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Impact de l'inflammation parodontale sur l'axe splénique cardiaque : étude des Neutrophils Extracellular Traps (NETs) et des microvésicules spléniques comme médiateurs des réponses procoagulantes et inflammatoires

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To my Grand-father

To my Family

To Joumana

Aknowledgements

"Through patience, great things are accomplished" Imam Ali (AS)

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Hanine El Itawi
Strasbourg, France

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LIST OF ABBREVIATIONS

ANO6	Anoctamine 6
ATP	Adenosine Tri-Phosphate
ADP	Adenosine di-phosphate
C12FDG	5-dodecanoylaminoFluoresein Di-β-D Galactopyranoside
CD	Cluster Differentiation
Chol	Cholesterol
C5	Convertase like enzyme
CHD	Coronary heart disease
CVD	Cardiovascular disease
DAPI	4,6-Diamidino-2-Phenylindole
DCs	Dendritic cells
DDR	Damage cell response
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ECs	Endothelial cells
EMPs	Endothelial cell-derived microparticles
EMVs	Endothelial cell-derived microvesicles
ERK	Extracellular signal regulated kinase

eNOS	Endothelial Nitric Oxide Synthase
FA	Fatty acids
GLA	Gamma-Carboxyglutamic Acid
GM-CSF	Granulocytes-Macrophages Colony-Stimulating Factor
GP	Glycoprotein
GTP	Guanosine triphosphate
Gvg	Gavage
HBSS	Hank's Balanced Salt Solution
H2O2	Hydrogen peroxide
HDL	High Density Lipoproteins
HUVEC	Human Umbilical Vein Endothelial Cells
HSP	Heat Shock Protein
ICAM-1	Intracellular adhesion molecule 1
IgE	Immunoglobulin E
IP	Intraperitoneal injection
IL	Interleukin
ISEV	International Society of Extracellular Vesicles
KLF2	Kruppel like Factor
LIG	Ligature
LIG-PG	Ligature + Porphyromonas gingivalis
LDL	Low Density Lipoproteins
LMPs	Leukocytes-derived microparticles

LMVs	Leukocytes-derived microvesicles
LAMP1	Lysosomal Associated Membrane Protein 1
LPS	Lipopolysaccharide
Mac-1	Macrophage antigen-1
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocomatibility Complex
miRNA	MicroRNA
MMPs	Metalloproteinases
MV	Microvesicle
MP	Microparticle
mMVs	Monocytes/ macrophage-derived microvesicles
mMPs	Monocytes/ macrophage-derived microparticles
mRNA	Messenger RNA
MZ	Marginal zone
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NET	Neutrophils extracellular traps
NF-*k*B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NKC	Natural killer cells
NLRP3	NOD-like receptor family pyrin domain-containing 3
nMPs	Neutrophils-derived microparticles
nMVs	Neutrophils-derived microvesicles

NO	Nitric Oxide
OX-LDL	Oxidized Low Density Lipoprotein
OMVs	Outer membrane microvesicles
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PG	Porphyromonas gingivalis
PE	Phosphatidylethanolamine
PL	Phospholipid
PMA/I	Phorbol Myristate Acetate/ Ionophore
PMPs	Platelet derived microparticles
PMVs	Platelet derived microvesicles
PS	Phosphatidylserine
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SASP	Senescence -Associated Secretory Phenotype
SA-β-Gal	Senescence -Asoociated Beta-Galactosidase
SM	Sphingomyelin
SMVs	Splenic Microvesicles
TCR	T-Cells Receptors
TF	Tissue Factor
TLRs	Toll-Like Receptors
TNF	Tumor Necrosis Factor

T.Denticola	Treponema Denticola
T. Forsythia	Tanerella Forsytia
VCAM-1	Vascular Cell Adhesion Molecule 1
WP	White pulp

RESUME DES TRAVAUX

Rate et cœur, un axe fonctionnel ?

La parodontite est une maladie inflammatoire buccale d'origine infectieuse très répandue et la principale cause de perte de dents chez l'adulte. Elle est associée à une destruction progressive des tissus parodontaux conduisant à la formation de poches parodontales.

Étant impliqué dans l'apparition et le développement de nombreuses pathologies systémiques telles que les maladies cardiovasculaires et l'athérothrombose, la parodontite a été associée à une aggravation cardiovasculaire *(Sanz M.et al 2020).*

La progression des connaissances sur la physiopathologie sous-jacente à ces maladies a permis de mettre en évidence des liens entre eux tant au niveau clinique qu'aux niveaux cellulaires et moléculaires, dont les mécanismes initiateurs et régulateurs restent mal connus.

Les microvésicules (MV) sont des fragments de membrane plasmique libérés en réponse au stress cellulaire qui agissent comme des effecteurs cellulaires. Elles sont émises de la membrane plasmique des cellules activées ou apoptotiques, initialement caractérisées par leur activité procoagulante et

qualifiées de "microparticules" *(Ridger et al, 2017)*. Elles sont émises par tous les types de cellules selon leur capacité de remodelage de la membrane plasmique. Dans le système vasculaire,d'origine endothéliale, plaquettaire, leucocytaire et de cellules musculaires lisses *(Boulanger et al, 2010)*

Initialement étudiées pour leurs propriétés procoagulantes sanguine, plus récemment, les microvésicules ont été reconnues comme ayant un rôle dans la communication cellule-cellule. *(Guillaume van Niel et al, 2018)* Elles sont actuellement reconnues comme de véritables effecteurs cellulaires car elles sont dotées d'une activité biologique intrinsèque. Elles sont capables de moduler de nombreuses réponses vasculaires telles que l'hémostase, l'inflammation, la vasomotricité, l'angiogenèse et la croissance vasculaire. Ces propriétés dépendent de l'origine cellulaire de la MV et du stress initiant leur libération et de leur capacité à se lier à la cellule cible. *(Cocucci E et al, 2009)*

Ainsi, dans le choc septique, les taux de MVs procoagulantes circulantes de neutrophiles sont corrélées à une hypercoagulabilité et à la présence de Neutrophil Extra cellular Traps (NETs) circulants*. (Steil et al 2016, DeLabranche et al 2017)*. Les NETs sont composés de chromatine décondensée qui portent certains constituants cytoplasmiques et des granules des neutrophiles qui les émettent. La Nétose des neutrophiles est probablement un mécanisme évolutif de défense contre la déssimination des agents pathogènes envahisseurs qui sont piégés dans les NETs *(Brinkmann et al 2016)*. En effet, les NETs ont été associés à la parodontite chez les

patients à risque cardiovasculaire, ainsi un axe splénocytaire avec invasion par les neutrophiles de la rate dans le cœur post-infarctus a été proposé (Moonen et al 2019) comme un nouveau mécanisme liant infection chronique et atteinte cardiovasculaire (Tian et al 2016).

Dans notre laboratoire, nous avons établi des méthodes d'extraction des MVs d'aorte de rat et démontré que ces MVs sont sensibles au microenvironnement vasculaire. Nous avons aussi montré que les MVs de rat d'âge moyen (48 semaines) ou âgé (92 semaines) isolées à partir de la rate sont pro-sénescentes et procoagulantes vis-à-vis de l'endothélium uniquement lorsqu'elles sont émises dans un microenvironnement proinflammatoire *(El Habhab et al 2020)* et (*Qureishi et al 2019*).

Par ailleurs, d'autres données antérieures du laboratoire ont montré que le LPS induit l'excrétion de MV procoagulants de la rate de rat capables de favoriser la sénescence endothéliale et une action pro-inflammatoire et réponses procoagulantes. (El Habhab et al 2020). L'infection par LPS du *Porphyromonas Gingivalis* ou par la bactérie inactivée conduit à l'émission de MVs proapoptotiques par les cellules endothéliales HUVEC (*Bugueno et al 2020*).

La faisabilité d'un contrôle pharmacologique de la libération de MV prosénescentes de la rate a été démontrée dans un modèle de rat. Le traitement oral par une formule optimisée d'oméga-3 s'est avéré protecteur à la fois contre le taux de libération des MV de la rate chez les rats d'âge moyen

et âgés, et contre leurs propriétés nocives prosénescentes envers l'endothélium coronaire. (Qureshi et al 2020)

Le travail de thèse vise à étudier l'impact des MVs et des NETs (neutrophil extracellular traps) comme acteurs de la réponse cellulaire induite par la parodontite et cause possible de la tendance thrombotique chez les patients. Des expériences sont conçues pour acquérir une meilleure connaissance des interactions entre l'infection locale des dents, la réponse de la rate et les tissus cardiovasculaires. Elles se concentrent sur le changement du phénotypage de l'endothélium qui est initialement une surface protectrice et devient pro-inflammatoire, procoagulant et prosénescent après contact avec les MVs émises localement en réponse à *P. gingivalis*.

Les hypothèses émises après examen de la littérature et en s'appuyant sur les données du laboratoires sont :

1- Les MVs excrétées en réponse à une infection par des agents pathogènes parodontaux sont des acteurs à distance expliquant l'impact de la parodontite sur l'aggravation des conditions vasculaires perpétuent l'inflammation vasculaire chronique.
2- La libération des MVs spléniques et leurs propriétés varient selon la sévérité de l'infection et / ou le type de lésion tissulaire
3- Les MV libérées par les splénocytes en réponse à *Porphyromonas Gingivalis* sont délétères pour les cellules endothéliales et agissent

comme des médiateurs cardiovasculaires pro-inflammatoires et prosénescents

4- Les microvésicules splénocytaires et les NETs libérés en réponse à *Porphyromonas Gingivalis* favorisent l'athérothrombose à des sites éloignés de l'infection dentaire

Les objectifs généraux du travail sont en conséquence :

1- Établir la preuve de concept dans un modèle murin de parodontite.
2- Caractériser la composition cellulaire de la rate et la libération des MVs par les splénocytes lors d'une infection parodontale par ligature combinée ou non à une infection locale par *Porphyromonas.gingivalis*.
3- Comparer les propriétés endothéliales proinflammatoires et procoagulantes des MVs tissulaires de souris dans ce modèle d'infection dentaire en utilisant la rate comme source majeure de MVs inflammatoires libérées en réponse à *P. gingivalis.*
4- Identifier les effets pro-sénescents des MVs sur l'endothélium coronaire en utilisant des cellules primaires dans un modèle de communication intercellulaire.
5- Identifier les NETs et les MVs comme acteurs circulants et distants de *P.Gingivalis* en comparant le modèle de ligature et l'injection intrapéritonéale de *P.gingivalis.*

6- Evaluer les répercussions de la parodontite sur le profil inflammatoire et procoagulant du tissu cardiaque.

Prolog

Spleen and heart contributing to pathological response in periodontitis.

Periodontitis is a highly prevalent, oral inflammatory disease of infectious origin and the primary cause of tooth loss in adults. It is associated with a progressive destruction of periodontal tissues leading to the formation of periodontal pockets.

Amazingly, periodontitis has been involved as a inducer in pathologies such as cardiovascular diseases and atherothrombosis, associated with cardiovascular worsened outcome [1].The evolution of the knowledge of physio pathological mechanisms underlying both diseases has made it possible to highlight cellular and molecular links , although these mechanisms remain to be deciphered.

The thesis work is investigating relationships between different effectors possibly contributing to a remote action of the local periodontitis.

Recently, Neutrophil Extracellular Traps (NETs), a mesh of decondensed DNA shed by neutrophils in response to local infection, and already known to contribute to hypercoagulability, have been associated with periodontitis in patients at cardiovascular risk. [2] Finally, leukocytes invasion of the spleen in the post-ischemic heart reperfusion has been proposed as a new mechanism

linking chronic infection and cardiovascular damage [3] possibly constituting a new spleen-cardiac deleterious axis.

Microvesicles (MVs) are plasma membrane fragments shed in response to cell stress that act as cellular procoagulant effectors. Previous data from the laboratory have shown that MVs may act as pro-inflammatory mediators promoting premature senescence of coronary artery endothelial cells [4,5] leading to endothelial dysfunction and the release of prosenescent MVs and that infection by LPS of *Porphyromonas Gingivalis,* the main pathogen in periodontitis leads to the emission of proapoptotic microvesicles (MVs) by HUVEC endothelial cells.[6] Previous *In vitro* data showed that LPS induces the shedding of procoagulant MVs from rat spleen that are able to promote endothelial senescence and a pro-inflammatory and procoagulant responses. [4] The feasibility of a pharmacological control of the release of prosenescent MVs from the spleen was further demonstrated in a rat model. Oral treatment by an optimized omega-3 formula was found protective against both the rate of the release of spleen MVs in middle-aged and aged rats, and their prosenescent noxious properties towards the coronary endothelium. [7]

The thesis work aims to study the impact of MVs and NETs (neutrophil extracellular traps) as actors of the remote cell response after periodontitis and

a possible cause of thrombotic tendency in patients. Experiments are designed to gain a better knowledge of the interactions between local teeth infection, the spleen response and the cardiovascular tissues. They focus on the endothelial switch from a protective to a proinflammatory and procoagulant and prosenescent surface mediated by *P. Gingivalis*-induced MVs.

Chapter I
Microvesicles: Extracellular vesicles shed from the activated plasma membrane

I.1 Background: Properties and characterization of extracellular vesicles

The International society of extracellular vesicles (ISEV) underwrites extracellular vesicle (EV) as the generic term for particles naturally released from the cell that are delimited by a lipid bilayer, precluding a functional nucleus and asserting they cannot replicate. [8]

Extracellular vesicles are designated in the literature as microparticles, microvesicles, or exosomes or apoptotic bodies.[9]

All extracellular vesicles are distinguished on the basis of their subcellular origin, their size, their content and the mechanism leading to their formation. [10] (see fig 1)

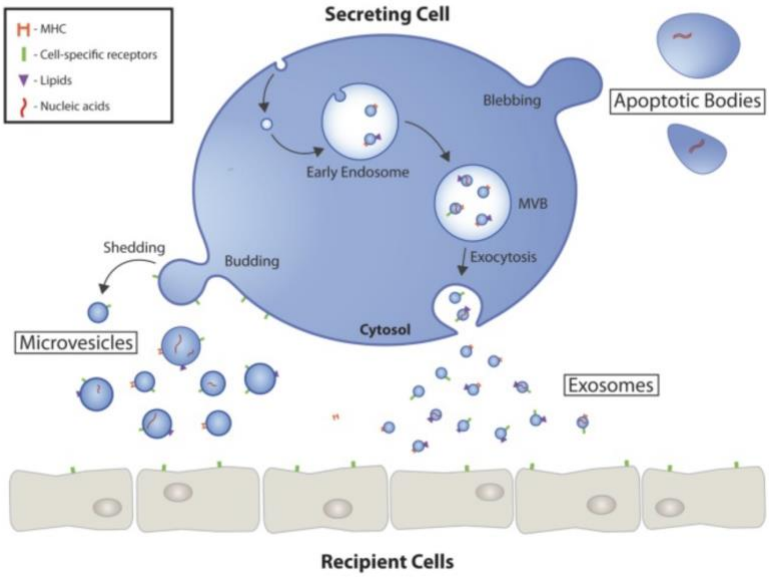


Fig 1: Schematic representation of the types of extracellular vesicles released by a cell. Exososomes, microvesicles and apoptotic bodies are secreted by the cell into the extracellular environment. Their biogenesis are different, exosomes are secreted by endocytosis,

microvesicles and apoptotic bodies are released by plasma membrane blebbing in response to cellular stress. [11](Gustafson et al 2017)

Exosomes

Exosomes are small, single-membrane, secreted organelles of ~30 nm to ~100 nm in diameter having the same form as the cell and enriched in proteins, lipids, nucleic acids, and glycoconjugates.[12] They are present in many biological fluids, including urine, [13] blood,[14] ascites, [15] and cerebrospinal fluid,[16] fractions of body fluids such as serum and plasma, and cultured medium of cell cultures . Exosomes are usually isolated by ultracentrifugation (100,000–200,000*g*).[17] Regarding their biochemical composition, exosomes are surrounded by a phospholipid membrane containing relatively high levels of cholesterol, sphingomyelin, and ceramide and containing detergent-resistant membrane domains (lipid rafts) [18]

Apoptotic bodies

Apoptosis is a highly regulated process of cell death. [19] During apoptosis, starting with the nuclear chromatin condensation, the process is followed by plasma membrane budding, progressing to the breakdown of the cellular content into distinct membrane enclosed vesicles termed apoptotic bodies or apoptosomes.[20]

Identified by Kerr, Wyllie in 1972[21] , apoptotic bodies are membrane structures that package cell organelles such as mitochondria, nuclear fragments, granules, cleaved DNA …[22]

They expose phosphatidylserine and contain DNA and RNA originating from nuclear membrane fragmentation.[23] (see table 1)

Table 1 Characteristics of various types of extracellular vesicles

Characteristic	Microparticles	Exosomes	Apoptotic bodies
Size	100–1000 nm	40–100 nm	>1 μm
Mechanism of formation	Outward blebbing of plasma membrane	Fusion of multivesicular bodies with plasma membrane	Cell shrinkage and death
How detected	Flow cytometry, capture-based assays and electron microscopy	Electron microscopy and Western blotting (characterization with exosome-enriched markers)	Flow cytometry and electron microscopy
Characteristic features	Annexin V-positivity and presence of cell-specific surface markers	LAMP1, CD63 and TSG101	Annexin V-positivity, DNA and permeable membrane
Composition	Protein, RNA and miRNA	Protein, RNA and miRNA	Protein, DNA, cell organelles, RNA and miRNA
Membrane properties	Externalized phosphatidylserine, rich in lipid rafts and impermeable	Rich in lipid rafts and impermeable	Externalized phosphatidylserine and permeable

Table 1: Classification of different types of extracellular vesicles and methods for their detection [24](Burger et al 2013)

Microvesicles (MVs)

Initially identified by Wolf 1967 as a platelet dust containing phospholipids, and capable of supporting coagulation,[25] microvesicles (MVs), also named microparticles, oncosomes [26] or ectosomes[27], are submicron fragments of the plasma membrane that spill into the extracellular space of cells under cellular stress conditions.[10] The ectosome nomenclature derives from the definition of ectocytosis introduced in 1991 by Stein and Luzio to describe the shedding of vesicles from the plasma membrane of stimulated neutrophils,[28] while

oncosomes are atypical large cancer-cell derived extracellular vesicles. However, when confirmation of the vesicles identity is achieved according to the recommendations of the ISEV 2018 norms, the most likely term to be used nowadays is microvesicles.[8]

MVs are released in biological fluids such as blood, urine, tears and saliva. In the blood, they circulate at low concentration in healthy individuals and their levels are modified in various cardiovascular and chronic diseases including diabetes, chronic renal failure, pre-eclampsia and hypertension. [24] Ranging in size between 100 to 1000 nm in diameter, they are released during cell-surface budding, and considered as physiological liposomes, since formed from the cell plasma membrane lipid bilayer. The MV membrane embeds a content of active proteins, signaling nucleotides, and metabolites as well as RNA, non-coding RNA and bioactive lipids.[9] MVs expose phosphatidylserine, a procoagulant phospholipid.[10] [30] (see fig 2 and table 2)

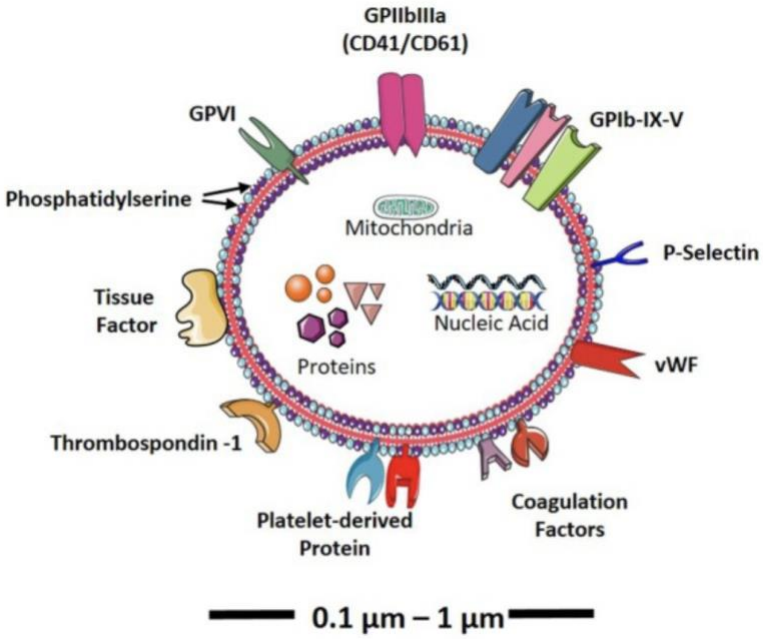


Fig 2: Shematic content and membrane of extracellular microvesicle released from platelet. (Zaldivia MTK et al 2017) [29]

Molecule	Type of cell producing microparticles	Target cell or environment
Receptor/membrane molecule		
Chemokine receptor CCR5 [72]	Peripheral blood mononuclear cells	Various cells
CXCR4 receptor [73,74]	Platelets	Various cells
Glycoprotein IIb/IIIa receptors [75]	Platelets	Neutrophils
Oncogenic receptor EGFRvIII (epidermal growth factor receptor variant III) [76]	Tumour cells	Various cells
Major histocompatibility complex (MHC) class II [77]	Immune cells	Immune cells
Tissue factor [48]	Monocytes	Platelets
Peroxisome proliferator-activated receptor gamma [78]	Platelets	Monocytes
Cytokines		
Interleukin-1beta [79–82]	Various cells	Various cells
Chemokine (C-C motif) ligand 5[83]	Platelets	Endothelial cells
Growth factors		
Vascular endothelial growth factor [84–85]	Platelets/tumour cells	Endothelial cells
Basic fibroblast growth factor [84,86]	Platelets/tumour cells	Endothelial cells
Platelet-derived growth factor [86]	Platelets	Endothelial cells
Lysis enzymes		
Matrix metalloproteinases [85]	Tumour cells	Extracellular matrix
Extracellular matrix metalloproteinase inducer [87]	Tumour cells	Extracellular matrix
Caspase 1 [88]	Monocyte	Smooth muscle cell
Lipids		
Arachidonic acid [89–91]	Platelets	Various cells
Platelet activated factor [92,93]	Various cells	Platelets
Ribonucleic acid (RNA)		
Messenger RNA [38,94]	Stem cells	Various cells
Micro RNA [95–97]	Stem cells	Various cells

Table 2: Table of bioactive molecules of extracellular microvesicle MVs.[31] *(Voukalis et al 2019)*

Plasma membrane phospholipid distribution

Eukaryotic cells are surrounded by a lipid bilayer subdivided into compartments with distinct structural and functional identities termed rafts. Where glycerophospholipids forms almost 70% of the total lipid content of mammalian cells, the other 30% consists of cholesterol, sphingomyelin, and glycosphingolipids. Among the phospholipids, phosphatidylcholine (PC) is the most prevalent and accounts for 40%–50% of the total,[32] whereas phosphatidylserine (PS) is the commensurate trifling constituent of most biological membranes.[33]

In addition, the content of conventional phospholipids varies among organelles. Membrane phospholipid asymmetry is characteristic of eukaryote cells, and is regulated by an active process by flipases and floppases. [34] In resting cells phosphatidylserine is sequestered in the inner leaflet of the plasma membrane. [35]

Phosphatidylserine

Phosphatidylserine (PS) species were first identified by Folch and coworkers in the 1940s. [36] Notwithstanding its low abundance in plasma membrane phospholipids , PS is of a substantial physiological importance owing to its unique physical and biochemical properties. It is the key to the recruitement of vitamin-K dependent coagulation factors [37] , it also signals important

events such as the internalization of viruses by host cells [33] and the clearance of apoptotic cells via the binding of lactadherin that is secreted by macrophages and bridges PS and α V β3, an integrin exposed by the macrophages.[38]

I.2 Mechanism of MV generation

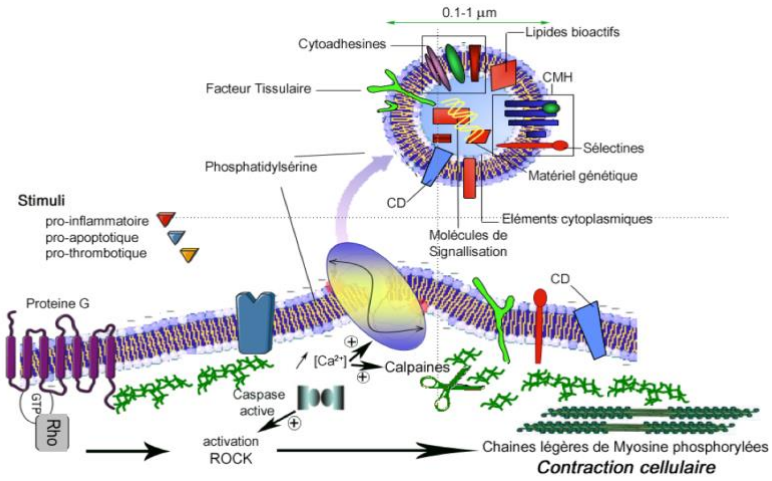
Loss of phospholipid asymmetry is a driving force for the membrane remodeling and generation of MVs.[10] Unlike exosome formation, MV formation involves plasma membrane budding [39]. Reorganization of the cytoskeleton and changes in membrane asymmetry of PS are key for the genesis of MV. In this respect, the dynamics of actin filaments seem crucial for the formation of MVs. [24] For instance, platelet activation leads to an increase in intracellular calcium, which in turn regulates the activity of transporters such as flippases, floppases and scramblases which are responsible for the movement of phospholipids between the two layers of the plasma membrane.[39] These proteins maintain the phospholipid asymmetry of the membrane at cell rest (fig 5). Activation of floppases and scramblases and inhibition of flippases in response to calcium influx induces exposure of PS at the outer leaflet of a cell membrane, whereas PS is sequestered in the inner leaflet of the plasma membrane at cell rest. The increase in cytoplasmic calcium concentrations causes the translocation of phosphatidylserine (PS), from the inner layer to the outer layer under the effect of a net floppase activity. At the same time, reverse anionic phospholipid

transporters (flippases) are inhibited by calcium. The transport of PS by floppases is very rapid while the reverse transport of other non-anionic phospholipids such as phosphatidylcholine is slower. The translocation of the PS creates a transient overload of the outer leaflet which leads to budding. Increased intracellular calcium also activates calpains and Rho kinases, responsible for cytoskeleton fragmentation, [10] thus, the membrane tension is not contained by the cytoskeleton and a MV exposing PS is released. [40] [41]

This process is sometimes considered protective because MV carry stress-induced deleterious signals from the parent cell. Thus, inhibition of apoptotic endothelial cell Rho kinase prevents the release of MP exporting active caspase 3 and promotes detachment and cell death. [42]

MVs are procoagulant because they carry PS which catalyzes the formation of the blood coagulation complexes and cellular effectors because they convey active proteins present in the cell at the time of their emission. [43] Bakouboula et al 2011. (see fig 3)

A-



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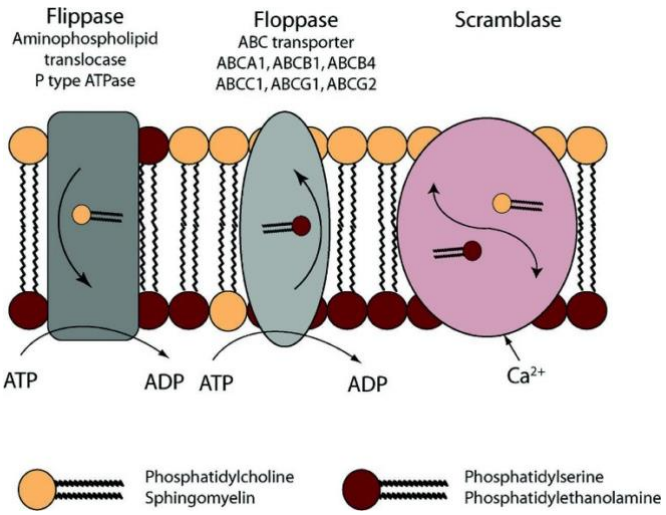

Fig 3: Mechanisms of generation of MVs in response to cell stimulation and membrane transporters A: cell response to stimuli: the activators bind to their specific receptor and induce a calcium influx. The increase in cytoplasmic calcium concentrations causes the translocation of phosphatidylserine (PS), from the inner layer to the outer layer under the effect of a net floppase activity. Proteases such as calpains and ROCK kinases are activated by high calcium concentrations and cause controlled proteolysis of the cytoskeleton, which promotes the release of MVs. B: Different types of phospholipid transporters in the membrane of eukaryotic cells. Flippase (repatriation of PC, floppases: externalization of PS, Scramblases:

mixing of phsopholipids between the layers) [44] *(Quazi et al 2011 & authorized by co-other Florence Toti).*

I.3 Methods of MV isolation from tissues, quantification and characterization

There are different strategies currently available to characterize EVs, including electron microscopy-based approaches and flow cytometry combined with antibodies against surface markers. Other strategies for studying EVs molecular signatures are based on molecular analysis of their cargo (mRNA, protein, or bioactive lipid content) by employing omics technologies aimed at the universal detection of mRNA species (transcriptomics), proteins (proteomics), lipids (lipidomics), and metabolites (metabolomics) [9] . Bulk analysis techniques, such as Western blots, ELISAs, mass spectrometry, and sequencing, and bead capture assays, have been widely used and instrumental in the field to date, associating EV phenotype (molecular cargo) with function [45].

As for MVs, the majority of clinical studies examine MVs isolated from plasma samples, which typically contain endothelial, leukocyte, platelet, and erythrocyte MVs. [39] Several approaches can be used for the detection and characterization of MVs with sometimes a pre-analytical isolation phase by high speed centrifugation and washing, before analysis by various methods such as prothrombinase enzymatic assay, spectrophotometric measurement

of MV proteins and RNA concentration [7] and other proposed methods listed in table 3 .

Protocol	Method of quantification	Advantages	Disadvantages
Flow cytometry	Fluorescence and light-scattering properties MPs in suspension	Available to most research facilities	Quantification of 100–400 nm may be imperfect
		Rapid	Cell origin identification is antibody-dependent
		Multiple antigens may be analysed in a single sample	
		MPs analysed on an individual basis	
Immunoassays	Immunocapture of MPs and quantification based on the presence of surface antigen	Available to most research facilities	Quantification is done in bulk
		No size restrictions	Quantifies based on a single antigen
			Does not allow for size determination
Functional assays	Procoagulant or prothrombinase activity of MPs	Available to most research facilities	Quantification is done in bulk
		Provides an indication of biological activity	Measures only a single biological activity
			Does not allow for size determination
Atomic force microscopy	Cantilever is used to scan the surface of MPs and tip displacement is related to surface properties	Allows for very accurate sizing of MPs	Non-universal technology
		Allows for three-dimensional view of MP structure	Determination of cell origin requires development of specialized antibody-coated surfaces
		May be used for quantification	Not conducive to large sample numbers
Nanoparticle tracking analysis	MPs are visualized by light microscopy and light scattering is observed; Brownian motion of individual particles is tracked by video	Clear idea of MP size	Utility of assay for quantification is unclear
		Allows for quantification	Non-universal technology
			May be time-consuming

Table 3: Different methods for MVs quantification [24] (Dylan Burger et al 2013)

According to the international society of extracellular vesicles (ISEV), there is no single optimal characterization method neither a recommendation on quantification of MVs, however, characterization of vesicles must be done using two technical complementary approaches[8].

Chapter II
Tissular and vascular Microvesicles: Biomarkers and cellular effectors

Inflammation is a major actor promoting altered vascular response and often associated with worsen cardiovascular outcome [46]. Plasma MVs are not only markers of cellular and tissue activation or damage but also actors of major physiological responses such as inflammation[47], atherosclerosis, [48,49] [30]cell survival and apoptosis [49], endothelial function, [50] [51] plasma membrane remodeling [52,53] and angiogenesis.[54] [30]

In the last decades, many studies explored the role of MVs as bioactive mediators, capable of raising and disseminating the signal responsible for their emission in the vessels and the extracellular space of the tissues in an autocrine or paracrine manner.[55] [49] [54,56,57] They do exhibit proinflammatory effects and induce the production of cytokines and chemokines while promoting activation of inflammatory cells.[24]

Circulating MVs mainly of platelet, leukocyte, and endothelial origin provide an additional phospholipidic surface for the assembly of blood coagulation factors and promote the coagulation cascade and thrombin generation. In the vasculature they constitute the principal reservoir of tissue factor (TF) activity, the celular initiator of blood coagulation. [58] Emerging as a key marker of global vascular damage, MVs bearing TF mediate noxious cellular responses in diabetic thrombophilia and cardiovascular complications and act as injurious amplifiers of various biological responses such as thrombogenicity and atherothrombotic plaque remodeling.[59]

II.1 Microvesicles and vascular inflammatory response

The endothelium is a major player in cardiovascular (CV) physiopathology.[60] In the healthy state, the endothelium is vasoprotective, mainly by the secretion of vasodilator agents such as nitric oxide (NO), the endothelium-derived relaxing factor [61] and prostacyclin,[62] and because the resting endothelium does not promote the assembly of blood coagulation factors (it does not expose PS and very little if any tissue factor). Endothelial dysfunction is an early pathogenic event of atherosclerosis, essential hypertension, and related cardiovascular disorders.[63]

The loss of cytoprotective NO makes cells susceptible to apoptosis. [64]Endothelial MVs are potential biomarkers of cardiovascular disease. In addition, many studies have reported that MVs can directly alter the endothelial function.[65]

According to the degree of the severity of pathological state associated with vascular dysfunction, the plasma concentrations of MVs are modified. Numerous clinical data indicate that plasma concentrations of endothelial-derived EMVs increase in obesity [66], physical inactivity [67], type 2 diabetes mellitus [68], end-stage renal disease [69], ischemic left ventricular dysfunction [70], and preeclampsia [71]. Furthermore, the phenotype of EMVs varies with the endothelial damage and reflects the initial stress. For instance, in response to platelet MVs, endothelial cells (ECs) release of pro-inflammatory cytokines

such as Il-6 or Il-8 cytokines and the expression of adhesion molecules ICAM-1,[72,73] VCAM-1 and E-selectin [73] . Interestingly, the majority of (EMVs) isolated from the site of coronary artery plaque during balloon angioplasty express the vascular cell adhesion molecule-1 (VCAM-1)[74]. Of note, VCAM-bearing endothelial cells or derived MVs were in higher amounts (70%-80%) when isolated from patients with Myocardial infarction as compared to stable coronary artery disease. Because VCAM-1 expression was absent on ECs in the peripheral circulation from these same subjects, authors claimed that VCAM expression at the membrane of cells or EMP appear as unique makers in the plaque tissue. At least they demonstrated that inflammatory markers accumulate in the plaque. Similarly, MVs generated in vitro from endothelial cells human coronary endothelial cells (HCAEC) exposed to high glucose concentration, show increased NADPH oxidase activity and ROS levels,[75] underlining their potential noxious role in diabetes and cardiovascular associated disorders .[76] [77]

In blood, MVs are most often mainly released from platelets and to a lesser extent from leukocytes and endothelial cells [78]. Plasma MVs are now also studied substantially as vascular effectors [79], and have been reported to be inflammatory effectors intensifying the secretion of a variety of pro-inflammatory mediators.[30] (see table 4)

Type of cell producing MPs	Target cell	Molecules involved in the pathogenesis of inflammation mediated by MPs
		Cytokines
Endothelial	Various inflammatory cells	IL-1β and TNF-α [113]
Leucocytes	Endothelial	IL-6 and MCP-1 [114]
T cells	Monocytes	IL-8, TNF-a and IL-1β [43,115]
		Adhesion molecules
Monocytes Platelet	Endothelial	Intercellular adhesion molecule-1, vascular cell adhesion molecule-1 E-selectin [91,116]
–	–	Lipids
Platelet	Endothelial	Thromboxane A2 and cyclooxygenase [89]
		Other
Polymorphonuclear	Macrophage	transforming growth factor beta1 [117]
–	–	Annexin V [118]
Monocytes	Macrophage	peroxisome proliferator-activated receptor gamma protein [119]
Polymorphonuclear	Various cells	complement proteins (C1q) [51]

IL: interleukin; TNF: tumour necrosis factor; MCP-1: monocyte chemoattractant protein-1.

Table 4: Microvesicles and inflammation [31] (Voukalis et al 2019)

II.2 Microvesicles in atherosclerosis and thrombosis

Atherosclerosis is a multifaceted pathology combining inflammatory responses as well as certain metabolic imbalance such as elevated LDL-cholesterol and resulting in the formation of thickening of the arterial intima and the formation of atherosclerotic plaque. [80] MVs are crucial actors in atherothrombosis, [48,49] [30] . I*n vitro*, plasma MVs foster the release of cytokines and cell adhesion molecules from endothelial cells and prompt the induction of tissue factor, the cellular initiator of the coagulation cascade.[81] Studies have shown that the coagulation capacity of platelet-derived MVs (PMVs) is 50 to100-fold higher than activated platelets. [82] An excessive amount of circulating PMVs has been observed in patients with atherosclerosis. [83] [84]

In the sequence of events following the initial MV-mediated coagulation, EMVs and leukocyte MVs will later carry the plasminogen activator uPA which enables the transformation of plasminogen into plasmin. MVs bearing uPA will

contribute to fibrinolysis cross-talk by docking into the growing thrombus to resorb it and also promote extracellular matrix remodelling during tissue healing.[85] Indeed, the cellular uPA borne by EMV and leukocyte-derived MVs promote the plasmin formation [86]. (see fig 4)

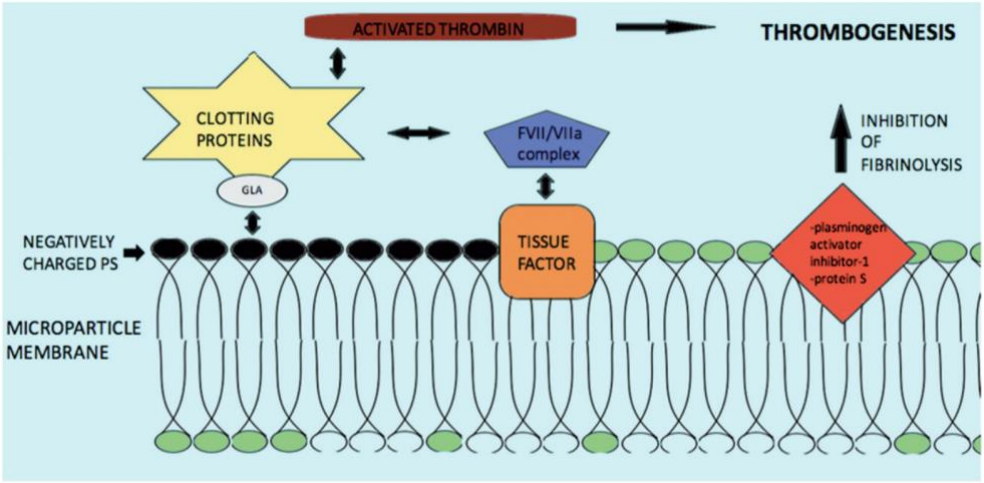

Fig 4: Mechanisms and molecules related with MVs-induced coagulation.
PS: phosphatidylserine; GLA:c-carboxyglutamic acid; clotting proteins factors VII, IX, X and prothrombin. PS electrostatically catalyzes the assembly of coagulation enzyme complexes by interacting with the GLA domain of the clotting factors called vitamin-K dependent factors. Tissue factor induced specifically at the surface of activated endothelial or leukocytes cells may also activate the coagulation cascade via the FVII/VIIa complex.[31] (Voukalis et al 2019)

Many studies focused on the correlation existing between the level of EMVs in the blood and cardiovascular diseases. [5,69,88] CD31+MVs plasma levels were correlated with a higher risk of major adverse cardiovascular and cerebrovascular events and the need for revascularization [89].The count of MVs of different origin, including EMV (CD31+, CD51/61+ and CD34+), Platelet MVs (CD42+), monocytes MVs(CD14+) and MV-derived from leukocytes

derived (CD45+) enabled the identification of characteristic patterns enabling the discrimination between patients with myocardial infarction (MI) from those with stable coronary artery disease and healthy volunteers.[90] Morel et al. described that patients with ST elevation myocardial infarction (STEMI) and unstable angina had higher circulating levels of procoagulant EMVs detected by CD105 labelling as compared with patients with stable angina [91] or acute coronary syndromes. Patients with acute ischemic stroke were found to have increased levels of platelet-derived MPs bearing the integrin GPIIb-IIIa during the acute phase and up to 6 months later, putting forward that thrombotic propensity might last more than the acute phase of the infarct.[92] Indeed GPIIb-IIIA is a platelet plasma membrane protein that enables the adhesion of platelets to fibrin and that is overexposed after platelet activation. In carotid, abdominal aorta and femoral arteries from patients with non-symptomatic atherosclerotic disease, elevated levels of circulating leukocyte-derived MVs (CD11a+) were measured [93] along with endothelial-derived MVs (CD105+). CD11a+ MVs concentrations correlated with the Framingham index that characterizes patients at cardiovascular risk and with higher carotid intima-media thickness assessed in 3 different vessels. Altogether, these data indicate the diagnosis value of MVs in the early detection of atherosclerotic disease [94].

Chapter III
Periodontitis: a local infection with cardiovascular consequences

III.1 Periodontitis: a clinical overview

Periodontitis, is a chronic inflammatory disease associated with the formation of a dysbiotic biofilm, progressive destruction of the tooth-supporting tissues such as the gingiva and the underlying alveolar bone [95] ultimately leading to tooth loss [96] and appraised as a silent disease. [97] Having various etiologic and contributory factors, [98] severe type of periodontitis affects approximately 11% of the world's population. [99] Mostly gram-negative anaerobic bacteria colonize subgingival biofilms. [100]Innate and adaptive immune response are triggered in periodontitis.[101]*Porphyromonas gingivalis, Treponema Denticola* and *Tanerella Forsythia,*known as the “red complex” in gingival plaque, are responsible for periodontitis progression.[102] [103]

III.2 *Porphyromonas gingivalis* (*P. gingivalis)*: Role in inflammation and immune response

P. gingivalis is a gram-negative anaerobic bacteria, able to overthrow the host response and to nurture the malfunction of the homeostatic state in a complement dependant manner. [104] It developed strategies to destabilize components of the host immune system such as Toll-like receptors and complement.

P. gingivalis undermines the innate immunity, demolishes the entire biofilm growth in the periodontium thus eliciting the homeostatic host-microbial balance, making it a keystone pathogen in periodontitis. It colonizes and set

the scene for inflammatory bone loss. *P. gingivalis* instigates dysbiosis and periodontitis in conventional mice due to the crucial enzymatic activity of the Arg-specific cysteine proteinases (gingipains) of *P. gingivalis* exhibiting complement C5 convertase-like activity.[105] (see fig 5)

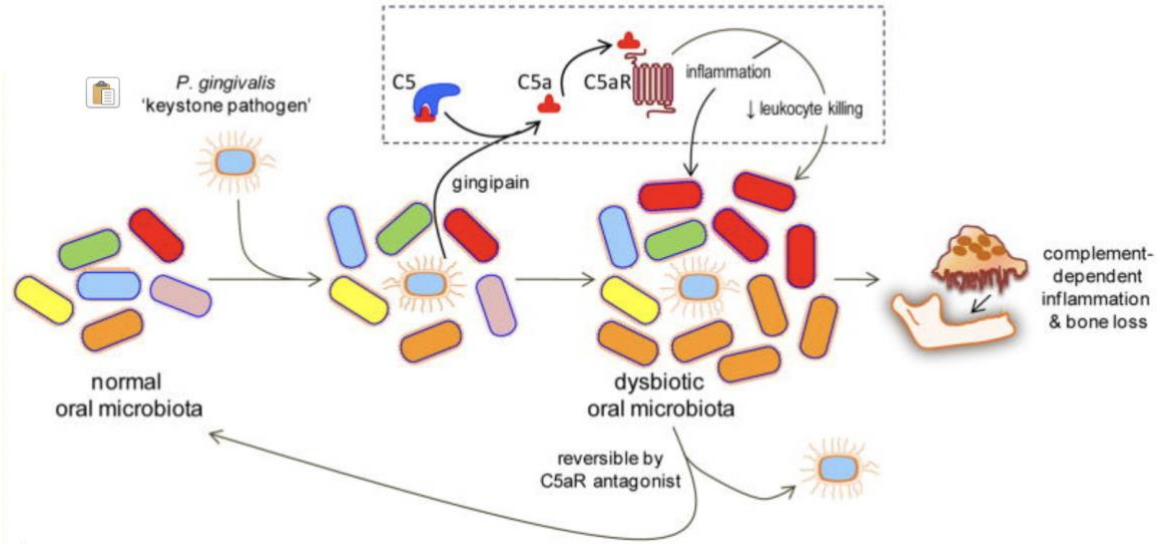

Fig 5: *P. gingivalis leads the inflammatory disease. The bacterium's gingipain, a C5 convertase-like enzyme cleaves C5 giving rise to high levels of C5a locally. C5a-induced activation of C5aR triggers inflammation and also is censoriously involved in a subversive crosstalk with TLR2 that impairs leukocyte killing.* [105] *(by Hajishengallis et al 2012)*

The possible mechanisms explaining the severity of the response are a remote action of the periodontal pathogens at distance, and/or the increased systemic inflammation mediated by pro-inflammatory cytokines such as interleukin (IL) -1, IL-6 and TNF-α released in the blood flow. [106] [6,107]

P. gingivalis harvests many virulence factors including lipopolysaccharide (LPS) [108] inducing Toll-like-receptor (TLR)-mediated inflammatory responses [109] and the recruitment of neutrophils and polymorphonuclear leukocytes (PMNs) at site of the initial infection [107]. The LPS from *P. gingivalis* activates TLR-4 or TLR-2, [110] paving the way for evasion to immune detection and favouring bacterial survival.

The unique ability of *P. gingivalis* to prompt TLR-2 signaling while TLR4-dependent recognition is significantly impaired, favors low-grade persistent infection. [111,112] At the vascular level, activation of TLR2 by LPS is associated with immunothrombosis, a mechanism that reinforces thrombotic pathways via exagerated local coagulation.

In animal models, *P. gingivalis* stimulation led to the up-regulation of TLR2 expression and to pro-inflammatory cytokine secretion *in vitro.* It was also evidenced that TLR2 is crucial for alveolar bone loss caused by *P. gingivalis* infection [113] as well as for *P. gingivalis* LPS-activated nitric oxide, TNF-α, and IL-6 production by macrophages .[114]

P. gingivalis employs both complement and TLR signaling to induce bacterial persistence. Recognized by the TLR2 on neutrophils, *P. gingivalis* activates the adaptor protein MyD88 initiating for bacterial elimination. However, *P. gingivalis* produces gingipains, enzymes that function as complement C5 convertases. C5a complement fragment binds to its receptor (C5aR), which induces the E3 ligase Smurf1 to ubiquitinate MyD88 and mark if for

proteosomal degradation, beneficially inhibiting bacteria killing. (see fig 6) Thrillingly, binding of *P. gingivalis* to the TLR2 also induces inflammation through a signaling pathway involving the adaptor molecule Mal and the enzyme phosphatidylinositol 3-kinase (PI-3K). Also, the same pathway can prevent actin polymerization through inhibition of the small GTPase RhoA and block phagocytosis.[115]

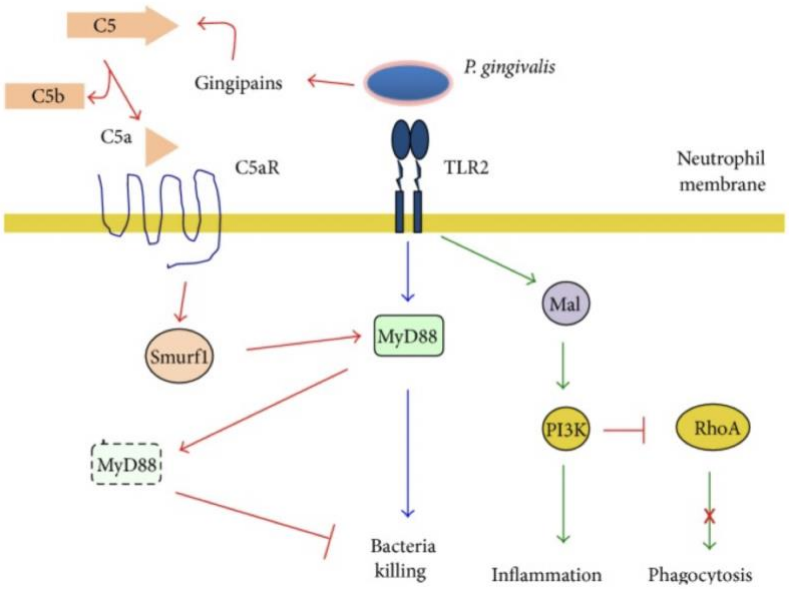

Fig 6: P. gingivalis employs both complement and TLR signaling to induce bacterial persistence. C5a: receptor for C5a.Mal adaptator protein; PI3K: phosphatidylinositol 3-kinase. .RhoA: small GTPase Rho.[115] *(by Cortes-Vieyra et al 2016)*

Neutrophils constitute the majority of leukocytes recruited to the gingival crevice or periodontal pockets, which are heavily invaded by subgingival microbial communities. [116] [117] Being indispensable for homeostatic immunity, [118] neutrophils are dysregulated in periodontitis, and can cause collateral

tissue damage through the release of toxic and/or inflammatory molecules or tissue degrading proteases.[119-121]

As stated above, P.gingivalis expresses ligands that activate the toll-like receptor-2/toll-like receptor-1 complex and enzymes (HRgpA and RgpB gingipains) with C5 convertase-like activity that generate high local concentrations of C5a ligand so that P.gingivalis coactivates C5a receptor and toll-like receptor-2 in neutrophils and macrophages.

In neutrophils, ubiquitination and proteasomal degradation of the MyD88, inhibits the host-protective antimicrobial signaling in response to the C5a receptor-1/TLR2 dependent release of TGFβ-1, that mediates MYdD88 ubiquitination by the E3 ubiquitin ligase Smurf1. Moreover, the C5a receptor-1/TLR2-2 cross-talk activates the PI3-kinase, which inhibits phagocytosis through suppression of RhoA GTPase activity and inhibition of actin polymerization and the induction cytokine production., In macrophages, P. gingivalis activates C5a receptor-1 and induces intracellular Ca 2+ influx, leading to a drastic enhancement of the cAMP-dependent signalling, otherwise weak when induced by TLR2 alone. cAMP-dependent protein kinase A (PKA) inhibits nuclear factor-kappaB and glycogen synthase kinase-3beta signaling, thereby suppressing inducible nitric oxide synthase activation (iNOS) and -oxidative destruction of the pathogen. (see fig 7)

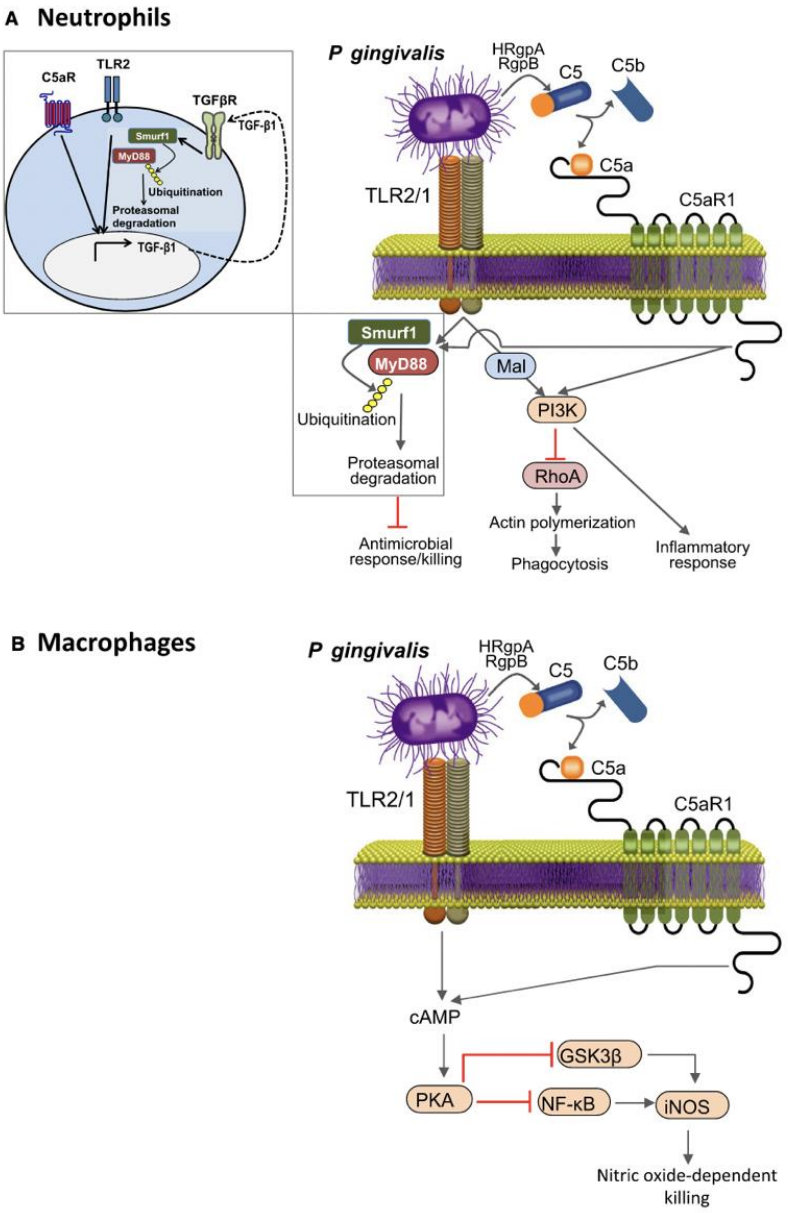

Fig 7: P. gingivalis-induced C5a receptor-1/toll-like receptor-2 crosstalk in neutrophils and macrophages. GSK, glycogen synthase kinase; iNOS, inducible nitric oxide synthase; Mal, myeloid differentiation primary response protein 88 adaptor-like; MyD88, myeloid differentiation primary response protein 88; NF-κB, nuclear factor-kappaB; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; TGF, transforming growth factor; TLR, toll-like receptor. [122] *(by Hajishengallis et al 2020)*

III.3 Periodontitis and endothelial dysfunction

Endothelial dysfunction occurs early in the pathogenesis of arterial disease, in response to a wide range of factors that have been shown to predict cardiovascular events in epidemiologic studies. [123] Periodontal diseases are associated with major systemic diseases such as cardiovascular diseases, diabetes, or adverse pregnancy outcomes. [124] For more than a decade, the link between systemic inflammation and periodontitis has been studied [125] , in particular by focusing on endothelial dysfunction. (see fig 8)

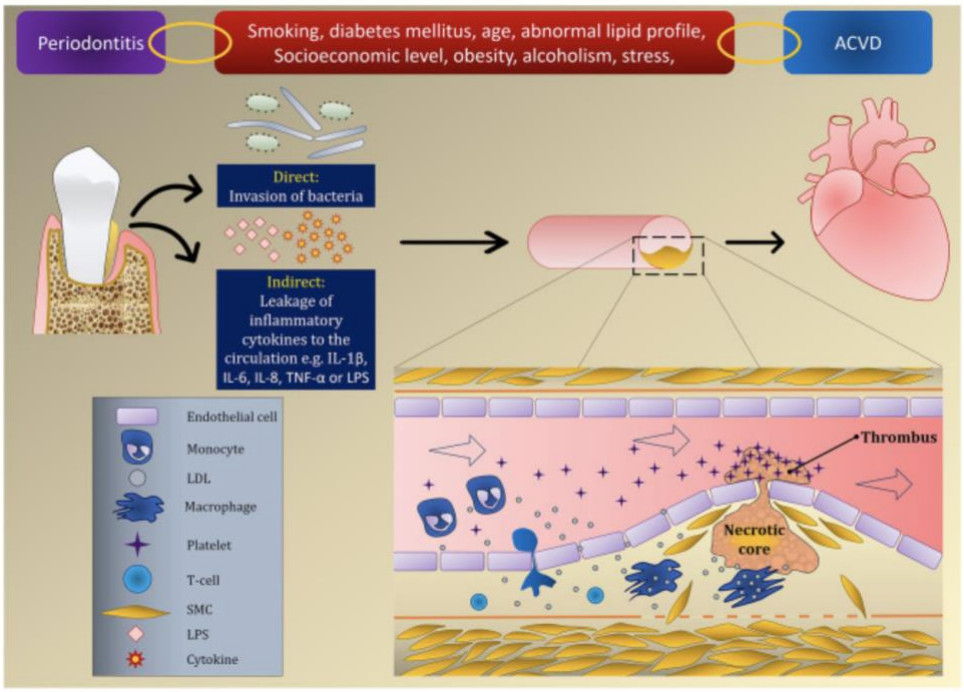

Fig 8: Link between periodontitis and atherosclerotic cardiovascular diseases induced by endothelial dysfunction[126] (by Zardawi et al 2021)

Different oral bacterial species have been identified in individual atheromas. [127] The mechanisms underlying bacterial colonization of plaque worsening atherosclerosis are activation of the immune system, bacteremia, direct

involvement of bacterial activated inflammatory mediators, pro-inflammatory cytokine involvement and predisposing factors that can be found in both periodontitis and atherosclerosis.[128]

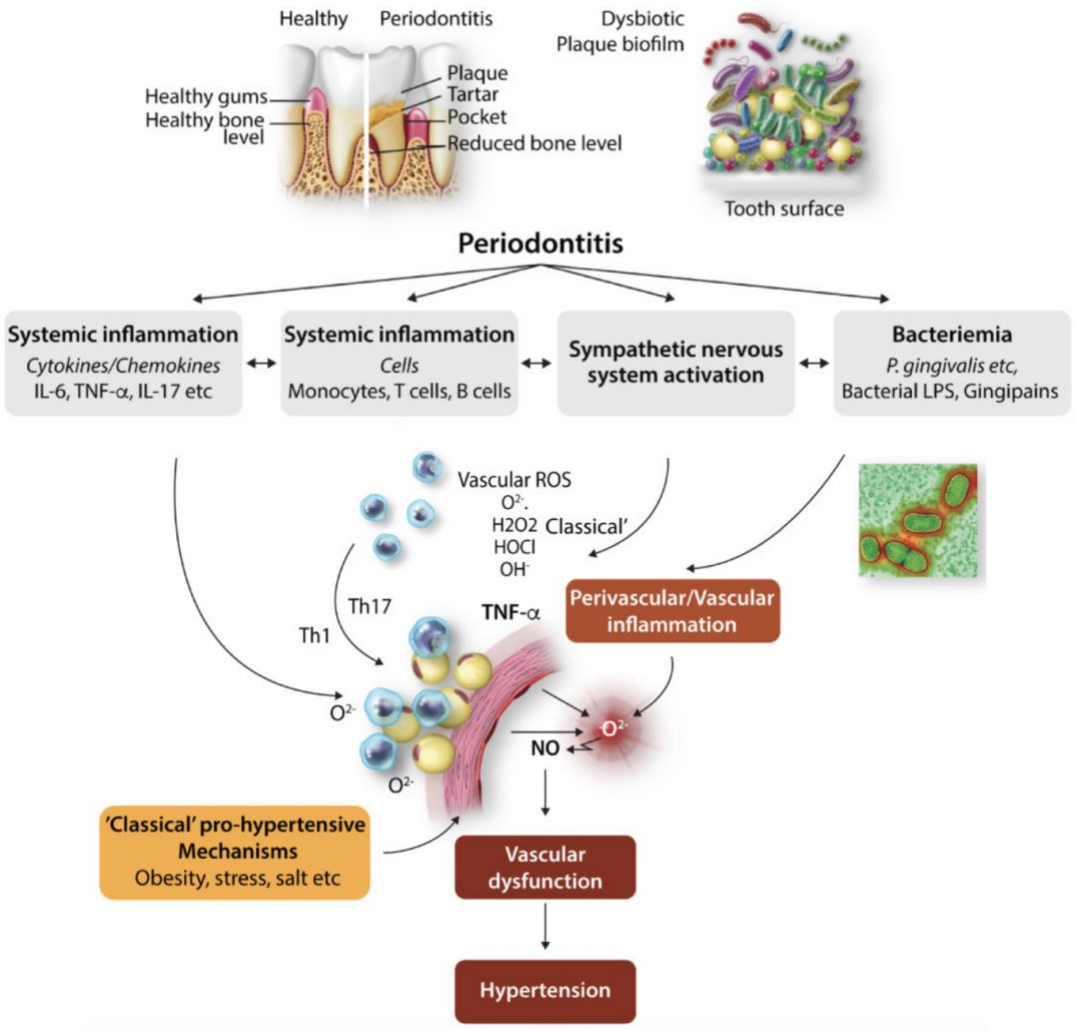

Fig 9: Periodontitis leads to hypertension and vascular dysfunction. Prospective mechanisms causally linking periodontitis with hypertension and vascular dysfunction. NO, nitric oxide; O-2, S, superoxide; Th, T-helper cells; ROS, reactive oxygen species[129] *(by Aguilera et al 2020)*

New approaches such as massive sequencing correlated findings from different samples, including atheroma and subgingival/oral samples, [130] thereby confirming that periodontal pathogens can reach and colonize

atheroma plaques. Living *P.gingivalis* and *Aggregatibacter actinomycetemcomitans* were found in atheromatous tissue.[131] Several in vitro studies indicates also that different periodontal pathogens, in particular, *P.gingivalis* colonize endothelial cells [6,132,133] .

The hypothesis of a remote pathogen action is also supported in animal models. Rabbits with experimentally induced periodontal disease, develop fatty streaks in the aorta.[134] In normocholesterolemic pigs, *P. gingivalis* induced aortic and coronary lesions,[135] while it accelerated atherosclerosis in murine models.[136]

Finally, in recent studies, the treatment of animals with patients sera induced endothelial dysfunction in mice, [137] and the up-regulation of ET-1, ICAM-1, E-selectin, IL-1, and sCD14,[138] all surrogate markers of the endothelial activation and inflammatory response. However, data on periodontitis direct outcomes in humans at risk of developing atherosclerosis remain scarce.

III.4 Periodontitis and systemic diseases: impact of *Porphyromonas Gingivalis* on cardiovascular disorders

Being involved in different systemic pathologies, including rheumatoid arthritis, cardiovascular pathologies, and neurodegenerative diseases, *P. gingivalis* is a clear evidence of correlations existing between oral health and systemic inflammation. [139] (see fig 10)

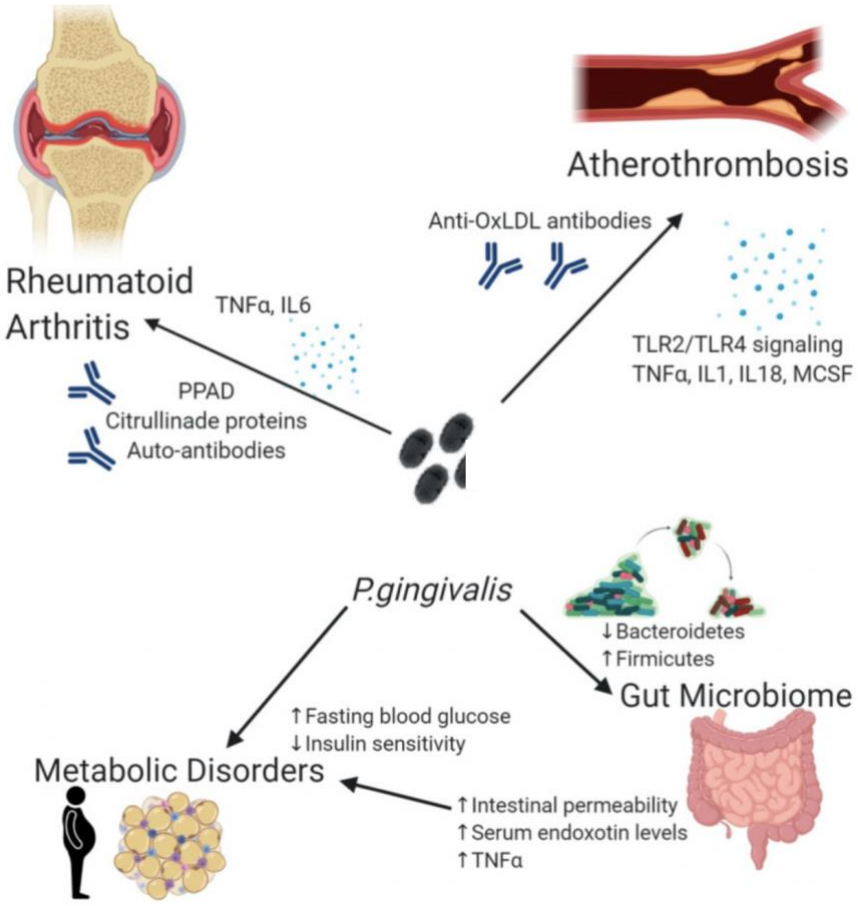

Fig 10 : *P. gingivalis emprises the progression of multiple chronic inflammatory diseases. Through the cross-reactive mediators in atherothrombosis, rheumatoid arthritis, the increased levels of systemic inflammation, gut microbiome dysbiosis, metabolic disorders. (↑ = increase ↓= decrease).* [140] *(by Mulhall et al 2020 & authorized by co-author Olivier Huck)*

P. gingivalis secretes a peptidyl arginine deiminase (PPAD) with a broad spectrum of action and particular interest in atherosclerotic lesions. [141] In addition, the periodontium of periodontitis patients with no signs of rheumatoid arthritis expresses citrullinated proteins. Furthermore, the serum from these patients contain higher levels of antibodies to citrullinated and non-citrullinated human peptides, suggesting that the overload in citrullination caused by periodontitis leads to a loss of tolerance to citrullinated peptides. This strongly

suggests a cross-reaction against citrullinated proteins of the joint, eventually favoring rheumatoid arthritis. [142] (see fig 11)

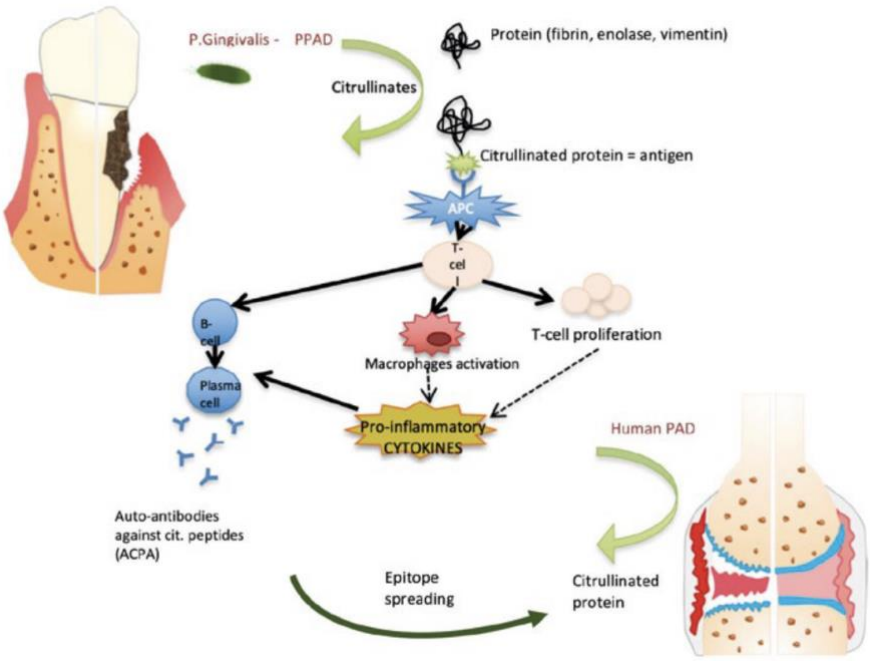

Fig 11: P. gingivalis in rheumatoid arthritis. P. gingivalis breaking immune tolerance to citrullinated proteins in rheumatoid arthritis (RA) by bacterial citrullination of proteins through PPAD, initiating an immune response leading to loss of tolerance for citrullinated joint proteins in the joints.

Abbreviations : PPAD: peptidyl arginine deiminase from P. gingivalis; APC: antigen presenting cell; TNF: tumour necrosis factor; IL: interleukin; GMCSF: granulocyte-macrophage colony-stimulating factor[143] (by Lopez-Oliva et al 2019)

Higher levels of autoantibodies against citrullinated proteins were assessed in patients with rheumatoid arthritis, they were targeting citrullinated histone 2B and correlated the presence of high scores of higher coronary artery calcification. The data suggest a potential role of sero-reactivity to citrullinated histones in atherosclerosis,[144] an hypothesis supported by growing evidences of the role of NETosis in immunothrombosis. Chronic oral infection by P.

gingivalis results in significant increases in oral bone resorption, aortic inflammation, viable bacteria in oral epithelium and aorta, and plaque development. *P. gingivalis* persists within vascular tissue through cell-to-cell transmission [145] and was reported to accelerate atherogenic plaque formation in ApoE mice fed with a high fat diet.[146]

In mice, periodontal pathogens induce myocarditis and/or myocardial infarction[147]. (see fig 12) Relationships between periodontal disease and myocardial infarction were hardly replicated or confirmed yet. [148] [149]

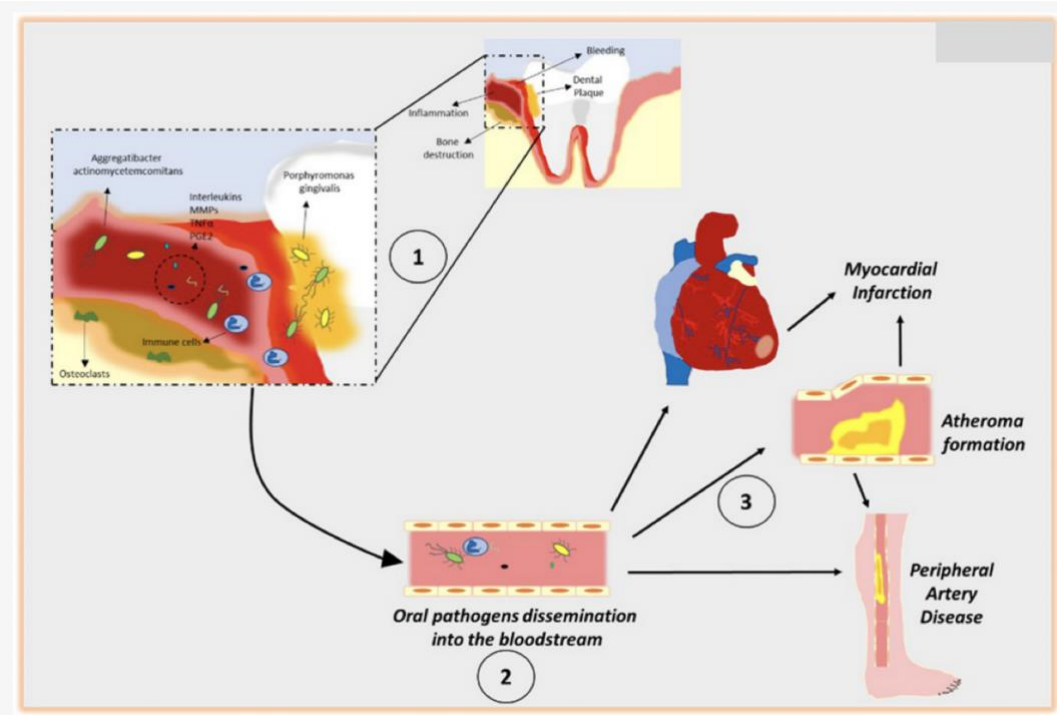

Fig 12: Possible inflammatory mechanisms linking periodontitis to Cardiovascular diseases (CVDs). (1) The imbalance in pathogens of the biofilm leads to gingival epithelium inflammation that releases inflammatory mediators, such as interleukins (ILs), prostaglandin E2 (PGE2), tumor necrosis factor alpha (TNF-α) and MMPs, that recruit immune cells. This inflammatory response induces alveolar bone reabsorption, by osteoclasts. (2) At a chronic

stage, oral pathogenic dissemination into the bloodstream leads to the onset of CVDs including atherosclerosis, myocardial infarction and peripheral artery disease (3).

III.5. *P.Gingivalis* impact on endothelial response

In vitro, the endothelial response to *P. gingivalis* has been assessed using HUVECs. LPS of *P.Gingivalis* or the bacteria itself, lead to the emission of proapoptotic microvesicles (MVs).[6] In addition, It was found that Gram-negative bacteria produce outer membrane vesicles (OMVs) at various stages of their growth, especially in the environment of infected tissues. [150] *P.gingivalis* OMVs were found more virulent than the parent bacteria[151] . OMVs strongly promote co-aggregation between different oral bacterial species that do not or only weakly copolymerize such as *Streptococcus*, *Actinomyces*, and *Candida albicans*. OMVs also inhibit and disperse biofilms in a gingipain-dependent manner, thereby creating a favorable environment for *P. gingivalis*. Moreover *P. gingivalis* OMVs enhance adhesion and invasion of epithelial cells by *Tannerella forsythia*[152]. (see fig 13)

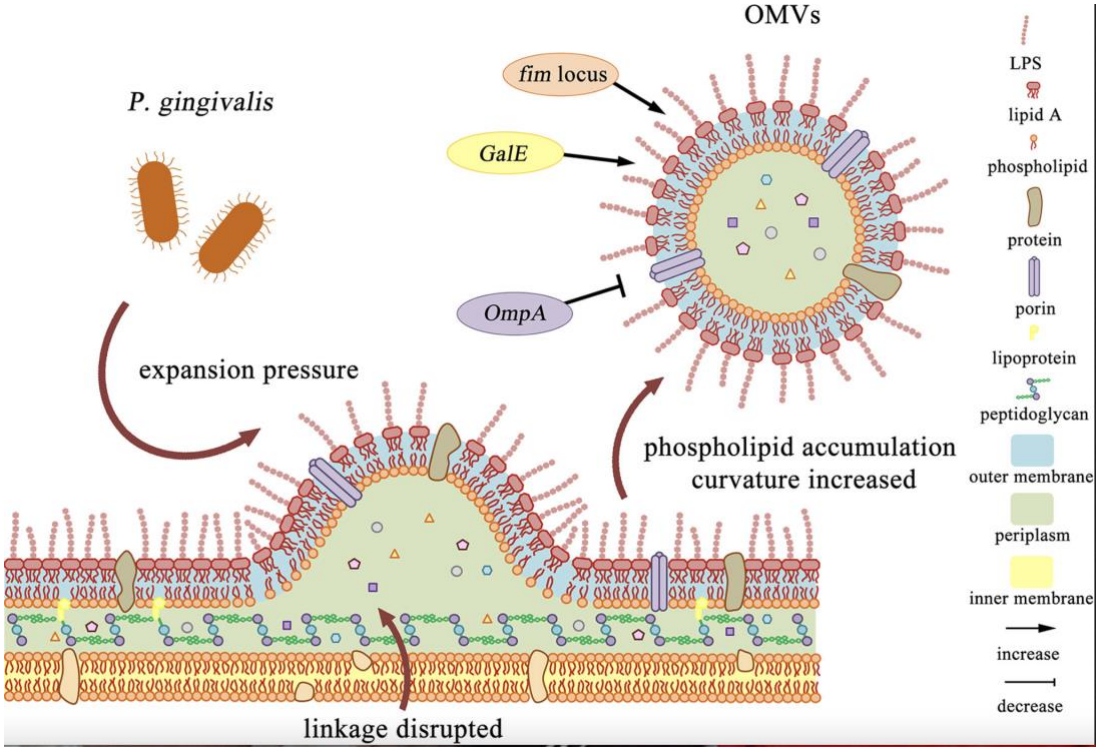

Fig 13: Production of P. gingivalis outer microvesicles (OMVs). 1. After the cell wall disruption, phospholipids accumulation in the outer membrane leaflets, and the expansion pressure continues to produce, which intensifies the further enrichment of outer membrane components. The linkage between the peptidoglycan and the outer membrane layer is disrupted, and finally P. gingivalis OMVs are formed. 2. The fim locus and GalE mutant strains reduced or even eliminated the production of P. gingivalis OMVs, while the OmpA mutant strain overproduce P. gingivalis OMVs. [153] *(By Zhang et al 2021)*

Bacteria associated with periodontitis can pervade into coronary vessels as well [154]. The major consequence of *P. gingivalis* infection is the proliferation of smooth muscle cells in the distal aorta and the risk of aneurysms.[155] It was found that *P. gingivalis* OMVs promote calcification of vascular smooth muscle cells in a concentration-dependent manner through ERK1/2-RUNX2. [156]*In vitro* and *in vivo* models showed that *P. gingivalis* OMVs significantly increase vascular permeability leading to vascular edema and mortality in a gingipain-dependent manner.[157]

These findings reinforce the hypothesis that P. gingivalis infection is a risk factor in cardiovascular diseases and in the development of atherosclerosis. [158] Nevertheless, the clinical assessment of all pathological aspects potentially supported by chronic gingival infection by *P. gingivalis* remains to be strengthen.

In addition, the respective contribution of the cellular damages and the sequential occurrence remain to be established.

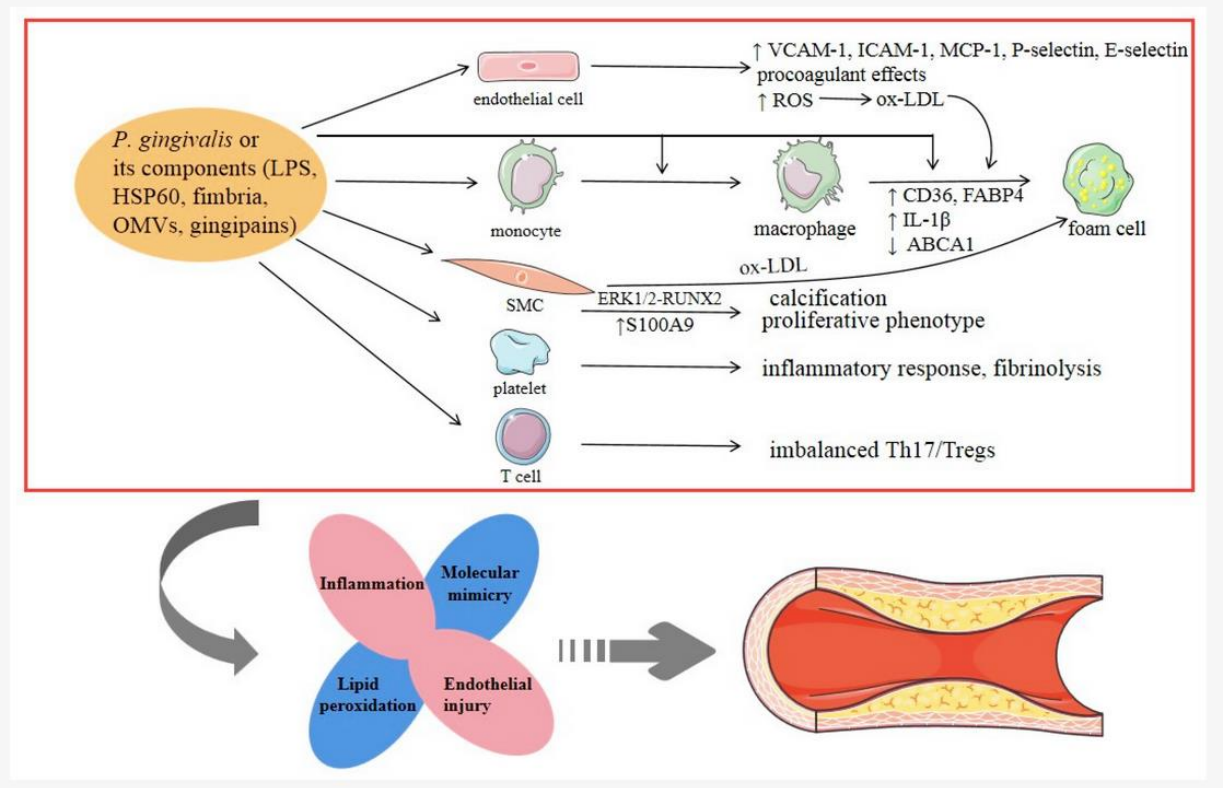

Fig 14: P.gingivalis-induced stimulation of accelerated atherosclerosis. Possible mechanisms: inflammation , endothelial injury, lipid peroxidation, and molecular mimicry. ox-LDL: oxidized low-density lipoprotein. [159] *(by Mai et al 2020)*

Chapter IV: The spleen

VI.1 Anatomy and cell composition

The spleen is the largest secondary lymphoid organ in the body and possesses a wide range of immunological functions.[160] It is divided by function and structure into the red and white pulp separated by the marginal zone (MZ) in rodents and the perifollicular zone in humans. Lacking afferent lymphatic vessels, all cells and antigens enter the spleen via the blood flow. The White pulp (WP) the primary immunologic region of the spleen in both species, and the red pulp (RP) constitutes the majority of the tissue with a distinct immune function. [161]

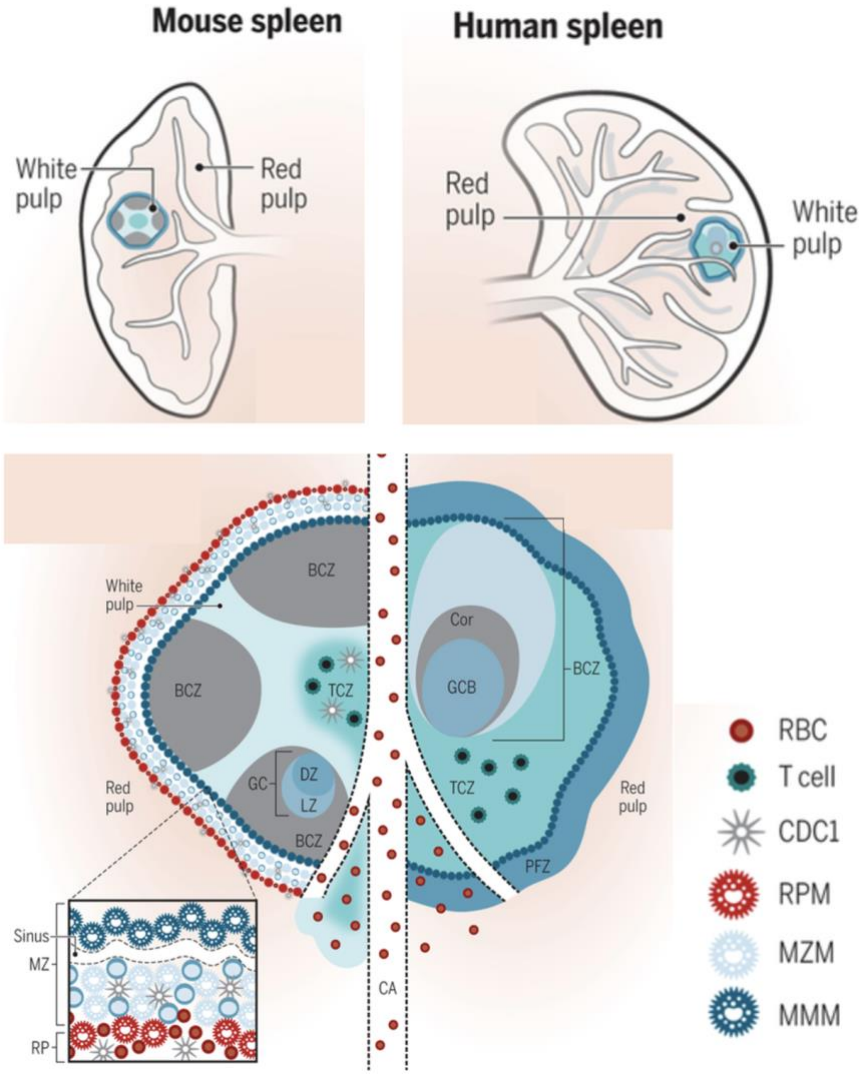

Fig 15: Mouse and human splenic immune cellular architecture.

Structural differences between the murine and human splenic immune system.

The organization of T cell zone (TCZ) and B cell zone (BCZ) follicles ,shown with light zone, LZ, and dark zone, DZ, organization in mouse spleen within the WP and the border between the WP and RP, the MZ (marginal zone) in mouse or perifollicular zone (PFZ) in human (dark blue outer ring). The extent to which the mouse MZ and human PFZ are analogous remains unknown. the precise layering and composition of macrophage subsets in the MZ is known for mice (bottom left box) CD169+ MMMs (dark blue) form a concentric ring around the WP with MZMs (light blue) and MZB cells (darker blue) but not for humans due to imaging techniques limitations. In humans, MZB cells surround activated B cells, containing a GC (In the human spleen on the right) and Corona ("Cor"). The homeostatic location of dendritic cell (DC) subsets in mice is shown (with cDC2s in the bridging channel, BC, and

cDC1s in the TCZ, MZ and RP, red pulp). Release of blood into the MZ of the WP from a central arteriole (CA) is shown. [160](Lewis et al 2019)

Immune cells in the spleen include different subsets of T and B cells, dendritic cells (DC) and macrophages making the spleen to be the most important organ for antibacterial and antifungal immune interactions, but also providing numerous other functions namely filtration, destruction of old red blood cells (RBCs) [162] and downstream activation of adaptive immune cell types. [163]

VI.2 Splenic Immune response and microvesicles

Spleen microvesicles (SMVs) are representative markers of pathophysiological conditions. Elevated levels of thrombocytes and their MVs were observed in splenectomized zebrafish.[164] In vitro, isolated rats SMV initiate an early pro-inflammatory, pro-coagulant and a pro-senescent response in endothelial coronary artery cells. [4] The SMV content and their noxious properties vary according to the age of the rat and could be counteracted by oral treatment. [7] In addition, injection of *P. gingivalis* significantly increased the shedding of procoagulant SMVs. In addition, the important role of neutrophil-derived MVs isolated from the spleen and inducing endothelial senescence was confirmed by immunodepletion.[4]

Chapter V: The heart

V. 1 Immune cells in the heart

The heart tissue is not only a muscle submitted to electrical pulses but also the host of a variety of cells. In addition to cardiomyoctes and mesenchymal cells, the cardiac tissue contains immune cells and all the cellular equipment of a vessel like endothelial cells, pericytes and circulating blood cells. The macrophages are the major immune cell population in the resting heart. They are found in the interstitium and around endothelial cells. Inflammatory monocytes and neutrophils are not present in the myocardial tissue but can be observed upon tissue damage. Mast cells, dendritic cells, B cells, NK cells and regulatory T cells are found sporadically in cardiac tissue.

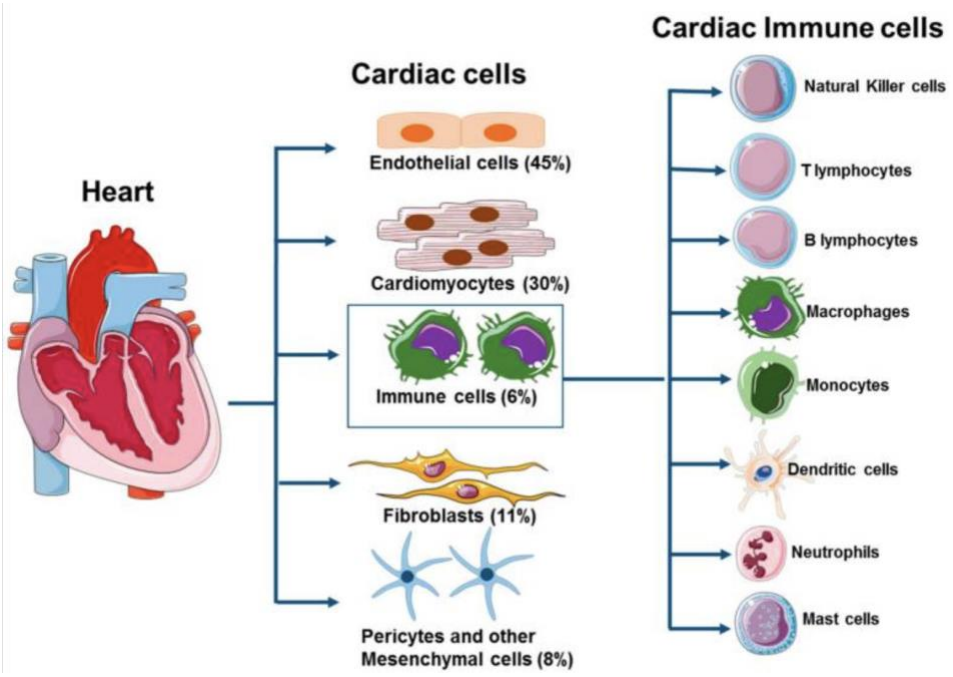

Fig 16: Cellular composition of the heart. The heart tissue contains different sub-types of immune cells depending on the physiopathological condition.

V.2 Evidences of inflammation in cardiac tissues

Inflammation has been implicated in the pathophysiology of various cardiac diseases. Heart failure and cardiomyopathy are associated with a 4–6-fold increase in atrial fibrillation prevalence.[165] In the pathophysiology of heart failure, inflammation modulates the cell signaling activation patterns associated with fibrosis, apoptosis and hypertrophy[166]. In addition, serum levels of tumor necrosis factor-α (TNF-α), were significantly in patients with elevation of diastolic heart failure and a history of atrial fibrillation suggesting an inflammatory signature and a possible paracrine effect.[167]
The inflammatory response to ischemia and reperfusion is a basic process for cellular debris removal and injured tissues repair. Similarly to the neurohormonal activation observed after acute myocardial infarction, inflammation might as well induce a systemic response that favors adverse remodelling.[168]

Through inflammatory processes, the NLRP3 inflammasome in the heart is activated by two independent steps: Initiating and triggereing. (see below)

V.3 Inflammatory signalling pathways in cardiac tissues

The inflammatory pathways in the heart trigger the inflammosome and down-stream apoptosis. Activation will be initiated through the TLR-dependent sensing of damage-associated molecular patterns (DAMP) exposed by pathogens or injured cells or tissues at the vicinity of the cardiac cells. This

“priming” of the inflamosome pathway initiates the NF-κB-dependent transcription and the up-regulation of various components of the inflamosome cascade like caspase-1, NLRP3 and ASC and the unmature forms of IL-1β, IL-18, (Pro-IL). Priming may also occur directly through NOD2 that also tagets NF-κB. The primary signal is necessary but insufficient to form the inflammasome in cardiomyocytes in the absence of the trigger signal. Eventually, the response will be amplified through P2X7 receptors activated by high concentrations of extracellular ATP released by activated or damaged cells. P2X7 receptors activation leads to K+ efflux, a step that triggers NLRP3 activation. Another mechanism triggering the inflamasome, is the lysosomal destabilization by indigestible material that leads to the leakage of the cathepsin B and the induction of K+ efflux favoring NLRP3 activation. Upon binding to the serine threonine kinase Nek7 that senses the K+ efflux, NLRP3 is activated by phosphorylation. In cardiomyocytes, the mitochondria count is high and their damage causes important oxidative stress through the release of ROS leading to the unfolded protein response also targetting NLRP3 via the capture of ROS by TXN further enabling the dissociation of the complex TXN/TXNIP. Mitophagy and autophagy maintain low levels of NLRP3 activation by effective clearance of mitochondrial debris that otherwise would favor lysosome destabilization.

After ischaemia, the activation of the inflammosome occurs upon binding of the tyrosine–protein kinase BTK and of apoptosis-associated speck-like protein

containing a CARD (ASC). The active NLRP3 oligomer functions as a platform for the polymerization of ASC into filaments, and in turn for the branching of caspase 1 filaments branches.

Finally, the activation of the inflammosome promotes the secretion of the pro-inflammatory cytokines IL-1 and IL-18 that are freed from their pro-peptide through the proteolytic cleavage by caspase 1. Gasdermin D (GSDMD) is also essential to interleukin secretion by forming a pore in the plasma cell membrane. Indeed, when cleaved by caspase 1 is able to oligomerize.

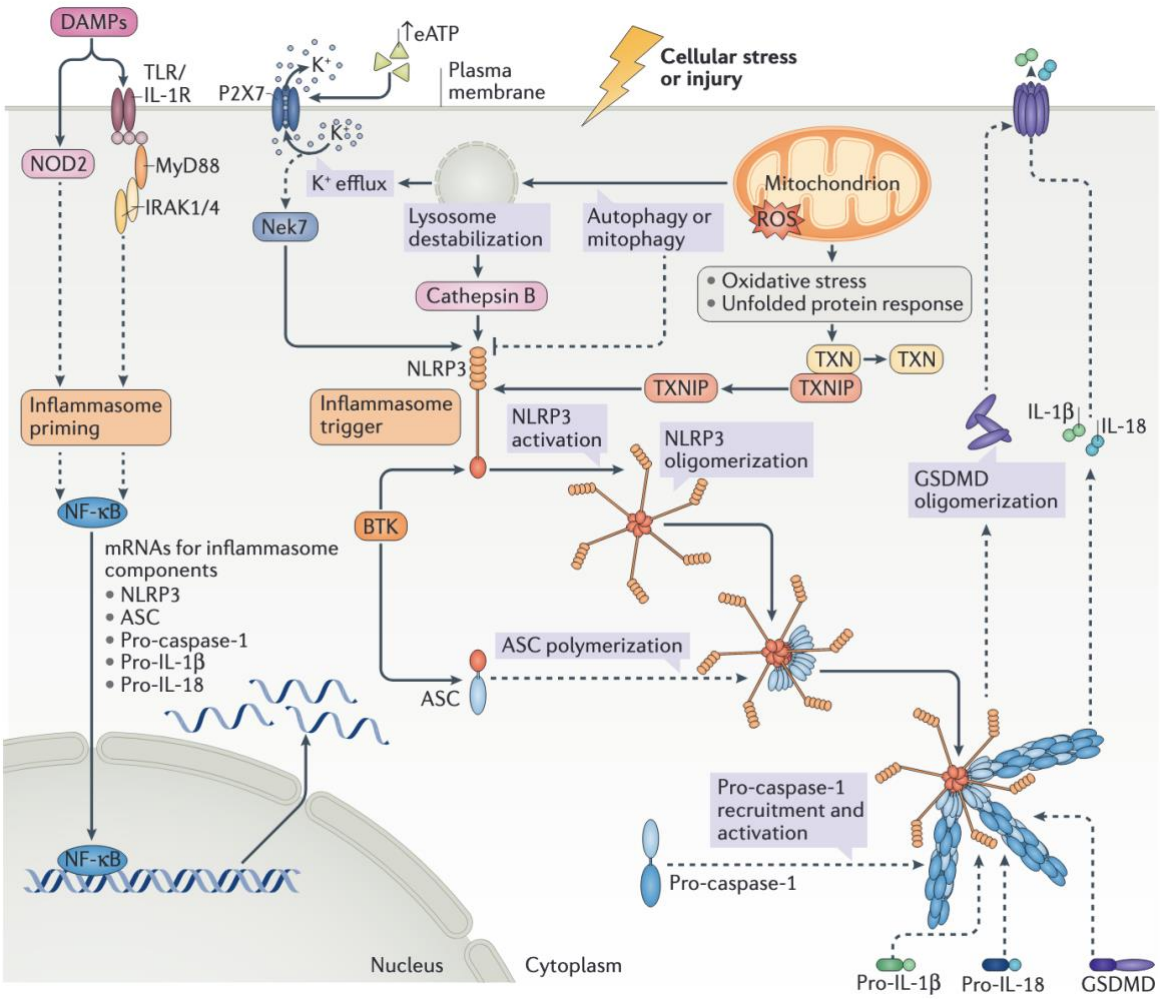

Fig 17: The formation of the inflammasome in the heart. [168]*(by S. Toldo and A. Abbate 2018) TLRs: Toll-like receptors; MyD88 myeloid differentiation primary response protein, and IRAKs: interleukin-1 receptor-associated kinases). NOD2: nucleotide-binding oligomerization domain-containing protein 2. TXNIP): Thioredoxin-interacting protein; (TRX) thioredoxin.*

Chapter VI:
Endothelial and immune senescence

VI.1 The different types of senescence

Aging is a universal, ingrained process distinguished by the progressive loss of tissue and organ function over time, resulting in progressive risk of pathologies, such as cardiovascular disorders, cancer, diabetes and neurodegenerative diseases. [169] The theory of aging affirms that human aging is the result of cellular aging, whereupon an enlarged proportion of cells reach senescence.[170] Originating from the Latin word senex, senescence means growing old. It has been demonstrated that senescent cells accumulate in tissues with age.[170]

Cellular senescence is defined as a cellular state of stable and long-term loss of proliferative capacity, withholding normal metabolic activity and viability. [171] It is an irreversible growth arrest that arises in response to persisting or sustained damaging stimuli, such as DNA damage, telomere shortening, telomere dysfunction and oncogenic stress leading to suppression of potentially dysfunctional, transformed, or aged cells.[172]

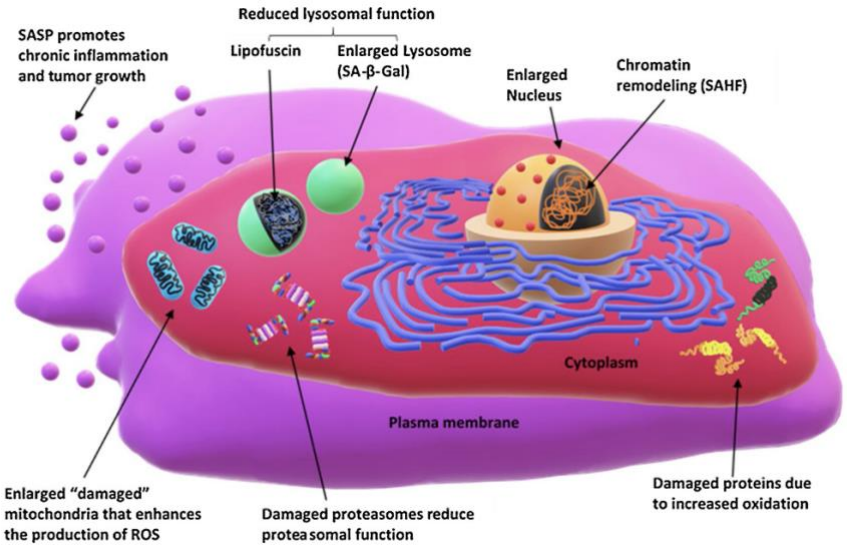

Fig 18: Signs of cellular senescence. [170]*(by Kamal et al 2020)*

Based on the stimuli's nature, cellular senescence can be categorized into telomere-dependent senescence and non-telomeric senescence respectively termed replicative senescence and stress-induced premature or accelerated senescence. [173] DNA damage, oncogenic mutations, reactive metabolites, mitogens, insulin-like growth factor 1 (IGF-1), telomere erosion, epigenetic stress, proteotoxic stress, and damage-associated molecular pattern proteins (DAMPs) are various types of senescent stimuli.

Characterized by a flat, expended morphology, and resistant to apoptosis, senescent endothelial cells also reveal changes in gene expression and chromatin organization and share common senescence-associated biomarkers such as senescence associated β-galactosidase (SA-β-gal) [174-176] and a senescence-associated secretory phenotype (SASP) that has IL-1α, IL-

6, NFκB, C/EBPβ, ROS, or GATA4 as regulators. “Senomorphics” block the harmful and deleterious effects of the SASP. As a counter-regulatory response, senescent cells up-regulate their anti-apoptotic pathways (SCAPs), thus promoting high resistance to apoptosis. In turn, SCAPs represent the targets of “senolytics” that selectively kill senescent cells by impairing the SCAPs. [177]

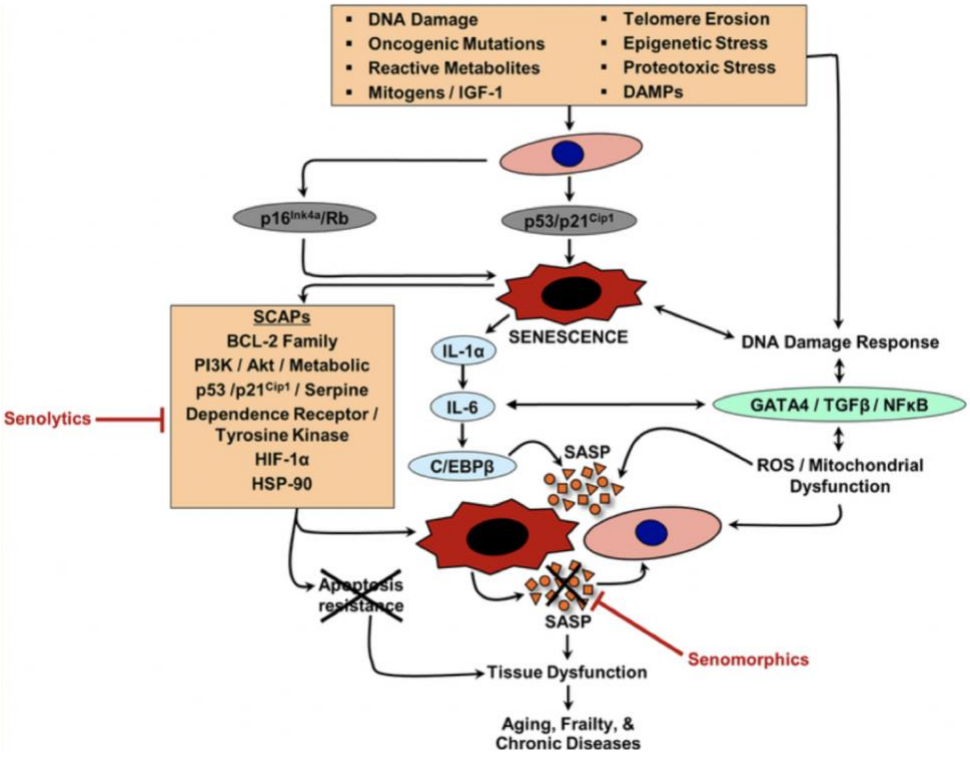

Fig 19: Mediators of senescence, SCAPs, the SASP, and effects of senescent cells. [177] *(by J. N. Farr and S. Khosla 2020)*

VI.2 Endothelial senescence in inflammation, atherosclerosis, thrombosis and aging

The Increase mean age of populations is a crucial challenge in the pathogenesis of cardiovascular disease (CVD) [178] [179]. Senescent ECs exhibit reduced endothelial nitric oxide (NO) production and increased endothelin-1 (ET-1), enhancing thrombosis, inflammation, and atherosclerosis with impairment of vessel tone, angiogenesis, and vascular integrity, [180] all driving the progression of CVD.[181] However, the molecular mechanisms of EC senescence, their initiation and control are not yet completely understood.

In advanced human atherothrombotic plaque lesions, there is an increased expression of senescent markers p16 and p21 easily observed in vascular smooth muscle cells (VSMCs), and associated with the large flattened cell shape and S-A βGalactosidase activity. Through replicative senescence, human VSMCs adopt an “osteoblast-like” phenotype and promote vascular calcification. In addition, they produce less collagen and secrete more proteases. As detailed above, senescent VSMCs secrete various pro-inflammatory cytokines as part of their senescence-associated secretory phenotype (SASP). These cytokines stimulate adjacent non-senescent ECs and VSMCs to express adhesion molecules and release cytokines, and/or trigger monocyte (MA) recruitment. Also VSMC senescence promotes fibrious cap thinning. Plaques manifest a direct evidence for replicative VSMC

senescence in human atherosclerosis, where telomere integrity, oxidative stress, plaque vulnerability and inflammation are potential triggers.[182]

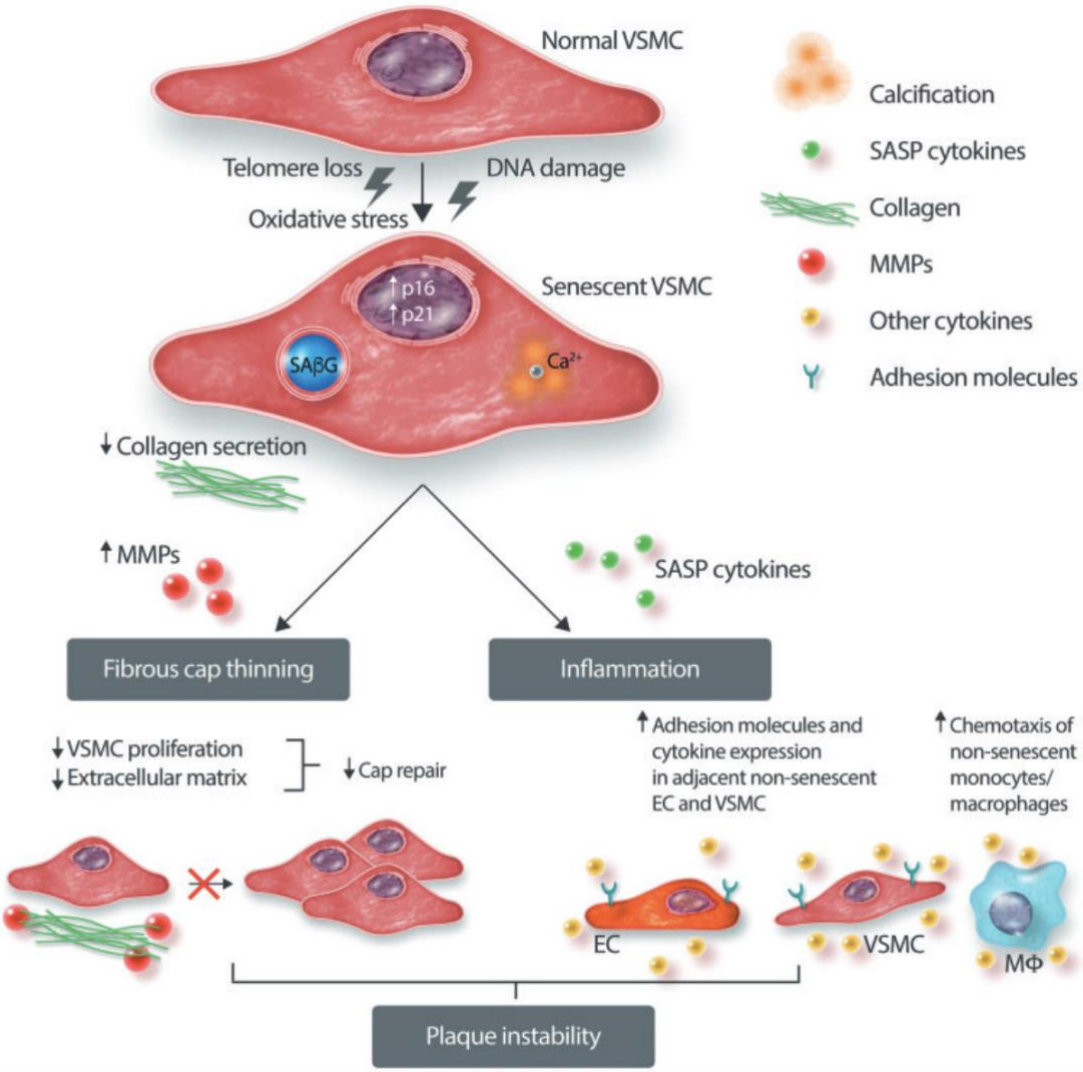

Fig 20: VSMC senescence in atherosclerosis. Through the establishment of the SASP, senescent VSMCs that are Unable to proliferate, put on the line plaque instability and inflammation. [182] *(by Gorenne et al 2006)*

VI.3 Microvesicles of leukocyte and endothelial origin as effectors of endothelial senescence

MVs from patients with coronary artery disease promote increased endothelial expression of SGLT1, SGLT2, VCAM-1, and the down-regulation of eNOS, and induce endothelial dysfunction and senescence. Previous data from the laboratory suggest the role of spleen-derived MVs in endothelial senescence. In rats, age was an indispensable trigger for the shedding of pro-senescent spleen-derived leukocytes microvesicles (SMVs) measured after spleen harvest. Such SMVs prompted premature senescence of primary coronary endothelial cells. The enhanced and progressive release of pros-senescent EMVs shed from middle-aged and old rat splenocytes suggested the feasability an early pharmacological control of the pro-senescent effects of SMVs. Indeed, oral treatment of the animals by omega-3 reduced and even abolished the pro-senescent action of the SMVs.[7] Using the same SMV endothelial cross-talk model in rats, SMVs generated from LPS or PMA/calcium ionophore treated splenocytes were able to trigger endothelial dysfunction and early senescence, suggesting that bacterial infection may also trigger endothelial senescence. [4]

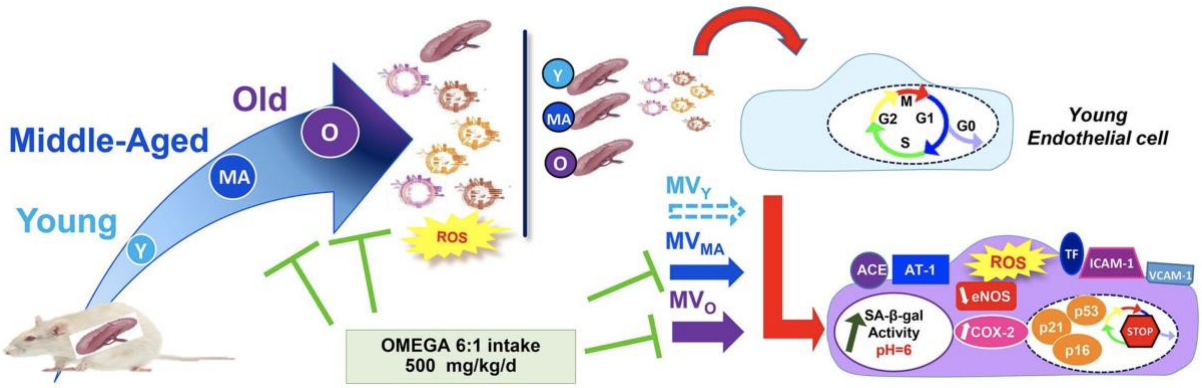

Fig 21: SMVs become prosenescent with ageing as measured in coronary endothelial primary cells. *ECs show a pro-inflammatory and procaogulant phenotype with over-expression of VCAm-1, TF, ICAM-1, ACE.and down-regulation of eNOS and over-expression of COS-2. MVy: SMVs isolated from young individuals, MV_{MA} from middle-aged rats, MV_O from old rats Intake of a specific formulation of omega-3 abolishes the noxious properties of SMVs (by Qureshi et al 2020 & authorized by the first author Qureshi AbdulWahid)*

VI.4 Immunosenescence

Immunosenescence is a process of immune dysfunction that happens within age. It remodels lymphoid organs and is associated with infections, autoimmune diseases, and malignant tumors. Degeneration of the thymus and declined IL-7 secretion are major factors of immunosenescence, followed by a decrease in telomere length and telomerase activity. [183] Senescent T cells show reduced SA-β-galactosidase activity, cytotoxic activity, expression of functional molecules such as IFN-γ, scarce proliferation, and also arrested cell cycle, due to an increase expression of the cyclin kinases, P16 and P21, and elevated ROS production. [183] Circular RNAs, and microRNAs were reported as specific T-cell senescence biomarker [184] [185]. As a proof of concept of the impact of immunosenescence, 7-week-old C57BL/6 irrradiated mice transplanted with

bone marrow cells from 48-week-old individuals showed less functional deterioration and histological tubular injury than those treated with 8-weeks old bone marrow cells. The parodoxical beneficial effect in the acute phase of kidney injury may have been due to a decreased inflammatory response possibly linked to the proportion of senescent BM cells . [186]

Nevertheless, the contribution of each immune cell line, belonging to either adaptative or innate immune responses, remains to be deciphered during immunosenescence.

When stimulated with proinflammatory stimulus such as LPS, neutrophils are the first recruited at the injury site with a quite short life-span. While their count increases with ageing, they show altered effector functions such as chemotaxis and intracellular killing but phagocytosis and their adhesive ability remain stable. Senescent neutrophils produce increased amount of metalloproteinase and cytokines , NF-kB continuous activation and PI3Kinase modified pathways.[187,188]

Natural killer cells as well as macrophages are also affected in immunosenescence. NK cells increase in number while their cytotoxicity decreases and macrophages proliferation becomes impaired.[188,189] As for B cells, antibodies diversity is limited, disabling the immune system from high affinity antibodies production. [187]

Together, all these age-related changes decrease effector functions and increase the susceptibility to infections and various pathological cell responses

like dysfunctional mitochondria, oxidative stress and NLRP3 inflammosomme activation. Inflamm-ageing is also associated with central obesity and increased gut permeability and contributes to the initiation and progression of atherosclerosis , diabetes, dementia peripheral vascular and chronic diseases.[190]

Chapter VII:
Neutrophils: a revised role in immunity and thrombotic disorders

VII.1 Neutrophils: a first line of defense against pathogens and actors of innate Immune response

Neutrophils are main actors in the innate immune system, they are heterogeneous in morphology, phenotype and function. [191] They are incessantly generated in the bone marrow from myeloid precursors and can reach up to 2×10^{11} cells produced daily.[192] During inflammation, neutrophils are recruited in tissues, and with time the cells undergo apoptosis and are removed by macrophages and dendritic cells.[193] In humans, 50–70% of circulating leukocytes are neutrophils, whereas only 10–25% are in mice169,170. Three types of neutrophil granules are formed consecutively during their maturation, and they are filled with pro-inflammatory and degradation proteins.[192,194,195] They are azurophilic (primary) granules, containing myeloperoxidase (MPO), specific (secondary) granules, containing lactoferrin, and gelatinase (tertiary) granules, containing matrix metalloproteinase 9 (MMP9).[196]

Different intra and extracellular mechanisms are implicated in pathogens elimination by neutrophils. First, neutrophils have a phagocytosis ability. After they are encapsulated in their phagosomes, pathogens are destroyed by ROS produced by NADPH oxygenase and/or cleaved by proteolytic proteins released by the granules (cathepsins, defensins, lactoferrin and lysozyme). [192,197]

Second, the antibacterial proteins, are also released from the neutrophil granules into the extracellular milieu via a specific process termed degranulation, thereby enabling the destruction of extracellular pathogens. Highly activated neutrophils can eliminate extracellular microorganisms by releasing neutrophil extracellular traps (NETs). [198]

VII.2 Netosis and formation of NETs

Netosis is the mechanism of neutrophil extracellular traps (NETs) formation.[199] NETs are made of decondensed chromatin that forms meshes of DNA fibers with approximately 200-nm pores[200]. NETs are coated with nuclear proteins (histones), granule proteins, like neutrophil elastase and myeloperoxidase, and cytosolic proteins such as S100 calcium-binding proteins A8, A9, and A12, as well as actin and α-actinin[201-203].

NET formation occurs via two processes: non-lytic or vital NETosis and lytic NETosis. Both lytic and non-lytic NETosis can cause thrombosis. In lytic fibrosis, PAD4 causes hypercitrullination of histones driving nuclear swelling due to chromatin decondensation, followed by release of NETs containing DNA and histones.

When Occurring without the necessity of cell lysis, the release of extracellular traps is mediated by vital NETosis.[204]

Upon stimulation, eosinophils [205], basophils [206], mast cells [207], and macrophages [208] were also described to release extracellular DNA.

Initiation of the NETosis signaling occurs through the ERK MAPKinase pathway that triggers nuclear activation of chromatin decondensation and the shattering of the nuclear membrane. The shedding chromatin fibers by passing through the cytoplasma embark bactericidal proteins like myeloperoxidase, neutrophil elastase and also bear citrullinated histones. Histone citrullination is mediated by peptidylarginine deiminase (PAD) and is mandatory for NET release**.**

VII.3 Neutrophils and NETs in sterile Inflammation

Inflammation in the absence of pathogens and their products is referred to as sterile inflammation. [210] Numerous stimuli may lead to sterile inflammation like ischemia reperfusion, macroscopic or particle-induced trauma. In this case, immune cells (DCs or macrophages) or nonhematopoietic cells (endothelial or epithelial cells) sense a variety of stimuli and initiate inflammatory pathways, some of them being shared with pathogen-induced inflammations (TLR, NALP3,) whereas others (RAGE, IL1-β-R) are distinct to sterile inflammation (see figure 24). In the end of the process, tissue repair relies on macrophages that clear necrotic or apoptotic neutrophils and produce factors that accelerate the healing process (resolvins, TGF-β). Growth factors released in the healing area by vascular cells (e.g., VEGF) also contribute to the restauration of the tissue. For instance, IL-4 produced by eosinophils is sensed by FAPs to mediate repair after acute skeletal muscle necrosis. Induced sterile

inflammation may trigger the adaptive immune responses, as in the case of organ transplantation.

Neutrophil recruitment in response to sterile inflammatory stimuli contributes to the immunological damages observed in many diseases, such as ischemic injuries/infarction, trauma, autoimmunity, drug-induced liver injury. As an ultimate neutrophil activation, Netosis takes place from 2–3 h up to 8 h from activation. About 20%–60% of isolated human neutrophils typically release NETs 2–4 h after contact with microbes or chemicals, however, they are able to respond within minutes when activated by LPS-stimulated platelets under conditions of flow.[212] It is worth noting that neutrophil stimulated in vitro by fMLP (N-formyl-Met-Leu-Phe) [213] release MVs but do not extrude NETs, suggesting that the two mechanisms are not directly dependent one from the other.

The mechanisms by which neutrophils respond to sterile tissue injury and cell death are of both homeostatic innate immune responses and pathogenic immune responses in disease.[214]

Neutrophils are leukocytes exposing CD11b and able to roll on the endothelium through interaction with different endothelial membrane proteins like E-Selectin, VCAM, ICAM . Tethered neutrophils are able to secrete chemokines (CXCLs) and interleukins (IL-8), and contribute to maintain the inflamed endothelial phenotype. In addition neutrophils also interacting with

naïve platelets via P-Selectin and Mac-1 binding promote platelet activation. Altogether, these interactions at the endothelial layer favor a procoagulant state at the vicinity of the site where neutrophils and maintain local inflammatory mediator production. Such mediators nurture cell activation and consecutive exposure of procoagulant phosphatidylserine as well as the induction of tissue factor (TF) finally exposed at the surface of both the inflamed endothelium and activated neutrophils. Importantly, TNF, IL-1 and ROS produced by the neutrophils extruding NETs are also inflammatory and oxidative mediators triggering ECs.

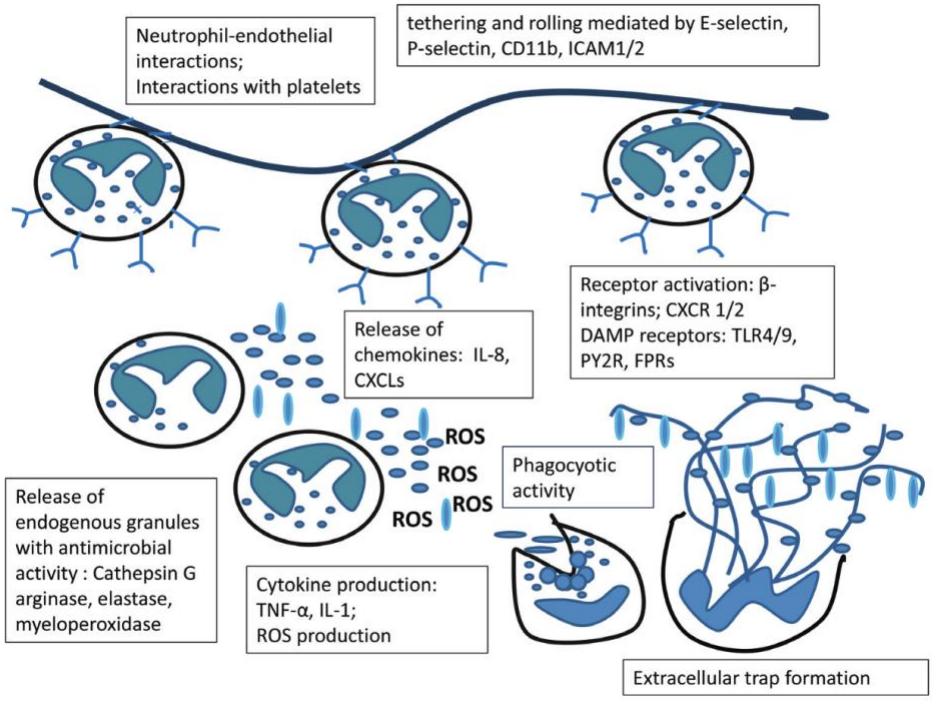

Fig 22: Neutrophils in response to sterile inflammation. Neutrophils produce NETs after activation by CD11b, integrin alpha M, ICAM, intercellular adhesion molecule; IL-8, interleukin-8 (chemokine receptor ligand 8); CXCLs, chemokine ligands; CXCR, chemokine receptor; DAMP, damage associated molecular pattern; TLR, Toll like receptor;IL-1, interleukin 1; PY2R, purinergic receptor; FPR, formyl peptide receptors; and TNFα, tumor necrosis factor-alpha.[212] *(by Edwards et al 2020)*

In murine models of deep vein thrombosis (DVT), it has been shown that the citrullinated histone H3 (citH3) born by NETs promotes a red blood cell and platelet rich thrombus.[215] Platelet activation by histones involves stimulation of platelet TLR2 and TLR4 receptors.[216] Activated platelets formed as a result of the NET-platelet interaction bind to neutrophils to promote NETosis.[217] This cycle favors thrombus formation.

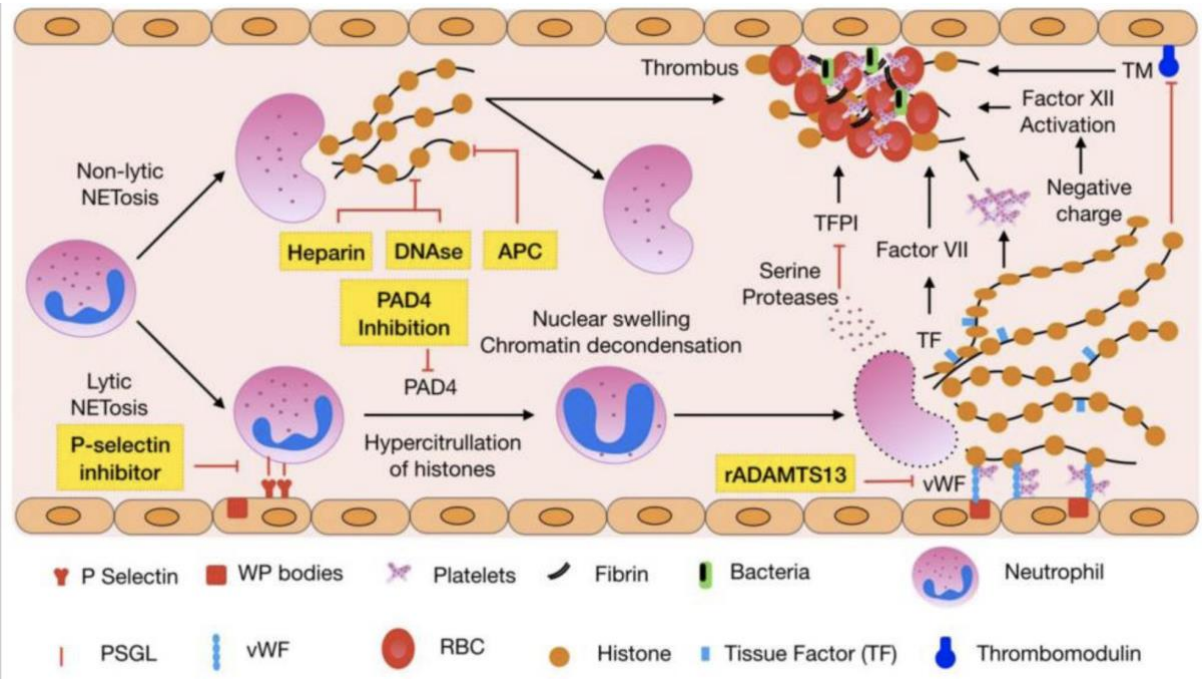

Fig 23: NET formation in thrombosis by lytic and non lytic NETosis *APC= activated protein C, DNAse= deoxyribonuclease, NET= neutrophil extracellular traps, PAD4= peptidylarginine deiminase, PSGL= P-selectin ligand 1, RBC= red blood cell, TF= tissue factor, TFPI= tissue factor pathway inhibitor, TM= thrombomodulin, vWF= von willebrand factor, WP= weibel palade bodies.* [218] *(by S. Kapoor, A. Opneja and L. Nayak 2018)*

Because DNA is a polyanionic molecule, a proportion of ots surface non covered by histones would offer docking sites for the recruitment of blood coagulation factors. These vitamin-K dependent factors would then bind to the exposed anionic charges via their Gla domain in the same way as they would on phosphatidylserine exposed by stimulated vascular cells. In a murine sepsis model by cecal-puncture ligation, NET-driven thrombin generation was abolished in factor XII knockout mice suggesting that the intrinsic coagulation pathway would act as an alternate process for coagulation.[219] Via interaction with von Willebrand Factor, a macromolecule that binds to platelets and endothelium, DNA-bound histones activate platelets and also exert an inhibitory action on thrombomodulin, the main endothelial anticoagulant membrane protein. Propensity to coagulation will be re-inforced by the release of granule serine proteases from the neutrophil undergoing NEtosis, that will inhibit the tissue factor inhibitor TFPI normally controlling the initial step of the coagulation cascade depending on tissue factor. Several strategies to limit NETs-driven coagulation have been proposed. They are based on 1) the use of DNAse to limit NET half-life at the vicinity or in the thrombus, 2) PAD4-inhibitors to reduce citrunillation of histones on chromatin fibers, 3) inhibitors of the interaction of platelets with vWF, like P-selectin inhibitors or ADAMS 13 that cleaves vWF, Recombinant proteins derived from the sequence of natural anticoagulants like activated protein C (APC) that interacts with TM to limit the generation of thrombin have been also evoked. Finally, heparin that enhances

the anticoagulant activity of antithrombin, a circulating natural anticoagulant trapping thrombin and factor Xa seems to have a dual benefit by also accelerating the activity of the DNAse 1, at least *in vitro.* [220]

VII.4 NETs and Microvesicles: tissue vascular partners in thrombogenicity

In humans, several studies demonstrated a positive correlation between the concentration of circulating NET markers and the atherosclerotic lesion size in different cohorts of patients. These markers contribute to enlarge the lesion size by attracting and activating monocytes and dendritic cells as well as releasing high amounts of inflammatory mediators, such as MMPs, NE, cathepsin G, and cathelicidin.[221] Once activated by platelet-derived CCL5 in early stages of atherogenesis, neutrophils adhere on arterial endothelial cells. The neutrophils degranulation products promote the endothelial recruitment and adhesion of monocytes via formyl-peptide receptors. Neutrophil proteins and NETs perpetuate local inflammation by activating dendritic cells and macrophages within the vessel wall. As a result, more LDL are converted into its oxidized form and facilitates foam cell formation and plaque growth. Finally, in late stages of atherothombosis, NETs can induce endothelial damage and promote the recruitment of new activated cells and potentially the erosion of the plaque.

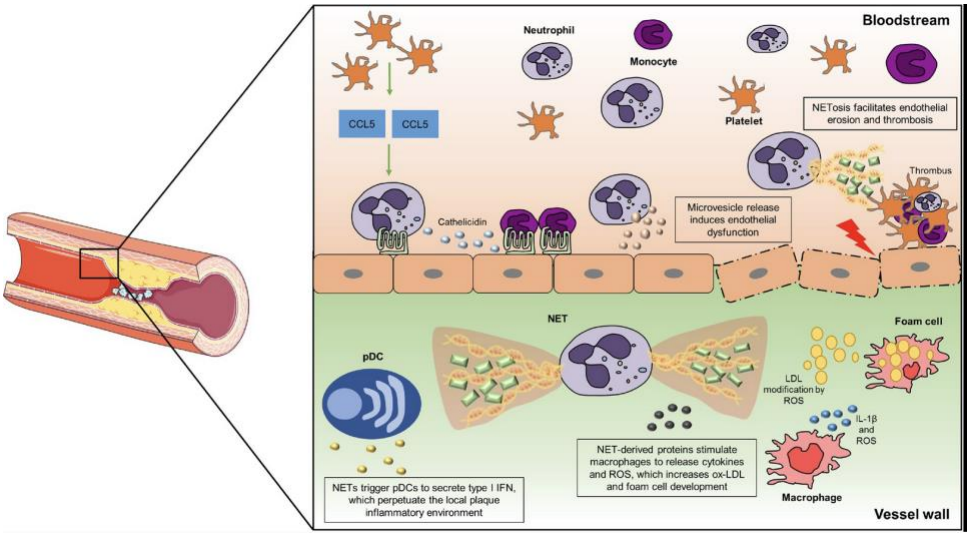

Fig 24: Role of neutrophils in atherosclerosis. CCL5, CC-chemokine ligand (CCL)5; IFN, interferon; IL, interleukin; LDL, low-density lipoprotein cholesterol; NET, neutrophil extracellular trap; Ox-LDL, oxidized low-density lipoprotein cholesterol; pDCs, plasmacytoid dendritic cells; ROS, reactive oxygen species. [221] *(by Bonaventura et al 2019)*

It is now demonstrated that, leucocyte-derived MVs induce endothelial activation thus enhancing protease and adhesion molecules expression resulting in endothelial dysfunction and leucocyte recruitment within the vessel wall.

Human polymorphonuclear neutrophils (PMNs) have the potency to release MVs and exosomes. [222] Such MVs contain granule proteins and surface membrane-expressed components such as complement receptors and CD59 released from the plasma membrane to the extracellular space.

Neutrophils-derived MVs express PS, annexin A1 and adhesion molecules such as CD11b. Of note, Annexin A1 has anti-inflammatory porperties by inhibiting phospholipase A2 (PLA2) and promotes neutrophil detachment as well as the phagocytosis of apoptotic neutrophils by macrophages. In fact,

Annexin A1 limits neutrophils extravasation and both exogenous and endogenous annexin A1 counter-act the action of activated effects innate immune cells, particularly the generation of proinflammatory mediators.

Protein analysis of neutrophil-derived MVs (NMVs) has revealed the presence of granule proteins such as myeloperoxidase, lactoferrin, elastase, matrix metallopeptidase 9, and proteinase 3. [223]. Altogether, data from the literature indicate that neutrophil-derived MVs, found at high levels at the injury site of injury and in bloodstream, amplify inflammatory processes with a variety of pathological outcomes ranging from autoimmune disorders to infectious conditions and atherosclerosis.[221]

El Habhab et al. showed that MVs shed from activated splenocytes prompt early pro-inflammatory, pro-coagulant and pro-senescent responses in coronary endothelial cells and that the depletion of neutrophil MVs from the culture medium preserved the endothelium. [4]

In summary, growing evidences of an association between neutrophil MVs and NETs open a large field of investigations to better understand the link between thrombosis and inflammatory responses and decipher the mechanisms and cellular partners involved in sterile and non-sterile inflammation.

Hypothesis

This work was undertaken to better understand how periodontitis can worsen the vascular conditions. In particular, the possibility that MVs shed in response to periodontal infection act as remote thrombogenic actors is by in the hypothesis. Altogether and based on the central assumption of a worsening of vascular conditions by perpetuated chronic vascular inflammation three hypothesis can be drawn:

1- The release of spleen MV and their properties vary with the severity of the infection and/or the type of tissue damage (mechanical vs. biological insult)
2- MVs released from splenocytes in response to *Porphyromonas Gingivalis* are deleterious to endothelial cells and act as pro-inflammatory cardiovascular mediators
3- Circulating spleen Microvesicles and NETs released in response to *Porphyromonas Gingivalis* favor atherothrombosis at sites remote from the dental Infection

Aims

To explore our hypothesis, we chose to establish the proof of concept in a murine model of periondontitis.

The following aims were defined:

1- To characterize the spleen cellular composition and the release of MVs by splenocytes upon periodontal infection using a silk ligature impregnated or not with P. Gingivalis.
2- To identify the pro-senescent effects of MVs on the coronary endothelium using an in vitro model.
3- To identify NETs and MVs as circulating and remote actors of *P.gingivalis* by comparing the ligature model and the intraperitoneal injection of *P.gingivalis* .

Results

Article 1

Isolation of Splenic Microvesicles in a Murine Model of Intraperitoneal Bacterial Infection

(Accepted manuscript) DOI: 10.3791/63480

Hanine El Itawi, Fareeha Batool, Céline Stutz, Abdul Wahid Qureshi, Fatiha El Zobairi, Olivier Huck, Florence Toti

(Published)

I-Background

The majority of clinical studies examine MVs isolated from plasma samples, which typically contain MVs of endothelial, leukocyte, platelet, and erythrocyte origin.[39] Several approaches can be used for the detection and characterization of MVs several requiring a preanalytical procedure.

The most commonly studied tissular MVs are those from atherosclerotic plaques, the cardiac tissue itself, or cancer tissues. [224,225] [226]

In mice, SMV assessment could be of particular interest in situations where active molecuiles or therapeutical devices are precious and limited in quantity, or for comparative purposes of different genetic modification in Knock-in or Knock out mice.

The study of spleen derived MVs (SMVs) in the context of cardiovascular dysfunction appears consistent with the recently evoked cardio splenic axis, as a new response driven by pericardiac inflammation post myocardial infarction. [227] [228] [229]

Indeed, based on the proposed link between periodontitis and enhanced cardiovascular risk, a part of the work explores the possibility of an elevated procoagulant and pro-inflammatory activity in spleen, pertaining to the remote action of microvesicles released after local gum infection by *P.gingivalis*. (see second manuscript)

The first objective of this study was to establish a methodological approach for the isolation of splenic MVs SMVs from a periodontal *P.gingivalis* infection murine model. In this context, we studied the variation of the biochemical parameters of MVs, in order to assess the relevance and correlation between the different methods for the quantification and characterization of these MVs. To achieve this aim, we first studied the different characteristics of SMVs isolated from young C57BL6 mice to establish a reliable procedure with minimal inter-individual variations in the assessment of SMVs. (see manuscript N°1 jove)

The first series of experiments also enabled to establish labelling procedures of the spleen tissue for further “en face” investigations (see methods for MV investigation part II)

In both manuscripts, C57BL6 mice were investigated and overall different subsets were assessed and compared either to untreated mice or to mice submitted to oral gavage. Mice were either submitted to local gum injury combined or not with *P.gingivalis* infection using silk threads eventuallysoabed with the bacterial suspension. Another group of mice consisted of intraperitoneally infected individuals, to mimick an acute and local infection known to recruit neutrophils.

In the laboratory we have established a model for the study of microvesicles isolated from the spleen as an alternative approach to investigate immune and leukocyte responses associated to vascular diseases and procoagulant

tendency. Indeed, in small animals, the blood volume is a limiting issue for extensive characterization of MVs as circulating surrogate makers of organ dysfunction. At the opposite, the murine spleen is a larger source of MVs.

II- Methodological approaches for MVs measurement in our laboratory:

Data from the laboratory obtained in rats, had confirmed that the spleen MVs concentration and cell origin profile reflects the physio-pathological conditions and could be an alternate approach to plasma MV assessment. Strikingly,oral treatment by a specific combination of omega 3 was able to modify the baseline level of SMVs generation, thereby adding a new interest to the model since it could be useful to evaluate pro-drugs.[7] Nevertheless, our previous data on the action of pathogens of SMV generation and phenotype profile were only established after in vitro LPS stimulation of isolated rat splenocytes.[4]

The approach chosen for the measurement of SMVs in extracts and washed final suspensions of SMVs was to select easy-to handle routine methods, like spectrophotometry, colorimetric assay of the protein content or TPRS (Tunable Resistive Pulse Sensing) that would spare minimal amount of the suspensions. They were compared to our gold standard prothrombinase assay (see article). Their respective advantages and limits are given in the table below.To establish the procedure, we compared animals treated by intraperitoneal injections of P. Gingivalis with untreated littermates, with the perpective of the secondary article dedicated to periodontitis.

METHODE	AVANTAGES	DESAVANTAGES
Dosage Prothrombinase 	• Dosing in series • Reproducibility • Sensitivity	• Expensive • No enumeration (PS surface eq) • No size identification
NanoDrop 	• Small quantity used for dosing • Automated, repeatable • Nucleic acids and proteins • Dosing in series • Immediate result	• No size identification • Sensitive to cellular stimulus (Different export of proteins)
Nanosizer 	• Size distribution • Peak homogeneity	• Long acquisition time • Definition of refractive index • Sensitive to aggregation and microcrystals. • No characterization of PS
Dosage colori-métrique au bleu 	• Measure of cytoplasmic and membrane proteins independently of their structure	• Result in one hour • Lysate (losses) • Sensitive to the amounts of aromatic AA. • Sensitive to cellular stimulus

Table 5: Comparison of different methods for MVs characterization

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Isolation of Splenic Microvesicles in a Murine Model of Intraperitoneal Bacterial Infection

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Abstract

Microvesicles (MVs) are submicron fragments released from the plasma membrane of activated cells that act as proinflammatory and procoagulant cellular effectors. In rats, spleen MVs (SMVs) are surrogate markers of pathophysiological conditions. Previous *in vitro* studies demonstrated that *Porphyromonas gingivalis* (*P. gingivalis*), a major periodontal pathogen, enabled the endothelial shedding and apoptosis while lipopolysaccharide (LPS) favors the shedding of splenocyte-derived microvesicles (SMVs). *In vivo* studies showed the feasibility of pharmacological control of SMV shedding. The present protocol establishes a standardized procedure for isolating splenic SMVs from the *P. gingivalis* acute murine infection model.*P. gingivalis* infection was induced in young C57BL/6 mice by intraperitoneal injection (three injections of 5 x 10^7 bacteria/week). After two weeks, the spleens were collected, weighed, and the splenocytes were counted. SMVs were isolated and quantified by protein, RNA, and prothrombinase assays. Cell viability was assessed by either propidium iodide or trypan blue exclusion dyes. Following splenocyte extraction, neutrophil counts were obtained by flow cytometry after 24 h of splenocyte culture. In *P. gingivalis*-injected mice, a 2.5-fold increase in spleen weight and a 2.3-fold rise in the splenocyte count were observed, while the neutrophils count was enhanced 40-fold. The cell viability of splenocytes from *P. gingivalis*-injected mice ranged from 75%-96% and was decreased by 50% after 24 h of culture without any significant difference compared to unexposed controls. However, splenocytes from injected mice shed higher amounts of MVs by prothrombinase assay or protein measurements. The data demonstrate that the procoagulant SMVs are reliable tools to assess an early spleen response to intraperitoneal *P. gingivalis* infection.

Introduction

Microvesicles (MVs), also termed microparticles or ectosomes, are plasma membrane fragments with a diameter of 0.1-1.0 µm released in body fluids and the extracellular space in response to various cell stimuli. First identified as platelet dust exposing procoagulant phospholipids, mostly phosphatidylserine (PSer), MVs constitute an additional surface for the assembly of the blood coagulation complexes[1,2]. The key role of circulating MVs as procoagulant effectors have been demonstrated in patients with Scott syndrome[2], a rare genetic disease that leads to dysfunctional PSer exposure and bleeding (**Supplementary Figure 1**). MVs have been extensively studied as circulating biomarkers of thrombotic risk in chronic diseases associated with cardiovascular disorders such as diabetes, chronic kidney disease, preeclampsia, and hypertension[3,4]. They are currently recognized as true cellular effectors in fluids or organ tissues like the myocardium[1]. Because they convey active proteins, lipids, and miRNA, they remotely modulate vascular responses such as hemostasis, inflammation, vascular angiogenesis, and vascular growth or tissue remodeling[5].

While clinical studies examine the prognosis value of MVs concerning risk factors, MVs isolated from the patient's fluids or tissue enable the *ex vivo* assessment of their effector properties[6]. The deciphering of the mechanisms governing MV biogenesis and cross-talk abilities is generally achieved in cell culture models to identify active molecules exposed by or encapsulated within the MVs and downstream signaling. The MV interactions with target cells will depend on membrane protein/protein binding, when appropriate counter-ligands are available, and/or lipid fusion[7].

Under physiological conditions, MVs circulating in the plasma mostly originate from vascular cells, as identified by the lineage cluster differentiation markers (CD)[8,9]. However, in pathology, notably in cancer[10] and graft rejection[11,12], MVs are shed from the damaged tissue and bear noxious procoagulant and proinflammatory features. These are detected in the systemic circulation, making them useful probes for monitoring protective or rejuvenation therapies, and possible pharmacological targets[13]. Because MVs circulate as a dynamic storage pool reflecting vascular and tissue cell homeostasis in health and disease, a better understanding of their remote action also needs to be investigated *in vivo*, after IV injection or nasal instillation in small animals[14,15]. Indeed, MVs have been considered major contributors to the intricate pathways coupling exaggerated inflammation and thrombosis[16].

Periodontitis is an inflammatory disease of infectious origin affecting tooth-supporting tissues[17,18] and is associated with thrombotic risk. It is characterized by gingival bleeding, alveolar bone destruction, tooth mobility and can ultimately lead to tooth loss. Periodontitis is highly prevalent worldwide and affects more than 50% of the population, with 11% presenting a severe form[19]. Periodontitis is induced by bacterial dysbiosis of the subgingival biofilms, which sustain an exacerbated inflammatory response that triggers tissue destruction[20]. Over the last decade, periodontitis has been linked to systemic diseases such as cardiovascular disorders, diabetes, and rheumatoid arthritis. The possible explanations are the action of the periodontal pathogens at a distance and/or the increased systemic inflammation mediated by

proinflammatory cytokines such as interleukin (IL)-1, IL-6, and TNF-α[21,22].

Among pathogens associated with the periodontitis onset and development, *Porphyromonas gingivalis (P. gingivalis)*[23] is found in most severe lesions that harvest several virulence factors, including lipopolysaccharide (LPS)[24] inducing Toll-like-receptor (TLR)-mediated inflammatory responses[25] and the recruitment of neutrophils and polymorphonuclear leukocytes (PMNs) at the site of the initial infection[26]. The LPS from *P. gingivalis* activates TLR-4 or TLR-2, facilitating immune detection and bacterial survival evasion[27]. At the vascular level, activation of TLR2 by LPS is associated with immunothrombosis. The unique ability of *P. gingivalis* to prompt TLR-2 signaling while TLR-4-dependent recognition is significantly impaired favors persistent low-grade infection[28,29].

In vivo procedures to study the MV responses to low-grade pathogen chronic and sustained infection are scarce. Methodological approaches for tissular MVs extraction are poorly described in the literature and generally concern the harvest of MVs from pathological tissues like solid tumors, liver steatosis, atherothrombotic plaques, or grafts[11,29,30], while the spleen senses bacteria and viruses in the bloodstream. It also stores erythrocytes, platelets, lymphocytes, monocytes, basophils, and eosinophils involved in the immune response. Granulocytes like neutrophils from the red pulp also generate reactive oxygen species (ROS) and proteases that destroy pathogens and prevent infection from spreading[31,32]. Amazingly, and to the best of our knowledge, spleen MVs are not investigated in the context of pathogen-induced tissue insults. There is a global unmet need to study the variations of tissular MVs in physiopathology.

In vitro data from our laboratory showed that LPS induces the shedding of procoagulant MVs from rat splenocytes, which induces the senescence of cultured coronary endothelial cells and promotes a proinflammatory and proinflammatory procoagulant endothelial phenotype[11]. The feasibility of the pharmacological control of the spleen MVs was further demonstrated after treating the animal with an optimized omega-3 formula. The oral gavage of middle-aged and aged rats was found to be protective against both the shedding of procoagulant MVs from splenocytes and their prosenescent noxious properties towards the coronary endothelium[33].

Compared to blood, the spleen offers the advantage of being an important source of leukocytes in one individual. In addition, a splenic-cardiac axis has recently been proposed[3,34], making the spleen a possible contributor to the infection-related cardiovascular risk of beneficial interest for pharmacological control. The assessment of the SMVs properties or release is key in understanding pathogen-induced or inflammatory responses with no alternative methodology to date. Interestingly, it can be achieved in treated animals and in different physiopathological models (age, hypertension, diabetes). Indeed, by comparing middle-aged and aged rats[33], the differences in spleen MVs properties and release can be evidenced following a simple 24 h splenocyte culture.

Given the above evidence of the alteration of the effector properties of spleen MVs and the feasibility of their pharmacological control in rats, a standardized protocol is described herein for the isolation of murine spleen MVs. The procedure would better fit in-depth investigations of the *in vivo* mechanisms supporting SMVs-mediated effects, eventually in engineered mice. The procedure was established in C57BL/6 mice using a local intraperitoneal infection by *P.*

gingivalis, to establish proof of a remote action of the pathogen on spleen MV (SMV) effector properties.

Protocol

All experimental protocols were approved by and followed the relevant guidelines of the local Ethics Committee (APAFIS#28745-2020121815051557) and animal care of the home University and of the INSERM. Male Young C57BL/6 mice, 6-8 weeks of age, were used for the experiments. Mice were regularly examined to evaluate pain and stress, and their weights were monitored daily. Unless otherwise stated, all extracting buffers and solutions must be sterile and at room temperature.

1. Animal preparation

1. Administer the mice with six intraperitoneal injections of *P. gingivalis* (PG) every 2 days for 2 weeks (three injections of 5 x 10^7 bacteria/week, **Supplementary File 1**).
2. Anesthetize the mice using a combination of 100 mg/kg of ketamine and 10 mg/kg of xylazine.
3. Sacrifice the anesthetized mice and harvest the spleen after laparotomy.
   NOTE: The specific conditions for *P. gingivalis* infection and the sacrifice of the animals for spleen harvest are detailed in the "animal experiment" section of **Supplementary File 1**. The schematic representation of the protocol steps is shown in **Figure 1**.

2. Extraction of splenocytes

1. Using a scalpel, mince the spleen tissue into ~1 mm slices in a Petri dish already filled with 3 mL of RPMI containing antibiotics (Streptomycin (100 U/mL)/Penicillin (100 U/mL), Fungizone (250 mg/mL), L-glutamine (2 mM)), and supplemented with 10% Fetal Bovine Serum (FBS) (see **Table of Materials**).
2. Transfer the slices to a sieve (see **Table of Materials**), which is from beforehand attached to a 50 mL polystyrene tube.
3. Use the plunger of a syringe as a pestle to crush the tissues on the sieve.
4. Rinse the sieve with the RPMI medium (3 mL).
5. Centrifuge the cell suspension eluate at 450 x *g* for 5 min at room temperature and discard the supernatant with caution by backflow.

3. Removal of erythrocytes*via* an osmotic shock

1. Add 1 mL of ACK buffer (see **Table of Materials**), mix well, incubate for 3 min at room temperature and shake gently by hand rotation.
2. Dilute by adding 9 mL of RPMI and mix well using aspiration and backflow.
3. Centrifuge at 450 x *g* for 5 min at room temperature.
4. Discard the supernatant by aspiration and backflow, and resuspend the pellet in 5 mL of RPMI medium.
5. Mix gently (aspiration/backflow) and eventually remove any lipidic debris by pipetting or using a sieve.

4. Adjustment of isolated splenocyte concentration

1. Fill a 1.5 mL microtube containing 900 µL of RPMI.
2. Using a micropipette, take 100 µL of the cellular suspension, add to the 1.5 mL microtube containing 900 µL of RPMI medium, and mix well.

3. Transfer 10 µL of the diluted cells into a microtube, add 10 µL of the trypan blue exclusion dye, and mix well.
4. Place 10 µL of the solution (step 3.3) into the counting slide of the automatic cell counter (see **Table of Materials**) and determine the cell count.
   NOTE: The device software gives two values: the total cell number and the living cell number and percentage.
5. Adjust the concentration of living cells to 3 or 5 x 10^6 cells/mL (V_{max} = 20 mL; max total capacity is 100 x 10^6 cells/mL).
6. Seed the cells in a 75 mL flask containing RPMI.
7. Incubate the flask vertically in a humidified incubator at 37 °C for 24 h.
   CAUTION: Trypan blue is a mutagenic and carcinogenic agent[35,36]. Ensure to wear gloves while counting, even outside of the hood. Immediately discard tips and fluids into containers and wash the bench surface.
   NOTE: If the collected cells are less than 50 x 10^6, use a 25 mL culture flask. The cell number should not vary much from one individual to the other. The percentage of living cells is generally superior to 80% at this step.

5. Isolation of splenocyte microvesicles

1. After 24 h, pour the flask content into a 50 mL polypropylene or polystyrene tube.
2. Centrifuge at 450 x *g* for 15 min at room temperature to remove cells and debris.
3. After centrifugation, keep 100 µL of the 1st supernatant (SN1) for an eventual measurement of the initial MV concentration to calculate the isolation yield. Note the SN1 volume.
4. Centrifuge the SN1 at 450 x *g* for 15 min at room temperature.
5. Discard pellet and centrifuge again at 450 x *g* for 15 min at room temperature. Rapidly withdraw and collect SN2 (note the volume).
6. Transfer SN2 into 1.8 mL microtubes with conical shape bottoms. Keep 100 µL of SN2 for an eventual measurement of the initial MV concentration to calculate the isolation yield in case too much debris eventually impairs MV measurement in SN1.
7. Add 5 mL of RPMI medium to the pellet (cells) and count the splenocytes.
   NOTE: The cell suspension can also be fixed for flow cytometry characterization in 0.1% paraformaldehyde (final concentration)[37].
8. Centrifuge SN2 at 14,000 x *g* for 1 h at 4 °C and eventually refrigerate the rotor in advance by running the empty rotor at 4 °C. This step generates SN3.
9. Immediately withdraw SN3 from each microtube by inverting over a single sterile 50 mL tube (under the hood). Note the SN3 volume.
10. Gently mix SN3 using a pipette under the hood. Keep aliquots for eventual measurements in the suspension.
    NOTE: The solution is now depleted of MVs but contains the soluble mediators and exosomes.
11. Scrape each microtube (closed) on a spiked rack to mechanically favor the resuspension of the MVs in the pellet.
12. Add 200 µL of Hank's balanced saline solution without Ca^{2+} and Mg^{2+} (HBSS) in the first microtube (see **Table of Materials**).

13. Resuspend the MV pellet of the first tube by gentle aspiration/backflow cycles (~10 times) using a 1 mL micropipette. Take great care to avoid foaming as this will promote the oxidation of the suspension vesicles and other moieties.
14. Harvest the first 200 µL of resuspended MVs and pour them into the second tube over the pellet. Gently mix as above, and then pour again into the third tube and mix gently. The suspension becomes more and more turbid from tube to tube. Close the third tube.
15. Initiate the collection of the next three microtubes as above (step 4.14).
16. Collect all the resuspended MVs in a single (or two) collector microtube (2 mL with conical shape bottom). Keep 50 µL for the eventual calculation of the yield of this step. Note the suspension volume.
17. Centrifuge the collector tube at 14,000 x *g* for 1 h at 4 °C. This step generates SN4.
18. Immediately discard the SN4 (pour over a supernatant collecting tube). Keep an aliquot of SN4 to calculate the yield of the purification step and gently resuspend the pellet in 500 µL of HBBS (without Ca^{2+} and Mg^{2+}). Keep a 50 µL aliquot of the resuspended pellet to calculate the purification yield and note its volume.
19. Store the resuspended pellet at 4 °C for up to one month for functional assays under sterile conditions or at -20 °C for structural assays.
20. Calculate the purification yield (see NOTE below) of each step or the whole procedure by measuring the concentration of MVs in supernatants and/or pellet suspensions.

NOTE: Purification Yield (%): ([MV1] x SN1 volume) / ([MV4] x final suspension volume) x 100.

This yield needs to be rationalized to the number of splenocytes isolated from the culture flask and counted in step 4.4. The present experiment's purification yield is ~70% starting from 111 x 10^6 cells and 60 mL of supernatant.

1. Evaluate the loss of SMVs caused by the procedure by measuring the SMVs remaining in the supernatants depleted of SMVs and mainly containing exosomes (SN2 and SN4).
2. Measure the MV concentration by TRPS or prothrombinase assay[38] for high sensitivity and specificity.

   CAUTION: Harvest the MVs after centrifugation by the rapid upside-down pouring of the supernatant. Waiting or using U-shape microtubes will prompt MV loss on the microtube wall. Never use a micropipette to discard the supernatant; the pellet can be aspirated instead. Never delay the process after the centrifuge is stopped. Overspeed centrifugation will result in contamination of the MV pellet by exosomes.

   NOTE: The centrifugation speeds and duration are optimized for an MV enrichment with minimal contamination by exosomes. This protocol is not reliable to study spleen monocyte/macrophage MVs because these cells can phagocyte and rapidly recapture their own MVs[39]. The protocol cannot be paused and restarted later because of loss of cells due to cell viability.

Representative Results

The data provided give a full representation of the whole procedure, using two main animal conditions: control untreated young C57BL/6 mice and their littermates subjected to six intraperitoneal (IP) PG injections every 2 days, for 2 weeks. They also show the remote action of an intraperitoneal PG injection on the spleen response after 2 weeks. Splenocyte microvesicles were quantified by either prothrombinase enzymatic assay or by measuring their proteins and RNA concentration by spectrophotometric, and the proportion of neutrophils was determined by flow cytometry[33,38] in the splenocyte suspension (sections 2-4 of **Supplementary File 1** and **Supplementary Figure 2**).

Spleen weight and cell number increase after 2 weeks of IP injection in *P. gingivalis*

In young adult mice, the PG injection leads to a significant 2.6-fold increase in spleen weight after 2 weeks, likely due to the immune response. Indeed, the total splenocyte count was also raised by 2.3 folds (**Figure 2**, n = 5 mice per each condition, $p < 0.0001$ by Student's T-test).

Splenocyte viability is not impaired by 2 weeks of injection in *P. gingivalis*

Similar viability rates were measured between treated and untreated mice after splenocyte isolation or 24 h after cell culture. Nevertheless, when the initial percentage of living cells at t0 was below 76%, viability was reduced in both groups by ~50% after 24 h. (see Ctl 1-3, **Table 1**, as an example). Conversely, and whatever the animal condition, the 24 h recovery was enhanced when the proportion of living cells reached 90% or more at t0, pointing at the crucial role of the first isolation steps (**Figure 3**).

Splenocyte neutrophil count is enhanced in *P. gingivalis*-injected mice

After double staining, neutrophils were identified by flow cytometry as $Mac1^+$ and $LYG\text{-}6\text{-}(Gr1)^+$ (see **Table of Materials** and section 4 in **Supplementary File 1**). In response to PG, the neutrophil spleen count was significantly enhanced by 40-folds just after cell isolation (t0, 4.8% ± 0.6 *vs*. 0.86% ± 0.06). Nevertheless, it is worth considering that after 24 h culture, the neutrophil proportion in the splenocyte suspension increases from 4.8% ± 0.6 to 14.67% ± 2.02 (**Figure 4**).

The shedding of MVs from splenocytes increases in mice infected by *P. gingivalis*

Following the protocol, we proved that the isolated splenocytes released more MVs when mice were subjected to PG infection. By comparison, to control individuals and the measurement assay, MVs shed in the supernatant were roughly tripled in PG-injected mice, using either the detection by prothrombinase assay that refers to the total surface of procoagulant anionic phospholipids borne by SMVs (**Figure 5A**), the protein content of SMVs measured by spectrophotometry (**Figure 5B**), or the RNA content of SMVs assessed by spectrophotometry (**Figure 5C**, n = 5, Prothrombinase: $p = 0.002$; protein content: $p = 0.003$; RNA content, $p = 0.008$*, by Student's T-test).* The higher fold-range extent was obtained using the prothrombinase assay that showed a very low background.

Interestingly, a correlation can be established between the SMVs protein content and SMVs measured by prothrombinase assay (**Supplementary Figure 3**). In addition, the SMVs size distribution did not vary as determined in preliminary experiments (**Supplementary Figure 4**) comparing SMVs from PG-stimulated and

unstimulated mice (median diameter 193 nm *vs*. 189 nm). These diameter values are close to those of SMVs isolated from rat splenocytes in response to a 24 h incubation with LPS (5 µg/mL) or Phorbol myristate acetate (25 ng/mL) and ionophore (1 µM), (median diameter, 222 nm *vs*. control 212 nm)[11] (**Supplementary Figure 5**). In addition, the protein content of the SMVs can be assessed by western blot, which enables the characterization of specific cytoplasmic or membrane proteins preferentially exported into the SMVs (**Supplementary Figure 5**).

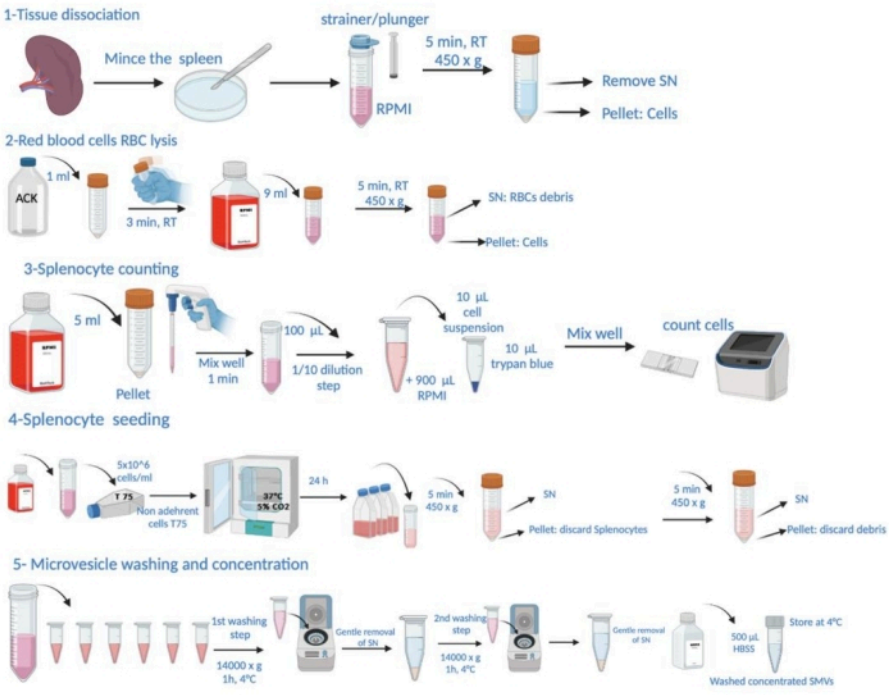

Figure 1: Schematic representation of the protocol steps. This consists of tissue dissection, red blood cell (RBC) lysis, splenocyte counting, seeding, microvesicle harvest from supernatant, washing and concentration determination. Please click here to view a larger version of this figure.

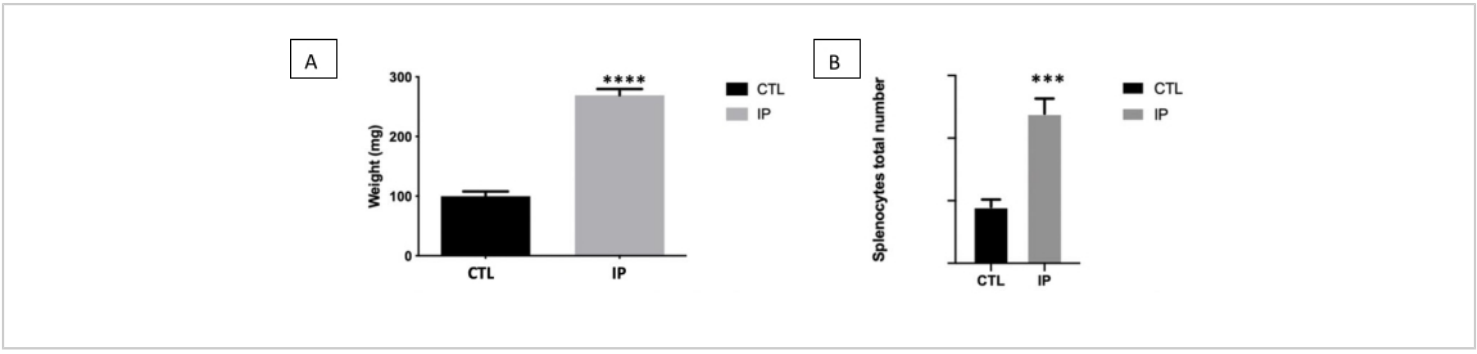

Figure 2: IP injection of *P. gingivalis* significantly enhances spleen weight (A) and splenocyte count (B). CTL: untreated mice, IP: mice subjected to PG injection for 2 weeks. Data are expressed as mean ± SEM. n = 5: ***: $p < 0.0001$ and 0.0005, respectively. Please click here to view a larger version of this figure.

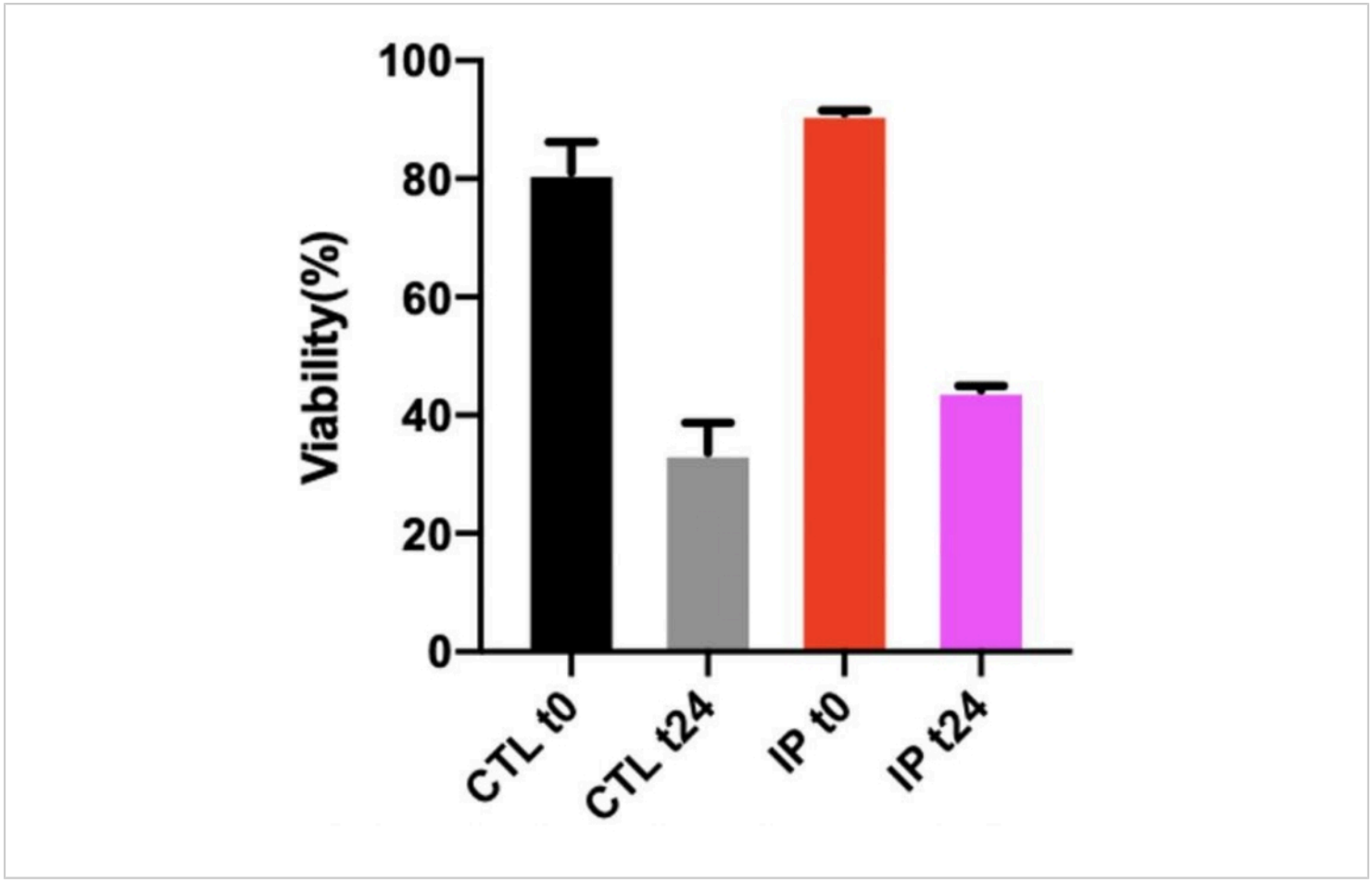

Figure 3: Splenocyte viability is reduced 24 h after splenocyte culture, whereas IP injection of *P. gingivalis* does not alter cell viability. CTL: untreated mice, IP: mice subjected to PG injection for 2 weeks, t0: viability measured immediately after isolation, t24: after 24 h culture. Data are expressed as mean ± SEM. n = 5, ***: $p < 0.0001$ by Student's T-test. Please click here to view a larger version of this figure.

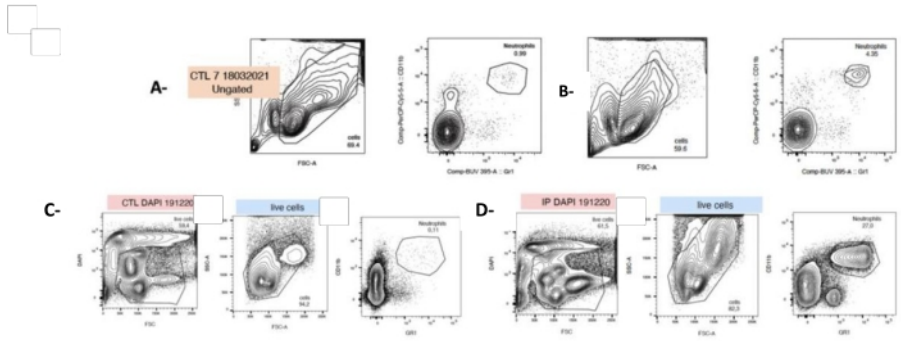

Figure 4: *P. gingivalis* injection increases the neutrophil count at t0 and after 24 h of cell culture. Cells were freshly isolated from the harvested spleen and labeled with fluorescent antibodies directed against granulocytes (LyG-6 Gr1) or neutrophils (LY6-C) and identified in the $CD11b^+$ gate that delineates monocytes, granulocytes, and neutrophils. Neutrophils were identified as $LY6\text{-}C^+$, $CD11b^+$, and LYG-6 Gr1 low. Representative plots showing SSC/FSC and $CD11b^+$, and LYG-6 Gr1 double staining for each condition at t0 (**A,B**) or 24h after seeding (**C,D**). A,C: untreated mice splenocytes; B,D: splenocytes from mice subjected to IP injection of *P. gingivalis*. FSC: Forward Scatter; SSC: Side Scatter; CTL: unexposed mice. n = 5, ***: $p < 0.0001$, 10000 events acquired per sample. Please click here to view a larger version of this figure.

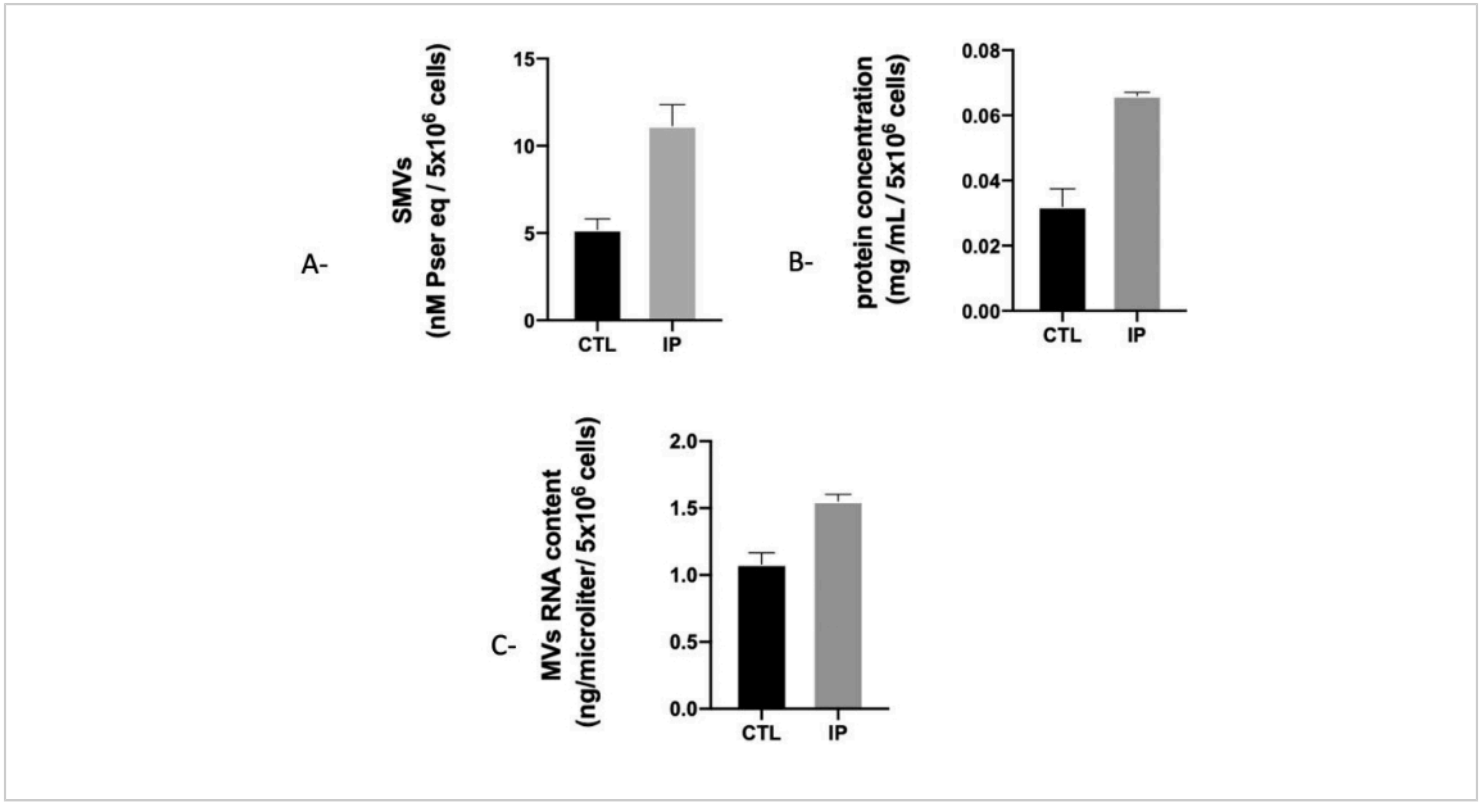

Figure 5: *P. gingivalis* favors enhanced MV splenocyte shedding after 24 h culture. Splenocytes were isolated from *P. gingivalis* infected and control mice and cultured for 24 h and SMVs measured by different assays. Black bars: untreated mice (CTL), grey bars (IP): mice subjected to *P. gingivalis* injection for 2 weeks. A: Quantification by prothrombinase assay, B: protein concentration of the intact SMVs determined by spectrophotometry, C: RNA content of SMVs determined by spectrophotometry. Data are expressed as mean ± SEM. n = 5, **: $p < 0.0001$, by Student's T-test. Please click here to view a larger version of this figure.

Animal condition	Spleen weight	Splenocytes number	Viability (%)	Viability (%)
			after cell isolation	
	(mg)	(10^6 cells)		After 24 h cell culture
			(t0)	
Ctl 1	108	25	75	24
Ctl 2	112	55	70	26
Ctl 3	118	52	73	26
Ctl 4	83	38	91	46
Ctl 5	78	55	96	46
IP 1	243	150	91	43
IP 2	250	138	93	46
IP 3	283	85	90	46
IP 4	270	100	91	43
IP 5	300	125	91	43

Table 1: Comparison of splenocyte viability rates between the treated and untreated mice after splenocyte isolation.

Supplementary Figure 1: How procoagulant MVs are procoagulant? For details, see **Supplementary File 1.** Please click here to download this File.

Supplementary Figure 2: The microparticles (MP) captured-based prothrombinase assay. For details, see **Supplementary File 1.** Please click here to download this File.

Supplementary Figure 3: Correlation plot between measurements of SMVs by prothrombinase assay and by spectrophotometric protein assay in untreated or *P. gingivalis* treated mice. Please click here to download this File.

Supplementary Figure 4: Analysis of the size distribution of SMVs isolated from mice (**A**) and rat splenocytes (**B**). The suspension of SMVs was obtained from the spleen of untreated mice (Control MVs) or individuals subjected to gum damage without LIG MVs or with local addition of *P. gingivalis* (LIG-PG MVs). SMVs from rats were harvested in the supernatant of isolated splenocytes subjected to a 24 h stimulation by LPS (LPS MVs) or PMA (PMA MVs) as described elsewhere[37]. Mean dia: Mean diameter; Mode dia: median value of the diameter. Measurements were performed using a 400 nanopore to assess the largest size distribution range. Please click here to download this File.

Supplementary Figure 5: Identification of the protein content of MVs from rat splenocytes isolated from young rats by western blot. For details, see **Supplementary File 1**. Please click here to download this File.

Supplementary File 1: Details on the experimental procedures and explanations for Supplementary Figures 1,2,5. Please click here to download this File.

Discussion

The present study confirms that the spleen is a major and reliable source of MVs with physiopathological relevance compared to other sources like blood, of limited volume in mice. Provided precautions are taken, the method is easy to set up and does not require expensive equipment. Since no alternate way other than *in vivo* assessment is available, the current model appears to be a valuable method to study the impact of pro-drugs on MV shedding. Importantly, the standardized protocol for harvesting murine splenic microvesicles (SMVs) presented here should fit the unmet needs for the *in vivo* study of the control of SMV-mediated effects, eventually in engineered mice after injection or nasal instillation.

The present method also shows that SMVs are relevant preclinical tools to investigate remote effects of local infections, like *P. gingivalis* IP infection. Indeed, using multiple approaches, significant variations between SMVs released from *P. gingivalis*-treated *vs*. untreated animals were measured, suggesting a remote effect of the local IP injection, already detectable after 2 weeks. Preliminary data from our laboratory using other remote *P. gingivalis* infection sites confirmed that *P. gingivalis* may initiate a 1.7-fold SMV elevation as early as day-7 post-infection.

It is also observed that the *P. gingivalis*-induced remote effects were confirmed by spleen analysis with significant variations in weight, cell viability, and total count. Furthermore, *P. gingivalis* prompted a 6.5-fold increase in the spleen neutrophil count when measured by immunostaining just after splenocyte isolation (t0). Accordingly, variations of the SMV cell origin profile would indicate the shedding of parental cells caused by the initial condition of the animal.

Because SMVs released in the supernatant of 24 h splenocyte cultures were tripled when isolated from IP-injected mice, they appear reliable biomarkers of remote spleen activation by *P. gingivalis,* making them eventual tools to monitor pharmacological control.

In vitro, the splenocytes culture can be performed using 10% FBS, depleted or not from exosomes. In the current experiments, no differences were observed, and variations between untreated splenocytes remained detectable to the same extent with both types of FBS.

In the present procedure, a 2-week duration did not lead to significant variations of the spleen weight in untreated mice. It is, however, important to keep in mind that the spleen weight may vary with age, as previously reported in a series of young, old, and middle-aged control rats.[1] In such a case, the SMV quantification needs to be expressed as a cell count ratio.

The present procedure was optimized for a murine model and suitable for further transgenic mice investigations. Although the major drawback of the murine model is a small spleen weight compared to rats[33], the adapted splenocyte and SMV isolation procedures described herein prevent cell damage and loss, while all contaminating erythrocytes are fully discarded. Washing is crucial for a good splenocyte purification yield and needs to be performed gently with no

more than 1 mL of ACK lysis buffer per spleen in 9 mL of RPMI to enable extensive elimination of red blood cells. This step may be repeated until the pellet is white. This procedure does not allow the investigation of monocytes and should be adapted for adherent cells in such eventuality.

After 24 h culture, the SMV concentration measured in cell culture supernatants reflects the balance between MV generation and MV recapture by the cultured splenocytes. Therefore, in adapted versions of the above procedure, special attention needs to be given to the presence of cells with professional or accessory phagocyte activity (monocytes, endothelial cells, etc.), and kinetics of the SMV release in supernatant should be carefully established accordingly.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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References

1. Loyer, X. et al. Intra-cardiac release of extracellular vesicles shapes inflammation following myocardial infarction. *Circulation Research.* **123** (1), 100-106 (2018).
2. Aupeix, K., Toti, F., Satta, N., Bischoff, P., Freyssinet, J. M. Oyxsterols induce membrane procoagulant activity in monocytic THP-1 cells. *Biochemical Journal.* **314** (3), 1027-1033 (1996).
3. Emami, S. *et al.* Antibiotic resistance pattern and distribution of pslA gene among biofilm producing Pseudomonas aeruginosa isolated from waste water of a burn center. *Jundishapur Journal of Microbiology.* **8** (11), e23669 (2015).
4. Burger, D. et al. Microparticles: biomarkers and beyond. *Clinical Science.* **124** (7), 423-441 (2013).
5. Braeckmans, K. et al. Sizing nanomatter in biological fluids by fluorescence single particle tracking. *Nano Letters.* **10** (11), 4435-4442 (2010).
6. Chironi, G. N. *et al.* Endothelial microparticles in diseases. *Cell and Tissue Research.* **335** (1), 143-151 (2009).
7. Meldolesi, J. Exosomes and ectosomes in intercellular communication. *Current Biology.* **28** (8), R435-R444 (2018).
8. Chen, T. S. *et al.* Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Research.* **38** (1), 215-224 (2010).
9. Pirro, M. et al. Microparticles derived from endothelial progenitor cells in patients at different cardiovascular risk. *Atherosclerosis.* **197** (2), 757-767 (2008).
10. Zahra, S., Anderson, J. A., Stirling, D., Ludlam, C. A. Microparticles, malignancy and thrombosis. *British Journal of Haematology.* **152** (6), 688-700 (2011).
11. Amoura, L. *et al.* Assessment of plasma microvesicles to monitor pancreatic islet graft dysfunction: Beta cell- and leukocyte-derived microvesicles as specific features

in a pilot longitudinal study. *American Journal of Transplantation.* **20** (1), 40-51 (2020).

12. De Rop, C. *et al.* Evaluation of tissue factor bearing microparticles as biomarkers in allogeneic stem-cell transplantation. *Transplantation.* **92** (3), 351-358 (2011).
13. de Abreu, R. C. et al. Native and bioengineered extracellular vesicles for cardiovascular therapeutics. *Nature Reviews Cardiology.* **17** (11), 685-697 (2020).
14. Beitler, J. R. *et al.* Advancing precision medicine for acute respiratory distress syndrome. *The Lancet Respiratory Medicine.* **10** (1), 107-120 (2022).
15. Porro, C. *et al.* Proinflammatory effect of cystic fibrosis sputum microparticles in the murine lung. *Journal of Cystic Fibrosis.* **12** (6), 721-728 (2013).
16. Boisrame-Helms, J. et al. Lipid emulsions differentially affect LPS-induced acute monocytes inflammation: in vitro effects on membrane remodeling and cell viability. *Lipids.* **49** (11), 1091-1099 (2014).
17. Kinane, D. F., Stathopoulou, P. G., Papapanou, P. N. Authors' reply: Predictive diagnostic tests in periodontal diseases. *Nature Reviews Disease Primers.* **3** (1), 1 (2017).
18. Kinane, D. F., Stathopoulou, P. G., Papapanou, P. N. Periodontal diseases. *Nature Reviews Disease Primers.* **3** (1), 1-14 (2017).
19. Kassebaum, D. K., Tedesco, L. A. The 21(st)-century dental curriculum: a framework for understanding current models. *Journal of Dental Education.* **81** (8), eS13-eS21 (2017).
20. Hajishengallis, G., Chavakis, T., Hajishengallis, E., Lambris, J. D. Neutrophil homeostasis and inflammation: novel paradigms from studying periodontitis. *Journal of Leukocyte Biology.* **98** (4), 539-548 (2015).
21. Suh, J. S. *et al.* Periodontitis-induced systemic inflammation exacerbates atherosclerosis partly via endothelial-mesenchymal transition in mice. *International Journal of Oral Science.* **11** (3), 21 (2019).
22. Bugueno, I. M. *et al.* Porphyromonas gingivalis triggers the shedding of inflammatory endothelial microvesicles that act as autocrine effectors of endothelial dysfunction. *Scientific Reports.* **10** (1), 1778 (2020).
23. Hajishengallis, G., Lamont, R. J. Breaking bad: manipulation of the host response by Porphyromonas gingivalis. *European Journal of Immunology.* **44** (2), 328-338 (2014).
24. Lamont, T., Worthington, H. V., Clarkson, J. E., Beirne, P. V. Routine scale and polish for periodontal health in adults. *The Cochrane Database of Systematic Reviews.* **12**, CD004625 (2018).
25. Huck, O. et al. Reduction of articular and systemic inflammation by Kava-241 in a Porphyromonas gingivalis-induced arthritis murine model. *Infection and Immunity.* **86** (9) e00356-18 (2018).
26. Sochalska, M., Potempa, J. Manipulation of neutrophils by Porphyromonas gingivalis in the development of periodontitis. *Frontiers in Cellular and Infection Microbiology.* **7**, 197 (2017).
27. Kocgozlu, L., Elkaim, R., Tenenbaum, H., Werner, S. Variable cell responses to P. gingivalis lipopolysaccharide. *Journal of Dental Research.* **88** (8), 741-745 (2009).

28. Slocum, C. et al. Distinct lipid a moieties contribute to pathogen-induced site-specific vascular inflammation. *PLOS Pathogens.* **10** (7), e1004215 (2014).
29. Thietart, S., Rautou, P. E. Extracellular vesicles as biomarkers in liver diseases: A clinician's point of view. *Journal of Hepatology.* **73** (6), 1507-1525 (2020).
30. Witek, R. P. et al. Liver cell-derived microparticles activate hedgehog signaling and alter gene expression in hepatic endothelial cells. *Gastroenterology.* **136** (1), 320-330 (2009).
31. Tahir, F., Ahmed, J., Malik, F. Post-splenectomy sepsis: a review of the literature. *Cureus.* **12** (2), e6898 (2020).
32. Hussain, M., Stover, C. M., Dupont, A. P. gingivalis in periodontal disease and atherosclerosis - scenes of action for antimicrobial peptides and complement. *Frontiers in Immunology.* **6**, 45 (2015).
33. Qureshi, A. W. et al. Ageing enhances the shedding of splenocyte microvesicles with endothelial pro-senescent effect that is prevented by a short-term intake of omega-3 PUFA EPA:DHA 6:1. *Biochemical Pharmacology.* **173**, 113734 (2020).
34. Dunford, A., Keramida, G., Anagnostopoulos, C. D., Michael Peters, A. The cardiosplenic axis: another obscure pathophysiological function of the spleen and its investigation using molecular imaging. *Nuclear Medicine Communications.* **38** (3), 205-208 (2017).
35. Ford, R. J., Becker, F. F. The characterization of trypan blue-induced tumors in Wistar rats. *The American Journal of Pathology.* **106** (3), 326-331 (1982).
36. Field, F. E. *et al.* Trypan blue: identification and teratogenic and oncogenic activities of its coloured constituents. *Chemico-Biological Interactions.* **16** (1), 69-88 (1977).
37. Covarrubias, R. et al. Optimized protocols for isolation, fixation, and flow cytometric characterization of leukocytes in ischemic hearts. *American Journal of Physiology - Heart and Circulatory Physiology.* **317** (3), H658-H666 (2019).
38. El Habhab, A. et al. Significance of neutrophil microparticles in ischaemia-reperfusion: Proinflammatory effectors of endothelial senescence and vascular dysfunction. *Journal of Cellular and Molecular Medicine.* **24** (13), 7266-7281 (2020).
39. Freyssinet, J. M. Cellular microparticles: what are they bad or good for? *The Journal of Thrombosis and Haemostasist.* **1** (7), 1655-1662 (2003).

Article 2

P. gingivalis alters spleen response in a murine periodontitis model

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Introduction

Periodontitis is a highly prevalent, oral inflammatory disease of infectious origin and the primary cause of tooth loss in adults. It is associated with a progressive destruction of periodontal tissues leading to the formation of periodontal pockets. Periodontitis has been evoked in the progression of physio-pathological mechanisms both at the clinical, cellular and molecular levels,[2] although the contributive mechanisms and how they interplay remain elusive.

Initially, identified by Wolf 1967 as a platelet dust containing phospholipids, and capable of supporting coagulation,[25] microvesicles (MVs), are submicron fragments of the plasma membrane that shed into the extracellular space in response to a variety of cellular stress conditions.[4] MVs are released in biological fluids such as blood, urine, tears and saliva. In the blood, they circulate at low concentration in healthy individuals and are elevated in various cardiovascular and chronic diseases including diabetes, chronic renal failure, pre-eclampsia and hypertension. [230] Ranging in size between 100 to 1000 nm in diameter, they are released during plasma membrane budding, and considered as physiological shuttles supporting cellular cross-talk. Indeed, the MV membrane embeds proteins, signaling nucleotides, and metabolites as well as RNA, non-coding RNA and bioactive lipids that were present at the site of

their emission.[231] MVs are considered pro-coagulant because they expose phosphatidylserine, a phospholipid that catalyzes the assembly of the complexes of blood coagulation .[232]

The spleen is the largest secondary lymphoid organ in the body and possesses a wide range of immunological functions [3]. It is an important source of MVs.[7] Previous data from the laboratory have shown that (i) spleen MVs may act as pro-inflammatory mediators promoting premature senescence of coronary artery endothelial cells, [7 4] leading to endothelial dysfunction and the secondary release of pro-senescent endothelial MVs and (ii) that the LPS of *Porphyromonas Gingivalis,* the main causative pathogen in periodontitis, induces to the shedding of proapoptotic endothelial-derived microvesicles (MVs) from cultured endothelial HUVEC.[6]

In vitro and in vivo data from the laboratory also identified *LPS* as an inducer of the shedding of pro-coagulant, pro-senescent and pro-inflammatory MVs from rat splenocytes.[4] The feasibility of a pharmacological control of the release of prosenescent MVs from the spleen was further demonstrated in a rat model of ageing. Oral treatment by an optimized omega-3 formula was found protective against both the increased release of SMVs isolated from middle-aged and aged rats, and their noxious endothelial prosenescent effects assessed in primary coronary endothelial cells . [7]

This work aims to study the impact and remoted effects of *P.gingivalis* local gum infection on the spleen tissue. Experiments are designed to gain a better knowledge of the interactions between local teeth infection and the spleen response.

Materials and methods

Animal experiment

8 weeks old C57BL6 mice (Janvier, Le Genest-Saint-Isle, France) were used in this study. Briefly, *P. gingivalis* (ATCC 33277) was cultured in anaerobic conditions in Brain heart Infusion medium (Bio-Rad, France) supplemented with hemine and menadione as described previously.

Experimental periodontitis was induced in mice gum with silk ligatures (LIG), *P.gingivalis* soaked ligatures*, P.gingivalis* oral gavage (LIG-PG) for 2 or 4 weeks. In some experiments of *P.gingivalis,* infection was performed by intraperitoneal injection (IP) of (5.10^7 bacteria) for 2 weeks. IP-treated mice were eventually treated by a PAD4-4 inhibitor injected simultaneously to IP (CL amidin, 4mg/ml)

Protocols were approved by the Ethics Committee “Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg (CREMEAS)” (APAFIS#28745-2020121815051557). Mice were examined to evaluate pain and stress, and their weight monitored daily. All infection procedures were repeated 3 times per week)

Mouse splenocyte isolation and culture

Splenocytes were obtained as detailed elsewhere (see manuscript 1)

After sacrifice, fresh mouse spleens were weighed, washed in PBS (phosphate-buffered saline, Lonza, USA) and cleaned from any fat debris. Under sterile conditions, spleens were cut using a scalpel into 1mm slices before homogenization by the plunger of a 2ml syringe used as a pestle and further filtered through 100-µm sterile strainers (Sarstedt, Germany). Following 450g centrifugation for 5 min, the cell pellet was re-suspended in a 1-ml ammonium-chloride-potassium erythrocyte lysis buffer for 5min, then completed with 9ml RPMI-1640 medium (PAN biotech) supplemented with L-glutamate (2mM), penicillin (100 U/mL), streptomycin (100 U/mL), fungizone (250 mg/mL) and 10% fetal bovine serum (FBS) (Dutscher). Cells were counted, seeded at 5×10^6 cells/mL in a 75 mL culture flask for adherent cells and incubated in humidified incubator at 37°C, with CO2 for 24 hours.

Characterization of splenocytes

Isolated splenocytes were characterized by flow cytometry (FACS 543 FORTESSA), according to a standardized protocol using APH7, BV786, PerCP-Cy5.5, Alexa Fluor700, BUV395 and eFluor 450 labelled monoclonal antibodies against mouse CD4, CD8, CD19, LY-6C, LYG-6 (GR-1), (BD Biosciences) and CD11b (Mac-1) (e-Biosciences). Data were analyzed by FlowJo.

Isolation and quantification of splenocytes microvesicles

After 24 hours culture, supernatants were collected under sterile conditions. Splenocytes and cell debris were discarded by centrifugation at 450 g, 15 min at room temperature. Supernatants were further centrifuged twice at 14000g, 60 min, 4°C, washed pelleted SMVs were concentrated in Hanks Balanced Salt Solution (HBSS, without phenol red, without calcium and magnesium, Lonza, Belgium) and stored at 4°C for a maximum of 1 month. Prothrombinase assay was performed to quantify SMVs after their capture on Annexin-A5 coated micro-wells using a microplate spectrophotometer in kinetic mode. Annexin-5 has high affinity for phosphatidylserine (PhtdSer) exposed at the surface of MVs. In this assay, PhtdSer is the rate-limiting factor of the generation of thrombin from prothrombin detected at 405 nm using a chromogenic substrate (PNAPEP-0216, cryopep, Montpellier, France) [40]. SMV concentration was referred to as nanomolar PhtdSer and was obtained by subtracting the OD values measured using isotype control biotynylatyed IgGs.

Protein content of splenic microvesicles

MVs protein content was measured by spectrophotometry using a Nanodrop (Thermo Scientific)

Characterization of oxidative stress in spleen tissues

Oxidative stress was determined in spleen tissue using DiHyroEthidium (DHE), a redox-sensitive fluorescent probe.Tissues were embedded in molds

containing Tissue-Tek optimum cutting temperature (OCT) compound (Sakura 4583, Leiden, Netherlands) and snap-frozen in liquid nitrogen, and cryosectionned (10μm) and mounted on slides. Slides were then incubated with DHE (2.5 μM) for 30 min, at 37°C in a dark slide humidified chamber. After 3 washings with PBS, slides were mounted under coverslip using fluorescence mounting medium (DAKO, USA), dried in dark and analyzed by confocal microscopy (Leica) with a 20X magnification lens. Level of oxidative stress was quantified using the Image J software.

Western blot

Spleen lysates (30 μg) were obtained by incubation in RIPA for 30 min on ice. SDS-PAGE electrophoresis was performed at 10% (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) at 100 volts for 2 hours (Bio-Rad). After transfer onto a nitrocellulose membrane (Amersham GE Healthcare, USA) for 30 min at 25 V, the aspecific sites were saturated by 1h incubation the membranes in a TBS solution (Euromedex, Strasbourg) containing 5% BSA and 0.1% of Tween 20. Membranes were then incubated overnight at 4°C with the primary antibodies directed against: VCAM-1 (1/1000; abcam, Cambridge, UK) and TF (Biomedica 11-4509). After washing, the a secondary antibody was incubated for 1 h at room temperature (1/10000, anti-rabbit or anti-mouse immunoglobulin G, Cell Signaling Technology). The detection of the bands was carried out by chemiluminescence (Fisher, Bioblock Scientific) and the analysis

of the optical density of each band performed by ImageQuant LAS 4000 imager (GE Healthcare). Optical densities are presented as a ratio, optical density of the band of interest to that of the GAPDH (1/10000; abcam Cambridge, UK) a maintenance protein.

Statistical analysis

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

Results

1- Impact of P.Gingivalis on spleen weight and cell count and cell viability

After 2 weeks no significant modification in spleen weight, viability or cell count could be observed between control individuals or those treated by ligature or ligatures impregnated or not with P. Gingivalis. Only IP injection led to a modification of spleen weight, independently of the co-injection of the PAD-inhibitor, suggesting a spleen cell proliferation or recruitment.

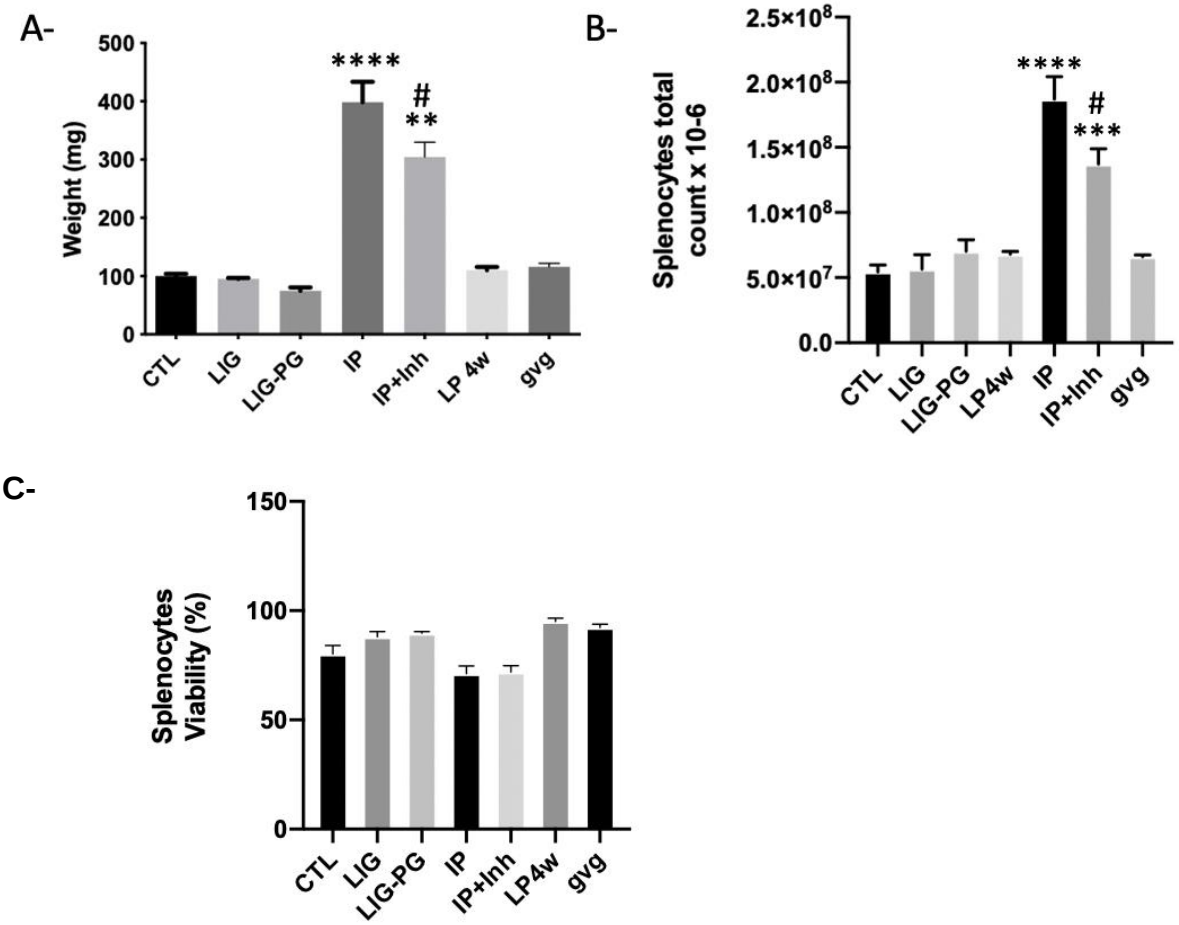


Fig 1: IP injection of P. gingivalis significantly enhances spleen weight (A) and splenocyte count (B) that is reduced by the PAD4 inhibitor.

*CTL: untreated mice, IP: mice submitted to P. Gingivalis IP injection for 2 weeks, LIG: mice submitted to 2 weeks gum ligation, LIG-PG : mice submitted to 2-weeks gum ligation with a thread impregnated with P.gingivalis, LP4W: mice submitted to gum ligation with a thread impregnated with P.gingivalis for 4-weeks , IP+I: mice submitted to simultaneous P.gingivalis and PAD4 inhibitor IP injections for 2-weeks, gvg: mice submitted to oral gavage for 2 weeks Data are expressed as mean± SEM. n=5: ***: p<0.0001 & 0.0001 versus CTL, respectively; #: p< 0.05 vs. IP.*

2- P. gingivalis increases the splenocyte ability to shed SMVs independently of histone citrullination

After 24h culture, only splenocytes extracted from individuals treated by intraperitoneal injection of P. Gingivalis showed enhanced ability to shed SMVs as measured by prothrombinase assay or protein content culture medium. SMVs concentration doubled as compared to the SMVs from splenocytes of control spleens (IP vs CTL, p< 0.01). Only the IP treatment enhanced the shedding of SMVs and the PAD4 inhibitor had no effect, suggesting that histone citrullination is not causative. Interestingly, when the *P. Gingivalis* treatment was extended from 2 weeks to 4 weeks, SMV shedding returned to the baseline levels of control individuals. Noteworthy, the delivery of *P. Gingivalis* by oral gavage was ineffective indicating that *P. Gingivalis* woud not activate spleen cells via a digestive route.

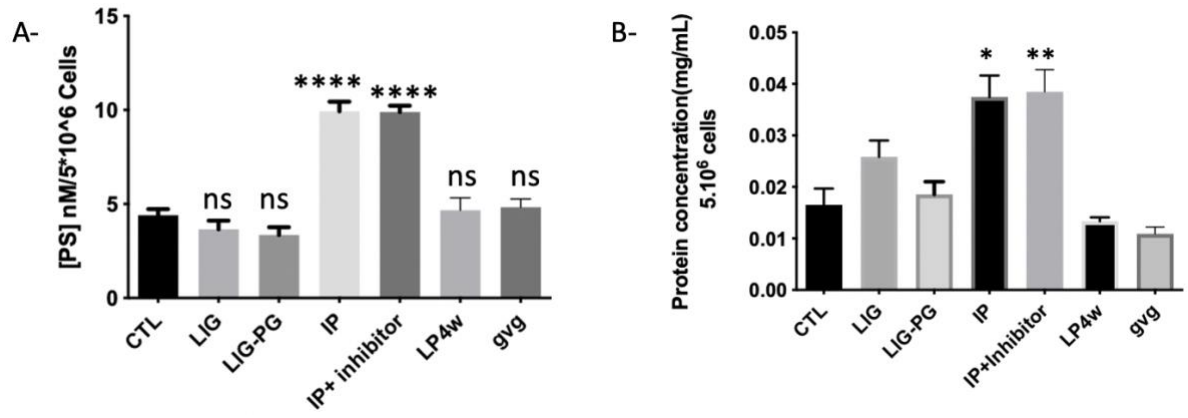


Fig 2: IP injection of P. gingivalis favors enhanced splenocyte shedding in vitro.

*SMVs were assessed by protein content, or prothrombinase assay. CTL: untreated mice, IP: mice submitted to P. Gingivalis injection for 2 weeks, LIG: mice submitted to gum ligation for 2 weeks, LIG-PG : mice submitted for 2 weeks to gum ligation with a thread impregnated with P.gingivalis, LP4W: mice submitted to gum ligation with a thread impregnated with P.gingivalis for 4 weeks, IP+I: mice submitted to simultaneous P.gingivalis and PAD4 inhibitor IP injections for 2-weeks, gvg: mice submitted to oral gavage for 2 weeks. Data are expressed as mean± SEM. n=5 ***: p<0.0001*

3. The cell composition of the spleen varies with the mode of administration of P. Gingivalis.

Modifications of cell composition in response to 2 weeks treatments was assessed by flowcytometry. Data indicate that even the gum ligature (LIG) is able to induce a significant elevation of spleen monocytes, neutrophils, although to a less extent as compared to P. Gingivalis IP injection that tripled monocytes counts and raised neutrophils by ten folds as expected following intense initiation of innate immunity. Compared to the sole ligature, impregnation of the thread by P. Gingivalis did not significantly alter the monocyte, neutrophil or granulocyte cell counts. By contrast, no modification

of the B, T4 and T8 lymphocyte counts could be observed in LIG nor LIG-PG after 2 weeks or even 4 weeks, suggesting a minor contribution of the adapatative immunity. Nevertheless, IP injection significantly reduced the T and B lymphocyte counts confirming the feasibility of their mobilization through vascular routes as compared to gavage. Interestingly, oral gavage deeply reduced the count of T8 lymphocytes as compared to IP injection.

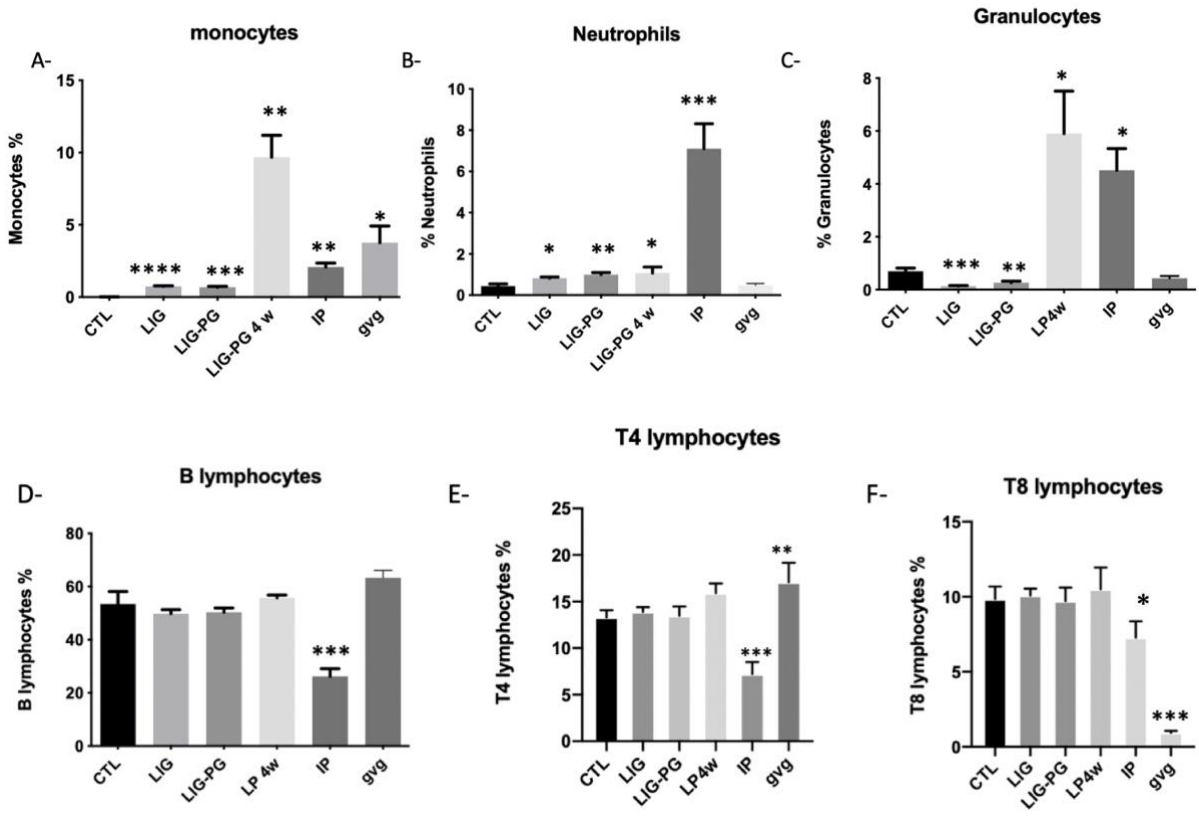


Fig 3: Spleen cell composition varies with the type of induction.

*Leukocyte counts were measured by flow cytometry of the spleen cells from mice treated for 2 weeks by either gum ligature (LIG) with or without P. Gingivalis thread impregnation (LIG-PG), intraperitoneal P. gingivalis injection (IP) or oral gavage (gvg). LP4w: and IP enhanced monocytes and neutrophils. , LP4W: mice submitted to gum ligation for 4 weeks with a thread impregnated with P.gingivalis. CTL: untreated mice. Data are expressed as mean± SEM. n=5 ****: p<0.0001, ***p<0.001, ** p<0.004*p<0.01, versus control.*

4- **Gum injury and P. gingivalis infection favor ROS accumulation in spleen tissue**

Compared to control spleens, gum ligature alone was able to trigger a 5-fold ROS accumulation as assessed by histological staining with dihydroethidium. Addition of P. gingivalis in threads further doubled values of ROS (LIG-PG), up to 75% of the highest ROS value observed after IP treatment. Interestigly, PAD4 drastically prevented the effect of intraperitoneal P. gingivalis, suggesting that NETosis is key to ROS accumulation in the spleen.

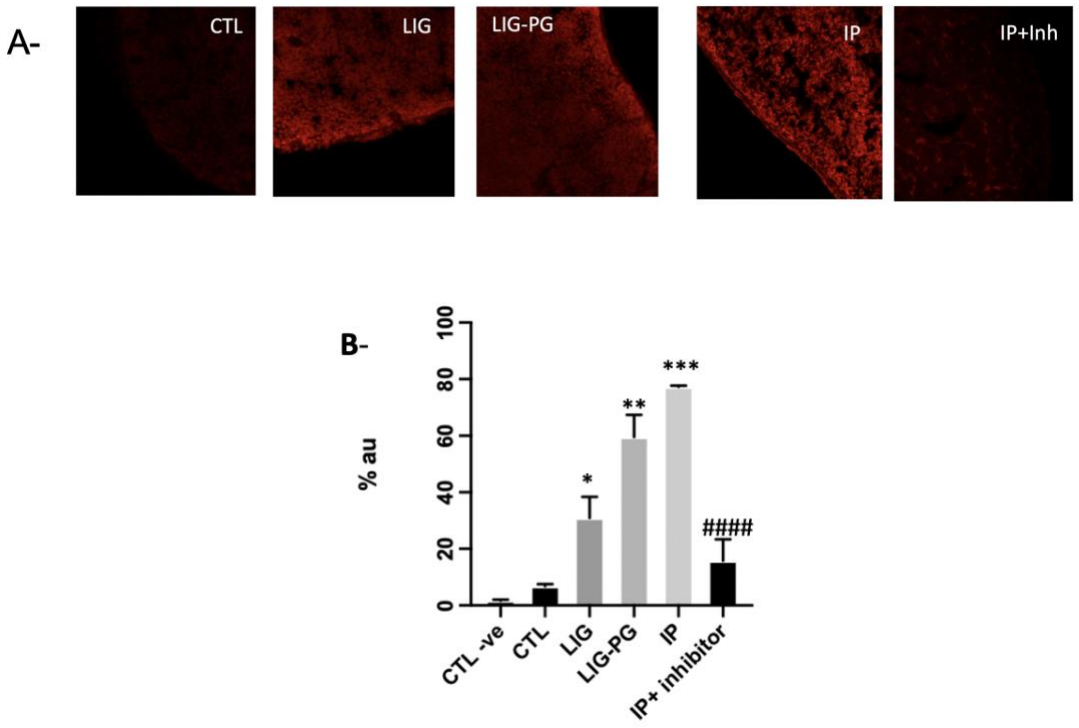


Fig 4: Upon gum injury and infection ROS accumulation increases in spleen tissue.

A: representative photographies of immuno-histological staining with the red fluorescent probe with DHE. CTL: untreated mice, IP: mice submitted to PG injection for 2 weeks, LIG: mice submitted to gum ligation for 2 weeks, LIG-PG : mice submitted to gum ligation for 2 weeks with a thread impregnated with P.gingivalis, IP+I: mice submitted to simultaneous P.gingivalis and PAD4 inhibitor

*injection for 2 weeks. Data are expressed as mean± SEM. n=5 ***: p<0.0001, **: p<0.001, *: p<0.03.#: p<0.0001 vs CTL.*

5- P.gingivalis enhances the pro-inflammatory response in spleen tissue

The activation of spleen cells was further characterized by assessment of the expression of VCAM-1, an inflammatory marker. In addition, tissue factor (TF) was assessed as an early indicator of leukocyte response to inflammation. As compared to control spleens, a 3-fold over-expression of VCAM-1 was observed in response to gum ligature (LIG), with or without P. gingivalis. IP injection of the pathogen did not induce higer VCAM-1 expression. Interestingly, TF was also overexpressed after 2 weeks of treatment, the highest value being observed with local gum injury combined to P.gingivalis soaked threads.

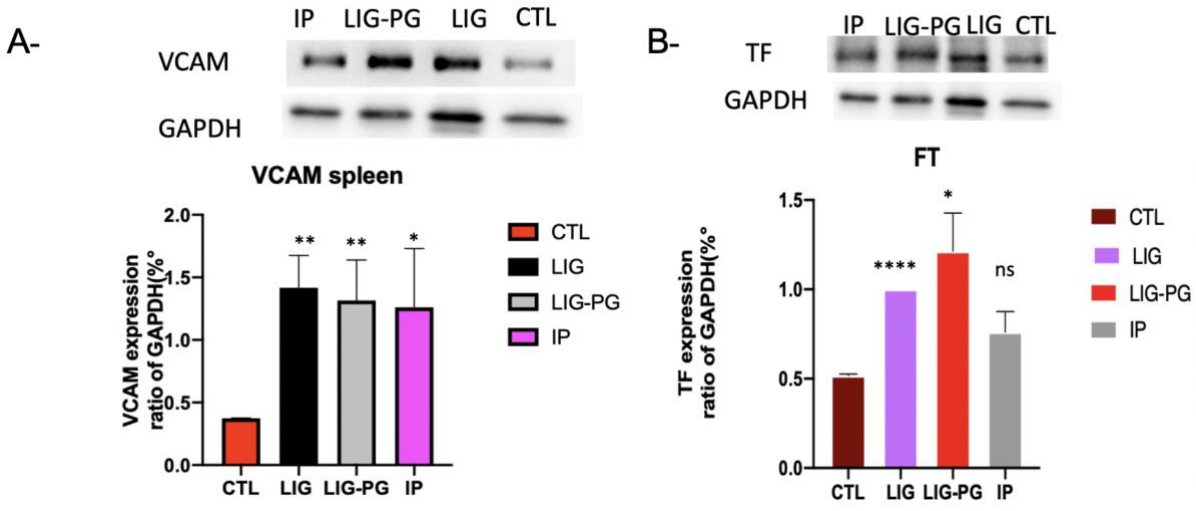


Fig5: Enhanced expression of VCAM-1 (A) and tissue factor (TF) in response to gum injury.

Upper panel: representative western blots. Lower panel: cumulative data. CTL: untreated mice, IP: mice submitted to P. Gingivalis injection for 2 weeks, LIG: mice submitted to gum ligation for 2 weeks, LIG-PG : mice submitted to gum ligation with a thread impregnated with P.gingivalis for 2 weeks.

*GAPDH: house-keeping protein. Data are expressed as mean± SEM. n=5 ****: p<0.0001, **: p<0.001, *: p<0.05 vs. CTL.*

Discussion

Neutrophils are key to the multiple spleen cell responses induced by P. Gingivalis gum insult

The present findings indicate that *P. gingivalis* is a strong inducer of the spleen response after only 2 weeks of treatment, highest values being observed when the pathogen is given by intraperitoneal injection. (figure 1)

While IP injection led to a drastic elevation after 2 to 4 weeks in spleen weight and cell count, they remained stable in individuals submitted to local gum ligature with soaked threads as compared to control animals. In all animal subsets cell viability did not differ from the baseline, suggesting that the above increase of weight was the consequence of either cell recruitment or cell proliferation. Interestingly, the simultaneous treatment by IP and a PAD 4 inhibitor that blunts histone citrunillation lead to a significant reduction of the spleen weight and total cell count thereby pointing at the possible role of NETosis.

Analysis of the cell composition revealed that the gum insult, eventually combined to P. gingivalis local infection, was also able to significantly modify the spleen cell composition, mainly cells involved into naïve immunity. Although to a lesser extent than IP infection, increased neutrophil and monocyte counts were found in LIG and LIG-PG subsets while granulocytes were decreased after 2 weeks. (see figure 4) When treatment was extented to 4 weeks only

monocytes and granulocytes were strongly elevated in LIG-PG thereby underlining the different kinetics in cell proliferation and recruitment. Conversely to the IP infection, gum insult did not induce any count modification of the cell involved in adaptative immunity after 2 or 4 weeks.

Gum insult early promotes spleen responses driven by naïve immunity

To confirm the spleen cell modification after 2-weeks gum insults, we measured by western blot the expression of VCAM-1, an endothelial inflammatory marker and TF, induced at the plasma membrane of activated monocytes, neutrophils and endothelial cells in response to inflammatory and procoagulant mediators. Interestingly, gum insult combined or not to P. gingivalis let to a drastic and similar over-expression of both VCAM-1 and TF, that nearly reached the values observed after IP injection of P. gingivalis. (figure 5) In addition, ROS accumulation in the spleen also indirectly confirmed the active contribution of neutrophils and granulocytes after gum insults. Altogether, data from LIG and LIG-PG subsets strongly suggest a key role of monocytes and neutrophils in accordance with the flow cytometry cell analysis. (see figures 3 & 5). Nevertheless, the significant induction of splenocyte shedding assessed by prothrombinase assay or protein content was only detected in response to intraperitoneal P. Gingivalis infection. Interestingly, PAD4 was not efficient in the limitation of IP-driven SMV shedding,thereby suggesting that the release of MVs in the spleen in response to IP injection was not dependent on NET

extrusion after 2 weeks. Therefore, SMVs are most likely from another cell origin than neutrophilsor released by non-extruding neutrophils in response to IP. The characterization of SMV cell origin would confirm the degree of activation of each contributing cell line.

Conclusion

Altogether data are highly suggestive of the strong and early contribution of the cells of naïve immunity in periodontitis and of a key role of neutrophils and of Netosis in the initiation and progression of periodontitis-driven spleen responses after 2 weeks of infection.

Complementary data

To further confirm the remote effects of gum insults described in the article, measurements have been initiated to detect

1) The properties of SMVs on coronary endothelial cells. The aim was to assess SMVs as possible mediators of a thrombotic tendency known to be associated with periodontitis in humans. For this purpose, we used a MV-mediated endothelial crosstalk model. The endpoint was the induction of endothelial senescence.

2) The heart inflammatory responses eventually driven by periodontitis by immunolabelling and western blot using heart lysates sampled from the different individuals that were analyzed in the article. We more particularly investigated markers of the endothelial inflammation (VCAM-I), the up-regulation of pro and anti-inflammatory cytokines and the expression of Gasdermin D, a recently identified contributor to the inflammosome.

Methods

Primary coronary endothelial cell culture

Primary coronary artery endothelial cells (ECs) were prepared from left coronary arteries of pig hearts, collected from local slaughterhouse (COPVIAL, Holtzheim, France) as described previously. [7] Left coronary arteries were

dissected and cleaned of any adhesive conjunctive tissues then flushed with PBS. After debris removal and blood excess, cleaned coronary arteries were treated with Collagenase type I (Gibco, life technologies corporation, USA) solution at 1 mg/ml prepared in MCDB-131 medium (Gibco, life technologies limited, UK), supplemented with streptomycin (100 U/ml), penicillin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM, all from Lonza,St Quentin en Yvelines, France) for 15 min at 37°C. Endothelial cells were then extracted into 50-mL falcon tubes by circular massage of arteries with frequent flushing with medium. The collected medium containing endothelial cells was then centrifuged (450g, 5min, at room temperature), supernatant was discarded and cells were re-suspended with complete MCDB-131 medium supplemented with 15% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM). Endothelial cells of 4-5 different coronary arteries were cultured in adherent T25 flask in humidified incubator at 37 °C, with 5% CO2. After 6 hours, cells were washed with PBS non-adherent cells removal and fresh complete medium was added. Thereafter, first passage ECs (P1ECs) were grown for 72 hours with medium changed every 48 hours.

Treatment of endothelial cells with SMVs

Following trypsinization (Trypsin, Gibco, Life Technologies SAS, St Aubin, France), P1 endothelial cells were seeded in 6-well plate at 65-75%

confluency and incubated with identical amounts of washed SMVs (30nM PhtdSer eq.) for 48 hours, P3 cells was our standard for senescence.

Measurement of Senescence-associated β -galactosidase activity

SAβ -gal activity was measured by flow cytometry using the C12FDG fluorogenic cell permeable substrate. Endothelial cells were alkalinized (PH raised to 6) with chloroquine (300 µmolar) for 1 hour, followed by 1 hour incubation with C12FDG (33 µmolar), a fluorogenic substrate of SAβ -gal. Cells were thereafter washed with ice cold PBS, harvested with trypsin and freshly analyzed using CellQuest software. (FACSscan, Becton Dickinson, San Jose, CA, USA). Light scattering parameters were set to eliminate dead cells and subcellular debris. The green C12-fluorescein signal was measured and SAβ -gal activity estimated using the mean fluorescence intensity (MFI) of the cell population. Auto-fluorescence gains were determined in unlabeled cells and set at the first logarithmic decade.[7]

Western blot

Heart lysates (30 µg) from the left ventricle were obtained by incubation in RIPA for 30 min on ice. SDS-PAGE electrophoresis was performed at 10% (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) at 100 volts for 2 hours (Bio-Rad). After transfer onto a nitrocellulose membrane (Amersham GE Healthcare, USA) for 30 min at 25 V, the aspecific sites were saturated by 1h incubation the membranes in a TBS solution (Euromedex, Strasbourg)

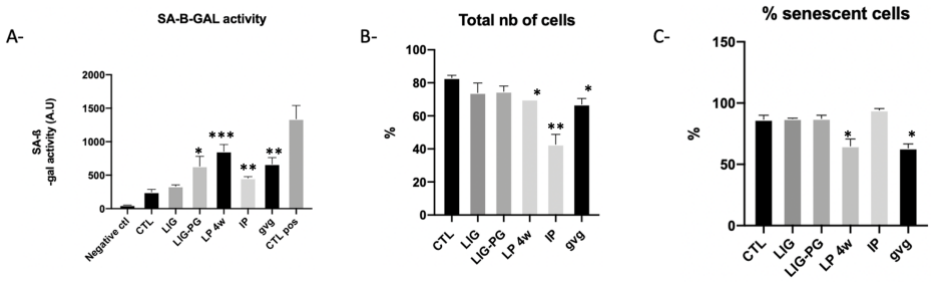
containing 5% BSA and 0.1% of Tween 20. Membranes were then incubated overnight at 4°C with the primary antibodies directed against: VCAM-1 (1/1000; abcam, Cambridge, UK) and TF (Biomedica 11-4509). After washing, the secondary antibody was incubated for 1 h at room temperature (1/10000, anti-rabbit or anti-mouse immunoglobulin G, Cell Signaling Technology). The detection of the bands was carried out by chemiluminescence (Fisher, Bioblock Scientific) and the analysis of the optical density of each band performed by ImageQuant LAS 4000 imager (GE Healthcare). Optical densities are presented as a ratio, optical density of the band of interest to that of the GAPDH (1/10000; abcam Cambridge, UK) a maintenance protein.

VCAM (1/1000; abcam, Cambridge, UK) Gasdermin (1/2500; abcam, Cambridge, UK) TNF-alpha (1/1000; abcam, Cambridge, UK) , IL-6 (1/1000; abcam, Cambridge, UK), IL-10 (1/1000; abcam, Cambridge, UK).

Results

1) Complementary data to establish the proof of concept of endothelial damage induced by SMVs

SMVs induce the senescence of primary coronary endothelial cells



D-

Conditions	Total nb of living cells	% senescent cells	Mean of senescent
Ctl	82	86	248
LIG	74	87	334
LIG-PG	75	87	634
LP 4w	70	65	854
IP	43	94	455
gvg	67	63	666

Fig 1: SMV generated in the presence of P.gingivalis induce endothelial senescence.

*CTL: untreated mice, IP: mice submitted to PG injection for 2 weeks, LIG: mice submitted to gum ligation for 2 weeks, LIG-PG : mice submitted to gum ligation for 2 weeks with a thread impregnated with P.gingivalis, IP+I: mice submitted to PG injection for 2 weeks with injection PAD4 inhibitor simultaneously Data are expressed as mean± SEM. n=5 ***: p<0.0001*

2) Complementary data to establish the proof of a remote heart damage prompted by P.gingivalis

P. gingivalis induces inflammation in heart tissue

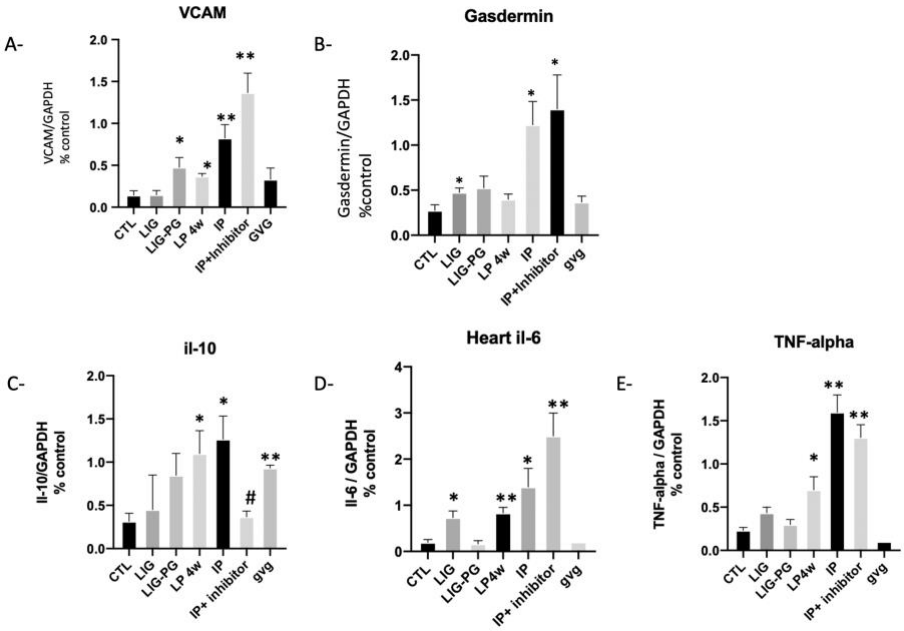


Fig: IP induces elevation of inflammatory markers in heart tissue: ***VCAM*** *(IP:0.8±0.1,CTL:0.2±0.05,p<0.0001),****Gasdermin (****IP:1.2±0.2,CTL 0.2±0.06 p=0.0009),****TNF-alpha(****IP:1.6± 0.2,CTL:2±0.03 p<0.0001),****IL-6****(IP 1.1±0.6,CTL 0.2±0.05 p<0.001)****, IL-10*** *(IP: 1.2±0.2 CTL 0.3±0.09, p=0.04).*

Discussion

Data clearly demonstrate that gum ligature with threads soaked in P. gingivalis induces high SMV-mediated endothelial senescence as compared to the SMV isolated from mice with IP infection This observation points at an eventual new mechanism possibly contributing to thrombotic tendency through remote paracrine MV effects. Indeed, senescent endothelial cells are characterized by an atherothrombogenic profile.

Analysis of western blots of heart extracts indicated major changes of the inflammatory profile. In response to IP *P.gingivalis* injection, PAD-4 only down-regulated **IL-10** (IP+INH:0.3 ±0.06, IP: 1.2±0.2), suggesting the impact of netosis on anti-inflammatory responses. After 2 weeks-ligature, P.gingivalis soaked thread significantly upregulated **VCAM** and **Gasdermin** (LP:0.5±0.1,p<0.0001;LP:0.6±0.1, p<0.001 respectively; CTL:0,2±0,055), while LIG alone had no effect. A 4-weeks sustained treatment raised the expression of **VCAM** (LP4W: 0.4±0.03, CTL 0.2±0.03 p<0.0001), **TNF-alpha** (LP4W: 0.7±0.1, CTL 0.2±0.03 p<0.0001) and **IL-6** (LP4w: 0.8±0.1, CTL 0.2±0.05 p<0.001). Strikingly, only the IL-10 expression reached the peak values induced by IP (LP4w: 1.1±0.2, IP: 1.2±0.2 vs CTL 0,3±0,09, p=0.04), strongly suggesting a delayed anti-inflammatory effect prompted by P.gingivalis. Indeed, gavage led to elevated IL-10 already after 2 weeks (0.9±0.03).

Interestingly, aside from the heart tissue responses after IP injection, mice submitted to gum insults showed a significant 2 folds elevation of VACM-I, suggesting a major endothelial insult (compared to 3 folds in the IP subset) Unfortunatly, variations of cytokines (IL-6 & IL-10) were less informative owing to variability and the balance between pro and anti-inflammatory interleukines could not be investigated after 2 weeks. Neverthless, the proinflammatory IL-6 was already elevated in response to ligature. Most probably the IL-10 assessmen by western blot is highly sensitive to the kinetics of the proinflammatory responses. The role of Netosis could be shown by the reduced IL-10 expression in mice treated with IP and inhibitor. The model also shows that heart inflammation is a chronic on-going process since a prolonged 4-weeks treatment enhances the up-regulation of TNF-alpha, VCAM-I, IL-6 as compared to LIG-PG for 2 weeks.

Conclusion

Taken together, all data confirm the hypothesis of a remote effect of P.gingivalis contributing to the gum insult. They also bring new evidences in the relationships between the spleen and the heart also termed the cardiac splenic axis. Responses.

Future experiments

To further confirm the remote effects of MVs and of Netosis in the initiation and progression of *P.gingivalis*-mediated effects, the plasma concentration of NETs could be measured as well as the variations of cytokines and of circulating microvesicles of leukocyte origin. For this purpose, pre-analytical sampling has been achieved using individuals of the seven animal subsets experimental procedures were established according to the following methods. The aims were to examine and confirm the eventual Netosis effects in the IP treated mice, to identify remote mediators in the plasma. In addition, lymph nodes of the abdomen were also harvested to examine early remote effects by RT-PCR.

Preparation of preanalytical samples for anticipated experiments

Intraperitoneal (IP) liquid collection

After mice anesthesia using a combination of 100mg/kg ketamine and 10mg/kg xylazine, injection of 2 ml of salt solution (NaCl) into the peritoneal sac was performed followed by a massage to the hall abdominal cavity using a 2ml syringe. Then, peritoneum, intraperitoneal liquid was collected slowly liquid (approximately 1,7 ml might be collected) and stored at -80°C for long storage.

Plasma preparation

After IP liquid collection, mice were sacrificed. Rapidly the blood was collected from the mice's heart and harvested on 0.129 M Na citrate ratio: 9 volume

blood per 1vol anticoagulant. The tube was well mixed by inverting then centrifuged rapidly the at 13000g for 3 min at room temperature. Supernatant was collected in a new Eppendorf and centrifuged again at 13000g for 3 min at room temperature.

Platelet free plasma (PFP) was then collected by rapid upside-down inverting in a 1.5 ml Eppendorf and stored at –80 °C.

After IP liquid and blood collection, the left part of the heart was isolated and stored at -80°C for long storage and the spleen was harvested after laparotomy.

Detection and quantification of NETs in the mice intraperitoneal liquid (IPL)

For comparative purposes, NETs will be characterized from the IPL of the different subsets and the and the activity of histones measured as a ratio of the NET length. After thawing, samples are centrifuged at 10,000g for 10 minutes. 50 µL of SN is recovered from washing. (SNL) and the pellet is suspended in 50 µL of PBS pH 7.4 or physiological solution. The obtained volumes are the separated into four 25 µL aliquots. 25 µl aliquot from the pellet and (SNL) are placed on slides, stained with 25 µL of DAPI or Hoetsch, incubated 10 min and then visualized under a fluorescence microscope. The remaining aliquot of each sample is used for elastase detection using the chromogenic substrate: MeOSuc-AAPV-pNA (Km 0.52 mM).

Dosage of cytokines in blood plasma

Cytokine assay was performed from blood samples collected during animal sacrifices. The assay of these cytokines was carried out using the Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D systems) consisting of profiling 40 different antibodies to mouse cytokines, chemokines, and acute phase proteins.

CXCL13/BLC/BCA-1	IL-5	M-CSF
C5a	IL-6	CCL2/JE/MCP-1
G-CSF	IL-7	CCL12/MCP-5
GM-CSF	IL-10	CXCL9/MIG
CCL1/I-309	IL-12 p70	CCL3/MIP-1 alpha
CCL11/Eotaxin	IL-13	CCL4/MIP-1 beta
ICAM-1	IL-16	CXCL2/MIP-2
IFN-gamma	IL-17	CCL5/RANTES
IL-1 alpha/IL-1F1	IL-23	CXCL12/SDF-1
IL-1 beta/IL-1F2	IL-27	CCL17/TARC
IL-1ra/IL-1F3	CXCL10/IP-10	TIMP-1
IL-2	CXCL11/I-TAC	TNF-alpha
IL-3	CXCL1/KC	TREM-1
IL-4		

The samples will be mixed with a cocktail of biotinylated detection antibodies, then incubated with the array membrane which is spotted in duplicate with capture antibodies to specific target proteins. After, captured proteins will be

visualized using chemiluminescent detection reagents. The signal produced will be proportional to the amount of analyte bound.

General discussion

In our study, we established the proof of concept of the impact of periodontitis on spleen and confirmed the interest of spleen in a C57BL6 murine model as a reliable and relevant source of MVs with no alternative method available for the procurement of large quantities of MVs. In addition, we confirmed that even after 2 weeks, the physio-pathological impact of any injury in the gum could be remotely sensed by the spleen, at much lower thresholds than those observed by intraperitoneal injection of the pathogen. (*El Itawi et al 2022*)

Interestingly, our standardized protocol for harvesting murine splenic microvesicles (SMVs) fits the unmet needs for the *in vivo* study of the control of SMV-mediated effects in engineered and transgenic mice.

Our findings indicate that *P. gingivalis* is a strong trigger for the shedding of pro-senescent spleen-derived microvesicles (SMVs) that were demonstrated to prompt senescence in primary coronary endothelial cells, on-line with our previous observation in the rat. [4,7]Our data are in accordance with the cardiac splenic axis that has been proposed to explain the link between inflammation and athero-thrombogenicity and in particular the pericardiac inflammation occurring post myocardial infarction. [227] [229] However, the role of cardiac or splenic Tregs lymphocytes and their constitution remains to be evaluated.[228]

Nevertheless, measurement of IL-17 and of its receptor has been undertaken by western blot and by antibody chips using cardiac extracts from individuals of all subsets. Detailed analysis will be necessary to compare plasma and

tissue expression levels of all cytokines and more specifically of those involved in the control of the innate and adaptative immune response.

Used as relevant preclinical tools in our investigation, SMVs showed remote effects of local infection by *P. gingivalis* using multiple approaches. We showed significant variations between SMVs released from *P. gingivalis*-treated *vs*. untreated animals, already detectable after 2 weeks. We observed that these remote effects were associated with significant variations in weight, cell viability, and most importantly of the cell composition of the spleen. Indeed, *P. gingivalis* prompted neutrophil count raise as assessed by immunostaining just after splenocyte isolation (t0) in mice submitted to gum injury and suggested a remote action caused by chronic infection.

Interestingly, ROS accumulation during *P. gingivalis* infection was reversed by a Netosis inhibitor after reaching it highest raise in response to IP infection.

In the supernatant of 24 h splenocyte cultures, released SMVs were tripled when isolated from IP-injected mice only. However, the SMVs shed from all the conditions were able to alter primary endothelial cells to various degree, indicating on-going effects potentially noxious after a prolonged exposure. Indeed, the prosenescent features of SMVs shed from LIG-PG, LP4w, IP and gvg mice observed in coronary artery primary endothelial cells confirms their authentic role as biomarkers of a remote spleen activation by *P. gingivalis.*

At the heart level, P.gingivalis mediated pro-atherothrombotic and pro-inflammatory markers in left ventricular heart extracts. IP induced elevation of all inflammatory markers in heart tissue including VCAM, Gasdermin,TNF-alpha, IL-6, IL-10. The Netosis inhibitor PAD-4 only down-regulated IL-10 suggesting the impact of netosis on anti-inflammatory responses. After 2 weeks-ligature, P.gingivalis soaked thread significantly upregulated VCAM and Gasdermin while LIG alone had no effect. A 4-weeks sustained treatment (LP4w) raised the expression of VCAM, TNF-alpha, IL-6. Strikingly, only the IL-10 expression reached the peak values induced by IP, strongly suggesting a delayed anti-inflammatory effect prompted by P.gingivalis while gavage led to elevated IL-10 already after 2 weeks.This confirms that our model as suitable for the study of remote cardiac effects by *P.gingivalis* local infection. Moreover, low but sustained pathogen-induced periodontitis alter the balance of heart inflammatory mediators where the control of Netosis appears valuable to preserve or delay heart tissue inflammation.

In the present procedure, a 2-week duration induced variations in the splenocytes population, ROS accumulation, prosenescent MVs shedding and heart inflammation. It is important to keep in mind that although the major drawback of the murine model is a small spleen weight as compared to rats, the present procedure was optimized for a murine model and suitable for further transgenic mice investigations.

Nevertheless, data confirm the model as suitable for the study of remote spleen and cardiac effects by *P.gingivalis* local or intraperitoneal infection.

Confirmation of our hypothesis of a remote action of periodontitis on spleen immune responses and noxious cardiac impact was obtained by examining spleen and cardiac tissues and the prosenescent effect of SMVs on endothelial cells. Owing to pandemia, samples remain to be assessed. In particular, the plasma circulating mediators, like circulating fragments of NETs and their enzyme activities remain to be measured. To confirm the early impact on the cardiac outcome, other proteins like MMP or NLRP3 associated to cardiac inflamosome could be of interest as markers of heart tissue remodeling. Finally, the role of the periodontitis in the recently proposed spleen-cardiac axis could be explored in the lymph nodes. Indeed, IL-17 related regulatory events could not be explored at the protein level in spleen or MV extracts, but remain accessible by RT-PCR.

Conclusion

P.gingivalis-associated periodontitis induces spleen activation that seems to be an important source of MVs, eventually associated with heart inflammation and the shedding of deleterious procoagulant SMVs. Our data, although preliminary, show the feasability of the evaluation of the noxious potency of circulating mediators such as MVs or NETs in individuals with periodontitis. Such results emphasize the need of periodontal management of patient at-risk. Moreover, the control of Netosis could be considered as an interesting strategy to preserve or delay the spleen inflammation.[233]

Annexe

Publications

1- Porphyromonas gingivalis triggers the shedding of inflammatory endothelial microvesicles that act as autocrine effectors of endothelial dysfunction.

Bugueno IM, Zobairi El-Ghazouani F, **El Itawi H**, Anglès-Cano E,Benkirane-Jessel, Toti F, Huck O. 2020 . Sci Rep **10**(1): 1778.

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

A.W Qureishi, R.Altamimy, El Habhab Ali, **El Itawi Hanine**,M.A.Farooq, F.ZOBAIRI,H.Hassan,L.Amoura, M.Kassem,C.Auger,V.Schini-Kerth,F.Toti. Biochem Pharmacol. 2020;173:113734.

3- Significance of Neutrophil Microparticles in Ischaemia-Reperfusion: Pro-inflammatory Effectors of Endothelial Senescence and Vascular Dysfunction

El Habhab A, Altamimy R, Abbas M, Kassem M, Amoura L, Qureshi AW**, El Itawi H**, Kreutter G, Khemais-Benkhiat S, Zobairi F, Schini-Kerth VB, Kessler L, Toti F . J Cell Mol Med. 2020;24(13):7266-81.

1- Isolation of Splenic Microvesicles in a Murine Model of Intraperitoneal Bacterial Infection.

H. EL Itawi, Fareeha Batool, Céline Stutz, Abdul Wahid Qureshi, Fatiha El Zobairi, Olivier Huck, Florence Toti. (Journal of visualized experiments Jove. Accepted manuscript 18 March 2022) DOI: 10.3791/63480)

Scientific communications

Poster presentation

1- Peridontal injury prompts remote spleen activation and the generation of spleen procoagulant microvesicles in a periodontitis murine model (International society for thrombosis and hemostasis ISTH – International online congress 17-23rd July 2021, granted a travel fellowship)

H. EL Itawi, S. Amissi, F. Batool, C. Stutz, F. El Ghazouani, A.B. Chaker, A.W. Qureshi, F. Moschovaki-Filippidou, C. Auger, E. Anglès-Cano, O. Huck, F. Toti

2- **Remote spleen activation in murine periodontitis prompts the generation of procoagulant and pro-senescent spleen microvesicles associated with early heart inflammation (European molecular biology lab congress : engineering in vascular biology. Barcelona, Spain 9-11 May 2022)**

***Hanine El Itawi*[1]** *,F. Batool [1], C. Stutz [1], F. Moschovaki Filippidou [1] ,F. El Ghazouani [1], A.B. Chaker [1], C. Auger [1], E. Angles Cano, [3] O. Huck [1,2], F. Toti [3]. 1-INSERM UMR 1260,CRBS, STRASBOURG University, 2-Faculté d'Odontologie,CRBS, STRASBOURG University, 3-INSERM UMR S-1140 , PARIS University, France*

3-Impact of periodontal inflammation on the cardiac tissues in a murine model. (International society for thrombosis and hemostasis ISTH congress. London 9-13 July 2021)

***Hanine El Itawi*[1]** *,F. Batool [1], C. Stutz [1], F. Moschovaki Filippidou [1] ,F. El Ghazouani [1],C. Auger [1],E.AnglesCano, [3]O.Huck [1,2],F.Toti [3]. 1-INSERM UMR 1260,CRBS, STRASBOURG University, 2-Faculté d'Odontologie,CRBS, STRASBOURG University, 3-INSERM UMR S-1140 , PARIS University, France*

Oral communication

Remote spleen activation after periodontal inflammation is mediated via netosis and prompts the generation of procoagulant microvesicles in an experimental murine model of periodontitis

Hanine El Itawi, Fatiha El Zobairi, Abdul Wahid Qureshi, Fareeha Batool, Céline Stutz, Ahmed Bey Chaker, Amissi Said, Cyril Auger, Olivier Huck, Florence Toti.

1st place prize winner for the best presentation of the Doctoral school ED414 days - University of Strasbourg 2020-2021

References

1. Sanz M, Marco Del Castillo A, Jepsen S, Gonzalez-Juanatey JR, D'Aiuto F, Bouchard P, Chapple I, Dietrich T, Gotsman I, Graziani F, et al. Periodontitis and cardiovascular diseases: Consensus report. *J Clin Periodontol*. 2020;47:268-288. doi: 10.1111/jcpe.13189
2. Moonen CG, Buurma KG, Faruque MR, Balta MG, Liefferink E, Bizzarro S, Nicu EA, Loos BG. Periodontal therapy increases neutrophil extracellular trap degradation. *Innate Immun*. 2020;26:331-340. doi: 10.1177/1753425919889392
3. Tian Y, Pan D, Chordia MD, French BA, Kron IL, Yang Z. The spleen contributes importantly to myocardial infarct exacerbation during post-ischemic reperfusion in mice via signaling between cardiac HMGB1 and splenic RAGE. *Basic Res Cardiol*. 2016;111:62. doi: 10.1007/s00395-016-0583-0
4. El Habhab A, Altamimy R, Abbas M, Kassem M, Amoura L, Qureshi AW, El Itawi H, Kreutter G, Khemais-Benkhiat S, Zobairi F, et al. Significance of neutrophil microparticles in ischaemia-reperfusion: Pro-inflammatory effectors of endothelial senescence and vascular dysfunction. *J Cell Mol Med*. 2020;24:7266-7281. doi: 10.1111/jcmm.15289
5. Abbas M, Jesel L, Auger C, Amoura L, Messas N, Manin G, Rumig C, León-González AJ, Ribeiro TP, Silva GC, 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;135:280-296. doi: 10.1161/circulationaha.116.017513
6. Bugueno IM, Zobairi El-Ghazouani F, Batool F, El Itawi H, Anglès-Cano E, Benkirane-Jessel N, Toti F, Huck O. Porphyromonas gingivalis triggers the shedding of inflammatory endothelial microvesicles that act as autocrine effectors of endothelial dysfunction. *Sci Rep*. 2020;10:1778. doi: 10.1038/s41598-020-58374-z
7. Qureshi AW, Altamimy R, El Habhab A, El Itawi H, Farooq MA, Zobairi F, Hasan H, Amoura L, Kassem M, Auger C, et al. Ageing enhances the shedding of splenocyte microvesicles with endothelial pro-senescent effect that is prevented by a short-term intake of omega-3 PUFA EPA:DHA 6:1. *Biochem Pharmacol*. 2020;173:113734. doi: 10.1016/j.bcp.2019.113734
8. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, Antoniou A, Arab T, Archer F, Atkin-Smith GK, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles*. 2018;7:1535750. doi: 10.1080/20013078.2018.1535750
9. Ratajczak MZ, Ratajczak J. Extracellular microvesicles/exosomes: discovery, disbelief, acceptance, and the future? *Leukemia*. 2020;34:3126-3135. doi: 10.1038/s41375-020-01041-z
10. Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat ML, Boilard E, Buzas EI, Caporali A, Dignat-George F, 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;117:1296-1316. doi: 10.1160/th16-12-0943

11. Gustafson D, Veitch S, Fish JE. Extracellular Vesicles as Protagonists of Diabetic Cardiovascular Pathology. *Front Cardiovasc Med*. 2017;4:71. doi: 10.3389/fcvm.2017.00071
12. Pegtel DM, Gould SJ. Exosomes. *Annu Rev Biochem*. 2019;88:487-514. doi: 10.1146/annurev-biochem-013118-111902
13. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A*. 2004;101:13368-13373. doi: 10.1073/pnas.0403453101
14. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol*. 2005;17:879-887. doi: 10.1093/intimm/dxh267
15. Keller S, Sanderson MP, Stoeck A, Altevogt P. Exosomes: from biogenesis and secretion to biological function. *Immunol Lett*. 2006;107:102-108. doi: 10.1016/j.imlet.2006.09.005
16. Vella LJ, Greenwood DL, Cappai R, Scheerlinck JP, Hill AF. Enrichment of prion protein in exosomes derived from ovine cerebral spinal fluid. *Vet Immunol Immunopathol*. 2008;124:385-393. doi: 10.1016/j.vetimm.2008.04.002
17. van der Pol E, Böing AN, Harrison P, Sturk A, Nieuwland R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol Rev*. 2012;64:676-705. doi: 10.1124/pr.112.005983
18. Wubbolts R, Leckie RS, Veenhuizen PT, Schwarzmann G, Möbius W, Hoernschemeyer J, Slot JW, Geuze HJ, Stoorvogel W. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem*. 2003;278:10963-10972. doi: 10.1074/jbc.M207550200
19. Xu X, Lai Y, Hua ZC. Apoptosis and apoptotic body: disease message and therapeutic target potentials. *Biosci Rep*. 2019;39. doi: 10.1042/bsr20180992
20. Akers JC, Gonda D, Kim R, Carter BS, Chen CC. Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. *J Neurooncol*. 2013;113:1-11. doi: 10.1007/s11060-013-1084-8
21. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*. 1972;26:239-257. doi: 10.1038/bjc.1972.33
22. Dive C, Gregory CD, Phipps DJ, Evans DL, Milner AE, Wyllie AH. Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta*. 1992;1133:275-285. doi: 10.1016/0167-4889(92)90048-g
23. Beyer C, Pisetsky DS. The role of microparticles in the pathogenesis of rheumatic diseases. *Nat Rev Rheumatol*. 2010;6:21-29. doi: 10.1038/nrrheum.2009.229
24. Burger D, Schock S, Thompson CS, Montezano AC, Hakim AM, Touyz RM. Microparticles: biomarkers and beyond. *Clin Sci (Lond)*. 2013;124:423-441. doi: 10.1042/CS20120309
25. Wolf P. The nature and significance of platelet products in human plasma. *Br J Haematol*. 1967;13:269-288. doi: 10.1111/j.1365-2141.1967.tb08741.x
26. Minciacchi VR, You S, Spinelli C, Morley S, Zandian M, Aspuria PJ, Cavallini L, Ciardiello C, Reis Sobreiro M, Morello M, et al. Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. *Oncotarget*. 2015;6:11327-11341. doi: 10.18632/oncotarget.3598

27. Cocucci E, Meldolesi J. Ectosomes and exosomes: shedding the confusion between extracellular vesicles. *Trends Cell Biol*. 2015;25:364-372. doi: 10.1016/j.tcb.2015.01.004
28. Stein JM, Luzio JP. Ectocytosis caused by sublytic autologous complement attack on human neutrophils. The sorting of endogenous plasma-membrane proteins and lipids into shed vesicles. *Biochem J*. 1991;274 (Pt 2):381-386. doi: 10.1042/bj2740381
29. Zaldivia MTK, McFadyen JD, Lim B, Wang X, Peter K. Platelet-Derived Microvesicles in Cardiovascular Diseases. *Front Cardiovasc Med*. 2017;4:74. doi: 10.3389/fcvm.2017.00074
30. Badimon L, Suades R, Arderiu G, Peña E, Chiva-Blanch G, Padró T. Microvesicles in Atherosclerosis and Angiogenesis: From Bench to Bedside and Reverse. *Front Cardiovasc Med*. 2017;4:77. doi: 10.3389/fcvm.2017.00077
31. Voukalis C, Shantsila E, Lip GYH. Microparticles and cardiovascular diseases. *Ann Med*. 2019;51:193-223. doi: 10.1080/07853890.2019.1609076
32. Vance JE, Tasseva G. Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells. *Biochim Biophys Acta*. 2013;1831:543-554. doi: 10.1016/j.bbalip.2012.08.016
33. Callahan MK, Popernack PM, Tsutsui S, Truong L, Schlegel RA, Henderson AJ. Phosphatidylserine on HIV envelope is a cofactor for infection of monocytic cells. *J Immunol*. 2003;170:4840-4845. doi: 10.4049/jimmunol.170.9.4840
34. van Meer G. Dynamic transbilayer lipid asymmetry. *Cold Spring Harb Perspect Biol*. 2011;3. doi: 10.1101/cshperspect.a004671
35. Marquardt D, Geier B, Pabst G. Asymmetric lipid membranes: towards more realistic model systems. *Membranes (Basel)*. 2015;5:180-196. doi: 10.3390/membranes5020180
36. Leventis PA, Grinstein S. The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys*. 2010;39:407-427. doi: 10.1146/annurev.biophys.093008.131234
37. Bevers EM, Comfurius P, van Rijn JL, Hemker HC, Zwaal RF. Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem*. 1982;122:429-436. doi: 10.1111/j.1432-1033.1982.tb05898.x
38. Kamińska A, Gajos K, Woźnicka O, Dłubacz A, Marzec ME, Budkowski A, Stępień E. Using a lactadherin-immobilized silicon surface for capturing and monitoring plasma microvesicles as a foundation for diagnostic device development. *Anal Bioanal Chem*. 2020;412:8093-8106. doi: 10.1007/s00216-020-02938-5
39. Morel O, Jesel L, Freyssinet JM, Toti F. Cellular mechanisms underlying the formation of circulating microparticles. *Arterioscler Thromb Vasc Biol*. 2011;31:15-26. doi: 10.1161/atvbaha.109.200956
40. Jy W, Horstman LL, Jimenez JJ, Ahn YS, Biró E, Nieuwland R, Sturk A, Dignat-George F, Sabatier F, Camoin-Jau L, et al. Measuring circulating cell-derived microparticles. *J Thromb Haemost*. 2004;2:1842-1851. doi: 10.1111/j.1538-7836.2004.00936.x
41. Chironi GN, Boulanger CM, Simon A, Dignat-George F, Freyssinet JM, Tedgui A. Endothelial microparticles in diseases. *Cell Tissue Res*. 2009;335:143-151. doi: 10.1007/s00441-008-0710-9
42. Abid Hussein MN, Böing AN, Sturk A, Hau CM, Nieuwland R. Inhibition of microparticle release triggers endothelial cell apoptosis and detachment. *Thromb Haemost*. 2007;98:1096-1107. doi: 10.1160/th05-04-0231

43. Bakouboula B, Morel O, Faller AL, Freyssinet JM, Toti F. Significance of membrane microparticles in solid graft and cellular transplantation. *Front Biosci (Landmark Ed)*. 2011;16:2499-2514. doi: 10.2741/3868
44. Quazi F, Molday RS. Lipid transport by mammalian ABC proteins. *Essays Biochem*. 2011;50:265-290. doi: 10.1042/bse0500265
45. Welsh JA, van der Pol E, Bettin BA, Carter DRF, Hendrix A, Lenassi M, Langlois MA, Llorente A, van de Nes AS, Nieuwland R, et al. Towards defining reference materials for measuring extracellular vesicle refractive index, epitope abundance, size and concentration. *J Extracell Vesicles*. 2020;9:1816641. doi: 10.1080/20013078.2020.1816641
46. Fioranelli M, Bottaccioli AG, Bottaccioli F, Bianchi M, Rovesti M, Roccia MG. Stress and Inflammation in Coronary Artery Disease: A Review Psychoneuroendocrineimmunology-Based. *Front Immunol*. 2018;9:2031. doi: 10.3389/fimmu.2018.02031
47. Gleizes C, Kreutter G, Abbas M, Kassem M, Constantinescu AA, Boisramé-Helms J, Yver B, Toti F, Kessler L. β cell membrane remodelling and procoagulant events occur in inflammation-driven insulin impairment: a GLP-1 receptor dependent and independent control. *J Cell Mol Med*. 2016;20:231-242. doi: 10.1111/jcmm.12683
48. Hulsmans M, Holvoet P. MicroRNA-containing microvesicles regulating inflammation in association with atherosclerotic disease. *Cardiovasc Res*. 2013;100:7-18. doi: 10.1093/cvr/cvt161
49. Guo Y, Tan J, Miao Y, Sun Z, Zhang Q. Effects of Microvesicles on Cell Apoptosis under Hypoxia. *Oxid Med Cell Longev*. 2019;2019:5972152. doi: 10.1155/2019/5972152
50. Shu Z, Tan J, Miao Y, Zhang Q. The role of microvesicles containing microRNAs in vascular endothelial dysfunction. *J Cell Mol Med*. 2019;23:7933-7945. doi: 10.1111/jcmm.14716
51. Laberge A, Arif S, Moulin VJ. Microvesicles: Intercellular messengers in cutaneous wound healing. *J Cell Physiol*. 2018;233:5550-5563. doi: 10.1002/jcp.26426
52. Morel O, Toti F, Jesel L, Freyssinet JM. Mechanisms of microparticle generation: on the trail of the mitochondrion! *Semin Thromb Hemost*. 2010;36:833-844. doi: 10.1055/s-0030-1267037
53. Morel O, Morel N, Jesel L, Freyssinet JM, Toti F. Microparticles: a critical component in the nexus between inflammation, immunity, and thrombosis. *Semin Immunopathol*. 2011;33:469-486. doi: 10.1007/s00281-010-0239-3
54. Burnouf T, Chou ML, Goubran H, Cognasse F, Garraud O, Seghatchian J. An overview of the role of microparticles/microvesicles in blood components: Are they clinically beneficial or harmful? *Transfus Apher Sci*. 2015;53:137-145. doi: 10.1016/j.transci.2015.10.010
55. Camussi G, Deregibus MC, Bruno S, Cantaluppi V, Biancone L. Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int*. 2010;78:838-848. doi: 10.1038/ki.2010.278
56. Muralidharan-Chari V, Clancy JW, Sedgwick A, D'Souza-Schorey C. Microvesicles: mediators of extracellular communication during cancer progression. *J Cell Sci*. 2010;123:1603-1611. doi: 10.1242/jcs.064386
57. Loyer X, Vion AC, Tedgui A, Boulanger CM. Microvesicles as cell-cell messengers in cardiovascular diseases. *Circ Res*. 2014;114:345-353. doi: 10.1161/circresaha.113.300858
58. Morel O, Toti F, Hugel B, Bakouboula B, Camoin-Jau L, Dignat-George F, Freyssinet JM. Procoagulant microparticles: disrupting the vascular homeostasis equation?

Arterioscler Thromb Vasc Biol. 2006;26:2594-2604. doi: 10.1161/01.Atv.0000246775.14471.26
59. Morel O, Jesel L, Abbas M, Morel N. Prothrombotic changes in diabetes mellitus. *Semin Thromb Hemost*. 2013;39:477-488. doi: 10.1055/s-0033-1343888
60. Leite AR, Borges-Canha M, Cardoso R, Neves JS, Castro-Ferreira R, Leite-Moreira A. Novel Biomarkers for Evaluation of Endothelial Dysfunction. *Angiology*. 2020;71:397-410. doi: 10.1177/0003319720903586
61. Bauer V, Sotníková R. Nitric oxide--the endothelium-derived relaxing factor and its role in endothelial functions. *Gen Physiol Biophys*. 2010;29:319-340.
62. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. *Circulation*. 2007;115:1285-1295. doi: 10.1161/circulationaha.106.652859
63. Giannotti G, Landmesser U. Endothelial dysfunction as an early sign of atherosclerosis. *Herz*. 2007;32:568-572. doi: 10.1007/s00059-007-3073-1
64. Zeng L, Zampetaki A, Margariti A, Pepe AE, Alam S, Martin D, Xiao Q, Wang W, Jin ZG, Cockerill G, et al. Sustained activation of XBP1 splicing leads to endothelial apoptosis and atherosclerosis development in response to disturbed flow. *Proc Natl Acad Sci U S A*. 2009;106:8326-8331. doi: 10.1073/pnas.0903197106
65. Liu ML, Williams KJ. Microvesicles: potential markers and mediators of endothelial dysfunction. *Curr Opin Endocrinol Diabetes Obes*. 2012;19:121-127. doi: 10.1097/MED.0b013e32835057e9
66. Esposito K, Ciotola M, Schisano B, Gualdiero R, Sardelli L, Misso L, Giannetti G, Giugliano D. Endothelial microparticles correlate with endothelial dysfunction in obese women. *J Clin Endocrinol Metab*. 2006;91:3676-3679. doi: 10.1210/jc.2006-0851
67. Navasiolava NM, Dignat-George F, Sabatier F, Larina IM, Demiot C, Fortrat JO, Gauquelin-Koch G, Kozlovskaya IB, Custaud MA. Enforced physical inactivity increases endothelial microparticle levels in healthy volunteers. *Am J Physiol Heart Circ Physiol*. 2010;299:H248-256. doi: 10.1152/ajpheart.00152.2010
68. Feng B, Chen Y, Luo Y, Chen M, Li X, Ni Y. Circulating level of microparticles and their correlation with arterial elasticity and endothelium-dependent dilation in patients with type 2 diabetes mellitus. *Atherosclerosis*. 2010;208:264-269. doi: 10.1016/j.atherosclerosis.2009.06.037
69. Amabile N, Guérin AP, Leroyer A, Mallat Z, Nguyen C, Boddaert J, London GM, Tedgui A, Boulanger CM. Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol*. 2005;16:3381-3388. doi: 10.1681/asn.2005050535
70. Bulut D, Maier K, Bulut-Streich N, Börgel J, Hanefeld C, Mügge A. Circulating endothelial microparticles correlate inversely with endothelial function in patients with ischemic left ventricular dysfunction. *J Card Fail*. 2008;14:336-340. doi: 10.1016/j.cardfail.2007.11.002
71. Meziani F, Tesse A, David E, Martinez MC, Wangesteen R, Schneider F, Andriantsitohaina R. Shed membrane particles from preeclamptic women generate vascular wall inflammation and blunt vascular contractility. *Am J Pathol*. 2006;169:1473-1483. doi: 10.2353/ajpath.2006.051304
72. Barry OP, Praticò D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. *J Clin Invest*. 1998;102:136-144. doi: 10.1172/jci2592
73. Nomura S, Tandon NN, Nakamura T, Cone J, Fukuhara S, Kambayashi J. High-shear-stress-induced activation of platelets and microparticles enhances expression of cell

adhesion molecules in THP-1 and endothelial cells. *Atherosclerosis*. 2001;158:277-287. doi: 10.1016/s0021-9150(01)00433-6
74. Radecke CE, Warrick AE, Singh GD, Rogers JH, Simon SI, Armstrong EJ. Coronary artery endothelial cells and microparticles increase expression of VCAM-1 in myocardial infarction. *Thromb Haemost*. 2015;113:605-616. doi: 10.1160/th14-02-0151
75. Jansen F, Yang X, Franklin BS, Hoelscher M, Schmitz T, Bedorf J, Nickenig G, Werner N. High glucose condition increases NADPH oxidase activity in endothelial microparticles that promote vascular inflammation. *Cardiovasc Res*. 2013;98:94-106. doi: 10.1093/cvr/cvt013
76. Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: what is the clinical significance? *Hypertension*. 2004;44:248-252. doi: 10.1161/01.HYP.0000138070.47616.9d
77. Rodrigo R, Prat H, Passalacqua W, Araya J, Guichard C, Bächler JP. Relationship between oxidative stress and essential hypertension. *Hypertens Res*. 2007;30:1159-1167. doi: 10.1291/hypres.30.1159
78. Lovren F, Verma S. Evolving role of microparticles in the pathophysiology of endothelial dysfunction. *Clin Chem*. 2013;59:1166-1174. doi: 10.1373/clinchem.2012.199711
79. Dickhout A, Koenen RR. Extracellular Vesicles as Biomarkers in Cardiovascular Disease; Chances and Risks. *Front Cardiovasc Med*. 2018;5:113. doi: 10.3389/fcvm.2018.00113
80. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005;352:1685-1695. doi: 10.1056/NEJMra043430
81. Mesri M, Altieri DC. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem*. 1999;274:23111-23118. doi: 10.1074/jbc.274.33.23111
82. Sinauridze EI, Kireev DA, Popenko NY, Pichugin AV, Panteleev MA, Krymskaya OV, Ataullakhanov FI. Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost*. 2007;97:425-434.
83. Fortin PR, Cloutier N, Bissonnette V, Aghdassi E, Eder L, Simonyan D, Laflamme N, Boilard E. Distinct Subtypes of Microparticle-containing Immune Complexes Are Associated with Disease Activity, Damage, and Carotid Intima-media Thickness in Systemic Lupus Erythematosus. *J Rheumatol*. 2016;43:2019-2025. doi: 10.3899/jrheum.160050
84. Tan KT, Tayebjee MH, Lim HS, Lip GY. Clinically apparent atherosclerotic disease in diabetes is associated with an increase in platelet microparticle levels. *Diabet Med*. 2005;22:1657-1662. doi: 10.1111/j.1464-5491.2005.01707.x
85. Lacroix R, Plawinski L, Robert S, Doeuvre L, Sabatier F, Martinez de Lizarrondo S, Mezzapesa A, Anfosso F, Leroyer AS, Poullin P, et al. Leukocyte- and endothelial-derived microparticles: a circulating source for fibrinolysis. *Haematologica*. 2012;97:1864-1872. doi: 10.3324/haematol.2012.066167
86. Dejouvencel T, Doeuvre L, Lacroix R, Plawinski L, Dignat-George F, Lijnen HR, Anglés-Cano E. Fibrinolytic cross-talk: a new mechanism for plasmin formation. *Blood*. 2010;115:2048-2056. doi: 10.1182/blood-2009-06-228817
87. Paone S, Baxter AA, Hulett MD, Poon IKH. Endothelial cell apoptosis and the role of endothelial cell-derived extracellular vesicles in the progression of atherosclerosis. *Cell Mol Life Sci*. 2019;76:1093-1106. doi: 10.1007/s00018-018-2983-9

88. Bernal-Mizrachi L, Jy W, Jimenez JJ, Pastor J, Mauro LM, Horstman LL, de Marchena E, Ahn YS. High levels of circulating endothelial microparticles in patients with acute coronary syndromes. *Am Heart J*. 2003;145:962-970. doi: 10.1016/s0002-8703(03)00103-0
89. Sinning JM, Losch J, Walenta K, Böhm M, Nickenig G, Werner N. Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. *Eur Heart J*. 2011;32:2034-2041. doi: 10.1093/eurheartj/ehq478
90. Stępień E, Stankiewicz E, Zalewski J, Godlewski J, Zmudka K, Wybrańska I. Number of microparticles generated during acute myocardial infarction and stable angina correlates with platelet activation. *Arch Med Res*. 2012;43:31-35. doi: 10.1016/j.arcmed.2012.01.006
91. Morel O, Hugel B, Jesel L, Mallat Z, Lanza F, Douchet MP, Zupan M, Chauvin M, Cazenave JP, Tedgui A, et al. Circulating procoagulant microparticles and soluble GPV in myocardial infarction treated by primary percutaneous transluminal coronary angioplasty. A possible role for GPIIb-IIIa antagonists. *J Thromb Haemost*. 2004;2:1118-1126. doi: 10.1111/j.1538-7836.2004.00805.x
92. Cherian P, Hankey GJ, Eikelboom JW, Thom J, Baker RI, McQuillan A, Staton J, Yi Q. Endothelial and platelet activation in acute ischemic stroke and its etiological subtypes. *Stroke*. 2003;34:2132-2137. doi: 10.1161/01.Str.0000086466.32421.F4
93. Chironi G, Simon A, Hugel B, Del Pino M, Gariepy J, Freyssinet JM, Tedgui A. Circulating leukocyte-derived microparticles predict subclinical atherosclerosis burden in asymptomatic subjects. *Arterioscler Thromb Vasc Biol*. 2006;26:2775-2780. doi: 10.1161/01.Atv.0000249639.36915.04
94. Chironi GN, Simon A, Boulanger CM, Dignat-George F, Hugel B, Megnien JL, Lefort M, Freyssinet JM, Tedgui A. Circulating microparticles may influence early carotid artery remodeling. *J Hypertens*. 2010;28:789-796. doi: 10.1097/HJH.0b013e328335d0a8
95. Darveau RP. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat Rev Microbiol*. 2010;8:481-490. doi: 10.1038/nrmicro2337
96. Papapanou PN, Sanz M, Buduneli N, Dietrich T, Feres M, Fine DH, Flemmig TF, Garcia R, Giannobile WV, Graziani F, et al. Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions. *J Periodontol*. 2018;89 Suppl 1:S173-s182. doi: 10.1002/jper.17-0721
97. Fischer RG, Lira Junior R, Retamal-Valdes B, Figueiredo LC, Malheiros Z, Stewart B, Feres M. Periodontal disease and its impact on general health in Latin America. Section V: Treatment of periodontitis. *Braz Oral Res*. 2020;34:e026. doi: 10.1590/1807-3107bor-2020.vol34.0026
98. Meyle J, Chapple I. Molecular aspects of the pathogenesis of periodontitis. *Periodontol 2000*. 2015;69:7-17. doi: 10.1111/prd.12104
99. Slots J. Life-threatening pathogens in severe/progressive periodontitis: Focal infection risk, future periodontal practice, role of the Periodontology 2000. *Periodontol 2000*. 2020;84:215-216. doi: 10.1111/prd.12375
100. Holt SC, Ebersole JL. Porphyromonas gingivalis, Treponema denticola, and Tannerella forsythia: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000*. 2005;38:72-122. doi: 10.1111/j.1600-0757.2005.00113.x
101. Hajishengallis G. The inflammophilic character of the periodontitis-associated microbiota. *Mol Oral Microbiol*. 2014;29:248-257. doi: 10.1111/omi.12065

102. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, Jr. Microbial complexes in subgingival plaque. *J Clin Periodontol*. 1998;25:134-144. doi: 10.1111/j.1600-051x.1998.tb02419.x
103. Slots J. Periodontitis: facts, fallacies and the future. *Periodontol 2000*. 2017;75:7-23. doi: 10.1111/prd.12221
104. Hajishengallis G. Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends Immunol*. 2014;35:3-11. doi: 10.1016/j.it.2013.09.001
105. Hajishengallis G, Darveau RP, Curtis MA. The keystone-pathogen hypothesis. *Nat Rev Microbiol*. 2012;10:717-725. doi: 10.1038/nrmicro2873
106. Suh JS, Kim S, Boström KI, Wang CY, Kim RH, Park NH. Periodontitis-induced systemic inflammation exacerbates atherosclerosis partly via endothelial-mesenchymal transition in mice. *Int J Oral Sci*. 2019;11:21. doi: 10.1038/s41368-019-0054-1
107. Sochalska M, Potempa J. Manipulation of Neutrophils by Porphyromonas gingivalis in the Development of Periodontitis. *Front Cell Infect Microbiol*. 2017;7:197. doi: 10.3389/fcimb.2017.00197
108. Lamont T, Worthington HV, Clarkson JE, Beirne PV. Routine scale and polish for periodontal health in adults. *Cochrane Database Syst Rev*. 2018;12:CD004625. doi: 10.1002/14651858.CD004625.pub5
109. Huck O, You J, Han X, Cai B, Panek J, Amar S. Reduction of Articular and Systemic Inflammation by Kava-241 in a Porphyromonas gingivalis-Induced Arthritis Murine Model. *Infect Immun*. 2018;86. doi: 10.1128/IAI.00356-18
110. Kocgozlu L, Elkaim R, Tenenbaum H, Werner S. Variable cell responses to P. gingivalis lipopolysaccharide. *J Dent Res*. 2009;88:741-745. doi: 10.1177/0022034509341166
111. Slocum C, Coats SR, Hua N, Kramer C, Papadopoulos G, Weinberg EO, Gudino CV, Hamilton JA, Darveau RP, Genco CA. Distinct lipid a moieties contribute to pathogen-induced site-specific vascular inflammation. *PLoS Pathog*. 2014;10:e1004215. doi: 10.1371/journal.ppat.1004215
112. Thietart S, Rautou PE. Extracellular vesicles as biomarkers in liver diseases: A clinician's point of view. *J Hepatol*. 2020;73:1507-1525. doi: 10.1016/j.jhep.2020.07.014
113. Papadopoulos G, Weinberg EO, Massari P, Gibson FC, 3rd, Wetzler LM, Morgan EF, Genco CA. Macrophage-specific TLR2 signaling mediates pathogen-induced TNF-dependent inflammatory oral bone loss. *J Immunol*. 2013;190:1148-1157. doi: 10.4049/jimmunol.1202511
114. Holden JA, Attard TJ, Laughton KM, Mansell A, O'Brien-Simpson NM, Reynolds EC. Porphyromonas gingivalis lipopolysaccharide weakly activates M1 and M2 polarized mouse macrophages but induces inflammatory cytokines. *Infect Immun*. 2014;82:4190-4203. doi: 10.1128/iai.02325-14
115. Cortés-Vieyra R, Rosales C, Uribe-Querol E. Neutrophil Functions in Periodontal Homeostasis. *J Immunol Res*. 2016;2016:1396106. doi: 10.1155/2016/1396106
116. Delima AJ, Van Dyke TE. Origin and function of the cellular components in gingival crevice fluid. *Periodontol 2000*. 2003;31:55-76. doi: 10.1034/j.1600-0757.2003.03105.x
117. Hajishengallis E, Hajishengallis G. Neutrophil homeostasis and periodontal health in children and adults. *J Dent Res*. 2014;93:231-237. doi: 10.1177/0022034513507956
118. Moutsopoulos NM, Konkel J, Sarmadi M, Eskan MA, Wild T, Dutzan N, Abusleme L, Zenobia C, Hosur KB, Abe T, et al. Defective neutrophil recruitment in leukocyte

adhesion deficiency type I disease causes local IL-17-driven inflammatory bone loss. *Sci Transl Med*. 2014;6:229ra240. doi: 10.1126/scitranslmed.3007696
119. Chapple IL, Matthews JB. The role of reactive oxygen and antioxidant species in periodontal tissue destruction. *Periodontol 2000*. 2007;43:160-232. doi: 10.1111/j.1600-0757.2006.00178.x
120. Ryder MI. Comparison of neutrophil functions in aggressive and chronic periodontitis. *Periodontol 2000*. 2010;53:124-137. doi: 10.1111/j.1600-0757.2009.00327.x
121. Eskan MA, Jotwani R, Abe T, Chmelar J, Lim JH, Liang S, Ciero PA, Krauss JL, Li F, Rauner M, et al. The leukocyte integrin antagonist Del-1 inhibits IL-17-mediated inflammatory bone loss. *Nat Immunol*. 2012;13:465-473. doi: 10.1038/ni.2260
122. Hajishengallis G. New developments in neutrophil biology and periodontitis. *Periodontol 2000*. 2020;82:78-92. doi: 10.1111/prd.12313
123. Lerman A, Zeiher AM. Endothelial function: cardiac events. *Circulation*. 2005;111:363-368. doi: 10.1161/01.Cir.0000153339.27064.14
124. Huck O, Buduneli N, Bravo D. Inflammatory Mediators in Periodontal Pathogenesis. *Mediators Inflamm*. 2019;2019:2610184. doi: 10.1155/2019/2610184
125. Linden GJ, Lyons A, Scannapieco FA. Periodontal systemic associations: review of the evidence. *J Periodontol*. 2013;84:S8-s19. doi: 10.1902/jop.2013.1340010
126. Zardawi F, Gul S, Abdulkareem A, Sha A, Yates J. Association Between Periodontal Disease and Atherosclerotic Cardiovascular Diseases: Revisited. *Front Cardiovasc Med*. 2020;7:625579. doi: 10.3389/fcvm.2020.625579
127. Figuero E, Sánchez-Beltrán M, Cuesta-Frechoso S, Tejerina JM, del Castro JA, Gutiérrez JM, Herrera D, Sanz M. Detection of periodontal bacteria in atheromatous plaque by nested polymerase chain reaction. *J Periodontol*. 2011;82:1469-1477. doi: 10.1902/jop.2011.100719
128. Bartova J, Sommerova P, Lyuya-Mi Y, Mysak J, Prochazkova J, Duskova J, Janatova T, Podzimek S. Periodontitis as a risk factor of atherosclerosis. *J Immunol Res*. 2014;2014:636893. doi: 10.1155/2014/636893
129. Muñoz Aguilera E, Suvan J, Buti J, Czesnikiewicz-Guzik M, Barbosa Ribeiro A, Orlandi M, Guzik TJ, Hingorani AD, Nart J, D'Aiuto F. Periodontitis is associated with hypertension: a systematic review and meta-analysis. *Cardiovasc Res*. 2020;116:28-39. doi: 10.1093/cvr/cvz201
130. Serra e Silva Filho W, Casarin RC, Nicolela EL, Jr., Passos HM, Sallum AW, Gonçalves RB. Microbial diversity similarities in periodontal pockets and atheromatous plaques of cardiovascular disease patients. *PLoS One*. 2014;9:e109761. doi: 10.1371/journal.pone.0109761
131. Rafferty B, Jönsson D, Kalachikov S, Demmer RT, Nowygrod R, Elkind MS, Bush H, Jr., Kozarov E. Impact of monocytic cells on recovery of uncultivable bacteria from atherosclerotic lesions. *J Intern Med*. 2011;270:273-280. doi: 10.1111/j.1365-2796.2011.02373.x
132. Deshpande RG, Khan MB, Genco CA. Invasion of aortic and heart endothelial cells by Porphyromonas gingivalis. *Infect Immun*. 1998;66:5337-5343. doi: 10.1128/iai.66.11.5337-5343.1998
133. Dorn BR, Dunn WA, Jr., Progulske-Fox A. Invasion of human coronary artery cells by periodontal pathogens. *Infect Immun*. 1999;67:5792-5798. doi: 10.1128/iai.67.11.5792-5798.1999
134. Jain A, Batista EL, Jr., Serhan C, Stahl GL, Van Dyke TE. Role for periodontitis in the progression of lipid deposition in an animal model. *Infect Immun*. 2003;71:6012-6018. doi: 10.1128/iai.71.10.6012-6018.2003

135. Brodala N, Merricks EP, Bellinger DA, Damrongsri D, Offenbacher S, Beck J, Madianos P, Sotres D, Chang YL, Koch G, et al. Porphyromonas gingivalis bacteremia induces coronary and aortic atherosclerosis in normocholesterolemic and hypercholesterolemic pigs. *Arterioscler Thromb Vasc Biol*. 2005;25:1446-1451. doi: 10.1161/01.ATV.0000167525.69400.9c
136. Gibson FC, 3rd, Hong C, Chou HH, Yumoto H, Chen J, Lien E, Wong J, Genco CA. Innate immune recognition of invasive bacteria accelerates atherosclerosis in apolipoprotein E-deficient mice. *Circulation*. 2004;109:2801-2806. doi: 10.1161/01.Cir.0000129769.17895.F0
137. Parvaneh M, Witting PK, Ku J, Moradi T, Eroglu E, Freedman B, Sutherland GT, McCorkindale A, Guennewig B, Choowong P, et al. Periodontitis induces endothelial dysfunction in mice. *Sci Rep*. 2021;11:14993. doi: 10.1038/s41598-021-94418-8
138. Bergandi L, Giuggia B, Alovisi M, Comba A, Silvagno F, Maule M, Aldieri E, Scotti N, Scacciatella P, Conrotto F, et al. Endothelial Dysfunction Marker Variation in Young Adults with Chronic Apical Periodontitis before and after Endodontic Treatment. *J Endod*. 2019;45:500-506. doi: 10.1016/j.joen.2019.01.018
139. Fiorillo L, Cervino G, Laino L, D'Amico C, Mauceri R, Tozum TF, Gaeta M, Cicciù M. Porphyromonas gingivalis, Periodontal and Systemic Implications: A Systematic Review. *Dent J (Basel)*. 2019;7. doi: 10.3390/dj7040114
140. Mulhall H, Huck O, Amar S. Porphyromonas gingivalis, a Long-Range Pathogen: Systemic Impact and Therapeutic Implications. *Microorganisms*. 2020;8. doi: 10.3390/microorganisms8060869
141. Janssen KM, Vissink A, de Smit MJ, Westra J, Brouwer E. Lessons to be learned from periodontitis. *Curr Opin Rheumatol*. 2013;25:241-247. doi: 10.1097/BOR.0b013e32835d833d
142. de Pablo P, Dietrich T, Chapple IL, Milward M, Chowdhury M, Charles PJ, Buckley CD, Venables PJ. The autoantibody repertoire in periodontitis: a role in the induction of autoimmunity to citrullinated proteins in rheumatoid arthritis? *Ann Rheum Dis*. 2014;73:580-586. doi: 10.1136/annrheumdis-2012-202701
143. Lopez-Oliva I, de Pablo P, Dietrich T, Chapple I. Gums and joints: is there a connection? Part two: the biological link. *Br Dent J*. 2019;227:611-617. doi: 10.1038/s41415-019-0723-7
144. Geraldino-Pardilla L, Giles JT, Sokolove J, Zartoshti A, Robinson WH, Budoff M, Detrano R, Bokhari S, Bathon JM. Association of Anti-Citrullinated Peptide Antibodies With Coronary Artery Calcification in Rheumatoid Arthritis. *Arthritis Care Res (Hoboken)*. 2017;69:1276-1281. doi: 10.1002/acr.23106
145. Li L, Michel R, Cohen J, Decarlo A, Kozarov E. Intracellular survival and vascular cell-to-cell transmission of Porphyromonas gingivalis. *BMC Microbiol*. 2008;8:26. doi: 10.1186/1471-2180-8-26
146. Li L, Messas E, Batista EL, Jr., Levine RA, Amar S. Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E-deficient murine model. *Circulation*. 2002;105:861-867. doi: 10.1161/hc0702.104178
147. Akamatsu Y, Yamamoto T, Yamamoto K, Oseko F, Kanamura N, Imanishi J, Kita M. Porphyromonas gingivalis induces myocarditis and/or myocardial infarction in mice and IL-17A is involved in pathogenesis of these diseases. *Arch Oral Biol*. 2011;56:1290-1298. doi: 10.1016/j.archoralbio.2011.05.012
148. Sidhu RK. Association between Acute Myocardial Infarction and Periodontitis: A Review of the Literature. *J Int Acad Periodontol*. 2016;18:23-33.

149. Liccardo D, Cannavo A, Spagnuolo G, Ferrara N, Cittadini A, Rengo C, Rengo G. Periodontal Disease: A Risk Factor for Diabetes and Cardiovascular Disease. *Int J Mol Sci*. 2019;20. doi: 10.3390/ijms20061414
150. Ellis TN, Kuehn MJ. Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol Mol Biol Rev*. 2010;74:81-94. doi: 10.1128/mmbr.00031-09
151. Mantri CK, Chen CH, Dong X, Goodwin JS, Pratap S, Paromov V, Xie H. Fimbriae-mediated outer membrane vesicle production and invasion of Porphyromonas gingivalis. *Microbiologyopen*. 2015;4:53-65. doi: 10.1002/mbo3.221
152. Zhang Z, Liu D, Liu S, Zhang S, Pan Y. The Role of Porphyromonas gingivalis Outer Membrane Vesicles in Periodontal Disease and Related Systemic Diseases. *Front Cell Infect Microbiol*. 2020;10:585917. doi: 10.3389/fcimb.2020.585917
153. Roier S, Zingl FG, Cakar F, Durakovic S, Kohl P, Eichmann TO, Klug L, Gadermaier B, Weinzerl K, Prassl R, et al. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. *Nat Commun*. 2016;7:10515. doi: 10.1038/ncomms10515
154. Zaremba M, Górska R, Suwalski P, Kowalski J. Evaluation of the incidence of periodontitis-associated bacteria in the atherosclerotic plaque of coronary blood vessels. *J Periodontol*. 2007;78:322-327. doi: 10.1902/jop.2006.060081
155. Wada K, Kamisaki Y. Roles of oral bacteria in cardiovascular diseases--from molecular mechanisms to clinical cases: Involvement of Porphyromonas gingivalis in the development of human aortic aneurysm. *J Pharmacol Sci*. 2010;113:115-119. doi: 10.1254/jphs.09r22fm
156. Yang WW, Guo B, Jia WY, Jia Y. Porphyromonas gingivalis-derived outer membrane vesicles promote calcification of vascular smooth muscle cells through ERK1/2-RUNX2. *FEBS Open Bio*. 2016;6:1310-1319. doi: 10.1002/2211-5463.12151
157. Farrugia C, Stafford GP, Murdoch C. Porphyromonas gingivalis Outer Membrane Vesicles Increase Vascular Permeability. *J Dent Res*. 2020;99:1494-1501. doi: 10.1177/0022034520943187
158. Nakano K, Wada K, Nomura R, Nemoto H, Inaba H, Kojima A, Naka S, Hokamura K, Mukai T, Nakajima A, et al. Characterization of aortic aneurysms in cardiovascular disease patients harboring Porphyromonas gingivalis. *Oral Dis*. 2011;17:370-378. doi: 10.1111/j.1601-0825.2010.01759.x
159. Mei F, Xie M, Huang X, Long Y, Lu X, Wang X, Chen L. Porphyromonas gingivalis and Its Systemic Impact: Current Status. *Pathogens*. 2020;9. doi: 10.3390/pathogens9110944
160. Lewis SM, Williams A, Eisenbarth SC. Structure and function of the immune system in the spleen. *Sci Immunol*. 2019;4. doi: 10.1126/sciimmunol.aau6085
161. Bronte V, Pittet MJ. The spleen in local and systemic regulation of immunity. *Immunity*. 2013;39:806-818. doi: 10.1016/j.immuni.2013.10.010
162. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5:606-616. doi: 10.1038/nri1669
163. Barral P, Sánchez-Niño MD, van Rooijen N, Cerundolo V, Batista FD. The location of splenic NKT cells favours their rapid activation by blood-borne antigen. *Embo j*. 2012;31:2378-2390. doi: 10.1038/emboj.2012.87
164. Radhakrishnan UP, Al Qaryoute A, Raman R, Jagadeeswaran P. Splenectomy in zebrafish: a new model for immune thrombocytopenia. *Platelets*. 2022;33:54-58. doi: 10.1080/09537104.2021.1882667

165. Andrade J, Khairy P, Dobrev D, Nattel S. The clinical profile and pathophysiology of atrial fibrillation: relationships among clinical features, epidemiology, and mechanisms. *Circ Res*. 2014;114:1453-1468. doi: 10.1161/circresaha.114.303211
166. Hofmann U, Frantz S. How can we cure a heart "in flame"? A translational view on inflammation in heart failure. *Basic Res Cardiol*. 2013;108:356. doi: 10.1007/s00395-013-0356-y
167. Putko BN, Wang Z, Lo J, Anderson T, Becher H, Dyck JR, Kassiri Z, Oudit GY. Circulating levels of tumor necrosis factor-alpha receptor 2 are increased in heart failure with preserved ejection fraction relative to heart failure with reduced ejection fraction: evidence for a divergence in pathophysiology. *PLoS One*. 2014;9:e99495. doi: 10.1371/journal.pone.0099495
168. Toldo S, Abbate A. The NLRP3 inflammasome in acute myocardial infarction. *Nat Rev Cardiol*. 2018;15:203-214. doi: 10.1038/nrcardio.2017.161
169. Childs BG, Durik M, Baker DJ, van Deursen JM. Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nat Med*. 2015;21:1424-1435. doi: 10.1038/nm.4000
170. Mohamad Kamal NS, Safuan S, Shamsuddin S, Foroozandeh P. Aging of the cells: Insight into cellular senescence and detection Methods. *Eur J Cell Biol*. 2020;99:151108. doi: 10.1016/j.ejcb.2020.151108
171. Sreekumar PG, Hinton DR, Kannan R. The Emerging Role of Senescence in Ocular Disease. *Oxid Med Cell Longev*. 2020;2020:2583601. doi: 10.1155/2020/2583601
172. Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. *Exp Gerontol*. 2000;35:927-945. doi: 10.1016/s0531-5565(00)00180-7
173. Zhao H, Darzynkiewicz Z. Biomarkers of cell senescence assessed by imaging cytometry. *Methods Mol Biol*. 2013;965:83-92. doi: 10.1007/978-1-62703-239-1_5
174. Bernardes de Jesus B, Blasco MA. Assessing cell and organ senescence biomarkers. *Circ Res*. 2012;111:97-109. doi: 10.1161/circresaha.111.247866
175. Chen JH, Ozanne SE, Hales CN. Methods of cellular senescence induction using oxidative stress. *Methods Mol Biol*. 2007;371:179-189. doi: 10.1007/978-1-59745-361-5_14
176. 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. *Nat Protoc*. 2009;4:1798-1806. doi: 10.1038/nprot.2009.191
177. Farr JN, Khosla S. Cellular senescence in bone. *Bone*. 2019;121:121-133. doi: 10.1016/j.bone.2019.01.015
178. Jia G, Aroor AR, Jia C, Sowers JR. Endothelial cell senescence in aging-related vascular dysfunction. *Biochim Biophys Acta Mol Basis Dis*. 2019;1865:1802-1809. doi: 10.1016/j.bbadis.2018.08.008
179. Rossman MJ, Kaplon RE, Hill SD, McNamara MN, Santos-Parker JR, Pierce GL, Seals DR, Donato AJ. Endothelial cell senescence with aging in healthy humans: prevention by habitual exercise and relation to vascular endothelial function. *Am J Physiol Heart Circ Physiol*. 2017;313:H890-h895. doi: 10.1152/ajpheart.00416.2017
180. Jia G, Aroor AR, DeMarco VG, Martinez-Lemus LA, Meininger GA, Sowers JR. Vascular stiffness in insulin resistance and obesity. *Front Physiol*. 2015;6:231. doi: 10.3389/fphys.2015.00231
181. Hohensinner PJ, Kaun C, Buchberger E, Ebenbauer B, Demyanets S, Huk I, Eppel W, Maurer G, Huber K, Wojta J. Age intrinsic loss of telomere protection via TRF1

reduction in endothelial cells. *Biochim Biophys Acta*. 2016;1863:360-367. doi: 10.1016/j.bbamcr.2015.11.034
182. Gorenne I, Kavurma M, Scott S, Bennett M. Vascular smooth muscle cell senescence in atherosclerosis. *Cardiovasc Res*. 2006;72:9-17. doi: 10.1016/j.cardiores.2006.06.004
183. Lian J, Yue Y, Yu W, Zhang Y. Immunosenescence: a key player in cancer development. *J Hematol Oncol*. 2020;13:151. doi: 10.1186/s13045-020-00986-z
184. Tahir S, Fukushima Y, Sakamoto K, Sato K, Fujita H, Inoue J, Uede T, Hamazaki Y, Hattori M, Minato N. A CD153+CD4+ T follicular cell population with cell-senescence features plays a crucial role in lupus pathogenesis via osteopontin production. *J Immunol*. 2015;194:5725-5735. doi: 10.4049/jimmunol.1500319
185. Wang YH, Yu XH, Luo SS, Han H. Comprehensive circular RNA profiling reveals that circular RNA100783 is involved in chronic CD28-associated CD8(+)T cell ageing. *Immun Ageing*. 2015;12:17. doi: 10.1186/s12979-015-0042-z
186. Lee J, Ko YS, Lee HY, Yang J, Oh S, Jo SK, Cho W, Kim MG. The role of senescence of bone marrow cells in acute kidney injury. *Kidney Res Clin Pract*. 2019;38:25-32. doi: 10.23876/j.krcp.18.0114
187. Elias R, Hartshorn K, Rahma O, Lin N, Snyder-Cappione JE. Aging, immune senescence, and immunotherapy: A comprehensive review. *Semin Oncol*. 2018;45:187-200. doi: 10.1053/j.seminoncol.2018.08.006
188. Ray D, Yung R. Immune senescence, epigenetics and autoimmunity. *Clin Immunol*. 2018;196:59-63. doi: 10.1016/j.clim.2018.04.002
189. Sebastián C, Herrero C, Serra M, Lloberas J, Blasco MA, Celada A. Telomere shortening and oxidative stress in aged macrophages results in impaired STAT5a phosphorylation. *J Immunol*. 2009;183:2356-2364. doi: 10.4049/jimmunol.0901131
190. Ferrucci L, Fabbri E. Inflammageing: chronic inflammation in ageing, cardiovascular disease, and frailty. *Nat Rev Cardiol*. 2018;15:505-522. doi: 10.1038/s41569-018-0064-2
191. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nat Rev Immunol*. 2019;19:255-265. doi: 10.1038/s41577-019-0141-8
192. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. 2010;33:657-670. doi: 10.1016/j.immuni.2010.11.011
193. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity*. 2005;22:285-294. doi: 10.1016/j.immuni.2005.01.011
194. Doeing DC, Borowicz JL, Crockett ET. Gender dimorphism in differential peripheral blood leukocyte counts in mice using cardiac, tail, foot, and saphenous vein puncture methods. *BMC Clin Pathol*. 2003;3:3. doi: 10.1186/1472-6890-3-3
195. Mestas J, Hughes CC. Of mice and not men: differences between mouse and human immunology. *J Immunol*. 2004;172:2731-2738. doi: 10.4049/jimmunol.172.5.2731
196. Faurschou M, Sørensen OE, Johnsen AH, Askaa J, Borregaard N. Defensin-rich granules of human neutrophils: characterization of secretory properties. *Biochim Biophys Acta*. 2002;1591:29-35. doi: 10.1016/s0167-4889(02)00243-4
197. Falloon J, Gallin JI. Neutrophil granules in health and disease. *J Allergy Clin Immunol*. 1986;77:653-662. doi: 10.1016/0091-6749(86)90404-5
198. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. 2013;13:159-175. doi: 10.1038/nri3399
199. Vorobjeva NV, Chernyak BV. NETosis: Molecular Mechanisms, Role in Physiology and Pathology. *Biochemistry (Mosc)*. 2020;85:1178-1190. doi: 10.1134/s0006297920100065

200. Pires RH, Felix SB, Delcea M. The architecture of neutrophil extracellular traps investigated by atomic force microscopy. *Nanoscale*. 2016;8:14193-14202. doi: 10.1039/c6nr03416k
201. Chapman EA, Lyon M, Simpson D, Mason D, Beynon RJ, Moots RJ, Wright HL. Caught in a Trap? Proteomic Analysis of Neutrophil Extracellular Traps in Rheumatoid Arthritis and Systemic Lupus Erythematosus. *Front Immunol*. 2019;10:423. doi: 10.3389/fimmu.2019.00423
202. Petretto A, Bruschi M, Pratesi F, Croia C, Candiano G, Ghiggeri G, Migliorini P. Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. *PLoS One*. 2019;14:e0218946. doi: 10.1371/journal.pone.0218946
203. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, Brinkmann V, Jungblut PR, Zychlinsky A. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog*. 2009;5:e1000639. doi: 10.1371/journal.ppat.1000639
204. Thiam HR, Wong SL, Wagner DD, Waterman CM. Cellular Mechanisms of NETosis. *Annu Rev Cell Dev Biol*. 2020;36:191-218. doi: 10.1146/annurev-cellbio-020520-111016
205. Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E, Schmid I, Straumann A, Reichenbach J, Gleich GJ, et al. Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nat Med*. 2008;14:949-953. doi: 10.1038/nm.1855
206. Morshed M, Hlushchuk R, Simon D, Walls AF, Obata-Ninomiya K, Karasuyama H, Djonov V, Eggel A, Kaufmann T, Simon HU, et al. NADPH oxidase-independent formation of extracellular DNA traps by basophils. *J Immunol*. 2014;192:5314-5323. doi: 10.4049/jimmunol.1303418
207. von Köckritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, Medina E. Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood*. 2008;111:3070-3080. doi: 10.1182/blood-2007-07-104018
208. Chow OA, von Köckritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL, Gallo RL, Monestier M, Wang Y, Glass CK, et al. Statins enhance formation of phagocyte extracellular traps. *Cell Host Microbe*. 2010;8:445-454. doi: 10.1016/j.chom.2010.10.005
209. Abi Abdallah DS, Denkers EY. Neutrophils cast extracellular traps in response to protozoan parasites. *Front Immunol*. 2012;3:382. doi: 10.3389/fimmu.2012.00382
210. Zindel J, Kubes P. DAMPs, PAMPs, and LAMPs in Immunity and Sterile Inflammation. *Annu Rev Pathol*. 2020;15:493-518. doi: 10.1146/annurev-pathmechdis-012419-032847
211. Shen H, Kreisel D, Goldstein DR. Processes of sterile inflammation. *J Immunol*. 2013;191:2857-2863. doi: 10.4049/jimmunol.1301539
212. Edwards NJ, Hwang C, Marini S, Pagani CA, Spreadborough PJ, Rowe CJ, Yu P, Mei A, Visser N, Li S, et al. The role of neutrophil extracellular traps and TLR signaling in skeletal muscle ischemia reperfusion injury. *Faseb j*. 2020;34:15753-15770. doi: 10.1096/fj.202000994RR
213. Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ*. 2011;18:581-588. doi: 10.1038/cdd.2011.1
214. McDonald B, Pittman K, Menezes GB, Hirota SA, Slaba I, Waterhouse CC, Beck PL, Muruve DA, Kubes P. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science*. 2010;330:362-366. doi: 10.1126/science.1195491

215. Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, Bhandari AA, Wagner DD. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost*. 2012;10:136-144. doi: 10.1111/j.1538-7836.2011.04544.x
216. Semeraro F, Ammollo CT, Morrissey JH, Dale GL, Friese P, Esmon NL, Esmon CT. Extracellular histones promote thrombin generation through platelet-dependent mechanisms: involvement of platelet TLR2 and TLR4. *Blood*. 2011;118:1952-1961. doi: 10.1182/blood-2011-03-343061
217. von Brühl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, Khandoga A, Tirniceriu A, Coletti R, Köllnberger M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med*. 2012;209:819-835. doi: 10.1084/jem.20112322
218. Kapoor S, Opneja A, Nayak L. The role of neutrophils in thrombosis. *Thromb Res*. 2018;170:87-96. doi: 10.1016/j.thromres.2018.08.005
219. Wang Y, Luo L, Braun O, Westman J, Madhi R, Herwald H, Mörgelin M, Thorlacius H. Neutrophil extracellular trap-microparticle complexes enhance thrombin generation via the intrinsic pathway of coagulation in mice. *Sci Rep*. 2018;8:4020. doi: 10.1038/s41598-018-22156-5
220. Leppkes M, Knopf J, Naschberger E, Lindemann A, Singh J, Herrmann I, Stürzl M, Staats L, Mahajan A, Schauer C, et al. Vascular occlusion by neutrophil extracellular traps in COVID-19. *EBioMedicine*. 2020;58:102925. doi: 10.1016/j.ebiom.2020.102925
221. Bonaventura A, Montecucco F, Dallegri F, Carbone F, Lüscher TF, Camici GG, Liberale L. Novel findings in neutrophil biology and their impact on cardiovascular disease. *Cardiovasc Res*. 2019;115:1266-1285. doi: 10.1093/cvr/cvz084
222. Tsai CY, Hsieh SC, Liu CW, Lu CS, Wu CH, Liao HT, Chen MH, Li KJ, Shen CY, Kuo YM, et al. Cross-Talk among Polymorphonuclear Neutrophils, Immune, and Non-Immune Cells via Released Cytokines, Granule Proteins, Microvesicles, and Neutrophil Extracellular Trap Formation: A Novel Concept of Biology and Pathobiology for Neutrophils. *Int J Mol Sci*. 2021;22. doi: 10.3390/ijms22063119
223. Hong CW. Extracellular Vesicles of Neutrophils. *Immune Netw*. 2018;18:e43. doi: 10.4110/in.2018.18.e43
224. Leroyer AS, Isobe H, Lesèche G, Castier Y, Wassef M, Mallat Z, Binder BR, Tedgui A, Boulanger CM. Cellular origins and thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol*. 2007;49:772-777. doi: 10.1016/j.jacc.2006.10.053
225. Loyer X, Zlatanova I, Devue C, Yin M, Howangyin KY, Klaihmon P, Guerin CL, Kheloufi M, Vilar J, Zannis K, et al. Intra-Cardiac Release of Extracellular Vesicles Shapes Inflammation Following Myocardial Infarction. *Circ Res*. 2018;123:100-106. doi: 10.1161/CIRCRESAHA.117.311326
226. Minciacchi VR, Freeman MR, Di Vizio D. Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. *Semin Cell Dev Biol*. 2015;40:41-51. doi: 10.1016/j.semcdb.2015.02.010
227. Dunford A, Keramida G, Anagnostopoulos CD, Michael Peters A. The cardiosplenic axis: another obscure pathophysiological function of the spleen and its investigation using molecular imaging. *Nucl Med Commun*. 2017;38:205-208. doi: 10.1097/MNM.0000000000000635
228. Xia N, Lu Y, Gu M, Li N, Liu M, Jiao J, Zhu Z, Li J, Li D, Tang T, et al. A Unique Population of Regulatory T Cells in Heart Potentiates Cardiac Protection From Myocardial Infarction. *Circulation*. 2020;142:1956-1973. doi: 10.1161/circulationaha.120.046789

229. Emami H, Singh P, MacNabb M, Vucic E, Lavender Z, Rudd JH, Fayad ZA, Lehrer-Graiwer J, Korsgren M, Figueroa AL, et al. Splenic metabolic activity predicts risk of future cardiovascular events: demonstration of a cardiosplenic axis in humans. *JACC Cardiovasc Imaging*. 2015;8:121-130. doi: 10.1016/j.jcmg.2014.10.009
230. Boulanger CM. Microparticles, vascular function and hypertension. *Curr Opin Nephrol Hypertens*. 2010;19:177-180. doi: 10.1097/MNH.0b013e32833640fd
231. Chen TS, Lai RC, Lee MM, Choo AB, Lee CN, Lim SK. Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res*. 2010;38:215-224. doi: 10.1093/nar/gkp857
232. Hugel B, Martínez MC, Kunzelmann C, Freyssinet JM. Membrane microparticles: two sides of the coin. *Physiology (Bethesda)*. 2005;20:22-27. doi: 10.1152/physiol.00029.2004
233. Kang M, Jordan V, Blenkiron C, Chamley LW. Biodistribution of extracellular vesicles following administration into animals: A systematic review. *J Extracell Vesicles*. 2021;10:e12085. doi: 10.1002/jev2.12085

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Impact de l'inflammation parodontale sur l'axe splénique cardiaque : étude des Neutrophils Extracellular Traps (NETs) et des microvésicules spléniques comme médiateurs des réponses procoagulantes et inflammatoires.

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Résumé

P.gingivalis (PG) induit la parodontite et est associée au risque cardiovasculaire. Les microvésicules (MV) sont des effecteurs pro-inflammatoires et coagulants libérés des membranes plasmiques. L'effet à distance de PG sur le cœur et la rate de souris est détecté par immuno-marquage, mesure de ROS, et émission de MVs splénocytaires (SMVs). Les lésions gingivales par fils (LIG) imbibés ou non de PG (LIG-PG) sont comparées au gavage (gvg) et à l'injection intrapéritonéale (IP). LIG et LIG-PG réduisent les granulocytes, doublent les monocytes et les neutrophiles spléniques, sans réponse adaptative. IP triple les monocytes, multiplie par 10 les neutrophiles, réduit par 2 lymphocytes T ou B. L'inhibiteur de PAD-4 réduit les ROS, suggérant une Nétose. In vitro, les SMVs de LIG-PG, IP et gvg induisent la sénescence endothéliale. Dans le cœur, VCAM, Gasdermin-D, IL-6 et TNF-α inflammatoires sont surexprimés pour IP et LIG-PG.

En conclusion, PG induit l'activation splénique et cardiaque à distance, l'émission de SMVs pro-coagulantes et -sénescentes favorisant le risque cardiovasculaire.

Résumé en anglais

Periodontitis, mainly induced by P.gingivalis (PG) is associated with cardiovascular risk. Microvesicles (MVs) are pro-inflammatory and -coagulant effectors shed from plasma membranes. We evaluated the remote inflammatory impact of PG on heart and spleen tissue by immunostaining, ROS formation, and on endothelial senescence. In mice, ligature and/or PG gum injury by threads (LIG) soaked or not with PG (LP,LP 4w) were compared to oral gavage (gvg) and intraperitoneal injection (IP). LIG and LIG-PG reduced spleen granulocytes, doubled monocytes and neutrophils with no adaptative responses in 2-4 weeks. IP tripled monocytes with 10 folds neutrophil rise and half T or B lymphocytes. PAD-4 inhibitor decreased ROS, pointing at Netosis contribution. Splenocytes MVs (SMVs) raised in IP. In vitro, LIG-PG, IP and gvg SMVs induced coronary endothelial senescence. In heart extracts, pro-inflammatory VCAM, Gasdermin-D, IL-6, and TNF-α were only up-regulated in IP and LIG-PG. Anti-inflammatory IL-10 raised in IP.

Altogether, PG induces remote spleen and heart activation, the shedding of pro-coagulant and -senescent SMVs possibly favoring cardiovascular risk.