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Ciblage des cellules endothéliales sénescentes à l'aide d'une approche théranostique : potentiel thérapeutique d'une formulation d'Oméga 3

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Strasbourg, France

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List of abbreviations

3H-dT	3-hthymidine
53BP1	P53-Binding Protein-1
5-HT	5-Hydroxy-Tryptamine
AA	Arachidonic acid
ACE	Angiotensin converting enzyme
Ach	Acetylcholine
ACS	Acute coronary syndrome
ADMA	Asymmetric dimethyl arginine
ADP	Adenosine di-phosphate
AF	Arterial fibrillation
AGE	Advance Glycation End-products
AHA	American heart association
Akt	Protein kinase B
ALA	Alpha linoleic acid
AMPK	Adenosine monophosphate activated protein kinase
Ang II	Angiotensin II
AP-1	Activator protein-1
Apo CIII	Apo-lipoprotein C3
APs	Action potentials
AT1	Angiotensin type-1
AT2	Angiotensin type-2
AT1R	Angiotensin type-1 receptor
ATP	Adenosine tri-phosphate
bFGF	Basic fibroblast growth factor
BH_2	Dihydrobiopterin
BH_4	Tetrahydrobiopterin
BK	Bradykinin
BP	Blood pressure
BrdU	Bromodeoxyuridine
C12FDG	5-dodecanoylaminoFluorescein Di-β-D-Galactopyranoside
Ca^{2+}	Calcium
CaM	Calmodulin
CaM Kinase II	Calcium/calmodulin-dependent protein kinase II
CCFs	Cytoplasmic Chromatin Fragments
CD	Cluster of differentiation 31

CDC42	Cell division control protein 42
CHD	Coronary heart disease
CO	Carbon monoxide
COX	Cyclo-oxygenase
CVD	Cardiovascular disease
Cyp-450	Cytochrome p-450
DAG	Diacylglycerol
DAPI	4,6-Diamidino-2-Phenylindole
DART	Diet and reinfarction trial
DDR	DNA Damage Response
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
EE	Ethyl ester
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
ER	Endoplasmic reticulum
ERK	Extracellular signal regulated kinase
ESS	Endothelial shear stress
ET-1	Endothelin-1
FAD	Flavin-adenine dinucleotide
FAs	Fatty acids
FFAs	Free fatty acids
FMD	Flavin mononucleotide
FP	F-prostanoid receptor
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GPR120	G-protein coupled receptor 120

GTP	Guanosine trisphosphate
H	Histamine
H_2O_2	Hydrogen peroxide
H_2S	Dihydrogen sulfide
HDL	High density lipoprotein
HETE	Hydroxy eicosatetraenoic acid
HF	Heart failure
HGMB1	High–mobility group box 1
HOCL	Hypochlorous acid
HNF	Hepatic nuclear factor
HSP90	Heat shock protein 90
ICAM-1	Intercellular adhesion molecule 1
IDL	Intermediate density lipoprotein
IGF-I	Insulin-like growth factor-1
i-ƙB	Inhibitor of ƙB
IK_{ca}	Intermediate conductance Calcium channel
IL-12	Interleukin-12
IL-1β	Interleukin-1β
IL-2	Interleukin-2
IL-6	Interleukin-6
IP	I-prostanoid receptor
IP_3	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
LCT	Long chain triglycerides
LDL	Low-density lipoproteins
L-NA	L-nitroarginine
LNAME	L-nitroarginine methyl ester
LOX	Lipo-oxygenase
LOX-5	Lipo-oxygenase 5

LPL	Lipoprotein lipase
LTs	Leukotrienes
LTB_4	Leukotriene B_4
LTB_5	Leukotriene B_5
LVH	Left ventricular hypertrophy
LXR	Liver X receptor
MAGs	Monoacylglycerols
MAPK	Mitogen-activated protein kinases
MaR	Maresins
MCP-1	Monocyte chemoattractant protein-1
MCT	Medium chain triglycerides
MGJs	Myoendothelial gap junctions
MI	Myocardial infarction
MMPs	Matrix Metalloproteinases
mTOR	Mammalian target of rapamycin
MWNT	Multi-walled nanotube
MUFA	Monounsaturated fatty acid
Na^+	Sodium
Na^+/Ca^{2+}	Sodium/calcium pump
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NE	Nano-emulsions
NF-Κb	Nuclear factor kappa-B
NIR	Near-infrared
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NPs	Nanoparticles
O/W	Oil-in-water
$O_2^{-\cdot}$	Superoxide anion
$OH^{\cdot}$	Hydroxyl radical
$OONO^-$	Peroxynitrite anion
ox-LDL	Oxidized-low density lipoprotein
P	Protectins
p16	Cyclin-dependent kinase inhibitor 2A
p21	Cyclin-dependent kinase inhibitor-1
p53	Tumor protein p53
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PECAM-1	Platelet–endothelial cell adhesion molecule-1
PEG	Polyethylene glycol

PFCE	Perfluoro-15-crown-5-ether
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PI3K	Phosphatidyl inositol 3 kinase
PIC	Phase inversion composition
PIT	Phase inversion temperature
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
PLs	Phospholipids
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PNPs	Polymeric nanoparticles
PP2A	Protein phosphatase 2
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
Rv	Resolvins
RXR	Retinoid X receptor
SA-β-Gal	Senescence associated β-galactosidase
SASP	Senescence-associated secretory phenotype
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
SOD	Superoxide dismutase
SPMs	Specialized pro-resolving lipid mediators

SPR	Surface plasmon resonance
SR	Sarcoplasmic reticulum
SWNT	Single walled nanotube
TAGs	Triacylglycerols
TF	Tissue factor
TG	Triglycerides
TGF- β	Transforming growth factor
TNF-α	Tissue necrosis factor-α
TP	Thromboxane prostanoid receptor
TRL	Triglycerides rich lipoproteins
TXs	Thromboxanes
TXA_2	Thromboxane A2
TXA_3	Thromboxane A3
TXB_2	Thromboxane B2
VCAM-1	Vascular cell adhesion molecule 1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VLDL	Very low-density lipoprotein
VSMC	Vascular smooth muscle cell
VSMCs	Vascular smooth muscle cells
vWF	Von Willebrand factor
W/O	Water-in-oil
W/O/W	Water-in-Oil-in-Water emulsions

Abstract/ Résumé

Abstract

Endothelial dysfunction and cardiovascular diseases

Cardiovascular diseases (CVDs) are the leading cause of mortality and morbidity around the globe, both in developed and developing countries. The incidence and prevalence of cardiovascular diseases such as atherothrombosis, hypertension, myocardial infarction and heart failure increases with age. CVDs represent a major public health issue with a significant cost burden, their incidence progresses with age and often is associated with a reduced quality of life.

The endothelium, the cell monolayer lining the luminal surface of blood vessels, has as a pivotal role in the control of vascular homeostasis. Strategically located, endothelial cells can regulate diverse vascular responses including vascular tone, platelet aggregation, thrombosis, leukocyte trafficking, inflammation and angiogenesis. Endothelial cells regulate vascular tone mostly through the induction of potent vasodilator mechanisms including the formation of nitric oxide (NO) and endothelium-dependent hyperpolarization. Besides regulating vascular tone, NO is a potent inhibitor of platelet activation that also prevents the expression of numerous pro-atherosclerotic and pro-thrombotic factors. In major types of cardiovascular disease, an endothelial dysfunction is observed initially that is characterized by an imbalance between the vasodilator mechanisms and the induction of endothelium-derived contracting factors, resulting in impaired endothelium-dependent vasodilatation. Since endothelial dysfunction is observed before changes in the arterial wall structure, it is thought to contribute to the initiation and the development of vascular pathologies.

Both experimental and clinical studies have indicated that CV risk factors such as ageing, are associated with an endothelial dysfunction, which is involved in the mechanisms underlying the development of atherosclerotic lesions, including the up-regulation of adhesion molecules, the increased secretion of chemokines and leukocyte adherence, increased cell permeability,

enhanced low-density lipoprotein oxidation, platelet activation, 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 level of 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.

Diets, cellular protection and cardiovascular health

Several epidemiological studies and clinical trials have shown the impact of diet on healthy lifespan. In patients at high cardiovascular risk, the incidence of adverse cardiovascular events such as stroke, myocardial infarction is reduced by 30% in subjects following a Mediterranean diet rich in unsaturated fatty acids and the intake of nuts or olive oil. In addition, numerous epidemiological or interventional studies have shown that consumption of food containing fish or fish oil rich in omega-3 polyunsaturated fatty acids (n-3 PUFAs) are associated with a reduced risk of recurrence of coronary heart disease or stroke and a reduced level of blood pressure in hypertensive patients. Similarly, the Japanese population had a reduced risk of CVDs by an Okinawa-type diet, rich in seafood containing polyunsaturated fatty acids (PUFAs), and more specifically in omega-3 PUFAs.

The dietary consumption of the major omega-3 PUFAs, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has been related to a reduced risk of morbidity/mortality due to CVDs. However, one of the major difficulties in demonstrating the benefit of omega-3 on

cardiovascular health during epidemiological or interventional studies lies in the variety of formulations of nutritional intake or of the degree of enrichment in EPA and DHA.

The omega-3 PUFAs could exert their beneficial effects on the cardiovascular system by modulation of the lipid metabolism and their anti-inflammatory properties due to the decrease in pro-inflammatory cytokines 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 has previously shown that the stimulation of the endothelial formation of NO by n-3 PUFAs is dependent on both the purity and the ratio of EPA and DHA. Indeed, we have 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 recently showed that chronic intake of EPA:DHA 6:1 for 2 weeks significantly improved the age-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 responses most likely by preventing vascular oxidative stress and premature endothelial senescence.

Due to the presence of multiple unsaturation, omega-3 PUFAs are prone to degradation which can result in the production of various reactive chemical species leading to loss of functionality.

Therefore, the present study investigated whether nanoencapsulation of EPA:DHA 6:1 followed by coating with gum is able to improve the stability of omega-3 PUFAs and hence, to enhance their beneficial effect at the endothelial function using *in vitro*, *ex vivo* and *in vivo* approaches on cultured endothelial cells, isolated porcine coronary arteries and middle-aged rats, respectively, and, if so, to determine the underlying mechanisms.

Methodology and principal results

EPA:DHA 6:1 was emulsified in water phase and coated with proteins and gum derivatives or used in native form.

Firstly, vascular reactivity studies were performed on left circumflex porcine coronary artery rings and changes in isometric tension were determined using organ chambers.

Secondly, NO formation was assessed in cultured porcine coronary artery endothelial cells using the fluorescent probe DAF-FM and confocal microscopy. The antiaggregatory effect of endothelial cells was assessed using suspensions of washed human platelets incubated in an aggregometer.

Thirdly, for the *in vivo* study, middle-aged male Wistar rats received by daily gavage 100 mg/kg of either native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles or water (control). After 1-week of treatment, the animals were euthanized. The main mesenteric artery and thoracic aorta were used for vascular reactivity studies, and the thoracic aorta for immunofluorescence and fluorescence histochemistry studies on frozen section.

The addition of coated EPA:DHA 6:1 nanoparticles to U46619-pre-contracted coronary artery rings induced concentration-dependent relaxations in rings with intact endothelium that were more sustained than those of the native EPA:DHA 6:1 form, whereas small relaxations were observed in denuded rings. The sustained endothelium-dependent relaxation to the coated EPA:DHA 6:1 nanoparticles was abolished by an eNOS inhibitor (L-NA) and not affected by

inhibition of either cyclooxygenases or EDH indicating the exclusive involvement of NO. In contrast, the relaxation in response to the native form was significantly reduced by indomethacin in rings with and in those without endothelium indicating the involvement of vasorelaxant prostanoids.

To assess the vasodilation effect of corn oil, an equivalent and isocaloric oil containing less than 1% of omega-3 PUFAs, coronary artery rings were pretreated with corn oil before the addition of coated EPA:DHA 6:1 nanoparticles. Corn oil did induce neither endothelium-dependent relaxation alone nor affect the relaxation to coated EPA:DHA 6:1 nanoparticles, indicating the specific effect to the optimized formulation of omega-3 in inducing endothelium-dependent relaxations.

Since coated EPA:DHA 6:1 nanoparticles promoted long-lasting endothelium-dependent NO-mediated relaxations, experiments have assessed their ability to cause a sustained formation of NO in coronary artery ECs over a 24-h period. The coated EPA:DHA 6:1 nanoparticles induced significantly higher levels of NO in endothelial cells as compared with the native form and bradykinin. In addition, the stimulatory effect of both the coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 was markedly reduced by L-NA indicating the involvement of NO.

To assess the ability of ECs to inhibit platelet aggregation in response to thrombin, ECs were cultured on Cytodex-3 microcarrier beads and treated with either coated nanoencapsulated or native EPA:DHA 6:1 before the addition to suspensions of washed human platelets.

Although the addition of low numbers of EPA:DHA 6:1-treated ECs did not affect thrombin-induced aggregation, a pronounced inhibitory effect was observed in response to the addition of a similar number of coated EPA:DHA 6:1 nanoparticles-treated ECs. Moreover, the inhibitory effect was abolished by an eNOS inhibitor indicating the involvement of NO.

The findings of the *in vivo* study indicate that, compared to young rats (12 weeks-old), ageing was associated with an endothelial dysfunction characterized by a blunted NO-mediated component, an increased contraction to phenylephrine in the main mesenteric artery and thoracic aorta, and an increased basal formation of vasoconstricting prostanoids in the thoracic aorta.

Treatment with both EPA:DHA 6:1 formulations significantly improved acetylcholine-induced relaxation whereas, only coated EPA:DHA 6:1 nanoparticles reduced significantly phenylephrine-induced contraction in the main mesenteric artery of middle-aged rats.

Similarly, treatment with coated EPA:DHA 6:1 nanoparticles improved acetylcholine-induced relaxation and significantly reduced phenylephrine-induced contraction in the thoracic aorta.

Next, we investigated the expression level of target proteins in the thoracic aorta by immunofluorescence staining in order to characterize the protective mechanisms of both EPA:DHA 6:1 formulations. Since ageing is associated with reduced NO-mediated relaxation, we studied the expression level of eNOS and nitrotyrosine, indicators of the NO pathway.

Expression level of eNOS and nitrotyrosine in middle-aged rats was significantly increased compared to young rats. Compared to native form, the 1-week intake of coated EPA:DHA 6:1 nanoparticles significantly normalized the expression level of eNOS and nitrotyrosine in the thoracic aorta of middle-aged rats.

In addition, we assessed the level of vascular and mitochondrial oxidative stress using the redox-sensitive fluorescent probe dihydroethidium (DHE) and MitoSOX, respectively. Compared to young rats, middle-aged rats showed an increased formation of vascular and mitochondrial oxidative stress in the thoracic aorta, which were significantly reduced by both the native form and the coated EPA:DHA 6:1 nanoparticles treatment.

As the increased level of vascular oxidative stress has been attributed, at least in part, to the local angiotensin system, we determined the expression level of the angiotensin II type 1

receptor (AT1R) and angiotensin-converting enzyme (ACE). Compared to young control rats, the aortic wall of middle-aged rats exhibited a significantly increased expression level of AT1R and ACE in the endothelium and vascular smooth muscle of the thoracic aorta, which were improved partially by the native EPA:DHA 6:1 treatment and normalized by the coated EPA:DHA 6:1 nanoparticles treatment.

Altogether, the present findings indicate that encapsulation of EPA:DHA 6:1 followed by coating with gum is associated with a prolonged ability to cause endothelium-dependent NO-mediated relaxations of isolated coronary arteries and the ability to induce a sustained formation of NO in cultured endothelial cells as demonstrated using an NO-sensitive fluorescent probe and by the antiaggregatory effect on platelet aggregation. Thus, nanoencapsulation of omega-3 PUFAs followed by coating appears as an attractive approach to better protect the vascular system.

In addition, a 1-week intake of coated EPA:DHA 6:1 nanoparticles significantly improved the ageing-related endothelial dysfunction in rats. The beneficial effect involves an improvement of the NO-mediated relaxations as well as a reduction of oxidative stress and the local vascular angiotensin system.

Further investigations are required to elaborate the age-related changes in vascular tissues and organs such as the vascular and cardiac remodeling and to evaluate the impact of omega-3 nanoformulation. Moreover, it will be interesting to identify the pertinent biological markers and to characterize the cellular and molecular mechanisms involved in the beneficial endothelial effects of EPA:DHA 6:1. Such markers would allow a better evaluation of the beneficial effects of the EPA:DHA 6:1 nanoformulation during future randomized clinical trials in patients with cardiovascular diseases or risk factors associated with endothelial dysfunction.

Résumé

Dysfonction endothéliale et maladies cardiovasculaires

Les maladies cardiovasculaires (MCV) sont la principale cause de mortalité et de morbidité dans le monde, tant dans les pays développés que dans les pays en cours de développement. L'incidence et la prévalence des MCV telles que l'athérothrombose, l'hypertension, l'infarctus du myocarde et l'insuffisance cardiaque augmentent avec l'âge. Les MCV représentent un enjeu majeur en santé publique avec un poids important des coûts et des besoins, leur incidence évolue avec l'âge et est souvent associée à une qualité de vie réduite.

L'endothélium, la monocouche cellulaire tapissant la surface luminale des 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) et l'hyperpolarisation dépendante de l'endothélium (EDH). En plus de la régulation du tonus vasculaire, le NO est un puissant inhibiteur de l'activation plaquettaire qui empêche également l'expression de nombreux facteurs pro-athérosclérotiques et pro-thrombotiques. Dans les principaux types de maladies cardiovasculaires, on observe initialement 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.

Des études expérimentales et cliniques ont indiqué que les facteurs de risque CV tels que le vieillissement, sont associés à un dysfonctionnement endothélial, qui est impliqué 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 par l'augmentation du stress oxydant vasculaire induit par la surexpression de la NAPDH oxydase, une source majeure 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.

Régime alimentaire, protection cellulaire et santé cardiovasculaire

De nombreuses études épidémiologiques ont montré l'impact de la diète sur la santé cardiovasculaire et sur la qualité de vie. Chez les patients à fort risque cardiovasculaire, l'incidence d'évènements cardiovasculaires adverses comme les accidents vasculaires cérébraux, l'infarctus du myocarde est réduit de 30% chez les sujets suivant un régime méditerranéen riche en acides gras insaturés ingérés sous la forme de noix ou d'huile d'olive.

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 récidive de la maladie coronarienne ou d'accident vasculaire cérébraux, et réduire la pression

artérielle chez les hypertendus. De même, la population japonaise semble être protégée des maladies cardiovasculaires par un régime de type Okinawa, riche en produits de la mer contenant des acides gras polyinsaturés (PUFAs), et plus particulièrement en oméga-3 PUFAs.

La consommation des acides gras oméga-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. Cependant, une des difficultés majeures dans la démonstration du bénéfice des oméga-3 sur la santé cardiovasculaire au cours des études épidémiologiques ou interventionnelles réside dans la variété des formulations, de l'apport nutritionnel ou du degré d'enrichissement en EPA et DHA.

Les n-3 PUFAs pourrait avoir un effet bénéfique sur le système cardiovasculaire en modulant le métabolisme lipidique et du fait de leur 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 montré que la stimulation de la fonction endothéliale par les oméga-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 oméga-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 que la prise chronique d'EPA:DHA 6:1 pendant 2 semaines améliore significativement la dysfonction endothéliale lié à l'âge chez le rat. L'effet bénéfique implique une amélioration des relaxations médiées à la fois par NO et par EDH ainsi qu'une réduction de la réponse contractile dépendante de l'endothélium très probablement en empêchant le stress oxydant vasculaire et la sénescence endothéliale prématurée.

En raison de la présence de multiples insaturations, les oméga-3 sont sujets à une dégradation qui peut entraîner la production de diverses espèces chimiques réactives conduisant à une perte de fonctionnalité. Par conséquent, la présente étude a examiné si la nanoencapsulation d'EPA:DHA 6:1 suivie d'un enrobage avec de la gomme est capable d'améliorer la stabilité des oméga-3 PUFAs et, par conséquent, d'améliorer leur effet bénéfique sur la fonction endothéliale en utilisant une approche *in vitro, ex vivo* et *in vivo* sur des cellules endothéliales cultivées, des artères coronaires porcines isolées et des rats d'âge moyen, respectivement, et si oui, pour déterminer les mécanismes sous-jacents.

Méthodologie et principaux résultats

EPA:DHA 6:1 est émulsionné en phase aqueuse et enrobé de protéines et de dérivés de gomme ou utilisé sous forme native.

Tout d'abord, des études de réactivité vasculaire sont réalisées sur des anneaux d'artère coronaire porcine et les changements de tension isométrique sont déterminés à l'aide de chambres à organes isolés.

Deuxièmement, la formation de NO est évaluée dans des cellules endothéliales cultivées d'artère coronaire porcine en utilisant la sonde fluorescente DAF-FM et la microscopie confocale. L'effet antiagrégant des cellules endothéliales est évalué en utilisant des suspensions de plaquettes humaines lavées et incubées dans un agrégomètre.

Troisièmement, pour l'étude *in vivo*, des rats Wistar mâles d'âge moyen ont reçu quotidiennement pendant 1 semaine par gavage 100 mg/kg soit de la forme native d'EPA:DHA 6:1, soit de la forme nanoencapsulée d'EPA:DHA 6:1, soit de l'eau (contrôle). Après une semaine de traitement, les rats sont euthanasiés. L'artère mésentérique principale et l'aorte thoracique sont utilisées pour les études de réactivité vasculaire, et l'aorte thoracique pour les études d'immunofluorescence et de fluorescence sur coupes congelées.

L'ajout de nanoformulation d'EPA:DHA 6:1 à des anneaux d'artère coronaire pré contractés avec l'U46619 a induit des relaxations dépendantes de la concentration dans les anneaux ayant un endothélium intact qui étaient plus importantes et soutenues que celles de la forme native d'EPA:DHA 6:1, alors que seulement de faibles relaxations ont été observées dans les anneaux dénudés d'endothélium.

La relaxation soutenue dépendante de l'endothélium induite par la nanoformulation d'EPA:DHA 6:1 a été abolie par un inhibiteur de la eNOS (L-NA) et n'est pas affectée par l'inhibition des cyclooxygénases ou de l'EDH indiquant l'implication exclusive de NO. En revanche, la relaxation en réponse à la forme native est

significativement réduite par l'indométacine dans les anneaux avec et dans ceux sans endothélium indiquant l'implication des prostaglandines vasorelaxantes.

Pour évaluer l'effet vasodilatateur de l'huile de maïs, une huile équivalente et isocalorique contenant moins de 1% des omega-3, les anneaux d'artères coronaires ont été prétraités avec l'huile de maïs avant l'addition d'EPA:DHA 6:1 nanoencapsulé. L'huile de maïs n'a induit ni la relaxation des artères coronaires ni affecté la relaxation dépendante de l'endothélium enduite par l'EPA:DHA 6:1 nanoencapsulé, indiquant l'effet spécifique à la formulation optimisée des omega-3 en induisant des relaxations endothélium-dépendantes.

Étant donné que la nanoformulation d'EPA:DHA 6:1 favorisait des relaxations dépendant de l'endothélium de longue durée et qui sont médiées par la composante NO, des expériences ont évalué leur capacité à provoquer une formation soutenue de NO dans les CEs cultivées de l'artère coronaire sur une période de 24 h. La nanoformulation d'EPA:DHA 6:1 a induit des niveaux endothéliaux de NO significativement plus élevés par rapport à la forme native et à la bradykinine. En outre, l'effet stimulant à la fois de la forme nanoencapsulée et la forme native d'EPA:DHA 6:1 est nettement réduit par la L-NA indiquant l'implication de la composante NO.

Pour évaluer l'effet anti-agrégant de CEs en réponse à la thrombine, les CEs sont cultivées à l'intérieur des billes de Cytodex-3 et traitées avec de la forme nanoencapsulée et la forme native d'EPA:DHA 6:1 avant l'addition à des suspensions de plaquettes humaines lavées.

Bien que l'ajout de faibles quantités de CEs traitées par la forme native d'EPA:DHA 6:1 n'ait pas affecté l'agrégation induite par la thrombine, un effet inhibiteur prononcé est observé en réponse à l'ajout d'un nombre similaire de CEs traitées par

la forme nanoencapsulée. De plus, l'effet inhibiteur de l'agrégation plaquettaire est aboli par un inhibiteur de la eNOS indiquant l'implication de NO.

Les principaux résultats de notre étude *in vivo* indiquent que, par rapport aux rats jeunes de 12 semaines, le vieillissement physiologique était associé à une dysfonction endothéliale caractérisée à la fois par une diminution de la composantes NO de relaxation en réponse à l'acétylcholine, une augmentation de réponses contractiles à la phényléphrine de l'artère mésentérique principale et de l'aorte thoracique, et une formation basale élevée de prostanoïdes vasoconstricteurs dans l'aorte thoracique.

Le traitement avec la forme nanoencapsulée et la forme native d'EPA:DHA 6:1 améliore significativement les relaxations en réponse à l'acétylcholine, tandis que seule la forme nanoencapsulée d'EPA:DHA 6:1 réduit de manière significative les contractions en réponse à la phényléphrine dans l'artère mésentérique principale des rats de moyen âge.

De même, le traitement avec la forme nanoencapsulée d'EPA:DHA 6:1 améliore les relaxations en réponse à l'acétylcholine et réduit significativement les contractions en réponse à la phényléphrine dans l'aorte thoracique.

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'aorte thoracique par immunofluorescence sur coupes congelées. Dans un premier temps, nous avons étudié l'expression de la eNOS et de la nitrotyrosine (voie du NO)

Par rapport aux rats jeunes, le vieillissement est associé à une augmentation significative de l'expression de la eNOS et de la nitrotyrosine

Par rapport à la forme native, la prise chronique de la forme nanoencapsulée d'EPA:DHA 6:1 normalise significativement le niveau d'expression de la eNOS et de la nitrotyrosine dans l'aorte thoracique de rats d'âge moyen.

Comme la dysfonction endothéliale est associée à un stress oxydant, nous avons évalué le niveau de stress oxydant vasculaire et mitochondrial de l'aorte thoracique à l'aide de la sonde fluorescente sensible redox dihydroethidium (DHE) et MitoSOX, respectivement. Par rapport aux jeunes rats, les rats d'âge moyen ont montré une augmentation significative de la fluorescence dans l'ensemble de la paroi vasculaire et mitochondrial, et cette augmentation est significativement prévenue par la prise chronique de la forme nanoencapsulée et de la forme native d'EPA:DHA 6:1.

Du fait que l'augmentation de stress oxydant est due, du moins partiellement, au système local de l'angiotensine, le niveau d'expression du récepteur de l'angiotensine II de type 1 (AT1R) et de l'enzyme de conversion de l'angiotensine (ACE) a été déterminé. En comparaison à des rats jeunes, les rats d'âge moyen montrent une augmentation significative des niveaux d'expression d'AT1R et d'ACE dans l'endothélium et le muscle lisse vasculaire de l'aorte thoracique, qui ont été partiellement améliorés par le traitement avec la forme native d'EPA:DHA 6:1 et normalisés par le traitement avec la forme nanoecapsulée d'EPA:DHA 6:1.

L'ensemble des résultats obtenus indique que l'encapsulation d'EPA:DHA 6:1 suivie d'un enrobage avec de la gomme est associée à une capacité prolongée à provoquer des relaxations dépendantes de l'endothélium et médiées par la composante NO des artères coronaires isolées et à la capacité d'induire une formation soutenue de NO dans les cellules endothéliales cultivées comme démontré en utilisant une sonde fluorescente sensible au NO et par l'effet inhibiteur de l'agrégation plaquettaire.

Ainsi, la nanoencapsulation d'oméga-3 PUFAs suivie d'un enrobage apparaît comme une approche intéressante pour mieux protéger le système vasculaire.

En outre, la prise chronique de la forme nanoencapsulée d'EPA:DHA 6:1 pendant une semaine améliore significativement la dysfonction endothéliale liée à l'âge chez le rat. Les effets bénéfique de la consommation chronique de la formulation EPA:DHA 6:1 impliquent une amélioration des composantes de relaxations NO, une diminution du stress oxydant et du système d'angiotensine vasculaire.

Des recherches supplémentaires sont nécessaires pour élaborer les changements liés à l'âge dans les tissus et organes vasculaires tels que le remodelage vasculaire et cardiaque et pour évaluer l'impact de l'oméga-3 nanoformulé. De plus, il sera intéressant d'identifier les marqueurs biologiques pertinents et de caractériser les mécanismes cellulaires et moléculaires impliqués dans les effets endothéliaux bénéfiques d'EPA:DHA 6:1. De tels marqueurs permettraient une meilleure évaluation des effets bénéfiques d'EPA:DHA 6:1 lors de futurs essais cliniques randomisés chez des patients atteints de maladies cardiovasculaires ou de facteurs de risque associés à un dysfonctionnement endothélial.

Chapter one

Vascular endothelium and cardio-vascular diseases

1. Vascular physiology

1.1. Vasculature architecture and function

The human cardiovascular system is mainly composed of the heart and a network of blood vessels. The blood vessel network assists in perfusing and draining organs and tissues in a circular system. After leaving the heart, the blood is filled with nutrients and oxygen, and traverse a passageway of smaller tubular network known as arteries, arterioles, and capillaries. As blood travels back to the heart it enters a network of small veins that gradually merge to form larger vessels.

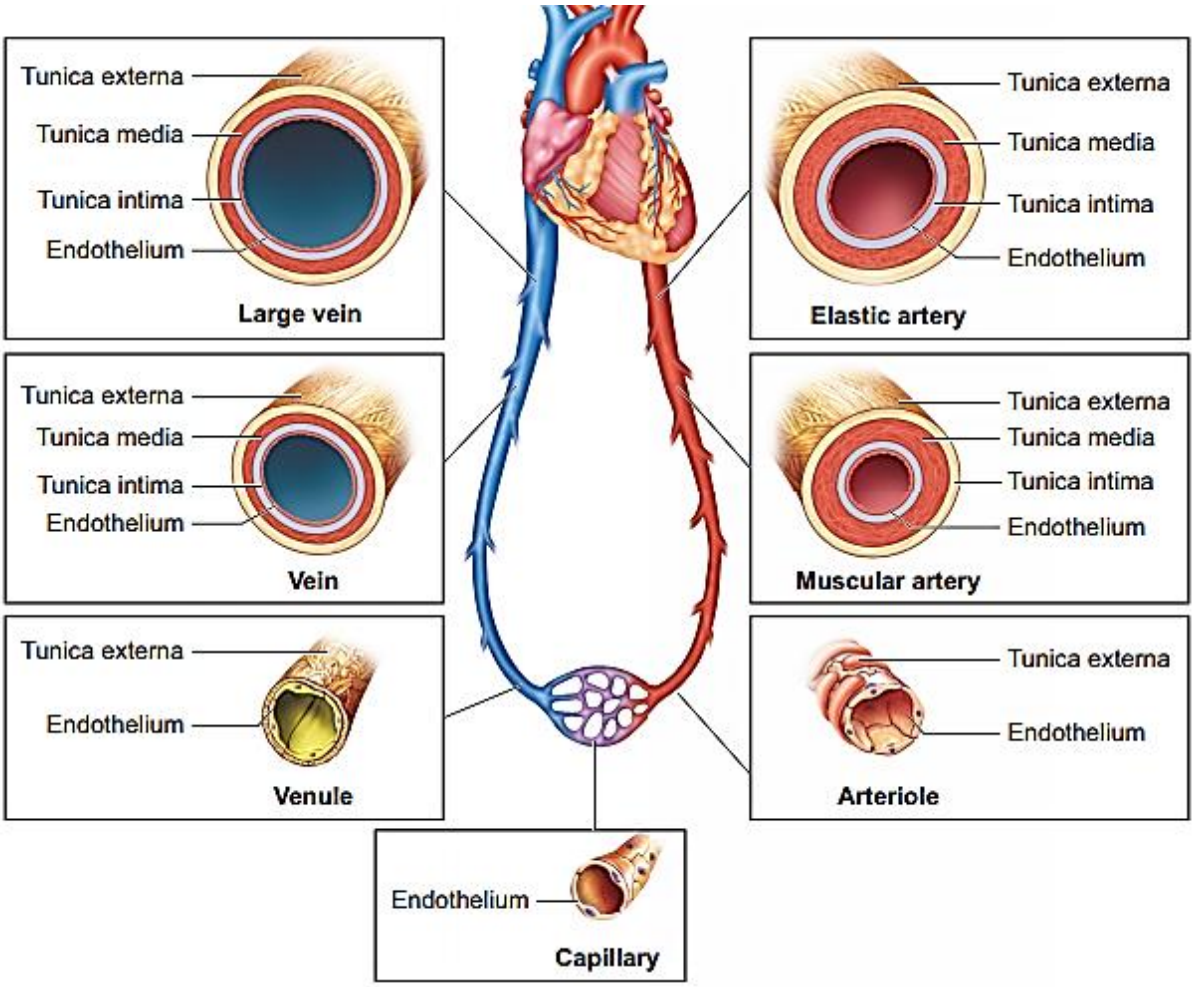
Throughout the cardiovascular system, the general architecture of all blood vessels except for capillaries, follows the same histological organization. The three structural layers from innermost to outermost are the tunica intima, tunica media and tunica adventitia that surround a central blood-containing space, the vessel lumen.

The tunica intima consists of an inner layer of endothelial cells (ECs) in direct contact with the blood as it flows through the lumen. The endothelium is supported by a delicate basement membrane, a thin layer of connective tissue and a layer of elastic fibers called the internal elastic lamina, which plays a crucial role in allowing better diffusion of substances within the arterial wall.

The tunica media is the middle layer composed of circularly organized strands of smooth muscle cells (SMCs) sustained by elastic fiber and connective tissue. This smooth muscle layer is responsible for controlling vascular resistance by neuronal and chemical mechanisms contributing to maintain blood pressure.

The tunica adventitia or tunica externa is composed of collagen, elastin and nerve fibers to reinforce and protect the vessels. In large vessels, the tunica adventitia contains a system of tiny arteries and veins known as the vasa vasorum providing nutrients for the external tissues of the blood vessel wall (Frederic H. Martini, Robert B. Tallitsch et al. 2018) (Figure 1).

A



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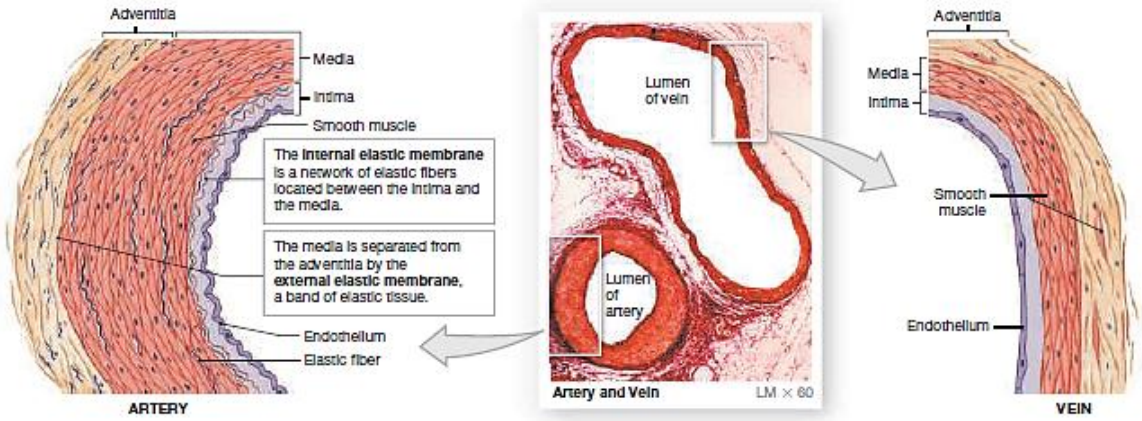

Figure 1. Structure of Blood Vessels (A) Differential types of blood vessels (Nadu 2018). (B) Histological comparison of arteries and veins (Frederic H. Martini, Robert B. Tallitsch et al. 2018).

1.2. Vascular endothelium: key role in the regulation of vascular tone

1.2.1 The endothelium

The endothelium is the innermost monolayer of cells, lining the luminal surface of the entire cardiovascular system. Covering a large surface area with approximately 1 to 6* 10^{13} cells of the human body, endothelial cells (ECs) are generally long, flat and slightly elongated in the direction of blood flow (Huttner IG and Gabbiani G 1983).

A dynamic balance of autocrine and paracrine mediators released by endothelial cells maintain homeostasis in response to hemodynamic, humoral and neural stimuli.

Due to its unique anatomical position which is in direct contact with the blood/lymph and the circulating cells, the endothelium is the chief governor of body functions including regulation of vascular tone, blood fluidity, platelet activation, fibrinolysis, solute exchange, inflammatory responses, angiogenesis and organ development and growth (Noyan Gokce and]oseph Loscalzo 2000).

ECs are versatile multifunctional cells by synthesizing and releasing bioactive substances such as vasoconstrictors (angiotensin II, endothelin-1 (ET-1), thromboxane (TXA2), reactive oxygen species (ROS)) and vasodilators (nitric oxide (NO), prostacyclin (PGI2)) (Aird 2007).

ECs inhibit vascular smooth muscle cells (VSMC) proliferation and have a non-thrombogenic surface that maintains blood in a fluid state (Galley and Webster 2004).

Additionally, they express markers such as endothelial nitric oxide synthase (eNOS), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, P-selectin, vascular endothelial cadherin (VE-cadherin) and von Willebrand factor (vWf) (Albelda and Buck 1990).

Another basic function of endothelium is the control of solutes exchange between blood and interstitial space. The transport across a semi permeable barrier is executed with two main mechanisms, paracellular for water and solute small molecules or transcellular for macromolecules (Mehta and Malik 2006).

1.2.2 Nitric oxide

Nitric oxide (NO) is the first acknowledged gaso-transmitter, a free radical with a half-life of few seconds (Furchgott and Zawadzki 1980).

In the vasculature, the endothelial NO synthase (eNOS) generates NO from the oxidation of the guanidine-nitrogen terminal of L-arginine to L-citrulline (Palmer, Ashton et al. 1988).

Endothelial NOS is constitutively expressed in endothelial cells in caveolae (small invaginations of cell membrane) and interacts with the coat protein, caveolin-1 to form an inactive complex (Minshall, Sessa et al. 2003). 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 (HSP90) (Dessy, Feron et al. 2010).

In the calcium (Ca^{2+})-dependent activation pathway, a receptor-mediated agonists such as acetylcholine (ACh), bradykinin (BK), histamine (H), thrombin, serotonin (5-HT), adenosine di-phosphate (ADP), noradrenaline and substance P increase the intracellular level of calcium (Ca^{2+}), which binds to calmodulin (CaM) and dissociates eNOS from caveolin-1 protein rendering the enzyme active (Tousoulis, Kampoli et al. 2012).

In the calcium (Ca^{2+})-independent activation pathway, protein kinase A (PKA), protein kinase B (Akt), protein kinase G (PKG), AMP-activated protein kinase A (AMPK), Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) and protein phosphatase 2A (PP2A) activate eNOS by phosphorylating Ser1177 residue (Mount, Kemp et al. 2007) in response to various stimuli such as vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-I), insulin, leptin, statin, fluid shear stress (Wang, Nagase et al. 2004) and oestrogens (Simoncini, Hafezi-Moghadam et al. 2000).

After diffusion from endothelial cells to adjacent vascular smooth muscle cells (SMC), NO as a signaling agent stimulates the soluble guanylyl cyclase (sGC) increasing intracellular cyclic 3',5'-guanylyl monophosphate (cGMP), which in turn reduces cytosolic [Ca2+], leading to relaxation of the SMCs. Besides vasodilation, NO inhibits SMC proliferation; prevents endothelial cell apoptosis and enhances endothelial cell proliferation (Lei, Vodovotz et al. 2013).

NO released towards the vascular lumen, suppresses platelet aggregation and prevents platelet, leukocyte and monocyte adhesion to endothelium contributing to the anti-atherothrombotic responses (Förstermann and Sessa 2012).

In addition to paracrine processes, NO controls intracellular functions such as reactive oxygen species (ROS) production, telomerase activity, mitochondria biogenesis, and low-density lipoprotein (LDL)-oxidation (Ghimire, Altmann et al. 2017) (Figure 2).

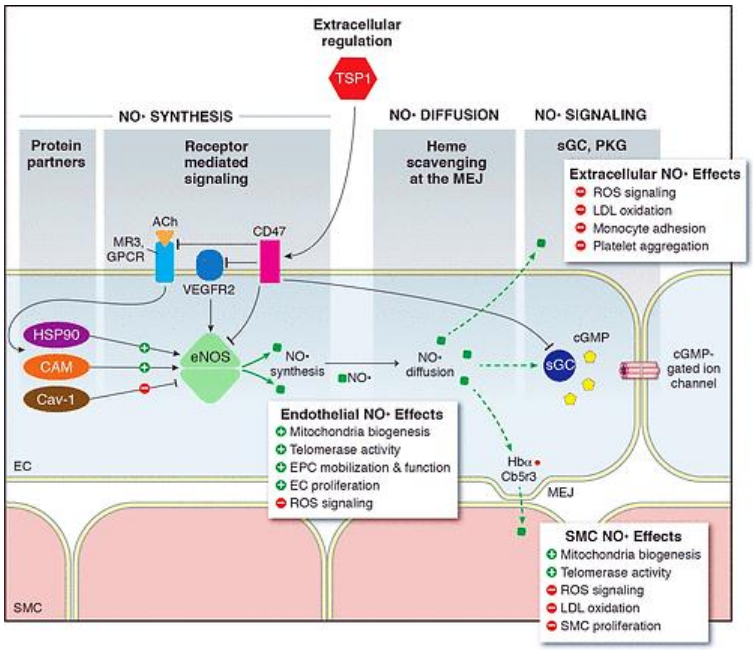

Figure 2. Nitric oxide (NO) regulation (Ghimire, Altmann et al. 2017).
Receptor-mediated signaling such as vascular endothelial growth factor receptor 2 (VEGFR2) and acetylcholine (ACh) as well as protein partners including heat shock protein 90 (HSP90), calmodulin (CaM), and caveolin-1 (Cav-1) regulate endothelial NO synthase (eNOS) activity and thus NO production in the vascular wall. Once generated, NO diffuses from endothelium to various cell types including vascular smooth muscle cells, platelets, and monocytes where it activates its receptor soluble guanylyl cyclase (sGC), increases production of cyclic guanosine monophosphate (cGMP), and controls cell function. In addition to paracrine processes, NO controls or alters intracellular functions such as mitochondria biogenesis, telomerase activity, reactive oxygen species (ROS) production, and low-density lipoprotein (LDL)-oxidation. Heme scavenging proteins, such as hemoglobin-_, are expressed in endothelial cells (EC) and serve as a "buffer" to control NO diffusion, a process that is regulated at endothelial (MEJ)-smooth muscle cell junctions by cytochrome b5 reductase 3 (Cb5r3). Extracellular "outside-in" signals, such as thrombospondin-1 (TSP1)-cluster of differentiation 47 (CD47) also have the ability to limit on NO signaling through inhibition of eNOS, sGC, and cGMP-dependent protein kinase (PKG) activity.
EPC, endothelial progenitor cells; MR3, muscarinic receptor 3; MEJ, myo-endothelial junction; SMC, smooth muscle cell; GPCR, G protein-coupled receptor.

2. Endothelial dysfunction and cardiovascular diseases

Endothelial dysfunction is a pathophysiological state with alterations in functional phenotype including a loss of NO bioavailability, vasodilation impairment, increased oxidative stress, enhanced endothelial permeability and reduced vascular reparation capacity, in addition to endothelial activation with procoagulant (PAI-1, TF), proliferative (ET-1, Ang II, TGF-β, PDGF) and proinflammatory (NF-Kb, TNF-α, IL-6, CRP, VCAM-1, ICAM-1) profile.

Endothelial dysfunction predisposes the vessel to vascular lesions, excessive vasoconstriction, inflammation and thrombosis, contributing to the progression of atherosclerosis.

Therefore, endothelial function assessment is an important prognostic marker of cardiovascular diseases (Favarato and da Luz 2018) (Figure 3).

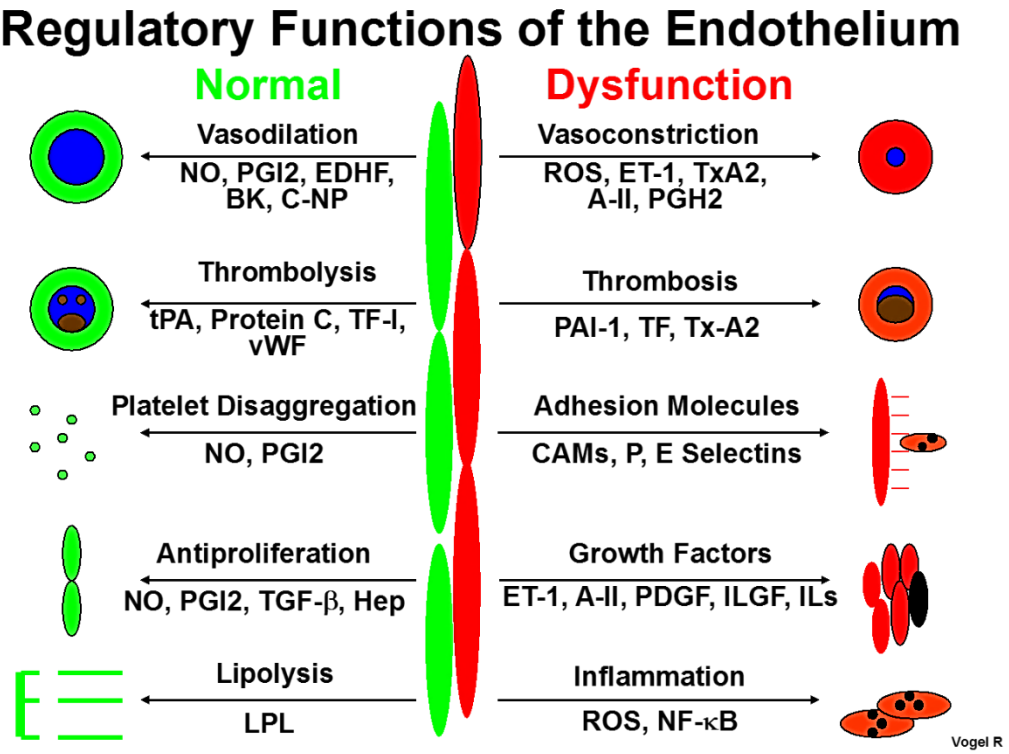

Figure 3. The differences between normal and dysfunctional endothelium (Robert A. Vogel).
NO indicates nitric oxide; PGI2, prostacyclin; EDHF, endothelium derived hyperpolarizing factor; BK, bradykinin; C-NP, C-type natriuretic peptide; ROS, reactive oxygen species; ET-1, endothelin-1; TXA2, thromboxane; Ang II, angiotensin II; PGH2, prostaglandin H2; t-PA, tissue-type plasminogen activator; vWF, von Willebrand factor; PAI-1, plasminogen activator inhibitor-1; TF, tissue factor; CAMs, cell adhesion molecules; TGF-β, transforming growth factor; PDGF, platelet derived growth factor; bFGF, basic fibroblast growth factor; ILGF, insulin-like growth factor; LPL, lipoprotein lipase; NF-κB: nuclear factor κB.

2.1. Underlined mechanisms of endothelial dysfunction

2.1.1. Disturbed shear stress

Shear stress is the force per unit area applied by the flowing blood on the endothelium of the arterial wall. Within the vasculature, the blood flow varies from a laminar type in straight arterial regions to an oscillatory type in branched and curved regions such as aorta arch area, so called atheroprone sites where atherosclerosis occurs frequently (Huang, Yang et al. 2017).

Shear stress plays a critical role in vascular homeostasis with an important modulation of endothelial function via activity of signaling pathway.

Keeping the ECs in health state, laminar shear stress promotes release of factors such as NO that inhibits coagulation, migration of leukocytes, and suppresses SMC proliferation, while simultaneously promoting EC survival (Traub and Berk 1998), up regulates the athero-protective and anti-inflammatory genes expression such as KLF2 (Pan 2009), and enhances EC migration to promote wound healing (Sprague, Luo et al. 1997).

Conversely, oscillating shear stress promotes atheroprone phenotype of ECs by impairing NO and PGI2 production associated with the expression of several pro-atherogenic genes (ICAM-1, VCAM-1, MCP-1, E-selectin, ET-1 and NF-κB) (Ohura, Yamamoto et al. 2003), an increased leukocyte adhesion, inflammation (Zakkar, Angelini et al. 2016) and EC proliferation (dela Paz, Walshe et al. 2012), thereby contributing to atherogenesis (Cunningham and Gotlieb 2005) (Figure 4).

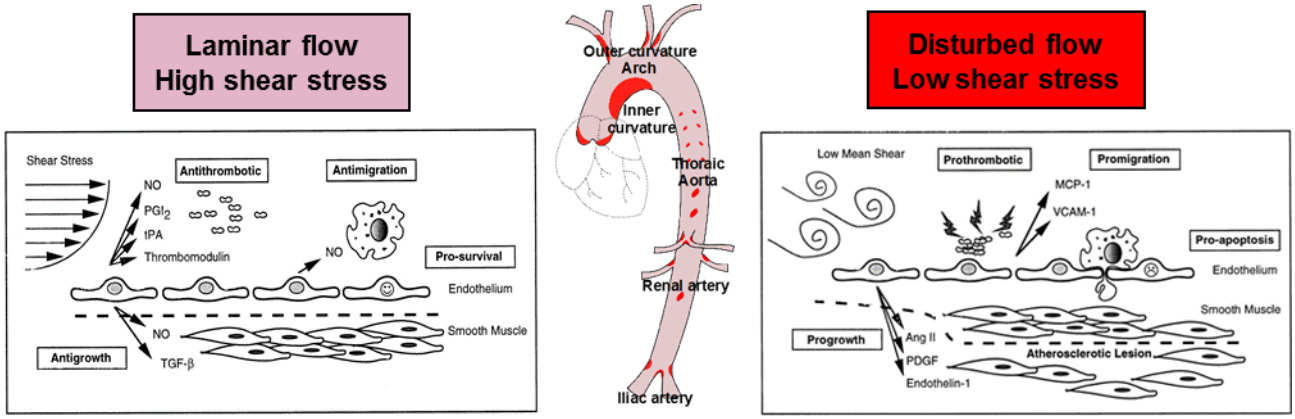

Figure 4. Endothelial cell biology and shear stress (Traub and Berk 1998).

2.1.2. Oxidative stress

Oxidative stress constitutes a unifying mechanism of injury process with an imbalance between the production of reactive oxygen species (ROS) and their elimination by the biological system.

ROS are mostly free radicals with an unpaired electron (superoxide anion (O_2^-), hydroxyl radical ($OH^·$), nitric oxide ($NO^·$), and lipid radicals) or have oxidizing effects including molecular oxygen such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and hypochlorous acid (HOCl), that contribute to oxidant stress.

Within the aerobic cell, the reactive intermediate superoxide anion (O_2^-) is produced by the mitochondrial electron chain, uncoupled eNOS, cyclooxygenases (COXs), NADPH oxidase, xanthine oxidase and Cytochrome p-450 (Cyp 450), then gives rise to several ROS molecules via radical chain reactions (Cai and Harrison 2000).

Excessive production of ROS results in oxidative damaging of vital macromolecules characterized by the oxidation of nucleic acids causing mutations and deletions, the peroxidation of lipids forming cytotoxic peroxides, proteins through nitrosylation, oxidation of amino acids or glycosylation leading to advanced glycation end-products (AGE) (Kregel and Zhang 2007)

Moreover, oxidative stress participates in vascular signaling and proatherogenic responses by modulating redox-sensitive transcription and transduction pathways. It alters both Akt kinase and activity of caspase inducing proliferation of ECs, stimulation of apoptotic signals and loss of ECs (Irani 2000), also interferes with gene expression modifying the transcription factors and results in cell cycle arrest via activation of p53/p21 pathway (Miyauchi, Minamino et al. 2004).

Importantly, increased oxidative stress activates a redox-sensitive transcription factor NF-κB and downstream genes as inflammatory cytokines and adhesion molecules (VCAM-1, ICAM-1 and MCP-1) exacerbating the oxidative/inflammatory cycle and facilitating endothelial cell injury and dysfunction (Badran, Ayas et al. 2014) (Figure 5).

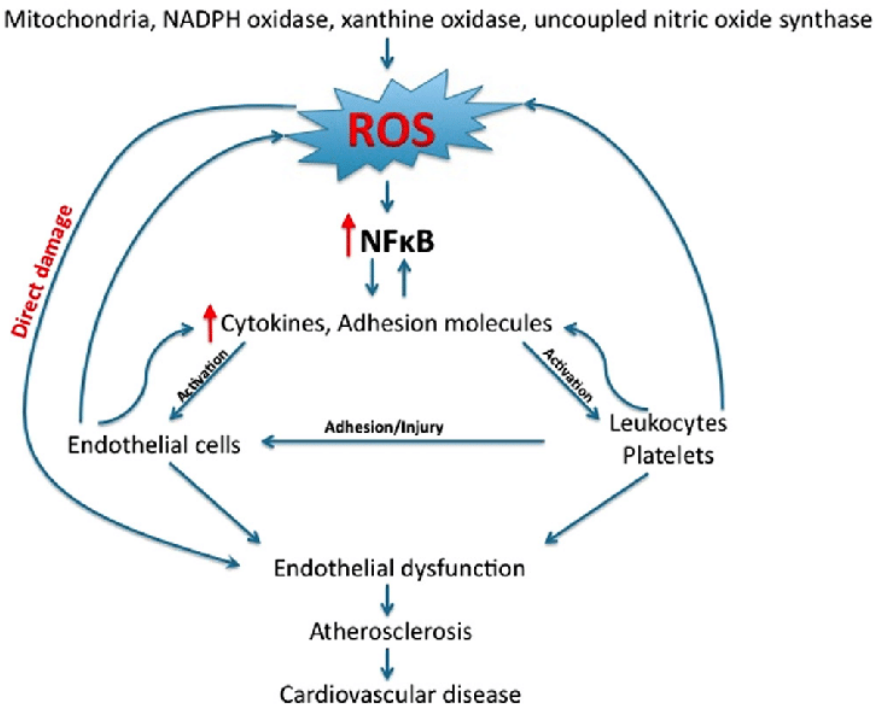

Figure 5. Oxidative stress and inflammation. Adopted from (Badran, Ayas et al. 2014).

2.1.3. Renin angiotensin system

Renin angiotensin system (RAS) is critical for vascular control mediated by angiotensin acting through its AT1 and AT2 receptors. Overproduction of angiotensin II (Ang II) is harmful towards vascular wall and disrupts the regulatory processes of the endothelium resulting in endothelial dysfunction (Stegbauer and Coffman 2011).

Ang II through AT1 receptors, stimulates NADPH oxidase via PKC dependent pathways, increasing generation of superoxide (O_2^-), peroxynitrite ($ONOO^-$) and diminishing $NO^\cdot$ bioavailability, leading to mitochondrial dysfunction and development of endothelial dysfunction (Doughan, Harrison et al. 2008).

Ang II upregulates the secretion of inflammatory cytokines such as cytokine tumor necrosis factor-alpha (TNF-α), activates nuclear factor (NF-kB) resulting in raised levels of cell adhesion molecules (VCAM-1, ICAM-1), monocyte chemoattractant protein-1 (MCP-1) and critically enhances the inflammatory process and promotes vascular disorders (Skultetyova, Filipova et al. 2007).

Ang II triggers accumulation of collagen and fibronectin in the vessel wall by activating the tissue growth factor β (TGFβ) leading to vascular fibrosis and remodeling (Yoon, Kim et al. 2016).

Ang II is also responsible for the induction of the ET-1 system that promotes vasoconstriction, increased coagulation, inflammation and cell proliferation (Barton 2014) (Figure 6).

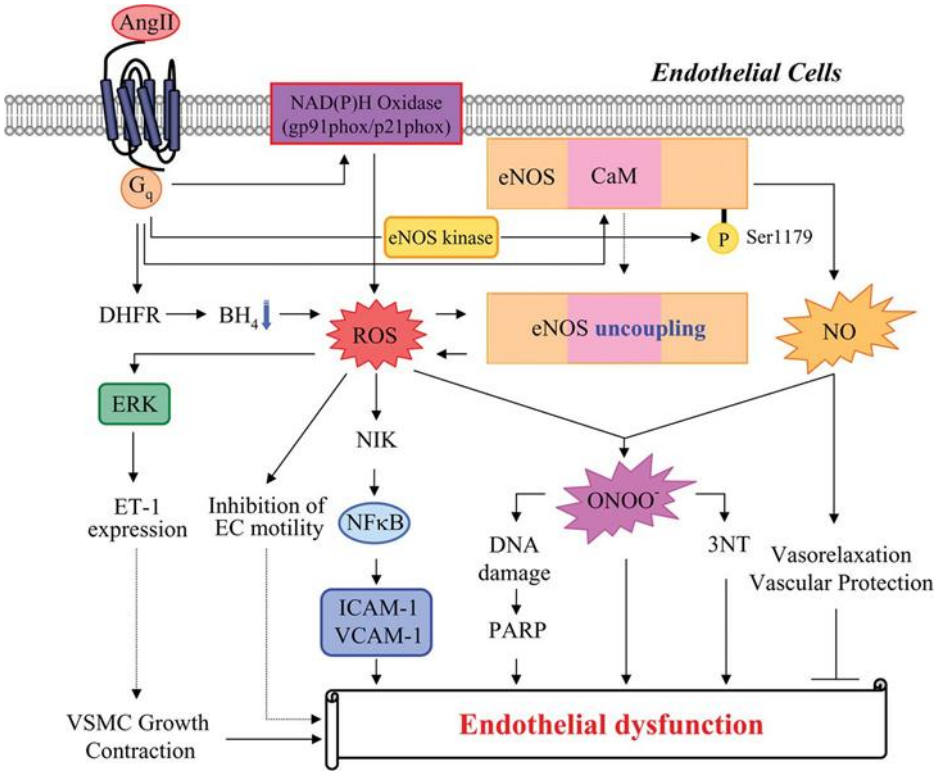

Figure 6. Role of angiotensin II signaling in endothelial dysfunction (Higuchi, Ohtsu et al. 2007).
Akt, protein kinase B; Ang II, angiotensin II; BH_4, tetrahydrobiopterin; CaM, calcium calmodulin; DHFR, dihydrofolate reductase; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal regulated kinase; ET-1, endothelin-1; G_q, G-protein q; NF-kB, nuclear factor kB; NO, nitric oxide; $ONOO^-$, peroxynitrite anion; PARP, poly adenosine diphosphate ribose polymerase; S1179, serine 1179; VSMC, vascular smooth muscle cell.

2.2. Pathophysiology of cardiovascular diseases

2.2.1. Atherosclerosis

Endothelial dysfunction comprises a defect in the bioavailability of NO due in part to an increased level of oxidative stress in response to cardiovascular risk factors such as hypertension, smoking, diabetes and hyperlipidemia (Chhabra 2009).

Endothelial dysfunction reflects a vascular phenotype prone to atherogenesis manifested as an increased ECs permeability, oxidation of low-density lipoproteins (LDLs), release of pro inflammatory cytokines and chemokines (MCP-1, TNF-alpha, IL-1β, GM-CSF), overexpression of adhesion molecules (VCAM-1, ICAM-1) facilitating the recruitment of

leukocytes, activation of platelets, and VSMCs migration and proliferation, promoting the initiation and progression of atherosclerosis (Herman and Moncada 2005) (Figure 7).

Furthermore, chronic endothelial dysfunction is linked with an erosion of the atherosclerotic plaque promoting plaque instability and acute vascular syndromes (Widlansky, Gokce et al. 2003).

In 1986, Ludmer et al used the acetylcholine test and reported the first evidence in humans of impaired endothelial vasodilator function in the course of coronary atherosclerosis (Ludmer, Selwyn et al. 1986).

Numerous studies in humans and apoE$^{-/-}$ mouse model have described the antiatherogenic role of NO. The enhancement of arginase activity, oxidative stress, oxidized LDLs , eNOS uncoupling contribute to atherosclerosis by reducing NO bioavailability (Chen, Ye et al. 2018).

Another clinical study proposes the potential contribution of low local endothelial shear stress (ESS) in the initiation and progression of coronary atherosclerosis driven mainly by endothelial dysfunction (Siasos, Sara et al. 2018).

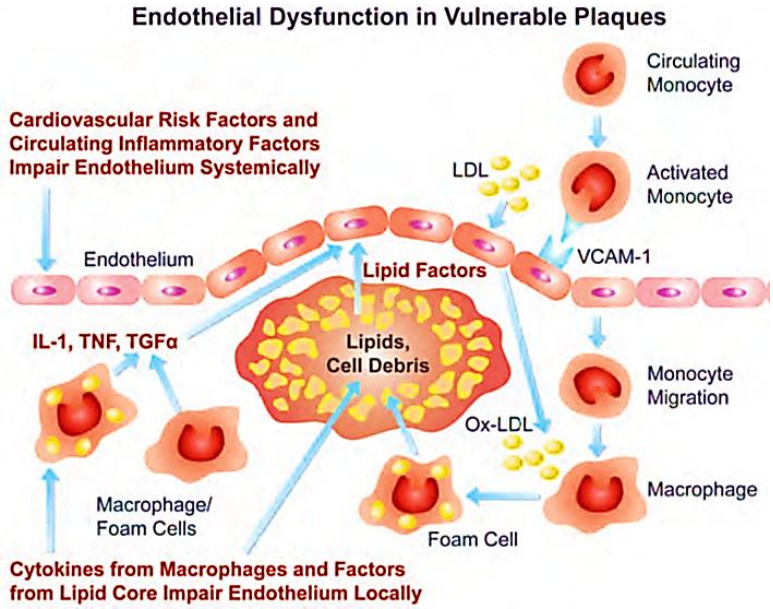

Figure 7. Vulnerable coronary atherosclerotic plaque (Ganz and Hsue 2013).
IL-1, interleukin-1; TGFa, transforming growth factor a; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule 1; LDL, low-density lipoprotein.

2.2.2. Hypertension

Essential hypertension is defined as a sustained rise in blood pressure that increases the risk for cardiac events such as stroke, myocardial infarction, atrial fibrillation, heart failure and peripheral artery disease (Whelton, Carey et al. 2018).

Endothelial dysfunction is strictly associated with hypertension and its complications resulting from an excessive vascular oxidative stress and a loss of the antiatherogenic and vasculoprotective effects of endothelium-derived NO (Dharmashankar and Widlansky 2010).

Rossi et al. showed that normotensive women with impaired endothelial function have a nearly six fold increased risk of developing hypertension (Rossi, Chiurlia et al. 2004).

In healthy normotensive humans, the administration of NOS inhibitors causes large increases in blood pressure (BP) that are in part sympathetically mediated (Sander, Chavoshan et al. 1999). Also, in knock-out mice that lack a functional eNOS gene, blood pressure level was more pronounced than in wild-type controls demonstrating the crucial role of endothelium-derived NO in maintaining a constant blood pressure level (Stauss, Gödecke et al. 1999).

(Lüscher and Vanhoutte 1986) have reported a decreased amplitude of endothelium-dependent relaxations in response to acetylcholine in contracted aortas from spontaneously hypertensive rats (SHR).

In addition, isolated carotid arteries from mice exposed to high blood pressure showed reductions in endothelium-dependent relaxation to acetylcholine, increases in vascular superoxide production, and increased NADPH oxidase activity (Vecchione, Carnevale et al. 2009).

In addition, Schiffrin et al reported an upregulation of Ang II receptors in mesenteric arteries of DOCA-salt hypertensive rats (Schiffrin, Thome et al. 1983). Ang II sustains hypertension through the activation of NADPH oxidase resulting in eNOS uncoupling, generation of peroxynitrite and also reduced extracellular superoxide dismutase (SOD) activity (Watson, Goon et al. 2008, Lob, Vinh et al. 2011).

The treatment of hypertensive patients with the Ang II receptor blocker “losartan” reduced elevated BP, decreased vascular oxidative stress and enhanced NO availability improving endothelial function (Sosa-C, Hernández-H et al. 2007).

2.2.3. Acute coronary syndrome

Acute coronary syndrome (ACS), including acute myocardial infarction and unstable angina, is a clinical state associated with a reduced blood flow to the heart in the coronary arteries. ACS is induced by the thrombus formation following the disruption of unstable atherosclerotic plaque (Peter Libby 2001) (Figure 8).

Endothelial dysfunction may play a fundamental role in the pathogenesis of ACS. Plaque destabilization, the detrimental process that predisposes the plaque to rupture, results from a complex interplay of inflammatory effects that involve cellular plaque components and various proinflammatory mediators. Endothelial dysfunction is characterized by an increased level of oxidative stress, reduced NO bioavailability promoting inflammatory processes and lipid-rich vascular sites. Endothelial dysfunction is characterized by an increase in procoagulant mediators and a reduction in the anticoagulant potential of the endothelium resulting in a thrombogenic vascular environment. Thus, a dysfunctional endothelium contributes to enhanced plaque destabilization and favors thrombus formation.

In addition, vasoconstrictive forces generated by metabolic stimuli such as platelet-derived mediators, endothelium-derived contractile factors (EDCF) and by endothelin-1, favor the physical disruption of coronary plaques (Bonetti, Lerman et al. 2003).

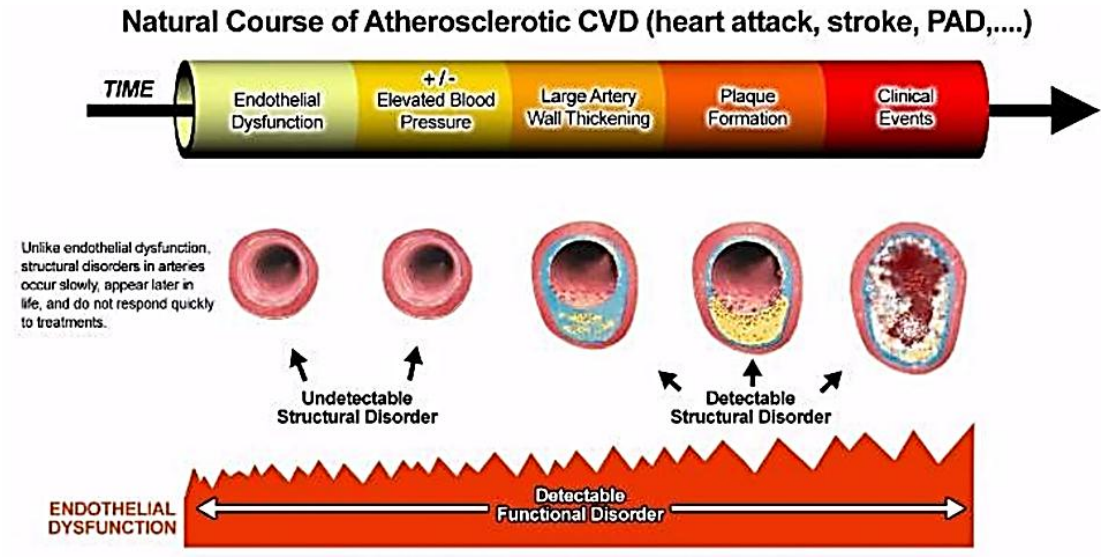

Figure 8. Endothelial dysfunction precedes structural disorders, plaque buildup, and clinical events (© Cenegenics Medical Institute of Phoenix, AZ).

2.2.4. Aging

Vascular aging characterizes the functional disturbances of the vascular wall and the development of structural alterations contributing to an impairment of endothelial function (Toda 2012) (Figure 9).

Cellular senescence is a characteristic of the irreversible growth arrest induced by repeated cell divisions over the time or prematurely by different stress factors (Rossman, Kaplon et al. 2017).

Endothelial senescence aggravates aging-related inflammation, oxidative stress, and endothelial dysfunction with a distinct phenotypic change and a loss of cellular homeostasis.

Senescent ECs demonstrate a reduction in NO availability, elevation of ROS (Minamino and Komuro 2007, Herrera, Mingorance et al. 2010), alterations in morphology, gene expression and secretory profile, also an altered expression of proteins which increase the onset of remodeling, contributing to an atherothrombotic vascular environment (Erusalimsky 2009).

ECs showed an increased expression of Senescence-Associated β-galactosidase (SA-β-gal) activity in the rabbit carotid artery (Fenton, Barker et al. 2001), aorta of Zucker diabetic rats (Chen, Brodsky et al. 2002), mice model of oxidative stress (Ota, Eto et al. 2008) and at sites overlapping atherosclerotic plaques of human aortic arch and coronary arteries (Vasile, Tomita et al. 2001, Minamino, Miyauchi et al. 2002).

The ageing process is linked to a chronic inflammatory state, referred as "inflamm-aging", it is associated with greater expression of NF-κB, cytokines (TNF-α, IL-1β, IL-6), adhesion molecules (ICAM-1, VCAM-1), elevations of C-reactive protein (CRP) and fibrinogen promoting the development of endothelial dysfunction (El Assar, Angulo et al. 2012).

Clinical studies have reported age-related endothelial dysfunction associated with a pro-inflammatory profile in vascular endothelial cells from healthy humans (Donato, Eskurza et al. 2007, Donato 2008, Donato, Gano et al. 2009).

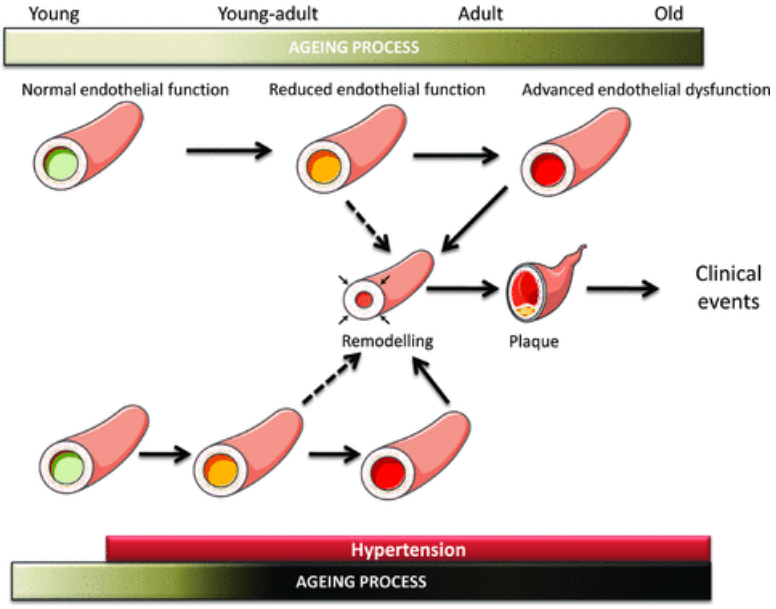

Figure 9. Schematic representation of the effect of the ageing process on vascular function
(Versari, Daghini et al. 2009).

3. Endothelial senescence

3.1. Cellular senescence

EC senescence is considered as a pivotal marker of the onset of age-related diseases such as, stroke, heart attack and diabetes (Barinda, Ikeda et al. 2020). Senescent cells arrest growth with an irreversible inability to proliferate exhibiting key changes in gene expression, morphology, and function (Kirkland and Tchkonia 2017).

Hayflick and Moorhead showed that normal cells stop dividing after limited number of populations doubling but remain viable. They also observed the decreasing number of divisions in cells derived from younger to older donors (Erusalimsky and Skene 2009).

Cellular senescence serves as beneficial in embryonic development, in tissue regeneration and as a protective mechanism against tumorigenesis. However, increased senescent cells in aged organisms generate a low-grade chronic inflammatory state supporting tumor progression (Bielak-Zmijewska, Grabowska et al. 2019).

3.2. Characteristics of Senescent cells

Distinctive characteristics of senescent cells have been observed in a variety of cells *in vitro* and *in vivo*, known as biomarkers of cellular senescence including morphology, genetics and secretory phenotype (Bernadotte, Mikhelson et al. 2016). The phenotype of senescent cells is highly heterogeneous, dynamic, and defined through various combinations of markers.

Senescent ECs are flat, enlarged and have an irregular shape with an altered plasma membrane which is associated with scaffolding protein caveolin-1 and Rho GTPases Rac1 and CDC42. Their nuclear integrity is damaged due to the loss of laminB1, resulting in cytoplasmic chromatin fragments (CCFs) (Herranz and Gil 2018).

Senescent ECs are metabolically active, they have an accumulation of dysfunctional mitochondria lowering ATP and elevating ROS production and an increased lysosomal mass linked to SA-β-Gal enzyme which is active at pH 6, independent of DNA synthesis distinguishing senescent cells from quiescent cells (Lorenzo Galluzzi and Kroemer 2013, Hernandez-Segura, Nehme et al. 2018) (Figure 10).

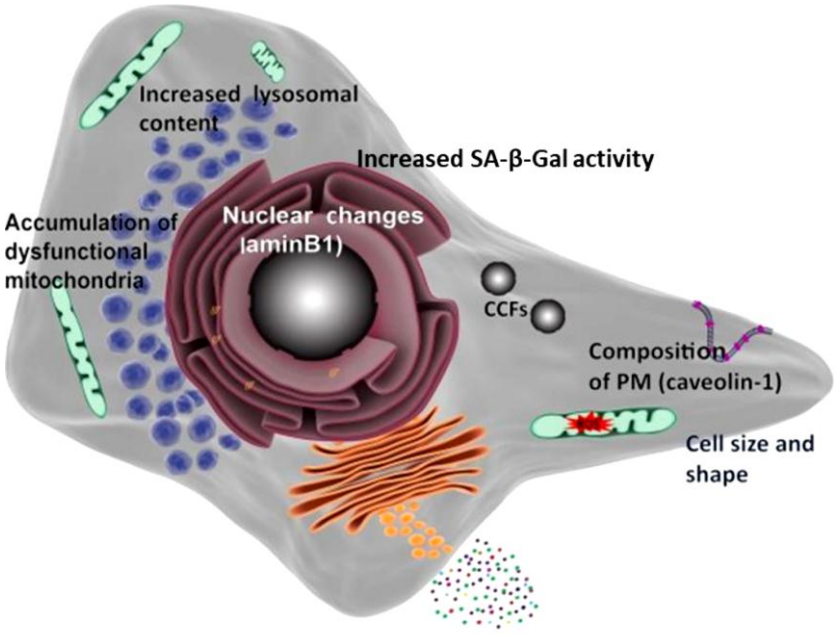

Figure 10. Hallmarks of morphological alterations (Hernandez-Segura, Nehme et al. 2018).
The molecular pathways of senescence result in morphological alterations. Senescent cells are enlarged and have an irregular shape; their nuclear integrity is compromised due to the loss of laminB1, which also leads to the appearance of cytoplasmic chromatin fragments (CCFs); they have an increased lysosomal content, which is often detected as high b-galactosidase activity; they have large but dysfunctional mitochondria that produce high levels of reactive oxygen species (ROS); and their plasma membrane (PM) changes its composition (for instance, upregulating caveolin-1).

Cellular senescence is induced in response to persistent DNA damage triggered by telomere shortening, oncogenic activity, oxidative stress, and cell-cell fusion.

DNA damage responses (DDR) include the nuclear foci of phosphorylated histone γ-H2AX, p53-binding protein 1 (53BP1) localized at double strand break sites (Bielak-Zmijewska, Grabowska et al. 2019, Shimizu and Minamino 2019).

Senescent cells are terminally arrested at G1 phase marked by high levels of cell cycle inhibitors, p53, $p21^{CIP1}$, and $p16^{Ink4a}$. Other markers indicate the proliferation disability like the down-regulated expression of Ki67, the lack of DNA incorporation of bromodeoxyuridine (BrdU) (Anat Biran, Lior Roitman et al. 2017) and the absence of HGMB1in the nucleus (Davalos, Kawahara et al. 2013) (Figure 11).

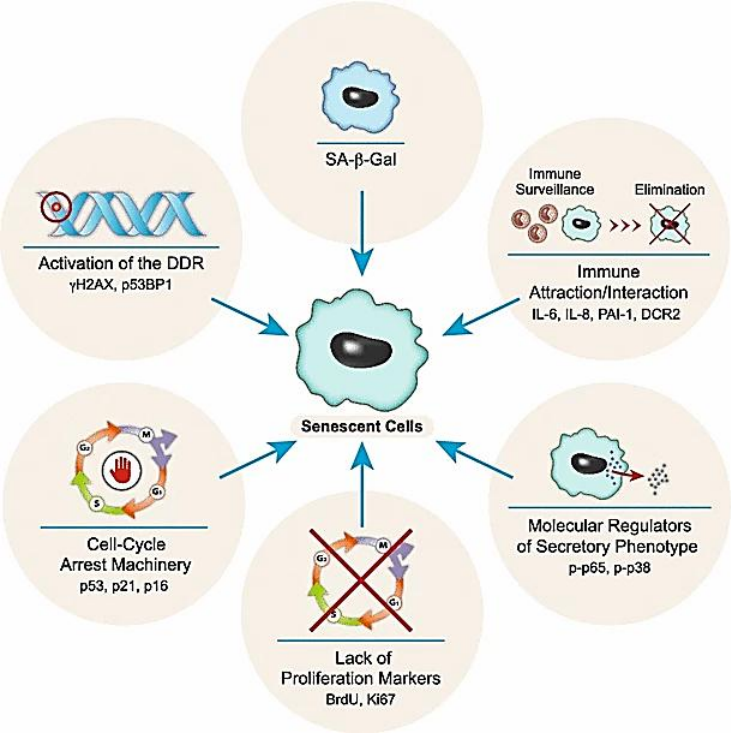

Figure 11. Markers of senescent cells (Burton and Krizhanovsky 2014).

The senescence is accompanied by alterations in chromatin structure forming the senescence-associated heterochromatin foci (SAHF). SAHF accumulation is associated with steady repression of E2F target genes and occurs only in irreversibly arrested cell cycle (Lorenzo Galluzzi and Kroemer 2013).

Moreover, senescent cells produce specific messaging secretomes that can disrupt tissue structure and alter tissue function, termed as the senescence-associated secretory phenotype (SASP) (figure 12).

The SASP comprises pro-inflammatory cytokines (IL-6, IFN-γ), chemokines (MCP-1), growth factors (VEGF, TGF-b, and GM-CSF), proteases (MMPs, PAI-1), soluble or shed receptors/ligands (EGF-R, ICAM, Fas, μPAR), non-protein soluble factors (NO, ROS, PGE2), insoluble extracellular matrix components (fibronectin, collagen, laminin) (Coppe, Desprez et al. 2010).

The secretory component is regulated by NF-kB, CCAAT/enhancer binding proteins-β transcription factors, via mammalian target of rapamycin (mTOR) and p38MAPK signaling (Watanabe, Kawamoto et al. 2017).

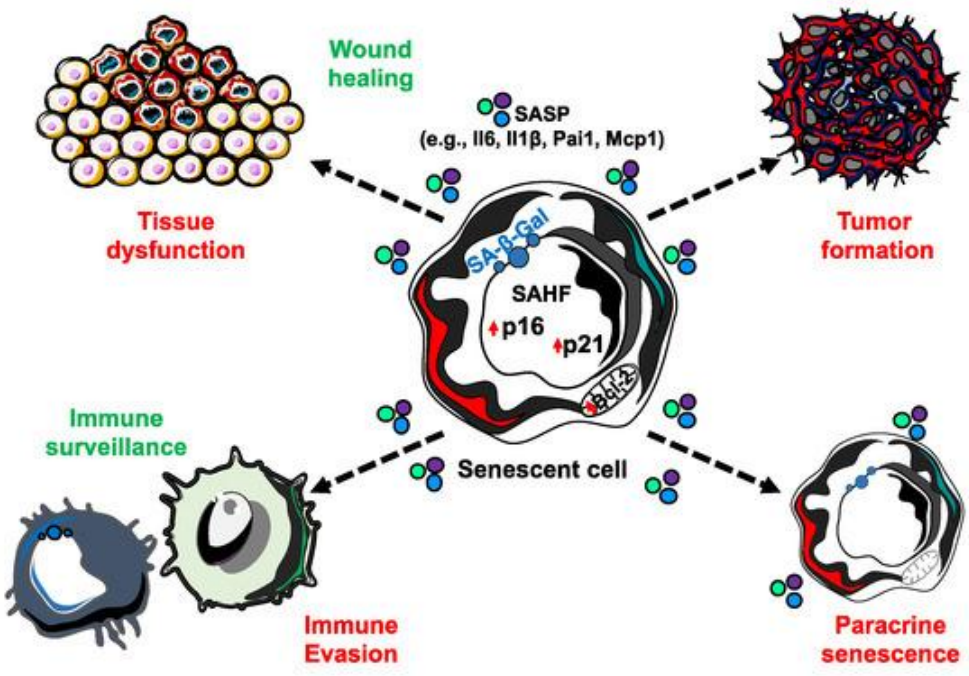

Figure 12. The biological effects of senescence-associated secretory phenotype (SASP) (Prieto, Graves et al. 2020).

Finally, senescent cells are resistant to apoptosis through prosurvival networks including ephrins, p21, BCL-2, HSP90, PI3K and plasminogen activated inhibitor-2 (Hernandez-Segura, Nehme et al. 2018).

Different methods are used to characterize senescent cells (Carracedo, Ramírez-Carracedo et al. 2018) (Table 1).

Characteristics	Markers	Regulation	Techniques
DNA replication (Senescent cells decline in DNA replication)	BrdU	↓	Fluorescence microscope
	^{3}H-dT	↓	Incorporation of radioactivity
	PCNA	↓	Immunostaining/Western blot
	Ki-67	↓	Immunostaining/Western blot
SA-β-gal activity (the SA-β-gal derives from the lysosomal β-galactosidase and reflects the increased lysosomal biogenesis)	X-gal substrate	↑	Light microscopy (production of blue precipitate)
	C12FDG (fluorogenic substrate	↑	Fluorescence microscopy (production of green fluorogenic color)
Cell cycle arrest proteins (early markers of DNA damage-induced senescence)	p16	↑	Western blot/immunostaining
	p21		
	p53		
	Cyclin D1		
	Lamin B1	↓	
SAHFs (reorganization of chromatin into discrete foci)	DNA dyes: DAPI	↑Presence of certain Heterochromatin-associated histone modifications	Fluorescence microscopy
SDF (different DNA repair proteins)	γ-H2AX:marker of DNA double strand breaks and genomic instability	↑	Fluorescence microscopy/Western blot
	53BP1: protein associated with DNA damage	↑	Fluorescence microscopy

Table 1. Senescence markers: regulation and detection (Carracedo, Ramírez-Carracedo et al. 2018)**.**
BrdU, 5-bromodeoxyuridine; ^{3}H-dT, ^{3}Hthymidine; PCNA, Proliferating cell nuclear antigen; SA-β-gal, Senescence-associated β-galactosidase; X-gal substrate, 5-bromo-4-chloro-3-indolyl-D-galactoside; C_{12}FDG, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside; SAHFs, senescence-associated heterochromatin foci; DAPI, 4′,6-diamidino-2-phenylindole; SDF, senescence-associated DNA damage foci; γ-H2AX; phosphorylated histone H2AX; 53BP1, p53-binding protein-1.

3.3. Mechanisms of senescence

In response to cellular damage instigated by a variety of intrinsic (telomere shortening, oxidative damage, mitochondrial dysfunction, oncogenes) or extrinsic (chemotherapeutic drugs, irradiation) stresses, cells can engage two effector pathways: p53-p21 and p16-RB to halt cell-cycle progression. If the damage is repaired, the cells can re-enter the cell-cycle. If the damage is unresolved, the cells can either die through apoptosis (cell death) or survive in the state of cellular senescence (Prieto, Graves et al. 2020) (Figure 13).

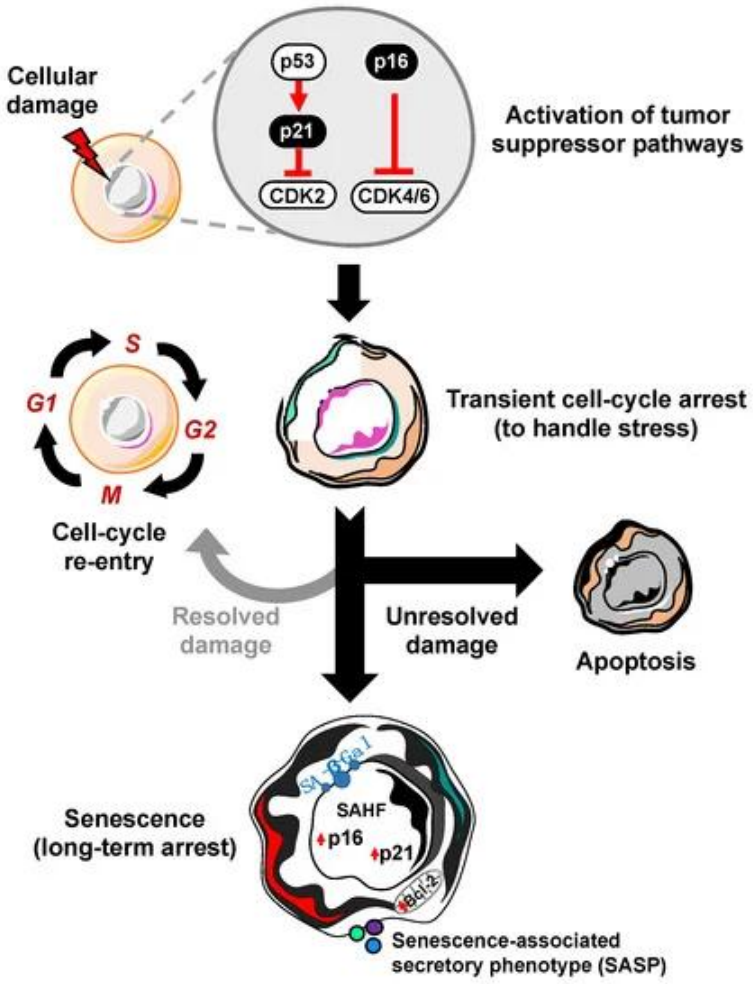

Figure 13. Process of cellular senescence (Prieto, Graves et al. 2020).

3.3.1. Replicative senescence

Telomeres are nucleoprotein structures with long repeated DNA sequence (TTAGGG), which protect chromosome ends. Replicative senescence is defined as the progressive shortening and dysfunction of telomeres due to successive rounds of cell division.

In addition to a gradual loss of telomeric DNA during cell division, telomeres are important targets for oxidative stress *in vitro* and *in vivo* and contribute to a persistent DNA damage response (DDR) that arrests growth permanently (Erusalimsky 2009, Hewitt, Jurk et al. 2012).

Hastings et al showed that after several passages endothelial cells display telomere attrition and express features of senescence such as b-galactosidase activity (Hastings, Qureshi et al. 2004).

3.3.2. Premature senescence

Senescence induced by cell stressors is an accelerated process, telomere independent and occurs without extensive cell proliferation, known as stress induced premature senescence (Kuilman, Michaloglou et al. 2010).

Endothelial senescence can be triggered by different stimuli such as angiotensin II (Hsu, Lin et al. 2018), high glucose (Kim 2019), thrombin (Hasan, Park et al. 2019), H_2O_2 (Xiao, Xu et al. 2019), Ox-LDL (Ming, Tang et al. 2016), cellular microparticles (Burger, Kwart et al. 2012), radiation (Lafargue, Degorre et al. 2017), homocysteine (Xiao-Hong, Chang-Qin et al. 2013) and ceramide (Venable and Yin 2009).

3.4. Endothelial senescence and endothelial dysfunction

Endothelial senescence is a strong inducer of endothelial dysfunction (Carracedo, Ramírez-Carracedo et al. 2018) which is outlined by reduced vasodilation, increased permeability, a pro-inflammatory state and pro-thrombotic properties (Endemann and Schiffrin 2004). Reduced eNOS activity and NO formation, increased oxidative stress, increased expression of adhesion molecules and pro-thrombotic factors directly correlates with endothelial dysfunction during senescence (Krouwer, Hekking et al. 2012). Up-regulated activity of NADPH oxidase (NOX) in senescent endothelial cells shifts eNOS towards a generation of superoxide anions, instead of NO, leading to dysfunction (Childs, Li et al. 2018). Kumar and colleagues have reported that overexpression of p53, cell cycle regulator, is associated with reduce expression of eNOS and thrombomodulin (TM) and stimulate the expression of plasminogen activator inhibitors-1 (PAI-1) and endothelin-1 (ET-1) (Kumar, Kim et al. 2011).

In-vivo administration of statins such as atorvastatin, effectively inhibited the endothelial senescence and improved endothelium-dependent relaxation (Gong, Ma et al. 2014).

3.5. Endothelial senescence and age-related cardiovascular diseases

In rodents and human, cells expressing one or multiple senescence markers have been found in different renewable tissue, including vasculature, rarely in young but more in aging and age-related chronic pathologies (Campisi and Di Fagagna 2007, Childs, Li et al. 2018, Karin, Agrawal et al. 2018). Sa-β-gal positive endothelial cells have been located in carotid and coronary atherosclerotic lesions at various stages of disease progression (Minamino, Miyauchi et al. 2002). Telomere length has been implemented as independent predictor of cardiovascular pathologies as it shortening has been observed in arteries from patients with age-associated coronary disease and endothelial dysfunction (Yeh and Wang 2016). In addition, increased number of senescent cells can impair vascular homeostasis and hence promote vascular ageing and age-related chronic diseases such as atherosclerosis (Minamino, Miyauchi et al. 2002). Reduced level of NO in senescent endothelial cells promotes vascular smooth muscle cells (VSMC) proliferation and production of collagen that leads to vasoconstriction and increasing the risk of angina pectoris and ischemia heart disease (Childs, Li et al. 2018). Increased accumulation of senescent endothelial cells have been reported in arteries of patients with abdominal aortic aneurysm (Cafueri, Parodi et al. 2012) and hypertension, which is a well established risk factor for the development of atherosclerotic disease. Therefore, senescent endothelial cells are key players in vascular ageing with deleterious effects on the cardiovascular system.

Chapter two

Omega-3 PUFAs and vascular health

1. Fatty acids

Fatty acids (FAs) are key components of the human body, having structural, functional and biological roles in cells, tissues and organs. They act as major constituents of cellular membrane and are interconnected with proteins in intra and intercellular pathways. They provide high calories responsible for almost 30% of energy production in humans.

Chemically, FAs are a diverse group of organic compounds, they are mostly carboxylic acids with an aliphatic chain of carbon atoms ranging from 4 to 36 atoms.

FAs are classified into three families based on the absence, presence or abundance of carbon-carbon double bonds, named as the saturated fatty acids (SFAs), the monounsaturated fatty acids (MUFAs) and the polyunsaturated fatty acids (PUFAs), respectively (Sokola-W and Czyz 2018, Eric R. Moellering 2019).

The biological effects of dietary FAs have been reported as beneficial for health. In particular, polyunsaturated fatty acids (PUFAs) are able to reduce cardio-vascular disease risk (Corazzin, Romanzin et al. 2019).

2. Omega-3 polyunsaturated fatty acids (n-3 PUFAs)

Omega-3 PUFAs (n-3 or w-3 PUFAs) are polyunsaturated fatty acids with a first unsaturation at the third position from the methyl terminal of the carbon chain.

Among many types of omega-3 PUFAs, the most significant in human physiology and nutrition are α-linolenic acid (ALA, C18:3 n-3) composed of 18 carbons with 3 double bonds, eicosapentaenoic acid (EPA, C20:5 n-3), 20 carbons with 5 double bonds and docosahexaenoic acid (DHA, C22:6 n-3), 22 carbons with 6 double bond (Sokoła-W, Wysoczański et al. 2018) (Figure 14).

In 1929, the biochemists Evans and Burr discovered the essential fatty acids. They showed that mammals cannot synthesize PUFAs due to lack of enzymes to create unsaturation at the desired carbon atoms. The essential fatty acids such as ALA are of primary importance for normal growth and must be obtained from dietary sources such as plants, seeds and vegetable oils (Kromhout, Yasuda et al. 2012).

The long chain omaga-3 PUFAs including EPA and DHA, are the main ALA metabolites generated via a series of desaturation and chain elongation steps. As the rate of biosynthesis of these highly unsaturated FAs is very low (less than 4%) and insufficient to fulfill the physiological requirements, they are termed “conditionally essential” (EPA, DHA) fatty acids and their dietary consumption is important (Shahidi 2018, Harwood 2019).

Several clinical studies have demonstrated the relevance of omega-3 PUFAs with the risk reduction for morbidity and mortality in various pathologies, particularly cardiovascular diseases (Sokoła-W, Wysoczański et al. 2018).

For example, omega-3 PUFAs modulate several key cellular processes associated with atherosclerosis, including attenuation of chemokine-driven monocytic migration and monocyte adhesion to endothelial cells (Yamada, Yoshida et al. 2008, Moss, Davies et al. 2016), inhibition of modified LDL uptake by macrophages (McLaren, Michael et al. 2011) and suppression of smooth muscle cell migration (Mizutani, Asano et al. 1997). Omega-3 PUFAs ameliorate atherosclerosis in apoE-/- or LDLr-/- mice by reducing plasma cholesterol and monocyte recruitment to aortic lesions (Brown, Zhu et al. 2012). In addition, omega-3 PUFAs attenuated the development of aortic aneurysms via inhibition of macrophage-mediated inflammation (Yoshihara, Shimada et al. 2015).

Furthermore, the n-3 PUFA-derived lipid mediators have served as lead compounds in drug development for the prevention and the treatment of cardiovascular events (Pazderka, Oliver et al. 2020).

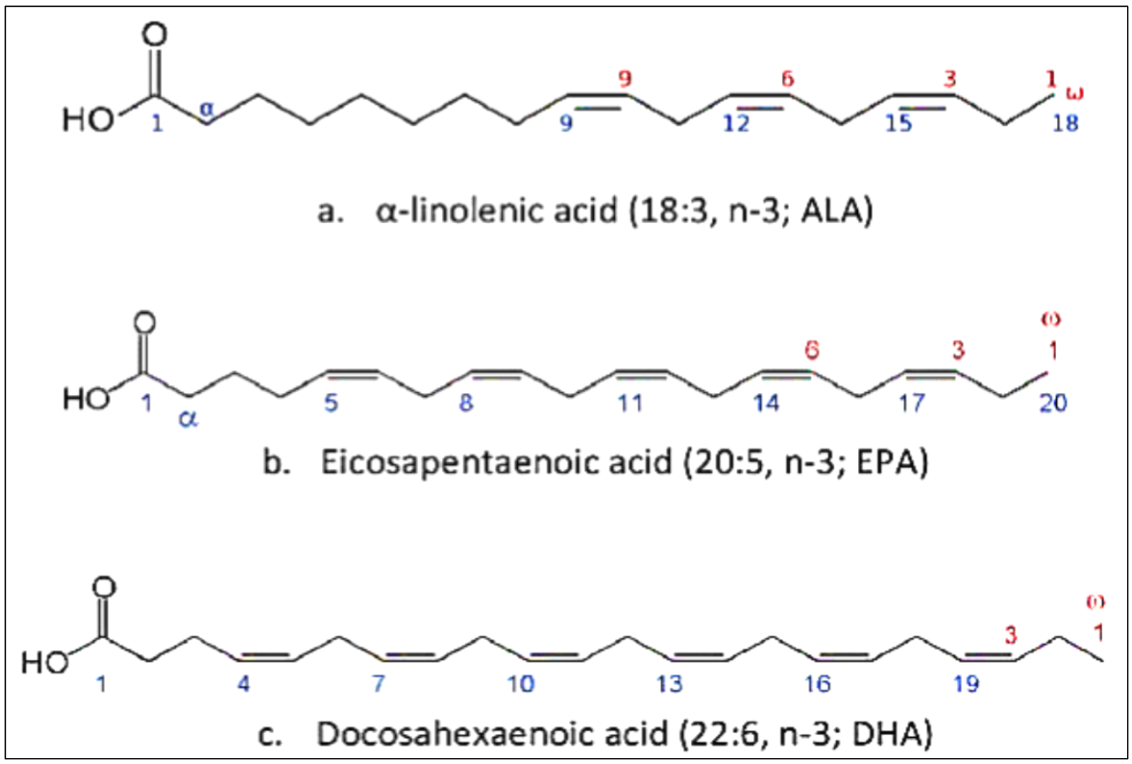

Figure 14. Chemical structures of important dietary omega-3 PUFAs (Khan, et al. 2015).

3. Dietary sources and intake of Omega-3 PUFAs

The ultimate vegetal sources of n-3 PUFAs include nuts such as walnuts, seeds such as chia seed, flax seed, rapeseed, camelina seed, perilla and vegetable oils (linseed, canola, hemp, soybean), which contain ALA as the main n-3 PUFAs (Sokola-W and Czyz 2018).

Regarding animal sources, fish is the richest source of long chain omega-3 PUFAs especially EPA and DHA, known as "marine omega-3". They are available in high concentrations in fatty fish (tuna, sardines, salmon, mackerel and herring) and in small amounts in shellfish, some marine mammals, crustaceans, and cephalopods (Shahidi 2018).

In addition, EPA and DHA are produced *de novo* in algae which are directly consumed by fish and then supplement the demand for n-3 PUFAs, for human nutrition (Harwood 2019).

Omega-3 fortified foods contain EPA and DHA and include dairy products, eggs, breads, pastas, salad dressings, spreads cereals, meats, juices and oils (Bowen, Harris et al. 2016). The regular consumption of omega-3 enriched foods, consumed over eight servings per day, providing between 50 and 150 mg EPA plus DHA per serving, increased the daily omega-3 intake of Australian adults from 200 mg/d to 960 mg/d. Also, it increased the EPA and DHA concentration in erythrocytes by 35 % and 53 % at 3 and 6 months respectively, after supplementation (Murphy, Meyer et al. 2007)

Other studies have reported that increases in plasma, platelet and mononuclear cell phospholipid content of omega-3 PUFAs can be achieved by consumption of omega-3 fortified foods (Mantzioris, Cleland et al. 2000). Additionally, the concentration of EPA and DHA expressed as the percentage of total fatty acid (omega-3 index) increased from 4 to 7 % over 6 months study, this improvement was associated with reduction in CVD risks (Murphy, Meyer et al. 2007).

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 servings of fatty fish 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).

4. Synthesis and metabolism of Omega-3 PUFAs

ALA is the precursor compound to the synthesis of long chain n-3 PUFAs. The bioconversion of ALA to stearidonic acid (SDA) requires a delta-6 desaturase, followed by an elongase of the microsomal system to form eicosatetraenoic acid, then EPA by the delta-5 desaturase. EPA as an active metabolic product, can be converted into DHA through delta-5 elongation, delta-4 desaturation and β-oxidation in the peroxisomes for chain shortening (Zarate, El Jaber-V et al. 2017) (Figure 15).

Furthermore, DHA is converted into specialized pro-resolving lipid mediators (SPMs), neuroprotectins and maresins, with anti-inflammatory and organ protective properties.

This process is limited within the body and active mostly in the liver and to a lesser extent in the cerebrovascular lumen, and astroglial cells.

The activity of enzymes involved in the metabolism of essential fatty acids (EFAs) is reduced by numerous factors including smoking, alcohol, stress (adrenaline), low insulin and deficiencies of minerals such as zinc, magnesium, pyridoxine, copper (Sokola-W and Czyz 2018).

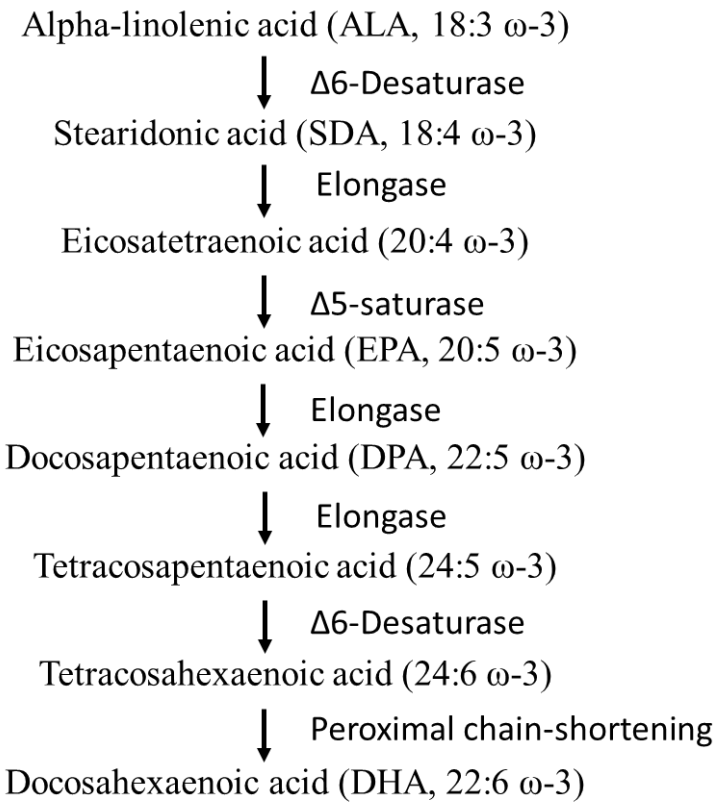

Figure 15. Metabolic pathway for the synthesis of omega-3 polyunsaturated fatty acids from α-linolenic acid (Shahidi 2018).

5. Bioavailability of Omega-3 PUFAs

Omega-3 PUFAs exist principally in various forms such as ethyl ester (EE), free fatty acid (FFAs), triacylglycerols (TAGs) or phospholipids (PLs) (Shahidi 2018).

Dietary omega-3 PUFAs are digested partially in stomach by gastric lipases to form diacylglycerol (DAG) and fatty acids (FAs) and a large emulsions of fat globules, followed by the action of bile salt and pancreatic lipases in the intestinal lumen, to release fatty acids (FAs) and monoacylglycerols (MAGs) which are absorbed by passive diffusion into enterocytes (Shi and Burn 2004).

However, omega-3 supplements containing ethyl ester forms of EPA and DHA are hydrolyzed by pancreatic carboxylic acid ester lipase to form FFAs for direct absorption (Shahidi 2018) (Figure 16).

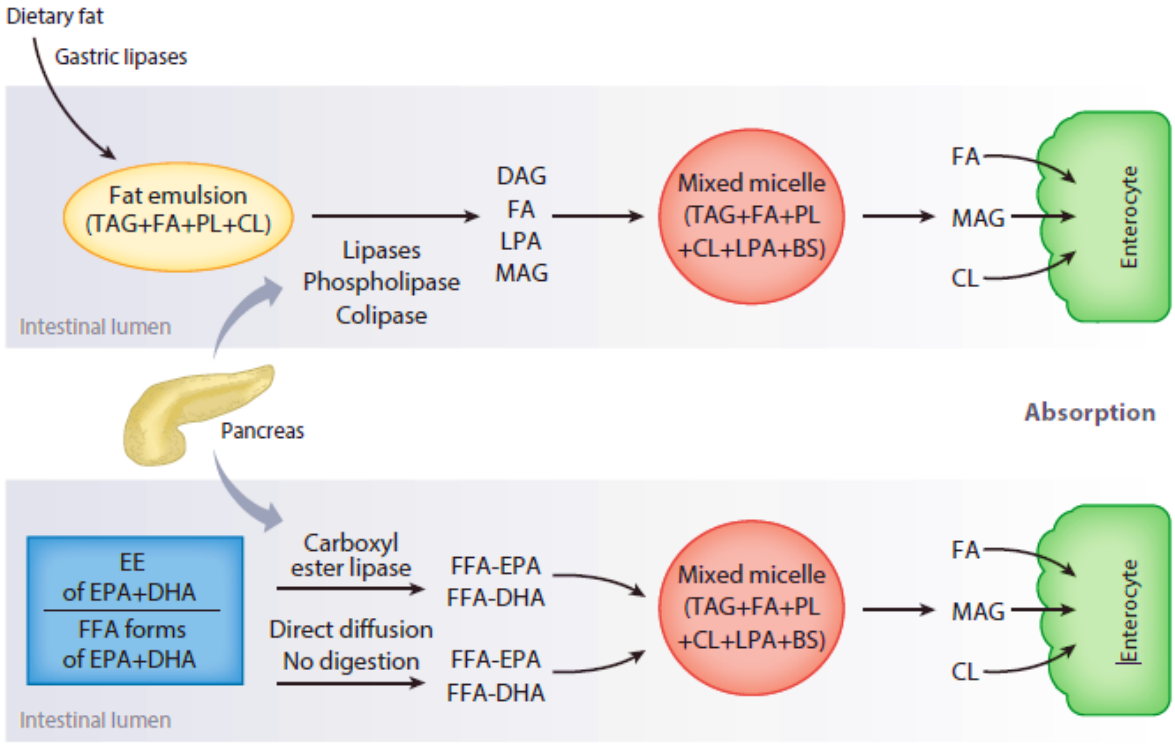

Figure 16. A schematic representation of dietary fat digestion and absorption of ethyl ester (EE) and free fatty acid (FFA) forms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Shahidi 2018).
BS, bile salt; CL, cholesterol; DAG, diacylglycerol; FA, fatty acid; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PL, phospholipid; TAG, triacylglycerol.

Absorption of n-3 PUFAs is affected by various factors, for example, the position at which they are attached to the triacylglycerol (TAG) backbone. Omega-3 PUFAs of fish oil are attached to the sn-2 position and directly absorbed as monoacylglycerols (MAGs) by passive

diffusion. Thus, they have a greater bioavailability than marine mammal oils, where long chain n-3 PUFAs are distributed in sn-1 and sn-3 positions and diffused actively via a protein mediator (Laidlaw, Cockerline et al. 2014).

In addition, the presence of dietary fats promotes the absorption of both EE and FFAs forms of n-3 PUFAs (Dyerberg, Madsen et al. 2010, Davidson, Johnson et al. 2012) by enhancing the activity of pancreatic enzymes (Schuchardt, Schneider et al. 2011).

Dietary omega-3 PUFAs containing the FFAs form of n-3 PUFAs have relatively higher bioavailability as compared to EE forms (Schuchardt, Schneider et al. 2011).

6. Mechanisms of Omega-3 PUFAs functions

Omega-3 PUFAs are characterized by the ability to incorporate into the cell membranes and exert their physiological functions by different pathways involving structural and functional alteration of cell membrane, modulation of ion channels, regulation of gene expression and generation of bioactive messengers (Figure 17).

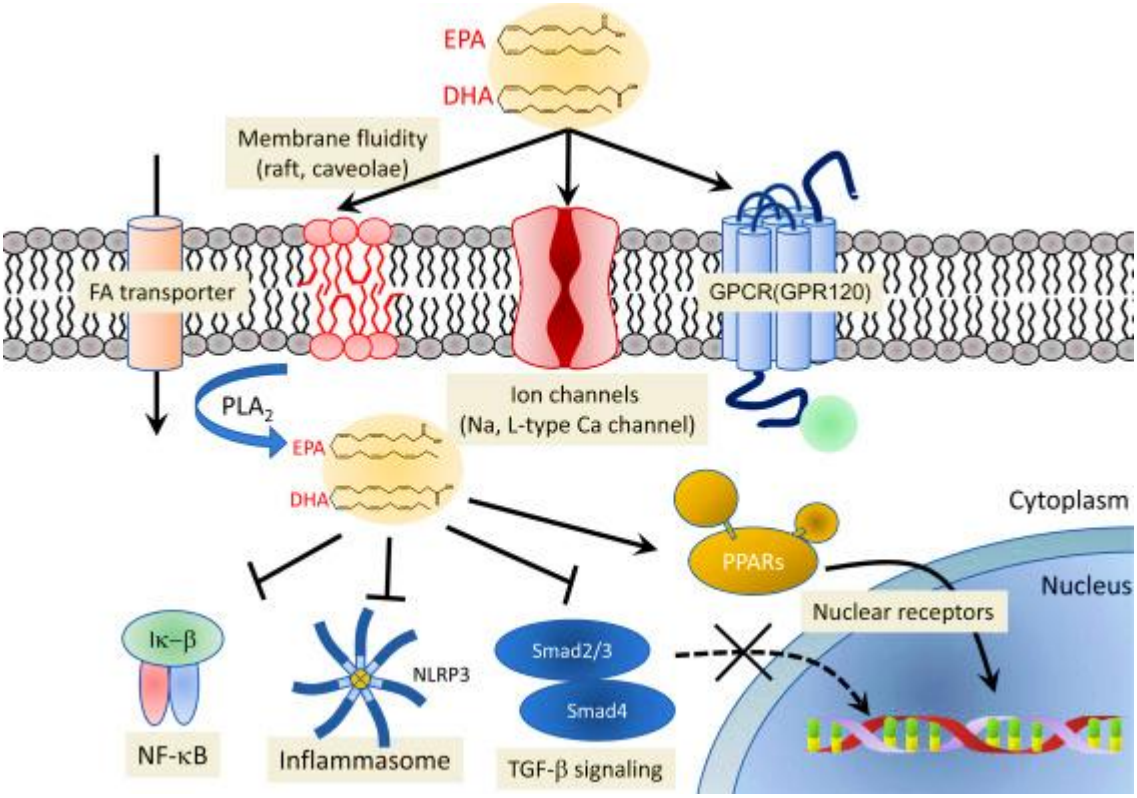

Figure 17. The proposed molecular mechanism of cardioprotection by omega-3 PUFAs (Endo and Arita 2016).

NF-κB: nuclear factor-κB; NLRP3: NOD-like receptor family, pyrin domain containing 3; PPARα/γ: peroxisome proliferator-activated receptor α/γ; GPR120: G protein-coupled receptor 120; TGF-β: transforming growth factor-β.

6.1. Structural and functional alteration of cell membrane

The cell membrane is a double layer of lipids, composed mainly of phospholipids which sustain the structural integrity of the cell along with proteins.

As part of the membrane phospholipids, FAs are important for mammalian cells, they assure the fluidity, flexibility, permeability of the membrane as well as the control of molecular transport and are an important class of intra and extracellular signaling molecules (Nagy and Tiuca 2017).

Omega-3 PUFAs can incorporate into the membrane phospholipids and alter membrane-cytoskeletal structure and function (Gerling, Mukai et al. 2019).

In addition, omega-3 PUFAs play a major role in altering the size and/or stability of lipid microdomains such as lipid rafts and caveolae in the plasma membrane and modify its lateral organization (Shaikh, Kinnun et al. 2015).

Furthermore, n-3 PUFAs perturb the recruitment and the activation of the signaling proteins necessary for T cell activation, such as the Src family kinases, PKC, LAT, Fas, PLC-γ1, and F-actin. Also, they suppress CD4+ T cell activation and differentiation by inhibiting mitochondrial translocation, IL-2 secretion and lymphoproliferation (Hou, McMurray et al. 2016).

6.2. Modulation of ion channels

Membrane-incorporated omega-3 PUFAs can modulate the cardiac ion channels involved in the genesis and/or maintenance of cardiac action potentials (APs).

Cardiomyocyte electrophysiology is directly modulated by omega-3 PUFAs altering function of the Na+ channel, L-type Ca+2 channel and Na+/Ca+2 exchanger. Such omega-3 PUFAs-mediated effects can influence the membrane depolarization and reduce the excitability of cardiac myocytes, cytosolic calcium levels, thereby limiting arrhythmia (Ferrier, Redondo et al. 2002, Xiao, Ma et al. 2006, Tribulova, Szeiffova Bacova et al. 2017)

In adult rat cardiomyocytes, EPA, DHA, and ALA inhibited the sodium current (Moreno, Macias et al. 2012).

Being the most potent n-3 PUFAs, the DHA acts to increase the activity of the Na+-K+-ATPase pump in the same membrane of heart and kidney tissues (Turner, Else et al. 2003).

Moreover, n-3 PUFAs supplementation is responsible for the antiarrhythmic properties reported in clinical trials focused on atrial fibrillation suggesting omega n-3 PUFAs to be used as an alternative therapy (Endo and Arita 2016).

6.3. Regulation of nuclear receptors and transcription factors

Omega-3 PUFAs are key mediators of lipid homeostasis as they bind to nuclear receptors triggering the transcription of genes encoding for metabolic and cellular processes (Echeverria, Ortiz et al. 2016).

Omega-3 PUFAs incorporate into nucleus and interact with nuclear receptors via the cytoplasmic fatty acid transporters (fatty acid binding protein) (Esteves, Knoll-Gellida et al. 2016). They are direct ligands of peroxisome proliferator-activated receptors (PPAR), hepatic nuclear factors (HNF), retinoid X receptor (RXR) and liver X receptors (LXR) in different organs (Papackova and Cahova 2015).

In particular, the activation of PPAR β/δ receptor stimulates FA β-oxidation and decreases circulating levels of free FAs and triglycerides (Echeverria, Ortiz et al. 2016).

EPA and DHA interact with G-protein coupled receptors (GPR43, GPR120) and prevent NF-kB signaling via the nuclear receptors PPAR-α/γ or by impeding I-κB phosphorylation, leading to a lower circulating concentration of inflammatory cytokines such as IL-1β, IL-6 and TNF-α.

In addition, EPA and DHA inhibit TGF-β1-induced smad2/3 nuclear translocation resulting in the reduction of cardiac remodeling. Also, they inhibit NLRP3 and prevent the formation of inflammasome involved in inflammatory response in myocardial infarction, ischemia reperfusion injury, and pressure overload-induced cardiac remodeling (Endo and Arita 2016).

6.4. Bioactive omega-3 derived eicosanoids

EPA and DHA, released into the cytoplasm by the hydrolytic action of phospholipase A2 (PLA2), are metabolized by COX, LOX and Cyp450 to generate eicosanoids messengers such as 3-series prostaglandins (PGI_3) and thromboxanes (TXs) and 5-series leukotrienes (LTs) (Endo and Arita 2016).

Furthermore, EPA and DHA are converted via COX and LOX pathways into specialized pro-resolving mediators (SPM) including resolvins (Rv), protectins (P) and maresins (MaR) (Calder 2017) (Figure 18).

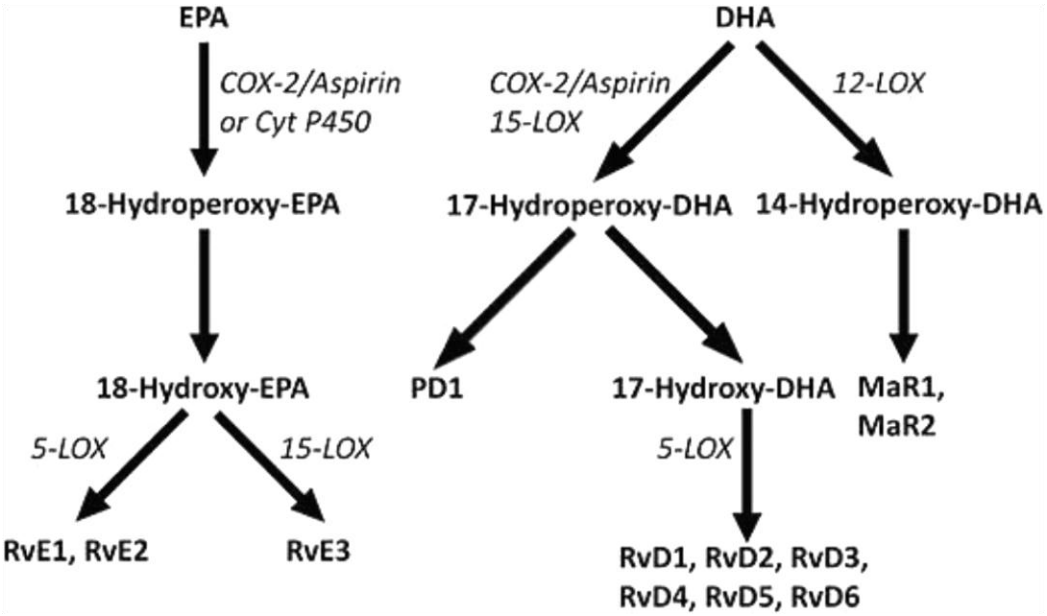

Figure 18. Pathways of synthesis of specialized pro-resolving mediators from EPA and DHA (Calder 2017).
COX, cyclooxygenase; Cyt P450, cytochrome P450; LOX, lipoxygenase; MaR, maresin; PD, protectin D; Rv, resolvin.

EPA-derived mediators including 18-hydroxy-eicosapentaenoic acid (18-HEPE), which confers cardioprotective effects in pressure overload-induced maladaptive remodeling and heart failure, 17,18-EpETE and E-series resolvins are involved in the resolution of inflammation via the inhibition of leukocyte trafficking, the reduction of inflammatory cytokines and the stimulation of macrophage phagocytosis and clearance of inflammatory cells (Ishihara, Yoshida et al. 2019) (Figure 19).

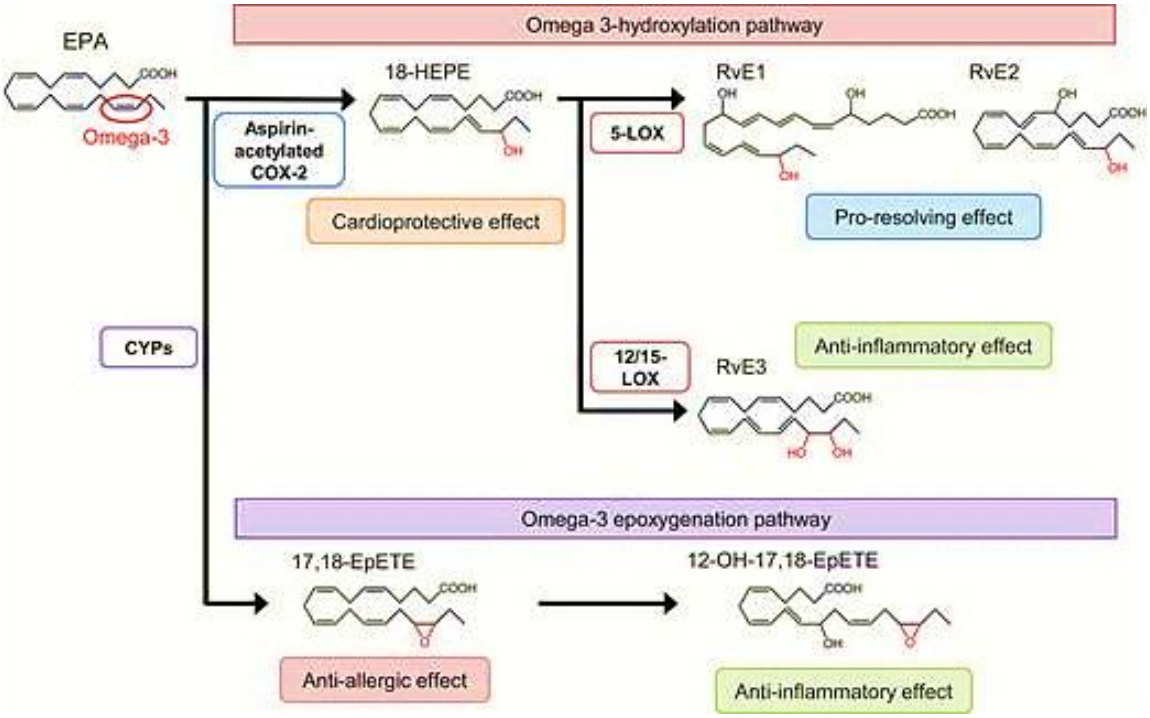

Figure 19. Metabolism of EPA and functions of derived metabolites (Ishihara, Yoshida et al. 2019).

Similarly, the DHA-derived D-series resolvins (RvD), protectins (PD) and maresins promote anti-inflammatory reactions and were reported to play a crucial role in atherosclerosis, oxidative injury, renal ischemic reperfusion injury and brain ischemia (Serhan 2014).

7. Omega-3 PUFAs and cardio vascular diseases

In both experimental and clinical studies, dietary omega-3 PUFAs intake has been shown to play an important role in reduced risk of coronary death and total mortality. Omega-3 PUFAs have beneficial cardiovascular effects through various actions, including triglyceride-lowering, lipid metabolism, platelet aggregation inhibition, anti-inflammatory effect, anti-hypertensive action, improved vascular endothelium function and cardiac hemodynamics (Nishizaki, Shimada et al. 2017). The American Heart Association (AHA) recommended consumption of two servings of fish per week to reduce the risk of coronary heart disease (CHD) and 1 g of EPA and DHA per day in patients with documented CHD (Ajith and Jayakumar 2019).

7.1. Hypertriglyceridemia

Hypertriglyceridemia is defined as high blood level of triglycerides TG and is independently associated with an increased risk of cardiovascular disease (CVD) (Backes, Anzalone et al. 2016). With high level of TG, there are elevations of TG-rich lipoproteins (TRL) (very-low-density lipoproteins [VLDL] plus chylomicrons) that up-regulate tumor necrosis factor-alpha, thereby inducing vascular cell adhesion molecule (VCAM)-1 expression in human aortic endothelial cells and monocyte adhesion and an increased permeability, which may contribute to atherosclerosis through the activation of pro-inflammatory, pro-coagulant, and pro-apoptotic pathways (Rosenson, Davidson et al. 2014).

Clinical trials have demonstrated the potential of omega-3 FAs to reduce the serum levels of TG and to provide efficacy and complementary benefits when administered as a combination therapy with statins in high-risk patients with hypertriglyceridemia (Backes, Anzalone et al. 2016).

Three studies of dietary omega-3 PUFAs evaluated lower doses (2.4–2.7 g/d EPA+DHA) in association with statin and reported 22% to 31% reductions in triglycerides (Vecka, Dušejovská et al. 2012, Maki, Yurko-Mauro et al. 2014, Hedengran, Szecsi et al. 2015).

Kinetic studies have demonstrated that n-3 FAs reduce hepatic secretion of triglyceride-rich lipoproteins. Ingestion of 3.4 g/d omega-3 PUFAs for one month induced a triglyceride lowering of 25-50% primarily through limited production of VLDL-TG in liver and secondarily increased clearance of VLDL (Shearer, Savinova et al. 2012).

Ingestion of pure fish oil or 15% of fish oil for four months reduced plasma levels of TG in mice by 40%-60% and was attributed to reduced endogenous synthesis of TG and through enhanced clearance of TG-rich particles from blood (Qi, Fan et al. 2008).

Moreover, omega-3 PUFAs-mediated decreased serum TG levels may be related to a reduction in intrahepatic fatty acid pools, a competitive blockade of hepatic enzymes (such as diacylgylcerol acyltranferase or phosphatidic acid phosphohydrolase) involved in the synthesis of TG, an enhanced fatty acid β-oxidation, and upregulation of lipoprotein lipase leading to an increased peripheral triglyceride clearance (Harris and Bulchandani 2006, Shearer, Savinova et al. 2012, Mori 2014) (Figure 20).

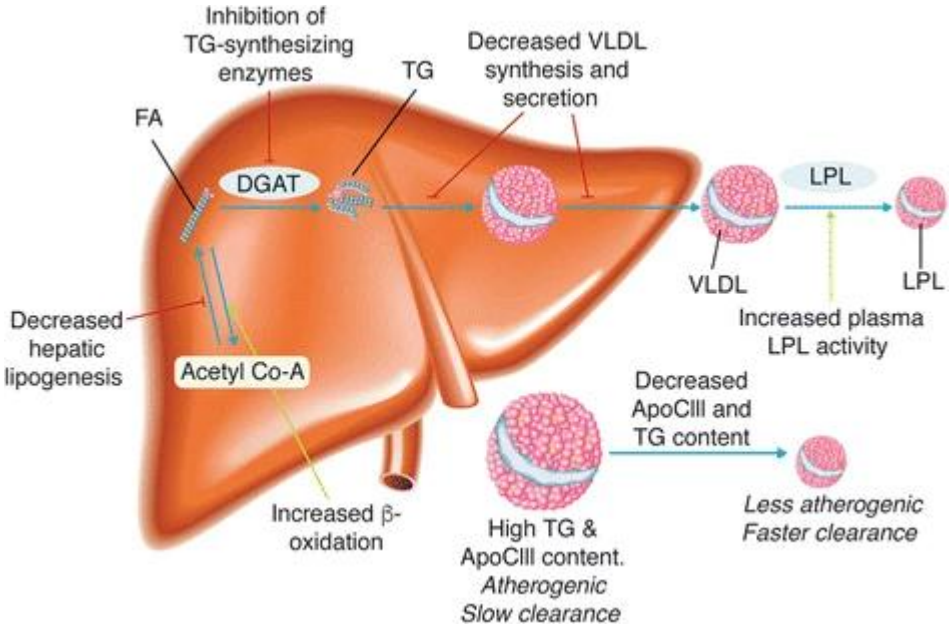

Figure 20. Proposed mechanisms of action of prescription formulations of long-chain omega-3 fatty acids (Backes, Anzalone et al. 2016).
ApoCIII apolipoprotein CIII, Acetyl Co-A acetyl coenzyme A, DGAT diglyceride acyltransferase; FA fatty acid, LPL lipoprotein lipase, TG triglyceride, VLDL very-low-density lipoprotein.

7.2. Inflammation

Omega-3 PUFAs acids have the potential to attenuate the inflammation targeting multiple mediators by reducing proinflammatory stimuli and by stimulating the resolution of inflammation (Figure 21).

Omega-3 PUFAs have an effect on the signaling pathways that control gene expression in inflammatory cells. EPA and DHA can blunt NF-kB signaling via the activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ) leading to a decreased production of inflammatory cytokines, chemokines and adhesion molecules (Calder 2017).

Omega-3 PUFAs displace arachidonic acid (AA) from the plasma membrane of endothelial cells, platelets and inflammatory cells, reducing the generation of pro-inflammatory metabolites including prostaglandin E2 (PGE_2), thromboxane B2, 4-series leukotriene (LTB_4) and providing alternative and less potent eicosanoids such as 3-series prostaglandin (PGE_3) and 5-series leukotriene (LTB_5) (Saini and Keum 2018, Ishihara, Yoshida et al. 2019).

In addition, EPA may compete with arachidonic acid (AA) as a substrate for the 5-lipoxygenase (5-LOX) enzyme to favor the formation of LTB_5, which is less biologically active at the BLT1 receptor compared with LTB4-associated chemoattraction of leukocytes limiting macrophage infiltration and production of classic pro-inflammatory cytokines.

Omega-3 PUFAs are effective actors in alleviating vascular inflammation by the production of specialized pro-resolving mediators (SPMs) which may have contributed to the beneficial effects of icosapent ethyl in REDUCE-IT (Bhatt, Steg et al. 2019).

The EPA-derived resolvins (RvE1) induce the resolution of inflammation through the receptor ERV1/ChemR23 and promote anti-inflammatory effects by inhibiting BLT1 receptor signaling leading to a reduced atherosclerosis and cardiovascular risk (Back and Hansson 2019).

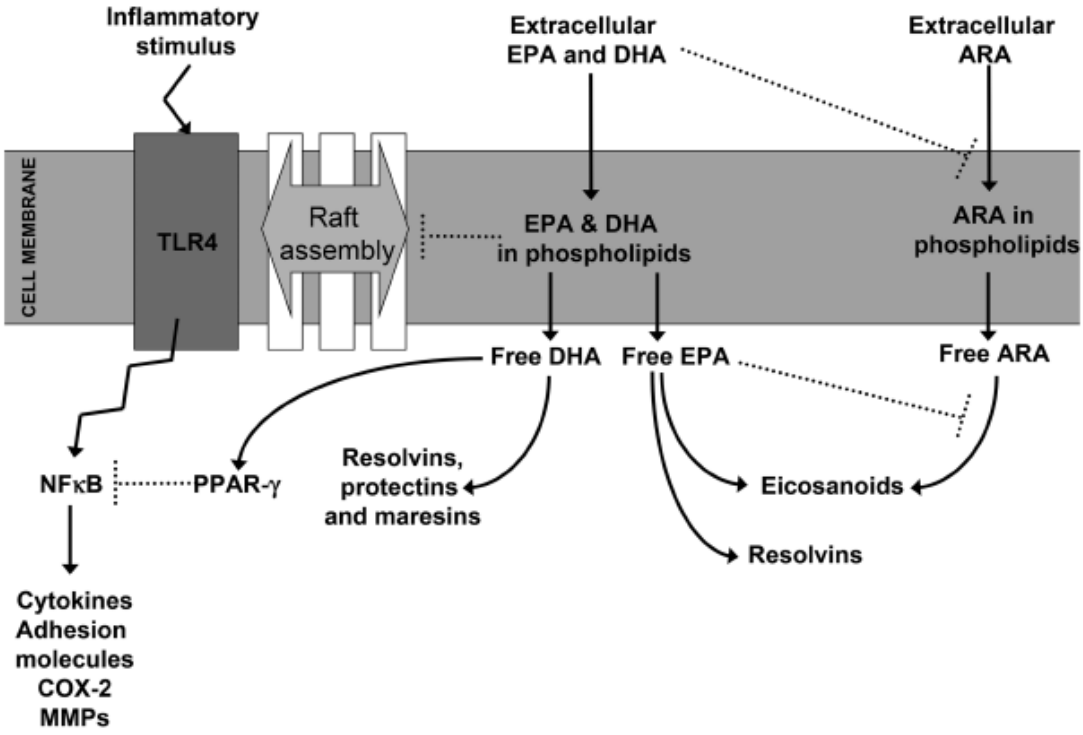

Figure 21. Depiction of some anti-inflammatory pathways triggered by EPA and DHA (Calder 2017).
ARA, arachidonic acid; COX, cyclooxygenase; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MMP, matrix metalloproteinase; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator-activated receptor. Dotted lines indicate inhibition

7.3. Endothelial dysfunction

A large number of epidemiological studies and clinical investigations revealed the protective effect of n-3 FAs on vascular endothelium and their role in the prevention of CVD, like myocardial infarction and stroke (Ander, Dupasquier et al. 2003).

Dietary intake of long chain omega-3 PUFAs prevented oxidative stress and inflammation associated with endothelial cell damage and dysfunction (Mori 2014). EPA and DHA increased eNOS activity and induced nitric oxide generation (Zgheel, Alhosin et al. 2014, Yamagata 2017)

Omega-3 PUFAs ameliorated endothelium dysfunction in chronic renal failure by increasing NO bioavailability, reducing the oxidative stress and pro-inflammatory cytokines production (Zanetti, Gortan Cappellari et al. 2017).

Daily intake of omega-3 PUFAs (2g) for two months improved endothelial function in subjects with stable ischemic heart failure (Oikonomou, Vogiatzi et al. 2019).

(Yamagata 2017) reported that DHA improves endothelial dysfunction by enhancing the activity and expression of eNOS and down-regulation of intracellular adhesion molecules-1 (ICAM-1) in cultured human endothelial cells *via* Akt/ERK/NF-kB signaling pathways.

(Mason, Dawoud et al. 2018) reported that treatment of HUVECs with EPA demonstrated a beneficial effect on endothelial function by increasing the endothelial NO/ONOO− release ratio and inhibiting oxidative stress mediated- eNOS uncoupling

In our lab, (Farooq, Gaertner et al. 2020) demonstrated that Intake of omega-3 formulation EPA:DHA 6:1 by old rats for 2 weeks attenuated endothelium dysfunction in the mesenteric artery via enhancing the production of endothelial NO and reducing oxidative stress.

Another colleague, (Gaertner, Auger et al. 2020) showed that EPA:DHA 6:1 improved age-related endothelial dysfunction in the femoral artery and vein of middle-aged rats.

7.4. Thrombosis

Omega-3 PUFAs are incorporated into platelet phospholipids and can regulate the platelet aggregation, activation and adhesion and clot formation contributing to a decrease in clinical atherothrombosis (Mori 2014).

Omega-3 PUFAs compete with arachidonic acid in platelet membrane and modify prostanoids profile of platelets generating lower ratio of proaggregatory thromboxanes A2 (TXA2) versus thromboxane A3 (TXA3) the analogous but substantially less biologically active EPA-derived metabolite (Knapp, Reilly et al. 1986, Mori 2014).

In vivo studies reported the capacity of n-3 PUFAs to inhibit COX-1 and COX-2 in platelets by the action of DHA-derived protectin DX, decreasing platelet activation and aggregation (Calvo, Martínez et al. 2017).

In patients with coronary artery disease undergoing percutaneous coronary intervention, omega-3 PUFAs decreased platelet activation and thrombin generation, improved fibrin clot properties (Gajos, Zalewski et al. 2011) and showed better response to antiplatelet agents in a dual therapy with aspirin and clopidogrel (Gajos, Rostoff et al. 2010).

7.5. Hypertension

Hypertension is considered as an independent, substantial risk factor for CVD and the leading cause of disability worldwide (Lamprea-M, Zelnick et al. 2018).

Several studies have shown that intake of long chain omega-3 PUFAs (EPA and DHA) can lead to clinically relevant blood pressure (BP) reductions by multiple mechanisms such as the increased production of endothelial NO, reduction of angiotensin-converting enzyme (ACE)

activity and angiotensin II formation, activation of the parasympathetic nervous system and inhibition of cyclooxygenase activity, suppressing the synthesis of proinflammatory cytokines and vasoconstrictor factors (Cicero, Ertek et al. 2009).

In addition, omega-3 PUFAs can lead to the BP lowering through the activation of large-conductance Ca+2-dependent K+ channels (BK channel) promoting vasodilatation (Hoshi, Wissuwa et al. 2013).

A meta-analysis including 36 studies with a median consumption of 3.7 g/day of fish oil over 2 weeks, showed a significant 2.1 mmHg reduction in systolic blood pressure (SBP) and 1.6 mmHg in diastolic blood pressure (DBP) in subjects >45 years and in hypertensive volunteers (Geleijnse, Giltay et al. 2002).

Another meta-analysis of 70 randomized clinical trials (RCTs) indicated that provision of EPA+DHA reduces SBP by 4.51mm Hg and DBP by 3.05mm Hg in non-treated hypertensive patients and a reduction of 1.25mm Hg in SBP and 0.46mm Hg in DBP in normotensive subjects (Miller, Van Elswyk et al. 2014).

8. Omega-3 PUFAs and clinical trials

The interest in cardioprotective effects of omega-3 PUFAs was raised many years ago due to the discovery of their potential in reducing the incidence of myocardial infarction and cardiovascular mortality among Greenland Inuits (Bang, Dyerberg et al. 1976).

Several clinical trials were conducted on omega-3 PUFAs following different criteria such as, health status, age, sex and number of participants, EPA and DHA dose, length of the follow-up, duration of supplementation and concurrent standard of care for cardiovascular disease (Gajos 2019).

8.1. Diet and Reinfarction Trial (DART)

The DART "Diet and Reinfarction Trial" was the first randomized controlled trial, published in 1989, to examine the effects of dietary omega-3 PUFAs in the secondary prevention of myocardial infarction (MI).

2033 men who had recovered from MI were randomly divided to 4 groups instructed to either i) reduce the fat intake and increase polyunsaturated to saturated fatty acid ratio, ii) increase in fatty fish intake, iii) increase in cereal fibre intake, or iv) control group with no specific instructions.

Patients allocated to receive fatty fish meal (200-400 g/week) or fish oil capsule (Maxepa 3 g/day) had experienced 29% reduction in 2 year all-cause mortality and 32% reduction in the incidence of reinfarction, as compared with control group (Burr 1989, Jain, Aggarwal et al. 2015, Bowen, Harris et al. 2016).

8.2. Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto micardico Prevenzione (GISSI-Prevenzione)

GISSI-Prevenzione trial "Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto micardico Prevenzione" was published in 1999, to assess the cardio-protective effects of omega-3 PUFAs in 11,323 patients (including 14.7% female) surviving a recent myocardial infarction (<3 months). The patients were randomly divided into 4 groups allocated to receive either i) omega-3 PUFAs (1 g/d), ii) Vitamin E (300 mg/d), iii) a combination of omega-3 plus vitamin E, or iv) control group with no treatment, with a follow-up duration of 3.5 years.

Patients treated with a lower dose of omega-3 PUFAs (1 g/d), had reported 41% reduction in total mortality at 3 months and 53% reduction in risk of sudden death within 4 months of starting therapy. The reductions in the risk of coronary, cardiovascular and sudden cardiac death remained significant at the end of the study (Investigators 1999, Marchioli, Barzi et al. 2002, Bowen, Harris et al. 2016).

8.3. GISSI-Heart Failure (GISSI-HF)

GISSI-HF "Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto micardico heart failure" was published in 2004, was the first double blind, randomized and placebo-controlled trial.

In total, 6975 patients (both men and women) with chronic heart failure (HF) were randomly assigned to receive either omega-3 PUFAs (1 g/d) or placebo, with a follow-up duration of 3.9 years.

The study reported that the long-term administration of 1 g/day omega-3 PUFAs was effective in reducing both all-cause mortality (9%) and admissions to hospital for cardiovascular reasons (8%) in patients with heart failure (Tavazzi, Maggioni et al. 2008, Bowen, Harris et al. 2016).

8.4. Japan EPA Lipid Intervention Study (JELIS)

JELIS "Japan EPA Lipid Intervention Study" was published in 2007, to evaluate the effects of statin plus EPA (1.8 g/day) versus statin alone in 18645 hyperlipidemic patients (total cholesterol > 6.5 mmol/L).

All patients were followed and monitored for 3.5 years for any major coronary event such as sudden cardiac death, myocardial infarction (fatal and non-fatal), and other nonfatal events such as unstable angina pectoris, angioplasty, stenting or coronary artery bypass grafting.

In the secondary prevention subgroup (patients with recent history of coronary artery disease), the dual therapy (EPA + statin) demonstrated a significant 19% reduction in major coronary events and a significant 28% reduction in the incidence of unstable angina, as compared with statin alone, whereas, the secondary prevention subgroup (patients without history of coronary artery disease) showed 18% non-significant reduction in coronary events (Yokoyama, Origasa et al. 2007, Bowen, Harris et al. 2016).

8.5. The Reduction of Cardiovascular Events with EPA-Intervention Trial (REDUCE-IT)

REDUCE-IT is a recent randomized, double blind, placebo-controlled trail, published in 2019, to evaluate the effects of icosapent ethyl (EPA ethyl ester) in reducing cardiovascular events.

In total, 8179 high risk patients (28.8% were female, median age= 64 years) with hypertriglyceridemia (TG level of 135-499 mg/dL and LDL-cholesterol levels of 41-100 mg/dL) and were already on statin therapy. Patients were randomly assigned to either icosapent ethyl EPA (4 g/d) or placebo that contains mineral oil to mimic the color and consistency of icosapent ethyl and were followed for 4.9 years.

The clinical end points of the study were cardiovascular death, nonfatal myocardial infarction, nonfatal stroke, coronary revascularization, or unstable angina.

Compared to the placebo, the combined therapy (EPA + statin) was associated with a 19% reduction in triglycerides and 25% reduction in major ischemic events, including cardiovascular death (Bhatt, Steg et al. 2019, Skulas-Ray, Wilson et al. 2019).

Chapter three

Nanomedical theranostics

1. Overview of nanoparticles

1.1. Nanoparticles: concepts and types

Nanoparticles (NPs) are defined as particulate dispersions or solid particles with a size in the range of 10-1000 nm (Mohanraj and Chen 2006).

NPs are composed of three layers i.e. i) The surface layer, which may be functionalized with a variety of small molecules, metal ions, surfactants and polymers. ii) The shell layer, which is chemically different material from the core in all aspects, and iii) The core, which is essentially the central portion of the NP (Khan, Saeed et al. 2019).

NPs occur in a great variety of shapes and are classified into different categories based on their physical and chemical characteristics. There are various types of pharmaceutical nanosystems divided into nanostructured polymer such as, organic based nanoparticles, dendrimer, micelles, drug conjugates and non polymer such as, carbon nanotubes, metallic NPs, silica NPs, ceramics NPs and quantum dots (Bhatia 2016) (Figure 22).

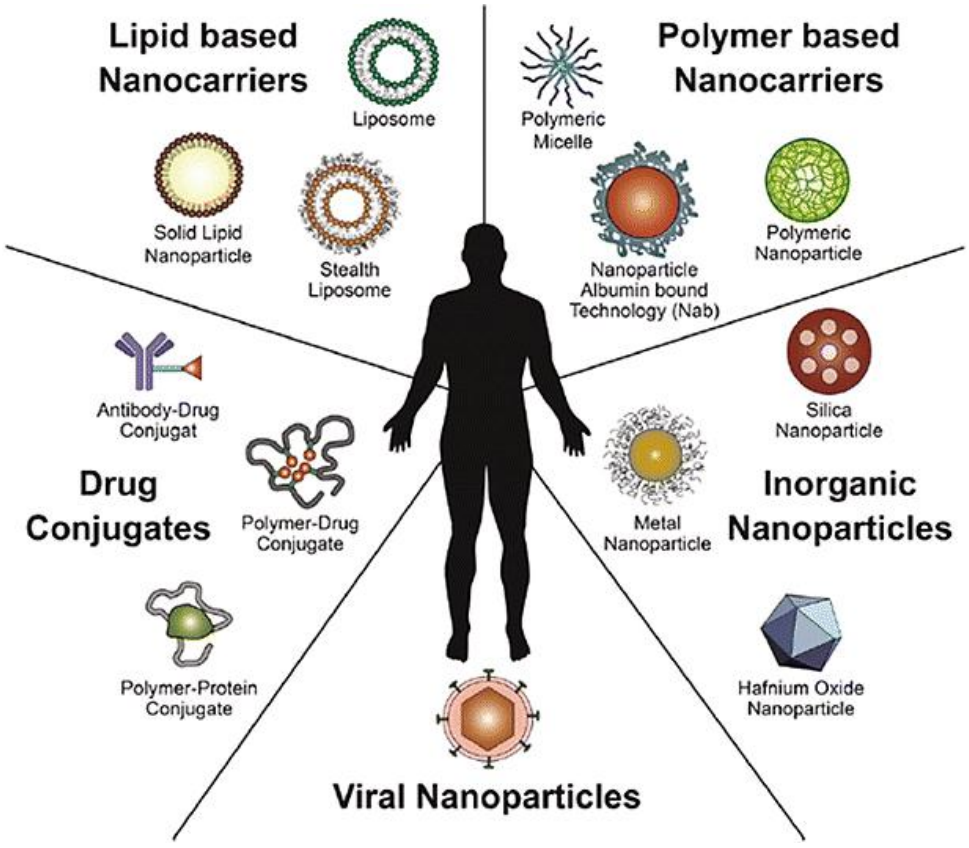

Figure 22. Schematic illustration of established nanotherapeutic platforms (Wicki, Witzigmann et al. 2015).

1.2 Nanoparticles properties

NPs have unique physicochemical characteristics that can be used to overcome the limitations found in traditional diagnostic agents and conventional therapies (Zhang, Gu et al. 2008) (Table 2).

The unique properties of NPs, such as ultra-small size, large surface area to volume ratio, high reactivity, and the ability to encapsulate various drugs, give them many advantages over their bulk counterparts. This includes tunable surface chemistry, efficient navigation of the complex in vivo environment, increased intracellular trafficking, and sustained circulation lifetimes and release of drug payload (Malam 2009, Xu 2015).

Moreover, NPs based specific drug targeting and delivery systems increase the stability of drug and formulation (Brewer, Coleman et al. 2011), enhance oral bioavailability (Goldberg, Vijayalakshmi et al. 2011), reduce the dose needed (Dutta and Jain 2007), reduce toxicity and other side effects and also improve the therapeutic index of the targeted drug (Bhatia 2016). Also, NPs help to increase the stability of drugs and possess convenient controlled drug release properties (Khan, Saeed et al. 2019).

Recently, biodegradable polymeric nanoparticles (PNPs), particularly those coated with hydrophilic polymer such as polyethylene glycol (PEG) known as long-circulating particles, have been used as potential drug delivery platforms because of their ability to circulate for a prolonged period of time and to deliver a higher concentration of pharmaceutical agent to a desired location and offer a significant improvement over traditional oral and intravenous methods of administration in terms of efficiency and effectiveness. These advantages have made (PNPs) desirable candidates for cancer therapy, delivery of targeted antibiotics, contraceptives and delivery of proteins, peptides and genes (Nagavarma, Yadav et al. 2012).

Table 2. Various characteristics and brief applications of nanosystems (Nahar, Dutta et al. 2006).

Types of Nanosystems	Size (nm)	Characteristics	Applications
Carbon nanotubes	0.5–3 diameter and 20–1000 length	Third allotropic crystalline form of carbon sheets either single layer (single walled nanotube, SWNT) or multiple layer (multi-walled nanotube, MWNT). These crystals have remarkable strength and unique electrical properties (conducting, semi conducting, or insulating)	Functionalization enhanced solubility, penetration to cell cytoplasm and to nucleus, as carrier for gene delivery, peptide delivery
Dendrimer	<10	Highly branched, nearly monodisperse polymer system produced by controlled polymerization; three main parts core, branch and surface	Long circulatory, controlled delivery of bioactives, targeted delivery of bioactives to macrophages, liver targeting
Liposome	50–100	Phospholipid vesicles, biocompatible, versatile, good entrapment efficiency	Long circulatory, offer passive and active delivery of gene, protein, peptide and various other
Metallic nanoparticles	<100	Gold and silver colloids, very small size resulting in high surface area available for functionalization, stable	Drug and gene delivery, highly sensitive diagnostic assays, thermal ablation and radiotherapy enhancement
Nanocrystals Quantum dots	2–9.5	Semi conducting material synthesized with II-VI and III-V column element; Size between 10 and 100 Å; Bright fluorescence, narrow emission, Broad UV excitation and high photo stability	Long term multiple color imaging of liver cell; DNA hybridization, immunoassay; receptor mediated endocytosis; labeling ofbreast cancer marker HeR 2 surface of cancer cells
Polymeric micelles	10–100 nm	Block amphiphilic copolymer micelles, high drug entrapment, payload, biostability	Long circulatory, target specifi c active and passive drug delivery, diagnostic value
Polymeric nanoparticles	10–1000	Biodegradable, biocompatible, offer complete drug protection	Excellent carrier for controlled and sustained delivery of drugs. Stealth and surface modified nanoparticles can be used for active and passive delivery of bioactives

2. Nano-emulsion: An advanced drug delivery system

2.1. Definition of Nano-emulsions

Nano-emulsions (NE) or lipid nanosized emulsions are dispersions of two immiscible phases which comprise nano sized droplets with diameter below 300 nm, typically ranging from 20-200 nm (Solans, Izquierdo et al. 2005, Anton and Vandamme 2011). They are spherical, very fine, kinetically stable, isotropic, and heterogenous systems (Rai, Mishra et al. 2018).

As nano-carriers, they provide the advantages of high interfacial area, transparency, low viscosity, long-term physical stability, both hydrophilic and hydrophobic drugs carrying potential, enhanced drug stability, greater transmucosal and transdermal drug delivery, fabrication of nanoparticles and hence improved bioavailability (Sonneville-Aubrun, Simonnet et al. 2004, Shafiq, Shakeel et al. 2007, Shafiq, Shakeel et al. 2007, Gorain, Choudhury et al. 2014)

2.2. Types of Nano-emulsions

Based on the constituents and relative distribution of dispersed phase and continuous phase, there are three types of nano-emulsions that can form: oil-in-water (O/W), water-in-oil (W/O), and a double nano-emulsions, so-called Water-in-Oil-in-Water emulsions (W/O/W) (Sharma, Mishra et al. 2013).

The O/W nano-emulsions (also called as “direct” or “water based” nano-emulsions) implicate the dispersion of oil droplets in continuous water phase (Rai, Mishra et al. 2018) favored by a water-soluble surfactant (Singh, Meher et al. 2017) in contrast to W/O nano-emulsions (also called as “inverse” or “oil based” nano-emulsions) where water droplets are dispersed in continuous oil phase (Rai, Mishra et al. 2018) which are favored by an oil soluble surfactant (Singh, Meher et al. 2017)

The double nano-emulsions (also called multiple nano-emulsions), consist of water droplets dispersed in continuous oil phase which itself is dispersed in another outermost continuous water phase (Ding, Serra et al. 2019).

2.3. Components of Nano-emulsions

Nano-emulsions are mainly composed of aqueous phase, oil and emulsifying agents (Jaiswal, Dudhe et al. 2015).

The aqueous phase is generally composed of water or some solutions including phosphate buffered saline, Ringer's solution, simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 6.8), the pH and ionic content of aqueous phase are important because of theirs strong influence on droplet size and physical stability of nano-emulsions (Gupta, Eral et al. 2016).

Considering the application and the different parameters such as drug solubility, oil toxicity, and route of administration, the most commonly used oils are long chain triglycerides (LCT), medium chain triglycerides (MCT) and short chain triglycerides (SCT), e.g., labrafac oil, omega oil, corn oil, castor oil, linseed oil, olive oil, soybean oil, sesame oil, etc (Chime, Kenechukwu et al. 2014).

The emulsifying agents or surfactants optimally stabilize the mixture of lipid and aqueous phase (Sharma, Hegde et al. 2017) in a low concentration by the quick absorption at the oil-water interface and reduction of the surface tension (Jaiswal, Dudhe et al. 2015).

The emulsifying agents can be classified into three types and they may include natural (sodium alginate), synthetic (polyethylene glycols) and semi-synthetic (hydroxyethyl cellulose) emulsifying agents (Chime, Kenechukwu et al. 2014).

2.4. Methods of preparation of Nano-emulsions

There are primarily two broad categories of techniques for the preparation of nano-emulsions, including i) High energy emulsification systems (involving high energy to increase surface area by size reduction) comprising ultrasonication, using high pressure homogenizers and micro fluidizers, and ii) Low energy emulsification methods (divert the intrinsic physicochemical properties of components to generate emulsion droplets in the nanometric range) consisting of spontaneous emulsification, phase inversion temperature method (PIT) and phase inversion composition method (PIC) (Roger, Cabane et al. 2010, Roger, Cabane et al. 2011, Solans and Solé 2012)

3. Applications of nanoparticles

3.1. Applications in drugs and medications

Nano-sized particles display unique, physical and chemical properties and represent an increasingly important material in the development of novel nanodevices which can be used in numerous physical, biological, biomedical and pharmaceutical applications (Martis, Badve et al. 2012, Nikalje 2015, Loureiro, G Azoia et al. 2016).

NPs have drawn considerable interest for their ability to deliver drugs to the precise action site at the therapeutically optimum degree and dose regimen, resulting in increased therapeutic efficiency of the drugs, minimized side effects and improved patient compliance (Alexis, Pridgen et al. 2008).

Liposomes have been used as a potential drug carrier to replace conventional dosage forms because of their unique advantages which include ability to protect drugs from degradation target to the site of action and reduce the side effects (Khan, Saeed et al. 2019).

Moreover, several forms of nanoparticles have shown promise in cancer treatment and offer another option for delivery of chemotherapeutic agents. With smartly designed NPs, targeted drug delivery at the tumor site do largely avoid the toxic effects to other healthy tissues and organs (Huang, Abraham et al. 2015, Shen, Ma et al. 2016).

The semiconductor and metallic NPs have immense potential for cancer diagnosis and therapy on account of their surface plasmon resonance (SPR) enhanced light scattering and absorption (Khan, Saeed et al. 2019).

In addition, micelles are also a great way to make insoluble drugs soluble due to their hydrophobic core and hydrophilic shell. If the micelle's surface is further PEGylated, it increases the ability of the nanocarriers to get through fenestrated vasculature of tumors and inflamed tissue through passive transport, thus resulting in higher drug concentration in tumors. As of now, several polymeric micelles containing anticancer drugs, are under clinical trials (Oerlemans, Bult et al. 2010) and one such system, Genexol-PM (paclitaxel) is approved for breast cancer patients (Zhang, Huang et al. 2014).

3.1.1. Applications of nano-emulsions as nano-carrier for lipophilic drugs

A great deal of research is ongoing in developing oil-in-water nano-emulsions to incorporate lipophilic drugs to overcome the solubility problems of poorly soluble drugs. The water insoluble drugs can be effectively delivered to the patients through nano vehicles.

In addition, the use of nano-emulsions as drug carriers enhanced bioavailability, reduced toxicity, improved stability and pharmacological activity, more sustained delivery and protection from physical and chemical degradation (Anton, Hallouard et al. 2016).

The development of nano-emulsions as drug carriers have been revealed as very promising system for the administration of drugs by various routes including oral, intravenous, topical, ocular, intranasal and pulmonary route because of the compatibility of their physico-chemical properties with the different administration routes (Table 3).

Table 3: Examples of nano-emulsions developed for lipophilic drug delivery.

Drug	Purpose	Indication	Route of administration	Reference
Candesartan	To improve oral bioavailability	Hypertension	Oral	(Gao, Zhang et al. 2011)
Dabigatran etexilate	To improve oral bioavailability	Stroke and thromboembolism	Oral	(Chai, Sun et al. 2016)
Cyclovirobu-xine D	To improve bioavailability	Arrythmias	Oral	(Ke, Hou et al. 2016)
Aspirin	To enhance analgesic and anti-inflammatory activity	Analgesic and antiinflammatory	Oral	(Tang, Sivakumar et al. 2012)
Insulin	To enhance oral absorption and efficacy	Diabetes	Oral	(Li, Tan et al. 2014)
Paclitaxel	To improve cellular uptake, bioavailability and antitumor activity	Cancer	Oral	(Choudhury, Gorain et al. 2014)
Docetaxel	To overcome poor solubility and hydrolytic instability	Cancer	Parenteral	(Venkateshwarlu, Prabhakar et al. 2010)
Fisetin	To improve bioavailability and anti-tumor activity	Cancer	Parenteral	(Ragelle, Crauste-M et al. 2012)
Nimesulide	To modulate the skin penetration	Analgesic and antiinflammatory	Topical	(Alves, Scarrone et al. 2007)
Loteprednol etabonate	To improve permeability, ocular bioavailability and sustained delivery	Inflammations of the eye	Ocular	(Patel, Nakrani et al. 2016)
Saquinavir mesylate	To improve penetration and brain targeting	AIDS	Intranasal	(Mahajan, Mahajan et al. 2014)

3.2. Biomedical imaging

Interest in theranostics is rapidly increasing due to the versatility of nanoparticle-based approaches. Theranostic probes are a class of agents that can simultaneously deliver diagnostic and therapeutic functions, enabling detection and treatment of diseases in a single procedure (Janib, Moses et al. 2010). This strategy has been realized in many classes of NPs including, drug conjugates, dendrimers, surfactant aggregates (micelles and vesicles), core-shell particles, and carbon nanotubes. By combining both therapeutic and imaging agent in one smart

formulation, it is possible to monitor the pathway and localization of these NPs at the target site as well as drug action to assess therapeutic response (Bhojani, Van Dort et al. 2010).

In particular, the o/w nano-emulsions are of interest for developing fluorescent nano carriers because their oily core provide high encapsulation of lipophilic dyes.

Klymchenko et al. have developed nano-emulsions encapsulating fluorescent dyes (based on 3-alkoxyflavone and Nile Red), in the oily core of the nano-droplets, and the dyes remained highly fluorescent within the nano-droplets even at high concentration (Klymchenko, Roger et al. 2012).

Texier et al. prepared lipid nanocarriers encapsulating cyanine dyes and showed that the dye at high concentration in the oily core of nano-droplets, preserved its efficient fluorescence, allowing successful cellular and *in vivo* imaging (Nogues, Goutayer et al. 2009).

Moreover, Rapoport et al. have demonstrated that biodegradable block copolymer stabilized perfluoro-15-crown-5-ether (PFCE) nano-emulsions are effective theranostic formulations with a very high potential for use in image-guided, ultrasound-mediated drug delivery (Rapoport, Mohan et al. 2011).

Attia et al have developed iodinated NEs for liver and spleen X-rays imaging (Attia, Anton et al. 2016).

3.3. Nutraceutical delivery

Nutraceuticals are food derived, standardized components with noticeable health benefits. They are commonly consumed as complement to provide extra health benefits and decrease risks of several chronic illnesses (Aggarwal, Van Kuiken et al. 2009).

Nutraceuticals are often lipophilic molecules, such as fat soluble vitamins (A, D, E and K), polyunsaturated lipids and their bioavailability is affected by food matrices interactions, aqueous solubility, degradation/metabolism, and epithelial permeability (McClements, Li et al. 2015).

Therefore, the use of nano-sized formulations improved the pharmacokinetic profile and bioavailability of nutraceuticals (Acosta 2009, McClements 2015)

In particular, Resveratrol is an important non-flavonoid polyphenol, naturally occurs in several plants. It is known for antioxidant, cardioprotective, anti-inflammatory and anticancer activities (Summerlin, Soo et al. 2015). Resveratrol has low solubility, with decent bioavailability, however, it is rapidly metabolized and eliminated from the body (Kapetanovic, Muzzio et al. 2011, Walle 2011).

Many nanoformulations of resveratrol have been reported including polymeric NPs (Sanna, Siddiqui et al. 2013), nano-emulsions (Sessa, Balestrieri et al. 2014) and liposomes (Catania, Barrajón-C et al. 2013).

The in vitro studies reported that nanoencapsulation of resveratrol increased the therapeutic efficacy in colon cancer cells (Feng, Zhong et al. 2017), prevented metastasis and pulmonary hemorrhage and inhibited murine melanoma tumor growth in mice model (Coradini, Lima et al. 2014).

Nanoformulation of resveratrol in biodegradable oil-core polymers potentiated the activity of resveratrol in clinical conditions associated with acute lung injury and respiratory failure (de Oliveira, de Sá Coutinho et al. 2019).

Co-encapsulation of resveratrol and lipoic acid into polymeric nanocapsules improved the antioxidant activity and is promising for application in topical formulations (Davies, Contri et al. 2020).

Aim of the study

It is now well established that the vascular endothelium has a key function in the control of the vascular homeostasis mostly via the release of vasoactive substances, which can regulate vascular tone, platelet function and inflammatory responses. Alteration of the endothelial cell function often contributes to the initiation of the pathogenesis of major cardiovascular diseases including dyslipidemia, diabetes, hypertension, coronary artery disease, peripheral vascular disease, chronic heart failure and also ageing. Endothelial dysfunction is characterized by a shift of the endothelial function towards blunted vasodilation, proinflammatory and prothrombotic responses driven by the sum of the different cardiovascular risk factors. Thus, the endothelium appears to be a most pertinent target for both preventive and regenerative therapies for cardiovascular diseases.

There is extensive evidence mostly based on pre-clinical studies and also on several clinical studies that fish oils and omega-3 polyunsaturated fatty acids (n-3 PUFAs) contribute to protect the cardiovascular system. The main omega-3 PUFAs involved in these protective effects are DHA and EPA. These PUFAs have been reported to reduce blood pressure and to improve the endothelial function as indicated by an increased NO bioavailability and a reduced level of vascular oxidative stress.

The host laboratory has shown that the vaso-protective effects of omega-3 PUFAs are dependent upon the ratio and purity of EPA and DHA. Moreover, the EPA:DHA ratio of 6:1 has been identified as a superior formulation inducing potent and sustained endothelium-dependent relaxations of artery rings involving both the NO- and EDH-mediated components of the relaxation (Zgheel faraj. 2014). In addition, the chronic intake of EPA:DHA 6:1 significantly reduced systolic blood pressure in angiotensin II-induced hypertensive rats, and improved the endothelial function and the vascular level of oxidative stress, (Niazi, Silva et al. 2017).

In addition, EPA:DHA 6:1 prevented the platelet-induced serotonin-mediated contractile responses in porcine coronary artery rings and human internal mammary artery rings, suggesting a beneficial effect of omega-3 PUFAs against platelets-induced vasospasms (Zgheel, Perrier et al. 2019). More recently, an *in vivo* study indicated that oral intake of EPA:DHA 6:1 for 2-weeks significantly improved the ageing-related endothelial dysfunction in rats. The beneficial effect involved an improvement of both the NO- and the EDH-mediated relaxations as well as a reduction of endothelium-dependent contractile responses most likely by preventing vascular oxidative stress and premature endothelial senescence. However, some reports questioned the effectiveness and stability of omega-3 compounds due to the high number of unsaturated double bonds that are highly sensitive to oxidative degradation.

Thus, the major goal of the present study was to investigate whether nanoencapsulation of EPA:DHA 6:1 followed by coating with gum is able to improve the stability of omega-3 PUFAs and, hence, to enhance their biological effect on the endothelial function using an *in vitro*, *ex vivo* and *in vivo* approaches involving cultured endothelial cells, isolated porcine coronary arteries and middle-aged rats, respectively.

More specifically the aims were to assess the biological responses of coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 on the endothelial function. These investigations aimed to respond to the following points:

1. To assess their ability to induce endothelium-dependent relaxations in rings of porcine coronary artery;
2. To evaluate their ability to enhance the endothelial formation of NO in cultured endothelial cells;
3. To determine their ability to potentiate the inhibitory effect of endothelial cells on platelet aggregation using washed human platelets;

4. To study the effect of their *in vivo* intake on ageing-related endothelial dysfunction as assessed in the main mesenteric artery rings and thoracic aorta rings of middle-aged rats;
5. To determine their effect on ageing-related vascular oxidative stress and the activation of the local angiotensin system.

Results

Article I

Nanoencapsulation of the omega-3 EPA:DHA 6:1 formulation enhances and sustains NO-mediated endothelium-dependent relaxations in coronary artery rings by perpetuating the endothelial formation of NO

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Abstract

The eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) formulation with a ratio of 6:1 is a potent stimulator of the endothelial formation of nitric oxide (NO). Since omega-3 products are unstable due to the high number of double bounds, we examined whether nanoencapsulation of EPA:DHA 6:1 followed by coating with gum increases their biological activity. Vascular reactivity was assessed using porcine coronary artery rings, the formation of NO in cultured endothelial cells (ECs) using DAF-FM and indirectly by platelet aggregation studies. Coated EPA:DHA 6:1 nanoparticles induced sustained relaxations of coronary artery rings that were greater in rings with than in those without endothelium, and more pronounced than with the native form. Treatment of ECs with coated EPA:DHA 6:1 nanoparticles caused greater and more sustained formation of NO and enhanced their anti-aggregatory effects. Thus, nanoencapsulation of EPA:DHA 6:1 is an attractive strategy to enhance the beneficial effect at the vascular endothelium.

Keywords: Omega-3**,** Nanoencapsulation, EPA:DHA 6:1, Endothelium, Nitric oxide

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality worldwide and account for approximately one-third of all deaths (Mozaffarian et al., 2015). The prevalence of CVD including coronary heart disease, heart failure, and stroke increases with age in both man and women in particular after 40 years of age (Benjamin et al., 2018). Given the heavy burden CVD present for health and economy, prevention and management of CVD is a prior target of ongoing research and clinical practice (Arnett et al., 2019).

Endothelial cells (EC) play a pivotal role in the control of vascular homeostasis mostly due to several vasoprotective mechanisms including nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) (Siasos, 2020). Endothelial dysfunction, a major early hallmark of CVD, is characterized by a reduced formation of nitric oxide (NO) and EDH and, often also, the appearance of endothelium-dependent contractile responses (EDCFs) involving vasoconstrictor prostanoids generated by the cyclooxygenase (COX) pathway (Gerasimos Siasos et al., 2015; Vanhoutte et al., 2009). Since endothelial dysfunction promotes excessive vasoconstriction, arterial remodeling, and atherothrombotic responses (G. Siasos et al., 2015), the regeneration of EC is a challenging therapeutic strategy to protect the cardiovascular system.

Several clinical and animal studies have shown that the consumption of omega-3 polyunsaturated fatty acids (PUFAs), including the two main compounds eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is associated with a beneficial effect on the cardiovascular system (DiNicolantonio et al., 2014; Iwamatsu et al., 2016; Kromhout et al., 2012). In 1980, Bang et al. (Bang et al., 1976) observed an inverse relation between the intake of a diet rich in long chain PUFAs and the incidence of CVDs among the Eskimo population of Greenland (Bjerregaard, 1991). Similarly, clinical trials such as DART (1989), GISSI-prevenzion (1999), GISSI-HF (2004) and JELIS (2007) showed a reduction of the risk of

cardiovascular deaths with daily consumption of omega-3 PUFAs (Bowen et al., 2016). More recently, the REDUCE-IT trial including patients with previously established cardiovascular diseases and a high level of triglycerides showed a 25% reduction of major cardiovascular events after ingestion of 4 g/day of icosapent ethyl EPA along with statin therapy (D. L. Bhatt et al., 2019; Gajos, 2019). The cardiovascular beneficial effect of omega-3 PUFAs has been attributable to their ability to reduce the level of triglycerides, platelet activation, pro-inflammatory responses, blood pressure, and also the improvement of the protective endothelial function. Of importance, the vasoprotective effect of omega-3 PUFAs is dependent on both the ratio and the purity of EPA and DHA preparations (Zgheel et al., 2014). Among several omega-3 formulations, EPA:DHA 6:1 caused pronounced NO-dependent relaxation of porcine coronary artery rings subsequent to the endothelial NO synthase (eNOS) activation via the Src/PI3-kinase/Akt and MAPKs pathways (Zgheel et al., 2014). In addition, EPA:DHA 6:1 reduced systolic blood pressure in angiotensin II-induced hypertensive rats and improved endothelial dysfunction and vascular oxidative stress (Niazi et al., 2017), and also improved established ageing-related endothelial dysfunction by targeting the AT1R/NADPH oxidase pathway (Muhammad A Farooq et al., 2020). Moreover, this formulation prevented effectively platelet-induced and serotonin-mediated contractile responses in porcine coronary artery and human internal mammary artery rings (Zgheel et al., 2019).

Since omega-3 PUFAs are highly sensitive to degradation due to the oxidation of the numerous double bounds, the aim of the study was to investigate whether nanoencapsulation of EPA:DHA 6:1 potentiates their beneficial effect on the endothelial function and to characterize the underlying mechanisms.

2. Materials and Methods

2.1. Omega-3 PUFAs formulations

The omega-3 EPA:DHA 6:1 (w/w) formulation was kindly provided by Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada). The coated EPA:DHA 6:1 nanoemulsion was prepared by emulsification of omega-3 fatty acids into a surfactant solution containing Tween 80, Span 80 and lecithin. The emulsion is further mixed with a gelatin and gummi arabicum solution solution. pH of the final emulsion was adjusted to 4.8 with 10% acetic acid.

2.2. Vascular Reactivity Studies

Vascular reactivity studies were performed using the left circumflex coronary artery of pig hearts collected from the local slaughterhouse (SOCOPA, Holtzheim, France). They were excised, cleaned of conjunctive tissues and cut into rings (4–5 mm in length). In some rings, the endothelium was removed mechanically by gently rubbing the lumen of the ring with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37 °C). The resting tension was set at 5 g before the assessment of changes in isometric tension. After the equilibration period, rings were exposed to a high K^+-containing Krebs bicarbonate solution (80 mM) until reproducible contractile responses were obtained. To assess the endothelial function, the rings were contracted with U46619 (10 nM) before the induction of a relaxation to bradykinin (0.3 µM). After washing and a 30-min resting period, coronary artery rings were contracted again with U46619 (10 nM) before assessment of the relaxation to either native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles in rings both with and without endothelium. Since preliminary investigations have shown that the development of an endothelial-dependent relaxation is slower and more sustained with the coated nanoformulation than with the native one, the

relaxation was evaluated in response to a single concentration of an omega-3 formulation and over a 60-min period. In some experiments, rings were incubated with a modulator for 30 min before the addition of U46619, and the subsequent relaxation to either native or coated EPA:DHA 6:1 nanoparticles.

2.3. Primary coronary artery endothelial cell culture

Left circumflex coronary arteries were excised, cleaned and flushed with PBS without calcium to remove remaining blood. Coronary arteries were treated with 1 mg/ml of collagenase type I (ThermoFisher) for 15 min at 37 °C. Thereafter, ECs were collected and cultured in T25 flasks containing MCDB 131 (Invitrogen, LifeTechnologies SAS, Courtaboeuf, France) medium supplemented with fungizone (2.5 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mM, all from Lonza, Levallois-Perret, France) and 15 % fetal bovine serum. Thereafter, ECs were grown to 80-90 % confluence over a 48-72-h period. Experiments were performed with cultured ECs at passage 1, which were exposed to serum-free culture medium for 2 h before the addition of bradykinin or EPA:DHA 6:1.

2.4. Detection of nitric oxide (NO) formation

ECs were cultured on 8-wells Lab-Tek® chambers and were exposed to serum-free culture medium for 2 h before the addition of bradykinin (100 nM) or native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles for 24 h. In some experiments, ECs were treated with either bradykinin (100 nM, 24 h) or N^{ω}-nitro-L-arginine (an inhibitor of eNOS, L-NA, 300 µM, 30 min) before the addition of either native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles for 24 h. Thereafter, ECs were exposed to DAF-FM diacetate (4-amino-5-methylamino-2',7'-difluororescein diacetate, 1 µM, a NO-sensitive fluorescent dye) for 20 min at 37 °C in the dark, followed by washing with PBS. After disassembling, slides were mounted with fluorescence mounting medium (Agilent Technologies France, Les Ulis, France) and

cover-slipped before being analyzed using confocal laser-scanning microscope (Leica SP2 UV DM IRBE, Heidelberg, Germany) with a 63x magnification lens. Quantification of fluorescence levels was performed using Image J software (version 1.49 for Windows, NIH).

2.5. Determination of platelet aggregation

Washed human platelets were provided by the Etablissement Français du Sang - Alsace (Strasbourg), and suspended in Tyrode buffer at 3.10^8 platelets/ml. Suspensions of washed platelets (450 µl) were incubated with continuous stirring (1000 r.p.m, 37 °C) in an aggregometer (Chronolog 490, Diagnostica Stago SAS, Asnière sur Seine, France). ECs were cultured on Cytodex-3 microcarrier beads, which were hydrated and sterilized according to the instructions supplied by the manufacturer (GE Healthcare Life Sciences, Strasbourg, France). ECs at passage 1 grown on Cytodex-3 beads were exposed to serum-free culture medium for 2 h before the treatment with either native EPA:DHA 6:1 or coated EPA:DHA 6:1 nanoparticles for 24 h. A volume of 10-35 µl of beads covered with ECs (about 2000 cells/ml) was added to suspensions of platelets for 1 min before the addition of thrombin (0.025 U/ml) to induce platelet aggregation. In some experiments, treated ECs with coated EPA:DHA 6:1 nanoparticles were incubated with L-NA (300 µM) for 30 min before the addition to the platelet suspension.

2.6. Statistical analysis

Results are presented as means ± S.E.M for n different experiments and analyzed by Graphpad Prism (Version 7). Mean values were compared between different groups by using Two-way or One-way ANOVA test followed by Bonferroni's Multiple Comparison *post hoc* test. Group differences were considered statistically significant at $P < 0.05$.

3. Results

The nanoemulsion was characterized for particle size and furthermore stability evaluation was conducted under accelerated conditions. The initial particle size of the nanoemulsion was 273.6 nm with a polydispersity index of 0.182. There was no significant change in particle size after a 1-month storage period at 40 °C. The results revealed a particle size of 275.2 nm and a PDI of 0.175 at the end of 1 month.

3.1. Coated EPA:DHA 6:1 nanoparticles cause sustained endothelium-dependent relaxations of coronary artery rings than the native form

To study the vasorelaxant effect of omega-3 EPA:DHA 6:1 formulations, porcine coronary artery rings with and without endothelium were contracted with U46619 before the addition of an omega-3 PUFAs formulation. Time course studies indicated that the relaxation to the native form from 0.1 to 0.4% v/v reached a maximal value after about 10 min and, thereafter, declined progressively towards baseline (Figures 1A-D). In contrast, relaxations to the coated EPA:DHA 6:1 nanoparticles increased progressively to reach a near maximal value at 60 min (Figures 1A-D). Relaxations to both native and coated EPA:DHA 6:1 nanoparticles were more pronounced in rings with than in those without endothelium (Figures 1A-D). Although relaxations at 10 min were similar in response to the native and the coated formulation, those at 60 min were markedly greater with the coated formulation at 0.1, 0.2, 0.3 and 0.4% v/v (the values were 39.7 ± 2.2 and 95.3 ± 1.2 for native and coated EPA:DHA 6:1 formulations at 0.4% v/v, respectively; Figures 1D,E,F).

3.2. The sustained endothelium-dependent relaxation to coated EPA:DHA 6:1 nanoparticles involves predominantly NO

Next, experiments were performed to determine the role of the different endothelium-derived relaxing factors in the relaxation induced by both EPA:DHA 6:1 formulations. The endothelium-dependent relaxation to coated EPA:DHA 6:1 nanoparticles at 0.3% v/v is abolished by N^{ω}-nitro-L-arginine (L-NA, an eNOS inhibitor) and affected neither by indomethacin (a non-selective cyclooxygenase inhibitor) nor by the inhibition of EDH responses using the combination of TRAM-34 plus UCL-1684 (inhibitors of IK_{Ca} and SK_{Ca}, respectively) indicating the exclusive involvement of NO (Figures 2A,C). In contrast, the relaxation to the native EPA:DHA 6:1 form was inhibited significantly by indomethacin in both rings with and without endothelium indicating the involvement of vasorelaxant prostanoids (Figure 2B). In addition to omega-3 PUFAs, experiments have also assessed the vasoactive effect of an isocaloric oil, corn oil, in rings with and without endothelium. The addition of corn oil to precontracted rings did affect neither vascular tone and nor the subsequent relaxation induced by coated EPA:DHA 6:1 nanoparticles at 0.3% v/v in rings with endothelium (Figures 2C,D). In rings without endothelium, the relaxation to coated EPA:DHA 6:1 nanoparticles at 0.3% v/v was not affected by corn oil during the first 20 min but, thereafter, was significantly reduced (Figure 2D).

3.3. Coated EPA:DHA 6:1 nanoparticles induce a sustained formation of NO in cultured endothelial cells

Previously, we have shown that EPA:DHA 6:1 is able to activate the PI3-kinase/Akt/eNOS pathway within 30 min in cultured coronary artery endothelial cells (Zgheel et al., 2014). Since a sustained endothelium-dependent relaxation was observed in response to coated EPA:DHA 6:1 nanoparticles, their ability to cause the formation of NO in coronary artery endothelial cells

over a 24-h period was assessed using DAF-FM, a fluorescence NO indicator, and compared to that induced by the native omega-3 formulation and the physiological agonist bradykinin. Coated EPA:DHA 6:1 nanoparticles increased the DAF-related fluorescence starting at a concentration of 0.001% v/v and reached about 2.5-fold at 0.003% v/v at 24 h whereas the native formulation increased the DAF signal only at 0.01% v/v (Figure 3). In addition, a stimulatory effect was also observed with bradykinin at 24 h, which, however, amounted to about 50% of that induced by the coated EPA:DHA 6:1 nanoparticles (0.003% v/v) (Figure 3). The stimulatory effect of both coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 at 0.3% v/v after 24 h was abolished by L-NA demonstrating the involvement of NO (Figure 4). L-NA also reduced the small stimulatory effect of bradykinin at 24 h. However, this effect did not reach statistical significance (Figure 4).

3.4. Coated EPA:DHA 6:1 nanoparticles potentiates the anti-aggregatory effect of ECs

Next, the potential of the omega-3 formulations to potentiate the antiaggregatory effect of ECs was determined by adding ECs cultured on Cytodex beads to suspensions of washed human platelets.

The addition of about 2000 ECs/ml to suspensions of platelets reduced by about 20% the sub-maximal aggregation induced by thrombin (0.025 U/ml, Figure 5). In contrast, the thrombin-induced platelet aggregation was abolished in the presence of coated EPA:DHA 6:1 nanoparticles-treated ECs and little affected by native EPA:DHA 6:1-treated ECs (Figure 5A). Moreover, pretreatment of coated EPA:DHA 6:1 nanoparticles-treated ECs with L-NA before their addition to the platelet suspension prevented the inhibitory effect demonstrating the involvement of NO (Figure 5B).

4. Discussion

Circulating levels of omega-3 PUFAs have been inversely associated with the risk of cardiovascular diseases. The Framingham Heart Study Offspring cohort reported that, a low content of omega-3 PUFAs in erythrocyte membranes has been associated with an increased risk of cardiovascular and all-cause mortality (Harris et al., 2018). Several clinical studies and meta-analyses have evaluated the effects of various omega-3 PUFAs-rich products (fish, fish oil, krill oil and marine oil) or purified omega-3 PUFAs such as EPA and DHA in primary and secondary prevention of cardiovascular disease (Aung et al., 2018; D. Bhatt et al., 2019; Deepak L Bhatt et al., 2019; Delgado-Lista, 2012; DiNicolantonio et al., 2014; Iwamatsu et al., 2016). Although some studies reported a beneficial effect of omega-3 PUFA supplementation on major cardiovascular endpoints (Delgado-Lista, 2012; Investigators, 1999; Maki et al., 2017), no such effect was observed in other studies (Aung et al., 2018; Enns et al., 2014). Such differences in outcomes might possibly relate to the use of different doses, omega-3 PUFA sources, the degree of purity, and the formulation of the omega-3 PUFA products (Kromhout, 2012). The beneficial effects of omega-3 PUFAs on the cardiovascular system have been attributable to several effects such as reduction of the chronic inflammatory response, inhibition of the thrombogenesis, reduction of hypertension, improvement of the myocardial function, and also an improvement of the pivotal protective endothelial function (Calvo et al., 2017; Mozaffarian & Wu, 2011). In the human body, PUFAs are prone to oxidation due to their exposure to free radicals and enzymes such as cyclooxygenases, lipoxygenases and cytochromes P450, and produce various reactive chemical species such as aldehydes and ketones that reduce their nutritional value and can exert adverse effects on human health. In addition, the lipid oxidation may induce oxidative stress and pro-inflammatory responses when the metabolites reach high concentrations (Tao, 2015). Therefore, the aim of the present study was to investigate whether

nanoencapsulation of EPA:DHA 6:1 followed by coating of the microparticles with gum is able to enhance their beneficial effect of the endothelial function.

The present findings indicate that EPA:DHA 6:1 induces concentration-dependent relaxations in porcine coronary artery rings, which were more prominent in rings with endothelium as compared to rings without endothelium. These findings are consistent with previous ones showing endothelium-dependent relaxations to EPA:DHA 6:1 in porcine coronary artery rings (Zgheel et al., 2014), in primary mesenteric artery rings of old rats (M. A. Farooq et al., 2020), and in femoral artery rings of middle aged rats (Gaertner et al., 2020). Furthermore, the findings indicate that nanoencapsulation of EPA:DHA 6:1 followed by coating of the nanoparticles with gum resulted in a progressively developing relaxing activity reaching a near maximal response over 60 min whereas the relaxing activity of the native form was transient reaching a maximal value after 10 min, and, thereafter, declining progressively towards baseline. The coated EPA:DHA 6:1 nanoparticles-induced relaxation was more sustained than that induced by the native form indicating that the encapsulation process promoted a persistent biological activity possibly by preventing the oxidative degradation of the omega-3 (Tao, 2015).

The sustained endothelium-dependent relaxation to the coated EPA:DHA 6:1 nanoparticles was abolished by an eNOS inhibitor (L-NA) and not affected by inhibition of either cyclooxygenases or EDH indicating the exclusive involvement of NO. In contrast, the relaxation in response to the native form was significantly reduced by indomethacin in rings with and in those without endothelium indicating the involvement of vasorelaxant prostanoids. The present findings are in agreement with our previous investigations showing that both the ratio and the purity of the EPA:DHA formulations are of major importance for the biological activity, as highly purified EPA:DHA 6:1 and 9:1 formulations demonstrated greater endothelium-dependent NO-mediated relaxation of porcine coronary artery rings than other ratios. Moreover, EPA:DHA 6:1 stimulated the redox-sensitive activation of Src/PI3-

kinase/Akt and MAPK pathways leading to eNOS phosphorylation at Ser1177 and the subsequent formation of NO within 30 min in cultured coronary artery endothelial cells (Zgheel et al., 2014). EPA:DHA 6:1 also induced endothelium-dependent NO-mediated relaxations and inhibited serotonin-induced contractile responses in human internal thoracic artery rings (Zgheel et al., 2019). The present findings also indicate that in contrast to EPA:DHA 6:1, corn oil did induce neither endothelium-dependent relaxation nor affect the relaxation to coated EPA:DHA 6:1 nanoparticles. Since coated EPA:DHA 6:1 nanoparticles promoted long-lasting endothelium-dependent NO-mediated relaxations, experiments have assessed their ability to cause a sustained formation of NO in coronary artery ECs over a 24-h period. The findings indicate coated EPA:DHA 6:1 nanoparticles induced significantly higher endothelial levels of NO as compared with the native form and bradykinin. In addition, the stimulatory effect of both the coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 was markedly reduced by L-NA indicating the involvement of NO. Thus, coated EPA:DHA 6:1 nanoparticles appears to be an interesting omega-3 PUFAs formulation promoting long-lasting release of NO from ECs. In addition, since ECs-derived NO is a strong inhibitor of platelet aggregation (Khemais-Benkhiat et al., 2020), we investigated the anti-aggregatory effect of cultured ECs treated with either coated nanoencapsulated or native EPA:DHA 6:1 on human platelets. Although the addition of low numbers of EPA:DHA 6:1-treated ECs did not affect thrombin-induced aggregation, a pronounced inhibitory effect was observed in response to the addition of a similar number of coated EPA:DHA 6:1 nanoparticles-treated ECs. Moreover, the inhibitory effect was abolished by an eNOS inhibitor indicating the involvement of NO**.**

In conclusion, the major novel findings of this study is that nanoencapsulation of EPA:DHA 6:1 followed by coating prolongs the ability of the omega-3 PUFA formulation to stimulate the endothelial formation of NO leading to a stronger platelet antiaggregatory response. They

further suggest that coated omega-3 PUFAs nanoformulations appear as an interesting approach to better protect the endothelial function and, hence, the cardiovascular system.

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Conflicts of interest:

The authors declare no conflict of interest.

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Figure legends

Figure 1: Coated EPA:DHA 6:1 nanoparticles promote greater and sustained endothelium-dependent relaxations than the native form in coronary artery rings. Coronary artery rings with or without endothelium were contracted with U46619 (10 nM) before the addition of either coated EPA:DHA 6:1 nanoparticles or native EPA:DHA 6:1 form and assessment of the relaxation over a 60-min period. Results are expressed in % relaxations as means ± SEM of 4-6 different experiments. * $P<0.05$ vs. Control (Coated EPA:DHA 6:1 nanoparticles with endothelium).

Figure 2: The coated EPA:DHA 6:1 nanoparticles-induced endothelium-dependent relaxation in porcine coronary artery rings involves predominantly NO and is not affected by corn oil. Coronary artery rings with or without endothelium were contracted with U46619 (10 nM) before the addition of either coated EPA:DHA 6:1 nanoparticles or native EPA:DHA 6:1 form. The role of prostanoids was assessed using indomethacin (10 µM, a non-selective COX inhibitor, A), of NO using N^{ω}-nitro-L-arginine (L-NA, 300 µM, an eNOS inhibitor), and of endothelium-derived hyperpolarization using TRAM-34 and UCL-1684 (1 µM each, inhibitors of IK_{Ca} and SK_{Ca}, respectively). (D) Coronary artery rings with and without endothelium were pre-incubated with corn oil (0.3% v/v) for 30 min before the addition of U46619, and the subsequent assessment of the relaxation to coated EPA:DHA 6:1 nanoparticles over a 60-min period. Results are expressed in % relaxations as means ± SEM of 5-6 different experiments. * $P<0.05$ vs. Control without endothelium.

Figure 3: Coated EPA:DHA 6:1 nanoparticles induce greater and sustained NO formation in endothelial cells compared to the native form. ECs were incubated with either bradykinin (100 nM), the native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles for 24 h. NO formation was assessed in ECs using the fluorescent probe DAF-FM (1 μM, a NO-sensitive fluorescent dye) and confocal laser-scanning microscope. Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as means ± SEM of 3-4 different experiments. $^{*}P < 0.05$ vs. Control. $^{\#}P < 0.05$ vs. Native EPA:DHA 6:1 form treated-ECs. Original magnification, 63x. Scale bar = 50 μm.

Figure 4: Native EPA:DHA 6:1 form and coated EPA:DHA 6:1 nanoparticles induce eNOS-derived NO formation in endothelial cells. ECs were incubated with either bradykinin (100 nM, 24 h) or N^{ω}-nitro-L-arginine (L-NA, 300 μM, 30 min) before the addition of either native EPA:DHA 6:1 form (0.3 % v/v, 24 h) or coated EPA:DHA 6:1 nanoparticles (0.3 % v/v, 24 h) . NO formation was assessed in ECs using the fluorescent probe DAF-FM (1 μM) and confocal laser-scanning microscope. Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as means ± SEM of n = 3. $^{*}P < 0.05$ vs. Control, $^{\#}P < 0.05$ vs. Bradykinin. $^{\$}P < 0.05$ vs. respective Control. Original magnification, 63x. Scale bar = 50 μm.

Figure 5: Coated EPA:DHA 6:1 nanoparticles-treated endothelial cells have a greater antiaggregatory effect than those treated with the native omega-3 formulation. ECs were cultured on Cytodex 3^{TM} beads and treated with either native EPA:DHA 6:1 form (0.003 %) or coated EPA:DHA 6:1 nanoparticles (0.003 %) for 24 h. Thereafter, (A) untreated or treated ECs were added to suspensions of platelets for 1 min before the induction of platelet aggregation with thrombin (0.025 U/ml). (B) ECs treated with coated EPA:DHA 6:1 nanoparticles were incubated with L-NA (300 µM, an eNOS inhibitor) for 30 min before the induction of platelet aggregation with thrombin. Representative platelet aggregation traces (upper panels) and corresponding quantitative analysis (lower panels). Results are expressed as mean ± SEM of 3-4 different experiments. *$P < 0.05$ vs. Control (no cells), $^{\#}P < 0.05$ vs. Control ECs, $^{\#}P < 0.05$ vs. Coated EPA:DHA 6:1 nanoparticles-treated ECs.

Figure 1

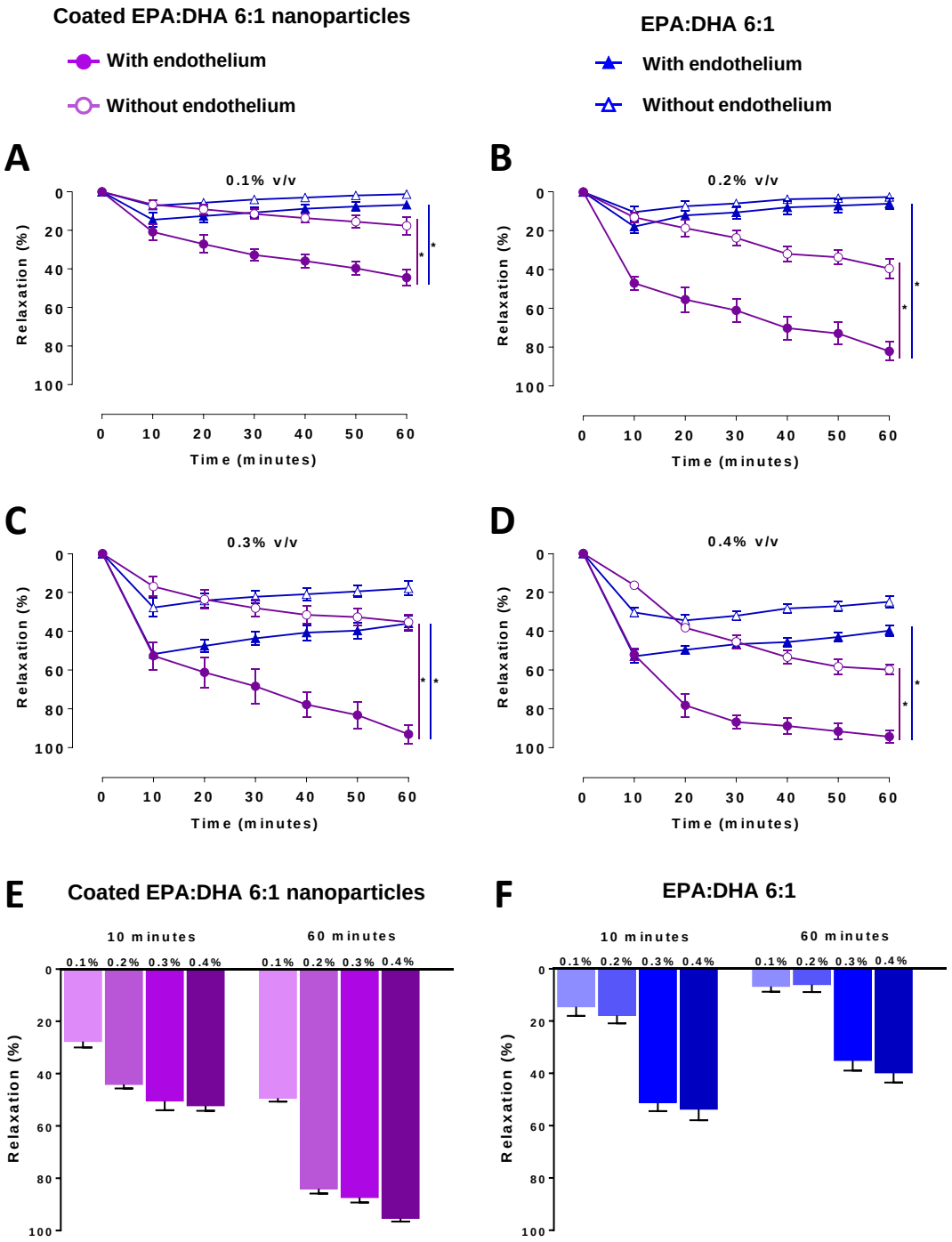

Coated EPA:DHA 6:1 nanoparticles
With endothelium
Without endothelium
EPA:DHA 6:1
With endothelium
Without endothelium
A
0.1% v/v
B
0.2% v/v
C
0.3% v/v
D
0.4% v/v
Relaxation (%)
Time (minutes)
E
Coated EPA:DHA 6:1 nanoparticles
F
EPA:DHA 6:1
10 minutes
60 minutes
0.1% 0.2% 0.3% 0.4%

Figure 2

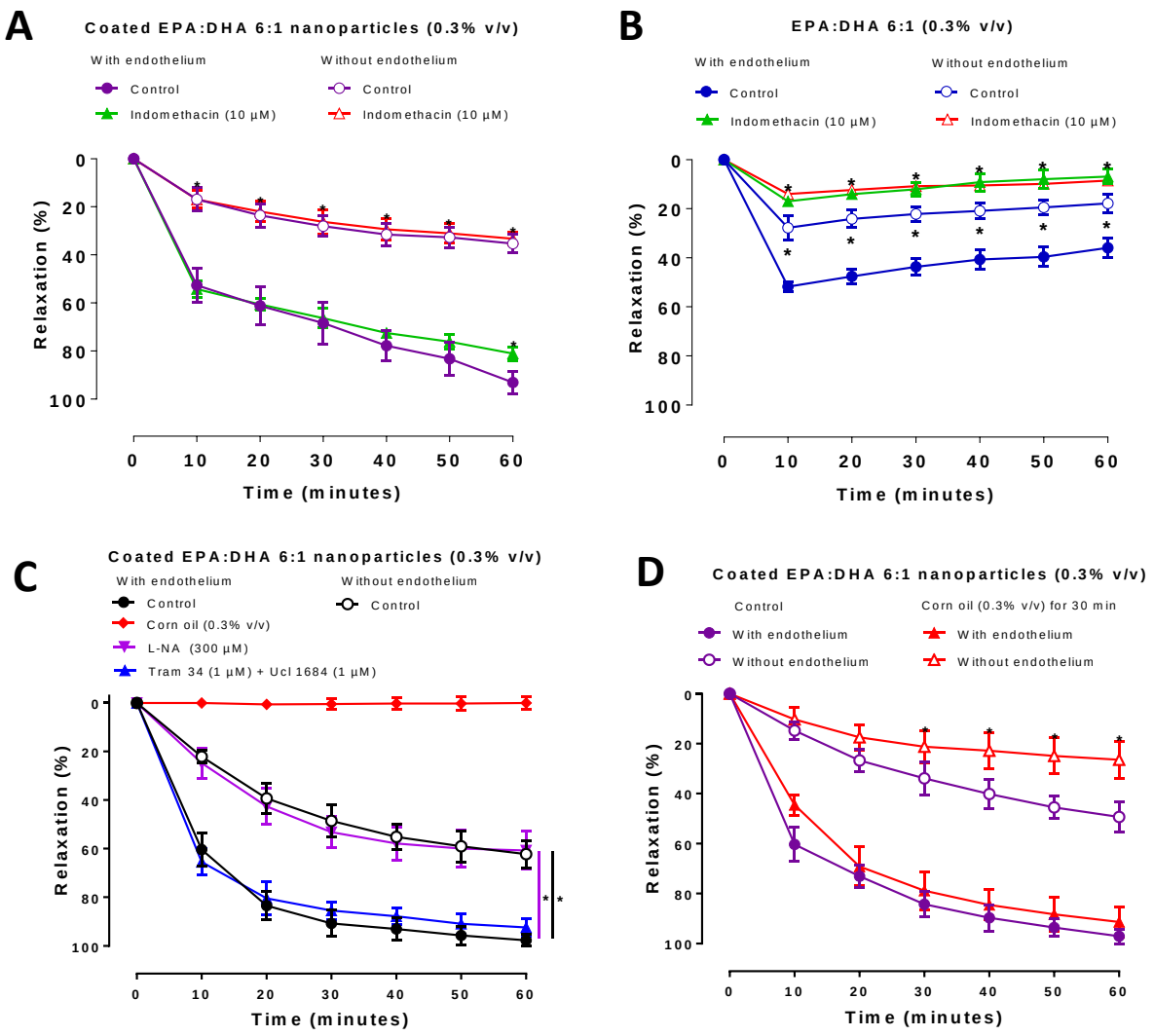
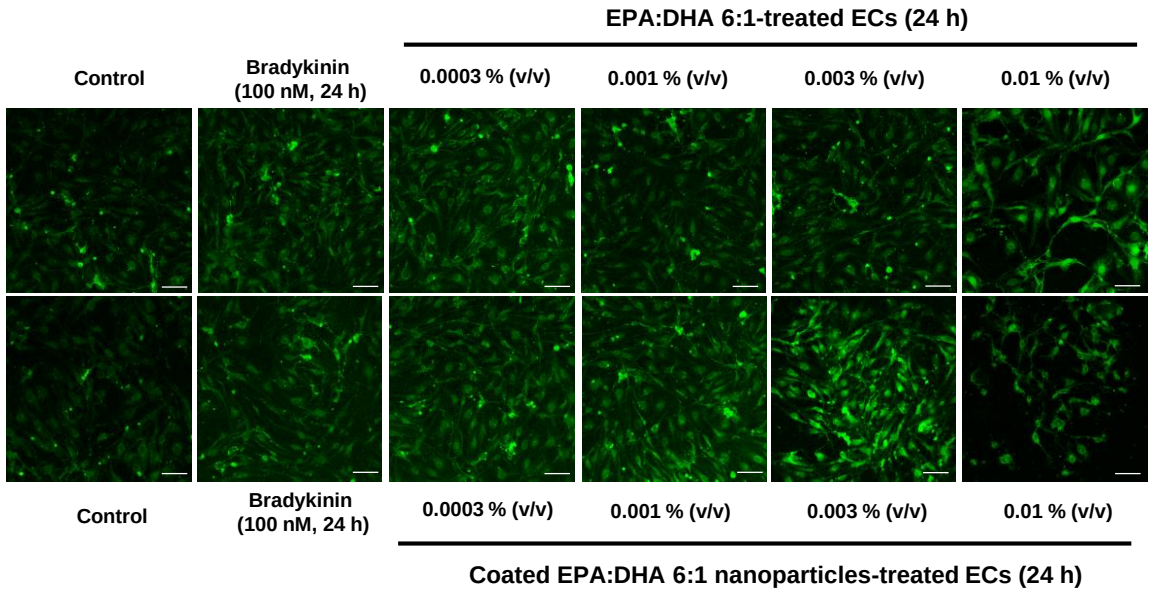

A
Coated EPA:DHA 6:1 nanoparticles (0.3% v/v)
With endothelium
Without endothelium
Control
Indomethacin (10 µM)
Relaxation (%)
Time (minutes)
B
EPA:DHA 6:1 (0.3% v/v)
C
Coated EPA:DHA 6:1 nanoparticles (0.3% v/v)
Corn oil (0.3% v/v)
L-NA (300 µM)
Tram 34 (1 µM) + Ucl 1684 (1 µM)
D
Coated EPA:DHA 6:1 nanoparticles (0.3% v/v)
Corn oil (0.3% v/v) for 30 min

Figure 3


EPA:DHA 6:1-treated ECs (24 h)
Control
Bradykinin (100 nM, 24 h)
0.0003 % (v/v)
0.001 % (v/v)
0.003 % (v/v)
0.01 % (v/v)
Control
Bradykinin (100 nM, 24 h)
0.0003 % (v/v)
0.001 % (v/v)
0.003 % (v/v)
0.01 % (v/v)
Coated EPA:DHA 6:1 nanoparticles-treated ECs (24 h)

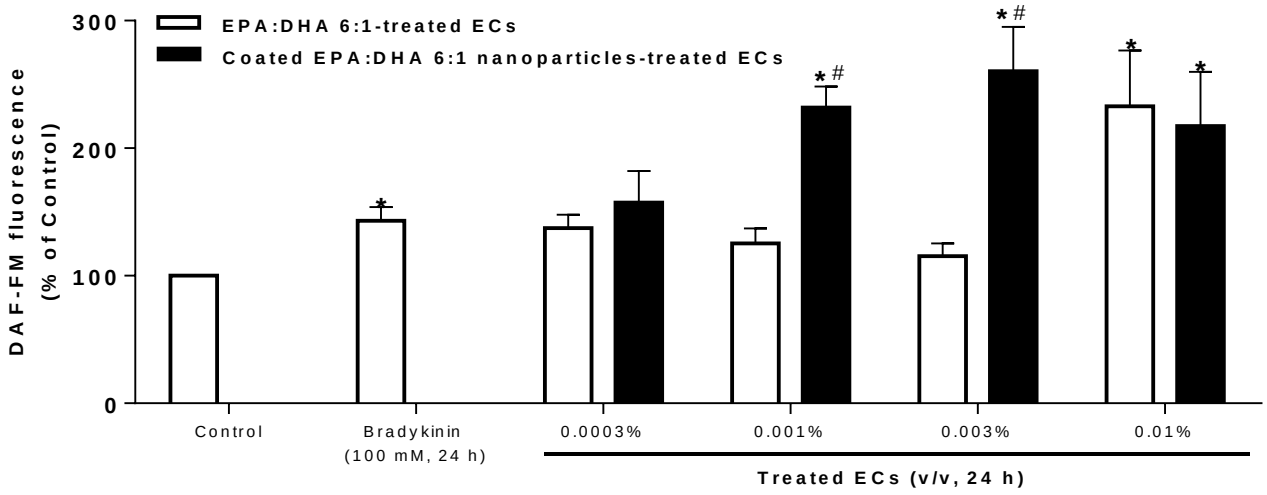

EPA:DHA 6:1-treated ECs
Coated EPA:DHA 6:1 nanoparticles-treated ECs
DAF-FM fluorescence (% of Control)
300
200
100
0
Control
Bradykinin (100 mM, 24 h)
0.0003%
0.001%
0.003%
0.01%
Treated ECs (v/v, 24 h)

Figure 4

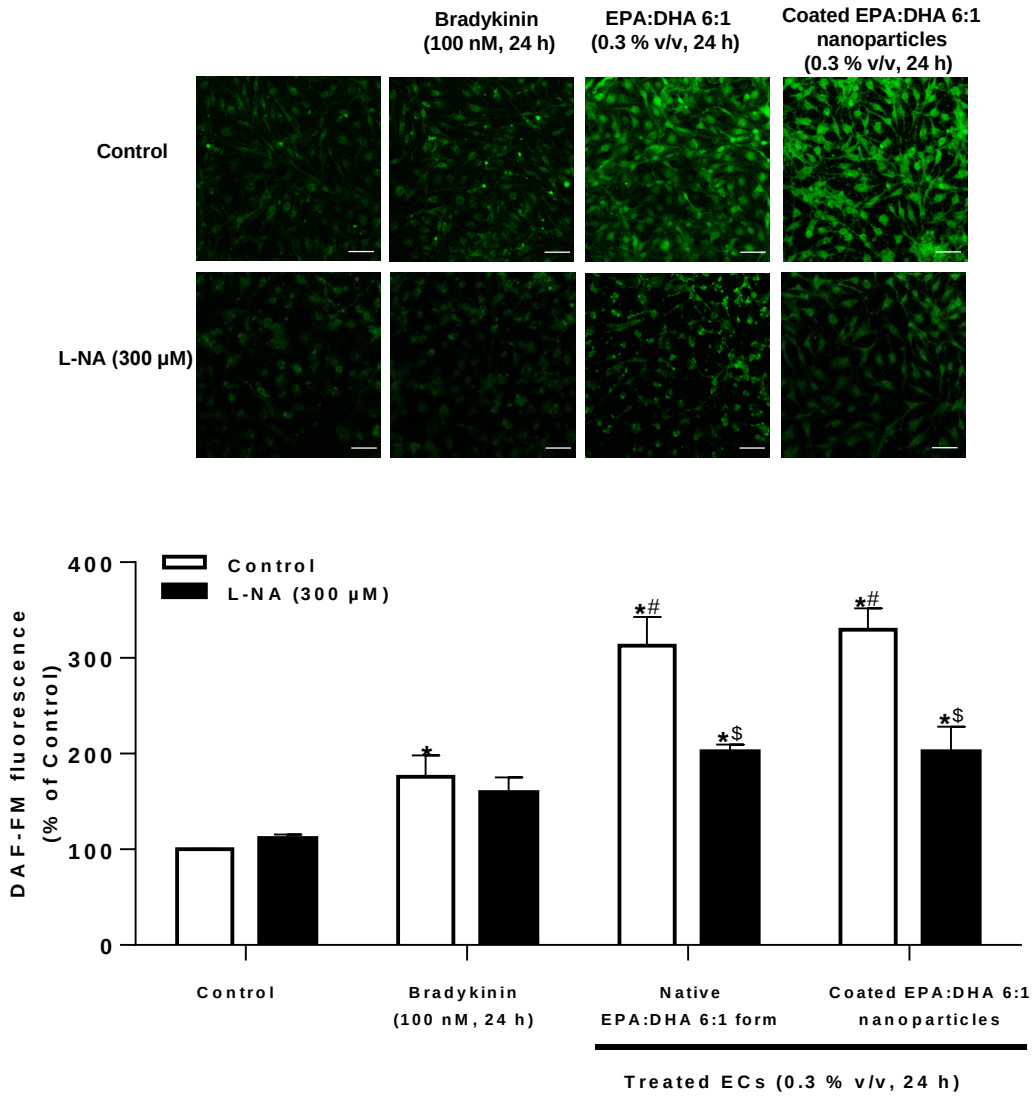
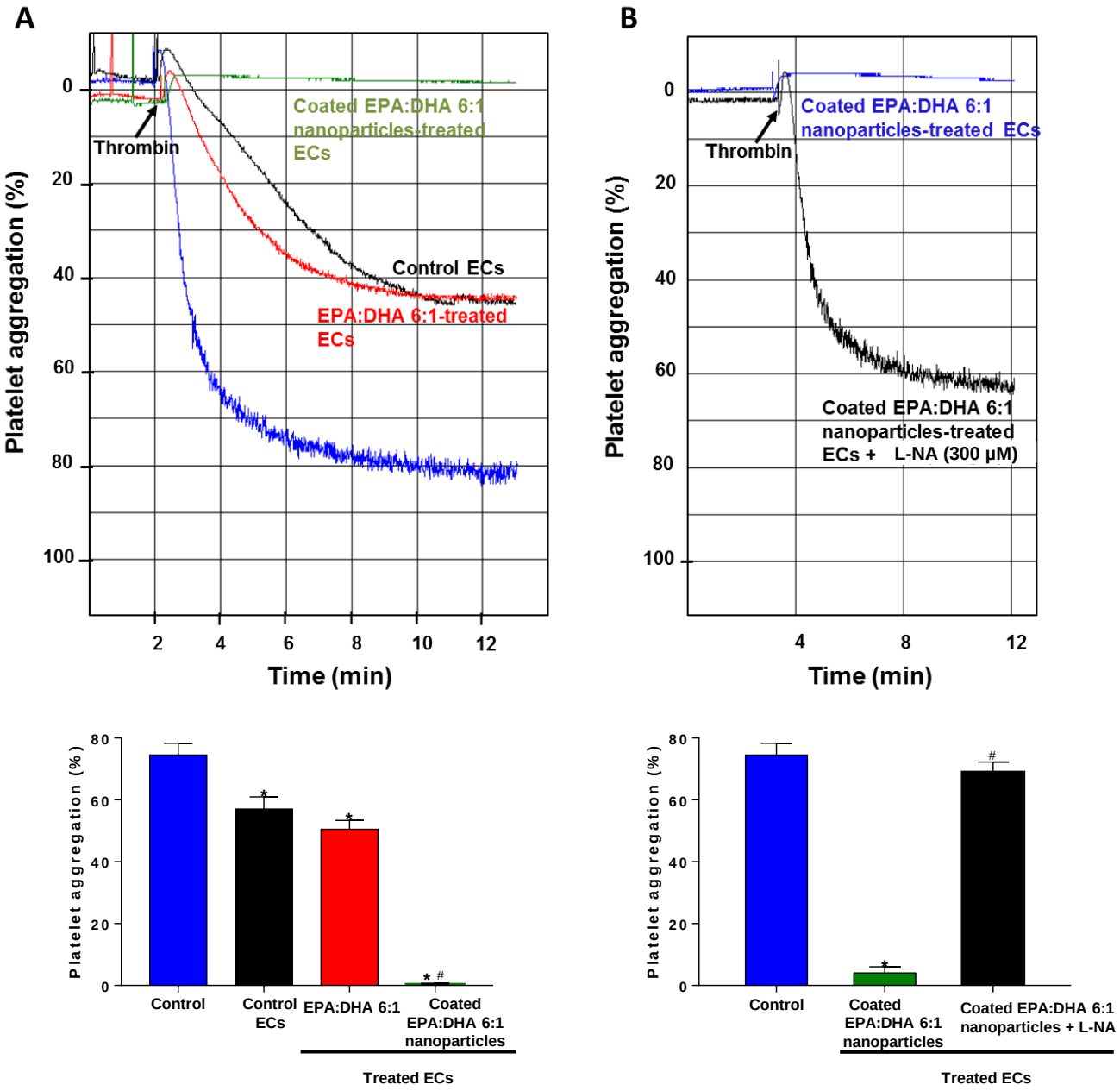

Bradykinin
(100 nM, 24 h)
EPA:DHA 6:1
(0.3 % v/v, 24 h)
Coated EPA:DHA 6:1
nanoparticles
(0.3 % v/v, 24 h)
Control
L-NA (300 µM)
Control
L-NA (300 µM)
DAF-FM fluorescence
(% of Control)
400
300
200
100
0
*
*#
*$
Control
Bradykinin
(100 nM, 24 h)
Native
EPA:DHA 6:1 form
Coated EPA:DHA 6:1
nanoparticles
Treated ECs (0.3 % v/v, 24 h)

Figure 5


A
Platelet aggregation (%)
0
20
40
60
80
100
Thrombin
Coated EPA:DHA 6:1 nanoparticles-treated ECs
Control ECs
EPA:DHA 6:1-treated ECs
2
4
6
8
10
12
Time (min)
B
Platelet aggregation (%)
0
20
40
60
80
100
Thrombin
Coated EPA:DHA 6:1 nanoparticles-treated ECs
Coated EPA:DHA 6:1 nanoparticles-treated ECs + L-NA (300 µM)
4
8
12
Time (min)
Platelet aggregation (%)
0
20
40
60
80
*
*
* #
Control
Control ECs
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Treated ECs
Platelet aggregation (%)
0
20
40
60
80
*
#
Control
Coated EPA:DHA 6:1 nanoparticles
Coated EPA:DHA 6:1 nanoparticles + L-NA
Treated ECs

Article II

Short-term intake of coated EPA:DHA 6:1 nanoparticles improves age-related endothelial dysfunction by enhancing endothelium-dependent relaxations and ameliorating oxidative stress: role of nitric oxide

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Abstract

Age-related vascular endothelial dysfunction as characterized by impaired endothelium-dependent relaxations and increased oxidative stress is a key event promoting the development of cardiovascular diseases (CVD). Omega-3 PUFAs have been shown to reduce cardiovascular risk, and EPA:DHA 6:1 has been identified as a superior formulation. The aim of the study was to investigate whether nanoencapsulation of EPA:DHA 6:1 is associated with an improved beneficial effect on age-related endothelial dysfunction and, if so, to determine the underlying mechanisms.

Thoracic aorta and main mesenteric artery from young (12 weeks) and middle-aged (50 weeks) control rats that had received daily by gavage 100 mg/kg of either native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles for 1-week were investigated. Vascular reactivity was assessed in thoracic aorta rings and main mesenteric artery rings using organ chambers. Oxidative stress and proteins level were assessed in the thoracic aorta using dihydroethidium and immunofluorescence staining, respectively.

Comparisons between young and middle-aged rats showed that ageing was associated with an endothelial dysfunction characterized by a reduced relaxation to acetylcholine and an increased contraction to phenylephrine. Endothelial dysfunction was associated with an increased level of vascular and mitochondrial oxidative stress, and of the local angiotensin system throughout the arterial wall of the thoracic aorta. Compared to the native EPA:DHA 6:1 form, the coated EPA:DHA 6:1 nanoparticles treatment improved to a greater extent the NO-mediated relaxations and reduced the contractile responses, and normalized vascular oxidative stress and the expression level of two major components of the local angiotensin system, namely, ACE and AT1R in the aged arterial wall.

The results indicate that the short-term administration of coated EPA:DHA 6:1 nanoparticles improves age-related endothelial dysfunction in middle-aged rats by improving the NO-

mediated endothelium-dependent relaxations, reduction of oxidative stress, and normalization of the local angiotensin system.

Keywords: Aging, EPA:DHA 6:1, Endothelial dysfunction, Oxidative stress, Nitric oxide

Introduction

The endothelium is a key regulator of vascular homeostasis through the release of several potent vasoactive factors that control vascular tone, smooth muscle cell proliferation, blood fluidity and inflammation. The endothelium-derived relaxing factors, which promote vascular protection, include nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarization (EDH) (Vanhoutte et al., 2009). Oxidative stress and impairment of vascular relaxation, key mechanisms of endothelial dysfunction and arterial damage, link these risk factors to vascular disease, arterial stiffness and aging (Donato et al., 2018). The age-related endothelial dysfunction is thought to be the prime contributor in the development of cardiovascular diseases (CVDs), even in the absence of other recognized risk factors including hypertension, atherosclerosis, hypercholesterolemia and diabetes (Lakatta, 2015). Hence, the age-related endothelial dysfunction provides an important therapeutic goal in preventing CVDs in the elderly people (Seals et al., 2011). Recent evidence suggests that endothelial senescence promotes blunted endothelium-dependent relaxations (Toda, 2012) with reduced formation of vasodilating factors including nitric oxide (NO) and endothelium-derived hyperpolarization (EDH), and an increased production of endothelium-derived contracting factors (EDCFs) and vascular oxidative stress (El Assar et al., 2012).

Omega-3 polyunsaturated fatty acids (PUFA) include two important components namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Both, EPA and DHA, have emerged as possible vasoprotective factors and are associated with lower cardiovascular mortality (Ajith & Jayakumar, 2019; Back, 2017) by ameliorating the endothelial dysfunction (Zehr & Walker, 2018).

Moreover, purified EPA and DHA formulations induce endothelium-dependent relaxations by activating two important pathways namely NO and endothelium-dependent hyperpolarization (EDH) in several types of blood vessels including the rat mesenteric artery and the porcine

coronary artery (Limbu et al., 2018; Zgheel et al., 2014). Moreover, intake of omega-3 EPA:DHA 6:1 has been shown to prevent endothelial dysfunction in angiotensin II-induced hypertension in rats (Niazi et al., 2017).

Since omega-3 PUFAs are highly sensitive to degradation due to the oxidation of the numerous double bounds, we have previously evaluated the beneficial effect of coated omega-3 PUFAs nanoformulations on the endothelial formation of NO using cultured endothelial cells. The findings indicate that nanoencapsulation of EPA:DHA 6:1 followed by coating prolongs the ability of the omega-3 PUFA formulation to stimulate the endothelial formation of NO leading to a stronger platelet antiaggregatory response.

Therefore, the aim of the present study was to investigate the ability of a short term oral intake of the omega-3 EPA:DHA 6:1 nanoformulation to improve endothelial dysfunction in middle-aged Wistar rats, and if so, to determine the mechanisms involved.

Materials and Methods

Ethics statement

This study conforms to the guide of animal care and use in laboratory, published by US institute of health (Bethesda, MD, USA; NIH publication number 85–23, revised 1996). The present protocol was authorized by the French Ministry of Higher Education, Research and Innovation and by the local Ethics Committee (Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg). All experiments were performed in a registered animal yard within Faculty of Pharmacy (Authorization number #7626-2016111715542930).

Preparation of omega-3 PUFAs

Formulations of omega-3 EPA:DHA ratios of 6:1 (w/w) were kindly provided by Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada) and the coated EPA:DHA 6:1 nanoemulsion by MyBiotech GmbH (Ueberherrn, Germany).

The coated EPA:DHA 6:1 nanoemulsion was prepared by emulsification of omega-3 PUFAs into a surfactant solution containing Tween 80, Span 80 and lecithin. The emulsion is further mixed with a gelatin and gummi arabicum solution. pH of the final emulsion was adjusted to 4.8 with 10% acetic acid.

***In vivo* treatment of rats**

Male Wistar rats (Janvier-labs, Le Genest-Saint-Isle, France) were kept in animal facility with controlled temperature (22 °C), 12 h light/dark cycle and were given free access to standard food and water from the age of 12 weeks until they were 50 weeks-old. They were then assigned to three groups and were administered daily by gavage with 100 mg/kg/day of either native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles, or tap water for 7 days. A group of 12 weeks-old rats was used as young control. After treatment, rats were weighed and euthanized by an intra-peritoneal injection of a mixture of ketamine/xylazine (120/10 mg/kg) before the collection of blood and organs.

Vascular reactivity studies

Vascular reactivity studies were performed using the main mesenteric artery and the thoracic aorta. Both arteries were cleaned of connective tissue and cut into rings (2–3 mm in length). Rings were suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25 and D-glucose 11, pH 7.4, 37 °C) for the assessment of changes in

isometric tension. The rings were stretched to an optimal resting tension of 1 g for the main mesenteric artery and 2 g for the thoracic aorta. After the equilibration period, the rings were exposed to high K^+-containing Krebs bicarbonate solution (80 mM) until reproducible contractile responses were obtained. To assess the endothelial function, the rings were precontracted with phenylephrine (PE, 1 µM) before the induction of a relaxation to acetylcholine (ACh, 1 µM). For the determination of contractile responses, rings were subjected to a concentration-contraction curves in response to PE. 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 characterise the different pathways involved in the relaxation, rings were incubated for 30 min with a pharmacological agent before the construction of a concentration-response curve. The role of NO-mediated relaxation was studied in rings incubated with both indomethacin (10 µM, a nonselective COX inhibitor) and TRAM-34 plus UCL-1684 (1 µM each, inhibitors of IK_{Ca} and SK_{Ca}, respectively) to inhibit the production of vasoactive prostanoids and the EDH-mediated relaxation, respectively. To study EDH-mediated relaxation, rings were incubated with indomethacin and N^{ω}-nitro-L-arginine (L-NA, 300 µM, an eNOS inhibitor) to prevent the release of vasoactive prostanoids and NO, respectively. To study the role of cyclooxygenase-derived prostanoids, rings were incubated with indomethacin (10 µM). To evaluate the vascular smooth muscle function, rings were incubated with indomethacin, L-NA and TRAM-34 plus UCL-1684, then contracted with PE (1 µM) before construction of a concentration-relaxation curve to either sodium nitroprusside (SNP, a NO donor) or levcromakalim (Lev, an ATP-sensitive potassium channels opener).

Immunofluorescence studies

Rings of the thoracic aorta were embedded in histomolds containing frozen section media (FSC 22, Leica Biosystems, Nanterre, France) and were snap-frozen in liquid nitrogen. Rings were cryosectioned at 14 µm and stored at -40 °C until use. Sections were defrosted with phosphate buffer saline (PBS) and fixed during 30 min with 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) and then incubated with blocking/permeabilizing buffer (PBS containing 1 % bovine serum albumin (BSA) (w/v) and 0.5 % Triton X-100 (w/v)) for 30 min at room temperature. After buffer removal, all sections, excluding negative controls, were incubated for 1 h at 4 °C with a primary antibody against eNOS (1/100, Cat. 610297, BD Transduction Laboratories, Le Pont de Claix, France), nitrotyrosine (1/500, Cat. 05-233, EMD Millipore, Billerica MA, USA), ACE (1/200, Cat.250450, Abbiotec, San Diego, USA) and AT1R (1/200, Cat. ab124505, Abcam, Paris, France). All sections were washed 3 times with PBS prior to incubation with either anti-rabbit or anti-mouse fluorescent secondary antibody (1/400, Alexa fluor 633 conjugate, Invitrogen) for 1 h at room temperature in the dark. After washing 3 times with PBS, sections were incubated with 1 mg/ml 4',6-diamidino-2'-phenylindole dihydrochloride (DAPI, Thermo Fisher) during 5 min at room temperature, in order to counterstain nuclei. Slides were washed 3 times with PBS and mounted under coverslip using fluorescence mounting medium (Dako, Agilent Technologies France, Les Ulis, France) and dried for 20 min at room temperature. Slides were then analyzed with confocal laser-scanning microscope (Leica SP2 UV DM IRBE; leica, Heidelberg, Germany) with a 20x magnification lens. The level of fluorescence was quantified by using Image J software (version 1.49 for Windows, NIH).

Determination of the level of oxidative stress

The level of ROS in the thoracic aorta and in the mitochondria were determined using the redox-sensitive fluorescent probe dihydroethidium (DHE, Invitrogen, ThermoFischer) and MitoSox probe (MitoSox^{TM}, Invitrogen, Thermofischer), respectively. Cryosections of thoracic aorta (25 µm) were defrosted with PBS and incubated with either DHE (2.5 µM) or MitoSox (2.5 µM) for 30 min at 37 °C in the dark. After washing 2 times with PBS, the sections were mounted under a cover-slip using fluorescence mounting medium (Dako, Agilent Technologies France, Les Ulis, France) and dried for 20 min at room temperature. Images were acquired using a confocal laser-scanning microscope (Leica SP2 UV DM IRBE; leica, Heidelberg, Germany) with a 20x magnification lens. Quantitative analysis was performed using Image J software (version 1.49 for Windows, NIH).

Statistical Analysis

Data are expressed as means ± standard error mean (S.E.M) for n different experiments and analyzed by Graphpad Prism (Version 7). Statistical variance between different groups was determined by applying Two-way Anova test for vascular reactivity studies and One-way Anova test for quantitative confocal microscopy results followed by Bonferroni's Multiple Comparison *post hoc* test. Group differences were considered statistically significant at $P < 0.05$.

Results

EPA:DHA 6:1 treatment improves the age-related endothelial dysfunction in the main mesenteric artery and the thoracic aorta

The endothelial and vascular function were assessed by vascular reactivity studies of the main mesenteric artery and the thoracic aorta. Rings from middle-aged rats showed significantly decreased relaxations in response to acetylcholine (0.1-10 μM) and increased contractions in response to increasing concentrations of phenylephrine (0.1-10 μM) in comparison with rings from young rats (Figures 1;4 A & B).

Treatment with both EPA:DHA 6:1 formulations, significantly improved acetylcholine-induced relaxation whereas, only coated EPA:DHA 6:1 nanoparticles reduced significantly phenylephrine-induced contraction in the main mesenteric artery of middle-aged rats (Figures 1 A & B). In the thoracic aorta, only the treatment with coated EPA:DHA 6:1 nanoparticles improved acetylcholine-induced relaxation and significantly reduced phenylephrine-induced contraction (Figures 4 A & B). In addition, neither ageing nor EPA:DHA 6:1 treatment affected the function of the vascular smooth muscle as indicated by similar concentration-dependent relaxations to SNP and Lev in the main mesenteric artery and the thoracic aorta (Figures 1;4 C & D).

Coated EPA:DHA 6:1 nanoparticles treatment improves the blunted NO-component of the relaxation in middle-aged rats

In the rings from the main mesenteric artery, the endothelium-dependent contractile responses to phenylephrine were not affected by indomethacin and were significantly reduced in the presence of indomethacin and TRAM-34 plus UCL-1684 in young and middle-aged rats (Figures 2 A & B). Treatment with coated EPA:DHA 6:1 nanoparticles, but not with native

EPA:DHA 6:1 form, increased the basal formation of NO, as demonstrated by the increased phenylephrine-induced contraction in presence of L-NA and indomethacin (Figures 2 C & D). The endothelium-dependent relaxations in response to acetylcholine were abolished by L-NA and not affected by indomethacin and TRAM-34 plus UCL-1684 in all groups, demonstrating the exclusive involvement of NO (Figure 3).

In the rings from the thoracic aorta, the contractile response to phenylephrine in middle-aged rats was significantly abolished by indomethacin indicating an age-related increase in the basal formation of vasoconstricting prostanoids (Figure 5 B).Treatment with coated EPA:DHA 6:1 nanoparticles, but not with native EPA:DHA 6:1 form, normalized the formation of vasoconstricting prostanoids and also increased the basal formation of NO, as indicated by the increased phenylephrine-induced contraction in presence of L-NA and indomethacin (Figures 5 C & D). In addition, the endothelium-dependent relaxation in response to acetylcholine were abolished by L-NA and not affected by indomethacin in all groups, indicating the exclusive involvement of NO (Figure 6).

EPA:DHA 6:1 treatment reduces vascular and mitochondrial oxidative stress in the thoracic aorta of middle-aged rats

As age-related endothelial dysfunction is associated with an increased level of oxidative stress (Dal-Ros et al., 2012; El Assar et al., 2013; Khodja et al., 2012), the formation of ROS in the thoracic aorta was assessed using the redox-sensitive fluorescent probe dihydroethidium. The dihydroethidium fluorescence signal was significantly increased throughout the arterial wall in middle-aged compared to young rats, indicating an increased formation of ROS (Figure 7 A). In addition, the level of mitochondrial oxidative stress was assessed using the redox-sensitive dye MitoSox. An increased signal was observed in the thoracic aorta of middle-aged rats compared to young rats (Figure 7 B).

The short term chronic intake of both EPA:DHA 6:1 formulations significantly reduced the level of vascular and mitochondrial oxidative stress. Compared to native EPA:DHA 6:1 form, the coated EPA:DHA 6:1 nanoparticles reduced to a significantly greater extent the level of ROS and MitoSox in the thoracic aorta of middle-aged rats (Figures 7 A & B).

EPA:DHA 6:1 treatment reduces the expression of eNOS and nitrotyrosine in the thoracic aorta of middle-aged rats

Since ageing is associated with reduced NO-mediated component of the relaxation, the expression of the proteins involved in NO pathway, was determined by immunofluorescence in the vascular wall of the thoracic aorta. eNOS was expressed only in the endothelium while nitrotyrosine, an indicator of the formation of peroxynitrites, was expressed throughout the vascular wall in the thoracic aorta.

Expression level of eNOS in middle-aged rats was significantly increased compared to young rats, demonstrating most likely a compensatory mechanism. Similarly, the sections of middle-aged rats showed high levels of nitrotyrosine compared to young rats. The short term chronic intake of both EPA:DHA 6:1 formulations improved the expression level of eNOS and nitrotyrosine. Compared to native EPA:DHA 6:1 form, the coated EPA:DHA 6:1 nanoparticles reduced to a significantly greater extent the expression level of eNOS and nitrotyrosine in the thoracic aorta of middle-aged rats (Figures 7 C & D).

EPA:DHA 6:1 treatment improves the age-related over-expression of two major component of angiotensin system in the thoracic aorta

As an angiotensin-converting enzyme (ACE) inhibitor and an angiotensin II type 1 receptor (AT1R) antagonist improved age-related endothelial dysfunction and excessive vascular ROS formation (Khodja et al., 2012; Mukai et al., 2002), the expression level of two major

components of the local angiotensin system, namely, ACE and AT1R, was assessed by immunofluorescence in the thoracic aorta of young and middle-aged rats. Aortic sections of middle-aged rats showed higher fluorescence signals of both ACE and AT1R in the endothelium and vascular smooth muscle than those of young rats. The short term chronic intake of both EPA:DHA 6:1 formulations significantly reduced the expression level of both AT1R and ACE. Compared to the native EPA:DHA 6:1 treatment, the coated EPA:DHA 6:1 nanoparticles treatment normalized signals of both ACE and AT1R in the middle-aged aorta to a similar level as those observed in the young aorta (Figures 8 A & B).

Discussion

With increasing age, the endothelium-dependent vasorelaxation is blunted due to an increased production of endothelium-derived contracting factors (EDCFs) and oxidative stress and/or inactivation/decreased vasodilator mechanisms including the formation of nitric oxide (NO) and endothelium-dependent hyperpolarization (EDH) (El Assar et al., 2012). The major findings of the present study indicate that daily oral intake of the coated EPA:DHA 6:1 nanoparticles for one week is able to regenerate to a greater extent than the native EPA:DHA 6:1 form the protective endothelial function, to reduce vascular and mitochondrial oxidative stress, to normalize the expression of eNOS and nitrotyrosine and to improve the local angiotensin system.

In the present study, we decreased the dose of EPA:DHA 6:1 compared to the dose of 500 mg/kg previously used in preclinical studies to assess the potency of the formulation to improve the endothelial function in a model of established age-related endothelial dysfunction (Farooq et al., 2020; Niazi et al., 2017). The dose of 100 mg/kg/day of native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles are equivalent to 1.14 g/day of omega-3 in a 70 kg human

(Reagan-Shaw et al., 2008). This dose is within the range of doses reported in different clinical studies, ranging from 0.18 to 10 g/day (Appel et al., 1993; Bhatt et al., 2019; Delgado-Lista et al., 2012; Enns et al., 2014; Miller et al., 2014).

The findings of the present study suggested that endothelial dysfunction might be associated with reduced endothelium-dependent relaxations in the main mesenteric artery and the thoracic aorta of 50 weeks-old rats. The present findings also indicate that age-related endothelial dysfunction is associated with vascular overproduction of ROS in the endothelium and vascular smooth muscle of the aorta and the mitochondria. Moreover, age-related endothelial dysfunction is associated with an increased local angiotensin system as suggested by the increased immunofluorescence signals of ACE and AT1R in the endothelium and vascular smooth muscle.

Previous studies have indicated that ageing is associated with progressive blunted endothelium-dependent relaxations whereas endothelium-independent relaxations to sodium nitroprusside remained unaffected (Egashira et al., 1993; Matz & Andriantsitohaina, 2003; Taddei et al., 1995; Toda, 2012). The age-related decline in endothelium-dependent relaxations is attributable, at least in part, with a decreased production or increased inactivation of vasodilating factors such as NO and EDH, and an augmented production oxidative stress (El Assar et al., 2012). Furthermore, aging and hypertension have an additive effect on 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 et al., 2001). Preclinical studies in different vascular beds of rats have shown that the age-related endothelial dysfunction is characterized by blunted NO-mediated relaxation only or also with a reduced EDH-component of the relaxation in the mesenteric artery (Dal-Ros et al., 2012; Idris Khodja et al., 2012), the aorta (van der Loo et al., 2000) and the coronary arterioles (Csiszar et al., 2002). The age-related endothelial dysfunction presents features similar to those

observed in other models such as the femoral artery from spontaneous hypertensive rats (SHR) rats (Puzserova et al., 2014), the mesenteric artery of angiotensin II-infused hypertensive rats (Niazi et al., 2017) and the mesenteric artery of cirrhotic rats (Rashid et al., 2018; Rashid et al., 2014).

The results indicate that in 50-weeks-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 (Farooq et al., 2020).

Treatment with low dose EPA:DHA 6:1 formulations significantly improved NO-mediated relaxations. Recovery of NO mediated relaxation in middle-aged rats were demonstrated by phenylephrine-induced contractile responses in the presence of an eNOS inhibitor, indicating an increased basal release of NO in the main mesenteric artery and thoracic aorta. Effects observed with the nano-formulation were more pronounced than those with the native form. These findings are consistent with our previous investigations showing that EPA:DHA 6:1 is a potent stimulator of the endothelial formation of NO in isolated porcine coronary arteries (Zgheel et al., 2014), human internal mammary artery (Zgheel et al., 2019) and main mesenteric artery of 20 months old rats (Farooq et al., 2020).

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 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 et al., 2013).

Dal-Ros *et al.* and Khodja *et al.* have reported the relationship between age and oxidative stress and the resultant dysfunction to endothelium using mesenteric arteries of middle-aged rats (Dal-Ros et al., 2012; Khodja et al., 2012). Increased ROS can either (1) inactivate NO directly by converting it into peroxynitrite anion (ONOO•-) or (2) decease its formation by causing oxidation of BH4, which is an important cofactor of eNOS activity. Oxidized BH4 leads to uncoupling of eNOS that further increases ROS production (Kirkwood & Kowald, 2012). Moreover, the increased expression level of eNOS in the old artery is mostly likely part of a compensatory mechanism subsequent to the reduced bioavailability of NO. When oxidative stress increases, bioavailability of NO decreases and a compensatory mechanism activates the expression of eNOS in the old artery. The findings of the present study indicate that low dose treatment with EPA:DHA 6:1 regenerates the endothelial function as indicated by improved endothelium-dependent relaxations mediated by NO and normalization of the vascular and mitochondrial level of oxidative stress and normalization of the expression level of eNOS and nitrotyrosine in the thoracic aorta, and that this effect is more pronounced with the coated EPA:DHA 6:1 nanoparticles.

The local vascular angiotensin system contributes to the induction of endothelial dysfunction and the associated vascular oxidative stress in *in vitro* and preclinical models of aging and hypertension (Dal-Ros et al., 2009; Harrison et al., 2003; Rajagopalan et al., 1996). Ageing has been associated with an increased expression of angiotensin II and ACE in the aorta of 30 months old rats (Challah et al., 1997) and non-human primates (Wang et al., 2003), whereas an age-related increase in expression of AT1R, angiotensin II and ACE was reported in the thoracic aorta of 24 months old mice (Yoon et al., 2016).

Moreover, the major role of the angiotensin system is underlined by the fact that treatment of old rats with either an ACE inhibitor or an AT1R antagonist resulted in an improvement of the endothelial dysfunction at least in part by a reduction of the level of oxidative stress (Goto et

al., 2000; Kansui et al., 2002; Mukai et al., 2002; Yasuo Kansui, 2002). In line with these studies, the present findings indicate a significantly increased expression of AT1R and ACE in the endothelium and vascular smooth muscle of aortic sections of middle-aged rats in comparison with young rats, indicating an activation of the local angiotensin system, which was improved partially by the native EPA:DHA 6:1 treatment and normalized by the coated EPA:DHA 6:1 nanoparticles treatment to a level similar to that observed in young rats. Similar observations to the EPA:DHA 6:1 treatment have been observed in old rats (Farooq et al., 2020) and Ang II-induced hypertensive rats (Niazi et al., 2017).

Taken together, the findings of the present study show that ageing is characterized by the development of an endothelial dysfunction mediated by decreased NO and EDH mediated relaxation and increased contractile responses. Moreover, the endothelial dysfunction is related to increased oxidative stress and activation of the local vascular angiotensin system.

The chronic oral intake of coated EPA:DHA 6:1 nanoformulation for one week is able to restore to a greater extent than the native formulation the protective endothelial function by increasing the NO component of the relaxation, by normalizing the local angiotensin system and the subsequent vascular level of oxidative stress. The major findings of this study is that nanoencapsulation of EPA:DHA 6:1 followed by coating prolongs the ability of the omega-3 PUFA formulation to improve the endothelial function. They further suggest that coated omega-3 nanoformulation appear as an interesting approach to better protect the endothelial function and, hence, provide specific therapeutic strategies for cardiovascular disease.

To determine the clinical relevance of the EPA:DHA 6:1, experiments were performed with human internal mammary artery (IMA) rings obtained from patients undergoing bypass surgery. EPA:DHA 6:1 inhibited the 5-HT-induced contractile responses in IMA rings and the inhibitory effect of EPA:DHA 6:1 was abolished by LNA. In addition, EPA:DHA 6:1 induced

concentration-dependent relaxations in human IMA rings that were abolished by LNA, indicating a pivotal role of the NO pathway (Zgheel et al., 2019).

In conclusion, EPA:DHA 6:1 is a potent inhibitor of 5-HT-induced contractile responses in IMA rings mainly due to its ability to stimulate the endothelial formation of NO.

These findings using the human internal mammary artery further highlight the potential protective effect of omega-3 PUFAs on the human vasculature and 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.

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Author contributions:

Conflicts of interest:

The authors declare no conflict of interest.

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Figure legends

Figure 1: Coated EPA:DHA 6:1 nanoparticles improve the age-related endothelial dysfunction in the main mesenteric artery. Rings were prepared from the main mesenteric artery and suspended in organ baths for the determination of changes in isometric tension. (A) To assess the contractile responses, rings were subjected to a concentration-contraction curves in response to phenylephrine. (B) To study the endothelium-dependent relaxation, rings were contracted with phenylephrine (PE, 1 µM) before the addition of increasing concentrations of acetylcholine (ACh). (C-D) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with PE (1 µM) 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 grams of contraction (A) or in % relaxations (B, C-D) as means ± SEM of 5-7 rats per group. * $P<0.05$ vs. Young rats, # $P<0.05$ vs. Middle-aged rats.

Figure 2: Coated EPA:DHA 6:1 increase the basal formation NO in the main mesenteric artery. (A-D) Rings were subjected to increasing concentrations of PE to construct a concentration-contractile response curve. In some baths, rings were incubated with either L-NA (eNOS inhibitor, 300 µM) or TRAM-34 plus UCL-1684 (inhibitors of endothelium dependent hyperpolarization, 1 µM each) for 30 min before contraction. All experiments were performed in the presence of indomethacin (10 µM) to prevent the formation of vasoactive prostanoids. Results are expressed in grams of contraction as means ± SEM of 5-7 rats per group. * $P<0.05$ vs. control without inhibitors.

Figure 3: Coated EPA:DHA 6:1 improve the blunted NO-component of the relaxation in the main mesenteric artery. Rings were contracted with phenylephrine (PE, 1 µM) before the addition of increasing concentrations of acetylcholine (ACh). In some baths, rings were exposed to a pharmacological agent for 30 min before contraction. To study the role of cyclooxygenase-derived prostanoids, rings were incubated with indomethacin (10 µM). NO-mediated relaxations were studied in the presence of N^{ω}-nitro-L-arginine (L-NA, 300 µM) and EDH - mediated relaxations were studied in the presence of TRAM-34 plus UCL-1684 (1 µM each). Results are expressed in % relaxations as means ± SEM of 5-7 rats per group. * $P<0.05$ vs. control without inhibitors.

Figure 4: Coated EPA:DHA 6:1 nanoparticles but not native EPA:DHA 6:1 improve the age-related endothelial dysfunction in the thoracic aorta. (A) To assess the contractile responses, rings were subjected to a concentration-contraction curves in response to phenylephrine. (B) To study the endothelium-dependent relaxation, rings were contracted with phenylephrine (PE, 1 µM) before the addition of increasing concentrations of acetylcholine (ACh). (C-D) The function of the vascular smooth muscle was assessed in rings with endothelium contracted with PE (1 µM) 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 grams of contraction (A) or in % relaxations (B, C-D) as means ± SEM of 5-7 rats per group. * $P<0.05$ vs. Young rats, # $P<0.05$ vs. Middle-aged rats.

Figure 5: Coated EPA:DHA 6:1 nanoparticles increase the basal formation NO and decreases vasoactive prostanoids in the thoracic aorta. (A-D) Rings were subjected to

increasing concentrations of PE to construct a concentration-contractile responses curve. In some baths, rings were incubated with either L-NA (eNOS inhibitor, 300 µM) or indomethacin (10 µM) for 30 min before contraction to study the NO-mediated component and vasoactive prostanoids, respectively. Results are expressed in grams of contraction as means ± SEM of 5-7 rats per group. * P<0.05 vs. control without inhibitors.

Figure 6: Coated EPA:DHA 6:1 nanoparticles improve the blunted NO-component of the relaxation in the thoracic aorta. Rings were incubated with either L-NA (eNOS inhibitor, 300 µM) or indomethacin (10 µM) for 30 min before contraction to phenylephrine (PE, 1 µM) and the subsequent relaxation to acetylcholine (ACh) to study the NO-mediated component and vasoactive prostanoids, respectively. Results are expressed in % relaxations as means ± SEM of 5-7 rats per group. * P<0.05 vs. control without inhibitors.

Figure 7: Coated EPA:DHA 6:1 nanoparticles improve the age-related increased oxidative stress in the thoracic aorta. (A-B) The level of ROS in thoracic aorta and the formation of mitochondrial superoxide were determined by fluorescence histochemistry using redox sensitive fluorescent probe dihydroethidium (DHE, 2.5 µM) and MitoSOX probe (2.5 µM), respectively. (C-D) The expression level of target proteins eNOS, nitrotyrosine were determined in cryosections of the thoracic aorta. Fluorescence signals were observed using a confocal laser-scanning microscope. Results are expressed as means ± SEM of 4 rats per group. * P<0.05 vs. Young rats, # P<0.05 vs. Middle-aged rats, §P < 0.05 vs. Native EPA:DHA 6:1 form. Scale bar = 50 µm.

Figure 8: Coated EPA:DHA 6:1 nanoparticles improve the age-related over-expression of the local angiotensin system in the thoracic aorta. The expression level of AT1 receptor

(AT1R) and angiotensin-converting enzyme (ACE) was determined by immunofluorescence in cryosections of the thoracic aorta and analysed by a confocal laser-scanning microscope. Results are expressed as means ± SEM of 4 rats per group. * $P<0.05$ vs. Young rats, # $P<0.05$ vs. Middle-aged rats, §$P < 0.05$ vs. Native EPA:DHA 6:1 form. Scale bar = 50 μm.

Figure 1

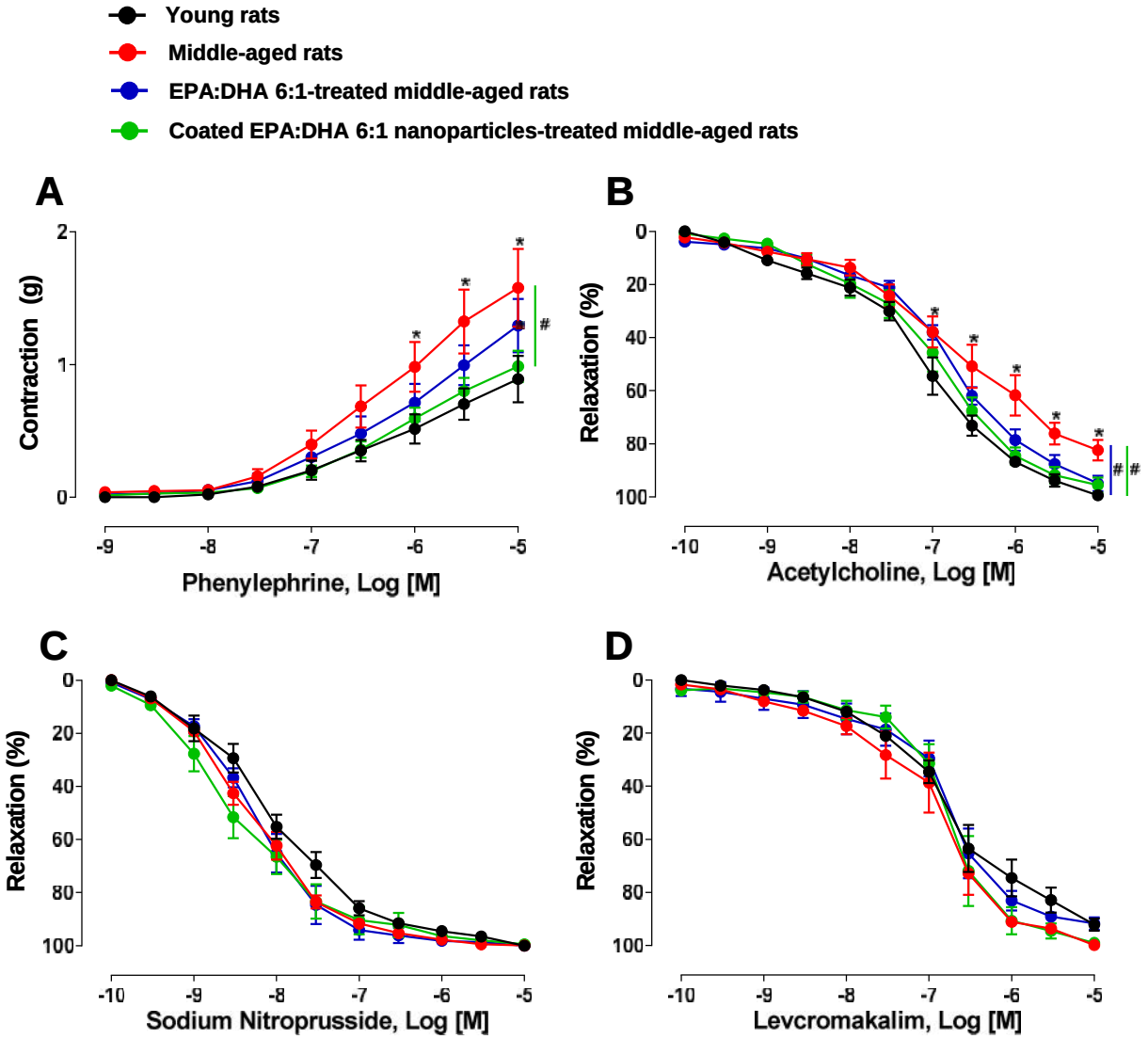

Young rats
Middle-aged rats
EPA:DHA 6:1-treated middle-aged rats
Coated EPA:DHA 6:1 nanoparticles-treated middle-aged rats
A
Contraction (g)
Phenylephrine, Log [M]
B
Relaxation (%)
Acetylcholine, Log [M]
C
Relaxation (%)
Sodium Nitroprusside, Log [M]
D
Relaxation (%)
Levcromakalim, Log [M]

Figure 2

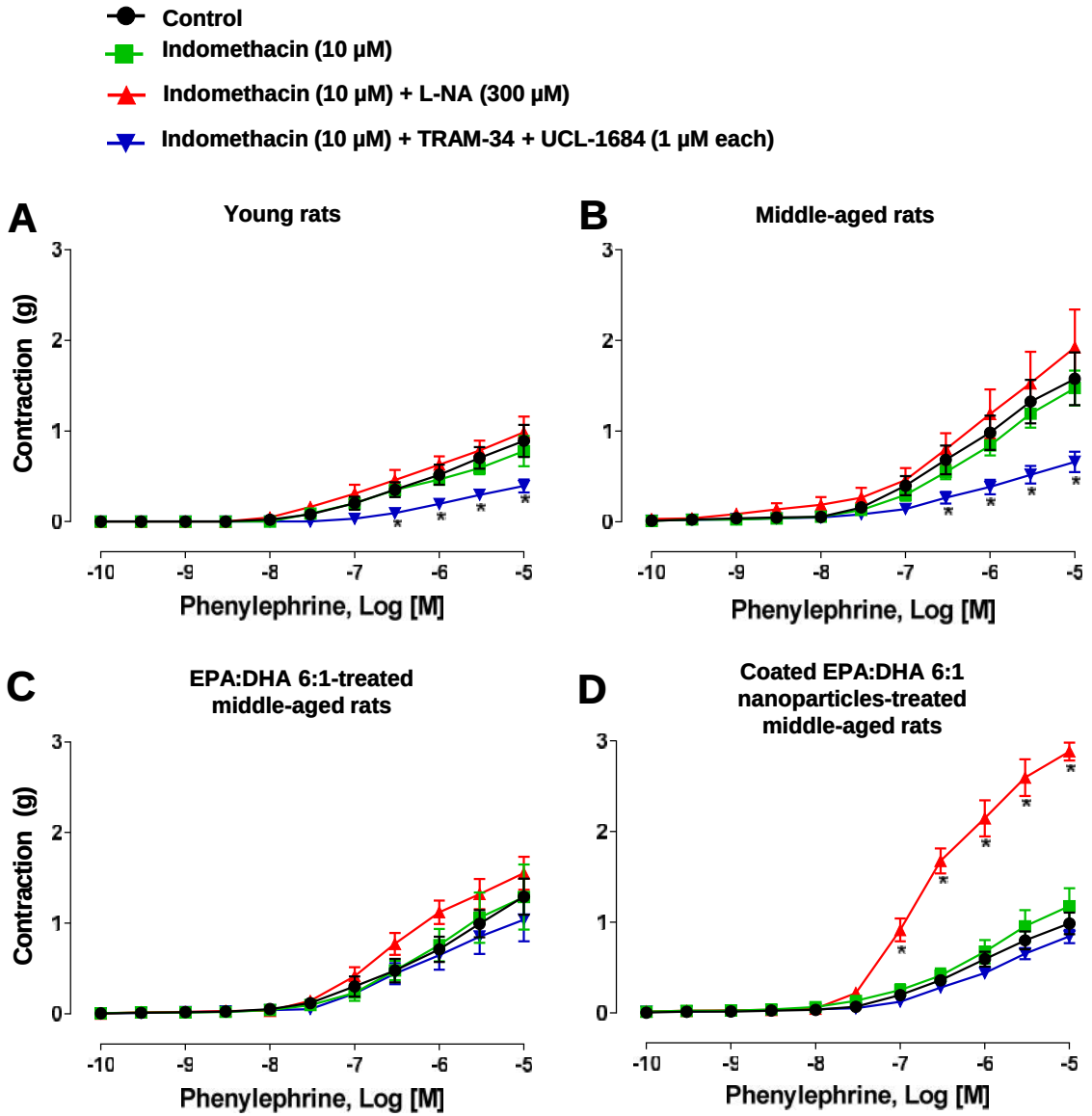

Control
Indomethacin (10 μM)
Indomethacin (10 μM) + L-NA (300 μM)
Indomethacin (10 μM) + TRAM-34 + UCL-1684 (1 μM each)
A
Young rats
B
Middle-aged rats
C
EPA:DHA 6:1-treated middle-aged rats
D
Coated EPA:DHA 6:1 nanoparticles-treated middle-aged rats
Contraction (g)
Phenylephrine, Log [M]
0
1
2
3
-10
-9
-8
-7
-6
-5
*

Figure 3

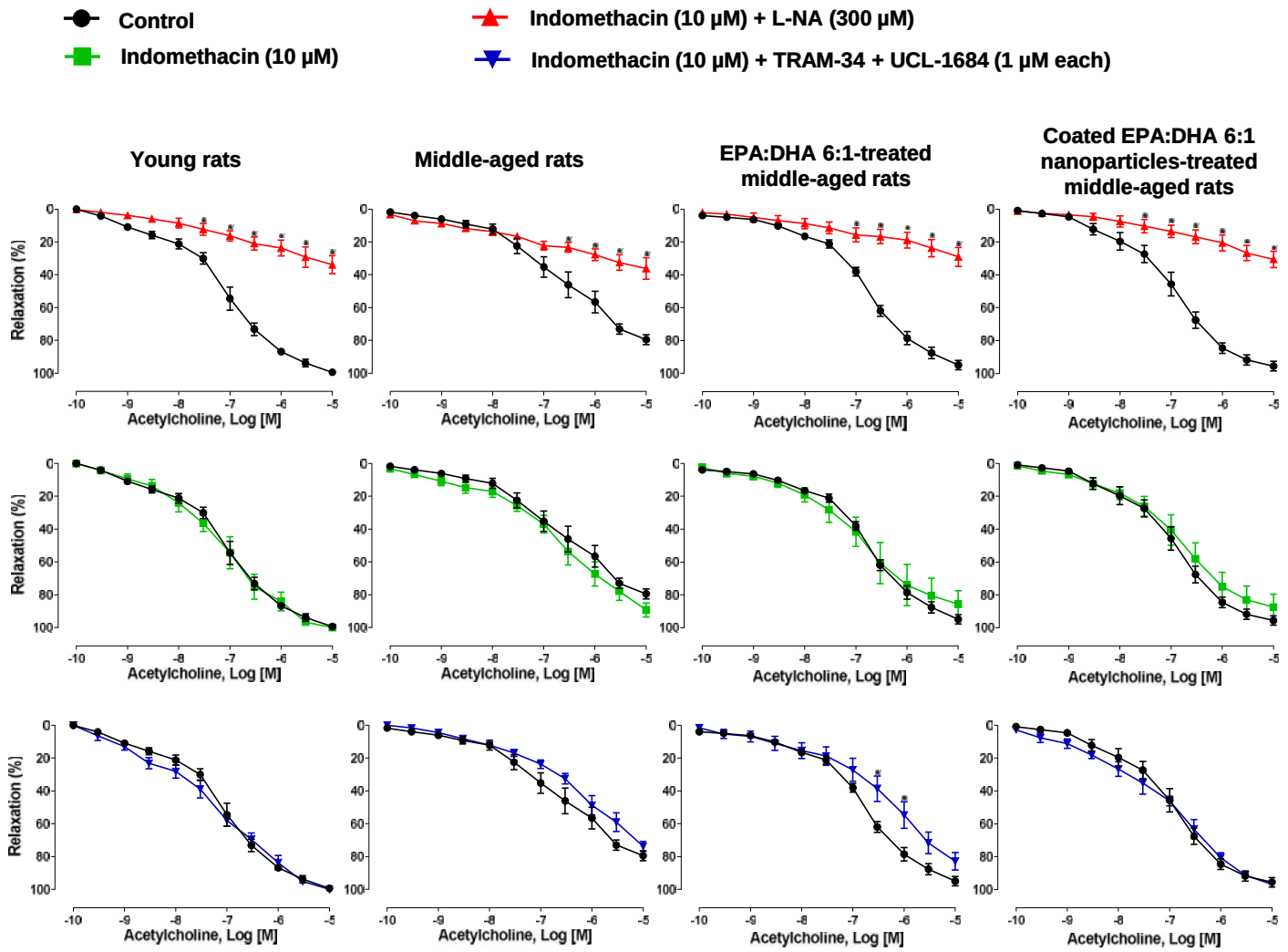

Control
Indomethacin (10 μM)
Indomethacin (10 μM) + L-NA (300 μM)
Indomethacin (10 μM) + TRAM-34 + UCL-1684 (1 μM each)
Young rats
Middle-aged rats
EPA:DHA 6:1-treated middle-aged rats
Coated EPA:DHA 6:1 nanoparticles-treated middle-aged rats
NO-mediated relaxations
Prostanoids-mediated relaxations
EDH-mediated relaxations
Relaxation (%)
Acetylcholine, Log [M]

Figure 4

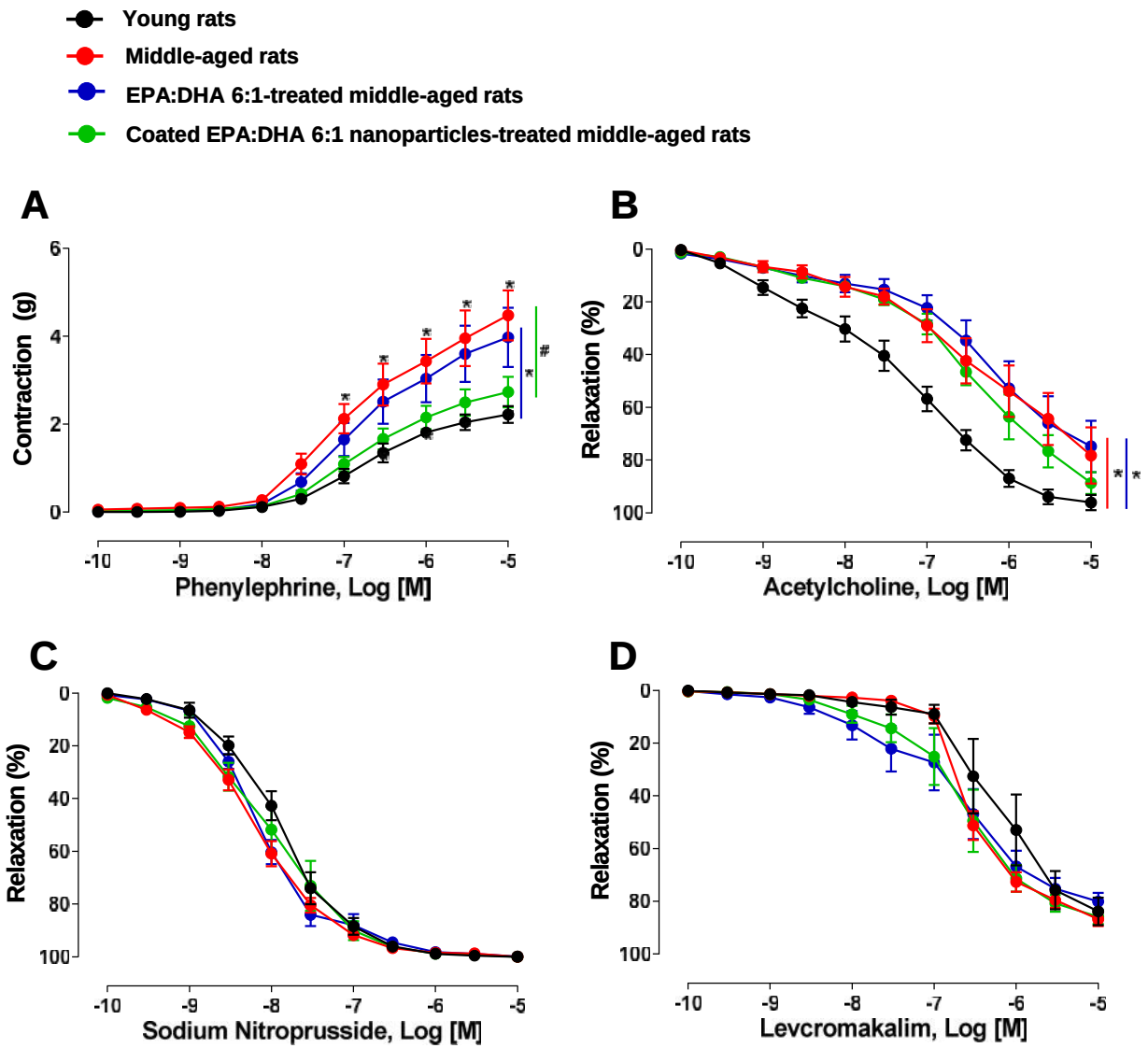

Young rats
Middle-aged rats
EPA:DHA 6:1-treated middle-aged rats
Coated EPA:DHA 6:1 nanoparticles-treated middle-aged rats
A
Contraction (g)
Phenylephrine, Log [M]
B
Relaxation (%)
Acetylcholine, Log [M]
C
Relaxation (%)
Sodium Nitroprusside, Log [M]
D
Relaxation (%)
Levcromakalim, Log [M]

Figure 5

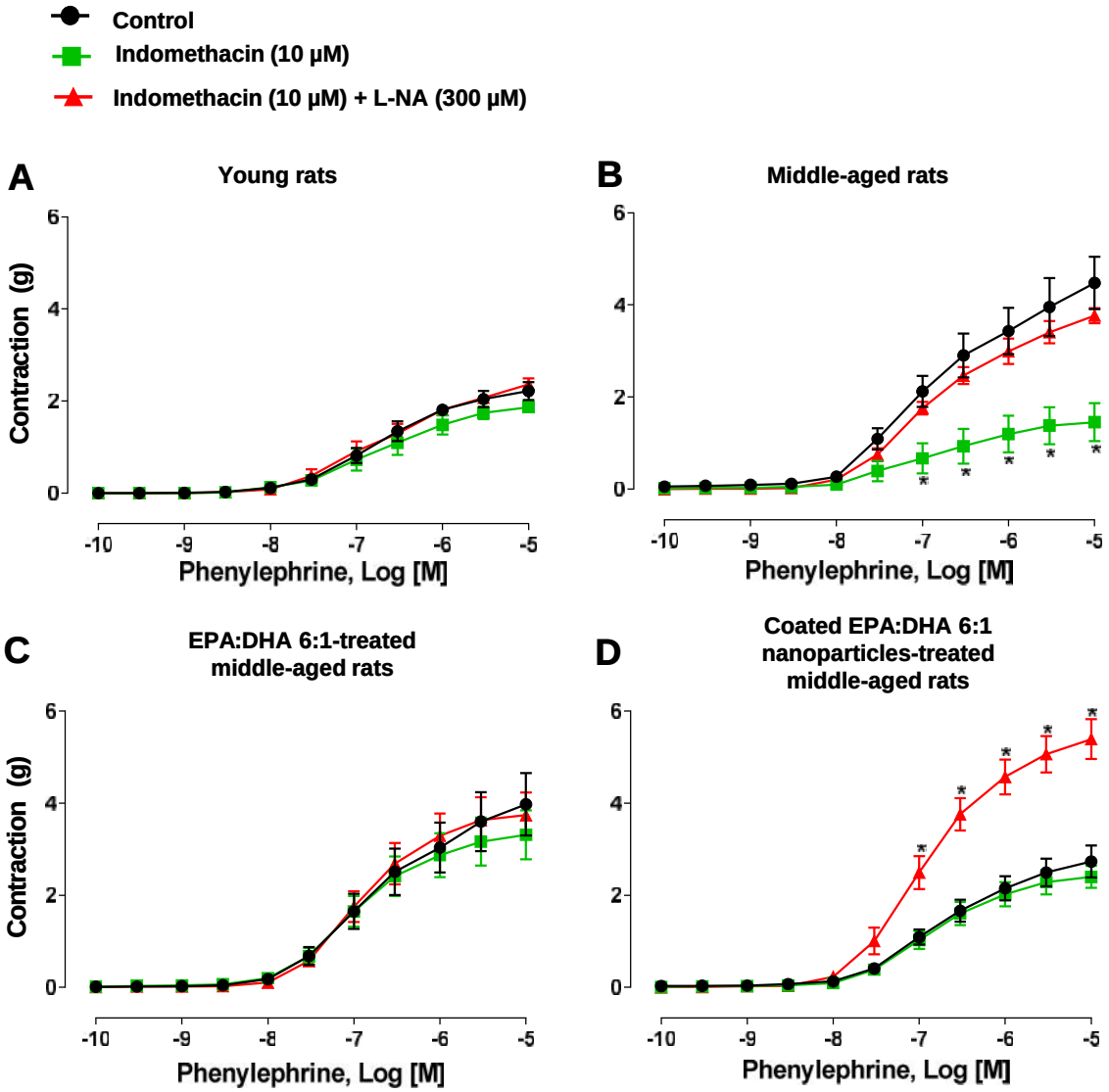

Control
Indomethacin (10 µM)
Indomethacin (10 µM) + L-NA (300 µM)
A
Young rats
B
Middle-aged rats
C
EPA:DHA 6:1-treated middle-aged rats
D
Coated EPA:DHA 6:1 nanoparticles-treated middle-aged rats
Contraction (g)
Phenylephrine, Log [M]
0
2
4
6
-10
-9
-8
-7
-6
-5
*

Figure 6

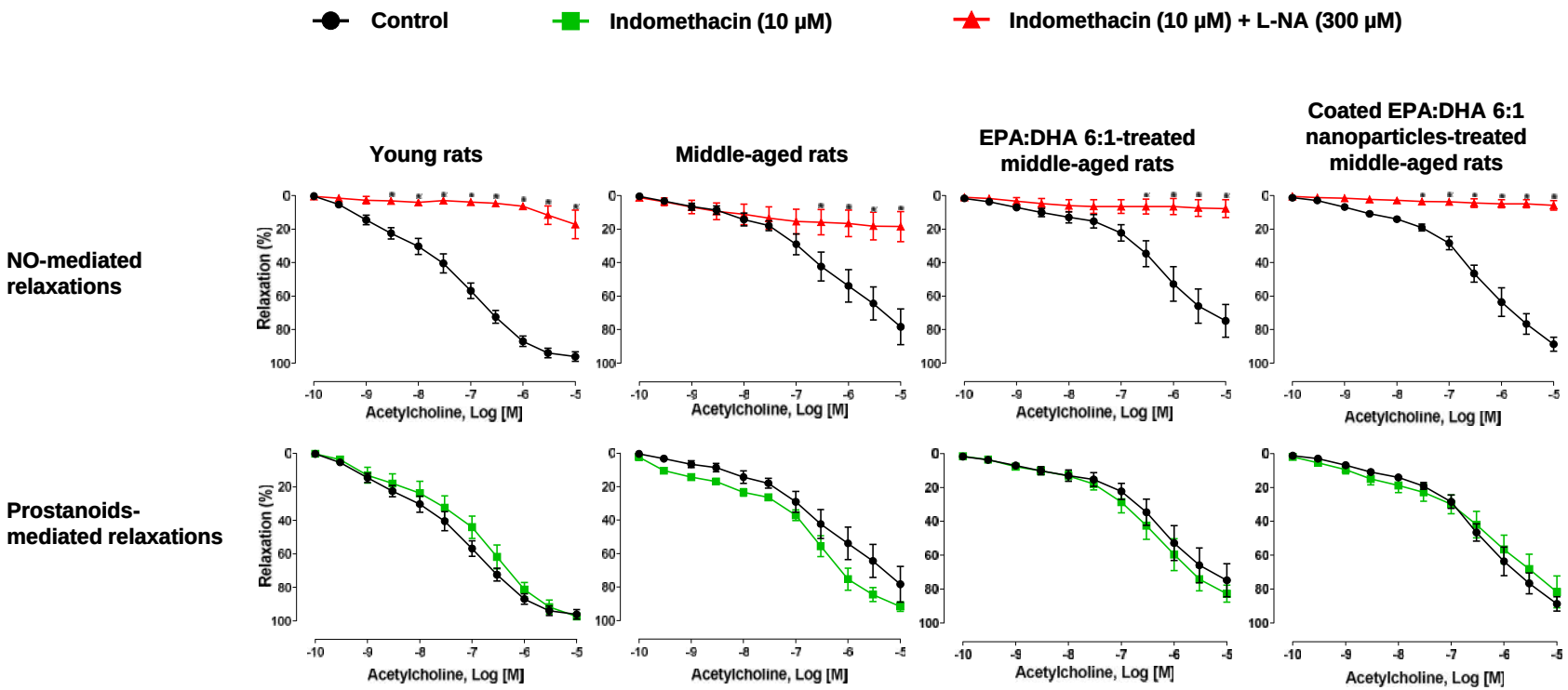

Control
Indomethacin (10 μM)
Indomethacin (10 μM) + L-NA (300 μM)
Young rats
Middle-aged rats
EPA:DHA 6:1-treated middle-aged rats
Coated EPA:DHA 6:1 nanoparticles-treated middle-aged rats
NO-mediated relaxations
Prostanoids-mediated relaxations
Relaxation (%)
Acetylcholine, Log [M]

Figure 7

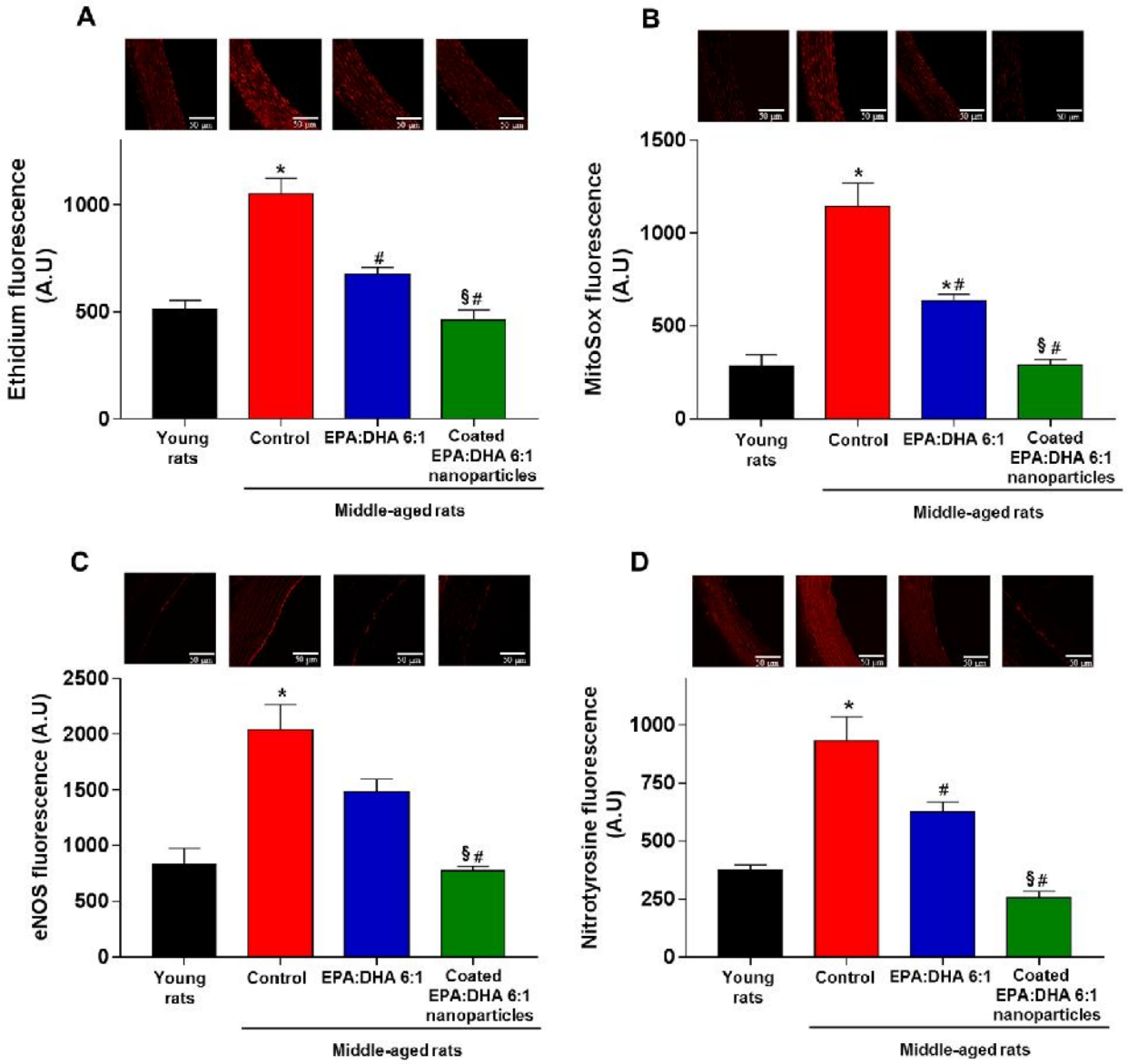
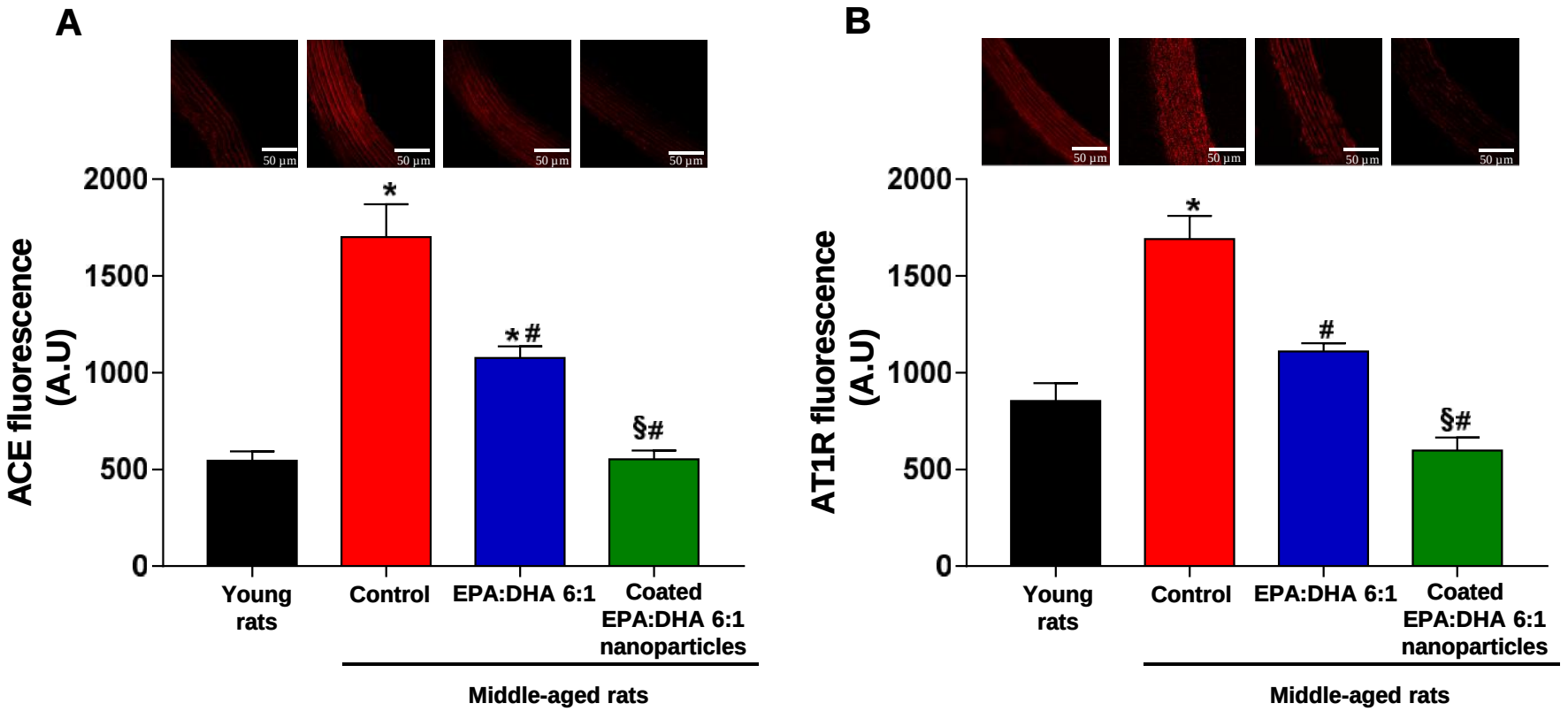

A
Ethidium fluorescence (A.U)
0
500
1000
*
#
§#
Young rats
Control
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Middle-aged rats
B
MitoSox fluorescence (A.U)
0
500
1000
1500
*
*#
§#
Young rats
Control
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Middle-aged rats
C
eNOS fluorescence (A.U)
0
500
1000
1500
2000
2500
*
§#
Young rats
Control
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Middle-aged rats
D
Nitrotyrosine fluorescence (A.U)
0
250
500
750
1000
*
#
§#
Young rats
Control
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Middle-aged rats

Figure 8


A
50 µm
50 µm
50 µm
50 µm
ACE fluorescence (A.U)
2000
1500
1000
500
0
*
*#
§#
Young rats
Control
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Middle-aged rats
B
50 µm
50 µm
50 µm
50 µm
AT1R fluorescence (A.U)
2000
1500
1000
500
0
*
#
§#
Young rats
Control
EPA:DHA 6:1
Coated EPA:DHA 6:1 nanoparticles
Middle-aged rats

Discussion and Perspectives

General discussion

Age-related endothelial dysfunction and cardiovascular diseases

Cardiovascular diseases are among the ailments that bring several health-related issues and are in the list of major diseases affecting human health mostly by promoting cerebrovascular and cardiac events (Celermajer et al., 2012). CVDs are the major cause of mortality worldwide in 2015 which is responsible for 17.7 million deaths. The number of deaths is expected to rise up to 23.6 million by 2030 (WHO, 2017). According to an estimation, 4% deaths in developed countries and 42% in developing countries are due to CVDs (Mendis, 2017). Major risk factors for developing CVDs are hyperlipidemia, diabetes mellitus, obesity, hypertension and insulin resistance. These risk factors promote the development of CVDs at an early age (Stewart et al., 2017). Irrespective of the fact that current advancements in the field of medicine has helped human to fight against several ailments, CVDs are still considered a global reason for morbidity and mortality especially in developing countries (Bansilal et al., 2015).

Endothelium, the inner most layer of blood vessels, regulates vascular tone by releasing various vasoactive factors. The endothelium maintains a balance between contraction and relaxation of vessels by secreting vasoconstricting and vasodilating factors. Moreover, endothelium secretes numerous mediators, regulates key functions such as thrombogenesis (i.e., von Willebrand factor, plasminogen activator inhibitor-1), coagulation and fibrinolysis (i.e., tissue plasminogen activator, prostacyclin), vascular proliferation, platelet gathering and disaggregation, and the expression of pro-inflammatory cytokines and adhesion molecules (Incalza et al., 2018).

Endothelial dysfunction is characterized by a reduced ability of the endothelium to induce vasodilation. Ageing is among the most important risk factor for endothelial dysfunction (Bermejo-Martin et al., 2018; Wang et al., 2019). It is now well established that aging is accompanied with reduced endothelium-dependent and unaltered endothelium independent vasorelaxation (Herrera et al., 2010; Toda, 2012). This decline in vasorelaxation with age is

due, at least in part, to reduced generation of relaxing factors, reduced bioavailability of endothelium-derived relaxing factors, and/or an over-production of contractile factors (El Assar et al., 2012). With increasing age, the NO component of endothelium-dependent relaxation of most vascular beds decreases, and in some types of blood vessels such as the mesenteric artery the blunted NO formation is accompanied by a reduced EDH component (Dal-Ros et al., 2012; Khodja et al., 2012).

Omega-3 fatty acids and cardiovascular health

Fish and fish oil are considered as rich sources of the omega-3 class of polyunsaturated fatty acids (Lane et al., 2014) and the most potent omega-3 PUFAs are EPA and DHA (Riediger et al., 2009). Numerous epidemiological, experimental and clinical studies indicate a beneficial effect of omega-3 PUFAs in lowering the incidence of myocardial infarction and cardiovascular mortality (Gajos, 2019). They also support the concept that omega-3 PUFAs have a beneficial effect in pathologies associated with cardiovascular risk factors like hypercholesterolemia, thrombosis, hypertension, arrhythmias, inflammation, oxidative stress and diabetes (Mozaffarian & Rimm, 2006). Indeed, an inverse correlation has been observed between the increased consumption of omega-3 PUFAs and the incidence of CVDs (Kromhout, 1989). Similarly, clinical trials such as DART (1989), GISSI-prevenzione (1999), GISSI-HF (2004) and JELIS (2007) showed a reduction of the risk of cardiovascular deaths with daily consumption of omega-3 PUFAs (Bowen et al., 2016). More recently, the REDUCE-IT trial including patients with previously established cardiovascular diseases and a high level of triglycerides showed a 25% reduction of major cardiovascular events after ingestion of 4 g/day of icosapent ethyl EPA along with statin therapy (Bhatt et al., 2019; Gajos, 2019). The cardiovascular beneficial effect of omega-3 PUFAs has been attributable to their ability to

reduce the level of triglycerides, platelet activation, pro-inflammatory responses, blood pressure, and also the improvement of the protective endothelial function.

The beneficial effects of EPA and DHA on the cardiovascular system is in part due to their protective effect on the endothelial function through the improvement of the NO bioavailability and the reduction of the level of vascular oxidative stress (Zanetti et al., 2017). In a 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 et al., 2012).

Our research team showed that the vasoprotective effect of omega-3 PUFAs is dependent on both the ratio and the purity of EPA and DHA preparations, thus, an EPA:DHA ratio of 6:1 caused potent endothelium-dependent relaxations by activating the redox-sensitive Src/PI3-kinase/Akt and MAPKs pathways leading to endothelial NO synthase activation and the endothelial formation of NO (Zgheel et al., 2014).

Moreover, chronic ingestion of EPA:DHA 6:1 improved endothelial function by a reduced systolic blood pressure in a model of angiotensin II-induced hypertensive rats (Niazi et al., 2017). In addition, our team recently showed that chronic intake of EPA:DHA 6:1 for 2 weeks significantly improved the ageing-related endothelial dysfunction in old rats. The beneficial effect involved an improvement of both the NO- and the EDH-mediated relaxations in the main mesenteric artery as well as a reduction of endothelium-dependent contractile responses most likely by preventing vascular oxidative stress and premature endothelial senescence (Farooq et al., 2020).

Apart from the reported benefits of omega-3 PUFAs, contradictory findings have also been reported. An increase in the LDLc fraction has been reported during omega-3 treatment of hyper triglyceridemia (Kim et al., 2011). As omega-3 PUFAs are prone to oxidative

modifications, their accumulation in LDL cholesterol may promote the formation of oxidized LDL, which is a known risk factor for atherogenesis and other lipid related disorders.

Studies by (Whitman et al., 1994), demonstrated that incorporation of omega-3 PUFA in an atherogenic diet increased the LDL oxidation *in vitro*.

The Center for Food Safety and Applied Nutrition, USA has reported the suspected risks of DHA and EPA consumption in excess of 3 g per day. It includes the possibility of an increased incidence of oxidative modifications of these fatty acid molecules into biologically active signaling molecules, elevated apoliproteins levels which are associated with LDL cholesterol and reduced glycemic control among diabetic and hyperlipidemic patients. In addition, oxidation of omega-3 PUFA can generate toxic aldehydes such as 4-hydroxy 2-hexenal (HHE), which can induce chronic inflammation and also form adducts with cellular macromolecules, contributing to degenerative pathologies. Thus, the safety of these omega-3 PUFAs regarding long-term consumption needs to be ensured (Narayanankutty et al., 2016).

Therefore, the major aim of the first study was to investigate whether nanoencapsulation of EPA:DHA 6:1 followed by coating with gum is able to improve the stability of omega-3 PUFAs and, hence, to promote a greater beneficial effect on the endothelial function. For this purpose, experiments were performed using an *in vitro* approach with cultured endothelial cells, and an *ex vivo* approach with isolated porcine coronary arteries, and the effect of the EPA:DHA 6:1 nanoformulation was compared to that of the native form.

The role of endothelium in omega-3 PUFAs-mediated vasodilation

The initial experiments aimed at investigating the role of endothelium in omega-3 PUFAs-induced relaxation of porcine coronary arteries using vascular reactivity studies. In coronary artery rings, endothelium removal led to partial attenuation of EPA:DHA 6:1-mediated relaxations of both formulations. These findings are consistent with previous ones that have

indicated the role of endothelium-dependent mechanisms in the vasorelaxation to omega-3 PUFAs (Omura et al., 2001; Zgheel et al., 2014). Furthermore, the findings indicate that nanoencapsulation of EPA:DHA 6:1 followed by coating of the nanoparticles with gum resulted in a progressively developing relaxing activity reaching a near maximal response over 60 min whereas the relaxing activity of the native form was transient reaching a maximal value after 10 min, and, thereafter, declining progressively towards baseline. The coated EPA:DHA 6:1 nanoparticles-induced relaxation was more sustained than that induced by the native form indicating that the encapsulation process promoted a persistent biological activity possibly by preventing the oxidative degradation of the omega-3 PUFAs (Tao, 2015).

Then, we characterized the mechanisms involved in the endothelium-dependent relaxation by investigating the role of NO, EDH and the prostanoid pathways. The findings demonstrated that the sustained endothelium-dependent relaxation to the coated EPA:DHA 6:1 nanoparticles was abolished by an eNOS inhibitor (L-NA) and not affected by inhibition of either cyclooxygenases or EDH indicating the exclusive involvement of NO. Thus, the coated EPA:DHA 6:1 nanoparticles can induce NO-mediated relaxations (Geleijnse et al., 2002; López et al., 2004; Raimondi et al., 2005).

The endothelium is involved in the production of several vasoactive factors including vasorelaxant COX-derived metabolites of AA such as PGI_2 that can elicit vasodilation (Bogatcheva et al., 2005). Both DHA and EPA compete with AA as substrates for COX enzymes resulting in the production of vasoactive metabolites involved in vasodilation (Schmitz & Ecker, 2008), indicating that this vasodilator pathway can also contribute to omega-3 PUFAs induced relaxation. COX metabolites of EPA have also been involved in vasodilatory responses (Engler et al., 2000). The mechanisms involved in endothelium-dependent relaxation can differ depending upon the omega-3 PUFA studied (EPA or DHA) (Mori et al., 2000).

In the present study, only the native form-induced relaxation was sensitive to COX inhibition. The relaxation in response to the native form was significantly reduced by indomethacin (a non-selective COX inhibitor) in rings with and in those without endothelium indicating the involvement of vasorelaxant prostanoids. In contrast, the endothelium-dependent relaxation to the coated EPA:DHA 6:1 nanoparticles was exclusively mediated by the NO pathway. This difference may be partly explained by a cross talk between NO and the production of PGI_2. The interactions between NO and the production of prostanoids may interfere in the kinetics of AA oxidation by altering the selectivity of COX for the substrate or by modifying the redox regulation of COX (Silva et al., 2017).

(Bakker & Sipkema, 1998) have demonstrated that AA induces COX-dependent vasodilation and indomethacin inhibited the response in endothelium intact rat arterioles. However, L-NA, which inhibits the generation of NO, reduced the vasodilation elicited by AA, which shows that NO plays a role in the metabolism of AA or at another level of the prostanoid cascade. High concentrations of NO inhibited the release of PGI_2 in bovine EC in a cGMP-dependent manner, possibly by lowering the activator calcium signal (Doni et al., 1988).

To assess the vasodilation effect of corn oil, an equivalent and isocaloric oil containing less than 1% of omega-3 PUFAs, coronary artery rings were pretreated with corn oil before the addition of coated EPA:DHA 6:1 nanoparticles. The findings indicate that corn oil did induce neither endothelium-dependent relaxation alone nor affect the relaxation to coated EPA:DHA 6:1 nanoparticles, demonstrating the specific effect to the optimized formulation of omega-3 in inducing endothelium-dependent relaxations.

Omega-3 PUFAs induced sustained NO generation in endothelial cells

Numerous studies have indicated a predominant role of NO in the omega-3 PUFAs-induced endothelium-dependent relaxation in different types of blood vessels including porcine coronary artery rings (Zgheel et al., 2014), primary mesenteric artery rings of old rats (Farooq et al., 2020), and femoral artery rings of middle aged rats (Gaertner et al., 2020). Since omega-3 PUFAs are potent inducers of the endothelial synthesis of NO, experiments have assessed the formation of NO in cultured coronary artery ECs treated with both formulations of EPA:DHA 6:1 and bradykinin used as a control, and evaluated their stimulatory effect over a 24-h period. The exposure of endothelial cells to EPA:DHA 6:1 for 24 h led to an enhanced generation of NO. This finding is in line with the literature reporting an increased NO formation following EPA and DHA supplementation in human and porcine coronary artery ECs (Stebbins et al., 2008; Zgheel et al., 2014).

Moreover, the stimulatory effect of the coated EPA:DHA 6:1 nanoparticles on the endothelial formation of NO was observed over a 24-h period and more pronounced than with the native form whereas bradykinin induced only a transient formation of NO. In addition, the stimulatory effect of both the coated EPA:DHA 6:1 nanoparticles and native EPA:DHA 6:1 was markedly reduced by L-NA indicating the involvement of NO.

Role of omega-3 PUFAs in the antiplatelet aggregatory effect of endothelial cells

Dietary intervention with 500 g of oil-rich fish per week for 4 weeks increased omega-3 PUFAs in plasma phospholipids and reduced platelet-monocyte aggregation in 14 healthy subjects (Din et al., 2008). A study in healthy adults reported that intake of fish oil providing 6 g of EPA/day (Li & Steiner, 1990), or supplementation with 3.6 g of omega-3 PUFAs for a period of 15 days, reduced platelet adhesion stimulated by ADP or thrombin (Andrioli et al., 1999). The *in vitro* incorporation of EPA and DHA into human washed platelet membranes reduced platelet

procoagulant activity and thrombus formation (Larson et al., 2013). The mechanism by which supplementation with omega-3 PUFAs decreased platelet aggregation, has been attributable to a decrease in thromboxane A_2 (a potent proaggregatory eicosanoid) and an increased in antiaggregatory prostaglandins (Wander & Patton, 1991). It has also been suggested that omega-3 PUFAs could reduce the aggregation of platelets by increasing the synthesis of NO in endothelial cells (Abeywardena & Head, 2001; Sagripanti & Carpi, 2000).

To assess the ability of ECs to inhibit platelet aggregation in response to thrombin, ECs were cultured on Cytodex-3 microcarrier beads and treated with either coated nanoencapsulated or native EPA:DHA 6:1 before the addition to suspensions of washed human platelets. Although the addition of low numbers of EPA:DHA 6:1-treated ECs did not affect thrombin-induced aggregation, a pronounced inhibitory effect was observed in response to the addition of a similar number of coated EPA:DHA 6:1 nanoparticles-treated ECs. Moreover, the inhibitory effect was abolished by an eNOS inhibitor indicating the involvement of NO**.**

Omega-3 PUFAs and age-related endothelial dysfunction

Age-related endothelial dysfunction can occur in both conductance and resistance arteries (Blackwell et al., 2004; Csiszar et al., 2007; Lesniewski et al., 2009). Preclinical studies have associated the age-related endothelial dysfunction with a blunted NO- and EDH-mediated relaxation in the rat mesenteric artery (Dal-Ros et al., 2012; Farooq et al., 2020; Khodja et al., 2012) and a blunted NO component in the aorta (Bernd van der Loo et al., 2000; Gong et al., 2014; Novella et al., 2013; Sotomayor et al., 2005). Vascular studies have reported that intake of omega-3 PUFAs can improve endothelial function and overall vascular tone through vasodilation mechanisms (Limbu et al., 2018). However, there is evidence indicating that vasodilation mechanisms can vary depending upon the size of blood vessels. For example, NO has been reported to be the main pathway involved in conductance arteries whereas EDH is

more predominant in the smaller resistance arteries (Hilgers et al., 2006; Sandow & Hill, 2000; Shimokawa et al., 1996).

Therefore, the present study involved the experiments in both conductance (rat aorta) and resistance arteries (rat mesenteric artery) for the characterization of the mechanisms underlying the EPA:DHA 6:1-induced vasodilation. The study focused on investigating the short-term effects of both EPA:DHA 6:1 formulations in middle-aged rat arteries following a low dose supplementation.

In the present study, we decreased the dose of EPA:DHA 6:1 from 500 mg/kg previously used in our preclinical studies to 100 mg/kg to assess the potency of the formulation to improve the endothelial function in a model of established age-related endothelial dysfunction (Farooq et al., 2020; Niazi et al., 2017). The dose of 100 mg/kg/day of either native EPA:DHA 6:1 form or coated EPA:DHA 6:1 nanoparticles are equivalent to 1.14 g/day of omega-3 PUFAs in a 70 kg human (Reagan-Shaw et al., 2008). Such a dose is within the range of doses reported in different clinical studies, ranging from 0.18 to 10 g/day (Appel et al., 1993; Bhatt et al., 2019; Delgado-Lista et al., 2012; Enns et al., 2014; Miller et al., 2014).

The results indicate that in 50-weeks-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 concentrations of ACh and PE respectively (Farooq et al., 2020).

Treatment with low dose of both EPA:DHA 6:1 formulations significantly improved NO-mediated relaxations. The recovery of NO generation in the blood vessels of middle-aged rats was demonstrated by the marked enhancement of the phenylephrine-induced contractile responses in the presence of an eNOS inhibitor, indicating an increased basal release of NO in the main mesenteric artery and thoracic aorta. The effects observed with the coated EPA:DHA 6:1 nanoparticles treatment were more pronounced than those with the native form. These

findings are consistent with our previous investigations showing that EPA:DHA 6:1 is a potent stimulator of the endothelial formation of NO in isolated porcine coronary arteries (Zgheel et al., 2014), human internal mammary artery (Zgheel et al., 2019) and main mesenteric artery of 20 months old rats (Farooq et al., 2020).

Engler demonstrated that the EPA-induced relaxation of the isolated rat aorta was sensitive to COX inhibition (Engler et al., 2000), which is consistent with the current *ex vivo* findings demonstrating an inhibitory effect of indomethacin on native EPA:DHA 6:1-induced relaxation. However, the *in vivo* study demonstrated that COX-derived metabolites are not involved in the relaxation mediated by both EPA:DHA 6:1 formulations in the isolated aorta and the mesenteric artery possibly due to the fact that they are less sensitive to oxidation *in vivo* than *ex vivo*.

Omega-3 PUFAs and age-related vascular oxidative stress

Aging is associated with an increased generation of ROS affecting the function of ECs (Herrera et al., 2010). An increased vascular level of oxidative stress promotes premature senescence and the development of cardiovascular diseases (Ungvari et al., 2008). Age-related increase in ROS, either by an excessive formation or a reduced inactivation, is a major event leading to endothelial dysfunction (El Assar et al., 2013; Higashi, 2009; Schieber & Chandel, 2014). Moreover, the increased expression level of eNOS in the middle-aged artery is mostly likely part of a compensatory mechanism subsequent to the reduced bioavailability of NO. When oxidative stress increases, bioavailability of NO decreases and a compensatory mechanism will activate the expression of eNOS in the old artery.

In the current study, we used dihydroethidium (DHE) and MitoSOX redox-sensitive fluorescent probes to evaluate the level of oxidative stress in the arterial wall and in the mitochondria, respectively. Results showed a significantly elevated ethidium and MitoSOX fluorescence in

the aortic sections of middle-aged rats compared to young rats demonstrating an increased level of vascular and mitochondrial oxidative stress with increasing age. The low dose treatment with EPA:DHA 6:1 regenerated the endothelial function as indicated by the normalization of the vascular and mitochondrial level of oxidative stress and also be the normalization of the expression level of eNOS and nitrotyrosine in the thoracic aorta, and that this effect is more pronounced with the coated EPA:DHA 6:1 nanoparticles treatment. The reduction of oxidative stress by EPA:DHA 6:1 is in line with the findings of Farooq *et al* showing a reduction of oxidative stress in the mesenteric artery and aortic sections of old rats after the 2-weeks oral intake of EPA:DHA 6:1 treatment (Farooq et al., 2020).

The improvement of the NO-mediated relaxation and the increased basal release of NO after the 1-week treatment of middle-aged rats with the EPA:DHA 6:1 nanoformulation could be partly due to a reduction in the ROS-mediated inactivation of NO and in eNOS uncoupling.

The local vascular angiotensin system contributes to the induction of endothelial dysfunction and the associated vascular oxidative stress in *in vitro* and preclinical models of aging and hypertension (Dal-Ros et al., 2009; Harrison et al., 2003; Rajagopalan et al., 1996). Ageing has been associated with an increased expression of angiotensin II and ACE in the aorta of 30 months old rats (Challah et al., 1997), whereas an age-related increase in expression of AT1R, angiotensin II and ACE was reported in the thoracic aorta of 24 months old mice (Yoon et al., 2016).

Moreover, treatment of old rats either with either an ACE inhibitor or an ATR1 antagonist improved endothelial dysfunction (Mukai et al., 2002). 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 the secondary branch mesenteric artery (Niazi et al., 2017). Similarly, the 2-weeks oral intake of EPA:DHA

6:1 treatment normalized the expression level of AT1R and ACE in the mesenteric artery and aortic sections of old rats (Farooq et al., 2020).

In line with these studies, the present findings indicate a significantly increased expression of AT1R and ACE in the endothelium and vascular smooth muscle of aortic sections of middle-aged rats in comparison with young rats, indicating an activation of the local angiotensin system, which was improved partially by the native EPA:DHA 6:1 treatment and normalized by the coated EPA:DHA 6:1 nanoparticles treatment to a level similar to that observed in young rats.

Altogether, the present findings indicate that nanoencapsulation of EPA:DHA 6:1 followed by coating with gum potentiates and sustains their ability to stimulate the endothelial formation of NO in cultured endothelial cells and isolated coronary arteries and, hence, enhances their vasodilatory and antiaggregatory properties. Thus, nanoencapsulation of omega-3 PUFAs followed by coating appears as an attractive approach to better protect the vascular system.

Moreover, a short term intake of coated EPA:DHA 6:1 nanoparticles significantly improved the ageing-related endothelial dysfunction in rats. The beneficial effect involves an improvement of the NO-mediated relaxations as well as a reduction of oxidative stress and the local vascular angiotensin system.

Conclusion and Perspectives

The present findings provide evidence that nanoencapsulation of omega-3 PUFAs potentiates and sustains their ability to stimulate the endothelial formation of NO in cultured endothelial cells and isolated coronary arteries and, hence, enhances their vasodilatory and antiaggregatory properties. Such a sustained formation of NO over, at least, a 24-h period is of major interest to better protect the vascular system. In contrast to the physiological agonist bradykinin that caused a bolus of NO formation for only about 30 min, a continuous low level of NO formation of about 2-fold has been suggested to be optimal to protect the cardiovascular system against the initiation and development of cardiovascular disease. Indeed, a 2-fold sustained endothelial formation of NO accounts for the beneficial effect of shear stress on the cardiovascular system (Davies, 2009). In addition, atheroprone arterial sites such as bifurcations and curvatures are characterized by a suboptimal endothelial formation of NO due to a reduced activation of endothelial cells by low shear (disturbed or turbulent blood flow) (Warboys et al., 2014). As a consequence of a reduced endothelial formation of NO, pro-inflammatory and pro-atherothrombotic responses are not counter-regulated and will promote the initiation of early atherosclerotic lesions that can progress to advanced plaques and, subsequently, lead to an atherothrombotic event (i.e., acute coronary syndrome, stroke). Thus, nanoformulations of omega-3 PUFAs appear as an attractive approach to protect atheroprone arterial sites at risk.

The *in vivo* investigations indicated that ageing-related endothelial dysfunction in rats is associated with a reduced endothelial NO formation and an increased level of oxidative stress throughout the arterial wall, which are in good agreement with previous studies (Farooq et al., 2020; Khodja et al., 2012). Treatment with 100 mg/kg/day of the EPA:DHA 6:1 nanoformulation for one week significantly improved the endothelial function and restored the NO-mediated relaxation in response to ACh and also increased the basal formation of NO in the main mesenteric artery and thoracic aorta of middle-aged rats. Moreover, the findings

indicate that the improvement of the endothelial function is associated with the normalization of the local angiotensin system, which contributes to the excessive level of oxidative stress (Farooq et al., 2020; Niazi et al., 2017).

Altogether, our findings indicate that intake of a low dose of nanoencapsulated omega-3 PUFAs (equivalent to 100 mg/kg/day of EPA:DHA 6:1) for a period as short as 1 week is associated with an improved endothelial function in middle-aged rats. They further indicate that the omega-3 PUFAs treatment is effective to improve an established ageing-related endothelial dysfunction in rats, and could be of potential relevance not only to improve a pathological arterial wall but possibly also to protect atheroprone arterial sites at early stages to promote healthy ageing. Further investigations are required to evaluate the potential of omega-3 formulations to improve the beneficial endothelial protective effect at arterial sites affected by disturbed blood flow and low shear stress in the presence of cardiovascular risk factors including ageing, hypertension, and diabetes. In addition, it would also be interested to evaluated the effect of omega-3 PUFAs nanoformulation on vascular and cardiac remodeling often contributing the cardiovascular disease. Furthermore, it would be of interest to evaluate whether EPA:DHA 6:1 metabolites are involved in the beneficial effects on the endothelial function and the vascular system.

Altogether, the present findings highlight the vasoprotective potential of omega-3 nanoformulation by restoring the endothelial formation of NO in ageing.

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Annexes

Scientific production

Publications

Lamia Remila, Eugenia Belcastro, Nazende Guenday-Tuereli, Sinhee Park, Ursula Houngue, Thierry Vandamme, Emre Tuereli, Paul Kerth, Cyril Auger, Valérie Schini-Kerth. **Nanoencapsulation of the omega-3 EPA:DHA 6:1 formulation enhances and sustains NO-mediated endothelium-dependent relaxations in coronary artery rings by perpetuating the endothelial formation of NO.** (Submitted to Journal of Functional Food, 2021).

Lamia Remila, Nazende Guenday-Tuereli, Ursula Houngue, Eugenia Belcastro, Christophe Bruckert, Thierry Vandamme, Emre Tuereli, Paul Kerth, Cyril Auger, Valérie Schini-Kerth. **Short-term intake of coated EPA:DHA 6:1 nanoparticles improves age-related endothelial dysfunction by enhancing endothelium-dependent relaxations and ameliorating oxidative stress: role of nitric oxide.** (In preparation)

Faraj Zgheel, Stéphanie Perrier, **Lamia Remila**, Jean-Philippe Mazzucotelli, Olivier Morel, Cyril Auger, Valérie B. Schini-Kerth. **EPA:DHA 6:1 is a superior omega-3 PUFAs formulation attenuating platelets-induced contractile responses in porcine coronary and human internal mammary artery by targeting the serotonin pathway via an increased endothelial formation of nitric oxide**. European Journal of Pharmacology, 2019;853:41-48.

Eugenia Belcastro, Asad Ur Rehman, **Lamia Remila**, Sin-Hee Park, Dal Seong Gong, Nicolas Anton, Cyril Auger, Olivier Lefebvre, Jacky G. Goetz, Mayeul Collot, Andrey S. Klymchenko, Thierry F. Vandamme, Valérie B. Schini-Kerth. **Fluorescent nanocarriers targeting VCAM-1 for early detection of senescent endothelial cells**. Nanomedicine: Nanotechnology, Biology and Medicine, 2021;43:11.

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Article III

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Fluorescent nanocarriers targeting VCAM-1 for early detection of senescent endothelial cells

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Abstract

Endothelial senescence has been identified as an early event in the development of endothelial dysfunction, a hallmark of cardiovascular disease. This study developed theranostic nanocarriers (NC) decorated with VCAM-1 antibodies (NC-VCAM-1) in order to target cell surface VCAM-1, which is overexpressed in senescent endothelial cells (ECs) for diagnostic and therapeutic purposes. Incubation of Ang II-induced premature senescent ECs or replicative senescent ECs with NC-VCAM-1 loaded with lipophilic fluorescent dyes showed higher fluorescence signals than healthy EC, which was dependent on the NC size and VCAM-1 antibodies concentration, and not observed following masking of VCAM-1. NC loaded with omega 3 polyunsaturated fatty acid (NC-EPA:DHA6:1) were more effective than native EPA:DHA 6:1 to prevent Ang II-induced VCAM-1 and p53 upregulation, and SA-β-galactosidase activity in coronary artery segments. These theranostic NC might be of interest to evaluate the extent and localization of endothelial senescence and to prevent pro-senescent endothelial responses.

Key words: Nanocarriers; Endothelial senescence and dysfunction; VCAM-1; Omega 3 polyunsaturated fatty acid

Cardiovascular diseases (CVDs) are the leading cause of death in the developed world and represent an immense clinical burden.[1] Atherothrombosis, a chronic and progressive inflammatory disease,[2] has been identified as the major contributor to the pathogenesis of CVDs including acute coronary syndrome and stroke.[3]

Although clinical scores, such as that derived from the Framingham Heart Study, are useful to evaluate the cardiovascular risk of patients, they may lose predictive value in the large segment of the population at intermediate risk.[4] Thus, there is a crucial need for novel diagnostic tools to identify early molecular changes associated with atherogenesis and to assess noninvasively the benefit of therapies, and also for new targeted and specific therapies. Recent findings have emphasized the potential role of nanoparticles, as promising tools for treatment of vascular disease, including atherothrombosis and its associated complications.[5] The recent development of ultrabright theranostic nanoparticles that may simultaneously act as carriers of both imaging payloads and therapeutic agents, has great promises for the future of personalized medicine.[6]

The atherogenesis process starts before childhood with the appearance of early lesions of atherosclerosis, which can progress into mature plaques impeding blood flow and optimal perfusion of target organs, and, ultimately, trigger an

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atherothrombotic event. Interestingly, the atherogenesis process is not a generalized alteration of the cardiovascular system but is targeting well-defined arterial sites at risk such as bifurcations and curvatures including the carotid and aorta-renal bifurcations.[7,8] Such atheroprone areas are characterized by an early development of endothelial dysfunction most likely as a consequence of their particular local flow behavior involving disturbed flow and low shear versus the atheroprotective areas characterized by laminar flow and high shear.[3,9] Indeed, high shear stress is a pivotal trigger of endothelial cell (ECs) protective mechanisms especially the formation of nitric oxide (NO), a potent vasodilator and inhibitor of atherothrombotic responses. Thus, ECs at athero-susceptible arterial sites are unlikely to protect adequately the arterial wall thereby accelerating the development of atherosclerotic lesions.[3]

Recent investigations support the concept that endothelial senescence is a determinant event leading to the development of endothelial dysfunction.[10,11] Cellular senescence is characterized by the hallmark marker senescence-associated β galactosidase activity, an irreversible cell cycle arrest,[12] and the induction of a pro-inflammatory senescence-associated secretory phenotype (SASP)[13,14,15] with increased expression of VCAM-1, MCP-1 and tissue factor, and down-regulation of the endothelial formation of NO, and is induced by pro-oxidant stimuli such as angiotensin II (Ang II) and elevated glucose concentration.[16,17] Increased numbers of senescent ECs have been observed overlying plaques in the human coronary artery[18] and the aortic arch.[19] The fact that overexpression of the senescence marker p53 selectively in the endothelium blunted NO formation and promoted endothelial dysfunction in rat aortic rings,[20] suggests that endothelial senescence leads to endothelial dysfunction. Among vasoprotective treatments, omega 3 polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to protect the cardiovascular system, in part, by causing a sustained endothelial formation of NO. Indeed, our previous studies indicated that EPA:DHA 6:1 is a superior omega 3 PUFA formulation to induce endothelium-dependent vasorelaxation in coronary artery rings, to cause eNOS activation in ECs, and to improve aging-related endothelial dysfunction.[21,22]

Thus, the main objective of the present study was to target endothelial senescence by taking advantage of a unique feature of the cell membrane of senescent ECs, the increased cell surface expression of VCAM-1. For this purpose, a core-shell nanoemulsions carrier decorated with antibodies (Abs) targeting VCAM-1 was generated and loaded with either lipophilic red or near-infrared (NIR) fluorescent dyes to enable detection of senescent ECs using fluorescence imaging or EPA:DHA 6:1 for delivery and regeneration of the protective endothelial function.

Methods

A detailed description of the experimental protocols and chemicals is provided in the Supplementary material.

Preparation of maleimide-decorated nanoemulsions: grafting of anti-VCAM-1 antibody and fluorescent dye loading

Oil and aqueous phases were prepared separately and heated at 90 °C for 30 min. Oil phase consists of polymer and 1% of dye based on Nile Red (NR668)[23] or NIR cyanine with a bulky counterion tetraphenylborate (Cy5.5-TPB)[24] dissolved in oil (Labrafac WL®), while the aqueous phase used was distilled water. 100 mg of maleimide modified polymer (synthesis described in supporting information) and 25 mg of dye/Labrafac WL® were mixed in a vial (4 ml). Then, 800 μl of hot distilled water was added and this mixture was homogenized by vortex for 30 s to obtain a primary emulsion and finally homogenized by ultrasonication to produce nanoemulsions (technical details are reported in the *Supplementary information* section). In some experiments, the non-functionalized nanocarriers were formulated with EPA:DHA 6:1 instead of Labrafac WL® as dispersed phase and distilled water as continuous phase, without any fluorescent probe. Furthermore, different amounts of the anti-VCAM-1 Ab were added into the diluted nanoemulsions to study the effect of increasing concentrations of the antibody on the extent of grafting. After Ab addition, the nanoemulsions were incubated for 24 h at 4-6 °C to cause attachment of the Ab onto the maleimide functions at the surface of the nanocarriers (NC-VCAM-1). The maleimide-decorated nanoemulsions, without grafting of the antibody at the surface of the nanocarriers (NC), were also used as a control formulation for the study.

Endothelial cell culture and preparation of coronary artery rings

ECs were isolated from freshly harvested porcine left circumflex coronary arteries using type I collagenase as described previously,[25] and cultured in MCDB 131 medium containing 15% fetal calf serum, fungizone (2.5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 5 L-glutamine (2 mM). ECs at passage 1 were exposed to serum-free culture medium for 2 h before the addition of Ang II to induce premature senescence. ECs passaged at a ratio of 1:3 until passage 3 to induce replicative senescence were also studied.[26] Coronary artery segments were incubated in RPMI without serum for 1 h before the addition of Ang II. Thereafter, they were embedded in FSC22 Frozen section medium and frozen.

Detection of senescence-associated β-galactosidase activity (SA-β-gal)

Senescence-associated β-galactosidase activity was assessed in porcine coronary artery segments by staining with X-gal solution as previously reported.[27]

Immunofluorescence studies and physicochemical characterization of nanocarriers

For immunofluorescence histochemistry, ECs and isolated healthy porcine coronary artery rings were evaluated using a Leica SP2 UV DM Irbe confocal laser-scanning microscope with 20× or 63×/OIL CS objectives. Quantification of fluorescence levels was performed with macros developed with Image J software. Nanocarrier physicochemical characteristics were

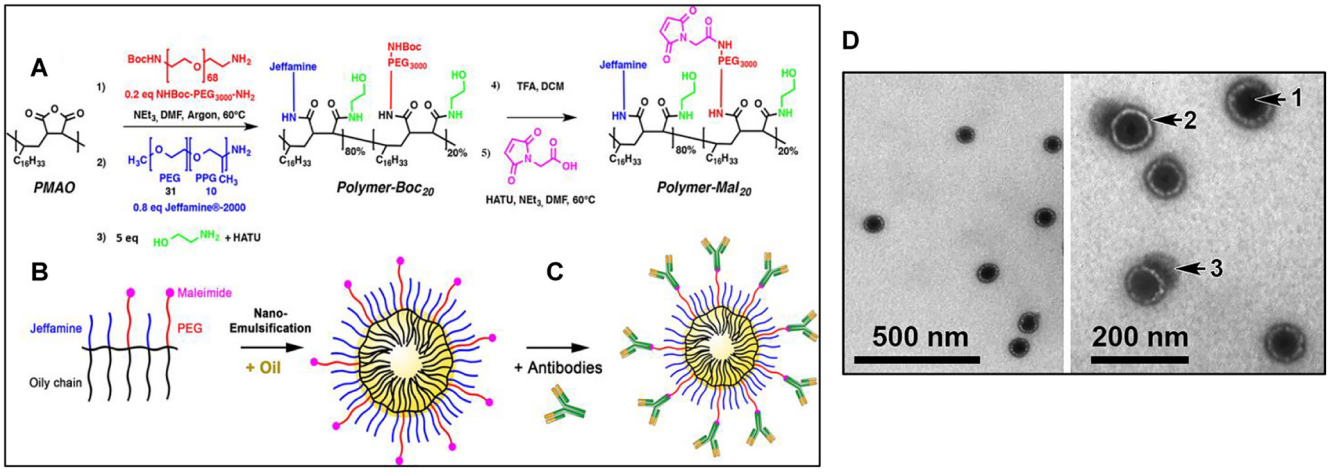

Figure 1. Design and chemical development of targeting lipid nanocarriers. **(A)** Synthesis of the maleimide decorated polymer, starting by a reaction between PMAO and Boc-NH-PEG3000-NH_2 on 20% of the anhydride functions of the polymer (1); the remaining 80% of the anhydride functions of the polymer were then reacted with Jeffamine-2000 (2); afterwards, ethanolamine was used to neutralize the unreacted carboxylic groups (3); then the deprotection of the amino functions of the Polymer-Boc_{20} was performed (4) followed by coupling of amino groups with the maleimidoacetic acid (5) resulting in the formation of Polymer-Mal_{20}. **(B)** Formation of nanoemulsions by ultrasonication method and **(C)** grafting of VCAM-1 Abs with the available maleimide functions on the surface of the nanocarriers. **(D)** TEM characterization of nanoemulsions formed by using polymer dissolved in 1% Cy5.5-TPB solution in Labrafac® as oil phase and distilled water as aqueous phase with spherical shape.

evaluated for the chemical optimization, such as the nanocarrier average size, the different amounts of Abs, the PEG/PEG-antibody ratio, the temperature stability, the use of different fluorescent probes and the targeting efficiency.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was assessed by one-way analysis of variance followed by Bonferroni's Multiple Comparison post hoc test using GraphPad Prism (Version 5). Group differences were considered statistically significant at $P < 0.05$.

Results

Synthesis of the amphiphilic polymer and evaluation of the structure by 1H NMR analysis

As a polymeric platform, PMAO is an alternated polymer (average M_n 30,000-50,000) with a repeating unit composed of a hydrophobic hydrocarbon chain (C_{18}) and a succinic anhydride function. As illustrated, an amphiphilic polymer (Polymer-Boc_{20}) was first synthesized through a reaction of PMAO with an amino-PEG_{3000} bearing a protected amino function (Boc-NH-PEG_{3000}-NH_2) on 20% of the anhydride functions (Figure 1, *A*). The remaining 80% of anhydride functions were then reacted with Jeffamine®2000, an amino/methoxy terminated PEG/PPG (poly(ethylene glycol)/poly(propylene glycol)) copolymer, to reduce the polymer hydrophobicity. Then the deprotection of the amino functions of Polymer-Boc_{20} was performed followed by coupling of the amino groups with the maleimidoacetic acid to result in the formation of Polymer-Mal_{20} (Figure 1, *A*). The progress of the reaction and the structure of the polymer were studied by performing NMR at each step of the synthesis of this amphiphilic polymer derivative (reported in Supplementary Information Figure S1). In Figure S1, ethanol is visible (not dry enough after dialysis) as well as the peak of NHBoc. The integration and comparison to the CH_3 of PEG show functionalization with PEG-NHBoc close to 20% (equal to 1.72 while 20% would correspond to a value of 1.8). This result proves that the ratio expected between non-functional and functional PEG chains is obtained. This value of 80% is confirmed with the integration of the NMR peak of the Me function (terminating the Jeffamines chains), as indicated in Figure S1. Then, the disappearance of the NHBoc peak in Figure S2 clearly confirmed the deprotection has been efficiently done. Comparison of two spectra is reported in Figure S3. On the other hand, NMR spectra with maleimide addition appear to be identical to the those after deprotection of Boc (Figure S4), likely due to screening of the numerous PEG chains present in these macromolecules. To summarize, NMR results have confirmed the attachment of Jeffamine® 2000 (80%) and PEG-NH_2 (20%) with the polymer network and confirmed the gradual building of the functional polymer.

Preparation of targeted fluorescent lipid nanocarriers

The multifunctional lipid nanocarriers were formulated by ultrasonication method. To render them fluorescent, their core was loaded with highly lipophilic dyes (NR668 or Cy5.5-TPB).[22,23] While NR668, operating in the red spectral range, is well adapted for fluorescence microscopy studies, the NIR dye Cy5.5-TPB is more suitable for *in vivo* and tissue imaging and they both display high stability against dye leakage in biological media.[28] The amphiphilic polymer derivative described above was used to stabilize the nanoemulsions and also to provide the facility to functionalize the nanocarriers after their formulation. During the formation of nanodroplets the amphiphilic polymer is expected

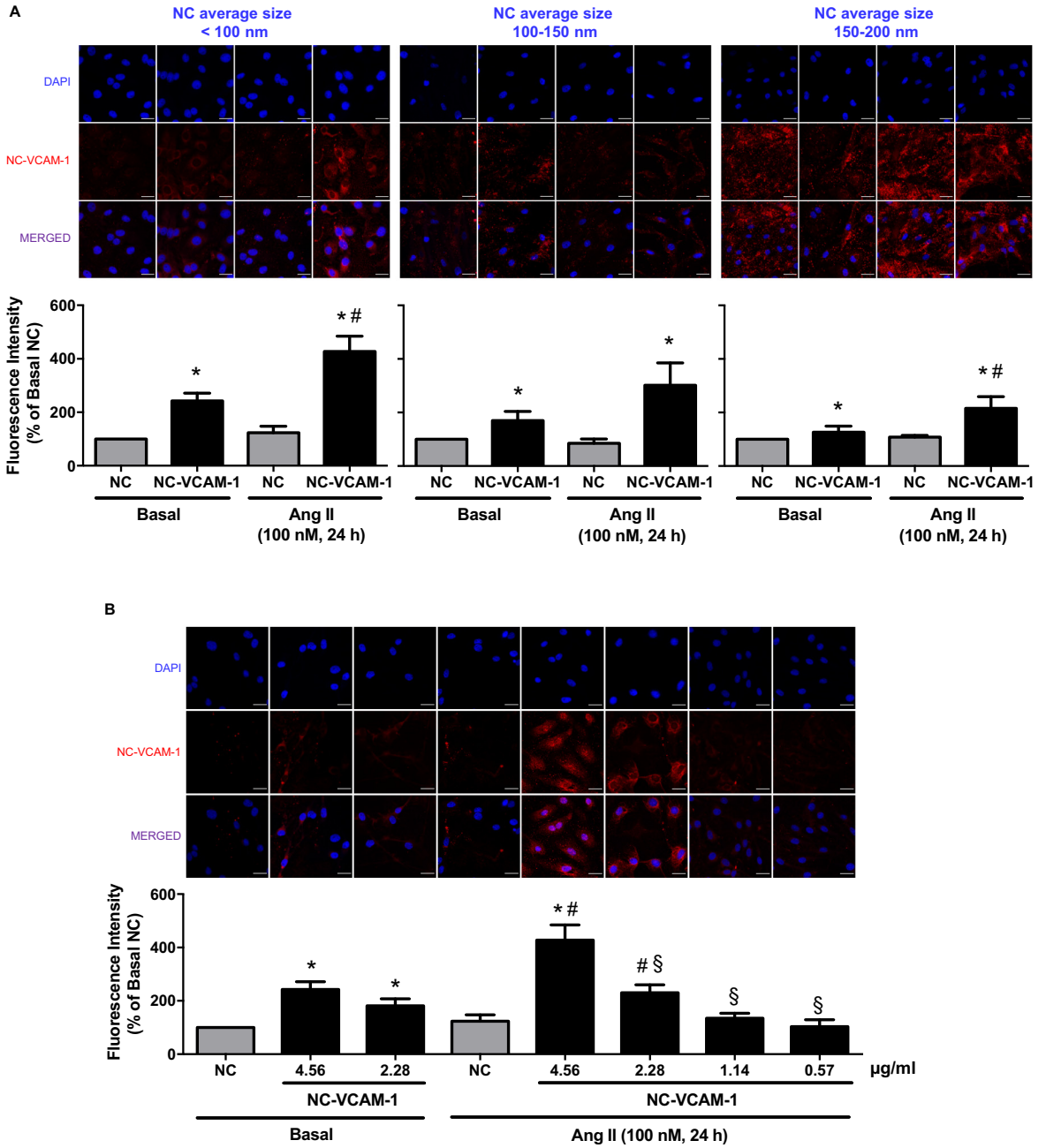

Figure 2. Optimization and evaluation of the theranostic potential of nanocarriers targeting surface VCAM-1 in a model of premature senescence. ECs are incubated with Ang II for 24 h to induce premature senescence and subsequently with either nanocarriers (NC) or nanocarriers decorated with VCAM-1 Abs (NC-VCAM-1) for 1 h. Experimental conditions tested NC with different diameters **(A)**, different concentrations of the anti-VCAM-1 Abs **(B)**, different storage temperatures **(C)**, and containing a specific designed lipophilic dye (NR668 or Cy5.5-TPB) **(D)**. NC and NC-VCAM-1 fluorescence staining appears in red and nuclei are stained with DAPI (blue). Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean ± SEM of n = 3-9. $^{*}P < 0.05$ vs respective NC, $^{\#}P < 0.05$ vs respective NC-VCAM-1, $^{\S}P < 0.05$ vs Ang II NC-VCAM-1 4.56 µg/ml. Original magnification, 63×. Scale bar = 20 µm.

to reorganize in such a way that the hydrophobic hydrocarbon chains remain in the apolar interior of the nanodroplets (oil phase) whereas the hydrophilic part of the polymer (J-2000 and maleimide-decorated PEG chains) is exposed towards the interface of the droplets (towards aqueous phase), as previously described.[29] This provides active maleimide functions at the surface of the nanodroplets which will ensure an anchorage of the VCAM-Abs, because the polymer is a part of the nanocarrier "shell" (Figure 1, *B*). Antibody decoration is performed by incubation of the functional maleimide-decorated nanoemulsions with the solution of anti-VCAM-1 Abs attaching the nanodroplets surface (Figure 1, *C*), giving rise to functionalized

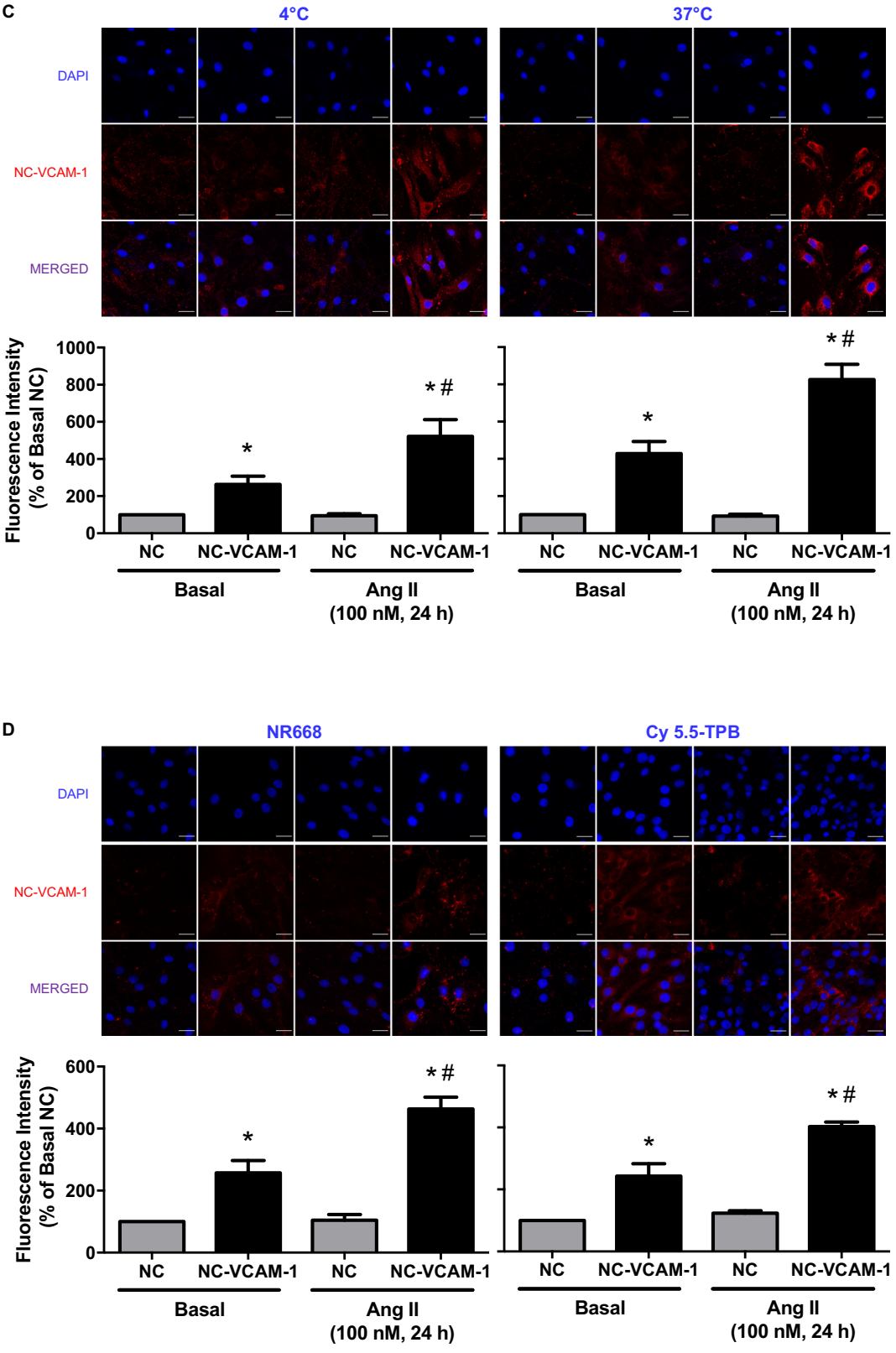

Figure 2. (*continued*).

nanocarriers. As a control formulation, nanoemulsions with a polymer without having the Boc-NH-PEG_{3000}-NH_2 component, i.e., PMAO:J-2000, 1:1 respectively were prepared. This formulation was prepared to compare the average hydrodynamic diameter as well as the surface charge on the droplets with the ones obtained with maleimide decorated nanocarriers.

Characterization of size and surface charge

The nanocarriers loaded with fluorescent probes (NR668 or Cy5.5-TPB) were characterized by DLS analysis. The average hydrodynamic diameter of the maleimide-decorated nanoemulsions was 96 ± 7 nm (n = 10). The hydrodynamic diameters obtained for nanocarriers developed by using Labrafac®, omega 3 EPA:DHA 6:1 oil and corn oil as dispersed phase were 96 nm, 80 nm and 190 nm, respectively. This difference is likely related to the nature of this oil impacting on the spontaneous emulsification process.[30] In the other two cases the average hydrodynamic diameters of the nanodroplets were below 100 nm, the optimal size for theranostic applications.[31] The average size of the nanodroplets obtained in the case of control formulation (PMAO: J2000 1:1) was considerably larger (around 160 nm) as compared to the maleimide-decorated nanodroplets. According to zeta-potential measurements, the surface charge on the nanoemulsion droplets, in the control formulation, was negative (−39 mV±1, n = 3) and the surface charge on the maleimide-decorated nanodroplets was positive and ranged from 10 to 15 mV, regardless of the nature of the oil used to formulate the nanocarriers (n = 8 for NR668/Cy5.5 TPB in Labrafac®, n = 3 for omega 3 EPA:DHA 6:1, n = 3 for corn oil). This change in the surface charge from negative to positive is most likely due to complete saturation of the carboxylic groups, followed by grafting of maleimide on the available sites.

TEM analysis of these nanocarriers (containing Cy5.5-TPB) is reported in Figure 1, *D*. The results confirmed the morphology and average size of the nanoemulsions given by DLS, i.e. below 100 nm. In addition, micrographs disclosed structural information, indeed coherent with the one expected: oil core (arrow 1 in Figure 1, *D*) is surrounded by a polymer shell (arrow 2). In some particles the presence of amorphous polymer can be observed (arrow 3). Interestingly, the bright aspect of the polymer shell is related to a pure and concentrated material, and this can be explained by the process itself. Initially the polymer is homogeneously solubilized in the oil phase, and then the emulsification process induces its concentration at the interface, making a concentrated shell. In some cases, excess of polymer (arrow 3) has induced amorphous domains. TEM pictures have confirmed a nanocarrier structure in line with the one eventually expected.

Optimization and evaluation of theranostics nanocarriers in in vitro senescence models

Size

In order to determine the importance of the size of VCAM-1 Ab-decorated nanocarriers, lipid nanocarriers with hydrodynamic diameters below 100 nm, 100-150 nm and 150-200 nm ranges were prepared by changing the time of formulation exposition to the ultrasonication process, and tested using control and Ang II-induced premature endothelial cell senescence. The highest fluorescence signal in cells was observed with the nanocarriers having sizes below 100 nm compared to those having sizes ranging from 100 to 150 nm and 150-200 nm (Figure 2, *A*). Thus, the targeting efficiency increased with the decrease in the size of the VCAM-1 Ab-decorated nanocarriers. Therefore, the lipid nanocarriers having diameter size below 100 nm were selected and used for further experiments. It is interesting to note that a better targeting efficiency is obtained for smaller particles. Indeed, for a similar amount of polymer and oils forming the nanocarriers, two different sizes mean a difference in particle number (concentration) and in the particle surface area. Thus, for the smaller particles, the particle concentration and surface area exposing specific ligands should be higher, which should favor targeting properties. In addition, smaller particles diffuse faster, allowing faster ligand/receptor interaction.

Different concentrations of anti-VCAM-1 antibodies

To find the optimum concentration of the Abs linked to the maleimide active sites at the surface of the nanocarriers for targeting, different concentrations of the anti-VCAM-1 Abs were tested and analyzed. Higher fluorescence signals were obtained with the formulation with the highest antibody concentration (4.56 μg/ml) in Ang II-induced endothelial cell senescence whereas low levels were observed in control cells (Figure 2, *B*), indicating that the targeting was directly dependent on the amount of VCAM-1 Abs decorating the nanocarriers. All further experiments were performed with NC-VCAM-1 generated with anti-VCAM-1 Abs at a concentration of 4.56 μg/ml.

Different temperature and encapsulated dye

To further characterize the targeting of the formulations and validate their stability, the role of the temperature was assessed. The fluorescent VCAM-1 Abs decorated nanocarriers targeted Ang II-induced senescent ECs within 1 h, at both 4 °C and 37 °C (body temperature) with minimal off-target cell surface interactions (Figure 2, *C*). Nanocarriers labeled with specially designed lipophilic derivatives of dyes either NR668 or Cy5.5-TPB were bright enough to allow the fine tracking of interactions with biological systems (Figure 2, *D*). Altogether, these results indicate that the different temperatures and fluorescent probes can be used to image active targeting of cells by the lipid nanocarriers.

Specific targeting of NC-VCAM-1 in premature and replicative senescence ECs

The targeting specificity of fluorescent VCAM-1 Abs decorated nanocarriers at 37 °C was evaluated by competition experiments using a VCAM-1 Ab to mask the VCAM-1 antigen. Nanocarriers decorated with VCAM-1 Abs strongly stained Ang II-induced senescent ECs and replicative senescence ECs (passage 3, Figure 3, *A*, *B*) whereas no such effects were observed following pretreatment of the cells with a VCAM-1 Ab demonstrating their uniqueness to selectively target membrane surface VCAM-1.

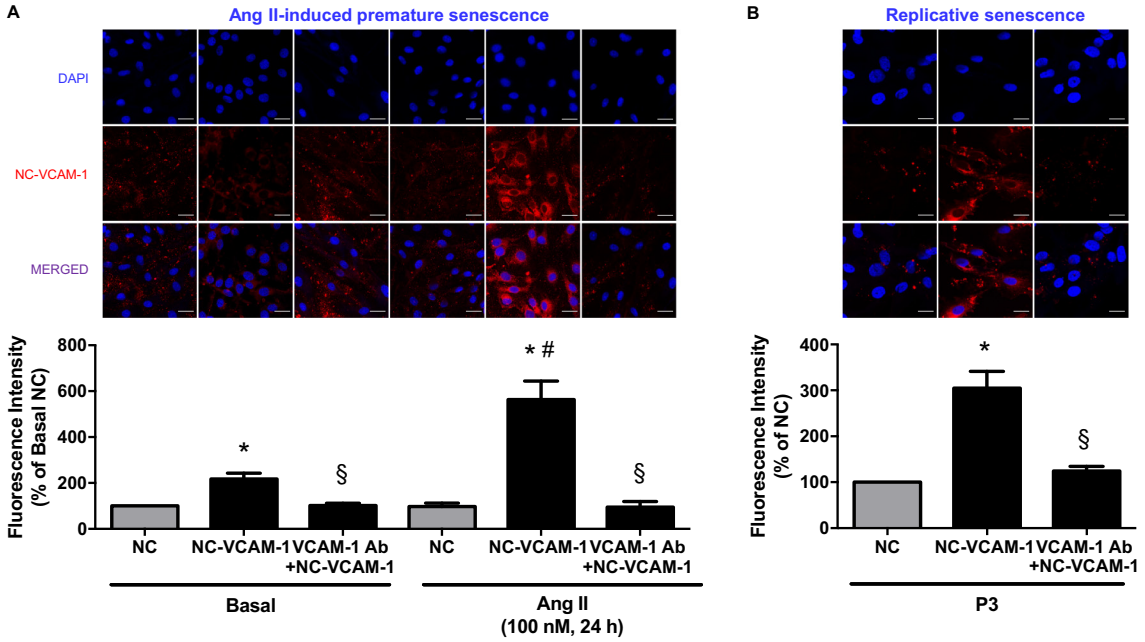

Figure 3. Selective targeting of the VCAM-1 decorated nanocarriers to premature and replicative senescent ECs. Nanocarriers decorated with VCAM-1 Abs (NC-VCAM-1) strongly stain ECs following their exposed to Ang II for 24 h to induce premature senescence **(A)**, and ECs at passage 3 (P3, replicative senescence) **(B)**. The targeting potential of NC-VCAM-1 is prevented by the previous exposure of ECs to the VCAM-1 Ab. NC and NC-VCAM-1 fluorescence staining appears in red and nuclei are stained with DAPI (blue). Results are shown as representative of immunofluorescence staining (upper panels) and corresponding cumulative data (lower panels). Data are expressed as mean ± SEM of n = 3-4. $^{*}P < 0.05$ vs respective NC, $^{\#}P < 0.05$ vs Basal NC-VCAM-1, $^{\S}P < 0.05$ vs respective NC-VCAM-1. Original magnification, 63×. Scale bar = 20 μm.

Protective effect of theranostics NC-EPA:DHA 6:1 in native endothelium of coronary artery segments

Since all investigations were performed with cultured ECs, experiments evaluated the protective effect of the omega 3 EPA:DHA 6:1 formulation and corn oil on Ang II-induced premature senescence in coronary artery segments, and to assess the impact of nanoencapsulation of the two oils. Exposure of coronary artery segments to Ang II upregulated the expression level of VCAM-1 and the senescence marker p53, and increased SA-β-gal activity predominantly in the endothelium (Figure. 4, *A*, *B*, *C*). The EPA:DHA 6:1 treatment significantly reduced the stimulatory effect of Ang II on VCAM-1 and SA-β-gal activity and reduced that on p53, which, however, did not reach statistical significance (Figure 4, *A*, *B*, *C*). The corn oil treatment blunted the stimulatory effect of Ang II on SA-β-gal activity and did not significantly affect that on VCAM-1and p53 (Figure 4, *A*, *B*, *C*). NC-EPA:DHA 6:1 markedly and significantly prevented Ang II-induced VCAM-1 and p53 upregulation in the endothelium of coronary artery segments, compared to the NC-CORN OIL (Figure 5, *A*, *B*). NC-EPA:DHA but not NC-CORN OIL also slightly but significantly reduced the VCAM-1 and p53 expression levels in control coronary artery segments (Figure 5, *A*, *B*).

Discussion

Atherosclerosis underlying major cardiovascular diseases, is a silent killer, which cannot be easily detected at an early stage by current imaging methods. Since the induction of endothelial senescence is thought to promote the development of endothelial dysfunction, an early event in the atherogenesis process, endothelial senescence appears to be an interesting target for both preventive and therapeutic interventions.[9,26,32,33] The major findings of the present study indicate the ability of nanocarriers decorated with VCAM-1 Abs and loaded with lipophilic fluorescent dyes to selectively and specifically target VCAM-1 overexpressed at the surface membrane of cultured senescent ECs compared to healthy ECs. They further show that nanoencapsulation of the vasoprotective omega 3 formulation EPA:DHA 6:1 is associated with enhanced inhibition of pro-atherosclerotic and pro-senescence responses in the native endothelium of isolated coronary arteries. Thus, fluorescent nanocarriers targeting a senescence-associated cell surface protein appear to be an attractive strategy to restore the protective endothelial function on the vascular system.

A low-grade inflammatory response is involved in the atherogenesis process affecting initially the endothelium with the appearance at the cell surface of several pro-atherothrombotic molecules including adhesion molecules that are tightly regulated both spatially and temporally.[3,34] Thus, these novel cell surface molecules are interesting targets for innovative diagnostics and therapeutics approaches in atherogenesis. Among potential candidates, VCAM-1 appears to be of particular interest since it is involved in the development of early atherosclerotic lesions[35,36] and has been observed on the endothelial cell surface of atheroprone areas before the onset of visible structural changes,[34] and increased levels of VCAM-1

expression have been associated with senescent ECs overlaying human atherosclerotic plaques.[18]

To date, several approaches have been described to image VCAM-1 and other cell adhesion molecules expression using radiolabeled antibodies,.[37,38] However, these agents usually result in modest target-to-background ratios,[37,38] limiting their use for *in vivo* cardiovascular imaging. Therefore, there is a need to develop alternative methods for imaging, ideally with improved sensitivity and resolution. Recently, nanoparticles have attracted a lot of interest as potential therapeutics, diagnostics, and theranostics due to their ability to target in a specific manner to the site of disease and to reduce the therapeutic load and, hence, side effects. The nanometric size of these materials and their surface functionalization characteristics preclude them from being readily cleared through the kidneys and decelerate the opsonization mechanism, thereby extending circulation in blood.[39]

Targeted nanoparticles like polymeric nanoparticles (e.g., PLGA), liposomes and nanoemulsions, have been shown to specifically accumulate in ECs, and suggested to be used for imaging or drug delivery applications.[5] In comparison to the other nanocarriers, nanoemulsions (the system used in the current study) are particularly attractive as drug delivery vehicles because of their non-toxic components, high loading capacity, biodegradability, good stability, low manufacturing cost and their potential to encapsulate hydrophilic, lipophilic, as well as amphiphilic drugs.[40–44] Such a system is a dispersion of two immiscible liquids (water-in-oil or oil-in-water), stabilized by a surfactant, which is a isotropically clear and thermo-dynamically stable liquid solution, usually with droplet diameter within the range of 10-500 nm.[30,45,46] However, it is not easy to chemically modify the surface of the nanoemulsions due to the challenges involved in the stabilization of the interface. Recent findings demonstrate that when the oil phase containing the polymer is in contact with the aqueous phase, the polymer PMAO as well as its derivatives with Jeffamine polyetheramines (J-1000 & J-2000), shows the ability to migrate at the oil–water interface and produce stable oil-in-water nanoemulsions in the absence of an organic solvent as well as surfactant.[29] In the current study, a strategy to functionalize nanoemulsions with antibodies based on PMAO polymer has been developed. Chemical modification of PMAO, by performing a systematic reaction of Jeffamine polyetheramine (Jeffamine® M2070) and a polyethylene glycol (PEG)-containing compound terminated by a reactive maleimide on each anhydride function of the polymer

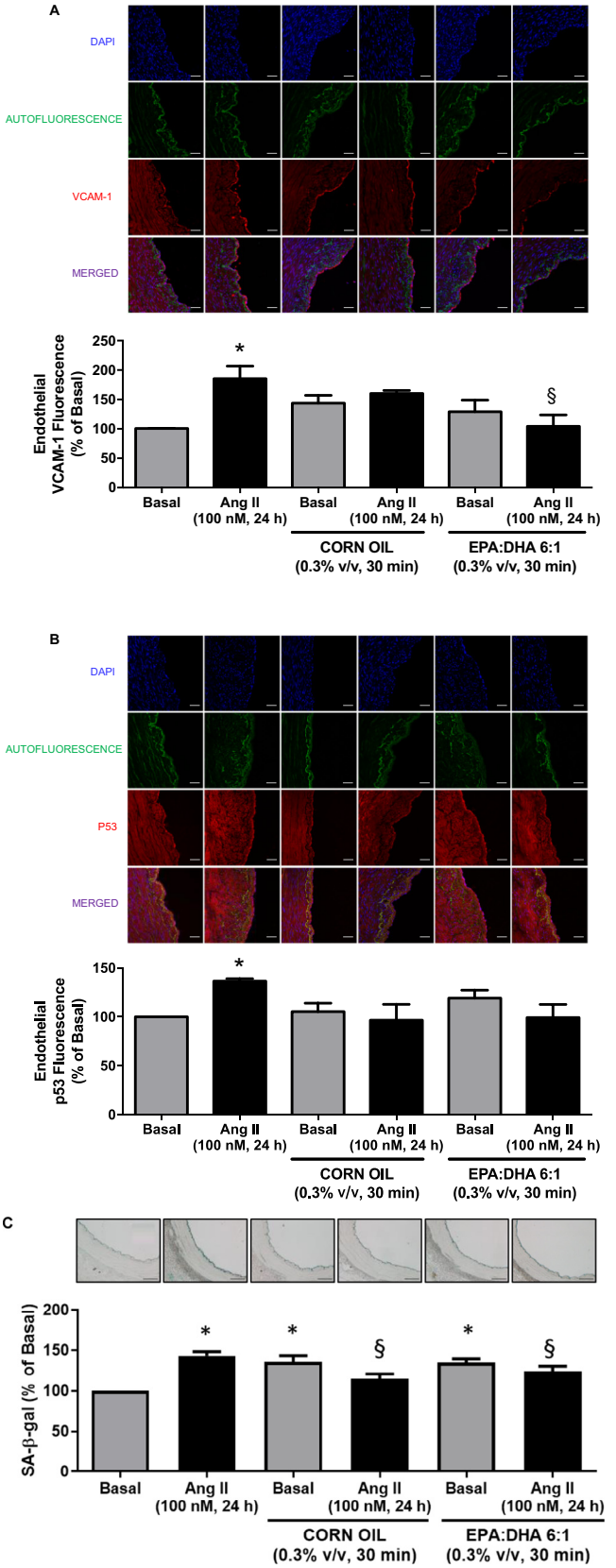

Figure 4. Native EPA:DHA 6:1 prevents the Ang II-induced up-regulation of VCAM-1 and SA-β-gal activity in the endothelium of coronary artery segments. Segments were either untreated, or exposed to CORN OIL or EPA:DHA 6:1 for 30 min before the addition of Ang II for 24 h. Thereafter, VCAM-1 and p53 signals were assessed by immunofluorescence, and SA-β-gal activity using X-gal. Representative images show VCAM-1 **(A)** and p53 **(B)** staining in red, nuclei in blue and autofluorescence in green (upper panels), and SA-β-gal activity (**C**, upper panels) and corresponding cumulative data of the endothelial signal (lower panels). Data are expressed as mean ± SEM of $n = 3$-4. $^{*}P < 0.05$ vs basal, $^{\S}P < 0.05$ vs Ang II. Original magnification, 20×. Scale bar = 50 μm **(A, B)**. Original magnification, 10×. Scale bar = 200 μm **(C)**.

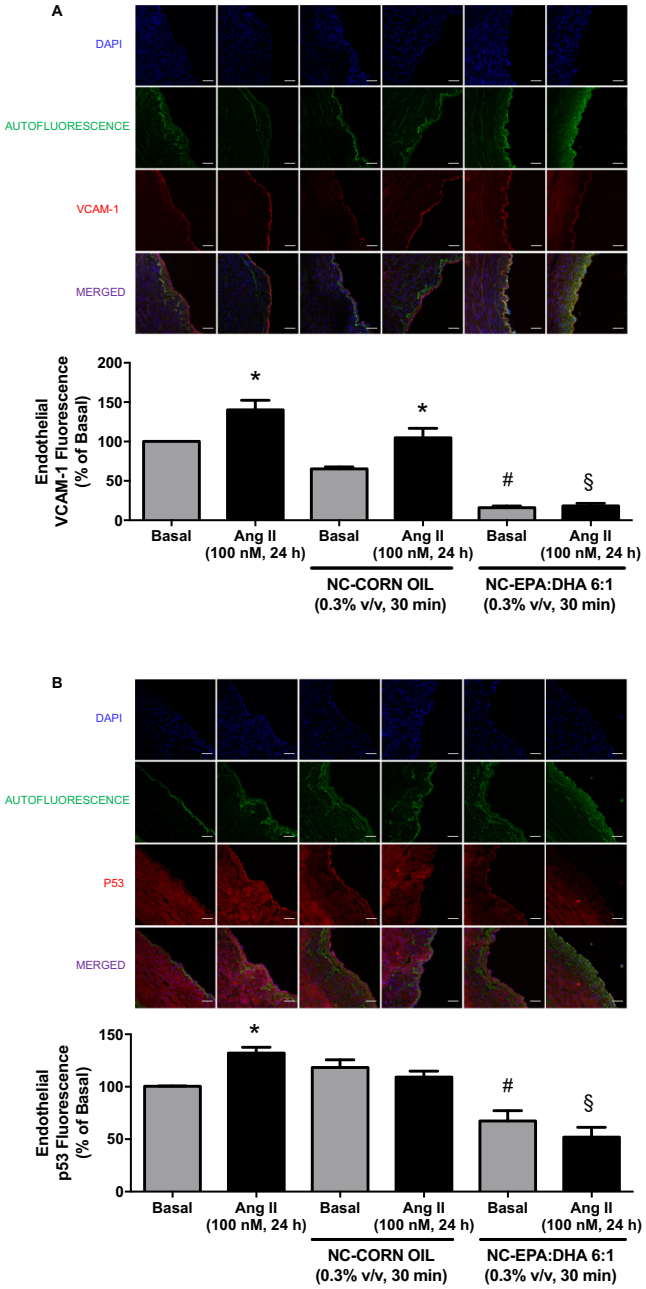

Figure 5. NC-EPA:DHA 6:1 strongly prevent the Ang II-induced up-regulation of VCAM-1 and p53 immunofluorescence signals in the endothelium of coronary artery segments. Segments were either untreated, or exposed to NC-CORN OIL or NC-EPA:DHA 6:1 for 30 min before the addition of Ang II for 24 h. Thereafter, the expression levels of VCAM-1 and p53 were assessed by immunofluorescence. Representative confocal immunofluorescence images showing VCAM-1 **(A)** and p53 **(B)** staining in red, nuclei in blue and autofluorescence in green (upper panels), and corresponding cumulative data of the endothelial signal (lower panels). Data are expressed as mean ± SEM of $n = 3$. $^{*}P < 0.05$ vs respective basal, $^{\#}P < 0.05$ vs untreated basal, $^{\S}P < 0.05$ vs Ang II. Original magnification, 20×. Scale bar = 50 μm.

has been made. The nanoemulsions are then developed using this newly synthesized polymer, with some PEG presenting maleimide at the surface of the nanodroplets to react with the cysteine function of the anti-VCAM-1 antibody, by ensuring a strong anchorage of the ligands since it will be a part of the nanocarriers as a "shell". The use of Jeffamine®2000 and PEG in this system, has great significance because the PEG-chains not only create steric repulsion among the neighboring nanodroplets and improve the interfacial stabilization but also have a well-recognized ability to i) reduce the uptake of the nanoemulsions by the immune system (after *in vivo* administration) and ii) inhibit the opsonization and phagocytosis processes, normally observed with conventional nanoemulsions, which in turn increases the circulation time of nanoemulsions in the body.[47,48] Another important chemical feature of this formulation is the type of fluorescent probe used. The probes studied, namely NR668 and Cy5.5-TPB, are specially designed to be highly lipophilic, resistant to dye leakage in biological media[28] and can be highly concentrated without excessive decrease of the fluorescence quantum yield.[23,24,49–52] As a result, nanocarriers are ultrabright and allow fine tracking of their interactions with biological systems.[23,24,49,50]

The biological evaluation of the fluorescent nanocarriers targeting VCAM-1 was assessed *in vitro* in premature and replicative senescent cultured ECs[26,33] and ex vivo in senescent native endothelium of isolated porcine coronary arteries. The nanoemulsion formulation has been optimized in relation to the size of the nanodroplets, which has an important impact on their interaction with living cells and also on the number of functional sites,[31,52] and the amount of the antibody to be attached to the maleimide groups available at the surface of the nanodroplets, which influences the efficient targeting of the targets.[39] These studies indicate that nanocarriers with average size below 100 nm and the highest antibody concentration tested (4.56 μg/ml) showed maximum fluorescence signals in senescent compared to healthy ECs, indicating that the targeting was directly dependent on the amount of VCAM-1 Abs decorating the nanocarriers. The targeting of senescent ECs by the nanocarriers is observed rapidly within 1 h, pronounced at body temperature and with minimal off-target cell surface interactions, and similar with the two fluorescent probes.

Among vasoprotective treatments, the omega 3 PUFAs, including EPA and DHA, have emerged as an attractive therapy to protect the cardiovascular system, by improving the endothelial function.[21] The beneficial effect of the omega 3 treatment is critically dependent on the purity level of the EPA and DHA, the EPA:DHA ratio, and also on the ability to protect the highly sensitive double bonds within their structures against oxidization.[21] Indeed, highly purified EPA:DHA 6:1 is a superior formulation than pure EPA, pure DHA and other EPA:DHA ratio including 1:1, causing a sustained endothelial formation of NO in cultured ECs and isolated artery rings including the human mammary artery.[21,53] The oral daily intake of EPA:DHA 6:1 has shown antihypertensive and improvement of the endothelial function in Ang II-treated rats, and has improved aging-related endothelial dysfunction by normalizing vascular levels of oxidative stress and restoring the endothelial formation of NO.[22] Such findings are in good agreement with those of the REDUCE-IT clinical trial indicating that the intake of pure EPA (4 g) provided about a 25% additional reduction in major adverse cardiovascular events in patients with elevated

cardiovascular risk treated with a statin.[54] Therefore, the present study has evaluated the effect of EPA:DHA 6:1 compared to that of corn oil, an isocaloric "control" lipid, both in the native form and after loading in non-functionalized nanocarriers on the Ang II-induced activation of the native endothelium using coronary artery segments. The Ang II treatment caused the appearance of pronounced VCAM-1 and p53 signals and increased SA-β-gal activity predominantly in the endothelium highlighting the particular susceptibility of the endothelium in the arterial wall towards pathological stimuli.[55] The stimulatory effect of Ang II on the endothelial VCAM-1 and p53 signals was reduced to a greater extent by the nanoencapsulated EPA:DHA 6:1 compared to the native omega-3 form, and also compared to nanoencapsulated and native corn oil. Thus these ex vivo investigations indicate a therapeutic potential of nanocarriers containing EPA:DHA 6:1 in the core to reach sufficient levels to restore the key protective endothelial function.

In conclusion, the present findings indicate the efficiency of fluorescent nanocarriers decorated with VCAM-1 Abs to selectively and specifically target VCAM-1 overexpressed at the membrane surface of cultured senescent ECs. They further indicate that nanoencapsulation of the vasoprotective and highly sensitive to oxidation EPA:DHA 6:1 leads to a greater beneficial effect towards the Ang II-induced dysfunction of native endothelium ex vivo. Additional *in vivo* studies are required to provide evidence of their monitoring and regenerative potentials of pathological endothelium overlying areas at risk. Ultimately, the developed platform could be applied to other targets, making it a useful scaffold for the development of novel targeted therapeutics and/or diagnostic tools.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nano.2021.102379.

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Targeting senescent endothelial cells using a theranostic approach: therapeutic potential of an Omega 3 formulation

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Résumé

La présente étude évalue la capacité de la nanoencapsulation d'EPA:DHA 6:1, à améliorer la stabilité des oméga-3 PUFAs, d'améliorer la fonction endothéliale et de mieux protéger le système vasculaire. La nanoencapsulation est associée à une capacité prolongée à provoquer des relaxations dépendantes de l'endothélium et médiées par la composante NO des artères coronaires isolées, et à la capacité d'induire une formation soutenue de NO dans les cellules endothéliales cultivées, conduisant à une réponse anti-agrégation plaquettaire plus forte.
En conclusion, la prise chronique de la forme nanoencapsulée d'EPA:DHA 6:1 améliore significativement la dysfonction endothéliale liée à l'âge chez le rat. Les effets bénéfique de la consommation chronique de la formulation EPA:DHA 6:1 impliquent une amélioration des composantes de relaxations NO, une prévention de l'activation du système d'angiotensine vasculaire local et une diminution du stress oxydant.

Mots clés :

Omega-3, Nanoencapsulation, EPA:DHA 6:1, Monoxide d'azote, Dysfonction endothéliale, Stress Oxydant

Résumé en anglais

The present study investigated whether nanoencapsulation of EPA:DHA 6:1 is able to improve the stability of omega-3 PUFAs and hence, to enhance their beneficial effect at the endothelial function and to better protect the vascular system. The nanoencapsulation of EPA:DHA 6:1 is associated with a prolonged ability to cause endothelium-dependent NO-mediated relaxations of isolated coronary arteries and the ability to induce a sustained formation of NO in cultured endothelial cells, leading to a stronger platelet antiaggregatory response.
In conclusion, chronic intake of coated EPA:DHA 6:1 nanoparticles significantly improved the ageing-related endothelial dysfunction in rats. The beneficial effect involves an improvement of the NO-mediated relaxations as well as preventing activation of the local vascular angiotensin system and the reduction of oxidative stress.

Key words:

Omega-3, Nanoencapsulation, EPA:DHA 6:1, Nitric oxide, Aging, Endothelial dysfunction, Oxidative stress.