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Splenocyte microvesicles are pro-senescent endothelial effectors: impact of age and protection by EPA/DHA 6/1, an optimized formulation of nutritional eicosapentaenoic and docosahexaenoic polyunsaturated fatty acids

Abdul Wahid Qureshi

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UNIVERSITÉ DE STRASBOURG



ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTÉ
Regenerative Nanomedicine – INSERM UMR 1260

THÈSE présentée par :
Abdul Wahid QURESHI

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Pharmacologie-Pharmacocinétique**

**Les microvésicules splénocytaires effecteurs de
la sénescence endothéliale : Impact de l'âge et
protection par apport nutritionnel d'une
formule optimisée d'acides gras poly-insaturés
eicosapentaénoïque et docosahexaénoïque,
EPA:DHA 6:1**

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“Great things are not done by one person. They’re done by a team of people” - Steve Jobs

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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
ACS	Acute Coronary Syndrome
ADMA	Asymmetric Dimethyl Arginine
ADP	Adenosine Di-Phosphate
ALA	α -Linoleic Acid
Ang II	Angiotensin II
ANO6	Anoctamine
aPL Antibodies	Anti-Phospholipid Antibodies
ASCEND	A Study of Cardiovascular Events in Diabetes
ATM	Ataxia Telangiectasia Mutated
ATP	Adenosine Tri-Phosphate
ATR-1	Angiotensin Type 1 Receptor
BCR	B-Cell Receptor
BMPs	Bone Morphogenetic Proteins
Bp	Base Pair
Bpm	Beats Per Minute
BrdU	Bromodeoxyuridine
BS	Bile Salt

C ₁₂ FDG	5-dodecanoylaminoFluorescein Di-β-D-Galactopyranoside
Ca	Calcium
CCFs	Cytoplasmic Chromatin Fragments
CD	Cluster Designation
CHD	Coronary Heart Disease
COX	Cyclooxygenases
cPLA ₂ α	Cytosolic Phospholipase a ₂ α
CVD	Cardiovascular diseases
DAMPs	Damage Associated Molecular Patterns
DAPI	4,6-Diamidino-2-Phenylindole
DART	Diet And Reinfarction Trial
DBP	Diastolic Blood Pressure
DCs	Dendritic cells
DDR	DNA Damage Response
DHA	Docosahexaenoic acid
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
DNA-SCARS	DNA Segments with Chromatin Alterations Reinforcing Senescence
DSBs	Double Stranded Breaks
ECM	Extracellular matrix
ECs	Endothelial Cells
EDCF	Endothelium-Derived Contractile Factors
EDH	Endothelium-Derived Hyperpolarization
EE	Ethyl Ester

EET	Epoxyeicosatrienoic
EGF	Epithelial Growth Factor
EMPs	Endothelial cell-derived microparticles
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic acid
EPCR	Endothelial Protein C Receptor
ERK1/2	Extracellular signal–regulated kinase ½
FA	Fatty Acids
FAO	Food and Agriculture Organization
FD	Fine Dust
FFAs	Free Fatty Acid
FGF	Fibroblast Growth Factor
FOXP3	Forkhead Box Protein P3
Fra-1	Fos-related antigen 1
GISSI	Gruppo Italiano per lo Studio della Sopravvivenza nell’Infarto miocardico
GISSI-HF	GISSI-heart failure
GLA	Gamma-Carboxyglutamic Acid
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GP	Glycoprotein
GPI	Glycosylphosphatidylinositol
GPR	G-Protein coupled receptors
H ₂ O ₂	Hydrogen peroxide
HDL	High Density Lipoproteins
HETE	Hydroxyeicosatetraenoic

HNF	Hepatic Nuclear Factors
HSC	Hematopoietic Stem Cells
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intercellular Adhesion Molecule 1
IgE	Immunoglobulin E
IL	Interleukin
IMA	Internal Mammary Artery
IRF	Interferon Regulatory Factor
JELIS	Japan EPA Lipid Intervention Study
LA	Linoleic Acid
LDL	Low Density Lipoproteins
IMPs	Lymphocyte-derived microparticles
LMPs	Leukocyte-derived microparticles
LOX	Lipoxygenases
LPA	Lysophosphatidic acid
LPS	Lipopolysaccharides
Mac-1	Macrophage antigen-1
MAGs	Monoacylglycerols
MAPK	Mitogen-Activated Protein Kinase
MaR	Maresins
MHC	Major Histocompatibility Complex
miR	MicroRNA
miRNA	Micro RNA
MLCK	Myosin Light Chain Kinases

MMPs	Metalloproteinases
mMPs	Monocyte/macrophage-derived microparticles
MP	Microparticle
mRNA	Messenger RNA
mTOR	Mammalian Target Of Rapamycin
MV	Microvesicle
NAC	N-Acetyl Cysteine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKC	Natural Killer Cells
NLRP3	NOD-like receptor family pyrin domain-containing 3
nMPs	Neutrophil-derived microparticles
NO	Nitric Oxide
Nox	NADPH Oxidase
ONOO-	Peroxynitrite
OX-LDL	Oxidized Low Density Lipoprotein
P	Protectin
PALS	Peri-Arterial Lymphoid Sheath
PAMPs	Pathogen Associated Molecular Patterns
PC	Phosphatidylcholine
PCBs	Polychlorinated biphenyls
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polycomb Repressive Complexes
PE	Phosphatidylethanolamine

PECAM	Platelet Endothelial Cell Adhesion Molecule
PG	Prostaglandin
PHA	Phytohemagglutinin
PHB1	Prohibitin-1
PI3K	Phosphoinositide 3-kinase
PI-3K	Phosphatidylinositol-3-kinase
PKC- Θ	Protein Kinase C Theta
PL	Phospholipid
PMA/I	Phorbol Myristate Acetate/Ionophore
PMPs	Platelet-derived microparticles
PPAR	Peroxisome Proliferator-Activated Receptors
PS	Phosphatidylserine
PUFAs	Polyunsaturated fatty acids
RAS	Renin-Angiotensin System
REDUCE-IT	The Reduction of Cardiovascular Events with EPA-Intervention Trial
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
Rv	Resolvins
RXR	Retinoid X Receptor
SAHF	Senescence-Associated Heterochromatin Foci
SASP	Senescence-Associated Secretory Phenotype
SA- β -Gal	Senescence-Associated Beta-Galactosidase
SBP	Systolic Blood Pressure
SDF	Senescence-associated DNA damage foci

SGLT	Sodium Glucose Linked Transporter
Shh	Sonic hedgehog
SIPs	Stress-Induced Premature Senescence
Sirt	Sirtuin
SM	Sphingomyelin
SOCE	Capacitative or Store-Operated Calcium Entry
SPMs	Specialized pro-resolving lipid mediators
SREBP-1c	Sterol Regulatory Element Binding Protein-1c
STAT	Signal Transducer and Activator of Transcription
TAGs	Triacylglycerols
TCR	T-Cells Receptors
TERT	Telomerase reverse transcriptase
TF	Tissue Factor
TF ⁺ MPs	Tissue factor bearing microparticles
TFPI	Tissue Factor Pathway Inhibitor
TG	Triglycerides
Th cells	Helper T-cells
TLRs	Toll-Like Receptors
TM	Thrombomodulin
TNF	Tumor Necrosis Factor
Treg	Regulatory T-cells
TRPC	Transient Receptor Potential Channel
TX	Thromboxane
VCAM-1	Vascular Cell Adhesion Molecule 1

VITAL	Vitamin D and omega-3 trial
VSMC	Vascular Smooth Muscle Cells
X-gal	5-bromo-4-chloro-3-indolyl-D-galactoside
Mm	Micro molar

Résumé en français

Introduction et état de l'art :

Sénescence endothéliale et maladies cardiovasculaires :

Les maladies cardiovasculaires (MCV) et le cancer sont les deux principales de mortalité et morbidité dans les pays riches et en voie de développement. L'incidence et la prévalence des de MCV comme l'hypertension, les maladies coronaires et l'insuffisance cardiaque augmentent avec l'âge. Du fait du vieillissement de la population, les MCV représentent un enjeu majeur en santé publique avec un poids important des coûts et des besoins.

L'endothélium, la monocouche cellulaire qui borde la lumière des vaisseaux, joue un rôle pivot dans l'hémostase et dans l'homéostasie vasculaire en contribuant à la régulation fine du de la coagulation et du tonus vasculaire, protégeant ainsi de la thrombose et d'un remodelage vasculaire excessif. La sénescence endothéliale liée à l'âge, caractérisée par un arrêt irréversible du cycle cellulaire, induit un stress oxydant et une dysfonction endothéliale, favorisant l'initiation et la progression des dommages cardiovasculaires.

Les microvésicules acteurs et effecteurs de l'hémostase et de l'homéostasie vasculaire

Les microvésicules (MVs) aussi appelées microparticules, sont des vésicules des fluides biologiques émises par la membrane plasmique des cellules en réponse à un stress cellulaire tel que l'inflammation, la sénescence, l'apoptose. Les MVs portent des protéines de la membrane plasmique, utiles pour la caractérisation de leur origine cellulaire, qui leur confèrent des propriétés de marqueurs circulants de dommage cellulaire à valeur diagnostique ou pronostique notamment dans les pathologies vasculaires et cardiaques, mais aussi des molécules bioactives, protéines, lipides, ARN, les transformant en effecteurs pathogéniques des maladies cardiovasculaires. En effet, les MVs sont par essence procoagulantes parce qu'elles exposent de la phosphatidylsérine, un aminophospholipide membranaire qui catalyse les réactions de la coagulation, propriété qui s'ajoute parfois à celle du facteur tissulaire (FT), l'initiateur cellulaire de la cascade de la coagulation. Notre groupe a montré qu'une proportion des MVs endothéliales circulantes chez les patients avec syndrome coronaire aigu se comporte en effecteur pro-sénescent induisant via un stress oxydant, l'activation du système angiotensine local et une réponse inflammatoire exacerbée de l'endothélium.

Outre les MVs d'origine endothéliale, les MVs d'origine leucocytaire ont été détectées comme cruciales dans l'homéostasie vasculaire par notre équipe et d'autres. Les MVs leucocytaires circulent en faible proportion chez les individus sains (<10%) et ont un double potentiel procoagulant car elles exposent le facteur tissulaire (cf. ci-dessus). Constitutives des thrombi en formation, ces MVs circulent à des taux élevés chez les patients avec des désordres cardiaques, métaboliques ou une hypercoagulabilité. Les MVs leucocytaires ont une valeur pronostique de risque élevé d'athéromatose chez les patients asymptomatiques. De fait, elles s'accumulent dans les plaques d'athérome. In vitro et dans les modèles animaux, les MVs leucocytaires apparaissent ainsi comme des effecteurs de la réponse vasculaire et de l'hémostase en véhiculant de nombreuses propriétés pro-inflammatoires pro-coagulantes, pro-angiogéniques, soulignant un rôle possible dans le couplage entre inflammation et thrombose.

Elles ciblent l'endothélium dans des modèles cellulaires et l'injection de MVs leucocytaires émises en réponse à un stress infectieux, apoptotique ou inflammatoire induit une réponse inflammatoire de la paroi vasculaire et du tissu myocardique et réduit la relaxation endothéliale-dépendante des aortes pré-contractée de souris.

Protection cellulaire contre le vieillissement et santé cardiovasculaire

De nombreuses études épidémiologiques ont montré l'impact de la diète sur la santé cardiovasculaire et sur la durée de la vie en bonne santé. Chez les patients à fort risque cardiovasculaire, l'incidence d'événements 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 même, la population japonaise serait 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 omega-3, PUFAs dont la troisième liaison carbone est insaturée. Ainsi, les acides eicosapentaénoïque (EPA) et docosahexaénoïque (DHA), diminueraient de 50% la mortalité coronaire chez les hommes sains et de 30% chez ceux ayant récemment subi un infarctus du myocarde. 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. De plus, les suspensions d'EPA et DHA ne sont pas utilisables *in vitro*, du fait de leur

forte toxicité cellulaire, l'étude de leurs effets nécessite donc des modèles animaux avec gavage. Notre équipe a montré un effet vasoprotecteur de la formulation optimisée EPA:DHA 6:1 qui limite le stress oxydant endothélial grâce à l'induction de la NO-synthase endothéliale via l'activation redox-sensible de la signalisation Src/PI3-kinase/Akt et des MAP-Kinases. Parallèlement, l'ingestion chronique d'EPA:DHA 6:1 réduit significativement la pression systolique dans un modèle d'hypertension induite par l'angiotensine II chez le rat.

Hypothèses et objectifs :

L'ensemble des données précédentes du laboratoire et celles d'autres équipes suggère que la dysfonction endothéliale favorise la perte du contrôle du tonus vasculaire. De plus, les travaux précédents de Ali ElHabhab, doctorant du laboratoire, ont montré que les MVs leucocytaires générées par stimulation de splénocytes de rat ont un effet pro-sénescence sur les cellules endothéliales primaires de coronaires. Nous avons cherché les mécanismes susceptibles d'expliquer le bénéfice cardiovasculaire des omega-3 en nous concentrant sur les interactions des MVs leucocytaires et des cellules endothéliales primaires de coronaires. Nos hypothèses étaient

- i) En condition pathologique, les MVs leucocytaires favorisent une sénescence endothéliale accélérée et un phénotype endothélial pro-inflammatoire et pro-coagulant
- ii) Les propriétés pro-sénescence des MVs leucocytaires évoluent avec l'âge.
- iii) L'ingestion d'omega-3 EPA:DHA 6:1 prévient la sénescence endothéliale en modifiant les propriétés des MVs leucocytaires qui interagissent avec l'endothélium.

Nos objectifs étaient la comparaison des propriétés pro-sénescence, pro-inflammatoires et pro-coagulantes des MVs leucocytaires de rats jeunes, âgés, ou d'âge moyen et l'étude de l'impact de l'ingestion d'une formulation optimisée d'omega-3 formulation (EPA:DHA 6:1) sur ces propriétés et l'identification des mécanismes moléculaires sous-jacents.

Méthodologie et principaux résultats :

Après prélèvement des rates de rats Wistar mâles, jeunes (12 semaines), d'âge moyen (M, 48 semaines) ou vieux (72 semaines), les splénocytes isolés ont été mis en culture. La capacité des splénocytes à émettre des MVs a été étudiée après 24 heures, leur origine

leucocytaire déterminée ainsi que leur propriétés pro-sénescences des SMVs sur des cellules endothéliales primaires de coronaires de porc en culture. L'induction de la sénescence endothéliale a été mesurée par l'activité de la Senescence-associated β -galactosidase activity (SA- β -gal) à l'aide d'un substrat fluorogénique le C₁₂FDG, en cytométrie en flux, et par l'expression des marqueurs protéiques de la sénescence p53, p21, p16 par western blot. La dysfonction endothéliale a été évaluée par la mesure du stress oxydant grâce à la sonde redox-sensible fluorescente dihydroéthidium (DHE) et aux marqueurs pro-inflammatoires, pro-coagulants ou potentiellement thrombogéniques par western blot et cytométrie. L'activité procoagulante des SMVs a été mesurée par dosage enzymatique prothrombinase. Parallèlement, l'effet d'une ingestion d'oméga-3 (7 jours, pour les rats d'âge moyen ; 14 jours pour les rats vieux) sur l'émission et les propriétés des SMVs a été mesuré dans chaque fratrie de rat. Les animaux ont ingéré 500 mg/kg/d de EPA:DHA 6:1, ou de EPA:DHA 1:1, ou de l'huile de maïs contrôle.

Impact de l'âge sur l'émission des MVs : L'émission de SMVs augmente proportionnellement à l'âge, comparativement aux rats jeunes, suggérant une susceptibilité membranaire accrue des splénocytes. De plus, leurs propriétés vis à vis de l'endothélium évoluent. En effet, l'ajout d'une concentration de 10 nM de SMVs aux cellules endothéliales coronaires primaires jeunes (P1 ECs) transforme leur phénotype lorsqu'elles proviennent de splénocytes de rats d'âge moyen ou âgés mais pas de rats jeunes : leur effet pro-sénescence est attesté par l'augmentation progressive de la SA- β -gal et une surexpression significative des marqueurs p21 et p16, impliqués dans la régulation du cycle cellulaire avec des réponses endothéliales aussi importantes que celles observées dans des cellules induites en sénescence soit par réplication successive ou à l'aide de peroxyde d'hydrogène (100 μ M). La réponse pro-inflammatoire et procoagulante est aussi significativement augmentée en réponse aux SMVs de rats âgés ou d'âge moyen avec une surexpression significative de facteur tissulaire, du récepteur de l'angiotensine et de son récepteur AT1R, des protéines pro-inflammatoires (VCAM-1, ICAM-1, NF- κ B, COX-2 mais pas COX-1) comparée aux rats jeunes. De plus, les SMVs de rats âgés ou d'âge moyen mais pas de rats jeunes induisent un stress oxydant détecté par DHE, et caractérisé d'origine cytoplasmique et mitochondriale par inhibition pharmacologique. A l'inverse, la NO synthase endothéliale protectrice est sous-exprimée comparativement aux cellules traitées par des SMVs de rats jeunes. De plus, seules les SMVs

de rats âgés réduisent la vaso-relaxation endothéliale dépendante d'artères aortiques de porc en réponse à la bradykinine.

L'ensemble des résultats suggère donc fortement que l'émission de SMVs augmente avec l'âge et favorise la sénescence des cellules endothéliales coronaires, déjà particulièrement prédisposées aux mécanismes d'athéromatose. En effet, les artères coronaires présentent des zones de courbures favorisant des perturbations de flux sanguin et où s'accumulent les plaques d'athérome. Comparativement à l'EPA :DHA 1 :1 ou à l'huile de maïs, l'ingestion d'EPA:DHA 6:1 prévient significativement l'émission des SMVs pro-sénescentes induite par l'âge et réduit leurs effets en limitant leur potentiel prooxydant, pro-inflammatoire et prothrombotique, et restent sans effet notable chez les rats jeunes. En outre, EPA:DHA 6:1 préserve les mécanismes protecteurs de la NO synthase endothéliale.

En conclusion, les MVs leucocytaires d'origine splénique induites par l'âge se comportent en effecteurs endothéliaux nocifs, pro-sénescents et pro-inflammatoires favorisant la dysfonction endothéliale et une réponse thrombogénique de l'endothélium coronaire spécifiquement contrecarrée par l'ingestion d'EPA:DHA 6:1.

Chapter 1: Ageing and its impact on the immune system

1.1 Ageing

Ageing is defined as an inevitable multifactorial biological process, characterized by progressive loss of physiological functions, allied with frailty and increased probability of death (Panagiotou, Neytchev et al. 2018). Age-related decline begins from third decade onwards and deterioration progresses with advancing age. Ageing-related phenotypes includes loss of muscle and bone mass and strength, abdominal fat gain, systemic functional decline, structural and mechanical alterations, age-related chronic diseases and frailty (Figure 1).

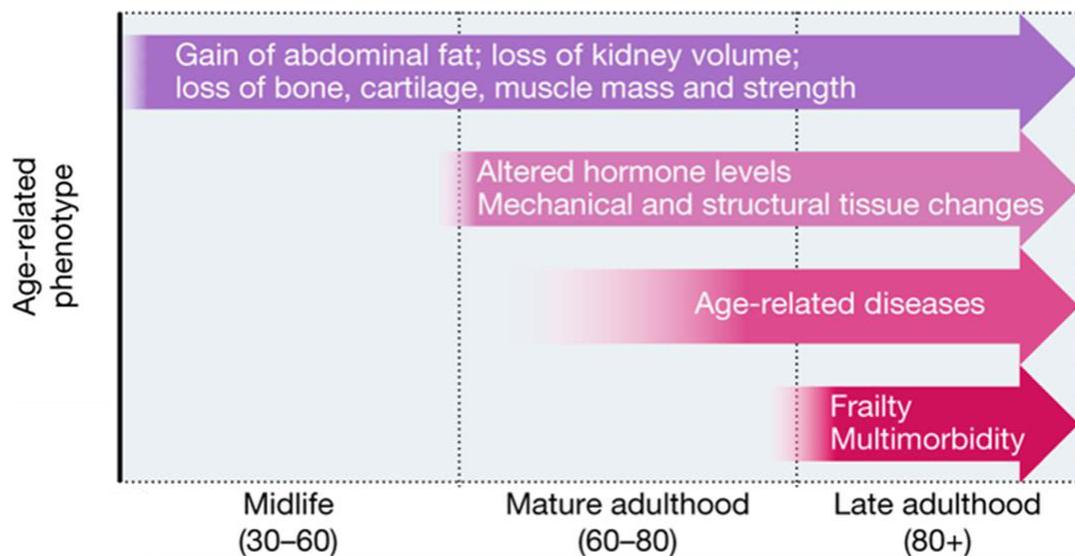


Figure 1. Timing and progression of ageing-associated phenotypes

Adapted from (Partridge, Deelen et al. 2018)

About half of the human deaths are attributed to chronic diseases associated with ageing. Ageing-associated increase in cellular oxidative stress, chronic low grade inflammation (inflamm-ageing), senescence, mitochondrial dysfunction, proteasome failure, impaired autophagy contributes to multi-systems loss of reserve and function, ultimately leading to incidence of multimorbidity among elderly (Figueira, Fernandes et al. 2016). Most conspicuous ageing-associated chronic diseases are cardiovascular diseases (CVD), diabetes, neurodegenerative diseases and cancer. CVD contributes for 39.6% of all ageing-related chronic diseases and CVD sharply increases after 40 years of age (Benjamin Emelia, Virani Salim et al. 2018, Fajemiroye, da Cunha et al. 2018).

All the mechanisms underlying ageing process are not yet understood. Still, extensive research has defined a set of hallmarks as a ground for better understanding

and ameliorate the late-life effects of ageing. This set includes indicators of altered cellular function, antagonistic responses to damage cellular function, and finally integrative hallmarks which are eventual culprits of clinical effects of ageing, mainly loss in organ dysfunction and homeostasis (Figure 2).

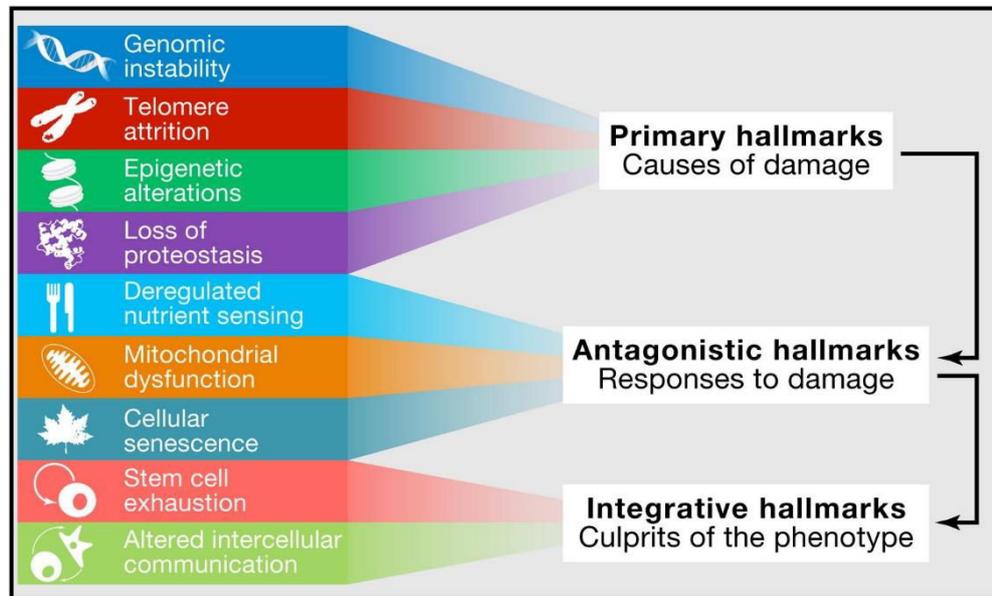


Figure 2. Hallmarks of ageing Adapted from (Aunan, Watson et al. 2016)

Since the last two centuries, human life expectancy has doubled in most developed countries due to better quality of food, hygiene, water, lifestyle, immunization, antibiotics and advance medical care. By year 2035, one fourth of world's population is anticipated to reach the age of 65 (Steenman and Lande 2017). However, despite increase in lifespan, there is not much increase in healthy, disease free lifespan. Between 2000 to 2015, on average, total life expectancy raised by 5 years throughout the world whereas healthy life expectancy has been raised by 4.6 years. On average, 16-20% of life is now spent with late-life morbidity. An unhealthy elderly population is a global challenge to society (Partridge, Deelen et al. 2018). Therefore, though it is not possible to abolish ageing, one of the major aims of current research is to reduce the length and severity of late-life morbidity.

1.2 Overview of immune system

The immune system integrates lymphoid organs, cytokines, cells, humoral factors and is responsible for recognition and eradication/neutralization of pathogens as well as

of abnormal self-cells (Parkin and Cohen 2001). Immune system is divided into innate and adaptive immune systems (Figure 3).

1.2.1 The innate immune system

The innate immune system, also called natural or native immune system, is the first line of immediate and non-specific defense mechanism. It comprises physical barriers (e.g. skin, epithelial cell layers etc.), immune cells (neutrophils, monocytes/macrophages, dendritic and natural killer cells) and responds without previous experience of its target. When it is overpowered, adaptive immune system will be activated (Nagaratnam and Adithya Nagaratnam 2019).

1.2.2 The adaptive immune system

The adaptive immune response or acquired immune system, functions *via* antigen specificity and immune memory. Adaptive responses primarily uses the antigen-specific receptors expressed on the surface of B cells and T cells to initiate targeted effector response. Targeted effector response can be due to activated T cells (cellular immunity) leaving lymphoid tissue and approaching to disease site, or to activated B cells (humoral immunity) releasing antibodies into blood, tissue fluids and finally to the infective focus (Nagaratnam and Adithya Nagaratnam 2019).

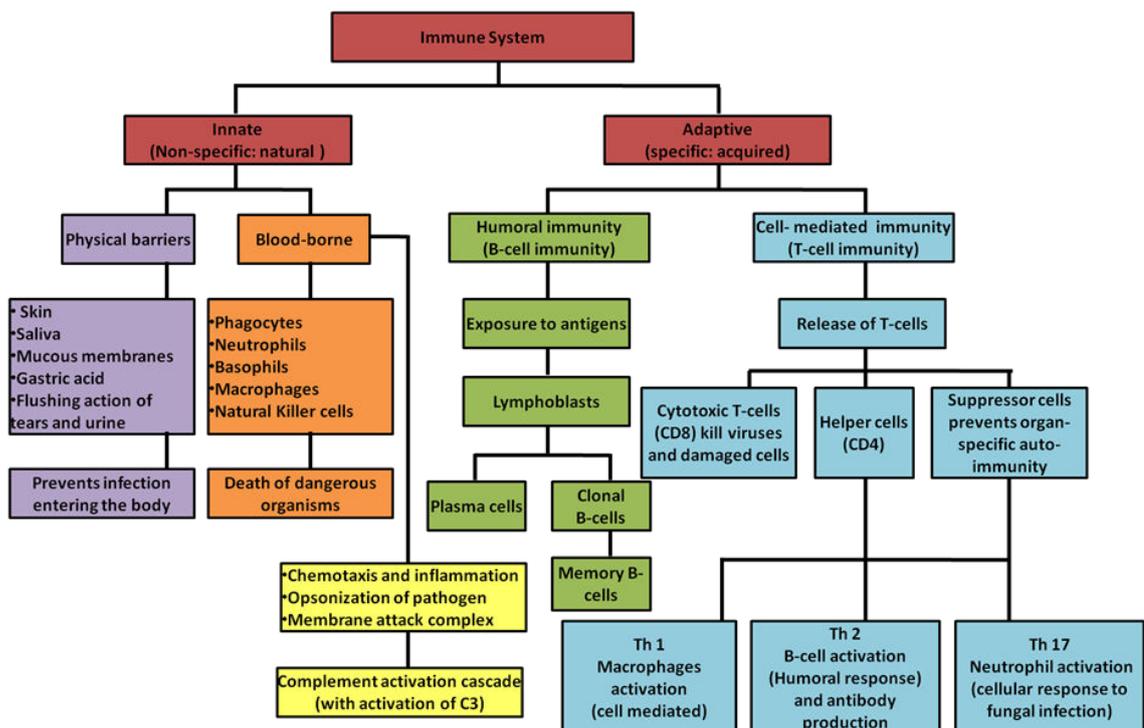


Figure 3. Overview of the immune system (Nagaratnam and Adithya Nagaratnam 2019)

1.2.3 Spleen, a rich source of leukocytes

In humans, spleen is 7-13 cm in length with a weight of about 150 g which may decrease with age. It is the largest lymphoid organ with important role in both innate and adaptive immune responses. Covered by a capsule of connective tissue, it is composed of branching arterial vessels, with smaller arterioles ending up into a venous sinusoidal system and a functional parenchyma made up of red and white pulp (Figure 4). Red and white pulp are separated by a thin layer of marginal zone. The red pulp is richly vascular specialized area composed of splenic cord, capillaries and venous sinuses that functions as a blood filter. The red pulp contains large aggregates of erythrocytes that favors blood viscosity. Altogether, the human spleen contains 200-250 mL of blood, representing 10% of the total red blood cells and 160 mL red blood cells can be expelled in response to exercise favoring oxygen fixation through enhanced plasma hemoglobin (Stewart and McKenzie 2002). The marginal zone contains B cells, marginal-zone macrophages, fibroblasts and dendritic cells (DCs). Cells from bloodstream enter and leave the white pulp through the marginal zone. The white pulp, consisting of lymphoid follicles and peri-arterial lymphoid sheath (PALS), is mostly rich in B and T lymphocytes and harbors the immunologic spleen function. Specific cluster designation (CD) and other markers defines the lymphoid cell immunophenotypes of the different spleen regions. (Stewart and McKenzie 2002, Mebius and Kraal 2005, Velasquez-Lopera, Correa et al. 2008, Pernar and Tavakkoli 2019).

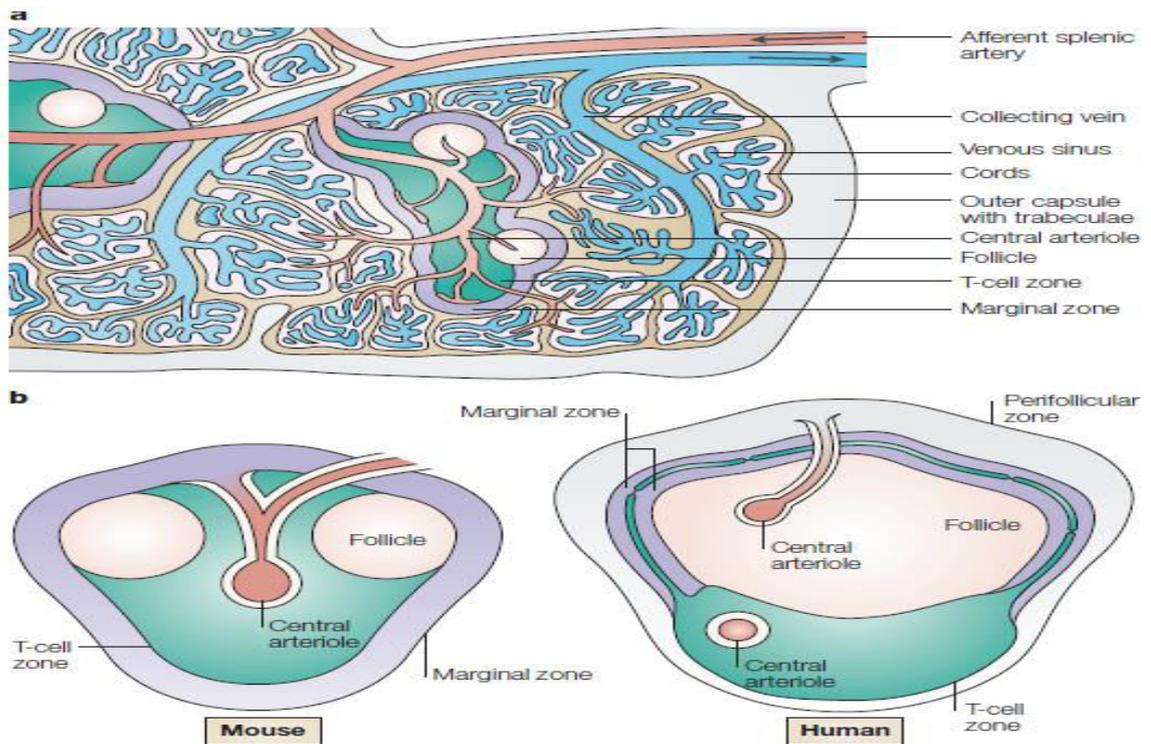


Figure 4: Structure of Spleen:

a) The afferent splenic artery branches into central arterioles, which are sheathed by white-pulp areas; these white-pulp areas consist of the T-cell zone (also known as the periarteriolar lymphoid sheath, PALS), arterioles and B-cell follicles. The arterioles end in cords in the red pulp, from where the blood runs into venous sinuses which collect into the efferent splenic vein. The larger arteries and veins run together in connective-tissue trabeculae, which are continuous with the capsule that surrounds the spleen. b) Comparison of the structure of the white pulp in rodents and primates. The main differences are found in the structure of the marginal zone, which surrounds the white pulp. In contrast to mice, humans have an inner and an outer marginal zone, which is surrounded by a large perifollicular zone. In the perifollicular

Altogether, the spleen serves various functions e.g. filtration, destruction of altered or old red blood cells, production of lymphocytes and monocytes, phagocytosis, storage of iron and viable blood cells (Stewart and McKenzie 2002).

1.3 Impact of ageing on immune system

Ageing-related effects on immune system are extensive and stretched from hematopoietic stem cells (HSC) and lymphoid progenitor cells in the thymus and bone marrow, and, to resident mature lymphocytes in secondary lymphoid organs including spleen (Montecino-Rodriguez, Berent-Maoz et al. 2013).

1.3.1 Immunosenescence

Ageing of the immune system or immunosenescence is characterized by multiple phenotypical and functional alterations in both innate and adaptive immune cells (Figure 5). Immunosenescence is most probably driven by sustained antigenic load (bacteria, virus, fungi, necrotic cell debris, toxins) with ageing, favoring inflammatory status and damage to various organs, at the onset of chronic diseases (Fulop, Dupuis et al. 2016, Nagaratnam and Adithya Nagaratnam 2019).

Neutrophils are the first recruit at the sites of injury and have short life-span until stimulated by some pro-inflammatory stimulus e.g. LPS (lipopolysaccharides). With ageing, the number of neutrophils are reported to be relatively higher with altered effector functions including chemotaxis, production of free radicals production and intracellular killing, while phagocytosis and adhesive ability of neutrophils remain stable during immunosenescence. Neutrophils in quiescent state, with sustained and increased production of cytokines, free-radical, metalloproteinase, concomitant activation of NF- κ B (nuclear factor of kappa B) and altered PI3K signaling pathways are indicators of altered effector functions (Elias, Hartshorn et al. 2018, Ray and Yung 2018).

Monocytes/macrophages that are critical regulators and effectors of inflammation also undergo age-related alterations in effector functions, for example, decreased antigen presentation, cytotoxicity and intracellular killing. PAMPs (pathogen-associated molecular patterns)-based activation of TLRs (Toll-like receptors), present on monocytes, macrophages, dendritic, epithelial and endothelial cells, and downstream immune signaling and phagocytosis is altered. With ageing reduced signaling through TLR1/2 heterodimers and decreased TLR-induced costimulatory expression is reported in monocytes (van Duin and Shaw 2007). Conversely, monocyte subpopulations, mainly with an inflammatory phenotype such as CD14⁺CD16⁺, are increased, leading to elevated pro-inflammatory cytokines (TNF-alpha, IL-1, IL-6) at the quiescent state. Age-associated DNA methylation and post-translational histone modifications along with transcription factors of forkhead box protein P3 (FOXP3), NF- κ B, interferon regulatory factor (IRF), and signal transducer and activator of transcription (STAT) families regulate inflammatory genes in monocytes Oxidation and phosphorylation of transcriptional factor STAT5a that is required for proliferation of macrophages is reduced in ageing. Macrophages exhibit elevated oxidative stress, shortened telomeres (replicative senescence), ultimately leading to impaired GM-CSF-dependent

macrophage proliferation. (Sebastian, Herrero et al. 2009, Desai, Grolleau-Julius et al. 2010, Fulop, Dupuis et al. 2016). Natural killer cells that protect from virus infection or cancerous cells are also affected by ageing. Their numbers are increased while their cytotoxic activity is reduced in the elderly (Ray and Yung 2018).

The adaptive immune system undergoes marked functional and phenotypical changes with ageing. Age-related involution of lymphoid tissue, reduced number of dendritic cells, continuous exposure to number of antigens, debilitation of naïve cell and accumulation of memory T cells contributes to overall altered adaptive immune system. Furthermore, the hematopoietic stem cells (HSC) are more inclined towards myeloid lineage at later part of life. Moreover, HSC lose self-renewal capacity due to increased oxidative stress-induced DNA damage. With ageing, the expression of pro-inflammatory chemokines and chemokines receptors on murine and human T cells is increased, thereby promoting chemotactic responses eventually pivotal in pathogenesis of ageing-related inflammatory diseases; including cardiovascular and autoimmune diseases (Desai, Grolleau-Julius et al. 2010, Ray and Yung 2018). Indeed, pro-inflammatory chemokines and chemokines receptors are important for migration of T cells into site of injury

Ageing-associated shortening of telomeres has been reported in T cells and limit their life-span. All human T cells, at birth, express a costimulatory receptor CD28 which plays a vital role in antigen based activation, division and survival. The ratio of CD28⁺/CD28⁻ is, particularly in CD8 subsets, decreases with ageing. Non-expression of CD28 is considered a marker of replicative senescent T cell, though all CD28⁻ cells are not senescent (Yu, Park et al. 2016).

Other than their numbers, most significant change in B cells with age is the narrowing of the diversity of the antibody response leading to impaired capability of the aged immune system to produce high affinity antibodies, clonal B cell expansion (Elias, Hartshorn et al. 2018). In elderly, the dramatic downfall of the B cell repertoire diversity

is strongly associated with general health status and an indicator of frailty (Gibson, Wu et al. 2009).

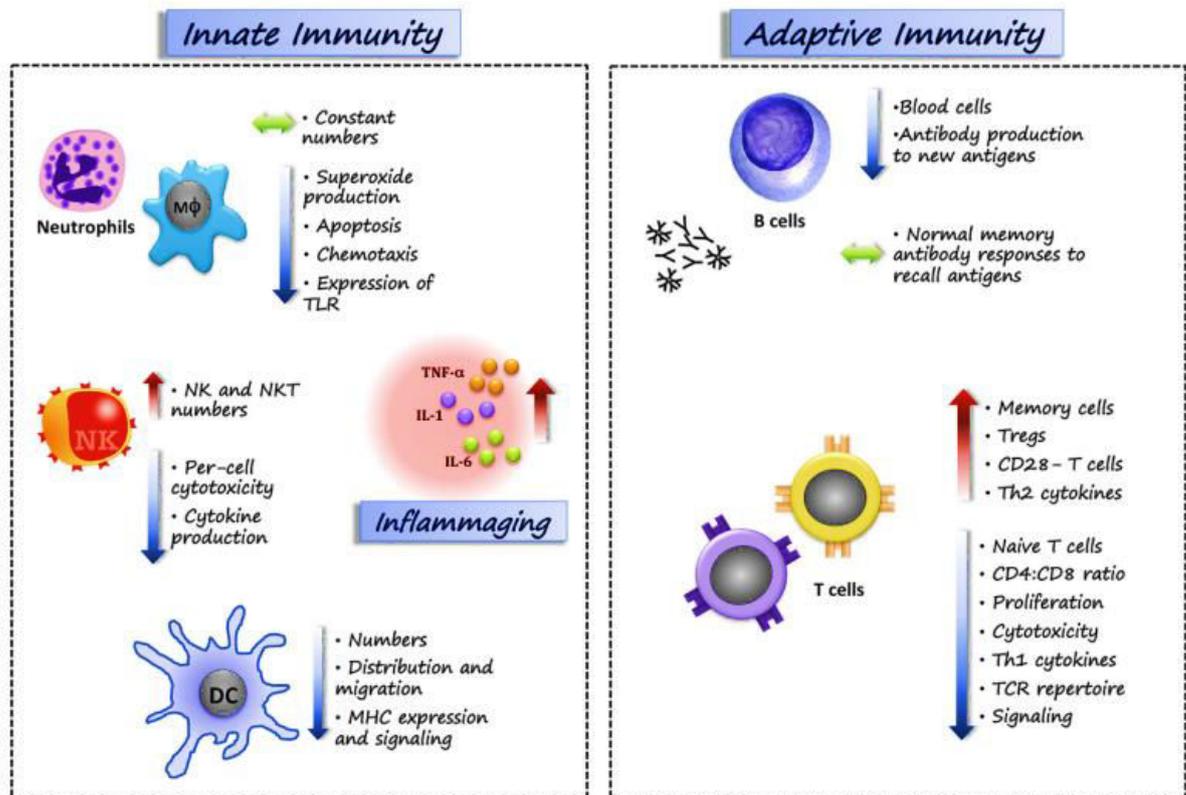


Figure 5. Age-related changes in the immune system (immunosenescence)

DC: dendritic cell; MHC: major histocompatibility complex; TLR: Toll-like receptors; NK: natural killer; Th: helper T cell; TCR: T-cell receptor; Treg: regulatory T cell. (Bauer and Fuente 2016)

All these age-related changes in innate and adaptive immune system favors decreased effector function, constant low-grade inflammation, increased susceptibility to infections and a greater risk for the development of several pathological conditions such as atherosclerosis, cancer, dementia, all sharing an inflammatory pathogenesis (Nagaratnam and Adithya Nagaratnam 2019).

1.3.2 Inflamm-ageing

Ageing is associated with constant low-grade inflammation, known as inflamm-ageing, a term first coined by Claudio Franceschi in 2000. This pro-inflammatory status among the elderly is characterized by high levels of circulating pro-inflammatory cytokines and proteins. High levels pro-inflammatory markers are detected among the majority of older individuals, even in the absence of risk or active clinical conditions.

Inflammation, a beneficial defense mechanism working to eliminate pathogens becomes detrimental to health, when sustained and prolonged in later life (Calder, Bosco et al. 2017).

Possible mechanisms of inflamm-ageing include genetic susceptibility, cellular senescence, central obesity, oxidative stress caused by dysfunctional mitochondria, increased gut permeability, changes to microbiota composition, NLRP3 inflammasome activation, immune cell dysregulation, and chronic infections (Figure 6) (Ferrucci and Fabbri 2018).

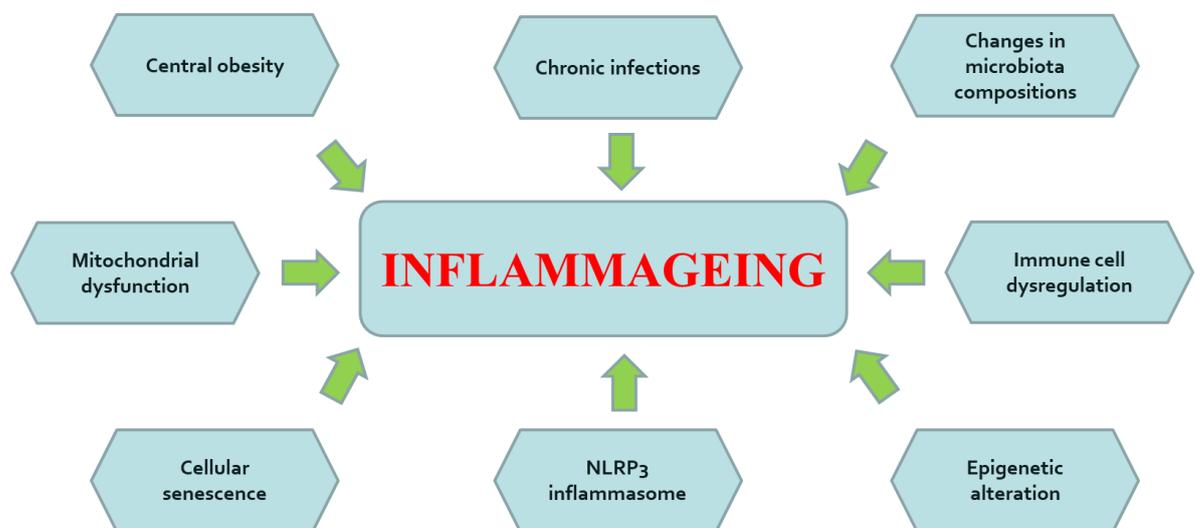


Figure 6. Mechanisms of inflamm-ageing. (Ferrucci and Fabbri 2018)

Multiple genetic variants have been identified in large populations that affect the concentration of pro-inflammatory mediators in blood. A gene-expression study conducted on whole-blood RNA samples from a large cohort in Europe and USA revealed that immune response and inflammation were the most highly up-regulated pathways in association with ageing. The contribution of miRNAs to inflamm-ageing is an active area of investigation with high translational potential. miRNAs which are non-coding, single-stranded RNAs modulate protein expression by interacting with mRNA. Specific miRNA in circulating cells, plasma or whole blood have been suggested to contribute to inflamm-ageing and related chronic diseases. For example, miR-126-3p inhibits endothelial inflammation, and low levels of miR-126-3p were found in patients with CVD and diabetes. Other miRNAs, such as miR-146 and miR-155, possibly present in microvesicles or other structures carrying miRNAs, promote inflamm-ageing by

induction of cellular senescence or modulating immune response (Olivieri, Albertini et al. 2015, Rea, Gibson et al. 2018).

Several studies have shown that ageing is associated with the exponential accumulation of senescent cells in different organs and tissues including skin, T lymphocytes, endothelium, kidney, liver, visceral fat, cardiac muscle, in both animals and humans. Senescent cells acquire a senescence-associated secretory phenotype (SASP) characterized by abnormal secretion of mediators such as interleukins, growth factors, chemokines, metalloproteinases and of other extracellular matrix molecules, all favoring a pro-inflammatory status and the development of cellular senescence in neighboring cells. As senescence is associated with ageing as well as inflammation and CVDs in aged individuals, it appears a strong candidate for contributor to inflamm-ageing (Fulop, Dupuis et al. 2016, Ferrucci and Fabbri 2018).

With advancing age, stressed cells, undergoing apoptosis, release large number of molecules known as damage associated molecular patterns (DAMPs) which includes ROS from dysfunctional or damaged mitochondria, oxidized LDL, microvesicles, mitochondrial DNA fragments or histones. Late removal of DAMPs can favor inflamm-ageing. For example, accumulated DAMPs activate NLRP3 inflammasome receptors up-regulating pro-inflammatory mediators (IL-1 β and IL-18). Indeed, ageing is associated with increased blood levels of IL-18 and blockade of NLRP3 signaling in mouse models extends healthspan by attenuating multiple age-related changes that are associated with inflamm-ageing (Youm, Grant et al. 2013). Moreover, increased levels of age-related ROS can also trigger inflammatory responses by activating NF- κ B signaling (Sies, Berndt et al. 2017, Ferrucci and Fabbri 2018).

Inflamm-ageing, indeed, acts as an accelerator for the emergence of age-associated chronic pathologies. For example, inflamm-ageing actively contributes to the initiation and progression of atherosclerosis, which is primary cause of stroke, coronary artery disease and peripheral vascular disease. Inflammation is also involved in pathophysiology of atrial fibrillation and post-myocardial infarction cardiac remodeling (Figure 7) (Fernández-Ruiz 2016, Ferrucci and Fabbri 2018).

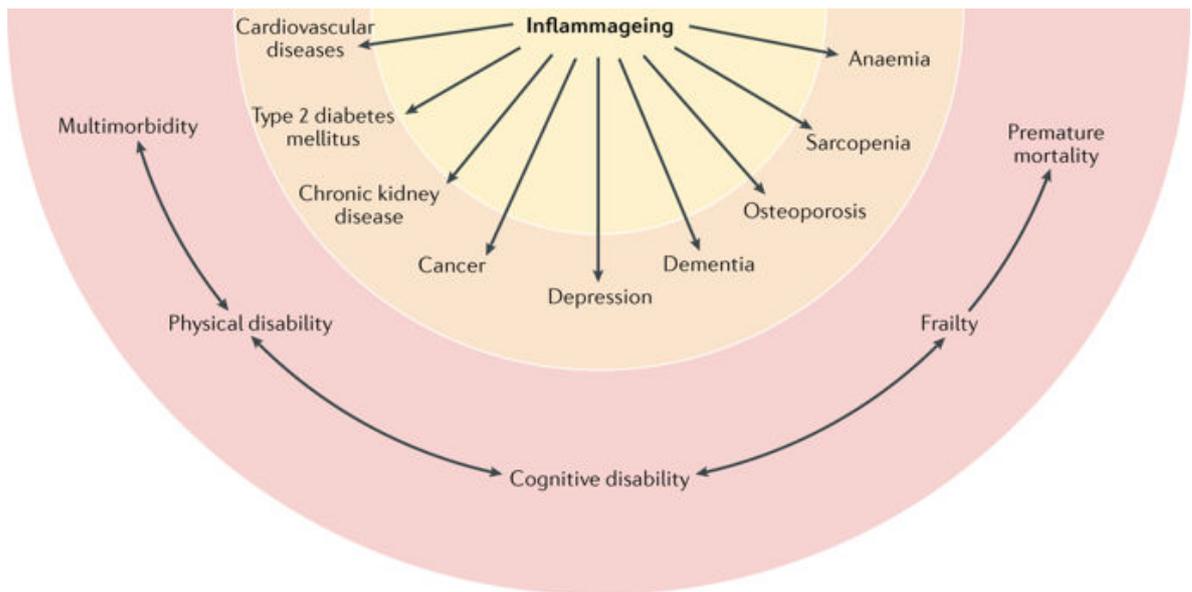


Figure 7. Inflamm-aging: a risk factor for multiple chronic diseases. (Ferrucci and Fabbri 2018)

1.4 Cell membrane asymmetry and fluidity: impact of ageing

The asymmetry of plasma membrane plays a key role in the maintenance of cellular functions. Under resting conditions, in eukaryotic cells, the phosphatidylserine (PS), phosphatidylethanolamine (PE) and aminophospholipids are situated in inner membrane leaflet of the bilayer whereas sphingomyelin and phosphatidylcholine (PC) are located in the outer leaflet. When asymmetry of membrane is lost, under stress conditions, PS detected at the outer leaflet of membrane, increasing the pro-coagulant potential of circulating cells and favoring the shedding of pro-coagulant microvesicles (Morel, Jesel et al. 2011). Cell asymmetry is also essential for cell viability. During apoptosis, exposure of PS at cell surface act as a signal recognition and removal by macrophages (Scott, Heberle et al. 2018).

Plasma membrane fluidity is determined superficially by phospholipid head groups at inner and outer membrane leaflets while in deep hydrophobic area of cell membrane by amount of cholesterol and unsaturated fatty acid side chains. Changes in membrane fluidity is associated with functional alteration of many integral receptors in plasma membrane (Ray, Kassan et al. 2016). In comparison to outer, inner leaflet of membrane has more fluidity because of higher PS content. Thus, alteration of asymmetry distribution of phospholipids in membrane can alter membrane fluidity and also function

of fundamental membrane proteins (Noble, Thomas et al. 1999). Membrane lipid rafts, which are microdomains of cholesterol, sphingolipids and scaffolding proteins, are key players in signal transduction, organizing cytoskeleton and vesicular trafficking (Lingwood and Simons 2010). Studies have shown link of immune cell function with functioning lipid raft as multiple immune cell receptors such as T-cell receptor (TCR), B-cell receptor (BCR) and high-affinity IgE receptor are dependent on efficiency of lipid raft for their activation and down-ward signaling (Rajendran and Simons 2005).

Alterations in cellular and intracellular membrane is one of the feature of ageing and many chronic medical conditions. Ageing is accompanied by changes in membrane's physical properties, lipid composition, receptor function and antigen presentation. Age-associated alteration in plasma membrane lipids including oxidation of lipids and loss of cholesterol leads to the loss of membrane lipids rafts and hence hamper the membrane bioactivity (Egawa, Pearn et al. 2016). Membrane phospholipids and their unsaturated fatty acids are especially sensitive to age-associated oxidative stress. In leukocytes, increased expression of PS on the outer leaflet of membrane in elderly is related to changes in subsets of lymphocytes, higher rate of apoptosis and alteration in the activity of membrane-bound transporters and ion channels (Noble, Thomas et al. 1999). Damaged cells and increased oxidative stress-mediated opening of calcium channels, resulting in increased calcium intracellular concentration, can activate membrane transporter like scramblases that rapidly destroy asymmetrical distribution of the membrane phospholipids (Figure 8) (Kodigepalli, Bowers et al. 2015, Nicolson and Ash 2017). Loss of asymmetry, in old age, is also correlated with increased membrane fluidity. So, age-related structural changes in plasma membrane of leukocytes has a role in altered function of membrane bound receptors and this could partially account for impaired immune responses and hemostasis, as witnessed with advancing age (Noble, Thomas et al. 1999, Nicolson and Ash 2017).

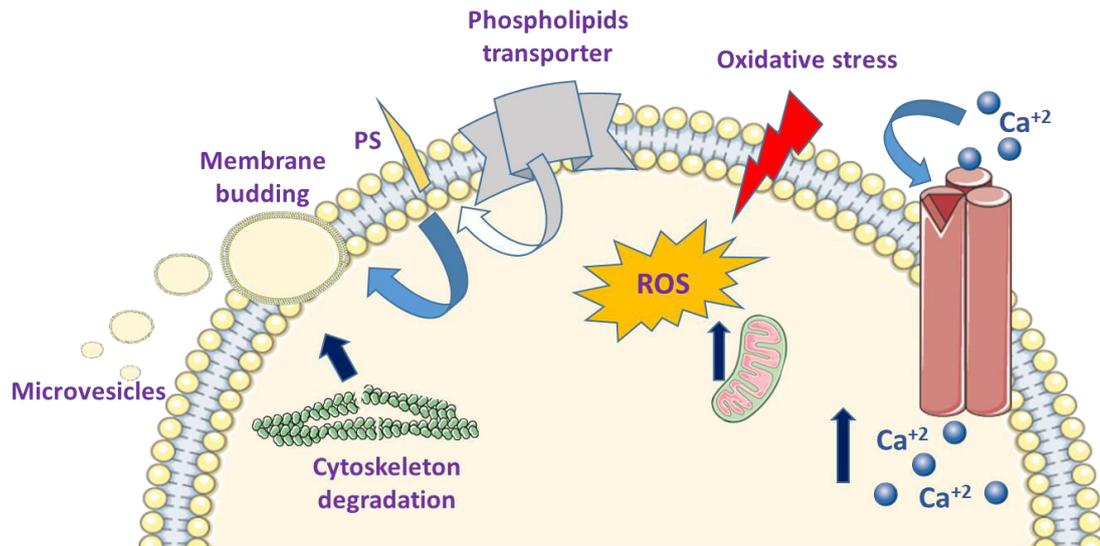


Figure 8. Ageing and plasma membrane remodeling

Increased oxidative stress and elevated intracellular accumulation of calcium promotes activation of plasma membrane transporter to alter the asymmetrical distribution of membrane phospholipids. Exposure of PS on surface of plasma membrane and cytoskeleton degradation favours membrane budding and release of microvesicles. PS: phosphatidylserine, ROS: reactive oxygen species

Chapter 2: Microparticles: overview and role in vascular damage

2.1 Overview of Microparticles

Microparticles, now commonly referred to as microvesicles (MVs), are small extracellular plasma membrane vesicles, 0.05-1 μ m in diameter, shed by activated, apoptotic and/or senescent cells (Figure 9) (Ridger, Boulanger et al. 2017). MVs were later identified as coagulant lipid rich particles and characterized by electron microscopy as small bilayer membrane vesicles released from activated platelets in plasma and serum and referred to as “platelet dust” possibly accounting for the “Platelet Factor 3” activity and now studied as vascular effectors extensively (Yáñez-Mó, Siljander et al. 2015, Said, Rogers et al. 2018).

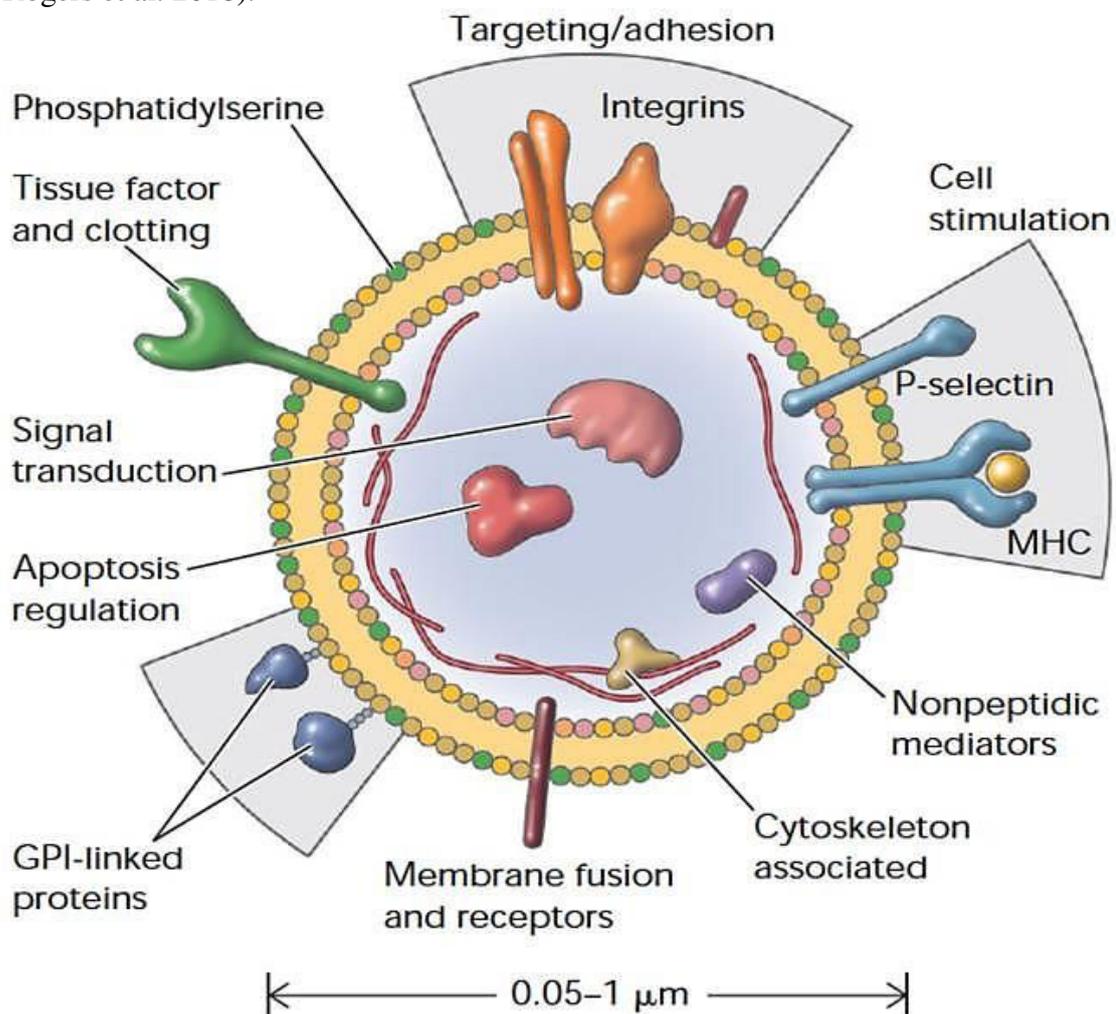


Figure 9. Cellular microparticles: a diffusing storage pool of bioactive effectors. Membrane microparticles are shed from the plasma membrane of stimulated cells. They harbor cytoplasmic proteins as well as bioactive lipids implicated in a variety of fundamental processes. MHC: Major histocompatibility complex, GPI: glycosylphosphatidylinositol (Hugel, Martinez et al. 2005)

MPs can be differentiated from other extracellular vesicles, including exosomes and apoptotic bodies, on the basis of their size, subcellular origin, content and mechanism of formation (Figure 10) (Ridger, Boulanger et al. 2017).

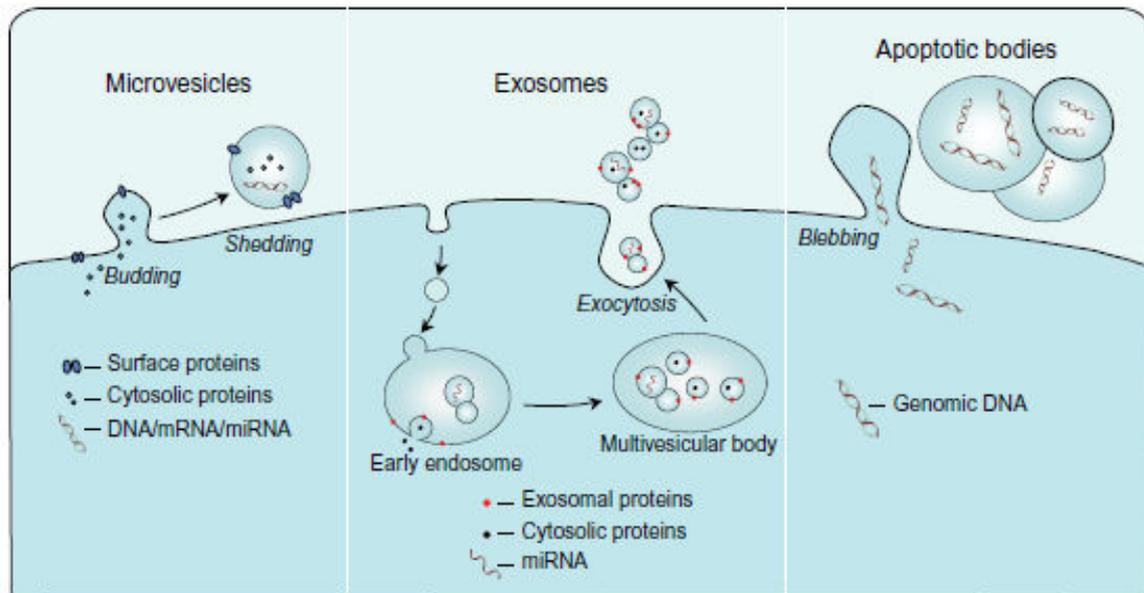


Figure 10. Schematic representation of the mechanisms of formation of microvesicles, exosomes, and apoptotic bodies (Lawson, Vicencio et al. 2016).

MPs are shed by cells in response to stress, after remodeling of the phospholipids of the plasma membrane, followed by budding of plasma membrane and shedding of microparticles into extracellular environment (Pollet, Conrard et al. 2018). For many decades, MPs were considered as inert debris resulting from the cellular destruction, growth or dynamic renewal. Later, they were recognized as pro-coagulant mediators in coagulation and, owing to their content (active lipids, nucleic acid, miRNA, proteins), as true vectors and mediators of biological messages (Mause and Weber 2010), in intercellular communication initially in cardiovascular issues (Benameur, Osman et al. 2019). Because MPs originate from the plasma membrane, they carry transmembrane and surface protein from parent cells that characterize their cell origin or even the initiator stress of tissue damage (Morel, Toti et al. 2006). MPs are not only markers of activation or cellular and tissue damage, but also actors of major physiological responses such as hemostasis, inflammation, cell survival and apoptosis, endothelial function, vascular remodeling and angiogenesis; some of them also altered by exosomes (Ridder, Sevko et al. 2015, Koenen and Aikawa 2018). The ubiquitous property of MPs is their pro-coagulant phenotype discovered and widely studied in cardiovascular diseases and hemostasis. By exposing phosphatidylserine, and sometimes tissue factor (TF), the MPs

constitutes a catalytic surface for the assembly and activation of blood coagulation complexes and thrombin generation (Morel, Jesel et al. 2011). Under normal physiological conditions, MPs do circulate in blood but at low concentration. However, in pathological conditions, the circulating levels of MPs derived from different origins, including platelets, leukocytes, endothelial cells, red blood cells and smooth muscle cells, are increased and are proportional to the severity of disease. Therefore, microparticles have potential use as biomarkers for the diagnosis and prognosis of different pathological conditions. Similarly, they appear as a pivotal target for the therapeutic follow-up in cardiovascular disease or even graft rejection (Amoura, Zobairi El-Ghazouani et al. 2019).

2.2 Mechanisms of formation of microparticles

The formation and release of microparticles mainly involves the loss of membrane phospholipids asymmetry and limited cytoskeleton cleavage and its re-organization (Figure 13).

2.2.1 Loss of asymmetry of membrane phospholipids and formation of Microparticles

In resting eukaryotic cells, the distribution of phospholipids in plasma membrane is asymmetrical which is key to maintain important cellular functions including membrane potential and cell activation (Ma, Poole et al. 2017, Ridger, Boulanger et al. 2017). In all eukaryotic cells, sphingomyelin (SM) and phosphatidylcholine (PC) are located in outer membrane leaflet while phosphatidylethanolamine (PE) and the negatively charged lipids phosphatidylserine (PS) and phosphatidylinositol (PI) within the inner leaflet (Figure 11). Other lipids, like cholesterol and ceramides, are present within both membrane leaflets but mostly enriched within inner leaflet (Marquardt, Geier et al. 2015).

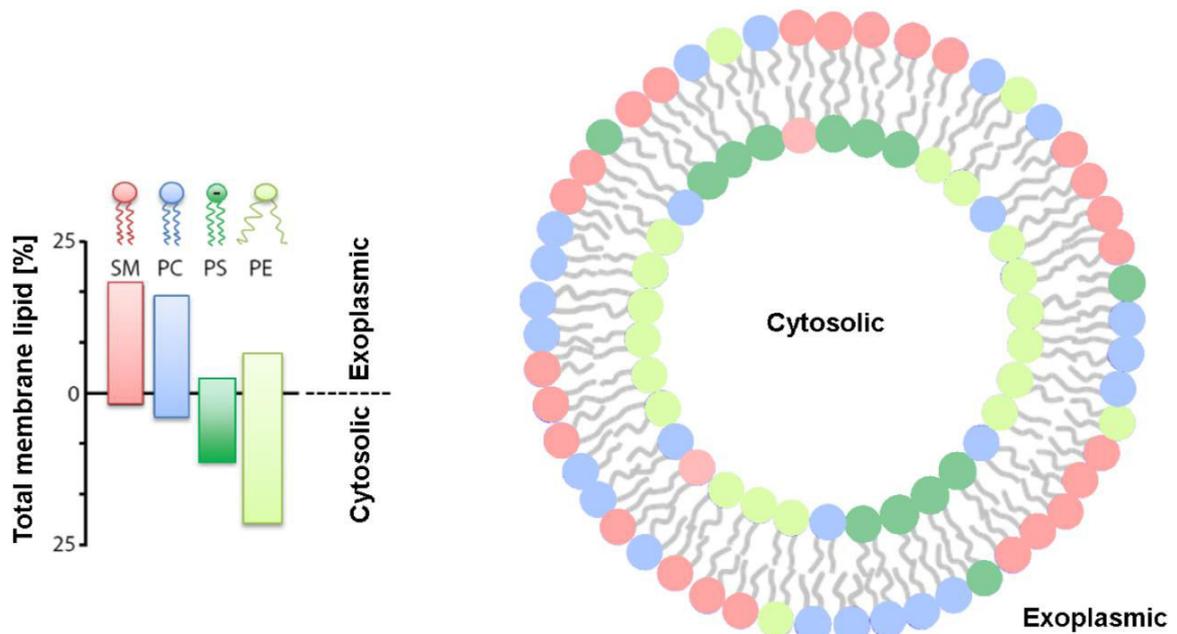


Figure 11. Asymmetrical distribution of phospholipids in red blood cell membrane
(Marquardt, Geier et al. 2015)

The asymmetrical distribution of phospholipids is not at equilibrium and is, therefore, maintained actively (requiring ATPs) by cells. There are three main classes of proteins involved in the control of asymmetrical distribution of membrane phospholipids i) ATP-dependent floppases, responsible for the outward transfer of ii) ATP-dependent flippases (P4 ATPases), that belong to the family of ABC transporters (ATP binding cassette), responsible for the inverse phospholipid transfer iii) Ca^{2+} -dependent scramblases, which swiftly transports phospholipids across the membrane in both directions (Figure 12) (Hankins, Baldrige et al. 2015). Of note, the ABC transporter (ABCA1) has been identified as occasional plasma membrane phospholipids translocator in vascular wall (Toti, Schindler et al. 1997, Hamon, Broccardo et al. 2000, Quazi and Molday 2011).

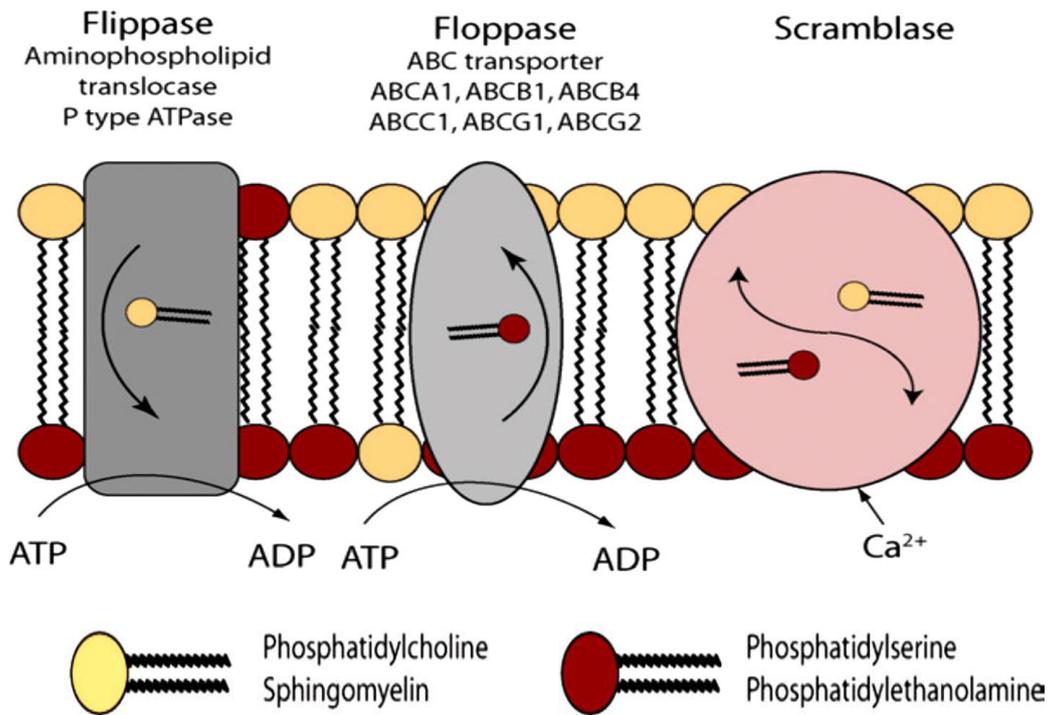


Figure 12. Functions of membrane lipid transporters (Quazi and Molday 2011)

Disruption or loss of asymmetrical distribution of phospholipid is the driving force for the membrane remodeling and generation of MPs (Ridger, Boulanger et al. 2017). During cell activation or apoptosis, the prominent change is the translocation of PS to the outer leaflet which precedes MP formation (Lee, Meng et al. 2013). Excess of negative charge is created at cell surface due to the rapid egress of PS which imbalances the molecular masses of the two membrane leaflets and is not counterbalanced at equal speed by PC transporters. The surface tension between the two leaflets is abolished by MP shedding. MP shedding is enabled because of the partial loss of the interaction between the cytoskeleton proteins and the inner leaflet resulting from its proteolysis. The link of MP shedding with PS externalization is confirmed in patients with Scott's syndrome, a rare human hereditary hemorrhagic disorder with defective platelet's pro-coagulant activity due to defective PS externalization ability, also accompanied by reduced MP shedding (Toti, Satta et al. 1996). Inability to externalize PS by patients with Scott's syndrome is due to mutated scramblase TMEM16-F (anoctamine, ANO6) but not due to, previously thought, phospholipid scramblase (PLSCR1) as PLSCR1 is not mutated in Scott's syndrome patients and calcium induced PS externalization is also not affected in mouse cells with knock out

PLSCR gene, confirming PLSCR is not responsible for inability to expose PS by Scott's syndrome patients (Hankins, Baldrige et al. 2015).

Externalization of PS is controlled by calcium-dependent imbalanced activities of flippases, floppases and scramblases. Increased cytosolic levels of calcium results in the inhibition of flippases which is followed by externalization of PS by overwhelming actions of floppases and/or scramblases with a lower phosphatidylcholine and sphingomyeline reverse transport. This initiates local transverse instability of the plasma membrane and shedding of MPs, also associated with lateral membrane reorganization and raft clustering (Benameur, Osman et al. 2019). The increased intracellular calcium levels, under stress or stimulation, results from opening of the membrane calcium ion channels, the depletion of intracellular calcium stocks, a phenomenon known as capacitative or store-operated calcium entry (SOCE) leading to significant increase in cytoplasmic calcium concentration (30-350 μM compared to 1 μM at basal state) (Kunzelmann-Marche, Freyssinet et al. 2001). Depolarization of the outer membrane of mitochondria also contributes to increased intracellular calcium levels *via* the opening of mitochondrial. The SOCE pathway including Orai channel 1 and mitochondrial STIM1 calcium sensors as well as other calcium channels such as TRPC6 (transient receptor potential channel), the P2X1 purinoreceptor in platelets, sustain the cell calcium concentration in the cytoplasm to maintain prolong cell stimulation (VARGA-SZABO, BRAUN et al. 2009).

2.2.2 Reorganization/degradation of cytoskeleton and formation of Microparticles

Following intracellular calcium influx and externalization of PS, membrane blebbing involves re-organization/degradation of the cytoskeleton constituents (talin, filamin, gelsolin, myosin, α -actinin) by cysteine proteases, like μ calpains and/or caspases. μ calpain is activated in response to elevated levels of cAMP and subsequent activation of protein kinase A, while caspases chiefly act *via* Rho kinase-dependent phosphorylation of the myosin light chain kinases (MLCK) (Ridger, Boulanger et al. 2017). Calpains are critical for neutrophil or platelet MP shedding, caspases for MP generation in vascular cells under apoptotic and activated conditions. The phosphorylation of MLCK causes actin/myosin-mediated contractile tension, which ultimately results in membrane bleb formation. In apoptotic endothelial cells, the phosphorylation of MLCK is mediated by

the serine/threonine kinase Rho-associated protein kinase- I (ROCK-I), one of the downstream effectors of the small GTP binding proteins, Rab22A or ARF6 (Ridger, Boulanger et al. 2017, Benameur, Osman et al. 2019). Caspase-3 also triggers Xkr8, a putative scramblase or caspase transducer that promotes PS externalization in the membrane of apoptotic cells. In platelets, inhibition of μ calpain prevented the shedding of MP while α IIb β 3-mediated destabilization of cytoskeleton resulted in MP shedding, confirming the importance of cytoskeleton integrity and of its interaction with membrane proteins in shedding of MP (Ridger, Boulanger et al. 2017).

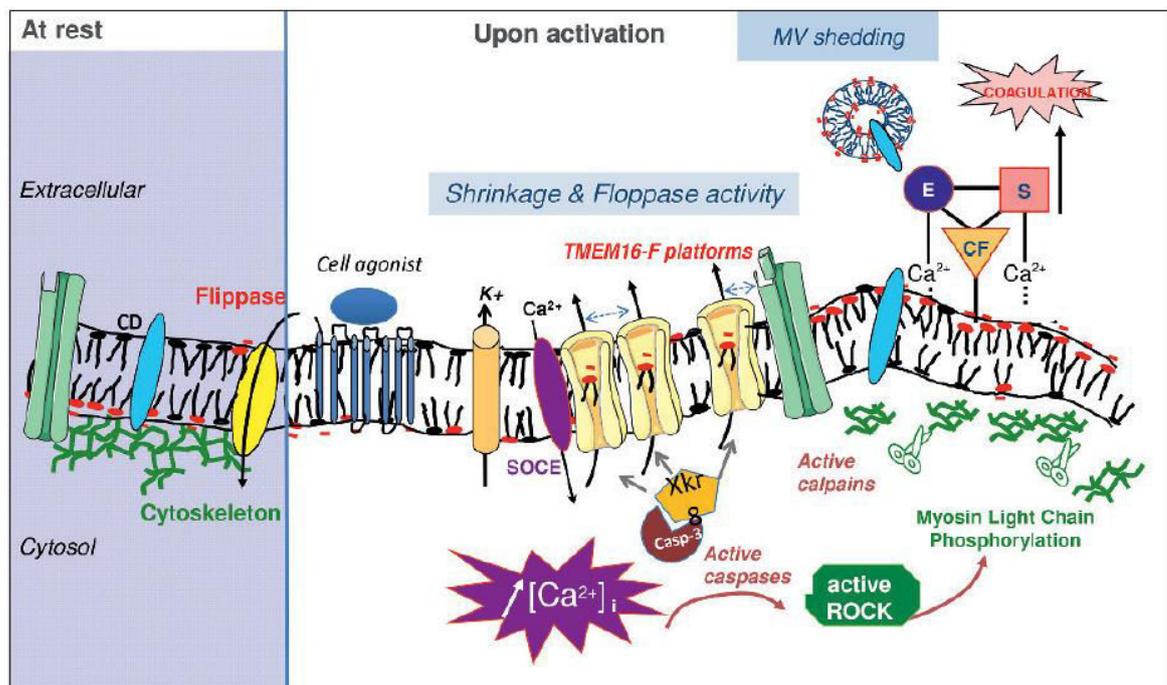


Figure 13. Floppase activity and facilitated transport of phosphatidylserine by TMEM16-F (ANO-6) and procoagulant MP shedding. *At rest, phosphatidylserine (PS) is translocated to the inner leaflet by flippase activity. Upon cell activation and calcium-dependent flippase inhibition, PS translocation to the outer leaflet is driven by TMEM-16F and local K^+ efflux prompts cell shrinkage and re-shaping. High calcium concentration promoted by Stored Operated Channels (SOCE) favors the constitution of TMEM16-F platforms by oligomerisation or interaction with other receptors like P2XR in the case of long term exposure to Ca^{2+} . Transient phospholipid imbalance between leaflets and the proteolysis of cytoskeleton by calpains and/or caspases lead to facilitated procoagulant MP shedding. Putative scramblase transducers as Xkr8 are activated by caspases and would trigger enhanced floppase activity. Exposed PS catalyzes the assembly of blood coagulation complexes at cell and MP surface. (E: Enzyme, S: Substrate, CF cofactor) (Ridger, Boulanger et al. 2017)*

2.3 Composition of MPs

MPs, shed by stimulated/apoptotic cells, carry components from parent's cells including proteins, lipids and nucleic contents (Figure 14)

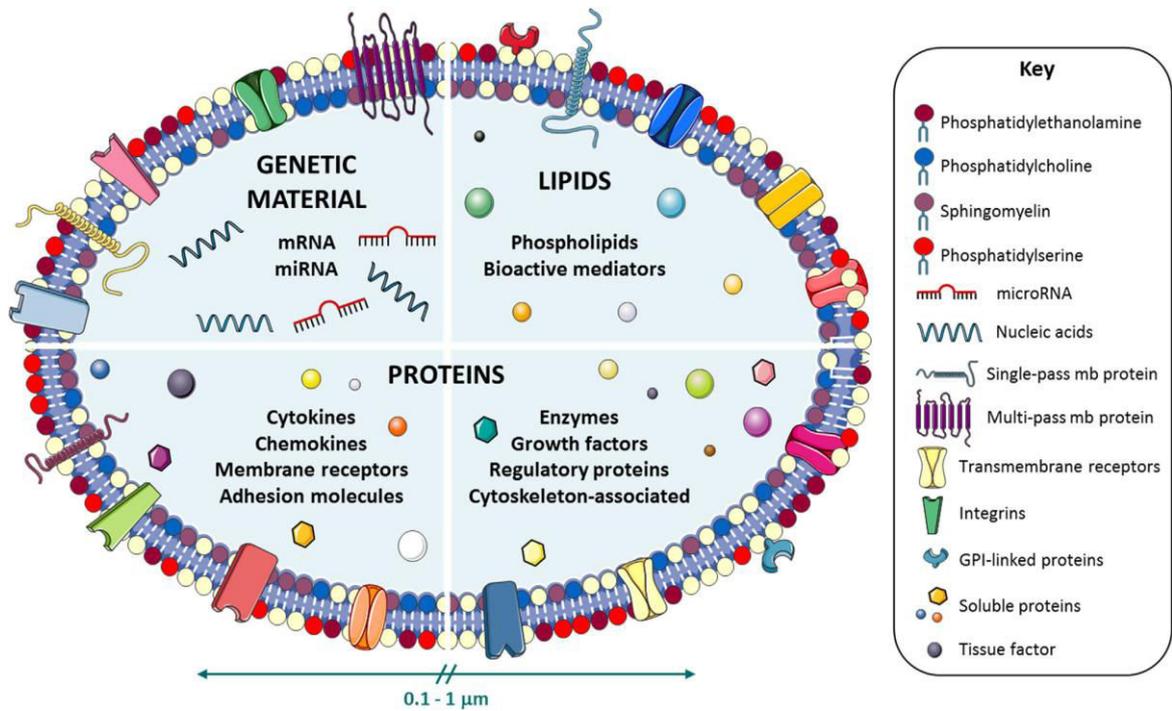


Figure 14. Composition of MPs.

MPs are loaded with distinct components of genetic material (nucleic acids, mRNAs, microRNAs), lipids (phospholipids and bioactive mediators), and proteins (chemokines, cytokines, membrane receptors, enzymes, adhesion molecules, growth factors, and cytoskeleton-associated and regulatory proteins) to eventually mediating intercellular communication (Koenen and Aikawa 2018).

2.3.1 Protein Content of MPs

Analysis of the MP protein content by proteomics has confirmed its relationship to cell origin and the stimulus initiating their generation (Benameur, Osman et al. 2019). For example, T-lymphocytes stimulated with phytohemagglutinin (PHA) for 72 h, followed by 24h-induction of apoptosis by phorbol-12-myristate-13 (PMA) and actinomycin D, leads to the release of MPs exposing morphogen sonic hedgehog (Shh) (Agouni, Mostefai et al. 2007) while T-lymphocytes only stimulated with actinomycin D, lack Shh expression (Mostefai, Agouni et al. 2008). Shh⁺MPs reduce NO bioavailability in endothelial cells and promote endothelial dysfunction in mouse aorta. Thereby confirming the close link between the MP inducer and its properties as cellular effector.

As biogenesis of MPs includes remodeling of plasma membrane and cytoskeleton re-arrangement/degradation, MPs carry proteins involved in their generation as well as cytoskeleton components. For example, the MPs from the tumoral LOX cell line harbor

ARF6, identified as regulator of their shedding (Muralidharan-Chari, Clancy et al. 2009). Actin is detected in MPs derived from RBCs and neutrophils (Pollet, Conrard et al. 2018).

MPs are enriched in several membrane proteins including adhesion molecules, like, P-selectin, glycoprotein Ib (GPIb) or integrins (GPIIb/IIIa). Characteristic membrane proteins can be used for MP detection, identification of the cell/stimulus from which they are generated (CD42a for platelets or CD3 for T-cells), and furthermore, on the basis of specific and differential expression of specific patterns between MPs from healthy/abnormal/activated or apoptotic cells, MPs can be used as a tool for the diagnosis and progression of diseases (Distler, Pisetsky et al. 2005). As apoptosis is characterized by nucleus condensation and fragmentation, apoptotic MPs would be identified on the basis of a high proportion of genetic and nuclear content (Saleh and Kabeer 2015). Other proteins include membrane receptors, transcription factors and bioactive enzymes such as metalloproteinases (MMPs), mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI-3K) and phospholipases A2, C and D, depending on the lineage, stress and species (Benameur, Osman et al. 2019).

2.3.2 Lipid content of MPs

The phospholipid composition of MPs varies with the cell origin, mechanism of its generation as well as the oxidation status of lipids (Pollet, Conrard et al. 2018). For instance, circulating MPs derived from platelets (PMPs) comprise of 20% sphingomyelin, 9% phosphatidylethanolamine (PE), 60% phosphatidylcholine (PC), 5% phosphatidylserine (PS) and minor quantities of other lipids including PAF and inositolphosphate (Weerheim, Kolb et al. 2002, Cognasse, Hamzeh-Cognasse et al. 2015). Furthermore, the MP lipid composition will vary with the parental cell niche and the degree of lipid oxidation (Fourcade, Simon et al. 1995, Huber, Vales et al. 2002). As a matter of proof, MPs from apoptotic or activated ECs have distinct lipid profile (Jimenez, Jy et al. 2003) and circulating MPs or MPs from the atherosclerotic plaque also differs (Leroyer, Isobe et al. 2007). The lipids composition and hence function of MPs from healthy subjects is different from those MPs which are generated and collected from patients with metabolic disturbances and dyslipidemias (Nomura, Inami et al. 2009). Although extensive data are lacking for all MP lineages, it was shown that the pro-coagulant activity of platelet MP is 50-100 folds higher than that of activated platelet. Thereby confirming their pro-coagulant feature (Sinauridze, Kireev et al. 2007).

2.3.3 Nucleic acid and miRNA content of MPs

The presence of functional mRNA in MP was described making them true vehicles for transfer of genetic material between cells (Deregibus, Cantaluppi et al. 2007, Jansen, Yang et al. 2013, Agouni, Andriantsitohaina et al. 2014). miRNAs are small non-coding RNAs which play a critical role in epigenetic regulation inside the cell or circulation associated with lipoproteins or packaged within exosomes or MPs. They control physiological and metabolic processes (Fernandes, Acuña et al. 2019) and are involved in many inflammatory, cardiovascular, and metabolic disorders (Fernandez-Hernando, Ramirez et al. 2013). In vivo administration of endothelial cell-derived MPs (EMPs) containing miR-126 can accelerate re-endothelialization in carotid arteries after the mice were exposed to the injury from electric endothelial denudation (Jansen, Yang et al. 2013, Chen, Li et al. 2018). Thrombin-mediated activation of platelets alters the miRNAs signature of platelets and their derived MPs with differential expression of miR-15a, miR-98, miR-339-3p, miR-365, miR-361-3p and miR-495 (Benameur, Osman et al. 2019), thereby confirming the cell niche and activation on the characteristic features of MPs during cardiovascular diseases (Loyer, Vion et al. 2014).

2.4 Mechanisms of interaction of MPs with recipient cells

In the last decade, many studies investigated the effector role of MPs as effector molecules. Initially, (Barry, Pratico et al. 1997) showed specific biological effects in endothelial cells targeted by platelet-MPs via transfer of bioactive lipids. Similarly, (Barry, Kazanietz et al. 1999) showed modulation of pro-coagulant responses in platelets following transfer of arachidonic acid between activated and resting platelets. Since then, numerous studies have confirmed MPs as very efficient mode of cell-cell communication and cargo delivery among different lineage including platelets, leukocytes and endothelial cells. Platelet-MPs-mediated transfer of adhesion molecule CD41 (integrin α -IIb) to endothelial cells results in pro-adhesive phenotype of endothelial cells (Barry, Pratico et al. 1998). MPs from platelets also transfers chemokines CCL5 (RANTES) to targeted endothelial cells *via* GPIIb/IIIa and JAM-A dependent pathways leading to recruitment of monocytes (Mause, von Hundelshausen et al. 2005). Furthermore, MPs isolated from atherosclerotic human plaques have been shown to transfer intercellular

adhesion molecule-1 (ICAM-I) to endothelial cells following membrane fusion, resulting in increased adhesion of monocytes (Rautou, Leroyer et al. 2011).

MPs can transfer biological messages. MPs can interact through multiple mechanism at each encounter *via* i) ligand/receptor type of interaction ii) transfer of surface proteins, receptors, ligands, genetic material, lipids iii) membrane-membrane fusion iv) internalization of MPs by target cell (Figure 15) (Ridger, Boulanger et al. 2017, Pollet, Conrard et al. 2018, Benameur, Osman et al. 2019).

In addition, MPs also trigger multiple signaling pathways that are time-orchestrated potentially making them versatile effectors depending on their acute or sustained interaction with the target cells. For example, MPs carrying morphogen Shh induced activation of morphogen signaling pathway in targeted endothelial cell resulting in rapid NO release and enhancement of angiogenic process (Agouni, Mostefai et al. 2007, Benameur, Osman et al. 2019), possible owing to their internalization and enhanced expression of antioxidant enzymes (Soleti, Lauret et al. 2012).

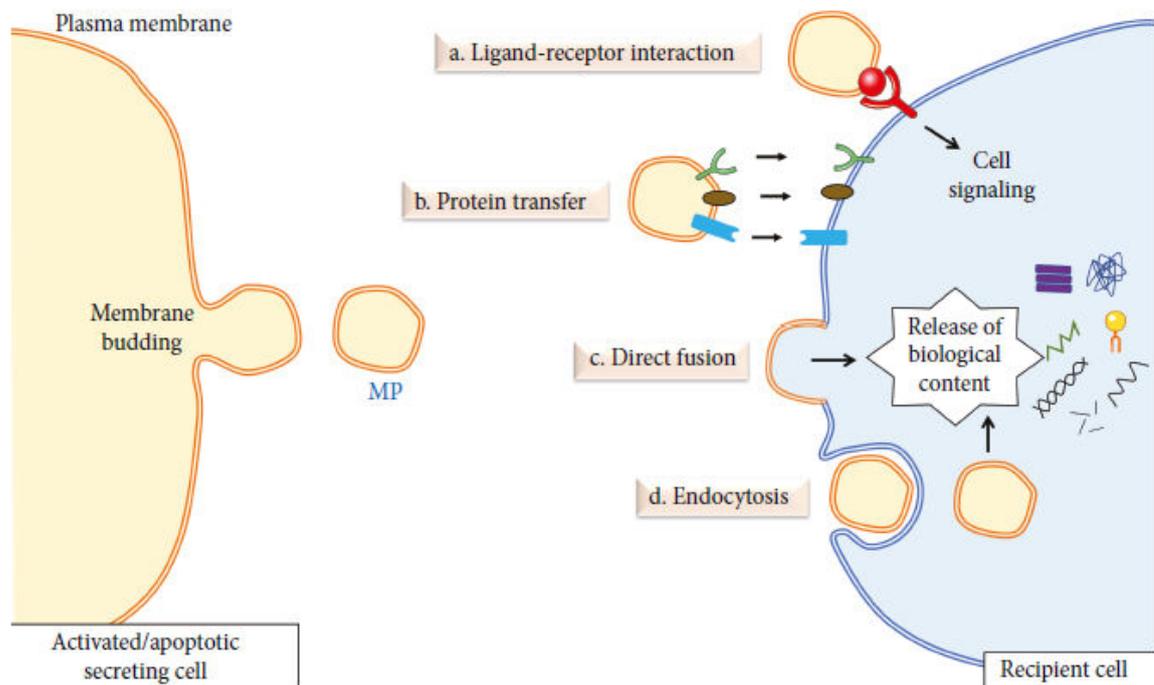


Figure 15. Mechanisms of interaction of MPs with recipient cells.

a) MPs can then directly interact with the recipient cell via ligand-receptor interaction which results in cell signaling b) transfer of proteins (adhesion molecules, MHC, and membrane receptors) from the MP vesicle to the surface of the recipient cell c) deliver the biological content of the MP to the recipient cell via either direct fusion of the MP with the plasma membrane of the recipient cell d) endocytosis. MHC: Major Histocompatibility Complex (Benameur, Osman et al. 2019).

2.5 MPs and vascular damage

MPs, once considered as inert particles, are now studied extensively as vascular effectors (Dickhout and Koenen 2018). Circulating MPs from different origins including platelets, leukocytes, erythrocytes and/or endothelial cells, not only act as biomarker of organ damage but also act as vascular effectors due to their capability to regulate endothelial function (Saleh and Kabeer 2015, Suades, Padro et al. 2015) and interaction between vascular cells involved in hemostasis, angiogenesis and vascular repair (Boulanger, Loyer et al. 2017, Chen, Li et al. 2018, Sluijter, Davidson et al. 2018).

2.5.1 Microparticles and endothelial dysfunction

Endothelium plays vital role in maintaining cardiovascular homeostasis by secreting endothelium-derived relaxing and contracting factors (Rajendran, Rengarajan et al. 2013). NO is the key endothelium-derived relaxing factor which plays a focal role in the maintenance of vascular tone. NO is also an inhibitor of inflammation, coagulation and oxidative stress. Diminished or reduced production of NO characterizes endothelial dysfunction, an early event in the progression of numerous cardiovascular diseases (Bauer and Sotnikova 2010).

Endothelium is the one of primary targets of circulating MPs (Boulanger Chantal 2016). Recent studies have reported that MPs can directly alter the endothelial function by increasing endothelial oxidative stress, reducing NO formation, inducing endothelial cell senescence and shifting endothelial cell towards pro-coagulant and pro-inflammatory phenotypes enabling stimulated platelets and monocyte adhesion (Neves, Rios et al. 2019). MPs from activated T-lymphocytes decrease the production of NO and promote oxidative stress in different sources of endothelial cells. Reduced NO production is the consequence of impaired endothelial NO synthase activity eventually involving phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2), and nuclear factor- κ -light-chain-enhancer of activated B cell (NF- κ B) pathways. These *in-vitro* observations were confirmed *in-vivo* by injection of MPs from human apoptotic T-lymphocytes resulted in impaired acetylcholine-evoked endothelial relaxation in mice aorta (Mostefai, Agouni et al. 2008).

Leukocytes MPs (LMPs) may originate from neutrophils (nMPs), monocytes/macrophages (mMPs), lymphocytes (B and T lymphocytes, IMPs). LMPs carry surface markers (such as CD11b, CD3, CD45) from parent cells and also harbor

cytosolic and membrane proteins (ICAM-1, metalloproteases, complement factor, PSGL-1, TF, interleukin-1 β) and bioactive lipids (phosphatidylserine, PS) that are implicated in regulation of various fundamental processes such as inflammation, coagulation, endothelial function, angiogenesis, apoptosis and vascular remodeling (Angelillo-Scherrer 2012, Ridger, Boulanger et al. 2017, Zara, Guidetti et al. 2019). In healthy individuals, circulatory levels of LMPs accounts for only 10% of total MP population in blood. Higher circulatory levels of LMPs during immune cell activation, leukocytes proliferative/apoptotic process and numerous cardiovascular, metabolic and inflammatory disorders sign leukocytes activation and suggest a key role in the coupling of thrombosis and inflammation (Ridger, Boulanger et al. 2017), most probably during prolong release (Benameur, Osman et al. 2019). In-vitro treatment of endothelial cells (ECs) with LMPs triggers the up-regulation of pro-inflammatory genes in endothelial cells (ECs), leading to production of cytokines and leukocytes-endothelial cells adhesion molecules (Angelillo-Scherrer 2012). Because of expression of L-selectin on their surface (Gasser, Hess et al. 2003), neutrophils MPs (nMPs) adhere to and activate endothelial cells to secrete interleukins and expose tissue factor (TF) on surface, prompting a pro-thrombotic and pro-inflammatory endothelial response with the risk of cardiovascular event (Chironi, Simon et al. 2010). Monocytes/macrophages MPs (mMPs) carry intercellular adhesion molecule (ICAM-1) and therefore, enhance vascular inflammation through leukocytes at the endothelium (Rautou, Leroyer et al. 2011). mMPs can activate endothelial cells by delivering IL-1 β (Wang, Williams et al. 2011). In addition, MPs from lymphocytes (lMPs) up-regulate NF- κ B and cyclooxygenase-2 (COX-2) in the endothelium and vessel wall .(Tesse, Martinez et al. 2005) Neutrophils MPs (nMPs) contains α M β 2 (Mac-1, macrophage antigen-1) which mediate interaction of MPs with resting platelets leading to platelets activation, increased P-selectin expression and perpetuate formation of thrombus (Pluskota, Woody et al. 2008). LMPs can also induce endothelial dysfunction by altering the balance between NO production and oxidative stress. Indeed, LMPs induced unbalanced oxidative stress in ECs associated with decreased NO levels and reduced endothelial NO synthase activity involving phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase 1/2 (ERK1/2) and NF- κ B (Angelillo-Scherrer 2012).

Endothelial MPs (EMPs) induced oxidative stress lead to the up-regulation of adhesion proteins in cultured venous or arterial endothelial cells (Paudel, Panth et al. 2016). Interestingly angiotensin II favors the release of pro-oxidant EMPs with enhanced

adhesion to macrophages and pro-inflammatory endothelial effects (Burger, Montezano et al. 2011). Dihydroethidium (DHE) staining and western blot of EMPs from murine aorta ECs showed expression of p22^{phox}, a subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and some NADPH that may fuel ROS production in MPs. In addition MPs were enriched in of p22^{phox} and Nox4 while poor in of p67^{phox} and Nox1 as compared to the EC concentration (Burger, Turner et al. 2016).

(Boulanger Chantal, Scoazec et al. 2001) showed that MPs circulating in blood of patients with myocardial infarction or non-ischemic syndrome impair endothelium-dependent relaxation in aortic rings of rats.

Compared to healthy patients, circulating level of MPs of leukocyte origin is significantly increased in patients with metabolic syndrome (Chironi, Simon et al. 2010). Injection of such MPs into mice resulted in impaired endothelium dependent relaxation, by increasing ROS release and by altering cyclooxygenase metabolites and monocyte chemotactic protein-1 via Fas/Fas-ligand pathway (Agouni, Ducluzeau et al. 2011). Moreover, circulating MPs isolated from the plasma of patients with diabetes (Tesse, Martinez et al. 2005, Rodrigues, Pietrani et al. 2018), myocardial infarction (Boulanger Chantal, Scoazec et al. 2001), valvular heart disease (Fu, Hu et al. 2015) and undergoing percutaneous coronary intervention (Ye, Shan et al. 2017) alter the endothelial function and alter the expression of eNOS in rat or mice vessel rings.

2.5.2 Microparticles and inflammation

Inflammation is a key regulator that links various risk factors of cardiovascular disease with altered arterial biology (Fioranelli, Bottaccioli et al. 2018). For instance, circulatory MPs from patients with obstructive sleep apnea or preeclampsia induced up-regulation of inflammatory COX-2 in human ECs and also in mice aorta (Priou, Gagnadoux et al. 2010, Tual-Chalot, Fatoumata et al. 2012). MPs have been well documented to be inflammatory effectors enhancing the secretion of various pro-inflammatory mediators. MPs of different origins such as leukocytes, endothelial and platelet circulate at higher levels during different inflammatory processes and can also trigger various pro-inflammatory pathways (Figure 16).

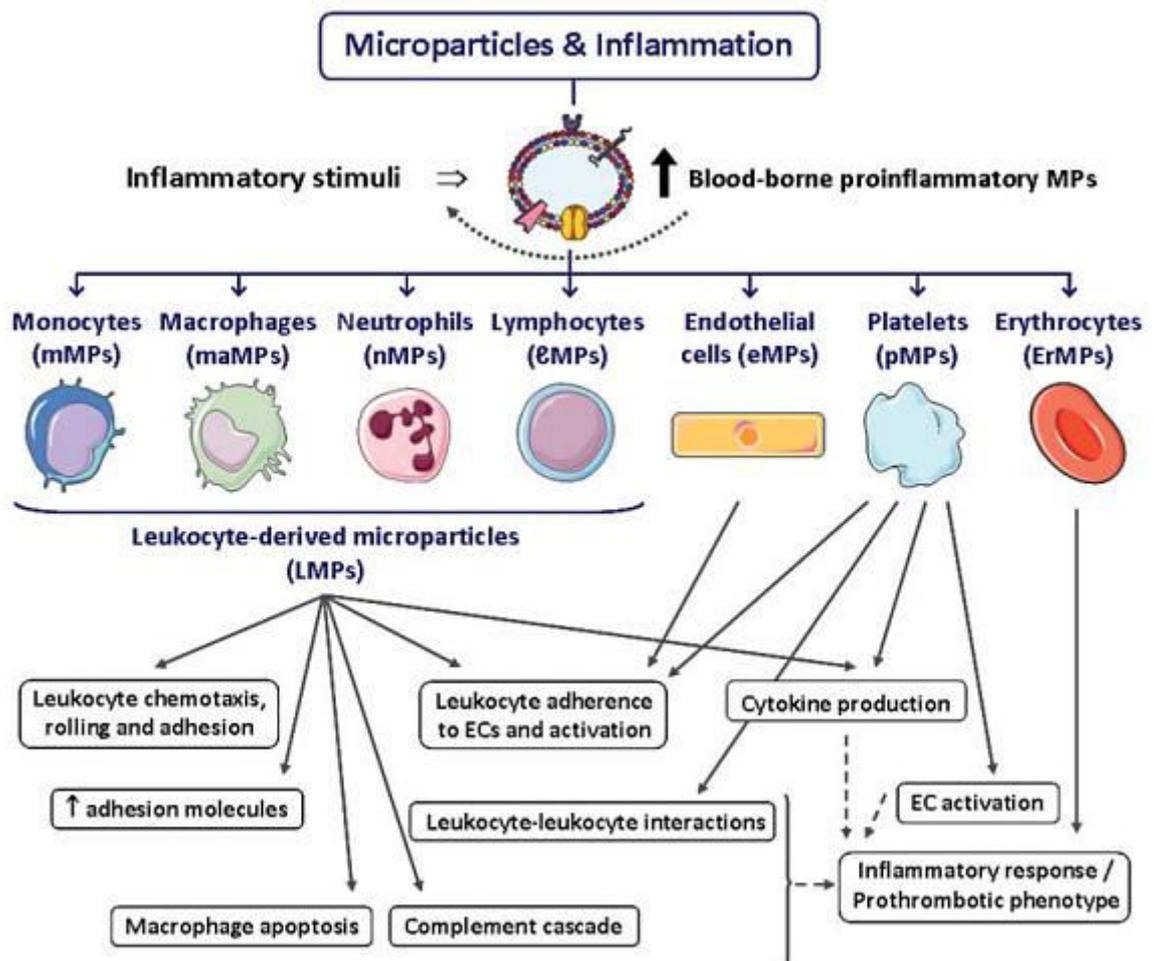


Figure 16. Cell-type specific MPs and inflammatory responses.

(Suades, Padro et al. 2015)

Endothelial MPs (EMPs) are the markers and effectors of vascular injury associated with vascular inflammatory and thromboembolic complications. Their levels are raised in inflammatory conditions such as atherosclerosis, coronary artery disease (CAD) and hypertension, suggesting inflammation has a role in vascular damage. (Deng, Wang et al. 2017). In response to oxidative stress, shed EMP carry active oxidized phospholipids which trigger activation of neutrophil and promote endothelial adherence of monocytes. This is an early and a key event in atherogenesis. Because EMP bear ICAM-1, PECAM-1 and VCAM-1 and constitute an additional adhesive surface they promote the adhesion of leukocytes to the endothelium, their incorporation into the thrombus (see below) (Paudel, Panth et al. 2016). Moreover, in the APOE^{-/-} mouse EMPs can also recruit leukocytes through surface expression of CD11b and contribute to overall

inflammatory process by targeting rafts (Burger, Montezano et al. 2011). Leukocytes MPs (LMPs) also play important role in vascular inflammation by enhancing release of cytokine from endothelial cells and promoting pro-inflammatory activity by inducing leukocytes recruitment and monocytes chemotaxis (Angelillo-Scherrer 2012, Suades, Padro et al. 2015, He, Tang et al. 2017). Monocytes MPs are an important route of mature IL-1 β secretion. IL-1 β conveyed by monocyte MPs is able to bind to receptor (IL-1R) and initiate inflammation, suggesting that MPs are likely to promote plaque growth of atheroma and vascular inflammation (Rautou, Leroyer et al. 2011, Paudel, Panth et al. 2016). Molecular mechanisms responsible for the pro-inflammatory effects of MPs involves activation of nuclear factor kappa light chain (NF-kB) and up-regulation of cyclooxygenase-2 (Tesse, Martinez et al. 2005, Scanu, Molnarfi et al. 2008, Neri, Armani et al. 2011). Platelets MPs (PMPs) from activated platelets contributes to inflammatory process by triggering leukocyte-endothelial cells cross talk through enhanced binding via p-selectin/p-selecting glycoprotein ligand-1 (PSLG-1) (Montoro-Garcia, Shantsila et al. 2014) and increasing the recruitment of immune cells such as natural killer cells, monocytes and lymphocytes on surface of endothelium (Cognasse, Hamzeh-Cognasse et al. 2015). In the hyperlipidemia mouse, PMPs promote monocytes adhesion to endothelium by depositing inflammatory mediators e.g. c-c chemokine ligand 5 (CCL5), increasing expression of ICAM-1 in monocytes and binding to monocytes through fractalkine (FKN)/FKN receptor (CX3CL1-CX3CR1) axis (Postea, Vasina et al. 2012, Montoro-Garcia, Shantsila et al. 2014). In addition, PMPs can also facilitate monocytes migration, tissue recruitment and differentiation into macrophages by activating monocytes via RANTES (regulated upon activation, normal T-cell expressed and secreted) pathways (Mause, von Hundelshausen et al. 2005).

In the atherothrombosis plaque, a proportion of MPs carry metalloproteases ADAM17 (TACE), and contribute to inflammation by promoting the secretion of TNF and the expression of endothelial TNF-R and endothelial protein c receptor (EPCR) (Canault, Leroyer et al. 2007).

2.5.3 Microparticles, coagulation and thrombosis

Microparticles are strong pro-coagulant actors due to the exposure of phosphatidylserine (PS) and tissue factor (TF) on their outer membrane leaflet (Morel, Toti et al. 2006). PS is an anionic phospholipid which catalyzes the assembly of

components of coagulation cascade due to an electrostatic interaction between negatively charged PS and positively charged gamma-carboxyglutamic acid (GLA) domains on clotting factor VII, IX, X and prothrombin (Figure 9) (Owens and Mackman 2011). The major source of MPs are originated from platelets and they account for 70 to 90% of total circulating MPs (Berckmans, Nieuwland et al. 2001, Hron, Kollars et al. 2007). These PS⁺ platelet MPs bear receptors for both von Willebrand factor and collagen, suggesting their potential role in primary hemostasis. Bleeding tendency in patients with Scott's syndrome and Castaman's defect is due to defect in the ability of activated platelets to transport PS to the surface of cell (Owens and Mackman 2011) and/or to shed MPs. The hemostatic role of MPs was further confirmed by (Hrachovinova, Cambien et al. 2003) who showed that by increasing the TF⁺MPs levels in circulation, bleeding was abolished in mouse model of hemophilia A.

Aside from the catalytic activity of PS⁺MPs that enables the formation of blood coagulation complexes, TF is another contributor to their pro-coagulant potential. TF is a receptor of high affinity for Factor VII/VII that acts as cellular initiator of coagulation (Mackman 2009, Cimmino and Cirillo 2018). The pro-coagulant property of MPs bearing TF increases dramatically. Under normal physiological conditions, binding of circulatory FVII/VIIa to TF is well regulated by tissue factor pathway inhibitor (TFPI) which is synthesized by endothelial cells and circulates in blood to inhibit inappropriate initiation of coagulation (Brummel-Ziedins and Mann 2018). Circulating level of TF⁺MPs, under normal conditions, is too low to contribute to thrombin and their TF activity is detectable only after inhibition of TFPI (Mooberry and Key 2016). However, under stimulation by TNF α or LPS, human umbilical vein endothelial cells (HUVEC) shed FT⁺MPs that shorten the coagulation time of normal plasma but not of Factor VII deficient plasma, confirming the FVII dependent pro-coagulant activity of TF in these MPs.

Furthermore, elevated levels of FT⁺MPs in numerous thrombotic conditions and incorporation of FT⁺MPs in growing thrombus indicates correlation between the expression of TF on the surface of MPs and their thrombogenicity in cardiovascular disease and associated disorders (Biro, Sturk-Maquelin et al. 2003). Previous findings showed that vessel wall is the sole source of TF that contribute to thrombosis in carotid artery injury model in mouse. However, (Reinhardt, von Brühl et al. 2008) reported that injecting mice with human monocytes TF bearing MPs increased the fibrin accumulation in carotid artery ligation model. Role of TF⁺MPs in thrombus formation is also studied in laser endothelial injury model of arteriole thrombosis (Falati, Gross et al. 2002)

observed recruitment of TF⁺MPs to the thrombus, accompanied by swift buildup of TF and fibrin upstream of the thrombus. Further they also showed that accumulation of TF was more at thrombus-vessel wall interface, indicating vessel wall TF also contribute to thrombus formation. In mice, kinetics of TF incorporation in growing thrombus was examined by Gross and colleagues and found that accumulation of TF⁺MPs of leukocyte origin into developing arteriolar thrombus is rapid and reach to peak within 60 sec after the initiation of endothelial injury (Gross, Furie et al. 2005). In patients with acute coronary syndrome (unstable angina and myocardial infarction) and patients undergoing angioplasty, the circulatory levels of pro-coagulant MPs and plasma levels of TF antigen is raised. In plaques, 50% of total isolated MPs were found to possess TF and 97% of the pro-coagulant activity of these MPs was due to TF (Mallat, Hugel et al. 1999). Furthermore, in comparison to MPs from blood of same patient, the plaque MPs showed higher thrombogenic potential (Leroy, Isobe et al. 2007, Owens and Mackman 2011).

In conclusion, circulating levels of pro-coagulant MPs appears to be the relevant index of the overall vascular status enabling the appraisal of the individual atherothrombotic risk.

2.5.4 Microparticles and endothelial senescence

During endothelial senescence from human coronary ECs (Abbas, Jesel et al. 2017) or from murine aortic ECs (Burger, Kwart et al. 2012), MPs are pro-senescent endothelial effectors. Endothelial MPs (EMPs) harvested from plasma of patients with acute coronary syndrome (ACS) and from aged porcine coronary artery endothelial cells induce premature endothelial senescence through angiotensin II-induced oxidative stress leading to activation of MAPKs and PI3-kinase/Akt pathways (Abbas, Jesel et al. 2017). Pro-senescent effect of EMPs was also associated with shedding of secondary pro-senescent and pro-coagulant MPs from these MPs-targeted cells which can act in auto- or paracrine fashion to further promote induction of senescence. Pro-senescent EMPs act *via* increase ROS through NADPH oxidase and mitochondrial respiratory chain pathways of ROS generation (Figure 17) (Burger, Kwart et al. 2012, Abbas, Jesel et al. 2017). Similarly, (Simoncini, Chateau et al. 2017) reported that sirtuin-1 (Sirt1) deficiency-mediated accelerated ageing and dysfunction of endothelial colony forming cells is accompanied by increased shedding of EMPs which promote senescence in naïve endothelial cells involving MAPK and pro-oxidant pathways.

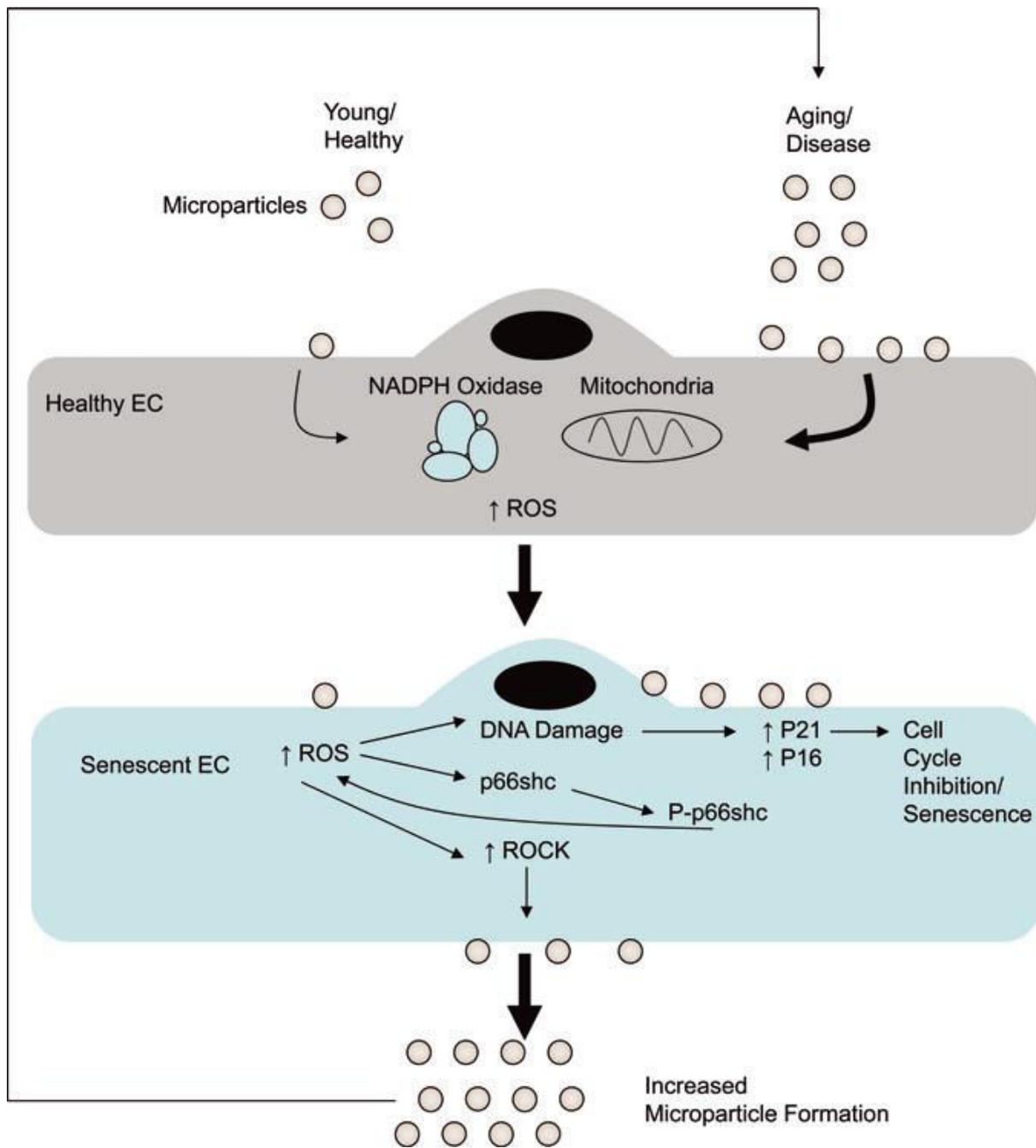


Figure 17. Putative mechanisms of MPs induced vascular cell aging and senescence.

MP formation is increased in aging or under stress (disease) conditions, MPs, in turn, promote oxidative stress in ECs via NADPH oxidase and mitochondria. Increased oxidative stress ultimately results in EC senescence through DNA damage, leading to expression of cell cycle inhibitors p16ink4a (p16) and p21cip1 (p21) and increased activation of p66Shc, a determinant of cell longevity. The resultant EC senescence leads to increased formation of secondary MPs via ROCK, which further promotes oxidative stress in a feed-forward fashion. (Burger, Kwart et al. 2012). (See also chapter 3)

Our team has shown that stimulation of leukocytes with LPS or PMA/I induce shedding of MPs (LMPs) which induce premature endothelial senescence through

activation of inflammatory and senescence-related molecular pathways and are associated with the shedding of secondary pro-coagulant EMPs (Thesis Ali El Habhab, February 2018). In addition, LMPs from stimulated leukocytes exaggerate the pro-senescent potential of high glucose on porcine coronary artery endothelial cells (Thesis Raed Altamimy, May 2018).

In conclusion, MPs contribute to vascular damage by inducing pro-inflammatory, pro-thrombotic and pro-senescent effects leading to endothelial dysfunction and increased risk of cardiovascular events (Figure 18).

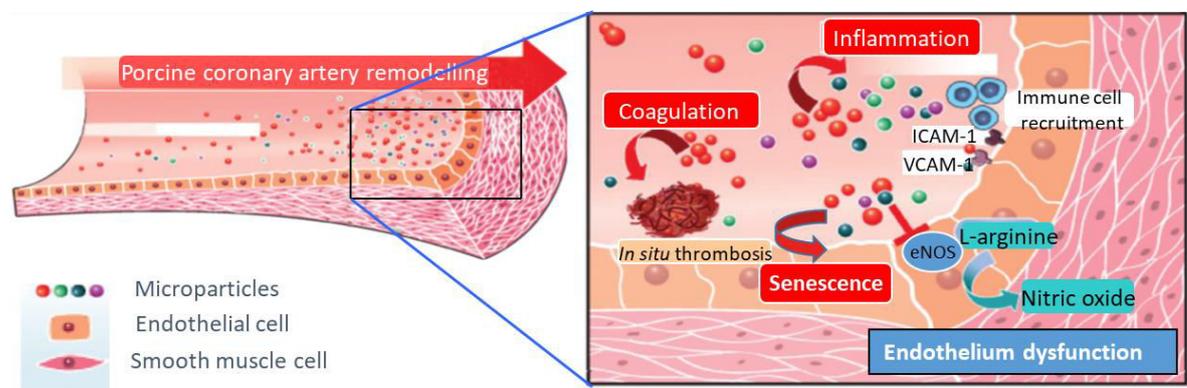


Figure 18. Processes involved in MPs-mediated endothelial dysfunction. (Amabile, Guignabert et al. 2013)

2.6 Microparticles and atherothrombosis

Detected at higher circulating levels, LMPs in asymptomatic patients were positively correlated with subclinical atherosclerotic burden (Chironi, Simon et al. 2006). Human atherosclerosis plaques contains MPs originating predominately from leukocytes and were also reported from smooth muscle cells, erythrocytes and endothelial cells (Leroyer, Isobe et al. 2007). Plaque LMPs contains bioactive protein such as CD40 ligand, major histocompatibility complex class 1 and II (MHC I and II), interleukin 1 β and intercellular adhesion molecule-1 (ICAM-1) which triggers activation of leukocytes, endothelial cell proliferation, monocytes adhesion and migration, and intraplaque neovascularization (Leroyer, Rautou et al. 2008, Rautou, Leroyer et al. 2011, Wang, Williams et al. 2011). Furthermore, plaque LMPs also carry various matrix metalloproteinase (MMP), for instance, MMP-1, MMP-8, MMP-13, suggesting that they

can induce plaque rupture. Monocyte/Macrophage MPs (mMPs) harboring P-selectin (CD15, P-selectin glycoprotein ligand-1), PS and TF may promote atherothrombosis at site of plaque rupture (Wang, Aikawa et al. 2013). In-vivo treatment of ApoE null mice with mMPs promoted infiltration of monocytes and leukocytes in vessel wall leading to the formation of plaque (Hoyer, Giesen et al. 2012). Together all, LMPs are not only markers of plaque development but they are also contributor to initiation and promoter of plaque pathogenesis, growth and rupture (Morel, Jesel et al. 2011, Wang, Aikawa et al. 2013).

Chapter 3: Endothelial senescence and vascular dysfunction

3.1 Overview of endothelium

Vessel wall is composed of three layers; tunica intima (innermost layer), tunica media (middle layer), tunica externa (outer layer). The vascular endothelium is a monolayer of endothelial cells (ECs), comprising of approx. 6×10^{13} endothelial cells, almost equal to 1kg organ of body, covers the luminal side of the entire circulatory system and acts as selective barrier/interface between the blood and vessel wall (Figure 19) (Favero, Paganelli et al. 2014, Cahill and Redmond 2016).

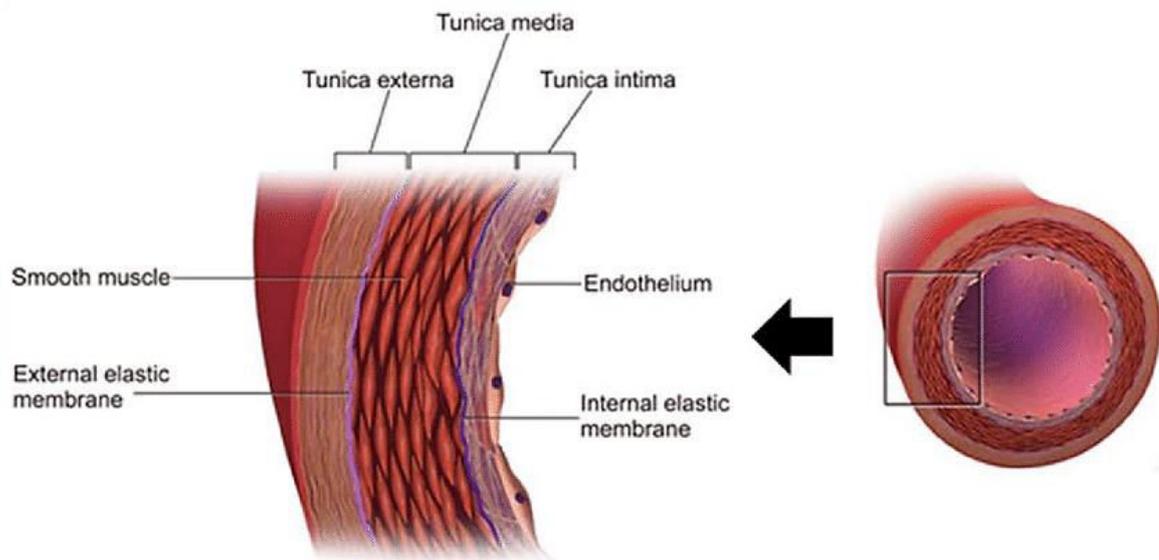


Figure 19. Vessel wall and endothelium (Weidmann 2015)

In 1865, the term “endothelium” was first coined by Wilhelm His, a Swiss anatomist. Initially considered as mere inert diffusion barrier, the important physiological role of endothelium was predicted by Florey. His observation of the ultrastructure of endothelial cells lead to extensive research and key discoveries in the field of vascular and cardiovascular disorders (Figure 20) by providing clues on their origin and progression of (Moncada 2018).

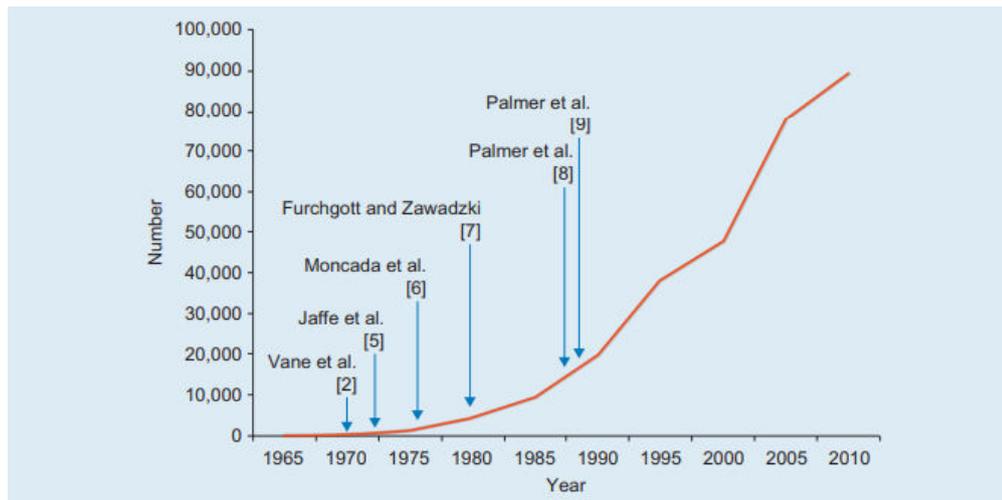


Figure 20. Endothelium-related key discoveries and number of publications/year.
(Moncada 2018)

Structure and function of the endothelium is important for the maintenance of vascular integrity (Murakami and Simons 2009). The endothelium has multiple and tightly controlled functions that are pivotal to hemostasis and vascular homeostasis through balanced actions of auto- and paracrine mediators including vasodilators, vasoconstrictors, coagulants and anti-coagulants, pro- and anti-inflammatory, fibrinolytic and anti-fibrinolytic, pro- and anti-oxidant and proangiogenic factors (Kazmi, Boyce et al. 2015). Multiple functions of endothelium include regulation of vascular tone and permeability, blood fluidity, inflammatory responses, platelet adhesion, angiogenesis, production of extracellular matrix products and smooth muscle cell proliferation (Figure 21) (Widmer and Lerman 2014). Vascular tone is regulated by the release of vasoactive mediators including vasodilating and vasoconstricting factors. The balance between vasodilating and vasoconstricting factors is important and tightly maintained at neutral state, favoring dilation or constriction by endothelium to ensure proper perfusion of organ. The vasodilatory response is predominantly regulated by the release of nitric oxide (NO), which is synthesized from L-arginine by the activity of endothelial nitric oxidase (eNOS) (Sandoo, van Zanten et al. 2010, Rajendran, Rengarajan et al. 2013).

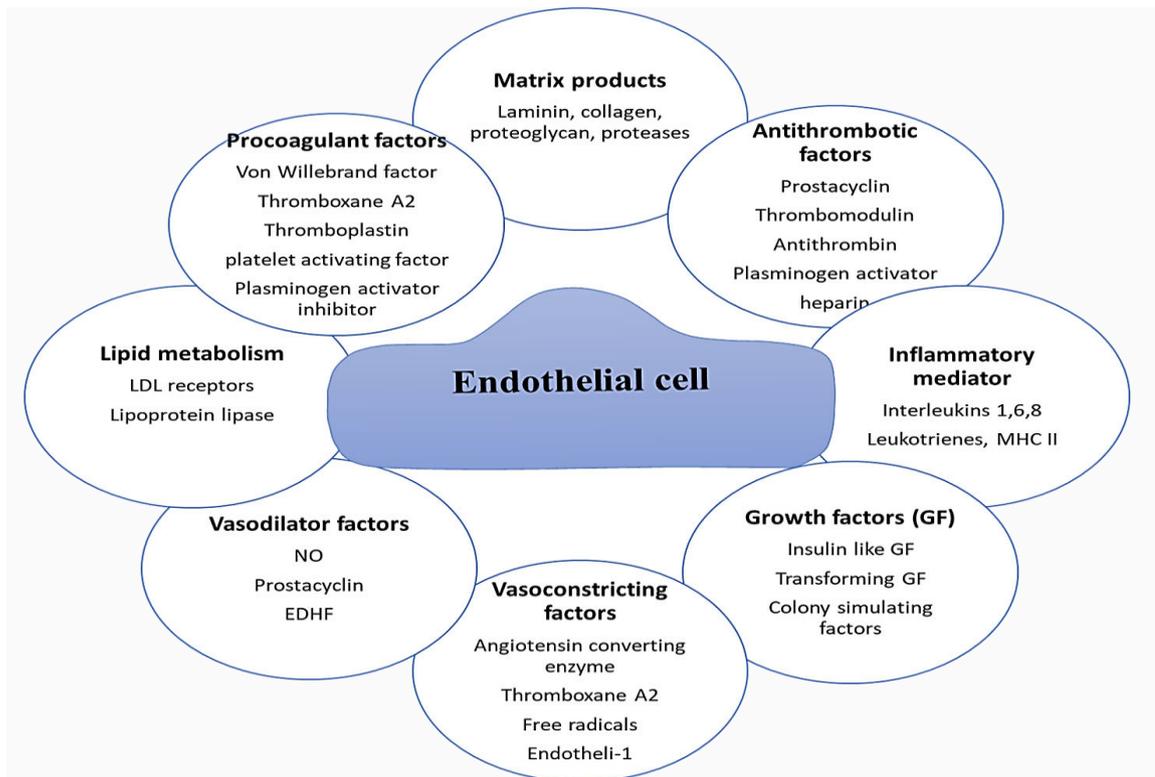


Figure 21. Functions of endothelial cells and mediators (Widmer and Lerman 2014)

Endothelial dysfunction, is characterized by reduced generation and bioavailability of NO, enhanced expression of adhesion molecules, increased generation of oxidative stress, enhanced permeability of the endothelium, impaired fibrinolytic ability and hemodynamic dysregulation. Endothelial dysfunction correlates with the initiation and progression of vascular disorders, and predicts cardiovascular diseases (Yuyun, Ng et al. 2018).

3.2 Cellular senescence

Cellular senescence is defined as an irreversible cell cycle arrest and is accompanied by changes in cell morphology, function and gene expression (Kirkland and Tchkonja 2017). Senescent cells shows arrest in G1 phase of cell cycle and are metabolically active but non-proliferative in response to mitogenic stimuli. The concept of cellular senescence was first described by Hayflick and Moorhead in nineteen sixties who observed that upon serial sub-cultivation, human diploid fibroblasts stop dividing

after limited number of population doubling. They also observed that cells from older donors showed fewer divisions as compared to cells from younger donors (Erusalimsky and Skene 2009). These observation lead to the concept of replicative senescence which was later found to be associated with the erosion of telomeres. Premature senescence can also be induced in response to external (chemical or physical) or internal (overstimulation of oncogenes, DNA damage, increased ROS, ER-stress and chromatin structure dysfunction) stimuli. This type of senescence is termed as stress-induced premature senescence (SIPS). SIPS is rapid, independent of telomeres shortening and does not in require extensive cell proliferation (Figure 22) (Bielak-Zmijewska, Grabowska et al. 2019, Melo Pereira, Ribeiro et al. 2019). Cellular senescence is observed during embryonic development, wound healing and acts as a protective mechanism against tumorigenesis. However, accumulation of senescent cells is in ageing would favor multiple age-related diseases including cardiovascular and chronic diseases (Bielak-Zmijewska, Grabowska et al. 2019).

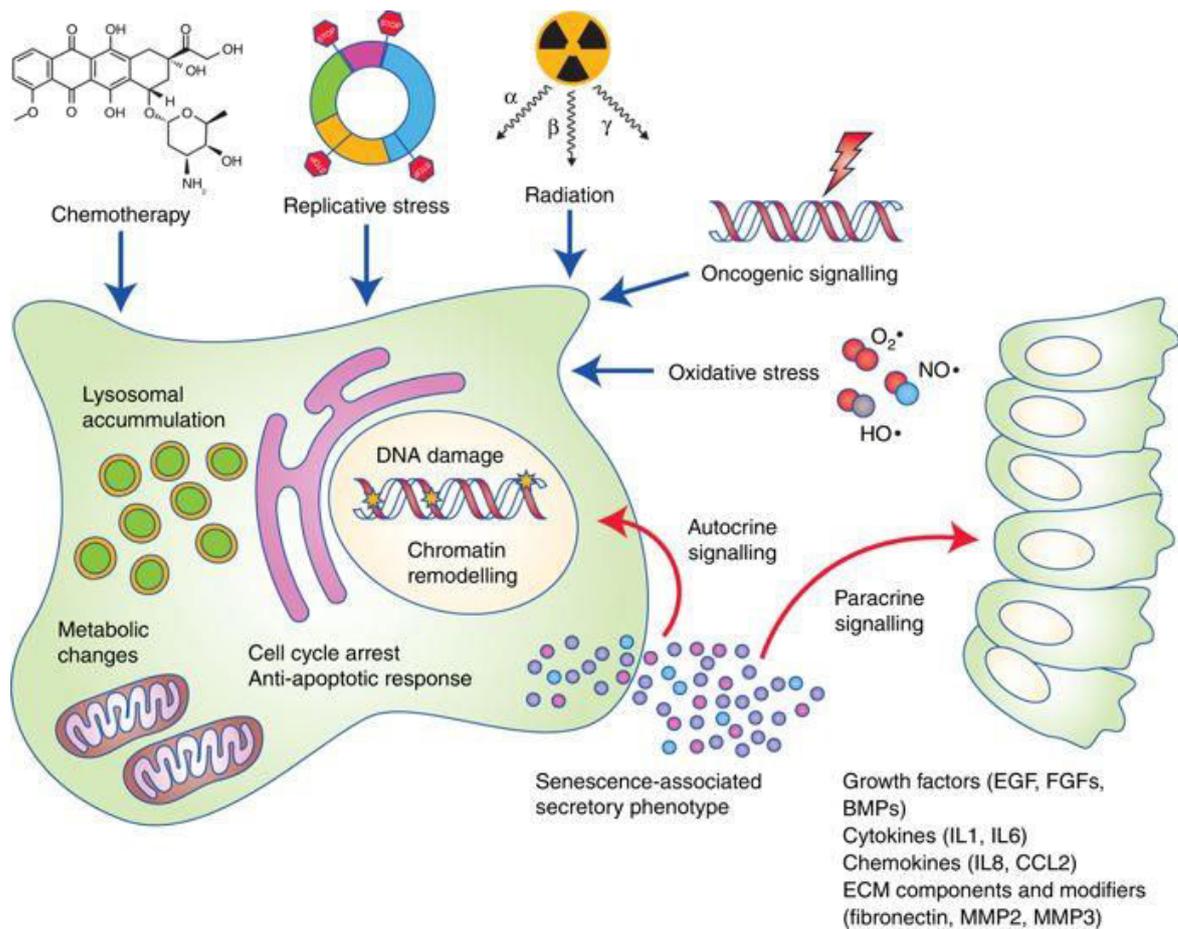


Figure 22. Overview of cellular senescence (Gonzalez-Meljem, Apps et al. 2018)

EGF: epithelial growth factor, FGFs: fibroblast growth factors, BMPs: bone morphogenetic proteins, MMPs: matrix metalloproteinases, ECM: extracellular matrix

3.3 Characteristics of Senescent cells

Distinctive characteristics of senescence cells have been observed in a variety of cells e.g. lymphocytes, keratinocytes, epithelial cells, glial cells, melanocytes, endothelial cells and even tissue stem cells (Tominaga 2015) including morphology, genetics and secretory phenotype (Figure 24).

Senescent endothelial cells are enlarged, flat and have irregular shape. Changes in shape of senescent cells are associated with scaffolding protein caveolin-1 and Rho GTPases Rac1 and CDC42. Due to loss of laminA/B, nuclear integrity is compromised with abnormal cytoplasmic chromatin fragments (CCFs). Together with increased lysosomal content, dysfunctional mitochondria and altered composition of plasma membrane are other senescence features (Figure 23) (Hernandez-Segura, Nehme et al. 2018). Increased lysosomal content is linked to the activity of a specific senescence-associated beta-galactosidase (SA- β -Gal) enzyme only active at pH 6m different from the β -galactosidase activity only detectable at pH 4 in non-senescent cell (Piechota, Sunderland et al. 2016).

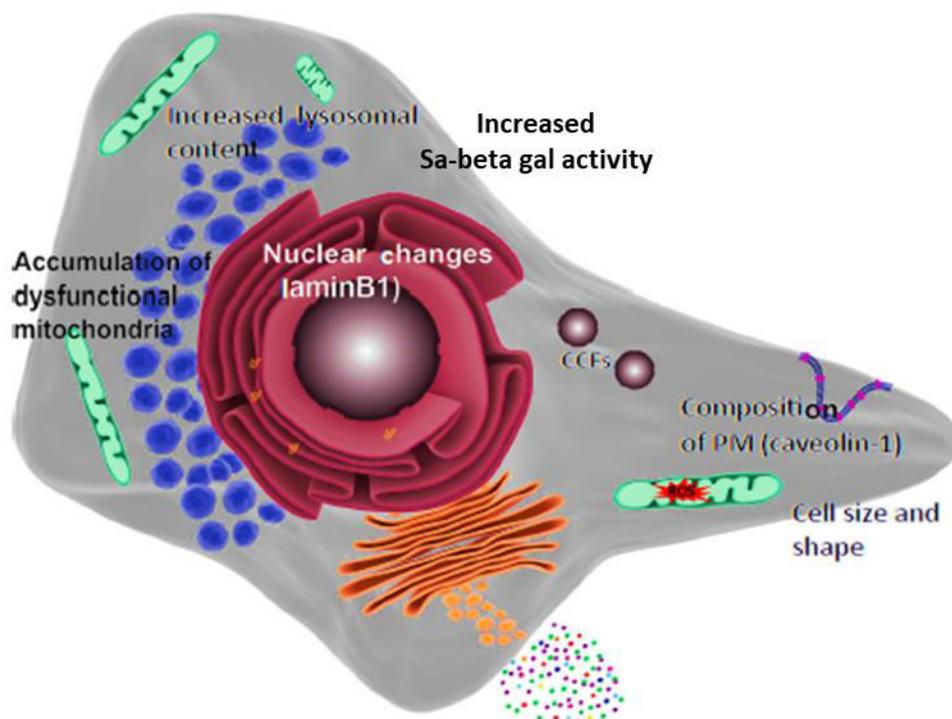


Figure 23. Hallmarks of morphological alterations

(Hernandez-Segura, Nehme et al. 2018)

Senescence-induced cell cycle arrest at G1 phase is marked by the up-regulation of key cell cycle regulators, p53, p21 and p16 (Bielak-Zmijewska, Grabowska et al. 2019). In addition, the down-regulated expression of Ki67, a protein which is strictly associated with cell proliferation and the lack of DNA incorporation of bromodeoxyuridine (BrdU), an analogue of thymidine sign the inability to proliferate (Biran, Zada et al. 2017).

One of the principal step for irreversible cell cycle arrest is the silencing of proliferation-promoting genes such as E2F target genes like cyclin A, mainly involved in S phase progression. Senescent cells have specialized foci of condensed heterochromatin, called as senescence-associated heterochromatin foci (SAHF). These non-transcribed sequences would act by sequestering proliferation –promoting cell cycle exit genes (Aird and Zhang 2013).

DNA damage response (DDR) is one the fundamental route to the induction of senescence. Senescent cells present increased double stranded breaks (DSBs) phosphorylated by H2AX (gamma-H2AX) histones by ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) proteins. Gamma-H2AX foci formation is the first stride in DNA repair proteins at the site of break and used as a biomarker of senescence-induced DNA damage (Kuo and Yang 2008, Bielak-Zmijewska, Grabowska et al. 2019).

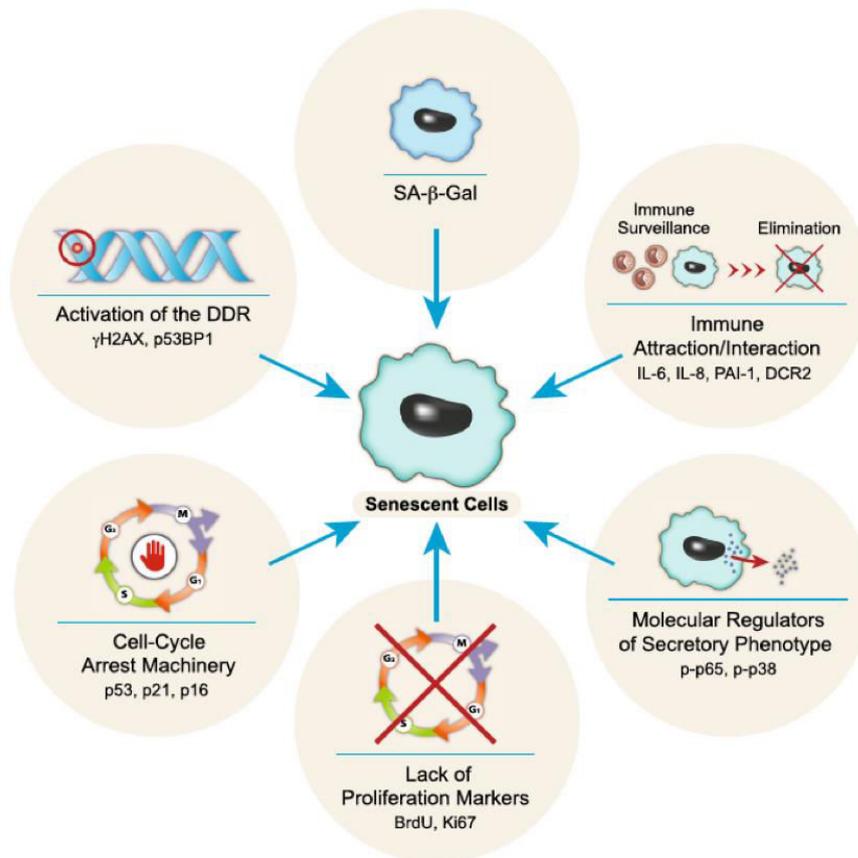


Figure 24. Markers of senescent cells (Burton and Krizhanovsky 2014)

Another important feature of senescent cells is the senescent-associated secretory phenotype (SASP) (Figure 25). SASP includes many soluble and insoluble factors like interleukins (IL-6), chemokines (MCP), proteases (MMPs, PAI), growth factors (EGF, NGF, VEGF), soluble or shed receptors/ligands (EGF-R, ICAM, Fas, μ PAR), non-protein soluble factors (NO, ROS, PGE₂), inflammatory factors (IFN- γ) and insoluble extracellular matrix components (fibronectin, collagen, laminin) (Coppé, Desprez et al. 2010). SASP induction is mainly regulated by nuclear factor-kB (NF-kB), CCAAT/enhancer binding proteins- β transcription factors, *via* mammalian target of rapamycin (mTOR) and p38MAPK signaling (Watanabe, Kawamoto et al. 2017).

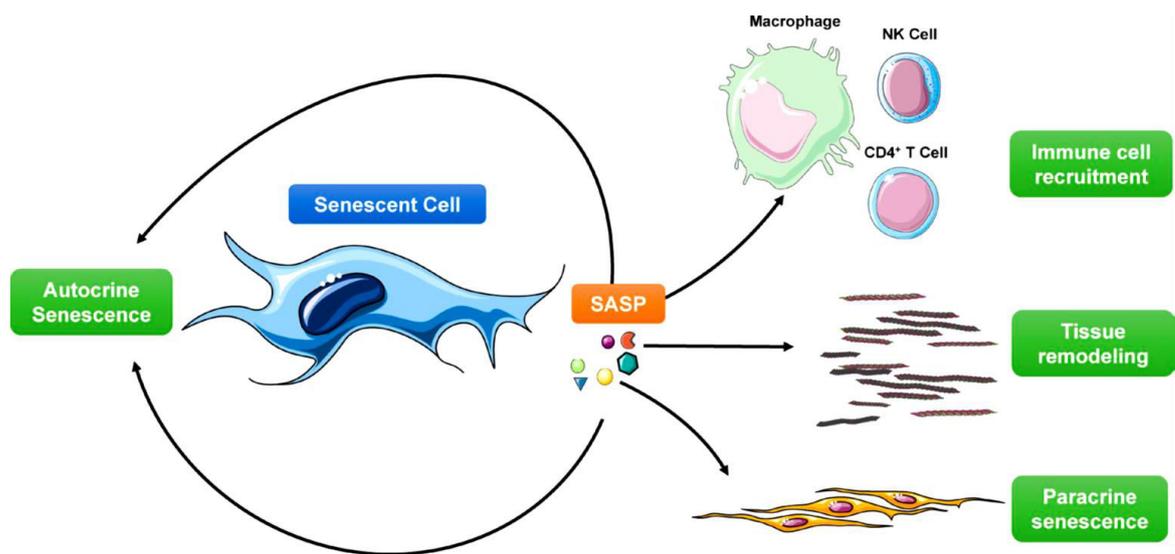


Figure 25. Functions of senescence-associated secretory phenotype (SASP)

(McHugh and Gil 2017)

Finally, pathways that regulate the secretory phenotype (e.g. p-p65 or p-p38) and immune surveillance-related genes and those involved in -survival response also contribute to senescence (DCR2, p-Akt, p-Erk) (Burton and Krizhanovsky 2014).

Different methods, in addition to the most common SA- β -gal activity, are used to characterize senescent cells (Table 1) (Carracedo, Ramírez-Carracedo et al. 2018).

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)
	C ₁₂ FDG (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; *3H-dT*, 3Hthymidine; *PCNA*, Proliferating cell nuclear antigen; *SA-β-gal*, Senescence-associated β-galactosidase; *X-gal substrate*, 5-bromo-4-chloro-3-indolyl-D-galactoside; *C12FDG*, 5-dodecanoylamino fluorescein 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

3.4 Mechanisms of senescence

3.4.1 Replicative and premature senescence

Replicative senescence is linked with progressive shortening and dysfunction of telomeres with each cycle of cell division (Kuilman, Michaloglou et al. 2010). Telomeres are the long repeated 10-15 kilo-bases DNA sequence (TTAGGG), located at the end of linear chromosomes that prevent genome instability (Vitorelli and Passos 2017). The synthesis of telomeric DNA is dependent on telomerase, an enzyme that catalyzes the addition of TTAGGG repeats to the 3' end of DNA chain. Most somatic cells lack the telomerase activity, combined to a lack of ability of DNA polymerase to replicate the end of lagging strand. Synthesis of DNA leads to gradual shortening of telomeric DNA with cell division (Erusalimsky and Skene 2009, Vitorelli and Passos 2017), resulting in the loss of 50-200 base pairs (bp) of telomeric repeat sequences per cell division (Muraki, Nyhan et al. 2012). Furthermore, higher GGG content of telomeric DNA makes it more susceptible to oxidative damage and single strand breaks. Telomere erosion compromises the DNA strand functional integrity and ultimately, results in the induction of DNA damage checkpoint response that arrest the cell cycle permanently (Erusalimsky and Skene 2009, Coluzzi, Colamartino et al. 2014).

The induction of premature senescence is rapid, independent of telomeres shortening and does not require extensive cell proliferation (Kuilman, Michaloglou et al. 2010). Premature senescence can be induced by radiation, alkylating agent, oxidizing compounds and drugs that can directly root a persistent DNA damage response leading to the activation of tumor suppressor pathways and hence stable cell cycle arrest (Erusalimsky and Skene 2009).

3.4.2 Cell cycle arrest

Stable cell growth arrest is regulated mainly by two tumor suppressor pathways: p53 and the p16/Rb (Figure 26). Activation of these pathways is triggered by persistent DDR (DNA damage response) instigated by external (chemotherapeutic drugs, irradiation) or internal (oxidative damage, mitochondrial dysfunction, oncogenes, telomere shortening insults (Herranz and Gil 2018).

3.4.2.1 p53/p21 and senescence

The activation of p53 is triggered by DNA damage (d'Adda di Fagagna 2008, Fumagalli, Rossiello et al. 2012) prompt the transcription of the cyclin-dependent kinase (CDK) inhibitor p21 which in turn blocks CDK4/6 activity, resulting in hypophosphorylated retinoblastoma (Rb) protein and cell cycle arrest (d'Adda di Fagagna 2008). P53 levels are also transiently raised in quiescent state to activate DNA repair processes, however, in senescence, the induction of p53 (Salama, Sadaie et al. 2014, Kruiswijk, Labuschagne et al. 2015) and downstream p21 is sustained because of the damage to repair-resistant sequences termed as DNA Segments with Chromatin Alterations Reinforcing Senescence (DNA-SCARS), in telomeres (Rodier, Munoz et al. 2011, Fumagalli, Rossiello et al. 2012). Owing to the key role of p53, additional regulation of p53 is also achieved *via* ARF, a product of INK4/ARF locus. In senescence, induction of ARF sequesters the ubiquitin ligase MDM2 and hence contributes to the raised levels of p53 by limiting its degradation by the proteasome (McHugh and Gil 2017).

3.4.2.2 The INK4/ARF locus and senescence

INK4/ARF locus comprises three tumor suppressor, p16^{INK4a}, ARF and p15^{INK4b}. p16^{INK4a} and ARF are encoded by the CDKN2A gene while p15^{INK4b} is encoded by CDKN2B (Herranz and Gil 2018). p16^{INK4a} and p15^{INK4b} are CDKIs that influence the cell cycle by binding and suppressing the CDK4/6 while ARF allows cross talk with p53/p21 pathway through inhibition of MDM2. Conversely, up-regulation of ARF expression in p53^{-/-} embryonic fibroblasts of mouse demonstrated that the expression of ARF can also be regulated by p53 through a negative feedback (Harris and Levine 2005). In young, non-senescent cells, the INK4/ARF locus is silenced epigenetically *via* deposition of trimethyl histone H3K27 acting as repressive marks (Herranz and Gil 2018). Methylation of H3K27 is guarded by polycomb repressive complexes (PCR1 and PCR2). Loss of PCR complexes due to reduction of their components such as BMI1, EZH2 or CBX7, results in the activation of p16^{INK4a} and thereby senescence. INK4/ARF locus is therefore a key senescence sensor and p16^{INK4a} is considered as surrogate marker of ageing (McHugh and Gil 2017).

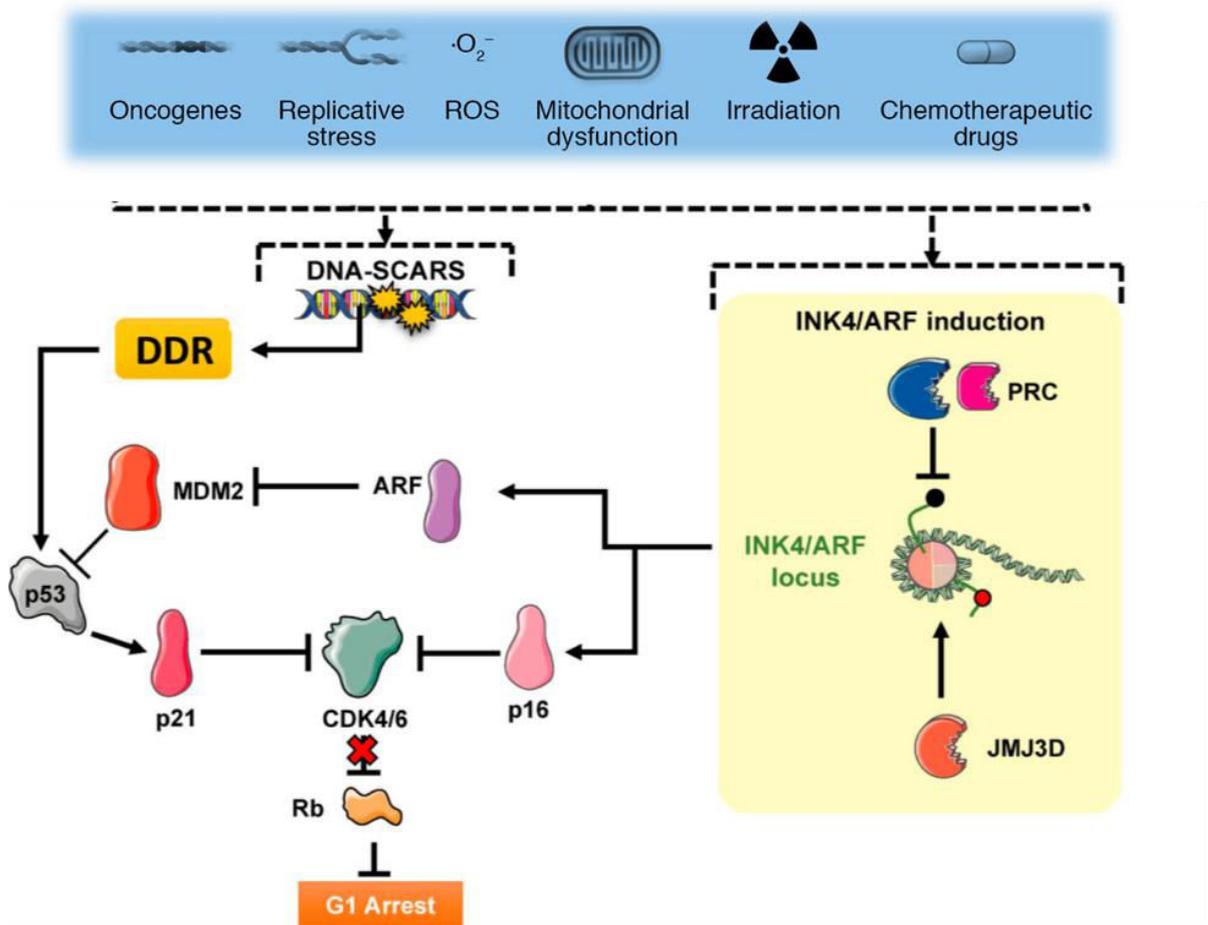


Figure 26. Pathways regulating senescence-mediated cell cycle arrest
(McHugh and Gil 2017)

3.5 Endothelial senescence

Under normal physiological conditions, endothelial cells are mostly quiescent and about 0.1% only are maintained at replicative status. Studies have shown that, in normal rats, the percentage of replication of endothelial cells in aorta at birth (13%) falls drastically at the age of 5-6 months (0.1%-0.3%). However, in response to some stress or injury, they can become activated and proliferative to replace or cover the dysfunctional/lost endothelial cells in the vessel. With ageing, this compensatory capability of endothelial cells is declined which indicates the endothelial cells possibly undergoes senescence (Tian and Li 2014).

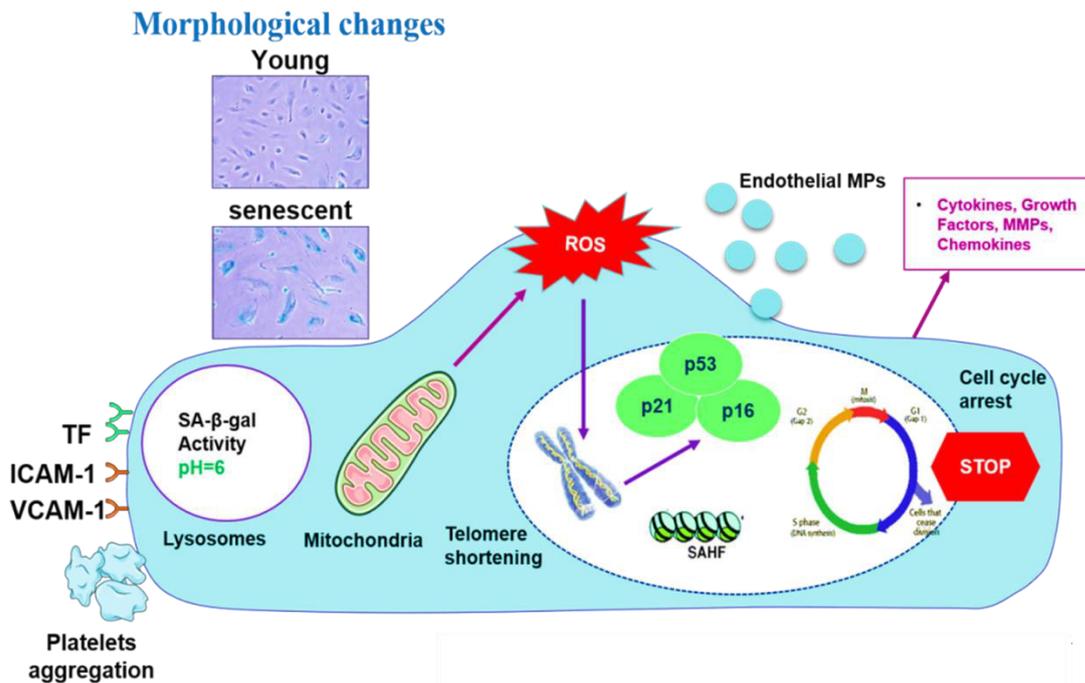


Figure 27. Characteristics of senescent endothelial cells

Endothelial cell senescence is associated with morphological alteration and increased oxidative stress, Sa-beta gal activity, up-regulation of pro-senescent, pro-inflammatory and pro-thrombotic proteins markers, accompanied by shedding of pro-coagulant microparticles

Endothelial cells have been reported to exhibit both replicative and premature senescence. Upon several passages endothelial cells have been shown positive staining for SA-β-gal activity and are associated with shortened telomeres, morphological alterations, modified gene expression and secretory phenotype (Figure 27) (Carracedo, Ramírez-Carracedo et al. 2018). Similarly, endothelial cells express features of senescence when exposed to different stimuli such as high glucose (Song, Yang et al. 2017), angiotensin II (Hsu, Lin et al. 2018), radiation (Khemais-Benkhiat, Idris-Khodja et al. 2016, Lafargue, Degorre et al. 2017), cellular microparticles (Burger, Kwart et al. 2012), homocysteine (Wang, Hu et al. 2010), oxidized low density lipoprotein (OX-LDL) and ceramides (Venable and Yin 2009) etc. In addition, elevated blood pressure and inflammation can also accelerate the induction of senescence *via* different mechanisms (Tian and Li 2014). OX-LDL accelerate the induction of senescence in human umbilical vein endothelial cells (HUVEC) *via* reduced expression of SIRT1 (Lei, Gu et al. 2014), Ang II *via* activation of mitogen-activated protein kinase (MAPK) (Shan, Bai et al. 2008), endothelial microparticles (EMPs) from plasma of acute coronary syndrome (ACS) patients *via* Ang II-mediated activation of MAPKs and PI3-kinase pathways (Abbas, Jesel et al. 2017), and high glucose *via* increased intracellular

oxidative stress (Maeda, Hayashi et al. 2015, Khemais-Benkhiat, Idris-Khodja et al. 2016).

3.6 Oxidative stress and endothelial senescence

Free radicals are highly reactive species which are constantly generated from endogenous and exogenous sources. Their harmful effects are neutralized by anti-oxidant defense mechanism (Liguori, Russo et al. 2018). Reactive oxygen species (ROS) are continuously produced under normal physiological conditions but their levels are raised when cell is under stress (Bodega, Alique et al. 2017). Oxidative stress is generated when the balance between production of reactive species and their neutralization by anti-oxidant defense is compromised (Liguori, Russo et al. 2018). Several studies have reported that oxidative stress is a potent inducer of endothelial senescence, generated by numerous inducers of endothelial cell senescence and involving telomere dependent or independent mechanisms (Figure 28) (Pole, Dimri et al. 2016). The rate of telomere erosion is dependent on oxidative stress as the guanine base of higher proportion being highly sensitive to oxidative damage (Grahame and Schlesinger 2012). In addition, increased ROS prevents the access of telomerase to telomere by promoting the translocation of a telomerase reverse transcriptase (TERT) from nucleus to cytoplasm (Haendeler, Hoffmann et al. 2003). Pro-atherogenic TNF- α reduces activity of telomerase via oxidative stress-mediated inhibition of PI3K/AKT pathway (Breitschopf, Zeiher et al. 2001). While ROS inhibition and translocation of NF- κ B prevents TNF- α derived premature cell cycle arrest in HUVEC (Khan, Awad et al. 2017). Modification of intracellular redox environment by treatment of endothelial cells with vitamin C analog (El Assar De La Fuente, Angulo Frutos et al. 2012), homocysteine (Xu, Neville et al. 2000), or by altering the glutathione redox cycle have confirmed the association between accelerated telomere shortening and oxidative stress (Erusalimsky 2009). Similarly, pre-treatment of porcine coronary artery endothelial cells with an anti-oxidant, N-acetyl cysteine (NAC), prevents replicative senescence, suggesting role of oxidative stress in induction of endothelial senescence (Khemais-Benkhiat, Idris-Khodja et al. 2016). Oxidative stress-mediated direct damage to genomic or mitochondria DNA, and activation of kinases or other redox-sensitive signaling proteins have also been implicated in induction of endothelial senescence (Erusalimsky 2009). Knocking-down

of prohibitin-1 (PHB1), an inner membrane constituent that is important for the mitochondrial functional integrity, in endothelial cells increased the mitochondrial ROS generation and induction of senescence involving ROS-dependent Akt activation (Schleicher, Shepherd et al. 2008).

Hydrogen peroxide (H_2O_2) induces premature senescence in human umbilical vein endothelial cells (HIVEC) which is attenuated by ginsenoside Rb1, a major constituent of ginseng (Liu, Chen et al. 2011). Sirt6, a critical regulator of senescence, is a nuclear, chromatin-bound protein also involved in inflammation and ageing. Partial knock-down of Sirt6 or H_2O_2 -mediated reduced expression of Sirt6 leads to the induction of a senescence phenotype in endothelial cells (Liu and Liu 2014).

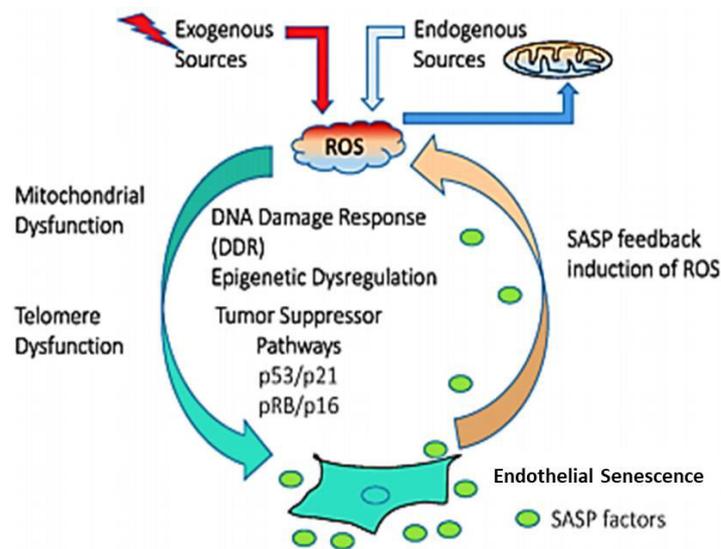


Figure 28: Actors of ROS-mediated endothelial senescence (Pole, Dimri et al. 2016)

3.7 Angiotensin system and endothelial senescence

Angiotensin II (Ang II), the prime effector molecule of the renin-angiotensin system (RAS), is a potent inducer of endothelial senescence (Khemais-Benkhiat, Idris-Khodja et al. 2016). A 30% prolongation of lifespan in AT1R knock-out mouse, compared to genetically match wild-type controls, was associated with reduce ROS generation, attenuated mitochondrial damage, improved cardiovascular morphology and enhanced expression of survival genes such as sirtuin-3 and nicotinamide phosphoribosyl transferase (Nguyen Dinh Cat, Montezano et al. 2013). Discovery of tissue-specific

components of RAS lead to the concept of the local angiotensin system acting eventually independently from circulating RAS (Nehme, Zouein et al. 2019). In Ang II-regulated cell proliferation and endothelial functions occur *via* the G-protein coupled angiotensin type I receptor (AT1R) (Kawai, Forrester et al. 2017) and coupled to various signaling mediators, including NADPH oxidase (Nox1, NOx2, Nox3, Nox4, Nox5) thereby promoting oxidative stress that may influence downstream senescence-associated signaling (Nguyen Dinh Cat, Montezano et al. 2013), accompanied by structural and functional changes in vessel function and eventually increased risk of a cardiovascular events (Li, Mi et al. 2019). Human umbilical vein endothelial cells (HUVEC) exposed to Ang II showed up-regulated expression of senescence-associated genes and increased SA- β -gal activity, associated with mitochondrial ROS (mtROS) production and further rise of the intracellular level of ROS triggering cell cycle inhibitors (de Cavanagh, Inserra et al. 2011, Li, Mi et al. 2019). Ang II acts *via* alternate mechanisms. For example, (Kim, Heo et al. 2012) have shown that Ang II can promote induction of endothelial senescence *via* inflammatory pathways such as the activation of NF- κ B. Long-time exposure of endothelial cells to Ang II may lead to 2 mechanisms of Ang II-mediated senescence; (i) by decreasing the expression of anti-ageing gene, Sirtuin-1 (Sirt1) (ii) by reducing the activity of telomerase (Li, Mi et al. 2019). Di Yang has reported that Ang II-mediated endothelial senescence is also dependent on the transcription factor Fos-related antigen 1 (Fra-1), the expression of which is also increased in atherosclerosis plaques. Silencing of Fra-1 in endothelial cells from rat aorta blocked the Ang II-mediated senescence and senescence-associated secretory phenotype. Furthermore, knock-down of Fra-1 inhibits the ageing phenotype in Ang II-infused mice (Yang, Xiao et al. 2019).

In addition, numerous studies have shown that different inducers of endothelial senescence propagate their effects *via* activation of angiotensin system. (Khemais-Benkhiat, Belcastro et al. 2019) have shown that high glucose concentration induces senescence *via* Ang II-induced oxidative stress leading to up-regulation of SGLT1 and SGLT2 expression in primary porcine coronary artery endothelial cells. (Sharma, Lee et al. 2019) have found and confirmed the role of the angiotensin system in fine dust (FD)-mediated increased SA- β -gal activity, cell cycle arrest, increased oxidative stress and reduced eNOS expression in endothelial cell by blocking all these effects with Losartan (AT1 blocker).

3.8 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). Reduced activity of eNOS and lower levels of NO in senescent human umbilical veins endothelial cells favors reduced vasodilation (Hayashi, Yano et al. 2008). Reduced endothelium dependent relaxation in aorta from aged or telomerase deficient (*Terc*^{-/-}) mice characterize the role of senescence in endothelial dysfunction while combination of superoxide dismutase mimetic and NADPH oxidase inhibitor (apocynin) improved endothelium-dependent relaxation in both aged/*Terc*^{-/-} mice indicates that senescence-mediated oxidative stress is involved in induction of endothelial dysfunction (Bhayadia, Schmidt et al. 2015). Furthermore, inhibition of telomere function in cultured human aortic endothelial cells (HAECs) induced the up-regulation of intracellular adhesion molecule-1 (ICAM-1) and down-regulated the activity of endothelial nitric oxide synthase (eNOS), suggesting that endothelial senescence causes endothelial dysfunction (Minamino, Miyauchi et al. 2002). 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). After series of population doubling (replicative senescence), in-vitro cultured endothelial cells showed reduced expression of another vasodilator, prostacyclin, explaining some reduction in vasodilation (Erusalimsky 2009). 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). Endothelial NO prevents up-regulation of TF on the surface of endothelium and inhibits adhesion, activation and aggregation of platelet and therefore limits the formation of thrombus. Replicative senescence in porcine coronary artery endothelial cells, accompanied by reduced NO formation and up-regulated expression of TF, promotes thrombogenicity and endothelial dysfunction (Silva, Abbas et al. 2017). Down-regulation of endothelial cell-cell junction proteins such as cytosolic phospholipase A2 α (cPLA2 α) were reported in

senescent HUVEC, showing that endothelial senescence also interfere with the formation and maintenance of tight junctions, hence, increase the permeability of endothelium (Krouwer, Hekking et al. 2012). In-vivo administration of statins such as atorvastatin, effectively inhibited the endothelial senescence and improved endothelium-dependent relaxation (Gong, Ma et al. 2014). Endothelial dysfunction, is characterized by reduced vasodilation, pro-thrombotic and pro-inflammatory state and it is an early hallmark of developing cardiovascular diseases (Zehr and Walker 2018). Spasms of coronary arteries, found most frequently at sites of atherosclerotic lesion, is an independent cardiovascular risk factor and is associated with endothelial dysfunction (Zaya, Mehta et al. 2014, MacAlpin 2015)

3.9 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 d'Adda di Fagagna 2007, Childs, Li et al. 2018, Karin, Agrawal et al. 2019). 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 cells from atherosclerotic plaque, the telomere are shorter in human aorta as compared to normal vessel (Katsuumi, Shimizu et al. 2018). The increase of senescent cells over time can age-associated DNA damage and/or prolonged exposure to other pro-senescent stimuli. Another possibility is that senescent cells clearance by the immune system is less effective with aging (Karin, Agrawal et al. 2019). Accumulation of senescent endothelial cells in age-related cardiovascular pathologies is not only a biomarker but also a strong promoter of diseases (Minamino, Miyauchi et al. 2002, Katsuumi, Shimizu et al. 2018). For example, pathological cardiovascular tissue contain elevated expression of stressor that induce senescence such as oxidative stress and telomere shortening (Childs, Li et al. 2018). Genetic invalidation of senescence effectors can blunt age-associated tissue loss of function (Baker, Perez-Terzic et al. 2008). Senescence inhibition or removal of senescent cells limits the age-

associated abnormalities in cardiovascular tissues (Childs, Gluscevic et al. 2017, Childs, Li et al. 2018, Karin, Agrawal et al. 2019). In turn, 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). Patients with ischemia heart disease have higher levels of Sa- β -gal activity on luminal side of the coronary arteries, suggesting accumulation of senescent endothelial cells (Katsuomi, Shimizu et al. 2018). Increased accumulation of senescent endothelial cells have also 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. Senescence of VSMCs and endothelial cells association with impaired vasodilation, enhanced vascular stiffness and hypertension have been demonstrated in murine model of genome instability. In contrast to non-hypertensive patients, increased binding of p53 to p21 promoter and 2-fold higher telomere uncapping has been reported in hypertensive patients (Morgan, Ives et al. 2014).

In conclusion, as senescent endothelial cells are key players in vascular ageing and age-associated cardiovascular complications, better understanding of underlying stressor or mechanisms can help to identify new protective approaches against their deleterious effects on the cardiovascular system.

Chapter 4: Omega-3 PUFAs and vascular health

4.1 Fatty acids

Fatty acids (FA) are the fundamental structural molecules, along with proteins and carbohydrates, tissues and organs. FA also serves as a vast source of energy and are responsible for synthesis of active lipids. FA are classified as saturated and unsaturated or polyunsaturated fatty acids (Kremmyda, Tvrzicka et al. 2011, Sokoła-Wysoczańska, Wysoczański et al. 2018).

4.2 Omega-3 polyunsaturated fatty acids (n-3 PUFAs)

omega-3 PUFAs (n-3 or ω -3 PUFAs) are polyunsaturated fatty acid and the first double bond is after 3rd carbon from the CH₃-end of the carbon chain (Figure 29) (Shahidi and Ambigaipalan 2018). Among many types of omega-3 PUFAs, the most important and common dietary omega-3 PUFAs are α -linoleic acid (ALA, C18:3 n-3), eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C20:6 n-3) (Sokoła-Wysoczańska, Wysoczański et al. 2018). Omega-3 PUFAs were discovered in 1929 by George Burr and his wife, and since then have been reported of health benefit including in normal growth and development (Scorletti and Byrne 2013). ALA is an essential fatty acid as it cannot be synthesized *de novo* and dietary intake is imperative (Kaur, Chugh et al. 2014). EPA and DHA are derivatives of ALA and their biosynthesis is dependent on the activity of enzymes responsible for inserting the double bonds. As the rate of biosynthesis of these long chain omega-3 PUFAs is very low (less than 4%) and insufficient to fulfill the physiological demand (Shahidi and Ambigaipalan 2018), they are termed “conditionally essential” (EPA, DHA) fatty acid (Scorletti and Byrne 2013). Recently, interest in omega-3 PUFAs is escalated due to their relevance with the reduction of risk of various diseases, particularly cardiovascular diseases (Bowen, Harris et al. 2016, Sokoła-Wysoczańska, Wysoczański et al. 2018).

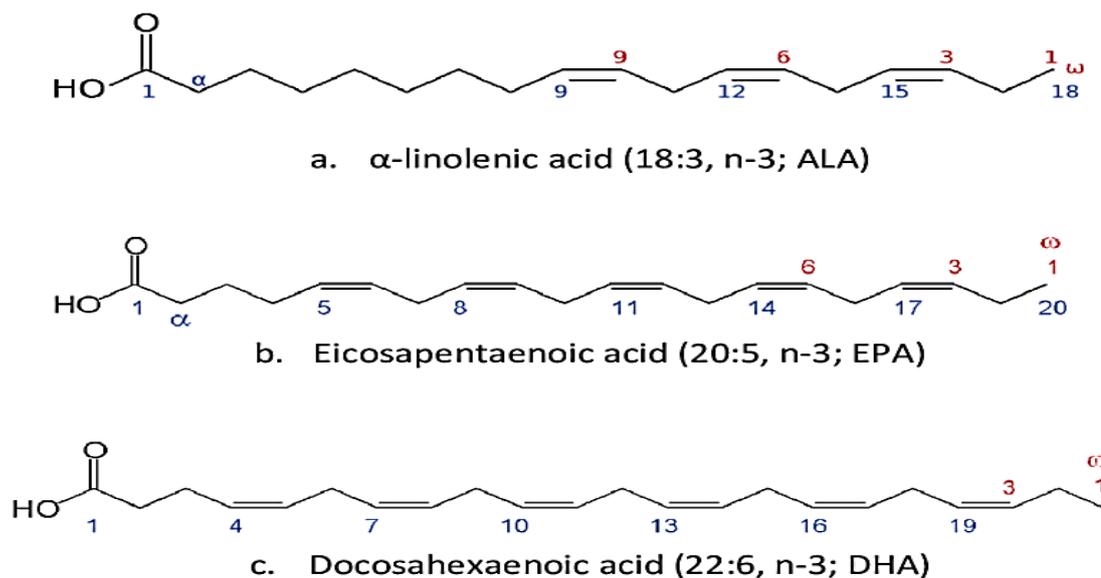


Figure 29. Chemical structures of important dietary omega-3 PUFAs.
(Khan, Moshir Rahman et al. 2015)

4.3 Dietary sources and intake of Omega-3 PUFAs

Primary source of ALA is plants, mainly nuts such as walnut, seeds such as chia seeds (*salvia hispanica*), vegetable oils (linseed, canola, hemp, soybean), eggs, dairy products and algae (Sokoła-Wysoczańska, Wysoczański et al. 2018). Flaxseed (linseed) oil contains high content of ALA (49.5g/100g) (Shahidi and Ambigaipalan 2018).

Endogenous conversion of ALA to EPA (0.2% - 8%) and then to DHA (0% - 4%) is limited, thus circulating and tissue levels of EPA and DHA are principally determined by their dietary consumption (Burdge, Jones et al. 2002). EPA and DHA are often termed as “marine omega-3” as the richest source of these LC omega-3 PUFAs is seafood especially fish (Usydus, Bodkowski et al. 2012). Flesh from fatty fish such as tuna, salmon and mackerel have higher amounts of omega-3 PUFAs as compared with flesh from lean fish such as cod, which, contrary to fatty fish, preferably stores lipids in liver than in flesh (Lane, Derbyshire et al. 2014). A single meal of salted mackerel contains high amount (4.57g/100g) of EPA and DHA (Shahidi and Ambigaipalan 2018).

Fish oils from cod flesh, halibut, and skipjack tuna contains highest amount of DHA (30% of total FAs), whereas the highest amounts of EPA (15–19% of total FAs) is found in cod flesh, flounder species, and haddock fish oil (Table 2). In addition to fish, some marine mammals, crustaceans, bivalves, and cephalopods and marine algae are also

among other sources of omega-3 PUFAs (Shahidi and Ambigaipalan 2018). Senanayake and fichtali have reported *Cryptocodinium cohnii* and *Schizochytrium* species as the two major algal sources of DHA (55% and 40% of total FAs, respectively). Furthermore, omega-3 PUFAs, especially EPA and DHA, are synthesized by phytoplankton, and algae are ultimately transferred through the food chain to fish and marine mammals (Alasalvar, Shahidi et al. 2002).

Fish oil	EPA %	DHA %	DPA %
Menhaden oil	18.3	9.6	1.8
Herring oil	7.5	6.8	0.75
Cod liver oil	12.2	12.7	1.7
Capelin oil	9.3	4.2	0.9
Skipjack tuna oil	11.1	29.1	0
Butterfish	5.1	10.8	2.4
Halibut oil	9.6	30.6	2.6
Mackerel oil	8	19.3	1.6
Salmon oil	6.2	9.1	1.8
Cod flesh oil	19.1	32.6	6.2
Yellow flounder oil	15	18.7	3.3
Winter flounder oil	14.4	20.1	3.8
Haddock	14.8	24.8	1.9

Table 2. Omega-3 PUFAs content in fish oils. (Shahidi and Ambigaipalan 2018)

Owing to cultural and dietary habits, average per day consumption of omega-3 PUFAs differs worldwide. Japanese are the top consumers with an average consumption of 5-6 g/day, Inuits with 3-4 g/day. Australians consume only 0.189 g/day whereas Europeans and North Americans are least consumers with as low as 0.15-0.25 g/day (Scorletti and Byrne 2013).

In addition to the beneficial effects, concerns have been raised due to potential harm from contaminants such as methylmercury, dioxins and polychlorinated biphenyls (PCBs), presents in some species of fish. In most species, mercury content is quite low, whereas some (albacore tune) have moderate (0.36 µg/g) and others (tilefish, swordfish, shark) have high (1 µg/g). However, benefits of a modest consumption of fish outweigh the potential risks (Mozaffarian and Wu 2011).

4.4 Metabolism and bioavailability of Omega-3 PUFAs

The conversion of ALA to SDA and subsequently to EPA, DPA and DHA requires desaturases, a microsomal elongase and peroxisomal β -oxidation for shortening of chain (Zárate, El Jaber-Vazdekis et al. 2017) (Figure 30). (Burdge and Calder 2005) reported that consumption of ALA significantly increased the levels of EPA and DPA in breast milk and plasma fraction (white blood cells, red blood cells and platelets) whereas only a minor increase was observed in the levels of DHA. Using a stable isotope, (Pawlosky, Hibbeln et al. 2001) showed conversion of ALA into EPA, DPA and DHA as 0.2%, 0.13% and 0.05% respectively. Furthermore, Food and Agriculture Organization (FAO 2010) report have revealed that the activity of desaturases is affected by low insulin and deficiencies of minerals such as zinc, magnesium, copper, limit the conversion of ALA to omega-3 PUFAs (Shahidi and Ambigaipalan 2018). The conversion of EPA and DHA are also affected by the concentration of omega-6, which tends to be 20 times higher in modern diet as compared to omega-3 (Kromhout, Yasuda et al. 2012). Precursors of omega-3 (alpha-linoleic acid, ALA) and omega-6 (linoleic acid, LA) compete for the same enzymes of their metabolic pathways (Figure 30). Hence, high intake of LA can reduce the availability of desaturases and elongase for the metabolism of ALA and the production of EPA and DHA. Furthermore, DHA, a derivative of omega-3 ALA, is further converted into specialized pro-resolving lipid mediators (SPMs), such as resolvins (D and E series), neuroprotectins and maresins, with anti-inflammatory and organ protective properties, whereas cyclooxygenases- and lipoxygenases-mediated biotransformation of arachidonic acid (AA), derivative of omega-6 LA, yields to thromboxane (2-series), prostaglandin (2-series), leukotrienes (4-series), lipoxins and derivatives of epoxyeicosatrienoic (EET) and hydroxyeicosatetraenoic (HETE), which are pro-inflammatory and pro-thrombotic (Schmitz and Ecker 2008). Amount, type and improper proportion of dietary fats would therefore increase the risk of diseases, including cardiovascular diseases and cancer, as well as weaken the immune system (Desnoyers, Gilbert et al. 2018, Sokoła-Wysoczańska, Wysoczański et al. 2018).

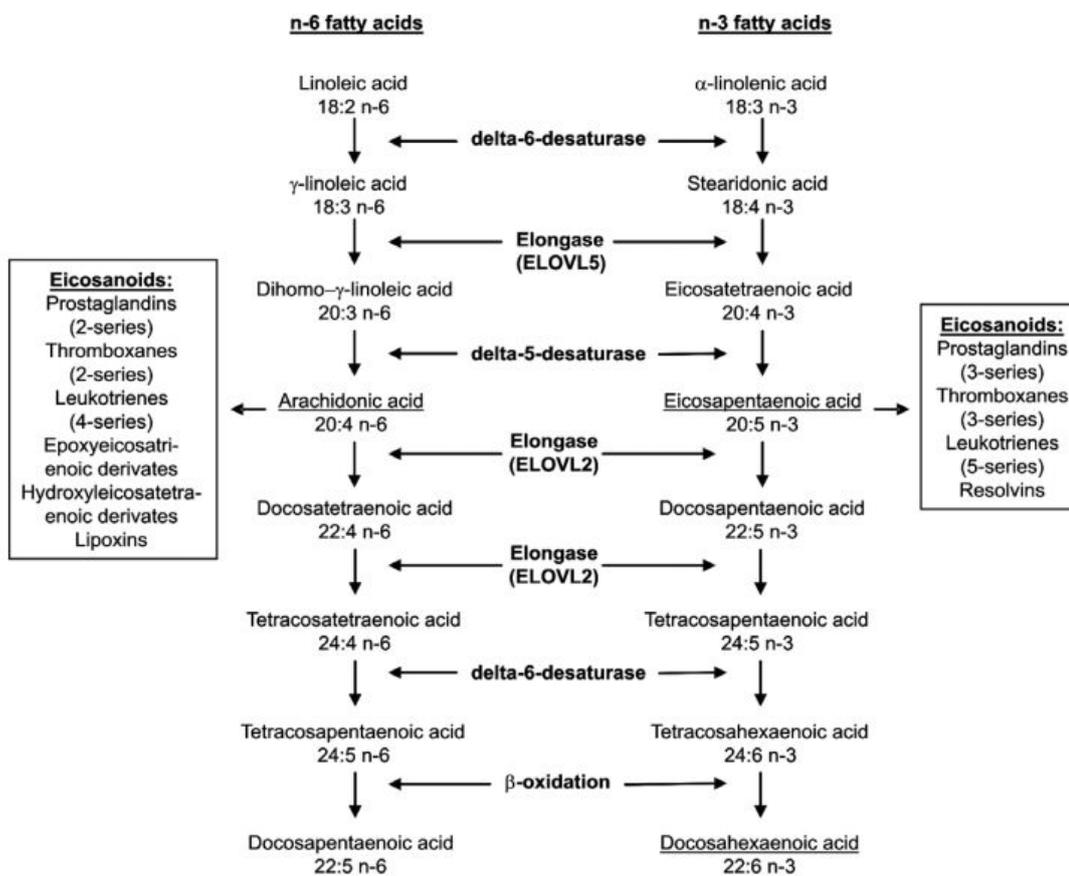


Figure 30. Metabolism of omega-3 and omega-6 PUFAs.

(Schmitz and Ecker 2008)

Omega-3 PUFAs are present in various forms such as free fatty acid (FFAs), ethyl ester (EE), triacylglycerols (TAGs) or phospholipids (PLs) (Shahidi and Ambigaipalan 2018). N-3 PUFAs in krill oil are mainly present in the form of triacylglycerol and free fatty acids. A substantial amount is also bound to phospholipids in cellular membrane. Bioavailability of n-3 PUFAs is affected by various factors, for instance, supplements containing the EE form of n-3 PUFAs have relatively lower bioavailability as compared to FFAs forms (Schuchardt, Schneider et al. 2011). The absorption of both EE and FFAs forms of n-3 PUFAs is enhanced by the presence of dietary fats (Davidson, Johnson et al. 2012). Their bioavailability is also affected by the position at which they are attached to the triacylglycerol (TAG) backbone. For example, n-3 PUFAs attached at *sn*-2 position of TAG are readily absorbed, as monoacylglycerols (MAGs), by passive diffusion. Fish oil have n-3 PUFAs, with greater bioavailability, located in *sn*-2 position as compared to marine mammal oils where attachment is at *sn*-1 and *sn*-3 position (Laidlaw, Cockerline

et al. 2014). Digestion of dietary omega-3 PUFAs starts in stomach where triacylglycerol forms are partially broken by gastric lipases into diacylglycerol and free fatty acids, forming large emulsion of fat globules. Complete digestion occurs in the intestinal lumen with the help of bile salt and pancreatic lipases, yielding free fatty acids and monoacylglycerol which subsequently diffuse passively into enterocytes (Shi and Burn 2004). Ethyl ester forms of EPA and DHA are mainly hydrolyzed by the pancreatic carboxyl ester lipase to release free fatty acids for absorption (Figure 31) (Shahidi and Ambigaipalan 2018).

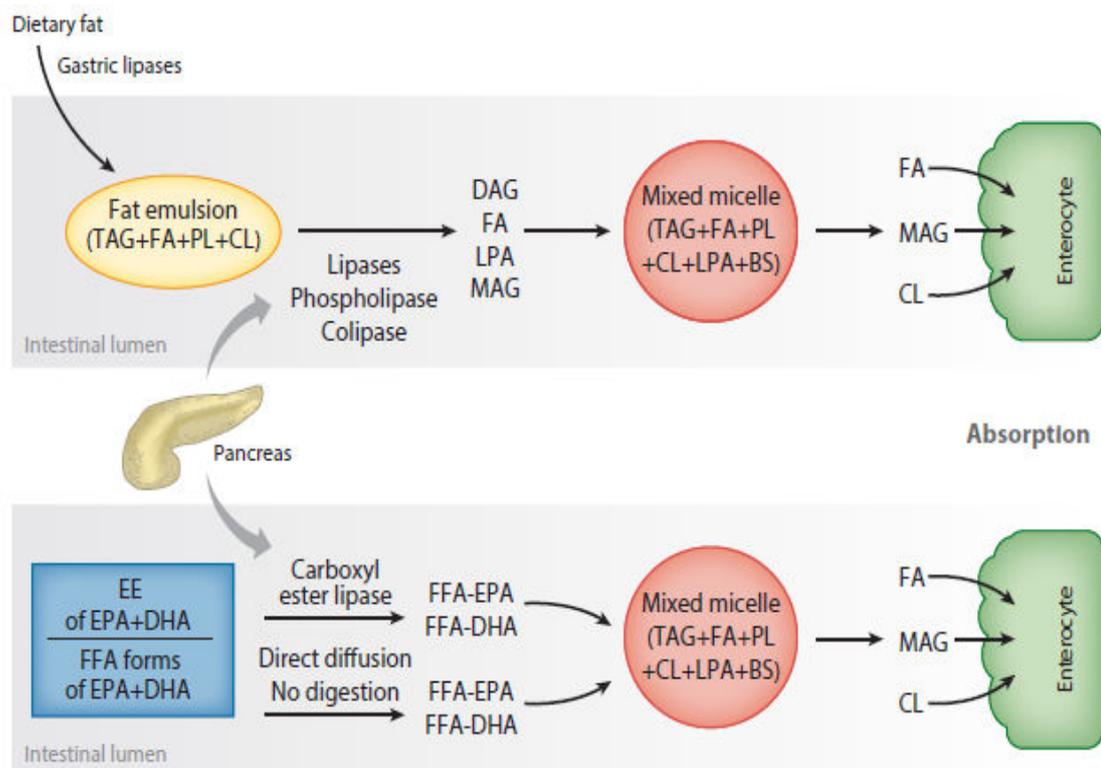


Figure 31. A schematic representation of dietary fat digestion and absorption of ethyl ester (EE) and free fatty acid (FFA) forms of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). (Shahidi and Ambigaipalan 2018)

BS, bile salt; CL, cholesterol; DAG, diacylglycerol; FA, fatty acid; LPA, lysophosphatidic acid; MAG, monoacylglycerol; PL, phospholipid; TAG, triacylglycerol.

4.5 Mechanisms of Omega-3 PUFAs functions

Omega-3 PUFAs have been reported to exert their physiological function through different mechanism involving alteration of cell membrane structure and function, modulation of ion channels, regulation gene expression and through generation of bioactive mediators (Figure 32).

4.5.1 Structural and functional alteration of cell membrane

The composition of phospholipids, the major constituents of cell membrane along with proteins, is pivotal for cell structure and function. Change in membrane phospholipid composition affects fluidity, permeability as well as interactions between membrane-bound protein and lipids of high impact on a myriad of physiological processes (Gerling, Mukai et al. 2019). Omega-3 PUFAs have the ability to modify the membrane phospholipid composition and alter fluidity, permeability and lateral organization of the membrane altering ions and substrate transport across the membrane (Adkins and Kelley 2010, Gerling, Mukai et al. 2019). Omega-3 PUFAs alter lipid microdomains such as lipid rafts and caveolae, which are the operational platforms for signaling pathways, endocytosis, ion channel kinetics and membrane and protein trafficking (Mozaffarian and Wu 2011). In T cells, omega-3 PUFAs incorporation is associated with a decreased production of interleukin-2 and reduced protein kinase C theta (PKC- Θ) signaling, pivotal for T cell life and death (Altman and Villalba 2003). Furthermore, it prevents LPS-mediated inflammation via altered dimerization and recruitment of Toll-like receptors 4 (TLR-4) and down-regulation of NF-kB (Endo and Arita 2016).

4.5.2 Modulation of ion channels

Numerous in-vitro and in-vivo studies have shown that incorporated omega-3 PUFAs can exert their physiological and pharmacological effects by altering the function of different ions channels (Endo and Arita 2016, Elinder and Liin 2017). For example, cardiomyocyte electrophysiology is directly modulated by omega-3 PUFAs altering function of the Na⁺ channel, L-type Ca²⁺ channel and Na⁺/Ca²⁺ 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).

Omega-3 PUFAs can also directly interact and modulate the ions channels and partner proteins. For example, in human cardiomyocytes, significant reduction in the inhibitory action of EPA on voltage-gated Na⁺ channel was observed after insertion of a single amino acid point mutation in the alpha subunit of the Na⁺ channel (Xiao, Ke et al. 2001).

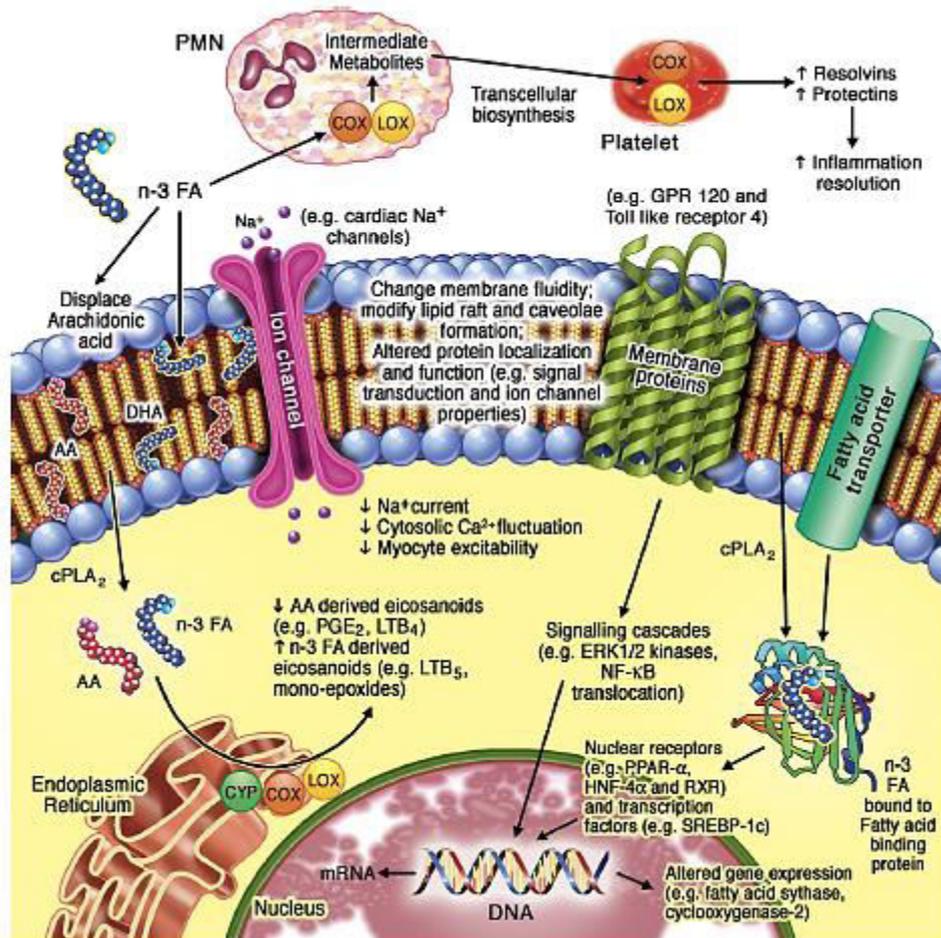


Figure 32. Molecular mechanisms of physiological effects of omega-3 PUFAs. (Mozaffarian and Wu 2011)

4.5.3 Alteration of nuclear receptors and transcription factors

Omega-3 PUFAs can alter gene expression by interacting with transcription factors. In mice hepatocytes, they bind sterol regulatory element binding protein-1c (SREBP-1c). They also are direct ligand for peroxisome proliferator-activated receptors (PPAR), retinoid X receptor (RXR), hepatic nuclear factors (HNF) and liver X receptors, in various organs such as lungs and kidney. All these nuclear receptors are pivotal

regulators of inflammation, lipid metabolism and glucose-insulin homeostasis (Mozaffarian and Wu 2011, Papackova and Cahova 2015). The interaction with nuclear receptors is facilitated by cytoplasmic fatty acid transporters (fatty acid binding protein), shuttling omega-3 PUFAs into nucleus (Esteves, Knoll-Gellida et al. 2016).

Membrane incorporated omega-3 PUFAs can exert their anti-inflammatory action via two G-protein coupled membrane receptors (GPR43, GPR120) (Cornall, Mathai et al. 2011) that limit the activation of NF-kB either *via* activation of PPAR or via inhibition of Ikb kinase (IKK), responsible for the Ikb α phosphorylation and dissociation, from NF-kB promoting its translocation of NF-kB into nucleus (Liu, Zhang et al. 2017, Desnoyers, Gilbert et al. 2018).

4.5.4 Omega-3 derived eicosanoids

Free fatty acids, released from membrane phospholipids by the hydrolytic action of cytosolic phospholipase A₂ (cPLA₂) (Kita, Shindou et al. 2019), are further metabolized by lipoxygenases (LOX), cyclooxygenases (COX) and epoxygenases (cytochrome P₄₅₀) to generate eicosanoids mediators including thromboxanes, leukotrienes and prostaglandins (Saini and Keum 2018). High incorporation of omega-6 PUFAs in membrane phospholipids mainly gives eicosanoids with pro-inflammatory, pro-thrombotic properties whereas replacement of omega-6 PUFAs with enhanced ingestion and incorporation of omega-3 PUFAs results in the generation of eicosanoids which are mainly less or anti-inflammatory (Table 2). Because of the opposite properties of their derived eicosanoids and dependency on same metabolic pathways (LOX, COX, CYP P450) for synthesis, the ratio of omega 3/6 in diet is crucial (Desnoyers, Gilbert et al. 2018).

	Arachidonic acid (n-6) derived messengers	←————→		EPA and DHA (n-3) derived messengers
		Physiological effects	Physiological effects	
Prostaglandins	PGD ₂			PGD ₃
	PGE ₂	Pro-arrhythmic	Anti-arrhythmic	PGE ₃
	PGF ₂			PGF ₃
	PGI ₂	Pro-arrhythmic	Anti-arrhythmic	PGI ₃
Thromboxanes	TXA ₂	Platelet activator	Platelet inhibitor	TXA ₃
	TXB ₂	Vasoconstriction	Vasodilatation	TXB ₃
Leukotrienes	LTA ₄			LTA ₅
	LTB ₄	Pro-inflammatory	Anti-inflammatory	LTB ₅
	LTC ₄			LTC ₅
	LTD ₄			LTD ₅
	LTE ₄			LTE ₅
Epoxyeicosatri-enoic derivatives	5,6-EET			
	8,9-EET			
	11,12-EET	Inflammatory		
	14,15-EET			
Hydroxyeicosatetra-enoic derivatives	5-HETE			
	12-HETE			
	15-HETE			
Lipoxins	LXA ₄			
Resolvins			Anti-inflammatory	RVE1
			Anti-inflammatory	RVD
Neuroprotectin			Anti-inflammatory	NPD1

Table 3. Omega-3 and -6 derived eicosanoids and their physiological effects.

(Schmitz and Ecker 2008)

Furthermore, a major advancement in the field of omega-3 PUFAs and their anti-inflammatory actions is attributed to the discovery and structural and functional elucidation of omega-3 PUFAs-derived specialized pro-resolving mediators (SPM) including resolvins (Rv), protectins (P) and maresins (MaR) (Calder 2017). Resolvins were first discovered by Dr. Serhan through their inhibitory actions on migration and infiltration of human leukocytes (Moro, Nagahashi et al. 2016). COX and LOX are the main pathways that operate in paracrine transcellular manner to synthesize SPM from EPA and DHA (Figure 33) (Molfini, Amabile et al. 2017). For example, during leukocyte-endothelial cell cross talk, conversion of EPA to 18R-hydro eicosapentaenoic acid (HEPE) occurs within endothelial cells. HEPE is then rapidly taken up and metabolized into RVE1 by activated leukocytes (Moro, Nagahashi et al. 2016).

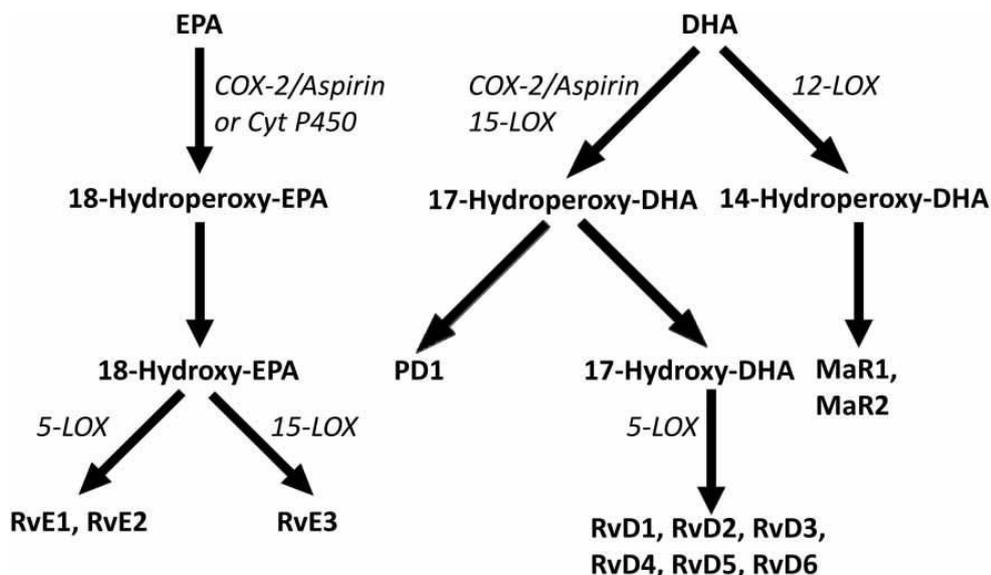


Figure 33. Pathways involved in production of SPMs from EPA and DHA
(Calder 2017)

In animals and humans blood SPM increases after ingestion of a diet rich in EPA and DHA (Barden, Mas et al. 2014). Anti-inflammatory and inflammation resolving effects of SPM have been extensively examined and demonstrated in models of including arthritis, colitis and asthma, *in-vivo* and *in-vitro* (Calder 2017). RvE1, RvD1 and protectin D1 inhibits transendothelial migration and hence infiltration of neutrophils to the sites of inflammation. RvD1 inhibits the production of IL-1 β and protectin D1 of both IL-1 β and TNF- α (Serhan, Chiang et al. 2008, Bannenberg and Serhan 2010, Serhan and Chiang 2013). In addition, DHA-derived protectins (PD), D-series resolvins (RvD) and maresins were reported to protect from renal ischemic reperfusion injury, brain ischemia, oxidative injury and atherosclerosis (Serhan 2014), making omega-3 derived SPM the emerging potential therapeutic candidate (Ishihara, Yoshida et al. 2019)

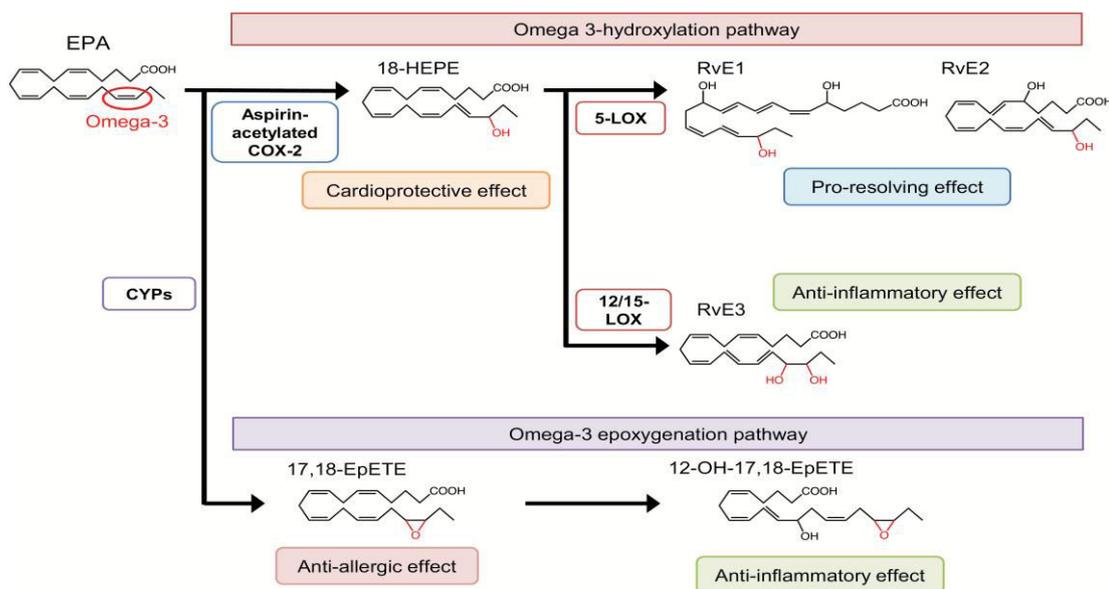


Figure 34. Metabolism of EPA and functions of derived metabolites

(Ishihara, Yoshida et al. 2019)

4.6 Omega-3 PUFAs and risk of CVDs

Numerous epidemiological, clinical and research data have shown the reduced risk of primary and secondary cardiovascular diseases after ingestion of omega-3 PUFAs, that trigger elevated blood pressure, thrombosis, inflammation, hyperlipidemia, endothelial dysfunction, peripheral artery resistance and myocardial dysfunction (Figure 35) (Bowen, Harris et al. 2016, Shahidi and Ambigaipalan 2018). The correlation was first observed by Dyerberg and colleagues in 1976 when they found that the incidence of CVD was low in Inuit of Greenland in comparison with the population of Denmark, the principle underlying difference being the diet rich in omega-3 fatty acids EPA and DHA (Yamagata 2017). Ingestion of 12% of fish oil, containing EPA (18%-19%) and DHA (12-13%), by rats showed protection against ischemia reperfusion injury in isolated rat hearts and reduced myocardial infarct size (Desnoyers, Gilbert et al. 2018). (Ajith and Jayakumar 2019) suggested, as recommended by The American Heart Association, consumption of two meals/per week of fish to reduce the risk of coronary heart disease (CHD) and 1g of EPA and DHA per day in patients with documented CHD.

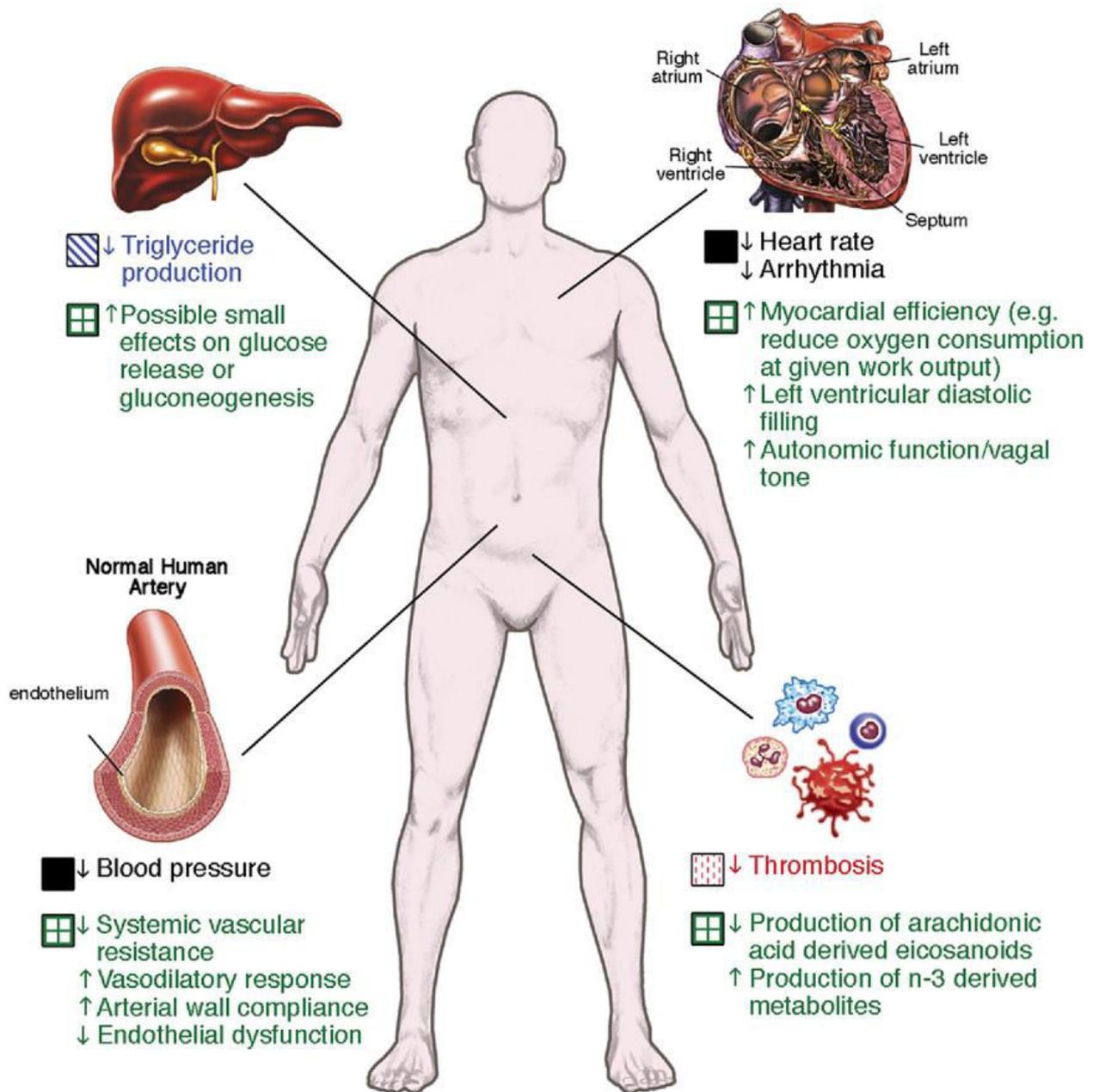


Figure 35. Omega-3 PUFAs targets in cardiovascular diseases (Mozaffarian and Wu 2011)

4.6.1 Blood pressure

Hypertension is one of the substantial risk factor for almost all cardiovascular diseases including coronary heart disease, stroke, sudden cardiac death, heart failure, valvular heart disease, left ventricular hypertrophy, atrial fibrillation, peripheral arteria disease and myocardial infarction (Kjeldsen 2018). Several studies have showed that fish oil or omega-3 PUFAs (EPA and DHA) intake can reduce blood pressure and augment the efficacy of anti-hypertensive drugs (Chobanian, Bakris et al. 2003, Miller, Van Elswyk et al. 2014). An epidemiological study including 4680 subjects from different

countries, such as Japan, China, UK and USA showed inverse correlation between dietary long chain omega-3 PUFAs intakes and blood pressure (Ueshima, Stamler et al. 2007). A randomized study has reported that two months ingestion of 4g/d of EPA and DHA by mild hypertensive patients lead to significant decrease in Systolic Blood Pressure (SBP) (from 154±4 to 148±5 mm Hg) and Diastolic Blood Pressure (DBP) (from 97±8 to 92±6 mm Hg) (Prisco, Paniccia et al. 1998, Colussi, Catena et al. 2017). A meta-analysis of 36 clinical studies concluded that consumption of fish oil (3.7g/d, 2 weeks) resulted in significant reduction of 2.1 mm Hg and 1.6 mm Hg in SBP and DBP, respectively, among elderly and hypertensive patients (Geleijnse, Giltay et al. 2002). Another meta-analysis of seventy randomized clinical trials (RCTs) refers to a reduction of SBP by 4.51mm Hg and DBP by 3.05mm Hg in non-treated hypertensive patients whereas blood pressure was also reduced among normotensive subject by a reduction of 1.25mm Hg in SBP and 0.46mm Hg in DBP (Miller, Van Elswyk et al. 2014). The average increase in SBP is 0.6mm Hg per year among adults (Appel, Giles et al. 2009) and omega-3 PUFAs-mediated reduction of 1.25mm Hg SBP among normotensive patients would delay the age-related rise in SBP by 2 years. Whereas, reduction of 4.51mm Hg SBP among non-medicated hypertensive patients can maintain the hypertension at lower stages without need of anti-hypertensive medication (Miller, Van Elswyk et al. 2014). (Cabo, Alonso et al. 2012) also demonstrated that doses of over 3g/day of omega-3 PUFAs are recommended to reduce blood pressure in older and hypertensive patients. Moreover, our team has recently showed that the ingestion of 5.67 g/day of optimized formulation of omega-3 PUFAs, EPA:DHA 6:1 for four weeks resulted in reduction of SBP by about 60% in Ang II-induced hypertensive rats.

Different mechanisms have been suggested to explain blood pressure lowering by omega-3 PUFAs such as the production of vasodilatory metabolites of omega-3 PUFAs (PGI₃, TXA₃) (Schmitz and Ecker 2008), prevention of angiotensin II-mediated vasoconstriction by reducing the expression of angiotensin converting enzyme (ACE) and AT₁R (Cabo, Alonso et al. 2012, Niazi, Silva et al. 2017, Yang, Shi et al. 2019), improvement of the endothelial function by reducing the oxidative stress and increasing the expression of functional eNOS favoring NO-mediated vasorelaxation, and the limitation of COX-derived endothelium-derived contractile factors (EDCF) production (Zgheel, Alhosin et al. 2014, Niazi, Silva et al. 2017). In addition, omega-3 PUFAs can also lower the blood pressure by enforcing vasodilatory effect via direct activation of

large-conductance Ca^{2+} -dependent K^+ channels (BK channel) in vascular smooth muscle (Hoshi, Wissuwa et al. 2013).

4.6.2 Heart rate

Heart rate is linked with many cardiovascular conditions, such as hypertension, stroke, coronary artery disease, ischemia heart disease and heart failure and presents a significant risk for the cardiovascular mortality (Tadic, Cuspidi et al. 2018). Increase of 10 beats per minute (bpm) in heart rate is correlated with 20% increased risk of sudden cardiac death (Perret-Guillaume, Joly et al. 2009). A meta-analysis by (Aune, Sen et al. 2017) showed that after an elevation of 10 bpm in resting heart rate increased the risks of heart failure onset by 18%, coronary artery disease by 7%, sudden cardiac death by 9%, stroke by 6% and of cardiovascular morbidity by 15%. In 12 cohort studies including 112680 subjects, resting heart rate of more than 80 bpm showed 44% increase in cardiovascular mortality as compared with lower bpm (Woodward, Webster et al. 2014).

The effects of omega-3 PUFAs on heart rate have been observed in various populations (Kang 2012). Consumption of fish, 3 or more than 3 times per week, showed reduction in heart rate by 3 bpm (Cabo, Alonso et al. 2012). The meta-analysis of 30 randomized trials showed that fish oil consumption is associated with heart rate lowering effect, dependent of treatment duration and heart rate baseline. Reduction by 2.5 bpm was observed when treatment lasted more than 12 weeks and heart rate baseline was more than 69 bpm while little effects (- 0.7 bpm) was reported with shorten treatment and baseline was less than 69 bpm (Mozaffarian, Geelen et al. 2005). Another randomized controlled trial including patients with previous myocardial infarction and ejection fractions of less than 40%, also showed that omega-3 PUFAs reduced the heart rate by 5 bpm and also improved heart rate recovery after exercise (O'Keefe, Abuissa et al. 2006). Moreover, 1g/d intake of EPA and DHA is linked with 2.3 bpm reduction corresponding to a 7.5% lower risk of sudden cardiac death (Mozaffarian, Prineas et al. 2006). The possible mechanism of heart rate lowering effect of omega-3 PUFAs is attributed to a direct inhibition of ion channels (L-type Ca^{2+} channels and membrane Na^+ channels) thereby lowering the resting membrane potential, ultimately, resulting in reduced electrical excitability of myocardium (Kang 2012).

4.6.3 Plasma lipids

Hyperlipidemia, high blood levels of triglycerides and cholesterol, is a major risk factor of cardiovascular (Nelson 2013, Zodda, Giammona et al. 2018). High density lipoproteins (HDL) are considered beneficial since they help in the removal of cholesterol from the plasma. Major risk of cardiovascular events is associated with low and very low density lipoproteins (LDL, VLDL) which are involved in the deposition of cholesterol in vessel walls and promote ischemic heart disease, atherosclerosis and even heart attack (Nelson 2013, Sokoła-Wysoczańska, Wysoczański et al. 2018). Numerous studies have demonstrated that omega-3 PUFAs reduce serum levels of triglycerides and can also augment the efficacy of anti-hyperlipidemic treatment. Elevated levels of TG presents CVDs risk, promote the release of pro-inflammatory cytokines, favors coagulation and impair fibrinolysis (Reiner 2017) and potential of omega-3 PUFAs to reduce the serum levels of TG, could constitute therapeutic option for hyperlipidemic patients (Kimmig and Karalis 2013). Moreover, treatment of patients with consistent high TG levels with combined therapy of 4g/d of omega-3 PUFAs (EPA 460mg, DHA 380 mg) and 40mg/d of simvastatin showed better effects than the intake of simvastatin alone (Davidson, Stein et al. 2007). Plasma levels of triglycerides (TG) after ingestion of 3.4 g/d omega-3 PUFAs for one month are reduced by 25-50% primarily through limited production of VLDL-TG in liver and secondarily increased clearance of VLDL (Shearer, Savinova et al. 2012). The reduced fatty acid (FA) lipogenesis resulting in shortage of substrate, an increased beta-oxidation of FA in chylomicrons leading to reduced transfer of non-esterified FA to the liver, an increased generation of phospholipids rather than TG and competitive blockade of enzymes (such as diacylglycerol acyltransferase or phosphatidic acid phosphohydrolase) involved in the production of TG, all contributes, at least in part, to omega-3 PUFAs-mediated decreased serum TG levels (Harris and Bulchandani 2006, Mori 2014). The reduction in plasma levels of TG by 40%-60% after ingestion of pure fish oil or 15% of fish oil for four months, was attributed in mice to reduced endogenous synthesis of TG principally and also through enhanced clearance of TG-rich particles from blood (Qi, Fan et al. 2008). DHA, an omega-3 PUFA, enhance blood clearance of TG by reducing the levels of Apo CIII protein, which is an inhibitor of the activity of lipoprotein lipase (Larsson, Vorrstö et al. 2013), through activation of PPAR α and inhibition of NF-kB (Adkins and Kelley 2010).

4.6.4 Inflammation

Inflammation is a protective mechanism but, if exacerbated and prolonged, a strong promoter of various pathologies (Rea, Gibson et al. 2018). Indeed, inflammation has been depicted as underlined mechanism in pathogenesis of cardiovascular diseases such as myocardial infarction, atherosclerosis, cardiac remodeling and myocarditis (Endo and Arita 2016). Recently, omega-3 PUFAs has gain much more attention due to their potential to attenuate inflammation *via* multiple mechanisms (Figure 37).

First, omega-3 PUFAs displace arachidonic acid (ARA) from the plasma membrane of endothelial cells, platelets and inflammatory cells, resulting in a decreased production of pro-inflammatory mediators such as prostaglandin E₂, thromboxane B₂, 5-hydroxyeicosatetraenoic acid (5-HETE), leukotriene B₄ and E₄. In addition, LOX and COX mediated metabolism of membrane incorporated omega-3 PUFAs leads to the production of anti-inflammatory mediators which are such as 3-series prostaglandins and 5-series leukotrienes (Figure 36) (Saini and Keum 2018).

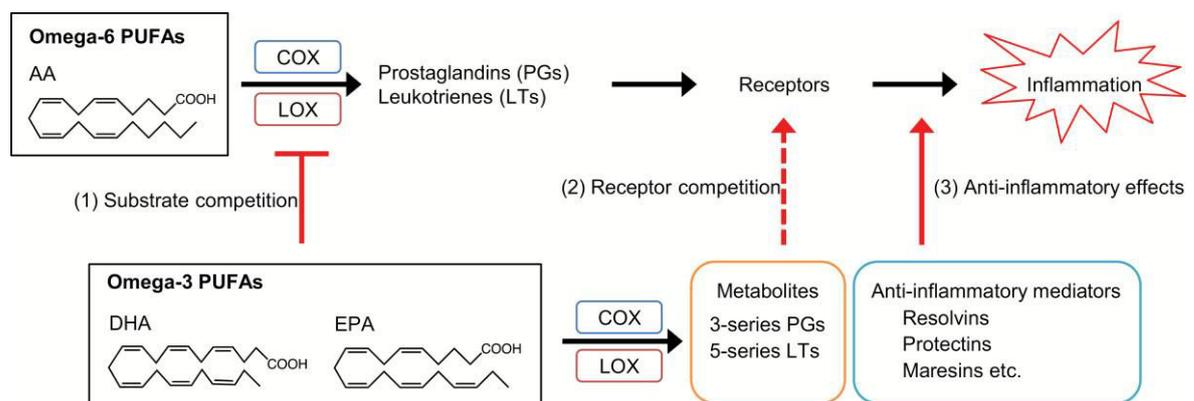


Figure 36. Role of omega-3 and omega-6 PUFAs in inflammation
(Ishihara, Yoshida et al. 2019)

Second, omega-3 PUFAs limit the production of classic pro-inflammatory cytokines, tumor necrosis factor-alpha (TNF- α), IL-1 and IL-6. In patients with non-ischemic dilated cardiomyopathy, ingestion of omega-3 PUFAs was linked to decreased concentration of circulating pro-inflammatory cytokines and improved left ventricular functional capacity (Endo and Arita 2016). Some reported studies have also shown that EPA and DHA concentration can also increase the production of interleukin-10 (IL-10) that has an anti-inflammatory effects (Calder 2015).

Third, omega-3 PUFAs reduce the expression of adhesion molecules (ICAM-1, VCAM-1) on the surface of endothelial cells and inflammatory cells such as monocytes, macrophages and lymphocytes, thereby preventing diapedesis (Miles, Wallace et al. 2000, Calder 2015, Calder 2017). In healthy men, supplementation of diet with omega-3 PUFAs resulted in the down-regulation of ICAM-1 at the surface of interferon- γ -stimulated monocytes (Calder 2017). Similarly, the adhesion of blood monocytes, harvested from omega-3 PUFAs-treated patients with peripheral artery disease, to cultured endothelial cells was significantly lowered (Luu, Madden et al. 2007). Furthermore, concentration of soluble ICAM-1 and VCAM-1 was also lowered in EPA and DHA supplemented elderly and patients with metabolic syndrome (Yamada, Yoshida et al. 2008).

Fourth, omega-3 PUFAs interfere with pro-inflammatory signaling pathways. For example, EPA/DHA as well as fish oil have been reported to reduce LPS-mediated activation of NF- κ B in human blood monocytes, cultured macrophages and dendritic cells. EPA and DHA can also blunt NF- κ B signaling via the activation of peroxisome proliferator-activated receptor-gamma (PPAR- γ) (Calder 2017).

Fifth, DHA-mediated inhibition of the recruitment of pro-inflammatory signaling proteins (TLR4, MyD88) into lipid rafts of the membrane of inflammatory cells would blunt the effects of inflammatory stimuli such as LPS (Wong, Kwon et al. 2009).

Sixth, omega-3 PUFAs prevent the NOD-like receptor family pyrin domain-containing 3 (NLRP3) responsible for inflammasome-based inflammation in myocardial infarction, ischemia reperfusion injury and cardiac remodeling (Endo and Arita 2016).

Seventh, through generation of specialized pro-resolving mediators including resolvins, protectins and maresin, omega-3 PUFAs are effective actors of resolution of inflammation (Calder 2017).

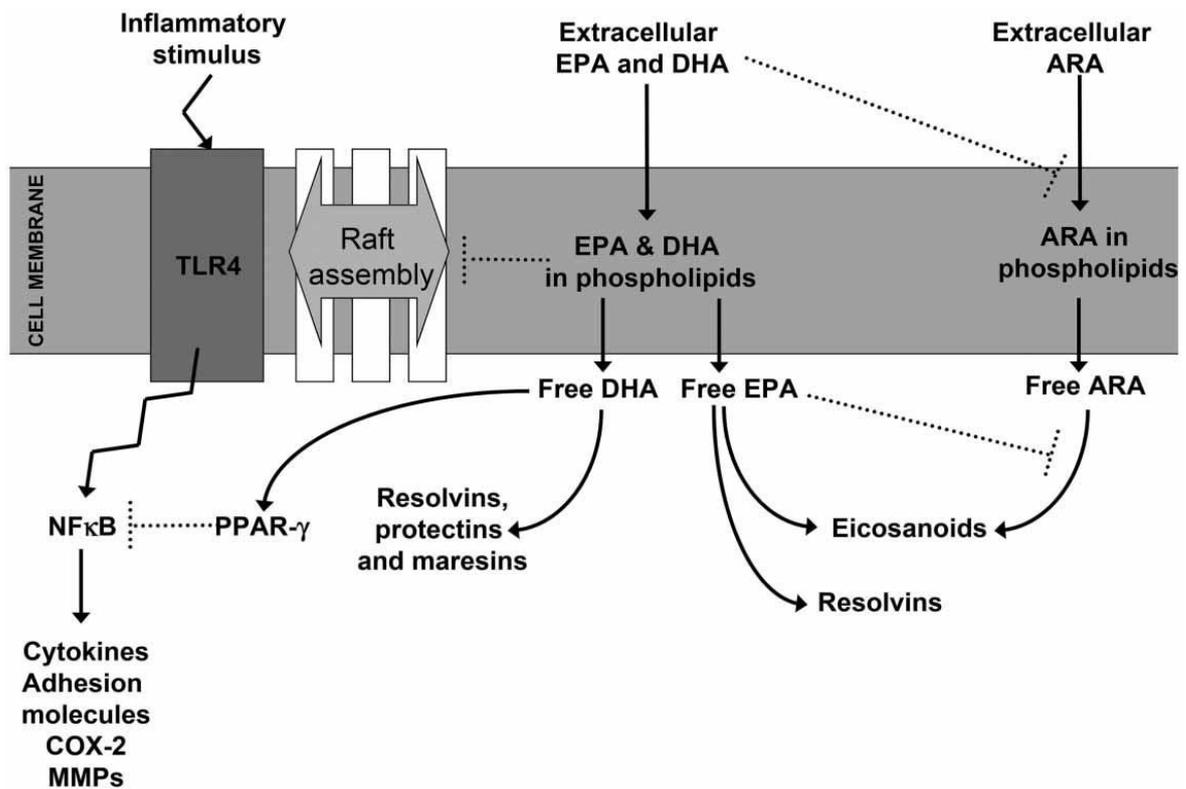


Figure 37. Depiction of some anti-inflammatory pathways triggered by EPA and DHA (Calder 2017)

4.6.5 Endothelial dysfunction

The endothelium, a monolayer of endothelial cells (ECs), play a fundamental role in vascular homeostasis. Endothelial cells regulate blood pressure, blood coagulation, inflammation, arterial stiffness and vascular permeability through a well-regulated synthesis and release of various bioactive mediators (such as NO, PGI₂, endothelin1, angiotensin II) and down/upregulation of various receptors, adhesion molecules and transporters (Yamagata 2017).

Various pre-clinical and clinical studies have reported that ingestion of omega-3 PUFAs improves endothelial dysfunction (Mori 2014) *via* enhancing the production of endothelial NO, reducing oxidative stress and preventing inflammatory and thrombotic processes. (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 culture human endothelial cells *via* Akt/ERK/NF-κB signaling pathways.

Omega-3 PUFAs improves endothelium dependent vaso-relaxation by enhancing the levels of NO, reducing the oxidative stress and pro-inflammatory cytokines production

as well as reduction of circulating levels of endogenous inhibitor of NOS, asymmetric dimethyl arginine (ADMA) (Zanetti, Gortan Cappellari et al. 2017). Through its antioxidant effects, omega-3 PUFAs (2.4g/day) significantly improved endothelial function and arterial stiffness in patients with metabolic syndrome (Jackova, Jedlickova et al. 2015). Ingestion of marine-derived omega-3 PUFAs (> 2g/day) has been reported to have improved endothelial function via anti-atherogenic and anti-thrombotic effects in patients with diabetes mellitus and dyslipidemia, conditions strongly associated with accelerated atherosclerosis and high CVD risk. In addition, owing to anti-thrombotic properties of omega-3 PUFAs, endothelial dysfunction was also shown to improve in patients with antiphospholipid syndrome (APS), a systemic autoimmune disease associated with recurrent episodes of thrombosis or/and obstetric morbidities and constant serum antiphospholipid antibodies (aPL). aPL binding to endothelial cells beta-2 glycoprotein I leads to endothelial dysfunction and thrombus formation (Felau, Sales et al. 2018). (Preston Mason 2019) Reported improvement of endothelial function by increasing the ratio of NO to peroxynitrite (ONOO⁻) without affecting the expression of eNOS which suggest protection against oxidative stress mediated- eNOS uncoupling by omega-3 PUFAs. In our lab, it has been demonstrated by (Zgheel, Alhosin et al. 2014) that among different omega-3 PUFAs formulations, EPA:DHA 6:1 was most potent stimulator of endothelial NO by activating eNOS *via* redox sensitive activation of Src/PI3K/Akt and MAPKs pathways and, to lesser extent, endothelium-derived hyperpolarization (EDH) in porcine coronary arteries. Another colleague, (Niazi, Silva et al. 2017) showed that omega-3 PUFA formulation EPA:DHA 6:1 attenuates endothelial dysfunction in ang II-induced hypertensive rats *via* inhibition of NADPH oxidase- and cyclooxygenase-derived oxidative stress. Recently, (Zgheel, Perrier et al. 2019) has also showed that omega-3 PUFA formulation EPA:DHA 6:1 significantly induced concentration-dependent relaxation in porcine coronary artery and human internal mammary artery (IMA) rings and effectively prevented platelets-induced 5-hydroxytryptamine (5-HT)-mediated contraction, most likely involving endothelial NO dependent mechanism and hence, may aid to secure cardiovascular system.

4.6.6 Thrombosis

Adhesion, activation and aggregation of platelets has a central role in hemostasis, however, uncontrolled activation and aggregation of platelets can promote thrombosis

and blood vessel occlusion at the site of atherosclerotic lesion, ultimately, leading to heart attack and stroke. Omega-3 PUFAs are incorporated in phospholipids membrane of platelets and hence, can regulate the platelets function and subsequently thrombosis (Adili, Hawley et al. 2018). Omega-3 PUFAs can exert their anti-thrombotic effects through different pathways. Direct activation of eNOS and increase level of NO is responsible for inhibition of platelets activation and aggregation (Abeywardena and Head 2001). EPA and DHA can inhibit activity of platelets by directly inhibiting the thromboxane receptors (Swann, Pelham et al. 1989). By incorporating into platelets membrane and replacing the arachidonic acid, omega-3 PUFAs shifts the prostanoids profile of platelets (less TXA₂, which is inducer of aggregation of platelet, more TXA₃ and PGI₃, which are anti-aggregatory in actions) towards anti-thrombotic effects (Kromhout, Yasuda et al. 2012, Mori 2014). In patient undergoing percutaneous coronary intervention, omega-PUFAs alters the production of thrombin, a potent platelet agonist, alters the properties of fibrin clot (Gajos, Zalewski et al. 2011). In addition, omega-3 PUFAs combined therapy with aspirin and clopidogrel enhance the response of platelets to clopidogrel after percutaneous coronary intervention (Gajos, Rostoff et al. 2010).

4.6.7 Arrhythmia

Cardiac arrhythmias, irregular heartbeats, are directly linked with sudden cardiac death (Adabag, Luepker et al. 2010). (Albert, Chae et al. 2003) have demonstrated that lowered incidence of sudden cardiac death is related with an improved variability in heart rate and reduce ventricular arrhythmias. Since late 1980s, the interrelation among omega-3 PUFAs intake, arrhythmias and sudden cardiac death has been demonstrated through observational and interventional studies (Saravanan, Davidson et al. 2010). Membrane lower incorporation with omega-3 PUFAs is associated with increased risk of ventricular fibrillation in post myocardial infarction patients (Aarsetoy, Ponitz et al. 2008). Omega-3 PUFAs have been reported to regulate the electrical activity of heart *via* regulation of ion channels and parasympathetic tone (Ajith and Jayakumar 2019). Reduced release of Ca⁺² from sarcoplasmic reticulum of myocytes and an increase in parasympathetic tone alleviate the tachyarrhythmias in rat myocytes (Das 2000, Negretti, Perez et al. 2000).

4.7 Omega-3 PUFAs and clinical trials

Owing to their beneficial cardiovascular effects in numerous observational studies, omega-3 PUFAs were assessed in randomized clinical trials for their potency to prevent various adverse cardiovascular outcomes.

4.7.1 Diet and Reinfarction Trial (DART)

DART was the first randomized clinical trial, published in 1989, to assess the cardio-protective effects of omega-3 PUFAs in patients with previous myocardial infarction. In total, 2033 men, after myocardial infarction, were randomly divided into groups with or without advice to increase the intake of omega-3 fatty acids and were followed for two years. The omega-3 fatty acids intake was achieved in the form of 2 servings per week of fatty fish (200-400g) or in some cases in the form of fish capsule (Mexepa, 3g/day). After 2 years of follow-up, the group advised to take fatty fish meal or fish oil capsule showed 29% reduction in all-cause mortality and the incidence of reinfarction and death due to ischemia cardiac events was reduced by 32% (Burr, Fehily et al. 1989, Jain, Aggarwal et al. 2015, Bowen, Harris et al. 2016).

4.7.2 Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico Prevenzione (GISSI-Prevenzione)

GISSI-Prevenzione trial, published in 1999, included 11,323 patients (including 14.7% female) with previous recent history of myocardial infarction (3 months). Patients were randomly divided into four groups as 1st receiving omega-3 PUFAs (1 g/d), 2nd group received treatment with Vitamin E (300 mg/d) , 3rd group was give combined therapy (omega-3 PUFAs plus vitamin E) and 4th group received no treatment. All patients were followed and monitored for 3.5 years for stroke, myocardial infarction or death. Patients in omega-3 PUFAs group showed reduction in risk of all cause and cardiac death by 41% and 53%, respectively, within 3 or 4 months of treatment. Similarly, 30% and 45% reduction in the risk of cardiovascular death and sudden cardiac death, respectively, was reported in omega-3 PUFAs treated group at the end of follow-up. The authors concluded that reduction of sudden cardiac death by omega-3 PUFAs is due to their anti-arrhythmic effects which is the prime reason for lowered risk of all-cause mortality (1999, Bowen, Harris et al. 2016).

4.7.3 GISSI-Heart Failure (GISSI-HF)

GISSI-HF, published in 2004, was the first double blind, placebo-controlled randomized trial. In total of 6975 patients (both men and women) with chronic heart failure (HF) were treated with either omega-3 PUFAs (1 g/d) or placebo. A follow-up of 3.9 years showed 9% reduction in all-cause mortality and 8% reduction in hospitalization due to cardiovascular events in the omega-3 treated group, compared to placebo (Bowen, Harris et al. 2016).

4.7.4 Japan EPA Lipid Intervention Study (JELIS)

A study conducted in Japan and published in 2007, examined the effects of statins, alone vs. in combination with EPA (1800 mg/d). In total 18,645 patients with hyperlipidemia (total cholesterol \geq 6.5 mmol/L), including patients with previous history of cardiovascular events, were randomly divided into two groups and were given either statin alone or in combination with EPA and were followed for 5 years. The endpoints of the study was any major coronary event such as sudden cardiac death, myocardial infarction (fatal or non-fatal) and other fatal or non-fatal events such as unstable angina pectoris, stenting, angioplasty or coronary artery bypass grafting. The combined therapy was found to reduce the worsen outcomes by 19%, as compared with statin alone. Subgroup analysis revealed that the reduction of major coronary events was significantly reduced in patients with recent history of coronary artery disease (secondary prevention) whereas, 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).

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

REDUCE-IT is a double blind, randomized, placebo-controlled trial which included 8179 patients with previously established cardiovascular disease or diabetes or other risk factors. All the patients had a fasting 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 and followed for 4.9 years. The composite of cardiovascular death, non-fatal stroke, non-fatal myocardial infarction, coronary revascularization or unstable angina was the end point of the study. Compared to the placebo, the omega-3 treated group showed 19% reduction in

triglycerides and 25% reduction in major cardiovascular events (Bhatt, Steg et al. 2019, Grzegorz 2019).

4.8 Omega-3 PUFAs and reduction of cardiovascular risk- a controversy

When there are many clinical trials which are in the favor that omega-3 PUFAs reduce the risk of cardiovascular disease, there are other clinical trials including ALPHA OMEGA, SU.FU.OM3, ASCEND, VITAL which could not give satisfactory evidence to proof the cardio-protective effects of omega-3 PUFAs. The different potential explanations for the no or little cardio-protective effects of omega-3 PUFAs concluded in these trials includes underpowered studies (small sample or low cardiovascular event rate), participants with background of high fish/seafood intake, age of the participants, length of the follow-up, suboptimal EPA and DHA dose, duration of supplementation and concurrent standard of care for cardiovascular disease. Based on the contrasting conclusion of all the clinical trials conducted on omega-3 PUFAs, it is clearly indicated that still more knowledge is required to determine the efficacy, mechanisms, formulation, dose and optimal targeted population of omega-3 PUFAs (Bowen, Harris et al. 2016, Grzegorz 2019).

Hypothesis and Aims

Cardiovascular diseases are the leading cause of death worldwide and age is one of the major risk factor for many chronic diseases including cardiovascular diseases. Age is associated with persistent activation of the immune system which leads to constant low grade inflammation, referred to as inflamm-ageing, which involves functional alteration of immune cells. Inflamm-ageing, indeed, is the accelerator for many chronic inflammatory diseases such as atherosclerosis which is the primary cause of stroke, coronary artery disease and peripheral vascular diseases.

Microvesicles, small membrane vesicles shed by apoptotic or activated cells, are the pro-senescent effectors of endothelial cells, promoting endothelial dysfunction and hence, increasing the risk of cardiovascular diseases (Abbas, Jesel et al. 2017, El Habhab, Abbas et al. 2017). Microvesicles are shed by different types of cells including platelets, endothelial cells and leukocytes. Leukocytes microvesicles are crucial to the vascular homeostasis and their circulatory levels are correlated with multiple chronic cardiovascular diseases. Human plaques have shown to be incorporated with microvesicles of more than 50% of leukocyte origin, showing strong link of involvement of leukocytes MVs in the initiation and progression of atherosclerotic plaque.

Omega-3 PUFAs, including the two most important EPA and DHA, have been reported in epidemiological, experimental and clinical studies to reduce the risk of primary and secondary cardiovascular disease. The beneficial effects of omega-3 PUFAs are, at least in part, could be related to their ability to improve endothelial function by reducing the vascular oxidative stress, increasing the endothelial formation of NO and reducing the circulatory levels of pro-coagulant microvesicles. In patients with post-myocardial infarction, omega-3 PUFAs have been shown to reduce the circulatory levels of pro-coagulant MVs of platelets and leukocytes origins (Del Turco, Basta et al. 2008). Moreover, our team has showed that the vaso-protective effects of omega-3 PUFAs are dependent upon the ratio and purity of EPA and DHA and the ratio of 6:1 of EPA and DHA is the most potent for maximal vasorelaxation. Omega-3 EPA:DHA 6:1 induced the vaso-relaxation in freshly isolated porcine coronary arteries through increased activation of eNOS via redox-sensitive Src/PI3-kinase/Akt (Zgheel, Alhosin et al. 2014). Moreover, omega-3 EPA:DHA 6:1 ingestion by angiotensin-induced hypertensive rats also showed improved endothelial function and more than 50% reduction in systolic blood pressure (Niazi, Silva et al. 2017). Recently, in an *ex-vivo* study using porcine coronary artery and human internal mammary artery, omega-3 EPA:DHA 6:1 have been reported to prevent the platelet-mediated serotonin-induced contractile response,

suggesting the protective role of omega-3 PUFAs against the platelets-induced vascular injuries (Zgheel, Perrier et al. 2019).

So, the hypothesis of our study was first that ageing is associated with increase shedding of MVs from leukocytes which are pro-senescent effectors of the endothelial cells and shift the endothelial cells towards pro-inflammatory and pro-coagulant phenotype, hence, promote endothelial and coronary artery dysfunction and second that Omega-3 PUFAs prevent the endothelial dysfunction and reduce the risk of cardiovascular disease by protecting the endothelium against pro-senescent MVs shed by aged leukocytes.

The aims of the study were

- Determine the impact of age on shedding of MVs and their pro-senescent potential
- Determine the impact of omega-3 EPA:DHA 6:1 intake on ability of spleen-derived leukocytes to shed microvesicles
- Determine the protective effects of EPA:DHA 6:1 intake against the pro-senescent and pro-inflammatory effects of leukocyte-derived MVs on endothelium

Results

Impact of EPA:DHA 6:1 on vascular ageing and role of microvesicles

Background

The debated benefits of omega 3 PUFA for cardiovascular health

Despite early epidemiological reports from the latter half of the last century in local country-side populations of Japan, Kreta or Inuit Greenland territories, the therapeutic benefits of omega-3 PUFAs have been difficult to demonstrate. The variety of the omega-3 PUFAs composition, regimen, duration of treatment, and clinical backgrounds of the patients may account for discrepancies with opposite conclusions on the secondary prevention of cardiovascular disease (Bowen, Harris et al. 2016, Grzegorz 2019, Li, Wahlqvist et al. 2019).

Furthermore, the aged-related risk combined to chronicity and progressive development of CVD requires large and time extended trials that are of financial burden for investigators and omega-3 producing companies.

Concomitant medication and the important proportion of omega-3 deficiency in the cohorts fed by western diet may have obscuring the benefit. Indeed, lower omega-3 index is a marker of increased propensity to secondary cardiovascular worsen outcome as reported in hypertensive rats with increased malignant arrhythmias (Bacova, Sec et al. 2013) and in humans (McNamara 2009).

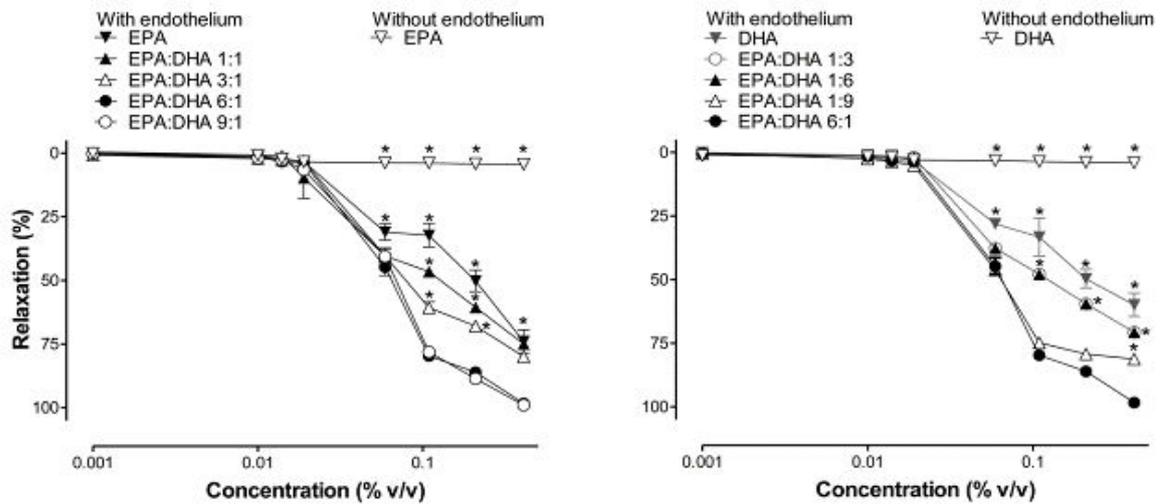
Another cause of discrepancies, at least in the USA, is the large proportion of soybean oil in the diet (9% of all calories) that may have impair the detection Omega-3 benefits since it leads to a high proportion of linoleic acid (LA) in the tissues favoring the downstream production of pro-inflammatory arachidonic acid (AA) at the expenses of EPA and DHA. Therefore, a simple reduction of such intake could have undetected beneficial effects of the nutritional change together with EPA DHA supplementation (Hibbeln, Nieminen et al. 2006, Harris 2018).

Interestingly, omega-3 deficiency has been associated in the past decade with enhanced mortality and morbidity in 12 risk models including cardiovascular and mental diseases and nutritional complementation estimated to diminish the world wide burden of cardiovascular mortality by at least 30% with 180 mg/day and 85%-99% with 500 mg/day representing 0.22% of energy intake (Hibbeln, Nieminen et al. 2006) and a lower omega 3 index measuring the proportion of EPA:DHA in the erythrocyte membrane seems indicative of worsen control of heart rate availability in vegans compared to omnivores(< 2.7 % vs. 8 %) (Hall 2017).

While the epidemiologic evidence of a cardiovascular protection is more acknowledged (Jain, Aggarwal et al. 2015), even in peripheral vascular diseases (Ramirez, Zahner et al. 2019), and recently strengthened by the REDUCE-IT trial (see introduction pages 95-96), the underlying mechanisms on how low concentrations of omega-3 in cell membranes may affect cardiac electrophysiology and chronic inflammation as well remain poorly understood, partly due to the difficulties in establishing omega-3 metabolites thresholds in blood and tissues, of great interest since they contribute to the homeostatic inflammatory response (Capó, Martorell et al. 2018).

Recent data on the vascular impact of omega-3 in animal models

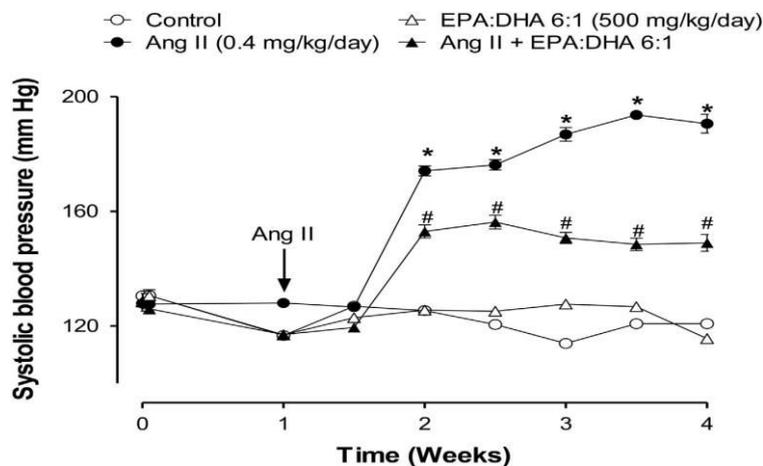
Previous data from our laboratory have shown the beneficial effects of new formulation omega-3 EPA:DHA 6:1 on vascular tone and blood pressure in healthy porcine normotensive coronary arteries (ex-vivo) and in hypertensive rats given a short-term omega-3 intake (see figures below). These data clearly indicate that the endothelial function was improved ex-vivo and in vivo at doses corresponding to those recommended by the cardiovascular scientific societies (AHA). Furthermore, the new formulation omega-3 EPA:DHA 6:1 was triggering the best protection of the endothelial function (Zgheel, Alhosin et al. 2014, Zgheel, Perrier et al. 2019)



Acetylcholine-induced endothelial-dependent relaxation of porcine coronary arteries and effects of different formulations of EPA and DHA

Coronary artery rings with endothelium were contracted with U46619 before the addition of increasing concentrations of an omega-3 fatty acid product. All experiments were performed in the presence of indomethacin (10 μ M) to prevent the formation of vasoactive prostanoids. Results are expressed as means \pm SEM of 5 different experiments. *P<0.05

Adapted from (Zgheel, Alhosin et al. 2014).



Effect of omega 3 EPA:DHA 6:1 on the systolic blood pressure in a rat model of hypertension induced by angiotensin II

10 weeks old Wistar rats received daily by gavage 500 mg kg⁻¹ per day of either EPA:DHA 6:1 or corn oil (control) for 1 week before administration of Ang II (0.4 mg kg⁻¹ per day) using mini osmotic pumps for 3 weeks. Blood pressure was monitored by tail-cuff sphygmomanometry. Results are expressed as means \pm S.E.M. of 8 rats per group. *P<0.05 vs. Control, #P<0.05 vs. Ang II. Adapter from (Niazi, Silva et al. 2017)

Furthermore, these above data also suggest that EPA:DHA 6:1 does not override the normal function of the vessel and endothelial cells but that in vascular disability it rather restores the healthy baseline.

Other data from the laboratory indicated a protection of the arteries against the oxidative stress and the up-regulation of angiotensin converting enzyme (ACE) and the AT1 receptor as well as pro-inflammatory markers such as COX-2. Omega-3 EPA:DHA 6:1 also normalized the expression of eNOS (Niazi, Silva et al. 2017) (Thesis Farooq, 2018)

Impact of ageing on vascular inflammation and endothelial injury

Inflamm-ageing is associated with progressive and sustained low grade activation of the immune system favoring the dysfunction of neutrophils, monocytes/macrophages, natural killer cells and B and T lymphocytes.

Neutrophils have been recently recognized as key drivers of the vascular inflammation through multiple pathways. Upon activation they release ROS and proteinases such as elastase, myeloperoxidases, the latter also attached to extracellular Traps (NETs) consisting of unpacked chromatin, useful to limit pathogen dissemination but a highly negative surface suitable for the assembly of coagulation complexes when the neutrophil is recruited at site of vessel damage. In non-pathogen low grade inflammation, the neutrophil interacts rapidly with the inflamed or damaged endothelium, most probably because of an initial up-regulation of endothelial adhesion molecules that enhance neutrophil recruitment, followed by diapedesis thereby amplifying the vascular inflammatory response. Furthermore, NETs, activated neutrophils and platelets promote hypercoagulability and thrombosis through feed forward interactions leading to thrombus growth. Multiple inflammatory mediators released by platelets and neutrophils have been involved in atherothrombosis or deep vein thrombosis including MVs. (Gomez-Moreno, Adrover et al. 2018).

Impact of ageing on endothelial senescence and dysfunction

Because premature endothelial senescence is caused by inflammatory mediators as well as flow disturbance, the question of the role of leukocyte MVs in endothelial senescence appears crucial especially in atherothrombosis. In a previous work from the

laboratory, Abbas et al demonstrated that pro-senescent MVs of endothelial origin circulate in the plasma of patients with cardiovascular events and that their ability to activate senescence was markedly dependent on shear rate (Abbas, Jesel et al. 2017). Furthermore, Ali El Habhab showed that MVs from activated splenocytes from rat but not from resting rats are pro-senescent, pro-inflammatory and pro-coagulant endothelial effectors on line with the current concept of cellular- and MV-driven hemostasis that involves platelet, leukocyte and endothelium interactions (Badimon, Suades et al. 2016) and in accordance with previous observation that endothelial senescence first develops at the sites of flow disturbances prone to atherothrombosis in animal models (Gimbrone and García-Cardena 2016).

Using spleen as a convenient and rich source of primary leukocytes we investigated the role of ageing in leukocyte MV shedding and in their pro-senescent abilities. Since, vascular protection via inhibition of endothelial oxidative stress and inflammatory responses at thrombogenic sites and restoration of endothelium-dependent vasorelaxation was observed in rats treated with various anti-oxidant extracts, we anticipated that omega-3 intake could be of similar benefit in the context of combined inflammaging and endothelial senescence by directly modifying the pro-senescent leukocyte MVs features.

Article

Ageing enhances the shedding of splenocyte microvesicles with endothelial pro-senescent effect that is prevented by a short-term intake of omega-3 PUFA EPA:DHA 6:1

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Abstract

Background: Ageing is associated with progressive endothelial senescence and dysfunction, and cardiovascular risk. Circulating endothelial microvesicles (MVs) are pro-senescent and pro-inflammatory endothelial effectors in acute coronary syndrome. Omega 3 PUFAs intake was claimed beneficial in cardiovascular prevention.

Purpose: To investigate whether the intake of the omega-3 formulation EPA:DHA 6:1 by middle aged and old rats reduces the shedding of pro-senescent microvesicles from cultured spleen leukocytes (SMVs) and clarify the underlying mechanisms in target coronary primary endothelial cells (ECs).

Methods: Middle-aged male Wistar rats (M, 48-week old) received 500 mg/kg/d of either EPA:DHA 6:1, EPA:DHA 1:1, or vehicle (CTL) for 7 days, old rats (72-week old) for 14 days. Spleen-derived leukocytes were prepared and cultured for 24 h and MVs collected from supernatants (SMVs). Cultured ECs were prepared from freshly isolated porcine coronary arteries. Senescence-associated β -galactosidase activity (SA- β -gal) was assessed by C12FDG, protein expression by Western blot analysis, oxidative stress by dihydroethidium using confocal microscopy, and procoagulant MVs by prothrombinase assay. The pro-senescent potential of SMVs from middle-aged rats was compared to young (Y, 12-week) and old (O) rats.

Results: The shedding of SMVs significantly increased with age and was inhibited by EPA:DHA 6:1 intake that also prevented ROS accumulation in spleen. Incubation of ECs with 10 nM SMVs from middle-aged and old rats but not those from young induced premature senescence after 48 h. The pro-senescent effect of M-SMVs was prevented by Losartan and associated with endothelial oxidative stress. M-SMVs induced an up-regulation of senescence markers (p16, p21, p53), pro-atherothrombotic (VCAM-1, ICAM-1, tissue factor) and pro-inflammatory markers (pNF- κ B, COX-2) and proteins of the angiotensin system (ACE, AT1-R). Conversely, endothelial NO synthase was down-regulated. Intake of EPA:DHA 1:1 and 6:1 by middle-aged rats decreased the SMVs shedding by 14% and 24%, respectively, EPA:DHA 6:1 reducing their pro-senescent action by 38%. Protection of ECs was not observed in response to M-SMVs from EPA:DHA 1:1 treated rats.

Conclusion: Ingestion of EPA:DHA 6:1 abolishes in middle-aged rats, and limits in old rats both the shedding of SMVs and their pro-senescent, pro-thrombotic and pro-inflammatory effects in ECs, most likely by triggering the local angiotensin system. EPA:DHA 6:1 may help to delay ageing-related endothelial dysfunction.

Introduction

Cardiovascular diseases (CVDs) contribute for 39.6% of all age-related chronic diseases (Fajemiroye, da Cunha et al. 2018). The age-driven production of reactive oxygen species (ROS) in the vessel wall is a well-known actor in vascular dysfunction (Tan, Norhaizan et al. 2018) partly caused by endothelial dysfunction. Endothelial senescence is emerging as a new cause of the progressive alteration of the vascular tone, appearing with age but also prematurely at thrombogenic vascular sites prone to flow disturbance such as coronary arteries. It is characterized by an irreversible cell cycle arrest, morphological changes and a pro-atherogenic endothelial phenotype, favoring a loss of endothelial-dependent vascular function and progressive cardiovascular damages accompanying hypertension or atherosclerosis (Herrera, Mingorance et al. 2010, Khemais-Benkhiat, Idris-Khodja et al. 2016). In senescent endothelial cells (ECs), P53 and down-stream cyclin-dependent kinase inhibitors P21 and P16 are up-regulated, reactive oxygen species (ROS) accumulate and redox-sensitive processes, among which the up-regulation of NADPH oxidase, cyclooxygenases (COXs) and the down-regulation of endothelial nitric oxide synthase (eNOS), prompt endothelial dysfunction ultimately shifting the hemostatic features of the healthy endothelium towards a pro-inflammatory, procoagulant and vasoconstricting phenotype. In addition, senescent ECs promote the release of endothelial microvesicles able to disseminate their pro-senescent signal (Burger, Kwart et al. 2012, Abbas, Jesel et al. 2017).

Microvesicles (MVs), are sub-micron plasma membrane vesicles shed by cells in response to stress. MVs convey active cytoplasmic proteins, lipids as well as miRNA that characterize the cell lineage and stress at the origin of their release (Benameur, Osman et al. 2019). In blood, they circulate as surrogate and pathogenic markers of primary and secondary cardiovascular risk (Koenen and Aikawa 2018). Indeed, circulating MVs constitute a storage pool of effectors of multiple cell origin orchestrating vascular cell cross-talk. MVs are pro-coagulant because they expose phosphatidylserine (Phtdser), an anionic phospholipid that catalyzes the assembly of blood coagulation complexes, and when stemmed from endothelial cells and monocyte, tissue factor (TF), the cellular initiator of blood coagulation (Morel, Jesel et al. 2011). MVs of endothelial origin from patients with acute coronary syndrome (ACS) have been shown to induce coronary endothelial cell dysfunction, premature senescence and thrombogenicity through the activation of the local angiotensin system (Abbas, Jesel et al. 2017).

Interestingly, leukocytes-derived MVs (LMVs), were shown crucial to thrombus growth and vascular remodeling (Swystun and Liaw 2016), their plasma level being correlated with the Framingham score in primary and secondary cardiovascular adverse outcomes (Chironi 2012). LMVs are recruited at the surface of the inflamed endothelium, constitute an additional source of TF and mediate cell interactions within the thrombus (Falati, Liu et al. 2003, Grover and Mackman 2018). In human atherosclerotic plaques, 55% of MVs originate from leukocytes (Leroyer, Isobe et al. 2007). Animal models of atherothrombosis or ischemia reperfusion indicate that LMVs contribute to tissue remodeling by limiting the endothelial NO and promoting redox-sensitive pathways, among which the up-regulation

of endothelial pro-inflammatory cytokines and of adhesion proteins favoring diapedesis. In mice, the injection of LMVs from diabetic patients alters the vascular tone by reducing the acetylcholine-evoked endothelial relaxation of aorta (Tesse, Martinez et al. 2005, Angelillo-Scherrer 2012). Altogether, TF⁺-MVs prompt coagulation and exacerbate inflammation within multiple amplification loops involving TF-driven cell responses (Leroyer, Anfosso et al. 2010). Interestingly, MV shedding seems linked to the lateral lipid organization of the plasma membrane, lipid rafts favoring their TF enrichment when released from activated macrophages (Pollet, Conrard et al. 2018).

The plasma membrane lipid composition of immune cells influences membrane fluidity, cell signaling and lipid mediator production. In humans consuming western diet, polyunsaturated fatty acids (PUFAs) in the plasma membrane of circulating neutrophils, lymphocytes, monocytes are mainly composed of 10%-20% of arachidonic acid, 0.5%-1% eicosapentaenoic acid (EPA) and 2%-4% docosahexaenoic acid (DHA). The intake of omega-3 PUFAs enhances the EPA /DHA membrane proportion at the expense of the n-6 PUFAs, mainly arachidonic acid and derived metabolites. It affects the membrane order, and ultimately raft functions and their dependent downstream signaling pathways (Calder 2010). Clinical trials and animal models have shown that EPA and DHA reduce the risk of cardiovascular diseases (DiNicolantonio, Niazi et al. 2014, Iwamatsu, Abe et al. 2016). Daily consumption of omega-3 PUFAs is protective against primary or secondary cardiovascular events (Kromhout, Yasuda et al. 2012). While EPA and DHA attenuate redox-driven DNA damages in aorta cells (Sakai and Ishida 2017), vasoprotection by omega-3 PUFAs was shown dependent on the EPA/DHA ratio. Maximal vaso-relaxation of healthy pig coronary artery is only obtained with omega-3 EPA:DHA 6:1 as compared to omega-3 EPA:DHA 1:1 (Zgheel, Alhosin et al. 2014). Intake improves the endothelial dysfunction and also reduces systolic blood pressure by 53% in angiotensin-II-induced hypertensive rats (Niazi, Silva et al. 2017). Still, the beneficial effects of omega-3 PUFAs in cardiovascular health are controversial. Clinical trials such as DART, GISSI-Prevenzione, GISSI-HF, JELIS have shown beneficial effects of omega-3 PUFAs in reducing the risk of cardiovascular diseases, whereas, others such as ALPHA OMEGA, SU.FU.OM3, ASCEND, VITAL did not observed promising proofs of reduction (Bowen, Harris et al. 2016, Abdelhamid, Brown et al. 2018). Most recently, the REDUCE-IT trial which included hyperlipidemic patients with previously established cardiovascular diseases and receiving statin therapy showed an efficient reduction of 19% in triglycerides and 25% reduction in major cardiovascular events after ingestion of 4 g/d of icosapent EPA and a follow-up of 4.9 years (Bhatt, Steg et al. 2019, Grzegorz 2019).

Strikingly, mechanisms of endothelial protection by omega-3 PUFAs against LMVs remain yet to be characterized. In post-myocardial 60 years-old patients, elevated levels of circulating MV levels of leukocyte and platelet origin were slightly but significantly lowered after 12 weeks supplementation with 5.2 g/day omega-3 PUFA (33% EPA 60%DHA w:w) given at 6 month distance (Del Turco, Basta et al. 2008), while their procoagulant potential was reduced *in vitro* and involved both TF-dependent

and TF-independent coagulation pathways. (Del Turco, Basta et al. 2008). However, it still remains unclear whether omega 3 PUFA prevent the release of LMVs or rather shifts noxious LMVs into cytoprotective vascular effectors and if so, how it is beneficial to the ageing endothelium.

In vitro, omega-3 PUFAs directly modulate ion channels endocytosis, trigger PPAR- α or HNF-4 α nuclear receptor dependent-responses, G-protein mediated-activation and ultimately ERK $\frac{1}{2}$ kinases and NF- κ B signaling. They also may alter cell responses by incorporating into the plasma membrane where they are converted by COXs and LOXs into key anti-inflammatory specialized pro-resolving lipids mediators, including D- and E-series resolvins and protectins, maresin, (Mozaffarian and Wu 2011, Molfino, Gioia et al. 2014, Sokoła-Wysoczańska, Wysoczański et al. 2018).

This study was aimed to investigate whether the intake of the omega-3 EPA:DHA 6:1 new formulation modifies the ability of ageing immune cells to release MV, and whether it prevents their pro-senescent action on the endothelium. MVs were isolated from the spleen of middle-aged (48 weeks), young (12 weeks) and old (72 weeks) rats. The ability of the spleen-derived leukocytes (SMVs) to promote premature endothelial senescence was examined in primary coronary endothelial cells (ECs).

Materials and Methods

Ethics statement

Wistar rats (Janvier-labs, Le Genest-St-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. Experiments were consistent with the guidelines on animal care and use in laboratory, published by US institute of health (NIH, publication no. 85-23, revised 1996) and were authorized by French Ministry of Higher Education and Research and by the local ethic 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 E-67-218-26).

Preparation of Omega-3 PUFAs

Purified formulations of omega-3 EPA and DHA were obtained from Pivotal Therapeutics, Inc. (Woodbridge, ON, Canada). EPA:DHA ratios of 6:1 and 1:1 (w/w) were prepared by mixing each EPA:DHA according to their relative purity under nitrogen flux to avoid oxidation, and stored in amber colored glass vials at 4°C.

Rat treatment

Middle-age male wistar rats (M, 48-week old) were weighed and assigned to four groups (9 rats/group). Rats were given 500 mg/kg/day oral dose of either EPA:DHA 6:1, EPA:DHA 1:1, corn oil as an isocaloric control without omega-3 or tap water for a week. Young (Y, 12-weeks old) and old (O, 72-

weeks-old) male wistar rats, respectively treated for 7 and 14 days, were used for comparative purpose. After treatment, rats were weighed and euthanized by I.P injection of pentobarbital (150 mg/kg).

Spleen-derived leukocytes culture (splenocytes)

Freshly isolated rat spleens were washed with sterile PBS (phosphate buffered saline, Lonza, USA), cleaned of any fat debris and weighed. Under sterile conditions, spleens were cut using razor blades into five or six large pieces further homogenized in PBS using the plunger of 2 mL syringes and filtered through a strainer (100- μ m Nylon, Falcon, USA) over a 50-mL tube. Filtrate was then centrifuged at 450g for 5-min. at room temperature (RT). The pellet was gently re-suspended in 3 mL ammonium chloride potassium (ACK) erythrocyte lysis buffer (0.15M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH 7.2-7.4), mixed under gentle shaking for 3 min, re-centrifuged (450g, 5min, RT) and re-suspended with RPMI medium-1640 (Gibco, life technologies limited, UK) supplemented with L-glutamate (2 mM), penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 mg/mL) and 15% fetal bovine serum (FBS) (Gibco, Saint Aubin, France). Cells were counted, seeded at 3×10^6 cells/mL in T75 flasks and incubated in humidified incubator at 37 °C, with 5% CO₂ for 24 hours.

Isolation, quantification and characterization of splenocyte microvesicles

After 24 hours culture, supernatants were collected under sterile conditions and splenocytes were counted using Trypan blue to determine the number of surviving cells. Splenocytes and cell debris were discarded by centrifugation at 450g, 15min, RT. Supernatants were centrifuged twice at 14,000g, 60min, 4 °C and washed SMVs pelleted and concentrated in Hanks Balanced Salt Solution (HBSS, without phenol red, without Ca⁺² and Mg⁺², Lonza, Belgium) and were stored at 4°C for a maximum of 1 month. Prothrombinase assay was performed to quantify SMVs after their capture on Annexin-A5 coated micro-wells using microplate spectrophotometer in kinetic mode. Annexin-5 has high affinity for phosphatidylserine (PhtdSer) exposed at the surface of MVs. In this assay, PhtdSer is the rate-limiting factor of the generation of thrombin from prothrombin detected at 405 nm using a chromogenic substrate (PNAPEP-0216, cryopep, Montpellier, France). SMV concentration was referred to as nanomolar PhtdSer equivalent (nM phtdser), by reference to a standard curve constructed with synthetic vesicles with known concentration of PhtdSer (Hugel, Zobairi et al. 2004). The cell origin of SMVs was characterized by capturing SMVs onto biotinylated antibodies against leukocytes CDs before quantification by pro-thrombinase assay as described by (Delabranche, Boisrame-Helms et al. 2013) using the following IgGs : anti-CD45 for leukocyte common antigen, anti-CD3 for T lymphocyte population, anti-CD4 for T helper cells, anti-CD8b for T cytotoxic cells, anti-CD161a for natural killer cells, anti-CD25 for IL-2 receptor T cells and splenic dendritic cells, anti-CD31, mainly for endothelial cells, anti-CD11b/c for monocyte/macrophage and granulocyte, anti-CD11b for neutrophil. Biotinylated monoclonal antibodies were insolubilized onto the streptavidin-coated microtitration

plates and incubated with SMVs. All antibodies were purchased from BD Pharmingen, San Jose, USA. The MVs concentration was obtained by subtracting the OD values measured using an isotype control biotinylated IgG.

Primary coronary artery endothelial cell culture

Primary coronary artery endothelial cells (ECs) were prepared from left circumflex coronary arteries of pig hearts, collected from local slaughterhouse (COPVIAL, Holtzheim, France) as described by (Abbas, Jesel et al. 2017). Briefly, left circumflex coronary arteries were dissected out of freshly slaughtered pig hearts, cleaned of any adhesive conjunctive tissues and flushed with PBS without calcium to remove all the remaining blood. Cleaned coronary arteries were then treated with Collagenase type I (Gibco, life technologies corporation, USA) solution at 1 mg/ml prepared in MCDB-131 medium (Gibco, life technologies limited, UK), supplemented with streptomycin (100 U/ml), penicillin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM, all from Lonza, St Quentin en Yvelines, France) for 15 min at 37°C. ECs were then extracted into 50-mL falcon tube by circular massage of arteries with frequent flushing with medium. Collected medium with ECs was then centrifuged (450g, 5min, RT), supernatant was discarded and cells were re-suspended with complete MCDB-131 medium supplemented with 15% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM). ECs per three different coronary arteries were cultured in adherent T25 flask in humidified incubator at 37 °C, with 5% CO₂. After 6 hours, cells were washed with PBS to remove any non-adherent cells and fresh complete medium was added (P0 ECs). Thereafter, first passage ECs (P1ECs) were grown for 72 hours with medium changed every 48 hours.

Treatment of ECs by splenocyte MVs

Following trypsinization (Trypsin, Gibco, Life Technologies SAS, St Aubin, France) P1ECs were seeded in 6-well plate at 65-75% confluency and incubated with either SMVs (10 or 30nM PhtdSer eq.) for 6 or 48 hours, H₂O₂ (100 µM for senescence for 24 h or 300 µM for apoptosis for 1 h) or, in some experiments, pharmacological modulators were added prior to SMVs e.g. NADPH oxidase inhibitor (VAS-2870, 5 µM), a cyclooxygenase inhibitor (indomethacin, 30 µM), mitochondrial combined inhibitors (myxothiazol, potassium cyanide and rotenone, 1 µM each) for 30 min or AT1R inhibitor (Losartan, 1 µM) for 1 h before application of SMVs.

Measurement of Senescence-Associated β-galactosidase activity

The fluorogenic cell permeable substrate C12FDG (5-dodecanoylaminofluorescein Di-β-D-galactopyranoside, Invitrogen, ThermoFisher, Illkirch, France) was used to determine the senescence-associated β-galactosidase activity (SA-β-gal) in treated ECs by flow cytometry, as described previously (Abbas, Jesel et al. 2017). Briefly, ECs were alkalinized (pH raised to 6) with chloroquine

(300 μ M) for 1 h, followed by 1 h incubation with C12FDG (33 μ M). Cells were thereafter washed with ice-cold PBS, harvested with trypsin and freshly analyzed using the CellQuest software (FACScan, Becton Dickinson, San Jose, CA, USA). Light scattering parameters were set to eliminate dead cells and subcellular debris. The green C12-fluorescein signal was measured and SA- β -gal activity was estimated using the mean fluorescence intensity (MFI) of the population. Auto-fluorescence gains were determined in unlabeled cells and set at the first logarithmic decade.

Western blot analysis

After SMV treatment, ECs were washed with cold PBS, and proteins were extracted by RIPA lysis buffer (20mMTris/HCl , 150mM NaCl, 1mM Na₃VO₄, 10mM sodium pyrophosphate , 0.01mM okadaic acid, 20mM, a tablet of protease inhibitor (Complete, Roche), and 1% Triton X-100 (Euromedex, Souffelweyershem, France). Proteins (15 μ g or 20 μ g) were separated by 10 % or 12 % SDS-PAGE at 100 V for 2 hours and further electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane (GE Healthcare, VWR, Fontenay-sous-Bois, France) at 100 V for 2 hours. Membrane non- specific binding sites were blocked in Tris-buffered saline (TBS) solution containing 5% BSA (Bovine Serum Albumin) and 0.1% Tween-20 (Euromedex) for 1 h at room temperature. Proteins of interest were probed with specific primary antibodies in blocking solution, anti-VCAM-1, anti-ICAM-1, anti-phosphorylated NF- κ B, anti-COX-1 & anti- COX-2 (1:1000 dilution, abcam, UK), anti-TF (1:1000, Sekisui Diagnostics, Germany), anti-eNOS (1:1000, BD Biosciences, France), anti-p21 & anti-p53 (1:1000; Santa Cruz Biotechnology, USA), anti-p16 & anti-ACE (1:500, abbiotec, USA), AT1R (1:500 dilution, Santa Cruz Biotechnology, USA), at 4°C overnight. Membranes were washed three times with TBS-T (TBS-Tween, Euromedex) and incubated with peroxidase-labelled secondary antibodies (anti-rabbit, anti-mouse, 3:10,000 dilution, cell signaling technology, USA) for 60 min, at room temperature. Pre-stained markers (protein ladder, Euromedex) were used for molecular mass determination. Immunostaining was revealed by chemiluminescence solution (ECL, Bio-Rad laboratories, USA). The chemiluminescence signal was recorded with ImageQuant LAS4000 system (GE Healthcare Europe GmbH, Velizy-Villacoublay, France) and analyzed using ImageQuant TL software (version 8.1, GE Healthcare). Quantitative normalization with respect to housekeeping protein (anti-GAPDH, 1:1000 dilution, abcam, UK) was done for each protein of interest.

Characterization of oxidative stress in spleen tissue and ECs

Oxidative stress in spleen tissue or ECs was determined using a redox-sensitive fluorescent probe, DiHydroEthidium (DHE). Spleen tissues, embedded in histo-molds containing Tissue-Tek optimum cutting temperature (OCT) compound (Sakura 4583, Leiden, Netherlands) and snap-frozen in liquid nitrogen, were cryosectioned (25 μ m) and mounted on slides. For ECs, PIECs were seeded in labtek chambers (millicell EZ slide, Ireland) and incubated with 10nM PhtdSer eq. SMVs in humidified

incubator at 37 °C, with 5% CO₂ for 6 hours. Slides (spleen tissue and ECs) were then incubated with DHE (2.5 μM) for 30min, at 37°C in a light protected humidified chamber. Slides were washed three times with PBS and mounted under coverslip using fluorescence mounting medium (DAKO, USA), dried in dark and analyzed with confocal laser-scanning microscope (Leica SP2 UV DM IRBE; leica, Heidelberg, Germany) with a 20X magnification lens. Level of oxidative stress was quantified by using Image J software.

To identify the sources of oxidative stress, ECs were pre-treated for 30 min, with NADPH oxidase inhibitor (5 μM VAS-2870), a cyclooxygenase inhibitor (30 μM indomethacin), antioxidant (1mM N-acetylcysteine), or a mixture of mitochondrial inhibitors of the respiratory chain (myxothiazol, potassium cyanide and rotenone, 1 μM each) prior addition of DHE alone (tissue) or combined with SMV (ECs).

Measurement of endothelial apoptosis

Apoptosis was measured by flow cytometry using double labeling with Annexin-5 and propidium iodide. ECs treated with SMVs (10 nM and 30 nM) for 48 h were incubated with fluorescent Annexin-5 (5 μg/mL ImmunoTools, Friesoythe, Germany) and 2.5 μg/ml propidium iodide (Miltenyi Biotec SAS, Paris, France) in dark for 15 min, at room temperature. The cell population with early apoptosis was defined as Annexin-5 stained and PI unlabeled. Fluorescence acquisition was performed by Guava EasyCyte Plus FlowCytometry System (Millipore). Analysis was constructed from a minimum of 2000 events.

Statistical Analysis

Data are expressed as mean ± standard error mean (S.E.M) for n different experiments and analyzed by Graphpad Prism 5. Statistical variance between two groups was determined by applying unpaired-T test. Group differences were considered statistically significant at P < 0.05.

Results

Microvesicle shedding from primary splenocytes increases with age and is associated with enhanced pro-senescent potential

Using freshly harvested rat spleens from young (Y), middle-aged (M) and old (O) rats as a convenient source of primary spleen-derived leukocytes thereafter termed splenocytes, we measured the microvesicle (SMVs) shedding in their supernatant after 24h culture as well as splenocyte survival. The proportion of surviving splenocytes was reduced by half in old compared to young splenocytes (figure 1c) while SMVs shedding significantly increased with age (3.3 folds in O, 1.7 folds in M vs. Y, p < 0.01) (figure 1a). The pro-senescent potential of SMVs was measured by Senescence-associated β

galactosidase activity (SA- β -gal activity) after 48h incubation of 10 nM SMVs with young primary coronary endothelial cells at passage 1 (P1ECs). SA- β -gal activity was detected by the conversion of its fluorogenic substrate C12FDG (Debacq-Chainiaux, Erusalimsky et al. 2009). Only SMVs from old and middle-aged rats induced significant premature endothelial senescence (O: 2 folds, M: 1.5 folds vs untreated P1 ECs, $p < 0.01$). Compared to untreated P1 ECs, SMVs from young rats had little or no effect on premature endothelial senescence (figure 1b). Correspondingly and on line with other reports, (Luceri, Bigagli et al. 2017) we also identified an age-related ROS formation in the original spleen tissues with a significant accumulation of ROS in middle-aged rats by DHE staining (M: 1959.91 A.U. vs Y: 1492.75 A.U., figure 2). Altogether, data suggest a progressive alteration of splenocytes with ageing that is associated with redox-sensitive mechanisms and leads to an increased shedding of pro-senescent SMVs. Since omega-3 PUFA were reported to incorporate in rafts and modulate the plasma membrane lateral organization of lymphocytes (Fan, Fuentes et al. 2018), we investigated the impact of an EPA:DHA short-term intake on SMVs and first focused on splenocytes freshly isolated from middle-aged rats.

EPA:DHA 6:1 intake reduces oxidative stress in middle-aged rats with no incidence on spleen or total bodyweight

Middle-aged rats were given 500 mg/kg/d EPA:DHA 6:1, EPA:DHA 1:1, or corn oil or tap water for 07 days before spleen harvest and splenocyte isolation. No significant changes were observed in weight of spleen or total bodyweight of rats after 7 days of treatment with either corn oil or omega-3 (figure 3a-b). Treatment with EPA:DHA 6:1, but not corn oil or EPA:DHA 1:1, significantly lowered the accumulation of age-related oxidative stress by 19.5% in spleen tissues of middle-aged rats (EPA:DHA 6:1 : 1575.9 vs control : 1959.91 A.U., $p < 0.05$, figure 3c).

EPA:DHA 6:1 intake by middle-aged rats prevents the splenocyte shedding

Intake of omega-3 EPA:DHA 6:1 and EPA:DHA 1:1, but not of corn oil, significantly reduced the shedding of procoagulant SMVs measured by prothrombinase assay in the splenocyte supernatants, by 24% and 14%, respectively, suggesting that omega-3 significantly limit plasma membrane remodeling *in situ*. (figure 4a) The shedding of neutrophils, T lymphocytes, natural killer cells, monocytes/macrophages and granulocytes, was approximately reduced by half, reaching ~ 60% decrease in MV release from natural killers and T lymphocytes (figure 4b and table 1), thereby suggesting that EPA:DHA 6:1 strongly targets cells of the innate immune system. By comparison, the low CD31⁺SMVs shedding remained statistically unchanged, confirming that, in this model, immune cells are the main contributors to MV release and prime targets of omega-3 PUFA.

EPA:DHA 6:1 intake blunts the endothelial pro-senescent impact of splenocyte-derived MVs

Young primary coronary endothelial cells (P1ECs) were incubated for 48 h with 10 nM PhtSer eq. of washed SMVs isolated from either one of the 4 different rat subsets (SMV_{CTL}, SMV_{C0}, SMV_{1:1}, SMV_{6:1}). SMV_{CTL} significantly increased the SA- β -gal activity by 55% in P1ECs ($p < 0.05$, SMV_{CTL} vs. P1ECs), reaching values similar to those induced by H₂O₂, a known inducer of premature endothelial senescence (figure 5a). SMV_{CTL} also induced significant up-regulation of senescence markers p53 (1.6 fold) and down-stream p21 and p16 (1.7 and 2 fold, respectively), thereby confirming the pro-senescent potential of SMVs detected by SA- β -gal activity (figures 5b, and above). EPA:DHA 6:1, but not corn oil or EPA:DHA 1:1, abolished the pro-senescent effect of SMVs by SA- β -gal activity (17.7 A.U. vs 10.8 A.U., $p < 0.05$, SMV_{6:1} vs. SMV_{CTL}) an observation that was also confirmed by assessment of the expression of p53, p21 and p16 senescence protein markers.

In addition, the fact that splenocyte supernatants depleted of SMV_{CTL} by high-speed centrifugation, did not induce significant SA- β -gal activity, confirmed that SMV_{CTL} were the true inducers of premature senescence in the cell medium. Of note, none of the SMV subsets affected the degree of apoptosis in P1ECs, confirming that SMVs specifically deliver a pro-senescent signal (figure 5a, 5c).

EPA:DHA 6:1 intake by middle-aged rats prevents the endothelial pro-inflammatory and pro-thrombotic responses induced by SMVs

We previously had reported that endothelial senescence is characterized by a pro-inflammatory and procoagulant phenotype (Abbas, Jesel et al. 2017), we therefore examined the eventual protection by omega-3 PUFA intake using the above SMV-induced senescence model. ECs were treated by 10 nM washed SMV_{CTL}, SMV_{C0}, SMV_{1:1}, or SMV_{6:1} for 6 hours or 48 hours and pro-inflammatory and pro-coagulant protein markers were assessed by western blot. After 48 h incubation, SMV_{CTL} induced the up-regulation of COX-2, but not COX-1 by 3 folds, ICAM-1 and TF by 1.5 fold, VCAM-1 by 1.6 fold (figure 6). Interestingly, a significant 1.9 fold rise in phosphorylated NF- κ B was detectable as early as 6 h after the addition of SMVs, pointing at a pivotal role of the inflammatory signaling in the development of senescence. Conversely, the expression of pro-inflammatory and procoagulant makers was abolished in the presence of SMV_{6:1} but not corn oil or SMV_{1:1}, indicating a specific effect of EPA:DHA 6:1 ($p < 0.05$ SMV_{6:1} vs. SMV_{CTL}, figure 6).

EPA:DHA 6:1 intake by middle-aged rats limits oxidative stress in SMVs-treated ECs

Since ROS early accumulated in middle-aged spleen and because oxidative stress contributes to endothelial senescence via reduced NO formation (Khemais-Benkhiat, Idris-Khodja et al. 2016), we further examined whether 10 nM SMVs could act as early contributors to oxidative stress in ECs and whether omega 3 intake would be beneficial. After 6 hours incubation, SMV_{CTL} significantly increased ROS accumulation by 2 folds in young P1ECs (SMV_{CTL}: 2673.65 A.U. vs. untreated P1ECs: 1315.37 A.U, $p < 0.01$). Consistent with the whole spleen tissue measurements (figure 7), P1ECs treated with

SMV_{6:1}, but not with SMV_{CO} or SMV_{1:1}, showed significantly lowered amounts of ROS, suggesting that omega-3 EPA:DHA 6:1 intake prevents the SMVs-induced oxidative stress. Pharmacological inhibition by Indomethacin, VAS-2870 and the mitochondrial respiration complex inhibitors significantly blunted the SMVs-induced formation of ROS, indicating that COXs, NADPH oxidase and mitochondria contribute to the SMVs-induced oxidative stress. Consistent with the observation, western blot analysis indicated that SMV_{CTL} down-regulated eNOS by 30% in PIECs after 48 h (SMV_{CTL} vs. untreated ECs, $p < 0.05$, figure 7). Altogether, omega-3 EPA:DHA 6:1, but not of corn oil or omega-3 EPA:DHA 1:1, was a cytoprotective treatment against SMVs-induced endothelial dysfunction.

EPA:DHA 6:1 intake by middle-aged rats reduces the SMVs-mediated activation of local angiotensin system in ECs

Because the local angiotensin system contributes to the induction of endothelial senescence and is associated with increased endothelial expression of the angiotensin converting enzyme (ACE) and its angiotensin type-1 receptors (AT1R) (Khemais-Benkhiat, Idris-Khodja et al. 2016), we investigated the impact of omega-3 intake. The expression of ACE and AT1-R was significantly up-regulated in PIECs treated with SMV_{CTL} for 48h (SMV_{CTL} vs. untreated PIEC, $p < 0.05$, figure 8a). Only SMV_{6:1} ingestion, reduced the ACE and AT1-R expression by 44% and 45%, respectively, suggesting that EPA:DHA 6:1, but not corn oil or EPA:DHA 1:1, prevents the SMVs-mediated activation of the local angiotensin system. Furthermore, the SMV_{CTL}-induced SA- β gal activity was reduced to baseline in the presence of Losartan, an AT1R antagonist, thereby indicating the pivotal role of the angiotensin system in the SMV-induced premature senescence (figure 8b).

Altogether, our data indicate that SMVs from middle-aged rats are endothelial pro-senescent effectors acting via redox-sensitive pathways that alter the endothelial protective features and can be abolished by a seven days omega-3 EPA:DHA 6:1 intake.

EPA:DHA 6:1 intake is beneficial to old rats

Because the shedding of SMVs was significantly higher in old rats, we also verified that a 14 days intake of only EPA:DHA 6:1, but not of corn oil, significantly reduced the ability of old splenocytes to shed SMVs. EPA:DHA 6:1 significantly reduced the shedding of SMVs by 39% while a symmetrical 22% splenocyte survival was observed as compared to treated rats ($p < 0.05$ O_{6:1} vs. O_{CTL}, figure 9a-b). Conversely, a 14 days corn oil ingestion had an inverse effect with a 25% fold increase in SMV release (figure 9a) and a reduced splenocyte survival (figure 9b). Using the SMV-induced senescence model, we observed a significant 35% reduction of the PICEs SA- β -gal activity in the presence of SMV_{6:1} (figure 9c).

Discussion

The present findings indicate that age is a strong and progressive trigger for the shedding of pro-senescent spleen-derived leukocytes microvesicles (SMVs) that prompt premature senescence in primary coronary endothelial cells, on line with our previous observation (El Habhab, Abbas et al. 2017). Senescence was characterized by SA- β -gal activity, pro-oxidative, pro-inflammatory and procoagulant and pro-atherogenic responses. Interestingly, the release of pro-senescent MVs was a characteristic feature of both old and middle-aged rat splenocytes, thereby suggesting the benefit of an early pharmacological control. We therefore investigated the possibility that a short-term omega-3 intake prevents the age-related SMV effects in middle-aged rats. The intake dose was equivalent to 5.67 g/day/70 kg body weight in humans (Reagan-Shaw, Nihal et al. 2008), on line with the 0.18 to 10 g/day ranges given in clinical studies (Calder 2007, Delgado-Lista, Perez-Martinez et al. 2012, Barden, Mas et al. 2014, Enns, Yeganeh et al. 2014).

The ratio of EPA:DHA Omega 3 is critical in the prevention of the SMV-induced premature senescence.

Using an original SMV-endothelial cross-talk model, we compared two different formulations of omega 3 and clearly demonstrate that only the EPA:DHA 6:1 formulation has strong anti-senescent effects. Omega-3 EPA:DHA 6:1 limited the release of splenocyte SMVs and significantly blunted their pro-senescent properties whereas EPA:DHA 1:1 had no effect. This observation is consistent with a previous ex-vivo study of healthy pig coronary arteries showing that the EPA:DHA 6:1 formulation is an optimal ratio to induce endothelium-dependent vaso-relaxation, predominantly involving eNOS and hence restoring NO availability (Zgheel, Alhosin et al. 2014). Interestingly, in hypertensive rats EPA:DHA 6:1 chronic intake limited hypertension and was also associated to an improved endothelial-dependent vaso-relaxation measured in mesenteric arteries (Niazi, Silva et al. 2017). Similarly, our data also indicate that the oxidative stress was specifically reduced by EPA:DHA 6:1 in spleen tissues from which SMVs were harvested, thereby confirming the cell organ targeting.

EPA:DHA 6:1 protection against age-related SMV-induced endothelial senescence and inflammaging.

As previously reported, inflammaging, characterized by the impairment of the immune system and chronic low-grade inflammation, contributes to persistent tissue damages altering both innate and adaptative immune cells. Accumulation of senescent cells in tissues also impacts tissue repair and amplifies the senescence associated secretory syndrome (SASP) favoring pro-inflammatory and pro-oxidative loops while soluble mediators as cytokines act as paracrine effectors. (Figueira, Fernandes et al. 2016).

Endothelial-derived and splenocyte-derived MVs were demonstrated pro-senescent autocrine (Burger, Kwart et al. 2012, Abbas, Jesel et al. 2017) and paracrine (El Habhab, Abbas et al. 2017) effectors of premature endothelial senescence and elevated circulating levels of endothelial and leukocyte-derived MVs are characteristic features of cardiovascular diseases and chronic inflammatory disorders like type 2 diabetes (Ridger, Boulanger et al. 2017, Pollet, Conrard et al. 2018).

In the present study, EPA:DHA6:1 omega 3 intake modifies the pattern of the cellular origin of spleen leucocyte MVs and strongly reduces the spontaneous release of MVs from the innate and adaptive immune systems. Furthermore, in middle-aged rats, the pro-senescent features of SMVs leading to coronary endothelial senescence are abolished by a short-term EPA:DHA6:1 intake, that completely blunts the endothelial dysfunction and SMV-borne pro-oxidative, pro-coagulant and pro-inflammatory potentials, most likely by reducing oxidative stress, enhancing NO bioavailability and preventing the activation of the local angiotensin system. In addition, the rapid blunting of the inflammatory stress via an early targeting of the NF- κ B activation would also contribute to prevent the up-regulation of TF, an early responsive gene with high thrombogenic potential (Grover and Mackman 2018).

Since endothelial senescence is promoted by flow disturbance favoring MV-endothelial interactions and early detected at coronary branches, it is tempting to speculate that EPA:DHA6:1 also blunts the pro-senescent signaling of LMVs at such endothelial sites prone to athero-thrombosis, thereby limiting the low grade but sustained endothelial inflammation in middle-aged and old rats and deferring the progression of cardiovascular events with age.

Ageing-related endothelial dysfunction and targets of EPA:DHA 6:1 Omega-3

We could identify NADPH oxidase cyclooxygenases (COXs) and the mitochondrial respiratory chain as the endothelial main sources of ROS accumulation during age-related SMV induced endothelial senescence. In a previous report from the team, EPA:DHA 6:1 was shown a potent inducer of NO formation targeting endothelial Src/PI3-kinase/Akt and MAPkinase pathways and eNOS activation (Zghele, Alhosin et al. 2014). Nevertheless, the EPA:DHA 6:1 direct targets remain poorly characterized, despite clear anti-inflammatory effects (Calder 2010). In the splenocyte membrane, non-exclusive mechanisms would be (i) the modification of the caveole lipid microenvironment (Li, Zhang et al. 2007) and/or membrane composition possibly leading to the accelerated endocytosis of TF, ACE or AT1, (ii) the modification of the lateral membrane proteo-lipidic domains (rafts) involved in pro-inflammatory signaling favoring cytokine release (Li, Wang et al. 2014), thereby blunting the downstream up-regulation of TF, ICAM, VCAM, or (iii) the sorting of noxious active proteins exported in the MV membrane. To the best of our knowledge, the impact of EPA:DHA on lipid transporters across the membrane bilayer remains to be deciphered.

Of note, the EPA:DHA 6:1 intake was not associated with any sign of bleeding nor had any effect on SMV-induced endothelial senescence in healthy young rats, confirming the absence of safety issues

after short-term intake and indicating that ageing cells are specific targets of treatment. An eventual explanation is the reported enhanced proportion of EPA in cells from old individuals. (Calder 2007).

Relevance to cardiovascular diseases

Angiotensin II is an inducer of premature endothelial senescence cells (Shan, Guo et al. 2014). Although we demonstrated that EPA:DHA 6:1 protection against SMV-induced senescence was blunted after ECs pretreatment with Losartan, an inhibitor of the AT1 receptor, the beneficial effect of EPA:DHA 6:1 and the ECs cytoprotection against SMV-induced premature senescence remains to be established in young and older hypertensive rats. Indeed, a spleen-cardiac axis was recently proposed, pointing at the role of immune cells and chronic low-grade inflammatory state in cardiovascular diseases. In addition, in aged patients with chronic heart failure, splenomegaly and a higher rate of peripheral monocytes were indicative of non-responders to resynchronization therapy and higher prevalence of new hospitalization due to disease progression. (Fujinami, Kondo et al. 2018). Furthermore, in a mouse model of prolonged cardiac ischemic insult, a cardio HMGB1-splenic RAGE signaling axis was evidenced during the infarct reperfusion, showing that cardiac tissues release mediators contributing to infarct exacerbation through the activation of splenic neutrophil in a FPR-1-dependent manner and their migration to the heart (Tian, Pan et al. 2016).

Conclusion

Ageing is associated with increased shedding of leukocyte-MVs, which are strong promoters of age-associated endothelial dysfunction due to their pro-senescent, pro-inflammatory and pro-atherothrombotic properties. Short-term ingestion of the omega-3 optimized formulation EPA:DHA 6:1 not only reduces the shedding of leukocyte-MVs but also protects the endothelium against their noxious properties, most probably by reducing oxidative stress, preserving eNOS and preventing the activation of the local angiotensin system. Moreover, spleen appears to be a convenient and rich source of leukocyte-MVs to assess their in vivo pharmacological control. The data further suggest that omega-3 EPA:DHA 6:1 may help to prevent or delay the age-related endothelial dysfunction.

Acknowledgments

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Figures and table legends

Table 1. Omega EPA:DHA 6:1 intake modifies the pattern of cell origin of SMVs

*SMV cell origin was determined after capture onto specific antibodies and quantification by prothrombinase assay. *: significant variation compared to untreated rat *:p<0.05*

Figure 1: Ageing enhances the release of pro-senescent MVs from splenocytes. *MVs were measured in the supernatant of freshly isolated primary splenocytes after 24 h culture by prothrombinase assay (n=5) simultaneously to splenocyte survival (n=4). Their pro-senescent ability was quantified by SA- β -GAL activity, measured in young primary coronary endothelial cells at passage 1 (PIECs) after incubation with 10nM SMVs from young (SMV_y), middle-aged (SMV_M) and old (SMV_O) rats for 48 h (n=4). *: p < 0.05, **: p < 0.01*

Figure 2: ROS accumulation in the ageing spleen.

*ROS were measured in snap-frozen samples by fluorescence microscopy using DHE. Each analysis is the mean of measurements performed in 4 individuals (4 sections/individual). *: p < 0.05*

Figure 3: Omega-3 EPA:DHA 6:1 intake prevents the spleen ROS accumulation in middle-aged rats. *Rats were given either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) or tap water (control) for 7 days. 3a-b: measurement of spleen (n=7) and body weight (n=9). 3c: ROS spleen accumulation measured after DHE staining by fluorescence microscopy in snap-frozen tissue samples of 4 individuals (4 sections/individual). 3d: assessment of ROS sources after incubation of each section with pharmacological inhibitors for 30 min before the addition of DHE.*

*IND: indomethacin, NAC: N-acetyl cysteine, VAS: VAS2870, NADPH oxidase inhibitor, MIT: Mitochondrial respiratory chain inhibitors, Neg DHE: auto-fluorescence level, control: absence of pharmacological inhibitor. *: p < 0.05*

Figure 4: Omega EPA:DHA 6:1 intake reduces SMVs shedding and modifies the pattern of their origin.

4a: *SMVs were measured by prothrombinase assay in the supernatant of freshly isolated splenocytes from middle-aged rat receiving a 7 day-treatment by either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) or tap water (control)(n=6). 4b: the SMV cell origin was determined after capture onto specific antibodies and measurement by prothrombinase assay (n=3). 4c: after 24 h culture survival of splenocytes freshly isolated from treated and untreated animals (n=4). *: p < 0.05, **: p < 0.01*

Figure 5: Omega EPA:DHA 6:1 14 day-intake protects the primary coronary endothelial cells against pro-senescent SMVs from middle-aged rats. Ten nM SMVs isolated from middle-aged rats treated by either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) or tap water (control) were incubated for 48 h with primary young endothelial cells (PIECs). **5a:** SA- β -GAL activity was measured in ECs by flow cytometry and compared to that of untreated PIECs or H₂O₂ treated ECs. Supernatant depleted of SMVs (SN-SMV) from untreated rats were also incubated with PIECs (n=4). **5b:** Western blot analysis of senescent markers p53 (n=5) and downstream p21 (n=3), p16 (n=7) in SMV-treated PIECs after 48 h. **5c:** measurement of apoptosis in SMV-treated ECs. ECs were treated with 10 nM and 30 nM SMVs for 48 h or H₂O₂ for 1 h as control of apoptosis ECs induction (n=4). * : $p < 0.05$, ** : $p < 0.01$

Figure 6: Omega EPA:DHA 6:1 intake prevents pro-inflammatory, pro-thrombotic responses of young primary ECs in response to SMVs from middle-aged rats. ECs were incubated for 48 h with 10 nM SMVs from middle-aged rats treated by either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) or tap water (control), for 48 h except for the measurement of NF- κ B expression that was performed after 6 h (n=4). * : $p < 0.05$, ** : $p < 0.01$

Figure 7: Omega EPA:DHA 6:1 intake by middle-aged rats prevents young ECs from SMV-induced oxidative stress and endothelial dysfunction. PIECs DHE staining was performed after 6 h incubation with 10 nM SMVs (n=4) and eNOS expression was assessed after 48 h of incubation (n=3). * : $p < 0.05$, ** : $p < 0.01$

Figure 8: AT1 receptors mediate SMV-induced endothelial senescence and omega EPA:DHA 6:1 intake by middle-aged rats prevents the SMV-induced up-regulation of ACE and AT1-R. SMVs were isolated from middle-aged rats that were treated by either EPA:DHA 6:1, EPA:DHA 1:1, Corn oil (CO) or tap water (control) for 7 days. **8a:** PIECs were incubated with 10 nM SMVs for 48 h and up-regulation of ACE (n=3) and AT1-R (n=5) was measured. **8b:** Prior to incubation of PIECs with SMVs for 48 h, PIECs were treated with Losartan for 30 min, and SA- β -GAL activity was performed (n=4). * : $p < 0.05$, ** : $p < 0.01$

Figure 9: Omega EPA:DHA 6:1 intake by old rats for 14 days counteracts the shedding of pro-senescent SMVs. old rats were given either omega EPA:DHA 6:1 (O_{6:1}), Corn oil (O_{CO}) or tap water (O_{CTL}) for 2 weeks before assessment of the SMVs shedding (n=5) (**9a**), splenocyte survival (n=5) (**9b**) and SMV-induced endothelial senescence assessed by SA- β -GAL activity (n=3) (**9c**). * : $p < 0.05$, ** : $p < 0.01$

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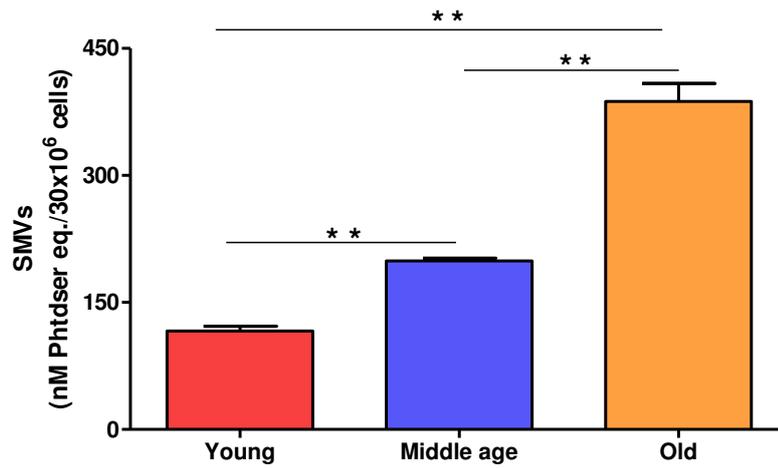
Table 1

SMVs Cell origin	SMVs Cell marker	Control	Omega 6:1	Omega 1:1	Corn oil
Total SMVs	AV ₅ ⁺	269 ± 11	192 ± 8	235 ± 8	279 ± 13
Monocytes/macrophages, granulocytes	CD11b/c ⁺	65 ± 5	39 ± 7*	54 ± 6	70 ± 5
T-helper cells	CD4 ⁺	70 ± 7	54 ± 13	72 ± 8	90 ± 13
Leukocyte common antigen	CD45 ⁺	60 ± 2	47 ± 4	65 ± 2.2	81 ± 2.6
Natural killer cells	CD161 ⁺	19.7 ± 1.3	8.2 ± 1.3*	20.9 ± 2	33 ± 3
Endothelial cells	CD31 ⁺	63 ± 5	53.5 ± 2.2	66 ± 10	89 ± 10
IL-2 receptor T-cells and splenic dendritic cells	CD25 ⁺	12.7 ± 1.3	6.5 ± 1*	9 ± 3	7 ± 2.2
Neutrophils	CD11b ⁺	37.8 ± 2.2	22 ± 2.7*	26 ± 4	41 ± 10
T-lymphocytes	CD3 ⁺	6.5 ± 0.8	2.8 ± 0.6*	4.8 ± 0.8	5.5 ± 0.5
Naïve T-cells	CD45RA ⁺	180 ± 20	114 ± 4.5*	125 ± 22	159 ± 10

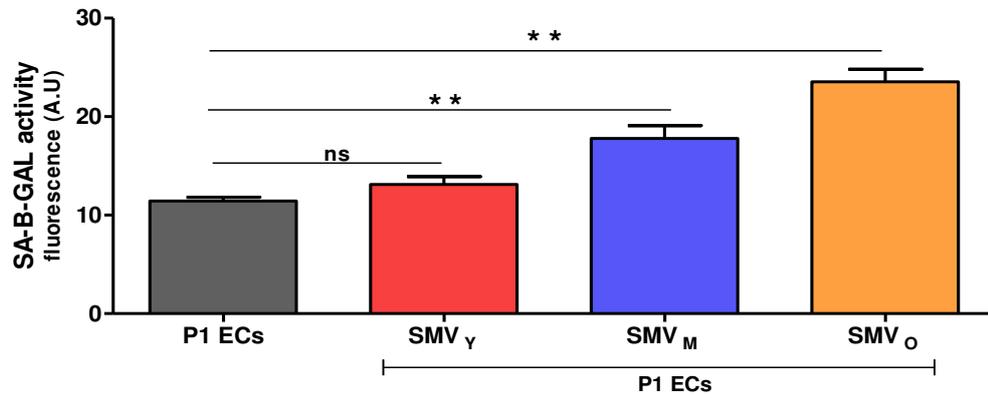
Figures

Figure 1

1a



1b



1c

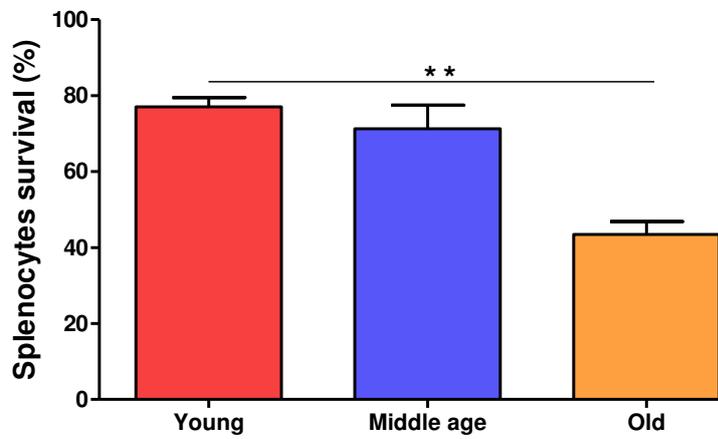


Figure 2

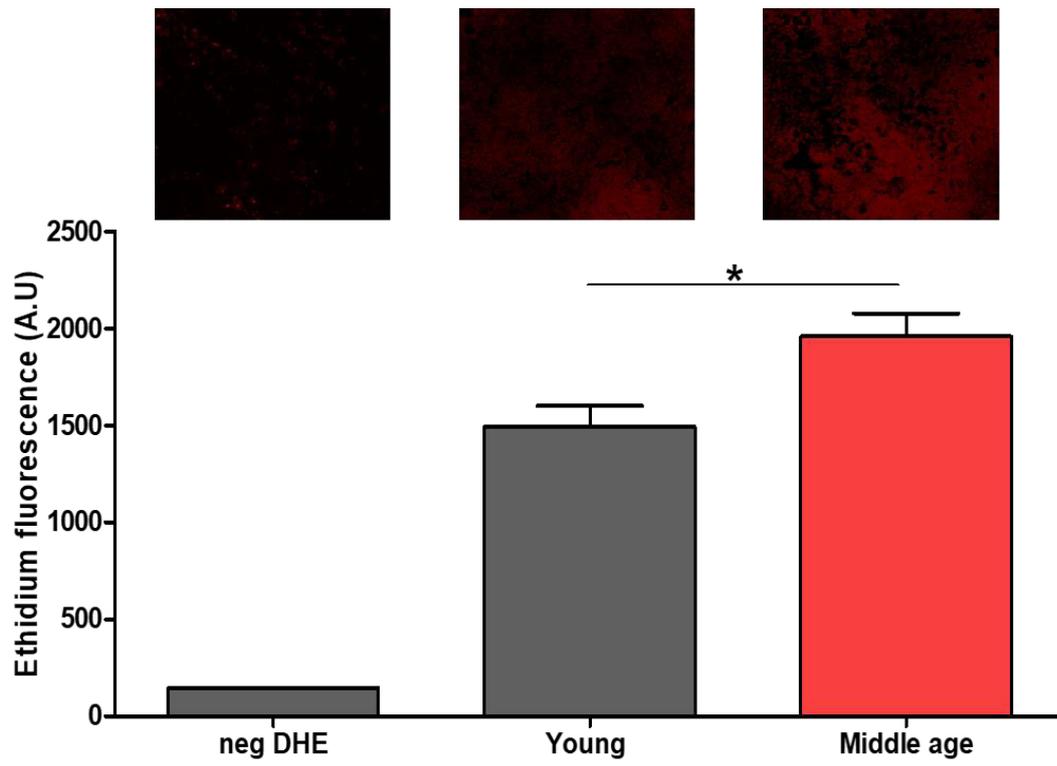


Figure 3

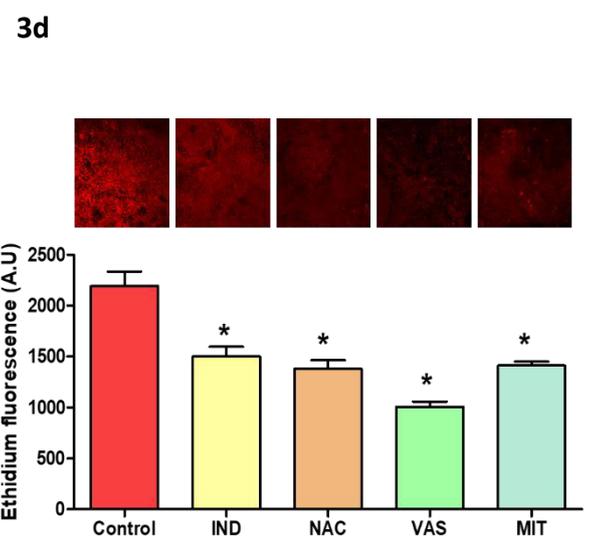
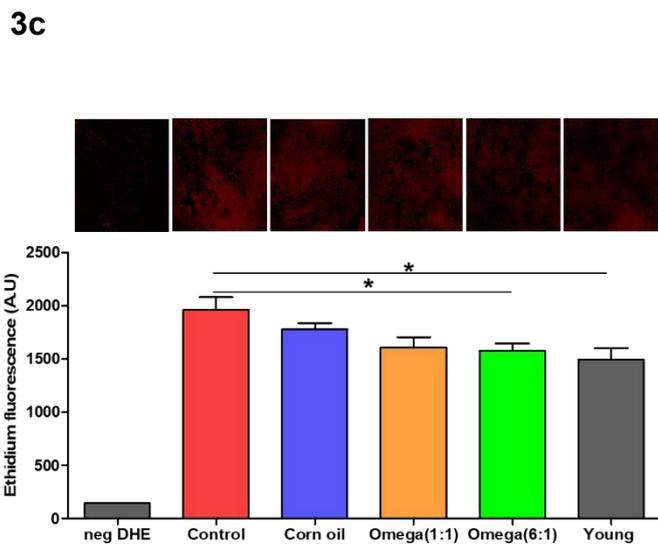
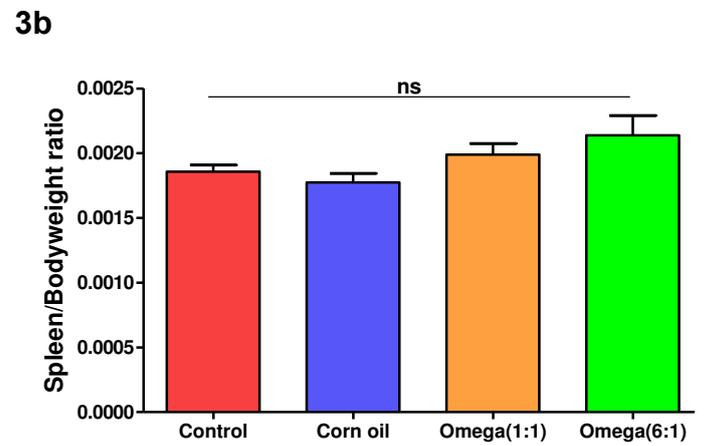
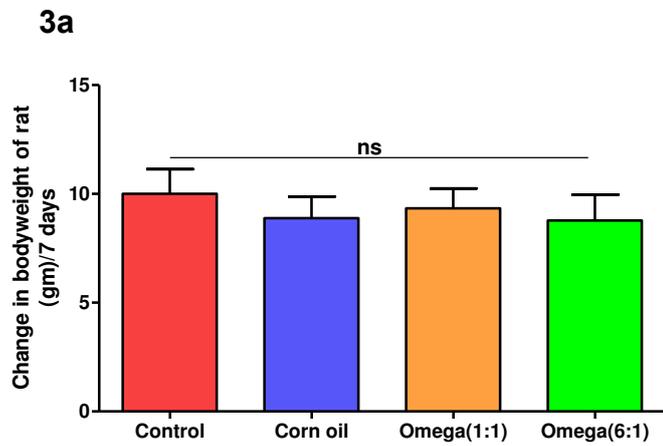
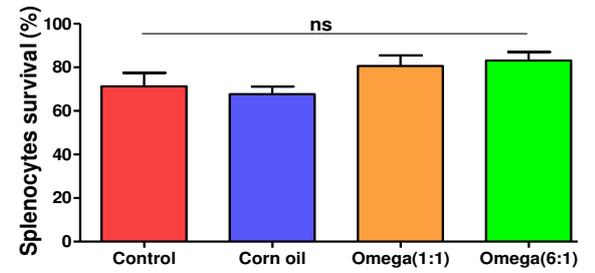
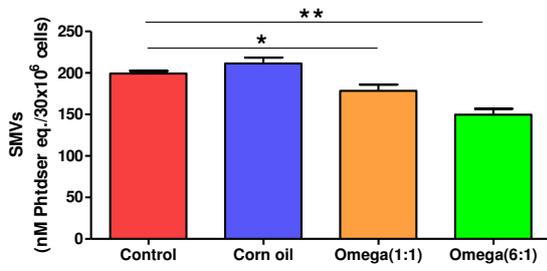


Figure 4

4a



4b

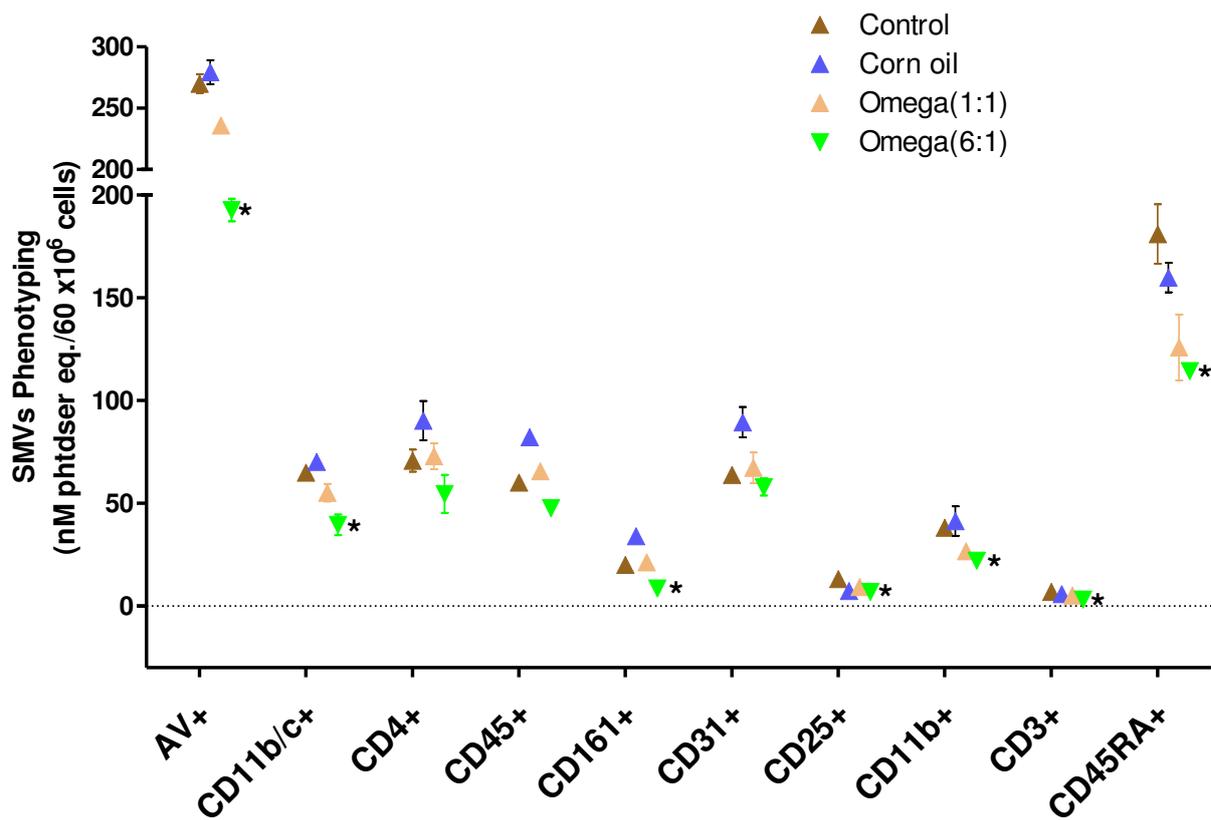
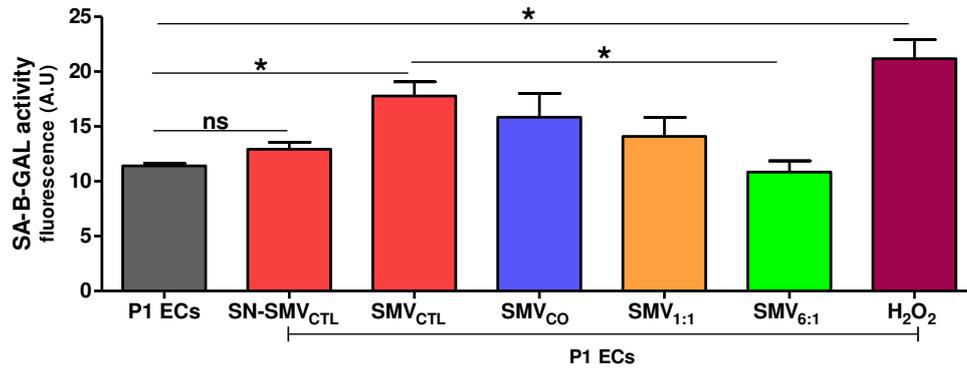
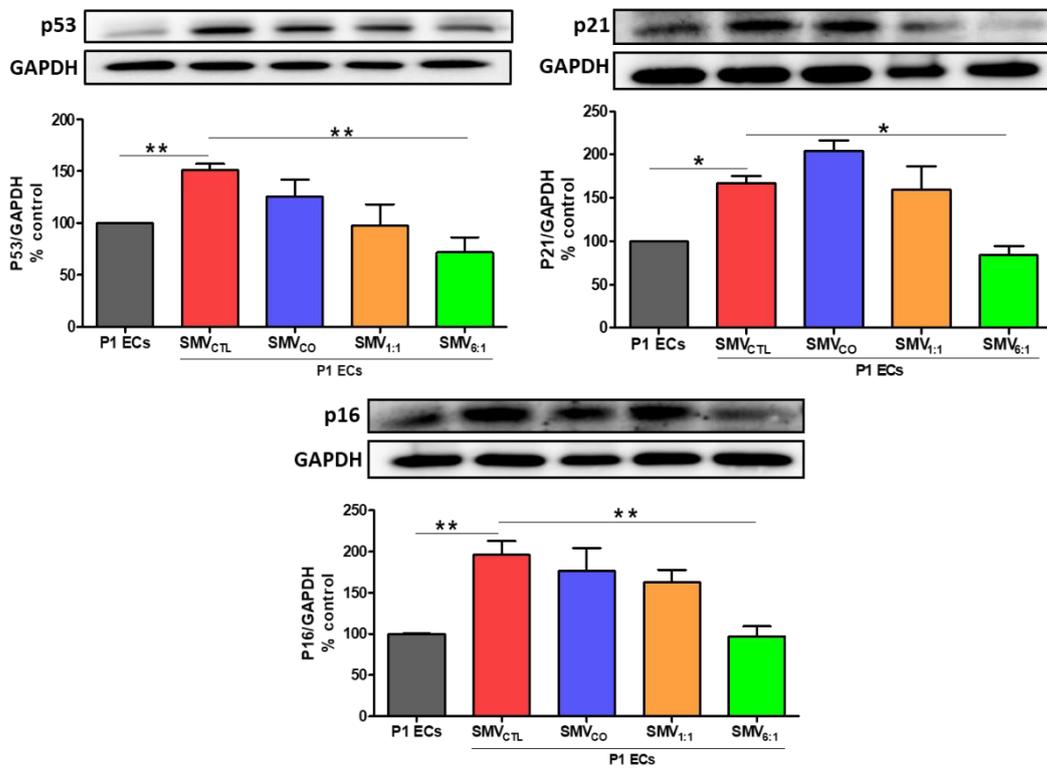


Figure 5

5a



5b



5c

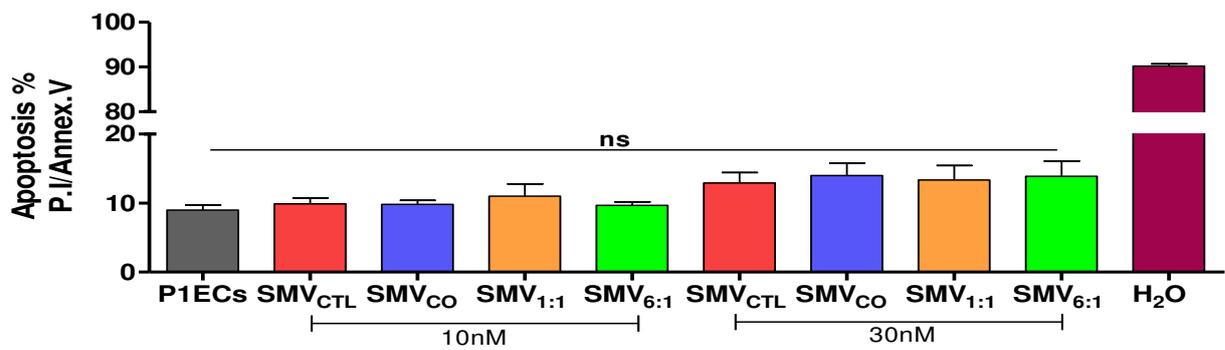


Figure 6

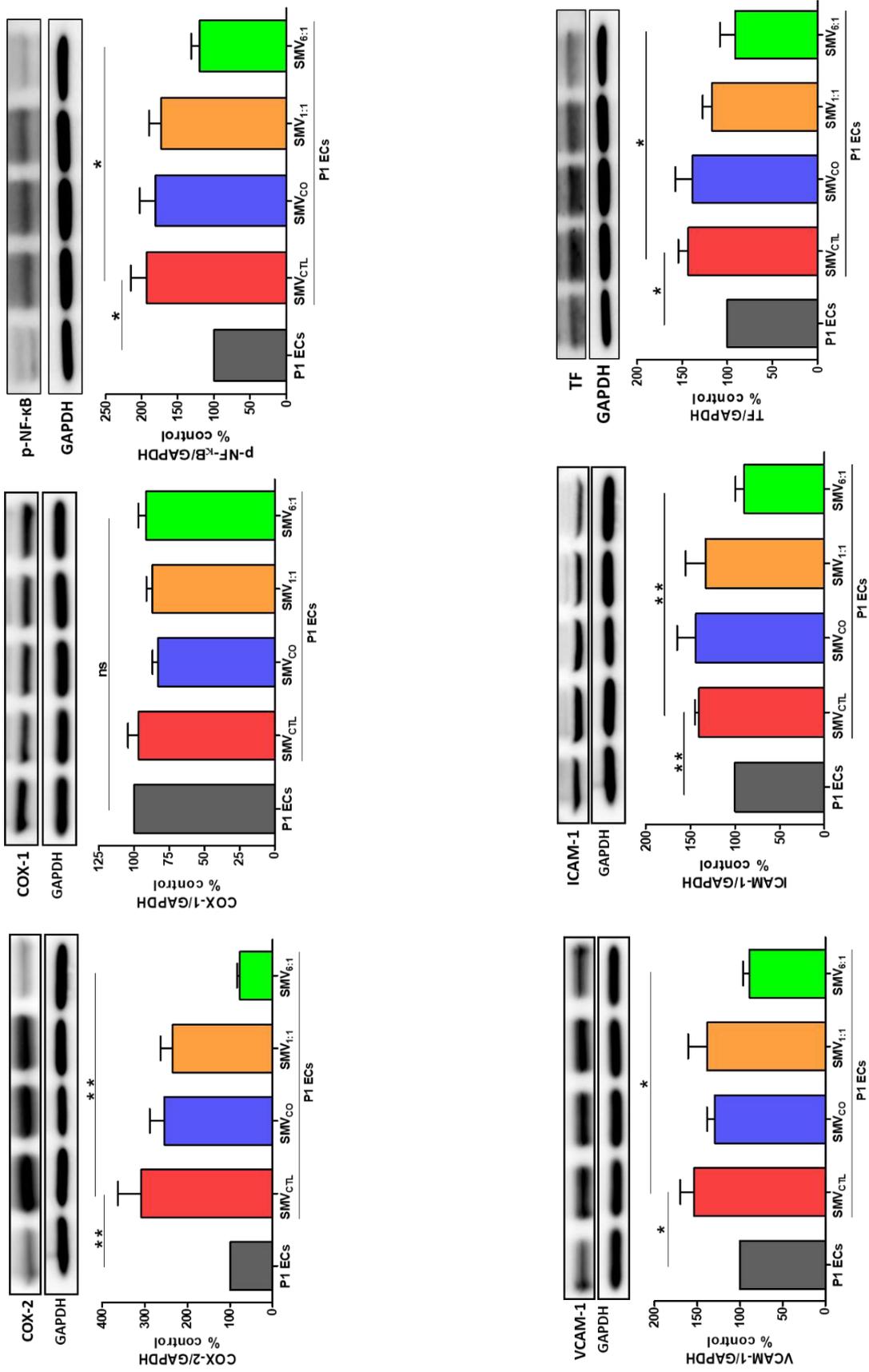


Figure 7

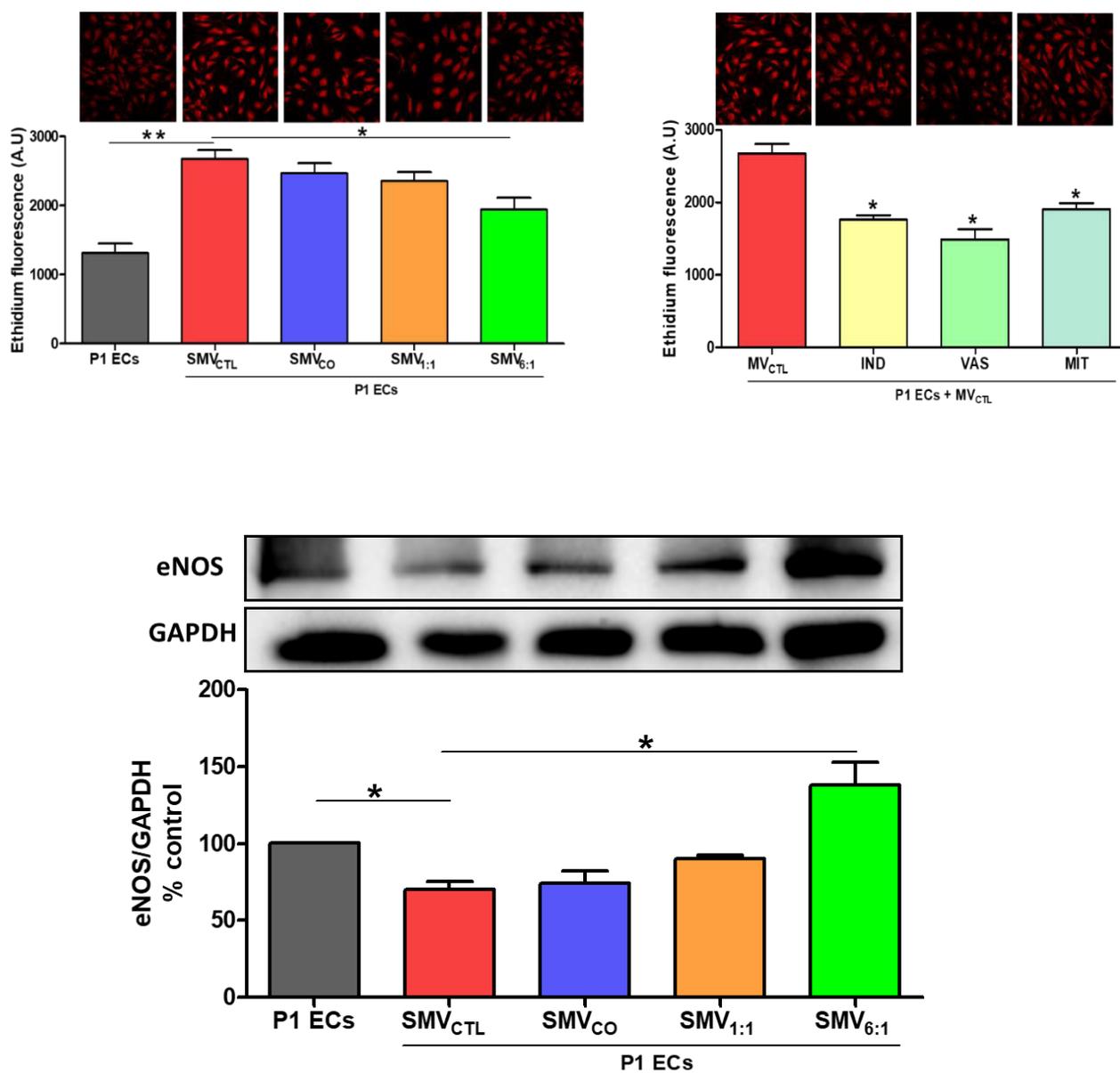
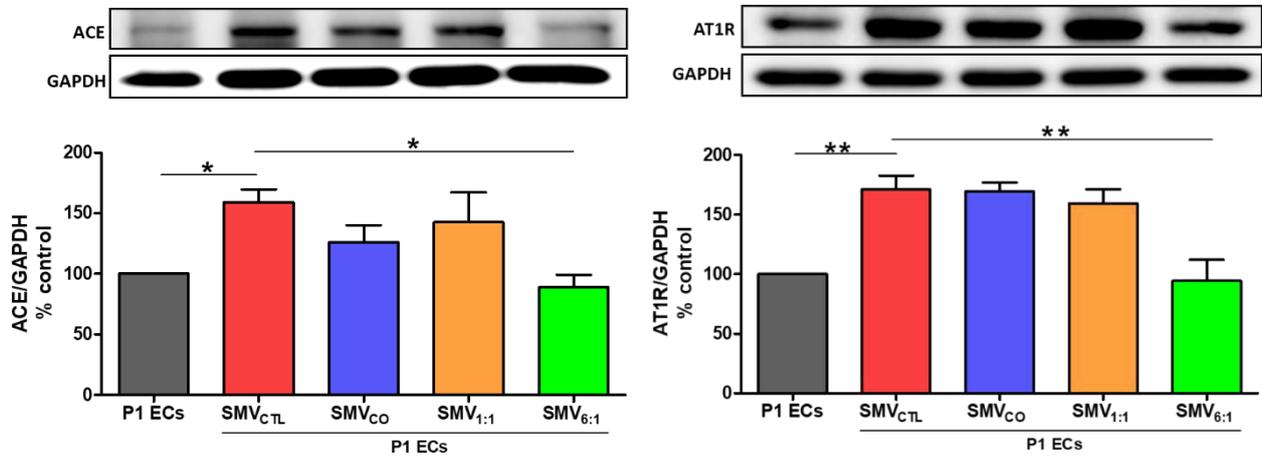


Figure 8

8a



8b

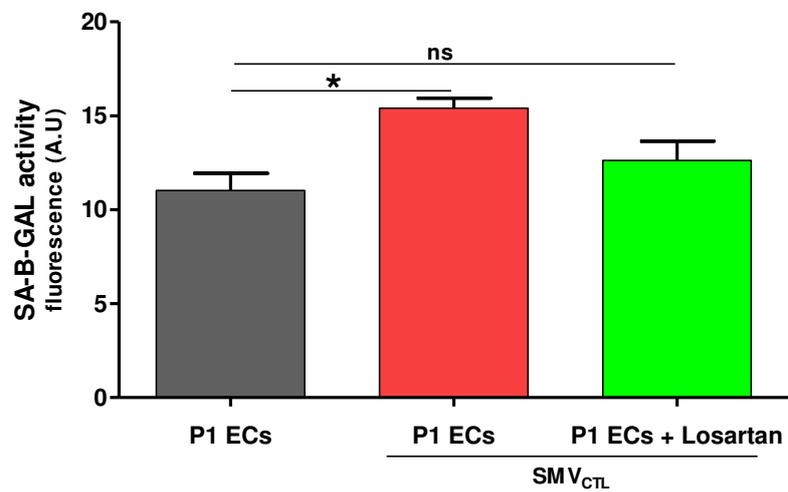
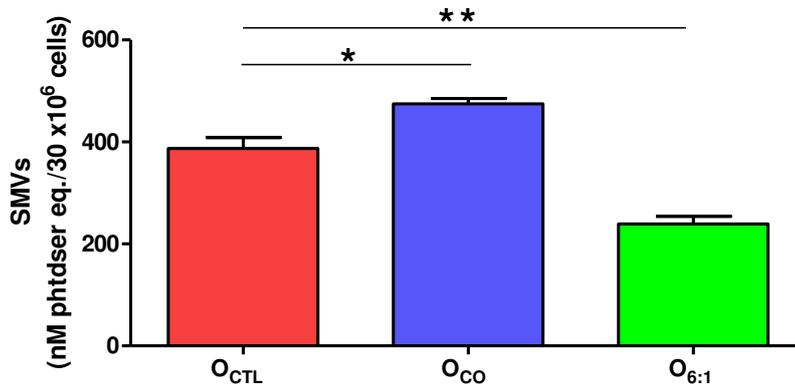
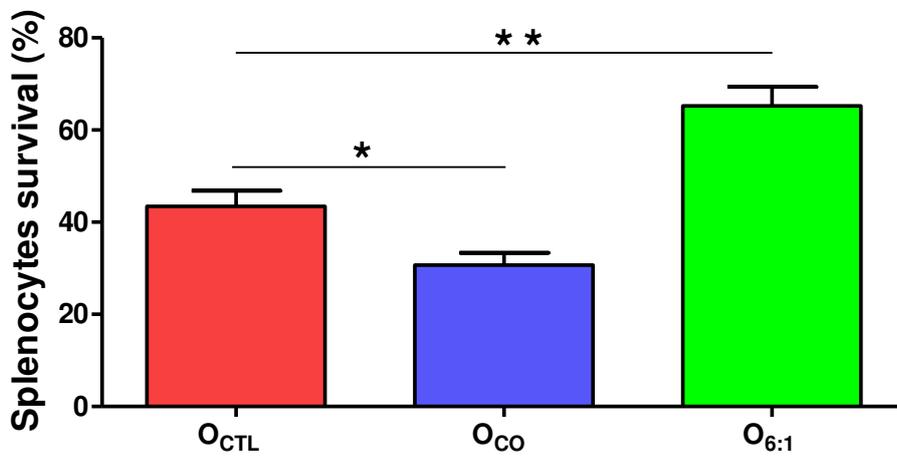


Figure 9

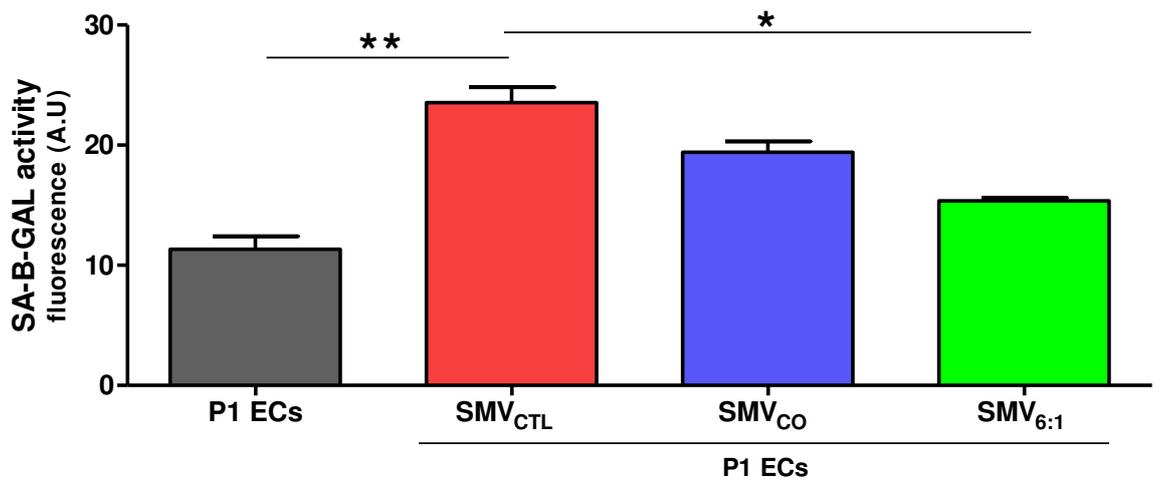
9a



9b



9c



General discussion, conclusion and further prospect

General discussion

Ageing and release of microvesicles

The present findings indicates that the ageing spleen is associated with increased shedding of microvesicles (MVs) from its constitutive cells, a main proportion of them being from leukocyte origin. A first possible cause would be age-associated increased cellular oxidative stress, senescence, low-grade inflammation (inflamm-ageing), mitochondrial dysfunction and epigenetic dysregulation, all favoring structural and functional alterations of the plasma membrane lipid composition and organization, impacting lipid rafts, function of phospholipid transporters and ion channels (Noble, Thomas et al. 1999, Egawa, Pearn et al. 2016, Figueira, Fernandes et al. 2016).

Among elderly, increased membrane PS externalization in circulating lymphocytes was previously linked with an increased rate of lymphocyte apoptosis, exaggerated activation of ion channels and phospholipid transporters (Noble, Thomas et al. 1999). Measurements of circulating MVs at different ages of life in healthy individuals are yet scarce. However, a consensus seems to emerge with increasing MVs levels during childhood (Proulle, Hugel et al. 2005), stabilization to lower levels in young adults and eventual increase at older age or in old cells (Leal, Adjobo-Hermans et al. 2018). In contrast, recently the total plasma concentration of extracellular vesicles (EVs) including microvesicles, exosomes and apoptotic bodies have been reported to decrease among elderly due to increased internalization by leukocytes (Eitan, Green et al. 2017), similarly CD144⁺EMV also decrease with age (Forest, Pautas et al. 2010).

In our study we determined the shedding of spleen-derived leukocytes (SMVs) of young, middle-aged and old rats and found that it was age-dependent suggesting a strong and progressive trigger for cell vesiculation. In addition our data indicate that abnormal age-associated plasma membrane phospholipid remodeling begins early in adulthood at least in a proportion of the spleen cells.

Since age-associated oxidative stress mediates calcium channels opening that results in calcium dependent activation of membrane phospholipids transporters rapidly disrupting the asymmetrical distribution of the membrane phospholipids (Kodigepalli, Bowers et al. 2015), it is tempting to conclude the activation rate of these transporters and channels contribute to phospholipid randomization across the plasma membrane and increases with age. In addition, phospholipid oxidation, eventually occurring with the loss of efficient

anti-oxidative pathways, is another contributor to membrane instability and to the dissemination of noxious monocyte MVs triggering the endothelium.(Huber, Vales et al. 2002) We therefore determined the level of ROS in spleen tissues and found that they already rise in middle-aged rats, confirming that the increased oxidative stress could be one of the trigger for altering the function of phospholipid transporters, proteolipid domains, and MVs shedding with age. However, a complete analysis of the redox status of the splenocyte membrane phospholipids and the lipid membrane composition remains to be performed to confirm the above observation. In addition, using different pharmacological inhibitors of ROS pathways, we were able to determine cyclooxygenases, NADPH oxidase and mitochondrial respiratory chain complex as major contributors of ROS in spleen tissue.

This raises the question of the eventual ROS content of SMVs at least of cytoplasmic origin and its variation with age (Larson, Hillery et al. 2014).

The specific contribution of each lineage to the spleen ROS accumulation by fluorescence microscopy is time consuming and requires a complete topography of the spleen. However, a clue to the identification of most activated cells would be given by the phenotype of the spleen MVs and its variation with age.

Release of MVs in spleen: an alternate approach to plasma MVs in animal models?

Under physiological conditions, leukocytes MVs represent a very small proportion less than 10% of total MVs in plasma (Hoyer, Nickenig et al. 2010). However, their significance and potential to participate in development and progression of chronic pathological conditions is evident from the fact that their circulatory levels are raised in numerous inflammatory diseases (Niazi, Silva et al. 2017) and constitute 55% of total MVs incorporated in the atherosclerotic plaque (Angelillo-Scherrer 2012). Their effector properties are however difficult to demonstrate due to limitations such as the net yield of leukocyte MVs isolation from plasma in small animals owing to extensive purification washings and MV loss to avoid exosome contamination. Isolation from rat spleen following a protocol established and validated by Ali El Habhab from our team (El Habhab, Abbas et al. 2017), circumvents this drawback since it is a rich source of leukocytes and even a more representative storage pool of leukocyte MVs taking into account the global immune response so as to analyze in vitro the pro-senescent features

of SMVs. Indeed, our data clearly show that spleen is a convenient source to study the impact of pharmacological modulators of immune responses mediated by MVs. Variations of plasma and spleen leukocyte-derived MVs remain to be described.

Since in our study, we used pooled MVs from splenocytes, identification of specific types of MVs responsible for pro-senescent effects could be done after sequential immunodepletion of each type of MVs, which remains challenging considering the need for positive and negative selection methods despite a sufficient yield in the SMV isolation. Indeed, each depletion round induces a global loss of MVs.

Finally, the leukocyte-derived MVs isolated from fresh spleen are in sufficient amount to allow their concentration and injection into the systemic circulation without induction of massive blood dilution possibly modifying the circulating storage pool of MVs through mass imbalance.

Leukocyte MVs are inducers of age-related vascular dysfunction

In our team, we evidenced that MVs are vascular effectors triggering the endothelium (Altamimy, Qureshi et al. 2018) as stated and reported by others (Abou-Saleh and S Kabeer 2015) (Lovren and Verma 2013). Endothelial (Abbas, Jesel et al. 2017) and leukocyte MVs were demonstrated pro-senescent effectors in coronary artery and aorta arteries endothelial cells (El Habhab, Abbas et al. 2017) (Burger, Montezano et al. 2011).

In accordance with our previous observations (El Habhab, Abbas et al. 2017), we demonstrated that SMVs are pro-senescent endothelial effectors and that their ability to induce endothelial senescence is age-dependent at least when using porcine coronary ECs as a target and by measuring SA-beta Gal activity. Confirmation was obtained by western blot demonstrating the increased expression of pro-senescent (p21, p16, p53), pro-inflammatory (Cox-2 but not COX-1, NF- κ B) and pro-thrombotic (ICAM-1, VCAM-1, TF) protein markers with minimal effect on the degree of endothelial apoptosis.

Assuming that all spleen lineages evolve on the same time-scale, measurement of splenocyte telomere length in young, middle-aged and old rats would also ascertain the progressive shortening with age and a replicative senescence process. Indeed, telomere shortening, DNA damage and increased oxidative stress also lead to the up-regulation of p53/p21/p16 signaling that characterize cellular senescence (Katsuumi, Shimizu et al. 2018).

Previous data from our laboratory have shown that circulating MVs from septic rats induce NF- κ B activation in vessel walls and cardiac tissues when injected to healthy individuals (Boisrame-Helms, Delabranche et al. 2014). Because activated NF- κ B also prompts senescence-associated secretory phenotype (SASP), we assessed NF- κ B activation in SMV-treated endothelial cells (Salminen, Kauppinen et al. 2012). We could confirm an early SMV-induced pro-inflammatory action 6 hours after cell treatment and 42 hours before the detection of SA-beta Gal activity and the up-regulation of other senescence markers, thereby confirming the prime role of the inflammatory pathways in the induction of endothelial senescence.

We also demonstrated that the pro-senescent effects are specifically mediated by SMVs which was evident from the fact that supernatants from spleen-derived cultured leukocytes, when deprived of SMVs, was unable to raise the level of SA- β -gal activity in young endothelial cells.

Because of its pleiotropic effects such as vasorelaxation, anti-inflammatory and anti-thrombotic effects, NO is considered as one of the chief controller of vascular homeostasis. Deficiency of NO is hallmark of endothelial dysfunction and hence increased risk of cardiovascular events (Yuyun, Ng et al. 2018). SMVs from middle-aged rats reduced the expression of eNOS in young endothelial cells suggesting that SMVs are potential inducer of endothelial dysfunction. This observation is consistent with previous reports showing that MVs from cultured apoptotic T-lymphocytes reduce endothelial NO formation due to reduce eNOS expression involving phosphatidylinositol-3-kinase (PI3K), extracellular signal-regulated kinase $\frac{1}{2}$ (ERK1/2) and NF- κ B pathways (Angelillo-Scherrer 2012). Owing to the shortage of samples, we chose not to confirm the drop of NO in SMV-induced endothelial senescence (Abbas, Jesel et al. 2017).

Oxidative stress and angiotensin II are potent inducer of endothelial senescence (Shan, Guo et al. 2014). ROS mediate both replicative and premature endothelial senescence in rat aortic and porcine coronary endothelial cells (Khemais-Benkhiat, Idris-Khodja et al. 2016, Abbas, Jesel et al. 2017). *In vitro*, MVs of porcine, rat or murine origin mediate endothelial senescence through generation of oxidative stress and multiple pathways (Burger, Kwart et al. 2012). In the present study, we observed that SMVs are pro-oxidant, and that activation of local angiotensin system is involved in the induction of SMV-mediated endothelial senescence. ROS increase was dependent on NADPH oxidase, cyclooxygenase activities and on the mitochondrial respiratory chain. Similarly, up-

regulated expression of angiotensin converting enzyme (ACE) and AT1-R in SMV-treated endothelial cells is in accordance with previous study reporting that MVs from acute coronary syndrome (ACS) patients induce endothelial senescence through AT1-R receptor, enhanced activity of ACE and redox-sensitive pathway (Abbas, Jesel et al. 2017). Role of angiotensin II and AT1-R was further confirmed by the fact that pre-treatment of young endothelial cells with Losartan, an AT1-R blocker, prevented the SMV-mediated increase in SA- β -gal activity.

Accordingly, age-related increased expression of ACE and AT1-R has previously been shown in in-vitro (replicative endothelial senescence in cultured primary porcine coronary artery endothelial cells) (Khemais-Benkhiat, Idris-Khodja et al. 2016) and in-vivo (aorta from aged mice) (Yoon, Kim et al. 2016) models.

Relevance to Cardiovascular diseases

Globally, cardiovascular disease (CVD) are the leading cause of death (Mc Namara, Alzubaidi et al. 2019) and despite all the advancement in assessment and treatment, deaths due to CVD are estimated to be raised to 24 million by 2030. This could be, at least in part, due to lacking of accurate prognostic indicators and insufficient knowledge of all pathways contributing to cardiovascular diseases (Saleh and Kabeer 2015). MVs are currently regarded as prominent biomarkers and effectors of vascular function (McVey and Kuebler 2018), although awaiting easy measurement and routine laboratory devices. In our study, age-associated elevation of SMVs with cardiovascular disease show endothelial pro-senescent, pro-inflammatory and pro-coagulant effects.

Reduced NO formation and increase oxidative stress is one of the major characteristics of endothelial dysfunction while circulatory levels of MVs of endothelial and leukocyte origin are featured in cardiovascular and chronic inflammatory disorders such as diabetes (Ridger, Boulanger et al. 2017, Pollet, Conrard et al. 2018). It has been assumed in the previous reports that circulating leukocyte-derived MVs were stemmed from blood cells. However, recent reports point at the role of spleen resident immune cells and low grade inflammatory state in cardiovascular diseases. The proposed model of physiological interactions between spleen and heart states that spleen activation favors disease progression during ischemia and that the ischemic cardiac tissue remodeling drives the splenic expansion of the white pulp together with the activation of splenocytes of selective immune cell subsets. In a mice model of myocardial infarction induced through ligation of left coronary artery, monocytes from spleen migrated and infiltrated in infarcted area (Swirski, Nahrendorf et al. 2009). Accumulation of

monocytes/macrophages from spleen at the site of myocardial infarct in atherosclerotic apoE knockout mice enhanced the infarct size and impaired the wound healing (Heusch 2019). Similarly, in chronic low grade inflammatory state, such as heart failure, increased number of peripheral monocytes was accompanied by splenomegaly (Glezeva, Voon et al. 2015, Fujinami, Kondo et al. 2018). In mice, a cardio-splenic axis was demonstrated during chronic ischemic heart failure that promoted activated splenocyte homing to the heart and abnormal tissue remodeling. In addition, the adoptive transfer of CD4+ spleen T cells from mice with heart failure induced left ventricle dysfunction 8 weeks later, suggesting that CD4+SMVs are potential paracrine effectors of cardiac injury (Prabhu 2018). Of note, a cardio-splenic axis was also evidenced in humans by positron tomography in ischemic disease and acute coronary syndrome. (Emami, Singh et al. 2015)

Omega-3-as a specific ageing therapy

The interest in cardio-protective benefits of omega-3 PUFAs was sparked nearly 50 years ago with the observation that Greenland Inuits have lower incidence of myocardial infarction and cardiovascular mortality (Grzegorz 2019). Since then, numerous clinical and experimental studies have reported the association between increased dietary intake of omega-3 PUFAs and beneficial effects on cardiovascular system (Mozaffarian and Rimm 2006, Roth and Harris 2010) and subsequent reduction in risk of cardiovascular event (Mori 2014). Among different types of omega-3 PUFAs, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) are the most potent omega-3 PUFAs (Riediger, Othman et al. 2009) and fish or fish oil is considered a rich source of omega-3 PUFAs (Lane, Derbyshire et al. 2014).

Previous studies have reported that omega-3 PUFAs improve endothelial function by increasing the endothelial NO formation and reduce the oxidative stress (Zanetti, Gortan Cappellari et al. 2017). Our team has showed that omega-3 PUFAs improved endothelial function in hypertensive (Niazi, Silva et al. 2017) and aged rats (Thesis Farooq, 2018) and their vaso-relaxing properties are dependent on both ratio and purity EPA and DHA. Indeed, highly purified omega-3 EPA:DHA 6:1 formulation was shown to be most potent vaso-protective ratio (Zgheel, Alhosin et al. 2014).

In the present study, we therefore investigated whether short-term intake of the omega-3 EPA:DHA 6:1 new formulation reduce the age-associated shedding of pro-senescent SMVs. Omega-3 EPA:DHA was given at a dose of 500 mg/kg body weight of rats, which is equivalent to 5.67 g/day/70 kg body weight in humans (Reagan-Shaw, Nihal et al.

2008), on line with the 0.18 to 10 g/day ranges given in various meta-analysis and clinical studies (Appel, Miller et al. 1993, Delgado-Lista, Perez-Martinez et al. 2012, Enns, Yeganeh et al. 2014, Miller, Van Elswyk et al. 2014). Corn oil was used as isocaloric control.

Our findings indicate that omega-3 EPA:DHA 6:1, but not corn oil, significantly reduce the MV-shedding ability of spleen-derived leukocytes in middle-aged and old rats with little or no significant effect on young rats, indicating that omega-3 EPA:DHA 6:1 specifically targets ageing cells as compared to EPA:DHA 1:1. An eventual explanation is the enhanced incorporation of EPA in cells from aged individuals (Calder 2007).

Since oxidative stress contributes to MV shedding through activation of membrane phospholipids transporters (Kodigepalli, Bowers et al. 2015), we verified if omega-3 EPA:DHA 6:1 alter age-dependent oxidative stress in spleen tissues using DHE staining. We observed that that omega-3 EPA:DHA 6:1 intake resulted in significant reduction in age-associated oxidative stress, suggesting the expected targeting of SMVs mother cells. Moreover, characterization of the origins of SMVs revealed that omega-3 EPA:DHA 6:1 reduces the spontaneous age-related shedding of SMVs of neutrophil, monocyte/macrophages, T-lymphocyte, natural killer cells and granulocytes origin, indicating that omega-3 EPA:DHA 6:1 targets cells of both innate and adaptive immune response. However, the eventual validation of specific immune cells targeting by omega-3 EPA:DHA 6:1 is possible by flow cytometric analysis of treated and un-treated spleen (Colovai, Giatzikis et al. 2004). Furthermore, knowing the direct effects of omega-3 PUFAs and its metabolites, the lipid profiling of SMVs from omega-3 EPA:DHA 6:1 treated-rats could be informative with respect to the potential of SMVs as carrier for omega-3 or its active metabolites such as resolvins. Of note, omega-3 EPA:DHA 6:1 did not induce any harmful effect e.g. bleeding in young rats, confirming the absence of safety issues with short-term intake of omega-3 EPA:DHA 6:1.

Using an original SMV-endothelial cross-talk model, we demonstrated that omega-3 EPA:DHA 6:1 strongly abolished the pro-senescent potential of SMVs. On contrary both omega-3 EPA:DHA 1:1 and corn oil produce little or no significant effect. The data indicate the superiority of omega-3 EPA:DHA 6:1 over omega-3 EPA:DHA 1:1 and is in accordance with previous *ex-vivo* study on porcine coronary arteries showing maximum endothelium-dependent vasorelaxation with omega-3 EPA:DHA 6:1 (Zgheel, Alhosin et al. 2014) and also with *in-vivo* study where omega-3 EPA:DHA 6:1 ingestion reduced the expression of senescence associated protein markers (p21, p16, p53) in mesenteric arteries from aged rats (Thesis Farooq MA, 2018). We mainly studied the modulation of

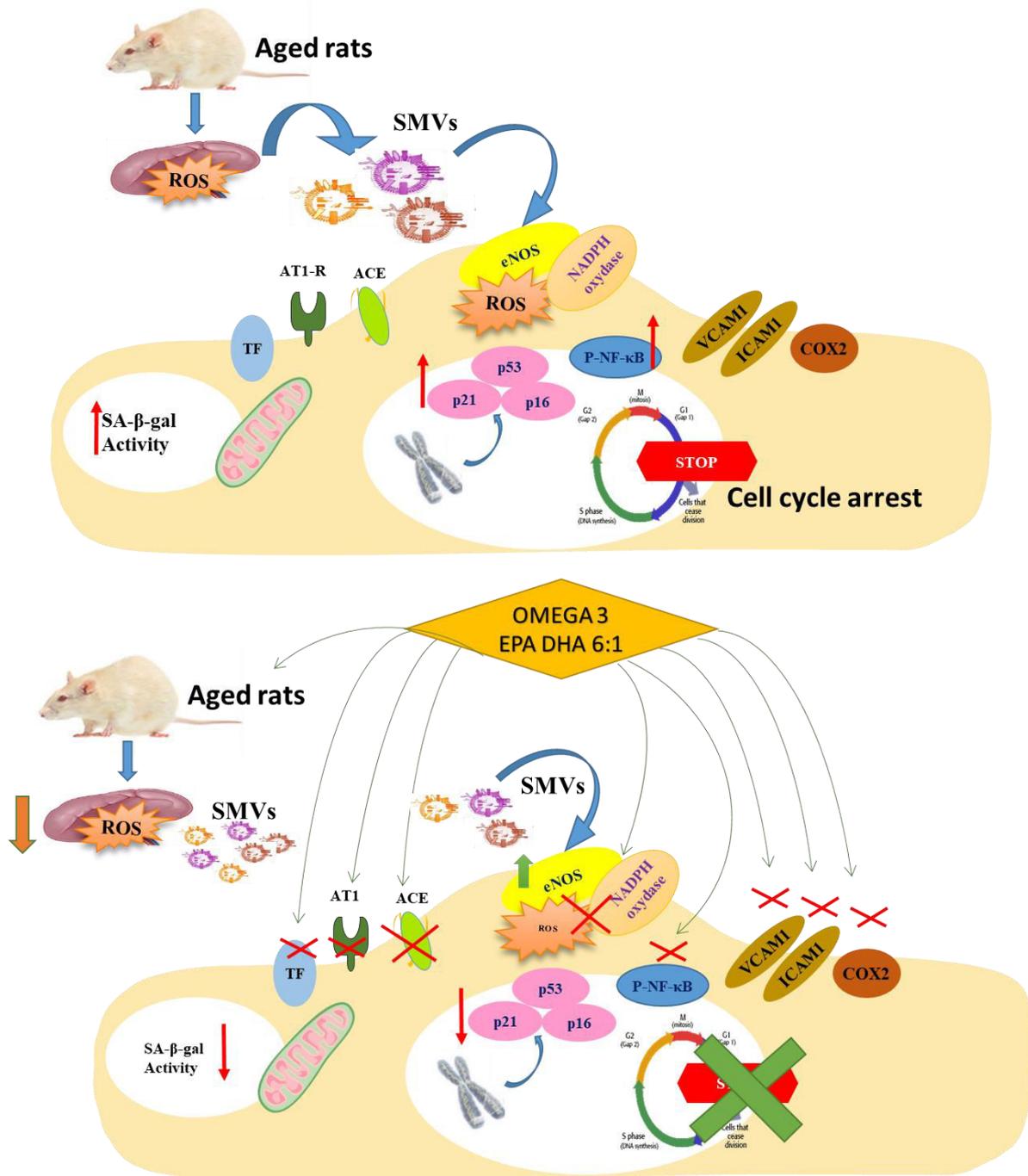
the pro-senescent effects of SMVs from middle-aged rats to examine whether an omega-3 treatment would be of benefit in mild vascular injury. However, the same procedure could be applied to old rats.

In accordance with the effects observed on spleen shedding, we could demonstrate that a short-term omega-3 intake modifies the SMV features even when isolated from middle-aged rats that have milder pro-senescent potential. The expression of pro-inflammatory COX-2 and pro-coagulant ICAM-1, VCAM-1 and TF was significantly reduced by omega-3 EPA:DHA 6:1 in SMV-treated young endothelial cells. In addition, omega-3 EPA:DHA 6:1 also prevented the early phosphorylation of NF- κ B which could possibly account for their anti-inflammatory effects. Indeed, omega-3 PUFAs incorporation into cellular membrane have been reported to reduce the activation of NF- κ B, involving two G-protein coupled receptors, GPR43 and GPR120 (Desnoyers, Gilbert et al. 2018). In addition, our data are in line with previous reports of omega-3 PUFAs limiting the endothelial expression of ICAM-1 and VCAM-1 in cytokine-induced endothelial dysfunction (Trommer, Leimert et al. 2017) and of COX-2 expression in LPS-treated human umbilical vein endothelial cells (Lee, Kim et al. 2009).

In line with the previous studies reporting that omega-3 PUFAs improves endothelial dysfunction by inducing NO formation through targeting Src/PI3-kinase/Akt and MAPKinase pathway and activation of eNOS (Zgheel, Alhosin et al. 2014), we demonstrate that omega-3 EPA:DHA 6:1 intake normalized the reduced expression of eNOS in SMV-treated endothelial cells. This effect could be associated with reduced oxidative stress as increased oxidative stress and reduced NO is a hallmark of endothelial dysfunction and this inverse relation is evident at the vascular regions prone to development of atherosclerosis (Davignon and Ganz 2004). Indeed, omega-3 EPA:DHA 6:1 clearly reduced the SMV-mediated increased endothelial ROS, consistent with the reported reduced ROS in human aortic endothelial cells and the increased expression of anti-oxidant molecules such as ferritin heavy chain, ferritin light chain, heme oxygenase-1, manganese superoxide dismutase and thioredoxin reductase 1 (Sakai and Ishida 2017). In our study, omega-3 EPA:DHA 6:1 intake also reduced the SMV-induced activation of the local angiotensin system that is also a contributor to oxidative stress through NADPH oxidase activity and a strong inducer of endothelial senescence and dysfunction (Li, Mi et al. 2019). Prevention against angiotensin-mediated endothelial dysfunction in SMV-treated young endothelial cells was significant only after omega-3 EPA:DHA 6:1 intake, not EPA:DHA 1:1 and was totally abolished in middle-aged rats as characterized by

western blotting, in accordance with previous observation of a protective effect in rat vessels (Niazi, Silva et al. 2017).

To complete the study and examine the possibility that omega 3 6:1 benefits are of relevance to a cardio-splenic axis developping with age, the plasma leukocyte-derived MVs phenotype should be confronted to the pattern of the SMVs in middle-aged and old rats and compared to those of young rats. Similarly, endothelial pro-senecent properties spleen-derived leukocyte MVs (SMVs) and circulating leukocyte-derived MVs could be compared.



Omega-3 EPA:DHA 6:1 protects against SMVs-induced endothelial senescence.

TOP) Age-related increase oxidative stress in spleen tissue is accompanied by increase shedding of SMVs. SMVs induced premature senescence in young porcine coronary artery endothelial cells (ECs) that was marked by increase Sa-β-gal activity and increased expression of senescence-related protein markers (p53, p21, p16). In addition, SMVs reduced the expression of eNOS and shifted ECs towards pro-inflammatory and pro-atherothrombotic phenotype, most probably involving oxidative stress and local angiotensin system. **Bottom)** Short-term omega-3 EPA: DHA 6:1 intake by aged rats reduced the age-related oxidative stress, shedding of SMVs and protected ECs against SMVs-mediated noxious effects.

Conclusion and further prospects

In conclusion, the present findings indicate an increased and progressive oxidative burden with age that is accompanied by shedding of spleen-derived leukocyte MVs (SMVs), of immune cells of both innate and adaptive immune systems. Because of their pro-senescent, pro-inflammatory and atherothrombogenic properties, SMVs are strong promoters of age-associated endothelial dysfunction that is characterized by reduced endothelial NO formation and increased oxidative stress.

In our model, omega-3 EPA:DHA 6:1 is a potent preventing therapy of age-associated endothelial dysfunction mediated by SMVs. In addition to a reduced oxidative stress in spleen tissues of middle-aged rats, omega-3 EPA:DHA 6:1 also reduced the spontaneous shedding of SMVs from aged spleen. Furthermore, omega-3 EPA:DHA 6:1 abolishes in middle-aged and attenuates in old rats, the noxious pro-senescent properties of SMVs and protects against the SMV-mediated endothelial shift towards pro-inflammatory and pro-thrombotic features. Omega-3 EPA:DHA 6:1, most probably, mediate its endothelium protective effects by reducing the oxidative stress, preserving the eNOS expression and preventing the activation of the local angiotensin system. Data further suggest that omega-3 EPA:EDA 6:1 may help to prevent or delay age-associated endothelial dysfunction. Finally, spleen appears to be a convenient source of leukocyte and leukocytes-derived MVs to assess pharmacological modulation of immune responses.

Nevertheless, since a splenic-cardiac axis has been recently proposed and inflamaging contributes to alteration of both innate and adaptative systems, characterization of MVs lipid mediators appears mandatory to better explain the action of PUFAs. For example, resolvins are the specific omega-3 PUFAs lipid metabolite which are well known actors in the resolution of inflammation (Serhan and Levy 2018). As SMVs from omega-3 EPA:DHA 6:1 rats was associated with lower expression of pro-inflammatory protein markers, suggesting that such SMVs would carry omega-3 metabolites with anti-inflammatory actions.

Further investigations are required to more extensively elaborate the age-related changes in spleen tissue especially with respect to shedding and functional properties of microvesicles. Many membrane phospholipids transporters (flippases, floppases, scramblases) have been identified that control the asymmetrical distribution of membrane

phospholipids and, hence, shedding of pro-coagulant microvesicles. It will be interesting to demonstrate the type of phospholipid transporter involved in the senescence-induced shedding of SMVs with age and identify which is targeted by omega-3 EPA:DHA 6:1 intake. Recently, a phospholipid floppase, TMEM16F or anoctamin 6, has been characterized as a transporter of phosphatidylserine (PS) to the external membrane leaflet (Bever and Williamson 2016). Impact of TMEM16F has been demonstrated in different conditions such as apoptosis, inflammation and coagulation (Baig, Haining et al. 2016, Simoes, Ousingsawat et al. 2018). Increased oxidative stress, nature of membrane phospholipids and increased intracellular concentration of Ca^{+2} are demonstrated as activators of TMEM16F (Schreiber and Ousingsawat 2018). ROS-mediated activation of TMEM16F is detected in cultured human lymphocytes as well as airways epithelial cells (Kmit, van Kruchten et al. 2013, Simoes, Ousingsawat et al. 2018). As we have demonstrated increased oxidative stress in spleen tissue from aging rats, it could be hypothesized that TMEM16F activation accounts for the increase shedding of SMVs. In addition, other membrane proteins mediating the externalization of phosphatidylserine such as Xkr8 (Suzuki, Imanishi et al. 2016) would also be of interest although they have been more related to apoptosis.

The fact that miRNA packed in microvesicles are involved in cellular cross-talk (Creemers, Tijssen et al. 2012, Xia, Zeng et al. 2018), suggest that the characterization of the miRNA content of SMVs from both treated and untreated rats would add some new hints on their mechanism of action. Many miRNAs are differentially expressed in senescent cell and aged tissue and consider biomarkers of senescence, ageing and multiple diseases including cardiovascular diseases (Creemers, Tijssen et al. 2012, Bu, Wedel et al. 2017). In several studies on ageing, blood samples have showed specific types of miRNAs as signature of ageing including miRNA-130a, miRNA-93, miRNA-126 (Olivieri, Capri et al. 2017). Additionally, characterization of the SMV content might enable their eventual detection in the plasma of cardiovascular patients and allow the study of the cardiosplenic axis.

Altogether, our data support the fact that short-term intake of omega-3 intake is of benefit against age-related damages in spleen and probably in the cardiovascular system. They also suggest that such intake could be of relevance at the early stages of cardiovascular diseases, as a alternate strategy to prolong a healthy ageing.

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Annexes

Scientific production

Publications:

Ageing enhances the shedding of splenocyte microvesicles with endothelial pro-senescent effect that is prevented by a short-term intake of omega-3 PUFA EPA:DHA 6:1

A.W. Qureshi, R. Altamimy, A. El Habhab, H. El Itawi, M.A. Farooq, F. El-Ghazouani, H. Hasan, C. Auger, V.B. Schini-Kerth, F. Toti.

Spleen neutrophil microparticles induce endothelial senescence and impair vascular relaxation of coronary arteries via inflammatory signaling.

Ali El Habhab, Raed Altamimy, Malak Abbas, Mohamad Kassem, Guillaume Kreutter, Lamia Amoura, **Abdul Wahid Qureshi**, Sonia Khemais-Benkhiat, Fatiha Zobairi, Valérie B.Schini-Kerth, Laurence Kessler, Florence Toti. (Submitted to Journal of Cellular and Molecular Medicine, June 2019)

Thrombin promotes premature atrial endothelial cell senescence leading to the induction of pro-infiltrative and pro-fibrotic responses: Role of the local angiotensin II/AT1 receptor system.

H. Hasan, S. Park, E. Belcastro, M. Abbas, **A.W. Qureshi**, M.A. Farooq, P. Ohlmann, F. Toti, C. Auger, V.B. Schini-Kerth, O. Morel, L. Jesel. (Submitted to the journal of Thrombosis and Haemostasis)

The omega-3 EPA:DHA 6:1 formulation improves ageing-related blunted endothelium-dependent relaxations and increased contractile responses in the mesenteric artery: role of oxidative stress and cyclooxygenases.

M.A. Farooq, L. Amoura, S. Gaertner, Z.R. Niazi, S. Park, **A.W. Qureshi**, M.H Oak, F. Toti, V.B. Schini-Kerth, C. Auger. (Redacted & will be submitted by the end of august 2019)

Empagliflozin, a sodium-glucose cotransporter inhibitor, improved heart remodeling and mesenteric artery endothelial function in the metabolic syndrome with HFpEF ZSF1 rat: role of cyclooxygenases.

S. Park, M.A. Farooq, S. Gaertner, C. Brucker, **A.W. Qureshi**, H. Lee, D. Benrahla, B. Pollet, D. Stephan, P. Ohlmann, E. Mayoux, C. Auger, O. Morel, V.B. Schini-Kerth. Under preparation

Oral communications:

Treatment of rats with the omega fatty acid 3 formulation EPA:DHA 6:1 decreases the leukocyte microparticles-induced endothelial pro-inflammatory responses and senescence.

A.W. Qureshi, R. Altamimy, A. El Habhab, L. Amoura, M. Kassem, S. Khemais, M. Farooq, H. Hasan, S. Park, F. El-Ghazouani, C. Auger, L. Kessler, V.B. Schini-Kerth, F. Toti. International meeting on ischemia reperfusion injury in transplantation (IMIRT), April 2018, Poitiers, France

Leukocyte-derived microparticles exaggerate endothelial senescence and vascular dysfunction induced by high glucose.

R.Altamimy, **A.W. Qureshi**, L. Amoura, A. El Habhab, H. El Itawi, M.Kassem, S.Khemais, B.Pollet, F.El-Ghazouani, C.Auger, V.B. Schini-Kerth, F.Toti. Printemps de la Cardiologie Recherche Fondamentale & Clinique, 4-6 April 2018, Montpellier, France.

Poster communications:

Intake of the omega 3 PUFAs formulation EPA:DHA 6:1 by aged rats reduced shedding of microvesicles from spleen-derived cultured leukocytes and their ability to promote senescence in endothelial cells.

A.W. Qureshi, R. Altamimy, A. El Habhab, L. Amoura, M. Kassem, S. Khemais, M. Farooq, H. Hasan, S. Park, F. El-Ghazouani, C. Auger, L. Kessler, V.B. Schini-Kerth, F. Toti. European Society of Cardiology (ESC), Aug-sept 2019, Paris, France.

Leukocyte-derived microparticles exaggerate endothelial senescence and vascular dysfunction induced by high glucose.

R.Altamimy, **A.W. Qureshi**, L. Amoura, A. El Habhab, H. El Itawi, M.Kassem, S.Khemais, B.Pollet, F.El-Ghazouani, C.Auger, V.B. Schini-Kerth, F.Toti. Printemps de la Cardiologie Recherche Fondamentale & Clinique, 4-6 April 2018, Montpellier, France.

**Les microvésicules splénocytaires
effecteurs de la sénescence
endothéliale : Impact de l'âge et
protection par apport nutritionnel d'une
formule optimisée d'acides gras poly-
insaturés eicosapentaénoïque et
docosahexaénoïque, EPA:DHA 6:1**

Résumé

L'âge conduit à une sénescence progressive des cellules endothéliales (ECs) favorisant une dysfonction endothéliale et vasculaire, souvent associée aux maladies cardiovasculaires. Nous avons étudié son impact sur l'émission de microvésicules (SMVs) par les leucocytes isolés de la rate de rats jeunes, d'âge moyen, ou vieux (J, MA, V) et caractérisé leurs propriétés pro-sénescences sur des EC primaires d'artères coronaires porcines. Avec l'âge, le stress oxydant s'accumule dans la rate et l'émission de SMVs augmente. Seules les SMVs de MA et O induisent une sénescence des ECs prématurée avec une activité Senescence-Associated- β -galactosidase et un stress oxydant augmentés, la surexpression des marqueurs p53, p21, p16, un phénotype pro-inflammatoire et pro-athérogène et une sous-expression de la eNOS. Un gavage de 7 jours avec des omega-3 EPA:DHA 6:1 mais pas EPA:DHA 1:1, réduit le stress oxydant splénique des MA et des O, et la vésiculation. EPA:DHA 6:1 abolit le caractère pro-sénescence des SMVs de MA, probablement via la réduction du stress oxydant et en prévenant l'activation du système angiotensine local.

Microvésicules ; Microparticules ; Omega-3 EPA:DHA 6:1 ; Sénescence endothéliale ; Age

Résumé en anglais

Ageing is associated with progressive endothelial senescence favoring endothelial and vascular dysfunction, often associated with cardiovascular diseases. We investigated in young, middle-aged and old rats (Y, MA, O) the impact of ageing on the shedding of spleen-derived leukocytes microvesicles (SMVs) and measured their pro-senescent effects in porcine primary coronary artery endothelial cells (ECs). Oxidative stress accumulates in spleen tissue and SMVs shedding increases with age. SMVs from MA, O but not Y rats induced premature endothelial senescence, with increased Senescence-Associated- β -galactosidase activity and up-regulated p53, p21, p16. SMVs shifted ECs towards a pro-inflammatory and pro-atherothrombotic phenotype with increased endothelial oxidative stress and down-regulated eNOS. Short-term intake of omega-3 EPA:DHA 6:1 but not EPA:DHA 1:1 reduced age-related oxidative stress and SMVs shedding in MA and O spleen tissues, and abolished SMVs-induced premature endothelial senescence in MA, most probably by reducing oxidative stress and preventing the activation of the local angiotensin system.

Microvesicles ; Microparticles ; Omega-3 EPA:DHA 6:1 ; Endothelial senescence ; Age