Auger C, Schini-Kerth VB. En préparation pour soumission en aout 2020.

Carissa edulis extract, a medicinal plant of Benin pharmacopoeia, induces potent endothelium-dependent relaxation of coronary artery rings involving predominantly nitric oxide. Houngue U, **Chaker AB**, Remila L, Bruckert C, Park S-H, Belcastro E, Bouchakour I, Tokoudagba JM, Auger C, Gbaguidi F, Schini-Kerth VB. En préparation.

Communications orales :

Evaluation of the expression level of sodium-glucose cotransporter (SGLT)1 and 2 in the internal thoracic aorta of bypass surgery patients. Algara-Suarez P, Matsushita K, Park SH, Bruckert C, **Chaker AB**, Belcastro E, Auger C, Jesel L, Ohlmann P, Morel O, Mazzucotelli JP, Schini-Kerth VB. 13th International Symposium on Mechanisms Of VasoDilatation & 7th International Symposium on Endothelium-Dependent Hyperpolarization, 20-22 May 2019, Rotterdam, The Netherlands.

Anthocyanin-rich blackcurrant intake improves age-related increased systolic blood pressure, vascular oxidative stress and endothelial dysfunction in rats: Role of SGLT 1 and 2-mediated vascular uptake of anthocyanins. **Chaker AB**, Algara-Suarez P, Remila L, Bruckert C, Park SH, Houngue U, Belcastro E, Qureshi AW, El Itawi H, Toti F, Schini-Kerth VB, Auger C. 5th IUPHAR World Conference on the Pharmacology of Natural Products, 5-7 December 2019, Hyderabad, India.

Communications affichées :

Oral intake of an anthocyanin-rich blackcurrant juice decreases systolic blood pressure and improves the endothelial function in the mesenteric artery of aged rats. **Chaker AB**, Remila L, Brucker C, Park SH, Houngue U, Qureshi AW, El Itawi H, Toti F, Schini-Kerth VB, Auger C. 13th International Symposium on Mechanisms Of VasoDilatation & 7th International Symposium on Endothelium-Dependent Hyperpolarization, 20-22 May 2019, Rotterdam, The Netherlands.

Ageing is associated with increased endothelial sodium-glucose cotransporter 1 expression at arterial sites at risk promoting enhanced anthocyanin accumulation and improved vascular oxidative stress. **Chaker AB**, Algara-Suarez P, Remila L, Bruckert C, Park SH, Houngue U, Belcastro E, Qureshi AW, El Itawi H, Toti F, Schini-Kerth VB, Auger C. European Society of Cardiology Congress 2019, 31 August-4 September 2019, Paris, France.

Empagliflozin treatment does not affect the hypertensive response to Ang II administration to rats but decreases oxidative stress in the arterial wall, and endothelial and cardiac dysfunction. Bruckert C, Remila L, Matsushita K, Auger C, Houngue U, **Chaker AB**, Park S, Algara-Suarez P, Belcastro E, Jesel L, Ohlmann P, Morel O, Schini-Kerth VB. European Society of Cardiology Congress 2020, 29 August – 01 September 2020, e-meeting.

Nanoencapsulation of EPA:DHA 6:1 potentiates the endothelium-dependent relaxation of coronary artery rings compared to the native form: role of NO, endothelium-dependent hyperpolarization end prostanoids. Remila L, Belcastro E, Guenday-Tuereli N, **Chaker AB**,

Vandamme T, Tureli E, Kerth P, Auger C, Schini-Kerth VB. Printemps de la cardiologie 2020, 28-30 October 2020, Grenoble, France.

Anthocyanin-rich blackcurrant intake by old rats improves blood pressure, vascular oxidative stress and endothelial dysfunction associated with SGLT1- and 2-mediated vascular uptake of anthocyanin. **Chaker AB**, Algara-Suarez P, Remila L, Bruckert C, Park S-H, Houngue U, Belcastro E, Qureshi AW, El Itawi H, Toti F, Schini-Kerth VB, Auger C. Printemps de la cardiologie 2020, 28-30 October 2020, Grenoble, France.

Carissa edulis extract, a medicinal plant of Benin pharmacopoeia, induces potent endothelium-dependent relaxation of coronary artery rings involving predominantly nitric oxide. Houngue U, **Chaker AB**, Remila L, Bruckert C, Park S-H, Belcastro E, Bouchakour I, Tokoudagba JM, Auger C, Gbaguidi F, Schini-Kerth VB. Congrés de la Société Française de Pharmacologie et de Thérapeutique, 14-17 juin 2021, Lille, France.

Chapter 1: cardiovascular diseases

Cardiovascular diseases

Cardiovascular diseases (CVDs) include pathologies that affect the heart and all blood vessels, such as atherosclerosis, heart rhythm disorders, high blood pressure, myocardial infarction, heart failure or even strokes. CVDs are the leading causes of death globally, taking an estimated 17.9 million lives each year, with a ratio of four out of five deaths currently occurring in low- and middle-income countries (WHO 2017). The increase in CVD that took place during the twentieth century is partially explained by a longer life expectancy, but also by economic and human development such as urbanization and industrialization (Steptoe, Rosengren et al. 2011). Cardiovascular disease will remain the leading cause of death worldwide in 2030, with an estimated 23 million deaths expected that year. In addition, CVDs have serious consequences which affect the quality of life of patients and present a high cost for society (Mathers and Loncar 2006). In France, it is estimated that around 120,000 people each year are affected by a myocardial infarction, and 130,000 by a stroke, and the expenses due to cardiovascular pathologies represent 20% of the annual health expenses reimbursed in France, i.e. more than 30 billion euros versus 14.5 billion for cancer (Lévy and Tedgui 2016).

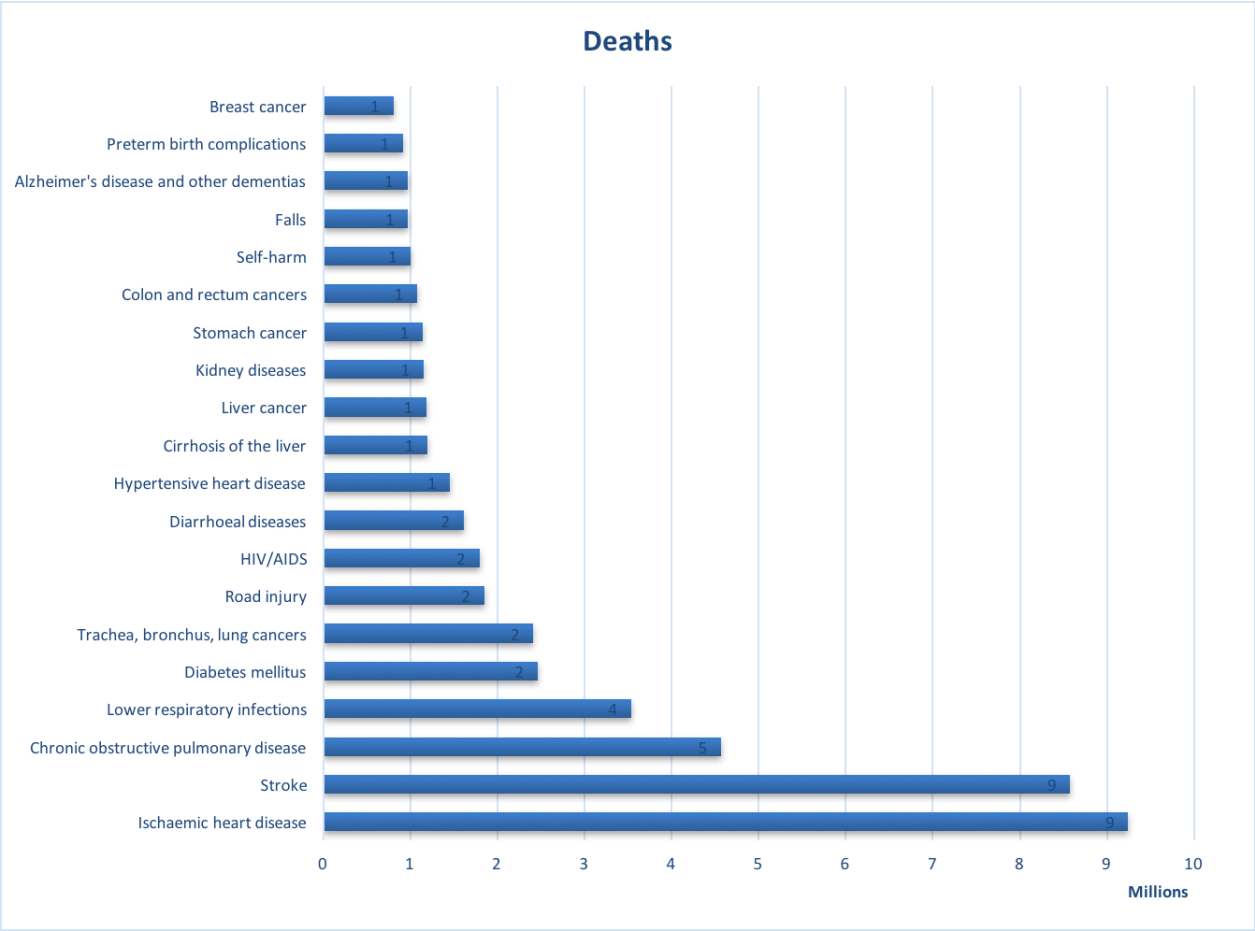

Figure 1 – Estimated causes of death worldwide in 2030. (WHO, 2018)

The mechanisms involved in the etiology of CVDs could vary depending on the disease and are not well defined in certain diseases such as heart failure or essential hypertension. Coronary artery disease, ischemic stroke, and peripheral artery disease are responsible of 85% of the total deaths due to CVD in women and 88% in men (Mendis, Puska et al. 2011) and are all secondary to the development of atherosclerosis.

Atherosclerosis is a chronic inflammatory disease of the arterial wall associated with the accumulation of lipids and cholesterol in the subendothelial space of arteries. Atherosclerotic lesions develops over many years and operates the link between CVDs and a high cholesterol level in the blood (Tedgui and Mallat 2006).

It is been reported by the WHO that over 80% of CVD and 90% of type 2 diabetes can be avoided (Waxman 2004). This prevention involves improving several risk factors through healthy food, exercise, stopping tobacco and limiting alcohol intake. Treating risk factors, such as high blood pressure, blood lipids and diabetes is also beneficial (McGill,

McMahan et al. 2008). Experience has shown that a multifactorial approach, one that takes into consideration all the risk factors, is probably the best strategy for the prevention of coronary heart disease (Anderson, Odell et al. 1991).

CVD Risk factors

Several epidemiological studies have reported an association between CVDs and several risk factors. In 1951, the first comprehensive study on CVD risk factors was published. The Framingham Heart Study (FHS), which began in 1948 with 5,209 adult subjects from Framingham, Massachusetts, is now on its fourth generation of participants (FHS 2005). The study has identified several risk factors that interact in a deleterious manner to have a cumulative impact on cardiovascular disease. More recently, the international Interheart study published in 2004 in The Lancet have further identified risk factors and their relative contribution to myocardial infarction in 52 countries, including low and middle-income countries (Yusuf, Hawken et al. 2004). Similarly, the Interstroke study involving 32 countries have reported the relative weight of risks factors for stroke (O'Donnell, Chin et al. 2016).

Risks factors associated to CVDs are generally classified as modifiable and non-modifiable risk factors.

Modifiable cardiovascular risk factors

Hypercholesterolemia

It is now admitted that the circulating cholesterol levels plays a major role in the genesis of atherosclerosis and that the reduction of its concentration in the blood constitutes a major therapeutic target to reduce the risk of cardiovascular disease (Gotto 2011). Observational studies showed that beyond a total cholesterol level of about 1.6 g/l there is a linear relationship between cholesterolemia and cardiovascular mortality (Pekkanen, Linn et al. 1990).

Cholesterol is a lipid classified as a sterol, or steroid alcohol, which is an essential element for cell life. Indeed, cholesterol is involved in the composition of all cell membranes where it plays functional roles. Cholesterol is also the precursor of several bioactive molecules including steroid hormones and bile acids. Like any lipid, insoluble in the blood due to its hydrophobic

nature, cholesterol is transported in the plasma in the form of lipoproteins (Cox and García-Palmieri 1990). There are several types of lipoproteins that have been characterized according to their size and composition in apolipoproteins. Amongst lipoproteins, low-density lipoproteins (LDL) are responsible for transport of cholesterol to tissues and is considered as "bad cholesterol" because its contribution to development of atherosclerosis. Conversely, the high-density lipoproteins (HDL), considered as "the good cholesterol", transport the excess of the cellular cholesterol back to the liver to be excreted in the bile. The imbalance in the level of blood lipids, which most often is characterized by an increase in plasma LDL cholesterol or triglycerides and/or a decrease in HDL cholesterol level, is known as dyslipidemia and is one of the most relevant risk factors and contributes intensively to the development of CVDs (Pappan and Rehman 2020). Studies have shown that established dyslipidemia increases the risk of heart disease through the increased level of circulating LDL which undergoes oxidative and structural changes leading to accumulation in the arterial wall and intervention of inflammatory cells (macrophage, monocytes). This promotes endothelial dysfunction triggered by the impairment of NO, the major regulator of vascular tone and inhibitor of vascular smooth muscle proliferation (Mahdy Ali, Wonnerth et al. 2012, Wengrofsky, Lee et al. 2019). Conversely, dyslipidemia therapy is considered an effective way to prevent the development of CVD. Up to now, statins have been found to be the most effective drugs for decreasing LDL. They reduce tissue cholesterol by inhibiting HMG-CoA reductase, a key enzyme involved in the cholesterol synthesis pathway, resulting in a significant decrease in circulating LDL (Zhou and Liao 2009). Beyond that, they also help to stabilize plaques by reducing inflammation and increasing collagen content in atherosclerotic plaques, and to slow the growth of total plaque volume (Crisby, Nordin-Fredriksson et al. 2001).

Another non-statin class called PCSK9 inhibitors have been intensively studied the last few years and considered as a new era of lipid lowering therapy (Chaudhary, Garg et al. 2017). PSCK9 operates by interacting with the hepatic LDL-receptor and promoting the receptor's degradation via hepatic lysosomes (Page and Watts 2016, Wengrofsky, Lee et al. 2019). PSCK9 inhibitors reduce PSCK9-mediated hepatic LDL-receptor turnover, extending its longevity and thus increasing LDL uptake. In combination with statins, PCSK9 inhibitors reduced plaque calcification and promoted VSMC plaque content while simultaneously decreasing the size of the lipid core, thus stabilizing the plaque (Wengrofsky, Lee et al. 2019)

Disease-related risk factors

Overweight and obesity

Obesity is associated with an increased risk of developing cardiovascular disease (CVD), particularly heart failure (HF) and coronary heart disease (CHD) (Carbone, Canada et al. 2019). An American study that evaluated mortality risk according to different patterns of adiposity, combining body mass index (BMI) with measures of central obesity, reported that the relative risk of coronary disease was of 1 for men with a body mass index lower than 23, 1.72 for overweight subjects having a BMI between 25 and 29, and 3.44 for obese subjects with a BMI higher than 33 (Coutinho, Goel et al. 2013). The mechanisms through which obesity increases CVD risk are multiple, involving mainly adipose tissue as an active endocrine and paracrine organ that releases a large number of cytokines and bioactive mediators such as pro-inflammatory cytokines (IL-6, TNF-a) (Van Gaal, Mertens et al. 2006). Moreover, approximately 60-70% of obese patients present a dyslipidemia (Feingold 2020). The lipid abnormalities in obese patients include high circulating levels of triglyceride and VLDL, which can promote not only the formation of atherosclerotic plaques but also induces an impairment in insulin sensitivity (Hardy, Czech et al. 2012)

Diabetes

Studies have showed that patients suffering from either type 1 or type 2 diabetes are highly exposed to several cardiovascular disorders (Martín-Timón, Sevillano-Collantes et al. 2014, Lee, Patel et al. 2015) and that the cardiovascular complications are the leading cause of diabetes-related morbidity and mortality in the world (A.D.B 1999). Indeed, compared to non-diabetics, diabetic patients have a 2 to 4 times increased cardiovascular risk. Although the exact pathophysiology of coronary artery diseases progression in patients with diabetes has not yet been determined, several studies supported the fact that chronic hyperglycemia resulting from insulin resistance is associated with glucose toxicity, which is the main cause of diabetic complications especially cardiovascular complications, *via* the induction of an oxidative stress (Kawahito, Kitahata et al. 2009). Indeed, the endothelial cells and hepatic cells take up glucose independently of insulin *via* glucose transporters (GLUT1 and GLUT2), thus allowing glucose to enter into the cell without a tight control which added to the absence of insulin signaling results in toxic effect (Battault, Renguet et al. 2020)

Hypertension

Hypertension is defined as persistent elevated blood pressure (BP) and is one of the most important risk factors for CVD. Indeed, approximately 54% of strokes and 47% of coronary heart diseases, worldwide, are attributable to high BP (Wu, Hu et al. 2015). The correlation between increase in BP level and cardiovascular risk is well established (Luo, Cheng et al. 2020). Some studies also suggest that prehypertensive patients with BP values in the 130–139/85–89 mmHg range present elevated risk of CVD compared with those with normotensive BP levels of 120/80 mmHg (Mancia and Grassi 2014).

Lifestyle related risk factors

Tobacco

Tobacco smoking is one of major leading causes of CVD morbidity and mortality for most of CVD subtypes (Banks, Joshy et al. 2019). Indeed, more than 1 of 10 deaths were attributed to smoking and tobacco use is responsible of more than 140,000 death from CVD annually (Control and Prevention 2010). Moreover, a relationship between the risk of CVDs and the duration and/or levels of exposure (number of cigarettes/day) have been reported (Banks, Joshy et al. 2019). A cigarette contains more than 7000 chemical substances and many of them, such as carbon monoxide, polycyclic aromatic hydrocarbons, nicotine and heavy metals, have known adverse effects on the vascular endothelium, dyslipidemia, and thrombotic factors leading to adverse cardiovascular events like CAD, stroke and myocardial infarction (Roy, Rawal et al. 2017). Accumulating evidences indicated that increased oxidative stress is closely connected to smoking as a risk factor for CAD. The amount of cigarettes smoked each day has a significant impact on the degree of oxidative damage and antioxidant defense, resulting in increased oxidative stress (Kamceva, Arsova-Sarafinovska et al. 2016).

Diet

There is much controversy surrounding the optimal diet for cardiovascular health, and the way people eat has changed greatly across the globe toward snacking and fast-food, with these eating behavior changes directly related to many cardiovascular events (Anand, Hawkes et al. 2015). Amongst the first epidemiological studies linking diet and CVDs, the 7 countries study by Ancel Keys has reported a positive correlation between consumption of fat in the diet and mortality by coronary heart disease (Keys 1953, Keys 1970). The study was further expanded

to 22 countries that showed a weaker correlation between fat contents of the diet and mortality by coronary heart disease (CHD) (Yerushalmy and Hilleboe 1957). Recent meta-analysis indicate that saturated fat consumption is associated with an increased risk of CHD mortality without significant effect on stroke (de Souza, Mente et al. 2015, Hooper, Martin et al. 2015). Similarly, consumption of *trans*-unsaturated fatty acids is associated with an increased risk of all-cause mortality and fatal and non-fatal CHD, but seems to have no significant effect on the risk of ischemic stroke (de Souza, Mente et al. 2015). Interestingly, the "natural" dietary *trans*-fats from ruminants (milk, cheese, butter, etc.) were not associated with increased risk of either all-cause mortality or CHD, but were also associated with a beneficial effect on risk of developing type 2 diabetes(de Souza, Mente et al. 2015). Conversely, *trans*-fats from industrial origin (e.g. hydrogenated fats in frying oil or in spreads) were associated with increased risk of fatal and non-fatal CHD (de Souza, Mente et al. 2015). In addition, dietary consumption of poly-unsaturated fats, and especially of omega-3, was associated with a reduced risk of CVDs (Mozaffarian, Micha et al. 2010). Moreover, a recent clinical trial has shown that chronic intake of an omega-3 fatty acids by patients at high risk of CVDs was associated with a strong reduction in risk of cardiovascular adverse events (Bhatt, Steg et al. 2019)

Apart from dietary fats, other components of the diet seem to have protective effects towards the cardiovascular system, and in particular vegetables and fruits. Indeed, the WHO attributed approximately 1.7 million deaths worldwide to low fruit and vegetable consumption (World Health 2017). The Mediterranean diet, rich in fruits and vegetables, unsaturated fat from olive oil, wholegrain products and with a reduced intake of refined sugars, meat and saturated fats, was associated with a reduced risk of CVDs. A meta-analysis showed that a better adhesion to Mediterranean diet was associated with a 9 % reduction in cardiovascular mortality (Sofi, Cesari et al. 2008). Furthermore, the PREDIMED trial demonstrated that the adhesion to Mediterranean diet resulted to a significant reduction of first cardiovascular adverse events by 30% in patients with high risk factors of CVDs (Estruch, Ros et al. 2013).

In addition, data from various studies suggest that decreasing dietary salt intake from the current global levels of 9–12 grams/day to the recommended level of 5 grams/day would have a major impact on blood pressure and CVD (Mendis, Puska et al. 2011).

Physical Activity

In the recent years, physical inactivity and sedentary behavior have emerged as an independent risk factor for CVDs (Lavie, Ozemek et al. 2019). Moreover, a low level of physical activity is associated with increased prevalence of CVDs risk factors such as obesity, T2D, hypertension and dyslipidemia (Lavie, Arena et al. 2015). In the Interheart study, the proportion of myocardial infarction attributable to physical inactivity was estimated at 12% (Yusuf, Hawken et al. 2004). Conversely, frequent exercise is highly associated with a decrease in cardiovascular mortality as well as the risk of developing a CVD (Nystoriak and Bhatnagar 2018). Moreover, the WHO indicated that the participation to 150 minutes of moderate physical activity each week (or equivalent) is estimated to reduce the risk of ischemic heart disease by approximately 30% and the risk of diabetes by 27%, making moderate exercising an effective way for prevention of CVD and associated risk factors (Mendis, Puska et al. 2011)

Unmodifiable cardiovascular risk factors

Aging

The life expectancy of the world population has dramatically increased over the last century, passing from 45 to 74 years for men and 82 for women. However, Aging is characterized by a progressive loss of organs functions, homeostatic regulation and vital cellular events which can be linked with the development of CVD (Grossen 2002, Fajemiroye, Cunha et al. 2018). Aging process is accompanied by a gradual increase in oxidative stress, physiologically due to an increased production of free radicals, that is aggravated by a decrease in dietary intakes of antioxidants in old subjects (Roussel and Ferry 2002). Some studies on aged rodent models presented a wide range of cardiovascular modifications also observed in humans (Figure 2), including vascular remodeling, high level of oxidative stress and inflammation, which promotes the development of coronary artery disease and stroke in the elderly patients (Chiao, Lakatta et al. 2016).

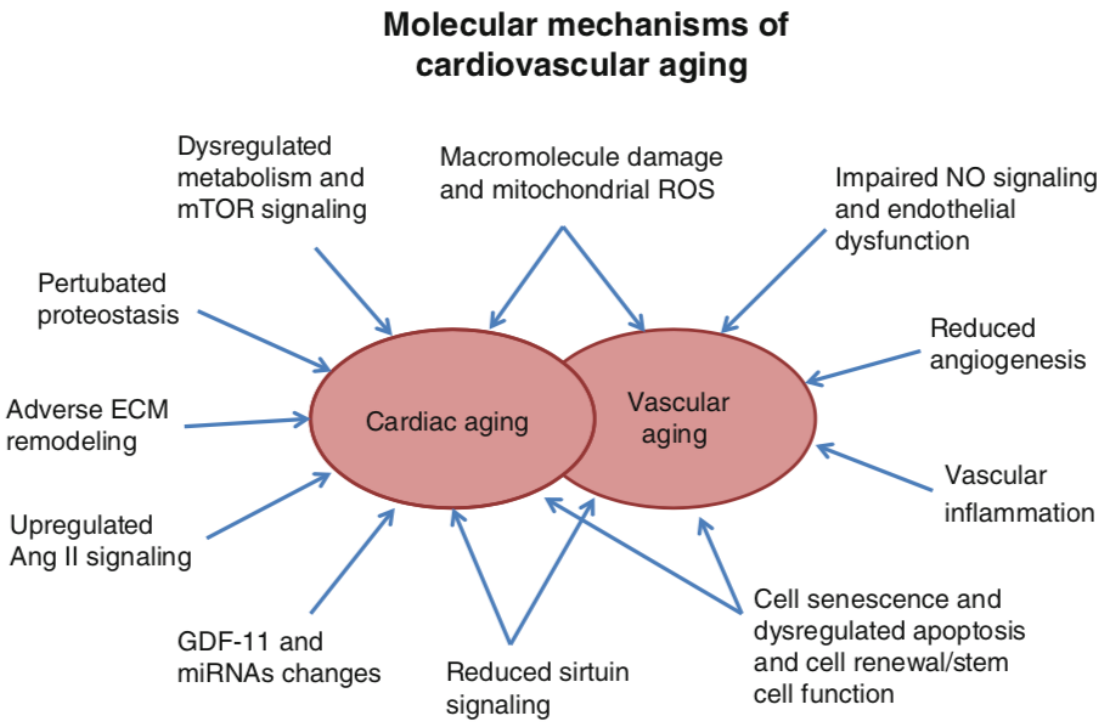

Figure 2 – Mechanisms that mediate cardiovascular aging. (Chiao, Lakatta et al. 2016)

Genetics

Genetic background that are associated with dysregulation of the cardiovascular system are associated with hereditary cardiovascular diseases or risk factors, including arrhythmia, congenital heart disease, cardiomyopathy, and hypercholesterolemia/dyslipidemia (ICUO 2020). Moreover, CHD and the subsequent heart attack, stroke or heart failure are sometimes reported in more than one family member, which indicates the presence of genetic risk factors (WHO 2004). Similarly, the data from the Framingham study suggest that CHD in a parent increases the risk of coronary disease in children by 30% (Lévy and Tedgui 2016).

Chapter 2: Role of endothelium in cardiovascular diseases

Structure of blood vessels

Most of blood vessels are made up of three layers, and their proportions vary among vascular categories. These layers are respectively called the adventitia (outer layer), the media (middle layer) and the intima (inner layer) (Figure 3) (Tucker and Mahajan 2017).

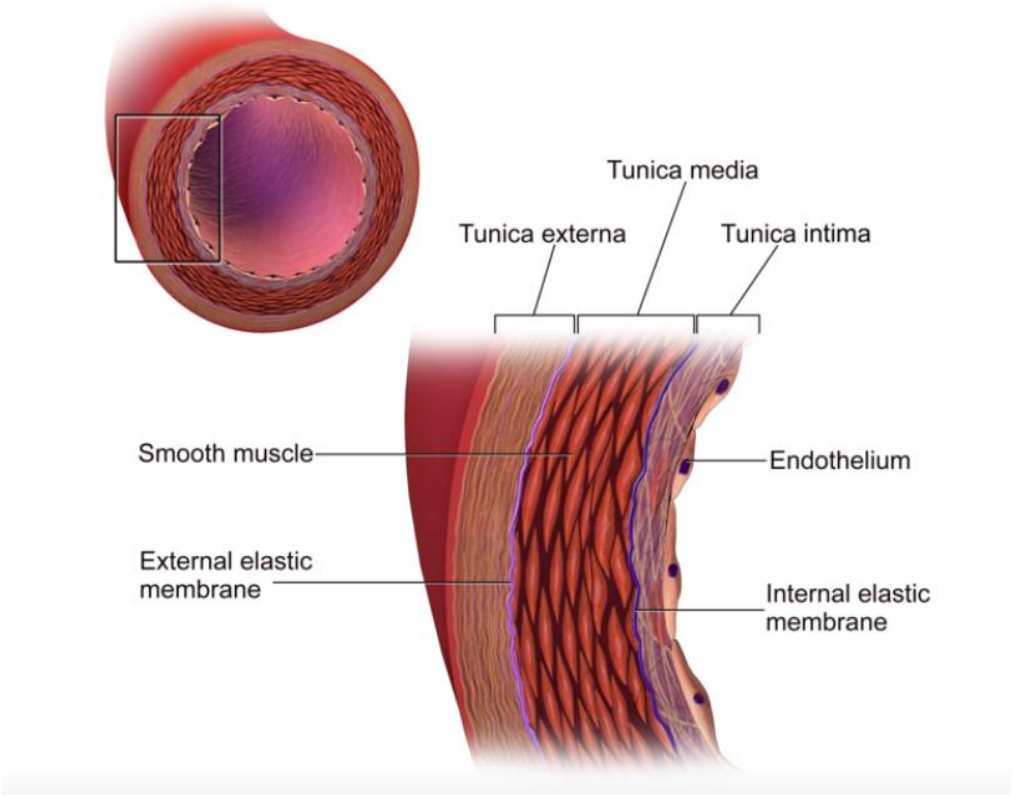

Figure 3 – Structure of the vascular wall, disposition of the three vascular layers (Blausen 2014)

The Adventitia

Also known as *tunica adventitia* or *tunica externa*, it is a layer mainly composed of collagen, elastic fibers and fibroblasts (Maleszewski, Lai et al. 2016). The organization of the adventitia is the same regardless of the type of vessel whereas the thickness could vary. It allows the packaging of vessels and participates in growth and repair of the vessel wall, and it mediates

communication between the vascular endothelial cells and smooth muscle cells (SMCs) and their local tissue environment (Majesky, Dong et al. 2011).

The media

Also called *tunica media*, the media is made up of extracellular constituents (elastic fibers, elastin fibrils, fibers and fibrils of collagen and proteoglycans) and mainly smooth muscle cells (SMCs). It is separated from the adventitia by the external elastic lamina and from the intima by the internal elastic lamina. Its main function is to ensure the maintenance of tone and vascular resistance (Zaromitidou, Siasos et al. 2016).

The intima

The intima is the innermost layer of blood vessels that is constituted of connective tissues supporting the endothelium at the luminal side. The endothelium is a single layer of endothelial cells, that covers the inner cellular lining of the blood vessels (arteries, veins and capillaries) and the lymphatic system, and therefore the luminal side of the endothelium interacts with the blood/lymph and the circulating cells, and the other side adjacent to the adventitia interacts with surrounding tissue (Félétou 2011). While for a long time this monolayer was assimilated to a simple "envelope" involved in the processes of hemostasis, the endothelium is now considered as an endocrine gland, but also as an integrator of the underlying tissue processes (Krüger-Genge, Blocki et al. 2019). It controls the degree of vascular relaxation and constriction by generating either vasconstricting molecules (vasoconstrictor prostanoids, angiotensin II, endothelin-1, ROS) or vasodilating bioactive molecules like nitric oxide (NO) in the blood in a continuous manner. Moreover, the endothelium is also directly involved in the regulation of vascular health through the inhibition of platelet aggregation and inflammation (Sandoo, van Zanten et al. 2010).

Arterial high-risk zones

Because of the structural complexity of blood vessels, certain segments may be more prone to damage. Variations in vessel diameter, branching angles, and wall composition can create localized areas of increased mechanical stress (Chiu and Chien 2011, Gimbrone and García-Cardeña 2016). Arterial high-risk zones are regions within the vascular system where anatomical features, mechanical stress, and disturbed blood flow converge to increase the likelihood of pathology, particularly atherosclerosis, aneurysms, and dissections (Chiu and Chien 2011, Gimbrone and García-Cardeña 2016). The aorta, the body's principal artery, exemplifies this heightened vulnerability through five key segments: (1) the aortic root and ascending aorta, subjected to high pulsatile stress and prone to root aneurysms or ascending dissections; (2) the aortic arch, where branching vessels and curvature create shear stress variations that favor plaque formation; (3) the descending thoracic aorta, consistently exposed to elevated pressure yet slightly lower than in the ascending portion; (4) the abdominal aorta (particularly infrarenal), where reduced flow velocity and turbulence at branch points promote atherosclerosis and abdominal aortic aneurysms; and (5) the iliac bifurcation, where branching angles can cause flow disturbances leading to plaque deposition and peripheral arterial disease (Mitchell and Schoen 2010). Across these zones, distinct hemodynamic factors such as high shear stress, altered flow patterns, or branching turbulence drive endothelial dysfunction and progressive vascular damage, culminating in severe clinical events like dissection, rupture, or ischemic complications (Chiu and Chien 2011, Gimbrone and García-Cardeña 2016).

Role of the endothelium

For many years, the endothelial cells were considered as a simple population of cells forming a separating barrier between the vascular space and the interstitium until Florey indicated that the endothelium is more than a barrier (Florey 1966). In 1980, Furchgott and Zawadzki pointed that the endothelium plays a central role in the acetylcholine-induced vasorelaxation (Furchgott and Zawadzki 1980). Indeed, the endothelium plays a pivotal role in the maintenance of the vascular homeostasis by releasing numerous factors including vasodilatory molecules such as nitric oxide and prostacyclin (PGi2) or vasoconstrictive factors

such as thromboxane A2 (TXA2), endothelin-1 (ET-1), angiotensin II (Ang II) and ROS (Vanhoutte, Shimokawa et al. 2017). The endothelium is also involved in other critical functions of the cardiovascular system, including fluid and solute exchange, coagulation, inflammatory responses and angiogenesis (Sandoo, van Zanten et al. 2010). Any disorder in the endothelial regulatory processes of the vascular homeostasis can cause an endothelial dysfunction which leads to a favorable environment for the development of cardiovascular diseases (Lerman and Zeiher 2005).

Endothelium-derived vasorelaxing factors

Nitric oxide

Nitric oxide is a free radical gas that exists naturally in the atmosphere during storms. It is also formed in an enzyme-catalyzed reaction between molecular oxygen and L-arginine. Furchgott and Zawadzki first identified an endothlium-derived relaxing factors in 1980 that was partially identified as NO by Ignarro et al. in 1988 (Ignarro, Buga et al. 1987). NO is the endogenous activator of the soluble guanylate cyclase (sGC) leading to the formation of cyclic GMP (cGMP) which plays the role of a second messenger in many cells, including smooth muscle cells and nerves. In vascular smooth muscle cells cGMP activates the protein kinase G, thus promoting the uptake of cytosolic calcium into the sarcoplasmic reticulum and the expulsion of calcium out of the cell leading to a decrease in the cytosolic calcium concentration and subsequent reduction of the activity of the calcium-dependent myosin light chain kinase (Zhao, Vanhoutte et al. 2015). Moreover, cGMP increased concentration also induces the opening of calcium-activated potassium channels leading to hyperpolarization of the smooth muscle cells. Finally, the increased cGMP concentration induces the activation of the myosin-light chain phosphatase leading to a decrease in contraction. The NO-mediated regulation of the vascular tone is important in resistance vessels because it contributes to the regulation of the blood pressure. Apart from its effect on vascular tone, the endothelium-derived NO plays a role in limiting the development of cardiovascular diseases by inhibiting vSMC proliferation, platelet adhesion and monocytes migration (Moncada, Palmer et al. 1989, Rang, Dale et al. 2003, Zhao, Vanhoutte et al. 2015). (Figure 4).

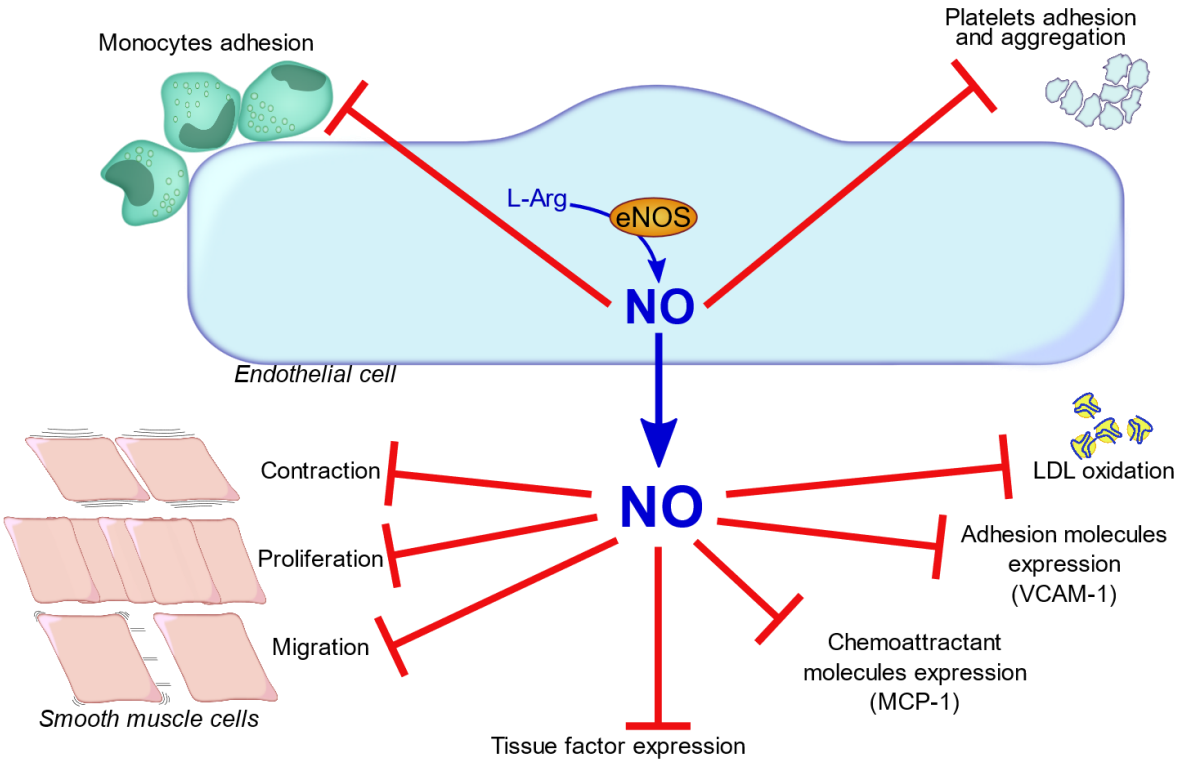

Figure 4 – : Vasoprotective effects of NO (VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; LDL, low density lipoproteins)

Biosynthesis of nitric oxide

A family of three isoforms of nitric oxide synthase (NOS) are behind the generation of NO. NOS from endothelial cells (eNOS or NOS-3) and from neurons (nNOS or NOS-1) are constitutively expressed enzymes whose activities are stimulated by increased cytosolic calcium concentration. The third isoform is an inducible NOS (iNOS or NOS-2) whose activity is independent of calcium. iNOS is involved in the immune system and its expression requires activation by pro-inflammatory stimuli (Förstermann and Sessa 2012). NOS requires tetrahydrobiopterin (BH4), among other co-factors. NOS enzymes are synthesized as monomers which need to form dimers to bind BH4 and L-arginine, before being able to catalyze NO production (Bendall, Douglas et al. 2014). Therefore, the coupling of NOS is important to produce NO. The monomers, which are not coupled with their cofactors or substrates, generate only superoxide anions instead of NO. This phenomenon is referred to as

"NOS uncoupling" (Lee, Bae et al. 2016). NO is synthetized in a cell by NOS, which hydroxylate a terminal guanidino-nitrogen of Arginine to generate *N*-hydroxy-L-arginine (NOHA) with the participation of NADPH as an electron source and the presence of O_2, carry out a five-electron two-stage oxidation of the L-arginine guanidine group with the formation of NO and L-citrulline (Stuehr 2004) (Figure 5).

Furthermore, the modulation of eNOS expression is influenced by several factors such as bradykinin, insulin, estrogen, VEGF-A and shear stress. In the endothelial cell, NO synthesis is activated via 2 different pathways: a cytosolic calcium (Ca^{2+})-dependent pathway and Ca^{2+}-independent pathway (PI3kinase / Akt pathway) (Fleming and Busse 2003, Devika and Jaffar Ali 2013). The activity of eNOS is most of the time regulated by the dephosphorylation of its inhibitory site at threonine 495, which is constitutively phosphorylated at basal state, thus allowing calcium/calmoduline to bind and by phosphorylation of serine 1177, an activator site, both promoting the formation of NO (Fleming 2010). The inhibition of serine 1177 phosphorylation by O-glycosylation has been shown to contribute to the decrease in NO formation (Matsumoto, Shimabukuro et al. 2014). NO is a potent vasodilator in both resistance and conductance arteries, where it diffuses to underlying vascular smooth muscle cells (VSMCs) to activates soluble guanylyl cyclase (sGc) by binding to its ferrous heme, leading to conversion from guanosine triphosphate (GTP) to cyclic guanosine monophosphate 3 ', 5' (cGMP) which, in turn, activates protein kinase G and modifies the level of phosphorylation of many target proteins to finally decrease the concentration of cytosolic calcium promoting relaxation (Evora, M Evora et al. 2012) (Figure 5). NO also has a direct inhibitory effect on the activity of Rho-kinase which controls the assembly of actin filaments of the cytoskeleton and the contraction of SMCs (Mills, Chitaley et al. 2002).

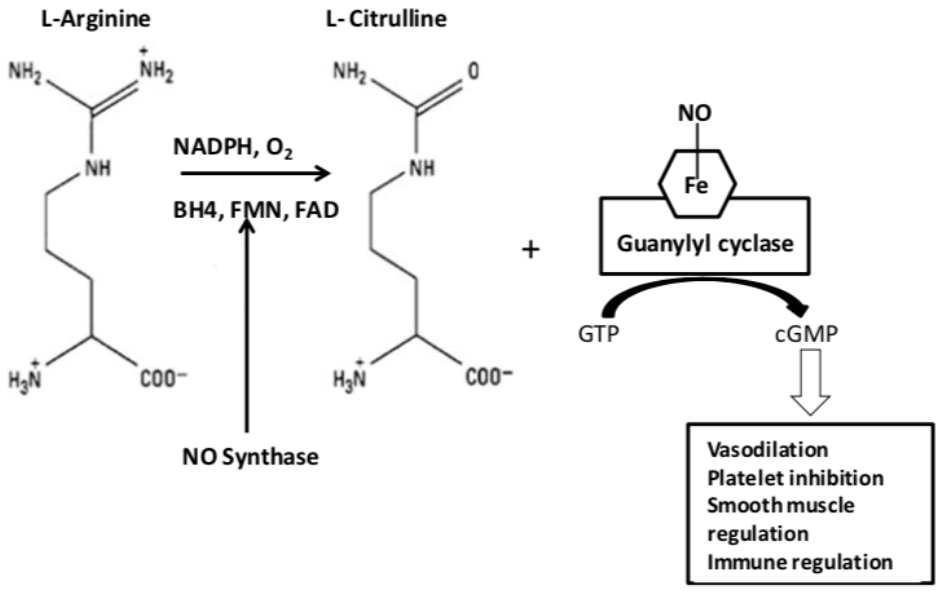

Figure 5 – Biosynthesis of nitric oxide and activation of sGC (Hu, George et al. 2013)

Prostacyclin (PGI_2)

Prostaglandin I_2 (PGI_2), or prostacyclin, is a prostaglandin that affects many organ systems. It is a member of the prostanoids group of eicosanoids that regulate vascular homeostasis, smooth muscle function, inflammation and platelet aggregation (Kelton and Blajchman 1980). Prostanoids are lipid autacoids derived from arachidonic acid by the sequential actions of phospholipase A_2, cyclooxygenase (COX), and specific prostaglandin (PG) synthases (Ricciotti and FitzGerald 2011). There are two main COX enzymes, COX1 and COX2, that vary in structure, distribution, function and localization in the cell and tissues. It was generally reported that COX1 is largely expressed in a constitutive way, while COX2 is induced during inflammation and vascular injury (Majed and Khalil 2012). PGI_2 is produced by endothelial cells and influences many vascular processes. Indeed, PGI_2 acts mainly on the prostacyclin (IP) receptor, but because of receptor homology, PGI_2 analogs such as iloprost may act on other prostanoid receptors with variable affinities. PGI_2/IP interaction stimulates G protein-coupled increase in cAMP and protein kinase A, resulting in decreased cytosolic calcium concentration, and could also cause an inhibition of the Rho kinase pathway, leading to vascular smooth muscle relaxation. In addition, PGI_2 intracrine signaling pathway may target nuclear

peroxisome proliferator-activated receptors and regulate gene transcription. PGI_2 counteracts the vasoconstrictor and platelet aggregation effects of thromboxane A_2 (TXA_2), and both prostanoids create an important balance in cardiovascular homeostasis.

Endothelium-dependent hyperpolarization

Nitric oxide and prostacyclin are not the only vasodilatation factors present in the endothelium. Indeed, in many vascular beds there is a progressive increase in the contribution of endothelium-dependent hyperpolarization (EDH) to the regulation of the vascular tone (Félétou and Vanhoutte 2006). EDH is associated with the hyperpolarization of both endothelial and vascular smooth muscle cells, and has a particular importance in the control of small resistance vessels, local organ blood flow, peripheral vascular resistance, and BP. The importance of EDH in the vasodilation and control of the vascular tone increases as arterial diameter decreases, causing greater dilation in the smaller distal than the bigger proximal uterine vessels (Possomato-Vieira and Khalil 2016). Although the exact nature of the factors involved in EDH is complex, the common features present potassium efflux from ECs through intermediate and small-conductance calcium-activated potassium channels (IK_{Ca} and SK_{Ca}, respectively) leading to the hyperpolarization of the plasma membrane of vascular smooth muscle either by an electrical coupling through the myoendothelial gap junction and/or the activation of potassium efflux in smooth muscle cells by inward rectifier potassium channel (K_{ir}) and/or Na^+/K^+ ATPase pump (Oyama and Node 2013, Vanhoutte, Shimokawa et al. 2017)

Endothelium derived vasocontraction factors

Endothelin-1

Endothelin-1 (ET-1) is a 21 amino acid linear peptide that was isolated by Yanagisawa in 1988 from cultures of porcine aortic endothelial cells (Yanagisawa, Kurihara et al. 1988). ET-1 is produced from a precursor that is converted to the active protein mainly by cleavage by an endothelin-converting enzyme (Varga and Lafyatis 2015). It has at least three isoforms called ET-1, ET-2 and ET-3 respectively. Among these three isoforms, ET-1 is a potent vasoconstrictor that is the only isoform released by vascular endothelial cells, and thus plays an important role in maintaining vascular tone (Davenport, Hyndman et al. 2016). ET-1, ET-2, and ET-3 work by binding to membrane receptors, called ET_A and ET_B. The ET_A receptor has a

greater affinity for ET-1 (100 times greater than for ET-3)(Maguire and Davenport 2015). The binding of endothelin to its receptor activates a G protein coupled to phospholipase C leading to an increase in the cytosolic concentration of calcium responsible for the contraction of vascular smooth muscle (Dinh-Xuan 2003). In addition, it is important to note that the ET_A receptor is mainly expressed in cardiomyocytes and vascular smooth muscle cells, but it is not present in vascular endothelial cells. However, the type B receptor (ET_B) is expressed mainly in vascular endothelial cells and in tubular epithelial cells of the nephron. It is also present in small amounts in vascular smooth muscle cells (Guan, VanBeusecum et al. 2015). This high density of ET_B receptors in the endothelium will allow, when stimulated, the release of relaxing factors such as NO and PGI_2. Thus, they oppose the vasoconstrictor effects mediated by the ET_A receptor and slow down the production of ET-1. Under normal physiological conditions, an increase in ET-1 secretion is quickly compensated for by a release of vasodilator factors allowing the control of the vascular tone (del Villar, Alonso et al. 2005, Kowalczyk, Kleniewska et al. 2015) .

Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) are reactive derivatives of O_2 metabolism, including superoxide anion, hydrogen peroxide, hydroxyl radical and singlet oxygen. All types of vascular cells produce ROS, primarily *via* cell membrane-associated NAD(P)H oxidase (Xu and Touyz 2006). ROS are produced by all the cellular components of the vascular wall including endothelial cells, smooth muscle cells, fibroblasts and immune cells. Accumulating evidence indicates that ROS serve as signaling molecules that modulate acute vascular functions such as vasodilation, vasoconstriction and vascular permeability (Staiculescu, Foote et al. 2014). In normal conditions, there is a dynamic balance between the generation of ROS and its elimination, promoting the normal function of cells, whereas under pathological conditions, the equilibrium changes to overproduction of ROS leading to increased oxidative stress that damages essential macromolecules such as proteins, lipids and nucleic acids (Zhang, Wang et al. 2016). The physiological reason for cellular ROS generation could be divided into two categories:

- the mitochondrial oxidative metabolism, that release ROS as a byproduct, or a waste product, of various other necessary reactions (Zhang, Wang et al. 2016).

- processes in cellular response to xenobiotics, cytokines, bacterial invasion and high stress, that generate ROS intentionally, either in molecular synthesis or in breakdown, as part of a signal transduction pathway, or as part of a cell defense mechanism (Zhang, Wang et al. 2016).

The generation of ROS in the endothelial cell involves many sources and pathways such as NADPH oxidases (NOX), uncoupled NO synthases, cyclooxygenases (COX), mitochondrial respiratory chain, cytochrome p450 (CYP450), xanthine oxidase and the peroxisomes (Di Meo, Reed et al. 2016). However, ROS are inactivated by various antioxidant such as the superoxide dismutases (SODs) which act exclusively on superoxide, whereas catalase and the peroxiredoxin (PRDX) enzymes act only on hydrogen peroxide (Holmström and Finkel 2014). Moreover, several dietary micronutrients have antioxidants properties, such as vitamin A (retinoids, carotenes), vitamins C and E (tocopherols), lycopene, lutein, ubiquinone, glutathione, and polyphenols including anthocyanins, catechins or procyanidins (Poljsak, Šuput et al. 2013, Auger and Schini-Kerth 2014).

The regulation of vascular tone by ROS is mainly due to the inactivation of relaxing factors. Indeed, the inactivation of NO by ROS is a key mechanism underlying the development of endothelial dysfunction, which in turn is an important contributor to cardiovascular disease pathophysiology (Cave, Brewer et al. 2006). ROS, and particularly superoxide anions, react with NO leading to reduction of NO bioavailability and of subsequent vasorelaxation. In addition, the peroxynitrite anions resulting from the interaction of NO and superoxide anions can lead to tyrosine nitration of proteins, thus modulating their activity, including the inactivation of prostacyclin synthase (Vanhoutte, Shimokawa et al. 2017).

Furthermore, ROS can directly induce vasocontraction of smooth muscle cells. Vasoconstriction in response to the increased production of ROS is mediated, at least in part, through the activation of the ryanodine receptors (RyRs) on the sarcoplasmic reticulum thus releasing calcium (Song, Makino et al. 2011). ROS also activate protein kinase C (PKC) which, together with ROS, inhibit membrane voltage-gated potassium channels (K_V) causing membrane depolarization and opening of membrane voltage-gated calcium channels (Ca_V). Additionally, ROS and PKC can also activate membrane store-operated calcium channels (SOC), both contributing to the increase in cytosolic calcium levels facilitating contraction

(Wang and Zheng 2010). Moreover, ROS can activate COX, leading to the formation of vasoconstricting prostanoids (Vanhoutte, Shimokawa et al. 2017)

Thromboxane A2 (TXA_2)

Endothelium might maintain vascular tone by controlling the balance between NO and COX-derived prostanoids (Lüscher and Vanhoutte 1986). Indeed, Luscher and Vanhoutte reported that ACh caused endothelium-dependent contractions in thoracic aorta isolated from spontaneously hypertensive rats (SHR), and that these contractions were normalized by indomethacin, an unspecific cyclooxygenase (COX) inhibitor. This involved the release of an endothelium-derived contracting factors, namely a COX-derived prostanoids, later identified as Thromboxane A_2.

Thromboxane A_2 (TxA_2) plays a very important role in the endothelium-dependent contractions of arteries. The arachidonic acid (AA) is metabolized by cyclooxygenase (COX) to form the unstable prostaglandin H_2 which is further converted into TxA_2 (Chen 2018). After being produced by thromboxane synthase (TxAS), TxA_2 ultimately stimulates the TxA_2/prostanoid (TP) receptor to induce vasoconstriction. The calcium ionophore A23187, the prostanoid precursor AA, or the muscarinic receptor agonist acetylcholine (ACh) can evoke endothelium-dependent contractions associated with TxA_2 (Maclouf and Bellucci 1986, Chen 2018).

Angiotensin II

Angiotensin II (Ang II) is produced from a protein called angiotensinogen mainly synthetize in the liver. This serpin is then cleaved by renin, an enzyme of the renin-angiotensin system (RAS) produced in the kidney, to form angiotensin I that seems to have no clear biological function and is an essential precursor for angiotensin II. As it passes in the bloodstream through the lungs and kidneys, it is further metabolized to produce angiotensin II by the action of angiotensin-converting enzyme (ACE) (Fountain and Lappin 2017). Moreover, cells localized in the vascular wall, such as endothelial cells and smooth muscle cells, are able to synthesize their own tissue-based components of the local RAS (Itoh, Kajikuri et al. 2003).

Angiotensin II (Ang II) is a potent multifunctional peptide acting on vascular receptors. The two types of Ang II receptors present in the heart and vascular smooth muscle and that are

responsible for signal transduction mediating the vasoconstrictive and vasoprotective action of Ang II are respectively the AT_1 and AT_2 receptors. The AT_1 receptor mediates the vasopressor activity of Ang II while the AT_2 receptor is associated with the vasoprotective actions of Ang II and tends to counteract the AT_1 effects (Kawai, Forrester et al. 2017). The Ang II/AT_1 Receptors pathway interact with several heterotrimeric G-proteins coupled to second messengers and cytosolic proteins, including phospholipase C (PLC), phospholipase A2 (PLA2), phospholipase D (PLD), and protein kinase C (PKC) (Nguyen Dinh Cat, Montezano et al. 2013), leading to the calcium-dependent phosphorylation of myosin and subsequent contraction of the vascular smooth muscle. This arterial smooth muscle contraction is responsible for raising blood pressure (Morris and Kahwaji 2019). In addition several evidences indicate that the Ang II/AT_1R pathway is a potent activator of NADPH oxidases (Nox), which are a major sources of ROS in vascular cells (Nguyen Dinh Cat, Montezano et al. 2013).

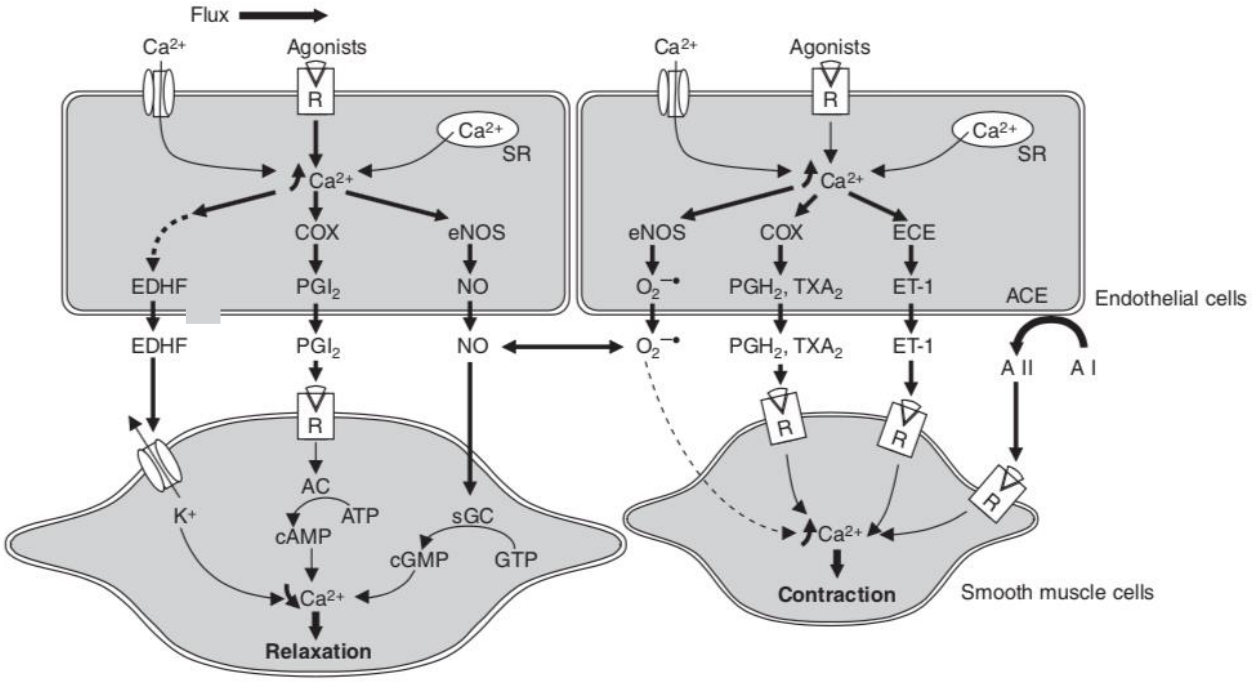

Figure 6 – Release of relaxing and contracting factors from endothelial cells and their effects on vascular smooth muscle cells (Shiogai, Stefanovska et al. 2010).

AC: adenylyl cyclase; ACE: angiotensin converting enzyme; ATP: adenosine triphosphate; A I: angiotensin I; A II: angiotensin II; cAMP: cyclic adenosine monophosphate; cGMP: cyclic guanosine monophosphate; COX: cyclo-oxygenase; ECE: endothelin converting enzyme; EDH: endothelium-derived hyperpolarization; eNOS: endothelial nitric oxide synthase; ET-1: endothelin-1; GTP: guanosine triphosphate; NO: nitric oxide; $O_2^{-\bullet}$: superoxide anions; PGH_2: prostaglandin H_2; PGI_2: prostacyclin; sGC: soluble guanylyl cyclase; SR: sarcoplasmic reticulum; TXA_2: thromboxane A_2.

Age-related endothelial dysfunction

As we mentioned in chapter 1, aging is one of the most important risk factors for cardiovascular diseases. During Aging, the cardiovascular system undergoes structural and functional changes which compromise endothelial health and promotes vascular pathologies that affect the ability to execute daily tasks (Donato, Machin et al. 2018). Cardiovascular parameters at rest change little with age in a healthy individual. They are still sufficient to ensure adequate blood flow. It is during intense efforts that the effects of aging on cardio-circulatory function are evident (Grossen 2002). This is attributable, in part, to the development of a vascular endothelial dysfunction triggered by a reduced peripheral artery endothelium-dependent dilation in response to different stimuli (Seals, Jablonski et al. 2011).

Indeed, a decrease in ACh-induced endothelium-dependent relaxation during aging has been observed in several vascular beds including human brachial artery (Taddei, Galetta et al. 2000), and rats' aorta and mesenteric artery (Matz, de Sotomayor et al. 2000) (Figure 7).

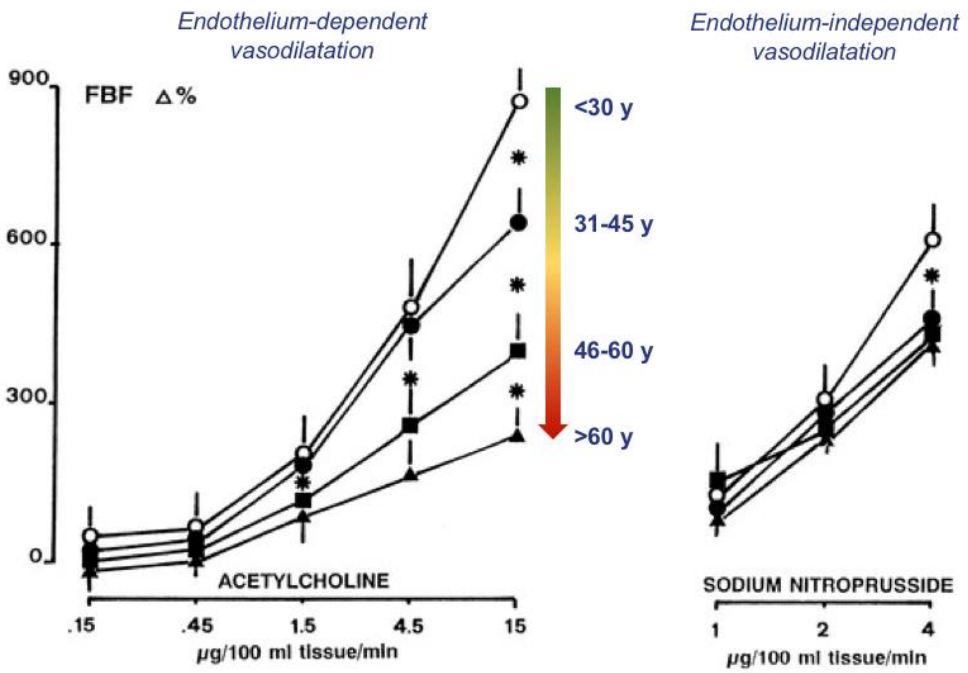

Figure 7 – Influence of age on forearm blood flow (FBF) induced by intra-arterial acetylcholine in normotensive subjects with different ages. Adapted from (Taddei, Virdis et al. 1995).

The age-related endothelial dysfunction is characterized by a reduced formation of vasorelaxant factors and an increased formation of vasoconstricting factors. Moreover, age-related endothelial dysfunction is associated with increased vascular oxidative stress and structural modification of the vascular wall.

Increased oxidative stress

Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and the body's antioxidant defense systems, potentially leading to damage to proteins, lipids, and DNA (Cai and Harrison 2000, Förstermann and Sessa 2012). In the endothelium, this excess of ROS notably impairs the function of endothelial nitric oxide synthase (eNOS). The activity of eNOS is strongly influenced by the redox-sensitive balance between tetrahydrobiopterin (BH4) and dihydrobiopterin (BH2) (Schmidt and Alp 2007). When BH4 levels decrease in favor of BH2, eNOS becomes "uncoupled," producing more superoxide anions ($O_2^{\cdot -}$) instead of nitric oxide (NO). Conversely, restoring adequate BH4 levels re-establishes normal NO production and reduces the formation of reactive species, contributing to vascular homeostasis and protecting the endothelium from oxidative stress (Augustin and Koh 2024). Accumulating evidences suggest that during Aging, oxidative stress is increased in

the body beyond the capacity of endogenous antioxidants to balance redox status, promoting the age-related endothelial dysfunction (Marín, Yubero-Serrano et al. 2013). Indeed, vascular aging is associated with vascular oxidative stress (Ungvari, Tarantini et al. 2018). In these conditions, ROS act as destructive agents affecting proteins, lipids and DNA, leading to cellular damage, tissue injury, and inflammation (Sena, Leandro et al. 2018, Sridevi, Budde et al. 2018).

Superoxide anion ($O^{\bullet -}_2$) is the major ROS produced in response to several stimuli (such as hyperglycemia, hyperlipidemia, and hypertension) that quickly combines with NO to produce peroxynitrite which has many proatherogenic effects, such as decreasing NO bioavailability and promoting oxidation of LDL-cholesterol, a major initiating events of atherosclerosis (Pellegrin, Mazzolai et al. 2009). In response to increased ROS formation, the endothelial cells became activated and produce vasoconstrictor agents such as thromboxane A2, endothelin-1, or prostaglandin H2 (Mudau, Genis et al. 2012, Sena, Leandro et al. 2018).

NADPH oxidase is the primary source of ROS in the vasculature and is functionally active in all cells within the vessel wall, including endothelial cells, VSMCs, fibroblasts and monocytes/macrophages (Cohen and Tong 2010, Konior, Schramm et al. 2014). The endothelial dysfunction is generally associated with overexpression and activation of the NADPH oxidases, due at least in part to the activation of the local angiotensin system. Angiotensin II (Ang II) stimulates NADPH oxidase both by increasing expression of NADPH oxidase subunits as well as by increasing ROS production and enhances arginase activity mainly through angiotensin II receptor type 1 (AT1R), leading to reduce NO bioavailability (Cohen and Tong 2010). Indeed, NADPH oxidase-mediated formation of superoxide anions is associated with the NO inactivation and $ONOO^-$ formation, and with the oxidization of the eNOS cofactor BH4 leading to eNOS uncoupling that will in turn generate further superoxide anions and decreases NO production (Chen, Ye et al. 2018).

In addition to the NADPH oxidases, mitochondrial oxidative phosphorylation produces superoxide anions that can be converted to H_2O_2 by the manganese-dependent superoxide dismutase (MnSOD) and subsequently to water by glutathione peroxidase 1. Under pathological conditions, due to insufficient ROS detoxification or excessive ROS production, mitochondrial dysfunction and oxidative stress have been linked with several cardiovascular diseases including atherosclerosis in human (Sena, Leandro et al. 2018).

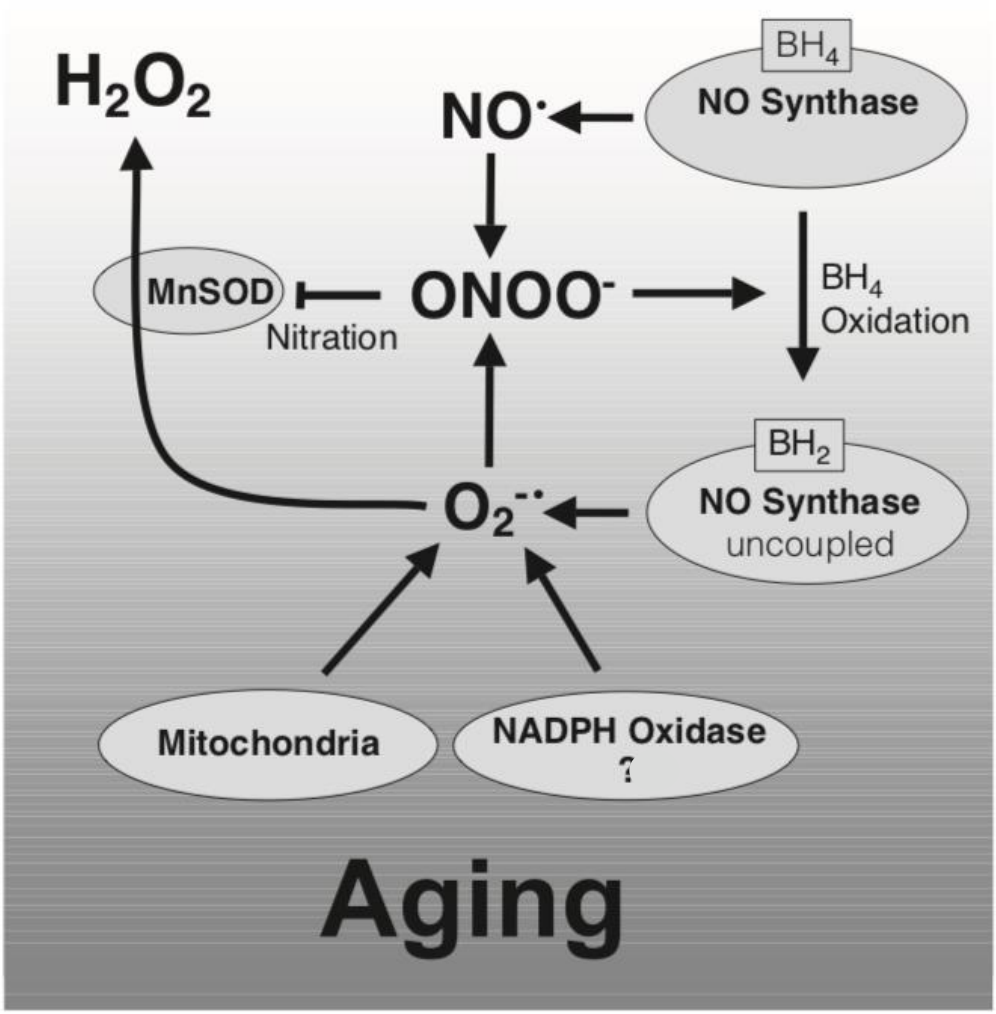

Figure 8 – Mechanisms of aging-induced oxidative stress in endothelial cells (Brandes, Fleming et al. 2005).

Decreased NO production

In a previous paragraph, we discussed the importance of nitric oxide in normal physiological situations and its role in preserving vascular homeostasis. We also said that any imbalance may lead to endothelial dysfunction and subsequently to cardiovascular diseases. Aging is associated with such imbalance, and most studies agree that the bioavailability and/or the generation of NOS-derived NO decreases with aging (Torregrossa, Aranke et al. 2011). The reduced NO bioavailability could be due to either inactivation by $O^{\bullet -}_2$ overproduction, as already described above, and/or to an reduced NO formation through altered eNOS expression/activity/pathway (Cau, Carneiro et al. 2012).

The alteration in the L-arginine-NO pathway could be related to reduced substrate availability or to the presence of an endogenous NO synthase inhibitor, such as asymmetric dimethyl-L-arginine (ADMA) (Taddei, Virdis et al. 2001). The deficit in L-arginine, the substrate of eNOS, has been shown to promote eNOS uncoupling and endothelial dysfunction (Piotr, Mariusz et al. 2016). The reduced NO bioavailability is associated with increased arginase expression/activity and the uncoupling of eNOS (Shatanawi, Lemtalsi et al. 2015). Indeed, arginase is a key enzyme in L-arginine catabolism. Two subtypes of arginase (ArgI and ArgII) directly compete with eNOS for L-arginine to catalyze its conversion to ornithine and urea, inducing the release of superoxide anions that further reduce NO bioavailability (Lee, Bae et al. 2016). The age-related upregulation of arginase II leads to NOS uncoupling and enhances ROS generation by consuming arginine, oxidizing BH4 cofactors, and/or increasing endogenous methylarginine in endothelial cells, and thereafter induces vascular dysfunction (Figure 9) (Chen, Ye et al. 2018) .

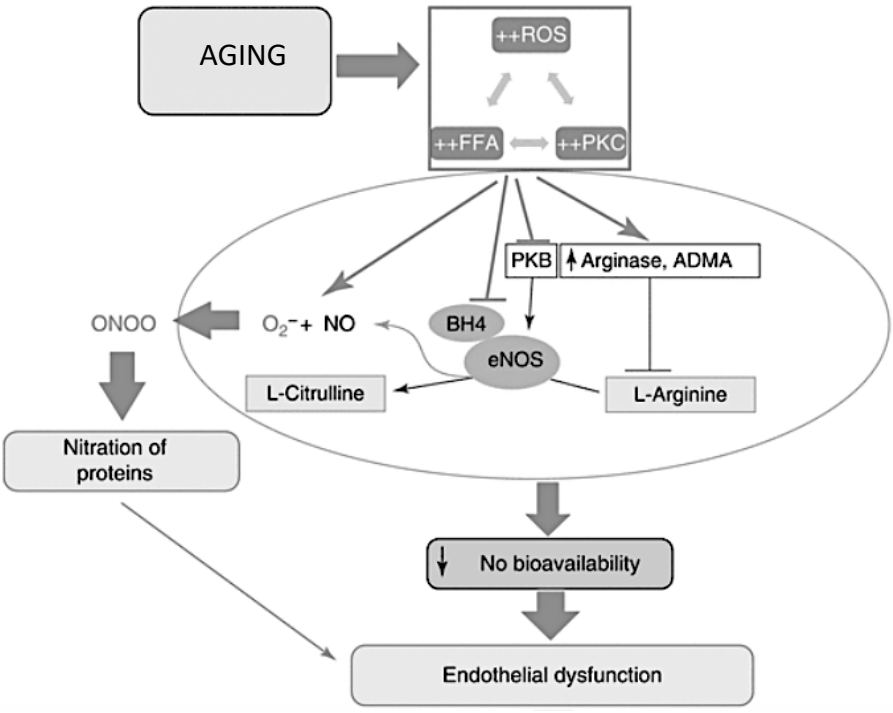

Figure 9 – Mechanisms leading to a decrease in NO bioavailability and subsequent vascular endothelial dysfunction

PKC: Protein kinase C, FFA: free fatty acids, PKB: protein kinase B

Decreased EDH

In addition to a reduced NO bioavailability, the age-related endothelial dysfunction is also characterized by reduced or abolished EDH-mediated vasodilatation in rodents (Long, Newaz et al. 2005, Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012, Farooq, Gaertner et al. 2020, Gaertner, Auger et al. 2020). Moreover, a study on human arteries showed that the EDH-mediated relaxations are impaired in large arteries with aging (Urakami-Harasawa, Shimokawa et al. 1997). Emerging evidences suggest that the alterations of endothelial ion channels such as SK_{Ca} channels, K_{ir} channels, calcium-activated chloride channels, and transient receptor potential vanilloid type 4 (TRPV4) channels contribute to the impairment of EDH during aging (Goto, Ohtsubo et al. 2018). The calcium-activated potassium channels (K_{Ca}) plays a major role in the EDH-mediated vasodilatations and the age-related oxidative stress, especially high level of H_2O_2, can inhibit the function of the endothelial K_{Ca} by modification of cysteine residue (Brakemeier, Kersten et al. 2003). The K_{Ca}-dependent vasodilatations mainly involve SK_{Ca} and IK_{Ca}, and a recent study has shown a significant impairment of SK_{Ca}-mediated relaxation in mesenteric arteries of Aging rats, which may be due to decreased expression or activity of SK_{Ca}, whereas IK_{Ca}-mediated relaxation remained unchanged (Kong, Man et al. 2015). Furthermore, the age-related decrease in EDH is also associated with a decreased expression of SK_{Ca}, IK_{Ca} and AT_2R, and an increased expression of AT_1R in the mesenteric arteries of rats (Idris Khodja, Chataigneau et al. 2012)

Increased endothelin-1

Animal studies provided clear evidences that Aging is correlated with an elevated activation of ET-1 pathway (Van Guilder Gary, Westby Christian et al. 2007). Several studies reported increased circulating levels of ET-1 associated with increased functional activity of endothelin converting enzyme (ECE) in aged rodents (Barton, Shaw et al. 1997). Moreover, contractions in response to ET-1 were assessed in numerous vascular beds and were found to be decreased only in arteries where endothelium-dependent relaxations were decreased (Barton, Shaw et al. 1997). Another study showed that the blocking of ET-1 receptors enhanced the ACh-induced endothelium-dependent vasodilatation in aged hypertensive patients, showing that an increased ET-1 activity leads to hypertension-related vasomotor dysfunction (Cardillo, Campia et al. 2002). In addition, an increased ET-1 system activity may contribute to

atherogenesis through the promotion of fibrosis and the inhibition of endothelial NO synthesis (Stauffer, Westby et al. 2008).

Prostanoids modulation

Endothelium-derived prostanoids such as prostaglandins (prostacyclin, PGI_2) and thromboxane A_2 play a pivotal role in mediating vasodilation and vasoconstriction, and thus in maintaining vascular homeostasis. Accumulating evidences indicate that the age-related changes in endothelial eicosanoids contribute to the decline in the endothelial function and are associated with pathological dysfunction (Qian, Luo et al. 2012). The imbalance between vasodilatory and vasoconstrictor prostanoids is mainly characterized by a progressive decreased production of the vasorelaxant prostacyclin and an increase in the production of cyclooxygenase (COX)-derived vasoconstricting factors (Félétou and Vanhoutte 2006). The role of COX-derived prostanoids in the age-related endothelial dysfunction is demonstrated by the fact that the age-related blunted endothelium-dependent relaxations are significantly improved by COXs inhibitors (Vanhoutte, Shimokawa et al. 2017). Moreover, a study on young and aged hamsters reported an endothelium-dependent contraction in the absence of eNOS inhibitors as well as a blunted endothelial-dependent relaxation in the aorta of old hamsters, supporting the idea that endothelium-dependent contractions are unmasked by a reduction in NO bioavailability in aged animals (Wong Siu, Leung Fung et al. 2009). In addition, indomethacin, a non-selective COX inhibitor, improved the relaxations in response to acetylcholine in humans isolated renal arteries from aged patients as well as the vasodilator response to the muscarinic agonist in the forearm of aging subjects (LÜScher, Cooke et al. 1987, Taddei, Virdis et al. 1995). An enhanced COX isoenzyme protein expression may be pointed out as a mechanism responsible for the increase participation of these vasoconstrictors prostanoids in the age-related endothelial dysfunction (Herrera, Mingorance et al. 2010). Interestingly, there seems to be differences between vessel types in the age-related modification in COX-derived prostanoids. Indeed, in the femoral artery and veins of middle-aged rats, the ACh-induced endothelium-dependent relaxation is blunted in veins but showed an increased contraction in arteries at high concentrations of ACh. Moreover, in the femoral artery, the ACh-induced contraction was significantly abolished in presence of either indomethacin or a selective inhibitor of COX-1 and worsen by a selective COX-2 inhibitor, indicating the involvement of COX-1 derived prostanoids in the age-related COX-mediated

endothelial dysfunction (Gaertner, Auger et al. 2020). Conversely, in the femoral vein of middle-aged rats, the ACh-induced relaxation was significantly improved by a selective inhibitor of COX-2, suggesting that COX-derived prostanoids are differently regulated in femoral artery and vein (Gaertner, Auger et al. 2020). Similarly, in old rat aortas, an increased production of TXA_2 has been detected and may contribute to the endothelial dysfunction.

Increased oxidative stress may be involved in the COX isoenzyme increased expression by editing the composition of the cellular lipid membranes and increases their peroxidation, which would affect lipid substrates availability. Subsequently, the hydroxyl radical derived from superoxide anions could have a positive impact on modulating the COX-2 protein expression, increasing the production of endoperoxide and other vasoconstrictor prostanoids (Herrera, Mingorance et al. 2010).

The renin-angiotensin system

Angiotensin II (Ang II) is the key effector molecule in the renin-angiotensin system (RAS). An upregulated renin-angiotensin system activity is linked with many vascular disorders and many studies pointed out an increased vascular expression of Ang II and ACE with aging (El Assar De La Fuente, Angulo Frutos et al. 2012). Furthermore, a role of the angiotensin system in the age-related endothelial dysfunction has been observed in experimental models (Idris Khodja, Chataigneau et al. 2012, Farooq, Gaertner et al. 2020). Indeed, Ang II is a potent inducer of endothelial dysfunction and vascular oxidative stress *via* the AT1R-mediated activation of the NADPH oxidases to generate superoxide anion. Excessive superoxide anion production promotes the uncoupling of endothelial NO synthase, which in turn reduces NO availability and enhances ROS production, and uncontrolled Ang II–dependent ROS generation takes place as a consequence of the age-associated activation of RAS (Conti, Cassis et al. 2012).

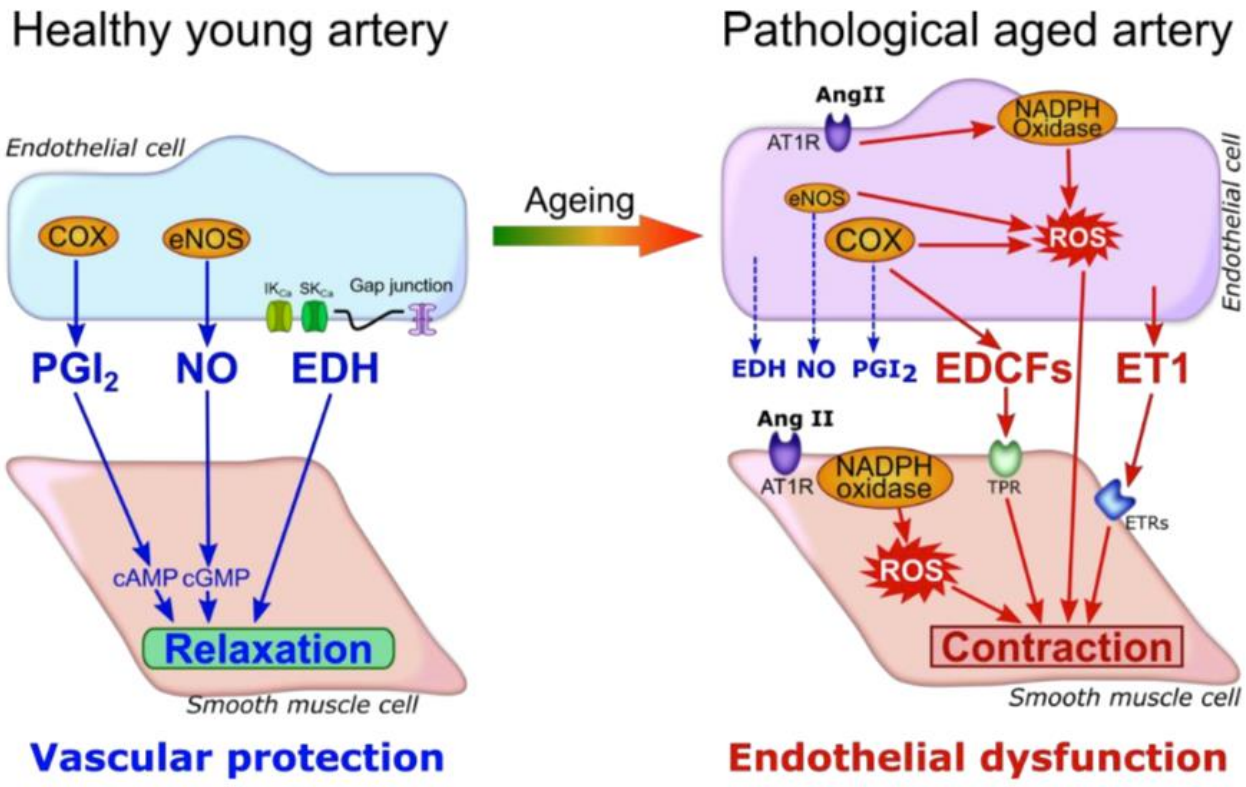

Figure 10 – Role of aging in promoting endothelial dysfunction in aged arteries

Aging and fibrosis

Fibrosis is the development of fibrous connective tissue as a restorative reaction to injury or damage (Anna Biernacka 2011). It is characterized by the aggregation of extracellular matrix (ECM) components, particularly collagen, at the injury site. Fibrosis is a critical component of wound healing and tissue recovery, while prolonged activation is highly counterproductive and a widespread pathological mechanism in cardiovascular diseases (Murtha, Morten et al. 2019). In the cardiovascular system, a progressive age-related deposition of collagen in the vascular wall and in the cardiac interstitial and perivascular space leads to a reduction of the myocardial and arterial compliances (Anna Biernacka 2011). Some evidences suggest that the renin-angiotensin system and generation of reactive oxygen species (ROS) may be involved in age-related fibrotic cardiac remodeling (Horn and Trafford 2016).

The age-related increase in oxidative stress stimulated by Ang II exerts its effects directly through its receptor 1 (AT1R) and indirectly through the induction of the expression of transforming growth factors (TGF) and in particular TGF-β *via* the Smad 2/3 pathway (Rodríguez-Vita, Sánchez-López et al. 2005). Indeed, Ang II induces a NADPH oxidase-mediated generation of ROS which activate TGF-β and upregulate its downstream fibrogenic effector, the connective tissue growth factor (CTGF) (Jiang, Liu et al. 2014). Moreover, some studies showed that the inhibition of the angiotensin system using ACE inhibitors and AT1R antagonists diminished the tissue expression of TGF-β and subsequent fibrosis, and that blockade of TGF-β decreased Ang II-induced ECM production (Border Wayne and Noble Nancy 1998). Furthermore, the TGF-β/Smad2/3 signaling pathway promotes fibroblast proliferation, phenotypic conversion to myofibroblasts and the production of extracellular matrix components including fibrillar collagen, fibronectin, and proteoglycans (Khalil, Kanisicak et al. 2017). In addition, ROS, Ang II and ET-1 induce the expression of adhesion molecules in the microvascular endothelium and of proinflammatory mediator such as MCP-1, which may induce and activate matrix metalloproteinases (MMPs) and induce an imbalance of MMPs and their inhibitors (TIMPs)(Anna Biernacka 2011) (Figure 11).

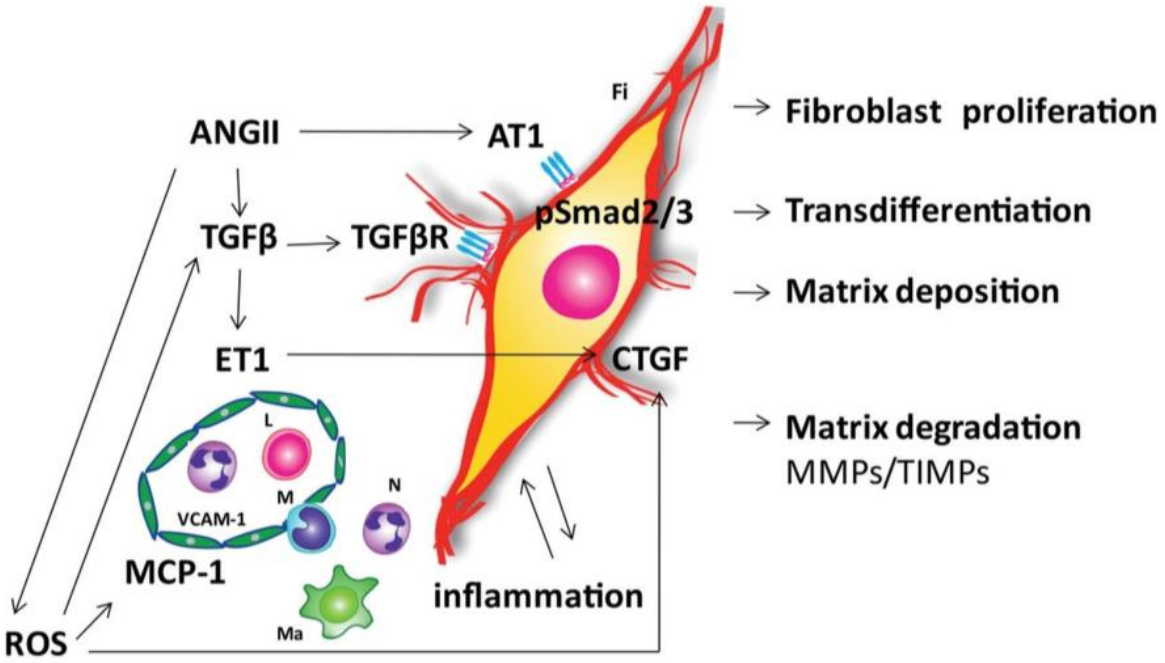

Figure 11 – Pathways involved in the pathogenesis of fibrosis in aged cardiovascular system (Anna Biernacka 2011)

Chapter 3: Endothelial senescence and role of SGLTs

Cellular senescence

Cellular senescence is a physiological process of the physiological aging defined as an irreversible cell cycle arrest, and it is accompanied by changes in cell morphology, function and gene expression. Senescent cells show arrest in the G_1 phase of the cell cycle and are metabolically active but non-proliferative in response to mitogenic stimuli (van Deursen 2014, Herranz and Gil 2018). It was first defined by Leonard Hayflick in 1961 that cultured fibroblasts showed a permanent proliferation cessation after a defined number of cell divisions (Hayflick and Moorhead 1961), although it was believed that cells were immortal and could multiply indefinitely in culture. The number of cycles the cell completed before entering an irreversible growth arrest is known as the "Hayflick Limit" or "replicative senescence" (Sell 2007). Hayflick's discovery of mortal cells paved the way for the discovery and understanding of the molecular pathways underlying cellular aging and marked the onset of cell aging or senescence.

It's important to notice that aging in itself is not a pathology as we do not die of aging but of the pathological complications associated with it, and controlled tissue renewal is essential for the viability of the organism (Verma 2014). However, senescence is observed during embryonic development, wound healing and acts as a protective mechanism against tumorigenesis. In fact, it is recognized as a powerful tumor suppressor mechanism because it stops the proliferation of cells at risk of malignant transformation and constitutes a strong barrier against cancer progression (Vijg, Maslov et al. 2008, van Deursen 2014).

Characteristics of the senescent cells

Senescent cells show several molecular and morphological changes that are observed during the process of aging. These changes are most often accompanied by a resistance to apoptotic signals, an increase in the activity of the lysosomal β-galactosidase enzyme, as well as cytomorphological changes (cell flattening, cytoplasmic vacuolization, increase in nuclear volume) and major alterations in the chromatin architecture and gene expression (Childs, Baker et al. 2014, Song, Lam et al. 2020).

During the initial phase of senescence, which is characterized by cell cycle arrest, we mainly observe an activation of the proteins p53, p21, p16 ^{INK4a} and pRB (Mijit, Caracciolo et al. 2020). During the early phase, cytomorphological modifications emerge (flattening and therefore enlargement of the cell), chromatin modifications (nucleation of heterochromatin foci associated with senescence (SAHF), loss of lamin B1), while the activity of lysosomal β-galactosidase increases significantly (Dimauro and David 2009). This feature is responsible for senescence-associated β-galactosidase (SA-β-gal) activity. Indeed, it has been shown that senescent cells express β-galactosidase activity detectable at pH 6.0 that differ from the acid β-galactosidase activity present in all cells and detectable only at pH 4.0 (Debacq-Chainiaux, Erusalimsky et al. 2009). At this stage, the cell already secretes a large number of pro-inflammatory molecules that are part of the senescence-associated secretory phenotype (SASP) (Borodkina, Deryabin et al. 2018). When senescence persists, cytomorphological and chromatin changes are accentuated and the nature of SASP changes, promoting chronic inflammation (Coppé, Desprez et al. 2010).

Premature senescence

Cellular senescence was originally identified as a process resulting from telomere shortening linked to successive mitoses (Bernadotte, Mikhelson et al. 2016). Since then, many studies have established that beyond this replicative senescence exists other cellular destinies grouped under the term of stress-induced premature senescence (SIPS), caused by various stimuli such as genotoxic stress, oxidative stress, overactivation of oncogenes or tissue damage, and which can also impact post-mitotic cells (Toussaint, Salmon et al. 2005).

Premature senescence is an active cytostatic program that is activated in response to high oxidative stress, proliferative or genotoxic stress, such as the expression of strong oncogenes, tumor suppressor loss, exposure to DNA damage, and reactivation of tumor suppressor pathways. Unlike replicative senescence, premature senescence can be induced irrespective of the replicative "age" of cells, is independent of telomere attrition, and cannot be overridden by restoration of telomerase activity (Bolden and Lowe 2015).

In 1997, the first example of oncogene-induced senescence was described, where forced expression of an oncogenic allele of Ras induced a senescence response in primary human and rodent cells that was accompanied by the induction of p53 and p16 (Mendelsohn, Howley

et al. 2014). Inactivation of p53 or p16 was sufficient to enable the proliferation of Ras-expressing rodent cells, and co-expression of adenoviral protein E1A and Ras was sufficient to enable senescence bypass in human cells (Serrano, Lin et al. 1997, Mendelsohn, Howley et al. 2014).

Endothelial senescence

The senescent phenotype in endothelial cells can be caused by a variety of factors, such as telomere injury, oxidative stress, and prolonged mitogenic stimulation (Regulski 2017). Several laboratories obtained firm evidence indicating that senescent endothelial cells accumulate after repeated balloon endothelial denudation of the rabbit carotid artery, an injury model that provokes endothelial and smooth muscle cell proliferation, and showed that replicative senescence promotes prothrombotic responses in endothelial cells (Fenton, Barker et al. 2001, Erusalimsky and Kurz 2006, Silva, Abbas et al. 2017).

Several lines of evidence indicate that endothelial cell senescence leads to age-related tissue dysfunction and is assumed to be caused by a decrease in regenerative ability, which finally favors disease development (Katsuumi, Shimizu et al. 2018). Studies on cultured endothelial cells have shown that the onset of senescence can be modulated by numerous factors affecting vascular function. These include mitogens, inflammatory molecules, angiotensin II, oxidants and antioxidants, nitric oxide, high glucose, advanced glycation end-products (AGEs), and mitochondrial dysfunction (Erusalimsky and Kurz 2006). Most of these factors influence senescence *via* two main processes: by altering the intracellular levels of cellular oxidative stress and/or by modulating telomerase activity (Erusalimsky 2009)

Oxidative stress

A number of atherogenic and inflammatory stimuli, as well as mitochondrial dysfunction, may lead to an increase in the levels of ROS, thus causing intracellular oxidative stress (Murphy 2013). ROS can induce senescence by acting at multiple levels. Besides affecting telomeres, they can damage genomic DNA. ROS can also damage mitochondrial DNA and other components of this organelle. Mitochondria by themselves generate ROS during normal aerobic metabolism, and some studies suggest that when their function is impaired they may increase their ROS output, thus increasing the oxidative burden of the cell (Di Meo, Reed et

al. 2016). In addition, ROS can act as mediators of oncogenic stress and in turn activate cytosolic stress response kinases and other signaling molecules which have been involved in senescence responses (Davalli, Mitic et al. 2016).

Oxidative stress caused by intracellular mediators such as mitochondria, NADPH oxidases, and the activity of lipoxygenases, cytochrome P450 family members, cyclooxygenase and other cellular oxidases primarily generate $O_2^{\bullet -}$ (superoxide anions) (Pole, Dimri et al. 2016). These mediators and other stressors including activated oncogenes such as H-RasV12 generate ROS, which is known to induce senescence in human and mouse cells (Ogrunc, Di Micco et al. 2014). The ROS-induced senescence *via* mitochondrial or non-mitochondrial pathways likely converge at the known molecular regulators of the cellular senescence (p53, pRB, p16, p21) regulating the expression of inflammatory cytokines, growth factors, and extracellular matrix components (Pole, Dimri et al. 2016, Mijit, Caracciolo et al. 2020). The subsequent modification of the cellular microenvironment creates a vicious cycle of oxidative stress and inflammation causing tissue dysfunction during aging. This process is known as the senescence associated secretory phenotype (SASP) (Figure 12) (Lopes-Paciencia, Saint-Germain et al. 2019)

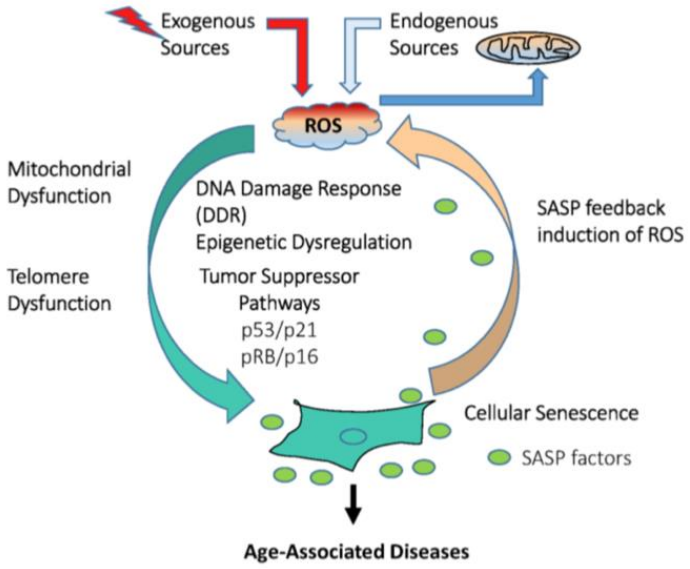

Figure 12 – Role of oxidative stress in cellular senescence (Pole, Dimri et al. 2016)

Modulation of Telomerase Activity

Telomeres are pieces of DNA on the ends of chromosomes that prevent them from end-end fusion or twisting, which might cause DNA to operate improperly. Telomeres shorten when cells duplicate, and this process is associated with senescence or cellular Aging (O'Connor 2008). The correlation between telomere length and replicative potential became a mechanistic link when it was demonstrated that the replicative potential of primary human fibroblasts can be extended indefinitely by artificially elongating telomeres (Aubert and Lansdorp 2008). Oxidative stress and oncogenes can also trigger telomere shortening and dysfunction, one of the hallmarks of Aging, that has been linked to several age-associated traits (normal and pathological) (López-Otín, Blasco et al. 2013).

The occurrence of endothelial cell senescence *in vivo* has also been inferred from examination of telomere length in the vasculature (Erusalimsky 2009). A number of independent studies have shown that telomeres in the endothelium shorten with age and that this erosion is more pronounced in atherosclerosis-prone areas (Khan, Chuturgoon et al. 2012). Substances that induce oxidative stress and have proatherogenic properties such as TNF-α and oxidized LDL were reported to reduce telomerase activity in endothelial cells in association with the inhibition of the PI3K/AKT pathway (Erusalimsky 2009) (Figure 13). In addition, increased generation of ROS has been shown to promote the translocation of TERT from the nucleus to the cytoplasm, thus preventing the enzyme from accessing the telomere (Zheng, Huang et al. 2019). Furthermore, evidence from other cell types suggest that oxidation of a TERT cysteine residue that is sensitive to the intracellular levels of glutathione may account, at least in part, for the decrease in activity that occurs in endothelial cells upon inhibition of glutathione synthesis or nitrosative stress (Kurz, Decary et al. 2004).

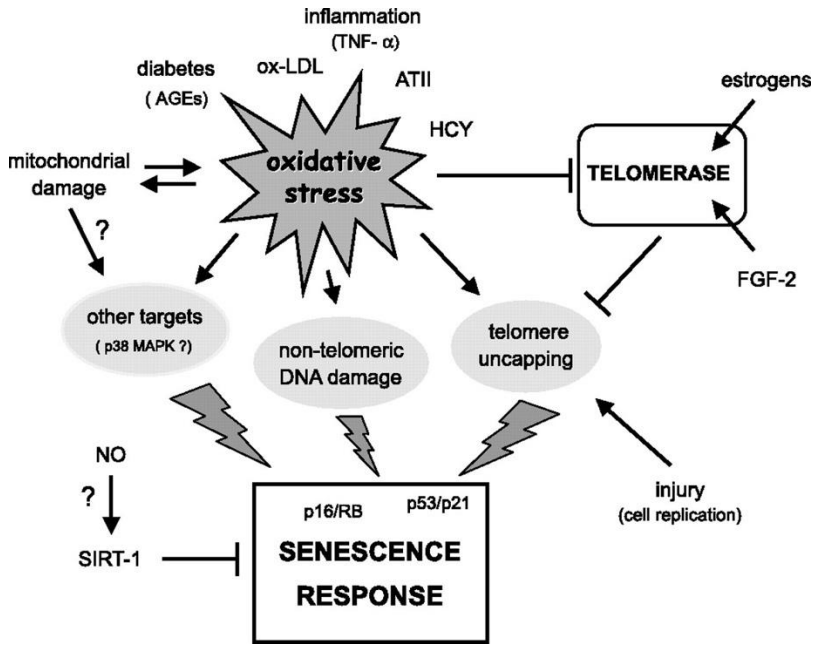

Figure 13 – Effect of oxidative stress on telomerase activity (Erusalimsky 2020)

Sodium glucose co-transporters

Despite the decline in their proliferative potential, senescent cells display a high metabolic activity and have been shown to acquire a more glycolytic state even in presence of high oxygen levels, in a way similar to cancer cells, which results in an upregulation in the expression sodium glucose transporters (Sabbatinelli, Prattichizzo et al. 2019, Khemais-Benkhiat, Belcastro et al. 2020). 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. The two well-known SGLT family members are SGLT1 and SGLT2 (Song, Onishi et al. 2016, Khemais-Benkhiat, Belcastro et al. 2020).

SGLT1

Is a protein encoded by the SLC5A1 gene which transports D-glucose and D-galactose with a similar affinity (Km = 0.5 mmol / l) and with a stoichiometry of 2 sodium / 1 sugar (Hediger, Budarf et al. 1989, Chen, Coady et al. 1995, Hirayama, Díez-Sampedro et al. 2001). SGLT1 is primarily expressed at the apical membrane of the brush border of epithelial cells in the small intestine and in the S3 segment of the proximal renal tubule, where it is responsible for the reabsorption of approximately 10% of filtered glucose. In addition to the intestine and kidney,

hSGLT1 mRNA has been detected in the heart, testes, prostate, colon, trachea, lung, brain, spinal cord, spleen, liver, uterus, pancreas and blood vessels (Poppe, Karbach et al. 1997, Zhou, Cryan et al. 2003). studies have also suggested that SGLT1 may facilitate the uptake of flavonoids such as quercetin-glucoside in the intestine and possibly other tissues where SGLT1 is expressed like epithelial cell (Arts, Sesink et al. 2002, Chen, Ma et al. 2014), Jin et al. demonstrated that delphinidin-3-O-glucoside, a prominent anthocyanin, can be transported into endothelial cells via SGLT1. This finding expanded the classical understanding of SGLT1 beyond its conventional role in glucose uptake. The study suggests that endothelial SGLT1 may directly facilitate the vascular protective actions of anthocyanins by delivering these compounds into the endothelium (Jin, Yi et al. 2013). In addition, Chen et al. explored the mechanism by which resveratrol, a well-known stilbene, enters endothelial cells and uncovered a potential SGLT1-mediated pathway. Although resveratrol generally exists as an aglycone, the observation that it can also be handled by SGLT1 highlights the transporter's broader substrate specificity. Such uptake could support the compound's anti-inflammatory and cardioprotective benefits at the vascular level (Chen, Yi et al. 2013). Moreover hi et al. (2016) showed that calycosin-7-O-glucoside, an isoflavone glycoside, is similarly dependent on SGLT1 for its absorption in the intestine. This underscores a pattern wherein the sugar conjugation of polyphenols enhances transporter affinity. Consequently, SGLT1 activity is a key factor in the effective delivery and bioactive potential of this class of phytoestrogens (Shi, Zheng et al. 2016).

SGLT2

Is a protein encoded by the SLC5A2 gene and is expressed in the apical membrane of epithelial cells of the brush border of the S1 and S2 segments of the proximal renal tubule, where it ensures the transport of glucose coupled with sodium with a stoichiometry of 1 sodium / 1 glucose and is responsible for the reabsorption of 90% of filtered glucose at the renal level (Kanai, Lee et al. 1994, Hummel, Lu et al. 2012).

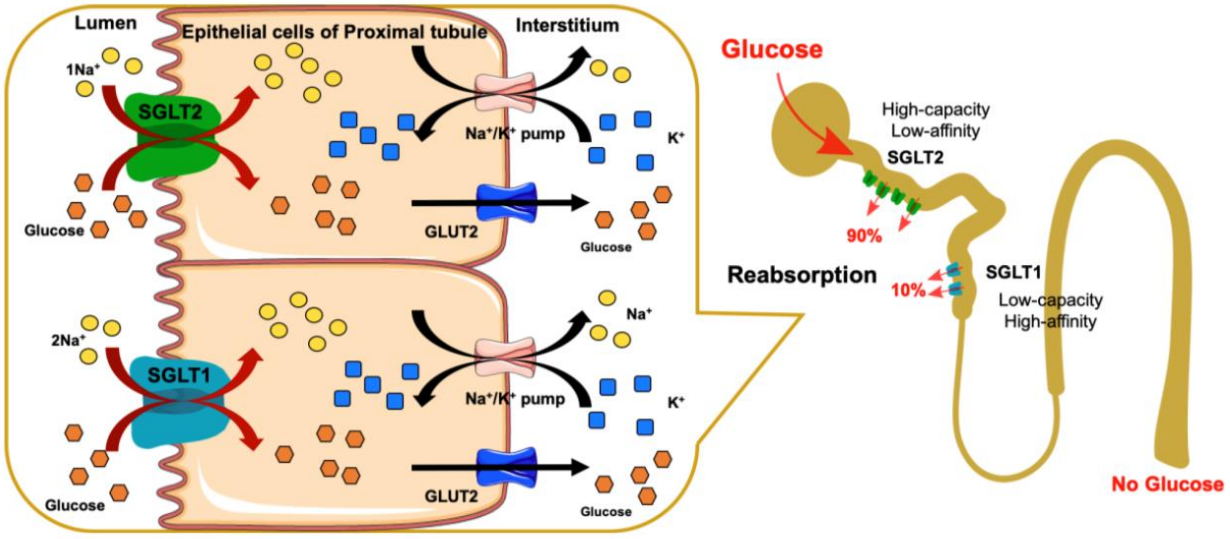

Figure 14 – Role of SGLT1 and 2 in normal renal physiology (Park 2019)

SGLT1 and SGLT2 expression under pathological conditions

It has been described that under pathological conditions such as cardiac ischemia, heart failure and chronic hyperglycemia, the glucose requirements of cardiomyocytes are increased (Tian and Abel 2001). Similarly, in the context of diabetes, glucose transport is increased in cells such as skeletal muscle cells, renal epithelial cells, hepatocytes and adipocytes (Bogan 2012). This increased transport is provided by the overexpression of various transporters including GLUTs, SGLT1 and SGLT2 (Wang, Levi et al. 2017). Moreover, stress-induced senescence sems to promote the expression of SGLT1 and 2 *in vivo* and *in vitro* (Kitada, Nakano et al. 2014, Madonna, Doria et al. 2020). Indeed, the redox-sensitive upregulation of SGLT1 and 2 was observed in cultured and native coronary artery endothelial cells and *in vivo* at arterial sites at risk, under pathological conditions such as hyperglycemic state, increased oxidative stress, endothelial senescence and dysfunction by promoting excessive glucose entry (Khemais-Benkhiat, Belcastro et al. 2020, Park, Farooq et al. 2020). In addition, circulating microparticles from coronary artery disease patients were reported to upregulate SGLT1 and 2 expression in endothelial cells (Park, Belcastro et al. 2019).

SGLT Inhibitors

SGLT inhibitors are a recently developed class of prescription drugs used for the treatment of type 2 diabetes through an increased urinary excretion of glucose. Most of the SGLT inhibitors such as canagliflozin, dapagliflozin and empagliflozin are targeting selectively SGLT2 whereas sotagliflozin is a dual inhibitor of SGLT1 and SGLT2 (Van Name 2019). Selectivity of SGLT inhibitors for SGLT2 over SLGT1 is important as 90% of the glucose is reabsorbed in the renal tubule by SGLT2. Moreover, the inhibition of SGLT1 in the intestine could lead to glucose-galactose malabsorption, a disease characterized by severe dehydration and diarrhea (Haider, Pathak et al. 2019).

The complications of type 2 diabetes are frequently linked to atherosclerotic cardiovascular disease, heart failure, and chronic kidney disease (Standl, Schnell et al. 2016). The established clinical efficacy of the SGLT inhibitors class on the cardiovascular complications of type 2 diabetes has resulted in consensus recommendations of the European society of cardiology in their recent guidelines (Cosentino, Grant et al. 2020). Indeed, clinical trials conducted to assess SGLT2 inhibitors safety and efficacy in diabetic patients with established vascular disease, such as EMPA-REG OUTCOME (Empagliflozin Cardiovascular Outcomes), demonstrated that patients who were at high risk of cardiovascular diseases and that were treated with SGLT2 inhibitors had an early reduction in major cardiovascular and renal outcomes (Zinman, Wanner et al. 2015) and these effects appear to be independent of glycemic control (Inzucchi, Kosiborod et al. 2018). However, the mechanisms involved in the cardiovascular protective effect of SGLT2 inhibitors remain largely unknowns. Studies suggested that this protective effect is due to the ability of empagliflozin to reduce senescence of cardiac cells, oxidative stress, blood pressure, autophagy (autophagic cell death) or to improve glucose control (Cowie and Fisher 2020, Madonna, Doria et al. 2020, Jiang, Xu et al. 2021). In addition, the dual SGLT1 and SGLT2 inhibitor sotagliflozin has been reported to reduce or abolish the glucose uptake in the intestine with a potential effect on reducing CVD events (Cefalo, Cinti et al. 2019). Indeed, the clinical study SOLOIST-WHF recently demonstrated a promising effect of sotagliflozin on cardiovascular events in patients with type 2 diabetes with heart failure, showing a reduction of adverse events in favor of sotagliflozin *versus* placebo (51 vs. 76.3 events/100 patients). So far, the study included 1222 patients but was interrupted prematurely because of the COVID pandemic (Bhatt, Szarek et al. 2020).

Chapter 4: Anthocyanins and cardiovascular health

Anthocyanins

The word Anthocyanin is a combination of two greek words, Anthos and Kyanos, meaning “the blue from flowers” (Anthos=Flower, and Kyanos=Blue). Indeed, anthocyanins are water-soluble pink to purple colored natural plant pigments belonging to the flavonoid subclass. Anthocyanins can be synthesized as secondary metabolites mainly in flower and fruits of many plants, including grapes (Crozier, Jaganath et al. 2009, Yang, Yuan et al. 2018). They are naturally found in glycosylated forms and are responsible for the colors in fruits and vegetables such as berries, currants, grapes, and some tropical fruits (Khoo, Azlan et al. 2017).

Anthocyanins are abundant in the human diet and can be found in red wine, certain varieties of cereals, vegetables (aubergines, cabbages, beans, onions and radishes), but they are most abundant in fruit (Del Rio, Rodriguez-Mateos et al. 2013). So far, over 600 anthocyanins were isolated from various plants species (de Pascual-Teresa and Sanchez-Ballesta 2008). They are derived from only about 30 different anthocyanidins, but the majority is based on either cyanidin (31%), delphinidin (22%), or pelargonidin (18%), peonidin, malvidin and petunidin (Martín, Navas et al. 2017).

Besides the use of anthocyanins as natural dyes as food colorants in the food industry and cosmetics and personal care products, including lipsticks, blushes, and hair dyes (Sigurdson, Tang et al. 2017), these colored pigments have potential beneficial health effects. Indeed, several studies including *in vitro* studies, experimental *in vivo* studies and clinical trials have shown that anthocyanins possess antioxidative and antimicrobial activities, improve visual and neurological health, and protect against cardiovascular diseases (Khoo, Azlan et al. 2017).

Structure and characteristics

Many structures and anthocyanins profiles have been reported in different plant species Anthocyanins are based on a single basic core structure (Figure 15), the flavylium cation (Pervaiz, Songtao et al. 2017). Chemically, anthocyanins are glycosylated polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrilium, usually with molecular weights ranging

from 400 to 1200 g/mole and containing two benzyl rings (A and B) and one heterocyclic benzopyran ring (C ring)(Bueno, Sáez-Plaza et al. 2012). Anthocyanins are usually conjugated to a single glucoside unit, but many anthocyanins contain two, three, or more sugars attached at multiple positions or by the C3 hydroxyl in C ring. The intensity and type of color of the anthocyanins is affected by several factors including the number of hydroxyl and methoxyl groups, with hydroxyl groups associated with a hypsochromic shift (towards blue) and methoxyl groups with a bathochromic shift towards red (Khoo, Azlan et al. 2017, Martín, Navas et al. 2017).

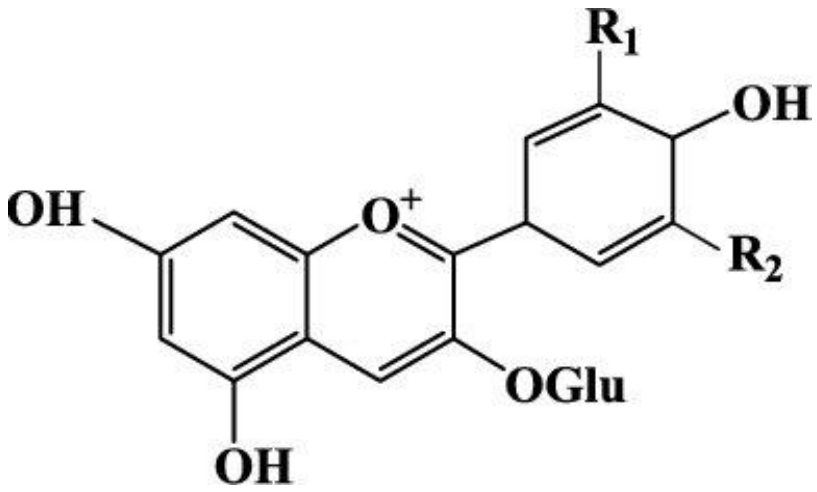

Figure 15 – General structure of anthocyanins (Eker, Aaby et al. 2020)

Anthocyanidin	Abbrev.	R_1	R_2	Some of the produced colors
Delphinidin	Dp	OH	OH	Purple, mauve and blue
Petunidin	Pt	OH	OCH_3	Purple
Malvidin	Mv	OCH_3	OCH_3	Purple
Cyanidin	Cy	OH	H	Magenta and crimson
Peonidin	Pn	OCH_3	H	Magenta
Pelargonidin	Pg	H	H	Orange salmon

Table 1 – Major anthocyanins present in fruits (Bueno, Sáez-Plaza et al. 2012)

Anthocyanins' stability

Due to the presence of the flavylium cation, anthocyanins are extremely instable and can be easily degraded to colorless or brown-color compounds in neutral or alkaline environment. Their color is influenced by the pH due to conversion between molecular forms, and eventually leading to degradation into chalcones at higher pH. In addition to pH, the stability of anthocyanins is influenced by a number of factors including temperature, oxygen concentration, light, molecular concentration, and the presence of copigments, metallic ions and enzymes (Jiang, Mao et al. 2019)

pH

pH has a significant effect on the color of anthocyanins because protonation and deprotonation of the molecule affect and breaks the conjugated double bond structure that gives the molecule its color (Kaimainen 2014). In acidic environments, the anthocyanins are predominantly in the red flavylium cation state, but as the pH rises to neutral, the deprotonated blue quinonoidal base, or the colorless hydroxyl adducts carbinol (pseudo) base are predominant and could lead to degradation. With increasing pH, anthocyanins normally fade to the point where they may appear colorless (pH 4.5) before finally turning purple or blue at pH> 6. (Jackman and Smith 1996). The structural transformations of anthocyanins due to pH changes are fundamental for their color and stability

Temperature

As most of chemical reactions, the anthocyanins stability and the rate of their degradation are strongly influenced by temperature, and thermal degradation usually follows a first-order reaction kinetics (Loypimai, Moongngarm et al. 2016). Prolonged exposure of anthocyanins to high temperature results in fission between the B and C rings of the chalcone, releasing the phenolic acid and aldehyde constituents. The thermal degradation of delphinidin aglycone, for example, leads to the generation of gallic acid and of a trihydrobenzaldehyde (McDougall, Dobson et al. 2005)

Other factors

Oxygen and light have been found to have a damaging effect on anthocyanins (Rein 2005). Moreover, it has also been conclusively demonstrated that anthocyanins structure can be

stabilized by their increased concentration and co-pigmentation reactions (phenomenon where pigmentation due to the presence of anthocyanins is enhanced by the presence of other colorless compounds, known as "co-pigments"). Increasing the anthocyanins concentration profoundly improves their stability through a process known as self-association. Moreover, co-pigmentation with other polyphenols, alkaloids, amino acids and organic acids stabilizes the chemical structure of anthocyanins (Rein 2005, González-Manzano, Santos-Buelga et al. 2008)

Anthocyanins in Blackcurrant juice and extract

Blackcurrant (Ribes nigrum L.) juice and extracts are widely recognized for their remarkably high concentrations of anthocyanins. Fifteen anthocyanin structures are reported from an extract of black currant berries (Slimestad and Solheim 2002). The anthocyanins were characterized by means of size exclusion chromatography, high-performance liquid chromatography, UV-visible spectroscopy, and electrospray mass spectrometry revealed that delphinidin-3-rutinoside, delphinidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside are the most present pigment in the extract with 97% (Slimestad and Solheim 2002). These anthocyanins not only offer potent antioxidant effects by scavenging free radicals but may also exert anti-inflammatory and cardioprotective influences when consumed as part of a balanced diet. In addition to anthocyanins, blackcurrant juice and extracts contain other phenolic molecules, such as flavonols (e.g., quercetin and myricetin) and phenolic acids (e.g., chlorogenic, caffeic, and ferulic acids), which further enhance the overall antioxidant profile (Mattila, Hellström et al. 2011). The interplay among these phenolic compounds appears to produce synergistic effects, potentially making blackcurrant-based products particularly beneficial for health promotion and disease prevention. Furthermore, factors like fruit ripeness, cultivar variation, and processing methods such as pasteurization or freeze-drying can influence the retention and stability of these bioactive constituents (Moyer, Hummer et al. 2002). Consequently, blackcurrant juice and extracts are increasingly explored as functional ingredients in the food and nutraceutical industries, where they are valued both for their robust flavor and their promising health benefits.

Bioavailability of anthocyanins

Studies involving individual anthocyanins have shown that anthocyanins amount in plasma is at most around 1% of the ingested quantity, due to limited intestinal absorption, while additional factors may contribute to the proposed low anthocyanins bioavailability in plasma and urine, such as high rates of cellular uptake, metabolism, and excretion (Milbury, Vita et al. 2010).

Indeed, in a study where pigs were fed diets supplemented with blueberries for 4 weeks, no anthocyanins could be detected in the plasma or urine of the fasted animals, whereas intact anthocyanins were detected in the liver, eyes, cortex, and cerebellum. The results suggest that anthocyanins can accumulate in tissues, including tissues beyond the blood–brain barrier (Kalt, Blumberg et al. 2008)

Another work , conducted by a French team on Wistar rats receiving a blackberry extract in the diet during 12 days, showed that anthocyanins derivatives (methylated anthocyanins and glucurono-conjugated derivatives) could be detected in different organs (bladder, prostate, testes, heart, and adipose tissue) (Felgines, Texier et al. 2009). The same study reported that the bladder contained the highest levels of anthocyanins, followed by the prostate. Prostate, testes, and heart contained native cyanidin-3-glucoside and a small proportion of cyanidin monoglucuronide. Cyanidin-3-glucoside and methylated derivatives were also present in adipose tissue.

Role of anthocyanins in cardiovascular health

Fruits and vegetables intake has been widely associated with reduced cardiovascular risk, highly likely due to the abundance and variety of bioactive compounds present (Yu, Zhang et al. 2014). The consumption of diets rich in natural bioactive components, in particular anthocyanins-rich products, has been the subject of a considerable number of studies because of their potential in improving cardiovascular health (Hassellund, Flaa et al. 2013).

Anthocyanins have been used as traditional or folk medicine around the world, where they provide a large array of health benefits (Figure 16) such as protection against liver injuries, reduction of blood pressure, improvement of eyesight, suppression of proliferation of cancer

cells, and cardiovascular disease prevention (Konczak and Zhang 2004). However, it should be noted that many studies use standardized extracts or isolated molecules that may not accurately reflect the complexity of whole foods and often utilize concentrations that exceed typical dietary intake, which can lead to an overestimation or underestimation of their physiological impact. In that regard, blackcurrants (Ribes nigrum) stand out due to their particularly high anthocyanin content, most notably cyanidin-3-O-glucoside and delphinidin-3-O-glucoside (Moyer et al., 2002). These berries also contain other beneficial compounds such as vitamin C, flavanols, and dietary fibers, which can work synergistically to enhance the health-promoting effects of anthocyanins. And thus, cardiovascular health

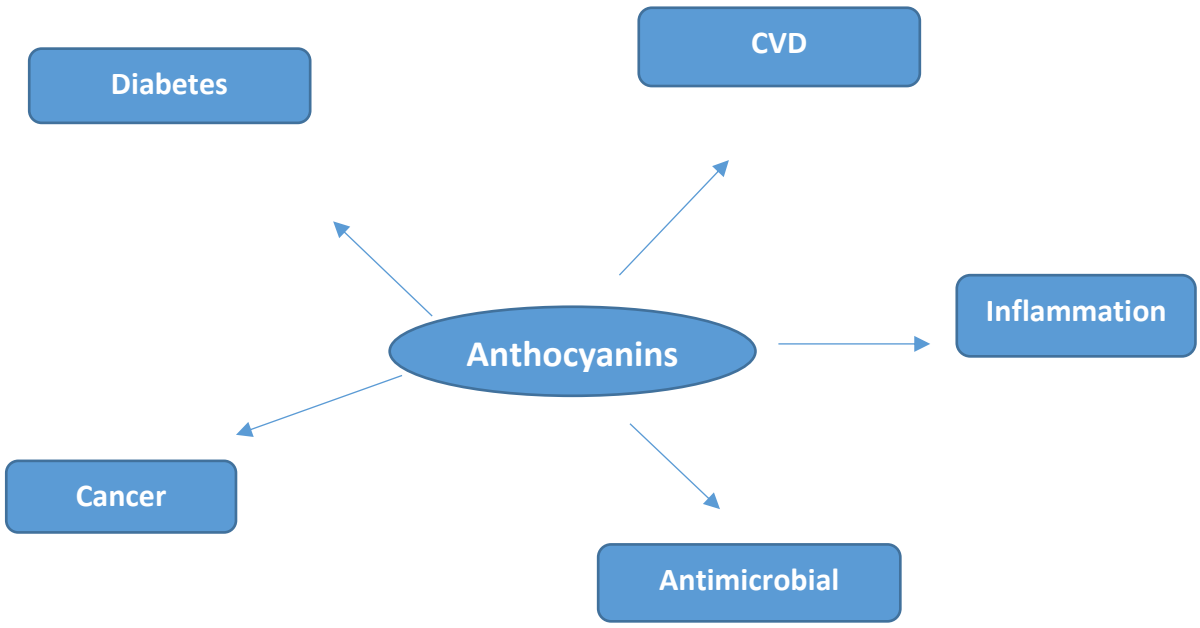

Figure 16 – Beneficial effects of anthocyanins on various disease

Antioxidant properties

Anthocyanins possess antioxidant effects in a wide range of chemical oxidation systems by virtue of two hydroxyl groups on the phenyl ring (Andriantsitohaina, Auger et al. 2012). The antioxidant mechanisms of the anthocyanins typically include the suppression of reactive species formation through their ability to scavenge radicals and chelate metal ions, and to inhibit pro-oxidant enzymes, thus preventing cellular oxidative damage (Pagano Patrick, Chanock Stephen et al. 1998). It was proposed that the direct antioxidant effect against

reactive oxygen species of anthocyanins could prevent LDL oxidation and the associated inflammatory responses, thus attenuating the development and progression of atherosclerosis (Tsuda, Shiga et al. 1996). However, more recent studies have revealed the implication of more complex molecular mechanisms of action, including modulation of gene expression, cell signaling, and miRNA expression (Krga and Milenkovic 2019). Indeed, several *in vitro* studies reported that anthocyanins and their microbial metabolites could modulate the expression of genes coding for both anti- and pro-oxidant enzymes (Pojer, Mattivi et al. 2013). Conversely, it has been shown that anthocyanins could increase the expression of genes encoding enzymes involved in the antioxidant defense in endothelial cells after exposure to different inflammatory mediators such as heme oxygenase 1 (HO-1) and NADPH quinone oxidoreductase 1 (NQO1) (Cimino, Speciale et al. 2013). Furthermore, a reduced expression of genes encoding NADPH-oxidase subunits, NOX2 and NOX4, was observed in endothelial cells isolated from diabetic mice following a 10-weeks supplementation with strawberries (Petersen, Bharat et al. 2018, Huang, Hutabarat et al. 2020). Similarly, genes encoding for antioxidant enzymes like glutathione reductase, thioredoxin reductase 1, and superoxide dismutases 1 and 2 were upregulated in the aortas of ApoE$^{-/-}$ mice after 20 weeks of dietary supplementation with blueberries (Wu, Kang et al. 2010). Beyond the important antioxidant role of anthocyanins in the endothelial cells, the latter data may also confirm the ability of anthocyanins to prevent endothelial dysfunction which may lead to the development of cardiovascular diseases such as atherosclerosis and inflammatory responses.

Vasodilatory properties

Several studies confirm the beneficial effects of anthocyanins and anthocyanin-rich products on the established biomarkers of CVD risk such as reduced NO bioavailability, inflammation, and endothelial dysfunction. They have been shown to have vasoprotective properties such as antioxidant, anti-inflammatory, anti-atherogenic and vasodilatory effects (Schini-Kerth, Auger et al. 2010, Auger and Schini-Kerth 2014).

Some studies have proposed that the beneficial effects of anthocyanins on hypertension and cardiovascular diseases could be due, at least in part, to their ability to promote endothelial function. In a recent study, blueberry anthocyanins protected endothelial function against high-glucose (HG) injury *via* antioxidant properties and mechanisms involving the activation

of phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. The study suggested that the vasodilator mechanism of anthocyanins seems to be associated with NO production by endothelial NOS subsequent to the activation of the PI3K/Akt pathway (Huang, Hutabarat et al. 2020). Moreover, several studies indicated that anthocyanins-rich berry juices, including blackcurrant, are able to induce potent endothelium-dependent relaxation in isolated arterial rings (Auger, Kim et al. 2011, Auger, Pollet et al. 2015, Auger, Pollet et al. 2015, Tabart, Auger et al. 2018). Interestingly, the potential of fruits juice to induce endothelium-dependent vasorelaxation in isolated arteries is correlated to their anthocyanins content, whereas they were no such correlation with other flavonoids subclasses (Tabart, Auger et al. 2018, Matute, Tabart et al. 2020).

In addition, a cross-over study including 12 hypercholesterolemic patients that were given 320 mg of anthocyanins isolated from berries for 12 weeks reported that anthocyanins supplementation increased the flow-mediated dilatation (28,4% vs 2,2% in control group) and the HDL-cholesterol level in plasma. The authors attributed this effect to the activation of the NO-cGMP signaling pathway, improvements in the serum lipid profile, and decreased inflammation (Zhu, Xia et al. 2011). Furthermore, the ingestion of red wine polyphenols that contains anthocyanins reduced angiotensin II-induced hypertension in rats and improved an established age-related endothelial dysfunction in old rats (Dal-Ros, Bronner et al. 2009, Idris-Khodja, Di Marco et al. 2013). In addition, several studies indicate that anthocyanins-rich products are able to improve the endothelial function in various experimental models (Auger and Schini-Kerth 2014).

Evidence from preclinical studies

Shaughnessy et al. showed that the treatment of young spontaneously hypertensive stroke-prone rats (SHRSP) with blueberry-enriched diet for 8 weeks resulted in a significant decrease in systolic blood pressure (178 ± 15 mmHg vs. 216 ± 11 mmHg for the control group). The authors concluded that dietary consumption of blueberries may be used to combat hypertension and improve cardiovascular disease prevention (Shaughnessy, Boswall et al. 2009).

A similar study investigated the effect of the consumption of a blueberry-enriched diet for 7 weeks on the vascular reactivity. The vasoconstriction induced by phenylephrine (Phe) in

aortic rings of rats was lower and the NO-mediated relaxations induced by acetylcholine were higher in the group fed with blueberry (Kalea, Clark et al. 2009). Other studies also reported a vasodilator effect of anthocyanins through the induction of endothelium-dependent relaxations (Xu, Ikeda et al. 2004, Idris-Khodja, Di Marco et al. 2013). Furthermore, interesting data showed that anthocyanins-rich blackcurrant juice intake by cirrhotic rats for 7 weeks prevented the endothelial dysfunction, and in particular the blunted EDH component, in mesenteric artery and the inflammatory response, at least in part, by attenuating the oxidative stress due to the activation of the angiotensin system (Rashid, Idris-Khodja et al. 2018). However, it's important to note that animal studies, including those using SHRSP rats, have inherent limitations in directly translating to human health outcomes due to species differences and the controlled laboratory conditions that may not fully reflect real-world dietary and physiological complexities.

Evidence from human studies

The Iowa Women's Health Study on 34,489 postmenopausal women found that eating strawberries and blueberries just once per week was associated with a significant reduction in death from cardiovascular disease over a 14 year period (Mink, Scrafford et al. 2007)

Another study on 93,600 healthy women from the Nurses' Health Study revealed a 34 % lower risk of myocardial infarctions in women who consumed at least three servings of blueberries and strawberries per week (Cassidy, Mukamal Kenneth et al. 2013). Additionally, anthocyanins have been shown to lower systolic blood pressure and arterial pressure, which can result in fewer cardiac events, such as a heart attack (Jennings, Welch et al. 2012).

Another interesting study on 120 dyslipidemic subjects (age 40-65 y) who were given capsules with 160 mg of 17 purified anthocyanins from bilberry and blackcurrant twice daily for 12 weeks showed a significant increase in HDL-cholesterol and cholesterol efflux in serum, and a decrease in LDL-cholesterol concentrations in the anthocyanins group compared to placebo. Thus, the authors concluded that anthocyanins are associated to a better efflux of cholesterol and of cGMP, which can lead to improvement of the endothelium-dependent vasodilation through the activation of the NO-cGMP signaling pathway (Qin, Xia et al. 2009).

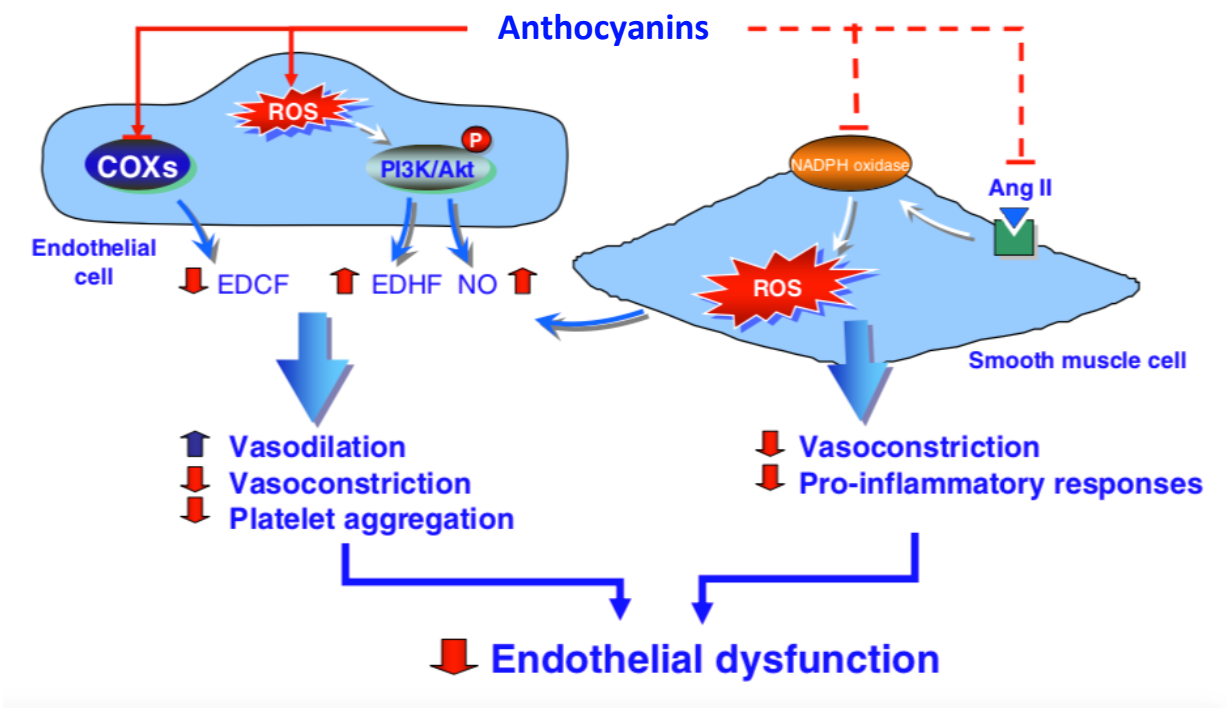

Figure 17 – Schematic summarizing the protective effect of anthocyanins on the arterial wall in major types of cardiovascular diseases (Schini-Kerth, Auger et al. 2010)

Anthocyanins in the endothelial cells (EC's)

Uptake of anthocyanins

The ability of anthocyanins to enter in the endothelial cells was first studied by Youdim et *al*. using bovine and human ECs incubated in the presence of an extract of elderberry containing only 4 anthocyanins. The results indicate that these compounds were found in the plasma membrane and the cytosol of endothelial cells. The proportion of anthocyanins incorporated in the EC was not uniform. Better incorporation was observed for the mono-glycoside derivatives compared to the diglycosides in the two compartments. The incorporation of elderberry extract by ECs significantly increased their resistance to oxidative stress caused by H_2O_2 (Youdim, Martin et al. 2000)

More recently, Jin et al. showed that delphinidin-3-*O*-glucoside (D3G) attenuated oxLDL-induced mitochondrial dysfunction *via* decreased reactive oxygen species (ROS) and superoxide anion generation, and inhibited apoptosis in HUVECs. Moreover, inhibition of SGLT1 activity by phlorizin or SGLT1 silencing by siRNA reduced D3G intake by HUVEC by approximately 96%, suggesting that D3G enter into endothelial cells via SGLT1 (Jin, Yi et al. 2013).

Aim of the study

We have seen through this bibliographic work that endothelial dysfunction is an early predictor and marker of cardiovascular diseases. Vascular aging is associated with endothelial dysfunction characterized by decreased endothelium-dependent relaxations in several vascular beds in humans such as brachial artery (Taddei, Virdis et al. 1995), and in the aorta (LÜScher, Cooke et al. 1987) and the mesenteric artery of rats (Idris Khodja, Chataigneau et al.

2012). Age-related endothelial dysfunction is also associated with an imbalance between the endothelial vasodilatory factors (NO, EDHF, PGI2) and vasoconstriction factors (endothelin-1, thromboxane A2, angiotensin II, superoxide anion) which control vascular homeostasis and oxidative stress.

We have seen that anthocyanin-rich products have considerable therapeutic potential in the cardiovascular field, at least through their vasodilator and antioxidant effects. The beneficial effects of anthocyanins like those in blackcurrant can be explained, in part, by their action on vascular health, in particular on endothelial function. In fact, anthocyanins are potent inducers of endothelium-dependent vascular relaxations by stimulating the formation of NO and endothelium-dependent hyperpolarization. Anthocyanins are also powerful antioxidants that may protect against vascular oxidative stress involved in the endothelial dysfunction associated with age.

In previous works we have shown that anthocyanins-rich blackcurrant juice is a potent inducer of endothelium-dependent relaxation (Auger, Pollet et al. 2015, Auger, Pollet et al. 2015, Lee, Khemais-Benkhiat et al. 2017). The anthocyanins exerts there vasorelaxant effect, at least in part, through the cellular entry by SGLTs and the subsequent activation of eNOS by the redox-sensitive Src/PI3K/Akt pathway (Auger, Pollet et al. 2015, Lee, Khemais-Benkhiat et al. 2017). Moreover, the chronic intake of anthocyanins-rich blackcurrant juice was able to prevent the endothelial dysfunction in the mesenteric artery of an experimental model of hepatopulmonary syndrome (Rashid, Idris-Khodja et al. 2018).

The objectives of this thesis work are

- To characterize the effect of aging on endothelial function in the mesenteric artery by evaluating endothelium-dependent vasodilator and contractile responses in an experimental model of physiological aging in rats
- To evaluate the potency of a chronic intake of anthocyanins-rich blackcurrant juice to improve the age-related endothelial dysfunction in aged rats
- To determine if the blackcurrant intake is associated with a tissular anthocyanins uptake in arteries, and in particular in the endothelium
- To identify the mechanisms underlying the age-related endothelial dysfunction as well as the beneficial effects of anthocyanins

Results

Anthocyanin-rich blackcurrant intake by old rats improves age-related increased blood pressure, vascular oxidative stress and endothelial dysfunction: role of SGLT-1 and -2-mediated vascular uptake of anthocyanins

Ahmed Bey Chaker [1,2], Paola Algara-Suárez [3], Lamia Remila [1,2], Christophe Bruckert [1,2], Sin-Hee Park [1,2], Ursula Houngue [1,2], Eugenia Belcastro [1,2], Jessica Tabart [4], Claire Kevers [4], Abdul Wahid Qureshi [1,2], Hanine El Itawi [1,2], Florence Toti [1,2], Valérie B. Schini-Kerth [1,2,*], Cyril Auger [1,2,*].

[1] *INSERM (French National Institute of Health and Medical Research), UMR 1260, Regenerative Nanomedicine, FMTS, Strasbourg, France*

[2] *University of Strasbourg, Faculty of Pharmacy, Illkirch-Graffenstaden, France*

[3] *Universidad Autónoma de San Luis Potosí, Facultad de Enfermería y Nutrición, México*

[4] *Plant Molecular Biology and Biotechnology, University of Liège, Liège, Belgium*

* V.B. Schini-Kerth and C. Auger are joint last authors.

Name and complete address of corresponding author:

Dr. Cyril Auger,

INSERM UMR 1260,

CRBS, University of Strasbourg

1, rue Eugène Boeckel

67084 Strasbourg Cedex

Phone: +33 3 68 85 42 45

Fax: +33 3 68 85 43 13

Email: cyril.auger@unistra.fr

Abstract

Ageing-related endothelial dysfunction and vascular oxidative stress early affect arterial sites at risk. Anthocyanin uptake via sodium-glucose co-transporter 1 (SGLT1) are potent inducers of the endothelial formation of nitric oxide (NO). This study examined whether anthocyanin-rich blackcurrant (ARB) improves the endothelial function in old rats.

Male Wistar rats (22-month-old) received ARB (60 and 120 mg/kg/day) in drinking water for 2 weeks. Young rats were used as control. Systolic blood pressure (SBP) was assessed by tail-cuff sphygmomanometry, vascular reactivity using organ chambers, protein expression by immunofluorescence, oxidative stress using dihydroethidium, and anthocyanin uptake using Neu reagent.

In old rats, age was associated with an increased SBP, an endothelial dysfunction characterized by an abolished endothelium-dependent hyperpolarization (EDH)-mediated relaxation and increased contractile response to phenylephrine in the mesenteric artery, and an increased vascular oxidative stress associated with the activation of angiotensin system, and increased expression of markers of vascular fibrosis and eNOS. All were improved by ARB 120 mg/kg/d treatment. SGLT1 immunofluorescence predominantly in the endothelium was more pronounced in the aortic arch than the aorta and higher in old than young rats, whereas SGLT2 was below detection level. The ARB treatment induced a dose-dependent accumulation of anthocyanin in the aorta and aortic arch. An ARB purified extract promoted *ex vivo* greater anthocyanin uptake mostly in the endothelium in the aortic arch than aorta, and in old compared to young rats. The anthocyanin uptake was inhibited to a greater extent by a dual SGLT1/2 inhibitor than by a selective SGLT2 inhibitor in the aorta of young and old rats. Both SGLT inhibitors reduced also *ex vivo* the age-related vascular oxidative stress.

The upregulation of SGLT1, and the greater SGLT1 and SGLT2-mediated uptake of anthocyanin predominantly in the endothelium at arterial sites at risk in old rats suggest that anthocyanin appear as interesting natural products to protect the endothelial function with increasing age.

Keywords: endothelial dysfunction, anthocyanin-rich blackcurrant, ageing, sodium-glucose co-transporters, arterial sites at risk

Introduction

Cardiovascular diseases (CVD) include pathologies that affect the heart and all blood vessels, such as atherosclerosis, heart rhythm disorders, high blood pressure, myocardial infarction, heart failure or even strokes. CVD are the number one cause of death globally, taking an estimated 17.9 million lives each year (WHO 2017). Age is an independent risk factor for CVD and the ageing process is associated with a gradual decrease in endothelial function (Taddei, Virdis et al. 1995). The age-related endothelial dysfunction has been associated with an increased vascular oxidative stress, a decreased bioavailability of NO in Human (Taddei, Virdis et al. 2001), and an reduced endothelium-derived hyperpolarization component of the endothelium-dependent relaxation in old rats (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012).

Fruits and vegetables intake has been widely associated with reduced cardiovascular risk, and decreased age-related oxidative stress high likely due to the abundance and variety of bioactive compounds present such anthocyanins (Yu, Zhang et al. 2014). Moreover, in experimental models of physiological ageing, the intake of a polyphenol-rich red wine extract has been associated with an improved endothelial function (Idris Khodja, Chataigneau et al. 2012)

Anthocyanins are water-soluble plant pigments present in the flesh, skin and roots of many colored fruits (Crozier, Jaganath et al. 2009), they are the most abundant in the human diet and can be found in red wine, tea, coffee, cocoa, certain varieties of cereals, vegetables and fruits, including red and black fruits and berries (Del Rio, Rodriguez-Mateos et al. 2013). Many clinical and preclinical studies have indicated that anthocyanin-rich products consumption is associated with improved endothelial function and with reduction of specific CVD risk factors, such as hypertension (Hassellund, Flaa et al. 2013, Oak, Auger et al. 2018).

Amongst anthocyanin-rich natural products, blackcurrant seems to be an interesting source as it contains limited numbers of anthocyanins, with only four of them constituting the large majority of the contents in flavonoids (Maatta, Kamal-Eldin et al. 2003, Borges, Degeneve et al. 2010). Interestingly, blackcurrant juices have been shown to be potent inducer of endothelium-dependent relaxation in isolated coronary arteries (Auger, Pollet et al. 2015, Auger, Pollet et al. 2015, Tabart, Auger et al. 2018, Matute, Tabart et al. 2020). In addition, anthocyanin-rich blackcurrant juice intake has been associated with improved endothelial dysfunction in an experimental model of hepatopulmonary syndrome (Rashid, Idris-Khodja et al. 2018).

The sodium-glucose co-transporters (SGLT) 1 has been reported to mediate the cellular entry of anthocyanin in the endothelial cells (Jin, Yi et al. 2013). However, very few studies have addressed the ability of anthocyanin to reach the endothelium after an oral intake.

Therefore, the aim of the present study is to assess whether the chronic intake of anthocyanin-rich blackcurrant is able to improve vascular and endothelial dysfunction in old rats with an established endothelial dysfunction, and if so, to evaluate the uptake of anthocyanin in the endothelium and the cellular and molecular pathways underlying the beneficial of anthocyanin-rich blackcurrant.

Materials and Methods

Preparation of blackcurrant extract (BCE)

Blackcurrant concentrated juice (ARB) was kindly provided by Eckes-Granini (Nieder-Olm, Germany, 67.3 °Bx,). 2.5 g of the concentrated juice was loaded on a column packed with 50 g of Sephadex LH-20 (GE healthcare 17-0090-01) conditioned in ultrapure water. The column was eluted successively with 250 ml of ultrapure water acidified with 0.1 % of trifluoroacetic

acid, 250 ml of acidified methanol (0.1 % trifluoroacetic acid), and 250 ml of aqueous acetone (60 % Acetone / 40 % ultrapure water). The anthocyanin-rich fraction was collected and dried under vacuum using a rotary evaporator temperature not exceeding 40°C. Column was further washed with 250 ml aqueous acetone (60 % Acetone / 40 % ultrapure water) and rinsed with ultrapure acidified water. Several extractions were done and pooled, and kept dry at -20 °C until use.

Chemical analysis of the blackcurrant juice and extract

RP-UPLC-PDA analyses were performed in a liquid Acquity chromatograph equipped with a PDA photodiode array detector (Waters) as described previously (Tabart et al. Nutrition, 2018). Separation was carried out using an Acquity UPLC BEH C18 steel cartridge (Waters), 100 mm x 2.1 mm, filled with 1.7 µm particles at 50°C. The elution gradient for anthocyanins analysis was performed from water/acetonitrile/formic acid (88:10:2, v/v/v) at time 0 to 75:23:2 at time 7 min. Flow rate was 0.2 mL/min. Absorbance was recorded at 518 nm. Standards of cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, and delphinidin-3-*O*-rutinoside were purchased from Extrasynthese (Genay, France).

In vivo treatment of rats

45 male Wistar rats (10 weeks-old) were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animals were kept in animals' facility with food and water given *ad libitum,* until they reached the desired age. 45 aged rats (22-months old) were randomly assigned to 3 groups of 15 rats each. 8 young (12-weeks old) male Wistar were used as control. Aged rats received either water or anthocyanins rich blackcurrant juice (ARB, 60 and 120 mg GAE/kg/day) in drinking water for two weeks while young rats received only water. After 2

weeks of treatment, rats were anaesthetized by an intra-peritoneal injection of a mixture of ketamine/xylazine (120/10 mg/kg) and blood was collected from the left ventricle by intracardiac puncture. After excision, organs were collected and weighted, the main mesenteric artery was placed in Krebs-bicarbonate solution for the subsequent determination of vascular reactivity using organ chambers, thoracic, abdominal aorta and aortic arch were used for immunofluorescence study and determination of vascular oxidative stress.

Blood pressure measurements

Systolic blood pressure was determined by tail-cuff sphygmomanometry 3 times per week during 2 weeks using the blood pressure analysis system (BP-2000 Serie II, Visitech Systems, Bioseb, Vitrolles, France). Prior blood pressure monitoring, rats were trained daily for one week to get used to the system.

Vascular reactivity study

The main mesenteric artery was dissected, cleaned of connective tissue and cut into rings (2-3mm) before suspension in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (composition in 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). The resting tension was set at 1 gram before determination of changes in isometric tension. After the equilibration period, rings were exposure to Krebs bicarbonate solution containing a high concentration of potassium (80 mM) until reproducible contractile responses were obtained. To check the presence and the functionality of the endothelium, rings were contracted with phenylephrine (PE, 1 µM) to approximately 80% of the maximal contraction induced by the high potassium solution

before addition of acetylcholine (ACh, 1 µM). After washout and a 30-minutes equilibration period, rings were subjected to a concentration-contraction curves in response to PE to assess the contractile responses. To assess the endothelium-dependent relaxations, rings were contracted with PE (1 µM) before the construction of concentration-relaxation curves in response to ACh. In some experiments, rings were incubated for 30 minutes with pharmacological agents before the construction of concentration-response curves. To study the role of cyclooxygenase-derived prostanoids, rings were incubated with indomethacin (10 µM, a nonselective COX inhibitor). To study NO-mediated relaxation rings were incubated with both indomethacin and TRAM-34 plus UCL-1684 (1 µm each, inhibitors of IK_{Ca} and SK_{Ca}, respectively) to inhibit the release of vasoactive prostanoids and the EDH-mediated relaxation, respectively. The role of EDH-mediated relaxation was studied in rings incubated with indomethacin and N^{ω}-nitro-L-arginine (L-NA, 300 µM, an eNOS inhibitor) to prevent the formation of vasoactive prostanoids and NO, respectively. To assess the vascular smooth muscle function, rings were contracted with PE (1 µM) before the construction of concentration-relaxation curves in response to either sodium nitroprusside (a NO donor) or levcromakalim (an ATP-sensitive potassium channels opener), in the presence of indomethacin, L-NA and TRAM-34 plus UCL-1684 to prevent the formation of vasoactive prostanoids, NO and EDH, respectively.

Ex vivo vascular anthocyanins quantification by confocal microscopy

After excision of the aorta and careful cleaning of connective tissues, rings of 3-4 mm of thoracic aorta and segments of the aortic arch cut at the level of the branches were washed in Krebs solution before incubation with Krebs containing 0.4% of 2-aminoethyl diphenylborinate (Neu reagent A) for 5 minutes at 37°C with 5% CO_2. Anthocyanins and neu

reagent react to create compounds with hyperchromic and bathochromic effects, thus make them detectable by confocal microscopy in the red channel. Subsequently, rings were washed with cold PBS, snap-frozen with liquid nitrogen in Histomolds containing frozen section compound (FSC 22, Leica Biosystems, Nanterre, France), before being cryosectioned at 14 µm and mounted on Superfrost plus slides (ThermoFisher) with Dako medium (fluorescence editing medium, Dako, Agilent Technologies France, Les Ulis, France) and dried for 20 minutes at room temperature. Slides were then analyzed on the same day using a confocal laser scanning microscope (Leica TSC SPE, Mannheim, Germany). Quantitative analysis of fluorescence was performed using Image J software (version 1.6 for Windows, NIH).

Ex vivo anthocyanins uptake stimulation and modulation

Segments of the aortic arch and rings of the thoracic aorta were washed with Krebs solution and placed in 24 wells plates containing MCDB131 culture medium (Gibco, ThermoFisher Scientific, Illkirch, France) supplemented with fungizone (250 µg/ml), penicillin (100 UI/ml), streptomycin (100 UI/ml), L-glutamine (2 mM, all from Lonza Levallois-Perret, France). Aortic arch segments and thoracic aorta rings were incubated with BCE (60µg/ml) for 3 minutes at 37°C with 5% CO_2. To assess the involvement of sodium glucose co-transporters (SGLT) 1 and 2 in anthocyanins uptake, thoracic aorta rings were incubated with either a dual SGLT1/2 inhibitor (Sotagliflozin, 0.1 µM) or a selective SGLT2 inhibitor (Empagliflozin, 0.1 µM) for 5 minutes at 37°C before addition of BCE. Aortic arch segments and thoracic aorta rings were then washed twice with PBS before further incubation with PBS containing 0.4% of 2-aminoethyl diphenylborate for 5 minutes at 37°C. Subsequently, rings were washed with cold PBS, snap-frozen with liquid nitrogen in Histomolds containing frozen section media

(FSC 22, Leica Biosystems), before being cryosectioned at 14 µm and mounted on Superfrost plus slides (ThermoFisher) with Dako mounting-medium (Agilent Technologies France, Les Ulis, France) and dried for 20 minutes at room temperature. Slides were then analyzed on the same day using a confocal laser scanning microscope (Leica TSC SPE). Quantitative analysis of fluorescence was performed using Image J software (version 1.6 for Windows, NIH).

Immunofluorescence studies

Rings of the thoracic aorta were embedded in Histomolds containing frozen section media (FSC 22, Leica Biosystems, Nanterre, France) and were snap-frozen in liquid nitrogen. Rings were cryosectioned at 14 µm and stored at -80°C until use. Sections were thawed with phosphate buffer saline (PBS) and fixed for 30 minutes with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) prior to blocking for 1 hours at room temperature with 1 % bovine serum albumin (BSA) in PBS containing 0.5% Triton X-100 to prevent nonspecific binding. All sections, excluding negative controls, were then incubated overnight at 4 °C with a solution of blocking buffer containing a primary antibody against either SGLT1 (1/100, AGT-031, Alomone Labs, Israel), SGLT2 (1/100, AGT-032, Alomone Labs; 1/100, Ab85626, Abcam, Paris, France ; sc393350, Santa Cruz Biotechnology), eNOS (1/100, BD 610297, BD Transduction Laboratories, Le Pont de Claix, France), MCP-1 (1/100, Ab9669, Abcam), p53 (1/80, sc-126, Santa Cruz Biotechnology, Clinisciences SA, Nanterre, France), ACE (1/200, #250450, Abbiotec, Clinisciences SA), AT1R (1/200, Ab124505, Abcam), TGF-β1 (1/150, Ab27969, Abcam), Collagen I (1/100, Ab34710, Abcam), MMP-2 (1/100, Ab86607, Abcam), or MMP-9 (1/100, Ab38898, Abcam). Next day, all sections were washed with PBS prior to incubation with a solution of blocking buffer containing a fluorescent secondary

antibody (Alexa Fluor 633 antirabbit or antimouse, Invitrogen, Fisher Scientific France, Illkirch, France) for 1 hour at room temperature in the dark, followed by washing with PBS and air drying for 15-20 minutes. Slides were then cover-slipped with Dako fluorescence mounting solution and dried for 20 minutes at room temperature. Slides were then analyzed with the help of a confocal laser scanning microscope (Leica TSC SPE, Mannheim, Germany). Quantitative analysis of fluorescence was performed using Image J software (version 1.6 for Windows, NIH).

Vascular oxidative stress detection

The vascular level of ROS and mitochondrial superoxide formation were determined using redox sensitive fluorescent probe dihydroethidium (DHE, Invitrogen, ThermoFischer) and MitoSOX probe (MitoSox^{TM}, Invitrogen, Thermofischer), respectively. Cryosections of thoracic aorta (25 µm) were thawed with PBS and incubated with either DHE (2.5 µM) or MitoSox (2.5 µM) for 30 minutes at 37°C in a humidified black box to protect from light. To assess the specificity of the signal, sections were treated with the antioxidant N-acetylcysteine (NAC, 3 mM) for 2 hours at 37°C prior to the addition of DHE. To determine the role of SGLT1 and/or 2 on vascular oxidative stress level, sections were pretreated with either a dual SGLT1/2 inhibitor LX4211 (Sotagliflozin, 0.1 µM) or a selective SGLT2 inhibitor (Empagliflozin, 0.1 µM) for 30 minutes at 37°C prior to incubation with DHE. The sections were then washed with PBS before mounting under a cover-slip in Dako medium. Slides were allowed to dry in the dark for 20 minutes before being analyzed on the same day with confocal laser scanning microscope. Quantitative analysis of fluorescence was performed using Image J software (version 1.6 for Windows, NIH).

Statistical analysis

Values were expressed as mean ± S.E.M. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's post-hoc test for vascular reactivity, and by two-tailed unpaired t-test for other data. Normality of the data was verified by Shapiro-Wilks test. Values were considered statistically different for $P<0.05$. All statistical analyses were performed using Graph Pad Prism software (version 8 for Windows, GraphPad Software, Inc., San Diego, CA, USA).

Results

ARB and BCE composition

The total polyphenol concentration of ARB was 6.81 ± 0.02 g GAE/L as measured by the Folin-Ciocalteu method and expressed as gallic acid equivalents (GAE).

RP-UPLC-PDA analysis of the ARB and BCE showed the presence of four major sugar-conjugated anthocyanins (Table 1), namely delphinidin-3-*O*-rutinoside, cyanidin-3-*O*-rutinoside, delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside), peonidin and several other minor compounds, including malvidin, delphinidin, peonidin-3-*O*-glucoside, petunidin and peonidin-3-*O*-rutinoside.

ARB chronic intake reduces systolic blood pressure in aged rats

Ageing was associated with a small but significant increase in systolic blood pressure by about 9.3 mmHg in old rats compared to young rats (135.8±1.1 vs. 126.5±1.0 mmHg, respectively). Systolic blood pressure was significantly lower in both groups treated with ARB (Figure 1). The ARB treatment significantly reduced systolic blood pressure by about 5.4 and 7.6 mmHg for the 60 and 120mg/kg/day treatment, respectively (Figure 1).

Effect of chronic intake of Anthocyanin rich-blackcurrant juice (ARB) on morphometric parameters in aged rats

The morphometric evaluation of rats and organs indicated that the weights of several organs including spleen, liver, kidney, heart were not significantly different before and after the treatment. However, after two weeks of treatment body weights were significantly lower in both ARB treated groups compared to control old rats (Table 2).

ARB chronic intake improves endothelial dysfunction in the main mesenteric artery

To assess the effect of 2 weeks intake of ARB on vascular function, vascular reactivity studies were performed in the main mesenteric artery, which present characteristics from both conductance and resistance arteries.

In young rats and old rats, the acetylcholine (ACh)-induced relaxations were not significantly affected by either indomethacin or indomethacin in combination with TRAM-34 plus UCL-1684, suggesting that ageing have no effect on the endothelial formation of either vasoactive prostanoids or NO in the present study (Figure 2A). In addition, the ACh-induced relaxation was completely abolished by the combination of indomethacin with L-NA in old rats, whereas it was only reduced in young rats (Figure 2A). This indicate that the EDH component of the endothelium-dependent relaxation is abolished by ageing in old rats.

In the absence of pharmacological agents, the ACh-induced relaxation in mesenteric artery rings were not significantly different between all groups (Figure 2B). However, in the presence of the combination of indomethacin with L-NA, the EDH component of the endothelium-dependent relaxation is abolished in old rats compared to young rats and is normalized in the ARB 120 mg/kg/d group (Figure 2B). In the ARB 60 mg/kg/d group, the EDH-mediated relaxation is improved but not significantly (Figure 2B). Regarding the NO-mediated relaxation, in the presence of the combination of indomethacin plus TRAM-34 and UCL-1684, ageing is not associated with a significant reduction of the ACh-induced relaxation (Figure 2B). Similarly, the ARB 120 mg/kg/d treatment does not have any effect on the NO-mediated relaxation (Figure 2B). However, we have observed an improved NO-mediated relaxation for the ARB 60 mg/kg/d treatment (Figure 2B).

Compared to young rats, the phenylephrine (PE)-induced contractions of mesenteric artery rings were significant increases in old rats either in the absence or presence of inhibitors of

the NO and EDH components (Figure 2 C). Treatment with ARB 120 mg/kg/day significantly reduced PE-induced contraction in the main mesenteric artery of old rats (Figure 2C).

The relaxation in response to sodium nitroprusside, a NO donor, were not different in either groups indicating that ageing is not associated with a reduce sensibility to NO of the vascular smooth muscle in mesenteric artery rings (Figure 2D). In contrast, ageing was associated with a small but significant increase in the relaxation in response to levcromakalim, an ATP-sensitive potassium channel opener, suggesting an increased sensitivity to EDH of the vascular smooth muscle of the mesenteric artery in old rats (Figure 2D). This could be an adaptative response to the abolishment of EDH in old rats. Furthermore, levcromakalim-induced relaxation was significantly potentiated in the mesenteric artery rings of rats treated with ARB 120 mg/kg/d (Figure 2D).

These results indicate that the ageing-related endothelial dysfunction is associated with an abolished EDH-mediated relaxation and an increased contractile response to PE. ARB treatment at 120 mg/kg/d normalized EDH-mediated relaxation and significantly reduced contractile response to PE in mesenteric artery rings.

ARB chronic intake is associated with a dose-dependent accumulation of anthocyanins in the vascular wall

Vascular accumulation of anthocyanins was determined in section of the thoracic aorta and rings of the aortic arch at sites of branching using the Neu A reagent (2-Aminoethyl diphenylborinate). The background signal was similar between young control rats and old untreated control rats in the vascular wall of either thoracic aorta, inner curvature or outer curvature of the aortic arch (Figure 3). Chronic intake of ARB induced a dose-dependent accumulation of anthocyanins in both aorta and aortic arch, with a basal accumulation of

anthocyanins increased by 2 folds in thoracic aorta in the ARB 120/mg/kg/day treated group compared to the ARB 60 mg/kg/day treated group. Similarly, signals were significantly higher in the ARB 120/mg/kg/day treated group compared to control groups in both inner and outer curvature of the aortic branching area and were higher than in the ARB 60 mg/kg/day treated group (Figures 3).

ARB chronic intake is associated with an increased expression of SGLT1 in the endothelium

We have demonstrated that SGLT1 and SGLT2 are overexpressed in senescent endothelial cells and in the endothelium at arterial site at risk (Khemais-Benkhiat, Belcastro et al. 2020, Park, Belcastro et al. 2021). Moreover, SGLT1 has been reported to be involved in the cellular uptake of anthocyanins in endothelial cells (Jin, Yi et al. 2013). Thus, SGLT1 levels in frozen sections of thoracic aorta and aortic arch branching were determined by immunofluorescence. SGLT1 fluorescence was observed mostly in the endothelium, and expression level was significantly higher in old rats compared to young rats, and further increased in the ARB 120/mg/kg/day group in both thoracic aorta and inner and outer curvatures of the aortic arch branching (Figure 4). The expression level for SGLT1 was higher in the outer curvature than the inner curvature in both old untreated and ARB 120 mg/kg/day treated rats (Figure 4), outer curvature being associated with a turbulent flow and low shear stress due to branching.

ARB chronic intake is associated with a dose-dependent increased capacity for uptake of anthocyanin in the endothelium

To assess the ability of the vascular tissues to incorporate anthocyanins, we exposed sections of the thoracic aorta and of the aortic arch to an *ex vivo* treatment with 60 µg/ml of

a purified blackcurrant extract (BCE) for 3 minutes. In all groups, treatment with BCE was associated with a significant increase in anthocyanin signal mostly in the endothelium (Figure 4). The signal was significantly higher in the inner and outer curvature of the aortic arch than in the thoracic aorta in all groups of rats (Figure 5). In line with SGLT1 expression levels (Figure 4), anthocyanin uptake was further increased in the ARB 120/mg/kg/day group compared to control old rats (Figure 5).

SGLT1 and SGLT2 are both involved in the anthocyanin uptake in the endothelium

To assess the involvement of SGLT1 and SGLT2 transporters in the uptake of anthocyanin in the endothelium, thoracic aorta sections were incubated with either a dual SGLT1/2 inhibitor (sotagliflozin) or a selective SGLT2 inhibitor (empagliflozin) before exposure to BCE 60 µg/ml. In all groups, SGLTs inhibition was associated with a significantly reduced uptake of anthocyanin in the endothelium (Figure 6). Interestingly, in old rats (untreated or treated with ARB 120 mg/kg/day) anthocyanin uptake inhibition with the dual SGLT1/2 inhibitor was smally but significantly more pronounced than with the specific SGLT2 inhibitor (Figure 6), indicating the involvement of both SGLT1 and SGLT2 in the cellular uptake of anthocyanin in endothelial cells.

ARB chronic intake is associated with improves the age-related vascular oxidative stress

Since age related endothelial dysfunction is associated with increased level of oxidative stress (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012, Marín, Yubero-Serrano et al. 2013, Farooq, Gaertner et al. 2020), vascular oxidative stress was determined in unfixed cryosections of the thoracic aorta using the redox-sensitive probe dihydroethidium. To assess the specificity of the signal, sections were incubated for 2 h with

the anti-oxidant N-acetylcysteine (NAC) before the addition of DHE. Compared to young rats, thoracic aorta from old rats showed significantly higher levels of ethidium fluorescence that were significantly reduced by NAC, indicating that ageing is associated with an increased vascular oxidative stress (Figure 7A). Chronic intake of ARB 120 mg/kg/day normalized the level of vascular oxidative stress in the thoracic aorta (Figure 7A). Similar results were obtained using the mitochondria superoxide indicator MitoSox (Figure 7B).
In addition, the level of oxidative stress in the section of thoracic aorta of untreated old rats was significantly reduced in presence of either a dual SGLTs inhibitor or the selective SGLT2 inhibitor (Figure 7C), indicating that SGLTs are involved in the ageing-related vascular oxidative stress.

ARB chronic intake is associated with improved endothelial health in the thoracic aorta

Previously published studies indicate that vascular ageing is associated with an overexpression of eNOS and markers of endothelial senescence and activation of the local angiotensin system in the endothelium (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012, Farooq, Gaertner et al. 2020). In the present study, ageing was associated with an increased expression of eNOS, monocyte chemoattractant protein 1 (MCP-1, marker of endothelial dysfunction), p53 (marker of endothelial senescence), and markers of activation of local angiotensin system, angiotensin converting enzyme (ACE) and angiotensin II type I receptor (AT1R) in the endothelium of untreated old rats compared to young rats (Figure 8). In addition, the endothelium of the thoracic aorta of old rats showed an increased expression level of markers of vascular inflammation (transforming growth factor β1, $TGF_{\beta 1}$), of fibrosis (collagen-I) and of remodeling (Matrix metalloproteinase 2 and 9, MMP-2 and MMP-9) compared to young rats (Figure 9). Chronic intake of ARB 120

mg/kg/day significantly reduced the age-related increased expression of all markers (Figures 8 and 9).

Disscussion

Ageing is an independent risk factor for the development of cardiovascular diseases (Dhingra and Vasan 2012) through, at least in part, the age-related endothelial dysfunction. The findings of the present study indicate that ageing is associated with an increased systolic blood pressure and an endothelial dysfunction mainly characterized by an abolished EDH-mediated relaxation and increased contractile responses in the main mesenteric artery of old rats. This age-related endothelial dysfunction was associated with an increased vascular oxidative stress due, at least in part, to the activation of the local angiotensin system. In line with previous studies in middle-aged and old rats (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012, Farooq, Amoura et al. 2017), the present findings also indicate that ageing is associated with an increased vascular oxidative stress and the endothelial activation of the local angiotensin system as indicated by the increased expression of ACE and AT1 receptors, which in turns could leads to the upregulation and activation of the vascular NADPH oxidase and subsequent increased vascular oxidative stress as reported previously (Dal-Ros, Bronner et al. 2009). Furthermore, ageing is also associated with an increased expression of markers of endothelial senescence and dysfunction, vascular inflammation and fibrosis in the endothelium of the main mesenteric artery of old rats.

In the present study, the old rats were treated with an anthocyanin-rich blackcurrant juice diluted in the drinking water at dose of either 60 or 120 mg/kg/day. Theses doses are in the range of doses reported in different preclinical studies day on the effect of anthocyanins, ranging from 50 to 200 mg/kg/ (Idris Khodja, Chataigneau et al. 2012, Qin and Anderson 2012, Wu, Gao et al. 2018, Nemes, Homoki et al. 2019). These dose are equivalent to 0.68

and 1.3 g/day of anthocyanins in a 70 kg human, for the 60 and 120 mg/kg/day doses, respectively (Reagan-Shaw, Nihal et al. 2008).

The 2-weeks short term oral intake of anthocyanin rich blackcurrant juice improved the age-related increase in systolic blood pressure, and the age-related endothelial dysfunction mainly by improving the EDH-mediated relaxations and decreasing the contractile responses. The improved endothelial dysfunction was associated with an improved vascular level oxidative stress, the normalization of the local angiotensin system and reduced expression of endothelial senescence and dysfunction, vascular inflammation and fibrosis in the endothelium of the main mesenteric artery of old rats treated with ARB.

The ARB-induced reduction in arterial blood pressure is consistent with many published clinical and preclinical studies reporting that chronic intake of anthocyanins is associated with reduced risk of hypertension and lower blood pressure in Humans (Cassidy, O'Reilly et al. 2011, Jennings, Welch et al. 2012, Oak, Auger et al. 2018). The beneficial effects of the anthocyanin-rich blackcurrant on the systolic blood pressure seem to be due, at least in part, to the improved endothelial function and the reduced vascular activation of the local angiotensin system. Indeed, the angiotensin system plays a pivotal role in the induction of endothelial senescence and dysfunction, as well as in the establishment of hypertension. Activation of the local angiotensin system by angiotensin II in rats is associated with an endothelial dysfunction characterized by a reduced EDH-mediated relaxation (Dal-Ros, Bronner et al. 2009) and with increased vasoconstriction evoked by activation of AT1R (Touyz and Schiffrin 2000). Anthocyanins have been reported to possess potent *in vitro* angiotensin-converting enzyme (ACE) inhibitory activity (Geng, He et al. 2010) and to suppress the mRNA and protein expressions of angiotensin type 1 receptors in mice and in

isolated vascular smooth muscle cells (Miyazaki, Ichiki et al. 2008). Anthocyanins and anthocyanin-rich products, including berries, also have been reported to improve endothelial dysfunction in various experimental models (for review, see (Auger, Said et al. 2016, Oak, Auger et al. 2018)). Moreover, anthocyanin-rich blackcurrant has been shown to improve endothelial dysfunction in the mesenteric artery of a rat model of cirrhosis-associated portal hypertension (Rashid, Idris-Khodja et al. 2018). Furthermore, intake of anthocyanins is also associated with improved endothelial function in Humans, as anthocyanins from berries improved flow-mediated dilation in several studies (Rodriguez-Mateos, Heiss et al. 2014).

In addition, the present study indicate that the age-related endothelial activation of the local angiotensin system was associated with an overexpression of p53, a marker of endothelial senescence. This is in line with a previous study that have shown that ageing was associated with increased expression of senescence markers, oxidative stress, expression of NADPH oxidase and activation of the local angiotensin system in the mesenteric artery of old rats (Farooq, Gaertner et al. 2020). Our previously published studies on endothelial cells have demonstrated that the development on endothelial senescence involves a pro-oxidant feed-forward loop with SGLTs and the local angiotensin system (Hasan, Park et al. 2019, Park, Belcastro et al. 2021). The age-related endothelial senescence phenotype is also associated with an overexpression of markers of vascular inflammation ($TGF_{\beta 1}$), fibrosis (collagen-I) and remodeling (MMP-2 and MMP-9). Moreover, another recent study reported that increased vascular oxidative stress is associated with an increased expression of markers of fibrosis and remodeling (collagen, fibronectin, TGFβ, MCP-1, MMP2 and MMP9) in stroke-prone spontaneously hypertensive rats (SHRSP) (Harvey, Montezano et al. 2017). The age-related vascular fibrosis and remodeling are driven by the endothelial senescence mainly through

reduced NO bioavailability (Soucy, Ryoo et al. 2006, Kim, Bugaj et al. 2009, Ihm, Jang et al. 2012), leading to the age-related increased arterial stiffness and subsequent increased blood pressure (Wen, Luo et al. 2015).

Treatment with ARB is associated with a reduction in the expression levels of all markers of endothelial senescence, fibrosis, remodeling and inflammation. This is in line with a cross-sectional study indicating that anthocyanin intake is associated with lower arterial stiffness and blood pressure in women (Jennings, Welch et al. 2012).

Taken together, these data indicate that ARB intake was able to improve an already established endothelial senescence and dysfunction, thus leading to reduced vascular pro-inflammatory, pro-fibrotic and pro-remodeling pathways that are involved in development of age-related cardiovascular diseases.

The strong novelty of the present study is that ARB intake for 2 weeks was associated with a dose-dependent accumulation of anthocyanins in the vascular tissue. While anthocyanins bioavailability was reported as very low, recent studies suggest that the actual bioavailability could be higher, and that circulating metabolites could reach the endothelium (Krga and Milenkovic 2019). However, whereas some studies have indicated that specific anthocyanin such as cyanidin-3-*O*-glucoside could enter the endothelial cells using *in vitro* cell models (Ziberna, Tramer et al. 2012, Jin, Yi et al. 2013), to our better knowledge it is the first time that an accumulation of anthocyanin is reported *in situ* after oral intake.

The vascular accumulation of anthocyanins in vascular tissues of old rats is associated with an increased expression of SGLT1, a sodium-glucose co-transporter reported for cellular entry of cyanidin-3-*O*-glucoside in endothelial cells (Jin, Yi et al. 2013). Despite using several commercially available antibodies directed against SGLT2, we were not able to detect a

signal for SGLT2 by immunofluorescence, suggesting that SGLT2 expression level is below detection level in the endothelium (data not shown). In a previous study on endothelial cells, we have shown that SGLT1 and 2 were involved in the cellular entry of glucose conjugated anthocyanin from blackcurrant (Lee, Khemais-Benkhiat et al. 2017). Inhibition of SGLTs was associated with a reduced redox-sensitive activation of the Akt/eNOS pathways in endothelial cells induced by blackcurrant anthocyanins (Lee, Khemais-Benkhiat et al. 2017). In the present study, the inhibition of SGLTs resulted in a reduced endothelial uptake of anthocyanins in sections of thoracic aorta, indicating that SGLTs is indeed involved in cellular entry of anthocyanins in the native endothelium. Furthermore, even if SGLT2 expression was not detected in endothelial cells using immunofluorescence, the reduction in the endothelial uptake of anthocyanins in presence of a selective SGLT2 inhibitor strongly suggest that SGLT2 are also involved in the cellular entry of anthocyanins in the endothelium. In recent studies, we have shown that SGLT2 is overexpressed in premature senescent endothelial cells, and that SGLTs and the local angiotensin system act as a pro-oxidant feed-forward loop in the development of endothelial senescence (Khemais-Benkhiat, Belcastro et al. 2020, Park, Belcastro et al. 2021). In addition, the SGLTs overexpression associated with endothelial senescence have been reported in aortic arch of rats, an arterial site at risks (Park, Belcastro et al. 2021). Taken together, these data suggest that anthocyanin could be an interesting complementary therapeutic tool to improve endothelial health in arterial site at risk with increased expression of SGLTs, thus allowing a natural targeting as cellular uptake of the beneficial anthocyanin will be increased specifically in these areas.

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Author contributions:

Conceptualization, V.B. S.-K. and C.A.; Methodology, V.B. S.-K. and C.A.; Validation, V.B. S.-K. and C.A.; Formal Analysis, A.B.C. and C.A.; Investigation, A.B.C., P.A.-S., L.R., C.B., S.-H. P., U.H., E.B., J.T., C.K., A.W.Q., H.E.-I., F.T.; Writing – Original Draft Preparation, A.B.C. and C.A.; Writing – Review & Editing, V.B.S.-K. and C.A.; Visualization, A.B.C. and C.A.; Supervision, V.B.S.-K. and C.A.; Project Administration, V.B.S.-K. and C.A.; Funding Acquisition, V.B.S.-K.

Institutional Review Board Statement:

This study was performed in accordance with the guidelines on animal care published by US institute of health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996), the European Union directive (Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010), and the French law. The protocol for this study was approved by the local Ethics Committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg) and authorized by the French Ministry of Higher Education, Research and Innovation (authorization #16593-2018090315391558).

Data Availability Statements

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy issues.

Conflicts of Interest:

The authors declare no conflict of interest.

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Table 1: Anthocyanin contents of ARB and BCE

Anthocyanins	ARB composition (mg/L)	BCE composition (mg/g)
Delphinidin-3-*O*-glucoside	1016.5 ± 66.0	17.49 ± 0.69
Delphinidin-3-*O*-rutinoside	4622.1 ± 422.0	76.25 ± 1.82
Cyanidin-3-*O*-glucoside	104.0 ± 11.4	6.11 ± 0.04
Cyanidine-3-*O*-rutinoside	948.8 ± 73.2	38.81 ± 0.20
Delphinidin	14.8 ± 0.4	0.10 ± 0.01
Peonidin-3-*O*-glucoside	19.6 ± 2.9	0.05 ± 0.00
Peonidin-3-*O*-rutinoside	0.00 ± 0.00	0.34 ± 0.01
Peonidin	60.6 ± 6.1	7.22 ± 0.11
Petunidin	23.9 ± 1.2	0.00 ± 0.00
Malvidin	34.5 ± 1.0	0.90 ± 0.01

Results are presented as means±S.D. of triplicate analysis

Table 2: ARB chronic intake reduces the body weight in old rats

	Young rats	Old rats	Old rats + ARB 60 mg/kg/d	Old rats + ARB 120 mg/kg/d
Body Weight (g)	350±14	790±31 *	756±32 *,#	753±24 *,#
Left kidney (% of body weight)	0.40±0.03	0.24±0.02	0.30±0.03	0.26±0.02
Right kidney (% of body weight)	0.39±0.03	0.26±0.02	0.30±0.03	0.27±0.02
Heart (% of body weight)	0.33±0.01	0.26±0.02	0.26±0.01	0.23±0.02
Spleen (% of body weight)	0.29±0.02	0.13±0.03	0.15±0.03	0.14±0.03
Liver (% of body weight)	4.22±0.11	2.82±0.22	2.89±0.14	2.63±0.21

Rat organs were weighed and indexed to the respective body weight at the end of the experiment. Results as expressed as mean ± SEM of 10 rats per group. *P<0.05 vs. young rats; # P<0.05 vs. Old rats

Figures legends:

Figure 1: ARB chronic intake reduces the age-related increase in blood pressure. Blood pressure was monitored by tail-cuff sphygmomanometry after 2 weeks of treatment with ARB. Results as expressed as mean ± SEM of 9-10 rats per group. *P<0.05 vs. Young rats; $^{\#}$ P<0.05 vs. Old rats

Figure 2: ARB chronic intake improves the age-related endothelial dysfunction in the main mesenteric artery.

Rings with endothelium were prepared from the main mesenteric artery and suspended in organ baths for the determination of changes in isometric tension. (A, B) To study the endothelium-dependent relaxation, rings were contracted with 1 µM phenylephrine (PE) before the addition of increasing concentrations of acetylcholine (ACh). (C, D) To assess the contractile responses, rings were subjected to a concentration-contraction curves in response to phenylephrine. In some baths, rings were exposed to a pharmacological agent for 30 min before contraction. To study the role of cyclooxygenase-derived prostanoids, rings were incubated with indomethacin (10 µM, a nonselective COX inhibitor). To study NO-mediated relaxation rings were incubated with both indomethacin and TRAM-34 plus UCL-1684 (1 µm each, inhibitors of IK_{Ca} and SK_{Ca}, respectively) to inhibit the release of vasoactive prostanoids and the EDH-mediated relaxation, respectively. The role of EDH-mediated relaxation was studied in rings incubated with indomethacin and N^{ω}-nitro-L-arginine (L-NA, 300 µM, an eNOS inhibitor) to prevent the formation of vasoactive prostanoids and NO, respectively.

(E) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with 1 µM PE before the construction of a concentration-relaxation curve to either either sodium nitroprusside (a NO donor) or levcromakalim (an ATP-sensitive potassium channels opener), in the presence of indomethacin, L-NA and TRAM-34 plus UCL-1684 to prevent the formation of vasoactive prostanoids, NO and EDH, respectively.

Results are expressed in % relaxations (A, B, E) or in grams of contraction (C, D) as means ± SEM of 4-10 rats per group. (A, C) * $P<0.05$ vs. control without inhibitors. (B, D, E) *$P<0.05$ vs. Young rats; $^{\#}$ $P<0.05$ vs. Old rats.

Figure 3: ARB chronic intake is associated with a dose-dependent increase in the endothelial accumulation of anthocyanins, especially at arterial sites at risk.

Basal vascular accumulation of anthocyanins was determined in arterial rings of the thoracic aorta and rings of the aortic arch at sites of branching using the Neu A reagent. Results are expressed as mean ± SEM of 4-7 rats per group. * P<0.05 vs. Old rats.

Figure 4: ARB chronic treatment increases the expression of SGLT1 in the endothelium of the thoracic aorta and aortic arch branching.

Determination of the expression level of SGLT1 was done in arterial rings of the thoracic aorta and rings of the aortic arch at sites of branching using immunofluorescence. All samples were

observed using a confocal laser-scanning microscope. Quantification of fluorescence levels was performed in the endothelium. Results are expressed as mean ± SEM of 4-7 rats per group. * P<0.05 vs. Young rats, # P<0.05 vs. Old rats, † P<0.05 vs. respective Inner.

Figure 5: ARB chronic intake is associated with an increased acute anthocyanins uptake in the endothelium at arterial sites at risk.

Acute vascular uptake of anthocyanins was determined in arterial rings of the thoracic aorta and rings of the aortic arch at sites of branching treated with blackcurrant polyphenolic extract (BCE, 60 µg/ml) for 3 minutes using the Neu A reagent before cryosection. Results are expressed as mean ± SEM of 4-7 rats per group. * P<0.05 vs. respective control; # P<0.05 vs. respective Old rats; † P<0.05 vs. respective thoracic aorta.

Figure 6: Inhibition of SGLT1 and 2 reduces the endothelial uptake of anthocyanins.

Acute endothelial uptake of anthocyanins was determined in arterial rings of the thoracic aorta incubated with blackcurrant polyphenolic extract (BCE, 60 µg/ml) for 3 minutes using the Neu A reagent before cryosection. Sections were incubated with either a dual inhibitor of SGLT1/2 (sotagliflozin, 100 nM) or a selective SGLT2 inhibitor (empagliflozin, 100 nM) for 5 min before the addition of BCE. All samples were observed using a confocal laser-scanning microscope. Quantification of fluorescence levels was performed in the endothelium. Results are expressed

as mean ± SEM of 4-7 rats per group. * P<0.05 vs. respective control; # P<0.05 vs. respective BCE treatment; † P<0.05 vs. respective BCE+SGLTs dual inhibitor treatment.

Figure 7: ARB chronic intake reduces the age-related increased vascular oxidative stress mediated, at least in part, by SGLTs.

Vascular oxidative stress was determined in unfixed cryosections of the thoracic aorta using the redox-sensitive probe dihydroethidium or the Mitochondrial Superoxide Indicator MitoSox™. (A) To assess the level of oxidative stress, some sections were incubated for 2 h with the anti-oxidant N-acetylcysteine (NAC, 3 mM) before the addition of DHE. (B) To assess the role of SGLT1 and SGLT 2 in the ageing-related increased vascular oxidative stress, sections were incubated with either a dual SGLTs inhibitor (sotagliflozin, 100 nM) or a selective SGLT2 inhibitor (empagliflozine, 100 nM) for 30 min before the addition of DHE. All samples were observed using a confocal laser-scanning microscope. Quantification of fluorescence levels was performed in the arterial wall. Results are expressed as mean ± SEM of 4 rats per group. * P<0.05 vs. Young rats; # P<0.05 vs. Old rats. *

Figure 8: ARB chronic intake improves the age-related up-regulation of markers of endothelial senescence and dysfunction, and of angiotensin system activation in the thoracic aorta.

Protein immunoreactive signals were determined in fixed cryosections of the thoracic aorta. The determination of the expression level of protein markers of endothelial function (eNOS, MCP-1), senescence (p53) and activation of the local angiotensin system (ACE, AT1R) was done by immunofluorescence and observed using a confocal laser-scanning microscope.

Quantification of fluorescence levels was performed in the endothelium. Results are expressed as mean ± SEM of 4-5 rats per group. * P<0.05 vs. Young rats, # P<0.05 vs. Old rats.

Figure 9: ARB chronic intake improves the age-related up-regulation of markers of vascular fibrosis and remodeling in the thoracic aorta.

Protein immunoreactive signals were determined in fixed cryosections of the thoracic aorta. The determination of the expression level of protein markers of vascular fibrosis (TGFβ1, Collagen-1) and remodeling (MMP-2 and MMP-9) was done using immunofluorescence and observed using a confocal laser-scanning microscope. Quantification of fluorescence levels was performed in the endothelium. Results are expressed as mean ± SEM of 4-5 rats per group. * P<0.05 vs. Young rats, # P<0.05 vs. Old + ARB.

Figure 1

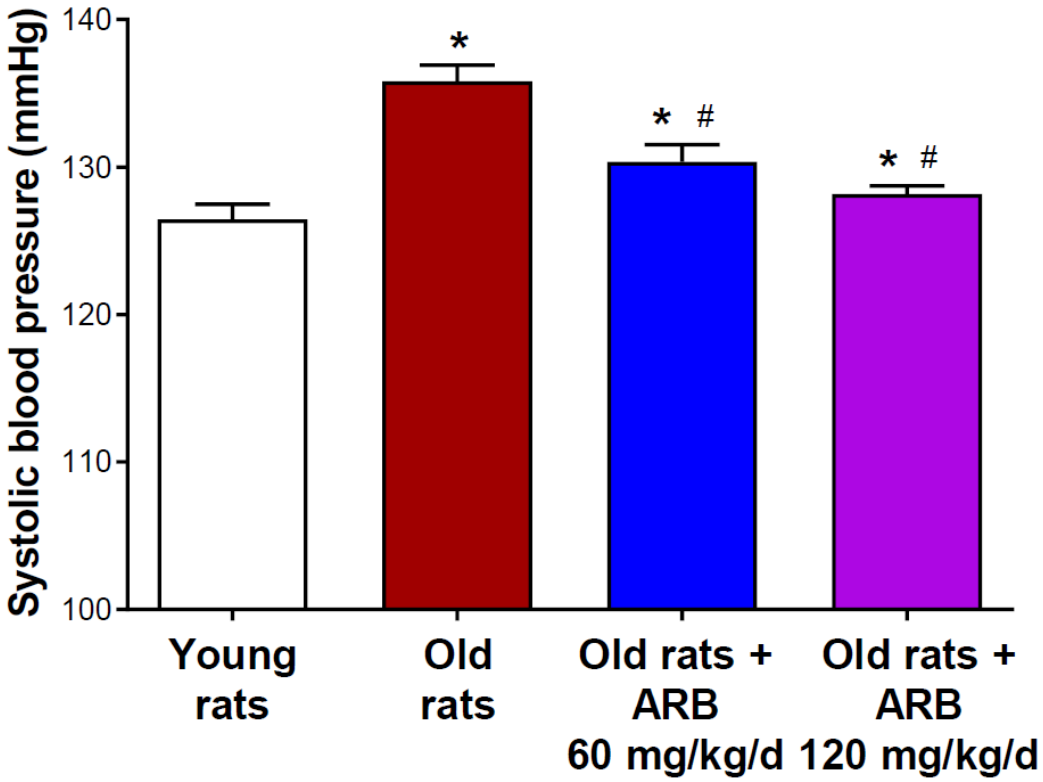

Systolic blood pressure (mmHg)
140
130
120
110
100
*
* #
* #
Young rats
Old rats
Old rats + ARB 60 mg/kg/d
Old rats + ARB 120 mg/kg/d

Figure 2

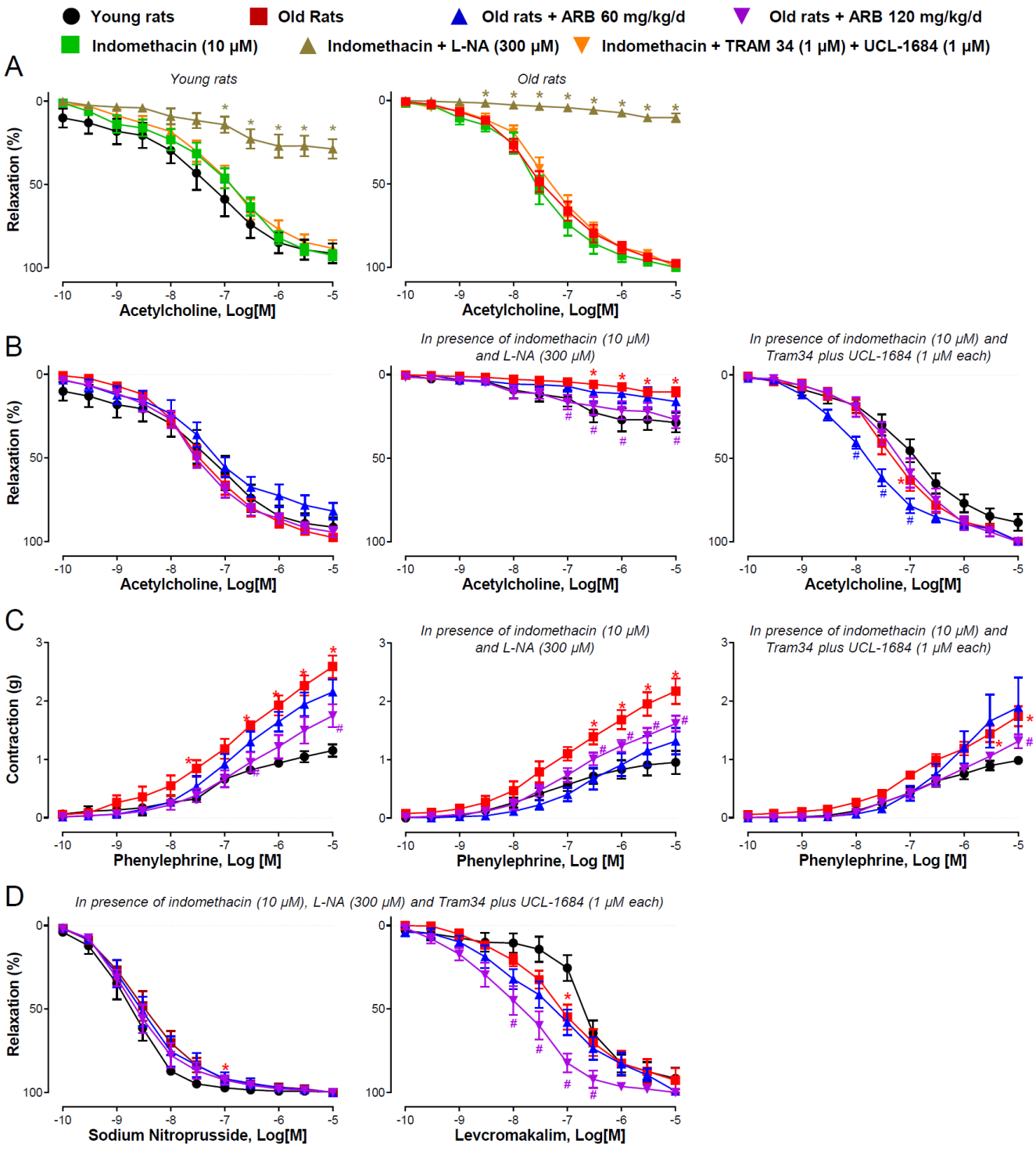

Young rats
Old Rats
Old rats + ARB 60 mg/kg/d
Old rats + ARB 120 mg/kg/d
Indomethacin (10 µM)
Indomethacin + L-NA (300 µM)
Indomethacin + TRAM 34 (1 µM) + UCL-1684 (1 µM)
A
Young rats
Old rats
Relaxation (%)
Acetylcholine, Log[M]
B
In presence of indomethacin (10 µM) and L-NA (300 µM)
In presence of indomethacin (10 µM) and Tram34 plus UCL-1684 (1 µM each)
C
Contraction (g)
Phenylephrine, Log [M]
D
In presence of indomethacin (10 µM), L-NA (300 µM) and Tram34 plus UCL-1684 (1 µM each)
Sodium Nitroprusside, Log[M]
Levcromakalim, Log[M]

Figure 3

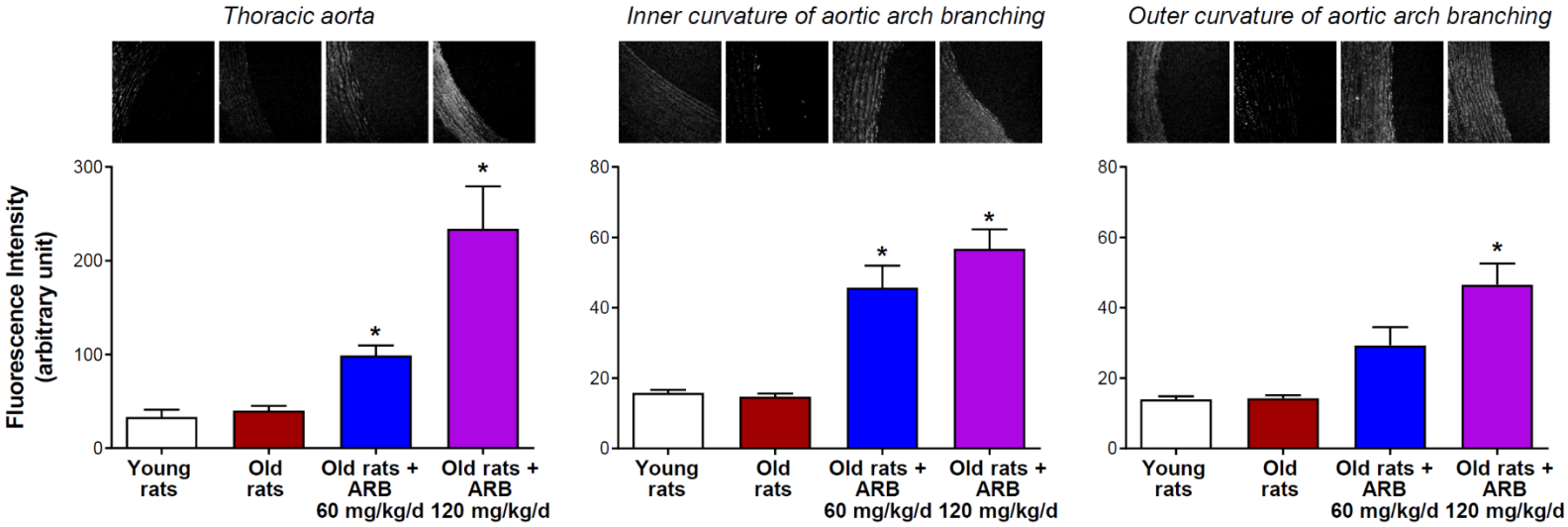

Thoracic aorta
Inner curvature of aortic arch branching
Outer curvature of aortic arch branching
Fluorescence Intensity (arbitrary unit)
300
200
100
0
80
60
40
20
0
*
Young rats
Old rats
Old rats + ARB 60 mg/kg/d
Old rats + ARB 120 mg/kg/d

Figure 4

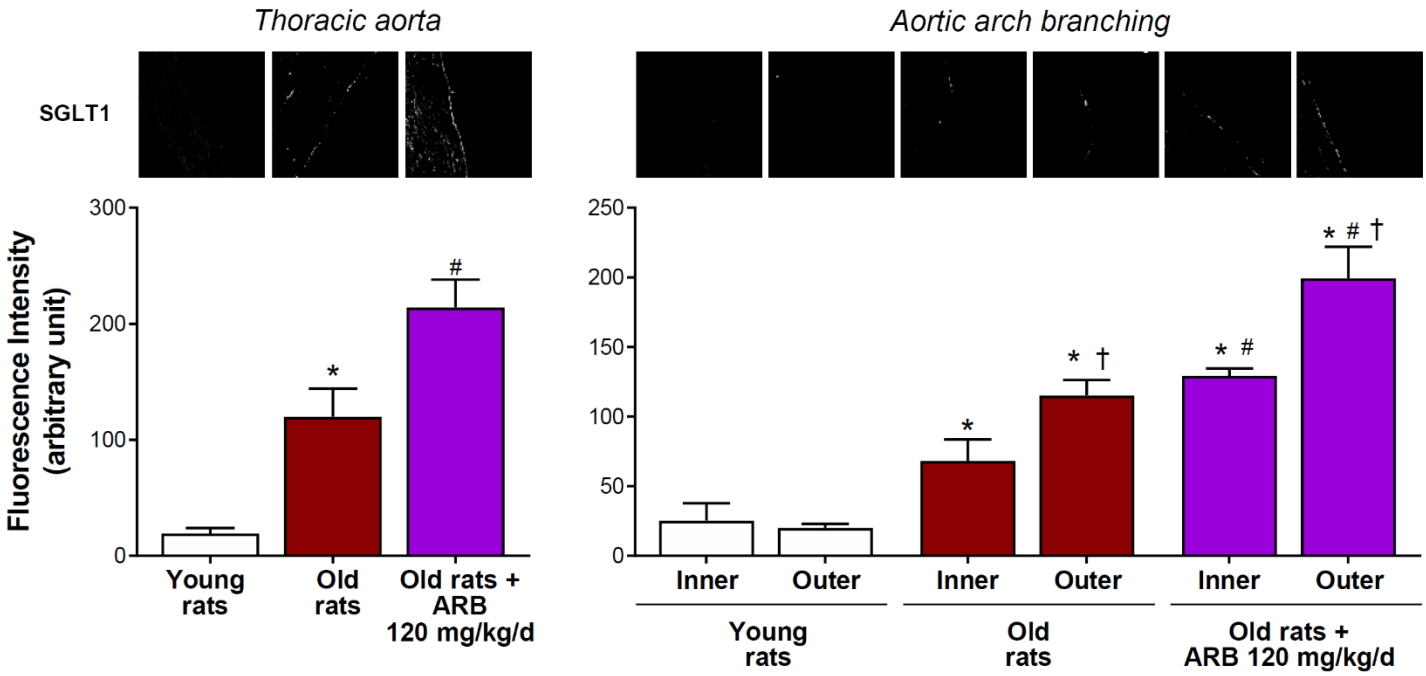

Thoracic aorta
Aortic arch branching
SGLT1
Fluorescence Intensity (arbitrary unit)
300
200
100
0
250
150
50
*
#
* †
* # †
Young rats
Old rats
Old rats + ARB 120 mg/kg/d
Inner
Outer

Figure 5

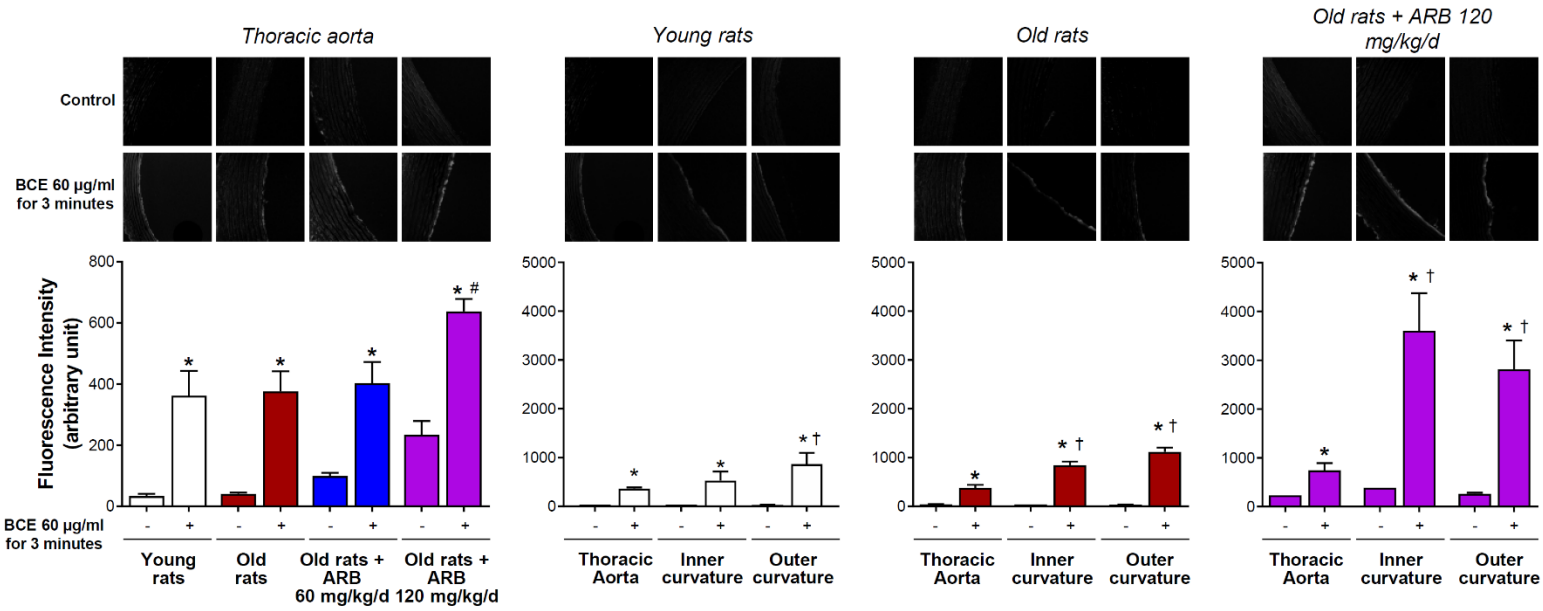

Thoracic aorta
Young rats
Old rats
Old rats + ARB 120 mg/kg/d
Control
BCE 60 µg/ml for 3 minutes
Fluorescence Intensity (arbitrary unit)
BCE 60 µg/ml for 3 minutes
Young rats
Old rats
Old rats + ARB 60 mg/kg/d
Old rats + ARB 120 mg/kg/d
Thoracic Aorta
Inner curvature
Outer curvature

Figure 6

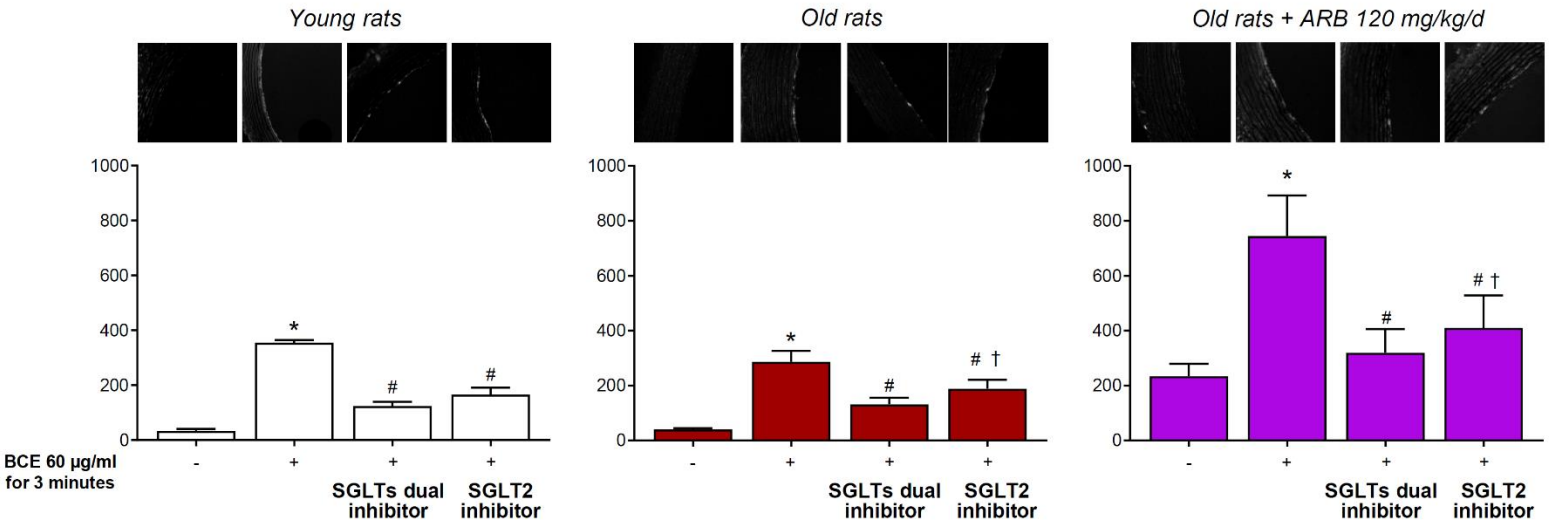

Young rats
Old rats
Old rats + ARB 120 mg/kg/d
1000
800
600
400
200
0
*
#
†
BCE 60 µg/ml
for 3 minutes
-
+
+
+
SGLTs dual inhibitor
SGLT2 inhibitor

Figure 7

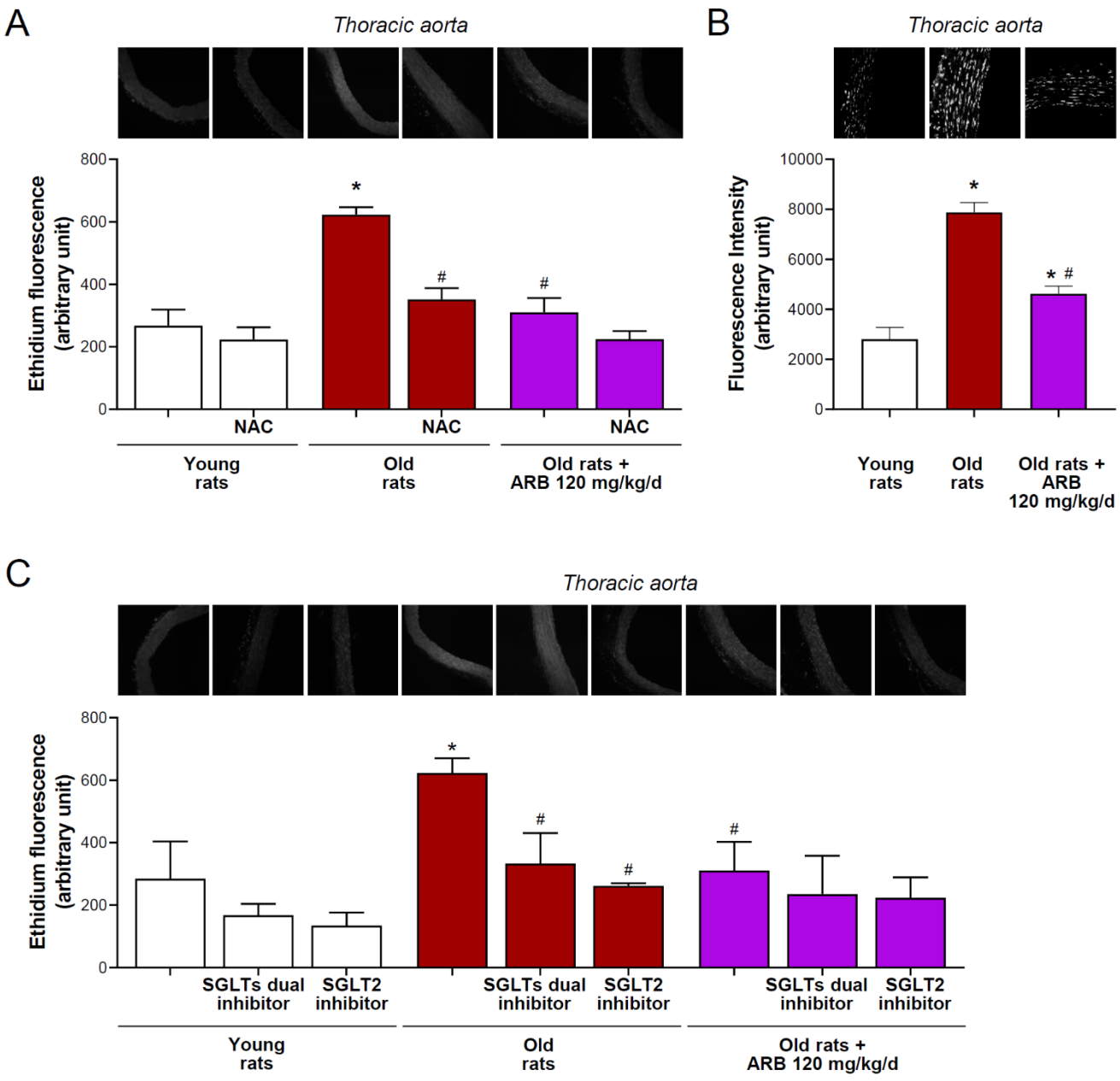

A
Thoracic aorta
Ethidium fluorescence (arbitrary unit)
800
600
400
200
0
*
#
#
NAC
NAC
NAC
Young rats
Old rats
Old rats + ARB 120 mg/kg/d
B
Thoracic aorta
Fluorescence Intensity (arbitrary unit)
10000
8000
6000
4000
2000
0
*
* #
Young rats
Old rats
Old rats + ARB 120 mg/kg/d
C
Thoracic aorta
Ethidium fluorescence (arbitrary unit)
800
600
400
200
0
*
#
#
#
SGLTs dual inhibitor
SGLT2 inhibitor
SGLTs dual inhibitor
SGLT2 inhibitor
SGLTs dual inhibitor
SGLT2 inhibitor
Young rats
Old rats
Old rats + ARB 120 mg/kg/d

Figure 8

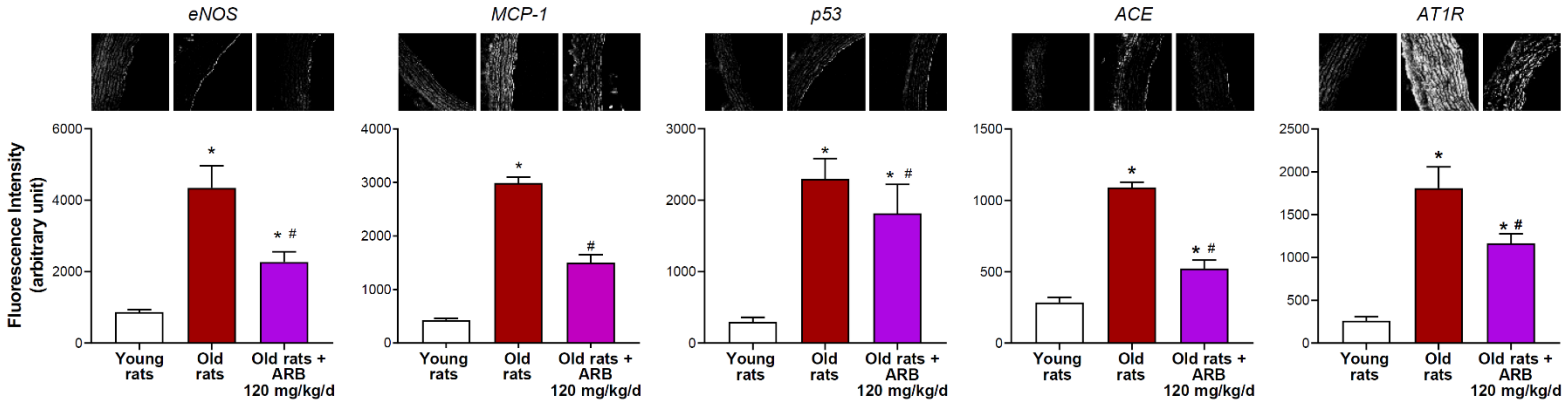
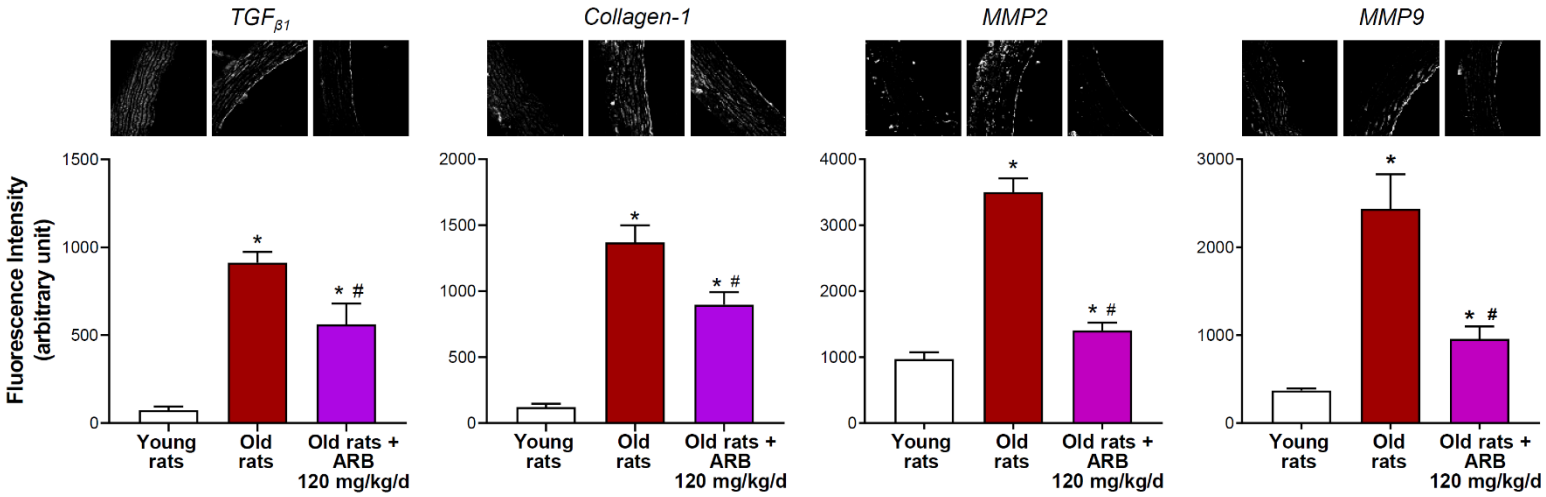

eNOS
MCP-1
p53
ACE
AT1R
Fluorescence Intensity (arbitrary unit)
Young rats
Old rats
Old rats + ARB 120 mg/kg/d

Figure 9


TGF$_{\beta 1}$
Collagen-1
MMP2
MMP9
Fluorescence Intensity (arbitrary unit)
1500
1000
500
0
2000
1500
1000
500
0
4000
3000
2000
1000
0
3000
2000
1000
0
*
* #
Young rats
Old rats
Old rats + ARB 120 mg/kg/d

Discussion

Aging is defined as a gradual decline in biological functions. It is associated with an increased incidence of CVDs, which are the leading cause of death in the elderly (Dai, Chen et al. 2012). Epidemiological studies show that regardless of the other risk factors, age is an independent risk factor for cardiovascular morbidity and mortality (Hajar 2017). Vascular aging is also associated with endothelial dysfunction which is considered to be a major risk factor for the development of cardiovascular pathologies (Hadi, Carr et al. 2005). The treatment or prevention of age-related endothelial dysfunction could be an efficient target for the prevention of cardiovascular pathologies since aging is associated with an increasing in blood pressure and blunted endothelium-dependent relaxations due to decreased production or increased inactivation of vasodilating factors such as NO and EDH, and an augmented production of EDCFs and oxidative stress (Farooq 2018).

In this work we focused on the protective effects of naturally occurring antioxidant substances, the anthocyanins, against age-related endothelial dysfunction in rats. Indeed, anthocyanins and anthocyanins-rich products may reduce CVD events such as cardiovascular death, coronary events, ischemia and improve the bioavailability of vasoprotective factors such as NO (Wallace 2011, Fairlie-Jones, Davison et al. 2017). We have shown that anthocyanins are potent molecules for both improving an established age-related endothelial dysfunction, at least in part by decreasing endothelial senescence.

Age-related endothelial dysfunction in old rats

One crucial aspect of this study was the selection of an appropriate rat model to recapitulate aspects of human aging. Wistar rats typically have a lifespan of about 2.5 to 3 years, and at 22 months old, they are considered to be in an advanced life stage corresponding to late adulthood in humans approx. 55-60 years old (Turturro, Witt et al. 1999). Using 22-month-old rats allowed us to capture the range of physiological and pathological processes associated with advanced vascular aging, such as endothelial dysfunction, elevated blood pressure, increased oxidative stress, and a heightened inflammatory status. Meanwhile, 8-week-old rats, considered early adults, served as a healthy control group, enabling us to assess age-dependent alterations in vascular function more accurately. Employing this age comparison (22 vs. 8 weeks) is a well-

established experimental approach to investigate the mechanisms and potential therapeutic targets of age-related cardiovascular dysfunction.

In the present work, we found that aging is associated with a small but significant increase in the arterial blood pressure in old rats compared to young controls. The age-related increase in blood pressure has always been taken as an unavoidable consequence of Aging that leads to pathologies and ultimately death. Indeed, the Framingham study showed that the raising in BP is one of the main risk factors of CVDs (FHS 2005). The rises in blood pressure with age is a major risk factor for cardiovascular, renal disease, and type 2 diabetes mellitus (Lawes, Bennett et al. 2004, Conen, Ridker et al. 2007, Xie, Atkins et al. 2016).

Increase in BP with age is mostly related to changes in arterial and arteriolar stiffness. Large artery stiffness (LAS) is mainly due to arteriosclerotic structural alterations and calcification (Pinto 2007). This leads to earlier reflected pressure waves from the arterioles towards the heart during BP wave propagation, thus increasing central SBP (Wang, Cheng et al. 2010).

In the present study, old rats aged of 22 months showed an increased SBP compared to the young rats, and the SBP was significantly reduced in old rats treated with ARB 120 mg/kg/day for two weeks. These results are in line with previous clinical and preclinical studies that showed that anthocyanins intake is associated with lower blood pressure and arterial stiffness. Indeed, a cross sectional study in 1898 women indicate that the higher quintile for intake of anthocyanins-rich products is associated with lower SBP and arterial stiffness (Jennings, Welch et al. 2012). In addition to a reduction of pro-fibrotic and pro-atherosclerotic mechanisms, the beneficial of anthocyanins on blood pressure could be due to a direct vasodilator effect. The intravenous injection of a blackcurrant juice produced a dose-dependent decrease in the mean blood pressure and heart rate of anesthetized rabbits that was associated with a potent vasorelaxant activity on isolated aortic rings (Branković, Miladinović et al. 2016).

Vascular reactivity

Our results allowed us to characterize the endothelial dysfunction in old Wistar rats mesenteric artery using acetylcholine and phenylephrine concentration-response curves. The results indicate

that while the NO component of the endothelium-dependent relaxation was not affected by aging, the EDH component was totally abolished in the mesenteric artery ring of old rats. Indeed, the response to ACh in presence of indomethacin and L-NA (inhibitors COX and NOS, respectively) failed to induce relaxation in 22-month-old rats, whereas it induces a relaxation of about 25 % in 8-week-old rats. The EDH component was significantly restored in the old Wistar rats treated with ARB 120/mg/kg/day. Thus, the EDH component appears to be very sensitive to the physiological process of aging in the mesenteric artery and could be significantly improved by a short chronic intake of anthocyanin-rich products. Various published studies have also reported that aging strongly affect the EDH-mediated relaxation in rat mesenteric artery (Dal-Ros, Bronner et al. 2012, Idris-Khodja, Auger et al. 2012, Idris-Khodja and Schini-Kerth 2012, Idris Khodja, Chataigneau et al. 2012, Dunn, Hilgers et al. 2017, Farooq, Gaertner et al. 2020), and polyphenols such as anthocyanins seem to be able to improve endothelial function in various experimental models associated with endothelial dysfunction (Idris-Khodja, Di Marco et al. 2013, Kim, Auger et al. 2013, Oak, Auger et al. 2018, Rashid, Idris-Khodja et al. 2018).

The age-related decline in endothelium-dependent relaxations is attributable, at least in part, to some modification of cellular events causing an augmented production of EDCFs, increased oxidative stress, and decreased production or increased inactivation of vasorelaxant factors such as NO and EDH (Dunn, Hilgers et al. 2017). The latter appear to be inducible by anthocyanins since our results showed an improvement in EDH-mediated relaxation in mesenteric artery, probably *via* the SK_{Ca} and IK_{Ca} channel expressed in endothelial cells that generate a hyperpolarization, which will be transmitted to the underlying vascular smooth muscle to induce relaxation (Kong, Man et al. 2015, Lee 2018).

In the present studies, age-related endothelial dysfunction was also associated with an increase in the contractile responses to PE in the mesenteric artery of old rats compared to young rats. The chronic intake of ARB 120mg/kg/day by old rats was associated with significant decreased contractile responses to PE, indicating that anthocyanins treatment may also reduce the overproduction of EDCFs induced by aging.

ARB chronic intake is associated with a dose-dependent increased capacity for uptake of anthocyanins in the endothelium

The major novel finding of the present study is that the chronic intake of ARB was associated with a dose-dependent accumulation of anthocyanins in the endothelium and vascular wall of the aorta and aortic arch of old rats. Moreover, aging was associated with an increased ability of the endothelium to uptake anthocyanins, and the ability was further increased by the ARB treatment. In addition, the *ex vivo* exposure to a purified extract of blackcurrant and the subsequent anthocyanins uptake was significantly inhibited by inhibitors of SGLT1 and 2 (sotagliflozin and empagliflozin), indicating the involvement of SGLT1 and 2 in anthocyanins uptake in the endothelial cells. The involvement of SGLT1 in the cellular uptake of anthocyanins in the endothelial cells is in line with a study that reported delphinidin-3-*O*-glucoside was transported into cultured endothelial cells in a temperature, concentration, and time-dependent manner via SGLT1 (Jin, Yi et al. 2013). We also showed that both SGLT1 and SGLT2 are involved in the cellular entry of blackcurrant-derived anthocyanins in endothelial cells (Lee, Khemais-Benkhiat et al. 2017). Previous bioavailability studies have indicated that anthocyanins are rapidly absorbed and eliminated in blood, urine, plasma and intestine with a low apparent bioavailability (Talavéra, Felgines et al. 2003, Kay 2006). To the better of our knowledge, the present study is the first to report that after chronic oral intake, anthocyanins could accumulate in the vascular tissue.

Furthermore, the increased ability for anthocyanins uptake induced by either aging and ARB treatment was associated with an upregulation of SGLT1 in both aorta and aortic arch, whereas SGLT2 signal was below detection level. These data are similar to our studies that showed that endothelial senescence is associated with an increased expression of both SGLT1 and SGLT2 *via* a redox-sensitive mechanisms involving the local angiotensin system (Khemais-Benkhiat, Belcastro et al. 2020, Park, Belcastro et al. 2021). Despite relatively low circulating concentrations of anthocyanins, the observed endothelial accumulation suggests that even modest plasma levels are sufficient to achieve high local concentrations in aging or senescent endothelial cells. This local enrichment of anthocyanins is critical because it can enhance NO bioavailability and formation. Accumulation in the endothelium may promote eNOS (endothelial nitric oxide

synthase) activity and reduce oxidative stress, thus favoring increased NO production (Freedman, Parker et al. 2001, Álvarez-Cilleros, Ramos et al. 2018). Consequently, the protective vascular effects we observed, including improved endothelium-dependent relaxation, may stem from a microenvironment in which anthocyanins concentrate to potentiate NO signaling, even if overall plasma levels remain relatively low.

ARB chronic intake is associated with reduced vascular oxidative stress

It was reported that aging and age-related endothelial dysfunction are associated with increased level of vascular oxidative stress. Although the mechanism underlying the aging-related oxidative stress remains to be determined, several lines of evidence support a role for the angiotensin system. Indeed, angiotensin II is a potent inducer of endothelial senescence and dysfunction, and of vascular oxidative stress (Wen, Gwathmey et al. 2012, Dikalov and Nazarewicz 2013, Khemais-Benkhiat, Idris-Khodja et al. 2016, Khemais-Benkhiat, Belcastro et al. 2020). In addition, the activation of the angiotensin system has been associated with an increased production of ROS *via* NADPH oxidase and mitochondrial dysfunction (Wen, Gwathmey et al. 2012). The present findings indicate that the age-related endothelial dysfunction is associated with elevated levels of ROS including an increased mitochondrial ROS production in the vasculature of aged rats signify heightened oxidative stress, as well as an upregulation of AT_1R and ACE, two components of the local angiotensin system in the arterial wall of the aorta from old rats. The reduction in the vascular ROS by the intake of ARB is associated with a decreased expression of ACE and AT1R, suggesting that anthocyanins decrease which may indicate a reduction in vascular oxidative, at least in part, by reducing the activation of the local angiotensin system. This is in line with previous results on the mesenteric artery of cirrhotic rats that showed that ARB intake was associated with a decreased vascular ROS associated with a reduced upregulation of the NADPH oxidase (Rashid, Idris-Khodja et al. 2018).

Previous studies have also indicated that intake of several other polyphenol-rich sources such as a red wine extract prevented vascular oxidative stress and endothelial dysfunction in the angiotensin II-induced hypertensive rats (Sarr, Chataigneau et al. 2006) and in old rats (Idris Khodja, Chataigneau et al. 2012), and tea catechin in the Otsuka Long-Evans Tokushima fatty rat

model of metabolic syndrome (Ihm, Jang et al. 2012). It's important to mention that one of the limitations of our approach is that it solely evaluates ROS formation (which is conventionally the strongest marker of oxidative stress), without any assessment of antioxidant defenses. Consequently, we cannot conclusively determine whether the interventions have truly mitigated overall oxidative stress. In order to draw a more robust conclusion, future studies should incorporate measurements of both oxidative biomarkers and antioxidant capacity.

ARB chronic intake is associated with improved endothelial health in the thoracic aorta

Furthermore, the present study indicate that aging is also associated with increased endothelial expression of pro-inflammatory (MCP-1) and profibrotic mediators (TGFβ), as well as an increased intimal expression of pro-remodeling enzymes (MMP 2 and 9) and deposition of collagen-1. Many studies have mentioned that with aging the vasculature undergoes structural and functional changes characterized by endothelial dysfunction, wall thickening, reduced distensibility, and arterial stiffening. Vascular stiffness results from fibrosis and extracellular matrix (ECM) remodeling (Ihm, Jang et al. 2012). In addition, Fornieri et al. highlighted the role of the extracellular matrix in the age-related modifications of the rat aorta (Fornieri, Quaglino et al. 1992). Another recent study using stroke-prone spontaneously hypertensive rats (SHRSP) reported a high vascular expression of collagen, fibronectin, TGFβ, MCP-1, MMP2 and MMP9 associated with increased ROS generation (Harvey, Montezano et al. 2017). The chronic intake of ARB significantly reduced the expression of MCP-1, MMP2, MMP9 and TGFβ in aorta of old rats. Our study suggests that daily intake of blackcurrant anthocyanins could reduce the expression of inflammatory mediators such as MCP-1 *in vivo*, resulting in the suppression of local arterial inflammation. Moreover, anthocyanins could also reduce the protein expression and activity of TGFβ, collagen-1, MMP-2 and MMP-9. Therefore, anthocyanins could preserve the integrity of the aortic wall structure and reduce the occurrence and development of age-related vascular fibrosis and its following cardiovascular complications. Indeed, Wang et al. reported that grape polyphenols play a protective role in elastase-induced Abdominal Aortic Aneurysm in mice *via* the reduction of inflammation (MCP-1) and decreased activity of matrix metalloproteinase 2 and 9,

which is in line with our study (Wang, Wang et al. 2017). Previous study linked the protective effect of anthocyanins to their ability to attenuate the oxidative stress, which is involved in process such as inducing inflammation leading to structural alteration of the arterial wall (Kim, Park et al. 2011, Wang, Wang et al. 2017). However, it may be other mechanisms of these effects of anthocyanins that remain to be determined in future studies.

Extrapolation to human health and proposed strategies

Although these findings were obtained in an animal model, they hold promise for human vascular health. Animal experiments often employ relatively high doses of anthocyanins (per kg body weight) compared to typical human dietary patterns, raising the question of dose extrapolation. By standard allometric scaling (Reagan-Shaw, Nihal et al. 2008), the 120 mg/kg/day dose of ARBJ used in old rats which contains 6.8g GAE/L translates to 1.36g/day for an adult human which is approximately around 150g/day of fresh fruits, the human-equivalent dose may surpass the average daily anthocyanin intake in populations with low fruit and vegetable consumption.

Evidence suggests that the average daily polyphenol intake in Western diets ranges from 500 to 1500 mg, which confirm that the dose we used is within this range, although anthocyanins constitute only a small fraction of this total (Del Rio, Rodriguez-Mateos et al. 2013). Typical anthocyanin intake estimates vary widely, from a few milligrams to tens of milligrams per day, depending on dietary habits (Zamora-Ros, Knaze et al. 2016). High consumers of berry-rich diets may reach well above 50 mg/day of anthocyanins (Kay and Holub 2002). Our data imply that even if plasma concentrations remain modest, repeated daily consumption of anthocyanin-rich foods such as berries, blackcurrants, and other deeply pigmented fruits could enable sufficient local endothelial accumulation of these compounds, thereby offering significant vasoprotective benefits.

From a clinical perspective, supplementation with anthocyanin-rich extracts or juices could serve as an adjunct therapy in individuals at risk for endothelial dysfunction (e.g., older adults or patients with metabolic syndrome). Future well-designed clinical trials should carefully assess human-equivalent doses, incorporate dietary evaluations to account for baseline anthocyanin

intake, and measure vascular outcomes via non-invasive methods (e.g., flow-mediated dilation, pulse-wave velocity), along with blood biomarker analysis for anthocyanin metabolites.

Conclusion and perspective

In conclusion, the major findings of this study are that chronic intake of 120 mg/kg/day of anthocyanins-rich blackcurrant juice for two weeks by old rats reduced systolic blood pressure by 7 mmHg and improved age-related endothelial dysfunction both by restoring abolished EDH-mediated relaxations and by reducing contractile responses. The vasoprotective properties of anthocyanins are associated with a reduction of the age-related vascular oxidative stress, activation of the local angiotensin system and expression of pro-inflammatory, pro-fibrotic and pro-remodeling factors. The beneficial effects of the chronic intake of anthocyanins seem to be due to the dose-dependent endothelial uptake of anthocyanins *via* the sodium-glucose transporters 1 and 2. These data suggest that anthocyanins could be an interesting complementary therapeutic approach to improve endothelial health in physio-pathological situations associated with premature endothelial senescence. As premature endothelial senescence is more pronounced in arterial site at risk and is characterized with increased endothelial expression of SGLTs, anthocyanins could allow a natural targeting as cellular uptake of the beneficial anthocyanins will be increased specifically in these areas.

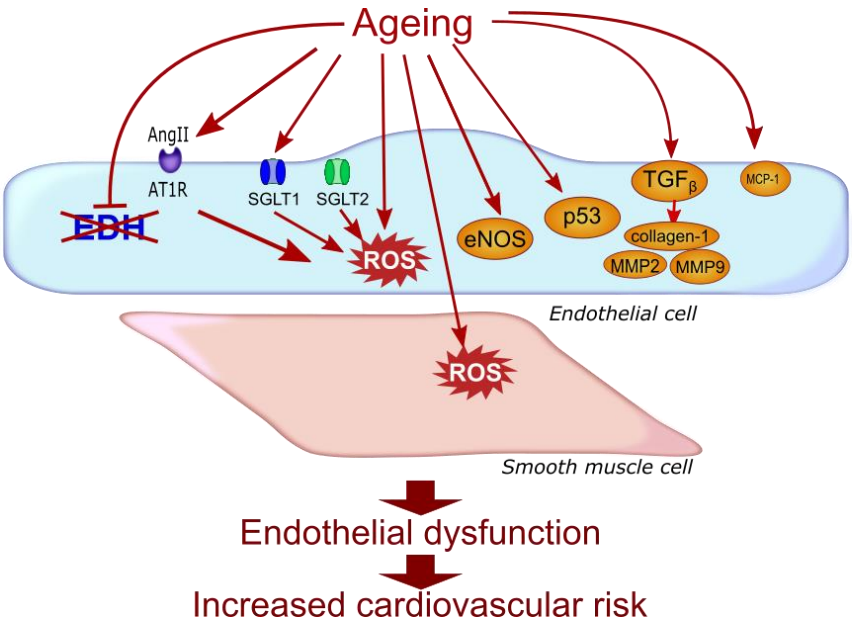


Figure 18 – Effect of aging on the endothelial function

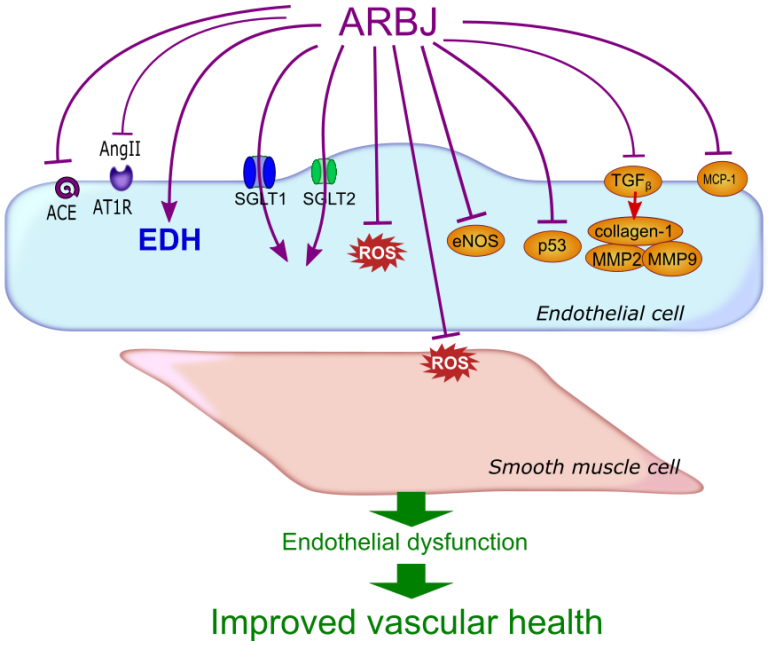


Figure 19 – The role of anthocyanins in improving endothelial dysfunction

The present study has produced interesting new data regarding the potential of anthocyanins in the management of cardiovascular diseases related to aging. However, further work is warranted to complete the present study.

Firstly, we have focused our present work on endothelial dysfunction in the mesenteric artery and in the aorta. However, whereas such arterial beds present similarities with other vascular beds, they are not generally involved in the development of cardiovascular diseases. Thus, further work should confirm the present results in more relevant vascular beds such as the coronary (micro)circulation for CAD, CHD and heart failure, the cerebral arteries for stroke, or the femoral vessels for peripheral arteriopathy and deep-vein thrombosis.

Our results indicate that both SGLT1 and SGLT2 are involved in the cellular uptake of anthocyanins in the endothelium. However, we were not able to determine the vascular expression of SGLT2 in the aortic tissues by immunofluorescence. The expression of SGLT2 thus needs to be assessed using complementary techniques such as Western blot analysis and RT-PCR.

Moreover, while we have shown that the chronic intake of the anthocyanin-rich blackcurrant is associated with a dose-dependent accumulation in vascular tissue, we currently lack information regarding the nature of the active molecules and of the cellular metabolites. Previous *in vitro* studies indicated that amongst the 4 major anthocyanins present in the blackcurrant, only the glucoside-conjugated derivatives of cyanidin and delphinidin enter the cell through SGLT and activate the redox-sensitive Src/PI3K/Akt pathway leading to eNOS activation (Lee, Khemais-Benkhiat et al. 2017). This suggests that metabolites of cyanidin and/or delphinidin could be the molecules that accumulate in endothelial cells after oral intake of blackcurrant, but characterization and formal identification must be conducted.

Lastly, further work will have to assess the relevance of the present finding for human age-related endothelial dysfunction and vascular alterations. Indeed, the current study has been conducted in Wistar rats under a controlled environment, including standard chow devoid of phenolic compounds. Thus, further studies should assess the potency of anthocyanins to improve the age-related endothelial dysfunction in middle-aged to aged humans that have a life-long dietary exposure to anthocyanins.

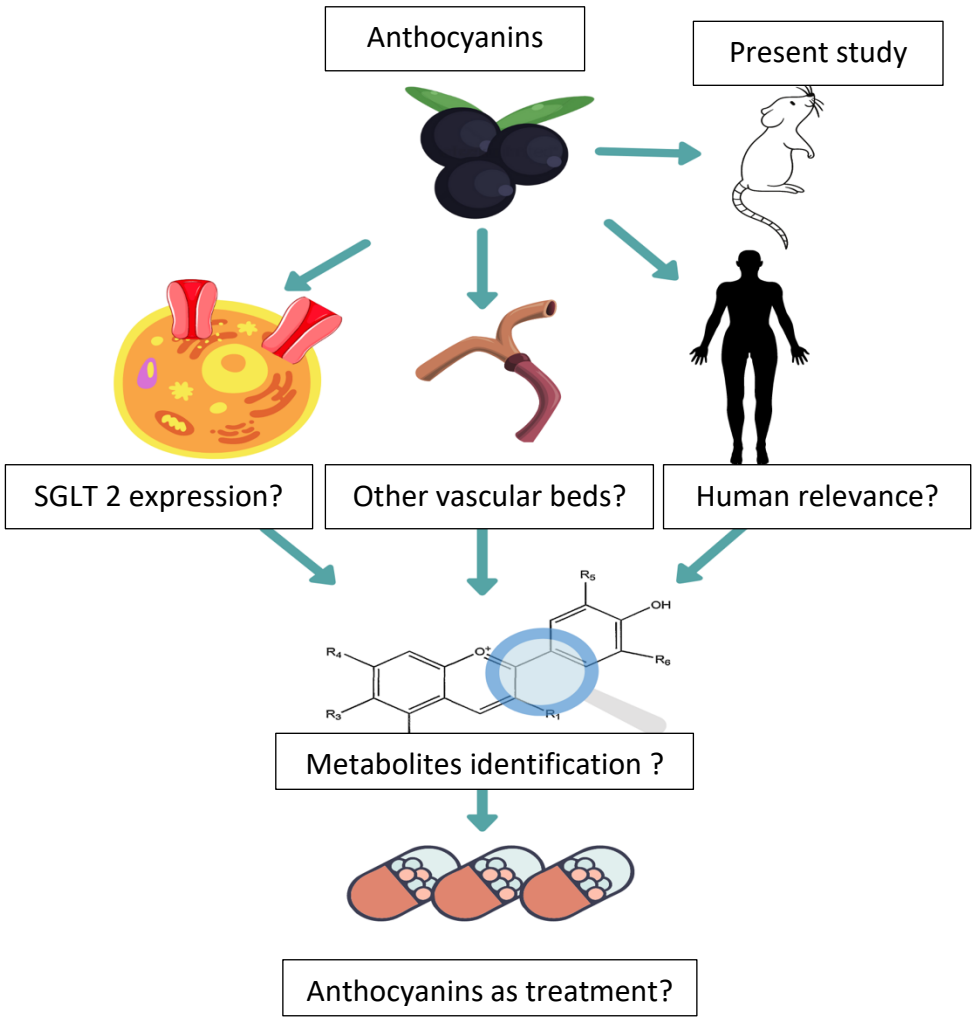


Figure 20 – Perspective of the present study

Discussion générale

Le vieillissement est défini comme un déclin progressif des fonctions biologiques. Il est associé à une incidence accrue de maladies cardiovasculaires, qui sont la principale cause de décès chez les personnes âgées (Dai, Chen et al. 2012). Les études épidémiologiques montrent en effet que l'âge est un facteur de risque indépendant de morbidité et de mortalité cardiovasculaires (Hajar 2017). Le vieillissement vasculaire est également associé à une dysfonction endothéliale qui est elle-même un facteur de risque majeur pour le développement de maladies cardiovasculaires (Hadi, Carr et al. 2005). Le traitement ou la prévention de la dysfonction endothéliale liée à l'âge serait une cible d'intérêt pour la prévention des maladies cardiovasculaires du fait que le vieillissement est associé à une augmentation de la pression artérielle et à une altérations des relaxations dépendantes de l'endothélium dues à une diminution de la production ou à une inactivation accrue de facteurs vasodilatateurs tels que le monoxyde d'azote (NO) ou l'hyperpolarisation dépendante de l'endothélium (EDH), à une production accrue de facteurs vasoconstricteurs dérivés de l'endothélium (EDCFs) et a un stress oxydant (Farooq 2018).

Dans cette étude, nous nous sommes intéressés plus particulièrement aux effets protecteurs de produits naturels, les anthocyanes, vis-à-vis de la dysfonction endothéliale liée à l'âge chez le rat. En effet, les anthocyanines et les produits riches en anthocyanes pourraient réduire les occurrences des événements cardiovasculaires comme les événements coronariens ou l'ischémie cérébrale (AVC) en améliorant la biodisponibilité des facteurs vasoprotecteurs tel que le NO (Wallace 2011, Fairlie-Jones, Davison et al. 2017). Nous avons montré que les anthocyanes sont des molécules efficaces pour améliorer une dysfonction endothéliale liée à l'âge établie, au moins en partie en diminuant la sénescence endothéliale.

La dysfonction endothéliale liée à l'âge chez les rats âgés

Dans notre étude, nous avons montré que le vieillissement est associé à une augmentation légère, mais néanmoins significative, de la pression artérielle systolique chez les rats âgés par rapport aux rats jeunes. Cette augmentation liée à l'âge de la pression artérielle a toujours été considérée

comme une conséquence inévitable du vieillissement cardiovasculaire conduisant à des maladies cardiovasculaires et finalement au décès. En effet, l'étude de la cohorte de Framingham a montré que l'augmentation de la pression artérielle est l'un des facteurs majeurs des maladies cardiovasculaires (FHS 2005). Cette augmentation de la pression artérielle avec l'âge est aussi un facteur de risque majeur les maladies cardiovasculaires et rénales, ainsi que pour le diabète de type 2 (Lawes, Bennett et al. 2004, Conen, Ridker et al. 2007, Xie, Atkins et al. 2016). L'augmentation de la pression artérielle avec l'âge est principalement due à des altérations dans la rigidité des artères et des artérioles. La rigidification des artères est principalement due à la calcification et aux remodelages liées à l'athérosclérose (Pinto 2007). Cela conduit une augmentation de la réflexion des ondes de pouls depuis les artérioles vers le cœur, augmentant ainsi la pression artérielle systolique (Wang, Cheng et al. 2010).

Dans cette étude, les rats âgés de 22 mois présentaient une augmentation de la pression artérielle qui était significativement réduite chez les rats recevant le jus cassis pendant 2 semaines. Ces résultats sont cohérents avec ceux d'études expérimentales et cliniques qui ont montré que la consommation d'anthocyanes est associée avec une diminution de la pression artérielle et de la rigidité artérielle. En effet, dans une étude épidémiologique portant sur 1898 femmes le plus haut quintile de consommation de produits riches en anthocyanes était associée avec une plus faible rigidité artérielle et une pression artérielle systolique plus basse (Jennings, Welch et al. 2012). En plus d'une réduction des mécanismes pro-fibrotique et pro-inflammatoire, l'effet bénéfique des anthocyanes sur la pression artérielle pourrait être dû à un effet vasodilatatoire direct. Ainsi, l'injection intra-veineuse d'un jus de cassis induit une diminution dépendante de la dose de la pression artérielle et du rythme cardiaque chez des lapins anesthésiés, ce qui était associé à une forte activité vasorelaxante dans des anneaux aortiques (Branković, Miladinović et al. 2016).

Réactivité vasculaire

Dans notre étude, nous avons caractériser la dysfonction endothéliale liée à l'âge dans les artères mésentériques des rats Wistar âgés en construisant des courbes concentration-réponses à l'acétylcholine et à la phényléphrine. Les résultats indiquent qu'alors que la composante NO de la relaxation dépendante de l'endothélium n'est pas affectée par l'âge, la composante EDH est

totalement abolie dans les anneaux d'artère mésentérique des rats âgés. En effet, en présence d'indométacine et de N^G-nitro-L-arginine (inhibiteurs des COXs et de la eNOS, respectivement), l'acétylcholine n'induit aucune relaxation chez les rats âgés, contre une relaxation d'environ 25% chez les rats jeunes. La composante EDH de la relaxation est significativement améliorée dans le groupe des rats âgés recevant les anthocyanes de cassis à la dose de 120 mg/kg/j. Ainsi, la composante EDH dans l'artère mésentérique semble particulièrement sensible au vieillissement physiologique et peut être significativement améliorée par une consommation sur un court terme de produits riches en anthocyanes. Plusieurs études publiées ont également rapportées que le vieillissement affectait fortement la composante EDH dans les artères mésentériques de rats (Dal-Ros, Bronner et al. 2012, Idris-Khodja, Auger et al. 2012, Idris-Khodja and Schini-Kerth 2012, Idris Khodja, Chataigneau et al. 2012, Dunn, Hilgers et al. 2017, Farooq, Gaertner et al. 2020), et que les polyphénols, dont les anthocyanes, pouvaient améliorer la fonction endothéliale dans plusieurs modèles expérimentaux présentant une dysfonction endothéliale (Idris-Khodja, Di Marco et al. 2013, Kim, Auger et al. 2013, Oak, Auger et al. 2018, Rashid, Idris-Khodja et al. 2018).

La diminution liée à l'âge des relaxations dépendantes de l'endothélium est attribuable, du moins en partie, à des altérations des processus cellulaires conduisant à une augmentation de la production d'EDCFs, à du stress oxydant et à une diminution de la production et/ou une augmentation de l'inactivation des facteurs vasorelaxants comme le NO ou l'EDH (Dunn, Hilgers et al. 2017). Ce dernier semble être induit par les anthocyanes, comme le montre nos résultats sur l'augmentation de la composante EDH, probablement via les canaux potassique SK_{Ca} et IK_{Ca} exprimés dans l'endothélium et qui génèrent une hyperpolarisation qui sera transmis aux cellules musculaires lisses sous-jacente pour induire la relaxation (Kong, Man et al. 2015, Lee 2018).

Dans notre étude, la dysfonction endothéliale liée à l'âge était aussi associée à une augmentation des réponses contractiles à la phényléphrine dans les anneaux d'artère mésentérique des rats âgés en comparaison des rats jeunes. La consommation chronique de jus de cassis à 120 mg/kg/j était associée à une diminution significative des réponses contractiles à la phényléphrine, indiquant que les anthocyanes pourraient aussi réduire la surproduction d'EDCFs liée au vieillissement.

La consommation chronique de jus de cassis est associée à une augmentation dose-dépendante de la capacité de l'endothélium à assimiler et accumuler les anthocyanes

L'apport majeur de notre étude est le résultat montrant que la consommation chronique du jus de cassis est associée de façon dépendante de la dose de l'accumulation d'anthocyanes dans l'endothélium et la paroi vasculaire de l'aorte et de la crosse aortique des rats âgés. De plus, le vieillissement est associé à une augmentation de la capacité de l'endothélium à assimiler les anthocyanes, et cette capacité est de surcroît augmentée par la consommation chronique du jus de cassis. Cette assimilation des anthocyanes suite à l'exposition ex vivo à un extrait de jus de cassis est significativement inhibées par les inhibiteurs des SGLT1 et SGLT2 (sotagliflozine et empagliflozine), indiquant une implication des SGLT1 et SGLT2 dans l'assimilation endothéliale des anthocyanes. Ce rôle du SGLT1 dans l'entrée des anthocyanes dans les cellules endothéliales est conforme aux résultats d'une étude qui a montré que la delphinidin-3-*O*-glucoside pénétrait dans les cellules endothéliales en culture via le SGLT1 de façon dépendante de la température, de la concentration et du temps (Jin, Yi et al. 2013). Nous avons également montré que SGLT1 et SGLT2 sont tous les deux impliqués dans l'entrée cellulaire des anthocyanes du cassis dans les cellules endothéliales en culture (Lee, Khemais-Benkhiat et al. 2017). A notre connaissance, notre étude est la première à montrer que les anthocyanes peuvent s'accumuler dans les tissues vasculaires après une consommation chronique de jus de cassis. Des études sur la disponibilité avaient indiqué que les anthocyanes sont rapidement absorbées dans le sang puis éliminés dans les urines et l'intestin avec une biodisponibilité apparente très faibles, sans accumulation tissulaire (Talavéra, Felgines et al. 2003, Kay 2006).

De plus, l'augmentation de la capacité d'assimilation des anthocyanes avec l'âge ou le traitement avec le jus de cassis est associée à une régulation positive du SGLT1 dans l'aorte thoracique et dans la crosse aortique, alors que le signal pour le SGLT2 semble sous le seuil de détection. Ces données sont similaires à celles de nos études indiquant que la sénescence endothéliale est associée à une augmentation de l'expression de SGLT1 et SGLT2 via un mécanisme redox-sensible

impliquant le système angiotensine local (Khemais-Benkhiat, Belcastro et al. 2020, Park, Belcastro et al. 2021).

La consommation chronique de jus de cassis est associée à une diminution du stress oxydant vasculaire lié à l'âge

Il a été indiqué que le vieillissement vasculaire et la dysfonction endothéliale liée à l'âge sont associées à une augmentation du stress oxydant vasculaire. Bien que le mécanisme à l'origine du stress oxydant et de la dysfonction endothéliale liés à l'âge reste à identifier, plusieurs faisceaux de preuves indiquent un rôle du système angiotensine local. En effet, l'angiotensine II est un puissant inducteur de la senescence et de la dysfonction endothéliale ainsi que du stress oxydant (Wen, Gwathmey et al. 2012, Dikalov and Nazarewicz 2013, Khemais-Benkhiat, Idris-Khodja et al. 2016, Khemais-Benkhiat, Belcastro et al. 2020). L'activation du système angiotensine est associé à une augmentation de la formation des espèces réactives de l'oxygène (ERO) *via* la NADPH oxydase et la dysfonction mitochondriale (Wen, Gwathmey et al. 2012). Les résultats de notre étude indiquent que la dysfonction endothéliale liée à l'âge est associée à un stress oxydant vasculaire, incluant une augmentation de la production mitochondriale d'ERO, ainsi qu'une surexpression de ACE et AT1R, deux composants du système angiotensine locale, dans la paroi vasculaire aortique des rats âgés. La diminution du stress oxydant vasculaire en réponse à la consommation chronique de jus de cassis est associée à une diminution d'expression d'ACE et AT1R, suggérant que les anthocyanes peuvent réduire le stress oxydant vasculaire, du moins en partie, par une réduction de l'activation du système angiotensine local. Ce résultat est cohérent avec ceux d'une étude précédente portant sur les artères mésentériques de rats cirrhotiques et qui indiquait que la consommation de jus de cassis était associé à une diminution du stress oxydant vasculaire liée à une réduction de la surexpression de la NADPH oxydase (Rashid, Idris-Khodja et al. 2018).

D'autres études ont également indiqué que la consommation de plusieurs sources riches en polyphénols, telles qu'un extrait de vin rouge, prévient contre le stress oxydant vasculaire et la dysfonction endothéliale dans des modèle d'hypertension induite par l'angiotensine II (Sarr, Chataigneau et al. 2006), chez le rat âgés (Idris Khodja, Chataigneau et al. 2012), ou encore avec

la catéchine dans le modèle de syndrome métabolique de rat obèse Otsuka Long-Evans Tokushima (Ihm, Jang et al. 2012). Il est important de mentionner que l'une des limites de notre approche réside dans le fait qu'elle évalue uniquement la formation des ROS (généralement considérée comme le principal marqueur du stress oxydatif), sans aucune évaluation des défenses antioxydantes. Par conséquent, nous ne pouvons pas déterminer de manière concluante si les interventions ont réellement atténué le stress oxydatif global. Afin de parvenir à une conclusion plus solide, les études futures devraient inclure à la fois la mesure de biomarqueurs de l'oxydation et de la capacité antioxydante.

La consommation chronique de jus de cassis est associée à une amélioration de la santé endothéliale et vasculaire dans l'aorte thoracique

De plus, notre étude indique que le vieillissement est associé à une augmentation de l'expression endothéliale de médiateurs pro-inflammatoire (MCP-1) et pro-fibrotique (TGFβ), de même qu'à une augmentation de l'expression des enzymes pro-remodelage (MMP-2 et -9) et du dépôt de collagene-1 dans l'intima. Plusieurs études ont montré qu'avec l'âge, l'arbre vasculaire subit des modifications structurales et fonctionnelles caractérisées par une dysfonction endothéliale, un épaississement pariétal, une diminution de la déformabilité et une augmentation de la rigidité artérielle. L'augmentation de la rigidité artérielle résulte d'une fibrose et d'un remodelage de la matrice extracellulaire (Ihm, Jang et al. 2012). De surcroit, Fornieri et collaborateurs ont souligné le rôle de la matrice extracellulaire dans le vieillissement vasculaire dans l'aorte de rat (Fornieri, Quaglino et al. 1992). Une autre étude sur des rats SHRSP (spontanément hypertendus et susceptible aux AVC) a montré qu'une forte expression vasculaire de collagène, fibronectine, TGFβ, MCP-1, MMP-2 et MMP-9 était associée à une augmentation de la formation des ERO (Harvey, Montezano et al. 2017). La consommation chronique de jus de cassis est aussi associée à une diminution d'expression pour MCP-1, MMP2, MMP9 et TGFβ dans l'aorte de nos rats âgés. Notre étude suggère que la consommation journalière de jus de cassis peut réduire l'expression de médiateurs pro-inflammatoire comme MCP-1 in vivo, conduisant à la suppression de l'inflammation artérielle locale. Ainsi, les anthocyanes pourraient préserver l'intégrité de la structure de la paroi artérielle et réduire le développement de la fibrose vasculaire liée à l'âge et

les complications cardiovasculaires en découlant. En effet, Wang et collaborateurs indiquent que les polyphénols du raisin jouent un rôle protecteur vis-à-vis de l'induction d'un anévrisme aortique abdominal par l'élastase *via* une réduction de l'inflammation et une diminution des activités des MMP-2 et -9 , ce qui est cohérent avec nos propres résultats (Wang, Wang et al. 2017). D'autres études précédentes ont lié l'effet protecteurs des anthocyanes à leur capacité à atténuer le stress oxydant, qui est impliqué dans des processus comme l'induction de l'inflammation conduisant aux altérations structurales de la paroi vasculaire (Kim, Park et al. 2011, Wang, Wang et al. 2017). Toutefois, il pourrait exister d'autres mécanismes impliqués dans l'effet bénéfiques des anthocyanes qui devront être déterminer dans de futures études.

Extrapolation à la santé humaine et stratégies proposées

Bien que ces résultats aient été obtenus à partir d'un modèle animal, ils sont porteurs d'espoir pour la santé vasculaire humaine. Les expériences chez l'animal utilisent souvent des doses relativement élevées d'anthocyanines (par kg de poids corporel) par rapport aux habitudes alimentaires humaines, ce qui soulève la question de l'extrapolation des doses. Selon la méthode de conversion allométrique standard (Reagan-Shaw et al., 2008), la dose de 120 mg/kg/jour d'ARBJ administrée à des rats âgés (contenant 6,8 g GAE/L) correspond à 1,36 g/jour pour un adulte humain, soit environ 150 g/jour de fruits frais. Cette dose équivalente humaine pourrait dépasser l'apport quotidien moyen en anthocyanines dans les populations dont la consommation de fruits et légumes est faible.

Les données disponibles suggèrent que l'apport quotidien moyen en polyphénols dans les régimes occidentaux varie de 500 à 1 500 mg, ce qui confirme que la dose que nous avons utilisée se situe dans cet intervalle, bien que les anthocyanines ne représentent qu'une petite fraction de cet ensemble (D. Del Rio et al., 2013). Les estimations de l'apport habituel en anthocyanines varient considérablement, allant de quelques milligrammes à des dizaines de milligrammes par jour, selon les habitudes alimentaires (Zamora-Ros et al., 2016). Les grands consommateurs de régimes riches en baies peuvent dépasser 50 mg/jour d'anthocyanines (C. D. Kay & Holub, 2002). Nos données suggèrent que même si les concentrations plasmatiques restent modestes, une consommation quotidienne répétée d'aliments riches en anthocyanines—tels que les baies, les

cassis et d'autres fruits intensément colorés—pourrait permettre une accumulation endothéliale suffisante de ces composés, offrant ainsi des bénéfices vasoprotecteurs significatifs.

D'un point de vue clinique, la supplémentation en extraits ou en jus riches en anthocyanines pourrait servir de thérapie adjuvante chez les personnes à risque de dysfonction endothéliale (par ex., les personnes âgées ou les patients atteints de syndrome métabolique). De futures études cliniques rigoureuses devront évaluer soigneusement les doses équivalentes chez l'humain, intégrer des évaluations alimentaires pour tenir compte de l'apport de base en anthocyanines et mesurer les effets vasculaires via des méthodes non invasives (p. ex. dilatation médiée par le flux, vélocité de l'onde de pouls), ainsi que les biomarqueurs sanguins des métabolites d'anthocyanines.

Conclusion et perspectives

En conclusion, cette étude indique que la consommation chronique de 120 mg/kg/j d'un jus de cassis riche en anthocyanes pendant deux semaines par des rats âgés est associée à une diminution de la pression artérielle systolique de 7 mmHg et une amélioration de la fonction endothéliale due à la fois à une restauration de la composante EDH de la relaxation et à une diminution des réponses contractiles. Les effets vasoprotecteurs des anthocyanes de cassis sont associés à une réduction du stress oxydant vasculaire, de l'activation du système angiotensine local et de l'expression des facteurs pro-inflammatoires, pro-fibrose et pro-remodelage liés à l'âge. L'effet bénéfique des anthocyanes semble être dû à l'accumulation endothéliale dépendante de la dose des anthocyanes *via* les co-transporteurs sodium-glucose SGLT1 et SGLT2. Ces résultats suggèrent que les anthocyanes pourraient constituer une approche complémentaire d'intérêt pour améliorer la santé endothéliale dans les situations pathophysiologique associées à une sénescence et une dysfonction endothéliales prématurées. Comme la senescence endothéliale prématurée est plus prononcée dans les zones artérielles à risque et qu'elle caractérisée par une surexpression des transporteurs SGLT1 et SGLT2, les anthocyanes pourraient permettre un ciblage naturel du fait de l'augmentation de l'assimilation cellulaire des anthocyanes spécifiquement dans ces zones à risques.

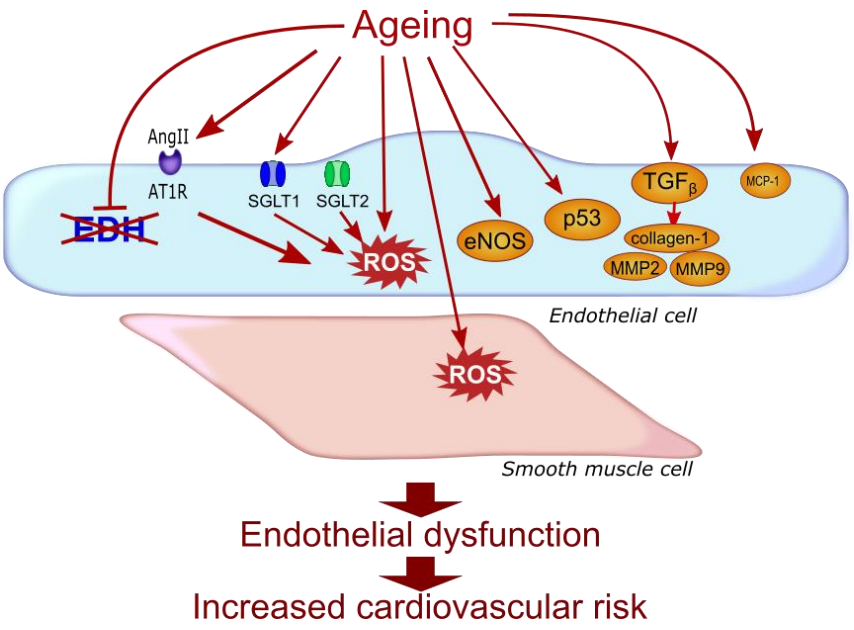


Figure 21 – Effets du vieillissement sur la fonction endothéliale et la santé vasculaire

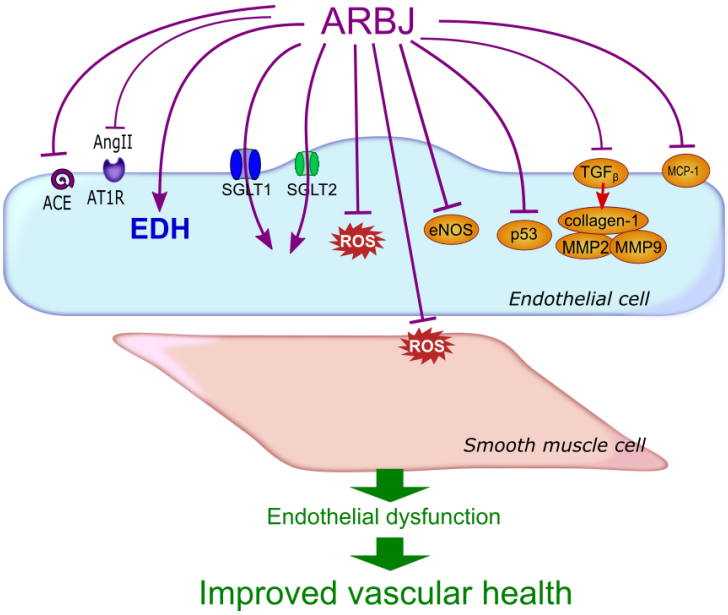


Figure 22 – Rôle des anthocyanes dans l'amélioration de la fonction endothéliale et de la santé vasculaire chez les rats âgés

Cette étude a amenée des nouvelles données d'intérêt concernant le potentiel des anthocyanes dans la prise en charge des maladies cardiovasculaire liées à l'âge. Toutefois, un travail plus poussé est nécessaire pour compléter cette étude.

Nous avons d'abord focalisé notre étude sur la dysfonction endothéliale dans l'artère mésentérique principale et dans l'aorte. Toutefois, bien que ces lits vasculaires présentent des similitudes avec d'autres lits vasculaires, ils ne sont généralement pas impliqués dans le développement des maladies cardiovasculaires liées à l'âge. Ainsi, de futurs travaux devront confirmer nos résultats dans des lits vasculaires plus pertinents comme la (micro)circulation coronaire pour les maladies coronariennes et l'insuffisance cardiaques, les artères cérébrales pour les AVC, ou les vaisseaux fémoraux pour les artériopathies des membres inférieures et les thromboses veineuses profondes.

De plus, nos résultats indiquent que SGLT1 et SGLT2 sont tous deux impliqués dans l'entrée et l'assimilation des anthocyanes dans les cellules endothéliales. Toutefois, nous n'avons pas été en mesure de déterminer l'expression vasculaire de SGLT2 dans les parois aortiques par immunofluorescence. Cette expression doit donc être déterminée à l'aide de techniques complémentaires comme le Western blot ou la RT-PCR.

Bien que nous ayons montré que la consommation chronique de jus de cassis riche en anthocyanes soit associée à une accumulation dépendante de la dose dans les tissues vasculaires, nous n'avons pour le moment aucune information concernant la nature des molécules actives et de leurs métabolites cellulaires. De précédentes études in vitro ont indiqué que parmi les 4 anthocyanes majeures du cassis, seuls les dérivés gluco-conjugués de la cyanidine et de la delphinidine peuvent passer par les SGLT pour aller activer la voie de signalisation redox-sensible Src/PI3-kinase/Akt conduisant à l'activation de la eNOS (Lee, Khemais-Benkhiat et al. 2017). Ceci suggère que les métabolites de la cyanidine et/ou de la delphinidine pourraient être les molécules qui s'accumulent dans les cellules endothéliales après la consommation chronique du jus de cassis, mais une caractérisation et une identification formelle reste à faire.

Enfin, de futurs travaux devront évaluer la pertinence de nos résultats vis-à-vis de la dysfonction endothéliale et des altérations vasculaire liées à l'âge chez l'homme. En effet, cette étude a été

faite chez le rat Wistar dans des conditions d'élevage très contrôlées, comprenant notamment une nourriture standardisée dépourvue de flavonoïdes. Les futurs travaux devront donc évaluer le potentiel des anthocyanes à améliorer la dysfonction endothéliale liée à l'âge chez des sujets humains allant d'un âge moyen à un âge avancé et qui ont été exposé tout au long de leurs vies à une prise alimentaire d'anthocyanes.

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Université de Strasbourg

Ahmed Bey CHAKER

Potential of anthocyanins to prevent age-related endothelial senescence and dysfunction

Résumé

Ces travaux évaluent la capacité des anthocyanes de cassis (ARB), des molécules induisant la fonction endothéliale et la formation de NO, à améliorer la dysfonction endothéliale liée à l'âge chez le rat. Le vieillissement est associé à une augmentation de pression artérielle, une dysfonction endothéliale, l'activation du système angiotensine, une augmentation du stress oxydant vasculaire et de l'expression endothéliale de facteurs pro-athérogène. La prise chronique d'ARB diminue la pression artérielle, améliore la fonction endothéliale, et réduit le stress oxydant vasculaire et l'expression des protéines pro-athérogènes. La prise d'ARB est associée à une accumulation d'anthocyanes dans la paroi vasculaire and une augmentation de l'assimilation endothéliale via les SGLTs. En conclusion, la prise chronique d'ARB améliore la dysfonction endothéliale liée à l'âge chez les rats âgés, probablement en réduisant l'activation du système angiotensine local et la senescence endothéliale.

Mots-clés : Vieillissement, Dysfonction endothéliale, Endothélium, Anthocyanes, Cassis, Stress Oxydant, Système angiotensine local, SGLTs, Senescence endothéliale

Résumé en anglais

Anthocyanin-rich blackcurrant juice (ARB) has been shown to induce sustained endothelial NO formation and subsequent vasorelaxation. This study examined if the intake of ARB improves an established ageing-related endothelial dysfunction. Ageing was associated with an increased systolic blood pressure, an endothelial dysfunction, increased vascular oxidative stress, activation of the angiotensin system and endothelial expression of pro-atherogenic factors. Chronic intake of ARB decreased the systolic blood pressure, improved the endothelial function, and reduced vascular oxidative stress and the expression of protein markers. ARB intake was associated with anthocyanin accumulation in vascular tissue and with increased uptake of anthocyanin in the endothelium via SGLTs. In conclusion, chronic intake of ARB improved the ageing-related endothelial dysfunction in old rats, most likely by preventing activation of the local angiotensin system and the endothelial senescence.

Keywords: Ageing, Endothelial dysfunction, Endothelium, Anthocyanins, Blackcurrant, Oxidative stress, Local angiotensin system, SGLTs, Endothelial senescence