Interactions between coronary artery endothelial cells and leukocyte MPs shed in response to E. coli lipopolysaccharide : in-vitro and ex-vivo studies of the impact of vascular ageing and of high glucose

Raed Adill Hannon Altamimy

UNIVERSITÉ DE STRASBOURG

ECOLE DOCTORALE 414-Sciences de la vie et de la santé

INSERM UMR-S 1260 Nanomédecine régénérative

THÈSE

Présentée par :

Raed ALTAMIMY

Soutenue le :18 *Mai 2018*

Pour obtenir le grade de : **Docteur de l'Université de Strasbourg**

Discipline/ Spécialité : **Immunology**

Interactions between coronary artery endothelial cells and leukocyte MPs shed in response to *E. coli* lipopolysaccharide: In-vitro and ex-vivo studies of the impact of vascular ageing and of high glucose

THÈSE dirigée par:

Mme Florence TOTI — Professeur, Université de Strasbourg

Rapporteurs externes:

Mr. Olivier BLANC-BRUDE — CR CNRS, INSERM UMR-S 970

Mme Isabelle LARTAUD — Professeur, Université de Lorraine

Examinateur interne:

Mr. Olivier HUCK — Professeur, Université de Strasbourg

Invitée:

Mme Valérie SCHINI-KERTH — Professeur, Université de Strasbourg

« Napoleon and other great men were makers of empires, but these eight men whom I am about to mention were makers of universes and their hands were not stained with the blood of their fellow men. I go back 2,500 years and how many can I count in that period? I can count them on the fingers of my two hands. Pythagoras, Ptolemy, Kepler, Copernicus, Aristotle, Galileo, Newton and Einstein and I still have two fingers left vacant »

George Bernard Shaw

Acknowledgements

First, I would like to thank the members of my thesis jury for evaluating my work under a unusual schedule.

From all my heart, I would also like to thank the supervisor of my thesis, Professor **Florence TOTI**. Thank you for trusting me and welcoming me in your team. Thank you for your support, enthusiasm and advice, thank you for guiding through these years, for your supervision and great availability throughout my thesis. Thank you for initiating me to a multidisciplinary and transversal approach. Thank you for the hours you spent correcting my mistakes. Without you this these work would not have been completed. **Thank you Chef !!!**

I am also very grateful to Professor Valerie Schini-Kerth and her research lab for generous support in achieving my professional goals. I would also like to thank my colleagues who supported me and helped me throughout my research. I would also like to thank the members of the team headed by Professor Laurence Kessler for their sustained support throughout my progress in research. I owe many thanks to Brigitte Pollet for helping me and advising me in vascular reactivity experiments with kindness and efficiency.

I am also greateful to all researchers, students and teachers that I met on my way during these years. They gave me much valuavle advices, comments and suggestions during incubation times and at smocking breaks….. In fact, this work reflects all these scientific conversations with my scientific fellows at the Faculty.

I would also like to thank my country Iraq, and the University of Thi-Qar, my University, who provided financial, health and moral support throughout the research period, and my Host country and especially the University of Strasbourg and the Ecole Doctorale Vie et Santé, for accepting my candidacy as a doctoral fellow.

Finally, I would not be presenting this work without my family support that gave me the strength to reach my up-most goal. And great thanks to the spirit of my mother that made me what I am now. As well I thank my dear wife who stood by me in difficult times ... I love you. And thanks to my children “Adyan, Ali, Jaafar, Homam, and who will come later” who made me stronger, I hope they will be proud of our family path.

Above all, I thank God for the inspiration and all the opportunities that made this possible.

France-Strasbourg

Raed Adill ALTAMIMY

18-05-2018

List of Abbreviations

AGEs	Advanced glycation end-products
Ang II	Angiotensin II
cAMP	Cyclic 3 ', 5' adenosine monophosphate
CD14	Cluster of Differentiation 14
CDK	Cyclin-dependent kinase
DCs	Dendritic Cells
DDR	DNA Damage Response
DT1	Type 1 diabetes
DT2	Type 2 diabetes
E. coli	*Escherichia coli*
ECs	Endothelial cells
EDHF	Endothelium-Derived Hyperpolarizing Factor
EMP	Endothelium Microparticle
EMPA	Empagliflozin
EVs	Extracellular Vesicles
HG	High Glucose
hTERC	Catalytic subunit of human telomerase RNA component
IK_{Ca}	Intermediate-conductance calcium-activated potassium channel
IL-6	interleukin-6
LBP	LPS-binding protein
LMP	Leukocyte-derived Microparticle
LPS	Lipopolysaccharides
LPS	lipopolysaccharide
MAPK	Mitogen-Activated Protein Kinase
MPs	Microparticles
mTOR	mammalian Target of Rapamycin
MVs	Microvesicles
PAMPs	pathogen-associated molecular patterns
PhChol	PhosphatidylCholine
PhEth	Phosphatidylethanolamine

PKC	Protein kinase C
PMA	phorbol 12-myristate 13-acetate
PRRs	pattern recognition receptors
PS	Phosphatidylserine
SA-β-gal	Senescence-associated beta-galactosidase
SAHF	senescence-associated heterochromatin foci
SASP	senescence-associated secretory phenotype
SHR	spontaneously hypertensive rats
SK_{Ca}	intermediate-small-conductance calcium -activated potassium channels
TF	Tissue factor, the cell initiator of blood coagulation cascade
TLR4	Toll-like receptor 4
TNF-α	tumor necrosis factor-α

List of Figures

Figure 1: Structure of the spleen
Figure 2: Structure of bacterial cell walls
Figure 3A-3B: Structure and biosynthesis of LPS in *E. coli*
Figure 4: LPS recognition by the TLR4/MD2/CD14 complex
Figure 5: The plasma membrane remodeling and MPs shedding
Figure 6: Phospholipid transporters
Figure 7: Models of phospholipid movements across the plasma membrane bilayer and MP release
Figure 8: Impact of circulating MPs with procoagulant and pro-inflammatory properties on the endothelium and vessel wall
Figure 9: A schematic representation of blood vessels and their components
Figure 10: Structure of the arterial wall
Figure 11: The different metabolic functions of endothelial cells and endothelial mediators
Figure 12 a: Figure 12: Endothelial nitric oxide synthase (eNOS) can be activated in calcium-dependent or- independent ways and favors vasodilatation through NO production
Figure 12 b: B1 and B2 signalling pathways in ECs and smooth muscle cells
Figure 13: Synthesis of endothelium-dependent relaxation and contraction pathways
Figure 14: Characteristics of senescent cells
Figure 15: mechanism of teleomer repair
Figure 16: Cell cycle regulation by the complex Cyclin Dependent Kinase- Cyclin

Table of contents

Résumé extensif en français……... 1
THESIS ENGLISH VERSION…….. 11
Introduction ……..... 12
I- Overview of the immune system and of its responses to *E. coli* lipopolysaccharide ..13
I-1. The spleen ...…….14
I-1.1 Immune cells and leukocytes in the spleen ……14
I-2 Overview of the Immune Response ……15
I-2.1 The innate immune system ……...15
I-2.2 The Adaptive immune system ……...16
I-3 *Escherichia coli* ……...16
I-3.1 Structure & Classification ……16
I-3.2 *E. coli* as a pathogen ……17
I-3-3 Lipopolysaccharide of *E. coli* ……17
I-3-3.1 Structure of lipopolysaccharide of *E. coli* ……18
I-3.3.2 Response of the immune cells to LPS: PAMPS and PRRs ……19
I-3-3.3 Bacterial virulence, LPS, severity of the disease and impact on therapy ……21
I-3.3.4 LPS/TLR4 signal pathway ……22
II- Microparticles and vascular damage ……24
II-1.1 Overview and physiopathological significance ……25
II-1.2 Membrane remodeling and vesiculation ……25
II-1.2.1 Phospholipid asymmetry of the plasma membrane in resting cells ……27
II-1.2.2 The phospholipid transporters ……27
II-1.2.3 Cell Activation causing MP shedding ……29
II-1.2.3(1) Calcium influx and disruption of membrane asymmetry ……29
II-1.2.3 (2) Calcium influx and degradation of the cytoskeleton ……29
II-2 Pathogenic effects of microparticles in vascular diseases ……30
II-2.1 Microparticles, coagulation and thrombosis ……31
II-2.2 Microparticles and Diabetes ……31
II-2.3 Microparticles, endothelial dysfunction and inflammation ……32
II-2.4 MPs and oxidative stress in the endothelium ……33
II-2.5 Microparticles and senescence ……34

III- Structure and Physiology of Endothelium 35
III-1 Blood vessels 36
III-2 Endothelium: overview of its function 36
III-3 Endothelium and vascular tone 39
III-3.1 Endothelium-derived vasorelaxing factors 39
III-3.1.1 Nitric Oxide 39
III-3.1.1(1) The kinin system 40
III-3.1.1(2) Bradykinin formation and physiological significance 41
III-3.1.1(3) Transduction of signals from BK receptors 41
III-3.1.1(4) BK receptors as therapeutical targets 43
III-3.1.2 Endothelium-derived hyperpolarizing factors (EDHF) 43
III-3.1.3 Prostacyclin (PGI2) 44
III-3.2 Endothelium-derived vasocontracting factors (EDCF) 44
III-3.2.1 Reactive oxygen species (ROS) 44
III-3.2.2 Thromboxane A2 & Prostaglandin H2 (TxA2 & PGH2) 46
III-3.2.3 Angiotensin 46
III-4 Age-related endothelial dysfunction 47
III-5 Hyperglycemia and vascular toxicity (glucotoxicity) 48
III-5.1 Hyperglycemia and endothelial and vascular dysfunction 48
III-5.1.1 Role of the angiotensin system in senescence induced by hyperglycemia..... 49
III-5.2 Hyperglycemia and inflammation 50
III-5.3 Hyperglycemia and oxidative stress 50
III-5.3.1 Auto-oxidation of glucose 50
III-5.3.2 Advanced glycation end-products (AGEs) 51
III-5.3.3 The polyol route 51
III-5.3.4 Activation of protein kinase C by diacylglycerol 52
III-5.3.5 The hexoamine pathway 52
IV- Endothelial senescence and vascular function 53
IV-1 Cellular senescence 54
IV-1.1 Characteristics of senescent cells 54
IV-1.2 Mechanisms of senescence 56
IV-1.2.1 Replicative senescence 56
IV-1.2.2 Premature senescence 57
IV-1.3 Mechanisms of cell cycle arrest in senescence 58

IV-2 Contribution of cellular senescence in vascular aging ……..….. 60
IV-2.1 Senescence and endothelial dysfunction ……..….. 61
IV-2.2 Oxidative stress at the origin of endothelial senescence ……..…….. 61
IV-2.3 Role of the Angiotensin System in Vascular Senescence ……..…. 63
IV-2.4 Hyperglycemia and endothelial senescence …….. 64
IV-2.4.1 Role of the angiotensin system in senescence induced by hyperglycemia…..... 65
Hypothesis and aims ……..…66
V-Results ……..……..…. 67
PART 1: Methodological approach to the study tissue MPs: MPs from splenocytes as a signature of the immune cells activation ……..……. 67
1-Background and proof of concept ……..…68
2- Methodological approach ……..…… 69
2-1 Variations of splenocyte-derived MPs (SR-MPs) with age and stimulus …..…..… 70
2-1.1 Ageing and LPS or PMA/I treatment reduce splenocyte recovery ……..... 70
2-1.2 Ageing and LPS or PMA/I treatment promote the release of SR-MPs ……...…. 70
2.2- Ageing and LPS or PMA/I treatment modify the pro-senescent effects of SR-MPs 71
2.3- High glucose concentration modify the ability of splenocytes to shed MPs ……..… 72
3- Impact of short and long term incubation for the investigation of SR-MP vascular effects: Preliminary studies with coronary artery rings ……..…. 73
4- Impact of glucose concentration for the investigation of SR-MP vascular effects: Preliminary studies with coronary artery rings ……..…. 74
Article 1 ……..…….. 76
Result Part2: Pharmacological protection of the endothelium against the pro-senescent action of SR-MPs in porcine coronary arteries at high glucose concentration …..…... 101
1- Background ……..... 102
1-1 Glucose transport by SGLT1 and SGLT2 ……..….. 102
1-1.1 SGLT1 ……..... 103
1-1.2 SGLT2 ……..... 103
1-2 SGLT1 and SGLT2 expression in diabetes and cardiovascular disorders ……..... 104
1-3 SGLT1 and SGLT2 and cellular dysfunction ……..……..... 105
2- Rationale ……..…... 105
3- Preliminary manuscript ……..…. 108

VI- General discussion and conclusion ……..……..116

Bibliography ……..…….. 119
Annexes …….. 152

Scientific production ……...……152

Résumé extensif en français

Contexte et état de l'art

Les microparticules, aussi appelées microvésicules, sont des fragments de la membrane plasmique émis en réponse à un stress cellulaire et retrouvées dans les fluides biologiques tels que le sang, l'urine, le liquide céphalo-rachidien, les larmes, les crachats ou les liquides bronchoalvéolaires.

Dans le sang, les microparticules (MPs) circulantes sont des marqueurs de l'activation ou de l'atteinte cellulaire et également des effecteurs capables de disséminer un message biologique à des cellules endothéliales ou vasculaires à distance de la cellule émettrice.

Ces dernières années, le laboratoire ainsi que d'autre équipes nationales et internationales a démontré que des concentrations élevées de MPs circulantes témoignent de la sévérité de la maladie qu'elle soit chronique ou aigue. (Ridger, Boulanger et al. 2017) De plus l'origine cellulaire elle-même de ces MPs varie avec l'évolution de la maladie comme par exemple dans le choc septique dans lequel les MPs d'origine endothéliale sont indicatives d'une évolution vers la coagulopathie, également associée à la présence de MPs d'origine neutrophilique. (Delabranche, Quenot et al. 2016, Delabranche, Stiel et al. 2017)
Dans le sang périphérique, les MPs circulantes sont majoritairement d'origine plaquettaire, leucocytaire, endothéliale ou érythrocytaire. Cependant, d'autres MPs émises par des organes hautement vascularisés sont détectables dans le flux sanguin périphérique, notamment en situation de stress cellulaire majeur, comme au cours de l'ischémie-reperfusion ou lors d'un remodelage tissulaire important de l'organe. (Bakouboula, Morel et al. 2012) (Bakouboula, Morel et al. 2008, Bal, Ederhy et al. 2010) Ainsi des MPs de cellules hépatocytaires sont détectées par exemple dans la stéatose alcoolique ou des MPs cellules béta du pancréas chez les patients diabétiques de type 1 en rejet de greffe d'îlots pancréatiques. (Toti, Bayle et al. 2011, Moreau, Toti et al. 2012, Rautou, Vion et al. 2013, Lemoinne, Thabut et al. 2014).

Du fait de leurs propriétés procoagulantes et proinflammatoires, les MPs ont été longtemps étudiées comme des effecteurs endothéliaux capables d'amplifier la réponse inflammatoire endothéliale et procoagulante, de favoriser la formation du thrombus voire la constitution de plaques d'athéromes. Ainsi, les MPs d'origine leucocytaires sont associées au risque primaire et secondaire d'athérothrombose. (Koga, Sugiyama et al. 2005, Chironi,

Simon et al. 2006) En effet, les MPs leucocytaires et plaquettaires portent des molécules actives et sont capables d'interagir avec les cellules et avec les MPs par interactions contre-récepteurs ligands notamment via les protéines d'adhésion, ou via Fas/ Fas ligand, ou même par fusion lipidique.

Les propriétés procoagulantes des MPs sont dues à la phosphatidylsérine un aminophospholipide anionique (Pser) et au facteur tissulaire (FT) initiateur cellulaire de la coagulation inductible dans les cellules endothéliales, monocytaires et neutrophilique principalement. La Pser est séquestrée dans le feuillet interne de la membrane plasmique au repos et transloquée dans le feuillet externe en réponse à l'influx calcique concomitant à la stimulation. Ainsi, la Pser constitue une surface anionique capable de focaliser les facteurs de la coagulation et leur assemblage, nécessaire à la production de l'enzyme ultime de la cascade : la thrombine qui clive le fibrinogène en fibrine constituant ainsi un maillage stabilisant le caillot ou le thrombus. Plus récemment la fonction hémostatique des MPs a été mise en évidence par le biais du syndrome de Scott, un syndrome hémorragique caractérisé par une incapacité de la membrane plasmique des plaquettes, érythrocytes et lymphocytes à émettre des MPs procoagulantes en réponse à une stimulation aigue, c'est à dire en situation de blessure vasculaire provoquée. (Toti and Freyssinet 2005, Lhermusier, Chap et al. 2011) D'autres données cliniques ou dans des modèles animaux indiquent que le risque hémorragique est aggravé en cas de cytopénie et de concentration faible de MPs circulantes. Ainsi, les MPs circulantes peuvent être considérées comme une surface additionnelle pour l'assemblage des complexes de la coagulation en situation de réponse hémostatique. Il est intéressant de noter que les MPs d'origine endothéliale et leucocytaire ont donc un double pouvoir procoagulant porté par la Pser et le FT.

La translocation de la Pser en réponse à l'activation cellulaire est le résultat de (i) l'inhibition des flippases qui au repos rapatrient toute Pser du feuillet externe vers le feuillet interne de la bicouche lipidique, et à (ii) l'activation des floppases responsables du transport inverse sous l'effet de l'influx calcique et des scramblases, brasseurs de phospholipides non spécifiques. Une floppase a récemment été identifiée grâce à l'identification de mutations dans le gène de l'anoctamine 6 (TMEM16F), chez les patients atteints du syndrome de Scott. (Toti and Freyssinet 2005) Cependant, les modalités d'action des différentes floppases restent mal connues, notamment en raison de transporteurs qui sont des floppases occasionnelles comme les transporteurs ABCA-1, MDR-1, MDR-2, et du fait d'un défaut d'outil

pharmacologiques réellement spécifiques de chaque transporteur. (Ridger, Boulanger et al. 2017).

Dans le contexte général des maladies associées aux désordres cardiovasculaires, les concentrations circulantes de MPs d'origine endothéliale sont généralement élevées comme dans le diabète, l'infarctus du myocarde, et l'hypertension artérielle pulmonaire.(Omoto, Nomura et al. 2002, Morel, Hugel et al. 2004, Davi and Ferroni 2005, Koga, Sugiyama et al. 2005, Koga, Sugiyama et al. 2006, Feng, Chen et al. 2010, Diehl, Aleker et al. 2011, Boulanger, Loyer et al. 2017) Plus récemment, le laboratoire a montré que les MPs émises par des cellules endothéliales de coronaires de porc induites en sénescence réplicative étaient des effecteurs pro-sénescents induisant un arrêt prématuré du cycle cellulaire. De plus, des MPs endothéliales pro-sénescentes circulaient chez les patients à risque cardiovasculaire élevé. Les propriétés pro-sénescentes des MPs endothéliales sont associées à une activation précoce et redox-sensible des MAP-kinases et de la PI-3kinase. (Abbas, Jesel et al. 2017) Ces résultats sont cohérents avec l'observation d'une atteinte de la fonction vasculaire endothélium-dépendante décrite dans les modèles animaux chez qui la sénescence endothéliale est détectée précocement aux bifurcations vasculaires prédisposant à la dysfonction endothéliale, comme la crosse aortique. (Sonia Khemais, Thèse de l'Université de Strasbourg 2017).

Le vieillissement est associé à une modification de l'ensemble du système immunitaire touchant tous les organes de l'immunité et affectant la production de lymphocytes B et T matures. On constate une perte progressive de leur fonction associée à la sénescence de certains sous-types et l'apparition de tumeurs et de maladies auto-immunes. Le rôle et l'importance des MPs émises par les cellules leucocytaires dans un contexte de sénescence cellulaire reste peu exploré, notamment en raison de la difficulté à obtenir des quantités suffisantes de MPs issues de cellules primaires. En effet, chez le petit animal les volumes sanguins ne sont pas suffisants pour autoriser des études de modèles de communication intercellulaire. Les MPs de cellules leucocytaires émises en conditions de stress cytokinique à partir de lignées immortalisées sont connues pour leurs propriétés pro-inflammatoires vis à vis de l'endothélium. Ainsi les MPs de cellules monocytaires constituent une voie majeure de sécrétion d'IL-1ß active tandis que les MPs lymphocytaires convoient Fas et Fas ligand. (Albanese, Meterissian et al. 1998, MacKenzie, Wilson et al. 2001).

Le laboratoire a également mis en évidence dans un modèle de communication intercellulaire induite par les MPs, que les MPs leucocytaires issues de la rate des rats étaient capables de déclencher une réponse pro-sénescente des cellules endothéliales coronaires sans initier d'apoptose, suggérant un effet spécifique. (Thèse Ali El Habhab, Université de Strasbourg, 2018). Par ailleurs, ces MPs splénocytaires sont majoritairement émises par des cellules de l'immunité innée ou adaptative (lymhocytes B, lymphocytes T, neutrophiles, monocytes, plaquettes)

Dans le contexte plus particulier du diabète, l'équipe a établi que les cellules béta sécrétrices d'insuline sont capables d'émettre des MPs porteuses de FT en réponse à un stimulus cytokininique. Ces MPs se comportent comme des effecteurs pro-inflammatoires et proapoptotiques et agissent au moins partiellement par des voies de signalisation dépendantes du récepteur GLP-1. (Gleizes, Constantinescu et al. 2014, Gleizes, Kreutter et al. 2016) Enfin, S. Khemais a établi au laboratoire que la sénescence des cellules endothéliales de coronaires était induite prématurément au bout de 4 jours en présence de forte concentrations de glucose (25 mM) et que les voies de signalisation impliquées sont redox-sensibles. (Khemais-Benkhiat, Abbas et al. 2016)

Il est important de noter que les cellules endothéliales en sénescence prématurée sont dysfonctionnelles et présentent un phénotype particulier pro-inflammatoire et procoagulant, que ce soit en réponse au glucose élevé ou en réponse aux MPs pro-sénescentes d'origine endothéliale ou leucocytaire.

Hypothèses et objectifs

L'ensemble de ces données ont conduit aux hypothèses suivantes:

- les MPs émises par les leucocytes contribuent à la sénescence endothéliale et au vieillissement prématuré du vaisseau
- Au cours du diabète, l'émission soutenue de MPs d'origine leucocytaire contribue à la sénescence prématurée de l'endothélium
- La protection pharmacologique de l'endothélium contre la sénescence prématurée induite par les MPs est une cible intéressante pour la protection vasculaire au cours du diabète

Pour explorer ces hypothèses nos objectifs ont été

- De valider une approche méthodologique pour l'isolement des MPs tissulaires en utilisant la rate comme source de MPs leucocytaires, les artères coronaires porcines comme source de MPs du tissus vasculaire
- D'étudier l'effet de l'âge et du glucose à forte concentration sur l'émission des MPs tissulaires
- De caractériser les propriétés pro-sénescentes des MPs isolées de la rate sur l'endothélium coronaire dans un modèle de sénescence induite par le glucose.
- D'établir l'effet de ces MPs sur la réponse vasculaire dépendante de l'endothélium, notamment dans des conditions de stress glucosé.

Résultats

I- Approche méthodologique pour l'étude des MPs tissulaires :

Nous avons choisi d'utiliser les cellules de rate du rat comme source de cellules immunes. L'avantage de cette méthode est la possibilité d'étudier des MPs d'animaux traités par des agents pharmacologiques. Le procédé est basé sur l'isolement de splénocytes après choc osmotique et leur conservation en milieu de culture avant une éventuelle stimulation par du LPS ou une combinaison de PMA/ionophore A23187 afin d'étudier les capacités des cellules à émettre des MPs en condition de stress. Les MPs sont ensuite isolées par des étapes de centrifugation différentielle afin d'obtenir une suspension de MPs concentrées et lavées issues de cellules non stimulées (SMP_{CTL}) ou stimulées par les agonistes (SMP_{LPS} , $SMP_{PMA/I}$)

Pour valider la fiabilité de la méthode d'extraction, nous avons comparé les concentrations et les propriétés des MPs émises à l'état basal par les splénocytes de rats jeunes, d'âge moyen et vieux après 24h de mise en culture. Les MPs ont ét mesurées soit par dosage prothrombinase, soit par dosage de leur contenu protéique. Les propriétés pro-sénescentes des MPs isolées ont été évaluées après application 48h à des cellules endothéliales (ECs) de coronaires de porc dans un modèle cellulaire visant à se rapprocher des conditions des premières lésions athéro-thrombotiques.

Les données montrent que la concentration de MPs émises à l'état basal (sans stimulation des splénocytes) augmente significativement avec l'âge et que la sénescence endothéliale mesurée par l'activité de la galactosidase spécifique de la sénescence (SA-ß galactosidase) est induite de manière significativement supérieure par les MPs de rats d'âge moyen et de rats âgés comparativement aux rats jeunes. Ainsi, les propriétés pro-sénescentes des MPs splénocytaires augmentent avec l'âge du rat et les splénocytes eux-mêmes ont une capacité à émettre des MPs qui augmente avec l'âge, ce dernier favorise aussi la production de MPs en réponse au PMA/I ou au LPS (PMA /I : 2.32 fois *vs.* 1.57 fois splénocytes de rats d'âge moyen *vs.* Rats âgés).

La cinétique d'émission des MPs semble être un événement très précoce que ce soit par les splénocytes ou par les anneaux d'artères coronaires. En effet après 1h, les MPs sont détectables et les concentrations élevées de glucose augmentent significativement leur émission (30 % après 90 min pour les MPs splénocytaires et 25 % après 50 min pour les anneaux d'artères).

En conclusion, la méthode d'extraction des MPs de la rate et des vaisseaux est fiable et reproductible. Quelle que soit la source tissulaire, les MPs sont procoagulantes (dosage prothrombinase) et la méthode conserve les propriétés effectrices des MPs qui reflètent l'état physiopathologique (pas d'effet pro-sénescent des MP de rat jeune), ou l'environnement du tissu (forte teneur en glucose). L'identification des mécanismes en jeu dans l'émission des MPs reste à réaliser dans les deux modèles avant d'envisager un conrtôle pharmacologique. La rate est une source importante de MPs chez les petits animaux.

II- Les concentrations élevées de glucose (« high Glucose, HG ») potentialisent l'effet pro-sénescent des MPs leucocytaires sur l'endothélium coronaire et sur le tonus vasculaire.

L'effet pro-sénescent et pro-inflammatoire des MPs splénocytaires SMP_{LPS}, $SMP_{PMA/I}$, SMP_{CTL} a été mesuré dans un modèle de sénescence endothéliale coronaireinduite par le glucose (25 mM appliqué pendant 96h).

Après 48h de traitement par le glucose, l'addition de SMP_{LPS}, $SMP_{PMA/I}$, double l'effet pro-sénescent du glucose mesuré par l'activité SA-ß galactosidase dans les cellules d'artères coronaires de porc. Les western blots indiquent que le HG potentialise la surexpression des marqueurs pro-sénescents p-16 et p21 induites par les SMP_{LPS}, $SMP_{PMA/I}$ mais pas aux SMP_{CTL}. Par ailleurs, si SMP_{LPS}, $SMP_{PMA/I}$ induisent un baisse modérée de l'expression de la eNOS et une surexpression modeste des protéines pro-inflammatoires COX-2, ICAM-1, VCAM-1, une synergie est également observée en présence de HG qui double voir triple la réponse induite par les SMP_{LPS}, $SMP_{PMA/I}$ alors que la réponse aux SMP_{CTL} n'augmente au plus que de 50 %, en présence de HG.

Pour confirmer la dysfonction endothéliale consécutive à la sénescence, et parce que les COX-2 sont susceptibles d'intervenir dans la réponse EDCF, les SMPs ont été appliquées à des anneaux d'artères coronaires de porc à différentes concentrations et en présence de milieu normoglucosé (NG) ou HG. Après 18h d'incubation (37 °C), la relaxation endothéliale-dépendante a été mesurée en réponse à la bradykinine. Les résultats indiquent que les SMP_{CTL} n'ont aucun effet sur le tonus vasomoteur mais que les SMP_{LPS}, $SMP_{PMA/I}$ réduisent la relaxation induite par la bradykinine en milieu NG et que le HG amplifie cet effet, alors que seul il n'altère pas la réponse après 18h d'incubation. L'impact endothélial a été confirmé par analyse immuno-histochimique des anneaux de coronaires qui montre une accumulation des ROS induite par les SMP_{LPS}, $SMP_{PMA/I}$ uniquement et qui est augmentée en

présence de HG. Au contraire, le HG diminue l'expression de la eNOS de manière drastique en présence de SMP_{LPS}, $SMP_{PMA/I}$. Alors que le HG seul augemente modérémment l'expression endothéliale de COX-2, ICAM-1 et VCAM-1 dans la monocouche endothéliale des anneaux, le HG potentialise drastiquement leur effet (multiplié par un facteur 2 ou 4).

En conclusion, les MPs de splénocytes stimulés par LPS ou PMA/I mais pas celles de cellules naives non stimulées sont des effecteurs endothéliaux pro-sénescents, pro-inflammatoires et pro-oxydants. L'effet optimum a été caractérisé après 48h d'exposition dans un modèle de cellules endothéliales primaires de coronaires de porc isolées, mais aussi dans un modèle d' anneaux d'artères coronaires de porc. Ces MPs conduisent à une dysfonction endothéliale caractérisée par à une capacité de relaxation dépendante de l'endothélium moindre après une exposition prolongée (18h). Les mécanismes restent à identifier.

III- Protection pharmacologique de l'endothélium contre les SMPs prosénescentes et la potentialisation de leurs effets par le glucose.

Parce que de fortes concentrations en glucose (HG) favorisent l'apparition d'une sénescence endothéliale dans les cellules endothéliales d'artères coronaires de porc ainsi que l'apparition d'un phénotype endothélial pro-inflammatoire et procoagulant, nous avons cherché à évaluer la réponse endothéliale-dépendante d'anneaux d'artères coronaires après incubation en présence de HG. S. Khemais a montré dans sa thèse que les transporteurs SGLT (Sodium Glucose transporters) sont impliqués dans la sénescence endothéliale induite par les fortes concentrations de glucose (HG, 25 mM). Dans notre série d'expériences, l'exposition prolongée au HG pendant 18h en présence d'inhibiteurs des transporteurs SGLT a indiqué que le HG diminue la réponse contractile à l'activateur U46619l et que les inhibiteurs des SGLT1 ou de SGLT2 abolissent complètement l'inhibition de façon endothéliale-dépendante aucune protection en absence d'endothélium). De plus, l'utilisation d'inhibiteurs pharmacologiques ajoutés 15 minutes avant la mesure indique que l'effet n'est pas dépendant des COX ou des ROS mais implique les canaux potassium dépendants SKa et IKa. Dans le tissu artériel, l'analyse des anneaux après 18h d'incubation montre que la concentration en glucose favorise l'accumulation des ROS dans le tissu et la surexpression des COX-2, de VCAM-1, ICAM-1 et la répression de l'expression de la eNOS dans la couche endothéliale de manière SGLT1 et 2 dépendante.

L'ensemble de ces données indique que la perte de vasocontraction dans les vaisseaux après 24h d'exposition prolongée à une forte concentration de glucose est dépendante des transporteurs SGLTs. L'analyse histologique suggère un mécanisme redox-sensible qui implique tout le tissu pariétal et la mesure des MPs émises par l'anneau aortique après moins de 2h suggère une action très précoce des inhibiteurs de SGLTs qui limitent également la production de MPs vasculaires endogènes (cf. paragraphe précédent). Les résultats devront être complétés par l'analyse d'autres marqueurs d'activation du tissu pariétal comme les récepteurs de l'angiotensine, l'enzyme de conversion de l'angiotensinogène ou le facteur tissulaire, tous impliqués dans la réponse athérothrombogénique du vaisseau et plus particulièrement de l'endothélium.

Conclusion générale

Ce travail démontre que l'étude des MPs tissulaires à partir d'organes est une bonne alternative à la mesure des MPs circulantes, particulièrement lorsque l'animal est de petite taille. Notre méthode d'extraction est suffisamment douce pour conserver les propriétés de biomarqueurs des MPs. Ainsi, Les MPs extraites de rate ont des propriétés différentes selon l'état physiopathologique ou le traitement du rat.

Nos mesures par différentes méthodes (dosage protéique ou dosage prothombinase), indiquent que l'âge ou la concentration en glucose modifient le degré d'émission des MPs par les tissus et cela de façon très précoce, mais aussi les propriétés pro-sénescentes et pro-indlammatoires des MPs examinées dans un modèle de sénescence endothéliale à partir de cellules primaires d'artères coronaires de porc ou directement par analyse histochimique d'anneaux d'artères coronaires. Enfin, dans notre modèle, seules les MPs émises en réponse à un stress induisent une dysfonction endothéliale, ce qui suggère également que les MPs leucocytaires sont une cible pharmacologique d'intérêt dans les maladies chroniques qui favorisent une activation cellulaire soutenue. Dans ce cadre, la contribution des transporteurs SGLT1/2 dans la réponse à long terme de l'endothélium inflammatoire au glucose reste à confirmer, notamment par la recherche de l'expression des miRNA et des proteines dans les tissus et cellules. Nos résultats suggèrent qu'une des stratégies thérapeutiques éventuelles serait de limiter la sénescence endothéliale et la libération de MPs endothéliales pro-sénescentes secondaires.

THESIS ENGLISH VERSION

Introduction

Humans and other mammals live numerous relationships with pathogenic and non-pathogenic microorganisms that may debilitate or contribute to ordinary homeostasis. The immune system continuously accommodates to protect the host, through early innate and late adaptive immune systems that incorporate self-non self discrimination to recognize and eventually eliminate pathogenic organisms.

The vessel is the route for immune cells of the innate response to target microorganisms at site of infection. However, immune responses are also the support of immunothrombosis that are deleterious to vascular health and may favour excessive inflammatory responses of the endothelium leading to a prothrombotic endothelial phenotype.

Numerous studies have shown that endothelium dysfunction associated with loss of endothelial vasoprotective properties, is one of the major factors contributing to vascular disease in the elderly and in chronic diseases with cardiovascular and thrombotic disorders such as diabetes. Recently, it has been suggested that endothelial senescence progressively occurring with aging may contribute to the endothelial dysfunction.

Chapter One

Overview of the immune system and of its responses to E. coli lipopolysaccharide

I-1 The spleen

The spleen is the largest filter in the body of blood, located in the abdomen and connected to the stomach (Kraal 1992, Steiniger and Barth 2000).. Figure (1). The spleen combines the cells of innate and adaptive immune system and also enables removal of the old erythrocytes from the bloodstream. This function, in combination with a highly regulated lymphocyte responses and proliferation, makes of the spleen the most important organ for antibacterial and antifungal immune interactions (Mebius and Kraal 2005) and leads to the effective removal of microorganisms transmitted by blood and cellular debris.

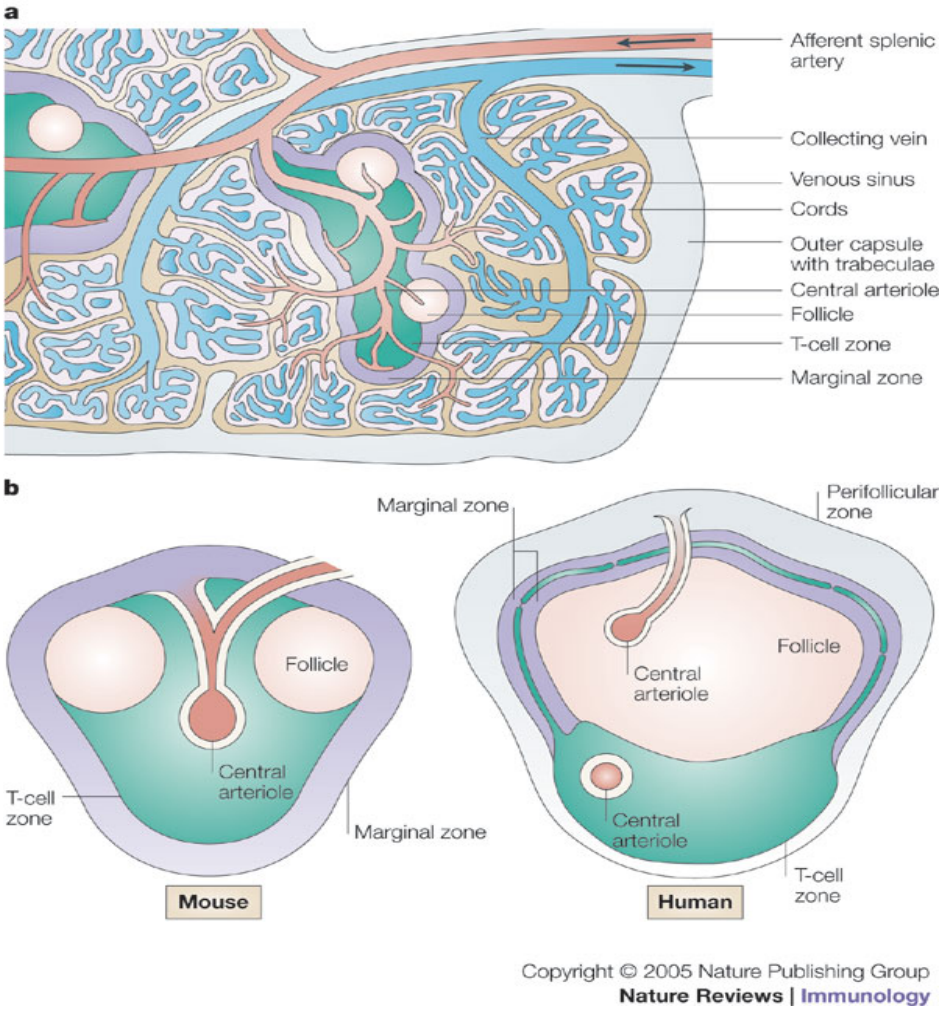


Figure 1: Structure of the spleen (Mebius and Kraal 2005) *The spleen is organized in regions called the red pulp and white pulp, which are separated by an interface called the marginal zone (MZ) (MacNeal 1929)*

I-1.1 Immune cells and leukocytes in the spleen:

Immune cells in the spleen include different subsets of T and B cells, dendritic cells (DCs) and macrophages. The macrophages of the red pulp are specialized in phagocytosis of aging red blood cells and regulate iron recycling and release. MZ Macrophages of the

marginal zone (MZ) and metallophilic macrophages express a unique set of pattern-recognition receptors and contribute to the removal of blood bacteria and viruses. Also, the MZ contains MZ B cells and dendritic cells (DCs) (Mebius and Kraal 2005). The spleen contains all major types of mononuclear phagocytes, including macrophages, DCs and monocytes. These cells identify pathogens and cellular stress, regulate tissue homeostasis and inflammatory responses, remove dead cells and foreign material, and shape adaptive immunity (Bronte and Pittet 2013). In addition to DCs and macrophages, natural killer T (NKT) cells of the spleen, which sense lipid antigens secrete cytokines and induce downstream activation of adaptive immune cell types (Barral, Sanchez-Nino et al. 2012), see **figure 1**.

I-2 Overview of the Immune Response

Immune responses rely on self-non self-discrimination to respond to invading microbes and other exogenous threats and can be divided in innate immune system and adaptive immune systems (Chaplin 2010).

I-2.1 The innate immune system

The innate immune response is essential for barrier against microbial pathogens. The innate immune system involves all aspects of host immune defense mechanisms including physical barriers, such as epithelial cell layers, the epithelial cilia, and the secreted mucus layer that overlays the epithelium. Another local barrier is obtained through the recruitment of neutrophils that shed neutrophil extracellular Traps (Nets) that constitute at site of bacterial infection a network of decondensed chromatin also carrying oxidases and proteases. The innate response also requires soluble proteins and bioactive small molecules that are either primarily present in biological liquids (such as the defensins and complement proteins) or which are released from cells as they are activated. Among them chemokines that attract inflammatory leukocytes, lipid mediators of inflammation, enzymes and bioactive amines that participate to tissue inflammation, cytokines that act as autocrine or paracrine cell effectors, and reactive free radical species. Finally, the innate immune system relies on membrane receptors that bind the molecular patterns expressed by microbial surfaces (Chaplin 2010).

There is a noticeable comparable pattern of gene expression in human peripheral blood mononuclear cells caused by bacterial lipid polysaccharides and killed bacteria although some

differences were observed according to each type of bacteria, most probably linked to discrepancies in bacterial virulence (Boldrick, Alizadeh et al. 2002).

I-2.2 The Adaptive immune system

The adaptive immune response relies on T cells and B lymphocyte cells (Medzhitov and Janeway 2000). Adaptive responses are basically based on specific antigen receptors expressed on the surfaces of T- and B-lymphocytes. The antigen-specific receptors of the adaptive response are encoded by the genes sorted by somatic rearrangement of germ-line gene elements to form T cell receptor and B cell receptor (Chaplin 2010).

I-3 *Escherichia coli*

I-3.1 Structure & Classification

Escherichia coli (*E. coli*) is one of the most common inhabitants of the human intestinal tract and considered to be an indicator of the quality of water and food contaminated with faeces.

E. coli is the predominant facultative anaerobic bacteria. The organism usually colonizes the infant's digestive system within hours of life, and then, *E. coli* and the host derive mutual benefit.

E. coli is the canonical specie from genus Escherichia within the family Enterobacteriaceae. Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups (Selander, Caugant et al. 1986, Herzer, Inouye et al. 1990) and that virulent extra-intestinal strains belong to group B2 and, to a lesser extent, to group D (Bingen, Picard et al. 1998, Boyd and Hartl 1998, Picard, Garcia et al. 1999, Johnson and Stell 2000), and can be divided into seven subgroups (A0, A1, B1, B22, B23, D1 and D2) according to the combination of the genetic markers (Escobar-Paramo, Le Menac'h et al. 2006).

Table 1: *E. coli* phylogenetic classification

Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacteriales
Family	Enterobacteriacae
Genus	*Escherichia*
Species	*coli*

E. coli can be recovered easily from clinical specimens on general or selective media under aerobic conditions at 37°C. *E. coli* in stool are most often recovered on MacConkey or eosin methylene-blue agar, which selectively grow members of the Enterobacteriaceae and allows differentiation of enteric organisms depending on the morphology. About 90% of *E. coli* strains are lactose positive whereas its 99% of *E. coli* strains are positive with indole test which is the single best test for differentiation from other members of the Enterobacteriaceae (Balows and Shadomy 1991).

I-3.2 *E. Coli* as a pathogen

Adam, in 1927, suspected that certain strains of *E. coli* were the cause of infantile diarrhea and identified several by a series of fermentation and biochemical tests (Adam 1927). Then Goldschmidt, Cooper and coll. isolated the strains from babies with diarrhea (Goldschmidt 1933, Cooper 1955). *E. coli* is a leading reason of infant diarrhea, killing hundreds of thousands of children annually worldwide (DeVinney, Gauthier et al. 1999, Anselmi, Toti et al. 2017). Various *E. coli* strains cause a wide range of extra-intestinal diseases, such as urinary tract infections, neonatal meningitis, diarrhea, septicaemia (Drasar 1974, Orskov and Orskov 1992), and some strains contain endotoxins that cause traveler's diarrhea and very. The term endotoxins refers to toxic materials, secreted toxins being known as ‘‘exotoxins” (Wang and Quinn 2010).

I-3-3 Lipopolysaccharide of *E. coli*

The outer membrane of *E. coli* is composed of two lipid monolayers, the inner consisting primarily of glycerophospholipids and similar in lipid composition to the two monolayers of the inner membrane. In contrast, lipopolysaccharide (LPS) is the major component of the outer monolayer of the outer membrane. (Figure 2)

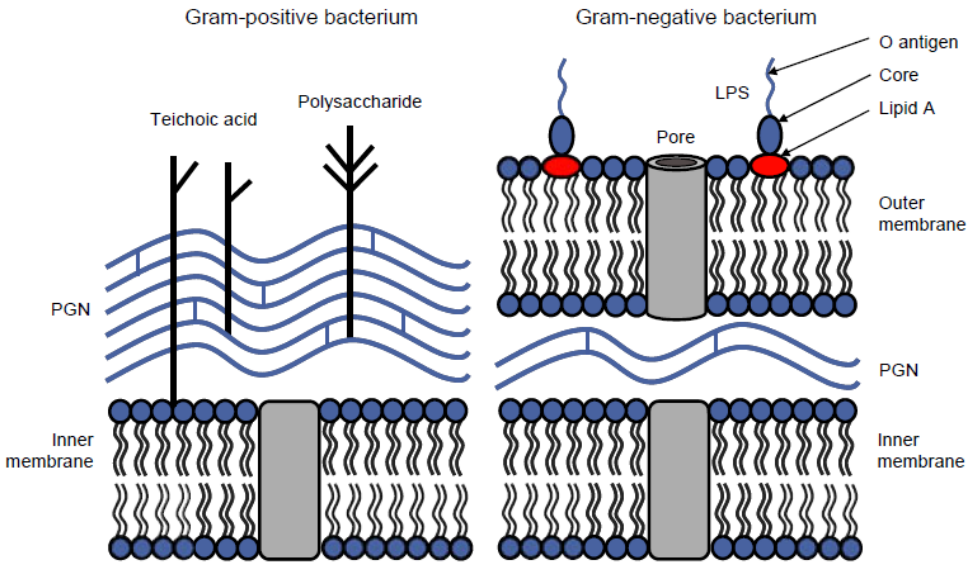


Figure 2: Structure of bacterial cell walls. *Only Gram-negative bacteria have an outer membrane that contains lipopolysaccharides (LPS)(Turner, Healey et al. 2012)*

I-3.3.1 Structure of lipopolysaccharide of *E. coli*

The LPS of *E. coli* is composed of three parts: lipid A, Core oligosaccharides, and O-antigen replicate (Figure 3).

Lipid A is a hydrophobic component of the LPS which is located in the outer leaf of the outer membrane, while core polysaccharides and O-antigen replicate on the surface of the bacterial cells (Holst 1999, Raetz and Whitfield 2002, Raetz, Reynolds et al. 2007). The core-lipid A and the O-antigen are synthesized at the cytoplasmic surface of the inner membrane, and then assembled in the periplasm to produce LPS molecules further exported across the inner membrane to the outer leaflet of outer membranes (Doerrler 2006).

The O-antigen consists of repeating oligosaccharide units (O units) each containing three to five or more monosaccharide residues such as glucose, galactose, mannose, rhamnose, fucose, and, rarely, dideoxyhexoses such as abequose, tyvelose, paratose, or colitose. O-antigen is synthesized by assembly of monosaccharide residues by glycosyltransferase. The O-antigens of LPS exhibit considerable diversity and is formed of homopolymers or heteropolymers. The connection of units in O-antigen may be branched or linear. In *E. coli*, the numbers of O-antigen groups are 164 (Wang and Quinn 2010).

Core oligosaccharides contribute to two distinct structures: the outer core of the O-antigen repeats and the inner core bound to lipid A. The structures of core oligosaccharides differ with *E. coli* strains R1, R2, R3, R4, and K-12, (Muller-Loennies, Lindner et al. 2002, Muller-

Loennies, Lindner et al. 2003) with some common core oligosaccharides that are keto-deoxyoctulosonate, heptose, D-galactose and D-glucose (Wang and Quinn 2010). Figure (3) Lipid A is the bioactive part of LPS and in *E. coli* it is a strong stimulator of the innate immune system (Golenbock, Hampton et al. 1991).

I-3.3.2 Response of the immune cells to LPS: PAMPS and PRRs

The innate immune system is the first line of host defense against pathogens that leads to a proinflammatory response. The innate immune response can differentiate self and the pathogens by pattern recognition receptors (PRRs). PRRs have common properties, first, PRRs can identify pathogen-associated molecular patterns (PAMPs), that are fundamental for the microorganism, therefore, difficult for the microorganism to alter at any stage of their life-cycle. The different innate immune cells of host organisms such as monocytes, macrophages, neutrophils and stem cells can identify LPS bound to a specific receptor exposed at their surfaces called toll-like receptor 4 (TLR4) (Poltorak, He et al. 1998, Medzhitov and Janeway 2000, Akira, Uematsu et al. 2006) (see below). Lipid A seems the portion of LPS mainly responsible for TLR4-mediated immune responses. The number and length of fatty acyl chains and the phosphate groups of lipid A determines the affinity for TLR4 (Loppnow, Brade et al. 1989, Rietschel, Kirikae et al. 1994) and its virulence (Montminy, Khan et al. 2006)

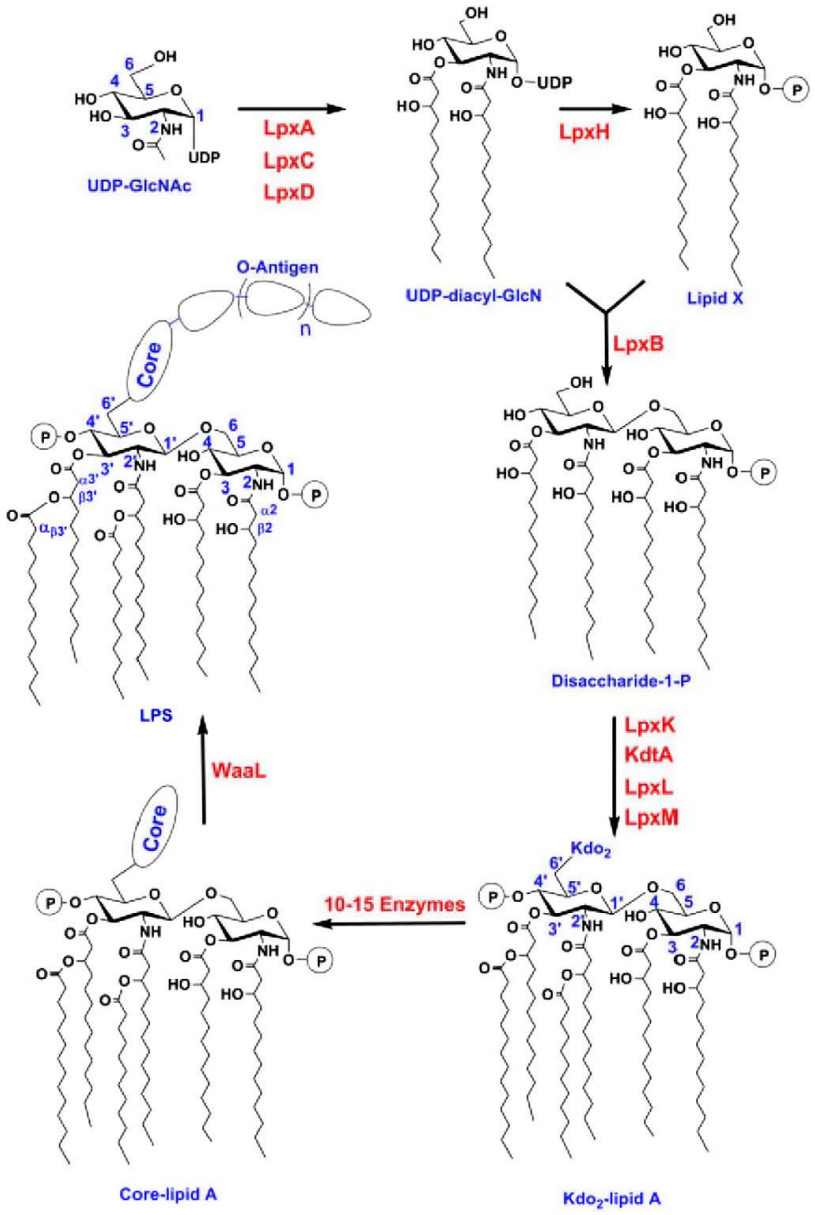


Figure 3: A: Structure and biosynthesis of LPS in *E. coli.* *For each reaction the substrate (blue) is converted by a specific enzyme (red). The structure of lipid A is shown in details, but structures of core oligosaccharides and O-antigen are simplified as symbols since there are many variations in these two regions (Raetz and Whitfield 2002, Raetz, Reynolds et al. 2007)*

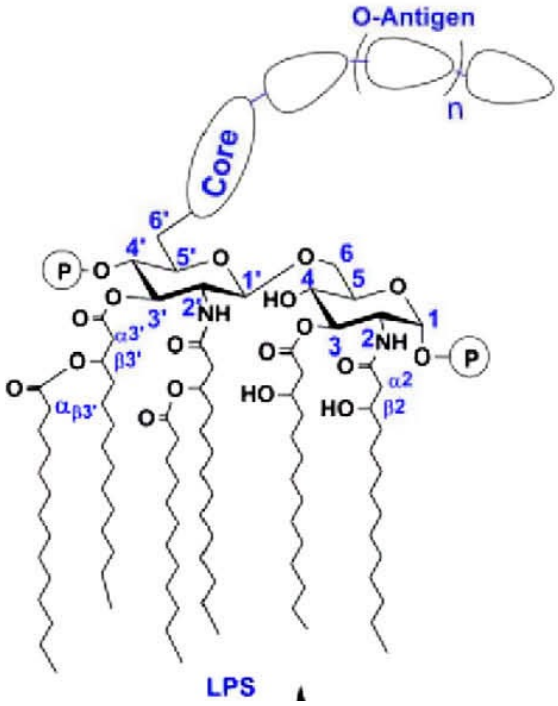


Figure 3B: Structure of LPS in *E. coli* (Raetz and Whitfield 2002, Raetz, Reynolds et al. 2007)

I-3.3.3 Bacterial virulence, LPS, severity of the disease and impact on therapy

Virulence is the ability of bacteria to infect the host and cause disease. It is characterized in terms of the number of infecting bacteria, the route of entry, the effects of host defense mechanisms, and intrinsic characteristics of the bacteria called virulence factors. In fact, the structure of LPS that varies from one bacterium to another, could itself affect the bacterial virulence (Wilkinson 1996). Since LPS is the first molecule barrier in Gram-negative bacteria, its structure is crucial for the sensibility of the pathogen to a variety of host proteins or metabolites, and to drugs (Whitfield and Trent 2014). Indeed, the LPS net electric charge is negative owing to different moieties such as acyl chains and phosphoethanolamine, that shields the bacteria from most antimicrobial peptides released by the host immune system that have a net positive charge and are termed "cationic antimicrobial peptides" (Kanazawa, Sato et al. 2009, Anaya-Lopez, Lopez-Meza et al. 2013). It also avoids recognition by TLR4 receptors (Wang and Quinn 2010) (see below). The excessive host responses to LPS and more specifically to Lipid A that is pro-inflammatory can lead to systemic inflammatory conditions from sepsis, to severe sepsis, and fatal septic shock. TLR4 pro-inflammatory signaling has also been associated with pathological responses to endogenous ligands in autoimmune

disorders and chronic inflammatory conditions accompanying development of atherosclerosis, neurodegenerative diseases, and others (O'Neill, Bryant et al. 2009, den Dekker, Cheng et al. 2010, Trotta, Porro et al. 2014).

I-3.3.4 LPS/TLR4 signal pathway

The Toll proteins were first detected in Drosophila, as necessary for the modeling of dorsal-central development of territories during embryogenesis (Anderson, Jurgens et al. 1985, Morisato and Anderson 1995). It was further recognized as pivotal in the initial stages of the innate immune response (Lemaitre, Nicolas et al. 1996, Cherry and Silverman 2006). Toll-like receptors (TLRs) are part of the PRR family and mediate pathogen-associated molecular pattern (PAMP)-induced signalling (Romani 2004). LPS can bind to TLR4 and activate the transcription factor nuclear factor κB (NF-κB), a key player in the inflammation process that up-regulates the expression of many genes (Imler and Hoffmann 2001, Wan and Lenardo 2010). The activation of TLR4 starts from an interaction of LPS with LPS-binding protein (LBP) circulating in blood. LBP facilitates extraction of LPS monomers from LPS aggregates by CD14 (Yu and Wright 1996, Iovine, Eastvold et al. 2002),a GPI-anchored glycoprotein at the surface of monocytes, macrophages, dendritic cells, and at a lower level, neutrophils (Goyert, Ferrero et al. 1988, Simmons, Tan et al. 1989). Subsequently, CD14 transfers the LPS to MD-2 (Lymphocyte antigen 96) in the TLR4/MD-2 complex (da Silva Correia, Soldau et al. 2001, Gioannini, Teghanemt et al. 2004, Gioannini, Teghanemt et al. 2005). By simultaneously binding to MD-2 and to the adjacent TLR4 receptor, LPS facilitates formation of TLR4/MD-2 complexes (Nagai, Akashi et al. 2002, Ohto, Fukase et al. 2007, Park, Song et al. 2009, Resman, Vasl et al. 2009). The TLR4 complex recruits two pairs of adaptor cytoplasmic proteins, TIRAP/MyD88 and TRAM/TRIF, to the TIR domain of the receptor by homotypic TIR–TIR interactions. MyD88 then recruits the IRAK4 and IRAK2 (or IRAK1) kinases so-called myddosome (Motshwene, Moncrieffe et al. 2009, Lin, Lo et al. 2010). This multimolecular complex triggers a signaling cascade leading to activation of NF-κB and MAP kinases and the up-regulation of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6). On the other hand, TRIF initiates a signaling pathway which leads to the expression of interferons (IFN) type I and IFN-inducible chemokines like IL-10, late-phase activation of NF-κB and MAP kinases follows (Kawai and Akira 2011).

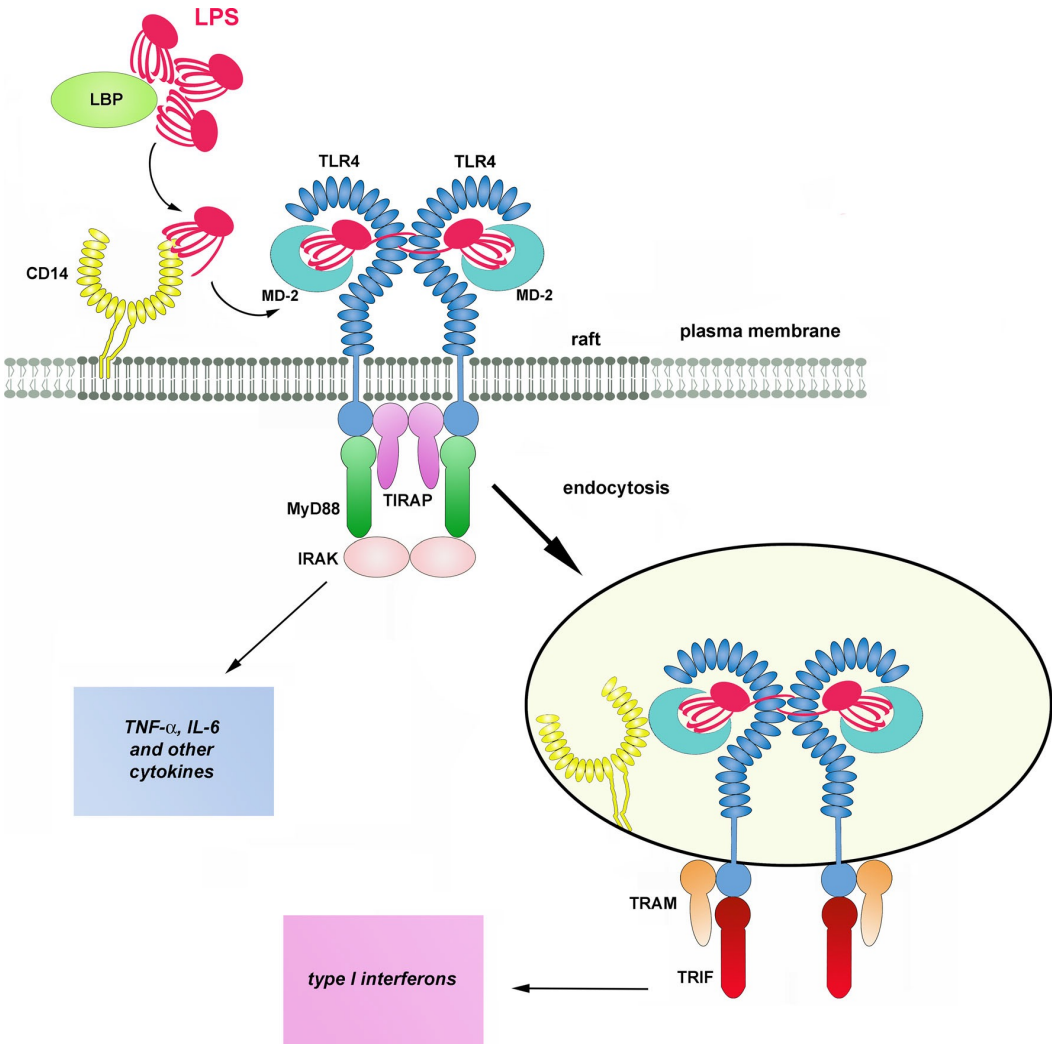


Figure 4: LPS recognition by the TLR4/MD2/CD14 complex. *LPS circulates in blood bound to LBP protein. The main LPS binding site is a large N-terminal highly hydrophobic pocket of CD14 monomers (Kim, Lee et al. 2005, Kelley, Lukk et al. 2013). The hydrophobic pocket of the adaptator protein MD-2 accommodates most of the lipid portion of LPS, however, the endotoxin interacts with the ectodomain of TLR4 molecule. Additionally, the phosphate groups of lipid A interact with positively charged amino acids of TLR4. Homotypic TIR–TIR domain interactions allow the formation of the signaling complex and the recruitment of downstream pro-inflammatory effectors leading to the up-regulation of cytokines and type 1 interferon. Adapted from* (Plociennikowska, Hromada-Judycka et al. 2015)

Chapter Two

Microparticles and vascular damage

II-1.1 Overview and physiopathological significance

The Microparticles (MPs) also termed microvesicles (MVs) are plasma membrane vesicles capable of transmitting a pro-inflammatory message to neighboring or remote target cells (Haddad 2002, Delabranche, Berger et al. 2012) (Bonello, Zahringer et al. 2007). Their diameter ranges from 50 nm to1µm and they contain cytoplasmic components such as proteins, lipids or nucleic acids. They form a storage pool of biologically active messengers that are able to diffuse into body fluids. MVs were first called "ectosomes" and their nature and origin are completely different from exosomes that are smaller vesicles released from the intracellular late endosomes. MP release occurs after plasma membrane phospholipid randomization between the inner and outer leaflets in response to cell activation, (Hugel, Martinez et al. 2005). Microparticles (MPs) are shed by cells in response to a variety of stimuli such as apoptosis, oxidative stress or inflammation. MPs are released into the surrounding extracellular matrix and tissues and into biological fluids. They exhibit the distinctive features of the plasma membrane of the cells they were stemmed from, essentially membrane proteins (CDs) that allow for the characterization of the MPs cell origin and also anionic phospholipids that confers them a procoagulant potential in blood. MPs may convey stress-specific activation markers. MP also convey biologically active molecules that were present in the plasma membrane or in the cytoplasm at the emission site (lipids, peptides, proteins, mRNA), and behave as cellular effectors (Morel, Jesel et al. 2011). Whatever the initial stimulation, MPs are reliable hallmarks of cell damage while cells they originate from remain sequestered in tissues or are already submitted to phagocytosis.

MPs also act as cellular effectors. They contribute to inflammatory and homeostatic responses, vascular remodeling and angiogenesis, cell survival, and apoptosis, and are known actors in atherothrombosis owing to their procoagulant properties (Haddad 2002). Indeed, hardly detectable in the peripheral blood of healthy individuals, procoagulant MP circulating at elevated levels are often associated with thrombotic propensity (Chironi, Simon et al. 2006, Morel, Toti et al. 2006), see below and figure 5.

II-1.2 Membrane remodeling and vesiculation

It is generally assumed that MPs form when the plasma membrane loses the asymmetrical distribution of lipids between the inner and outer leaflets. Under resting conditions, phosphatidylserine (PS) is located exclusively in the inner monolayer (Morel,

Jesel et al. 2011). After stimulation, there is a rapid egress of aminophospholipid to the outer leaflet, with decreased activity of flippase while floppase activity is induced.

Owing to a slower compensating phosphaticylcholine transport from the outer to the inner leaflet a transient mass imbalance of the exoplasmic leaflet occurs at the expense of the cytoplasmic one (Morel, Toti et al. 2006). Ultimately, membrane budding is resolved by the release of MPs that expose PS and hijack membrane antigens and cytoplasm components. The dynamic balance of cell stimulation, cell proliferation, and death within the vessels is reflected by the variation in MP populations that constitute a vascular storage pool. Furthermore, MPs modulate a variety of cell responses such as cell death, vasomotricity, redox signaling, inflammation, thrombosis, angiogenesis and tissue remodelling.

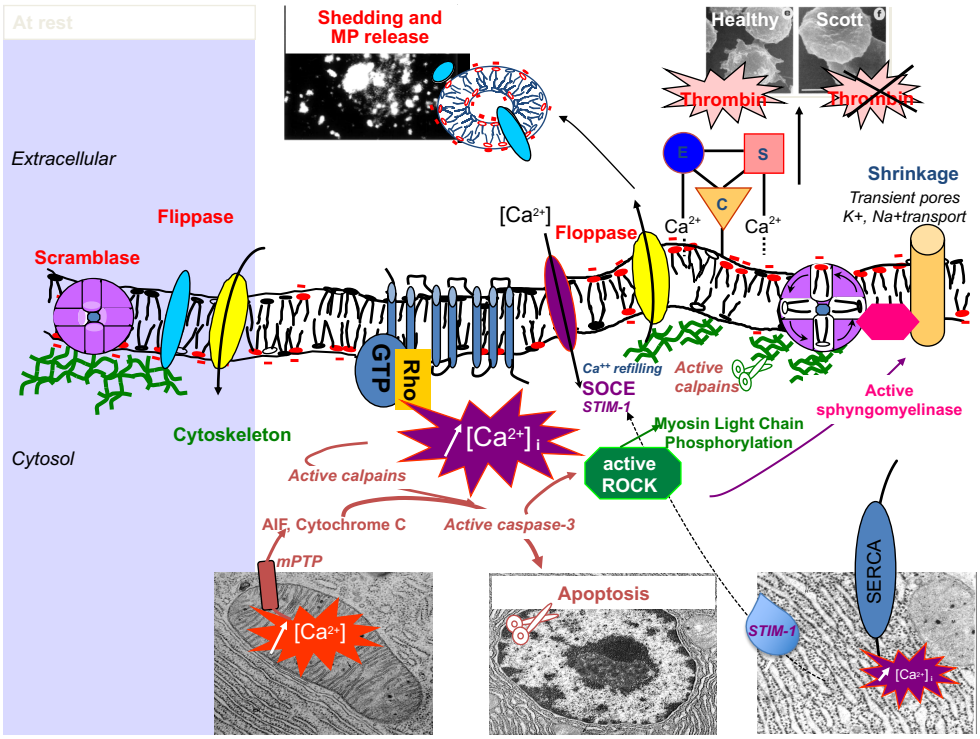


Figure 5: The plasma membrane remodeling and MPs shedding *Phospholipids in the plasma membrane of resting cells present an asymmetric distribution between the two leaflets (left panel). Randomization of phospholipids between the two leaflets of the membrane requires cytoskeleton reorganization involving calcium-activated proteases such as caspases and calpains. A sustained increase in intracellular calcium ions is mandatory for PS exposure during apoptosis, ultimately associated with changes in mitochondrial permeability. The exact interplay between ion transport across the membrane, transient transmembrane pores, and Stored Operated Channels calcium Entry (SOCE) deserves further investigations to gain insight into a potential pharmacological approach to limit anionic phospholipid exposure in thrombotic disorders.* (Morel, Jesel et al. 2011)

II-1.2.1 Phospholipid asymmetry of the plasma membrane in resting cells

Plasma membrane fluidity is an important factor in maintaining cell function and responses to its environment. It is dependent on both temperature and lipid composition. Phospholipids, glycolipids and cholesterol ensure the selectivity of the membrane barrier. The main phospholipids of the plasma membrane are: phosphatidylcholine (PhChol), phosphatidylethanolamine (PhEth), the phosphatidylserine (PS) and sphingomyelin and their proportions vary with the cell lineage. The chain length and number of unsaturated links of fatty acids determines the flexibility and thickness of the membrane. PS and PhEth are mainly located in the inner leaflet Sphingomyelin and PhChol are found on the outer leaflet (Agger, Christensen et al. 2008). The distribution of the membrane lipids is the result of an active process under the dependence of complementary phospholipids transporters governing either the inward (flip) or the outward (flop) translocation. Any appearance of PS or phosphatidylethanolamine on the outer leaflets is counteracted by a reverse transport to the inner leaflet by an aminophospholipid translocase with flippase activity that maintains the normal resting phospholipid distribution (Morel, Jesel et al. 2011). The lateral movements of proteins and phospholipids in the same monolayer are part of the membrane's fluidity. This transverse diffusion allows the formation of functional platforms known as "lipid rafts" that enhance the concentration of specific receptors and phospholipids, PS, being recruited at their vicinity (Kunzelmann-Marche, Freyssinet et al. 2002). Of note, PS exposure at cell surface is a signal for phagocytosis and a common feature of stimulated, apoptotic and senescent cells. In addition, differentiated cells and cancer cells expose higher proportion of PS than their counterparts.

II-1.2.2 The phospholipid transporters

Basically, the transverse movements between the two layers phospholipids of the plasma membrane are rare and slow. However, the transmembrane transport of phospholipids play an important role in diverse cellular processes such as the maintenance of the plasma membrane, and asymmetry, vesicular trafficking, the regulation of the functions of membrane proteins, phagocytosis, coagulation, and apoptosis (Coleman, Quazi et al. 2013). Lipid asymmetry is generated primarily by selective synthesis of lipid on one side of the membrane. Because passive lipid trans bilayer diffusion is slow, a number of proteins have evolved to either dissipate or maintain this lipid gradient. These proteins fall into three classes: 1) ATP-dependent transporter called "flippases", which is highly selective for PS and functions to keep this lipid sequestered from the cell surface, 2) ATP-dependent transporter

called "floppases, their activity has been associated with ABC class of transmembrane transporters, and finally the third ATP-independent transporter called "scramblases", nonspecific carrier which ensures homogeneous distribution of lipids reformed in the endoplasmic reticulum or in the plasma membrane of a selected cell (Daleke 2003).

Under conditions of stress and calcium influx, flippase inactivation; scramblase and floppase activation causes membrane phospholipid randomization and the exposure of PS by the outer leaflets (Segawa, Kurata et al. 2014). This outsourcing of PS is the expression of early apoptosis or cell activation and a signed for phagocytosis (Fadok, Warner et al. 1998).

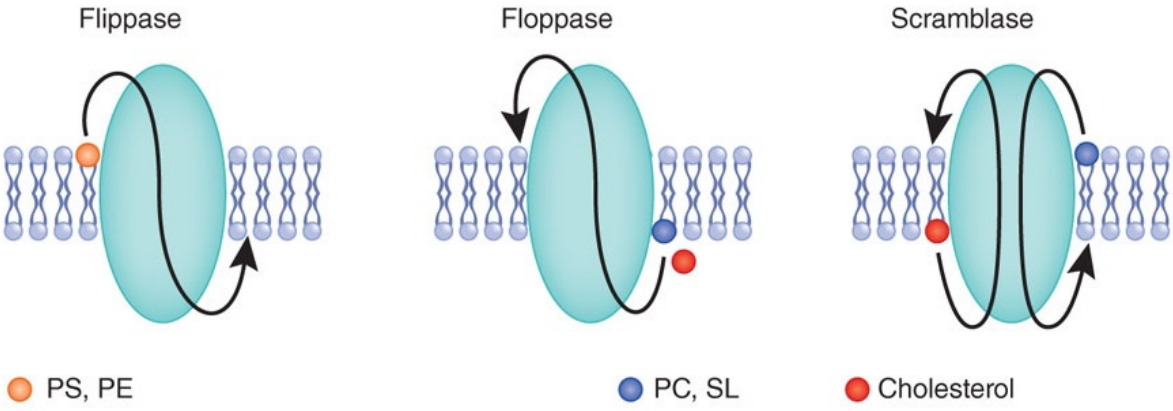


Figure 6 : Phospholipid transporters (Clark 2011)
Transport by flippases is ATP-dependent it leads to the translocation of the phosphatidylserine (PS) and, less significantly, phosphatidylethanolamine (PE), from the outer leaflet to the inner leaflet of the Plasma Membrane. Symmetrically, floppases use ATP to transport phosphatidylcholine (PC), sphingolipid (SL) and cholesterol from cytoplasmic to the extracellular leaflet, against the concentration gradient. The activity of the calcium-dependent scramblases ensures a non-specific movement of the phospholipids according to their concentration gradient.

Interestingly, in Scott syndrome, a rare bleeding disorder characterized by altered flip-flop and PS translocation at the outer leaflet of the plasma membrane of red blood cells, lymphocytes and platelets, MP shedding is also impaired testifying to the hemostatic function of MPs. (Toti, Satta et al. 1996). However, no deficiency in activity or mutation of the identified scamblases or in flippases could be evidenced. Instead, altered ABC-A1 transporters that are occasional floppases led to impaired so-called scramblase activity. Recently, mutation of the gene of the anoctamine 6, a calcium-dependent chloride channel, was identified in homozygous and heterozygous-type Scott patients, indicating that other lipid transporters are involved in fli-flop and MP shedding. (Sohal, Mockett et al. 2002, De Luca d'Alessandro, Bonacci et al. 2011, Lopez-Otin, Blasco et al. 2013)

II-1.2.3 Cell Activation causing MP shedding

II-1.2.3(1) Calcium influx and disruption of membrane asymmetry

Many stimuli can lead to cell activation and an immediate or rapid MP release in response to acute stress, or to a slower but sustained MP shedding as the result of cell death or differentiation. Stimuli include endotoxin, non-esterified cholesterol, thrombin, hypoxia, growth factors and inflammatory molecules (IL-1β, TNF-α, Fas, etc.). Cell activation is accompanied by a large and sustained increase of intracellular calcium (30-350 µM against 1 µM at baseline). The intracellular calcium concentration increase is consecutive to the release of intracellular stores and to calcium influx (Martinez and Freyssinet 2001). TRPC6 (transient receptor potential channel), the SOCE (store-operated channel entry) channels that are activated by the emptying of the intracellular stores, but also other calcium channels, promote the secondary calcium influx into the cell (Munnix, Harmsma et al. 2003). In addition, depolarization of the outer membrane of the mitochondria causes the opening of the mitochondrial pores and the release of calcium into the cytosol. Increased calcium concentrations inhibit flippase activity and stimulate floppase and scramblase activities (Wolfs, Comfurius et al. 2005). Activation of the cells simultaneously leads to a faster transfer of PS to the outer leaflet. As a result, the breakdown of membrane asymmetry is the main mechanism involved in the emission of MPs. Most likely mechanisms involved in flip-flop depend on the type of stress and duration, on the physical characteristics of the plasma membrane of the cell lineage, on the membrane proteins that may also act on its curvature and on cytoskeleton proteins that interact with the inner leaflet anionic phospholipids (Lhermusier, Chap et al. 2011), see below.

II-1.2.3 (2) Calcium influx and degradation of the cytoskeleton

The excess of the intracellular calcium promotes a calcium-dependent proteolytic activity of calpain and other cysteine proteases like caspases. Local protein degradation of the cytoskeleton like filamin-1, gelosine, talin and myosin facilitates microparticle budding (Morel, Jesel et al. 2011). It is enhanced by caspase activation in the case of apoptotic stress. At the initial steps of apoptosis, the concentration of MPs is directly related to the activation of caspases: caspase-3 cleaves the Rho kinase ROCK-1 that phosphorylates myosin light chain (MLC), inducing the cytoskeleton remodelling and prompting the release of MPs. ROCK-2 is similarly activated in thrombin-activated Endothelial cells(Coleman, Sahai et al. 2001) (Cabelof, Raffoul et al. 2002),Figure 7

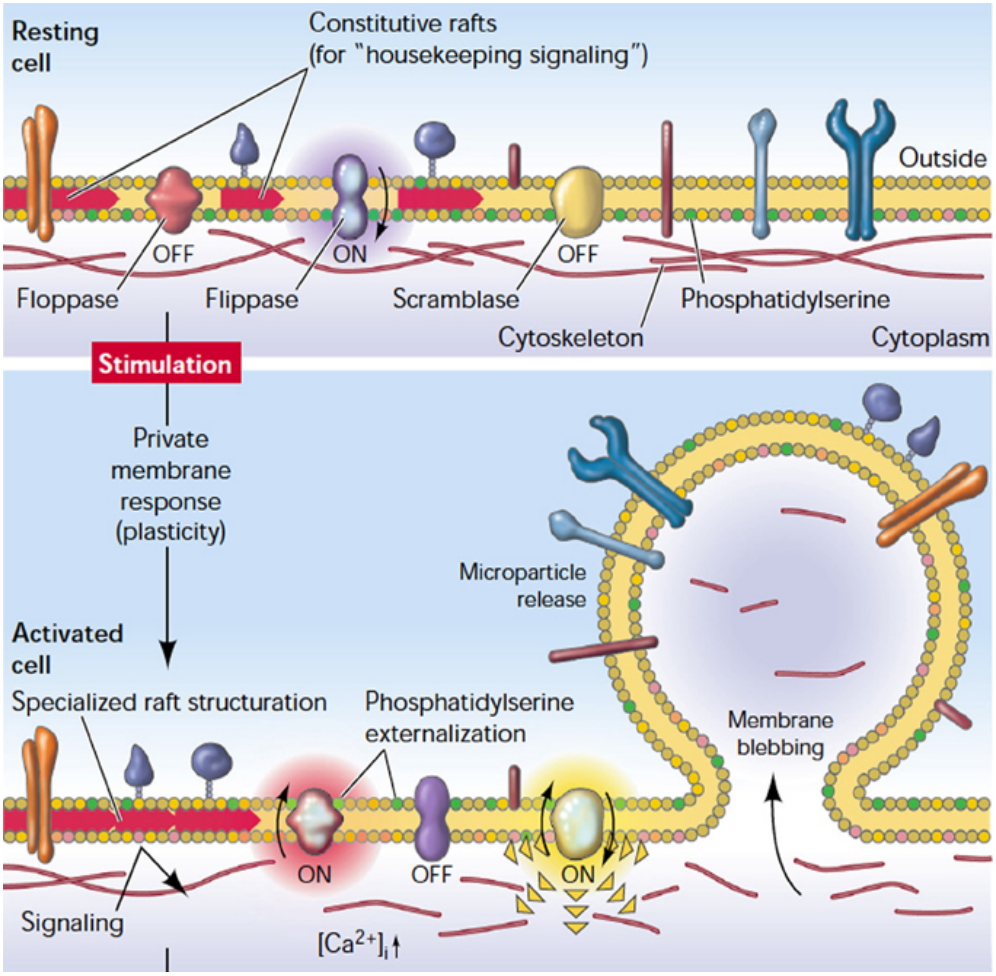


Figure 7 : Models of phospholipid movements across the plasma membrane bilayer and MP release (Hugel, Martinez et al. 2005).*Following stimulation and enhance in intracellular calcium concentration, the activity of phospholipid transporters is altered. The activation of floppase and inhibition of flippase activities results in an accumulation of PS at the outer leaflet of the plasma membrane.*

II-2 Pathogenic effects of microparticles in vascular diseases

Recent data have confirmed that MPs released from senescent endothelial cells play a key role in vascular health and disease and that they may orientate the ability of the vessel to maintain vascular homeostasis (Sabatier, Camoin-Jau et al. 2009). Ins pathological vessel, two distinct groups of MP can be distinguished: (i) circulating MPs released from endothelial and circulating blood cells and (ii) MPs sequestered within the atherosclerotic plaque or in the vessel wall. MP from the plaque are eventually shed by senescent or apoptotic cells recruited into the lipid core and may eventually be released in blood flow after plaque rupture (Beckman and Ames 1998, Morel, Morel et al. 2008).

II-2.1 Microparticles, coagulation and thrombosis

The most established property of MPs is their ability to promote coagulation. Circulating MP levels are elevated in hypercoagulability disorders. Several studies have shown that the amount of circulating MPs, in particular of platelet, endothelial and leukocyte origin, is related to the severity of the cardiovascular disease or disorder like pulmonary hypertension (Wallace 1994, Ward 1994, Breen and Murphy 1995). The procoagulant properties of MPs, mainly due to the exposure of PhSer or TF expression in inducible cells (monocytes and endothelial cells mainly), play a central role in the prothrombotic effect of MPs. Conversely, in the rare bleeding disorder called Scott syndrome, flip-flop and MP shedding are defective, thereby confirming the hemostatic role of MPs in healthy individuals. (Hagen 2003)

In a mice model of endothelial-injury mediated thrombosis, Owens *et al.* have shown in vivo that TF-bearing MPs are incorporated into the growing thrombus (Owens, Passam et al. 2012). In addition, TF^{+}-MPs are elevated in various thrombotic disorders, which affect the arterial or venous beds, or microcirculation. In the arterial compartment, within the atherosclerotic plaque, MPs from macrophages or smooth muscle cells harbor at least 90% of tissue factor activity (Mallat, Benamer et al. 2000) while they were almost undetectable in adjacent healthy tissue or in the mammary artery (Canault, Leroyer et al. 2007). A recent study has suggested that circulating endothelial MPs are predictive of plaque instability in carotid patients (Wekesa, Cross et al. 2014).

II-2.2 Microparticles and Diabetes

Plasma pro-coagulant MPs are elevated in diabetes type 2 (T2DM). Cimmino and coll. have observed a higher rate of TF-related MPs correlated with increased markers of activation of coagulation (Santilli, Marchisio et al. 2016). In addition, the increase in pro-coagulant MP in DT1 patients is correlated with HbA1c, suggesting that the pro-coagulant potential of MPs is associated with the loss of glycemic control, an eventual cause of cellular damage or senescence (Sabatier, Darmon et al. 2002). Endothelial dysfunction is a central pathogenic mechanism in the development of vascular complications related to diabetes. The endothelial-derived MP concentrations increase during thrombotic complications in DT2 patients and EMP convey TF (Tsimerman, Roguin et al. 2011). Ettelaie *et al*. have shown that TF-bearing MPs, released by renal mesangial cells in response to glucose, induce capillary formation in human dermal endothelial cells (Ettelaie, Su et al. 2008).

Taken together, these data suggest that circulating MPs of endothelial origin and/or bearing TF promote a cardiovascular risk profile in diabetes (Sinning, Losch et al. 2011). However, the potential mechanisms involved in the increased release of MPs in diabetes remain to be characterized. In leptin receptor-deficient diabetic mice the high endothelial MP level was positively correlated with blood glucose concentration and infarct volume after ischemic brain injury (Chen, Chen et al. 2011). Thus, elevated levels of endothelial MPs have been associated with increased levels of plasma-oxidized LDL in T2 DM patients, suggesting that oxidized LDLs contribute to vesiculation of the endothelial membrane (Nomura, Shouzu et al. 2004).

II-2.3 Microparticles, endothelial dysfunction and inflammation

Various proinflammatory stimuli cause the release of proinflammatory MPs. This process may be considered a form of sterile inflammation, which involves the production of pro-inflammatory mediators such as cytokines and chemokines and promotes the secondary recruitment of inflammatory cells (Chen and Nunez 2010). Mesri *et al.* have shown that leukocyte MPs promote the release of inflammatory cytokines, IL-6 and MCP-1 and the induction of TF via the JNK1 pathway (Mesri and Altieri 1999). In addition, T lymphocyte MPs have been reported to induce the production of TNF-a and IL-1β by monocytes (Scanu, Molnarfi et al. 2008). Monocyte-derived MPs stimulate IL-8 and MCP-1 production in human bronchial epithelial cells (Neri, Armani et al. 2011), and both monocyte and endothelial MPs promote the production of IL-6 and MCP-1 in human podocytes (Eyre, Burton et al. 2011). In this respect, treatment with platelet MPs is associated with increased expression of ICAM-1 in cultured endothelial cells promoting leukocyte recruitment (Barry, Pratico et al. 1998). In addition, monocyte MPs activate the NF-kB pathway and increase the expression of adhesion molecules (Wang, Williams et al. 2011). Endothelial MPs have been shown to bind to monocytes and promote their transendothelial migration (Jy, Minagar et al. 2004). In a model of acute pulmonary injury involvement in rats, the inherent pro-inflammatory effect of endothelial MPs was associated with endothelial dysfunction, acute lung injury, increased levels of systemic and alveolar pro-inflammatory cytokines (IL-1β and TNF-α), neutrophile infiltration into the perivascular space and histological lesions (Densmore, Signorino et al. 2006, Buesing, Densmore et al. 2011). Similarly, injection of circulating MPs from septic patients in mice results in increased expression of iNOS, COX-2, and NF-kB in the heart and lungs, leading to vascular dysfunction (Mastronardi, Mostefai et al. 2011). Similar pro-inflammatory properties of MPs from septic rats were identified and were found sensitive to

pharmacological modulation (Boisrame-Helms, Delabranche et al. 2014) indicating that they may constitute a therapeutic target or tool depending on the signal they convey. In humans, the relationship between the inflammatory state, the release of MPs and the extent of atherosclerosis has been demonstrated in asymptomatic patients with metabolic syndrome (Chironi, Simon et al. 2006), see figure 8.

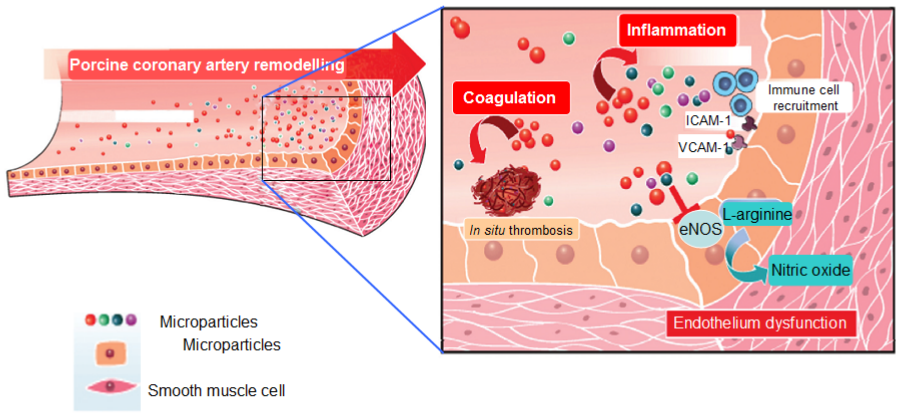


Figure 8 : Impact of circulating MPs with procoagulant and pro-inflammatory properties on the endothelium and vessel wall (Amabile, Guignabert et al. 2013)

II-2.4 MPs and oxidative stress in the endothelium

Several studies have highlighted the properties of MPs as cellular effectors of oxidative stress in blood vessels. Tual *et al*. have shown that hypoxic circulating MPs promote endothelial dysfunction in rat aorta and pulmonary arteries by reducing NO production, a key regulator of endothelial survival. In addition, these MPs exhibited tissue specificity with oxidative stress preferentially induced in pulmonary endothelial cells (Tual-Chalot, Guibert et al. 2010). The deleterious action of the MPs is confirmed in vivo by the injection into mice of circulating MPs isolated from patients with the metabolic syndrome which reduces the endothelium-dependent relaxation and the expression of eNOS and hence also the production of NO confirmed in endothelial cell cultures (Agouni, Lagrue-Lak-Hal et al. 2008). Cheng et al., have shown in vitro that circulating endothelial MPs cause endothelial dysfunction via Nox4 expression and ROS production in human umbilical vein endothelial cells (HUVECs) (Cheng, Wang et al. 2013). Other studies specified a role for T lymphocyte MPs that decrease NO production and increase oxidative stress in endothelial cells via

xanthine oxidase (Mostefai, Agouni et al. 2008). Monocyte MPs induce endothelial production of ROS, mainly superoxide anion, parallel to rapid expression of von Willebrand factor at the endothelial surface, and induction of functional TF, via NADPH oxidase, mitochondria, xanthine oxidase and cyclooxygenase (Essayagh, Xuereb et al. 2007, Terrisse, Puech et al. 2010). Brodsky *et al*. initially showed that endothelial MPs decrease NO production in rat aortic rings and was associated with the impaired endothelial function (Brodsky, Zhang et al. 2004). All of these findings identify endothelial dysfunction mediated different sources of MP. Abbas et al. have identified the role of oxidative stress in autocrine endothelial dysfunction induced by senescent MPs and likely to circulate in the plasma of patients with the acute coronary syndrome. These MPs induce ROS accumulation and increased expression of NADPH oxidase subunits in young endothelial cells (Abbas, Jesel et al. 2017).

II-2.5 Microparticles and senescence

Several studies have suggested a relationship between endothelial senescence and MPs. Burger's team showed in a mouse model that treatment of primary aortic endothelial cells by MPs or H2O2 was associated with a nonproliferative phenotype, meaning that MPs induce premature senescence, which was established via the NADPH oxidase and mitochondrial ROS measurement expression (Burger, Kwart et al. 2012). Brodsky *et al*. showed that the development of accelerated vasculopathy in Zucker diabetic rats was characterized by a 6-fold increase in the number of senescent endothelial cells, an increase in MP levels, a decrease in NO production and vasorelaxation (Brodsky, Gealekman et al. 2004).

Chapter Three

Structure and Physiology of Endothelium

III-1 Blood vessels

Blood vessels play an essential role in the vascular homeostasis through a complex network comprised of arteries, capillaries, and veins (Chiu and Chien 2011). Blood vessels constituted of distinct histological regions containing smooth muscle cells and elastin. The tunica intima includes a simple layer of lining the vessel cavity and the internal elastic lamina membrane which is highly elastic, enriched in collagen fibers and supported by a basement membrane (or basal lamina). In close juxtaposition to the endothelial cells, perivascular cells or pericytes can differentiate into a wide variety of cells (fibroblast, adipocytes and smooth muscle cells). They act as scaffold system providing balanced microenvironment for the endothelial cells lacking blood supply (Chakravarthy and Archer 1992).

The tunica media consists of smooth muscle cells and elastic connective tissue. It is separated from adventitia by external elastic lamina. The media is responsible for vasoconstriction and vasodilation, the two physiological phenomena responsible for the regulation of vascular tone.

The last and outermost layer of the blood vessel is the tunica adventitia, composed of collagen and elastic fibers (Gutterman 1999). Its inner side is surrounded by elastic lamina. It consists of nerves and lymphatic vessels, and contains small blood vessels known as vaso vasorum. The vasa vasorum consist of arterioles and provides the adequate blood supply for areas far from the lumen (Pugsley and Tabrizchi 2000), see figure 9.

III-2 Endothelium: overview of its function

The term endothelium was coined in 1865 by the anatomist Wilhelm His, who differentiated the inner lining of body cavities from the epithelium. The endothelial monocellular layer separates all tissues from the circulating blood (Minami and Aird 2005, Gori, Dragoni et al. 2007). In the shape, endothelial cells (ECs) are generally flat (Gori, Dragoni et al. 2007). The endothelium is characterized by structural and functional heterogeneity as the shape and organization of cells vary across the blood vessels tree. Blood vessel endothelium traverses each and every tissue, but each vascular bed has unique structural and functional properties (Aird 2007). It is heterogeneous, and are classified into three types: 1) the continuous endothelium found in most arteries, veins, and capillaries of the brain, skin, lungs, heart, and muscles. ECs are contiguous and maintained by narrow junctions at the continuous basement membrane. 2) The fenestrated endothelium is also connected to a continuous basement membrane but is characterized by the existence of trans-cellular pores

50 to 60 nm wide. 3) The discontinuous endothelium, which is associated with a poorly structured basement membrane, is characterized by large fenestrations of 100 to 200 nm in width. This type of endothelium is found largely in the liver, spleen and bone marrow (Aird 2007).

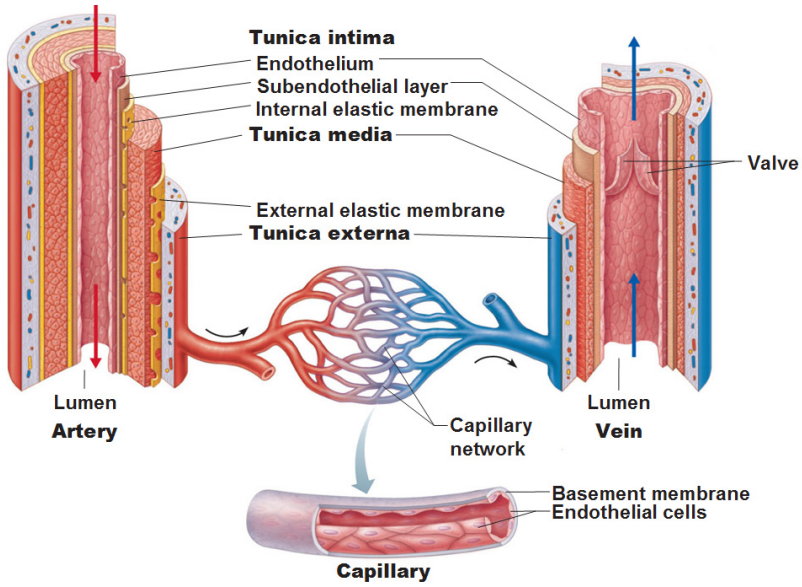


Figure 9: A schematic representation of blood vessels and their components. Adapted from Wayne W. La Morte, MD, PhD, MPH, Boston University School of Public Health.

EC thickness varies from less than 0.1 µm in capillaries and veins to 1 µm in the aorta (Florey 1966, Aird 2007). ECs are key player in maintaining vascular homeostasis. The healthy endothelium is characterised by a hemocompatible phenotype and anti-inflammatory and vasodilatory properties whereas endothelial dysfunction is characterised by reduced vasodilatory responses to flow or agonists, is inflamed and procoagulant (Celermajer, Sorensen et al. 1994, Dohi, Kojima et al. 1995, Deanfield, Halcox et al. 2007). ECs are in direct contact with the blood and form a selective barrier between the vessel lumen and the surrounding tissues. ECs contain multiple transport systems for amino acids and glucose (Galley and Webster 2004). They express or secrete matrix products, anticoagulant or anti-thrombotic factors, fibrinolysis activators, growth factors such as insulin growth factor, inflammatory mediators and are involved in the control of vascular tone by soluble mediators or the expression of different receptors for vasoconstrictors or dilators (van Hinsbergh 2012). The endothelial function is gradually penetrated with ageing (Taddei, Virdis et al. 1995, Favero, Paganelli et al. 2014). A key mechanism responsible for the impairment of endothelial function with ageing is thought to be the loss of the endothelium derived nitric

oxide (NO), a vaso-relaxing factor, via its interaction with accumulated ROS to form peroxynitrite. Peroxynitrite oxidises BH4, an primary cofactor for NO synthesis by endothelial nitric oxide synthase (eNOS), to its inactive form resulting in reduced NO production (Sindler, Delp et al. 2009), see below. The endothelial dysfunction means damage to vascular endothelial, its an initial step in the development and progression of atherosclerosis, and the pivotal underlying pathology in the arterial wall (Vanhoutte 2009, Gutierrez, Flammer et al. 2013).

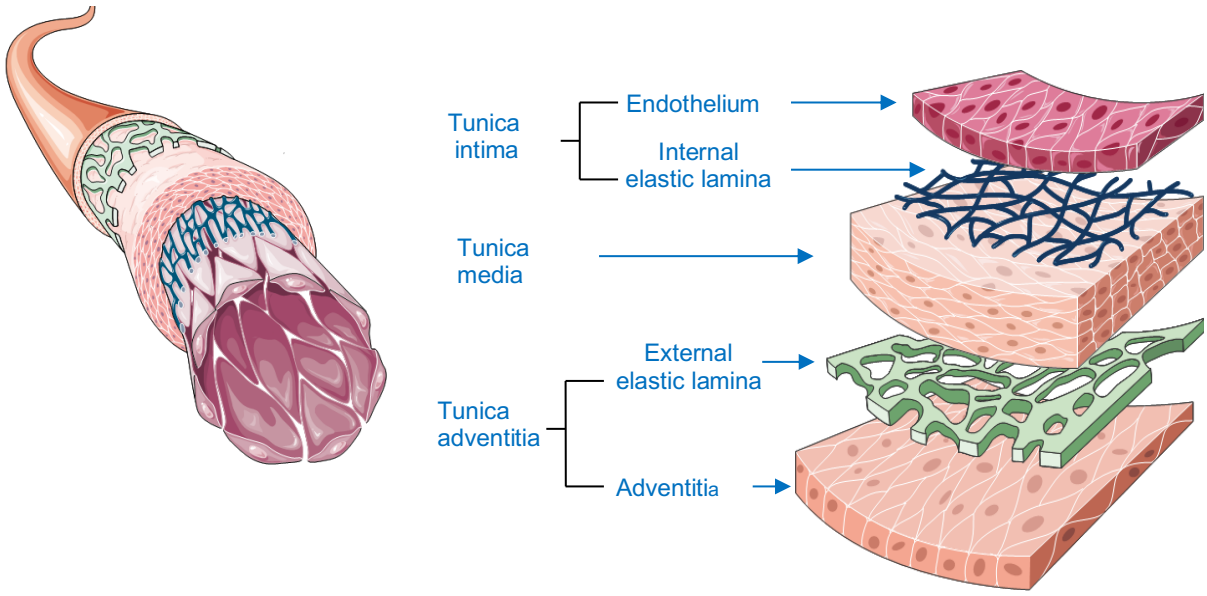


Figure 10: Structure of the arterial wall. *The arterial wall is composed of three layers: tunica intima, tunica media, and tunica external. (Gutterman 1999)*

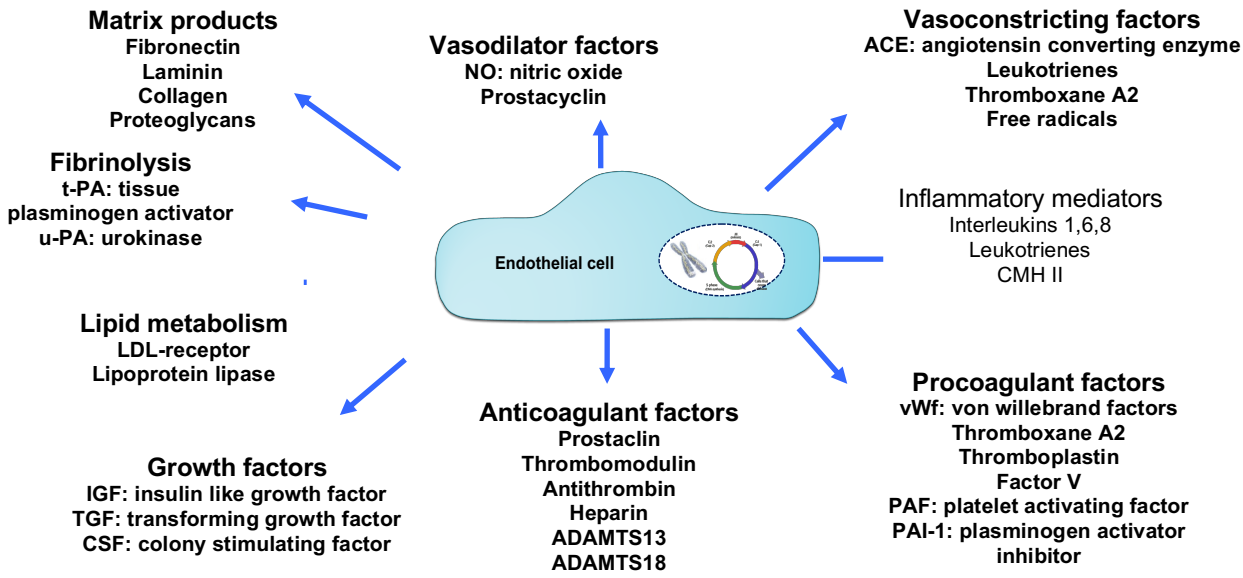


Figure 11 : The different metabolic functions of endothelial cells and endothelial mediators (Galley and Webster 2004)

III-3 Endothelium and vascular tone

The multiple functions of vascular endothelium include regulation of vessel integrity, tissue growth and metabolism, vascular growth and remodeling, cell adhesion, immune responses, angiogenesis, hemostasis and vascular permeability. The endothelium is the main player in the regulation of vascular tone, controlling tissue blood flow and maintaining blood fluidity and inflammatory responses (Feletou and Vanhoutte 2006, Moncada and Higgs 2006). Endothelial cells control vascular tone by releasing several relaxing factors, including nitric oxide (NO), endothelium-derived hyperpolarizing factors (EDHF), and prostacyclin. NO, produced by endothelial NO synthase (eNOS), is the principal mediator of acetylcholine (ACh)-induced, endothelium-dependent, vasodilatation in large conduit arteries. However, in small resistance arteries, including those of mesenteric, renal, and coronary circulation, EDHF is of increasingly greater significance with decreasing vessel diameter in agonist-elicited vasodilatation (Wang, Borrego-Conde et al. 2003, Park, Capobianco et al. 2008, Zhou, Qing et al. 2010, Kang 2014).

III -3.1 Endothelium-derived vasorelaxing factors

III -3.1.1 Nitric Oxide

Nitric oxide (NO) is a soluble and diffusible gas, a highly reactive free radical that acts as a messenger molecule in various physiological and pathological processes (Palmer, Ferrige et al. 1987, Moncada, Palmer et al. 1991). NO is formed by the oxidation of L-arginine through the activation of three different NO synthase (NOS) isoforms, including neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS) (Sessa, Harrison et al. 1992, Xie, Cho et al. 1992). In addition, a fourth type of the mitochondrial NOS (mtNOS). Its occurrence and function has been confirmed in many tissues and it seems to be a crucial regulator of mitochondrial function (Ghafourifar and Cadenas 2005). The expression of eNOS is preferential in ECs but eNOS is also described in smooth muscle cells, cardiomyocytes, neurons, and bone cells (Lei, Vodovotz et al. 2013). The loss of endothelial NO function is associated with several cardiovascular disorders which is due either to decreased production or to increased degradation of NO (Davignon and Ganz 2004). eNOS plays a key role in the physiological regulation of the cardiovascular system since abnormalities in its productions and/or bioavailability accompany or even precede diseases such as hypertension, atherosclerosis, and angiogenesis associated disorders (Moncada and Higgs 2006, Pacher, Beckman et al. 2007, Vanhoutte 2009, Vanhoutte, Shimokawa et al. 2009).

The eNOS/NO functioned as powerful angiogenic mediators and can induce endothelial cell proliferation, migration, and tube formation (Kimura and Esumi 2003, Zhao, Vanhoutte et al. 2015). eNO synthase is activated by intracellular calcium in response to changes in mechanical distension (shear stress) caused by blood flow to the vascular wall or via receptor-mediated processes. Released NO activates soluble guanylate cyclase (GC) in smooth muscle cells, converting GTP to cGMP. This activates protein kinases that lead to inhibition of calcium flux in smooth muscle cells, and decreased calcium-calmodulin stimulation of the myosin light chain kinase thereby reducing smooth muscle tension and causing vasodilation (Harrison 1997), see figure 12.

Agonists, such as acetylcholine, bradykinin and histamine, bind specific endothelial receptors that mediate the elevation of intracellular calcium concentrations which activates eNOS to produce nitric oxide (NO). Activation of eNOS also occurs independently of the calcium concentration via eNOS phosphorylation. The responses to hemodynamic shear stress and hormones are mediated mainly through this calcium-independent pathway (see figure 12). ECs from old donors are more sensitive to apoptotic stimuli and produce less NO in response to physiological stimuli. In aging aorta, the reduced NO production seems associated with calcium-independent e-NOS activation

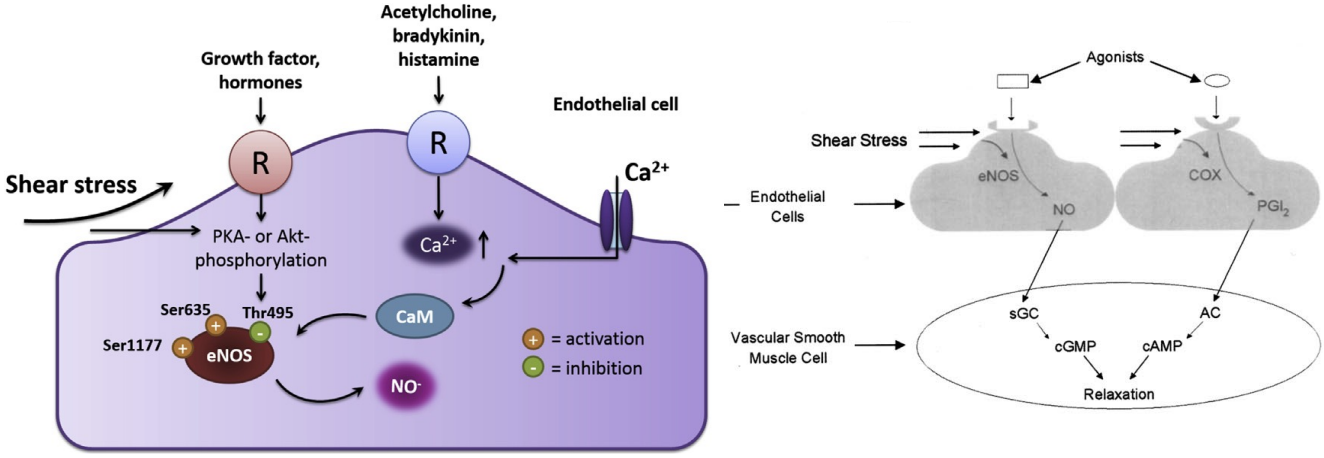


Figure 12 a: Endothelial nitric oxide synthase (eNOS) can be activated in calcium-dependent or- independent ways and favors vasodilatation through NO production (Zhao, Vanhoutte et al. 2015).

III-3.1.1(1) The kinin system

Biologically active kinins, including bradykinin (BK) and Lys(0)- BK, are short-lived peptide circulating mediators predominantly generated by the enzymatic action of kallikreins on kininogen precursors (Howl and Payne 2003) and they potent endothelium-dependent vasodilators that contribute to vasodilation and hypotension. The kinin system consists of

substrates (kininogens) and plasma and tissue kallikreins their specific activators producing the two vasoactive peptides bradykinin and kallidin. The effects of kinins are mediated via B1 and B2 specific receptors. BK is key for blood pressure regulation and in inflammatory reactions. It acts through its ability to elevate vascular permeability and to cause vasodilatation of arteries and veins of the gut, aorta, uterus, and urethra. BK also triggers the release of other mediators such as nitric oxide in inflammatory (Wu, Akaike et al. 2002). The kinin system is involved in many clinical situations including septic shock, heart diseases, respiratory allergic reactions, hypertension and its treatment, hypotensive transfusion reactions, pancreatitis, hereditary and acquired angioedema, Alzheimer disease and liver cirrhosis with ascites (Agostoni and Cugno 2001). Kinins act via their G protein-coupled receptors.

III-3.1.1(2) Bradykinin formation and physiological significance

The levels of kinin peptides in tissues are higher than in blood, confirming the primary tissue localization of the kallikrein-kinin system. Kallikreins are produced by the inactive precursors prekallikreins after activation in plasma-mediated by Factor XII (Hageman factor). Kallidin is transformed into bradykinin by the enzymatic action of a plasma aminopeptidase. Many studies measuring bradykinin and kallidin peptides demonstrated the differential regulation of the plasma and tissue kallikrein-kinin systems. In addition, studies have demonstrated that kinin peptide levels are enhanced in the heart of rats with myocardial infarction, in tissues of diabetic and spontaneously hypertensive rats, and in the urine of patients with interstitial cystitis, suggesting a potential role for kinin peptides in the tissue response to such pathological conditions (Leeb-Lundberg, Marceau et al. 2005).

III-3.1.1(3) Transduction of signals from B1 and B2 BK receptors

BK interacts with its G protein-coupled B1 and B2 receptors and initiates the elevation of intracellular calcium concentration and involves several mediators such as phospholipase C, prostagladins, protein kinwase and phospholipase A (Blaukat 2003). The hyperpolarizing phase of the cell response to BK may be due to inositol 1,4,5- trisphosphate-dependent release of stored Ca2+ into the cytoplasm, which activates Ca2+-dependent K+ channels. The depolarizing phase of the cell response to bradykinin is due largely to inhibition of M channels, thereby decreasing K+ efflux from cells and, to a lesser extent, to activation of Ca2+-dependent ion channels and Ca2+ channels (Higashida, Streaty et al. 1986). The mitochondrial ATP-sensitive K+ channels act as downstream targets of PKC (Li and Sato 2001). BK B2 receptors can mediate cardioprotective preconditioning of guinea pig hearts by

the dual activation of PKC and PI3-K. In addition, the binding of BK to its B2 receptor induces IL-6 expression in airway smooth muscles cells via ERK1/2 and p38 MAP kinase signaling pathways. Moreover, the IL-6 secretion by BK proved to be sensitive to corticosteroids and was regulated by Thi2-cytokines (Huang, Tliba et al. 2003).

The bradykinin receptors B1 and B2 are important mediators of cardiovascular homeostasis. However, the tissue distribution of BK receptors is wide, and they are detected in tissues such as uterus, intestine, kidney, heart, and aorta. The B2 receptor would play a key role in cardiovascular pathologies with hypertensive component. The activation of B2 receptors by bradykinin promotes the release of prostacyclin and NO production by activating endothelial NO synthase (eNOS)(Luckhoff, Busse et al. 1987, Schini, Boulanger et al. 1990, Vanhoutte, Zhao et al. 2016). This increase is partially associated with enhanced phosphorylation/dephosphorylation of eNOS by many protein kinases and phosphatases (Bae, Kim et al. 2003). The B2 receptor is involved in the acute phase of inflammation and of somatic and visceral pain. Conversely, the B1 receptor participates in the chronic phase of these responses and is likely to play a strategic role in diseases with a strong immune component such as rheumatoid arthritis, multiple sclerosis, septic shock and diabetes (Gabra, Couture et al. 2003).

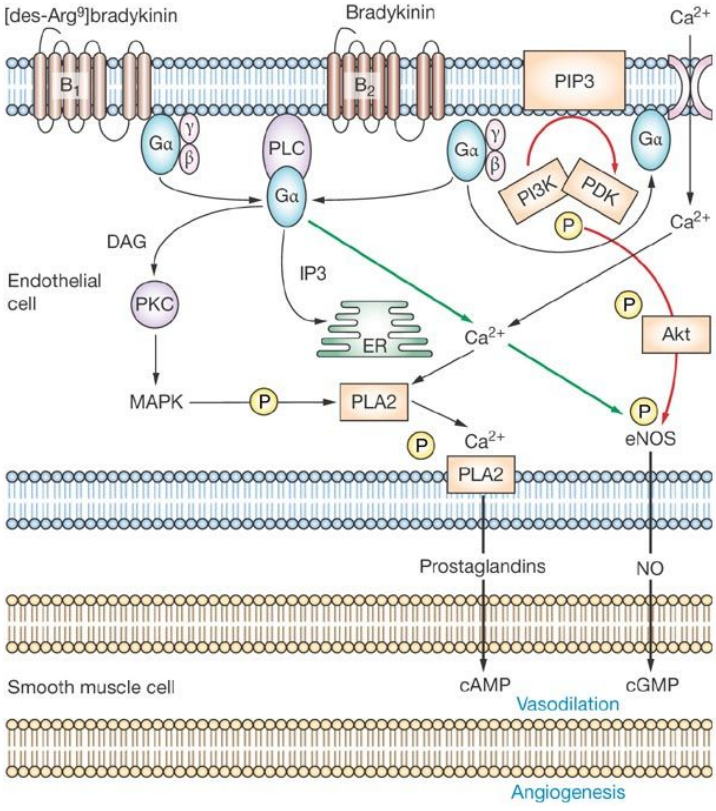


Figure 12 b: B1 and B2 signalling pathways in ECs and smooth muscle cells (Madeddu, Emanueli et al. 2007)

III-3.1.1(4) BK receptors as therapeutical targets

In many animal and human models, it has been shown that the stimulation of BK B2 receptors is not only involved in causing inflammation, pain and tissue injury but also brings powerful cardioprotective mechanisms. Either exogenous administration of BK or locally increased BK concentrations as a consequence of the inhibition of its metabolic breakdown by angiotensin converting enzyme inhibitors, reveals the significant contribution of BK in powerful cardioprotective mechanisms (Heitsch 2003) mainly caused through vasorelaxant, anti-hypertrophic and anti-atherosclerotic endothelial mediators such as NO, prostaglandins and tissue-type plasminogen activator. Such mediators are elevated by ischaemic preconditioning and in insulin sensitivity. Thus, BK B2 receptor agonists may have value in the treatment and prevention of various cardiovascular disorders such as ischaemic heart disease, hypertension, left ventricular hypertrophy, ventricular remodeling, and congestive heart failure as well as diabetic disorders. However, this potential should be balanced with the pro-inflammatory signalling of B1 receptors in chronic diseases. (see above)

III-3.1.2 Endothelium-derived hyperpolarizing factors (EDHF)

The key effector in control of endothelium-dependent EDHF-evoked vasorelaxation in small arteries is hyperpolarization through opening of various types of potassium and calcium-activated K+ channels (KCa) (Feletou and Vanhoutte 2006) (Zhou, Qing et al. 2010, Mori, Suzuki et al. 2011, Kang 2014, Seki, Goto et al. 2017). Although its nature still raises many questions, its mechanism of action implies an increase in the intracellular concentration of calcium, followed by the opening of calcium-dependent potassium channels of low and medium conductance, SKCa and IKCa respectively (Vanhoutte, Zhao et al. 2016).

Activation of IK_{Ca} and small conductance SK_{Ca} favors potassium ions release from ECs, leading to their hyperpolarization. Low concentrations of potassium ions in the extracellular space can activate inwardly rectifying K+ (KIR) channels and Na+/K+-ATPase to cause hyperpolarization of smooth muscle cells through the release of potassium ions by smooth muscle cells (Feletou, Verbeuren et al. 2009). EDHF can be induced by various agonists such as acetylcholine or bradykinin or calcium ionophore A23187 or thapsigargin, an inhibitor of sarco / endoplasmic reticulum Ca^{2+} ATPase (SERCA) that are responsible of calcium re-uptake by the endoplasmic reticulum.

III-3.1.3 Prostacyclin (PGI2)

In addition to the release of NO, ECs can also induce, under certain conditions, the release of prostacyclin (PGI2), the main metabolite of arachidonic acid (AA). It is produced by the sequential actions of phospholipase A2 (PLA2), cyclooxygenase-1 (COX-1) and prostacyclin synthase (PGIS). The synthesis of PGI2 can be stimulated by shear forces, hypoxia, or following activation of membrane receptors by thrombin, bradykinin, and growth factors. This synthesis is calcium-dependent and regulated by different signaling pathways, such as phosphorylation modification of certain transcription factors. For example, binding of thrombin to its PAR-1 receptor activates MAPKs which causes phosphorylation of serine 505 and activation of cytosolic PLA2, leading to an increase in the release of AA and the rapid synthesis of PGI2 by COX-1. In addition, activation of PAR-1 and PAR-2 increases the activity of the NF-κB transcription factor, which promotes inducible COX-2 expression and leads to sustained synthesis of PGI2 (Wheeler-Jones 2008). PGI2 acts primarily via the prostacyclin (PI) membrane receptor to stimulate soluble adenylyl cyclase to form cyclic 3 ', 5' adenosine monophosphate (cAMP) which, in turn, activates protein kinase A-dependent pathways and may also cause vasodilatation via inhibition of Rho kinase and drop of calcium concentration. Under physiological conditions, the vasodilatory properties of PGI2 are often masked by those of NO (Vanhoutte, Zhao et al. 2016). However, in pathological conditions such as diabetes, the effect of PGI2 seems to be preponderant. Indeed, PGI2 participates actively in the acetylcholine-induced relaxations in the diabetic dog coronary artery (Gebremedhin, Koltai et al. 1988) and in the diabetic mouse aorta (Shen, Ye et al. 2003). In addition, oral administration of prostacyclin analogue normalizes endothelium-dependent acetylcholine relaxation in the aorta of diabetic rats (Matsumoto, Morishita et al. 2002).

III-.3.2 Endothelium-derived vasocontracting factors (EDCF)

III-3.2.1 Reactive oxygen species (ROS)

Oxidative stress is characterized by the excessive generation of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical and peroxynitrite. ROS are important mediators of inflammation that are the principle for immune response and host defenses. ROS are produced and released from ECs under physiological conditions and involved in vascular homeostasis. However, excessive ROS production is observed in hypertension, diabetes mellitus, acute and chronic inflammatory diseases and aging. A variety of enzymes contributes to ROS production including cytochrome P450, cyclooxygenases (COXs), lipoxygenases, uncoupled-eNOS, xanthine oxidase, NADPH

oxidase (p22phox, p47phox, gp91phox). In addition, the mitochondrial electron transport chain is an important source of endothelial ROS under physiological or pathological conditions (Griendling and Ushio-Fukai 1997). Mitochondria play a key role in production, because the majority of cellular ROS are generated by oxidative phosphorylation, a mitochondrial process in which electrons are extracted from NADH and FADH and then transferred to molecular oxygen by a chain of 4 complex enzymatic assays for the phosphorylation of ADP in ATP and the reduction of molecular oxygen in water. ROS are by-products of several metabolic and enzymatic pathways and highly reactive due to the presence of unpaired electrons in their outer layer. These electrons can react directly with oxygen acceptors to generate ROS.

It has been suggested that less than 2% of total oxygen consumption is diverted for ROS generation (Chance, Sies et al. 1979). Low concentrations of ROS are necessary for proper cell function (Werner and Werb 2002). Produced in excess, ROS interact with a large number of molecules: proteins, lipids, polysaccharides and nucleic acids, which can irreversibly alter the function of the target molecule and thus disrupt the cellular response.

The production of ROS is often self-amplified. For example, ROS released during the respiratory process damage mitochondrial DNA, which, unlike nuclear DNA, does not have a repair mechanism. The mutations thus induced in the mitochondrial genome cause functional abnormalities of the respiratory chain favoring an excessive production of ROS (Miquel 1998). In addition, the superoxide anion can undergo exaggerated production of hydrogen peroxide H_2O_2 (Bienert, Schjoerring et al. 2006). The latter does not have unpaired electrons and is more stable than the superoxide anion. It diffuses everywhere in the cell and gives rise, via reactions of the "Fenton" type, to the generation of most deleterious of the ROS, the radical hydroxyl (•OH) (Valko, Leibfritz et al. 2007).

$$H_2O_2 + Fe^{2+} \longrightarrow \ \bullet OH + OH^- + Fe^{3+}$$

$$H_2O_2 + Cu^{2+} \longrightarrow \ \bullet OH + OH^- + Cu^{3+}$$

The main contribution of oxidative stress to the atherosclerotic burden in human or in animal models has been supported by earlier works that have shown an improvement of various parameters of the endothelial function under antioxidants therapies (Kanani, Sinkey et al. 1999, Aubin, Carrier et al. 2006, Holowatz, Thompson-Torgerson et al. 2007, Liu, You et al. 2007).

III -3.2.2 Thromboxane A2 & Prostaglandin H2 (TxA2 & PGH2)

T_XA_2 and PGH_2 are generated subsequently to the metabolism of arachidonic acid (AA) by COX-1 and COX-2 in endothelial cells (Oates, FitzGerald et al. 1988). PGH_2 is the precursor of the prostaglandins E_2 ($PGE_{2\alpha}$), F_2 ($PGF_{2\alpha}$) and T_XA_2. By thromboxane synthase in platelets, PGH2 synthesizes TXA2, that promotes platelet activation and aggregation, vasoconstriction, and smooth muscle proliferation. In smooth muscle cells, it causes an increase of intracellular Ca2+ elevation via binding to thromboxane-prostanoid (TP) receptors and favors vasoconstriction (Vanhoutte and Tang 2008). In normal vessels, regulation of TXA2 and PGI2 is essential since they have opposing functions in maintaining of vascular homeostasis. In particular, their ratio seems to be more important than their absolute amounts in pulmonary vascular diseases (Caughey, Cleland et al. 2001).

III-3.2.3 Angiotensin

Angiotensin II (Ang II) is a powerful vasoconstrictive hormone of the renin-angiotensin system (RAS), which is formed after Ang I is converted to Ang II by the Angiotensin I Converting Enzyme (ACE). Ang II is a multifunctional hormone: in addition to its ability to cause vasoconstriction, it acts on cell growth, hormone secretion, and neuronal activities. In general, RAS is involved participates in various cardiovascular pathologies such as left ventricular hypertrophy, post-infarct remodeling, or neointima formation (Zhuo and Li 2011). Ang II effect is mediated through the G-protein coupled receptors AT1 and AT2 (Horiuchi, Akishita et al. 1999). AT1 receptor is involved in the regulation of vascular cell proliferation or cell death (Csikos, Gallinat et al. 1997). AT2 participates in the transduction of various anti-apoptotic or survival pathways. Within the vascular wall, Ang II is also known to be a potent stimulant for ROS production through NADPH oxidase activation (Lerman 2007).

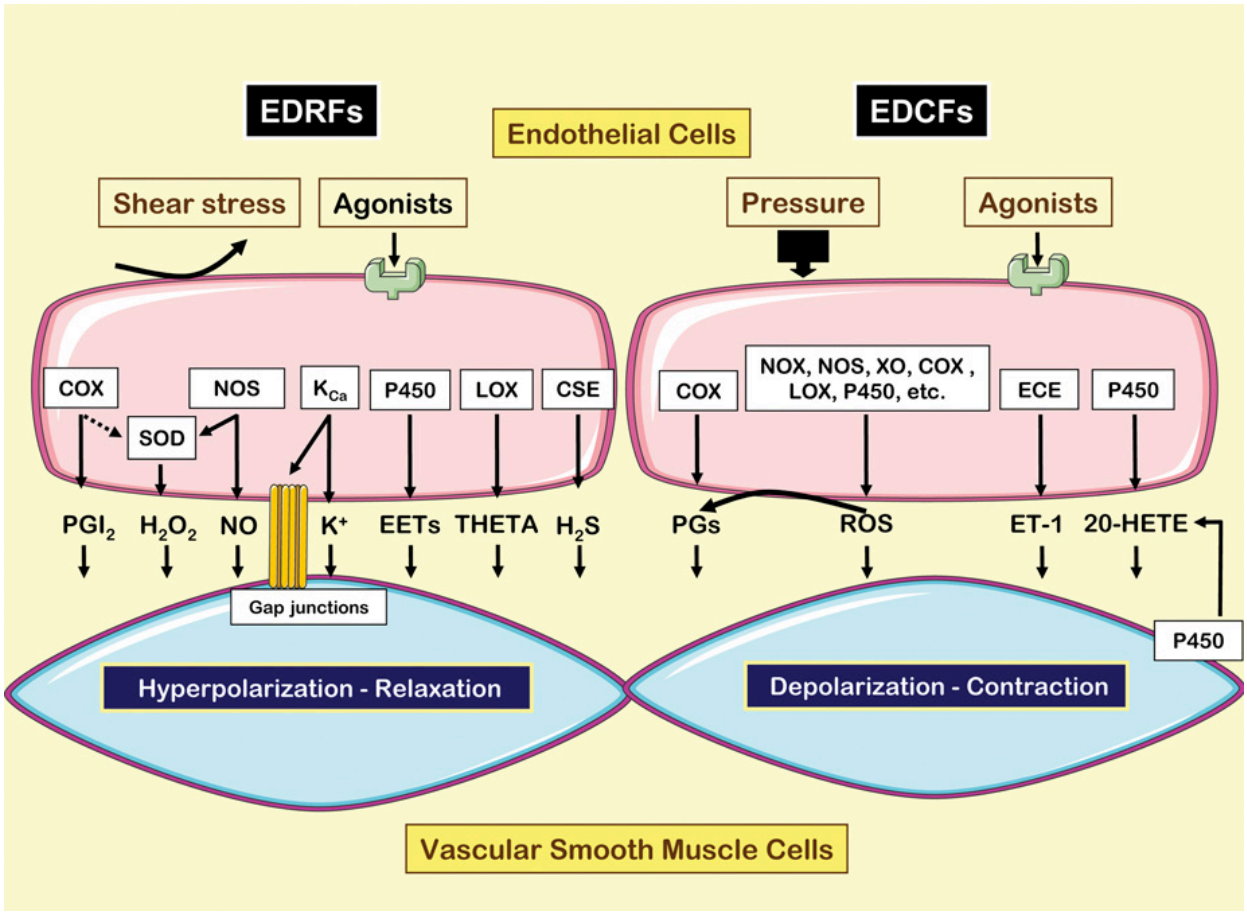


Figure 13: Synthesis of endothelium-dependent relaxation and contraction pathways. EDRFs: Endothelium Derived Relaxation Factors; EDCFs: Endothelium Derived Contraction Factors (from Feletou M. and Vanhoutte P. et al, 2009)(Feletou and Vanhoutte 2009)

III-4 Age-related endothelial dysfunction

Fluid-induced vasodilation, an indicator of the health status of the endothelium, gradually decreases in humans after 50 years (Taddei, Virdis et al. 2001). Epidemiological studies have identified age as the leading risk factor for cardiovascular morbidity and mortality (Lakatta and Levy 2003).(Shih, Lee et al. 2011). Aging is characterized by a gradual change in the structure and function of the vessel leading to a dysfunction of ECs and CML. The thickness of the vascular wall increases two to three times between 20 and 90 years and the media shows an increase in collagen and elastin content, which increases vascular resistance and the risk of atherosclerosis (Lakatta and Levy 2003). A meta-analysis showed that a 0.1 mm increase in intima-media thickness (IMT) of the carotid artery (a marker of vascular disease used clinically) is associated with an 18% increase in risk stroke and 15% risk of myocardial

infarction (Lorenz, Markus et al. 2007). The old CML acquire a proliferative, migratory and secretory phenotype. Old ECs show low eNOS expression and decreased NO production, accompanied by increased formation of ROS and pro-inflammatory molecules, vasoconstrictor prostanoids and endothelin-1 (Sato, Kaji et al. 1993, Minamino, Miyauchi et al. 2002).

ECs isolated from older donors show a decrease in NO synthesis in response to mechanical stimuli or agonists, accompanied by increased sensitivity to apoptotic stimuli (Matz, Schott et al. 2000, Hoffmann, Haendeler et al. 2001). The loss of PI3K / Akt-dependent eNOS phosphorylation appears to be the primary mechanism for reducing NO production in aged rat aortas (Smith and Hagen 2003).

The older vessels show impaired endothelial relaxation. This has been observed in different vascular beds, such as the rat mesenteric artery and the brachial artery in humans, and involves a reduction in both NO and EDH components and is often associated with an increase in EDCF responses involving COXs (Vanhoutte and Tang 2008). A decrease in calcium-dependent potassium channel expression has also been observed in elderly rat coronary arteries (Marijic, Li et al. 2001, Herrera, Mingorance et al. 2010).

III-5 Hyperglycemia and vascular toxicity (glucotoxicity)

The mechanisms linking hyperglycemia to CV events have not been fully elucidated. However, a triad consisting of vascular inflammation, oxidative stress and endothelial dysfunction is considered central and creates a vicious circle favorable to the establishment of a pro-atherothrombotic endothelium. Substantial clinical and experimental evidence demonstrated that impaired endothelium-dependent relaxation existed consistently in both conduit and resistance arteries of diabetes mellitus (Leo, Joshi et al. 2012, Freidja, Vessieres et al. 2014, Salheen, Panchapakesan et al. 2015).

III-5.1 Hyperglycemia and endothelial and vascular dysfunction

Chronic hyperglycemia induces endothelial dysfunction characterized by a decrease in endothelium-dependent relaxation in different vascular beds (De Vriese, Verbeuren et al. 2000, Shi and Vanhoutte 2009). Several factors seem to contribute to endothelial dysfunction related to diabetes.

Reduction of NO bioavailability, due to a decrease in the expression and phosphorylation of eNOS (Matsumoto, Shimabukuro et al. 2014) and increased production of ROS (Alves-Lopes,

Neves et al. 2016) constitute a major cause of dysfunction. In murine diabetic models, a decreased expression of eNOS is observed in the heart (Lei, Li et al. 2013, Peng, Chen et al. 2015), in the ECs of cerebral vessels (Poittevin, Bonnin et al. 2015), glomerular (Hou, Huang et al. 2014) and retina (Omae, Nagaoka et al. 2013). Conversely, overexpression of eNOS in transgenic diabetic mice improves cerebral vascular reactivity (Chandra, Mohan et al. 2016), and administration of substances that can increase endogenous production of NO (L-arginine or arginase inhibitors) in diabetic animals, improve cardiomyopathy, preserve renal function (Claybaugh and Decker 2014, Hsu, Lee et al. 2015) and restores the blood flow of the posterior ischemic limbs (Bir, Pattillo et al. 2014, Kant, Kumar et al. 2015).

An increase in EDCF production has also been observed in diabetic animals. Contractions mediated by EDCF are increased in the conductance arteries, such as the aorta and renal artery (Kagota, Yamaguchi et al. 2000) and in the arteries of resistance such as the mesenteric artery diabetic rats (Vessieres, Guihot et al. 2013).

Another contributor to diabetes-related endothelial dysfunction is ET-1b that promotes vasoconstriction and inflammation. Glycemic control by administration of Metformin to diabetic rats has been shown to prevent activation of the endothelin system with normalization of ET1 plasma levels and decreased expression of the ET A receptor in the mesenteric artery (Sachidanandam, Hutchinson et al. 2009). In addition, ET receptor antagonists prevent vascular remodeling and cerebrovascular dysfunction in the middle cerebral arteries of Goto-Kakizaki diabetic rats (Harris, Hutchinson et al. 2005, Kelly-Cobbs, Elgebaly et al. 2011). Administration of ACE inhibitors improves endothelial function measured by FMD in type 1 diabetic patients with normal blood pressure (Arcaro, Zenere et al. 1999), indicating a key role of the RAS.

III-5.1.1 Role of the angiotensin system in senescence induced by hyperglycemia

Meta-analysis of several clinical trials showed that inhibition of RAS decreased the number of new diabetic patients (Abuissa, Jones et al. 2005, Yusuf, Ostergren et al. 2005). In addition, clinical trials have shown a beneficial CV effect of ACE inhibitors and Sartans in diabetics.

Chen et al., showed that EC exposure to high glucose (HG) increases SA-β-gal activity and mitochondrial oxidative stress, accompanied by overexpression of the conversion enzyme, and AT-receptor 1 of Ang II. The treatment of ECs with ACE inhibitors (benazepril) or Sartans (Losartan) inhibits HG-induced senescence and mitochondrial oxidative stress, suggesting a key role of the RAS system in glucose-induced endothelial senescence. Thus, the

deleterious effects of chronic hyperglycemia on vascular function could contribute to the development of cardiovascular disease (CVD) in diabetics. (see also endothelial senescence, chapter IV)

III-5.2 Hyperglycemia and inflammation

High levels of cytokines and inflammatory factors such as TNFα, interleukin-6, soluble ICAM, MCP-1 protein and soluble E-selectin have been found in the serum of diabetic patients and animals (Fukui, Tanaka et al. 2013, Leguina-Ruzzi, Pereira et al. 2015, Wu, Liang et al. 2016). Indeed, hyperglycemia via ROS generation induces the expression of redox-sensitive genes (the major redox-sensitive transcription factor is NF-kB) leading to a pro-inflammatory phenotype by overexpression of adhesion molecules (VCAM-1) and pro-inflammatory cytokines (IFNγ), and pro-coagulant by overexpression of tissue factor (FT), leading to recruitment and infiltration of monocytes, and activation of the coagulation cascade. All of these events create an endothelial and vascular environment that is conducive to atherothrombosis, and in a higher proportion in patients with T2D than in patients with degree of CV risk similar but free from diabetes.

III-5.3 Hyperglycemia and oxidative stress

In diabetes, the increase in oxidative stress is attributed to the increased production of ROS and the alteration of antioxidant defenses. Indeed, an increase in the expression of NADPH oxidase (Kowluru, Kowluru et al. 2014, Shida, Nozawa et al. 2014, Li, Wang et al. 2015) and COX-2 (Roy, Kim et al. 2015), as well as a decrease in the expression of manganese superoxide dismutase (SOD) (Wang, Li et al. 2015) have been observed in the vessels of T2D patients and of diabetic animals. For instance, vitamin C significantly improved endothelium-dependent vasodilation in these patients (Ting, Timimi et al. 1996). The oxidative stress linked to hyperglycemia is of multiple origine.

III-5.3.1 Auto-oxidation of glucose

Glucose, in its ene-diol form, in the presence of transition metals, gives rise to an anionic radical ene-diol; this, by reducing the molecular oxygen, releases superoxide anions with the concomitant formation of a carbonyl compound. The superoxide anion can be

disproportionated to hydrogen peroxide which in turn produces hydroxyl radicals in the presence of transition metals (Delattre, Bonnefont-Rousselot et al. 1999).

III-5.3.2 Advanced glycation end-products (AGEs)

AGEs are formed in hyperglycemic conditions and during aging and contribute to the pathophysiology of vascular diseases of diabetes (Schmidt, Yan et al. 1995, Rojas and Morales 2004). The most common AGEs in diabetic vessels are relatively inert Nε-carboxymethyl-lysine and hydroimidazolones, and highly reactive derivatives of methyl-glyoxal and 3-deoxyglucosone. Indeed, by binding to their membrane receptors (RAGEs), members of the family of immunoglobulin-like proteins, AGEs cause the activation of multiple signaling pathways targeting NADPH oxidase, p21, MAPK and the NF-kB transcription and target genes, thus promoting oxidative stress and an inflammatory response. Furthermore, soluble AGEs activate monocytes. Inhibition of RAGEs in an accelerated atherosclerosis model in diabetes transgenic mice, completely suppresses vascular lesions, without changes in plasma lipid or glycemia (Schmidt, Yan et al. 1999). Elevated serum AGEs in patients with T2D were inversely correlated with the degree of endothelial-dependent vasodilation in the brachial artery as measured by high resolution vascular ultrasound (Tan, Chow et al. 2002).

III-5.3.3 The polyol route

Glucose metabolism is activated by high intracellular glucose concentrations. First aldose reductase, which normally has the function of reducing toxic aldehydes to inactive alcohols, reduces glucose to sorbitol using NADPH as a cofactor, sorbitol is then metabolized to fructose by sorbitol dehydrogenase that uses NAD + as a cofactor. Sorbitol is a highly hydrophilic polyhydroxylated alcohol that accumulates in the cytosol with osmotic consequences that modify the intracellular tonicity by production of intracellular osmolytes to counterbalance the extracellular hypertonicity. The fructose produced by the polyol route is phosphorylated to fructose-3-phosphate then decomposed into 3-deoxyglucosone, a powerful glycosylating agent that enters the AGEs formation pathway. Finally, Consumption of NADPH by the aldose reductase limits its availability for the glutathione reductase, an enzyme that regenerates reduced glutathione, a critical antioxidant.

III-5.3.4 Activation of protein kinase C by diacylglycerol

PKC plays an important role in endothelial dysfunction (Hink, Li et al. 2001, Cosentino, Eto et al. 2003). It induces the production of ROS by stimulating NADPH oxidase. The treatment of ECs and aortic SMCs with phorbol myristic acid (PMA), a PKC activator, increases the production of ROS that is reversed by diphenylene iodonium, an inhibitor of NADPH oxidase (Inoguchi, Li et al. 2000). Hyperglycemia causes vascular accumulation of DAG, Diacylglycerol, the physiological activator of PKC, thereby contributing to cardiovascular pathologies related to diabetes (Geraldes and King 2010). High levels of DAG have been found in the aorta and heart of diabetic rats (Inoguchi, Battan et al. 1992). Exposure of ECs and aortic SMCs to elevated glucose levels increases DAG levels and ROS production that is inhibited by GF109203X, a specific inhibitor of PKC (Inoguchi, Li et al. 2000) thereby testifying to the central role of PKC in response to high glucose. Indeed, oral intake of LY333531, an oral inhibitor of PKCβ, improves the glomerular filtration rate, and retinal circulation in diabetic rats (Ishii, Jirousek et al. 1996). In humans, Chererithrin, another PKC inhibitor, reduced superoxide production by 25% in the internal mammary artery segments of non-diabetic patients compared to a significantly 60% reduction in diabetic patients, suggesting that inhibition of PKC decreases vascular ROS production in vessels from diabetic patients.

III-5.3.5 The hexosamine pathway

When the intracellular glucose concentration is high, it is metabolized by glycolysis to glucose-6-phosphate, then to fructose-6-phosphate before entering the glycolytic pathway. However, part of fructose-6-phosphate is diverted to another signaling pathway and converted to glucosamine-6-phosphate by the Glutamine Fructose-6 Phosphate Amidotransferase (GFAT) that further promotes the synthesis of uridine diphosphate-N-acetylhexosamine (UDP-GlcNAc), a substrate for N- or O-glucosamination of many proteins and transcription factor.

Substantial evidence indicates that hyperglycemia-induced ROS production can promote telomere shortening, DNA damage, and trigger a p53-dependent response. Indeed, hyperglycemia in diabetic patients has been shown to be a crucial factor in accelerating the progression of endothelial senescence. (see chapter IV)

Chapter Four

Endothelium Senescence and vascular function

IV-1 Cellular senescence

Cell senescence was first defined by Leonard Hayflick in 1961who evidenced that fibroblasts stopped proliferating after a number of cell divisions (Hayflick and Moorhead 1961). The number of cycles performed by the cell before discontinuation of the division was called "Hayflick Limit" and marked the onset of cell aging or senescence, thereby determining the lifespan.

Senescence is defined as the physiological process of biological aging, associated with the progressive degradation of an organism's functions, caused by the accumulation of dysfunctional cells. Aging itself is not a disease, death occurring rather from pathological complications associated to ageing rather tat ageing itself because the renewal of tissues is essential to the viability of the body. Senescence is recognized as a potent tumor suppressor a mechanism that stops the proliferation of cells at risk of malignant transformation making of senescence a strong barrier against cancer progression (Vijg, Maslov et al. 2008). The ambivalence of senescence lies in the early benefit of tumor proliferation and the late deleterious effect related to aging, this duality is called antagonistic pleiotropy.

At the cellular level, senescence is characterized by an irreversible cell cycle arrest leading to a non-proliferation state accompanied by a set of modifications, at the level of the morphological, functional, and gene expression level.

IV-1.1 Characteristics of senescent cells

Although senescent cells are metabolically active, their arrest in the G1 phase of the cell cycle has many consequences. From a morphological point of view, the senescent cells flatten out and their cell volume increases. Increased protein synthesis via the mTOR-dependent signalling has been proposed to explain these changes (Fingar, Salama et al. 2002, Mamane, Petroulakis et al. 2006). Others suggested changes in the structure of the cytoskeleton (Nishio, Inoue et al. 2001).

A common feature to senescent cells is the increase in the number and activity of lysosomes. This characteristic is at the origin of the increased β-galactosidase activity associated with senescence (SA-β-gal). Indeed, it has been shown that senescent cells express detectable β-galactosidase activity at pH 6.0 that is different from the acidic β-galactosidase activity present in normal cells and detectable at pH 4.0 (Debacq-Chainiaux, Erusalimsky et al. 2009).

Senescent cells exhibit excessive gene expression resulting in a secretion profile of many pro-inflammatory cytokines, chemokines, growth factors, and proteases called the senescence-

associated secretory phenotype (SASP). SASP confers a strong ability to senescent cells to interact with their environment, to promote and enhance inflammation, which plays a major role in age-related diseases such as atherosclerosis (Chung, Cesari et al. 2009, Sikora, Arendt et al. 2011).

In addition, some SASP proteins may favour cell cycle arrest, and the secretion of including inflammatory cytokines (interleukins) and chemokines (Kuilman, Michaloglou et al. 2008).

At the nuclear level, the structure of chromatin determines whether the gene is transcriptionally active (euchromatin) or silent (heterochromatin). Senescent cells have a characteristic foci of condensed heterochromatin, termed SAHF (senescence-associated heterochromatin foci) (Narita, Nunez et al. 2003) that are detectable as clusters after DAPI labelling of the DNA and are distinct from the homogeneous staining in non-senescent cells. Because the heterochromatin structure mainly depends on histone modifications by acetylation or methylation, acetylation of lysine 9 or methylation of lysine 4 of histone H3K9, is an exclusive marker of SAHF. SAHFs are associated with the down regulation of genes regulated by the E2F transcription factor, such as cyclins, and to the occurrence of several pathways involving p16 or p53 proteins.

It should be noted that many concepts related to senescence have been developed from studies of human fibroblasts and some immortalized cell lines. Therefore, their relevance should be questioned for primary endothelial cells.

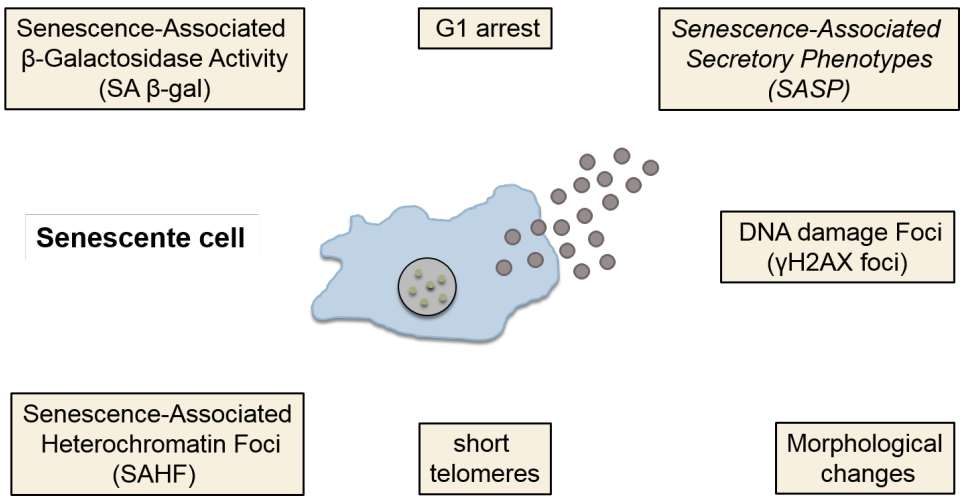


Figure 14: Characteristics of senescent cells. *Senescent cells are flattened, they secrete inflammatory mediators, present DNA damages and high Senescent-associated ß galactosidase activity. They are maintained in G1 phase and are dysfunctional.*

IV-1.2 Mechanisms of senescence

IV-1.2.1 Replicative senescence

Replicative senescence is observed after a number of divisions (Erusalimsky and Skene 2009) and is associated with physiological aging and would be triggered by the progressive shortening of telomeres, a DNA sequence located at the end of chromosomes, and by a dysfunctional telomerase. Indeed, the cells lose between 50 to 300 nucleotides telomeric after each division (Levy, Allsopp et al. 1992, Allsopp, Chang et al. 1995), until critical length, which triggers cell cycle arrest. Due to a defect in the DNA replication machinery, the so-called "end replication problem", conventional DNA polymerases are unable to replicate the end of the distal DNA strand, resulting in a gradual loss of nucleotides at the telomeres. This observation is at the origin of the hypothesis of the "mitotic clock".

The telomeres consist of the same six nucleotides repeat sequence (TTAGGG) that extends over a length of 10 to 15 kilobases. The single-stranded 3 'end of the distal strand is inserted into the double-stranded DNA molecule at the telomere level, forming a T-loop which no longer allows the outgoing single-strand end to appear, thus preventing its recognition as a break, by the machinery of DNA repair, which can lead to cell death. This conformation is stabilized by a set of specialized binding proteins called shelterin, a heterodimeric complex of six proteins, TRF1, TRF2, Pot1-TPP1, Rap1, and Tin2. The synthesis of telomeric DNA requires the presence of a particular DNA polymerase, a telomerase, a ribonucleoprotein complex consisting of an enzymatic subunit, hTERT (catalytic subunit of human telomerase reverse transcriptase) which catalyzes the addition of the TTAGGG sequence to the 3 'end of the DNA by virtue of its reverse transcriptase activity using a RNA primer present in the regulatory subunit, hTERC (Blackburn 2000, McEachern, Krauskopf et al. 2000). The majority of somatic cells have a very low level of telomerase, unlike germ cells or tumor cells. The inhibition of telomerase by RNAi induces the arrest of tumor growth (Li, Crothers et al. 2005). In contrast, overexpression of telomerase in mice increases their life expectancy (Gonzalez-Suarez, Samper et al. 2001).

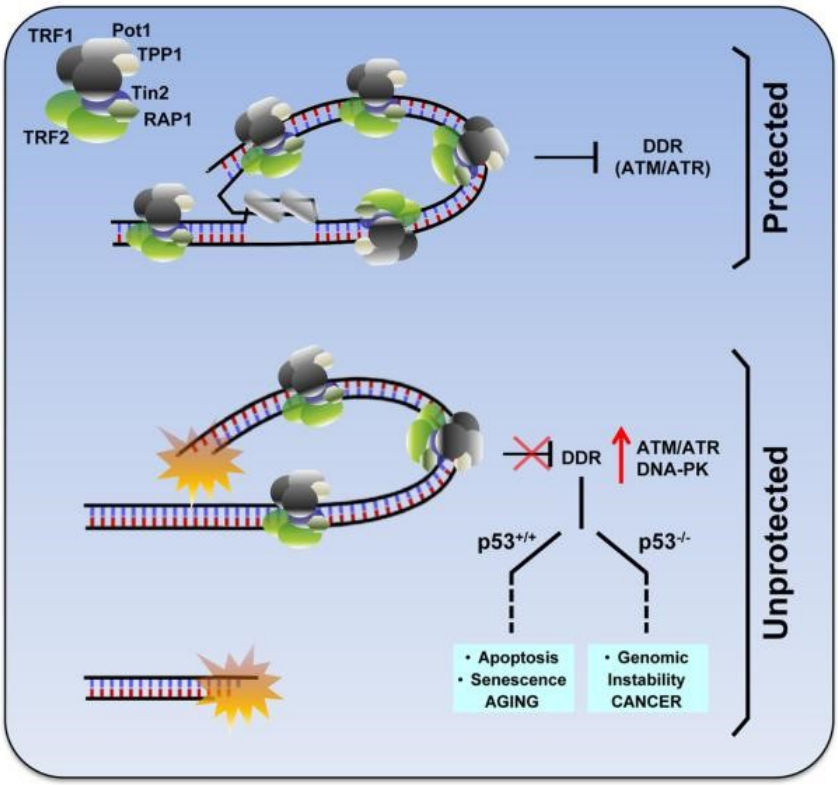


Figure 15 : Mechanism of telomer repair *The shortening of telomeres induces senescence (Herrera, Mingorance et al. 2010)*. (DDR: DNA damage response, ATM: ataxia telangiectasia mutated, ATR: ATM and Rad3-related

IV-1.2.2 Premature senescence

Other studies have reported that the same cells able to enter replicative senescence after a certain number of divisions, could also present senescence, despite a small number of cell divisions, suggesting the existence of other inducers of senescence independently shortening of telomeres. Telomer extension by telomerase overexpression in human fibroblasts has been reported not to protect against senescence observed after exposure to UV, γ-irradiation, or H2O2, confirming the existence of a senescence mechanism independent of telomerase action (Gorbunova, Seluanov et al. 2002). Indeed premature senescence is also inducible in response to 2 different cellular stress (Erusalimsky 2009): Stress-Induced Premature Seniors (SIPS) including oxidative stress or DNA damage, and OIS (Oncogene-Induced Senescence) involving persistent mitogenic stimulation or exposure to radiation.

The development of premature endothelial senescence in pathological conditions proves to be an early response to stress induced by various risk factors such as diabetes, hypertension, smoking or hyperglycemia. In fact, cardiovascular risk factors are associated with an increase

in oxidative stress levels (Csiszar, Ungvari et al. 2002). Thus, aortic fragments from spontaneously hypertensive rats (SHR) show an increase in SA-β-gal labeling, associated with an increase in phosphorylation of the γH2AX histone and overexpression of p53, compared to control rats Kyoto. Similarly, SA-β-gal labeling was observed in the thoracic aorta of Zucker's obese diabetic rats (ZDF) but remains undetectable in their lean counterparts (ZL) (Chen, Brodsky et al. 2002).

The distinction between replicative senescence, induced by telomere shortening, or stress-induced premature senescence remains delicate. It is very likely that both types of senescence may contribute jointly to pathological processes in vivo. Excessive telomere shortening has been observed in circulating white blood cells of patients with hypertension (Jeanclos, Schork et al. 2000), coronary atherosclerosis (Samani, Boultby et al. 2001, Fitzpatrick, Kronmal et al. 2007), myocardial infarction (Brouilette, Singh et al. 2003), and diabetes (Jeanclos, Krolewski et al. 1998) suggesting that cardiovascular risk factors could also affect the replicative potential of cells in vivo, and accelerate the physiological aging process (Chen and Goligorsky 2006). It has been shown that premature senescence ECs from atherosclerotic patients exhibited the same pathways components than replicative senescence. This may be related to the fact that atherothrombosis is an inflammatory process characterized, among other things, by a so-called "restorative" hyperproliferation of ECs and CML, which can explain the shortening of telomeres observed in these cells. In addition, in atherosclerotic patients, the development of EC senescence correlates with the duration of exposure to cardiovascular risk factors and the severity of the disease rather than with age. Overexpression of telomerase in these cells stabilizes telomere length and slows the onset of senescence (Voghel, Thorin-Trescases et al. 2007).

IV-1.3 Mechanisms of cell cycle arrest in senescence

When DNA damage is detected, the cell cycle is momentarily interrupted until repair or, if appropriate, entry into senescence or apoptosis. Both the replicative and premature senescence mechanisms often involve the p16/Rb or p53/p21 tumor suppressor pathway, which orchestrates cell cycle arrest. The p53 protein regulates apoptosis via the activation of the proapoptotic protein BAX (Miyashita and Reed 1995), and senescence via activation of the p21 cell cycle inhibitor (el-Deiry, Tokino et al. 1993). In response to shortening of the telomeres or after DNA damage, p53 is phosphorylated on the serine residue by the ATM, or on the serine residue by the CHK2 kinase (ATM and CHK2 being the proteins of the DDR: DNA Damage Response), which stabilizes p53 preventing its binding to its main inhibitor, mdm2. The ubiquitylation of p53 by mdm2 causes its degradation in the

proteasome. Moreover, the acetylation of p53 on the lysine residues 382 and 320 allows its binding to the DNA and increases its transcriptional activity allowing the overexpression of its main target gene in senescent cells, p21 / WAF1 (Webley, Bond et al. 2000), see figure 15.

The p21 protein is a Cip / Kip family cyclin dependent kinase inhibitor, it binds to the cyclin kinase CDK2, preventing the formation of the cyclin E-CDK2 complex and the G1-S transition (Harper, Adami et al. 1993), see figure 16 below. CDKs are serine-threonine kinases, while cyclins lack enzymatic activity and serve as a regulatory subunit for CDKs. The cell enters and leaves the cell cycle according to the balance between synthesis and degradation of cyclins, whose synthesis is not constant. Inhibition of these complexes results in cell cycle arrest.

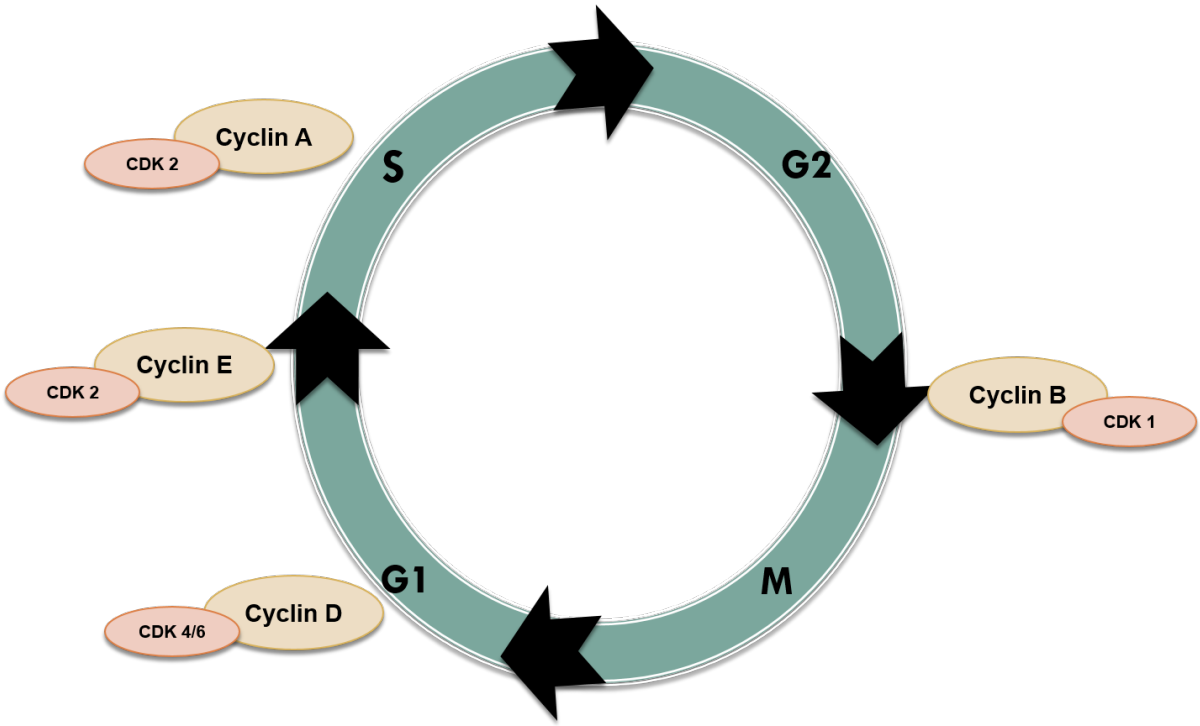


Figure 16 : Cell cycle regulation by the complex Cyclin Dependent Kinase- Cyclin

Control points of the cell cycle are regulated by specific cyclin-CDK (cyclin-dependent kinase) complexes. CDKs are serine-threonine kinases, while cyclins lack enzymatic activity and serve as a regulatory subunit for CDKs. The cell enters and leaves the cell cycle according to the balance between synthesis and degradation of cyclins, whose synthesis is not constant. Inhibition of these complexes results in cell cycle arrest. CDK inhibitors, CKIs, are divided into INK4 and the Cip / Kip families. During phase G1, an important target of cyclin-CDK complexes is the retinoblastoma protein (Rb). In its dephosphorylated active form, it forms a complex with the transcription factor E2F and repress the expression of many genes involved in the regulation of the cycle. Phosphorylation of Rb releases the E2F transcription factor which binds to the promoters of different genes involved in the progression of the cycle and allows the entry into mitosis (Schafer 1998).

The p16 protein is a CDK inhibitor involved in senescence. Overexpression of p16 occurs after p21 activation and proliferation arrest, emphasizing the essential role of p21 in the initiation of senescence (Alcorta, Xiong et al. 1996, Stein, Drullinger et al. 1999, Dulic, Beney et al. 2000). p16 belongs to the INK4a family. It interacts with CDK4 and CDK6 to induce cell cycle arrest in G1 phase, dependent on Rb protein (Parry, Bates et al. 1995). Unlike p21, p16 is not under the control of p53. It is regulated by the RAS-RAF-MEK pathway.

There is also a debate about the need and contribution of each of the tumor suppressor pathways to initiate senescence. In human fibroblasts both pathways can be involved (Itahana, Zou et al. 2003). It has been proposed that separate senescence programs may progress in parallel, resulting in "mosaic cultures," with some cells expressing p21 or other p16s or both (Ben-Porath and Weinberg 2005).

IV-2 Contribution of cellular senescence to vascular aging

Many studies confirm an association between aging-related pathologies and mediators of cellular senescence. Clearance of p16-positive senescent cells, using a transgene, INK-ATTAC, to induce apoptosis of cells expressing p16, prolonged the life span of mice, and attenuated age-related deterioration of several organs such as kidney, heart and adipocytes (Baker, Smyth et al. 2014).

Several research groups have examined *in vivo* the hypothesis that vascular cell senescence may contribute to the pathogenesis of cardiovascular disease (Burrig 1991) notably using cytochemical analysis SA β-gal. In animals, an increase in the number of SA-β-gal positive cells with overexpression of p53, p21 and p16 and high oxidative stress was observed in the aortic endothelium of obese and diabetic Zucker rats (Chen, Brodsky et al. 2002, Brodsky, Gealekman et al. 2004). In addition, Fenton *et al*. detected positive SA-β-gal endothelial cells in rabbit carotid arteries after repeated balloon endothelial denudation. All of these studies confirm the existence of endothelial senescence in vivo and its contribution to the pathogenesis of cardiovascular diseases (Fenton, Barker et al. 2001, Minamino, Miyauchi et al. 2002).

Histological examination of human atherosclerotic plaques confirmed the presence of SA-β-gal-positive ECs and smooth muscle cells, that also showed overexpression of p53, p21 and p16 and short telomeres. In addition, senescent endothelial cells have also been identified in coronary arteries of patients with ischemic heart disease, whereas no SA-β-gal positive cells have been observed in the internal mammary arteries of the same patients (Minamino,

Miyauchi et al. 2002), highlighting a role of the endothelial territory and the existence of possible areas at risk of senescence. In another study, the accumulation of senescent cardiomyocytes with significant telomere shortening was detected by endomyocardial biopsy in the hearts of elderly patients with cardiomyopathy (Chimenti, Kajstura et al. 2003).

IV-2.1 Senescence and endothelial dysfunction

Whatever the origin of the senescence (replicative or premature), the resulting functional alterations, lead to a pro-inflammatory and pro-thrombotic phenotype, known to have a role in the cardiovascular diseases associated with aging and associated disorders like diabetes or hypertension.

Several studies have confirmed the role of senescence in the development of endothelial dysfunction. Basal NO production by human umbilical vein endothelial cells (HUVEC) decreases with the doubling of the population (Sato, Kaji et al. 1993). Similarly, NO production in response to shear forces is also decreased in senescent human aortic endothelial cells (HAEC) (Matsushita, Chang et al. 2001). In addition, production of prostacyclin is also decreased in EC after several serial passages (replicative senescence), or after incubation with H2O2 (premature senescence), while thromboxane A2 production is increased. One study has shown by a more direct approach that targeted p53 overexpression in the endothelium is associated with decreased endothelium-dependent relaxation in aortic rings, with reduced NO bioavailability, whereas sodium nitroprusside, a NO donor, could trigger relaxation. The role of senescence in the induction of endothelial dysfunction has been confirmed by other approaches showing that the prevention of senescence improves endothelial dysfunction. Indeed, the extension of telomeres by ectopic expression of hTERT (catalytic subunit of telomerase) in human ECs in culture, prolonged their lifespan and protected against endothelial dysfunction (Yang, Chang et al. 1999, Minamino, Miyauchi et al. 2004). Similarly, transfection of human aortic EC by telomerase induced a young phenotype, delayed age-related deficits in eNOS expression, and increased NO production (Matsushita, Chang et al. 2001).

IV-2.2 Oxidative stress at the origin of endothelial senescence

The theory of free radical contribution to aging dates back to the middle of the 20th century, when it was discovered that ROS, traditionally considered too reactive to exist in biological systems, can be formed *in situ*, in response to ionizing radiation, or oxygen, and are

responsible for deleterious responses. Harman proposed that ROS be endogenously formed from oxygen metabolism and play a vital role in the aging process (Harman 1956).

Kapahi *et al*. Observed a direct correlation between the longevity of different animal species and their antioxidant defenses (Kapahi, Boulton et al. 1999). However, most clinical trials have failed to demonstrate any significant beneficial effect of antioxidants (α-tocopherol, vitamin C and / or E) on the morbidity and mortality associated with cardiovascular disease (Steinberg and Witztum 2002).

Numerous in vitro experimental data indicate that oxidative stress induces senescence following oxidative damage to DNA. Thus, the oxidation rate of the DNA measured by the release of 8-dihydroxyguanine is increased in senescent cells (Chen, Brodsky et al. 2002). Oxidative stress increases telomere shortening (von Zglinicki, Pilger et al. 2000), probably because of the particular richness of telomer DNA, in guanidic bases particularly vulnerable to oxidation.

The culturing of human CML in a hypoxic environment prolongs their lifespan and increases the activity of telomerase, compared to those cultivated under normoxic conditions (Minamino and Komuro 2002). Furthermore, the addition of an antioxidant, N-acetyl cysteine (NAC) in the culture medium, prevents the replicative senescence of primary coronary pork CEs (Khemais-Benkhiat, Idris-Khodja et al. 2016). Similarly, NAC significantly delays senescence in isolated arterial segments from patients with severe coronary artery disease, activates the telomerase catalytic subunit (hTERT), and inhibits telomere attrition (Voghel, Thorin-Trescases et al. 2007).

Conversely, culturing smooth muscle cells of rat thoracic aortas under hyperoxic conditions (40% O2) induces DNA damage and up-regulates markers of senescence. Hyperoxia causes accelerated shortening of telomeres (66 base pairs lost at 20% O2 versus 486 base pairs at 40% O2), accompanied by p53 activation and premature senescence (Vaziri, West et al. 1997).

In fact, the senescence induced by the oxidative stress constitutes a model of accelerated or premature senescence frequently used. Chronic exposure of human umbilical vein endothelial cells (HUVEC) to non-cytotoxic doses of hydrogen peroxide or to an inhibitor of glutathione synthesis, buthionine sulfoximine, accelerates the onset of senescence after 30 versus 46 doublings of population (Kurz, Decary et al. 2004). Similarly, the exposure of ECs of porcine coronary arteries to oxygen peroxide (H2O2) increases SA-β-galactosidase activity and accelerates the onset of expression of cell cycle inhibitors (Abbas, Jesel et al. 2017). Symmetrically, reducing the activity of intracellular peroxides with a scavenger, alpha-

phenyl-t-butyl-nitrone, reduces the degree of telomere shortening and increases cell lifespan (von Zglinicki, Pilger et al. 2000).

IV-2.3 Role of the Angiotensin System in vascular senescence

Benigni *et al.* have shown that inactivation of the Ang II AT-1 receptor increases longevity in mice. When all wild-type mice died, 85% of AT-1R KO mice were still alive (Benigni, Corna et al. 2009) suggesting that the angiotensin system plays a key role in the aging process itself.

The components of the renin-angiotensin system have been shown to be increased in the tissues of older animals (Wang, Takagi et al. 2003), and inhibition of RAS by sartans or ACE inhibitors, shows a protective effect against age-related vascular diseases in rodents, such as hypertension and atherosclerosis (de Cavanagh, Piotrkowski et al. 2004, Basso, Paglia et al. 2005), and limits the development of age-related cardiovascular disease in humans (Brown and Hall 2005).

Ang II is indeed likely to contribute to age-related vascular diseases such as atherosclerosis by promoting the senescence of SMCs and ECs (Kunieda, Minamino et al. 2006, Shan, Guo et al. 2014).

Herbert *et al.* demonstrated that Ang II induces senescence of human SMCs in response to DNA damage caused by ROS. This response involved both replicative and premature pathways of senescence as this induction was associated with telomere shortening, however, transfection of SMC by the hTERT subunit of telomerase was not sufficient to prevent induced senescence by Ang II. Only pre-incubation of cells with Losartan, an AT-1R antagonist, or catalase, an antioxidant, prevented Ang II-induced senescence and associated DNA damage (Herbert, Mistry et al. 2008). These results suggest that Ang II, via the AT-1R receptor and the production of ROS, causes DNA damage in SMCs that cause cell cycle arrest and senescence. In vitro, Ang II decreases the telomerase activity of endothelial progenitor cells, an effect that is prevented by superoxide dismutase, again suggesting the involvement of oxidative stress (Imanishi, Hano et al. 2005).

Several molecular mechanisms have been proposed to explain the induction of vascular senescence by Ang II. The stimulation of endothelial progenitor cells by Ang II increases the expression of the gp91phox subunit of NADPH oxidase, thus contributing to oxidative stress, as evidenced by the formation of peroxynitrite (Imanishi, Hano et al. 2005). Exposure of human umbilical vein endothelial cells (HUVEC) to Ang II induces senescence via the production of mitochondrial ROS released by loss of mitochondrial membrane potential ($\Delta\Psi m$) and membrane redistribution of cytochrome C. These mitochondrial effects are

prevented by benazepril, a converting enzyme inhibitor or Losartan. Symmetrically, HUVEC treatment by a mitochondrial transition pore activator (mPTP), carbonyl cyanide 3-chlorophenylhydrazone (CCCP) induces mitochondrial damage similar to that induced by Ang II, accompanied by an increase in markers of senescence. However, these effects are not prevented by benazepril or Losartan, confirming the role of mitochondrial damage in the induction of senescence by Ang II in HUVECs.

In addition to the induction of oxidative stress, Ang II disrupts the intracellular redox-sensitive balance by decreasing antioxidant defenses. Ang II down-regulates the expression of catalase by preventing the binding of the FoxO1 transcription factor (forkhead box O1) to the promoter of the catalase gene (Xiong, Salazar et al. 2010).

Other actors and molecular mechanisms are involved in senescence in response to Ang II such as activation of small G Ras proteins, MAPKs, and transcription factors such as the nuclear factor NF-kB and AP-1 were also described (Blagosklonny 2003).

IV-2.4 Hyperglycemia and endothelial senescence

Recently, the arguments have multiplied in favor of the involvement of endothelial senescence as an early event of diabetes-related endothelial dysfunction. Senescence has been described in diabetic rat vessels and in ECs exposed to high concentrations of glucose (Yokoi, Fukuo et al. 2006, Bhatt, Lim et al. 2013). An increase in SA-β-gal activity was observed in the ECs of the aorta of mice made diabetic treated by streptozotocin , and in the aorta of Zucker diabetic rats (Inoguchi, Li et al. 2000, Yokoi, Fukuo et al. 2006).

In diabetic patients, measurement of leukocyte telomeres has been used as a surrogate for vascular senescence. The telomere length was significantly shorter in type 2 diabetics compared to control subjects, and inversely proportional to levels of oxidative DNA damage (Sampson, Winterbone et al. 2006).

In vitro, many teams have shown an induction of endothelial senescence by "high glucose in the incubation media. Hayashi et al., showed that exposure of HUVECs to high glucose concentrations increases SA-β-gal activity and ROS production, and decreases telomerase activity. These effects were accompanied by decreased expression of eNOS and endothelial NO production. All of these effects are prevented by treatment with L-arginine, L-citrulline and antioxidants (vitamin C and E) alone or in combination, as well as by overexpression of eNOS in transfected ECs, suggesting an important role for NO in the prevention of endothelial senescence during diabetes (Hayashi, Matsui-Hirai et al. 2006).

Interestingly, the young endothelial cells cultured on a glycated collagen matrix show an increase in SA-β-gal activity and the expression of p53 without telomere shortening nor a decrease in telomerase activity. These effects are associated with a decrease in NO production despite a 3-fold increase in eNOS expression, and an increase in nitrotyrosine-modified proteins. All these effects are prevented and reversed by the antioxidant treatment, ebselen or by a superoxide dismutase analogue. These results indicate that the reduction in the bioavailability of NO, the excess of peroxynitrite and / or superoxide anions observed in diabetes are the cause of premature endothelial senescence induced by hyperglycemia (Chen, Brodsky et al. 2002).

Meta-analysis of several clinical trials showed that inhibition of RAS decreased the number of new diabetic patients (Abuissa, Jones et al. 2005, Yusuf, Ostergren et al. 2005). In addition, clinical trials have shown a beneficial CV effect of ACE inhibitors and Sartans in diabetics. Thus, it is possible that the angiotensin system is involved in the development of vascular dysfunction during diabetes.

IV-2.4.1 Role of the angiotensin system in senescence induced by hyperglycemia

Chen et al., Showed that EC exposure to high glucose (HG) increases SA-β-gal activity and mitochondrial oxidative stress, accompanied by overexpression of the conversion enzyme, and AT-receptor. 1 of Ang II. The treatment of ECs with ACE inhibitors (benazepril) or Sartans (losartan) inhibits HG-induced senescence and mitochondrial oxidative stress, suggesting a key role of the RAS system in glucose-induced endothelial senescence (S. Khemais, 2016). Thus, the deleterious effects of chronic hyperglycemia on vascular function could contribute to the development of cardiovascular disease (CVD) in diabetics via exaggerated AT1 –dependent senescence.

Hypothesis and aims

Because MPs are markers of vascular damage occurring during acute events such as myocardial infarction, but also identified during other chronic disorders with cardiovascular issues such as diabetes, we reasoned that

- **Microparticles contribute to the endothelial senescence and consecutive dysfunction in vascular aging**
- **Microparticles contribute to the premature endothelial dysfunction in diabetes**
- Endothelial pharmacological protection against premature senescence or dysfunction should prevent MP-induced vascular damage

To explore these hypothesis our aims were:

- **To set and validate a methodological approach for the isolation of tissue MPs as a source of immune cells-derived MPs or of coronary artery-derived MPs**
- **To measure the endothelial pro-senescent properties of these MPs**
- **To measure the properties of these MPs on endothelial-dependent vascular reactivity**

Results

Results Part 1:

Methodological approach to the study of tissue MPs:

MPs from splenocytes as a signature of the immune cells activation and vascular effectors

1-Background and proof of concept

One of the most recognized results of aging is reduced immune function (Thompson, Shay et al. 2003). The effects of aging on the immune system are widespread and affect the rate at which naive B and T cells are produced the composition and quality of the mature lymphocyte pool (Thompson, Shay et al. 2003).

In healthy individuals, leukocyte-derived MPs (LMPs) represent a small fraction (<10%) of the total MP population in blood (Hoyer, Nickenig et al. 2010). Plasma levels of LMPs significantly raise in patients with cardiovascular, atherosclerosis, hypertension, and diabetes mellitus (Owens and Mackman 2011, Angelillo-Scherrer 2012).

Few studies is septic rats have indeed shown that circulating circulating leukocyte-derived MPs are elevated and a characteristic feature of a pro-inflammatory and procoagulant state. (Mostefai, Agouni et al. 2008, Boisrame-Helms, Delabranche et al. 2014) In patients with septic shock, elevated MPs of neutrophile and endothelial origin typify underlying disseminated intravascular coagulation and patients outcome may be related to the Netosis, i.e. to the proportion of neutrophils that have release decondensed chromatin to limit bacterial spread.(Delabranche, Stiel et al. 2017)

Although circulating MPs of leukocyte origin are a signature of the pro-inflammatory and procoagulant vascular response, their properties as vascular effectors remain difficult to study in animal models owing to technical limitations: the purification yields from the plasma remain low in order to avoid exosome contamination, their half-life is not well characterized and varies with species and pathological condition, making the identification of the smallest proportion of leukocyte-derived MPs, such as neutrophil-derived MP, difficult to assess.

To circumvent this difficulty, we chose to harvest immune MPs from the spleen of rats. The methods offer the advantage of a possible assessment of a pharmacological control of the MP release by various treatments targeting either plasma membrane remodelling or specific leukocyte sub-population. To validate the model, we compared the release of MPs generated from splenocytes, under conditions mimicking innate or adaptive immune responses and studied their variations with ageing. The method is based on the harvest of rat spleen tissues that were submitted to standardized extraction procedures to obtain spleen–derived leukocytes. After a first osmotic elimination of red blood cells, splenocytes were cultured for 24h before collection of MPs in the cell supernatants.

To assess the ability of splenocytes to release SR-MPs we compared the amount and properties of MPs generated at baseline or after stimulation by *E. Coli* LPS or by a

combination of calcium ionophore and PMA to mimic innate and adaptative immune response triggered by LPS or PKC-dependent pathways. This method was previously developed in the laboratory by Ali El Habhab and Sonia Khemais (thesis in 2018 and 2017). In addition, Ali El Habhab demonstrated that SR-MPs are inducers of endothelial senescence without triggering EC apoptosis.

Other members of the laboratory have investigated the MP release from the vessel wall. All data will be compiled to publish a methodological approach for the analysis of tissue MPs, an area of expertise that remains to be developed. The manuscript of compiled data is in preparation with co-authors (see annexes and discussion)

2- Methodological approach

Previous data from our group and others have shown that circulating vascular MPs levels remain stable in middle age rats whereas onset of pancreatic islets damage could be observed in histological sections, suggesting that early age-related organ alterations may be specific and hardly detectable in blood flow. (Faggioli, Wang et al. 2012)

In a first set of experiments and to validate the method, we examined the variations of splenocyte-derived MPs (SR-MPs) obtained from splenocytes freshly isolated from the spleen of young, middle age and old Wistar rats.

In a second set of experiments the method was optimized and was further applied to the study of the interactions between leukocyte MPs and endothelial cells in a model of high glucose-induced endothelial senescence, in order to further examine whether high glucose concentration could potentiate the pro-senescent effect of SR-MPs. Endothelial-dependent vascular response to high glucose and SR-MPs was also assessed. Data are summarized in an original manuscript

2-1- Variations of splenocyte-derived MPs (SR-MPs) with age and stimulus

Spleens were isolated from young, middle-age and old Wistar rats (8, 24, 72 weeks, respectively). After osmotic treatment to eliminate red blood cells, 3 millions splenocytes/ mL were seeded in RPMI culture medium in the absence or presence of 5 µg/ml LPS from E. Coli, or a combination of PMA (25 ng/ml / 1 µM A23189 calcium ionophore (PMA/I), see following manuscript).

2-1.1 Ageing and LPS or PMA/I treatment reduce splenocyte recovery

After 24h incubation of splenocytes in culture medium, a reduced recovery of splenocytes from old or middle age rats *vs.* young rats with respectively 14.4 %, 31.83%, 62.04% loss (see figure below). Whatever the age, both inducers led to a significantly reduced recovery of splenocytes (untreated cells from young rats: 14.4 % loss vs. 25.4 % after 24h treatment with LPS and 42.08% by the combination PMA/ A23189 calcium ionophore (PMA/I); middle-age rats untreated cells: 31.83 % loss vs. + LPS: 43.2%, + PMA/I :, 61.01% ; old rats untreated cells : 62.04% loss vs. + LPS: 85.52% , + PMA/I : 72.03%).

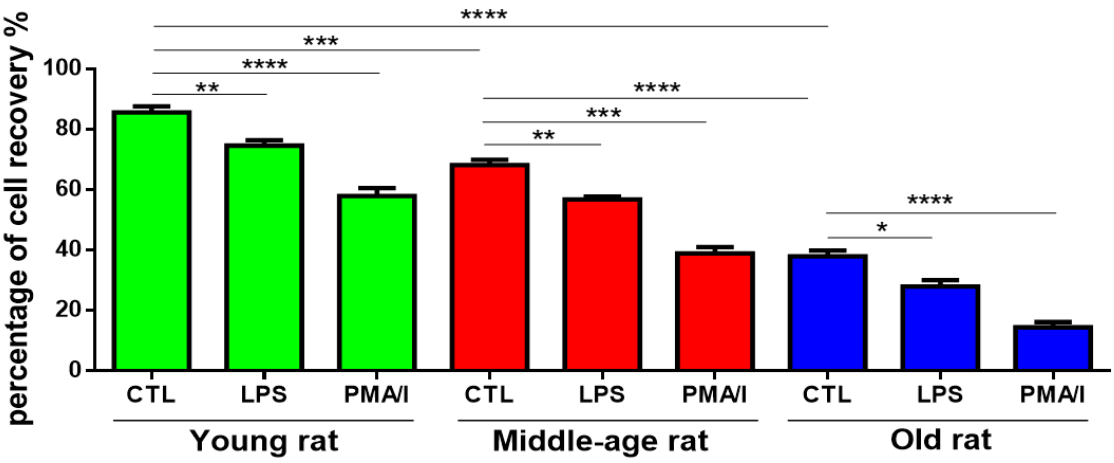


Figure 1. Effect of age, LPS or PMA/calcium ionophore treatment on the splenocyte recovery. *CTL: unstimulated splenocytes*

2-1.2 Ageing and LPS or PMA/I treatment promote the release of SR-MPs

Compared to young rat cells, splenocytes from middle age and old rats release a significant higher amount of SR-MPs after 24h culture (2- and 3.5 – fold enhancement respectively). Whatever the age, LPS or PMA/I) enhanced significantly the SR-MP release

(approximatively 1,3 and 2- fold enhancement by LPS or PMA/I respectively in young or middle age rats, and 2.8 enhancement by PMA/I in cells from old rats).

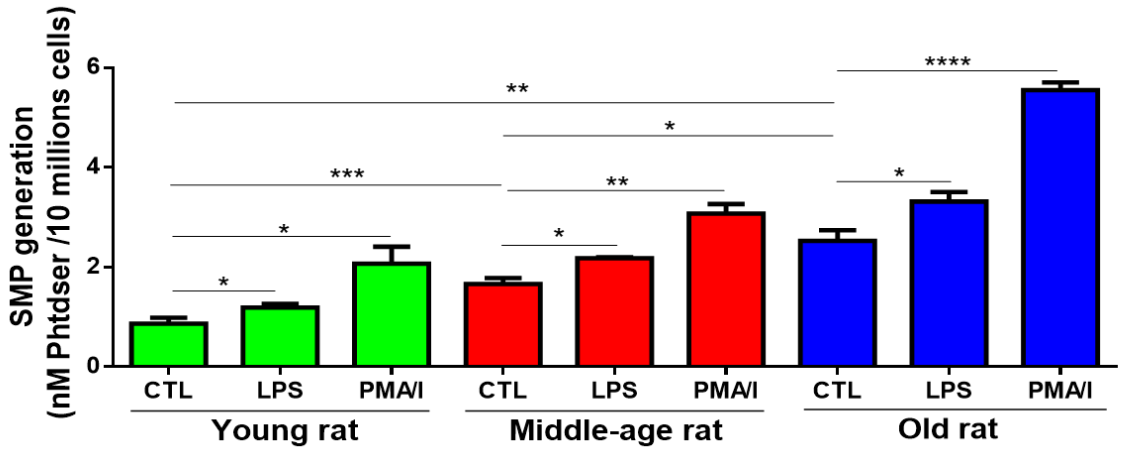


Figure 2. Effect of age, LPS or PMA/calcium ionophore treatment on the release of MPs by splenocytes

2-2 Ageing and LPS or PMA/I treatment modify the pro-senescent effects of SR-MPs

To determine whether the properties of SR-MPs vary with age, SR-MPs were isolated from the spleens, cultured in medium for 24h as above, isolated from supernatant before incubation with porcine primary coronary endothelial cells. All MP preparations were applied at the identical concentration (10 nM). After 48h, senescence was measured in ECs by the ability of Senescence-Associated galactosidase to cleave a fluorogenic substrate (figure 3).

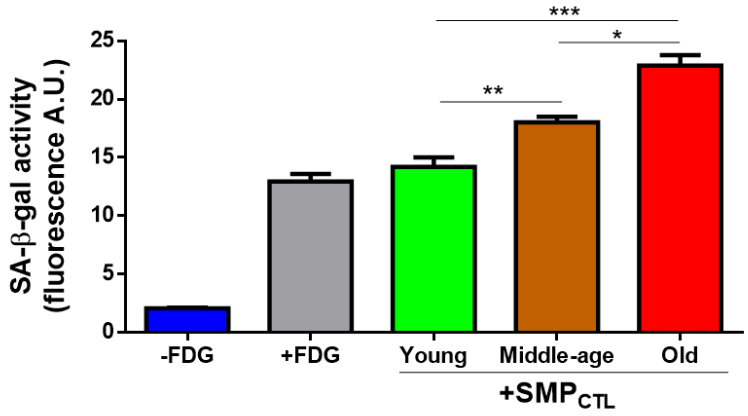


Figure 3. Impact of the age on SR-MPs induced endothelial senescence. *Senescence was measured by flow cytometry using the FDG fluorogenic substrate of senescence-associated -β galactosidase (SA- β Gal activity). –FDG: unlabelled ECs, + FDG: labelled ECs. SMP$_{CTL}$: SR-MPs isolated from the spleen without any inducer of MP release*

Taken together, these data indicate that SR-MPs are indicators of the spleen tissue damage:

- *The ability of splenocytes to release SR-MPs decreases with age*
- *SR-MP release in response to LPS and PMA/I is higher than baseline*
- *SR-MPs acquire significant endothelial pro-senescent property with age*

2-3 High glucose concentration modify the ability of splenocytes to shed MPs

As shown in the figure below a concentration of 5 millions splenocytes /mL induces a drop of the medium glucose concentration as early as 5 hours after splenocyte culture in RPMI medium containing 11 mM glucose (normal glucose, NG) or 10h in RPMI containing 25 mM glucose (high glucose, HG). Therefore, we selected 3 millions/mL splenocytes to produce MPs in both HG and NG culture media in order to limit glucotoxicity that might alter the number and/or properties of a proportion of the released MPs.

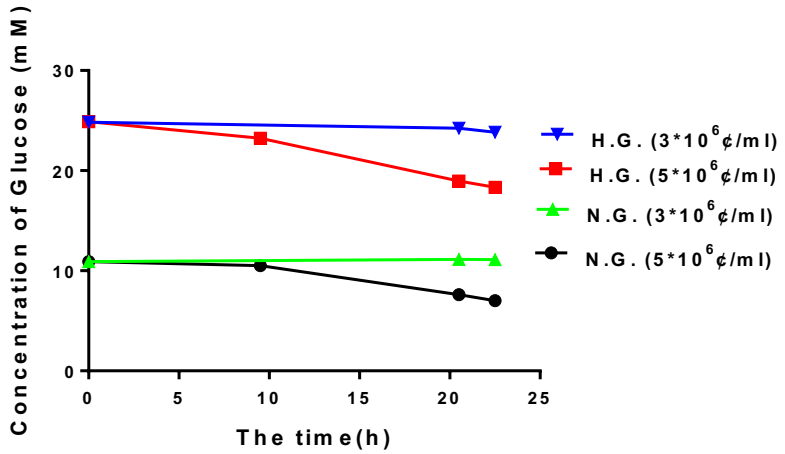


Figure 4. Kinetics of glucose consumption by splenocytes incubated in high (HG) and low glucose (LG) over 24 h. *Cells were incubated at different concentrations*

To further confirm that SR-MP shedding from splenocytes is dependent on their environment, we incubated 5 million/mL splenocytes in NG or HG for 30 min and extracted the MPs after additional 20 min (17 rpm, RT) rotating of the cell suspension, before application of the standardized centrifugation isolation procedure (800g, and twice 14000 g).

Early discrepancy between the two media was evidenced after a total exposure of 40 min. Compared to NG, HG led to a significant increase in MP shedding that mirrored a drop of

splenocyte recovery. However, the enhancement did not the optimal effect obtained by treatment with a combination of phorbol myristate acetate and calcium ionophore (PMA/I)

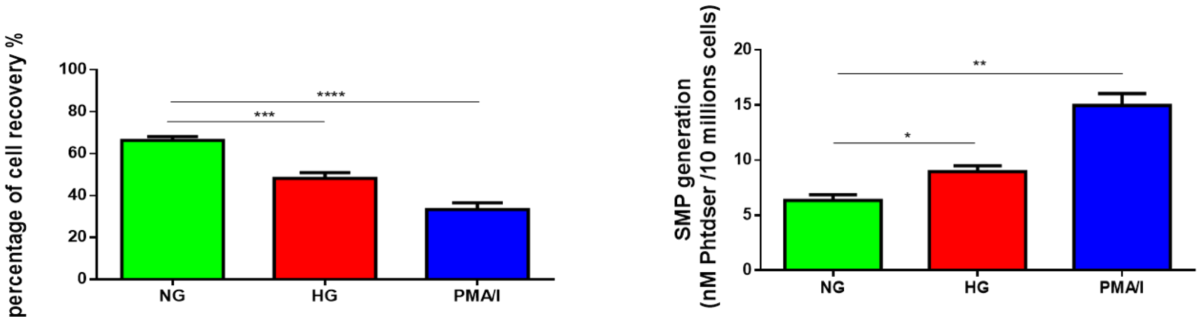


Figure 5. Early alteration of splenocyte recovery and MP shedding in high (HG) and low glucose (LG). *Measurements were performed after 40 min incubation and compared to stimulation by a combination of phorbol myristate acetate and calcium ionophore known to prompt optimal SMP release.*

These data confirm that spleen-derived MPs are reliable indicators of the severity of the alterations of membrane remodelling:

- **SR-MP shedding is enhanced in response to exogenous pro-inflammatory mediators (PMA/I) and in the presence of high glucose concentration.**
- **Alterations of SR-MP shedding prompted by exogenous mediators are initiated within the first hour of incubation.**

Having optimized the conditions of MP generation from splenocytes and their isolation, we took advantage of this important source of MPs derived from primary immune cells to study their effector properties in hyperglycemia-induced endothelial senescence.

3- Impact of short and long term incubation for the investigation of SR-MP vascular effects: Preliminary studies with coronary artery rings

We had to optimize the classical vascular reactivity study procedures in order to be able to investigate MP effects, owing to the mandatory longer time of incubation. In preliminary data we measured bradykinin-induced relaxation after 6h, 12h, 18h, and 24h incubation in RPMI containing 11 mM glucose. Data indicate that under our conditions of incubation (37°C,

sterile and humide atmosphere), vascular relaxation is achieved at lower bradykinin concentration. Because the relaxation response was sharp we applied a progressive bradykinin concentration between 10^{-10} M- 7.1 10^{-9} M. (see figure 5 bis below)

RPMI was selected because other tested media like MCDB led to higher variability in relaxation values. Similarly, supplementation of media with foetal bovine serum (10 % in RPMI or 15% in MCDB) led to higher variability in vascular relaxation. To circumvent this drawback the optimal incubation procedure included RPMI without serum and a gentle hand shaking of the incubation plate, every 3 hours.

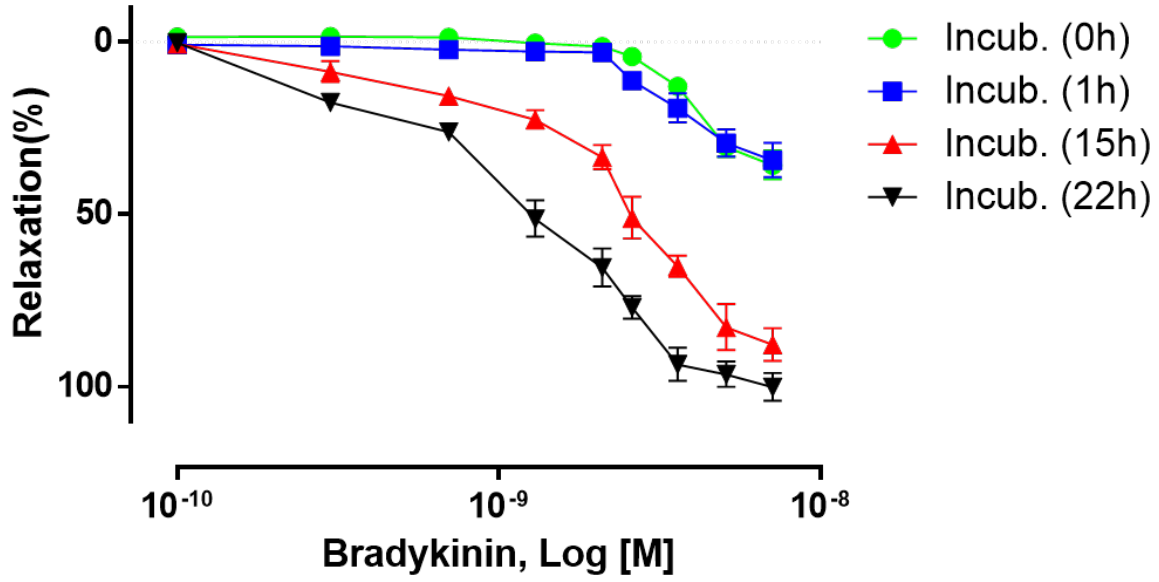


Figure 5bis. Effect of incubation time on the endothelial-dependent relaxation of porcine coronary artery rings in response to bradykinin. *Measurements were performed after 1h, 15h and 22h of incubation in RPMI medium without serum at 37°C under humid atmosphere. Ablation of the endothelium before adding bradykinin completely abolished vasorelaxation. Green curve: control (no incubation at 37°C, rings were kept in Krebs solution at 4°C during the time of incubation of the other samples)*

4- Impact of glucose concentration for the investigation of SR-MP vascular effects: Preliminary studies with coronary artery rings

Because glucose concentration might interfere with the endothelial-dependent relaxation, we examined whether glucose concentration could vary with the incubation time in the limited incubation volume of the microtitration wells. Glucose concentration was measured in 0.5 ml, 1 mL, 5 mL RPMI after 1h, 6h, 12h, 18h, and 24 hours. We could observe that 1 mL

incubation medium lost 0.22 mM glucose per hour in the presence of 1 ring of porcine coronary artery (2-3 mm width) (see figure 5 ter below). For comparison purposes between high and low glucose concentrations, incubation media were ready-to-use in order to avoid any modification of other reactants of the medium. Optimal incubation time was 18h (without MPs) that enabled clear differences between high and low glucose effects on the vascular response, either by vascular reactivity or by measuring protein expression (see also part 2). Finally, to avoid misinterpretation of the data, wells in which the incubation medium colour turned to yellow were suspected of contamination and discarded. However, it has to be noted that a switch of colour could also be observed after glucose shortage. To prevent contamination, extensive washing of the ring prior incubation was performed either with RPMI or sterile Krebs buffer (4-5 times at room temperature and under the hood).

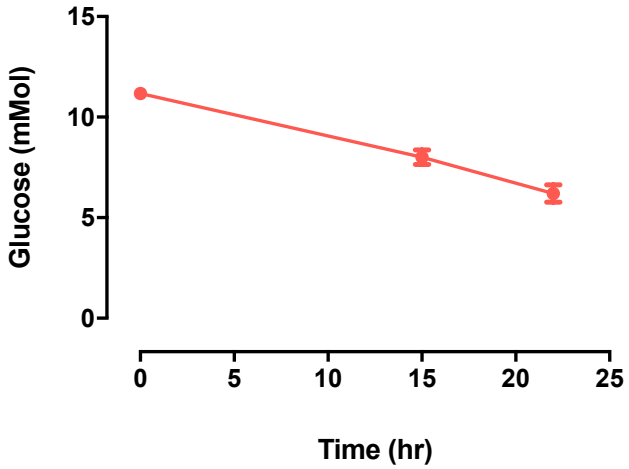


Figure 5 ter. Kinetics of glucose consumption by the porcine coronary artery rings. *Measurements were performed after 1h, 15h and 24h of incubation in RPMI medium containing 11 mM without serum at 37°C under humid atmosphere. Concentration was measured as mM of glucose in the culture medium (1 mL containing 1 ring 2-3 mm length) at each time. N=6 experiments, each measure performed in triplicate (6 different individuals).*

High glucose potentiates the pro-senescent effect of leukocyte-derived MPs in isolated ECs and in the vessel wall: Impact on endothelial-dependent control of the vascular tone

Altamimy R, Qureshi A, Amoura L, El Habhab A, EL Itawi H, Kassem M, Khemais S, Pollet B, Zobairi F, Auger C, Schini-Kerth V, Toti F.

Summary

Introduction Microparticles (MPs) are plasma membrane vesicles and vascular effectors. We have shown that (i) leukocyte-derived MPs shed in response to stress are pro-inflammatory, procoagulant and prosenescent endothelial effectors, (ii) high glucose induces premature endothelial senescence.

Objective: To determine the combined noxious effects of leukocyte-derived MPs endothelial and high glucose on endothelial senescence and dysfunction.

Methods: Leukocyte MPs were isolated from rat splenocytes with 5 mg/ml LPS (MP_{LPS}), 25-ng/ml PMA/1mM A23187 ionophore (MP_{PMAi}), or vehicle (MP_{CTL}). Porcine coronary artery endothelial cells (ECs) at passage 1 were incubated for 48 h with 1-30 nM MPs in high or low glucose concentration (HG 25 mM, NG 5.5 mM). Senescence-associated β-galactosidase (SA-ß-GAL) activity was assessed by C12FDG, protein expression by Western blot analysis. Pig coronary artery rings were pre-incubated with HG or NG for 12 h prior to addition of 1-30 nM MPs for 12h. Bradykinin (BK)-induced endothelium-dependent relaxations were assessed in organ chambers, and staining of target proteins by confocal microscopy.

Results: At 10 nM, MP_{LPS} and $MP_{PMA/I}$ enhanced SA-ß -GAL activity both by about 2-fold in NG, and respectively 3 and 3.7-fold in HG. The expression of senescence markers p21, p16 doubled and that of eNOS decreased 2-fold. MP_{PMAi} and MP_{LPS} induced a concentration-dependent inhibition of BK-induced relaxation, inhibition by respectively 10 nM and 30 nM, being 65 % in NG, amounting to about 85% in HG, whereas 30 nM MP_{CTL} had no effect. MP_{PMAi} and MP_{LPS} reduced eNOS expression by 60 % in NG and 80 % in HG. Conversely, VCAM-1, COX-2 was up regulated.

Conclusion: Leukocyte-derived MPs enhance HG-induced alteration of the endothelial function by inducing premature senescence and might contribute to vascular dysfunction in diabetes patients.

Introduction

The pathogenesis of atherosclerosis is highly accelerated by impaired glucose tolerance and diabetes. Insulin resistance can significantly contribute to early vascular dysfunction in patients with type 2 diabetes (Natali, Toschi et al. 2006). It has been estimated that more than 65% of adult diabetic patients die from cardiovascular disease or stroke (Grundy, Benjamin et al. 1999). Microparticles (MPs) are plasma membrane fragments released by stimulated cells. MPs of endothelial and leukocyte origin circulate at high levels in patients with cardiovascular disorders. Because they convey bioactive proteins and lipids and RNA to remote cells they also act as cellular effectors. (Bonello, Zahringer et al. 2007)In metabolic syndrome, circulating leukocyte-derived MPs were shown of prognosis value in sub-clinical atherothrombosis, and they accumulate into the atherosclerotic plaque. They were shown to favor a procoagulant and pro-inflammatory state of endothelial cells in MP-mediated cross-talk models. (Salminen, Kaarniranta et al. 2012)

In diabetes, manifestations of the vascular disease are characterized by an imbalance of vasodilation and vasoconstriction, increased arterial stiffness, and pathological remodeling (Tabit, Chung et al. 2010). High levels of pro-inflammatory cytokines and procoagulant endothelial-derived MPs circulate at high level in diabetic patients reflecting a pro-inflammatory state and endothelial damage or dysfunction. (Sabatier, Darmon et al. 2002)

Endothelial dysfunction, defined as vasoconstriction rather than arterial vasodilation after stimulation with endothelium-dependent agents such as acetylcholine or bradykinin is associated with early stages of atherosclerosis (Furchgott and Zawadzki 1980, Bonetti, Lerman et al. 2003) and characterized by endothelial dysfunction with reduced of bioavailability of vasodilators, in particular, nitric oxide (NO), whereas contracting-derived endothelium factors (Lerman and Burnett 1992) increase. There is strong evidence that hyperglycemia is deleterious to endothelial function (Taylor and Poston 1994, Dorigo, Fraccarollo et al. 1997, Jin and Bohlen 1997, Williams, Goldfine et al. 1998), especially with long-term exposure. However, a few studies have demonstrated the effects of high glucose with short-term exposure. (Williams, Goldfine et al. 1998, MacKenzie, Cooper et al. 2008, El-Awady, El-Agamy et al. 2014).

The presence of endothelial dysfunction is a strong risk for development of coronary artery disease and worsened outcome (Halcox, Schenke et al. 2002). Most studies have

investigated endothelial dysfunction and its determinants in peripheral arteries and only a few studies directly measured local changes in vascular reactivity, particularly with regard to coronary vasculature (Ong, Athanasiadis et al. 2014).

Endothelial senescence is a main contributor to vascular ageing (Erusalimsky 2009, Wang and Bennett 2012) and is accelerated by hyperglycemia-induced generation of reactive oxygen species (ROS)(Doles, Storer et al. 2012). The superoxide radical generated by NADPH oxidase system is both a main source of ROS and a main contributor to the development of diabetes-associated endothelial dysfunction (Ding, Aljofan et al. 2007, Giacco and Brownlee 2010, Doles, Storer et al. 2012). Thus, hyperglycemia-induced ROS formation and decreased bioavailability of NO help promote the shortening of telomere length, increase genomic instability and growth arrest, increase DNA damage, which leads to premature senescence in endothelial cells (Hoffmann, Haendeler et al. 2001, Hayashi, Matsui-Hirai et al. 2006, Yokoi, Fukuo et al. 2006, Ota, Eto et al. 2008, Zhong, Zou et al. 2010, Matsui-Hirai, Hayashi et al. 2011). In an amplification loop, endothelial senescence leads to the release of pro-senescent MPs and pro-senescent endothelial MPs circulate in the plasma of patients at cardiovascular risk. (Abbas, Jesel et al. 2017)

In diabetes mellitus, changes in barrier function and adhesion of leukocytes and other circulating cells at the surface of the inflamed endothelium of the artery would favor endothelial dysfunction and apoptosis and the secondary release of endothelial procoagulant MPs. In addition, hyperglycemia prompts the apoptosis of the endothelial cells from the pancreatic islet vessel (Lorenzi and Cagliero 1991, Baumgartner-Parzer, Wagner et al. 1995, Cines, Pollak et al. 1998, Ho, Liu et al. 2000, Ido, Carling et al. 2002, Favaro, Miceli et al. 2008).

In the present work, we studied the pro-senescent effects of MPs freshly isolated from the spleen and of high glucose concentration on endothelial senescence and dysfunction. MPs were isolated from freshly isolated splenocytes stimulated by LPS or a combination of phorbol myristate acetate and ionophore to mimic pro-inflammatory conditions, and incubated with primary coronary artery endothelial cells and with coronary artery rings to assess endothelial dysfunction.

Materials & Methods

Animals

Animals Male Wistar rats (Janvier-labs, Le Genest-St Isle, France) were stored in a temperature control room (22°C) and maintained on a standard 12-h light/dark cycle (lights on at 07:00 am) with free access to food and water. The experiments are consistent with the Guide to Care and the Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and were authorized by the French Ministry of Higher Education and Research and by the local ethic committee (Comité d'éthique en Matière d'expérimentation animale de Strasbourg, authorization 03799.01). All animal experiments were carried out in a registered animal yard within the Faculty of Pharmacy (Authorization number E-67-218-26).

Splenocyte isolation and culture

Spleens were withdrawn, weighed and placed in a 10-cm petri dish containing 10 ml sterile phosphate-buffered saline (PBS). All further steps were realized under sterile conditions. The spleen tissue was homogenized using a 2-ml rubber syringe plunger and the homogenate filtered through 100-µm sterile cell strainers into a 50-ml tube to remove cell debris. Following centrifugation at 300 g for 5 min, the supernatant was removed, and the cell pellet gently tapped for re-suspension in a 5-ml ammonium-chloride potassium (ACK) erythrocyte lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, and 0.1 mM Na2EDTA, pH 7.4) for 5 min. After centrifugation of remaining cells (300g, 5 min) and washing in PBS, the final cell suspension was re-suspended in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 2 mM L-glutamate, 10 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% heat-inactivated (56°C, 30 min) fetal bovine serum (FBS) (Gibco, Saint Aubin, France). Cells seeded into T75 culture flask at 3.10^6 cells/mL were maintained in complete RPMI-1640 medium at 37°C in a 5% CO2 humid atmosphere until MP harvest.

Generation, isolation, quantification and characterization of MPs from splenocytes suspension

Splenocytes were stimulated by 5 µg/ml of lipopolysaccharide (LPS) (from Escherichia coli 0127:B8; Sigma) or a combination of PMA (25 ng/ml; Enzo) and A12387 calcium ionophore (1 µM; Sigma) at 37°C in a humid atmosphere of 5% CO2 for 24 h. After 24h, recovered

splenocytes were numbered for each condition. MP concentration was measured by prothrombinase assay in the supernatant of splenocytes in response to LPS (SMP_{LPS}) and PMA/I ($SMP_{PMA/I}$) as compared to MPs assessed in the supernatant of untreated splenocytes (SMP_{CTL}). MPs were isolated and washed by differential centrifugation of the suspension. Briefly, splenocytes and cellular debris were discarded by a first centrifugation step at 800 g for 15 min at room temperature and the supernatant submitted to a second double centrifugation step at 14 000 g and 4°C for 60 min. The suspension of washed concentrated splenocyte-derived MPs (SMP) was re-suspended in Hanks Balanced Salt Solution (HBSS) and was kept until use for less than one month at 4°C. MP measurement was performed by prothrombinase assay after capture onto Annexin-A5 coated micro-wells using a microplate spectrophotometer set in kinetic mode. In this assay, phosphatidylserine is the rate-limiting factor of the generation of thrombin from prothrombin detected at 405 nm using a chromogenic substrate (PNAPEP0216, cryopep, Montpellier, France). MPs concentration was referred to as phosphatidylserine equivalent (nM PhtdSer eq.), by reference to a standard curve made with synthetic vesicles of known amounts of PhtdSer (Jy, Horstman et al. 2004).

Isolation and Culture of Coronary Artery Endothelial Cells (ECs)

Pig hearts were collected from the local slaughterhouse (COPVIAL, Holtzheim, France) and (ECs) were isolated from the left circumflex coronary arteries as described previously (Khemais-Benkhiat, Idris-Khodja et al. 2016). Briefly, left circumflex coronary arteries were excised from the fresh heart, cleaned of adhesive conjunctive tissues, and the remaining blood was flushed with cold phosphate-buffered saline (PBS) without calcium. Subsequently, ECs were isolated by filling the artery with a type I collagenase (Worthington Biochemicals Corp., Lakewood, NJ) solution at 1 mg/mL dissolved in MCDB131medium (Life Technologies SAS, St Aubin, France) supplemented with penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 mg/mL), and L-glutamine (2 mM) (all from Lonza, St Quentin en Yvelines, France) for 15 minutes at 37°C. ECs were collected in the effluent after circular massage of the arteries and the remaining cells were flushed with the medium. Collected ECs were gently centrifuged at 400 g, the medium was removed, and pelleted cells were suspended in complete MCDB131 medium solution supplemented with 15% fetal bovine serum. ECs from three different arteries were seeded in a T25 flask and expanded at 37°C in a 5% CO2 humidified atmosphere for 5-6, then removing the medium containing non-adherent cells and replaced with a new medium, grown for 48-72 hours (passage 0).

Endothelial cell treatments

ECs at passage 1 (P1 ECs) were seeded at 75%-80% confluence in multi-well plates and were pre-incubated with high or low glucose concentration (HG 25 mM, NG 5.5 mM) for 48h prior to addition SMP_{CTL}, SMP_{LPS}, or $SMP_{PMA/I}$ (10 nM) for an additional period of 48h. In all cases, the medium was changed every 24 h.

Fluorescence detection of senescence-associated β-galactosidase (SA-β-gal) activity

To measure Senescence-associated β-galactosidase activity (SA-β-gal) in ECs by flow cytometry (FACScan, Becton Dickinson, San Jose, CA, USA), we used the fluorogenic substrate C_{12}FDG (5 dodecanoylaminofluorescein Di-β-D-galactopyranoside, Invitrogen, Life Technologies SAS) as previously described (Debacq-Chainiaux, Erusalimsky et al. 2009). This compound is membrane-permeable and nonfluorescent, however, it emits green fluorescence upon excitation and remains confined within the cell after hydrolysis of the galactosyl residues by β-galactosidase. Briefly, ECs were pre-treated with chloroquine (300 μM) for 1 h to raise the pH of the internal lysosomes to approximately 6. C_{12}FDG (33 μM) was then added for 1 h. At the end of the incubation period, ECs were washed with ice-cold PBS, the following trypsinization and analyzed immediately using a flow cytometer (FACScan, BD Biosciences, San Jose, CA). Data were acquired and analyzed using the CellQuest software (Becton Dickinson). Light scatter 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 median fluorescence intensity (MFI) of the population. Autofluorescence was assessed in unlabeled cells to C_{12}FDG, was negligible.

Western blot analysis

After treatment, cells were washed twice with PBS and then lysed in extraction buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 20 mM NaF, 0.01 mM okadaic acid, 1% Triton X-100 (Euromedex, Souffelweyershem, France), a tablet of complete protease inhibitor (Roche). Total proteins (20 μg or 30 μg) were separated on 8% or 12% sodium dodecyl sulfate-polyacrylamide gels at 100V for 2h and transferred electrophoretically in polyvinylidene difluoride membranes (GE healthcare, Vélizy Villacoublay, France) at 100V for 2 h. Membranes were incubated with blocking buffer (Tris-buffered saline solution (TBS) and 0.1% Tween 20 [Euromedex]) containing 3% bovine serum albumin for 1 h. For detection of proteins, membranes were incubated overnight at 4°C

with blocking buffer containing the respective primary antibody: rabbit polyclonal anti-eNOS (diluted 1:1.000; BD Biosciences, Le Pont de Claix, France), mouse monoclonal anti-p21 (diluted 1:500; Santa Cruz Biotechnology), mouse monoclonal anti-p16 (diluted 1:500; Santa Cruz Biotechnology), mouse monoclonal COX-2 (diluted 1:500 dilution; BD Biosciences), mouse monoclonal anti-ICAM1 (1:1000 dilution, Abcam, UK), rabbit monoclonal anti-VCAM1 (1:1000 dilution, Abcam, UK), Anti β-actin (1:20000 dilution). After washing, membranes were incubated with the secondary antibody (peroxidase-labeled antirabbit and anti-mouse immunoglobulin G, dilution of 1:5000; Cell Signaling Technology) at room temperature for 1 h. Pre-stained markers (Invitrogen, France) were used for molecular mass determination. Immunoreactive bands were detected by enhanced chemiluminescence and their density analyzed using the ImageQuant acquisition system and analysis software (LAS4000 and ImageQuant TL 8.1, Amersham, UK). The amount of protein in each lane was normalized to the housekeeping protein β-actin before the analysis was performed.

Immunofluorescence staining of coronary artery rings

To characterize the mechanism underlying the HG and/or SMP-induced endothelial dysfunction, the expression level of several proteins involved in redox-sensitive and inflammatory pathways were determined by immunofluorescence in sections of the coronary artery. For this purpose, porcine coronary arteries (4–5 mm length) were embedded in Tissue Tek OCT (Sakura 4583, Leiden, The Netherlands) and frozen in a nitrogen bath for cryostat sections at 14 μm. Sections were air dried for 15 min and stored at −80 °C until use. Sections were first fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), washed and treated with 10% milk in phosphate buffered saline containing 0.1% Triton X-100 for 1h at room temperature to block non-specific binding. Coronary artery sections were then incubated overnight at 4 °C with an antibody directed against either eNOS (1/1000, cat: 610297, BD Transduction Laboratories, Le Pont de Claix, France), VCAM1 (1:1000 dilution, 134047, Abcam, UK) and COX-2 (1/250 dilution, 15191, Abcam, UK). Negative controls were treated with suspensions deleted of primary antibodies. Sections were then washed with phosphate-buffered saline, three washes were followed by incubation with the fluorescent secondary antibody (1/400, Alexa 633-conjugated goat antirabbit or anti-mouse IgG, A-21070 and A-21050, Thermo Fisher, Illkirch, France) for 2 h at room temperature in the dark before being washed with phosphate-buffered saline and mounted in Dako fluorescence mounting medium (Dako S3023, Les Ulis, France) and cover-slipped before being evaluated by confocal microscopy using a confocal laser-scanning microscope

(Leica TSC SPE, Mannheim, Germany). Quantification of fluorescence levels in ECs was performed using Image J software (version 1.49p for Windows, US National Institutes of Health) after delineating the endothelial monolayer. For each condition, cumulative data were calculated as the mean of measurements on 6 sections and represent n=7 experimental data sets performed on separate occasions.

Determination of vascular oxidative stress

Porcine coronary artery was cleaned of connective tissue and flushed with PBS without calcium to remove remaining blood, cut into rings (4–5 mm in length), pre-incubated for 12 h under sterile conditions with HG or LG prior to addition of different concentrations of SMPs for 12h. After were embedded in OCT compound (Sakura Finetek, Villeneuve d'Ascq, France) and frozen in a nitrogen bath for cryostat sections. The redox-sensitive fluorescent dye dihydroethidium (DHE, 2.5 μM) was applied onto 25 μm unfixed cryosections of coronary arteries for 30 min at 37°C in a light protected humidified chamber. Section were then washed three times, mounted in (Dako, Les Ulis, France) and cover-slipped. The level of fluorescence in each section was examined under a confocal laser-scanning microscope (Leica SP2 UV DM IRBE; leica, Heidelberg, Germany) with a 20X magnification lens. Quantification of fluorescence levels was performed using the ImageJ software. For each condition, cumulative data were calculated as the mean of measurements on 6 sections and represent n=7 experimental data sets performed on separate occasions.

Vascular reactivity

Vascular reactivity studies were done in isolated porcine coronary arteries and vascular reactivity was assessed as indicated previously (Anselm, Chataigneau et al. 2007, Auger, Kim et al. 2010). Briefly, the coronary artery was cleaned of connective tissue and flushed with PBS without calcium to remove remaining blood, cut into rings (2–3 mm in length), pre-incubated 12 h under sterile conditions with HG or NG in RPMI-1640 medium (Sigma, St. Louis, MO) prior addition of different concentrations of SMPs for 12h and then suspended in organ baths containing oxygenated (95% O2, 5% CO2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH2PO4 1.18, MgSO4 1.18, CaCl2 1.25, NaHCO3 25, and D-glucose 11, pH 7.4, at 37 ° C) for the determination of changes in isometric tension (basal tension 5 g). The integrity of the endothelium was checked with bradykinin (0.3 mM).

After equilibration and functional tests, rings were precontracted with U46619 (1–60 nM), a thromboxane A2 receptor agonist, before construction of concentration-response curves to bradykinin. Relaxations are expressed as a percentage of the contraction to U46619. For each condition, cumulative data were calculated as the mean of measurements performed at least on seven separate experiments.

Statistical Analysis

The data are expressed as mean ± standard error mean (S.E.M.) and analyzed using GraphPad Prism5® (Prism5 Graphpad Company, La Jolla, CA, USA). Statistical analysis between two groups was carried out using one-way ANOVA test followed by the Tukey's multiple comparisons test. A P value <0.05 was considered significant. Experiments were conducted in at least in three separate experiments.

Results

SMP_{LPS} or $SMP_{MPA/I}$ are specific inducers of premature senescence in young ECs and high glucose potentiates the pro-senescent effect of SMP_{LPS} or $SMP_{PMA/I}$.

SMP_{LPS} or $SMP_{PMA/I}$ but not SMP_{CTL} applied at similar concentration (10 nM) significantly enhanced SA-ß -GAL activity by about 2-fold in NG, indicating a specific pro-senescent effect of SMPs released from stimulated splenocytes (SMP_{LPS} or $SMP_{PMA/I}$ *vs.* SMP_{CTL}, $p<0.010$, $p<0.0016$, respectively). As reported in a previous work from our team, exposure of untreated ECs to HG favored a two-fold increase in SA-ß -GAL activity compared to NG, and SMP_{CTL} had no effect. However, the combination of HG and SMP_{LPS} or $SMP_{PMA/I}$ led to additional SA-ß -GAL activity with respectively 50 % elevation and 2-fold enhancement compared to HG treated ECs ($p<0.0117$, HG *vs.* NG). In addition, after incubation with SMP_{LPS} or $SMP_{PMA/I}$ in NG, a significant 2-fold enhancement in the expression of senescence protein markers p16, p21 was measured by western blot, and the expression of both proteins increased by about 4-fold in HG ($p<0.0200$, HG *vs.* NG; $p<0.0012$, SMP_{LPS} or $SMP_{PMA/I}$ *vs.* SMPCTL, figure 2). Of note, addition to ECs of the conditioned medium of splenocytes deleted from SMPs by high-speed centrifugation had no effect on SA-ß -GAL activity and did

not modify the protein expression of relevant markers of senescence thereby excluding an effect of a truly soluble moiety.

High glucose potentiates the SMP_{LPS} or $SMP_{MPA/I}$ induced down-regulation of eNOS and favors ROS formation in coronary artery.

SMP_{LPS} or $SMP_{PMA/I}$ prompted a significant 2-fold and 4-fold down-regulation of eNOS in ECs grown respectively in NG ($p<0.0014$ vs. untreated ECs or SMP_{CTL}, n=4) or HG medium ($p<0.0001$ vs. untreated ECs or SMP_{CTL}, n=4). This observation was confirmed in porcine coronary rings pre-incubated with HG or NG for 12 h prior addition of SMPs for additional 12h incubation. SMP_{LPS} or $SMP_{PMA/I}$ reduced the eNOS expression measured in the endothelial monolayer of the rings by 60 % in NG (*vs.* untreated rings, $p<0.0014$, $p<0.0001$ respectively, n=7) and by 80 % in HG ($p<0.0001$ vs. untreated rings, n=7), suggesting a blunted formation of NO. Ethidium fluorescence probing of ROS in porcine coronary artery showed that both SMP_{LPS} and $SMP_{PMA/I}$ significantly increased ROS formation by 40 % in NG, which was doubled in HG medium. (figures 2 and 3)

SMP_{LPS} or $SMP_{MPA/I}$ are endothelial pro-inflammatory effectors ECs in response to HG

Western blots of ECs lysates indicated that SMP_{LPS} or $SMP_{PMA/I}$ induced a significant ~2-fold up-regulation of the endothelial expression of COX-2, ICAM-1 and VCAM-1 in NG ($p<0.0001$, SMP_{LPS} or $SMP_{PMA/I}$ vs. SMP_{CTL}, n=4) amounting to about 4 fold for $SMP_{PMA/I}$ in HG ($p<0.0001$ $SMP_{PMA/I}$ HG vs. SMP_{CTL} NG, n=4, figure 4A). The specific pro-inflammatory effect of SMP_{LPS} or $SMP_{PMA/I}$ was confirmed by immunostaining of the endothelial monolayer of the coronary rings (figure 4B) with a significant ~2-fold up-regulation of COX-2, and VCAM-1 in NG induced by SMP_{LPS} or $SMP_{PMA/I}$ ($p<0.0086$, SMP_{LPS} or $SMP_{PMA/I}$ vs. SMP_{CTL}, n=7), amounting a 4-fold increase in rings treated with $SMP_{PMA/I}$ in HG ($p<0.0001$, $SMP_{PMA/I}$ vs. SMP_{CTL}).

SMP_{LPS} or $SMP_{PMA/I}$ inhibit bradykinin induced-endothelial relaxation in a concentration-dependent manner

Either SMP_{LPS} or $SMP_{PMA/I}$ induced a concentration-dependent inhibition of BK-induced relaxation, $SMP_{PMA/I}$ being more efficient with a 50% inhibition measured in response to 10 nM concentration *vs.* 30 nM for SMP_{LPS} in NG ($p<0.0001$). Of note, SMP_{CTL} had no effect, even at 30 nM (figure 5A). However, the noxious effect of $SMP_{PMA/I}$ and SMP_{LPS} was more pronounced in HG ($SMP_{PMA/I}$: 85% inhibition *vs.* SMP_{CTL} and SMP_{LPS}: 80 % *vs.* SMP_{CTL},

p<0.0001, figure 5B). The MP-driven inhibition of relaxation was also confirmed by the fact that truly soluble 5 µg/ml of LPS or 25 ng/ml /1 µM of PMA/I remained unable to alter vascular reactivity in response to bradykinin. In addition, MP lysates had no effect, indicating the pivotal role of the MP membrane structure in the endothelial targeting. It has to be noted that HG alone did not modify the bradykinin-induced relaxation.

Discussion

Previous works from our laboratory had shown that replicative or premature endothelial senescence occurs via redox-sensitive pathways (Khemais-Benkhiat, Idris-Khodja et al. 2016) and can be triggered after 4-days incubation in 25 mM Glucose supplemented medium. Because leukocyte-derived MPs have been described as pro-inflammatory effectors favoring the expression of ICAM-1 and VCAM-1 at the endothelial surface, (Salminen, Kaarniranta et al. 2012), eventually contributing to a prothrombotic phenotype of ECs, we examined the endothelial impact of MPs isolated from primary splenocytes in a model of high glucose-induced endothelial senescence using primary coronary ECs. We further assessed the impact the endothelial dysfunction induced by microparticles and high-glucose on the vascular tone of porcine coronary arteries using bradykinin-induced vasorelaxation.

Taken together our data demonstrate that MPs generated from unstimulated splenocytes have little, if any, effect on the ECs and that MPs isolated from splenocyte stimulated either by PMA-ionophore or by LPS, act as specific pro-senescent and pro-inflammatory endothelial effectors.

In the present work endothelial-dependent bradykinin induced relaxation was impaired after 24h long-term exposure to HG only in the presence of SMP_{LPS} and $SMP_{PMA/I}$ suggesting that high glucose was potentiating the effect of SMPs, possibly through glucose-induced exaggerated senescence favouring enhanced endothelial dysfunction and the expression of pro-inflammatory VCAM-1 and ICAM-1 that are ligands for leukocyte recruitment. Indeed while only SMP_{LPS} and $SMP_{PMA/I}$ were able to prompt enhanced senescence and oxidative stress in ECs and coronary rings at high glucose concentration, all SMPs did prompt the significant up-regulation of pro-inflammatory COX-2, ICAM-1 and VCAM-1, although SMP_{CTL} were less efficient. This observation suggests that the inflamed endothelium plays a key role at the early stage of SMP-induced endothelial senescence and dysfunction even at low degree of leukocyte stimulation.

Because Bradykinin increases endothelial nitric oxide (NO) production by activating endothelial NO synthase (eNOS) (Bae, Kim et al. 2003), we assessed of eNOS protein expression in porcine coronary artery by confocal fluorescence microscopy, and we found that both SMP_{LPS} and $SMP_{PMA/I}$ induced a significant 2-fold decrease in eNOS expression in NG that was magnified to a 4-fold drop in HG.

Our data demonstrate that high glucose reduces bradykinin-induced endothelial-dependent relaxation of the coronary artery, favors coronary endothelial senescence and the accumulation of ROS and a pro-inflammatory endothelial development of arteriosclerotic plaques. They are in accordance with previous data indicating that high glucose (20 mM) impair bradykinin -induced relaxation of human subcutaneous arteries within 2 hours whereas opposite results were obtained in human mesenteric arteries (MacKenzie, Cooper et al. 2008). Of note, exposure of aortic rat rings to high glucose (44 mM) for 3h led to the attenuation of phenylephrine-induced contraction (El-Awady, El-Agamy et al. 2014), suggesting that HG may alter the vascular competence by altering both contraction and relaxation-dependent pathways.

Altogether our data demonstrate that the glucotoxicity exerts noxious effects on coronary artery endothelial cell function by acting as a potentiator of the pro-senescent effects of leukocyte MPs, provided they were generated in response to a pro-inflammatory stimulus. They also indicate that exposure to high glucose concentration also triggers endothelial inflammation and ROS formation, a process known to favor leukocyte recruitment at coronary sites prone to athero-thrombosis. In addition, our model could prove useful to investigate new endothelial pharmacological targets and protect the endothelium from prosenescent mediators eventually conveyed by leukocyte MPs.

Figure legends

Figure 1: SMP_{LPS} or $SMP_{PMA/I}$ but not SMP_{CTL} induce SA-β GAL activity in young ECs and high glucose potentiates the pro-senescent effect of SMP_{LPS} or $SMP_{PMA/I}$. ECs were pretreated in 25 mM (HG) or 5.5 mM normal glucose (NG) culture medium during 48h before an additional 48h treatment with SMPs. SA-β GAL activity was further measured by flow cytometry using the fluorescent substrate FDG. **SMP_{LPS}:** SMPs from LPS-stimulated splenocytes, **$SMP_{PMA/I}$:** SMPs from PMA/I-stimulated splenocytes, **SMP_{CTL}** were harvested from the supernatant of unstimulated splenocytes. FDG-: unlabeled ECs

Figure 2: SMP_{LPS} or $SMP_{PMA/I}$ but not SMP_{CTL} induce the up-regulation of senescence markers p21, P16 and down-regulate the expression of eNOS. High Glucose potentiates the SMP_{LPS} or $SMP_{PMA/I}$ effects. ECs were pre-treated in 25 mM (HG) or 5.5 mM normal glucose (NG) culture medium during 48h before an additional 48h treatment with SMPs (A). Expression of p21 and down-stream p16 senescent makers was assessed by western blot as a ratio of the expression of ß actin. Upper panel: representative blot, lower panel: cumulative data of 4 separate experiments. Porcine coronary rings were pre-incubated in HG or NG culture medium for 12 h prior addition of SMPs for additional 12h incubation (B). eNOS expression by the endothelial monolayer was measured by immunofluorescence staining of ring sections. Upper panel cumulative data of 7 separate experiments performed likewise. **SMP_{LPS}:** SMPs from LPS-stimulated splenocytes, **$SMP_{PMA/I}$:** SMPs from PMA/I-stimulated splenocytes, **SMP_{CTL}** were harvested from the supernatant of unstimulated splenocytes.

Figure 3: SMP_{LPS} or $SMP_{PMA/I}$ but not SMP_{CTL} induce ROS formation in coronary artery rings and high glucose exaggerates the pro-oxidant effect of SMPs. Porcine coronary rings were pre-incubated in HG (25 mM glucose) or NG (5.5 mM glucose) culture medium for 12 h prior addition of SMPs for additional 12h incubation. ROS formation was assessed through ethidium fluorescence by microscopy. Data are expressed as fold increase in fluorescence. Lower panel example of section staining, Upper panel cumulative data of 7 experiments performed likewise. **SMP_{LPS}:** SMPs from LPS-stimulated splenocytes, **$SMP_{PMA/I}$:** SMPs from PMA/I-stimulated splenocytes, **SMP_{CTL}** were isolated from the supernatant of unstimulated splenocytes.

Figure 4: High glucose is a potent enhancer of the endothelial up-regulation of inflammatory markers COX-2, VCAM-1, ICAM-1 by SMP_{LPS} or $SMP_{PMA/I}$ and is less efficient in the presence of SMP_{CTL}. The expression of inducible COX-2, of ICAM-1 and VCAM-1 was assessed by western blot in ECs grown in 25 mM high glucose (HG) or 5.5 mM normal glucose (NG) medium (4-A) and by immunofluorescence microscopy in the endothelial monolayer of coronary artery rings (4-B). ECs were pre-treated in NG or HG culture medium during 48h before an additional 48h treatment with SMPs. Expression pro-inflammatory protein makers was assessed by western blot as a ratio of the expression of ß actin. Porcine coronary rings were pretreated 12 hours in HG or NG medium and further incubated with SMPs for 12h. **SMP_{LPS}:** SMPs from LPS-stimulated splenocytes, **$SMP_{PMA/I}$:** SMPs from PMA/I-stimulated splenocytes, **SMP_{CTL}** were harvested from the supernatant of unstimulated splenocytes.

Figure 5: SMP_{LPS} or $SMP_{PMA/I}$ and not SMP_{CTL} impair aortic ring relaxation induced by bradikynin and HG enhances the noxious effect. Rings (2–3 mm in length), were pre-incubated 12 h under sterile conditions with HG or NG in RPMI-1640 medium (Sigma, St. Louis, MO) prior addition of different concentrations of SMPs for 12h and then suspended in organ baths containing oxygenated buffer for analysis of the bradykinin-induced relaxation. In a first set of experiments rings were incubated with SMP_{LPS} (5 nM-30nM) SMP_{LPS} (2 nM-10nM) in 5.5 mM normal glucose (NG) culture medium (figure 5A). In a second set of experiments $SMP_{PMA/I}$, SMP_{LPS} were incubated at 10 nM in high glucose culture medium (25 mM). SMP_{CTL} were incubated at 10nM or 30 nM concentration as indicated on the graph (figure 5B)

Figures

Figure 1

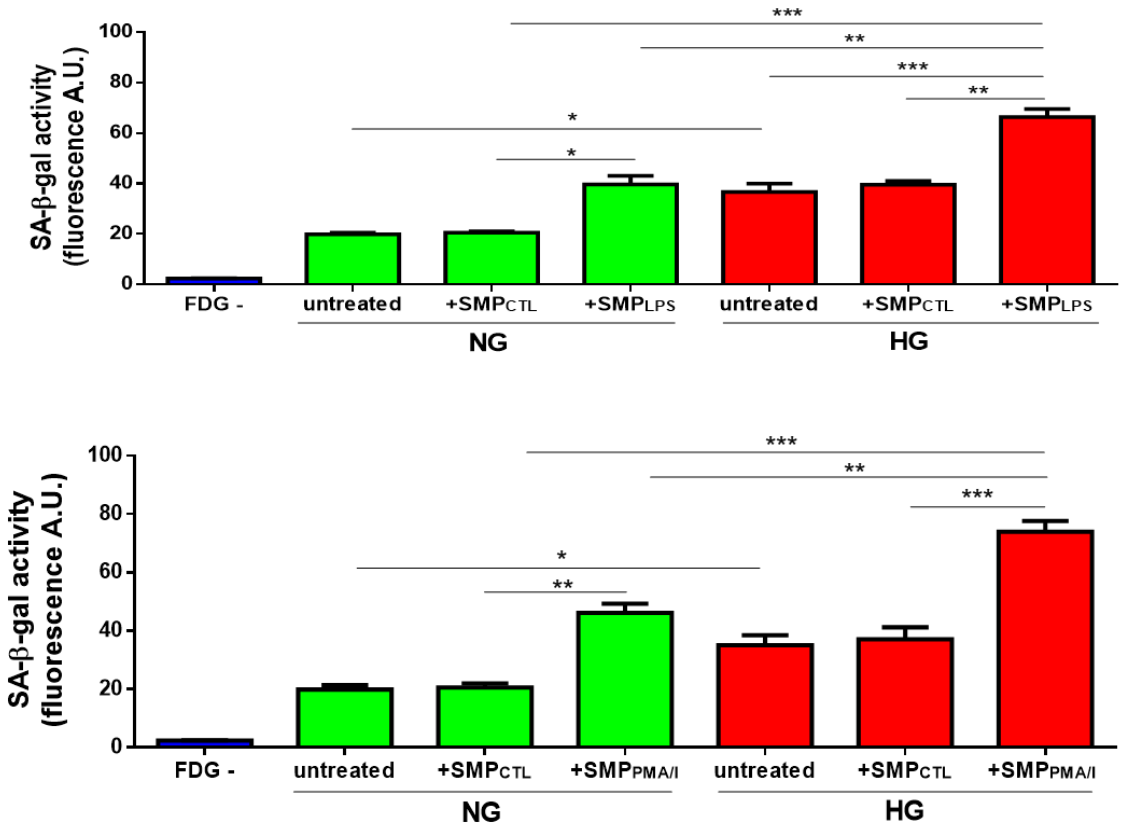


Figure 2A

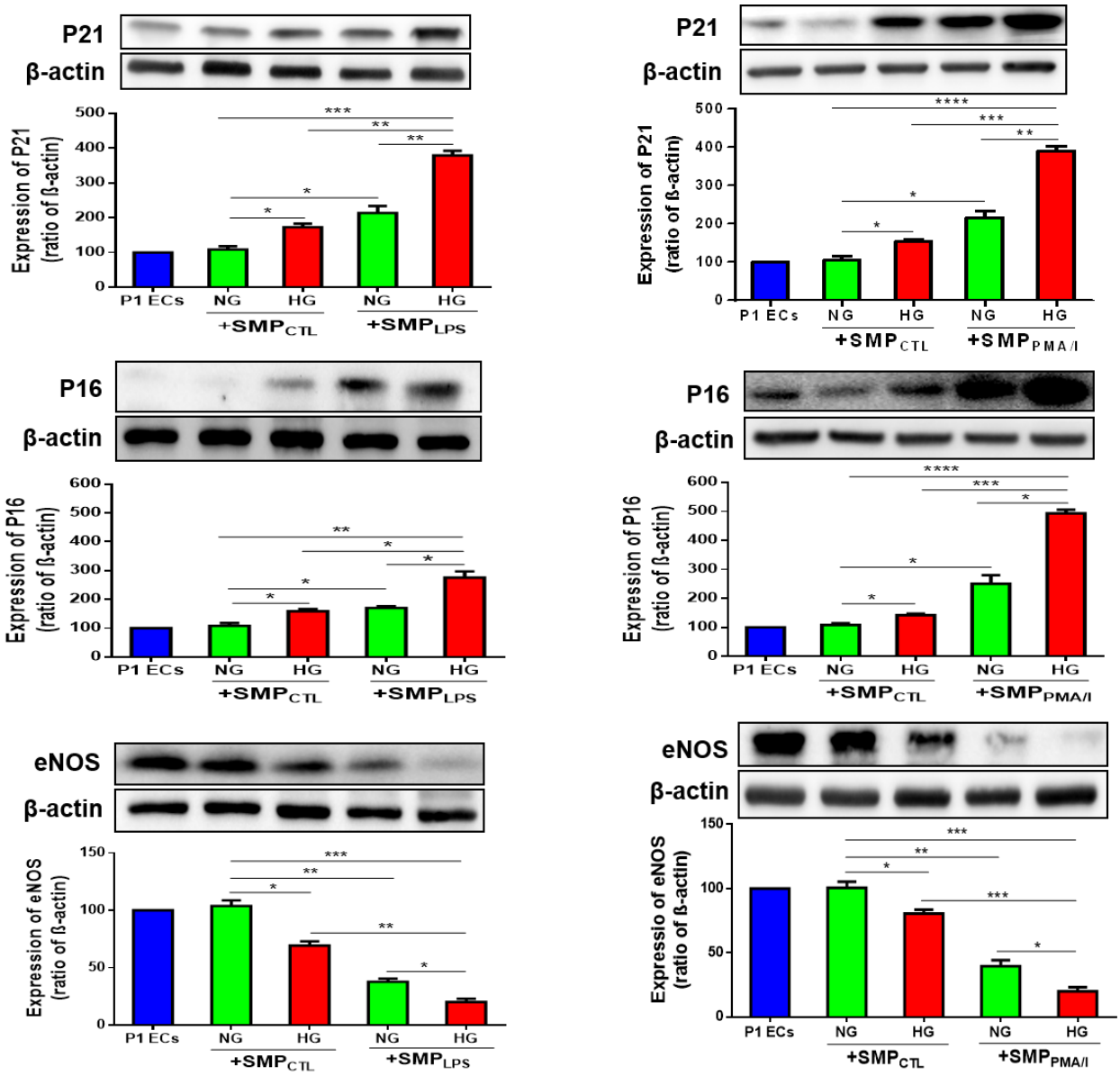

P21
β-actin
Expression of P21 (ratio of β-actin)
P1 ECs
NG
HG
+SMP$_{CTL}$
+SMP$_{LPS}$
+SMP$_{PMA/I}$
P16
Expression of P16 (ratio of β-actin)
eNOS
Expression of eNOS (ratio of β-actin)

Figure 2B

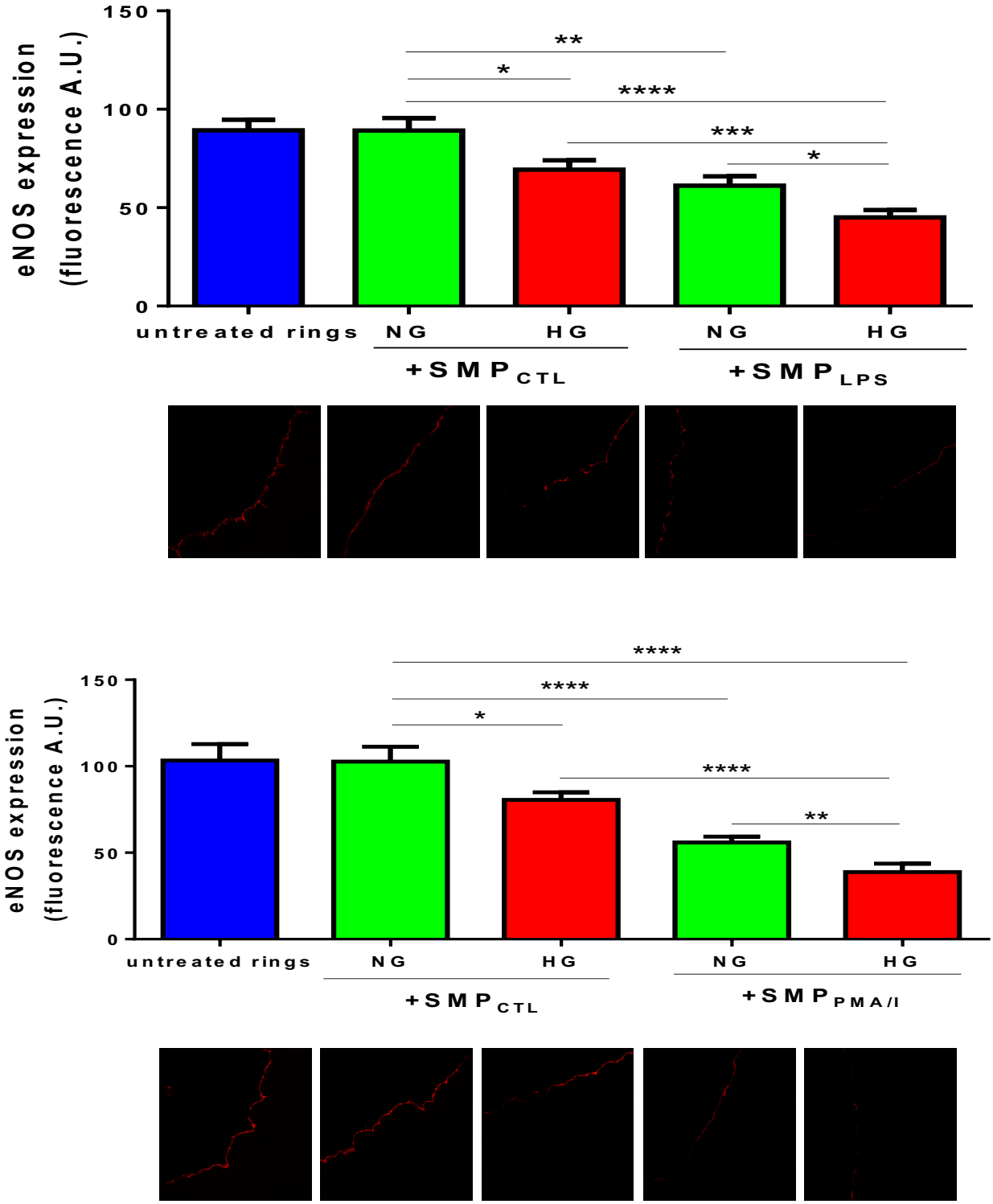
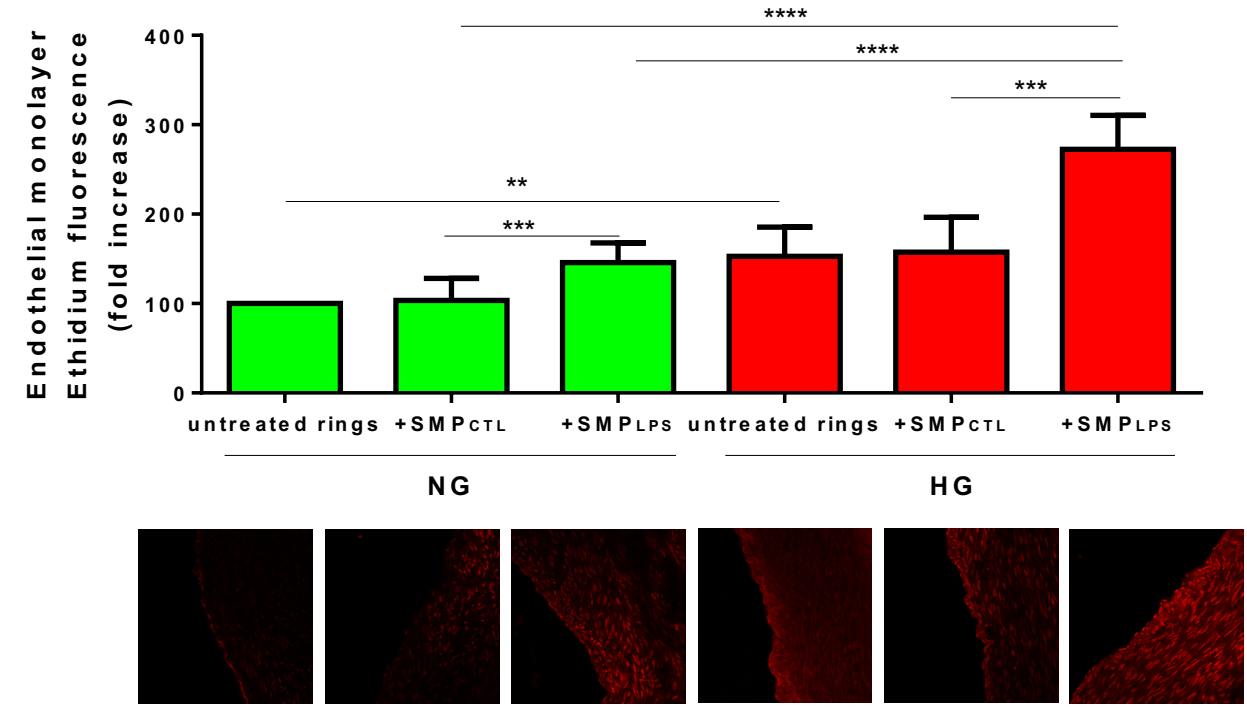

eNOS expression
(fluorescence A.U.)
150
100
50
0
**
*
****
***
*
untreated rings
NG
HG
NG
HG
+SMP CTL
+SMP LPS
****
****
*
****
**
untreated rings
NG
HG
NG
HG
+SMP CTL
+SMP PMA/I

Figure 3


Endothelial monolayer
Ethidium fluorescence
(fold increase)
400
300
200
100
0
****
****
***
**
***
untreated rings
+SMPCTL
+SMPLPS
untreated rings
+SMPCTL
+SMPLPS
NG
HG

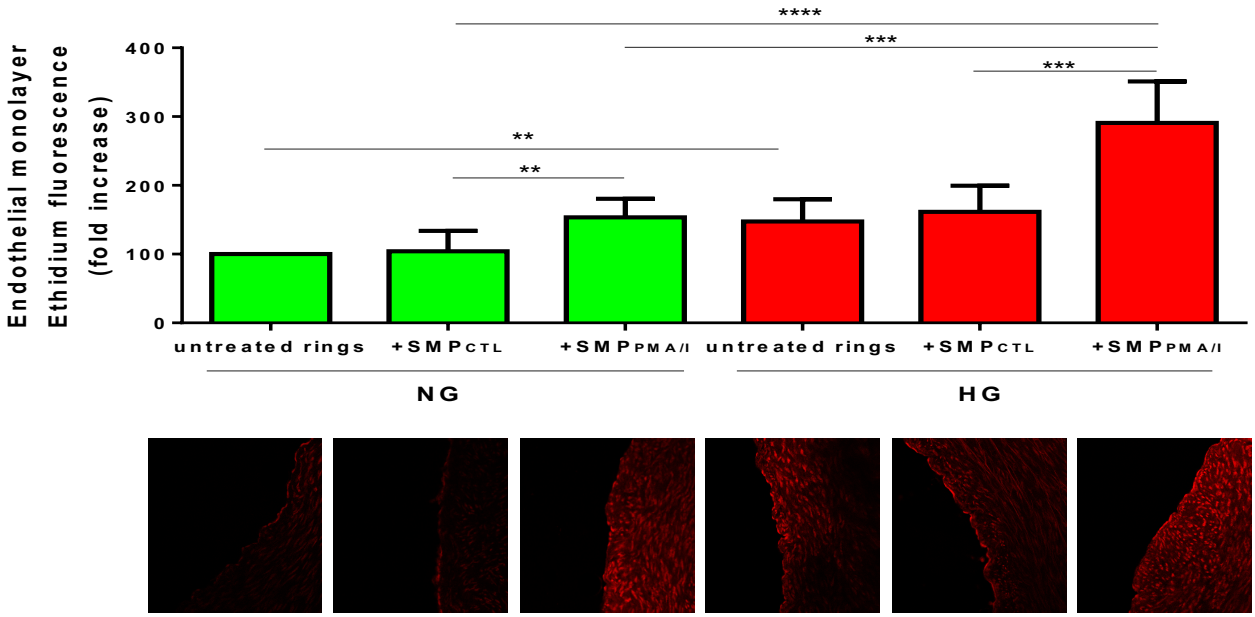

Endothelial monolayer
Ethidium fluorescence
(fold increase)
400
300
200
100
0
****
***
***
**
**
untreated rings
+SMPCTL
+SMPPMA/I
untreated rings
+SMPCTL
+SMPPMA/I
NG
HG

Figure 4 A

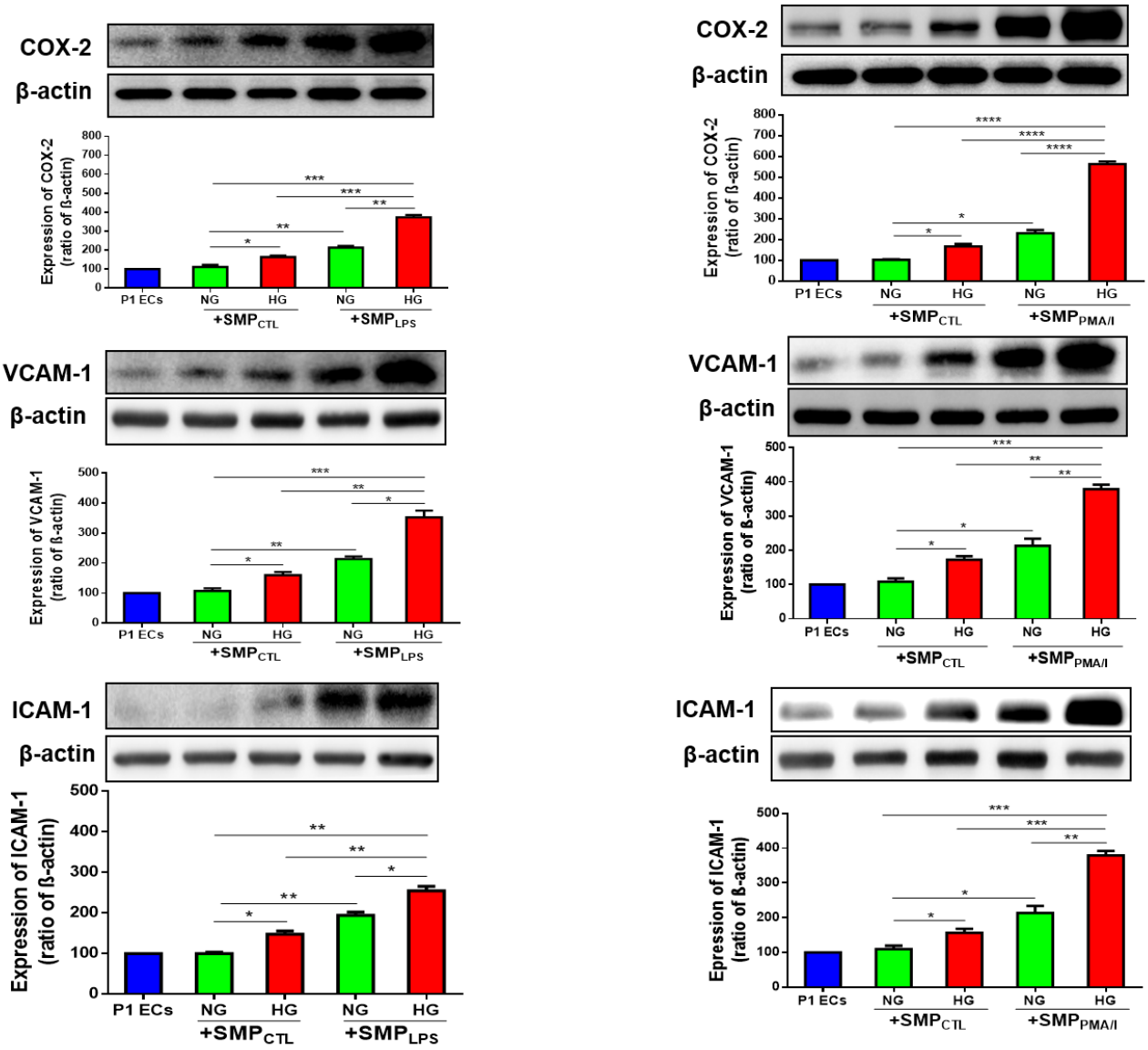
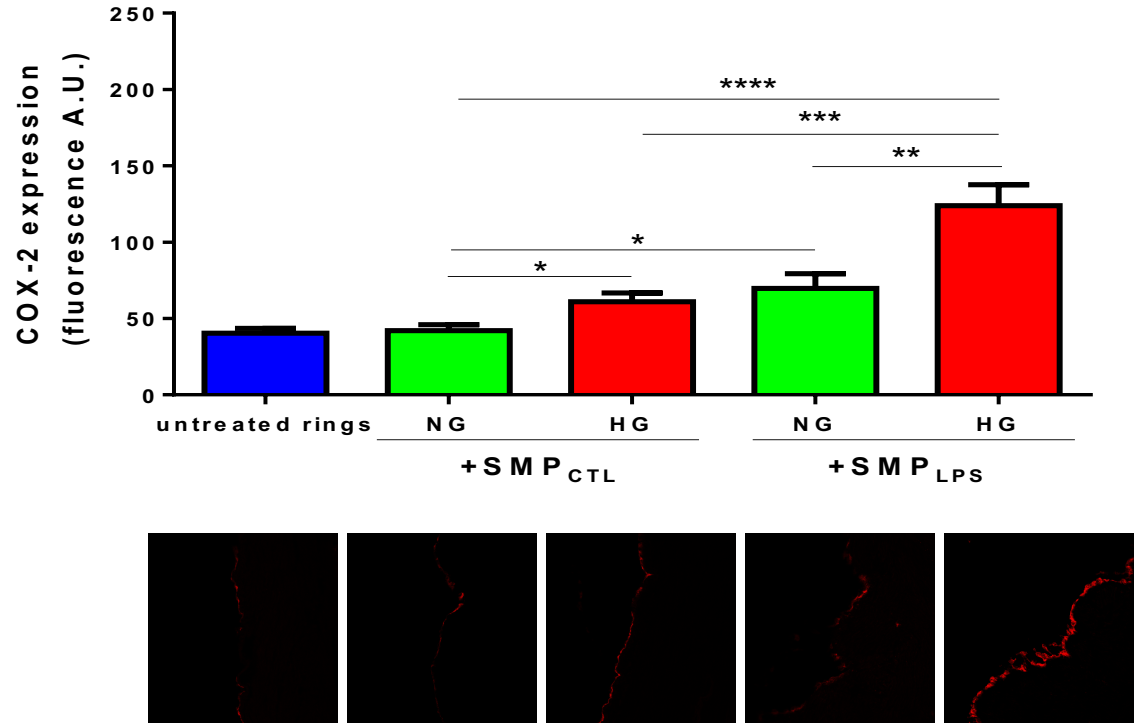
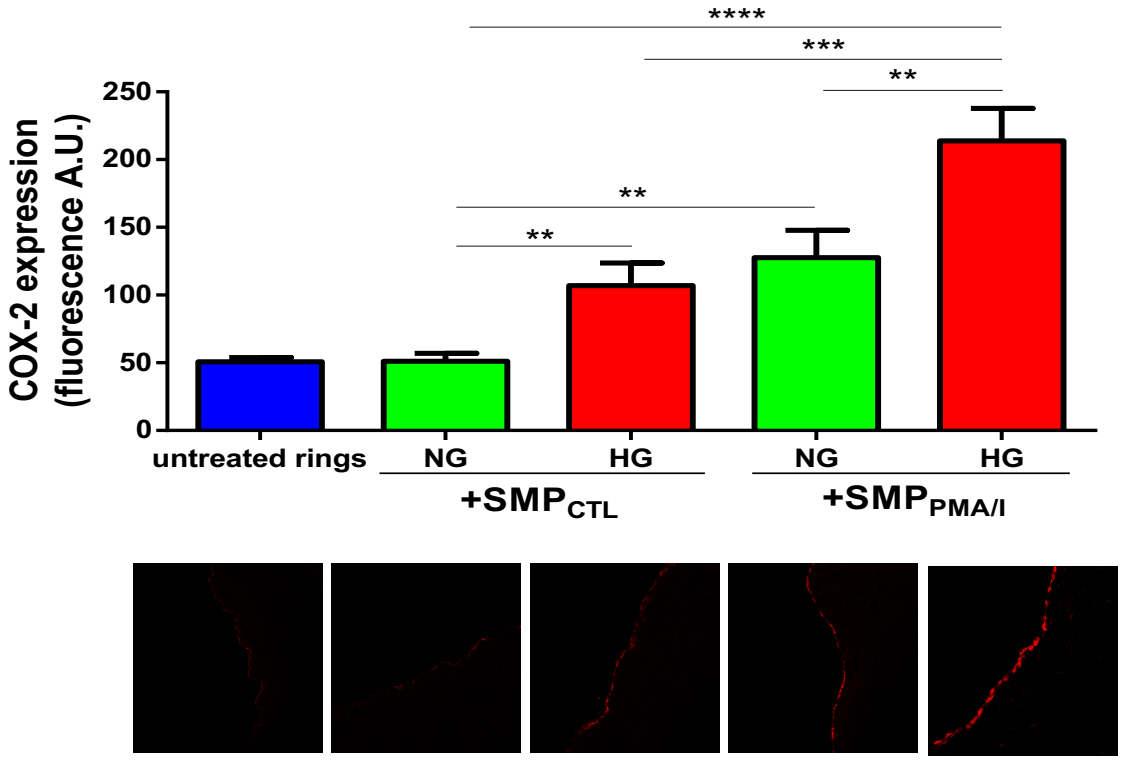
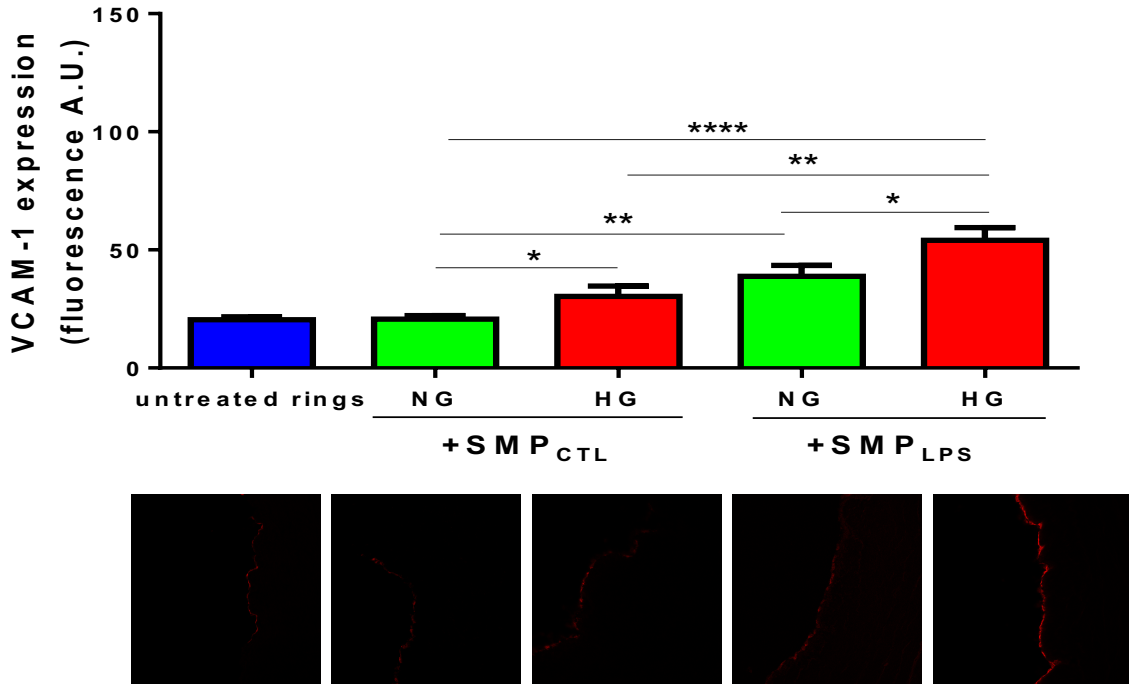

COX-2
β-actin
Expression of COX-2 (ratio of ß-actin)
P1 ECs
NG
HG
NG
HG
+SMP$_{CTL}$
+SMP$_{LPS}$
+SMP$_{PMA/I}$
VCAM-1
β-actin
Expression of VCAM-1 (ratio of ß-actin)
ICAM-1
β-actin
Expression of ICAM-1 (ratio of ß-actin)
Epression of ICAM-1 (ratio of ß-actin)

Figure 4 B


COX-2 expression (fluorescence A.U.)
250
200
150
100
50
0
****
***
**
*
*
untreated rings
NG
HG
NG
HG
+SMP CTL
+SMP LPS


COX-2 expression (fluorescence A.U.)
250
200
150
100
50
0
****
***
**
**
**
untreated rings
NG
HG
NG
HG
+SMPCTL
+SMPPMA/I


VCAM-1 expression
(fluorescence A.U.)
150
100
50
0
****
**
*
**
*
untreated rings
NG
HG
NG
HG
+SMP CTL
+SMP LPS

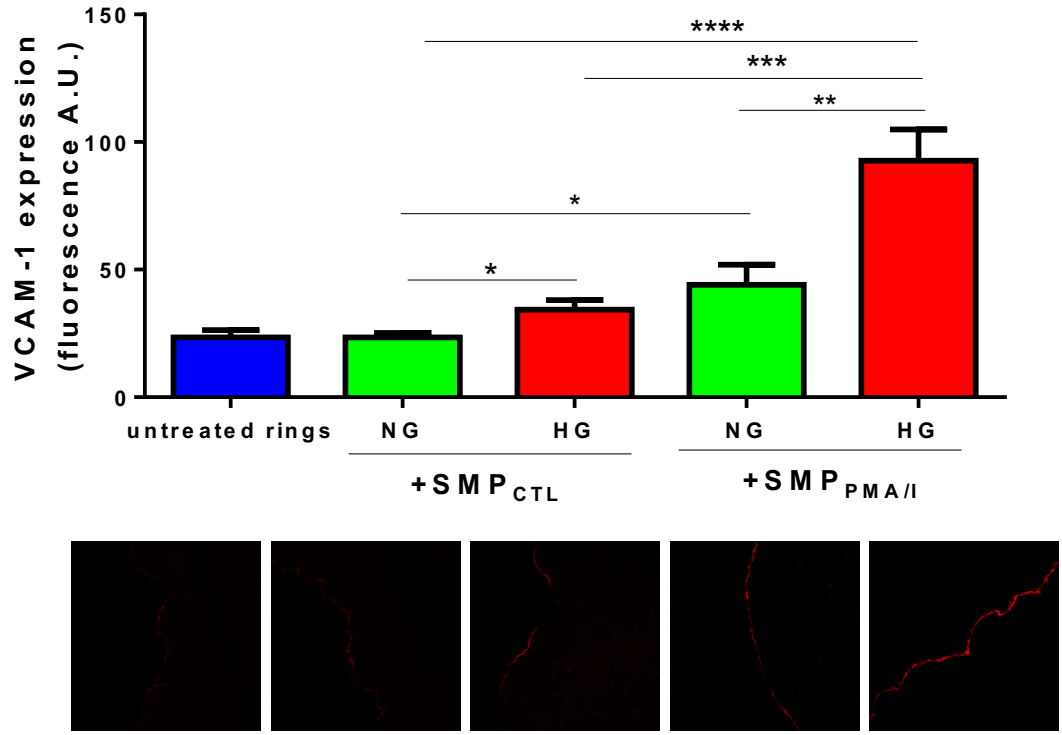

VCAM-1 expression
(fluorescence A.U.)
150
100
50
0
****
***
**
*
*
untreated rings
NG
HG
NG
HG
+SMP CTL
+SMP PMA/I

Figure 5 A

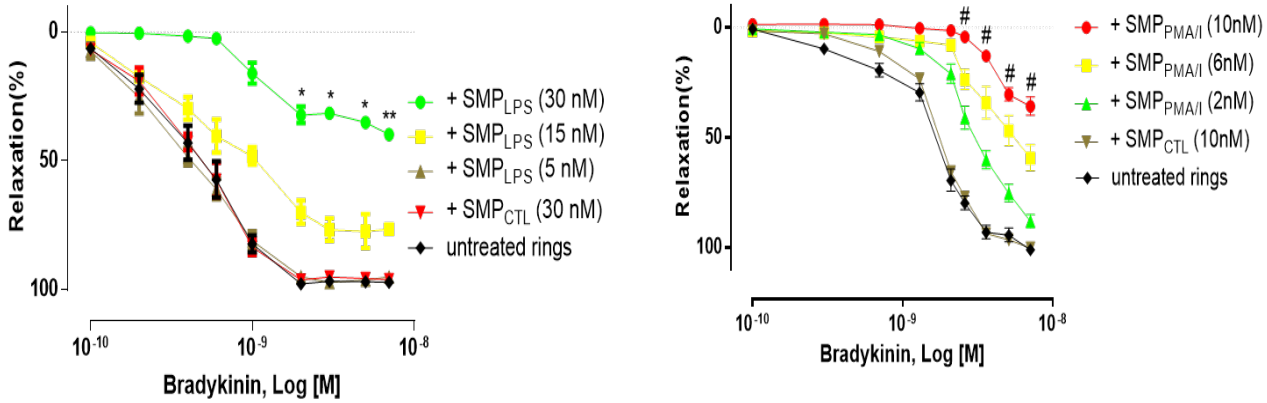
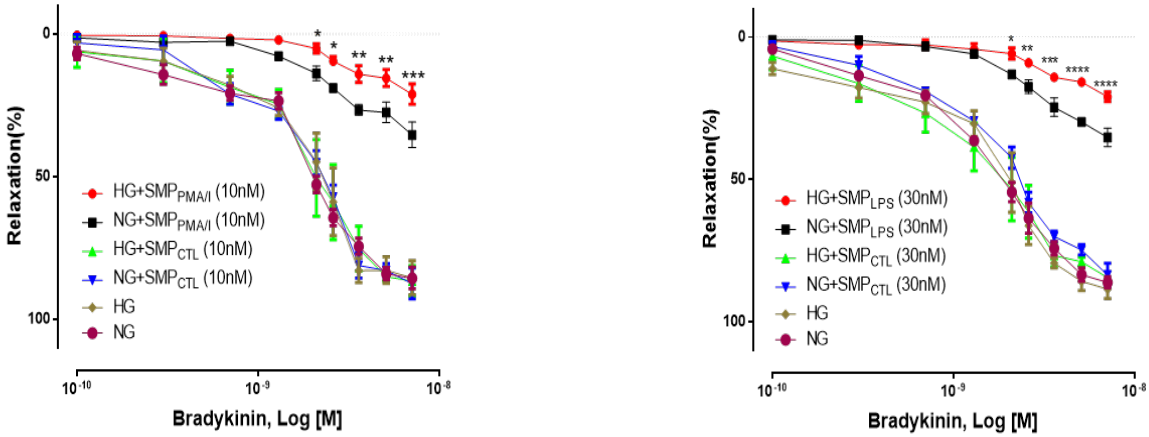


Figure 5 B



Bibliography

1. Natali A, Toschi E, Baldeweg S, Ciociaro D, Favilla S, Sacca L, et al. Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes. Diabetes. 2006;55(4):1133-40. doi: 55/4/1133 [pii]. PubMed PMID: 16567539.
2. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, et al. Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association. Circulation. 1999;100(10):1134-46. Epub 1999/09/08. PubMed PMID: 10477542.
3. Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat ML, et al. Microvesicles in vascular homeostasis and diseases. Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology. Thromb Haemost. 2017;117(7):1296-316. Epub 2017/06/02. doi: 10.1160/TH16-12-0943. PubMed PMID: 28569921.
4. Angelillo-Scherrer A. Leukocyte-derived microparticles in vascular homeostasis. Circulation research. 2012;110(2):356-69. doi: 10.1161/CIRCRESAHA.110.233403. PubMed PMID: 22267840.
5. Tabit CE, Chung WB, Hamburg NM, Vita JA. Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications. Rev Endocr Metab Disord. 2010;11(1):61-74.
6. Sabatier F, Darmon P, Hugel B, Combes V, Sanmarco M, Velut JG, et al. Type 1 and type 2 diabetic patients display different patterns of cellular microparticles. Diabetes. 2002;51(9):2840-5.
7. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction: a marker of atherosclerotic risk. Arteriosclerosis, thrombosis, and vascular biology. 2003;23(2):168-75. Epub 2003/02/18. PubMed PMID: 12588755.
8. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature. 1980;288(5789):373-6. Epub 1980/11/27. PubMed PMID: 6253831.
9. Lerman A, Burnett JC, Jr. Intact and altered endothelium in regulation of vasomotion. Circulation. 1992;86(6 Suppl):Iii12-9. Epub 1992/12/11. PubMed PMID: 1424046.
10. Taylor PD, Poston L. The effect of hyperglycaemia on function of rat isolated mesenteric resistance artery. British journal of pharmacology. 1994;113(3):801-8.
11. Dorigo P, Fraccarollo D, Santostasi G, Maragno I. Impairment of endothelium-dependent but not of endothelium-independent dilatation in guinea-pig aorta rings incubated in the presence of elevated glucose. British journal of pharmacology. 1997;121(5):972-6.
12. Jin JS, Bohlen HG. Non-insulin-dependent diabetes and hyperglycemia impair rat intestinal flow-mediated regulation. The American journal of physiology. 1997;272(2 Pt 2).
13. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy MA, Simonson DC, et al. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. Circulation. 1998;97(17):1695-701.
14. El-Awady MS, El-Agamy DS, Suddek GM, Nader MA. Propolis protects against high glucose-induced vascular endothelial dysfunction in isolated rat aorta. J Physiol Biochem. 2014;70(1):247-54.
15. MacKenzie A, Cooper EJ, Dowell FJ. Differential effects of glucose on agonist-induced relaxations in human mesenteric and subcutaneous arteries. British journal of pharmacology. 2008;153(3):480-7.

16. Halcox JP, Schenke WH, Zalos G, Mincemoyer R, Prasad A, Waclawiw MA, et al. Prognostic value of coronary vascular endothelial dysfunction. Circulation. 2002;106(6):653-8. Epub 2002/08/07. PubMed PMID: 12163423.

17. Ong P, Athanasiadis A, Borgulya G, Vokshi I, Bastiaenen R, Kubik S, et al. Clinical usefulness, angiographic characteristics, and safety evaluation of intracoronary acetylcholine provocation testing among 921 consecutive white patients with unobstructed coronary arteries. Circulation. 2014;129(17):1723-30. Epub 2014/02/28. doi: 10.1161/circulationaha.113.004096. PubMed PMID: 24573349.

18. Erusalimsky JD. Vascular endothelial senescence: from mechanisms to pathophysiology. Journal of applied physiology (Bethesda, Md : 1985). 2009;106(1):326-32. Epub 2008/11/28. doi: 10.1152/japplphysiol.91353.2008. PubMed PMID: 19036896; PubMed Central PMCID: PMCPmc2636933.

19. Wang JC, Bennett M. Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence. Circulation research. 2012;111(2):245-59. Epub 2012/07/10. doi: 10.1161/circresaha.111.261388. PubMed PMID: 22773427.

20. Triggle CR, Ding H. A review of endothelial dysfunction in diabetes: a focus on the contribution of a dysfunctional eNOS. Journal of the American Society of Hypertension : JASH. 2010;4(3):102-15. Epub 2010/05/18. doi: 10.1016/j.jash.2010.02.004. PubMed PMID: 20470995.

21. Ding H, Aljofan M, Triggle CR. Oxidative stress and increased eNOS and NADPH oxidase expression in mouse microvessel endothelial cells. Journal of cellular physiology. 2007;212(3):682-9. Epub 2007/04/20. doi: 10.1002/jcp.21063. PubMed PMID: 17443690.

22. Giacco F, Brownlee M. Oxidative stress and diabetic complications. Circulation research. 2010;107(9):1058-70. Epub 2010/10/30. doi: 10.1161/circresaha.110.223545. PubMed PMID: 21030723; PubMed Central PMCID: PMCPmc2996922.

23. Hoffmann J, Haendeler J, Aicher A, Rossig L, Vasa M, Zeiher AM, et al. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circulation research. 2001;89(8):709-15. Epub 2001/10/13. PubMed PMID: 11597994.

24. Hayashi T, Matsui-Hirai H, Miyazaki-Akita A, Fukatsu A, Funami J, Ding QF, et al. Endothelial cellular senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(45):17018-23. Epub 2006/11/01. doi: 10.1073/pnas.0607873103. PubMed PMID: 17075048; PubMed Central PMCID: PMCPmc1629003.

25. Yokoi T, Fukuo K, Yasuda O, Hotta M, Miyazaki J, Takemura Y, et al. Apoptosis signal-regulating kinase 1 mediates cellular senescence induced by high glucose in endothelial cells. Diabetes. 2006;55(6):1660-5. Epub 2006/05/30. doi: 10.2337/db05-1607. PubMed PMID: 16731828.

26. Ota H, Eto M, Kano MR, Ogawa S, Iijima K, Akishita M, et al. Cilostazol inhibits oxidative stress-induced premature senescence via upregulation of Sirt1 in human endothelial cells. Arterioscler Thromb Vasc Biol. 2008;28(9):1634-9. Epub 2008/06/17. doi: 10.1161/ATVBAHA.108.164368. PubMed PMID: 18556572.

27. Zhong W, Zou G, Gu J, Zhang J. L-arginine attenuates high glucose-accelerated senescence in human umbilical vein endothelial cells. Diabetes research and clinical practice. 2010;89(1):38-45. Epub 2010/04/20. doi: 10.1016/j.diabres.2010.03.013. PubMed PMID: 20398956.

28. Matsui-Hirai H, Hayashi T, Yamamoto S, Ina K, Maeda M, Kotani H, et al. Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence in vitro and in vivo: a relationship between telomeres and nitric oxide. The Journal of pharmacology

and experimental therapeutics. 2011;337(3):591-9. Epub 2011/03/02. doi: 10.1124/jpet.110.177584. PubMed PMID: 21357660.

29. Abbas M, Jesel L, Auger C, Amoura L, Messas N, Manin G, et al. Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging and Thrombogenicity: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways. Circulation. 2017;135(3):280-96.

30. Baumgartner-Parzer SM, Wagner L, Pettermann M, Grillari J, Gessl A, Waldhausl W. High-glucose--triggered apoptosis in cultured endothelial cells. Diabetes. 1995;44(11):1323-7. PubMed PMID: 7589831.

31. Favaro E, Miceli I, Bussolati B, Schmitt-Ney M, Cavallo Perin P, Camussi G, et al. Hyperglycemia induces apoptosis of human pancreatic islet endothelial cells: effects of pravastatin on the Akt survival pathway. The American journal of pathology. 2008;173(2):442-50. Epub 2008/07/05. doi: 10.2353/ajpath.2008.080238. PubMed PMID: 18599614; PubMed Central PMCID: PMCPmc2475781.

32. Ho FM, Liu SH, Liau CS, Huang PJ, Lin-Shiau SY. High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3. Circulation. 2000;101(22):2618-24. PubMed PMID: 10840014.

33. Ido Y, Carling D, Ruderman N. Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation. Diabetes. 2002;51(1):159-67. Epub 2002/01/05. PubMed PMID: 11756336.

34. Lorenzi M, Cagliero E. Pathobiology of endothelial and other vascular cells in diabetes mellitus. Call for data. Diabetes. 1991;40(6):653-9. Epub 1991/06/01. PubMed PMID: 2040380.

35. Cines DB, Pollak ES, Buck CA, Loscalzo J, Zimmerman GA, McEver RP, et al. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood. 1998;91(10):3527-61. Epub 1998/06/20. PubMed PMID: 9572988.

36. Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biro E, Nieuwland R, et al. Measuring circulating cell-derived microparticles. J Thromb Haemost. 2004;2(10):1842-51.

37. Khemais-Benkhiat S, Idris-Khodja N, Ribeiro TP, Silva GC, Abbas M, Kheloufi M, et al. The Redox-sensitive Induction of the Local Angiotensin System Promotes Both Premature and Replicative Endothelial Senescence: Preventive Effect of a Standardized Crataegus Extract. The journals of gerontology Series A, Biological sciences and medical sciences. 2016;71(12):1581-90.

38. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nature protocols. 2009;4(12):1798-806.

39. Auger C, Kim JH, Chabert P, Chaabi M, Anselm E, Lanciaux X, et al. The EGCg-induced redox-sensitive activation of endothelial nitric oxide synthase and relaxation are critically dependent on hydroxyl moieties. Biochemical and biophysical research communications. 2010;393(1):162-7.

40. Anselm E, Chataigneau M, Ndiaye M, Chataigneau T, Schini-Kerth VB. Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS. Cardiovascular research. 2007;73(2):404-13.

41. Bae SW, Kim HS, Cha YN, Park YS, Jo SA, Jo I. Rapid increase in endothelial nitric oxide production by bradykinin is mediated by protein kinase A signaling pathway. Biochemical and biophysical research communications. 2003;306(4):981-7. Epub 2003/06/25. PubMed PMID: 12821139.

Results Part 2:

Pharmacological protection of the endothelium against the pro-senescent action of SR-MPs in porcine coronary arteries at high glucose concentration

1- Background

The endothelial dysfunction observed in the macro and microsvaisseaux of patients with DT2 diabetes patients are the consequence of specific mechanisms pertaining to diabetes namely hyperglycemia but also to other CV risk factors.

In the Multiple Risk Factor Intervention Trial (MRFIT, 347,978 people) the absolute risk of CVD death was about 3-fold higher in diabetics than in non-diabetics, and this risk increased with the combination of CV risk factors (Stamler, Vaccaro et al. 1993). Therefore, the therapeutic management of diabetes should take into account the glycemic balance but also CV risk factors associated with diabetes and possibly the contribution of endothelial dysfunction and senescence. In the ADVANCE interventional study, a reduction of systolic blood pressure of 5.6 mmHg in diabetic patients receiving a combination of perindopril and indapamide resulted in a 9% reduction in macro and microvascular major, compared to those who received a placebo (Patel, Chalmers et al. 2005).

Glucose homeostasis is largely mediated by glucose uptake from dietary carbohydrates in the small intestine and by filtration and reabsorption into the kidney. Recently, gliflozins a new class of antidiabetes drugs that inhibit renal glucose re-uptake by SGLT1 and SGLT2 receptors, were shown to favour glucosuria and lower blood glucose independently of glycemia. A striking outcome of the different clinical trials was the early and major decrease of cardiovascular morbidity and mortality that was significant within the first 3 months after initiation of the treatment (EMAPREG,CANVAS,DECLARE).

Since the lipid bilayer of cell membranes is impermeable to this hydrophilic molecule, specialized transport proteins are required for its passage from the extracellular medium to the intracellular medium. Two types of glucose transporters, GLUTs and SGLTs are known, at least 14 GLUTs and 12 SGLTs have been identified (Wright, Loo et al. 2011).

1-1 Glucose transport by SGLT1 and SGLT2

SGLTs transport glucose across cell membranes against its concentration gradient. It is an active, so-called secondary transport that uses the energy created by a sodium gradient. It should be noted that the sodium gradient is maintained by the Na + / K + ATPase pump and stabilizes the membrane potential.

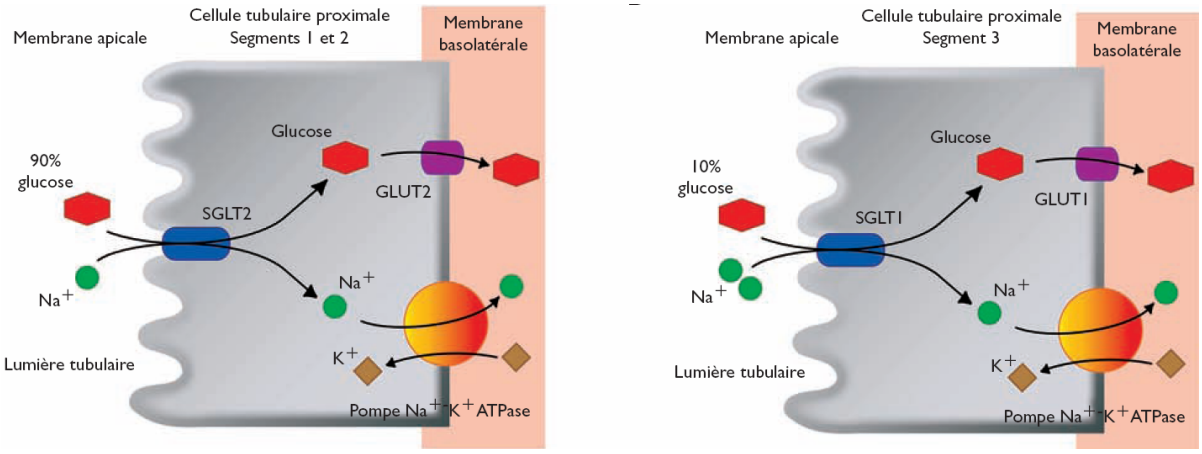


Figure 14: Glucose transport via SGLTs in renal tubule (Beaud, Pruijm et al. 2015) *Wright's and colleagues, described in the early 1980s, the existence of a co-transporter of Sodium-coupled glucose can be inhibited by phlorizine, a natural substance used as a research tool to induce kidney glycosuria.*

1-1.1 SGLT1 is mainly expressed in the apical membrane of epithelial cells of the brush border of the small intestine, and in the S3 segment of the renal proximal tubule, where it is responsible for the reabsorption of approximately 10% of the filtered glucose. hSGLT1 mRNA has been detected in the heart, testes, prostate, colon, trachea, lung, brain, spinal cord, spleen, liver, the uterus, pancreas and blood vessels (Poppe, Karbach et al. 1997, Zhou, Cryan et al. 2003, Chen, Williams et al. 2010). In humans, mutations in the SGLT1 gene are responsible for a rare autosomal recessive disorder: intestinal glucose-galactose malabsorption (GGM) (Turk, Kerner et al. 1996, Martin, Wang et al. 2000) causing severe osmotic diarrhea by excess of unabsorbed glucose and galactose in the intestinal lumen,

1-1.2 SGLT2 is expressed by the apical membrane of the brush border epithelial cells of the S1 and S2 segments of the proximal renal tubule, it transports sodium-coupled glucose with 1 Na + stoichiometry. / 1 glucose (Kanai, Lee et al. 1994) and is responsible for the reabsorption of 90% of the glucose filtered at the renal level. hSGLT2 mRNA was detected in the testes, cerebral arteries and cerebellum and pancreas (Zhou, Cryan et al. 2003, Chen, Williams et al. 2010, Wright, Loo et al. 2011, Bonner, Kerr-Conte et al. 2015). SGLT2 transports glucose from the glomerular filtrate into epithelial cells of the tubule and GLUT2 facilitates the release of glucose through the basolateral membrane to the interstitial fluid from which it will be captured by the peritubular capillaries to reach the bloodstream (Abdul-Ghani, Norton et al. 2011). Mutations in the SGLT2 sequence are responsible for familial renal glycosuria (FRG) (Calado, Sznajer et al. 2008, Santer and Calado 2010). This benign

genetic defect causes persistent glycosuria, with glucose excretion up to 160 g / day, without hypoglycaemia.

1-2- SGLT1 and SGLT2 expression in diabetes and cardiovascular disorders

In cardiac ischemia and heart failure the glucose requirements of cardiomyocytes are increased (Tian, Gong et al. 2001). In a diabetes context, the glucose transport is enhanced in skeletal muscle cells, renal epithelial cells, hepatocytes and adipocytes hence to GLUTs and SGLT1 and SGLT2 overexpression (Banerjee, McGaffin et al. 2009, Balteau, Tajeddine et al. 2011, Bogan, Rubin et al. 2012).

In human cardiac tissue, the expression of SGLT1 is increased in subjects with diabetes and ischemic cardiomyopathy, possibly as a result an adaptive change in response to a reduction in cardiac expression of GLUT1 and GLUT4. Interestingly, the myocardial expression of SGLT1 seems to increase with age with a level of SGLT1 mRNA gradually increased between 2 and 20 weeks in mice (Banerjee, McGaffin et al. 2009).

Glucose is the main modulator of SGLT1 gene expression in the gut and kidney (Lescale-Matys, Dyer et al. 1993, Vayro, Wood et al. 2001), but in brain ECs, SGLT1 would maintain adequate glucose concentration under only hypoglycemia conditions.

Interestingly, SGLT2 transporters are overexpressed by proximal tubule epithelial cells in diabetic patients (Rahmoune, Thompson et al. 2005), presumably following an adaptive response to the increase in glomerular glucose filtration., the overexpression of SGLT2 increasing the capacity for renal glucose reabsorption. Inhibitors of SGLT2 therefore decrease the maximal capacity of glucose reabsorption by the renal proximal tubule, in the context of diabetes. As for the expression of SGLT1 in diabetes, the observations diverge. Studies have provided evidence of increased, unchanged, or reduced expression and / or activity of SGLT1 in animal models of diabetes, or in vitro under high glucose conditions (Vallon and Thomson 2012).

The expression of SGLT1 and SGLT2 transporters is also enhanced after exposure of renal epithelial cells to HG and associated with increased secretion of pro-inflammatory cytokines (IL-6, TNF-α) that in turn also induce the auctocrine induction expression of SGLT2 but not of SGLT1, indicating an autocrine modulation of SGLT2 expression by cytokines (Maldonado-Cervantes et al., 2012), on line with the pro-inflammatory status of diabetes

patients. In diabetic patients, the plasma and urinary levels of IL-6 and TNF-α are high, and correlate with the progression of diabetic nephropathy (Navarro-Gonzalez and Mora-Fernandez 2008). SGLT2 expression is also increased in epithelial cells of the renal tubule of hypertensive rats (Bautista, Manning et al. 2004) and in the kidney DT2 animal models and in diabetic subjects (Freitas, Anhe et al. 2008, Vallon 2011), suggesting its relevance as a pharmacological target to reduce the cardiovascular morbidity and restore normal glycemia. (Rahmoune, Thompson et al. 2005).

1-3- SGLT1 and SGLT2 and cellular dysfunction

At the cellular level, SGLT1 seems involved in the activation of NADPH oxidase by "high glucose" in cardiomyocytes (Balteau, Tajeddine et al. 2011), whereas glucose metabolites (O-linked N-acetylglucosamine, glucose 6-phosphate) play no role, thereby suggesting that SGLT1 is key in NADPH oxidase activation and ROS production in response to high glucose, independently of glucose metabolism. (Balteau, Tajeddine et al. 2011).

Hydrogen peroxide and insulin induce SGLT2 overexpression in renal epithelial cells. The increased glucose uptake and ROS generation induced by insulin is prevented by pre-treatment with the NAC antioxidant, suggesting a key role of ROS in regulating SGLT2 expression (Nakamura, Matsui et al. 2015).

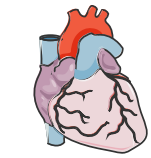
Renal tubular cells of patients with diabetic nephropathy have a senescent phenotype (Verzola, Gandolfo et al. 2008) that may be involved in the pathophysiology of diabetic nephropathy and hyperglycemia causes renal tubular epithelial cell senescence by an SGLT2-dependent mechanism in a model of streptozotocin-induced type 1 diabetes mellitus in mice. *In vitro,* the exposure of human proximal tubular cells to high glucose concentrations also increases the expression of senescence markers, that was reduced in the presence of SGLT2 siRNA, (Kitada, Nakano et al. 2014).

2- Rationale

The levels of endothelial cell-derived microparticles (EMP) are significantly increased in diseases with systemic endothelial damage as seen in diabetes mellitus and coronary artery disease (Rautou, Vion et al. 2011). We therefore anticipated that the vessel itself would contribute to autocrine dysfunction through the release of pro-senescent EMPs. Taken together, the above data from the literature, our data reported in part 1, and those from other

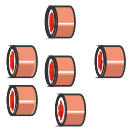
members of the laboratory (Sonia Khemais, thesis 2017), suggest that SGLT2 is key to hyperglycemia-induced endothelial senescence and dysfunction in the vessel. Main hypothetical mechanisms would include endothelial ROS accumulation, pro-inflammatory phenotype, enhanced cytokine secretion, recruitment of leukocytes and derived MPs, leading to SGLT2 overexpression. Although SGLT1 involvement is less ascertained, we chose to explore the effects of a SGLT1/2 inhibitor and of a SGLT2 specific inhibitor to examine the role high glucose-induced endothelial senescence on the pro-senescent action of SR-MPs in coronary artery rings. Rings were incubated for 18h with high glucose under strict sterile conditions before assessment of the senescence and pro-inflammatory markers and endothelial-dependent vascular reactivity. These unusual conditions were chosen as a pre-requisite since they enable the measurement of SR-MP vascular effects.

Preliminary data that show the optimization of the model and the first results are described in the following pages. They concern the effect of high glucose and SGLT inhibitors (SGLTi)on the release of MPs from the coronary ring, on the endothelial-dependent contraction and on the expression of pro-inflammatory and pro-senescent markers using standardized ring incubation procedure (18h) and the standardized MP extraction procedure from islosated splenocytes described in part 1. SGLTi were incubated simultaneously were also explored (see figure below).

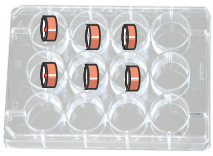


Porcine heart

Left anterior descending coronary artery

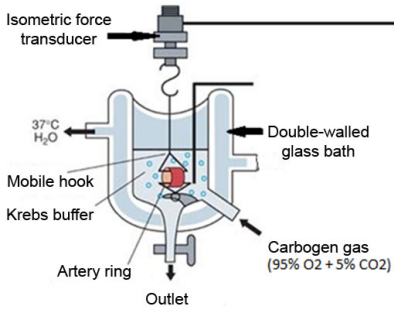


Standardized 2-3 mm fragments
4-5 mm fragments



Incubation 18h
37°C in 5 mL RPMI (CO_2)
± High Glucose
± SGLTs inhibitors

Tissue labelling
histological analysis



U46619-induced contraction

OCT inclusion

Confocal microscopy

Endothelial dysfunction and inflammatory markers COX-2, eNOS, VCAM

Protein extraction

Western blot

Inflammation markers (ICAM-1,VCAM-1)

3- Preliminary manuscript

Materials and methods

Generation, isolation, quantification and characterization of MPs from porcine coronary artery

Washed porcine coronary artery fresh artery were cut using a device containing a succession of fixed razor blades (1mm) then incubated with (HG or NG) on a rolling device (17 rpm) in RPMI-1640 medium (Sigma, St. Louis, MO) at room temperature for 1h30 min. After centrifugation at 800 g, 20°C for 15 min to discard cell debris and a further double centrifugation step at 14000 g, 4°C for 5min (two times), the washed isolated MPs were re-suspended in HBSS (Hanks Balanced Solution and their concentration was determined by prothombinase assay or Coomassie protein assay.

Vascular reactivity

Porcine coronary artery rings (2-3 mm were incubated for 18h at high glucose concentration (HG) or normal concentration (HG 25 mM, NG 11 mM) in RPMI-1640 medium (Sigma, St. Louis, MO). In some experiments, rings were incubated with pharmacological agents : LX4211 (Lexicon), a dual SGLT2/1 (10-7M), a highly selective SGLT2 inhibitor (SGLT2i, 10-7M). After incubation, rings were washed in HBSS, and U46619-induced endothelium-dependent contraction was assessed in organ chambers. (Anselm, Chataigneau et al. 2007, Auger, Kim et al. 2010). Rings were suspended in organ baths containing oxygenated (95% O2, 5% CO2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH2PO4 1.18, MgSO4 1.18, CaCl2 1.25, NaHCO3 25, and D-glucose 11, pH 7.4, at 37 ° C) for the determination of changes in isometric tension (basal tension 5 g). The integrity of the endothelium was checked with bradykinin (0.3 mM). After equilibration and functional tests, rings were contracted with U46619 (10^{-9} –10^{-6}M).

Western blot analysis

After 24h incubation of the rings with HG and NG medium, rings were washed twice with HBSS and directly frozen in liquid nitrogen and store at -80°C. The ring tissue was grinded and then lysed in extraction buffer (20 mM Tris/HCl [pH 7.5], 150 mM NaCl, 1 mM Na_3VO_4, 10 mM sodium pyrophosphate, 20 mM NaF, 0.01 mM okadaic acid, 1% Triton X-100 (Euromedex, Souffelweyershem, France), a tablet of complete protease inhibitor (Roche).

Total proteins (20 µg or 30 µg) were separated on 8% or 12% sodium dodecyl sulfate-polyacrylamide gels at 100V for 2h and transferred electrophoretically in polyvinylidene difluoride membranes (GE healthcare, Vélizy Villacoublay, France) at 100 V for 2 h. Membranes were incubated with blocking buffer (Tris-buffered saline solution [TBS] and 0.1% Tween 20 (Euromedex) containing 3% bovine serum albumin for 1 h. For detection of proteins, membranes were incubated overnight at 4°C with blocking buffer containing the respective primary antibody: rabbit polyclonal anti-eNOS (diluted 1:1.000; BD Biosciences, Le Pont de Claix, France), mouse monoclonal anti-p21 (diluted 1:500; Santa Cruz Biotechnology), mouse monoclonal anti-p16 (diluted 1:500; Santa Cruz Biotechnology), mouse monoclonal COX-2 (diluted 1:500 dilution; BD Biosciences), mouse monoclonal anti-ICAM1 (1:1000 dilution, Abcam, UK), rabbit monoclonal anti-VCAM1 (1:1000 dilution, Abcam, UK), Anti β-actin (1:20000 dilution). After washing, membranes were incubated with the secondary antibody (peroxidase-labeled antirabbit and anti-mouse immunoglobulin G, dilution of 1:5000; Cell Signaling Technology) at room temperature for 1 h. Pre-stained markers (Invitrogen, France) were used for molecular mass determination. Immunoreactive bands were detected by enhanced chemiluminescence and their density analyzed using the ImageQuant acquisition system and analysis software (LAS4000 and ImageQuant TL 8.1, Amersham, UK). The amount of protein in each lane was normalized to the housekeeping protein, β-actin before the analysis was performed.

Immunofluorescence studies

Immunofluorescence labelling was performed in sections (4–5 mm length) of the coronary artery that were embedded in Tissue Tek OCT (Sakura 4583, Leiden, The Netherlands) and frozen in a nitrogen bath for cryostat sections at 14 µm. Sections were air dried for 15 min and stored at −80 °C until use. Sections were first fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA), washed and treated with 10% milk in phosphate buffered saline containing 0.1% Triton X-100 for 1h at room temperature to block non-specific binding. Coronary artery sections were then incubated overnight at 4 °C with an antibody directed against either eNOS (1/1000, cat: 610297, BD Transduction Laboratories, Le Pont de Claix, France), VCAM1 (1:1000 dilution, 134047, Abcam, UK) and COX-2 (1/250 dilution, 15191, Abcam, UK). For negative controls, it has been deleted primary antibodies. Sections were then washed with phosphate-buffered saline, three washes were followed by incubation with the fluorescent secondary antibody (1/400, Alexa 633-conjugated goat antirabbit or anti-mouse IgG, A-21070 and A-21050, Thermo Fisher, Illkirch, France) for

2 h at room temperature in the dark before being washed with phosphate-buffered saline and mounted in Dako fluorescence mounting medium (Dako S3023, Les Ulis, France) and cover-slipped before being evaluated by confocal microscopy using a confocal laser-scanning microscope (Leica TSC SPE, Mannheim, Germany). Quantification of fluorescence levels in ECs was performed using Image J software (version 1.49p for Windows, US National Institutes of Health) after delimitation of the endothelial monolayer.

Determination of vascular oxidative stress

Porcine coronary artery was cleaned of connective tissue and flushed with PBS without calcium to remove remaining blood, cut into rings (4–5 mm in length), pre-incubated for 12 h under sterile conditions with HG or NG prior to addition of different concentrations of SMPs for 12h. After were embedded in OCT compound (Sakura Finetek, Villeneuve d'Ascq, France) and frozen in a nitrogen bath for cryostat sections. The redox-sensitive fluorescent dye dihydroethidium (DHE, 2.5 µM) was applied onto 25 µm unfixed cryosections of coronary arteries for 30 min at 37°C in a light-protected humidified chamber. Section were then washed three times, mounted in (Dako, Les Ulis, France) and cover-slipped. The level of fluorescence in each section was examined under a confocal laser-scanning microscope (Leica SP2 UV DM IRBE; leica, Heidelberg, Germany) with a 20X magnification lens. Quantification of fluorescence levels was performed using the ImageJ software.

Results

MPs release from freshly isolated splenocytes is enhanced by high glucose and prevented by simultaneous incubation with SGLT2i

We examined whether HG induced and increased microparticles (MPs) shedding from porcine coronary artery rings. Compared to NG, HG induced an early and significant 0.41 fold enhancelent in microparticles (MPs) shedding measured in the medium after 50 min incubation, whereas incubation with the SGLTi inhibitor restored baseline level of MP sehedding. Symetrically, cell recovery was reduced in HG and restored in the presence of the 10^{-7} M SGLTi inhibitor, thereby strongly suggesting an protective effect-mediated by SGLT2 targetting

x

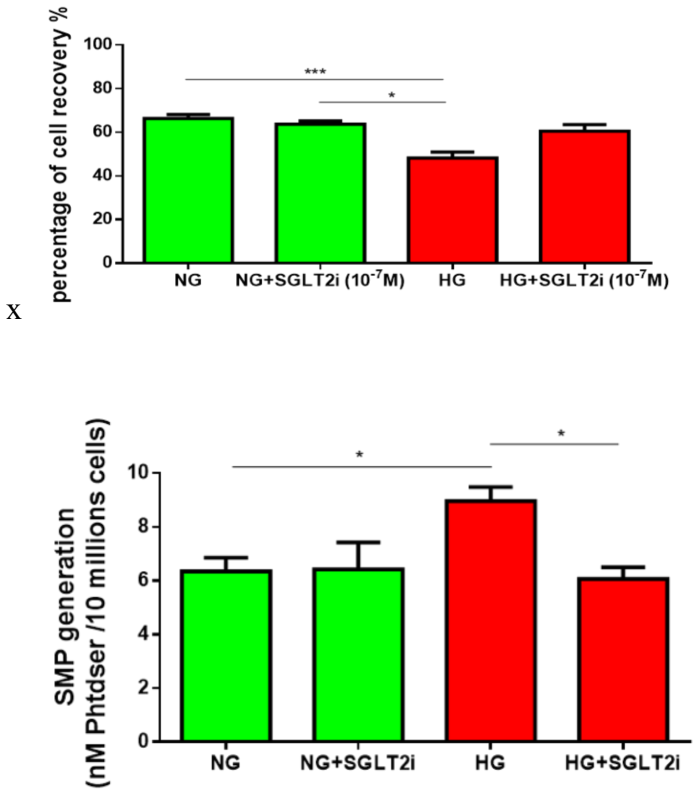


Figure 1 : Splenocyte recovery (up) and MP shedding (down) after 50 min incubation at high glucose (HG) or normal glucose (NG) concentration in the presence of 10-7 M SGLT2i. *MPs were measured by prothrombinase assay, n=6*

Inhibition of the U46619-induced contraction by HG is endothelial-dependent

Incubation of the coronary rings in 25 mM glucose (HG) during 18h inhibited U46619-induced contraction by 77.28% compared to Low Glucose medium (11 mM, LG), whereas 10^{-7}M SGLT2i significantly limited the HG inhibitory effect down to 26.92%, suggesting a SGLT2-mediated effect. The endothelial-dependence of the HG-mediated effect was confirmed by removal of the endothelial monolayer that led to a contraction response identical to that observed in LG rings. Of note, removal of the monolayer of LG-incubated rings did not alter the contractile response. Furthermore, replacement of the SGLT2i by a dual inhibitor of SGLT1 and SGLT2 did not restore contraction. Taken together these data suggested that HG induced a paradoxical inhibition and of the coronary vaso-contraction mediated by endothelial SGLT2 (see figure 2).

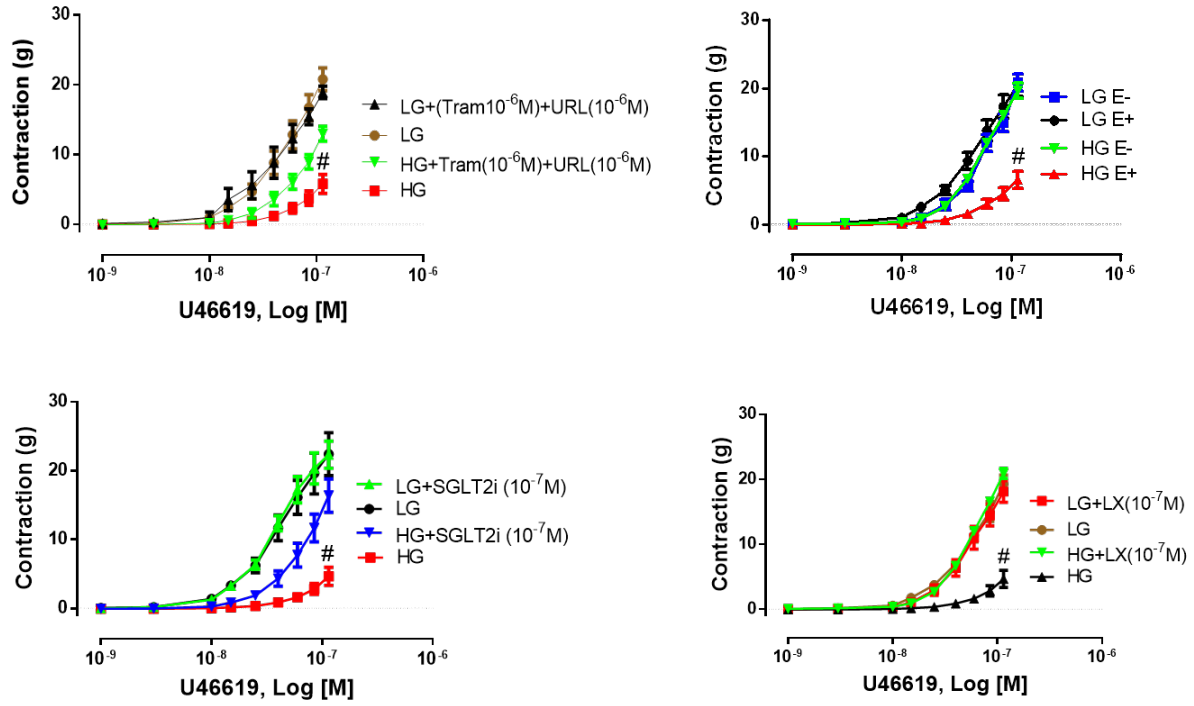


Figure 2 : HG reduces the vasocontraction of coronary artery rings in an endothelial dependent manner and SGLTi2 inhibitor prevents its effects. *10-7 M SGLT2nhibitor or dual SGLT1/2. Tram : inhibitor of* SK_{Ca} *and URL: inhibitor of* IK_{Ca}. Both inhibitors were added *in the water bath to abolish EDHF. E- removed endothelial monolayer, E+ with monolayer. Inhibitors of LG 11 mM Glucose, HG, 25 mM glucose, n=6*

SGLT2i prevents ROS accumulation induced by HG in response in coronary artery endothelial cells and prevents eNOS down regulation.

After 24 h incubation, HG prompted a 50 % increase of the endothelial ROS in coronary artery sections measured by the redox-sensitive fluorescent probe dihydroethidium (DHE) that was reduced by SGLT2i to baseline observed in LG. In addition a 40 % down refgulation of eNOS was also observed, that was counteracted by SGLTi2 (figures 3 and 4)

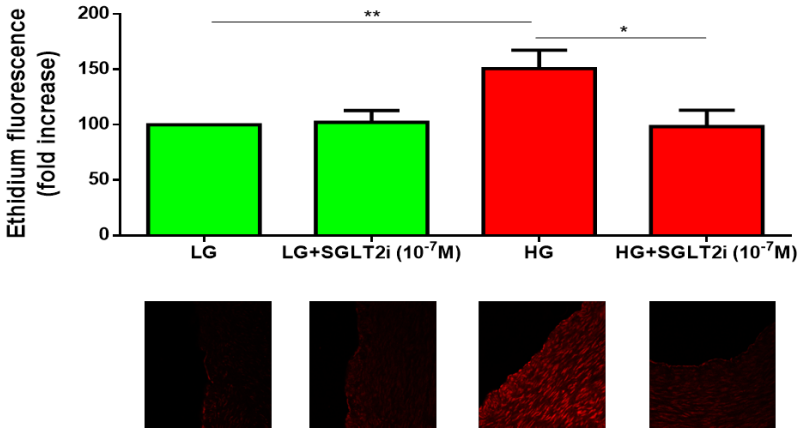


Figure 3 : HG favors ROS accumulation in the endothelial layer of coronary rings that is prevented by SGLTi inhibitor. *10-7 M SGLT2inhibitor LG: 11 mM glucose HG: 25 mM glucose , n=6*

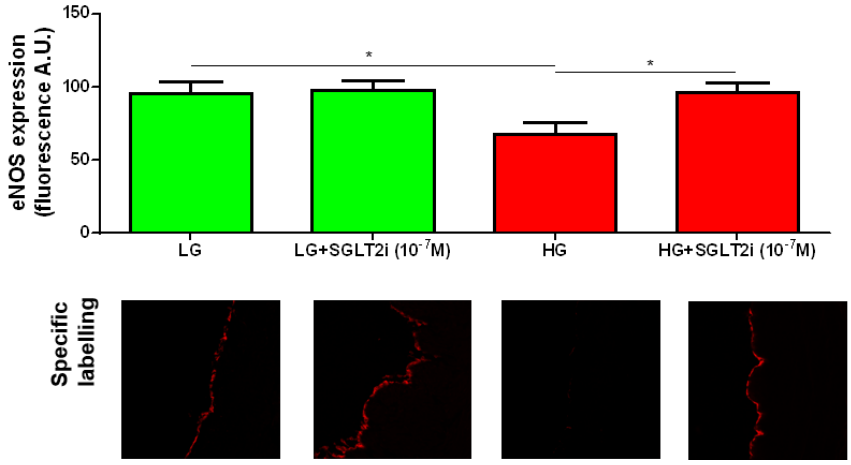


Figure 4 : HG favors the down regulation of eNOS in the endothelial layer of coronary rings that is prevented by SGLTi inhibitor. *10-7 M SGLT2inhibitor LG: 11 mM glucose HG: 25 mM glucose , n=6*

SGLT2i prevents the HG-induced up-regulation of pro-adhesion and pro-inflammatory proteins VCAM-1, ICAM-1 and inducible COX-2 in coronary rings

HG also induced a significant endothelial VCAM-1 (+50%), ICAM-1 (+50%), and COX-2 by (+200%) measured by western blots, whereas SGLT2i blunted the HG-mediated effect.

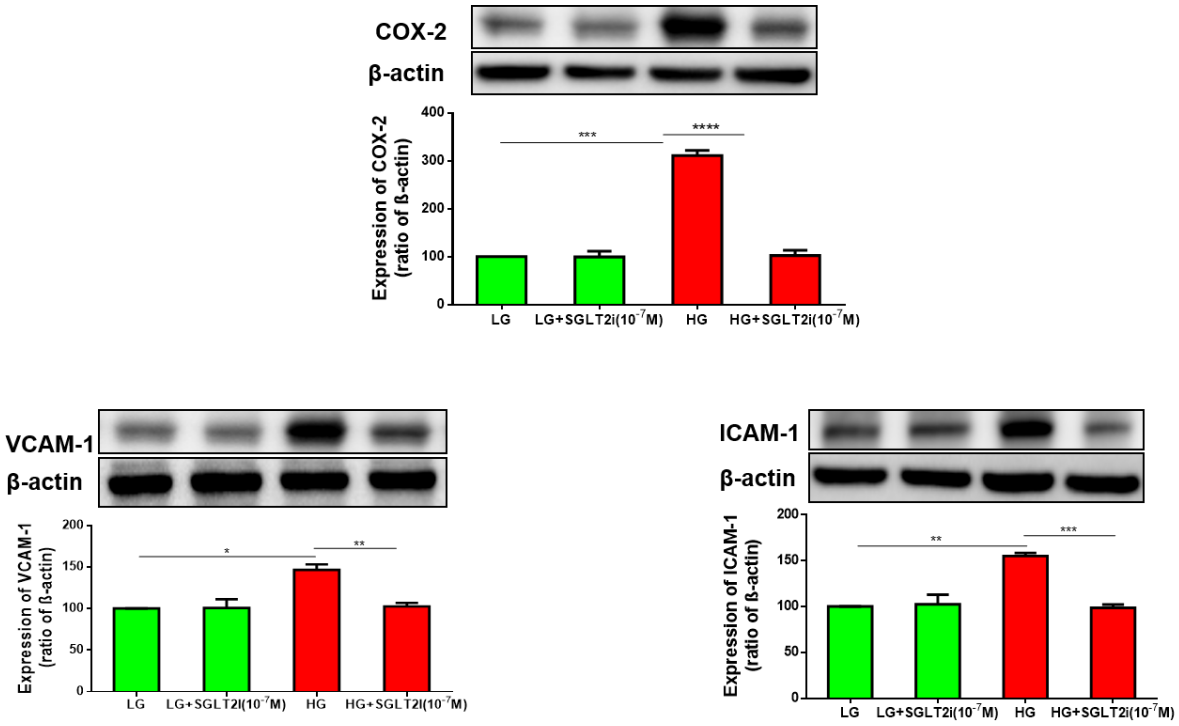


Figure 5: HG favors the up-regulation of pro-inflammatory proteins in coronary rings that is prevented by SGLTi inhibitor. *10-7 M SGLT2inhibitor LG: 11 mM glucose HG: 25 mM glucose, n=6, upper panels representative western blot, lower panel cumulative data of 6 experiments performed likewise.*

Conclusion

In this second set of preliminary experiments, we confirmed that overnight incubation of coronary ring with high glucose (HG) led to a major alteration of the vascular competence with a specific and paradoxical loss of vaso contraction that was endothelium-dependent and appears SGLT2 mediated.

Indeed, the addition of SFGLT2i that was incubated for 18h also completely abolished the HG-induced loss of vaso-contraction whereas Tram and UCL inhibitors of EDHF added in the water bath partially counteracted the loss of contractile response to U46619.

It is tempting to anticipate that part of the HG-mediated dysfunction of the endothelium could be the consequence of premature senescence as reported in endothelial ECs by S. Khemais (Thesis 2017). However, the data are two preliminary to draw a conclusion on the mechanisms mediated by SGLT2 and to determine whether they are only supported by the endothelium or pro-senescent derived EMPs in a autocrine amplification loop, as already described in a model of isolated coronary endothelial cells by M. Abbas. (Abbas, Jesel et al. 2017).

Further measurements of SA- Gal activity in the OCT sections or western blot analysis of p21, P16 senescence markers could bring some information.

Since high levels of cytokines and inflammatory factors such as TNFα, interleukin-6, soluble ICAM protein have been found in the serum of diabetic patients and animals (Wu, Liang et al. 2016), we also assessed inflammatory markers induced by HG in porcine coronary artery and the possibility of their inhibition by SGLT2-. Since high levels of cytokines and inflammatory factors such as TNFα, interleukin-6, soluble ICAM protein have been found in the serum of diabetic patients and animals (Wu, Liang et al. 2016), we further assessed inflammatory markers induced by HG in porcine coronary artery and the possibility of their inhibition with EMPA.

Some limitations to the model remain to be solved before examining the effect of SR-MPs on coronary rings:

- The small quantity of medium to eventually measure MP release
- The high sensitivity to endotoxins
- The lack of SGLT2 and SGLT1 efficient antibodies or probes for the identification of tissue SGLTs
- The low level of expression of SGLT1 and SGLT2.

VI -General discussion and conclusion

It seems that one of the key factors in ageing is the oxidative stress, resulting from the imbalance between the excessive production of reactive oxygen species (ROS). Free radicals promote oxidative damage to cellular macromolecules (DNA, lipids and proteins) (Haddad 2002, Bonello, Zahringer et al. 2007, Venkatesan, Mahimainathan et al. 2007). The ability to repair DNA damage is significantly reduced (50-75%; $P<0.01$) with age (Cabelof, Raffoul et al. 2002), and less responsive to oxidative stress (Edwards, Rassin et al. 1998).

In addition to the redox-sensitive alterations, 'inflammaging', i.e. pro-inflammatory phenotype that accompanies aging in mammals (Salminen, Kaarniranta et al. 2012) has been described. Indeed, exaggerated inflammatory responses contribute to the pathogenesis of chronic diseases like type 2 diabetes and obesity, two conditions that contribute to aging and cardiovascular risk (Barzilai, Huffman et al. 2012). As a proof of concept, genetic and pharmacological inhibition of NF-κB signaling prevents age-related features in different mouse models (Osorio, Barcena et al. 2012, Tilstra, Robinson et al. 2012)

The aim of our work was to better understand the relationships between high glucose-induced vascular damage, inflammaging and endothelial dysfunction.

For this purpose, we chose to use microparticles from spleen cells as the signature of immune-competence of the immune cells of young and aged rats to examine whether their interactions with the endothelium could accelerate the process of senescence under conditions mimicking hyperglycemia.

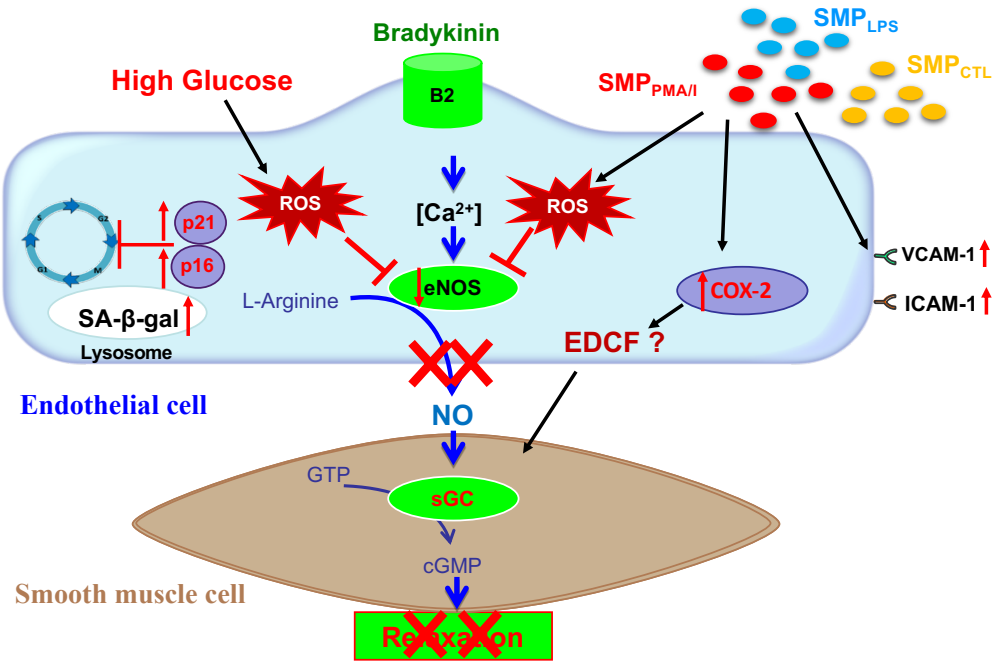
The choice of primary cell sources enabled the identification of the pro-senescent action of leukocyte MPs on isolated coronary endothelial cells and also in coronary rings. Interestingly, high glucose was a specific enhancer of all LMP-mediated alterations of the endothelial phenotype and function that were characterized by enhanced expression of inflammatory and oxidative markers.

In addition, we could demonstrate that ageing is indeed altering the tissue environment since we could measure a progressive MP shedding with the age of the spleen. Finally, we

could measure HG-mediated dysfunction that was characterized by the paradoxical loss of endothelial-dependent contraction, most probably mediated through SGLT2 receptors.

Our data are on line with the description of enhanced inflammatory responses, and immune senescence favoring the decline of the immune system with age (Deeks 2011).

Many *in vitro* studies have confirmed that MPs derived from different cell origins could induce endothelial dysfunction (Brodsky, Zhang et al. 2004, Mezentsev, Merks et al. 2005). In our work we showed that SR-MPs from stimulated splenocytes induced ROS accumulation and eNOS down-regulation, two causes of endothelial dysfunction. Nevertheless the exact molecular redox-sensitive mechanisms at the origin of the HG and SR-MP mediated senescence remain to be deciphered.



HG potentiates the pro-senescent effects of SMPs from stimulated splenocytes in coronary ECs and favors endothelial dysfunction. *This figure that summarizes our data. It indicates that conversely to MPs isolated from stimulated splenocytes (SMP$_{PMA/I}$ or SMP$_{LPS}$), MPs from naïve splenocytes (SMP$_{CTL}$) do not exert an effect on endothelial-dependent vascular responses. We observed the up-regulation of inflammation and senescence markers and the down regulation of e-NOS in isolated coronary endothelial cells or in the endothelial layer of coronary tissues and ROS accumulation. We also observed a reduction of the endothelial-dependent relaxation to bradykinin in response to SMP$_{PMA/I}$ or SMP$_{LPS}$ and at high glucose concentration. Our data also suggest that MPs target the EDCF response while reducing the endothelial relaxation in response to bradykinin.*

Because within the vessel, MPs act as procoagulant and pro-inflammatory effectors in amplification loops favouring the dissemination of noxious signals to endothelial cells, one further step of the present work would be to examine the impact of pro-senescent leukocyte MPs on the secondary release of procoagulant MPs by the endothelium. In addition, the identification of the molecular mediators of the SR-MP that initiated the accelerated endothelial senescence remain to be identified eventually by comparison with their counterparts isolated from unstimulated SR-MPs.

Finally, in our model, while the spleen appears an interesting tool as a source of primary cells eventually modulated by the animal treatment, it has the drawback of constituting an heterogeneous population of immune cells. To further identify the cell origin of the noxious pro-senescent SR-MPs their phenotype should be characterized before selective depletion of distinct MP population could be envisioned to confirm specific interactions with the endothelium.

Bibliography

"The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus." Diabetes Care **1997;20:1183–1202**.

Abbas, M., L. Jesel, C. Auger, L. Amoura, N. Messas, G. Manin, C. Rumig, A. J. Leon-Gonzalez, T. P. Ribeiro, G. C. Silva, R. Abou-Merhi, E. Hamade, M. Hecker, Y. Georg, N. Chakfe, P. Ohlmann, V. B. Schini-Kerth, F. Toti and O. Morel (2017). "Endothelial Microparticles From Acute Coronary Syndrome Patients Induce Premature Coronary Artery Endothelial Cell Aging and Thrombogenicity: Role of the Ang II/AT1 Receptor/NADPH Oxidase-Mediated Activation of MAPKs and PI3-Kinase Pathways." Circulation **135**(3): 280-296.

Abdul-Ghani, M. A., L. Norton and R. A. Defronzo (2011). "Role of sodium-glucose cotransporter 2 (SGLT 2) inhibitors in the treatment of type 2 diabetes." Endocr Rev **32**(4): 515-531.

Abuissa, H., P. G. Jones, S. P. Marso and J. H. O'Keefe, Jr. (2005). "Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers for prevention of type 2 diabetes: a meta-analysis of randomized clinical trials." J Am Coll Cardiol **46**(5): 821-826.

Adam, A. (1927). "Dyspepsiekoli. Zur Frage der bakteriellen Atiologie der sogenanntenalimentaren Intoxikation. ." Jahrb. Kinderheilkunde **116**: 8-40.

Agger, K., J. Christensen, P. A. Cloos and K. Helin (2008). "The emerging functions of histone demethylases." Curr Opin Genet Dev **18**(2): 159-168.

Agostoni, A. and M. Cugno (2001). "[The kinin system: biological mechanisms and clinical implications]." Recenti Prog Med **92**(12): 764-773.

Agouni, A., A. H. Lagrue-Lak-Hal, P. H. Ducluzeau, H. A. Mostefai, C. Draunet-Busson, G. Leftheriotis, C. Heymes, M. C. Martinez and R. Andriantsitohaina (2008). "Endothelial dysfunction caused by circulating microparticles from patients with metabolic syndrome." Am J Pathol **173**(4): 1210-1219.

Aird, W. C. (2007). "Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms." Circ Res **100**(2): 158-173.

Aird, W. C. (2007). "Phenotypic heterogeneity of the endothelium: II. Representative vascular beds." Circ Res **100**(2): 174-190.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.

Albanese, J., S. Meterissian, M. Kontogiannea, C. Dubreuil, A. Hand, S. Sorba and N. Dainiak (1998). "Biologically active Fas antigen and its cognate ligand are expressed on plasma membrane-derived extracellular vesicles." Blood **91**(10): 3862-3874.

Bakouboula, B., O. Morel, A. L. Faller, J. M. Freyssinet and F. Toti (2012). "Significance of membrane microparticles in solid graft and cellular transplantation." Front Biosci **17**: 2499-2514.
Albanese, J., S. Meterissian, M. Kontogiannea, C. Dubreuil, A. Hand, S. Sorba and N. Dainiak (1998). "Biologically active Fas antigen and its cognate ligand are expressed on plasma membrane-derived extracellular vesicles." Blood **91**(10): 3862-3874.

Alcorta, D. A., Y. Xiong, D. Phelps, G. Hannon, D. Beach and J. C. Barrett (1996). "Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts." Proc Natl Acad Sci U S A **93**(24): 13742-13747.

Allsopp, R. C., E. Chang, M. Kashefi-Aazam, E. I. Rogaev, M. A. Piatyszek, J. W. Shay and C. B. Harley (1995). "Telomere shortening is associated with cell division in vitro and in vivo." Exp Cell Res **220**(1): 194-200.

Alves-Lopes, R., K. B. Neves, A. C. Montezano, A. Harvey, F. S. Carneiro, R. M. Touyz and R. C. Tostes (2016). "Internal Pudental Artery Dysfunction in Diabetes Mellitus Is Mediated by NOX1-Derived ROS-, Nrf2-, and Rho Kinase-Dependent Mechanisms." Hypertension **68**(4): 1056-1064.

Amabile, N., C. Guignabert, D. Montani, Y. Yeghiazarians, C. M. Boulanger and M. Humbert (2013). "Cellular microparticles in the pathogenesis of pulmonary hypertension." Eur Respir J **42**(1): 272-279.

Anaya-Lopez, J. L., J. E. Lopez-Meza and A. Ochoa-Zarzosa (2013). "Bacterial resistance to cationic antimicrobial peptides." Crit Rev Microbiol **39**(2): 180-195.

Anderson, K. V., G. Jurgens and C. Nusslein-Volhard (1985). "Establishment of dorsal-ventral polarity in the Drosophila embryo: genetic studies on the role of the Toll gene product." Cell **42**(3): 779-789.

Angelillo-Scherrer, A. (2012). "Leukocyte-derived microparticles in vascular homeostasis." Circ Res **110**(2): 356-369.

Anselm, E., M. Chataigneau, M. Ndiaye, T. Chataigneau and V. B. Schini-Kerth (2007). "Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS." Cardiovasc Res **73**(2): 404-413.

Anselmi, L., L. Toti, C. Bove and R. A. Travagli (2017). "Vagally-mediated effects of brainstem dopamine on gastric tone and phasic contractions of the rat." American journal of physiology. Gastrointestinal and liver physiology: ajpgi 00180 02017.

Arcaro, G., B. M. Zenere, F. Saggiani, M. G. Zenti, T. Monauni, A. Lechi, M. Muggeo and R. C. Bonadonna (1999). "ACE inhibitors improve endothelial function in type 1 diabetic patients with normal arterial pressure and microalbuminuria." Diabetes Care **22**(9): 1536-1542.

Aubin, M. C., M. Carrier, Y. F. Shi, J. C. Tardif and L. P. Perrault (2006). "Role of probucol on endothelial dysfunction of epicardial coronary arteries associated with left ventricular hypertrophy." J Cardiovasc Pharmacol **47**(5): 702-710.

Auger, C., J. H. Kim, P. Chabert, M. Chaabi, E. Anselm, X. Lanciaux, A. Lobstein and V. B. Schini-Kerth (2010). "The EGCg-induced redox-sensitive activation of endothelial nitric oxide synthase and relaxation are critically dependent on hydroxyl moieties." Biochem Biophys Res Commun **393**(1): 162-167.

Bae, S. W., H. S. Kim, Y. N. Cha, Y. S. Park, S. A. Jo and I. Jo (2003). "Rapid increase in endothelial nitric oxide production by bradykinin is mediated by protein kinase A signaling pathway." Biochem Biophys Res Commun **306**(4): 981-987.

Baker, W. L., L. R. Smyth, D. M. Riche, E. M. Bourret, K. W. Chamberlin and W. B. White (2014). "Effects of sodium-glucose co-transporter 2 inhibitors on blood pressure: a systematic review and meta-analysis." J Am Soc Hypertens **8**(4): 262-275.e269.

Bakouboula, B., O. Morel, A. L. Faller, J. M. Freyssinet and F. Toti (2012). "Significance of membrane microparticles in solid graft and cellular transplantation." Front Biosci **17**: 2499-2514.

Bakouboula, B., O. Morel, A. Faure, F. Zobairi, L. Jesel, A. Trinh, M. Zupan, M. Canuet, L. Grunebaum, A. Brunette, D. Desprez, F. Chabot, E. Weitzenblum, J. M. Freyssinet, A. Chaouat and F. Toti (2008). "Procoagulant membrane microparticles correlate with the severity of pulmonary arterial hypertension." Am J Respir Crit Care Med **177**(5): 536-543.

Bal, L., S. Ederhy, E. Di Angelantonio, F. Toti, F. Zobairi, G. Dufaitre, C. Meuleman, Z. Mallat, F. Boccara, A. Tedgui, J. M. Freyssinet and A. Cohen (2010). "Circulating procoagulant microparticles in acute pulmonary embolism: a case-control study." Int J Cardiol **145**(2): 321-322.

Balows, A., W. J. Hausler, K. L. Herrmann, H. D. Isenberg, and H. J. and Shadomy (1991). "Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.".

Balteau, M., N. Tajeddine, C. de Meester, A. Ginion, C. Des Rosiers, N. R. Brady, C. Sommereyns, S. Horman, J. L. Vanoverschelde, P. Gailly, L. Hue, L. Bertrand and C. Beauloye (2011). "NADPH oxidase activation by hyperglycaemia in cardiomyocytes is independent of glucose metabolism but requires SGLT1." Cardiovasc Res **92**(2): 237-246.

Banerjee, S. K., K. R. McGaffin, N. M. Pastor-Soler and F. Ahmad (2009). "SGLT1 is a novel cardiac glucose transporter that is perturbed in disease states." Cardiovasc Res **84**(1): 111-118.

Barral, P., M. D. Sanchez-Nino, N. van Rooijen, V. Cerundolo and F. D. Batista (2012). "The location of splenic NKT cells favours their rapid activation by blood-borne antigen." Embo J **31**(10): 2378-2390.

Barry, O. P., D. Pratico, R. C. Savani and G. A. FitzGerald (1998). "Modulation of monocyte-endothelial cell interactions by platelet microparticles." J Clin Invest **102**(1): 136-144.

Barzilai, N., D. M. Huffman, R. H. Muzumdar and A. Bartke (2012). "The critical role of metabolic pathways in aging." Diabetes **61**(6): 1315-1322.

Basso, N., N. Paglia, I. Stella, E. M. de Cavanagh, L. Ferder, M. del Rosario Lores Arnaiz and F. Inserra (2005). "Protective effect of the inhibition of the renin-angiotensin system on aging." Regul Pept **128**(3): 247-252.

Baumgartner-Parzer, S. M., L. Wagner, M. Pettermann, J. Grillari, A. Gessl and W. Waldhausl (1995). "High-glucose--triggered apoptosis in cultured endothelial cells." Diabetes **44**(11): 1323-1327.

Bautista, R., R. Manning, F. Martinez, C. Avila-Casado Mdel, V. Soto, A. Medina and B. Escalante (2004). "Angiotensin II-dependent increased expression of Na+-glucose cotransporter in hypertension." Am J Physiol Renal Physiol **286**(1): F127-133.

Beaud, F., M. Pruijm, A. Humbert, M. Burnier and A. Zanchi (2015). "[Renal aspects of sodium glucose cotransporter 2 inhibitors]." Rev Med Suisse **11**(463): 488-492.

Beckman, K. B. and B. N. Ames (1998). "The free radical theory of aging matures." Physiol Rev **78**(2): 547-581.

Ben-Porath, I. and R. A. Weinberg (2005). "The signals and pathways activating cellular senescence." Int J Biochem Cell Biol **37**(5): 961-976.

Benigni, A., D. Corna, C. Zoja, A. Sonzogni, R. Latini, M. Salio, S. Conti, D. Rottoli, L. Longaretti, P. Cassis, M. Morigi, T. M. Coffman and G. Remuzzi (2009). "Disruption of the Ang II type 1 receptor promotes longevity in mice." J Clin Invest **119**(3): 524-530.

Bhatt, M. P., Y. C. Lim, Y. M. Kim and K. S. Ha (2013). "C-peptide activates AMPKalpha and prevents ROS-mediated mitochondrial fission and endothelial apoptosis in diabetes." Diabetes **62**(11): 3851-3862.

Bienert, G. P., J. K. Schjoerring and T. P. Jahn (2006). "Membrane transport of hydrogen peroxide." Biochim Biophys Acta **8**: 994-1003.

Bingen, E., B. Picard, N. Brahimi, S. Mathy, P. Desjardins, J. Elion and E. Denamur (1998). "Phylogenetic analysis of Escherichia coli strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains." J Infect Dis **177**(3): 642-650.

Bir, S. C., C. B. Pattillo, S. Pardue, G. K. Kolluru, X. Shen, T. Giordano and C. G. Kevil (2014). "Nitrite anion therapy protects against chronic ischemic tissue injury in db/db diabetic mice in a NO/VEGF-dependent manner." Diabetes **63**(1): 270-281.

Blackburn, E. H. (2000). "Telomeres and telomerase." Keio J Med **49**(2): 59-65.

Blagosklonny, M. V. (2003). "Cell senescence and hypermitogenic arrest." EMBO Rep **4**(4): 358-362.

Blaukat, A. (2003). "Structure and signalling pathways of kinin receptors." Andrologia **35**(1): 17-23.

Bogan, J. S., B. R. Rubin, C. Yu, M. G. Loffler, C. M. Orme, J. P. Belman, L. J. McNally, M. Hao and J. A. Cresswell (2012). "Endoproteolytic cleavage of TUG protein regulates GLUT4 glucose transporter translocation." J Biol Chem **287**(28): 23932-23947.

Boisrame-Helms, J., X. Delabranche, S. E. Degirmenci, F. Zobairi, A. Berger, G. Meyer, M. Burban, H. A. Mostefai, B. Levy, F. Toti and F. Meziani (2014). "Pharmacological modulation of procoagulant microparticles improves haemodynamic dysfunction during septic shock in rats." Thromb Haemost **111**(1): 154-164.

Boldrick, J. C., A. A. Alizadeh, M. Diehn, S. Dudoit, C. L. Liu, C. E. Belcher, D. Botstein, L. M. Staudt, P. O. Brown and D. A. Relman (2002). "Stereotyped and specific gene expression programs in human innate immune responses to bacteria." Proc Natl Acad Sci U S A **99**(2): 972-977.

Bonello, S., C. Zahringer, R. S. BelAiba, T. Djordjevic, J. Hess, C. Michiels, T. Kietzmann and A. Gorlach (2007). "Reactive oxygen species activate the HIF-1alpha promoter via a functional NFkappaB site." Arterioscler Thromb Vasc Biol **27**(4): 755-761.

Bonetti, P. O., L. O. Lerman and A. Lerman (2003). "Endothelial dysfunction: a marker of atherosclerotic risk." Arterioscler Thromb Vasc Biol **23**(2): 168-175.

Bonner, C., J. Kerr-Conte, V. Gmyr, G. Queniat, E. Moerman, J. Thevenet, C. Beaucamps, N. Delalleau, I. Popescu, W. J. Malaisse, A. Sener, B. Deprez, A. Abderrahmani, B. Staels and F. Pattou (2015). "Inhibition of the glucose transporter SGLT2 with dapagliflozin in pancreatic alpha cells triggers glucagon secretion." Nat Med **21**(5): 512-517.

Boulanger, C. M., X. Loyer, P. E. Rautou and N. Amabile (2017). "Extracellular vesicles in coronary artery disease." Nat Rev Cardiol **14**(5): 259-272.

Boyd, E. F. and D. L. Hartl (1998). "Chromosomal regions specific to pathogenic isolates of Escherichia coli have a phylogenetically clustered distribution." J Bacteriol **180**(5): 1159-1165.

Breen, A. P. and J. A. Murphy (1995). "Reactions of oxyl radicals with DNA." Free Radic Biol Med **18**(6): 1033-1077.

Brodsky, S. V., O. Gealekman, J. Chen, F. Zhang, N. Togashi, M. Crabtree, S. S. Gross, A. Nasjletti and M. S. Goligorsky (2004). "Prevention and reversal of premature endothelial cell senescence and vasculopathy in obesity-induced diabetes by ebselen." Circ Res **94**(3): 377-384.

Brodsky, S. V., F. Zhang, A. Nasjletti and M. S. Goligorsky (2004). "Endothelium-derived microparticles impair endothelial function in vitro." Am J Physiol Heart Circ Physiol **286**(5): H1910-1915.

Brodsky, S. V., F. Zhang, A. Nasjletti and M. S. Goligorsky (2004). "Endothelium-derived microparticles impair endothelial function in vitro." Am J Physiol Heart Circ Physiol **286**(5).

Bronte, V. and M. J. Pittet (2013). "The spleen in local and systemic regulation of immunity." Immunity **39**(5): 806-818.

Brouilette, S., R. K. Singh, J. R. Thompson, A. H. Goodall and N. J. Samani (2003). "White cell telomere length and risk of premature myocardial infarction." Arterioscler Thromb Vasc Biol **23**(5): 842-846.

Brown, B. and A. S. Hall (2005). "Renin-angiotensin system modulation: the weight of evidence." Am J Hypertens **18**(9 Pt 2): 127s-133s.

Buesing, K. L., J. C. Densmore, S. Kaul, K. A. Pritchard, Jr., J. A. Jarzembowski, D. M. Gourlay and K. T. Oldham (2011). "Endothelial microparticles induce inflammation in acute lung injury." J Surg Res **166**(1): 32-39.

Burger, D., D. G. Kwart, A. C. Montezano, N. C. Read, C. R. Kennedy, C. S. Thompson and R. M. Touyz (2012). "Microparticles induce cell cycle arrest through redox-sensitive processes in endothelial cells: implications in vascular senescence." J Am Heart Assoc **1**(3): 22.

Burrig, K. F. (1991). "The endothelium of advanced arteriosclerotic plaques in humans." Arterioscler Thromb **11**(6): 1678-1689.

Cabelof, D. C., J. J. Raffoul, S. Yanamadala, C. Ganir, Z. Guo and A. R. Heydari (2002). "Attenuation of DNA polymerase beta-dependent base excision repair and increased DMS-induced mutagenicity in aged mice." Mutat Res **500**(1-2): 135-145.

Calado, J., Y. Sznajer, D. Metzger, A. Rita, M. C. Hogan, A. Kattamis, M. Scharf, V. Tasic, J. Greil, F. Brinkert, M. J. Kemper and R. Santer (2008). "Twenty-one additional cases of familial renal glucosuria: absence of genetic heterogeneity, high prevalence of private mutations and further evidence of volume depletion." Nephrol Dial Transplant **23**(12): 3874-3879.

Canault, M., A. S. Leroyer, F. Peiretti, G. Leseche, A. Tedgui, B. Bonardo, M. C. Alessi, C. M. Boulanger and G. Nalbone (2007). "Microparticles of human atherosclerotic plaques enhance the shedding of the tumor necrosis factor-alpha converting enzyme/ADAM17 substrates, tumor necrosis factor and tumor necrosis factor receptor-1." Am J Pathol **171**(5): 1713-1723.

Caughey, G. E., L. G. Cleland, P. S. Penglis, J. R. Gamble and M. J. James (2001). "Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: selective up-regulation of prostacyclin synthesis by COX-2." J Immunol **167**(5): 2831-2838.

Celermajer, D. S., K. E. Sorensen, D. J. Spiegelhalter, D. Georgakopoulos, J. Robinson and J. E. Deanfield (1994). "Aging is associated with endothelial dysfunction in healthy men years before the age-related decline in women." J Am Coll Cardiol **24**(2): 471-476.

Chakravarthy, U. and D. B. Archer (1992). "Endothelin: a new vasoactive ocular peptide." Br J Ophthalmol **76**(2): 107-108.

Chance, B., H. Sies and A. Boveris (1979). "Hydroperoxide metabolism in mammalian organs." Physiol Rev **59**(3): 527-605.

Chandra, S. B., S. Mohan, B. M. Ford, L. Huang, P. Janardhanan, K. S. Deo, L. Cong, E. R. Muir and T. Q. Duong (2016). "Targeted overexpression of endothelial nitric oxide synthase in endothelial cells improves cerebrovascular reactivity in Ins2Akita-type-1 diabetic mice." J Cereb Blood Flow Metab **36**(6): 1135-1142.

Chaplin, D. D. (2010). "Overview of the immune response." J Allergy Clin Immunol **125**(2 Suppl 2): 980.

Chen, G. Y. and G. Nunez (2010). "Sterile inflammation: sensing and reacting to damage." Nat Rev Immunol **10**(12): 826-837.

Chen, J., S. V. Brodsky, D. M. Goligorsky, D. J. Hampel, H. Li, S. S. Gross and M. S. Goligorsky (2002). "Glycated collagen I induces premature senescence-like phenotypic changes in endothelial cells." Circ Res **90**(12): 1290-1298.

Chen, J., S. Chen, Y. Chen, C. Zhang, J. Wang, W. Zhang, G. Liu, B. Zhao and Y. Chen (2011). "Circulating endothelial progenitor cells and cellular membrane microparticles in db/db diabetic mouse: possible implications in cerebral ischemic damage." Am J Physiol Endocrinol Metab **301**(1): 19.

Chen, J. and M. S. Goligorsky (2006). "Premature senescence of endothelial cells: Methusaleh's dilemma." Am J Physiol Heart Circ Physiol **290**(5): H1729-1739.

Chen, J., S. Williams, S. Ho, H. Loraine, D. Hagan, J. M. Whaley and J. N. Feder (2010). "Quantitative PCR tissue expression profiling of the human SGLT2 gene and related family members." Diabetes Ther **1**(2): 57-92.

Cheng, F., Y. Wang, J. Li, C. Su, F. Wu, W. H. Xia, Z. Yang, B. B. Yu, Y. X. Qiu and J. Tao (2013). "Berberine improves endothelial function by reducing endothelial microparticles-mediated oxidative stress in humans." Int J Cardiol **167**(3): 936-942.

Cherry, S. and N. Silverman (2006). "Host-pathogen interactions in drosophila: new tricks from an old friend." Nat Immunol **7**(9): 911-917.

Chimenti, C., J. Kajstura, D. Torella, K. Urbanek, H. Heleniak, C. Colussi, F. Di Meglio, B. Nadal-Ginard, A. Frustaci, A. Leri, A. Maseri and P. Anversa (2003). "Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure." Circ Res **93**(7): 604-613.

Chironi, G., A. Simon, B. Hugel, M. Del Pino, J. Gariepy, J. M. Freyssinet and A. Tedgui (2006). "Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects." Arterioscler Thromb Vasc Biol **26**(12): 2775-2780.

Chiu, J. J. and S. Chien (2011). "Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives." Physiol Rev **91**(1): 327-387.

Chung, H. Y., M. Cesari, S. Anton, E. Marzetti, S. Giovannini, A. Y. Seo, C. Carter, B. P. Yu and C. Leeuwenburgh (2009). "Molecular inflammation: underpinnings of aging and age-related diseases." Ageing Res Rev **8**(1): 18-30.

Cines, D. B., E. S. Pollak, C. A. Buck, J. Loscalzo, G. A. Zimmerman, R. P. McEver, J. S. Pober, T. M. Wick, B. A. Konkle, B. S. Schwartz, E. S. Barnathan, K. R. McCrae, B. A. Hug, A. M. Schmidt and D. M. Stern (1998). "Endothelial cells in physiology and in the pathophysiology of vascular disorders." Blood **91**(10): 3527-3561.

Clark, M. R. (2011). "Flippin' lipids." Nat Immunol **12(5)**: 373-375.

Claybaugh, T. and S. Decker (2014). "L-Arginine Supplementation in Type II Diabetic Rats Preserves Renal Function and Improves Insulin Sensitivity by Altering the Nitric Oxide Pathway." **2014**: 171546.

Coleman, J. A., F. Quazi and R. S. Molday (2013). "Mammalian P4-ATPases and ABC transporters and their role in phospholipid transport." Biochim Biophys Acta **3**: 555-574.

Coleman, M. L., E. A. Sahai, M. Yeo, M. Bosch, A. Dewar and M. F. Olson (2001). "Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK I." Nat Cell Biol **3**(4): 339-345.

Cooper, M. L., Walters, E. W., and Keller, H. M. (1955). "Detection of a new serotype of E. coli 0127 :B8 associated with acute diarrhea in infants." J. Bact., **69**: 689-694.

Cosentino, F., M. Eto, P. De Paolis, B. van der Loo, M. Bachschmid, V. Ullrich, A. Kouroedov, C. Delli Gatti, H. Joch, M. Volpe and T. F. Luscher (2003). "High glucose causes upregulation of cyclooxygenase-2 and alters prostanoid profile in human endothelial cells: role of protein kinase C and reactive oxygen species." Circulation **107**(7): 1017-1023.

Csikos, T., S. Gallinat and T. Unger (1997). "Extrarenal aspects of angiotensin II function." Eur J Endocrinol **136**(4): 349-358.

Csiszar, A., Z. Ungvari, J. G. Edwards, P. Kaminski, M. S. Wolin, A. Koller and G. Kaley (2002). "Aging-induced phenotypic changes and oxidative stress impair coronary arteriolar function." Circ Res **90**(11): 1159-1166.

da Silva Correia, J., K. Soldau, U. Christen, P. S. Tobias and R. J. Ulevitch (2001). "Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. transfer from CD14 to TLR4 and MD-2." J Biol Chem **276**(24): 21129-21135.

Daleke, D. L. (2003). "Regulation of transbilayer plasma membrane phospholipid asymmetry." J Lipid Res **44**(2): 233-242.

Davi, G. and P. Ferroni (2005). "Microparticles in type 2 diabetes mellitus." J Thromb Haemost **3**(6): 1166-1167.

Davignon, J. and P. Ganz (2004). "Role of endothelial dysfunction in atherosclerosis." Circulation **109**(23 Suppl 1).

de Cavanagh, E. M., B. Piotrkowski and C. G. Fraga (2004). "Concerted action of the renin-angiotensin system, mitochondria, and antioxidant defenses in aging." Mol Aspects Med **25**(1-2): 27-36.

De Luca d'Alessandro, E., S. Bonacci and G. Giraldi (2011). "Aging populations: the health and quality of life of the elderly." Clin Ter **162**(1): e13-18.

De Vriese, A. S., T. J. Verbeuren, J. Van de Voorde, N. H. Lameire and P. M. Vanhoutte (2000). "Endothelial dysfunction in diabetes." Br J Pharmacol **130**(5): 963-974.

Deanfield, J. E., J. P. Halcox and T. J. Rabelink (2007). "Endothelial function and dysfunction: testing and clinical relevance." Circulation **115**(10): 1285-1295.

Debacq-Chainiaux, F., J. D. Erusalimsky, J. Campisi and O. Toussaint (2009). "Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo." Nat Protoc **4**(12): 1798-1806.

Deeks, S. G. (2011). "HIV infection, inflammation, immunosenescence, and aging." Annu Rev Med **62**: 141-155.

Delabranche, X., A. Berger, J. Boisrame-Helms and F. Meziani (2012). "Microparticles and infectious diseases." Med Mal Infect **42**(8): 335-343.

Delabranche, X., J. P. Quenot, T. Lavigne, E. Mercier, B. Francois, F. Severac, L. Grunebaum, M. Mehdi, F. Zobairi, F. Toti, F. Meziani and J. Boisrame-Helms (2016). "Early Detection of Disseminated Intravascular Coagulation During Septic Shock: A Multicenter Prospective Study." Crit Care Med **44**(10): e930-939.

Delabranche, X., L. Stiel, F. Severac, A. C. Galoisy, L. Mauvieux, F. Zobairi, T. Lavigne, F. Toti, E. Angles-Cano, F. Meziani and J. Boisrame-Helms (2017). "Evidence of Netosis in Septic Shock-Induced Disseminated Intravascular Coagulation." Shock **47**(3): 313-317.

Delattre, J., D. Bonnefont-Rousselot, M. Bordas-Fonfredre and M. Jaudon (1999). "[Diabetes mellitus and oxidative stress]." Ann Biol Clin (Paris) **57**(4): 437-444.

den Dekker, W. K., C. Cheng, G. Pasterkamp and H. J. Duckers (2010). "Toll like receptor 4 in atherosclerosis and plaque destabilization." Atherosclerosis **209**(2): 314-320.

Densmore, J. C., P. R. Signorino, J. Ou, O. A. Hatoum, J. J. Rowe, Y. Shi, S. Kaul, D. W. Jones, R. E. Sabina, K. A. Pritchard, Jr., K. S. Guice and K. T. Oldham (2006). "Endothelium-derived microparticles induce endothelial dysfunction and acute lung injury." Shock **26**(5): 464-471.

DeVinney, R., A. Gauthier, A. Abe and B. B. Finlay (1999). "Enteropathogenic Escherichia coli: a pathogen that inserts its own receptor into host cells." Cell Mol Life Sci **55**(6-7): 961-976.

Diehl, P., M. Aleker, T. Helbing, V. Sossong, M. Germann, S. Sorichter, C. Bode and M. Moser (2011). "Increased platelet, leukocyte and endothelial microparticles predict enhanced coagulation and vascular inflammation in pulmonary hypertension." J Thromb Thrombolysis **31**(2): 173-179.

Ding, H., M. Aljofan and C. R. Triggle (2007). "Oxidative stress and increased eNOS and NADPH oxidase expression in mouse microvessel endothelial cells." J Cell Physiol **212**(3): 682-689.

Doerrler, W. T. (2006). "Lipid trafficking to the outer membrane of Gram-negative bacteria." Mol Microbiol **60**(3): 542-552.

Dohi, Y., M. Kojima, K. Sato and T. F. Luscher (1995). "Age-related changes in vascular smooth muscle and endothelium." Drugs Aging **7**(4): 278-291.

Doles, J., M. Storer, L. Cozzuto, G. Roma and W. M. Keyes (2012). "Age-associated inflammation inhibits epidermal stem cell function." Genes Dev **26**(19): 2144-2153.

Dorigo, P., D. Fraccarollo, G. Santostasi and I. Maragno (1997). "Impairment of endothelium-dependent but not of endothelium-independent dilatation in guinea-pig aorta rings incubated in the presence of elevated glucose." Br J Pharmacol **121**(5): 972-976.

Drasar, B. S., and M. J. Hill (1974). "Human intestinal flora, Academic Press, Ltd., London, United Kingdom.": 36–43.

Dulic, V., G. E. Beney, G. Frebourg, L. F. Drullinger and G. H. Stein (2000). "Uncoupling between phenotypic senescence and cell cycle arrest in aging p21-deficient fibroblasts." Mol Cell Biol **20**(18): 6741-6754.

Edwards, M., D. K. Rassin, T. Izumi, S. Mitra and J. R. Perez-Polo (1998). "APE/Ref-1 responses to oxidative stress in aged rats." J Neurosci Res **54**(5): 635-638.

El-Awady, M. S., D. S. El-Agamy, G. M. Suddek and M. A. Nader (2014). "Propolis protects against high glucose-induced vascular endothelial dysfunction in isolated rat aorta." J Physiol Biochem **70**(1): 247-254.

el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein (1993). "WAF1, a potential mediator of p53 tumor suppression." Cell **75**(4): 817-825.

Erusalimsky, J. D. (2009). "Vascular endothelial senescence: from mechanisms to pathophysiology." J Appl Physiol (1985) **106**(1): 326-332.

Erusalimsky, J. D. and C. Skene (2009). "Mechanisms of endothelial senescence." Exp Physiol **94**(3): 299-304.

Escobar-Paramo, P., A. Le Menac'h, T. Le Gall, C. Amorin, S. Gouriou, B. Picard, D. Skurnik and E. Denamur (2006). "Identification of forces shaping the commensal Escherichia coli genetic structure by comparing animal and human isolates." Environ Microbiol **8**(11): 1975-1984.

Essayagh, S., J. M. Xuereb, A. D. Terrisse, L. Tellier-Cirioni, B. Pipy and P. Sie (2007). "Microparticles from apoptotic monocytes induce transient platelet recruitment and tissue factor expression by cultured human vascular endothelial cells via a redox-sensitive mechanism." Thromb Haemost **98**(4): 831-837.

Ettelaie, C., S. Su, C. Li and M. E. Collier (2008). "Tissue factor-containing microparticles released from mesangial cells in response to high glucose and AGE induce tube formation in microvascular cells." Microvasc Res **76**(3): 152-160.

Eyre, J., J. O. Burton, M. A. Saleem, P. W. Mathieson, P. S. Topham and N. J. Brunskill (2011). "Monocyte- and endothelial-derived microparticles induce an inflammatory phenotype in human podocytes." Nephron Exp Nephrol **119**(3): 18.

Fadok, V. A., M. L. Warner, D. L. Bratton and P. M. Henson (1998). "CD36 is required for phagocytosis of apoptotic cells by human macrophages that use either a phosphatidylserine receptor or the vitronectin receptor (alpha v beta 3)." J Immunol **161**(11): 6250-6257.

Faggioli, F., T. Wang, J. Vijg and C. Montagna (2012). "Chromosome-specific accumulation of aneuploidy in the aging mouse brain." Hum Mol Genet **21**(24): 5246-5253.

Favaro, E., I. Miceli, B. Bussolati, M. Schmitt-Ney, P. Cavallo Perin, G. Camussi and M. M. Zanone (2008). "Hyperglycemia induces apoptosis of human pancreatic islet endothelial cells: effects of pravastatin on the Akt survival pathway." Am J Pathol **173**(2): 442-450.

Favero, G., C. Paganelli, B. Buffoli, L. F. Rodella and R. Rezzani (2014). "Endothelium and its alterations in cardiovascular diseases: life style intervention." Biomed Res Int **801896**(10): 26.

Feletou, M. and P. M. Vanhoutte (2006). "Endothelial dysfunction: a multifaceted disorder (The Wiggers Award Lecture)." Am J Physiol Heart Circ Physiol **291**(3): 21.

Feletou, M. and P. M. Vanhoutte (2006). "Endothelium-derived hyperpolarizing factor: where are we now?" Arterioscler Thromb Vasc Biol **26**(6): 1215-1225.

Feletou, M. and P. M. Vanhoutte (2009). "EDHF: an update." Clin Sci (Lond) **117**(4): 139-155.

Feletou, M., T. J. Verbeuren and P. M. Vanhoutte (2009). "Endothelium-dependent contractions in SHR: a tale of prostanoid TP and IP receptors." Br J Pharmacol **156**(4): 563-574.

Feng, B., Y. Chen, Y. Luo, M. Chen, X. Li and Y. Ni (2010). "Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus." Atherosclerosis **208**(1): 264-269.

Fenton, M., S. Barker, D. J. Kurz and J. D. Erusalimsky (2001). "Cellular senescence after single and repeated balloon catheter denudations of rabbit carotid arteries." Arterioscler Thromb Vasc Biol **21**(2): 220-226.

Fingar, D. C., S. Salama, C. Tsou, E. Harlow and J. Blenis (2002). "Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E." Genes Dev **16**(12): 1472-1487.

Fitzpatrick, A. L., R. A. Kronmal, J. P. Gardner, B. M. Psaty, N. S. Jenny, R. P. Tracy, J. Walston, M. Kimura and A. Aviv (2007). "Leukocyte telomere length and cardiovascular disease in the cardiovascular health study." Am J Epidemiol **165**(1): 14-21.

Florey (1966). "The endothelial cell." Br Med J **2**(5512): 487-490.

Freidja, M. L., E. Vessieres, B. Toutain, A. L. Guihot, M. A. Custaud, L. Loufrani, C. Fassot and D. Henrion (2014). "AGEs breaking and antioxidant treatment improves endothelium-dependent dilation without effect on flow-mediated remodeling of resistance arteries in old Zucker diabetic rats." Cardiovasc Diabetol **13**(55): 1475-2840.

Freitas, H. S., G. F. Anhe, K. F. Melo, M. M. Okamoto, M. Oliveira-Souza, S. Bordin and U. F. Machado (2008). "Na(+) -glucose transporter-2 messenger ribonucleic acid expression in kidney of diabetic rats correlates with glycemic levels: involvement of hepatocyte nuclear factor-1alpha expression and activity." Endocrinology **149**(2): 717-724.

Fukui, M., M. Tanaka, T. Senmaru, M. Nakanishi, J. Mukai, M. Ohki, M. Asano, M. Yamazaki, G. Hasegawa and N. Nakamura (2013). "LOX-1 is a novel marker for peripheral artery disease in patients with type 2 diabetes." Metabolism **62**(7): 935-938.

Furchgott, R. F. and J. V. Zawadzki (1980). "The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine." Nature **288**(5789): 373-376.

Gabra, B. H., R. Couture and P. Sirois (2003). "[Functional duality of kinin receptors in pathophysiology]." Med Sci (Paris) **19**(11): 1101-1110.

Galley, H. F. and N. R. Webster (2004). "Physiology of the endothelium." Br J Anaesth **93**(1): 105-113.

Gebremedhin, D., M. Z. Koltai, G. Pogatsa, K. Magyar and P. Hadhazy (1988). "Influence of experimental diabetes on the mechanical responses of canine coronary arteries: role of endothelium." Cardiovasc Res **22**(8): 537-544.

Geraldes, P. and G. L. King (2010). "Activation of protein kinase C isoforms and its impact on diabetic complications." Circ Res **106**(8): 1319-1331.

Ghafourifar, P. and E. Cadenas (2005). "Mitochondrial nitric oxide synthase." Trends Pharmacol Sci **26**(4): 190-195.

Giacco, F. and M. Brownlee (2010). "Oxidative stress and diabetic complications." Circ Res **107**(9): 1058-1070.

Gioannini, T. L., A. Teghanemt, D. Zhang, N. P. Coussens, W. Dockstader, S. Ramaswamy and J. P. Weiss (2004). "Isolation of an endotoxin-MD-2 complex that produces Toll-like receptor 4-dependent cell activation at picomolar concentrations." Proc Natl Acad Sci U S A **101**(12): 4186-4191.

Gioannini, T. L., A. Teghanemt, D. Zhang, E. N. Levis and J. P. Weiss (2005). "Monomeric endotoxin:protein complexes are essential for TLR4-dependent cell activation." J Endotoxin Res **11**(2): 117-123.

Gleizes, C., A. Constantinescu, M. Abbas, H. Bouhadja, F. Zobairi, L. Kessler and F. Toti (2014). "Liraglutide protects Rin-m5f beta cells by reducing procoagulant tissue factor activity and apoptosis prompted by microparticles under conditions mimicking Instant Blood-Mediated Inflammatory Reaction." Transpl Int **27**(7): 733-740.

Gleizes, C., G. Kreutter, M. Abbas, M. Kassem, A. A. Constantinescu, J. Boisrame-Helms, B. Yver, F. Toti and L. Kessler (2016). "beta cell membrane remodelling and procoagulant events occur in inflammation-driven insulin impairment: a GLP-1 receptor dependent and independent control." J Cell Mol Med **20**(2): 231-242.

Goldschmidt, R. (1933). "Untersuehungen sur Atiologie der Durchfallserkrankungen des Siiuglings." Jahr. Kinderheilkunde **139**: 318-358.

Golenbock, D. T., R. Y. Hampton, N. Qureshi, K. Takayama and C. R. Raetz (1991). "Lipid A-like molecules that antagonize the effects of endotoxins on human monocytes." J Biol Chem **266**(29): 19490-19498.

Gonzalez-Suarez, E., E. Samper, A. Ramirez, J. M. Flores, J. Martin-Caballero, J. L. Jorcano and M. A. Blasco (2001). "Increased epidermal tumors and increased skin wound healing in transgenic mice overexpressing the catalytic subunit of telomerase, mTERT, in basal keratinocytes." Embo j **20**(11): 2619-2630.

Gorbunova, V., A. Seluanov and O. M. Pereira-Smith (2002). "Expression of human telomerase (hTERT) does not prevent stress-induced senescence in normal human fibroblasts

but protects the cells from stress-induced apoptosis and necrosis." J Biol Chem **277**(41): 38540-38549.

Gori, T., S. Dragoni, G. Di Stolfo and S. Forconi (2007). "Endothelium and haemorheology." Ann Ist Super Sanita **43**(2): 124-129.

Goyert, S. M., E. Ferrero, W. J. Rettig, A. K. Yenamandra, F. Obata and M. M. Le Beau (1988). "The CD14 monocyte differentiation antigen maps to a region encoding growth factors and receptors." Science **239**(4839): 497-500.

Griendling, K. K. and M. Ushio-Fukai (1997). "NADH/NADPH Oxidase and Vascular Function." Trends Cardiovasc Med **7**(8): 301-307.

Grundy, S. M., I. J. Benjamin, G. L. Burke, A. Chait, R. H. Eckel, B. V. Howard, W. Mitch, S. C. Smith, Jr. and J. R. Sowers (1999). "Diabetes and cardiovascular disease: a statement for healthcare professionals from the American Heart Association." Circulation **100**(10): 1134-1146.

Gutierrez, E., A. J. Flammer, L. O. Lerman, J. Elizaga, A. Lerman and F. Fernandez-Aviles (2013). "Endothelial dysfunction over the course of coronary artery disease." Eur Heart J **34**(41): 3175-3181.

Gutterman, D. (1999). "Adventitia-dependent influences on vascular function." Am J Physiol **277(4 Pt 2):H**: 1265-1272.

Haddad, J. J. (2002). "Antioxidant and prooxidant mechanisms in the regulation of redox(y)-sensitive transcription factors." Cell Signal **14**(11): 879-897.

Hagen, T. M. (2003). "Oxidative stress, redox imbalance, and the aging process." Antioxid Redox Signal **5**(5): 503-506.

Halcox, J. P., W. H. Schenke, G. Zalos, R. Mincemoyer, A. Prasad, M. A. Waclawiw, K. R. Nour and A. A. Quyyumi (2002). "Prognostic value of coronary vascular endothelial dysfunction." Circulation **106**(6): 653-658.

Harman, D. (1956). "Aging: a theory based on free radical and radiation chemistry." J Gerontol **11**(3): 298-300.

Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi and S. J. Elledge (1993). "The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases." Cell **75**(4): 805-816.

Harris, A. K., J. R. Hutchinson, K. Sachidanandam, M. H. Johnson, A. M. Dorrance, D. W. Stepp, S. C. Fagan and A. Ergul (2005). "Type 2 diabetes causes remodeling of cerebrovasculature via differential regulation of matrix metalloproteinases and collagen synthesis: role of endothelin-1." Diabetes **54**(9): 2638-2644.

Harrison, D. G. (1997). "Cellular and molecular mechanisms of endothelial cell dysfunction." J Clin Invest **100**(9): 2153-2157.

Hayashi, T., H. Matsui-Hirai, A. Miyazaki-Akita, A. Fukatsu, J. Funami, Q. F. Ding, S. Kamalanathan, Y. Hattori, L. J. Ignarro and A. Iguchi (2006). "Endothelial cellular

senescence is inhibited by nitric oxide: implications in atherosclerosis associated with menopause and diabetes." Proc Natl Acad Sci U S A **103**(45): 17018-17023.

Hayflick, L. and P. S. Moorhead (1961). "The serial cultivation of human diploid cell strains." Exp Cell Res **25**: 585-621.

Heitsch, H. (2003). "The therapeutic potential of bradykinin B2 receptor agonists in the treatment of cardiovascular disease." Expert Opin Investig Drugs **12**(5): 759-770.

Herbert, K. E., Y. Mistry, R. Hastings, T. Poolman, L. Niklason and B. Williams (2008). "Angiotensin II-mediated oxidative DNA damage accelerates cellular senescence in cultured human vascular smooth muscle cells via telomere-dependent and independent pathways." Circ Res **102**(2): 201-208.

Herrera, M. D., C. Mingorance, R. Rodriguez-Rodriguez and M. Alvarez de Sotomayor (2010). "Endothelial dysfunction and aging: an update." Ageing Res Rev **9**(2): 142-152.

Herzer, P. J., S. Inouye, M. Inouye and T. S. Whittam (1990). "Phylogenetic distribution of branched RNA-linked multicopy single-stranded DNA among natural isolates of Escherichia coli." J Bacteriol **172**(11): 6175-6181.

Higashida, H., R. A. Streaty, W. Klee and M. Nirenberg (1986). "Bradykinin-activated transmembrane signals are coupled via No or Ni to production of inositol 1,4,5-trisphosphate, a second messenger in NG108-15 neuroblastoma-glioma hybrid cells." Proc Natl Acad Sci U S A **83**(4): 942-946.

Hink, U., H. Li, H. Mollnau, M. Oelze, E. Matheis, M. Hartmann, M. Skatchkov, F. Thaiss, R. A. Stahl, A. Warnholtz, T. Meinertz, K. Griendling, D. G. Harrison, U. Forstermann and T. Munzel (2001). "Mechanisms underlying endothelial dysfunction in diabetes mellitus." Circ Res **88**(2): E14-22.

Ho, F. M., S. H. Liu, C. S. Liau, P. J. Huang and S. Y. Lin-Shiau (2000). "High glucose-induced apoptosis in human endothelial cells is mediated by sequential activations of c-Jun NH(2)-terminal kinase and caspase-3." Circulation **101**(22): 2618-2624.

Hoffmann, J., J. Haendeler, A. Aicher, L. Rossig, M. Vasa, A. M. Zeiher and S. Dimmeler (2001). "Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide." Circ Res **89**(8): 709-715.

Holowatz, L. A., C. S. Thompson-Torgerson and W. L. Kenney (2007). "Altered mechanisms of vasodilation in aged human skin." Exerc Sport Sci Rev **35**(3): 119-125.

Holst, O. (1999). "Chemical structure of the core region of lipopolysaccharides." In Endotoxin in Health and Disease (Brade, H.,Opal, S.M., Vogel, S.N. & Morrison, D.C., eds),: pp. 115–154. Marcel Dekker, Inc., New York.

Horiuchi, M., M. Akishita and V. J. Dzau (1999). "Recent progress in angiotensin II type 2 receptor research in the cardiovascular system." Hypertension **33**(2): 613-621.

Hou, N., N. Huang, F. Han, J. Zhao, X. Liu and X. Sun (2014). "Protective effects of adiponectin on uncoupling of glomerular VEGF-NO axis in early streptozotocin-induced type 2 diabetic rats." Int Urol Nephrol **46**(10): 2045-2051.

Howl, J. and S. J. Payne (2003). "Bradykinin receptors as a therapeutic target." Expert Opin Ther Targets **7**(2): 277-285.

Hoyer, F. F., G. Nickenig and N. Werner (2010). "Microparticles--messengers of biological information." J Cell Mol Med **14**(9): 2250-2256.

Hsu, Y. C., P. H. Lee, C. C. Lei, C. Ho, Y. H. Shih and C. L. Lin (2015). "Nitric oxide donors rescue diabetic nephropathy through oxidative-stress-and nitrosative-stress-mediated Wnt signaling pathways." J Diabetes Investig **6**(1): 24-34.

Huang, C. D., O. Tliba, R. A. Panettieri, Jr. and Y. Amrani (2003). "Bradykinin induces interleukin-6 production in human airway smooth muscle cells: modulation by Th2 cytokines and dexamethasone." Am J Respir Cell Mol Biol **28**(3): 330-338.

Hugel, B., M. C. Martinez, C. Kunzelmann and J. M. Freyssinet (2005). "Membrane microparticles: two sides of the coin." Physiology **20**: 22-27.

Ido, Y., D. Carling and N. Ruderman (2002). "Hyperglycemia-induced apoptosis in human umbilical vein endothelial cells: inhibition by the AMP-activated protein kinase activation." Diabetes **51**(1): 159-167.

Imanishi, T., T. Hano and I. Nishio (2005). "Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress." J Hypertens **23**(1): 97-104.

Imler, J. L. and J. A. Hoffmann (2001). "Toll receptors in innate immunity." Trends Cell Biol **11**(7): 304-311.

Inoguchi, T., R. Battan, E. Handler, J. R. Sportsman, W. Heath and G. L. King (1992). "Preferential elevation of protein kinase C isoform beta II and diacylglycerol levels in the aorta and heart of diabetic rats: differential reversibility to glycemic control by islet cell transplantation." Proc Natl Acad Sci U S A **89**(22): 11059-11063.

Inoguchi, T., P. Li, F. Umeda, H. Y. Yu, M. Kakimoto, M. Imamura, T. Aoki, T. Etoh, T. Hashimoto, M. Naruse, H. Sano, H. Utsumi and H. Nawata (2000). "High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C--dependent activation of NAD(P)H oxidase in cultured vascular cells." Diabetes **49**(11): 1939-1945.

Iovine, N., J. Eastvold, P. Elsbach, J. P. Weiss and T. L. Gioannini (2002). "The carboxyl-terminal domain of closely related endotoxin-binding proteins determines the target of protein-lipopolysaccharide complexes." J Biol Chem **277**(10): 7970-7978.

Ishii, H., M. R. Jirousek, D. Koya, C. Takagi, P. Xia, A. Clermont, S. E. Bursell, T. S. Kern, L. M. Ballas, W. F. Heath, L. E. Stramm, E. P. Feener and G. L. King (1996). "Amelioration of vascular dysfunctions in diabetic rats by an oral PKC beta inhibitor." Science **272**(5262): 728-731.

Itahana, K., Y. Zou, Y. Itahana, J. L. Martinez, C. Beausejour, J. J. Jacobs, M. Van Lohuizen, V. Band, J. Campisi and G. P. Dimri (2003). "Control of the replicative life span of human fibroblasts by p16 and the polycomb protein Bmi-1." Mol Cell Biol **23**(1): 389-401.

Jeanclos, E., A. Krolewski, J. Skurnick, M. Kimura, H. Aviv, J. H. Warram and A. Aviv (1998). "Shortened telomere length in white blood cells of patients with IDDM." Diabetes **47**(3): 482-486.

Jeanclos, E., N. J. Schork, K. O. Kyvik, M. Kimura, J. H. Skurnick and A. Aviv (2000). "Telomere length inversely correlates with pulse pressure and is highly familial." Hypertension **36**(2): 195-200.

Jin, J. S. and H. G. Bohlen (1997). "Non-insulin-dependent diabetes and hyperglycemia impair rat intestinal flow-mediated regulation." Am J Physiol **272**(2 Pt 2).

Johnson, J. R. and A. L. Stell (2000). "Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise." J Infect Dis **181**(1): 261-272.

Jy, W., L. L. Horstman, J. J. Jimenez, Y. S. Ahn, E. Biro, R. Nieuwland, A. Sturk, F. Dignat-George, F. Sabatier, L. Camoin-Jau, J. Sampol, B. Hugel, F. Zobairi, J. M. Freyssinet, S. Nomura, A. S. Shet, N. S. Key and R. P. Hebbel (2004). "Measuring circulating cell-derived microparticles." J Thromb Haemost **2**(10): 1842-1851.

Jy, W., A. Minagar, J. J. Jimenez, W. A. Sheremata, L. M. Mauro, L. L. Horstman, C. Bidot and Y. S. Ahn (2004). "Endothelial microparticles (EMP) bind and activate monocytes: elevated EMP-monocyte conjugates in multiple sclerosis." Front Biosci **9**: 3137-3144.

Kagota, S., Y. Yamaguchi, K. Nakamura and M. Kunitomo (2000). "Altered endothelium-dependent responsiveness in the aortas and renal arteries of Otsuka Long-Evans Tokushima Fatty (OLETF) rats, a model of non-insulin-dependent diabetes mellitus." Gen Pharmacol **34**(3): 201-209.

Kanai, Y., W. S. Lee, G. You, D. Brown and M. A. Hediger (1994). "The human kidney low affinity Na+/glucose cotransporter SGLT2. Delineation of the major renal reabsorptive mechanism for D-glucose." J Clin Invest **93**(1): 397-404.

Kanani, P. M., C. A. Sinkey, R. L. Browning, M. Allaman, H. R. Knapp and W. G. Haynes (1999). "Role of oxidant stress in endothelial dysfunction produced by experimental hyperhomocyst(e)inemia in humans." Circulation **100**(11): 1161-1168.

Kanazawa, K., Y. Sato, K. Ohki, K. Okimura, Y. Uchida, M. Shindo and N. Sakura (2009). "Contribution of each amino acid residue in polymyxin B(3) to antimicrobial and lipopolysaccharide binding activity." Chem Pharm Bull **57**(3): 240-244.

Kang, K. T. (2014). "Endothelium-derived Relaxing Factors of Small Resistance Arteries in Hypertension." Toxicol Res **30**(3): 141-148.

Kant, V., D. Kumar, D. Kumar, R. Prasad, A. Gopal, N. N. Pathak, P. Kumar and S. K. Tandan (2015). "Topical application of substance P promotes wound healing in streptozotocin-induced diabetic rats." Cytokine **73**(1): 144-155.

Kapahi, P., M. E. Boulton and T. B. Kirkwood (1999). "Positive correlation between mammalian life span and cellular resistance to stress." Free Radic Biol Med **26**(5-6): 495-500.

Kawai, T. and S. Akira (2011). "Toll-like receptors and their crosstalk with other innate receptors in infection and immunity." Immunity **34**(5): 637-650.

Kelley, S. L., T. Lukk, S. K. Nair and R. I. Tapping (2013). "The crystal structure of human soluble CD14 reveals a bent solenoid with a hydrophobic amino-terminal pocket." J Immunol **190**(3): 1304-1311.

Kelly-Cobbs, A., M. M. Elgebaly, W. Li and A. Ergul (2011). "Pressure-independent cerebrovascular remodelling and changes in myogenic reactivity in diabetic Goto-Kakizaki rat in response to glycaemic control." Acta Physiol (Oxf) **203**(1): 245-251.

Khemais-Benkhiat, S., M. Abbas, N. Nguyen, C. Auger, F. Toti, E. Mayoux, L. Kessler and V. Schini-Kerth (2016). "L'empagliflozine, un inhibiteur spécifique de SGLT2, prévient la sénescence induite dans les cellules endothéliales par des concentrations élevées de glucose." SFD, Congress

Khemais-Benkhiat, S., N. Idris-Khodja, T. P. Ribeiro, G. C. Silva, M. Abbas, M. Kheloufi, J. O. Lee, F. Toti, C. Auger and V. B. Schini-Kerth (2016). "The Redox-sensitive Induction of the Local Angiotensin System Promotes Both Premature and Replicative Endothelial Senescence: Preventive Effect of a Standardized Crataegus Extract." J Gerontol A Biol Sci Med Sci **71**(12): 1581-1590.

Kim, J. I., C. J. Lee, M. S. Jin, C. H. Lee, S. G. Paik, H. Lee and J. O. Lee (2005). "Crystal structure of CD14 and its implications for lipopolysaccharide signaling." J Biol Chem **280**(12): 11347-11351.

Kimura, H. and H. Esumi (2003). "Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis." Acta Biochim Pol **50**(1): 49-59.

Kitada, K., D. Nakano, H. Ohsaki, H. Hitomi, T. Minamino, J. Yatabe, R. A. Felder, H. Mori, T. Masaki, H. Kobori and A. Nishiyama (2014). "Hyperglycemia causes cellular senescence via a SGLT2- and p21-dependent pathway in proximal tubules in the early stage of diabetic nephropathy." J Diabetes Complications **28**(5): 604-611.

Koga, H., S. Sugiyama, K. Kugiyama, H. Fukushima, K. Watanabe, T. Sakamoto, M. Yoshimura, H. Jinnouchi and H. Ogawa (2006). "Elevated levels of remnant lipoproteins are associated with plasma platelet microparticles in patients with type-2 diabetes mellitus without obstructive coronary artery disease." Eur Heart J **27**(7): 817-823.

Koga, H., S. Sugiyama, K. Kugiyama, K. Watanabe, H. Fukushima, T. Tanaka, T. Sakamoto, M. Yoshimura, H. Jinnouchi and H. Ogawa (2005). "Elevated levels of VE-cadherin-positive endothelial microparticles in patients with type 2 diabetes mellitus and coronary artery disease." J Am Coll Cardiol **45**(10): 1622-1630.

Kowluru, R. A., A. Kowluru, R. Veluthakal, G. Mohammad, I. Syed, J. M. Santos and M. Mishra (2014). "TIAM1-RAC1 signalling axis-mediated activation of NADPH oxidase-2 initiates mitochondrial damage in the development of diabetic retinopathy." Diabetologia **57**(5): 1047-1056.

Kraal, G. (1992). "Cells in the marginal zone of the spleen." Int Rev Cytol **132**: 31-74.

Kuilman, T., C. Michaloglou, L. C. Vredeveld, S. Douma, R. van Doorn, C. J. Desmet, L. A. Aarden, W. J. Mooi and D. S. Peeper (2008). "Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network." Cell **133**(6): 1019-1031.

Kunieda, T., T. Minamino, J. Nishi, K. Tateno, T. Oyama, T. Katsuno, H. Miyauchi, M. Orimo, S. Okada, M. Takamura, T. Nagai, S. Kaneko and I. Komuro (2006). "Angiotensin II induces premature senescence of vascular smooth muscle cells and accelerates the development of atherosclerosis via a p21-dependent pathway." Circulation **114**(9): 953-960.

Kunzelmann-Marche, C., J. M. Freyssinet and M. C. Martinez (2002). "Loss of plasma membrane phospholipid asymmetry requires raft integrity. Role of transient receptor potential channels and ERK pathway." J Biol Chem **277**(22): 19876-19881.

Kurz, D. J., S. Decary, Y. Hong, E. Trivier, A. Akhmedov and J. D. Erusalimsky (2004). "Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells." J Cell Sci **117**(Pt 11): 2417-2426.

Lakatta, E. G. and D. Levy (2003). "Arterial and cardiac aging: major shareholders in cardiovascular disease enterprises: Part II: the aging heart in health: links to heart disease." Circulation **107**(2): 346-354.

Leeb-Lundberg, L. M., F. Marceau, W. Muller-Esterl, D. J. Pettibone and B. L. Zuraw (2005). "International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences." Pharmacol Rev **57**(1): 27-77.

Leguina-Ruzzi, A., J. Pereira, K. Pereira-Flores, J. P. Valderas, D. Mezzano, V. Velarde and C. G. Saez (2015). "Increased RhoA/Rho-Kinase Activity and Markers of Endothelial Dysfunction in Young Adult Subjects with Metabolic Syndrome." Metab Syndr Relat Disord **13**(9): 373-380.

Lei, J., Y. Vodovotz, E. Tzeng and T. R. Billiar (2013). "Nitric oxide, a protective molecule in the cardiovascular system." Nitric Oxide **35**: 175-185.

Lei, S., H. Li, J. Xu, Y. Liu, X. Gao, J. Wang, K. F. Ng, W. B. Lau, X. L. Ma, B. Rodrigues, M. G. Irwin and Z. Xia (2013). "Hyperglycemia-induced protein kinase C beta2 activation induces diastolic cardiac dysfunction in diabetic rats by impairing caveolin-3 expression and Akt/eNOS signaling." Diabetes **62**(7): 2318-2328.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart and J. A. Hoffmann (1996). "The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults." Cell **86**(6): 973-983.

Lemoinne, S., D. Thabut, C. Housset, R. Moreau, D. Valla, C. M. Boulanger and P. E. Rautou (2014). "The emerging roles of microvesicles in liver diseases." Nat Rev Gastroenterol Hepatol **11**(6): 350-361.

Leo, C. H., A. Joshi, J. L. Hart and O. L. Woodman (2012). "Endothelium-dependent nitroxyl-mediated relaxation is resistant to superoxide anion scavenging and preserved in diabetic rat aorta." Pharmacol Res **66**(5): 383-391.

Lerman, A. and J. C. Burnett, Jr. (1992). "Intact and altered endothelium in regulation of vasomotion." Circulation **86**(6 Suppl): Iii12-19.

Lerman, L. O., A. Lerman (2007). "All oxidase roads lead to angiotensin, too." Arterioscler Thromb Vasc Biol **27(4)**: 703-704.

Lescale-Matys, L., J. Dyer, D. Scott, T. C. Freeman, E. M. Wright and S. P. Shirazi-Beechey (1993). "Regulation of the ovine intestinal Na+/glucose co-transporter (SGLT1) is dissociated from mRNA abundance." Biochem J **291 (Pt 2)**: 435-440.

Levy, M. Z., R. C. Allsopp, A. B. Futcher, C. W. Greider and C. B. Harley (1992). "Telomere end-replication problem and cell aging." J Mol Biol **225**(4): 951-960.

Lhermusier, T., H. Chap and B. Payrastre (2011). "Platelet membrane phospholipid asymmetry: from the characterization of a scramblase activity to the identification of an essential protein mutated in Scott syndrome." J Thromb Haemost **9**(10): 1883-1891.

Li, J., J. J. Wang and S. X. Zhang (2015). "NADPH oxidase 4-derived H2O2 promotes aberrant retinal neovascularization via activation of VEGF receptor 2 pathway in oxygen-induced retinopathy." J Diabetes Res **2015**: 963289.

Li, S., J. Crothers, C. M. Haqq and E. H. Blackburn (2005). "Cellular and gene expression responses involved in the rapid growth inhibition of human cancer cells by RNA interference-mediated depletion of telomerase RNA." J Biol Chem **280**(25): 23709-23717.

Li, Y. and T. Sato (2001). "Dual signaling via protein kinase C and phosphatidylinositol 3'-kinase/Akt contributes to bradykinin B2 receptor-induced cardioprotection in guinea pig hearts." J Mol Cell Cardiol **33**(11): 2047-2053.

Lin, S. C., Y. C. Lo and H. Wu (2010). "Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling." Nature **465**(7300): 885-890.

Liu, Y. H., Y. You, T. Song, S. J. Wu and L. Y. Liu (2007). "Impairment of endothelium-dependent relaxation of rat aortas by homocysteine thiolactone and attenuation by captopril." J Cardiovasc Pharmacol **50**(2): 155-161.

Lopez-Otin, C., M. A. Blasco, L. Partridge, M. Serrano and G. Kroemer (2013). "The hallmarks of aging." Cell **153**(6): 1194-1217.

Loppnow, H., H. Brade, I. Durrbaum, C. A. Dinarello, S. Kusumoto, E. T. Rietschel and H. D. Flad (1989). "IL-1 induction-capacity of defined lipopolysaccharide partial structures." J Immunol **142**(9): 3229-3238.

Lorenz, M. W., H. S. Markus, M. L. Bots, M. Rosvall and M. Sitzer (2007). "Prediction of clinical cardiovascular events with carotid intima-media thickness: a systematic review and meta-analysis." Circulation **115**(4): 459-467.

Lorenzi, M. and E. Cagliero (1991). "Pathobiology of endothelial and other vascular cells in diabetes mellitus. Call for data." Diabetes **40**(6): 653-659.

Luckhoff, A., R. Busse, I. Winter and E. Bassenge (1987). "Characterization of vascular relaxant factor released from cultured endothelial cells." Hypertension **9**(3): 295-303.

MacKenzie, A., E. J. Cooper and F. J. Dowell (2008). "Differential effects of glucose on agonist-induced relaxations in human mesenteric and subcutaneous arteries." Br J Pharmacol **153**(3): 480-487.

MacKenzie, A., H. L. Wilson, E. Kiss-Toth, S. K. Dower, R. A. North and A. Surprenant (2001). "Rapid secretion of interleukin-1beta by microvesicle shedding." Immunity **15**(5): 825-835.

MacNeal, W. J. (1929). "The circulation of blood through the spleen pulp." Arch. Pathol **7**: 215–227.

Madeddu, P., C. Emanueli and S. El-Dahr (2007). "Mechanisms of disease: the tissue kallikrein-kinin system in hypertension and vascular remodeling." Nat Clin Pract Nephrol **3**(4): 208-221.

Mallat, Z., H. Benamer, B. Hugel, J. Benessiano, P. G. Steg, J. M. Freyssinet and A. Tedgui (2000). "Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes." Circulation **101**(8): 841-843.

Mamane, Y., E. Petroulakis, O. LeBacquer and N. Sonenberg (2006). "mTOR, translation initiation and cancer." Oncogene **25**(48): 6416-6422.

Marijic, J., Q. Li, M. Song, K. Nishimaru, E. Stefani and L. Toro (2001). "Decreased expression of voltage- and Ca(2+)-activated K(+) channels in coronary smooth muscle during aging." Circ Res **88**(2): 210-216.

Martin, M. G., J. Wang, R. S. Solorzano-Vargas, J. T. Lam, E. Turk and E. M. Wright (2000). "Regulation of the human Na(+)-glucose cotransporter gene, SGLT1, by HNF-1 and Sp1." Am J Physiol Gastrointest Liver Physiol **278**(4): G591-603.

Martinez, M. C. and J. M. Freyssinet (2001). "Deciphering the plasma membrane hallmarks of apoptotic cells: phosphatidylserine transverse redistribution and calcium entry." BMC Cell Biol **2**(20): 17.

Mastronardi, M. L., H. A. Mostefai, F. Meziani, M. C. Martinez, P. Asfar and R. Andriantsitohaina (2011). "Circulating microparticles from septic shock patients exert differential tissue expression of enzymes related to inflammation and oxidative stress." Crit Care Med **39**(7): 1739-1748.

Matsui-Hirai, H., T. Hayashi, S. Yamamoto, K. Ina, M. Maeda, H. Kotani, A. Iguchi, L. J. Ignarro and Y. Hattori (2011). "Dose-dependent modulatory effects of insulin on glucose-induced endothelial senescence in vitro and in vivo: a relationship between telomeres and nitric oxide." J Pharmacol Exp Ther **337**(3): 591-599.

Matsumoto, K., R. Morishita, N. Tomita, A. Moriguchi, K. Yamasaki, M. Aoki, K. Matsumoto, T. Nakamura, J. Higaki and T. Ogihara (2002). "Impaired endothelial dysfunction in diabetes mellitus rats was restored by oral administration of prostaglandin I2 analogue." J Endocrinol **175**(1): 217-223.

Matsumoto, S., M. Shimabukuro, D. Fukuda, T. Soeki, K. Yamakawa, H. Masuzaki and M. Sata (2014). "Azilsartan, an angiotensin II type 1 receptor blocker, restores endothelial

function by reducing vascular inflammation and by increasing the phosphorylation ratio Ser(1177)/Thr(497) of endothelial nitric oxide synthase in diabetic mice." Cardiovasc Diabetol **13**: 30.

Matsushita, H., E. Chang, A. J. Glassford, J. P. Cooke, C. P. Chiu and P. S. Tsao (2001). "eNOS activity is reduced in senescent human endothelial cells: Preservation by hTERT immortalization." Circ Res **89**(9): 793-798.

Matz, R. L., C. Schott, J. C. Stoclet and R. Andriantsitohaina (2000). "Age-related endothelial dysfunction with respect to nitric oxide, endothelium-derived hyperpolarizing factor and cyclooxygenase products." Physiol Res **49**(1): 11-18.

McEachern, M. J., A. Krauskopf and E. H. Blackburn (2000). "Telomeres and their control." Annu Rev Genet **34**: 331-358.

Mebius, R. E. and G. Kraal (2005). "Structure and function of the spleen." Nat Rev Immunol **5**(8): 606-616.

Medzhitov, R. and C. Janeway, Jr. (2000). "Innate immunity." N Engl J Med **343**(5): 338-344.

Mesri, M. and D. C. Altieri (1999). "Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway." J Biol Chem **274**(33): 23111-23118.

Mezentsev, A., R. M. Merks, E. O'Riordan, J. Chen, N. Mendelev, M. S. Goligorsky and S. V. Brodsky (2005). "Endothelial microparticles affect angiogenesis in vitro: role of oxidative stress." Am J Physiol Heart Circ Physiol **289**(3): H1106-1114.

Minami, T. and W. C. Aird (2005). "Endothelial cell gene regulation." Trends Cardiovasc Med **15**(5): 174-184.

Minamino, T. and I. Komuro (2002). "Role of telomere in endothelial dysfunction in atherosclerosis." Curr Opin Lipidol **13**(5): 537-543.

Minamino, T., H. Miyauchi, T. Yoshida, Y. Ishida, H. Yoshida and I. Komuro (2002). "Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction." Circulation **105**(13): 1541-1544.

Minamino, T., H. Miyauchi, T. Yoshida, K. Tateno and I. Komuro (2004). "The role of vascular cell senescence in atherosclerosis: antisenescence as a novel therapeutic strategy for vascular aging." Curr Vasc Pharmacol **2**(2): 141-148.

Miquel, J. (1998). "An update on the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications." Exp Gerontol **33**(1-2): 113-126.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." Cell **80**(2): 293-299.

Moncada, S. and E. A. Higgs (2006). "Nitric oxide and the vascular endothelium." Handb Exp Pharmacol **176**(1): 213-254.

Moncada, S., R. M. Palmer and E. A. Higgs (1991). "Nitric oxide: physiology, pathophysiology, and pharmacology." Pharmacol Rev **43**(2): 109-142.

Montminy, S. W., N. Khan, S. McGrath, M. J. Walkowicz, F. Sharp, J. E. Conlon, K. Fukase, S. Kusumoto, C. Sweet, K. Miyake, S. Akira, R. J. Cotter, J. D. Goguen and E. Lien (2006). "Virulence factors of Yersinia pestis are overcome by a strong lipopolysaccharide response." Nat Immunol **7**(10): 1066-1073.

Moreau, F., F. Toti, F. Bayle, T. Berney, H. Egelhofer, M. Chastre, M. J. Richard, M. Greget, D. Masson, F. Zobairi, P. Y. Benhamou, L. Kessler and G. group (2012). "Rescue of a pancreatic islet graft after steroid therapy." Transplantation **93**(3): e10-11.

Morel, O., B. Hugel, L. Jesel, F. Lanza, M. P. Douchet, M. Zupan, M. Chauvin, J. P. Cazenave, J. M. Freyssinet and F. Toti (2004). "Sustained elevated amounts of circulating procoagulant membrane microparticles and soluble GPV after acute myocardial infarction in diabetes mellitus." Thromb Haemost **91**(2): 345-353.

Morel, O., L. Jesel, J. M. Freyssinet and F. Toti (2011). "Cellular mechanisms underlying the formation of circulating microparticles." Arterioscler Thromb Vasc Biol **31**(1): 15-26.

Morel, O., N. Morel, J. M. Freyssinet and F. Toti (2008). "Platelet microparticles and vascular cells interactions: a checkpoint between the haemostatic and thrombotic responses." Platelets **19**(1): 9-23.

Morel, O., F. Toti, B. Hugel, B. Bakouboula, L. Camoin-Jau, F. Dignat-George and J. M. Freyssinet (2006). "Procoagulant microparticles: disrupting the vascular homeostasis equation?" Arterioscler Thromb Vasc Biol **26**(12): 2594-2604.

Mori, A., S. Suzuki, K. Sakamoto, T. Nakahara and K. Ishii (2011). "Role of calcium-activated potassium channels in acetylcholine-induced vasodilation of rat retinal arterioles in vivo." Naunyn Schmiedebergs Arch Pharmacol **383**(1): 27-34.

Morisato, D. and K. V. Anderson (1995). "Signaling pathways that establish the dorsal-ventral pattern of the Drosophila embryo." Annu Rev Genet **29**: 371-399.

Mostefai, H. A., A. Agouni, N. Carusio, M. L. Mastronardi, C. Heymes, D. Henrion, R. Andriantsitohaina and M. C. Martinez (2008). "Phosphatidylinositol 3-kinase and xanthine oxidase regulate nitric oxide and reactive oxygen species productions by apoptotic lymphocyte microparticles in endothelial cells." J Immunol **180**(7): 5028-5035.

Motshwene, P. G., M. C. Moncrieffe, J. G. Grossmann, C. Kao, M. Ayaluru, A. M. Sandercock, C. V. Robinson, E. Latz and N. J. Gay (2009). "An oligomeric signaling platform formed by the Toll-like receptor signal transducers MyD88 and IRAK-4." J Biol Chem **284**(37): 25404-25411.

Muller-Loennies, S., B. Lindner and H. Brade (2002). "Structural analysis of deacylated lipopolysaccharide of Escherichia coli strains 2513 (R4 core-type) and F653 (R3 core-type)." Eur J Biochem **269**(23): 5982-5991.

Muller-Loennies, S., B. Lindner and H. Brade (2003). "Structural analysis of oligosaccharides from lipopolysaccharide (LPS) of Escherichia coli K12 strain W3100 reveals a link between inner and outer core LPS biosynthesis." J Biol Chem **278**(36): 34090-34101.

Munnix, I. C., M. Harmsma, J. C. Giddings, P. W. Collins, M. A. Feijge, P. Comfurius, J. W. Heemskerk and E. M. Bevers (2003). "Store-mediated calcium entry in the regulation of phosphatidylserine exposure in blood cells from Scott patients." Thromb Haemost **89**(4): 687-695.

Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto and K. Miyake (2002). "Essential role of MD-2 in LPS responsiveness and TLR4 distribution." Nat Immunol **3**(7): 667-672.

Nakamura, N., T. Matsui, Y. Ishibashi and S. Yamagishi (2015). "Insulin stimulates SGLT2-mediated tubular glucose absorption via oxidative stress generation." Diabetol Metab Syndr **7**: 48.

Narita, M., S. Nunez, E. Heard, M. Narita, A. W. Lin, S. A. Hearn, D. L. Spector, G. J. Hannon and S. W. Lowe (2003). "Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence." Cell **113**(6): 703-716.

Natali, A., E. Toschi, S. Baldeweg, D. Ciociaro, S. Favilla, L. Sacca and E. Ferrannini (2006). "Clustering of insulin resistance with vascular dysfunction and low-grade inflammation in type 2 diabetes." Diabetes **55**(4): 1133-1140.

Navarro-Gonzalez, J. F. and C. Mora-Fernandez (2008). "The role of inflammatory cytokines in diabetic nephropathy." J Am Soc Nephrol **19**(3): 433-442.

Neri, T., C. Armani, A. Pegoli, C. Cordazzo, Y. Carmazzi, S. Brunelleschi, C. Bardelli, M. C. Breschi, P. Paggiaro and A. Celi (2011). "Role of NF-kappaB and PPAR-gamma in lung inflammation induced by monocyte-derived microparticles." Eur Respir J **37**(6): 1494-1502.

Nishio, K., A. Inoue, S. Qiao, H. Kondo and A. Mimura (2001). "Senescence and cytoskeleton: overproduction of vimentin induces senescent-like morphology in human fibroblasts." Histochem Cell Biol **116**(4): 321-327.

Nomura, S., A. Shouzu, S. Omoto, M. Nishikawa, T. Iwasaka and S. Fukuhara (2004). "Activated platelet and oxidized LDL induce endothelial membrane vesiculation: clinical significance of endothelial cell-derived microparticles in patients with type 2 diabetes." Clin Appl Thromb Hemost **10**(3): 205-215.

O'Neill, L. A., C. E. Bryant and S. L. Doyle (2009). "Therapeutic targeting of Toll-like receptors for infectious and inflammatory diseases and cancer." Pharmacol Rev **61**(2): 177-197.

Oates, J. A., G. A. FitzGerald, R. A. Branch, E. K. Jackson, H. R. Knapp and L. J. Roberts, 2nd (1988). "Clinical implications of prostaglandin and thromboxane A2 formation (1)." N Engl J Med **319**(11): 689-698.

Ohto, U., K. Fukase, K. Miyake and Y. Satow (2007). "Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa." Science **316**(5831): 1632-1634.

Omae, T., T. Nagaoka, I. Tanano and A. Yoshida (2013). "Adiponectin-induced dilation of isolated porcine retinal arterioles via production of nitric oxide from endothelial cells." Invest Ophthalmol Vis Sci **54**(7): 4586-4594.

Omoto, S., S. Nomura, A. Shouzu, M. Nishikawa, S. Fukuhara and T. Iwasaka (2002). "Detection of monocyte-derived microparticles in patients with Type II diabetes mellitus." Diabetologia **45**(4): 550-555.

Ong, P., A. Athanasiadis, G. Borgulya, I. Vokshi, R. Bastiaenen, S. Kubik, S. Hill, T. Schaufele, H. Mahrholdt, J. C. Kaski and U. Sechtem (2014). "Clinical usefulness, angiographic characteristics, and safety evaluation of intracoronary acetylcholine provocation testing among 921 consecutive white patients with unobstructed coronary arteries." Circulation **129**(17): 1723-1730.

Orskov, F. and I. Orskov (1992). "Escherichia coli serotyping and disease in man and animals." Can J Microbiol **38**(7): 699-704.

Osorio, F. G., C. Barcena, C. Soria-Valles, A. J. Ramsay, F. de Carlos, J. Cobo, A. Fueyo, J. M. Freije and C. Lopez-Otin (2012). "Nuclear lamina defects cause ATM-dependent NF-kappaB activation and link accelerated aging to a systemic inflammatory response." Genes Dev **26**(20): 2311-2324.

Ota, H., M. Eto, M. R. Kano, S. Ogawa, K. Iijima, M. Akishita and Y. Ouchi (2008). "Cilostazol inhibits oxidative stress-induced premature senescence via upregulation of Sirt1 in human endothelial cells." Arterioscler Thromb Vasc Biol **28**(9): 1634-1639.

Owens, A. P., 3rd and N. Mackman (2011). "Microparticles in hemostasis and thrombosis." Circ Res **108**(10): 1284-1297.

Owens, A. P., 3rd, F. H. Passam, S. Antoniak, S. M. Marshall, A. L. McDaniel, L. Rudel, J. C. Williams, B. K. Hubbard, J. A. Dutton, J. Wang, P. S. Tobias, L. K. Curtiss, A. Daugherty, D. Kirchhofer, J. P. Luyendyk, P. M. Moriarty, S. Nagarajan, B. C. Furie, B. Furie, D. G. Johns, R. E. Temel and N. Mackman (2012). "Monocyte tissue factor-dependent activation of coagulation in hypercholesterolemic mice and monkeys is inhibited by simvastatin." J Clin Invest **122**(2): 558-568.

Pacher, P., J. S. Beckman and L. Liaudet (2007). "Nitric oxide and peroxynitrite in health and disease." Physiol Rev **87**(1): 315-424.

Palmer, R. M., A. G. Ferrige and S. Moncada (1987). "Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor." Nature **327**(6122): 524-526.

Park, B. S., D. H. Song, H. M. Kim, B. S. Choi, H. Lee and J. O. Lee (2009). "The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex." Nature **458**(7242): 1191-1195.

Park, Y., S. Capobianco, X. Gao, J. R. Falck, K. C. Dellsperger and C. Zhang (2008). "Role of EDHF in type 2 diabetes-induced endothelial dysfunction." Am J Physiol Heart Circ Physiol **295**(5): 12.

Parry, D., S. Bates, D. J. Mann and G. Peters (1995). "Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product." Embo j **14**(3): 503-511.

Patel, A., J. Chalmers and N. Poulter (2005). "ADVANCE: action in diabetes and vascular disease." J Hum Hypertens **19 Suppl 1**: S27-32.

Peng, X., R. Chen, Y. Wu, B. Huang, C. Tang, J. Chen, Q. Wang, Q. Wu, J. Yang, H. Qiu and Q. Jiang (2015). "PPARgamma-PI3K/AKT-NO signal pathway is involved in cardiomyocyte hypertrophy induced by high glucose and insulin." J Diabetes Complications **29**(6): 755-760.

Picard, B., J. S. Garcia, S. Gouriou, P. Duriez, N. Brahimi, E. Bingen, J. Elion and E. Denamur (1999). "The link between phylogeny and virulence in Escherichia coli extraintestinal infection." Infect Immun **67**(2): 546-553.

Plociennikowska, A., A. Hromada-Judycka, K. Borzecka and K. Kwiatkowska (2015). "Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling." Cell Mol Life Sci **72**(3): 557-581.

Poittevin, M., P. Bonnin, C. Pimpie, L. Riviere, C. Sebrie, A. Dohan, M. Pocard, C. Charriaut-Marlangue and N. Kubis (2015). "Diabetic microangiopathy: impact of impaired cerebral vasoreactivity and delayed angiogenesis after permanent middle cerebral artery occlusion on stroke damage and cerebral repair in mice." Diabetes **64**(3): 999-1010.

Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, M. Freudenberg, P. Ricciardi-Castagnoli, B. Layton and B. Beutler (1998). "Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene." Science **282**(5396): 2085-2088.

Poppe, R., U. Karbach, S. Gambaryan, H. Wiesinger, M. Lutzenburg, M. Kraemer, O. W. Witte and H. Koepsell (1997). "Expression of the Na+-D-glucose cotransporter SGLT1 in neurons." J Neurochem **69**(1): 84-94.

Pugsley, M. K. and R. Tabrizchi (2000). "The vascular system. An overview of structure and function." J Pharmacol Toxicol Methods **44**(2): 333-340.

Raetz, C. R., C. M. Reynolds, M. S. Trent and R. E. Bishop (2007). "Lipid A modification systems in gram-negative bacteria." Annu Rev Biochem **76**: 295-329.

Raetz, C. R. and C. Whitfield (2002). "Lipopolysaccharide endotoxins." Annu Rev Biochem **71**: 635-700.

Rahmoune, H., P. W. Thompson, J. M. Ward, C. D. Smith, G. Hong and J. Brown (2005). "Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes." Diabetes **54**(12): 3427-3434.

Rautou, P. E., A. C. Vion, N. Amabile, G. Chironi, A. Simon, A. Tedgui and C. M. Boulanger (2011). "Microparticles, vascular function, and atherothrombosis." Circ Res **109**(5): 593-606.

Rautou, P. E., A. C. Vion, D. Valla and C. M. Boulanger (2013). "Circulating platelet derived microparticles are not increased in patients with cirrhosis." J Hepatol **59**(4): 912.

Resman, N., J. Vasl, A. Oblak, P. Pristovsek, T. L. Gioannini, J. P. Weiss and R. Jerala (2009). "Essential roles of hydrophobic residues in both MD-2 and toll-like receptor 4 in activation by endotoxin." J Biol Chem **284**(22): 15052-15060.

Ridger, V. C., C. M. Boulanger, A. Angelillo-Scherrer, L. Badimon, O. Blanc-Brude, M. L. Bochaton-Piallat, E. Boilard, E. I. Buzas, A. Caporali, F. Dignat-George, P. C. Evans, R. Lacroix, E. Lutgens, D. F. J. Ketelhuth, R. Nieuwland, F. Toti, J. Tunon, C. Weber and I. E.

Hoefer (2017). "Microvesicles in vascular homeostasis and diseases. Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology." Thromb Haemost **117**(7): 1296-1316.

Rietschel, E. T., T. Kirikae, F. U. Schade, U. Mamat, G. Schmidt, H. Loppnow, A. J. Ulmer, U. Zahringer, U. Seydel, F. Di Padova and et al. (1994). "Bacterial endotoxin: molecular relationships of structure to activity and function." Faseb J **8**(2): 217-225.

Rojas, A. and M. A. Morales (2004). "Advanced glycation and endothelial functions: a link towards vascular complications in diabetes." Life Sci **76**(7): 715-730.

Romani, L. (2004). "Immunity to fungal infections." Nat Rev Immunol **4**(1): 1-23.

Roy, S., D. Kim, C. Hernandez, R. Simo and S. Roy (2015). "Beneficial effects of fenofibric acid on overexpression of extracellular matrix components, COX-2, and impairment of endothelial permeability associated with diabetic retinopathy." Exp Eye Res **140**: 124-129.

Sabatier, F., L. Camoin-Jau, F. Anfosso, J. Sampol and F. Dignat-George (2009). "Circulating endothelial cells, microparticles and progenitors: key players towards the definition of vascular competence." J Cell Mol Med **13**(3): 454-471.

Sabatier, F., P. Darmon, B. Hugel, V. Combes, M. Sanmarco, J. G. Velut, D. Arnoux, P. Charpiot, J. M. Freyssinet, C. Oliver, J. Sampol and F. Dignat-George (2002). "Type 1 and type 2 diabetic patients display different patterns of cellular microparticles." Diabetes **51**(9): 2840-2845.

Sachidanandam, K., J. R. Hutchinson, M. M. Elgebaly, E. M. Mezzetti, A. M. Dorrance, K. Motamed and A. Ergul (2009). "Glycemic control prevents microvascular remodeling and increased tone in type 2 diabetes: link to endothelin-1." Am J Physiol Regul Integr Comp Physiol **296**(4): R952-959.

Salheen, S. M., U. Panchapakesan, C. A. Pollock and O. L. Woodman (2015). "The DPP-4 inhibitor linagliptin and the GLP-1 receptor agonist exendin-4 improve endothelium-dependent relaxation of rat mesenteric arteries in the presence of high glucose." Pharmacol Res **94**: 26-33.

Salminen, A., K. Kaarniranta and A. Kauppinen (2012). "Inflammaging: disturbed interplay between autophagy and inflammasomes." Aging (Albany NY) **4**(3): 166-175.

Samani, N. J., R. Boultby, R. Butler, J. R. Thompson and A. H. Goodall (2001). "Telomere shortening in atherosclerosis." Lancet **358**(9280): 472-473.

Sampson, M. J., M. S. Winterbone, J. C. Hughes, N. Dozio and D. A. Hughes (2006). "Monocyte telomere shortening and oxidative DNA damage in type 2 diabetes." Diabetes Care **29**(2): 283-289.

Santer, R. and J. Calado (2010). "Familial renal glucosuria and SGLT2: from a mendelian trait to a therapeutic target." Clin J Am Soc Nephrol **5**(1): 133-141.

Santilli, F., M. Marchisio, P. Lanuti, A. Boccatonda, S. Miscia and G. Davi (2016). "Microparticles as new markers of cardiovascular risk in diabetes and beyond." Thromb Haemost **116**(2): 220-234.

Sato, I., K. Kaji, I. Morita, M. Nagao and S. Murota (1993). "Augmentation of endothelin-1, prostacyclin and thromboxane A2 secretion associated with in vitro ageing in cultured human umbilical vein endothelial cells." Mech Ageing Dev **71**(1-2): 73-84.

Scanu, A., N. Molnarfi, K. J. Brandt, L. Gruaz, J. M. Dayer and D. Burger (2008). "Stimulated T cells generate microparticles, which mimic cellular contact activation of human monocytes: differential regulation of pro- and anti-inflammatory cytokine production by high-density lipoproteins." J Leukoc Biol **83**(4): 921-927.

Schafer, K. A. (1998). "The cell cycle: a review." Vet Pathol **35**(6): 461-478.

Schini, V. B., C. Boulanger, D. Regoli and P. M. Vanhoutte (1990). "Bradykinin stimulates the production of cyclic GMP via activation of B2 kinin receptors in cultured porcine aortic endothelial cells." J Pharmacol Exp Ther **252**(2): 581-585.

Schmidt, A. M., S. D. Yan and D. M. Stern (1995). "The dark side of glucose." Nat Med **1**(10): 1002-1004.

Schmidt, A. M., S. D. Yan, J. L. Wautier and D. Stern (1999). "Activation of receptor for advanced glycation end products: a mechanism for chronic vascular dysfunction in diabetic vasculopathy and atherosclerosis." Circ Res **84**(5): 489-497.

Segawa, K., S. Kurata, Y. Yanagihashi, T. R. Brummelkamp, F. Matsuda and S. Nagata (2014). "Caspase-mediated cleavage of phospholipid flippase for apoptotic phosphatidylserine exposure." Science **344**(6188): 1164-1168.

Seki, T., K. Goto, K. Kiyohara, Y. Kansui, N. Murakami, Y. Haga, T. Ohtsubo, K. Matsumura and T. Kitazono (2017). "Downregulation of Endothelial Transient Receptor Potential Vanilloid Type 4 Channel and Small-Conductance of Ca2+-Activated K+ Channels Underpins Impaired Endothelium-Dependent Hyperpolarization in Hypertension." Hypertension **69**(1): 143-153.

Selander, R. K., D. A. Caugant, H. Ochman, J. M. Musser, M. N. Gilmour and T. S. Whittam (1986). "Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics." Appl Environ Microbiol **51**(5): 873-884.

Sessa, W. C., J. K. Harrison, C. M. Barber, D. Zeng, M. E. Durieux, D. D. D'Angelo, K. R. Lynch and M. J. Peach (1992). "Molecular cloning and expression of a cDNA encoding endothelial cell nitric oxide synthase." J Biol Chem **267**(22): 15274-15276.

Shan, H., D. Guo, X. Li, X. Zhao, W. Li and X. Bai (2014). "From autophagy to senescence and apoptosis in Angiotensin II-treated vascular endothelial cells." Apmis **122**(10): 985-992.

Shen, B., C. L. Ye, K. H. Ye and J. J. Liu (2003). "Mechanism underlying enhanced endothelium-dependent vasodilatation in thoracic aorta of early stage streptozotocin-induced diabetic mice." Acta Pharmacol Sin **24**(5): 422-428.

Shi, Y. and P. M. Vanhoutte (2009). "Reactive oxygen-derived free radicals are key to the endothelial dysfunction of diabetes." J Diabetes **1**(3): 151-162.

Shida, T., T. Nozawa, M. Sobajima, H. Ihori, A. Matsuki and H. Inoue (2014). "Fluvastatin-induced reduction of oxidative stress ameliorates diabetic cardiomyopathy in association with improving coronary microvasculature." Heart Vessels **29**(4): 532-541.

Shih, H., B. Lee, R. J. Lee and A. J. Boyle (2011). "The aging heart and post-infarction left ventricular remodeling." J Am Coll Cardiol **57**(1): 9-17.

Sikora, E., T. Arendt, M. Bennett and M. Narita (2011). "Impact of cellular senescence signature on ageing research." Ageing Res Rev **10**(1): 146-152.

Simmons, D. L., S. Tan, D. G. Tenen, A. Nicholson-Weller and B. Seed (1989). "Monocyte antigen CD14 is a phospholipid anchored membrane protein." Blood **73**(1): 284-289.

Sindler, A. L., M. D. Delp, R. Reyes, G. Wu and J. M. Muller-Delp (2009). "Effects of ageing and exercise training on eNOS uncoupling in skeletal muscle resistance arterioles." J Physiol **587**(Pt 15): 3885-3897.

Sinning, J. M., J. Losch, K. Walenta, M. Bohm, G. Nickenig and N. Werner (2011). "Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes." Eur Heart J **32**(16): 2034-2041.

Smith, A. R. and T. M. Hagen (2003). "Vascular endothelial dysfunction in aging: loss of Akt-dependent endothelial nitric oxide synthase phosphorylation and partial restoration by (R)-alpha-lipoic acid." Biochem Soc Trans **31**(Pt 6): 1447-1449.

Sohal, R. S., R. J. Mockett and W. C. Orr (2002). "Mechanisms of aging: an appraisal of the oxidative stress hypothesis." Free Radic Biol Med **33**(5): 575-586.

Stamler, J., O. Vaccaro, J. D. Neaton and D. Wentworth (1993). "Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the Multiple Risk Factor Intervention Trial." Diabetes Care **16**(2): 434-444.

Stein, G. H., L. F. Drullinger, A. Soulard and V. Dulic (1999). "Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts." Mol Cell Biol **19**(3): 2109-2117.

Steinberg, D. and J. L. Witztum (2002). "Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis?" Circulation **105**(17): 2107-2111.

Steiniger, B. and P. Barth (2000). "Microanatomy and function of the spleen." Adv Anat Embryol Cell Biol **151**: 1-101.

Tabit, C. E., W. B. Chung, N. M. Hamburg and J. A. Vita (2010). "Endothelial dysfunction in diabetes mellitus: molecular mechanisms and clinical implications." Rev Endocr Metab Disord **11**(1): 61-74.

Taddei, S., A. Virdis, L. Ghiadoni, G. Salvetti, G. Bernini, A. Magagna and A. Salvetti (2001). "Age-related reduction of NO availability and oxidative stress in humans." Hypertension **38**(2): 274-279.

Taddei, S., A. Virdis, P. Mattei, L. Ghiadoni, A. Gennari, C. B. Fasolo, I. Sudano and A. Salvetti (1995). "Aging and endothelial function in normotensive subjects and patients with essential hypertension." Circulation **91**(7): 1981-1987.

Tan, K. C., W. S. Chow, V. H. Ai, C. Metz, R. Bucala and K. S. Lam (2002). "Advanced glycation end products and endothelial dysfunction in type 2 diabetes." Diabetes Care **25**(6): 1055-1059.

Taylor, P. D. and L. Poston (1994). "The effect of hyperglycaemia on function of rat isolated mesenteric resistance artery." Br J Pharmacol **113**(3): 801-808.

Terrisse, A. D., N. Puech, S. Allart, P. Gourdy, J. M. Xuereb, B. Payrastre and P. Sie (2010). "Internalization of microparticles by endothelial cells promotes platelet/endothelial cell interaction under flow." J Thromb Haemost **8**(12): 2810-2819.

Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson and K. Fukuda (2003). "Mortality associated with influenza and respiratory syncytial virus in the United States." Jama **289**(2): 179-186.

Tian, J., X. Gong and Z. Xie (2001). "Signal-transducing function of Na+-K+-ATPase is essential for ouabain's effect on [Ca2+]i in rat cardiac myocytes." Am J Physiol Heart Circ Physiol **281**(5): H1899-1907.

Tilstra, J. S., A. R. Robinson, J. Wang, S. Q. Gregg, C. L. Clauson, D. P. Reay, L. A. Nasto, C. M. St Croix, A. Usas, N. Vo, J. Huard, P. R. Clemens, D. B. Stolz, D. C. Guttridge, S. C. Watkins, G. A. Garinis, Y. Wang, L. J. Niedernhofer and P. D. Robbins (2012). "NF-kappaB inhibition delays DNA damage-induced senescence and aging in mice." J Clin Invest **122**(7): 2601-2612.

Ting, H. H., F. K. Timimi, K. S. Boles, S. J. Creager, P. Ganz and M. A. Creager (1996). "Vitamin C improves endothelium-dependent vasodilation in patients with non-insulin-dependent diabetes mellitus." J Clin Invest **97**(1): 22-28.

Toti, F., F. Bayle, T. Berney, H. Egelhofer, M. J. Richard, M. Greget, D. Masson, F. Zobairi, P. Y. Benhamou and L. Kessler (2011). "Studies of circulating microparticle release in peripheral blood after pancreatic islet transplantation." Transplant Proc **43**(9): 3241-3245.

Toti, F. and J.-M. Freyssinet (2005). "An ABC for Scott syndrome?" Blood **106**(2): 396-a-397.

Toti, F., N. Satta, E. Fressinaud, D. Meyer and J. M. Freyssinet (1996). "Scott syndrome, characterized by impaired transmembrane migration of procoagulant phosphatidylserine and hemorrhagic complications, is an inherited disorder." Blood **87**(4): 1409-1415.

Trotta, T., C. Porro, R. Calvello and M. A. Panaro (2014). "Biological role of Toll-like receptor-4 in the brain." J Neuroimmunol **268**(1-2): 1-12.

Tsimerman, G., A. Roguin, A. Bachar, E. Melamed, B. Brenner and A. Aharon (2011). "Involvement of microparticles in diabetic vascular complications." Thromb Haemost **106**(2): 310-321.

Tual-Chalot, S., C. Guibert, B. Muller, J. P. Savineau, R. Andriantsitohaina and M. C. Martinez (2010). "Circulating microparticles from pulmonary hypertensive rats induce endothelial dysfunction." Am J Respir Crit Care Med **182**(2): 261-268.

Turk, E., C. J. Kerner, M. P. Lostao and E. M. Wright (1996). "Membrane topology of the human Na+/glucose cotransporter SGLT1." J Biol Chem **271**(4): 1925-1934.

Turner, M. L., G. D. Healey and I. M. Sheldon (2012). "Immunity and inflammation in the uterus." Reprod Domest Anim **4**: 402-409.

Valko, M., D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur and J. Telser (2007). "Free radicals and antioxidants in normal physiological functions and human disease." Int J Biochem Cell Biol **39**(1): 44-84.

Vallon, V. (2011). "The proximal tubule in the pathophysiology of the diabetic kidney." Am J Physiol Regul Integr Comp Physiol **300**(5): R1009-1022.

Vallon, V. and S. C. Thomson (2012). "Renal function in diabetic disease models: the tubular system in the pathophysiology of the diabetic kidney." Annu Rev Physiol **74**: 351-375.

van Hinsbergh, V. W. (2012). "Endothelium--role in regulation of coagulation and inflammation." Semin Immunopathol **34**(1): 93-106.

Vanhoutte, P. M. (2009). "Endothelial dysfunction: the first step toward coronary arteriosclerosis." Circ J **73**(4): 595-601.

Vanhoutte, P. M. (2009). "How We Learned to Say NO." Arterioscler Thromb Vasc Biol **29**(8): 1156-1160.

Vanhoutte, P. M., H. Shimokawa, E. H. Tang and M. Feletou (2009). "Endothelial dysfunction and vascular disease." Acta Physiol **196**(2): 193-222.

Vanhoutte, P. M. and E. H. Tang (2008). "Endothelium-dependent contractions: when a good guy turns bad!" J Physiol **586**(22): 5295-5304.

Vanhoutte, P. M., Y. Zhao, A. Xu and S. W. Leung (2016). "Thirty Years of Saying NO: Sources, Fate, Actions, and Misfortunes of the Endothelium-Derived Vasodilator Mediator." Circ Res **119**(2): 375-396.

Vayro, S., I. S. Wood, J. Dyer and S. P. Shirazi-Beechey (2001). "Transcriptional regulation of the ovine intestinal Na+/glucose cotransporter SGLT1 gene. Role of HNF-1 in glucose activation of promoter function." Eur J Biochem **268**(20): 5460-5470.

Vaziri, H., M. D. West, R. C. Allsopp, T. S. Davison, Y. S. Wu, C. H. Arrowsmith, G. G. Poirier and S. Benchimol (1997). "ATM-dependent telomere loss in aging human diploid fibroblasts and DNA damage lead to the post-translational activation of p53 protein involving poly(ADP-ribose) polymerase." Embo j **16**(19): 6018-6033.

Venkatesan, B., L. Mahimainathan, F. Das, N. Ghosh-Choudhury and G. Ghosh Choudhury (2007). "Downregulation of catalase by reactive oxygen species via PI 3 kinase/Akt signaling in mesangial cells." J Cell Physiol **211**(2): 457-467.

Verzola, D., M. T. Gandolfo, G. Gaetani, A. Ferraris, R. Mangerini, F. Ferrario, B. Villaggio, F. Gianiorio, F. Tosetti, U. Weiss, P. Traverso, M. Mji, G. Deferrari and G. Garibotto (2008). "Accelerated senescence in the kidneys of patients with type 2 diabetic nephropathy." Am J Physiol Renal Physiol **295**(5): F1563-1573.

Vessieres, E., A. L. Guihot, B. Toutain, M. Maquigneau, C. Fassot, L. Loufrani and D. Henrion (2013). "COX-2-derived prostanoids and oxidative stress additionally reduce endothelium-mediated relaxation in old type 2 diabetic rats." PLoS One **8**(7): e68217.

Vijg, J., A. Y. Maslov and Y. Suh (2008). "Aging: a sirtuin shake-up?" Cell **135**(5): 797-798.

Voghel, G., N. Thorin-Trescases, N. Farhat, A. Nguyen, L. Villeneuve, A. M. Mamarbachi, A. Fortier, L. P. Perrault, M. Carrier and E. Thorin (2007). "Cellular senescence in endothelial cells from atherosclerotic patients is accelerated by oxidative stress associated with cardiovascular risk factors." Mech Ageing Dev **128**(11-12): 662-671.

von Zglinicki, T., R. Pilger and N. Sitte (2000). "Accumulation of single-strand breaks is the major cause of telomere shortening in human fibroblasts." Free Radic Biol Med **28**(1): 64-74.

Wallace, S. S. (1994). "DNA damages processed by base excision repair: biological consequences." Int J Radiat Biol **66**(5): 579-589.

Wan, F. and M. J. Lenardo (2010). "The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives." Cell Res **20**(1): 24-33.

Wang, D., L. J. Borrego-Conde, J. R. Falck, K. K. Sharma, C. S. Wilcox and J. G. Umans (2003). "Contributions of nitric oxide, EDHF, and EETs to endothelium-dependent relaxation in renal afferent arterioles." Kidney Int **63**(6): 2187-2193.

Wang, G., W. Li, Q. Chen, Y. Jiang, X. Lu and X. Zhao (2015). "Hydrogen sulfide accelerates wound healing in diabetic rats." Int J Clin Exp Pathol **8**(5): 5097-5104.

Wang, J. C. and M. Bennett (2012). "Aging and atherosclerosis: mechanisms, functional consequences, and potential therapeutics for cellular senescence." Circ Res **111**(2): 245-259.

Wang, J. G., J. C. Williams, B. K. Davis, K. Jacobson, C. M. Doerschuk, J. P. Ting and N. Mackman (2011). "Monocytic microparticles activate endothelial cells in an IL-1beta-dependent manner." Blood **118**(8): 2366-2374.

Wang, M., G. Takagi, K. Asai, R. G. Resuello, F. F. Natividad, D. E. Vatner, S. F. Vatner and E. G. Lakatta (2003). "Aging increases aortic MMP-2 activity and angiotensin II in nonhuman primates." Hypertension **41**(6): 1308-1316.

Wang, X. and P. J. Quinn (2010). "Lipopolysaccharide: Biosynthetic pathway and structure modification." Prog Lipid Res **49**(2): 97-107.

Ward, J. F. (1994). "The complexity of DNA damage: relevance to biological consequences." Int J Radiat Biol **66**(5): 427-432.

Webley, K., J. A. Bond, C. J. Jones, J. P. Blaydes, A. Craig, T. Hupp and D. Wynford-Thomas (2000). "Posttranslational modifications of p53 in replicative senescence overlapping but distinct from those induced by DNA damage." Mol Cell Biol **20**(8): 2803-2808.

Wekesa, A. L., K. S. Cross, O. O'Donovan, J. F. Dowdall, O. O'Brien, M. Doyle, L. Byrne, J. P. Phelan, M. D. Ross, R. Landers and M. Harrison (2014). "Predicting carotid artery disease and plaque instability from cell-derived microparticles." Eur J Vasc Endovasc Surg **48**(5): 489-495.

Werner, E. and Z. Werb (2002). "Integrins engage mitochondrial function for signal transduction by a mechanism dependent on Rho GTPases." J Cell Biol **158**(2): 357-368.

Wheeler-Jones, C. P. (2008). "Regulation of endothelial prostacyclin synthesis by protease-activated receptors: mechanisms and significance." Pharmacol Rep **60**(1): 109-118.

Whitfield, C. and M. S. Trent (2014). "Biosynthesis and export of bacterial lipopolysaccharides." Annu Rev Biochem **83**: 99-128.

Wilkinson, S. G. (1996). "Bacterial lipopolysaccharides--themes and variations." Prog Lipid Res **35**(3): 283-343.

Williams, S. B., A. B. Goldfine, F. K. Timimi, H. H. Ting, M. A. Roddy, D. C. Simonson and M. A. Creager (1998). "Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo." Circulation **97**(17): 1695-1701.

Wolfs, J. L., P. Comfurius, J. T. Rasmussen, J. F. Keuren, T. Lindhout, R. F. Zwaal and E. M. Bevers (2005). "Activated scramblase and inhibited aminophospholipid translocase cause phosphatidylserine exposure in a distinct platelet fraction." Cell Mol Life Sci **62**(13): 1514-1525.

Wright, E. M., D. D. Loo and B. A. Hirayama (2011). "Biology of human sodium glucose transporters." Physiol Rev **91**(2): 733-794.

Wu, J., T. Akaike, K. Hayashida, Y. Miyamoto, T. Nakagawa, K. Miyakawa, W. Muller-Esterl and H. Maeda (2002). "Identification of bradykinin receptors in clinical cancer specimens and murine tumor tissues." Int J Cancer **98**(1): 29-35.

Wu, J., Z. Liang, J. Zhou, C. Zhong, W. Jiang, Y. Zhang and S. Zhang (2016). "Association of Biomarkers of Inflammation and Endothelial Dysfunction with Fasting and Postload Glucose Metabolism: A Population-Based Prospective Cohort Study Among Inner Mongolians in China." Can J Diabetes **40**(6): 509-514.

Xie, Q. W., H. J. Cho, J. Calaycay, R. A. Mumford, K. M. Swiderek, T. D. Lee, A. Ding, T. Troso and C. Nathan (1992). "Cloning and characterization of inducible nitric oxide synthase from mouse macrophages." Science **256**(5054): 225-228.

Xiong, S., G. Salazar, A. San Martin, M. Ahmad, N. Patrushev, L. Hilenski, R. R. Nazarewicz, M. Ma, M. Ushio-Fukai and R. W. Alexander (2010). "PGC-1 alpha serine 570 phosphorylation and GCN5-mediated acetylation by angiotensin II drive catalase down-regulation and vascular hypertrophy." J Biol Chem **285**(4): 2474-2487.

Yang, J., E. Chang, A. M. Cherry, C. D. Bangs, Y. Oei, A. Bodnar, A. Bronstein, C. P. Chiu and G. S. Herron (1999). "Human endothelial cell life extension by telomerase expression." J Biol Chem **274**(37): 26141-26148.

Yokoi, T., K. Fukuo, O. Yasuda, M. Hotta, J. Miyazaki, Y. Takemura, H. Kawamoto, H. Ichijo and T. Ogihara (2006). "Apoptosis signal-regulating kinase 1 mediates cellular senescence induced by high glucose in endothelial cells." Diabetes **55**(6): 1660-1665.

Yu, B. and S. D. Wright (1996). "Catalytic properties of lipopolysaccharide (LPS) binding protein. Transfer of LPS to soluble CD14." J Biol Chem **271**(8): 4100-4105.

Yusuf, S., J. B. Ostergren, H. C. Gerstein, M. A. Pfeffer, K. Swedberg, C. B. Granger, B. Olofsson, J. Probstfield and J. V. McMurray (2005). "Effects of candesartan on the development of a new diagnosis of diabetes mellitus in patients with heart failure." Circulation **112**(1): 48-53.

Zhao, Y., P. M. Vanhoutte and S. W. Leung (2015). "Vascular nitric oxide: Beyond eNOS." J Pharmacol Sci **129**(2): 83-94.

Zhong, W., G. Zou, J. Gu and J. Zhang (2010). "L-arginine attenuates high glucose-accelerated senescence in human umbilical vein endothelial cells." Diabetes Res Clin Pract **89**(1): 38-45.

Zhou, E., D. Qing and J. Li (2010). "Age-associated endothelial dysfunction in rat mesenteric arteries: roles of calcium-activated K(+) channels (K(ca))." Physiol Res **59**(4): 499-508.

Zhou, L., E. V. Cryan, M. R. D'Andrea, S. Belkowski, B. R. Conway and K. T. Demarest (2003). "Human cardiomyocytes express high level of Na+/glucose cotransporter 1 (SGLT1)." J Cell Biochem **90**(2): 339-346.

Zhuo, J. L. and X. C. Li (2011). "New insights and perspectives on intrarenal renin-angiotensin system: focus on intracrine/intracellular angiotensin II." Peptides **32**(7): 1551-1565.

Annexes

Scientific production

Publications

Altamimy R, Qureshi A, Amoura L, El Habhab A, Itawi H, Kassem M, Khemais S, Pollet B, Zobairi F, Auger C, Schini-Kerth V, Toti F. High glucose potentiates the pro-senescent effect of leukocyte-derived MPs in isolated ECs and in the vessel wall: Impact on endothelial-dependent control of the vascular tone., *submitted to PLOs one April 2018*

Ali El Habhab, **Raed Altamimy**, Malak Abbas, Mohamad Kassem, Guillaume Kreutter, Lamia Amoura, Sonia Khemais-Benkhiat, Fatiha Zobairi, Valérie B. Schini-Kerth, Laurence Kessler, Florence Toti. Significance of immune cell derived microparticles in ischemia reperfusion: Induction of endothelial senescence and vascular dysfunction. Submitted to J Cell Transplantation, under revision

A. Qureshi, **R. Altamimy**, A. El Habhab, L. Amoura, M. Kassem, S. Khemais, M. Farooq, H. Hasan, P. Sin-Hee, F. El-Ghazouani, C. Auger, L. Kessler, V. Schini-Kerth, F. Toti. 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. In preparation.

L. Amoura, **R. Altamimy**, H. Itawi, A. El Habhab, M. Kassem, H. Merdji, S Khemais-Benkhiat, AW. Qureshi, S. Park, Sahraoui, Schini-Kerth V, F. Meziani, Kessler L, Toti F. Tissue microparticles as methodological tools in the pharmacological assessment of thrombogenicity. In preparation.

Amoura l, Zobairi F, **Altamimy R**, Kassem M, El Habhab A, Meyer N, Kreutter G, Sahraoui S, Toti F, Kessler L. Circulating microvesicles of the detection of pancreatic islet dysfunction in transplanted patients : a prospective study. in preparation

Oral Communication

Altamimy R, Qureshi A, Amoura L, El Habhab A, Itawi H, Kassem M, Khemais S, Pollet B, Zobairi F, Auger C, Schini-Kerth V, Toti F. Leukocyte-derived microparticles exaggerate endothelial senescence and vascular dysfunction induced by high glucose. oral and poster presentation, Congress of Printemps de la Cardiologie Recherche Fondamentale & Clinique. Montpellier 4- 6 April 2018, round table.

A. Qureshi, **R. Altamimy**, A. El Habhab, L. Amoura, M. Kassem, S. Khemais, M. Farooq, H. Hasan, P. Sin-Hee, F. El-Ghazouani, C. Auger, L. Kessler, V. Schini-Kerth, F. Toti. 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. International Meeting On Ischemic Reperfusion Injury (IMIRT), Poitiers, 17-20th April 2018 .

Poster Communications

Altamimy R, Qureshi A, Amoura L, El Habhab A, Itawi H, Kassem M, Khemais S, Pollet B, Zobairi F, Auger C, Schini-Kerth V, Toti F. Leukocyte-derived microparticles exaggerate endothelial senescence and vascular dysfunction induced by high glucose. oral and poster presentation, Congress of Printemps de la Cardiologie Recherche Fondamentale & Clinique. Montpellier 4- 6 April 2018

UNIVERSITÉ DE STRASBOURG

Raed ALTAMIMY

Interactions between coronary artery endothelial cells and leukocyte MPs shed in response to E. coli lipopolysaccharide: In-vitro and ex-vivo studies of the impact of vascular ageing and of high glucose

Résumé

Les microparticules (MP) sont des vésicules de la membrane plasmique émises après stress cellulaire. Nous avons étudié le rôle des MPs leucocytaires extraites de la rate de rats comme marqueur du vieillissement et effecteurs de la senescence et de la dysfonction endothéliales induites par les fortes concentrations de glucose (HG). L'émission basale de MP augmente avec l'âge qui favorise leur génération en réponse au LPS ou au PMA/ionophore A23187 (MP_{LPS}, $MP_{PMA/I}$). Les MP de rats âgés mais pas de jeunes induisent la sénescence de cellules endothéliales primaires d'artères coronaires (AC) de porc. MP_{LPS} ou $MP_{PMA/I}$ de rats jeunes, mais pas MP_{CTL} (cellules non traitées) réduisent la relaxation dépendante de l'endothélium d'anneaux d'AC en réponse à la bradykinine avec sous-expression de eNOS, surexpression de COX2, ICAM-1, VCAM-1. HG favorise l'émission des MP de rate. Dans les AC en HG, la vasoconstriction en réponse au U46619l est diminuée de manière dépendante du SGLT1/2 et de l'EDHF.

Microvesicules tissulaires, microparticules tissulaires, sénescence et dysfonction endothéliale.

Résumé en anglais

Microparticles (MP) are plasma membrane vesicles shed from stimulated cells. We investigated whether leukocyte MP extracted from rat spleen are reliable markers of aging and effectors of high glucose (HG)-induced endothelial senescence and dysfunction. Data indicate that ageing enhances MP shedding from spleen cells of middle-age and aged rats and raises MP release in response to LPS, or to PMA and ionophore A23187. Of note, MP from aged but not young rats induced senescence of porcine coronary artery primary endothelial cells. In young rats, MP_{LPS}, $MP_{PMA/I}$ but not from resting cells (MP_{CTL}) reduced the endothelial-dependent relaxation of coronary artery rings (CAR) in response to bradykinin with down-regulation of eNOS, up-regulation of COX-2, ICAM-1, VCAM-1. HG enhanced early and late MP release from spleen cells. Prolonged exposure to HG potentiated endothelial dysfunction in CAR and altered vasoconstriction in response to U46619l in a SGLT1/2 and EDHF dependent manner.

Tissue microvesicles, tissular microparticles, endothelial dysfunction and senescence