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# Lipid mediators of the resolution of inflammation in vascular ageing

Matthieu Bardin

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### Thèse

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DOCTEUR DE L'UNIVERSITE DE LORRAINE

Mention : « Sciences de la Vie et de la Santé »

par **Matthieu BARDIN**

## Résolution de l'inflammation par les médiateurs lipidiques dans le vieillissement vasculaire

Le 18 mai 2022

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## **Abbreviations**

AA: arachidonic acid

Abn-CBD: abnormal-cannabidiol

ApoE: apolipoprotein E

cAMP: cyclic adenosine monophosphate

CD: cluster of differentiation

ChemR23: chemerin receptor 23

COX: cyclooxygenase

CVDs: cardiovascular diseases

DAB: 3,3'-diaminobenzidine

DAPI: di aminido phenyl indol

DHA: docosahexaenoic Acid

DNA: deoxyribonucleic Acid

DPA: docosapentaenoic acid

EC: endothelial cells

ECM: extracellular matrix

EndMT: endothelial-to-mesenchymal transition

eNOS: endothelial nitric oxide synthase

ELISA: enzyme-linked immunosorbent assay

EPA: eicosapentaenoic acid

EPR: electron paramagnetic resonance

EV: extracellular vesicles

FCCP: carbonyl cyanide 4- [trifluoro methoxy] phenylhydrazone

FITC: fluorescein isothiocyanate

GPCR: G protein-coupled receptor

GPR18: G protein-coupled receptor 18

Hb: haemoglobin

HBSS: Hanks' balanced salt solution

HO-1: haem oxygenase-1

IGF: insulin like growth factor

IL: interleukin

iNOS: inducible nitric oxide synthase

LDL: low density lipoprotein  
Ldlr: low-density lipoprotein receptor  
LOX: lipoxygenase  
LT: leukotriene  
LX: lipoxin  
MAPK: mitogen-activated protein kinases  
MaR1: maresin 1  
MMP: matrix metalloproteinase  
n-3 PUFA: omega-3 polyunsaturated fatty acid  
n-6 PUFA: omega-6 polyunsaturated fatty acid  
NAGLy: N-arachidonylglycine  
NO: nitrogen monoxide  
OCR: oxygen consumption rate  
OCT: optimal cutting temperature compound  
oxLDL: oxidized LDL  
PBS: phosphate buffer saline  
PCR: polymerase chain reaction  
PD: protectin D  
PDGF: platelet-derived growth factor  
PFA: paraformaldehyde  
PG: prostaglandins  
PMN: polymorphonuclear leukocytes  
PPi: inorganic pyrophosphate  
PS: phosphatidylserine  
RBC: red blood cells  
RNA: ribonucleic acid  
ROS: reactive oxygen species  
RvD1: resolvin D1  
RvD2: resolvin D2  
RvE1: resolvin E1  
SE: senescent erythrocytes  
SPM: specialized pro-resolving lipids mediators  
TBHP: *tert*-Butyl hydroperoxide

TBS: tris buffer saline

TBS-T: TBS-tween

TG: triglycerides

TGF- $\beta$ : transforming growth factor  $\beta$

TLR: toll like receptor

TNF: tumor necrosis factor

TX: thromboxane

VCAM: vascular cell adhesion protein

VSMCs: vascular smooth muscle cells

WHO: world health organization

$\alpha$ SMA:  $\alpha$  smooth muscle actin

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## Résumé en français

### Situation du sujet

Les maladies cardiovasculaires sont la première cause de décès au niveau mondiale causant près de 18 millions de décès par ans selon l'Organisation Mondiale de la Santé. Un tiers de ces décès survient chez des personnes âgées de moins de 70 ans. En France les maladies cardiovasculaires sont la seconde cause de décès avec près de 140 000 morts par an.

D'ici 2050 la proportion de la population âgée de plus de 60 ans sera triplée. Avec ce vieillissement de la population mondiale les prédictions sont alarmantes puisque de plus en plus de personnes vont développer des maladies cardiovasculaires. Cet effet est d'autant plus visible dans les pays industrialisés à cause de l'augmentation du nombre de cas de diabète de type 2, des mauvaises habitudes alimentaires, de la consommation d'alcool et de la sédentarité.

Notre système cardiovasculaire permet au sang de circuler dans tout le corps et d'apporter oxygène et nutriments aux organes. L'athérosclérose est une maladie cardiovasculaire caractérisée par le dépôt de LDL contenant du cholestérol dans la paroi vasculaire. Ces particules de LDL vont s'oxyder et activer les cellules endothéliales et ainsi permettre le recrutement de monocytes qui vont se différencier en macrophages et phagocyter ces particules de LDL oxydées. Lors du développement de l'athérosclérose notre cœur est le premier touché au niveau de la racine aortique suivi par les artères coronaires et l'aorte. L'aorte, comme tous les vaisseaux de la macro-circulation, est organisée en 3 couches, de l'intérieur vers l'extérieur : l'intima, la media et l'adventice.

L'adventice est la couche la plus externe des vaisseaux, sa matrice extracellulaire est composée majoritairement de collagène fibrillaire qui apporte un support physique aux vaisseaux. Cette couche est innervée et irriguée grâce à des micro-vaisseaux afin d'apporter oxygène et nutriments aux cellules les plus éloignées de la lumière du vaisseau.

La media est constituées d'un seul type cellulaire, les cellules musculaires lisses vasculaires, lesquelles sont enchevêtrées dans les lames élastiques qui donnent aux vaisseaux leur élasticité et leur permet de résister au stress mécanique provoqué par le compartiment sanguin.

L'intima est la couche la plus interne des vaisseaux, elle est directement au contact du sang et est constituée d'une unique couche de cellules endothéliales. Cette couche cellulaire forme une barrière entre la paroi vasculaire et le compartiment sanguin et est également capable de réguler la tonicité vasculaire de par ses sécrétions de substances vasodilatatrices. Dans des conditions physiologiques les cellules endothéliales sont organisées parallèlement au flux sanguin, cette organisation est due au stress mécanique et à l'étirement cyclique faisant suite aux battements du cœur.

Le cœur possède son propre système circulatoire appelé la circulation coronaire. Il y a deux artères coronaires majeures, l'artère coronaire gauche qui irrigue la partie gauche du cœur et l'artère coronaire droite qui irrigue la partie droite du cœur. Une fonction réduite des artères coronaires affecte directement le cœur en diminuant les apports en oxygène et en nutriments au muscle cardiaque. Cela affecte la capacité du cœur à faire circuler le sang et peut mener à des angines, des crises cardiaques voir même la mort. Le développement de l'athérosclérose induit un rétrécissement des artères par les dépôts de lipides dans la paroi vasculaire ce qui va entraîner une diminution du débit sanguin. Les cellules vont alors manquer d'oxygène et une ischémie apparaît. La rupture de ces plaques va engendrer la formation d'un caillot bloquant le flux sanguin et engendrant une crise cardiaque.

Au niveau de l'insertion de l'aorte dans le cœur on retrouve la valve aortique aussi appelé la racine aortique. Cette valve tricuspide s'ouvre et se ferme à chaque battement de cœur permettant l'éjection du sang tout en empêchant son reflux dans le cœur. Une perte de fonction de cette valve se caractérise par une régurgitation du sang dans le ventricule gauche pendant la diastole et résultant en une dilatation du ventricule gauche.

Lors du développement de l'athérosclérose plus la tailles des lésions augmentent plus et le nombre de macrophages est important et l'inflammation est elle aussi plus grande. Les macrophages qui ont phagocytés les LDL oxydés se transforment en cellules spumeuses et l'inflammation devient chronique.

Lorsque les lésions sont à un stade avancé on retrouve en leur sein des amas de cellules mortes formant les cœurs nécrotiques. Ces lésions avancées présentent également des calcifications, parfois sous forme de microcalcifications qui rendent les plaques plus aptes à la rupture que

des calcifications bien établies.

Lorsque les lésions sont bien établies elles sont isolées de la circulation sanguine par une chape fibreuse, constitué de collagène, qui constitue également une protection contre la rupture.

Un des traitements majeurs de l'athérosclérose repose sur les statines de par leur action sur les lipides circulants. Des études prenant pour cible l'inflammation chronique ont également montrés des effets bénéfiques. Des essais cliniques utilisant des acides gras oméga-3 ont montré des effets bénéfiques en réduisant l'occurrence des évènements cardiovasculaires.

Un grand nombre de types cellulaires différents sont impliqués dans le développement de l'athérosclérose, parmi elles se trouvent les lymphocytes, les granulocytes, les macrophages, les cellules endothéliales, les cellules musculaires lisses vasculaires et les érythrocytes.

Les lymphocytes représentent 20% des cellules immunitaires présentes au sein des plaques athérosclérotiques. L'activation des lymphocytes T et leur polarisation en lymphocytes Th1 initie la formation des plaques et permet leur croissance, ce sous type est également associé à la déstabilisation des lésions et à la rupture des plaques.

Les neutrophiles granulocytes représentent une cible importante pour les médiateurs lipidiques et les chimiokines. Ils ont été décrits comme jouant un rôle clé dans le développement de l'athérosclérose et contribuent à la rupture des plaques à cause de leur forte expression de protéases.

Les macrophages jouent des rôles divers, ils participent à l'amplification de l'inflammation, la stimulation du système immunitaire mais agissent également sur les mécanismes anti-inflammatoires. Ils étaient auparavant décrits comme étant divisés en deux sous-types : les macrophages M1 qui sont pro-inflammatoires et les macrophages M2 décrit comme anti-inflammatoires. Depuis lors, de nouveaux sous-types de macrophages ont été décrits au sein des lésions athérosclérotiques tels les macrophages M2a, b et c ou encore les macrophages M4, Mox, Mhem et MHb. Ces différents types de macrophages ont tous une action particulière au sein des lésions.

Les cellules endothéliales ont diverses fonctions en conditions physiologiques. En plus de ré-



guler le tonus vasculaire elles évitent la coagulation du sang en inhibant les plaquettes et régulent le passage des cellules immunitaires. Elles sont de ce fait un acteur important de la régulation de la réponse inflammatoire et leur dysfonction est le premier signe détectable de changement au sein de la paroi artérielle qui va mener au développement de l'athérosclérose.

Les cellules musculaires lisses vasculaires n'ont aucune interaction avec le compartiment sanguin en condition physiologiques. Ces cellules sont caractérisées par l'expression de protéines spécifiques leur permettant de conserver leur capacité contractile. Le phénotype contractile est caractérisé par une faible capacité de prolifération, de migration et de synthèse de ces cellules. Avec le développement de l'athérosclérose les cellules musculaires lisses vasculaires perdent leur phénotype contractile et donc l'expression de leurs protéines spécifiques. Elles gagnent alors des capacités migratoires, prolifératives et peuvent synthétiser diverses molécules de la matrice extracellulaire tel le collagène.

Les érythrocytes quant à eux représentent 90% des cellules sanguines, durant la formation des lésions de l'athérosclérose les cellules sanguines entrent en collision avec la paroi artérielle. Ces cellules sont capables de pénétrer la paroi vasculaire et ont un effet direct sur les cellules musculaires lisses vasculaires. Des études ont montré que l'hème libre provenant des érythrocytes est capable d'oxyder les particules de LDL présentes dans la paroi vasculaire et donc de participer au développement de l'athérosclérose. Les cellules musculaires lisses vasculaires sont également capables de phagocyter ces érythrocytes ce qui engendre un stress oxydatif important. Le stress oxydatif étant un élément clé du développement de l'athérosclérose, l'entrée d'érythrocytes dans la paroi vasculaire est donc un mécanisme clé dans le développement de la maladie.

L'inflammation chronique est caractéristique de l'athérosclérose, la résolution de cette inflammation fait appel à des mécanismes actifs qui sont déréglés avec cette maladie. Après la formation de lésions la réponse inflammatoire et sa résolution sont divisées en deux phases. La première phase est la phase d'initiation de l'inflammation dans laquelle il est observé une augmentation de la quantité de molécules pro-inflammatoires y compris des médiateurs lipidiques pro-inflammatoires dérivés des acides gras oméga-6. La seconde phase correspond à la résolution de l'inflammation dont l'objectif est de stopper l'inflammation pour un retour à l'homéostasie. Cette résolution de l'inflammation passe par certaines molécules dont l'interleukine 10 et les

médiateurs lipidiques pro-résolutifs. Dans ces mécanismes la balance entre médiateurs lipidiques pro-inflammatoires et pro-résolutifs est primordiale pour déterminer si la résolution de l'inflammation va être mise en place ou si l'on va se diriger vers une inflammation chronique et donc progression de la maladie. Ces médiateurs lipidiques pro-résolutif dérivent des acides gras oméga-3 EPA, DPA et DHA tandis que les acides gras oméga-6 vont produire des médiateurs lipidiques pro-inflammatoire, à l'exception des lipoxines qui ont un pouvoir pro-résolutif. Tous ces médiateurs sont produits par une succession de réactions complexes qui font intervenir des lipoxigénases et des cyclooxygénases.

Ces médiateurs exercent leurs actions via des récepteurs transmembranaires spécifiques qui font partie de la famille des récepteurs couplés aux protéines G. La résolvine D2 (RvD2) a pour récepteur GPR18 tandis que la résolvine E1 (RvE1) a pour récepteur ChemR23.

Le récepteur GPR18 est exprimé par un certain nombre de type cellulaire dont les cellules du système immunitaire. La fixation de la RvD2 sur GPR18 a montré chez des macrophages de souris un effet anti-inflammatoire. Il a également été montré qu'un traitement à la RvD2 induit un changement phénotypique des macrophages du sous-type pro-inflammatoire M1 vers le sous-type pro-résolutif M2. La RvD2 a également montré des effets sur des cellules musculaires lisses aortique de souris et de veine humaine en réduisant leur migration et leur production de cytokines pro-inflammatoires. Utilisée en traitement combinatoire avec la maresine 1, un autre médiateur lipidique pro-résolutif, le RvD2 agit en limitant le développement de l'athérosclérose.

L'expression du récepteur ChemR23 a elle aussi été démontrée dans un certain nombre de type cellulaire incluant les macrophages et les cellules musculaires lisses vasculaires. La fixation de la RvE1 sur ChemR23 a montré des effets d'inhibition de formation de calcifications sur des cellules musculaires lisses vasculaires et a également montré des effets protecteurs contre l'athérosclérose. La RvE1 promeut également un changement phénotypique des macrophages en les polarisant vers le sous-type M2.

### **Hypothèses et objectifs du travail**

Par le passé l'observation des populations Inuit a montré qu'ils étaient moins sujets au déve-

loppement de maladies cardiovasculaires. Cette observation a été relié à leur grande consommation de poissons dans lesquels une grande quantité d'acides gras oméga-3 est présente. De nos jours, il est connu que les acides gras oméga-3 et les médiateurs lipidiques qui en dérivent ont un effet bénéfique sur le vieillissement cardiovasculaire.

Dans cette étude nous avons cherché à étudier l'effet des médiateurs lipidiques pro-résolutifs sur le vieillissement cardiovasculaire et plus précisément l'effet de la RvD2 dans le cadre de l'athérosclérose. Plus précisément les objectifs sont les suivants :

- Explorer l'impact d'un traitement à la RvD2 sur les mécanismes impliqués dans le développement de l'athérosclérose dans un modèle de cellules musculaires lisses aortiques humaines.
- Evaluer l'effet d'un traitement à la RvD2 sur le développement précoce de l'athérosclérose en utilisant un modèle de vieillissement accéléré chez la souris
- Evaluer la présence du récepteur GPR18 et de la RvD2 dans un modèle d'artères coronaires humaines obtenus de donneurs sains et athérosclérotiques.

### **Méthodologie générale**

Dans cette étude nous avons utilisé des cellules musculaires lisses aortiques humaines afin de montrer l'expression du récepteur GPR18 par immunofluorescence. Le développement de l'athérosclérose engendrant divers changements chez les cellules, nous avons étudié l'effet de la RvD2 sur le métabolisme des cellules à travers le prisme de leur respiration en utilisant des inhibiteurs de la chaîne respiratoire mitochondriale et la technologie Seahorse. Le stress oxydatif étant primordiale lors du développement de la maladie nous avons étudié ce stress via un modèle de phagocytose de globules rouges sénescents et mesuré le stress oxydatif qu'ils peuvent provoquer via une sonde fluorescente. Enfin, l'effet de la RvD2 sur la migration des cellules a été évalué en utilisant un kit avec des inserts qui permet plus de reproductibilité que le test de la cicatrice.

L'effet de la RvD2 sur le vieillissement cardiovasculaire a été étudié en utilisant un modèle de souris invalidées pour l'expression de l'apolipoprotéine E et nourries avec un régime riche en graisses et enrichi en cholestérol. Les souris ont été assignées aléatoirement à un des quatre groupes (contrôle, RvD2, O-1918 et O-1918+RvD2). A l'âge de 8 semaines les souris ont été pesées chaque semaine et injectées 3 fois par semaine avec le traitement correspondant à leur groupe (véhicule -éthanol-, RvD2 100 ng et/ou O-1918 2 mg/kg). Après 4 semaines de traitement le sang a été prélevé et analysé, les globules ont été incubés avec la sonde CMH et congelés pour analyse de la production de ROS par spectroscopie EPR. Le cœur coupé et congelé en OCT pour réaliser plus tard des coupes et des colorations à l'huile rouge pour révéler les lésions de la racine aortique. L'aorte thoracique a été prélevée, fixé au paraformaldéhyde et ouverte en face pour révéler les lésions par coloration à l'huile rouge. Les cellules intrapéritonéales ont été récupérées par lavages afin d'évaluer la capacité phagocytaire des macrophages intrapéritonéaux. Les ARNs des rates ont été extraits afin d'évaluer la production de cytokines pro-inflammatoires et la composition des lésions en macrophages a été étudié par immunomarquage.

Les patients issus de la cohorte d'artères coronaires humaines ont été séparés en 3 groupes pour les immunomarquages : sain, strie lipidique et athéromateux. Pour les dosages par ELISA des résolvines les patients ont été séparés en 2 groupes : sain et athéromateux.

L'évaluation de l'expression des récepteur GPR18 et ChemR23 a été réalisée par immunomarquages et le dosage des résolvines D2 et E1 ont été réalisés sur des milieux conditionnée préparés à partir des prélèvements d'artères coronaires.

## **Résultats obtenus**

Les expériences menées sur les modèles *in vitro* ont permis de mettre en évidence l'expression du récepteur GPR18 par les cellules musculaires lisses vasculaires aortiques humaines et au niveau de la média de l'aorte chez la souris. Le traitement à la RvD2 n'a pas montré d'effet significatif sur le métabolisme des cellules ou leur capacité à produire des radicaux libres ni leur migration.

Le traitement à base de RvD2 utilisé chez des souris invalidées pour l'apolipoprotéine E (ApoE)

et nourries avec un régime riche en gras enrichi en cholestérol n'a montré aucun effet sur la numération sanguine des souris, ainsi que sur leur taux de cholestérol et triglycérides circulants. En revanche les macrophages péritonéaux des souris traitées avec la RvD2 ont montré une capacité de phagocytose plus grande que les macrophages des souris contrôles, cet effet a été annulé par l'addition de O-1918, un antagoniste du récepteur GPR18. Les souris traitées à la RvD2 ont montré une diminution significative de la production de ROS par les érythrocytes des souris, ainsi que des lésions et des cœurs nécrotiques plus petits au sein de leur racine aortique mais ne présentent pas d'effet sur le développement des lésions dans l'aorte thoracique par rapport aux souris contrôles.

Les études menées dans des milieux conditionnés préparés à partir d'artères coronaires humaines nous ont permis de mettre en évidence une plus haute concentration en RvD2 ainsi qu'une plus faible concentration en RvE1 dans les milieux préparés à partir d'artères athéromateuses par rapport à des artères saines.

Les immunomarquages réalisés sur des coupes d'artères coronaires nous ont permis de mettre en évidence la colocalisation de CD68 avec ChemR23 and GPR18 ainsi que la colocalisation d' $\alpha$ SMA avec GPR18 and ChemR23.

Enfin, la classification des marquages en 3 catégories (pas exprimé, faible expression, expression modérée) a permis de montrer au sein des artères coronaires humaines une augmentation de la quantité de CD68 présent dans les lésions avec la progression de la maladie. L'expression de GPR18 et ChemR23 augmente dans les stades précoces de l'athérosclérose (strie lipidique) pour diminuer dans les lésions matures.

## **Discussion**

Dans cette thèse nous avons étudié l'effet de la RvD2, médiateur lipidique pro-résolutif dérivé de l'acide gras oméga-3 DHA, sur les mécanismes du vieillissement cardiovasculaire et plus particulièrement dans le cas de l'athérosclérose. Les principaux résultats obtenus sont 1) la RvD2 diminue le développement des lésions athérosclérotiques dans un modèle de souris ApoE<sup>-/-</sup> avec un régime riche en graisse, 2) le traitement à la RvD2 augmente la phagocytose des macrophages péritonéaux des souris ApoE<sup>-/-</sup>, 3) les artères coronaires humaines athéromateuses synthétisent plus de RvD2 que les artères coronaires saines 4) l'expression du récepteur de la RvD2, GPR18, augmente dans les stades précoces de l'athéroscléroses et diminue ensuite dans

les lésions matures.

Bien que des études aient été menées sur l'action de la RvD2 sur les cellules musculaires lisses vasculaires, l'expression de GPR18 sur ces cellules n'était pas établie contrairement au récepteur de la RvE1, ChemR23, dont l'expression par ces cellules avait été démontrée. L'athérosclérose induisant un changement phénotypique des CMLVs d'un phénotype contractile à un phénotype synthétique induit un changement de fonction des cellules et donc de leur métabolisme. La mesure du métabolisme de nos CMLVs prolifératives en présence de RvD2 n'a pas montré de différence avec les cellules non traitées. De même, le traitement n'a pas induit de différence significative sur la capacité de migration des cellules. Des études réalisées sur des cellules musculaires de la veine saphène humaine et des cellules musculaires aortiques murines ont montré que la RvD2 diminue la migration des cellules.

Le stress oxydatif étant un mécanisme primordial au développement de l'athérosclérose, des études ont montré que les globules rouges jouent un rôle important dans la génération de ce stress oxydatif. Des traitements *in vitro* et *in vivo* ont montré que la RvD2 diminue la formation de ROS et donc le stress oxydatif, cet effet a été observé dans nos conditions expérimentales par la mesure des ROS produits par les érythrocytes de nos souris.

Afin de mieux comprendre les mécanismes d'action de la RvD2 sur le développement de l'athérosclérose nous avons utilisé un modèle de vieillissement accéléré constitué de souris ApoE<sup>-/-</sup> nourries avec un régime riche en graisse enrichie en cholestérol. Ces souris ont été traitées avec de la RvD2 et/ou du O-1918. Bien que des études aient montré qu'une supplémentation en acide gras oméga-3 augmente le taux d'hémoglobine et diminue le taux de triglycérides, le traitement à la RvD2 n'a pas modifié ces paramètres suggérant que la RvD2 n'agit pas sur l'hématopoïèse, ni sur le taux circulant de triglycérides.

La phagocytose des cellules apoptotiques est un mécanisme primordial dans l'athérosclérose permettant d'augmenter la stabilité des lésions. Des études ont montré que le traitement de macrophages avec des médiateurs lipidiques pro-résolutifs (SPMs) augmente leur capacité de phagocytose. Nous avons montré que les macrophages péritonéaux de nos souris traitées avec la RvD2 avaient une capacité de phagocytose plus grande que ceux des souris contrôles. De plus, les macrophages des souris traitées avec le traitement combinatoire RvD2 + O-1918 ont une capacité de phagocytose égale à celle des souris contrôles. Ces résultats indiquent que la RvD2

augmente la capacité de phagocytose des macrophages en agissant via GPR18.

Le cholestérol et les triglycérides circulants sont responsables du développement de l'athérosclérose. Des études *in vivo* ont montré que l'inactivation de récepteur des SPMs augmentait le développement de l'athérosclérose alors qu'une supplémentation avec des SPMs tels que la RvE1 permettent de diminuer le développement des lésions. Notre modèle nous a permis de mettre en évidence que le traitement à la RvD2 diminuait le développement des plaques athérosclérotiques ainsi que la formation des cœurs nécrotiques. Cet effet n'a pas été observé au sein de l'aorte thoracique du fait de lésions trop précoces.

Le développement de l'athéroscléroses est caractérisé par une inflammation chronique et l'incapacité de la résolution de celle-ci. Parmi les mécanismes en cause, il a été montré que les enzymes impliquées dans la synthèse des SPMs sont moins actives et moins exprimées avec le développement de la maladie.

La mesure des résolvines E1 et D2 dans des milieux conditionnés préparés à partir d'artères coronaires humaines saines et athéromateuses a permis de mettre en évidence une diminution de la synthèse de la RvE1 dans les artères athéromateuses comparées aux artères contrôles. L'effet inverse est observé pour la RvD2 supportant le fait que la RvD2 a un rôle primordial à jouer dans la lutte contre le développement de l'athérosclérose.

Nous avons montré par des immunomarquages que les récepteur GPR18 et ChemR23 voient leur expression augmenter à un stade précoce de l'athérosclérose (strie lipidique) puis diminuer dans les lésions matures. Ces résultats vont dans le sens de la littérature où il a été montré que le récepteur de la résolvine D1, GPR32, voit son expression diminuer dans les carotides athéromateuses en comparaison aux carotides saines. Cette diminution d'expression des récepteurs participe à cette incapacité de résoudre l'inflammation induite par l'athérosclérose et donc au développement de la maladie.

## **Conclusions générales et perspectives**

Dans ce travail de thèse nous avons étudié l'effet de la RvD2 sur le développement de l'athérosclérose. Nos résultats montrent que la RvD2 possède un potentiel non négligeable dans les processus de protection contre le vieillissement cardiovasculaire et qu'approfondir nos connaissances sur ces mécanismes d'actions pourrait être bénéfique.

Dans cette étude nous avons observé que la RvD2 diminue la formation des plaques athéromateuses et augmente la capacité de phagocytose des macrophages. Nous avons également observé chez l'humain une augmentation de la production de RvD2 avec l'apparition de plaque d'athérome suggérant un rôle majeur de la RvD2 dans les processus de lutte contre le développement de l'athérosclérose.

L'action de la RvD2 sur les cytokines pro-inflammatoires reste à déterminer dans notre modèle ainsi que son action sur la stabilité des plaques athéromateuses. L'utilisation d'un modèle de souris GPR18<sup>-/-</sup> transplantées avec la moelle de souris GPR18<sup>+/+</sup> permettrait une meilleure compréhension de l'action du couple GPR18-RvD2 sur les cellules immunitaires ainsi que leur rôle dans l'inflammation et sa résolution. De plus, mieux connaître les voies de signalisation impliquées pourrait permettre de développer des traitements allant directement cibler ces voies de signalisations.



## **I. Introduction**

### **1. Cardiovascular ageing and diseases generalities**

Cardiovascular diseases (CVDs) are a major cause of death worldwide, according to the World Health Organization (WHO), causing almost 18 million deaths per year with more than 4 out of 5 of these deaths occurring by heart attack or stroke. People under the age of 70 represent a third of these deaths. CVDs regroup disorders of the heart and blood vessels. In France, CVDs are the second cause of death with more than 140 000 deaths per year.

Due to the world population getting older more and more people will develop CVDs, predictions estimate that by 2030 around 23.6 million people could develop these diseases. This effect could be due essentially to the fact that the world population above 60 years old will triple between 2000 and 2050 to reach 2 billion [1]. With normal ageing we observe a progressive modification and deterioration of structure and vascular function named arteriosclerosis which constitute a major risk factor for CVDs. In industrialized countries the increase of type 2 diabetes (which is one of the leading cause of early CVDs), metabolic disorders, and risk factors like tobacco smoking, bad food habits, alcohol and a sedentary lifestyle are also contributory factors, thus we can easily understand why CVDs cases are increasing to match the alarming predictions [2].

### **2. Aorta arterial wall and heart structure**

The cardiovascular system allows blood to circulate throughout the whole body and for the blood to transport nutrients and oxygen, among others. This system is composed mainly of the heart, arteries, arterioles, and capillaries. Cardiovascular diseases like atherosclerosis appear first in the heart, at the aortic root level, in the aorta and in coronary arteries.

The aorta is the main artery of the body and like all the other vessels from the macro-circulation is organized in three different layers from inside to outside: intima, media, and adventitia. The aorta is divided in two parts, the thoracic aorta, and the abdominal aorta. The thoracic aorta is itself divided in three parts: the ascending aorta extending from the aortic valve to the beginning of the bronchocephalic arterial trunk, the aortic arch and the descending aorta which ends at the aortic orifice of the diaphragm.

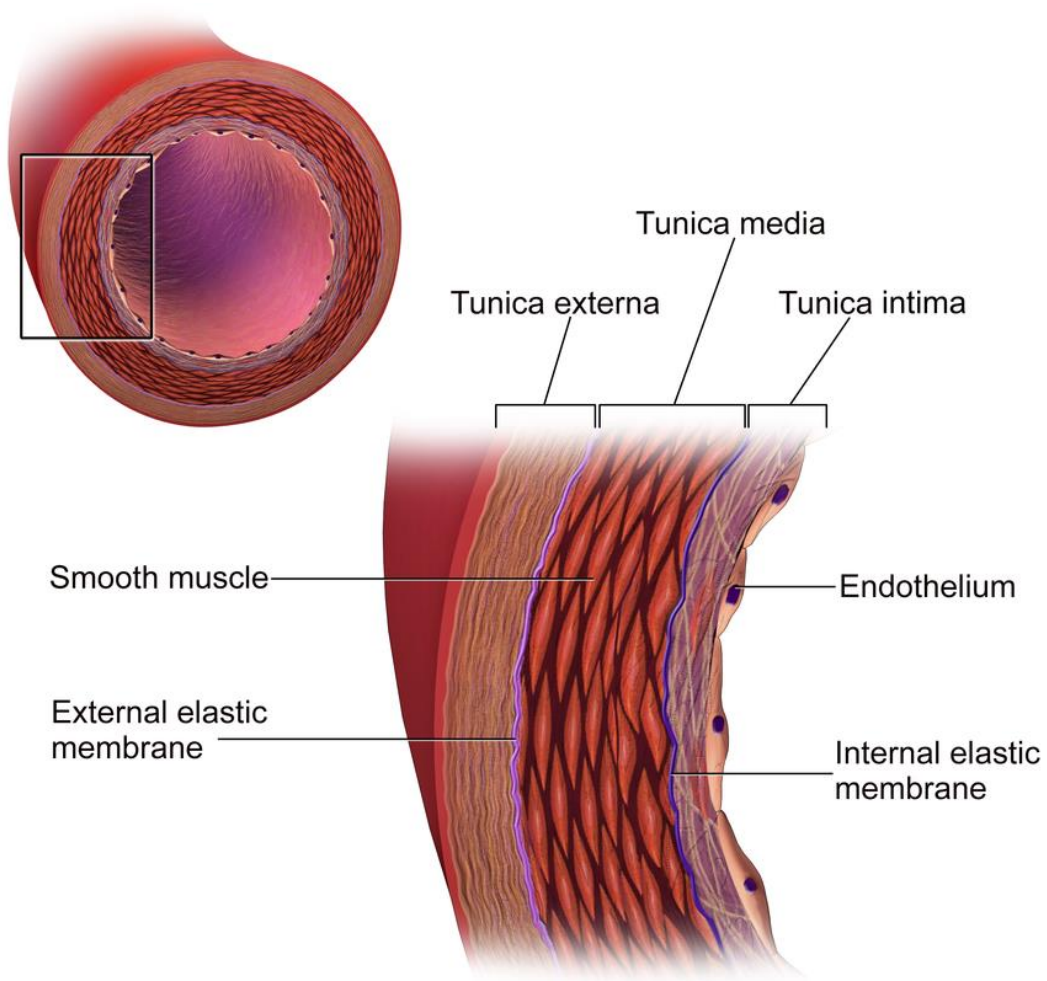


Figure 1: Representation of the structure of arterial wall.

*The vascular wall is composed of three layers: the tunica intima (intima), tunica media (media) and tunica externa (adventitia). Upper left: Representation of a whole artery in cross section. Bottom right: magnification of a part of the artery showing a representation of the different layers which compose an artery. Image courtesy: [https://commons.wikimedia.org/wiki/File7Blausen\\_0055\\_ArteryWallStructure.png](https://commons.wikimedia.org/wiki/File7Blausen_0055_ArteryWallStructure.png).)*

### **a. Aorta arterial wall**

#### **i. Adventitia**

The adventitia is the outermost layer of the artery wall, its extracellular matrix (ECM) is composed mainly of fibrillar collagen which provides physical support to the vessels (Figure 1). Fibroblasts are the main cell type found in this layer and are responsible for production,

organization, and degradation of the ECM but are also able to produce growth factors, cytokines, and chemokines. Nerves are present inside the adventitia as well as micro-vessels which help delivery of oxygen and nutrients to the cells far from the arterial lumen. These micro-vessels are also an entrance point for inflammatory cells [3,4].

## **ii. Media**

The media is constituted of several elastic laminae between which are located vascular smooth muscles cells (VSMCs), the only cell type present in this layer in healthy vessels. These elastic laminae give the vessel resistance to mechanical stress from the blood compartment. Lamellar units are formed by VSMCs and elastic laminae and their number are proportional to vessel diameter. The media is separated from the intima by the internal elastic membrane and from the adventitia by the external elastic membrane (Figure 1). This tunic is also the thickest of all three tunics [3]. A part will be dedicated to VSMCs later in this document (see I.4.e).

## **iii. Intima**

The intima is a layer composed of a unique layer of endothelial cells (EC). Its internal part, called the sub-endothelium, is in contact with the media (Figure 1). The main role of the endothelium is to form a barrier between the vascular wall and the blood compartment. This single layer of endothelial cells is metabolically active and is also extremely selective. Its production of nitrogen monoxide (NO) and prostacyclin (PGI<sub>2</sub>) regulates essential functions, these substances being two active vasodilators. The endothelium also exhibits anti-thrombotic characteristics and serves as an impermeable barrier to the parietal transport of molecules and to biological attacks from circulating blood. Due to the high variability of blood flow and pressure into the aorta, the endothelium is therefore constantly under high mechanical shear stress as well as cyclic stretching. These stresses will impose on this layer its organization, so under physiological conditions endothelial cells are aligned parallel to the blood flow [3]. A part will be dedicated to ECs later in this document (see I.4.d).

## **b. Heart structure**

### **i. Coronary arteries**

The heart has its own vascular system to deliver blood and nutrients to its muscles, this circulation is called the coronary circulation. Heart muscle is irrigated by two main coronary arteries, the left coronary artery and the right coronary artery, originating from the aorta. The right coronary artery and its arterioles supply blood to the right side of the heart, the smallest part of the heart that only pumps blood toward the lungs. The left coronary artery irrigates the whole left side of the heart, this side being the largest due to its function to pump blood to the rest of the body [5] (Figure 2A).

Reduced functions of the coronary arteries affect the heart itself due to the decrease in oxygen and nutrient supply. It can affect the ability of the heart to transport blood to organs body and have serious impact on health, eg like angina, myocardial infraction and even death.

When a state of heart failure occurs, the body will respond by a series of mechanisms aiming to compensate the failure of the myocardium. At the cardiac level there is an acceleration of heart rate (tachycardia), a dilatation of the left ventricle in order to maintain a sufficient systolic ejection volume and hypertrophy of the cardiac muscle to reduce wall pressure. At the peripheral level there is activation of the adrenergic system (tachycardia and inotropic effect, peripheral vasoconstriction and stimulation of the renin-angiotensin-aldosterone system) synthesis of endothelin and secretion of prostaglandins. These compensatory mechanisms are harmful in the long term for both the heart and the vascular system already weakened by years of damage.

In acute decompensated heart failure, blood perfusion and oxygen delivery to end organs need to be reestablish. Treatments against acute mitral-regurgitation (diuretic, vasodilatations, blood thinners) and noninvasive positive ventilation are used as for acute decompensated heart failure.

With atherosclerosis developing lipid deposits in the vascular wall which will cause narrowing of the coronary arteries that can begin to limit blood flow. Blood flow limitation causes a lack of oxygen and essential nutriments transportation to cells and will cause ischemia of the heart muscle. The cells can die from this lack of oxygen and cause a myocardial infraction commonly referred to as a heart attack. High-grade narrowing of the coronary arteries induces transient ischemia which leads to ventricular arrhythmia terminating in ventricular fibrillation which often leads to death (Figure 2B). Atherosclerosis plaque rupture in coronary arteries will result in

the formation of a thrombus that will completely block blood flow resulting in a heart attack.

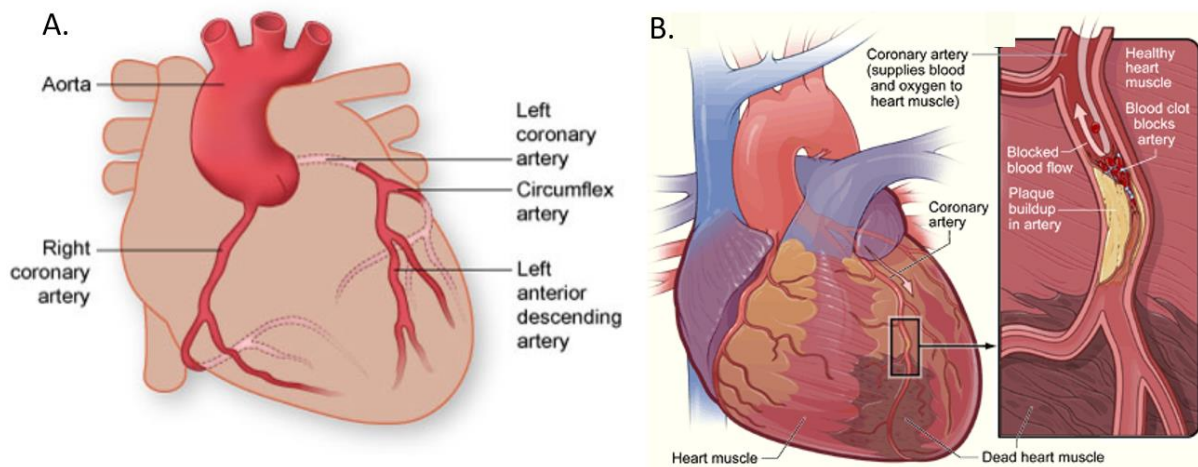


Figure 2: heart and coronary arteries representation.

*Representation of a human heart with the aorta and the two main coronary arteries and the region irrigated by them (A) (© Texas Heart Institute). Representation of a coronary artery which undergo ischemia due to atherosclerosis narrowing the artery (B) (obtained from <https://www.nhlbi.nih.gov/health-topics/heart-attack>).*

## ii. The aortic valve

The aortic valve is situated at the insertion of the aorta inside the heart (Figure 3). This valve is an avascular tissue composed of three leaflets. These leaflets are moving parts attached to the aortic wall able to open and close with every heartbeat. The leaflets are opened during the systole, allowing blood expulsion from the left ventricle to the aorta and are closed during the diastole, preventing regurgitation of blood in the left ventricle, and also allowing better refilling for the left ventricle [6]. Loss of function of the aortic valve leads to acute aortic insufficiency characterized by blood regurgitation into the left ventricle during the diastole resulting in a dilatation of the left ventricle.

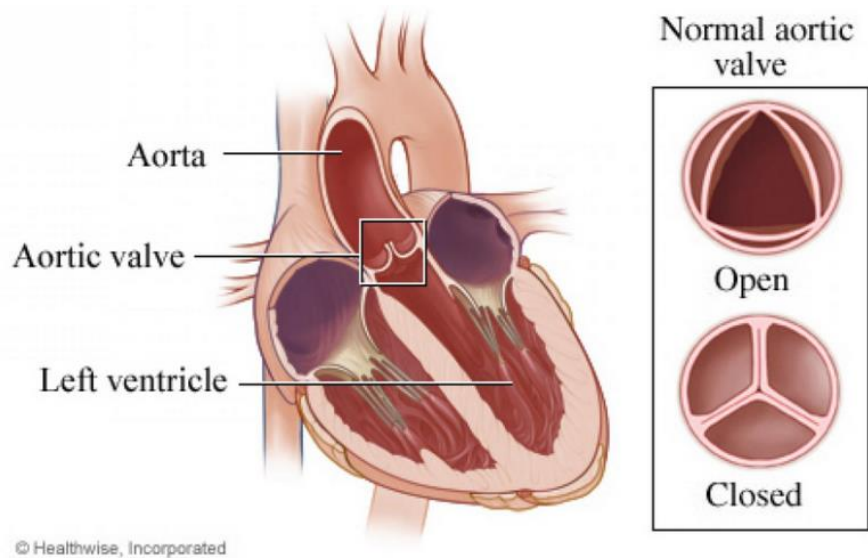


Figure 3: Representation of a heart longitudinally cut.

*This image shows the left ventricle, aorta insertion into the heart and leaflet position (left) and non-pathologic leaflet open and closed (right) (© Healthwise, Incorporated).*

### 3. Atherosclerosis Development

#### a. Definition of atherosclerosis

Atherosclerosis is a disease which is characterized by infiltration and deposition of cholesterol-containing LDL within the vascular wall during the early stages. It is then followed by a pronounced infiltration of inflammatory cells into the damaged area [7]. Cerebrovascular atherosclerosis is one of the causes of stroke whereas atherosclerosis is the underlying cause of coronary artery disease [8]. First stage of the disease, characterized by LDL infiltration in the vascular wall, is called fatty streak. As lesions size increase lesions are then classified as atheroma.

#### b. Early stages

The first step of the disease is the initiation of atherosclerotic plaque formation by cholesterol-containing LDL infiltration into the vascular wall through the intima [9]. By the action of myeloperoxidase, lipoxygenase and reactive oxygen species LDL particles located in the subendothelial space are oxidized [10]. This accumulation of oxLDL into the vascular wall lead to an activation of the endothelial cells whereby the expression of adhesion molecules, such as VCAM-1, is increased [11] (Figure 4A). Circulating monocytes are recruited to these damaged

areas as a response to this oxLDL accumulation. After their entry into the damaged area, monocytes differentiate into macrophages and start phagocytosing oxLDL particles [12] (Figure 4B).

### **c. Disease progression**

More and more oxLDL particles enter the vascular wall leading macrophages to produce inflammatory cytokines in bigger quantities and then enhancing recruitment of inflammatory cells. Activated macrophages which phagocytize oxLDL differentiate into foam cells and stay trapped in the vascular wall. This leads to the characteristic chronic inflammation process of atherosclerosis and drives formation and maturation of atherosclerotic plaque [13] (Figure 4B).

Dendritic cells present in the lesion take up antigens and migrate to secondary lymphoid organs where they can present the antigen to naïve T-cell through MHC class II antigen presentation. These T-cells can differentiate into subtypes which will play different roles in atherosclerotic plaque development. B-cells play a role in the response mediate against atherosclerotic plaque, it has been proved by the detection of anti-oxLDL antibodies in atherosclerotic plaque [14]. Vaccination against oxLDL showed a beneficial effect in preclinical animal models [15,16]

### **d. Advanced stages**

Advanced atherosclerotic lesions are characterized by the presence of dead cells forming a necrotic area in the lesions [17] (Figure 4C). Dead cell accumulation results from two process: an increase in the number of apoptotic cells and a decrease in efferocytosis meaning less clearance of apoptotic cells [18]. Advanced atherosclerotic plaques exhibit calcifications. Microcalcifications play a critical role in plaque stability and are a risk factor for rupture in comparison with bigger well-established calcifications [19,20]. Calcified nodules form within the plaques and present impaired ECM deposition, EVs released by macrophages, generation of sites for dystrophic mineralization and hydroxyapatite crystals formation by VSMCs followed by VMSCs differentiation into osteoblast-like cells [21].

### **e. Fibrous cap**

The fibrous cap covers the atherosclerotic plaque and keeps it isolated from the circulating

blood and constitutes a protection against plaque rupture. The fibrous cap is stable and composed of VMSCs and collagen (Figure 4C). Local degradation of these collagen fibres by the action of MMPs, increased calcifications, and VMSCs death weakens the plaque which is more prone to rupture [22]. An absence of endothelium can cause atherosclerotic plaque erosion and thrombus formation without any sign of plaque rupture [23] (Figure 4D).

If plaque rupture occurs, atherosclerotic plaque content is released into vessel lumen and interacts with bloodstream. This will lead to a local platelet aggregation and thrombus formation [22,24]. If the thrombus causes coronary artery occlusion it will cause a myocardial infarction; if the thrombus occlusion occurs in a cerebrovascular artery it will result in a stroke.

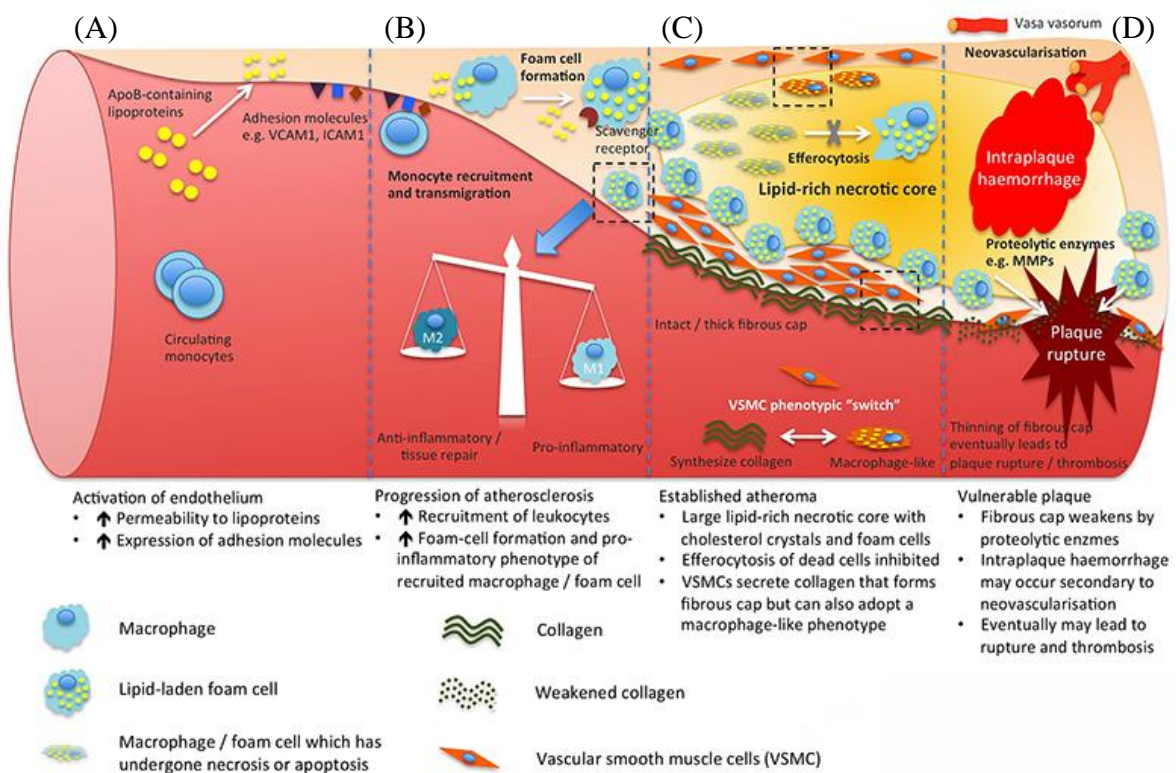


Figure 4: Atherosclerosis development process.

*Illustration showing atherosclerosis development stages, starting from cholesterol accumulation in the vascular wall and induction of the expression of adhesion molecules. Circulating monocytes are recruited through these molecules and enter the vascular wall where they will phagocytize cholesterol particles and transform into foam cells. The subtype mainly found into the lesion are the pro-inflammatory M1 subtype. VSMCs migrate to the intima and start synthesizing the fibrous cap and display impaired while a lipid rich necrotic core is forming. In the end, proteolytic enzymes, will disrupted the fibrous cap leading to plaque rupture, from Oxford Textbook of Medicine: Cardiovascular Disorders [25].*



#### **4. Atherosclerosis treatment**

Atherosclerosis treatment relies on lipid management by statins [26]. It has been shown that statins have a clear effect on reducing inflammation, but evidence suggests that their beneficial effect on atherosclerosis is not due to their effect on inflammation but to their potential to reduce lipid [27].

Reducing cardiovascular risk by targeting inflammation is an option that has already been studied. A recent clinical trial based on this hypothesis showed that targeting pro-inflammatory cytokines could serve as a potential treatment to reduce cardiovascular risk [28]. Patients with a history of myocardial infarction were recruited for this study. The results showed that patients who received Canakinumab, a monoclonal antibody targeting the IL-1 $\beta$ , presented a lower risk of recurrent cardiovascular events compared to the patients who received placebo. The study showed that one of the adverse secondary effects of this treatment against IL-1 $\beta$  was that patients were more prone to neutropenia and presented more deaths caused by sepsis or infection. Another clinical trial has shown that giving a low dose of colchicine, an anti-inflammatory drug, to patients with a chronic coronary disease reduce cardiovascular risk compared to placebo treated patients [29], showing beneficial effect of targeting inflammation in atherosclerosis.

It has been shown recently that ischemic events and cardiovascular death (4.3% for treated patients vs 5.2% for placebo treated patients, P=0.03) were significantly decreased with an omega-3 polyunsaturated fatty acid (n-3 PUFA) eicosapentaenoic acid treatment [30].

A large variability was found on the effect of n-3 PUFA treatments on cardiovascular disease risk and events during clinical trials. This variability could come from a lack of uniformity and quality control of the fish oil used for the different studies and/or the difficulty to show a beneficial effect of the n-3 PUFA treatment on individuals already under CVD or diabetes drugs [31].

#### **5. Cell types implicated in disease progression**

##### **a. Lymphocytes**

Lymphocytes represent up to 20% of the immune cells present in atherosclerotic lesions, the most abundant type of immune cells being macrophages. T lymphocyte activation and their

polarization into the Th1 subset regulates initiation and growth of atherosclerotic plaques and is also associated with lesion destabilization and rupture [32].

Studies have shown that anti-inflammatory cytokines such as TGF- $\beta$  and IL-10 can suppress the activity of T lymphocytes and modulate atherogenesis in mice [33–35]. The TGF $\beta$  is described as an immunosuppressive molecule activated by pro-inflammatory signals, and can also be activated by the pro-resolving lipids mediators. Indeed, in a model of ApoE<sup>-/-</sup> mice fed a high fat diet during 4 months with RvD2 and maresin-1 injection significantly increased TGF $\beta$  circulating levels [36]. Disruption of TGF- $\beta$  in dendritic cells CD11c<sup>+</sup> [37] and T lymphocytes CD4<sup>+</sup> [38] identified this cytokine as an actor to reduce atherosclerosis progression. Using an ApoE knockout mice for the TGF- $\beta$  signalling pathway model it was shown that the leukotriene pathway is one of the most upregulated in the T lymphocytes derived from atherosclerotic lesions of these mice [39]. The presence of a subset of regulatory T lymphocytes (Treg) which act by controlling effector cells has been shown [40]. Coming from this observation, immunization against atherosclerosis has been developed using the inactivated tuberculosis vaccine. In a mouse model, protection against atherosclerosis development was associated with induction of immunomodulation which is characterized by expansion of Treg cells [41]. Elimination of Treg cells delays clearance of cholesterol rich lipoproteins and is also responsible for an elevation of cholesterol concentrations in the plasma by direct action on hepatocytes [42]. This data indicate that T lymphocyte regulation of atherosclerosis is not only efficient within the plaque.

The presence of B lymphocytes in an atherosclerotic lesion has been demonstrated. Whereas B1 lymphocytes produce natural antibodies which confer protection against atherosclerosis [43], B2 cells have been shown in favour of atherosclerosis development [44].

## **b. Granulocytes**

An important target for lipid mediators and chemokines is neutrophil granulocytes [45]. Neutrophils can enter into atherosclerotic lesions as a part of the blood component of the intraplaque haemorrhage or enter by diapedesis [46]. In the model of Ldlr<sup>-/-</sup> mice it has been shown that hyperlipidaemia triggered by neutrophils is positively correlated with early atherosclerosis development [47]. Neutrophil count has also been described as an independent risk factor for cardiovascular disease in an epidemiological study supporting the idea that neutrophils are key

actors in atherosclerosis progression. Neutrophils are also a cell type with high proteases expression, these proteases being able to degrade proteins present in the plaque and the fibrous cap which can contribute to plaque rupture [48].

### **c. Macrophages**

Macrophages are a type of mononuclear white blood cell of the immune system. These cells can phagocytize dead and apoptotic cells and pathogens to protect the host against injuries and infection. These large phagocytic cells are found in all tissues where they patrol for potential pathogens, apoptotic and dead cells. Circulating monocytes can enter tissues and differentiate into macrophages. Macrophages play a very important role in amplifying inflammation and stimulating the immune system but also in the anti-inflammatory process. These cells are also of critical importance in atherosclerosis development and progression. With disease progression macrophages lose their efferocytic capacity, apoptotic cells accumulate in the vascular wall and will form necrotic areas.

Macrophages are historically divided into two subpopulations: the M1 subtype and the M2 subtype. M1 macrophages secrete pro-inflammatory molecules, they enhance and sustain the inflammatory response, this results in tissue damage and sometimes in impaired tissue healing [49]. M1 macrophages play an important role in protection against pathogens and produce high levels of TNF $\alpha$ , IL-1 $\beta$ , IL-6 and IL-12 and low levels of IL-10, they are also promoters of the Th1-type immune response [50] (Figure 5A). Whereas M2 macrophages produce anti-inflammatory cytokines, such as IL-10 and transforming growth factor- $\beta$  (TGF- $\beta$ ). These molecules promote the resolution of inflammation by enhancing clearance of apoptotic cells (efferocytosis), decreasing the immune response and promoting tissue healing and repair [51]. M2 macrophages are classified into several subtypes such as M2a, b and c. M2a macrophages contribute to tissue repair by secreting pro-fibrotic factors such as fibronectin, insulin like growth factor (IGF) and TGF- $\beta$  [52–56]. Exposure to IL-1 receptor agonists or TLR ligands and immune complexes trigger M2b macrophages [57], these macrophage subtypes produce proinflammatory molecules such as IL-1, IL-6 and TNF $\alpha$  but also anti-inflammatory cytokines such as IL-10 and TGF- $\beta$ 1. IL-10 and glucocorticoids trigger M2c macrophages. M2b and M2c macrophage subtypes are referred as “regulatory macrophages” whereas M2a macrophages are referred as “wound healing macrophages” [57]. However, all M2 macrophages exhibit an anti-

inflammatory cytokine profile and produce low levels of IL-12 and high levels of IL-10 [58]. Studies showed that M2 macrophages could play a role in atherosclerosis regression in a mouse model [59,60].

In atherosclerotic lesions CD68<sup>+</sup> macrophages are characterized according to their CD14 expression, CD14 being a pro-inflammatory marker [61]. The concept of M1 and M2 macrophages could, in this case, be more complicated in atherosclerotic lesions than it was first thought. CD14<sup>-</sup> macrophages were found to express the formyl peptide 2 and the A type lipoxin receptor (FRP2/ALX) and the chemokine receptor CCR7 which mediate respectively efferocytosis and emigration from the lesions.

The M4 macrophage subtype is identified by a loss of expression of CD163 and expression of CD68, S100A8 and MM7 (Figure 5 D). This subtype is associated with plaque instability in atherosclerosis [62]. In advanced plaque another macrophage subtype named M(Hb) has been identified. Differentiation into this macrophage subtypes occurs because of intraplaque haemorrhage which results in the release of free haemoglobin (Hb) and its uptake through CD163 and they are resistant to lipid accumulation (Figure 5B).

In murine atherosclerotic lesion another subtype of macrophages induced by oxLDL have been identified, this subtype was termed “mox” macrophages [63]. These macrophages express haem oxygenase-1 and present low phagocytic and chemotactic capacities and they display an anti-oxidant gene expression profile (Figure 5C). A specific resolution-phase macrophage subtype with a unique inflammatory profile has been described, these macrophages are controlled by cAMP levels [64].

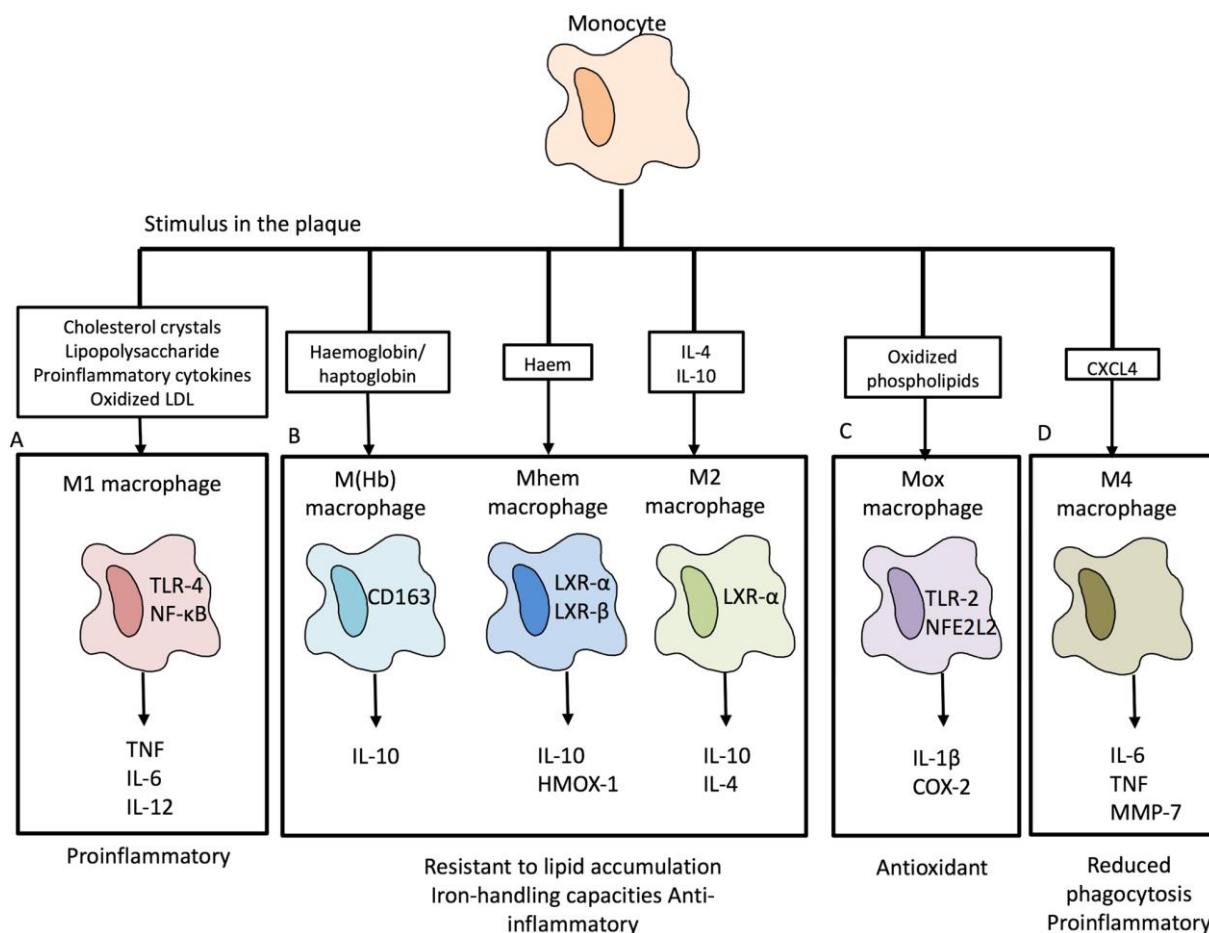


Figure 5: Schematic representation of the main macrophage subtypes found in atherosclerotic lesions.

*Representation of the macrophage subtypes, the stimulus inducing their polarization in each subtype and their role in the plaque. (A) M1 pro-inflammatory macrophages polarization is induced by cholesterol crystal, LPS, pro-inflammatory cytokines and oxLDL. This subtype synthesizes TNF, IL-6 and IL-12 among pro-inflammatory cytokines. (B) M(Hb), Mhem and M2 macrophages polarization are induced respectively by Haemoglobin/haptoglobin, Haem and IL-4/IL-10. They synthesize respectively IL-10, IL-10/HMOX-1 and IL-10/IL-4. These macrophages subtypes are resistant to lipid accumulation, can handle iron release and anti-inflammatory. (C) Mox macrophages are a special class of antioxidant macrophages induced by oxidized phospholipids that synthesize IL-1 $\beta$  and COX-2. (D) M4 macrophages polarization is induced by CXCL4, they are pro-inflammatory and have a reduce phagocytic capacity. They synthesize IL-6, TNF and MMP-7 [65].*

#### **d. Endothelial cells**

Endothelial cells compose the unique cell layer of the intima layer; these cells line the entire cardiovascular system from the heart to the smallest capillary. ECs can regulate vascular tone by releasing vasoactive factors such as nitric oxide and prostacyclin as vasodilators and thromboxane and endothelin-1 as vasoconstrictive factors. The role of these cells is also to avoid blood coagulation via inhibition of platelet and coagulation activations. These cells regulate the passage of immune cells and materials into and out of the circulating blood. ECs secrete PDGF-B, an important molecule to recruit pericytes. In mutant mice lacking the expression of PDGF-B pericytes are missing in many regions and this results in the development of microaneurysm during development of embryonic blood vessels [66].

Due to their capacity to let white blood cells enter the vascular wall, ECs are important mediators of the inflammatory response. Endothelial cell dysfunction is the earliest detectable change in the arterial wall that leads to atherosclerotic lesion development [23,67]. During atherosclerosis development, ECs are chronically activated by lipid accumulation in the vessel wall, exposure to inflammatory markers and turbulent blood flow [68].

The endothelium response to inflammatory stimuli has been identified has a potential target in cardiovascular diseases. Activated ECs can transdifferentiate into mesenchymal like cells in a process known as endothelial-to-mesenchymal transition (EndMT) in addition to being able to produce chemokines that communicate with resident and circulating inflammatory cells and to express leukocyte adhesion molecules. These cells express both EC and mesenchymal markers (intermediate state referred as partial EndMT) and can in some cases lose their EC identity, delaminate from the endothelium and contribute to the VSMC population and to the fibroblast population in the plaque [69,70].

#### **e. Vascular smooth muscle cells**

Smooth muscle cells are the major cell population of arteries and have no direct interaction with blood in physiological conditions, this is called the concept of immune-privilege of the media [71]. These cells are adherent, differentiated, and mononuclear. This differentiation is characterized by expression of specific proteins allowing VSMCs to maintain a contractile tone, regulate blood pressure and distribution of blood flow. Even if VSMCs have no contact with the

blood compartment due to the “immuno-privilege” because of stresses and pressure gradients elements from circulating blood can reach VSMCs.

Vascular smooth muscle cells are also able to synthesize their own extracellular matrix made up of fibrillar proteins such as elastin, collagen, glycoproteins, and proteoglycans. Matrix organization, composition, and the number of ECM-VSMC interactions influences the physical properties (strength and elasticity) of the tissue as well as VSMCs’ responses to mechanical stresses (flow and pressure). VSMCs’ phenotypic plasticity permits the cells to adapt their responses depending on the stimuli. Vascular smooth muscle cells can be divided in two main types: a contractile phenotype responsible for muscle tone control and a secretory phenotype (Figure 6).

#### **i. Secretory phenotype**

During vasculogenesis and in certain pathological conditions, such as atherosclerosis, VSMCs are characterized by a migratory and proliferative phenotype. These cells are also able to synthesize ECM molecules such as collagen, pro-elastin, and proteoglycans. This phenotype is associated with a loss of differentiation of the VSMCs and a decrease and in some cases a loss of expression of differentiation markers such as calponin, SM22 $\alpha$  and the  $\alpha$ -smooth muscle actin ( $\alpha$ SMA).

#### **ii. Contractile phenotype**

The contractile phenotype represents the differentiated phenotypes of smooth muscle cells and is characterized by low synthetic, migratory and proliferative capacities. The level of VSMC differentiation is determined by the level of expression of some proteins also responsible for their contractile function and so responsible for contraction and vascular tone [72]. Among these proteins associated with the differentiated phenotypes of VSMCs we can cite calponin, SM22 $\alpha$  and the  $\alpha$ -smooth muscle actin. This differentiated phenotype is reversible and VSMCs can switch phenotypes depending on several biological parameters such as cell-ECM, receptor-ligand, cell-cell interactions and of course with vascular pathologies [72]. This phenotype switch is regulated by epigenetic factors as well as by the local environment (redox imbalance, cholesterol accumulation in vascular wall).

Smooth muscle cells are said to be plastic and can switch to other phenotypes. It has been shown that VSMCs can switch to a macrophage-phenotype able to phagocytize apoptotic cells or red blood cells [73], when switching to this phenotype the cells can also phagocytize cholesterol that has accumulated into the vascular wall during atherosclerosis formation and turn into foam cells. These cells will then die and form necrotic cores in the lesions [17].

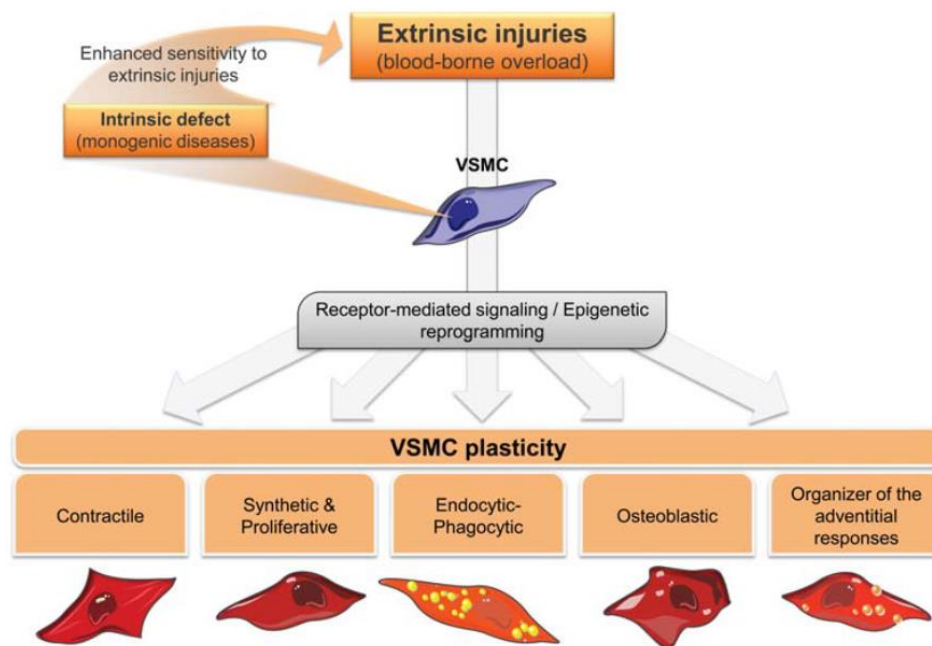


Figure 6: Schematic representation of VSMCs plasticity.

*Representation of VSMC plasticity showing the causes of this plasticity and the different phenotypes these cells can switch to, from JB Michel & al, Cardiovasc Res, 2012 [74].*

Vascular smooth muscle cells are of major importance in atherosclerosis development. During disease development, cholesterol-containing LDL infiltrate and accumulate in the vascular wall between the intima and the media. Due to this proximity, VSMCs are able to phagocytize these particles that will turn them into foam cells and are also exposed to pro-inflammatory cytokines synthesized by macrophages present into the lesions.

#### f. Erythrocytes

Red blood cells (RBCs), also referred as erythrocytes, represent 99% of the circulating blood cells. The cytoplasm of the cell is rich in haemoglobin which is an iron-containing molecule able to bind oxygen and thus transport O<sub>2</sub> and CO<sub>2</sub> throughout the body. In mammals, erythrocytes are cells which lack a nucleus that develop in the bone marrow.



During atherosclerosis formation, blood cells, including platelets, leukocytes and RBC, enter in collision with the arterial wall [75]. RBC can penetrate the vascular wall and have a direct effect on VMSCs. Studies have demonstrated that free haem increases LDL oxidation and participates in endothelial injury [76]. It has also been shown that free haemoglobin is toxic for the arterial wall [77]. Haem oxygenase-1 (HO-1) has been shown to reduce oxidative stress as well as monocyte recruitment and vascular dysfunction. Mice deficient for HO-1 were more susceptible to develop hyperglycaemia and faster vascular ageing [78]. Individuals with lower HO-1 expression displayed elevation of pro-inflammatory monocytes and a disturbed endothelial function which indicated that these individuals are more prone to develop cardiovascular events [79], [80].

Other studies demonstrated that intraparietal RBCs, through hematoma and haemorrhages, can contribute to lipid core growth due to their membrane cholesterol content [81,82]. Human VSMCs can perform both efferocytosis [83] and endocytosis [84] due to their phenotypic plasticity, those two capacities being associated with atheroma development. Internalization by VMSCs of phosphatidylserine (PS) exposing cells through the PS-receptor has been demonstrated. With a model of aged RBCs, also called senescent erythrocytes (SE), VSMCs are able to phagocytize those SE [83,85]. Delbosco & al demonstrated in 2017 the presence of iron and haemoglobin deposits in early atherosclerotic lesions in human aortas; using a glycophorin-A staining, which is a RBC membrane marker. They showed the presence of RBC in early aortic lesions. By studying oxidative stress they showed increased oxidative stress in early atheroma lesions and that VMSCs produced more reactive oxygen species (ROS) after phagocytosis of SE [85]. ROS and oxidative stress being one of the mechanism responsible for atherosclerosis development this study supported that aged RBC could play a key role in lesion development.

## **6. Resolution of inflammation**

### **a. Definition**

There are several hallmarks of chronic inflammation in cardiovascular diseases like infiltration into the vascular walls of inflammatory cells, lipoproteins, and lipids. The resolution

of inflammation is an active process that actively resolves and stops the inflammatory response by promoting efferocytosis and also by decreasing leukocyte recruitment [86,87]. After damage the inflammation/resolution response is divided into two phases. First, there is initiation of inflammation, this phase is characterized by infiltration of immune cells into the damaged area. Still in the initiation phase there is an increase of pro-inflammatory molecule synthesis including pro-inflammatory lipid mediators derived from the omega-6 fatty acid arachidonic acid, the leukotrienes and prostaglandins [88]. The second phase is the resolution of inflammation, the objective is to stop the inflammatory process and bring the environment back to homeostasis using active mechanisms. Several molecules mediate the resolution of inflammation such as IL-10 and specialized pro-resolving lipid mediators (SPMs) [89], these last being of particular importance for this thesis work. These specialized pro-resolving lipid mediators, synthesized from long chain PUFA and more specifically from n-3 PUFA for most of them, activate signalling pathways through specific G protein-coupled receptors (GPCRs)[90]. In case this signalling pathways fails to resolve inflammation, tissue damage and inflammation will persist and may become chronic [86] (Figure 7).

The balance between pro-inflammatory lipid mediators derived from n-6 PUFA and SPMs is a critical point in disease apparition and development. It has been showed that in vulnerable regions of human atherosclerotic lesions the LTB<sub>4</sub>/RvD1 ratio is higher, these regions being more prone to rupture [91].

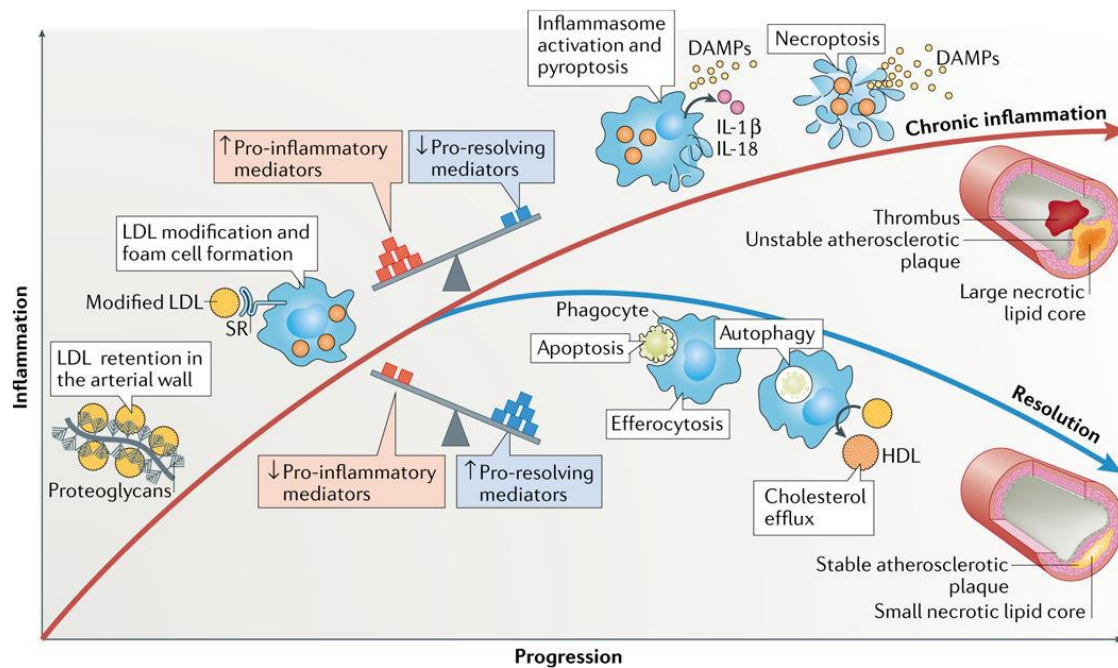


Figure 7: Schematic representation of the mechanisms involved in the resolution of inflammation versus chronic inflammation in atherosclerosis.

*Different mechanisms are involved in atherosclerosis progression under a pro-inflammatory profile (red line) and under a pro-resolutive profile (blue line). It starts with LDL retention in the arterial wall, LDL modification and then foam cell formations. If the balance is in favour of pro-inflammatory markers, the inflammasome is activated. This leads to chronic inflammation (red line). If the balance is in favour of pro-resolutive mediators, there is an increase phagocytosis of apoptotic cells an efflux of cholesterol leading to resolution of the inflammation characterized by a small necrotic core and a stable atherosclerotic plaque [86].*

### **b. Pro-resolving lipid mediators**

A class of fatty acid derived bioactive lipids with unique effects have been classified and named specialized pro-resolving lipid mediators (SPMs) [92]. These mediators are synthesized from omega-3 fatty acids eicosapentaenoic acid (EPA) docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) and from the omega-6 fatty acid arachidonic acid (AA). Among them are molecules such as E-series and D-series resolvins, obtained respectively from EPA and DHA, maresins and protectins. Another type of SPMs is synthesized and obtained from the omega-6 fatty acid arachidonic acid and are included in the lipoxin family. These bioactive lipids mediators can be synthesized by a wide variety of cell types but macrophages and neutrophils are the primary source of synthesis [92]. SPMs' biosynthesis is a complex succession of reaction

in which the main enzymes involved are the 5-lipoxygenase (5-LOX), 12-LOX and 15-LOX (Figure 8). Overexpression of 12/15-LOX in a model of ApoE<sup>-/-</sup> mice fed a chow diet was shown to be atheroprotective [93].

It was reported that patients with vascular diseases such as atherosclerosis and coronary artery disease exhibit low levels of SPMS in the plasma, including low levels of aspirin-triggered lipoxin A4 (LXA4) [94,95].

With Lovaza supplementation, which consist of a combination on ethyl ester of omega-3 fatty acid, patients exhibit after one year of treatment reduced levels of triglycerides and increased RvD6, RvE2 and AT-PD-1 levels compared to patients who did not receive the treatment [95].

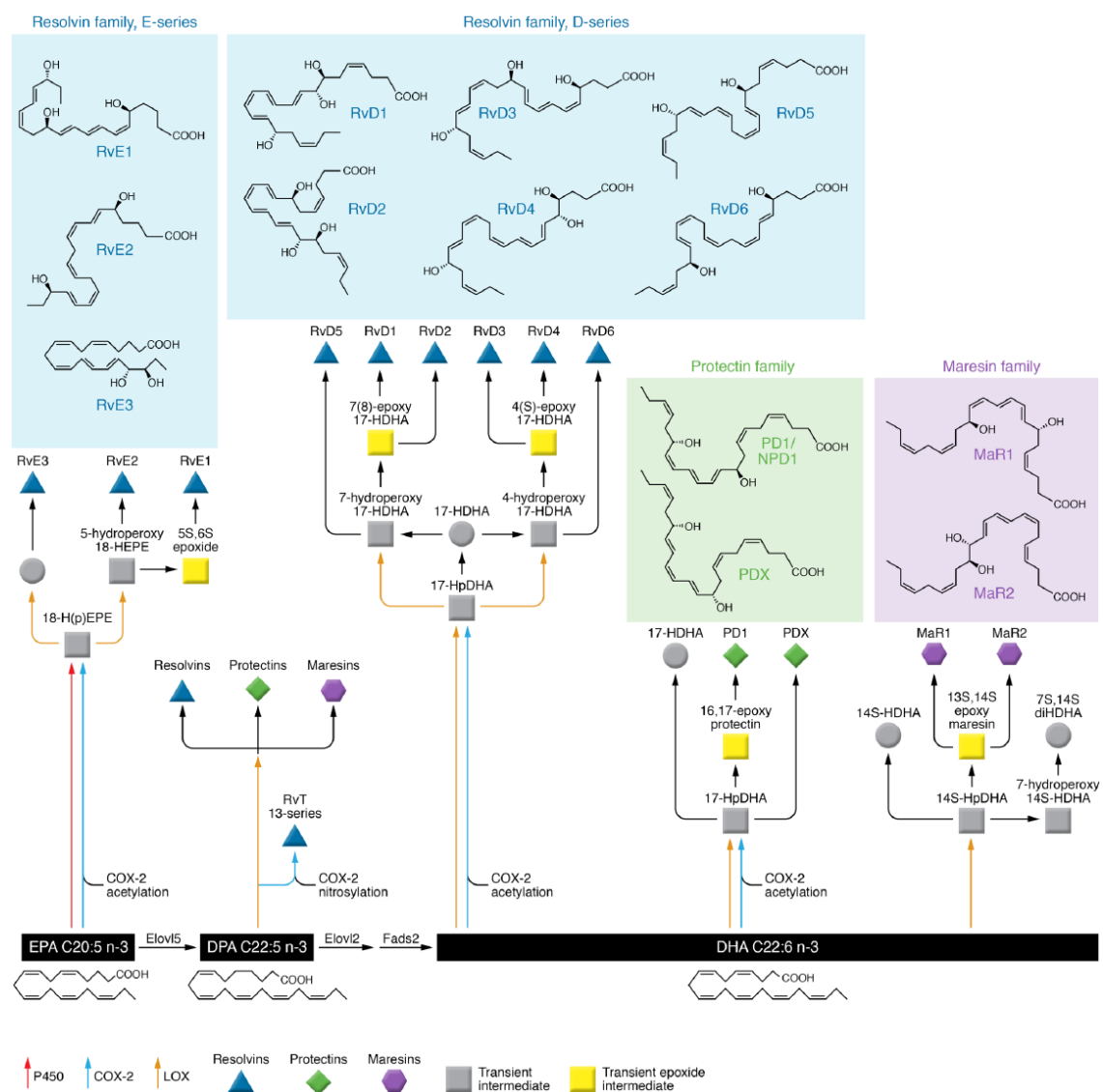


Figure 8: Specialized pro-resolving lipid mediators biosynthesis pathway.

*Schematic representation of specialized pro-resolving lipid mediator synthesis from the different n-3 PUFA and enzymes implicated, from omega-3 fatty acids and enzymes involved in the*

process. On the left side, mediators synthesized from EPA. On the right side, mediators synthesized from DHA [96].

### **c. Pro-inflammatory mediators**

Arachidonic acid, a 20-carbon fatty acid, is a constituent present in all cells but is not freely available. Stimulation by various cellular agonist and nonspecific stimuli like damage or injury will lead to AA release from membrane phospholipid stores. After its release in the cytosol AA can be metabolized in oxygenated products that will act in an autocrine/paracrine manner on target cells at nanomolar levels. Prostaglandins (PG) and thromboxane (TX), formed by cyclooxygenase (COX), leukotrienes (LT) and lipoxins, formed by lipoxygenases (LOXs) are parts of the formed products [97,98].

Brezinski *et al.*, showed that following angioplasty, levels of pro-inflammatory LT were increased in human coronary blood [99]. In human, a correlation was found between levels of plasma LTs and atherosclerotic plaque instability [100]. Another study demonstrated that LTB<sub>4</sub> increases intimal hyperplasia [101] as well as monocyte recruitment and their differentiation into foam cells [102]. Several variants of genes encoding for proteins and enzymes involved in LT synthesis exist. Among them there are variants of the 5 lipoxygenase (5-LOX), 5-LOX activated protein and LTA<sub>4</sub> hydrolase. Expression of these variants has been linked to an increase incidence of atherosclerosis, stroke, and myocardial infraction in humans [103–106].

Dwyer *et al.*, showed that individuals with a specific variant of 5-LOX fed with a diet rich in AA develop more carotid atherosclerosis compared to individuals with a nonvariant 5-LOX. They also showed that the same population given a diet rich in omega-3 fatty acids had less carotid atherosclerosis compared with a control population [107]. In human atherosclerotic lesions it was shown that the vulnerable ones had significantly less 5-LOX derived SPMs and an imbalance between SPMs and LT compared to stable plaque regions [91].

Two main isoforms of COX are involved in conversion of AA, COX-1, and COX-2. COX-1 is constitutively expressed in most cells types and tissues, whereas COX-2 expression is induced when cells are challenged with a pro-inflammatory stimuli [108]. More enzymes are involved in PG and TXA production as prostaglandin synthase and thromboxane synthase. However, the differential expression of these enzymes determines which mediator will be produced by the

cells, mast cells will mainly produce PGD<sub>2</sub> while macrophages will produce PGE<sub>2</sub> and TXA<sub>2</sub> [109]. Interestingly, it has been shown that during the onset phase of inflammation, PGE<sub>2</sub> indirectly acts in favour of the resolving of inflammation by inducing the transcription of enzymes required for the generation of other classes of SPMs such as the resolvins, protectins [89,110–112], and lipoxins [113].

## 7. Resolvins receptors

Resolvins and SPMs in general, exert their action through specific receptors part of the G-Protein coupled receptors (GPCRs) family. ChemR23 serve as receptor for the resolvin E1 (RvE1), and GPR18 for the resolvin D2 (RvD2) [114] (Figure 9).

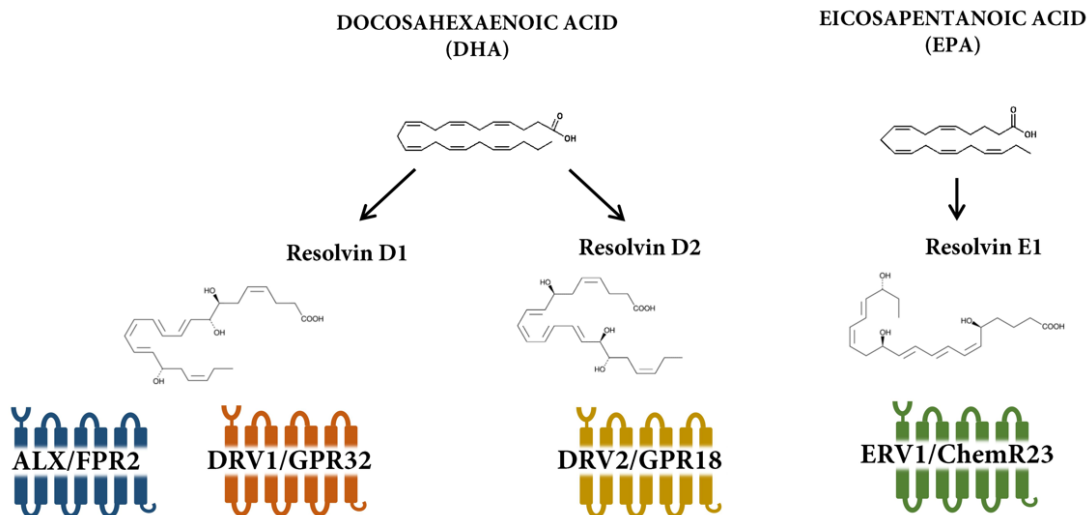


Figure 9: Resolvins and their receptors.

*Adapted from Pirault & Bäck, 2018 [114]. Left: Resolvin D2 derived from DHA and its receptor GPR18. Right: Representation of RvE1 derived from EPA and its receptor ChemR23.*

### a. GPR18/RvD2

GPR18 was firstly described in 1996 as an orphan receptor on the mice chromosome 14 [115], its human homolog on chromosome 13 was described a year later with high expression in spleen and testis [116]. GPR18 expression was later shown in leukocytes at the same time as its affinity with the specialized pro-resolving lipid mediator RvD2 [117]. GPR18 can be activated by several other ligands such as a metabolite of the endocannabinoid anandamide called N-arachidonylglycine (NAGly) [118], synthetic ligand as abnormal-cannabidiol (Abn-CBD) as well as

a partial agonist, O-1918, which can be used as a pharmacological tool to inhibit GPR18 signalling pathway activated by ligand fixation [119] (Figure 10).

Resolvin D2 receptor, GPR18, expression has been shown in several cells of the immune system and participates in development and homing of the CD8 $\alpha$ <sup>+</sup> lymphocytes in the small intestine. It has been shown in GPR18 deficient mice that the migratory capacities of these cells is decreased and also that their number is reduced [120]. In opposition to the beneficial effect described for CD8 $\alpha$ <sup>+</sup>, GPR18 has been described as pro-inflammatory in the mouse macrophage cell line RAW264.7. An increase in GPR18 expression was shown in M1 phenotype polarized macrophages supporting this pro-inflammatory effect [121]. However, even if GPR18 stimulation by NAGly on RAW macrophages increases apoptosis and caspase-3 expression, if mouse macrophages derived from bone marrow under a pro-inflammatory stimuli are treated with RvD2 a decrease in inflammasome activation is observed [121]. RvD2 was shown to stimulate revascularization in a murine model of hind limb ischemia while resolving inflammation induced by this revascularization [122]. Mice peritoneal macrophages treated with RvD2 showed less maturation of pro-IL-1 $\beta$  into mature and secreted IL-1 $\beta$ , this effect was blocked when the cells were treated with O-1918 in addition of RvD2 supporting a receptor-mediated response. RvD2 has also be described as inducing phenotype switch toward pro-resolving M2 macrophages in a model of self-resolving peritonitis induced by zymosan-A activation of the inflammasome [123]. In a coeliac ligation puncture induced sepsis model the GPR18-RvD2 pathway showed a protective effect and an increase in phagocytic capacity from macrophages, an effect that was absent in GPR18 deficient mice [124].

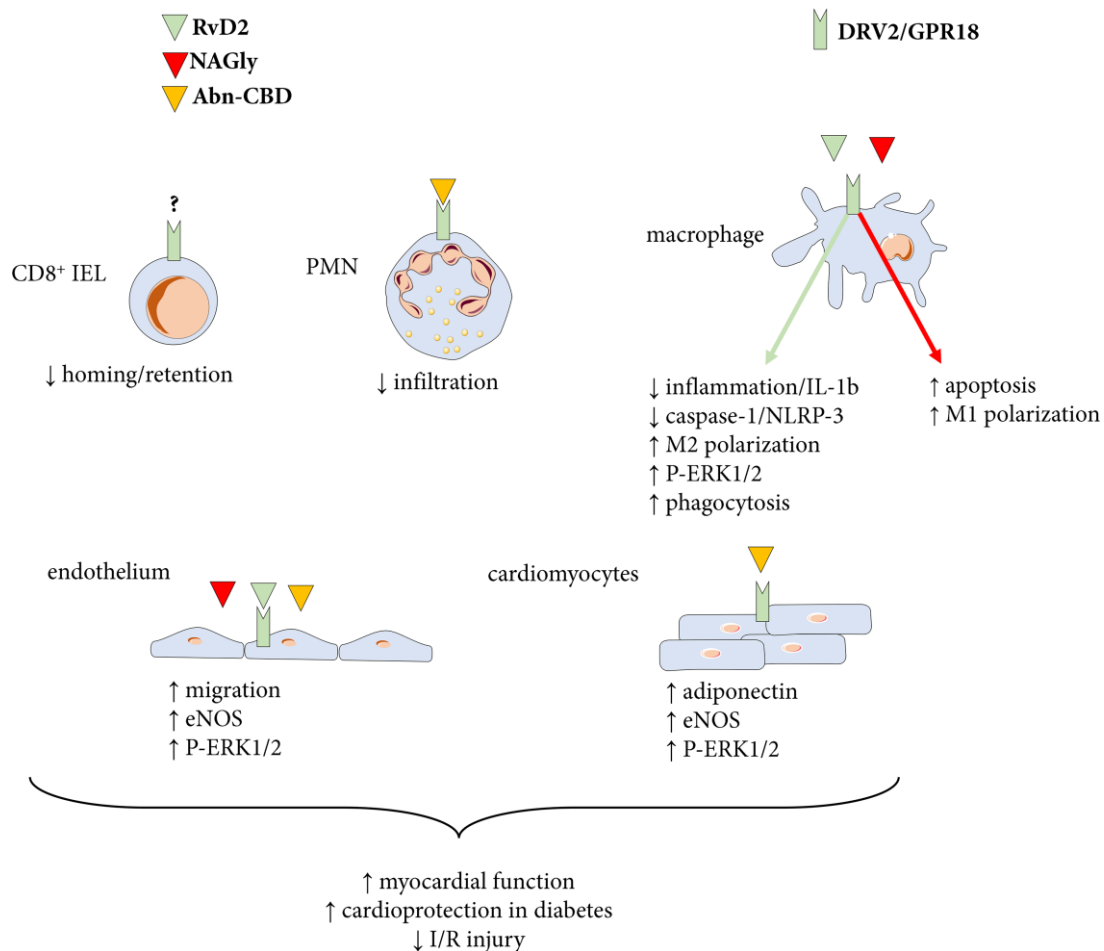


Figure 10: Effects of GPR18 ligands on different cell types.

Top: effect of the different GPR18 ligands of immune cells. Fixation of Abn-CBD through GPR18 on PMN decreased their infiltration in the tissues. Fixation of RvD2 through GPR18 on macrophages decreased inflammation and decrease the activation of caspase1/NLRP-3 axis while increasing phosphorylation of ERK1/2 and M2 polarization. Fixation of NAGly through GPR18 on macrophages increased their apoptosis and M1 polarization. Bottom: effect of the ligands on endothelium (left) and cardiomyocytes (right). Fixation of these 3 ligands through GPR18 on endothelial cells induced an increase in migration, eNOS production and phosphorylation of ERK1/2 whereas fixation of Abn-CBD on cardiomyocytes increased their production of adiponectin, eNOS and phosphorylation of ERK1/2 [114].

Using VMSCs isolated from the human greater saphenous vein Miyahara *et al.*, showed that RvD2 treatment attenuates VSMCs proliferation and migration but also acts to reduce pro-inflammatory gene expression in VSMCs [125]. A model of balloon angioplasty on rabbit femoral arteries showed that RvD2 treatment modulated the acute vascular injury response by reducing leukocytes infiltration and inflammatory gene expression (TNF $\alpha$ , MCP-1, IL-1 $\alpha$ ). The



same treatment also showed a capacity to reduce ROS production, and attenuated neointimal hyperplasia following balloon injury in the femoral arteries [125].

A study showed that ApoE<sup>-/-</sup> mice fed a high fat diet for 4 weeks, 8 weeks or 4 months had reduced SPM concentrations in aortic extracts but higher levels of pro-inflammatory mediators increasing with time on this diet. With the decline of maresine-1 (MaR1) and RvD2 during atheroprogession these two compounds were tested as an experimental treatment. This combination treatment using RvD2 and MaR1 was able to prevent atheroprogession in this model and induced a pro-resolving macrophage phenotype [36].

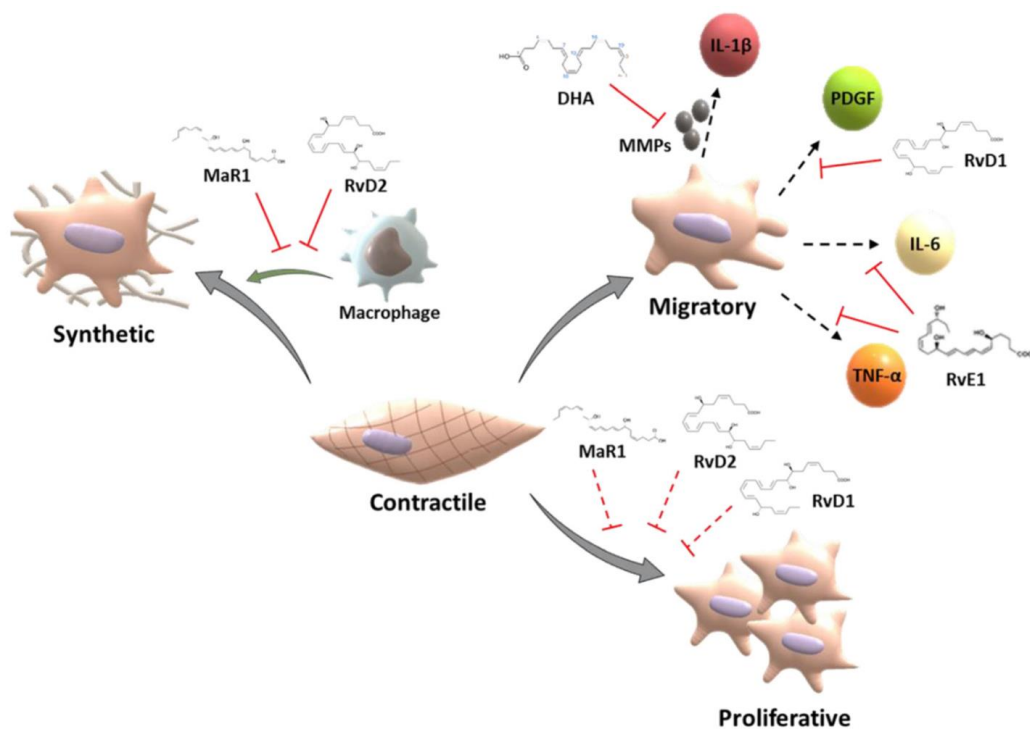


Figure 11: SPMs effects on vascular smooth muscle cells.

*Adapted from Carracedo & al. Illustration showing actions of different SPMs on synthetic, proliferative and migratory VSMCs. [126]*

### b. ChemR23/RvE1

ChemR23 is a GPCR coupled to the G protein G<sub>i/o</sub>. ChemR23 activation leads to the inhibition of the adenylyl cyclase and is followed by an accumulation of cAMP, intracellular calcium release and phosphorylation of mitogen-activated protein kinases (MAPK) [127,128].

ChemR23 expression has been shown on several cell types including macrophages [129], dendritic cells [130], natural killer cells [131], endothelial cells [132] and VSMCs [94]. ChemR23 was first described as a chemerin receptor [127], the receptor was later shown to have a similar affinity for RvE1 [133].

Studies showed that RvE1 inhibits VSMC calcification and protects against atherosclerosis development [126]. RvE1 also promotes macrophage polarization into M2 macrophages by inducing expression of a cluster of differentiation (CD) 36, CD163, CD206 and Arginase-1 [134] (Figure 12).

In atherosclerosis, through ChemR23, RvE1 modifies macrophage behaviour [135] and reduces lesion development [136]. In ApoE<sup>-/-</sup> mice fed a high fat diet ChemR23 deletion lead to increased atherosclerotic lesion size [135]. Administration of a RvE1 treatment in a mouse model in the context of intimal hyperplasia induced by femoral wire injury reduced neointima formation after arterial injury by actively promoting M2 macrophage polarization [134,137].

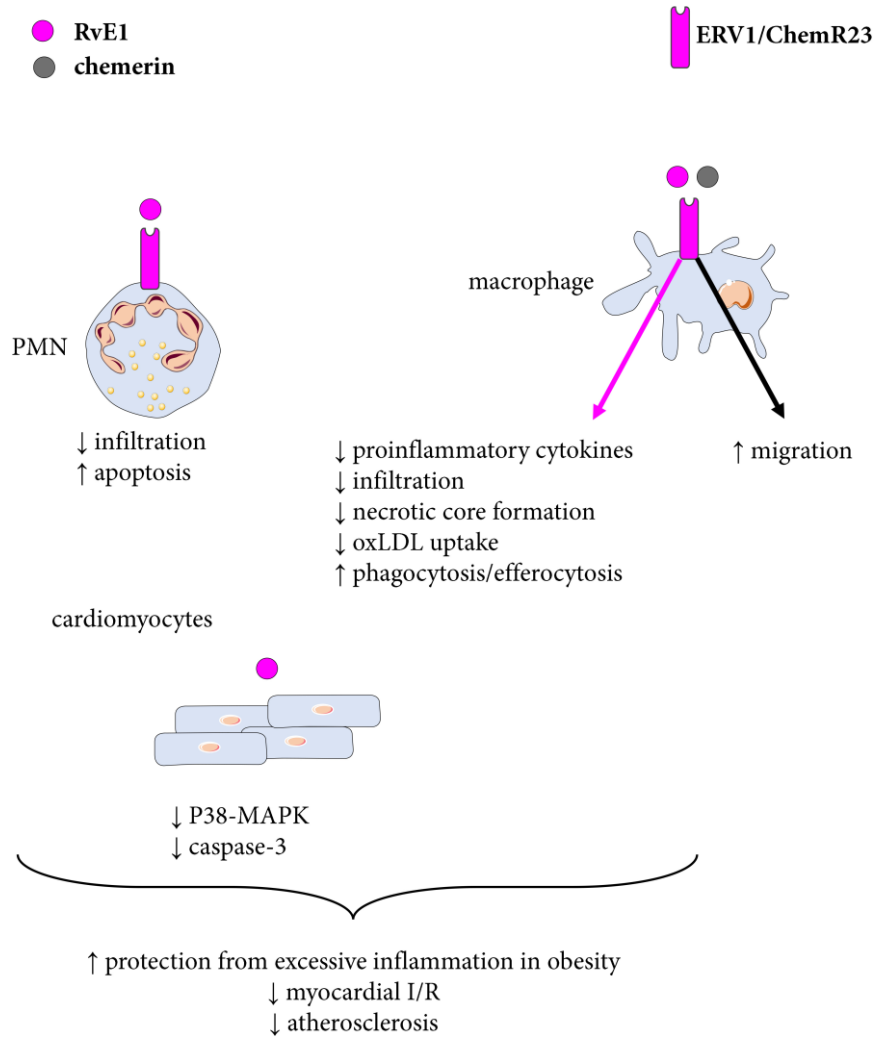


Figure 12: Effects of ChemR23 ligands on different cell types.

*Top: effect of the different ChemR23 ligands on immune cells. Fixation of RvE1 on PNM decreased their infiltration in tissues and increased their apoptosis whereas fixation of RvE1 on macrophages increased their phagocytic and efferocytic capacities and decreased their production of pro-inflammatory cytokines, their infiltration into tissues, oxLDL uptake and necrotic core formation. Fixation of chemerin through ChemR23 on macrophages increased their migration. Fixation of RvE1 on cardiomyocytes decreased the caspase-3 and P38-MAPK axis [114].*

## II. Hypothesis and objectives

In the past, observation of the Inuit population showed that they were less likely to develop cardiovascular diseases. This observation was linked to a high intake in fish in which there is an important amount of n-3 PUFA. It is now well established that n-3 PUFA and especially pro-resolving lipid mediators derived from them have a beneficial effect on cardiovascular system ageing. Several of these SPMs have been studied and shown beneficial effects *in vivo* and *in vitro* on cardiovascular system ageing and in limiting associated diseases development as well as implicated mechanisms.

We hypothesized that RvD2 and its receptor, GPR18, may play a protective role in the early stages of atherogenesis.

The aim of this work was to determine the impact of RvD2 on mechanisms involved in atherosclerosis development.

More specifically, the objectives of the study were:

- I. To explore the impact of a RvD2 treatment on various mechanisms involved in atherosclerosis development using human vascular smooth muscle cells.
- II. To establish the impact of a RvD2 treatment on early atherosclerosis development in a mouse model of early vascular ageing.
- III. To determine the expression of GPR18 and the presence of RvD2 in human coronary arteries from healthy donors and donors with atherosclerosis.

### **III. Methods**

#### **1. *In vitro* experiments**

##### **a. Cells and treatments**

Human aortic VSMCs were obtained from Lonza, cultivated in the specific medium for these cells also obtained from Lonza. The cells were used between passage 5 and 8.

RvD2 (solubilized in ethanol) was obtained from Cayman Chemicals while O-1918 was obtained from Tocris and reconstituted in absolute ethanol before use. PDGF-BB was obtained from Sigma and reconstituted in PBS.

##### **b. Immunofluorescence**

Human aortic VSMCs were fixed with 4% PFA; mice thoracic aortas were fixed with 4% PFA, embedded with OCT and cut in 5  $\mu$ M thick section before performing immunofluorescence staining using the following protocol.

Sections were washed with buffer (0.1% TBS-T) for 5 minutes before adding blocking solution for 30 minutes (5% BSA diluted in buffer). Primary antibody was diluted in blocking solution and incubated overnight at 4°C. Sections are then washed 3 times for 5 minutes with buffer before incubation with secondary antibody (1:300) diluted in blocking solution. Sections were washed 3 times with buffer before DAPI staining and mounting with aqueous medium.

##### **c. Seahorse experiments**

VSMCs cellular oxidative respiration was measured with the Seahorse XFp bioenergetic assay using the Seahorse Cell Mito Stress Test Kit (Agilent Technologies). Human aortic VSMCs were seeded onto a XFp microplate and let to adhere overnight. The next day medium is removed and changed for the specific medium assay and treated of not with RvD2 100 nM. The OCR was measured at a steady state, after which the machine sequentially added oligomycin (1  $\mu$ M), carbonyl cyanide 4- [trifluoro methoxy] phenylhydrazone (FCCP; 0.5  $\mu$ M), and a mixture of rotenone (0.5  $\mu$ M) and Antimycin A (1  $\mu$ M) into the wells to obtain the maximal and non-mitochondrial respiration rates according to the manufacturer's instructions (Figure 13A).

Glucose consumption was assayed using the Glycolytic rate assay, with injection of a mixture of rotenone (0.5  $\mu\text{M}$ ) and Antimycin A (1  $\mu\text{M}$ ) and 2-DG (Figure 13B).

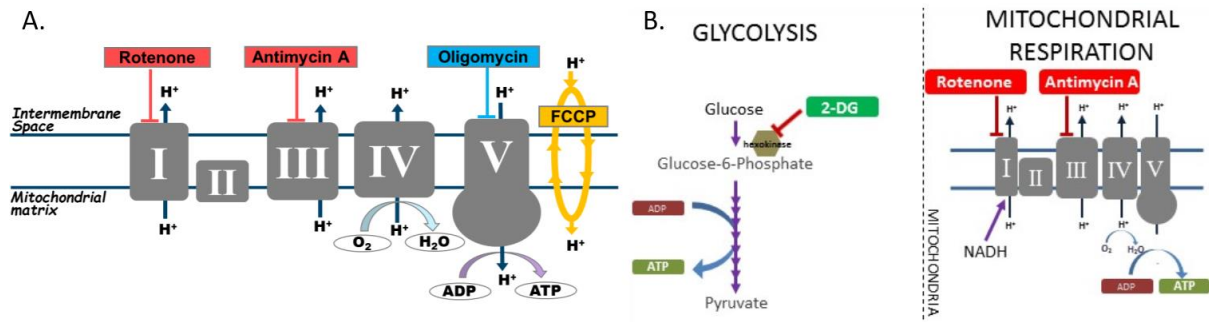


Figure 13: Illustration showing action of the different inhibitors used for metabolism assay. Action of the inhibitors used for the cell mito stress kit (A) and for the glycolytic rate assay (B); © Agilent technologies.

#### d. Senescent erythrocytes obtention

Blood was drawn from healthy volunteers and anti-coagulated with heparin. Erythrocytes are prepared using the following protocol. Blood was centrifuged at 120 g for 15 minutes then supernatant was discarded, and erythrocytes only were kept. Erythrocytes pellet was then suspended in 2% dextran (vol/vol) for 15 min before supernatant was discarded and pellet washed 4 times with 0.1% penicillin/streptomycin. Erythrocytes were then suspended in 0.1% glucose in HBSS and incubated at 37°C for at least 5 days. Senescence was checked by flow cytometry using Annexin V and CD235a to check population purity.

#### e. Senescent erythrocytes uptake

Human aortic VSMCs were seeded in 8-well labtek at 7500 cells per well. The next day 10<sup>6</sup> senescent erythrocytes or fresh erythrocytes (negative control) were added in each well and incubated for 48 hours. Wells are then washed 5 times with HBSS before staining using DAB and observation under a light microscope.

#### f. ROS production measurement

Human aortic VSMCs were seeded in a black 96-well plate with clear flat bottom at 5000 cells per well. The next day, VSMCs were pre-treated with RvD2 (100 nM) during 15 minutes before

adding  $10^6$  senescent erythrocytes per well and incubate with treatment for 48 hours. After 2 days, the 5 mM CM-H2DCFDA probe was diluted into OPTI-MEM to obtain a 5  $\mu$ M solution. Cell culture medium was discarded and well washed before adding 100  $\mu$ L of CM-H2DCFDA at 5  $\mu$ M per well, TBHP and CM-H2DCFDA-containing OPTI-MEM was used for positive control. Cells were incubated at 37°C for 45 minutes in the dark before discarding solution and washing. Fluorescence intensity was then read using a microplate reader (Ex 480 nm; Em 530 nm).

### **g. Migration assay**

Migration assays were performed using the kit Oris™ Cell migration assay. Human aortic VSMCs were seed in a 96-well plate with cell seeding stoppers at the density of 15000 cells per well. Cells were let to adhere overnight before adding treatments, PDGF-BB 10 ng/mL and/or RvD2 100 nM and cells seeding stoppers are removed. After 24 hours cells are fixed with 4% PFA, stained with 0.5% crystal violet before observation under a light microscope with the detection mask from the kit. Cells within the migration area are then counted.

## **2. *In vivo* experiments**

### **a. Authorization and treatments**

Animals' studies were carried out accordance with guidelines from the Directive 2010/63/EU of the European Parliament and were approved by the Ethical Committee of Nancy and by the French government. ApoE<sup>-/-</sup> mice on a C57Bl/6J were obtained from Taconic Biosciences (USA) and used at the aged of 8 weeks after 2 weeks of acclimatation into the animal facility. These mice, known for their early vascular ageing and early atherosclerotic lesion development, were fed a high fat (21%) +1.25% cholesterol obtained from Snniff (Germany) for 4 weeks on the purpose to enhance atherosclerotic lesion formation. All along these 4 weeks mice were intraperitoneally injected 3 times a week with 100  $\mu$ L of saline solution containing ethanol only for the control group, RvD2 at the dose of 100 ng per mouse for the RvD2 group, O-1918 at 2 mg/mL for the O-1918 group and O-1918 + RvD2 injected at the same time at the same concentration used for the previous group for the O-1918 + RvD2 group. O-1918 was chosen as a GPR18 antagonist as it was previously shown to block RvD2 protective effect in a model of

cerebral ischemia reperfusion [138].

GPR18 knockout mice were obtained from Taconic Biosciences (USA).

RvD2 (solubilized in ethanol) was obtained from Cayman Chemicals while O-1918 was obtained from Tocris and reconstituted in absolute ethanol before use.

### **b. Sample collection**

Animal were anesthetized with 4% isoflurane.

Blood was drawn from the carotid and anticoagulated with citrate. Whole blood was centrifuged at 190 g for 10 minutes at 20°C then at 2 000 g for 10 minutes at 20°C. Plasma was transferred into another tube before being centrifuged at 13 000 g for 30 minutes at 4°C. Plasma was aliquoted and stored at -80°C until use.

After the second centrifugation red blood cells were kept for use in another experiment.

Abdominals aortas and spleen were cleaned on ice cold saline solution before being snap frozen in liquid nitrogen and stored at -80°C. Thoracic aortas were cleaned and fix in 4% PFA for 24 hours. The next day the aortas were washed 3 times in PBS to eliminate all the PFA and then stored at 4°C.

The heart is cut in two parts; heart apex was cut and snap frozen in liquid nitrogen while aortic root was embedded in OCT and froze for later analysis (Figure 14).



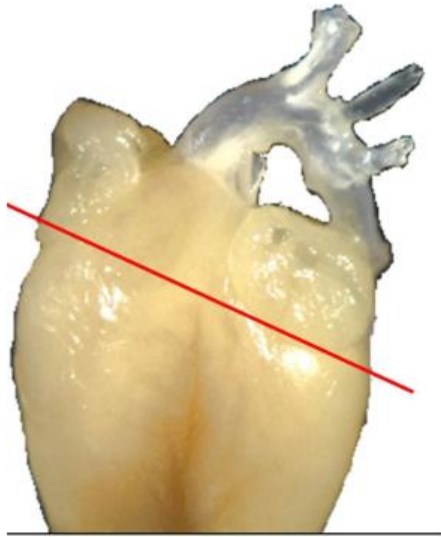


Figure 14: Photography showing how to cut heart for aortic root sectioning.

*Photography showing how to cut the heart (red line) before OCT embedding for aortic root sectioning and analysis (image obtained from DAUGHERTY LAB, Saha Cardiovascular Research Center, University of Kentucky).*

#### **c. Blood analysis**

Blood count was performed on this total blood using an automated haematology analyser ABXmicros60 (Horiba). Food was taken away 6 hours before measuring triglycerides levels using a glucose meter (Accutrend® Plus mmol/L) capable to measure triglycerides and lactate using specialized strips after a small incision at animals' tail. Cholesterol levels were measured in mice plasma using ELISA assay (MyBiosource, San Diego, USA, item MBS269999), mice were fed ad libidum before blood was drawn.

#### **d. Phagocytosis assay**

Mice were intraperitoneally washed by injecting 5 mL PBS to obtain peritoneal cells. Supernatant was centrifuged and cells were resuspended in 1 mL of medium before being counted. Cells were then plated in a black 96-well plate with clear bottom at  $10^5$  cells per well. 24 hours later cells were treated with FITC-zymosan (Invitrogen) ( $10^6$  particles per  $10^5$  cells) during 45 minutes before washing with PBS. Cells were then fixed with 4% PFA for 15 minutes. Nucleus were stained with DAPI (Sigma). Cells were observed under a fluorescence microscope.

Fluorescence quantification was performed with ImageJ software.

#### **e. Atherosclerotic lesion detection**

Thoracic aortas from mice are used to study atherosclerotic lesion formation. En face opening was performed before the staining. Oil Red-O is solubilized in isopropanol (0.3 g for 10 mL) and filtrated. Working solution is prepared by a mix of oil Red-O:water (6:4). To stain them, aortas were put 5 minutes into 70% ethanol then 15 minutes into oil Red-O stain. Aortas were washed in 70% ethanol 5 minutes then 10 minutes into distilled water before cleaning the fat left outside the vessel. After aortas were put between a slide and a coverslip with aqueous mounting medium

#### **f. RNA extraction and retro-transcription**

Total RNAs were extracted from mice spleen and abdominal aorta and purified using a RNeasy mini kit (Qiagen). Samples were prepare using the following protocol.

Samples were placed into labelled tubes and 700  $\mu$ L of QIAzol was added in each tube. Tubes are placed into the tissue lyzer and the tissues are disrupted 2 to 3 times at 30 Hz until complete dissolution. Tube content was transferred to a new tube before adding 140  $\mu$ L of chloroform. Tubes were vigorously agitated for 15 seconds before being centrifuged at 12000 g for 15 minutes at 4°C. the top clear layer is transferred to a new tube before RNA purification. Samples are mixed with 1 volume of 70% EtOH before being transferred in purification columns. Columns are centrifuged, flow through discarded and wash with 350  $\mu$ L of RW1 buffer before incubation with DNase mix. Columns are then washed with 350  $\mu$ L of RW1 buffer, 2 times with 500  $\mu$ L of RPE buffer before adding of 30  $\mu$ L of RNA free water. RNA concentration is measured using a nanodrop.

RNAs are then retro transcribed into cDNA and stored at 4°C before use.

#### **g. Real-Time PCR**

Real-Time quantitative PCR was performed on 384-well plate using 20 ng of cDNA and was performed on a 7900HT Fast Real-Time PCR system (Thermo fisher Scientific) with TaqMan

Assay-on-Demand probes (Thermo Fisher Scientific).

### h. Oxidative Stress

Reactive Oxygen Species production into mice erythrocytes was evaluated using EPR spectroscopy. Whole blood was collected from the carotid and anti-coagulated with citrate. Whole blood was centrifuged, and erythrocytes pellet was then washed 3 times with PBS-/- (followed each time by centrifugation at 1000 g, 10 minutes, 4°C). Incubations with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (200 μM, Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany) were performed to evaluate the production of reactive oxygen species. In brief, 5 μl of washed erythrocytes were added to 1 mL CMH:KREBS/HEPES solution, (1:1, v/v), mixed well, and incubated for 30 minutes at 37°C with gentle shaking. Incubations were stopped by freezing the samples on dry ice. Samples were stored at -80°C until ROS production was quantified with electron paramagnetic resonance (EPR) using Bruker E-Scan M system (Bruker, Billerica MA, USA) as previously with the following settings: center field 1.99 g, microwave power 1 mW, modulation amplitude 9 G, sweep time 10 seconds, number of scans 10, field sweep 60 G. The EPR spectrums are expressed as fold change from baseline.

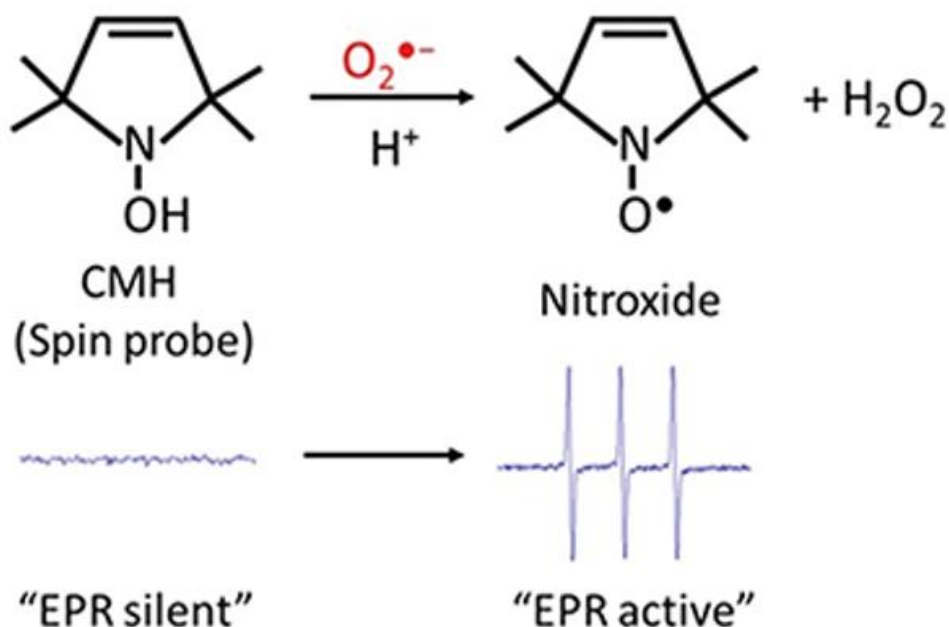


Figure 15: Image depicting the functioning of EPR spectroscopy.

*Illustration showing how the CMH probe react with ROS and can be use to measure oxidative stress using EPR spectroscopy [139].*

**i. Aortic root sectioning**

Heart was cut as seen in figure 16A before being embedded in OCT. These aortic roots embedded in OCT were sectioned using a cryostat CryoStar™ NX70 (Thermo Scientific) in 10 μM thick sections.

OCT block were mount on the specimen holder, while cutting sections were check under a light microscope. The first sections are only heart muscle (Figure 17A), while progressing in sectioning sections were regularly checked under the microscope. First the “worms” will appear (Figure 17B) followed by the apparition of two compartments (Figure 17C). The vessel wall will become thicker meaning cups are getting closer. When the first cups appears you can count this point as zero and adjust cutting angle to have the three cups at the same level (Figure 17D).

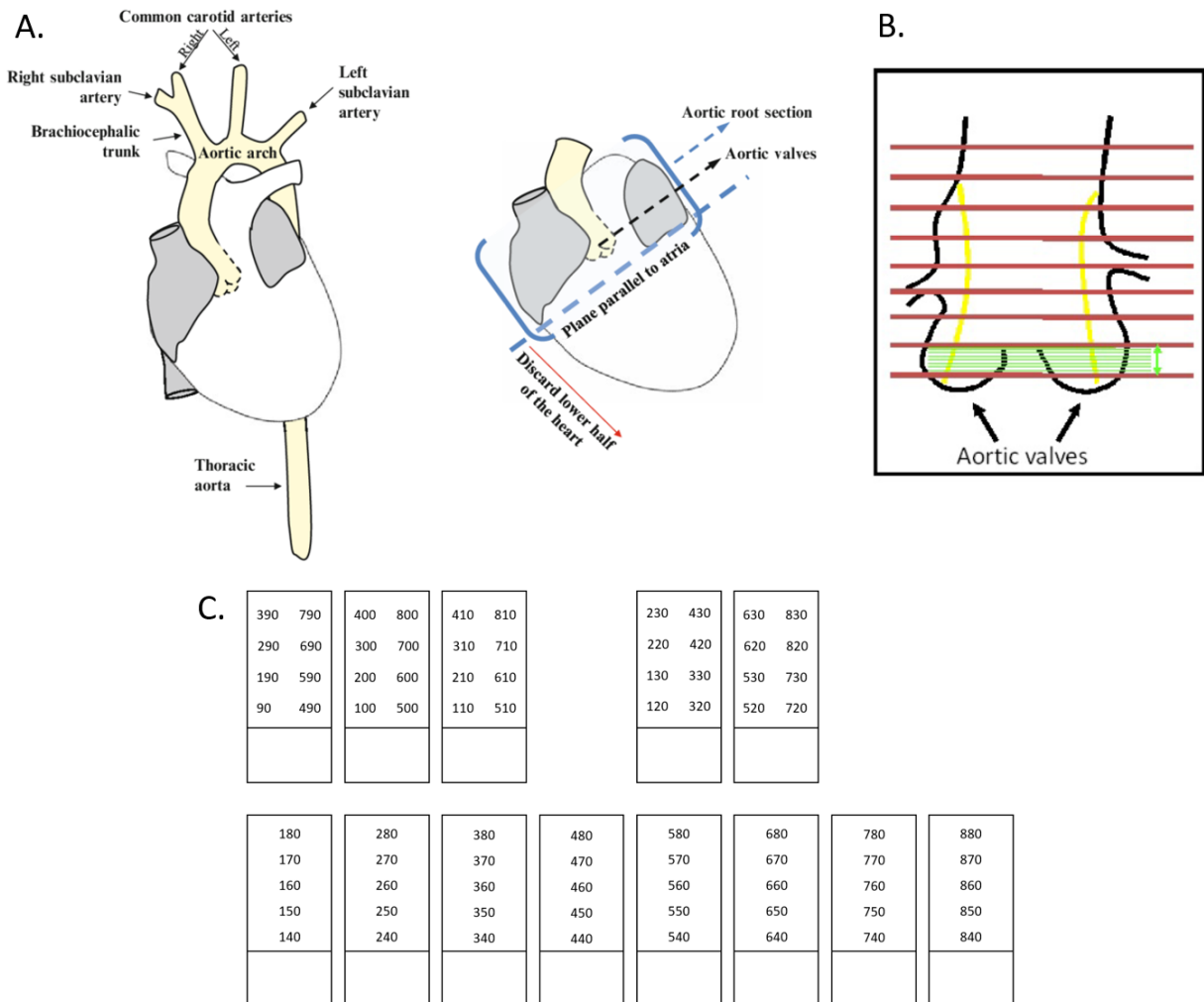


Figure 16: Aortic root sectioning.

*Illustration showing how heart must be prepared before OCT embedding for aortic root sectioning (A), how the aortic root will be cut, red lines represent 100  $\mu$ M (B) and section distributions on the different slides (C).*

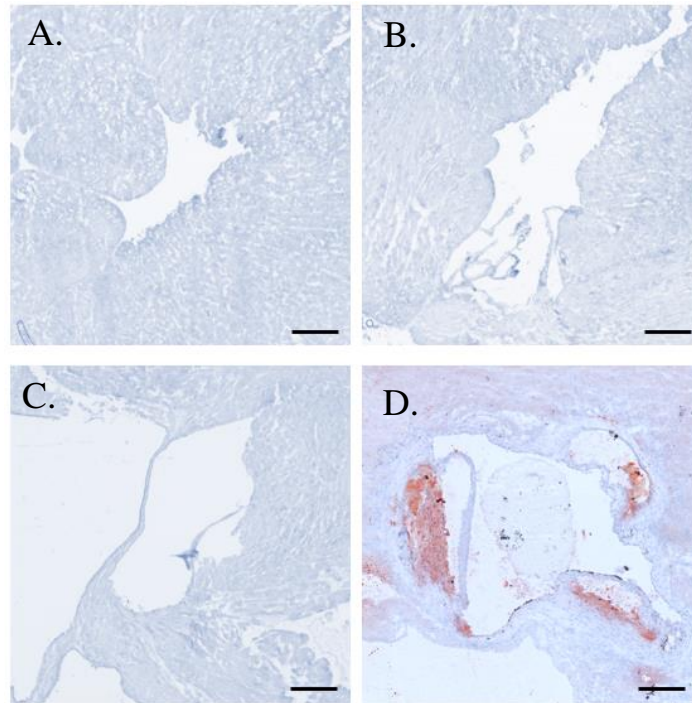


Figure 17: Photography of sectioned heart at different levels during aortic root sectioning. *On the beginning of the sectioning only myocardium is present (A). While progressing in the heart the “worms” appears and will later form the aorta and the pulmonary artery (B). The aorta will form a close compartment (C), finally the three cups will be present (D). Scale = 200  $\mu$ m.*

#### **j. Aortic Root Oil Red-O staining**

Saturated Oil Red-O solution was prepared by adding 1 g of powder in 100 mL of isopropanol. Working solution was then prepared by mixing 24 mL of saturated solution with 16 mL of distilled water. Sections used for Oil Red-O staining were fixed 20 minutes in 4% PFA before being washed with distilled water. Slides were stained with Oil Red-O working solution for 15 minutes before being washed 3 times for 5 minutes in water and counter stained with Haematoxylin QS for 45 seconds. Sections were then rinsed in warm tap water until nuclei become blue and mounted with aqueous mounting medium.

### **k. Evaluation of atherosclerotic lesions**

Atherosclerotic lesion in the aortic arch was determined by en face analysis. Dissected aortic arches were fixed in 4% PFA, cut longitudinally and stained with Oil Red-O solution (Sigma-Aldrich). Images were acquired using a light microscope (Nikon Eclipse Ci). Total area of the aortic arch and area of the atherosclerotic lesions were quantified using ImageJ (NIH).

Aortic roots were embedded in OCT (Leica) and frozen at  $-80^{\circ}\text{C}$ . 10  $\mu\text{m}$  thick cryosections were cut in serial and were collection starting from the aortic valve cusps and fixed either with 4% PFA or acetone. PFA fixed section were collected every 100  $\mu\text{m}$  from the aortic valve cusps were stained with Oil Red-O (Sigma-Aldrich). Images were acquired with a light microscope equipped with a DC300 camera (Leica).

Aortic root lesions were determined in a blinded fashion using ImageJ (NIH) and expressed as lesion surface area relative to total aortic root surface. The area of necrotic cores, defined as unstained zones devoid of cells or extracellular matrix, was measured in a blinded fashion using ImageJ (NIH). Lesion composition was analysed using immunohistochemistry in lesion-matched sections. In both cases, ie aortic root lesion area and necrotic core area, exclusive criteria were decided as followed: if two or more sections were missing between 200 and 600  $\mu\text{m}$  mice were excluded, if one section was missing value was imputed by the mean of the previous and the following value.

### **l. Immunostainings**

Sections used for immunohistochemistry were fixed 5 minutes in acetone before being store at  $-80^{\circ}\text{C}$  until use. Immunohistochemistry on acetone fixed frozen sections was performed using the ABC-method using the following protocol. Sections were circled with a wax pen and washed with buffer (0.1% TBS-T) before quenching of endogenous peroxidase using 0.3%  $\text{H}_2\text{O}_2$  for 30 minutes. Sections were washed before incubation with Avidin then Biotin solution (Vector Labs) for 15 minutes each. Blocking solution (5% horse serum diluted in buffer) was added for 30 minutes before adding of primary antibody incubated overnight at  $4^{\circ}\text{C}$ . Slides were washed with buffer before adding secondary antibody for 1 hour at room temperature. Sections were then washed with buffer before incubation with Avidin-Biotin-Complex-Po/AP 30 minutes at room temperature. After washing sections were stained using DAB for 7 minutes at room temperature and counter stained with Haematoxylin Qs for 45 seconds. Slides were

rinsed in “warm” tap water until nuclei became blue then dehydrated and mounted with medium.

### **m. Lesion composition**

Lesion composition in macrophages subtypes was studied using macrophages markers. Sections were incubated with antibodies against CD68 (1:20 000; Sorotec), CD206 (1:50; BioRad), Arginase-1 (1:50, Abcam), iNOS (1:100, Abcam) and isotype (1:100 Serotec).

## **3. Human studies**

### **a. Samples obtention**

Paraffin embedded samples and conditioned medium from human coronary arteries were a generous gift from Professor Jean-Baptiste Michel.

Post-mortem human coronary artery walls, including healthy and pathology at different stages of atherosclerotic diseases were collected from the Inserm human CV biobank (BB-0033-00029, U 1148, X. Bichat hospital, Paris), included in the European network BBMRI-ERIC, in accordance with the French regular and ethical rules (BioMedicine Agency convention DC2018-3141) and the principles of the declaration of Helsinki. Approval was obtained from the French Biomedical Agency (ABM, PFS09-007 & PFS17-002) and the Institutional Ethical Review board (SC09-09-66). Tissues were obtained from deceased organ donors for kidney and/or hepatic transplantation, in the absence of therapeutic uses for the heart. For immunohistochemistry, human arterial tissues were fixed with 4% (w/v) buffered formaldehyde solution prepared by depolymerisation of paraformaldehyde and imbedded in paraffin. Serial 5  $\mu\text{m}$  thick sections were performed.

Conditioned media were prepared from small pieces of tissue (about 2  $\text{mm}^3$ ) were weighed and incubated for 24 hours in RPMI culture medium containing 1% L-glutamine, 1% penicillin, streptomycin, and amphotericin at 37°C (5% CO<sub>2</sub>) and without serum. For standardization, the volume of medium was adjusted to sample wet weight (6 mL per gram of tissue). Conditioned media were collected, centrifuged (14 000 g for 15 minutes at 4°C), and stored at -80°C until further analysis as previously described [140].

## **b. ELISA Assay**

Resolvin E1 (Abbkine) and D2 (Cayman Chemicals) ELISA measurements in conditioned medium from human coronary arteries were performed according to manufacturer instructions. Results were then read between 405 and 420 nm using a microplate reader.

## **c. Immunohistochemistry**

Sections used were 5 µm thick. Immunohistochemistry on paraffin sections was performed using the ABC-method using the following protocol (see III.A.12.)

Immunohistochemistry was performed using primary antibodies against GPR18 (1:100; Bioss Antibodies), ChemR23 (1:100; Abcam), CD68 (1:8 000; Abcam) and αSMA (1:100; Sigma).

## **4. Statistical analysis**

Data are presented as mean ± SEM. GraphPad Prism 8.0.2 was used for data analysis and figure preparation. Differences between two groups were analysed using unpaired student *t*-test and paired *t*-test for erythrocytes ROS comparison. Differences between four groups were analysed using one-way ANOVA with Tukey correction for multiple comparison. Atherosclerotic lesion in thoracic aorta were analysed using Kruskal-Wallis test. To test for differences in aortic root lesion area and necrotic cores area two-way ANOVA followed by Tukey multiple comparison test was used. Significance was accepted at  $P < 0.05$ . Phagocytosis assay was analyzed using linear regression and comparison between the groups was performed by multilinear regression. Chi square was used to evaluate difference between group in human immunohistochemistry classification.



## IV. Results

### 1. Effect of resolvin D2 on VSMCs

#### a. GPR18 expression

GPR18 was shown to be expressed on primary cultured human aortic VSMCs (figure 18A). Thoracic aortas isolated from mice were also used to observe GPR18 expression. GPR18 was found to be expressed in the media of these aorta (figure 18B), i.e., the part of the aorta containing elastic laminae and the VSMCs.

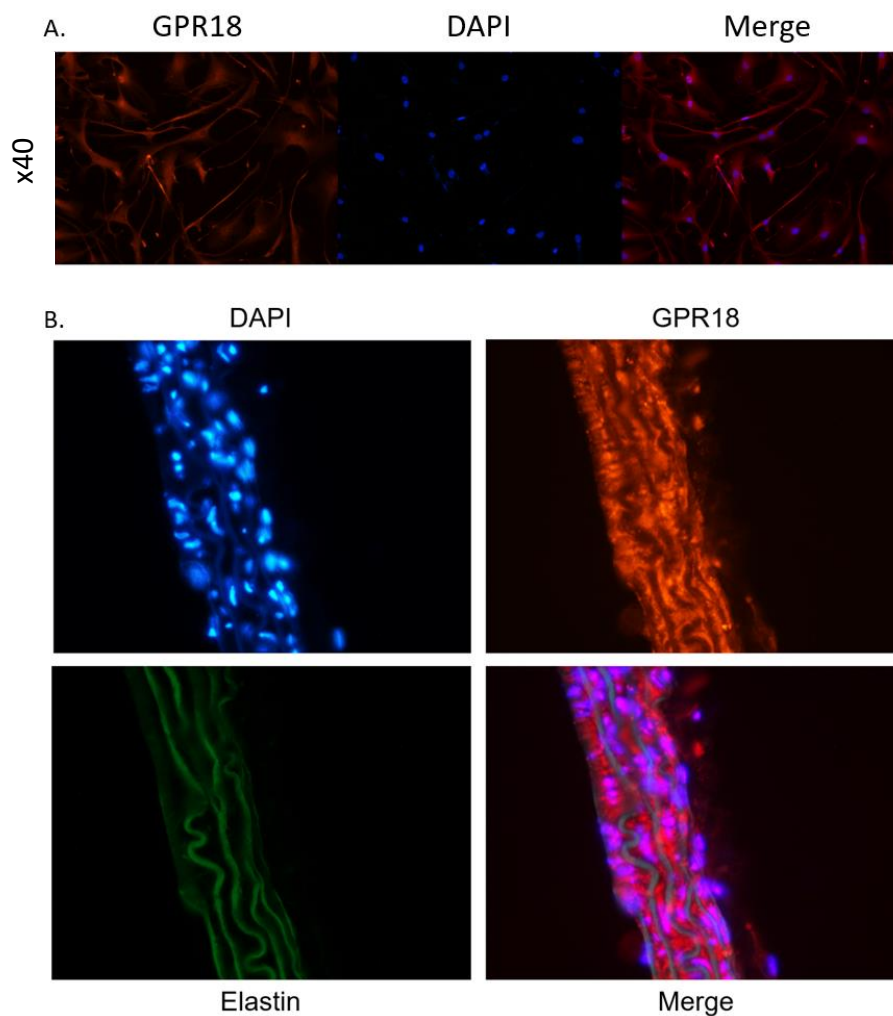


Figure 18: GPR18 expression.

*Representative immunofluorescence micrographs of human aortic VSMCs (A) and mice thoracic aorta showing GPR18 expression (B).*

## b. Effect of RvD2 on human VSMCs metabolism

Evidence indicates that n-3 PUFA and molecules derived from these have an effect on metabolism. Proliferative VSMCs were treated or not with RvD2 100 nM and their metabolism was measured using Seahorse technology.

Using mitochondrial respiratory chain inhibitors cells oxygen consumption rate (OCR) and extracellular acidification rate (data not shown) were measured. RvD2 treatment did not induce any significant modifications in respiratory capacity of the cells (Figure 19 top). Glycolysis of the cells was measured using the same device and inhibitor of the mitochondrial respiratory chain and an inhibitor of the enzyme involved in glycolysis. Glycolytic capacity was similar in the presence and absence of RvD2 (Figure 19 bottom).

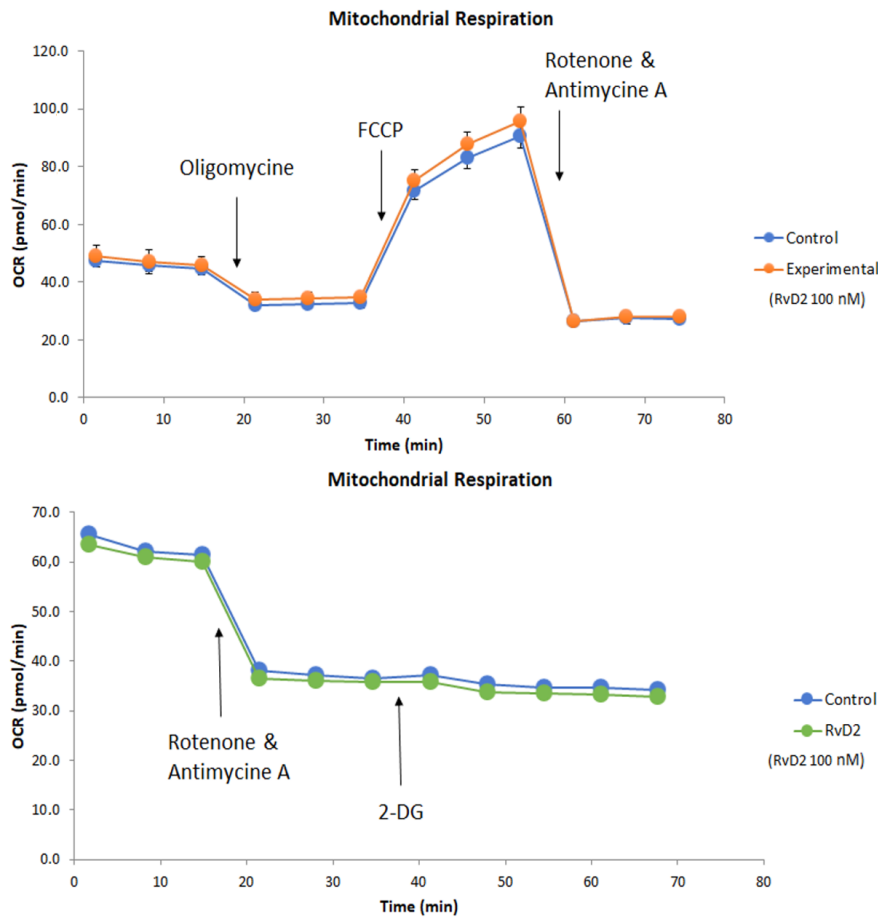


Figure 19: Metabolism measurement using Seahorse assay.

*Top: measurement of OCR using the cell mito stress assay kit (n=3). Bottom: OCR measurement using the glycolytic rate assay (n=2).*

### c. Senescent erythrocytes uptake

Among mechanisms involved in atherosclerosis lesion development, oxidative stress has been shown to play a key role. Due to their presence in mature lesions and to their peroxidase-like activity erythrocytes have been shown to play a role in this oxidative stress induced lesion development. In order to study the oxidative stress induced by erythrocytes in mature atherosclerotic lesions, erythrocyte uptake by human aortic VSMCs was first assayed. Staining using the peroxidase like activity showed no staining in cells not incubated with erythrocytes, neither in the cells incubated for 48 hours with fresh erythrocytes. Cells incubated 48 hours with senescent erythrocytes showed an internalization of the erythrocytes (Figure 20).

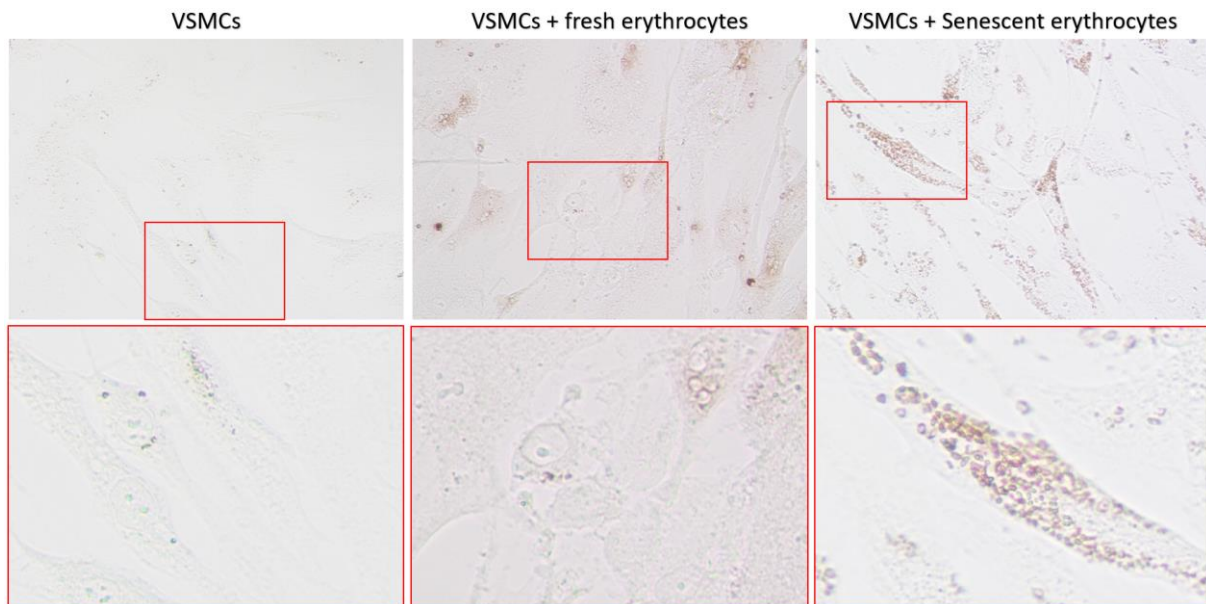


Figure 20: Senescent erythrocytes uptake by human aortic VSMCs.

*Representative image of VSMCs stained after 48 hours of incubation with senescent erythrocytes (right), fresh erythrocytes (middle) and VSMCs without erythrocytes (left). In red are shown the zoomed area of each condition.*

### d. Evaluation of ROS production by VSMCs

As erythrocytes are known to induce oxidative stress in atherosclerotic lesions, senescent erythrocytes (SE) were used *in vitro* to reproduce this effect on human aortic VSMCs. After showing that SE were internalized by the human aortic VSMCs, ROS production induced by their internalization was studied. Cells were incubated 48 hours before incubation with the CM-

H2DCFDA probe to reveal ROS. TBHP was used as an oxidative positive control. Interestingly in this experiment we failed to induce oxidative stress by SE internalization as previously shown [85]. ROS production was not significantly different in the absence or presence of RvD2 (100 nM) (Figure 21).

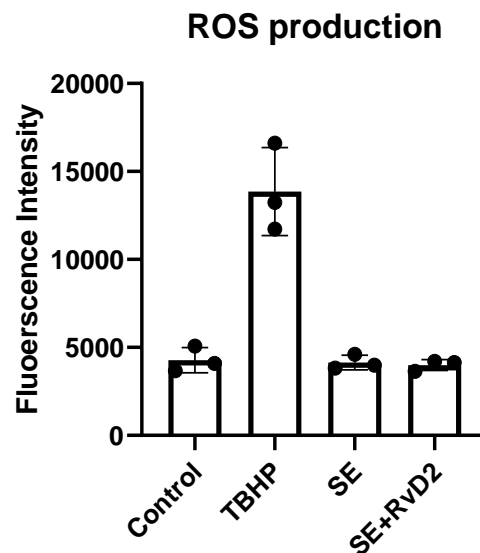


Figure 21: ROS production induced by senescent erythrocytes in VSMCs.

*Graph showing ROS production measurement in VSMCs. TBHP is used as a positive control. Our experiment did not show any significant changes in ROS production with the different treatments (n=3).*

#### **e. Effect of RvD2 on VSMC migration**

With atherosclerosis development a phenotypic switch is observed in VSMCs switching from a contractile to a synthetic phenotype characterized among others by an increased migratory capacity. Human aortic VSMCs migratory capacity was studied using cells seeded in a 96-well plate. Cells were treated with PDGF-BB (10 ng/mL) and/or RvD2 (100 nM). Left panel of figure 22 shows representative images of the migration area. PDGF-BB was used to induce cell migration in comparison to the control. Investigating the effect of RvD2 on these cells showed a trend of the treatment alone to induce cell migration but in combination with PDGF-BB a trend of decreased migration in comparison to PDGF-BB alone was observed (Figure 22 right panel). However, due to spreading of the number of cells in the migration area results were not significant.

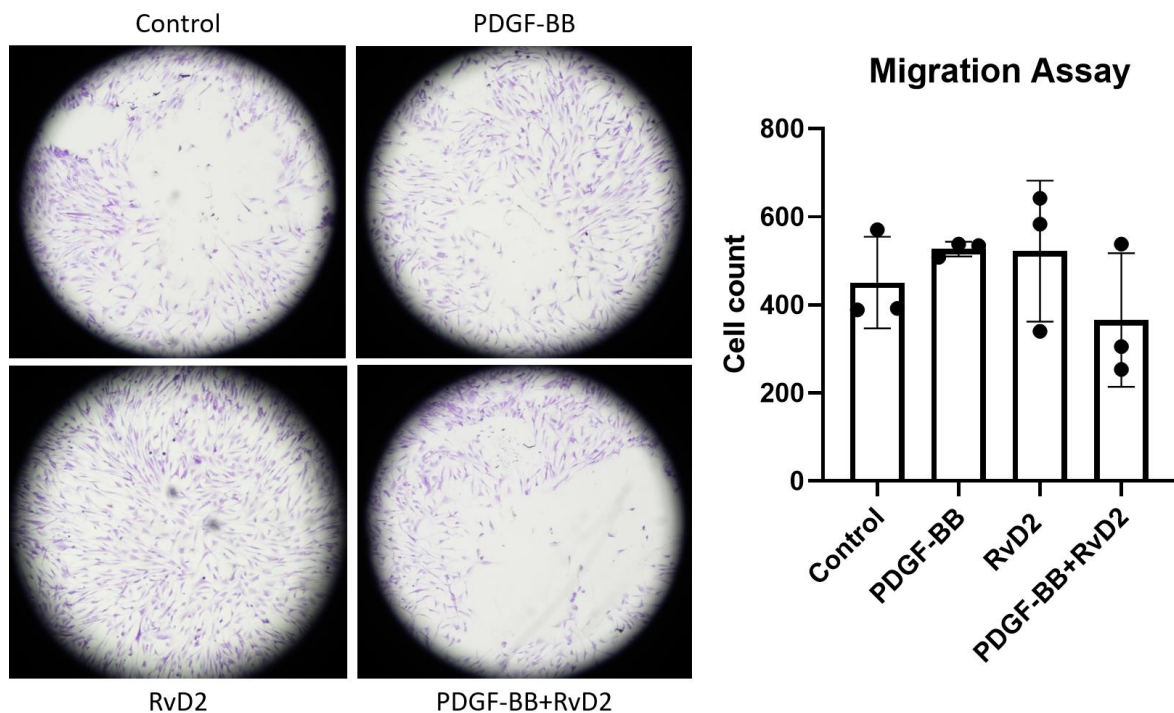


Figure 22: Human aortic VSMCs migration assay.

*Representative images of migration areas for each treatment (left), diagram shows the results of 3 independent experiments (right). Statistical analysis was performed using a one-way ANOVA test.*

## 2. Effect of rRvD2 on atherosclerosis development in ApoE<sup>-/-</sup> mice

### a. Weight gain induced by the diet

The ApoE<sup>-/-</sup> mice were fed a high fat diet enriched in cholesterol. Mice were weighed every week starting from the beginning of the protocol until the end. Weight was expressed as a gain in grams from day zero at each weighing. Statistical analysis showed no significant differences between the different groups at each weighing day. These results indicate that a 4 weeks treatment with RvD2 and/or O-1918 did not influence the weight of ApoE<sup>-/-</sup> mice fed a high fat diet (Figure 23).

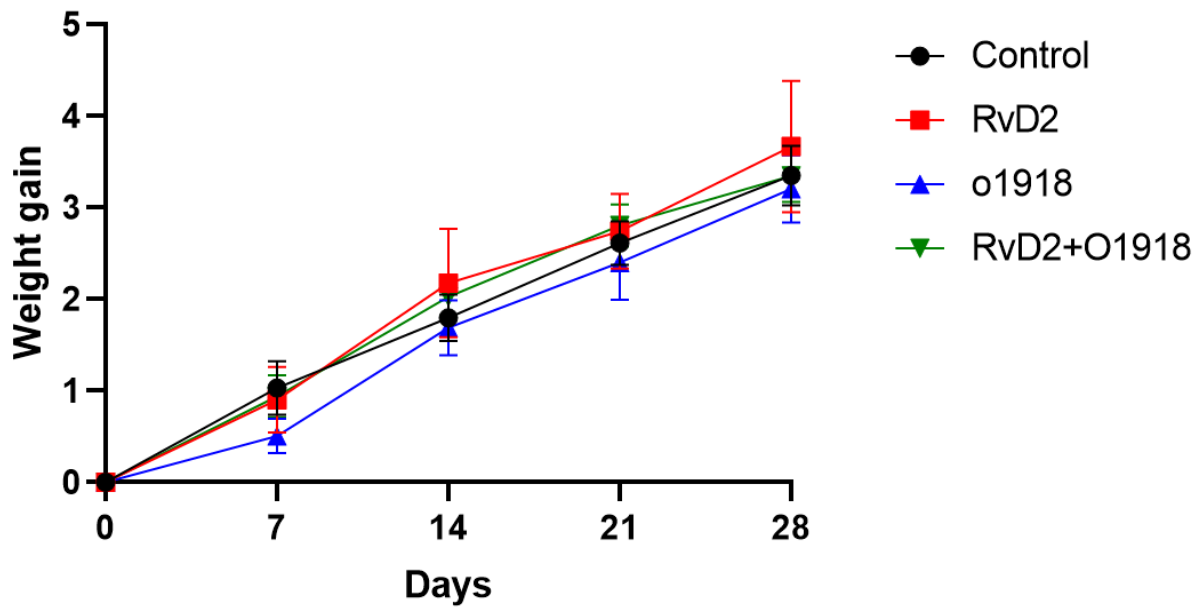


Figure 23: ApoE<sup>-/-</sup> mice weight.

Mice weights were expressed as weight gain on weighing day compared to the first day of the protocol (n=16). Statistical analysis was performed using a two-way ANOVA test.

#### b. ApoE<sup>-/-</sup> mice blood analysis

ApoE<sup>-/-</sup> mice blood under each treatment did not differ for erythrocytes, platelet, immune cells count, or any other parameter measured. These results indicate that our treatment did not induce any modification in blood cell composition (Table 1).

|  | Control     | RvD2        | O-1918      | O-1918+RvD2 |
|--|-------------|-------------|-------------|-------------|
| White blood cells - 10 <sup>3</sup> /mm <sup>3</sup> | 5.46 ± 0.82 | 6.37 ± 1.58 | 5.48 ± 0.71 | 5.98 ± 1.36 |
| Red blood cells - 10 <sup>6</sup> /mm <sup>3</sup>   | 8.01 ± 0.15 | 7.72 ± 0.2  | 7.82 ± 0.19 | 8.04 ± 0.23 |
| Haemoglobin - g/dl                                   | 11.7 ± 0.22 | 11.5 ± 0.21 | 11.5 ± 0.26 | 11.7 ± 0.33 |
| Haematocrit - %                                      | 36.6 ± 0.72 | 36.2 ± 0.71 | 35.5 ± 0.8  | 36.9 ± 1.1  |
| Platelet - 10 <sup>3</sup> /mm <sup>3</sup>          | 525 ± 38.97 | 544 ± 24.1  | 500 ± 39.1  | 494 ± 32.86 |
| MCV - μm <sup>3</sup>                                | 45.8 ± 0.14 | 46.8 ± 0.58 | 46.1 ± 0.2  | 46.0 ± 0.16 |
| MCHC - g/dl  | 32.0 ± 0.15 | 31.9 ± 0.14 | 32.0 ± 0.08 | 31.8 ± 0.12 |
| MPV - μm <sup>3</sup>                                | 6.45 ± 0.6  | 5.62 ± 0.25 | 5.38 ± 0.32 | 5.30 ± 0.21 |
| Lymphocytes - 10 <sup>3</sup> /mm <sup>3</sup>       | 3.63 ± 0.36 | 3.66 ± 0.34 | 4.13 ± 0.55 | 4.76 ± 1.19 |
| Monocytes - 10 <sup>3</sup> /mm <sup>3</sup>         | 0.31 ± 0.07 | 0.25 ± 0.05 | 0.36 ± 0.08 | 0.33 ± 0.09 |
| Granulocytes - 10 <sup>3</sup> /mm <sup>3</sup>      | 0.98 ± 0.17 | 0.76 ± 0.09 | 0.84 ± 0.09 | 0.89 ± 0.12 |

Table 1: Treatment did not induce modifications in ApoE<sup>-/-</sup> mice blood count.

Treatments did not alter blood count in ApoE<sup>-/-</sup> mice. Values expressed as mean ± SEM. n=16

per group. MCV: Mean corpuscular volume; MCHC: Mean corpuscular haemoglobin concentration; MPV: Mean platelet volume. Statistical analysis was performed using a one-way ANOVA test.

Cholesterol levels were measured at the end of the experiments. Neither GPR18 activation with RvD2 nor inhibition with O-1918 did not modify cholesterol levels (Figure 24A). After 3 weeks of treatment with RvD2 and/or O-1918 ApoE<sup>-/-</sup> mice fed with a high fat diet, circulating TG levels were measured. GPR18 activation or inhibition with RvD2 or O-1918 did not modify TG levels after 3 weeks of treatment (Figure 24B).

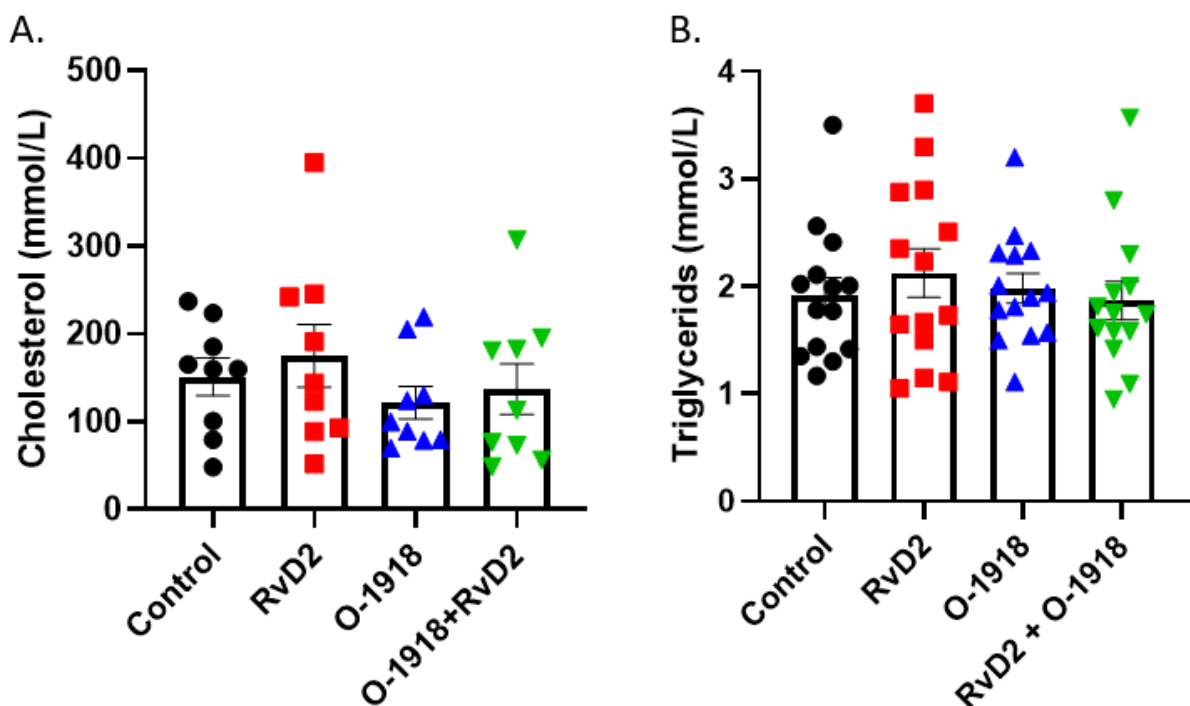


Figure 24: Treatments did not affect cholesterol and triglycerides levels in circulating blood. (A) Cholesterol measurement was performed on plasma using ELISA assay (n=9). (B) Triglycerides levels were measured in circulating blood using strips and the Acutrend ® Plus device (n=14). Statistical analysis was performed using a one-way ANOVA test.

### c. Peritoneal macrophages phagocytosis

Phagocytosis and clearance of apoptotic cells is one of the main function of macrophages. With lesions development, macrophages exhibited an impaired phagocytic capacity. Due to a variation in the number of cells obtained from the different mouse batches, the FITC fluorescence

for all technical repeats (n=6 per mouse) were plotted against the number of cells in each evaluated area. Representative images are shown in figure 25A.

The linear regression for controls (n=60 from n=10 mice, adjusted  $r^2=0.94$ ) had a slope of 0.51, whereas the linear regression for RvD2-treated mice (n=60 from n=10 mice, adjusted  $r^2=0.80$ ) had a slope of 0.95 (Figure 25B). Multilinear regression determined that phagocytosis ( $P=0.009$ ) but not cell number ( $P=0.16$ ) significantly predicted the control vs RvD2 groups from a linear combination of the independent variables. The treatment with a combination of RvD2 with O1918 reverted regression to the control (slope: 0.55, adjusted  $r^2=0.79$ , n=60 from n=10 mice).

Phagocytosis of peritoneal macrophages derived from WT and GPR18<sup>-/-</sup> mice was performed in the presence of RvD2 in culture media. There was a trend for the separation of the two groups but analysis by multiple linear regression was not able to detect any significant differences due to a low number of mice used for this preliminary experiment (Figure 25C).



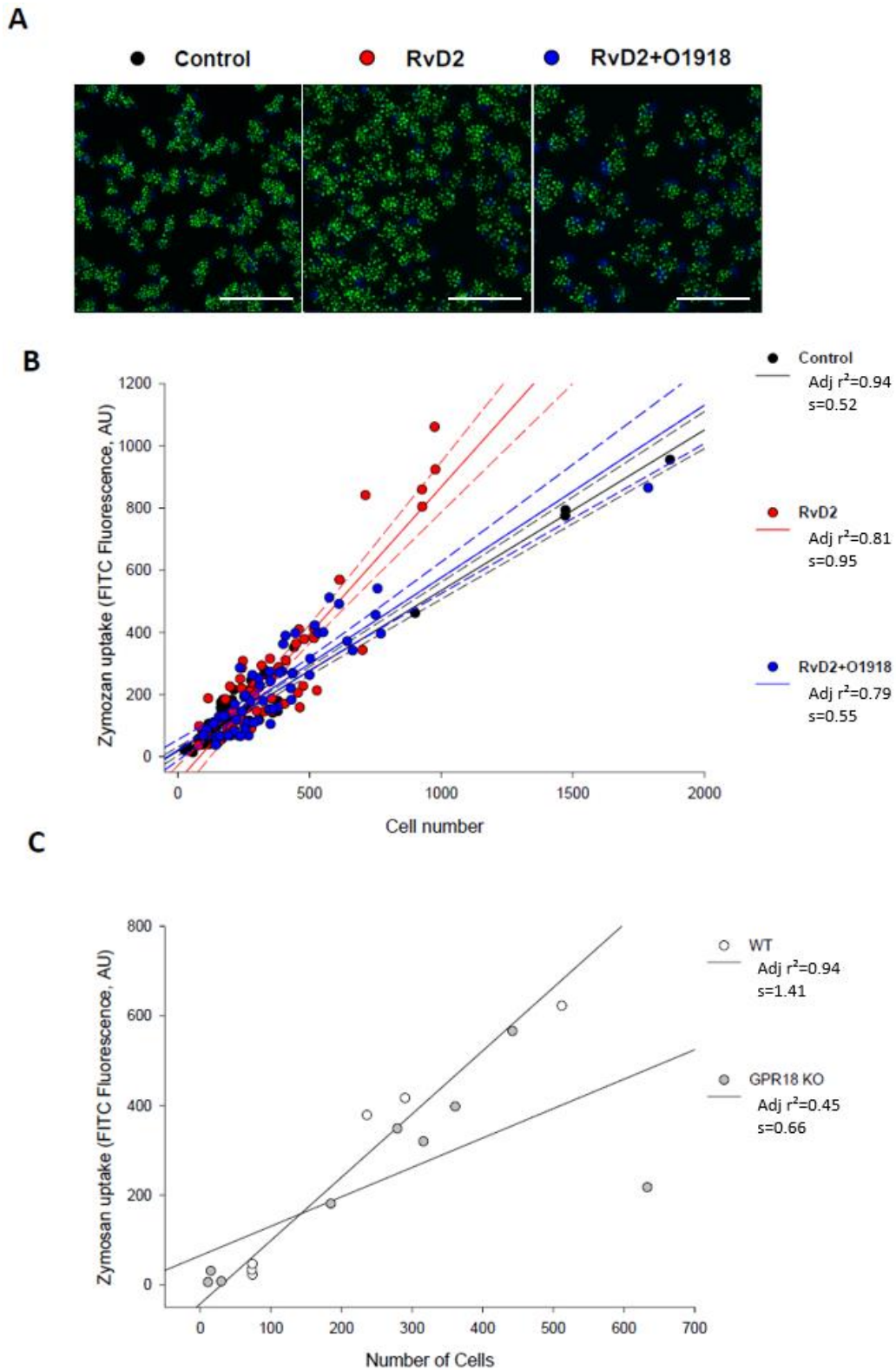


Figure 25: Peritoneal macrophages phagocytosis assay.

Representative images of peritoneal macrophages after zymosan-FITC label particles phagocytosis (A). phagocytosis results expressed as fluorescence intensity in function of cell number for  $ApoE^{-/-}$  mice;  $n=10$  mice per group (B) and for peritoneal macrophages from  $GPR18^{+/+}$  and  $GPR18^{-/-}$  treated with RvD2;  $n=2-3$  mice per group (C). Scale = 50  $\mu\text{m}$ .

#### d. Erythrocyte ROS production

Erythrocytes are major contributors to ROS production triggering vascular ageing and play a key role in atherosclerosis development. RvD2 treatment induced a significant decreased of ROS production in erythrocytes compared to erythrocytes obtained from control mice was observed (Figure 26A, top panel). O-1918 and combined treatment did not induce any significant effect on ROS production in mice erythrocytes (Figure 26A, bottom panel), representative curves are shown in figure 26B.

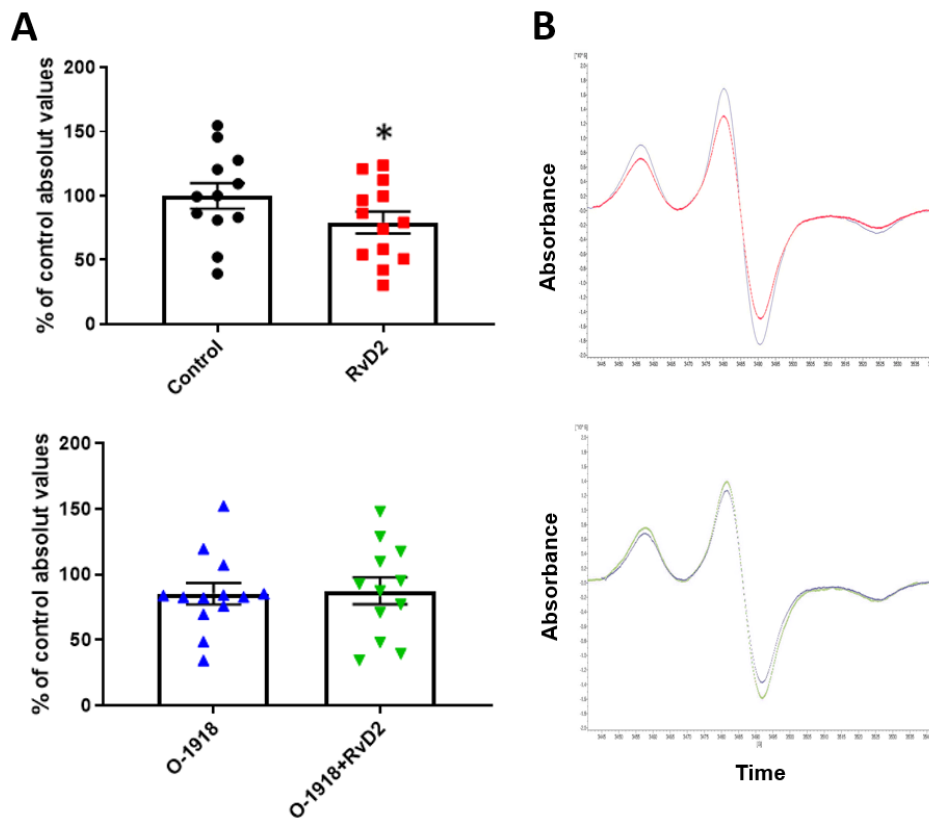


Figure 26: ROS produced by erythrocytes obtained from ApoE<sup>-/-</sup>.

*Erythrocytes were incubated with the CMH-hydrochloride probe and ROS were measured using EPR spectroscopy, results are presented in left panel, n=11-12 per group. Representative curves obtained are presented in right panel. Statistical analysis was performed using paired t-test.*

## e. Atherosclerotic lesion development

### i. Atherosclerotic lesion development in the aortic root

Evaluation of the RvD2 effect on atherosclerotic lesion development was evaluated in the aortic root of ApoE<sup>-/-</sup> mice. Figure 27A shows results of atherosclerosis quantification in the aortic root. Mice treated with either RvD2 or O-1918 exhibited a significant decrease in atherosclerosis development in comparison to the control group (Figure 27A). Group separation in absence and in presence of O-1918 showed a decrease in atherosclerosis development in mice treated with RvD2 alone compared to the control group (Figure 27B, left panel, P<0.001). Mice treated with the combined treatment of RvD2 and O-1918 did not exhibit any significant differences in atherosclerotic lesion development compared to mice treated with O-1918 alone (Figure 27B, right panel, P=0.417).

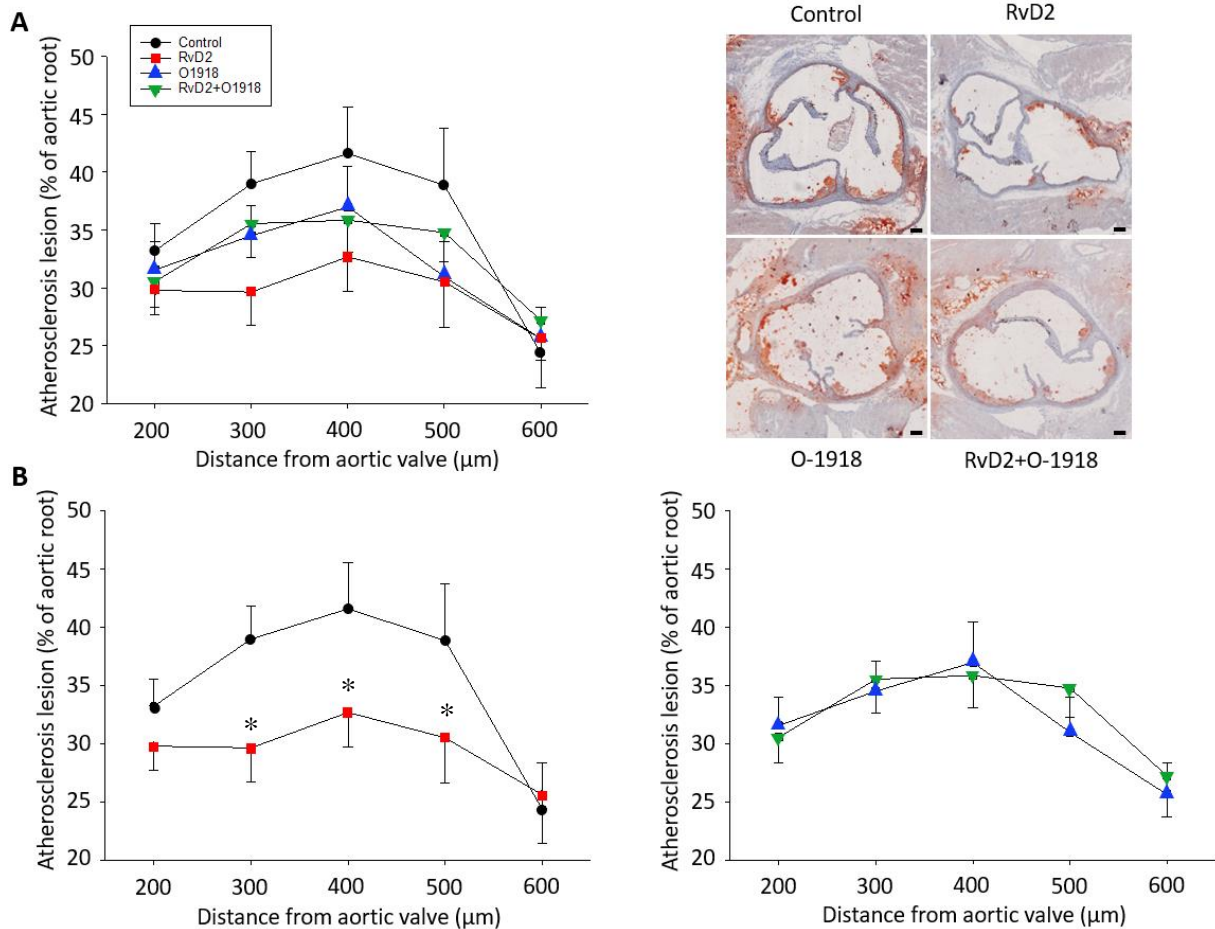


Figure 27: Resolvin D2 decrease aortic root atherosclerotic lesion development in ApoE<sup>-/-</sup> mice. *Quantification of aortic root lesion (A, left panel) and representative images of aortic root*

*lesions in the 400  $\mu\text{m}$  level (A, right panel). Separation of curves from the mice treated without O-1918 (B, left panel) and the groups of mice treated with O-1918 (B, right panel). n=15-16 per group. Scale = 100  $\mu\text{m}$ . Statistical analysis was performed using a two-way ANOVA test followed by Tukey multiple comparison test.*

## **ii. Necrotic area in lesion from the aortic root**

With atherosclerotic lesions becoming bigger as the disease progresses the area formed of dead cells, the necrotic cores, appears. As we showed in the previous part that RvD2 treated mice exhibited smaller atherosclerotic lesions in comparison to control mice, necrotic core areas in the lesions were measured. Figure 28A (left panel) shows the result of necrotic core quantification in the lesions of the aortic root, the right panel shows representative images of the lesion with necrotic cores, i.e., part of the lesion without cells or ECM. Separation of the groups of mice treated or not with O-1918 shows that mice treated with RvD2 exhibited a significantly lower necrotic surface in their lesion compared to control mice (Figure 28B, left panel,  $P=0.0112$ ), whereas mice treated with the combined treatment of RvD2 and O-1918 did not exhibit any differences in necrotic area size compared to mice treated with O-1918 alone (Figure 28B, right panel,  $P=0.5912$ ).

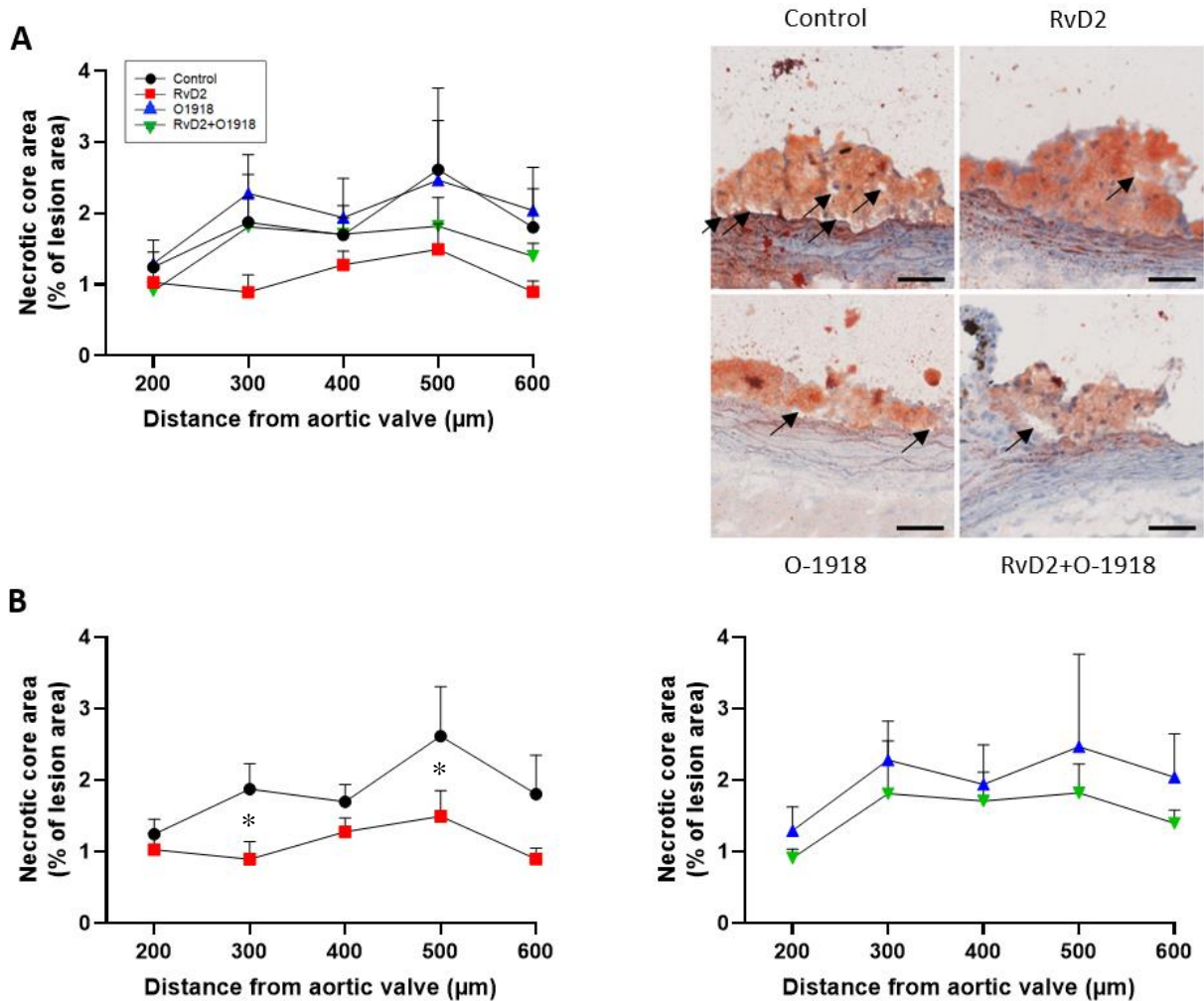


Figure 28: Resolvin D2 treatment decrease necrotic cores area in aortic root lesions in ApoE<sup>-/-</sup> mice.

Quantification of necrotic core area in the lesions of aortic root (A, left panel) and representative images of necrotic core areas in the 400 µm level, black arrows indicate necrotic core (A, right panel), scale = 50 µm. Separation of curves from the mice treated without O-1918 (B, left panel) and the groups of mice treated with O-1918 (B, right panel); n=11-14 per group. Statistical analysis was performed using a two-way ANOVA test followed by Tukey multiple comparison test.

### iii. Atherosclerotic lesion development in the thoracic aorta

To investigate the effect of the RvD2 on atherosclerotic lesion development we measured plaques in the thoracic aorta of our ApoE<sup>-/-</sup> mice. Figure 29A shows representative images of the thoracic aorta of our mice stained to detect atherosclerotic lesions. In the descending aorta lesions were small. Atherosclerotic lesions quantified in the thoracic aorta covered

0.858±0.167% of the vascular wall in the control group with a trend to lower lesion proportions in the RvD2-treated group (0.774±0.102%, P=0.602). O-1918 treatment did not induce any significant effect but a trend to increase lesion proportion (1.057±0.215%, P=0.205) and the combined treatment a trend in decreasing lesion proportion (0.604±0.071%, P=0.453). Figure 29B shows the results of the quantification of the lesion surface in the descending aorta.

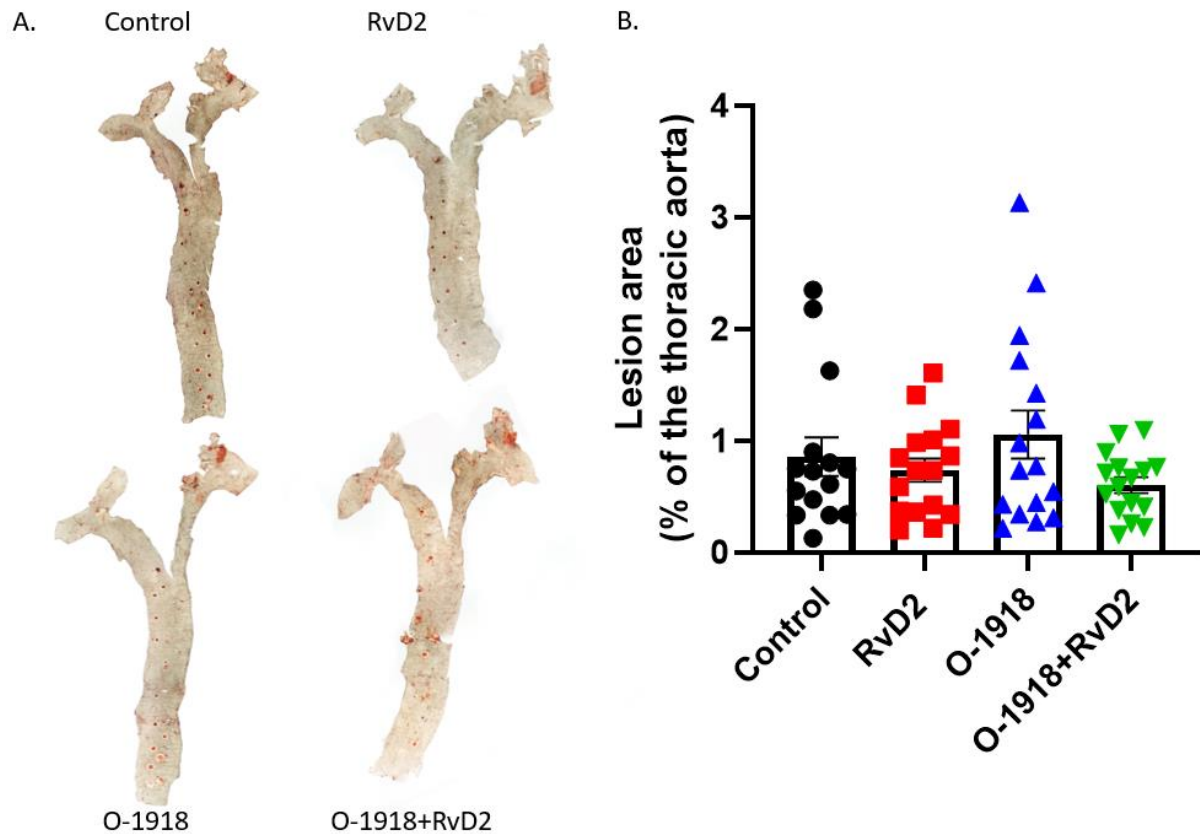


Figure 29: Resolvin D2 treatment did not affect early atherosclerotic lesion development in the thoracic aorta in ApoE<sup>-/-</sup> mice.

Representative images of the thoracic aorta after en face opening and Oil Red-O staining (A). Data from the lesion area in the thoracic aorta presented as mean±SEM. n=15-16 per group (B). Statistical analysis was performed using Kruskal-Wallis test.

#### f. Atherosclerotic lesion composition

Specialized pro-resolving lipid mediators are able to act on macrophage polarization and being able to induce a phenotypic switch from a pro-inflammatory subtype (M1) to a pro-resolving subtype (M2); marker expression of these two subtypes have been investigated in aortic root

lesions. CD68 and iNOS were used to detect pro-inflammatory macrophages (M1 subtype) inside the lesions whereas the arginase-1 and CD206 were used to detect the pro-resolving macrophages (M2 subtype).

Expression of these markers was quantified in aortic root lesions, results obtained in control group for each marker are shown in figure 30B. Representative images are shown in figure 30A. Quantification of iNOS mRNA expression in mouse aorta showed a decrease in iNOS mRNA expression in RvD2 treated mice *versus* control mice (Figure 30C), and no effect of RvD2 on iNOS mRNA expression in presence of O-1918 (figure 30D).

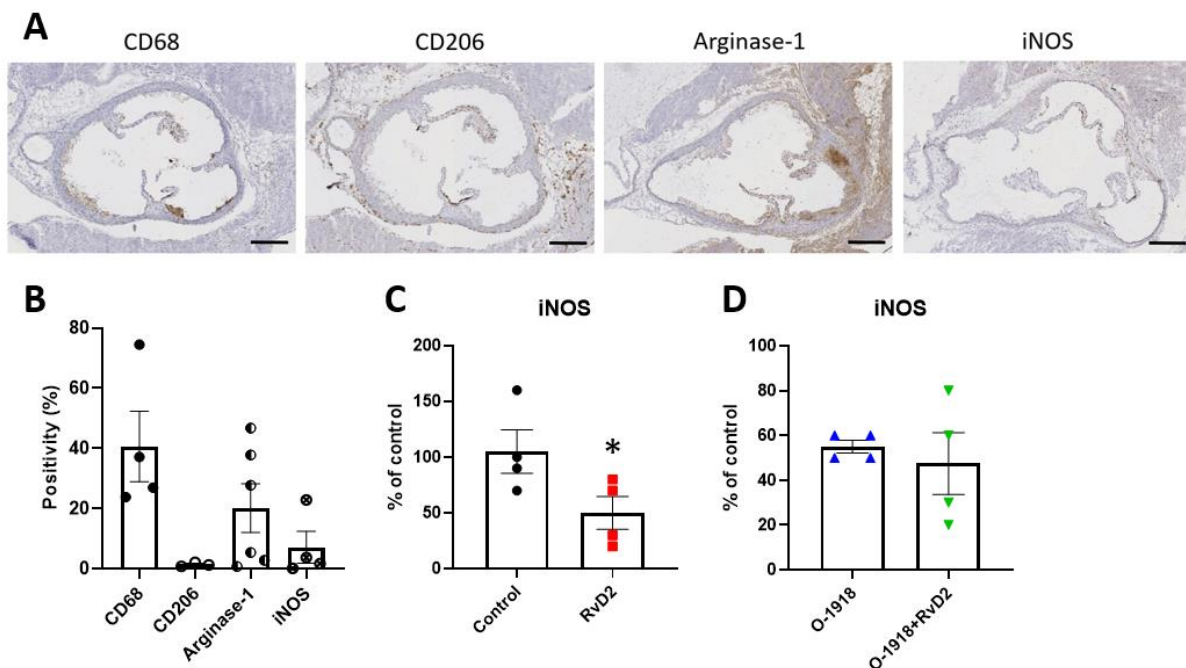


Figure 30: Macrophages subtypes in aortic root lesions.

(A) representative images obtained from immunohistochemistry for each marker in control group; scale = 250  $\mu$ m (B) quantification of the surface stained in aortic root lesion for each marker in control group. (C-D) Real-Time quantitative PCR against iNOS in ApoE<sup>-/-</sup> mice aorta.

### g. Marker expression in the spleen

Inflammation being a hallmark of atherogenesis and having previously demonstrated in this manuscript that RvD2 treatment decreased atherosclerosis development in ApoE<sup>-/-</sup> mice fed a high fat diet, inflammatory marker expression was studied by Real-Time quantitative PCR in mice spleens. RvD2 treatment did not induce any significant modification of inflammatory marker expression in mice spleens, neither did the other treatments (Figure 31A - C). Neither

RvD2, O-1918 nor the combined treatment induced modification of GPR18 expression (Figure 31D).

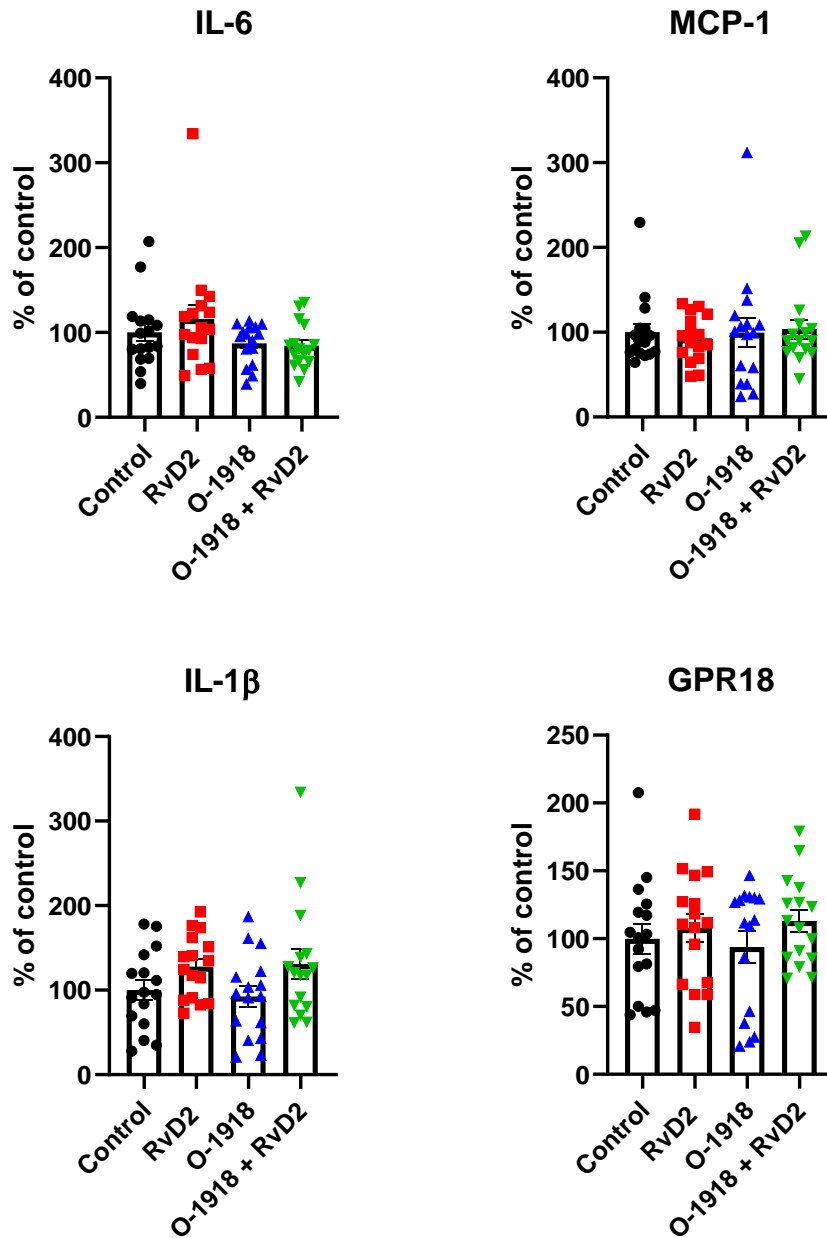


Figure 31: GPR18 and inflammatory markers expression in ApoE<sup>-/-</sup> mice spleen.

*GPR18 (D) and inflammatory markers IL-6 (A), MCP-1 (B) and IL-1β (C) expression in mice spleen were studied using Real-Time PCR. n=16 per group. Statistical analysis was performed using a one-way ANOVA test.*



### 3. Human coronary arteries studies

In addition to these *in vitro* and *in vivo* experiments, I was given access to a unique biobank of healthy and atherosclerotic human coronary arteries as well as conditioned media prepared from these arteries. The use of these samples at different stages of the disease allowed a better understanding of RvD2 action in atherosclerosis development and progression.

#### a. Resolvin measurement in conditioned media from human coronary arteries

##### i. Resolvin E1 measurement

RvE1 levels in conditioned media prepared from endarterectomies of  $n=7$  healthy coronary arteries (from 100% male, mean age  $42\pm 18.7$  years) and  $n=23$  atherosclerotic coronary artery segments (from 100% male, mean age  $59\pm 8.4$  years) were measured using an ELISA assay. RvE1 was found significantly higher in conditioned media derived from healthy coronary arteries compared with atherosclerotic coronary arteries (Figure 32).

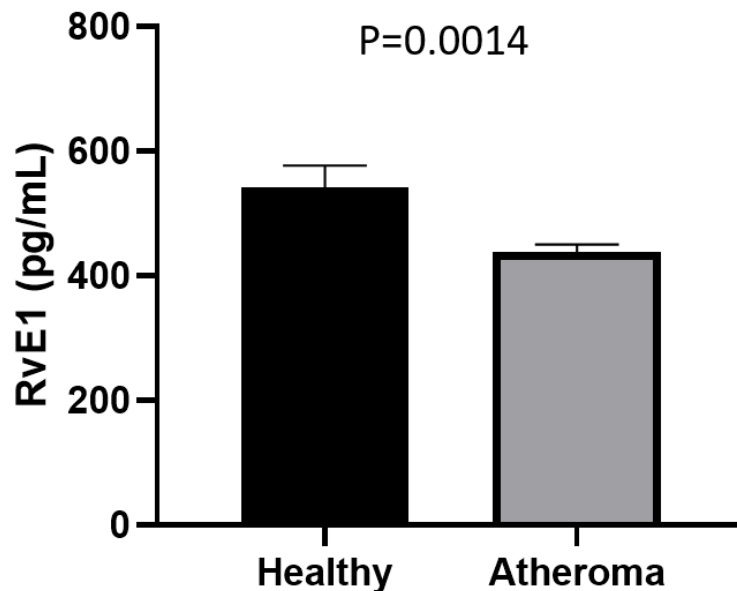


Figure 32: RvE1 levels in condition media from healthy and atherosclerotic coronary arteries. RvE1 levels were measured using resolvin E1 ELISA assay in condition media prepared from healthy and atherosclerotic coronary arteries;  $n = 7$  in the healthy group and  $n = 23$  in the atheroma group. Statistical analysis was performed using unpaired student t-test.

## ii. Resolvin D2 measurement

RvD2 levels were measured using conditioned media prepared from endarterectomies of  $n=7$  healthy coronary arteries (from 100% male, mean age  $42\pm 18.7$  years) and  $n=23$  atherosclerotic coronary artery segments (from 100% male, mean age  $59\pm 8.4$  years). RvD2 was found significantly higher in conditioned media derived from healthy coronary arteries compared with atherosclerotic coronary arteries (Figure 33).

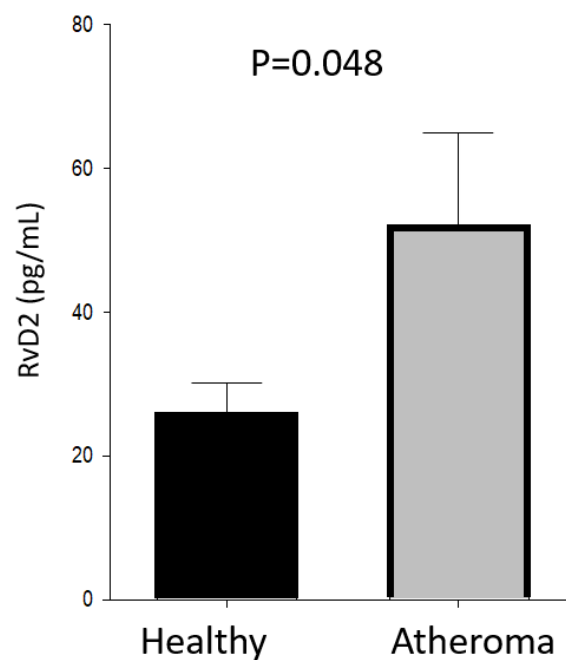


Figure 33: RvD2 levels in condition media from healthy and atherosclerotic coronary arteries. *RvD2 levels were measured using resolvin D2 ELISA assay in condition media prepared from healthy and atherosclerotic coronary arteries;  $n = 7$  in the healthy group and  $n = 23$  in the atheroma group. Statistical analysis was performed using unpaired student *t*-test.*

### b. Markers' expression in human coronary arteries

In order to examine the potential response of human coronary arteries to RvD2, GPR18 expression was detected in sections from these arteries. GPR18 expression was detected by immunostaining in 41 of 42 human coronary segments examined.  $\alpha$ SMA, CD68, GPR18 and ChemR23 staining were performed in consecutive sections. These stainings in consecutive sections suggest a colocalisation of CD68 with GPR18 and ChemR23, representative images are shown in figure 35. Figure 36 shows representative images of  $\alpha$ SMA, GPR18 and ChemR23

staining suggesting colocalisation between these markers.

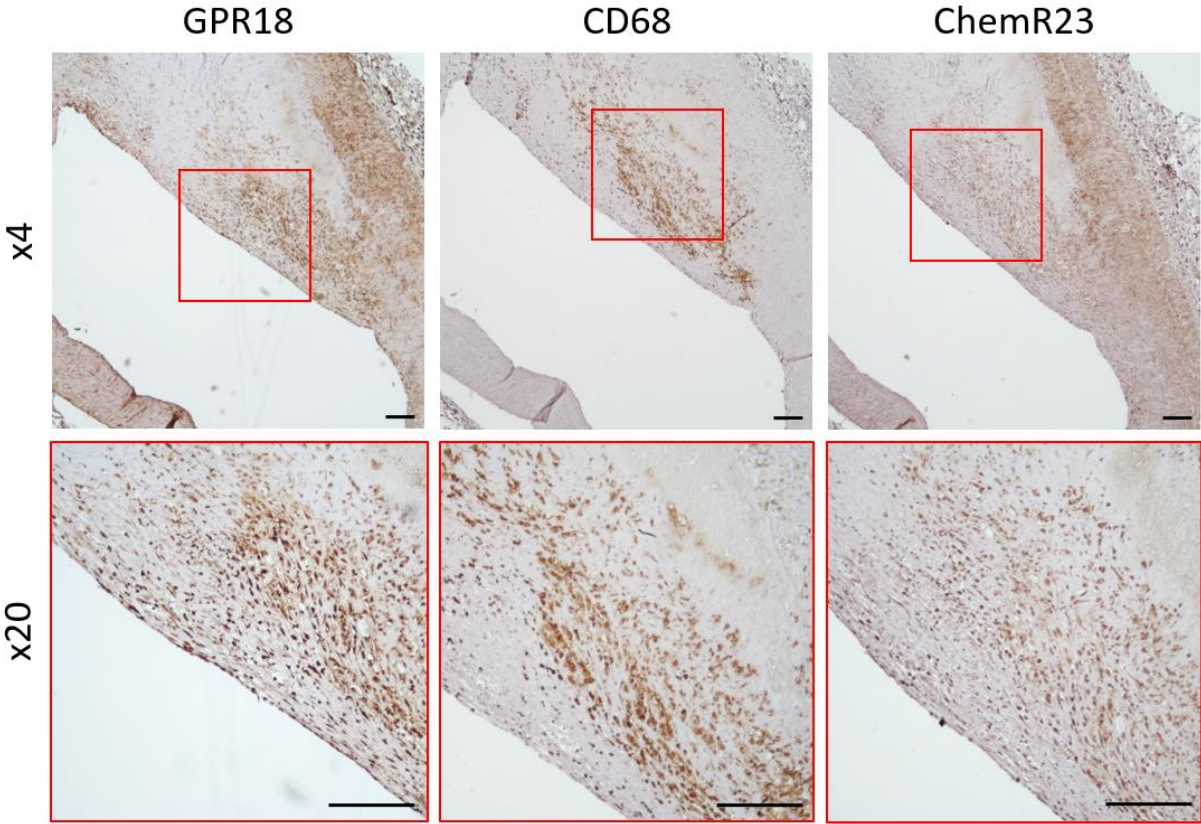


Figure 34: Evidence of co-localization of CD68 with GPR18 and ChemR23 in human coronary arteries.

*Representative images from the same patient of immunostaining for CD68, GPR18 and ChemR23 showing potential co-localisation of the markers; scale = 100  $\mu$ m.*

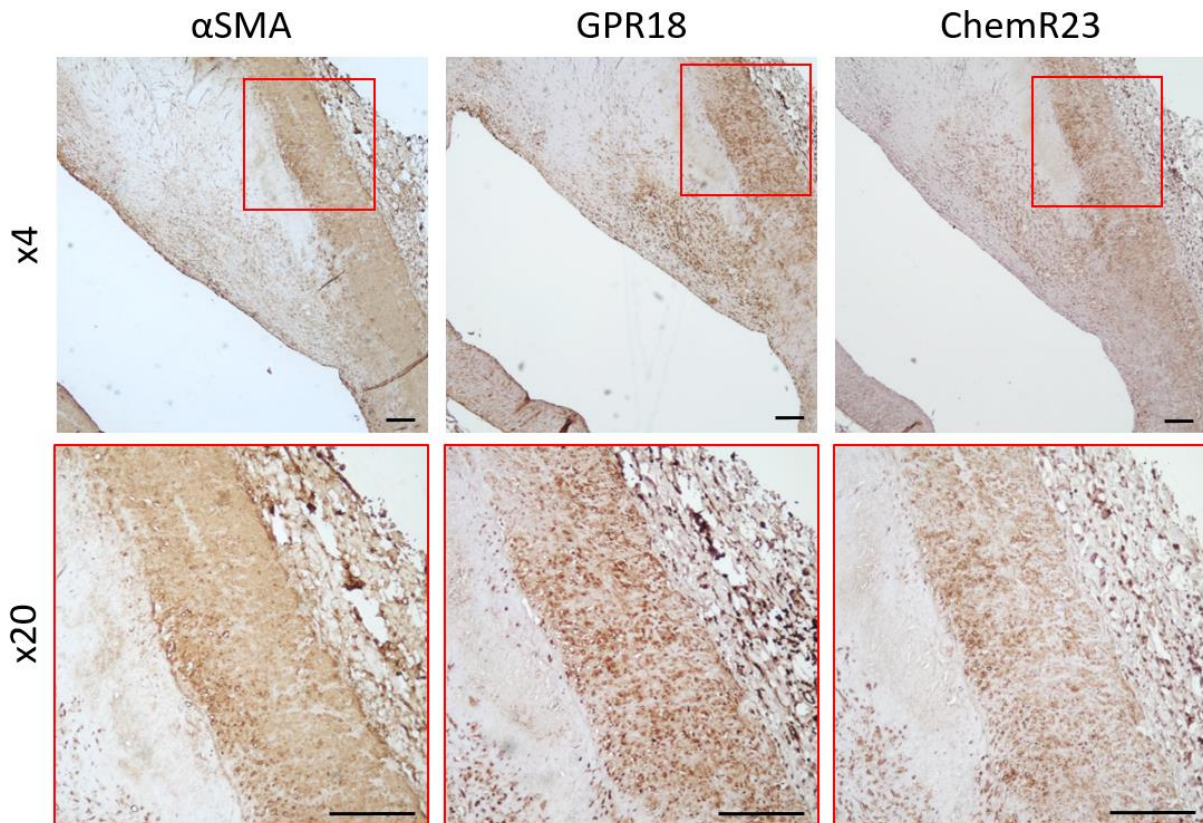


Figure 35: Evidence of co-localization of  $\alpha$ SMA with GPR18 and ChemR23 in human coronary arteries.

*Representative images from the same patient of immunostaining for  $\alpha$ SMA, GPR18 and ChemR23 showing potential co-localisation of the markers; scale = 100  $\mu$ m.*

Immunostaining of GPR18,  $\alpha$ SMA, CD68 and ChemR23 were categorized in three groups as negative (light blue), low expression (blue), or moderate expression (dark blue). These stainings were performed on n=42 sections (74% males, mean age of 57.6 years), representative images are shown in Figure 37.

CD68 immunostainings had a trend to gradually increase during disease progression from healthy (n=14, 57.1% males, mean age of 56.6 years) through fatty streaks (n=9, 40% males, mean age of 60.7 years) to established atherosclerotic lesions (n=19, 96.1% males, mean age of 55.9 years). In the same coronary segments, GPR18 in contrast exhibited a bell-shaped expression pattern with increased expression at early stages of atherosclerosis (fatty streaks) followed by decreased expression in established atherosclerotic lesions. ChemR23 follows a same pattern of expression as GPR18, while  $\alpha$ SMA expression stays stable during the different stages of disease progression (Figure 37).

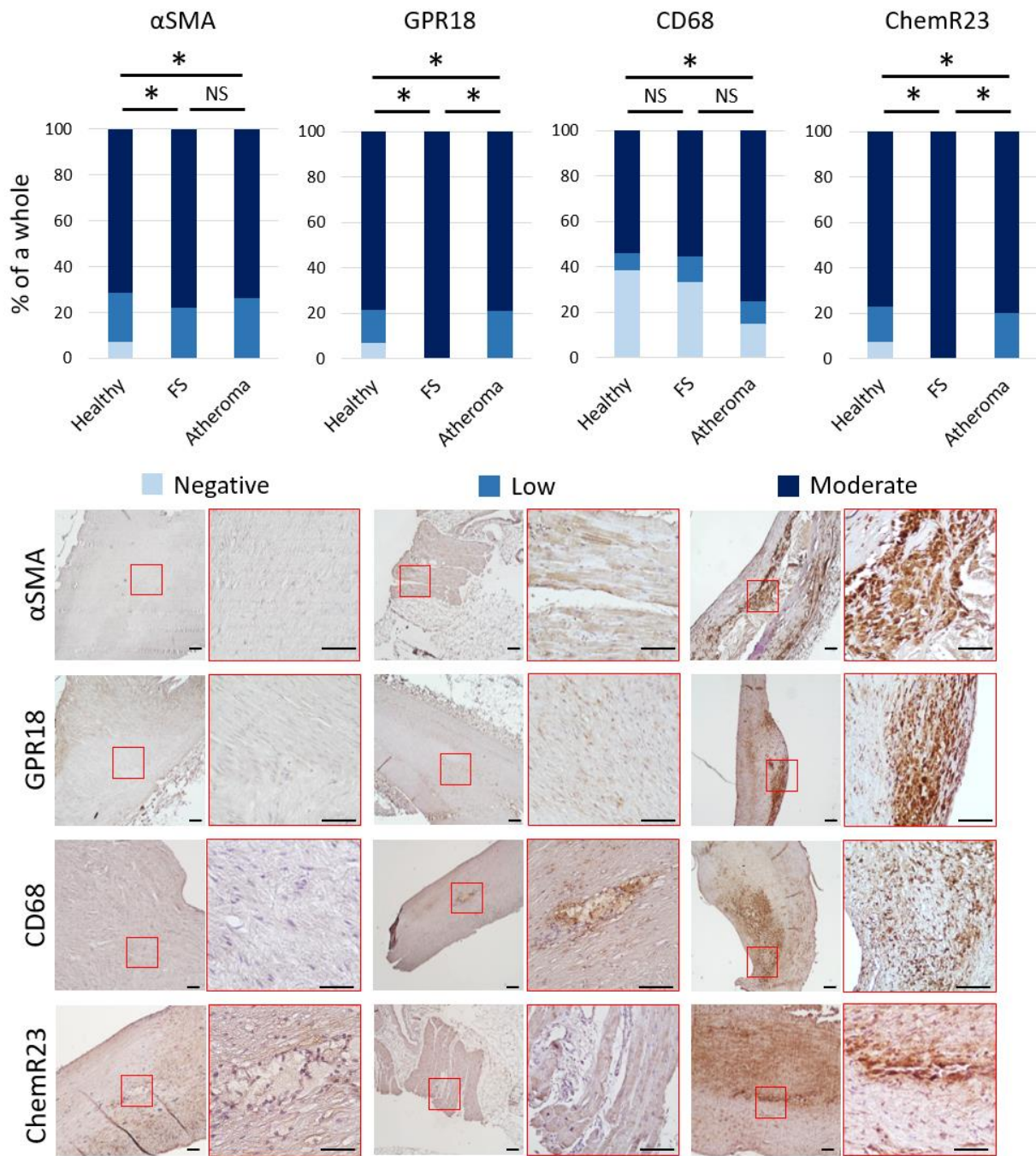


Figure 36: Markers' classification in human coronary arteries.

Top: Semi-quantitative classification of the four markers studied in the human coronary arteries. Bottom: representative images of each marker for each category of the classification; FS: Fatty Streak; scale = 100 μm; n = 14 in the healthy group, n = 9 in the fatty streak group and n = 19 in the atheroma group.

## Discussion

The effect of lipid mediators derived from n-3 PUFA on inflammation resolution mechanisms occurring during vascular ageing and especially in atherosclerosis development was the aim of this thesis. This work was performed with a particular focus on RvD2, derived from the n-3 PUFA DHA, and its receptor GPR18. Three different models were used for this work: 1) an *in vitro* model using human aortic VSMCs, 2) an *in vivo* model with of ApoE<sup>-/-</sup> mice fed a high fat diet enriched in cholesterol and treated with RvD2 and/or O-1918, a compound described as an antagonist of GPR18, and 3) a study on a biobank of human coronary arteries and associated conditioned media.

In order to study the effect of RvD2 on VSMCs we first wanted to demonstrate GPR18 expression in our models. GPR18 was found to be expressed in a very limited number of tissues and according to a gene expression data base it is only known to be expressed by a few organs such as bone marrow and lymphoid tissues, male tissues, gastro intestinal tract, and urinary bladder [141]. GPR18 expression on VSMCs was not mentioned in this data base even if experiments were conducted on the effect of its ligands, RvD2, on this cell type. We showed the expression of GPR18 in our model of cultured human aortic VSMCs and in the media of murine aorta.

During atherosclerosis development there is a phenotypic switch of the VSMCs from a contractile phenotype to a synthetic phenotype. This switch implicates that cells ensure different functions, meaning that cell metabolism can be affected. Little is known about the effect of GPR18 on metabolism, it was recently reported by Kotańka *et al* that some synthetic GPR18 ligands can have an effect on body weight and metabolic parameters in a model of rat with excessive eating [142], whereas no study reported an effect of RvD2 on weight or metabolic disorders. Other lipid mediators derived from n-3 PUFA have been suggested to act on metabolism, such as RvE1 which was reported to have an effect on liver transcriptional profile acting on the expression of genes implicated in glucose homeostasis and insulin sensitivity [143]. Administration of resolvin D1 (RvD1) to mice adipose tissue has been reported to increase adiponectin secretion and decrease leptin secretion leading to improved plasma glucose levels and a better insulin sensitivity [144]. These studies provide evidence of SPM action on metabolic disorders and indicate a potential effect of RvD2 on metabolism. To assess the effect of RvD2 on metabolism we measured the oxygen consumption rate of human aortic VSMCs in the presence and

in the absence of RvD2. The results of this experiments showed no significant effect of RvD2 treatment on VSMCs respiration despite using a common concentration of RvD2 used *in vitro*. Due to lack of studies on the effect of resolvin on VSMC metabolism it is difficult to evaluate the effect of RvD2, we can hypothesize that if RvD2 did not act directly on VSMC metabolism it can act indirectly on it by regulation of plasmatic glucose levels and insulin sensitivity.

Switching from the contractile phenotype from the synthetic phenotype also leads to an increase of cell migratory capacity. Using a model of cultured aortic VSMCs isolated from mice, Akagi *et al* demonstrated that RvD2 was able to reduce cell migration in a dose dependent manner [145]. The same effect was observed with the RvE1 using rat VSMCs [137]. Miyahara *et al* showed that an *in vitro* treatment of RvD2 on human VSMCs isolated from the greater saphenous vein decreased cell migration in a dose dependent manner [125]. The dose of PDGF use in our experiment failed to significantly enhance cell migration, but interestingly a trend to decrease cell migration was observed when cells treated with PDGF-BB were also treated with RvD2. This difference of effect from our experiments in comparison to the literature can be due to the different cell origins used in our study but also to the concentration of PDGF use.

Oxidative stress is a key mechanism in atherosclerosis development and lesion growth. It has been shown that glycophorin A, a RBC membrane marker, is present in early lesions and that VSMCs are able to phagocytize senescent erythrocytes but not fresh erythrocytes [85,146]. This internalization of senescent erythrocytes was shown to induce an increase in ROS production in human VSMCs [85]. No study reported RvD2 influencing ROS production using this model of senescent erythrocytes, but studies reported an effect of RvD2 on superoxide production. RvD2 was also reported to reduce oxidative stress *in vivo* in a rabbit model of injured arteries [125] and *in vitro* in a model of mice aortic VSMCs [145]. No previous study has reported the use of EPR spectroscopy to determine the effect of SPMs on ROS production. This technique was used to determine ROS production in other models such as studying ROS production in VSMCs isolated from male and female Sprague-Dawley rats [147]. Our cells showed the same capacities in phagocytizing erythrocytes, fresh erythrocytes were not internalized while senescent erythrocytes were internalized by the VSMCs. Surprisingly even with senescent erythrocyte internalization no increase in oxidative stress was found in our model. This discordance in results previously shown using a similar model of human aortic VSMCs and senescent eryth-

rocytes could indicate that in reality erythrocytes were not internalized but retained at cell surface. We investigated ROS production by erythrocytes obtained from ApoE<sup>-/-</sup> mice using EPR spectroscopy. The use of EPR to investigate RvD2 treatment on ROS production showed a significant decrease in ROS production by erythrocytes in mice treated with RvD2 compared to control mice. As RvD2 is known to decrease ROS production, these results indicate that RvD2 is able to affect and decrease the oxidative stress induced by erythrocytes in atherogenesis and could represent another atheroprotective mechanism of the RvD2.

In order to investigate the role of RvD2 on atherogenesis we used a model of ApoE<sup>-/-</sup> mice fed a high fat diet enriched in cholesterol as a model of early vascular ageing. Nnamonu *et al* reported a trend in gaining weight in albinos rat supplemented with omega-3 fatty acid in a dose dependent manner but this increase was not significant [148]. Other studies with omega-3 fatty acid supplementation conducted in animal and human showed the opposite effect, supplementation supported the reduction of accumulation of fat in tissues [149–151]. Our mice were weighed once a week during the four weeks of experiments. All the mice gained weight during the duration of the diet, and we did not observe any significant differences in the gain of weight between the different groups. In previous studies effects of omega-3 supplementation on weight were reported, the difference between our results and those that have been reported before can be explained by the fact that previous studies used EPA and/or DHA as supplement while we used only RvD2. As the molecule used is different, the receptor implicated is different and so are the signaling pathways activated by the fixation of the ligand on its receptor. Moreover, using n-3 PUFA can lead to the synthesis of the whole spectrum of SPMs that will activate their own receptors.

Omega-3 fatty acids and SPMs derived from them are known to have a particular effect on immune cells. It has been shown that n-3 PUFA supplementation in a model of the albino rat had a time and dose effect on increasing haemoglobin, white blood cells, mean cell volume and mean cell haemoglobin but no significant effect on red blood cells and mean cell haemoglobin concentration [148]. Another study reported an effect of n-3 PUFA supplementation on RBC formation by increasing their number [152]. Analysis of the blood of our four groups of mice showed no significant effect on all of the parameters measured. Once again previous studies used EPA and DHA supplementation while we used only RvD2. This result can indicate that RvD2 does not play a role in haematopoiesis.



Other blood parameters are of particular importance in cardiovascular diseases such as plasma cholesterol and triglycerides levels. Patients with coronary artery disease were supplemented during 1 year with a daily intake of n-3 PUFA, the result of the study showed that after one year these patients exhibited significantly lower triglyceride levels compared with patients not supplemented [95]. Omega-3 fatty acids were shown to reduce triglycerides by reducing hepatic secretion of TG-rich lipoprotein [153], DHA has also been shown to be more efficient than EPA in reducing triglycerides levels [154]. Measurement of cholesterol and triglycerides in the blood of our mice showed no effect of the treatments on their levels indicating that RvD2 atheroprotective effect is not due to an ability to reduce cholesterol and TG levels in blood in our model.

Apoptotic cell clearance by macrophages is of particular importance in atherosclerotic lesion to determine their stability, a defect in clearance leads to a bigger necrotic core and more unstable lesions. RvD2 was shown to enhance *E. coli* phagocytosis of macrophages derived from bone marrow [124], while RvD1 was shown to enhance phagocytosis of peritoneal macrophages [155]. RvD2 has also been described to have the potential to induce a phenotypic switch in macrophage polarization from the pro-inflammatory M1 subtype to the pro-resolutive M2 subtype [123]. M2 macrophages are characterized by an increased phagocytic capacity leading to a better clearance of apoptotic cells compared to M1 macrophages [51]. These different subtypes can be detected using specific markers such as CD68 and iNOS for the M1 subtype and CD206 and arginase-1 for the M2 subtype. It has been shown that mice invalidated for the receptor ChemR23 present bigger atherosclerotic lesions than wild type mice. Mice invalidated for ChemR23 expression also exhibited atherosclerotic lesions with a high expression of CD68 expressing cells [135]. We investigated the effect of RvD2 on the phagocytic capacity of peritoneal macrophages. In this study we found that zymosan uptake by peritoneal macrophages was increased in mice treated with RvD2, this effect was not observed in mice treated with a combination of RvD2 and O-1918. These results are in accordance with the literature where RvD2 is described as an enhancer of phagocytosis. Using macrophages from WT mice and GPR18<sup>-/-</sup> mice treated in vitro with RvD2 we saw a trend in increasing phagocytosis in peritoneal macrophages from WT mice but not from knockout mice, this result is supported by the fact that Chiang *et al* reported a similar effect on the phagocytic capacity of bone marrow derived macrophages [124]. No study has depicted the effect of O-1918 on phagocytosis but the GPR18 antagonist has been described to block RvD2 effects on ischemia reperfusion indicating an effect mediated by GPR18 [138]. A similar effect was observed with macrophages which

are not expressing GPR18. These data indicate that the effect of RvD2 on phagocytosis is mediated by GPR18. On the other hand, results obtained from immunostainings in the aortic root and from real-time quantitative PCR in mice abdominal aorta to determine lesion composition in macrophage showed a decrease in iNOS expression with RvD2 treatment compared to control mice. This result indicates that RvD2 treatment act on macrophages by inducing their polarization into M2 subtype. Taken together, phagocytosis and lesion composition results indicate that RvD2 treatment enhance the phagocytic capacity of macrophages by polarizing them into M2 subtype.

With high levels of circulating cholesterol and triglycerides, atherosclerotic lesions will appear and develop starting from the aortic root to then gain the thoracic aorta. SPMs are known to have a beneficial effect on cardiovascular diseases and delay their development. GPR32, the human receptor for RvD1, was found to be atheroprotective in a model of ApoE<sup>-/-</sup> mice fed a high fat diet by reducing the size of the atherosclerotic lesion and necrotic core areas in the aortic root as well as reducing atherosclerotic lesions coverage of the thoracic aorta [155]. A combined treatment of RvD2 and maresin 1, another SPMs derived from DHA, in ApoE<sup>-/-</sup> mice fed a high fat diet has a similar effect on atherosclerosis development by decreasing lesion size and necrotic core area [36]. In our experimental mice treated with RvD2 a significantly lower development of lesion in the aortic root compared to control mice was observed. Interestingly a similar effect was observed in mice treated with O-1918. Mice treated with RvD2 also exhibited a smaller necrotic core area compared with control mice. The observation that RvD2 decreased atherosclerotic lesion development and necrotic core area in the aortic root is in accordance with other studies reporting beneficial effects of SPMs on atherosclerosis development [36,136,155]. The observed effect of O-1918 needs further investigation. Indeed, O-1918 treatment is known to upregulate circulation of pro-inflammatory cytokines including IL-1 $\alpha$ , IL-2 IL-17 $\alpha$  and IL-18 in a model of diet-induced obesity [156]. Moreover, even if O-1918 is widely used as an antagonist for GPR18, this molecule is also described as a partial agonist. O-1918 is also described as a putative antagonist of the receptor GPR55 [119]. In our study RvD2 treatment did not induce a significant effect on lesion development in the thoracic aorta. Other SPMs have been reported to act on lesion development in the thoracic aorta, such as RvD1 [155]. In our protocol, mice exhibited low coverage of the thoracic aorta by atherosclerotic lesions, thus, we can hypothesize that in our model lesion development was at a really early stages and so no effect can be shown at this stage.

Chronic inflammation is a hallmark of atherosclerosis, characterized by an upregulation of inflammatory marker production. O-1918 was not found to alter GPR18 expression in both red and white gastrocnemius skeletal muscles in a model of the diet-induced obesity rat [157] but was found to upregulate pro-inflammatory cytokine production [156]. The spleen is an organ rich in immune cells in which the presence of hypercholesterolemia and atherosclerosis and a change in T cell population is observed [158]. We investigated the mRNA expression of GPR18 and inflammatory markers (IL-6, MCP-1, IL-1 $\beta$ ) in the spleen of our mice and found no modification of expression between the different groups. We can hypothesize that in this model of early atherosclerosis development inflammation was insufficiently triggered to observe an effect of RvD2.

Circulating SPMs play a key a role in the resolution of inflammation and in cardiovascular disease progression. With atherosclerosis development there is the onset of chronic inflammation, indicating a failure in the resolution of inflammation coming from the fact that enzymes producing SPMs are less expressed and present a lower activity. Ho *et al* reported in 2010 that aspirin-triggered a lipoxin A4 level decrease with peripheral arterial disease [94]. Viola *et al* showed that during atherosclerosis progression in mice RvD2 levels decrease as the disease progresses [36]. RvE1 levels have been shown to be significantly lower in aortic valves with calcification compared to non-calcified ones [159]. By measuring RvE1 with an ELISA assay in conditioned media prepared from healthy and atherosclerotic human coronary arteries we found a significant decrease in RvE1 production in patients with atheroma. On the other hand, measuring RvD2 levels in conditioned media from the same patient showed the opposite, that is to say that patients with atheroma exhibit a significantly higher level of RvD2 than healthy patients. Our finding that RvE1 levels decrease with atherosclerosis are in accordance with the literature data indicating a defect in inflammation resolution. The finding of RvD2 higher levels in patients with atherosclerosis compared to healthy controls is in discordance with what have been shown in aortic lipid extracts from ApoE<sup>-/-</sup> mice fed a high fat diet [36]. This leads to the conclusion that in human coronary artery RvD2 plays a key protective role against atherosclerosis, and with disease progression RvD2 production increases in order to protect against the disease without being able to stop its progression.

With atherosclerosis progression and concomitant impaired inflammation resolution observed with disease progression, enzymes involved in SPMs production are less expressed and less

active. This defect of inflammation resolution could also be due to a loss of SPMs receptor expression. We showed that GPR18 is expressed by VSMCs and it has been shown before that macrophages also express this receptor [121]. ChemR23 has been shown to be expressed by both macrophages and VSMCs [135]. Yanofsky *et al* showed in 2021 no differences in ChemR23 between stable and unstable carotid atherosclerotic plaque [160] whereas GPR32, the RvD1 receptor, was shown to have a significantly lower expression in human carotid atherosclerotic lesions compared with control arteries [155]. By staining GPR18, ChemR23, CD68 and  $\alpha$ SMA on human coronary arteries section from healthy arteries, arteries with fatty streak and arteries with atheroma we showed a potential colocalization of the markers studied. Our findings suggest a colocalization of GPR18 and ChemR23 with CD68 indicating that these two receptors are expressed by macrophages present in lesions. On the other hand, the suggestion that GPR18 and ChemR23 are also colocalized with  $\alpha$ SMA suggest that both ChemR23 and GPR18 are expressed by human coronary arteries smooth muscle cells. These data indicate that SPM action on inflammation resolution is not only mediated by immune cells but also by VMSCs. By classifying our staining for our four markers in three categories (i.e., not express, low expression and moderate expression) we found no changes in  $\alpha$ SMA during atherosclerosis progression but an increase in CD68 expression in the lesion as the disease progress. Interestingly GPR18 and ChemR23 showed the same pattern of expression with an increased expression in arteries with fatty streaks compared to healthy ones and a decreased expression in atherosclerotic lesions compared to those with fatty streaks. An increase in CD68 expression in lesions indicates an increase of inflammation as the disease progresses. This indicates that with the apparition of atherosclerotic lesions a defence mechanism is activated in response to lipid deposits and to inflammation by increasing expression of SPMs' receptors. With disease progression and failure to resolve this inflammation a loss of receptor expression is observed. This participates to impair the capacity to resolve inflammation and participates in disease progression.

## **General conclusions and perspectives**

The role of RvD2 in atherosclerosis development was explored in this thesis. Our findings show that RvD2 has significant potential in the process of protection against cardiovascular aging and in deepening our knowledge of these mechanisms of action could be beneficial. In this study we observed that RvD2 decreases the formation of atherosclerotic lesions and increases

the phagocytic capacity of macrophages. We have also observed in humans an increase in the production of RvD2 in coronary arteries with atherosclerotic plaques, suggesting a major role for RvD2 in the process of defence against atherosclerosis development.

RvD2 action on circulating pro-inflammatory cytokines remains to be investigated as well as its action on plaque stability. Signalling pathways activated following GPR18 activation by RvD2 remain unclear and will need further investigation. Using a model of GPR18<sup>-/-</sup> mice transplanted with bone marrow from GPR18<sup>+/+</sup> could permit a better understanding of GPR18-RvD2 actions on immune cells and their role in inflammation and its resolution. Moreover, a better understanding of signalling pathways implicated could allow to develop specific drugs targeting the same pathways.

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## The resolvin D2 – GPR18 axis is expressed in human coronary atherosclerosis and transduces atheroprotection in apolipoprotein E deficient mice

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### ABSTRACT

Chronic inflammation in atherosclerosis reflects a failure in the resolution of inflammation. Pro-resolving lipid mediators derived from omega-3 fatty acids reduce the development of atherosclerosis in murine models. The aim of the present study was to decipher the role of the specialized proresolving mediator (SPM) resolvin D2 (RvD2) in atherosclerosis and its signaling through the G-protein coupled receptor (GPR) 18. The ligand and receptor were detected in human coronary arteries in relation to the presence of atherosclerotic lesions and its cellular components. Importantly, RvD2 levels were significantly higher in atherosclerotic compared with healthy human coronary arteries. Furthermore, apolipoprotein E (ApoE) deficient hyperlipidemic mice were treated with either RvD2 or vehicle in the absence and presence of the GPR18 antagonist O-1918. RvD2 significantly reduced atherosclerosis, necrotic core area, and pro-inflammatory macrophage marker expression. RvD2 in addition enhanced macrophage phagocytosis. The beneficial effects of RvD2 were not observed in the presence of O-1918. Taken together, these results provide evidence of atheroprotective pro-resolving signalling through the RvD2-GPR18 axis.

### 1. Introduction

Atherosclerosis is in addition to elevated cholesterol-rich lipoproteins closely linked to chronic inflammation [1]. The immune responses of atherosclerosis include for example recruitment and activation of leukocytes, macrophage polarization, and synthesis of pro-inflammatory cytokines [2]. Advanced atherosclerotic lesions are characterized by the presence of a necrotic core, reflecting a malfunctioning efferocytosis and leading to insufficient clearance of apoptotic cells [3]. Taken together, the atherosclerotic lesion immune characteristics are concordant with a failure in the resolution of inflammation [4].

Resolution of inflammation is an active process to limit inflammatory activation to bring back the vascular wall to a homeostasis state. This protective mechanism can be mediated by for example lipid specialized

pro-resolving lipid mediators (SPM) [5], which signal through specific G protein-coupled receptors (GPR) [6]. In the context of atherosclerosis, exogenous administration of SPM has been consistently shown to reduce atherosclerosis in murine models [7–9]. Genetic modulation of SPM receptor expression in murine atherosclerosis has however generated contradictory results, indicating both beneficial effects [10,11] and exacerbated disease [12,13] transduced through these receptors. The latter observations reflect the importance of the balance between pro-inflammatory and pro-resolving ligands for the SPM receptors to determine the downstream signalling [7,14,15].

Among SPM, D-series resolvins, synthesized from the omega-3 polyunsaturated fatty acid (n-3 PUFA) docosahexaenoic acid (DHA), display protective effects against atherosclerosis progression, [8,11] which has also been demonstrated for their receptors ALX/FPR2 [7] and

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GPR32 [11]. Resolvin D2 (RvD2) promotes macrophage polarization towards a reparative phenotype, leading to plaque stabilization [8]. GPR18 was identified as receptor for RvD2 [16]. This receptor belongs to the orphan class A family and was identified as part of the endocannabinoid system, which plays a major role in energy homeostasis (modulation of caloric intake, nutrient transport, cellular metabolism, and energy storage) [17]. How the specific pathway involving RvD2 and GPR18 is affecting atherosclerosis has not been previously investigated.

The aim of this study was to decipher the implication of the RvD2-GPR18 axis in atherosclerosis. We explored the ligand and receptor in human coronary arteries in relation to the presence of atherosclerotic lesions and its cellular components. We furthermore treated hyperlipidemic mice with RvD2 with and without the GPR18 antagonist O-1918, to establish RvD2-induced effects through GPR18 on atherosclerotic plaque development, pro-inflammatory responses, and phagocytic activity. The study identified GPR18 and its ligand RvD2 in human coronary atherosclerosis and showed that this RvD2 reduced murine atherosclerosis. The effects of RvD2 were not observed in the presence of O-1918, indicating atheroprotective pro-resolving signalling through the RvD2-GPR18 axis.

## 2. Material and methods

### 2.1. Human tissues

Human coronary artery walls, including healthy and diseased arteries at different stages of atherosclerotic diseases, were collected from the Inserm human CV biobank (BB-0033-00029, U 1148, X. Bichat Hospital, Paris), included in the European network BBMRI-ERIC, in accordance with the French regular and ethical rules (BioMedicine Agency convention DC2018-3141) and the principles of the declaration of Helsinki. Approval was obtained from the French Biomedical Agency (ABM, PFS09-007 & PFS17-002) and the Institutional Ethical Review board (SC09-09-66). Tissues were obtained from deceased organ donors for kidney and/or hepatic transplantation in the absence of therapeutic uses for the heart. When collected, healthy coronary arteries were macroscopically analysed by a trained vascular surgeon (J-BM) to ensure that these samples were not pathological (absence of fatty streak or atherosclerosis plaques after longitudinal opening). For immunohistochemistry, human arterial tissues were fixed with 4% (w/v) buffered formaldehyde solution prepared by depolymerisation of paraformaldehyde and imbedded in paraffin. Serial 5 µm thick sections were collected.

Conditioned media were prepared from small pieces (about 2 mm<sup>3</sup>) of tissue, weighed, and incubated for 24 h in serum-free RPMI culture medium containing 1% L-glutamine, 1% penicillin, streptomycin, and amphotericin at 37°C (5% CO<sub>2</sub>). For standardization, the volume of medium was adjusted to sample wet weight (6 mL per gram of tissue). Conditioned media were collected, centrifuged (14000g for 15 min at 4°C), and stored at -80°C until further analysis as previously described [18]. RvD2 concentrations in conditioned media were measured by ELISA (Cayman Chemical, Ann Arbor, USA, item 501120).

### 2.2. Animals

ApoE<sup>+/+</sup> mice on a C57BL/6J background were obtained from Charles River (Wilmington, USA) and fed a standard diet. For the experimental protocols, 8–9 weeks old ApoE<sup>-/-</sup> mice on a C57BL/6J background from Taconic Biosciences (Rensselaer, USA) were used. Housing and experiments were conducted in accordance with the French regulations and the experimental guidelines of the European Community (Directive 2010/63/EU). Animals were housed under standard conditions (22°C, day-night cycle of 12 h, 70% humidity) and given free access to high fat diet (HFD) containing 1.25% cholesterol (E15749-347; Ssniff, Soest, Germany). The protocols were approved by the Animal Ethics Committee of Lorraine, France (#9411-2017032718404787 v4).

Mice were fed HFD for 4 weeks and injected 3 times per week (IP, 100 µL) with the following: (1) vehicle consisting of saline solution and EtOH 2.8% (control group), (2) RvD2 (100 ng per mouse in 2.8% EtOH) (from Cayman Chemical, Ann Arbor, USA), (3) O-1918 in 2.8% EtOH (2 mg/kg) (from Tocris, Bristol, UK) or (4) a combination of RvD2 and O-1918. Mice were put to death after isoflurane anaesthesia (1.5% in 1 L/min oxygen) followed by cervical dislocation. Blood, heart, aorta, and peritoneal macrophages were collected for the experiments detailed below.

### 2.3. Measurements of plasma lipid and RvD2 levels

Triglyceride levels were measured using a glucose meter (Accutrend® Plus mmol/L), capable to measure triglycerides using specialized strips. Cholesterol levels were measured in mice plasma using ELISA assay (MyBiosource, San Diego, USA, item MBS269999). RvD2 concentrations in murine plasma samples were measured by ELISA (Cayman Chemical, Ann Arbor, USA, item 501120).

### 2.4. Evaluation of atherosclerotic lesions

Atherosclerotic lesion burden in the aortic arch and the thoracic aortas was determined by *en face* analysis. Dissected aortic arches were fixed in 4% PFA, cut longitudinally, and stained with Oil Red-O solution (Sigma-Aldrich, Saint-Louis, USA). Images were acquired using a light microscope (Nikon Eclipse Ci). Total area of the thoracic aorta and area of the atherosclerotic lesions, respectively, were quantified using ImageJ (NIH) to express the relative lesion area as percentage of the thoracic aorta area. Aortic roots were embedded in OCT (Leica, Wetzlar, Germany) and frozen at -80°C. Serial, 10 µm thick cryosections were collected, starting from the appearance of the aortic valve cusps, and fixed either with 4% PFA or acetone. PFA fixed section, collected every 100 µm from the aortic valve cusps, were stained with Oil Red-O (Sigma-Aldrich, Saint-Louis, USA). Images were acquired with a light microscope equipped with a DC300 camera (Leica, Wetzlar, Germany).

The area of the aortic root lesions was determined in a blinded fashion using ImageJ (NIH) and expressed relative to the total aortic root area. The area of necrotic cores, defined as unstained zones devoid of cells or extracellular matrix, was measured in a blinded fashion using ImageJ (NIH). In both cases, *i.e.* relative aortic root lesion area and relative necrotic core area, exclusion criteria were decided as follows: if two or more sections were missing between 200 and 600 µm past the aortic valve cusps, mice were excluded; if one section was missing, the value was imputed by the mean of the previous and the following value.

### 2.5. Immunohistochemistry

Human coronary artery histological sections were stained with anti-GPR18 antibody (Bioss Antibodies, Boston, USA; 1:100 dilution), anti-CD68 antibody (Abcam, Cambridge UK; 1:8000 dilution). Murine atherosclerotic lesion composition was analysed using immunohistochemistry in level-matched sections with anti-CD68 antibody (Serotec, UK; 1:20000), anti-iNOS antibody (Abcam, Cambridge, UK; 1:100), anti-Arginase-1 antibody (Abcam, Cambridge, UK; 1:50), and anti-CD206 antibody (Serotec, UK; 1:50) or an isotype control (Serotec, UK; 1:100) and secondary antibodies (Vector Labs, Newark, USA; 1:200) using a standard avidin-biotin-peroxidase complex method. 3,3'-diaminobenzidine (Vector Labs, Newark, USA) was then used as the substrate to localize the primary antibodies. The preparations were counterstained with haematoxylin, mounted with Permount (Merck, Darmstadt, Germany) and examined by light microscopy.

### 2.6. Quantitative real-time PCR

Total RNA was isolated from the abdominal aorta using QIAzol (Qiagen, Hilden, Germany) and Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). RNA concentrations were measured

spectrophotometrically using Nanodrop 1000 (Thermo Fisher Scientific, Waltham, USA), and reverse transcription was performed using the High-capacity RNA-to-cDNA kit (Thermo Fischer Scientific, Waltham USA). Quantitative real-time PCR was performed on a 7900HT Fast Real-Time PCR system (Thermo Fisher Scientific, Waltham, USA) with the following TaqMan Assay-on-Demand probes (Thermo Fisher Scientific, Waltham USA): iNOS (Mm00440502\_m1), CD68 (Mm03047343\_m1), CD206 (Mm01329359\_m1), and Arg-1 (Mm00475988\_m1) were used to evaluate expression, and HPRT (Mm03024075\_m1) was used as housekeeping gene. Results are expressed as % of the control using the  $\Delta$ Ct method.

### 2.7. Phagocytosis assay

Mice were intraperitoneally washed by injecting 5 mL PBS to obtain peritoneal macrophages. Cells were then plated in a black 96-well plate with clear bottom at  $10^5$  cells per well. After 24 to 48 hrs, cells were treated with FITC-labelled zymosan particles (Invitrogen, Carlsbad, USA) ( $10^6$  particles per  $10^5$  cells) for 45 min before they were washed with PBS. The cells were then fixed with 4% PFA for 15 min, the nuclei were stained with DAPI, and the cells were observed under a fluorescence microscope. Quantification of fluorescence and nuclei count were performed with ImageJ software. Data are presented as fluorescence intensity plotted against the number of cells.

### 2.8. Erythrocyte isolation and incubation for ROS measurements

Whole blood was collected from the carotid artery into citrated tubes and centrifuged. The erythrocytes pellet was washed 3 times with PBS<sup>-/-</sup> (followed each time by centrifugation at 1000g, 10 min, 4 °C) and incubated with the spin probe 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (200  $\mu$ M, Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany) to evaluate the production of reactive oxygen species. In brief, 5  $\mu$ L of washed erythrocytes were added to 1 mL CMH:KREBS/HEPES solution, (1:1, v/v), mixed well, and incubated for 30 min at 37 °C with gentle shaking. Incubations were stopped by freezing the samples on dry ice. Samples were stored at -80 °C until ROS production was quantified with electron paramagnetic resonance (EPR) using Bruker E-Scan M system (Bruker, Billerica, USA) as previously described [28] with the following settings: center field 1.99 g, microwave power 1 mW, modulation amplitude 9 G, sweep time 10 s, number of scans 10, field sweep 60 G. The EPR spectrums are expressed % of control.

### 2.9. Statistical analysis

Data are presented as mean  $\pm$  SEM. For comparison between two groups, non-normally distributed data were compared using a Mann-Whitney Rank Sum test, whereas normally distributed data were compared by a two-tailed either paired or unpaired Student *t* test. One-way ANOVA was performed when comparing multiple groups. For the aortic root lesions and necrotic core analysis, two-way ANOVA followed by Tukey's multiple comparison was performed,  $p < 0.05$  was considered as statistically significant. Phagocytosis assay was analyzed using linear regression and comparison between the groups was performed by multivariable linear regression. Chi square test was used to evaluate differences between groups in human immunohistochemistry classification. Statistical analysis was performed using Graphpad prism 8.0.2 software.  $p < 0.05$  was considered as statistically significant.

## 3. Results

### 3.1. Resolvin D2 and GPR18 in human coronary artery atherosclerosis.

Conditioned media was prepared from endarterectomies of  $n = 7$  healthy coronary arteries (100% male, mean age  $42 \pm 18.7$  years) and  $n$

$= 23$  atherosclerotic coronary artery segments (100% male, mean age  $59 \pm 8.4$  years). The concentrations of RvD2 were significantly higher in conditioned media derived from patients with atherosclerotic coronary arteries compared with healthy coronary arteries (Fig. 1A). GPR18 immunostaining was detected in 41 of 42 human coronary segments examined. Staining in consecutive sections indicated co-localization of GPR18 and CD68 immunoreactivity (Fig. 1B).

The immunostainings for GPR18 and CD68 were categorized into negative (light blue), low expression (blue), and moderate expression (dark blue) for  $n = 42$  sections (74% males, mean age  $57.6 \pm 7.1$  years) (Fig. 1C and D). The CD68 immunostainings trended to gradually increase during disease progression from healthy ( $n = 14$ , 57.1% males, mean age  $56.6 \pm 7.3$  years) through fatty streaks ( $n = 9$ , 40% males, mean age  $60.7 \pm 8.8$  years) to established atherosclerotic lesions ( $n = 19$ , 96.1% males, mean age  $57.7 \pm 5.9$  years). In contrast, GPR18 exhibited in the same coronary segments a bell-shaped expression pattern with increased expression at early stages of atherosclerosis (fatty streaks) followed by decreased expression similar to controls in established atherosclerotic lesions (Fig. 1D).

### 3.2. Resolvin D2 decreased murine aortic root atherosclerosis through GPR18

Plasma levels of RvD2 were significantly lower in HFD-fed ApoE<sup>-/-</sup> mice compared with chow-fed ApoE<sup>+/+</sup> mice ( $105 \pm 7.8$  pg/mL;  $n = 15$  vs.  $533 \pm 108$  pg/mL;  $n = 4$ ;  $p < 0.05$ ). Quantification of atherosclerotic plaque burden in the aortic root are shown in Fig. 2A-B. ApoE<sup>-/-</sup> mice were subjected to intraperitoneal injections with either RvD2 or O-1918 alone or in combination followed by quantification of atherosclerotic plaque burden in the aortic root (Fig. 2AB) and thoracic aorta (C). Separating groups of mice in the absence or presence of the GPR18 antagonist O-1918 revealed that RvD2 significantly decreased atherosclerosis compared to the control group (Fig. 2A,  $p < 0.001$ ). In contrast, RvD2 in combination with O-1918 did not significantly alter the relative atherosclerotic lesion size compared with mice treated with O-1918 alone (Fig. 2B,  $p = 0.42$ ). The *en face*-quantified atherosclerotic lesion burden in the aortic arch and thoracic aorta was low without significant differences between the groups (Fig. 2C). There was no significant difference between the groups for blood cell counts, red blood cells parameters (Table 1), cholesterol or triglycerides (Table 2).

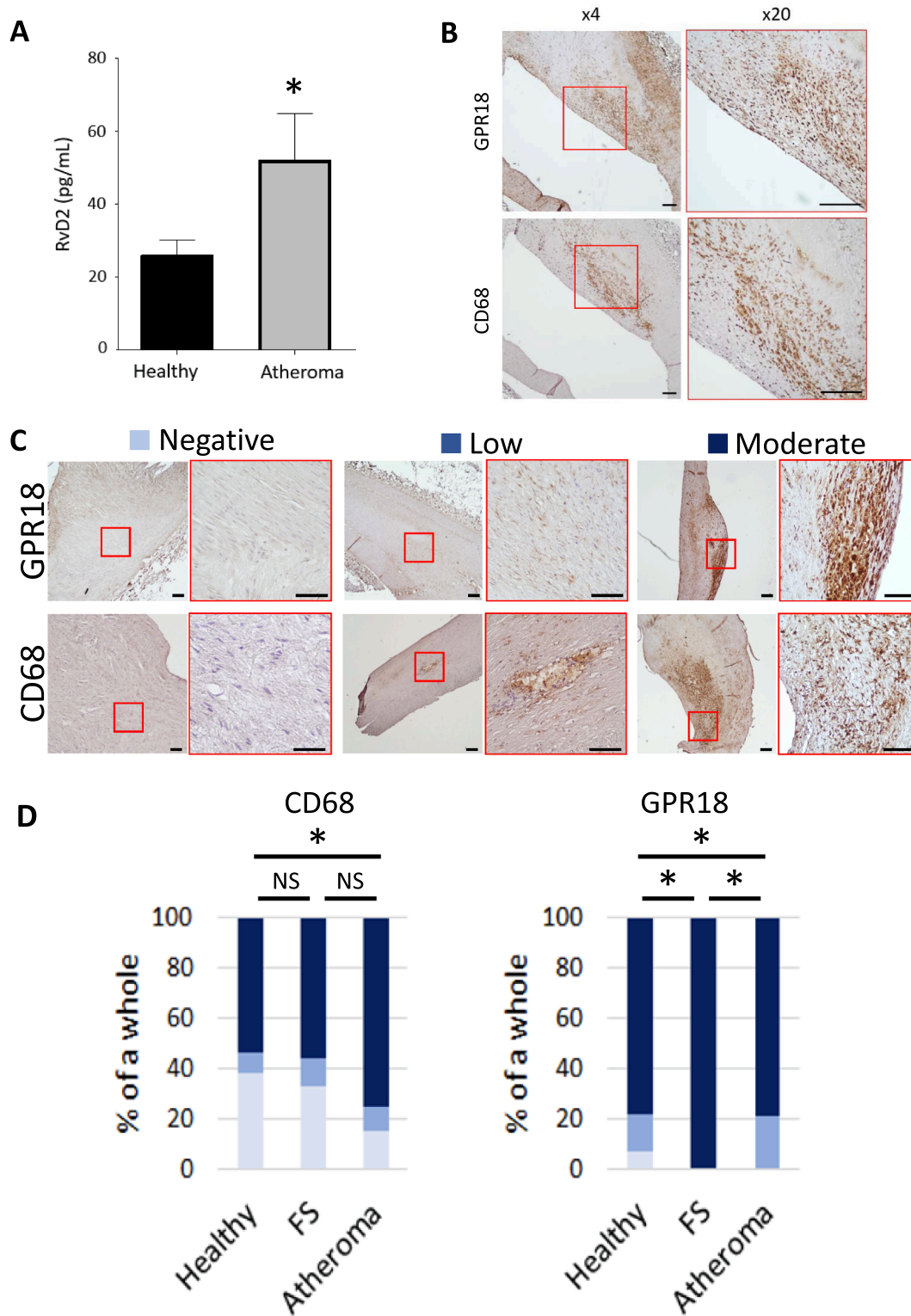
### 3.3. Necrotic core area decreased following RvD2 treatment

Necrotic core areas were measured in the aortic root (Fig. 3). In mice treated with RvD2, necrotic core areas were significantly reduced compared to mice from the control group [ $p = 0.011$ ] (Fig. 3A). In O-1918-treated mice, necrotic core areas were not significantly altered by the combination of O-1918 and RvD2 compared with O-1918 alone [ $p = 0.59$ ] (Fig. 3B).

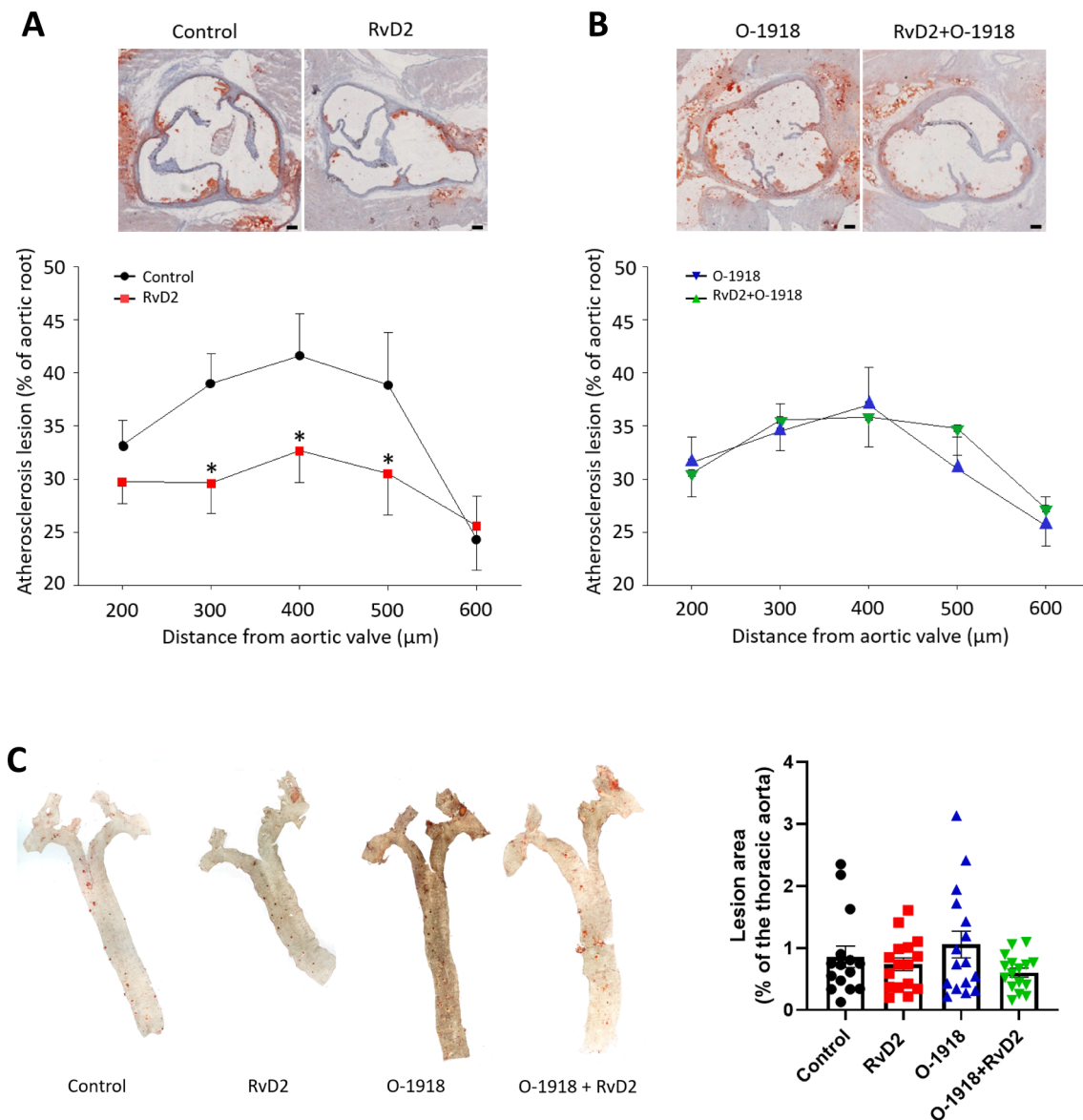
### 3.4. Plaque macrophage phenotypes

Since GPR18 expression was present in CD68 positive areas of human atherosclerotic lesions (Fig. 1B), macrophage markers were monitored in murine lesions. Aortic root sections positive for CD68 were stained for M2 macrophages markers and showed low expression of the M2 marker CD206 and a highly variable expression pattern for Arginase-1 (Arg-1). Staining for the M1 marker iNOS yielded a positivity of approximately 10% of the lesion area (Fig. 3C and D). To provide a quantification of the effects induced by the treatment on lesional macrophage subtypes, qPCR analysis was performed and revealed a significant decrease of aortic iNOS expression in the aortas of RvD2-treated compared with vehicle-treated mice (Fig. 3E). In mice treated with O-1918, aortic iNOS expression was not significantly altered by RvD2 (Fig. 3F). The qPCR analysis of aortic expression of CD68, CD206, and Arg-1 did not reveal any significant difference between the groups (data not shown).





**Fig. 1. Biosynthesis of resolvins D2 and expression of GPR18 receptor in human coronary arteries.** A. Measurement of resolvins D2 (RvD2) in conditioned media prepared from human healthy and atherosclerotic coronary endarterectomies. n = 7 in the healthy group and n = 23 in the atheroma group. B. Representative micrographs from atheroma coronary artery patient immunostained for GPR18 and CD68. C. Representative micrographs of coronary arteries with different classification of GPR18 and CD68 expression. D. Proportion of expression of CD68 and GPR18, respectively, in healthy, fatty streaks (FS), and atheroma arteries. Scale bar = 100  $\mu$ m. n = 14 in the healthy group, n = 9 in the fatty streak group, and n = 19 in the atheroma group. Data are presented as mean  $\pm$  SEM. \*p < 0.05, NS: not significant, differences in the RvD2 concentration were evaluated using a Mann-Whitney test, differences between groups in human immunohistochemistry classification were evaluated using Chi square test.



**Fig. 2.** Effect of RvD2 treatment on murine atherosclerosis development in the aortic root mediated through GPR18. **A.** Quantification of relative aortic root lesion size in ApoE<sup>-/-</sup> mice treated with RvD2 vs control mice (n = 11–15 per group). \*p < 0.05 vs Control (Two-way ANOVA followed by Tukey's multiple comparison) Representative images of aortic root sections stained with oil red O are shown above the graph. Scale bar = 100  $\mu\text{m}$ . **B.** Quantification of relative aortic root lesion size in ApoE<sup>-/-</sup> mice treated with O-1918 vs O-1918 + RvD2 treated mice (n = 11–15 per group). (Two-way ANOVA: NS) Representative images of aortic root sections stained with oil red o are shown above the graph. Scale bar = 100  $\mu\text{m}$ . **C.** Representative images of *en face*-stained thoracic aortas (left) and quantification of the relative plaque area (n = 15–16 per group). (One-way ANOVA: NS) Data are presented as mean  $\pm$  SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.5. Macrophage phagocytosis and red blood cell-dependent ROS formation

The ability of macrophages for phagocytosis is central in inflammation resolution. Therefore, we isolated peritoneal macrophages from the four groups of mice and measured uptake of zymosan. Zymosan uptake was linearly related to the cell density. Representative images are shown in Fig. 4A. Because the cell numbers varied between experiments, the FITC fluorescence for all technical repeats (n = 6 per mouse) were plotted against the number of cells in each evaluated area. The linear regression for controls (n = 60 from n = 10 mice, adjusted  $r^2 = 0.94$ ) had a slope of 0.51, whereas the linear regression for RvD2-treated mice (n = 60 from n = 10 mice, adjusted  $r^2 = 0.80$ ) had a slope of 0.95 (Fig. 4B). Multiple linear regression determined that phagocytosis (p = 0.009) but not cell number (p = 0.16) significantly predicted the control *versus*

RvD2-treated groups from a linear combination of the independent variables. The treatment with a combination of RvD2 with O-1918 reverted regression to the control (slope: 0.55, adjusted  $r^2 = 0.79$ , n = 60 from n = 10 mice) (data not shown).

Uptake mechanisms in atherosclerosis also involve red blood cells. Their ability to release haemoglobin followed by haeme oxidation induces a pro-atherogenic oxidant stimulus. Red blood cell-dependent ROS formation measured by EPR indicated a significantly reduced ROS production by red blood cells derived from RvD2- compared with vehicle-treated mice. (Fig. 4C). In contrast, ROS production in mice treated with O1918 was similar in the absence and presence of RvD2 treatments (Fig. 4C), representative curves of ROS measurements are shown in Fig. 4C.

**Table 1**

Blood cell count from high fat diet-fed ApoE<sup>-/-</sup> mice treated with vehicle (Control), RvD2, O-1918 or RvD2 + O-1918, respectively.

|  | Control     | RvD2        | O-1918      | O-1918 + RvD2 | p    |
|--|-------------|-------------|-------------|---------------|------|
| White blood cells - 10 <sup>3</sup> /mm <sup>3</sup> | 5.46 ± 0.82 | 6.37 ± 1.58 | 5.48 ± 0.71 | 5.98 ± 1.36   | 0.94 |
| Red blood cells - 10 <sup>6</sup> /mm <sup>3</sup>   | 8.01 ± 0.15 | 7.72 ± 0.2  | 7.82 ± 0.19 | 8.04 ± 0.23   | 0.64 |
| Hemoglobin - g/dl                                    | 11.7 ± 0.22 | 11.5 ± 0.21 | 11.5 ± 0.26 | 11.7 ± 0.33   | 0.87 |
| Hematocrit - %                                       | 36.6 ± 0.72 | 36.2 ± 0.71 | 35.5 ± 0.8  | 36.9 ± 1.1    | 0.86 |
| Platelet - 10 <sup>3</sup> /mm <sup>3</sup>          | 525 ± 38.97 | 544 ± 24.1  | 500 ± 39.1  | 494 ± 32.86   | 0.73 |
| MCV - μm <sup>3</sup>                                | 45.8 ± 0.14 | 46.8 ± 0.58 | 46.1 ± 0.2  | 46.0 ± 0.16   | 0.13 |
| MCHC - g/dl  | 32.0 ± 0.15 | 31.9 ± 0.14 | 32.0 ± 0.08 | 31.8 ± 0.12   | 0.67 |
| MPV - μm <sup>3</sup>                                | 6.45 ± 0.6  | 5.62 ± 0.25 | 5.38 ± 0.32 | 5.30 ± 0.21   | 0.15 |
| Lymphocytes - 10 <sup>3</sup> /mm <sup>3</sup>       | 3.63 ± 0.36 | 3.66 ± 0.34 | 4.13 ± 0.55 | 4.76 ± 1.19   | 0.68 |
| Monocytes - 10 <sup>3</sup> /mm <sup>3</sup>         | 0.31 ± 0.07 | 0.25 ± 0.05 | 0.36 ± 0.08 | 0.33 ± 0.09   | 0.81 |
| Granulocytes - 10 <sup>3</sup> /mm <sup>3</sup>      | 0.98 ± 0.17 | 0.76 ± 0.09 | 0.84 ± 0.09 | 0.89 ± 0.12   | 0.70 |

Results are expressed as mean ± SEM. MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MPC, mean platelet volume.

**Table 2**

Cholesterol and triglyceride blood levels from high fat diet-fed ApoE<sup>-/-</sup> mice treated with vehicle (Control), RvD2, O-1918 or RvD2 + O-1918, respectively.

|                        | Control     | RvD2        | O-1918      | O-1918 + RvD2 | p    |
|------------------------|-------------|-------------|-------------|---------------|------|
| Cholesterol (mmol/L)   | 151 ± 21.2  | 175 ± 35.6  | 122 ± 18.4  | 137 ± 28.6    | 0.55 |
| Triglycerides (mmol/L) | 1.92 ± 0.17 | 2.12 ± 0.23 | 1.98 ± 0.14 | 1.87 ± 0.18   | 0.77 |

Results are expressed as mean ± SEM.

#### 4. Discussion

The results of the present study point to the RvD2-GPR18 axis transducing atheroprotective effects. First, the ligand and receptor were identified in human coronary atherosclerotic lesions. Second, RvD2 treatment of mice decreased atherosclerosis burden and necrotic core size. Third, decreased aortic iNOS expression and increased macrophage phagocytosis were observed in RvD2-treated mice. Fourth, the RvD2-induced effects were not observed in the presence of the GPR18 antagonist O-1918. Taken together, these results suggest GPR18 activation by RvD2 as a possible driver of beneficial actions through an inflammation resolution in atherosclerosis development.

SPM may play a key role in inflammation resolution and in cardiovascular disease progression. The balance between pro-inflammatory and pro-resolving lipid mediators is of particular relevance since previous studies have identified a decreased SPM to leukotriene ratio in human vulnerable atherosclerotic lesions [14] and in association with subclinical atherosclerosis [19]. This is the first report of RvD2 release from human atherosclerotic lesions in conditioned media prepared from human coronary arteries. Previous reports indeed confirmed the ability of human atherosclerotic lesion to metabolize DHA into D-series resolvins, although RvD2 was undetectable from carotid arteries [14]. In the present study, an increased RvD2 release was observed from coronary arteries affected by atheroma. These findings are in contrast to the decreased levels of RvD2 with progression of murine atherosclerosis [8], which was confirmed in the present study.

Even if the specificity of RvD2 measurements by ELISA compared

with LC-MS/MS identification, the low cross-reactivity with structurally similar lipid mediators (e.g. 17-HDHA and RvD1) taken together with the shared biological activities between RvD2 stereoisomers support that that measured changes in lipid mediators between groups are comparable between the two platforms [20]. An increased RvD2 synthesis in atherosclerotic lesions is further supported when interrogating the STARNET (Stockholm-Tartu Atherosclerosis Reverse Networks Engineering Task) containing data on increased expression of the RvD2 biosynthetic 5- and 15 lipoxygenases in human atherosclerotic aortic root samples compared with atherosclerosis-free internal mammary artery [21].

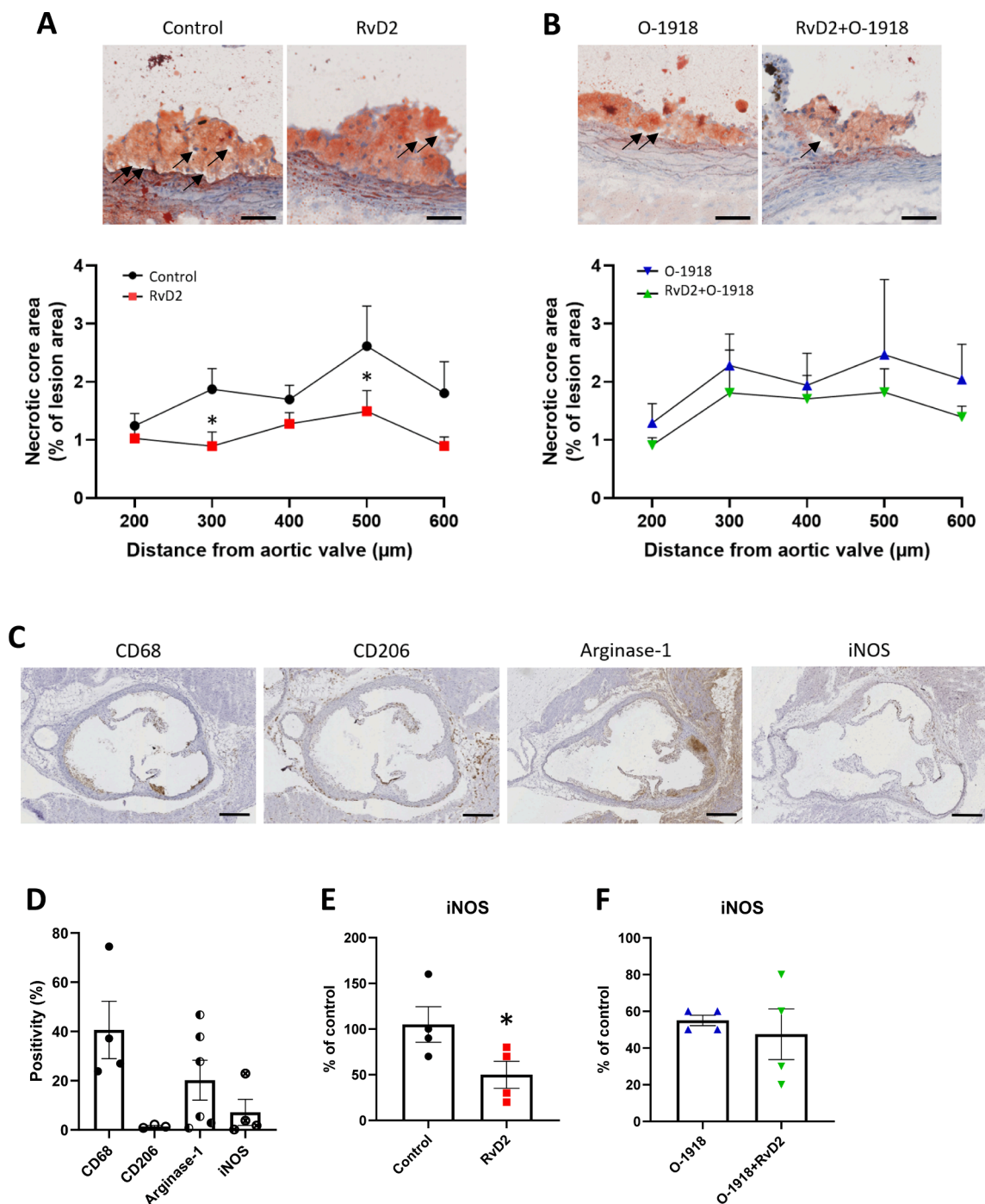
The next step in the regulation of pro-resolving effects is the expression of functional SPM receptors. We localized GPR18 expression in human atherosclerotic coronary arteries within CD68 positive areas, indicating macrophages as possible effectors of RvD2-induced effects. However, while the expression of CD68 was increasing with the coronary lesion progression, GPR18 exhibited an initial increase but decreased expression at more advanced stages. These results indicate that a functional RvD2 response towards a resolution of inflammation may be limited by a loss of GPR18 expression.

The notion of a beneficial role of RvD2 in atherosclerosis was raised by limitation of atheroprotection in mice treated with a combination of the two DHA metabolites RvD2 and maresin 1 [8]. However, the individual role of the two SPM and their receptors have not been previously investigated. Our results extend the findings by demonstrating that RvD2 treatment attenuated atherosclerosis. The GPR18 antagonist O-1918 exerted a tendency towards beneficial effects. O-1918 is however not a selective antagonist for GPR18 and has been reported to act also at GPR55 [22]. The decreased atherosclerosis by O-1918 in the present study may hence reflect effects on endocannabinoid signaling, which in addition to cannabinoid (CB) receptors can be transduced through GPR18 and GPR55. Importantly, RvD2 did not affect the atherosclerosis development in the presence of O-1918, pointing to RvD2 atheroprotective action being mediated by its ligation with GPR18.

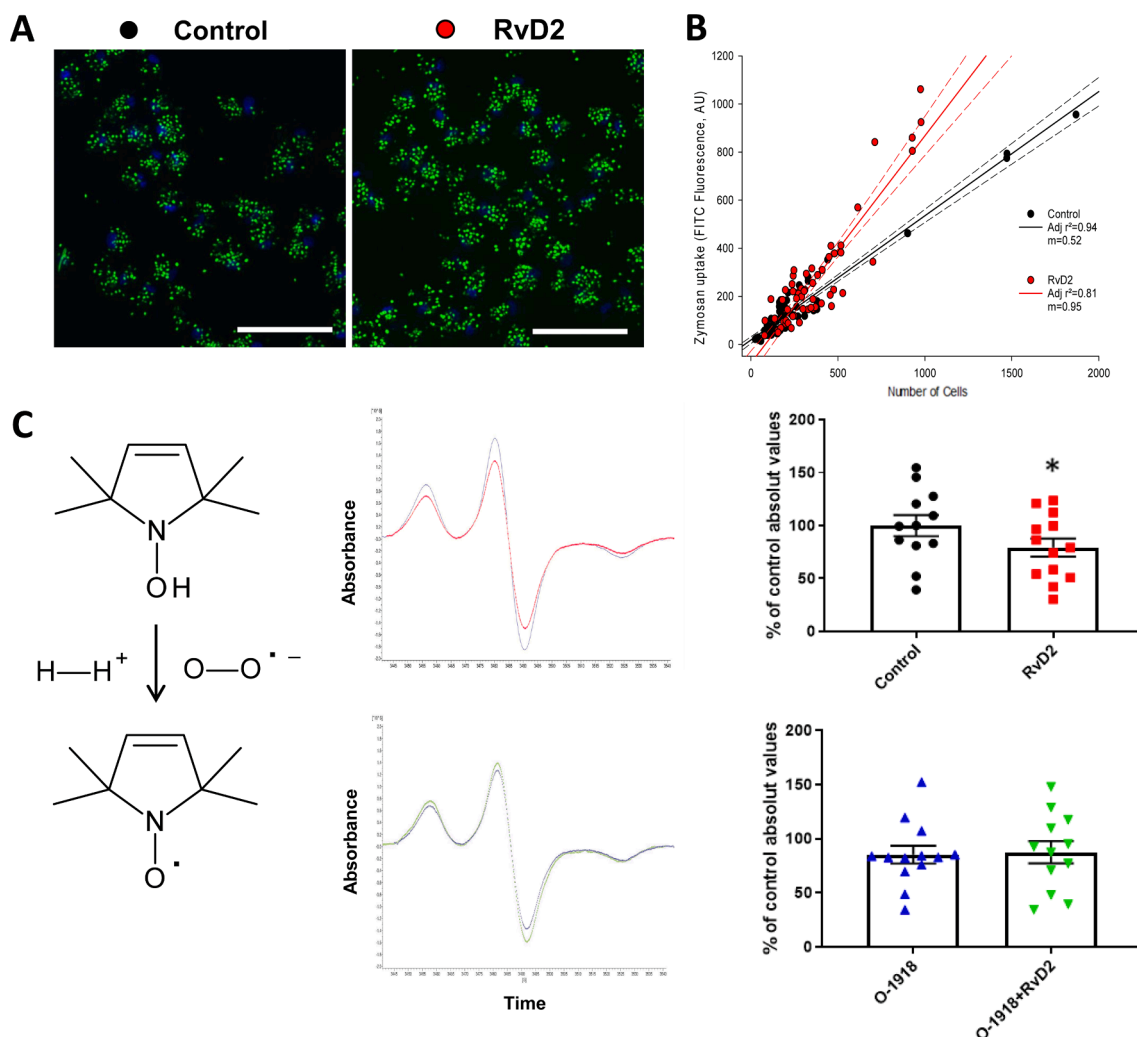
Another complexity of the SPM ligand-receptor interaction is the presence of pro-inflammatory agonists for SPM receptors. For example, the RvD1 and LXA4 receptor ALX/FPR2 activated by formylated bacterial peptides and pro-inflammatory serum amyloid A (SAA) transduces a pro-atherogenic response reflected by atheroprotection in mice lacking the murine ALX/FPR2 homologue when SAA prevails [15] but blunted effects of exogenous LXA4 administration [7]. Likewise, deletion of the RvE1 receptor ERV1/ChemR23 has been reported to either reduce [13] or enhance [10] atherosclerosis progression, which may depend on agonist availability for receptor ligation.

In addition to smaller lesion size, the necrotic core area was reduced in mice treated with RvD2, whereas RvD2 was devoid of significant effects on the necrotic core in the presence of the GPR18 antagonist O-1918. This is in line with the increased necrotic core in ChemR23 deficient ApoE<sup>-/-</sup> mice, [10], which may reflect the macrophage phenotypic change from pro-inflammatory towards pro-resolving subtypes [23,24] and increased phagocytic activity [10]. In line with the colocalization of GPR18 with macrophages in human plaques, we detected a decreased aortic iNOS expression in response to RvD2 in the absence but not in the presence of GPR18 antagonism, suggesting less pro-inflammatory macrophage phenotypes. RvD2 has previously been shown to facilitate macrophage differentiation towards pro-resolving phenotypes [25]. In addition, we found in macrophages isolated from the different groups of mice that RvD2 treatment resulted in an increased phagocytic activity which is a key mechanism of inflammation resolution and of importance to limit plaque development and enhance its stability [26]. The observation that RvD2 treatment did not alter cholesterol and triglycerides levels, which are of key importance in atherosclerosis development and progression, supports RvD2-induced atheroprotective effects that enhance macrophage phagocytosis.

Oxidative stress is another key mechanism in atherosclerosis development [27], in which erythrocytes play a major role [28]. When



**Fig. 3.** Relative necrotic core area and histological characterization of atherosclerotic plaques following RvD2 treatment and GPR18 inhibition, respectively. **A.** Quantification of the relative necrotic core area in aortic root plaques from ApoE<sup>-/-</sup> mice treated with RvD2 vs control mice (n = 11–14 per group). \*p < 0.05 vs Control (Two-way ANOVA followed by Tukey's multiple comparison). Representative images are shown above the graph. Scale bar = 50 μm. Black arrows indicate necrotic areas. **B.** Quantification of the relative necrotic core area in aortic root plaques from ApoE<sup>-/-</sup> mice treated with O-1918 vs O-1918 + RvD2 treated mice (n = 11–14 per group) (Two-way ANOVA: NS). Quantification of the relative necrotic core area in aortic root plaques from ApoE<sup>-/-</sup> mice treated with O-1918 vs O-1918 + RvD2 treated mice (n = 11–14 per group). (Two-way ANOVA: NS). Representative micrographs of immunohistochemistry staining with Hematoxylin are shown. Scale bar = 50 μm. Black arrows indicate necrotic areas. **C.** Representative micrographs of immunohistochemistry staining in the aortic root stained for CD68, CD206, Arginase-1, and iNOS, respectively, scale bar = 250 μm. **D.** Quantification of immunohistochemistry staining intensity in aortic roots stained for CD68, CD206, Arginase-1, and iNOS. **E.** Comparison of iNOS mRNA expression in the abdominal aorta obtained from control and RvD2-treated ApoE<sup>-/-</sup> mice (n = 4 per group). **F.** Comparison of iNOS mRNA expression in the abdominal aorta obtained from O-1918 and O-1918 + RvD2-treated ApoE<sup>-/-</sup> mice. Data are presented as mean ± SEM. Differences between two groups were evaluated using an unpaired student t test, \*p < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 4. Macrophage phagocytosis and red-blood cell-dependent formation of reactive oxygen species.** **A.** Representative images of peritoneal macrophages from ApoE<sup>-/-</sup> mice incubated with zymosan-FITC particles. Scale bar = 100  $\mu$ m. **B.** Quantification of phagocytosis expressed as zymosan uptake per number of macrophages. (n = 10 per group). **C.** Illustration showing oxidation of CMH-hydrochloride probe by ROS into EPR visible species (left). Representative curves obtained by EPR spectroscopy from the measurement of ROS produced by red blood cells (middle) and measurement of ROS production by red blood cell following CMH-hydrochloride incubation (right) (n = 11–12 per group). \*p < 0.05 vs Control (paired student *t* test). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phagocytized by vascular smooth muscle cells, the internalization of erythrocytes is responsible for a strong oxidative stress [29]. The use of EPR to investigate the RvD2-GPR18 axis on ROS production indicated a significant decrease in ROS production by erythrocytes in mice treated with RvD2 compared to control mice.

Since RvD2 and/or O-1918 treatments did not affect plaque size in the thoracic aorta while more advanced lesions in the aortic root were affected, the RvD2-GPR18 axis can be assumed to play a role in the progress of the disease rather than in its initiation. In human, atherosclerotic plaques progress from the aortic arch toward the thoracic aorta and are localized in areas with blood flow disturbance (e. g., posterior intercostal arteries) [30]. In this model of short time high fat diet-feeding, we mimicked the development of early plaque development as thoracic aortas plaques were barely detectable.

Taken together the data of the present study suggest that RvD2 protects against atherosclerosis through GPR18 in plaques at a certain stage of immune cell infiltration. Since RvD2 production was preserved in human coronary arteries while GPR18 was dysregulated, the notion of facilitating GPR18 signalling may represent a novel mean to obtain atheroprotection through a functional resolution of inflammation.

#### Author contribution

MBa, AM, JL, NM, SCP performed experiments and analyzed data. JBM provided human tissue sections and analyzed histology sections. MBa, MBä, SCP, NM, HA, VR, PL, BF designed and supervised the study. MBa, MBä, SCP, JL wrote the manuscript. All authors contributed to the final version of the manuscript.

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## **Résumé**

L'athérosclérose est une maladie cardiovasculaire qui se développe avec l'âge et qui est caractérisée par un dépôt de lipides au sein de la paroi artérielle pouvant à terme obstruer l'artère. Le développement de l'athérosclérose est accompagné par une inflammation chronique. Les acides gras oméga-3 ont montrés de fortes capacités protectrices contre le développement de maladies cardiovasculaires notamment en permettant la résolution de l'inflammation.

Afin d'étudier le rôle de la résolvine D2 (RvD2), un médiateur lipidique pro-résolutif dérivé de l'acide gras oméga-3 acide docosahexaénoïque, des souris déficientes pour l'apolipoprotéine E (ApoE) nourries avec un régime gras ont été utilisées. Ces souris ont reçu un traitement à la RvD2 et/ou au O-1918, un antagoniste du récepteur de la RvD2, le récepteur GPR18. La RvD2 diminue significativement de la taille des lésions au sein de la racine aortique. De plus, les mesures du cholestérol et des triglycérides circulants n'ont montré aucun effet des traitements sur ces deux paramètres. La capacité de phagocytose des macrophages intrapéritonéaux isolée des souris traitées à la RvD2 est également augmentée.

Sur des coupes d'artères coronaires humaines l'étude de l'expression du récepteur de la RvD2 montre une colocalisation avec  $\alpha$ SMA et CD68 suggérant respectivement une expression des récepteurs par les cellules musculaires lisses vasculaires et par les macrophages. Le dosage de la résolvine D2 dans des milieux conditionnés préparés à partir d'artères coronaires humaines a montré une augmentation de la synthèse de RvD2 dans les milieux obtenus à partir de donneur athéromateux comparé aux donneurs sains.

Ces résultats suggèrent que la RvD2 a un effet protecteur contre le développement de l'athérosclérose et que son mécanisme d'action ne passe pas par la diminution des concentrations de cholestérol et de triglycérides circulants. Les résultats obtenus avec les expériences de phagocytoses indiquent que l'effet athéroprotecteur de la RvD2 pourrait provenir de son action sur les macrophages en augmentant leur capacité de phagocytose. Une meilleure compréhension de ces voies de signalisation permettrait le développement de nouveaux traitements pour lutter contre les complications de l'athérosclérose.

**Mots clés :** athérosclérose, médiateurs lipidiques pro-résolutifs, macrophages, GPR18, RvD2

## **Abstract:**

Atherosclerosis is a cardiovascular disease developing with ageing characterized by lipid deposition inside the vascular wall and arteries narrowing. Atherosclerosis development is followed appearance of a chronic inflammation. Omega-3 fatty acids have a protective effect against cardiovascular diseases development, in particular by allowing the resolution of inflammation.

In order to study resolvin D2 (RvD2) actions, a pro-resolving lipid mediator derived from omega-3 fatty acid docosahexaenoic acid, apolipoprotein E (ApoE) deficient mice fed a high fat diet were used. These mice were treated with RvD2 and/or O-1918, an antagonist for GPR18, the RvD2 receptor. RvD2 treatment significantly decreased atherosclerotic lesions and necrotic cores in aortic root. Furthermore, treatments showed no impact on blood cholesterol and triglycerides levels. Phagocytosis assay on peritoneal macrophages showed that RvD2 increased phagocytosis.

On sections from human coronary arteries, studying the expression of GPR18 indicates a colocalization between with  $\alpha$ SMA and CD68 suggesting expressions by vascular smooth muscle cells and macrophages of this receptor. Resolvin measurement in conditioned media prepared from human coronary arteries showed an increase in RvD2 synthesis in conditioned media obtained from atherosclerotic arteries compared to healthy ones.

These results suggest that RvD2 has a protective effect against atherosclerosis development independent from circulating cholesterol and triglycerides levels. Results obtained from phagocytosis assay indicate that RvD2 protective effect may come from its actions on macrophages phagocytic capacity. A better understanding of these signaling pathway would allow to develop new drugs against atherosclerosis complications.

**Keywords:** atherosclerosis, specialized pro-resolving lipid mediators, macrophages, GRP18, RvD2