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Potential of omega-3 EPA/DHA 6/1 to ameliorate ageing-related endothelial dysfunction

Muhammad Akmal Farooq

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UNIVERSITÉ DE STRASBOURG



ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ

Regenerative Nanomedicine – INSERM UMR 1260

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Pharmacocinétique

**Potential of omega-3 EPA:DHA 6:1 to
ameliorate ageing-related endothelial
dysfunction**

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
ABC	ATP binding cassette transporter
ACE	Angiotensin converting enzyme
ACE2	Angiotensin converting enzyme 2
ACh	Acetylcholine
ADMA	Asymmetric dimethyl arginine
AF	Arterial fibrillation
AGE	Advance Glycation End-products
Akt	Protein kinase B
ALA	Alpha linoleic acid
AMPK	Adenosine monophosphate activated protein kinase
Ang I	Angiotensin I
AP-1	Activator protein-1
Apo CIII	Apo-lipoprotein C3
AT1R	Angiotensin type-1 receptor
BH₂	Dihydrobiopterin
BH₄	Tetrahydrobiopterin
BK	Bradykinin
BK_{Ca}	Large conductance Ca ²⁺ -activated potassium channels
BP	Blood pressure
Ca²⁺	Calcium
CaM	Calmodulin
CaM Kinase II	Calcium/calmodulin-dependent protein kinase II
Ca_v	Voltage-gated calcium channels
cGMP	Cyclic guanosine monophosphate
CHD	Coronary heart disease
CO	Carbon monoxide

COX	Cyclo-oxygenase
COX-1	Cyclo-oxygenase-1
COX-2	Cyclo-oxygenase-2
CVD	Cardiovascular disease
Cyp-450	Cytochrome p-450
DART	Diet and reinfarction trial
DHA	Docosahexaenoic acid
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
DP	D-prostanoid receptor
ECs	Endothelial cells
EDCF	Endothelium-derived contractile factors
EDH	Endothelium-dependent hyperpolarization
EDRF	Endothelium-derived relaxing factors
EET	Epoxy-eicosaicosatrienoic acid
EETs	Epoxy-eicosatrienoic acid derivatives
EGRF	Epidermal growth factor
eNOS	Endothelial nitric oxide synthase
EP	E-prostanoid receptor
EPA	Eicosapentaenoic acid
E_{pac}	Cyclic AMP-activated exchange protein
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ET-1	Endothelin-1
ET-2	Endothelin-2
ET-3	Endothelin-3
ET_A	Endothelin-1 receptor A
ET_B	Endothelin-1 receptor B
FAD	Flavin-adenine dinucleotide
FMD	Flavin mononucleotide
FP	F-prostanoid receptor

GPR120	G-protein coupled receptor 120
GTP	Guanosine trisphosphate
H	Histamine
H₂O₂	Hydrogen peroxide
H₂S	Dihydrogen sulfide
HDL	High density lipoprotein
HETE	Hydroxy eicosatetraenoic acid
HSP90	Heat shock protein 90
ICAM	Intercellular adhesion molecule
IDL	Intermediate density lipoprotein
i-κB	Inhibitor of κB
IK_{ca}	Intermediate conductance Calcium channel
IL-12	Interleukin-12
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IP	I-prostanoid receptor
IP₃	Inositol trisphosphate
JAKs	Janus kinases
JELIS	Japan eicosapentaenoic acid lipid intervention study
K_{ca}	Calcium activated potassium channel
K_{ir}	Inwardly rectifying potassium channel
K_v	Voltage-gated potassium channel
LA	Linoleic acid
LCFA	Long chain fatty acids
LDL	Low-density lipoproteins
L-NA	L-nitroarginine
LNAME	L-nitroarginine methyl ester
LOX	Lipo-oxygenase
LOX-5	Lipo-oxygenase 5

LPL	Lipoprotein lipase
LTB₄	Leukotriene B ₄
LTB₅	Leukotriene B ₅
LVH	Left ventricular hypertrophy
MCP-1	Monocyte chemoattractant protein-1
MGJs	Myoendothelial gap junctions
MUFA	Monounsaturated fatty acid
Na⁺	Sodium
Na⁺/Ca²⁺	Sodium/calcium pump
NF-κB	Nuclear factor kappa-B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
O₂^{·-}	Superoxide anion
OH[·]	Hydroxyl radical
Omega-3 PUFA	Omega-3 polyunsaturated fatty acids
OONO⁻	Peroxynitrite anion
p16	Cyclin-dependent kinase inhibitor 2A
p21	Cyclin-dependent kinase inhibitor-1
p22^{phox}	Membranal subunit of NADPH oxidase
p47^{phox}	Cytosolic subunit of NADPH oxidase
p53	Tumor protein p53
PGD₂	Prostaglandin D ₂
PGE₂	Prostaglandin E ₂
PGE₃	Prostaglandin E ₃
PGF_{2α}	Prostaglandin F _{2α}
PGH₂	Prostaglandin H ₂
PGI₂	Prostacyclin
PGI₃	Prostaglandin I ₃
PI3K	Phosphatidyl inositol 3 kinase
PKA	Protein kinase A

PKC	Protein kinase C
PKG	Protein kinase G
PLA₂	Phospholipase A ₂
PLC	Phospholipase C
PLD	Phospholipase D
PPAR	Peroxisome proliferator activated receptor
PPARα	Peroxisome proliferator activated receptor- α
PUFA	Polyunsaturated fatty acid
PUFAs	Polyunsaturated fatty acids
RAS	Renin angiotensin system
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
R_yR_s	Ryanodine receptors
SA-β-Gal	Senescence associated β -galactosidase
SC-560	Cyclo-oxygenase-1 inhibitor
SFA	Saturated fatty acid
sGC	Soluble guanylyl cyclase
SHR	Spontaneously hypertensive rats
SK_{ca}	Small conductance Ca ²⁺ activated K ⁺ channels
SMC	Smooth muscle cell
SOC	Store-operated channels
SOD	Superoxide dismutase
SR	Sarcoplasmic reticulum
TF	Tissue factor
TG	Triglycerides
TNF-α	Tissue necrosis factor- α
TP	Thromboxane prostanoid receptor
TXA₂	Thromboxane A ₂
TXA₃	Thromboxane A ₃
TXB₂	Thromboxane B ₂
VCAM	Vascular cell adhesion molecule

VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell
VSMCs	Vascular smooth muscle cells

Abstract / Résumé

Abstract

Cardiovascular diseases are the leading cause of death worldwide, both in developed and developing countries. As the incidence of cardiovascular diseases is increasing with age, the ageing of the population will be associated with an increase in cardiovascular diseases in the future, accounting, at least in part, for the World Health Organization prediction of cardiovascular diseases remaining the leading cause of death worldwide.

The endothelium, the monocellular layer lining all blood vessels, is a pivotal organ in maintenance of the vascular homeostasis. Indeed, endothelial cells contribute to the fine regulation of the vascular tone and to the protection against thrombosis and vascular remodeling, mainly through the release of potent vasoprotective factors such as nitric oxide (NO), the endothelium-dependent hyperpolarization (EDH), or the prostacyclin PGI₂.

Many preclinical and clinical studies have shown that development of cardiovascular diseases and risk factors, such as ageing, are associated early with 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.

Over the last decades, numerous epidemiological studies have reported that diets could play a role in the development of cardiovascular diseases. For example, a recent interventional study has shown that compared to a western diet rich in saturated fat, a Mediterranean diet rich in unsaturated fat from olive oil or nuts could decrease the incidence of a primary cardiovascular adverse event (myocardial infarction or stroke) by 30 % in subjects with high cardiovascular risk factors. Moreover, several epidemiological studies and clinical trials have shown that dietary intake of fish, fish oil or omega-3 polyunsaturated fatty acids (n-3 PUFAs) could reduce secondary events of coronary heart disease and stroke, and could also decrease hypertension. The dietary consumption of the major omega-3 PUFAs, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has also been related to a reduced risk of cardiovascular disease morbidity/mortality. The omega-3 PUFAs could exert their beneficial effects on the cardiovascular system by modulation of the lipids metabolism and their anti-inflammatory properties due to the decrease in pro-inflammatory cytokine production and the formation of anti-inflammatory metabolites including resolvins, protectins and maresins.

In addition, the omega-3 PUFAs could protect the cardiovascular system, at least in part, by exerting a protective effect on the endothelial function. Indeed, EPA and DHA are able to induce the activation of the endothelial nitric oxide synthase (eNOS), the enzyme responsible of the formation of NO, the most potent endothelium-derived vasoprotective factor.

Our research team previously show that the stimulation of the endothelial function by n-3 PUFAs is dependent on both the purity and the ratio of EPA and DHA. Indeed, we tested several EPA:DHA ratio (from 9:1 to 1:9) and shown that the EPA:DHA 6:1 formulation is a potent stimulator of the endothelial formation of NO, and to a lesser extent, of an increased endothelium-dependent hyperpolarization (EDH) response. The induction of the endothelial formation of NO by omega-3 fatty acids is mediated by redox-sensitive activation of the Src/PI3-kinase/Akt and MAPKs pathways leading to eNOS activation, which is dependent on the ratio and amount of the EPA:DHA in the formulation.

Moreover, our team demonstrated in a subsequent study that the chronic oral intake of the EPA:DHA 6:1 formulation was able to prevent the hypertension and endothelial dysfunction induced by angiotensin II infusion in rats. The beneficial effect of EPA:DHA 6:1 was mediated by an improvement of both the NO- and the EDH-mediated relaxations and a reduction of endothelium-dependent contractile response, most likely by preventing the oxidative stress induced by the up-regulation of the local angiotensin system.

As several clinical studies have shown that omega-3 PUFAs are able to decrease cardiovascular risks in patients with documented first cardiovascular events, we were looking to assess the potency of the EPA:DHA 6:1 formulation to improve the endothelial function in a pathophysiological situation associated with an established endothelial dysfunction.

The aim of the present study was to determine whether a short-term chronic oral intake of the optimized EPA:DHA 6:1 formulation is able to improve the ageing-related endothelial dysfunction in rats, and if so, to determine the underlying mechanisms.

For this study, 20 month-old male Wistar rats received by daily gavage 500 mg/kg BW of either corn oil, EPA:DHA 6:1 formulation or water (control) for 2 weeks. After 2 weeks of gavage, the animals were euthanized and the organ were collected. The main mesenteric artery was used for vascular reactivity studies, and main mesenteric artery and aorta were used for immunofluorescence and fluorescence histochemistry studies on frozen section, and for western blot analysis of protein expression.

The major results of our study show that, compared to young rats (12 weeks-old), ageing was associated with an endothelial dysfunction characterized by a blunted NO-mediated component, an abolished endothelium-dependent hyperpolarization (EDH)-mediated component, and also by the induction of endothelium-dependent contractile responses (EDCF, in the presence of NO and EDH inhibitors) sensitive to indomethacin (a cyclooxygenase inhibitor) in response to acetylcholine. Intake of EPA:DHA 6:1 for 2 weeks was able to improve the NO-mediated relaxations and to decrease the endothelium-dependent contractile responses, but had no effect on the EDH-mediated component. Intake of corn oil for 2 weeks had no effect on the ageing-related endothelial dysfunction.

To better characterize the molecular mechanisms involved in the protective effects of EPA:DHA 6:1 intake, we performed quantitative analysis of protein expression in the mesenteric artery and aorta by immunofluorescence. Firstly, we studied the expression of eNOS (NO component of relaxation), and cyclooxygenases (COXs, involved in EDCFs). Compared to the young rats, ageing is significantly associated with an increased expression of eNOS, COX-2, the inducible isoform of COXs, while down-regulating the expression of COX-1, the constitutive isoform of COXs in the arterial wall of the mesenteric artery. The

intake of EPA:DHA 6:1 normalized the expression levels of eNOS, COX-1, and COX-2, whereas corn oil had no significant effect.

As endothelial dysfunction is associated with a vascular oxidative stress, we measured the level of oxidative stress in the vascular wall of the mesenteric artery and aorta using the redox-sensitive fluorescent probe dihydroethidium (DHE). Compared to young rats, old rats show significant increases of DHE fluorescence throughout the vascular wall, which was significantly prevented by the EPA:DHA 6:1 intake. Moreover, the age-related increased vascular oxidative stress was due, at least in part, to the activities of uncoupled eNOS, COX-1 and COX-2, NADPH oxidase, and mitochondrial respiratory chain in the vascular wall of the aorta.

As the increased vascular oxidative stress has been attributed, at least in part, NADPH oxidase activity, we determined the expression level of NADPH oxidase subunits p22^{phox} and p47^{phox}. Compared to young control rats, the aortic wall of aged rats exhibit a significantly increased expression level of p22^{phox} and p47^{phox}, and the EPA:DHA 6:1 treatment normalized the expression of both NADPH oxidase sub-units.

Finally, we assess the level of premature endothelial senescence in the aortic wall. The expression levels of the senescence markers p53, p21 and p16 were significantly increased in aged rats compared to young rats. The intake of EPA:DHA 6:1 for 2 weeks normalized the expression levels of senescence markers.

Altogether, the present findings indicate that intake of EPA:DHA 6:1 significantly improved the ageing-related endothelial dysfunction in rats. The beneficial effect involves an improvement of both the NO- and the EDH-mediated relaxations as well as a reduction of endothelium-dependent contractile response most likely by preventing vascular oxidative stress and premature endothelial senescence.

Résumé

Les maladies cardiovasculaires représentent la première cause de mortalité dans le monde, que cela soit dans les pays développés ou ceux en cours de développement. L'incidence des maladies cardiovasculaires augmentant avec l'âge, le vieillissement des populations est associée à une augmentation des maladies cardiovasculaires dans futur participant, du moins partiellement, à expliquer les prédictions de l'Organisation Mondiale de la Santé montrant que les maladies cardiovasculaires resteront la première cause de mortalité dans la monde.

L'endothélium, la monocouche cellulaire tapissant les vaisseaux sanguins, est un organe central dans la régulation de l'homéostasie vasculaire. En effet, les cellules endothéliales ont un rôle clé dans le maintien du tonus vasculaire et dans la protection contre la thrombose et le remodelage vasculaire, principalement grâce à la formation et libération de puissants facteurs vasoprotecteurs tels que le monoxyde d'azote (NO), l'hyperpolarisation dépendante de l'endothélium (EDH), ou la prostacycline PGI₂.

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 plaquettaire, 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 NADPH 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.

Au cours des dernières décennies, de nombreuses études épidémiologiques ont montré que l'alimentation pouvait jouer un rôle dans le développement des maladies cardiovasculaires. Ainsi, une récente étude interventionnelle a démontré que, par rapport au régime occidental riche en graisses saturées, un régime Méditerranéen riche en graisse non-saturées issu de l'huile d'olive ou des fruits secs à coques permettait de réduire de 30% l'incidence d'un premier évènement cardiovasculaire aigu (infarctus du myocarde ou accident vasculaire cérébral ischémique) chez des sujets à haut risque cardiovasculaire. De plus, plusieurs études épidémiologiques ou d'intervention ont montré que la consommation alimentaire de poisson, d'huile de poisson ou d'acides gras polyinsaturés omega-3 (n-3 PUFAs) pouvait diminuer les risques de récurrence de la maladie coronarienne ou d'accident vasculaire cérébraux, et diminuait la pression artérielle chez les hypertendus. La consommation des acides gras omega-3 PUFAs majeurs, à savoir l'acide eicosapentaénoïque (EPA) et l'acide docosahexaénoïque (DHA), a aussi été associée à une réduction de la morbi-mortalité cardiovasculaire. Les n-3 PUFAs pourraient avoir un effet bénéfique sur le système cardiovasculaire en modulant le métabolisme lipidique et du fait de leurs propriétés anti-inflammatoires passant par une diminution de la production de cytokines pro-inflammatoires et une formation de métabolites anti-inflammatoires dont les résolvines, les marésines et les protectines.

De plus, les omega-3 PUFAs pourraient protéger le système cardiovasculaire, du moins en partie, en protégeant la fonction endothéliale. En effet, l'EPA et le DHA sont capables d'induire l'activation de la NO synthase endothéliale (eNOS), l'enzyme produisant le NO, le plus puissant des facteurs vasoprotecteurs issus de l'endothélium.

Notre équipe de recherche a récemment démontré que la stimulation de la fonction endothéliale par les omega-3 PUFAs dépend à la fois du ratio et du degré de pureté de la formulation en EPA et DHA. En effet, nous avons évalué la capacité de plusieurs formulations EPA:DHA (allant de 1:9 à 9:1) et nous avons montré que la formulation EPA:DHA 6:1 est un puissant activateur de la formation endothéliale de NO, et de façon moindre de l'augmentation de la réponse d'hyperpolarisation dépendante de l'endothélium (EDH). L'induction par les omega-3 PUFAs de la formation endothéliale de NO due à l'activation de

la eNOS via les voies de signalisation redox-sensibles Src/PI3-kinase/Akt et MAPKs, est dépendante du ratio et de la quantité de EPA et DHA dans la formulation.

De plus, notre équipe a récemment montré dans une étude suivante que la consommation orale de la formulation EPA:DHA 6:1 prévenait la survenue de l'augmentation de pression artérielle et de la dysfonction endothéliale induite par l'infusion d'Angiotensine II chez des rats. L'effet bénéfique de l'EPA:DHA 6:1 passait par une amélioration des relaxations dépendante du NO et de l'EDH et par une diminution des réponses contractiles dépendantes de l'endothélium, probablement dues à la prévention du stress oxydant vasculaire induit par l'activation du système angiotensine local.

Du fait que les études cliniques montrent que les oméga-3 PUFAs sont capables de diminuer les risques cardiovasculaires chez des patients avec un premier événement cardiovasculaire documenté, nous avons cherché à évaluer le potentiel de la formulation EPA:DHA 6:1 à améliorer la fonction endothéliale dans une situation physiopathologique associée à une dysfonction endothéliale installée.

L'objectif de la présente étude est de déterminer si une consommation chronique à court terme de la formulation optimisée EPA:DHA 6:1 est capable d'améliorer la dysfonction endothéliale liée à l'âge chez le rat, et le cas échéant, de déterminer les mécanismes sous-jacents.

Pour cette étude, des rats Wistar mâles de 20 mois ont reçu quotidiennement pendant 2 semaines par gavage 500 mg/kg soit d'huile de maïs, soit de la formulation EPA:DHA 6:1, soit d'eau (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 dysfonction endothéliale caractérisée à la fois par une diminution de la composante NO de relaxation en réponse à l'acétylcholine, une abolition de la composante EDH de la relaxation, et aussi par une augmentation de réponses contractiles dépendantes de l'endothélium (EDCFs) sensible à l'indométacine (un inhibiteur des cyclooxygénases) en réponse à l'acétylcholine. La

consommation chronique de EPA:DHA 6:1 pendant 2 semaines améliore significativement les relaxations dépendantes du NO et les EDCFs, mais n'a pas d'effet sur la composante EDH de la relaxation. La consommation d'huile de maïs pendant 2 semaines n'a pas eu d'effet sur la dysfonction endothéliale liée à l'âge.

Afin de mieux caractériser les mécanismes moléculaires impliqués dans l'effet protecteur de la formulation EPA:DHA 6:1, des analyses quantitatives des niveaux d'expression de protéines ont été effectués dans l'artère mésentérique et l'aorte par immunofluorescence sur coupes congelées. Dans un premier temps, nous avons étudié l'expression de la eNOS (composante NO de la relaxation) et des cyclooxygénases (COXs, impliquées dans les réponse EDCFs).

Par rapport aux animaux jeunes, le vieillissement est associé à une augmentation significative de l'expression de la eNOS et de COX-2, la forme inductible des COXs, et une diminution significative de l'expression de COX-1 dans la paroi vasculaire de l'artère mésentérique. La prise chronique de EPA:DHA 6:1 normalise l'expression de la eNOS, de COX-1 et de COX-2, alors que la prise d'huile de maïs n'a pas eu d'effet significatif.

Comme la dysfonction endothéliale est associée à un stress oxydant vasculaire, nous avons évalué le niveau de stress oxydant la paroi vasculaire de l'artère mésentérique et de l'aorte à l'aide de la sonde fluorescente redox-sensible dihydroéthidium (DHE). Par rapport aux rats jeunes, le vieillissement est associé à une augmentation significative de la fluorescence dans l'ensemble de la paroi vasculaire, et cette augmentation est significativement prévenue par la prise chronique de la formulation EPA:DHA 6:1 mais pas par l'huile de maïs. De plus, l'augmentation du stress oxydant vasculaire liée à l'âge dans la paroi aortique est due, au moins partiellement, à l'activité de la eNOS découplée, de la COX-1 et de la COX-2, de la NADPH oxydase et de la chaîne respiratoire mitochondriale.

Du fait que l'augmentation de stress oxydant est due, du moins partiellement, à une surexpression de la NADPH oxydase, les niveaux d'expression des sous-unités p22^{phox} et p47^{phox} de la NADPH oxydase ont été déterminés. En comparaison des rats jeunes, les rats âgés montrent une augmentation significative des niveaux d'expression des sous-unités p22^{phox} et p47^{phox} dans la paroi de l'aorte, et le traitement avec la formulation EPA:DHA 6:1 normalise l'expression des sous-unités de la NADPH oxydase.

De plus, le niveau de sénescence des cellules endothéliales a été évalué dans la paroi aortique. Le niveau d'expression des marqueurs de sénescence p53, p16 et p21 est significativement augmenté dans l'endothélium des rats âgés, comparativement aux rats jeunes. La prise orale

de la formulation EPA:DHA 6:1 pendant 2 semaines a permis de normaliser le niveau d'expression de ces markers de sénescence.

L'ensemble des résultats obtenus indique que la prise chronique de la formulation optimisée EPA:DHA 6:1 pendant 2 semaines améliore significativement la dysfonction endothéliale liée à l'âge chez le rat. Les effets bénéfiques de la consommation chronique de la formulation EPA:DHA 6:1 impliquent une amélioration des composantes de relaxations NO, une diminution des réponses contractiles dépendantes de l'endothélium, probablement via la diminution du stress oxydant vasculaire et de la sénescence endothéliale.

Scientific production

Publications:

The omega-3 EPA:DHA 6:1 formulation improves ageing-related blunted endothelium-dependent relaxations and increased contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases. **M.A. Farooq**, L. Amoura, S. Gaertner, Z.R. Niazi, S. Park, A.W. Qureshi, M.H Oak, F. Toti, V.B. Schini-Kerth, C. Auger. Under preparation.

EPA:DHA 6:1 prevents age-related endothelial dysfunction linked to cyclooxygenase pathway in rat femoral vessels: a way to prevent venous endothelial dysfunction mediated thrombosis? S. Gaertner, **M.A. Farooq**, B. Pollet, J. Graaf, S. Khemais-Benkhiat, S. Schrevens, Z.R. Niazi, A.W. Qureshi, S. Park, F. Toti, D. Stephan, N. Boehm, C. Auger, V. B. Schini-Kerth. Under preparation.

Activation of thromboxane prostanoid receptors mediates age-related endothelial dysfunction in the rat femoral vein: a potential mechanism increasing the risk of venous thromboembolism? S. Gaertner, C. Auger, **M.A. Farooq**, B. Pollet, J. Graaf, S. Khemais-Benkhiat, L. Amoura, S. Park, F. Toti, D. Stephan, N. Boehm, V. B. Schini-Kerth. Under preparation.

Femoral vessels premature aging and venous endothelial dysfunction in young ZSF-1 rats: key role of cyclooxygenases and potential risk for venous thrombosis induced by cumulative cardiovascular risk factors for atherosclerosis. S. Gaertner, **M. A. Farooq**, B. Pollet, J. Graaf, S. Khemais-Benkhiat, L. Amoura, S. Park, F. Toti, C. Auger, D. Stephan, N. Boehm, V. B. Schini-Kerth. Under preparation.

Empagliflozin, a sodium-glucose cotransporter inhibitor, improved heart remodeling and mesenteric artery endothelial function in the metabolic syndrome with HFpEF ZSF1 rat: role of cyclooxygenases. S. Park, **M.A. Farooq**, S. Gaertner, C. Brucker, A.W. Qureshi, H. Lee, D. Benrahla, B. Pollet, D. Stephan, P. Ohlmann, E. Mayoux, C. Auger, O. Morel, V.B. Schini-Kerth. Under preparation.

Treatment of rats with the omega fatty acid 3 formulation EPA:DHA 6:1 decreases the leukocyte microparticles-induced endothelial pro-inflammatory responses and senescence. A. Qureshi, R. Altamimy, A. El Habhab, L. Amoura, M. Kassem, S. Khemais, **M. Farooq**, H. Hasan, P. Sin-Hee, F. El-Ghazouani, C. Auger, L. Kessler, V. Schini-Kerth, F. Toti. Under preparation.

Thrombin promotes premature atrial endothelial cell senescence leading to the induction of pro-infiltrative and pro-fibrotic responses: Role of the local angiotensin II/AT1 receptor system. H. Hasan, S.H. Park, E. Belcastro, M. Abbas, A.W. Qureshi, **M.A. Farooq**, P. Ohlmann, F. Toti, C. Auger, V. Schini-Kerth, O. Morel, L. Jesel. Under preparation.

Oral communications:

The omega-3 EPA:DHA 6:1 formulation improves ageing-related blunted endothelium-dependent relaxations and increased contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases. **M.A. Farooq**, L. Amoura, S. Gaertner, Z.R. Niazi, S. Park, A.W. Qureshi, M.H Oak, F. Toti, V.B. Schini-Kerth, C. Auger. NutRedOx Cost action, 2017, Strasbourg, France

The omega-3 EPA:DHA 6:1 formulation improves ageing-related blunted endothelium-dependent relaxations and increased contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases. **M.A. Farooq**, L. Amoura, S. Gaertner, Z.R. Niazi, S.

Park, A.W. Qureshi, M.H Oak, F. Toti, V.B. Schini-Kerth, C. Auger. ICMAN/IUPHAR Joint meeting on natural products, 2017, Aberdeen, UK

Communications affichées :

Printemps de Cardiologie (ESC) 2017, Nantes, France, poster commenté

Journee Campus d'Illkirch (JCI) 2017, Illkirch, France, poster commenté

European Symposium on Vascular Biomaterials (ESVB) 2017, Strasbourg, France

ED days 2017, Strasbourg, France

DS days 2018, Strasbourg, France

The physiology of the endothelium

1 -THE PHYSIOLOGY OF THE ENDOTHELIUM

1.1 General anatomy of blood vessels

The circulatory system comprises the heart and all the blood vessels including arteries, veins, arterioles, capillaries and venules. Generally, the structure of blood vessels can be characterized into three distinct layers that are respectively the intima, media and adventitia (Figure 1). The intima, also called *tunica interna*, is the inner most layer of a blood vessel and is characterized by the presence of the endothelium supported by the presence of connective tissue and the internal elastic lamina. The media, also called *tunica media*, is a bulk of smooth muscle cells (SMC) with layers of elastic fibers that increase in thickness with the size of the vessel. The adventitia, also called *tunica externa* or *tunica adventitia*, is the external protective layer of the vessel containing elastin, collagen, fibroblasts, macrophages, nerve endings, *vasa vasorum* and protective fibers (Mulvany and Aalkjaer 1990).

The thickness and structure of each layer differs with the size and location of blood vessels. Large arteries contain a higher number of elastic fibers in the media while muscular arteries contain a higher number of smooth muscle cells. The capillaries contains only the endothelial layer and a basal membrane with connective tissue (Pais, Meiselman et al. 2010).

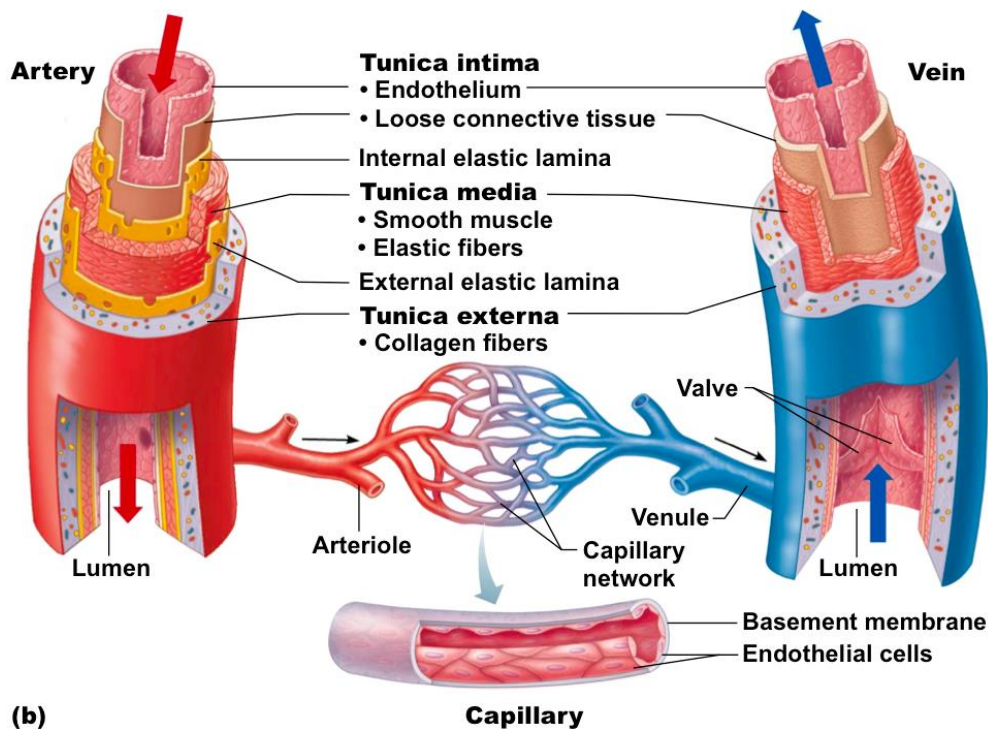


Figure 1: Structure of blood vessels (digikalla.info)

1.2 The role of the endothelium in the regulation of the vascular tone

Endothelium is a monolayer of endothelial cells (ECs) which lines the entire luminal surface of the circulatory system and acts as a selective barrier between blood and the surrounding tissues. The endothelium plays an important role in the regulation of blood flow and homeostasis by releasing bioactive molecules including vasoconstricting (angiotensin II, reactive oxygen species, endothelin, vasoconstrictor prostanoids) and vasodilating (nitric oxide, prostacyclin, endothelium derived hyperpolarization) factors in response to various neurotransmitters, hormones, vasoactive compounds and mechanical stimuli (Sandoo, van Zanten et al. 2010). Endothelium also controls vascular smooth muscle cells (VSMC) proliferation and exchange of molecules between plasma and the interstitial fluid, playing a key role in pro- and anticoagulant mechanisms (Galley and Webster 2004). Lack of endothelial regulation of the vascular homeostasis characterizes the endothelial dysfunction which results in a favorable environment for the development of vascular complications ultimately leading to cardiovascular diseases (CVDs) (Lerman and Zeiher 2005).

1.2.1 Endothelium-derived relaxing factors (EDRF)

1.2.1.1 Nitric Oxide (NO)

In 1980, Furchgott and Zawadzki discovered that in response to acetylcholine (ACh) the endothelium releases a substance responsible for smooth muscle relaxation in the vascular wall (Furchgott and Zawadzki 1980). This endothelium-derived relaxing factor (EDRF) was later identified as nitric oxide (NO), the first identified gaseous signaling molecule. NO not only has an important role in the regulation of vascular tone but it also plays a key role in vascular health through its vaso-protective mechanisms like the inhibition of leukocyte adhesion, platelet aggregation and the prevention of the expression of numerous pro-inflammatory and pro-thrombotic mediators such as monocyte chemoattractant protein (MCP-1), tissue factor (TF) and adhesion molecules. Thus, NO maintains the fluidity of blood by preventing platelet aggregation and monocyte adhesion (Gewaltig and Kojda 2002) (Figure 2).

NO is generated from L-Arginine by the action of NO synthase (NOS). NOS according to its location or type of function can be divided into three isoforms, namely the neuronal

NOS (nNOS, NOS I or bNOS) in the nervous tissue, the cytokine-inducible NOS (iNOS or NOS II) and the endothelial NOS (eNOS or NOS III). The eNOS and nNOS isoforms are constitutively expressed in their respective tissues, while iNOS is generally not expressed in unstimulated cells. The eNOS isoform is responsible for the major portion of NO produced in the vascular system in physiological situations. For the formation of NO from arginine, eNOS forms homodimers that need to bind an essential cofactor, the tetrahydrobiopterin (BH₄) (Forstermann and Sessa 2012). A reduced bioavailability of the co-factor BH₄, mainly through its oxidation to BH₂, as well as a lack of the substrate L-arginine, could result in the “uncoupling” of NOS leading to the formation of superoxide anion and hydrogen peroxide (Fleming 2010). The NOS are multi-domain enzymes containing a C-terminal reductase domain and N-terminal oxygenase domain. The oxygenase domain contains the binding sites for haem, BH₄ and L-arginine whereas the reductase domain contains the binding sites for NADPH, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and CaM. Binding of CaM to its binding site facilitates the transfer of electrons from NADPH at the reductase domain towards haem at the oxygenase domain via FAD and FMN, enabling the haem to bind with O₂ and cause its activation via reduction, which then oxidizes L-arginine to L-citrulline and NO (Fleming 2010, Forstermann and Sessa 2012).

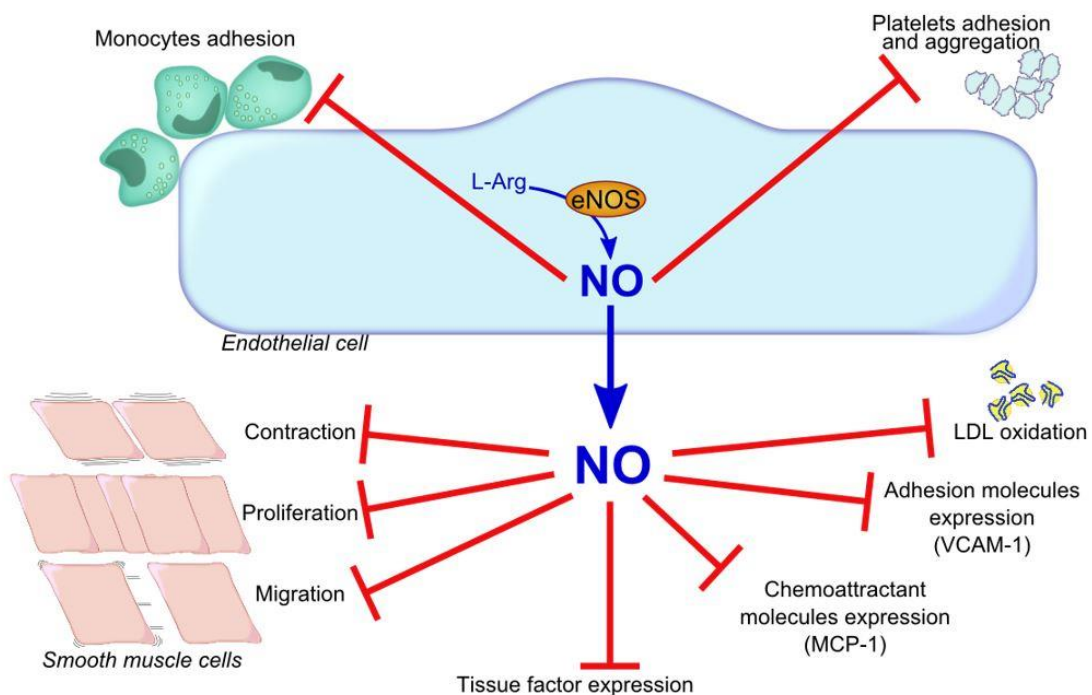


Figure 2: Vaso-protective effects of NO (VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; LDL, low density lipoproteins)

During pre-activation phase eNOS is localized in the caveolae (small invaginations of cell membrane) bound to caveolin-1 protein. The dissociation of eNOS from the caveolin-1 delocalize eNOS to the cytosol where it is activated by cofactors, phosphorylation by post-translational enzymes and association with heat shock protein 90 (Dessy, Feron et al. 2010). Under calcium (Ca^{2+})-dependent activation, in response to increases in intracellular Ca^{2+} , calmodulin detaches eNOS from caveolin-1 protein rendering the enzyme active. Ca^{2+} mobilizing agonists such as acetylcholine (ACh), bradykinin (BK) and histamine (H) activate the eNOS in the same manner. (Vanhoutte, Zhao et al. 2016). The other pathway of eNOS activation also called Ca^{2+} -independent pathway is through phosphorylation of eNOS by the action of different kinases. Indeed, cyclic AMP-dependent protein kinase A (PKA), protein kinase B (Akt), AMP-activated protein kinase A (AMPK), protein kinase G (PKG), Ca^{2+} /calmodulin-dependent protein kinase II (CaM kinase II) cause activation of eNOS by phosphorylation of the Ser1177 residue. However, the phosphorylation on the different residue can have opposing effects as phosphorylation of Ser1177, Ser635 and Ser617 leads to eNOS activation whereas phosphorylation of Thr495 and Ser116 have an inhibitory effect (Bauer, Fulton et al. 2003, Fleming 2010) (Figure 3). Mechanical forces (shear stress), vascular endothelial growth factor (VEGF) and hormones (estrogen, insulin) cause activation of eNOS by phosphorylation via PI3K/Akt pathway (Dudzinski and Michel 2007, Forstermann and Sessa 2012).

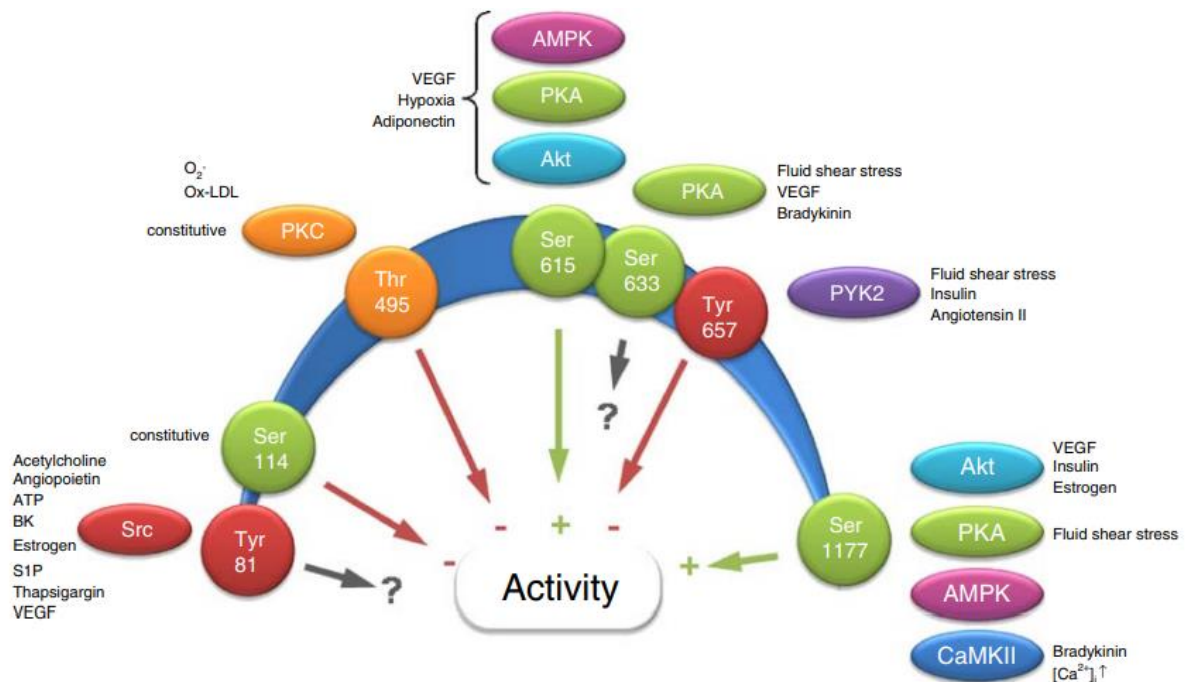


Figure 3: Activation and inhibitory sites of eNOS (Numbers refer to human sequence) (Fleming 2010)

After its formation in the endothelium, NO could diffuse freely into the adjacent SMC's where it activates the soluble guanylyl cyclase (sGC) catalyzing the conversion of 5'-guanylyl triphosphate (GTP) to cyclic 3',5'-guanylyl monophosphate (cGMP) (Figure 4). The resulting cGMP activates the protein kinase G (PKG) which decreases free cytosolic Ca^{2+} by inhibiting inositol trisphosphate (IP_3)-activated release from sarcoplasmic reticulum (SR) and its influx from extracellular spaces through activation of large conductance calcium-activated potassium channels (Furchgott and Vanhoutte 1989). PKG also facilitates the uptake of Ca^{2+} into SR through phosphorylation of phospholamban (a protein in the membrane of SR) which in turn activates SR calcium-ATPase (Gao 2010). This depletion of cytosolic Ca^{2+} in SR decreases the activity of calcium-dependent myosin light chain kinase resulting in interruption of contractile process (Zhao, Vanhoutte et al. 2015).

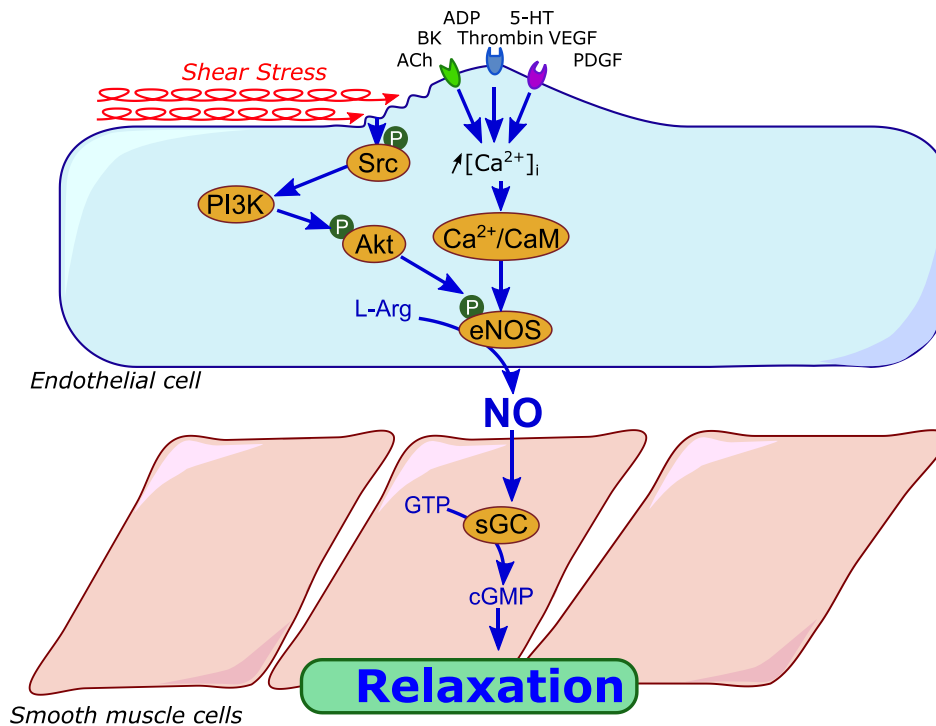


Figure 4: Nitric oxide-mediated vasorelaxation pathway (ACh, acetylcholine; BK, bradykinin; ADP, adenosine diphosphate; 5-HT, serotonin; PDGF, platelet derived growth factor; VEGF, vascular endothelial growth factor; Src, sarcoma-family kinases; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; L-Arg, L-arginin; Ca²⁺/CaM, calcium calmodulin; eNOS, endothelial nitric oxide synthase; NO, Nitric oxide; sGC, soluble guanylyl cyclase; GTP, guanosine 5-trisphosphate; cGMP, cyclic guanosine 3-5 monophosphate)

1.2.1.2 Prostacyclin (PGI₂)

Prostacyclin is regarded as a potent vasodilator and inhibitor of platelet aggregation playing a vasoprotective role (Coleman, Smith et al. 1994). Prostacyclin is a member of prostanoids which are metabolites of arachidonic acid (AA). Arachidonic acid is produced from the hydrolysis of membrane phospholipids catalyzed by phospholipase A₂ (Funk 2001). Biosynthesis of prostacyclin in endothelial cells involve conversion of AA to endoperoxides by cyclooxygenases (COXs), which are subsequently converted to prostacyclin by the enzyme prostacyclin synthase (Tang and Vanhoutte 2009).

After its production in the endothelial cell, PGI₂ is released via the ATP-binding cassette transporters (ABC) in the extracellular space between ECs and SMCs. PGI₂ produces

its vasodilatory and anti-aggregatory effect by acting on the IP₁ receptors which are membrane G-protein coupled receptors. IP₁ activates the enzyme adenylyl cyclase converting ATP to cAMP, which subsequently activates protein kinase A (PKA). PKA reduces availability of free Ca²⁺ by inhibiting its release and promoting its uptake by the sarcoplasmic reticulum (SR) in the SMCs favoring vasorelaxation (Mitchell, Ahmetaj-Shala et al. 2014). cAMP also activates an exchange protein (Epac) which, together with PKA, inhibits the proliferation of SMCs (Hewer, Sala-Newby et al. 2011). Moreover, PGI₂ also facilitates the release of NO from ECs hence promoting the vasodilatory action of NO (Shimokawa, Flavahan et al. 1988).

1.2.1.3 *Endothelium-dependent hyperpolarization (EDH)*

In addition to NO and prostacyclin (PGI₂), EDH is an important contributor for the endothelium-dependent vasorelaxations. EDH induces vasorelaxation which is resistant to the inhibitors L-nitroarginine methyl ester (LNAME) and indomethacin (inhibitors of eNOS and cyclooxygenases, respectively) (Takaki, Morikawa et al. 2008). EDH is not a single agent, but rather it's a group of diffusible factors (K⁺ ions, H₂O₂, H₂S, CO, ROS, different peptides and derivatives of COX, LOX and cytochrome p-450 pathway) influencing the action of different K⁺ channels causing hyperpolarization of SMCs (Figure 5) (El Assar, Angulo et al. 2012). Chemical nature and the potency of EDH depends on the location and size of different arteries mainly influencing the resistant arteries and smaller vessels (Ozkor and Quyyumi 2011). Indeed, the contribution of EDH to the regulation of the vascular tone increases as the diameter of the artery decreases (Leung and Vanhoutte 2015).

EDH works mainly through two pathways, diffusible and contact-mediated. Diffusible mechanism is characterized by diffusion of different agents (K⁺ ions, epoxyeicosatrienoic acids (EETs), C-type natriuretic peptide, H₂O₂ and H₂S) from ECs to the adjacent SMCs causing the activation of large conductance Ca²⁺-activated K⁺ channels (BK_{Ca}) rendering the SMCs hyperpolarized (Goto, Ohtsubo et al. 2018).

Whereas the contact-mediated mechanism of hyperpolarization involves the activation of small conductance calcium-activated potassium channels (SK_{Ca}) and intermediate conductance calcium-activated potassium channels (IK_{Ca}) in response to rise in intracellular Ca²⁺ in the ECs and direct transfer of hyperpolarization from ECs to the SMCs via

myoendothelial gap junctions (MGJs) (Feletou and Vanhoutte 2009, Goto, Ohtsubo et al. 2018).

Activation of SK_{Ca} and IK_{Ca} in the endothelial cells causes hyperpolarization of ECs by increasing the K^+ ions in the extracellular space between endothelium and SMCs activating inwardly rectifying K^+ channels (Kir) and Na^+/K^+ -ATPase rendering the SMCs hyperpolarized (Garland, Hiley et al. 2011).

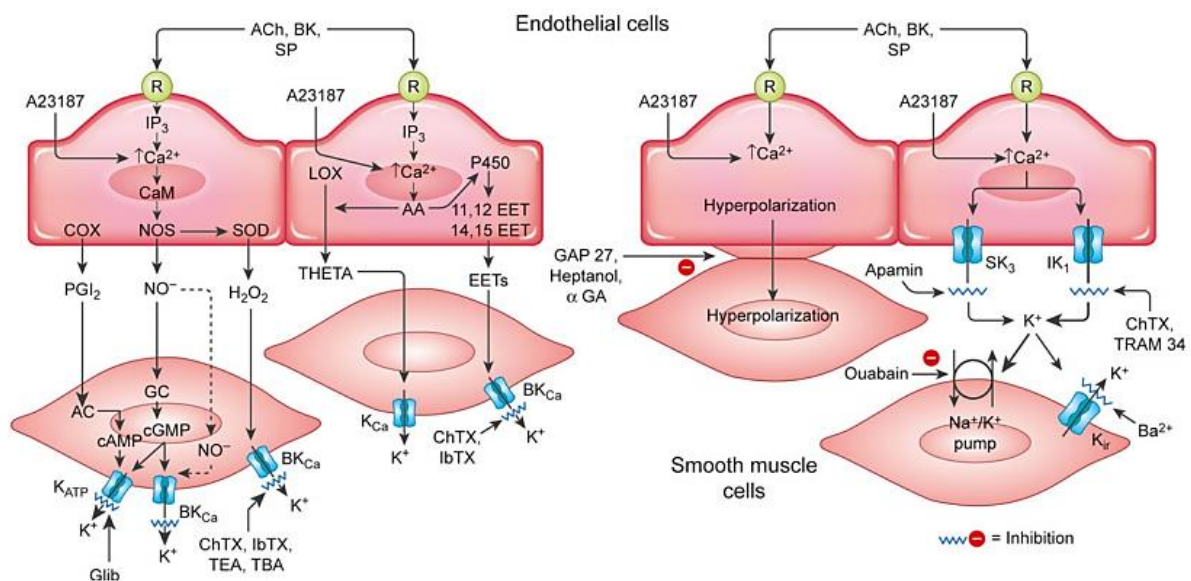


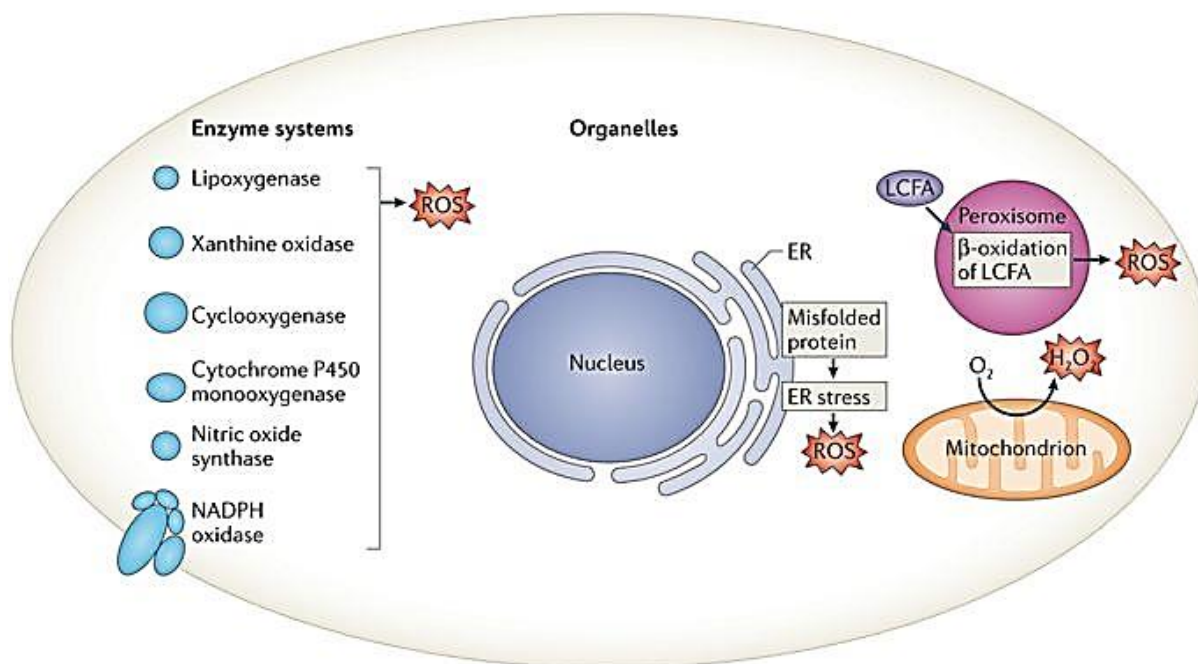
Figure 5: Endothelium-derived hyperpolarization-mediated vasodilation (AA, arachidonic acid; AC, adenylyl cyclase; ACh, acetylcholine; Ba^{+2} , barium; BK, bradykinin; Ca^{2+} , intracellular calcium concentration; BK_{Ca} , large conductance Ca^{2+} -activated K^+ channel; CaM, calcium calmodulin; cAMP, cyclic adenosine mono phosphate; cGMP, cyclic guanosine mono phosphate; ChTX, charybdotoxin; COX, cyclo-oxygenase; EETs, epoxyeicosatrienoic acids; αGA , 18 α -glycyrrhetic acid; GAP 27, connexin inhibitor; Glib, glibenclamide; H_2O_2 , hydrogen peroxide; IbTX, iberiotoxin; IK_1 , intermediate conductance K^+ channel 1; IP_3 , inositol trisphosphate; K^+ , potassium; K_{ATP} , ATP sensitive K^+ channels; K_{Ca} , Ca^{2+} activated K^+ channels; LOX, lipo-oxygenase; Na^+/K^+ , sodium/potassium pump; NO^- , nitrite anion; P450, cytochrome p-450; SK_{Ca} , small-conductance Ca^{2+} -activated K^+ channel subtype 3; NOS, nitric oxide synthase; PGI_2 , prostacyclin; SK_3 , small conductance K^+ channel 3; SOD, superoxide dismutase; SP, substance P; TBA, tetra-butyl ammonium; TEA, tetra-ethyl ammonium) (Vanhoutte, Shimokawa et al. 2017).

1.2.2 Endothelium-Derived Contractile Factors (EDCF)

1.2.2.1 *Reactive Oxygen Species (ROS) and Oxidative Stress*

Under normal physiological conditions, reactive oxygen species (ROS) are the byproducts of aerobic metabolism which serve as important signaling molecules in biological processes for normal cell functioning. ROS includes superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}) (Schieber and Chandel 2014). In healthy state there is a dynamic equilibrium between the production of ROS and its removal, promoting the normal functioning of cell. Under pathological conditions the balance shifts towards the overproduction of ROS, resulting in an increased oxidative stress damaging the important macromolecules such as proteins, lipids and nucleic acids (Schieber and Chandel 2014, Zhang, Wang et al. 2016). In the endothelial cells, the main sources of ROS are NADPH oxidases (NOX), NO synthases, cyclooxygenases (COX), mitochondrial respiratory chain, cytochrome p-450 (Cyp-450), xanthine oxidase and the peroxisomes (Figure 6), whereas ROS are inactivated mainly by the action of the superoxide dismutase (SOD) (Holmstrom and Finkel 2014).

ROS is an important contributor of EDCF-dependent contraction of vascular smooth muscle cells (VSMCs) (Yang, Feletou et al. 2003). Vasoconstriction in response to the increased production of ROS is mediated through the activation of the ryanodine receptors (RyRs) on the SR releasing Ca^{2+} . ROS also activates protein kinase C (PKC) which, together with ROS, inhibits membrane voltage-gated potassium channels (K_v) causing membrane depolarization and opening of membrane voltage-gated Ca^{2+} channels (Ca_v), additionally ROS and PKC can also activate membrane store-operated Ca^{2+} channels (SOC), both contributing to the increase in intracellular Ca^{2+} levels facilitating contraction (Wang and Zheng 2010). ROS is also responsible for impairment of the NO-mediated relaxation by its direct conversion into peroxynitrite anion ($OONO^-$), or decreased formation due to uncoupling of eNOS or oxidation of its cofactor BH_4 to BH_2 (Incalza, D'Oria et al. 2018). ROS also affects EDH-mediated relaxations by influencing the activity of K^+ channels (Kusama, Kajikuri et al. 2005) and modifying the modulation of hyperpolarization through myoendothelial gap junctions (Griffith, Chaytor et al. 2005).



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Figure 6: Cellular sources of reactive oxygen species (ER, endoplasmic reticulum; H₂O₂, hydrogen peroxide; LCFA, long chain fatty acids; ROS, reactive oxygen species) (Holmstrom and Finkel 2014).

1.2.2.2 *Thromboxane (TXA₂) and Prostacyclin (PGI₂)*

In contrast to its vasodilatory effect, ACh can also lead to endothelium-dependent contractions. These contractions are totally abolished in arterial rings pre-treated with indomethacin, indicating the involvement of the COX pathway (Gluais, Lonchampt et al. 2005). Moreover, pre-treatment with selective COX-2 inhibitor shows only partial or no inhibition of the contractile response to ACh, whereas COX-1 inhibitor shows significant or complete inhibition of contractile response pointing towards dominating role of COX-1 (Feletou, Huang et al. 2011). COX-mediated conversion of arachidonic acid culminates in the production of intermediate endoperoxides (PGH₂), which is the precursor of all the prostaglandins. Further important metabolites of PGH₂ include prostacyclin (PGI₂), thromboxane (TXA₂), prostaglandin E₂ (PGE₂), prostaglandin D₂ (PGD₂), prostaglandin F_{2α} (PGF_{2α}) produced by the action of their respective synthases (Bos, Richel et al. 2004). After their production, prostaglandins diffuse out of the endothelium and act on the G-protein coupled prostanoid receptors named IP, TP, EP, DP and FP respectively (Tsuboi, Sugimoto et al. 2002) (Figure 7).

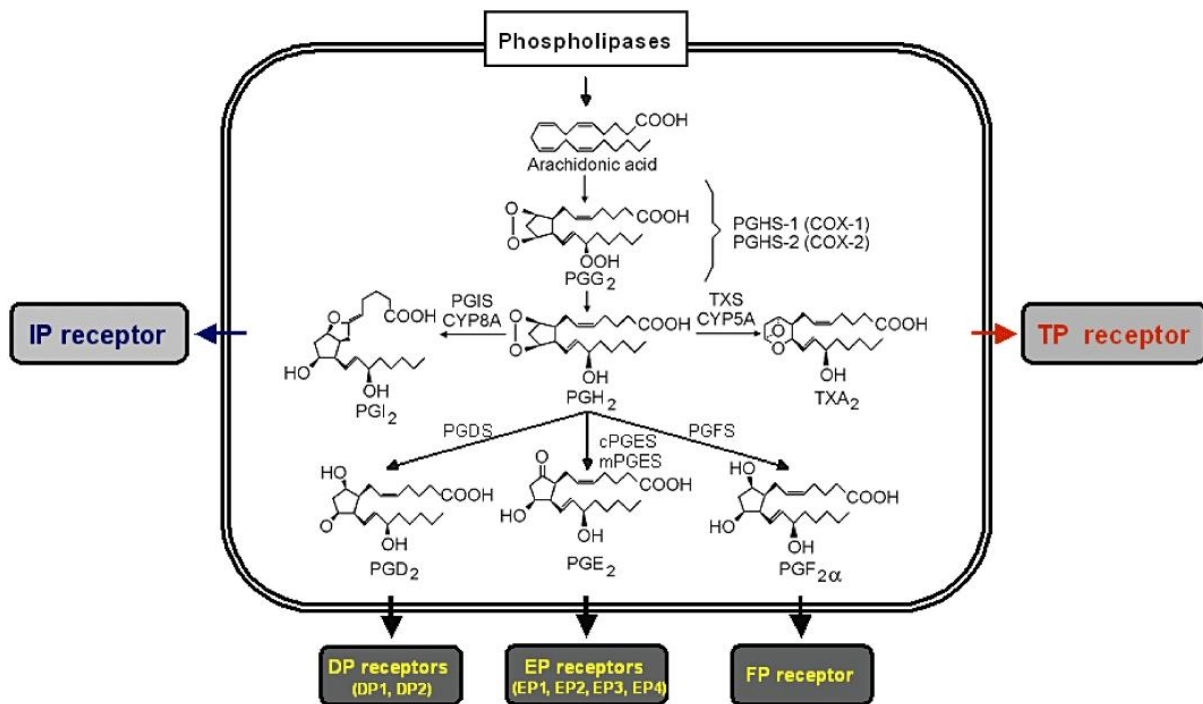


Figure 7: Cyclooxygenase-derived prostanoids and their respective receptors (DP, D prostanoid; EP, E prostanoid; FP, F prostanoid; IP, I prostanoid; PGD₂, prostaglandin D₂; PGDS, prostaglandin D synthase; PGE₂, prostaglandin E₂; PGES, prostaglandin E synthase; PGF_{2α}, prostaglandin F 2α; PGFS, prostaglandin F synthase; PGG₂, prostaglandin G₂; PGH₂, endoperoxides; PGI₂, prostacyclin; PGIS, prostacyclin synthase; TP, T prostanoid; TXA₂, thromboxane A₂; TXS, thromboxane synthase (Feletou, Huang et al. 2011).

TXA₂ and PGI₂ play the most important role in the mediation of COX-dependent EDCF via the activation of TP receptors which is responsible for diverse physiological and pathophysiological roles including VSMC contraction, platelet aggregation, expression of adhesion molecules and promotion of infiltration of monocytes and macrophages (Figure 8) (Feletou, Huang et al. 2010, Matsumoto, Goulopoulou et al. 2015). Upon its activation, TP receptor causes vasoconstriction by increasing intracellular Ca²⁺ levels through opening of receptor-operated and voltage-gated Ca²⁺ channels and Rho-kinase-mediated activation of myofilaments (Tang and Vanhoutte 2009).

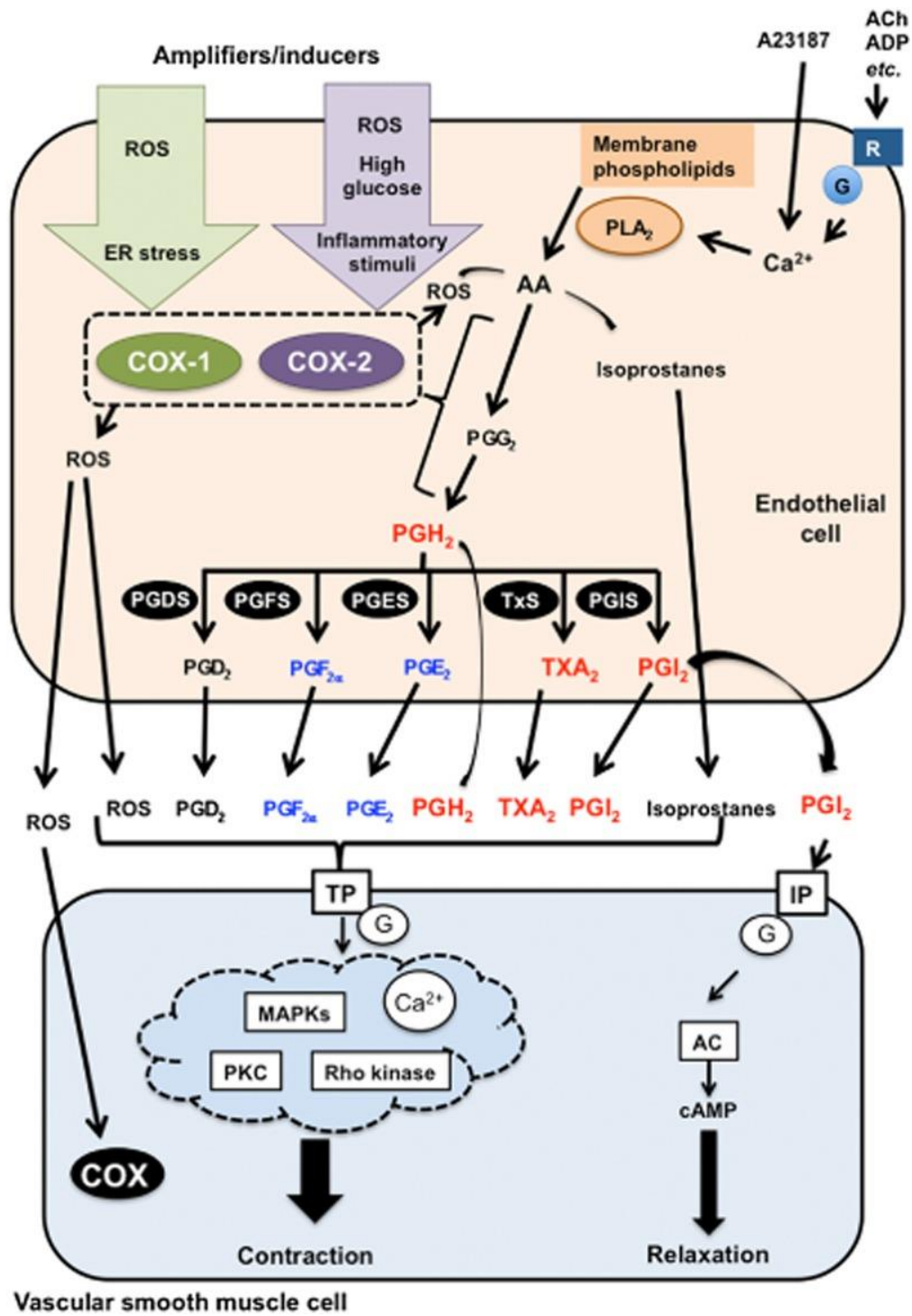


Figure 8: Major EDCF-mediated signaling pathways (Matsumoto, Gouloupoulo et al. 2015)

1.2.2.3 *Angiotensin II (Ang II)*

Angiotensin II (Ang II) is an octapeptide which is an important and multifunctional hormone of the renin-angiotensin system (RAS) accountable for the regulation of blood pressure and vascular homeostasis. Its biosynthesis involves production of its precursor angiotensinogen in the liver which is subsequently cleaved by renin to the biologically inactive angiotensin I (Ang I). Conversion of Ang I to the biologically active Ang II is catalyzed by the angiotensin converting enzyme (ACE) or chymases (Ma, Kam et al. 2010). Ang II is further convertible to a vasodilator metabolite Angiotensin (1-7) by the angiotensin converting enzyme 2 (ACE2) or the plasma endopeptidases (Schindler, Bramlage et al. 2007). Regulatory function of Ang II include blood pressure, plasma volume, thirst response, vascular tone, vascular remodeling, cellular proliferation and activation of sympathetic nervous system (Loiola, Fernandes et al. 2011). Ang II produce its action through two main receptors, the angiotensin type 1 and 2 receptors (AT1R & AT2R, respectively). AT1R are located on the membranes of endothelial cells, cardiomyocytes, VSMCs, nerve endings, conductive tissues, liver, lung and kidney whereas AT2R are located on the endothelial cells, VSMCs and fibroid tissue of heart, myometrium and kidney (Regitz-Zagrosek, Fielitz et al. 1998, Allen, Zhuo et al. 1999).

Ang II produce its vascular effects mainly through AT1R (Figure 9). AT1R are G-protein coupled receptors which further activate several other pathways. Vasoconstrictor action of AT1R is mediated via activation of voltage gated Ca^{+} channels, phospholipase C (PLC), phospholipase D (PLD), phospholipase A-2 (PLA₂). Moreover, AT1R receptor activate extracellular signal regulated kinase (ERK) cascade, platelet derived growth factor (PDGF), epidermal growth factor (EGFR), insulin receptor pathway, and tyrosine kinases and MAPK pathway related to Src family of proteins, proline rich tyrosine kinase 2, focal adhesion kinase and Janus kinases (JAKs). Increased production of ROS is also associated with AT1R via activation of NADPH oxidase (Touyz and Schiffrin 2000).

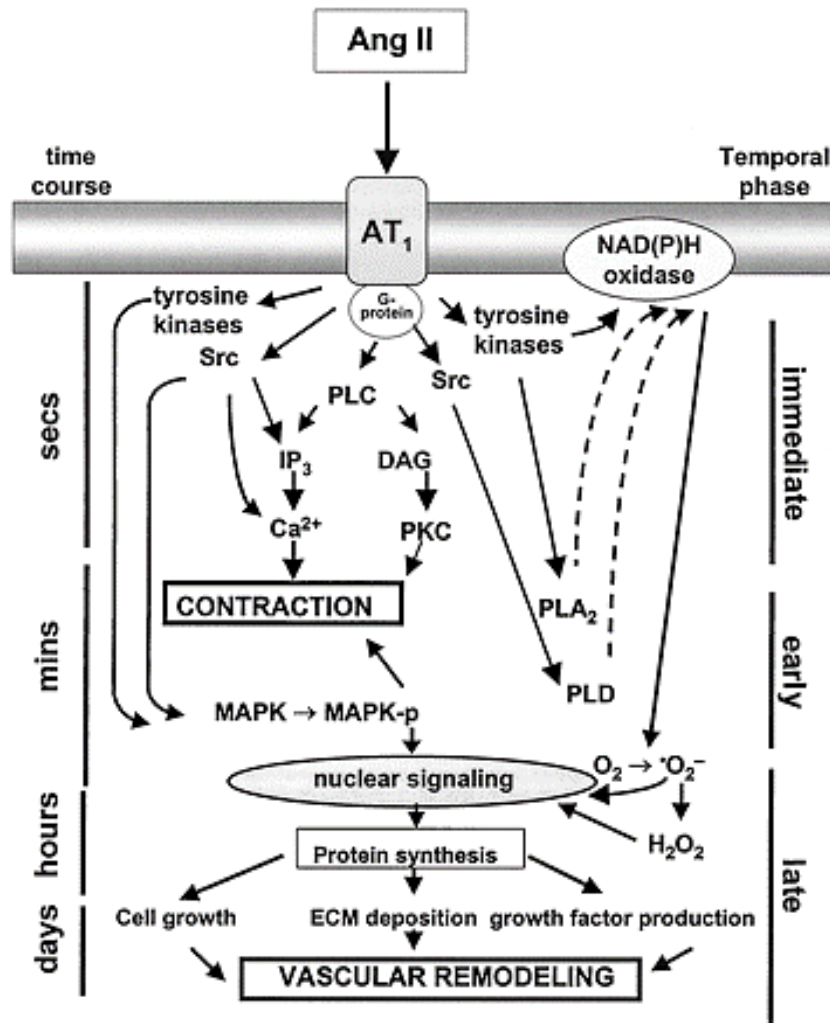


Figure 9: Angiotensin type 1 receptor-mediated actions (Ca²⁺, calcium; DAG, diacylglycerol; IP₃, inositol trisphosphate; MAPK, mitogen activated protein kinase; PKC, protein kinase C; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; Src, sarcoma-family kinases) (Touyz and Schiffrin 2000)

1.2.2.4 Endothelin-1 (ET-1)

ET-1 is a potent vasoconstrictor peptide produced and released by the endothelium along with its isoforms ET-2 and ET-3. But ET-1 is most important for cardiovascular system-related functions (Kedzierski and Yanagisawa 2001). ET-1 produce its effects through the activation of G-protein coupled ET receptors having two subtypes, ET_A and ET_B (Hynynen and Khalil 2006). ET_A receptors are only located on SMCs, whereas ET_B receptors are located on the endothelium and SMCs (El Assar, Angulo et al. 2012). ET_A receptor causes accumulation of IP₃ via activation of PLC leading to increased intracellular Ca²⁺ and vasoconstriction. ET_B receptors also act by increasing intracellular Ca²⁺ producing site-

specific actions. In SMCs producing vasoconstriction like AT1R and in endothelium Ca^{2+} dependent activation of eNOS and COX releasing NO and PGI_2 leading to vasodilation (Luscher and Barton 2000, Hynynen and Khalil 2006). ET-1 can also produce indirect vasoconstriction through activation of endothelium-derived TXA_2 (Bohm and Pernow 2007).

**AGE AND
AGE-RELATED
CARDIOVASCULAR
RISK**

2 -AGE AND AGE-RELATED CARDIO-VASCULAR RISK

2.1 Increase in cardiovascular risk with Age

Cardiovascular diseases (CVDs) are one of the major causes of death worldwide. Only in Europe they are responsible for over 4 million deaths annually (Townsend, Wilson et al. 2016). There is an increase in annual cases of CVDs including atherosclerosis, hypertension, ischemic injury, myocardial infarction, stroke, heart fibrosis and hypertrophy, and CVDs account for 39.6% of the all the age-related diseases (Fajemiroye, da Cunha et al. 2018). The risk of developing a CVD rise sharply after the age of 40 with a 50% risk of having chronic CVD, 85% for hypertension and 20% for chronic heart failure (Figure 10) (Lakatta 2015, Benjamin, Virani et al. 2018). Due to increased longevity and decreasing fertility, world population of aged individuals is rising, one fourth of the population is predicted to be reaching the age of 65 by the year 2035. Being the major risk factor for CVDs, ageing will thus cause a dramatic increase in their prevalence (Steenman and Lande 2017).

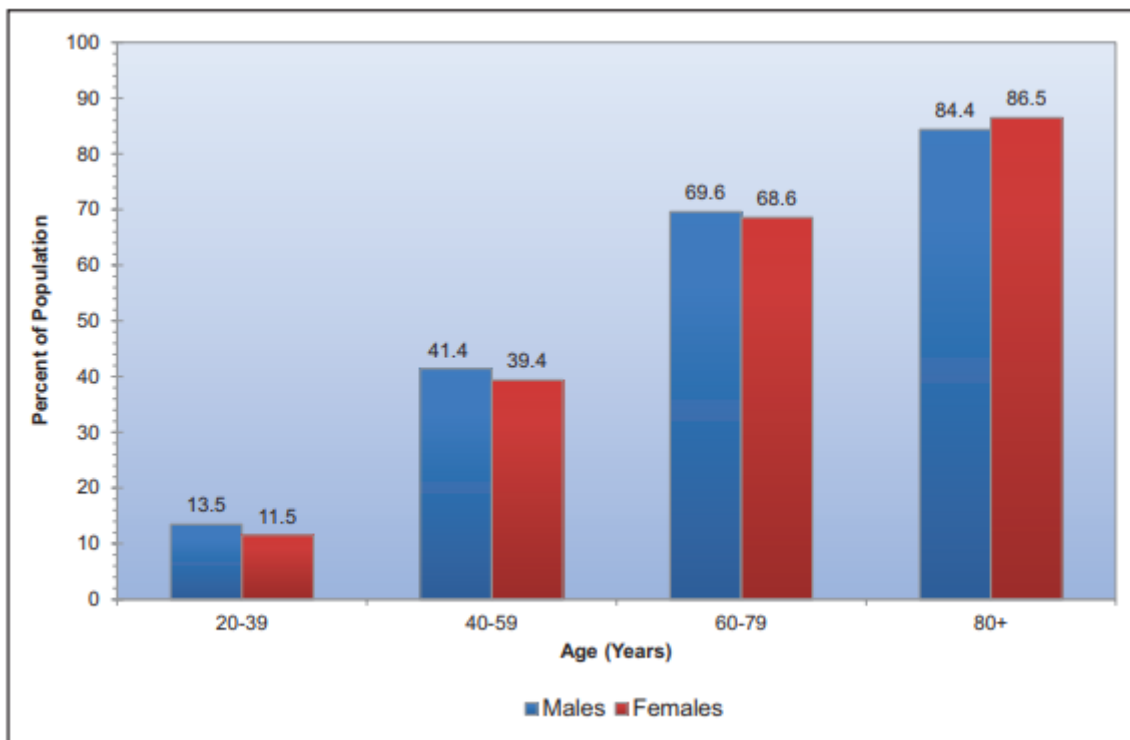


Figure 10: Prevalence of CVDs with increasing age. Data includes coronary heart disease, heart failure, stroke and hypertension. (Benjamin, Virani et al. 2018)

Ageing is associated with a reduction in both the function and the homeostatic regulation of vital cellular events affecting the regulatory interplay within and between cells (DiLoreto and Murphy 2015). Vascular ageing is generally characterized by a remodeling of vascular wall including thickening of vascular layers and an increased arterial stiffness. These changes are opposed by release of vasoprotective mediators such as NO, EDH and prostacyclin from vascular endothelium (Brandes, Fleming et al. 2005). Therefore, to completely understand the vascular ageing, knowledges about the age-related endothelial dysfunction is very important

2.2 The age-related endothelial dysfunction

Any chemical, structural or functional change on a molecular level which can undermine the vascular regulatory role of endothelium is called endothelial dysfunction. From several human and animal studies of last four decades on different vascular beds, it is evident that ageing is associated with blunted endothelium-dependent relaxations, while the endothelium-independent relaxations in response to sodium nitroprusside in the SMC remained un affected (Egashira, Inou et al. 1993, Taddei, Viridis et al. 1995, Matz and Andriantsitohaina 2003, Toda 2012). The age-associated decline in endothelium-dependent relaxations is attributable, at least in part, to modifications of cellular pathways causing a decreased bioavailability of endothelium-derived relaxing factors, either due to reduced formation or to increased inactivation, and an increased production EDCFs (El Assar, Angulo et al. 2012). Ageing is also associated with increased production of ROS affecting the normal functioning of endothelial cells (Davalli, Mitic et al. 2016) (Figure 11).

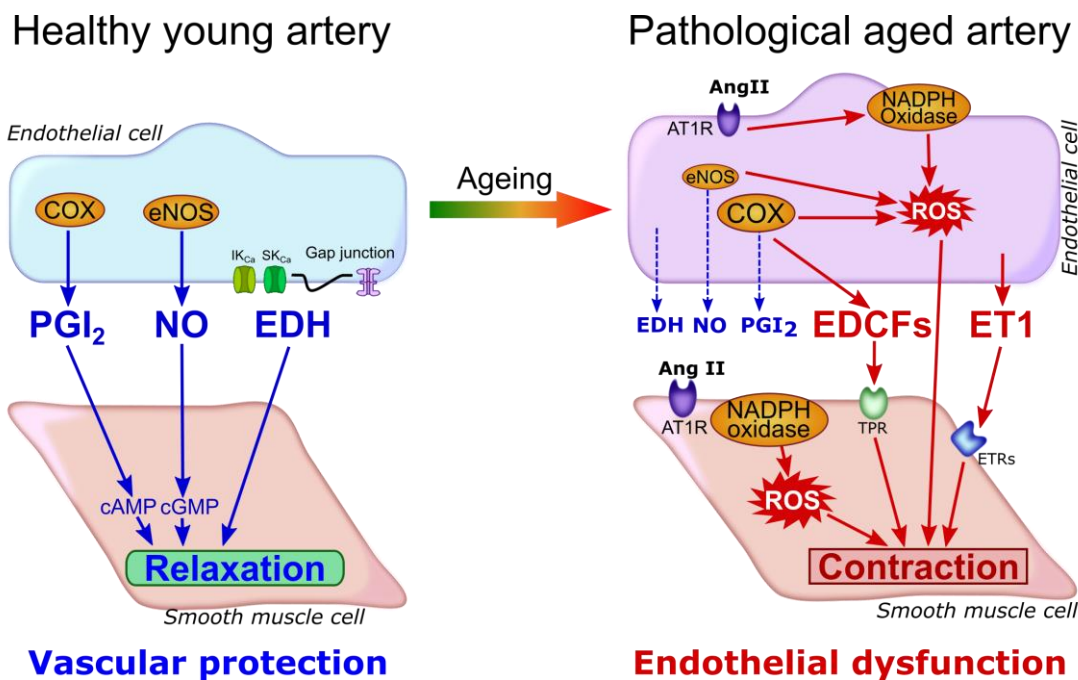


Figure 11: Age-related endothelial dysfunction

2.2.1 Increased ROS and oxidative stress

Under normal conditions, the cellular formation of ROS is under a tight control due to a balance between its creation and elimination, and its controlled presence is necessary for different signaling pathways which are necessary for healthy functioning of cells. Ageing is associated with an increased production of ROS from the main cellular sources (mitochondrial respiratory chain, NOX, COX, NOS, xanthine oxidase and Cyp-450) and/or a decreased inactivation by antioxidant defenses (Enzymatic: SODs, glutathione peroxidase, catalase; Non-enzymatic: vitamin C, vitamin E, Uric acid) causing an increased oxidative stress (Afanas'ev 2010, El Assar, Angulo et al. 2012). Oxidative stress results in oxidative damaging of vital macromolecules (proteins, lipids, polysaccharides, nucleic acids) and interference with signaling pathways leading to the disruption of the normal functioning of cells. Moreover, oxidative stress has a self-amplifying capability by producing more ROS while damaging other molecules. For example, elevated production of ROS during mitochondrial respiration can damage mitochondrial DNA, which lack repair capability like nuclear DNA, thus leading to the development of mutations causing anomalies in the respiratory chain and uncoupling that produce more ROS (Loeb, Wallace et al. 2005).

Similarly, oxidative stress in endothelial cells could lead to the oxidation of the eNOS essential cofactor BH₄ into BH₂, thus leading to eNOS uncoupling and subsequent increased ROS production (Fleming 2010).

The oxidative damages to macromolecules are characterized by the oxidation of nucleic acids causing mutations and deletions, the peroxidation of lipids leading to the formation of cytotoxic peroxides, proteins through nitrosylation, oxidation of amino acids or glycosylation leading to advanced glycation end-products (AGE). Oxidative stress also interferes with gene expression through the modification of transcription factors that can lead to cell cycle arrest or apoptosis *via* activation of pathways such as p53/p21 (Figure 12) (Kregel and Zhang 2007). Impact of ROS on different endothelial mediators of vascular tone will be discussed individually.

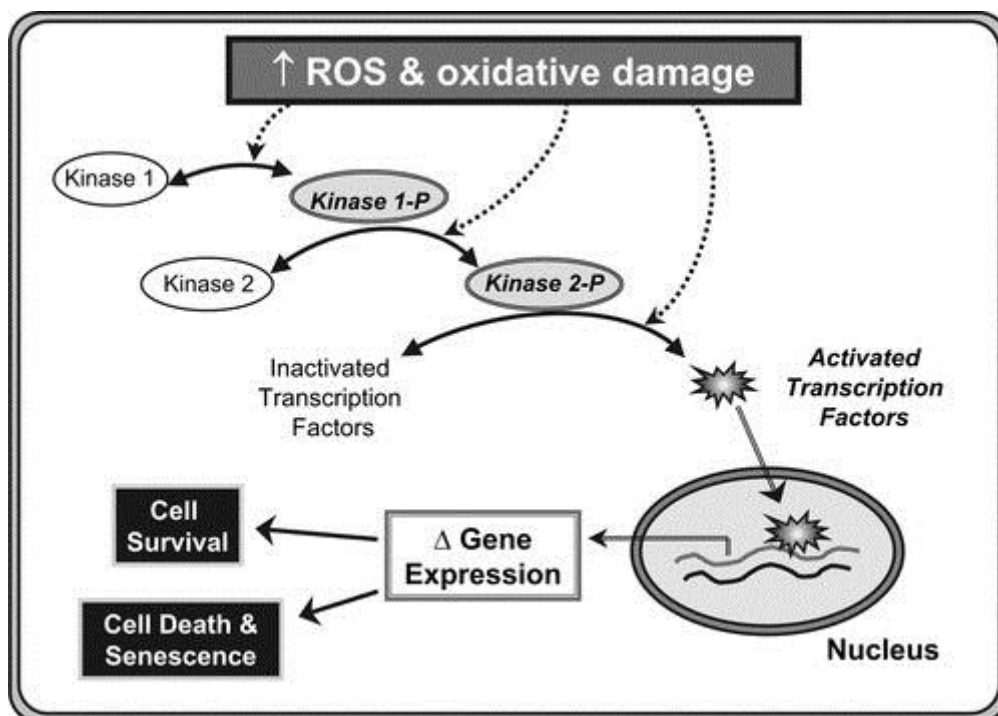


Figure 12: Oxidative stress can affect gene expression by modification of transcription factors (Kregel and Zhang 2007)

2.2.2 Decreased NO bioavailability

As discussed in the previous chapter, the protective role of NO in the vascular homeostasis and health is very important and includes vasodilation, inhibition of SMC proliferation and migration, of platelet aggregation, and of the expression of pro-inflammatory and pro-thrombotic mediators. Ageing is associated with a disruption of the balance between the NO production and its degradation, thus reducing its bioavailability (Brandes, Fleming et al. 2005). The reduction in NO bioavailability critically undermines the endothelial function protecting the vascular integrity, leading to an increased risk of developing CVDs like atherosclerosis, hypertension and diabetic vasculopathy (Sagach, Bondarenko et al. 2006).

2.2.2.1 *Decrease production of NO*

Ageing-related decreased production of NO can be related to either a deduced availability of the precursor, an improper functioning of eNOS due to uncoupling or its inhibition by endogenous inhibitor. Previous studies did not show any evidence of age-related decrease in L-arginine availability (precursor of NO) but one study showed an improvement of the endothelial function in old humans through oral administration of L-arginine for 14 days, characterized by significantly higher flow mediated dilation and plasma level of L-arginine in comparison with placebo (Bode-Boger, Muke et al. 2003). The age-related decreased bioavailability of NO could also be due to reduced formation of NO by eNOS. Indeed, ageing is associated with an increased bioavailability of the endogenous inhibitor of NOS called asymmetric dimethyl arginine (ADMA), that reduce NO formation by inhibiting L-arginine attachment to its binding site on NOS (Schulze, Maas et al. 2005).. Moreover, age-related oxidative stress leads to the uncoupling of eNOS through oxidation of BH₄ to inactive BH₂ or the nitrosylation of eNOS molecules, thus leading to the formation of ROS instead of NO (Yang, Huang et al. 2009). Furthermore, ageing is also associated with a reduced PI3K/Akt-dependent phosphorylation of serine 1177 (human) and serine 1176 (rats) residue responsible for eNOS activation, and an enhanced phosphorylation at threonine 494 residue responsible for the inhibition of eNOS (Cau, Carneiro et al. 2012). However, there is no strong evidence of correlation between eNOS expression and ageing. Indeed, several studies have reported either an increased (Cernadas, Sanchez de Miguel et al. 1998, Chou, Yen et al. 1998, Smith, Visioli et al. 2006), decreased (Challah, Nadaud et al. 1997, Yoon,

Cho et al. 2010) or unmodified expression of eNOS with increasing age (Sun, Huang et al. 2004, Rodriguez-Manas, El-Assar et al. 2009, Yang, Huang et al. 2009). .

2.2.2.2 *Increased inactivation of NO*

Age-related increase in oxidative stress is responsible for the direct inactivation of NO by its reaction with $O_2^{\cdot-}$ converting it to the radical peroxynitrite anion ($ONOO^{\cdot-}$), which due to its high reactivity with biomolecules (DNA, lipids, proteins) is called a reactive nitrogen species (RNS). In contrast to $O_2^{\cdot-}$, $ONOO^{\cdot-}$ has higher cellular penetrability causing easier oxidative modification of macromolecules, especially proteins by the nitrosylation of tyrosine and cysteine residues. $ONOO^{\cdot-}$ also causes oxidation of BH_4 and subsequent uncoupling of eNOS causing further amplification of the ROS production. These reactions produce cellular events ranging from small modification to severe damages causing cellular death (Figure 13) (Cau, Carneiro et al. 2012, El Assar, Angulo et al. 2012). The nitrosylation of tyrosine gives nitrotyrosine, whose level is considered as an important marker of oxidative injury to the cells. Previous studies have shown that an increased production of $ONOO^{\cdot-}$ during the ageing process (Francia, delli Gatti et al. 2004) and increased levels of nitrotyrosine in aged vessels (Csiszar, Ungvari et al. 2002, Rodriguez-Manas, El-Assar et al. 2009, Tian, Yan et al. 2010). Moreover, scavenger of $ONOO^{\cdot-}$ reversed the age-related blunted vasodilation in old rats (van der Loo, Labugger et al. 2000).

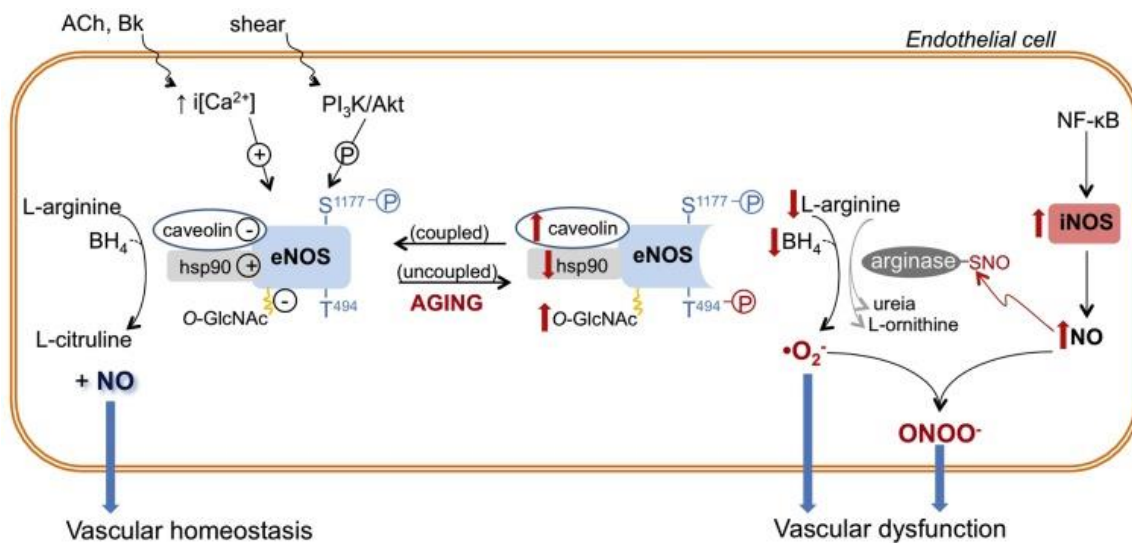


Figure 13: Role of NOS in age-related endothelial dysfunction (Akt, protein kinase B; BH₄, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; hsp90, heat shock protein 90; i[Ca²⁺], intracellular calcium; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor κB; NO, nitric oxide; O₂⁻, superoxide anion; ONOO⁻, peroxynitrite anion; PI₃K, phosphoinositide 3-kinase; S1177, serine 1177; T494, threonine 494) (Cau, Carneiro et al. 2012)

2.2.3 Decreased EDH

Ageing is associated with a partially or completely blunted EDH-mediated vasodilatation in rats (Brandes, Fleming et al. 2005, Long, Newaz et al. 2005). Moreover, a clinical study in 1999 showed negative correlation between EDH-mediated relaxation and age in Humans (Urakami-Harasawa, Shimokawa et al. 1997). The calcium-activated potassium channels (K_{Ca}) play a major role in the EDH-mediated vasodilatations and the age-related oxidative stress, especially high level of H₂O₂, can inhibit the function of the endothelial K_{Ca} by modification of cysteine residue (Brakemeier, Eichler et al. 2003, Tang, Garcia et al. 2004). 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 ageing rats, which may be due to decreased expression or activity of SK_{Ca}, while IK_{Ca}-mediated relaxation remained unchanged (Kong, Man et al. 2015). Moreover the age-related decrease in EDH is also associated with a decreased expression of SK_{Ca}, IK_{Ca}, and angiotensin II AT₂R and an increased expression of AT₁R in the mesenteric arteries of rats (Idris Khodja, Chataigneau et al. 2012).

2.2.4 Increased EDCF

In addition to decrease in EDRF, ageing is associated with an increased production of EDCF, characterized by an endothelium-dependent contractile response induced by acetylcholine. COX pathway plays a major role in the production of EDCF, whereas renin-angiotensin system (RAS) and endothelin-1 are also important contributors to endothelium-dependent contraction (El Assar, Angulo et al. 2012).

2.2.4.1 *COX-derived prostanoids*

In healthy vessels, the production of vasodilatory and vasoconstrictor prostanoids is a tightly regulated balance. Ageing is associated with a shift of this balance towards the overproduction of vasoconstricting prostanoids and/or the downregulation of vasorelaxing ones (Singh, Prasad et al. 2002, Qian, Luo et al. 2012). Indeed, previous studies in rats have reported an age-related increase in the level of vasoconstrictor TXA₂ in aorta (Matz, de Sotomayor et al. 2000, Kang, Rajanayagam et al. 2007) and impairment of PGI₂ synthesis in aorta and muscle feed arteries (Woodman, Price et al. 2003, Tang and Vanhoutte 2008). Similarly, the expression of PGI₂ in human cultured endothelial cells decreases with serial passages (Tokunaga, Yamada et al. 1991). Moreover, in Humans the PGI₂-mediated vasodilatation in response to exercise hyperaemia is almost abolished in aged subjects and isolated mesenteric microvessels of subjects older than 60 years shown an abolished COX-derived indomethacin-sensitive vasorelaxation and an increased in COX-derived indomethacin-sensitive vasoconstriction associated with an increased oxidative stress in the vascular wall (Schrage, Eisenach et al. 2007, Rodriguez-Manas, El-Assar et al. 2009). The age-related change in the production of different prostanoids has been proposed to concern mainly the decrease in vasodilating PGI₂ and increase in the vasoconstricting untransformed PGH₂, as their levels in the vascular tissue are 10-100 fold higher than the other prostanoids (Figure 14). The involvement of COX-derived prostanoids, and in particular PGH₂, in age-related increased EDCF is further strengthened by the abolishment of the contractile response to ACh in the presence of a TP receptor antagonist (Rodriguez-Manas, El-Assar et al. 2009). Furthermore, prostanoids receptors expression is also affected by ageing, mainly through the reduced expression of IP receptors whereas the expression of TP receptors is increased (Qian, Luo et al. 2012). In addition, the age-related increased vascular oxidative stress can lead to the activation of COX (Vanhoutte, Feletou et al. 2005), leading to an increased formation of prostanoids endoperoxides resulting in an increased stimulation of TP receptors.

However, the involvement of COX isoforms COX-1 and COX-2 in the age-related endothelial dysfunction is unclear at the moment. Indeed, some authors have reported an improvement of endothelial function with COX-1 inhibitor (Shi, Man et al. 2008) or COX-2 inhibitor (de Sotomayor, Perez-Guerrero et al. 2005). Similarly, the effect of ageing on the vascular expression COX-1 and COX-2 has no consensus (Heymes, Habib et al. 2000, Stewart, Zhang et al. 2000, Briones, Montoya et al. 2005, Shi, Man et al. 2008).

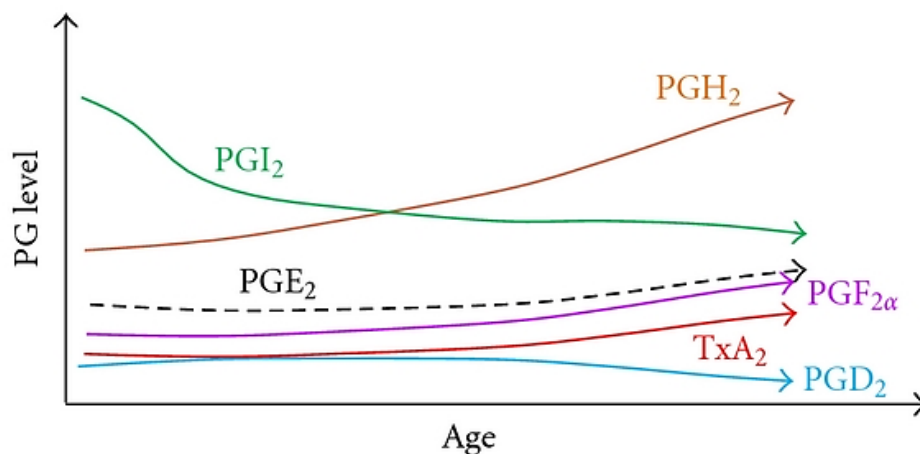


Figure 14: Age-related change in production of different prostanoids (PG, prostaglandin; PGD₂, prostaglandin D₂; PGE₂, prostaglandin E₂; PGF_{2α}, prostaglandin F_{2α}; PGI₂, prostacyclin; TxA₂, Thromboxane A₂) (Qian, Luo et al. 2012)

2.2.4.2 Renin-angiotensin system

Renin-angiotensin system plays a critical role in vascular health, pathology and ageing mainly through the angiotensin II-mediated actions. In the thoracic aorta of mice, ageing is associated with an increased expression of ACE, angiotensin II and AT1 receptors and a decreased expression of ACE2 and AT2 receptors in the vessels, shifting the balance towards vasoconstriction and vascular damage (Yoon, Kim et al. 2016). Overactivation of AT1R by angiotensin II is associated with an augmented generation of ROS through the activation of NOX and the oxidative uncoupling of eNOS, thus making angiotensin II a potent inducer of oxidative stress and endothelial dysfunction (Figure 15) (El Assar, Angulo et al. 2012, Idris Khodja, Chataigneau et al. 2012). Involvement of the angiotensin II pathway is further strengthened by the fact that treatments with ACE and AT1R inhibitors resulted in a better endothelial function through improvement of both NO- and EDH-mediated relaxations and

the reduction of vascular oxidative stress in old rats (Goto, Fujii et al. 2000, Kansui, Fujii et al. 2002). Angiotensin II causes downstream activation of tissue growth factor β (TGF β) responsible for accumulation of collagen and fibronectin in the vessel wall, leading to vascular thickness (Yoon, Kim et al. 2016). Moreover, Angiotensin II is also responsible for the induction of the ET-1 system, further contributing to the development of the endothelial dysfunction (d'Uscio, Shaw et al. 1998).

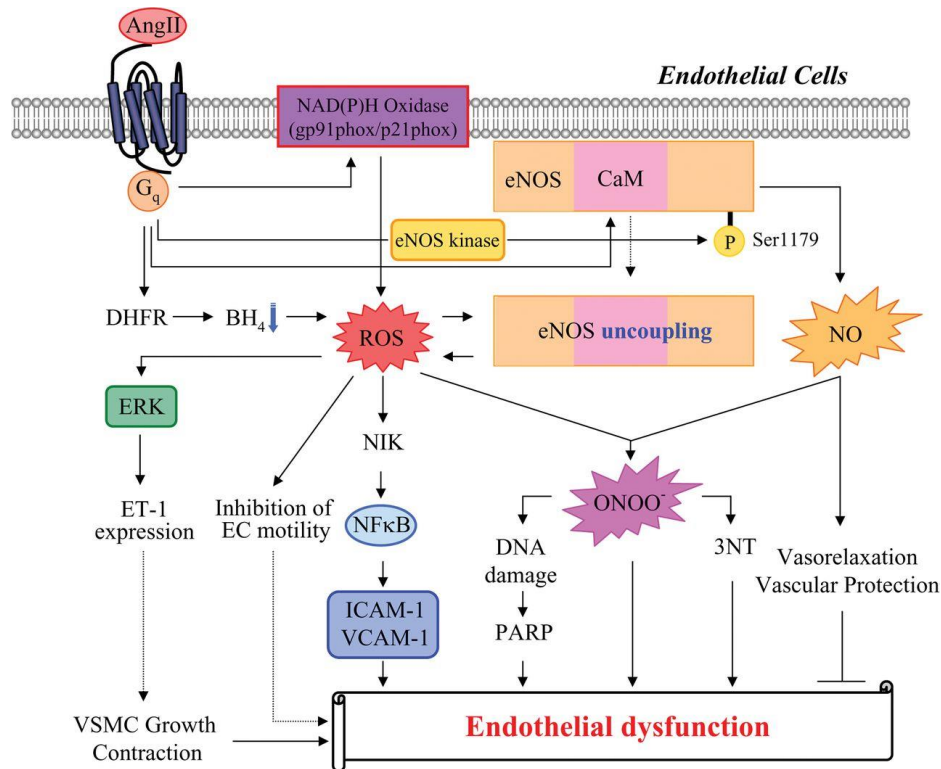


Figure 15: Role of angiotensin II signaling in endothelial dysfunction (Akt, protein kinase B; Ang II, angiotensin II; BH₄, tetrahydrobiopterin; CaM, calcium calmodulin; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal regulated kinase; ET-1, endothelin-1; G_q, G-protein q; NF- κ B, nuclear factor κ B; NO, nitric oxide; ONOO⁻, peroxynitrite anion; PARP, poly adenosine diphosphate ribose polymerase; S1179, serine 1179; VSMC, vascular smooth muscle cell) (Higuchi, Ohtsu et al. 2007)

2.2.4.3 Endothelin-1

Ageing is associated with increased endothelin-1-dependent vasoconstriction and ET_A receptor-mediated signaling in humans and rats (El Assar, Angulo et al. 2012). Plasma concentration of ET-1 and endothelial expression of ET_A receptors were found to be higher in older men and pre-treatment with ET_A receptor blocker resulted in 60% improvement of the

endothelium-dependent vasodilatation in old mice (Donato, Gano et al. 2009). In aged humans increased ET-1 activity was suggested to be linked with augmented production of superoxide (Van Guilder, Westby et al. 2007) and expression of ET-1 was reported to be positively correlated with levels of nitrotyrosine (Donato, Gano et al. 2009) but clear link between them has not been established. The angiotensin II and the ET-1 systems are closely related to each other and promote vasoconstriction, increased coagulation, inflammation and cell proliferation (Barton 2014). Activation of the ET-1 pathway has been associated to several cardiovascular risk factors including ageing, hypercholesterolemia, hyperglycemia, insulin resistance and obesity (Barton 2014) (Figure 16).

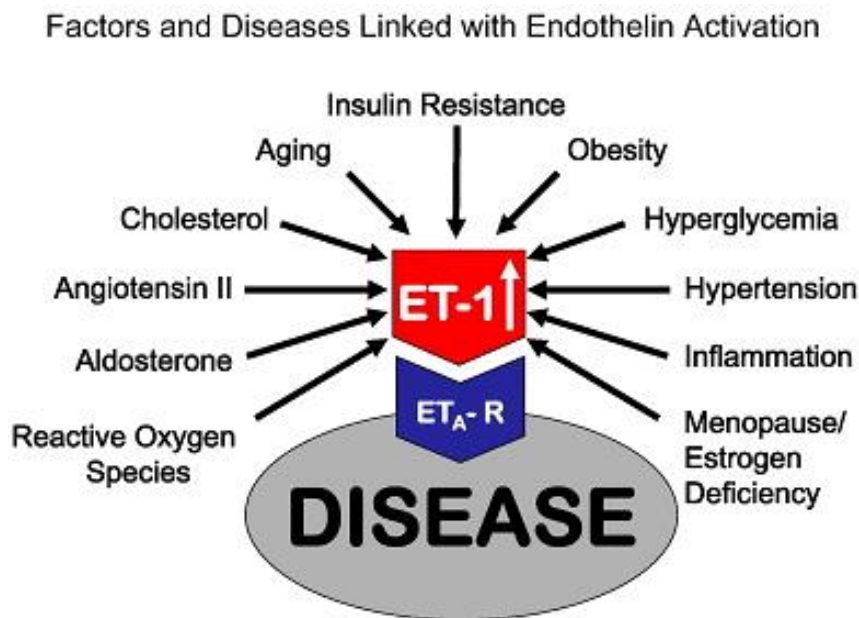


Figure 16: Factors and diseases responsible for ET-1 pathway activation (Barton 2014)

2.2.5 Role of inflammation in age-related endothelial dysfunction

Ageing is associated with a chronic pro-inflammatory state, also termed inflamm-aging, characterized by continuous stress of antigenic load. Physiological ageing is associated with higher levels of inflammatory mediators such as cytokines (TNF- α , IL-1 β , IL-6) and C-reactive proteins (Assar, Angulo et al. 2016). This age-related inflammation is interconnected with the age-related oxidative stress due to the ROS-dependent activation of the transcription factors NF- κ B and AP-1 resulting in the overexpression of cytokines (TNF- α , IL-1, IL-6) adhesion molecules (ICAM, VCAM) and proinflammatory enzymes (iNOS, COX-2) leading to inflammation and this inflammatory environment in turn augments ROS production, promoting the development of the endothelial dysfunction (Figure 17) (El Assar, Angulo et al. 2012). Human studies have also reported age-related increase in the expression of NF- κ B and inflammatory cytokines in vascular endothelial cells obtained by endovascular biopsy from brachial artery or peripheral veins (Donato, Eskurza et al. 2007, Donato, Black et al. 2008). Age-related upregulation of TNF- α is linked to endothelial apoptosis (Csiszar, Ungvari et al. 2004) and TNF- α inhibition causes arterial dilatation and decreased expression of ICAM-1 and iNOS (Arenas, Xu et al. 2006). Moreover, inflammation is associated with activation of iNOS, which has a 100-1000 times higher catalytic activity than eNOS. Age-related upregulation of iNOS is associated with higher levels of NO which undergo ROS-dependent inactivation, producing more nitrosative stress causing increased destruction of vital macromolecules (Loscalzo 2000). Conversely, the selective inhibition of iNOS resulted in a partial improvement of the endothelial function in human isolated mesenteric arteries from aged subjects (Rodriguez-Manas, El-Assar et al. 2009). Role of inflammatory cells such as neutrophils, eosinophils and monocytes is also important in vascular ageing. While remaining in their normal ranges an inverse correlation was reported between their count and arterial blood flow in old individuals (Walker, Seibert et al. 2010).

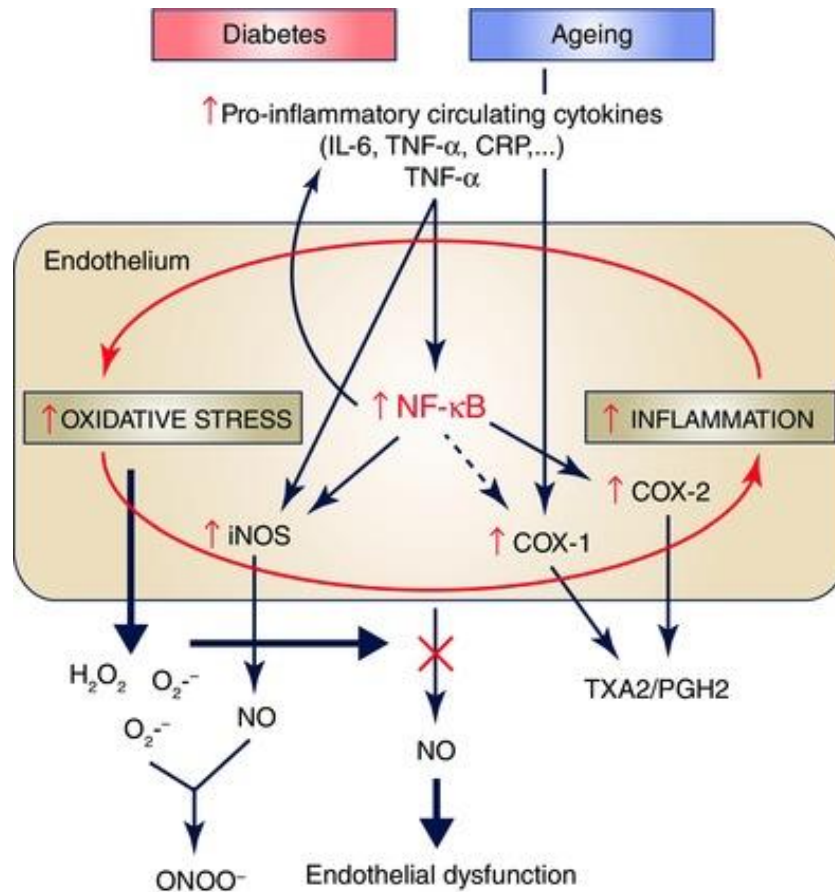


Figure 17: Role of age-related pro-inflammatory environment in endothelial dysfunction (Assar, Angulo et al. 2016)

2.2.6 Role of senescence in age-related endothelial dysfunction

Following a variable number of divisions or cellular stress, cells can go into an irreversible state of cell cycle arrest where they are still metabolically active, this state is termed as cellular senescence. Senescent cells show distinct morphological and functional changes leading to a loss of cellular homeostasis (Herrera, Mingorance et al. 2010). Senescence of vascular endothelial cells and its implications in endothelial dysfunction is an emerging topic of interest.

Two type of cellular senescence have been described. Firstly, the repeated cell divisions cause the shortening of telomeres that are considered as protector of DNA. When telomere reaches a critical length, it induces senescence *via* the activation of the p53/p21 pathway, and this phenomenon is called replicative senescence. Recently, it has been shown that senescence can be induced prematurely by different stress factors like oxidative stress, DNA

alterations, activation of local angiotensin system, radiations, high glucose and reduced NO bioavailability, leading to the activation of the p16 retinoblastoma protein pathway, and this phenomenon is termed as premature senescence (Campisi and d'Adda di Fagagna 2007, Erusalimsky 2009, Bhayadia, Schmidt et al. 2016).

Senescence of vascular endothelial cells is directly associated to age-related endothelial dysfunction because senescent endothelial cells show decrease in level of NO formation, eNOS activity, PGI₂ release and increase in ROS, TXA₂ and ET-1 production (Minamino and Komuro 2007, Herrera, Mingorance et al. 2010). Senescent endothelial cells also demonstrate alterations in morphology, gene expression and secretory profile such as an overexpression of inflammatory cytokines, adhesion molecules and an altered expression of proteins involved in the remodeling of the extracellular matrix, making the vascular environment more atherothrombogenic (Erusalimsky 2009).

More recently, our research group has reported that after serial passaging, cultured porcine coronary endothelial cells showed an increased presence of the senescence marker SA- β -gal (Senescence-Associated β -galactosidase) activity associated to an upregulation of p53, p21 and p16, an activation of the local angiotensin system and an upregulation of NADPH oxidase leading to a cellular oxidative stress (Khemais-Benkhiat, Idris-Khodja et al. 2016, Silva, Abbas et al. 2017). Endothelial senescence has also been reported *in vivo* using SA- β -gal activity as a histochemical marker in endothelial cells of rabbit carotid artery (Fenton, Barker et al. 2001), aortas of diabetic rats (Chen, Brodsky et al. 2002), and in atherosclerotic plaques of human aorta and coronary arteries (Vasile, Tomita et al. 2001, Minamino, Miyauchi et al. 2002).

OMEGA-3 PUFAs AND THEIR ROLE IN VASCULAR HEALTH

3 - OMEGA-3 PUFAS AND THEIR ROLE IN VASCULAR HEALTH

3.1 Lipids

Generally, lipids are defined as hydrophobic molecules which are soluble in organic solvents. More precisely from synthetic or structural point of view, lipids are hydrophobic or amphipathic molecules which are produced by carbon anion- and carbocation-based condensation of thioesters and isoprene units, respectively (Fahy, Subramaniam et al. 2005).

Lipids is a class of important biomolecules playing important roles at the cellular level as an energy source, structural components of cell membrane and mediators of different signaling pathways. Representing approximately 50% of the cell membrane structure, lipids affect membrane fluidity, orientation and diversity of membrane bound proteins influencing the action of receptors and membrane-bound enzymes. Therefore, careful dietary intake and lipid homeostasis is vital for good health and its disruption can cause life threatening pathologies (Oresic, Hanninen et al. 2008).

Lipids are a diverse group of chemical compounds. Due to high complexity of structural variability, lipids are classified into 8 groups: fatty acids, glycerolipids, glycerophospholipids, sphingolipids, saccharolipids and polyketides (keto-acyl condensation), sterol lipids and prenol lipids (isoprene condensation) (Fahy, Subramaniam et al. 2005).

3.2 Fatty Acids

Being the substantial part of cellular structure along with proteins and carbohydrates, fatty acids are very important from the biological point of view. They also serve as a source of energy responsible for almost 30% of energy production in humans. Chemically, fatty acids are carboxylic acids with an aliphatic chain of carbon atoms ranging from 4 to 36 atoms. Due to absence, presence or abundance of carbon-carbon double bonds, fatty acids can be termed as saturated, unsaturated or polyunsaturated (Tvrzicka, Kremmyda et al. 2011).

3.2.1 Saturated fatty acids

Fatty acids having a straight chain of carbon atoms lacking any double bond are termed as saturated fatty acids (SFA). SFAs range from liquid to semisolids at room temperature,

their fluidity decreasing with increasing number of carbons. Based upon number of carbon atoms, they can further sub-divided into short (1-6 carbons), medium (7-12 carbons) and long chain (13 or more carbons) SFA (Schonfeld and Wojtczak 2016). Relating to their size, small and medium chain SFA are readily absorbed in the gastrointestinal track and undergo hepatic oxidation, whereas long chain SFA enter the lymphatic circulation after being packed into chylomicrons. Dietary consumption of long chain SFA in high amounts is associated with an increased risk of coronary heart disease and raised low density lipoproteins (LDL) plasma level (Briggs, Petersen et al. 2017). The cardiovascular effects of short and medium chain SFA are still under investigation and the few existing studies show inconsistent results (Kris-Etherton and Fleming 2015). The most abundant dietary long chain SFAs are myristic (C14:n-0), palmitic (C16:n-0) and stearic (C18:n-0) acid, and are mainly found in coconut oil, palm kernel oil, butter, cocoa butter, soybean, sunflower oil and meat (Micha and Mozaffarian 2010).

3.2.2 Monounsaturated fatty acids

Fatty acids with a single double bond in a straight chain of carbon atoms are called monounsaturated fatty acids (MUFA). To indicate the position of double bond in the fatty acid, its name is followed by (CX:Y n-Z) where X is number of carbon atoms, Y is number of double bonds and Z is number of the first carbon of the first double bond from the methyl end of chain (Scorletti and Byrne 2013). For example, oleic acid (C18:1 n-9) (18 carbons, 1 double bond and double bond is between 9th and 10th carbon). Most abundantly consumed dietary MUFA are oleic (C18:1 n-9), palmitoleic (C16:1 n-7) and vaccenic acid (C18:1 n-7). Endogenously synthesized MUFA are myristoleic (C14:1 n-5), gondoic (C20:1 n-9), erucic (C22:1 n-9) and nervonic acid (C24:1 n-9). Oleic acid is the most abundant dietary MUFA found predominantly in olive oil, peanut oil, canola oil, safflower oil, rapeseed oil, sweet almond oil, hazelnut oil and avocado oil (Tvrzicka, Kremmyda et al. 2011). Substitution of SFA with MUFA in the diet is associated with a lower risk of cardiovascular diseases, metabolic syndrome, and the promotion of healthy lipid profile and reduction of obesity. Moreover, oleic acid is reported to be antithrombotic, antiatherogenic, promoter of higher HDL/LDL ratio and to decrease lipoperoxidation (Riccardi, Giacco et al. 2004, Gillingham, Harris-Janz et al. 2011).

3.2.3 Polyunsaturated fatty acids

Fatty acids having a carbon chain with two or more double bonds are termed as polyunsaturated fatty acids (PUFAs). Among PUFAs, the (n-3) and (n-6) fatty acids having first unsaturation at 3rd carbon and 6th carbon, also termed as omega-3 and omega-6 fatty acids, have received special attention (Figure 18). As mammals could not synthesized n-3 and n-6 PUFAs due to lack of enzymes to create unsaturation at the desired carbon atoms, they are often termed as essential fatty acids (linoleic and α -linolenic acids) or conditionally essential (eicosapentaenoic and docosahexaenoic acids), and their dietary intake is necessary (Scorletti and Byrne 2013).

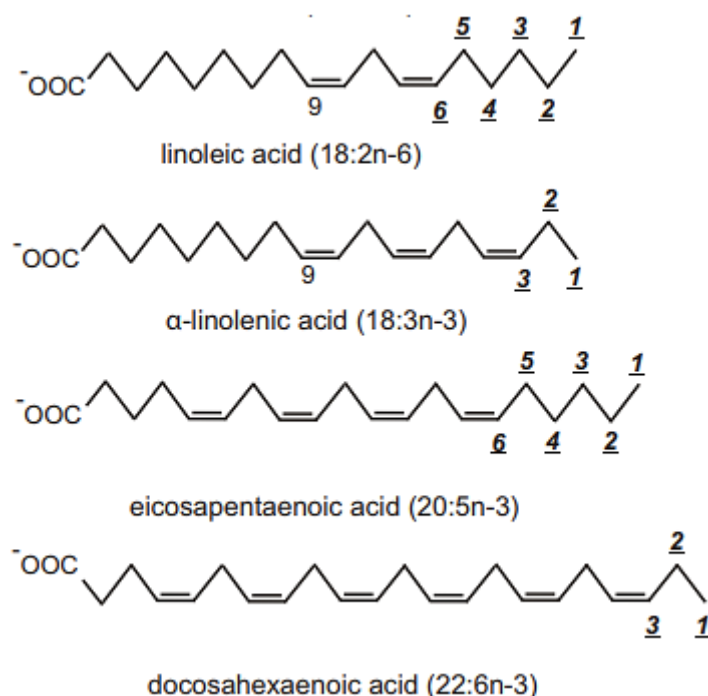


Figure 18: Important polyunsaturated fatty acids (Tvrzicka, Kremmyda et al. 2011)

3.2.4 Trans-fatty acids

trans MUFA such as elaidic (C18:1 n-9 t) and *trans*-vaccenic (C18:1 n-7 t) acid, which have opposite configuration of hydrogen atoms at the double bond (*trans* configuration), show higher melting point resulting in more rigid structure. *trans* MUFAs are not beneficial, and they have biological effects like SFA. They are normally produced during improper hydrogenation of vegetable oils and are considered more atherogenic than SFA (Tvrzicka, Kremmyda et al. 2011).

3.3 Omega-3 or n-3 PUFAs

The most common dietary omega-3 PUFAs is α -linoleic acid (C18:3 n-3, ALA), other long chain omega-3 PUFAs include eicosapentaenoic acid (C20:5 n-3, EPA), docosapentaenoic acid (C22:5 n-3, DPA), and docosahexaenoic acid (C22:6 n-3, DHA). Since their discovery in 1929 by George Burr and his wife, omega-3 PUFAs have been reported to exert several beneficial effects on health (Scorletti and Byrne 2013). Dietary intake of omega-3 fatty acids is reported to be associated with decreased risk factor of cardiovascular diseases and complications (Nair, Leitch et al. 1997, Rizos, Ntzani et al. 2012).

3.3.1 Synthesis and bioconversion

Plants can produce omega-3 PUFAs by the conversion of omega-6 linoleic acid (LA) to α -linoleic acid (ALA) with the help of the delta-15 desaturase enzyme. But as humans and other animals lack this enzyme, ALA is considered as an essential fatty acid (Scorletti and Byrne 2013). Animals can then synthesize other long chain omega-3 fatty acids such as EPA and DHA mainly from the hepatic bioconversion of ALA, but as this conversion is limited, EPA and DHA are often referred to as conditionally essential fatty acids (Calder and Yaqoob 2009). The bioconversion of ALA involves a delta-6 desaturase-mediated desaturation of ALA to form stearidonic acid (C18:3 n-3), followed by a chain elongation by the action of an elongase to form eicosatetraenoic acid (C20:4 n-3). It undergoes further desaturation by the delta-5 desaturase to form EPA (C20:5 n-3) that can be further converted to DHA (C22:6 n-3) through two elongations, one desaturation and one β -oxidation (in peroxisomes) with DPA (C22:5 n-3) as an intermediate of the first elongation (Calder and Yaqoob 2009) (Figure 19).

The bioconversion of omega-6 LA through the same enzymatic pathway result in the production of arachidonic acid (C20:4n-6, AA) and docosapentaenoic acid (C22:5 n-6) (Figure 19). Further cyclooxygenases- (COX) and lipoxygenases-mediated (LOX) bioconversion of AA result in the formation of the 2-series prostaglandins and thromboxanes, the 4-series leukotrienes, lipoxins, EET and HETE derivatives which are pro-inflammatory and atherogenic whereas metabolites of EPA and DHA, the 3-series prostaglandins and thromboxanes, the 5-series leukotrienes, protectins and resolvins are less inflammatory and vaso-protective in nature (Schmitz and Ecker 2008).

As omega-3 and omega-6 PUFAs undergo bioconversion by the same enzymatic pathway, they compete for these enzymes, thus limiting the bioconversion of omega-3

PUFAs. The first step of the conversion of LA to gamma-LA and/or the conversion of ALA to stearidonic acid is especially considered as a rate limiting step (Scorletti and Byrne 2013). Therefore, an increased dietary consumption of omega-3 PUFAs can shift the balance towards the production of less inflammatory and pathogenic metabolites (Scorletti and Byrne 2013).

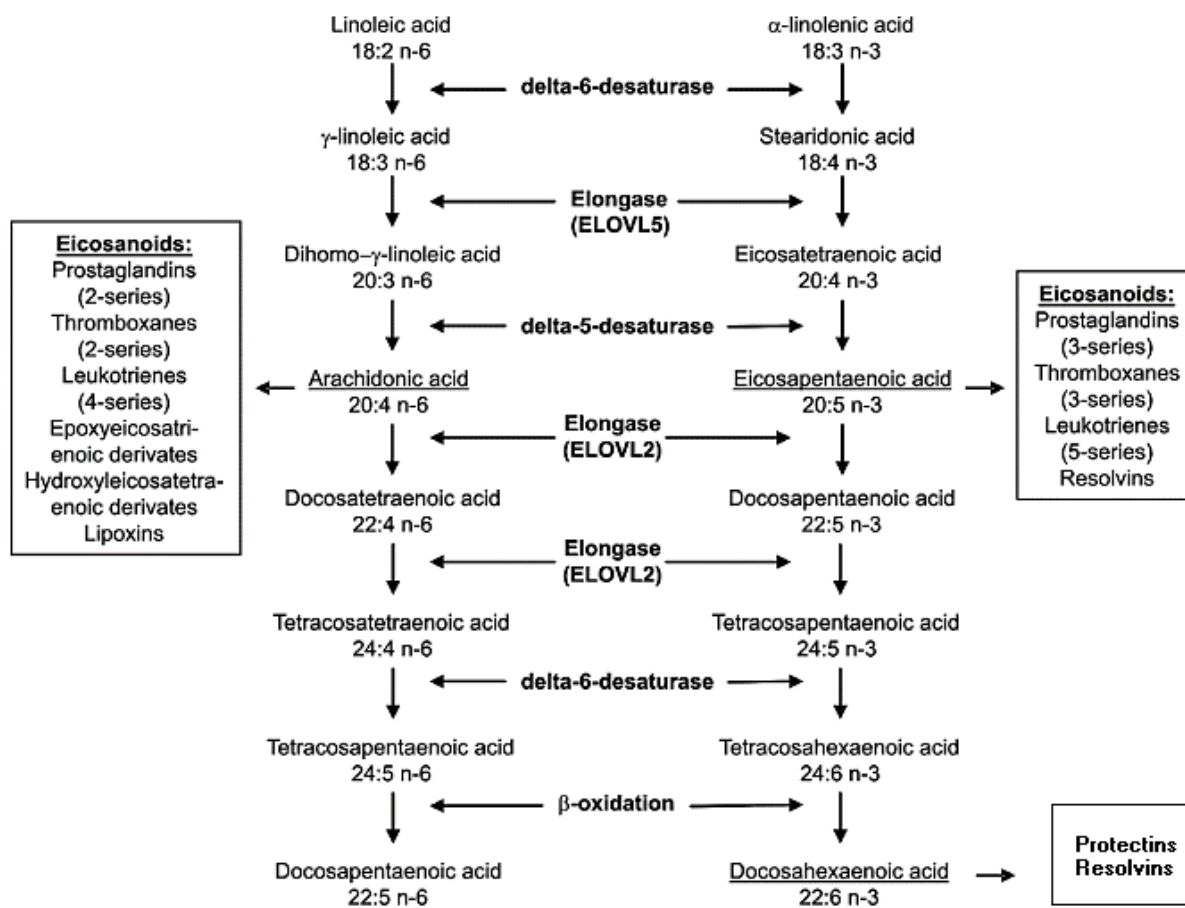


Figure 19: Bioconversion of linoleic acid and α -linolenic acid (Schmitz and Ecker 2008)

3.3.2 Dietary sources and intake

From plant origin, ALA is the main omega-3 PUFAs abundantly present in green leaf vegetables, seeds and nuts. Rich sources of ALA includes linseeds, flaxseeds, soybean, rapeseeds, walnuts and their oils. ALA is also present in small amounts in corn oil, sunflower oil, safflower oil (Burdge and Calder 2006, Scorletti and Byrne 2013).

The major animal source of omega-3 PUFAs is seafood, especially fish that is the most common source of long chain EPA and DHA. Flesh from fatty fishes (salmon, mackerel, tuna,

sardine and herring) contains higher concentration of omega-3 PUFAs than the flesh of lean fishes (cod), who contrary to fatty fish store lipids in the liver instead of the flesh. A single meal of fatty fish can provide 1.5-3 g of omega-3 fatty acids whereas the same portion of lean fish gives only 0.2-0.3 g. The lipids extracted from the flesh or livers of fish is generally called fish oil and is rich in omega-3 fatty acids (approximately 30%) along with fat soluble vitamins (A and E), palmitic acid and palmitoleic acid, and small amount of AA. The relative proportion of EPA and DHA also varies from fish to fish; e.g. cod liver oil contains more EPA whereas tuna oil contains more DHA (Calder and Yaqoob 2009). Algal oils are also an alternative source of omega-3 PUFAs (Lane, Derbyshire et al. 2014).

Average daily intake of omega-3 PUFAs varies from region to region due to cultural and dietary habits. Japanese have the highest mean intake of 5-6 g/day, followed by Eskimos with 3-4 g/day, whereas Australians consume only 0.189 g/day and Europeans and North Americans as low as 0.15-0.25 g/day (Scorletti and Byrne 2013).

Recommendations in 2002 update of Institute of Medicine for daily intake of ALA are 1.6 g for men and 1.1 g for women, whereas those for EPA and DHA are 0.25-2 g (2 fish meals/week) (Elmadfa and Kornsteiner 2009). According to the recommendations of the American Heart Association, one person should consume two fatty fish meals per week to reduce the risk of hypertriglyceridemia and CVDs, and for the treatment of hypertriglyceridemia the recommended intake of EPA and DHA is 2-4 g/day (Kris-Etherton, Harris et al. 2003).

3.4 Effects of Omega-3 PUFAs on Molecular Level

3.4.1 Alteration of cell membrane structure and function

The membrane lipid environment strongly influences the functioning of cells and organelles. The cell membrane is a lipid-bilayer structure composed mainly of phospholipids containing different fatty acids, and is imbedded with different membrane proteins. Omega-3 PUFAs gets incorporated into the membrane phospholipids, thus affecting membrane fluidity, permeability as well as the localization and function of different membrane proteins (Adkins and Kelley 2010). In particular, the omega-3 PUFAs impact the structure and the function of microdomains including lipid rafts and caveolae, which are the operational platforms of different cellular functions such as signaling pathways, cholesterol transport, endocytosis and

kinetics of ion channels. Membrane-incorporated omega-3 PUFAs can also act as substrates for different enzymes like COX, LOX and Cyp450 leading to the formation of vaso-protective metabolites (Figure 20) (Mozaffarian and Wu 2011). Previous studies have reported that omega-3 PUFAs is associated with the inhibition of PKC-theta lipid raft signaling and IL-2 production (Fan, Ly et al. 2004), and the inhibition of the lipopolysaccharide-mediated inflammation through a decreased recruitment and dimerization of toll like receptor-4 (Lee, Plakidas et al. 2003).

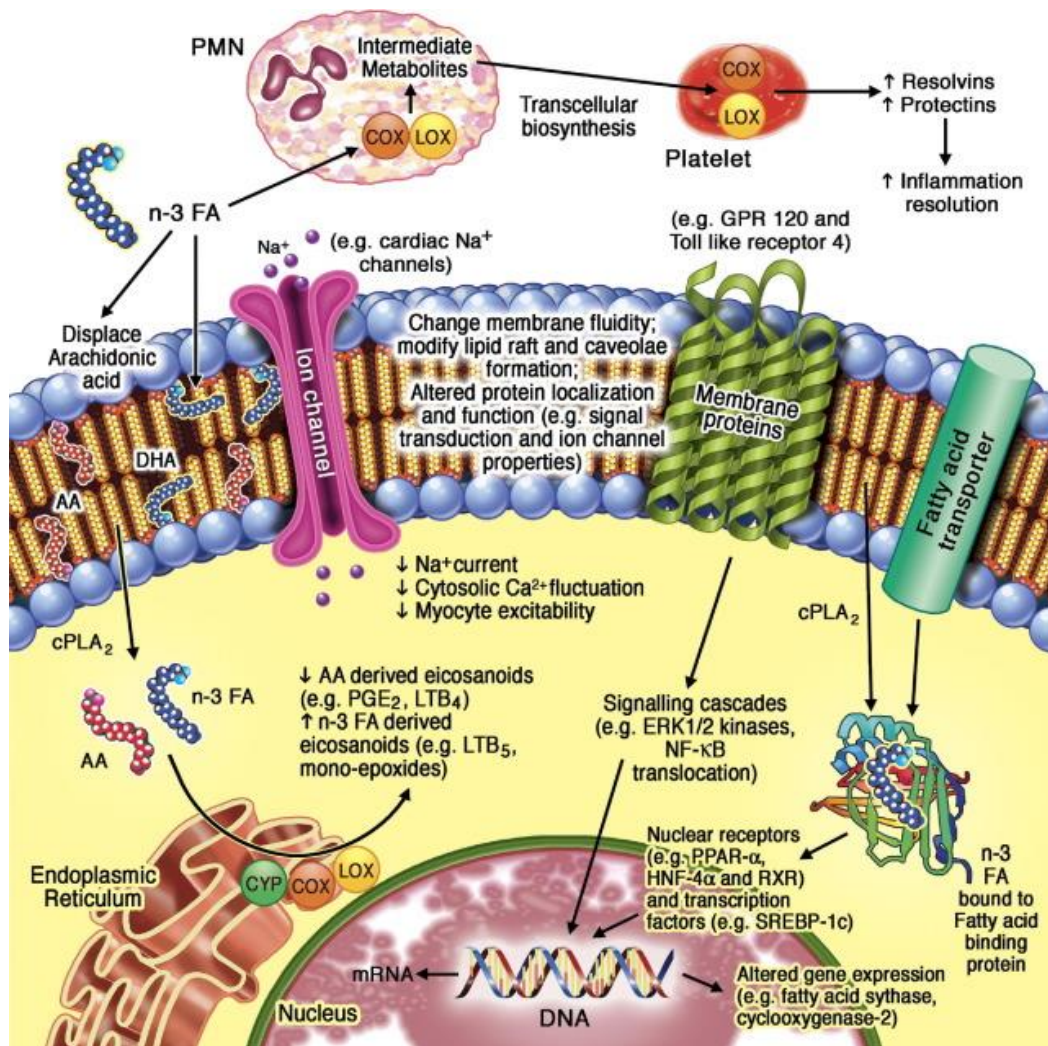


Figure 20: Molecular level effects of omega-3 PUFAs (Mozaffarian and Wu 2011)

3.4.2 Alteration of ion channel function

Membrane-incorporated omega-3 PUFAs can alter the function of membrane ion channels leading to decreases electrical excitability of the cell. In cardiomyocytes, omega-3

PUFAs alter electrophysiology via the functional alteration of membrane Na⁺ channels, Na⁺/Ca²⁺ exchanger and L-type Ca²⁺ channels (Ferrier, Redondo et al. 2002, Xiao, Ke et al. 2004, Dujardin, Dumotier et al. 2008). The combined effect of these modifications causes a reduced myocyte activation by increasing the threshold for membrane depolarization and decreasing the intracellular Ca²⁺ fluctuations, thus exerting anti-arrhythmic effect. Moreover, several clinical trials have suggested omega-3 PUFAs could be used as an alternative therapy for cardiac arrhythmias (Endo and Arita 2016).

3.4.3 Alteration of nuclear factors and transcription factors

The activation of NF-κB is associated with an increased gene expression of inflammatory mediators like cytokines (IL-1β, IL-2, IL-6, IL-12 and TNF-α), chemokines (MCP-1), inflammatory enzymes (iNOS and COX₂) and adhesion molecules (ICAM-1 and VACM-1) (Ghosh and Karin 2002). Omega-3 PUFAs decrease the activation of NF-κB through the inhibition of the phosphorylation of I-κB, its regulatory protein, via the activation of the G protein-coupled receptor-120 (GPR120) signaling pathway and the inhibition of toll like receptor-4 signaling pathway, resulting in a decreased production of inflammatory mediators and enzymes (Adkins and Kelley 2010, Endo and Arita 2016).

In addition, omega-3 PUFAs act as ligands of peroxisome proliferator-activated receptors (PPAR). The omega-3 PUFAs-mediated activation of PPAR-α and PPAR-γ receptors causes the inhibition of NF-κB binding activity, subsequently inhibiting the expression of inflammatory mediators such as IL-1β, IL-6 and TNF-α. The activation of PPAR-α receptor is also associated with an enhanced expression of fatty acid oxidation genes resulting in decreased triglycerides levels in the liver and plasma (Adkins and Kelley 2010).

3.4.4 Omega-3 PUFAs derived eicosanoids

Membrane phospholipids are hydrolyzed by the action of PLA₂ to release free fatty acids into the cytoplasm where they are metabolized by COX, LOX and Cyp450 to produce different eicosanoids. High membrane content of omega-6 fatty acids (AA) gives mainly eicosanoids such as COX-mediated 2-series prostaglandins (PGE₂, PGI₂, TXA₂), LOX-5 mediated 4-series leukotrienes and LOX-12 mediated epoxyeicosatrienoic acid (EET), hydroxy-eicosatetraenoic acid (HETE) and lipoxin-A₄ which are mainly vasoconstrictor, pro-inflammatory, platelets pro-aggregatory and pro-atherogenic. Dietary intake of omega-3 fatty acids result in the displacement of AA from cell membrane. Omega-3 PUFAs, and especially

3.5 Risk Factor Reduction for CVDs

Dietary intake of omega-3 PUFAs is associated with a decreased risk of CVDs risk factors such as elevated blood pressure, hyperlipidemia, arrhythmia, thrombosis, inflammation and endothelial dysfunction, increased vascular resistance and decreased myocardial efficacy (Bowen, Harris et al. 2016) (Figure 21).

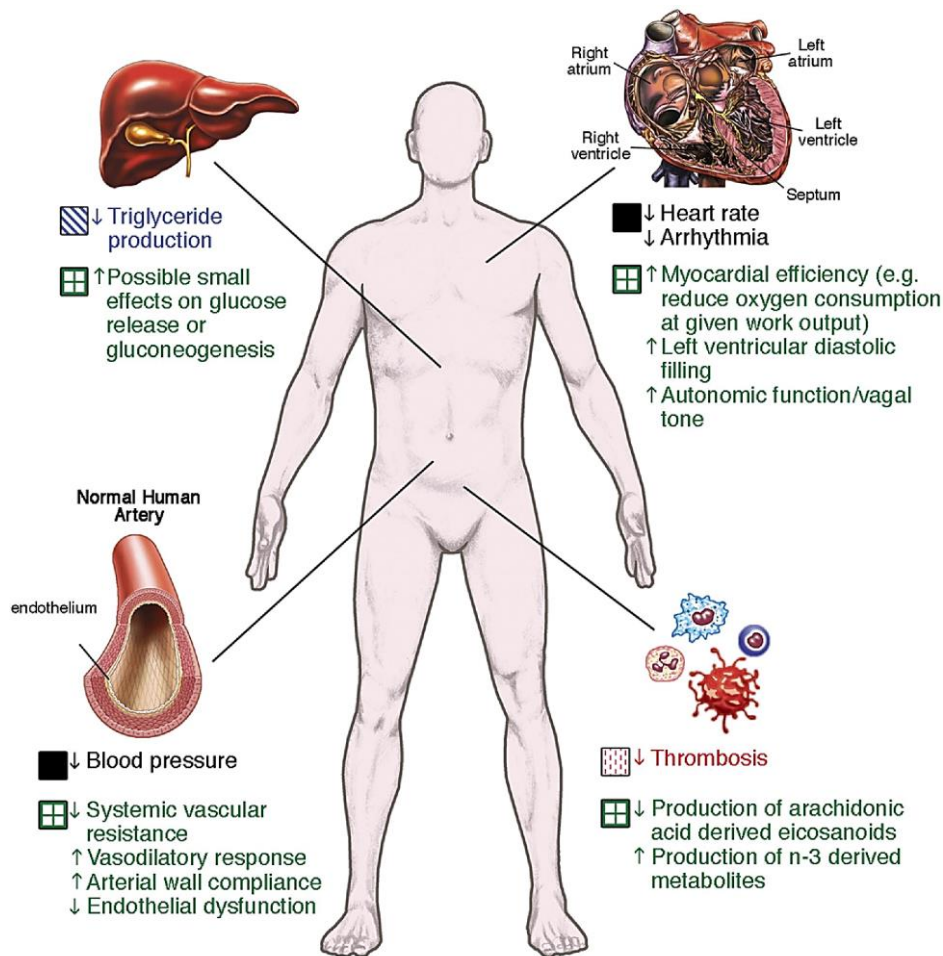


Figure 21: Mechanisms involved in risk reduction of cardiovascular diseases (Mozaffarian and Wu 2011)

Kromhout showed a strong correlation between the daily intake of omega-3 PUFAs-rich sea food and the reduction of risk for cardiovascular diseases, 50% reduction in risk of coronary heart disease was shown with a daily intake of 40-50g of sea food per day (Kromhout 1989) (Figure 22).

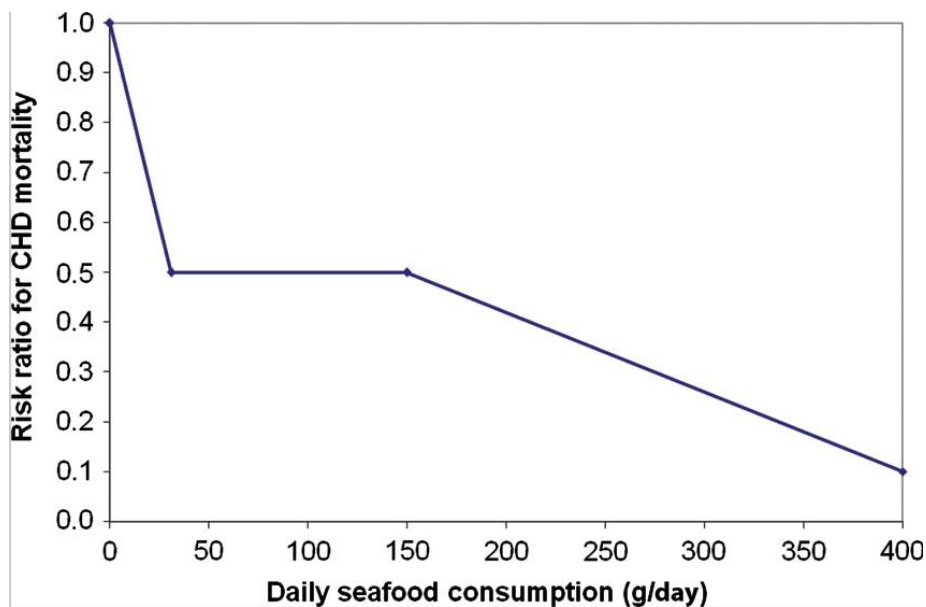


Figure 22: Association between consumption of sea food and risk for coronary heart disease (Kromhout 1989).

3.5.1 Blood Pressure

High blood pressure (BP) or hypertension is considered as the most important risk factor for cardiovascular diseases including stroke, myocardial infarction, heart failure, CHD, cardiac arrhythmia, atrial fibrillation (AF), left ventricular hypertrophy (LVH), valvular heart disease and peripheral arterial disease (Kjeldsen 2018). Omega-3 PUFAs intake through consumption of fish meals or fish oil supplements is associated with a lowering of BP. Indeed, dietary intake of 15 g/day of omega-3 PUFAs for 4 weeks has been shown to reduce systolic blood pressure by about 6.5 mmHg in mildly hypertensive patients (Knapp and FitzGerald 1989). Moreover, a meta-analysis of 31 clinical trials on 1536 subjects showed an average decrease of 3 and 1.5 mmHg in systolic and diastolic blood pressure, respectively (Morris, Sacks et al. 1993). Similarly, other meta-analyses have showed that the intake of more than 2 g/day of EPA+DHA for at least 3 weeks reduce systolic blood pressure by about 1.25 and 4.51 mmHg in normotensive and hypertensive subjects, respectively, whereas at least 3 g/day of omega-3 PUFAs for 3 weeks reduced SBP by about 1.5 and 5.5 mmHg in normotensive subjects and untreated moderately hypertensive patients, respectively (Appel, Miller et al. 1993, Miller, Van Elswyk et al. 2014). A recent meta-analysis studies concluded that to

achieve an antihypertensive effect of omega-3 PUFAs , relatively high doses (more than 3 g/day) are required (Cabo, Alonso et al. 2012).

The possible mechanism of this BP lowering effect is due, at least in part, to the production of omega-3 PUFAs-related vasodilatory prostaglandins such as PGI₃ and TXA₃ (Schmitz and Ecker 2008), the suppression of the Ang II-mediated vasoconstriction by a reduced expression of ACE and AT1 receptors (Cabo, Alonso et al. 2012, Niazi, Silva et al. 2017), the decreased oxidative stress, the increased NO-mediated vasorelaxation by activation of eNOS (Zgheel, Alhosin et al. 2014), and the decreased production of COX-derived EDCF (Niazi, Silva et al. 2017).

3.5.2 Plasma Lipids

Hyperlipidemia, characterized by higher blood levels of triglycerides (TG) and cholesterol, is also considered as one of the major risk factors of developing CVDs. Due to their insolubility in aqueous phase such as the plasma, lipids are transported through plasma associated to proteins in the form of particles called lipoproteins. Therefore, hyperlipidemia is classified based on lipoproteins such as high-density lipoproteins (HDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL), very low-density lipoproteins (VLDL) and chylomicrons. Among these, elevated LDL is considered most important risk factor for CVDs (Nelson 2013).

Dietary intake of omega-3 PUFAs is associated with a dose-dependent reduction of blood TG levels. Indeed, a daily intake of 4 g of omega-3 PUFAs is reported to lower serum TG by about 25-30%. Both EPA and DHA seems to be able of lowering TG and can serve as an alternative therapy for hyperlipidemia. The TG lowering effect of omega-3 PUFAs may be related to a reduced hepatic production of VLDL (which give LDL and IDL by lipolysis) due to a reduced fatty acid lipogenesis causing a shortage of substrate, an enhanced β -oxidation of fatty acids in chylomicrons causing a lower delivery of non-esterified fatty acids to the liver, a competitive inhibition of hepatic enzymes related to TG production and an enhanced hepatic production of phospholipids instead of TG (Mori 2014). DHA is associated with a reduced concentration of pro-atherogenic small dense LDL particles (Kelley, Siegel et al. 2007). DHA is also associated with a reduction of Apo CIII protein level which inhibits lipoprotein lipase (LPL), thus enhancing clearance of TG from blood. The regulation of Apo CIII mediated by the omega-3 PUFAs is characterized by the activation of PPAR α causing the downregulation of Apo CIII and by the inhibition of NF- κ B which upregulates Apo CIII (Adkins and Kelley

2010). Omega-3 PUFAs also reduces the peak of postprandial lipemia after a fatty meal, which is characterized by the production of pro-atherogenic and pro-thrombogenic lipoproteins (Jain, Aggarwal et al. 2015).

3.5.3 Heart rate

Increased heart rate is associated with sudden cardiac death. An increase of heart rate by 10 beats per minutes (bpm) increases the risk of cardiac death by 20%. Moreover, the risk of cardiac death is doubled in individuals having a heart rate of more than 90 bpm as compared to the ones with less than 90 bpm (Perret-Guillaume, Joly et al. 2009).

Omega-3 PUFAs intake is associated with a decrease in resting heart rate (O'Keefe, Abuissa et al. 2006). Moreover, the ingestion of daily fishmeal by obese hypertensive patients showed a reduction of 4.3 bpm in the awake heart rate (Bao, Mori et al. 1998). However, DHA but not EPA is associated with the heart rate reduction (Mori 2014). The possible mechanism of heart rate reduction is a direct effect of omega-3 PUFAs on myocytes through the incorporation into the cell membrane, thus altering their electrophysiology by the inhibition of L-type Ca^{2+} channels and membrane Na^{+} channels, leading to reduced excitability and altered parasympathetic activity and vagal tone (Mozaffarian, Geelen et al. 2005).

3.5.4 Arrhythmia

Irregularities in the myocardial electrical activity leading to irregular heartbeats is termed as cardiac arrhythmia. Cardiac arrhythmias are among major risk factors for sudden cardiac death (Adabag, Luepker et al. 2010). Clinical studies including GISSI-Prevenzione reported the beneficial role of omega-3 PUFAs in the prevention of arrhythmia-associated cardiac death (Endo and Arita 2016). The possible mechanism is most probably due to be modification of membrane ion channel via the incorporation into myocyte membrane, the prolongation of the refractory period via the inhibition of voltage-gated Na^{+} channels and the reduction of cytosolic free Ca^{2+} via inhibition of L-type Ca^{2+} channels. Omega-3 PUFAs also increase the vagal tone thus altering the autonomic activity in the myocardium (Kromhout, Yasuda et al. 2012).

3.5.5 Thrombosis

Platelet aggregation leading to clot formation, also known as thrombosis, is risk factor for cardiac death via thrombo-embolism. Higher bleeding tendency due to reduced thrombogenesis is reported in eskimos and is associated with the high intake of fish (Bang and Dyerberg 1980). Omega-3 PUFAs are associated with the modification of prostanoids profile causing the inhibition of platelet aggregation. Omega-3 PUFAs intake modify the AA/omega-3 ratio in platelet membrane thus causing decreased production of TXA₂, a potent inducer of platelet aggregation, and increased production of EPA-derived TXA₃ and PGI₃ which exert anti-aggregatory activity (Kromhout, Yasuda et al. 2012, Mori 2014). Moreover, the LOX-mediated dihydroxylation of DHA results in production of the protectin DX which can cause the inhibition of COX-1 and COX-2 in platelets leading to a decrease in their pro-aggregatory potential (Calvo, Martínez et al. 2017).

3.5.6 Inflammation

Inflammation is associated with an increased risk of cardiovascular diseases including atherosclerosis, endothelial dysfunction, myocarditis, myocardial infarction, aortic dissection and remodeling (Endo and Arita 2016). Omega-3 fatty acids have anti-inflammatory and immunomodulatory effects targeting the multiple mediators of inflammation. They exerts their effects through the modification of eicosanoid profile, reduction of the production of inflammatory cytokines, modulation of the immune cells activity and of the production of pro-resolution lipid-derived prostanoids (Mori 2014). Omega-3 fatty acids can modify the eicosanoids balance by reducing membrane ratio of AA/omega-3 leading to a reduce formation of AA-derived pro-inflammatory prostanoids including PGE₂, TXB₂, LTB₄ and eicosatrienoic acid derivatives and an increased formation of omega-3 PUFAs-related products exerting less pro-inflammatory activity such as PGE₃ and LTB₅ (Kromhout, Yasuda et al. 2012). Omega-3 PUFAs also reduce the LTB₄-associated chemoattraction of leukocytes. Moreover, omega-3 PUFAs have been shown to cause a GPR120- and PPAR α -mediated inhibition of NF- κ B, subsequently leading to a decreased production of inflammatory cytokines, chemokines and adhesion molecules. The resolution of inflammation by omega-3 PUFAs is also related to the production of omega-derived mediators such as resolvins, protectins and maresins (Calder 2013). In addition, fish oil intake has been associated to a reduced proliferation of T-cells leading to reduced inflammatory responses (Thies, Nebe-von-Caron et al. 2001).

3.5.7 Endothelial function

As discussed earlier, the vascular endothelium plays an important role in the maintenance of the vascular health, and its dysfunction is considered as a hallmark of cardiovascular diseases. Endothelial dysfunction is generally characterized by a decreased production of EDRF, especially NO, and an increased production of EDCF. Moreover, the endothelial dysfunction could be associated to an increased vascular oxidative stress.

Several clinical and preclinical studies have shown that omega-3 PUFAs intake is associated with an improvement of the endothelial function (Mori 2014). Indeed, omega-3 PUFAs are reported to augment NO production through the activation of eNOS by several pathways. EPA induced eNOS activation by stimulating the AMP-activated protein kinase (AMPK) (Wu, Zhang et al. 2012) and by increasing the dissociation of eNOS from caveolin (Omura, Kobayashi et al. 2001). Whereas several studies showed that EPA and DHA can induce the activation of eNOS, the potency of the induction of eNOS is dependent both on the purity and the formulation of EPA and DHA (Zgheer, Alhosin et al. 2014). Indeed, previous study from the laboratory have shown that the potency of EPA:DHA formulation to induce the activation of the endothelial function is due to a redox-sensitive activation of the Src/PI3-kinase/Akt pathway leading to the activating phosphorylation of eNOS on the Ser1177 residue. Moreover, the ability of the EPA:DHA formulation to induce the activation of eNOS was dependent on both the quantity of omega-3 and on the ratio, with EPA:DHA 6:1 and 9:1 being the most active formulations.

In addition, omega-3 PUFAs intake also decrease the circulating concentration of the ADMA, an endogenous inhibitor of eNOS, thus leading to a reduced inhibition of eNOS, in spontaneously hypertensive rats (Raimondi, Lodovici et al. 2005). Similarly, omega-3 PUFAs intake is also reported to decrease oxidative stress in hypertensive rats, possibly due to a decreased activation of the local angiotensin system and NADPH oxidase activity (Niazi, Silva et al. 2017). Moreover, omega-3 PUFAs can increase superoxide dismutase (SOD) activity in endothelial cells (Shen, Shen et al. 2012). Omega-3 PUFAs intake can also decrease EDCFs in hypertensive rats, most probably through a normalization of the expression of COX-1 and COX-2 (Niazi, Silva et al. 2017). Moreover, omega-3 PUFAs caused the reduction of inflammation via decreased activity of NF- κ B leading decreased production of adhesion molecules and inflammatory cytokines (Zanetti, Grillo et al. 2015). All these actions lead to improvement of endothelial function and vascular health.

3.6 Clinical Studies on Omega-3 PUFAs

Since several observational studies have reported a beneficial effect of omega-3 PUFAs on the cardiovascular system, their potency to prevent major adverse cardiovascular events were assessed in randomized clinical trials.

3.6.1 DART 1989

It was the first randomized controlled trial on the cardiovascular protective effects of omega-3 PUFAs. This “Diet and Reinfarction Trial (DART)” aimed to determine the effect of omega-3 PUFAs in post-myocardial infarction patients. In total, 2033 male patients were randomly assigned to 4 groups which were advised to either i) lower their fat intake, ii) increase polyunsaturated to saturated fatty acid ratio, iii) increase their consumption of fatty fish, or iv) were without any advice. The omega-3 fatty acids were consumed in the form of 2 servings of fatty fish per week (ii) or as 1.5 g/day in form of fish oil capsules (iii). The patients were followed for 2 years to monitor total death or ischemic cardiac event. The Fatty fish or fish oil groups showed a 27.6 % reduction in total deaths and a 32% reduction in deaths due to ischemic cardiac event (Burr, Fehily et al. 1989, Bowen, Harris et al. 2016).

3.6.2 GISSI-Prevention 1999

This randomized controlled trial called “Gruppo Italiano Per lo Studio della Sopravvivenza nell’Infarto Miocardico Prevenzione” was performed on 11323 post-myocardial patients. Patients were randomly assigned into four groups receiving supplementation with either omega-3 PUFAs as fish oil providing 850 to 882 mg/day of EPA and DHA, 300 mg/day of vitamin E, a combination of omega-3 plus vitamin E, or no supplementation. The endpoints of death, myocardial infarction or stroke were monitored for 3.5 years in each group. In the fish oil group, a 41% reduction in the risk of all cause death and a 53% reduction in the risk of cardiac death were reported after 3 and 4 months of treatment, respectively. Similarly, a 30% reduction in the risk of cardiovascular death and 45% reduction in the risk of sudden death was reported at the end of the study for the omega-3 treatment (1999, Bowen, Harris et al. 2016).

3.6.3 GISSI-HF 2004

“GISSI-Heart Failure” was a first large randomized, double blinded and placebo-controlled trial to assess the beneficial effects of omega-3 PUFAs in symptomatic heart failure

patients. Total of 6975 patients with chronic heart failure were treated either with 850-882 mg/ml EPA and DHA or the placebo. After the patients were followed for an average of 3.9 years, the study reported a 15% reduction in all-cause mortality, 20% reduction in cardiovascular hospitalization or 1252 deaths. Omega-3 PUFAs treated group showed 9% reduction in all-cause mortality and 8% reduction in cardiovascular hospitalization (Tavazzi, Maggioni et al. 2008, Bowen, Harris et al. 2016).

3.6.4 JELIS 2007

In this study called “Japan EPA Lipid Intervention Study (JELIS)”, 18645 hyperlipidemic patients with a plasma total cholesterol level of 6.5 mmol/L or higher were treated with 1800 mg/ day of EPA on top of statins or with statins alone. They were followed for an average of 4.6 years for any major coronary event including sudden cardiac death, myocardial infarction, angina pectoris and treatment interventions like angioplasty, stenting or coronary artery bypass. EPA group patients with a history of coronary artery diseases showed a significant 19% reduction in the major coronary events (secondary prevention), whereas in the patients with no history of coronary artery disease (primary prevention) the reduction of 18% in major coronary events was not significant (Yokoyama, Origasa et al. 2007, Bowen, Harris et al. 2016).

4-AIM OF THE STUDY

Cardiovascular diseases are the major of death worldwide and should remain the leading cause of mortality and morbidity over the next few decades. Age is an important risk factor for the development of cardiovascular diseases associated with increased thickness and stiffness of arterial walls. In addition, ageing is associated with a decreased regulation of vascular tone due to an age-related endothelial dysfunction. The endothelial dysfunction is generally characterized by a reduced formation of vasoprotective factors including nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH), and an increased production of vasoconstricting factors such as cyclooxygenase (COX)-derived metabolites of arachidonic acid involved in the endothelium-dependent contractile factors.

The dietary intake of omega-3 polyunsaturated fatty acids (PUFAs), including the two major compounds eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to reduce the risk of cardiovascular diseases in several epidemiological and both primary and secondary prevention studies. The beneficial effects of omega-3 PUFAs could be due, at least in part, to their ability to activate the endothelial function. Indeed, purified formulations of EPA and DHA are able to induce potent and sustained endothelium-dependent relaxations of isolated artery rings involving both the NO- and EDH-mediated components of the relaxation. Moreover, a previous study by our research team has indicated that the omega-3 PUFAs-induced endothelium-dependent vasorelaxation is dependent on both the purity and ratio of EPA:DHA, with EPA:DHA ratio of 6:1 and 9:1 being superior formulations (Zgheel et al., 2014).

In a previous study by our research team, EPA, DHA and different ratio of EPA and DHA (EPA:DHA 9:1, 6:1, 3:1, A 1:1, 1:3, 1:6 and 1:9) were assessed for their potency to induce endothelium-dependent relaxations and omega-3 EPA:DHA 6:1 was the most potent formulation for the induction of both NO- and EDH-mediated responses in porcine coronary artery rings. Moreover, the cellular mechanism was described as the activation of eNOS through the redox-sensitive activation of the Src/PI3-kinase/Akt pathway (Zgheel, Alhosin et al. 2014). In addition, the oral intake of the omega-3 EPA:DHA 6:1 formulation has been shown to partially but significantly prevent the angiotensin II-induced hypertension and associated endothelial dysfunction (Niazi et al., 2017). The major goal of this study was to determine whether a 2-weeks chronic intake of the omega-3 PUFAs EPA: DHA 6:1 formulation can improve the endothelial function in presence of an already established age-related endothelial dysfunction in 20 months-old rats.

More specifically the aims were

1. To study the effect of EPA:DHA 6:1 on the endothelium-dependent relaxations in mesenteric artery rings.
2. To evaluate the ability of EPA:DHA to reduce endothelium-dependent contractile responses in mesenteric artery rings.
3. To determine the effect of EPA:DHA 6:1 on the age-related vascular oxidative stress.
4. To study the effect of EPA:DHA 6:1 on the activation of the angiotensin system and the induction of senescence in the vascular wall.

5-RESULTS

The omega-3 fatty acids formulation EPA:DHA 6:1 improves ageing-related endothelial dysfunction by enhancing endothelium-dependent relaxations and reducing contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases

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The endothelium plays a central role in the regulation of the vascular homeostasis through the synthesis and secretion of various potent vasoconstricting and vasodilating factors including NO and EDH. In addition, they also exert potent anti-atherothrombotic effects by decreasing the endothelial expression of adhesion molecules, limiting smooth muscle cell proliferation and migration, and inhibiting monocyte adhesion and platelet aggregation. Thus, endothelial dysfunction is an early event for the development and progression of most of the cardiovascular diseases such as hypertension, atherosclerosis leading to myocardial infarction, stroke and peripheral artery diseases, and heart failure. Endothelial dysfunction is generally characterized by impaired endothelium-dependent relaxations involving a reduced NO bioavailability, a decreased EDH component of relaxation and an increased production of endothelium-derived contractile factors (EDCF).

Various studies demonstrate that regular intake of fish products and dietary consumption of fish or fish oil rich in omega-3 PUFAs, particularly EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) has been related to a reduced risk of cardiovascular disease morbidity/mortality.

The aim of the present study was to determine whether chronic intake of EPA:DHA 6:1 improves ageing-related endothelial dysfunction, and, if so, to determine the underlying mechanisms.

The omega-3 fatty acids formulation EPA:DHA 6:1 improves ageing-related endothelial dysfunction by enhancing endothelium-dependent relaxations and reducing contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases

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ABSTRACT

Objectives: Omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acids (DHA) have been shown to protect the cardiovascular system and to cause potent endothelium-dependent nitric oxide (NO)-mediated relaxations, with EPA:DHA 6:1 being a superior formulation. The aim of the present study was to determine whether chronic intake of EPA:DHA 6:1 improves ageing-related endothelial dysfunction, and, if so, to determine the underlying mechanisms.

Methods: Old Male Wistar rats (20 months-old) received daily by gavage 500 mg/kg/day of either EPA:DHA 6:1, corn oil or water for 2 weeks. Young male Wistar rats (12 weeks-old) were used as control. Main mesenteric artery reactivity was assessed using organ chambers, proteins level by immunofluorescence, and oxidative stress using dihydroethidium.

Results: In the main mesenteric artery, ageing was associated with an endothelial dysfunction characterized by a reduced relaxation to acetylcholine (ACh) involving a blunted NO-mediated component (as assessed in the presence of indomethacin, UCL-1684 and TRAM-34) whereas the endothelium-dependent hyperpolarization (EDH, as assessed in the presence of indomethacin and N^o-nitro-L-arginine)-mediated component was abolished, and also by the induction of endothelium-dependent contractile responses (EDCFs, as assessed in the presence of N^o-nitro-L-arginine, UCL-1684 and TRAM-34) sensitive to indomethacin (a cyclooxygenase inhibitor). Endothelial dysfunction was associated with an increased level of vascular oxidative stress and expression of cyclooxygenase-2 (COX-2) and the downregulation of COX-1 throughout the vascular wall of the mesenteric artery, and with an increased expression of eNOS, activation of the local angiotensin system and increased expression of senescence markers p53, p16 and p21 in the endothelium. Chronic intake of EPA:DHA 6:1 improved the NO-mediated relaxations and reduced EDCFs, vascular oxidative stress and normalized the expression of protein markers in aged arterial wall.

Conclusions: The present findings indicate that 2-week intake of EPA:DHA 6:1 by very old rats improved the ageing-related endothelial dysfunction. The beneficial effect of EPA:DHA 6:1 involves an improved NO-mediated relaxation and a reduction of endothelium-dependent contractile responses most likely by preventing activation of the local angiotensin system and the subsequent vascular oxidative stress.

INTRODUCTION

Cardiovascular diseases such as ischemic heart disease and stroke are one of the major causes of deaths, causing 32% of deaths worldwide in 2013 (Roth *et al.*, 2015). Age is an important risk factor for the development of cardiovascular diseases. Ageing of the vascular system results in increased thickness and stiffness of arterial walls and decreased regulation of vascular tone involving an age-related endothelial dysfunction (North *et al.*, 2012). The vascular endothelium plays a very important role in cardiovascular health by mainly producing potent vaso-protective agents including nitric oxide (NO) and the endothelium-dependent hyperpolarization (EDH). Ageing is associated with an endothelial dysfunction characterized by a decreased production of these vaso-protective agents, as well as an increased production of endothelium-derived contractile factors (EDCF) and vascular oxidative stress (Dal-Ros *et al.*, 2012; El Assar *et al.*, 2012; Idris Khodja *et al.*, 2012). Moreover, ageing is associated with an increased formation of reactive oxygen species (ROS) through multiple cellular sources including NADPH oxidase, cyclooxygenases (COXs), mitochondrial respiratory chain and uncoupled endothelial nitric oxide synthase (eNOS) (Dal-Ros *et al.*, 2012; Idris Khodja *et al.*, 2012).

Several studies have shown that omega-3 polyunsaturated fatty acids (PUFA), including the two main compounds named eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can improve the endothelial dysfunction in different pathological conditions and reduce the risk of cardiovascular diseases (Tousoulis *et al.*, 2014; Zanetti *et al.*, 2017; Zehr *et al.*, 2018). Formulations of purified EPA and DHA can directly induce endothelium-dependent relaxations mainly through activation of the NO and EDH pathways (Limbu *et al.*, 2018). Moreover, our previous study demonstrated that amongst several omega-3 PUFAs formulations, EPA:DHA 6:1 was identified as a potent stimulator of the endothelial formation of nitric oxide (NO), and also, to a lesser extent, of endothelium-dependent hyperpolarization

(EDH) in porcine coronary arteries (Zgheel *et al.*, 2014). The increased endothelial NO formation is mediated by a redox-sensitive activation of Src/PI3-kinase/Akt and MAPKs pathways leading to endothelial NO synthase (eNOS) activation, which is dependent on both ratio and purity of the EPA:DHA formulation (Zgheel *et al.*, 2014). The oral intake of the omega-3 EPA:DHA 6:1 formulation has been shown to partially but significantly prevent the angiotensin II-induced hypertension and associated endothelial dysfunction in Wistar rats (Niazi *et al.*, 2017).

Therefore, the aim of the present study was to investigate the possibility that a 2 weeks oral intake of the omega-3 EPA:DHA 6:1 formulation may improve an established age-related endothelial dysfunction in Wistar rats, and if so, to determine the mechanisms involved.

METHODS

Ethic 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) and the French Legislation. The protocole for this study was approved by the local ethics committee (Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg, approval #7626-2016111715542930 v2).

Preparation of Omega-3 PUFA

Purified formulations of EPA and DHA were obtained from Pivotal therapeutics, Inc. (Woodbridge, ON, Canada). EPA:DHA 6:1 (w/w) ratio was prepared by mixing purified formulations according to their relative purity and the resultant solution was then aliquoted in amber colored vials. All the process was done under nitrogen flux to avoid oxidation of omega-3 PUFA.

In vivo treatment of Rats

Thirty Wistar rats were kept in the animal facility and given free access to standard diet and tap water from the age of 12 weeks until they were 20 months-old. They were then divided randomly into 3 groups and were administered daily by gavage with 500 mg/kg/day of either omega-3 EPA:DHA 6:1, corn oil (isocaloric control without omega-3) or tap water (untreated control) for 14 days. A group of 12 weeks-old rats was used as young control. After 14 days of treatment, rats were euthanized by an intraperitoneal injection of a lethal dose of pentobarbital (150 mg/kg) before collection of organs.

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% O₂, 5% CO₂) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.18, CaCl₂ 1.25, NaHCO₃ 25 and D-glucose 11, pH 7.4, 37°C) for the determination of changes in isometric tension. Rings were allowed to stabilize for 30-45 minutes at a pretension of 1 g before the reactivity of the vascular smooth muscle was assessed in response to Krebs buffer containing 80 mM of potassium. The integrity of the endothelium was checked with acetylcholine (ACh 1 μM) after a sub-maximal contraction (about 70%) with phenylephrine (PE 1 μM). To assess the endothelium-dependent relaxations, rings were contracted with PE (1 μM) before the construction of concentration-relaxation curves in response to ACh. To assess the contractile responses, rings were subjected to a concentration-contraction curves in response to PE. In some experiments, 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, an unselective COX inhibitor). To study NO-mediated relaxation, rings were incubated in the presence of indomethacin and TRAM-34 plus UCL-1684 (1 μM each, inhibitors of IK_{Ca} and SK_{Ca}, respectively) to prevent the formation of

vasoactive prostanoids and EDH-mediated relaxation, respectively. The EDH-mediated relaxation was studied in rings incubated with indomethacin and N^o-nitro-L-arginine (L-NA, 300 μM, an eNOS inhibitor) to prevent the formation of vasoactive prostanoids and NO, respectively. To study endothelium-dependent vasoconstrictor factors (EDCFs), rings were exposed to L-NA and TRAM-34 plus UCL-1684 to prevent NO and EDH, respectively, with or without indomethacin, before pre-contraction by about 30% with PE and the subsequent construction of a concentration-contraction curve in response to ACh. To assess the endothelium-independent relaxations, rings were contracted with PE (1 μM) before the construction of concentration-relaxation curves in response to either sodium nitroprusside (SNP, a NO donor) or levcromakalim (Lev, an ATP-sensitive potassium channels opener) in the presence of indomethacin, L-NA and TRAM-34 plus UCL-1684.

Immunofluorescence studies

Rings of the main mesenteric artery and of the thoracic aorta were embedded in histo-molds containing Tissue-Tek optimum cutting temperature (OCT) compound (Sakura 4583, Leiden, The Netherlands) and were snap-frozen in liquid nitrogen. Rings were cryosectioned at 14 μm and stored at -80°C until use. Sections were defrosted with phosphate buffer saline (PBS) and fixed for 1 hour with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) prior to blocking for 2 hours at room temperature with 10 % non-fat powdered milk in PBS containing 0.1% 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 COX-1 (1/250, Cat. ab109025, Abcam, Paris, France), COX-2 (1/200, Cat. Ab15191, Abcam), eNOS (1/100, Cat. 610297, BD Transduction Laboratories, Le Pont de Claix, France), p16 (1/200, Cat. db018, Delta Biolabs, California, USA), p21 (1/100, Cat. Sc817, Santa Cruz Biotechnology, Clinisciences, Nanterre,

France), p53 (1/100, Cat. Sc6243, Santa Cruz Biotechnology), p22^{phox} (1/100, Cat. Sc11712, Santa Cruz Biotechnology), p47^{phox} (1/100, Cat. Sc7660, Santa Cruz Biotechnology), AT1R (1/200, Cat. Sc1173, Santa Cruz Biotechnology) and ACE (1/200, Cat.250450, Abbiotec, San Diego, USA). Next day, all sections were washed with PBS prior to incubation with a solution of blocking buffer containing a fluorescent secondary antibody (Alexafluor 633 anti-rabbit or anti-mouse) for 2 hours 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 (Ref. S3023, Dako, California, USA) 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 1.49 for Windows, NIH).

Determination of *in situ* level of ROS

ROS was determined using red-ox sensitive fluorescent probe dihydroethidium (DHE). 25 μ m cryosections of mesenteric artery or aorta were defrosted with PBS and incubated with DHE (2.5 μ M) for 30 minutes at 37°C in a black box to protect from light. To determine the sources of ROS, aorta sections were pretreated with inhibitors such as N-acetylcysteine (antioxidant), indomethacin (inhibitor of COX), NS-398 (COX-2 inhibitor), SC-560 (COX-1 inhibitor), L-NA (inhibitor of eNOS), VAS-2870 (inhibitor of NADPH oxidase) and inhibitors of mitochondrial respiratory chain (KCN, myxothiazole, potassium cyanide and rotenon) 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 fluorescence solution. Slides were allowed to dry in the dark for 20 minutes before being analyzed with confocal laser scanning microscope. Quantitative analysis was performed using Image J.

Statistical Analysis

Values were expressed as mean \pm S.E.M. Statistical analysis was performed using unpaired T test using Graph Pad prism 5. Values were considered statistically for $P < 0.05$.

RESULTS

2-weeks chronic intake of EPA:DHA 6:1 improves the age-related endothelial dysfunction

To assess the effect of a 2-weeks chronic oral intake of the omega-3 EPA:DHA 6:1 formulation on an established age-related endothelial dysfunction, vascular reactivity study was performed on rings from the main mesenteric artery. After precontraction with PE (1 μ M), rings from old rats showed significantly decreased relaxations in response to ACh (0.1-10 μ M) in comparison with rings from young rats (Figure 1A). Similarly, rings from old rats showed significantly increased contractions in response to increasing concentrations of PE (1-10 μ M) in comparison with those from young rats (Figure 1B). Treatment with EPA:DHA 6:1, but not with corn oil, significantly improved ACh-induced relaxation and reduced PE-induced contraction in the main mesenteric artery of old rats (Figure 1A & 1B).

To assess the amount of EDCF, rings were preincubated with L-NA and TRAM-34 plus UCL-1684 to prevent NO- and EDH-mediated relaxation. Rings from untreated old rats showed significantly higher contraction in response to ACH as compared to rings from young rats (Figure 1C) and this contractile response was abolished in presence of indomethacin (Figure 1D) showing the increased formation of COX-derived EDCFs in the main mesenteric artery of old rats. Treatment with EPA:DHA 6:1 partially, but significantly, reduced the EDCF-mediated contractions in the main mesenteric artery of old rats, whereas corn oil was without effect.

In addition, the endothelium-independent relaxations in response to either SNP or Lev was not significantly altered with ageing, indicating that the age-related endothelial dysfunction

does not involve altered responses of the vascular smooth muscle in the main mesenteric artery (Figure 1E and 1F).

2-weeks chronic intake of EPA:DHA 6:1 improves the blunted NO-component of the relaxation and decrease the formation of EDCF

In the rings from the main mesenteric artery of young rats, ACh-induced relaxation was significantly reduced in presence of L-NA, and slightly but significantly reduced TRAM-34 plus UCL-1684, indicating the involvement of both NO- and EDH-mediated components of the endothelium-dependent relaxation (Figure 2A). In comparison, rings from untreated old rats showed an abolished relaxation in presence of L-NA, whereas TRAM-34 plus UCL-1684 were without effect, indicating that the age-related endothelial dysfunction is characterized by a reduced NO-mediated component of the relaxation and an abolished EDH-mediated relaxation (Figure 2B). Treatment of old rats with EPA:DHA 6:1 or corn oil had no effect of the relaxation in presence of either L-NA or TRAM-34 plus UCL-1684, indicating that the improved ACh-induced relaxation in the EPA:DHA 6:1 group is due, at least in part, to improved NO-mediated relaxation whereas the EDH-mediated relaxation remains abolished (Figure 2 C & D).

In rings from the main mesenteric artery of old rats, the PE-induced contraction was significantly reduced in presence of indomethacin, indicating an age-related increase in the basal formation of COX-derived vasoconstricting prostanoids (Figure 2F). Treatment with EPA:DHA 6:1, but not with corn oil, suppress the effect of indomethacin on the PE-induced contraction, suggesting a normalization of vasoconstricting prostanoids formation, and the increased contractile response to PE in presence of L-NA suggest an increased basal formation on NO in the main mesenteric artery, as indicated by (Figure 2 G & H).

2-weeks chronic intake of EPA:DHA 6:1 improves the age-related altered expression of eNOS and COXs in the vascular wall of the mesenteric artery and aorta

As the age-related endothelial-dysfunction in the mesenteric artery was characterized by a reduced NO-mediated component of the relaxation and an increased formation of COX-derived EDCF, the expression of the proteins involved in these pathways, the eNOS and COXs, respectively, were determined by immunofluorescence in the vascular wall of both the main mesenteric artery and thoracic aorta. eNOS was expressed only in the endothelium, whereas COX-1 and COX-2 were expressed throughout the vascular wall in both the mesenteric artery and the aorta in all groups. Compared to sections of young rats, the sections of old rats showed an increased expression of eNOS and COX-2, and a decreased expression of COX-1 (Figure 3). Treatment of old rats with EPA:DHA 6:1, but not corn oil, normalized the expression of eNOS, COX-2 and COX-1 in the endothelium of both mesenteric artery and thoracic aorta (Figure 3).

2-weeks chronic intake of EPA:DHA 6:1 improves the age-related vascular oxidative stress

Since aging related endothelial dysfunction is associated with increased level of oxidative stress (Dal-Ros *et al.*, 2012; Idris Khodja *et al.*, 2012; El Assar *et al.*, 2013), the level of oxidative stress was assessed by histochemistry in the vascular wall of mesenteric artery and thoracic aorta. Compared to young rats, mesenteric artery and aorta from untreated and corn oil treated old rats showed significantly higher levels of ethidium fluorescence, indicating an increased formation of ROS (Figure 4 A & B). Treatment of old rats with EPA:DHA 6:1, but not corn oil, normalized the level of oxidative stress in the sections of both mesenteric artery and thoracic aorta (Figure 4 A & B).

Moreover, the level of oxidative stress in the aorta of old rats was significantly reduced in presence of either N-acetylcystein (NAC, an antioxidant), indomethacin (non-selective COXs inhibitor), SC-560 (a selective COX-1 inhibitor), NS-398 (a selective COX-2 inhibitor), VAS-2870 (a NADPH oxidase inhibitor), L-NA (NOS inhibitor) or potassium cyanide plus rotenone plus myxothiazol (inhibitors of the mitochondrial respiratory chain). This indicates the involvement of COX-1 and COX-2, uncoupled eNOS, NADPH oxidase and mitochondrial respiratory chain in the age-related increased vascular oxidative stress (Figure 4C).

2-weeks chronic intake of EPA:DHA 6:1 improves the age-related increased expression of NADPH oxidase

As the age-associated vascular oxidative stress involves NADPH oxidase, a major cellular source of ROS in cardiovascular disease risk (Lassegue *et al.*, 2010), we assessed the level of expression of the NADPH oxidase subunits p47^{phox} and p22^{phox} in the vascular wall of aortic sections. Compared to sections from young rats, the expression level of both p47^{phox} and p22^{phox} was significantly increased in sections from old rats. Treatment of old rats with EPA:DHA 6:1, but not corn oil, normalized the expression level of both subunits (Figure 5).

2-weeks chronic intake of EPA:DHA 6:1 improves the age-related activation of the local angiotensin system in the vascular wall

The angiotensin system is involved in aging-related endothelial dysfunction and overproduction of ROS via an AT₁ receptor-mediated upregulation of NADPH oxidase (Idris Khodja *et al.*, 2012), we checked the expression level of two major components of the local angiotensin system, the angiotensin-converting enzyme (ACE) and the angiotensin II type 1 receptor (AT1R). Compared to sections of young rats, aortic sections of untreated old rats showed an increased expression of both ACE and AT1R (Figure 6). Treatment of old rats

with EPA:DHA 6:1, but not corn oil, normalized the expression level of both ACE and AT1R (Figure 6).

2-weeks chronic intake of EPA:DHA 6:1 improves the age-related premature endothelial senescence

Recent studies have reported that the age-related endothelial dysfunction and vascular oxidative oxidative stress are associated with an increased endothelial senescence as indicated by the overexpression of the senescence markers p53, p21 and p16 (Rossman *et al.*, 2017). Compared to sections of young rats, aortic sections of untreated old rats showed an increased expression of both p53, p21 and p16 throughout. Treatment with EPA:DHA 6:1, but not corn oil, normalized the expression level of both p53, p21 and p16 (Figure 7).

Discussion

The findings of the present study indicate that ageing is associated with an endothelial dysfunction mainly characterized by a blunted NO-mediated relaxation, an abolished EDH-mediated relaxation, and an increased production of COX-derived EDCF 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. The 2-weeks short term oral intake of the EPA:DHA 6:1 ameliorated the age-related endothelial dysfunction mainly by improving the NO-mediated relaxations and decreasing the formation of EDCF. The present findings also indicate that ageing is associated with a vascular overproduction of ROS through multiple sources including COXs, uncoupled eNOS, NADPH oxidase and mitochondrial respiratory chain. Furthermore, vascular ageing caused an enhanced activation of the local angiotensin system as indicated by the increased expression of ACE and AT1 receptors, leading to the upregulation of the NADPH oxidase and increased vascular senescence. The intake of EPA:DHA 6:1 decreased the vascular level oxidative stress, at least in part, by the normalization of the angiotensin system.

The dose of 500 mg/kg/day is consistent with the dose previously used in preclinical studies on the effect of omega-3 PUFAs (Niazi *et al.*, 2017). This dose is equivalent to 5.67 g/day of omega-3 in a 70 kg human (Reagan-Shaw *et al.*, 2008), which is within the range of doses reported in different clinical studies, ranging from 0.18 to 10 g/day (Appel *et al.*, 1993; Delgado-Lista *et al.*, 2012; Enns *et al.*, 2014; Miller *et al.*, 2014).

Previous studies have indicated that the age-related endothelial dysfunction is characterized by blunted NO- and EDH-components of the endothelium-depend relaxation. An age-associated decrease in NO-mediated relaxation was reported in the mesenteric artery (Dal-Ros *et al.*, 2012), femoral arteries (Puzserova *et al.*, 2014), aorta (van der Loo *et al.*, 2000) and coronary arterioles (Csiszar *et al.*, 2002) of rats. Similarly, an age-related decrease in the

EDH-component was also reported in the rat mesenteric artery (Dal-Ros *et al.*, 2012; Idris Khodja *et al.*, 2012). Our present study indicates that ageing is associated with an abolished EDH-component whereas the NO-component was partially but significantly decreased, which is consistent with a previous study in middle-aged rats (Idris Khodja *et al.*, 2012). The 2-weeks treatment with a dose of 500 mg/kg/day of EPA:DHA 6:1 caused a significant improvement of NO-mediated relaxations whereas it has no effect on the EDH-component. The EPA:DHA 6:1-induced improvement of the NO pathway is also evident from the fact that the PE-induced contractions are increased in the presence of an eNOS inhibitor, indicating an increased basal release of NO in the main mesenteric.

The present results also indicate that the age-related endothelial dysfunction is characterized by an increased EDCF-mediated contraction which was abolished in the presence of indomethacin, showing the dominant role of COX-derived vasoconstricting prostanoids. Previous studies have showed that ageing is associated with a shift in the balance of COX-derived prostanoids from production of vasodilatory prostanoids, such as prostacyclin (PGI₂), towards vasoconstricting prostanoids such as thromboxane A₂ (TXA₂). Indeed, the age-related decreased in PGI₂ and/or increased formation of COX-derived vasoconstricting prostanoids were reported in rat femoral artery (Shi *et al.*, 2008) and in rat mesenteric artery and aorta (de Sotomayor *et al.*, 2005), as well as in isolated human mesenteric arteries (Rodriguez-Manas *et al.*, 2009) and in clinical studies (Taddei *et al.*, 1997; Vanhoutte *et al.*, 2005; Schrage *et al.*, 2007). The involvement of COX-2 in the age-related increased in EDCF has already been reported as a upregulation of COX-2 in the mesenteric arteries of aged rats (Stewart *et al.*, 2000), and as the fact that the selective inhibition of COX-2 improves the endothelial dysfunction in the mesenteric arteries and aorta of aged rats (de Sotomayor *et al.*, 2005). The oral intake of EPA:DHA 6:1 partially but significantly reduced the EDCF response in old rats, probably due to the decreased overexpression of COX-2 in the vascular

wall. The EPA:DHA 6:1 has been shown to decrease the indomethacin-sensitive EDCF response associated to a normalized expression of COX-2 in the mesenteric of angiotensin II-induced hypertensive rats (Niazi *et al.*, 2017). Similarly, the oral intake of EPA induced a reduction in EDCF response and in COX-2 overexpression in the mesenteric artery of type 2 diabetic Otsuka Long-Evans Tokushima fatty (OLETF) rats (Matsumoto *et al.*, 2009).

An increased vascular oxidative stress has been shown to be involved the vascular damage related to ageing and to various cardiovascular diseases and risk factors such as hypertension and diabetes (Valko *et al.*, 2007; Ungvari *et al.*, 2008). The role of an increased oxidative stress in the age-related endothelial dysfunction and blunted NO-component of the endothelium-dependent relaxation has already been reported in the rat mesenteric arteries of middle-aged rats (Dal-Ros *et al.*, 2012; Idris Khodja *et al.*, 2012). An increased formation of ROS can cause a direct inactivation of NO by chemically converting it to peroxynitrite anion (ONOO⁻) and can decrease the production of NO by the oxidation of BH₄, the most important cofactor of eNOS, thus leading to the uncoupling of eNOS and further production of ROS by the uncoupled eNOS (Yang *et al.*, 2009). The uncoupling of eNOS is strongly suggested by the fact that the vascular oxidative stress is reduced in the presence of the eNOS inhibitor L-NA and that the reduced NO-mediated component of the relaxation even when the eNOS is overexpressed in old rats. The increased expression of eNOS in old rats could be a compensatory mechanisms induced by the reduced bioavailability of NO due to increased oxidative stress. The EPA:DHA 6:1 treatment improved the endothelium-dependent NO-mediated relaxation and increased the basal formation of NO, probably by reducing the vascular oxidative stress as shown by the decreased ethidium staining in the mesenteric artery and aortic sections.

The local vascular angiotensin system plays a major role in the induction of the endothelial dysfunction and the associated vascular oxidative stress (Rajagopalan *et al.*, 1996; Dal-Ros *et*

al., 2009). Indeed, studies have reported that the treatment with ACE or AT1R inhibitors caused an improvement of the endothelial dysfunction in aged rats, in part by reducing the vascular oxidative stress (Goto *et al.*, 2000; Kansui *et al.*, 2002; Mukai *et al.*, 2002). Moreover, AT1R knocked out mice showed a prolonged lifespan due, at least in part, to an improvement of the cardiovascular health mainly through a reduction of oxidative stress (Benigni *et al.*, 2009). The induction of the vascular oxidative stress and the subsequent endothelial dysfunction is due to an AT1R-mediated activation of NADPH oxidase (Rajagopalan *et al.*, 1996; Harrison *et al.*, 2003). In addition, the activation of the angiotensin system has been associated with an increased production of mitochondrial ROS *via* the NADPH oxidase (Dikalov *et al.*, 2013). Indeed, the depletion of the NADPH oxidase subunit p22^{phox} decreased the angiotensin II-induced production of ROS in mitochondria isolated from endothelial cells from bovine aorta, while the inhibitor of NADPH oxidase apocynin reversed the angiotensin II-induced mitochondrial dysfunction (Doughan *et al.*, 2008). In the present study, the reduced ethidium fluorescence in the presence of the NADPH oxidase inhibitor VAS-2870, as well as the upregulation of the expression of NADPH oxidase subunits p22^{phox} and p47^{phox}, indicate the involvement of NADPH oxidase in the age-related vascular oxidative stress. Moreover, the increased expression of ACE and AT1R shows the increased activation of the local angiotensin system, consistent with previous study in rats of increasing age (Yoon *et al.*, 2016). Similarly, the fact that inhibitors of the mitochondrial respiratory chain reduced the ethidium fluorescence in aortic sections from old rats suggest that the NADPH oxidase-induced mitochondria-derived oxidative stress could be involved in vascular ageing in rats. The reduction in the vascular oxidative stress by the intake of EPA:DHA 6:1 is associated with the decreases expression of NADPH oxidase subunits p22^{phox} and p47^{phox}, ACE and AT1R, suggesting that EPA:DHA 6:1 decrease the vascular oxidative in part by reducing the activation of the local angiotensin system and the subsequent upregulation of NADPH

oxidase. These results are consistent with the reported reduction of the vascular oxidative stress through the reduction of the local angiotensin system and NADPH oxidase by the intake of EPA:DHA 6:1 in the angiotensin II-induced hypertensive rats (Niazi *et al.*, 2017). In addition, the age-related endothelial dysfunction has been associated with an induction of premature endothelial senescence causing cell cycle arrest (Herrera *et al.*, 2010; Jane-Wit *et al.*, 2012). Several cellular stresses including oxidative stress, angiotensin II, radiations, high glucose, DNA damage and reduced availability of NO are associated with the induction of premature endothelial senescence, which cause the upregulation of the cyclin-dependent kinase inhibitors p53, p21 and p16 leading to the cell cycle arrest (Erusalimsky, 2009). Moreover, serial passaging of cultured endothelial cells studies induce endothelial senescence associated with an upregulation of the angiotensin system and NADPH oxidase, and an increased oxidative stress (Khemais-Benkhiat *et al.*, 2016; Silva *et al.*, 2017). The present study shows that ageing is associated with the upregulation of p53, p21 and p16 in the aortic wall, suggesting an age-related vascular senescence, and EPA:DHA 6:1 seems to reduce the age-related senescence probably by decreasing the angiotensin system activation and the subsequent oxidative stress. This could also be due to a direct reduction of oxidative stress as a recent study has shown that omega-3 PUFA can prevent the hydrogen peroxide-induced senescence in endothelial cells from human aorta (Sakai *et al.*, 2017).

Taken together, the present findings indicate that ageing is associated with the development of an endothelial dysfunction characterized by a blunted NO-mediated relaxation, an abolished EDH-mediated relaxation, and an increased formation of COX-derived EDCF. Furthermore, the endothelial dysfunction is associated with increased oxidative stress, activation of the local angiotensin system, expression of NADPH oxidase and premature senescence in the vascular wall. A short term intake of the omega-3 PUFA EPA:DHA 6:1 formulation for 14 days was able to significantly improve the endothelial function by increasing the NO-

mediated component of the relaxation and by decreasing the formation of EDCF. The beneficial effect of EPA:DHA 6:1 on the endothelial function may be due to the normalization of the local angiotensin system and of the subsequent oxidative stress and premature senescence.

REFERENCES

Appel LJ, Miller ER, 3rd, Seidler AJ, Whelton PK (1993). Does supplementation of diet with 'fish oil' reduce blood pressure? A meta-analysis of controlled clinical trials. *Archives of internal medicine* **153**(12): 1429-1438.

Benigni A, Corna D, Zoja C, Sonzogni A, Latini R, Salio M, *et al.* (2009). Disruption of the Ang II type 1 receptor promotes longevity in mice. *The Journal of clinical investigation* **119**(3): 524-530.

Csiszar A, Ungvari Z, Edwards JG, Kaminski P, Wolin MS, Koller A, *et al.* (2002). Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function. *Circulation research* **90**(11): 1159-1166.

Dal-Ros S, Bronner C, Auger C, Schini-Kerth VB (2012). Red wine polyphenols improve an established aging-related endothelial dysfunction in the mesenteric artery of middle-aged rats: role of oxidative stress. *Biochemical and biophysical research communications* **419**(2): 381-387.

Dal-Ros S, Bronner C, Schott C, Kane MO, Chataigneau M, Schini-Kerth VB, *et al.* (2009). Angiotensin II-induced hypertension is associated with a selective inhibition of endothelium-derived hyperpolarizing factor-mediated responses in the rat mesenteric artery. *The Journal of pharmacology and experimental therapeutics* **328**(2): 478-486.

de Sotomayor MA, Perez-Guerrero C, Herrera MD, Jimenez L, Marin R, Marhuenda E, *et al.* (2005). Improvement of age-related endothelial dysfunction by simvastatin: effect on NO and COX pathways. *British journal of pharmacology* **146**(8): 1130-1138.

Delgado-Lista J, Perez-Martinez P, Lopez-Miranda J, Perez-Jimenez F (2012). Long chain omega-3 fatty acids and cardiovascular disease: a systematic review. *The British journal of nutrition* **107 Suppl 2**: S201-213.

Dikalov SI, Nazarewicz RR (2013). Angiotensin II-induced production of mitochondrial reactive oxygen species: potential mechanisms and relevance for cardiovascular disease. *Antioxidants & redox signaling* **19**(10): 1085-1094.

Doughan AK, Harrison DG, Dikalov SI (2008). Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction. *Circulation research* **102**(4): 488-496.

El Assar M, Angulo J, Rodriguez-Manas L (2013). Oxidative stress and vascular inflammation in aging. *Free radical biology & medicine* **65**: 380-401.

- El Assar M, Angulo J, Vallejo S, Peiro C, Sanchez-Ferrer CF, Rodriguez-Manas L (2012). Mechanisms involved in the aging-induced vascular dysfunction. *Frontiers in physiology* **3**: 132.
- Enns JE, Yeganeh A, Zarychanski R, Abou-Setta AM, Friesen C, Zahradka P, *et al.* (2014). The impact of omega-3 polyunsaturated fatty acid supplementation on the incidence of cardiovascular events and complications in peripheral arterial disease: a systematic review and meta-analysis. *BMC cardiovascular disorders* **14**: 70.
- Erusalimsky JD (2009). Vascular endothelial senescence: from mechanisms to pathophysiology. *Journal of applied physiology* **106**(1): 326-332.
- Goto K, Fujii K, Onaka U, Abe I, Fujishima M (2000). Angiotensin-converting enzyme inhibitor prevents age-related endothelial dysfunction. *Hypertension* **36**(4): 581-587.
- Harrison DG, Cai H, Landmesser U, Griendling KK (2003). Interactions of angiotensin II with NAD(P)H oxidase, oxidant stress and cardiovascular disease. *Journal of the renin-angiotensin-aldosterone system : JRAAS* **4**(2): 51-61.
- Herrera MD, Mingorance C, Rodriguez-Rodriguez R, Alvarez de Sotomayor M (2010). Endothelial dysfunction and aging: an update. *Ageing research reviews* **9**(2): 142-152.
- Idris Khodja N, Chataigneau T, Auger C, Schini-Kerth VB (2012). Grape-derived polyphenols improve aging-related endothelial dysfunction in rat mesenteric artery: role of oxidative stress and the angiotensin system. *PloS one* **7**(2): e32039.
- Jane-Wit D, Chun HJ (2012). Mechanisms of dysfunction in senescent pulmonary endothelium. *The journals of gerontology. Series A, Biological sciences and medical sciences* **67**(3): 236-241.

Kansui Y, Fujii K, Goto K, Abe I, Iida M (2002). Angiotensin II receptor antagonist improves age-related endothelial dysfunction. *Journal of hypertension* **20**(3): 439-446.

Khemais-Benkhiat S, Idris-Khodja N, Ribeiro TP, Silva GC, Abbas M, Kheloufi M, *et al.* (2016). The Redox-sensitive Induction of the Local Angiotensin System Promotes Both Premature and Replicative Endothelial Senescence: Preventive Effect of a Standardized Crataegus Extract. *The journals of gerontology. Series A, Biological sciences and medical sciences* **71**(12): 1581-1590.

Lassegue B, Griendling KK (2010). NADPH oxidases: functions and pathologies in the vasculature. *Arteriosclerosis, thrombosis, and vascular biology* **30**(4): 653-661.

Limbu R, Cottrell GS, McNeish AJ (2018). Characterisation of the vasodilation effects of DHA and EPA, n-3 PUFAs (fish oils), in rat aorta and mesenteric resistance arteries. *PloS one* **13**(2): e0192484.

Matsumoto T, Nakayama N, Ishida K, Kobayashi T, Kamata K (2009). Eicosapentaenoic acid improves imbalance between vasodilator and vasoconstrictor actions of endothelium-derived factors in mesenteric arteries from rats at chronic stage of type 2 diabetes. *The Journal of pharmacology and experimental therapeutics* **329**(1): 324-334.

Miller PE, Van Elswyk M, Alexander DD (2014). Long-chain omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid and blood pressure: a meta-analysis of randomized controlled trials. *American journal of hypertension* **27**(7): 885-896.

Mukai Y, Shimokawa H, Higashi M, Morikawa K, Matoba T, Hiroki J, *et al.* (2002). Inhibition of renin-angiotensin system ameliorates endothelial dysfunction associated with aging in rats. *Arteriosclerosis, thrombosis, and vascular biology* **22**(9): 1445-1450.

Niazi ZR, Silva GC, Ribeiro TP, Leon-Gonzalez AJ, Kassem M, Mirajkar A, *et al.* (2017). EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase- and COX-derived oxidative stress. *Hypertension research : official journal of the Japanese Society of Hypertension* **40**(12): 966-975.

North BJ, Sinclair DA (2012). The intersection between aging and cardiovascular disease. *Circulation research* **110**(8): 1097-1108.

Puzserova A, Ilovska V, Balis P, Slezak P, Bernatova I (2014). Age-related alterations in endothelial function of femoral artery in young SHR and WKY rats. *BioMed research international* **2014**: 658479.

Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, *et al.* (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *The Journal of clinical investigation* **97**(8): 1916-1923.

Reagan-Shaw S, Nihal M, Ahmad N (2008). Dose translation from animal to human studies revisited. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **22**(3): 659-661.

Rodriguez-Manas L, El-Assar M, Vallejo S, Lopez-Doriga P, Solis J, Petidier R, *et al.* (2009). Endothelial dysfunction in aged humans is related with oxidative stress and vascular inflammation. *Aging cell* **8**(3): 226-238.

Rossmann MJ, Kaplon RE, Hill SD, McNamara MN, Santos-Parker JR, Pierce GL, *et al.* (2017). Endothelial cell senescence with aging in healthy humans: prevention by habitual exercise and relation

to vascular endothelial function. *American journal of physiology. Heart and circulatory physiology* **313**(5): H890-H895.

Roth GA, Huffman MD, Moran AE, Feigin V, Mensah GA, Naghavi M, *et al.* (2015). Global and regional patterns in cardiovascular mortality from 1990 to 2013. *Circulation* **132**(17): 1667-1678.

Sakai C, Ishida M, Ohba H, Yamashita H, Uchida H, Yoshizumi M, *et al.* (2017). Fish oil omega-3 polyunsaturated fatty acids attenuate oxidative stress-induced DNA damage in vascular endothelial cells. *PloS one* **12**(11): e0187934.

Schrage WG, Eisenach JH, Joyner MJ (2007). Ageing reduces nitric-oxide- and prostaglandin-mediated vasodilatation in exercising humans. *The Journal of physiology* **579**(Pt 1): 227-236.

Shi Y, Man RY, Vanhoutte PM (2008). Two isoforms of cyclooxygenase contribute to augmented endothelium-dependent contractions in femoral arteries of 1-year-old rats. *Acta pharmacologica Sinica* **29**(2): 185-192.

Silva GC, Abbas M, Khemais-Benkhiat S, Burbán M, Ribeiro TP, Toti F, *et al.* (2017). Replicative senescence promotes prothrombotic responses in endothelial cells: Role of NADPH oxidase- and cyclooxygenase-derived oxidative stress. *Experimental gerontology* **93**: 7-15.

Stewart KG, Zhang Y, Davidge ST (2000). Aging increases PGHS-2-dependent vasoconstriction in rat mesenteric arteries. *Hypertension* **35**(6): 1242-1247.

Taddei S, Virdis A, Mattei P, Ghiadoni L, Fasolo CB, Sudano I, *et al.* (1997). Hypertension causes premature aging of endothelial function in humans. *Hypertension* **29**(3): 736-743.

Tousoulis D, Plastiras A, Siasos G, Oikonomou E, Verveniotis A, Kokkou E, *et al.* (2014). Omega-3 PUFAs improved endothelial function and arterial stiffness with a parallel antiinflammatory effect in adults with metabolic syndrome. *Atherosclerosis* **232**(1): 10-16.

Ungvari Z, Buffenstein R, Austad SN, Podlutzky A, Kaley G, Csiszar A (2008). Oxidative stress in vascular senescence: lessons from successfully aging species. *Frontiers in bioscience : a journal and virtual library* **13**: 5056-5070.

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology* **39**(1): 44-84.

van der Loo B, Labugger R, Skepper JN, Bachschmid M, Kilo J, Powell JM, *et al.* (2000). Enhanced peroxynitrite formation is associated with vascular aging. *The Journal of experimental medicine* **192**(12): 1731-1744.

Vanhoutte PM, Feletou M, Taddei S (2005). Endothelium-dependent contractions in hypertension. *British journal of pharmacology* **144**(4): 449-458.

Yang YM, Huang A, Kaley G, Sun D (2009). eNOS uncoupling and endothelial dysfunction in aged vessels. *American journal of physiology. Heart and circulatory physiology* **297**(5): H1829-1836.

Yoon HE, Kim EN, Kim MY, Lim JH, Jang IA, Ban TH, *et al.* (2016). Age-Associated Changes in the Vascular Renin-Angiotensin System in Mice. *Oxidative medicine and cellular longevity* **2016**: 6731093.

Zanetti M, Gortan Cappellari G, Barbetta D, Semolic A, Barazzoni R (2017). Omega 3 Polyunsaturated Fatty Acids Improve Endothelial Dysfunction in Chronic Renal Failure: Role of eNOS Activation and of Oxidative Stress. *Nutrients* **9**(8).

Zehr KR, Walker MK (2018). Omega-3 polyunsaturated fatty acids improve endothelial function in humans at risk for atherosclerosis: A review. *Prostaglandins & other lipid mediators* **134**: 131-140.

Zgheel F, Alhosin M, Rashid S, Burban M, Auger C, Schini-Kerth VB (2014). Redox-sensitive induction of Src/PI3-kinase/Akt and MAPKs pathways activate eNOS in response to EPA:DHA 6:1. *PloS one* **9**(8): e105102.

Figure legends

Figure 1: EPA:DHA 6:1 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) To study the endothelium-dependent relaxation, rings were contracted with 1 μ M phenylephrine (PE) before the addition of increasing concentrations of acetylcholine (ACh). (B) To assess the contractile responses, rings were subjected to a concentration-contraction curves in response to phenylephrine. (C-D) To study endothelium-dependent vasoconstrictor factors (EDCFs), rings were exposed to L-NA (N^0 -nitro-L-arginine, 300 μ M, an eNOS inhibitor) and TRAM-34 plus UCL-1684 (1 μ m each, inhibitors of IKCa and SKCa, respectively) to prevent NO and EDH, respectively, with (D) or without (C) indomethacin (10 μ M, an unselective COX inhibitor), before pre-contraction by about 30% with PE and the subsequent construction of a concentration-contraction curve in response to ACh. (E-F) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with 1 μ M PE before the construction of a concentration-relaxation curve either sodium nitroprusside (SNP, a NO donor) or levcromakalim (Lev, an ATP-sensitive potassium channels opener) in the presence of L-NA and TRAM-34 plus UCL-1684 and indomethacin to prevent the contribution of NO, EDH and vasoactive prostanoids, respectively. Results are expressed in % relaxations (A, D-F) or in grams of contraction (B) as means \pm SEM of 10 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats control.

Figure 2: EPA:DHA 6:1 improves the blunted NO-component of the relaxation and decrease the formation of EDCF

Rings with endothelium were prepared from the main mesenteric artery and suspended in organ baths for the determination of changes in isometric tension. (A-D) To study the

endothelium-dependent relaxation, rings were contracted with 1 μ M phenylephrine (PE) before the addition of increasing concentrations of acetylcholine (ACh). (E-H) 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 μ M, an unselective COX inhibitor). To study the NO-mediated component, rings were incubated with N^o-nitro-L-arginine (L-NA, 300 μ M, an eNOS inhibitor). The EDH-mediated component was studied in rings incubated with TRAM-34 plus UCL-1684 (1 μ m each, inhibitors of IK_{Ca} and SK_{Ca}, respectively). Results are expressed in % relaxations (A-D) or in grams of contraction (E-H) as means \pm SEM of 10 rats per group. * $P < 0.05$ vs. control without inhibitors.

Figure 3: EPA:DHA 6:1 improves the age-related up-regulation of eNOS and COX-2, and the down-regulation of COX-1 in the mesenteric artery and aorta

Protein immunoreactive signals were determined in unfixed cryosections of the main mesenteric artery and of thoracic aorta. The determination of the expression level of eNOS, COX-1 and COX-2 was done by immunofluorescence and analysed by confocal microscope. Results are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats control.

Figure 4: EPA:DHA 6:1 improves the age-related increased vascular oxidative stress in the mesenteric artery and aorta

Vascular oxidative stress was determined in unfixed cryosections of the main mesenteric artery (A) and of thoracic aorta (B-C) by fluorescence histochemistry using the redox-sensitive probe dihydroethidium, and analysed by confocal microscope. (C) To determine the

sources of ROS, aortic sections were pretreated with inhibitors including N-acetylcysteine (antioxidant, 1 mM), indomethacin (inhibitor of COX, 10 μ M), NS-398 (COX-2 inhibitor 3 μ M), SC-560 (COX-1 inhibitor, 0.3 μ M), L-NA (inhibitor of eNOS, 100 μ M), VAS-2870 (inhibitor of NADPH oxidase, 10 μ M) and inhibitors of mitochondrial respiratory chain (myxothiazole 0.5 μ M, potassium cyanide 1 μ M and rotenone 1 μ M) for 30 minutes at 37°C prior to incubation with DHE. Results are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats control.

Figure 5: EPA:DHA 6:1 improves the age-related up-regulation of NADPH oxidase subunits p22^{phox} and p47^{phox} in the thoracic aorta

Protein immunoreactive signals were determined in unfixed cryosections of thoracic aorta.

The determination of the expression level of p22^{phox} and p47^{phox} was done by

immunofluorescence and analysed by confocal microscope. Results are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats control.

Figure 6: EPA:DHA 6:1 improves the age-related activation of the local angiotensin system in the thoracic aorta

Protein immunoreactive signals were determined in unfixed cryosections of the thoracic aorta.

The determination of the expression level of the component of the angiotensin system ACE and AT1R was done by immunofluorescence and analysed by confocal microscope. Results

are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats control.

Figure 7: EPA:DHA 6:1 improves the age-related up-regulation of senescence markers in the thoracic aorta

Protein immunoreactive signals were determined in unfixed cryosections of the thoracic aorta. The determination of the expression level of the senescence markers p53, p16 and p21 was done by immunofluorescence and analysed by confocal microscope. Results are expressed as means \pm SEM of 4-5 rats per group. * $P < 0.05$ vs. Young rats, # $P < 0.05$ vs. Old rats control.

Figure 1

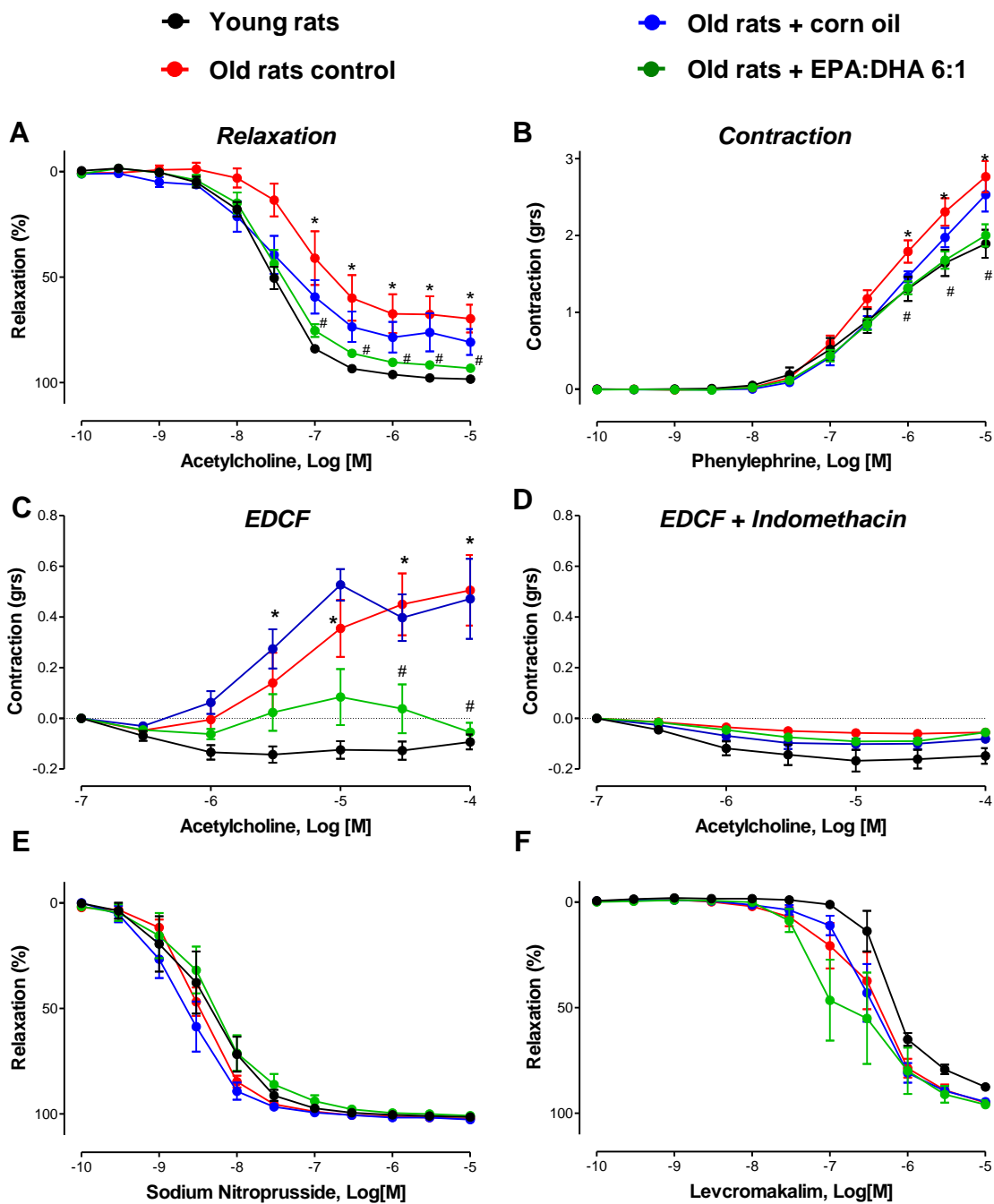


Figure 2

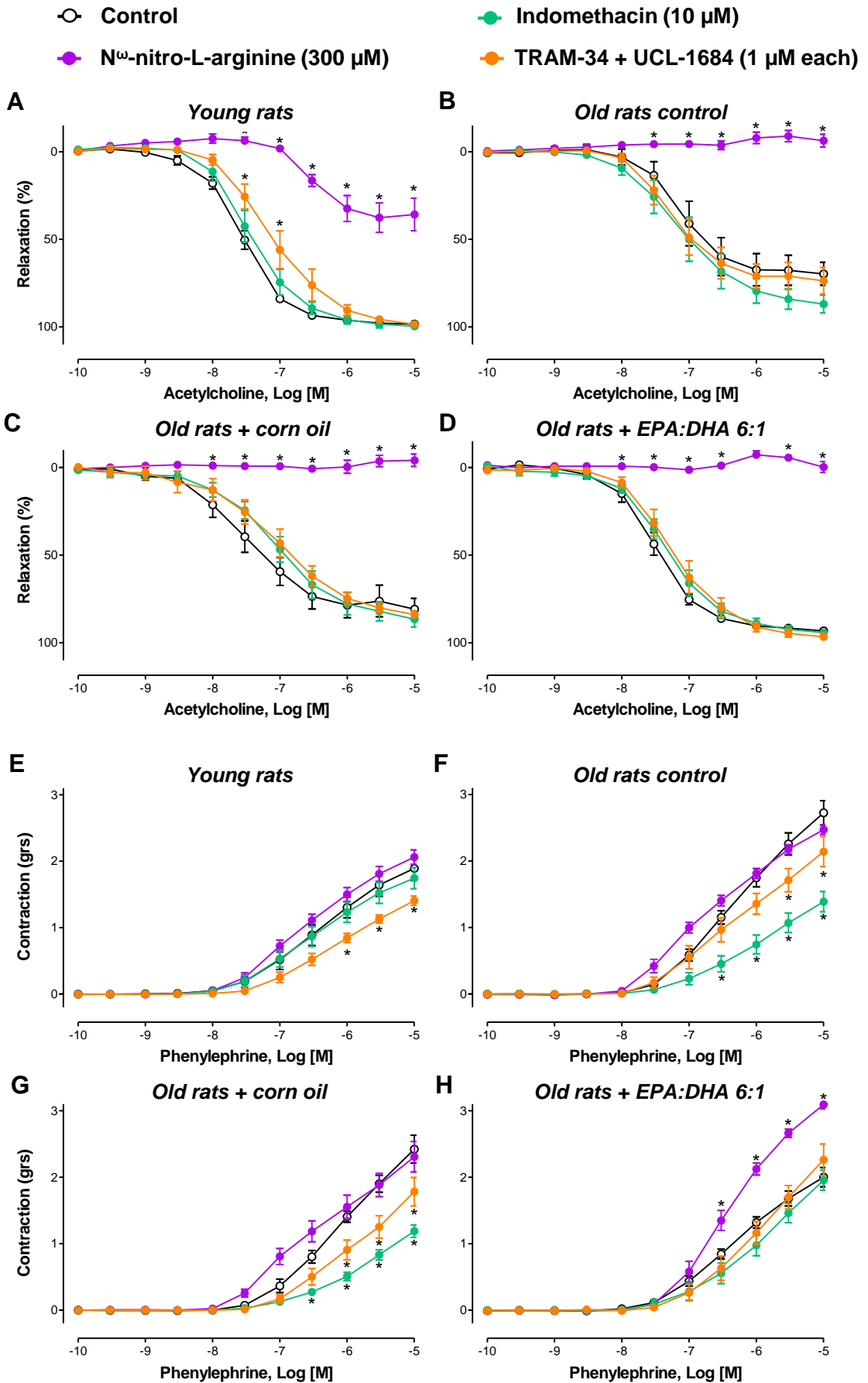


Figure 3

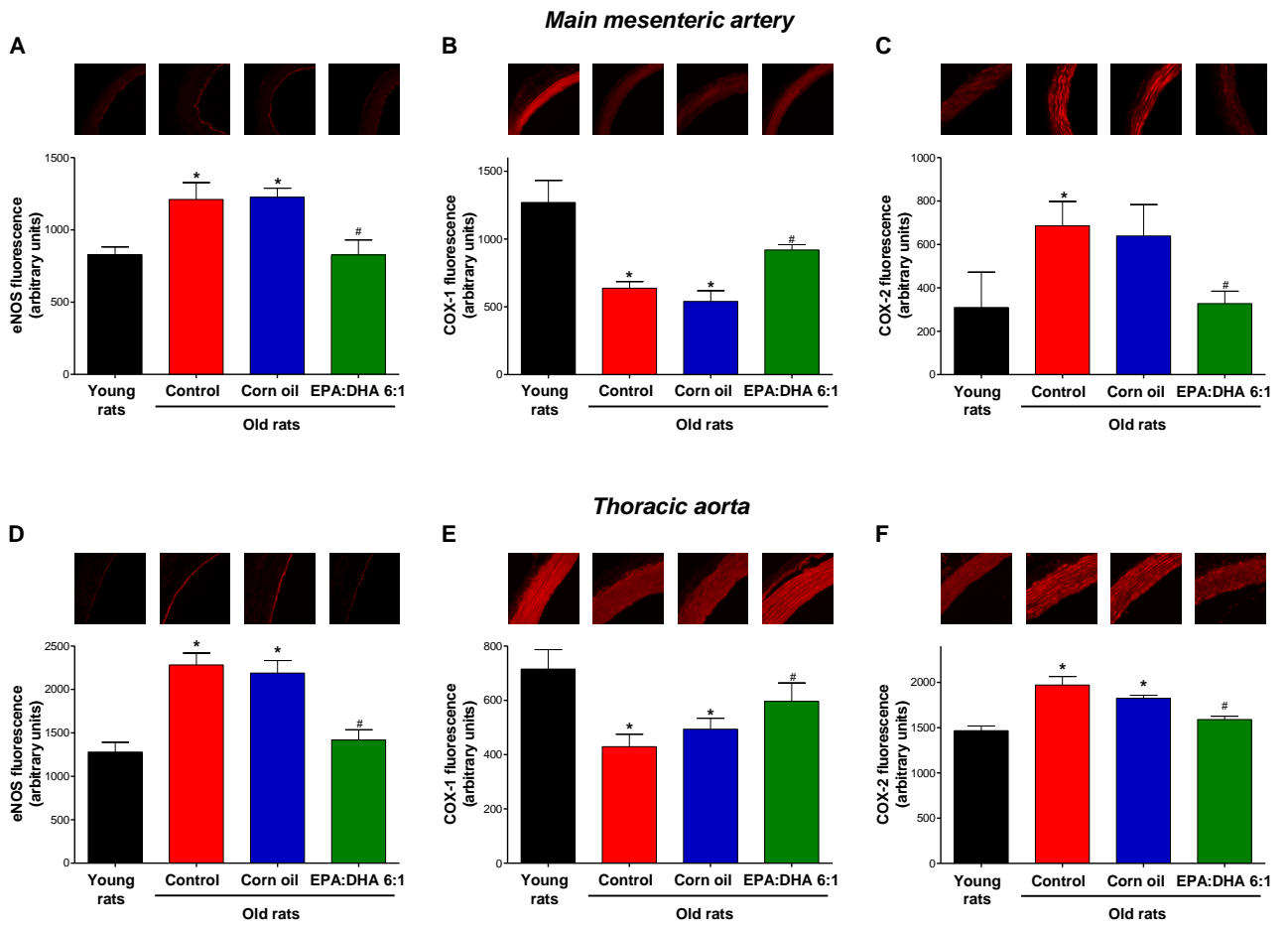


Figure 4

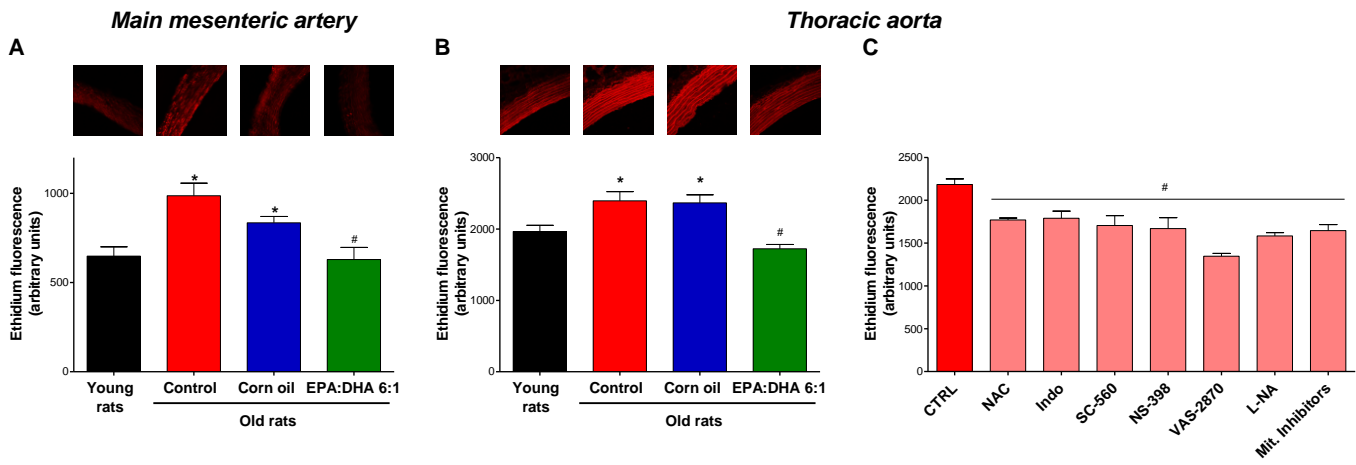


Figure 5

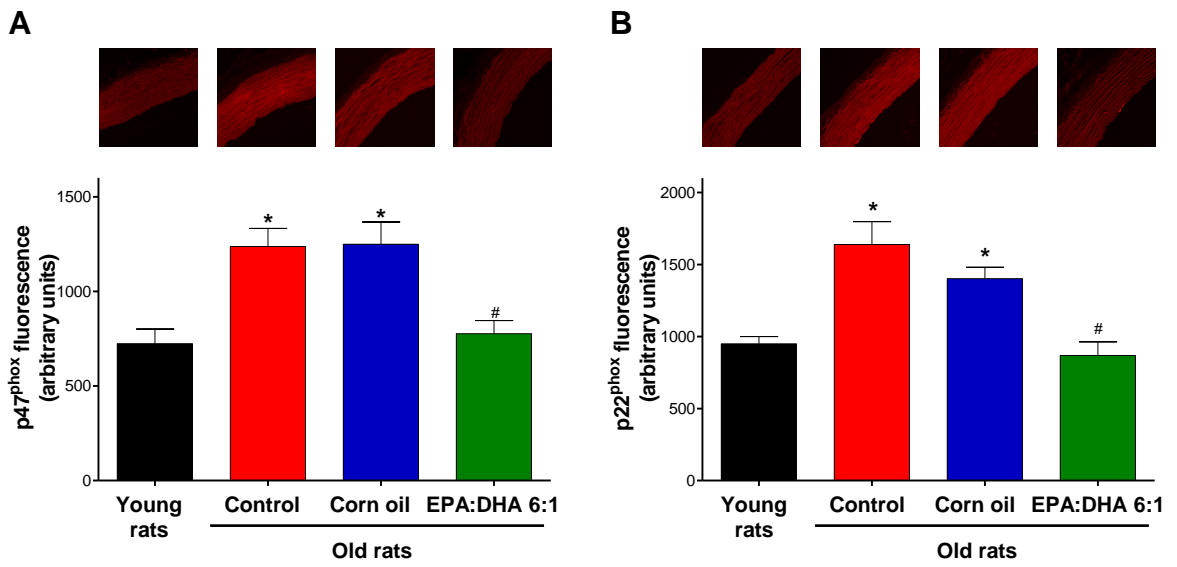


Figure 6

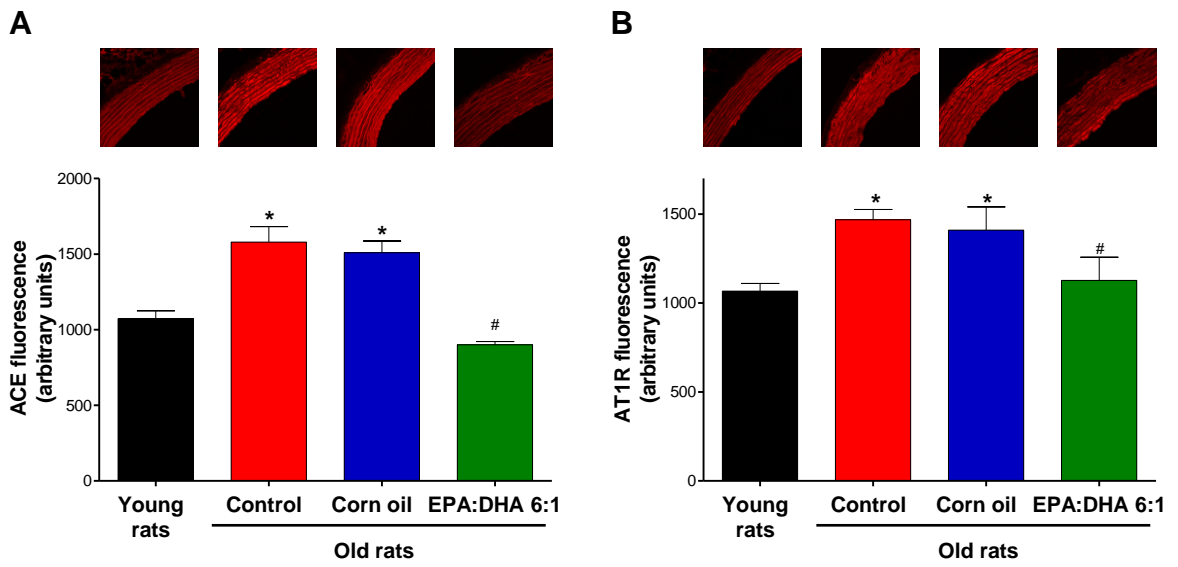
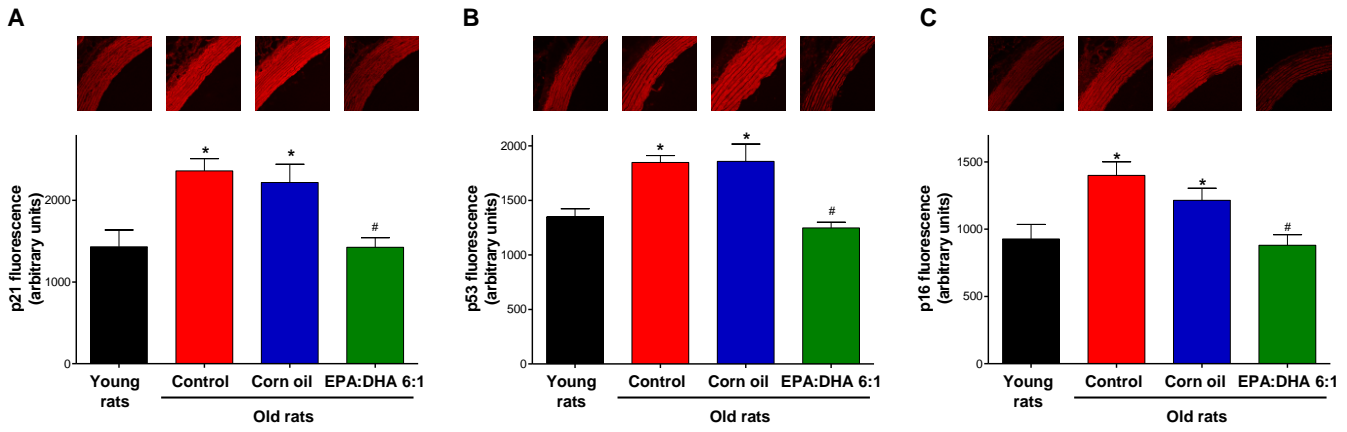


Figure 7



Results and conclusion

The major results of our study show that that ageing is associated with an endothelial dysfunction mainly characterized by a blunted NO-mediated relaxation, an abolished EDH-mediated relaxation, and an increased production of COX-derived endothelium-derived contractile factors (EDCFs) in response to acetylcholine in the main mesenteric artery of old rats. The 2-weeks short term oral intake of the EPA:DHA 6:1 ameliorated the age-related endothelial dysfunction mainly by improving the NO-mediated relaxation and decreasing the formation of EDCF, whereas the EDH-mediated relaxation remains abolished. To better characterize the molecular mechanisms involved in the beneficial effects of EPA:DHA 6:1 intake, we performed quantitative analysis of protein expression in the mesenteric artery and thoracic aorta by immunofluorescence. The age-related endothelial dysfunction was associated with an increased expression of eNOS and COX-2, and a decreased expression of COX-1. The present findings also indicate that ageing is associated with a vascular overproduction of ROS through multiple sources including COXs, uncoupled eNOS, NADPH oxidase and mitochondrial respiratory chain. Furthermore, vascular ageing caused an enhanced activation of the local angiotensin system as indicated by the increased expression of ACE and AT1 receptors, leading to the upregulation of the NADPH oxidase and increased vascular senescence. The intake of EPA:DHA 6:1 decreased the vascular oxidative stress, at least in part, by the normalization of the angiotensin system. In addition, the present study reports that ageing is associated with the upregulation of the senescence markers p53, p21 and p16 in the aortic wall, suggesting an age-related vascular senescence, and EPA:DHA 6:1 seems to reduce the age-related senescence probably by decreasing the angiotensin system activation and the subsequent oxidative stress.

Altogether, the present findings indicate that ageing is associated with the development of an endothelial dysfunction characterized by a blunted NO-mediated relaxation, an abolished EDH-mediated relaxation, and an increased formation of COX-derived EDCF. This endothelial dysfunction is associated also with increased vascular oxidative stress, activation of the local angiotensin system, expression of NADPH oxidase and premature senescence in the vascular wall. A short-term 2-weeks intake of the omega-3 PUFA EPA:DHA 6:1 was able to significantly improve the endothelial function through increased NO-mediated relaxation and decreased EDCF response. The beneficial effect of

EPA:DHA 6:1 on the endothelial function could be due to the normalization of the local angiotensin system and of the subsequent oxidative stress and premature senescence.

6-DISCUSSION AND PERSPECTIVES

6-GENERAL DISCUSSION

6.1 The age-related endothelial dysfunction

Endothelial dysfunction is generally characterized by a decreased ability of the endothelium to induce vasodilation in response to chemical or mechanical stimulus (Matz, de Sotomayor et al. 2000). The age-related endothelial dysfunction is considered as a major contributor in the development of cardiovascular diseases, even in the absence of other established risk factors like hypertension, diabetes, hypercholesteremia and atherosclerosis (Lakatta 2015). Therefore, the age-related endothelial dysfunction represents an interesting therapeutic target for the prevention of CVDs in the elderly people (Seals, Jablonski et al. 2011). From preclinical and clinical studies, it is evident that ageing is associated with an increasing blunted endothelium-dependent relaxations whereas endothelium-independent relaxations to sodium nitroprusside remained unaffected (Egashira, Inou et al. 1993, Taddei, Viridis et al. 1995, Matz and Andriantsitohaina 2003, Toda 2012). The age-related decline in endothelium-dependent relaxations is attributable, at least in part, to some modification of cellular events causing a decreased production or increased inactivation of vasodilating factors such as NO and EDH, and an augmented production of EDCFs and oxidative stress (El Assar, Angulo et al. 2012). Indeed, clinical studies on coronary artery and forearm blood flow reported a decline in the endothelium-dependent relaxations in response to ACh infusion with increasing age (Egashira, Inou et al. 1993, Taddei, Viridis et al. 1995). Furthermore, ageing is an additional risk factor of endothelial dysfunction, with the age-related decline in NO-mediated relaxations being present in both normotensive and hypertensive subjects, but with an accelerated dysfunction in the later (Taddei, Viridis et al. 2001). Preclinical studies in different vascular beds of rats have associated the age-related endothelial dysfunction with a blunted NO-mediated relaxation alone or together with reduced EDH-component of the relaxation in mesenteric arteries (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012), aorta (van der Loo, Labugger et al. 2000) and coronary arterioles (Csiszar, Ungvari et al. 2002). The age-related endothelial dysfunction thus presents features similar to those observed in other models such as the femoral artery from SHR rats (Puzserova, Ilovska et al. 2014), the mesenteric artery of angiotensin II-infused hypertensive rats (Niazi, Silva et al. 2017) and the mesenteric artery of cirrhotic rats (Rashid, Idris Khodja et al. 2014, Rashid, Idris-Khodja et al. 2018).

In our present study, we have used acetylcholine and phenylephrine concentration-response curves to characterize the endothelium-dependent relaxations, the EDCF formation and the vascular contractile responses in the main mesenteric artery of young and aged rats. The results indicate that in 20-months-old rats, the age-related endothelial dysfunction is characterized by a significantly blunted endothelium-dependent relaxation and an increased vascular contraction in response to increasing concentration of acetylcholine (ACh) and phenylephrine (PE) respectively. Moreover, the EDH-mediated component of the relaxation was totally abolished in old rats whereas the NO-mediated component was partially, but significantly, reduced as shown by the absence of relaxation in the presence of indomethacin and TRAM-34 plus UCL-1680 (inhibitors of prostacyclin and EDH, respectively) and the reduced relaxation in response to acetylcholine in the presence of indomethacin and L-NA (inhibitors of prostacyclin and eNOS, respectively). These results are in line with previous studies showing that EDH-mediated component are strongly affected by ageing in the rat mesenteric artery (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012).

In addition, significantly augmented levels of EDCF were found while assessing the endothelium-dependent contraction in response to increasing concentration of ACh in the presence of TRAM-34 plus UCL-1684 and L-NA (blocking NO and EDH, respectively). The EDCF response was abolished by the addition of indomethacin indicating the involvement of COX-derived prostanoids. This finding is also strengthened by the significant reduction of PE-induced contractile response after the pretreatment with indomethacin in the mesenteric artery of old rats. This is consistent with the fact that ageing has been associated with a shift in the balance between vasodilatory and vasoconstrictor prostanoids in favor of the later. Indeed, human *in vitro* and *in vivo* studies have shown an age-related decline in formation of vasodilatory prostacyclin and increase in COX-derived vasoconstrictor prostanoids (Taddei, Viridis et al. 1997, Vanhoutte, Feletou et al. 2005, Schrage, Eisenach et al. 2007, Rodriguez-Manas, El-Assar et al. 2009). The hypothesis of an involvement of COX-derived prostanoids is further strengthened with the improvement of the relaxations by the use of TP-receptor antagonist in humans (Rodriguez-Manas, El-Assar et al. 2009). In the present study, the immunofluorescence experiments showed an augmented expression of COX-2 in the mesenteric artery and aortic sections from old rats, which is in agreement with a previously published study (Stewart, Zhang et al. 2000). The upregulation of COX-2 can account for the increased EDCF as the EDCF response has shown to be related with an increased formation

of the COX-2-derived PGF_{2α} in aged hamsters (Wong, Leung et al. 2009). Conversely, the selective COX-2 inhibitor (NS-398) caused an improvement of the endothelial dysfunction in aorta and mesenteric artery of aged rats (de Sotomayor, Perez-Guerrero et al. 2005).

Increased vascular oxidative stress and its link with vascular damage has been described in ageing and different cardiovascular diseases and risk factors like hypertension and diabetes (Ungvari, Buffenstein et al. 2008). The age-related increased ROS production leading to oxidative stress is a major contributor in the endothelial dysfunction and can be due to either an increased ROS production and/or a reduced inactivation of ROS by the cellular defense mechanisms (El Assar, Angulo et al. 2013). The infusion of vitamin C (antioxidant) is associated with an augmentation of the Ach-induced increase in forearm blood flow in old and hypertensive humans (Taddei, Viridis et al. 2001). We used the dihydroethidium (DHE) probe to assess the levels of ROS in the aortic and mesenteric artery sections. As DHE has a high membrane permeability, it can react with intracellular superoxide anions and give rise to ethidium which gives red fluorescence upon excitation (Owusu-Ansah, Yavari et al. 2008). The present findings showed a significantly higher ethidium staining in the sections of untreated old rats as compared to young rats, indicating an increased oxidative stress.

The vascular oxidative stress can involve several cellular sources including the NADPH oxidase, the xanthine oxidase, the uncoupled eNOS, the COXs and the mitochondrial respiratory chain (El Assar, Angulo et al. 2012). Our study showed a decreased ethidium staining in the aortic sections of old rats after pretreatment with either indomethacin (a COX inhibitor), SC-560 (a selective COX-1 inhibitor), NS-398 (a selective COX-2 inhibitor), L-NA (a NOS inhibitor), VAS-2870 (a NADPH oxidase inhibitor) and the combination of KCN, rotenone and myxothiazol (inhibitors of mitochondrial respiratory chain), indicating the implication of multiple sources of ROS in the age-related vascular oxidative stress. The ethidium staining was most strongly inhibited by VAS-2870, suggesting that NADPH oxidase may play a major role in the age-related oxidative stress. Previously published studies have showed that an increased NADPH oxidase activity is associated with the age-related endothelial dysfunction in humans, and that the inhibition of NADPH oxidase with apocynin resulted in an improved endothelial function (Rodriguez-Manas, El-Assar et al. 2009). Moreover, an age-related increase in the expression of NADPH oxidase subunits NOX-1 and p22^{phox} was reported in the mesenteric artery of middle-aged rats (Idris Khodja,

Chataigneau et al. 2012), which are consistent with the increased expression of p22^{phox} and p47^{phox} subunits of NADPH oxidase in aortic sections of untreated old rats observed in the present study.

The NADPH oxidase produce ROS mainly in the form of superoxide anions that can decrease NO bioavailability by chemically converting it to peroxynitrite anion (ONOO⁻). Moreover, peroxynitrite anions and superoxide anions can further increase ROS production and decrease NO production by “uncoupling” eNOS *via* the oxidation of BH₄, an essential cofactor of eNOS, into BH₂ (Yang, Huang et al. 2009, Forstermann 2010). The infusion of BH₄ in human subjects resulted in an improvement of the endothelial function (Vasquez-Vivar, Kalyanaraman et al. 2003). The uncoupling of eNOS can explain the decreased ethidium staining in aorta sections in presence of L-NA, and the increased expression of eNOS in aorta and mesenteric artery sections of old rats could be due to a mechanism to compensate the reduced bioavailability of NO. An increased expression of eNOS has been reported in aorta and mesenteric arteries of old rats (Cernadas, Sanchez de Miguel et al. 1998, Briones, Montoya et al. 2005). Moreover, the blunted EDH-mediated component of relaxation can be explained, at least in part, by a functional impairment of calcium-activated potassium channels (SK_{Ca} and IK_{Ca}) by oxidative stress, resulting in an impairment of the electrical activity responsible of the endothelial hyperpolarization (Behringer, Shaw et al. 2013). Moreover, previous studies in mesenteric artery of middle-age rats have linked an age-related decrease in EDH with an increased oxidative stress and a decreased expression of SK_{Ca} and IK_{Ca} (Idris Khodja, Chataigneau et al. 2012).

Angiotensin II plays an important role in the regulation of the cardiovascular system mainly through the activation of its type 1 and 2 receptors (AT1R and AT2R, respectively). An increased angiotensin II activity has been shown to be associated with an endothelial dysfunction and the augmented vascular oxidative stress via an AT1R-mediated activation of NADPH oxidase (Rajagopalan, Kurz et al. 1996, Harrison, Cai et al. 2003, Niazi, Silva et al. 2017). The major role of the angiotensin system is underlined by the fact that treatment of old rats with either ACE inhibitor or AT1R antagonist resulted in an improvement of the endothelial dysfunction at least in part by a reduction of the oxidative stress (Goto, Fujii et al. 2000, Kansui, Fujii et al. 2002, Mukai, Shimokawa et al. 2002). Furthermore, AT1R knocked-out mice evidenced a prolongation of lifespan *via* an improvement of the cardiovascular morphology, a decreased ROS production and the normalization of

mitochondrial function (Benigni, Corna et al. 2009). Also, ageing has been associated with an increased expression of angiotensin II and ACE in rats (Challah, Nadaud et al. 1997) and non-human primates (Wang, Takagi et al. 2003), whereas an age-related increase in expression of AT1R, angiotensin II and ACE was reported in mice (Yoon, Kim et al. 2016). In line with these studies, the present results showed a significantly increased expressions of AT1R and ACE in aorta sections of untreated old rats in comparison with young rats, indicating an activation of the local angiotensin system. The infusion of angiotensin II in rats induced an endothelial dysfunction associated with an increased oxidative stress due, at least in part, to an upregulation of AT1R and NADPH oxidase subunits p22^{phox} and p47^{phox} (Niazi, Silva et al. 2017). An increased angiotensin II activity is also associated with increased production of mitochondrial ROS via NADPH oxidase (Dikalov and Nazarewicz 2013). Moreover, the depletion of the NADPH oxidase subunit p22^{phox} was associated with a decreased production of ROS in mitochondria isolated from angiotensin II-treated endothelial cells of bovine aorta, whereas the inhibitor of NADPH oxidase apocynin reversed the angiotensin II-induced mitochondrial dysfunction (Doughan, Harrison et al. 2008). The involvement of an angiotensin II-induced formation of ROS from the mitochondrial respiratory chain is also consistent with our results showing that the age-related vascular oxidative stress is reduced in presence of inhibitors of the mitochondrial respiratory chain.

Recent studies reported that premature senescence could be associated with endothelial dysfunction and development of cardiovascular diseases. Senescence is a vital mechanism which prevent the replication of cells containing damaged DNA. Senescence is characterized by a cessation of cell division due to cell cycle arrest associated with an upregulation of the cyclin-dependent kinase inhibitors p53, p21 and p16. The senescence can be induced prematurely by different cellular stressors (Campisi and d'Adda di Fagagna 2007). Premature endothelial senescence has been reported in response to oxidative stress, angiotensin II, radiations, high glucose, DNA damage and reduced availability of NO (Erusalimsky 2009). Cell culture studies on cultured porcine coronary endothelial cells showed that senescence and upregulation of p53, p21 and p16 were induced after serial passages, and were related to an activation of the local angiotensin system leading to the upregulation of NADPH oxidase and the subsequent cellular oxidative stress (Khemais-Benkhiat, Idris-Khodja et al. 2016, Silva, Abbas et al. 2017). The present findings indicate that the age-related endothelial dysfunction is associated with an upregulation of the senescence markers p53, p16 and p21 in

the aortic sections from untreated old rats. Similarly, an increased expression of these markers was found in endothelial cells from old men compared to those of young men (Rossman, Kaplon et al. 2017).

6.2 Beneficial effects of omega-3 PUFAs

Numerous clinical and experimental studies have associated the dietary intake of omega-3 PUFAs with a beneficial effect on cardiovascular system (Mozaffarian and Rimm 2006, Roth and Harris 2010), and a decreased risk of cardiovascular diseases and risk factors like hypercholesteremia, thrombosis, hypertension, arrhythmias, inflammation, oxidative stress and diabetes (Mozaffarian and Wu 2011, Mori 2014). Indeed, an inverse correlation has been observed between the increased consumption of omega-3 PUFAs and the incidence of CVDs (Kromhout 1989). The long chain omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been reported to be the most potent omega-3 PUFAs (Riediger, Othman et al. 2009). On the molecular level, omega-3 PUFAs produce these effects by alterations of the function of cell membrane and receptors associated with it, functional alterations of ion channels, alterations of prostanoids profile by competing with arachidonic acid as a precursor of COX- and LOX-derived products, and by modifications of nuclear receptors and transcription factors (Mozaffarian and Wu 2011, Calvo, Martínez et al. 2017). Seafood is considered as a rich source of long chain omega-3 fatty acids, and especially fatty fishes (salmon, mackerel, tuna, sardine and herring). Moreover, fish oils, which consist of the lipids extracted from flesh or liver of fish, is also a rich source of omega-3 PUFAs (Lane, Derbyshire et al. 2014).

The beneficial effects of omega-3 PUFAs on cardiovascular disease is in part due to their beneficial effects on the endothelial dysfunction through the improvement of the NO bioavailability and the reduction of the vascular oxidative stress (Zanetti, Gortan Cappellari et al. 2017). In meta-analysis of different clinical studies, omega-3 PUFAs intake has been shown to improve the endothelial function without affecting endothelium-independent vasodilation in humans (Wang, Liang et al. 2012). Previous studies by our research team have shown that the long chain omega-3 fatty acids EPA and DHA induced potent endothelium-dependent relaxations in porcine coronary artery rings, and that the potency to induce relaxation was dependent on both the purity and ratio of the EPA:DHA formulation.

Indeed, the use of high purity EPA:DHA formulations indicated that the 6:1 and 9:1 ratios were most potent inducer of endothelium-dependent relaxation (Zgheel, Alhosin et al. 2014). The endothelium-dependent relaxation in porcine coronary artery rings induced by the EPA:DHA 6:1 formulation was due to the activation of both the NO and EDH-mediated component of the relaxation *via* a redox-sensitive activation of the Src/PI3-kinase/Akt pathway (Zgheel, Alhosin et al. 2014).

For the present study, we use the purified EPA:DHA 6:1 formulation to assess its potency to improve the endothelial function in a model of established age-related endothelial dysfunction. The EPA:DHA 6:1 formulation was given at the dose 500 mg/kg which is equivalent to 5.67 g in a 70 kg human (Reagan-Shaw, Nihal et al. 2008), and this dose is in line with reported clinical trials and meta-analysis, where doses range from 0.18 up to 10 g/d of various omega-3 products (Appel, Miller et al. 1993, Delgado-Lista, Perez-Martinez et al. 2012, Enns, Yeganeh et al. 2014, Miller, Van Elswyk et al. 2014). Corn oil was used as a gavage control using an isocaloric oil with a low content of omega-3 fatty acids (less than 1 % w/w compared to >95 % for the EPA:DHA 6:1 formulation).

In a previous study, the oral intake of the purified EPA:DHA 6:1 formulation by angiotensin II-infused rats was able to significantly prevent the angiotensin II-induced hypertension and endothelial dysfunction in secondary branch mesenteric artery. EPA:DHA 6:1 prevented the blunting of both the NO- and EDH-mediated relaxations, and prevented the increase in EDCFs, most likely by preventing NADPH oxidase- and COX-derived oxidative stress (Niazi, Silva et al. 2017).

The present findings show that a short term 2-weeks intake of EPA:DHA 6:1 by aged rats with an established endothelial dysfunction caused a significant improvement in the endothelium-derived relaxations mainly through improving the NO-mediated component of the relaxation, whereas the EDH-component remained abolished (Figure 23). EPA:DHA 6:1 caused significant improvement of basal release of NO as evidenced by the significantly increased PE-induced contractile response in presence of the NOS inhibitor L-NA. The improvement of NO-mediated relaxations and the increased basal release of NO after the 2-weeks treatment with EPA:DHA 6:1 could be partly due to a reduction in the ROS-mediated inactivation of NO and in eNOS uncoupling. Moreover, the indomethacin-sensitive EDCF was partially, but significantly, reduced probably due to the decreased over-expression of

COX-2. The treatment with EPA:DHA 6:1 also significantly decrease the age-related vascular oxidative stress as indicated by the reduced ethidium fluorescence in rings from the main mesenteric artery and aorta. This decrease in the age-related vascular oxidative stress may be due to the decreased activation of the local angiotensin system, and the subsequent downregulation of the NADPH oxidase. Indeed, the EPA:DHA 6:1 treatment reduced the over-expression of two components of the angiotensin system, namely ACE and AT1R, as well as of the subunits of NADPH oxidase p22^{phox} and p47^{phox}. The reduction of oxidative stress by EPA:DHA 6:1 is in line with the findings of clinical study showing a reduction of oxidative stress in atherosclerotic patients after the intake of an omega-3 treatment (Hassan Eftekhari, Aliasghari et al. 2013). The EPA:DHA 6:1 treatment also showed a decreased expression of senescence markers p53, p21 and p16, which can be related to the ability of the EPA:DHA 6:1 treatment to reduce the increased angiotensin system activity and oxidative stress, two cellular stresses that have previously been reported to induce endothelial senescence. This hypothesis is consistent with fact that the senescence level and the expression of these senescence markers were decreased after the treatment with antioxidant and inhibitors of ACE and AT1R in a model of induced senescence in cultured endothelial cells (Khemais-Benkhiat, Idris-Khodja et al. 2016).

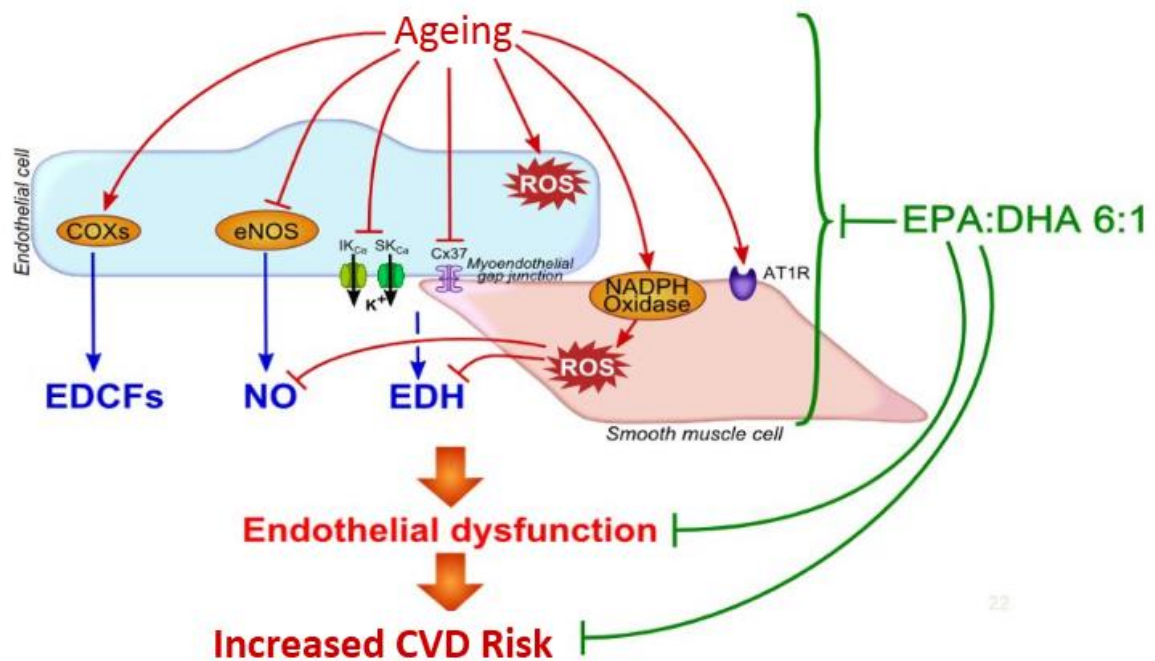


Figure 23: Short term oral intake of omega-3 PUFA EPA:DHA 6 :1 improves the age-related endothelial dysfunction in rats

In addition, the age-related increase in vascular oxidative stress causes the activation of the transcription factors NF- κ B and AP-1 leading to an upregulation of pro-inflammatory cytokines (TNF- α , IL-1, IL-6), adhesion molecules (ICAM, VCAM) and enzymes (iNOS, COX-2). This pro-inflammatory environment can further induce the formation of ROS leading to a worsening of the age-related endothelial dysfunction (El Assar, Angulo et al. 2012). Omega-3 intake increase omega-3 to omega-6 ratio of cell membrane by incorporation into membrane phospholipids. This result in an increased competition between arachidonic acid and omega-3 for the COX and LOX enzymes, resulting in a shift of balance from AA-derived pro-inflammatory eicosanoids (PGE₂, TXB₂, LTB₄ and eicosatrienoic acid derivatives) towards omega-3 PUFAs-derived less inflammatory PGE₃ and LTB₅ (Kromhout, Yasuda et al. 2012). Moreover, omega-3 PUFAs also reduce the LTB₄-associated chemoattraction of leukocytes. The anti-inflammatory action of omega-3 PUFAs could also be due to their ability to induce a GPR120- and PPAR α -mediated inhibition of NF- κ B,

subsequently leading to a decreased production of inflammatory cytokines, chemokines and adhesion molecules. In addition, fish oil intake has been associated to a reduced proliferation of T cells leading to reduced inflammatory responses (Thies, Nebe-von-Caron et al. 2001). Furthermore, the resolution of inflammation by omega-3 PUFAs is also related to the production of omega-3-derived mediators such as resolvins, protectins and maresins (Calder 2013).

6.3 Conclusion and perspectives

In conclusion, the present work has assessed the potency of an EPA:DHA 6:1 formulation to improve the endothelial function in a model of established age-related endothelial dysfunction. In the main mesenteric artery, the age-related endothelial dysfunction was characterized by a blunted NO-mediated component of the endothelium-dependent relaxation, an abolished EDH-mediated component, and by an increased production of COX-derived EDCF. The age-related endothelial dysfunction was associated to an up-regulation of eNOS and COX-2 and a down-regulation of COX-1. Moreover, the endothelial dysfunction was associated with an increased vascular oxidative stress and increased expression of senescence markers, most probably due to an activation of the local angiotensin system and the subsequent up-regulation of NADPH oxidase.

A short term oral intake of 500 mg/kg/day of the EPA:DHA 6:1 formulation significantly improved the endothelial function through an increase in the NO-mediated relaxation and basal formation of NO, and a decreased formation of COX-derived EDCF response, whereas EDH-mediated component remained abolished. The improvement of the endothelial function was associated with a decreased oxidative stress due, at least in part, to a normalization of the angiotensin system activation and the subsequent downregulation of NADPH oxidase and senescence markers.

Further studies are needed to determine the nature of the metabolites involved in the beneficial effects of EPA:DHA 6:1 on the endothelial function and the vascular system in general (Figure 24). Firstly, the impregnation of omega-3 fatty acids in the vascular tissues which needs to be clarified to identify the active compounds. In addition, a metabolomics approaches is needed to determine i) which pro-inflammatory and vasoconstricting prostanoids are involved in the age-related endothelial dysfunction and ii) which active metabolites of omega-3 fatty acids such as resolvins, protectins, thromboxane A₃, leukotriene 5 and lipoxins are released after the EPA:DHA 6:1 treatment. Furthermore, it would be of interest to assess the potential of omega-3 to prevent and/or delay the vascular and cardiac remodeling associated with cardiovascular diseases with increasing age.

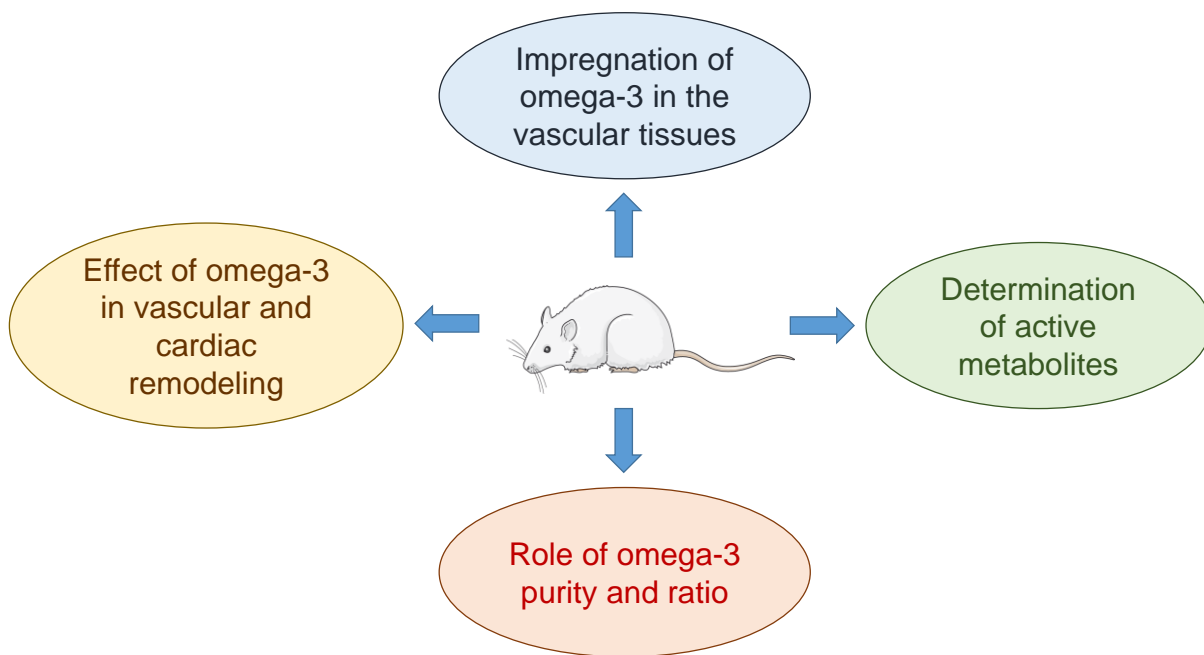


Figure 24: Perspective in preclinical studies

On the basis of vasoprotective results established by the current study, a clinical study can be designed to determine the potential of EPA:DHA 6:1 to reduce cardiovascular risk factors associated with ageing (Figure 25).

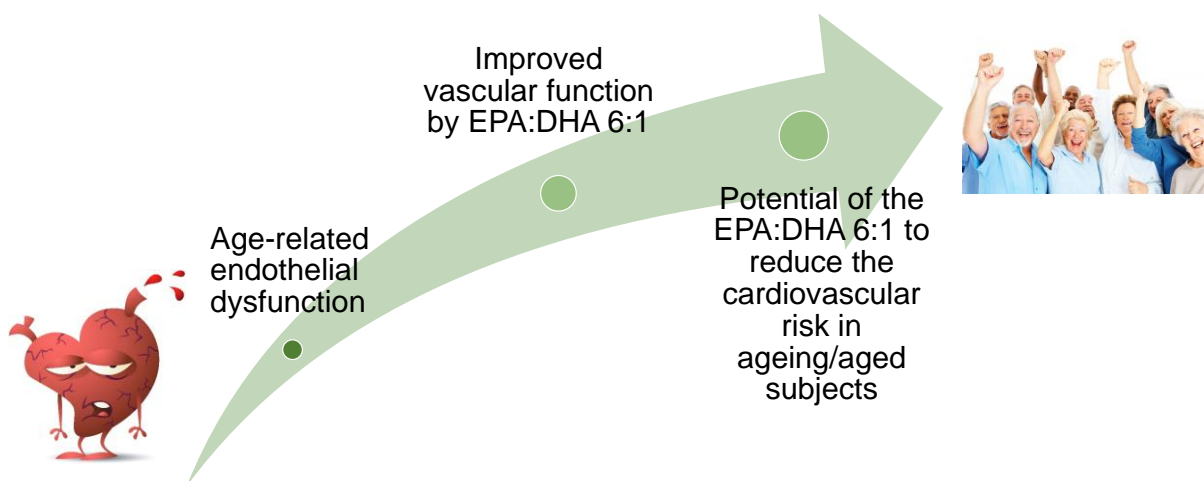


Figure 25: Clinical Perspectives

7-DISCUSSION ET PERSPECTIVES

7- DISCUSSION GENERALE

7.1 La dysfonction endothéliale liée à l'âge

La dysfonction endothéliale se caractérise généralement par une diminution de la capacité de l'endothélium à induire une vasodilatation en réponse à des stimuli chimiques ou mécaniques. (Matz, de Sotomayor et al. 2000). La dysfonction endothéliale liée à l'âge est considérée comme un facteur majeur dans le développement des maladies cardiovasculaires avec l'âge, et ce même en l'absence d'autres facteurs de risques tels que l'hypertension artérielle, le diabète, les dyslipidémies ou l'athérosclérose (Lakatta 2015). De ce fait, la dysfonction endothéliale liée à l'âge constitue une cible thérapeutique d'intérêt dans la prévention des maladies cardiovasculaires chez les sujets âgés (Seals, Jablonski et al. 2011). Des études précliniques et cliniques ont montré que le vieillissement est associé à une réduction des relaxations dépendantes de l'endothélium, alors que les relaxations indépendantes de l'endothélium en réponse au nitroprussiate de sodium ne sont pas affectées (Egashira, Inou et al. 1993, Taddei, Viridis et al. 1995, Matz and Andriantsitohaina 2003, Toda 2012). La diminution des relaxations dépendantes de l'endothélium au cours du vieillissement peut être attribué, du moins partiellement, à des modifications des réponses cellulaires entraînant une diminution de la production ou une inactivation des facteurs vasodilatateurs tels que le NO ou l'EDH, et une augmentation de la production des EDCFs et du stress oxydant (El Assar, Angulo et al. 2012). En effet, des études cliniques sur le flux sanguin dans les artères coronaires et l'avant-bras ont montré une diminution des relaxations dépendantes de l'endothélium en réponse à l'infusion d'acétylcholine au cours du vieillissement (Egashira, Inou et al. 1993, Taddei, Viridis et al. 1995). De plus, le vieillissement est un facteur de risque individuel additionnel pour la dysfonction endothéliale, la diminution des relaxations induites par le NO se retrouvant à la fois chez les sujets normotendus et les patients hypertendus, mais avec une dysfonction plus précoce chez ces derniers (Taddei, Viridis et al. 2001). Les études précliniques dans plusieurs lits vasculaires chez le rat ont aussi montré que la dysfonction endothéliale liée à l'âge était associée à une diminution des relaxations induites par le NO seule ou avec une diminution de la composante EDH de la relaxation dépendante de l'endothélium dans les artères mésentériques (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012), l'aorte (van der Loo, Labugger et al. 2000) et les artérioles

coronaires (Csiszar, Ungvari et al. 2002). Cette dysfonction endothéliale liée à l'âge présente donc des caractéristiques similaires aux dysfonction endothéliales observées dans d'autres modèles comme dans l'artère fémorale de rats spontanément hypertendus (Puzserova, Ilovska et al. 2014), l'artère mésentérique de rats hypertendus par infusion d'angiotensine II (Niazi, Silva et al. 2017) ou l'artère mésentérique de rats cirrhotiques (Rashid, Idris Khodja et al. 2014, Rashid, Idris-Khodja et al. 2018).

Lors de notre étude, nous avons construit des courbes concentration-réponse à l'acétylcholine et à la phényléphrine pour caractériser les relaxations dépendantes de l'endothélium, la formation d'EDCFs et les réponses contractiles vasculaires dans l'artère mésentérique principale de rats jeunes et âgés. Les résultats montrent que chez les rats âgés de 20 mois, la dysfonction endothéliale liée à l'âge est caractérisée par une atténuation des relaxations dépendantes de l'endothélium et une augmentation des contractions vasculaires en réponses à des concentrations croissantes d'acétylcholine (ACh) et de phényléphrine (PE), respectivement. De plus, la composante EDH de la relaxation est totalement abolie chez les rats âgés alors que la composante NO est partiellement, mais significativement, réduite comme l'indique respectivement l'absence de relaxation en présence d'indométacine et du mélange TRAM-34 plus UCL-1684 (inhibiteurs de la voie de la prostacycline et de l'EDH), et la réduction de relaxation en présence d'indométacine et de L-NA (inhibiteurs de la voie de la prostacycline et du NO). Ces résultats sont cohérents avec ceux obtenus dans des études antérieures qui ont montré que la composante EDH de la relaxation dépendante de l'endothélium était fortement affectée par le vieillissement dans l'artère mésentérique de rat (Dal-Ros, Bronner et al. 2012, Idris Khodja, Chataigneau et al. 2012).

De surcroît, une forte augmentation des réponses EDCF a été mise en évidence par la contraction dépendante de l'endothélium en réponses à des concentrations croissantes d'acétylcholine en présence de L-NA et du mélange TRAM-34 plus UCL-1684 (bloquant les composante NO et EDH, respectivement). L'abolition des réponses EDCF en présence d'indométacine indique l'implication des prostanoïdes dérivés de la voie des cyclooxygénases. Ce résultat est renforcé par la réduction significative des réponses contractiles induite par la PE observées en présence d'indométacine dans l'artère mésentérique de rat. Ceci est en adéquation avec le fait que le vieillissement a été associé à un déséquilibre de la balance entre les prostanoïdes vasodilatateurs et vasoconstricteurs en faveur de ces derniers. En effet, des études *in vitro* et *in vivo* chez l'homme ont indiqué que le vieillissement était associé à une diminution de la formation de la prostacycline

vasodilatatrice et une augmentation de la formation des prostanoïdes dérivés de la voie des cyclooxygénases (Taddei, Viridis et al. 1997, Vanhoutte, Feletou et al. 2005, Schrage, Eisenach et al. 2007, Rodriguez-Manas, El-Assar et al. 2009). Cette hypothèse d'une implication des prostanoïdes dérivés de la voie des cyclooxygénases est encore renforcé par le fait que l'on observe une amélioration des relaxations en présence d'antagoniste du récepteur TP chez l'homme (Rodriguez-Manas, El-Assar et al. 2009). Dans la présente étude, les données d'immunofluorescence montrent une augmentation de l'expression de la COX-2 dans les sections d'artère mésentérique et d'aorte des rats âgés, ce qui est cohérent avec les études précédemment publiées (Stewart, Zhang et al. 2000). L'augmentation d'expression de la COX-2 peut expliquer en partie l'augmentation des réponses contractiles EDCF car il a été montré que ces dernières étaient liées à une augmentation de la formation de $\text{PGF}_{2\alpha}$ par la voie COX-2 chez les hamsters âgés (Wong, Leung et al. 2009). A l'inverse, l'inhibiteur sélectif de la COX-2 (NS-398) permet d'améliorer la dysfonction endothéliale dans l'aorte et l'artère mésentérique de rats âgés (de Sotomayor, Perez-Guerrero et al. 2005).

L'augmentation du stress oxydant vasculaire et son lien avec les dommages vasculaires ont été rapportés dans le cadre du vieillissement et de divers facteurs de risque de maladies cardiovasculaires tels que l'hypertension artérielle et le diabète (Ungvari, Buffenstein et al. 2008). L'augmentation de la formation d'espèces réactives de l'oxygène (ERO) liée au vieillissement est un facteur majeur de la dysfonction endothéliale et elle pourrait être due à la fois à une augmentation de la formation des ERO et/ou à une diminution de l'inactivation de ces ERO par les mécanismes de défenses antioxydant cellulaires (El Assar, Angulo et al. 2013). Ainsi, l'infusion de vitamine C est associée avec une augmentation du flux sanguin dans l'avant-bras en réponse à l'ACh chez des sujets âgés et hypertendus (Taddei, Viridis et al. 2001). La sonde redox-sensible dihydroéthidium (DHE) nous a permis de mesurer le niveau de formation des ERO dans des sections congelées d'aorte et d'artère mésentérique. Du fait de la grande perméabilité membranaire du DHE, il peut pénétrer dans la cellule pour réagir avec les ERO intracellulaire, conduisant à la formation d'éthidium donnant une forte fluorescence dans le rouge (Owusu-Ansah, Yavari et al. 2008). Les résultats obtenus montrent une augmentation significative du marquage à l'éthidium dans les sections des rats âgés non traités par rapport aux rats jeunes, indiquant une augmentation du stress oxydant vasculaire.

Le stress oxydant vasculaire peut provenir de plusieurs sources cellulaires dont la NADPH oxydase, la xanthine oxydase, la eNOS découplée, les COXs et la chaîne respiratoire mitochondriale (El Assar, Angulo et al. 2012). La présente étude montre une diminution du

marquage à l'éthidium dans les sections aortiques après traitements avec soit l'indométacine (un inhibiteur de COX), SC-560 (un inhibiteur sélectif de COX-1), NS-398 (un inhibiteur sélectif de COX-2), L-NA (un inhibiteur des NOS), VAS-2870 (un inhibiteur de la NADPH oxydase) ou la combinaison de KCN, roténone et myxothiazole (inhibiteurs de la chaîne respiratoire mitochondriale), indiquant l'implication de plusieurs sources d'ERO dans le stress oxydant vasculaire lié à l'âge. Le marquage éthidium était le plus fortement réduit en présence de VAS-2870, suggérant que la NADPH oxydase joue un rôle majeur dans le stress oxydant lié à l'âge. Des études précédemment publiées ont montré qu'une augmentation de l'activité NADPH oxydase est associée avec la dysfonction endothéliale liée à l'âge chez l'homme, et que l'inhibition de la NADPH oxydase par l'apocynine conduit à une amélioration de la fonction endothéliale (Rodriguez-Manas, El-Assar et al. 2009). De plus, une augmentation liée à l'âge de l'expression des sous-unités de la NADPH oxydase NOX-1 et p22^{phox} a été décrite dans l'artère mésentérique de rats d'âge moyen (Idris Khodja, Chataigneau et al. 2012), ce qui concorde avec l'augmentation d'expression des sous-unités p22^{phox} et p47^{phox} de la NADPH oxydase dans les sections d'aorte des rats âgés non-traités observée dans la présente étude.

La production d'ERO par la NADPH oxydase est principalement sous forme d'anion superoxyde qui peut réduire la biodisponibilité du NO en le transformant chimiquement en anion peroxy-nitrite (ONOO⁻). De plus, l'anion peroxy-nitrite et l'anion superoxyde peuvent conduire à une diminution de la formation de NO et à une formation supplémentaire d'ERO en « découplant » la eNOS via l'oxydation du BH₄, un cofacteur essentiel de la eNOS, en BH₂ (Yang, Huang et al. 2009, Forstermann 2010). L'infusion de BH₄ à des sujets humains a conduit à une amélioration de la fonction endothéliale (Vasquez-Vivar, Kalyanaraman et al. 2003). Le découplage de la eNOS pourrait expliquer la diminution du marquage à l'éthidium dans le section d'aorte en présence de L-NA, et la surexpression de la eNOS dans l'aorte et l'artère mésentérique des rats âgés pourrait être due à un mécanisme de compensation de la réduction de la biodisponibilité du NO. Une augmentation de l'expression de la eNOS a précédemment été décrite dans l'aorte et l'artère mésentérique de rats âgés (Cernadas, Sanchez de Miguel et al. 1998, Briones, Montoya et al. 2005). En outre, la diminution de la composante EDH de la relaxation pourrait être expliquée, du moins en partie, par un dysfonctionnement des canaux potassiques activés par le calcium (SK_{Ca} et IK_{Ca}) du au stress oxydant, conduisant à un affaiblissement des activités électriques à l'origine de l'hyperpolarisation de l'endothélium (Behringer, Shaw et al. 2013). Ainsi, des études

précédentes ont associé une diminution de la composante EDG liée à l'âge à une augmentation du stress oxydant et à une diminution de l'expression des canaux SK_{Ca} and IK_{Ca} dans l'artère mésentérique de rats moyennement âgés (Idris Khodja, Chataigneau et al. 2012).

L'angiotensine II joue un rôle important dans la régulation du système cardiovasculaire principalement par l'activation de ces récepteurs de type 1 et 2 (AT1R et AT2R, respectivement). Une augmentation de l'activité angiotensine II a été associée à une dysfonction endothéliale et une augmentation du stress oxydant vasculaire *via* une activation de la NADPH oxydase induite par AT1R (Rajagopalan, Kurz et al. 1996, Harrison, Cai et al. 2003, Niazi, Silva et al. 2017). Le rôle clé du système angiotensine est mis en évidence par le fait que le traitement de rats âgés soit par un inhibiteur de l'enzyme de conversion de l'angiotensine (ECA) ou par un antagoniste du récepteur AT1R conduisait à une amélioration de la dysfonction endothéliale due, au moins partiellement, à une diminution du stress oxydant (Goto, Fujii et al. 2000, Kansui, Fujii et al. 2002, Mukai, Shimokawa et al. 2002). De surcroît, les souris KO pour AT1R ont montré une augmentation de leur durée de vie *via* une amélioration de la morphologie cardiovasculaire, une diminution de la formation des ERO et la normalisation de la fonction mitochondriale (Benigni, Corna et al. 2009). De plus, le vieillissement a été associé à une augmentation de l'expression de l'angiotensine II et de l'ECA chez le rat (Challah, Nadaud et al. 1997) et les primates non-humains (Wang, Takagi et al. 2003), alors qu'une augmentation liée à l'âge de l'expression de AT1R, de l'angiotensine II et de l'ECA a été rapportée chez la souris (Yoon, Kim et al. 2016). Conformément à ces études, les présents résultats ont montré une augmentation significative des expressions de AT1R et de l'ECA dans les sections d'aorte de rats âgés non traités par rapport aux rats jeunes, indiquant une activation du système angiotensine local. L'infusion d'angiotensine II chez le rat induit une dysfonction endothéliale associée à une augmentation du stress oxydant vasculaire due, du moins en partie, à une surexpression de AT1R et des sous-unités de la NADPH oxydase p22^{phox} et p47^{phox} (Niazi, Silva et al. 2017). Une augmentation de l'activité angiotensine II est aussi associée à une production accrue d'ERO mitochondriaux par la NADPH oxydase (Dikalov and Nazarewicz 2013). De plus, la déplétion de la sous-unité p22^{phox} de la NADPH oxydase a été associée à une diminution de la formation d'ERO dans les mitochondries isolées de cellules endothéliales d'aorte de bovine, alors que l'inhibiteur de la NADPH oxydase, l'apocynine, annule la dysfonction mitochondriale induite par l'angiotensine II (Doughan, Harrison et al. 2008). L'implication de la formation d'ERO par la chaîne respiratoire mitochondriale induite par l'angiotensine II est aussi en accord avec les

présents résultats montrant que le stress oxydant lié à l'âge est réduit en présence des inhibiteurs de la chaîne respiratoire mitochondriale.

Des études récentes rapportent que la sénescence prématurée pourrait être associée à une dysfonction endothéliale et au développement de maladies cardiovasculaires. La sénescence est un processus vital qui empêche la réplication de cellules contenant un ADN endommagé. Elle est caractérisée par un arrêt de la division cellulaire due à un arrêt du cycle cellulaire associé à une surexpression des inhibiteurs des kinases dépendantes des cyclines p53, p21 et p16. La sénescence peut de plus être induite par des facteurs de stress cellulaires (Campisi and d'Adda di Fagagna 2007). Ainsi, une sénescence endothéliale prématurée a été décrite en réponse au stress oxydant, à l'angiotensine II, aux radiations, aux concentrations élevées en glucose, aux dommages à l'ADN et à la réduction de biodisponibilité du NO (Erusalimsky 2009). Les études sur des cultures de cellules endothéliales d'artères coronaires de porc ont montré que la sénescence et une surexpression de p53, p21 et p16 étaient induits par des repiquages en série, et qu'elles étaient associées à une activation du système angiotensine locale menant à une surexpression de la NADPH oxydase et au stress oxydant en résultant (Khemais-Benkhiat, Idris-Khodja et al. 2016, Silva, Abbas et al. 2017). Les présents résultats indiquent que la dysfonction endothéliale liée à l'âge est associée à une surexpression des marqueurs de sénescence p53, p16 et p21 dans les sections d'aorte des rats âgés non traités. De même, chez l'homme, une augmentation de l'expression de ces marqueurs a été identifiée dans des cellules endothéliales de sujets âgés en comparaison avec celles de sujets jeunes (Rossman, Kaplon et al. 2017).

7.2 Effets bénéfiques des acides gras polyinsaturés oméga-3

De nombreuses études expérimentales et cliniques ont associé la prise alimentaire d'acides gras polyinsaturés (AGPI) oméga-3 avec un effet bénéfique sur le système cardiovasculaire (Mozaffarian and Rimm 2006, Roth and Harris 2010), et une diminution du risque de maladies cardiovasculaires et de leurs facteurs de risques tels que l'hypercholestérolémie, l'athéromatose, l'hypertension, les arythmies, l'inflammation, le stress oxydant et le diabète (Mozaffarian and Wu 2011, Mori 2014). En effet, une corrélation inverse a été observée entre l'augmentation de la consommation des AGPI oméga-3 et l'incidence des maladies cardiovasculaires (Kromhout 1989). Les acides gras oméga-3 à chaînes longues, l'acide eicosapentaénoïque (EPA) et l'acide docosahexaénoïque (DHA), ont

été décrit comme étant les AGPI oméga-3 les plus efficaces (Riediger, Othman et al. 2009). Au niveau moléculaire, les AGPI oméga-3 produisent leurs effets en agissant sur le fonctionnement des membranes cellulaires, des récepteurs membranaires et des canaux ioniques associés, en altérant le profil de formation des prostanoïdes en entrant en compétition avec l'acide arachidonique en tant que précurseurs des produits dérivés des voies des COX et des LOXs, et en modifiant l'activité de certains récepteurs nucléaires et facteurs de transcription (Mozaffarian and Wu 2011, Calvo, Martínez et al. 2017). Les poissons et les fruits de mer sont des sources alimentaires riches en AGPI oméga-3 à chaînes longues, en particulier les poissons gras comme le saumon, le maquereau, le thon, la sardine et le hareng. De plus, les huiles de poissons, qui sont les lipides extraits de la chair et/ou du foie des poissons, sont des sources importantes en AGPI oméga-3 (Lane, Derbyshire et al. 2014).

Les effets bénéfiques des AGPI omega-3 sur le système cardiovasculaire sont en partie dus à leurs effets bénéfiques sur la dysfonction endothéliale par l'amélioration de la biodisponibilité du NO et la réduction du stress oxydant vasculaire (Zanetti, Gortan Cappellari et al. 2017). Dans les méta-analyses d'études cliniques, la consommation d'AGPI omega-3 améliore la fonction endothéliale sans affecter les vasodilatations indépendantes de l'endothélium (Wang, Liang et al. 2012). Des études précédentes de notre groupe ont montré que les oméga-3 à chaînes longues EPA et DHA induisent des fortes relaxations dépendantes de l'endothélium dans des anneaux d'artère coronaire de porc, et que la capacité à induire ces relaxations dépendait à la fois de la pureté et du ratio du mélange des formulations EPA:DHA. En effet, le recours à des formulations EPA:DHA hautement purifiées a indiqué que les ratios 6:1 et 9:1 étaient les plus puissants inducteurs des relaxations dépendantes de l'endothélium (Zgheel, Alhosin et al. 2014). De plus, la relaxation dépendante de l'endothélium induite par la formulation EPA:DHA 6:1 dans des anneaux d'artère coronaire de porc est due à l'activation des deux composantes NO et EDH de la relaxation *via* une activation redox-sensible de la voie Src/PI3-kinase/Akt (Zgheel, Alhosin et al. 2014).

Pour cette étude, nous avons utilisé la formulation purifiée EPA:DHA 6:1 afin de déterminer sa capacité à améliorer la fonction endothéliale dans un modèle présentant une dysfonction endothéliale liée à l'âge établie. La formulation EPA:DHA 6:1 a été donnée à la dose de 500 mg/kg, ce qui équivaut à une dose de 5,67 g pour un homme de 70 kg (Reagan-Shaw, Nihal et al. 2008), et cette dose est cohérente avec les doses rapportées pour les études cliniques et les méta-analyses, doses allant de 0,18 à 10 g/j de divers produits contenant des oméga-3 (Appel, Miller et al. 1993, Delgado-Lista, Perez-Martinez et al. 2012, Enns,

Yeganeh et al. 2014, Miller, Van Elswyk et al. 2014). L'huile de maïs a été choisi comme un contrôle pour le gavage avec une huile iso-calorique contenant un faible taux d'oméga-3 (moins de 1 % par rapport à plus de 95 % pour formulation EPA:DHA 6:1).

Dans une étude précédente, le traitement *per os* de rats infusés à l'angiotensine II avec la formulation purifiée EPA:DHA 6:1 a permis de prévenir significativement l'hypertension et la dysfonction endothéliale induite par l'angiotensine II dans les branches secondaires de l'artère mésentérique. La formulation EPA:DHA 6:1 a aussi permis de prévenir significativement la diminution des relaxations induites par les composantes NO et EDH, et l'augmentation des réponses contractiles EDCFs, très probablement en prévenant le stress oxydant dues à la NADPH oxydase et aux COXs (Niazi, Silva et al. 2017).

Les présents résultats indiquent que la prise de la formulation EPA:DHA 6:1 pendant 2 semaines par des rats âgés présentant une dysfonction endothéliale établie conduit à une amélioration des relaxations dépendantes de l'endothélium principalement par l'amélioration de la composante NO, alors que la composante EDH reste abolie (Figure 23). La formulation EPA:DHA 6:1 améliore significativement la formation basale de NO ainsi que le montre l'augmentation des réponses contractile induites par la PE en présence de L-NA, l'inhibiteur de NOS. L'amélioration des relaxations induites par le NO et l'augmentation de la formation basale de NO après 2 semaines de traitements avec la formulation EPA:DHA 6:1 pourrait être partie dues à la diminution de l'inactivation du NO et du découplage de la eNOS induite par les ERO. De plus, la composante EDCF sensible à l'indométacine est réduite partiellement, mais significativement, probablement du fait de la réduction de la surexpression de COX-2. Le traitement avec la formulation EPA:DHA 6:1 a aussi également réduit significativement le stress oxydant lié à l'âge comme l'indique la diminution du niveau de fluorescence de l'éthidium dans les sections d'aorte et d'artère mésentérique. Cette réduction du stress oxydant vasculaire lié à l'âge pourrait être due à une diminution de l'activation du système locale de l'angiotensine, et de la surexpression de la NADPH oxydase en résultant. En effet, le traitement avec la formulation EPA:DHA 6:1 a réduit la surexpression de deux composants du système angiotensine, à savoir l'ECA et AT1R, de même que les deux sous-unités de la NADPH oxydase p22^{phox} et p47^{phox}. Cette réduction du stress oxydant avec le traitement EPA:DHA 6:1 est en accord avec les conclusion d'une étude clinique qui a montré une réduction du stress oxydant chez les patients athérosclérotiques après la prise d'un traitement d'oméga-3 (Hassan Eftekhari, Aliasghari et al. 2013). Le traitement avec la formulation EPA:DHA 6:1 a aussi induit une diminution de l'expression des marqueurs de senescence

p53, p21 et p16, ce qui peut être reliée à la capacité du traitement EPA:DHA 6:1 à réduire l'activité du système angiotensine locale et le stress oxydant vasculaire, deux stress cellulaire connus pour induire la sénescence endothéliale. Cette hypothèse est cohérente avec le fait que le niveau de sénescence et l'expression des marqueurs de sénescence ont été réduits après un traitement avec un antioxydant et des inhibiteurs de l'ECA et de AT1R dans un modèle de sénescence induite dans des cellules endothéliales en culture (Khemais-Benkhiat, Idris-Khodja et al. 2016).

De surcroît, l'augmentation liée à l'âge du stress oxydant vasculaire cause l'activation des facteurs de transcription NF- κ B et AP-1 menant à la surexpression de cytokines pro-inflammatoires (TNF- α , IL-1, IL-6), de molécules d'adhésion (ICAM, VCAM) et d'enzymes (iNOS, COX-2). Cet environnement pro-inflammatoire peut encore davantage induire la formation d'ERO conduisant une exacerbation de la dysfonction endothéliale liée à l'âge (El Assar, Angulo et al. 2012). La prise d'oméga-3 augmente le ratio omega-3/omega-6 ratio dans la membrane cellulaire par incorporation dans les phospholipides membranaires. Cela mène à une compétition entre l'acide arachidonique et les oméga-3 pour l'utilisation par les COX et les LOX, conduisant à un déséquilibre dans la balance entre les eicosanoïdes pro-inflammatoires dérivés de l'acide arachidonique (PGE₂, TXB₂, LTB₄ et les dérivés de l'acide eicosatriénoïque) au profit des PGE₃ et LTB₅ moins pro-inflammatoires dérivés des oméga-3 (Kromhout, Yasuda et al. 2012). De plus, les AGPI omega-3 réduisent également la chimio-attraction des leucocytes par le LTB₄. L'action anti-inflammatoire des AGPI oméga-3 peut aussi être due à leur capacité à induire l'inhibition de NF- κ B induite par GPR120 et PPAR α , aboutissant à une diminution de la production des molécules d'adhésion, chimiokines et cytokines inflammatoires. En outre, la consommation d'huile de poisson a été associée à une réduction de la prolifération des lymphocytes T menant à une diminution des réponses inflammatoires (Thies, Nebe-von-Caron et al. 2001). Enfin, la résolution de l'inflammation par les AGPI omega-3 est aussi liée à la formation de médiateurs dérivés des omega-3 tels que les résolvines, protectines et marésines (Calder 2013).

7.3 Conclusion et perspectives

En conclusion, les travaux présentés ici ont évalué la capacité d'une formulation EPA:DHA 6:1 à améliorer la fonction endothéliale dans un modèle de dysfonction endothéliale liée à l'âge établie. Dans l'artère mésentérique, la dysfonction endothéliale liée à l'âge est caractérisée par une réduction de la composante NO de la relaxation dépendante de l'endothélium, une abolition de la composante EDH, et une augmentation de la production d'EDCF provenant de la voie des COX. La dysfonction endothéliale liée à l'âge est associée à une surexpression de la eNOS et de COX-2 et une diminution d'expression de COX-1. De plus, la dysfonction endothéliale est associée à une augmentation du stress oxydant vasculaire et une augmentation de l'expression des marqueurs de sénescence, très probablement dues à une activation du système angiotensine locale et la surexpression de NADPH oxydase en résultant.

Un traitement à court terme avec 500 mg/kg de la formulation EPA:DHA 6:1 a significativement amélioré la fonction endothéliale de par l'augmentation de la relaxation due à la composante NO et de la formation basale de NO, et de par la réduction de la formation des réponses EDCF dérivés de la voie des COX, alors que la composante EDH de la relaxation restait abolie. L'amélioration de la fonction endothéliale est associée à une diminution du stress oxydant due, du moins partiellement, à la normalisation de l'activation du système angiotensine locale et la réduction d'expression de la NADPH oxydase et des marqueurs de sénescence en résultants.

Des études complémentaires sont nécessaires pour déterminer la nature des métabolites impliqués dans l'effet bénéfiques de la formulation EPA:DHA 6:1 sur la fonction endothéliale et sur le système vasculaire en général (Figure 24). Tout d'abord, l'imprégnation des acides gras oméga-3 dans les tissus vasculaires doit être évaluée pour identifier les composés actifs. De plus, une approche en métabolomique permettra de déterminer i) la nature des prostanoïdes pro-inflammatoires et vasoconstricteurs impliqués dans la dysfonction endothéliale liée à l'âge, et ii) la nature des métabolites actifs des oméga-3 tels que les résolvines, protectines, thromboxane A₂, leukotriène 5 and lipoxines libérés après le traitement avec la formulation EPA:DHA 6:1. Enfin, il serait intéressant d'évaluer la capacité des oméga-3 à prévenir et/ou ralentir les remodelages vasculaire et cardiaque associés avec les maladies cardiovasculaire liée au vieillissement.

Sur la base des effets vasoprotecteurs démontrés lors de la présente étude, une étude clinique pourrait être construite pour évaluer le potentiel de la formulation EPA:DHA 6:1 à réduire les facteurs de risques cardiovasculaire associés au vieillissement (Figure 25).

REFERENCES

- GISSI Investigators (1999). "Dietary supplementation with n-3 polyunsaturated fatty acids and vitamin E after myocardial infarction: results of the GISSI-Prevenzione trial. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico." Lancet **354**(9177): 447-455.
- Adabag, A. S., R. V. Luepker, V. L. Roger and B. J. Gersh (2010). "Sudden cardiac death: epidemiology and risk factors." Nat Rev Cardiol **7**(4): 216-225.
- Adkins, Y. and D. S. Kelley (2010). "Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids." J Nutr Biochem **21**(9): 781-792.
- Afanas'ev, I. (2010). "Signaling and Damaging Functions of Free Radicals in Aging-Free Radical Theory, Hormesis, and TOR." Aging Dis **1**(2): 75-88.
- Allen, A. M., J. Zhuo and F. A. Mendelsohn (1999). "Localization of angiotensin AT1 and AT2 receptors." J Am Soc Nephrol **10 Suppl 11**: S23-29.
- Appel, L. J., E. R. Miller, 3rd, A. J. Seidler and P. K. Whelton (1993). "Does supplementation of diet with 'fish oil' reduce blood pressure? A meta-analysis of controlled clinical trials." Arch Intern Med **153**(12): 1429-1438.
- Arenas, I. A., Y. Xu and S. T. Davidge (2006). "Age-associated impairment in vasorelaxation to fluid shear stress in the female vasculature is improved by TNF-alpha antagonism." Am J Physiol Heart Circ Physiol **290**(3): H1259-1263.
- Assar, M. E., J. Angulo and L. Rodriguez-Manas (2016). "Diabetes and ageing-induced vascular inflammation." J Physiol **594**(8): 2125-2146.
- Bang, H. O. and J. Dyerberg (1980). "The bleeding tendency in Greenland Eskimos." Dan Med Bull **27**(4): 202-205.
- Bao, D. Q., T. A. Mori, V. Burke, I. B. Puddey and L. J. Beilin (1998). "Effects of dietary fish and weight reduction on ambulatory blood pressure in overweight hypertensives." Hypertension **32**(4): 710-717.
- Barton, M. (2014). "Aging and endothelin: determinants of disease." Life Sci **118**(2): 97-109.
- Bauer, P. M., D. Fulton, Y. C. Boo, G. P. Sorescu, B. E. Kemp, H. Jo and W. C. Sessa (2003). "Compensatory phosphorylation and protein-protein interactions revealed by loss of function and gain of function mutants of multiple serine phosphorylation sites in endothelial nitric-oxide synthase." J Biol Chem **278**(17): 14841-14849.
- Behringer, E. J., R. L. Shaw, E. B. Westcott, M. J. Socha and S. S. Segal (2013). "Aging impairs electrical conduction along endothelium of resistance arteries through enhanced Ca²⁺-activated K⁺ channel activation." Arterioscler Thromb Vasc Biol **33**(8): 1892-1901.
- Benigni, A., D. Corna, C. Zoja, A. Sonzogni, R. Latini, M. Salio, S. Conti, D. Rottoli, L. Longaretti, P. Cassis, M. Morigi, T. M. Coffman and G. Remuzzi (2009). "Disruption of the Ang II type 1 receptor promotes longevity in mice." J Clin Invest **119**(3): 524-530.
- Benjamin, E. J., S. S. Virani, C. W. Callaway, A. M. Chamberlain, A. R. Chang, S. Cheng, S. E. Chiuve, M. Cushman, F. N. Delling, R. Deo, S. D. de Ferranti, J. F. Ferguson, M. Fornage, C. Gillespie, C. R. Isasi, M. C. Jimenez, L. C. Jordan, S. E. Judd, D. Lackland, J. H. Lichtman, L. Lisabeth, S. Liu, C. T. Longenecker, P. L. Lutsey, J. S. Mackey, D. B. Matchar, K. Matsushita, M. E. Mussolino, K. Nasir, M. O'Flaherty, L. P. Palaniappan, A. Pandey, D. K. Pandey, M. J. Reeves, M. D. Ritchey, C. J. Rodriguez, G. A. Roth, W. D. Rosamond, U. K. A. Sampson, G. M. Satou, S. H. Shah, N. L. Spartano, D. L. Tirschwell, C. W. Tsao, J. H. Voeks, J. Z. Willey, J. T. Wilkins, J. H. Wu, H. M. Alger, S. S. Wong, P. Muntner, E.

- American Heart Association Council on, C. Prevention Statistics and S. Stroke Statistics (2018). "Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association." Circulation **137**(12): e67-e492.
- Bhayadia, R., B. M. Schmidt, A. Melk and M. Homme (2016). "Senescence-Induced Oxidative Stress Causes Endothelial Dysfunction." J Gerontol A Biol Sci Med Sci **71**(2): 161-169.
- Bode-Boger, S. M., J. Muke, A. Surdacki, G. Brabant, R. H. Boger and J. C. Frolich (2003). "Oral L-arginine improves endothelial function in healthy individuals older than 70 years." Vasc Med **8**(2): 77-81.
- Bohm, F. and J. Pernow (2007). "The importance of endothelin-1 for vascular dysfunction in cardiovascular disease." Cardiovasc Res **76**(1): 8-18.
- Bos, C. L., D. J. Richel, T. Ritsema, M. P. Peppelenbosch and H. H. Versteeg (2004). "Prostanoids and prostanoid receptors in signal transduction." Int J Biochem Cell Biol **36**(7): 1187-1205.
- Bowen, K. J., W. S. Harris and P. M. Kris-Etherton (2016). "Omega-3 Fatty Acids and Cardiovascular Disease: Are There Benefits?" Curr Treat Options Cardiovasc Med **18**(11): 69.
- Brakemeier, S., I. Eichler, A. Knorr, T. Fassheber, R. Kohler and J. Hoyer (2003). "Modulation of Ca²⁺-activated K⁺ channel in renal artery endothelium in situ by nitric oxide and reactive oxygen species." Kidney Int **64**(1): 199-207.
- Brandes, R. P., I. Fleming and R. Busse (2005). "Endothelial aging." Cardiovasc Res **66**(2): 286-294.
- Briggs, M. A., K. S. Petersen and P. M. Kris-Etherton (2017). "Saturated Fatty Acids and Cardiovascular Disease: Replacements for Saturated Fat to Reduce Cardiovascular Risk." Healthcare (Basel) **5**(2).
- Briones, A. M., N. Montoya, J. Giraldo and E. Vila (2005). "Ageing affects nitric oxide synthase, cyclooxygenase and oxidative stress enzymes expression differently in mesenteric resistance arteries." Auton Autacoid Pharmacol **25**(4): 155-162.
- Burdge, G. C. and P. C. Calder (2006). "Dietary alpha-linolenic acid and health-related outcomes: a metabolic perspective." Nutr Res Rev **19**(1): 26-52.
- Burr, M. L., A. M. Fehily, J. F. Gilbert, S. Rogers, R. M. Holliday, P. M. Sweetnam, P. C. Elwood and N. M. Deadman (1989). "Effects of changes in fat, fish, and fibre intakes on death and myocardial reinfarction: diet and reinfarction trial (DART)." Lancet **2**(8666): 757-761.
- Cabo, J., R. Alonso and P. Mata (2012). "Omega-3 fatty acids and blood pressure." Br J Nutr **107** Suppl 2: S195-200.
- Calder, P. C. (2013). "Omega-3 polyunsaturated fatty acids and inflammatory processes: nutrition or pharmacology?" Br J Clin Pharmacol **75**(3): 645-662.
- Calder, P. C. and P. Yaqoob (2009). "Understanding omega-3 polyunsaturated fatty acids." Postgrad Med **121**(6): 148-157.
- Calvo, M. J., M. S. Martínez, W. Torres, M. Chávez-Castillo, E. Luzardo, N. Villasmil, J. Salazar, M. Velasco and V. Bermúdez (2017). "Omega-3 polyunsaturated fatty acids and

- cardiovascular health: a molecular view into structure and function." Vessel Plus **1**(3): 116-128.
- Campisi, J. and F. d'Adda di Fagagna (2007). "Cellular senescence: when bad things happen to good cells." Nat Rev Mol Cell Biol **8**(9): 729-740.
- Cau, S. B., F. S. Carneiro and R. C. Tostes (2012). "Differential modulation of nitric oxide synthases in aging: therapeutic opportunities." Front Physiol **3**: 218.
- Cernadas, M. R., L. Sanchez de Miguel, M. Garcia-Duran, F. Gonzalez-Fernandez, I. Millas, M. Monton, J. Rodrigo, L. Rico, P. Fernandez, T. de Frutos, J. A. Rodriguez-Feo, J. Guerra, C. Caramelo, S. Casado and F. Lopez (1998). "Expression of constitutive and inducible nitric oxide synthases in the vascular wall of young and aging rats." Circ Res **83**(3): 279-286.
- Challah, M., S. Nadaud, M. Philippe, T. Battle, F. Soubrier, B. Corman and J. B. Michel (1997). "Circulating and cellular markers of endothelial dysfunction with aging in rats." Am J Physiol **273**(4 Pt 2): H1941-1948.
- Chen, J., S. V. Brodsky, D. M. Goligorsky, D. J. Hampel, H. Li, S. S. Gross and M. S. Goligorsky (2002). "Glycated collagen I induces premature senescence-like phenotypic changes in endothelial cells." Circ Res **90**(12): 1290-1298.
- Chou, T. C., M. H. Yen, C. Y. Li and Y. A. Ding (1998). "Alterations of nitric oxide synthase expression with aging and hypertension in rats." Hypertension **31**(2): 643-648.
- Coleman, R. A., W. L. Smith and S. Narumiya (1994). "International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes." Pharmacol Rev **46**(2): 205-229.
- Csiszar, A., Z. Ungvari, J. G. Edwards, P. Kaminski, M. S. Wolin, A. Koller and G. Kaley (2002). "Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function." Circ Res **90**(11): 1159-1166.
- Csiszar, A., Z. Ungvari, A. Koller, J. G. Edwards and G. Kaley (2004). "Proinflammatory phenotype of coronary arteries promotes endothelial apoptosis in aging." Physiol Genomics **17**(1): 21-30.
- d'Uscio, L. V., S. Shaw, M. Barton and T. F. Luscher (1998). "Losartan but not verapamil inhibits angiotensin II-induced tissue endothelin-1 increase: role of blood pressure and endothelial function." Hypertension **31**(6): 1305-1310.
- Dal-Ros, S., C. Bronner, C. Auger and V. B. Schini-Kerth (2012). "Red wine polyphenols improve an established aging-related endothelial dysfunction in the mesenteric artery of middle-aged rats: role of oxidative stress." Biochem Biophys Res Commun **419**(2): 381-387.
- Davalli, P., T. Mitic, A. Caporali, A. Lauriola and D. D'Arca (2016). "ROS, Cell Senescence, and Novel Molecular Mechanisms in Aging and Age-Related Diseases." Oxid Med Cell Longev **2016**: 3565127.
- de Sotomayor, M. A., C. Perez-Guerrero, M. D. Herrera, L. Jimenez, R. Marin, E. Marhuenda and R. Andriantsitohaina (2005). "Improvement of age-related endothelial dysfunction by simvastatin: effect on NO and COX pathways." Br J Pharmacol **146**(8): 1130-1138.
- Delgado-Lista, J., P. Perez-Martinez, J. Lopez-Miranda and F. Perez-Jimenez (2012). "Long chain omega-3 fatty acids and cardiovascular disease: a systematic review." Br J Nutr **107** **Suppl 2**: S201-213.

- Dessy, C., O. Feron and J. L. Balligand (2010). "The regulation of endothelial nitric oxide synthase by caveolin: a paradigm validated in vivo and shared by the 'endothelium-derived hyperpolarizing factor'." Pflugers Arch **459**(6): 817-827.
- Dikalov, S. I. and R. R. Nazarewicz (2013). "Angiotensin II-induced production of mitochondrial reactive oxygen species: potential mechanisms and relevance for cardiovascular disease." Antioxid Redox Signal **19**(10): 1085-1094.
- DiLoreto, R. and C. T. Murphy (2015). "The cell biology of aging." Mol Biol Cell **26**(25): 4524-4531.
- Donato, A. J., A. D. Black, K. L. Jablonski, L. B. Gano and D. R. Seals (2008). "Aging is associated with greater nuclear NF kappa B, reduced I kappa B alpha, and increased expression of proinflammatory cytokines in vascular endothelial cells of healthy humans." Aging Cell **7**(6): 805-812.
- Donato, A. J., I. Eskurza, A. E. Silver, A. S. Levy, G. L. Pierce, P. E. Gates and D. R. Seals (2007). "Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB." Circ Res **100**(11): 1659-1666.
- Donato, A. J., L. B. Gano, I. Eskurza, A. E. Silver, P. E. Gates, K. Jablonski and D. R. Seals (2009). "Vascular endothelial dysfunction with aging: endothelin-1 and endothelial nitric oxide synthase." Am J Physiol Heart Circ Physiol **297**(1): H425-432.
- Doughan, A. K., D. G. Harrison and S. I. Dikalov (2008). "Molecular mechanisms of angiotensin II-mediated mitochondrial dysfunction: linking mitochondrial oxidative damage and vascular endothelial dysfunction." Circ Res **102**(4): 488-496.
- Dudzinski, D. M. and T. Michel (2007). "Life history of eNOS: partners and pathways." Cardiovasc Res **75**(2): 247-260.
- Dujardin, K. S., B. Dumotier, M. David, M. Guizy, C. Valenzuela and L. M. Hondeghem (2008). "Ultrafast sodium channel block by dietary fish oil prevents dofetilide-induced ventricular arrhythmias in rabbit hearts." Am J Physiol Heart Circ Physiol **295**(4): H1414-1421.
- Egashira, K., T. Inou, Y. Hirooka, H. Kai, M. Sugimachi, S. Suzuki, T. Kuga, Y. Urabe and A. Takeshita (1993). "Effects of age on endothelium-dependent vasodilation of resistance coronary artery by acetylcholine in humans." Circulation **88**(1): 77-81.
- El Assar, M., J. Angulo and L. Rodriguez-Manas (2013). "Oxidative stress and vascular inflammation in aging." Free Radic Biol Med **65**: 380-401.
- El Assar, M., J. Angulo, S. Vallejo, C. Peiro, C. F. Sanchez-Ferrer and L. Rodriguez-Manas (2012). "Mechanisms involved in the aging-induced vascular dysfunction." Front Physiol **3**: 132.
- Elmadfa, I. and M. Kornsteiner (2009). "Fats and fatty acid requirements for adults." Ann Nutr Metab **55**(1-3): 56-75.
- Endo, J. and M. Arita (2016). "Cardioprotective mechanism of omega-3 polyunsaturated fatty acids." J Cardiol **67**(1): 22-27.
- Enns, J. E., A. Yeganeh, R. Zarychanski, A. M. Abou-Setta, C. Friesen, P. Zahradka and C. G. Taylor (2014). "The impact of omega-3 polyunsaturated fatty acid supplementation on the incidence of cardiovascular events and complications in peripheral arterial disease: a systematic review and meta-analysis." BMC Cardiovasc Disord **14**: 70.

- Erusalimsky, J. D. (2009). "Vascular endothelial senescence: from mechanisms to pathophysiology." J Appl Physiol (1985) **106**(1): 326-332.
- Fahy, E., S. Subramaniam, H. A. Brown, C. K. Glass, A. H. Merrill, Jr., R. C. Murphy, C. R. Raetz, D. W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M. S. VanNieuwenhze, S. H. White, J. L. Witztum and E. A. Dennis (2005). "A comprehensive classification system for lipids." J Lipid Res **46**(5): 839-861.
- Fajemiroye, J. O., L. C. da Cunha, R. Saavedra-Rodriguez, K. L. Rodrigues, L. M. Naves, A. A. Mourao, E. F. da Silva, N. E. E. Williams, J. L. R. Martins, R. B. Sousa, A. C. S. Rebelo, A. Reis, R. D. S. Santos, M. L. Ferreira-Neto and G. R. Pedrino (2018). "Aging-Induced Biological Changes and Cardiovascular Diseases." Biomed Res Int **2018**: 7156435.
- Fan, Y. Y., L. H. Ly, R. Barhoumi, D. N. McMurray and R. S. Chapkin (2004). "Dietary docosahexaenoic acid suppresses T cell protein kinase C theta lipid raft recruitment and IL-2 production." J Immunol **173**(10): 6151-6160.
- Feletou, M., Y. Huang and P. M. Vanhoutte (2010). "Vasoconstrictor prostanoids." Pflugers Arch **459**(6): 941-950.
- Feletou, M., Y. Huang and P. M. Vanhoutte (2011). "Endothelium-mediated control of vascular tone: COX-1 and COX-2 products." Br J Pharmacol **164**(3): 894-912.
- Feletou, M. and P. M. Vanhoutte (2009). "EDHF: an update." Clin Sci (Lond) **117**(4): 139-155.
- Fenton, M., S. Barker, D. J. Kurz and J. D. Erusalimsky (2001). "Cellular senescence after single and repeated balloon catheter denudations of rabbit carotid arteries." Arterioscler Thromb Vasc Biol **21**(2): 220-226.
- Ferrier, G. R., I. Redondo, J. Zhu and M. G. Murphy (2002). "Differential effects of docosahexaenoic acid on contractions and L-type Ca²⁺ current in adult cardiac myocytes." Cardiovascular Research **54**(3): 601-610.
- Fleming, I. (2010). "Molecular mechanisms underlying the activation of eNOS." Pflugers Arch **459**(6): 793-806.
- Forstermann, U. (2010). "Nitric oxide and oxidative stress in vascular disease." Pflugers Arch **459**(6): 923-939.
- Forstermann, U. and W. C. Sessa (2012). "Nitric oxide synthases: regulation and function." Eur Heart J **33**(7): 829-837, 837a-837d.
- Francia, P., C. delli Gatti, M. Bachschmid, I. Martin-Padura, C. Savoia, E. Migliaccio, P. G. Pelicci, M. Schiavoni, T. F. Luscher, M. Volpe and F. Cosentino (2004). "Deletion of p66shc gene protects against age-related endothelial dysfunction." Circulation **110**(18): 2889-2895.
- Funk, C. D. (2001). "Prostaglandins and leukotrienes: advances in eicosanoid biology." Science **294**(5548): 1871-1875.
- Furchgott, R. F. and P. M. Vanhoutte (1989). "Endothelium-derived relaxing and contracting factors." FASEB J **3**(9): 2007-2018.
- Furchgott, R. F. and J. V. Zawadzki (1980). "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine." Nature **288**(5789): 373-376.
- Galley, H. F. and N. R. Webster (2004). "Physiology of the endothelium." Br J Anaesth **93**(1): 105-113.

- Gao, Y. (2010). "The multiple actions of NO." Pflugers Arch **459**(6): 829-839.
- Garland, C. J., C. R. Hiley and K. A. Dora (2011). "EDHF: spreading the influence of the endothelium." Br J Pharmacol **164**(3): 839-852.
- Gewaltig, M. T. and G. Kojda (2002). "Vasoprotection by nitric oxide: mechanisms and therapeutic potential." Cardiovasc Res **55**(2): 250-260.
- Ghosh, S. and M. Karin (2002). "Missing pieces in the NF-kappaB puzzle." Cell **109** **Suppl**: S81-96.
- Gillingham, L. G., S. Harris-Janzen and P. J. Jones (2011). "Dietary monounsaturated fatty acids are protective against metabolic syndrome and cardiovascular disease risk factors." Lipids **46**(3): 209-228.
- Gluais, P., M. Lonchampt, J. D. Morrow, P. M. Vanhoutte and M. Feletou (2005). "Acetylcholine-induced endothelium-dependent contractions in the SHR aorta: the Janus face of prostacyclin." Br J Pharmacol **146**(6): 834-845.
- Goto, K., K. Fujii, U. Onaka, I. Abe and M. Fujishima (2000). "Angiotensin-converting enzyme inhibitor prevents age-related endothelial dysfunction." Hypertension **36**(4): 581-587.
- Goto, K., T. Ohtsubo and T. Kitazono (2018). "Endothelium-Dependent Hyperpolarization (EDH) in Hypertension: The Role of Endothelial Ion Channels." Int J Mol Sci **19**(1).
- Griffith, T. M., A. T. Chaytor, L. M. Bakker and D. H. Edwards (2005). "5-Methyltetrahydrofolate and tetrahydrobiopterin can modulate electrotonically mediated endothelium-dependent vascular relaxation." Proc Natl Acad Sci U S A **102**(19): 7008-7013.
- Harrison, D. G., H. Cai, U. Landmesser and K. K. Griendling (2003). "Interactions of angiotensin II with NAD(P)H oxidase, oxidant stress and cardiovascular disease." J Renin Angiotensin Aldosterone Syst **4**(2): 51-61.
- Hassan Eftekhari, M., F. Aliasghari, M. A. Babaei-Beigi and J. Hasanzadeh (2013). "Effect of conjugated linoleic acid and omega-3 fatty acid supplementation on inflammatory and oxidative stress markers in atherosclerotic patients." ARYA Atheroscler **9**(6): 311-318.
- Herrera, M. D., C. Mingorance, R. Rodriguez-Rodriguez and M. Alvarez de Sotomayor (2010). "Endothelial dysfunction and aging: an update." Ageing Res Rev **9**(2): 142-152.
- Hewer, R. C., G. B. Sala-Newby, Y. J. Wu, A. C. Newby and M. Bond (2011). "PKA and Epac synergistically inhibit smooth muscle cell proliferation." J Mol Cell Cardiol **50**(1): 87-98.
- Heymes, C., A. Habib, D. Yang, E. Mathieu, F. Marotte, J. Samuel and C. M. Boulanger (2000). "Cyclo-oxygenase-1 and -2 contribution to endothelial dysfunction in ageing." Br J Pharmacol **131**(4): 804-810.
- Higuchi, S., H. Ohtsu, H. Suzuki, H. Shirai, G. D. Frank and S. Eguchi (2007). "Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology." Clin Sci (Lond) **112**(8): 417-428.
- Holmstrom, K. M. and T. Finkel (2014). "Cellular mechanisms and physiological consequences of redox-dependent signalling." Nat Rev Mol Cell Biol **15**(6): 411-421.
- Hynynen, M. M. and R. A. Khalil (2006). "The vascular endothelin system in hypertension--recent patents and discoveries." Recent Pat Cardiovasc Drug Discov **1**(1): 95-108.

- Idris Khodja, N., T. Chataigneau, C. Auger and V. B. Schini-Kerth (2012). "Grape-derived polyphenols improve aging-related endothelial dysfunction in rat mesenteric artery: role of oxidative stress and the angiotensin system." PLoS One **7**(2): e32039.
- Incalza, M. A., R. D'Oria, A. Natalicchio, S. Perrini, L. Laviola and F. Giorgino (2018). "Oxidative stress and reactive oxygen species in endothelial dysfunction associated with cardiovascular and metabolic diseases." Vascul Pharmacol **100**: 1-19.
- Jain, A. P., K. K. Aggarwal and P. Y. Zhang (2015). "Omega-3 fatty acids and cardiovascular disease." Eur Rev Med Pharmacol Sci **19**(3): 441-445.
- Kang, K. B., M. A. Rajanayagam, A. van der Zyppe and H. Majewski (2007). "A role for cyclooxygenase in aging-related changes of beta-adrenoceptor-mediated relaxation in rat aortas." Naunyn Schmiedebergs Arch Pharmacol **375**(4): 273-281.
- Kansui, Y., K. Fujii, K. Goto, I. Abe and M. Iida (2002). "Angiotensin II receptor antagonist improves age-related endothelial dysfunction." J Hypertens **20**(3): 439-446.
- Kedzierski, R. M. and M. Yanagisawa (2001). "Endothelin system: the double-edged sword in health and disease." Annu Rev Pharmacol Toxicol **41**: 851-876.
- Kelley, D. S., D. Siegel, M. Vemuri and B. E. Mackey (2007). "Docosahexaenoic acid supplementation improves fasting and postprandial lipid profiles in hypertriglyceridemic men." Am J Clin Nutr **86**(2): 324-333.
- Khemais-Benkhiat, S., N. Idris-Khodja, T. P. Ribeiro, G. C. Silva, M. Abbas, M. Kheloufi, J. O. Lee, F. Toti, C. Auger and V. B. Schini-Kerth (2016). "The Redox-sensitive Induction of the Local Angiotensin System Promotes Both Premature and Replicative Endothelial Senescence: Preventive Effect of a Standardized Crataegus Extract." J Gerontol A Biol Sci Med Sci **71**(12): 1581-1590.
- Kjeldsen, S. E. (2018). "Hypertension and cardiovascular risk: General aspects." Pharmacol Res **129**: 95-99.
- Knapp, H. R. and G. A. FitzGerald (1989). "The antihypertensive effects of fish oil. A controlled study of polyunsaturated fatty acid supplements in essential hypertension." N Engl J Med **320**(16): 1037-1043.
- Kong, B. W., R. Y. Man, Y. Gao, P. M. Vanhoutte and S. W. Leung (2015). "Reduced activity of SKC a and Na-K ATPase underlies the accelerated impairment of EDH-type relaxations in mesenteric arteries of aging spontaneously hypertensive rats." Pharmacol Res Perspect **3**(3): e00150.
- Kregel, K. C. and H. J. Zhang (2007). "An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations." Am J Physiol Regul Integr Comp Physiol **292**(1): R18-36.
- Kris-Etherton, P. M. and J. A. Fleming (2015). "Emerging nutrition science on fatty acids and cardiovascular disease: nutritionists' perspectives." Adv Nutr **6**(3): 326S-337S.
- Kris-Etherton, P. M., W. S. Harris, L. J. Appel and A. H. A. N. C. A. H. Association (2003). "Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association." Arterioscler Thromb Vasc Biol **23**(2): 151-152.
- Kromhout, D. (1989). "N-3 fatty acids and coronary heart disease: epidemiology from Eskimos to Western populations." J Intern Med Suppl **731**: 47-51.

- Kromhout, D., S. Yasuda, J. M. Geleijnse and H. Shimokawa (2012). "Fish oil and omega-3 fatty acids in cardiovascular disease: do they really work?" Eur Heart J **33**(4): 436-443.
- Kusama, N., J. Kajikuri, T. Yamamoto, Y. Watanabe, Y. Suzuki, H. Katsuya and T. Itoh (2005). "Reduced hyperpolarization in endothelial cells of rabbit aortic valve following chronic nitroglycerine administration." Br J Pharmacol **146**(4): 487-497.
- Lakatta, E. G. (2015). "So! What's aging? Is cardiovascular aging a disease?" J Mol Cell Cardiol **83**: 1-13.
- Lane, K., E. Derbyshire, W. Li and C. Brennan (2014). "Bioavailability and potential uses of vegetarian sources of omega-3 fatty acids: a review of the literature." Crit Rev Food Sci Nutr **54**(5): 572-579.
- Lee, J. Y., A. Plakidas, W. H. Lee, A. Heikkinen, P. Chanmugam, G. Bray and D. H. Hwang (2003). "Differential modulation of Toll-like receptors by fatty acids: preferential inhibition by n-3 polyunsaturated fatty acids." J Lipid Res **44**(3): 479-486.
- Lerman, A. and A. M. Zeiher (2005). "Endothelial function: cardiac events." Circulation **111**(3): 363-368.
- Loeb, L. A., D. C. Wallace and G. M. Martin (2005). "The mitochondrial theory of aging and its relationship to reactive oxygen species damage and somatic mtDNA mutations." Proc Natl Acad Sci U S A **102**(52): 18769-18770.
- Loiola, R. A., L. Fernandes, R. Eichler, C. Passaglia Rde, Z. B. Fortes and M. H. de Carvalho (2011). "Vascular mechanisms involved in angiotensin II-induced venoconstriction in hypertensive rats." Peptides **32**(10): 2116-2121.
- Long, D. A., M. A. Newaz, S. S. Prabhakar, K. L. Price, L. D. Truong, L. Feng, W. Mu, A. O. Oyekan and R. J. Johnson (2005). "Loss of nitric oxide and endothelial-derived hyperpolarizing factor-mediated responses in aging." Kidney Int **68**(5): 2154-2163.
- Loscalzo, J. (2000). "What we know and don't know about L-arginine and NO." Circulation **101**(18): 2126-2129.
- Luscher, T. F. and M. Barton (2000). "Endothelins and endothelin receptor antagonists: therapeutic considerations for a novel class of cardiovascular drugs." Circulation **102**(19): 2434-2440.
- Ma, T. K., K. K. Kam, B. P. Yan and Y. Y. Lam (2010). "Renin-angiotensin-aldosterone system blockade for cardiovascular diseases: current status." Br J Pharmacol **160**(6): 1273-1292.
- Matsumoto, T., S. Goulopoulou, K. Taguchi, R. C. Tostes and T. Kobayashi (2015). "Constrictor prostanoids and uridine adenosine tetraphosphate: vascular mediators and therapeutic targets in hypertension and diabetes." Br J Pharmacol **172**(16): 3980-4001.
- Matz, R. L. and R. Andriantsitohaina (2003). "Age-related endothelial dysfunction : potential implications for pharmacotherapy." Drugs Aging **20**(7): 527-550.
- Matz, R. L., M. A. de Sotomayor, C. Schott, J. C. Stoclet and R. Andriantsitohaina (2000). "Vascular bed heterogeneity in age-related endothelial dysfunction with respect to NO and eicosanoids." Br J Pharmacol **131**(2): 303-311.
- Micha, R. and D. Mozaffarian (2010). "Saturated fat and cardiometabolic risk factors, coronary heart disease, stroke, and diabetes: a fresh look at the evidence." Lipids **45**(10): 893-905.

- Miller, P. E., M. Van Elswyk and D. D. Alexander (2014). "Long-chain omega-3 fatty acids eicosapentaenoic acid and docosahexaenoic acid and blood pressure: a meta-analysis of randomized controlled trials." Am J Hypertens **27**(7): 885-896.
- Minamino, T. and I. Komuro (2007). "Vascular cell senescence: contribution to atherosclerosis." Circ Res **100**(1): 15-26.
- Minamino, T., H. Miyauchi, T. Yoshida, Y. Ishida, H. Yoshida and I. Komuro (2002). "Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction." Circulation **105**(13): 1541-1544.
- Mitchell, J. A., B. Ahmetaj-Shala, N. S. Kirkby, W. R. Wright, L. S. Mackenzie, D. M. Reed and N. Mohamed (2014). "Role of prostacyclin in pulmonary hypertension." Glob Cardiol Sci Pract **2014**(4): 382-393.
- Mori, T. A. (2014). "Omega-3 fatty acids and cardiovascular disease: epidemiology and effects on cardiometabolic risk factors." Food Funct **5**(9): 2004-2019.
- Morris, M. C., F. Sacks and B. Rosner (1993). "Does fish oil lower blood pressure? A meta-analysis of controlled trials." Circulation **88**(2): 523-533.
- Mozaffarian, D., A. Geelen, I. A. Brouwer, J. M. Geleijnse, P. L. Zock and M. B. Katan (2005). "Effect of fish oil on heart rate in humans: a meta-analysis of randomized controlled trials." Circulation **112**(13): 1945-1952.
- Mozaffarian, D. and E. B. Rimm (2006). "Fish intake, contaminants, and human health: evaluating the risks and the benefits." JAMA **296**(15): 1885-1899.
- Mozaffarian, D. and J. H. Y. Wu (2011). "Omega-3 Fatty Acids and Cardiovascular Disease: Effects on Risk Factors, Molecular Pathways, and Clinical Events." Journal of the American College of Cardiology **58**(20): 2047-2067.
- Mukai, Y., H. Shimokawa, M. Higashi, K. Morikawa, T. Matoba, J. Hiroki, I. Kunihiro, H. M. Talukder and A. Takeshita (2002). "Inhibition of renin-angiotensin system ameliorates endothelial dysfunction associated with aging in rats." Arterioscler Thromb Vasc Biol **22**(9): 1445-1450.
- Mulvany, M. J. and C. Aalkjaer (1990). "Structure and function of small arteries." Physiol Rev **70**(4): 921-961.
- Nair, S. S., J. W. Leitch, J. Falconer and M. L. Garg (1997). "Prevention of cardiac arrhythmia by dietary (n-3) polyunsaturated fatty acids and their mechanism of action." J Nutr **127**(3): 383-393.
- Nelson, R. H. (2013). "Hyperlipidemia as a risk factor for cardiovascular disease." Prim Care **40**(1): 195-211.
- Niazi, Z. R., G. C. Silva, T. P. Ribeiro, A. J. Leon-Gonzalez, M. Kassem, A. Mirajkar, A. Alvi, M. Abbas, F. Zgheel, V. B. Schini-Kerth and C. Auger (2017). "EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase- and COX-derived oxidative stress." Hypertens Res **40**(12): 966-975.
- O'Keefe, J. H., Jr., H. Abuissa, A. Sastre, D. M. Steinhaus and W. S. Harris (2006). "Effects of omega-3 fatty acids on resting heart rate, heart rate recovery after exercise, and heart rate variability in men with healed myocardial infarctions and depressed ejection fractions." Am J Cardiol **97**(8): 1127-1130.

- Omura, M., S. Kobayashi, Y. Mizukami, K. Mogami, N. Todoroki-Ikeda, T. Miyake and M. Matsuzaki (2001). "Eicosapentaenoic acid (EPA) induces Ca(2+)-independent activation and translocation of endothelial nitric oxide synthase and endothelium-dependent vasorelaxation." FEBS Lett **487**(3): 361-366.
- Oresic, M., V. A. Hanninen and A. Vidal-Puig (2008). "Lipidomics: a new window to biomedical frontiers." Trends Biotechnol **26**(12): 647-652.
- Owusu-Ansah, E., A. Yavari and U. Banerjee (2008). "A protocol for in vivo detection of reactive oxygen species."
- Ozkor, M. A. and A. A. Quyyumi (2011). "Endothelium-derived hyperpolarizing factor and vascular function." Cardiol Res Pract **2011**: 156146.
- Pais, E., H. J. Meiselman and T. Alexy (2010). "Characteristics of Blood Vessels." Exercise Physiology: From a Cellular to an Integrative Approach **75**: 183.
- Perret-Guillaume, C., L. Joly and A. Benetos (2009). "Heart rate as a risk factor for cardiovascular disease." Prog Cardiovasc Dis **52**(1): 6-10.
- Puzserova, A., V. Ilovska, P. Balis, P. Slezak and I. Bernatova (2014). "Age-related alterations in endothelial function of femoral artery in young SHR and WKY rats." Biomed Res Int **2014**: 658479.
- Qian, H., N. Luo and Y. Chi (2012). "Aging-shifted prostaglandin profile in endothelium as a factor in cardiovascular disorders." J Aging Res **2012**: 121390.
- Raimondi, L., M. Lodovici, F. Visioli, L. Sartiani, L. Cioni, C. Alfarano, G. Banchelli, R. Pirisino, E. Cecchi, E. Cerbai and A. Mugelli (2005). "n-3 polyunsaturated fatty acids supplementation decreases asymmetric dimethyl arginine and arachidonate accumulation in aging spontaneously hypertensive rats." Eur J Nutr **44**(6): 327-333.
- Rajagopalan, S., S. Kurz, T. Munzel, M. Tarpey, B. A. Freeman, K. K. Griendling and D. G. Harrison (1996). "Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone." J Clin Invest **97**(8): 1916-1923.
- Rashid, S., N. Idris-Khodja, C. Auger, C. Kevers, J. Pincemail, M. Alhosin, N. Boehm, M. Oswald-Mammosser and V. B. Schini-Kerth (2018). "Polyphenol-Rich Blackcurrant Juice Prevents Endothelial Dysfunction in the Mesenteric Artery of Cirrhotic Rats with Portal Hypertension: Role of Oxidative Stress and the Angiotensin System." J Med Food **21**(4): 390-399.
- Rashid, S., N. Idris Khodja, C. Auger, M. Alhosin, N. Boehm, M. Oswald-Mammosser and V. B. Schini-Kerth (2014). "0104: Blackcurrant juice prevents endothelial dysfunction and vascular oxidative stress in the mesenteric artery of cirrhotic rats with hepatopulmonary syndrome." Archives of Cardiovascular Diseases Supplements **6**: 16.
- Reagan-Shaw, S., M. Nihal and N. Ahmad (2008). "Dose translation from animal to human studies revisited." FASEB J **22**(3): 659-661.
- Regitz-Zagrosek, V., J. Fielitz and E. Fleck (1998). "Myocardial angiotensin receptors in human hearts." Basic Res Cardiol **93 Suppl 2**: 37-42.
- Riccardi, G., R. Giacco and A. Rivellese (2004). "Dietary fat, insulin sensitivity and the metabolic syndrome." Clinical nutrition **23**(4): 447-456.

- Riediger, N. D., R. A. Othman, M. Suh and M. H. Moghadasian (2009). "A systemic review of the roles of n-3 fatty acids in health and disease." J Am Diet Assoc **109**(4): 668-679.
- Rizos, E. C., E. E. Ntzani, E. Bika, M. S. Kostapanos and M. S. Elisaf (2012). "Association between omega-3 fatty acid supplementation and risk of major cardiovascular disease events: a systematic review and meta-analysis." JAMA **308**(10): 1024-1033.
- Rodriguez-Manas, L., M. El-Assar, S. Vallejo, P. Lopez-Doriga, J. Solis, R. Petidier, M. Montes, J. Nevado, M. Castro, C. Gomez-Guerrero, C. Peiro and C. F. Sanchez-Ferrer (2009). "Endothelial dysfunction in aged humans is related with oxidative stress and vascular inflammation." Aging Cell **8**(3): 226-238.
- Rossmann, M. J., R. E. Kaplon, S. D. Hill, M. N. McNamara, J. R. Santos-Parker, G. L. Pierce, D. R. Seals and A. J. Donato (2017). "Endothelial cell senescence with aging in healthy humans: prevention by habitual exercise and relation to vascular endothelial function." Am J Physiol Heart Circ Physiol **313**(5): H890-H895.
- Roth, E. M. and W. S. Harris (2010). "Fish oil for primary and secondary prevention of coronary heart disease." Curr Atheroscler Rep **12**(1): 66-72.
- Sagach, V., A. Bondarenko, O. Bazilyuk and A. Kotsuruba (2006). "Endothelial dysfunction: Possible mechanisms and ways of correction." Exp Clin Cardiol **11**(2): 107-110.
- Sandoo, A., J. J. van Zanten, G. S. Metsios, D. Carroll and G. D. Kitas (2010). "The endothelium and its role in regulating vascular tone." Open Cardiovasc Med J **4**: 302-312.
- Schieber, M. and N. S. Chandel (2014). "ROS function in redox signaling and oxidative stress." Curr Biol **24**(10): R453-462.
- Schindler, C., P. Bramlage, W. Kirch and C. M. Ferrario (2007). "Role of the vasodilator peptide angiotensin-(1-7) in cardiovascular drug therapy." Vasc Health Risk Manag **3**(1): 125-137.
- Schmitz, G. and J. Ecker (2008). "The opposing effects of n-3 and n-6 fatty acids." Prog Lipid Res **47**(2): 147-155.
- Schonfeld, P. and L. Wojtczak (2016). "Short- and medium-chain fatty acids in energy metabolism: the cellular perspective." J Lipid Res **57**(6): 943-954.
- Schrage, W. G., J. H. Eisenach and M. J. Joyner (2007). "Ageing reduces nitric-oxide- and prostaglandin-mediated vasodilatation in exercising humans." J Physiol **579**(Pt 1): 227-236.
- Schulze, F., R. Maas, R. Freese, E. Schwedhelm, E. Silberhorn and R. H. Boger (2005). "Determination of a reference value for N(G), N(G)-dimethyl-L-arginine in 500 subjects." Eur J Clin Invest **35**(10): 622-626.
- Scorletti, E. and C. D. Byrne (2013). "Omega-3 fatty acids, hepatic lipid metabolism, and nonalcoholic fatty liver disease." Annu Rev Nutr **33**: 231-248.
- Seals, D. R., K. L. Jablonski and A. J. Donato (2011). "Aging and vascular endothelial function in humans." Clin Sci (Lond) **120**(9): 357-375.
- Serhan, C. N. (2014). "Pro-resolving lipid mediators are leads for resolution physiology." Nature **510**(7503): 92-101.
- Shen, J., S. Shen, U. N. Das and G. Xu (2012). "Effect of essential fatty acids on glucose-induced cytotoxicity to retinal vascular endothelial cells." Lipids Health Dis **11**: 90.

- Shi, Y., R. Y. Man and P. M. Vanhoutte (2008). "Two isoforms of cyclooxygenase contribute to augmented endothelium-dependent contractions in femoral arteries of 1-year-old rats." Acta Pharmacol Sin **29**(2): 185-192.
- Shimokawa, H., N. A. Flavahan, R. R. Lorenz and P. M. Vanhoutte (1988). "Prostacyclin releases endothelium-derived relaxing factor and potentiates its action in coronary arteries of the pig." Br J Pharmacol **95**(4): 1197-1203.
- Silva, G. C., M. Abbas, S. Khemais-Benkhiat, M. Burban, T. P. Ribeiro, F. Toti, N. Idris-Khodja, S. F. Cortes and V. B. Schini-Kerth (2017). "Replicative senescence promotes prothrombotic responses in endothelial cells: Role of NADPH oxidase- and cyclooxygenase-derived oxidative stress." Exp Gerontol **93**: 7-15.
- Singh, N., S. Prasad, D. R. Singer and R. J. MacAllister (2002). "Ageing is associated with impairment of nitric oxide and prostanoid dilator pathways in the human forearm." Clin Sci (Lond) **102**(5): 595-600.
- Smith, A. R., F. Visioli, B. Frei and T. M. Hagen (2006). "Age-related changes in endothelial nitric oxide synthase phosphorylation and nitric oxide dependent vasodilation: evidence for a novel mechanism involving sphingomyelinase and ceramide-activated phosphatase 2A." Aging Cell **5**(5): 391-400.
- Steenman, M. and G. Lande (2017). "Cardiac aging and heart disease in humans." Biophys Rev **9**(2): 131-137.
- Stewart, K. G., Y. Zhang and S. T. Davidge (2000). "Aging increases PGHS-2-dependent vasoconstriction in rat mesenteric arteries." Hypertension **35**(6): 1242-1247.
- Sun, D., A. Huang, E. H. Yan, Z. Wu, C. Yan, P. M. Kaminski, T. D. Oury, M. S. Wolin and G. Kaley (2004). "Reduced release of nitric oxide to shear stress in mesenteric arteries of aged rats." Am J Physiol Heart Circ Physiol **286**(6): H2249-2256.
- Taddei, S., A. Viridis, L. Ghiadoni, G. Salvetti, G. Bernini, A. Magagna and A. Salvetti (2001). "Age-related reduction of NO availability and oxidative stress in humans." Hypertension **38**(2): 274-279.
- Taddei, S., A. Viridis, P. Mattei, L. Ghiadoni, C. B. Fasolo, I. Sudano and A. Salvetti (1997). "Hypertension causes premature aging of endothelial function in humans." Hypertension **29**(3): 736-743.
- Taddei, S., A. Viridis, P. Mattei, L. Ghiadoni, A. Gennari, C. B. Fasolo, I. Sudano and A. Salvetti (1995). "Aging and endothelial function in normotensive subjects and patients with essential hypertension." Circulation **91**(7): 1981-1987.
- Takaki, A., K. Morikawa, M. Tsutsui, Y. Murayama, E. Tekes, H. Yamagishi, J. Ohashi, T. Yada, N. Yanagihara and H. Shimokawa (2008). "Crucial role of nitric oxide synthases system in endothelium-dependent hyperpolarization in mice." J Exp Med **205**(9): 2053-2063.
- Tang, E. H. and P. M. Vanhoutte (2008). "Gene expression changes of prostanoid synthases in endothelial cells and prostanoid receptors in vascular smooth muscle cells caused by aging and hypertension." Physiol Genomics **32**(3): 409-418.
- Tang, E. H. and P. M. Vanhoutte (2009). "Prostanoids and reactive oxygen species: team players in endothelium-dependent contractions." Pharmacol Ther **122**(2): 140-149.
- Tang, X. D., M. L. Garcia, S. H. Heinemann and T. Hoshi (2004). "Reactive oxygen species impair Slo1 BK channel function by altering cysteine-mediated calcium sensing." Nat Struct Mol Biol **11**(2): 171-178.

- Tavazzi, L., A. P. Maggioni, R. Marchioli, S. Barlera, M. G. Franzosi, R. Latini, D. Lucci, G. L. Nicolosi, M. Porcu, G. Tognoni and H. F. I. Gissi (2008). "Effect of n-3 polyunsaturated fatty acids in patients with chronic heart failure (the GISSI-HF trial): a randomised, double-blind, placebo-controlled trial." Lancet **372**(9645): 1223-1230.
- Thies, F., G. Nebe-von-Caron, J. R. Powell, P. Yaqoob, E. A. Newsholme and P. C. Calder (2001). "Dietary supplementation with gamma-linolenic acid or fish oil decreases T lymphocyte proliferation in healthy older humans." J Nutr **131**(7): 1918-1927.
- Tian, J., Z. Yan, Y. Wu, S. L. Zhang, K. Wang, X. R. Ma, L. Guo, J. Wang, L. Zuo, J. Y. Liu, L. Quan and H. R. Liu (2010). "Inhibition of iNOS protects endothelial-dependent vasodilation in aged rats." Acta Pharmacol Sin **31**(10): 1324-1328.
- Toda, N. (2012). "Age-related changes in endothelial function and blood flow regulation." Pharmacol Ther **133**(2): 159-176.
- Tokunaga, O., T. Yamada, J. L. Fan and T. Watanabe (1991). "Age-related decline in prostacyclin synthesis by human aortic endothelial cells. Qualitative and quantitative analysis." Am J Pathol **138**(4): 941-949.
- Touyz, R. M. and E. L. Schiffrin (2000). "Signal transduction mechanisms mediating the physiological and pathophysiological actions of angiotensin II in vascular smooth muscle cells." Pharmacol Rev **52**(4): 639-672.
- Townsend, N., L. Wilson, P. Bhatnagar, K. Wickramasinghe, M. Rayner and M. Nichols (2016). "Cardiovascular disease in Europe: epidemiological update 2016." Eur Heart J **37**(42): 3232-3245.
- Tsuboi, K., Y. Sugimoto and A. Ichikawa (2002). "Prostanoid receptor subtypes." Prostaglandins Other Lipid Mediat **68-69**: 535-556.
- Tvrzicka, E., L.-S. Kremmyda, B. Stankova and A. Zak (2011). "Fatty acids as biocompounds: Their role in human metabolism, health and disease-a review. Part 1: Classification, dietary sources and biological functions." Biomedical Papers of the Medical Faculty of Palacky University in Olomouc **155**(2).
- Ungvari, Z., R. Buffenstein, S. N. Austad, A. Podlutzky, G. Kaley and A. Csiszar (2008). "Oxidative stress in vascular senescence: lessons from successfully aging species." Front Biosci **13**: 5056-5070.
- Urakami-Harasawa, L., H. Shimokawa, M. Nakashima, K. Egashira and A. Takeshita (1997). "Importance of endothelium-derived hyperpolarizing factor in human arteries." J Clin Invest **100**(11): 2793-2799.
- van der Loo, B., R. Labugger, J. N. Skepper, M. Bachschmid, J. Kilo, J. M. Powell, M. Palacios-Callender, J. D. Erusalimsky, T. Quaschnig, T. Malinski, D. Gygi, V. Ullrich and T. F. Luscher (2000). "Enhanced peroxynitrite formation is associated with vascular aging." J Exp Med **192**(12): 1731-1744.
- Van Guilder, G. P., C. M. Westby, J. J. Greiner, B. L. Stauffer and C. A. DeSouza (2007). "Endothelin-1 vasoconstrictor tone increases with age in healthy men but can be reduced by regular aerobic exercise." Hypertension **50**(2): 403-409.
- Vanhoutte, P. M., M. Feletou and S. Taddei (2005). "Endothelium-dependent contractions in hypertension." Br J Pharmacol **144**(4): 449-458.

- Vanhoutte, P. M., H. Shimokawa, M. Feletou and E. H. Tang (2017). "Endothelial dysfunction and vascular disease - a 30th anniversary update." Acta Physiol (Oxf) **219**(1): 22-96.
- Vanhoutte, P. M., Y. Zhao, A. Xu and S. W. Leung (2016). "Thirty Years of Saying NO: Sources, Fate, Actions, and Misfortunes of the Endothelium-Derived Vasodilator Mediator." Circ Res **119**(2): 375-396.
- Vasile, E., Y. Tomita, L. F. Brown, O. Kocher and H. F. Dvorak (2001). "Differential expression of thymosin beta-10 by early passage and senescent vascular endothelium is modulated by VPF/VEGF: evidence for senescent endothelial cells in vivo at sites of atherosclerosis." FASEB J **15**(2): 458-466.
- Vasquez-Vivar, J., B. Kalyanaraman and P. Martasek (2003). "The role of tetrahydrobiopterin in superoxide generation from eNOS: enzymology and physiological implications." Free Radic Res **37**(2): 121-127.
- Walker, A. E., S. M. Seibert, A. J. Donato, G. L. Pierce and D. R. Seals (2010). "Vascular endothelial function is related to white blood cell count and myeloperoxidase among healthy middle-aged and older adults." Hypertension **55**(2): 363-369.
- Wang, M., G. Takagi, K. Asai, R. G. Resuello, F. F. Natividad, D. E. Vatner, S. F. Vatner and E. G. Lakatta (2003). "Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates." Hypertension **41**(6): 1308-1316.
- Wang, Q., X. Liang, L. Wang, X. Lu, J. Huang, J. Cao, H. Li and D. Gu (2012). "Effect of omega-3 fatty acids supplementation on endothelial function: a meta-analysis of randomized controlled trials." Atherosclerosis **221**(2): 536-543.
- Wang, Y. X. and Y. M. Zheng (2010). "ROS-dependent signaling mechanisms for hypoxic Ca(2+) responses in pulmonary artery myocytes." Antioxid Redox Signal **12**(5): 611-623.
- Wong, S. L., F. P. Leung, C. W. Lau, C. L. Au, L. M. Yung, X. Yao, Z. Y. Chen, P. M. Vanhoutte, M. Gollasch and Y. Huang (2009). "Cyclooxygenase-2-derived prostaglandin F2alpha mediates endothelium-dependent contractions in the aortae of hamsters with increased impact during aging." Circ Res **104**(2): 228-235.
- Woodman, C. R., E. M. Price and M. H. Laughlin (2003). "Selected Contribution: Aging impairs nitric oxide and prostacyclin mediation of endothelium-dependent dilation in soleus feed arteries." J Appl Physiol (1985) **95**(5): 2164-2170.
- Wu, Y., C. Zhang, Y. Dong, S. Wang, P. Song, B. Viollet and M. H. Zou (2012). "Activation of the AMP-activated protein kinase by eicosapentaenoic acid (EPA, 20:5 n-3) improves endothelial function in vivo." PLoS One **7**(4): e35508.
- Xiao, Y. F., Q. Ke, Y. Chen, J. P. Morgan and A. Leaf (2004). "Inhibitory effect of n-3 fish oil fatty acids on cardiac Na⁺/Ca²⁺ exchange currents in HEK293t cells." Biochem Biophys Res Commun **321**(1): 116-123.
- Yang, D., M. Feletou, N. Levens, J. N. Zhang and P. M. Vanhoutte (2003). "A diffusible substance(s) mediates endothelium-dependent contractions in the aorta of SHR." Hypertension **41**(1): 143-148.
- Yang, Y. M., A. Huang, G. Kaley and D. Sun (2009). "eNOS uncoupling and endothelial dysfunction in aged vessels." Am J Physiol Heart Circ Physiol **297**(5): H1829-1836.
- Yokoyama, M., H. Origasa, M. Matsuzaki, Y. Matsuzawa, Y. Saito, Y. Ishikawa, S. Oikawa, J. Sasaki, H. Hishida, H. Itakura, T. Kita, A. Kitabatake, N. Nakaya, T. Sakata, K. Shimada,

- K. Shirato and E. P. A. I. i. s. I. Japan (2007). "Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis." Lancet **369**(9567): 1090-1098.
- Yoon, H. E., E. N. Kim, M. Y. Kim, J. H. Lim, I. A. Jang, T. H. Ban, S. J. Shin, C. W. Park, Y. S. Chang and B. S. Choi (2016). "Age-Associated Changes in the Vascular Renin-Angiotensin System in Mice." Oxid Med Cell Longev **2016**: 6731093.
- Yoon, H. J., S. W. Cho, B. W. Ahn and S. Y. Yang (2010). "Alterations in the activity and expression of endothelial NO synthase in aged human endothelial cells." Mech Ageing Dev **131**(2): 119-123.
- Zanetti, M., G. Gortan Cappellari, D. Barbetta, A. Semolic and R. Barazzoni (2017). "Omega 3 Polyunsaturated Fatty Acids Improve Endothelial Dysfunction in Chronic Renal Failure: Role of eNOS Activation and of Oxidative Stress." Nutrients **9**(8).
- Zanetti, M., A. Grillo, P. Losurdo, E. Panizon, F. Mearelli, L. Cattin, R. Barazzoni and R. Carretta (2015). "Omega-3 Polyunsaturated Fatty Acids: Structural and Functional Effects on the Vascular Wall." Biomed Res Int **2015**: 791978.
- Zgheel, F., M. Alhosin, S. Rashid, M. Burban, C. Auger and V. B. Schini-Kerth (2014). "Redox-sensitive induction of Src/PI3-kinase/Akt and MAPKs pathways activate eNOS in response to EPA: DHA 6: 1."
- Zgheel, F., M. Alhosin, S. Rashid, M. Burban, C. Auger and V. B. Schini-Kerth (2014). "Redox-sensitive induction of Src/PI3-kinase/Akt and MAPKs pathways activate eNOS in response to EPA:DHA 6:1." PLoS One **9**(8): e105102.
- Zhang, J., X. Wang, V. Vikash, Q. Ye, D. Wu, Y. Liu and W. Dong (2016). "ROS and ROS-Mediated Cellular Signaling." Oxid Med Cell Longev **2016**: 4350965.
- Zhao, Y., P. M. Vanhoutte and S. W. Leung (2015). "Vascular nitric oxide: Beyond eNOS." J Pharmacol Sci **129**(2): 83-94.

Potential of omega-3 EPA:DHA 6:1 to ameliorate ageing-related endothelial dysfunction

Résumé

La présente étude évalue la capacité de la formulation d'oméga-3 EPA:DHA 6:1, une formulation capable d'induire la formation continue de monoxyde d'azote par la NO synthase endothéliale, à améliorer la dysfonction endothéliale liée à l'âge établie chez le rat. La dysfonction endothéliale liée à l'âge est caractérisée par une altération des composantes de la relaxation et une augmentation des réponses contractiles dépendantes de l'endothélium. L'âge augmente le stress oxydant vasculaire, l'expression de la NADPH oxydase, COX-2, eNOS, ACE, AT1R, et des marqueurs de senescence, alors que la COX-1 est sous-exprimé. La formulation EPA:DHA 6:1 améliore la composante NO, diminue l'EDCF et le stress oxydant vasculaire, et normalise l'expression des protéines cibles. En conclusion, la consommation chronique de EPA:DHA 6:1 améliore la dysfonction endothéliale liée à l'âge chez le rat, probablement en prévenant l'activation du système angiotensine locale et le stress oxydant en résultant.

Mots-clés: Vieillesse, Endothélium Vasculaire, Monoxyde d'azote, Acides Gras Poly-Insaturés oméga-3, Stress Oxydant, Système Angiotensine Local

Résumé en anglais

EPA:DHA 6:1 omega-3 formulation has been shown to induce a sustained endothelial NO synthase-derived formation of nitric oxide. This study examined if the intake of EPA:DHA 6:1 improves an established ageing-related endothelial dysfunction. Ageing-related endothelial dysfunction was characterized by a blunted NO-mediated component of relaxation, abolished EDH-mediated component and increased COX-derived endothelium-dependent contractile responses. Ageing increased vascular oxidative stress, expression of NADPH oxidase subunits, COX-2, eNOS, ACE, AT1R, and senescence markers, whereas COX-1 was down-regulated. Chronic intake of EPA:DHA 6:1 improved the NO-mediated relaxations, reduced EDCFs, vascular oxidative stress and normalized the expression of protein markers. In conclusion, chronic intake of EPA:DHA 6:1 prevented the ageing-related endothelial dysfunction in old rats, most likely by preventing activation of the local angiotensin system and the subsequent vascular oxidative stress.

Keywords: Ageing, Vascular Endothelium, Nitric Oxide, Omega-3 Polyunsaturated Fatty Acids, Oxidative Stress, Local Angiotensin System