Étude du rôle des monocytes / macrophages et des micro-ARNs dans les anévrismes de l'aorte abdominale

Juliette Raffort

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UNIVERSITÉ CÔTE D'AZUR

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SCIENCES DE LA VIE ET DE LA SANTÉ

THÈSE DE DOCTORAT

Étude du rôle des monocytes/ macrophages et des micro-ARNs dans les anévrismes de l'aorte abdominale

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Étude du rôle des monocytes-macrophages et des micro-ARNs dans les anévrismes de l'aorte abdominale

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Titre : Etude du rôle des monocytes- macrophages et des micro-ARNS dans les anévrismes de l'aorte abdominale

Résumé

L'anévrisme de l'aorte abdominale (AAA) représente un problème majeur de santé publique et est associé à des taux extrêmement élevés de mortalité en cas de rupture aortique. Il est classiquement associé à l'athérosclérose et aux facteurs de risque cardiovasculaires, à l'exception du diabète qui jouerait un rôle protecteur dans la maladie. Bien que certaines études aient montré une infiltration de macrophages et une modification de l'expression des micro-ARNs dans la paroi anévrismale, leurs rôles respectifs dans le développement de l'AAA ne sont pas encore totalement élucidés. Même si les modèles expérimentaux classiques sont utiles pour adresser cette question, ils ne miment pas parfaitement la physiopathologie humaine. Nous avons développé un nouveau modèle d'AAA associant application topique d'élastase et neutralisation systémique du TGFβ permettant de reproduire les principales caractéristiques de l'AAA humain et induisant une rupture aortique.
Les objectifs de ce travail étaient de: -1/ Caractériser le phénotype des monocytes/ macrophages dans ce modèle murin d'AAA. -2/ D'étudier l'expression des micro-ARNs dans ce modèle. -3 / Les mécanismes impliqués dans la relation négative entre diabète et AAA étant à ce jour peu connus, le 3ème objectif était de mettre en place une étude clinique afin d'étudier l'expression des micro-ARNs chez des patients diabétiques ayant un AAA.
L'application d'élastase associée à la neutralisation du TGFβ chez des souris C57/Bl6j entrainait une augmentation significative de la densité de macrophages infiltrés dans le tissu aortique anévrismal. L'analyse des tissus aortiques a montré des modifications significatives des marqueurs des macrophages pro-inflammatoires dits « M1 » et des marqueurs des macrophages impliqués dans la réparation tissulaire, dits « M2 ». Afin de mieux comprendre le rôle des macrophages dans ce modèle murin, des injections de clodronate ont été réalisées pour dépléter ces cellules. L'injection de clodronate diminuait de façon significative la dilatation anévrismale et prévenait la rupture. Ceci était associé à une préservation de la matrice extracellulaire et une modification de l'expression de certains marqueurs des macrophages, dont l'arginase-1 (ARG1), molécule impliquée dans la réparation tissulaire. La proportion de macrophages exprimant l'ARG1 augmentait en fonction de la sévérité de l'AAA. Enfin, la neutralisation du TGFβ induisait une diminution significative d'un type particulier de monocytes dits « Sat-Mono», impliqués dans la fibrose. Cette étude a ainsi montré le rôle des macrophages dans le développement et la rupture anévrismale avec une modification de leur phénotype. 752 micro-ARNs ont ensuite été analysés dans le tissu aortique, ce qui a permis d'identifier les micro-ARNs dont l'expression était modifiée dans ce modèle par rapport au groupe contrôle. Enfin, l'expression des micro-ARNs a été recherché chez l'homme en mettant en place une étude clinique. Bien que l'AAA chez l'homme soit classiquement associé à l'athérosclérose et aux facteurs de risque cardiovasculaire, il est paradoxalement négativement associé au diabète. Les mécanismes en cause sont encore peu connus. Nous avons comparé l'expression des micro-ARNs entre des patients diabétiques et non-diabétiques présentant un AAA. Cette étude pilote a permis d'identifier des cibles potentielles impliquées dans l'association négative entre diabète et AAA.

Mots clés : anévrisme aorte, micro-ARNs, macrophages

Title: Monocytes/ macrophages and micro-RNAs in abdominal aortic aneurysm

Abstract

Abdominal aortic aneurysm (AAA) is a major public health concern and is associated with extremely high rates of mortality in case of aortic rupture. AAA is most often associated with atherosclerosis and cardiovascular risk factors, except diabetes that may rather play a protective role in the disease. Even though several studies have highlighted an infiltration of macrophages and changes of the expression of micro-RNAs in the aneurysmal wall, their role in AAA is still not fully understood. While experimental animal models are very useful to address this question, none of them perfectly mimics human pathophysiology. We recently created a new murine model of AAA based on topic application of elastase on the aorta associated with systemic TGFβ neutralization which reproduces the main human features of AAA and leads to fatal aortic rupture. The aims of this study were: -1/ Characterize the phenotype of monocytes/ macrophages in this murine model of AAA. -2/ Study the expression of micro-RNAs in this model. -3/ As the mechanisms involved in the negative association between diabetes and AAA are still poorly known, the third goal was to mount a clinical study to compare the expression of micro-RNAs between diabetic and non-diabetic patients with AAA.
Topic application of elastase associated with systemic TGFβ neutralization in C57/Bl6j male mice led to a significant increase of macrophage infiltration in the aneurysmal tissue. This was associated with changes of the gene expression of the markers of the pro-inflammatory macrophages, called “M1” and of the macrophages involved in wound healing, called “M2”. To investigate the role of macrophages in this model, we used liposomes containing clodronate injections to deplete these cells. This led to significant decrease of the aortic dilatation and prevented rupture. This was associated with a better preservation of the extracellular matrix and significant changes in the gene expression of the markers of macrophages including arginase-1 (ARG1), a molecule involved in would healing. The proportion of macrophages expressing ARG1 increased with the severity of the AAA. At last, TGFβ neutralization led to a significant decrease of a population of macrophages involved in fibrosis, called “Sat-Mono”. This study highlighted the role and the phenotypic changes of macrophages during AAA development. We then analyzed the expression of 752 micro-RNAs in the aneurysmal aortic tissue which allowed identifying the micro-RNAs whose expression varied in the murine model. At last, the expression of micro-RNAs was investigated in patients with AAA. We compared the expression of micro-RNAs between diabetic and non-diabetic patients with AAA. This pilot study led to the identification of micro-RNAs that could potentially represent new targets involved in the negative association between diabetes and AAA.

Keywords: aortic aneurysm, micro-RNAs, macrophages

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Article in press

2018 - « Diabetes and aortic aneurysm: current state of the art »
Cardiovascular Research *(IF :5.8)*
J. Raffort*, F. Lareyre, M. Clément, R. Hassen-Khodja, G. Chinetti, Z. Mallat*
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2017 - « Monocytes and macrophages in abdominal aortic aneurysm »
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J. Raffort, F. Lareyre, *M. Clément, R. Hssen-Khodja, G. Chinetti, Z. Mallat*
Nat Rev Cardiol. 2017 Aug;14(8):457-471. doi: 10.1038/nrcardio.2017.52. Epub 2017 Apr 13. Review.

2017: « TGFβ blockade induces a human-like disease in a non-dissecting mouse model of abdominal aortic aneurysm »
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F. Lareyre, M. Clément, ***J. Raffort****, S. Pohlod, M. Patel, B. Esposito, L. Masters, A. Finigan, M. Vandestienne, N. Stergiopulos, S. Taleb, B. Trachet, Z. Mallat*
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2018 - « Differential micro-RNA expression in diabetic patients with abdominal aortic aneurysm »
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2018- British Atherosclerosis Society- Cambridge- Royaume-Uni
«Differential micro-RNA expression in diabetic patients with abdominal aortic aneurysm»
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2018- Congrès annuel de la Société Française de Chirurgie Vasculaire, Nice – France
« La neutralisation du TGF β module le phénotype des macrophages dans le modèle murin d'anévrisme induit par l'élastase et entraine une augmentation de l'arginase-1 »
F. Lareyre, ***J. Raffort****, M. Clément, L. Masters, G. Chinetti, E. Jean-Baptiste, A. R. Hassen-Khodja, Z. Mallat*

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« Le TGFβ protège de la rupture aortique dans le modèle murin d'anévrisme induit par l'élastase »
F. Lareyre, M. Clément, M. Patel, ***J. Raffort****, R. Assil, A. Finigan, L. Master, R. Hassen-Khodja, Z. Mallat.*

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2016- BHF Cambridge Centre of Excellence – Cardiovascular Event, Cambridge – Royaume-Uni
« La neutralisation du TGFβ induit une rupture anévrismale chez la souris. »
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LISTE DES ABREVIATIONS

AAA : anévrisme de l'aorte abdominale

ADN : acide désoxyribonucléique

ARG1: arginase1

ARNm: ARN messager

C/EBP$_{\beta}$: CCAAT/ enhancer-binding protein-β

CCL: CC-chemokine ligand

CCR : CC-chemokine receptor

CEACAM1: carcinoembryonic antigen-related cell adhesion molecule 1

Chi3l1: chitinase 3 like 1

CMLs : cellules musculaires lisses

CX$_3$CL1: CX$_3$C-chemokine ligand 1

CX$_3$CR1: CX$_3$C-chemokine receptor 1

E : élastase

EI : élastase inactivée

HbA1c: hémoglobine glyquée A1c

HDL: High-Density-Lipoprotein

HIF1α: hypoxia-inducible factor 1α

IFNγ: interferon γ

IL-1β: interleukin-1β

KLF4: Kruppel-like factor 4

LDL: Low-Density-Lipoprotein

Ldlr: low-density lipoprotein receptor

LTB4: leukotriene B4

LY6C: lymphocyte antigen 6C

LPS: lipopolysaccharide

MEC: matrice extra-cellulaire

MGL1: macrophage galactose-type lectin 1

miR: micro-ARN

MHC II : major histocompatbility complex class II

MSR1: macrophage scavenger receptor type 1

MMPs: métallo-protéinases

NF-κB: nuclear factor κB

NK : natural killer

NOS2: nitric oxide synthase 2

NR4A1: nuclear receptor subfamily 4 group A member 1

O.C.T: Optimal Cutting Temperature compound

PBS : Phosphate Buffered saline

PBMCs: cellules sanguines mononuclées circulantes

PFA : paraformaldehyde

qPCR : quantitative polymerase chain reaction

RAGE: receptor for advanced glycation end products

RISC: RNA Induced Silencing Complex

Sat-Mono: segregated-nucleus-containing atypical monocytes

STAT6: signal transducer and activator of transcription 6

TGFβ : Transforming Growth Factor-β

TIMPs: Tissue Inhibitor of Matrix Metalloprotéinases

TNFα: Tumor Necrosis Factor

VEGF: Vascular Endothelial Growth Factor

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INTRODUCTION

1. Anévrisme de l'aorte abdominale (AAA)

1.a. Définition, épidémiologie et prise en charge

L'anévrisme de l'aorte abdominale (AAA) correspond à une dilatation d'un segment plus ou moins long de l'aorte dans sa portion abdominale, avec une perte du parallélisme de ses bords [1-3]. En pratique, le diagnostic d'AAA est porté lorsque la dilatation aortique est supérieure à 30 mm de diamètre [1,3]. De par sa fréquence et sa gravité, l'AAA représente un problème majeur de Santé Publique. Différentes études épidémiologiques ont estimé la prévalence de l'AAA entre 4 et 8% chez les hommes et entre 0.7% et 2.2% chez les femmes de plus de 60 ans [1,4]. La maladie est le plus souvent asymptomatique et découverte de façon fortuite au cours de la réalisation d'un examen d'imagerie ou peut être révélée par la survenue de complications. La principale complication des AAA est la rupture anévrismale, associée à des taux de mortalité très élevés. En effet, malgré l'évolution et les progrès des techniques chirurgicales, une méta-analyse estimait la mortalité à 48% des patients opérés pour un AAA rompu [5]. D'autres études rapportaient des taux similaires avec 34.3% et 38% de mortalité à 90 jours après traitement chirurgical d'AAA rompus par voie ouverte ou endovasculaire [6]. La mortalité annuelle globale liée à l'AAA a été estimée à 6 200 personnes en moyenne aux Etats-Unis et 5300 personnes au Royaume Uni [7].

Du fait de l'extrême gravité de la pathologie en cas de rupture et d'une prévalence plus élevée chez l'homme, les recommandations actuelles ont montré l'intérêt du dépistage de l'AAA par échographie chez les hommes de plus de 60 ans [1]. A l'heure actuelle, le traitement curatif de l'AAA est uniquement chirurgical et peut être réalisé par voie ouverte ou endovasculaire [1,3]. En pratique, la décision de traiter les patients repose sur la balance entre le risque opératoire et le

risque de croissance et de rupture de l'AAA [1,3]. Les anévrismes symptomatiques nécessitent une prise en charge en urgence. Pour les AAA asymptomatiques, il est en général admis que les AAA de petite taille (inférieurs à 40 mm de diamètre) et à faible risque de rupture nécessitent une surveillance régulière ; les AAA de plus grande taille (supérieurs à 54 mm de diamètre) ou à haut risque de rupture constituent une indication chirurgicale. Aucun traitement pharmacologique n'a à ce jour montré une efficacité suffisante pour être couramment utilisé dans la prise en charge des patients atteints d'AAA. Cela souligne la réelle nécessité de mieux comprendre les mécanismes aboutissant au développement de la maladie dans le but d'identifier de nouvelles cibles thérapeutiques.

1.b. Facteurs de risque

Les études épidémiologiques ont permis d'identifier différents facteurs de risque aboutissant au développement, à la croissance et à la rupture de l'AAA [1,4]. L'AAA est le plus souvent associé à l'**athérosclérose**. L'athérosclérose correspond à un remaniement pathologique des artères de gros et moyens calibres à l'origine de nombreuses maladies cardiovasculaires tels que l'insuffisance coronaire, la sténose carotidienne ou l'artériopathie des membres inférieurs. Bien que les liens entre AAA et athérosclérose ne soient pas totalement élucidés, ces deux pathologies présentent des facteurs de risque communs.

Tout d'abord, l'incidence de l'AAA augmente avec l'**âge** et il est estimé que le risque de développer un AAA augmente de 40% tous les 5 ans au-delà de 65 ans [8]. Le **sexe masculin** a été identifié comme un facteur de risque, la prévalence de l'AAA étant en moyenne 6 fois plus élevée chez l'homme [9]. Les causes du risque d'AAA plus élevé chez l'homme ne sont pas complètement comprises mais seraient probablement plurifactorielles et en partie liées à des facteurs hormonaux, génétiques et environnementaux prédisposants [4].

Le **tabagisme** est un facteur de risque bien établi et les fumeurs présentent un risque relatif de développer un AAA de 7.6 [10,11]. De plus, le tabagisme favorise la croissance anévrismale [12].

Les **dyslipidémies** sont un facteur de risque classique d'athérosclérose. L'association entre dyslipidémies et AAA est complexe. En effet, différentes études ont montré qu'un taux sérique de cholestérol total élevé était associé à un risque plus élevé d'AAA [13-15]. Cependant, une autre étude épidémiologique rétrospective n'a pas reproduit ces résultats et a identifié un effet protecteur des concentrations élevées du HDL-cholestérol [16]. Réciproquement, la baisse du HDL-cholestérol est associée à une dilatation anévrismale plus importante tant chez l'homme que dans un modèle animal [17,18]. Le traitement des dyslipidémies peut également lui-même influencer le développement de l'AAA même si l'impact des traitements hypolipémiants n'est pas encore clairement compris. Certaines études montrent un effet protecteur des statines sur la croissance anévrismale alors que d'autres rapportent un risque accru de développer un AAA [15,19].

L'**obésité** a été identifiée comme un facteur de risque indépendant d'AAA, différentes études épidémiologiques ayant montré une association positive entre l'indice de masse corporelle, la circonférence abdominale et le risque d'AAA [20,21].

L'**hypertension artérielle** constitue un facteur de risque bien établi d'athérosclérose mais l'association entre hypertension et risque d'AAA reste faible [22,23]. En effet, bien qu'une élévation de la tension artérielle ait été identifiée comme un facteur de risque indépendant de rupture aussi bien chez l'homme que chez la femme [24], l'hypertension semble associée au risque d'AAA uniquement chez la femme [15]. De plus, l'hypertension favorise la croissance anévrismale dans certains modèles expérimentaux [25] mais son effet chez l'homme n'a pas été démontré.

A l'inverse des autres maladies cardiovasculaires, il existe paradoxalement une association négative entre **diabète** et anévrisme, suggérant un effet protecteur de ce dernier sur l'AAA [26,27].

Les patients diabétiques présentent une prévalence, une incidence, une croissance anévrismale ainsi qu'un risque de rupture plus faible que les patients non diabétiques [26,27].

Enfin, les **antécédents familiaux** constituent un facteur de risque d'AAA. La probabilité qu'un jumeau homozygote d'une personne atteinte d'AAA développe la maladie a été estimé à 24% [28]. Un antécédent familial d'un parent du premier degré est associé à un risque multiplié environ par 2 de développer un AAA [29]. Cette prédisposition génétique est complexe impliquant plusieurs gènes de susceptibilité [30,31]. Des études d'associations pangénomiques (genome wide association studies) ont permis d'identifier différents variants associés à l'AAA [32]. De plus, certaines mutations du gène codant pour la fibrilline 1 ou encore le récepteur du Transforming Growth Factor-β (TGFβ) ont été mis en évidence chez les patients ayant un anévrisme syndromique tel que le syndrome de Marfan ou encore le syndrome de Loeys-Dietz [33].

1.c. Physiopathologie

1.c.1. Structure de la paroi aortique normale

D'un point de vue anatomique, l'**aorte** est une artère qui nait du ventricule gauche au niveau du cœur et qui est classiquement divisée en plusieurs segments (Figure 1). L'aorte thoracique correspond à la portion sus-diaphragmatique et comprend trois parties : aorte ascendante, crosse de l'aorte et aorte descendante. L'aorte abdominale correspond à la portion naissant sous le diaphragme jusqu'à la bifurcation iliaque. La dilatation anévrismale peut être observée sur la portion thoracique et/ou abdominale de l'aorte. Néanmoins, chez l'homme, l'anévrisme survient le plus souvent au niveau de l'aorte abdominale sous rénale.

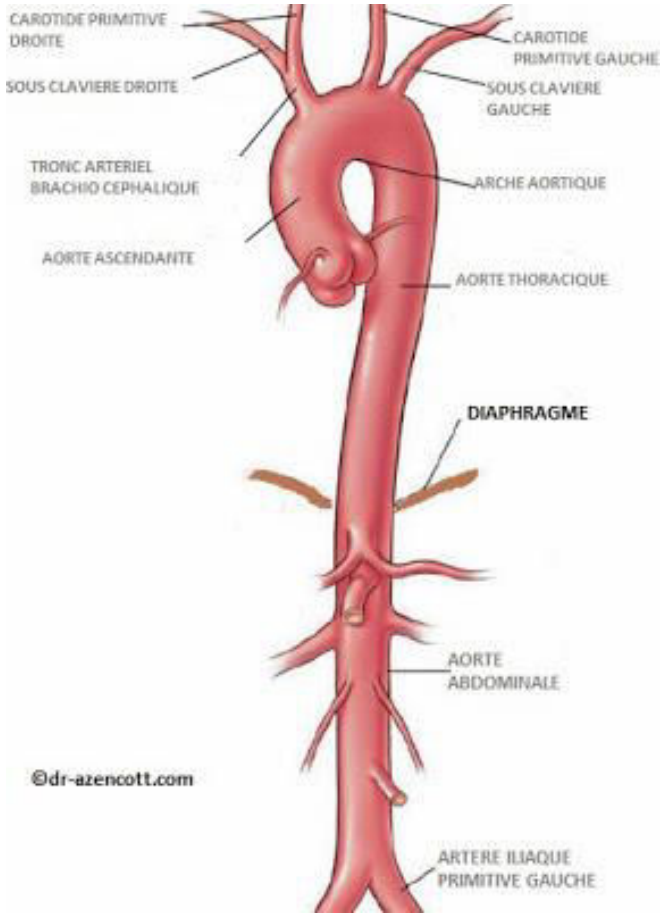

Figure 1 : Schéma illustrant les principaux segments aortiques

A l'état physiologique, l'aorte possède une paroi tubulaire aux bords parallèles. Celle-ci est composée de 3 tuniques principales, de l'intérieur vers l'extérieur : l'intima, la média et l'adventice (Figure 2).

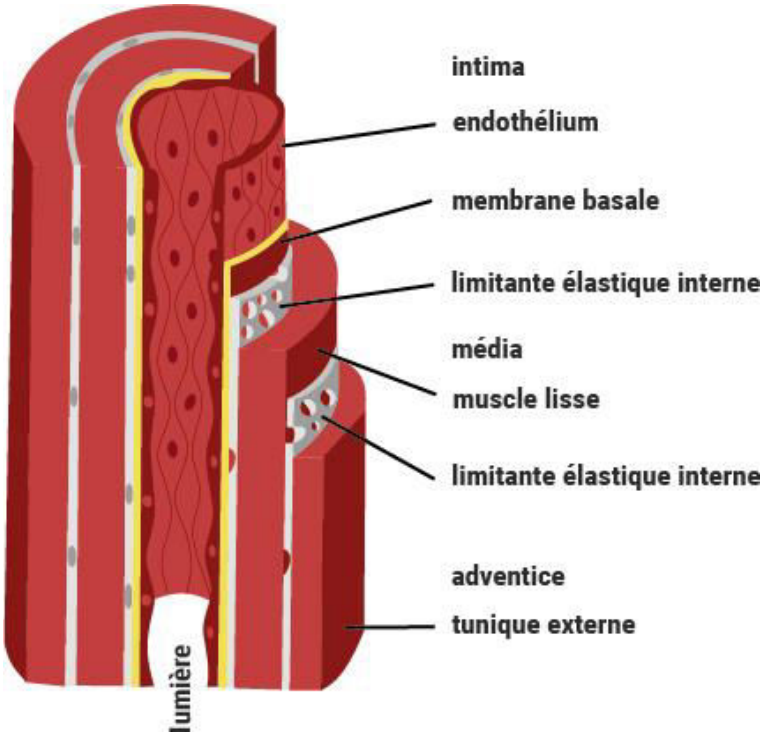

Figure 2 : Organisation de la paroi artérielle normale

L'**intima** est la tunique la plus interne. Elle est constituée d'une monocouche de cellules endothéliales ainsi que d'un tissu conjonctif sous endothélial. L'intima constitue à la fois une barrière physiologique et une surface d'échange entre le sang circulant et les couches sous-endothéliales. Elle est très sensible aux stimuli mécaniques imposés par l'écoulement sanguin et régule en conséquence le remodelage de la paroi vasculaire. Elle joue un rôle clé dans la régulation de la vasomotricité et de la coagulation. Elle est séparée de la média par la lame limitante élastique interne.

La **média** est la couche intermédiaire délimitée par deux lames limitantes élastiques (interne et externe). Elle est constituée d'un réseau tridimensionnel de cellules musculaires lisses (CMLs) disposées en anneaux et de composants de la matrice extra-cellulaire synthétisés par les CMLs elles-mêmes [34]. Les CMLs jouent un rôle fondamental dans la structure de l'artère en régulant le tonus vasculaire nécessaire au maintien de la pression artérielle et à la perfusion des tissus [35]. Les CMLs ont une grande plasticité et peuvent adopter différents phénotypes. Les CMLs différenciées ayant un phénotype contractile sont caractérisées par l'expression de différentes

protéines du cytosquelette telles que l'actine et la myosine permettant la contraction des cellules [35]. Sous l'influence de facteurs environnementaux, les CMLs peuvent se dé-différencier vers un phénotype prolifératif et sécrétoire [36,37]. En cas de lésion vasculaire, elles ont la capacité de se dé-différencier, d'augmenter leur prolifération, leur migration ainsi que la synthèse de protéines de la matrice-extra-cellulaire pour favoriser la réparation tissulaire [35]. Elles participent également à la néo-angiogénèse jouant ainsi un rôle clé dans le remodelage vasculaire [38]. Elles sont à l'origine de la synthèse des protéines de la matrice extra-cellulaire.

La **matrice extra-cellulaire (MEC)** est composée d'un assemblage de macromolécules et constitue une charpente dans laquelle sont intégrées les CMLs. Les CMLs synthétisent ainsi la MEC qui les entoure et en retour cette matrice influence le phénotype des cellules [39]. Elle est principalement composée de 4 grandes familles : les collagènes, les fibres élastiques, les glycoprotéines de structure et les protéoglycanes.

- Les **collagènes** sont des protéines fibreuses volumineuses représentant la majorité des protéines de la MEC. Ils assurent la résistance aux forces d'étirement et de tension et contribuent à la rigidité de l'artère. Le collagène est formé de 3 chaînes alpha assemblées en triple hélice. Chaque chaîne alpha est constituée d'une séquence répétitive caractéristique composée de 3 acides aminés Glycine-X-Y, X et Y étant le plus souvent la proline et l'hydroxyproline [40]. Les chaînes alpha sont synthétisées sous forme de pro-peptides dans le réticulum endoplasmique, puis s'assemblent en triple hélice pour former le pro-collagène. Les molécules de pro-collagène sont sécrétées par exocytose puis clivées pour former les molécules de collagène. Ces dernières peuvent ensuite s'auto-assembler en fibrilles ou en réseau. Plus d'une quarantaine de chaînes alpha différentes ont été identifiées et leur assemblage dans des combinaisons variées a permis de définir différents types de collagène [41,42]. Les principaux types de collagène retrouvés au niveau de la paroi artérielle s'organisent en fibrilles et correspondent au collagène de type I, III et V.

- Les **fibres élastiques** sont composées de molécules d'élastine associées à des glycoprotéines de structure. Elles sont organisées en lames concentriques et assurent la souplesse et l'élasticité de la paroi artérielle. L'élastine est synthétisée sous la forme d'un précurseur hydrophobe, la tropo-élastine. Après sécrétion, les molécules de tropo-élastine s'assemblent entre elles pur former un polymère insoluble conférant l'élasticité à la paroi artérielle [43].

- Les **glycoprotéines de structure** correspondent à des protéines sur lesquels sont greffées de courtes chaînes glucidiques jouant un rôle clé dans l'organisation de la MEC et dans les interactions entre les cellules et la MEC [44]. Il en existe une grande variété dont notamment la fibronectine, les laminines, les nidogènes, les ténascines et l'ostéopontine.

- Enfin, les **protéoglycanes** sont des molécules constituées d'une partie protéique sur laquelle sont accrochées un ou plusieurs chaînes polysaccaridiques appelées glycosaminoglycanes (GAG) [45,46]. Elles sont caractérisées par la présence de groupements carboxyliques et sulfates, leur conférant une forte densité de charge négative. Elles sont très hydrophiles et sont capables d'attirer et de fixer des quantités importantes d'eau de par leurs propriétés osmotiques. Leur propriété d'hydratation permet de conférer à la paroi artérielle une résistance aux forces de compression.

L'**adventice** est la tunique la plus externe de la paroi artérielle et est séparée de la média par la lame limitante élastique externe. Elle est composée d'un tissu conjonctif riche en fibres de collagène, principalement produite par des fibroblastes. Les fibres de collagène permettent de protéger les vaisseaux et de les ancrer aux structures environnantes. L'adventice est parcourue de vaisseaux nourriciers appelés *vasa vasorum* [47]. On y également trouve également des terminaisons nerveuses ainsi que des cellules immunitaires.

1.c.2. Principaux mécanismes aboutissant à l'AAA

Les mécanismes contribuant au développement de l'AAA sont complexes et plurifactoriels, impliquant un remodelage de l'ensemble de la paroi artérielle aboutissant à la dilatation et à la perte du parallélisme de celle-ci (Figure 3).

L'AAA est associé à un **remodelage de la MEC**. Histologiquement, il existe une fragmentation et une destruction des fibres de collagène et d'élastine de la MEC, ce qui va contribuer à fragiliser la paroi et favoriser la rupture [48,49]. A l'état physiologique, il existe un équilibre entre la synthèse des protéines de la MEC et leur dégradation par des protéases telles que les cathépsines, les cystéine-protéases, les sérines protéases ou les métallo-protéinases (MMPs). Au cours de l'AAA, il existe une augmentation de l'activité de ces protéases entrainant une dégradation accrue des composants de la MEC, diminuant la résistance et l'élasticité de la paroi et favorisant la dilatation aortique. Ces protéases sont produites par les CMLs ainsi que par les cellules immunitaires infiltrant la paroi aortique dont les macrophages et les polynucléaires neutrophiles. Leur rôle a été confirmé dans différents modèles expérimentaux où leur délétion génétique avait un effet protecteur sur la formation d'AAA [21,50-52]. De plus, chez l'homme l'expression sérique de certaines MMP comme la MMP-9 est augmentée chez les patients porteurs d'AAA par rapport aux sujets contrôle [53,54]. L'activité des MMPs est physiologiquement régulée par des inhibiteurs nommés Tissue Inhibitor of Matrix Metalloprotéinase (TIMPs). Au cours de l'AAA, il existe un déséquilibre entre les protéases et leurs inhibiteurs dont les TIMPs favorisant la dégénérescence protéolytique [55,56]. Il a été observé chez l'homme une diminution de l'expression des TIMP-1 et TIMP-2 dans le tissu anévrismal comparé au tissu aortique sain [57]. Le rôle des inhibiteurs de protéases a été confirmé dans différents modèles expérimentaux où leur surexpression protégeait de l'AAA [58-60].

En plus de la dégradation de la MEC, la média subit de profonds remaniements liés à l'**altération de l'homéostasie des CMLs**. Le rôle des CMLs est complexe puisqu'elles sont nécessaires à la production des composantes de la MEC mais sont également capables de

sécréter des facteurs pro-inflammatoires ainsi que des protéases favorisant la dégradation de la paroi [61]. La modulation de leur phénotype joue ainsi un rôle clé dans la pathogénèse anévrismale. Au cours de l'AAA, il existe une diminution de la densité en CMLs au niveau de la paroi aortique [62,63]. Cette perte des CMLs est en partie liée à une augmentation de leur apoptose favorisée par la sécrétion de facteurs pro-inflammatoires tels que le TNFα, l'IFNγ, l'IL-1β, de perforines et de l'activation de signaux de mort par les cellules immunitaires [63-65]. Le stress oxydatif et la production de radicaux libres impacte également la survie des CMLs en favorisant leur apoptose [66]. Enfin, la dégradation protéolytique induit une altération des interactions entre les cellules et la MEC, diminuant l'ancrage des CMLs et favorisant leur destruction par anoïkis [67,68].

La **réponse inflammatoire** et l'**infiltration de cellules immunitaires** au sein de la paroi aortique jouent un rôle clé dans la genèse de l'AAA. Cela implique aussi bien les cellules de l'immunité innée comme les macrophages, les polynucléaires neutrophiles, les cellules NK ou les mastocytes, que les cellules impliquées dans la réponse immune adaptative comme les lymphocytes T et B [69-72 73]. Ces cellules proviennent à la fois de la circulation sanguine et des *vasa vasorum* [74-76] et vont pénétrer et infiltrer la média et l'adventice. Elles sont responsables de la sécrétion de nombreux facteurs inflammatoires comme des cytokines, des leucotriènes, des chemokines ou des immunoglobulines [70,71] qui vont contribuer à remodeler la paroi artérielle. Leur recrutement au sein de la paroi artérielle est favorisé par la dégradation de la MEC et va être entretenu et amplifié par les cellules immunitaires elles-mêmes [77-79]. Chez l'homme, les cellules prépondérantes dans l'infiltrat inflammatoire sont principalement les macrophages ainsi que les lymphocytes B et T [80,81].

Le développement de l'AAA chez l'homme est le plus souvent associé à la formation d'un **thrombus** au sein de la lumière artérielle. Son apparition est favorisée par les modifications hémodynamiques liées à la formation de l'AAA. La paroi aortique en regard du thrombus est

en général plus fine et la dégradation de la MEC y est accrue. Le thrombus est en effet une source importante de protéases dont la MMP-9 qui va contribuer à dégrader la paroi [82]. De plus, il est riche en polynucléaires neutrophiles qui vont sécréter des facteurs pro-inflammatoires contribuant ainsi au recrutement d'autres leucocytes [82,83]. Le thrombus intra-luminal joue un rôle critique dans l'aggravation de l'AAA puisque sa croissance aggrave la dilatation anévrismale et favorise la rupture [84].

Enfin, la formation de nouveaux vaisseaux au sein de la paroi aortique, processus nommé **néo-angiogenèse,** va jouer un rôle déterminant dans la progression et la rupture de l'AAA [85,86]. Parmi les médiateurs impliqués dans la néo-angiogenèse, le Vascular Endothelial Growth Factor (VEGF) est augmenté dans le tissu anévrismal aussi bien chez l'homme que dans les modèles expérimentaux [87,88]. L'utilisation d'agents anti-angiogéniques dont des inhibiteurs de la voie de signalisation du VEGF a montré un rôle protecteur dans la survenue d'AAA [87,89].

Bien que les principales caractéristiques physiopathologiques aboutissant à la formation, la progression et la rupture anévrismale aient été décrites, les mécanismes cellulaires et moléculaires ne sont pas encore totalement élucidés.

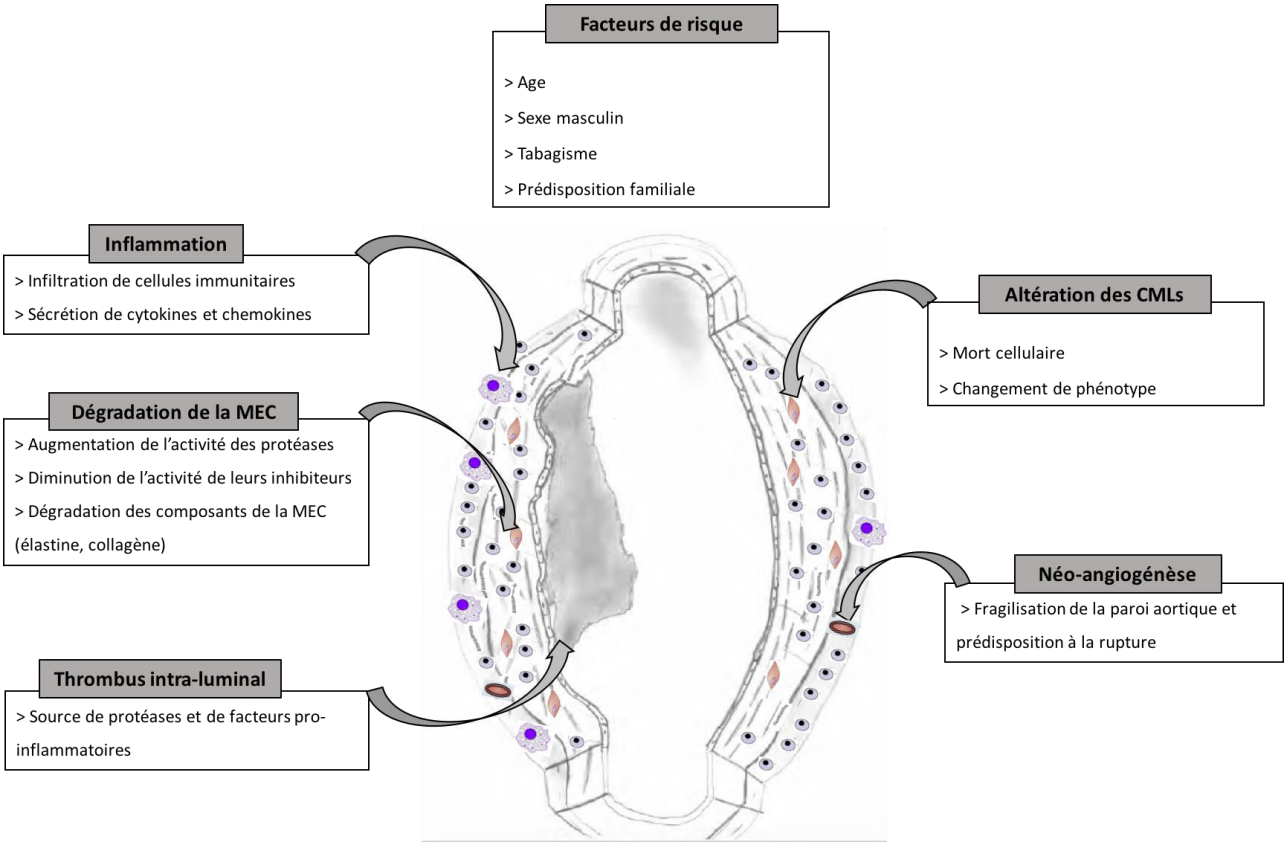

Figure 3 : Principaux mécanismes impliqués dans la pathogénèse de l'AAA (adapté d'après Raffort et al. [90])

1.d. Modèles expérimentaux d'AAA

1.d.1. Principaux modèles d'AAA

En vue de mieux comprendre les mécanismes impliqués dans la pathogenèse anévrismale, différents modèles animaux d'AAA ont été développés [91-94]. Ces modèles peuvent être induits dans différentes espèces animales dont le cochon, le mouton, le lapin, le cygne ou encore le rat et la souris [93]. Les modèles murins ont l'avantage d'être relativement peu coûteux, faciles à mettre en place et permettent de réaliser des manipulations génétiques. De par ces avantages, ils sont le plus souvent utilisés. On distingue 3 grands types de modèles en fonction de la méthode d'induction de l'AAA : les modèles chirurgicaux, génétiques, ou chimiques.

Les **modèles chirurgicaux** permettent d'induire directement l'AAA en imposant une contrainte mécanique sur la paroi aortique. Différentes techniques ont été développées comme notamment l'utilisation de patchs veineux ou synthétiques après réalisation d'une incision au niveau de la paroi aortique qui permettent d'entrainer une dilatation anévrismale [95,96]. Une autre méthode d'induction d'AAA consiste à réaliser une xénogreffe en transplantant du tissu aortique d'une espèce sur l'aorte abdominale d'un animal d'une autre espèce [97-99]. La réaction immune engendrée par la transplantation va induire une dégradation de la paroi aortique à l'origine de l'anévrisme. Bien que ces modèles soient utiles, ils ne reproduisent pas parfaitement les caractéristiques de la physiopathologie humaine et nécessitent l'utilisation d'animaux de grande taille, limitant leur utilisation en pratique [91].

Les **modèles génétiques** présentent l'avantage de permettre d'étudier spécifiquement un gène ou une voie de signalisation dans la pathologie anévrismale. Ils sont le plus souvent induits chez la souris en raison de la facilité technique pour manipuler le génome dans cette espèce. Ces modèles ciblent des voies de signalisation clé de la pathogénèse anévrismale comme des molécules impliquées dans le remodelage de la MEC incluant les TIMP et les MMPs, ou d'autres cibles impliquées dans la réponse inflammatoire comme le leukotriene B4 (LTB4) [93]. Ces modèles sont très utiles car ils permettent une approche mécanistique. Néanmoins, chez l'homme, bien qu'une prédisposition familiale puisse favoriser le développement de certains AAA, les mécanismes sont complexes impliquant plusieurs gènes de susceptibilité.

De par leur facilité d'utilisation, les **modèles chimiques** sont actuellement les plus couramment réalisés, dont les principaux sont induits par l'application d'élastase ou de chlorure de calcium ou à la perfusion d'angiotensine II.

- Le **modèle à l'élastase** a été décrit pour la première fois chez le rat et consistait à perfuser de l'élastase pancréatique porcine pendant 2 heures après exposition chirurgicale de l'aorte [100]. Depuis, le protocole a été adapté et peut être utilisé chez la souris avec des temps de perfusion

plus courts entre 5 et 15 minutes [50,101]. L'élastase peut aussi directement être appliquée au niveau de l'adventice [102]. L'élastase va induire une dégradation des fibres élastiques ainsi qu'un recrutement de cellules inflammatoires au sein de la paroi aortique aboutissant à la formation d'un AAA dans les 14 jours [50,100]. Bien qu'un thrombus intraluminal soit observé chez le rat, il est en général absent chez la souris [103]. La croissance anévrismale ainsi que le taux de rupture peuvent être très variables [100,104,105] et influencés par l'efficacité du lot d'élastase utilisée [106]. Ce modèle nécessite une certaine expérience de l'opérateur pour exposer l'aorte et perfuser ou appliquer l'élastase et ne reproduit pas parfaitement la physiopathologie humaine.

- L'application de **chlorure de calcium** au niveau de l'adventice a été initialement développée chez le lapin puis adaptée chez la souris [107]. Les dépôts de calcium au niveau de la paroi entrainent une dégradation des fibres élastiques, favorisent l'apoptose des CMLs et l'infiltration de cellules inflammatoires dans la paroi aortique. Ces modifications sont à l'origine d'une dilatation anévrismale après 2 à 4 semaines [107,108]. Néanmoins, ce modèle ne permet pas la formation d'un thrombus intra-luminal et n'induit pas de rupture.

- L'**infusion d'angiotensine II** présente l'avantage d'être plus simple à réaliser techniquement [91]. Initialement développé par Daugherty et al., le modèle consiste à implanter une pompe osmotique au niveau sous-cutané délivrant de l'angiotensine II pendant 2 à 4 semaines [109]. Il peut être utilisé chez des souris génétiquement modifiées déficientes en ApoE (ApoE-/-) ou en low-density lipoprotein receptor-/- (Ldlr-/-). Ces deux types de souris sont caractérisées par la présence d'une hypercholestérolémie spontanée ou apparaissant après un régime riche en graisses, respectivement, entrainant le développement de lésions d'athérosclérose [110,111]. L'infusion d'angiotensine II va induire la formation d'un AAA et reproduire certaines caractéristiques de la physiopathologie humaine comme un remodelage de la MEC ainsi qu'une infiltration de cellules inflammatoires [109,112]. En revanche, contrairement à ce qui est observé chez l'homme, l'AAA est plus souvent localisé dans la région aortique

supra-rénale. De plus, le modèle est associé à des micro-déchirures de l'intima entrainant une dissection médiale [113]. La dissection correspond à une séparation des couches composant la paroi aortique entrainant une infiltration de sang à l'origine d'un hématome intra-mural. Chez l'homme, l'AAA survient le plus souvent indépendamment de toute dissection et le thrombus est intra-luminal et non intra-mural. Alors que l'hyperlipidémie augmente l'incidence de l'AAA, elle n'est pas indispensable pour son induction. En conséquence, le modèle a aussi été utilisé chez des souris C57BL/6 [114].

L'infusion d'angiotensine II induit une réponse inflammatoire au sein de la paroi aortique associée à une augmentation de l'expression de certaines cytokines dont le **Transforming Growth Factor-β (TGFβ)** [115]. Le TGFβ joue un rôle complexe dans la pathogénèse anévrismale. En effet, l'activité du TGFβ est augmentée dans le tissu aortique chez les patients atteints d'anévrismes thoraciques syndromiques tels que le syndrome de Marfan ou le syndrome de Loeys-Dietz [116-119] ainsi que chez les patients atteints d'anévrismes thoraciques non syndromiques [120]. Les études expérimentales dans des modèles animaux d'anévrisme thoraciques dont le syndrome de Marfan ont initialement suggéré un effet pathogène de l'activation de la voie de signalisation du TGFβ dans le développement d'une dissection et d'une dilatation anévrismale [121,122]. Néanmoins, ce concept est actuellement remis en cause, différentes études ayant suggéré un effet protecteur du TGFβ sur la paroi vasculaire [123]. Le TGFβ joue notamment un rôle important dans le remodelage de la MEC en stimulant la synthèse du collagène [124,125]. Une étude dans un modèle d'anévrisme induit par xénogreffe chez le rat a montré un effet protecteur de la surexpression du TGFβ, permettant de stabiliser la dilatation anévrismale grâce à une préservation de la MEC et une diminution de l'infiltration de cellules inflammatoires [126]. Dans le modèle d'AAA induit par l'infusion d'angiotensine II, la neutralisation du TGFβ par l'injection systémique d'anticorps anti-TGFβ dans des souris C57BL/6 était associée à une augmentation de la dilatation anévrismale et entraînait des

dissections aboutissant à une rupture aortique fatale, suggérant ainsi un rôle protecteur du TGFβ [127]. Cet effet était associé à une augmentation de l'activité des MMP-9 et 12, une dégradation accrue de la MEC, une perte des CMLs, ainsi qu'une infiltration de cellules inflammatoires au sein de la paroi aortique [127]. Ainsi l'infusion d'angiotensine II associée à la neutralisation du TGFβ a été proposé comme un modèle d'AAA disséquant et de rupture [127].

- D'autres modèles chimiques induisant des AAA disséquant ont été décrits. Parmi ceux-ci, l'utilisation de β-aminopropionitrile, un **inhibiteur de la lysyl-oxidase**, a montré son efficacité dans l'induction d'AAA chez le rat et la souris [91,128,129]. La lysyl-oxidase est une enzyme produite par les CMLs impliquée dans la synthèse du collagène [130]. Son inhibition entraine une dégradation de la MEC et une réaction inflammatoire au sein de la paroi, à l'origine de la dilatation anévrismale. Enfin, l'utilisation d'**agonistes du récepteurs aux minéralocorticoïdes** associés à un régime riche en sel chez de souris C57BL/6 est un autre modèle d'AAA disséquant associé à une dégradation de la MEC, une perte des CMLs et une infiltration de cellules immunitaires au sein de la paroi aortique [131].

1.d.2. Description du modèle à l'élastase associé à la neutralisation du TGFβ

Bien qu'utiles, les modèles expérimentaux décrits jusqu'à présent ne miment pas parfaitement la physiopathologie humaine et peu permettent d'induire une rupture [91]. Dans ce contexte, nous avons émis l'hypothèse que la neutralisation du TGFβ dans le modèle d'AAA non disséquant induit par l'élastase allait aggraver la dilatation anévrismale et induire une rupture aortique [132]. Ce travail a fait l'objet d'un article publié dans Arteriosclerosis, Thrombosis and Vascular biology (Annexe 1). La technique consiste à réaliser une application topique d'élastase au niveau de l'adventice de l'aorte abdominale sous-rénale chez des souris C57BL/6. Sous

anesthésie générale, une laparotomie est réalisée afin d'exposer l'aorte et d'y appliquer de l'élastase porcine pendant 5 minutes (Figure 4).

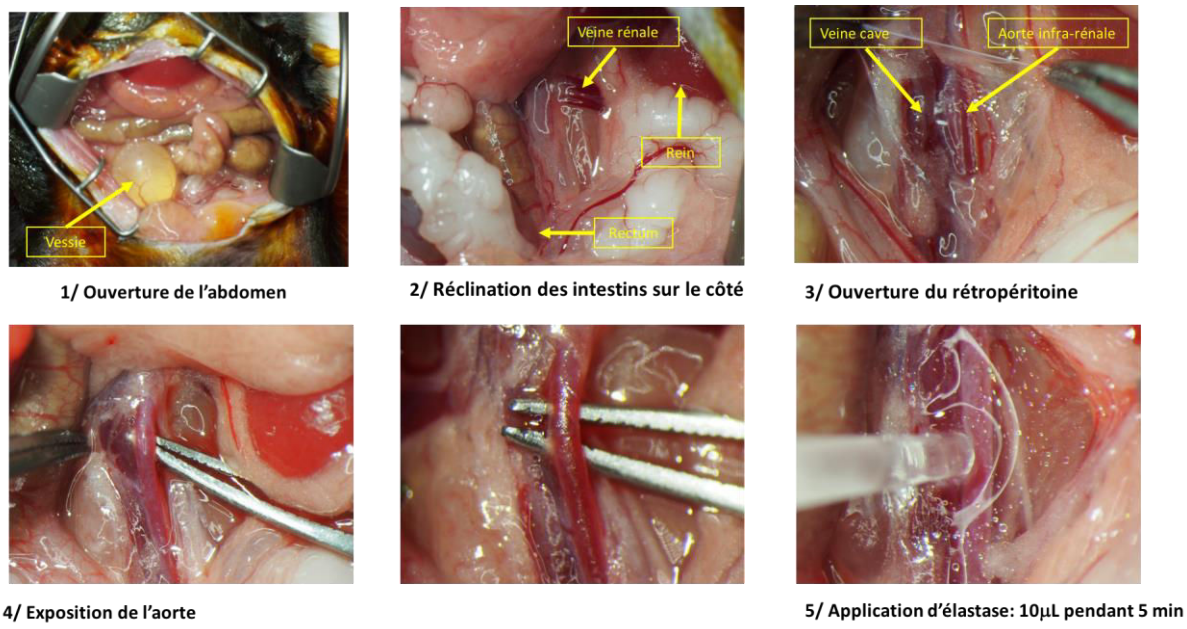

Figure 4 : Images illustrant les étapes techniques pour réaliser l'application d'élastase au niveau de l'adventice de l'aorte abdominale sous-rénale

La neutralisation du TGFβ est réalisée grâce à l'injection intrapéritonéale d'anticorps anti-TGFβ avant la fermeture de l'animal le jour de l'intervention, puis 3 fois/semaine pendant 14 jours. La neutralisation du TGFβ aggrave la dilatation anévrismale par rapport à l'application d'élastase seule et entraine des ruptures aortiques mortelles, avec un taux de survie de 57% à 14 jours [132]. Ce modèle permet de reproduire les principales caractéristiques physiopathologiques observées chez l'homme. En effet, l'AAA est créé dans la région aortique sous-rénale et peut évoluer vers la rupture. L'inhibition du TGFβ induit un remodelage vasculaire important avec une infiltration de cellules inflammatoires, une dégradation de la MEC impliquant une destruction des fibres d'élastine et de collagène ainsi qu'une augmentation de l'activité des MMPs. De plus, il existe une diminution de la densité en CMLs au sein de la

média, une augmentation de la néo-angiogenèse dans l'adventice ainsi que la formation d'un thrombus intraluminal [132]. Enfin, l'analyse *ex-vivo* des tissus aortiques par imagerie par synchrotron en haute résolution a permis de montrer que la croissance anévrismale impliquait une dégradation de la paroi aortique en l'absence de dissection médiale engendrant une rupture aortique transmurale mortelle [132].

2. Rôle des monocytes et des macrophages dans les AAA

Les modèles animaux ont permis de mieux comprendre certains mécanismes clés de la pathogenèse anévrismale dont le rôle de la réponse inflammatoire [69-73]. Etant donné que les macrophages représentent une des populations cellulaires prépondérante de l'infiltrat inflammatoire au sein de la paroi aortique [80,81], nous nous sommes particulièrement intéressés à leur rôle. J'ai écrit une revue de la littérature publiée dans Nature Review Cardiology afin de faire le point sur l'état des connaissances sur leurs rôles, leurs origines et leurs fonctions au cours des différents stades de développement de l'AAA [133] (Annexe 2).

2.a. Notion générale sur les monocytes

Les monocytes correspondent à une population de leucocytes produits par la moelle osseuse et identifiables par leur morphologie, l'expression de certains marqueurs ainsi que par leur fonction. Chez l'homme, 3 principaux types de monocytes peuvent être distingués grâce à l'expression des marqueurs de surface CD14 et CD16 [134,135] (Tableau 1). Les **monocytes dits « classiques »** CD14++CD16- représentent environ 90% des monocytes circulants. Ils expriment fortement le CC-chemokine receptor 2 (CCR2) et faiblement le CX_3C-chemokine receptor 1 (CX_3CR1). Ils sont caractérisés par l'expression de différents gènes impliqués dans

la phagocytose et la réponse immunitaire innée dirigée contre les agents infectieux [135,136]. Ils expriment également fortement des gènes codant pour des protéines impliquées dans l'angiogenèse, la cicatrisation et la coagulation suggérant leur rôle dans la réparation tissulaire [137]. Les **monocytes dits « non classiques »** sont CD14+CD16++, expriment faiblement le CCR2 et fortement le CX_3CR1 [134,135]. Ils sont principalement impliqués dans la surveillance immunitaire grâce à leur fonction de « patrouillage » [135,136]. Enfin, un **phénotype intermédiaire,** CD14++CD16+, a été décrit et est principalement impliqué dans la phagocytose et la réponse pro-inflammatoire.

Chez la souris, les monocytes sont caractérisés par l'expression des marqueurs CD11b et CD115 ainsi qu'une faible expression du marqueur macrophagique F4/80 [138,139]. L'expression du marqueur lymphocyte antigen 6C (LY6C) permet de distinguer différents phénotypes. Les monocytes exprimant fortement LY6C ($LY6C^{high}$) sont l'équivalent des monocytes classiques observés chez l'homme et ont principalement un rôle pro-inflammatoire. Les monocytes exprimant faiblement LY6C ($LY6C^{low}$) sont l'équivalent des monocytes non classiques et sont principalement impliqués dans la surveillance et la réparation tissulaire [138,139].

Une nouvelle population de monocytes appelée **« segregated-nucleus-containing atypical monocytes » (Sat-Mono)** a récemment été identifiée chez la souris [140]. Ces monocytes présentent certaines caractéristiques morphologiques proches des polynucléaires neutrophiles et dérivent d'un progéniteur commun aux granulocytes/macrophages. Ils sont caractérisés par l'expression des marqueurs CD11b+ Ly6C- Ceacam1+ Msr1+ et sont impliqués dans le processus de fibrose [140].

Types de monocytes	Marqueurs de surface		Facteurs induisant le phénotype	Facteurs produits	Fonctions
	Homme	Souris			
Classique	CD14++CD16-	$LY6C^{high}$	CCL2/CCR2	TNF (souris), IL-1	Pro-inflammatoire
Intermédiaire	CD14++ CD16+	$LY6C^{int}$	CCL2/CCR2, CCL5/CCR5	TNF (homme), IL-1	Pro-inflammatoire
Non classique	CD14+CD16++	$LY6C^{low}$	NR4A1, CX_3CL1/ CX_3CR1	IL-10	Surveillance, réparation tissulaire
Sat-Mono	?	CD11b+LY6C- CEACAM1+ MSR1+	C/EBPβ	TNF	Fibrose

Tableau 1 : Principaux types de monocytes chez l'homme et la souris (adapté selon Raffort et al. [133])

CEACAM1: carcinoembryonic antigen-related cell adhesion molecule 1
CCL : CC-chemokine ligand
CCR : CC-chemokine receptor
C/EBP_{β}: CCAAT/ enhancer-binding protein-β
CX_3CL1: CX_3C-chemokine ligand 1
CX_3CR1: CX_3C-chemokine receptor 1
IL: interleukin
LY6C : lymphocyte antigen 6C
MSR1: macrophage scavenger receptor type 1
NR4A1: nuclear receptor subfamily 4 group A member 1
TNF : Tumor necrosis factor

2.b. Rôles des monocytes dans les AAA

Différentes études cliniques ont montré une modification du phénotype des monocytes circulants chez les patients atteints d'AAA, suggérant leur rôle potentiel dans la maladie. En effet, les patients ayant un AAA présentent une proportion plus importante de monocytes intermédiaires ainsi qu'une diminution des monocytes classiques par rapport aux sujets sains [141,142]. Les résultats sont plus controversés concernant les monocytes non classiques, une étude ayant montré une augmentation de leur proportion alors que d'autres auteurs n'ont pas mis en

évidence de différence significative [143]. Une analyse transcriptomique et protéomique des macrophages dérivés des cellules sanguines mononuclées a montré une différence de profile entre des patients atteints d'AAA et des patients souffrant d'artériopathie oblitérante des membres inférieurs mais n'ayant pas d'AAA [144]. Parmi les molécules identifiées, certaines sont impliquées dans des processus relevant pour la pathologie anévrismale comme la réponse inflammatoire ou le remodelage de la MEC.

Les études expérimentales ont montré une augmentation des monocytes circulants CCR2+ chez les souris ApoE-/- recevant une infusion d'angiotensine II pendant 14 jours [145]. Les taux de monocytes circulants $LY6C^{high}$ et $LY6C^{low}$ étaient plus élevés à J3 et J7 chez les souris ayant développée un AAA après 28 jours d'infusion d'angiotensine II comparé aux souris n'ayant pas développé d'AAA [146]. De plus, l'utilisation de certains traitements, comme l'administration d'angiopoietine-2, permettant de limiter la dilatation anévrismale induite sous infusion d'angiotensine II, était associée à une diminution des monocytes circulants $LY6C^{high}$ [147]. L'ensemble de ces résultats suggère un effet potentiellement pathogène des monocytes circulants $LY6C^{high}$. Néanmoins, la fonction des différents phénotypes de monocytes circulants dans le développement de l'AAA est loin d'être totalement élucidée et l'implication de certaines populations récemment identifiées comme les Sat-Mono a été très peu décrite [133].

2.c. Notions générales sur les macrophages

Il existe différents phénotypes de macrophages. De façon schématique, les **macrophages dits « M1 »** sont considérés comme pro-inflammatoires et sont caractérisés par la production d'enzymes protéolytiques et de cytokines pro-inflammatoires comme le TNF, l'IL6, l'IL12, l'IL1β, NOS2 ou CCL2 [69,148] (Tableau 2). Les **macrophages dits « M2 »** ont plutôt un rôle anti-inflammatoire et sont impliqués dans le remodelage de la matrice et la réparation tissulaire

grâce à la sécrétion de différents facteurs tels que l'IL-10 ou le TGFβ [148]. Bien que cette classification entre macrophages M1 et M2 soit bien définie *in vitro*, la distinction de ces phénotypes *in vivo* est difficile à établir puisque les facteurs stimulant la différenciation en macrophages M1 ou M2 sont souvent co-exprimés dans les tissus et les marqueurs utilisés ne sont pas spécifiques d'un phénotype donné [148] [149]. La caractérisation du phénotype des macrophages *in vivo* est en réalité complexe et correspond à un spectre d'activation des macrophages que l'on peut étudier en combinant différents marqueurs [148].

Types de macrophages	**Marqueurs de surface**		**Facteurs induisant le phénotype**	**Facteurs produits**	**Fonctions**
	Homme	**Souris**			
M1	HLA-DR+, CD80, CD86	MHC II+, CD80, CD86	LPS, IFNγ, TNFα, NF-κB, HIF1α	TNF, IL-6, IL-1β, NOS2, CCL2	Pro-inflammatoire
M2	CD163, CD200R, CD206, stabiline 1	CD163, MGL1, stabiline 1	IL-10, TGFβ, IL-4, IL-13, KLF4, STAT6	IL-10, TGFβ	Anti-inflammatoire, réparation tissulaire

Tableau 2 : Principaux types de macrophages chez l'homme et chez la souris (adapté selon Raffort et al. [133])

CCL2: C-C chemokine ligand
HIF1α: hypoxia-inducible factor 1α
HLA-DR : human leukocyte antigen-DR
IFN : interferon
IL: interleukin
KLF4: Kruppel-like factor 4
LPS: lipopolysaccharide
MHC II : major histocompatbility complex class II
MGL1: macrophage galactose-type lectin 1
NF-κB: nuclear factor κB
NOS2: nitric oxide synthase 2
TGF: tumor growth factor
TNF : tumor necrosis factor
TGFβ: tumor growth factor β
STAT6: signal transducer and activator of transcription 6

2.d. Phénotype des macrophages dans les AAA

Différentes études ont montré une infiltration de macrophages dans le tissu anévrismal chez l'homme [80,150] ainsi que dans les modèles animaux [109,127,151] [50,152,153]. Les macrophages s'infiltrent dans les 3 tuniques de la paroi aortique avec une localisation préférentielle au niveau de l'adventice et du thrombus intraluminal [154,155].

Afin de mieux comprendre l'accumulation des macrophages au sein de la paroi aortique, certains auteurs ont analysé la composition cellulaire de tissus anévrismaux humains après avoir séparé la média et l'adventice [154]. Il était observé une diminution des macrophages M1 en faveur des macrophages M2 au niveau de l'adventice dans le tissu anévrismal comparé au tissu contrôle [154]. Une autre étude a également montré une augmentation de la densité en macrophages M2 dans le tissu aortique anévrismal humain [156]. En revanche, les auteurs observait une localisation préférentielle des macrophages M1 dans l'adventice alors que les macrophages M2 était prépondérant dans le thrombus intraluminal [156]. Cette discordance de résultats pourrait être en partie liée à la différence de méthodologie utilisée pour définir les macrophages M1 et M2 (choix des marqueurs), soulignant la difficulté de clairement caractériser leur phénotype *in vivo*. De plus, il est possible que les échantillons utilisés correspondaient à des stades différents d'AAA et que le phénotype des macrophages évolue au cours du développement de la maladie.

Des études expérimentales ont été réalisées en vue de mieux comprendre la polarisation des macrophages dans l'AAA. Une première étude a montré une augmentation du ratio M1/M2 dans le tissu aortique supa-rénal entre J7 et J10 après infusion d'angiotensine II dans des souris ApoE-/- et cet effet était maintenu jusqu'au 28ème jour [157]. D'autres auteurs ont ensuite étudié la polarisation des macrophages dans modèle d'AAA avec un suivi plus long et ont montré qu'après 84 jours d'infusion d'angiotensine II, l'expression du CD206, un marqueur des

macrophages M2 était plus importante que l'expression de NOS2 (un marqueur des macrophages M1), suggérant que l'évolution de l'AAA est associée à une différenciation préférentielle des macrophages vers un phénotype de type M2 [158]. La polarisation des macrophages vers un phénotype M1 ou M2 au cours de l'AAA est un phénomène complexe et plurifactoriel, potentiellement influencé par différents facteurs dont les modifications hémodynamiques, l'expression de cytokines et chemokines, la formation du thrombus intraluminal ou encore l'apparition de micro-dissections dans la paroi aortique [133].

2.e. Rôle des macrophages dans les AAA

Les macrophages jouent un rôle majeur dans le remodelage de la paroi aortique au cours de l'AAA en régulant le remodelage de la MEC, la réponse inflammatoire et en étant impliqués dans le processus de réparation tissulaire. Il a été observé chez l'homme que l'expression des cathepsines, des MMPs ou des TIMPs dans les macrophages dérivés des monocytes circulants chez les patients atteints d'AAA était modifiée par rapport aux sujets contrôles [144]. De plus, l'expression des MMPs et des cathepsines co-localisait avec les macrophages dans le tissu anévrismal, suggérant le rôle de ces derniers dans le remodelage de la MEC [73,159]. Ce rôle a été confirmé par les études expérimentales dans des souris déficientes en MMP-9 (MMP-9-/-) [50,160]. Ces souris étaient protégées contre le développement de l'AAA et la reconstitution des macrophages exprimant MMP-9 par la réalisation d'une greffe de moelle osseuse ou d'une injection de macrophages péritonéaux restaurait la formation de l'AAA. Les macrophages sont capables de produire de nombreux facteurs impliqués dans la réponse inflammatoire et leur activité est elle-même influencée par ces médiateurs [161-163]. Un exemple caractéristique au cours de l'AAA est illustré par le rôle de l'IL-6. Les macrophages infiltrés dans la paroi aortique sont une source majeure de la production d'une chemokine, le CXCL1, qui favorise l'attraction et

le recrutement des polynucléaires neutrophiles capables de sécréter de l'IL-6 [164]. Au cours de l'AAA, l'augmentation de l'expression de l'IL-6 au niveau de la paroi aortique va favoriser la différenciation des monocytes vers des macrophages sécrétant le CCL2, qui en retour accroit le recrutement des monocytes [150]. En plus de leur rôle dans le remodelage de la MEC et la réponse inflammatoire, les macrophages sont impliqués dans la régulation du stress oxydatif ainsi que dans l'élimination des débris, modulant ainsi la réparation tissulaire de la paroi aortique [133]. La modulation de leur phénotype au cours des différents stades de développement de l'AAA peut avoir à la fois un effet pathogène et protecteur, soulignant la complexité de leur rôle qui n'est pas encore totalement élucidé.

3. Rôle des micro-ARNs dans les AAA

3.a. Notions générales sur les micro-ARNs

Les micro-ARNs (miRs) sont des ARNs non codants de petite taille (21 à 25 nucléotides) jouant un rôle majeur dans la régulation post-transcriptionnelle de l'expression génique [165]. Ils sont transcrits à partir du génome nucléaire, et après plusieurs étapes de maturation ils exercent leur action dans le cytoplasme en agissant sur des ARN messagers (ARNm) cibles (Figure 5A).

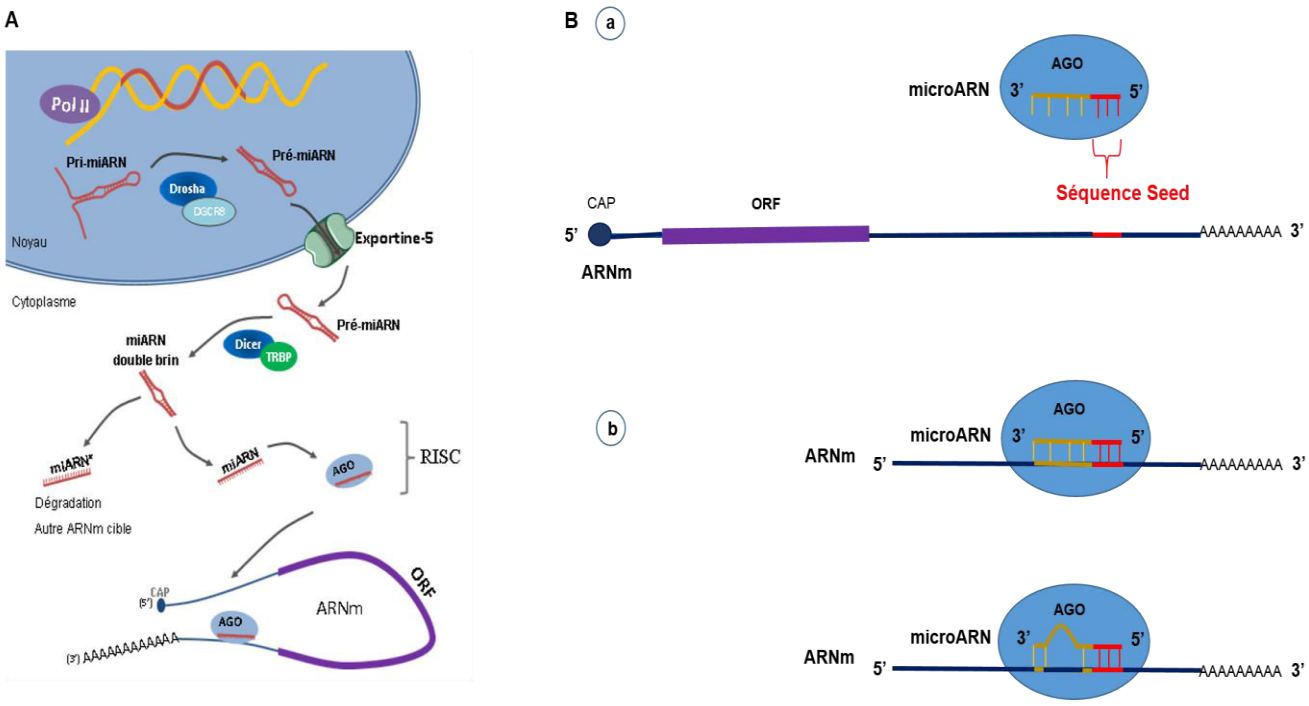

Figure 5 : Biogenèse et appariement des miRs.

(A) Les miRs sont principalement synthétisés par l'ARN polymerase II sous forme de pri-miRs. Ils sont maturés par l'enzyme Drosha, associée à DGCR8 en pré-miRs. Les pré-miRs sont transportés dans le cytoplasme par l'exportine 5. Ils sont maturés par l'enzyme Dicer, associée à son cofacteur TRBP et forment alors un double brin d'ARN. Un brin s'associe à la protéine argonaute (AGO) pour former le complexe RISC (RNA Induced Silencing Complex). Ce dernier va cibler un ARNm et inhiber sa traduction principalement. L'autre brin est dégradé ou va cibler un autre ARNm. (B) Représentation de l'appariement du miR sur l'ARNm a. Représentation de la séquence « seed » du miR. Elle doit être parfaitement complémentaire à l'ARNm cible et s'étend sur 2 à 8 nucléotides. b. Le reste de la séquence du miR s'apparie à l'ARNm de façon parfaitement complémentaire ou non entrainant sa dégradation et/ou inhibant sa traduction.

En s'appariant de façon parfaitement complémentaire ou non dans la région 3' UTR de certains ARNm cibles, les miRs sont capables de réprimer leur traduction et/ou d'induire leur dégradation (Figure 5B) [166]. Du fait de l'appariement imparfait, un miR est capable d'agir sur différents ARNm. Réciproquement, un ARNm cible peut être régulé par plusieurs miRs différents. De par leur propriété, les miRs jouent un rôle dans la régulation de nombreux processus. Leur implication a été montrée dans la prolifération et la différenciation cellulaire, l'apoptose, la réponse immunitaire ou encore le métabolisme [165]. Ils jouent un rôle clé dans la

régulation du remodelage vasculaire et leur implication a été montré dans de nombreuses pathologies cardio-vasculaires [167-169].

Les miRs exercent ainsi principalement leur action dans le cytoplasme, à l'intérieur des cellules. Cependant, des études récentes ont mis en évidence la présence de miRs à l'extérieur des cellules dans de nombreux liquides biologiques dont le sang, la salive, les urines, les larmes, ou encore le liquide céphalo-rachidien [170]. Dans ces liquides, la stabilité de ces miRs est rendue possible grâce à différentes formes de transport les rendant résistants aux RNAses [171]. En effet, ils ont été identifiés comme étant associés à des protéines telles que la protéine argonaute, ou à des lipoprotéines comme les HDL. Ils peuvent également être encapsulés dans différents types de vésicules se distinguant par leur taille et leur provenance comme les microparticules, les exosomes ou les corps apoptotiques. Le rôle des miRs extra-cellulaires est encore peu connu. Pour certains, ces miRs seraient issus de la mort cellulaire ; pour d'autres, ils seraient sécrétés spécifiquement et participeraient à la communication inter-cellulaire [171]. De par leur grande stabilité dans le milieu extra-cellulaire, les miRs ont été proposés comme biomarqueurs de différentes pathologies et notamment dans les maladies cardiovasculaires [172].

3.b. Variation de l'expression des micro-ARNs chez les patients atteints d'AAA

Afin de réaliser une synthèse de l'état des connaissances du rôle des miRs dans les AAA, j'ai écrit une revue de la littérature publiée dans Cardiovascular Research [173] (Annexe 3).

Différentes études chez l'homme ont montré une dérégulation du profile des miRs dans le tissu anévrismal comparé au tissu aortique sain (pour revue [173,174]). L'expression de certains miRs (miR-21 13-15, miR-205, 378 14, miR-126, 20a, 27a, 92a, 221, 222, let-7f, 155, 146a, 223, 124a 15) [175-177] était augmentée, alors que d'autres miRs étaient diminués (miR-133a, 331-3p, 30c-2*, 204 16, 24, 27b, 17) [178,179]. Des résultats contradictoires ont été trouvés pour certains

miRs (miR-133b, 29b) [175-178]. Cette discordance pourrait être due à des différences dans la méthodologie utilisée telle que les caractéristiques des patients inclus et la technique utilisée pour analyser l'expression des miRs. En plus d'une dérégulation au niveau du tissu aortique, il a été observé une différence d'expression des miRs dans le sang total, le sérum ou encore le plasma entre des patients atteints d'AAA et des patients sans AAA, suggérant l'intérêt potentiel des miRs en tant que biomarqueurs non invasifs [179-182]. Certains miRs pourraient en effet être des marqueurs de la croissance anévrismale, une étude ayant montré une association entre le diamètre anévrismal et l'expression des miR-340, 20a, 148a, 125b, 195 dans le plasma [182]. De plus, une différence de profile d'expression des miRs a été observée entre des patients ayant une croissance anévrismale rapide par rapport à ceux ayant une croissance anévrismale lente [183], suggérant l'intérêt des miRs circulants en tant que marqueurs pronostiques. Les études cliniques ont l'avantage d'adresser directement l'intérêt potentiel des miRs pour la pratique médicale. Néanmoins, la spécificité de la variation des miRs dans le sang des patients présentant un AAA reste à évaluer, puisque différentes études ont montré que le profil des miRs peut être influencé par l'âge, le sexe, la présence de comorbidités ou de facteurs de risque cardiovasculaires [184-187]. De plus, les études cliniques ne permettent pas une approche mécanistique dans le but de comprendre le rôle des miRs dans les AAA.

3.c. Rôle des micro-ARNs dans les AAA

Les études expérimentales ont permis de montrer l'implication des miRs dans les mécanismes physiopathologiques clés de l'AAA tels que le remodelage de la MEC, la réponse inflammatoire ou l'homéostasie des cellules dans la paroi vasculaire [173]. La dérégulation de certains miRs dans le tissu anévrismal a un rôle pathogène dans le développement de l'AAA. Par exemple, le miR-24 est diminué dans le tissu anévrismal par rapport au tissu aortique sain

dans les modèles d'AAA induits par l'élastase et l'infusion d'angiotensine II [179]. L'inhibition du miR-24 par injection d'un antagoniste augmentait la dilatation anévrismale et l'incidence de la rupture [179]. Ceci était associé à une augmentation de la chitinase 3 like 1 (Chi3l1), un facteur inflammatoire ciblé par le miR-24. La surexpression du miR-24 avait un effet opposé sur l'expression de Chi3l1, était associé à une diminution de l'infiltration des macrophages et atténuait la dilatation anévrismale. A l'inverse, la dérégulation de certains miRs exerce un effet protecteur compensatoire dans le développement de l'AAA. C'est le cas notamment du miR-29b dont l'expression était diminuée dans le tissu anévrismal dans le modèle à l'élastase et à l'angiotensine II [188]. Ceci était associé à une augmentation de la densité de collagène et d'élastine. L'inhibition du miR-29b par injection d'antagoniste augmentait l'expression génique de l'élastine et du collagène entrainant une diminution significative de la croissance anévrismale.

Ainsi, les études sur modèles animaux permettent de mieux comprendre le rôle des miRs et de tester des stratégies thérapeutiques en inhibant ou en augmentant leur expression. Ils permettent de limiter l'impact des potentiels facteurs de confusion pouvant influencer l'expression des miRs comme la présence de comorbidités ou de facteurs de risque cardiovasculaires. Ils offrent également l'opportunité de rechercher la potentielle implication des miRs dans les mécanismes moléculaires associant les facteurs de risque et la survenue d'AAA.

4. Effet du diabète dans les AAA

4.a. Données épidémiologiques

Alors que l'AAA est classiquement associé à l'athérosclérose et aux facteurs de risque cardiovasculaires correspondants, il est paradoxalement négativement associé au diabète. En effet, l'incidence ainsi que la prévalence de l'AAA est plus faible chez les patients diabétiques par rapport aux sujets non diabétiques [26,189-194]. De plus, les patients diabétiques présentent des AAA de plus petite taille, avec un diamètre aortique significativement inférieur par rapport aux sujets non diabétiques [189,191,195,196]. Les patients diabétiques ont également un risque de rupture plus faible par rapport aux patients non diabétiques [197]. Le diabète est caractérisé par une hyperglycémie chronique. La glycémie impacte sur le développement de l'AAA, comme évoqué par l'existence d'une relation inverse entre la glycémie à jeûn et le diamètre anévrismal [198,199]. Alors que la glycémie est variable au cours de la journée, l'hémoglobine glyquée A1c (HbA1c) est le reflet de l'équilibre glycémique des 3 derniers mois [200]. Une association inverse a été mise en évidence entre la croissance anévrismale et le taux d'HbA1c, suggérant un effet protecteur du diabète dans l'AAA. Une meilleure compréhension des mécanismes cellulaires et moléculaires impliqués dans la relation négative entre diabète et AAA pourrait permettre d'identifier de nouvelles cibles thérapeutiques.

4.b. Mécanismes impliqués dans l'association négative entre diabète et AAA

Afin de mieux comprendre les mécanismes impliqués dans la relation négative entre diabète et AAA, j'ai réalisé une revue de la littérature publiée dans Cardiovascular Research (Annexe 4). Différentes études ont montré l'impact du diabète sur la composition de la MEC. En effet, l'analyse protéomique des artères de patients diabétiques a montré une augmentation de certains

composants de la MEC comme le collagène alpha1 et alpha2 de type IV, la gamma1 et la beta2 laminine [201]. De plus, le diabète peut influencer la composition en protéoglycanes au niveau de la paroi aortique. En effet, il a été observé dans des cellules humaines mésangiales que l'expression de la décorine, un protéoglycane riche en leucine, augmente en réponse à de fortes concentrations en glucose [202]. La décorine est capable d'interagir avec le TGFβ pour réguler l'organisation de la MEC et du réseau de fibres de collagène et pourrait limiter le développement de l'AAA en préservant la MEC [203,204]. En plus d'impacter sur les composants de la MEC, le diabète influence leur turn-over en régulant l'expression des protéases. En effet, il a été observé une diminution de la sécrétion de la MMP-9 après incubation de monocytes avec de la média extraite à partir de patients diabétiques, comparé à des patients non diabétiques [196]. Les études expérimentales corroborent cette observation où l'effet protecteur du diabète sur le développement de l'AAA était associé à une diminution de l'expression et de l'activité de la MMP-9 et une préservation de l'élastine [205,206]. Les concentrations sériques de MMP-2 et MMP-9 chez les patients diabétiques sont plus faibles que chez les patients non diabétiques et pourraient contribuer à l'effet protecteur du diabète [207]. Néanmoins, l'impact du diabète est complexe et n'est pas totalement élucidé. Paradoxalement, les taux sériques d'HbA1c sont positivement corrélés à la concentration de la MMP-9 chez les patients diabétiques [207]. De plus, certaines études rapportent une augmentation de l'expression et de l'activité de la MMP-9 dans des monocytes humains exposés à de fortes concentrations en glucose [208].

L'hyperglycémie chronique chez les patients diabétiques aboutit à la formation de produits de glycation qui résultent de la glycoxidation non enzymatique des protéines [209]. Une étude a montré que les produits de glycation comme la pentosidine étaient significativement diminués dans le tissu anévrismal comparé au tissu aortique sain [210]. Les patients diabétiques avaient une concentration de pentosidine plus élevée dans le tissu anévrismal par rapport aux patients non diabétiques. La concentration en pentosidine avait tendance à être inversement corrélée au

diamètre anévrismal, suggérant un effet protecteur des produits de glycation. Cependant, l'effet des produits de glycation sur l'AAA est en réalité bien plus complexe, puisqu'ils sont à la fois capables d'influencer la structure tri-dimensionnelle des protéines impactant la composition de la MEC, et d'activer différentes voies de signalisation par l'intermédiaire de récepteurs de surface tels que les receptor for advanced glycation end products (RAGE). Différentes études expérimentales ont montré l'effet pathogène de l'activation de cette voie de signalisation dans le développement de l'AAA [211,212].

Le diabète impacte sur la réponse inflammatoire et différentes études suggèrent un rôle pro-inflammatoire au niveau des tissus vasculaires [213]. L'impact du diabète sur la réponse inflammatoire au cours de l'AAA est encore peu connu et les résultats des différentes études sont assez contradictoires. Par exemple, les études expérimentales sur modèles animaux ont montré une diminution de l'infiltration des macrophages dans les souris diabétiques par rapport aux contrôles [205,206,214]. En revanche, chez l'homme, il a été observé une infiltration plus importante de macrophages dans le tissu anévrismal des patients diabétiques par rapport aux patients non diabétiques et celle-ci était corrélée avec les concentrations sériques de glucose [215]. Aucune différence n'était constatée concernant l'infiltration des lymphocytes T entre les patients diabétiques et les non diabétiques.

Le diabète a également un impact sur la régulation de la néo-angiogenèse [216,217] et pourrait influencer le développement de l'AAA par ce mécanisme. De plus, le diabète affecte la structure du thrombus intra-luminal, la densité de ce dernier étant plus importante chez les patients diabétiques [218]. Ceci pourrait contribuer à le rendre plus résistant et à diminuer l'activité des protéases, limitant le développement de l'AAA.

Enfin, le diabète régule finement l'homéostasie des CMLs. La morphologie des CMLs chez les patients diabétiques diffère des patients non diabétiques avec un aspect rhomboide et une désorganisation du réseau des fibres d'actine, suggérant un phénotype dédifférencié [219].

L'analyse histologique des tissus anévrismaux de patients diabétiques a montré une diminution du marquage de l'actine, suggérant une diminution du contenu en CMLs dans le tissu ou une dé-différenciation de celles-ci [210]. Néanmoins, l'impact du diabète sur les CMLs n'est pas complètement compris. D'autres études ont en effet montré un effet stimulant du diabète sur la prolifération des CMLs par l'activation de la voie de signalisation ERK ou la sécrétion de facteurs mitogènes [220,221].

Outre l'action directe du diabète sur les mécanismes clés impliqués dans le développement de l'AAA, l'utilisation des traitements anti-diabétiques eux-mêmes influence la formation de l'AAA. Une étude incluant 1269 patients atteints d'AAA a montré que l'utilisation de traitements anti-diabétiques était associée à une diminution de la croissance anévrismale de 56% [222]. Une autre étude incluant 4468 patients ayant un anévrisme et les comparant avec 4468 témoins appariés suggère également l'effet protecteur des médicaments anti-diabétiques [223]. L'utilisation de metformine, de sulfonylurées ainsi que de thiazolidinediones était associée à un risque plus faible de développer un anévrisme [223]. Les études expérimentales ont permis de confirmer l'effet protecteur de la metformine et des thiazolidinediones sur le développement de l'AAA [224] [196,225,226].

Ainsi, le diabète et son traitement jouent un rôle complexe dans le développement de l'AAA et les mécanismes moléculaires impliqués sont encore peu connus. A notre connaissance, le rôle potentiel des miRs dans l'association entre diabète et AAA n'a encore jamais été étudié.

5. Objectifs

L'anévrisme de l'aorte abdominale est le plus souvent associé à l'athérosclérose et aux facteurs de risque cardiovasculaires à l'exception du diabète. Bien que certaines études aient montré une infiltration de macrophages et une modification de l'expression des miRs dans la paroi anévrismale, leurs rôles respectifs dans le développement de l'AAA ne sont pas encore totalement élucidés. Bien qu'utiles pour adresser cette question, les modèles expérimentaux classiques ne miment pas parfaitement la physiopathologie humaine. Nous avons développé un nouveau modèle d'AAA associant application topique d'élastase et neutralisation systémique du TGFβ permettant de reproduire les principales caractéristiques de l'AAA et induisant une rupture aortique. Dans ce contexte, les objectifs de ce travail étaient de : -1/ Caractériser le phénotype des monocytes/ macrophages dans le modèle murin d'AAA induit par l'application topique d'élastase associée à la neutralisation du TGFβ. -2/ Etudier l'expression des miRs dans ce modèle murin. -3/ Enfin, les mécanismes impliqués dans la relation négative entre diabète et AAA étant à ce jour peu connus, le 3ème objectif de cette thèse était de mettre en place une étude clinique afin d'étudier l'expression des miRs chez des patients diabétiques ayant un AAA.

MATERIEL ET METHODES

1. Etude expérimentale

1.a. Souris et nourriture

Les souris utilisées sont des mâles de la souche C57BL/6 (Charles River Laboratoire®, Royaume-Uni) de 8 semaines, élevées en animalerie entre 22 et 24°C, avec un cycle nycthéméral de 12 heures. L'accès à l'eau et à la nourriture (Arie Block®, Woorden, Hollande) sont *ad libitum*.

Les souris sont anesthésiées grâce à une induction à l'isoflurane 2% couplé à l'oxygène 2%. Durant l'intervention chirurgicale, une administration de 1.6-1.8% d'isoflurane permet le maintien de l'anesthésie. La technique chirurgicale d'induction de l'AAA consiste à injecter de l'élastase en péri-adventitielle au niveau de l'aorte abdominale sous rénale. L'élastase porcine de type I (6UI/mg, Sigma Aldrich®) utilisée au cours de la chirurgie est préalablement filtrée à 0.45µm. Afin de la rendre inactive pour constituer le groupe contrôle, l'élastase est chauffée (30 minutes à 95°C) avant d'être de nouveau filtrée à 70 µm sur « Cell strainer ».

La première injection intrapéritonéale d'anticorps anti-TGFβ (100µg/souris, BioXcell®) est effectuée avant la fermeture de l'animal le jour de l'intervention. Les souris sont contrôlées 2 fois par jour et l'anticorps anti-TGFβ est injecté 3 fois/semaine pendant 14 jours.

Trois groupes expérimentaux étaient créés : le groupe contrôle recevant de l'élastase inactivée (EI), les souris recevant uniquement de l'élastase (E) et les souris recevant de l'élastase associée à l'anti-TGFβ (E + anti- TGFβ) [132].

La déplétion des monocytes/ macrophages est réalisée dans le groupe E + anti-TGFβ par une injection intraveineuse de 150 µl d'une solution de liposomes contenant du PBS ou du clodronate 24 heures avant le sacrifice des souris.

1.b. Suivi des souris par échographie

Le suivi échographique des souris est effectué avant la procédure, à 7 et à 14 jours. Un écho-cardiographe Vevo 2100 (VisualSonics®) équipé d'une sonde MS550D 22–55 MHz est utilisé. Durant la réalisation de l'examen, une anesthésie générale à l'isoflurane 1.6-1.8% est administrée au moyen d'un cône nasal adapté. Après placement de la souris en supination sur une plateforme chauffante à 37°C, du gel échographique est utilisé comme interface entre la sonde et la peau. Des coupes sagittales en mode B au niveau de l'aorte abdominale sont réalisées afin de permettre une mesure du diamètre aortique (en mm).

1.c. Collection des tissus

A 7, 10 ou 14 jours, les souris sont sacrifiées et les organes prélevés afin d'être analysés. Le sang est prélevé par ponction intracardiaque (ventricule droit) et collecté sur un tube à l'EDTA (plasma) ou sur un tube sec (sérum). L'oreillette droite est ouverte et le ventricule gauche est perfusé avec une solution tampon « Phosphate Buffered saline » (PBS, Sigma Aldrich®) à 4°C afin d'éliminer le sang et de conserver les tissus intacts.

Pour les analyses histologiques, les tissus sont prélevés et fixés dans du paraformaldéhyde (PFA) 4% pour 12 heures maximum. Le lendemain, les tissus sont nettoyés puis incubés dans une solution de sucrose à 30% pour une durée de 10 heures avant d'être inclus dans de

« l'Optimal Cutting Temperature compound » (O.C.T. Compound®, Leuven, Netherlands). Les tissus sont coupés au cryostat par section de 10µm conservées dans un congélateur à -80°C. Pour les analyses de biologie moléculaire, les tissus sont nettoyés dans du PBS et immédiatement plongés dans de l'azote liquide pour être ensuite conservés à -80°C.

1.d. Histologie

Après avoir rincé les lames dans du PBS afin d'éliminer l'O.C.T, une solution de perméabilisation contenant 0.1% de Triton X-100 et 0.1% de citrate de sodium est ajoutée sur les coupes d'aortes pendant 2 minutes. Après un nouveau rinçage au PBS, les coupes sont incubées avec l'anticorps primaire. Les sections aortiques sont ensuite incubées après lavage au PBS avec l'anticorps secondaire également dilué au 1/200. Les marquages sont visualisés à l'aide d'une caméra-vidéo Leica DFC340 FX (Leica Microsystems®) connectée à un microscope fluorescent (Leica DMRB®, logiciel LAS®version 2.8.1).

1.e. Cytométrie en flux

1.e.1. Isolement des cellules

Les aortes abdominales des souris sont digérées par une solution enzymatique (Elastase 1 mg/ml, Worthington; Collagenase D, 0.2 mg/ml, Roche; Dispase I, 0.2 U/ml, Stemcell; DNAse I, 0.2 mg/ml, Roche; in RPMI 1640) à 37°C pendant 45 minutes. L'activité enzymatique est stoppée en ajoutant du PBS à 4°C. Les cellules de la rate et les cellules sanguines sont isolées en utilisant une solution de lyse des globules rouges (tampon de lyse Ammonium-Chloride-

Potassium ACK, Life Technologies®). Les suspensions cellulaires sont ensuite passées sur un filtre de 70 µm « Cell strainer ».

1.e.2. Marquage des cellules

Les cellules sont incubées avec une solution de blocage (Fc block solution ebiosciences, clone 93, dilution 1/200) pendant 10 minutes à 4°C, puis marquées avec les anticorps spécifiques. Après un nouveau lavage, les cellules sont analysées par cytométrie en flux sur le LSR-FORTESSAII équipé de 4 lasers (405, 488, 561 and 640 nm). Les données sont analysées à l'aide du logiciel BD FACS Diva 6.0 de chez BD Biosciences® et du logiciel FlowJo de chez TreeStar®.

1.f. Biologie moléculaire

Les tissus sont broyés et pulvérisés après agitation avec des billes. L'extraction des ARNs est réalisée en utilisant les kits d'extraction RNAeasy mini kit (Qiagen). La transcription inverse est effectuée en utilisant le QuantiTect Reverse transcription kit (Qiagen). Pour l'analyse des miRs, les ARNs sont extraits au Trizol® (Invitrogen), puis reverse transcrits en utilisant le kit miRCURY LNA Universal RT microRNA PCR (Exiqon Qiagen®). Le profil d'expression de 752 miRs chez l'homme et chez la souris est réalisé en utilisant le kit miRCURY LNA Universal RT microRNA PCR, panel I and II V4 (Exiqon- Qiagen®). Le screening par qPCR est réalisé par Exiqon. La qualité des échantillons est vérifiée en utilisant l'ADNc de synthèse UniSp6 lors de la transcription inverse et en analysant les Ct obtenus pour les miR-103, 23a, 30c, 451 et 142-3p. Les miRs-103, 23a et 30c sont exprimés dans la majorité des échantillons biologiques à un niveau stable [227]. Le miR-451 est fortement exprimé dans les globules

rouges [228]; le miR-142-3p est fortement exprimé par les thrombocytes [229]. Les qPCR sont réalisées avec du Syber Green (qPCR mix, Applied Biosystems) sur des plaques de 384 puits et analysées sur une machine 7900HT Fast Real-Time PCR System (Applied Biosystems). L'analyse de l'expression génique est effectuée selon la méthode des ΔΔCt. L'expression des miRs est normalisée par rapport à l'expression de l'ARNsn U6, l'ARNr 5S et l'ARN RNU5G.

1.g. Analyse statistique

Les tests statistiques utilisés pour répondre à chaque objectif de cette thèse sont détaillés ultérieurement dans les partie I, II et III.

2. Etude clinique

Afin d'évaluer la relevance des résultats expérimentaux pour la pathologie humaine, nous avons mis en place une étude clinique dans le Service de Chirurgie Vasculaire au CHU de Nice permettant de créer une collection biologique incluant des patients atteints d'anévrisme de l'aorte. Les autorisations auprès de la Direction de la Recherche Clinique et de l'Innovation et du Comité Ethique (Comité de Protection Sud Méditerranée V) ont été obtenues afin de collecter le sang total, le sérum, le plasma, les cellules sanguines mononuclées circulantes (PBMCs) ainsi que le tissu aortique des patients opérés d'un anévrisme de l'aorte. En parallèle, l'accord du Centre de Coordination des prélèvements d'organes et de l'Agence de Biomédecine a été obtenu afin de collecter du tissu aortique contrôle à visée scientifique chez des donneurs d'organes en état de mort encéphalique. Le détail des caractéristiques cliniques des patients inclus sont présentées ultérieurement dans la partie III.

PARTIE I :

CARACTERISATION DU PHENOTYPE DES MACROPHAGES DANS LE MODELE MURIN D'AAA INDUIT PAR L'ELASTASE ET LA NEUTRALISATION DU TGFβ

Cette partie présente les résultats de 2 articles :

- "TGFβ (Transforming Growth Factor-β) Blockade Induces a Human-Like Disease in a Nondissecting Mouse Model of Abdominal Aortic Aneurysm." (Annexe 1)

Lareyre F, Clément M, Raffort J, Pohlod S, Patel M, Esposito B, Master L, Finigan A, Vandestienne M, Stergiopulos N, Taleb S, Trachet B, Mallat Z.

Arterioscler Thromb Vasc Biol. 2017 Nov;37(11):2171-2181.

PMID: 28912363

- "TGFβ (transforming growth factor-β) neutralization finely tunes macrophage phenotype in elastase induced abdominal aortic aneurysm and is associated with an increase of arginase-1 expression in the aorta."

Raffort J, Lareyre F, Clément M, Moratal C, Jean-Baptiste E, Hassen-Khodja R, Burel-Vandenbos F, Bruneval P, Chinetti G, Mallat Z.

Accepté dans Journal of Vascular Surgery

1. Résultats

1.a. Validation des conditions expérimentales

Afin d'étudier l'effet de la neutralisation du TGFβ sur l'AAA induit par l'application d'élastase, 3 groupes expérimentaux ont été créés : le groupe contrôle recevant de l'élastase inactivée (EI), le groupe recevant de l'élastase seule (E) et le groupe recevant de l'élastase avec l'anti-TGFβ (E + anti- TGFβ). Le suivi échographique des souris a permis de montrer une augmentation significative du diamètre aortique à J7 dans le groupe E et E + anti-TGFβ. Alors qu'avec l'application d'élastase seule, la dilatation anévrismale restait stable entre J7 et J14, la neutralisation du TGFβ entrainait une progression significative de la dilatation (Figure 6A et 6B). La mesure ex vivo du diamètre externe de l'aorte abdominale à J14 à l'aide d'un vernier gradué (caliper) a permis de confirmer ces résultats (Figure 6C). La neutralisation du TGFβ pouvait provoquer une rupture aortique survenant entre J9 et J14, entrainant une mortalité de 50% à J14 (Figure 6D). Les analyses histologiques ont permis de confirmer la dégradation de la MEC avec une dégradation des fibres élastiques (Figure 6F).

Nous avons montré une que la neutralisation du TGFβ induisait une augmentation significative de l'infiltration des cellules inflammatoires au sein de la paroi aortique, avec notamment une augmentation des lymphocytes T (Figure 7A), des macrophages (Figure 7B) et des neutrophiles (Figure 7C). L'analyse par cytométrie en flux a permis de caractériser la composition cellulaire de l'aorte abdominale et a montré que les macrophages représentaient la population cellulaire prépondérante (Figure 7D).

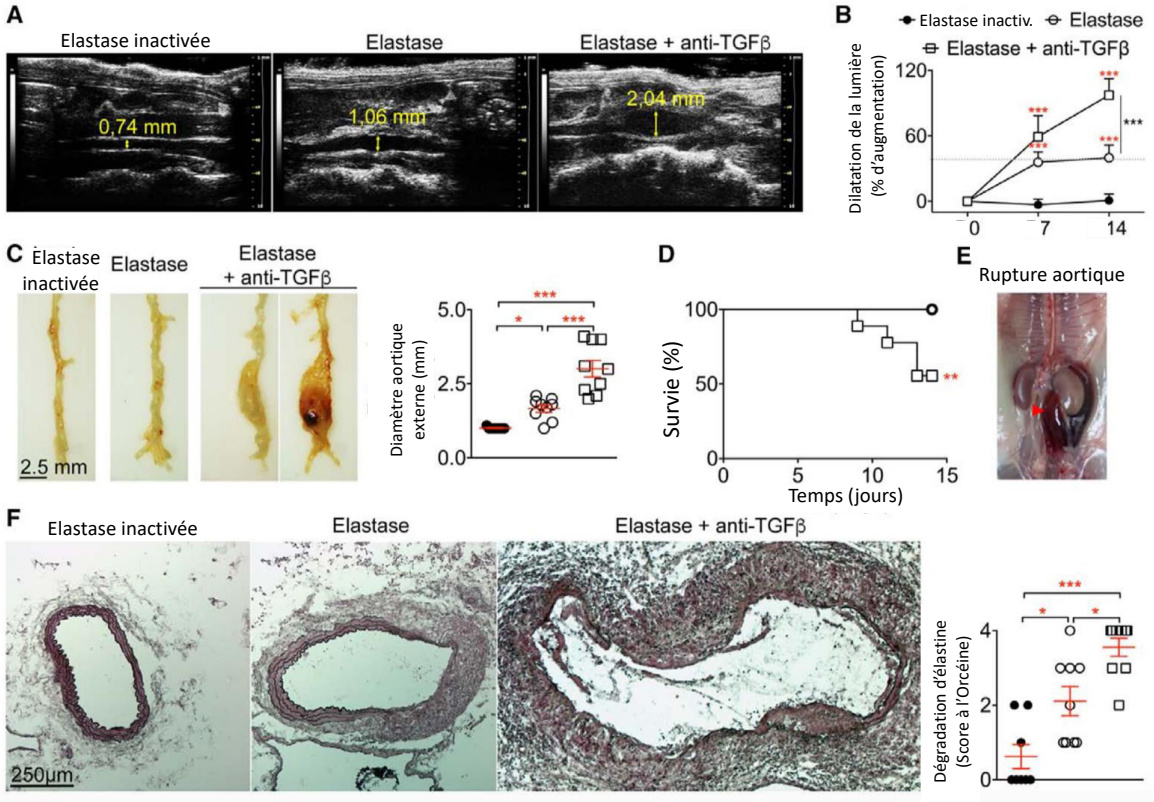

Figure 6 : La neutralisation du TGFβ aggrave la dilatation anévrismale et induit une rupture aortique fatale.

(A) Images représentatives de l'échographie de l'aorte abdominale à J14. (B) Quantification du diamètre aortique au cours du temps (n=9 par groupe). Analyse avec Two-Way ANOVA et test post hoc de Tukey. (C) Images représentatives et quantification du diamètre aortique externe (mm) mesuré au caliper à J14 (n=9 par groupe). Test de Kruskal Wallis suivi d'un test de Dunn. (D) Courbe de survie (n=9 par groupe). Test de Log Rank (E) Images représentatives d'une rupture aortique avec un hématome rétropéritonéal après nécropsie. (F) Images représentatives de la coloration à l'orcéine et quantification de la dégradation de l'élastine à J14 (n=9 par groupe). Test de Kruskal Wallis suivi d'un test de Dunn.
*** P<0.001

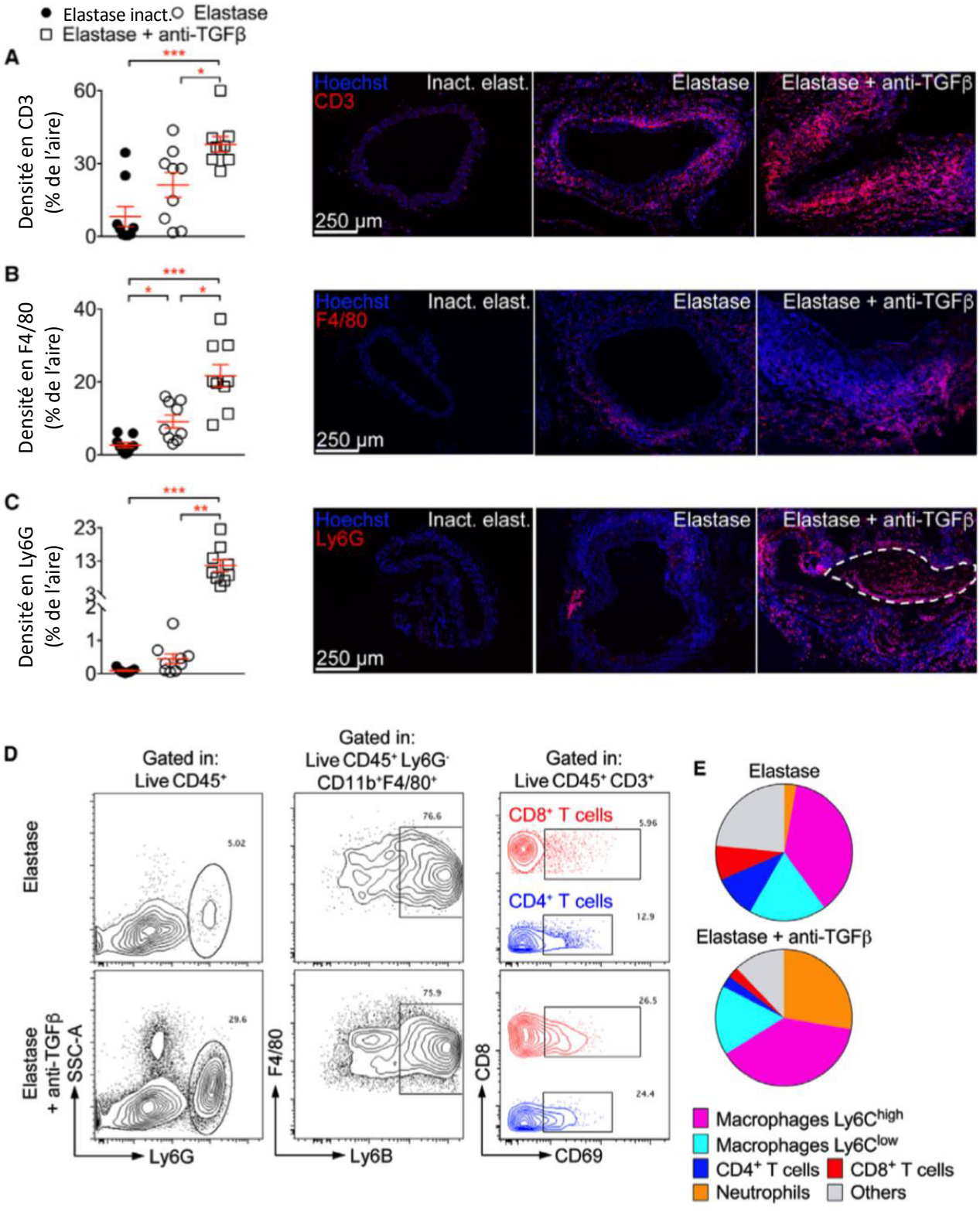

Figure 7 : La neutralisation du TGFβ après application d'élastase induit une infiltration de cellules inflammatoires au sein de la paroi aortique.

Les coupes en cryosection des aortes de souris à J14 (n=9 par groupe) ont été marquées pour les lymphocytes T (A), les macrophages (B) et les neutrophiles (C). (A-C) La quantifications de ces cellules a été réalisée à 14 jours (n=9 par groupe). Test de Kruskall-Wallis suivi d'un test de Dunn. Dans la figure (C), les ligne pointillées soulignent le thrombus intra-luminal. (D) et (E) Analyse en cytométrie de flux des cellules aortiques $CD45^+$ à 14 jours (n=4/groupe). (D) Flow chart représentant les neutrophiles, les macrophages et les lymphocytes T dans les groupes élastase et élastase + anti-TGFβ. (E) Distribution des cellules immunitaires aortique dans les groupes élastase et élastase + anti-TGFβ.
***P<0.001
**P<0.01
*P<0.05

1.b. Caractérisation du phénotype des macrophages infiltrés dans la paroi aortique

Afin d'étudier l'effet de la neutralisation du TGFβ sur le phénotype des macrophages infiltrés dans la paroi aortique, nous avons analysé l'expression génique des marqueurs M1 (CCL2, NOS2, CCL5, IL1β, IL6) et M2 (ARG1, Ym1/2, FIZZ1, MGL2) au niveau de l'aorte abdominale (Figure 8). A J7, l'expression de CCL2, NOS2, CCL5, IL1β, ARG1 et Ym1/2 était significativement augmentée après application d'élastase, avec ou sans neutralisation du TGF β, par rapport au groupe contrôle. L'expression de FIZZ1 était significativement diminuée. A J14, l'expression de CCL2 et de NOS2 restaient élevée dans les groupes ayant reçu de l'élastase. Par rapport au groupe ayant reçu de l'élastase seule, la neutralisation du TGFβ entrainait une augmentation significative de l'expression de l'IL6, l'IL1β, l'ARG1 et une diminution de l'expression de FIZZ1 et MGL2.

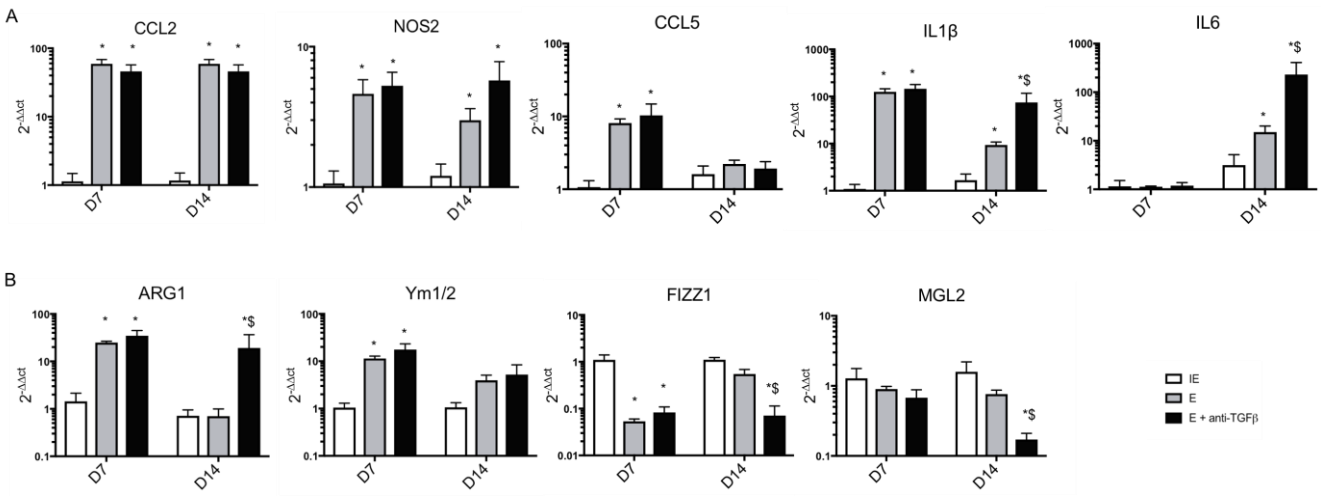

Figure 8 : Cinétique de l'expression génique des marqueurs M1 et M2 dans le tissu aortique après application d'élastase et neutralisation du TGFβ.

(A) Expression génique des marqueurs M1. (B) Expression génique des marqueurs M2.
Résultats obtenus à partir de 3 expériences indépendantes (n= 4 à 15 par groupe). Test de Kruskal Wallis suivi d'un test de Dunn.
IE: élastase inactivée
E: élastase
E + anti-TGFβ: élastase associée à la neutralisation du TGFβ
D7: 7ème jour
D14: 14ème jour
* P<0.05 vs élastase inactivée
$ P<0.05 vs élastase

Afin de déterminer si l'expression des marqueurs M1/M2 était liée à la présence de monocytes/ macrophages infiltrés dans l'aorte, nous avons déplété les monocytes/ macrophages en injectant du clodronate chez des souris recevant de l'élastase + anti- TGFβ. Comme attendu, l'injection de clodronate entrainait une réduction drastique des monocytes $Ly6B^{high}$ et des monocytes $Ly6B^{low}$ dans le sang ainsi qu'une diminution des macrophages infiltrés dans la rate et la paroi aortique (Figure 9A). La déplétion des macrophages infiltrés dans la paroi aortique après injection de clodronate a été confirmée par l'analyse histologique des coupes aortiques montrant l'absence de marquage F4/80 après injection de clodronate (Figure 9B). La déplétion

en macrophages provoquait une réduction significative de la dilatation anévrismale (Figure 9C) et prévenait la rupture aortique entrainant un taux de survie dans 100% des cas (Figure 9D). Ceci était associé à une meilleure préservation des fibres d'élastine et de collagène (Figure 9E et 9F). Ces résultats suggèrent un effet globalement pathogène des macrophages dans ce modèle. De façon intéressante, la déplétion en macrophages réduisait de façon significative l'expression de certains marqueurs M1 comme l'IL6 et l'IL1β et de certains marqueurs M2 comme l'ARG1 et Ym1/2 (Figure 9G). Ces résultats suggèrent que les macrophages sont en partie responsables de l'expression de ces facteurs dans le tissu aortique.

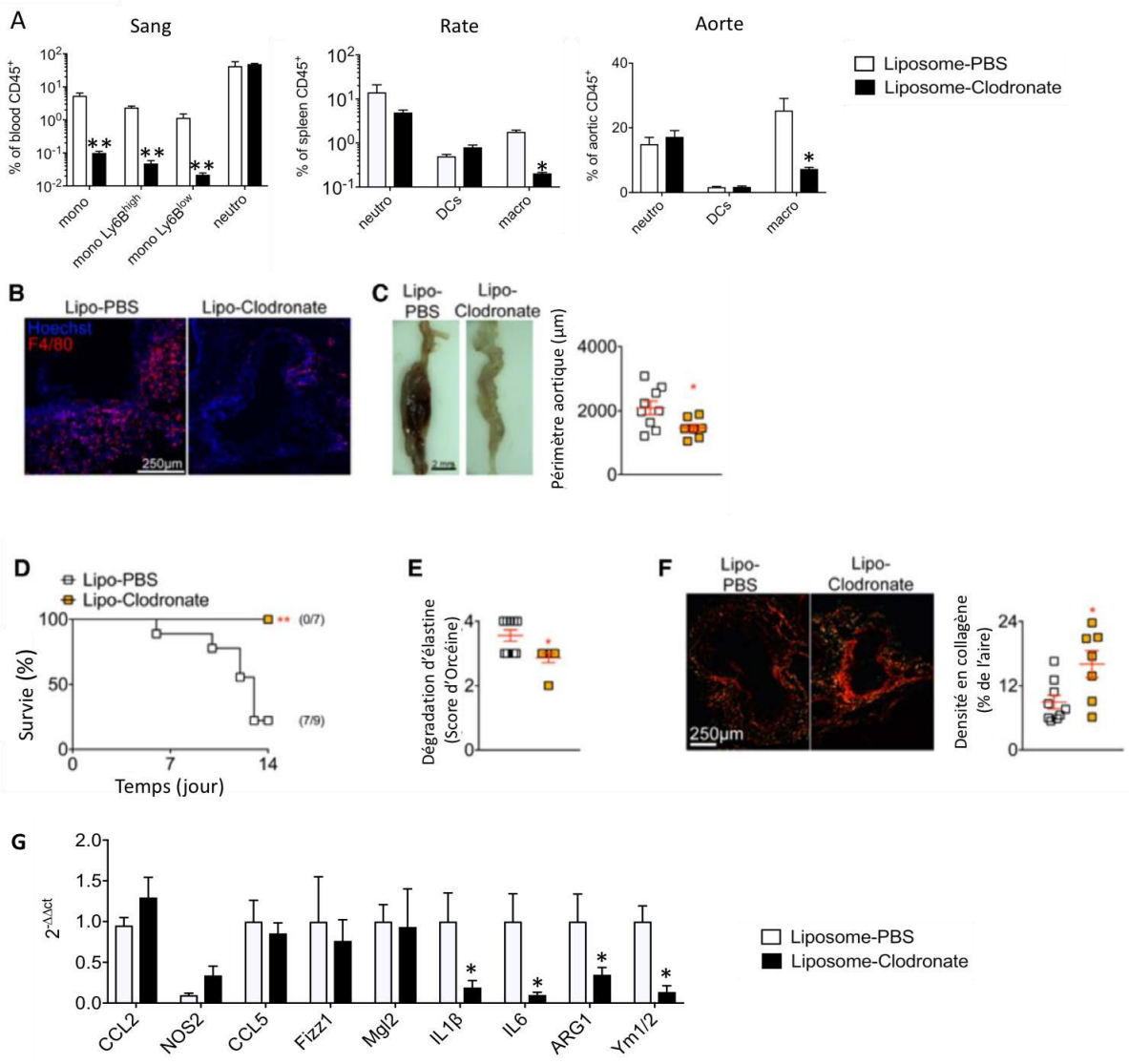

Figure 9 : La déplétion des monocytes/ macrophages après injection de clodronate chez les souris E + anti- TGFβ limite la dilatation aortique, prévient la rupture et modifie l'expression de certains marqueurs M1 et M2.

(A) Quantification par cyotométrie en flux des neutrophiles, des cellules dendritiques, des monocytes et des macrophages dans le sang, la rate et le tissu aortique. Résultats obtenus à partir de souris traitées par élastase + anti-TGFβ avec injection de clodronate ou de PBS et sacrifiées à J7.
(E) Expression génique relative des marqueurs M1 et M2 dans le tissue aortique
DCs: dendritic cells
macro: macrophages
mono: monocytes
neutro: neutrophils
* P<0.05
** P<0.01

Alors que l'expression et le rôle de certains marqueurs comme l'IL6, l'IL1β, l'IL10 ou le TGFβ ont bien été étudiés dans l'AAA [123,132,150,230], il existe peu de données sur l'ARG1. L'ARG1 est une enzyme qui catalyse la dernière étape du cycle de l'urée et différentes études ont suggéré son rôle dans le remodelage et la réparation tissulaire [231].

Afin de confirmer les résultats sur l'expression génique de l'ARG1 dans le tissu aortique, nous avons analysé son expression protéique par immunofluorescence (Figure 10A). Ceci a permis de confirmer son augmentation dans le groupe E + anti-TGFβ par rapport au groupe E et au groupe contrôle. L'analyse histologique nous a permis de confirmer que l'expression de l'ARG1 co-localisait avec les macrophages (Figure 10B). Par rapport aux souris traitées par élastase seule, la neutralisation du TGFβ augmentait significativement la proportion de macrophages exprimant l'ARG1 au niveau de l'aorte (Figure 10C). De plus, nous avons caractérisé la sévérité de l'AAA dans le groupe élastase + anti-TGFβ en fonction de l'aspect macroscopique de la dilatation aortique (grade II : dilatation aortique, grade III : dilatation aortique d'aspect bulbaire contenant du thrombus, grade IV : rupture). L'infiltration de neutrophiles augmentait avec la sévérité de l'anévrisme. Plus l'anévrisme était sévère, plus la densité en macrophages exprimant l'ARG1 était élevée (Figure 10D).

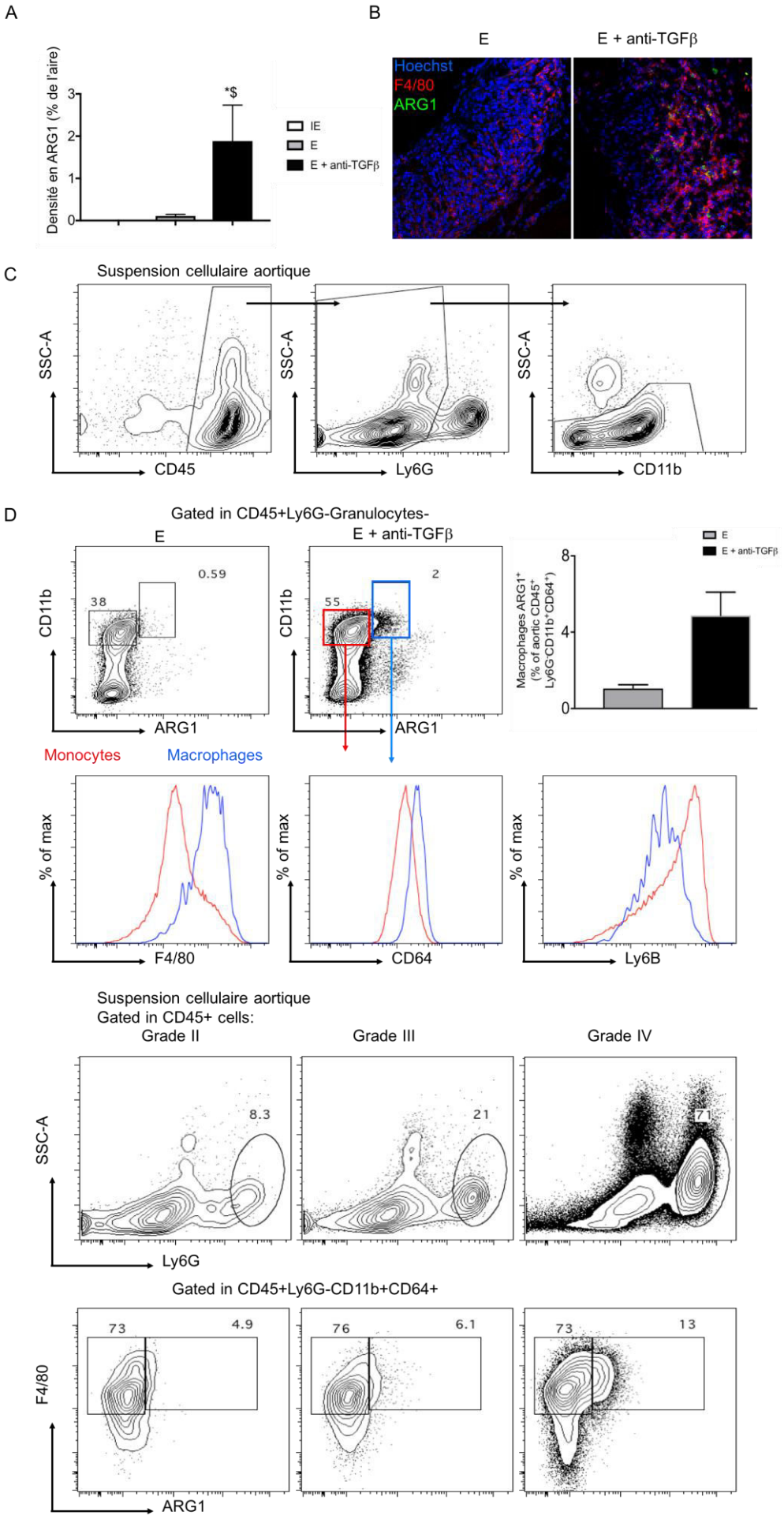
A
Densité en ARG1 (% de l'aire)
*$
IE
E
E + anti-TGFβ
B
E
E + anti-TGFβ
Hoechst
F4/80
ARG1
C
Suspension cellulaire aortique
SSC-A
CD45
Ly6G
CD11b
D
Gated in CD45+Ly6G-Granulocytes-
E
E + anti-TGFβ
CD11b
ARG1
38
0.59
55
2
Monocytes
Macrophages
Macrophages ARG1+ (% of aortic CD45+ Ly6G-CD11b+CD64+)
% of max
F4/80
CD64
Ly6B
E
Suspension cellulaire aortique
Gated in CD45+ cells:
Grade II
Grade III
Grade IV
SSC-A
Ly6G
8.3
21
7
F
Gated in CD45+Ly6G-CD11b+CD64+
F4/80
ARG1
73
4.9
76
6.1
73
13

Figure 10. : La neutralisation du TGFβ induit une augmentation des macrophages exprimant l'ARG1.

(A) Quantification de l'expression protéique de l'ARG1 dans le tissue aortique anévrismal. Résultats obtenus à partir de 10 souris par groupe sacrifiées à J14. (B) Images représentatives du co-marquage F4/80 et ARG1 en immunofluorescence. Test de Kruskal Wallis suivi d'un test de Dunn. (C) Graphiques illustrant la stratégie d'identification des macrophages dans la suspension cellulaire issue des aortes. Les macrophages ont été identifiés parmi les cellules CD45+Ly6G-SSC^{low} (excluant les granulocytes). (D) Graphiques illustrant l'expression de CD11b et ARG1 dans les cellules CD45+Ly6G-SSC^{low}. Les cellules CD11b+ARG1- (en rouge) et CD11b+ARG1+ (en bleu) ont ensuite été analysées pour l'expression de F4/80, CD64 et Ly6B. Les cellules CD11b+ARG1- ont un phénotype proche des monocytes (Ly6B^{high}CD64^{int}F4/80^{int}) alors que les cellules CD11b+ARG1+ s'apparentent aux macrophages (Ly6B^{int}CD64^{high}F4/80^{high}). (E) Graphiques illustrant l'infiltration des neutrophiles dans le tissu aortique en fonction du grade de l'AAA dans les souris élastase + anti-TGFβ. (F) Graphiques illustrant l'expression de F4/80 et ARG1 dans les cellules aortiques CD45+Ly6G-CD11b+CD64+.
IE: élastase inactivée
E: élastase
E + anti-TGFβ: élastase associée à la neutralisation du TGFβ
D7: 7ème jour
D14: 14ème jour
* P<0.05 vs élastase inactivée
$ P<0.05 vs élastase

L'expression de l'ARG1 au cours de l'AAA étant peu connue, nous avons analysé son expression protéique sur des coupes anévrismales humaines (Figure 11). Alors qu'aucun marquage n'était observé dans le tissu aortique sain, des cellules exprimant l'ARG1 étaient identifiées dans le tissu anévrismal, avec une localisation préférentielle au niveau de l'adventice.

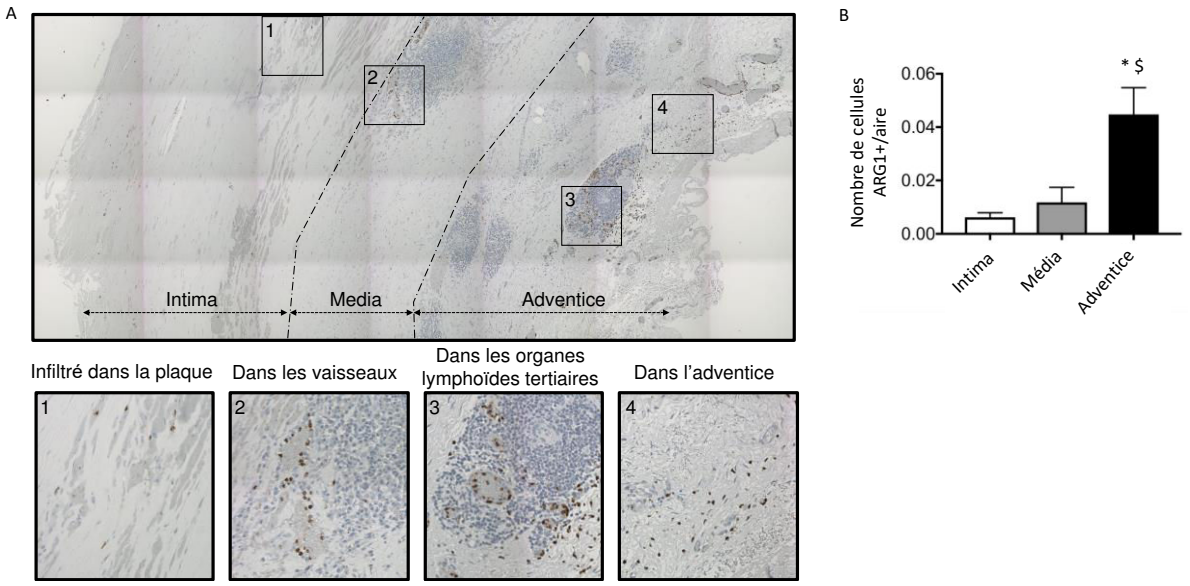

Figure 11: L'expression protéique de l'ARG1 est augmentée dans le tissu anévrismal humain.

(A) Image représentative de l'expression protéique de l'ARG1 dans le tissu anévrismal infra-rénal humain. (B) Quantification de l'expression protéique de l'ARG1dans le tissu aortique anévrismal (n=10). Test de Kruskal Wallis suivi d'un test de Dunn.
* P<0.05 vs intima
$ P<0.05 vs media

1.c. Caractérisation d'une population particulière de monocytes dits « Sat-Mono »

Une étude récente ayant identifié un nouveau type de monocytes appelés Sat-Mono, caractérisés par l'expression des marqueurs CEACAM1+MSR1+Ly6B/C-F4/80-MAC1+ et jouant un rôle clé dans la fibrose [140], nous avons caractérisé cette population dans le modèle murin à J4 et J11 (Figure 12). Par rapport à l'application d'élastase seule, la neutralisation du TGFβ entrainait une diminution significative des Sat-Mono dans la moelle osseuse, le sang ainsi que l'aorte.

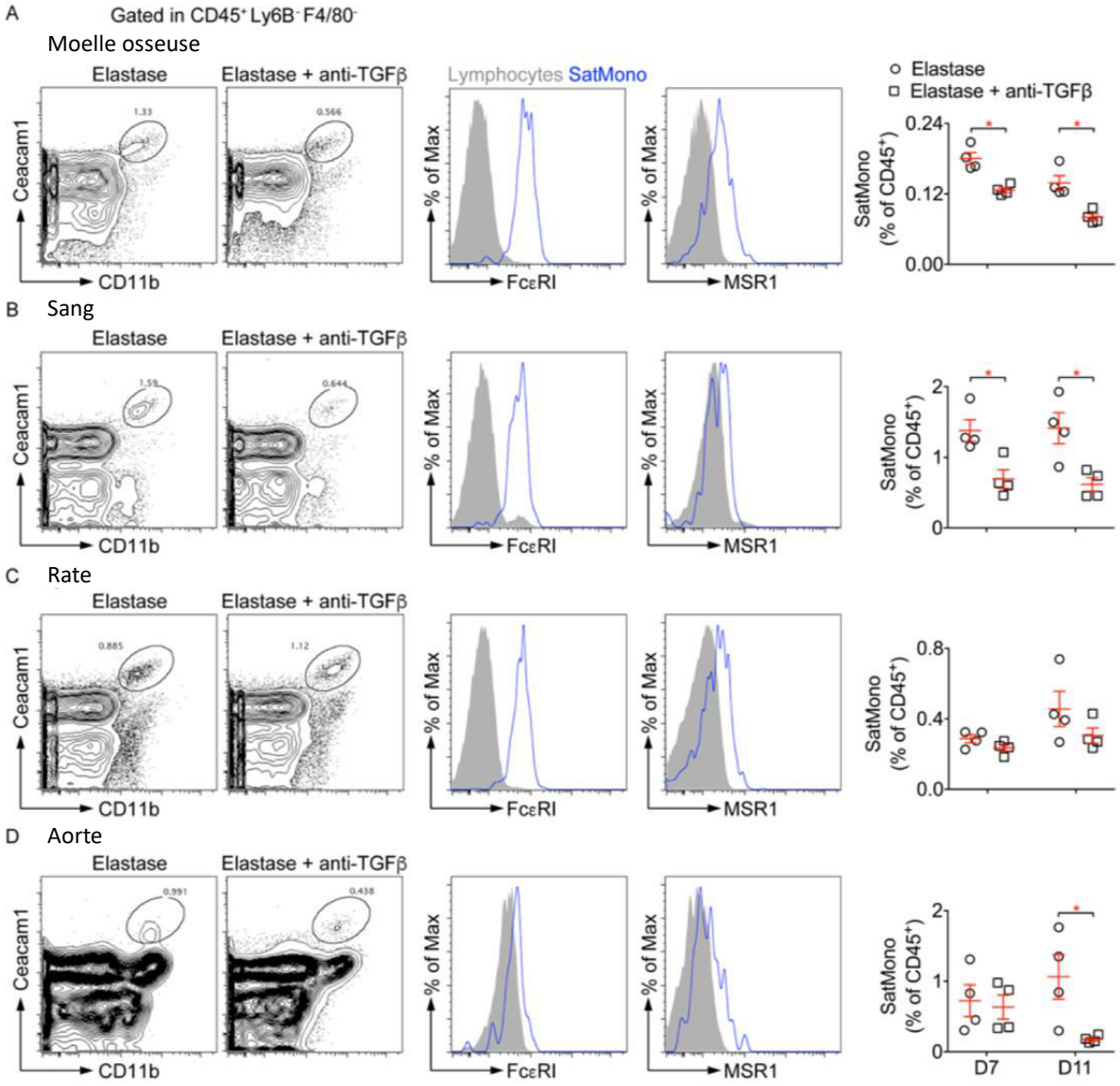

Figure 12 : La neutralisation du TGFβ induit une diminution des Sat-Mono.

Analyse par cytométrie en flux des Sat-Mono dans la moelle osseuse (A), le sang (B), la rate (C) et l'aorte (D) à J4 et J11 après application d'élastase avec ou sans neutralisation du TGFβ. Les Sat-Mono ont été identifiés comme des cellules CD45+, Ly6B-, F4/80-, CD11b+, Ceacam1+ et analysé pour l'expression du FcεRI (n= 4 par groupe). Test de Mann-Whitney
* P<0.05

2. Discussion

Le but de cette étude était de caractériser le phénotype des macrophages dans le modèle murin d'AAA induit par l'application d'élastase et la neutralisation du TGFβ. L'utilisation d'une stratégie de déplétion par injection de clodronate nous a permis d'identifier leur rôle et de mieux comprendre si l'expression des marqueurs M1 et M2 dans l'aorte était principalement liée aux macrophages. Nos résultats montrent qu'au cours du développement de l'AAA, il existe une augmentation de l'expression des marqueurs M1 (CCL2, NOS2, CCL5) alors que les marqueurs M2 sont diminués (FIZZ1, MGL2) ou augmentés (ARG1, Ym1/2). Au cours des dernières années, différentes études expérimentales ont exploré la polarisation des macrophages dans le modèle d'AAA induit par l'angiotensine II. Une première étude avait caractérisé les macrophages M1 et M2 par cytométrie en flux grâce à l'expression des marqueurs NOS2 et Ym1/2 [157]. Les auteurs avaient mis en évidence une augmentation du ratio M1/M2 dans l'aorte supra-rénale des souris ApoE-/- après 7 jours d'infusion d'angiotensine II et cet effet était maintenu à 28 jours. Une deuxième étude a montré que l'infusion d'angiotensine II sur une durée totale de 84 jours entrainait l'expansion de l'AAA ainsi que la rupture, qui était associée avec une augmentation de l'infiltration des macrophages dans la paroi aortique [158]. Les auteurs ont identifié sur coupes histologiques les macrophages grâce aux marquages CD68 et MOMA2 et ont montré que l'expression du marqueur M2 CD206 dans la paroi aortique était plus fréquente que celle du marqueur M1 NOS2. Ces résultats suggéraient que l'orientation plus tardive des macrophages vers un phénotype de type M2 résultait d'un mécanisme compensateur pour prévenir l'expansion de l'AAA [133]. Nos résultats indiquent que la stricte dichotomie entre macrophages M1 et M2 ne s'applique pas *in vivo* et qu'il existe en réalité une modulation complexe du phénotype des macrophages au cours de l'AAA. De plus, les marqueurs M1 et M2 ne sont pas exclusivement

exprimés par les macrophages. Ceci pourrait expliquer, du moins en partie, pourquoi l'expression de certains marqueurs tels que CCL2, NOS2, CCL5, FIZZ1 et MGL2 ne variait pas significativement après injection de clodronate. Enfin, l'expression des marqueurs M1 et M2 variait au cours du temps, soulignant l'intérêt de réaliser une cinétique.

L'injection de clodronate diminuait la dilatation anévrismale et protégeait de la rupture grâce à une meilleure préservation de la MEC. Le clodronate entrainait une déplétion des monocytes circulants et des macrophages infiltrés dans la paroi aortique dérivés des monocytes circulants. Nos résultats suggèrent un effet globalement pathogène de ce type de macrophage dans l'AAA. Néanmoins, le rôle des macrophages résidents reste à explorer.

La neutralisation du TGFβ provoquait également une diminution des Sat-Mono dans la moelle osseuse, le sang ainsi que l'aorte. Bien que des études complémentaires soient nécessaires pour mieux comprendre leur rôle dans l'AAA, étant donné le rôle des Sat-Mono dans la fibrose, leur diminution pourrait contribuer aggraver la dilatation anévrismale [140].

Alors que l'expression et le rôle de certains marqueurs M1 et M2 tels que l'IL6, l'IL1β, l'IL10 ou le TGFβ a été bien étudié [123,132,150,230], l'expression de l'ARG1 a été moins explorée. La neutralisation du TGFβ augmentait l'infiltration de macrophages exprimant l'ARG1 et celle-ci corrélait avec la gravité de l'AAA. L'ARG1 est une enzyme impliquée dans la conversion de l'arginine en urée et en ornithine [231]. L'ornithine est un précurseur à la production de la proline, un acide aminé utile à la synthèse de protéines riches en proline telles que le collagène, constituant indispensable de la MEC. Différentes études ont montré que la supplémentation en arginine augmentait le dépôt de collagène chez l'homme et sur modèle animal et favorisait la réparation tissulaire [232,233]. De plus, des études *in vitro* ont montré que l'ARG1 favorisait la prolifération des CMLs et sa surexpression dans les CMLs inhibait la sécrétion de TNFα induite par le LPS et inhibait la migration des monocytes [234,235]. L'ARG1 pourrait donc potentiellement jouer un rôle dans la pathogénèse des AAA via un effet sur le remodelage de la MEC,

l'homéostasie des CMLs ou encore la régulation de la réponse inflammatoire. Alors que l'expression de l'ARG1 augmente avec la progression anévrismale, des études expérimentales complémentaires avec des souris déficientes en ARG1 sont nécessaires pour déterminer si l'augmentation de l'ARG1 a un effet pathogène ou compensateur sur le développement de l'AAA. Enfin, l'AAA est le plus souvent associé à l'athérosclérose [4] et différentes études ont exploré le rôle de l'ARG1 dans cette pathologie. Une première étude a montré une augmentation de l'expression de l'ARG1 dans les macrophages de lapins génétiquement prédisposés à développer peu d'athérosclérose, comparé à un groupe fortement prédisposé à développer de l'athérosclérose, suggérant le rôle de l'ARG1 dans la résistance à l'athérosclérose [236]. En plus des macrophages, l'ARG1 peut aussi être détectée dans les cellules endothéliales et les CMLs [237]. Une étude a montré que même si la délétion spécifique de l'ARG1 dans les cellules hématopoïétiques augmentait la formation de cellules spumeuses, il n'y avait pas d'effet significatif sur la taille ou la stabilité de la plaque ainsi que le contenu en macrophages dans la racine aortique [238]. Afin de déterminer si l'augmentation de l'ARG1 dans le tissu aortique ou l'augmentation des macrophages exprimant l'ARG1 jouent un rôle dans l'AAA, il serait utile d'utiliser des souris spécifiquement invalidées pour l'ARG1 dans les macrophages. L'ensemble de ces résultats souligne le rôle complexe de l'ARG1 qui pourrait varier selon le type cellulaire. Des études complémentaires sont nécessaires afin de mieux comprendre son rôle dans la formation et la progression de l'AAA.

PARTIE II :

ETUDE DE L'EXPRESSION DES MICRO-ARNs DANS LE MODELE MURIN D'AAA INDUIT PAR L'ELASTASE ET LA NEUTRALISATION DU TGFβ

Cette partie fait l'objet de résultats préliminaires servant de base pour la réalisation d'expériences complémentaires au laboratoire.

1. Résultats

1.a. Profil de l'expression des micro-ARNs

Afin d'explorer l'expression des miRs dans les aortes de souris ayant un AAA induit par l'application d'élastase et la neutralisation du TGFβ, une analyse du profil de 752 miRs de souris a été réalisée dans les groupes des souris contrôles, élastase et élastase + anti-TGFβ sacrifiées à J14 (n=3 par groupe). Sur 752 miRs explorés, 338 étaient détectables dans tous les échantillons et le nombre moyen de miRs détectés par échantillon était de 413 (Figure 13A). La qualité des échantillons a été contrôlée en analysant les Ct obtenus pour l'ADNc UniSp6 ajouté pendant la transcription inverse et les Ct obtenus pour les miR-103, 23a, 30c, 451 and 142-3p (Figure 13B). Les miR-103, 23a et 30c sont connus pour être exprimés dans la majorité des échantillons biologiques à un niveau stable. Le miR-451 est fortement exprimé par les globules rouges et le miR-142-3p par les thrombocytes. Les Ct obtenus pour ces contrôles étaient similaires entre les échantillons, témoignant de leur comparabilité.

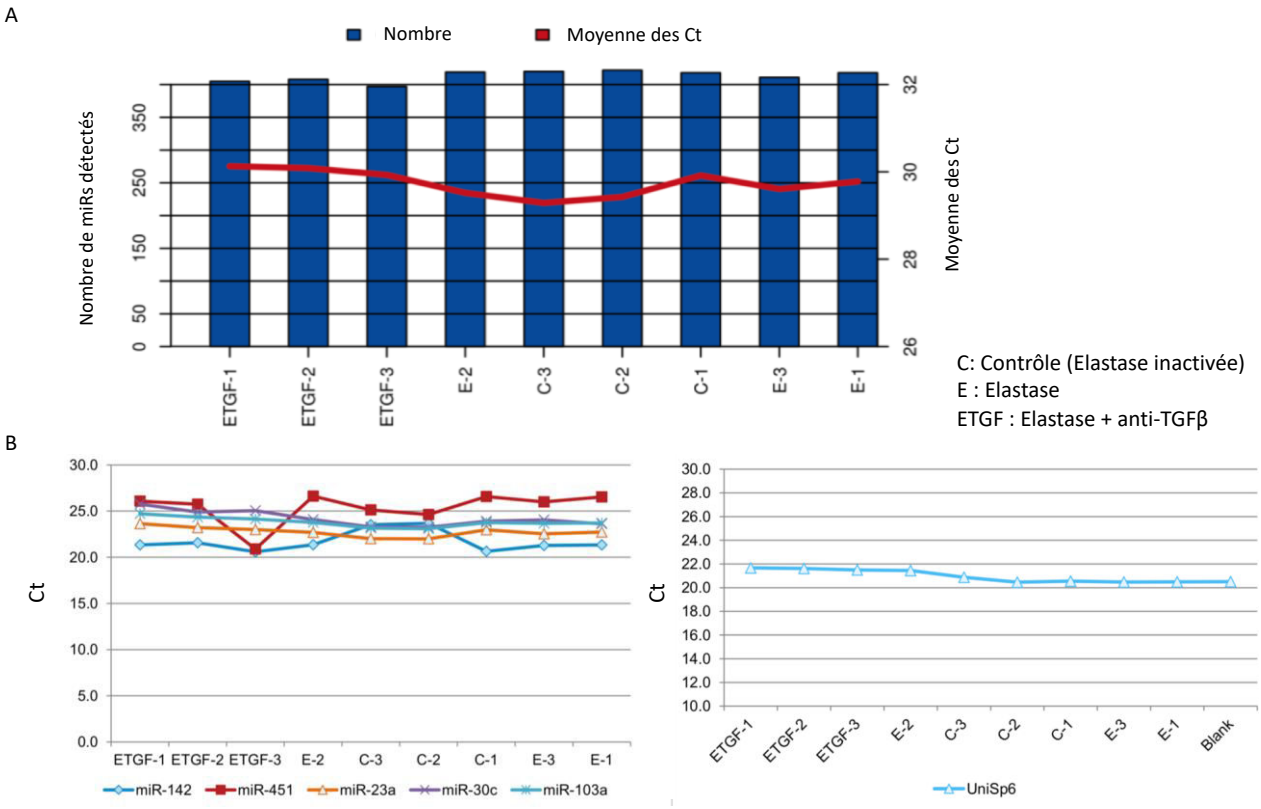

Figure 13 : Contenu en miRs et contrôles de la qualité des tissus aortiques utilisés pour le profil d'expression des miRs

(A) Graphique illustrant le contenu en miRs. Les barres représentent le nombre de miRs détectés et les lignes illustrent les moyennes des Ct obtenues pour les miRs exprimés. Légende des échantillons
(B) Ct obtenus pour les miRs-142, 451, 23a, 30c, 103a et l'ADNc de synthèse UniSp6.

Les profils d'expression des miRs sont présentés et illustrés dans le Figure 2. Le heat map (Figure 14A) et l'analyse en composante principale (Figure 14B) montrent que les échantillons peuvent effectivement être regroupés en 3 classes : contrôles, élastase et élastase + anti-TGFβ.

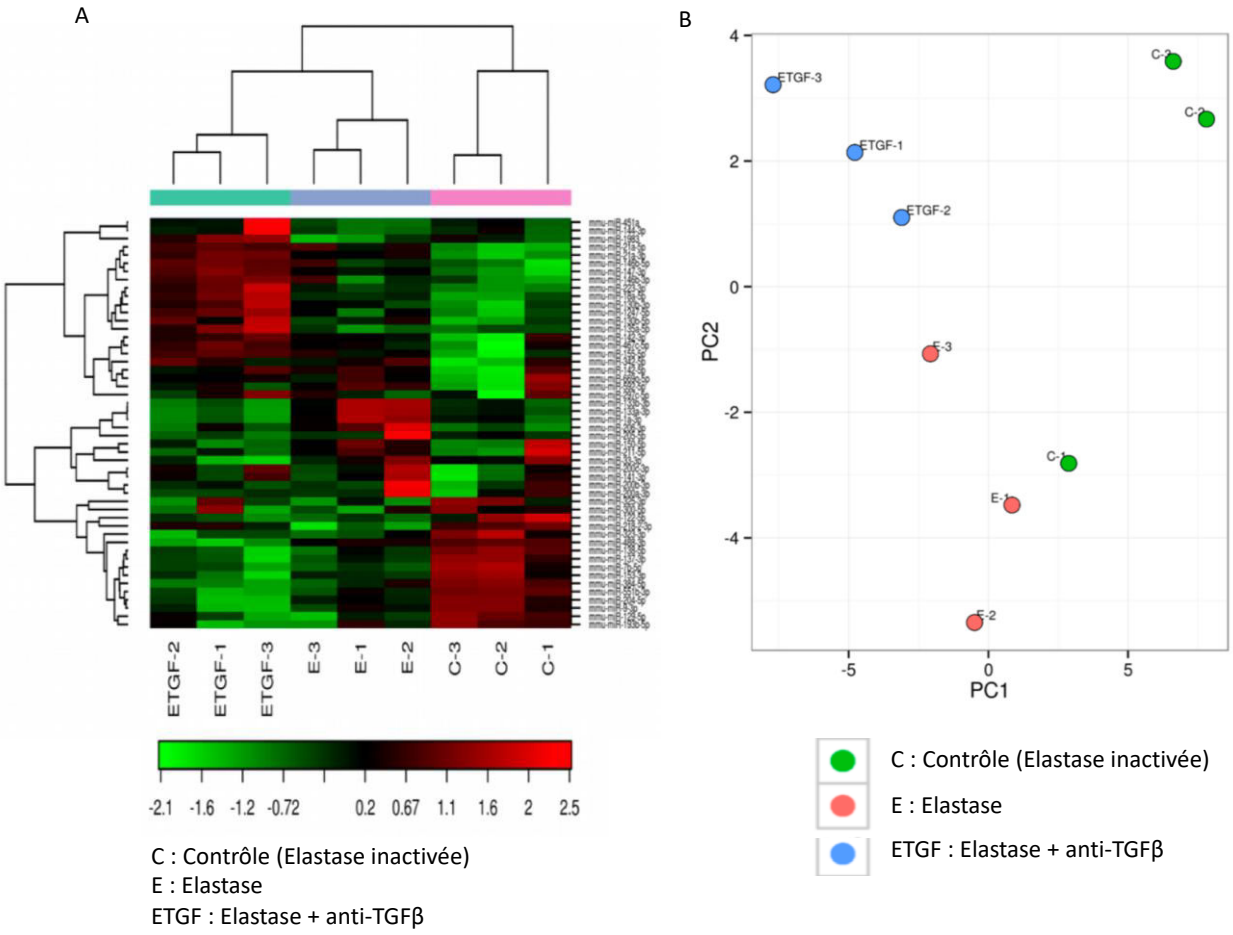

Figure 14 : Profil d'expression des miRs dans le tissu aortique (n= 3 par groupe).

(A) Heat map réalisé en groupant les échantillons sur la base des 50 miRs ayant les déviations standards les plus élevées. (B) Analyse en composante principale réalisée en groupant les échantillons sur la base des 50 miRs ayant les déviations standards les plus élevées.

1.b. Confirmation de l'expression des micro-ARNs

A partir du profil d'expression des miRs et de la revue de la littérature, nous avons sélectionné certains candidats sur les critères suivants : miRs les plus différentiellement exprimés entre les groupes contrôle et élastase + anti-TGFβ, miRs dont le rôle dans l'AAA n'a pas encore été décrit dans un modèle murin, miRs dont la variation au cours de l'AAA a été identifiée chez l'homme (soit dans la littérature, soit dans le profil d'expression que nous

avons réalisé en parallèle au laboratoire sur tissus humains). Deux miRs d'intérêt ont ainsi été retenus : miR-18a-3p et 223-3p. L'expression de ces miRs a été confirmée dans le tissu aortique à J7 et J14 à partir de 3 expériences indépendantes et a également été recherchée dans le sérum (Figure 15). L'expression du miR-18a-3p était significativement augmentée dans le tissu aortique des souris élastase + anti-TGFβ dès J7 et était maintenue à J14 par rapport aux souris contrôles. De façon intéressante, l'expression de ce miR dans le sérum était également augmentée à J7. L'expression du miR-223-3p était significativement augmentée dans le tissu aortique ainsi que dans le sérum des souris recevant de l'élastase et de l'élastase associée à la neutralisation du TGFβ par rapport aux contrôles.

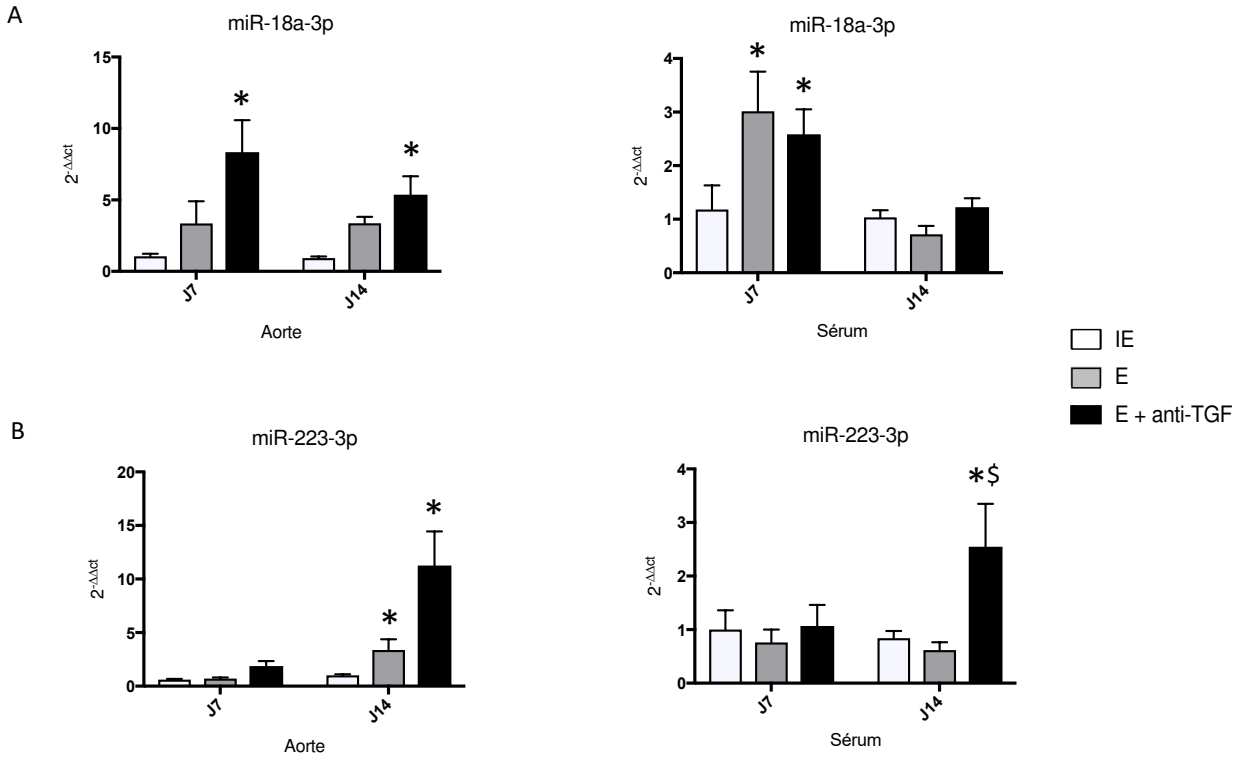

Figure 15 : Expression relative des miRs dans le tissu aortique et le sérum.

(A) Expression dans le tissu aortique (n= 4 à 10 par groupe, à partir de 3 expériences indépendantes). (B) Expression dans le sérum (n=4 à 6 par groupe, à partir de 2 expériences indépendantes)
* P<0.05 vs élastase inactivée
$ P<0.05 vs élastase

2. Discussion

Ces résultats préliminaires ont permis d'identifier deux miRs candidats qui pourraient jouer un rôle important dans le développement de l'AAA. Le miR-18a-3p a été identifié dans différentes maladies cardiovasculaires et son rôle dans l'AAA n'est pas encore connu. Le miR-18a-5p est impliqué dans la régulation de la transition endothéliale- mésenchymateuse en condition de fortes concentrations en glucose dans les cellules endothéliales humaines et pourrait ainsi jouer un rôle dans la fibrose myocardique observée chez les patients diabétiques [239]. Une autre étude a montré que la surexpression du miR-18 dans les cardiomyocytes avait un effet protecteur sur l'hypertrophie cardiaque induite par l'hypertension [240]. Le miR-18a est également impliqué dans la réponse immunitaire en régulant la différenciation des lymphocytes T auxiliaires 17 (Th17) [241]. Les Th17 jouent un rôle dans la réponse inflammatoire et sont impliqués dans différentes maladies auto-immunes [242]. L'inhibition de la différenciation vers un phénotype Th17 avait un effet protecteur sur la formation d'AAA induite par infusion d'angiotensine II [243]. Enfin, le miR-18a-3p a été identifié dans différentes pathologies tumorales comme l'hépatocarcinome ou le glioblastome [244-246].

Le miR-223-3p a été mis en évidence dans la pathologie anévrismale humaine. Son expression est en effet augmentée dans le tissu aortique chez les patients atteints d'AAA ou de TAA par rapport aux contrôles [177,247]. De plus, le miR-223-3p était augmenté dans le plasma des patients ayant un AAA avec une croissance rapide comparativement aux patients ayant un AAA de croissance lente [183]. Son rôle a été identifié dans différentes maladies cardiovasculaires. Il est en effet surexprimé au cours de l'infarctus du myocarde et son inhibition diminuait la survenue d'arythmie liée à l'ischémie [248]. Il est également impliqué dans l'insuffisance cardiaque où l'augmentation de son expression limitait l'hypertrophie ventriculaire droite et améliorait la fonction cardiaque après hypoxie [249]. Différentes études

ont également montré son rôle dans certains cancers ou encore les maladies inflammatoires chroniques intestinales via un effet sur la prolifération cellulaire et la réponse inflammatoire [250-254].

Alors que les miR-18a-3p et 223-3p sont impliqués dans certaines maladies cardiovasculaires, leur rôle dans l'AAA est à ce jour inconnu. Des expériences complémentaires au laboratoire sont en cours de réalisation. Afin de mieux comprendre le rôle des miRs identifiés, des injections d'antago-miRs vont être réalisées dans le modèle murin induit par l'élastase et la neutralisation du TGFβ. Ces antago-miRs se lient de façon complémentaire au miR d'intérêt, formant un duplex stable et empêchant le miR d'exercer son effet sur sa cible [255]. De plus, des souris invalidées pour le miR-223-3p sont disponibles et il est prévu d'étudier le développement et la croissance de l'AAA dans ce modèle.

Bien que des études complémentaires soient nécessaires pour comprendre leur rôle dans la pathogénèse anévrismale, les résultats sur l'expression des miRs nous ont permis d'identifier 2 candidats qui pourraient potentiellement représenter une cible thérapeutique dans l'AAA. Leur variation dans le sérum des souris suggère leur intérêt potentiel en tant que biomarqueurs. Enfin, ces 2 candidats sont potentiellement relevant pour la pathologie humaine puisqu'ils ont été décrits chez des patients porteurs d'AAA pour le miR-223-3p et identifiés comme étant différentiellement exprimés dans le tissu anévrismal humain dans notre laboratoire.

PARTIE III :

ETUDE DE L'EXPRESSION DES MICRO-ARNs CHEZ LES PATIENTS DIABETIQUES PRESENTANT UN AAA

Cette partie a fait l'objet de la rédaction d'un article soumis dans Biochemia Medica.

"Differential micro-RNA expression in diabetic patients with abdominal aortic aneurysm."

Fabien Lareyre, Marc Clément, Claudine Moratal, Xavier Loyer, Elixène Jean-Baptiste, Réda Hassen-Khodja, Giulia Chinetti, Ziad Mallat, Juliette Raffort

1. Résumé

Introduction : Alors que le diabète est un facteur de risque cardiovasculaire bien établi, il est paradoxalement négativement associé à l'AAA. L'implication potentielle des miRs dans cette association n'a encore jamais été étudiée. Le but de cette étude pilote était de comparer l'expression des miRs entre des patients diabétiques et des non diabétiques présentant un AAA.

Matériel et méthodes : Une étude prospective a été menée dans le Service de Chirurgie Vasculaire du CHU de Nice comparant des patients diabétiques et des sujets non diabétiques appariés ayant un AAA. Un prélèvement veineux sanguin était réalisé afin de collecter le sérum, le plasma et les cellules sanguines mononuclées. Le tissu aortique anévrismal était recueilli chez les patients bénéficiant d'une chirurgie ouverte. Un profile d'expression de 752 miRs a été réalisé sur les cellules sanguines mononuclées (miRCURY LNA Universal RT

microRNA PCR, Exiqon- Qiagen®) (n=3 par groupe). Les miRs différentiellement exprimés (P <0.05) entre patients diabétiques et non diabétiques ont été sélectionnés puis analysés dans le sérum, le plasma et le tissu aortique anévrismal.

Résultats : Parmi les 752 miRs explorés, 275 étaient détectables dans tous les échantillons. Quatre miRs étaient significativement différentiellement exprimés dans les cellules sanguines mononuclées entre patients diabétiques et non diabétiques : 3 étaient surexprimés (miR-144-3p, 20a-5p and 188-3p) and 1 sous-exprimé (miR-548k). La surexpression des miR-144-3p, 20a-5p and 188-3p dans les cellules sanguines mononuclées a été confirmée dans a cohorte entière et les miR-144-3p et 20a-5p étaient également augmentés dans le tissu anévrismal et le sérum respectivement. L'expression du miR-20a-5p dans les cellules sanguines mononuclées était corrélée à la concentration en fructosamine (r=0.62, p=0.006).

Conclusion : Trois miRs étaient significativement sur-exprimés dans les cellules sanguines mononuclées des patients diabétiques ayant un AAA et certains miRs étaient également dérégulés dans le tissu anévrismal et le sérum. Même si des études complémentaires sont nécessaires afin d'établir leur rôle, ils pourraient potentiellement représenter une nouvelle cible thérapeutique.

2. Article

Differential micro-RNA expression in diabetic patients with abdominal aortic aneurysm

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What this study adds:

Even though diabetes represents a major cardiovascular risk factor, it is negatively associated with abdominal aortic aneurysm. The mechanisms underlying this negative association are still poorly known. This study investigated for the first time miR expression between diabetic and non-diabetic patients with AAA. Three miRs were identified to be differentially expressed in peripheral blood mononuclear cells and their expression was also explored in serum, plasma and aneurysmal tissue. Even if further studies are required to determine their direct role in AAA formation and progression, they could represent interesting new targets.

Abstract:

Objectives: Even though diabetes represents a major cardiovascular risk factor, it is negatively associated with abdominal aortic aneurysm (AAA) development and the potential implication of micro-RNAs (miRs) has so far never been addressed. The aim of this pilot study was to compare miR expression between diabetic and non-diabetic patients with AAA.

Design: A prospective pilot study was performed at the University Hospital of Nice and included 20 men with infrarenal AAA. Ten diabetic patients were included and compared to 10 age- and sex-matched non-diabetic patients.

Materials: Fasting blood samples were collected and peripheral blood mononuclear cells (PBMCs), sera and plasma were extracted. Full thickness aneurysmal aortic tissues were collected in patients who underwent open surgical repair.

Methods: A profiling analysis of 752 human miRs was performed from PBMCs of diabetic and non-diabetic patients (n=3 per group) using miRCURY LNA Universal RT microRNA PCR (Exiqon- Qiagen®). miRs that showed significant differential expression ($P < 0.05$) were selected and further analyzed in the entire cohort in PBMCs, plasma, sera (n=10 per group) and aneurysmal tissue.

Results: Among the 752 miRs screened in PBMCs, 275 were detected in all samples. Four miRs were significantly differentially expressed in diabetic patients compared to non-diabetics: 3 were upregulated (miR-144-3p, 20a-5p and 188-3p) and 1 downregulated (miR-548k). The upregulation of miR-144-3p, 20a-5p and 188-3p in PBMCs was confirmed in the entire cohort and miR-144-3p and 20a-5p were also increased in aneurysmal tissue and serum, respectively. The expression of miR-20a-5p in PBMCs was correlated with fructosamine concentration (r=0.62, p=0.006).

Conclusions: Three miRs were identified to be significantly upregulated in PBMCs of diabetic patients with AAA, some of them being also differentially expressed in aneurysmal tissue and serum. Even if further studies are required to determine their direct role in AAA formation and progression, they could represent interesting new targets.

Introduction

Abdominal aortic aneurysm (AAA) is most often associated with atherosclerosis and cardiovascular risk factors (1, 2). The main pathophysiological features involve inflammatory cell infiltration, extracellular matrix (ECM) remodeling, impairment of vascular smooth muscle cell homeostasis as well as intraluminal thrombus formation (3, 4). Molecular pathways involved are complex and micro-RNAs (miRs) have emerged as major regulators of gene expression and have been shown to play key roles in AAA formation (5, 6). Patients with AAA present a differential miR expression profile in aneurysmal aortic tissue as well as in plasma and serum compared to controls (5, 7-9). In addition, experiments in animal models have unraveled the role of the deregulated miRs, some being protective, and others having a pathogenic effect on AAA development (5, 7, 9-11).

Intriguingly, epidemiological studies identified a negative association between diabetes and AAA (12-15). While diabetes represents a major cardiovascular risk factor, it appears to exert

a protective effect on AAA formation (12). The identification of the molecular and cellular pathways responsible for this protection could lead to innovative therapeutic approaches. The potential implication of miRs in the inverse association between diabetes and AAA has never been addressed so far. The aim of this pilot study was to compare miR expression between diabetic and non-diabetic patients with AAA.

Material and methods

Study population

Twenty men with AAA were prospectively included in the Department of Vascular Surgery at the University Hospital of Nice between June and December 2016. Ten diabetic patients were included and compared to 10 age- and sex-matched non-diabetic patients. The study was approved by the institutional local ethics committee (Comité de Protection des Personnes Sud Méditerranée V) and was conducted in conformity with the Declaration of Helsinki. All patients were informed and gave written consent. Inclusion criteria were men between 50 and 80 years with infrarenal AAA. Aneurysms caused by specific etiologies including infectious, genetic or auto-immune diseases were excluded. Diabetes was defined based on medical records and the use of antidiabetic drugs. All diabetic patients suffered from the type 2 form of the disease, were treated with oral anti-diabetic drugs and none required insulin treatment. The characteristics of the patients were prospectively collected and included age, sex, the presence of cardiovascular risk factors, treatments, fasting glycemia, fructosamine, insulin and C-peptide concentrations. The aneurysmal diameter was systematically determined using the CT-scan images. Data were collected using manuscript and electronic medical records, as well as a computer software program (Clinicom®). Imaging data were recorded and extracted from the Picture Archiving and Communication System (PACS®) multiple modalities software.

Sample collection

For all patients, blood samples were collected in Vacutainer® tubes with serum separator gel and clot activator and in tubes coated with EDTA after 8 to 12 hours fasting. They were centrifuged at 20°C for 10 minutes at 2200 rpm. Sera and plasma were aliquoted and immediately stored at -80°C until further use. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from 10 mL of venous blood using Histopaque (Sigma®). Briefly, blood was diluted in phosphate buffer saline (PBS) containing EDTA, overlaid onto Histopaque, centrifuged at 2200 rpm for 25 min at room temperature. Mononuclear cell fraction was harvested and washed 3 times in PBS. Final pellet was immediately snap frozen and stored at -80°C. Full thickness aortic wall tissue specimens were collected in patients who underwent open surgical repair. Tissues were harvested at the site of maximal aneurysmal dilatation, were immediately snap-frozen in liquid nitrogen and stored at -80°C.

RNA extraction

The aortic tissues were pulverized using beads and shaker. For aortic tissue and PBMCs, total RNAs were isolated using Trizol® reagent (Invitrogen). For serum and plasma, total circulating RNA was extracted using miRNeasy® Mini Kit (Qiagen), according to the manufacturer's instructions. RNA quality control was performed using Nanodrop 2000 (Thermofisher scientific).

miR expression

A profiling analysis was performed from PBMCs of diabetic and non-diabetic patients (n=3 per group) and 752 human micro-RNAs were screened using miRCURY LNA Universal RT microRNA PCR, panel I and II V4 (Exiqon- Qiagen®). The screening was carried out by

Exiqon. Sample quality was checked using the cDNA synthesis control (UniSp6) added during the reverse transcription and analysing the Ct levels obtained for miR-103, 23a, 30c, 451 and 142-3p. Fold changes for RT-PCR were calculated according to the method of 2-delta-delta Ct. miR that showed significant differential expression in PBMCs between diabetics and non-diabetics were selected and further analyzed in the entire cohort in PBMCs, plasma, sera (n=10 per group) and aneurysmal tissue. miR expression was quantified using miRCURY LNA Universal RT microRNA PCR (Exiqon Qiagen®) according to the manufacturer's instructions. qPCRs were performed on a 7500HT Fast Real-Time PCR System (Applied Biosystems). miR expression was normalized to U6 snRNA together with 5S rRNA and RNU5G. Relative expression was calculated using the 2-delta-delta Ct method followed by geometric average.

Statistical analysis

Results for continuous variables were expressed as median (interquartile ranges) and compared with the Mann-Whitney test. Categorical variables were expressed as number of patients and percentage and compared with the Fisher's exact test. Correlations were determined by non-parametric univariate linear regression (Spearman's rank test). A two-sided P value <0.05 was considered as significant. Statistical analyses were performed using GraphPad Prism® software (version 7.00, La Jolla California USA).

Results

A screening was performed comparing miR expression in PBMCs between age- and sex-matched diabetic and non-diabetic patients with AAA (n=3 per group, Supplemental Table S1). Among the 752 human miRs screened, 275 were detected in all samples and the average number of assays detected per sample was 340 (Supplemental Figure S1-A). Sample quality

was checked and samples were comparable with similar raw Ct values obtained for UniSp6, miR-103, 23a, 30c, 451 and 142-3p (Supplemental Figure S1-B). The profiles obtained for each sample were compared and illustrated in Figure 1. The heat map, the volcano plot and the principal component analysis indicated that samples formed weak clusters between diabetic and non-diabetic patients based on miR expression in PBMCs. Four miRs were identified as significantly differentially expressed in diabetic patients compared to non-diabetics: 3 were upregulated (miR-144-3p, miR-20a-5p, miR-188-3p) and 1 downregulated (miR-548k) (Table 1).

The expression of the 4 miRs identified was further validated in 10 diabetic patients with AAA compared to 10 non-diabetic patients with AAA. The median values of AAA diameters were respectively 49 mm and 50 mm, p=0.7244. Treatments did not significantly differ among the groups and all diabetic patients were treated with oral anti-diabetic drugs. General characteristics of the study population including the age and the presence of cardiovascular risk factors were similar among the groups (Table 2). The upregulation of miR-144-3p, miR-20a-5p, and miR-188-3p expression previously observed in diabetic patients with AAA was confirmed on the PBMCs of this cohort, whereas no significant difference was observed for miR-548k (Figure 2). The expression of the 3 validated miRs was further investigated in plasma, serum and aneurysmal tissue. Expression of miR-20a-5p was significantly upregulated in the serum of diabetic patients compared to non-diabetics (Figure 2.C) and miR-144-3p was significantly upregulated in the aneurysmal tissue (Figure 2.D). No difference was observed in plasma (Figure 2.B).

To investigate factors influencing the upregulation of miRs identified in PBMCs of diabetic patients, the association between glycaemic parameters and miR expression was analysed in the whole cohort using Spearman's correlation coefficient (Table 3). The expression of miR-20a-5p in PBMCs was significantly correlated with fructosamine concentration (r=0.62,

p=0.006). No significant correlation was observed between glycaemic parameters and miR-144-3p or miR-188-3p expression.

Discussion

This pilot study reports for the first time a comparison of miR expression between diabetic and non-diabetic patients with AAA. We found a significant upregulation of miR-144-3p, 20a-5p and 188-3p in PBMCs of diabetic patients. The level of miR-144-3p and miR-20a-5p were also significantly increased in aneurysmal tissue and serum respectively.

Several studies have addressed the expression of miR-144 in the setting of diabetes. miR-144 was expressed in PBMCs of diabetic patients and was one of the miR commonly shared between patients with type 1, type 2 and gestational diabetes mellitus (16). A meta-analysis of controlled profiling studies further revealed that miR-144 has been found upregulated in blood and in muscle of type 2 diabetic patients compared to non-diabetics (17). Furthermore, plasma level of miR-144 in type 2 diabetic patients was higher in Swedes than in Iraqis, suggesting that its expression in circulating blood could be related to ethnicity (18). Taken together, these results are concordant with our findings of an upregulation of miR-144-3p in PBMCs and aneurysmal tissue of diabetic patients. No significant difference was observed in plasma and serum. This could potentially be due to the small cohort studied which may have limited the statistical power of the analysis. To the best of our knowledge, the expression and the role of miR-144-3p in AAA has never been investigated so far. Several studies highlighted its role in vascular homeostasis through regulation of hematopoiesis and vascular development (19), and regulation of contractile differentiation of smooth muscle cells (20). Moreover, miR-144 has been identified as a potential regulator of transforming growth factor-β (TGF-β) signaling pathway during liver fibrosis (21). Even if further studies are required, these cellular and molecular pathways are potentially relevant for AAA pathogenesis and could be involved in the negative association between diabetes and AAA.

We found that miR-20a-5p level was higher in PBMCs and serum of diabetic patients with AAA compared to non-diabetics. In addition, the expression of miR-20a-5p in PBMCs positively correlated with fructosamine concentration. As fructosamine reflects the average glycemic status over the preceding 2 to 3-week period (22), these results suggest that miR-20a-5p expression is partly modulated by mid-term glucose homeostasis. Another study investigated circulating miR-20a-5p and also found an increased level in patients with gestational diabetes mellitus compared to controls (23). miR-20a expression in abdominal aortic aneurysmal tissue was increased compared to ascending normal aorta (24), and its level in plasma inversely correlated with AAA diameter (25). Its precise mechanistic effect in AAA pathogenesis is still unknown but several studies highlighted its role in angiogenesis (26, 27). Its overexpression in endothelial cells impaired sprouting, suggesting an anti-angiogenic action (26). However, another study revealed that miR-20a was induced upon vascular endothelial growth factor (VEGF) treatment in endothelial cells and contributed to VEGF-induced angiogenic responses (28), underlining a complex effect on angiogenesis. As neoangiogenesis is a key-mechanism in AAA development and progression to aortic rupture (29), the upregulation of miR-20a observed in diabetic patients could potentially have a protective role in the disease. Furthermore, the role of miR-20a in vascular remodeling has been exemplified in the hypoxia-induced mouse model of pulmonary hypertension (30). In that study, the treatment of mice with antagomiR-20a reduced wall thickness of small pulmonary arteries and reduced right ventricular hypertrophy. In vitro experiments further revealed that transfection of antagomiR-20a in human pulmonary arterial smooth muscle cells reduced their proliferation. Thus, miR-20a could also affect AAA pathogenesis through the regulation of vascular smooth muscle cell homeostasis.

Our results also showed increased expression of miR-188-3p in PBMCs of diabetic patients with AAA. A previous study identified miR-188 in the neck biopsies of patients with AAA

(8). Another experimental study revealed an upregulation of miR-188 in the left ventricle of diabetes-induced rats (31). Its expression and its role in the setting of diabetes and aneurysm pathology have been so far poorly investigated. Further studies are required to determine whether miR-188-3p is a simple marker or a player in the inverse association observed between diabetes and AAA.

Based on the results of this study, several perspectives can be suggested. First, the pathophysiological mechanisms underlying differential miR expression in diabetic patients with AAA remain to be determined. Experimental in vivo studies using specific miR modulators are required to validate the role of the identified miRs, and their potential contribution to the protective effect of diabetes on AAA formation. Finally, our results were obtained in a small cohort of patients, and need further confirmation in a larger cohort.

Conclusion

To the best of our knowledge, this is the first study to compare miR expression between diabetic and non-diabetic patients with AAA. Three miRs (miR-144-3p, 20a-5p and 188-3p) were significantly upregulated in PBMCs of diabetic patients, and miR-144-3p and 20a-5p were also increased in aneurysmal tissue and serum, respectively. Even if their role in AAA formation and progression remains to be determined, they could represent new therapeutic targets. Further studies are required to investigate the mechanisms behind the differential expression of those miRs in diabetic patients with AAA, and determine their direct role in AAA formation and progression.

Author contribution

All authors confirm they contributed to the intellectual content of this article including conception and design. They revised the manuscript and approved the final version.

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Conflicts of interest: none declared

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Tables

miR name	SD PBMC diabetic	SD PBMC non-diabetic	Average dCt PBMC diabetic	Average dCt PBMC non-diabetic	Fold change	P value
hsa miR-548k	0.27	0.18	-4.8	-3.5	-2.5	0.0034
hsa miR-144-3p	0.77	0.71	3.5	1.5	4.1	0.028
hsa miR-20a-5p	0.0023	0.068	5.4	5.2	1.2	0.034
hsa miR-188-3p	0.37	0.40	-3.2	-4.2	2.0	0.038

Table 1: Identification of miR significantly differentially expressed between PBMCs from diabetic and non-diabetic patients with AAA (n=3 per group)

Values are standard deviation (SD) across the groups, followed by average normalized Ct values for each group and fold change between the two groups.

PBMCs: peripheral blood mononuclear cells

Characteristics	Non-diabetic patients with AAA (n=10)	Diabetic patients with AAA (n=10)	P value
Age (years)	67 (66 – 74.5)	73 (63.8 – 75.3)	0.6160
Body mass index (kg/m^2)	25.6 (22.2 – 27.6)	25.8 (24.4 – 28.8)	0.6665
Overweight or obesity	4 (40%)	5 (50%)	0.9999
Arterial hypertension	8 (80%)	8 (80%)	0.9999
Dyslipidemia	3 (30%)	5 (50%)	0.6499
Smoking	7 (70%)	8 (80%)	0.9999
Coronary artery disease	4 (40%)	2 (20%)	0.6285
Ejection fraction	60 (57.5 – 68)	60 (55 – 62)	0.3657
AAA diameter (mm)	50 (43.3 – 54.5)	49 (38 – 57)	0.7244
Use of statins	6 (60%)	8 (80%)	0.6285
Use of antiplatelets	7 (70%)	7 (70%)	0.9999
Use of anti-hypertensive drugs	8 (80%)	8 (80%)	0.9999
Use of oral anti-diabetic drugs	0 (0%)	10 (100%)	0.0001

Use of insulin therapy	0 (0%)	0 (0%)	NA
Fasting glycaemia (mmol/L)	5.8 (4.7 – 7.9)	6.4 (4.4 – 8.6)	0.8024
Fructosamine (µmol/L)	236.5 (222.5 – 255)	274 (232.5 – 312.5)	0.1629
C-peptide (nmol/L)	0.78 (0.45 – 1.23)	1.02 (0.52 – 1.12)	0.8633
Insulin (mU/L)	7 (3.8 – 16.3)	6 (3.5 – 13)	0.7950

Table 2: Characteristics of the study population

Values are median (interquartile range) or n (%). % are expressed compared to the group.

NA: Not applicable

Glycaemic parameters	**miR-144-3p**	**miR-20a-5p**	**miR-188-3p**
Fasting glycaemia (mmol/L)	r= -0.21, p= 0.47	r= 0.09, p=0.76	r= 0.17, p=0.56
Fructosamine (µmol/L)	r= 0.30, p= 0.23	r= 0.62, p= 0.006 *	r= 0.12, p= 0.65
C-peptide (nmol/L)	r= -0.18, p= 0.49	r= 0.22, p=0.39	r= 0.15, p= 0.57
Insulin (mU/L)	r= -0.23, p= 0.38	r= 0.20, p=0.43	r= 0.03, p= 0.88

Table 3: Correlation between miR expression in PBMCs and glycemic parameters (n=20).

r: Spearman correlation coefficient value

* $p<0.05$

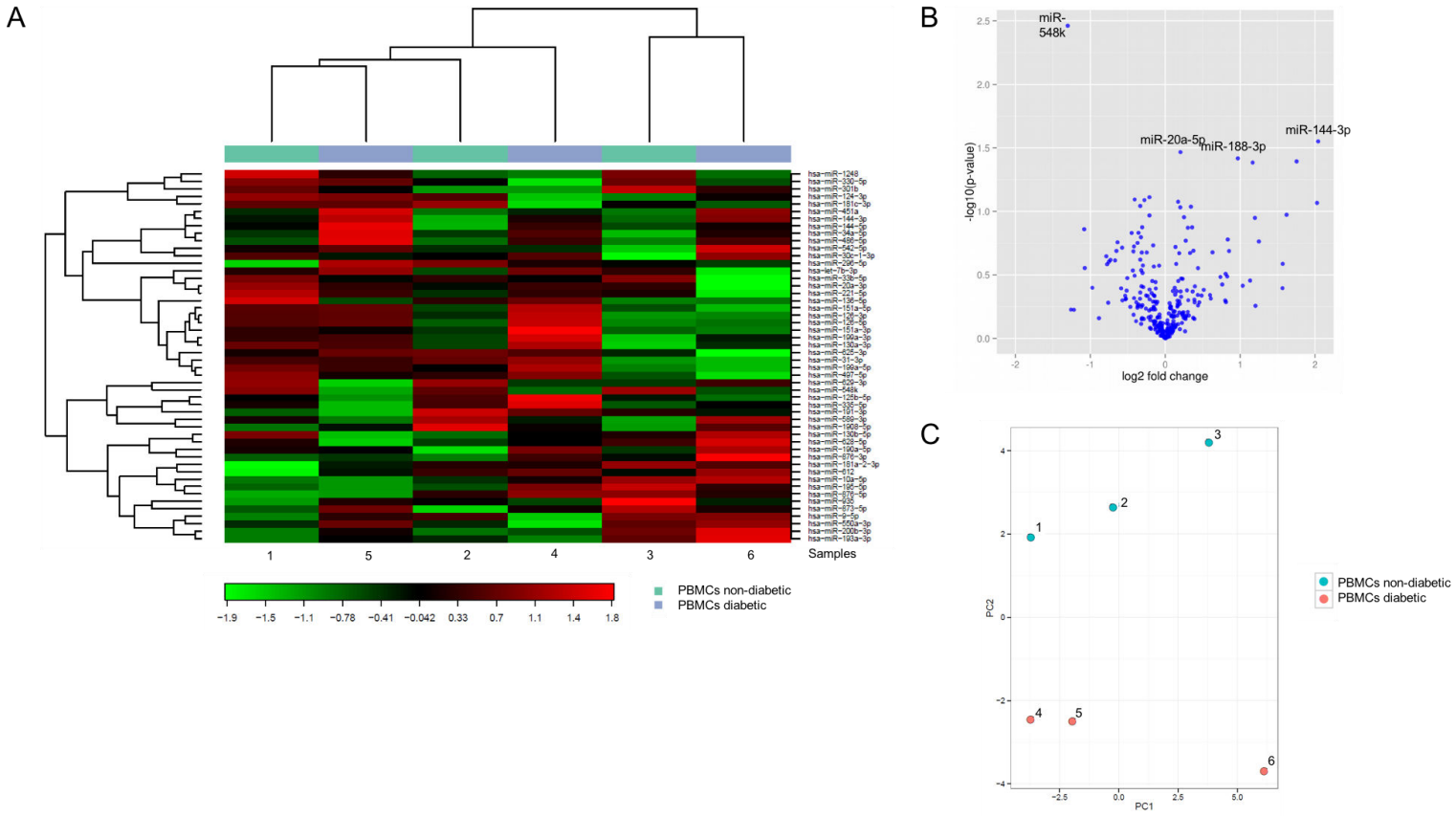


Figure 1: Screening of miR expression in PBMCs (n=3 per group)

(A) Heat map and unsupervised hierarchical clustering. The clustering was performed on all samples and on the top 50 miRs with highest standard deviation. Samples 1, 2, 3 and 4, 5, 6 were respectively obtained from non-diabetic and diabetic patients with AAA. (B) Volcano plot showing relationship between the p values and the ddCt. (C) Principal Component (PC) Analysis Plot. The clustering was performed on all samples and on the top 50 miRs with highest standard deviation.

PBMCs: peripheral blood mononuclear cells

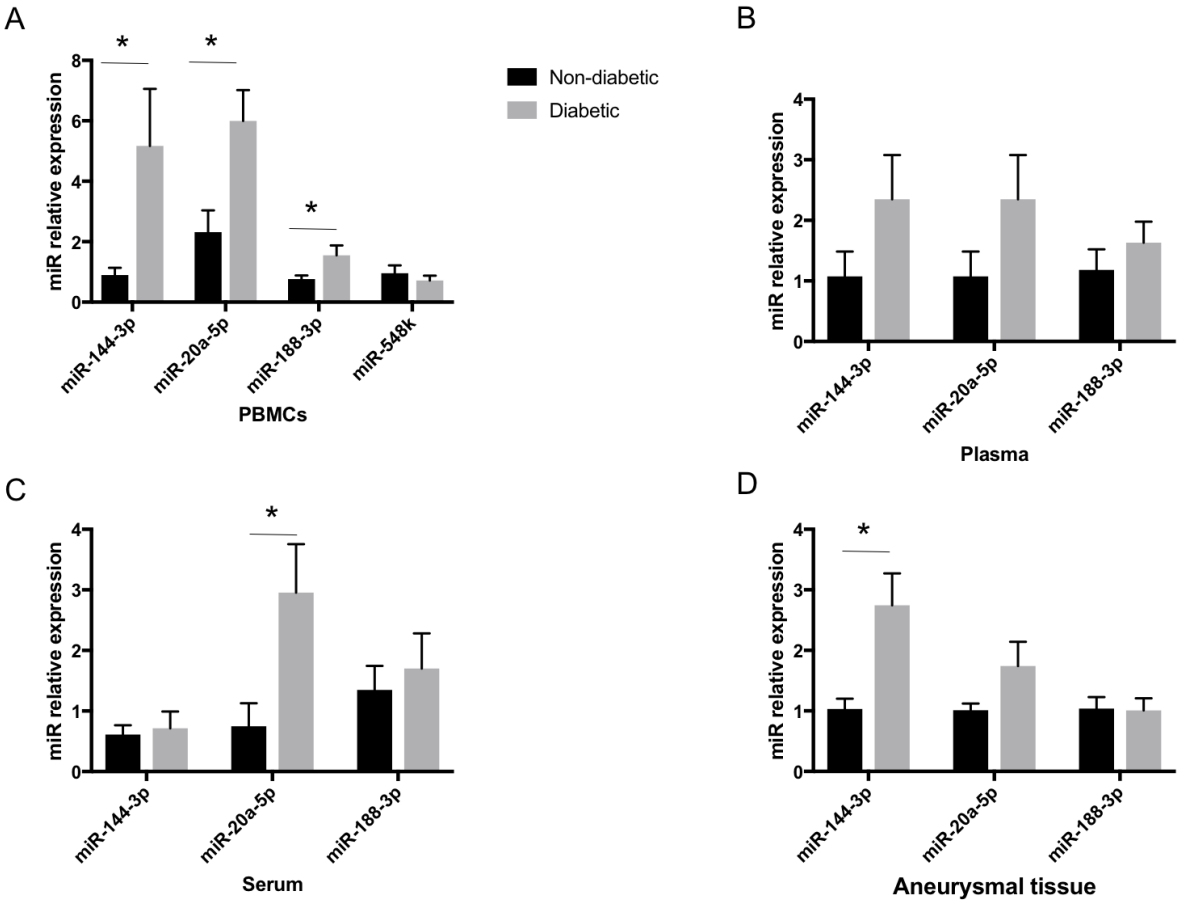


Figure 2: miR relative expression in diabetic and non-diabetic patients with AAA.

(A) miR expression in PBMCs (n=10 per group). (B) miR expression in plasma (n=10 per group). (C) miR expression in serum (n=10 per group). (D) miR expression in aneurysmal tissue (n=3 per group).

Data are expressed as means +/- standard error of means (SEM)

* $p<0.05$

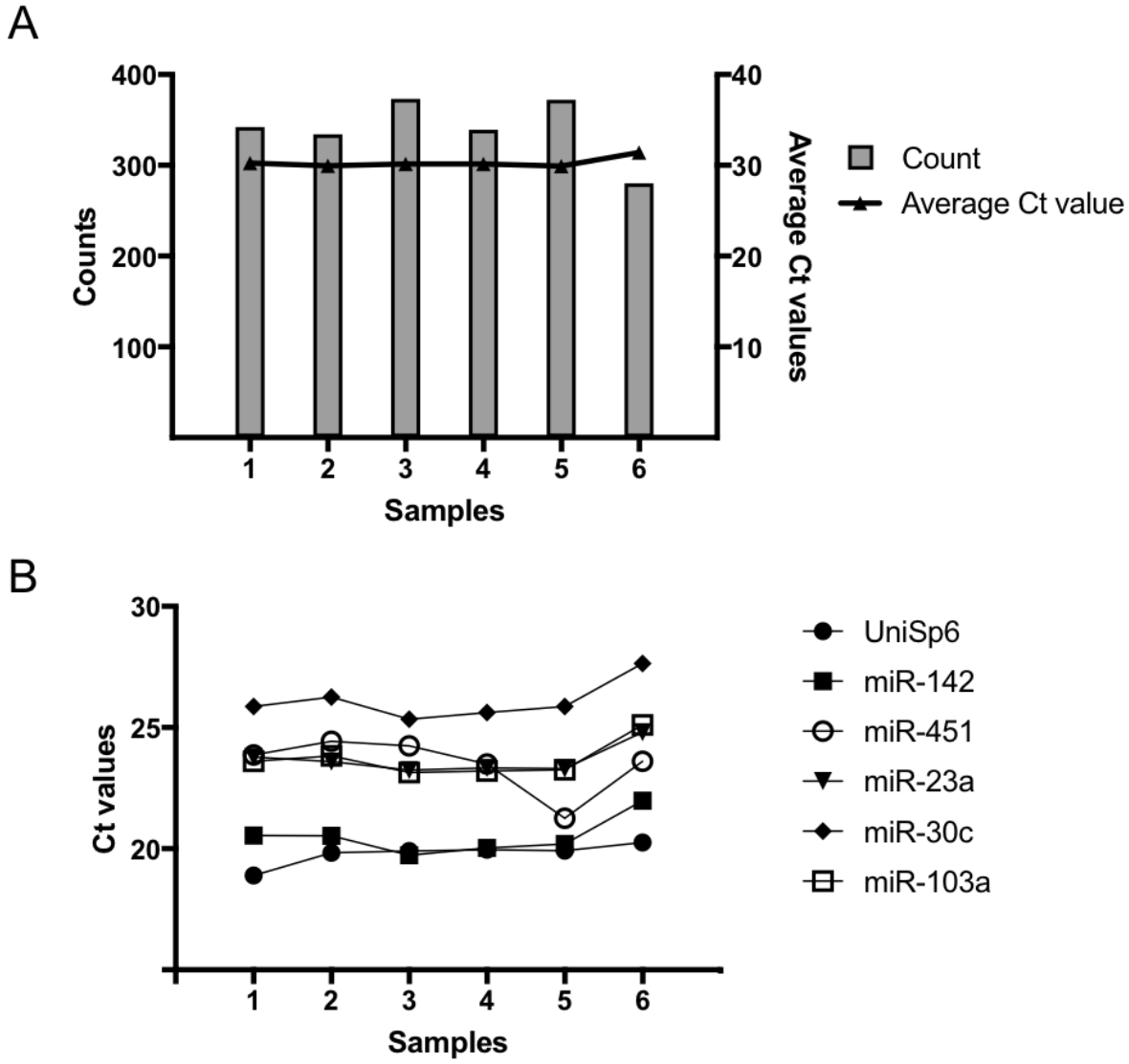
PBMCs: peripheral blood mononuclear cells

Supplemental

Characteristics	Non-diabetic patients with AAA (n=3)	Diabetic patients with AAA (n=3)	P value
Age (years)	66 (61 – 66)	73 (54 – 75)	0.7143
AAA diameter (mm)	56 (52 – 70)	60 (50 – 66)	0.9999

Supplemental table S1: Characteristics of the groups used for the screening of miR expression in PBMCs

Values are median (interquartile range).



Supplemental Figure S1: miR content and sample quality controls of PBMCs used for miR screening

(A) Graphical illustration of the miR content. The bars represent number of miR detected and the line shows the average Ct value for the expressed miRs. Samples 1, 2, 3 and 4, 5, 6 were respectively obtained from non-diabetic and diabetic patients with AAA. (B) Raw Ct values obtained for the cDNA synthesis control (UniSp6) and miR-142, 451, 23a, 30c, 103a.

DISCUSSION GENERALE ET CONCLUSION

L'AAA représente un problème majeur de santé publique, responsable d'un taux de mortalité très élevé en cas de rupture aortique. L'AAA est le plus souvent lié à l'athérosclérose et aux facteurs de risque cardiovasculaires, à l'exception du diabète qui jouerait un rôle protecteur dans la pathologie. A l'heure actuelle, aucun traitement pharmacologique n'existe, soulignant la nécessité de mieux comprendre les mécanismes impliqués dans l'initiation, la croissance et la rupture anévrismale. Bien qu'utiles, les modèles expérimentaux classiques ne reproduisent pas parfaitement la physiopathologie humaine. Notre laboratoire a récemment mis au point un modèle murin d'AAA non disséquant induit par l'application topique d'élastase au niveau de l'adventice associée à la neutralisation systémique du TGFβ. Ce modèle permet de reproduire les principales caractéristiques physiopathologiques observées chez l'homme. En effet, l'AAA est créé dans la région aortique sous-rénale et peut évoluer vers la rupture.
L'inhibition du TGFβ induit un remodelage vasculaire important avec une infiltration de cellules inflammatoires, une dégradation de la MEC, une diminution de la densité en CMLs, une augmentation de la néo-angiogenèse dans l'adventice ainsi que la formation d'un thrombus intra-luminal.
Dans ce modèle, il existait une augmentation de l'infiltration des macrophages au sein de la paroi aortique, avec une modification de l'expression des marqueurs M1 et M2, une augmentation des macrophages exprimant l'ARG1 et une diminution des Sat-Mono. De façon intéressante, la déplétion en macrophages avait un effet protecteur sur la croissance et la rupture aortique. Nous avons ensuite analysé l'expression des miRs dans le tissu aortique et mis en évidence une modification de leur profile. Deux miRs d'intérêt (miR-18a-3p et 223-3p) ont été sélectionnés. Ces deniers étaient significativement augmentés dans le tissu

aortique et étaient également dérégulés dans le sérum, suggérant leur intérêt potentiel en tant que biomarqueurs. De plus, ils ont été identifiés comme également dérégulés dans le tissu aortique humain. A l'issue de ces résultats, le rôle de ces miRs dans la pathogénèse anévrismale grâce à l'utilisation d'injection d'antago-miRs et de souris Knock out sera étudié. Leur lien avec les macrophages sera recherché en analysant l'expression de ces miRs dans le tissu aortique après injection de clodronate et en réalisant des études *in vitro* afin de moduler leur expression et d'étudier leur effet sur le phénotype et l'activité des macrophages.

Enfin, nous avons mis en place une étude clinique incluant des patients atteints d'AAA et permettant de réaliser une collection biologique incluant le sérum, le plasma, les cellules sanguines mononuclées et le tissu aortique. L'implication potentielle des miRs dans la relation négative observée entre diabète et AAA n'ayant à ce jour encore jamais été explorée, nous avons réalisé une étude pilote comparant des patients diabétiques et non diabétiques atteints d'AAA. Trois miRs d'intérêt ont été identifiés comme différentiellement exprimés dans les cellules sanguines mononuclées et certains étaient également dérégulés dans le sérum et le tissu aortique. A l'issue de ces résultats, il serait intéressant de déterminer plus précisément dans quels types de cellules sanguines mononuclées ces miRs sont dérégulés, à savoir les lymphocytes ou les monocytes/ macrophages. De plus, des études *in vitro* seraient intéressantes afin de mieux comprendre leur rôle dans l'AAA et de déterminer s'ils sont impliqués dans la relation négative entre diabète et AAA.

En conclusion, notre travail a montré la complexité de la régulation du phénotype des macrophages et de l'expression des miRs dans un nouveau modèle murin d'AAA induit par l'élastase et la neutralisation du TGFβ et a permis d'identifier chez l'homme des miRs potentiellement impliqués dans l'association négative entre diabète et AAA. A long terme, ces résultats pourraient permettre d'identifier de nouveaux biomarqueurs et de nouvelles cibles thérapeutiques utiles pour la prise en charge des patients atteints d'AAA.

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ANNEXE

Annexe 1: TGFβ (Transforming Growth Factor-β) Blockade Induces a Human-Like Disease in a Nondissecting Mouse Model of Abdominal Aortic Aneurysm.

TGFβ (Transforming Growth Factor-β) Blockade Induces a Human-Like Disease in a Nondissecting Mouse Model of Abdominal Aortic Aneurysm

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Objective—Current experimental models of abdominal aortic aneurysm (AAA) do not accurately reproduce the major features of human AAA. We hypothesized that blockade of TGFβ (transforming growth factor-β) activity—a guardian of vascular integrity and immune homeostasis—would impair vascular healing in models of nondissecting AAA and would lead to sustained aneurysmal growth until rupture.

Approach and Results—Here, we test this hypothesis in the elastase-induced AAA model in mice. We analyze AAA development and progression using ultrasound in vivo, synchrotron-based ultrahigh resolution imaging ex vivo, and a combination of biological, histological, and flow cytometry-based cellular and molecular approaches in vitro. Systemic blockade of TGFβ using a monoclonal antibody induces a transition from a self-contained aortic dilatation to a model of sustained aneurysmal growth, associated with the formation of an intraluminal thrombus. AAA growth is associated with wall disruption but no medial dissection and culminates in fatal transmural aortic wall rupture. TGFβ blockade enhances leukocyte infiltration both in the aortic wall and the intraluminal thrombus and aggravates extracellular matrix degradation. Early blockade of IL-1β or monocyte-dependent responses substantially limits AAA severity. However, blockade of IL-1β after disease initiation has no effect on AAA progression to rupture.

Conclusions—Endogenous TGFβ activity is required for the healing of AAA. TGFβ blockade may be harnessed to generate new models of AAA with better relevance to the human disease. We expect that the new models will improve our understanding of the pathophysiology of AAA and will be useful in the identification of new therapeutic targets.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2017;37: 2171-2181. DOI: 10.1161/ATVBAHA.117.309999.)**

Key Words: aneurysm ■ animals ■ humans ■ immune system ■ models

The occurrence of abdominal aortic aneurysm (AAA) is a significant cause of morbidity and mortality in Western countries. According to the UK National Health Service AAA screening program, the prevalence of AAA is 1.2% in all men at age 65 irrespective of any other risk factor.[1] AAA affects a much larger proportion of men >65 years of age, particularly in the presence of a family history of AAA and a positive history of smoking.[1,2] In contrast, a history of diabetes mellitus is associated with a strong protection against the occurrence and progression of AAA.[1–3]

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AAA is responsible for >11 000 deaths per year in the United States and has been consistently found to resist medical treatment. Aortic wall repair through either endovascular or open surgical procedures is the only option available, with a relatively high risk of early or late morbidity and mortality.[2,4]

The pathophysiology of AAA is complex and associates proteolysis of the extracellular matrix (ECM), degeneration and loss of vascular smooth muscle cells (VSMCs), alteration of endothelial cells, and formation of intraluminal thrombus (ILT), along with an infiltration and activation of innate and adaptive immune cells.[5] The absence of resolution of those processes leads to progressive AAA growth and culminates in AAA rupture when the aortic wall is unable to sustain the hemodynamic overload. Of note, in contrast to thoracic aortic aneurysms,[6] medial dissection is a rare event in AAA, which

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Nonstandard Abbreviations and Acronyms	
AAA	abdominal aortic aneurysm
ECM	extracellular matrix
ILT	intraluminal thrombus
SatM	segregated nucleus-containing atypical monocyte
TGFβ	transforming growth factor-β
VSMC	vascular smooth muscle cell

rather progresses to a complete rupture of the thin aortic wall, usually at a stage of advanced aortic dilatation.[7]

A variety of animal models have been developed to better understand the pathophysiology of AAA. Strikingly, however, most models fail to reproduce or combine the major features of human AAA. Currently, most experimental models of AAA are performed in rodents, particularly mice, and can be divided into 2 main categories.[8] The first category includes models that induce medial aortic dissection rather than true aortic aneurysmal dilatation, and lead to hemorrhage into a false channel (intramural hemorrhage), which may ultimately rupture. This is the case of the angiotensin II-infusion model, initially described by the Daugherty et al[9] groups in mice with genetic susceptibility to hyperlipidemia and atherosclerosis,[10] but also models of lysyl oxidase inhibition[11–13] and those combining mineralocorticoid receptor activation with high salt.[14] Despite the many advantages of those dissecting AAA models, medial dissection is not a major feature of human AAA and is rarely an initiating event in the disease process.[8] Rather, most human AAAs are characterized by aneurysmal dilatation of the aorta without false channel formation and culminate in a rupture of the thin aortic wall.[7] The second category of experimental AAAs includes models that induce abdominal aortic dilatation without medial dissection, as is the case for the widely used elastase-induced or calcium chloride-induced AAA models.[8] However, those models produce moderate aortic dilatation in mice and do not induce aortic wall rupture. Finally, although leukocyte infiltration, ECM degradation, and medial degeneration are observed in almost all the abovementioned models, ILT is frequently absent. Thus, there is currently no validated AAA model in mice that reproduces and combines the major features of human AAA: sustained aneurysmal dilatation of the native aorta without false lumen formation, associating ECM degradation, medial degeneration, leukocyte infiltration, ILT formation, and culminating in transmural rupture of the thin aortic wall.

Studies on the mechanisms of aortic aneurysm and dissection have shed light on the importance of TGFβ (transforming growth factor-β) activity in the maintenance of aortic wall integrity. In mice, genetic disruption of TGFβ signaling in VSMCs early during the disease process promotes the development of aneurysms and dissections in the thoracic aorta.[15,16] Furthermore, blockade of TGFβ activity aggravates ECM degradation, medial degeneration, leukocyte infiltration, and increases the susceptibility to both thoracic and abdominal aortic dissection in mice infused with angiotensin II.[17,18] Finally, promotion of TGFβ activity limits the development and expansion of experimental AAAs.[19] Based on these observations, we hypothesized that TGFβ may be the critical factor that maintains aortic wall integrity in mouse models of nondissecting AAA (eg, elastase-induced abdominal aortic injury) and that blockade of TGFβ activity in those settings would aggravate aneurysmal aortic dilatation, induce ILT formation, and promote aortic rupture, thereby reproducing the major features of complicated human AAA.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Inhibition of TGFβ Activity Induces Abdominal Aortic Rupture After Elastase Injury

We combined topical application of elastase on the abdominal aorta[20–22] with systemic blockade of TGFβ activity using a neutralizing mouse monoclonal anti-TGFβ.[17,18] As already described,[20–22] periaortic elastase significantly increased aortic diameter (compared with inactive elastase) as assessed by serial in vivo ultrasound imaging (Figure 1A and 1B) and direct caliper measurement of the external diameter of the aorta at the time of euthanization (Figure 1C). Interestingly, although aortic dilatation did not progress after day 7 in the group receiving active elastase only (Figure 1B), concomitant inhibition of TGFβ activity dramatically reduced Smad2/3 phosphorylation (Figure I in the online-only Data Supplement), steadily enhanced aortic enlargement at the site of elastase application throughout the duration of the experiment (Figure 1B), and eventually led to sudden death because of aortic rupture (Figure 1D and 1E). Staining of aortic sections with Orcein revealed significantly enhanced elastin degradation in mice cotreated with elastase and anti-TGFβ (Figure 1F). These results were reproduced in hyperlipidemic *Apoe*$^{-/-}$ mice (Figure IIA in the online-only Data Supplement). Systolic blood pressure was not affected by the interventions and was similar between all experimental groups (Figure IIB in the online-only Data Supplement). Our data identify a critical role for TGFβ in preserving aortic wall integrity in response to an AAA-inducing insult. The steady increase of AAA diameter over the course of the experiment and the occurrence of AAA rupture in mice treated with anti-TGFβ antibody strongly suggest impaired resolution of the inflammatory process and altered tissue healing in the absence of TGFβ activity (below). This is further supported by our finding that TGFβ blockade initiated 14 days after periaortic elastase application—a stage of nonprogressing or resolving AAA—rapidly induced AAA destabilization and led to fatal aortic rupture (Figure III in the online-only Data Supplement).

Synchrotron-Based Ultrahigh Resolution Imaging Reveals Features of Human AAA

To characterize this new mouse model of AAA rupture, we used synchrotron-based ultrahigh resolution ex vivo imaging on tissue samples from mice that had been euthanized at different time points and assessed in details aortic diameter, elastin degradation, adventitial thickness, and the occurrence of ILT, medial dissection, or rupture (Figures 2 and 3). The medial perimeter was highest for locations at which elastin degradation was most outspoken (Figure 2A and 2D; Figure IVA in the online-only Data Supplement). Both medial perimeter (Figure 2A through 2C) and elastin degradation (Figure 2C through 2F) were already

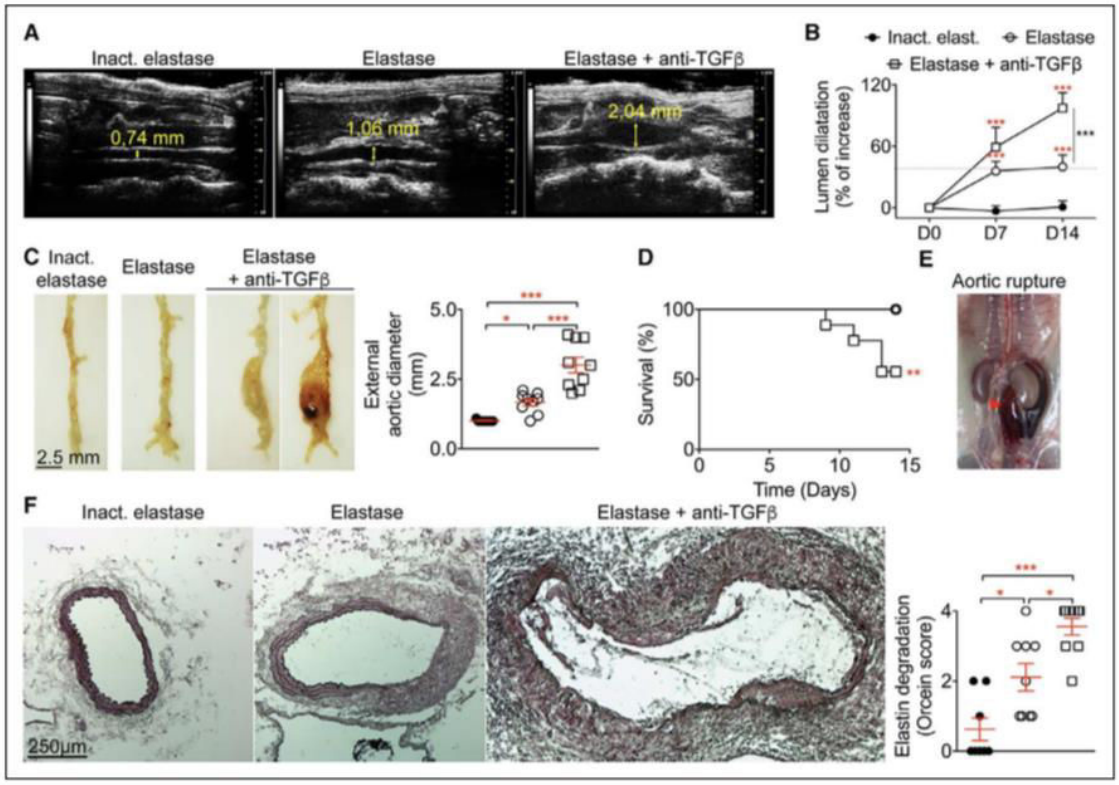

Figure 1. Blockade of TGFβ (transforming growth factor-β) activity in mice increases the severity of elastase-induced abdominal aortic aneurysm and induces aortic rupture. **A** and **B**, Representative ultrasound images at day 14 (D14), and quantification of lumen dilation over time in the 3 groups (n=9 per group). Two-way ANOVA and Tukey post hoc analysis. Red stars: ***P<0.001 D7 or D14 vs D0; Black stars: ***P<0.001 elastase vs elastase+anti-TGFβ at D14. **C**, Representative images and quantification of external aortic diameter (mm) by caliper at D14 (n=9 per group; Kruskal–Wallis+uncorrected Dunn test). *P<0.05 inactive elastase vs elastase, ***P<0.001 inactive elastase vs elastase+anti-TGFβ and elastase vs elastase+anti-TGFβ. **D**, Survival curve (n=9 per group; log-rank, Mantel–Cox test) and representative image of aortic rupture in situ (red arrow shows the hematoma) after necropsy (**E**). **P<0.01 elastase vs elastase+anti-TGFβ. **F**, Representative images of Orcein staining and quantification of elastin degradation at D14 (n=9 per group). Kruskal–Wallis+uncorrected Dunn test; *P<0.05 inactive elastase vs elastase and elastase vs elastase+anti-TGFβ, ***P<0.001 inactive elastase vs elastase+anti-TGFβ.

elevated 3 days after elastase application, followed by adventitial thickening (Figure 2G through 2I) and the occurrence of ILT (Figure 2J through 2L). In nonruptured AAA, elastin degradation did not change after day 3 (Figure 2D and 2E), whereas medial perimeter (Figure 2A and 2B), adventitial thickening (Figure 2G and 2H), and ILT (Figure 2J and 2K) still increased progressively at later time points. We observed that animals with ruptured AAA had substantially larger medial perimeters (Figure 2C), extensive elastin degradation (Figure 2F), and larger ILT volumes (Figure 2I) but only moderate although significant increase in adventitial thickness (Figure 2L) compared with animals with intact AAA. Synchrotron-based ultrahigh resolution analysis throughout the length of the AAA ruled out the occurrence of medial delamination/dissection and attributed fatal aortic death to complete transmural rupture of the media (Figure 3A). ILT presence was found both in the main aorta and the side branches (Figure 3A; Figure IVB and IVC in the online-only Data Supplement) and was confirmed on aortic sections (Figure 3B). ILT stained positive for platelets (anti-CD41), leukocytes (Hoechst), and fibrin/fibrinogen as shown by confocal microscopy (Figure 3C). Platelets were also found in the aortic wall, in between the medial and adventitial layers, probably because of hemorrhage (Figure 3D).

Aortic Tissue Remodeling in the Elastase/Anti-TGFβ Model

By microscopy on sections from day 14, we confirmed that the combination of elastase and anti-TGFβ significantly enhanced the accumulation of blood/iron in the aortic wall (Figure 4A) and induced profound aortic wall remodeling with smooth muscle cell loss in the media (Figure 4B), adventitial neovascularization (Figure 4B), increased accumulation of apoptotic cells (Figure 4C), as well as increased matrix metalloproteinase activity (Figure 4D) and reduced collagen content (Figure 4E), as compared with the elastase-only group. Proliferation as assessed by Ki67 staining was detected in both immune and stromal cells of vessels subjected to elastase application but was not different between elastase and elastase+anti-TGFβ groups (Figure V in the online-only Data Supplement).

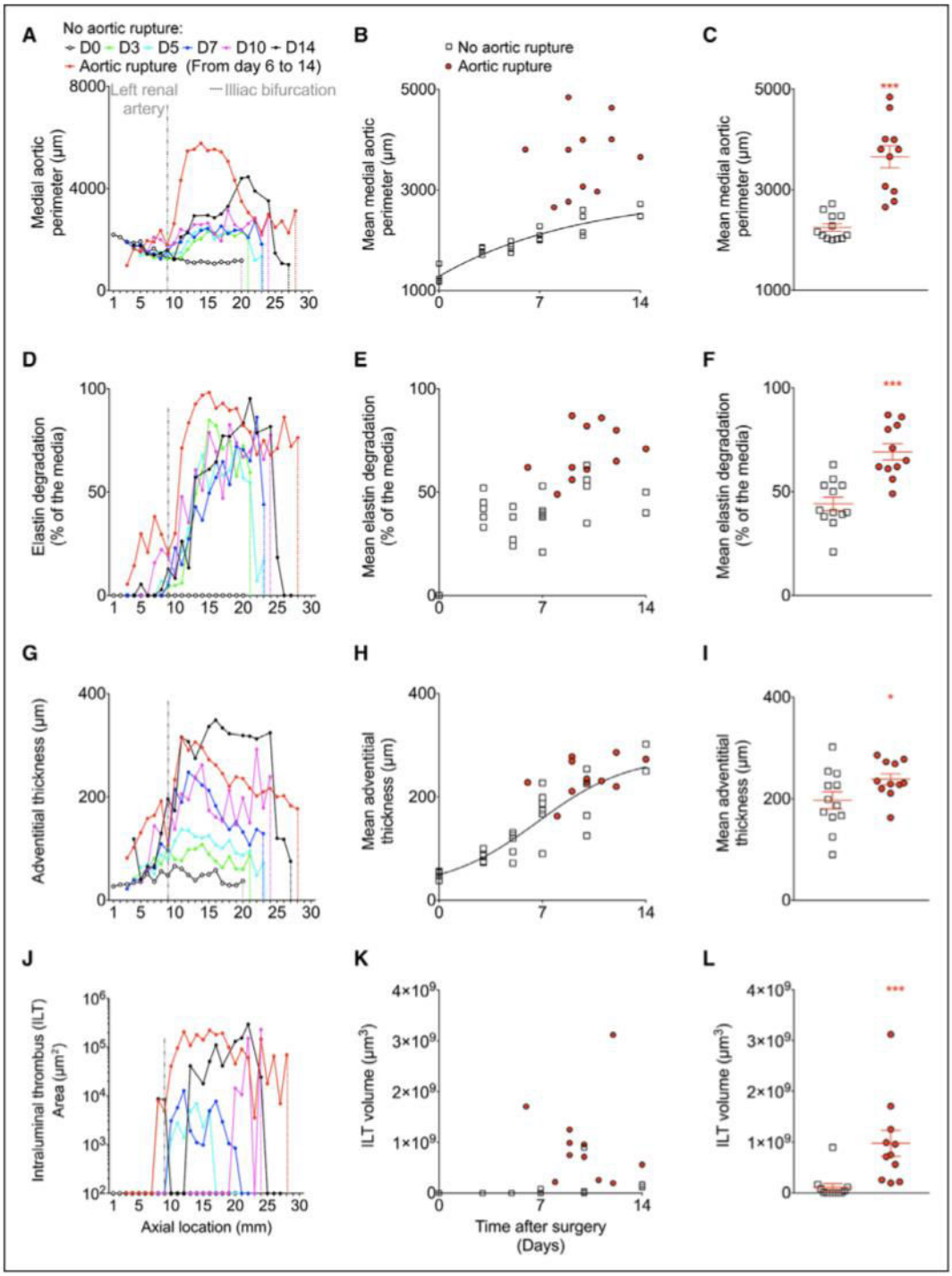

Figure 2. Synchrotron-based analysis of elastase-induced injury in the presence of anti-TGFβ (transforming growth factor-β). Digital analysis of medial aortic perimeter (**A**–**C**), elastin degradation (**D**–**F**), adventitial thickening (**G**–**I**), and intraluminal thrombosis (ILT; **J**–**L**) after synchrotron imaging. **A**, **D**, **G**, and **J**, **Left** dotted line represents the location of the left renal artery; **right** (colored) dotted lines represent the iliac bifurcation. Note that the distance between the left renal artery and the iliac bifurcation increased with the progression of abdominal aortic aneurysm. **C**, **F**, **I**, and **L**, No aortic rupture: live mice between day 7 (D7) and D14 (n=12); aortic rupture: death occurred between D6 and D14 (n=11). Mann–Whitney *U* test. **P*<0.05, ****P*<0.001 no aortic rupture vs aortic rupture.

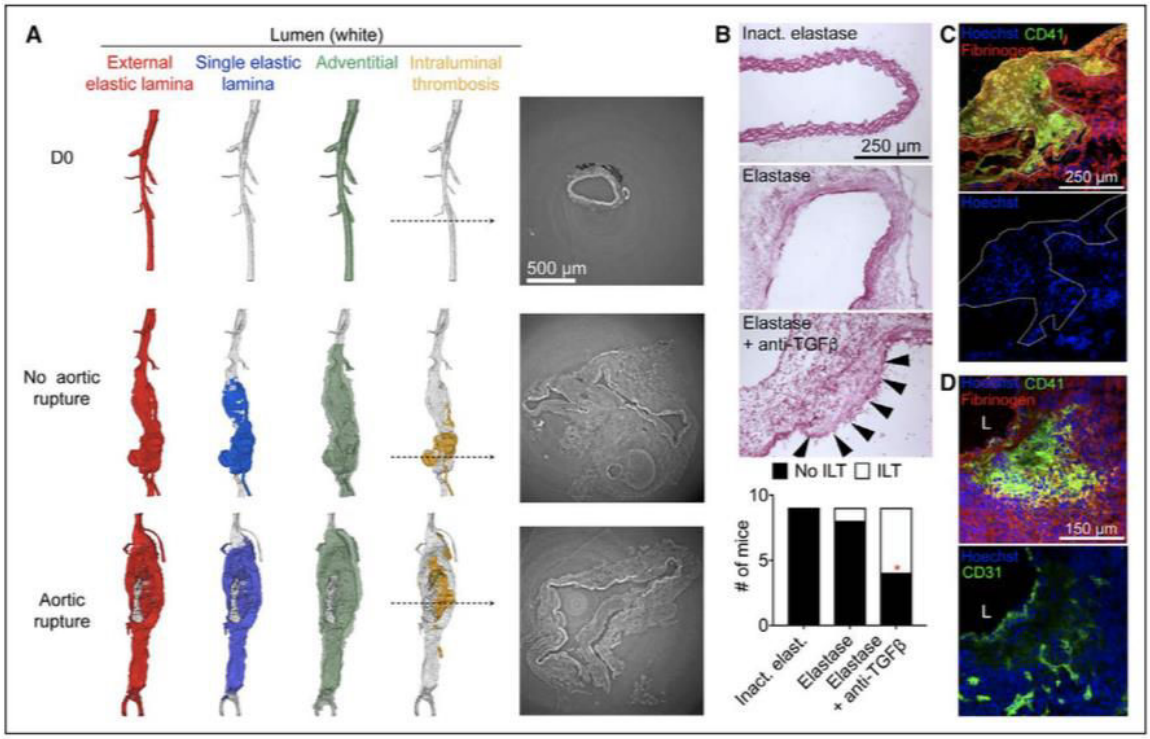

Figure 3. Blockade of TGFβ (transforming growth factor-β) activity after elastase injury induces elastic degradation, adventitial remodeling, aortic rupture, and intraluminal thrombus (ILT) formation. **A**, Representative 3-dimensional segmentation and raw synchrotron pictures of a normal aorta (**top** row), an aneurysmal aorta (**middle** row), and a ruptured aneurysm (**bottom** row). Elastic laminae, adventitial remodeling, and ILT formation are color-coded. **B**, Representative images of ILT analysis in the 3 groups and quantification of the presence of ILT. χ^2 test; $^*P<0.05$ elastase vs elastase+anti-TGFβ. **C**, Characterization of ILT by immunofluorescent staining showing the accumulation of platelets (CD41-green), fibrin/fibrinogen (red), and cells (Hoechst+) in the lumen side of the aortic wall. **D**, Representative picture showing the accumulation of platelets in the aortic wall and the formation of neovessels (CD31+) on serial sections.

Inhibition of TGFβ Activity Increases Immune Cell Accumulation in the Aorta After Elastase Injury

Inflammation is another feature of AAA remodeling and rupture in humans.[5] Using fluorescent microscopy and flow cytometry analysis, we characterized the inflammatory infiltrate present in aortic samples at day 14. TGFβ inhibition after periaortic elastase application dramatically increased the accumulation of T cells, macrophages, and neutrophils, as compared with aortic samples treated with elastase only (Figure 5). Flow cytometry analysis suggested that macrophages were mostly derived from monocytes, given their high expression of Ly6B (Figure 5D) and that the T-cell population was composed of both $CD4^+$ and $CD8^+$ cells with an activated phenotype ($CD69^+$; Figure 5D). We also noticed that, as described in human AAA, the ILT of mice treated with elastase+anti-TGFβ was rich in neutrophils (Figure 5C).

TGFβ Blockade Limits the Accumulation of Profibrotic Segregated Atypical Monocytes

Akira et al recently identified a segregated nucleus-containing atypical monocyte (SatM) subset derived from a committed progenitor downstream of $Ly6B/C^-Fc\varepsilon RI^+$ granulocyte/macrophage progenitors. SatM are $CEACAM1^+MSR1^+Ly6B/C^-F4/80^-MAC1^+$ monocytes that differentiate under the control of C/EBPβ, home to injured tissues during the healing phase, and play a crucial role in tissue fibrosis.[23] SatM do not produce TGFβ; however, C/EBPβ deficiency, which eliminates SatM, is associated with substantial reduction of TGFβ production.[23] Therefore, we tested the impact of TGFβ blockade on the accumulation of SatM after elastase injury. Interestingly, we found a substantial reduction of SatM in the bone marrow, blood, and aortic wall of mice treated with neutralizing anti-TGFβ antibody and periaortic elastase, compared with periaortic elastase only (Figure VI in the online-only Data Supplement). Our results support an important role for TGFβ in SatM homeostasis, with potentially important consequences on the healing response.

Monocytes/Macrophages Are Necessary for the Induction of AAA Rupture After Elastase and Anti-TGFβ Treatment

Given the imbalance between inflammatory monocytes and SatM in mice treated with anti-TGFβ, we hypothesized that monocyte/macrophage depletion using clodronate liposomes would limit disease severity and reduce the occurrence of aortic rupture. Treatment with clodronate liposomes effectively depleted systemic monocytes (Figure 6A) and aortic macrophages (Figure 6B) in comparison with phosphate-buffered saline–loaded liposomes. Monocyte/macrophage depletion

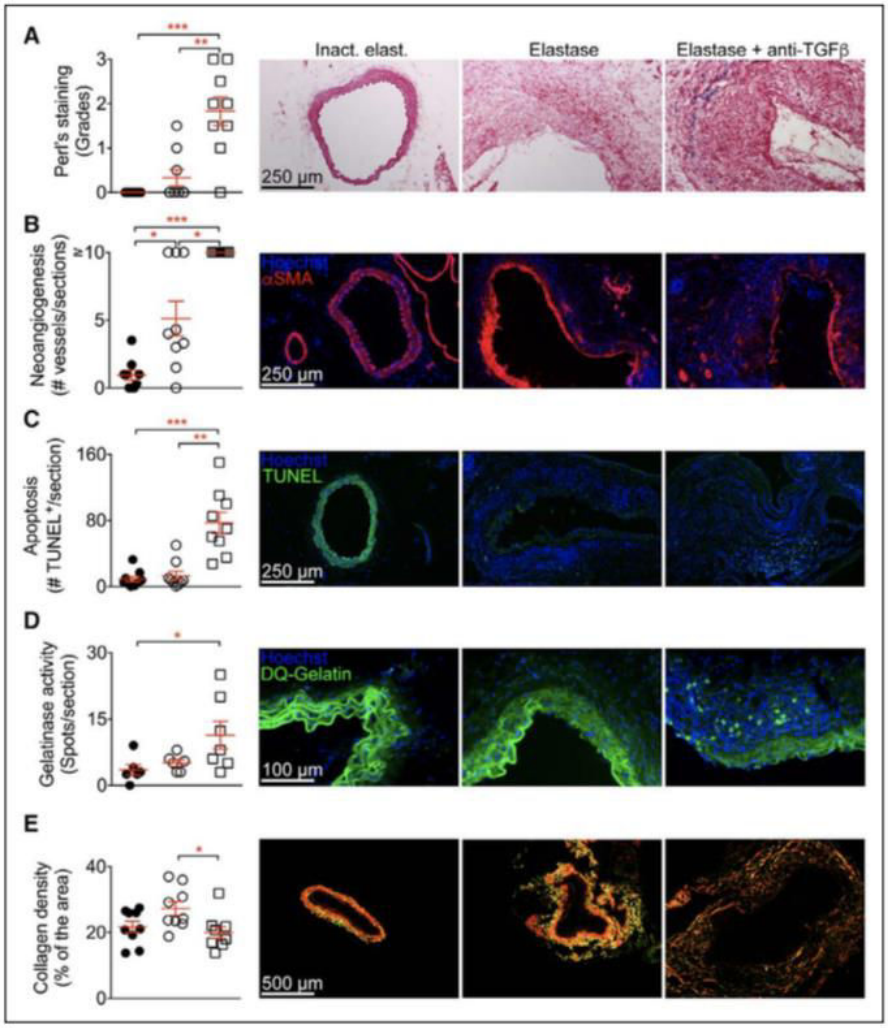

Figure 4. Blockade of TGFβ (transforming growth factor-β) activity after elastase injury reproduces histological features of human abdominal aortic aneurysm. Aortic cryosections from mice at day 14 (n=9 per group) after surgery were stained for iron accumulation (**A**, Perl staining), the presence of smooth muscle cell (SMC) to assess neovascularization and medial SMCs (αSMA [smooth muscle cell actin] staining, **B**), apoptosis (TUNEL [terminal deoxynucleotidyl transferase dUTP nick-end labeling] staining, **C**), gelatinase activity (DQ-Gelatin, **D**), and collagen (Sirius red staining, **E**). Each staining was quantified in the 3 groups of mice (n=9 per group). Kruskal–Wallis+uncorrected Dunn test. In **A**, ***P<0.001 inactive elastase vs elastase+anti-TGFβ, **P<0.01 elastase vs elastase+anti-TGFβ. In **B**, ***P<0.001 inactive elastase vs elastase+anti-TGFβ, *P<0.05 elastase vs elastase+anti-TGFβ and inactive elastase vs elastase. In **C**, ***P<0.001 inactive elastase vs elastase+anti-TGFβ, **P<0.01 elastase vs elastase+anti-TGFβ. In **D**, *P<0.05 inactive elastase vs elastase+anti-TGFβ. In **E**, *P<0.05 elastase vs elastase+anti-TGFβ.

did not abrogate aneurysm development (Figure 6C) but led to a marked reduction of AAA severity (Figure 6C) and completely protected against AAA rupture (Figure 6D). This was associated with a significant reduction of elastin degradation (Figure 6E) and increased collagen content (Figure 6F).

Role of IL-1β in the Initiation Versus Progression of AAA

To gain more insight into the inflammatory response, we analyzed mRNA expression of cytokines (by quantitative polymerase chain reaction) in the aortic tissue and found that both proinflammatory (tumor necrosis factor, interleukin [IL]-6, IL-1β, and CXCL1) and anti-inflammatory (TGFβ and IL-10) cytokines were induced after elastase injury (Figure VII in the online-only Data Supplement). Tumor necrosis factor, IL-1β, and CXCL1 reached high levels at day 7, but their expression was attenuated at day 14. IL-6 expression showed a late increase between day 7 and day 14. IL-10 and TGFβ increased at day 7, with variable expression of IL-10 at day 14. TGFβ blockade on top of elastase application led to sustained elevations and higher levels of IL-1β, CXCL1, and IL-6 at day 14 after aortic injury but did not alter tumor necrosis factor and IL-10 expression. We also detected a slightly higher aortic TGFβ expression in the elastase+anti-TGFβ at day 14, compared with the elastase-only group.

The kinetics of IL-1β expression in the aortic wall suggested a differential effect in the initiation versus progression

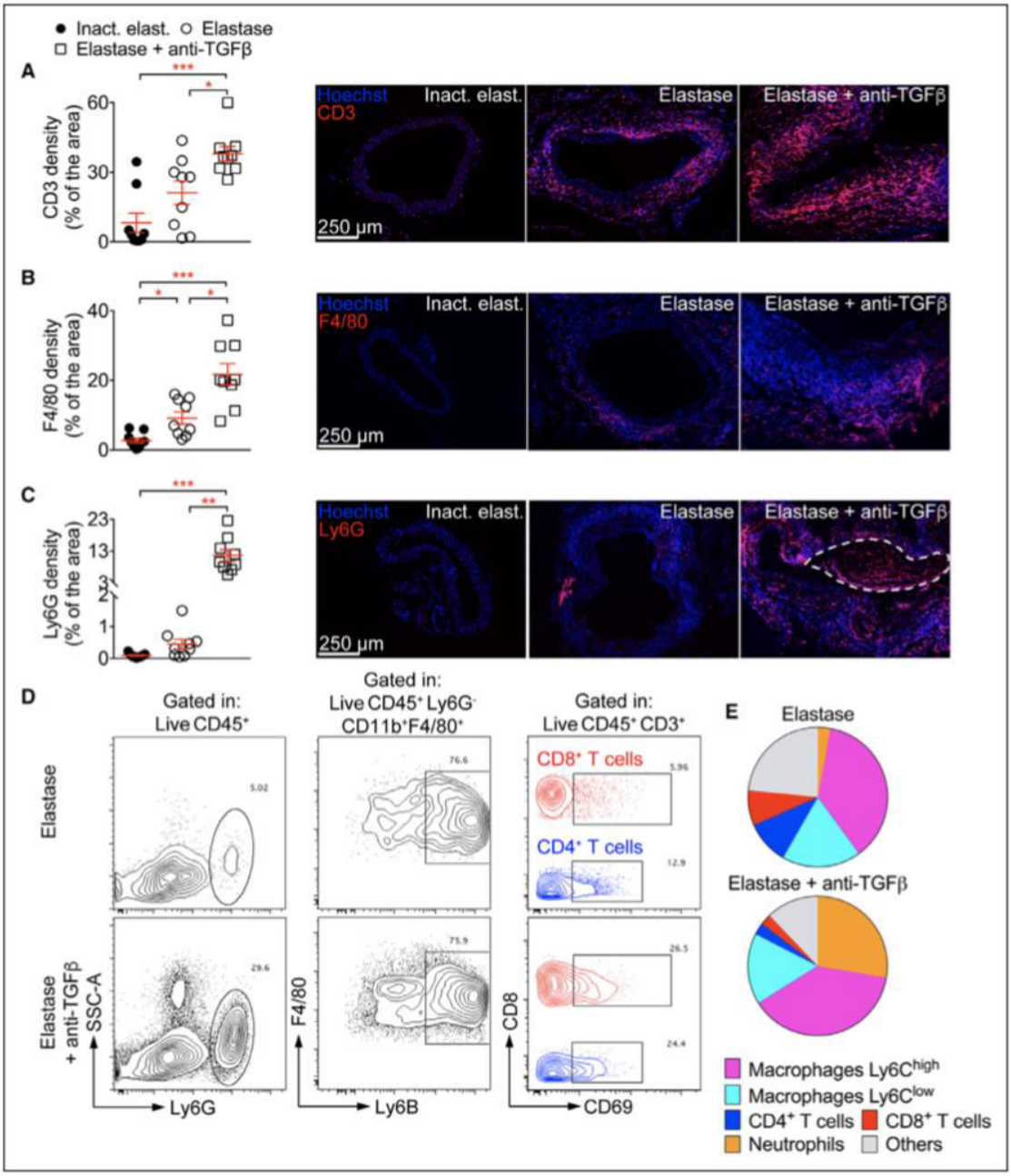

Figure 5. Blockade of TGFβ (transforming growth factor-β) activity sustains elastase-induced inflammation in the aorta. Aortic cryosections from mice at day 14 (n=9 per group) after surgery were stained for T cells (**A**), macrophages (**B**), and neutrophils (**C**). **A**–**C**, Quantifications are shown at day 14 (n=9 per group). Kruskal–Wallis+uncorrected Dunn test. In **C**, the dotted line circles the intraluminal thrombus. **D** and **E**, Flow analysis of aortic CD45+ cells at day 14 (n=4 per group). Representative flow chart showing neutrophils, macrophages, and T-cell analysis in the aorta of mice treated with elastase or elastase+anti-TGFβ (**D**), and distribution of immune cells in the aorta of each group (**E**). In **A**, ***P<0.001 inactive elastase vs elastase+anti-TGFβ, *P<0.05 elastase vs elastase+anti-TGFβ. In **B**, ***P<0.001 inactive elastase vs elastase+anti-TGFβ, *P<0.05 elastase vs elastase+anti-TGFβ and inactive elastase vs elastase. In **C**, ***P<0.001 inactive elastase vs elastase+anti-TGFβ, **P<0.01 elastase vs elastase+anti-TGFβ.

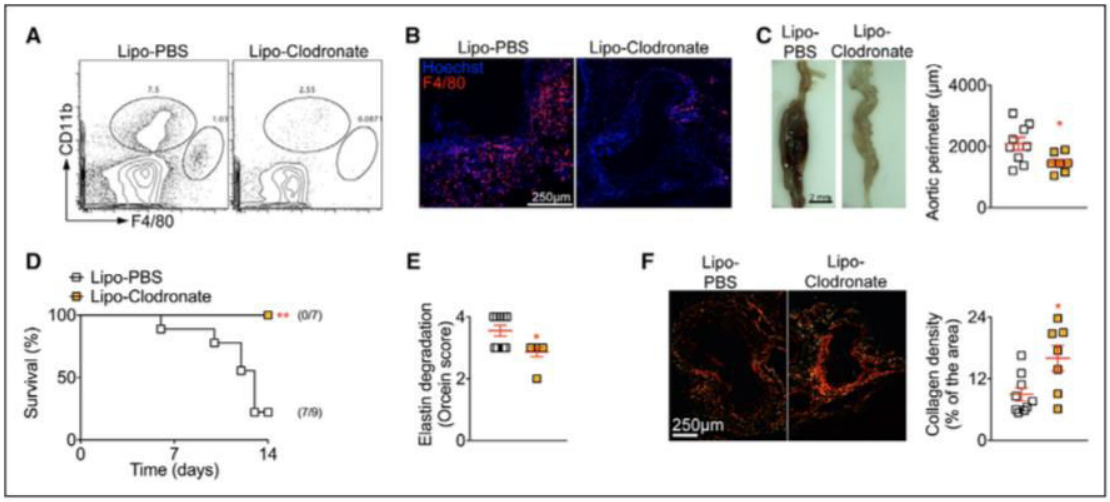

Figure 6. Depletion of monocytes/macrophages using clodronate liposomes in mice treated with elastase+anti-TGFβ (transforming growth factor-β) prevents aortic rupture. **A**, Representative flow data of macrophages in the spleen 5 d after liposome injection (n=5 per group). **B**, Representative immunofluorescent staining of macrophages in the aorta on day 14 after surgery (n=7–9 per group). **C**, Representative macroscopic picture and quantification of aortic perimeter of aorta at day 14 after surgery (n=7–9 per group). Mann–Whitney *U* test. **P*<0.05 liposome-phosphate-buffered saline (PBS) vs liposome-clodronate. **D**, Survival curves of mice injected with liposomes (n=7–9 mice per group). Log-rank, Mantel–Cox test. ***P*<0.01 liposome-PBS vs liposome-clodronate. **E** and **F**, Analysis of elastin and collagen degradation using Orcein staining (**E**) and Sirius red (**F**), respectively. Mann–Whitney *U* test (n=7–9 mice per group). In **E** and **F**, **P*<0.05 liposome-PBS vs liposome-clodronate.

of AAA. To directly assess the implication of IL-1β in aortic dilatation and rupture, we compared IL-1β knockout (KO) and wild-type mice. We confirmed the absence of IL-1β in the plasma of IL-1β KO mice and found that CXCL1 and IL-10 were also significantly reduced, suggesting a role for IL-1β in the production of these cytokines (Figure VIIIA in the online-only Data Supplement). At day 5 after injury, IL-1β KO mice showed a reduction of circulating neutrophils (Figure VIIIB in the online-only Data Supplement), in accordance with the reduction of plasma CXCL1 levels. As compared with wild-type mice, IL-1β KO mice showed a marked protection against aortic dilatation (Figure 7A) and were totally protected against AAA rupture induced by elastase and anti-TGFβ treatment (Figure 7B). Moreover, the injury induced by elastase and TGFβ blockade was dramatically reduced in IL-1β KO mice (Figure VIIIC through VIIIF in the online-only Data Supplement). To address the role of IL-1β in the progression and complications of established AAA, we used a mouse monoclonal antimouse IL-1β antibody. The antibody significantly blocked the induction of CXCL1 by VSMCs in response to recombinant IL-1β in vitro (Figure IXA in the online-only Data Supplement) and significantly reduced plasma levels of CXCL1 and IL-6 in vivo while impairing IL-1β clearance (Figure IXB in the online-only Data Supplement). Importantly, however, neutralization of IL-1β with this monoclonal anti–IL-1β

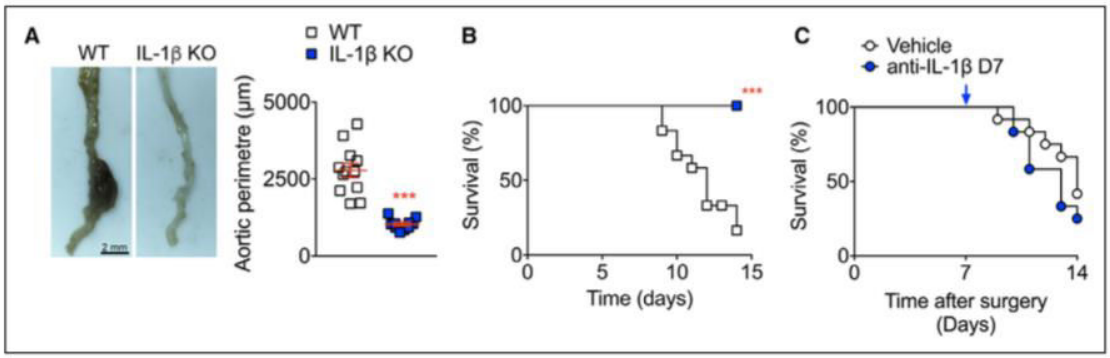

Figure 7. Blocking IL-1β does not prevent aortic rupture of established abdominal aortic aneurysm. **A**, Representative macroscopic picture and quantification of aortic perimeter of aorta at day 14 after surgery (n=10–12 per group). Mann–Whitney *U* test. **B**, Survival curves of mice (n=10–12 per group). Log-rank, Mantel–Cox test. **C**, Wild-type (WT) mice were injected (or not) on day 7 with a neutralizing mouse antimouse IL-1β antibody. Injections were repeated on days 9 and 11. Survival curves of mice (n=12 per group). Log-rank, Mantel–Cox test. In **A** and **B**, ****P*<0.001 WT vs IL-1β knockout (KO).

antibody starting at day 7 after elastase+anti-TGFβ did not the limit the progression of established AAA toward fatal rupture (Figure 7C). Taken together, our data indicate that IL-1β is important for the initiation of AAA but is dispensable for the progression and complications of established AAA.

Discussion

Major features of human AAA include ECM degradation, medial smooth muscle cell loss and degeneration, infiltration and activation of immune cells, ILT formation, progressive aortic dilatation in the absence of medial dissection, and finally aortic wall rupture. It is noteworthy that despite the variety of experimental models of AAA, none has been reported to combine the major features of human AAA. Most models either induce early medial dissection, not transmural aortic wall rupture, or lead to self-contained aortic dilatation. Furthermore, most models do not induce ILT formation. A rare occurrence of aortic rupture has been reported in elastase-induced AAA in rats, not mice, but those rare ruptures occur early after elastase application and are because of an overactive exogenous elastase rather than a progressive aneurysmal aortic disease that culminates in aortic wall rupture. Recently, Lu et al[22] reported the development of a chronic model of AAA in mice after a combination of periaortic elastase application and continuous oral administration of the lysyl oxidase inhibitor BAPN (beta-aminopropionitrile). AAA development in that model was associated with thrombus formation and spontaneous rupture. Despite those interesting human-like aneurysm features, the authors provided no evidence for the absence of medial dissection, and there was no evidence that the few late ruptures had arisen from the aortic wall itself; in fact, some deaths were attributed to rupture or dissection of small arteries.[22] Moreover, BAPN is known to induce medial dissections, not transmural ruptures, which lead to intramural, not intraluminal, hemorrhage and thrombus formation.[11–13]

In contrast to previous models, the AAA model we describe here in detail associates all the major features of human AAA and is potentially of great interest to our understanding of the pathophysiology of AAA development, progression, and complications.

It is remarkable to observe that the transition from a model inducing a self-contained aortic dilatation to a human-like model of sustained aneurysmal growth culminating in rupture is because of the blockade of a single cytokine, TGFβ. This supports a critical role of TGFβ signaling in the maintenance of aortic wall integrity and the protection against AAA progression and complications. Mutations in the TGFβ signaling pathway are responsible for severe forms of thoracic aortic aneurysms and dissections in humans,[24] and some studies reported associations between single-nucleotide polymorphisms in the TGFβ signaling components and AAA.[25] Moreover, human AAA biopsies frequently show loss of 1 copy of TGFβ receptor type 2 exon 8 in association with a substantial reduction of gene expression, suggesting an acquired impairment in the TGFβ pathway and local downregulation of TGFβ signaling.[26] The major sources of TGFβ during AAA development remain to be identified. Immune cells, VSMCs, (myo)fibroblasts, and platelets may all contribute to TGFβ production.

How does TGFβ activity control AAA dilatation and propensity to rupture? TGFβ is well known to control major biological processes involved in AAA, including ECM remodeling, VSMC survival and proliferation, and the activation of the immuno-inflammatory response.[25] We show here that at least some of the protective effect occurs through the control of the innate immune response and more particularly, the balance between inflammatory monocytes/macrophages and profibrotic SatM. The specific role of the latter monocyte subset will require further investigation. Moreover, other innate immune cells like neutrophils, or adaptive immune cells like T cells, may cooperate with monocytes/macrophages and probably contribute to the process. Note that our study does not exclude a role for TGFβ activity in vascular cells and (myo) fibroblasts. However, deletion of TGFβ receptor signaling in VSMC did not lead to aggravation of AAA after elastase application in mice.[27] The latter result is surprising given the major role of VSMC-restricted TGFβ signaling in the protection against thoracic aortic aneurysms.[15,16,28]

Our study identifies IL-1β as an important cytokine involved in the prevention of AAA development, which is an agreement with previous studies performed in less-severe models of the disease.[29] Surprisingly, however, IL-1β blockade did not affect the progression of established AAA toward rupture, suggesting a limited role (if any) of IL-1β in advanced stages of the disease. The role of IL-1 pathway in human AAA is not well understood. Genetic upregulation of IL-1 receptor antagonist in humans is associated with increased, not reduced, susceptibility to AAA.[30] Moreover, a recent placebo-controlled randomized trial failed to show any benefit of canakinumab (a neutralizing monoclonal anti–IL-1β antibody) on AAA progression in patients with small AAAs (between 40 and 50 mm; clinicaltrials.gov, NCT02007252). The trial included a limited number of patients in each group (31 Canakinumab and 33 placebo), and only 2 of 3 patients completed the 12-month treatment period. These unexpected human data are in disagreement with previous mouse data that suggested a detrimental role for IL-1 signaling in the progression of AAA, using the elastase model.[29] However, they seem to be in agreement with our findings of a limited role of IL-1β in established AAA, using the present elastase+anti-TGFβ model, further highlighting the clinical translational relevance of this new mouse model.

Another important point to consider is the chronicity and sustainability of AAA development and growth over time. Most available experimental models of nondissecting AAA induce a development of AAA for 7 days and are rapidly self-contained with no or little AAA progression thereafter; in fact, they tend to heal with time in the absence of sustained injury. The elastase+BAPN model described recently allows the development of a chronic aortic aneurysm and is worth of further consideration.[22] However, there is no solid evidence yet that it is not driven by early medial dissection or that sudden death is because of true aortic wall rupture. In this context, our identification of TGFβ as a critical mediator of AAA healing is likely to open new avenues for the development of chronic models of nondissecting AAA characterized by an impaired healing process and sustained growth, until aortic rupture. Our current elastase/anti-TGFβ model produces a human-like AAA and a high percentage of AAA rupture within 14 days. We think that this model can be adapted to produce a nonresolving inflammation that will sustain AAA

growth for a longer period, for example, through controlled blockade of TGFβ over time (varying the frequency and dose of anti-TGFβ), better mimicking the progression of human AAA. It is expected that in such a chronic smoldering model, the pathogenic mechanisms of AAA growth may differ from our current understanding of disease pathogenesis.

Finally, the new model described here may be useful to address the role of specific features of AAA, for example, ILT formation in AAA progression and susceptibility to rupture. Moreover, it will be interesting to examine whether blockade of TGFβ activity may be useful in promoting features of human-like AAA in other experimental models, like the CaCl2 model.

In conclusion, we have shown that current experimental mouse models of AAA can be improved to better reproduce major features of human AAA and may be harnessed to predict response to therapy in the human disease setting. We expect that optimized models will provide a better understanding of the pathophysiology of AAA and will be useful to identify and test new and relevant therapeutic targets.

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Disclosures

None.

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Highlights

- Endogenous TGFβ (transforming growth factor-β) activity is required for the healing of abdominal aortic aneurysm (AAA).
- Systemic blockade of TGFβ in the elastase-AAA model in mice induces a transition from a self-contained aortic dilatation to a model of sustained aneurysmal growth, associated with the formation of an intraluminal thrombus, and culminates in fatal transmural aortic wall rupture.
- Early blockade of IL1β limits AAA severity. However, blockade of IL1β after disease initiation has no effect on AAA progression to rupture.
- TGFβ blockade may be harnessed to generate new models of AAA with better relevance to the human disease.

Annexe 2: Monocytes and macrophages in abdominal aortic aneurysm

Monocytes and macrophages in abdominal aortic aneurysm

Juliette Raffort[1–4], Fabien Lareyre[1,4,5], Marc Clément[1], Réda Hassen-Khodja[4,5], Giulia Chinetti[3,4] and Ziad Mallat[1,2]

Abstract | Abdominal aortic aneurysm (AAA) is a life-threatening disease associated with high morbidity, and high mortality in the event of aortic rupture. Major advances in open surgical and endovascular repair of AAA have been achieved during the past 2 decades. However, drug-based therapies are still lacking, highlighting a real need for better understanding of the molecular and cellular mechanisms involved in AAA formation and progression. The main pathological features of AAA include extracellular matrix remodelling associated with degeneration and loss of vascular smooth muscle cells and accumulation and activation of inflammatory cells. The inflammatory process has a crucial role in AAA and substantially influences many determinants of aortic wall remodelling. In this Review, we focus specifically on the involvement of monocytes and macrophages, summarizing current knowledge on the roles, origin, and functions of these cells in AAA development and its complications. Furthermore, we show and propose that distinct monocyte and macrophage subsets have critical and differential roles in initiation, progression, and healing of the aneurysmal process. On the basis of experimental and clinical studies, we review potential translational applications to detect, assess, and image macrophage subsets in AAA, and discuss the relevance of these applications for clinical practice.

Aortic aneurysm is a focal dilatation of the aorta associated with substantial morbidity and mortality as a result of complications such as aortic rupture[1] (BOX 1). Although remarkable progress has been achieved in both open and endovascular surgery, curative pharmacological treatments for this condition are still lacking[2], highlighting a real need to improve our knowledge of the mechanisms involved in development and progression of aortic aneurysm. Although vessel dilatation can occur in the abdominal or thoracic aorta, the most common form of aneurysm in humans occurs in the infrarenal region of the abdominal aorta, which is the subject of this Review.

Detailed pathological and imaging studies of human abdominal aortic aneurysm (AAA), together with the development of several animal models of the disease, have led to the characterization of the main mechanisms underlying AAA development and its complications[3,4]. Pathological features include extracellular matrix (ECM) degradation and loss of vascular smooth muscle cells (VSMCs), associated with adventitial and medial inflammatory cell infiltration, all contributing to vascular remodelling and weakening of the aortic wall. Innate and adaptive immune cells, including neutrophils, macrophages, mast cells, natural killer cells, dendritic cells, B cells, and T cells, have been identified in aortic aneurysms and have been shown to contribute to aortic aneurysm development[5]. In this Review, we focus on monocytes and macrophages in aortic aneurysm, given the critical and distinct roles of these cells in several steps of the vascular response to injury, and provide a summary of current knowledge on monocyte and macrophage origin and roles in AAA development and its complications, highlighting potential translational applications targeting monocytes and macrophages.

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Blood monocytes and AAA

Circulating blood monocytes originate in the bone marrow and are a heterogeneous leukocyte population with various subsets that differ in morphology, surface markers, gene-expression profile, and functions. In humans, three main subsets can be distinguished on the basis of the expression of the cell surface markers CD14 and CD16 (FIG. 1). Classical monocytes, defined as $CD14^{++}CD16^{-}$, usually represent up to 90% of blood monocytes[6,7]. Classical monocytes have high surface levels of the CC-chemokine receptor CCR2 and CD62L (also known as L-selectin), and low levels of the CX_3C-chemokine receptor CX_3CR1 (REFS 6–8). This monocyte subset is characterized by high expression levels of genes

Key points

- Most of the macrophages that accumulate in the aneurysmal aortic wall originate from circulating monocytes; however, some macrophages in abdominal aortic aneurysms (AAAs) might originate from aortic tissue-resident macrophages
- The main factors involved in macrophage accumulation in the AAA wall include chemokines and cytokines produced in response to tissue injury, products of extracellular matrix degradation, and microenvironmental conditions
- Monocytes and macrophages have distinct phenotypes during the development and progression of AAA, with major implications for monocyte and macrophage activation and biological functions in AAA
- Macrophages have both pathogenic and reparative roles in AAA through their involvement in extracellular matrix remodelling, in promotion and resolution of inflammation, and in various aspects of the tissue-healing response
- State-of-the-art translational applications are available that can be improved and harnessed for the use of monocytes and macrophages as diagnostic and prognostic biomarkers, and as therapeutic targets in AAA

coding for antimicrobial and phagocytosis-related proteins, emphasizing the major role of classical monocytes in innate immune responses[7,8]. In addition, classical monocytes express genes encoding proteins involved in angiogenesis, wound healing, and coagulation, suggesting a potential role of this subset in tissue repair[9] (FIG. 1). Nonclassical monocytes are defined as $CD14^{+}CD16^{++}$, with low surface levels of CCR2 and high levels of CX_3CR1 (REFS 6–8). Owing to their vascular patrolling properties, nonclassical monocytes are involved in innate immune surveillance of tissues[7,8]. An intermediate phenotype, characterized by high CD14 levels with low CD16 levels ($CD14^{++}CD16^{+}$) and with pro-inflammatory and phagocytic properties, has also been described[6–8].

In mice, monocyte subsets — which express CD11b (also known as Mac-1 or integrin α_M) and CD115 (also known as CSF1R) with low surface levels of the macrophage marker F4/80 — are classified according to the surface levels of lymphocyte antigen 6C (LY6C) into two major subsets that are similar to the major human subsets in terms of chemokine receptor expression and function[10,11] (FIG. 1). $LY6C^{high}$ monocytes are equivalent to human classical monocytes and are thought to promote pro-inflammatory responses. By contrast, $LY6C^{low}$ monocytes have functions similar to human nonclassical monocytes, and are involved in patrolling, immune surveillance, and tissue repair[8,10,11].

Monocytosis (increased number of monocytes in blood) has been associated with a number of cardiovascular diseases including atherosclerosis and myocardial infarction[7,8,12,13]. In aortic aneurysm, clinical studies revealed substantial modifications in peripheral blood monocyte subsets and phenotypes, suggesting the involvement of monocytes in the disease. Patients with AAA have a higher proportion of circulating, intermediate, $CD14^{++}CD16^{+}$ monocytes, concomitant with a decrease in the number of classical, $CD14^{++}CD16^{-}$ monocytes, compared with healthy individuals[14,15]. Results for nonclassical $CD14^{+}CD16^{+}$ monocytes are less consistent, with one study showing an increase of this subset[14], whereas no significant differences were found in another study[14,15]. Proteomic and transcriptomic analyses of macrophages derived from human peripheral blood monocytes showed differences in the protein and gene expression profiles of these cells between patients with AAA and patients with peripheral arterial disease without AAA[16]. Among those differentially expressed were mRNAs and proteins related to pathophysiological mechanisms relevant to AAA, such as ECM remodelling or inflammation, suggesting a specific role of monocyte-derived macrophages in AAA pathogenesis.

To gain further insight into the pathological relevance of blood monocytes in AAA, investigators have used animal models of AAA (BOX 2). An increase in circulating classical $CCR2^{+}$ monocytes was observed in *Apoe*$^{-/-}$ mice infused with angiotensin II for 14 days[17]. Given the capacity of $CCR2^{+}$ monocytes to migrate to sites of injury and to inflamed tissues[18], the increase in this monocyte subset in blood after angiotensin II infusion suggests that $CCR2^{+}$ monocytes contribute to the vascular inflammatory response. Another study reported higher circulating levels of $LY6C^{high}$ monocytes and $LY6C^{low}$ monocytes at day 3 and day 7 after angiotensin II infusion, respectively, in mice that later developed AAA after 28 days of angiotensin II infusion compared with mice that did not develop AAA[19]. Remarkably, in mice that did not develop AAA after angiotensin II infusion, monocyte levels did not increase and remained stable over time[19]. Moreover, in *Apoe*$^{-/-}$ mice the efficacy of some treatment strategies (such as administration of recombinant angiopoietin 2) in attenuating angiotensin II-induced aortic dilatation and rupture was associated with a reduction in circulating levels of $LY6C^{high}$ inflammatory monocytes[20]. Interestingly, mice deficient in myeloid differentiation primary response protein 88 (MYD88) — a critical adaptor protein required for signalling downstream of several Toll-like receptors (TLRs), as well as receptors of the interleukin (IL)-1 family of cytokines — showed reduced redistribution of monocytes from $LY6C^{low}$ to $LY6C^{high}$ in both peripheral blood and spleen after angiotensin II infusion, which was associated with significant attenuation of AAA formation[21]. The data suggest a pathogenic role of $LY6C^{high}$ monocytes, but a potentially less critical role for $LY6C^{low}$ monocytes in the development of AAA. Mice deficient in the transcription factor nuclear receptor subfamily 4 group A member 1 (NR4A1), which lack $LY6C^{low}$ monocytes[22], had increased aortic dilatation and elastin disruption

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Box 1 | **Abdominal aortic aneurysm**

Definition
Abdominal aortic aneurysm (AAA) is defined as a focal dilatation of the aorta (generally an infrarenal diameter of ≥3 cm), associated with a loss of arterial wall parallelism.

Epidemiology
The prevalence of AAA is 1.34% in men aged 65 years[168], irrespective of any other risk factor. In the presence of risk factors, AAA prevalence is up to 6% in men[1] and 1.3% in women aged >65 years[169]. An estimated 2% of all deaths are AAA-related (11,000 deaths/year in the USA), and AAA is associated with catastrophic rates of death (up to 80%) in the event of aortic rupture[1].

Risk factors
AAA is frequently associated with atherosclerosis. Risk factors of AAA include age, male sex, smoking, a family history of aortic aneurysm, hypertension, and low HDL-cholesterol levels[1]. By contrast, diabetes mellitus is a protective factor[170,171]. Genome-wide association studies for AAA reported several disease-specific risk loci[172–175].

Pathophysiology
AAA is characterized by extracellular matrix degradation, impairment of vascular smooth muscle cell functions, and ultimately loss of vascular smooth muscle cells. These features are associated with infiltration of immune cells in adventitial and medial layers, and lead to an expansive remodelling of the aortic wall with progressive loss of aortic wall function and development of an intraluminal thrombus. If unabated, those processes severely weaken the aortic wall, and lead to microdissections or severe complications such as aortic rupture[1].

Current treatment
Treatment for AAA is currently based on surgical intervention, including endovascular repair or open surgical repair[169]. The decision to treat AAA relies on a balance between the risk of disease progression and aortic rupture and the operative risks[169]. Aortic wall repair is associated with a fairly high risk of early or late morbidity and mortality[176].

in response to angiotensin II[23], suggesting a protective role for LY6C^{low} monocytes against AAA development. Nevertheless, the phenotype of NR4A1-deficient mice could also be a result of a role of NR4A1 in VSMCs[23] or to functional alteration in the remaining LY6C^{high} monocytes and bone-marrow-derived macrophages in the absence of NR4A1 (REFS 24,25). The latter hypothesis can potentially be addressed by use of mice with deletion of an *Nr4a1* superenhancer subdomain that differentially controls and decouples the role of NR4A1 in LY6C^{low} monocyte development from the regulation of *Nr4a1* expression in macrophages[26].

Taken together, these results point to a potential role of circulating monocytes in regulating vascular inflammation and remodelling during the development of AAA, and suggest that determination and modulation of blood monocyte numbers has predictive and therapeutic value (see below). Interestingly, a study published in 2017 reported the identification of a novel atypical monocyte subset (CD11b$^+$LY6C$^-$CEACAM1$^+$MSR1$^+$), called segregated-nucleus-containing atypical monocytes (SatM), which derive from LY6C$^-$FcεRI$^+$ SatM progenitors downstream of granulocyte–macrophage progenitors[27] (FIG. 1). SatM have granulocyte characteristics, require CCAAT/enhancer-binding protein (C/EBP)-β for their differentiation, and have a critical role in fibrosis[27]. We suspect that this monocyte subset has a human equivalent, with functions in tissue repair during AAA. In summary, multiple monocyte subsets exist, with various and distinct functions that warrant detailed investigation in future studies in the context of AAA.

Efferocytosis
Process by which apoptotic cells are removed by phagocytic cells.

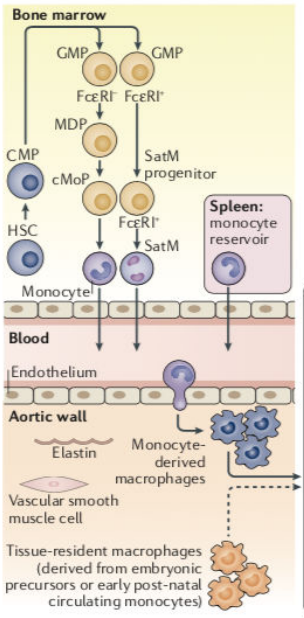
Macrophage characterization in AAA

Over the past 2 decades, several macrophage phenotypes have been described in inflamed tissues, with a particular focus on the dichotomy between 'classically activated' M1 macrophages and 'alternatively activated' M2 macrophages[28–30]. M1 macrophages are considered to be pro-inflammatory and are characterized by the production of proteolytic enzymes and pro-inflammatory cytokines such as tumour necrosis factor (TNF), IL-6, IL-12, IL-1β, nitric oxide synthase 2 (NOS2), or the CC-chemokine CCL2[5,28]. By contrast, M2 macrophages have anti-inflammatory roles through secretion of factors such as IL-10 or transforming growth factor-β (TGFβ), and are involved in ECM remodelling and tissue repair[28]. A specific pathway for the generation of NOS2$^+$ macrophages from a subset of LY6C^{high} monocytes was described in 2016, indicating that specific subsets of monocytes might have the capacity to generate functionally distinct macrophage subsets[31]. However, although the classification of macrophage subsets is well defined *in vitro*, distinction of macrophage subsets *in vivo* is less clear and more problematic given that separate, selective M1 and M2 stimuli do not occur *in vivo*, and the markers used to differentiate macrophage subsets lack specificity[28,32]. Therefore, macrophage subsets *in vivo* correspond to a spectrum of activation and are characterized by a combination of markers[28].

Several studies have reported the presence of macrophages in the aneurysmal aortic wall both in human aortic sections[33] and in animal models of AAA[34–39] (FIG. 1). Macrophages accumulate in the three layers of the aneurysmal aorta wall, but accumulate predominantly in the adventitia and the intraluminal thrombus[40,41]. Dutertre *et al.* analysed the composition of human aneurysmal tissues by flow cytometry after separation of media and adventitia and showed an increased proportion of M2 macrophages — defined as CD15$^-$CD14^{high} with high expression levels of the M2 markers CD163, CD206 (also known as MRC1), and the tyrosine kinase receptor MERTK (involved in efferocytosis of apoptotic cells) — in the adventitia of aneurysmal tissue compared with control tissue, whereas the proportion of M1 macrophages (CD15$^+$CD14^{low} and negative for CD163, CD206, and MERTK) decreased[40]. Another study on human aortic aneurysmal sections showed a higher cellular density of M2 macrophages (defined as CD68$^+$CD206$^+$) than of M1 macrophages (defined as CD68$^+$CD206$^-$) in the aortic wall[42]. However, the M1 subset was preferentially located in the adventitia, whereas M2 macrophages were predominant in the intraluminal thrombus[42]. Isolation of each macrophage subset by laser capture microdissection revealed that gene and protein expression of peroxiredoxin 1 (involved in redox status) was higher in M1 macrophages than in M2 macrophages[42]. These results contrast with the findings of Dutertre *et al.*[40], and might be explained by differences in the methods and markers used to identify M1 and M2 macrophages. Additionally, the samples might represent different stages of the disease, and macrophage infiltration and polarization might evolve during the various steps of aneurysm development and

its complications. Both studies, however, suggest potentially distinct roles of macrophage subsets in disease development and aortic remodelling.

Macrophage polarization has also been studied in animal models of AAA. Increased accumulation of total macrophages, with a higher M1/M2 ratio, was observed in the suprarenal aorta of *Apoe*$^{-/-}$ mice after 7–10 days of angiotensin II infusion, and the high M1/M2 ratio was maintained during the 28 days of the experiment[43]. The reasons for this M1 shift are not fully understood, but a few mechanisms have been suggested, including upregulation of TLR4 by angiotensin II[43] and a role for ECM remodelling and elastin degradation products, particularly the VGVAPG repeat sequence[44]. Compared with *Apoe*$^{-/-}$ mice infused with angiotensin II for 28 days, prolonged infusion for an additional 56 days led to increased aneurysm expansion and propensity to rupture, with an increase in CD68^{+} macrophages in the AAA wall[45]. Positive immunostaining in the AAA wall for the M2 marker CD206 was more common than for the M1-like marker NOS2 (REF. 45), suggesting that end-stage aneurysm is associated with a shift towards M2 polarization. Given the role of M2 macrophages in tissue repair and wound healing, we can hypothesize that the shift towards the M2 phenotype might result from a compensatory effect to prevent AAA expansion and rupture. The mechanisms behind the M2 polarization are not well understood. Intriguingly, although angiotensin II promotes the infiltration of LY6C^{high} monocytes in the aortic wall, angiotensin II might also directly limit the development of M1-type macrophages and promote an M2 phenotype[46]. In addition to angiotensin II, the coagulation factor XIII A chain (factor XIIIa) has been identified to have an important role in mediating gene expression during M2 polarization[47], and production of this transglutaminase is induced in adventitial



Cell subset	Surface markers		Main inducers or polarization/ survival factors	Main output products	Functions
	Human	Mouse			
Blood monocytes					
Classical	CD14^{++}CD16^{-}	LY6C^{high}	CCL2/CCR2	TNF (mice), IL-1	Pro-inflammatory phenotype
Intermediate	CD14^{++}CD16^{+}	LY6C^{int}	CCL2/CCR2, CCL5/CCR5	TNF (human), IL-1	Pro-inflammatory phenotype
Nonclassical	CD14^{+}CD16^{++}	LY6C^{low}	NR4A1, CX_3CL1/CX_3CR1	IL-10	Crawling, patrolling, tissue repair
SatM	?	CD11b^{+}LY6C^{-} CEACAM1^{+} MSR1^{+}	C/EBPβ, TGFβ?	TNF	Fibrosis
Macrophages in AAA					
M1-like	HLA-DR^{+}, CD80, CD86	MHC II^{+}, CD80, CD86	LPS, IFNγ, TNF, NF-κB, HIF1α	TNF, IL-6, IL-1β, NOS2, CCL2	Pro-inflammatory phenotype
M2-like	CD163, CD200R, CD206 (also known as MRC1), stabilin 1	CD163, MGL1, stabilin 1	IL-10, TGFβ, IL-4, IL-13, KLF4, STAT6	IL-10, TGFβ	Anti-inflammatory, reparatory phenotype
HA-mac	CD163^{high}, HLA-DR^{low}	CD163^{high}, MHC II^{low}	Haemoglobin, haptoglobin, NRF2, ATF1	HMOX1, IL-10	Haemoglobin clearance, anti-inflammatory
Osteoclast-like	RANK, calcitonin receptor	RANK, calcitonin receptor	TNF, RANKL, calcium phosphate	Proteases	Extracellular matrix degradation

Figure 1 | **Origin of macrophages in the aneurysmal aortic wall.** Most macrophages accumulating in the aneurysmal aortic wall probably derive from circulating monocytes, which are produced in the bone marrow and can be mobilized from peripheral reservoirs such as the spleen. A contingent of macrophages might also originate from tissue-resident macrophages developed from embryonic precursors and early postnatal circulating monocytes. The diverse origin of macrophages leads to the development of macrophage subsets with distinct phenotypes, with major implications for macrophage activation and biological functions in abdominal aortic aneurysm (AAA) development and progression. ATF1, activating transcription factor 1; C/EBPβ, CCAAT/enhancer-binding protein-β; CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CEACAM1, carcinoembryonic antigen-related cell adhesion molecule 1; CMP, common myeloid progenitor; CX_3CL1, CX_3C-chemokine ligand 1; CX_3CR1, CX_3C-chemokine receptor 1; cMoP, common monocyte progenitor; FcεRI, high-affinity immunoglobulin E receptor; GMP, granulocyte–macrophage progenitor; HIF1α, hypoxia-inducible factor 1α; HLA-DR, human leukocyte antigen-DR; HMOX1, haem oxygenase 1; HSC, haematopoietic stem cell; IFNγ, interferon-γ; IL, interleukin; int, intermediate; KLF4, Krüppel-like factor 4; LPS, lipopolysaccharide; LY6C, lymphocyte antigen 6C; MDP, macrophage and dendritic cell progenitor; MGL1, macrophage galactose-type lectin 1; MHC II, major histocompatibility complex class II; MRC1, macrophage mannose receptor 1; MSR1, macrophage scavenger receptor type 1; NF-κB, nuclear factor-κB; NOS2, nitric oxide synthase 2; NRF2, nuclear factor erythroid 2-related factor 2; NR4A1, nuclear receptor subfamily 4 group A member 1; RANK, receptor activator of nuclear factor-κB; RANKL, RANK ligand; SatM, segregated-nucleus-containing atypical monocytes; STAT6, signal transducer and activator of transcription 6; TGFβ, transforming growth factor-β; TNF, tumour necrosis factor.

Box 2 | **Animal models of AAA**

Several experimental models of abdominal aortic aneurysm (AAA) have been developed, each reproducing some of the main features of human AAA[167].

Nondissecting AAA models

Nondissecting AAA is generated mainly through local perfusion and/or administration in the aortic wall of porcine pancreatic elastase or calcium chloride, which leads to aortic dilatation in association with extracellular matrix degradation and induction of a pro-inflammatory response[167,177,178].

Dissecting AAA models

Dissecting AAA models in mice can be generated using several methodologies, including systemic infusion of angiotensin II[35], with or without inhibition of transforming growth factor-β (TGFβ)[34], or with or without inhibition of lysyl-oxidase (through chronic administration of β-aminopropionitrile[179]), but also through combined administration of a mineralocorticoid-receptor agonist and high salt[180]. These models are characterized by the induction of medial dissection and are also associated with extracellular matrix degradation and vascular inflammation[167,181]. The angiotensin II infusion model usually induces a dissecting AAA located to the suprarenal aorta[182], but the addition of TGFβ or lysyl-oxidase inhibition induces a high incidence of dissection in the thoracic aorta[34,179].

CXCR3$^+$ macrophages in response to blood-flow impairment[48,49]. CXCR3$^+$ macrophages accumulate in the aneurysmal aortic wall[49]. It is tempting to hypothesize that in advanced aortic aneurysms, the reduction of shear stress as a result of profound alterations of aortic wall geometry and haemodynamics promotes the production of factor XIIIa in adventitial CXCR3$^+$ macrophages to limit expansive wall remodelling. Other mechanisms of M2-like polarization might involve pathways related to apoptotic cell efferocytosis[50], or clearance of red blood cells after haemorrhage. Indeed, haemorrhage-associated macrophages, including HA-mac, M(Hb), and Mhem macrophages, have been identified in areas of intraplaque haemorrhage and participate in haemoglobin clearance and tissue remodelling[30,51]. These macrophages are characterized by the expression of CD163 — which is involved in haemoglobin–haem uptake and endocytosis of haemoglobin–haptoglobin complexes — and CD163 activation leads to the induction of an anti-inflammatory phenotype in macrophages[52,53]. CD163 gene expression and protein levels are increased in human aneurysmal wall compared with healthy aorta, and CD163 is mainly found in areas rich in haemosiderin adjacent to neoangiogenic microvessels in the adventitia[15]. Therefore, similarly to M2 macrophages, anti-inflammatory HA-mac macrophage subsets, characterized by high surface levels of CD163 and low surface levels of HLA-DR, also accumulate in the adventitial layer of AAA[15,40].

In summary, on the basis of the results of studies in mice and humans, we can hypothesize that early stages of AAA are associated with a predominant infiltration of macrophages whose characteristics are close to the M1 phenotype and that probably enhance vascular inflammation. We suspect that, at least in mice, some macrophages, such as NOS2$^+$ macrophages, might directly derive from a selective subset of LY6C^{high} monocytes[31]. This M1 polarization could contribute to increased aortic dilatation and adverse vascular remodelling (see below). When the disease evolves, several changes in the artery wall, such as altered haemodynamics, distinct chemokine/cytokine expression profile, intraluminal thrombus formation, or the occurrence of microdissections, might lead to the recruitment of distinct monocyte subsets with reparative potential, such as LY6C^{low} monocytes or SatM monocytes, or might influence macrophage polarization and promote anti-inflammatory and pro-resolving macrophage phenotypes to heal the aortic wall and maintain vascular integrity.

Origin of macrophages in the AAA wall

For decades, the assumption was that tissue macrophages originate from bone-marrow-derived monocytes that extravasate into tissues[54,55]. However, advances in macrophage ontogeny have substantially revisited and refined this concept. Tissue-resident macrophages originate from embryonic progenitor cells that migrate into the tissues during embryonic and fetal life, and in the steady state the majority of tissue-resident macrophages are self-maintained during adulthood, independently of bone-marrow progenitors[54,55]. This embryonic origin and steady-state self-maintenance also applies to cardiac-resident macrophages[56] and vascular-resident macrophages[57]. Local proliferation of tissue macrophages is involved in several cardiovascular processes[58,59], and a greater cell-proliferation index has been reported in macrophages of aneurysmal aortas compared with control aortas[40]. However, cell proliferation can occur in both tissue-resident macrophages and macrophages derived from circulating monocytes that infiltrate injured vessels[60]. Therefore, whether vascular-resident macrophages have any relevant contribution to aortic remodelling during aneurysm development is still unclear (FIG. 1).

Most of the available evidence, derived in part from bone-marrow transplantation experiments, points to an important contribution of monocyte-derived macrophages to macrophage accumulation in AAA. Transplantation of bone-marrow cells expressing green fluorescent protein into wild-type mice showed that a proportion of adventitial macrophages originated from bone-marrow-derived monocytes recruited in response to increased expression of CXCL12 in the aorta after local application of calcium chloride[61]. Experiments in *Apoe*$^{-/-}$ mice infused with angiotensin II also indicate that most of the macrophages in the aneurysmal wall derive from circulating monocytes[19]. Interestingly, angiotensin II infusion leads to an increase in blood LY6C^{high} monocytes at day 3 in parallel to the decrease of this monocyte subset in the spleen, with no significant changes in the bone marrow[19], suggesting that monocytes and macrophages that accumulate in the abdominal aorta are mobilized from the spleen by angiotensin II. This hypothesis is corroborated by the observation that splenectomy in *Apoe*$^{-/-}$ mice infused with angiotensin II induces a decrease in aortic macrophage accumulation[19,62]. Investigation of the mechanisms involved in monocyte mobilization from the spleen revealed that monocyte mobilization did not result from a direct mechanical contraction of the spleen in response to angiotensin II, and that splenic B lymphocytes were required for monocyte mobilization[19].

These results show that monocyte and macrophage mobilization from peripheral reservoirs is an important and complex mechanism that contributes to aortic inflammation, and merits further investigation of the molecular mechanisms.

Consistent with an important role of circulating monocytes in the pathogenesis of AAA, monocyte depletion in mice (using clodronate-containing liposomes) substantially limits macrophage accumulation in the aortic wall and reduces the development and severity of AAA[19,63,64]. Intriguingly, deficiency of monocyte and macrophage populations (as a result of a spontaneous inactivating mutation in *Csf1*) did not prevent AAA formation in *Apoe*$^{-/-}$ mice and, unexpectedly, induced intramural haemorrhage in the thoracic aortas in both *Apoe*$^{-/-}$ and *Apoe*$^{+/+}$ backgrounds[65]. These results should be interpreted with caution, given the low number of *Csf1*-mutant mice used in the study and the unusually low percentage of angiotensin II-induced AAAs in the *Csf1*$^{+/+}$ control mice[65]. However, an important difference between clodronate depletion and *Csf1*-mutant mice is that clodronate depletes only circulating monocytes and does not affect vascular-associated resident macrophages of the adventitia, whereas *Csf1*-mutant mice are also deficient in arterial-resident macrophages[57]. Therefore, to speculate that arterial-resident macrophages have essential roles in preserving vascular integrity is tempting.

Monocyte recruitment to the AAA wall

Chemokine/chemokine-receptor pathways. The three major chemokine/chemokine-receptor pathways controlling recruitment of circulating LY6C^{high} and LY6C^{low} monocyte subsets in atherosclerosis (CCL2/CCR2, CCL5/CCR5, and CX_3CL1/CX_3CR1)[66] are the most studied in the context of AAA. Expression and secretion of CCL2 (which promotes LY6C^{high} recruitment) from aortic explants is increased in response to angiotensin II infusion compared with control mice[33,67]. Indeed, an amplification loop between CCL2 and IL-6 has been described in the aortic wall following angiotensin II infusion, in which IL-6 and CCL2 production by aortic adventitial fibroblasts initiates monocyte recruitment, monocytes then differentiate into macrophages and stimulate the proliferation of aortic adventitial fibroblast, leading to additional cytokine and chemokine production in a vicious circle[68]. Both global CCR2 deficiency and specific deficiency of CCR2 in leukocytes decreased aortic dissection and aneurysm formation compared with wild-type mice in the model of angiotensin II infusion, and were associated with a decreased number of aortic macrophages and an inhibitory effect on inflammatory cytokine expression in mice[33,67]. Similarly, global CCL2 deficiency, as well as CCL2 deficiency in bone-marrow-derived cells, had protective effects in the model of elastase-induced aneurysm, and was associated with a drastic reduction in aortic M1-like macrophages compared with wild-type mice[69]. These results confirm the role of LY6C^{high} monocytes and the CCL2/CCR2 axis in macrophage accumulation and the promotion of vascular inflammation during the development of aortic aneurysm.

The role of the other chemokine pathways has received less attention. Deletion of *Cx3cr1* led to partial protection against fatal aortic dissection in a model of angiotensin II and anti-TGFβ infusion[34]. Interestingly, CCR2 and CX_3CR1 were sequentially and differently produced in the aortic wall during early stages of AAA development, and this differential production was associated with early predominance of LY6C^{high} monocyte over LY6C^{low} monocyte recruitment in the abdominal aorta[19]. Inhibition of CCL5–CXCL4 heterodimer formation was associated with a reduction in monocyte and macrophage infiltration into the AAA wall compared with vehicle-treated mice in an elastase-induced aneurysm model[70]. However, deletion of *Ccr5* did not alter aortic disease in a model of calcium chloride-induced AAA[71].

In addition to monocyte recruitment, chemokines might also modulate macrophage retention in the tissue. Regulators of G-protein signalling (RGS) proteins target GTPase activity of chemokine receptors that are coupled to $G\alpha_i$ subunits, and desensitize receptor signalling[72]. Interestingly, *RGS1* is highly expressed in human AAA tissue compared with nonaneurysmal vascular tissue[73]. RGS1-deficient mice showed reduced leukocyte trafficking, increased macrophage chemotaxis, and lower macrophage retention into the aortic wall compared with control mice, which was associated with a protection against aneurysm formation and progression[73].

Selectins. In addition to chemokines and cytokines, the selectin family is known for its role in immune cell interaction with the vessel wall[74]. L-Selectin deficiency impaired neutrophil recruitment and macrophage accumulation in the AAA wall, and suppressed elastase-induced AAA formation[75]. As neutrophils are recruited to the aortic wall before macrophages in wild-type mice, impaired macrophage accumulation in the AAA wall might be attributed, at least in part, to diminished neutrophil recruitment and activation in L-selectin-deficient mice compared with wild-type mice. Indeed, neutrophils can promote monocyte recruitment through cytokine secretion, neutrophil extracellular trap formation, or secretion of granule proteins, thereby inducing adhesion and migration of monocytes to the site of inflammation[76].

Risk factors and AAA microenvironment. What induces the production of chemokines and chemotactic products in the vascular wall during development of AAA? Several risk factors of AAA, including smoking and hypertension, have been associated with inflammatory activation of the vascular wall[77,78]. Moreover, ECM remodelling, changes in haemodynamics and circumferential stress, or the occurrence of microdissections and intramural haemorrhage during progression of AAA might further modulate inflammatory cell recruitment and activation. These mechanisms have been addressed in several experimental studies. Macrophages accumulate predominantly in regions of medial disruption on the adventitial side, as indicated by elastin fragmentation, suggesting that ECM components act as chemotactic stimuli[45]. *In vitro* experiments showed that soluble proteins extracted from human AAA tissue enhanced

migration of the human monocyte cell line U937 in a concentration-dependent manner[79]. The researchers attributed the monocyte chemotactic activity in the AAA extracts to the presence of elastin degradation products and intact cell-surface receptors for elastin-related ligands[79]. Therefore, elastin degradation products might promote monocyte recruitment into the aneurysmal wall, which in turn will secrete proteolytic enzymes that further degrade the tissue. A variety of other molecules associated with ECM remodelling, including osteopontin, thrombospondin 1, and plasminogen, have also been shown to affect monocyte and macrophage recruitment, either directly or indirectly[80–82]. ECM degradation might lead to microdissections and intramural haemorrhage, which could further recruit more monocytes and macrophages into the aortic wall. Indeed, increased migration of human monocytes *in vitro* was reported in response to haemoglobin and to conditioned medium obtained from human adventitial AAA[15].

Macrophage infiltration can also be modulated by the intraluminal microenvironment, particularly by changes in haemodynamic conditions. Macrophage accumulation in the elastase-induced aneurysm model was inversely correlated to luminal flow and wall shear stress[83]. High aortic flow and wall shear stress were associated with increased medial macrophage apoptosis. Interestingly, *Ccl2* mRNA expression in the aorta was inversely correlated with the level of blood flow[83] and might partly explain the reduction in macrophage accumulation in the media observed in animals with high flow. The abluminal microenvironment, such as the periaortic adipose tissue, might also influence macrophage infiltration. Obesity is associated with AAA[84], and periaortic fat volume correlated with the dimensions of both thoracic and abdominal aortas, suggesting that local fat accumulation contributes to aortic remodelling[85]. Indeed, periaortic adipose explants from abdominal aortas of mice fed a high-fat diet showed greater CCL2 release and higher capacity to induce migration of peritoneal macrophages than explants from mice fed a low-fat diet[86].

Risk factors and changes in the AAA microenvironment might also directly affect activation and adhesive and migratory properties of blood monocytes. For example, thrombomodulin and CD14 production in monocytes modulates monocyte production of inflammatory mediators, monocyte adhesion to endothelial cells, and monocyte chemotaxis. In mice, systemic CD14 deficiency or myeloid cell-specific thrombomodulin deficiency attenuate macrophage accumulation in the aortic wall in response to systemic angiotensin II infusion or local application of calcium chloride, respectively, and significantly prevent AAA development[87,88]. Human studies confirm that circulating monocytes of patients with AAA have higher expression levels of lymphocyte function-associated antigen 1 (LFA1; also known as integrin $\alpha_L\beta_2$) and CD11b compared with monocytes from healthy individuals[89]. Moreover, *in vitro* assays showed that blood monocytes from patients with AAA had higher adhesion to an endothelial monolayer and higher capacity for transmigration through the endothelium than monocytes from healthy individuals[89].

Role of macrophages in AAA

Extracellular matrix degradation. Macrophage accumulation in the aneurysmal aortic wall has a crucial role in the response to tissue injury. Macrophages profoundly alter the balance between destructive tissue remodelling and reparative tissue healing through control of ECM remodelling and their role in both promotion and resolution of the inflammatory response[90].

ECM remodelling results from a balance between the activities of proteases and their inhibitors. During AAA development the levels of proteases such as cathepsins and matrix metalloproteinases (MMPs) increase, and deficiency in these enzymes protects from AAA formation[37,91–93]. Conversely, enhanced AAA formation is observed in mice deficient in tissue inhibitors of metalloproteinases (TIMPs)[94]. Cathepsin S protein levels and *MMPs* and *TIMPs* expression levels are increased in monocyte-derived macrophages from patients with AAA compared with patients with peripheral arterial disease but no AAA[16]. Moreover, increased protein levels of MMPs and cathepsins colocalized with macrophages in the aneurysmal wall, suggesting a role for monocyte-derived and macrophage-derived proteases in ECM remodelling during aneurysm formation[16,95,96]. This hypothesis is supported by mechanistic studies in animal models of AAA. *Mmp9*-knockout mice were protected against aneurysm formation, and reconstitution of MMP9-competent macrophages through bone-marrow transplantation or reinfusion of wild-type peritoneal macrophages re-established the susceptibility to aneurysm formation[37,91]. Both mRNA and protein levels of MMP9 differ between M1 and M2 macrophages in the aneurysmal wall, suggesting that macrophages differentially regulate ECM degradation depending on their phenotype and activation profile[42]. Another study identified macrophages with osteoclast markers in human aneurysmal aortas, and these osteoclast-like macrophages had higher protease activity than undifferentiated macrophages[97]. Inhibition of osteoclastogenic differentiation by bisphosphonate treatment in an AAA animal model inhibited aneurysm development, supporting the contribution of osteoclast-like macrophages to AAA pathogenesis[97]. Other studies identified previously unsuspected roles for telomerase reverse transcriptase (TERT) in the regulation of macrophage proteolytic activity during AAA formation through the modulation of *Mmp2* expression[98].

Another process related to ECM degradation is macrophage invasiveness and migration within the tissue. A study published in 2017 showed increased formation of podosomes in macrophages infiltrating the aneurysmal wall[99]. Gene and protein levels of Krüppel-like factor (KLF) 5 were increased in macrophages in murine and human AAA[99]. KLF5 promoted podosome formation and macrophage migration by upregulation of *Myo9b* transcription and inhibition of RHOA signalling, and the presence of KLF5 increased the susceptibility to experimental AAA[99]. However, whether podosome formation *per se* is required for KFL5-dependent pro-aneurysmal effects in macrophages is still unclear.

Podosomes
Actin-based structures that dynamically protrude from the plasma membrane, and are recognized as prominent sites of attachment to, and degradation of, the extracellular matrix.

Inflammatory response. In addition to ECM remodelling, inflammation is one of the main features of aneurysm pathophysiology[100]. Macrophages both produce and respond to inflammatory mediators[101–103]. Macrophages in the aneurysmal wall are a major source of the chemokine CXCL1, which attracts and recruits neutrophils that produce IL-6 (REF. 104). As mentioned above, increased IL-6 levels during aneurysm formation promote monocyte differentiation into activated macrophages that secrete CCL2, which in turn promotes more monocyte recruitment into the aorta[33]. Cytokines are crucial mediators of inflammation and important regulators of various immune and nonimmune cells in the aortic wall. Most of the knowledge on the direct roles of cytokines in AAA comes from studies in animals with global deficiency rather than monocyte-selective and macrophage-selective deficiency in the cytokine of interest or in the pathways that lead to cytokine production. Total deletion of components of the canonical inflammasome pathway (*Nlrp3*, *Pycard*, *Casp1*, or *Il1b*)[105,106] or total deletion of *Tnf*[107] or *Il6* (REF. 108) substantially limits AAA development. Conversely, total deletion of *Il10* — which encodes an immunosuppressive cytokine that reduces MMP activation and preserves ECM integrity — substantially aggravates AAA formation[109]. Interestingly, a negative correlation was found between IL-6 and IL-10 production in human AAA explant cultures, and the balance between these two cytokines seems to modulate the immune response in the aortic wall[110].

A better proof of the direct contribution of leukocytes, particularly macrophages, to the inflammatory process in AAA is exemplified in an animal model with myeloid cell-specific deficiency of KLF6. KLF6 is a transcription factor robustly produced in macrophages[111]. Mice heterozygous for systemic *Klf6* deletion, as well as mice with myeloid cell-specific deletion of *Klf6*, have a phenotype of exacerbated AAA, with increased infiltration of macrophages and increased *Il6* expression in the aortic wall[112]. The investigators showed that granulocyte–macrophage colony-stimulating factor (GM-CSF) is a direct target of KLF6, and increased production of GM-CSF by macrophages contributed to AAA development[112].

Regulation of macrophage inflammatory activity by a variety of factors other than chemokines and cytokines can also alter the development of AAA. Both murine and human macrophages infiltrating the aneurysmal aortic wall have high levels of angiopoietin-related protein 2 (ANGPTL2)[113]. Treatment of ANGPTL2-deficient macrophages with exogenous ANGPTL2 increases the expression of pro-inflammatory cytokine genes and *Mmp9* (REF. 113), suggesting that ANGPTL2 regulates macrophage activity in AAA in an autocrine manner. This theory is supported by decreased inflammation as well as decreased *Mmp9* expression and enzyme activity in macrophages from ANGPTL2-deficient mice, along with reduced AAA development, compared with wild-type mice[113]. Another potential mediator of macrophage activity is peroxisome proliferator-activated receptor (PPAR)-δ. PPARδ activation in macrophages attenuated the incidence and severity of AAA in experimental models, concomitantly with a decrease in inflammatory mediators such as CCL2 and IL-1β[114].

A study published in 2017 demonstrated a major role for NOS2 in VSMCs in mediating the development of thoracic aortic aneurysms[115]. The investigators suggested that high NOS2 levels promote elastin degradation and potentially VSMC contraction[115], although the exact pathogenic mechanisms are still unclear. A subset of macrophages expresses high levels of NOS2 (REF. 31), and high NOS2 levels are found in the AAA wall[116]. NOS2 inhibition did not affect aortic dilatation in a mouse model of elastase infusion[117], but significantly limited AAA formation in a more-severe elastase-induced model in rats[118,119]. Moreover, angiotensin II markedly stimulates the accumulation of $NOS2^+$ macrophages in the aorta[120], particularly under conditions of reduced TGFβ signalling[64], and NOS2 production by these macrophages directly contributes to angiotensin II-induced aortic AAA and dissection, in part through enhanced MMP activation and ECM degradation[64].

Tissue healing and repair. In addition to their roles in promoting inflammation and ECM degradation, macrophages are important for tissue remodelling during the healing response. Tissue debris and toxic products such as extracellular haemoglobin[121] that accumulate in the aortic wall during AAA development can be engulfed by macrophages[42]. Macrophage differentiation towards the HA-mac phenotype is associated with enhanced haemoglobin uptake and increased secretion of the anti-inflammatory cytokine IL-10 (REF. 15), pointing to a protective role of some macrophage subsets during AAA, mediated by haemoglobin clearance, regulation of oxidative stress, and resolution of the inflammatory response.

Of note, despite their function in the clearance of toxic products, macrophages can generate large amounts of reactive oxygen species. However, the effect of oxidative stress on macrophage activity during AAA seems to be more complex than initially anticipated. For example, the protection against AAA formation observed in thrombomodulin-deficient mice was partly attributed to decreased generation of reactive oxygen species by macrophages[88]. A major source for the generation of reactive oxygen species in macrophages is the NADPH oxidase, which comprises transmembrane and cytosolic subunits, including NCF1 (also known as $p47^{phox}$) and NOX2 (also known as gp91)[122]. Global deficiency of NCF1 in $Apoe^{-/-}$ mice decreased the incidence of angiotensin II-induced AAA, and was associated with decreased generation of reactive oxygen species and reduced aortic macrophage infiltration into the AAA wall compared with NCF1-proficient $Apoe^{-/-}$ mice[123]. By contrast, both systemic deficiency of NOX2 and *Nox2*-specific deletion in bone-marrow-derived cells under a $Ldlr^{-/-}$ background reduced the level of reactive oxygen species in AAA lesions, but unexpectedly aggravated AAA and promoted an M1 phenotype in macrophages, with increased production of IL-1β and MMP9, compared with NOX2 proficient $Ldlr^{-/-}$ mice[124].

Canonical inflammasome pathway
Innate immune system receptors/sensors that promote the maturation of IL-1β through the activation of caspase 1.

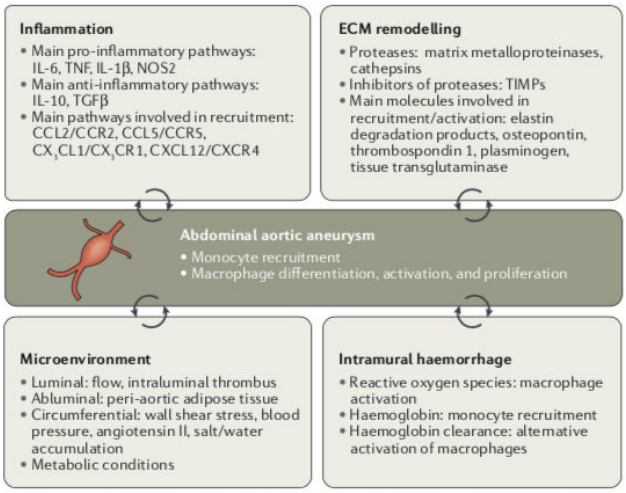


Figure 2 | **Role of macrophages in AAA and main factors modulating macrophage activity.** Macrophages accumulating in the aneurysmal aortic wall have crucial roles in the pathogenesis of abdominal aortic aneurysm (AAA) and its complications through their effects in extracellular matrix (ECM) remodelling, promotion and resolution of inflammation, and their involvement in various aspects of the tissue healing response. In turn, monocyte and macrophage recruitment, and macrophage accumulation, proliferation, and activation are modulated by chemokines and cytokines produced in response to tissue injury, by products of ECM degradation, and by microenvironmental conditions (such as blood flow, circumferential stress, thrombus, and peri-aortic fat). CCL, CC-chemokine ligand; CCR, CC-chemokine receptor; CX_3CL1, CX_3C-chemokine ligand 1; CX_3CR1, CX_3C-chemokine receptor 1; CXCR4, CXC chemokine receptor type 4; IL, interleukin; NOS2, nitric oxide synthase 2; TGFβ, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases; TNF, tumour necrosis factor.

A study published in 2016 suggested a role for NOX2 in promoting production of high-mobility group protein B1 (HMGB1) in macrophages, which was associated with overactivation of the adaptive T_H17 immune response and aggravation of AAA in the experimental mouse model of elastase perfusion[125]. Therefore, the role of macrophage-dependent oxidative stress in AAA needs further investigation.

To summarize, macrophages have both pathogenic and protective functions in AAA pathophysiology through their involvement in ECM remodelling, inflammation, and oxidative stress (FIG. 2). Regulation of these functions is complex and might change during the different stages of disease development and progression, and is influenced by the local microenvironment. In addition to the macrophage roles described above, we believe that other macrophage functions are worth investigating in the context of AAA pathogenesis. For instance, macrophages are involved in the clearance of apoptotic debris[126]. Impaired efferocytosis profoundly alters the development and progression of atherosclerosis[127,128] and ischaemic cardiovascular diseases[129,130], and efferocytosis is probably highly relevant to tissue remodelling and repair in the context of AAA. $CXCR3^+$ macrophages that produce coagulation factor XIIIa accumulate in the adventitia of human AAAs in a CXCL10-dependent manner[89]. We believe that $CXCR3^+$ macrophages contribute to matrix crosslinking and to limiting aneurysmal expansion of the aortic wall. $CXCR3^+$ macrophages can also co-express CD163 (REF. 49), suggesting additional roles in haemorrhage clearance and induction of an anti-inflammatory environment. A study published in 2016 demonstrated the contribution of perivascular macrophages to vessel permeability in nonpathological conditions[131]. Wall thickening during AAA is often associated with the occurrence of microdissections, and to hypothesize a role for adventitial macrophages in regulating vascular permeability during AAA is tempting. Finally, skin macrophages regulate salt-dependent volume retention through vascular endothelial growth factor C (VEGFC)-dependent regulation of the lymph capillary network[132]. Whether aortic wall macrophages also have such a role is still unknown and merits further exploration, given the proposed role of interstitial fluid pressure regulation in the pathogenesis of aortic aneurysm and the susceptibility to aortic dissection[133].

Potential translational applications

Monocytes and macrophages as diagnostic and prognostic biomarkers of AAA. The role of monocytes and macrophages in the development and complications of AAA suggests interesting perspectives for translational applications (BOX 3). Variations in circulating monocyte subsets during AAA, as well as changes in monocyte activation status[14,16,89], suggest that these parameters could be useful as noninvasive biomarkers. Nevertheless, further studies are required to address the correlation between these variations and the characteristics of the aneurysm, such as aneurysm morphology, size, or risk of rupture. Moreover, the specificity of these variations should be explored, because the phenotype and activation status of circulating blood monocytes can be altered in a wide range of diseases, including cardiovascular and inflammatory pathologies[7,8,12].

In addition to their potential use as circulating biomarkers, experimental studies indicate that macrophages could be used as imaging targets. Macrophage activity can be tracked with MRI by detecting phagocytosis of ultrasmall superparamagnetic iron oxide particles (USPIO). The use of this technique in experimental models of AAA showed colocalization of USPIO with phagocytic cells in the adventitia[36], suggesting a potential utility of this technique in assessing the effect of macrophage-dependent processes on the development and progression of AAA *in vivo*. Nevertheless, AAA surrounding tissues, such as the liver, can also uptake USPIO, which can create a challenge to the acquisition of correct and reliable imaging of the aorta *in vivo*. Furthermore, USPIO turnover in aortic macrophages needs to be determined before any potential use of these particles in multiple serial imaging of the inflammatory response during the progression of AAA.

Another targeted approach is PET–CT imaging with ^{18}F-labelled crosslinked iron oxide (CLIO) nanoparticles[62]. This technique was used in *Apoe*$^{-/-}$ mice infused with angiotensin II, and *ex vivo* analysis of the aortas by flow cytometry concluded that 90% of the PET

High-mobility group protein B1
Nonhistone nuclear protein that regulates gene expression; can be translocated to the cytosol and secreted as a pro-inflammatory mediator through activation of Toll-like receptors or advanced glycosylation end-products receptors.

Matrix crosslinking
Process by which chemical bonds are formed between adjacent molecules composing the matrix.

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Somatostatin receptors
G-protein-coupled receptors that are expressed in various tissues including the central nervous system, immune system, and gastrointestinal tissues. Somatostatin receptor type 2 is highly expressed on macrophages that accumulate in inflammatory tissues.

signal detected in aneurysms was due to uptake of nanoparticles by monocytes and macrophages[62], supporting the reliability of the method for macrophage quantification in the aortic wall *in vivo*. Early PET–CT imaging revealed that the signal was increased in aneurysms that grew over time compared with aneurysms that did not increase in diameter[62]. As the doses of CLIO nanoparticles used in experimental models are lower than the currently approved clinical doses, the results are highly encouraging for translational applications.

^{18}F-fluorodeoxyglucose (FDG) is a marker that accumulates in cells with high metabolic activity, which led researchers to consider ^{18}F-FDG as a potential marker of vessel wall inflammation[134]. However, only a few small clinical studies have addressed the usefulness of ^{18}F-FDG PET–CT imaging in the context of AAA. A review comparing the results of six clinical studies revealed heterogeneous results on the association between ^{18}F-FDG uptake and AAA growth[135]. ^{18}F-FDG uptake can be higher in the presence of intraluminal thrombus and in AAA regions subjected to high mechanical stress[136]. One study on USPIO-MRI showed that AAA growth was higher in patients with focal USPIO uptake in the aneurysmal wall than in patients with diffuse or no USPIO uptake[137]. Another study compared ^{18}F-FDG PET–CT imaging with USPIO-enhanced MRI in the same cohort of patients[138]. Although both ^{18}F-FDG PET–CT and USPIO-MRI uptake detected vascular inflammation in association with AAA, the particle distribution was distinct, with ^{18}F-FDG activity mainly detected in the shoulder region, whereas USPIO uptake was more apparent in the main body of the aneurysm[138]. These results suggest that the two techniques are complementary in the characterization of AAAs, with ^{18}F-FDG uptake reflecting glycolytic activity and potentially M1-like inflammatory pathogenic properties and USPIO detection more probably reflecting phagocytic activity and potentially a better indicator of the reparative function of macrophages. Given the complexity of macrophage roles in the pathogenesis of AAA, further investigation will be useful to validate the use of these imaging techniques to predict AAA progression or risk of rupture before their potential use in medical practice.

In the near future, other imaging tools that better identify macrophages will be available. For example, PET tracers such as DOTATATE, which can be labelled with either ^{68}Ga or ^{64}Cu, have macrophage-binding properties owing to a specific binding affinity for somatostatin receptors[139]. Studies in animal models demonstrated the uptake of ^{68}Ga-DOTATATE in atherosclerotic plaques[139], and a PET–CT clinical study in patients with cancer found an association between increased ^{68}Ga-DOTATATE uptake in large arteries and cardiovascular risk factors[140]. Interestingly, a PET–MRI study in patients with carotid artery plaques showed a correlation between *Cd163* expression and ^{64}Cu-DOTATATE uptake, suggesting a selective detection of alternatively-activated macrophages by DOTATATE[141]. In 2017, PET imaging with ^{18}F-Macroflor — a small-size, renally-excreted, polyglucose nanoparticle that has high binding affinity for macrophages — was used successfully to detect tissue-resident macrophages or newly-infiltrated macrophages in several experimental models of atherosclerosis and myocardial infarction[142]. No study has been published on the use of ^{64}Cu-DOTATATE or ^{18}F-Macroflor for imaging AAA. We believe that PET–MRI with ^{64}Cu-DOTATATE or ^{18}F-Macroflor warrant further investigation in this context.

Monocytes and macrophages as therapeutic targets in AAA. Several potential strategies are available to target monocyte and/or macrophage recruitment, accumulation, and activation during the progression of AAA, although most of the approaches lack selectivity for the monocyte–macrophage lineage. Inhibition of chemokine axes has shown efficacy in limiting monocyte recruitment in animal models of AAA, such as blockade of either CXCL12/CXCR4 or CCL2/CCR2 signalling pathways[61,63]. Further studies showed that CCL2/CCR2-specific inhibition in leukocytes or in bone-marrow-derived cells protected against aneurysm formation[69,143]. By contrast, global CCL2 deficiency or systemic CCL2 blockade was less protective or even worsened aneurysm formation, respectively[69,143]. The reasons for these unexpected findings are unclear. Nevertheless, the data suggest that future research should be oriented to test drugs that inhibit the CCL2/CCR2 pathway in specific cell subsets to optimize therapeutic efficiency and to limit adverse effects. For example, targeting CCR2 degradation in circulating monocytes with small interfering RNA nanoparticles prevented monocyte accumulation at sites of inflammation[144]. This treatment has been

Box 3 | Targeting monocytes and macrophages in AAA

Blood monocytes and macrophages can be used as biomarkers and imaging targets, with potential diagnostic and/or prognostic value in abdominal aortic aneurysm (AAA), or as therapeutic targets to treat and/or limit AAA growth and its complications.

Biomarkers for diagnosis and progression of AAA

- Analysis of blood monocytes:
  - Subset distribution
  - Gene and protein expression profiles
- Imaging
  - USPIO-MRI, myeloperoxidase-MRI
  - ^{18}F-labelled crosslinked iron oxide (CLIO) nanoparticles-PET–CT
  - ^{18}F-fluorodeoxyglucose-PET–CT
  - DOTATATE-PET or PET–MRI
  - ^{18}F-Macroflor-PET or PET–MRI

Therapeutic targets

- Modulation of cytokine and chemokine pathways
  - Individual blockade of chemokine pathways, such as CCL2/CCR2, CCL5/CCR5, and CXCL12/CXCR4
  - Broad chemokine blockade with evasins
  - Blockade of cytokines, such as interleukin (IL)-1β, IL-6, or tumour necrosis factor
- Modulation of macrophage phenotype and activity
  - Immunotherapy: promotion of M2 macrophages, mesenchymal stem cells, supplementation with regulatory T cells, low-dose IL-2 to enhance endogenous regulatory T cells
  - Resolvins
  - Inhibition of nitric oxide synthase 2
  - Bisphosphonate
  - Activator of peroxisome proliferator-activated receptor-δ, glycolysis inhibitors

tested in several cardiovascular disease settings including atherosclerosis, myocardial infarction, and myocarditis, and has demonstrated beneficial effects[144,145]. Testing the same therapeutic strategy in the context of AAA would be interesting. The protective effect of other drugs on AAA has also been linked to modulation of the CCR2 pathway. For example, administration of everolimus, an immunosuppressive drug mostly used in drug-eluting stents, protected mice against angiotensin II-induced aortic dilatation through a decrease in the number of bone-marrow monocytes and circulating $CCR2^+$ monocytes[17].

In addition to CCL2/CCR2, treatments targeting the CCL5/CCR5 axis might also be useful. Treatment of mice with MKEY, an inhibitor of CXCL4–CCL5 heterodimer formation, limited AAA enlargement and was associated with a reduction of mural macrophage infiltration and elastin degradation[70]. MKEY treatment suppressed leukocyte migration to the aorta without affecting leukocyte migration to lymphoid organs such as lymph nodes and spleen[70]. Further investigations are required to substantiate the feasibility and safety of this approach, but these results are encouraging for potential translational applications in AAA. Indeed, some CCR5-antagonist drugs are already approved and are currently used in medical practice in the setting of HIV infection, and several clinical studies of CCL5/CCR5 antagonists have been completed or are ongoing in other disease settings[146,147]. Of note, chemokine axes are highly redundant. The development of evasins (chemokine-binding proteins) with broad C-binding, CC-binding, and CXC-binding properties[148] might enable a better and more efficient targeting of monocyte and macrophage recruitment in AAA.

Targeting cytokines is not completely selective for monocytes and macrophages, but this approach merits consideration in translational approaches. Tocilizumab is a humanized, monoclonal antibody specific for the IL-6 receptor that has proven efficacy in the treatment of rheumatoid arthritis[149]. IL-6 is an important regulator of inflammation and macrophage activation in AAA[33], and Mendelian randomization studies provided strong evidence for the involvement of the IL-6 receptor pathway in human AAA[150] and human coronary artery disease[151,152]. Therefore, we believe that testing tocilizumab or other inhibitors of the IL-6 pathway (including IL-6 trans-signalling) in the context of AAA is worth exploring. Blockade of IL-1β has shown efficacy in experimental models of AAA[106]. However, a randomized, double-blind, placebo-controlled, clinical trial in patients with infrarenal AAAs of 40–50 mm showed lack of efficacy of the monoclonal anti-IL-1β antibody ACZ885 (canakinumab) on aneurysmal growth rate ($n=31$) compared with placebo ($n=33$)[153]. Although these results do not exclude the involvement of the IL-1 pathway in AAA (the IL-1 receptor can be activated by ligands other than IL-1β), the lack of efficiency of canakinumab should prompt us to reconsider our understanding of IL-1β in the pathogenesis of AAA. Of note, Mendelian randomization studies did not confirm the causality between the IL-1 pathway and the occurrence of cardiovascular diseases, including AAA[154]. However, the study in AAA addressed the relationship between genetic variants of *IL-1RN* and cardiovascular diseases[154]; therefore, drawing valid conclusions about the causality of the individual IL-1α or IL-1β cytokines in disease development is not possible. Another potential explanation for the disappointing clinical results of IL-1β inhibition is that anti-IL-1β therapy might be useful only in a subset of patients with AAA with a high inflammatory burden. Combining state-of-the-art diagnostic approaches to detect AAA with an active inflammatory component (see above) with a more-targeted, anti-inflammatory therapy might prove useful in improving the management of patients with AAA. Another example of such a combined diagnostic and therapeutic approach is the detection of increased uptake of ^{18}F-FDG in AAA, indicative of high glycolytic activity, followed by the administration of a glycolysis inhibitor to limit macrophage activation[155]. This approach has proven to be successful in animal models of AAA[155], and might warrant consideration for future translation into the human setting.

Modulation of macrophage phenotype and activity is another therapeutic option. Experimentally, intravenous injection of M2 macrophages had a protective effect on calcium chloride-induced aneurysm, as demonstrated by improved survival, decreased aortic dilatation, and elastin preservation[44]. Therefore, therapeutic strategies or drugs with known effect on macrophage polarization might be of interest. For example, D-series resolvins increase the number of M2 macrophages and decrease the number of M1 macrophages, and administration of D-series resolvins either before or after induction of experimental AAA halted disease development and progression[156]. In experimental models of AAA, treatment with mesenchymal stem cells reduced pathogenic NOX2 activation in macrophages, although the underlying mechanisms remain unclear[125]. Regulatory T (T_{reg}) cells control the inflammatory response in AAA[109]. Several studies showed that administration of T_{reg} cells or endogenous induction of T_{reg} cells with IL-2 had protective effects against aneurysm development, which were associated with reduced macrophage infiltration in the aorta[157–159]. *In vitro* experiments further showed that T_{reg} cells induced a decrease in macrophage CCL2 secretion and protease expression and activity, in part through the induction of IL-10 production[158]. In addition, T_{reg} cells downregulated M1-associated genes and upregulated M2-associated genes in macrophages[159], making T_{reg} cells attractive candidates for immunotherapy in AAA.

Finally, systemic treatments targeting macrophage polarization have provided encouraging results, but advances in the understanding of the effect of biomaterials on macrophage phenotype should offer new perspectives[160]. Several studies revealed a previously unappreciated effect of roughness, size, shape, and chemical composition of biomaterials on macrophage phenotype[160]. These findings could offer new opportunities to modulate M1/M2 polarization locally in order to limit aneurysm expansion.

Evasins
Class of chemokine-binding proteins, initially isolated from salivary glands of ticks, that can inhibit chemokine binding to their receptors, and might have either a narrow or a broad binding spectrum.

D-series resolvins
Family of mediators derived from omega-3 essential fatty acid precursors; involved in the resolution phase of inflammation.

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Future research

On the basis of findings from experimental and clinical studies, several future directions for fundamental and translational research can be suggested. Even if different subsets of monocytes and macrophages have been identified, their distinct origins, roles, and functions seem more complex than previously anticipated, and several aspects remain to be elucidated. Are specific monocyte subsets wired to differentiate into specific macrophage subsets? Or can monocytes differentiate into any macrophages subset depending on the local microenvironment? What is the precise role in AAA of recently identified subsets such as SatM monocytes or vascular tissue-resident macrophages? Are the various subsets

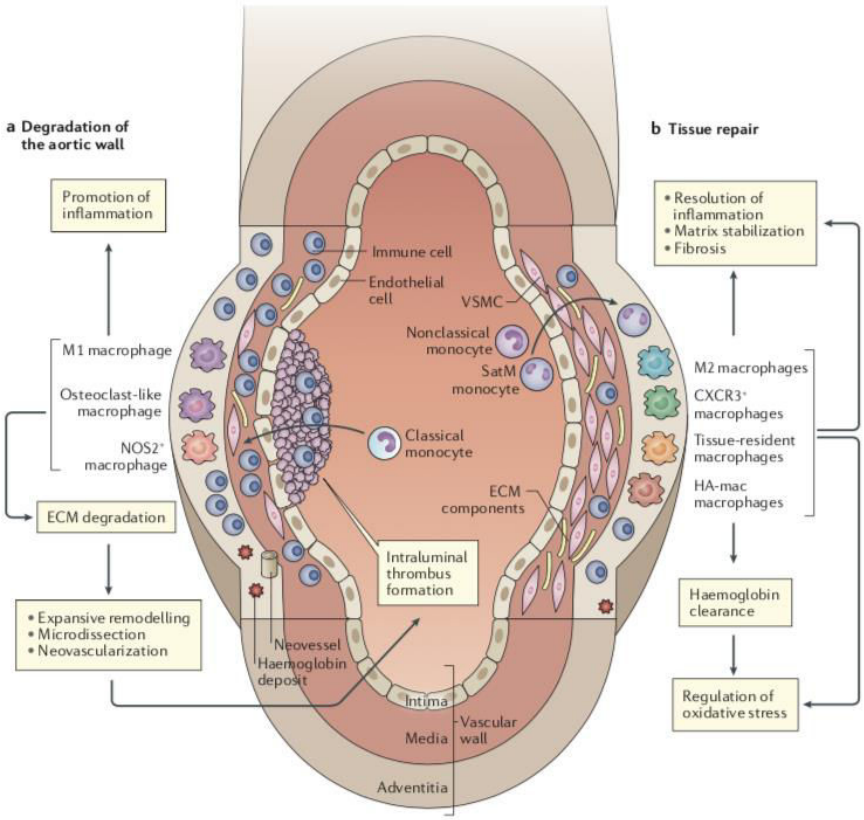

Figure 3 | **Hypothesized roles of monocyte and macrophage subsets in AAA pathogenesis. a** | Abdominal aortic aneurysm (AAA) lesions are characterized by extracellular matrix (ECM) degradation, loss of vascular smooth muscle cells (VSMCs), and immune cell infiltration. Classical monocytes infiltrate all layers of the vascular wall. Monocyte differentiation into several types of macrophages, including M1 macrophages, nitric oxide synthase 2 ($NOS2)^+$ macrophages, and osteoclast-like macrophages is associated with enhanced production of pro-inflammatory mediators and matrix-degrading proteases, which weaken the aortic wall and promote expansive aortic remodelling. Eventually, this process favours microdissections and intramural haemorrhage, neoangiogenesis, and intraluminal thrombus formation, and might further enhance immune cell infiltration and activation. If these processes are not contained, they might ultimately promote aortic wall rupture. **b** | The development and progression of AAA can be delayed through compensatory mechanisms that limit AAA expansion and maintain vascular integrity. M2 macrophages can contribute to the resolution of inflammation and promotion of tissue healing owing to their capacity to secrete anti-inflammatory mediators and profibrotic factors. HA-mac macrophages are involved in haemoglobin clearance, exert anti-inflammatory actions, and regulate oxidative stress, and can contribute to aortic tissue repair. Other macrophage subsets such as CXC-chemokine receptor type 3 $(CXCR3)^+$ macrophages can limit expansive aortic wall remodelling by producing transglutaminase factor XIIIa. Nonclassical monocytes can give rise to M2, haemorrhage-associated (HA-mac), or $CXCR3^+$ macrophages, but all these tissue healing-associated macrophages might also arise from other monocyte subsets or from locally differentiated tissue-resident, adventitial macrophages. Other monocyte subsets, such as segregated-nucleus-containing atypical monocytes (SatM), might be involved in tissue healing and repair through their profibrotic properties.

directly representative of stages of disease development and progression? Do subsets closely correlate with clinical outcomes? Could the different subsets be used in daily practice as diagnostic or prognostic tools, or as therapeutic targets?

Epidemiological studies have highlighted the association between AAA and risk factors including age, male sex, smoking, hypertension, and low HDL-cholesterol levels[1]. Interestingly, these factors can influence monocyte and macrophage activity and function[30,161–166]. Additional research is required to explore the molecular pathways that might link these risk factors with the contribution of monocytes and macrophages to AAA. The development of experimental models of AAA over the past decade has offered great opportunities to study the role of monocytes and macrophages in AAA pathogenesis. However, none of these models perfectly reproduces the human features of AAA, highlighting a real need to develop new and more relevant models[167].

Conclusions

Several experimental and clinical studies demonstrate a role of monocytes and macrophages in all stages of AAA formation, from initial development to the occurrence of microdissections and rupture (FIG. 3). In the past decade, we witnessed an impressive advance in our understanding of the cellular and molecular mechanisms involved in monocyte mobilization and recruitment to sites of vascular injury, and the mechanisms that control macrophage differentiation, proliferation, activation, and phenotype switch. This knowledge is now backed by the development of state-of-the-art imaging techniques to detect and track monocyte and macrophage accumulation and activation *in vivo*, the development of new, anti-inflammatory therapies, as well as additional technological improvements that will enable selective manipulation of various macrophage functions *in vivo*. We truly believe that the combination of such innovative approaches will improve the stratification and management of patients with AAA.

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Annexe 3: Micro-RNAs in abdominal aortic aneurysms: insights from animal models and relevance to human disease

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REVIEW

Micro-RNAs in abdominal aortic aneurysms: insights from animal models and relevance to human disease

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Abstract Abdominal aortic aneurysm (AAA) is a major health concern and may be associated with high rates of mortality linked to acute complications. Diagnosis and treatment are, respectively, based on imaging and surgical techniques. Drug-based therapies are still mostly ineffective, which highlight a real unmet need. Major pathophysiological mechanisms leading to aneurysm formation involve inflammatory processes, degradation of the extracellular matrix, and loss of smooth muscle cells. However, the precise cellular and molecular pathways are still poorly understood. Recently, microRNAs have emerged as major intracellular players in a wide range of biological processes, and their stability in extracellular medium within microvesicles has led to propose them as mediators of intercellular crosstalk and as potential biomarkers and therapeutic targets in a variety of disease settings. To date, several studies have been performed to address the involvement of micro-RNAs (miRs) in aneurysm formation and complications. Here, we discuss the roles and implications of miRs in animal models and their relevance to human AAA.

Keywords Abdominal aortic aneurysm • Micro-RNAs • Human studies • Animal models

1. Introduction

Abdominal aortic aneurysm (AAA) is defined as a focal dilatation of the aorta. Affecting up to 8% of men[1] and 1.3% of women over 65 years old,[2] the disease is often asymptomatic but may become life-threatening, especially when revealed by an acute rupture.[3] Epidemiological studies highlight the frequent association of AAA with atherosclerosis and some corresponding risk factors.[1,4,5] However, the mechanisms underlying AAA formation and progression may differ, particularly with regard to the degree of vascular remodelling in the media layer, and are not completely understood. AAA formation results from a complex process involving vascular and immune cell subsets.[6] Pathological features include adventitial and medial inflammatory cell infiltration associated with a degradation of extracellular matrix (ECM) components, including elastin and collagen, by an array of proteases such as matrix metalloproteinases (MMPs). Impairment of vascular smooth muscle cell (VSMC) homeostasis favours their (de)differentiation to a secretory phenotype with alteration of migratory, proliferative, and survival functions, which may significantly impact vascular remodelling and weaken the aortic wall. Additional alterations of endothelial cell function and formation of an intraluminal thrombus most probably contribute to pathological aortic wall remodelling and disease progression.

Over the past decades, micro-RNAs (miRs) have emerged as major post-transcriptional regulators of gene expression[7] and have been shown to play key roles in vascular biology and cardiovascular diseases.[8–10] In this context, clinical and experimental studies have been performed to better understand the implications of miRs in AAA pathophysiology.

The aim of our review is to discuss the interest, limits, and relevance of models to investigate the roles of miRs in AAA.

2. Human studies

2.1 Clinical relevance

The treatment of AAA is currently based on surgical intervention as no drug has proved yet enough benefit. In practice, the decision to treat relies on a balance between the risk of progression or rupture and the operative risks.[4,5] However, despite the identification of some risk factors (e.g. large aneurysm diameter, high growth rate, association with female gender, smoking, or hypertension), the risk of progression

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and rupture can still be difficult to predict. While some biomarkers have been reported to be associated with AAA, none of them has proved enough sensitivity or specificity to be used in daily medical practice.[11,12] Thus, a real need emerged to develop new tools and help clinicians evaluate the risk of progression, rupture, and post-therapeutic outcomes.

2.2 miRs as potential biomarkers of AAA

In that context, several clinical studies have been set up to identify new biomarkers of AAA (*Table 1*). The first landmark supporting the idea that miRs could reflect the disease was the observation of a differential miR expression in aneurysmal tissue compared with intact aortic samples. Indeed, some were found up-regulated in aneurysmal tissue (miR-21,[15,16,22] miR-205, 378,[16] and miR-126, 20a, 27a, 92a, 221, 222, let-7f, 155, 146a, 223, 124a),[22] others down-regulated (miR-133a, 331-3p, 30c-2*, 204,[13] 24, 27b),[21] whereas some (miR-133b, 29b) appeared either increased or decreased.[13,14,16,22] Note that the differentially expressed miRs substantially varied between studies. In a few cases, a similar variation in expression was observed for miR-21 and miR-155 in aneurysmal aortic tissue compared with their proper controls.[15,16,18,22] In contrast, divergent results were found for miR-29b and miR-133b,[13,14,16,22] which could be partly explained by differences in methodologies used, including patient population, stage of the disease, and technical approaches. While most of the above studies compared miR expression in the aorta between patients with or without AAA, one study investigated miR expression in aortic samples exclusively from patients with AAA.[18] Interestingly, the authors found that the composition and the expression of miRs are different between aortic tissue obtained from the body (at the site of maximum AAA dilatation) and the neck (defined as macroscopically non-dilated aorta), revealing a variation in miR expression within the aneurysmal tissue itself.

In contrast, some investigators studied miR expression in some specific structures present in AAA such as adventitial tertiary lymphoid organs (ATLOs).[20] As ATLOs are mainly composed of immune cell subsets,[30] it is not surprising that the authors observed a differential miR expression in ATLOs compared with VSMCs from non-aneurysmal aorta. Although ATLOs appear to play a role in atherosclerosis and AAA development, they are not systematically observed in atherosclerotic and aneurysmal tissues.[20,31] Hence, miR expression could reflect the presence of ATLOs and inflammatory processes occurring within the adventitial layer during AAA. Besides, these results suggest that cell-specific miR expression should be analysed in order to fully understand their role. In another study, some authors addressed this question and found a differential expression of miR-516a-5p and miR-1260 in VSMCs cultured from AAA tissue when compared with VSMCs from non-aneurysmal aortas.[17]

While the observation of a deregulated miR expression profile in aortic tissue and specific cell subsets during AAA offers perspective to identify new biomarkers of the disease, their potential use in clinical practice is hardly possible using aortic samples as they can only be collected by invasive procedures. The discovery of circulating miRs in different biological fluids has led to propose them as non-invasive biomarkers in a wide range of pathologies including cardiovascular diseases.[32] Interestingly, a differential miR expression was observed between patients with AAA and controls, both in whole blood[19] and in plasma.[19,21,23,24]

Some investigators also found a differential miR expression in plasma (miR-15a-3p, 30a-5p) and in serum (miR-155) between patients with AAA and those with peripheral arterial disease (PAD),[18,20] suggesting that circulating miRs may display distinct patterns in AAA compared with other cardiovascular diseases. However, in another study investigating other miRs, no difference in miR profile could be detected between patients with AAA and PAD,[19] suggesting that changes in the expression of some miRs could be linked to a common pathological process such as atherosclerosis. The identification of similar miR patterns in distinct cardiovascular diseases underlines the need to test their specificity for a potential use in medical practice. Addressing this question in a cohort of 120 individuals, some investigators found that for three miRs (miR-191-3p, 455-3p, and 1281) investigated, the sensitivity to diagnose AAA varied from 76.7 to 95% and the specificity from 96.7 to 100%.[23] Even if further investigations are required on larger cohorts, the results are encouraging and may lead to the validation of reliable biomarkers of AAA. Furthermore, circulating miRs could be potential biomarkers of AAA growth, as suggested by the association between aortic diameter and plasma levels of miR-340, 20a, 148a, 125b, and 195.[24]

However, only a few studies identified the same disease-related miRs in peripheral blood and results were heterogeneous, as demonstrated by divergent circulating levels for miR-155[18,22] and miR-29b.[19,22] The observed difference could be partly explained by the nature of the blood sample used, as the expression of some miRs can vary between plasma and serum.[33]

Additional points need also to be more deeply investigated. First, the origin of circulating miRs is not totally elucidated. According to the current knowledge, they could derive from cellular degradation and/or be released through various modes of vesiculation (e.g. microparticles or exosomes).[34] Besides, circulating miRs measured in blood could come from a wide range of tissues and cell subsets. Even if some miRs misexpressed in aortic samples were also deregulated in the circulation such as miR-20a,[22,24] miR-155,[18,22] miR-29b,[14,22] and miR-124a, -223, -126, and -146a,[22] the pathways responsible for their deregulation in aortic samples and blood need to be further investigated and potential links identified. Also, the role of differentially expressed miRs remains to be established in order to better appreciate their relevance as biomarkers or causal factors in AAA pathophysiology.

2.3 Roles of miRs in the pathophysiology of human AAA

Intracellular miRs mainly regulate gene expression through binding to their target messenger RNA, inhibiting their translation, or inducing their degradation.[7] Several studies identified predicted targets of deregulated miRs using bioinformatics approach and many of them appear to be involved in biological pathways relevant to AAA development such as inflammation, ECM remodelling, apoptosis, or cell proliferation.[13,17,19]

Some investigators provided direct *in vitro* experimental evidence for the regulation of the theoretical targets by the identified miRs, and further addressed gene expression or protein levels of the potential miR targets *in vivo*. Interestingly, a correlation was found between some deregulated miRs in aneurysmal aorta and expression of their putative targets involved in inflammation, as demonstrated by the opposite variation between miR-155 and cytotoxic T-lymphocyte-associated protein 4 expression,[18] miR-24 and chitinase 3-like 1 (Chi3l1),[21] miR-146a, 124, 223, 126 and inflammatory cytokines,[22] or miR-516-5p and levels of interleukin-3 and vascular endothelial growth factor A secreted by VSMCs.[17] Besides, several studies observed a decrease of some miRs (miR-1260, 29b) in aneurysmal aorta, in association with significant changes in protein or gene expression of ECM

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Table 1 Summary of human studies on miRs in aortic aneurysms

Population		Samples	Main miRs identified	References
AAA	Screening study – Five patients with infrarenal AAA undergoing elective surgical repair – Five controls from cadaveric donors Validation study – Thirty-six patients with AAA undergoing surgical repair (25 elective and 11 ruptured) – Seven controls from cadaveric donors	Aorta: full thickness aortic wall	– Differential miR expression between patients with AAA and controls: → Decreased: miR-133b, 133a, 331-3p, 30c-2*, 204	Pahl *et al.*[13]
	– Fifteen patients with infrarenal large AAA (59–68 mm) undergoing surgical repair – Five controls from organ donors	Aorta: full thickness aortic wall	– Differential miR expression between patients with AAA and controls: → Decreased: miR-29b	Maegdefessel *et al.*[14]
	– Thirteen patients with large AAA (57–68 mm) undergoing surgical repair (eight active smokers and five non-smokers) – Five controls from organ donors	Aorta: full thickness aortic wall	– Differential miR expression between patients with AAA and controls. → Increased: miR-21	Maegdefessel *et al.*[15]
	– Five patients with AAA who underwent surgery – Four controls from patients without AAA	Aorta: formalin-fixed paraffin-embedded sections	– Differential miR expression between patients with AAA and controls. → Increased: miR-205, 21, 133b, 378	Kim *et al.*[16]
	Screening study – Six patients with AAA undergoing surgical open repair – Six patients from men undergoing liver transplantation Validation study – Ten patients with AAA undergoing surgical open repair (six elective AAA and four ruptured AAA) – Ten controls	Aorta: medial wall dissected from luminal thrombus and adventitia, culture of VSMCs	– Differential miR expression between patients with AAA and controls. → Increased: miR-516a-5p → Decreased: miR-1260	Cheuk *et al.*[17]
	– Ten patients with large infrarenal AAA (>50 mm) undergoing surgical open repair	Aorta: full thickness of the aortic wall Body biopsy: at the site of maximum AAA dilatation Neck biopsy: at the site of macroscopically non-dilated AAA neck	– miRs found exclusively in AAA body: miR-96, 9, 105, 33 – miRs found exclusively in AAA neck: miR-189, 151, 154, 154*, 153, 188, 147, 183, 199b – Differential miR expression between AAA body and AAA neck. → Increased: miR-155, 28, 30a-3p, 150, 302b*, 93, 99a	Biros *et al.*[18]
	– Ten patients with large infrarenal AAA (>50 mm) undergoing surgical open repair – Ten patients with PAD as controls	Serum	– miRs found exclusively in patients with AAA, not in controls: miR-220, 10a, 23b – Differential miR levels between patients with AAA and patients with PAD. → Increased: miR-155	
	Initial study – Nineteen patients with infrarenal AAA undergoing surgical open repair – Ten controls from cadaveric donors Validation study – Twenty-two patients with AAA infrarenal undergoing surgical open repair – Seventeen controls from cadaveric donors – Three patients with aorto-occlusive disease	Aorta: full thickness of the aortic wall	– No significant difference observed between patients with AAA and controls	Stather *et al.*[19]
	Initial study – Fifteen patients with large AAA (>54 mm)	Whole blood	– Differential miR level between patients with AAA and controls. → Decreased: miR-15a, 196b	

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Table I *Continued*

Population	Samples	Main miRs identified	References
– Ten healthy controls Validation study – Forty patients with small AAA (30–54 mm) – Forty patients with large AAA (>54 mm) – Forty patients with AAA who had surgical repair (samples collected 6 months after open or endovascular repair) – Forty patients with PAD – Forty healthy controls		– Differential miR level between patients with PAD and controls. → Decreased: miR-let-7e, 15a, 196b → Increased: miR-411	
– Thirty-six patients with small AAA (30–54 mm) – Thirty-six patients with large AAA (>54 mm) – Thirty-five patients who had AAA repair (samples collected 6 months after open or endovascular repair) – Thirty-five patients with PAD – Twenty-eight healthy controls	Plasma	– Differential miR level between patients with AAA and controls. → Decreased: miR-196b but significance not maintained after binary logistic regression	
– Twenty patients with infrarenal AAA undergoing surgical open repair – Fourteen controls from organ donors	Aorta: whole tissue or extraction of areas rich in adventitial tertiary lymphoid organs (ATLOs)	– Differential miR expression in ATLOs compared with VSMCs from non-aneurysmal aorta. → Increased: 489-3p → Decreased: miR-15a-3p, 30a-5p	Spear *et al.*[20]
– Twenty-four patients with AAA requiring surgery (>50 mm or increasing more than 10 mm during the past 6 months) – Eighteen patients with PAD	Plasma	– Differential miR level between patients with AAA and patients with PAD. → Decreased: miR-15a-3p, 30a-5p	
– Twenty-two patients with infrarenal large AAA (52–115 mm) undergoing elective surgical open repair – Fourteen controls from organ donors	Aorta: full thickness aortic wall	– Differential miR expression between patients with AAA and controls. → Decreased: miR-24, 27b	Maegdefessel *et al.*[21]
– Fifty-four patients with small AAA (45–67 mm) – Fifty-one patients with large AAA (69–150 mm) – Twenty-two patients with peripheral vascular occlusive disease (PVOD) – Fifty-two controls	Plasma	– Differential miR level between patients with AAA and controls. → Decreased: miR-24 – Differential miR level between patients with AAA and patients with PVOD. → Decreased: miR-24	
– Thirteen patients with infrarenal AAA undergoing elective surgical open repair – Seven controls from ascending normal aorta from patients undergoing aortic valve replacement surgery	Aorta: full thickness aortic wall	– Differential miR expression between patients with AAA and controls. → Increased: miR-126, 20a, 27a, 92a, 221, 222, let-7f, 21, 155, 146a, 223, 124a, 29b	Kin *et al.*[22]
– Twenty-three patients with AAA – Seventeen patients with coronary artery disease (CAD) – Twelve healthy volunteers	Plasma	– Differential miR level between AAA and controls. → Decreased: miR-124a, 29b, 155, 223, 126, 146a – Differential miR expression in plasma between AAA and CAD. → Decreased: miR-124a, 29b, 155, 223, 21	
Screening study – Ten patients with AAA – Ten healthy controls Validation study – Sixty patients with AAA – Sixty healthy controls	Plasma	– Differential miR level between patients with AAA and controls. → Increased: miR-191-3p, 455-3p, 1281	Zhang *et al.*[23]

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Table I *Continued*

Population		Samples	Main miRs identified	References
	– Thirty-two patients with AAA – Forty-one controls	Plasma	– Inverse association of miR level with the presence of AAA and the aortic diameter: miR-195	Zampetaki *et al.*[24]
TAA	– Thirty patients with ascending TAA + tricuspid aortic valves (TAV) undergoing surgery – Ten controls from ascending non-aneurysmal aorta of heart donors and coronary artery bypass graft patients	Aorta: full thickness aortic wall	– Differential miR expression between patients with TAA + TAV and controls. → Decreased: miRs -1, 21, 29a, 133a, 486-5p	Jones *et al.*[25]
	– Ten patients with ascending TAA + tricuspid aortic valves (TAV) undergoing surgery – Ten controls patients from non-aneurysmal aortas of coronary artery bypass graft patients	Aorta: full thickness aortic wall	– Differential miR expression between patients with TAA + TAV and controls. → Increased: miR-145	Pei *et al.*[26]
	– Ten patients with non-familial non syndromic ascending TAA undergoing surgery – Ten controls from ascending non-aneurysmal aorta of heart transplant recipients	Aorta: full thickness aortic wall	– Differential miR expression between patients with TAA and controls. → 99 differentially expressed miRs identified by microarray	Patuzzo *et al.*[27]
	– Seventy-nine patients with TAA + bicuspid aortic valve (BAV) undergoing surgery – Thirty patients with TAA + tricuspid aortic valve (TAV) undergoing surgery – Thirty-three controls from non-aneurysmal aorta of coronary bypass surgery patients	Aorta: full thickness aortic wall	– Differential miR expression between patients with TAA + BAV, with TAA + TAV and controls. → Increased: miR-29b	Boon *et al.*[28]
	– Twenty-one patients with ascending TAA + bicuspid aortic valves (BAV) undergoing surgery – Twenty-one patients with ascending TAA + tricuspid aortic valve (TAV) undergoing surgery – Ten controls from non-aneurysmal ascending aorta of heart transplant donors or recipients	Aorta: full thickness aortic wall	– Differential miR expression between patients with TAA + BAV and controls. → Decreased: miR-143 → Increased: miR-29a, 145 – Differential miR expression between patients with TAA + TAV and controls. → Decreased: miR-1, 133a, 143 → Increased: miR-21 – Differential miR expression between patients with TAA + TAV and patients with TAA + BAV → Decreased: miR-1 → Increased: miR-21	Ikonomidis *et al.*[29]
		Plasma	– Differential miR level between patients with TAA + BAV and controls. → Decreased: miR-145 → Increased: miR-133a – Differential miR expression between patients with TAA + TAV and controls. → Decreased: miR-21, 29a, 143 → Increased: 133a	

AAA, abdominal aortic aneurysm; BAV, bicuspid aortic valves; CAD, coronary artery disease; PAD, peripheral arterial disease; PVOD, peripheral vascular occlusive disease; TAA, thoracic aortic aneurysm; TAV, tricuspid aortic valves; VSMCs, vascular smooth muscle cells.

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components such as collagen.[14,17] Thus, clinical studies revealed concomitant deregulation of some miRs and their putative targets, some of them being key players in mechanisms involved in AAA formation, including inflammation, fibrosis, or ECM remodelling, suggesting potentially causal roles of miRs in the pathophysiology of human AAA.

2.4 Limits of clinical studies

Clinical studies have highlighted the potential implication of miR-regulated processes in the pathophysiology of AAA. However, comparison of the results among different studies reveals a low overlap of differentially regulated miRs. Thus, several considerations should be taken into account when designing clinical studies in order to minimize potential bias in data collection, analysis, and interpretation.

The first point relates to the population studied and the inclusion criteria. Pathophysiological characteristics including age, sex, the presence of comorbidities, or associated cardiovascular risk factors may impact on miR expression[35–38] and act as confounding elements. Such factors should be clearly identified and controlled for during study design and in the final statistical analysis.

Additional considerations relate to sample collection. First, human aortic tissue can only be collected from patients who undergo a surgical intervention and as a consequence, analysis of aortic miR expression was most often performed on small cohorts. Obtaining proper aortic controls can even be more challenging. Studies used aortic samples from different origin: (i) patients undergoing liver transplantation or surgical valve repair,[17,22] (ii) organ donors,[14,15,20,21] and (iii) cadaveric donors.[13,19] However, although miRs are highly stable in both tissues and biological fluids,[39,40] their stability in postmortem conditions is not well known.[41] While in the majority of studies aortic control samples were taken from the abdominal aorta, one study obtained samples from the ascending aorta.[22] Note that under physiological conditions, thoracic and abdominal aortas are subjected to different haemodynamic loads and differ in histological and biochemical composition and in the embryological origin of their VSMCs.[42,43] In the pathological setting, AAA and thoracic aortic aneurysm (TAA) are characterized by distinct epidemiological patterns and aetiological factors.[44] While a few miRs deregulated in AAA such as miR-21, miR-29b, and miR-133a were also differentially expressed in TAA compared with controls,[14–16,22,25,27–29] specific miRs other than those identified in AAA could be identified in TAA, including differential expression of miR-1, 29a, 143, 145, and 486-5p.[25–27,29] As such, it is highly plausible that the pattern of miR expression may differ between the abdominal and thoracic aorta, both in health and disease. Thus, appropriate control samples should come from the same aortic region. In other studies, some investigators did not collect aortic samples from patients without AAA and made direct comparisons between miRs expressed in the body of AAA and in the neck.[18] The use of the neck as control to study miR expression presents the advantage to be perfectly matched with the samples obtained from the body of the AAA. However, histological analysis revealed that even if biopsies from the neck show relative preservation of intimal and medial structures, the morphology is not completely normal, as demonstrated by inflammatory cells infiltrated in the adventitial layer.[45] As a consequence, it is possible that miR expression in the neck of AAA differ from intact non-aneurysmal aorta.

Compared with aortic tissues, blood samples have the advantage to be easier to collect, allowing to study larger cohorts of patients with AAA. Nevertheless, a circulating miR profile may differ depending on the nature of the sample[33] and can be altered by treatments commonly prescribed to patients with AAA such as anticoagulants, antiplatelets,[46] antidiabetic agents,[47] or cholesterol-lowering drugs.[48] Also, the numerous methods used for miR detection and data normalization can potentially impact on miR profile.[49,50]

As a conclusion, clinical studies on miRs in AAA offer interesting opportunities to explore their potential use as biomarkers and to study their role in AAA pathophysiology. However, the methodology used differed between the studies, making difficult any direct comparison between the results. Besides, clinical studies present several limitations in addressing the precise cellular and molecular effects of miRs in AAA development. First, aortic samples can only be taken from patients who have surgical indication, which means that they have advanced or complicated lesions. This is a major limitation to the study of the role of miRs in the early stages of AAA development. Secondly, while some correlations between miRs and their putative targets have been identified, it is difficult to determine whether miR deregulation has a causal effect on AAA development or on the contrary results from a compensatory mechanism to regulate it. Hence, the need to use animal models, which allow detailed mechanistic approaches to the disease process.

3. Animal studies

3.1 Animal models of AAA used to study miRs

Several experimental models of AAA have been developed over the past decades including genetic, surgical, and chemically induced models in rodents or larger animals.[51–53] Surgical models directly induce mechanical injury within the arterial wall leading to AAA, but require technically difficult procedures and expose the animals to prolonged anaesthesia. In that context, due to their efficiency to induce AAA, their relative technical simplicity, and low cost, studies on miRs have been exclusively performed on chemically induced AAA in rodents (*Table 2*).

Angiotensin II infusion into apolipoprotein E-deficient mice (angiotensin II/apoE$^{-/-}$) has been, to date, the model the most commonly used to study miRs in AAA.[14–16,21,24,54] This model usually induces AAA located in the suprarenal aorta within 28 days and reproduces some of the major features observed in AAA pathophysiology such as medial degradation with ECM remodelling, inflammation, and association with atherosclerosis.[57,58] While hyperlipidaemia increases incidence, it is not required for the induction of AAA. As a consequence, some investigators used angiotensin II infusion in C57BL/6 mice.[28] Another commonly used chemically induced model of AAA is the elastase perfusion model. The technique requires a surgical intervention to introduce a catheter in the infrarenal aorta and perfuse pancreatic porcine elastase for 5–15 min.[59] The perfusion of elastase induces a destruction of medial elastic tissue and inflammation, leading to infrarenal AAA formation within 14 days.[60] At last, some investigators used a third model in rats, consisting in the application of calcium chloride in combination with collagenase on the adventitial layer of the aorta between the renal branches and the iliac bifurcation.[55] Calcium chloride application provokes calcium deposition throughout the media, disruption of ECM, and induction of inflammatory responses, leading to infrarenal AAA development between 2 and 4 weeks.[61]

3.2 Roles of miRs in AAA pathophysiology and as therapeutic tools

The studies performed so far in animal models have provided interesting results. First, differential miR expression between the aneurysmal and control aorta was observed in all the models described

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Table 2 Main miRs identified in animal models of aortic aneurysms and comparison with miRs dysregulated in human

	Animal models	Main miRs dysregulated in animal models validated by qPCR	miRs observed in animal models also dysregulated in patients with aortic aneurysm
AAA	Angiotensin II infusion in apoE$^{-/-}$ mice	– Differential miR expression between aneurysmal whole aortic tissue and controls. → Increased: [15]:miR-21 [24]:miR-195 → Decreased: [14]:miR-29b [21]:miR-24 [54]:miR-26a – Differential miR expression between endothelium of aneurysmal aorta and controls. → Increased: [16]:miR-21, 1, 133b, 207, 378, 1957, 712, 205 → Decreased: [16]:miR-1952, 1249 – Differential miR expression in plasma between mice with AAA and controls. → Decreased: [21]:miR-24	– Differential miR expression between abdominal aneurysmal whole aortic tissue and controls. → Increased: [15]:miR-21 [22]:miR-21, 146a, 29b, 221, 222 [16]:miR-21, 133b → Decreased: [14]:miR-29b [21]:miR-24 [13]:miR-133b – Differential miR level in plasma between patients with AAA and controls. → Decreased: [21]:miR-24 [24]:miR-195 [22]:miR-29b – Differential miR level in plasma between patients with AAA and coronary artery disease. → Decreased: [22]:miR-29b
	Angiotensin II infusion in C57BL/6 mice	Differential miR expression between aneurysmal whole aortic tissue and controls. → Increased: [28]:miR-29b	
	Elastase perfusion in ApoE$^{-/-}$ mice	Differential miR expression between aneurysmal whole aortic tissue and controls. → Increased: [54]:miR-21 → Decreased: [54]:miR-26a	
	Elastase perfusion in C57BL/6 mice	Differential miR expression between aneurysmal whole aortic tissue and controls. → Increased: [15]:miR-21 → Decreased: [14]:miR-29b [21]:miR-24 – Differential miR level in plasma between mice with AAA and controls: → Decreased: [21]:miR-24	
	Calcium chloride and collagenase application in Sprague–Dawley rats	Differential miR expression between aneurysmal whole aortic tissue and controls. → Increased: [55]:miR-19a, 19b, 132, 221	
TAA	Fibulin-4(R/R) knockdown mice	Differential miR expression between aneurysmal aortic tissue and controls. → Increased: miR-29a, 29b, 29c[28]	– Differential miR expression between aneurysmal whole aortic tissue (from patients with TAA + BAV) and controls. → Increased: miR-29a[29]
	Fbn1(C1039G/+) mice	Differential miR expression between aneurysmal whole aortic tissue and controls. → Increased: miR-29b[56]	– Differential miR expression between patients with TAA and controls. → Increased: miR-29b[27]

AAA, abdominal aortic aneurysm; BAV, bicuspid aortic valve; ApoE$^{-/-}$, apolipoprotein E-deficient mice; qPCR, quantitative PCR; TAA, thoracic aortic aneurysm; TAV, tricuspid aortic valve.

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above.[14–16,21,24,28,54,55] Furthermore, several studies reported inverse correlations between miR levels and the expression of their putative targets, involved in key mechanisms of AAA pathophysiology. As an example, miR-29b expression was decreased in aneurysmal aortic wall and negatively correlated with collagen and elastin gene expression,[14] which strongly suggested a role of miR-29b in ECM remodelling. In another study, a decrease of miR-24 was observed concomitantly with an increase of its putative target Chi3l1, pinpointing the potential role of miR-24 in inflammation.[21] Similarly, a decrease of miR-26a in aneurysmal aortic tissue was reported to occur in parallel to an increase of several predicted targets, including genes involved in cell cycling, apoptosis, proliferation, migration, or cytokine production.[54]

While correlation between miR expression and their predicted targets can be studied in both human and animal models, animal studies offer the great possibility to modulate miR expression and study its direct impact on the disease process. miR modulation consists in overexpressing an miR using an miR-mimic or inhibiting its expression with an antago-miR. miR-mimics are double-stranded oligonucleotides in which one of the strand is the mature miR sequence. In the cell, miR biogenesis machinery recognizes the miR-mimic and processes it, delivering the mature miR strand and leading to its overexpression.[62] In contrast, antago-miRs are synthetic single-stranded RNA analogues complementary to miRs, which hybridize specifically with the target miR, inhibiting its effect.[63]

Modulating miR expression allows for better understanding of their roles. Several studies highlighted the potential contribution of miRs to AAA development. As an example, miR-712 and its homologue miR-205 were increased concomitantly with: (i) a decrease of two MMP inhibitors tissue inhibitor of metalloproteinase 3 (TIMP3) and reversion-inducing cysteine-rich protein with kazal motifs (RECK) and (ii) an increase of MMP activity in the abdominal aortic endothelium of angiotensin II-treated apoE$^{-/-}$ mice compared with controls.[16] Silencing miR-712 or miR-205 reversed the down-regulation of TIMP3 and RECK, decreased MMP activity and elastin fragmentation, preventing AAA formation. Hence, miR-712 and miR-205 misexpression seems to play a causal effect in aortic endothelium promoting AAA progression through targeting key molecules involved in ECM remodelling.

Another study found a decrease of miR-24 in AAA tissue compared with non-diseased aorta, in both elastase and angiotensin II/apoE$^{-/-}$ models.[21] In the elastase model, miR-24 inhibition using an intraperitoneal injection of lenti-anti-miR-24 increased aortic dilatation and incidence of rupture, concomitantly with an increase of Chi3l1. In contrast, miR-24 overexpression using a lenti-premiR-24 injection revealed opposite effects on Chi3l1 expression, decreased macrophage infiltration, and attenuated AAA dilatation. Similar results were found in angiotensin II/apoE$^{-/-}$ model, suggesting that miR-24 contributes to AAA development through the regulation of inflammatory processes via targeting Chi3l1.

While the above-mentioned studies revealed a contributing role of some miRs to AAA formation, others have suggested protective or compensatory roles for a few miRs. For example, miR-29b expression was decreased in infrarenal aortic tissue of both elastase and angiotensin II/apoE$^{-/-}$ models, concomitantly with an increase of collagen and elastin.[14] Further inhibition of miR-29b through an anti-miR-29b injection boosted the increase of collagen and elastin gene expression, resulting in a significant reduction of AAA expansion. Another study corroborated this finding and reported a decrease of aortic diameter and enhanced survival after systemic inhibition of miR-29b in angiotensin II/apoE$^{-/-}$ mice.[24] Overexpressing miR-29b through administration of premiR-29b showed opposite effects, increasing AAA development.[14] These results indicate that the decrease of miR-29b observed in aortic tissue may result from a compensatory mechanism to counterregulate AAA expansion. In another study, miR-21 was found increased in AAA tissue of elastase and angiotensin II/apoE$^{-/-}$ models.[15] miR-21 overexpression increased cell proliferation and decreased apoptosis in the aortic wall, associated with a reduction of phosphatase and tensin homologue (PTEN) protein and activation of AKT which led to protection against AAA expansion. In contrast, antago-miR-21 injection increased PTEN expression and promoted AAA growth. Hence, miR-21 up-regulation observed in AAA tissue appears to be a physiological response elicited to counterregulate and prevent AAA expansion.

Thus, several miRs have been identified as causally linked to the development and complication of AAA in animal models, offering the perspective to use miRs as therapeutic tools and targets. Nevertheless, further studies in animal models need to be performed before potential applications can be translated to the clinical arena. First, the sustainability of the biological effect of miR modulation needs to be further investigated as several studies observed a steady decrease in the biological effect during the 28-day follow-up period.[14,15] Secondly, the delivery and uptake of miR modulators need to be well characterized in order to improve selective targeting and limit potential side effects. Analysis of aortic localization and effects of some miR modulators after systemic injection suggested that they were almost exclusively limited to the site of injury in the elastase model.[14,15,21] However, their uptake in the aortic wall in other AAA models needs to be further characterized. Systemic administration of some miR modulators of miR-29b or miR-21 was associated with biological effects in other organs such as the heart, the liver, or the kidney,[14,15,24] which may induce substantial side effects. Hence, local administration could be a step forward to limit undesirable effects. Interestingly, some investigators studied *in vivo* local inhibition of some miRs using an aortic isograft model.[24] The technique consists in harvesting the aorta, incubating it with miR modulators, and then reimplanting it into the carotid artery of recipient mice. While the technique resulted in effective local modulation of miR expression, it cannot be extrapolated for a potential use as a therapeutic tool in patients as it requires aortic tissue removal and reimplantation. In practice, surgical intervention physically excludes the aneurysm using a synthetic prosthesis.[4,5] Thus, investigating local modulation of miR expression through drug administration via the prosthesis would be of great interest. To date, experiments using stent grafts for surgical repair of AAA have been mostly performed on big animals such as dogs and pigs given the larger size of the aorta.[64,65] Nevertheless, graft implantation in smaller animals like rats has been developed[66] and performed in animals with induced AAA.[67] In this context, a recent study addressed the feasibility of local miR modulation using an anti-miR-21-coated stent.[68] To investigate the role of miRs in in-stent restenosis, the authors used a model in which balloon-injured and stented human internal mammary arteries were anastomosed to the abdominal aorta of Rowett nude rats. Interestingly, anti-miR-21–coated stents reduced in-stent restenosis compared with bare metal stents and screening for off-target effects did not reveal any miR-21 expression changes in kidney, liver, heart, or lung. Hence, stent graft implantation in murine models and the development of stents coated with miR modulators offer great perspectives for the testing and translation of miR-based therapeutic strategies to combat AAA.

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3.3 Impact of risk factors on miRs and consequences for AAA development

While in human studies the presence of associated risk factors may potentially confound results interpretation, animal studies offer the advantage of a homogeneous background and the possibility to investigate the contribution of individual risk factors to miR expression and AAA development. Among the risk factors of AAA, smoking is considered as one of the most important.[4,5] As previously mentioned, miR-21 expression is increased in the aneurysmal aortic segments of both elastase and angiotensin II mouse models.[15] Interestingly, subcutaneous nicotine administration during 60 days boosted the increase of miR-21 in both models. The overall results of the study suggested that miR-21 up-regulation was a physiological protective response to prevent AAA expansion.

The impact of ageing has also been addressed. Investigators reported an increase of miR-29b expression in aortic tissue of aged compared with young mice.[28] miR-29b expression is decreased in the aneurysmal aorta of both elastase and angiotensin II/apoE$^{-/-}$ models, and its down-regulation seems to play a protective role on AAA formation.[14] Interestingly, an increase of miR-29b expression was observed in the aneurysmal aorta of aged wild-type mice infused with angiotensin II.[28] Furthermore, inhibition of miR-29b expression in this model prevented the increase of aortic diameter induced by angiotensin II. These results suggest that down-regulation of miR-29b could result from a physiological response to prevent AAA expansion and that ageing could, at least partly, contribute to AAA development through an impairment of that mechanism. Hence, animal models offer the possibility to better understand the direct roles of miRs and the molecular mechanisms that link risk factors identified in epidemiological studies to AAA development.

3.4 miRs in other forms of aortic aneurysm

Aortic aneurysm may also involve the thoracic aorta. TAA develops frequently at younger ages and are often linked to hereditary influence and display distinct pathological features compared with AAA.[44] Only a few studies investigated miR expression in animal models of TAA: fibulin-4$^{R/R}$ knockdown and Fbn1 (C1039G/+) Marfan mouse model.[28,56] miRs differentially expressed within the aneurysmal aortic wall during TAA differed from those identified in AAA, as demonstrated by changes in expression of miR-29a and miR-29c in TAA, whereas no variation was observed in miR-21.[28,56] In contrast to AAA animal models, miR-29b expression was found increased in thoracic aneurysmal aortas,[28,56] and played a causal role as demonstrated by the prevention of early aneurysm development induced after blockade of miR-29b in Marfan mice.[56]

3.5 Relevance of animal models to AAA

Animal models provide interesting opportunities to study miRs in AAA. However, it is necessary to identify the potential confounding factors that may alter miR expression profile and downstream miR effects in each model. For example, hypertension, which develops in response to infusion of angiotensin II,[69] may be associated with modification of miR expression in the aorta[70] and may therefore account, at least in part, for changes of miR profile observed in angiotensin II-infused mice. The use of apoE$^{-/-}$ mice, which develop hypercholesterolaemia and atherosclerosic lesions,[71] may also be a confounding factor, given the potential direct impact of atherosclerotic changes on miR expression profile in the aortic wall.[72] Thus, data reproducibility between different animal models of AAA is worth of evaluation.

A few studies investigated the same miRs in different models of AAA. Interestingly, consistent results of miR expression in the aneurysmal aorta were found between different models for miR-21 and miR-29b,[14–16,54] and their effects on the disease process were reproducible.[14,15] Note that while a decrease of miR-29b was observed in aortic tissue in both elastase and angiotensin II/apoE$^{-/-}$ models,[14] one study reported an increase of its expression in the aorta of 18-month wild-type C57BL/6 mice infused with angiotensin II.[28] However, that difference may be attributed to the impact of ageing on the aorta. The concordance of results between different animal models suggests that the identified miRs are strongly linked to the pathophysiology of AAA, and less to the direct effect of AAA-inducing agents or to the presence of associated disease states such as hypertension, hypercholesterolaemia, or atherosclerosis. Nevertheless, it is possible that divergences between animal models are underestimated due to the fact that inconsistent results among different models in the same study are less likely to be published.

The relevance of animal models to human AAA pathophysiology also needs to be addressed.

The mouse models described above all reproduce some of the human features of AAA such as higher incidence in males than in females. They also mimic some of the histological characteristics of human lesions including medial degradation, ECM remodelling, and inflammatory responses.[57,60,61] However, each model has its own specificities, which may make it differ from the human disease.

Infusion of angiotensin II in ApoE$^{-/-}$ is associated with the development of atherosclerosis, hypertension, and hypercholesterolaemia, which are risk factors frequently identified in human AAA.[4,5] Death related to aortic ruptures can be observed in the model,[73] allowing to study the mechanism leading to this complication. Nevertheless, aneurysm formation tends to occur in the suprarenal aorta, while the most frequent location of AAA in humans is infrarenal. In addition, rupture observed in that animal model is favoured by the rapid development of medial dissection associated with the formation of transmural thrombi and haematomas.[73,74] In human AAA, medial dissection is not common, in contrast to rupture, and thrombi usually appear in the intraluminal part of the aorta.[75] Compared with angiotensin II infusion, elastase perfusion model has the advantage to directly induce AAA in the infrarenal aorta.[59,60] However, incidence of rupture is rare. The calcium chloride model does not lead to thrombus formation or rupture, which are major features of the human disease.

Dissimilarities of each of the models with the human disease may question the translational relevance of the results obtained in mice. Interestingly, some miRs identified in animal studies were also observed to be dysregulated in humans (*Table 2*). This is, for example, the case of miR-21,[15,16,22,54] mir-221, miR-146a, and miR-222,[22,55] miR-24,[21] or miR-205,[16] which were similarly modulated in aneurysmal tissue of mice and humans.

However, the expression of other miRs revealed more heterogeneous results. This was the case for miR-133b, which was increased in endothelial cells of murine aneurysmal aorta,[16] but was reported to be either increased[16] or decreased[13] in human aneurysmal aorta compared with normal aortic tissue. Note that in the animal study, investigators only used the endothelial part of the aorta, while in both human studies the whole wall tissue was processed to extract RNAs. Heterogeneous results were also found for miR-29b, which was decreased in two different animal models,[14] while its expression

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was either decreased[14] or increased[22] in human aneurysmal aorta. miR-195 was increased in the aorta of angiotensin II/apoE$^{-/-}$ mice, but when its circulating level was investigated in human plasma, an inverse association was found between miR-195 and the presence of AAA.[24] Note that the origin of the biological samples used to determine miR-195 expression differed, which may explain the opposite variation of this miR between mice and humans.

Thus, even if animal models are useful to study the role of miRs in AAA development, dissimilarities of expression were found for some miRs between humans and animals, which can be attributed to differences in methodology and/or pathophysiological features. Moreover, even if miRs are well conserved, their regulation may differ among species, which may account for some of the observed differences.[76]

4. Future directions

We believe that a few issues should be taken into account in future work. Most studies addressed the expression of a limited number of miRs and followed a hypothesis-driven approach. As changes in miRs do not occur in isolation, it should be useful to follow, at least in pilot studies, an unbiased discovery-driven approach looking at all existing miRs, with the aim to account for the formidable complexity of the disease and try to identify novel miR signatures (combinations of miRs). This may not only improve the sensitivity and specificity of circulating miRs as potential biomarkers of AAA, but may also inform about the deregulation of selective and previously unsuspected biological pathways involved in the disease.

In both clinical and experimental studies addressing vascular miR expression, the role of miRs was mainly investigated in whole aortic tissue samples. To fully understand their complex role in AAA pathophysiology, it would be useful to specifically study miR expression in the different structures of the aortic wall and in specific cell subsets. The use of laser capture microdissection that allows for the extraction of very precise tissue structures and cells[77] could be very useful in that purpose. This can be coupled with advanced flow cytometry-based cell sorting and RNA sequencing techniques to gain deeper insights into

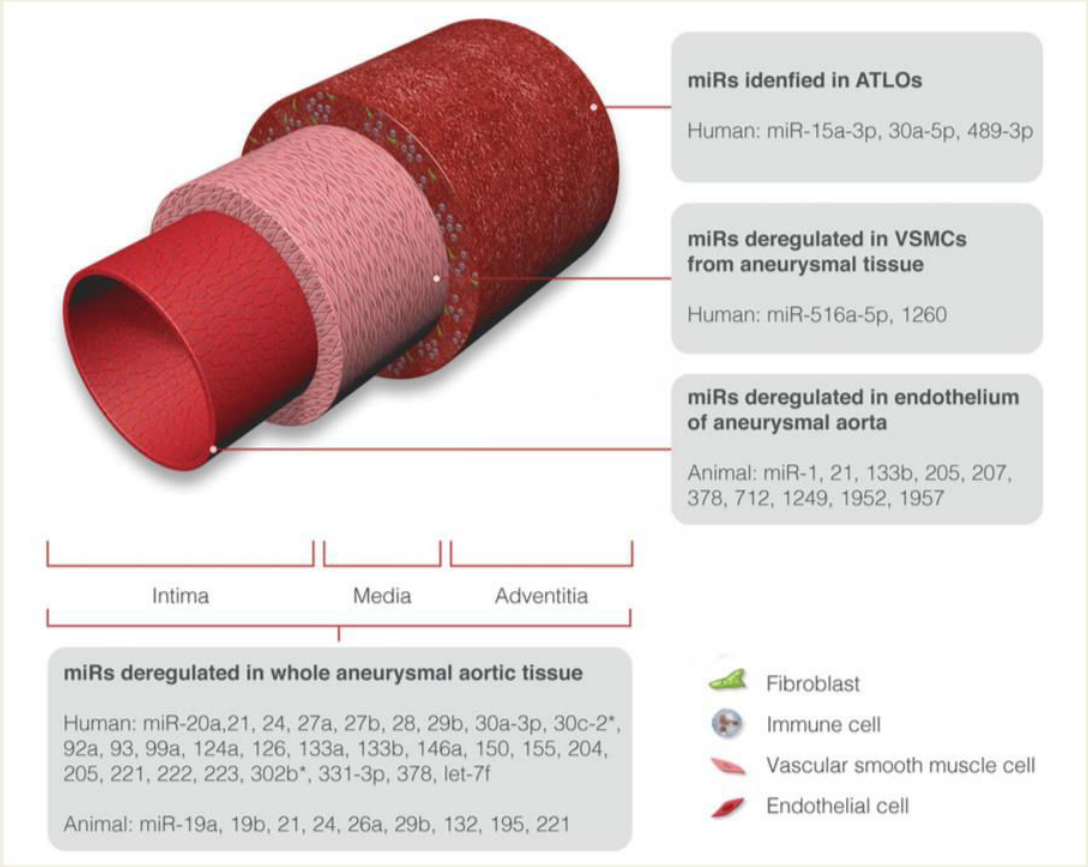

Figure 1 Main miRs identified in aortic structures during AAA. mRs are differentially expressed during AAA, whether analysed in whole aortic tissue, or in distinct aortic layers such as the endothelium or the VSMC layer. mRs have also been identified in artery tertiary lymphoid organs that form in the adventitial layer during AAA. AAA, abdominal aortic aneurysm; ATLOs, artery tertiary lymphoid organs; VSMC, vascular smooth muscle cells; miRs, micro-RNAs.

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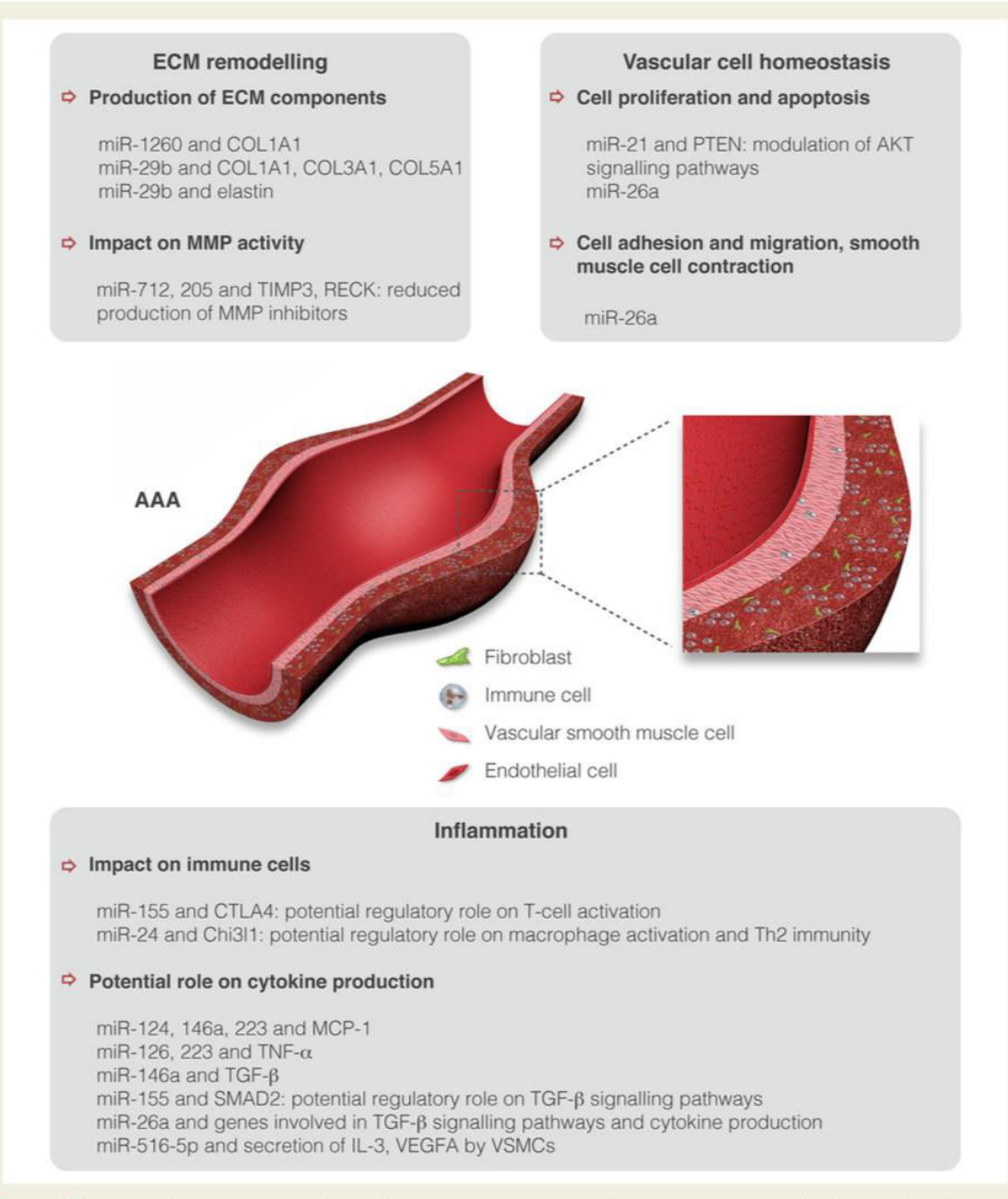

Figure 2 Implication of miRs in pathogenic pathways of AAA revealed by concomitant variation of miRs and their predicted targets. miRs are involved in ECM remodelling through their impact on the production of ECM components such as elastin or collagen or via a regulatory role on MMP activity. They regulate vascular cell homeostasis by targeting key molecules involved in cell proliferation, apoptosis, adhesion, and migration. They are also involved in the inflammatory process that characterizes AAA through their modulatory effects on immune cells and cytokine production. AAA, abdominal aortic aneurysm; Chi3l1, chitinase 3-like 1; COL1A1, collagen type 1, alpha 1; COL3A1, collagen type 3, alpha 1; COL5A1, collagen type 5, alpha 1; CTLA4, cytotoxic T-lymphocyte-associated protein; ECM, extracellular matrix; IL-3, interleukin-3; MCP-1, monocyte chemoattractant protein-1; MMP, metalloproteinase; PTEN, phosphatase and tensin homologue; RECK, reversion-inducing cysteine-rich protein with kazal motifs; TGF-β, transforming growth factor beta; Th2, T-helper lymphocyte, type-2; TIMP3, tissue inhibitor of metalloproteinase-3; TNF-α, tumour necrosis factor alpha; VEGFA, vascular endothelial growth factor A; miRs, micro-RNAs; VSMC, vascular smooth muscle cell.

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the expression profile and biological pathways altered in specific locations and specific cell types, both systemically and within the aneurysmal wall.

Finally, the low level of consistency among the small clinical studies underlines the need to perform multicentre studies on larger cohorts of patients, providing enough power to detect a significant result after adjustment for multiple covariates and confounding factors. In this regard, longitudinal studies would be a great asset to investigate miR variation, roles, and implications during disease progression. It could offer the opportunity to explore miRs as biomarkers of progression and risk of rupture, but also as biomarkers of therapeutic outcome.

5. Conclusion

Human and animal studies show that distinct miRs are differentially expressed within the aneurysmal wall (*Figure 1*) and may play causal roles in AAA formation (*Figure 2*). Basic experimental and translational approaches present advantages and limitations, but may complement each other. Human studies offer the possibility of identifying selective miRs as potential biomarkers and players in AAA pathophysiology. However, the investigation of their precise roles may be limited and impacted by several confounding factors. Hence, the need to standardize study design and methods used, at least for miR analysis. Animals are more suitable to mechanistic studies and interventional approaches, which should allow for a better comprehension of the precise roles of miRs in AAA pathophysiology. There is an immense need to develop better animal models that more closely reproduce the human pathophysiological features of AAA. New models of the disease have been developed over the past few years,[53] some of which have led to a reconsideration of the role of major pathways in disease pathogenesis.[78] However, we believe that the current models are highly perfectible and research should still be directed towards this aim. Graft implantation in rodents and coated stent technology are under development and would offer new opportunities to test therapeutic strategies based on miR modulation. No doubt that major advances in the understanding of miR functions and regulations are to be expected and could offer innovative perspectives to identify, treat, and improve the daily management of patients with AAA.

Authors' contributions

All authors confirmed that they have contributed to the intellectual content of this article and have approved the final version.

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Annexe 4: Diabetes and aortic aneurysm: current state of the art

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REVIEW

Diabetes and aortic aneurysm: current state of the art

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Abstract

Aortic aneurysm is a life-threatening disease due to the risk of aortic rupture. The only curative treatment available relies on surgical approaches; drug-based therapies are lacking, highlighting an unmet need for clinical practice. Abdominal aortic aneurysm (AAA) is frequently associated with atherosclerosis and cardiovascular risk factors including male sex, age, smoking, hypertension, and dyslipidaemia. Thoracic aortic aneurysm (TAA) is more often linked to genetic disorders of the extracellular matrix and the contractile apparatus but also share similar cardiovascular risk factors. Intriguingly, a large body of evidence points to an inverse association between diabetes and both AAA and TAA. A better understanding of the mechanisms underlying the negative association between diabetes and aortic aneurysm could help the development of innovative diagnostic and therapeutic approaches to tackle the disease. Here, we summarize current knowledge on the relationship between glycaemic parameters, diabetes, and the development of aortic aneurysm. Cellular and molecular pathways that underlie the protective effect of diabetes itself and its treatment are reviewed and discussed, along with their potential implications for clinical translation.

Keyword Diabetes • Aneurysm • Pathophysiology

Introduction

Aortic aneurysm corresponds to a localized enlargement of the aorta resulting from a weakening of the aortic wall. The disease is often asymptomatic but is associated with high rates of mortality, especially when complications such as aortic rupture occur.[1] Despite significant advances in our understanding of the disease mechanisms, the only curative treatment available today relies on endovascular or open surgical repair; drug-based therapies are still lacking.[1] The disease may occur both in the thoracic and abdominal parts of the aorta, the most frequent localization being the abdominal infrarenal aorta. Thoracic and abdominal aortas differ in structure, biochemical composition, smooth muscle cell origin and biology, and even if abdominal and thoracic aneurysms share common mechanisms, they both have distinct features.[2] The pathologic processes that lead to abdominal aortic aneurysm (AAA) formation are complex, and involve alteration and loss of vascular smooth muscle cells (VSMCs), in association with inflammatory cell infiltration, extracellular matrix (ECM) remodelling, and intraluminal thrombus (ILT) formation.[3,4]

Thoracic aortic aneurysms (TAA) frequently develop at younger ages, are often linked to genetic mutations such as Marfan syndrome or Loeys-Dietz syndrome, and are frequently associated with aortic dissection.[5] Alterations of ECM and VSMC tone play important roles in TAA, but the inflammatory component and VSMC apoptosis are less prominent than in AAA.[2,5,6] Despite these differences, epidemiological studies have paradoxically highlighted an inverse relationship between diabetes mellitus (DM) and both TAA and AAA.[3,7] The aim of our review is to summarize the current knowledge on the pathophysiological mechanisms that underlie the inverse association between diabetes and aortic aneurysm.

Diabetes and the epidemiology of aortic aneurysm

Diabetes mellitus corresponds to a heterogeneous disease characterized by a chronic hyperglycaemia and is generally classified in several categories including mainly type 1 (T1D) and type 2 diabetes (T2D).[8] Although

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DM represents a major cardiovascular risk factor,[8,9] the vast majority of epidemiological studies have paradoxically identified an inverse association between DM and the prevalence and incidence of AAA.[10–16] Indeed, several reports have shown that diabetic patients develop smaller AAA, as demonstrated by significantly lower aortic diameters compared to non-diabetic subjects.[10,17] Besides, several studies highlighted lower growth rates of AAA in diabetic patients compared to controls.[11,13,18,19] In practice, the decision to treat AAA takes into consideration the risk of rupture. As large aortic diameter and high growth rate represent major risk factors of rupture,[7] it is not surprising that a negative association between DM and aneurysm rupture was identified.[20]

While DM appears as a protective factor of AAA formation and expansion, the prognosis and outcome after AAA treatment also differs between diabetics and non-diabetic patients. Heterogeneous results were found among different studies, some reports revealing increased operative mortality in diabetics,[11,21] others showing no difference,[12,22] and some reporting lower mortality.[15] Besides, morbidity following AAA repair was analysed, and DM was identified either as a negative or protective factor of specific post-operative outcomes. Higher rates of complications such as myocardial infarction, infection, or pancreatitis were observed in diabetic patients after AAA open repair,[22] and higher incidence of device-related complications following endovascular AAA repair were found.[21] On the opposite, DM had a protective effect on AAA growth and re-interventions after endovascular repair,[23] but no significant difference in the occurrence of neck dilatation or type 1 endoleaks was identified between diabetics and controls.[24]

Similar to AAA, a negative association was identified between DM and TAA.[25,26] A meta-analysis combining four studies which included patients with ascending TAA, thoraco-abdominal aneurysms, and non-syndromic TAA confirmed the negative association between diabetes and TAA.[25] However, the impact of diabetes on TAA growth is still unknown and its effect on post-operative outcome has been poorly investigated. It will be important to better explore the impact of diabetes in the different forms of TAA distinguishing ascending and descending TAA, thoraco-abdominal aneurysms, as well as TAA caused by genetic disorders in order to fully understand the link between these disease states.

Glycaemic parameters and aortic aneurysm

Glucose homeostasis results from a complex mechanism involving two main hormones secreted by the pancreas, insulin, and glucagon, which have opposite effects.[8] The impairment of glucose regulation leads to chronic hyperglycaemia, which characterizes DM. In practice, diabetes is biologically defined as fasting plasma glucose (FPG) >7.0 mmol/L (>126 mg/dL) or as a 2-h plasma glucose value after a 75 g oral glucose tolerance test (OGTT) >11.1 mmol/L (>200 mg/dL)[8] or as a glycated haemoglobin A1c (HbA1c) >6.5%.[8]

It is noteworthy that in the wide majority of epidemiological studies on AAA, DM was defined as a known history of the disease according to the patient's declaration and to medical records. However, Hjellestad *et al.*[27] found that according to OGTT and HbA1c values, half of the patients with AAA and DM were unaware of their DM diagnosis. Hence, the exclusive reliance on history of disease and disease records could lead to an underestimation of DM in the studied cohorts. Moreover, most of the studies did not distinguish between T1D and T2D diabetic patients. Given the distinct characteristics of pathophysiological patterns between these two forms of DM,[8] it is possible that their effects and relationships with AAA differ. Also, the relationship between AAA development and the pre-diabetic state is still poorly understood, underlining the need for a better investigation of the association between AAA and the different forms and stages of DM.

Nevertheless, several studies investigated the relationship between glycaemic parameters and AAA characteristics. An inverse correlation was found between fasting glucose and aortic diameter, suggesting a relationship between glucose metabolism and AAA formation.[17,28] However, no association was found between serum levels of glucose and aortic expansion in a cohort of 198 patients followed up to 3 years.[19] While fasting blood glucose reflects short-term glucose regulation, HbA1c is representative of the glycaemic status over the past 3 months.[8] In this regard, an inverse association between the growth rate of AAA and the level of HbA1c was found both in individuals with and without DM, suggesting a mechanistic relationship between long-lasting elevated blood glucose levels and AAA progression.[18]

Another study found no association between fasting blood glucose measured at mean age 33 and aortic diameter measured at age 65 years.[29] Moreover, Taimour *et al.*[30] revealed that AAA prevalence did not differ between 65-year-old men who were newly diagnosed as T2D patients and non-diabetic men, and HbA1c level did not correlate with AAA diameter in newly diagnosed T2D patients. These results suggest that mechanisms linking altered glucose metabolism and a decreased risk of AAA are not relevant until late in life or until they are sustained long after the diagnosis of DM.

While short- and long-term glucose levels have been investigated in the context of AAA, the relationships between AAA characteristics and parameters of glucose regulation, such as insulin or glucagon, have so far received little attention. One study did not identify any association between fasting insulin and AAA,[28] whereas another reported a positive correlation between C-peptide and AAA diameter in T2D patients.[30] C-peptide is a biomarker used in medical practice to assess endogenous insulin secretion and is also useful to indirectly evaluate insulin resistance. Nevertheless, the significance and direct role of C-peptide in AAA pathogenesis are still poorly understood. Moreover, the link between glycaemic parameters and TAA has not been investigated so far.

Animal models of diabetes and aortic aneurysm

Several experimental models have been developed to understand the impact of DM on aneurysm. Chemically induced AAA models are schematically categorized in non-dissecting and dissecting models and reproduce main human pathophysiological features even if they present some limits.[31] Non-dissecting AAA models can be created through perfusion or application of porcine pancreatic elastase or calcium chloride.[31] These models reproduce some of the main features of human AAA, including VSMC loss, inflammatory cell infiltration, ECM degradation, and aortic dilatation. However, they are self-contained and do not progress to rupture. Dissecting AAA models are characterized by intramural delamination leading to haematoma formation and aortic dilation and are often associated with dilatation and dissection of the thoracic aorta.[31] They can be induced by infusion of angiotensin II, with or without inhibition of transforming growth factor (TGF)-β or lysyl-oxidase, or through administration of a mineralocorticoid-receptor agonist and high salt. Animal models of genetically triggered TAA (Marfan or Loeys-Dietz syndromes) have also been developed through mutation or deletion of the incriminated gene, such as fibrillin-1 or TGF-β ligands and receptors.[32]

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Table 1 Experimental models used to study aneurysm development in diabetic mice

Diabetes model	Aneurysm model	Main results	References
T1D: streptozotocin administration	$CaPO_4$-induced aneurysm in the carotid artery of C57BL/6 mice	Decreased AAA formation Decreased macrophage infiltration	Tanaka *et al.*[36]
	Elastase infusion in the abdominal aorta of C57BL/6 mice	Decreased AAA formation Decreased macrophage infiltration associated with decreased PAI-1 aortic gene expression	Dua *et al.*[34]
		Decreased AAA formation Decreased macrophage infiltration associated with a decrease of mural neovascularity and MMP9 activity and a better elastin preservation	Miyama *et al.*[35]
	Angiotensin II infusion in *ApoE*$^{-/-}$ mice	Decreased AAA formation Better elastin preservation	Miyama *et al.*[35]
	Angiotensin II infusion in *CDA1*/*ApoE*$^{-/-}$ mice	Development of AAA in diabetic *CDA1*/*ApoE*$^{-/-}$ mice, not observed in diabetic *ApoE*$^{-/-}$ or diabetic wild type mice Increased macrophage infiltration and MMP12 expression, reduced collagen expression and TGF-β signalling in diabetic *CDA1*/*ApoE*$^{-/-}$ mice	Li *et al.*[37]
T2D: KK-Ay mice	$CaPO_4$-induced aneurysm in the carotid artery	Decreased aneurysm formation Decreased macrophage infiltration	Tanaka *et al.*[36]

In parallel, several animal models of DM have been developed, allowing to study the impact of the disease on AAA development. T1D can be induced by administration of streptozotocin or alloxan, which affects pancreatic beta cell survival and leads to chronic hyperglycaemia.[33] Streptozotocin-induced T1D had a protective effect on elastase-induced AAA development, as demonstrated by decreased aortic diameter and decreased AAA growth.[34,35] Similar results were obtained in calcium phosphate-induced aneurysm model and after angiotensin II perfusion in *Apoe*$^{-/-}$ mice.[35,36]

Models of T2D tend to reproduce human features of insulin resistance and/or beta cell failure and can be associated with obesity.[33] Obese animal models of T2D include monogenic models such as mice deficient in leptin signalling pathway or polygenic models. Among them, KK-Ay mice develop severe hyperinsulinaemia and insulin resistance associated with impairment of pancreatic islet function. Non-obese models of T2D, such as Goto-Kakizaki (GK) rats, are also available allowing the study of disease mechanisms in a lean colony.[33] Similarly to humans, T2D exerts a protective effect on aneurysm development in animal models, as demonstrated by a decrease of calcium phosphate-induced aneurysm formation in KK-Ay mice.[36] The induction of aortic aneurysm in different models of diabetic mice has offered the opportunity to better understand the link between these two diseases (*Table 1*). Unfortunately however, among the previously described animal models of diabetes and AA, only a few were used to address that link, and most of them were restricted to chemically induced AAA and T1D models.

Mechanistic effects of diabetes on aortic aneurysm

ECM remodelling

Several studies addressed the impact of DM on vascular wall structure (*Figure 1*). In the absence of difference in aortic lumen diameter, aortic wall stress of the abdominal aorta was shown to be 20% lower in T1D patients compared to controls, with 22% larger aortic intima-media thickness, which significantly correlated with the duration of DM.[38] A study integrated protein–protein interaction and genetic interaction to examine biological pathways related to DM, aneurysm, and atherosclerosis.[39] The study identified a set of 16 proteins with high brokerage values (i.e. essential for the interconnectedness of the network), and this network was enriched in proteins involved in cell matrix-adhesion, supporting a role for ECM alterations in the link between DM and reduced risk of aneurysm. ECM is a dynamic network composed of basement membrane, collagen, elastin, and proteoglycans that contribute to tissue organization and structure. Interestingly, proteome analysis of non-atherosclerotic arteries (free of adventitia) from T2D patients showed increased amounts of the basement membrane and ECM components including alpha1-type IV and alpha2-type IV collagen, gamma1, and beta2 laminin.[40] These results suggest distinct properties of ECM in diabetic patients and could partly be involved in the protective effect on AAA development. In addition to the fibrous proteins, the background of the ECM is composed of glycosaminoglycans (GAG), which are complex polysaccharides covalently linked to ECM proteins in the form of proteoglycans. GAGs are categorized into four classes according to their disaccharide units, including chondroitin sulfate/dermatan sulfate, heparan sulfate, keratin sulfate, and hyaluronan. Biglycan, a member of chondroitin sulfate/dermatan sulfate, is abundant in normal human aorta and its expression is decreased in abdominal aortic aneurysmal tissue.[41] Moreover, biglycan gene defects cause an X-linked syndromic form of severe TAA and dissection.[42] To better understand the role of biglycan in aneurysmal pathology, experimental models were used. Biglycan deficiency in *Ldlr*$^{-/-}$ mice aggravated angiotensin II-induced aortic aneurysm, with increased rates of vascular mortality and rupture, and induced the development of descending TAA.[43] This was associated with breaks in medial elastin as well as a decrease in dense collagen fibre network in the aortic wall. In addition, biglycan deficiency in BALB/cA mice induced

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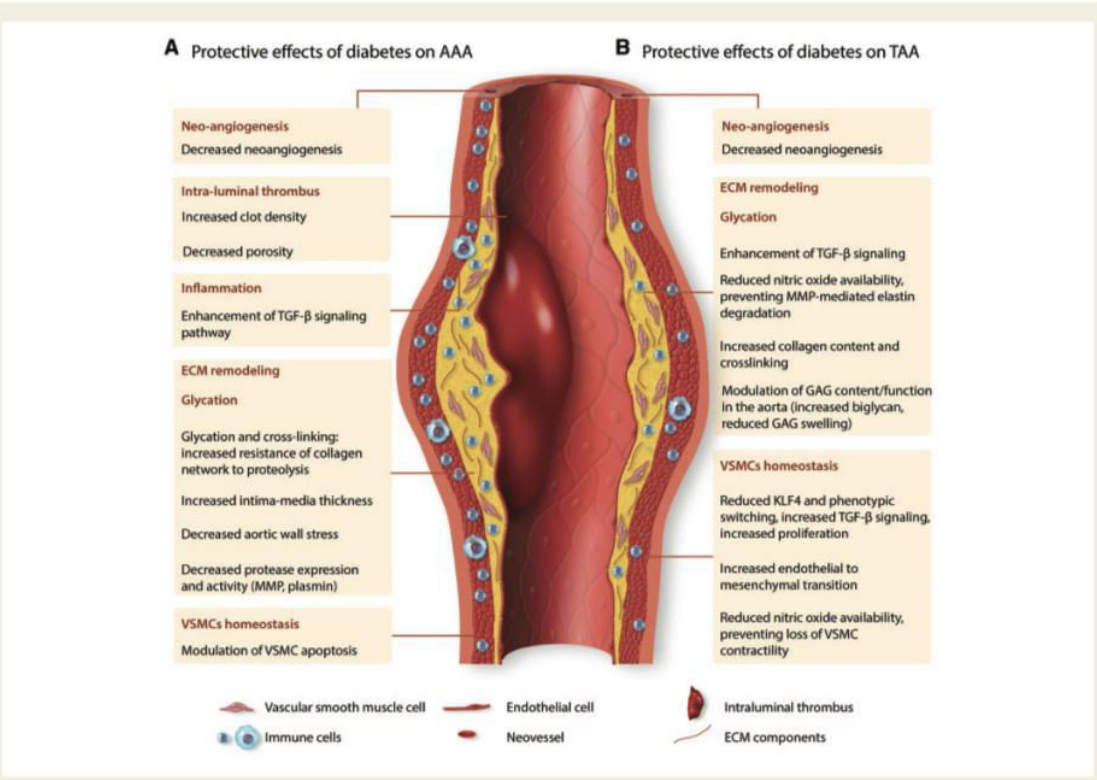

Figure 1 Hypothesized protective effects of diabetes mellitus in AAA and TAA. The main mechanisms that underlie the protective effect of diabetes in the pathogenesis of aortic aneurysm are mediated through effects on aortic mural neoangiogenesis, intraluminal thrombus formation, inflammation, glycation, extracellular matrix remodelling, and vascular smooth muscle homeostasis. Even though AAA and TAA share common pathophysiological mechanisms, they are characterized by distinct features. The main pathways underlying the protective effect of diabetes mellitus may differ between AAA and TAA.

mortality due to spontaneous aortic dissection and rupture.[44] Transmission electron microscopy and biomechanical testing revealed abnormalities of collagen fibrils with marked variations in size and shape associated with reduced tensile strength. Other studies pointed to interactions between biglycan and cytokines, particularly TGF-β, which plays a central role in both TAA and AAA.[45,46] Biglycan can regulate TGF-β signalling pathway[47] and TGF-β can enhance biglycan expression,[48] suggesting a mutual and positive feedback interaction to preserve the ECM and protects the arterial wall against aneurysm development and dissection. Although the direct impact of diabetes on biglycan expression in the aneurysmal aortic wall is still uncertain, diabetes is associated with an upregulation of Cell Division Autoantigen 1 (CDA1) which enhances TGF-β signalling pathway and contributes to the protective effect of diabetes on AAA formation.[37] DM can also alter the production, degradation and deposition of other GAGs in the aorta, with additional consequences on ECM remodelling as well as the structural and physical properties of the arterial wall. Decorin, a small leucin-rich repeat proteoglycan, is increased in response to high-glucose concentration, interacts with TGF-β to regulate matrix organization and collagen matrices and may reduce aneurysm formation in part through modulation of ECM remodelling.[49–51] On the other hand, substantial accumulation of (foci of) GAGs, mainly hyaluronan, aggrecan and versican, is frequently associated with the progression and severity of TAA.[52–54] These GAG foci generate important osmotic pressures with localized increases in intramural stress, and are thought to initiate medial delamination.[53] We speculate that an imbalance between GAGs and the connective tissue in the aorta of diabetic patients in favour of increased collagen deposition, may limit the susceptibility of the aorta to intralamellar Donnan swelling pressures caused by focal accumulation of GAGs,[55] thereby limiting the risk of aneurysmal dilatation and dissection, particularly in the thoracic aorta.

ECM remodelling also results from a balance between the synthesis of ECM components and their degradation by proteases including cathepsins, metalloproteinases (MMPs), and serine proteases. In some studies, the protective effect of DM on experimental AAA was associated with a decrease of MMP9 expression and activity, and a relative elastin preservation compared to controls.[34,35] This is corroborated by the observation of decreased MMP9 secretion by activated monocytes exposed to aortic media extracted from diabetic patients compared to non-diabetics.[19] To further address the mechanisms involved, *in vitro* studies were performed using murine macrophage cell line RAW264.7 treated

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with tumour necrosis factor alpha (TNF alpha) and calcium phosphate ($CaPO_4$) to mimic conditions of $CaPO_4$-induced AAA formation.[36] Hyperglycaemic conditions (incubation with glucose 15.5 mmol/L) attenuated TNF/$CaPO_4$-induced elevation of MMP9 at the gene, protein and activity levels, in a Nr1h2-dependent mechanism.[36] However, others reported an induction of MMP expression and activity, including MMP9 in human monocyte-derived macrophages, and MMP1/MMP2 in human endothelial cells, under higher glucose concentrations (25 mmol/L).[56] MMP-2 and MMP-9 serum concentrations were lower in T2D patients compared to non-diabetics and hyperglycaemia during OGTT induced a decrease in MMP9. However, HbA1c paradoxically correlated with MMP9 in T2D patients.[57] Hence, the effect of hyperglycaemia on MMP regulation is complex and would require further studies to fully understand it. It is also important to note the conflicting evidence regarding the role of MMP collagenases in aneurysm formation, and more particularly the increased susceptibility to angiotensin II-induced dissecting AAA in hypercholesterolaemic mice with MMP9 deficiency or genetically engineered resistance to collagenases,[58,59] some of these effects being related to altered biomechanical properties of the aortic wall due to altered collagen deposition and fibre orientation. In addition to MMPs, serine proteases such as plasmin represent one of the major proteolytic enzymes involved in ECM remodelling. Plasmin activity results from the activation of plasminogen by plasminogen activators (PA), and this pathway is inhibited by plasminogen activator inhibitor-1 (PAI-1).[60] Interestingly, AAA development was decreased in diabetic mice compared to controls, concomitantly with an increase of plasma PAI-1 level and aortic PAI-1 gene expression, and a decrease of plasmin generation.[34] However, the mechanisms behind PAI-1 modulation in diabetic mice, and the direct link with reduced AAA development have not been explored.

Glycation and advanced glycation end products

DM and chronic hyperglycaemia lead to an increase of advanced glycation end products (AGEs), which result from non-enzymatic glycoxidation of proteins and peptides after contact with aldose sugar.[61] These products include non-cross-linking AGEs such as carboxymethyllysine (CML) and carboxyethyllysine (CEL), and cross-linking AGEs such as pentosidine. Interestingly, concentrations of pentosidine, CML and CEL were in general lower in AAA biopsies compared to non-aneurysmal aortic tissues.[62] When comparing diabetic patients to non-diabetics, an increase of pentosidine concentration was observed in AAA aortic samples and its concentration showed a tendency toward a negative correlation with AAA diameter, suggesting a protective effect of cross-linking AGEs. Besides, a significant decrease of carboxyterminal telopeptide of collagen type 1, and a tendency toward a decrease of collagen type 1C-telopeptide was observed in glycated AAA tissues compared to non-glycated AAA tissues. As those collagen telopeptides are products from the degradation of mature type 1 collagen by MMPs and cysteine proteases, and no difference was found in protease activity (including MMP2, MMP9, cathepsin A, B, and S) between AAA biopsies of diabetics compared to non-diabetics, the results suggest that glycation of AAA tissue protects the collagen network and increases its resistance to proteolysis. The precise effects of glycation and cross-linking on ECM remodelling were further investigated by *in vitro* studies on THP1 cell lines and peripheral blood mononuclear cells (PBMCs).[19] Interestingly, glycated bovine serum albumin (BSA) and glycated monomer collagen enhanced the secretion of MMP9 by THP1 cells. In contrast, glycated collagen lattices and cross-linked collagen lattices reduced the secretion of MMP2 and MMP9 by both THP1 cells and PBMCs. These results underline the complexity of the effect of glycation on monocytes and ECM remodelling, which may differ depending on the type of glycated protein.

In addition to direct modification of protein structure, AGEs can lead to the activation of several signalling pathways through various cell surface receptors. The most known of those receptors is the multi-ligand receptor for advanced glycation end products (RAGE), which has been identified in various cells including monocytes/macrophages, endothelial, and VSMCs.[63] Experimental studies unravelled the complex role of AGEs and their receptor RAGE in angiotensin II-induced AAA model using $Apoe^{-/-}$ $RAGE^{-/-}$ mice. RAGE deficiency decreased the incidence of AAA, and this was associated with a decrease of MMP9 activity and cellularity of the adventitia. Further *in vitro* studies revealed that stimulation with RAGE ligands increased MMP9 gene expression as well as MMP9 activity in murine macrophage cell line RAW 264.7.[64] Another study using transgenic mice overexpressing the human S100A12 (a ligand of RAGE known to be increased in DM) reported progressive dilatation of the aorta with elastin fibre disruption, fibrosis, and loss of VSMCs within the medial layer, along with an increase of MMP2 protein expression.[65] Besides, analysis of human samples revealed that S100A12 protein was expressed in all cases of thoracic aortic type A dissection, in 25% of stable TAA, but was not detected in non-aneurysmal control aortic tissue, consistent with a potential pathogenic role in human aortic disease.[66] Interestingly, investigation of the association between RAGE gene polymorphisms and AAA revealed that the 82S allele of RAGE was a risk factor for AAA.[67] Taken together, these results suggest that the impact of glycation on protein structure could partly account for the protective effect of DM on AAA formation (*Figure 1*), whereas its effect through RAGE signalling pathway may exert a pathogenic effect on the aortic wall and contribute to AAA development.

As glycation affects AAA development, investigators examined if measurement of AGEs could be of interest as a biomarker in AAA. Interestingly, skin accumulation of AGEs measured by auto fluorescence was found to be higher in patients with AAA compared to controls.[68] However, this association was lost when adjusted for cardiovascular risk factors. No association was found between AAA progression and serum CML concentration.[19] Besides, no correlation was found between pentosidine concentration and AAA diameter, and there was no correlation between plasma levels of pentosidine and its levels in AAA biopsies.[62] It is reasonable to hypothesize that AGEs are formed in other tissues, making the systemic measurement less representative of the local effect in the AAA wall. This field of research is still in its infancy, and new clinical studies are currently investigating the types and locations of AGEs in aortic tissues and in several biological fluids of patients with AAA, and will probably provide a better understanding of the complex role of glycation in this disease.[69]

Inflammation

Over the past decades, several studies revealed a close link between metabolism and immunity; alterations of the immune system contribute to the pathogenesis of DM and conversely, metabolic alterations leading to DM modulate the inflammatory response.[70] The enhanced state of immune activation in DM, whether chronic autoimmunity in T1D or chronic low-grade inflammation in T2D with enhanced production of inflammatory mediators, is not expected to protect against the development and/or progression of aortic aneurysm. Most of the studies point to a promoting role of DM on vascular inflammation through several mechanisms, including increased oxidative and endoplasmic reticulum stress responses, and impaired activation of protective anti-inflammatory

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pathways (reduced endothelial nitric oxide synthase, insulin resistance in vascular cells, etc.).[71] It should be kept in mind, however, that this is not a black-and-white situation, and strong counter-regulatory mechanisms (e.g. TGF-β) may still operate and may even be promoted, systemically and locally, under diabetic conditions. Monocyte recruitment to the endothelium is finely regulated by glycaemic conditions. A study in Goto-Kakizaki rats (GK-rat), a genetic non-obese model of T2D, revealed that fluctuation of blood glucose enhanced monocyte adhesion to the thoracic aortic endothelium, an effect possibly due to increased gene expression of connecting segment (CS)-1 and very-late acting antigen (VLA)-4.[72] On the other hand, administration of insulin and nateglinide (known to stimulate insulin secretion) in GK-rats reduced post-prandial hyperglycaemia, thus attenuating blood glucose fluctuation, and induced a decrease of monocyte adhesion to endothelial cells.[73] The results suggest a promoting effect of DM on vascular inflammation. However, diabetes alters the differentiation of haematopoietic stem cells towards monocytes and macrophages, in part through upregulation of DNMT1 and hypermethylation of major transcription regulators PU.1, NOTCH1, and KLF4,[74] which may limit the inflammatory process. Furthermore, increased monocyte recruitment may not favour AAA development unless monocytes differentiate into pro-inflammatory macrophages. However, DM or hyperglycaemic conditions have been shown to modulate macrophage phenotype towards either a pro-[74–76] or anti-inflammatory state,[77] through various mechanisms including upregulation of acyl-CoA synthetase 1[75] or regulation of AKT/mTOR and ERK pathways.[77] The pro-inflammatory phenotype appears to be prevalent after short-term diabetes and in association with a hypercholesterolaemic setting,[75] whereas the anti-inflammatory phenotype was observed after several months of diabetes induction.[77] Thus, increased monocyte recruitment into the injured artery wall in DM may not lead invariably to increased accumulation of pro-inflammatory macrophages. Furthermore, glycation has been shown to alter monocyte/macrophage function toward an anti-inflammatory phenotype, reducing interleukin (IL)-6 production as shown in lipopolysaccharide-activated THP1 monocytes incubated with glycated collagen lattices and cross-linked collagen lattices.[19] As IL-6 deficiency limits both TAA and AAA development in mice, and Mendelian randomization approaches supported the involvement of the IL6 receptor pathway in human AAA,[78] the effect of glycation on IL-6 may contribute to the protective effect of DM in aortic aneurysm.

Studies in animal models of AAA revealed a decrease of macrophage infiltration in diabetic mice compared to controls.[34–36] In contrast, a higher macrophage infiltration was observed in AAA wall of diabetic compared to non-diabetic patients who underwent AAA surgical repair and was correlated with serum glucose concentration.[79] AAA diameter was similar between diabetics and non-diabetics, and no significant difference was observed for T-lymphocyte infiltration. The discrepancy between those human and animal studies underlines the complex impact of diabetes on inflammation and suggests that the protective effect of DM on AAA development and progression may not be linked to its impact on inflammatory pathways (*Figure 1*). Another plausible explanation is that a higher level of inflammation is required to overcome the resistance of the aorta of diabetic patients to aneurysm development.

Aortic mural neoangiogenesis

Aortic mural neoangiogenesis is involved in TAA and AAA development, and in the progression to aortic rupture.[80] Interestingly, a decrease of mural neovascularity was observed in the aneurysmal wall of diabetic mice compared to controls.[35] Studies in animal models revealed that DM inhibits neoangiogenesis in aortic tissue, impairs ischaemia-induced neovascularization,[81] and impairs vascular endothelial growth factor pathway induction by hypoxia-inducible factor 1.[82] Similar mechanisms may be involved during AAA. Angiogenesis inhibitors limit AAA progression in a dissecting angiotensin II model, possibly through reduced immune cell infiltration[83] and reduced interstitial oedema,[55] suggesting that the protective effect of DM on AAA and TAA could partly be attributed to a decrease of neovascularization (*Figure 1*).

VSMC homeostasis

VSMCs have the ability to switch from a contractile phenotype to a secretory phenotype and impact on vascular homeostasis in physiological and pathological states.[84] Several studies addressed the impact of DM on VSMCs.[84] First, the morphology of saphenous vein VSMCs collected from T2D patients differ from non-diabetics, with a rhomboid morphology suggesting a dedifferentiated phenotype.[85] Focal adhesions characterized by vinculin immunostaining were increased in VSMCs from diabetic patients, with a disparate organization of the alpha actin network. Histologic analysis of tissues from patients with AAA suggested decreased staining for alpha-smooth muscle actin in diabetic patients compared with non-diabetics.[62] The significance of this finding is unclear and may reflect either reduced VSMC number or increased VSMC dedifferentiation. Although some studies reported less proliferation in VSMCs from T2D compared to non-diabetics,[85] most of the studies are in favour of a promoting effect of DM on VSMC proliferation through activation of ERK signalling pathways and the secretion of mitogenic factors.[86,87] Note that VSMCs were harvested from the saphenous vein and from the internal mammary arteries, respectively, which may explain, at least in part, the discrepancy between the studies. Experimental studies in animal models revealed persistent phenotypic changes and altered inflammatory gene expression in aortic VSMCs cultured from diabetic mice.[88] High glucose and palmitic acid induce epigenetic changes in aortic VSMCs that promote VSMC 'dysfunction'. For example, miR-504 is upregulated in VSMC under those conditions, leading to inhibition of contractile gene expression, while increasing migration, proliferation, and inflammatory gene expression, through effects on Grb10 and Egr2.[89] The study fell short however of establishing a direct link between diabetic conditions, epigenetic alterations, and VSMC dedifferentiation. Moreover, the term VSMC 'dysfunction' is too vague and may be misleading. For example, inflammatory changes in VSMCs under diabetic conditions may be expected to be conducive for aortic aneurysm development and progression. In contrast, acquisition of migratory and proliferative properties by VSMCs may have a protective healing and stabilizing role. Phenotypic switching of VSMC is controlled by the transcription factor KLF4[90] and is an important feature of aortic aneurysms.[91] Selective deletion of KLF4 in VSMCs reduces the development and severity of aortic aneurysm in both dissecting and non-dissecting mouse models of the disease.[91] Intriguingly, hyperglycaemia reduces KLF4 expression in VSMCs and arteries of mice and patients with diabetes, in part through Foxoa2-dependent upregulation of miR-29c.[92] The results are consistent with the upregulation of miR-143/145 (a major regulator of KLF4 expression[93]) in VSMCs (from saphenous vein) of patients with diabetes, and suggest a potential protective role of diabetes against AAA and TAA through limitation of KLF4 induction. Along this line of thinking, increased activation of the TGF-β signalling pathway under diabetic conditions, both systemically and locally in aortic VSMCs,[94,95] may also play an important protective role. TGF-β is required for the induction and maintenance of VSMC differentiation and homeostasis. Genetic mutations in the TGF-β signalling pathway predispose to TAA in humans,[96]

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and selective deletion of TGF-β signalling pathway in VSMCs promotes TAA development and dissection in mice.[97–99] Beyond a direct effect on VSMC differentiation, TGF-β signalling in VSMCs appears to limit their production of IL-1β,[100] which further contributes to the protection against aneurysm formation. Another pathway involved in the protective effect of TGF-β may be related to the regulation of VSMC contractility.[101] Genetic mutations in genes affecting VSMC contractility predispose to TAA in humans,[102] and alteration of VSMCs contractility has been suggested to contribute to aortic dissection in mice with disrupted TGF-β signalling.[103] In this regard, it is interesting to note that the reduction of KLF4 expression in VSMCs of diabetic mice and diabetic patients was associated with increased expression of contractile proteins.[92] Others have also reported increased expression of contractile genes and proteins in VSMCs under elevated glucose levels, related in part to activation of protein kinase C, Rho/Rho-kinase and actin polymerization.[104] Given the contribution of VSMC contractility to microvascular tone and ECM contraction, and the proposed roles of the latter physiological pathways in aortic wall homeostasis and its resistance to aneurysm formation and dissection,[55] those changes in VSMCs induced by, or promoted under diabetic conditions may further account for the protection against aortic aneurysm.

Endoplasmic reticulum (ER) stress induction in aortic VSMCs is thought to promote cell death and aneurysm,[105] a pathway that might be counter-regulated by induction of autophagy.[106] High-glucose conditions may favour the induction of ER stress in aortic VSMCs.[107] However, DM also highly promotes AGE-dependent VSMC proliferation through induction of autophagy,[108] which may overcome its deleterious effect on ER stress.

Endothelial to mesenchymal transition, which corresponds to the process by which endothelial cells are able to switch to a myofibroblastic phenotype in response to injury, is impaired in TAA.[109] As diabetes is associated with enhanced endothelial to mesenchymal transition in the vascular tissue, this process could potentially be involved in the inverse association between diabetes and aneurysm. Finally, a recent study unravelled a detrimental role of nitric oxide (as a result of NOS2 activation) in promoting medial aortic degeneration and dilatation in a mouse model of Marfan syndrome.[110] The mechanisms may involve an impact of nitric oxide on VSMC contractility and MMP9-mediated elastin fragmentation. It is therefore tempting to hypothesize that reduced nitric oxide bioavailability in diabetic setting may limit this pathogenic process. In general, the impact of DM on VSMC phenotype during aneurysm has not been thoroughly investigated so far (*Figure 1*), and the available studies did not specifically distinguish between VSMCs derived from the thoracic and the abdominal aortic region. This is of major importance given the distinct embryonic origins of VSMCs in AAA and TAA, and the distinct pathological features of aortic aneurysm in those different locations.

Intraluminal thrombus formation

ILT formation is a factor contributing to AAA development. The wall underlying the thrombus is thinner and exhibits increased signs of degradation. The rate of thrombus growth also correlates with aneurysm growth and rupture.[111] Interestingly, glucose concentration is associated with altered clot structure, with a higher clot density and decreased porosity reported in T2D patients compared to controls.[112] This may confer increased clot resistance to fibrinolysis in diabetic patients. As ILT is a source of protease release and activation, it can be hypothesized that a more compact but less active clot could limit AAA expansion in diabetic patients (*Figure 1*). In TAA, thrombus may develop within the aortic wall after dissection but it is not considered as an important factor in the initiation of dissection.

Effects of antidiabetic treatments

Treatment of DM relies on lifestyle measures associated with blood glucose lowering drugs.[9] Insulin administration is the main therapy for T1D patients. Pharmacological treatments mostly used in T2D include insulin sensitizers such as biguanides (metformin) or thiazolidinediones (rosiglitazone, pioglitazone), drugs stimulating insulin secretion such as sulfonylureas or meglitinides, and drugs with incretin effects such as glucagon-like-peptide-1 (GLP-1) receptor agonists (i.e. liraglutide, exenatide, lixisenatide) or dipeptidyl peptidase 4 (DPP-IV) inhibitors (i.e. alogliptin).

Intriguingly, epidemiological studies have revealed that mechanisms conferring a protective effect of DM on AAA are not only related to the pathophysiology of DM but also to antidiabetic treatments. A study including 1269 patients with AAA showed that the use of antidiabetic drugs was associated with a 56% reduction in AAA growth rate, and this association was independent of confounding factors including other therapeutic agents.[113] In addition, a nested case–control analysis including 4468 patients with AAA and 4468 matched controls revealed that metformin, sulfonylurea, and thiazolidinedione use was associated with a lower risk of developing aneurysm.[114] The negative association between metformin use and AAA enlargement and growth was confirmed in other studies.[115,116] No significant association was found between metformin use and the risk of rupture.[117]

Experimental studies were performed to better understand the cellular and molecular pathways underlying the negative association between antidiabetic use and AAA development. Studies using the elastase-induced AAA model in normoglycaemic mice suggest a protective role of metformin, with preservation of VSMCs and aortic medial elastin, associated with decreased inflammatory cell infiltration[115] (*Figure 2*).

However, lower levels of ECM components or basement membrane proteins, including alpha-chains of type IV collagen, collagen type XVIII, laminin-gamma-1, laminin-beta-2, and nidogen-1 were observed in arteries from T2D patients treated by metformin compared to non-metformin users.[39] Taken together, these results point to a complex effect of metformin on the pathophysiological events of AAA development and progression.

Several experimental studies addressed the impact of thiazolidinediones on AAA development (*Figure 2*). This drug class improves insulin sensitivity through activation of the nuclear transcription factor peroxisome proliferator-activated receptor (PPAR)-gamma and plays important roles in regulating inflammation by antagonizing several inflammatory transcription factors AP-1, STAT, and NF-kappaB in various immune cells.[118,119] Although treatment with thiazolidinediones has been associated with adverse hepatic, cardiovascular, osteogenic, and carcinogenic effects, their use in T2D is still debated.[119] Treatment with pioglitazone as well as treatment with PPAR-alpha agonists (fenofibrate) reduced aortic diameter in the angiotensin II/*Apoe*$^{-/-}$ model, and this effect was associated with reduced osteopontin (OPN) staining in the aorta and reduced macrophage infiltration.[120] As OPN is a chemotactic factor for macrophages and its deficiency improves AAA,[121] the protective effect of pioglitazone could be partly due to downregulation of OPN. Treatment with rosiglitazone also reduced AAA formation and abolished death caused by rupture in the angiotensin II/*Apoe*$^{-/-}$ model.[122,123] This selective agonist of PPAR-gamma decreased angiotensin II-induced expression of E-selectin, TNF-α and IL-6 in the aorta,

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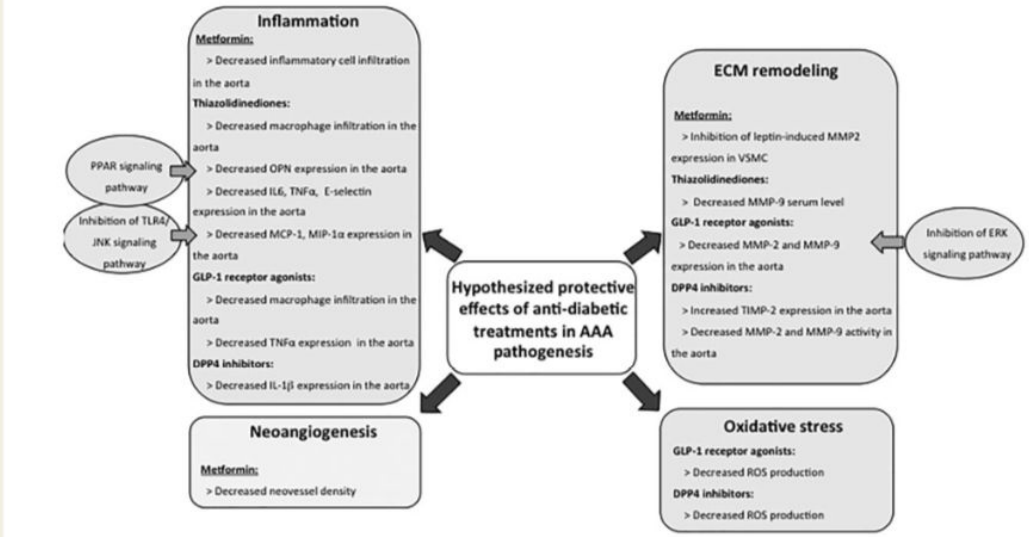

Figure 2 Hypothesized protective effects of antidiabetic treatments in AAA. Antidiabetic treatments may contribute to the negative association observed between diabetes and AAA through protective effects on inflammation, neoangiogenesis, extracellular matrix remodelling, and oxidative stress.

which may in part explain the protective effect given the known roles of those pro-inflammatory mediators in experimental AAA.[4] In addition, rosiglitazone inhibited angiotensin II-induced JNK activation, modulated the inflammatory process by downregulating TLR4, and suppressed the production of TLR4/JNK dependent chemokines Monocyte Chemoattractant Protein-1 and MIP1 alpha, which may further explain its protective effect on AAA development.[122] At last, as rosiglitazone reduced serum levels of MMP9 in T2D patients, its protective effect on AAA development could potentially be linked with ECM preservation.[124]

The action of drugs with incretin effect including GLP-1 receptor agonists (i.e. lixisenatide, liraglutide) and DPP-IV inhibitors (i.e. alogliptin, sitagliptin, MKEY0620) has also been investigated (*Figure 2*). Subcutaneous administration of GLP-1 receptor agonist lixisenatide reduced $CaCl_2$-induced AAA development through antioxidant, anti-inflammatory, and ECM preservation effects, as revealed by decreased reactive oxygen species (ROS), reduced macrophage infiltration and inhibition of TNF, MMP2, and MMP9 gene expression in the aortic wall.[125] These effects were associated with a decrease of ERK expression in the AAA wall suggesting the involvement of this signalling pathway in the protective effect of GLP-1 analogues. The protective effect of this drug was confirmed in another study where administration of liraglutide reduced AAA formation in *Apoe*$^{-/-}$ mice infused with angiotensin II.[126] The treatment increased circulating active GLP-1 and was associated with a preservation of elastin content, a decrease of macrophage infiltration, and a reduction of MMP2 and MMP9 expression. *In vitro* studies on monocytic U937 cells further confirmed the effect of the GLP-1 analogue, where pre-treatment with liraglutide decreased angiotensin II-induced ROS formation, MMP2 and MMP9 activities, and abolished angiotensin II-induced monocytic recruitment in a concentration dependent manner.[126]

Other studies investigated the effect of DPP-IV inhibitors (alogliptin, sitagliptin, MKEY0620) and revealed the protective effect of this class of drugs on AAA development[126–128] (*Figure 2*). DPP-IV gene expression was increased in the aneurysmal aortic wall, and immunostaining for DPP-IV (also known as CD26) showed it was expressed in macrophages.[127] Besides, plasma DPP-IV activity was increased, and plasma active GLP-1 concentration decreased in *Apoe*$^{-/-}$ mice after angiotensin II infusion compared to controls.[126] As expected, the administration of DPP-IV inhibitors decreased DPP-IV activity and increased plasma active GLP-1 concentration.[126,128] Intriguingly, DPP-IV inhibitors did not affect blood glucose concentration[126,127] and plasma insulin levels,[128] suggesting that the protective effect of DPP-IV inhibitors against AAA development may not be related to an incretin effect. DPP-IV inhibitors induced a dose dependent increase of elastin content, associated with a decrease in ROS production, and a reduction of the expression and gelatinolytic activities of MMP2 and MMP9 in the aneurysmal aortic wall.[127] In another study, DPP-IV inhibitors reduced the incidence of AAA and the protective effect was associated with reduced aortic expression of IL1β and increased tissue inhibitor of metalloproteinase 2 (TIMP2).[128] Additional treatment with an incretin receptor antagonist did not reverse the protective effect of DPP-IV inhibitors,[128] confirming that DPP-IV inhibitors limit AAA formation in an incretin independent manner. In addition to GLP-1, DPP-IV has various substrates, at least *in vitro*, including gastrointestinal hormones, neuropeptides, cytokines (IL-1β and IL-2), and chemokines (CCL5),[129] which could potentially account for the protective effect of DPP-IV inhibitors.

While oral antidiabetic drugs were associated with a protective effect in experimental AAA, insulin therapy had an intriguing opposite effect, negating the protective effect of hyperglycaemia (induced by streptozotocin injection) on elastase-induced AAA formation.[35]

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To conclude, the mechanism involved in the protective effect of antidiabetic drugs may not be directly related to an effect on blood glucose or insulin secretion. It may rather be due to improvement of insulin resistance, and modulation of other pathways, including inflammation, oxidative stress, and ECM remodelling (*Figure 2*). The impact of antidiabetic treatments on TAA development and progression is still unknown.

Conclusion and perspectives

Epidemiologic and experimental studies demonstrated a negative association between DM and aortic aneurysm formation and expansion. DM contributes to vascular tissue remodelling and has profound impact on several pathways relevant to aneurysm development, including ECM remodelling, inflammation, VSMC homeostasis, aortic mural neoangiogenesis, and ILT formation. Despite sharing a few common mechanisms, AAA and TAA display distinct features, and the main mechanisms underlying the protective effect of DM may slightly differ between AAA and TAA (*Figure 1*). The protective effect of diabetes on AAA may involve the role of glycation and cross-linking on ECM remodelling, the anti-inflammatory properties of TGF-β signalling pathway, and the impact on ILT formation, neoangiogenesis, and VSMC apoptosis. In addition to DM itself, antidiabetic drugs interfere with the pathophysiological mechanisms of aneurysm and may contribute partly to the protective effect observed in diabetic patients. In TAA, the protective effect of diabetes may involve the balance between collagen and GAG contents in the media and the resulting consequences on intramural stress distribution, the regulation of neoangiogenesis, the prevention of the deleterious effect of nitric oxide on aortic degeneration through reduced nitric oxide availability, and the promotion of protective TGF-β signalling in VSMCs, VSMC proliferation and endothelial to mesenchymal transition. Note, however, that the effect of antidiabetic drugs on TAA formation is still unknown, and merits appropriate investigation.

In the limelight of current knowledge, future directions can be proposed. First, DM is a complex disease classified in different types whose pathophysiological mechanisms differ. Future clinical studies should distinguish T1D from T2D patients to better understand the link of each type of DM with aortic aneurysm formation and progression. In parallel, the vast majority of experimental studies were performed in animal models mimicking T1D. More studies are needed in T2D animal models, with detailed investigation of the cellular and molecular mechanisms at play. Besides, the impact of intermediate states such as prediabetes is still unknown and remains to be elucidated. While the effect of hyperglycaemia itself has been investigated, the impact of major hormones involved in glucose homeostasis such as insulin and glucagon is poorly understood. Moreover, the molecular and cellular effects of DM in modulating the distinct pathophysiological mechanisms of AAA and TAA require further investigation. Given the availability of experimental models of DM and aortic aneurysm, and given the potential perspective to identify and develop new therapeutic tools based on modulation of glucose metabolism, the challenge is undoubtedly worth it.

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