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Sénescence, remodelage tissulaire et membranaire, risque thrombotique au cours de la Fibrillation Auriculaire

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LISTE DES ABREVIATIONS

ADMA	dimethylarginine asymétrique
Ang II	angiotensine II
ARA2	inhibiteurs de l'angiotensine II
AT1 récepteur	récepteur AT1 à l'angiotensine
AVC	accident vasculaire cérébral
CaMKII	protéine kinase 2 Ca^{2}+/calmodulin dépendante
cav-1	cavéoline 1
COX	cyclooxygénase
CRP	C-reactive protein
ET-1	endothéline 1
FA	fibrillation atriale
FC	fréquence cardiaque
FDRCV	facteur de risque cardio-vasculaire
FGF	fibroblast growth factor
FMD	flow-mediated dilation
FT	facteur tissulaire
GC	guanylate cyclase
GP	graisse péricardique
H2O2	peroxydes d'hydrogène
HTA	hypertension artérielle
ICAM	Intercellular Adhesion Molecule 1
IEC	l'enzyme de conversion
IL	interleukine
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein
MEC	matrice extra-cellulaire
MMP	métalloprotéinase
MP	microparticule
MPO	myélopéroxydase
NAV	nœud auriculo-ventriculaire
NO	monoxyde d'azote
NOS	NO synthase
$O2^{-}$	anions superoxides
OD	oreillette droite
OG	oreillette gauche
ONOO-	peroxynitrite
PA	potentiel d'action
PAF	platelet-activating facteur
PAF	FA paroxystique
PAI-1	inhibiteur de l'activateur du plasminogène
PAR	protease activated receptor
PDE	phosphodiestérase

PDGF	platelet-derived growth factor
PECAM-1	platelet endothelial cell adhesion molecule
PGH2	prostaglandine H2
PLA2	phospholipase A2
PmAF	FA permanente
PNN	polynucléaires neutrophiles
PS	phosphatidylsérine
ROS	espèces réactives de l'oxygène
RP	retinoblastoma protein
RS	rythme sinusal
SA-Béta-GAL	Senescence Associated béta-galactosidase
SMA	smooth muscle actin
SOD	superoxyde dismutase
SRA	système rénine-angiotensine
SS	shear stress
TGF-ß	transforming growth factor ß
TIMP	tissue-inhibitor of MMP
TNF α	système rénine-angiotensine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TXA2	thromboxane A2
VCAM-1	Vascular cell adhesion protein 1
VE-cadhérine	vascular endothelial cadherin
VEGF	vascular endothelial growth facteur
VEGF	vascular endothelial cell growth factor
VP	veine pulmonaire
vWF	facteur de von Willebrand

I. Résumé

Etat de la question

1-La fibrillation auriculaire

La fibrillation auriculaire (FA) est un trouble du rythme au niveau atrial qui s'accompagne d'une augmentation de la morbi-mortalité en particulier en raison de ses complications emboliques dont l'accident vasculaire cérébral est la plus sévère. Le risque de développement d'une insuffisance cardiaque et d'une cardiopathie rythmique contribue au pronostic péjoratif de ce trouble du rythme.

De récentes études ont identifié de nouveaux facteurs de risque comme l'inflammation, le stress oxydant et des facteurs génétiques dans la genèse de la FA. Elle induit une dysfonction endothéliale, une majoration du statut inflammatoire et une activation plaquettaire. Le rôle de l'apoptose et de l'activation cellulaire, notamment endothéliale, dans la détermination d'un sur-risque thrombotique associé à la FA reste méconnu.

2-FA et sur-risque thrombotique

Sur le plan mécanique, la perte de la systole auriculaire est associée à une stase sanguine et à une diminution importante des forces de cisaillement. Quel que soit le facteur déclenchant, l'activation cellulaire au sens large, est associée à une libération de microparticules membranaires procoagulantes dans le compartiment vasculaire. L'endothélium est une surface hémocompatible dont la thrombogénicité se développe avec l'âge et les agressions inflammatoires. Les microparticules (MP) amplifient la réponse thrombogénique en délivrant un message pro-coagulant, pro-inflammatoire et pro-sénescent à la cellule endothéliale.

Dans la FA, comme dans d'autres pathologies associées à un risque thrombotique accru, une élévation importante des taux de microparticules procoagulantes dans la circulation veineuse a pu être démontrée. Dans le flutter auriculaire, nous avons pu montrer une génération importante de MP procoagulantes d'origine plaquettaire et leucocytaire associée à une activation de la coagulation au cours des ablations par radiofréquence (oreillette droite). Si l'impact clinique de cette activation de la coagulation reste probablement modeste lorsque les procédures d'ablation sont réalisées dans les cavités droites, cette majoration du statut thrombotique pourrait être plus critique lorsque les procédures d'ablation par radiofréquence sont faites dans les cavités gauches.

3-FA et remodelage

Si le mécanisme initiateur de la FA a été bien identifié, les mécanismes participant à la pérennisation du trouble du rythme sont beaucoup plus complexes. Des extrasystoles venant des veines pulmonaires dans l'oreillette gauche constituent le déclencheur de l'arythmie. Les techniques ablatives visant à une isolation électrique des veines pulmonaires constituent une avancée thérapeutique majeure et validée. La FA entraine tout d'abord un remodelage électrique puis structurel qui va modifier le substrat de l'arythmie à savoir le tissu atrial de l'oreillette gauche. Peu à peu de la fibrose va se développer créant par là même des modifications des propriétés électrophysiologiques favorisant la pérennisation de la FA. Les facteurs de risque cardiovasculaire, en particulier l'hypertension artérielle, des facteurs génétiques, un état inflammatoire, le syndrome d'apnée du sommeil vont également favoriser ce remodelage tissulaire qui entraine peu à peu une dilatation de l'oreillette gauche favorisant par là-même la recrudescence de l'arythmie par un mécanisme auto-entretenu. Comprendre les mécanismes du remodelage atrial et pouvoir le limiter constitue une étape majeure de la prise en charge de la FA.

4-Difference de statut thrombotique entre les oreillettes

Dans la FA, les raisons pour lesquelles la plupart des thrombi se forment dans l'oreillette gauche (OG) restent débattues. Des **don**nées récentes suggèrent qu'une activation plaquettaire plus intense dans l'OG pourrait expliquer le risque accru de formation de thrombus dans l'OG et non dans l'oreillette droite (OD). Parallèlement au lien unissant stase sanguine et formation de thrombus, il a été suggéré que le sur-risque thrombotique dans la FA pourrait s'expliquer par une dysfonction endothéliale majeure, une expression accrue de facteur tissulaire (FT) associée à une activation plaquettaire et à l'inflammation. Les MP circulantes sont de petites vésicules émises par un bourgeonnement

membranaire de la cellule en réponse à une activation, un stress ou un message pro-apoptotique. Ces MP sont libérées dans le compartiment vasculaire. Ces MP procoagulantes circulantes, principalement d'origine plaquettaire, leucocytaire ou endothéliale fournissent une surface phospholipidique additionnelle pour l'assemblage des complexes de la coagulation, favorisant par là-même la cascade de coagulation et la génération de thrombine. Les MP d'origine endothéliale et plaquettaire retrouvées à taux élevés dans l'AVC ischémique et associées à la taille de l'infarctus et au pronostic clinique ont été proposées comme marqueur de risque ischémique. De plus il a été décrit une augmentation de ces MP circulantes dans la FA persistante par rapport au rythme sinusal chez des patients comparables en terme de facteur de risque cardiovasculaire.

5-FA et senescence

Sur-risque thrombotique et remodelage tissulaire dans la FA impliquent des mécanismes communs comme l'apoptose, la senescence, l'inflammation et la dysfonction endothéliale. A ce jour, l'implication des processus de sénescence dans le sur-risque thrombotique et le remodelage tissulaire associée à la FA reste parfaitement méconnue.

Le vieillissement vasculaire est un facteur majeur de développement de fibrillation auriculaire, de remodelage des oreillettes et de développement de complications thrombotiques. Si, sur le plan épidémiologique, les liens entre vieillissement et thrombogénicité sont bien caractérisés, les mécanismes précis à l'échelle cellulaire et moléculaire restent largement spéculatifs. Jusqu'alors, aucun modèle de culture primaire de cellules endothéliales auriculaires n'avait été développé. Les données permettant de comprendre la physiologie de la cellule endothéliale auriculaire sont principalement issues d'extrapolations obtenues à partir de cellules endothéliales vasculaires. Une des principales limites de ces extrapolations est la reconnaissance d'une spécialisation possible de la cellule endothéliale en fonction de chaque territoire vasculaire et de ses propriétés rhéologiques. A titre d'illustration, le stretch (forces de cisaillement) joue un rôle majeur dans la survie, la senescence vasculaire et l'apoptose endothéliale. Ces forces de cisaillement, basses dans les cavités auriculaires en rythme sinusal sont encore probablement considérablement réduites en cas de développement de fibrillation auriculaire. A l'échelle cellulaire, la sénescence endothéliale est un mécanisme de réponse au stress caractérisé par un retrait permanente du cycle cellulaire et par l'acquisition de propriétés morphologiques et phénotypiques distinctes. Largement étudiée dans des modèles expérimentaux, l'importance des processus de sénescence a plus récemment été établie en pathologie cardiovasculaire notamment au cours des processus d'athérothrombose.

<u>Hypothèses et objectifs du travail</u>

Notre hypothèse est que la FA favorise un état prothrombotique et que les MPs y contribuent en fournissant une surface catalytique pour les réactions de la coagulation. Par ailleurs les MPs constitueraient une signature de l'activation cellulaire dans l'enceinte de l'oreillette accessible par la caractérisation de leur origine cellulaire. Dans ce contexte, les différentes techniques ablatives de FA pourraient avoir des impacts différentiés sur l'activation cellulaire et la génération de MPs dans l'oreillette. Par ailleurs, l'oreillette en FA présente un remodelage tissulaire d'abord électrique, contractile puis structurel (pathologique ou thérapeutique après ablation). Nous émettons l'hypothèse que ces mécanismes favorisent la sénescence endothéliale définie comme l'arrêt du cycle cellulaire. En effet la sénescence endothéliale est associée à un stress oxydant et à l'augmentation de l'expression de facteur tissulaire, initiateur cellulaire de la coagulation.

Les objectifs de ce travail sont:

1-Evaluer dans les deux oreillettes le sur-risque thrombotique dans la FA en mesurant la concentration des MPs procoagulantes et en déterminant leur origine cellulaire qui constitue une signature de l'activation cellulaire afin de déterminer une éventuelle différence du statut pro-thrombotique entre oreillettes droite et gauche.

2- Evaluer l'impact des techniques ablatives de la FA par radiofréquence dans l'oreillette gauche sur l'activation cellulaire et plaquettaire en mesurant la concentration des MP et comparer les effets de deux types de cathéters d'ablation.

3- Identifier la présence d'une sénescence dans la FA en analysant l'expression protéique de p53, p16, marqueurs clé de la sénescence cellulaire, du facteur tissulaire qui lui est associé, et des marqueurs de remodelage tissulaire dans les auricules de patients en FA et en rythme sinusal, prélevés lors d'une chirurgie cardiaque.
4- Mettre au point un modèle de sénescence réplicative et prématurée de cellules endothéliales auriculaires extraites d'oreillettes gauches de porc et étudier les modifications phénotypiques associées au développement de la senescence afin de comprendre la physiologie de la cellule endothéliale auriculaire.

Résultats

1- Pas de différence de statut pro-thrombotique entre l'oreillette gauche et droite de patients présentant de la FA.

Vingt-deux patients présentant de la FA paroxystique ou persistante symptomatique hospitalisés pour ablation de FA ont été prélevés dans l'OD puis dans l'OG après cathétérisme trans-septal avant l'ablation afin d'évaluer l'activation cellulaire et plaquettaire et des marqueurs d'inflammation dans les 2 oreillettes.
Aucune différence n'a été observée entre les deux oreillettes concernant les taux de MP totales, leucocytaires et plaquettaires. Les MP d'origine endothéliale étaient modestement augmentées dans l'OD par rapport à l'OG. L'activité Facteur tissulaire portées par les MP était également modérément augmentée dans l'OD par rapport à l'OG. Il n'y avait pas de corrélation entre le niveau des MP, l'âge, les diamètres des oreillettes ni la durée de l'histoire de FA. L'agrégation plaquettaire induite par le collagène était également plus élevée dans l'OD. Il n'y avait pas de différence entre les oreillettes concernant le niveau d'agrégation plaquettaire induite par l'ADP, ni la P-selectine, ni l'expression de PAC-1 (GPIIb/IIIa activé).
Cette étude montre l'absence de différence de statut pro-thrombotique dans l'OG par rapport à l'OD. De façon surprenante, les dommages endothéliaux et l'agrégation plaquettaire induite par le collagène sont même plus élevés dans l'OD allant à l'encontre des hypothèses émises quant à une activation plaquettaire majorée dans l'OG. L'aspect anatomique de l'OG avec en particulier l'auricule gauche de morphologie très variable et plus ou moins emboligène associé à un remodelage tissulaire plus marqué dans l'OG pourrait expliquer la propension des thrombi à se développer dans l'OG.

- Jesel L, Arentz T, Herrera-Siklody C, Trenk D, Zobairi F, Abbas M, Weber R, Minners J, Toti F, Morel O. **Do atrial differences in endothelial damage, leukocyte and platelet activation, or tissue factor activity contribute to chamber-specific thrombogenic status in patients with atrial fibrillation?** *Journal of cardiovascular electrophysiology 2014 Mar;25(3):266-70*

2- Caractérisation des dommages tissulaires et de leur importance sur la réaction inflammatoire, la myonécrose et la réponse prothrombotique dans l'ablation de FA et comparaison de deux types de cathéters de radiofréquence.

Dans l'ablation de FA, le cathéter irrigué ouvert est le plus utilisé. Il a été montré que le refroidissement de l'électrode permettait pendant l'ablation d'obtenir une énergie délivrée plus importante et une augmentation de la taille de la lésion. Des données expérimentales suggèrent que les cathéters à irrigation interne, donc sans ouverture du liquide vers le patient, auraient plus de risque de formation de thrombus sur l'électrode et donc de risque embolique.
Nous avons donc évalué l'impact de procédures d'ablation de FA en radiofréquence avec ces deux types de cathéters sur l'activation cellulaire et plaquettaire, les dommages tissulaires et l'inflammation.
Soixante patients ont été prélevés avant, juste après l'ablation dans l'oreillette droite puis à 24h et 48h sur voie veineuse périphérique. Un tirage au sort a réparti les patients en 2 groupes, cathéter à irrigation interne et cathéter à irrigation ouverte.

Les paramètres procéduraux étaient similaires dans les 2 groupes. Les dommages tissulaires (hs-troponine T et CPK) étaient maximum à 24h puis diminuaient après 24h, de façon similaire dans les 2 groupes. Les MP totales et plaquettaires augmentaient significativement à 24h et 48h. Les MP leucocytaires augmentaient de façon retardée par rapport aux MP totales. Il n'y avait pas de modification significative des MP endothéliales après l'ablation. Aucune différence n'était observée entre les 2 cathéters sur les taux de MP. L'activation plaquettaire (P-selectine, GPIIB/IIIa) majorée après l'ablation était similaire dans les deux groupes. La réponse inflammatoire augmentait significativement jusqu'à 48h sans différence entre les 2 groupes.
Cette étude montre que l'ablation de FA par radiofréquence entraine le même profil de dommages cellulaires, la même réaction inflammatoire et une majoration du statut pro-thrombotique similaire quel que soit le type de cathéter utilisé irrigué ouvert ou fermé.

- Laurence Jesel, Claudia Herrera Siklódy, Malak Abbas, Dietmar Trenk, Christian Stratz, Christian M. Valina, Reinhold Weber, Jan Minners, Valérie Schini-Kerth, Florence Toti, Thomas Arentz, Olivier Morel. **A Prospective Randomized Study Comparing an Open Irrigated Tip and an Internal Irrigated Tip Radiofrequency Catheter in atrial fibrillation ablation**.
  Soumis au Canadian Journal of Cardiology

3-FA et senescence

Une étude a montré dans un modèle animal qu'une senescence prématurée des myofibroblastes pouvait agir dans la régulation des mécanismes fibrotiques. Il n'y a pas de données dans la littérature sur la FA.

Nous avons essayé d'établir le lien entre FA et sénescence en analysant l'expression protéique de p53, marqueur clé de la sénescence cellulaire, du facteur tissulaire et des marqueurs de remodelage tissulaire (MMP-9) sur des auricules de patients en FA et en rythme sinusal, prélevés lors d'une chirurgie cardiaque. 147 patients ont été inclus dans l'étude. 21/147 patients présentaient de la FA paroxystique ou persistante. Ils ont été appariés à 21 patients en rythme sinusal ayant le même âge, des facteurs de risque cardiovasculaire identiques et des traitements similaires (Statines, ARA2 et IEC). Des westerns blots ont été réalisés afin d'étudier l'expression protéique de p53, marqueur de senescence, de MMP-9 impliquée dans le remodelage tissulaire, de facteur tissulaire (FT), initiateur de la coagulation et de eNOS, marqueur de la fonction endothéliale.
Les deux groupes de patients étaient similaires sur tous les paramètres cliniques, biologiques et échocardiographiques sauf la surface de l'oreille gauche en échographie était significativement plus dilatée dans le groupe en FA. L'expression de FT et de p53 était significativement augmentée dans le groupe en FA. MMP-9 était également augmentée dans le groupe en FA et la eNOS diminuée sans atteindre de signification statistique. MMP-9 était corrélée à l'âge, à la masse VG et au score de CHA2DSVASc. P53 était corrélée à l'expression de FT. La surface de l'OG n'était corrélée ni à l'expression de p53, ni de MMP-9 ni de FT.
Le seul facteur prédictif d'une élévation de la p53 était une histoire de FA paroxystique ou permanente ou de la FA au moment de la chirurgie. L'expression protéique de FT, MMP-9 et p53 ont été étudiée selon que le patient présentait du rythme sinusal (RS), de la FA paroxystique ou persistante. Il existait une augmentation progressive et significative de l'expression de ces 3 marqueurs entre le RS, la FA paroxystique puis la FA permanente indiquant que plus la pathologie est sévère plus le remodelage est important, la senescence marquée ainsi que le risque pro-thrombotique.
Cette étude montre l'existence d'une senescence majorée dans la FA. Il existe une incrémentation de la senescence associée au risque thrombotique et au remodelage tissulaire entre les patients en RS puis en FA paroxystique puis en FA permanente.

- Jesel Laurence[a,b] MD[§], Abbas Malak[a] PhD[§], Kindo Michel[b] MD, PhD, Hira Hasan[a], Zahid Niazi[a] PhD, Auger Cyril[a] PhD, Guillaume Manin MD[b], Sin Hee Park[a], Ohlmann Patrick[b] MD, PhD, Mazzucotelli Jean Philippe [b] MD, PhD, Schini-Kerth Valérie B[a] PhD*, Morel Olivier[a,b] MD, PhD*, Toti Florence[a] PhD*. **Impact of atrial fibrillation progression on human atrial senescence burden as determined by p53 and p16 expression**

4-Mise au point d'un modèle original de culture primaire de cellules endothéliales auriculaires extraites d'oreillettes gauches de porc. Etude des modifications phénotypiques associées au développement de la senescence.

A ce jour, l'implication des processus de sénescence dans le développement de la thrombogénicité et du remodelage tissulaire associée à la fibrillation auriculaire reste méconnue. Plusieurs travaux ont mis en exergue l'importance de l'angiotensine II dans le remodelage vasculaire associé à la fibrillation auriculaire via une stimulation des récepteurs AT1 et une activation des MAP kinases. Néanmoins, l'implication de l'Angiotensine II dans le développement de la senescence auriculaire reste à caractériser.

Dans un premier temps, nous avons développé un modèle original de culture primaire de cellules endothéliales auriculaires extraites à partir d'oreillettes gauches de porc. Ces cellules sont obtenues après trypsinisation de la cavité auriculaire puis les cellules endothéliales surnageantes sont recueillies et mises en culture.

Un modèle de sénescence réplicative, mise en évidence par une augmentation de l'expression de la SA ßgal, l'augmentation de l'expression de p53 un inducteur de sénescence, l'augmentation de p16 et p21, des inhibiteurs kinases cycline-dépendants a pu être caractérisé. La sénescence des cellules endothéliales auriculaires a également pu être induite par le L-NAME, un inhibiteur de la NO synthase, l'H_2O_2, un inducteur puissant de stress oxydant et de la sénescence vasculaire et par l'Angiotensine II.

Dans un second temps, nous nous sommes attachés à caractériser les modifications phénotypiques associées au développement de la sénescence endothéliale auriculaire. La sénescence endothéliale auriculaire s'accompagne d'une dysfonction endothéliale mise en évidence par une diminution de l'expression de la NO synthase. Cette sénescence s'accompagne de l'acquisition d'un phénotype pro-thrombotique caractérisé par (i) une augmentation de la libération de microparticules membranaires pro-coagulantes (ii) une augmentation de l'expression du facteur tissulaire, initiateur cellulaire majeur de la coagulation (iii) une augmentation de l'agrégabilité des plaquettes à la surface de la cellule endothéliale sénescente, probable reflet de la diminution de la production du monoxyde d'azote (NO). Les modifications pro-adhésives ou pro-inflammatoires sont illustrées par une augmentation de l'expression des cytoadhésines VCAM-1 et ICAM-1 qui jouent un rôle important dans l'adhésion et la diapédèse des cellules inflammatoires au sein des cavités cardiaques. Il existe par ailleurs une augmentation de l'expression de la métalloproteinase (MMP-2) qui joue un rôle important dans la protéolyse interstitielle impliquée dans le remodelage auriculaire associé à la fibrillation auriculaire. De plus, l'augmentation de l'expression du récepteur AT1 de l'Angiotensine II et de l'enzyme de conversion de l'angiotensine à la surface des cellules endothéliales auriculaires sénescentes témoigne de l'activation locale de ce système au cours du vieillissement.

Nous nous sommes enfin intéressés à la possibilité de réguler sur le plan pharmacologique ce phénomène de vieillissement des cellules endothéliales auriculaires. Dans ce modèle, le losartan, un inhibiteur des récepteurs AT1 de l'angiotensine II tout comme les inhibiteurs de l'enzyme de conversion inhibent le développement de la sénescence endothéliale auriculaire.

Au total, ce travail a permis le développement d'un modèle original de culture primaire de cellules endothéliales auriculaires permettant une compréhension approfondie de la physiologie de la cellule endothéliale auriculaire. Ce modèle permet notamment l'étude des modifications phénotypiques associées au développement de la senescence. La sénescence endothéliale auriculaire est associée à l'acquisition d'un phénotype pro-thrombotique (génération de microparticules pro-coagulantes, expression accrue du facteur tissulaire, majoration de l'agrégation plaquettaire), pro-inflammatoire

(augmentation de l'expression des cytoadhésines ICAM-1 et VCAM-1), protéolytique (MMP-2) et à l'activation locale du système local de l'angiotensine. Enfin, la possibilité de réguler pharmacologiquement la sénescence endothéliale auriculaire via l'inhibition des récepteurs AT1 de l'Angiotensine II a pu être établie.

- Jesel Laurence[a,b] MD[§], Abbas Malak[a] PhD[§], Hira Hasan, Auger Cyril[a] PhD, Guillaume Manin[b], Sin Hee Park[a], Ohlmann Patrick[b] MD, PhD, Schini-Kerth Valérie B[a] PhD*, Morel Olivier[a,b] MD, PhD*, Toti Florence[a] PhD*. **Endothelial atrial cells senescence induces thrombogenicity, inflammation and metalloproteinase expression: Role of Ang II/ATI receptor pathway**

Conclusion et perspectives

Nos travaux ont permis de mettre en évidence que les MPs contribuent à un état pro-thrombotique et pro-inflammatoire dans la FA et dans l'ablation de ce trouble du rythme. Leur activité protéolytique susceptible de favoriser la migration embolique mais possiblement aussi le remodelage tissulaire devra être étudiée dans des modèles spécifiques. Nous avons également montré l'importance de la sénescence endothéliale dans l'altération tissulaire des oreillettes en FA qui évolue avec la sévérité du remodelage associé à la progression du trouble du rythme. Notre modèle cellulaire devrait permettre de comprendre la physiologie de la cellule endothéliale auriculaire lors de la FA en évaluant l'effet de MP isolées de patients en FA.

II. Etat de la question

II. Etat de la question

1. La fibrillation auriculaire

1.1 Généralités.

1.1.1 Epidémiologie et définitions

La fibrillation auriculaire (FA) est un trouble du rythme au niveau des oreillettes. C'est la plus fréquente des arythmies avec une prévalence de 1% dans la population jeune et jusqu'à 9% chez les patients de plus de 75 ans. Il est estimé que sa prévalence sera multipliée par 3 vers 2050 [1]. La FA n'est pas un trouble du rythme bénin. Elle s'accompagne d'une augmentation de la morbi-mortalité, actuellement 3.5% par an [2], en particulier en raison de ses complications emboliques dont l'accident vasculaire cérébral est la plus sévère et du risque de développer une insuffisance cardiaque et une cardiopathie rythmique. Les AVC d'origine cardio-embolique sont également plus sévères avec une mortalité et des séquelles neurologiques plus importants que les AVC d'autres origines. En cas de FA, il a clairement été établi un risque majeur d'altération des fonctions cognitives et de survenue d'une démence par rapport aux patients en rythme sinusal normal (RS) et ce sans AVC constitué[3, 4]. La contraction anarchique de l'oreillette a comme conséquence une diminution du remplissage du ventricule gauche et une baisse du débit cardiaque de 20 à 30% d'où la dyspnée d'effort très souvent rapportée par le patient. La cadence ventriculaire rapide et irrégulière peut dans certains cas entraîner une véritable cardiopathie rythmique avec altération sévère de la fraction d'éjection pouvant conduire à un choc cardiogénique. Sa caractéristique est d'être réversible à la restauration du rythme sinusal. Tous ces éléments conduisent à en faire un véritable problème de santé publique ce d'autant que le coût de santé lié à cette pathologie croissante est majeur. Une étude française a montré en 2011 une prévalence entre 600.000 et un million de patients en FA dont deux tiers de plus de 75 ans. L'incidence était de 110.000 à 230.000 nouveaux cas /an d'où l'importance d'optimiser les thérapies qui apportent un bénéfice clinique et économique net[5]. Déjà en 2004, l'étude COCAF[6] soulignait un taux d'hospitalisation et de décès supérieur dans la FA permanente par rapport à la FA paroxystique et un coût majeur lié à l'hospitalisation et aux médicaments.

La FA entraine un rythme irrégulier au niveau ventriculaire. L'oreillette en fibrillation présente une contraction désynchronisée à une fréquence entre 400 et 600 bpm qui est transmise par le nœud auriculo-ventriculaire (NAV) au ventricule à une cadence dépendant des propriétés électrophysiologiques du NAV. Le patient la ressent le plus souvent sous forme de palpitations irrégulières, d'esssoufflement ou de sensations de mal être. Le patient peut être également totalement asymptomatique et la survenue d'un évènement cardio-embolique peut être la première manifestation du trouble du rythme.

Il existe des causes réversibles de FA et une fois l'épisode passé, le risque de récurrence n'est pas établi. L'hyperthyroïdie en est la 1ére cause suivie de l'intoxication éthylique aigue, de toute pathologie cardiaque aigue (infarctus du myocarde, péricardite, myocardite) ou de survenue en post-chirurgie cardiaque ou pulmonaire.
La FA peut être familiale et certains facteurs génétiques (gènes et polymorphisme génétiques) ont été décrits.[7, 8] L'analyse génétique dans la FA à contrario de certaines canalopathies ou cardiopathies ne fait pas partie de la prise en charge thérapeutique actuelle du trouble du rythme.

1.1.2 Facteurs favorisants et estimation du risque associé à la FA

Les facteurs de risque cardiovasculaire favorisent la survenue de FA en particulier l'hypertension artérielle (HTA) en favorisant le remodelage structurel de l'oreillette. Ils interviennent aussi dans l'évaluation du risque thrombotique associé. Le score CHA2DS2VASC (figure 1) permet de calculer un score de risque de survenue d'AVC en contexte de FA. Pour un score ≥ 1, une anticoagulation peut être envisagée, le fait d'être une femme n'intervenant que si un autre facteur de risque est présent.[9]

CHA2DS2-VASc Risk	Score
CHF or LVEF ≤ 40%	1
Hypertension	1
Age ≥ 75	2
Diabetes	1
Stroke/TIA/ Thromboembolism	2
Vascular Disease	1
Age 65 - 74	1
Female	1

Figure 1 : Score CHA2DS2-VASC (ESC Guidelines 2012)[9]

La FA peut survenir sur cœur sain et souvent les veines pulmonaires (VP) sont impliquées comme déclencheurs mais elle peut également survenir en contexte de cardiopathie et les mécanismes impliqués sont alors plus complexes. Toute atteinte cardiaque structurelle peut entraîner de la FA qui n'a du coup pas le même pronostic. La cardiopathie hypertrophique obstructive ou post-HTA, les cardiopathies valvulaires (mitrale) et la cardiopathie hypokinétique sont les plus fréquentes.

On distingue la « FA non-valvulaire » de la « FA valvulaire ». En effet toute pathologie de la valve mitrale, en particulier le rétrécissement mitral entraîne une dilatation de l'oreillette gauche avec un remodelage majeur et la survenue irrémédiable de FA. Une FA est considérée comme « valvulaire » s'il existe un rétrécissement mitral ou une prothèse valvulaire mitrale[10].

1.1.3 Les différents types de FA

Il existe 3 types de FA. La FA paroxystique est caractérisée par des épisodes récurrents de moins de 7 jours selon la définition[9]. La FA persistante est récurrente soutenue et nécessite une cardioversion pharmacologique ou électrique pour restaurer le rythme sinusal. La FA est permanente quand elle date de plus de 1 an et quand il y a échec de toutes les thérapeutiques pour maintenir le RS. Cette classification s'applique à des épisodes de FA d'une durée >30 secondes, pour lesquelles une origine secondaire ne peut être mise en évidence.

De nombreux facteurs cliniques favorisent soit l'initiation soit la pérennisation de la FA avec des impacts propres sur des mécanismes cellulaires sous-tendant la FA (figure 2).

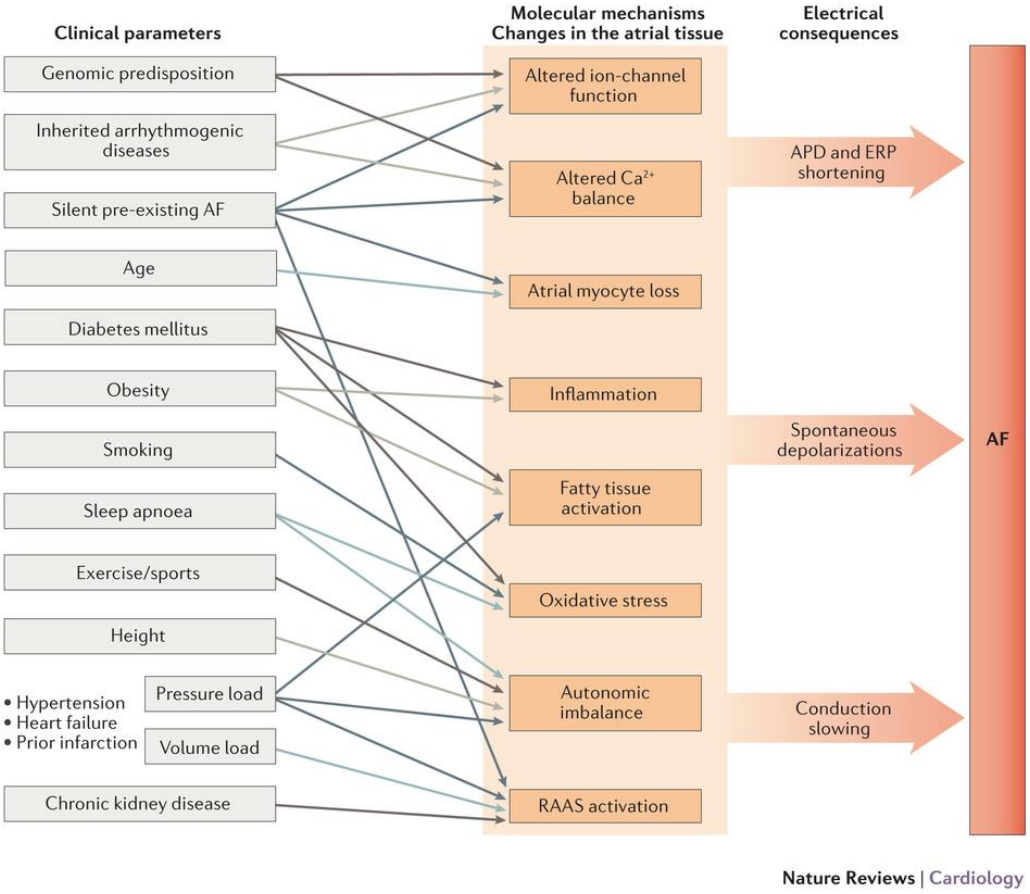

Figure 2: Hypothétiques mécanismes d'interactions cliniques associées à la FA

APD, action-potential duration; ERP, effective refractory period; RAAS, renin–angiotensin–aldosterone system. [2]

1.2 Mécanismes de la FA

Pour exister, une arythmie a besoin d'un substrat où elle se propage, d'un déclencheur, le « trigger », et d'un modulateur. C'est le triangle de Coumel. En 1998, l'équipe de Michel Haïssaguerre montrait que la FA s'initiait par des battements ectopiques à partir les veines pulmonaires (VP) dans l'oreillette gauche (OG)[11] . Dans la FA, le substrat est l'OG, le trigger sont les extra-systoles venant des VP, le modulateur est le système nerveux autonome.
Dès lors, une attention accrue a été consacrée au rôle des veines pulmonaires (VP), de la paroi postérieure de l'oreillette gauche (OG) et de l'innervation locale par le système nerveux autonome. De plus, cela a renforcé le concept selon lequel la FA nécessite un élément déclencheur *(trigger)* et un substrat anatomique capables d'initier et de maintenir l'arythmie. En parallèle, des travaux récents ont également mis en évidence le rôle d'autres mécanismes, tel que la réentrée électrique, le remodelage du tissu atrial ou l'influence du système nerveux autonome[12] (fig. 2).

1.2.1 Réentrées multiples

Jusqu'à la fin des 1980, l'hypothèse de multiples ondelettes de réentrée était généralement reconnue comme le mécanisme principal de la FA. Selon cette hypothèse, la FA résulterait du fractionnement de fronts d'ondes se propageant dans les oreillettes, entraînant des «ondelettes filles» qui s'auto-entretiennent [12](fig. 3A). D'après ce modèle, la quantité d'ondes présentes à un moment donné dépend de la longueur d'onde du circuit de réentrée (qui est le produit de la vitesse de conduction atriale et de la période réfractaire) et de la masse atriale. En effet, la persistance de la FA est favorisée par une augmentation de la masse atriale, un raccourcissement de la période réfractaire et une diminution de la vitesse de conduction. En pratique, la stimulation vagale diminue la période réfractaire,
entraînant une diminution de la taille des circuits permettant ainsi à une quantité supérieure de circuits de coexister dans un même espace, favorisant ainsi le maintien de l'arythmie. En revanche, les traitements anti-arythmiques augmentent la longueur d'onde, diminuant ainsi le nombre de circuits et pouvant donc entraîner la disparition de la FA[13]. A noter également que la procédure chirurgicale du Maze a été développée à partir de ce modèle et du concept que le maintien de la FA nécessite un nombre critique d'ondes de réentrées, chacune nécessitant une masse critique de tissu atrial[14] .

1.2.2 Foyers déclenchants

Haïssaguerre et collaborateurs[11, 15] ont décrit en 1994 une série initiale de trois patients avec une FA traitée par ablation par radiofréquence. Dans chaque cas, il fut démontré que la FA provenait d'une «source focale». Ce résultat suggéra donc que chez certains patients, la FA pouvait être causée par un déclencheur *(trigger)* focal, dont l'ablation pouvait faire disparaître l'arythmie. Ces foyers ectopiques rapides étaient localisés en grande majorité au niveau des orifices des VP. Ces observations ont conduit les investigateurs à systématiquement tenter le traitement de la FA paroxystique par la cartographie et l'ablation individuelle de foyers avec une activité ectopique. Par la

suite, d'autres foyers ont également été identifiés au niveau de la paroi postérieure de l'OG, de l'oreillette droite, de la veine cave supérieure, du ligament de Marshall, de la crista terminalis et du sinus coronaire[11] (fig. 3B).

Les foyers se situent dans des manchons de tissu musculaire atrial s'étendant dans ces structures. Les mécanismes impliqués dans la production de l'activité ectopique par ces manchons musculaires, ainsi que le mécanisme exact de l'initiation de la FA par l'activité rapide ne sont à ce jour pas résolus. Il a cependant été démontré que les VP présentent une hétérogénéité des périodes réfractaires effectives qui pourrait favoriser la formation de réentrées[16]. Par ailleurs, sur la base d'études animales et histologiques, des mécanismes tels que la réentrée, l'automaticité augmentée ou l'activité déclenchée ont été proposés[17]. Il est tout à fait probable que ces trois mécanismes soient chacun impliqués dans des proportions différentes avec un foyer ectopique (mécanisme d'automaticité anormale ou activité déclenchée) qui déclencherait une réentrée initialement autour des VP, se généralisant par la suite en réentrées multiples dans les oreillettes.

1.2.3 Rotors

Des travaux effectués chez les animaux (chèvre, chien) ont suggéré que dans certains cas, la FA pouvait être maintenue par un *rotor* qui est une onde spiralée rotationnelle mobile à haute fréquence (sorte de tornade), située habituellement au niveau de la paroi postérieure de l'OG et entraînant une distribution spatiale de gradients de fréquences[18, 19]. Selon cette hypothèse, des fronts d'ondes rapides successives issues d'un foyer ectopique au niveau d'une VP peuvent, en présence d'un substrat hétérogène approprié, se briser et engendrer deux vortex tournant en sens contraires. A terme, seul un de ces rotors persiste, devenant ainsi une machine à haute fréquence qui maintient la FA en transmettant une conduction fibrillatoire au reste des oreillettes. Ainsi, des foyers déclenchants situés au niveau ou proches des VP pourraient initier des rotors suite à la conduction anormale d'une impulsion et de la rupture du front d'onde. Un rotor situé à n'importe quel niveau de l'OG qui générerait des ondes en spirales à une fréquence extrêmement élevée, pourrait alors devenir la source dominante responsable du maintien de la FA. Récemment, l'équipe d'Haissaguerre, grâce à une technique de cartographie non invasive (Cardioensight), a montré l'existence de régions motrices dans la FA persistante dont le nombre augmente avec la durée de la FA [20]. Cette nouvelle technologie permet de mieux cibler les zones d'intérêt pour guider l'ablation de FA (figure 4).

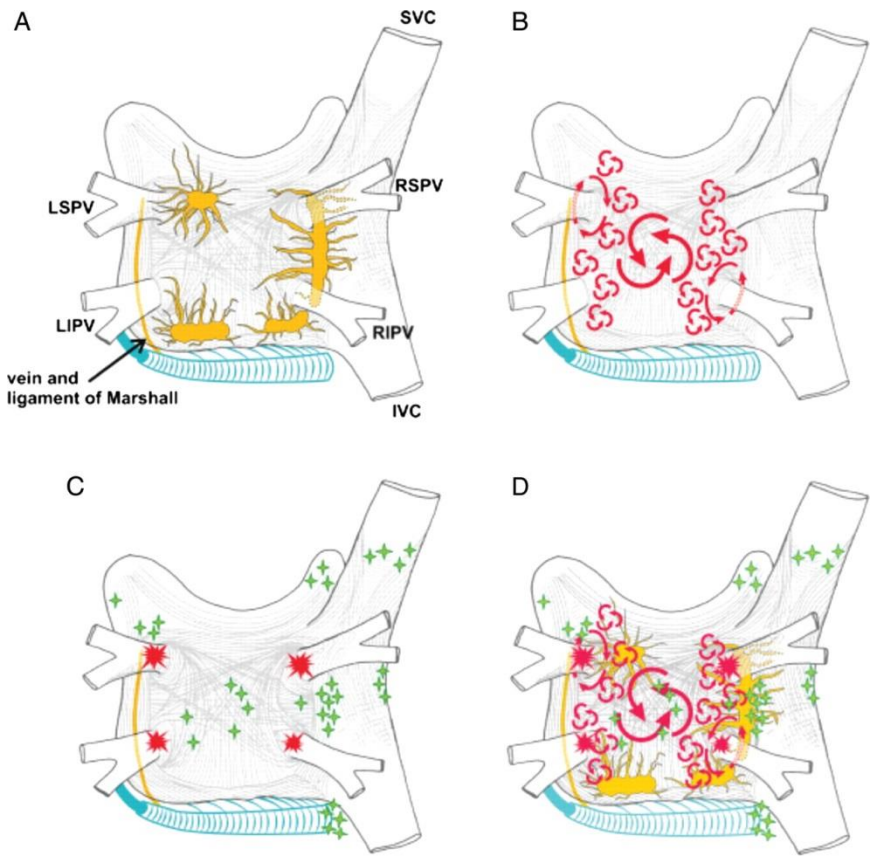

Figure 3 : Structure et mécanismes de la FA. [12]
A. Représentation schématique des oreillettes en vue postérieure. L'extension des fibres musculaires dans les VP est indiquée. Le sinus coronaire (représenté en bleu) est enveloppé par des fibres musculaires connectées aux oreillettes. La veine et le ligament de Marshall (également en bleu) cheminent depuis le sinus coronaire jusqu'à la région entre la VP supérieure gauche l'appendice auriculaire gauche. Les circuits de micro et macro réentrée jouant un rôle dans l'initiation et le maintien de la FA sont représentés par les flèches rouges.
B. Sites communs des foyers déclenchant dans les VP (rouge) ainsi que des foyers non-veineux pulmonaires (en vert).
C. Représentation (en jaune) des quatre principaux plexus ganglionnaires du système nerveux autonome (supérieur gauche, inférieur gauche, antérieur droit et inférieur droit).
D. Composite des différents mécanismes de la FA.

VPSG = veine pulmonaire supérieure gauche; VPIG = veine pulmonaire inférieure gauche; VPSD = veine pulmonaire supérieure droite; VPID = veine pulmonaire inférieure droite; VCS = veine cave supérieure; VCI = veine cave inférieure.

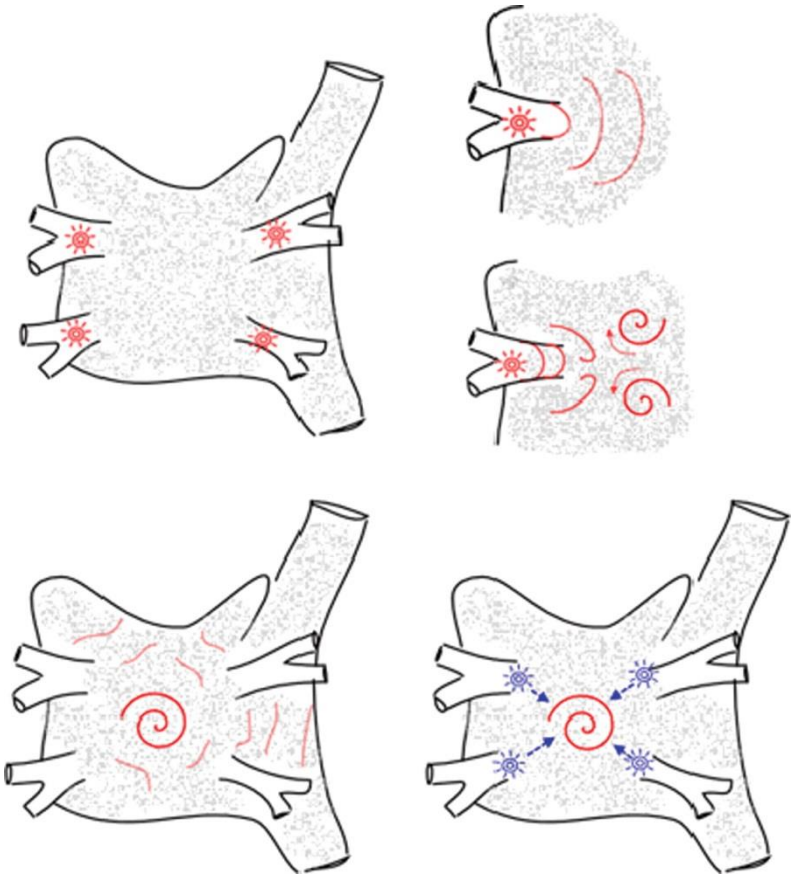

Figure 4 : Représentation schématique illustrant la manière dont des foyers déclenchant mènent à l'initiation de rotors. A terme, le remodelage atrial entraîne d'autres foyers et la perpétuation de la réentrée.[11]

1.3 L'ablation

1.3.1 Définition et énergies utilisées

L'ablation est une technique curative des arythmies et fait partie de leur prise en charge quotidienne. Le but de l'ablation est de convertir des tissus normalement actifs sur le plan électrique en tissus cicatriciels électriquement inactifs. A l'heure actuelle la radiofréquence est la source d'énergie la plus souvent utilisée pour réaliser les lésions d'ablation qui doivent être transmurales pour être effectives. Un courant électrique est délivré à l'extrémité du cathéter d'ablation entraînant un échauffement des tissus en regard. La température supérieure à 48°C produit une dessiccation tissulaire, dénaturation des protéines et coagulation du sang. La lésion va se transformer en cicatrice fibreuse homogène bien déterminée. La taille de la lésion est fonction de l'énergie délivrée (puissance du générateur), la surface de l'électrode, le contact entre l'électrode et l'endocarde et la durée d'application du courant. La cryothérapie, une autre source d'énergie est actuellement fréquemment utilisée dans l'ablation de FA. Un ballon gonflé à l'entrée des VP et

occlusif est refroidi par de l'azote entraînant une lésion circonférentielle autour des VP. D'autres systèmes existent utilisant d'autres sources d'énergie, plus rarement utilisés ou en voie de développement

1.3.2 Technique et stratégie d'ablation

Idéalement, le traitement de la FA devrait avoir comme cible les différents mécanismes physiopathologiques, pouvoir éliminer la FA ainsi que d'autres arythmies atriales concomitantes, restaurer le rythme sinusal ainsi que rétablir la fonction contractile et chronotrope, et finalement être associée à un minimum de complications ainsi qu'à des effets pronostiques favorables au long cours. Les traitements anti-arythmiques actuels présentent une efficacité limitée et sont fréquemment associés à des effets secondaires importants, rendant la prise en charge pharmacologique souvent décevante[21]. Les progrès réalisés dans la compréhension de certains des mécanismes à l'origine de la FA ont mené à l'ablation par radiofréquence, dont le principe est l'interruption de la conduction de l'activité électrique entre les tissus contenant les déclencheurs arythmogènes, le plus fréquemment les VP et les oreillettes, et la modification du substrat atrial responsable du maintien de la FA.

Les premières procédures d'ablation par radiofréquence cherchaient à reproduire le Maze chirurgical (compartimentation chirurgicale de l'OG) par la création de lésions linéaires dans l'endocarde atrial [22]. Les modestes taux de succès (40% à 50%) associés à un risque élevé de complications ont freiné l'enthousiasme pour cette technique. Cependant, la découverte que des potentiels issus des VP provoquaient la FA, et la démonstration que l'élimination de ces foyers pouvait terminer l'arythmie provoqua un regain d'intérêt majeur pour ce type de procédure [11]. La technique, qui visait initialement à ablater des foyers individuels au niveau des VP, évolua par la suite vers l'isolement électrique circonférentielle de la musculature des VP [23] (fig. 5A), puis fut encore modifiée par l'incorporation de plusieurs lésions linéaires, notamment au niveau de l'OG postérieure et de l'isthme mitral (fig. 5B, 5C). Finalement, l'ablation d'électrogrammes complexes fractionnés (multiples potentiels de bas voltage au niveau des oreillettes) a également montré des résultats positifs (fig. 5D) [24]. Il est probable que les stratégies actuelles d'ablation par radiofréquence aient un impact autant sur les éléments déclenchants que sur le substrat capable du maintien de l'arythmie Le taux de succès est variable selon que la FA est paroxystique ou persistante (de 56% à 87% des patients sans récidive de FA à 1 an)[25],[26], avec parfois la nécessité de réitérer la procédure.

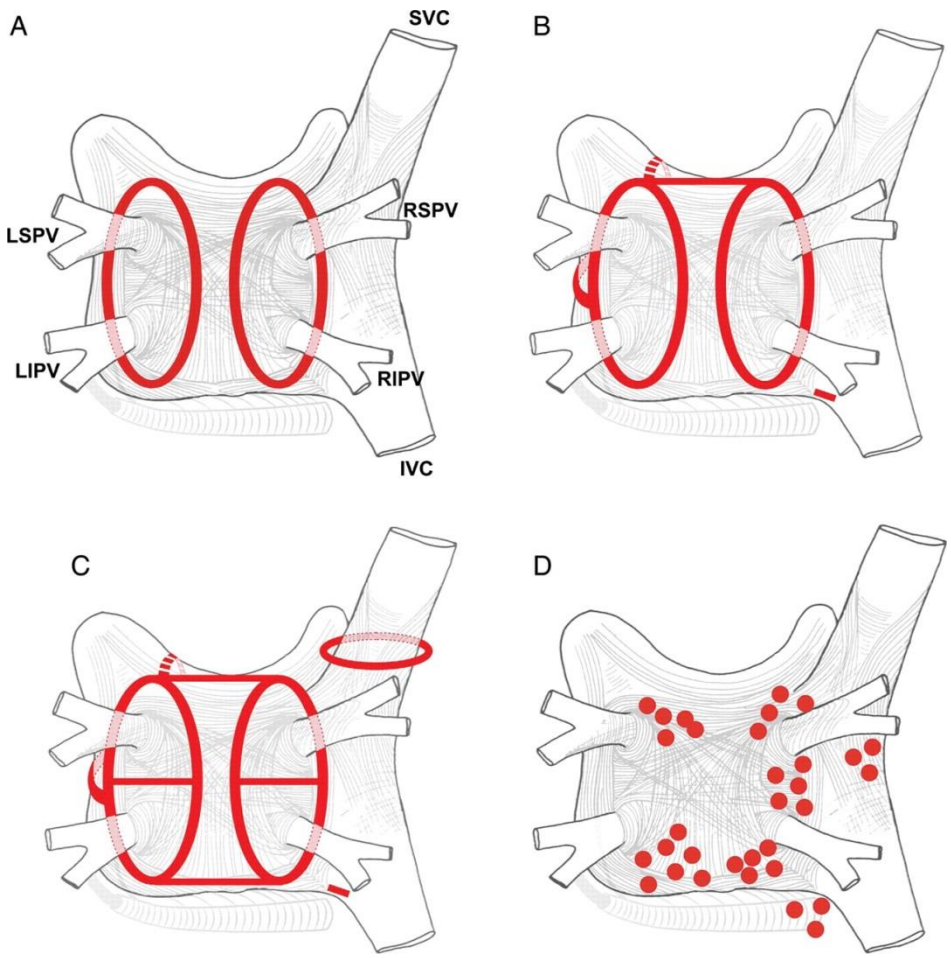

Figure 5: Représentation schématique des types de lésions effectuées lors de l'ablation de FA. [12]

A Lésions d'ablations circonférentielles autour des VP gauches et droites. L'objectif primaire de cette stratégie est l'isolement électrique de la musculature des VP.
B Sites les plus fréquents d'ablations linéaires. Ceux-ci comprennent des lésions encerclant les VP gauches et/ou droites, une ligne de «l'isthme mitral» connectant la valve mitrale et la lésion autour des VP gauches au niveau de la VP inférieure gauche, et une lésion linéaire antérieure connectant soit la «ligne du toit» ou la lésion circonférentielle droite ou gauche et l'anneau mitral antérieur. Une lésion linéaire au niveau de l'isthme cavo-tricuspidien est aussi représentée. Cette lésion est généralement effectuée chez les patients présentant un flutter atrial isthmo-dépendant clinique ou induit lors de l'examen électrophysiologique.
C Similaire à B, mais avec des lésions additionnelles entre les VP supérieures et inférieures, produisant en une lésion en «8». L'isolement de la veine cave supérieure est effectué si un foyer à ce niveau est démontré. Certains opérateurs isolent empiriquement la veine cave supérieure.
D Certains des sites d'ablations lorsque des électrogrammes complexes fractionnés sont visés.

1.3.3 Résultats de l'ablation

En clinique, l'intérêt des techniques ablatives n'est plus à démontrer et elles peuvent être proposées en même première intention dans la FA paroxystique et persistante depuis les recommandations de l'ESC 2012 [27] (figure 6, 7).

	CLASS	LEVEL
INDICATIONS FOR CATHETER ABLATION of AF		
Symptomatic AF refractory or intolerant to at least one Class 1 or 3 antiarrhythmic medication		
Paroxysmal: Catheter ablation is recommended*	I	A
Persistent: Catheter ablation is reasonable	IIa	B
Longstanding Persistent: Catheter ablation may be considered	IIb	B
Symptomatic AF prior to initiation of antiarrhythmic drug therapy with a Class 1 or 3 antiarrhythmic agent		
Paroxysmal: Catheter ablation is reasonable	IIa	B
Persistent: Catheter ablation may be considered	IIb	C
Longstanding Persistent: Catheter ablation may be considered	IIb	C

Figure 6 : Recommandations de l'ablation de FA (ESC 2012)
Classe I preuves cliniques d'efficacité et de bénéfice. Classe II preuves cliniques divergentes sur l'intérêt et l'utilité, (a) poids des données en faveur et devrait être considéré ; (b) utilité et efficacité moins bien établies, peut être considéré. Classe III Avis général de non-efficacité. N'est pas recommandé.

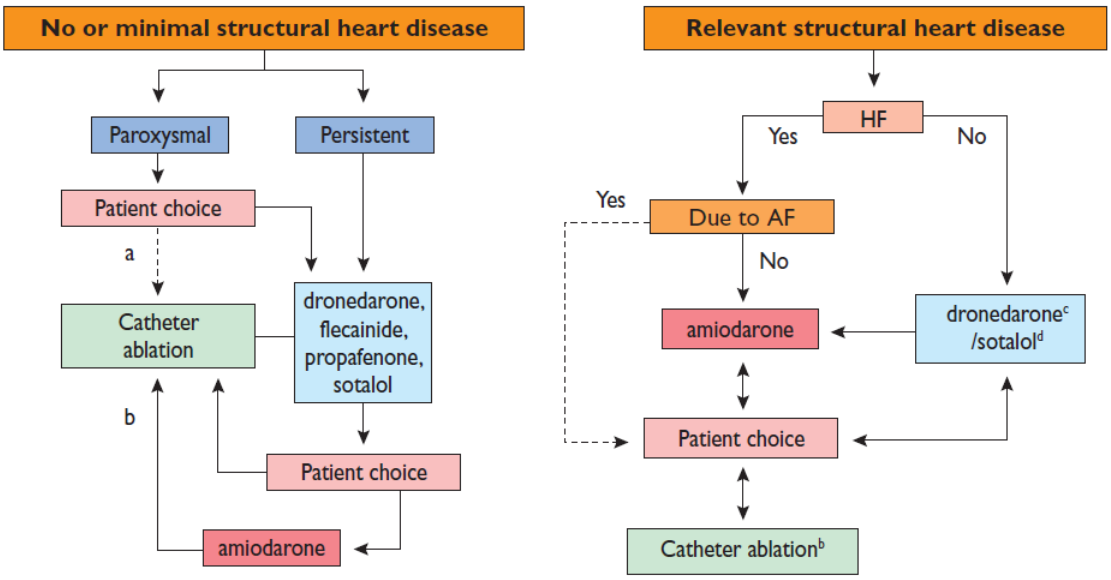

Figure 7: Recommandations ESC 2012: anti-arythmiques ou ablation pour le contrôle du rythme dans la FA.[27]

Plusieurs études ont montré à 12 mois un maintien du rythme sinusal de façon très supérieure chez le patient ablaté par rapport au patients traité par anti-arythmiques, 55.9% vs 8.7% dans CACAF[26], 75% vs 7% dans A4[28]. La mortalité et les taux d'hospitalisation après ablation sont également très inférieurs comparé au traitement anti-arythmique, probablement à cause de l'effet pro-arythmogène de ces

médicaments[21]. L'équipe de Bordeaux a été dans les premières à montrer l'effet bénéfique de l'ablation de FA persistante dans l'insuffisance cardiaque avec une amélioration de la fraction d'éjection ventriculaire gauche de 35 à 55% et une diminution des diamètres ventriculaires[11]. Déjà en 2005, Reant et al [29] a mis en évidence chez le patient ablaté pour une FA isolée sans cardiopathie, un remodelage inverse au niveau atrial et ventriculaire gauche avec diminution de leurs diamètres ainsi qu'une amélioration de la fonction diastolique (figure 8). Ce remodelage positif sera confirmé par plusieurs études également dans l'ablation de FA persistante [30] parallèlement à une amélioration des symptômes et de la qualité de vie[31, 32].

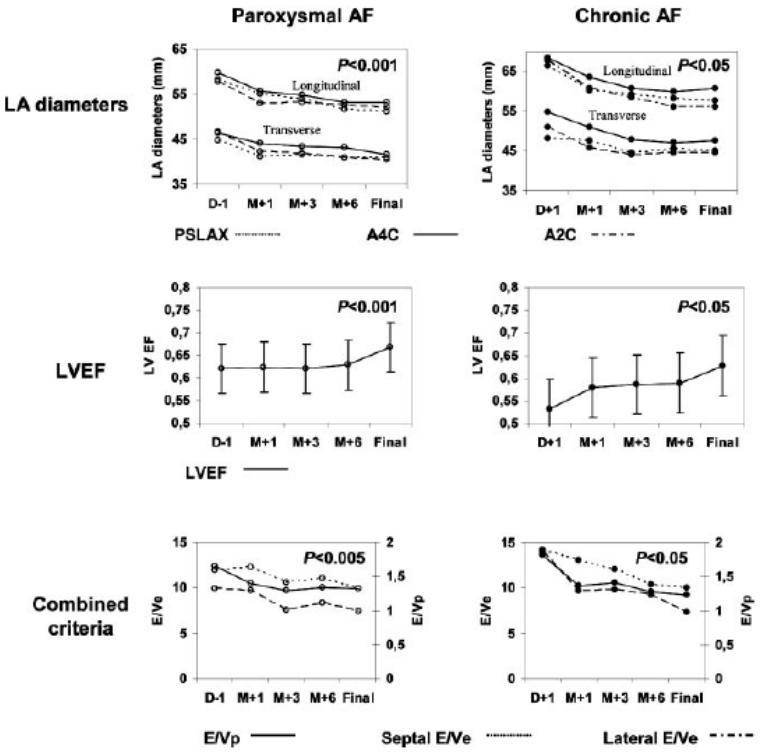

Figure 8 : Remodelage significatif et progressif de l'OG et amélioration de la FEVG et des diamètres ventriculaires après 11 mois de suivi après ablation de FA. Etude de 48 patients. Dans la 1ère phase (0 à 3 mois), la taille de l'OG diminue et la FEVG reste stable pour la FA paroxystique et augmente pour la FA persistante. Dans la 2ème phase (3-12 mois), les dimensions OG restent stables et la FEVG augmente.[29]

Ces techniques ablatives ont un taux de récidive à 3 mois de 20 à 30% pour la FA paroxystique et de 30 à 50% pour la FA persistante en fonction des études, du follow-up et des critères de succès considérés (anti-arythmiques ou pas). Très souvent une 2ème procédure voire plus améliore le taux de succès aux alentours de 80-90% en fonction du type de FA. Une reconnection électrique des VP est souvent objectivée en post-ablation du fait d'une lésion d'ablation insuffisamment étendue une fois la phase inflammatoire post-ablation terminée.

Par contre, le suivi à plus long terme montre dans les 2 types de FA un taux de récidive supérieur à 5 ans par rapport à ceux objectivés à un an de la procédure[33]. Dans la FA paroxystique où les extra-systoles des VP sont le mécanisme initiateur majeur, la reconnection électrique des VP à distance de

la procédure d'ablation est également très souvent retrouvée en cas de récidives tardives témoignant d'un remodelage tissulaire à ce niveau. La « réablation » de ces reconnections donnent d'ailleurs de bons résultats. Dans la FA persistante, les mécanismes de récidive sont plus complexes et sont également le reflet d'un remodelage du tissu atrial. Il témoigne des altérations dynamiques du substrat sous-jacent de même que l'impact des pathologies associées comme l'HTA, le diabète, l'apnée du sommeil sur l'histoire naturelle de la FA[34].

La taille de l'OG, la présence de fibrose détectée à l'IRM, la présence d'une cardiopathie et la durée de l'histoire de FA ont d'ailleurs été bien corrélés au risque de récidive. L'intérêt de certaines molécules à effet anti-fibrotique comme les inhibiteurs de l'enzyme de conversion (IEC) ou les inhibiteurs de l'angiotensine II (ARA2) et bien entendu le contrôle de tous les facteurs de risque favorisant le remodelage atrial ont été évoqués dans la modulation de ce dernier le plus précocement possible. Contrôler et limiter le remodelage atrial en prévention primaire et secondaire constituerait une avancée clinique majeure dans la prise en charge de la FA.

2. Le remodelage atrial

L'expérience clinique suggère que la FA paroxystique progresse fréquemment en FA persistante, et plus la FA persiste, plus il est difficile de rétablir le rythme sinusal ou de le maintenir après cardioversion. Si le mécanisme initiateur de la FA a été bien identifié, les mécanismes participant à la pérennisation du trouble du rythme sont beaucoup plus complexes. Des études animales ont montré que la FA modifie certaines propriétés au niveau des oreillettes afin de favoriser son propre maintien, menant ainsi au principe selon lequel *la « FA engendre la FA »*[35]. Le remodelage atrial est donc causé par les pathologies associées et par la FA elle-même et peut pré-exister à la FA.

La FA entraine par elle-même un remodelage électrique puis structurel qui va modifier le « substrat » de l'arythmie à savoir le tissu atrial de l'oreillette gauche. Peu à peu des zones fibreuses vont se développer créant par là même des modifications des propriétés électrophysiologiques favorisant la pérennisation de la FA. Les facteurs de risque cardiovasculaire, en particulier l'hypertension artérielle, des facteurs génétiques, un état inflammatoire, le syndrome d'apnée du sommeil vont également favoriser ce remodelage tissulaire qui entraine peu à peu une dilatation de l'oreillette gauche favorisant par là-même la recrudescence de l'arythmie par un mécanisme auto-entretenu[2]. Comprendre les mécanismes du remodelage atrial et pouvoir le limiter constitue une étape majeure dans l'amélioration de la prise en charge de la FA.

2.1 Le remodelage électrique

La première étape du remodelage est le *remodelage électrique* et consiste en des modifications des canaux ioniques et de l'équilibre intracellulaire du calcium, résultant en un raccourcissement du potentiel d'action (PA) et des périodes réfractaires effectives[36]. Ce phénomène commence déjà quelques minutes après le début de la FA, et devient complet en 24 à 48 heures. La FA induit une

réduction notable des courant Ca^{2+} type-L expliquant le raccourcissement du PA et la perte d'adaptation physiologique de la durée du PA à la fréquence. Les courant potassiques Ito entre autres sont également diminués[36].

Cependant, suite à cette période, la probabilité d'une persistance de la FA continue à s'accroître, suggérant l'implication de mécanismes additionnels augmentant la vulnérabilité par rapport à la FA. La réversibilité du remodelage électrique a été étudiée après retour en rythme sinusal (RS) chez l'animal et chez l'homme. Même après une période prolongéee de FA, le raccourcissement de la période réfractaire et la diminution de l'adaptation à la fréquence reste complètement réversible[36]. Le fait que la période réfractaire atriale redevienne normale seulement après quelques jours de RS a d'importantes implications cliniques. Ainsi, les récurrences de FA survenant plus d'une semaine après la cardioversion ne peuvent s'expliquer par des anomalies de la repolarisation dues au remodelage électrique.

Il n'a pas encore été clairement établi si une fréquence atriale rapide prolongée conduit à un ralentissement de la conduction atriale. Chez le chien, après 42 jours de stimulation, une diminution de 25% de la vitesse de conduction dans l'oreillette a été rapportée[37]. Chez la chèvre, cela n'a pas été retrouvé[38]. De même, le rôle des jonctions cellulaires dit « gap junctions » de l'oreillette dans le ralentissement de la vitesse de conduction n'est pas clair. Elvan[39] rapporte une augmentation de l'expression de la connexine 43 chez le chien alors que chez l'homme, une diminution a été objectivée[40]. Dans un modèle de chèvre[41], seule une diminution de la connexine 40 est retrouvée. Même si les gap junctions jouent un rôle important dans la conduction, il faut une diminution de plus de 40% de l'expression des connexines pour affecter la propagation de l'influx électrique[41]. Quoiqu'il en soit il est fort probable que le remodelage des gap junctions soit impliqué dans la création du « substrat » de la FA persistante et que le raccourcissement du PA ne soit pas le seul élément impliqué dans le développement de la FA. Il a été suggéré qu'un 2ème facteur était impliqué dans le remodelage, c'est l'augmentation d' « anisotropie tissulaire » due aux changements dans l'expression des protéines des gap junction ou due à de la fibrose tissulaire comme montré sur un modèle canin d'insuffisance cardiaque[42] où des régions de conduction lente favorisent la stabilité de la FA.

2.2 Le remodelage contractile

Le *remodelage contractile* est aussi lié à des altérations du calcium intracellulaire et survient également dès le début de l'arythmie. Il a été montré que la myolyse associée à la dégradation des protéines contractiles était liée à l'activation induite par la FA de cystéines protéases responsables de la perte de l'appareil contractile via la voie des calpaines (dégradation de troponine)[36, 43].

Ce processus semble mener à la paralysie atriale avec une augmentation de la compliance, aboutissant en une dilatation de l'oreillette en l'espace de plusieurs jours à plusieurs semaines. En échocardiographie, il a été montré que la dysfonction contractile de l'oreillette était corrélée à la durée de la FA et que plusieurs semaines voire mois sont nécessaires pour restaurer la fonction auriculaire

[44, 45]. Après 2 semaines de FA, la fonction contractile est rétablie dès 24h de RS mais une FA de plus de 6 semaines nécessitera plus d'un mois. Ceci explique que les complications thromboemboliques puissent survenir juste après la cardioversion mais également après plusieurs semaines même si aucun thrombus n'était présent avant la cardioversion. Il a été établi qu'une FA persistante, induisait des changements supplémentaires responsables d'une dysfonction contractile atriale impliquant une très nette réduction des courants calciques[46] sans mobilisation des voies dépendantes des récepteurs ß-adrénergiques[47]. Ces modifications de force contractile ont été documentées en échocardiographie.

2.3 Le remodelage structurel

L'augmentation du stress pariétal lié au remodelage contractile semblerait être à son tour responsable d'un *remodelage structurel*. Actuellement, il est difficile d'estimer la contribution des différents mécanismes de remodelage dans l'augmentation de probabilité de la persistance de la FA[36].

Le remodelage structurel est considéré comme le principal contributeur à l'initiation et à la persistance de la FA.

Dans la FA, les changements de structures du myocyte atrial observés dans des modèles animaux et chez l'homme incluent une augmentation de taille, une accumulation périnucléaire de glycogène, une altération des sarcomères (myolyse), la modification de l'expression des connexines, le changement de forme des mitochondries, une perturbation du réticulum sarcoplasmique, le changement de la quantité et la localisation des protéines de structure cellulaire (figure 9). Ces modifications morphologiques chez l'animal seraient une conséquence d'adaptation physiologique à une surcharge calcique chronique et d'un stress métabolique plutôt que la manifestation d'une dégénérescence car elles ne s'accompagnent pas d'apoptose (figure10).[36]

Le premier changement structurel apparait après une semaine de FA et progresse avec le temps.

La dilatation atriale est un élément pronostique majeur de la FA et de son ablation. Il est corrélé chez l'homme à la sévérité de la maladie sous-jacente mais également à la charge en FA. Dans un modèle de chèvre stimulée à 240 bpm au niveau atrial, Schoonderwoerd [48] note l'absence de dilatation de l'oreillette dans le groupe de chèvre où la cadence ventriculaire a été contrôlée à 80 bpm alors que dans le groupe où ventricule et oreillette sont rapidement stimulés, l'oreillette est dilatée. Ceci suggère qu'une cadence ventriculaire rapide associée entraînant une insuffisance cardiaque congestive et une surcharge volémique est un déterminant important de la dilatation atriale.

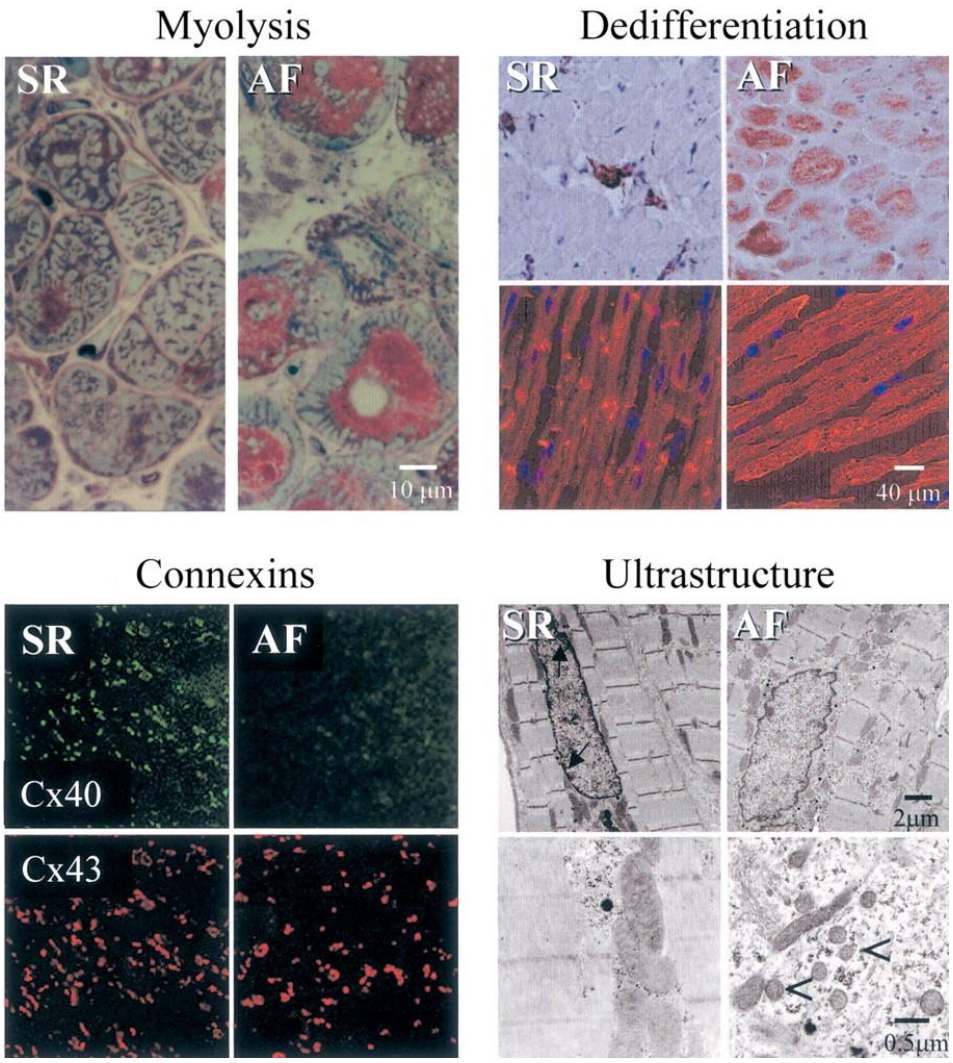

Figure 9 : Remodelage structurel des myocytes atriaux après 4 mois de FA chez la chèvre.
Les images de gauche sont issues de chèvres en RS (SR) et celles de droite de chèvres en FA chronique (AF).
Microscopie (en haut à gauche) montre des cellules avec myolyse sévère (perte des sarcomères : marquage bleu) et accumulation de glycogène (rouge).
L'immuno-marquage des protéines structurelles (en haut à droite) démontre la dé-différentiation du tissu myocardique atrial avec une augmentation du marquage de l'actine-α fœtale de cellules musculaires lisses (marquage rouge sur les images du haut). Pendant la FA, la desmine myocytaire (coloration rouge) perd son aspect strié dans le cytoplasme et disparait au niveau des disques intercalés, le marquage de desmine n'est plus présent (image en haut à droite)
En bas à gauche, les protéines des gap junctions sont révélées par immuno-fluorescence. Le marquage de la Cx40 (coloration verte) et Cx43 (coloration rouge) révèle une nette réduction de l'expression de la Cx40 et pas de changement de celle de la Cx43.
Microscopie électronique (en bas à droite) : les changements de l'organisation cellulaire du myocyte atrial incluent au cours de la FA, un noyau à distribution plus homogène de chromatine (agglomération normale de chromatine au niveau de la membrane nucléaire indiquée par des flèches dans l'image SR sur la photo du haut. Pendant la FA, plusieurs petites mitochondries en forme de beignets peuvent être trouvées (flèches en bas à droite) d'après Allessie, Ausma et Van der Velden)[36, 41]

Dans les modèles de FA induite par la stimulation atriale rapide (chèvre), les cardiomyocytes atriaux ont une taille augmentée après 9 à 23 semaines comme dans les modèles d'insuffisance cardiaque[36].

Un autre mécanisme adaptatif contribuant au processus de remodelage est la dé-différentiation impliquant un changement d'expression génique vers un phénotype plus fœtal. Certains gènes

spécifiques du développement sont ré-exprimés comme l'α-actine squelettique, la chaine lourde de la ß-myosine, le peptide natriurétique atrial. La réexpression de la chaine lourde de la ß-myosine pourrait s'intégrer dans un programme d'utilisation plus efficace de l'énergie. Ceci a été étudié dans des modèles animaux et vérifié sur des biopsies d'oreillettes humaines. Les signes de dédifférentiation sont la myolyse, l'accumulation de glycogène, les changements des mitochondries, la dispersion de la chromatine et la perte du réticulum sarcoplasmique et correspondrait dans la FA à un mécanisme adaptatif d'un processus demandant de grandes quantités d'énergie (figure 9).

En revanche, chez l'homme la situation est plus complexe et les données plus limitées. En effet de l'apoptose a été mise en évidence (réactivité TUNEL, expression de bcl-2 sur des biopsies atriales…) associée à un degré de fibrose interstitielle important dans des oreillettes de patients en FA. Comparées aux modèles animaux où la FA est le plus souvent induite par une stimulation atriale rapide sur des animaux adultes, les modifications structurelles beaucoup plus importantes observées chez l'homme pourraient être liées au vieillissement et aux pathologies cardiovasculaires associées[36].

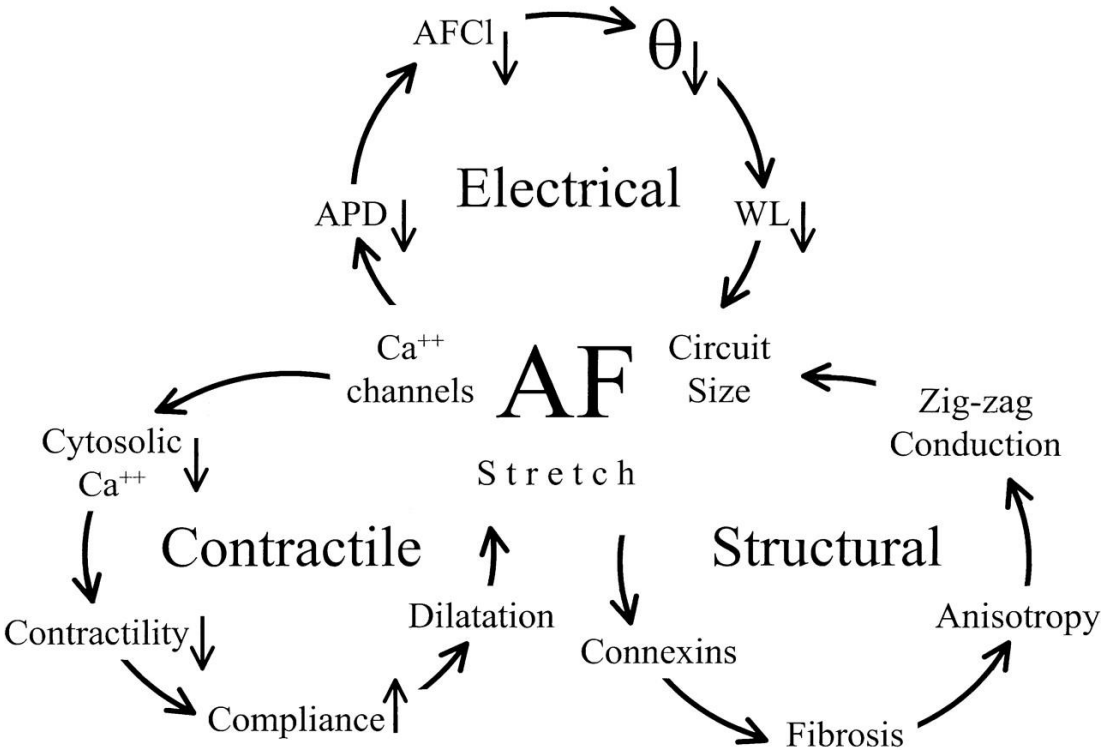

Figure 10 : Trois mécanismes proposés contribuant au remodelage atrial dans la FA.
La régulation négative des canaux Ca^{2+} type-L est considérée comme la 1ère cause de remodelage électrique et contractile. L'étirement (stretch) du myocarde atrial qui est le résultat de la perte de contractilité et de l'augmentation de compliance des oreillettes en fibrillation est supposé agir comme un stimulus du remodelage structurel de l'oreillette.
En conséquence, le « substrat » électro-anatomique de la FA consiste en des oreillettes dilatées permettant des circuits intra-atriaux de petites tailles dus à la réduction de la longueur d'onde (raccourcissement de la période réfractaire et ralentissement de la conduction) et à une augmentation non uniforme de l'anisotropie tissulaire (conduction zig-zag).[36]
APD : durée du potentiel d'action, AF Cl : longueur du cycle de la FA, WL : longueur d'onde, **θ** : conduction.

2.4 La fibrose et ses acteurs

2.4.1 Physiopathologie

La fibrose, caractéristique principale du remodelage et important « substrat » de la FA, est le résultat d'une production excessive de matrice extra-cellulaire (MEC) composée principalement de fibroblastes, de fibres élastiques et de collagène. Dans les modèles animaux avec une stimulation atriale rapide sans cadence ventriculaire contrôlée et dans des modèles d'insuffisance cardiaque, la fibrose est retrouvée de façon constante[36].

Les myofibroblastes sont des fibroblastes possédant la particularité d'exprimer l'actine α-SMA (smooth muscle actin). Ils jouent un rôle important dans la séquence de recrutement et de la prolifération des cellules, de la réparation tissulaire et pour la reconstitution du tissu conjonctif comme par exemple dans l'infarctus du myocarde où un tissu cicatriciel comble l'espace occupé par les cellules mortes et préserver l'intégrité de l'organe[49]. Par contre, un programme de réparation hyperactive entraînerait une fibrose pathologique. L'angiotensine II (Ang II), l'endothéline-1 (ET-1), le transforming growth factor ß (TGF-ß) et le facteur de croissance dérivé de la plaquette (PDGF) ont une action synergique pour induire l'activation de fibroblastes interstitiels résidents, favoriser la persistance de myofibroblastes et d'induire l'expression d'une grande variété de composants de la MEC comme le collagène type I favorisant ainsi l'initiation et le maintien du processus fibrotique.[49]

Les protéines de la MEC sont des composants non-structuraux qui modulent la réponse des facteurs de croissance au vieillissement, à la charge mécanique, à la régénération et favorisent l'angiogenèse, l'inflammation, la réparation tissulaire et la fibrose[50]. Les fragments de la MEC remodelée (en particulier sous l'action des métalloprotéinases) constituent des molécules bioactives appelées matrikines qui sont des ligands pour les intégrines leucocytaires et pour d'autres récepteurs de cellules activées favorisant ainsi le recrutement séquentiel des cellules sécrétrices des protéines du tissu conjonctif[51]. Le collagène matriciel est un réservoir dynamique dont l'importance dépend du taux de synthèse et de dégradation des protéines de la MEC. Le taux de renouvellement de la MEC affecte ainsi les voies de signalisation des cardiomyocytes, fibroblastes, cellules endothéliales et leucocytes en favorisant un environnement inflammatoire qui accélère encore le remodelage

2.4.2 Le TGF-ß1

Le TGF-ß1 est une cytokine pro-fibrotique qui joue un rôle dans la réparation tissulaire et dans la fibrose pathologique[49]. Il est produit principalement par les fibroblastes myocardiques et les macrophages. C'est le ligand canonique des récepteurs à activité sérine-thréonine kinase qui initie la voie Smad-dépendante impliquée dans de nombreuses fonctions du récepteur TGF-ß1, telles l'angiogénèse, la cicatrisation, la différentiation cellulaire et l'immuno-suppression[49] (figure11).

Le TGF-ß lors d'un phénomène normal de réparation entraine la différentiation de fibroblastes en myofibroblastes et favorise la déposition de MEC i) en régulant de façon positive l'expression les

gènes de MEC et des inhibiteurs des métalloprotéinases matricielles (TIMP) ii) en supprimant l'expression des gènes des métalloprotéinases (MMP) [49]. Une fois la réparation faite, le myofibroblaste doit disparaître. S'il persiste, il induit une fibrose pathologique[52]

Dans la fibrose cardiaque et le remodelage, le TGF-ß provient initialement des cellules immunes et est ensuite produit par les myofibroblastes [53, 54].

Dans un modèle d'infarctus de rat, l'inhibition du récepteur du TGF-ß diminue la dysfonction systolique et le remodelage du ventricule gauche[55]. Par contre, dans une autre étude des anti-corps anti-TGF-ß diminuaient la production de collagène, augmentaient l'expression des MMP et aggravaient le remodelage ventriculaire résultant en une augmentation de la mortalité [56].

Ces résultats contradictoires sont probablement en relation avec la complexité et l'intrication des voies de signalisation couplée à la voie canonique Smad-dépendante du récepteur du TGF-ß. Ce récepteur est la molécule canonique des récepteurs à activité sérine thréonine kinase (RSTK) qui ne sont actifs que sous forme oligo-dimérique et dont la spécificité de signalisation dépend de la combinaison des 12 isoformes (6 de type I, 6 de type II) et de 29 ligands (TGF-ß, activines…). Si l'oligomère de type II qui lie le TGF-ß possède une activité ST kinase constitutive, c'est l'assemblage avec le récepteur de type I qui initiera son activité kinasique inductible et la phosphorylation des protéines R-SMAD (smad 1,2,3,5) qui après assemblage aux coSmad constituent un complexe de facteurs transcriptionnels inhibant la prolifération cellulaire, favorisant la synthèse des protéines de la MEC et la production de Smad7, protéine régulatrice capable d'inhiber la voie smad en ciblant dans un rétro-contrôle négatif les coSmad. Ainsi toute baisse de la concentration cellulaire de smad7 favorise une réponse excessive au TGF-ß. C'est ce qui a été objectivé dans le cas de la fibrose rénale du diabétique.[57] En effet, smad7 est aussi le co-activateur de la synthèse de IK_B dans les fibroblastes limitant la réponse inflammatoire aux cytokines grâce à la complexation de IK_B à NFκB, facteur transcriptionnel pro-inflammatoire. Ainsi chez le diabétique, les fortes concentrations de cytokines circulantes mobilisent smad7 pour le contrôle IK_B-dépendant de la réponse inflammatoire privant la voie smad de son rétrocontrole négatif et favorisant la synthèse des protéines de la MEC. Chez le rat, l'expression de smad7 dans les fibroblastes est augmentée en réponse au TGF-ß et entraîne une baisse de production de collagène I et III. Smad 7 et le récepteur type I du TGF-ß sont peu exprimés dans le tissu cicatriciel puis dans le tissu myocardique à distance de la lésion d'infarctus.[58]

Dans la FA permanente, une concentration importante de protéines de TGF-ß1 a été retrouvée dans les oreillettes[59] comparée au patient en RS. De plus, le TGF-ß1 a été associé au remodelage atrial dans un modèle de souris sur-exprimant TGF-ß1 dans le cœur. Ces souris montraient une fibrose atriale et une induction de FA favorisée[60].

Le TGF-ß induit la différentiation du myofibroblaste en fibroblaste cardiaque de façon dépendante de la NADPH oxydase[61] favorisant la production de ROS susceptibles d'activer les MAPkinases qui sont redox sensibles et ainsi augmenter la réponse au TGF-ß[62].

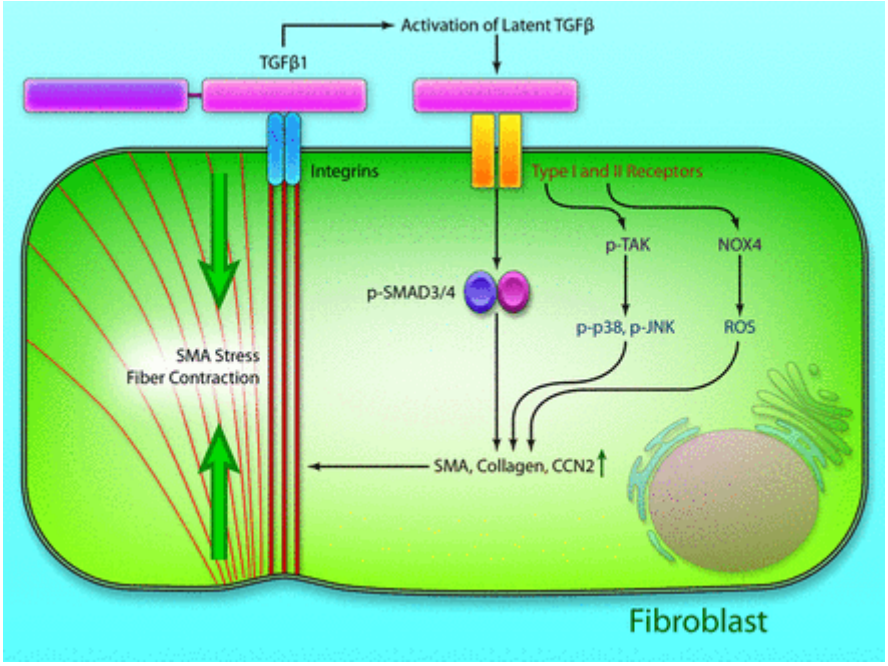

Figure 11 : La voie du Transforming growth factor-β (TGF-β) dans le fibroblaste. TGF-β est une cytokine fibrogénique majeure. Le TGF-β est synthétisé et secrété par les fibroblastes et se lie à son récepteur ALK5 qui sous forme hétérodimérique est alors capable d'initier la voie smad ciblant des gènes pro-inflammatoires producteurs d'interleukines et de cytokines. Le TGF-β phosphoryle aussi la TAK-1, une kinase capable d'activer la voie des MAPkinases c-jun et p38 dans les myofibroblastes. De plus, le TGF-β induit un stress oxydant dépendant de la NADPH oxydase (NOX 4) entraînant l'expression de gènes fibrogéniques comme α-smooth muscle actine (α-SMA), collagène et CCN2.[49]

2.4.3 L'Angiotensine II

L'angiotensine II (AngII), peptide vasoconstricteur et causant l'augmentation de la pression artérielle, est retrouvée à concentration élevée dans les cœurs fibreux[63]. Chez le rat ayant de la fibrose myocardique, AngII est exprimée et activée par les macrophages et les myofibroblastes[64]. Dans les cardiomyocytes et les fibroblastes, la fixation l'AngII sur son récepteur AT-1 induit l'expression de TGF-ß1. In vivo TGF-ß1 est associé à l'hypertrophie ventriculaire et à la fibrose[65]. Dans les fibroblastes cardiaques, l'AngII induit l'expression de collagène dépendante de TGF-ß1 et d'une kinase contrôlée par la production NF-κB dépendante de l'IL-6[66], [67] (figure 12).

Par ailleurs dans le fibroblaste atrial, l'AngII augmente également la sécrétion de CCN2, un membre de la famille des protéines matricielles non structurelles, retrouvé dans la fibrose cardiaque post-infarctus ou l'hypertrophie, via les MAPK/ TGF-ß1/TNFαF6[68].L'inhibition de CCN2 dans le fibroblaste cardiaque entraîne la réduction d'expression des chémokines induites par le « stretch », des MMP2 et 9, des protéines de la MEC et des connexines 43.[69]

Les inhibiteurs du récepteur de l'angiotensine comme le losartan ont montré une certaine efficacité dans la réduction de la fibrose cardiaque chez l'animal et chez l'homme [70, 71].

L'Ang II agit également par des effets pro-inflammatoires entraînant l'augmentation de cytokines pro-inflammatoires (IL-6, TNFα), des molécules d'adhésion, la protéine chemoattractante-1 (MCP-1) et des sélectines (P-selectine). Par la libération des chémokines, l'AngII peut initier le recrutement des neutrophiles[72]. L'expression des récepteurs de l'AngII a été associée à une infiltration leucocytaire et à une apoptose atriale augmentée[73].

L'AngII va être également impliquée dans l'activation de plusieurs MMP et du TXA2 (produit par les plaquettes activées) soit par un effet direct soit par un effet médié par l'IL-6 [74]. De plus, l'Ang II en favorisant la production de ROS contribue à la perte de NO endothélial et de ses capacités vasoprotectrices du tonus vasculaire physiologique.Elle entraîne aussi l'augmentation de la synthèse du PAI-1, témoin d'une dysfonction endothéliale susceptible de perturber la fibrinolyse au cours de la FA[75].

L'Ang II se situe donc à l'interface de l'inflammation, du remodelage tissulaire, de la thrombose, de l'apoptose et de la dysfonction endothéliale tous impliqués dans la FA.

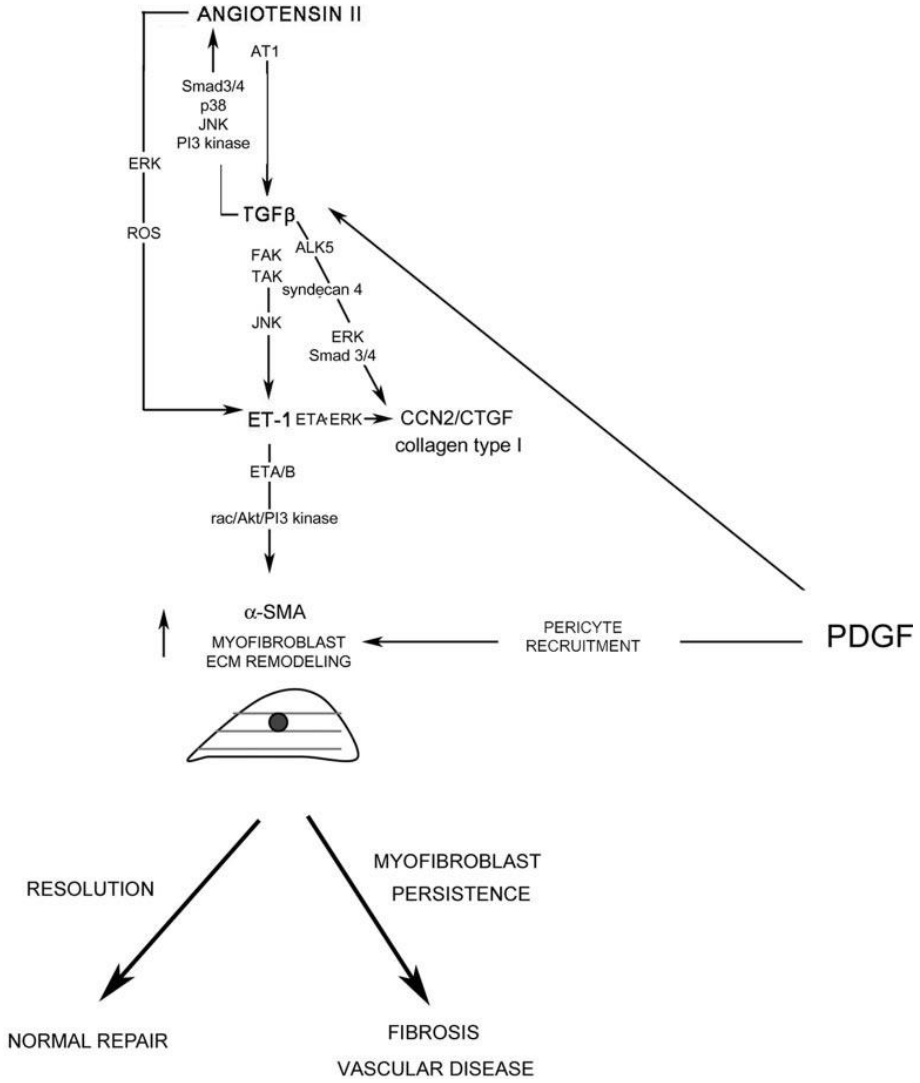

Figure 12 : Schéma des interactions entre cytokines pro-fibrotiques
L'Ang II induit le TGFß, CCN2/CTGF et ET-1 directement, TGFß induit ET-1 et CCN2/CTGF; ET-1 induit CCN2/CTGF. TGFß induit également le récepteur de l'angiotensine augmentant ainsi la stimulation par l'Ang II. De façon collective, ces protéines favorisent l'activation des fibroblastes et la

formation des myofibroblastes. Dans la réparation tissulaire normale, les myofibroblastes disparaissent et la fonction de l'organe est restaurée. La persistance des myofibroblastes conduit à une pathologie.[75]
FAK : adhésion focale kinase; Akt, V-akt murine thymoma viral oncogene homolog 1.

2.4.4 L'endothéline-1

L'endothéline-1 (ET-1) est principalement produite par la cellule endothéliale mais peut aussi être produite par les macrophages, les cardiomyocytes et les fibroblastes. La fixation d'ET-1 à son récepteur entraine une augmentation de la production de collagène dans le fibroblaste cardiaque[49, 76]. Le développement de la fibrose cardiaque et de l'hypertrophie en réponse à l'Ang II est arrêté chez la souris déficiente en ET-1 sur les cellules endothéliales vasculaires[77]. Ces données suggèrent que l'Ang II induit l'expression d'ET-1 d'une façon redox-sensible par l'intermédiaire de la synthèse de médiateurs pro-inflammatoires comme l' IL-6 et MCP-1.
De plus, des taux élevé d'ARNm d'ET-1 dans le tissu atrial ont été retrouvé dans la FA[78]. Wang rapporte que des taux élevés d'ET-1 avant ablation de FA sont associés à la récidive de FA à long terme seulement chez des patients ayant une FA paroxystique[79]. De plus, un taux plasmatique élevé d'ET-1 était corrélé à la taille de l'OG. Il est probable que le rôle d'ET-1 varie en fonction du type de FA comme le montre une diminution d'expression des récepteurs ET-A et ET-B dans l'oreillette entre la FA paroxystique et la FA persistante[80].

2.4.5 Le platelet-derived growth facteur (PDGF)

Les PDGF sont une famille complexe de protéines qui se fixent à leur récepteur PDGF α ou ß. Après l'infarctus du myocarde, le PDGF est augmenté dans la cellule endothéliale, les macrophages et les myofibroblastes[49]. L'augmentation de l'expression de ses récepteurs coincide avec l'angiogénèse, l'inflammation et la réponse fibrotique dans le myocarde infarci, témoin de leur participation à la réparation tissulaire[81]. Le PDGF induit la synthèse de TGF-ß et exerce son effet pro-fibrotique par cette voie. Dans un modèle de cœur de souris en surcharge de pression, l'injection d'anticorps neutralisants du récepteur PDGFα atténue la fibrose atriale alors que PDGF-AA, un agoniste stimule la fibrose atriale et favorise la FA dans le cœur normal[82].

2.4.6 Les métalloprotéinases (MMP)

L'initiation des processus de fibrose est également liée à la présence dans la MEC de MMPs et de leurs inhibiteurs endogènes TIMPs (tissue inhibitors of metalloproteinase)[49]. Les MMPs sont sécrétées par les fibroblastes, les cellules musculaires lisses, les cellules endothéliales et les myocytes[83]. Ce sont des enzymes protéolytiques des constituants de la MEC (collagénases, gélatinases).

Dans la FA, un changement dans l'expression des MMPs et des TIMPs a été associé aux concentrations sériques de marqueurs du turnover du collagène. Le « stretch » des cardiomyocytes atriaux induit une augmentation du calcium intracellulaire entrainant l'expression calcineurine-dépendante des MMP-2 et 9. Il augmente également l'expression de TIMP-1 mais la proportion de MMPs liées à leur inhibiteur TIMP-1 est diminuée suggérant une augmentation de l'activation des MMP[84].

L'augmentation de l'activité MMP inductrice de fibrose peut paraître paradoxale puisqu'on s'attendait à la dégradation du collagène matriciel. En fait les MMP favorisent la production des matrikines et le recrutement exacerbé de cellules de la réparation et de la cicatrisation et le dépôt anarchique du collagène synthétisé de manière non contrôlée conduisant à un contenu collagénique de la MEC de mauvaise qualité[83].

Les MMP ont également été impliquées dans l'état d'hypercoagulabilité dans la FA en raison de plusieurs interactions avec la cascade de coagulation et surtout la plasmine[85]. Gramley[86] montre que plus la durée de FA est importante, plus l'activité de MMP- 2 et MMP-9 dans l'oreillette augmente et plus l'expression de PAI-1 circulant et TIMP diminue, suggérant un lien entre MMP et sur-risque thrombotique dans la FA.

3. Stress oxydant et dysfonction endothéliale dans la FA

Le stress oxydant est impliqué dans la pathogénie de la FA. Si la plupart des facteurs favorisant la survenue de FA sont associés au stress oxydant, la FA elle-même l'induit. Le stress oxydant aurait donc un rôle majeur dans l'initiation et la pérennisation de la FA.

3.1 L'endothélium vasculaire

L'endothélium, l'interface entre le sang circulant et la paroi du vaisseau a un rôle de barrière sélective contrôlant le transport de différentes substances et le transit des leucocytes de part et d'autres[87]. La cellule endothéliale présente une activité métabolique complexe et secrète plusieurs substances impliquées dans le contrôle des fonctions hémostatiques et vasculaires[88, 89] :

-des produits de la matrice extra-cellulaire : fibronectine, lamine, collagène

-des facteurs anti-thrombotique : prostacycline, thrombomoduline, anti-thrombine, héparine, facteur du plasminogène

-des facteurs pro-coagulant : facteur de von Willebrand, thromboxane A2, platelet-activating facteur (PAF), inhibiteur de l'activateur du plasminogène (PAI-1), microparticules (MP)

-des facteurs de croissance : insulin-like growth facteur, transforming growth facteur (TGFß)

-des médiateurs de l'inflammation : interleukine 1, 6, 8

-des vasodilatateurs : monoxyde d'azote (NO), prostacycline,

-des vasoconstricteurs : enzyme de conversion de l'angiotensine, ET-1, radicaux libres

-des éléments du métabolisme lipidique : récepteur LDL et lipoprotéine lipase (wrzosek 2009)

La cellule endothéliale exprime plusieurs marqueurs, PECAM-1 (platelet endothelial cell adhesion molecule) ou CD31, VEGF R2 (vascular endothelial growth facteur receptor 2), VE-cadhérine (vascular endothelial cadherin) ou CD105 et du facteur de von Willebrand (vWF).
L'homéostasie vasculaire dans des conditions physiologiques implique des mécanismes complexes faisant intervenir tous ces éléments.

3.2 Le monoxyde d'azote

Le monoxyde d'azote (NO) est un médiateur cellulaire qui joue un rôle majeur dans la régulation de la fonction cardiaque. Il est synthétisé par toutes les cellules cardiaques et est très diffusible. Généré par les cardiomyocytes, il peut exercer un effet autocrine ou modifier les propriétés des cardiomyocytes adjacents. Le NO produit à partir des autres cellules (endocarde, endothélium, éléments sanguins circulant, cellules du système nerveux autonome) peut exercer un effet direct sur les cardiomyocytes et un effet indirect en modulant le débit coronaire et la transmission par le système nerveux autonome[89].

Le NO est formé à partir de la L-arginine par la NO synthase (NOS) qui est Ca^{2+}-calmoduline dépendante pour le type1 et 3, et indépendante du Ca^{2+} pour le type2[88, 89] (figure 13).
Il existe 3 isoformes de NO synthase : NOS1 (neuronal nNOS), iNOS inductible ou NOS2, NOS3 endothéliale (eNOS). Dans le cœur, NOS1 et NOS3 sont exprimées constitutivement et iNOS uniquement en situation inflammatoire ou pathologique. La eNOS est surtout présente dans l'endothélium, dans l'endocarde et est faiblement détectée dans le cardiomyocytes ou dans les plaquettes et monocytes. La nNOS est exprimé dans le cardiomyocyte, système nerveux autonome cardiaque et les ganglions intracardiaques. Dans le cardiomyocyte au repos, nNOS est localisée majoritairement dans le réticulum sarcoplasmique, eNOS dans les caveolines, iNOS est ubiquitaire dans le cytosol.
Dans les conditions normales, le NO produit par la NOS se lie à la guanylyl cyclase soluble favorisant la production de GMPc dont la concentration est déterminante pour l'activité d'une kinase GMPc-dépendante (PKG) et de nombreuses phosphodiestérases (PDEs). Le NO peut aussi réguler la fonction cardiaque par des mécanismes non dépendants du GMPc par la nitration de la tyrosine ou nitrosylation des résidus cystéines, mécanismes impliqués dans la modulation de l'activité de plusieurs canaux ioniques[90].
Dans certaines conditions pathologiques, il peut y avoir un découplage du cofacteur de la NOS (BH4) conduisant à la production d'anions superoxydes (O2-), puissants oxydants.

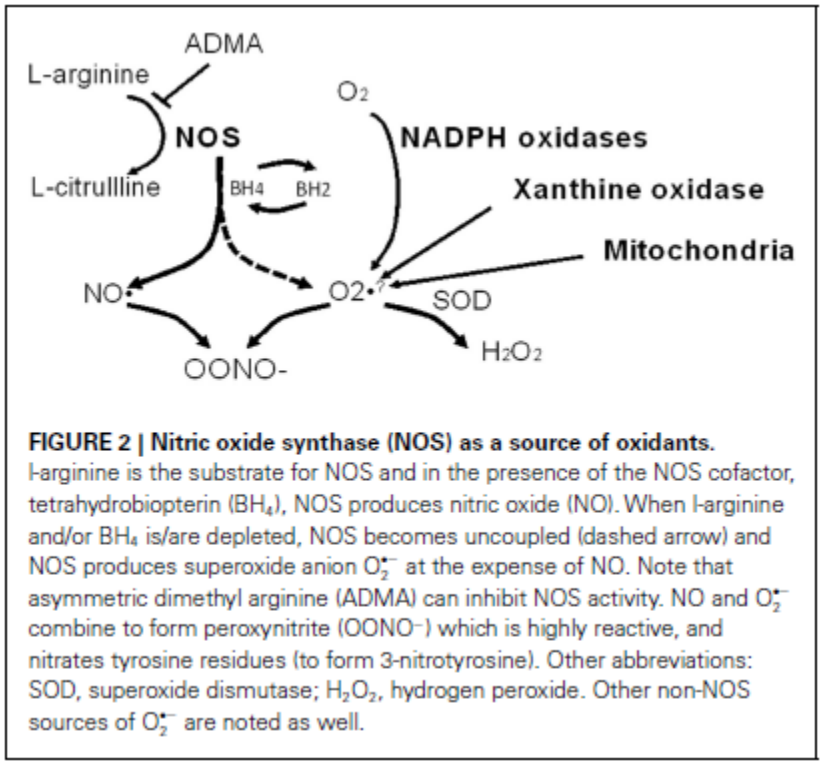

FIGURE 2 | Nitric oxide synthase (NOS) as a source of oxidants. l-arginine is the substrate for NOS and in the presence of the NOS cofactor, tetrahydrobiopterin (BH_4), NOS produces nitric oxide (NO). When l-arginine and/or BH_4 is/are depleted, NOS becomes uncoupled (dashed arrow) and NOS produces superoxide anion $O_2^{\bullet-}$ at the expense of NO. Note that asymmetric dimethyl arginine (ADMA) can inhibit NOS activity. NO and $O_2^{\bullet-}$ combine to form peroxynitrite ($OONO^-$) which is highly reactive, and nitrates tyrosine residues (to form 3-nitrotyrosine). Other abbreviations: SOD, superoxide dismutase; H_2O_2, hydrogen peroxide. Other non-NOS sources of $O_2^{\bullet-}$ are noted as well.

Figure 13 : NO synthase, source de substances oxydantes[89]

3.3 Le NO dans l'endothélium

Dans l'endothélium, le NO est un puissant vasodilatateur produit constitutivement par la cellule endothéliale. En plus de son rôle dans le tonus vasculaire, il inhibe l'activation plaquettaire, l'adhésion des leucocytes et a un effet anti-thrombotique et anti-apoptotique. C'est un déterminant majeur de la viabilité, longévité de l'endothélium vasculaire[89]. La NO synthase endothéliale produit le NO à partir d'un acide aminé cationique (L-arginine). L'enzyme est activée par l'augmentation des concentrations en calcium intracellulaire en réponse à des changements de distension mécanique (shear stress) causé par le flux sanguin sur la paroi vasculaire ou via des processus impliquant des récepteurs. Le NO libéré active en quelques ms la guanylate cyclase (GC) dans les cellules musculaires lisses qui transforment le GTP en GMPc susceptible d'activer des canaux calciques GMPc-dépendants, la protéine kinase GMPc-dépendante (inhibition de l'entrée du flux calcique dans la cellule musculaire lisse) et d'être dégradé par certaines phosphodiestérases (PDE5). Dans la cellule musculaire lisse, la PKG I phosphoryle la phosphatase de la chaine légère de la myosine. La phosphorylation de la chaine légère de la myosine diminue en conséquence, réduisant la contraction des cellules musculaires lisses et provoquant la vasodilatation[91]. Le NO est également impliqué dans l'inhibition de la thrombose en inhibant l'adhésion, l'activation plaquettaires et la sécrétion induite d'agoniste[92]. La production de NO est augmentée par le shear stress causé par le flux sanguin. La production réduite

ou la diminution de la biodisponibilité du NO a été impliquée dans la pathogénie de l'hypertension artérielle pulmonaire primitive et dans plusieurs pathologies vasculaires comme l'athérosclérose[93].

Les nitrites et les anions nitrates sont les produits finaux d'oxydation du NO et reflètent l'activité endogène de la NOS. Le NO produit réagit rapidement avec les acides aminés, les ions et les anions superoxides ($O2^-$), espèces réactives de l'oxygène (ROS)[94]. Le NO, chélateur de $O2^-$ agit donc comme une molécule anti-oxydante et sa biodisponibilité est réduite en cas de niveau élevé de $O2^-$.

Lors du vieillissement endothélial, l'activité de la NO synthase est réduite en raison de l'augmentation d'interaction avec la cavéoline et la diminution de la phosphorylation de la sérine. De plus, la diminution de la disponibilité de la L-arginine induit un découplage de la eNOS ce qui entraine une modification de l'enzyme qui favorise la génération d' $O2^-$ contribuant à la dysfonction vasculaire[95] (figure 14).

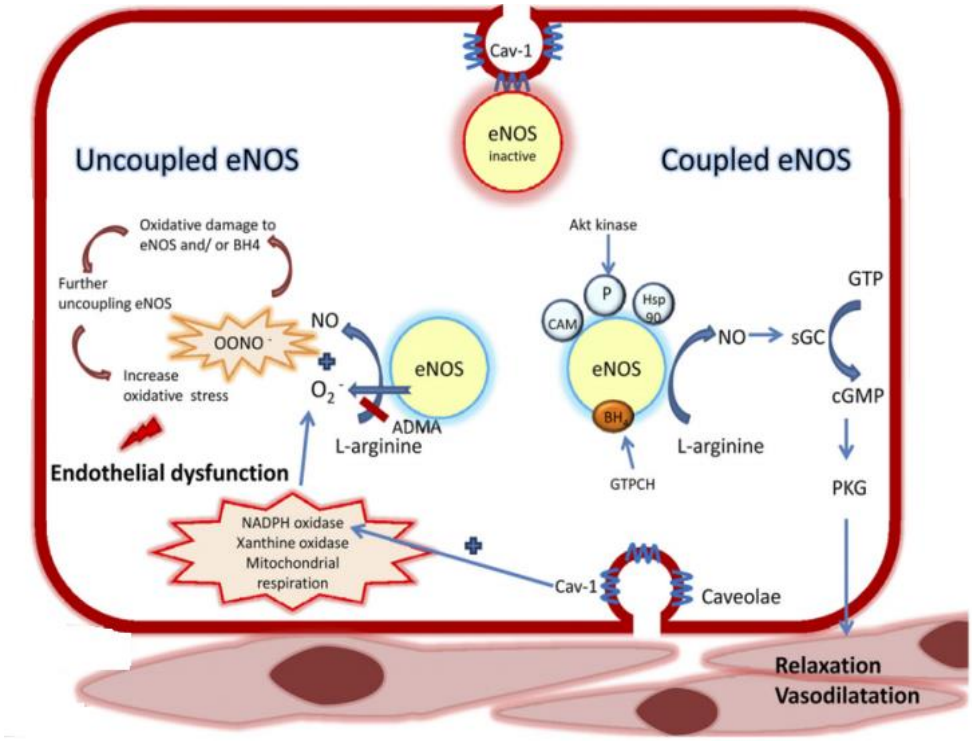

Figure 24 : Rôle central du découplage de la eNOS dans la pathogénie de la dysfonction endothéliale

La eNOS est localisée au niveau des cavéolines de la membrane plasmatique. Dans la cellule endothéliale, eNOS est inactive quand elle est liée à la cavéoline 1 (cav-1) et sa sérine 116 est phosphorylée tandis que les cystéines 96 et 101 sont nytrosylées. Quand elle devient active, la eNOS se dissocie de cav-1 et se lie à la calmoduline (CAM) et à la heat shock protein 90 (Hsp90) et ensemble à la phosphorylation de sites serine (e.g., Ser1177). La eNOS fonctionnelle est un dimère (appelé couplage de la eNOS). Tetrahydrobiopterine (BH4), un co-factor essentiel de la eNOS, est nécessaire à son activité optimale. BH4 facilite le transfert d'électrons dérivés de la NADPH à partir du domaine réductase de la eNOS au domaine oxygénase pour convertir la L-arginine en NO et L-citrulline. L'interaction entre L-arginine et dimethylarginine asymétrique (ADMA) est une compétition directe pour la eNOS.

Quand la disponibilité de L-arginine ou de BH4 ne permet pas le couplage optimal, la eNOS découplée conduit à une production diminuée de NO et à une génération paradoxale de superoxydes. De plus l'interaction entre NO et superoxydes conduit à la formation de péroxynitrite, un puissant oxydant, qui oxyde BH4, renforçant ainsi le découplage en un cercle vicieux suivi d'une dysfonction endothéliale. [96]

3.4 Le NO dans le cardiomyocyte

Dans le cardiomyocyte, le NO a un effet sur les canaux ioniques. Par exemple, le NO peut augmenter ou diminuer I_{Na}, courant qui génère la dépolarisation rapide du potentiel d'action (PA) et détermine l'excitabilité cardiaque et la vitesse de conduction[97]. Sur le canal I_{Ca}, il exerce des effets opposés. En cas d'exposition à l'isoprotérénol, durant l'ischémie–reperfusion ou en cas de FA chronique, le NO diminue I_{Ca} entrant, donc l'augmentation de Ca^{2+} dans le réticulum sarcoplasmique et les lésions cardiaques induites par le Ca^{2+}, constituant par là-même un mécanisme cardioprotecteur[98]. Le NO module également l'activité de plusieurs canaux potassiques sortants (qui déterminent le potentiel de repos de la membrane, la fréquence cardiaque et la forme et durée du potentiel d'action) comme I_{to}, I_{Kur}, I_{Kr}, I_{Ks}...

3.5 NOS et effets du NO sur les arythmies cardiaques

La dysfonction endothéliale coronaire et les inhibiteurs de NOS diminuent la quantité de NO dans le flux coronaire et augmentent l'incidence et la sévérité des arythmies ventriculaires dans des modèles d'ischémie-reperfusion chez le rat[99] alors que la L-arginine et les donneurs de NO, même à des doses faiblement vasodilatatrices diminuent la fibrillation ventriculaire induite par l'ischémie-reperfusion chez le rat et chien[100]. Beaucoup de données suggèrent que le NO produit par la eNOS exerce un effet cardioprotecteur[90]. Des souris $NOS3^{-/-}$ ne montrent pas de différence sur les temps de conduction à l'ECG ou en intracardiaque mais la fréquence cardiaque est plus lente que chez le type sauvage. Mais la digoxine qui favorise l'entrée calcique en ciblant les ATPases Na+/K+, produit plus d'extrasystole ventriculaire ou de tachycardie ventriculaire chez les souris $NOS3^{-/-}$[101].La eNOS exercerait un effet cardioprotecteur contre les arythmies médiées par le système ß-adrénergique en inhibant I_{Ca} et en limitant les concentrations calciques intracellulaires élevées. La nNOS exercerait également un effet protecteur contre l'ischémie-reperfusion et les arythmies induites par le Ca^{2+}[102].

Les VP contiennent un endothélium, des cardiomyocytes et des cellules musculaires lisses qui produisent du NO. Il a été montré que le NO pouvait réguler l'arythmogénicité des VP par un feedback mécano-électrique[103]. Parce que les VP induisent des arythmies atriales par une augmentation de l'activité déclenchée, il est possible que le NO joue un rôle important dans l'activité des VP. Lin YK[104] montre sur des cardiomyocytes de VP de chien, que les donneurs de NO diminuent la fréquence spontanée des cardiomyocytes et supprime les post-dépolarisations tardives impliquées dans les extra-systoles initiatrices impliquant le NO dans la genèse de la FA.

Dans la FA chronique, plusieurs données suggèrent que la quantité de NO et sa biodisponibilité sont notablement réduits dans l'oreillette. Dans des modèles animaux, la stimulation rapide de l'oreillette diminue l'expression d'eNOS endocardique et la biodisponibilité du NO dans l'oreillette gauche (pas dans la droite) probablement suite également à une diminution de l'activité eNOS dans des conditions de flux sanguin turbulent[105]. La FA chronique augmente aussi l'activité de la NADPH atriale et de la formation de peroxynitrite et diminue les concentrations plasmatiques de nitrite/nitrate et le GMPc

plaquettaires[106]. De plus, les NOS contribuent à la production atriale de superoxydes suggérant que la dysfonction des NOS pourrait jouer un rôle important dans les lésions atriales de stress oxydant et le remodelage électrophysiologique atrial caractérisé par un raccourcissement de la durée du PA atrial et de la période réfractaire[107]. Le NO module les courants ioniques Na+, Ca²+, et K+ impliqués dans ce remodelage électrique. La production réduite de NO induite par la FA chronique favorise les courants potassiques sortants ce qui raccourcirait le plateau du PA atrial contribuant au remodelage électrophysiologique qui maintient l'arythmie (figure 15). Dans la FA chronique, la sous-unité α1C du canal Ca²+ type-L est hypernitrosylé ce qui diminue I_{Ca} et exerce ainsi un effet cardioprotecteur en atténuant la surcharge calcique.
Certains polymorphismes des gènes NOS prédisposeraient à la survenue de FA[108].

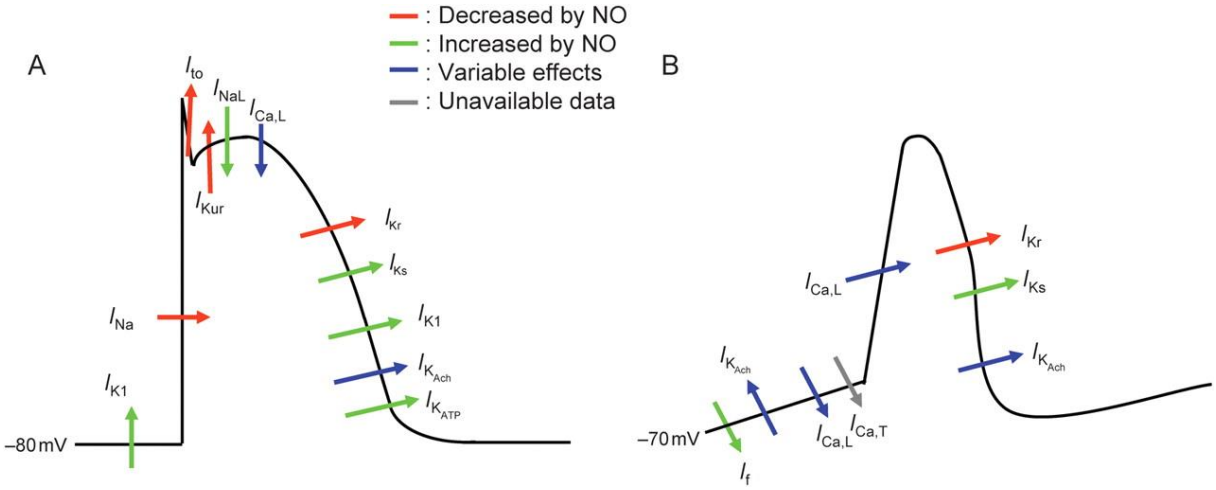

Figure 15 : Schémas du potentiel d'action cardiaque dans (A) cellules atriales et ventriculaires et (B) cellules du noeud sinusal montrant les effets du NO sur les courants participant à sa génèse. Les flèches indiquent la direction des mouvements ioniques alors que les couleurs indique l'augmentation (vert), la diminution (rouge) ou un effet variable (bleu) du NO.[90]

3.6 Les facteurs vasoconstricteurs dérivés de l'endothélium

3.6.1 ROS

3.6.1.1 Production et effets des ROS

Les ROS sont produits et libérés par la cellule endothéliale dans des conditions physiologiques et participent à l'homéostasie vasculaire. Une trop grande production de ROS est objectivée dans certaines situations pathologiques comme l'hypertension, le diabète, les maladies inflammatoires aigues ou chroniques et le vieillissement[88].
Les ROS sont d'importants médiateurs de l'inflammation, essentiels pour la réponse immune. Dans le lit vasculaire, plusieurs enzymes contribuent à la production de ROS : le cytochrome P450, les

cyclooxygénases (COX-1 et 2), la lipooxygénase, la xanthine-oxydase, la NADPH oxydase (p22phox, p47phox, gp91phox) dans le cytosol. De plus, la chaine de transport des électrons dans la mitochondrie a été identifiée comme une importante source de ROS dans la cellule endothéliale dans des conditions physiologiques et pathologiques[109]. Les ROS modifient des protéines redox sensibles entrainant un gain ou une perte de fonction. Plus récemment, il a été montré que ces biomolécules pouvaient propager leur signal dans d'autres compartiments cellulaires en ciblant des facteurs de transcription redox sensibles[88].

Les ROS peuvent avoir un effet vasoconstricteur direct en facilitant la mobilisation du Ca^{2+} cytosolique[110]. De plus les ROS particulièrement O^{2}- potentialisent la réponse contractile en réduisant la biodisponibilité du NO[111] ou en activant la COX dans la cellule musculaire lisse[112]. Ils sont associés aux troubles de la relaxation dépendants de l'endothélium.

3.6.1.2 Contrôle de la génération des ROS

La dégradation d'O^{2}- peut être catalysée par la superoxyde dismutase (SOD) en H2O2 ensuite transformé en eau et oxygène par la catalase. La gluthation peroxydase dégrade également H2O2 en H2O[113]. Ces enzymes ont une action anti-oxydante. Une interaction entre NO et les anions superoxydes (O^{2}-) peut être mise en évidence par la présence de peroxynitrite (ONOO-) qui sont eux-mêmes de puissants oxydants impliqués dans les maladies cardio-vasculaires. Plusieurs études en montrant l'amélioration de la fonction endothéliale par des traitements anti-oxydants ont souligné l'importance du stress oxydant dans l'athérosclérose sur des modèles animaux et chez l'homme[114, 115].

3.6.1.3 FA et ROS

La FA est associée au stress oxydant dans l'oreillette et des modifications irréversibles d'oxydation des protéines ont été mises en évidence dans la FA permanente liées principalement à une production accrue d'anions superoxydes (O2-). Mais les peroxydes d'hydrogène (H2O2), les peroxynitrites (ONOO-), dérivés hydroxyl (OH) et le NO sont également impliqués. La NADPH oxydase 2 (NOX2) est la première source de production d'O2- dans le myocyte atrial humain[107].

Il est intéressant de noter un changement des sources de production de ROS et du statut de la NOS dans le temps au cours de la FA. Dans un modèle de chèvre en FA sur 6 mois, la production de O2- par la NADPH oxydase est augmentée dans l'OG après 2 semaines de FA alors qu'à 6 mois, la source principale d'O2- était liée à la mitochondrie et au découplage de la NOS[116]. Dans la FA permanente, H2O2 est prépondérant, lié à une surexpression de la super oxyde dismutase 1 (SOD1)[117]. Il existe aussi des modifications irréversibles des protéines des myofilaments du sarcomère liées à l'oxydation pouvant conduire à une perte de fonction ayant une implication clinique certaine[118].

Dans la FA, l'oxydation de la protéine kinase 2 Ca^{2+}/calmodulin dépendante (CaMKII) induite par l'angiotensine II favorise la fuite de Ca^{2+} du réticulum sarcoplasmique via le récepteur de la ryanodine conduisant à une susceptibilité accrue à la FA chez la souris[119].

3.6.2 Thromboxane A2

Le TXA2 est un produit du métabolisme de l'acide arachidonique par les COX-1 et 2 de la cellule endothéliale. Dans la plaquette, il est synthétisé à partir de la prostaglandine H2 (PGH2) par la thromboxane synthase et favorise l'agrégation plaquettaire, la vasoconstriction et la prolifération de cellules musculaires lisses. Dans la cellule musculaire lisse, il provoque une augmentation du Ca^{2+} intracellulaire en se liant au récepteur thromboxane-prostanoid pour induire la vasoconstriction[120].
Au niveau cardiaque, le TXA2 injecté dans l'OG de lapin anesthésié est capable d'induire par une action directe une arythmie ventriculaire sans induction d'ischémie, ni vasoconstriction, ni modification du débit coronaire. Le cardiomyocyte du ventricule de lapin exprime le récepteur du TXA2 qui stimulé induit une augmentation du Ca^{2+} intracellulaire[121]. Il n'y a pas de donnée sur le TXA2 dans la FA.

3.6.3 Angiotensine II

L'angiotensine (AngII) est un peptide puissant vasoconstricteur du système rénine-angiotensine (SRA) qui est formé à partir de l'angiotensine I par l'enzyme de conversion de l'angiotensine I (ACE). L'Ang II est impliquée dans plusieurs fonction : vasoconstriction, croissance, sécrétion hormonale. Le SRA est impliqué dans plusieurs pathologies : hypertrophie ventriculaire gauche, remodelage post-infarctus, formation néointimale. Ang II exerce ses effets après fixation sur ses récepteurs AT1 et AT2. Le récepteur AT1, récepteur à 7 domaines trans-membranaires couplé à une protéine G[122] intervient dans la régulation de la prolifération cellulaire dans le compartiment vasculaire[123]. La fonction du récepteur AT2, toujours débattue, semble liée à la transduction de plusieurs voies de signal anti-apoptotique ou de survie cellulaire impliquant le NO (figure 16).

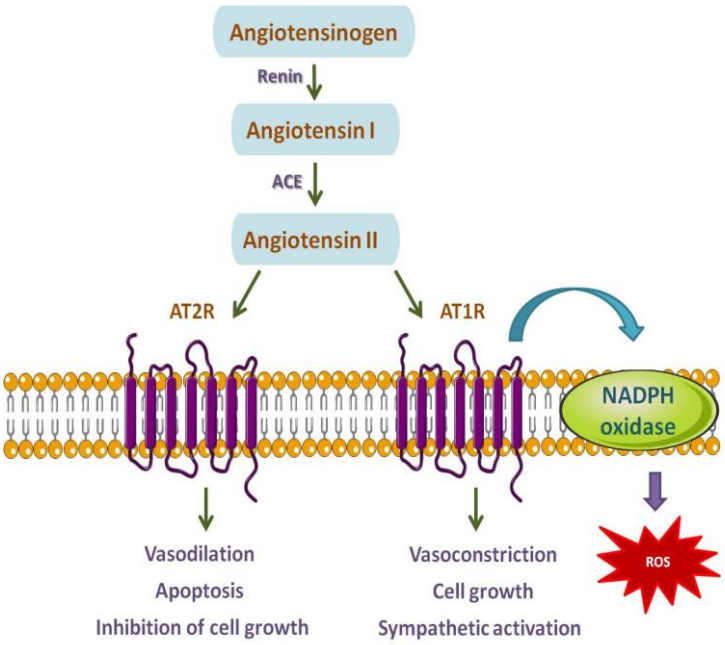

Figure 16 : Le système rénine-angiotensine-aldostérone.

ACE = enzyme de conversion de l'angiotensine; AT1R= récepteur type 1 de l'angiotensine II; AT2R= récepteur type 2 de l'angiotensine II. Ang II stimule la NADPH oxydase, via le récepteur de type 1(AT1) de l'angiotensine, ce qui entraîne la génération de ROS.

Par ses actions vasoconstrictrices, mitogène, pro-inflammatoire et pro-fibrotique, l'Ang II contribue à altérer le tonus vasculaire, la dysfonction endothéliale, le remodelage structurel et l'inflammation vasculaire, caractéristiques des dommages vasculaires dans l'hypertension, le diabète et l'athérosclérose[124]. L'Ang II est un puissant inducteur de dysfonction endothéliale et de stress oxydant vasculaire dépendant de l'activation de la NADPH oxydase[125] (figure 17).

Chez des rats hypertendus, l'Ang II en se liant au récepteur AT1 réduit la production de NO endothélial et l'hyperpolarisation de la cellule[126]. L'Ang II contribue à la dysfonction endothéliale dépendante de l'âge. En effet, une expression supérieure du récepteur AT1 a été observée dans des aortes de vieux rats comparés à des jeunes et dans des artères mammaires internes de sujets âgés[127]. Chez le rat âgé normotendu, la réduction de la dysfonction endothéliale (relaxation médiée par EDHF) associée à l'âge est observée après traitement par inhibiteur de l'enzyme de conversion ou par antagoniste des récepteur AT1[128]. De plus, l'espérance de vie de souris porteuses d'une invalidation du gène du récepteur AT1 est supérieure à celle des souris de phénotype sauvage et une réduction significative du stress oxydant était objectivée dans le cœur, l'aorte et le rein.

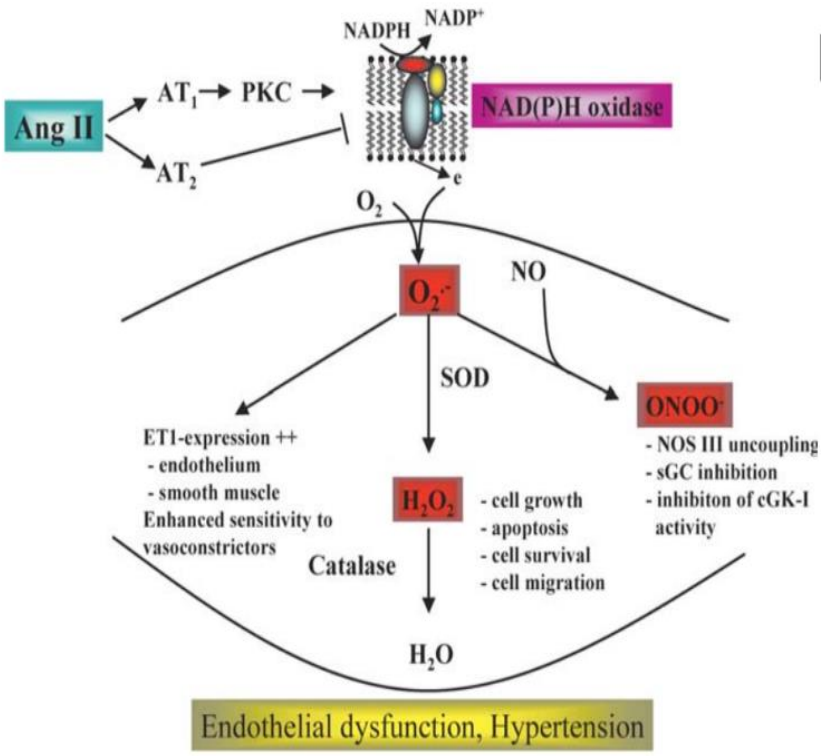

Figure 17 : Schémas explicitant les mécanismes sous-jacents de l'hypertension et de la dysfonction endothéliale induite par l'angiotensine II.

En plus de ses actions vasoconstrictrice et hypertensive, l'Ang II est impliquée dans la fibrose myocardique (figure 18). Chez des rats ayant de la fibrose myocardique, Ang II est exprimée et activée par les macrophages et les myofibroblastes[129].

L'Ang II induit une expression de TGF-ß1 dans les cardiomyocytes et les fibroblastes dépendante de son récepteur AT1. In vivo le TGF-ß1 est responsable de l'hypertrophie et de la fibrose[65]. Dans les fibroblastes cardiaques, Ang II induit l'expression de collagène via TGF-ß1 et une kinase régulée par un mécanisme dépendant de l'IL-6[66]. La capacité de l'Ang II à induire l'expression d'IL-6 semble NFkappa B-dépendante[67]. Récemment, le concept d'un effet pro-fibrotique de l'Ang II IL-6 dépendant a été renforcé par l'observation chez des souris invalidée pour le gène de l'IL-6, d'une résistance à l'hypertension induite par un régime hypersodé, à l'hypertrophie cardiaque, à l'induction d'une dysfonction cardiaque, à l'inflammation et à la fibrose provoquée par l'AngII[130].

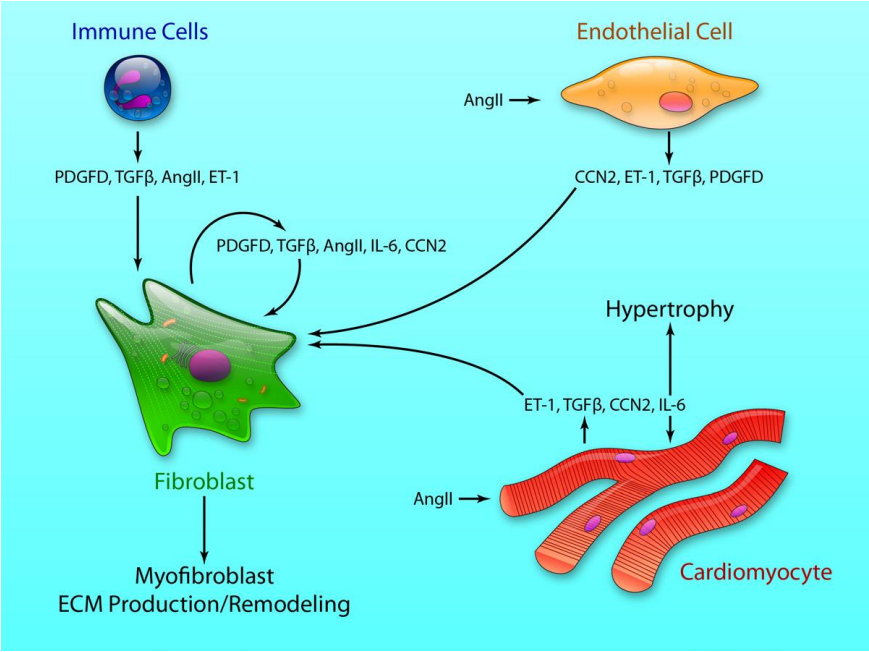

Figure 18 : Interactions cellulaires contribuant à la fibrose cardiaque. Les protéines pro-fibrotiques proviennent de plusieurs sources cellulaires différentes (cellules endothéliales, immunes ou fibroblastes). Cependant, l'effecteur cellulaire final est le myofibroblaste. [49]
Ang-II angiotensine II; ECM, matrice extra-cellulaire; ET, endotheline-1; IL, interleukine; PDGFD, platelet-derived growth factor D; and TGF-β, transforming growth factor-β

3.7 Rôle du stress oxydant dans le remodelage tissulaire

Les sources principales de ROS dans les cellules cardiaques sont la mitochondrie, la xanthine oxydase, la NADPH oxydase et la NO synthase découplée de son coactivateur BH4. Les ROS activent les MAP kinases et la voie Jak/Stat dans les cellules cardiaques induisant ainsi l'expression des MMP. *In vitro* les péroxydes d'hydrogène H2O2 induisent la production de MMP par la cellule endothéliale ou le fibroblaste cardiaque[131, 132]. La production d'anions superoxydes induite par les cytokines dans le cardiomyocyte ou le macrophage augmente la transcription de MMP MAPK-dépendante[133]. *In vivo et ex vivo*, le clivage du pro-peptide pro-MMP-2 et 9 permettant la sécrétion des protéases est redox sensible (H2O2, peroxynitrite et HOCl).
De plus, la synthèse du collagène par le fibroblaste cardiaque est dépendante de la xanthine oxydase[132]. La différentiation pro-fibrotique des fibroblastes en myofibroblastes est également provoqué par les ROS[134]. Ainsi, la différentiation du fibroblaste en myofibroblaste induite par les peroxynitrites est NFκB-dépendante et aboutit à la production de TGFß1, de fibronectine et de collagène I par la cellule différentiée[135].

4. Inflammation au cours de la FA

4.1 Les acteurs de l'inflammation dans la FA

Le rôle de l'inflammation dans l'initiation de la FA a été initialement associé aux états inflammatoires comme la myocardite, la péricardite ou en post-chirurgie cardiaque. Cependant la FA contribue aussi à l'inflammation de manière propre.
Ainsi des infiltrats inflammatoires ont été identifiés dans des biopsies atriales de patients en FA en l'absence de cardiopathie[136] ainsi que des taux circulants élevés de marqueurs inflammatoires comme la CRP, l'IL-2 ,6 et 8, le tumeur necrosis facteur α (TNF α)[137].
Dans la FA, l'activation des leucocytes est considérée comme une cause majeure de l'inflammation. Cytokines et chémokines orchestrent l'activation et la diapédèse des leucocytes et sont des médiateurs de la pérennisation de la FA.

4.1.1 La CRP

La CRP est synthétisée par le foie et par le tissu adipeux. Elle favorise le chimiotactisme des monocytes favorisant l'expression du récepteur des CC chémokines Elle induit également la sécrétion de FT par les monocytes et l'expression de PAI-1 circulant favorisant ainsi un état pro-thrombotique. Marott[138] a identifié un lien entre taux de CRP et FA. Des taux élevés de CRP ont été associés aux récurrences de FA après cardioversion[139] . Des niveaux élevés de CRP plasmatique ont été significativement associés à la présence de contraste spontané dans l'oreillette gauche en échocardiographie suggérant un lien entre l'inflammation et l'état pro-thrombotique de la FA[140]. Henningsen[141] a également montré que la CRP diminuait chez les patients ayant bénéficié d'une ablation de FA avec succès et que des taux élevés de CRP avant ablation constituaient un facteur prédictif de récidive post-ablation.

4.1.2 Le facteur Nécrosant des tissus (TNFα)

Le TNFα principalement synthétisé par les monocytes et les macrophages est une molécule pro-inflammatoire dont l'expression est régulée dans de nombreuses maladies cardiovasculaires dont la FA. Il va stimuler la voie du NFkB. Le TNFα serait impliqué plutôt dans la pathogénie de la FA chronique. Des taux supérieurs de TNFα ont été objectivés chez des patients en FA persistante par rapport à des patients en FA paroxystique[137]. De plus, des patients en FA valvulaire montrent des taux plus élevés de TNFα et une infiltration leucocytaire plus importante avec plus de fibrose que les patients valvulaire en rythme sinusal[142]. Dans une cohorte de patients en FA chronique non valvulaire suivie pendant 3 ans, il a été montré que le TNFα était un facteur prédictif significatif d'AVC[143].

4.1.3 Les interleukines et chimiokines

L'IL-2 est principalement produite par les lymphocytes T activés et est un facteur de croissance des lymphocytes T et des cellules NK. Des taux bas d'IL-2 plasmatique ont été associés à un succès de la cardioversion pharmacologique chez des patients en FA récente[144].

L'Il-6, une cytokine pro-inflammatoire est produite par les cellules immunes, par les monocytes et macrophages et par la cellule endothéliale, la cellule musculaire lisse vasculaire et le cardiomyocyte ischémié. L'IL-6 stimule la synthèse de plusieurs protéines comme la CRP, le fibrinogène et contre-régule le TNFα et l'IL-1β. L'IL-6 induit également la synthèse de FT, de fibrinogène et de facteur de von Willebrand dans la cellule endothéliale[145]. Elle a été impliquée dans la génération et la pérennisation de la FA[146]. Des niveaux élevés d'IL-6 ont été corrélés à la présence et à la durée de la FA ainsi qu'à un diamètre de l'OG augmenté chez le patient en FA. Plusieurs études ont corrélé des taux élevés d'IL-6 à la survenue de FA en post-pontage, post-cardioversion ou post-ablation par radiofréquence[141, 147]. Marcus[148] a objectivé chez des patients en flutter des niveaux élevés de CRP et IL-6 qui diminuaient après l'ablation du flutter.

L'IL-8 est synthétisée par plusieurs types cellulaires comme les monocytes, macrophages, hépatocytes, fibroblastes et cellules endothéliales[145]. C'est un important activateur et puissant chémoattractant des neutrophiles favorisant ainsi les lésions d'organes induites par les neutrophiles. Elle renforce l'activation endothéliale et a été directement impliquée dans la modulation des interactions plaquettes/plaquettes et plaquettes/leucocytes dans plusieurs états inflammatoires et pro-thrombotiques[149]. Liuba[150] a montré les taux d'IL-8 plus élevés chez des patients en FA permanente comparé à des patients en FA paroxystique.

Le MCP-1 (monocyte chemoattractant protéine) est une CC dont l'expression peut être favorisée par plusieurs médiateurs de l'inflammation IL-1, IL-4, VEGF et PDGF. Les principales sources de MCP-1 sont le monocyte et le macrophage. Il exerce un puissant chimiotactisme et active les leucocytes. Des taux élevés de MCP-1 ont été rapportés dans la FA surtout chez des patients ayant un thrombus de l'auricule. Li montre que les taux de MCP-1 étaient indépendamment associés à la FA qu'elle soit paroxystique ou permanente[137].

4.2 Couplage inflammation et thrombose

La rhéologie, la dysfonction endothéliale et l'inflammation concourent à favoriser un état pro-thrombotique dans la FA notamment par la production du FT inductible par les cytokines dans les monocytes et la cellule endothéliale mais aussi par un degré d'activation plaquettaire supérieur et une concentration de fibrinogène plasmatique accrue[151, 152].

L'altération de la fonction endothéliale contribue à l'état pro-inflammatoire et thrombotique. Les protéines sur-exprimées comme le vWF et la P-sélectine soluble favorisent le recrutement des cellules du système immunitaire à l'endothélium et contribuant au développement de l'état pro-inflammatoire et pro-thrombotique.

Une corrélation étroite a été objectivée entre les taux plasmatiques d'IL-6 et l'expression du FT dans l'OG et l'OD chez des patients en FA[145]. La CRP contribue également à l'état inflammatoire de l'endothélium en favorisant l'expression de MCP-1 conduisant au chimiotactisme et à la sécrétion de FT à partir du monocyte.

Dans la FA, l'IL-6 favoriserait l'agrégation plaquettaire en réponse à la thrombine[152]. Les interactions entre CD40-L plaquettaires et CD40 leucocytaires sont également susceptibles de favoriser l'expression de FT et la formation du thrombus[153, 154].

La P-sélectine molécule d'adhésion et son ligand PSGL-1 ont un rôle important dans l'interaction plaquette/monocyte pendant la formation du thrombus. Ayashi[155] a démontré que l'induction de FA augmentait l'expression de P-sélectine sur la plaquette dans les minutes qui suivent le déclenchement de la FA pour initier une interaction plaquettes/monocytes. De plus, la P-sélectine est aussi surexprimée par les monocytes et détectée dans les MP circulantes, suggérant que l'interaction des plaquettes et des leucocytes avec l'endothélium, dépendante du contre-ligand PSLG-1, serait renforcée et participerait à l'incrémentation du thrombus.

L'ensemble de ces données laissent à penser que les biomarqueurs de l'inflammation pourraient aider à la stratification du risque embolique dans la FA et démontre clairement l'interaction entre l'inflammation et la thrombose.

4.3 Inflammation, dysfonction endothéliale et remodelage tissulaire dans la FA

Les leucocytes sont une source importante de ROS au site de l'inflammation, en particulier les polynucléaires neutrophiles (PNN) qui sont le 1er type cellulaire à infiltrer les tissus[156]. Les PNN infiltrants libèrent des ROS et de la myélopéroxydase (MPO) qui induisent l'activation d'autres PNN dans le tissu infiltré. Les Pro-MMP sont converties par la MPO et les ROS en MMP actives qui dégradent la MEC amplifiant le recrutement leucocytaire dépendant des fragments protéiques et favorisent la différentiation des fibroblastes en myofibroblastes et un turnover de la MEC accéléré en créant un milieu inflammatoire qui favorise encore le remodelage et conduit à la fibrose.

La MPO secrétée par les PNN est retrouvée en concentration élevée dans l'oreillette de patients en FA[157]. La MPO produit des ROS à partir d'H2O2 ce qui conduit à une diminution de la biodisponibilité du NO et à une dysfonction endothéliale (figure 19).

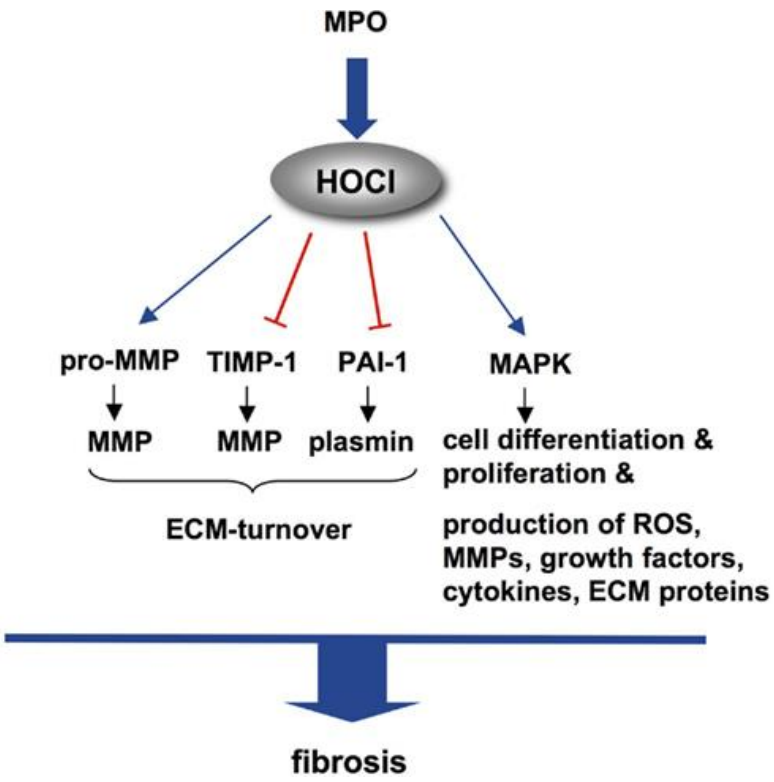

Figure 19 : Cibles moléculaires de HOCl induisant de la fibrose. Dans les PNN, la MPO produit du HOCl qui induit la conversion de la pro-MMP en MMP active, inactive TIMP-1 et PAI-1. Il en résulte un turnover accéléré de la MEC.
Abbreviations: ECM, extracellular matrix; HOCl, hypochlorous acid; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PAI-1, plasmin activator-inhibitor-1; ROS, reactive oxygen species; TIMP-1, tissue-inhibitor of MMPs.[156]

La nitration des protéines dépendante de MPO favorise aussi l'apoptose de cardiomyocyte dans un modèle de dysfonction cardiaque post traumatique[158]. MPO régule l'expression des MMP et son activation est liée à la consommation en NO. Wang a montré[159] que la baisse de biodisponibilité du NO induisait une sécrétion redox-dépendante de MMP-2 active dans les cœurs isolés de rat. La MPO secrétée et associée aux membranes des neutrophiles agit comme un modulateur autocrine en interagissant avec l'intégrine CD11b-CD18 et en activant la voie des p38 MAPkinases indépendamment de l'activité MPO propre[160]. La MPO joue donc un rôle important dans le turnover de la MEC et dans la fibrose en agissant sur le cardiomyocyte mais aussi en amplifiant l'activation neutrophilique, la production d'élastase par les PNN et d'autres enzymes protéolytiques (MMP) dans les tissus cardiaques (figure 20) liant dans la FA, inflammation, stress oxydant et fibrose.
L'Ang II en plus de ses effets directs profibrotiques sur le fibroblaste cardiaque et le cardiomyocyte et en plus de stimuler la libération de MMP, est capable d'activer les leucocytes, de favoriser la dégranulation des PNN[157] de manière MPO-dépendante. En effet, chez des souris invalidées pour le gène de la MPO, le recrutement leucocytaire dans l'oreillette est limité et les souris protégées de la FA

et de la fibrose. De même, l'invalidation de l'intégrine CD11b protège les souris de la fibrose et de l'induction de FA confirmant l'importance du recrutement leucocytaire dans le développement de la fibrose cardiaque au cours de la FA[161].

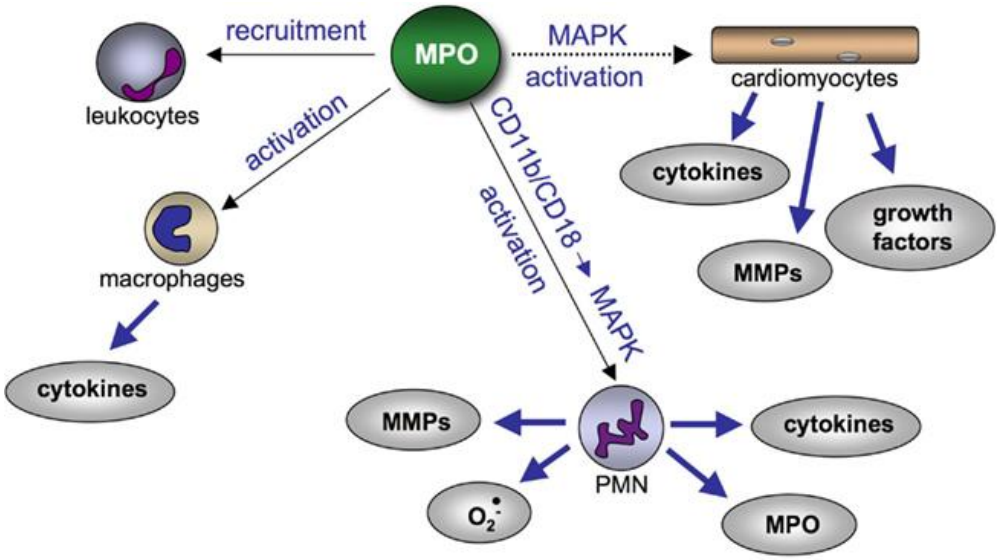

Figure 20 : Effets de la MPO sur les cellules cardiaques et les leucocytes contribuant au remodelage fibrotique.
MPO favorise le recrutement des leucocytes et initie la secrétion de cytokines redox sensible à partir des macrophages. En sus de l'activité MPO, la liaison de la MPO secrétée favorise à la surface du neutrophile une activation autocrine intégrine-dépendante (activation de MAPKs dans le PNN via les integrines CD11b/CD18) avec la sécretion de MMPs actives, la production d'ions superoxides et la secrétion de cytokines. La MPO pourrait aussi activer des voies pro-fibrotiques dans le cardiomyocytes médiées par les MAPKinases (pointillé).
Abréviations: MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; MPO, myeloperoxidase.[156]

4.4 Rôle de la graisse péri-atriale

Récemment la graisse péricardique (GP) en contact direct avec le myocarde adjacent a été impliquée dans la survenue de FA. La GP peut être visualisée et quantifiée au scanner et en IRM. La GP constitue un réservoir important de cytokines et d'adipocytokines qui ont une véritable fonction endocrine. L'abondance de ce tissu adipeux est un facteur prédictif indépendant de survenue de FA[162] et est associé à la charge en FA[163]. Un lien possible a été proposé entre cette GP richement innervée et le système nerveux autonome situé sur le mur postérieur de l'OG avec un impact sur la variation de la FC et les troubles du rythme[164].

La GP s'accumule principalement à l'antrum des VP, sur le mur antérieur, le toit, le plancher et la valve mitrale, régions cibles fréquentes de l'ablation de FA. Le tissu adipeux épicardique pourrait contribuer à la fibrose en libérant des facteurs pro-fibrotiques tels les cytokines inflammatoires, des facteurs de croissance et des MMP comme il a été observé en post-chirurgie cardiaque[165, 166]. En effet, le tissu adipeux épicardique est susceptible d'induire la fibrose atriale chez le rat dans un modèle d'organoculture[166].

Une des adipokines secrétée, l'activine A de la famille du TGFß induit de la fibrose dans l'oreillette. La quantité d'activine dans la GP est élevée dans l'insuffisance cardiaque et chez le patient obèse avec un diabète de type-2[167]. Non seulement ce tissu favorise la fibrose mais l'interaction des lymphocytes T et CD8 concourerait également à sa transformation en tissus fibreux[168].

En plus d'avoir un effet profibrotique , la GP secrète des cytokines inflammatoires associées à la FA telles l'IL-6, IL-8, IL-1ß et le TNFα. De plus, les adipocytes secrètent le MCP-1, une chimiokine recruteuse de monocytes également producteurs de cytokines pro-inflammatoires[169]. Girerd[170] a montré dans une étude évaluant la GP par scanner chez des patients en FA une association entre le volume de GP et des concentrations plasmatiques élevées d'ICAM et vWF, marqueur de la dysfonction endothéliale, particulièrement importante dans la FA persistante.

Le tissu adipeux est donc probablement une source de cytokines pro-inflammatoires qui peuvent diffuser dans le myocarde voisin. La GP est également une source importante de ROS liée à l'activité oxydante majeure du tissu et favorisant ainsi la progression du « substrat » de la FA.

La perte pondérale[171, 172] a un effet sur la charge en FA et diminue la nécessité des anti-arythmiques. Chez le patient obèse, l'état inflammatoire latent, l'activité du système rénine-angiotensine augmenté et des taux élevés d'endothéline et de TGFß1 peuvent contribuer à la progression du substrat atrial[173].

Les causes de l'accumulation de GP sont inconnues. Il est à noter que la stimulation atriale rapide induit chez le cochon l'expression de plusieurs gènes adipocytaires dans l'oreillette susceptibles de contrôler l'accumulation de tissu adipeux péricardique et dont le profil d'expression similaire est retrouvé dans les oreillettes de patients en FA persistante[174].

Ces données confirment un rôle important du tissu adipeux cardiaque dans la pathogénie de la FA bien que son rôle précis et les mécanismes sous-jacents restent encore à définir.

5. FA et sur-risque thrombotique

De récentes études ont identifié de nouveaux facteurs de risque comme l'inflammation, le stress oxydant et des facteurs génétiques dans la genèse de la FA. Elle induit une dysfonction endothéliale, une majoration du statut inflammatoire et une activation plaquettaire. Le rôle de l'apoptose et de l'activation cellulaire, notamment endothéliale, dans la survenue d'un sur-risque thrombotique associé à la FA reste méconnu.

5.1 Pourquoi y a-t-il un état pro-thrombotique dans la FA ?

L'association entre FA et risque d'accident vasculaire cérébral est connu depuis longtemps. Le risque de formation de thrombus dans la FA est multifactoriel et la stase sanguine associée à des concentrations de thrombine augmentée, dans une OG peu contractile n'est pas le seul élément pouvant l'expliquer. Il existe dans la FA un état pro-thrombotique majeur impliquant une dysfonction endothéliale, une stase sanguine anormale et une hémostase perturbée impliquant coagulation, plaquettes et fibrinolyse.

L'anatomie de l'OG est caractérisée par la présence de l'auricule dont l'anatomie prédispose à la stase sanguine et constitue l'endroit où le thrombus se forme dans la plupart des cas. La stase sanguine suite à la perte de la systole atriale lors de la FA dans l'OG est un autre élément contribuant à la genèse du thrombus. Cette stase peut être attestée en échographie trans-oesophagienne par la présence d'un contraste spontané dont la présence est considérée comme un risque indépendant de survenue de thrombus[175, 176].

Le sur-risque thrombotique dans la FA est complexe et multifactoriel et va s'expliquer par des phénomènes d'apoptose, de génération de microparticules (MP) pro-coagulantes et la présence d'un état pro-inflammatoire avec des concentrations élevées de CRP, d'IL-6 et l'induction de l'expression endothéliale et leucocytaire de FT. L'IL-6 augmente aussi la production de plaquettes et leur sensibilité à la thrombine[177]. Ces éléments participent au couplage inflammation/ thrombose dans la FA.

Les modifications de la MEC et le remodelage tissulaire avec l'activation des MMP va participer à cet état pro-thrombotique, favoriser la fibrose, l'infiltration de l'endocarde et la formation du thrombus[178].

Enfin la dysfonction endothéliale avec la perte de biodisponibilité du NO qui normalement prévient l'agrégation plaquettaire et dérégule la production de PAI-1[179], l'implication du système-rénine-angiotensine et son impact sur l'inflammation, la fibrose, la fibrinolyse renforcent le risque thrombotique dans la FA.

5.2 Les microparticules pro-coagulantes (MP)

L'endothélium est une surface hémocompatible dont la thrombogénicité se développe avec l'âge et les agressions inflammatoires. Sur le plan mécanique, la perte de la systole auriculaire est associée à une stase sanguine et à une diminution importante des forces de cisaillement qui diminuent la production de NO vasoprotecteur. Quel que soit le facteur déclenchant, l'activation cellulaire au sens large, est associée à une libération de microparticules membranaires pro-coagulantes dans le compartiment vasculaire. Ce sont des petites vésicules de membrane plasmique émises par les cellules en réponse à une activation, à l'apoptose ou à une lésion thermique. Dans la circulation sanguine, les MP sont émises par la cellule apoptotique ou stimulée et sont la caractéristique d'un dommage tissulaire[180]. Longtemps considérées comme débris cellulaire, les MP constituent un élément clé de la réponse hémostatique car elles exposent de la phosphatidylsérine (PS), un phospholipide anionique dit pro-

coagulant car il est nécessaire à l'assemblage des complexes de la coagulation. Les MP circulantes, provenant principalement des plaquettes, des leucocytes et des cellules endothéliales fournissent une surface phospholipidique additionnelle favorisant la cascade de coagulation et la génération de thrombine. Dans la circulation sanguine, elles sont le réservoir principal de facteur tissulaire (FT) circulant et actif, principal initiateur de la thrombose[181]. Les MP ont déjà été décrites dans les pathologies athérothrombotiques et des données récentes suggèrent leur rôle potentiel dans l'état pro-thrombotique de la FA. Les microparticules (MP) amplifient la réponse thrombotique en délivrant un message pro-coagulant, pro-inflammatoire et pro-sénescent à la cellule endothéliale.

5.3 Apoptose et FA

Quand elle est exposée à un stress, la mort cellulaire survient secondairement à une augmentation de la perméabilité membranaire et à un remodelage. Les concentrations cytosoliques croissantes de Ca^{2+} permettent au cytosquelette de se cliver via l'activation des caspase et de la calpaïne ce qui conduit à une externalisation à la membrane cellulaire d'aminophospholipides pro-coagulants normalement internalisés. Ce processus de remodelage membranaire conduit au bourgeonnement de MPs qui expriment de la phosphatidylsérine (PS) à leur surface[182].

Dans l'oreillette, tous les types de cellules (endothéliale, cardiomyocytes, fibroblastes) peuvent être exposé à la mort cellulaire programmée. Un taux élevé d'apoptose a été détecté dans le coeur humain ou dans les modèles animaux en FA[183].

Il est vraisemblable que les phénomènes pro-apoptotiques commencent assez tôt dans les processus de développement de la FA et du remodelage tissulaire. Dans des modèles animaux d'induction de FA par stimulation, l'apoptose et l'infiltration leucocytaire surviennent tôt et précèdent le remodelage structurel[73]. Dans la cellule endothéliale apoptotique, l'exposition de PS à la membrane précède généralement la fragmentation de l'ADN[184] et est associée à la formation de MP qui exprime à sa surface lorsque la cellule est endothéliale ou leucocytaire, la PS et le FT[182]. En fonction du degré d'apoptose, l'exposition de PS par les cellules apoptotiques augmentera l'activité pro-coagulante phospholipide-dépendante de 25 à 60% évaluée par la mesure du complexe tenase et réduit l'activité anticoagulante de la thrombomoduline, des héparanes sulfates exposés à la surface de la cellule endothéliale[184]. De plus, les cellules apoptotiques endothéliales favorisent l'adhésion aux plaquettes non activées et leur activation suggérant une boucle d'amplification pro-thrombotique[184]. La FA paroxystique est associée à une activation plaquettaire locale[155] et une dysfonction endothéliale[151]. Pendant la FA, le FT, principal inducteur cellulaire de la coagulation est sur-exprimé dans le tissu endothélial lésé où il existe de plus une infiltration de cellules inflammatoires tandis que les cellules mononucléaires circulantes sont en nombre supérieur.

5.4 Inducteurs de mort cellulaire dans la FA

5.4.1 La fréquence cardiaque

La fréquence cardiaque (FC) est probablement le 1er responsable des dommages cellulaires pendant la FA. Plusieurs études ont rapporté que les dommages cellulaires liés à la FC était complètement réversibles quand la FC est contrôlée par des drogues ou l'ablation[185]. Dans un modèle canin de FA induite par une stimulation atriale prolongée, il a été observé la présence d'apoptose dans le tissu atrial avec mise en évidence d'une activation des caspase-3, de Bax et des calpaines concommitante d'une inhibition de la voie anti-apoptotique bcl-2[186]. La perte progressive des cardiomyocytes dans l'insuffisance cardiaque est attribuée à l'apoptose[187, 188]. Dans un modèle d'insuffisance cardiaque induite par la stimulation ventriculaire rapide, l'augmentation d'apoptose était corrélée à des taux d'épinéphrine importants et à la baisse du débit cardiaque[187].

A l'inverse, dans les modèles animaux avec une stimulation atriale rapide mais avec une cadence ventriculaire contrôlée, il n'y avait pas d'induction significative d'apoptose dans l'oreillette. Il apparait donc qu'une cadence ventriculaire élevée est un déterminant majeur de mort cellulaire[189]. Bien que les mécanismes de la cardiopathie rythmique ne soient pas clairement élucidés, les mécanismes possibles sont une surcharge calcique associée à l'inhibition de la Ca^{2+}-ATPase du réticulum sarcoplasmique, le stress oxydant, les dommages mitochondriaux et l'inflammation conduisant à une majoration de l'apoptose[190].

5.4.2 Apoptose et shear stress

Le shear stress (SS) est également un déterminant majeur d'apoptose endothéliale. Le maintien d'un flux laminaire physiologique exerce en effet protecteur du lit vasculaire in vivo par la libération de substances qui favorisent l'anticoagulation, inhibent l'inflammation et induisent la vasodilatation comme le NO[191]. Par exemple, le SS diminue l'activité du FT de la cellule endothéliale en augmentant la sécrétion d'inhibiteur de la voie du FT. Comme la FA conduit à une perte de la contraction organisée et à un flux sanguin turbulent dans l'OG, l'hypothèse a été émise que la FA puisse causer une diminution de l'expression de la NOS dans le tissu endothélial de l'oreillette. La baisse de NO favorise l'expression de PAI-1 et l'agrégation plaquettaire et contribue à la thrombose dans l'OG comme cela a été montré dans un modèle de cochon[105]. In vitro, il a été montré qu'un SS oscillatoire pulsatile induisait un phénotype procoagulant de la cellule endothéliale associée à un doublement de l'activité du FT favorisant la thrombose[192].

5.4.3 Apoptose et hypoxie

Un autre inducteur d'apoptose est l'hypoxie. En effet, le rythme irrégulier de la FA peut être responsable d'un débit coronaire diminué insuffisant pour les besoins augmentés en O2 pendant la FA. La FA est associée à une surexpression atriale de marqueurs hypoxiques et angiogéniques comme les facteurs inductibles à l'hypoxie et le VEGF (vascular endothelial cell growth factor)[193] en parallèle avec le développement de la fibrose. Cette fibrose contribue à l'hypoxie en perturbant la diffusion de l'O2 et en induisant par là-même une ischémie provoquant un signal d'angiogénèse.

5.4.4 Le stretch atrial

Un « stretch » mécanique augmenté au niveau de l'oreillette est également un puissant médiateur d'apoptose et de remodelage tissulaire pendant la FA. Le « stretch » est associé à des changements de la charge calcique pendant la FA conduisant à une augmentation du Ca^{2+} intracellulaire lié aux canaux calciques de type-L et au récepteur de la ryanodine. In vitro, le « stretch » des cardiomyocytes ventriculaires induit la production d'anions superoxydes et les ROS modulent la réponse apoptotique au « stretch » mécanique[189].

5.4.5 L'inflammation et le stress oxydant

En plus du phénotype pro-thrombotique, les cellules endothéliales acquièrent également un phénotype pro-inflammatoire et pro-adhésif. La séquestration dans le tissu atrial de cytokines inflammatoires comme CRP et IL-6 observés chez les patients en FA ou en flutter suggèrent que ces protéines peuvent activer des voies toxiques conduisant à l'apoptose ou au remodelage tissulaire[194]. Une étude récente montre que non seulement la FC mais également l'inflammation et le stress oxydant peuvent déclencher l'apoptose du cardiomyocyte pendant la FA[195]. Chez les patients dont la CRP était plus élevée, une up-régulation de iNOS était observée. La corrélation entre l'expression de iNOS, les péroxynitrites et l'étendue de l'apoptose suggère un lien entre le statut inflammatoire, le stress oxydant et la mort cellulaire. La CRP agirait suite à un déficit en NO comme un puissant inducteur de l'émission de MP par les cellules endothéliales et contribuant à la thrombogénicité[196] Une boucle d'amplification est fournie par les MP également capables de véhiculer la CRP[197].

5.5 Des taux de MP augmentés dans la FA

Dans la FA, il a été mis en évidence une augmentation de la génération de MP pro-coagulantes[153, 198]. L'augmentation progressive des taux de MP pro-coagulantes des patients en RS sans facteur de

risque cardiovasculaire comparée aux patients en RS avec facteurs de risque cardiovasculaire puis chez les patients en FA. Il existe un doublement du taux de MP chez les patients en FA permanente par rapport à des sujets à facteurs de risque égaux. La présence de FA était un facteur prédictif significatif d'augmentation de MP[198].

L'induction de FA active les plaquettes dans les minutes qui suivent et augmente significativement l'expression de P-sélectine sur les plaquettes et sur les MP tandis que son récepteur PSLG-1 est surexprimé par les cellules mononucléaires[155]. L'augmentation d'un taux circulant de MP pendant la FA reflète l'état d'hypercoagulation qui pourrait contribuer à la thrombose atriale. Cependant, chez les patients en FA le lien direct entre l'émission importante de MP et le risque d'AVC n'a pas été montré.

L'origine des MP pro-coagulantes circulantes pendant la FA est plaquettaire ou endothéliale. La quantité de MP plaquettaires mesurées dans le sang augmente chez le patient en FA[155, 199, 200]. Choudhury[153] a montré que les patients en FA avaient des taux plus importants de MP plaquettaires et de P-sélectine soluble que les volontaires sains en RS. Cependant il n'y avait pas de différence observée quand on appariait les patients avec facteurs de risque suggérant que ces modifications peuvent être conséquences des comorbidités cardiovasculaires sous-jacentes plutôt que de la FA elle-même. Si les MP d'origine endothéliale sont plus élevées chez les patients en FA que chez les patients sans FDRCV, les différences ne persistent pas après ajustement à ces facteurs. Il existe une relation significative entre les MP d'origine endothéliale, la pression artérielle systolique, le diabète, l'hypercholestérolémie et la maladie coronaire[198]. Chez des patients en FA, il a été montré après traitement par digitalique qu'il existait des taux élevé de MP endothéliales portant CD62E ou CD31 suggérant une atteinte endothéliale. En effet la digitaline affecte le métabolisme du Ca^{2+} intracellulaire régulateur majeur de la fonction endothéliale[199].

5.6 Rôle potentiel des MP dans la FA

5.6.1 Thrombogénicité

Dans les vaisseaux, le remodelage rapide de la bicouche lipidique de la membrane plasmique (flip-flop) caractérise la réponse procoagulante physiologique en favorisant l'exposition de PS normalement séquestrée dans le feuillet interne de la bicouche[182]. L'exposition de la PS peut être induite par les MP et par d'autres éléments sanguins circulant ou cellules du système vasculaire.

Les MP favorisent la génération de thrombine en catalysant l'assemblage des complexes de la coagulation et initient la cascade de coagulation (FT)[182]. Le FT a une forte affinité pour le facteur VII et va favoriser sa conversion en facteur VIIa, réaction accélérée en présence de phospholidides anioniques et de Ca^{2+} aboutissant à la formation de thrombine, enzyme finale de la coagulation transformant le fibrinogène en fibrine. Dans la FA, un taux élevé de facteur de von Willebrandt (vWF) circulant est aussi objectivé mais aussi son expression par l'endothélium atrial et surtout auriculaire gauche a été mis en évidence. L'interaction plaquettaire GPIb-vWF initie l'arrêt de la plaquette sur la surface vasculaire et contribue à l'émission de MP plaquettaires qui accélèrent encore la formation du

thrombus[201]. Les MP plaquettaires contribuent aussi à l'interaction et au recrutement des monocytes à l'endothélium inflammé et favorise la diapédèse via la libération de RANTES, une CC chémokine[202].
Il est clairement établi que les MP leucocytaires portent une forte activité FT[181]. Dans un modèle expérimental murin de thrombose veineuse, les MP leucocytaires circulantes sont négativement corrélées au poids du thrombus suggérant leur incorporation dans le thrombus. Les MP plaquettaires étaient significativement corrélées à la croissance de ce thrombus[203] indiquant le rôle pivot de l'activation plaquettaire. Il est à noter que les neutrophiles activés émettent aussi des MP porteuses de MAC-1, responsables de l'activation plaquettaire de manière GP-1bα et P-sélectine-dépendante[204].
L'induction de FA active les plaquettes en quelques minutes, augmente l'expression de P-sélectine sur les plaquettes et les MP. Les interactions avec les monocytes étant initiées via la P-sélectine exposée par les MP leucocytaires expliquent possiblement la sur-expression FT par les monocytes[155].

5.6.2 Inflammation

L'interaction plaquette/leucocytes explique l'augmentation de la réponse inflammatoire dans la FA. En effet, dans des conditions inflammatoires, les leucocytes émettent des MP leucocytaires dont la concentration est doublée chez le sujet sain et qui se comportent en effecteurs endothéliaux pro-coagulants. Ces MP induisent l'expression de FT actif et de ICAM-1 et VCAM-1 à la surface cellulaire[205]. Outre ces 2 dernières protéines d'adhésion, les MP induisent la sécrétion de cytokines pro-inflammatoires à partir de l'endothélium comme IL-6 et MCP-1. Il a d'ailleurs été montré que CRP et IL-6 stimulaient la production de FT à partir des monocytes in vitro[206]. Les MP peuvent transférer la CRP pro-inflammatoire à la surface de la cellule suggérant un rôle possible des MP dans le transport et la libération de CRP dans la circulation[196].
Les MP leucocytaires induisent aussi une augmentation rapide de l'expression de vWF à la surface endothéliale favorisant l'adhésion transitoire de plaquettes non activées[207]. Ces 2 observations suggèrent un rôle possible des MP dans des boucles d'amplification du dommage vasculaire induit par les MP pro-inflammatoires. Burger[208] a également montré que les MP endothéliales induite pas l'Ang II stimulaient la surexpression de molécules d'adhésion (V-CAM, PECAM).

5.6.3 Dommages endothéliaux et stress oxydant

Le NO est un facteur important de survie de la cellule endothéliale. Les MP des lymphocytes T altèrent la fonction endothéliale[209] en inhibant l'expression de la eNOS et en induisant la surexpression des cavéoline-1. Burger[208] a également montré que l'Ang II entraîne la libération redox-sensible de MP endothéliales qui stimulent la production de ROS endothéliales et la réponse inflammatoire. En conditions de stress oxydant, les MP endothéliales contiennent des ROS qui induisent une interaction cellules endothéliales/monocytes. Essayag a aussi montré que les MP issues des cellules musculaires lisses apoptotiques peuvent également diminuer la production de NO

contribuant à la dysfonction endothéliale[210]. De la même manière, les MP plaquettaires entraînent une surexpression de cytoadhésines dans les monocytes et les cellules endothéliales favorisant l'adhésion cellulaire et l'inflammation[211].

5.6.4 Remodelage

Les MMP de la MEC et leurs inhibiteurs régulent en partie le remodelage tissulaire[212]. Comme les MP émises par les cellules apoptotiques portent des MMP actives, il est possible que les MP participent à la protéolyse de la MEC et au remodelage de l'OG[180]. *In vitro et in vivo*, la thrombine et le CD40L ont un effet synergique sur l'expression de MMP-10 au niveau de la cellule endothéliale et des MP ce qui pourrait représenter un nouveau lien entre l'inflammation, la thrombose et le remodelage tissulaire[213]. *In vitro*, les MP endothéliales sont capables de se lier aux protéines de la MEC comme la fibronectine et montrent une activité MMP-2 alors que leurs inhibiteurs endogènes TIMP1 et 2 restent en phase soluble[214]. Ces données indiquent que les MP sont susceptibles de se concentrer dans la MEC et de la dégrader, soulignant une relation possible entre MP et remodelage électrophysiologique objectivé dans la FA. Okumura a d'ailleurs récemment montré que les MMP-2 et non la CRP était le principal facteur prédictif de récurrence de FA après ablation[215].

5.6.5 MP et AVC

Comme un taux de MP circulantes élevé est retrouvé dans la FA, il a été suggéré que ces MP pouvaient se comporter comme des inducteurs de thrombus dans l'AVC où leur concentration notamment d'origine plaquettaire augmente[216]. Dans une autre étude, les MP endothéliales étaient liées à la taille de l'infarctus cérébral et au pronostic clinique[217]. Les source des MP endothéliales pendant l'AVC sont encore débattues. Elles peuvent provenir de la stimulation endothéliale systémique ou atriale ou de l'ischémie cérébrale locale. Ces données suggèrent que les MP pourraient servir de moyens de surveillance du risque thrombotique dans la FA.

5.7 L'hypercoagulabilité peut induire le remodelage tissulaire dans la FA

En plus des potentielles implications des MP porteuses de MMP dans le remodelage, il a été montré que les facteurs de coagulation favorisaient le remodelage dans la FA. En effet, la thrombine, une serine protéase, la dernière enzyme de la cascade de la coagulation, est directement impliquée dans la réparation tissulaire et le remodelage car elle favorise la transdifférentiation mésenchymateuse endothéliale qui transforme des cellules endothéliales en myofibroblastes capables de secréter la MEC dans les embryons de poulet et par un mécanisme PAR-dépendant[218]. La thrombine exerce également un effet sur les jonctions cellulaires endothéliales, sur la cellule endothéliale et sur la migration des cellules musculaires lisses et leur prolifération dépendante de son récepteur PAR-1

(protease activated receptor) et de la PLA2c[219]. La transcription et l'activité de la MMP-2 induite par la thrombine peut également contribuer au changement structurel dans l'oreillette[220] comme le fait l'augmentation de collagène secrété par les fibroblastes induite par la thrombine.
Il a également été montré que le facteur Xa favorisait la prolifération des fibroblastes, la migration et différentiation en myofibroblastes via l'activation du récepteur PAR-2 directement activé par le facteur Xa sans passer par la thrombine. Le facteur Xa stimule la prolifération de fibroblastes et de cellules musculaire lisses. Cette voie permet l'expression d'IL-6, IL-8, de MCP-1 et de TGFß dépendant de ERK1/2[221]. Très récemment, l'équipe de Schotten[222] a montré que l'état d'hypercoagulabilité de la FA entrainait une réponse pro-fibrotique (augmentation du TGFß), pro-inflammatoire (augmentation du MCP-1) dans le fibroblaste atrial. Ces effets sont atténués par le dabigatran, un inhibiteur direct de la thrombine. L'hypercoagulabilité favorise le développement du substrat de la FA chez des souris transgéniques à phénotype pro-coagulant. Dans un modèle de chèvre en FA, la nadroparine (HBPM fraxiparine) atténuait aussi la fibrose atriale et la complexité du substrat de la FA soulignant que l'anticoagulation prévient non seulement l'AVC mais inhibe aussi le développement d'un substrat pour la FA.

6. FA et senescence

6.1 Introduction et définitions

Le vieillissement vasculaire est un facteur majeur de développement de fibrillation auriculaire, de remodelage des oreillettes et de développement de complications thrombotiques. Si, sur le plan épidémiologique, les liens entre vieillissement et thrombogénicité sont bien caractérisés, les mécanismes précis à l'échelle cellulaire et moléculaire restent largement spéculatifs.
Le sur-risque thrombotique et le remodelage tissulaire dans la FA impliquent des mécanismes communs comme l'apoptose, l'inflammation, la sénescence et la dysfonction endothéliale. A ce jour, le rôle de la sénescence endothéliale dans le sur-risque thrombotique et le remodelage tissulaire associée à la FA reste parfaitement méconnue.

La sénescence correspond à un arrêt irréversible du cycle cellulaire et peut être déclenchée par différents stress cellulaires comme les dommages de l'ADN, le stress oxydant ou l'activation d'oncogènes[223]. L'induction de sénescence à court terme a un rôle bénéfique dans la suppression des tumeurs, les phénomènes de cicatrisation et possiblement le développement embryonnaire. La présence à long terme de cellules sénescentes dans les tissus favorise les pathologies liées à l'âge et la tumorigénèse. Ces cellules sénescentes s'accumulent dans les tissus âgés, restent métaboliquement actives mais ne peuvent proliférer pouvant par là-même empêcher la régénération tissulaire ou la dysfonction d'organe.
Récemment, il a été suggéré que la senescence endothéliale contribuait au développement progressif de la dysfonction endothéliale lors du vieillissement[224]. La sénescence a déjà été décrite il y a 40 ans

comme un processus limitant la prolifération de la cellule humaine normale en culture après 50 à 70 doublements de population, ces cellules sont arrêtées irréversiblement en phase G1 du cycle cellulaire et ne répondent plus aux stimuli prolifératifs mitogéniques[225, 226] bien que conservant une proportion d'activité métabolique. Elles acquièrent des propriétés morphologiques et phénotypiques distinctes.

Dans le compartiment vasculaire, la sénescence cellulaire peut être vue comme un mécanisme qui limite les dommages tissulaires et la tumorigénicité. Dans des conditions physiologiques, le système immunitaire est capable d'éliminer les cellules sénescentes favorisant la suppression tumorale et la cicatrisation. Les cellules sénescentes s'accumulent dans les tissus avec l'âge en raison d'un niveau élevé de formation de cellules sénescentes soit par défaut du système immunitaire vieillissant ou par une clearance diminuée[223]. L'existence au long cours de cellules sénescentes dans les tissus peut générer des pathologies liées à l'âge et contribue à expliquer le déclin de l'organe avec le temps[227].

La sénescence cellulaire peut être induite par différents stimuli qui partagent tous les mêmes voies pour initier et pérenniser un programme soutenu de sénescence[228]. La sénescence peut être la conséquence d'un raccourcissement des télomères (sénescence réplicative) ou par d'autres stimuli indépendants du raccourcissement des télomères qui induisent de façon permanente de la sénescence (sénescence prématurée).

6.2 Sénescence et effet suppresseur de tumeur

Échapper au processus de sénescence constitue une étape de la tumorigenèse qui permet aux cellules cancéreuses de proliférer[223]. Le facteur physiologique principal responsable du dysfonctionnement des télomères dans les cellules somatiques humaines est l'accumulation des divisions cellulaires. Au cours de la division cellulaire, des motifs TTAGGG sont perdus en raison de l'incapacité de la machinerie conventionnelle à répliquer les extrémités linéaires des chromosomes. Cette perte d'ADN télomérique est compensée par une transcriptase inverse spécialisée, la télomérase, qui assure la synthèse *de novo* des répétitions télomériques sur les télomères préexistants, maintenant ainsi leur longueur. Le raccourcissement des télomères au-dessous d'une taille minimale critique est une source d'instabilité génétique et serait à la base de la sénescence réplicative. Il a été proposé qu'après épuisement de leur stock d'ADN télomérique, les chromosomes seraient reconnus comme une simple « cassure » de l'ADN activant les systèmes de réparation. En accord avec cette hypothèse, l'entrée en sénescence s'accompagne d'une augmentation de la fonction transactivatrice de p53 et de l'accumulation de p21, un inhibiteur des kinases dépendantes des cyclines en aval, codé par *WAF1*, gène cible de p53. De plus, le déclenchement et le maintien de l'arrêt réplicatif dépendent de p53.

Les suppresseurs de tumeur p16 et p21 sont des médiateurs de l'arrêt du cycle cellulaire et de la senescence.

Plusieurs signaux de stress, comme les dommages causés à l'ADN (cytotoxicité…), le stress oxydant et l'expression d'oncogène (*ras* activé), activent un programme de sénescence non télomérique, mais

qui est morphologiquement et biochimiquement semblable à la sénescence réplicative. La sénescence peut également être déclenchée in vitro par la délétion de suppresseur de tumeur comme PTEN, un facteur de transcription qui régule la stabilité de p53 en l'induisant.

Elle peut être également causée en culture cellulaire quand les cellules explantées d'un organe et placées en culture sont en concentration anormale de nutriments et en l'absence de voisinage cellulaire et de matrice extracellulaire[229].

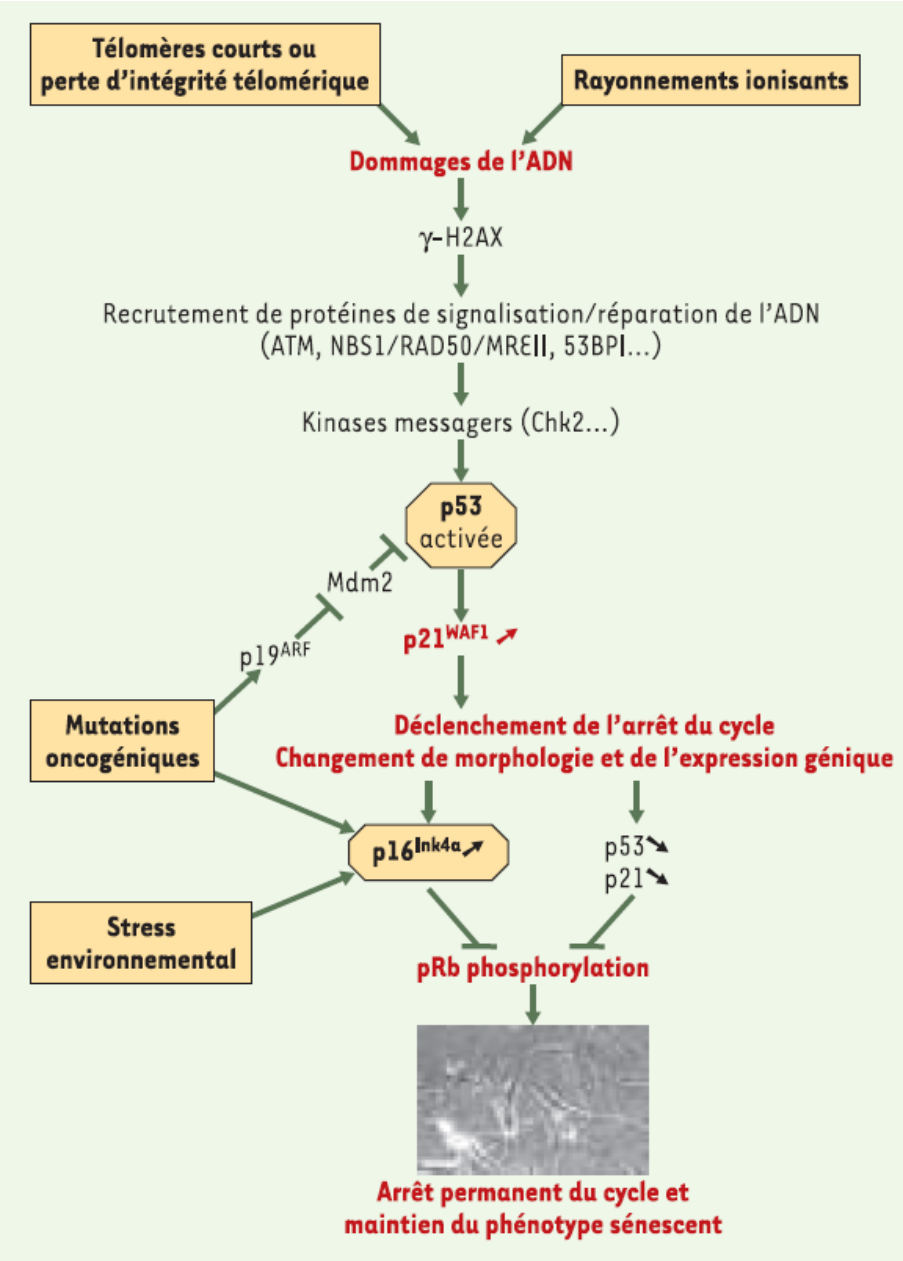

Figure 21 : Mécanismes de l'arrêt permanent du cycle cellulaire lors de la sénescence. Divers stimuli peuvent activer un programme commun de sénescence. Le raccourcissement des télomères est le stimulus physiologique le mieux décrit dans l'induction de la sénescence. L'expression d'oncogènes activés peut également déclencher la sénescence et représenterait une autre façon par laquelle la sénescence est un mécanisme suppresseur de tumeur. Le programme effecteur de la sénescence comprend les voies p53/p21WAF1 et p16INK4a/pRb. p53 serait activée par les kinases ATM/Chk2 (médiateurs des systèmes de réparation des dommages de l'ADN) ou par p19ARF (médiateur de la voie signalétique activée par ras oncogénique chez la souris). Le facteur p16INK4a serait activé par le stress imposé par des conditions inadéquates de culture et par une stimulation oncogénique déréglée.[230]

6.3 Rôle de la voie de signalisation p53/p21

La p53 est un facteur de transcription, suppresseur de tumeur indispensable au maintien de l'intégrité cellulaire, considérée comme « gardien du génôme » dont le gène est situé sur le chromosome 17[230].
Elle se fixe de manière spécifique aux régions régulatrices de gènes dont elle contrôle l'expression. Dans une cellule normale, en l'absence de tout stress, il y a très peu de p53, car celle-ci n'est pas nécessaire au fonctionnement de la cellule. Cette absence de p53 est due à l'enzyme mdm2, une E3 ubiquitine ligase qui, en se fixant sur p53, induit sa destruction dans le protéosome. En réponse au stress, l'association entre p53 et mdm2 est abolie ce qui conduit à une augmentation de la quantité de p53 libre dans la cellule. C'est la « phase d'activation » qui est suivie d'une « phase de modifications » post traductionnelles qui active la fonction de facteur de transcription.
L'ensemble de ce programme pourra conduire soit à l'arrêt du cycle cellulaire soit à l'apoptose. L'arrêt du cycle cellulaire permet à la cellule de stimuler ses mécanismes de réparation, dont certains sont directement activés par p53. Lorsque la réparation est effectuée, le taux de p53 retourne à la normale et le cycle cellulaire reprend. Si les dommages subis par la cellule sont irréversibles, p53 induit l'apoptose (ce qui conduit à son élimination)[231] en favorisant l'expression l'expression du gène de Bax, protéine pro-apoptotique et de p21, p27, p47, p15, p16, p18 et 19 qui sont des inhibiteurs de kinases cyclines-dépendante CKDI. La surexpression de p21 et p16 peut aussi être p53 indépendante[226, 232].
L'activation de ces voies inactive toutes les CDK et empêche la phosphorylation des protéines RB (retinoblastoma protein) conduisant à l'arrêt du cycle cellulaire

6.4 Caractéristiques de la sénescence et biomarqueurs

Les cellules sénescentes montrent différentes caractéristiques morphologiques et biochimiques dont la détection permet l'identification de la sénescence in vivo et in vitro (figure 22).
L'arrêt du cycle cellulaire (1ère caractéristique de la sénescence) ne peut être réversé par des stimuli physiologiques[233]. Plusieurs voies pour réverser cet arrêt et ré-entrer dans le cycle cellulaire ont été décrites. L'inactivation de la voie p53 permet une réversion de la sénescence et certaines interleukines peuvent mettre fin à cet arrêt[234] Cette réversibilité peut impliquer l'expression de p16. Les suppresseurs de tumeur p16 et p21 sont des médiateurs de l'arrêt du cycle cellulaire et de la sénescence. Du fait que ni p16 ni p21 ne sont strictement nécessaires pour l'induction et le maintien du programme de sénescence, leur valeur prédictive est limitée quand analysée isolément[235].

L'activité enzymatique de la Senescence Associated béta-galactosidase (SA-Béta-GAL) est le marqueur de sénescence le plus utilisé[236]. La β-galactosidase normalement active à pH 4,5 dans les lysosomes est capable de dégrader le composé X-gal dans des conditions non optimales (pH 6) dans les seules cellules sénescentes du fait de leur forte activité lysosomiale[237]. L'activité SA-Béta-GAL n'est pas complètement spécifique car certaines études montrent qu'on peut obtenir un marquage positif avec des cellules en forte confluence après une culture cellulaire prolongée[238]. Cependant le

SA-βgal reste le marqueur le plus souvent utilisé pour identifier des cellules sénescentes *in vitro* comme *in vivo*.

In vitro, les cellules ayant atteint le plateau de la courbe de croissance présentent de nombreuses caractéristiques phénotypiques spécifiques de la sénescence. Ces changements phénotypiques sont :

- augmentation de la taille des cellules et étalement sur leur support
- augmentation de la taille des noyaux, parfois accompagnée d'une polynucléation
- changements dans la structure de la chromatine (foyers d'hétérochromatine, *Senescence Associated Hetero-chromatine Foci* (SAHF)
- augmentation de l'activité lysosomiale pouvant être associé à une augmentation des activités autophagiques (formation de vésicules à double membrane qui contiennet une activité lysosomiale importante et jouent un rôle important dans la régulation des protéines maintenant l'homéostasie)
- accumulation d'agrégats lipido-protéiques (lipofuscine)
- désensibilisations des cellules aux stimuli de facteurs de croissance et d'apoptose
- métabolisme actif permettant l'expression de transcriptomes, protéomes et sécrétomes spécifiques.

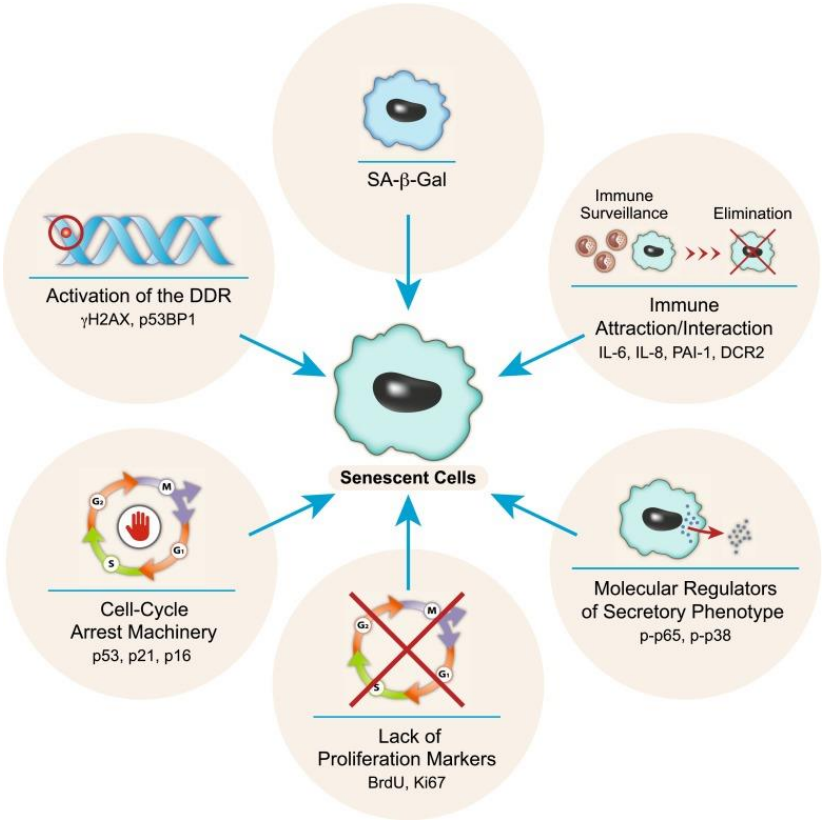

Figure 22 : Caractéristiques biochimiques et morphologiques des cellules sénescentes. Les marqueurs d'identification de la cellule sénescente : l'utilisation de plusieurs marqueurs moléculaires représentant des caractéristiques différentes de la cellule sénescente sont nécessaires pour les identifier. Les marqueurs sont divisés en plusieurs catégories selon leur fonction. Une combinaison de différents marqueurs augmente la validité de leur identification. [239]

6.5 Autre impact physiologique des cellules sénescentes in vivo

En plus de leur implication dans la suppression de tumeur, les cellules sénescentes jouent un rôle bénéfique dans des pathologies non cancéreuses en limitant la fibrose. Par exemple, dans le foie, les dommages tissulaires stimulent les cellules hépatiques stellaires qui prolifèrent et secrètent de la matrice extracellulaire (collagène) et forment une cicatrice fibreuse. La prolifération de ces cellules induit la sénescence cellulaire conduisant à une sécrétion limitée réduisant la fibrose. Les cellules sénescentes sont ensuite éliminées par les cellules immunes comme les natural killer (NK). Quand les mécanismes conduisant à l'élimination cellulaire médiée par les NK est défaillante, la fibrose est augmentée[240, 241]. Ainsi l'induction de sénescence dans les cellules hépatiques stellaires prévient les dommages hépatiques et favorise la fonction hépatique. Dans le pancréas, les mêmes phénomènes ont été observés[242].

De même, CCN1, une protéine de la matrice extracellulaire qui s'accumule dans la MEC des tissus lésés cutanés induit en se liant avec l'intégrine alpha 6 béta1 et les héparan sulfate une sénescence rédox-sensible des fibroblastes avec activation de p53 et de p16 conduisant à l'expression de gènes anti-fibrotiques. Au cours du processus de réparation, ce mécanisme protège d'une fibrose excessive le tissu de granulation où s'accumulent les fibroblastes et favorise la cicatrisation[243].

La sénescence cellulaire est un processus qui limite les dommages tissulaires dans une multitude de tissus du moins à court terme. Elle initie la réparation tissulaire et restaure le tissu dans un état proche de celui pré-lésionnel. Cependant, une sénescence cellulaire prolongée, lorsque les cellules immunes n'éliminent pas les cellules sénescentes, la dysfonction cellulaire caractéristique de la cellule sénescente provoque un remodelage pathologique des tissus et conduit à la fibrose[223].

6.6 Impact pathologique des cellules sénescentes

Si l'induction de sénescence est bénéfique dans la prévention de la tumorigénèse et de la limitation des dommages tissulaires, l'effet à long terme de l'accumulation dans les tissus des cellules sénescente favorise les pathologies liées à l'âge.

La présence de cellules sénescentes impacte de 3 manières le tissu[223] : elle empêche la régénération tissulaire en raison de l'incapacité à proliférer, elle entraîne une dysfonction cellulaire puis tissulaire et elle a un effet sur son micro-environnement.

Il est probable que les protéines et molécules secrétées par la cellule sénescente joue un rôle pivot dans la progression des pathologies liées à l'âge caractéristique d'un état d'« inflammation locale stérile » qui induit des dommages dégénératifs et hyperplasiques à long terme [223].

Par exemple, les voies de signalisation dépendantes des récepteurs de l'IL-8 et IL-6 sont nécessaires à l'induction de sénescence en réponse à l'oncogène BRAF[244]. Récemment dans un modèle murin de surcharge en pression, il a été montré une surexpression p53 dépendante de la protéine d'adhésion ICAM-1 dans les cellules endothéliales du coeur en insuffisance cardiaque sous le contrôle du système nerveux sympathique, suggérant un état inflammatoire de l'endothélium[245].

Le stress oxydant joue un rôle majeur dans la sénescence, en provoquant le raccourcissement des télomères induisant la réponse aux dommages de l'ADN. L'élévation des ROS, majoritairement de source mitochondriale, a été associé à la sénescence réplicative et prématurée induite par le stress et les oncogènes[246],[247]. La suppression du stress oxydant ou de l'hypoxie préserve la longueur des télomères et prolonge la durée de survie de la cellule[248]. Cependant les mécanismes impliquant les ROS restent mal définis. Dans la cellule endothéliale, à la dysfonction associée à la sénescence est associée à une diminution de l'expression de eNOS et de formation de NO et les MPs émises par les cellules sénescentes de souris ont été décrites comme effecteurs autocrines et d'une sénescence ROS-dépendante[249] (Burger,J Am Heart Assoc 2012), tandis que d'autres chercheurs du laboratoire ont identifié une mobilisation précoce des MAP-kinases dans ces mécanismes rédox impliquant le système local de l'angiotensine. (M. Abbas, thèse de sciences)

Le remodelage de cette matrice est un important mécanisme dans la régulation de la différentiation cellulaire, de l'angiogénèse et de la cicatrisation et son intégrité fonctionnelle est nécessaire au maintien d'une niche tissulaire physiologique, un déséquilibre du contenu protéique de la MEC ou sa lyse partielle par les MMPs ou collagénases secrétées par les cellules sénescentes provoquant un état pathologique[223].

L'accumulation de molécules pro-inflammatoires ou pro-thrombotiques comme IL-1α, ICAM-1 ou de l'inhibiteur-1 de l'activateur du plasminogène (PAI-1) dans l'environnement des cellules sénescentes qui les secrètent, modifie l'environnement tissulaire suggérant un rôle dans le vieillissement accéléré des tissus[250],[251, 252]. Par exemple, dans un modèle murin de vieillissement accéléré, les cellules musculaires lisses vasculaires sénescentes expriment et sécrètent par un mécanisme dépendant du degré de dommage de l'ADN et associé à une surexpression de la p16, des protéines identifiées dans la différentiation des ostéogénique (RUNX-2, alkaline phosphatase (ALP), type I collagen and BMP-2, prélamine A) et susceptibles de favoriser la calcification vasculaire au cours des pathologies cardiovasculaires[239, 253] . Récemment dans un modèle murin de constriction aortique[254], une activité SA-Bétagalactosidase, et la surexpression de p53 et p16 a été spécifiquement mise en évidence dans les régions fibrotiques péri-vasculaires chez les animaux soumis à constriction aortique. L'invalidation des gènes de p53/p16, majorait la fibrose induite après constriction aortique et la dysfonction cardiaque, tandis qu'une surexpression cardiaque du gène de CCN1 (cf ci-dessus), inducteur de sénescence prématurée, réalisée par transfert adénoviral protégeait la fonction cardiaque et diminuait la fibrose de 50%. Ces données suggèrent que la sénescence prématurée de myofibroblastes cardiaques constitue un mécanisme anti-fibrotique physiologiquement pertinent.

Dans la fibrillation auriculaire où des réponses cellulaires inflammatoires, pro-fibrotiques et apoptotiques favorisent le remodelage tissulaire, aucune donnée sur le rôle potentiel de la sénescence n'est disponible. Seule une étude utilisant les puces à cDNA a décrit dans le tissu atrial de patients en FA, une expression augmentée des gènes du récepteur AT2 de l'AngII, de p21 et de p27 et une expression diminuée du gène de Bcl-2 protecteur de l'apoptose comparativement à celle de patients en RS. Cet état pro-sénescent était vérifié au niveau protéique et associé à une fragmentation de l'ADN des cardiomyocytes indiquant la concommitance de processus apoptotiques et pro-sénescents[255].

III. Hypothèses et objectifs du travail

III. Hypothèses et objectifs du travail

Le risque de formation de thrombus dans la FA est multifactoriel et fortement associé à une perturbation des paramètres de la triade de Virchow mais aussi à des dysfonctions des cellules vasculaires (sénescence endothéliale, musculaire, leucocytaire) et à une réponse inflammatoire. De plus, une hémostase perturbée dont la localisation atriale constitue une niche tissulaire amplificatrice avec un recrutement plaquettaire et neutrophilique favorise un remodelage tissulaire pro-fibrotique. Par ailleurs, l'oreillette en FA présente un remodelage tissulaire électrique, contractile puis structurel (pathologique ou après ablation thérapeutique) également associé à un stress oxydant, et à des réponses inflammatoires et procoagulantes.

Notre hypothèse est que dans la FA, les MPs sont des biomarqueurs reflétant le degré d'activation ou d'apoptose des cellules circulantes de la paroi vasculaire mais aussi des effecteurs cellulaires autocrines et paracrines. Ces MPs contribueraient à la sénescence endothéliale, induiraient l'expression d'un profil d'expression protéique thrombogénique par les cellules (facteur tissulaire, ICAM, AT-1, AT-2) mais aussi, en fourniraient une surface catalytique pour les réactions de la coagulation, dont la présence prolongée est favorisée par la stase sanguine dans l'auricule ou l'oreillette.

Dans ce contexte, la niche environnementale que constitue chaque oreillette (OG vs OD) pourrait influer sur le profil cellulaire et les propriétés des MPs. De même, les différentes techniques ablatives de FA pourraient avoir des impacts spécifiques sur l'activation cellulaire, la génération de MPs dans l'oreillette et sur leurs propriétés d'effecteurs tissulaires.

Les objectifs de ce travail sont:

1-Mesurer et déterminer dans les deux oreillettes les MPs procoagulantes et leur origine cellulaire afin de déterminer une éventuelle différence du statut pro-thrombotique entre les oreillettes droite et gauche dans la FA.

2- Evaluer l'impact des techniques ablatives de la FA par radiofréquence sur l'activation cellulaire dans l'oreillette gauche en mesurant la concentration des MP et comparer les effets de deux types de cathéters d'ablation sur le statut thrombotique.

3- Identifier la sénescence dans les auricules de patients en FA et en rythme sinusal en analysant l'expression protéique de différents marqueurs clés tels p53, FT, le stress oxydant dans les tissus de l'auricule prélevés chez les patients bénéficiant d'une chirurgie cardiaque.

4- Mettre au point un modèle in vitro de sénescence réplicative et prématurée de cellules endothéliales auriculaires extraites d'oreillettes gauches de porc et étudier les modifications phénotypiques associées au développement de la senescence, y compris la génération de MPs, afin de comprendre la physiologie de la cellule endothéliale auriculaire.

IV. Exposé des travaux

Première partie: Fibrilation auriculaire et statut thrombotique: Existe-t-il des différences selon l'oreillette ou la technique d'ablation ?

Dans la FA, les causes de formation de la plupart des thrombi dans l'oreillette gauche (OG) restent débattues. Des données récentes suggèrent qu'une activation plaquettaire supérieure dans l'OG favoriserait la formation de thrombus dans l'OG et non dans l'oreillette droite (OD)[256].
Outre l'évidente stase sanguine, une dysfonction endothéliale majeure, favorisant l'expression induite de facteur tissulaire (FT), le recrutement et l'activation plaquettaire et leucocytaire ainsi que la réponse inflammatoire ont été évoquées.[201]
Par ailleurs, les techniques ablatives sont susceptibles de favoriser la formation d'un thrombus au cours de la procédure, notamment sur l'électrode des cathéters à irrigation interne. Nous avons donc évalué l'impact de procédures d'ablation de FA en radiofréquence avec deux types de cathéters sur l'activation cellulaire et plaquettaire, les dommages tissulaires et l'inflammation. Un tirage au sort a réparti les patients en 2 groupes, cathéter à irrigation interne et cathéter à irrigation ouverte.

Les MPs, outil non invasif de détection d'un dommage cellulaire potentiellement thrombotique

Pour évaluer l'éventuel sur-risque thrombotique chez les patients en FA, nous avons choisi une méthode non invasive : la mesure des MPs pro-coagulantes accessibles par prélèvement sanguin, et la caractérisation de leur origine cellulaire qui constitue une signature de l'atteinte cellulaire et du stress cellulaire à l'origine de l'émission des MPs[257]. En effet, les concentrations des MPs sanguines varient avec le degré de sévérité clinique de la maladie, comme le laboratoire et d'autres l'ont démontré dans l'HTAP[258]. La méthode de dosage est une technique originale basée sur la capture des MPs sur annexine-5 (MP totales) ou sur un anticorps spécifique de la lignée cellulaire émettrice[259]. Cette méthode fonctionnelle garantit le caractère pro-coagulant des MPs grâce à une mesure par dosage prothrombinase qui est strictement dépendant de la quantité de phosphatidylsérine exposée à la surface des MPs. En outre, cette méthode de détection est indépendante de la taille des MPs[260]. Une variante de la méthode permet le dosage de l'activité facteur tissulaire des MPs (cf article 1).

Les MP constituent un réservoir dynamique d'effecteurs pro-coagulants dont les concentrations circulantes et l'origine cellulaire sont la résultante de leur production sous l'effet du stress cellulaire, de leur capture par les cellules circulantes, dans des agrégats, ou à la surface des cellules endothéliales inflammatoires ou activées comme les MPs plaquettaires qui favorisent par le dépôt endothélial de RANTES le recrutement monocytaire[257]. Elles sont pro-coagulantes en raison de la phosphatidylsérine, un phospholipide anionique de la bicouche de la membrane plasmique exposé à leur surface, et de l'éventuelle présence du facteur tissulaire. La phosphatidylsérine constitue une surface phospholipidique additionnelle pour l'assemblage des complexes de la coagulation et la

génération de thrombine, tandis que le facteur tissulaire, est le seul facteur de la coagulation membranaire induit en réponse à un stress et initiateur de la cascade. Dans les désordres cardiovasculaires, les MPs circulantes sont majoritairement d'origine plaquettaire et leucocytaire et l'élévation des concentrations de MPs endothéliales est souvent caractéristique bien qu'elles constituent une population minoritaire[181]. Les MPs d'origine endothéliale et plaquettaire sont retrouvées à taux élevés dans l'AVC ischémique et associées à la taille de l'infarctus et au pronostic clinique et constituent donc un marqueur potentiel de risque ischémique[217, 261]. Dans la FA persistante, ces MPs circulent à des concentrations supérieures à celles mesurées chez des patients en rythme sinusal présentant des facteur de risque cardiovasculaires similaires[198].

Dans le premier article, les concentrations de MPs (Totales, plaquettaires, leucocytaires, endothéliales et porteuses de FT) ont été mesurées localement dans la cavité atriale, de façon à comparer les concentrations locales, potentiellement modulées en cas de rétention dans la cavité ou d'activation cellulaire intense du tissu atrial. Vingt-deux patients présentant de la FA paroxystique ou persistante symptomatique hospitalisés pour ablation de FA ont été prélevés dans l'OD puis dans l'OG après cathétérisme trans-septal avant l'ablation afin d'évaluer l'activation cellulaire et plaquettaire.

Dans le deuxième article, les MPs ont été mesurées chez soixante patients prélevés avant, juste après l'ablation dans l'oreillette droite puis à 24h et 48h après sur voie veineuse périphérique.

Dans les deux cas les MPs pro-coagulantes d'origine endothéliale, plaquettaire et leucocytaire ont été recherchées pour évaluer le statut thrombotique et aussi l'éventuelle présence d'une réponse inflammatoire ou immune. Les mesures de MPs ont été confrontées à celles de l'activation plaquettaire évaluée par l'immunomarquage de GPIIbIIIa et de P-sélectine à la surface des plaquettes prélevées dans les mêmes conditions que les MPs et par le degré d'activabilité des plaquettes dans le plasma veineux. D'autres marqueurs circulants de l'inflammation ou du dommage tissulaire ont été recherchés en parallèle. (CRP, CK, Troponine T)

Article 1 :

Do Atrial Differences in Endothelial Damage, Leukocyte and Platelet Activation, or Tissue Factor Activity Contribute to Chamber-Specific Thrombogenic Status in Patients with Atrial Fibrillation?

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Do Atrial Differences in Endothelial Damage, Leukocyte and Platelet Activation, or Tissue Factor Activity Contribute to Chamber-Specific Thrombogenic Status in Patients with Atrial Fibrillation?

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Atrial Differences in Endothelial Damage, Leukocyte and Platelet Activation. ***Background:*** **Thrombi form mainly in the left rather than the right atria of patients with atrial fibrillation (AF), the reason of this predilection being unknown.**

Objective: **The purpose of this study was to investigate whether atrial-specific differences in endothelial damage, leukocyte activation, platelet stimulation, and tissue factor activity occur in patients with AF.**

Methods: **Twenty-two patients (16 men, 6 women; age 56 ± 8 years; 16 paroxysmal AF, 6 persistent AF) with AF undergoing pulmonary vein isolation were investigated. Blood samples from the left and the right atrium were obtained at the start of the procedure. Microparticles (MPs) released by apoptotic/stimulated cells were measured by capture assays. Their procoagulant abilities were quantified by functional prothrombinase and tissue factor assays and their cellular origin were determined (endothelium, platelet, leukocyte). Platelet reactivity was evaluated by whole blood flow cytometry for expression of platelet P-selectin (CD62P), active glycoprotein IIb/IIIa receptor (PAC-1). Platelet aggregation was evaluated using ADP, TRAP and collagen-induced whole blood aggregometry.**

Results: **There were no atrial-specific differences in the levels of total procoagulant MPs, leukocyte-derived-MPs or platelet-derived MPs. Conversely, endothelial-derived MPs and tissue factor activity and collagen-induced platelet aggregation were slightly elevated in the right atrium ($P < 0.05$).**

Conclusions: **Our data show no evidence for increased thrombogenic status in the left atrium that would account for its greater propensity for thrombus formation in patients with AF.** *(J Cardiovasc Electrophysiol, Vol. 25, pp. 266-270, March 2014)*

apoptosis, arrhythmia, microparticles, stroke, thrombosis, tissue factor

Introduction

In atrial fibrillation (AF), the reasons why most of the thrombi form in the left atrium are mainly unknown. Recent data have suggested that enhanced platelet reactivity within the left atria may explain why AF is associated with an increased risk of thrombus formation in the left but not the right atrium.[1] Besides the classical paradigm linking stasis to platelet activation and thrombus formation, advances in the understanding of the purported mechanisms for thrombogenesis in AF have suggested a key role for endothelial damage/apoptosis, tissue factor (TF) expression together with platelet activation and inflammation.[2-8] Circulating procoagulant microparticles (MPs) are small-membrane vesicles that are shed from virtually all cells in response to activation, thermic injury,[9,10] and/or apoptosis.[8,11] Apart from being a reliable probe of vascular injury, circulating procoagulant MPs mainly of platelet, leukocyte, and endothelial origin provide an additional phospholipidic surface for the assembly of blood coagulation factors, thereby promoting the coagulation cascade and thrombin generation. In the vasculature they constitute the principal reservoir of TF activity, the main initiator of blood coagulation.[11] Because high levels of platelets and endothelial-derived MPs are identified during stroke and are associated with infarct size and clinical outcome, they are proposed to be a potent marker of ischemic risk.[8,12-14] In keeping with the important role of circulating procoagulant MPs in the hypercoagulable state observed in AF, a stepwise increase of procoagulant MPs has been seen in patients in

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sinus rhythm without cardiovascular risk factors to patients in sinus rhythm with cardiovascular risk factors to patients with AF, a 2-fold elevation of procoagulant MP levels being demonstrated in patients with permanent or persistent AF with respect to age-matched controls.[8,15]

In this study we sought to investigate whether atrial-specific differences in endothelial damage, leukocyte activation, platelet stimulation, and TF activity could explain the increased thrombotic propensity described in the left atrium of patients with AF.

Methods

Patients Characteristics

Between April 2007 and April 2009, 22 patients with highly symptomatic, drug-refractory paroxysmal or persistent episodes of AF referred to our center for catheter ablation were enrolled to undergo transseptal isolation of the PVs either using an open irrigated-tip RF catheter or a cryoballoon catheter. In patients with persistent AF, cardioversion was performed 6 weeks before the ablation procedure under an intensified antiarrhythmic drug treatment to maintain the patient in sinus rhythm. Exclusion criteria were longstanding persistent AF (longer than 12 months), LA diameter >55 mm, intracardiac thrombi documented by transesophageal echocardiography (TEE), myocardial infarction or cardiac surgery in the previous 3 months, and previous ablation for AF.

The ethical committee of the University of Freiburg approved the study protocol, and written informed consent was obtained from all patients.

Oral Anticoagulation

Oral anticoagulation was reduced 2 days prior to the intervention to achieve an international normalized ratio between 1.8 and 2.5. CT or MRI of the LA was performed the day before the ablation procedure. Antiarrhythmic drug treatment was suspended for the day of the ablation procedure, restarted the following day, and stopped after at least 1 month. The interventions were performed under general anesthesia with remifentanil and propofol. Depth of anesthesia was measured by bispectral index. TEE was performed to exclude thrombi in the left atrium and to guide transseptal puncture. After transseptal puncture, and after blood sampling, heparin was given IV to maintain an ACT between 300 and 350 seconds in both treatment groups.

Laboratory Examinations

Citrated blood samples were collected in the right atrium and after transseptal puncture in the left atrium before starting ablation, for assessment of cell damage, platelet activation, and inflammatory markers.

Isolation of Circulating MPs

Platelet-poor plasma (PPP) containing circulating MPs was obtained by double centrifugation as previously described[16] and stored at −80 °C. Procoagulant MPs were captured onto insolubilized annexin V and their phosphatidylserine (PhtdSer) content was measured by functional prothrombinase assay. Results were expressed as PhtdSer equivalent (PhtdSer Eq.) by reference to a standard curve. Duplicate measurements were performed in 1 single series of assays.

Search for the Cellular Origin of Circulating MPs and Determination of Their Procoagulant Potential

Various cell type origins were identified using biotinylated monoclonal antibodies insolubilized onto streptavidin-coated micro titration plates and incubated with PPP (anti-CD11a for leukocytes, anti-CD105 [endoglin] for endothelial cells, anti-GP-Ib for platelets). Identical batches of antibodies were used throughout all assays. After washing, antibody-captured procoagulant MPs were quantified by prothrombinase assay. Background values obtained with corresponding irrelevant IgGs were subtracted. Variations in measurements using this assay were routinely below 10% regardless of the pathological issue and of the capture system (annexin V or antibodies).

Assessment of TF Activity Harbored by Microparticles

Microparticles bearing TF were isolated by capture onto insolubilized biotinylated-specific antibody to human TF and quantified using a standardized TF activity assay (Innovin, Dade Behring, Margburgh, Germany). Values are expressed as femtomoles of active TF.

Platelet Function

Optical aggregometry

Turbidimetric aggregometry was assessed using a 4-channel Bio/Data PAP4 aggregometer (Mölab, Hilden, Germany). Platelet-rich plasma was prepared by centrifugation of citrated venous blood (10 mL blood to be sampled) at 750 × *g* for 2 minutes and adjustment to 275–325 × 10^3 thrombocytes/μL by dilution with platelet poor plasma (PPP) from the same patient. ADP (5/20 μmol/L; Sigma, Munich, Germany), collagen (1.0/2.5 mg/L; Nycomed Pharma, Unterschleissheim, Germany), and a thrombin receptor activating peptide (TRAP, 25 μmol/L; Bachem, Bubendorf, Switzerland) were added to induce aggregation. Light transmission in PRP was recorded for 5 minutes. Maximum aggregation within the incubation period, final aggregation 5 minutes after addition of stimulants as well as the slope of the aggregation curves were determined from the recordings.

Flow cytometry

ADP-induced surface expression of P-selectin and activated GP IIb/IIIa were determined by triple color flow cytometry as previously described.[27] Platelets were stained in whole blood with fluorescein-isothiocyanate tagged PAC-1 (recognizing activated GP IIb/IIIa receptors, Becton Dickinson, Germany); phycoerythrin-tagged anti-CD62P (recognizing P-selectin) and phycoerythrin-cyanin 5.1 tagged anti-CD41 (recognizing total GP IIb/IIIa receptors, Beckman Coulter, Germany) monoclonal antibodies. Platelets were incubated with the antibodies and ADP at a final concentration of 20 μmol/L for 30 minutes. Thereafter, 300 μL of paraformaldehyde 1% was added for fixation.

Statistical Analysis

All data were expressed as mean ± SD or number (%) for continuous and categorical variables, respectively. Differences between the left and right atrium were compared by

TABLE 1
Patient Characteristics

Patients	N = 22
Mean age (years)	56 ± 8
Male	73%
Paroxystic atrial fibrillation	73%
Persistent atrial fibrillation	27%
BMI (kg/m^2)	27 ± 4
Hypertension	45%
Diabetes mellitus	5%
Cl creat (mL/min)	107 ± 32
History of AF (years)	5 ± 3
Organic heart disease	9%
LVEF (%)	63 ± 6
End diastolic diameter (mm)	51 ± 4
End systolic diameter (mm)	35 ± 7
Left atrium diameter (mm)	40± 4
CHA_2DS_2-VASc score	
0	32% (7)
1	36% (8)
2	32% (7)

paired Student *t*-test or Wilcoxon ranks test as appropriated. Categorical variables were compared by chi-square analysis or with the Fisher exact test for cell size less than 5. A P value of <0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS software (Version 13.0, SPSS Inc., Chicago, IL, USA).

Results

Patient Characteristics

The baseline clinical characteristics of the study participants are shown in Table 1. 73% were male. 73% were symptomatic of paroxysmal AF. 45% had hypertension and 5% diabetes mellitus. The mean history of AF was 5 ± 3 years. Echocardiographic data showed normal parameters. A total of 32% had a CHA_2DS_2-VASc score of 0, 36% of 1 and 32% of 2.

Microparticles and Procoagulant Activities Within the Atrium

No atrial-specific differences in the levels of total procoagulant MPs, leukocyte-derived MPs or platelet-derived MPs were found. Conversely, endothelial-derived MPs (CD105$^+$) were slightly more elevated in the right atrium compared to left atrium (RA 0.9 ± 0.5 vs LA 0.8 ± 0.4 nm PhtdSer Eq.; P = 0.041) (Table 2).

TF activity displayed by MPs was slightly increased in the right atrium (148 ± 68 vs 131 ± 62 femtomoles; P = 0.002) (Table 2).

No correlation between MP levels, age, atrium diameters, and AF history duration could be found.

Platelet Aggregation and Activation Within the Atrium

Collagen-induced platelet aggregation was higher in the right atrium (collagen 1 mg/L in RA 48 ± 31 % vs LA 37 ± 29%; P = 0.035). Platelet aggregation induced by ADP was comparable within both chambers. No differences in platelet P-selectin, activated GPIIb/IIIa (PAC-1 expression) were seen (Table 3).

Discussion

The main finding of this study is the absence of a heightened thrombotic status within the left atrium of AF patients. Unexpectedly, endothelial damage, TF activity and collagen-induced platelet aggregation appeared slightly more elevated in the right atrium. These results challenge the current paradigm explaining enhanced thrombotic status within the left atrium by chamber differences in platelet activation or thrombin generation.[1,17]

Microparticles in AF

During AF, several mechanisms, such as high ventricular heart rate, low or oscillatory shear stress, stretch, hypoxia, inflammation, and oxidative stress, are potent inducers of apoptotic cell death or platelet activation[8,18-21]

TABLE 2
Measurement of Circulating Procoagulant Microparticles (MPs) and Tissue Factor Activity within the Right and the Left Atrium in Patients with Atrial Fibrillation

	Right Atrium	Left Atrium	P
Total procoagulant MPs captured onto annexin V	5.9 ± 3.8	4.7 ± 2.9	0.360
Platelet-derived MPs captured onto anti-GPIb antibody (nM PhtSer Eq.)	3.6 ± 3.2	3.5 ± 1.9	0.145
Leukocyte-derived MPs captured onto anti-CD11a antibody (nM PhtSer Eq.)	3.4 ± 1.1	3.2 ± 1.2	0.147
Endothelial-derived MPs captured onto anti-CD105 antibody (nM PhtdSer Eq.)	0.9 ± 0.5	0.8 ± 0.4	**0.041***
Tissue factor activity harbored by MPs (femtomolar)	148 ± 68	131 ± 62	**0.002***

TABLE 3
Assessment of Platelet Reactivity within the Right and the Left Atrium in Patients with Atrial Fibrillation

	Right Atrium	Left Atrium	P
Maximal platelet aggregation induced by collagen (1 mg/L)	48 ± 31	37 ± 29	**0.035***
Maximal platelet aggregation induced by ADP (5 μM)	67 ± 20	58 ± 25	0.212
Platelet P-selectin expression following ADP 20 μM stimulation	23 ± 9	24 ± 9	0.455
Activated GPIIb/IIIa (PAC-1) receptors following ADP 20 μM stimulation	30 ± 9	35 ± 10	0.133

Blood samples were processed immediately (>10 minutes). Turbidimetric aggregometry were performed on platelet poor plasma following collagen or ADP stimulation. Expression of P-selectin, and activated GPII/bIIIa (PAC-1-FITC) was determined by flow cytometry. Paired comparisons were made using a Wilcoxon rank test.

that lead to the shedding of procoagulant MPs within the vasculature.[15,22-24] As key regulators of cell–cell cross-talk and important mediators of inflammatory, thrombogenic, and proteolytic pathways, MPs may directly or indirectly contribute to the amplification loops involved in AF.[8] Several *in vivo* models using *intravital* real-time microscopy have demonstrated that, at sites of endothelium damage, the swift recruitment of procoagulant MPs, possibly harboring TF, through P-Selectin/PSGL-1 interactions, contributes to the concentration of TF activity above a threshold enabling coagulation to be triggered and the growth of the thrombus.[25]

Regional Differences in Procoagulant Potential

In this study, we could not find any elevation in the prothrombotic status within the left atrium. In our hands, endothelium damage, collagen-induced platelet aggregation and TF activity displayed by circulating MPs appeared slightly elevated within the right atrium. Previous observations by the groups of Sanders and Yamamoto have suggested that regional differences in platelet activation or thrombin generation might explain enhanced thrombogenicity within the left atrium.[1,17] In patients with mitral stenosis, elevated concentrations of fibrinopeptide A or thrombin and antithrombin complex pointing to enhanced thrombin generation were found within the left atrium. Conversely, no regional differences in platelet factor 4, beta-thromboglobulin (markers of platelet activation), D-dimer or plasmin-alpha 2-plasmin inhibitor complex (markers of fibrinolysis) could be observed.[17] In a paper by Willoughby *et al*., significant elevation in P-selectin expression and ADP-induced aggregation could be observed within the left atrium.[1] However, regional differences in platelet activation could not be confirmed in a very recent paper published by the same group.[7] Because they lead to less significant complications (subclinical pulmonary embolism vs stroke), it is likely that thrombi developing within the right heart are less frequently detected. According to De Divitiis *et al*., thrombi within the right atrium might not be so rare (6.7% within the right atrium vs 12.2% in the left atrium). In this study, spontaneous echo contrast was found in the nearly same extent in the right atrium and in the left atrium (57% and 66%, respectively).[26]

Other Hypotheses

AF predisposes to stroke because it fulfills Virchow's triad of thrombogenesis, whereby thrombus formation occurs in the presence of "vessel wall abnormalities" (now recognized as representing structural heart disease), "abnormal blood flow" (as evidenced by stasis in association with this arrhythmia), and "abnormal blood constituents" (given the abnormalities of clotting and platelets that are present in AF).[2]

If the thrombotic status is not enhanced in left atrium, how could we explain the higher prevalence of thrombi? Very recent data have shown that differences in atrial morphologies are an important factor of the risk of stroke; non-"chicken wings" morphologies being associated with a consistent increase of the risk of stroke[27] especially in low CHA_2DS_2-VASc score patients.[28] In the latter work, a "cauliflower" morphology, defined as a main lobe less than 4 cm long without forked lobes, was significantly more common in stroke patients.[28] Another explanation could rely on differences in appendage remodeling. Although AF is associated with depressed right atrial appendage (RAA) and left atrial appendage ejection velocities, anatomic RAA remodeling appears to be absent. Larger RAA width and lack of anatomic remodeling may partially explain the lower prevalence of RAA thrombus found among patients with AF.[29]

Study Limitations

This work is monocentric and has the inherent limitations of any small series. Atrial concentrations of MPs were not assessed in a control group without AF. However, as many other confounding factors such as age, diabetes mellitus, hypertension, smoking, heart failure, and aortic stenosis are known contributors of MPs shedding, intra individual comparisons are more suitable than inter individual ones to assess local differences in thrombogenic factors. Most of the patients were at low thrombotic risk (CHA_2DS_2-VASc score 0–2). In this study, most of the patients were in sinus rhythm when blood sampling was performed. We could not exclude that the measurement of MPs in patients in long standing sustained AF or permanent AF could have led to different results. The *in situ* impact of AF induction on MP shedding and platelet aggregation was not established. The lack of randomization of the initial site of blood sampling (right vs left) constitutes a potential bias that should be taken into account in the interpretation of the data. Indeed, we could not exclude that transseptal puncture per se could affect the generation of MPs. However, such an effect would have led to an overestimation of the levels measured within the left atrium. Given the short time lapse between right and left sided sampling, it is unlikely that hemodilution due to fluid perfusion could have led to an underestimation of the levels measured within the left atrium. Moreover, previous data have indicated that transseptal puncture itself does not cause platelet reactivity.[1]

Finally, we could not exclude that the sampling through the sheaths might have led to an artefactual elevation of the biomarkers. However, artefactual platelet and cellular activation caused by the sampling procedure itself was kept as low as possible by discarding the first 20 mL of blood.

Conclusion

In patients with AF, endothelial damage, procoagulant microparticles release, TF activity, and platelet activation was not more pronounced within the left atrium. Our data did not substantiate the view that chamber-specific enhanced thrombogenic status could be a reliable explanation for the increased propensity for thrombus formation observed in the left atrium in patients with paroxysmal or persistent AF.

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Remarque conclusive sur le travail (article 1)

Les résultats de notre travail prospectif, sont en faveur d'une absence de différence de statut pro-thrombotique dans l'OG par rapport à l'OD que ce soit par la mesure des MPs ou l'étude des marqueurs plaquettaires.

Chez les patients en FA, aucune différence significative de concentration de MP totales, leucocytaires et plaquettaires entre les deux oreillettes n'a été mesurée. L'agrégation plaquettaire induite par le collagène était également plus élevée dans des échantillons de l'OD, ce qui peut indiquer un nombre de plaquettes non activées supérieure et aucune différence dans le degré d'exposition de P-selectine ou de GPIIb/IIIa nécessaires à l'adhésion des plaquettes à l'endothélium ou au fibrinogène n'est retrouvée. L'ensemble est donc en faveur d'une activation plaquettaire similaire dans les deux oreillettes.
Par contre, les MP d'origine endothéliale ou porteuses d'activité FT étaient modestement mais significativement augmentées dans l'OD par rapport à l'OG, suggérant une contribution de l'endothélium atrial à la thrombogénicité de l'oreillette droite, à l'encontre des hypothèses émises.

Cette observation paradoxale est peut-être à reconsidérer à l'aune d'une sous-estimation des thrombi formés dans l'OD chez les patients en FA. En effet, ces thrombi n'entraînent pas de complication notable et sont probablement sous-détectés. De Devitiis[262] a montré que les thrombi de l'OD ne sont pas si rares (6.7% dans l'OD vs 12.2% dans l'OG) et que du contraste spontané en échocardiographie était retrouvé dans à peu près les mêmes proportions dans les deux cavités. L'aspect anatomique de l'OG, qui n'est pas étudié ici, avec en particulier l'auricule gauche de morphologie très variable et plus ou moins emboligène (celle en « choux fleur »[263] ou « non en aile de poulet »[264] étant plus souvent retrouvée chez les patients ayant présenté un AVC) associé à un remodelage tissulaire plus marqué dans l'OG est une des causes possibles de la formation accrue des thrombi dans l'OG rapportée dans différentes études. L'anatomie de l'auricule droit est très différente, en particulier sa base d'implantation est plus large avec un remodelage moins important en FA que le gauche même si en FA les vitesses d'éjection sont altérées de façon identique ce qui explique également une moindre propension à développer des thrombi dans l'OD[265].

Il faut également noter que l'analyse quantitative des marqueurs d'activation plaquettaire reste délicate en présence de microthrombi puisqu'une partie des plaquettes ou des MPs peut être piégée dans les agrégats et rester inaccessible. Il se peut qu'une partie des MPs ou des plaquettes soit séquestrée dans l'espace de l'auricule. Par ailleurs, bien que la répartition des patients entre les scores CHADS 0, 1, 2 soit homogène, on ne peut exclure que les différences dans le degré de sévérité de la FA masque certaines variations des MPs ou des autres marqueurs.

Article 2 :

Caractérisation des dommages tissulaires et de leur importance sur la réaction inflammatoire, la myonécrose et la réponse prothrombotique dans l'ablation de FA et comparaison de deux types de cathéters de radiofréquence.

"A Prospective Randomized Study Comparing an Open Irrigated Tip and an Internal Irrigated Tip Radiofrequency Catheter in atrial fibrillation ablation"

Laurence Jesel, MD; Claudia Herrera Siklódy; Malak Abbas; Dietmar Trenk; Christian Stratz; Christian M. Valina; Reinhold Weber; Jan Minners; Valérie Schini-Kerth; Florence Toti; Thomas Arentz; Olivier Morel

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Abstract: Background: Irrigated tip radiofrequency (RF) ablation catheters are widely used for pulmonary vein isolation (PVI). Internal irrigated tip (IIT) catheters have the advantage of lower risk of fluid volume overload, especially in heart failure patients. However, it has been suggested that they might have a higher thromboembolic risk.
Objective: We aimed to compare both systems safety by measuring sensitive laboratory markers of platelet activation, cell damage, and inflammation after PVI using either OIT or IIT.
Methods: Sixty patients with symptomatic AF referred for PVI (57±4 yrs, 44 males, 32 paroxysmal, 28 persistent AF) were randomly assigned to PVI using either an OIT (Thermocool®, Biosense) or an IIT(Chilli II®, Boston Scientific). Systemic markers of cell damage (total procoagulant microparticles (MP) and MP of various cellular origin, troponin T, CK), platelet activation (ADP aggregometry, P-selectin and GPIIb/IIIa) and inflammatory response (hs-CRP) were monitored before and 8, 24, 48 hours after ablation.
Results: PVI induced a significant increase in CPK, hs-TnT, microparticles, markers of platelet activation and inflammation. Maximum values were observed at 24h for tissue damage markers and platelet activation. Hs-CRP and leukocyte-derived MP were still increasing at 48h. There was no significant difference in these parameters between OIT or IIT groups. No significant difference in procedure and fluoroscopic time, complication, success rate was observed.
Conclusions: PVI using either OIT or IIT resulted in a similar pattern of tissue damage, platelet activation and inflammatory response. No difference in clinical outcome between the two catheters suggests an identical safety profile in PVI.

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Cover Letter

Dear Stanley Nattel, MD
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Electrophysiology, University of Montreal
Cardiologist and Director of Electrophysiology Research Program, Montreal Heart Institute
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We would much appreciate if you would be willing to consider our original research article entitled « **A Prospective Randomized Study Comparing an Open Irrigated Tip and an Internal Irrigated Tip Radiofrequency Catheter in atrial fibrillation ablation"** by Jesel and coworkers for publication in ***Canadian Journal of Cardiology***.

Irrigated tip radiofrequency (RF) ablation catheters are widely used for pulmonary vein isolation (PVI). Internal irrigated tip (IIT) catheters have the advantage of lower risk of fluid volume overload, especially in heart failure patients. However, it has been suggested that they might have a higher thromboembolic risk. In this randomized trial, we aimed to compare both systems safety by measuring sensitive laboratory markers of platelet activation, cell damage, coagulation and inflammation after PVI using either OIT or IIT. In this trial, PVI using either OIT or IIT resulted in a comparable increase in markers of cell damage, platelet function and inflammatory response. These data suggest a similar safety profile in PVI with both catheters.

All authors have participated in the work and have reviewed and agree with the content of the article. None of the article contents are under consideration for publication in any other journal or have been published in any journal. No portion of the text has been copied from other material in the literature (unless in quotation marks, with citation). I am aware that it is the author's responsibility to obtain permission for any figures or tables reproduced from any prior publications, and to cover fully any costs involved. Such permission must be obtained prior to final acceptance.
Pr Arentz received grants from Boston Scientifics. Other authors disclose any financial association that might pose a conflict of interest in connection with the submitted article.

We are looking forward to hearing from you.

Yours sincerely,
Laurence Jesel MD

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A Prospective Randomized Study Comparing an Open Irrigated Tip and an Internal Irrigated Tip Radiofrequency Catheter in atrial fibrillation ablation

Open vs closed irrigated tip catheter in AF ablation

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Abstract

Background: Irrigated tip radiofrequency (RF) ablation catheters are widely used for pulmonary vein isolation (PVI). Internal irrigated tip (IIT) catheters have the advantage of lower risk of fluid volume overload, especially in heart failure patients. However, it has been suggested that they might have a higher thromboembolic risk.

Objective: We aimed to compare both systems safety by measuring sensitive laboratory markers of platelet activation, cell damage, and inflammation after PVI using either OIT or IIT.

Methods: Sixty patients with symptomatic AF referred for PVI (57±4 yrs, 44 males, 32 paroxysmal, 28 persistent AF) were randomly assigned to PVI using either an OIT (Thermocool®, Biosense) or an IIT(Chilli II®, Boston Scientific). Systemic markers of cell damage (total procoagulant microparticles (MP) and MP of various cellular origin, troponin T, CK), platelet activation (ADP aggregometry, P-selectin and GPIIb/IIIa) and inflammatory response (hs-CRP) were monitored before and 8, 24, 48 hours after ablation.

Results: PVI induced a significant increase in CPK, hs-TnT, microparticles, markers of platelet activation and inflammation. Maximum values were observed at 24h for tissue damage markers and platelet activation. Hs-CRP and leukocyte-derived MP were still increasing at 48h. There was no significant difference in these parameters between OIT or IIT groups. No significant difference in procedure and fluoroscopic time, complication, success rate was observed.

Conclusions: PVI using either OIT or IIT resulted in a similar pattern of tissue damage, platelet activation and inflammatory response. No difference in clinical outcome between the two catheters suggests an identical safety profile in PVI.

Key words: atrial fibrillation, thrombosis, stroke, leukocyte, platelet activation, endothelium, microparticles

Introduction

Transvenous pulmonary vein isolation (PVI) is a widely adopted treatment for atrial fibrillation (AF). Irrigated tip radiofrequency ablation catheters are commonly used for this type of procedure in the left atrium (LA) (1-3). It has been shown that, by cooling the electrode, there is increased energy delivery into the tissue, thus increasing lesion size (4). It has been shown for OIT-catheters that the risk of thrombus formation is reduced as compared to non-irrigated catheters. Internal irrigated tip (IIT) catheters have the advantage of a lower risk of volume overload, especially in patients with heart failure. However, experimental data suggested that IIT-catheters could carry a higher thromboembolic risk (5). We aimed to compare the safety profile of open irrigated tip (OIT) and internal irrigated tip (IIT) RF catheters in PVI. We determined the impact of ablation on platelet activation, tissue damage and inflammation after PVI using either OIT or IIT catheters.

Patients and Methods

Patients

Sixty patients with symptomatic, drug-refractory paroxysmal or persistent AF referred to our center were randomly assigned to undergo PVI using either an open irrigated-tip radio-frequency catheter (OIT group) or an internal irrigated tip catheter (IIT group). Exclusion criteria were longstanding persistent AF (>12 months), LA diameter >55mm, intracardiac thrombi found on transesophageal echocardiography (TEE) or cardiac surgery in the previous 3 months and previous AF ablation. The study was approved by the ethics committee of the University of Freiburg (Germany). Written informed consent was obtained from all patients.

Electrophysiological Study and Ablation Procedure

Oral anticoagulation was reduced 2 days prior to the intervention (INR 1.8-2.5). LA computerized tomography was performed the day before the procedure. In patients with persistent AF, cardioversion was performed 6 weeks before the ablation procedure under antiarrhythmic drug treatment to keep the patient in sinus rhythm.

Antiarrhythmic drugs were suspended the day of the procedure, restarted the following day and stopped after 2 months.
The interventions were performed under general anaesthesia with TEE to exclude LA thrombi and to guide transseptal puncture (TS). After TS, heparin was given intravenously to maintain an activated clotting time between 300 and 350 seconds in both groups. Surface and bipolar endocardial electrograms were monitored and stored on a computer-based digital amplifier/recorder system (C.R. Bard, Inc., Lowell, USA).

In both groups, ablation consisted of circumferential PVI of both ipsilateral veins using the NavX™ System (St Jude Medical, St Paul, USA). In the OIT group, an open-irrigated tip catheter (Thermocool™, Biosense Webster, Diamond Bar, USA), with energy settings of 25-35 Watts with an irrigation flow of 17 ml/min was used. In the IIT group, an internal irrigated tip catheter (Chilli II™, Boston Scientific), with energy settings of 25-35 Watts with the recommended flow rate was used.
Procedural success defined as complete electrical PVI, including a 30-minutes waiting time from the last application to exclude early conduction recovery. Disappearance of PV potentials was verified with a circular mapping catheter (Optima™, St Jude Medical, St Paul, MN, USA).

Post-ablation care and Follow Up

After the procedure, treatment with a vitamin K antagonist was restarted and intravenous heparin was administered until an international normalized ratio $\geq$ 2.5. Anticoagulation was continued for at least 3 months. On the first post-procedural day, patients underwent surface ECG, transthoracic echocardiography, and 24-hour Holter monitoring. Antiarrhythmic drugs were stopped two months after the procedure.
One, 3, 6 and 12 months after the procedure, the patients were seen by the referring cardiologist for 24-hour Holter monitoring. Patients with AF symptoms but no documentation on ECG or Holter monitoring were provided with an event monitor for 8 weeks.
Freedom from AF was defined as no symptoms of AF and no atrial arrhythmias lasting >30 seconds on Holter monitoring after a single procedure, without antiarrhythmic drugs, after a 3 months blanking period.

Laboratory Examinations

Citrated blood samples were collected before and after ablation. Microparticles (MP) were measured before TS and immediately after ablation from the right atrium, and 24 and 48 hours after the procedure. High-sensitive troponin T (hs-TNT), platelet activation, platelet reactivity by optical aggregometry, expression of surface proteins P-selectin and activated glycoprotein (GP IIb/IIIa) and inflammatory markers (high-sensitive C-reactive protein (hs-CRP) expected to show a later response, were assessed before TS and 8, 24 and 48 hours after the procedure.

Tissue Damage

Direct tissue damage was determined by measurement of CK, CK-MB, high-sensitive troponin T (hs-TnT) and procoagulant MPs.

CK and CK-MB plasma concentrations were determined using classical isoenzyme determination.

Cardiac troponin T in plasma was determined using the Elecsys Troponin T hs STAT assay on a COBAS autoanalyzer (Roche Diagnostics, Germany). The limit of blank (LoB) of the assay was 3 ng/L. Baseline levels of troponin T below the limit of blank were substituted by 0.5*LoB (i.e. 1.5 ng/L) for statistical analysis.

Isolation of circulating MPs, cellular origin and determination of their procoagulant potential

Platelet-poor plasma (PPP) containing circulating MP was obtained by double centrifugation as previously described (6) and stored at -80°C until assay. Procoagulant MP were captured onto insolubilized annexin V, and their phosphatidylserine (PhtdSer) content was measured by functional pro-thrombinase assay. Variations in MP measurements were routinely less than 10% regardless of the pathologic status and the capture system (6). Results were expressed as PhtdSer equivalent (PhtdSer Eq.) by reference to a standard curve. Duplicate measurements were performed in one single series of assays.

Biotinylated annexin V has strong affinity for procoagulant phosphatidylserine accessible at the microparticle surface. Because phosphatidylserine is a ubiquitous feature of procoagulant

MPs, the latter provide information on the total amount of circulating microparticles, regardless of their cellular origin. The quantity of microparticles captured onto biotinylated monoclonal antibodies gives additional indication on their cellular origin and the cellular response to vascular stress. The following biotinylated monoclonal antibodies were used in this study: CD11a (leukocytes), CD105/endoglin (endothelial cells and leukocytes) and GPIb (platelets). During MPs quantitation, the background reactivities to isotypic irrelevant IgG were subtracted from all values.

Platelet Reactivity

Light transmission aggregometry was performed using a four-channel Bio/Data PAP4 aggregometer (Mölab, Germany). Platelet-rich plasma was prepared by centrifugation of citrated venous blood (10 ml blood to be sampled) at 750g for 2 minutes and adjustment to 275-325x10^3 thrombocytes/µl by dilution with platelet-poor plasma from the same patient. Adenosine diphosphate (ADP, final concentration 5 µmol/L; Sigma, Munich, Germany), was added to induce aggregation. Light transmission in platelet-rich plasma was recorded for 5 minutes. Maximum aggregation within the incubation period and final aggregation 5 minutes after addition of stimulants were determined. The coefficient of variation of our light transmission aggregometry assay is 6.1% (7).

Flow cytometry

Surface expression of P-selectin and activated GP IIb/IIIa were determined by a four-channel flow cytometer equipped with a 488 nm argon laser (FACSCalibur, Becton Dickinson, Germany) as previously described (7). Platelets were stained in whole blood with fluorescein-isothiocyanate labeled PAC-1 (activated GP IIb/IIIa receptors; Becton Dickinson, Germany), phycoerythrin-labeled anti-CD62P (P-Selectin; Beckman Coulter, Germany) and phycoerythrin-cyanin 5.1 labeled anti-CD41 (total GP IIb/IIIa receptors; Beckman Coulter, Germany) monoclonal antibodies. Whole blood was incubated with antibodies for 30 minutes. Thereafter, 300 µL phosphate-

buffered saline (Sigma-Aldrich, Germany) containing 1% paraformaldehyde was added for dilution and fixation. Flow cytometric analysis was performed within 24 hours and 10,000 events were analyzed from each sample.

Flow-Jo software (Version V8.7.1 X; Tree Star Inc, USA) was used for analysis of raw data obtained from the flow cytometer.

Inflammatory Response

A latex particle-enhanced immunoassay on a COBAS autoanalyzer (CROPL3 assay, Roche Diagnostics) was used for determination of high-sensitive C-reactive protein (hs-CRP). The LoB of the assay was 0.3 mg/L.

Statistical Analysis

Continuous variables were provided as mean ± SD and were compared by Student *t*-test. Categorical variables were compared by chi-square analysis or with the Fisher exact test for cell size less than 5. Time courses versus baseline and comparison of the effect of OIT versus IIT catheters on markers of cell damage, platelet activation and inflammatory response over time were performed using a general linear model and applying a Bonferroni correction for repeated measures. Freedom from recurrent AF was determined by Kaplan-Meier analysis with the log-rank test. The PASW software package (version 18;SPSS Inc, Chicago, USA) was used. $P < 0.05$ using the 2-tailed test was considered significant.

Results

The clinical and baseline characteristics of patients treated with an OIT or an IIT catheter are listed in Table 1.

The 60 patients, mainly males (73.3%) described an AF history of 5.7 ± 3.9 years and a mean number of 2.6 ± 1.8 unsuccessful antiarrhythmic drug treatments. 32 patients presented with paroxysmal AF and 28 with persistent AF. 19 patients (32%) had structural heart disease and 28 (47%) had arterial hypertension. There was no difference in clinical and baseline characteristics between the two groups.

Procedural parameters

Procedural time and fluoroscopic time were comparable using either OIT or IIT. Radiofrequency application time was similar with both catheters on left and right veins. All pulmonary veins were successfully isolated. The mean fluid perfusion through OIT catheter was 1241±580 ml. No patients in the OIT group experienced major acute post-procedural complication, whereas in IIT group, 1 patient developed a femoral pseudoaneurysm and 2 had major bleeding. No embolic event was observed. There was no statistical difference between groups (Table 2).

Cellular damage

Maximal values of all different laboratory parameters are summarized in Table 3. Myocardial damage, demonstrated by a significant hs-troponin T and CK release, was observed after PVI with both catheters. Maximum values were noted at 24 hours and decreased thereafter. These 2 markers followed the same pattern. There was no difference between OIT and IIT groups either in peak values or kinetics (Figure 1). CK-MB peak was earlier at 6 hours after ablation but no difference could be found between the 2 study groups.

OIT and IIT catheters both induced release of procoagulant MPs captured on annexin V, reaching a peak 24 hours after the procedure and decreasing thereafter. Procoagulant platelet-derived MPs showed the same kinetic. Leukocytes-derived MPs increased significantly at 24 and 48 hours after the procedure with a delayed kinetic compared to MPs captured on annexin V. No significant modification of endothelial-derived MPs could be observed after ablation procedure. There was no significant difference in procoagulant MPs shedding between OIT and IIT catheter groups (Figure 2).

Platelet activation

Attested by optical aggregometry, surface expression of P-selectin and GPIIb/IIIa, a significant platelet activation was measured 24 and 48 hours after ablation in OIT and IIT groups. But no significant difference could be observed between groups (Figure 3).

Inflammatory response

A significant inflammatory response was detected in both groups as shown by an increase in hs-CRP 24 and 48 hours after the procedure. There was no significant difference between the two groups (Figure 4).

Follow-up

After a median follow-up of 12 months (range 3-29 months), 24 patients (80%) from the OIT group and 19 patients (63%) from the IIT group were free of AF without antiarrhythmic drugs (p= 0.17) (Figure 5).

Discussion

The main finding of this study is that PVI by radiofrequency ablation produces the same pattern of cellular damage, inflammatory response and subsequent prothrombotic state using either open or closed irrigated tip catheters.

RF induces circulating cellular activation and inflammatory response

When exposed to hyperthermia, cell injury occurs via increased membrane permeability, leading to the exposure of procoagulant aminophospholipids and to the shedding of microparticles. Activated cell membranes and derived MPs provide an

additional phospholipidic surface, enabling the assembly of the tenase and prothrombinase complex, leading to thrombin generation (8-9). Pioneering work by Bulava and coworkers in 2004 clearly demonstrated that radiofrequency (RF) catheter isolation of the pulmonary veins (PV) induces a systemic procoagulant state, as reflected by fibrin turnover, fibrinolysis activation and endothelial damage (10). In our experience in patients undergoing RF ablation of common atrial flutter or undergoing PVI by cryotherapy or RF, a significant rise in platelet- and leukocyte-derived procoagulant MPs is observed both immediately after and 24 hours after the procedure (6,11). Furthermore, significant changes in vWF Ag, PAI-1, and D-dimer occur, supporting the idea that activation of the coagulation occurs despite the use of anticoagulants (6). Another study depicts the kinetics of platelet activation following RF or cryoablation (11). Together with activated platelets and leukocytes, platelet derived MPs contribute to the initiation and acceleration of clotting reactions at the site of endothelial injury. PMPs are also able to stimulate neutrophils in vitro, possibly explaining the time course of leukocyte-derived MP release observed during RF ablation. Moreover, platelet-derived MP could contribute to monocyte adhesion and tissue infiltration (12). Various experimental models of thrombosis have demonstrated that the swift recruitment of leukocyte-derived MPs harboring tissue factor activities is mandatory to promote thrombus development (13).

The interplay between platelets and leukocytes leading to leuko-platelet conjugates could also contribute to an enhancement of the inflammatory process observed in AF (9). RF ablation induces necrosis followed by inflammatory infiltrates leading to a fibrotic scar. Post-ablation inflammation has been linked to healing of RF ablation with production of cytokine (IL-6), C-reactive protein and tissue remodeling as determined by metalloproteinase secretion (MMP-9) (14,15).
In the present study, the two catheters yielded the same pattern of MP shedding, platelet activation and inflammatory response. CRP and leukocyte-derived MP still increase at 48 hours. Saline infusion inside or outside of the catheter, known to alter lesion size or geometry, does not influence tissue damage.

Lesion size

Several previous studies have suggested that irrigated tip catheters produce larger lesions compared to non-cooled-tip catheters (16-18). In the work by Dorwarth et al., OIT and IIT catheters yielded the same volume of lesion measured ex vivo, but a smaller lesion diameter was seen using the IIT catheter (19). Other groups provided conflicting reports. According to Yokoyama et al., no difference between the two catheters in lesion depth, impedance nor tissue temperature at 20 or 30 W could be found (5). Everett showed that most of the volume of the lesion created by an OIT catheter is due to the lesion width rather than the depth whereas, for an IIT catheter, lesion depth plays a larger role (20).

In our study, we saw no difference in lesion size as assessed by markers of cardiomyocyte necrosis (hs-troponin T, CK) or circulating microparticles between the two catheters. Accordingly, the extent of the inflammatory response observed following post PV isolation was in the same range in both groups. We have verified that procedure time, RF ablation time on left and right side of PV were comparable between groups. After a mean follow-up of 12 months, there was no statistically significant difference in clinical outcomes. Golden et al. reported a cohort of 195 patients ablated with internal irrigated catheter. Event-free survival was 73.6% with one ablation; i.e. comparable to studies with open irrigated catheters (21).

Thrombus formation

Rare, but potentially life-threatening complications, such as thromboembolism, can occur during PV isolation (22). In atrial fibrillation, tissue damage, platelet and leukocyte activation and stasis contribute to the development of thrombi (9). Whether the type of catheter influences the extent of thrombus formation remains controversial. According to Yokoyama, a higher incidence of thrombus formation occurred when using a closed irrigated catheter (5). Our findings challenge the perceived thrombotic propensity associated with a closed irrigated catheter. In our hands, no difference in procoagulant platelet-derived MPs levels, expression of platelet activation markers such as P-selectin and GPIIbIIIa and platelet aggregation induced by ADP could be found between groups. The present data are in line with

recent observations by Everett and al. demonstrating in an ex vivo model that thrombus formation occurred to the same extent with open and closed irrigated catheters until a power of 50 Watts (20). In the present cohort, no neurological clinical event was observed in either group. These results are in line with the study by Golden et al. reporting a low rate (2/195 patients) of thrombotic complications with clinically significant neurological events during PVI procedures using IIT catheters, which is comparable to studies using OIT catheters (21). MRI-detected silent cerebral ischemia during AF ablation using RF, known to occur around 14% (23) of the cases would constitute an important step to confirm the relevance of these results.

Clinical implication

Open irrigated tip catheters are the most widely used catheters for AF ablation. In OIT, the requirement of saline perfusion to achieve thermodilution increases intravascular volume, potentially leading to left atrium pressure overload and to pulmonary edema. Catheters having multiple pores of irrigation which require less fluid like Thermo cool SF™ have partially improved this problem of fluid overload.
The increasing development of persistent AF ablation procedures with extensive left atrium defragmentation or lines raises the interest of new tools, catheters that could induce wider RF lesions with fewer applications. Our findings of no difference in the pro-thrombotic state and inflammatory response with IIT could be of interest in the conception of new catheters.

Study limitation

Intra-procedural blood sampling was performed from the vascular sheath in the right atrium and post-procedural blood sampling was from a peripheral vein. The site and method of sampling could have influenced the measurement of biomarkers, but would have affected both groups in the same manner.
Our study was not powered to assess clinical differences between the two catheters. Large prospective randomized trials comparing the two catheters in AF ablation with clinical endpoints are still necessary.

Conclusion

In this randomized, prospective study, open irrigated and closed irrigated catheters in PVI yielded a similar pattern of tissue damage, platelet activation and inflammatory response. There was no difference in clinical outcome between the two catheters suggesting an identical safety profile in PVI.

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Figures legend

Figure 1: Time course of high-sensitive (hs) troponine T, CK and MB levels in both groups. Curves display mean and standard deviation (open irrigated tip catheter (OIT) in *blue*, internal irrigated catheter (IIT) in *orange*).

Figure 2: Rise of the different microparticle (MP) types in both groups. Curves display mean and standard deviation (open irrigated tip catheter (OIT) in *blue*, internal irrigated catheter (IIT) in *orange*). **A**: Total circulating microparticles. **B**: Platelet-derived microparticles. **C** : Leukocyte-derived microparticles. **D** : Endothelial-derived microparticles.

Figure 3: Time course of platelet activation parameters in both groups. Curves display mean and standard deviation (open irrigated tip catheter (OIT) in *blue*, internal irrigated catheter (IIT) in *orange*). **A**: Surface expression of activated GPIIb/IIa. **B**: Adenosine diphosphate-induced light transmission aggregometry. **C**: Surface expression of P-selectin.

Figure 4: Time course of high-sensitive (hs) C-reactive protein levels in both groups. Curves display mean and standard deviation (open irrigated tip catheter (OIT) in *blue*, internal irrigated catheter (IIT) in *orange*).

Figure 5: Arrhythmia-free survival curve in both groups (open irrigated tip catheter (OIT) in *blue*, internal irrigated catheter (IIT) in *orange*).

Figure 1

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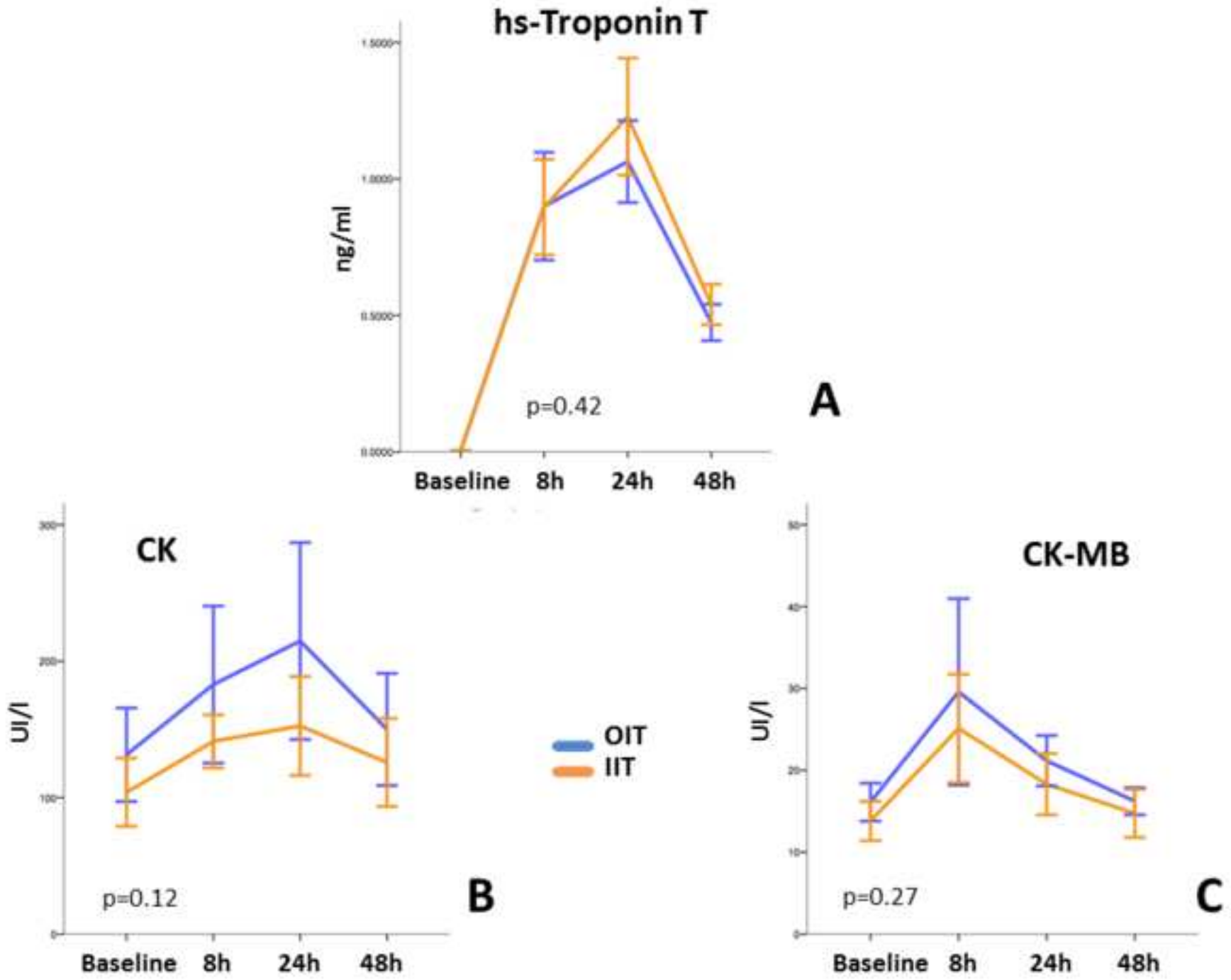

Figure 2
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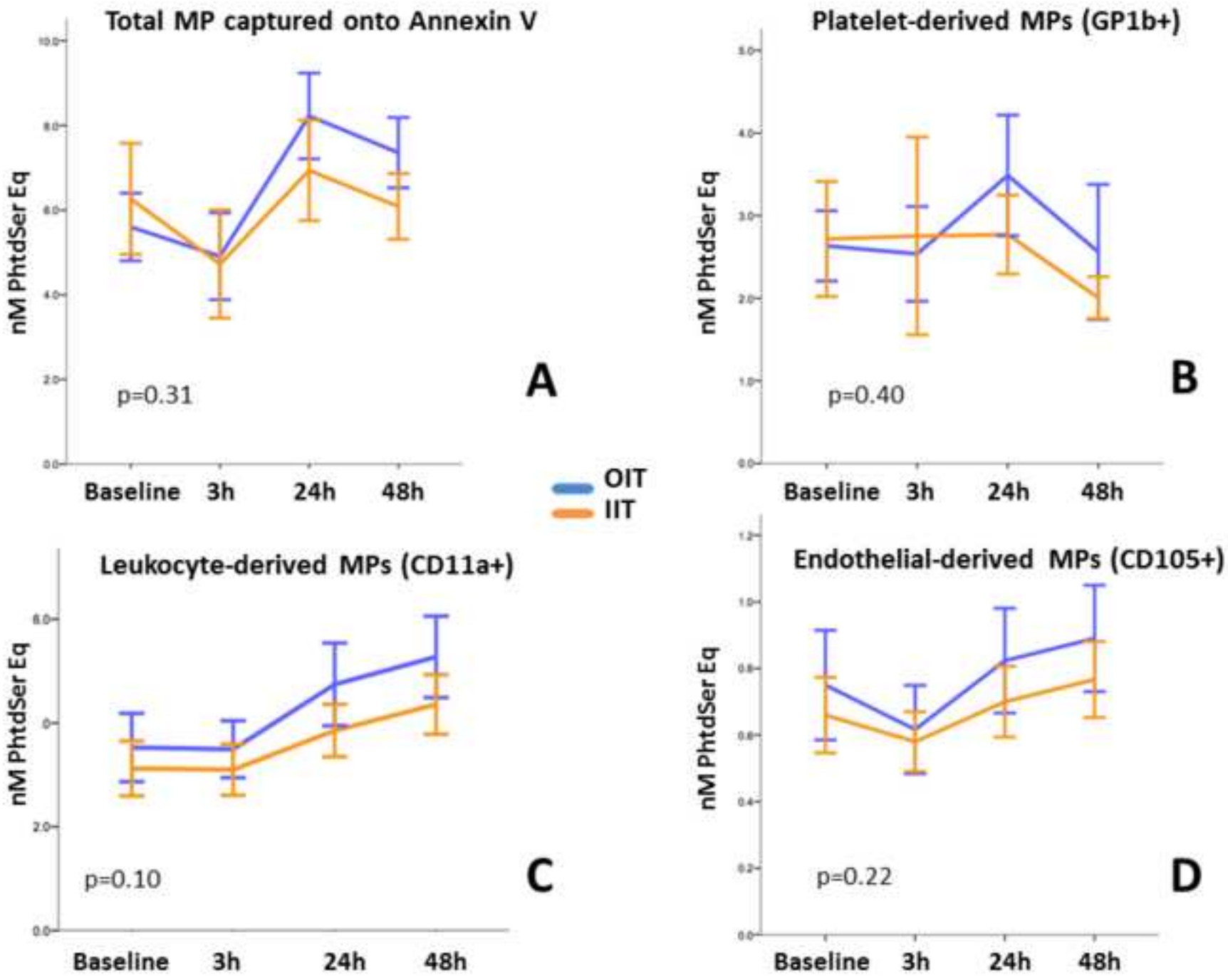

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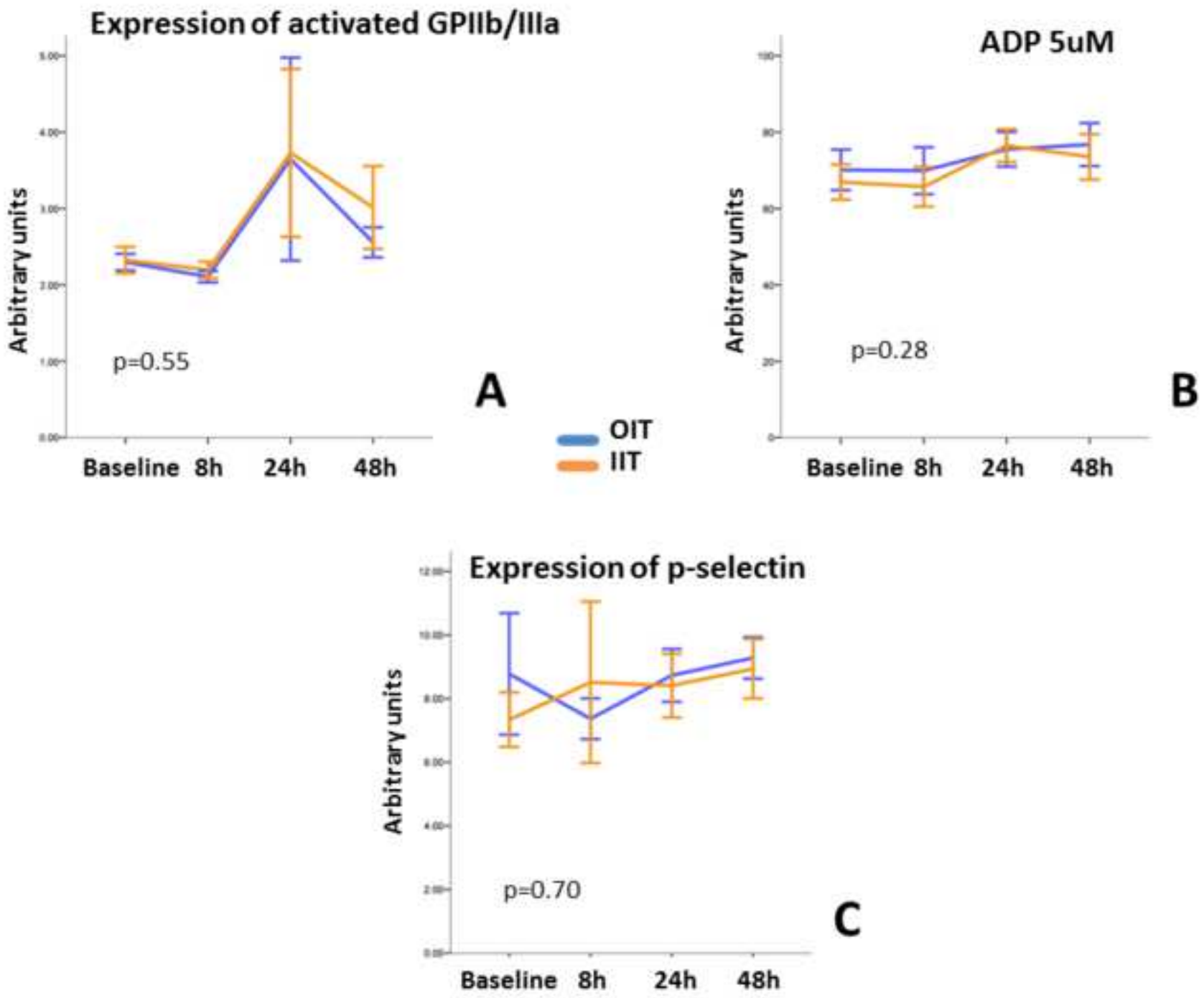

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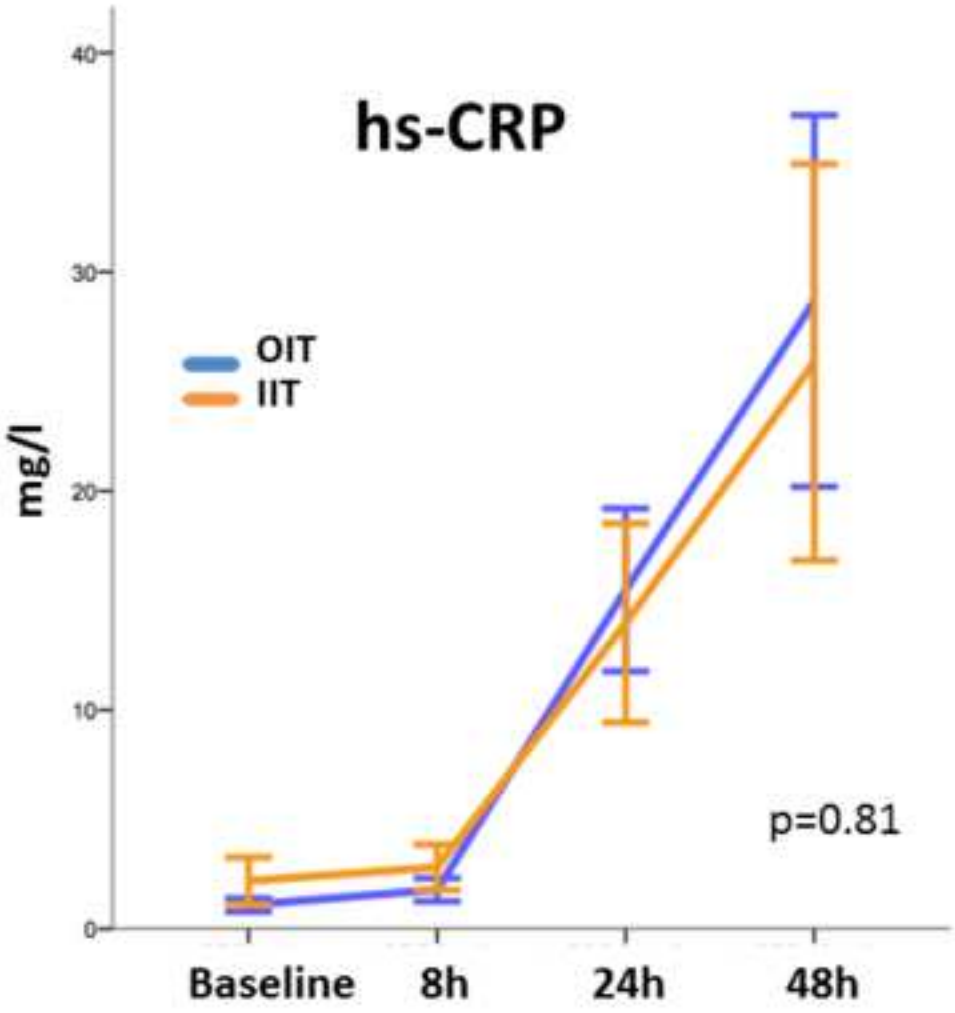

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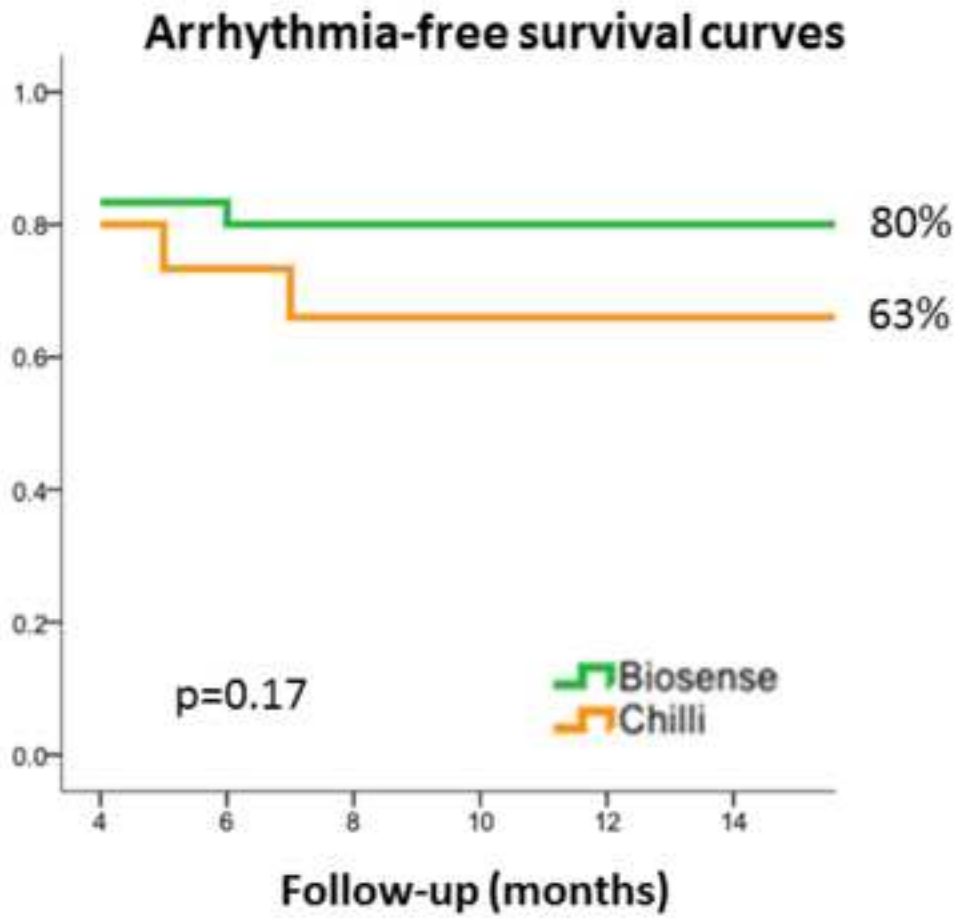

Table 1: Patients characteristics.

	Open Irrigated tip catheter (n=30)	Internal Irrigated tip catheter (n=30)	*p* value
Mean age (y)	55±10	59±9	0.15
Males	23 (76.73%)	21 (70%)	0.56
Paroxysmal AF	17 (56.7%)	15 (50.0%)	0.61
History of AF (y)	5.6±4.2	5.8±3.6	0.87
Failed AAD	2.7±2.2	2.4±1.3	0.63
Heart disease (%)	11 (36.7%)	8 (26.7%)	0.41
	CHD 4	CHD 6	
	HCMP1	HCMP 0	
	Tachymyopathy 2	Tachymyopathy 2	
	Valvular 4	Valvular 0	
Hypertension (%)	14 (46.7%)	14 (46.7%)	1.00
LA (mm)	40.0±5.5	39.5±4.4	0.66

AF: atrial fibrillation; AAD: antiarrhythmic drugs; CHD: coronary heart disease; HCMP: hypertensive cardiomyopathy; LA: left atrium

Table 2: Procedural parameters.

	Open Irrigated tip catheter (n=30)	Internal Irrigated tip catheter (n=30)	*p*
Mean procedure time (min)	200±46	191±49	0.49
Mean fluroroscopy time (min)	37.0±16	36±12	0.71
RF time (min)	52±13	50±11	0.74
Complications	1 (3.3%) 1 minor bleeding	4 (13.3%) 1 minor bleeding 2 major bleeding 1 pseudoaneurysm	0.20

Table 3: Maximal values of all different laboratory parameters after AF ablation using either OIT or IIT.

	OIT (n=30)	IIT (n=30)	*p*
High-sensitive troponin T (ng/mL)	1.06±0.4	1.23±0.57	0.42
CK (UI/l)	214±140	152±68	0.12
CK-MB (UI/l)	21±6	18±7	0.27
Total MP captured onto Annexin V (nM PhtdSer Eq)	8.2±2.7	6.9±3.2	0.31
Platelet-derived MPs (nM PhtdSer Eq)	3.5±2	2.8±1.3	0.40
Leukocyte-derived MPs (nM PhtdSer Eq)	5.3±2.1	4.4±1.5	0.10
Endothelial-derived MPs (nM PhtdSer Eq)	0.9±0.4	0.8±0.3	0.22
Surface expression of activated GP IIb/IIIa	3.6±3.5	3.7±2.9	0.55
Optical aggregometry	77±15	73±16	0.28
Surface expression of P-selectin	9.3±1.7	8.9±2.3	0.70
High-sensitive C-reactive protein (mg/l)	29±23	26±24	0.81

OIT= open irrigated tip catheter; IIT = internal irrigated tip catheter; GP= glycoprotein; MP= microparticle; PhtdSer= phosphatidylserine

Remarque conclusive sur le travail (article 2)

Dans l'ablation de FA, le refroidissement de l'électrode du cathéter irrigué permet d'obtenir une énergie délivrée plus importante et une augmentation de la taille de la lésion. Il a également été observé qu'avec des cathéters irrigués ouverts, le risque de formation de thrombus pendant l'ablation était diminué par rapport aux cathéters non irrigués. Des données expérimentales suggèrent que les cathéters à irrigation interne, donc sans ouverture du liquide vers le patient, auraient plus de risque de formation de thrombus sur l'électrode et donc de risque embolique[266].

Cette étude prospective démontre que les MPs sont de bons indicateurs de l'atteinte tissulaire et des marqueurs tissulaires. En effet on constate une augmentation significative des dommages tissulaires, des concentrations de MPs, des marqueurs d'activation plaquettaire (P-selectine, GPIIB/IIIa) et des marqueurs sanguins de dommage tissulaire (hs-troponine T et CPK) qui est maximale à 24h dans les 2 groupes de patients traités par cathéter ouvert ou fermé. De manière intéressante aucune augmentation des MPs endothéliale n'est détectable.
Cette étude apporte aussi des données nouvelles sur la séquence des réponses cellulaires induite par l'ablation de la FA. La cinétique d'évolution des concentrations des MPs détectées dans le sang périphérique à 24 et 48h montre que les MP leucocytaires apparaissent de façon retardée par rapport aux MPs totales ou plaquettaires dont les taux décroissent à 48h. Cette observation est à rapprocher des mesures de la CRP circulante dont l'augmentation persiste à 48h avec des taux 20-30 fois supérieure à la valeur de référence quelle que soit la technique ablative. Il est possible que l'interaction entre MP plaquettaires et leucocytaires soit impliquée dans cette cinétique et qu'une partie des MPs plaquettaires circulantes échappe au dosage du fait de leur incorporation par interaction P-sélectine/PSLG-1 dans des agrégats leuco-plaquettaires[267].

Cette étude montre que l'ablation de FA par radiofréquence entraine le même profil de dommages cellulaires, la même réaction inflammatoire et favorise un statut pro-thrombotique similaire quel que soit le type de cathéter utilisé, irrigué ouvert ou fermé. Aucune différence significative en terme de complication, succès primaire et suivi n'a été observée.

Deuxième partie: Fibrilation auriculaire et sénescence cellulaire: rôle de l'endothélium.

Dans la première partie des travaux exposés nous avons pu mettre en évidence un statut thrombotique de la FA qui ne semblait pas dépendre de la localisation atriale droite ou gauche mais qui pouvait être dépendant de l'atteinte endothéliale (cf articles 1 et 2). C'est pourquoi nous avons cherché à mieux comprendre le rôle de l'endothélium dans l'oreillette en recherchant les signes d'une sénescence susceptible de favoriser l'acquisition de caractères thrombogéniques et aussi de modifier le tissu de l'oreillette notamment par la sécrétion d'enzymes protéolytiques.

Le troisième article examine l'expression dans l'oreillette de marqueurs protéiques de la sénescence (p53,p16), du remodelage tissulaire (MMP-9), de la tendance pro-coagulante (FT), et de la fonction endothéliale (eNOS) par western blot et par marquage immunohistologie. Les auricules de 21 patients en FA paroxystique (PAF) ou permanente (PmAF) et de 21 patients appariés en rythme sinusal (SR) ont été récoltés et analysés selon leur rythme, en 3 groupes PAF, PmAF, et RS. Les résultats confirment une progression de la sénescence avec la sévérité de la FA : L'expression des protéines pro-coagulantes et pro-sénescentes et protéasiques augmente de la PAF à la PmAF tandis que l'expression de la eNOS protectrice contre la dysfonction endothéliale diminue.

Le quatrième article décrit et caractérise un modèle cellulaire d'exploration des mécanismes moléculaires de la sénescence endothéliale des cellules de l'oreillette gauche. Outre le profil caractéristique des protéines exprimées au cours de la sénescence endothéliale (baisse de la eNOS, sur-expression du facteur tissulaire), le modèle a confirmé un rôle central du système de l'angiotensine local avec une surexpression du récepteur AT-1 de l'angiotensine et de l'enzyme de conversion de l'angiotensine, confirmant des données établies au laboratoire avec des cellules endothéliales de coronaires de porc mais aussi par Burger avec des cellules endothéliales aortiques de souris[208]. De manière très intéressante la cellule endothéliale auriculaire sénescente sur-exprime également les MMP-2 et MMP-9 ainsi que le TGFβ, impliqués dans le remodelage tissulaire.

Article 3 :

Impact of atrial fibrillation progression on human atrial senescence burden as determined by p53 and p16 expression

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Impact of atrial fibrillation progression on human atrial senescence burden as determined by p53 and p16 expression

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Total word count:

Abstract

Background: Atrial fibrillation (AF) occurs with increasing age. Whilst the link between aging and thrombogenicity is well established, the cellular and molecular mechanisms are still unknown. Cellular senescence is characterized by the acquisition of a pro-inflammatory and pro-thrombotic profile. In AF, the role of senescence in tissue remodeling and pro-thrombotic state is unclear.

In this study, we investigated the link between AF, and senescence by comparing the expression of senescence markers (p53,p16), with pro-thrombotic and inflammatory proteins like tissue factor (TF), the matrix metalloproteinase-9 (MMP-9) and endothelial NO synthase (eNOS), in the human appendages from patients in AF and sinus rhythm (SR).

Methods and results: Right appendages of 147 patients undergoing open-heart surgery (valvular AF patients were excluded) were harvested. Patients were classified according to heart rhythm. 21 patients presenting paroxysmal (PAF) or permanent AF (PmAF) were matched with 21 SR patients according to CHA2DS2VASC score and drug treatment. Protein expression was assessed by western blot of tissue lysates. The expression of p53, p16 and TF was significantly increased in AF compared to SR group (0.91±0.31 vs 0.58±0.31; p=0.001; 0.76±0.32 vs 0.35±0.18; p=0.0001 and 0.88±0.32 vs 0.68±0.29; p=0.045 respectively). Expression of eNOS was lower in AF. (0.25±0.15 vs 0.35±0.12; p=0.023). There was a stepwise increase of p53, p16, TF and MMP-9 and a progressive decrease of eNOS between SR, PAF and PmAF. AF was the only predictive factor of p53 and p16 elevation by multivariate analysis.

Conclusions: The study brings new evidences indicating that AF progression is strongly related to human atrial senescence burden and points at a strong link between senescence, thrombogenicity, endothelial dysfunction and atrial remodeling.

Key words: atrial fibrillation, senescence, tissue factor, endothelial dysfunction, atrium

Introduction

Atrial fibrillation (AF), the most frequent cardiac arrhythmia is associated with increased morbidity and mortality due mainly to heart failure and embolic complications. This arrhythmia is well known to occur more frequently with increasing age. Vascular aging is pivotal in AF development. During AF, inflammation, apoptosis, endothelial dysfunction and platelet activation contribute to create a pro-thrombotic state and atrial remodeling[1]. While the link between aging and thrombogenicity is well established, the cellular and molecular mechanisms are still unknown.

Premature cellular senescence is an irreversible form of cell-cycle arrest that can be triggered by various cellular stresses, including deoxyribonucleic acid damage, oxidative stress and oncogene activation[2]. It is characterized by morphological changes with the acquisition of pro-inflammatory and pro-thrombotic profile. Senescent cells are found in aged tissue where they remain viable and metabolically active but they are unable to proliferate despite the presence of mitogens. Their role and effect in tissue is not well understood.

p53, a protein with a central role in DNA repair and cell cycle regulation is pivotal in the induction of senescence[2]. Physiological p53 activity prevents from cancer and protects from aging whereas excessive p53 activation is detrimental to healthy aging. Increased p53 expression induces cardiac inflammation and altered systolic function in an animal model of heart failure[3]. Other data obtained in a murine model have highlighted a key role of p53 and p16 in the regulation of pro-fibrotic signaling through premature senescence of myofibroblasts[4]. In AF, the role of senescence in atrial remodeling and the development of a pro-thrombotic state remains unclear.

In this study, we investigated the link between AF and senescence through the assessment of protein expression in the tissue lysates of human appendages from patients in AF and in sinus rhythm. The expression of protein p53, p16 a down-stream modulator of cell cycle arrest, tissue factor (TF), the cellular initiator of the coagulation also up-regulated in senescent endothelial cells, MMP-9, a matrix metalloproteinase, a protein involved in

extracellular matrix remodeling, and eNOS, the pivotal endothelial NO synthase that participates to redox-induced senescence and endothelial dysfunction when down-regulated. Immunohistological staining was also performed on appendages sections.

Material and methods

Patients

Between October 2013 and October 2015, all patients undergoing open-heart surgery in our medical center (Nouvel Hôpital civil, University hospital of Strasbourg, France) were screened for participation in this study. Surgery was mainly CABG or aortic valve replacement. Patients with mitral valve surgery were excluded to avoid valvular AF. 147 patients gave informed consent in accordance with the local ethics committee (reference number EK2044). Patients with relevant comorbidities such as malignancies, chronic inflammatory diseases or acute infections were excluded.

Patients were classified according to heart rhythm and duration of AF in 3 groups, sinus rhythm (SR), paroxysmal AF (PAF) and permanent AF (PmAF). Out of 147 patients included, 21 presented paroxysmal or permanent atrial fibrillation. These 21 patients were matched with 21 sinus rhythm patients according to criteria of CHA2DS2VASC score (age, sexe, hypertension, diabetes, vascular disease, congestive heart failure, stroke or systemic embolism) and drug treatment (AT2 blockers or ACE inhibitors and statins). All patients underwent medical history recording, physical examination, coronary angiography, transthoracic echocardiography (LVEF, LA volume, left and right pressure, valvulopathy…), 12-lead electrocardiography (ECG) recording, and routine hematologic and biochemical blood tests prior to the surgical intervention. All data were collected.

Paroxysmal atrial fibrillation (AF) was defined as self-terminating but recurrent atrial fibrillation of less than 7 days and permanent AF as chronic AF of at least 12 months duration with failed or unattempted cardioversion.

Sample preparation

Right atrial appendages (RAA) were harvested during cannulation of the RAA in OHS (a step performed to connect the patient to the heart-and-lung machine) and immediately collected.to avoid any additional risk pf the withdrawal procedure to the patient. The samples were immediately minced and snap-frozen in liquid nitrogen for western blot. One piece, was kept and embedded in OCT (Optimal cutting temperature) for immunofluorescence histological analysis. Frozen samples were stored at -80°C.

Western blot analysis

Proteins were extracted from appendages as described previously[5] and 30 mg total proteins were separated by SDS-PAGE (12 % acrylamide, Euromedex, Souffelweyersheim, France) at 100 V for 2 h and transferred electrophoretically onto PVDF membranes (GE healthcare, VWR, Fontenay-sous-Bois, France) at 100 V for 120 min. Aspecific binding sites were saturated by incubation of the membranes for 1 h with Tris-buffered saline solution (TBS, Euromedex) containing 3% bovine serum albumin and 0.1 % Tween 20. Membranes were probed overnight at 4 °C with blocking solution containing specific antibodies directed against proteins of interest. The antibodies used were either mouse anti-TF (1:1000, Sekisui Diagnostics, Sekisui Virotech GbmH, Rüsselsheim, Germany), anti-eNOS (1:1000, BD Biosciences, Le Pont de Claix, France), anti-human p16 (1:1000; Santa Cruz Biotechnology), anti-human p53 (1:1000; Santa Cruz Biotechnology) and anti-human MMP-9 (1:10000 Abcam).

Thereafter, membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and density signals of each band were detected using enhanced Clarity Western ECL Substrate (170-0561, BIO-RAD, United States). Membranes were incubated with a mouse polyclonal anti-beta tubulin antibody for normalization purposes. Results were expressed as a ratio of beta tubulin density using the ImageQuant acquisition system and analysis software (LAS4000 and ImageQuant TL 8.1, Amersham).

Immunofluorescence studies

Right atrial appendages obtained from patients were embedded in Tissue-Tek OCT (Sakura 4583, Leiden, Netherlands) and were cryosectioned at 14 µm. Sections were air dried for 15 min and stored at -80 °C until use. Sections were first fixed with paraformaldehyde at 4% (Electron microscopy sciences, Hatfield, PA, USA), washed and treated with either 10% milk or 5% goat serum in PBS containing 0.1% Triton X-100 for 1 h at room temperature to block non-specific binding. Atrial appendages sections were then incubated overnight at 4 °C with an antibody directed against either p53 (1:1000; Santa Cruz Biotechnology), p16 (1:1000; Santa Cruz Biotechnology) and eNOS (1/50, cat: 610297, BD Transduction Laboratories, Le Pont de Claix, France), cat: APC064, Alomone Labs, Jerusalem, Israel).

For negative controls, primary antibodies were omitted. Sections were then washed with PBS, incubated with the fluorescent secondary antibody (1/400, Alexa 633-conjugated goat anti-rabbit or anti mouse IgG, A-21070 and A-21050, Thermo Fisher, Illkirch, France) for 2 h at room temperature in the dark before being washed with PBS and mounted in Dako fluorescence mounting medium (Dako S3023, Les Ulis, France) and cover-slipped before being evaluated by confocal microscopy using a confocal laser-scanning microscope (Leica TSC SPE-Mannheim, Germany). Quantification of fluorescence levels was performed using Image J software (version 1.49p for Windows, US National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Continuous variables are expressed as mean ± SD or median (interquartile range); categorical variables are expressed as frequencies and percentages. Continuous variables were analyzed for normal distribution using the Shapiro-Wilk test. Continuous variables between paired groups were compared by paired Student *t* test or by non parametric test as appropriate. Fisher exact test was used for comparison of categorical variables. To determine predictive factors of p53 or p16 elevation, the cohort was split into two subgroups according to p53 or p16 elevation (above or below the median value). Univariate and multivariate analysis of p53 or p16 elevation was done using bivariate logistic regression analysis. Variables with $p < 0.05$ in univariate analysis were entered into a stepwise ascending multivariate analysis. The results of the binary logistic regression are presented as hazard ratios (HR), their 95% confidence intervals (CIs), and p values. A p value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS version 13.0 software (SPSS Inc., Chicago,Illinois).

Results

1. Patients characteristics

Twenty one patients with AF were compared to 21 matched patients in sinus rhythm. There was 11 paroxysmal (PAF) and 10 persistent AF (PsAF). At the time of surgery, 4 paroxysmal AF patients presented with ongoing AF. Baseline clinical characteristics are summerized in Table 1.

Age, body mass index, sexe and cardiovascular risk factors were equally distributed. Euroscore I and II were more elevated in AF group without reaching statistical significance.

There was also more significant mitral valvulopathy (grade I or II) in the AF group. The repartition between CHA2DS2VASc group 0-1, 2-3 and ≥ 4 was similar in the two groups.

There was no difference between the two groups concerning drug treatment (statin, ACE inhibitor, AT2 blockers nor ßblockers). As expected, patients from AF group were more often under vitamin K antagonists (VKA) treatment whereas patients from sinus group were more often treated with aspirin.

No difference in echocardiographic parameters could be evidenced except left atrium surface which was larger in AF group. The groups were also similar concerning biological parameters. Data are summarized in Table 1.

2. p53, p16, TF, MMP-9 are upregulated and eNOS is down-regulated in the appendages of patients with FA

Western blot analysis of the appendages lysates indicated that the expression of p53, and p16 were significantly increased in AF group compared to SR group (0.91±0.31 vs 0.58±0.31; p=0.001 and 0.76±0.32 vs 0.35±0.18; p= 0.0001) as well as that of TF, the main cellular activator of the blood coagulation (AF: 0.88±0.32 vs RS:0.68±0.29; p=0.045). A trend to enhanced MMP-9 expression was also detected. Conversely, eNOS expression significantly decreased in appendages samples from AF patients compared to those from SR (figure 1). The degree of MMP-9 expression was correlated to age (r=0.538; p<0.001), left ventricular mass (r=0.487; p=0.002) and CHA2DS2VASc score (r=0.638; p<0.001). The amount of p53 was correlated to TF expression (r=0.533; p<0.001) but not to CHA2DS2VASc score nor to age. P16 was correlated to p53 (r=0.388; p=0.011) and inversely to eNOS (r=-0.335; p=0.034).(figure 1)

Interestingly, LA surface was not correlated to p53, p16, MMP-9 nor TF expression but to CHA2DS2VASc (r=0.359; p=0.0048). Data are summarized in Table 2.

The data were confirmed by immunohistological analysis of the fresh-frozen OCT-embedded right appendage sections. p53 and p16 staining was strong in AF patients samples compared to matched SR samples. Conversely, eNOS, immunohistochemical staining was stronger in SR compared to the AF matched samples (figure 2).

3. p53, p16, MMP-9 and TF are differenrially expressed in SR, PAF and PmAF

Protein expression in appendage lysates was evaluated by western blot according to SR, PAF and PmAF. A stepwise increase in p53, p16, MMP-9 and TF could be evidenced from SR to PAF and PmAF. Conversely, expression of eNOS significantly decreased gradually from SR to PmAF (figure 3). Most interestingly, MMP-9 was clearly evidenced as elevated in PmAF vs SR or PAF, a data that was not evidenced by comparing SR and FA (see figures 1 and 3). Data are summarized in Table 3.

4. **Predictive factors of p53 and p16 elevation**

No relationship between age, cardiovascular risk factors, biological and echocardiographic parameters and Euroscore and p53 or p16 expression could be established. History of AF (paroxysmal or permanent), permanent AF and AF at the time of surgery were evidenced as predictive factors of p53 elevation by univariate analysis. By contrast, paroxysmal AF was not predictive of p53 elevation. As expected, p16, the down-stream cyclin-dependent kinase inhibitor was associated with p53 enhanced expression. By multivariate analysis, AF was the only predictive factor of p53 or of p16 enhanced expression (Tables 4 and 5).

Discussion

The major findings of the present study indicate that the progression of atrial fibrillation is strongly related to human atrial senescence burden as determined by p53 and p16 expression. The stepwise increase of senescence (p53, p16), pro-thrombotic (TF) and pro-remodeling (MMP-9) markers observed between sinus rhythm, paroxysmal and permanent atrial fibrillation points toward multiple interactions in the human atrium, that enhance the senescence burden, through atrial extracellular matrix remodeling, thrombogenicity and other putative mediators involved in the progression of AF.

Senescence burden and atrial fibrillation

Although the link between ageing and AF burden has been extensively characterized in epidemiological studies, little is known about the alteration of atrial tissue during senescence. While the occurrence and mechanisms of cellular senescence has been explored *ex vivo*, or in cultured cells, it is only recently that the importance of this mechanism has been appreciated in cardiovascular diseases. The putative role of senescence in AF has up to now, attracted less attention. We therefore explored a new paradigm linking senescence, atrial remodeling and substratum alteration that pave the way to AF onset and perpetuation. Kim and co-workers first reported an enhanced p21 expression together with the up-regulation of pro-apoptotic genes in human tissue samples from patients in permanent AF[6]. P21 protein, along with down-stream p16 is a cyclin-dependent kinase inhibitor that contributes to the inhibition of CDK2 and CDK1 complexes and functions as a regulator of cell cycle progression[2]. P21 and p16 gene expression are tightly regulated by the tumor suppressor protein p53, that mediates the p53-dependent cell cycle G_1 phase arrest in response to a variety of stress stimuli.

Initially considered as a stress and damage response phenomenon resulting in an irreversible cell cycle arrest, cellular senescence is also characterized by reduced migratory

behaviour, and the appearance of distinct morphological and functional changes that could contribute to the impairment of cellular homeostasis. More recently, several studies have emphasized that senescent vascular cells, also secrete pro-inflammatory and pro-remodeling factors, which in turns may alter tissue environment[7, 8]. To underscore a possible link between senescence burden and the extent of AF, we first characterized p53 and p16 expression in human atrial samples obtained in patients treated by cardiac surgery. Increased p53 and p16 atrial expression could be evidenced in patients with AF history consistent with the enhanced p21 expression described by Kim[6]. In our study, the stepwise increase in p53 and p16 expression between sinus rhythm, paroxysmal AF, permanent AF is clearly suggestive of a link between senescence and AF progression. In an attempt to characterize predictive factors of the senescent burden as assessed by p53 and p16 expression, logistic regression analysis was performed. While no relationship between age, cardiovascular risk factors, valvular or coronary disease, transthoracic echocardiographic finings including atrium surface, CHADS2VASC2 score and senescent markers could be established, a strong relationship between AF and senescence could be evidenced. In the setting of AF, atrial senescence can be induced by a number of factors such as low or oscillatory shear stress, that contribute to an endothelial NO synthase decrease, and enhanced angiotensin II, hypoxia, inflammation or redox mediated stress . Up to now, controversies remain on the role of p53/p16/p21 pathway in the regulation of cardiac homeostasis. According to Maejima[9], NO could attenuate ischemia/reperfusion mediated apoptosis through the up-regulation of p21 protein. Clearance of p16 positive cells was reported to attenuate age-related deterioration of several organs without apparent side effects, including heart[10]. Other authors have pointed at the importance of the p53 pathway in the regulation of a pro-fibrotic pattern, premature senescence of myofibroblasts being viewed as an essential antifibrotic mechanism[4]. Another study has suggested the implication of p53-induced inflammation in heart failure[3]. Whether this up-regulation of p53 signaling pathway is deleterious or confers cytoprotection of atrial tissue in AF is not clear and remained to be investigated. Although the precise study of the mechanistic pathways linking senescence to

atrial tissue remodeling in AF is far beyond the scope of the present study, our data collected from human appendages clearly demonstrate a tight relationship between senescence burden and AF progression.

Senescence is associated with endothelial dysfunction and enhanced tissue factor expression

Previous data have emphasized that endothelial senescence is characterized by a huge oxidative stress along with a pronounced down-regulation of eNOS expression and endothelial formation of NO[11]. Complex interplay between senescence and oxidative stress are illustrated by the fact that reactive oxygen species (ROS) can directly induce telomeres shortening, the initial step of cellular senescence or take part *per se* to the reduction of NO bioavailability[12]. In the present study, the inverse correlation between eNOS and p16 expression possibly witnesses the relationship between senescence, oxidative stress and endothelial dysfunction. The present observation of a decline in eNOS expression as AF progresses is consistent with previous findings showing that AF causes atrial endocardial dysfunction leading to decreased nitric oxide (NO) concentration and down-regulation of eNOS [13, 14] and that successful pulmonary vein isolation enables improvement of endothelial dysfunction as measured by flow-mediated vasodilaton in AF patients (FMD)[15]. In chronic AF, down-regulation of eNOS and increased proportion of ROS mainly originating from NADPH oxidase, have also been involved in electrical remodeling. The diminished bioavaibility of NO induces a shortening of action potential duration in modulating sodium, calcium and potassium channels[16]. Oxidative stress induces also a pro-fibrotic state favoring differentiation of fibroblasts in collagen-secreting myofibroblasts[17, 18] that lead to left atrium remodeling enabling AF perpetuation. Besides its role in the control of vascular tone and the regulation of endothelial survival, NO plays a determinant role in platelet aggregation at endothelial surface.

Another key factor in pro-coagulant propensity is TF, the main initiator of cell coagulation. Recent insights from our group have highlighted the view that enhanced TF expression, together with pro-coagulant MP release and blunted NO-mediated inhibition of platelet aggregation are characteristics features of endothelial senescence. In the present work, the rise of atrial TF expression parallels AF progression and is indicative of an enhanced pro-thrombotic state. Although the cause of TF elevation could not be established in this observational study, the correlation between p53 and TF expression is another indication of a the pro-thromnotic feature of endothelial senescence. In the setting of AF, recent data have emphasized the view that alteration in atrial hemodynamics caused by the AF rhythm disturbance may promote a pro-thrombotic propensity through enhanced TF expression[19]. Altogether, these findings highlight the view of a progressive endothelial senescence and dysfunction, that favors thrombotic propensity and the development and establishment of AF.

MMP-9 expression, as a surrogate marker of atrial remodeling and atrial fibrillation

Many observational studies have pointed at the expression of MMP, particularly MMP-2 and 9, and TIMPs (tissue inhibitors of matrix metalloproteinases) in atrial tissues or their concentration in the peripheral blood in AF[20]. MMP are secreted by fibroblasts, endothelial cells and cardiomyocytes[21] and belong to a class of pro-inflammatory and pro-angiogenic factors that control tissular remodeling by regulating extracellular matrix degradation and release of growth factors such as TGF-B that triggers a pro-fibrotic signaling. Recent data have also underscored that TGF-β1 increased p16 expression and senescence through production of mitochondrial reactive oxygen species and decreased expression of various anti-oxidants proteins[22]. The present observation of an enhanced expression of MMP-9 expression in human atrial tissue from AF patients is consistent with previous findings by Gramley F and coworkers who observed a rise in MMP-2 and MMP-9 expression with AF progression[23]. In a model of aging mice, recent studies have depicted enhanced metalloproteinase expression concomitant with the development of diastolic dysfunction, as a

surrogate marker of cardiac fibrosis[24] and that MMP-9 deletion attenuates the age-related decline in diastolic function in part by reducing TGF-ß pro-fibrotic signaling[25]. Senescence prompts the secretion of MMP and collagenase from human aged fibroblasts thereby promoting the degradation of the extracellular matrix[26, 27]. In the present work, no correlation between senescent markers and MMP-9 expression could be stablished. MMP-9 levels were correlated to age, left ventricular mass, CHA2DS2VASc score as a possible illustration of the complex network linking ageing, arterial hypertension and atrial remodeling.

Study limitations

Assessment of protein expression in appendages was limited to western blot and immunohistological staining analysis. Other important markers involved in atrial remodeling (ROS, TGF-ß, TIMPs, inflammation) were not investigated not the activity of Senescence-associated beta galactosidase. Although we rigorously tried to match our study controls (AF vs SR), we could not exclude that significant bias remained. However, it should be emphasized that baseline characteristics were not significantly different between groups. Finally, owing to the observational nature of the present study, no mechanistic insight on the link between AF, senescence, pro-thrombotic and pro-remodelling pattern could be provided. As a consequence, the present data should mainly be considered as “hypothesis generating” and should be further investigated in appropriate models.

Conclusion

This study provides compelling evidences indicating that AF progression is strongly related to human atrial senescence burden as determined by p53 and p16 expression. The stepwise increase of senescence (p53, p16), pro-thrombotic (TF) and pro-remodeling (MMP-9) markers observed between sinus rhythm, paroxysmal and permanent AF points toward a possible network that links in human atrium, senescence burden, endothelial dysfunction, thrombogenicity and atrial remodeling.

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Table 1: Baseline characteristics

Variables	Sinus Rhythm	AF	*p*
Patients demographics			
Age (yrs)	68±11	70±12	0.630
Sexe (male)	13 (61.9)	13 (61.9)	1
HTA (no/%)	14 (66.7)	13 (61.9)	0.747
Diabetes	7 (33.3)	8 (38.1)	0.747
Tabac	7 (33.3)	7 (33.3)	1
LVEF<40%	1	1	
Vascular disease	6 (28.6)	4 (19)	0.469
Body Mass Index	28.4±5.7	30.5±6.9	0.301
Euroscore I	4.2±3.1	6.8±5.1	0.056
Euroscore II	1.9±1.8	3.5±3.2	0.060
Valvulopathy	12 (57.1)	19 (90.5)	**0.014**
Aortic valvulopathy	11 (52.4)	13 (61.9)	0.533
Mitral valvulopathy	2 (9.5)	9 (42.9)	**0.014**
CHA2DS2VASc			
0-1	6	5	
2-3	8	7	
≥4	7	9	
Drug treatment			
Bblokers	12 (57.1)	15 (71.4)	0.334
ACE inhibitor AT2 bloker	15 (71.4)	11 (52.4)	0.204
Aspirin	14 (66.7)	2 (9.5)	**<0.001**
VKA	2 (9.5)	14 (66.7)	**<0.001**
Statin	12 (57.1)	11 (52.4)	
Echographic data			
LA surface (cm²)	23±9	36±19	**0.017**
RA surface (cm²)	18±7	23±9	0.147
LVEF (%)	62±8	60±8	0.407
EDLVD (cm)	51±8	53±10	0.497
LV mass	133±41	127±40	0.647
Biological parameters			
Hb g/l	13.5±1.1	13.5±1.6	0.992
Leukocytes	6677±2131	7191±2004	0.426
Fibrinogen g/l	3.4±0.5	3.6±0.9	0.582
Creatinine clearance	87±30	78±33	0.449

Table 2: MMP9, p53, p16, TF and eNOS expression in sinus rhythm and AF subsets. Results are expressed as fold increased compared to the density of the beta tubulin in each appendage lysate

Variables	Sinus Rhythm (SR)	Atrial fibrillation (AF)	p
MMP9	0.73 ± 0.29	0.81±0.34	0.617
P53	0.58 ± 0.31	0.91±0.31	**0.001**
P16	0.35±0.18	0.76±0.32	**0.0001**
TF	0.68±0.29	0.88±0.32	**0.045**
eNOS	0.35±0.12	0.25±0.15	**0.023**

Table 3: MMP9, p53, p16, TF and eNOS expression according to sinus rhythm, paroxysmal AF, permanent AF. Results are expressed as fold increase compared to the density of the beta tubulin in each appendage lysate

	Sinus rhythm (SR)	Paroxysmal AF (PAF)	Permanent AF (PmAF)	p (SR/parox)	P (SR/perm)	P (PAF/PmAF)
MMP9	0.73 ± 0.29	0.65 ± 0.35	0.96± 0.27	0.499	**0.047**	**0.023**
P53	0.58 ± 0.31	0.78± 0.30	1.04 ± 0.29	0.107	**< 0.001**	0.059
P16	0.35±0.18	0.73±0.30	0.78±0.35	**0.0001**	**0.002**	0.120
TF	0.68 ± 0.29	0.76 ±0.26	1.00 ± 0.34	0.542	**0.010**	0.081
eNOS	0.35±0.12	0.29±0.17	0.22±0.13	0.135	**0.01**	0.210

Table 4: Predictors of p 53 elevation: univariate and multivariate analysis

Variable	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
	HR	**95% CI**	**p**	**HR**	**95% CI**	**p**
Age	1.011	0.959-1.066	0.679			
AF	10.240	2.475-42.370	**0.001**	7.849	1.330-46.333	**0.023**
Permanent AF	15.000	1.685-133.551	**0.015**			
Paroxysmal AF	1.600	0.413-6.193	0.496			
AF at the time of surgery	8.000	2.012-31.803	**0.003**			
Arterial hypertension	1.231	0.38-4.358	0.748			
Diabetes mellitus	0.533	0.148-1.922	0.336			
Smoker	0.538	0.422-5.606	0.514			
Female sexe	1.083	0.288-4.081	0.906			
LVEF < 40%	2.105	0.176-25.170	0.556			
Vasculopathy	0.333	0.073-1.527	0.157			
Chads2Vasc2	1.085	0.760-1.549	0.652			
Statins	0.699	0.433-1.129	0.143			
ACE AT2 inhibitors	0.667	0.190-2.334	0.526			
Valvulopathy	2.125	0.515-8.770	0.297			
AO valvulopathy	0.677	0.198-2.312	0.534			
Mi valvulopathy	2.125	0.515-8.770	0.297			
Coronaropahy	0.300	0.083-1.081	0.066			
Euroscore I	1.089	0.937-1.266	0.267			
Euroscore II	1.063	0.844-1.338	0.604			
Hb	1.165	0.754-1.800	0.492			
Leukocytes	1.309	0.936-1.831	0.115			
Fibrinogen	1.009	0.425-2.398	0.983			
Creat clearance	1.003	0.995-1.010	0.493			
LV mass	1.002	0.986-1.018	0.829			
LA surface	1.032	0.973-1.094	0.297			
P16	16.121	1.461-177.851	0.023	1.986	0.109-36.312	0.643

Table 5: Predictors of p 16 elevation: univariate and multivariate analysis

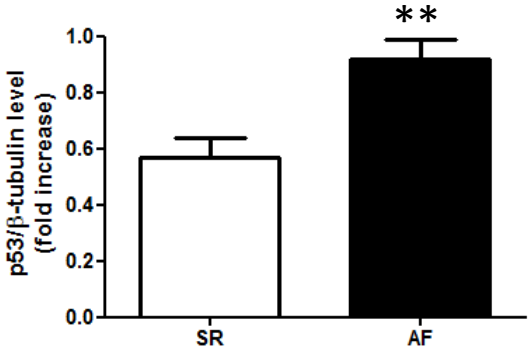
Variable	UNIVARIATE ANALYSIS			MULTIVARIATE ANALYSIS		
	HR	**95% CI**	**p**	**HR**	**95% CI**	**p**
Age	1.010	0.959-1.065	0.698			
AF	18.062	0.871-84.283	**<0.001**	15.741	2.863-86.556	**0.002**
Permanent AF	3.000	0.655-13.747	0.157			
Paroxysmal AF	8.636	1.593-46.807	**0.012**			
AF at the time of surgery	13.600	3.091-59.831	**0.001**			
Arterial hypertension	0.533	0.148-1.922	0.336			
Diabetes mellitus	0.813	0.229-2.877	0.748			
Smoker	1.538	0.422-5.606	0.514			
Female sexe	0.686	0.182-2.589	0.578			
LVEF < 40%						
Vasculopathy						
Chads2Vasc2	1.050	0.737-1.498	0.786			
Statins	1.250	0.274-5.705	0.773			
ACE AT2 inhibitors	0.284	0.076-1.063	0.062			
Valvulopathy						
AO valvulopathy						
Mi valvulopathy	1.280	0.322-5.088	0.726			
Coronaropahy	0.677	0.198-2.312	0.534			
Euroscore I	1.036	0.899-1.194	0.624			
Euroscore II	1.091	0.861-1.383	0.469			
Hb	0.937	0.609-1.441	0.768			
Leukocytes	1.153	0.847-1.569	0.366			
Fibrinogen						
Creat clearance	1.004	0.993-1.014	0.490			
LV mass	0.997	0.982-1.014	0.750			
LA surface	1.028	0.972-1.087	0.331			
P53	7.842	1.167-52.711	**0.034**	1.532	0.133-17.610	0.732

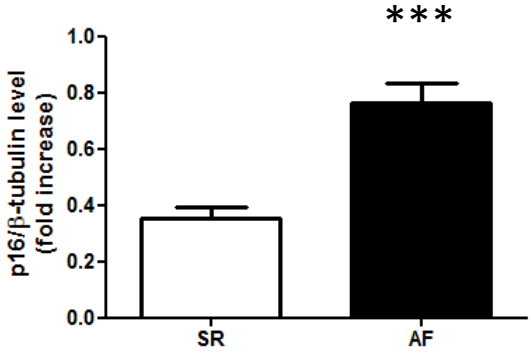
Figure 1: Expression of p53, p16, TF, eNOS and MMP-9 in appendage from patients with sinus rhythm or atrial fibrillation (SR: sinus rhythm, AF: atrial fibrillation)
Protein expression was determined by westen blots of 3 different lysates. Results are expressed as a ration of beta-tubulin expression.

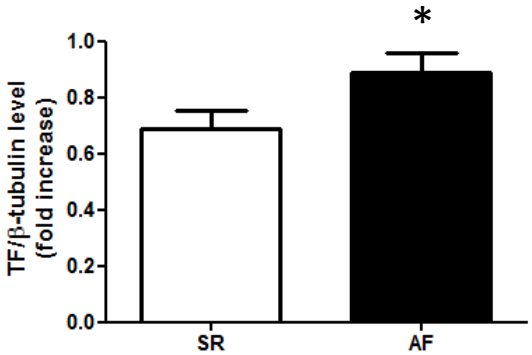
Figure 2: Expression of p53, p16 and eNOS proteins in right appendages sections from patients with sinus rhythm or atrial fibrillation (SR: sinus rhythm, AF: atrial fibrillation)
Immunohistologiacal labelling of appendage sections was analyzed by confocal immunofluorocytometry.

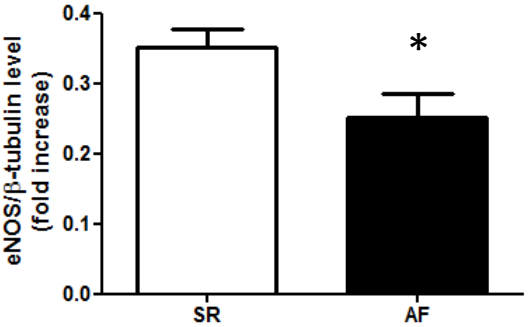
Figure 3: p53, p16, TF, eNOS and MMP-9 expression according to sinus rhythm, paroxysmal or permanent atrial fibrillation (SR: sinus rhythm, AF: atrial fibrillation)

Figure 1


**
p53/β-tubulin level
(fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
SR
AF


***
p16/β-tubulin level
(fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
SR
AF


*
TF/β-tubulin level
(fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
SR
AF


*
eNOS/β-tubulin level
(fold increase)
0.4
0.3
0.2
0.1
0.0
SR
AF

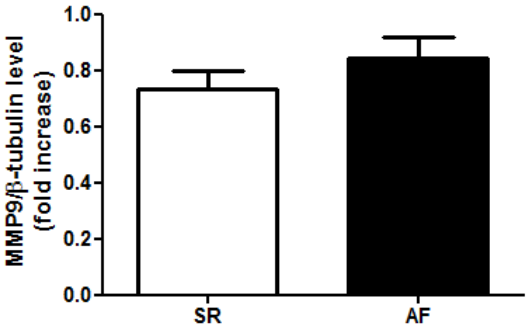

MMP9/β-tubulin level
(fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
SR
AF

Figure 2

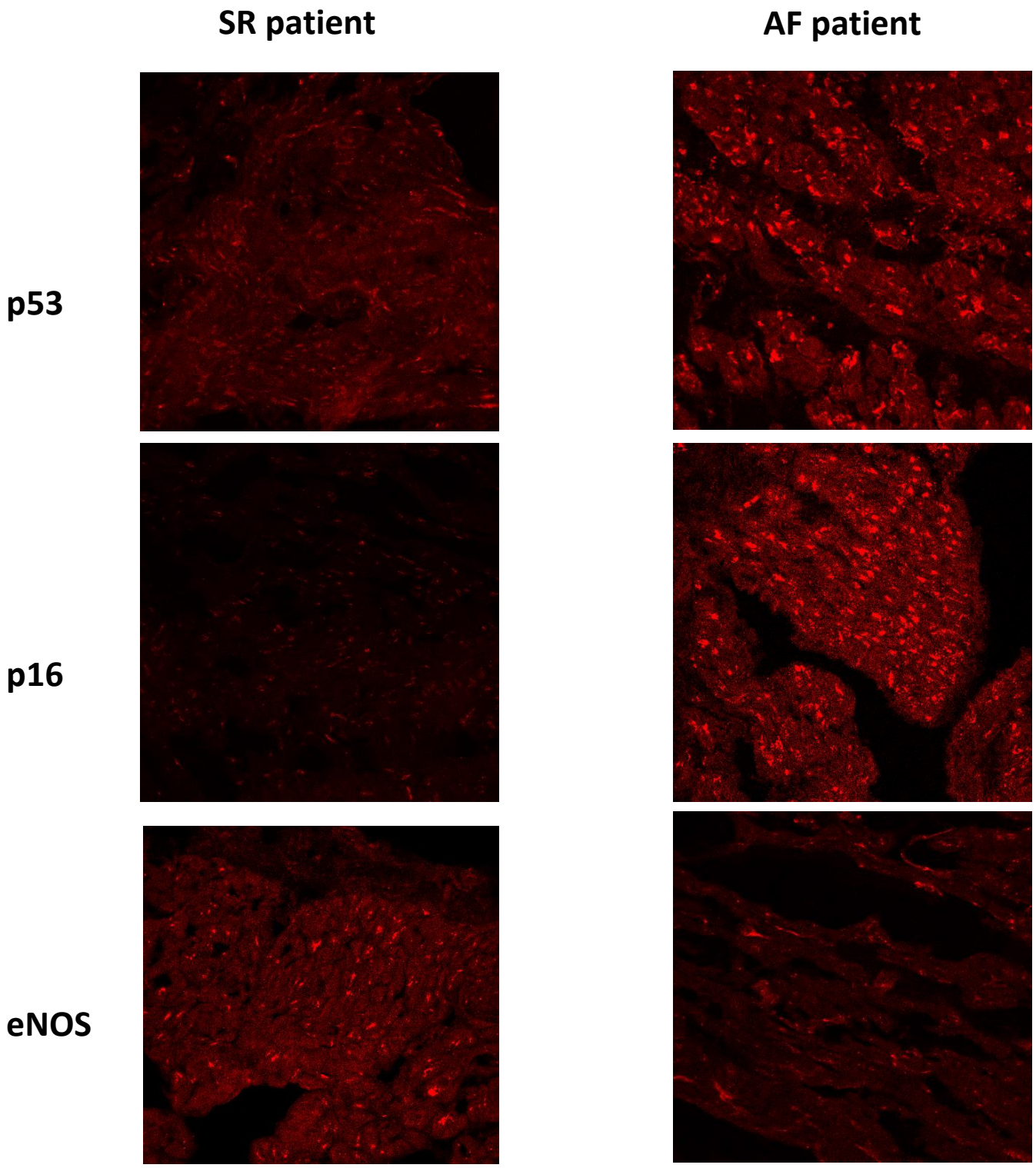
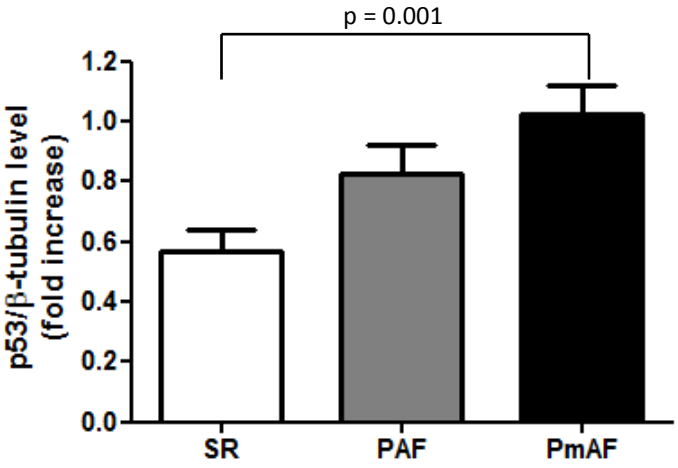
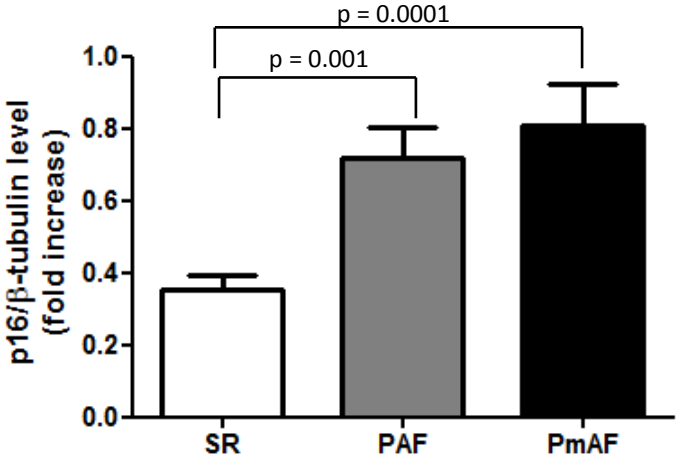
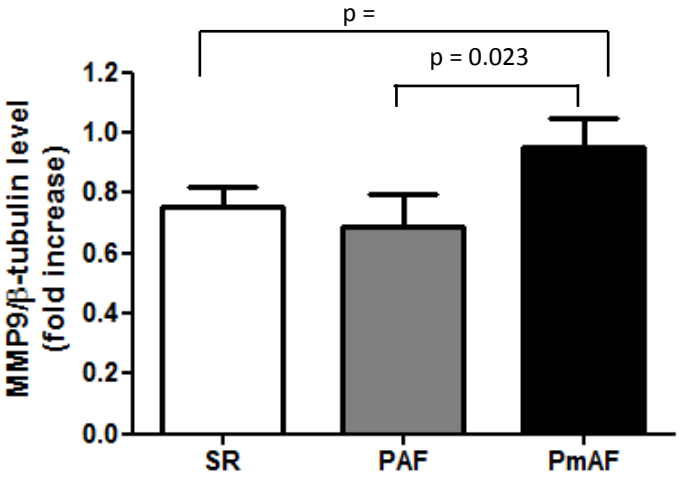
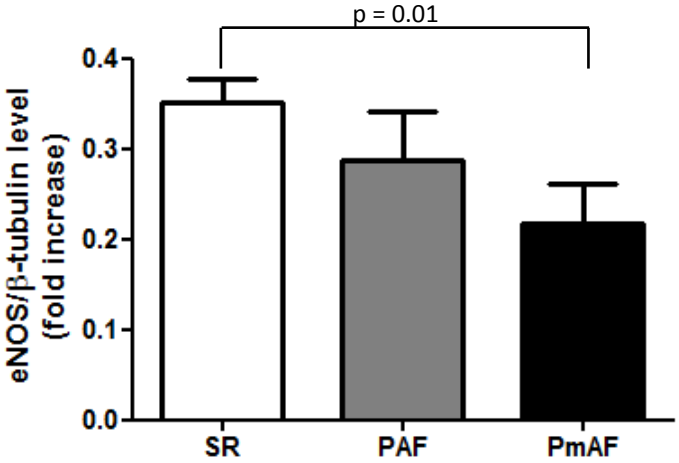


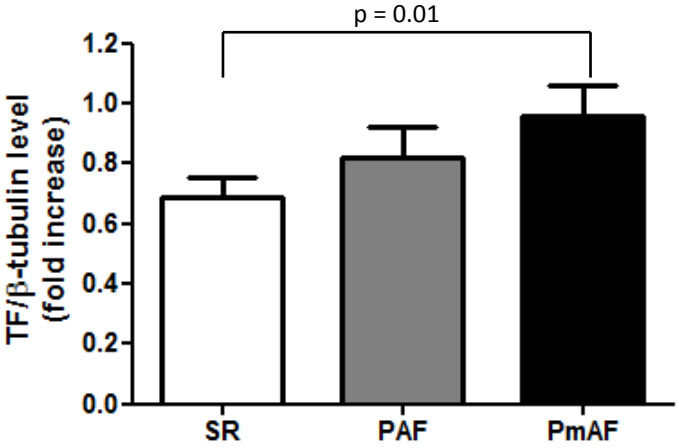
Figure 3 : Expression of p53, p16, MMP-9, eNOS and TF according to sinus rhythm, paroxysmal AF, permanent AF











Remarque conclusive sur le travail (article 3)

Nos résultats sont fortement évocateurs d'une sénescence apparaissant progressivement avec la sévérité de la FA. En effet, la surexpression des protéines caractéristiques p53 et p16 croit du rythme sinusal à la FA permanente de même que la baisse de l'expression de la eNOS suggérant l'établissement progressif d'un défaut de biodisponibilité du NO. Il reste à confirmer que cette dernière dysfonction endothéliale est associée à un stress oxydant excessif comme dans les autres modèles de sénescence et à identifier la source de ce stress, notamment en examinant l'expression des oxydases cellulaires ou mitochondriales et en mesurant le contenu tissulaire en ROS par RPE.

Ces travaux sont à rapprocher des données exposées dans la première partie et indiquant un état pro-thrombotique dans la FA. En effet, si l'analyse histologique des oreillettes n'évalue pas *stricto sensu* le risque thrombotique associé à la sénescence dans la FA, nous avons retrouvé la sur-expression caractéristique du facteur tissulaire, initiateur cellulaire de la coagulation, connue dans la sénescence endothéliale vasculaire et qui s'accompagne alors de la sur-expression de cytoadhésines[208].

Nous avons également mis en évidence une sur-expression progressivement croissante de la MMP-9 entre le RS et la PmAF suggérant un lien entre remodelage tissulaire et sénescence atriale.

Article 4 :

Mise au point d'un modèle original de culture primaire de cellules endothéliales auriculaires extraites d'oreillettes gauches de porc. Etude des modifications phénotypiques associées au développement de la senescence.

Atrial endothelial cells senescence induces thrombogenicity, inflammation and extra-cellular matrix remodeling: Role of Ang II/ATI receptor pathway

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Atrial endothelial cells senescence induces thrombogenicity, inflammation and extra-cellular matrix remodeling: Significance of the Ang II/ATI receptor pathway

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Short title: Atrial endothelial senescence and thrombogenicity

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Abstract

Background: Ageing is a main determinant of atrial remodeling that paves the way to atrial fibrillation and enhanced thrombogenicity. Preclinical studies on endothelial atrial cells are conspicuously lacking. As a result, the initial cellular triggers for clotting, inflammation and proteolysis in atrial fibrillation remain speculative. This study characterized phenotypical changes associated with atrial endothelial cells senescence and examined the role of Angiotensine II (AngII) /AT1R pathway.

Methods and results: Senescence, as assessed by β-galactosidase activity (SA-β-gal), was induced by passaging of porcine left atrium endothelial cells from passage P1 to P4. Replicative senescence, exposure to L-NAME, an eNOS inhibitor, H_2O_2 or Ang II were characterized by up-regulation of p53, a key regulator in cellular senescence, p21 and p16, key cyclin-dependent kinase inhibitors. Senescent atrial cells phenotype was characterized by (i) cell thrombogenicity through tissue factor up-regulation, shedding of procoagulant microparticles, eNOS down-regulation and reduced NO-mediated inhibition of platelet aggregation (ii) cell adhesion through up-regulation of VCAM-1 and I-CAM-1 (iii) proteolysis and fibrosis remodeling through MMP-2, 9 and TGFß1 expression (iv) up-regulation of the local Ang II system through enhanced AT1 receptors and ACE expression at endothelium atrial cell surface. Losartan, an AT1 receptor antagonist, and ACE inhibitor prevented atrial endothelial cell senescence.

Conclusions: These data provide evidences indicating that atrial endothelial senescence promotes thrombogenicity, inflammation, matrix remodeling and up-regulation of the local Ang II system. They further suggest that targeting Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial phenotypical alteration associated with ageing.

Keywords: senescence, thrombosis, atrial fibrillation, endothelium, microparticles

Introduction

Ageing is a main determinant of atrial remodeling that contributes to atrial fibrillation (AF) initiation and perpetuation and enhanced thrombogenicity. Because one of the main consequences of AF is thrombotic stroke, the hypothesis that AF *per se* is a pro-thrombotic disease was raised [1]. Evidences in favor of this paradigm include increased levels of plasma markers of a pro-thrombotic state such as vWF, fibrinogen, D-Dimer and pro-coagulant microparticles (MPs) in the plasma of AF patients [1, 2]. Although the link between ageing and thrombogenicity was extensively characterized in epidemiological studies, little is known about phenotypical changes associated with endothelial atrial cells senescence that could promote clot formation. Preclinical studies on endothelial atrial cells are conspicuously lacking. In vitro studies so far have been hampered by the inability to isolate and culture endothelial cells from the atria. As a result, the initial cellular triggers for clotting, inflammation and proteolysis induction in AF remain mainly speculative. Endothelial cell senescence and dysfunction are the hallmark of cardiovascular disease [3, 4]. Cellular senescence is a stress and damage response resulting in an irreversible cell cycle arrest and the appearance of distinct morphological and functional changes associated with impairment of cellular homeostasis. Senescent vascular endothelial cells also secrete pro-inflammatory and pro-remodeling factors, which in turn may alter tissue environment [3, 4]. In senescent endothelial cells increased ROS concentration and decreased endothelial NO synthase expression reduce NO availabilty [5]. In cultures and tissues senescence-associated ß-galactosidase (SA-ß-gal) activity, senses the increased lysosomal mass from senescent cell and is a canical marker of senescence [5]. The senescence-inducing signals engage either or both the p53/p21 and p16/retinoblastoma protein tumour suppressor pathways, as the final effectors of the senescence program. Endothelial senescence can be induced by a number of factors implicated in vascular pathologies. In the setting of AF, inflammation, oxidative stress, angiotensin II, shear stress, increased mechanical stretch, hypoxia are pivotal to atrial remodeling. Rhythm disturbance induced low and oscillatory shear stress can result in blunted NO formation[6], induction of endothelial-derived MP release [7] and endothelial

senescence via a p53-dependent pathway [8].Previous studies have demonstrated a key role of angiotensin II in the pathogenesis of AF[9]. Ang II type 1 receptor (AT1) activation is known to induce the activation of Mitogen activated protein kinase (MAPK) which in turn favors atrial remodeling through fibroblast proliferation, cellular hypertrophy and apoptosis. The importance of Ang II in atrial remodeling was further emphasized by studies demonstrating enhanced Ang II production during tachypacing in canine hearts and arrhythmogenic atrial structural remodeling mediated by 38 MAK kinase phosphorylation[10]. Because ageing is a main determinant of AF, we developped and characterized an original model of replicative senescence in primary culture of porcine left atrium endothelial cells (AEC). The typical loss of NO availability was investigated by NO-inhibition of platelet agregation and variations of the protein expression pattern were analyzed by western blot. Pro-coagulant, -inflammatory and -senescent markers mainly TF, AT-1 and ACE, eNOS, VCAM and ICAM, were assessed as well as metalloproteases MMP-2 and MMP-9. In addition, the release of procoagulant microparticles was measured in AEc supernatants as a signature of senescence. Since angiotensin II has been reported to be a potent inducer of endothelial senescence, oxidative stress, MP release and thrombogenicity [11, 12] in vascular cells, we have examined the possibility that the local angiotensin II/AT1 receptor pathway prompts premature atrial endothelial senescence in young AECs.

Materials and methods

Cell culture

Pig hearts were collected from the local slaughterhouse (COPVIAL, Holtzheim, France) and ECs were isolated from left atria. In order to obtain an entire left atrium, heart-lung blocks were dissected. Left ventricle was removed at the level of the mitral valve and pulmonary veins were ligated. The remaining blood was flushed with cold PBS without calcium. Subsequently, endothelial cells (ECs) were isolated by filling the left atrium with a type I collagenase (Worthington, Serlabo Technologies, Entraigues sur la Sorgue, France) solution

at 1 mg/ml dissolved in MCDB131 medium with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1mM, all from Lonza, St Quentin en Yvelines, France) for 40 min at 37°C. Endothelial cells were collected in the effluent after massages of the atrium walls and the remaining cells were flushed with medium. Collected cells were gently centrifuged at 600 g, the medium was removed and cells were suspended in MCDB131 medium supplemented with 15% fetal calf serum, penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM). Cells from two different left atria were seeded in a T25 flask and cultivated at 37 °C in a humidified incubator with 5 % CO2. The endothelial cells adhering to the bottom of the flask quickly were selected by removing the medium with non-adherent cells 5-6 hours after seeding. The medium was changed every 48 h.

At regular intervals, ECs were sequentially passaged from P1 to P4 by splitting at a ratio of 1:3 using trypsin treatment until senescence was reached (Gibco-BRL, Grand Island, N.Y., USA). With respect to the high shear stress encountered in artery circulation (10-20 dynes/cm^2), statics conditions are closed to the rheological conditions evidenced in AF and characterized by low and oscillatory shear stress (< 3 dynes/cm^2). Premature EC senescence was induced either by H_2O_2 (100 µM), L-NAME (1 mM), angiotensin II (10 µM) treatment. In some experiments, ECs were exposed to a pharmacological modulators for at least 24 hours.

Chemicals

Unless indicated, all chemicals and solvents were from Sigma-Aldrich (Sigma-Aldrich SARL, St Quentin Fallavier, France). Angiotensin II was purchased from Enzo Life Sciences (Villeurbanne, France). Losartan (10 µM) and perindoprilat (10^{-7} M).

MPs isolation and measurement

Microparticles (MPs) were collected from conditioned medium of senescent cells by sequential centrifugation under sterile conditions as described previously [13]. Briefly, cells and

cellular debris were discarded by a 2-steps 800 g centrifugation for 15 min at room temperature, and the supernatant was further centrifuged at 13 000 g and 4 °C for 60 min. The final suspension was kept in Hanks balanced solution (HBSS) less than 30 days at 4 °C. MPs measurement was performed by prothrombinase assay after capture onto Annexin-5 as previously described using microplate spectrophotometric reader set in kinetic mode, at 405 nm [14]. This capture-based assay allows extensive washing of captured MPs, taking advantage of the high affinity of Annexin-5 for phosphatidylserine (Phtdser) exposed at MPs surface. MPs concentration was referred to as Phtdser equivalent, by reference to a standard curve made with synthetic vesicles of known amounts of PhtdSer [14]. In some experiments, the MP free supernatant was centrifuged during 4 h at 25 000 g to discard exosomes and assayed as a control of truly soluble content of the conditioned medium.

Assessment of senescence indicators

Senescence-associated β-galactosidase (SA-β-gal) activity was measured by flow cytometry using C_{12}FDG a fluorogenic substrate of the enzyme as described elsewhere (citer le papier de schini déjà publié), endotheial NO synthase (eNOS) expression by Western, and the production of endothelial NO through the inhibition of platelet aggregation under standardized conditions using an aggregometer.(see below)

Detection of SA-β-gal activity

Senescence-associated β-galactosidase activity (SA-β-gal) was measured by flow cytometry in cells labelled with the fluorogenic cell permeable substrate C12FDG (5-dodecanoylaminofluorescein Di-β-D-galactopyranoside, Invitrogen, ThermoFisher, Illkirch, France) following the methods previously described [15]. Briefly, endothelial cells were pretreated with chloroquine (300 µM) for 1 h before addition of C12FDG (33 µM). After 1 h incubation, cells were washed with ice-cold PBS, trypsinized and analyzed immediately using a flow cytometer (FACScan, Becton Dickinson , San Jose, CA, USA). Data were acquired and analyzed with CellQuest software (Becton Dickinson). Light scatter parameters were set

to eliminate dead cells and subcellular debris. The green C12-fluorescein signal was measured and SA-β-gal activity was estimated using the mean fluorescence intensity (MFI) of the population. Auto-fluorescence gains were determined in unlabelled cells and set at the first logarithmic decade.

Western blot analysis

Levels of protein expression in cells were measured by Western blot analysis. Proteins were extracted from cells as described previously[5], and 30 mg total proteins were separated by SDS-PAGE (12 % acrylamide, Euromedex, Souffelweyersheim, France) at 100 V for 2 h and transferred electrophoretically onto PVDF membranes (GE healthcare, VWR, Fontenay-sous- Bois, France) at 100 V for 120 min. Aspecific binding sites were saturated by incubation of the membranes for 1 h with Tris-buffered saline solution (TBS, Euromedex) containing 3% bovine serum albumin and 0.1 % Tween 20. Membranes were probed overnight at 4 °C with blocking solution containing specific antibodies directed against proteins of interest. The antibodies used were either mouse anti-TF (1:1000, Sekisui Diagnostics, Sekisui Virotech GbmH, Rüsselsheim, Germany), anti-eNOS (1:1000, BD Biosciences, Le Pont de Claix, France), rabbit anti-human p21 (1:1000; Santa Cruz Biotechnology, Clinisciences, Nanterre, France), anti-human p16 (1:1000; Santa Cruz Biotechnology), anti-human p53 (1:1000; Santa Cruz Biotechnology), anti-AT1R (1:1000, Santa Cruz Biotechnology), anti-ACE (1:500; Abbiotec, Clinisciences, Nanterre, France), TGF ß (1:1000 Abcam), MMP-2 and MMP-9 (1:10000 Abcam), ICAM (1:20000 Abcam), VCAM (1:20000 Abcam).

Thereafter, membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and density signals of each band were detected using enhanced Clarity Western ECL Substrate (170-0561, BIO-RAD, United States). Membranes were incubated with a mouse polyclonal anti-beta tubulin antibody for normalization purposes. Results were expressed as a ratio of beta tubulin density using the ImageQuant acquisition system and analysis software (LAS4000 and ImageQuant TL 8.1, Amersham).

Platelet aggregation

Platelets freshly isolated from healthy human blood and suspended in Tyrode-albumin buffer at 300 000 platelet/µl were obtained from the local blood transfusion center (Etablissement

Français du Sang-Alsace, Strasbourg). For aggregation measurement, 450 µl of platelet suspension was incubated under constant stirring into a small glass vial at 37°C in an aggregometer (Chromo-Log 490D, Chrono-Log, Stago BNL, Netherland). For each batch of platelets, a concentration-response curve was constructed with U-46619 (9,11-dideoxy-9α,11α-methanoepoxy Prostaglandin F2α, Cayman Chemicals, Interchim, Montluçon, France), a Thromboxane A2 receptor agonist. To assess the anti-aggregant potential of NO-released from endothelial cells, ECs at indicated passages were grown on Cytodex™ 3 beads (GE healthcare) and exposed or not to L-NAME for 24 h. Pre-treated or P3 ECs on Cytodex™ 3 beads were added to the platelets at the indicated concentration and incubated for 3 min before induction of aggregation by U46619 to obtain 60-70% aggregation.

Statistical analysis

Statistical analysis were done by one-way analysis of variance (ANOVA) followed by the Tukey's post-hoc tests or Student's t test for the comparisons of groups using GraphPad Prism (version 5.01 for Windows, GraphPad Software, San Diego, USA). Statistical significance was considered for $P < 0.05$.

Results

Replicative passaging of atrial endothelial cells and eNOS inhibition promotes senescence

Replicative passaging of AECs induced a progressive increase in the number of cells with SA-β-gal activity (Figure 1A). Likewise, H_2O_2, a strong inducer of premature endothelial senescence [11], or L-NAME, an eNOS inhibitor, induced AECs senescence, confirming a redox sensitive nature of the pathway (Figure 1B). Senescence was also witnessed by a strong up-regulation of p53, a key regulator in cellular senescence, and of down-stream p21 and p16, key cyclin-dependent kinase inhibitors, in P4 AECs and in L-NAME and H2O2

treated P1 AECs (Figure 1C). We had previously verified that the induction of replicative senescence did not heighten the level of apoptosis as previously reported in porcine aortic endothelial cells (Abbas, Circulation 2016 in revision).

Atrial endothelial senescence, endothelial dysfunction and thrombogenicity

Because vascular endothelial senescence is usually characterized by endothelial dysfunction and a reduced formation of NO [16, 17], the expression of eNOS within atrial endothelial cells was assessed. A significant down-regulation of eNOs expression could be established by Western Blot analysis in P4 AECs compared to P1 (Figure 2A). Given the key role of NO in the modulation of platelet aggregation, we investigated the ability of AECs to inhibit platelet aggregation. Senescent P3 AECs, L-NAME treated P1 AECs significantly enhanced platelet aggregation suggesting a blunted formation of NO (Figure 2B). In addition, senescence shifted the endothelial phenotype to a pro-coagulant status in P4 AECs as evidenced by the 3-fold enhancement in pro-coagulant MPs release and TF expression (Figure 3). In addition H2O2 also prompted MP release and TF up-regulation (Figure 3).

The local angiotensin system mediates premature atrial endothelial senescence, with prothrombotic pattern

We measured a 3-fold up-regulation of AT-1 in P4 AEcs compared to P1 and the redox sensitive nature of ACE at AT-1 up-regulation was evidenced in L-NAME and H2O2 treated cells P1 cells (figure 4A). Since Ang II was reported a strong inducer of senescence and MPs formation through enhanced oxidative stress in cultured murine aortic ECs [11], we examined the possibility of a redox-sensitive senescence induction by the local angiotensin system in Ang II treated young P1 cells 24h after incubation. As shown in Figure 4B, 10^{-7}M Ang II up-regulated p53, p21 and p16 (figure 4B). In addition, procoagulant TF factor expression was highly expressed in Ang II treated young P1 cells 24h after incubation (figure 5).

Since Ang II-induced premature senescence was associated with the up-regulation of AT1R, ACE (Figure 6A), experiments were performed to determine whether Losartan, an AT1 receptor inhibitor or Perindoprilat, an ACE inhibitor could prevent atrial ECs senescence in Ang II induced-P1 cells. Both Losartan and Perindoprilat were potent inhibitors of AECs senescence (figure 6B) pointing at the local angiotensin system as a senescent mediator.

Atrial endothelial senescence is associated with a characteristic pro-adhesive and tissue remodeling pattern

To underscore the impact of atrial senescence on cell adhesion and remodeling, variation of the expression of key cytoadhesins and MMPs were investigated between P1 and P4. A 3-fold enhanced redox-sensitive expression of ICAM-1, V-CAM-1 and MMP-2 and MMP-9 could be established (Figure 7). In addition, in premature Ang-II induced senescence an identical pro-adhesive and proteolytic protein pattern was identified but also a pro-fibrotic phenotype with an enhanced expression of TGF-ß1, a pivotal mediator in the control of tissue remodeling during cardiac fibrosis (figure 8).

Discussion

The present findings indicate that atrial endothelial senescence promotes thrombogenicity, inflammation, proteolysis, fibrosis and up-regulation of the local Ang II system. They further suggest that targeting Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial phenotypical alteration associated with senescence.

Atrial endothelial senescence is associated with endothelial dysfunction and a pro-thrombotic pattern

The potential role of senescence in endothelial dysfunction has lately attracted a lot of

interest using cultured ECs and experimental models of atherosclerotic diseases[3], its impact during arrhythmogenic remodeling associated with AF being unraveled. Both premature EC senescence, as observed in response to Ang II or H_2O_2, and replicative senescence are characterized by the association of oxidative stress with a pronounced down-regulation of eNOS expression and of NO endothelial formation[4, 5]. The present findings further extents these previous reports by showing that replicative senescence promotes, besides blunted NO formation, a pronounced pro-thrombotic pattern in senescent AECs as indicated by their reduced ability to prevent platelet aggregation, the upregulation of the expression of TF protein and the shedding of procoagulant MPs. We and others have recently demonstrated that senescent EC-derived MPs act as effective mediators propagating a pro-senescent and pro-thrombotic message to neighboring ECs and contribute locally to thrombus formation [11]. Besides representing a surrogate marker of vascular and endothelial dysfunction, circulating MPs, evidenced at elevated levels in AF [2, 18] or within the hours following radiofrequency or cryoablation procedures [19, 20] might also contribute to endothelial dysfunction and thrombotic propensity of atrial cells. Because procoagulant MPs circulate at high levels during AF or during ablation procedures associated with thromboembolic complications, MPs might behave as potent effectors of stroke events during AF. Indeed, circulating levels of endothelial MPs were related to infarct size and clinical outcome in patients with acute ischaemic stroke [21].

If the link between ageing and endothelial dysfunction appears robustly established, the relationship between ageing and the propensity to favor arterial thrombosis independently of other relevant risk factors remains controversial. In a recent investigation using organ chamber experiments, arterial thrombosis as induced by photochemical injury was unchanged in old mice and no changes of arterial TF expression or activity, platelet activation over ageing could be established [22]. By contrast, another report has demonstrated that H_20_2, a potent senescent inducer, promotes ageing related platelet hypereactivity and thrombosis in mice [23]. Other recent data have emphasized the view that alteration in atrial

hemodynamics caused by the AF rhythm disturbance may promote a prothrombotic endothelial pattern through enhanced TF expression [24]. However since those data were obtained using primary ECs of human aortic origin, extrapolation of those results to the behavior of the porcine atrial ECs should be taken with caution.

Atrial endothelial senescence is associated with a pro-adhesive pattern

Besides the progressive establishment of a pro-thrombotic profile, pro-adhesive and pro-inflammatory proteins are other characteristic features of senescent cells, summarized by the term “senescent associated secretory phenotype” that includes enhanced expression of VCAM-1 and ICAM-1 and secretion of various chemokines by vascular ECs [4]. The relevance of the inflammatory response was recently suggested by the colocalization between enhanced macrophages infiltration and thrombus burden within atrial tissues of AF patients [25] and by the enrichment in monocyte-platelet aggregates or CD11b expression in AF patients with proven thrombus formation [26]. Other recent insights came from studies showing that (i) the extent of periatrial epicardial fat, a marker of AF burden and outcome after AF ablation is associated with enhanced expression of ICAM-1 and vWF levels [27] or that (ii) inflammation of the epicardial fat predicts the occurrence of paroxysmal AF[28]. In AF, several reports have underlined a possible nexus between the pro-thrombotic state and inflammation. For instance, recent data from a large cohort obtained in patients with AF on anticoagulation, have demonstrated that biomarkers of inflammation were significantly associated with an increased risk of mortality [29].

The present findings indicate that atrial ECs senescence is associated with the up-regulation of various cytoadhesins including VCAM-1 and ICAM-1. Besides the induction of senescence, other agonists of VCAM-1 expression by endothelial cells are represented by Toll-like receptor 4 signaling that was demonstrated to promote atrial thrombogenesis [30]. Enhanced expression of intercellular adhesion molecule-1 (ICAM-1) on the endothelial cell surface critically regulates the binding of inflammatory cells that express CD11b/CD18

integrins (Mac1) and constitute a prerequisite for subsequent leukocyte extravasation. Ang II is also a potent stimulus for polynuclear neutrophils activation and CD11b/CD18 expression[31] that could contribute to enhanced transmigration of inflammatory cells cytoadhesins at the surface of senescent ECs. In line with this view, endothelial senescence was demonstrated to alter tight junction integrity thereby promoting inflammatory cells transendothelial migration [32]. A nice demonstration of the major role played by leukocyte infiltration in atrial remodeling and AF vulnerability came from the invalidation of CD11 gene which protected mice from AF vulnerability [31]. These data are consistent with the recent demonstration of a tight relationship between the enlargement of the left atrium and the occurrence of inflammatory cells infiltration in AF patients[33]. Altogether, these data suggest that the pro-adhesive pattern associated with premature atrial endothelial senescence may contribute to leukocyte infiltration and atrial remodeling.

Atrial endothelial senescence induces enhance MMP expression: relevance to fibrosis-associated senescence

Plethora of observational studies have point out the relationship between MMP and TIMPs (tissue inhibitors of matrix metalloproteinases) expression as measured in atrial tissues or in the peripheral blood and the occurrence of AF [34]. The human MMP belong to a class of pro-inflammatory and pro-angiogenic factors that control tissue remodeling through the tuning of extracellular matrix degradation and the release of TGF-β that triggers a pro-fibrotic pathway. Atrial fibrosis leads to atrial remodeling that contributes to AF persistence. In aging mice, recent studies have depicted an enhanced metalloproteinase expression concomitant with the development of diastolic dysfunction, as a surrogate marker of cardiac fibrosis[35] . Furthermore, the deletion of matrix metalloproteinase-9 attenuates the age-related decline in diastolic function in part by reducing TGF-ß pro-fibrotic signaling [36]. Recent data have also underscored that TGF-β1 increased p16 expression and senescence through the production of mitochondrial ROS and owing to a decreased expression of various anti-oxidants proteins

[37]. Other data have emphasized the relationship between endothelial senescence, as induced by Sirtuin depletion, and the development of a pro-fibrotic phenotype. In our work, the elevation of MMP release observed in atrial senescent cells is consistent with previous findings obtained in bovine aortic ECs [38]. Altogether, these data emphasize the view that atrial ECs senescence could contribute to cardiac tissue remodeling through MMPs-mediated extracellular matrix degradation.

Ang II/ AT1 receptor system regulates atrial endothelial senescence

Several reports have pointed out the paramount role of Ang II in atrial remodeling, its involvement in fibrosis, arrhythmogenic substrate development being extensively described. By contrast, its impact on atrial endothelial cell function remains uncharacterized. The fact that Ang II is a potent inducer of vascular endothelial senescence[12] prompts us to investigate the hypothesis that the local angiotensin system contributes AECs senescence. The present findings indicate that Ang II, at concentrations that could be reached in pathophysiological issues, promotes AECs premature senescence to the same extent that one obtained with H202, a well-described inducer of senescence and L-NAME, an eNOS inhibitor, pointing at eNOS dependence and NO availability in the control of senescence pathways in response to AngII. The importance of the Ang II/AT1R pathway in the induction of premature senescence was emphasized by the demonstration that both losartan, an AT1R inhibitor and perindolaprilat, an ACE inhibitor abolish the induction of premature senescence. Altogether, these findings highlight a pivotal role of the local angiotensin system in the induction of premature endothelial senescence via activation of AT1R.

Conclusion

These data provide compelling evidences indicating that atrial endothelial senescence promotes thrombogenicity, through enhanced tissue factor expression, blunted NO-mediated inhibition of platelet aggregation and procoagulant MPs release. Senescence also induces

inflammation, proteolysis, fibrosis and up-regulation of the local Ang II system. They further suggest that targeting Ang II/AT1R pathway may be a promising therapeutic strategy to delay atrial endothelial phenotypical alteration associated with ageing.

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Disclosures

None

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Figure legends

Figure 1: Replicative passaging of AECs induces a progressive increase in the number of senescent cells. (A)

Senescence was measured by SA-β-gal activity (A) and evidenced by the expression of p53, p21 and p16 from P1 to P4 by western blot (C). In control experiments, treatment with H_2O_2 and L-NAME induced AECs senescence in P1 AECs with increased SA-β-gal activity (B) and up-regulated p53, p21 and p16 (C). Lower panels cumulative data of densitometry analysis. Upper panels: representative blot of 3 experiments performed likewise with lower line showing β-tubulin labelling. Results expressed as mean ± SD.

Figure 2: A. Down-regulation of eNOs in senescent AECs examined after four replicative passages. Western blots from AEcs lysates were realized using young P1 and old P4 cells. **B.** P3 AECs and L-NAME treated P1 AECs significantly enhanced platelet aggregation in a dose dependent manner suggesting a blunted formation of endothelial NO.

Figure 3 : Senescence shifted the endothelial phenotype to a pro-coagulant status

Senescence shifted the endothelial phenotype to a pro-coagulant status in P4 AECs as evidenced by the enhancement in pro-coagulant MPs release and TF expression and in P4 AECs or L-NAME and H2O2-treated P1 AECs (lysates was analyzed by western blot). Lower panels cumulative data of densitometry analysis. Upper panels: representative blot of the 3 experiments performed likewise with lower line showing β-tubulin labelling. Results expressed as mean ± SD.

Figure 4 : Angiotensine II induces endothelial senescence in young P1 AECs.

Cells were treated with 10-7 or 10-5 M angiotensin II (respectively grey and black bars) during 24h, n=3. **A.** P4 AECs and P1 AECs exposed to either L-NAME or H_2O_2 had an

increased expression of ACE and AT1R compared with untreated P1 AECs. **B**. Ang II up-regulated p53, p21 and p16.

Figure 5: Angiotensine II induces increase in procoagulant TF factor expression in young P1 cells 24h after incubation.

Figure 6: Angiotensin II- induced AEc senescence is associated with the up-regulation of the local angiotensin system.

A. AT-1 R and ACE expression was measured by western blot in cell lysates from P1 AECs treated or not with 10-7 and 10-5 M Angiotensin II. Lower panels cumulative data of densitometry analysis. Upper panels: Representative blot of the 3 experiments performed likewise with lower line showing β-tubulin labelling. Results expressed as mean ± SD. **B.** SA-beta gal activity was measured in P4 cells in the presence of Losartan or perindoprilat.

Figure 7 : Enhanced expression of ICAM-1, V-CAM-1 and MMP-2 and MMP-9 in senescent P4 atrial endothelial cells

Protein expression in P4 AECs or L-NAME and H2O2-treated or untreated P1 AECs lysates was analyzed by western blot. Lower panels cumulative data of densitometry analysis. Upper panels: Representative blot of the 3 experiments performed likewise with lower line showing β-tubulin labelling. Results expressed as mean ± SD.

Figure 8: Ang II induced expression of ICAM-1, V-CAM-1, MMP-2 and MMP-9 and TGF-ß1 in P1 AEcs.

ICAM-1, V-CAM-1, MMP2 and MMP-9 and TGF-ß1 expression was measured by western blot in cell lysates from P1 AECs treated or not with 10-7 and 10-5 M Angiotensin II. Lower panels cumulative data of densitometry analysis. Upper panels: Representative blot of the 3 experiments performed likewise, with lower line showing β-tubulin labelling

Figure 1

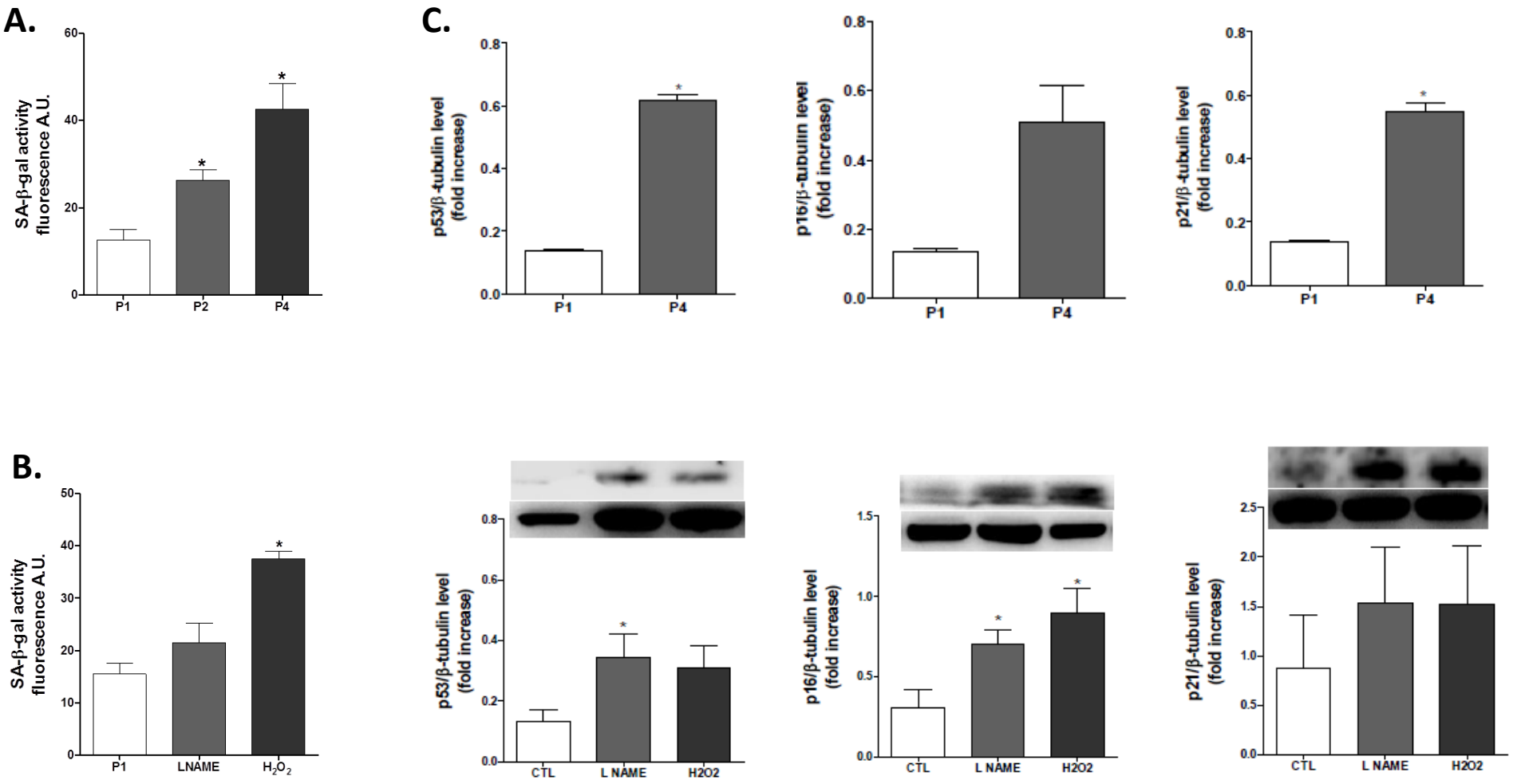
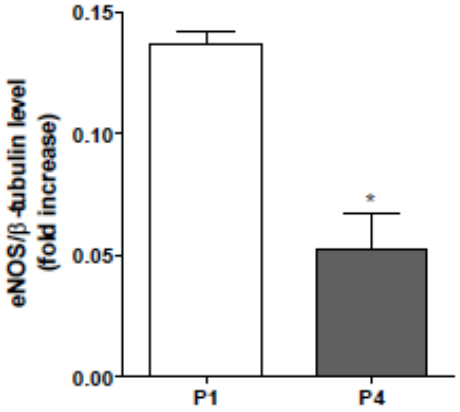

A.
SA-β-gal activity fluorescence A.U.
60
40
20
0
P1
P2
P4
C.
p53/β-tubulin level (fold increase)
p16/β-tubulin level (fold increase)
p21/β-tubulin level (fold increase)
0.8
0.6
0.4
0.2
0.0
P1
P4
B.
SA-β-gal activity fluorescence A.U.
50
40
30
20
10
0
P1
LNAME
H_2O_2
p53/β-tubulin level (fold increase)
p16/β-tubulin level (fold increase)
p21/β-tubulin level (fold increase)
1.5
1.0
0.5
0.0
2.5
2.0
CTL
L NAME
H2O2

Figure 2

A



B

Trace 1: 0 ECs
Trace 2: 10* 10^4 ECs
Trace 3: 15* 10^4 ECs
Trace 4: 20* 10^4 ECS

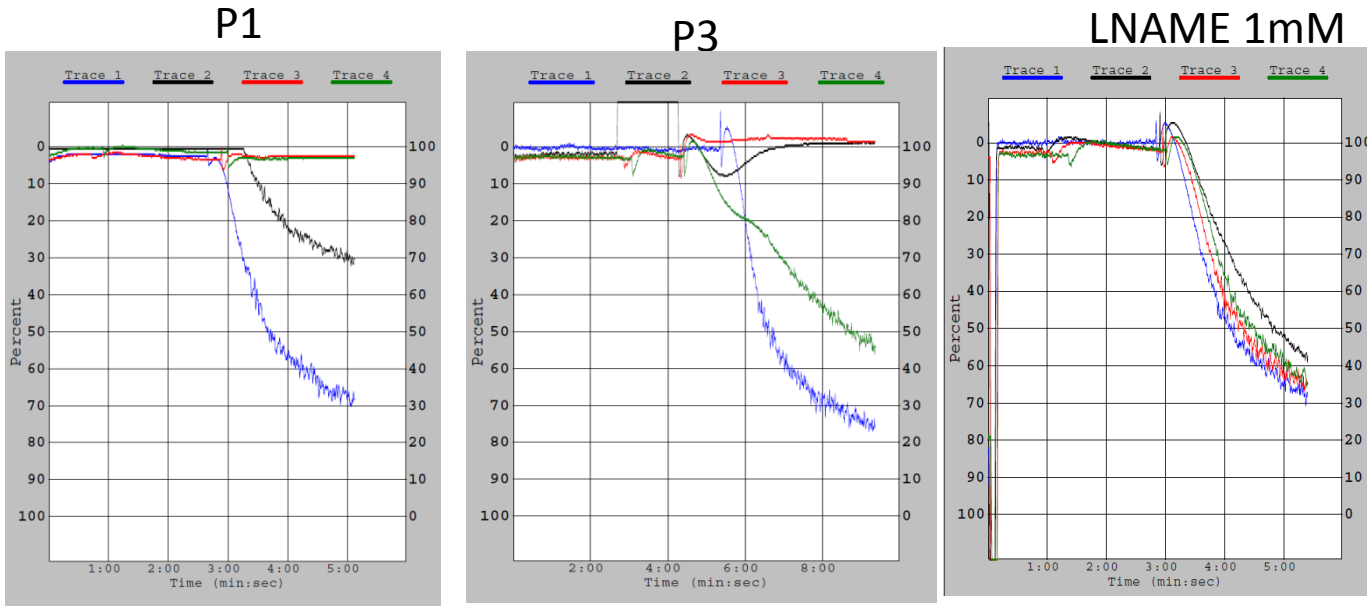
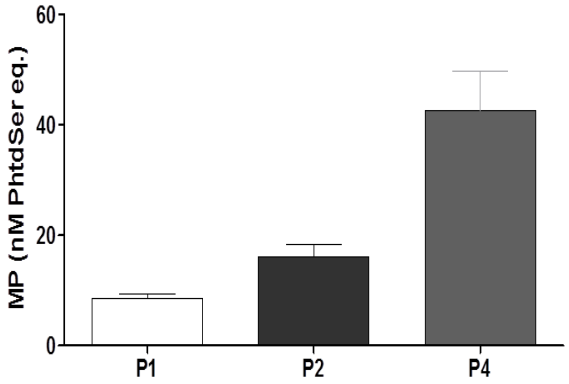
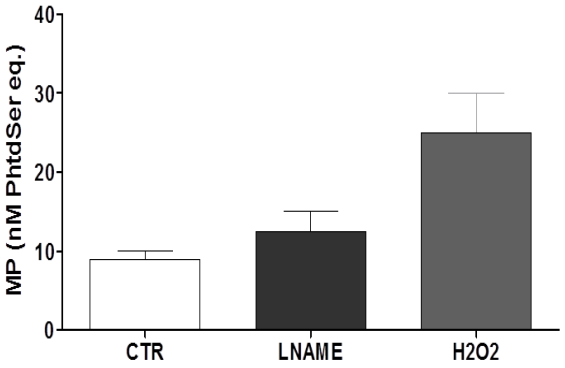
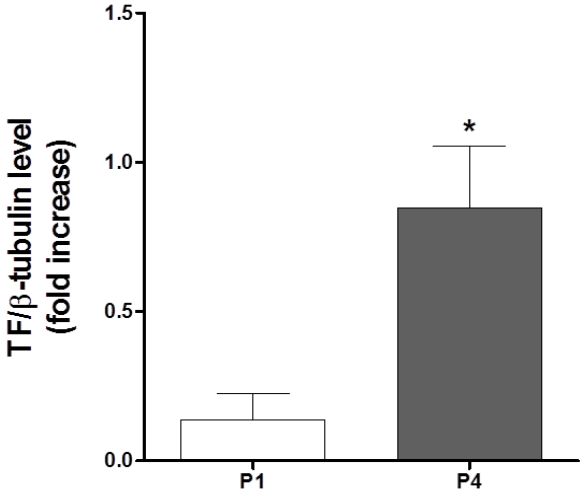


Figure 3


MP (nM PhtdSer eq.)
60
40
20
0
P1
P2
P4


MP (nM PhtdSer eq.)
40
30
20
10
0
CTR
LNAME
H2O2


TF/β-tubulin level
(fold increase)
1.5
1.0
0.5
0.0
*
P1
P4

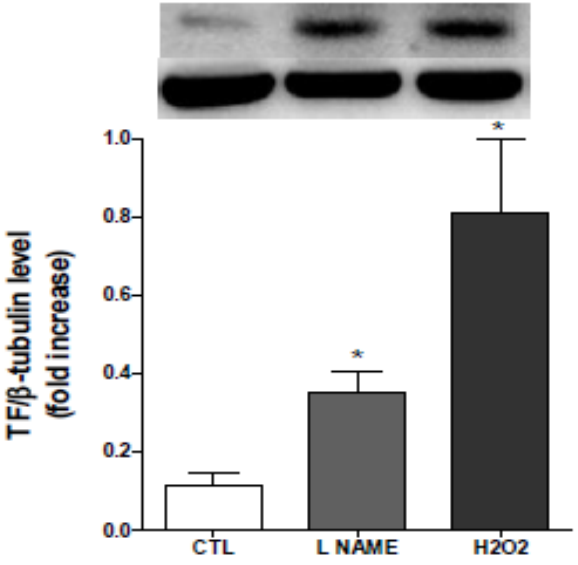

TF/β-tubulin level
(fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
CTL
L NAME
H2O2

Figure 4

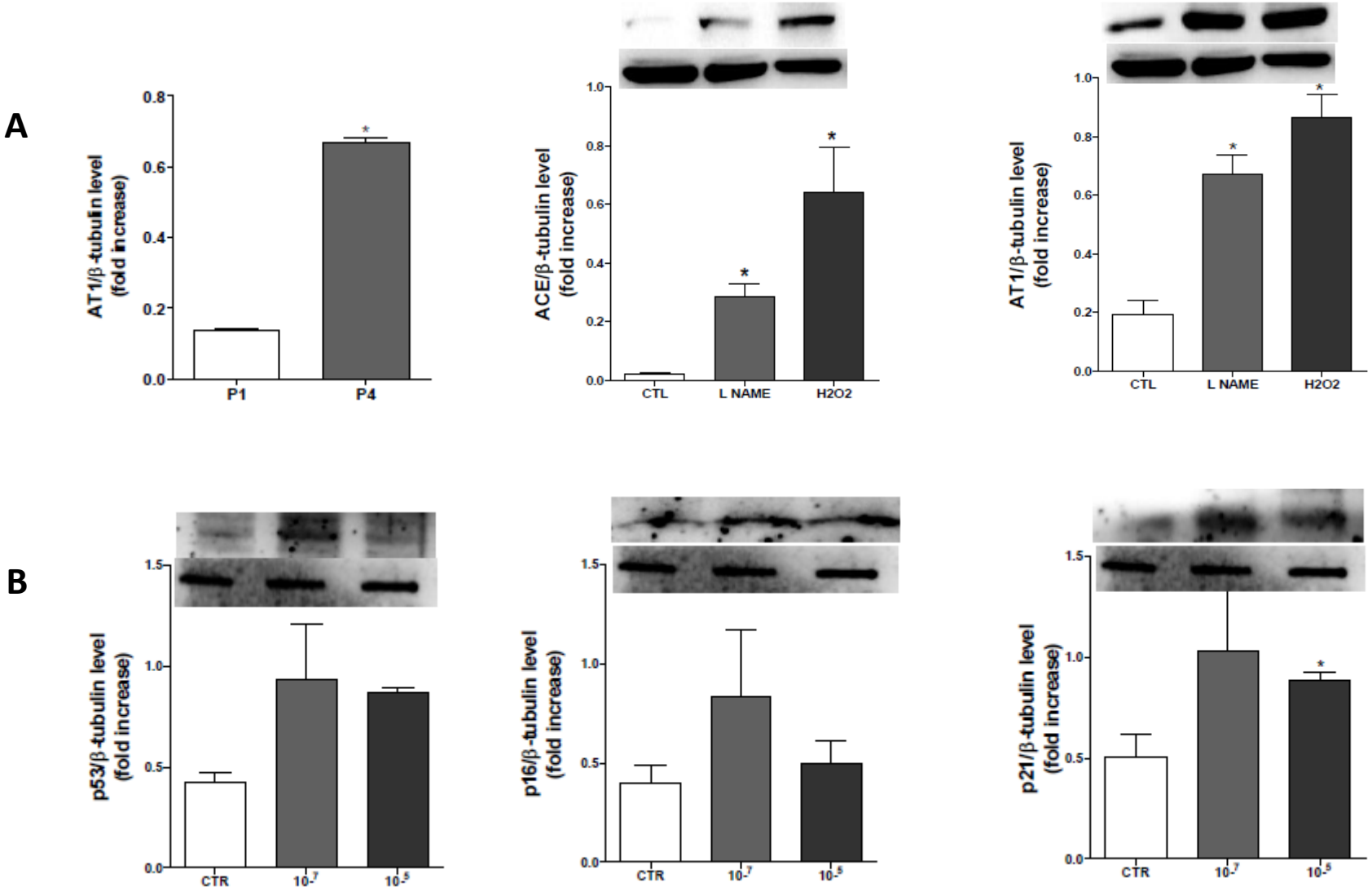

A
AT1/β-tubulin level (fold increase)
0.0
0.2
0.4
0.6
0.8
P1
P4
ACE/β-tubulin level (fold increase)
1.0
CTL
L NAME
H2O2
AT1/β-tubulin level (fold increase)
B
p53/β-tubulin level (fold increase)
1.5
CTR
10-7
10-5
p16/β-tubulin level (fold increase)
p21/β-tubulin level (fold increase)

Figure 5

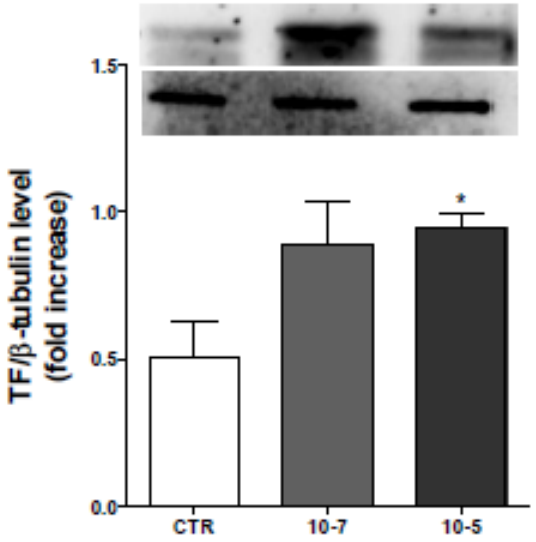


Figure 6

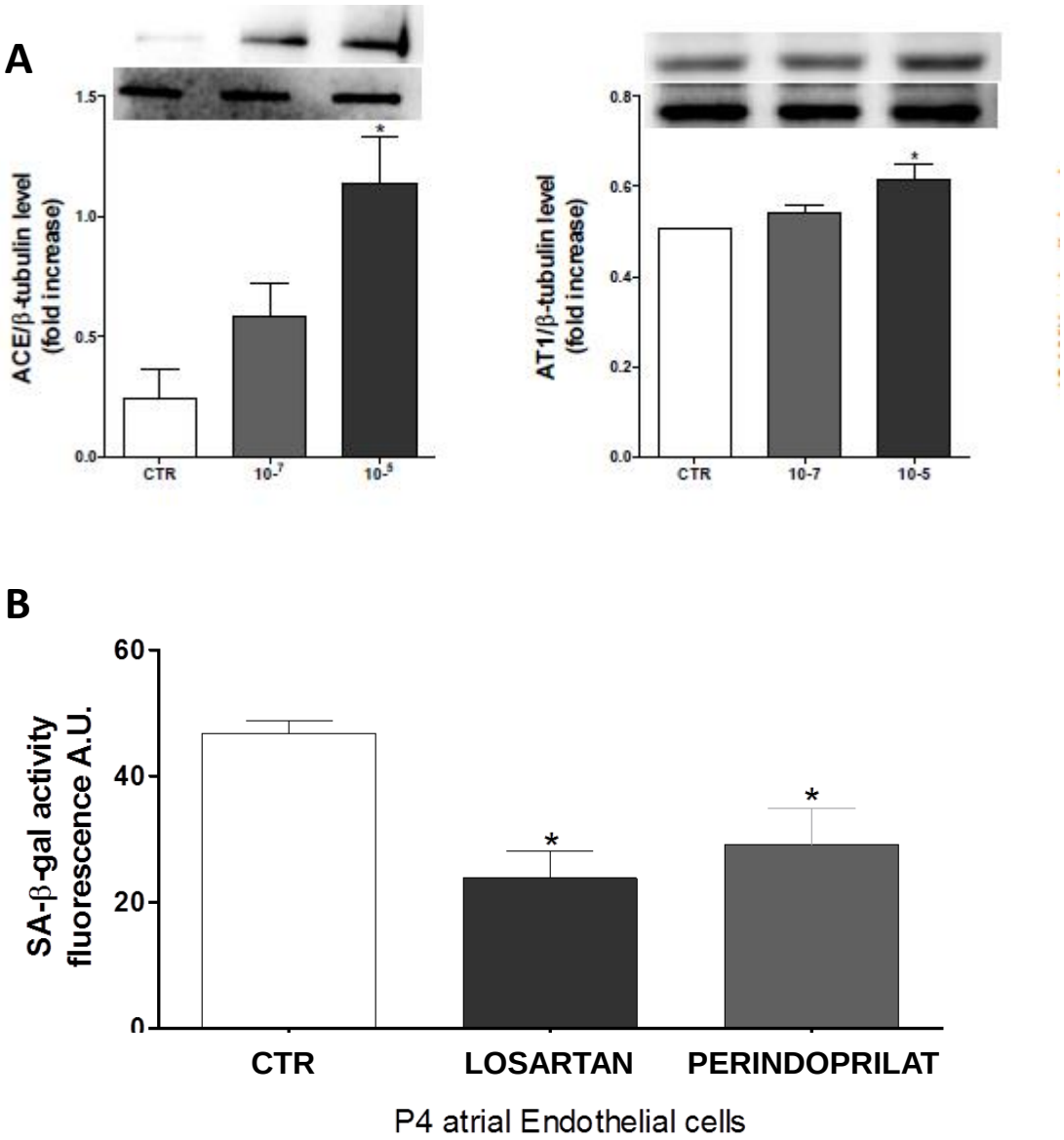

A
ACE/β-tubulin level (fold increase)
1.5
1.0
0.5
0.0
CTR
10-7
10-5
AT1/β-tubulin level (fold increase)
0.8
0.6
0.4
0.2
0.0
CTR
10-7
10-5
B
SA-β-gal activity fluorescence A.U.
60
40
20
0
CTR
LOSARTAN
PERINDOPRILAT
P4 atrial Endothelial cells

Figure 7

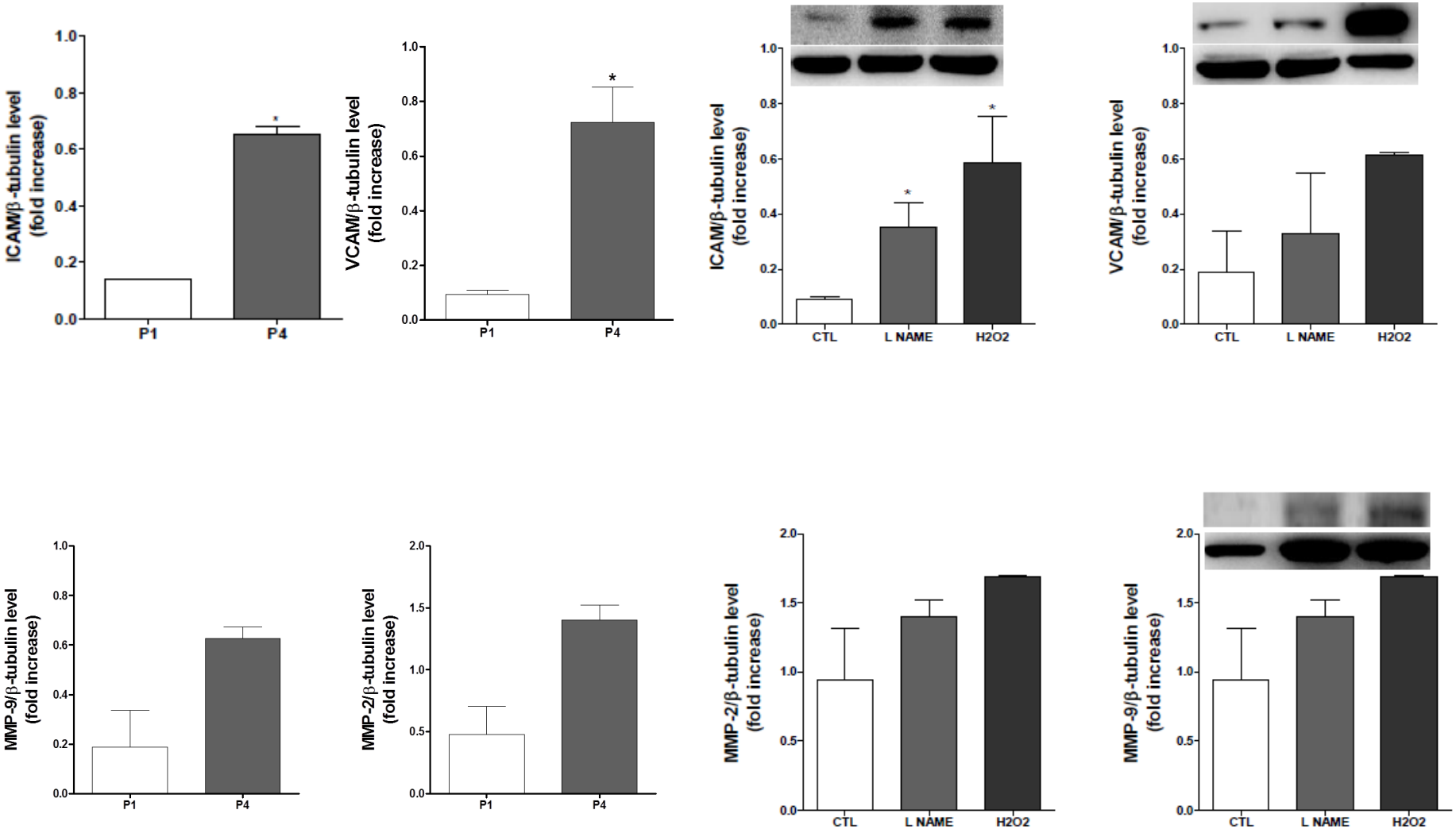
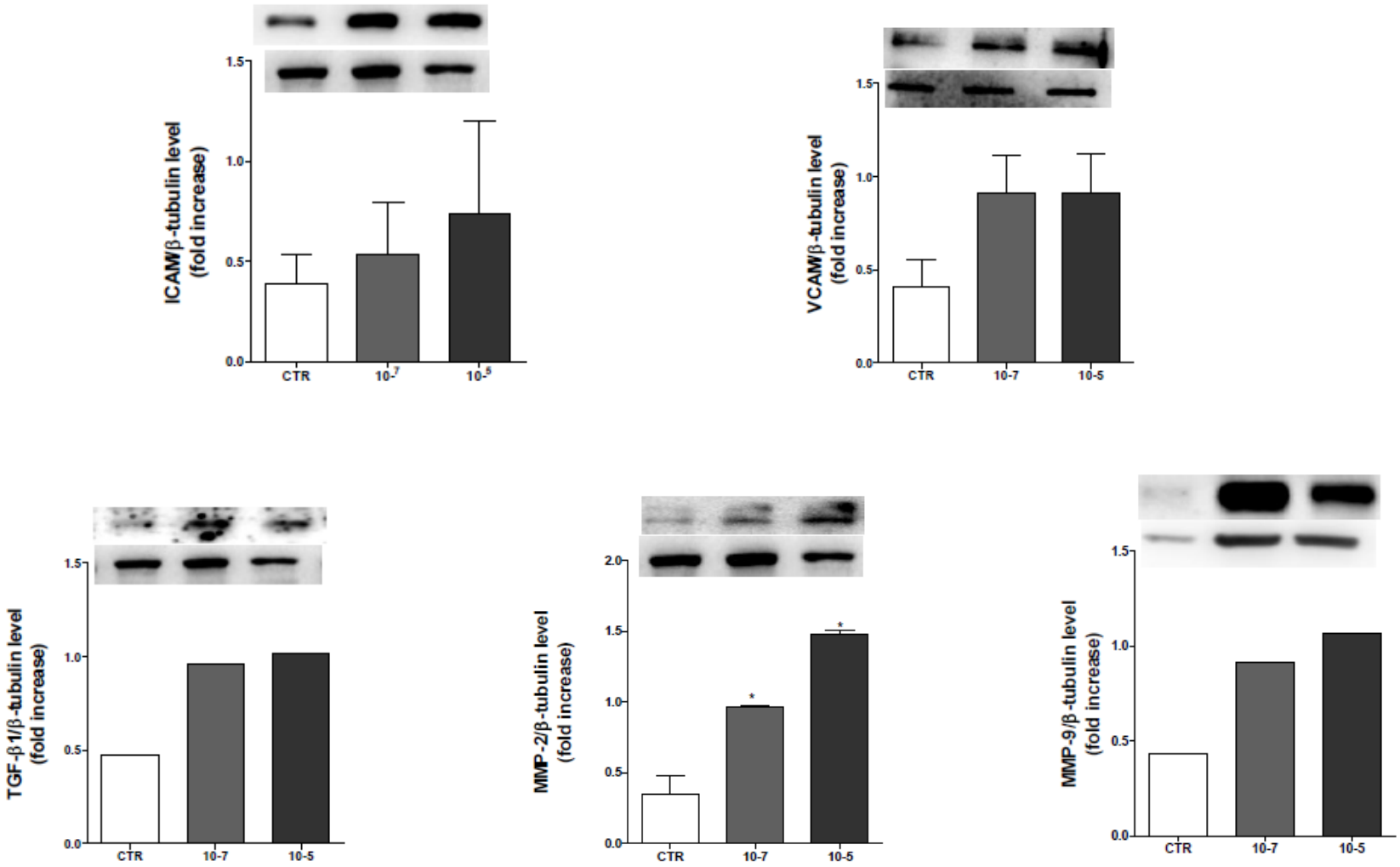

ICAM/β -tubulin level (fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
*
P1
P4
VCAM/β-tubulin level (fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
*
P1
P4
ICAM/β-tubulin level (fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
*
*
CTL
L NAME
H2O2
VCAM/β-tubulin level (fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
CTL
L NAME
H2O2
MMP-9/β-tubulin level (fold increase)
1.0
0.8
0.6
0.4
0.2
0.0
P1
P4
MMP-2/β-tubulin level (fold increase)
2.0
1.5
1.0
0.5
0.0
P1
P4
MMP-2/β-tubulin level (fold increase)
2.0
1.5
1.0
0.5
0.0
CTL
L NAME
H2O2
MMP-9/β-tubulin level (fold increase)
2.0
1.5
1.0
0.5
0.0
CTL
L NAME
H2O2

Figure 8


ICAM/β-tubulin level
(fold increase)
1.5
1.0
0.5
0.0
CTR
10-7
10-5
VCAM/β-tubulin level
(fold increase)
1.5
1.0
0.5
0.0
CTR
10-7
10-5
TGF-β1/β-tubulin level
(fold increase)
1.5
1.0
0.5
0.0
CTR
10-7
10-5
MMP-2/β-tubulin level
(fold increase)
2.0
1.5
1.0
0.5
0.0
*
*
CTR
10-7
10-5
MMP-9/β-tubulin level
(fold increase)
1.5
1.0
0.5
0.0
CTR
10-7
10-5

Remarque conclusive sur le travail (article 4)

Dans ce travail nous avons caractérisé les conditions méthodologiques strictes permettant de développer une sénescence réplicative à l'aide de cellules endothéliales de l'oreillette gauche de porc fraîchement isolées. Outre la confirmation des caractéristiques surexpressions des protéines p53, p21 et p16 dans les cellules sénescentes P4 et de l'activité SA-β galactosidase, nous avons détecté la progressive sur-expression du facteur tissulaire parallèlement à la sous-expression de la eNOS. La baisse de la biodisponibilité du NO a été confirmée par une inhibition de l'agrégation plaquettaire qui était réduite en présence des cellules sénescentes comparativement aux cellules jeunes. Il reste à vérifier que cette perte de NO s'accompagne d'un stress oxydant et à identifier les protéines oxydantes et par inhibition pharmacologique la source cytoplasmique ou mitochondriale de ROS.

La eNOS semble centrale dans le processus de sénescence des cellules auriculaires qui émettent de manière eNOS dépendante des MPs procoagulantes. De plus, l'Ang II induit une sénescence prématurée des cellules jeunes dans des proportions similaires à celle induite par privation de eNOS (L-NAME) ou par perte de la biodisponibilité du NO provoquée par un stress oxydant majeur induit par l'H2O2. Enfin, la sur-expression du récepteur AT1 et de l'enzyme de conversion de l'angiotensine (ACE) témoigne de l'activation locale de ce système au cours du processus de sénescence et ouvre des perspectives thérapeutiques. En effet, la sénescence induite par l'Ang II promeut un phénotype pro-thrombotique avec l'augmentation du FT et l'émission de MP et pro-adhésif avec l'expression de ICAM et VCAM-1. De plus, l'Ang II induit la surexpression du TGFß dans la cellule sénescente, une réponse connue dans le cardiomyocyte et le fibroblaste[65]. Enfin, les cellules sénescentes sur-expriment les MMP-2 et 9 susceptibles de fragmenter la MEC.

La caractérisation de ce modèle de sénescence endothéliale auriculaire réplicative ou induite est un outil indispensable pour l'exploration des effets des MPs circulantes au cours de la FA, de la modélisation des mécanismes sous-jacents et de leur éventuelle modulation pharmacologique.

V. Discussion générale

V. Discussion générale

Dans une première partie de nos travaux, nous avons utilisé les MPs comme marqueurs pro-coagulants et non invasifs d'un état d'activation cellulaire. Nos travaux ont mis en évidence la présence de concentration élevées de MPs susceptibles de contribuer à état pro-thrombotique et pro-inflammatoire chez les patients en FA. Cet état a été confirmé par la mesure de marqueurs indépendants de l'activation plaquettaire ou de marqueurs circulants de l'inflammation comme la CRP. Les MPs en tant que signature d'un état procoagulant, n'ont pas permis de mettre en évidence de différence de statut thrombotique entre les deux oreillettes au cours de la FA. Notre travail a également montré que l'ablation par radiofréquence utilisant un cathéter irrigué ouvert ou fermé augmente les concentrations de MPs non seulement *in situ* dans les heures qui suivent l'acte thérapeutique mais aussi 24 à 48 heures après, dans la circulation systémique.

Sur le versant tissulaire de l'exploration de la FA, nous avons remarqué l'importance de la sénescence dans l'altération tissulaire des oreillettes en FA. La senescence évolue parallèlement à la sévérité du remodelage tissulaire lui-même associé à la progression du trouble du rythme. Dans notre modèle de sénescence réplicative de la cellule endothéliale d'oreillette gauche de porc, la sénescence endothéliale est associée à une dysfonction cellulaire, à l'acquisition d'un phénotype pro-thrombotique (génération de microparticules pro-coagulantes, expression du FT), pro-inflammatoire (augmentation de l'expression des cytoadhésines ICAM-1 et VCAM-1), protéolytique (MMP-2 et 9), pro-fibrotique (TGFß1) et à l'activation du système local de l'angiotensine. Enfin, la possibilité de réguler pharmacologiquement la sénescence endothéliale auriculaire en ciblant les récepteurs AT1 de l'Angiotensine II a pu être établie *in vitro.*

1. Les microparticules dans la FA : témoin et acteur possible de l'activation cellulaire, le remodelage tissulaire, la thrombogénicité et la sénescence

1.1 MP marqueur de l'état pro-thrombotique dans la FA

La contribution des MPs pro-coagulantes à l'état pro-thrombotique dans la FA est très fortement suggérée par l'observation de Ederlhy et al[198] qui a mesuré des concentrations supérieures de MP pro-coagulantes circulantes chez les patients FA comparées à celles des patients en RS avec facteur de risque cardiovasculaire, elles-mêmes supérieures à celles de patients en RS sans facteur de risque cardiovasculaire. Les MPs circulantes étaient deux fois plus élevées chez les patients en FA permanente par rapport à des sujets à facteurs de risque équivalents. De plus, la présence de FA était statistiquement un facteur prédictif significatif d'augmentation des concentrations de MPs.

Nous avons recherché une éventuelle différence de statut prothrombotique entre l'oreillette droite et l'oreillette gauche en mesurant les MPs *in situ*. Willoughby[256] a en effet décrit une augmentation significative de l'expression de la P-sélectine et de l'agrégation plaquettaire induite par l'ADP dans l'oreillette gauche. Cependant, ces variations régionales de l'activation plaquettaire n'étaient pas confirmées dans une autre étude de la même équipe en 2013[268]. Nos résultats obtenus dans une population très homogène de patients en FA à faible risque vasculaire sont en faveur d'une absence de différence de statut pro-thrombotique entre les deux oreillettes, du moins dans cette population.

Les MPs participent probablement également à l'état pro-coagulant lié aux facteurs de risque cardiovasculaire préexistant à la survenue de la FA. En effet, dans la cohorte Framingham, les MP circulantes d'origine endothéliale ont été corrélées à la présence de facteur de risque cardiovasculaire en particulier la dyslipidémie, ce qui souligne l'impact de ces facteurs de risque sur l'intégrité de la fonction endothéliale. [269] En effet, dans la FA, les taux plasmatiques MP plaquettaires et de P-sélectine soluble sont supérieurs à ceux de volontaires sains en RS[270], suggérant une corrélation entre activation plaquettaire et MP circulantes. Cependant, ces variations de concentrations de MPs sont une possible conséquence des comorbidités sous-jacentes plutôt que de la FA elle-même car elles disparaissent lorsque l'on apparie les patients avec les facteurs de risque.

Pour établir un rôle spécifique des MPs dans la FA il faudrait établir un modèle d'évaluation fonctionnelle des propriétés des MPs circulantes. Nous avons donc développé un modèle de sénescence réplicative ou prématurée qui permet la mise en évidence de différents marqueurs de la dysfonction et de la sénescence endothéliale (cf article 4). Ce modèle validé par notre travail permettra l'étude des propriétés pro-sénescentes, inflammatoires ou thrombotiques de MPs isolées de patients en FA grâce à la mesure de leurs effets cellulaires.

1.2 Les microparticules : caractéristiques et cinétique dans les procédures d'ablation

Un travail préalable dans l'ablation de flutter [271] nous avait permis de mettre évidence que l'ablation par radiofréquence dans l'OD induisait un état pro-thrombotique avec une augmentation des MP principalement plaquettaires et leucocytaires associée à celle du vWF Ag, de PAI-1, témoins d'une altération du phénotype secrétoire endothélial et des D-dimères signant l'activation de la coagulation malgré une anticoagulation optimale. [271] Lors des ablations de FA dans l'OG, l'état pro-thrombotique exacerbé expliquerait en partie les complications emboliques de ces procédures qu'il s'agisse d'AVC clinique ou de lésions cérébrales silencieuses[272]. Gaita[273] a montré que la technologie utilisée pour l'ablation de FA pouvait avoir une incidence sur les lésions cérébrales thromboemboliques silencieuses. L'équipe de T.Arentz a observé que ces lésions cérébrales étaient similaires en radiofréquence (cathéter irrigué ouvert) et en cryothérapie mais majorées avec le système PVAC[274]. Nous avons montré que l'ablation par radiofréquence (cathéter irrigué ouvert) et la cryothérapie dans

l'OG généraient des MP avec des cinétiques d'émission similaires, et s'accompagnait de degrés d'activation plaquettaire et inflammatoire équivalents. Cette observation allait à l'encontre d'un meilleur profil de sécurité apporté par la cryothérapie[275]. Nous avons établi la cinétique des MP après ablation de FA par 2 cathéters de radiofréquence irrigués, l'un ouvert, l'autre fermé, et établi une augmentation précoce des concentrations de MP totales et plaquettaires (2h) et plus retardée des MP leucocytaires. Cette augmentation, associée à une activation plaquettaire et à un statut inflammatoire sans modification notable des MP endothéliales était similaire pour les deux types de cathéters.
A nouveau, les dommages tissulaires et l'activation cellulaire similaires allaient à l'encontre de l'idée que les cathéters irrigués fermés entraîneraient plus de complications thromboemboliques. Bien entendu, même si dans notre étude, aucun évènement thromboembolique n'a été observé dans aucun des groupes, une étude de l'ischémie cérébrale silencieuse en IRM aurait un grand intérêt pour confirmer ces données et leur intérêt pour le décryptage des mécanismes précoces des conséquences thromboemboliques de l'ablation.

Le développement croissant de l'ablation de FA, en particulier persistante, comportant la nécessité de lésions thérapeutiques plus extensives dans l'OG crée le besoin de nouveaux outils d'ablation plus efficaces, autorisant des lésions plus étendues réalisées en des temps d'ablation plus courts. Nos données pourraient être intéressantes pour la conception de nouveaux cathéters. D'une manière similaire à la démonstration de concentrations de MPs circulantes reflétant le degré de sévérité de la pathologie[258], il serait intéressant de vérifier si notre technique de dosage est suffisamment sensible pour confirmer que la quantité de MPs produites est proportionnelle à l'étendue de la lésion d'ablation et/ou que les caractéristiques de ces MPs reflètent le degré de la réponse inflammatoire dans l'oreillette. La mesure des MP serait alors un outil d'évaluation de nouveaux cathéters. Enfin, la caractérisation des cinétiques d'apparition des différentes populations (plaquettaires ou leucocytaires) et de leur contenu en protéines actives (caspases, MMPs) favoriserait notre compréhension de la séquence cellulaire du remodelage tissulaire post-ablation. (cf ci-dessous)

1.3 Place des MP en tant qu'effecteur endothélial putatif dans la FA

Dans les modèles de FA, l'induction par stimulation rapide de l'OG active les plaquettes en quelques minutes, augmente l'expression de P-sélectine à la surface plaquettaire et des MPs favorisant le recrutement monocytaire[155]. Les interactions avec l'endothélium sont aussi renforcées par les concentrations élevées en vWF associées à la FA [201]. De plus, les MPs contribuent au couplage inflammation thrombose : les MPs plaquettaires grâce à un mécanisme de "rolling" sont capables de déposer RANTES et de favoriser ainsi la diapédèse de l'endothélium inflammé[202]. Il est aussi établi que les MP leucocytaires portent une forte activité FT[181] qui contribue à l'incrémentation du thrombus.
Dans le contexte spécifique d'une inflammation soutenue post-procédure d'ablation, attestée dans notre travail par la persistance d'une concentration plasmatique en hs-CRP croissante 48h post-ablation, la question de l'impact des MPs d'origine endothéliale sur l'état de la cellule endothéliale

reste posée. En effet, *in vitro* la CRP induit une perte de NO endothéliale[196] favorisant l'émission des MPs sénescentes (cf article 4). De plus, sous l'action de la CRP injectée au rat, les concentrations plasmatiques en MPs endothéliales augmentent parallèlement au détachement de cellules endothéliales qui deviennent circulantes[196]. Toutefois, ces derniers travaux n'ayant pas exploré le contenu en protéines et molécules actives des MPs isolées conjointement aux exosomes dans les surnageants cellulaires, l'hypothèse d'une MP endothéliale circulante pourvoyeuse d'un signal pro-inflammatoire CRP-dépendant reste irrésolue. Plus certainement, il a été démontré que les MP endothéliales stimulent la sécrétion de cytokines pro-inflammatoires par l'endothélium inflammé comme l'IL-6 et MCP-1 et contribuent à un effet amplificateur autocrine[276] .

L'absence de variation des concentrations de MPs endothéliales observée post-ablation dans notre étude laisse ouverte l'hypothèse d'une recapture locale et rapide de ces MPs par les cellules avoisinantes ou le tissu sous-jacent. En effet, les cellules endothéliales sont des phagocytes occasionnels et le recrutement des monocytes/ macrophages est une des composantes de la cicatrisation du tissu.

Si la réaction inflammatoire post-ablation est liée à la constitution de la lésion d'ablation, elle est source de récidive précoce de FA après la procédure. Des thérapeutiques à visée anti-inflammatoire comme la colchicine ont d'ailleurs montré un effet protecteur contre la survenue de récidives précoces de FA après isolation des VP et semblent impliquer la CRP et l'IL-6[277]. Nous avons des travaux en cours visant à évaluer l'effet modulateur potentiel des corticoïdes sur les taux de MP en post-ablation de FA.

1.4 Microparticules et couplage entre dysfonction endothéliale, inflammation, thrombogénicité, et remodelage tissulaire au cours de la FA

Les MPs apparaissent comme des éléments du couplage inflammation thrombose dans la FA, et sont également impliquées dans l'induction du stress oxydant et du remodelage, autres facteurs favorisant l'initiation et la pérennisation de la FA. En effet, les données de notre laboratoire et ceux de Burger[208] obtenues *in vitro* montrent que les cellules endothéliales sénescentes aortiques ou coronaires émettent de manière redox sensible des MP endothéliales pro-sénescentes qui se comportent comme des effecteurs pro-inflammatoires.
Dans la circulation sanguine, la contribution des MP leucocytaires dans le processus de sénescence n'est pas exclue, notamment dans les conditions de forte stimulation inflammatoire. Ces MPs sont aussi capables d'altérer la fonction endothéliale[209] via une régulation négative de la eNOS et l'activation de NF-KB et des Cox dans les tissus myocardiques et vasculaires. (cf paragraphe ci - dessous)[278].

1.4.1 Microparticules et remodelage tissulaire

Les MMP de MPs endothéliales sénescentes sont donc des acteurs probables de la protéolyse de la MEC et du remodelage de l'OG au cours de la FA. En effet, la capacités des MPs endothéliales à convoyer des protéines conservant leur activité protéolytique a été mise en évidence depuis plusieurs années[279]. En réponse aux facteurs pro-angiogéniques, au FGF (fibroblast growth factor-2) ou au VEGF, les MPs endothéliales sont rapidement émises (4h) et sont enrichies en MMP actives leurs conférant des capacités d'effecteur autocrine dans des matrigels tridimensionnels[280]. De même, la thrombine et le CD40L, acteurs de la réponse inflammatoire et pro-coagulante favorisent de manière synergique la transcription de la MMP-10 endothéliale et l'émission de MPs enrichies en MMP-10 et CD40L, susceptibles de contribuer à un nouveau couplage entre l'inflammation, la thrombose et le remodelage tissulaire[213].

Au cours de la FA, les MMPs participent à la fragmentation protéolytique de la MEC qui favorise la fibrose. Dans une étude *in vitro* des effets d'une suspension de MP et d'exosomes issus de cellules endothéliales de la microcirculation, Lozito a montré que les MPs portant l'intégrine αV se lient aux protéines constitutives de la MEC comme la fibronectine, et y focalisent une activité MMP-2 importante lorsqu'elles sont émises après un stress cytokinique ou en condition d'hypoxie. De plus, les MMPs sont protégées de l'action de leurs inhibiteurs endogènes TIMP1 et TIMP-2 lorsqu'elles sont convoyées par les MPs[214]. De manière similaire, Taraboletti[280] avait décrit que les cellules endothéliales macrovasculaires émettaient après 4 h de stimulation par des facteurs pro-angiogéniques des MPs convoyant des MMPs actives à leur surface, les inhibiteurs TIMP-1 et TIMP-2 et une intégrine β1.

Ces données soulignent le rôle d'effecteur local des MP dans le remodelage tissulaire et suggèrent fortement des interactions précoces et rapides entre les MPs émises par les cellules endothéliales atriales sénescentes et le tissu atrial, susceptibles de concourir au remodelage tissulaire au cours de la FA.

1.4.2 Microparticules catalyseurs de la production de thrombine et modulateurs des effets PAR-1 dépendants

Les MPs en tant que surface catalytique des réactions de la coagulation favorisent la génération accrue de thrombine au site de leur adhésion endothéliale. Outre l'action pro-coagulante directe de la thrombine qui active rapidement les plaquettes adhérentes au collagène et polymérise le fibrinogène en fibrine, l'enzyme terminale de la cascade de la coagulation est également susceptible d'activer des voies inflammatoires PAR-1 dépendantes dans les cellules endothéliales favorisant un effet pro-fibrotique. En effet, selon la voie de signalisation mobilisée en aval par les protéines G trimériques, la

thrombine induit la sur-expression PAR1-dépendante de cytokines pro-inflammatoires (IL-1, IL-6 and TNF-α) et de molécules d'adhésion E-selectin, P-selectin, ICAM-1, VCAM-1). La thrombine active aussi,la cascade des caspases et augmente la perméabilité membranaire par une activation PAR1-dépendante de NF-KB, le facteur de transcription pro-inflammatoire[281].

Dans la FA, Chang et al[282] ont démontré un effet endothélial pléiotrope de la thrombine qui diminue de manière PAR1-dépendante les battements spontanés des veines pulmonaires (VP) de lapin et augmente la tension diastolique de l'OG et le potentiel de repos membranaire avec une diminution de la durée du PA. Un inhibiteur de PAR-1 limitait les réponses de l'endothélium à la thrombine (VP et oreillette) et le Dabigatran protégeait les VP par son action inhibitrice de l'enzyme. L'ensemble souligne la dualité de l'action de la thrombine sur le remodelage électrique et structurel au cours de la FA .

Récemment Spronk et al.[222] ont émis l'hypothèse et démontré que l'hypercoagulabilité observée au cours de la FA, et en particulier la concentration anormalement élevée de thrombine, avait des actions sur d'autres cellules du tissus atrial. *In vitro* la thrombine induit la secrétion de MCP-1 pro-inflammatoire et du TGFß pro-fibrotique par le fibroblaste atrial. De plus, un traitement anticoagulant ciblant la thrombine ou le facteur Xa protégeait des complications emboliques la souris et la chèvre en FA et inhibait aussi le développement du "substrat" de la FA.

De façon surprenante, la MMP-1 est capable d'activer le récepteur PAR-1 d'une manière similaire à la thrombine. La MMP-1 active les voies calcium-dépendantes canoniques et thrombogéniques de PAR-1 initiées par la thrombine qui se traduisent également par une augmentation de la perméabilité membranaire. L'élastase neutrophilique, une autre protéase susceptible d'être déversée dans l'environnement de la lésion auriculaire après le recrutement leucocytaire, clive un site activateur de PAR-1 différent et réoriente le récepteur vers une signalisation intracellulaire non canonique en activant des voies pro-inflammatoires dépendantes des MAP-kinases, mais qui ne modifient pas la perméabilité de la barrière membranaire[283].

Ces données soulignent le couplage entre l'état pro-coagulant, l'inflammation et le remodelage électrique et structurel au cours de la FA et l'intérêt de traitements ciblant précocément ces différentes réponses. La littérature récente indique une possible modulation pharmacologique de la réponse endothéliale utilisant les ligands dits biaisés du récepteur PAR-1. Ces peptides modifiés miment l'action des néoligands de PAR-1 spécifiques de chaque clivage activateur. Ainsi certains ligands biaisés du récepteur en protégeant spécifiquement la cellule de la réponse inflammatoire induite par les protéases du "substrat" de la FA (MMPs, élastase neutrophilique), apporteraient également l'avantage d'une diminution de la proportion de récepteurs PAR1 disponibles pour la stimulation par la thrombine et s'opposeraient à ses conséquences thrombogéniques et à l'induction d'une perméabilité membranaire thrombine-dépendante favorable à la diapédèse et à l'amplification d'une remodelage

tissulaire inapproprié[284].

2. Sénescence et FA

De nombreuses études suggèrent que la survenue de FA est étroitement liée à l'âge bien que les mécanismes cellulaires et moléculaires impliqués soient inconnus. La sénescence prématurée est définie comme un arrêt irréversible du cycle cellulaire déclenché par différents stress cellulaires. L'induction de sénescence à court terme a un rôle bénéfique dans la suppression des tumeurs, la cicatrisation des blessures. A long terme, la présence de cellules sénescentes dans les tissus favorise le développement de pathologies et la tumorigénèse. Ces cellules sénescentes, métaboliquement actives mais non prolifératives empêchent par là même la régénération tissulaire et favorisent la dysfonction d'organe car leur activité métabolique est dysfonctionnelle. Si le rôle de la sénescence dans certaines pathologies d'organes comme le foie ou le pancréas a pu être impliqué, dans la FA, il n'existe aucune donnée.
Nous avons recherché les marqueurs de sénescence dans les auricules dans un groupe de patients en FA et développé un modèle cellulaire de sénescence de la cellule atriale pour l'étude des mécanismes sous-jacents de la sénescence propres à ces cellules.

2.1 Mise en évidence de la sénescence dans la FA

L'analyse des modifications d'expression génique dans du tissu atrial humain de patients en RS et en FA par Kim NH[255] a identifié une régulation positive de gènes pro-apoptotiques , du gène de la p21, un inhibiteur de kinase cycline-dépendant, régulateur de la progression du cycle cellulaire activé par la p53. En accord avec ces données, nous avons mis en évidence une sur-expression protéique de p53 et de son effecteur p16 dans des auricules de patients en FA, comparativement à ceux des patients en RS. Alors qu'aucun lien entre l'âge, les facteurs de risque cardiovasculaire, le score CHADS2VASC2 n'est statistiquement confirmé, nos données montrent une association significative entre FA et sénescence. La sénescence semble s'établir concomitamment à la progression du trouble du rythme puisque le degré de sénescence augmente du stade paroxystique au stade permanent. (cf paragraphe ci-dessous). Cependant, il reste à établir si les mécanismes de sénescence dépendant de p53 sont délétères ou au contraire cytoprotecteurs dans la FA. En effet, il faut noter que les marqueurs de sénescence ont été identifiés dans les lysats du tissu atrial, et ne sont donc pas restreints à la seule sénescence endothéliale et mettent en jeu un environnement cellulaire et tissulaire complexe. (cf paragraphe ci-dessous)

2.2 Sénescence, inflammation et remodelage

Plusieurs études montrent l'implication de la sénescence dans des pathologies du système cardiovasculaire. Très récemment, pour explorer les conséquences de l'accumulation de cellules sénescentes dans les organes, Baker DJ[285] a mis au point un transgène pour induire l'apoptose de cellules exprimant p16 chez la souris. La clairance de ces cellules entraînait un retard de tumorigénèse et atténuait la détérioration liée à l'âge de plusieurs organes comme le rein, le cœur et la graisse. En 2007, Sano et al[286] montrait que les propriétés anti-angiogéniques de p53 pouvaient avoir un rôle fondamental dans la transition de l'hypertrophie cardiaque à l'insuffisance cardiaque. Plus récemment, des travaux ont souligné dans la cellule vasculaire sénescente des changements de profil sécrétoire avec une augmentation de facteurs pro-inflammatoire favorisant le remodelage tissulaire[287] avec une sur-expression d'IL-1α[250], des molécules d'adhésion comme ICAM-1 ou de l'inhibiteur-1 de l'activateur du plasminogène (PAI-1)[251].

Yoshida Y[245] a mis en évidence l'implication de p53 dans l'inflammation au cours de l'insuffisance cardiaque ayant comme conséquence une altération de la fraction d'éjection du VG. La sur-expression de p53 dans la cellule endothéliale cardiaque du myocarde en insuffisance cardiaque favorisait l'expression d'intégrines telles ICAM-1 susceptibles de recruter les leucocytes. Dans notre modèle de cellules endothéliales d'oreillette gauche de porc l'induction de la sénescence s'accompagne également d'un phénotype pro-adhésif avec augmentation de l'expression d'ICAM et VCAM. De plus, nous avons identifié un profil secrétoire pro-remodelage avec une expression accrue de MMP-2 et MMP-9 et pro-fibrotique avec l'augmentation d'expression de TGFß1, susceptibles de favoriser la dégradation de la matrice extracellulaire comme déjà observé dans les fibroblastes sénescents [288, 289].

Les données de notre étude sont établies à partir de lysats du tissu atrial, et ne sont donc pas restreintes à la sénescence endothéliale. D'autres cellules, comme les fibroblastes, sont susceptibles d'entrer en sénescence au cours du vieillissement de l'oreillette et de contribuer par leur action sur la MEC à la sénescence endothéliale. Wu[290] a objectivé dans des cellules multipotentes mésenchymales pouvant se différentier en plusieurs types cellulaires dont les cardiomyocytes, que le TGF-ß1 pouvait induire la sénescence et la production de ROS d'origine mitochondriale.

Il est actuellement difficile d'établir si la sénescence est cause ou conséquence de cet état pro-fibrotique et pro-oxydant coexistant dans la FA à un instant donné. Dans les auricules de patients en FA, nous montrons avec la progression de la FA une augmentation du degré de sénescence qui est associée à une sur-expression des MMP-9. Ces données suggèrent un rôle potentiel de la sénescence dans le remodelage tissulaire induite par la dégradation protéolytique de la MEC au cours de la FA.

2.3 Sénescence, dysfonction endothéliale et phénotype pro-thrombotique

La survenue de FA augmente avec l'âge et le risque de complication thromboembolique également. Le risque d'AVC est évalué classiquement par le score de CHA2DS2VASC et plus récemment un nouveau score de risque ABC (age, biomarkers, clinical history)[291] est proposé. Quel que soit le score, l'âge est un élément majeur du calcul du risque embolique. A ce jour, la contribution des mécanismes de sénescence au sur-risque thrombotique associée à la FA reste parfaitement méconnue.

2.3.1. Stress oxydant et sénescence

Plusieurs études ont souligné que la sénescence endothéliale était caractérisée par un stress oxydant majeur avec une régulation négative de l'expression de la eNOS et de la biodisponibilité du NO. La relation entre stress oxydant et sénescence est illustrée par le fait que les ROS peuvent induire un raccourcissement des télomères, et participer à la baisse du NO[246]. Dans notre modèle cellulaire, les cellules endothéliales atriales sénescentes montraient effectivement au stade P4 une expression de eNOS diminuée par rapport aux cellules jeunes P1. Dans notre étude comparant auricules de patients en RS et en FA, la corrélation inverse entre eNOS et p16 témoigne d'un lien entre sénescence, dysfonction endothéliale et stress oxydant. En clinique,il a été montré que la FA altère la fonction vasoprotéctrice NO-dépendante de l'endothélium par mesure de la FMD (flow-mediated dilation) et que celle ci est améliorée par l'isolation des veines pulmonaires [292].A côté de son rôle majeur dans le contrôle du tonus vasculaire et la survie de la cellule endothéliale, le NO joue un rôle protecteur en limitant l'agrégation plaquettaire à la surface de la cellule endothéliale, protection qui est largement diminuée dans la cellule sénescente.(cf article 4) En effet, les cellules atriales induites en sénescence réplicative ont une expression de la eNOS réduite favorisant la perte de biodisponibilité du NO. Dans notre modèle, le rôle éventuel du stress oxydant dans la perte de NO endothélial au cours de la sénescence reste à confirmer par l'analyse des concentrations et sources de ROS.

2.3.2. Facteur tissulaire et sénescence

Un autre acteur majeur associé à la sénescence endothéliale est le facteur tissulaire. Dans les auricules de patients en FA, nous avons pu montrer que la progression de la FA s'accompagnait d'un degré de sénescence important, d'une dysfonction endothéliale probable mais aussi du statut pro-thrombotique avec une augmentation de l'expression de FT, initiateur de la coagulation. La corrélation entre l'expression de p53 et le FT signe un lien entre sénescence et état pro-thrombotique. En accord avec cette hypothèse, Simmers[293] a récemment, montré que les perturbations de flux typiques de la FA favorisent l'expression de FT dans les cellules endothéliales. Dans notre modèle, les cellules au stade P4 présentent une augmentation de l'expression de FT et de l'émission de MP pro-coagulantes suggérant un phénotype pro-coagulant de la cellule endothéliale atriale sénescente.

Les cellules sénescentes contribuent également indirectement à l'état pro-thrombotique par leur capacité réduite à inhiber efficacement l'agrégation plaquettaire.
L'ensemble de ces données suggère fortement une contribution de la sénescence endothéliale atriale dans la dysfonction endothéliale et l'état pro-thrombotique associés à la FA.

2.4 Rôle des MP dans la sénescence

Dans un modèle de cellule endothéliale aortique de souris, Burger et al[249] a mis en évidence que l'émission de MP par la cellule endothéliale sénescente était augmentée. Nous avons établi qu'elles sont aussi un indicateur du degré de sénescence dans le modèle de sénescence endothéliale atriale et des données du laboratoire montrent un comportement similaire des cellules endothéliales de coronaires de porc en sénescence (Thèse de Malak Abbas).
Les MPs émises par les cellules endothéliales sénescentes ont aussi une action autocrine pro-sénescente. Les MP sont un inducteur redox-dépendant de sénescence dans les cellules endothéliales aortiques de souris et de coronaire de porc. Les données du laboratoire indiquent que les MP issues de cellules endothéliales de coronaires de porc sénescentes (P4) et les MP endothéliales de patients présentant un syndrome coronarien aigu induisent la sénescence par des mécanismes redox-sensibles impliquant le système local de l'angiotensine.

Cet effet autocrine et progressif suggère un rôle possible des MP dans la propagation de la dysfonction endothéliale et du statut pro-thrombotique dans le tissu atrial.

2.5 La cellule endothéliale au cœur des processus pathologiques impliqués dans la FA

Dans la FA, le stress oxydant, l'inflammation et l'hypercoagulabilité concourent à créer un état propice à la formation de thrombus et au remodelage tissulaire selon une boucle d'auto-amplification, favorisant la pérennisation du trouble du rythme. Le remodelage tissulaire et la formation de fibrose sont l'étape finale de la progression de la FA. Passée un certain volume ou une certaine quantité de fibrose, l'oreillette gauche ne sera plus capable de retrouver un rythme sinusal. En comparant les auricules de patients en FA et en RS, nous avons pu constater la présence de sénescence associée à la FA, ce qui est décrit pour la première fois. Ce processus de sénescence semble accompagner les phénomènes de remodelage tissulaire, d'état pro-thrombotique et de stress oxydant dans la progression de la pathologie rythmique. Les mécanismes sous-tendant ces liens sont encore à établir. L'étude de notre modèle de cellule endothéliale atriale a mis en évidence que la sénescence entraînait un phénotype pro-thrombotique, pro-inflammatoire, pro-adhésif, pro-fibrotique et pro-remodelage suggérant i) le rôle majeur de la cellule endothéliale dans l'interface entre le sang et ses éléments circulants et le tissu sous-jacent ii) le rôle potentiel que pourrait jouer la sénescence dans la thrombose, le stress oxydant, l'inflammation et le remodelage tissulaire au cours de la FA.

3. Le système rénine angiotensine

3.1 Un rôle central dans l'initiation et la pérennisation de la FA

Plusieurs paramètres cliniques comme l'hypertension, l'insuffisance cardiaque, l'apnée du sommeil, l'insuffisance rénale sont connus pour favoriser l'activation de ce système rénine-angiotensine et favoriser la survenue de FA[2]. Le système rénine angiotensine a directement été impliqué dans l'initiation et la pérennisation de la FA[294-296].

Le rôle pro-fibrotique de l'angiotensine constitue un de ses effets majeurs. Il a été montré dans les cardiomyocytes et les fibroblastes, que l'AngII induisait l'expression de TGF-ß1 via son récepteur AT1. *In vivo*, le TGF-ß1 est responsable de l'hypertrophie et de la fibrose[65] myocardique et est impliqué dans la régulation des MMP[52].

L'Ang II augmente aussi la production de cytokines pro-inflammatoires (IL-6, TNFα), des molécules d'adhésion (VCAM-1), des sélectines et protéines chémoattractantes (MCP-1, P-sélectine)[72, 201, 297]. Par ailleurs, dans le système local au niveau de la cellule endothéliale l'Ang II en se fixant à son récepteur AT1, induit une dysfonction endothéliale et un stress oxydant dépendant de la NADPH oxydase.

Ces données placent le système rénine-angiotensine local au cœur des mécanismes qui concourent à la FA. Plusieurs études cliniques ont d'ailleurs montré un bénéfice clinique des antagonistes des récepteurs de l'Ang II. Une méta-analyse[298] en 2010 regroupant 23 études et 87048 patients a confirmé que l'inhibition du système rénine-angiotensine réduisait d'environ 33% la survenue de FA. L'efficacité en prévention primaire se retrouvait chez les patients hypertendus et insuffisants cardiaques mais pas en post-infarctus. En prévention secondaire, les molécules étaient souvent administrées avec des anti-arythmiques et leurs effets pourraient être synergiques ou du moins interagir avec les anti-arythmiques. En 2011, une étude sur 9016 patients avec l'irbesartan[299] s'avérait décevante avec l'absence de diminution des évènements cardiovasculaires chez les patients présentant de la FA après 4 ans de suivi. L'étude ANTIPAF trial[300] après 1 an de traitement par olmesartan montrait l'absence de diminution du nombre d'épisodes de FA chez des patients présentant de la FA paroxystique sur coeur sain. Dans l'ablation de FA, plusieurs études ont été menées avec des résultats très variables en faveur d'un effet ou non de ces traitements[301-303]. Récemment dans une étude sur 256 patients ablatés de FA paroxystique et traités pendant 3 mois après l'ablation, Wang Q[304] retrouve une nette diminution des récidives à 3 mois mais également à un an. Ces données laissent à penser qu'il existe un effet des inhibiteurs du système rénine-angiotensine sur le remodelage mais à des degrés variables, possiblement en raison des pathologies hétérogènes sous-jacentes associées à la FA.

3.2 Un rôle central dans la sénescence endothéliale

L'implication de l'Ang II dans les mécanismes inflammatoires, pro-oxydants, pro-thrombotiques et fibrotiques retrouvés au cours de la FA est bien établie. Son rôle dans la sénescence endothéliale identifié *in vitro* [249] nous a conduit à l'hypothèse que l'activation locale du système rénine–angiotensine favoriserait l'induction de sénescence de la cellule endothéliale auriculaire au cours de la FA.

Dans notre modèle de sénescence atriale réplicative, l'Ang II à des concentrations pertinentes en pathologie induit une sénescence dans des proportions similaires à celle induite par privation de eNOS (L-NAME) ou par perte de la biodisponibilité du NO provoquée par un stress oxydant majeur induit par l'H2O2. De plus, la sur-expression du récepteur AT1 et de l'enzyme de conversion de l'angiotensine (ACE) témoigne de l'activation locale de ce système au cours du processus de sénescence.

La sénescence induite par l'Ang II promeut un phénotype pro-thrombotique avec l'augmentation du FT et l'émission de MP et pro-adhésif avec l'expression de ICAM et VCAM-1. De plus, l'Ang II induit la surexpression du TGFß dans la cellule sénescente, une réponse connue dans le cardiomyocyte et le fibroblaste[65]. Enfin, les cellules sénescentes surexpriment les MMP-2 et 9, une réponse qui a été considérée comme responsable d'une prolongation de la durée de la FA [86].

Ces données mettent en évidence que l'Ang II induit un état sénescent de la cellule endothéliale atriale qui s'accompagne d'un phénotype pro-thrombotique, pro-adhésif, pro-fibrotique et protéolytique suggérant i) une potentielle implication de la sénescence induite par l'Ang II dans les mécanismes délétères retrouvés au cours de la FA ii) un rôle majeur de la cellule endothéliale dans l'interface entre le sang, ses éléments circulants et le reste du myocarde sous-jacent dans la diffusion du message favorisant le remodelage atrial.

La possibilité d'une modulation pharmacologique de la sénescence endothéliale auriculaire induite par l'Ang II a été confirmée in vitro par la prévention de l'induction de sénescence en présence de losartan, un inhibiteur des récepteurs AT1, ou par les inhibiteurs de l'enzyme de conversion de l'angiotensine I. L'Ang II favorise aussi l'émission de MP par les cellules endothéliales atriales qui sont capables d'effet autocrines NADPH oxydase et ROS dépendants sur les cellules avoisinantes [19 45].

Les données actuelles de la littérature et du laboratoire montrent un mode d'action similaire de l'Ang II sur la sénescence de cellules endothéliale de coronaires et d'oreillette gauche de porc ou d'aorte de souris et une action pro-sénescente des MPs émises par la cellule endothéliale sénescente. L'hypothèse d'une action pro-sénescente des MPs plasmatiques d'origine notamment endothéliale a pu être vérifiée à l'aide de MP de patients présentant un syndrome coronarien aigu. Elles induisaient une sénescence de manière dépendante du stress oxydant et du système rénine angiotensine local. Le système rénine-angiotensine constitue donc une cible d'intérêt pour prévenir la perennisation des processus de sénescence dans l'oreillette soutenue par l'émission de MPs procoagulantes et

effecteurs pro-sénescents qui favorisent et les changements phénotypiques de la cellule endothéliale atriale et participent à la dysfonction endothéliale et à l'altération du tissu atrial avec le vieillissement.

VI. Conclusions et perspectives

Nos travaux s'inscrivent dans une démarche de recherche translationnelle développant des outils méthodologiques et des observations cliniques et biologiques pour mieux décrypter les mécanismes cellulaires et moléculaires associés à la FA et au cours du vieillissement. Nous avons mis en évidence que les MPs reflètent et contribuent à un état d'hypercoagulabilité et pro-inflammatoire au cours de la FA. Leurs concentrations *in situ* dans la cavité atriale ou circulantes témoignent d'un état pro-thrombotique. Les concentrations en MPs évoluent parallèlement à l'augmentation de l'activation cellulaire et plaquettaire. Si les deux cathéters d'ablation par radiofréquence étudiés révèlent en post-procédure des effets superposables sur le plan du risque thrombotique attesté par des cinétiques similaires d'apparition des MPs, l'impact clinique des MP serait particulièrement intéressant à évaluer sur le plan clinique en particulier sur les lésions ischémiques silencieuses objectivées à l'IRM. Leur activité protéolytique susceptible de favoriser la migration embolique mais possiblement aussi le remodelage tissulaire devra être étudiée dans des modèles spécifiques. La modulation pharmacologique des MP par des corticoïdes en per et post-ablation est en cours d'évaluation.

Nous avons également montré l'importance de la sénescence dans l'altération tissulaire des oreillettes en FA qui évolue avec la sévérité du remodelage associé à la progression du trouble du rythme. Il est difficile actuellement de savoir si ces processus de sénescence constituent un facteur délétère ou cytoprotecteur.

Nous avons caractérisé un modèle cellulaire de sénescence réplicative ou induite de la cellule endothéliale auriculaire permettant d'identifier l'apparition d'un phénotype pro-thrombotique, pro-inflammatoire, pro-adhésif, de le quantifier ainsi que de mesurer la sécrétion des protéases et cytokines impliquées dans la fibrose et le remodelage tissulaire. Grâce à ce modèle, qui permet d'évaluer des modulateurs pharmacologiques de la dysfonction endothéliale nous avons établi que le système rénine-angiotensine semble jouer un rôle majeur dans les mécanismes initiateurs de la sénescence endothéliale auriculaire et constitue une cible d'intérêt pour en limiter les conséquences sur l'altération du tissu atrial au cours du vieillissement. Ce modèle permettra également d'analyser l'effet de MP isolées de patients en FA selon leur score de CHA2DS2-VASc.

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VIII. Annexes

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Review

Microparticles in atrial fibrillation: A link between cell activation or apoptosis, tissue remodelling and thrombogenicity

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ABSTRACT

Microparticles (MPs) are small membrane vesicles that are shed from virtually all cells in response to stress. Widely described in atherothrombotic diseases, recent data suggest a role for circulating MPs in the hypercoagulable state associated with supraventricular tachyarrhythmia. During atrial fibrillation, several mechanisms, such as high ventricular heart rate, low or oscillatory shear stress, stretch, hypoxia, inflammation and oxidative stress, are potent inducers of apoptotic cell death, which leads to the shedding of procoagulant MPs within the vasculature. As key regulators of cell–cell cross-talk and important mediators of inflammatory, thrombogenic and proteolytic pathways, MPs directly or indirectly contribute to the amplification loops involved in atrial fibrillation. Because high levels of platelets and endothelial-derived MPs are identified during stroke and are associated with infarct size and clinical outcome, they are proposed to be a potent marker of ischaemic risk. During pulmonary vein isolation, the additional increases of platelet and leukocyte MP levels suggest the extent of tissue damage and reflect a transient activation of the coagulation cascade that could favour ischaemic stroke. Conversely, the observed decreases of several apoptotic markers some months after the restoration of sinus rhythm suggest that the extent of apoptotic processes is reversible and might enable restoration of haemostasis. In this review, we will summarise the current evidence supporting the roles of apoptosis and cell activation in the development of the prothrombotic state observed in atrial fibrillation, with a particular focus on procoagulant MPs.

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1. Introduction

Circulating MPs are small membrane vesicles that are shed from virtually all cells in response to activation, apoptosis or thermic injury. In the vasculature, MPs shed by stimulated or apoptotic cells are hallmarks of tissue damage; the degree of shedding correlates to the proportion of stimulated cells. Having long been viewed as cellular dust, MPs are now considered a key component in the haemostatic response [1]. Circulating MPs, mainly from platelets, leukocytes and endothelial cells, provide an additional phospholipid surface to support the assembly of blood coagulation factors, thereby promoting the coagulation cascade and thrombin generation. In the vasculature, they are the main carriers of circulating tissue factor, the principal initiator of vascular thrombosis [1]. MPs are widely described in atherothrombotic diseases, and recent advances suggest they have a role in the hypercoagulable state associated with supraventricular tachyarrhythmia [2–5]. Due to their inflammatory, thrombogenic, proteolytic, and endothelium-damaging properties, MPs directly or indirectly contribute to the noxious amplification loops associated with the marked thrombotic propensity observed in atrial fibrillation (AF) [2,6] (Fig. 1). Moreover, because high levels of platelet- and endothelial-derived MPs are identified during stroke and are associated with infarct size and clinical outcome, they are proposed to be a potent marker of ischaemic risk [7–10].

In this review, we will summarise the current evidence supporting the roles of apoptosis and cell activation in the development of the prothrombotic state observed in AF, with a particular focus on procoagulant microparticles.

Apoptosis in atrial fibrillation

When exposed to stress, cell death occurs secondary to increased membrane permeability and remodelling [11]. Rising cytosolic Ca^{2+} concentrations allow cytoskeleton cleavage through calpain [12] and caspase activation, which leads to the externalisation of normally internalised procoagulant aminophospholipids on the cell membrane. This process of membrane remodelling ultimately results in the

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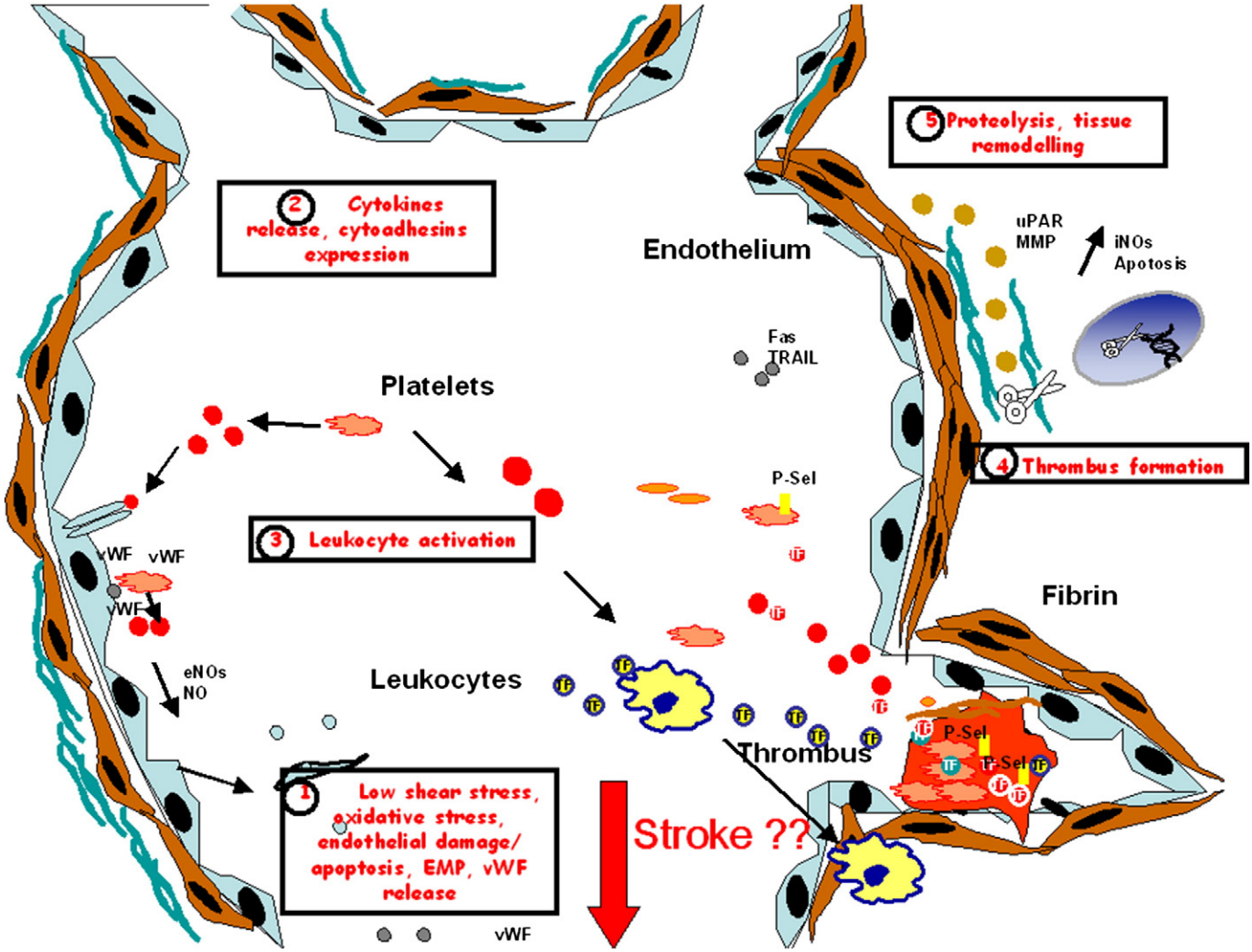

Fig. 1. Deleterious role of circulating and trapped MPs in atrial fibrillation. During atrial fibrillation, low or oscillatory shear stress, hypoxia, stretch, inflammation and oxidative stress are likely to promote apoptotic cell death and the release of circulating microparticles. Endothelial damage is characterised by the release of several marker such as endothelial-derived microparticles (EMPs) or vWF antigen [1]. Behaving as noxious shuttles of biological information, circulating MPs promote cytokines release, cytoadhesins expression, leukocyte activation and endothelial damage [2,3]. At sites of endothelial damage, the swift recruitment of MPs harbouring tenase, prothrombinase and tissue factor activities are mandatory for the growth of the thrombus [4] and could be a determinant of the risk of stroke. Within the atrial tissue, MPs shed by apoptotic cells harbouring functional metalloproteinase or uPAR that lead to plasmin generation could contribute to tissue remodelling through fibrillar matrix proteolysis [5].

shedding of MPs that express phosphatidylserine (PS) at their outer leaflet [11].

Within the atrium, all of the cellular lineages, including endothelial cells, fibroblasts and cardiomyocytes, are potentially subjected to programmed cell death. Increased apoptosis has been detected in tissue samples from animal and human hearts with AF [13]. Compared to tissues from patients in sinus rhythm, fibrillating atria have significantly more myolysis, nuclear alteration, and apoptosis activation [13,14]. The detection of elevated serum levels of apoptosis mediators, including Fas in patients with AF, is another illustration of the importance of programmed cell death during AF [15]. The degree of myolysis and an increased apoptotic pattern could be significant predictors for the development of postoperative AF [16]. Indeed, atrial histology shows degenerative changes that may correlate with advanced age and left atrial enlargement.

It is likely that apoptotic processes begin relatively early in the course of AF and associated tissue remodelling. Evidence from experimental models of pacing-induced AF suggests that apoptosis, leukocyte infiltration, and increased cell death occur early and precede arrhythmogenic structural remodelling [17]. In endothelial cells undergoing cell death, membrane phosphatidylserine (PS) typically precedes DNA fragmentation [18] (Fig. 2). The process of membrane remodelling that occurs during apoptosis is ultimately resolved by the shedding of membrane microparticles that express various effector signalling proteins for the thrombotic processes, including PS and tissue factor (TF)[1]. Depending on the stage of apoptosis, the exposure of procoagulant phospholipids at the outer leaflet of activated or apoptotic cells induces an increase of the activity of the tenase complex by 25% to 60%. Endothelial apoptosis could be associated with enhanced expression of TF antigen, or activity under proinflammatory conditions when cells were pre-treated by lipopolysaccharide. Apoptosis induction is also associated with a swift decrease of various anticoagulant activities [18]. Consequently, the induction of endothelial apoptosis leads to a significant increase in thrombin formation. Moreover, apoptotic endothelial cells become proadhesive to non-activated platelets and promote their slight activation, suggesting an amplification pathway to thrombosis. [19]. Consistent with this paradigm, acute-onset AF is associated with local cardiac platelet activation [20] and endothelial dysfunction [21]. During AF, tissue factor, which is the main inducer of blood coagulation, is overexpressed in damaged

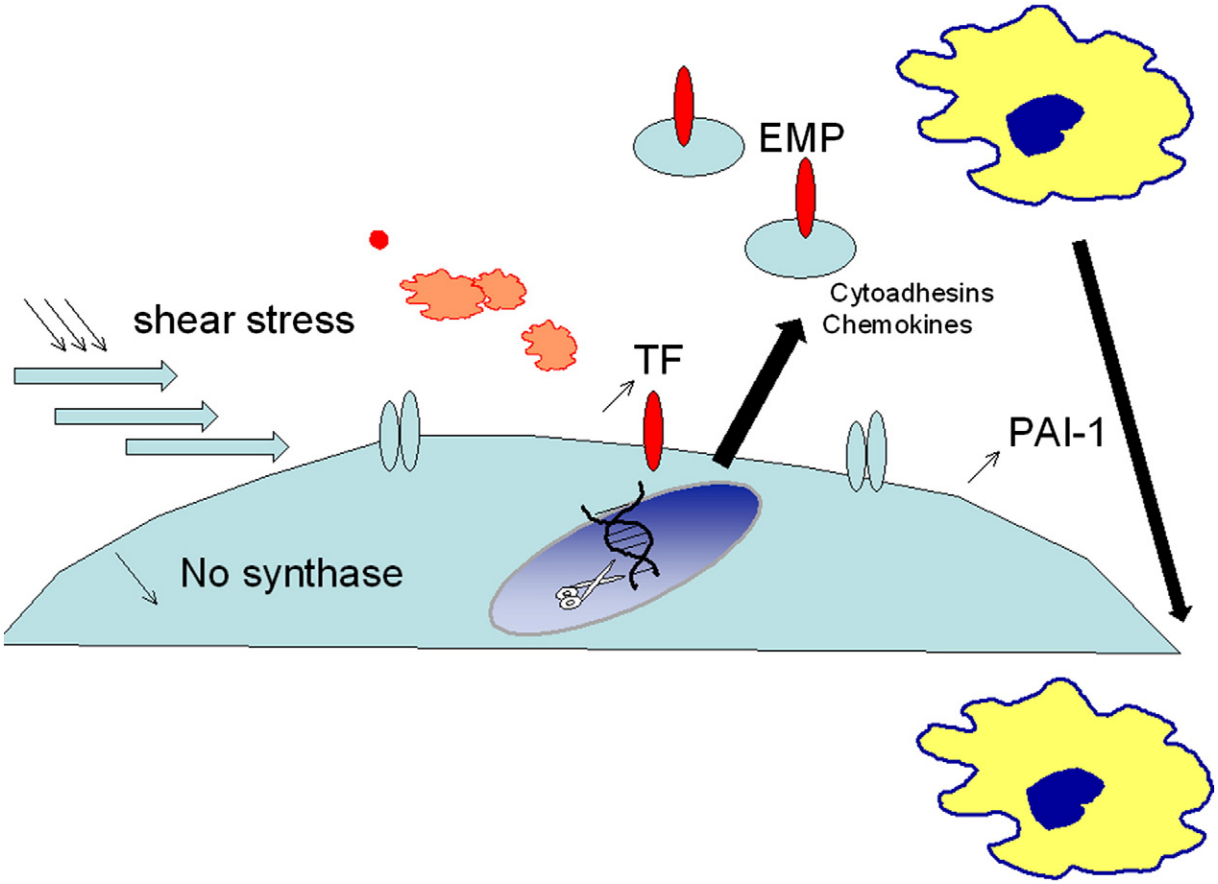

Fig. 2. Endothelial damage in atrial fibrillation. During atrial fibrillation, low or oscillatory shear stress contributes to decrease endothelial NO synthase activity and the bioavailability of nitric oxide a major determinant of endothelial cell survival. Cell activation is associated with plasma membrane remodelling, the exposure of procoagulant aminophospholipid such as phosphatidylserine (PS) and the release of procoagulant endothelial-derived microparticles (EMP) and vWF normally located in the Weibel Palade bodies. PS provides an additional phospholipidic surface enabling the assembly of the coagulation enzyme complex such as tenase and prothrombinase finally resulting in thrombin generation. Under inflammatory conditions, endothelial cell activation is also characterised by the enhancement of tissue factor activity, possibly harboured by cell-derived MPs. Endothelial activation is also characterised by an enhanced expression of PAI-1, an inhibitor of the fibrinolysis process and by the reduction of various anticoagulant pathways. Chemokines release, cytoadhesins expression and the augmentation of endothelium permeability contribute to the recruitment and the trapping of inflammatory cells within the atrial tissue.

endothelial tissue, which is characterised by histologic evidence of denuded endothelial matrix. Also, damaged tissue is associated with the presence of inflammatory cells [22] and increased circulating mononuclear cells [20].

2. Inductors of cell death during atrial fibrillation

2.1. Heart rate

Heart rate (HR) is probably the main contributor of cell damage during atrial fibrillation. Several clinical studies have consistently reported that the cellular damage caused by high HR is completely reversible when the HR is controlled by means of drugs or catheter ablation [23]. In vitro, tachypacing of atrial cardiomyocytes increases caspase activity, a crucial effector of the apoptotic process. In line with this paradigm, several animal models have demonstrated that rapid pacing enables the induction of congestive heart failure, thereby triggering atrial cell death [17,24]. For instance, in a canine model of AF induced by prolonged atrial pacing, characteristic features of apoptosis are observed and are associated with caspase-3, Bax and calpain activation concomitant with inhibition of the anti-apoptotic bcl-2 pathway [25]. Likewise, significantly elevated expression of apoptotic molecules (Fas and Fas ligand) has been observed in cardiac tissue from the atria and ventricles of dogs subjected to rapid ventricular pacing over a 4-week period with respect to values measured in tissue samples retrieved from control, non-paced hearts [26]. In pigs, burst atrial pacing led to development of persistent AF and associated tachycardiomyopathy. Histological changes corresponding to these ultrastructural effects include extensive myolysis, variable fibrosis, with the greatest effects seen within the ventricles, and apoptosis [24]. Accordingly, it was previously demonstrated that cardiac pacing in dogs produces an increase in myocardial monocyte infiltration, monocyte chemoattractant protein-1 expression, and renin–angiotensin system and local matrix metalloproteinase activity. The progressive loss of valuable cardiomyocytes in heart failure is attributed to apoptosis, a result of various ongoing metabolic and neurohumoral insults [27,28]. In this model, the observed increase in apoptosis was correlated with elevated norepinephrine levels and the decrease of cardiac output. Conversely, in animal models of rapid atrial pacing with a controlled ventricular rate, no significant cell death is induced within the atria. It is thus likely that a rapid ventricular rate is a crucial determinant for the onset of cell death in AF [29]. In line with this view, clinical studies have suggested that the main predictor of tachycardia-induced cardiomyopathy was represented by the ventricular rate [30]. Although the precise mechanisms involved in this tachycardia-induced cardiomyopathy are still not fully characterised, possible mechanisms rely on (i) calcium overload, possibly associated with the inhibition of sarcoplasmic reticulum Ca^{2+}-ATPase (ii) oxidative stress [31] (iii) mitochondrial damage and (iv) inflammation leading to enhanced stunning and/or apoptosis [24,27,28]. In the specific context of atrial fibrillation, other authors have suggested that down regulation or altered function of L-type

Ca^{2+}-channel and an increased Ca^{2+} extrusion via the Na^{+}/ Ca^{2+} exchanger could be the main factor responsible for the depressed contractility in remodelled atria [32].

2.2. Shear stress

Shear stress (SS) is a major determinant of endothelial apoptosis, and physiological laminar fluid SS promotes endothelial cell survival and quiescence through NO release. Maintenance of a physiological laminar SS is crucial for normal vascular structure and function, and this type of SS exerts vascular protective effects in vivo through the release of substances that promote anticoagulation, inhibit inflammation, and induce vasodilation [33]. For instance, shear stress decreases endothelial cell tissue factor activity by augmenting the secretion of tissue factor pathway inhibitor [34]. Because AF leads to a loss of organised atrial contraction and to turbulent blood flow in the left atrium, it was hypothesised that AF may cause a down regulation of NOS gene expression and function in the left atrial endocardium. The subsequent decrease in NO concentration may lead to increased expression of PAI-1 and platelet aggregation and ultimately contribute to thrombosis in the left atrium (LA). In a pig model of AF induced by rapid atrial pacing, the loss of atrial contraction and consequent reduction in shear stress appeared to down regulate LA expression of nitric oxide synthase with a corresponding decrease in nitric monoxide bioavailability, a key factor for endothelial cell survival [35]. This marked decrease in endocardial NOS expression and NO bioavailability was associated with an increase in PAI-1 expression in the left atrium. These data suggest that organised atrial contraction is needed to maintain normal endocardial expression of NOS. It is likely that loss of this antithrombotic enzyme contributes to the thromboembolic phenomena commonly observed in AF. Accordingly, another report has documented decreased plasma levels of NO_2^- and NO_3^- (NO_x) in patients with atrial fibrillation. AF is associated with a decrease in eNOS expression and a consequent decrease in NO concentration on the endocardial surface of the left atrium [35]. It is also likely that an oscillatory shear stress characterises the haemodynamic environment of AF as opposed to the unidirectional shear stress typical of sinus rhythm. In vitro vascular models have clearly demonstrated that this pulsatile oscillatory shear stress induces the pro-coagulant phenotype of endothelial cells associated with a 2-fold increase of TF activity, which may favour the formation and progression of thrombotic lesions [36] in the left atrium and atrial appendage.

2.3. Hypoxia

Another important inducer of cell death is hypoxia. Irregular RR intervals, as seen in AF, might be responsible for the decreased coronary blood flow reserve that is insufficient to meet the increased O_2 demand in AF. AF is closely associated with atrial up-regulation of hypoxic and angiogenic markers, such as hypoxia-inducible factor and vascular endothelial cell growth factor [37], in parallel with the development of fibrosis. It is conceivable that fibrosis might lead to an increased O_2 diffusion distance, thereby inducing ischaemic signalling, which, in turn, leads to angiogenesis. Although still controversial, these findings support the possibility of a vicious cycle in which structural remodelling (i.e., fibrosis), AF, and hypoxia interact with each other to promote the chronicity of atrial fibrillation.

2.4. Stretch

Converging data have suggested that increased mechanical stretch is a potent mediator of apoptosis and tissue remodelling during AF. Stretch has been linked to changes in calcium handling during AF, leading to Ca^{2+} overload. L-type calcium current (Ical), and ryanodine receptors are thought to be involved in the increased intracellular calcium levels observed in vitro in ventricular cardiomyocytes [29]. Stretch-induced apoptosis of ventricular cardiomyocytes is blunted by the inhibition of Na^{+}/Ca^{2+} exchangers, L-type Ca^{2+}-channels and ryanodine receptors [38]. In vitro, ventricular cardiomyocyte stretch increases superoxide anion production, whilst reactive oxygen species modulate the apoptotic response to mechanical stretch [29]. It is likely that the cellular loss induced by apoptotic cell death contributes to noxious amplification loops that further increase the stretch on remaining cells.

2.5. Inflammation and oxidative stress

Beside the creation of a prothrombotic profile by phenotypic modifications of endothelial cells, other characteristic features of activated endothelial cells are represented by proadhesive and proinflammatory changes [39].

The sequestration of inflammatory cytokines, such as CRP and IL-6, observed in patients with atrial flutter or AF [40] suggests that these proteins could activate noxious pathways leading to cell death or tissue remodelling. One recent study found that not only heart rate but also inflammation and oxidative stress could trigger cardiomyocyte apoptosis during human AF [41]. In that study, the apoptotic index and caspase 3 staining were predominantly enhanced in the right atrium of patients with permanent AF with respect to patients with sinus rhythm. When subdividing the cohort into two groups according to CRP level, a significant up-regulation of inducible nitric oxide synthase was observed in patients with higher CRP (>5 mg/L). The correlation of inducible nitric oxide synthase expression, 3 nitrotyrosine (a biological marker of peroxynitrite) expression and the extent of cell death suggest a link between inflammatory status, oxidative stress and cell death. Interestingly, CRP was recently demonstrated to act via NO deficiency as a potent inductor of microparticle shedding by endothelial cells, possibly contributing to thrombogenicity [42]. Another amplification loop is provided by MPs, able to carry and deliver CRP [43].

The relevance of the inflammatory and oxidative stress pathway was nicely demonstrated in clinical practice: various clinical studies have demonstrated that enhanced oxidative stress or inflammatory response is associated with early [44] or late AF recurrence [45,46]; corticosteroids [47] and colchicine [48] have been shown to reduce inflammatory status and to prevent AF recurrence after radiofrequency ablation.

3. Chamber specific cell and platelet activation in atrial fibrillation

In patients with AF, thrombi predominantly form in the left rather than the right atrium. The reasons for this remain incompletely understood. Previous data by Sanders and coworkers have suggested that, in patients with AF, left atrial platelet reactivity is increased compared to the right atria and peripheral circulation. According to this view, the presence of chamber-specific platelet activation may explain, in part, the propensity for left atrial thrombus formation in patients with AF [49]. In our experience, no differences in MPs, tissue factor or various markers of platelet activation could be detected between the chambers (Jesel et al. unpublished data). Because they led to less meaningful complications (sub-clinical pulmonary embolism vs. stroke), it is likely that thrombi developing within the right heart are less frequently detected. According to de Divitiis, the detection of thrombin within the right atrium is not rare (6.7% within the right atrium vs. 12.2% in the left atrium). In this study, spontaneous echo contrast was found at nearly same extent in the right and left atrium (57% and 66%, respectively) [50]. Very recent data has underlined that differences in atrial morphologies are an important risk factor for stroke; non «chicken wings» morphologies being associated with a consistent increase of the risk of stroke [51]. Although AF is associated with depressed right atrial appendage (RAA) and left atrial appendage ejection velocities, anatomic RAA remodelling appears to be absent. The larger RAA width and lack of anatomic remodelling may partially explain the

substantially lower prevalence of RAA thrombus found among patients with AF [52].

4. Increased levels of MPs during atrial fibrillation

Given the primary importance of cell activation and cell death during AF, several groups, including ours, hypothesised that a sustained and enhanced generation of microparticles (MPs) could occur during AF and contribute to enhanced thrombogenicity [2,3]. It has been suggested that the pathophysiology of thromboembolism in AF is multifactorial and fulfils Virchow's triad [53]. Moreover, it has been postulated that AF is associated with a prothrombotic or hypercoagulable biologic state. In fact, abnormal blood flow, together with vessel wall and haemostatic abnormalities, has been described in AF. However, the role of confounding factors, including those of vascular risk, must be taken into account. For instance, in a case–control study from the Framingham cohort that compared matched subjects with and without AF, haemostatic parameters, such as vWF antigen, t-PA, and PAI-1, were no longer associated with AF in multivariate analysis adjusted for cardiovascular risk factors [54].

5. Procoagulant MPs

Consistent with the important role of circulating MPs in the hypercoagulable state observed in AF, a stepwise increase of procoagulant MPs has been seen from patients in sinus rhythm without cardiovascular risk factors to patients in sinus rhythm with cardiovascular risk factors to patients with AF. In our experience, a 2-fold elevation of procoagulant MP levels was demonstrated in patients with permanent or persistent AF with respect to age-matched controls [2]. On multiple regression analysis, the presence of AF was a strong predictor of annexin-V positive MPs, a reliable marker of PS expression at the outer leaflet of MPs.

Acute induction of AF activates platelets within minutes and significantly increases the expression of P-selectin on platelets and derived MPs together with an overexpression of the counter-receptor on mononuclear cells [20]. Therefore, the increase of circulating procoagulant MPs in persistent or permanent AF might reflect a hypercoagulable state that could contribute to atrial thrombosis and thromboembolism [2]. It should, however, be recognised that in AF patients, strong evidence supporting a direct link between enhanced shedding of procoagulant MPs and increased risk of stroke is still lacking.

5.1. Source of procoagulant MPs during AF: Platelet-derived MPs

The level of platelet-derived MPs (PMPs) measured in the peripheral venous blood increases in patients with AF [4,20,55], especially in patients with rheumatic mitral stenosis [56] or those treated with digoxin [4]. In a small case–control study, Azzam and Zagloul demonstrated a two-fold increase of platelet-derived MPs in patients with chronic rheumatic mitral stenosis and AF with respect to values measured in controls. That study documented a direct relationship between mitral stenosis severity and PMPs level [56], suggesting that stasis or alterations in atrial haemodynamics could favour the shedding of PMPs. In a larger trial, Choudhury and co-workers showed that patients with AF had far higher amounts of PMPs and soluble P-selectin than healthy volunteers in sinus rhythm [3]. However, no difference was observed between patients with AF and disease-matched controls, suggesting that the abnormal changes detected were a consequence of underlying co-morbidities rather than AF itself [3]; these findings are consistent with previous data obtained by our group [2].

5.2. Endothelial-derived MPs

In our experience, the shedding of endothelial-derived MPs (EMPs) during AF appears to reflect the presence of associated cardiovascular risk factors rather than AF itself. Whereas EMP levels are higher in AF patients than in patients without cardiovascular risk factors, differences do not persist after adjustment for potential cofounders. Significant relationships between EMP level, systolic blood pressure, diabetes, hypercholesterolaemia and coronary heart disease exist [2]. Because intracellular calcium is a key regulator of endothelial cell function, endogenous digitalis-like substances affect such biological processes in endothelial cells, suggesting that these therapies are potent inducers of endothelial blebbing during AF. In patients with non-valvular damage-induced AF, elevated levels of endothelial-derived MPs harbouring the CD62E or CD31 phenotypes are observed after treatment by digitalis, supporting the hypothesis of specific endothelial damage. Significant increases of PMPs or leuko-platelet conjugates are also detected. Interestingly, differences in EMP levels persist after adjustment for potential confounding factors, such as age, congestive heart failure, and coronary heart disease [4].

6. Impact of ablation on cell activation and apoptotic processes

6.1. Acute phase

Because thermic injury is a potent inducer of cell death/activation, several groups have investigated the impact of radiofrequency ablation on tissue damage and platelet, leukocyte and endothelial activation. Table 1 summarises the main studies that have assessed the impact of ablation and treatment on cell activation/apoptosis. When exposed to hyperthermia, cell injury occurs via increased membrane permeability and remodelling. Pioneering work by Bulava and co-workers in 2004 clearly demonstrated that radiofrequency (RF) catheter isolation of the pulmonary veins (PV) induced a systemic procoagulant state, as reflected by fibrin turnover, fibrinolysis activation and endothelial damage [57]. In our experience with patients undergoing radiofrequency ablation of common atrial flutter [5] a significant rise in platelet- and leukocyte-derived procoagulant MPs is observed both immediately after and 24 h after the procedure (Fig. 3). Furthermore, significant changes in vWF Ag, PAI-1, and D-dimer occur, supporting the idea that the activation of the coagulation cascade occurs despite the wide use of anticoagulants [5]. Another study nicely depicts the kinetics of platelet activation following RF or cryoablation [58]. Taken together, these observations clearly indicate that RF ablation induces a prothrombotic state and the release of platelet- and leukocyte-derived procoagulant MPs. Whereas the activation of blood coagulation could be viewed as clinically marginal in right-sided procedures, its relevance in left-sided procedures remains to be established. Rare but potentially life-threatening complications, such as thromboembolism, can occur during PV isolation [59]. Therefore, we recently investigated the extent of tissue damage and the shedding of procoagulant MPs in AF patients undergoing PV isolation by RF or cryoablation [60]. With respect to other methods, cryoablation reduces endothelial disruption and thrombus formation [61]. In our experience, both techniques induce cell damage and activation characterised by the shedding of procoagulant PMPs, myonecrosis and an inflammatory state [60] within 8 h–48 h. Conversely, no significant changes in EMP levels are detected. In our experience, the extent of tissue damage and the subsequent prothrombotic state induced by RF appeared to be similar to that observed after cryoablation [60] in terms of thromboembolic complications [62]. Until now, the impact of various anticoagulant protocols on MP shedding and coagulation activation has not been investigated. In healthy volunteers, the new oral anticoagulant Dabigatran was shown to substantially reduce TF expression and MP shedding [63]. The clinical impact of such cellular activation associated with a transient hypercoagulable state has been difficult to assess. It should, however, be emphasised that treatment using extensive and highly complex RF ablation can induce a systemic inflammatory response within 12–48 h and is associated with the development of severe pulmonary oedema in some patients [64].

Table 1
Impact of treatment and ablation procedures on cell damage/apoptosis/repair, platelet activation, coagulation and inflammation in patients with supraventricular tachyarrhythmia.

Clinical setting	Treatment	Cell damage/apoptosis/repair	Platelet activation	Coagulation/fibrinolysis	Myonecrosis	Inflammation	References
Non-valve damage-induced Atrial fibrillation	Digoxin	↑ EMPs levels	↑ PMPs	ND	ND	↑ platelet–leukocyte aggregates	Chirinos et al.
Atrial fibrillation	Radiofrequency/ cryoablation	↑ LMP (delayed), EMP = (no differences between RF and cryoablation)	↑ PMPs (early), ↑ P-Selectin and GPIIbIIIa expression (no differences between RF and cryoablation)	↑ Prothrombinase activity displayed by MPs (no differences between RF and cryoablation)	↑ TnT (no differences between RF and cryoablation)	↑ hs-CRP, (no differences between RF and cryoablation)	Herrera et al.
Atrial fibrillation	Radiofrequency					↑↑↑ CRP, ↑↑↑ leukocytes, SIRS	Weber et al.
Atrial fibrillation	Radiofrequency	↑ vWF Ag	ND	↑ D-dimer, ↑ t-PA, ↓ PAI-1	ND	ND	Bulava et al.
Atrial fibrillation	Epicardial ablation	↓↓ Fas, TRAIL at 3 and 6 months	ND	ND	ND	ND	Osmancik et al.
Atrial fibrillation	Cardioversion	↑ CPC	ND	ND	ND	ND	Watson et al.
Common flutter	Radiofrequency	↑ LMP (delayed), EMP =, ↑ vWF Ag	↑ PMPs (early)	↑ Prothrombinase activity displayed by MPs, ↑ D-dimer, ↓ PAI-1	ND	ND	Jesel et al.
Common flutter	Radiofrequency/ cryoablation	ND	↑ P-Selectin and GPIIbIIIa expression (less platelet activation with cryoablation)	ND	↑ TnT (less myonecrosis with cryoablation)	ND	Hochholzer et al.

t-PA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1; vWF: von Willebrand factor; LMP: leukocyte-derived microparticles (MPs); PMP: platelet-derived MPs; EMPs: endothelial-derived MPs; TnT: Troponin T; hs-CRP: high-sensitivity C-reactive protein; CPC: circulating progenitor cells; SIRS: systemic inflammatory response syndrome; ND: not determined.

6.2. Chronic phase

If the cell activation and transient hypercoagulable state observed during the acute phase of ablation mainly reflect tissue damage and membrane remodelling induced by RF or cryoablation, the restoration of sinus rhythm could contribute to decreasing the extent of apoptosis. In an attempt to evaluate the reversibility of apoptosis in patients with AF alone, one study investigated surrogate blood markers of cell death, such as apoptosis-stimulating fragment (Fas) and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), following surgical epicardial ablation [65]. In that study, concentrations of both Fas and TRAIL decreased in the serum of successfully ablated patients, whereas no significant changes were seen in patients with recurrent AF. These data highlight that the restoration and maintenance of sinus rhythm could be associated with a mid-term decrease of apoptosis [65]. Changes in the extent of cell activation and apoptosis during the acute and chronic phases following ablation are summarised in Table 1.

7. Potential role of MPs during atrial fibrillation

In the clinical setting of AF, two important pools of MPs are worth considering when studying pathophysiological amplification loops: (i) the pool of MPs circulating in the blood and (ii) the tissue pool of MPs sequestered within the atria (Fig. 1).

7.1. Thrombogenicity

In the vasculature, rapid phospholipid membrane remodelling and PS egress characterise a physiologically relevant procoagulant response. Activated/apoptotic vascular cells, together with MPs, harbour PS, which provides an additional phospholipid surface enabling the assembly of the tenase and prothrombinase complexes that lead to thrombin generation. PS plays a key catalytic role by promoting the assembly of the vitamin K-dependent enzyme complexes of the coagulation cascade. Vitamin K-dependent factors (FIX, FX and FII) bind to accessible PS through their gamma-carboxyglutamyl residues [1]. PS exposure on the surface of activated/adherent platelets is part of the physiological response at a wound site. PS exposure can also be triggered by MPs and other activated blood and vascular cells. Local concentrations of focused enzymes, cofactors (FVIIIa and FVa) and zymogens enable the fulfilment of the kinetic constraints for optimal thrombin generation that stimulate platelets in an amplification loop, which is the basis of a swift haemostatic response.

The involvement of MPs in thrombin generation also results from their capacity to harbour, deliver, and induce TF activity. Circulating MPs are the main reservoir of blood-borne TF activity. In the course of AF characterised by atrial endothelial damage and increased vWF release [53]. It is likely that GPIbalpha-vWF interactions not only initiate platelet arrest onto reactive vascular surfaces but also contributes to the generation of procoagulant MPs that further enhance thrombus formation (Fig. 3) [1].

Together with activated platelets and leukocytes, PMPs contribute to the initiation and acceleration of clotting reactions at site of endothelial injury. PMPs are also able to stimulate neutrophils in vitro, possibly explaining the time course of leukocyte-derived MP release observed during RF ablation. PMPs contribute to monocyte adhesion to the inflamed endothelium and further diapedesis through the delivery of RANTES, a CC chemokine [66]. LMPs are reliable probes of leukocyte stimulation that could play a specific role during RF ablation. By comparison to PMPs and D dimers, we observed a delayed rise in LMPs peaking on day 1 after ablation of common atrial flutter [5]. At the onset of thrombogenesis, it is likely that the recruitment of LMPs harbouring TF is mandatory, whereas the level of PMPs mostly reflects the extent of platelet stimulation. In animal models, circulating LMPs have a pivotal role in the formation of thrombi [67]. In an experimental

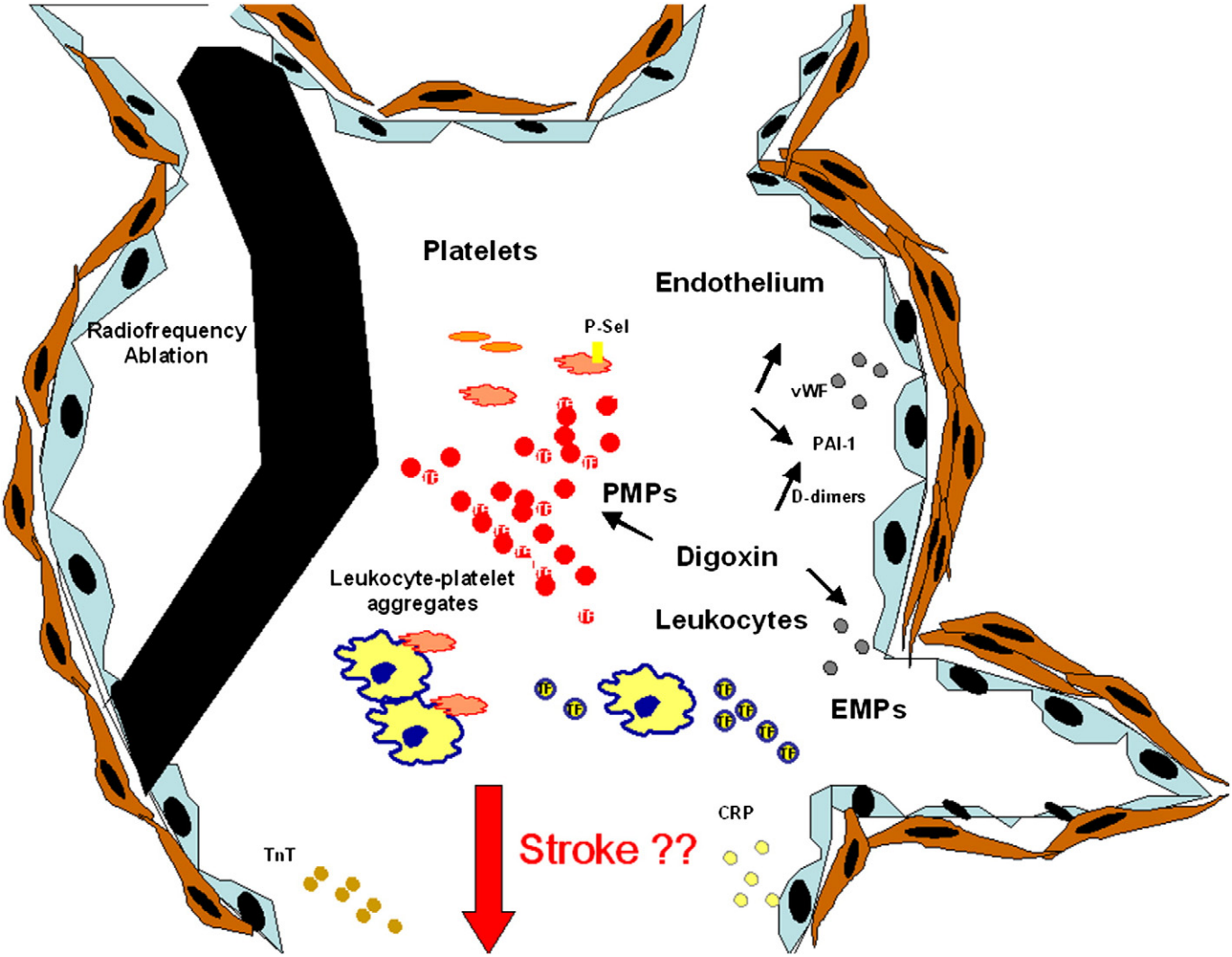

Fig. 3. Impact of ablation procedures during pulmonary vein isolation on tissue damage, inflammation, and cell activation/apoptosis. In patients undergoing radiofrequency ablation a significant rise in platelet and leukocyte-derived procoagulant MPs could be evidenced after the procedure. Myonecrosis and inflammation could be demonstrated by the rise of troponin, CRP or by the formation of leuko-platelet aggregates. Furthermore, significant changes in vWF Ag, PAI-1, and D-dimers occurred, testifying to the activation of blood coagulation that occurs in spite of the wide use of anticoagulants. In our experience, during pulmonary vein isolation, the extent of tissue damage and subsequent prothrombotic state induced by RF energy appeared in the same range of that one observed with cryoenergy in line with the occurrence of thromboembolic complications using either one of these energies. Up to now, the impact of this transient hypercoagulable state induced by the procedure on the thromboembolic complications is unknown. The use of digoxin, associate with intracellular calcium overload could also contribute to the shedding of endothelial and platelet-derived MPs and the formation of leuko-platelet aggregates. Conversely, the midterm restoration of sinus rhythm is associated with the decrease of several apoptotic markers that could testify to the restoration of the vascular and tissular normal homeostasis.

mouse model of venous thrombosis, circulating LMPs are negatively correlated with thrombus weight, suggesting their early incorporation within the growing thrombus [68]. Conversely, PMPs were positively correlated with thrombus growth [68]. In acute myocardial infarction, sequestered inflammatory cells together with LMPs harbouring TF appear to be crucial for the formation of the thrombus. Independent of the eventual role played by PMPs, LMPs are the main contributor of TF activity in the vicinity of the arterial thrombus [69]. Several animal models using intravital microscopy to monitor thrombus development have demonstrated that LMPs, harbouring TF, are recruited to endothelial lesion sites via the P-selectin/PSGL-1 interaction before leukocyte infiltration. LMPs would also promote thrombus growth through the activation and recruitment of rolling platelets at the vicinity [67]. In view of recent data, the role of MPs in the integrated interactions between leucocyte and platelets during permanent AF is consistent with the mechanisms of thrombus growth described above. Acute onset AF was observed to activate platelets within minutes; interactions with monocytes being initiated through platelet P-selectin and PSLG-1 exposed by leucocyte MPs and possibly explaining TF overexpression by monocytes [20].

7.2. Inflammation

The interplay between platelets and leukocytes leading to leuko-platelet conjugates could also contribute to an enhancement of the inflammatory process. [4]. Mesri and Altieri afforded the first hint suggesting an MP-mediated prothrombotic pathway under inflammatory conditions. This team showed that circulating LMPs were up-regulated in vivo and a stress signalling pathway was activated in endothelial cells, leading to increased TF activity [70]. Additionally, MPs can stimulate the release of proinflammatory endothelial cytokines, including IL-6 and MCP-1, and they can induce the expression of the ICAM-1, VCAM-1 and E-selectin cytoadhesins molecules [70]. MPs can also transfer pro-inflammatory monomeric CRP (mCRP) to the cell surface, suggesting a possible mCRP transport/delivery role of MPs in the circulation [43].

Monocyte-derived MPs augment the expression of active TF by endothelial cells, and a rapid increase in vWF expression on the cell surface promotes transient adhesion of non-activated platelets to the endothelial surface [71]. In vitro experiments demonstrate that these two major cell responses are under the control of reactive oxygen species delivered by MPs (see below). Two observations indicate a possible role for MPs in the coupling of apoptosis and inflammation vascular cell responses.

7.3. Endothelial damage and oxidative stress

NO, a key component in endothelial cell survival, is also targeted by MPs. Apoptotic T lymphocytes impair endothelium-dependent relaxation through eNOS down-regulation and caveolin-1 overexpression [72]. MPs derived from apoptotic smooth muscle cells can also diminish NO production, contributing to endothelial dysfunction [73]. Under oxidative stress, EMPs contain oxidised phospholipids that trigger monocyte–endothelial interactions. Similarly, PMPs up-regulate cytoadhesins expression in monocytes and endothelial cells through the delivery of arachidonic acid, thus reinforcing cell adhesiveness [74].

7.4. Atrial remodelling

Histological studies have tested the hypothesis that enhanced generation of extracellular matrix metalloproteins or their inhibitors could contribute to tissue remodelling in AF [75]. Because MPs shed by apoptotic cells harbour functional metalloproteinase [1], one cannot exclude that they contribute to the proteolysis of the fibrillar matrix, LA enlargement and LA overstretching. Both in vitro and in vivo, thrombin and CD40L have a strong synergistic effect on endothelial cell and MP expression of MMP-10, which may represent a new link between inflammation/thrombosis and tissue remodelling [76]. In vitro, endothelial-derived MPs are able to bind to extracellular matrix proteins, such as fibronectin, and show MMP-2 activity whilst their specific inhibitors TIMP-1 and TIMP-2 remain in the soluble phase [77]. Taken together, these results indicate that MPs interact with extracellular matrices, where they localize and activate MMP-2 to modify the pericellular tissue. These data support the potential relevance of MPs as actors in matrix and electrophysiological remodelling in AF settings [77]. In line with this view, a recent clinical study has underlined that MMP-2 and not CRP is the main predictor of AF recurrence [78]. The possible role of MPs in atrial tissue and its relationship with respect to circulating MPs are currently under investigation in our laboratory [79].

7.5. MPs and stroke

Because elevated circulating levels of procoagulant MPs are detected during AF or during procedures associated with thromboembolic complications, it was suggested that MPs might behave as potent effectors of the stroke process during AF. A first study in the early 1990s by Lee and co-workers suggested a direct contribution of PMPs to brain ischaemic complications and stroke [7]. Another recent study has confirmed the elevation of platelet-derived MPs during stroke [80]. Besides platelet activation marker, vWF, an endothelial cell damage marker, was found to predict the risk of stroke and vascular events [81]. More recently, circulating levels of EMPs were found to be related to infarct size and clinical outcome in patients with acute ischaemic stroke [8]. Uncertainty remains concerning the origin of EMPs during stoke, but they have at least three possible sources: (i) general endothelial stimulation, (ii) atrial stimulation and (iii) local brain ischaemia or stimulation. Since circulating procoagulant MPs are elevated at baseline in patients with permanent atrial fibrillation, the additional rise of platelet- and leukocyte-derived MPs induced by the RF procedure may substantially alter haemostasis. To the best of our knowledge, the impact of the per procedural rise in procoagulant MPs on thromboembolic complications is unknown. It should, however, be emphasised that in approximately 50% of ablation procedures, the levels of PMPs and LMPs peak in the same ranges as those observed in patients with acute myocardial infarction [82]. Overall, these data provide evidence that the monitoring of MPs may be a valuable tool when studying the thrombotic risk associated with AF, particularly during ablation procedures.

8. Conclusion

In the course of atrial fibrillation, several mechanisms, such as high ventricular heart rate, low or oscillatory shear stress, stretch, hypoxia, inflammation and oxidative stress, are potent inducers of apoptotic cell death, which leads to the shedding of procoagulant MPs within the vasculature. MPs behave as biological cargo enabling cell interactions, and they represent one of the elements linking cell death, inflammation, and thrombosis during AF. MPs may also be a determinant of stroke risk. Beyond the well-described procoagulant properties of MPs, recent advances suggest that MPs sequestered within tissue harbouring metalloproteinase or functional urokinase (one enzyme that enables the transformation of plasminogen into plasmin) contribute to the proteolysis of the fibrillar extracellular matrix and subsequent tissue remodelling. During AF ablation procedures, the additional increases in PMPs and LMPs levels testify to the extent of tissue damage and reflect a transient hypercoagulable state that may favour ischaemic stroke. Conversely, the observed decreases of several apoptotic markers some months after the restoration of sinus rhythm suggest that the extent of apoptosis is reversible and might enable the restoration of haemostasis. In clinical practice, the use of MPs to assess the thrombotic risk or atrial remodelling is of potential interest but requires further validation.

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The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology.

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Cellular damage, platelet activation, and inflammatory response after pulmonary vein isolation: A randomized study comparing radiofrequency ablation with cryoablation

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BACKGROUND Experimental data suggest that use of cryoablation in pulmonary vein isolation (PVI) is associated with less cell damage and less thrombus formation compared to radiofrequency (RF) energy.

OBJECTIVE The purpose of this study was to test the hypothesis that cryoablation significantly reduces markers of cell damage, platelet activation, and inflammation in patients undergoing PVI for treatment of atrial fibrillation (AF).

METHODS Sixty patients with symptomatic drug-resistant AF (age 56 ± 9 years, 48 males, 38 with paroxysmal AF) were randomly assigned to undergo PVI using either an open irrigated-tip RF catheter or a cryoballoon. Markers of cell damage (high-sensitive troponin T [hs-TnT], microparticles), platelet activation (platelet reactivity by aggregometry, expression of platelet surface proteins P-selectin and activated glycoprotein [GP] IIb/IIIa), and inflammatory response (high-sensitive C-reactive protein [hs-CRP]) were determined before and up to 48 hours after the procedure.

RESULTS PVI resulted in a significant rise in hs-TnT, microparticles, markers of platelet activation, and hs-CRP over time, with distinct temporal patterns for each parameter. However, after Bonferroni correction for repeated measurements, no significant differences were noted in these parameters between patients treated with cryoablation or RF energy. Procedural time was significantly shorter in patients treated with cryoballoon (177 ± 30 minutes vs 200 ± 46 minutes, $P = .03$), with no differences in fluoroscopic time, periprocedural complications, or success rate.

CONCLUSION Cryoablation and RF energy result in a comparable rise of markers of cell damage, platelet activation and inflammatory response. The data do not support the concept of an improved safety profile for cryoablation in PVI.

KEYWORDS Atrial fibrillation; Catheter ablation; Cryoballoon; Inflammation; Microparticles; Platelet activation; Tissue damage

ABBREVIATIONS **AF** = atrial fibrillation; **GP** = glycoprotein; **hs-CRP** = high-sensitive C-reactive protein; **hs-TnT** = high-sensitive troponin T; **LoB** = limit of blank; **MP** = microparticle; **PhtdSer** = phosphatidylserine; **PV** = pulmonary vein; **PVI** = pulmonary vein isolation; **RF** = radiofrequency

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Introduction

Transvenous pulmonary vein isolation (PVI) is a new effective treatment for atrial fibrillation (AF).[1–3] However, rare but potentially life-threatening complications such as thromboembolism,[4,5] pulmonary vein (PV) stenosis,[6], left atrium–esophageal fistula,[7] and inflammatory syndromes[8] have been described. In an attempt to reduce the incidence of complications, alternatives to the traditionally used radiofrequency (RF) energy have been investigated. Cryoenergy has been used since the mid-1970s for the surgical management of arrhythmia substrates.[9,10] It was first introduced as a catheter-based technology in 2000.[11] Cryoablation has been shown to result in lesions characterized by dense homogeneous fibrosis with well-delineated border zones and preservation of the extracellular matrix and tensile strength,[12] as well as to reduce endothelial disruption and thrombus formation.[13,14] Therefore, cryoablation appears to represent the ideal energy for safe creation of lesions in the left atrium. To further assess the differential effects of cryoablation vs RF energy in patients undergoing PVI, we measured markers of tissue damage, platelet activation, and inflammatory response before and up to 48 hours after the procedure. We hypothesized that cryoablation would be associated with a favorable safety profile compared to RF energy.

The first 2 authors contributed equally to this study. This study received financial support from CryoCath. Dr. Herrera Siklódy received consulting and speaking fees from Medtronic, Biosense Webster, and St. Jude. Dr. Arentz received consulting and speaking fees from Medtronic and St. Jude. ClinicalTrials.gov identifier: NCT00773539. **Address reprint requests and correspondence:** Dr. Claudia Herrera Siklódy, Herz-Zentrum Bad Krozingen, Südring 15, 79189 Bad Krozingen, Germany. E-mail address: claudia.h.siklody@gmail.com.

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Patients and methods

Between April 2007 and April 2009, 60 patients with highly symptomatic, drug-refractory paroxysmal or persistent episodes of AF referred to our center were randomly assigned to undergo transseptal PVI using either an open irrigated-tip radiofrequency catheter (RF group) or a cryoballoon catheter (Cryo group). Exclusion criteria were long persistent AF (>12 months), left atrial diameter >55 mm, intracardiac thrombi determined by transesophageal echocardiography, myocardial infarction or cardiac surgery in the previous 3 months, and previous ablation for AF. The study was approved by the ethics committee of the University of Freiburg (Germany), and written informed consent was obtained from all patients.

Electrophysiologic study and ablation procedure

Oral anticoagulation was stopped 2 days prior to the intervention to achieve an international normalized ratio between 1.8 and 2.5. Computerized tomography or magnetic resonance imaging of the left atrium was performed the day before the ablation procedure. In patients with persistent AF, cardioversion was performed 6 weeks before the ablation procedure under an intensified antiarrhythmic drug treatment to maintain the patient in sinus rhythm. Antiarrhythmic drug treatment was suspended the day of the ablation procedure, restarted the following day, and stopped after 2 months.

The interventions were performed with the patient under general anesthesia with remifentanil and propofol. Depth of anesthesia was measured by bispectral index. Transesophageal echocardiography was performed to exclude thrombi in the left atrium and to guide transseptal puncture. After transseptal puncture, heparin was given intravenously to maintain an activated clotting time between 300 and 350 seconds in both treatment groups. Surface ECGs and bipolar endocardial electrograms were monitored and stored on a computer-based digital amplifier/recorder system (CR Bard Inc, Lowell, MA, USA).

In the RF group, ablation consisted of circumferential PVI of both ipsilateral veins using the NavX System (St. Jude Medical, St. Paul, MN, USA) with an open irrigated-tip catheter (ThermoCool, Biosense Webster, Diamond Bar, CA, USA), with energy settings of 25–35 W. A detailed description of the ablation technique has been previously published.[15] In the Cryo group, PVI was performed under transesophageal echocardiographic guidance using the Arctic Front balloon (CryoCath, Medtronic, Minneapolis, MN, USA). The size of the balloon was chosen based on the patient's anatomy. PV diameters ≤18 mm were treated with a 23-mm balloon and those >18 mm were treated with a 28-mm balloon. The procedure has been described previously.[16] In brief, the balloon was inflated in front of each PV ostium and then advanced to achieve PV occlusion documented by color Doppler. Each PV was frozen twice over 5 minutes. During ablation of right-sided PVs, phrenic nerve pacing was performed from a catheter located in the superior vena cava in order to promptly detect phrenic nerve injury. In case of persistent conduction after cryoballoon ablation, electrical isolation was completed with focal applications using a 10-mm cryoablation catheter (Freezor MAX, CryoCath, Medtronic). RF procedures were performed by 4 operators (T.A., R.W., D.K., C.H.S.) and cryoballoon ablation procedures by 2 operators (T.A., C.H.S.). All operators had sufficient expertise in each technique.

Procedural success was complete electrical PVI for both groups, including a 30-minute waiting time from the last application to exclude early conduction recurrences. Disappearance of PV potentials was verified with a circular mapping catheter (Optima, St. Jude Medical). If potentials were still recorded, differential pacing was performed to prove entrance and exit block. No further ablation beyond PVI was allowed during the index procedure.

Postablation care and follow-up

After the procedure, treatment with a vitamin K antagonist (phenprocoumon) was restarted, and intravenous heparin was administered until the international normalized ratio was ≥2.5. Anticoagulation was continued for at least 3 months. On the first postprocedural day, patients underwent surface ECG, transthoracic echocardiography, and 24-hour Holter monitoring. Antiarrhythmic drugs were stopped 2 months after the ablation procedure. One and three months after the procedure, the patients were seen by the referring cardiologist for 24-hour Holter monitoring. Six months after the procedure, Holter monitoring and exercise testing were performed during a follow-up visit at our center. Patients with symptoms of AF but no documentation on ECG or Holter monitoring were provided with an event monitor for 8 weeks. Reevaluation of symptoms and 24-hour Holter monitoring were performed 12 months after the procedure, and all patients were contacted at the end of the study.

Freedom from AF was defined as no symptoms of AF and no atrial arrhythmias lasting >30 seconds on Holter monitoring after a single procedure, without antiarrhythmic drugs, and after a 3-month blanking period.

Laboratory examinations

Citrated blood samples were collected before and after ablation. Microparticles (MPs) were measured before transseptal puncture and immediately after ablation from the right atrium, and 24 hours and 48 hours after the procedure. High-sensitive troponin T (hs-TNT), platelet activation (platelet reactivity by optical aggregometry, expression of surface proteins P-selectin, and activated glycoprotein [GP] IIb/IIIa) and inflammatory markers (high-sensitive C-reactive protein [hs-CRP] were expected to show a later response and were assessed before transseptal puncture, and 8 hours, 24 hours, and 48 hours after the procedure.

Tissue damage

Direct tissue damage was determined by measurement of hs-TnT and procoagulant MPs.

High-sensitive cardiac troponin T

Cardiac troponin T in plasma was determined using the Elecsys Troponin T hs STAT assay on a COBAS autoanalyzer (Roche Diagnostics, Mannheim, Germany). The limit of blank (LoB) of the assay was 3 ng/L. Baseline levels of troponin T below the limit of blank were substituted by 0.5*LoB (ie, 1.5 ng/L) for statistical analysis. CK and CK-MB were not used for assessment of myocardial damage because enzyme activity is selectively damaged by RF energy but not by cryoenergy.[17]

Isolation of circulating MPs

Platelet-poor plasma containing circulating MP was obtained by double centrifugation as previously described[18] and stored at −80°C until assay. Procoagulant MPs were captured onto insolubilized annexin V, and their phosphatidylserine (PhtdSer) content was measured by functional prothrombinase assay. Variations in MP measurements were routinely less than 10% regardless of the pathologic status and the capture system.[19] Results were expressed as PhtdSer equivalent (PhtdSer Eq.) by reference to a standard curve. Duplicate measurements were performed in one single series of assays.

Cellular origin of circulating MP and determination of their procoagulant potential

Biotinylated annexin V has strong affinity for procoagulant phosphatidylserine accessible at the MP surface. Because phosphatidylserine is an ubiquitous feature of procoagulant MPs, the latter provide information on the total amount of circulating procoagulant MPs, regardless of their cellular origin. The quantity of MPs captured onto biotinylated monoclonal antibodies gives additional indication on their cellular origin and the cellular response to vascular stress. The following biotinylated monoclonal antibodies were used in this study: CD11a (leukocytes), CD105/endoglin (endothelial cells and leukocytes), and GP Ib (platelets). During MP quantitation, the background reactivities to isotypic irrelevant IgG were subtracted from all values.

Platelet reactivity

Light transmission aggregometry was performed using a 4-channel Bio/Data PAP4 aggregometer (Mölab, Hilden, Germany). Platelet-rich plasma was prepared by centrifugation of citrated venous blood (10-mL blood to be sampled) at 750*g* for 2 minutes and adjustment to 275–325 $\times$ 10^3 thrombocytes/μL by dilution with platelet-poor plasma from the same patient. Adenosine diphosphate (final concentration 5 μmol/L; Sigma, Munich, Germany) was added to induce aggregation. Light transmission in platelet-rich plasma was recorded for 5 minutes. Maximum aggregation within the incubation period and final aggregation 5 minutes after addition of stimulants were determined. The coefficient of variation of our light transmission aggregometry assay is 6.1%.[20]

Flow cytometry

Surface expression of P-selectin and activated GP IIb/IIIa were determined by a 4-channel flow cytometer equipped with a 488-nm argon laser (FACSCalibur, Becton Dickinson, Heidelberg, Germany) as previously described.[20] Platelets were stained in whole blood with fluorescein-isothiocyanate labeled PAC-1 (activated GP IIb/IIIa receptors; Becton Dickinson), phycoerythrin-labeled anti-CD62P (P-selectin; Beckman Coulter, Krefeld, Germany), and phycoerythrin-cyanin 5.1 labeled anti-CD41 (total GP IIb/IIIa receptors; Beckman Coulter, Krefeld, Germany) monoclonal antibodies. Whole blood was incubated with antibodies for 30 minutes. Thereafter, 300 μL of phosphate-buffered saline (Sigma-Aldrich, Taufkirchen, Germany) containing 1% paraformaldehyde was added for dilution and fixation. Flow cytometric analysis was performed within 24 hours, and 10,000 events were analyzed from each sample.

Flow-Jo software (version V8.7.1 X; Tree Star Inc, Ashland OR, USA) was used for analysis of raw data obtained from the flow cytometer.

Inflammatory response

A latex particle–enhanced immunoassay on a COBAS autoanalyzer (CROPL3 assay, Roche Diagnostics) was used for determination of hs-CRP. The LoB of the assay was 0.3 mg/L.

Statistical analysis

Based on a previous study comparing changes of platelet function between cryoablation and RF ablation of right-sided cavotricuspid isthmus,[21] we assumed a 75% relative reduction in the number of activated platelets with cryoablation. A power calculation using a 2-tailed α value of 0.05 and a power of 80% resulted in a sample size of 30 patients per group. Continuous variables are given as mean $\pm$ SD and were compared by Student *t*-test. Time courses of parameters vs baseline were compared using a general linear model and applying a Bonferroni correction for repeated measures. Total effect of the ablation procedures on biomarkers, MPs, and parameters of platelet reactivity were assessed by calculation of the area under the curve within 48 hours using the linear trapezoidal rule. Categorical variables were compared by χ^2 analysis or with the Fisher exact test for cell size <5. Freedom from recurrent AF was determined by Kaplan-Meier analysis with the log-rank test. The PASW software package (version 18; SPSS Inc, Chicago, IL, USA) was used. P <.05 using the 2-tailed test was considered significant.

Results

The clinical and baseline characteristics of patients treated with cryoablation or RF ablation are listed in Table 1. After an AF history of 5.2 $\pm$ 3.6 years and a mean number of 2.8 $\pm$ 1.0 unsuccessful antiarrhythmic drugs, 38 patients presented with paroxysmal AF and 22 with persistent AF. Nineteen patients (32%) had a history of structural heart disease, and 27 (45%) had arterial hypertension.

Procedural parameters

Procedural time was significantly shorter using the cryoballoon (177 $\pm$ 30 minutes) compared to using RF catheters

Table 1 Patient characteristics

	Cryoballoon (n = 30)	Radiofrequency ablation (n = 30)	*P* value
Mean age (y)	57 ± 8	56 ± 10	.517
Males	25 (83.3%)	23 (76.7%)	.519
Paroxysmal AF	21 (70.0%)	17 (56.7%)	.284
History of AF (y)	4.2 ± 2.7	5.6 ± 4.2	.124
Failed AAD	2.9 ± 1.8	2.7 ± 2.2	.658
Organic heart disease (%)	8 (26.7%)	11 (36.7%)	.405
	CHD 3 HOCM 1 Tachymyopathy 1 Valvular 2 DCMP 1	CHD 4 HCMP 1 Tachymyopathy 2 Valvular 4	
Hypertension (%)	13 (43.3%)	14 (46.7%)	.795
Left atrium (mm)	41.4 ± 4.3	40.0 ± 5.5	.280

AAD = antiarrhythmic drugs; AF = atrial fibrillation; CHD = coronary heart disease; DCMP = idiopathic dilated cardiomyopathy; HCMP = hypertensive cardiomyopathy; HOCM = hypertrophic cardiomyopathy.

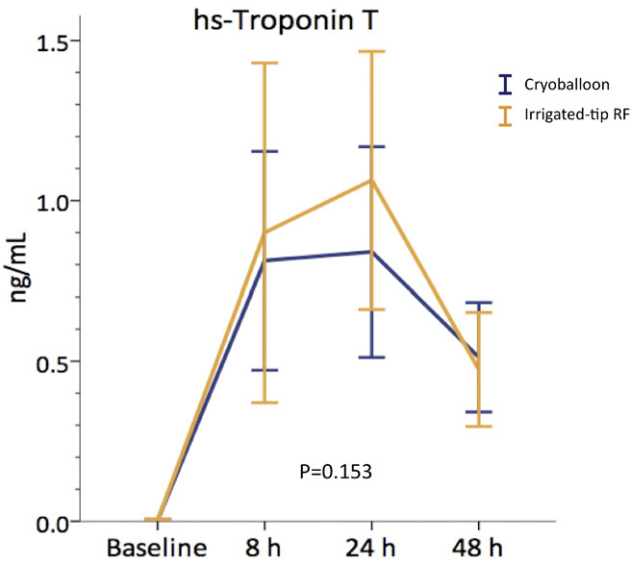

Figure 1 Time course of high-sensitive (hs) troponin T levels in both groups. Curves display mean and standard deviation (cryoenergy in *blue,* irrigated-tip radiofrequency [RF] energy in *yellow).*

(200 ± 46 minutes, $P = .028$), but fluoroscopy time was similar (38 ± 12 minutes vs 37 ± 16 minutes respectively, $P = .827$). RF was applied over 52 ± 21 minutes to achieve isolation of all 4 veins (total delivered energy 87,584 ± 35,953 J). In the Cryo group, 21 patients were ablated using a 28-mm balloon, 7 with a 23-mm balloon, and 2 using both sizes. Ten patients required additional focal cryoablations to complete PVI. Cryoenergy was delivered with the balloon over 44 ± 6 minutes, and with the focal cryocatheter in 10 patients over 19 ± 12 minutes. All PVs could be successfully isolated. The fluid balance during the procedure was comparable in both groups (+2.0 ± 1.0 L in the Cryo group vs +2.2 ± 1.3 L in the RF group, $P = .512$). None of the patients in the RF group experienced acute postprocedural complications, whereas in the Cryo group 1 patient developed a groin hematoma, 1 a femoral pseudoaneurysm, and 2 a transient phrenic nerve palsy. Only 1 patient (in the Cryo group) complained of pericardial pain. He presented with no pericardial rub, and routine echocardiography showed no pericardial effusion. Procedural parameters are summarized in Table 2.

Table 2 Procedural parameters

	Cryoballoon (n = 30)	Radiofrequency ablation (n = 30)	*P* value
Mean procedural time (min)	177 ± 30	200 ± 46	.028
Mean fluoroscopy time (min)	37.7 ± 11.8	37.0 ± 16.2	.827
Complications	4 (13.3%) 1 groin bleeding 1 pseudoaneurysm 2 phrenicus	0 (0.0%)	.052

Cellular damage

Myocardial damage as indicated by a significant increase in hs-TnT was observed both after cryoablation and after RF ablation (Figure 1). The total release of hs-TnT over time as calculated by the area under the time course up to 48 hours after ablation provided similar figures for both methods (32.7 ± 11.5 ng/mL*h^{-1} vs 37.8 ± 15.3 ng/mL*h^{-1} for Cryo and RF, respectively; $P = .153$). Compared to patients who did not need supplementary focal cryoablations, patients who received touch-ups displayed a slightly lower (but not statistically significant) TnT curve (29.8 ± 7.2 ng/mL*h^{-1} vs 33.8 ± 12.7 ng/mL*h^{-1}, $P = .41$).

Both cryoablation and RF induced release of procoagulant MPs captured onto annexin V, reaching a peak 24 hours after the procedure and decreasing thereafter. Procoagulant platelet-derived MPs followed the same pattern. A progressive increase in procoagulant leukocyte-derived MPs was demonstrated, with a significant increase of leukocyte-derived MPs observed between 24 h and 48 hours after the procedure. Conversely, no significant elevation of endothelial-derived MPs could be evidenced following AF ablation. There were no significant differences in the shedding of procoagulant MPs between Cryo and RF ablation (Figure 2).

Platelet activation

Significant activation of platelets could be detected 24 and 48 hours after ablation in both groups (optical aggregometry, surface expression of P-selectin and GP IIb/IIIa). No significant differences were found between treatment groups (Figure 3).

Inflammatory response

There was a significant inflammatory response after both cryoablation and RF as represented by an increase in hs-

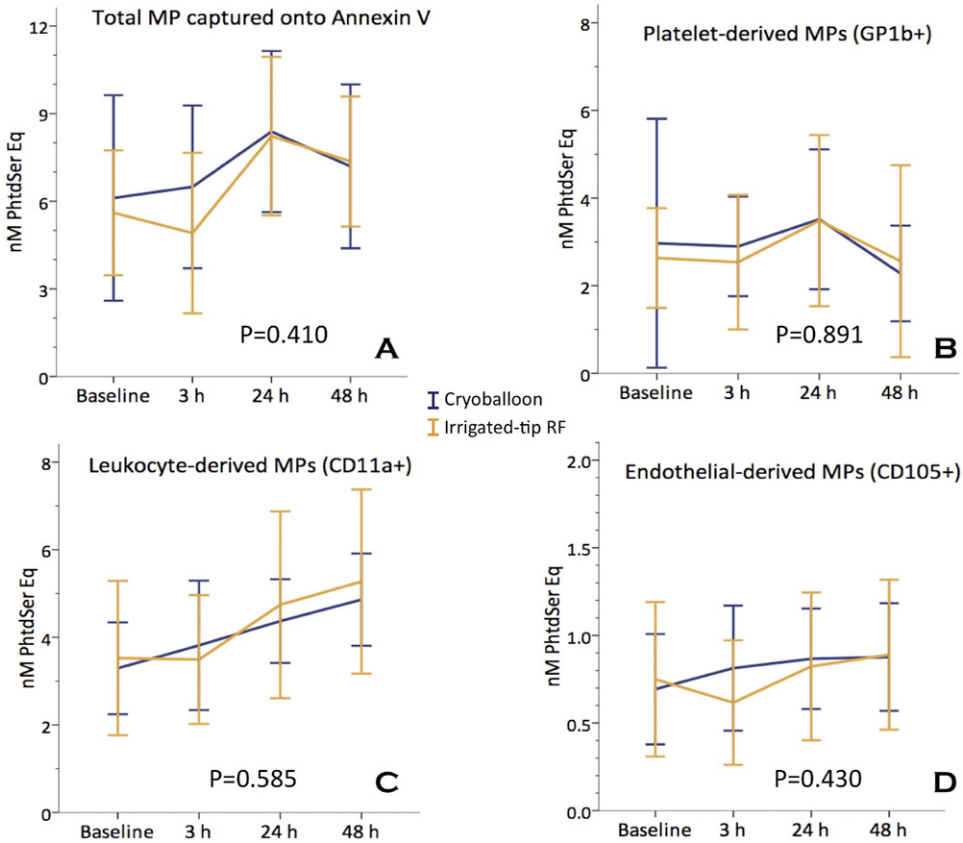

Figure 2 Rise of the different microparticle (MP) types in both groups. Curves display mean and standard deviation (cryoenergy in *blue,* irrigated-tip radiofrequency [RF] energy in *yellow).* **A:** Total circulating microparticles. **B:** Platelet-derived microparticles. **C:** Leukocyte-derived microparticles. **D:** Endothelial-derived microparticles.

CRP 24 and 48 hours after the procedure. There was a trend toward a higher peak value after 48 hours after RF ablation (28.6 ± 22.7 mg/L vs cryoablation 20.5 ± 23.3 mg/L, P = .175), with no statistically significant differences after correction for repeated measurements (Figure 4).

Table 3 provides an overview of the global effect of ablation on the different laboratory parameters in both groups.

Follow-up

After median follow-up of 12 months (range 3–29 months), 24 patients (80%) after RF ablation and 19 (63%) after cryoballoon ablation were free of AF without antiarrhythmic drugs (P = .15; Figure 5). One patient in the Cryo group presented with typical AV nodal reentrant tachycardia that was successfully ablated 2 months after the index procedure; another patient had a single recurrence of AF 10 months after catheter ablation in the context of a manifest hyperthyroidism. Two patients in the RF group developed an atypical atrial flutter: one had a right-sided scar flutter and the other one a focal atrial tachycardia from the superior vena cava. Both of these patients could be successfully ablated. The rest of the patients (9 in the Cryo group and 4 in the RF group) presented with an AF recurrence and underwent reisolation of the PV.

Discussion

In this randomized study, we showed that PVI procedures produce tissue damage, inflammatory response, and platelet activation. Both cryoenergy and RF energy resulted in a comparable rise in all laboratory markers, not supporting a safety benefit of cryoenergy over RF.

Influence of lesion size

This finding contrasted with our previous findings, suggesting reduced platelet activation after ablation of cavotricuspid-dependent atrial flutter using cryoenergy.[21] However, in that study, troponin release was significantly lower after cryoablation than after RF ablation, suggesting a lesser extent of lesions. In addition, recently published randomized trials using different energies for treatment of typical right-sided flutter showed a significantly higher recurrence rate after focal cryoablation than after RF ablation, indicating smaller and less stable lesions.[22] Tse et al[23] also reported significant platelet activation after PVI using RF ablation but not after cryoablation. However, in their work, only the upper PVs were segmentally isolated using a focal cryocatheter. In contrast, performance of circumferential PVI using the cryoballoon, as in the present study, produced a significant rise of troponin, comparable to the one observed after RF ablation. Cryolesions created with this new ablation tool, therefore, seem to be larger than those created

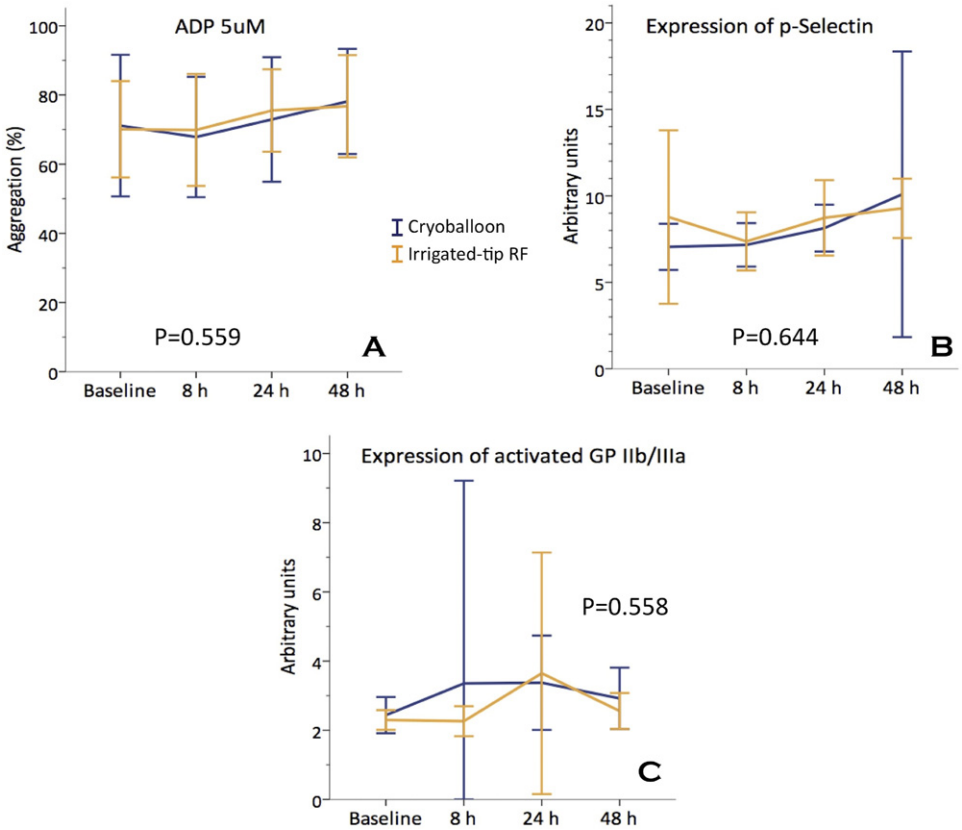

Figure 3 Time course of platelet activation parameters in both groups. Curves display mean and standard deviation (cryoenergy in *blue,* irrigated-tip radiofrequency [RF] energy in *yellow).* **A:** Adenosine diphosphate-induced light transmission aggregometry. **B:** Surface expression of P-selectin **C:** Surface expression of activated GP IIb/IIIa.

with focal catheters. Myocardial necrosis has been described to induce platelet activation in other clinical situations.[24] In addition to the size of the myocardial lesion (as assessed by levels of hs-TnT), its biological consequences in terms of release of MPs, platelet activation, and inflammation was monitored for the first time in a clinical study. Our data indicate that endothelial damage and consecutive platelet activation and inflammation are less dependent on the type of energy used and more on the size of the lesion induced.

Microparticles

Circulating procoagulant MPs are small membrane vesicles that are shed from virtually all cells in response to activation, thermic injury, and/or apoptosis. Apart from being a reliable probe of vascular injury, circulating procoagulant MPs, mainly of platelet, leukocyte, and endothelial origins, provide an additional phospholipidic surface for the assembly of blood coagulation factors, thereby promoting the coagulation cascade and thrombin generation. Using real-time intravital microscopy to monitor thrombus growth at sites of vascular injury, several reports have highlighted that the swift recruitment of leukocyte-derived MPs at the edge of the thrombus are mandatory for its development.[25,26] These properties suggest an important procoagulant potential of circulating procoagulant MPs, which could contribute to initiation or perpetuation of thrombotic clinical conditions. Being detectable at low levels in healthy individuals, elevated levels of circulating procoagulant MPs could be evidenced in various thrombotic settings such as AF.[27] In addition, in patients with acute ischemic stroke, circulating levels of MPs were found to be related to infarct size and clinical outcome.[28]

We recently described a similar pattern of vascular damage and intense platelet and leukocyte stimulation after RF ablation of atrial flutter.[18] The present study shows the same results after PVI procedures. Moreover, the extent of tissue damage and subsequent prothrombotic state induced by RF energy appeared in the same range of that observed with cryoenergy, explaining the occurrence of thromboembolic complications using either one of these energies.[29–31] A strict anticoagulation regime is therefore mandatory after PVI using RF energy as well as cryoenergy.

Platelet activation

RF ablation procedures bear a low but clinically significant risk of thromboembolic events.[32] Previous studies have already shown that RF ablation induces activation of platelet function.[21,33] *In vitro* studies pointed out that cryolesions displayed lower thrombus formation than RF lesions.[14] Further clinical studies found lower platelet activation using cryoenergy compared to RF energy,[21,23] suggesting that

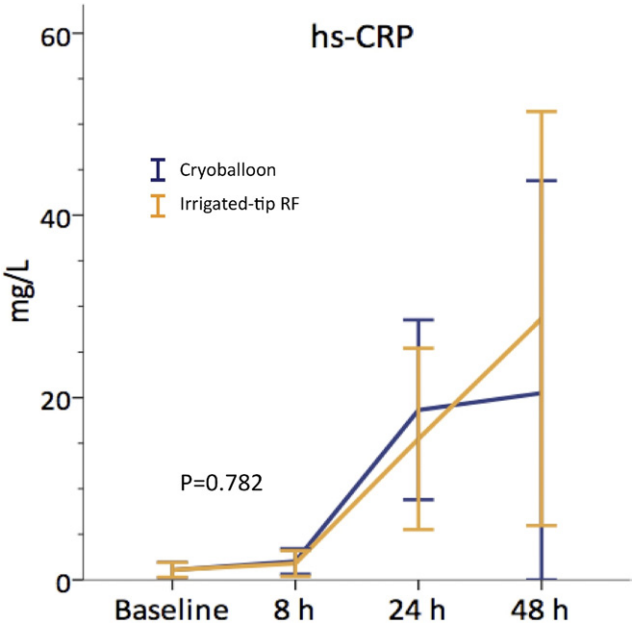

Figure 4 Time course of high-sensitive (hs) C-reactive protein levels in both groups. Curves display mean and standard deviation (cryoenergy in *blue,* irrigated-tip radiofrequency [RF] energy in *yellow).*

cryoenergy might be a safer alternative to RF energy, especially for left-sided ablations.

In our study, we could once more confirm that catheter ablations result in enhanced platelet activation. However, our data showed a similar level of activation using both cryoenergy and RF energy, probably related to the size of the lesions created by the cryoballoon, comparable to those created by RF catheters.

Inflammatory response

Previous studies using RF have demonstrated that ablation triggers inflammation response, frequently associated with generalized edema.[8] Histopathologic studies have established that RF ablation induces necrosis followed by inflammatory infiltrates leading to a fibrotic scar.

In the present study, we confirmed that leukocyte-derived MPs, a reliable probe of leukocyte stimulation, and hs-CRP significantly rose following ablation. However, no differences could be evidenced between patients ablated with RF energy or cryoenergy.

Clinical implications

Cryoballoon ablation is a powerful method for creating circumferential lesions around the PVs, as indicated by significant hs-TnT release and a clinical success rate comparable to PVI performed with a standard irrigated-tip catheter. Moreover, procedural time was significantly shorter using the cryoballoon. However, the safety arguments initially favoring the use of cryoenergy might not be valid when using the cryoballoon. We could not detect any important difference in tissue damage, platelet activation, or inflammatory processes after cryoballoon or irrigated-tip RF procedures. These results are consistent with the recently presented clinical data from the STOP-AF trial comparing cryoballoon PVI with a medical treatment.[30] In this study, cryoballoon PVI was highly effective, with a clinical success rate of 70%, but showed relatively frequent complications such as phrenic nerve palsy (22/163 patients [13.5%]), PV stenosis (5 patients [3.1%]), and thromboembolic events (7 patients [4.3%]), which were not anticipated previously. In our study, clinical complications (1 groin bleed, 1 pseudoaneurysm, 2 transient phrenic nerve palsies) occurred in the Cryo group only. The higher incidence of groin complications may be due to the use of a bigger 15F sheath compared to the 8.5F sheath used for the RF catheter.

In summary, PVI with the cryoballoon requires the same caution as use of an irrigated-tip RF catheter in order to prevent complications. Ablation should be performed in the antrum of the vein to prevent PV stenosis. A sufficient anticoagulation regimen should be assured. In addition, an antiplatelet treatment might be useful in order to preclude thromboembolic events.[21] Moreover, pacing of the phrenic nerve should be performed during cryoablation of right-sided veins to avoid phrenic nerve palsy.

Study limitations

Cryoballoon and irrigated-tip RF PVI involve use of a sheath and a catheter of very different sizes. Those technical disparities could have influenced platelet activation and inflammation parameters.

Intraprocedural blood sampling was performed from the vascular sheath in the right atrium and postprocedural blood sampling from a peripheral vein. The site and method of sampling could have influenced the measurement of biomarkers, but it would have affected both groups in the same manner.

Table 3 Summary of global effect of ablation (area under curve) on all different laboratory parameters

	Cryoballoon (n = 30)	Radiofrequency ablation (n = 30)	*P* value
High-sensitive troponin T ($ng/mL*h^{-1}$)	32.7 ± 11.5	37.8 ± 15.3	.153
Total MP captured onto annexin V (nM $PhtdSer*h^{-1}$)	362 ± 107	341 ± 91	.410
Platelet-derived MPs (nM $PhtdSer*h^{-1}$)	146 ± 51	144 ± 68	.891
Leukocyte-derived MPs (nM $PhtdSer*h^{-1}$)	207 ± 44	217 ± 87	.585
Endothelial-derived MPs (nM $PhtdSer*h^{-1}$)	40.8 ± 12.5	37.7 ± 17.2	.430
Optical aggregometry	3292 ± 898	3410 ± 597	.559
Surface expression of P-selectin	398 ± 108	409 ± 78	.644
Surface expression of activated GP IIb/IIIa	152 ± 87	140 ± 75	.558
High-sensitive C-reactive protein ($mg/L*h^{-1}$)	648 ± 449	680 ± 436	.782

GP = glycoprotein; MP = microparticle; PhtdSer = phosphatidylserine.

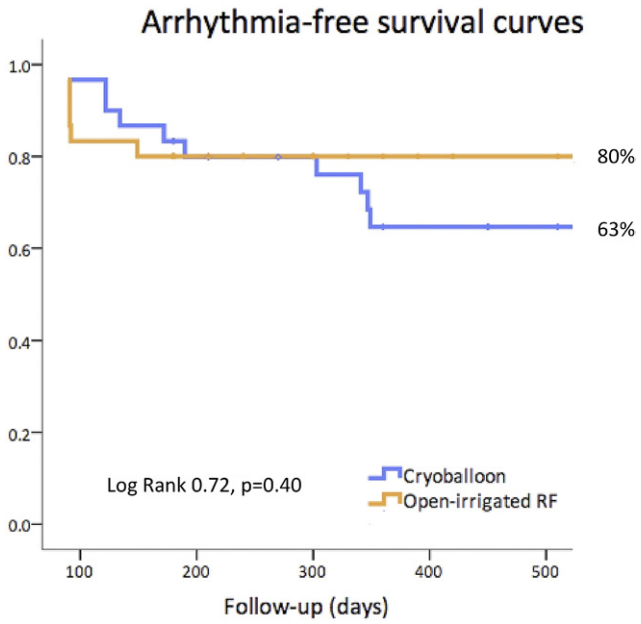

Figure 5 Arrhythmia-free survival curve in both groups (cryoenergy in *blue,* irrigated-tip radiofrequency [RF] energy in *yellow).*

Our study was not powered to assess clinical differences between the two energies. Large-scale prospective randomized trials comparing cryoballoon and irrigated-tip catheters for PVI using clinical endpoints are necessary.

Conclusion

In this prospective randomized trial, the cryoballoon and the irrigated-tip RF catheter induced a similar rise in systemic sensitive laboratory markers of cell damage, platelet activation, and inflammation after PVI. Lesion size rather than ablation method appears to be responsible for the magnitude of the systemic procoagulant and inflammatory response.

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Radiofrequency Catheter Ablation of Atrial Flutter Induces the Release of Platelet and Leukocyte-Derived Procoagulant Microparticles and a Prothrombotic State

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Background: *To assess the extent of endothelium, platelet, and leukocyte damage and coagulation activation induced by radiofrequency catheter ablation (RF) of atrial flutter. In the vasculature, procoagulant microparticles (MPs) are reliable markers of vascular damage. They provide an additional phospholipidic surface, enabling the assembly of the enzyme complexes of blood coagulation and consequent thrombin generation.*

Methods: *MPs were measured in the venous blood of 33 patients with isthmus-dependent atrial flutter undergoing RF before (RF_0), immediately after (RF_1), and at day 1 (RF_2) thereafter. Concentrations of PAI-1, vWF, and D-dimers were simultaneously determined. MPs procoagulant activities were determined using a functional prothrombinase assay. RF induces an early rise of platelet-derived MPs (platelet), vWF Ag, and D-dimers levels, which is concomitant with the decrease of PAI-1 concentrations. Conversely, no significant changes in endothelial-derived MPs could be evidenced. At RF_2, sustained elevation of leukocytes-derived MPs, vWF, and D-dimers testified to an ongoing prothrombotic status.*

Conclusion: *RF ablation of common flutter induces a prothrombotic state and the release of platelet and leukocyte-derived procoagulant microparticles. Whereas this activation of blood coagulation could be viewed as clinically marginal in right-sided procedures, its relevance in left-sided procedures should be established. (PACE 2009; 32:193–200)*

apoptosis, endothelial activation, leukocyte, inflammation, coagulation

Introduction

Radiofrequency catheter ablation (RF) is a widely used treatment of most cardiac arrhythmias including atrial flutter.[1–3] The need for radiofrequency ablation to achieve a critical lesion size has justified the development of catheters enabling the creation of lesions of sufficient volume and depth through tissue contact. Although such characteristics could be considered beneficial with respect to the success rate of the procedure, the electrode-blood pool interface could also lead to significant tissular injury and consequent blood coagulation activation. As a consequence, energy delivery can lead to clot formation, with thrombotic events including stroke being reported in 0.6–5.6% of RF procedures, especially in left-sided ones.[3] The cause of these complications remains incompletely understood, although several groups have suggested excess thermal injury or repeated energy deliveries as potent contributors. Indeed, RF induces detectable tissue damage including myocardial necrosis and platelet activation,[2,3] whereas its impact on endothelial injury and leukocyte activation—key participants in the initiation of thrombotic processes—remains poorly investigated. When exposed to hyperthermia, cell injury occurred through increased membrane permeability and remodeling.[4] The rise of the cytosolic Ca^{2+} concentrations allows cytoskeleton cleavage through calpain activation and the exposure at the external leaflet of the membrane of procoagulant aminophospholipid normally located at the inner one. Membrane budding

**Equal contribution.

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is ultimately resolved through the release of microparticles (MPs) that expose phosphatidylserine (PhtdSer), a procoagulant aminophospholipid. In the vasculature, MPs shed by stimulated or apoptotic cells are hallmarks of tissue damage, the degree of shedding correlating to the proportion of stimulated cells. Having long been viewed as cellular dust, MPs are now considered a key component in the hemostatic response. Indeed, when exposed to blood flow, MPs constitute a reservoir of highly thrombogenic material. They provide an additional phospholipidic surface necessary for the assembly of the characteristic enzyme complexes of the coagulation cascade and disseminate tissue factor activity, the main cell inducer of blood coagulation.[5,6] Widely described in atherothrombotics diseases,[5–12] recent advances suggest a role for MPs in the procoagulant state associated with supraventricular tachyarrhythmia.[13] Moreover, high levels of platelet and endothelial-derived MPs were identified during stroke[14–16] and were associated with infarct size and clinical outcome.[15]

In this prospective study, we sought to determine the impact of RF ablation using cooled-tip catheters or standard ones on MPs phenotypes and procoagulant activities. In addition, the link between procoagulant MPs levels and the procedure characteristics (energy delivered, burns numbers) was investigated.

Ablation Therapy

Two 7-French sheaths were inserted into the right femoral vein using Seldinger technique. A deflectable duodecapolar catheter (Stablemapper, Medtronic, Minneapolis, MN, USA) was positioned in the lateral right atrium with the distal pole in the coronary sinus. Ablation was performed with a nonirrigated 8-mm tip RF ablation catheter (Stinger 8 mm, Bard, Lowell, MA, USA) or with an open-irrigated 4-mm tip RF ablation catheter (Celsius thermo-cool, Biosense Webster, Diamond Bar, CA, USA). The catheter was irrigated using 0.9% saline infusion at a pump flow rate of 17–40 mL/min during RF delivery (Cool Flow, Biosense Webster) to achieve a temperature of 40°C. A linear lesion from the tricuspid valve annulus to the inferior vena cava was achieved by point-by-point ablation for a maximum duration of 60 seconds each until a complete bidirectional block of cavotricuspid isthmus was present and persistent over 30 minutes. RF energy was delivered using a Stockert generator (Biosense Webster) for the irrigated catheter with a maximum power output of 35 W and temperature limit of 48°C and using an AttakR-II (Medtronic Inc.) for the nonirrigated catheter with a maximum temperature of 60°C and a power limited to 50 W. All patients received local anesthesia at venous access site and were awake during treatment.

Procedure and Blood Sampling

Blood samples were drawn by puncture of an antecubital vein with a 19-gauge needle using minimal venous occlusion before (RF_0), immediately after (RF_1), and the day after (RF_2). To ascertain that the generation of procoagulant MPs during RF ablation was the result of induced cardiac tissue damage and not an artifact due to platelet and cell activation owing to blood sampling through the vascular access, we have verified in 11 patients undergoing radiofrequency ablation for atrioventricular nodal reentrant tachycardias or accessory pathways with minimal tissue damage (burn numbers = 5.2 ± 4.8, mean burn duration = 3.9 ± 3.4 [min]) that no significant elevation of procoagulant MPs captured onto Annexin V occurred during the procedure. To avoid artefactual platelet activation and subsequent thrombin generation, the first 5 mL of blood were systematically discarded. Blood samples were then collected on 12.9 mM citrate. All blood samples were processed within 2 hours. Platelet-poor plasma samples (PPP) containing circulating MPs were obtained by double centrifugation as previously described and immediately frozen at −80°C.

Materials

Monoclonal antibody (mAb) to human platelet glycoprotein GP-Ib was previously characterized and biotinylated.[17] Monoclonal antibody (mAb) to human CD105 (RD Systems, USA) was biotinylated in the lab as previously described.

Biotinylated mAb to CD31 was from CALTAG Laboratories (Burlingame, CA, USA), biotinylated mAb to CD11a and corresponding irrelevant biotinylated immunoglobulin were from Leinco Technologies (Ballwin, MO, USA). Human prothrombin (FII) was from Hyphen BioMed (Andresy, France), activated factor X (FXa) from Biogenic S.A. (Mauguio, France). Factor Va was from American Diagnostica (Stamford, CT, USA). Biotinylated recombinant human annexin V was prepared as reported elsewhere.[17] High binding capacity streptavidin-coated microtitration plates and Chromozym TH were from Roche Diagnostics (Mannheim, Germany).

Isolation of Circulating Microparticles, Search for Their Cellular Origin, and Determination of Their Procoagulant Potential

Two MP isolation procedures by capture on either insolubilized annexin V or specific antibodies were used as described elsewhere.[18] Both ligands (antibodies or annexin V) were

separately insolubilized onto streptavidin-coated microtitration plates before incubation with patients' PPP. This capture system allows extensive washing, ensuring specific MP binding.[18] Each ligand has its advantage in the assessment of circulating MPs and comparison of their circulating levels in clinical subsets. Biotinylated Annexin V is a reliable probe of PhtdSer accessibility at MP surface. Because PhtdSer is an ubiquitous feature of procoagulant MPs, the quantity of MPs captured on annexin-V provides information on the total amount of circulating procoagulant MPs, regardless of their cellular origin. The quantity of MPs captured onto biotinylated monoclonal antibodies gives additional indication on (i) the cellular origin and (ii) on the cell response to vascular stress, provided that it is reflected by membrane antigens eventually borne by MPs. In this study, biotinylated monoclonal antibodies to various cell types or phenotypes (anti-CD11a for leukocytes, anti-CD105 [endoglin] for endothelial cells, anti-CD31, a dual probe for apoptotic endothelial cells and platelets, anti-GP-Ib for platelets) were used.[18] Identical batches of antibodies were used throughout all assays. Background values in the quantification of MPs (see below) were obtained with corresponding irrelevant IgGs and subtracted.

After PPP incubation and washings, annexin V- and antibody-captured procoagulant MPs were quantified by functional prothrombinase assay. The assay measures the PhtdSer content of MPs by virtue of its ability to promote the activation of prothrombin to thrombin in a calibrated purified system ensuring that PhtdSer is the rate-limiting parameter of the reaction. Briefly, immobilized MPs were incubated with factor Xa (50 pmol/L), factor Va (360 pmol/L), prothrombin (1.3 μmol/L), and 2.3 mmol/L $CaCl_2$ in TBS-Ca^{2+} containing 45 μM human serum albumin at 37°C. Generated thrombin was assessed by the linear absorbance changes resulting from the cleavage of a chromogenic substrate (380 μmol/L). Results were expressed as PhtdSer equivalent (PhtdSer Eq.) by reference to a standard curve constructed using liposomes of known concentration, containing 33% PhtdSer and 67% phosphatidylcholine (mol/mol).[18] Duplicate measurements were performed in all cases.

This solid-phase capture combined with prothrombinase assay provides a functional assessment of the procoagulant abilities of the isolated MPs, regardless of the capture ligand. It does not allow the capture of lipoproteins[8] and the presence of TF on captured MPs does not alter values corresponding to PhtdSer content. Truly soluble forms of membrane antigens do not generate prothrombinase activity.[18] No direct comparison between capture by annexin V and antibodies can be afforded because affinities for the respective ligands and incubation times are different in the two capture steps. Variations in measurements using this assay are routinely below 10% (measurement of an identical PPP sample on 15 separate occasions), regardless of the pathological issue and of the capture system (annexin V or antibodies).

Miscellaneous Measurements

Quantitative determination of plasma von Willebrand factor antigen (vWF:Ag) and D-dimers was realized by immunoturbidimetric assays. PAI-1 plasma concentrations were determined using a synthetic chromogenic substrate method.

Statistical Analysis

Results are expressed as mean ± SD. Statistical analysis was carried out using Wilcoxon test or the Mann-Whitney U test for nonparametric variables, depending on paired or unpaired groups. For normally distributed data, the paired or unpaired Student's *t*-test was used. Fisher's exact test was used for categorical variables. Correlation was assessed using a Spearman rank coefficient test. A p value < 0.05 was considered statistically significant. All analyses were performed using SPSS 13.0 software (Chicago, IL, USA).

Results

Patients

Clinical and procedural characteristics and treatments are listed in Table I. Paroxysmal common atrial flutter was present in 11 patients, persistent flutter in 22 patients. Cavotricuspid ablation was considered successful in 28/33 patients. No clinical thromboembolic events could be recorded during the procedure or at the 30-day follow-up. Markers levels or kinetics were not significantly influenced by the type of anticoagulation (OAC vs UFH). Furthermore, we verified that markers were not significantly elevated at baseline in patients with persistent flutter.

Kinetics of vWF, D-Dimers, and PAI-1 after Radiofrequency Ablation in the Whole Population

With respect to the values measured at baseline, RF ablation induces the release of vWF antigen, a marker of endothelium damage, immediately after the end of the procedure and reaching a peak the day after. The vWF levels at peak were found related to endothelial-derived MPs ($CD105^+$-MPs) levels ($r = 0.469$; $P = 0.006$) but not to plaletet-derived MPs ($GPIb^+$-MPs). Baseline levels of vWF were the only predictor of D-dimer elevation (OR 1.023 95% CI [1.003–1.043]; $P = 0.025$). The elevation of D-dimers—a marker of ongoing activation of coagulation and

Table I.

Baseline Demographic, Clinical and Procedural Characteristics of the Study Cohort

	Whole Population (N = 33)	RF Ablation Using Irrigated Catheter (N = 19)	RF Ablation Using Nonirrigated Catheter (N = 14)	P Value
Age	66 ± 16	64 ± 6	69 ± 6	0.027
Men	24 (72%)	14 (73%)	10 (71%)	1
Arterial hypertension	16 (48)	10 (52)	6 (42%)	1
Diabetes mellitus	7 (21)	6 (31%)	1 (7%)	0.195
Dyslipidemia	12 (36)	7 (36)	6 (42%)	1
Persistent atrial flutter	23 (69%)	10 (52%)	13 (92%)	0.004
OAC before	24 (72%)	16 (84%)	8 (57%)	0.122
Left ventricular ejection fraction	56 ± 14	52 ± 17	61 ± 7	0.068
Left atrial diameter (mm)	46 ± 8	46 ± 10	45 ± 5	0.581
Burn duration (min)	21 ± 13	25 ± 14	15 ± 7	0.029
Temperature	58 ± 12	50 ± 10	68 ± 7	<0.001
Burn numbers	25 ± 17	30 ± 19	16 ± 8	0.010
Maximum power (W)	45 ± 11	47 ± 5	43 ± 16	0.464
Success	28 (85%)	14 (73%)	14 (100%)	0.057

fibrinolysis—was more substantial at RF_1 than later (RF_2). Conversely, a significant fall of PAI-1 concentrations, an inhibitor of fibrinolysis processes, could be observed at RF1, values being at RF_2 similar to those observed before the procedure (Fig. 1). At RF_1, D-dimers and PAI-1 concentrations were found inversely related ($r = -0.64$; $P < 0.01$).

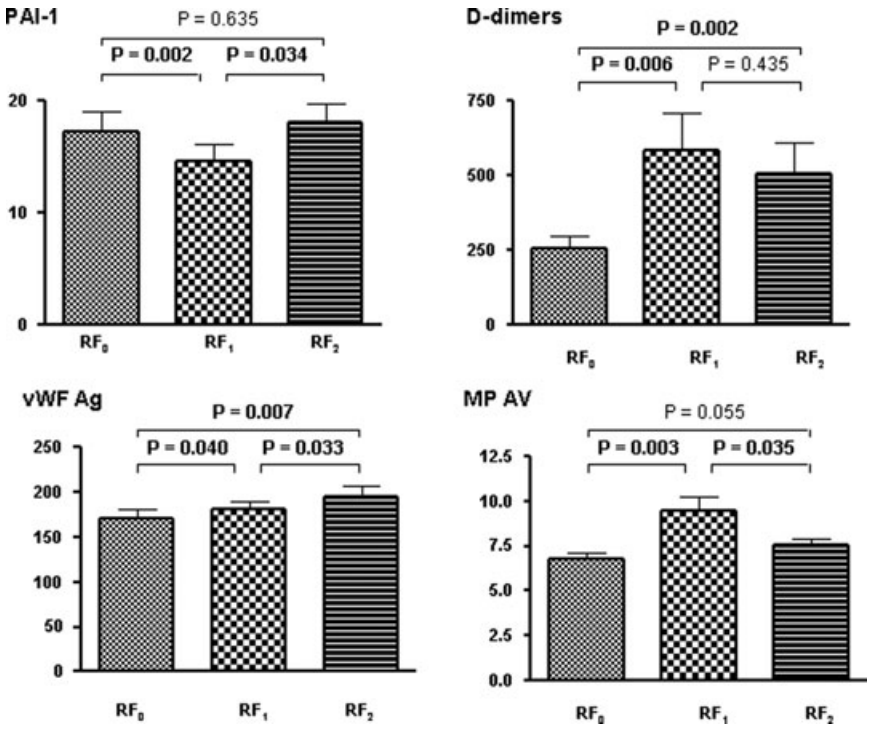

Figure 1. *Plasma levels of PAI-1, D-dimers, vWF Ag, and procoagulant microparticles captured onto annexin V (MP AV) before, immediately after (RF1), and at day 1 (RF2) after ablation in patients treated with radiofrequency ablation.*

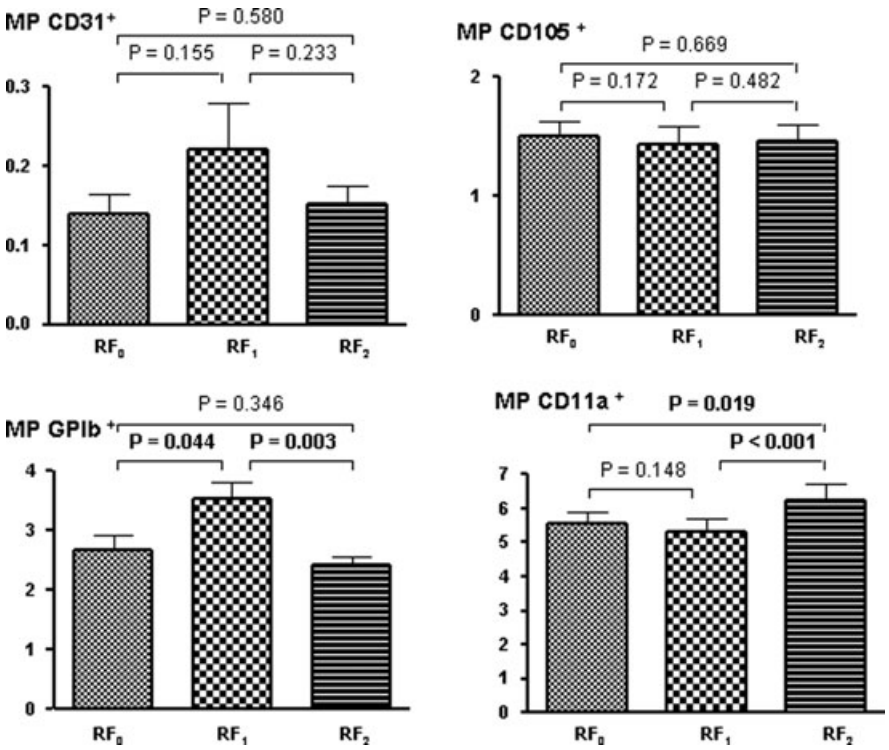

Figure 2. *Plasma levels of procoagulant endothelial-derived MPs (CD31$^+$-MPs and CD105$^+$-MPs), platelet-derived MPs (CPIb$^+$-MPs), and leukocyte-derived MPs (CD11a$^+$-MPs) before, immediately after (RF1), and at day 1 (RF2) after ablation in patients treated with radiofrequency ablation.*

Kinetics of Procoagulant Release after Radiofrequency Ablation in the Whole Population

In patients undergoing RF ablation, the time course of procoagulant microparticles captured onto annexin V (Total MPs) and platelet-derived MPs displayed an early peak after the procedure (RF_1) and decreased thereafter (RF_2). The increase in leukocyte-derived MPs was delayed with the maximal increase occurring on the first postprocedural day (RF_2). Conversely, changes in procoagulant MPs isolated by capture onto CD31 (platelet and apoptotic endothelium) or CD105 (endoglin, endothelium) did not reach statistical significance (Fig. 2). Burn numbers and duration correlated with Total MP levels measured on the first postprocedural day (RF_2) (respectively, $r = 0.396$; $P = 0.025$ and $r = 0.398$; $P = 0.027$). However, no relationship between the total energy delivered and MPs levels could be established.

Comparison between Irrigated and Nonirrigated Catheters

Microparticles, D-dimers, PAI-1, and vWF levels after the procedure were not significantly different in patients treated with irrigated or nonirrigated catheters (Table II).

Discussion

In this prospective study, we depict the release of procoagulant MPs following radiofrequency ablation of common atrial flutter. Whereas no differences in endothelial-derived MPs could be observed, a significant rise in platelet and leukocyte-derived procoagulant MPs could be evidenced after the procedure. Furthermore, significant changes in vWF Ag, PAI-1, and D-dimers occurred, testifying to the activation of blood coagulation in spite of the wide use of anticoagulants.

Platelet Stimulation, Coagulation Activation, and Endothelium Damage during Radiofrequency Ablation

Previous reports have demonstrated that RF ablation induced significant platelet activation measured by indirect markers or P-selectin membrane expression.[3,19,20] With respect to other markers of platelet activation released upon granule secretion or after lysis at membrane surface, the shedding of procoagulant MPs testifies to drastic platelet stimulation. Immediately after the

Table II.

Kinetics of vWF, D-dimers, PA-1, and Microparticles Levels after Radiofrequency Ablation of Atrial Flutter

	Whole Population (N = 33)	RF Ablation Using Irrigated Catheter (N = 19)	RF Ablation Using Nonirrigated Catheter (N = 14)	P Value
INR	1.76 ± 5	1.84 ± 0.56	1.65 ± 0.62	0.375
Platelet count ($\times 10^3/\mu$L)	214 ± 45	218 ± 50	209 ± 40	0.589
Leukocyte count ($\times 10^9$/L)	6.317 ± 1.111	6.220 ± 1.237	6.421 ± 993	0.632
vWF Ag (U/mL)				
RF_0	170 ± 61	187 ± 62	148 ± 53	0.067
RF_1	180 ± 54	192 ± 61	165 ± 41	0.145
RF_2	195 ± 67	210 ± 67	175 ± 66	0.153
D-dimers (μg/L)				
RF_0	257 ± 215	216 ± 136	312 ± 288	0.264
RF_1	581 ± 721	488 ± 614	707 ± 855	0.425
RF_2	504 ± 593	456 ± 400	570 ± 798	0.631
PAI-1 (U/mL)				
RF_0	17 ± 10	18 ± 11	15 ± 9	0.358
RF_1	14 ± 8	15 ± 9	13 ± 6	0.425
RF_2	18 ± 9	19 ± 9	15 ± 8	0.181
MP captured onto AV				
RF_0	6.7 ± 1.9	6.9 ± 1.8	6.6 ± 2.1	0.686
RF_1	9.5 ± 4.2	8.9 ± 2.7	10.3 ± 5.8	0.430
RF_2	7.5 ± 1.8	7.9 ± 1.3	7.1 ± 2.3	0.308
Platelet-derived MP ($GPIb^+$)				
RF_0	2.6 ± 1.4	2.6 ± 1.0	2.7 ± 1.8	0.818
RF1	3.5 ± 1.5	3.3 ± 0.9	3.8 ± 2.2	0.587
RF_2	2.4 ± 0.8	2.3 ± 0.8	2.4 ± 0.9	0.829
Endothelium-derived MP ($CD105^+$)				
RF_0	1.5 ± 0.7	1.5 ± 0.8	1.4 ± 0.5	0.427
RF_1	1.4 ± 0.8	1.5 ± 0.9	1.3 ± 0.6	0.211
RF_2	1.4 ± 0.8	1.6 ± 0.9	1.3 ± 0.6	0.818
MP originating from platelet and endothelial cells ($CD31^+$)				
RF_0	0.13 ± 0.13	0.09 ± 0.12	0.20 ± 0.13	0.030
RF_1	0.22 ± 0.31	0.18 ± 0.11	0.26 ± 0.49	0.600
RF_2	0.15 ± 0.12	0.15 ± 0.12	0.14 ± 0.13	0.750
Leukocyte-derived MPs ($CD11a^+$)				
RF_0	5.5 ± 2.1	5.5 ± 2.3	5.5 ± 1.8	0.932
RF_1	5.3 ± 2.1	5.3 ± 2.5	5.2 ± 1.3	0.860
RF_2	6.2 ± 2.6	6.7 ± 2.0	5.5 ± 2.1	0.178

procedure, platelet-derived MPs levels were in 17 patients (51%) above the mean value observed at the acute phase of myocardial infarction (2.82 nM PhtdSer Eq.).[21] Because they disseminate various bioactive effectors originating from the parent cells, MPs tune endothelial or blood cell functions and regulate biological responses involved in vascular homeostasis. Indeed, regardless of their cellular origin, shed MPs, rich in procoagulant aminophospholipd, provide in the vasculature an additional surface necessary for the assembly of tenase and prothrombinase complexes and consequent thrombin generation. Under shear stress conditions, the interaction between GPIbalpha-vWF not only initiates platelet arrest onto reactive vascular surfaces, but also contributes to the generation of procoagulant MPs that further enhances thrombus formation.[22,23] Furthermore, MPs' involvement in thrombin generation also results from their capacity to harbor, deliver, or induce tissue-factor activity. Together with activated platelets and leukocytes (see below), platelet-derived MPs play an important role in focusing the hemostatic response to

the region of injury but also initiate and accelerate the subsequent clotting reaction.

In this study, the extent of endothelium damage induced by RF ablation appears moderate, the rise of vWF levels contrasting with the absence of rise in levels of endothelial-derived MPs. This observation may indicate detection limits in the measurement of procoagulant endothelial-derived MPs, which depend on the following: (i) the level of endothelial marker borne by MPs, (ii) the antibody affinity, and (iii) the availability of the antigen (steric hindrance or limited proteolysis leading to an unrecognized epitope). Alternatively, endothelial-derived MPs could bind to target cells exposing appropriate counter-ligand. Nevertheless, the induced tissue damage was associated with the trigger of blood coagulation. Of note, in spite of the wide use of anticoagulants, significant elevation in D-dimers occurred, whereas PAI-1—the natural inhibitor of tissular plasminogen activator—concentration decreased, as a possible result of its consumption. The inverse correlation observed at day 1 between D-dimers and PAI-1 levels ($r = -0.64$; $P < 0.01$) is in line with this view. At RF_1, peak values of D-dimers were in nine patients (27%) above the threshold used in our institution for the detection of thromboembolic state (500 μg/L).

Inflammation Response and Leukocyte Activation during Radiofrequency Ablation

In this study, we showed that leukocyte-derived MPs, a reliable probe of leukocyte stimulation, are significantly elevated on day 1 after ablation. With respect to the kinetics of platelet-derived MPs or D-dimers, the rise of leukocyte-derived MPs appears delayed, reaching a peak on day 1. Apart from their role in the initiation of blood coagulation, platelet-derived MPs could also contribute to the activation of neutrophils explaining the time course in the release of leukocyte-derived MPs. For instance, platelet-derived MPs are also able to bind, activate, and aggregate neutrophils *in vitro*.[24] In addition, platelet-derived MPs could contribute to monocyte adhesion and tissular infiltration through the delivery of RANTES, a CC chemokine to the inflamed endothelium.[25] Previous studies have demonstrated that RF ablation of common flutter triggers inflammation response. Histopathologic studies have established that RF ablation induces necrosis followed by inflammatory infiltrates leading to a fibrotic scar. Inflammation following RF ablation has been linked to healing of RF ablation with production of cytokine (IL-6), C-reactive protein, and metalloproteinase secretion (MMP-9).[1,2] Leukocyte-derived MPs are not solely a probe of leukocyte stimulation but behave also as a key component in the initiation of the coagulation. In animal models, several reports underline a pivotal role for the circulating leukocyte-derived MPs in the formation of the thrombus.[26] Leukocyte-derived MPs, possibly harboring TF activity, were found to be recruited at sites of vascular lesion, through P-selectin/PSGL-1 interactions[26,27] before leukocyte infiltration. TF transfer through leukocyte-derived MPs incorporated into the growing thrombus would thus make adherent platelets capable of triggering and propagating thrombosis.[26,27]

Clinical Implications

This study demonstrates that RF ablation induces a prothrombotic state and the release of platelet and leukocyte-derived procoagulant microparticles. Whereas this activation of blood coagulation could be viewed as clinically marginal in right-sided procedures, its relevance in left-sided procedures remains to be established. Since circulating procoagulant MPs were reported elevated at baseline in patients with permanent atrial fibrillation, the additional rise of platelet and leukocyte-derived MPs induced by the RF procedure could alter substantially the homeostasis equation. This issue should be investigated in atrial fibrillation patients undergoing pulmonary vein isolation. The impact of the rise in procoagulant MPs on thromboembolic complications is unknown at the present time. It should, however, be emphasized that in approximately 50% of the procedures, the levels of platelet-derived MPs were at their peak in the same range as those observed in patients with acute myocardial infarction.[21] In addition, in patients with stroke, circulating levels of microparticles were found related to infarct size and clinical outcome.[15] Overall, this study provides evidence that the monitoring of MPs levels could be an interesting tool when studying tissular damage induced by ablation procedures.

Study Limitations

This work is monocentric and has the limitations of any small series. The extent of cardiomyocytes damage using troponin levels was not established. The absence of randomization to determine the use of irrigated and nonirrigated catheter constitutes a limitation when interpreting these data. Microparticles and coagulation markers were not measured in the direct vicinity of the damaged tissue (right atrium) but in a peripheral vein. Therefore, we could not exclude the possibility that the measurement of these markers in the right atrium may have led to the detection of higher levels.

Conclusion

In patients under anticoagulant therapy, RF ablation of common flutter induces a prothrombotic state and the release of platelet and leukocyte-derived procoagulant microparticles. Whereas this activation of blood coagulation could be viewed as clinically marginal in right-sided procedures, its relevance in left-sided procedures should be established.

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REVIEW

Microparticles: a critical component in the nexus between inflammation, immunity, and thrombosis

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Abstract Plasma membrane remodeling characterized by phosphatidylserine exposure and consecutive microparticle (MP) shedding is an ubiquitous process enabling the clearance of senescent cells and the maintenance of tissue homeostasis. MPs are released as fragments from the budding plasma membrane of virtually all eukaryotic cell types undergoing stimulation or apoptosis and may be considered a broad primitive response to stress. MP release is dependent on cytoskeleton degradation pathways involving caspases, requires a sustained increase in intracellular calcium triggering K^+ and Cl^- efflux and is possibly tuned by mitochondria permeability changes. Because they convey a broad spectrum of bioactive molecules, circulating MPs may serve as shuttles promoting cellular cross talk in various pathological settings such as inflammation or immunity-induced thrombotic disorders. If the drastic shedding of procoagulant MPs appears clearly noxious in thrombotic disorders or in some models of inflammation-induced coagulopathy, this does not necessarily endorse their invariably harmful nature. In the vessel, endothelial cytoprotection reported in the early regulation of inflammation-induced coagulopathy is emblematic of the beneficial effects provided by MPs. In addition, MPs would prove beneficial in the prevention of blood leakage. Because of their multiple properties that are characteristic of a private response of the parental cell, MPs could act as cytoprotective and anti-inflammatory agents through the delivery of activated protein C or annexin 1 and could contribute to the limitation of vascular hyporeactivity. Owing to their ability to cargo bioactive signals, MPs could be viewed as an integrated communication network enabling the coordination of complex cellular responses in biological fluids and the maintenance of the homeostasis equation. A better understanding of the molecular mechanisms involved in MP shedding would pave the way of a new pharmacological approach aiming at the control of MP-driven cellular responses.

Keywords Phosphatidylserine · Ca^{++} · Thrombosis · Apoptosis · Platelet · Scott syndrome · Immunity inflammation · Tissue factor · TMEM16F

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Introduction

Microparticles (MPs) are released from the budding plasma membrane of virtually every eukaryotic cell types after stimulation or apoptosis. They can also result from the resealing of membrane fragments disrupted during cell

necrosis. In multicellular organisms, mostly functioning in a minimal energy consumption mode with permanent recycling, MPs are thought to reflect a dynamic balance between cell proliferation, stimulation, and death [1]. At the surface of senescent or apoptotic cells, the exposure of phosphatidylserine (PS) constitutes a recognition signal that enables phagocytosis clearance in multicellular organisms [2]. Because the randomization of membrane lipid asymmetry is an important physiological process, it is likely that the machinery for accomplishing it is common to all eukaryotic cells [3]. Owing to their smaller size and therefore greater ability to diffuse more rapidly and transiently escape phagocytosis, MPs survive longer than the activated cells they stem from. Detectable in small amounts in the peripheral blood of healthy individuals, elevated concentrations of MPs originating from platelet, granulocyte, erythrocyte, and endothelial lineages are associated with a variety of pathophysiological issues including thrombosis, inflammation, sepsis, malaria, metabolic disorder, auto-immune disease, trauma, cancer, or sickle-cell disease. MPs convey a broad spectrum of bioactive molecules thought to contribute to cell cross talk. Because they display autoantigens, such as RNA and DNA, in a highly immunostimulatory manner, they may also act as potent autoadjuvants. Released in the vasculature or in other biological fluids, MPs may tune cellular interactions and behave as key regulators in the nexus between inflammation, immunity and thrombosis. The aim of this review is to (1) describe the molecular mechanisms possibly governing MP generation (2) discuss the role of MPs in the nexus between inflammation–immunity and coagulopathies.

Generation of microparticles by stimulated cells

In eukaryotic cells, each of the two leaflets of the plasma membrane bilayer has a specific lipid composition. Aminophospholipids [PS and phosphatidyl-ethanolamine (PE)] are more specifically segregated in the inner leaflet, whereas phosphatidylcholine (PC) and sphingomyelin are enriched in the external one. The maintenance of this asymmetric distribution or its loss is the consequence of opposite transports, their balance being under control of specific transporters governing inward (flip) or outward (flop) translocation [4]. Many years after this first observation, the identity and actual biological function of proteins involved in lipid translocation remains yet incomplete. The maintenance of lipid asymmetry relies on an adenosine triphosphate (ATP)-dependent transporter, the aminophospholipid translocase, that specifically and rapidly conveys PS and PE from the outer to the inner leaflet and that is inhibited by calcium ions [5]. Because transbilayer phospholipid transport is slow in the absence of a translocator, lipid asymmetry is stable in quiescent cells [3].

Membrane remodeling is a crucial event in cell response and is involved in apoptosis, hemostasis, and cell clearance that is tightly regulated. To provide an adequate procoagulant surface upon stimulation, at least one, and probably more than one, transporters facilitate the rapid egress of PS at the outer leaflet. Possible translocators are members of the floppase family (outward-directed transporters including the ATP-binding cassette transporter A1, ABCA1), and the so-called calcium-dependent scramblase that facilitate bidirectional movement between membrane leaflets [6–8]. Cell stimulation, on the broad acceptance of the term, triggers increased cytosolic [Ca^{2+}] concentration and the outward transport of PS that is enabled by the concerted activity of lipid transporters. Aminophospholipids are rapidly translocated to the outer leaflet, flippase activity is reduced while an overwhelming floppase activity and the activation of the scramblase that favors phospholipid exchanges between the two leaflets, regardless of the chemical nature of the polar head group, account for transient phospholipids mass imbalance between the two leaflets. Surface tension and membrane budding are ultimately resolved into MP shedding.

The procoagulant nature of micropartcicles

PS exerts a key catalytic role by promoting the assembly of the vitamin K-dependent enzyme complexes of the coagulation cascade. Its exposure by the surface of stimulated/adherent platelets is part of the physiological hemostatic response at site of the wound, also triggered by MPs and other activated blood and vascular cells. Vitamin K-dependent factors (FVII, FIX, FX, and FII) bind to accessible PS through their γ-carboxyglutamyl residues. Local concentrations of focused enzymes, cofactors (FVIIIa and FVa) and zymogens enable the fulfillment of the kinetic constraints for optimal thrombin generation that will in turn stimulate platelets in an amplification loop at the basis of the rapidity of the hemostatic response [4]. Platelets, with a high rate of floppase and scramblase activity behave as true sensors that trigger blood coagulation at site of vascular injury [3]. In the vasculature, MPs shed from platelets provide an additional phopholipidic surface with approximately 50–100-fold higher procoagulant activity [9]. In other cell types, such as T cells or EBV transformed cells, the scrambling rate is intermediate, the lowest range being measured in erythrocytes [3]. The relevance of "flip-flop" to hemostasis, is suggested from the phenotype of Scott syndrome, a rare bleeding disorder in which both PS exposure and MP shedding are deficient [10, 11]. Severe to moderate bleeding episodes, generally provoked, constitute

the clinical phenotype, with no other apparent disorders in homozygous-like patients. Very recently, a hereditary bleeding disorder with a deficient platelet procoagulant activity was identified in German Shepherd dogs with features of Scott syndrome [12]. As yet, the paucity of human cases has hindered genetic identification of the trait. Phospholipid scramblase 1 and the transporter ABCA1 are putative aminophospholipid floppases that were not confirmed the defective element responsible for Scott syndrome.

Factors governing plasma membrane remodeling and microparticle shedding

Calcium homeostasis and PS exposure

Calcium concentration in the cytosol is tightly regulated and an essential factor in the regulation of aminophospholipid transbilayer migration (Fig. 1). The Ca^{2+}-dependent mechanisms associated with rapid phospholipid redistribution include a variety of processes, such as the activation of outward PS transporters, inhibition of the inward aminophospholipid translocase, and cytoskeleton degradation. Notably, cell shrinkage itself that accompanies stimulation-driven ions and water fluxes across the membrane modifies lipid packing in both leaflets and PS redistribution between leaflets. Intracellular Ca^{2+} rises as a consequence of its release from internal stores and of its entry across the plasma membrane. In platelets, agonist-induced stimulation of different receptors leads to the activation of phospholipase C isoforms, which hydrolyze phosphoinositide-4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). Ca^{2+} IP3 releases Ca^{2+} from the intracellular stores through IP3 receptor channels, while DAG is involved in Ca^{2+} entry from the extracellular compartment. The agonist-induced Ca^{2+}release from stores is followed by a Ca^{2+} influx through the plasma membrane, a particular mode of refilling referred to as store-operated calcium entry (SOCE). Prurigenric $P2X_1$ channel are another source of calcium entry in platelets [13]. Stromal interacting protein 1 (STIM1), a transmembrane protein located in the tubular dense system and the platelet equivalent to endoplasmic reticulum (ER), was recently assessed as a Ca^{2+} sensor that detects release from the ER. When translocated to the platelet plasma membrane, it is in turn able to trigger a sustained influx of extracellular calcium. Orail1 was very recently identified as the principal actor of SOCE sensored by STIMI-1 (Fig. 1). In mice expressing a mutated form of Orai1 (R93W), a marked inhibition of platelet SOCE together with impaired agonist-induced cytosolic [Ca^{2+}] could be evidenced (Fig. 1). The relevance of the STIM-1/Orai1 SOCE pathway in thrombosis was underlined by the fact that platelet deficiency in either Orai1 or STIM1 protects against collagen-dependent arterial thrombus formation and brain infarction in vivo [14, 15]. Conversely, one surprising finding is that platelets from $Stim1^{-/-}$ and $Orai1^{-/-}$ mice, despite a complete lack of SOCE are still able to fulfill most of their physiological functions. Whilst the Ca^{2+} release from internal stores appears to be sufficient to trigger shape change, integrin activation and granule release, the Orai1 pathway would be of particular relevance in coagulant activity, MP shedding, and the stabilization of the thrombus. These observations fit well with the phylogenic perspective that platelets have to trigger a rapid response to stimulation. It appears reasonable that basic platelet functions would be regulated without the delay of an indirect SOCE process, which involves a molecular coupling that necessitates up to 15 s to be accomplished [13, 16].

Another important regulatory protein triggered by calcium is calmodulin (CaM). Upon binding of Ca^{2+}, CaM affinity for downstream target proteins like kinases or phosphatases increases. This wide array of kinases includes myosin light chain kinase, CaM-dependent protein kinases I, II, and IV, and calcineurin [7]. Best known as an immune-suppressor in vivo, CsA mechanistically inhibits calcineurin by forming a complex with its effector proteins cyclophilins (see below). Interestingly, earlier studies have reported that cyclosporin A (CsA) abrogates ABCA1-dependent cholesterol efflux [17, 18]. Very recently, Karwatsky et al. have demonstrated that cholesterol efflux to apoA-I in ABCA1-expressing cells is regulated by Ca^{2+}-dependent calcineurin signaling [19].

Cytoskeleton, membrane lateral organization and PS exposure

Cytoskeleton integrity is believed to participate in the maintenance of membrane asymmetry and cell shape, its cleavage being therefore a facilitator of membrane budding in stimulated cells. Cytoplasmic caspases and calpains are Ca^{2+}-dependent thiol proteases that contribute to cytoskeleton reorganization by favoring talin, filamin, and gelsolin cleavage. Whereas baseline calpain activity appears insufficient, agonist-induced Ca^{2+} influx was found necessary for maximal protease activity and cytoskeleton degradation [20]. Caspase-3 was also demonstrated a major factor in cytoskeleton reorganization. It mediates the cleavage of ROCK I, a rho-kinase acting by myosine light chain phosphorylation that induces cell membrane contraction, shrinkage, and MP release [21]. Independently of cell death, ROCK-II, another Rho-kinase isoform, was demonstrated to be activated by caspase-2 following endothelial cell stimulation by thrombin and to be involved in MP shedding [22]. Interestingly, prolonged exposure of endothelial cells to thrombin triggers the expression of a cell-

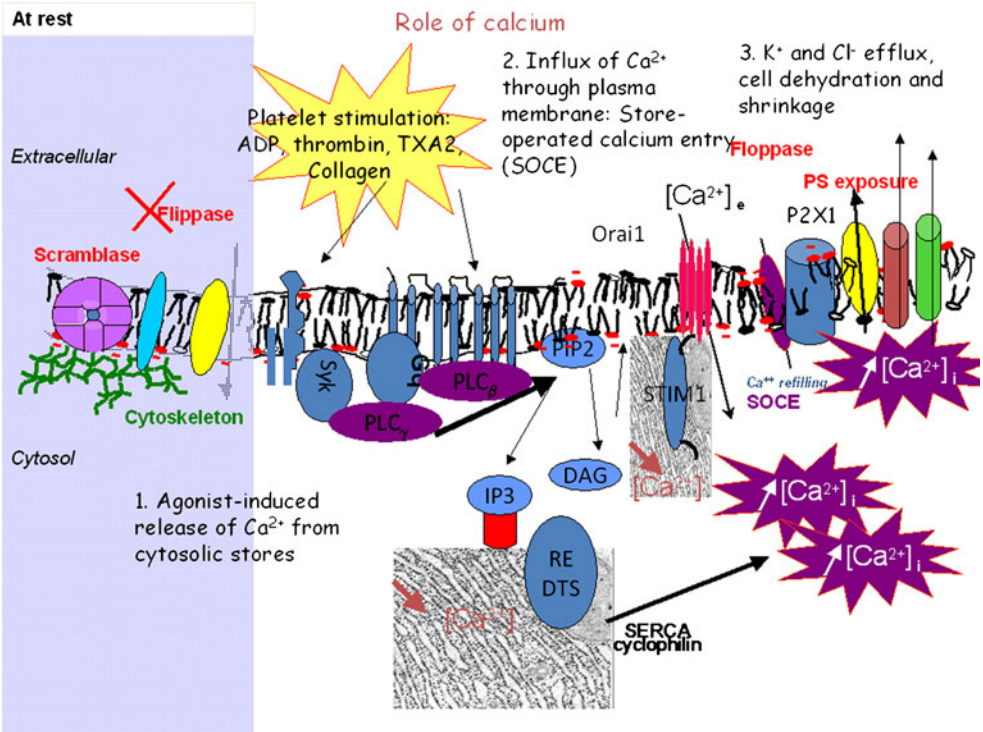

Fig. 1 Role of calcium in the formation of microparticles. In platelets, activation can occur through different pathways that culminate in the activation of phospholipase C isoforms and the production of diacylglycerol (*DAG*) and inositol triphosphate (*IP3*). The binding of IP3 to its receptors located in the dense tubular system mediates Ca^{2+} release from cytosolic stores. In turn, the resulting decline in Ca^{2+} store content, sensored by the stromal interacting molecules 1 (*STIM-1*) triggers a sustained influx of extracellular calcium by a mechanism known as store-operated calcium entry. It was recently demonstrated that Orai1 is the principal Ca^{2+} entry channel involved in store-operated calcium entry. The role of direct-operated calcium channel such as the $P2X_1$ prurinoreceptor needs further investigation. Interestingly in transgenic mouse that overexpresses $P2X_1$ receptor increased PS exposure was observed. The raise of calcium is supposed to promote PS exposure through several mechanisms including flippase inhibition and the activation of floppase and scamblase. In addition, the elevation of Ca^{2+} cytosolic concentration could activate the K^+ Gardos channel and the TMEM16F Cl^- channel. Water follows K^+ and Cl^- efflux inducing cell dehydration and shrinkage. In two patients with Scott syndrome 3 identified mutations in TMEM16F predicted the truncation of the protein and premature termination of the protein devoid of its calcium sensing domain

associated and of a soluble form of TRAIL that participates in the release of MPs [23].

Rafts that are organized as functional transbilayer domains through the specific recruitment of particular proteins and lipids may also contribute to the membrane response [24]. PS exposure is dramatically reduced after raft disruption by methyl-cyclodextrin and proteins with strong cytoskeleton interactions are represented to a lesser extent in MPs [25]. In this view, a given stimulus could thus be expected to elicit a "private" membrane response after raft reorganization leading to inclusive or exclusive protein sorting. Such lateral remodeling would explain how MPs of the same cellular origin may have different protein and lipid compositions [4]. As an illustration, CD63 and P-selectin (CD62) were found increased in PS^+-MPs whereas GPIb (CD42b) was less represented [26]. The evidence of an increased expression of platelet activation markers (CD62P and CD 63) on PS^+-MPs could suggest that those particular MPs are preferential markers of platelet stimulation [26]. Indeed, data from Perez-Pujol and coworkers suggest that the exposure of PS is accompanied by the selective shedding of MPs and that their phenotype is dependent on the stress that elicited their release [27, 28].

Physical mechanisms involved in PS exposure and MP shedding

Challenging the current paradigm of a membrane remodeling driven by specific lipid transporters, other authors have suggested that the rapid diffusion of lipids across the plasma membrane could be the consequence of physical constraints resulting from the formation of blebs or transient transmembrane pores triggered by ion fluxes. Indeed, following stimulation by a Ca^{2+} ionophore, K^+ and Cl^- ions leave the cell, water follows, and the cell shrinks. According to Eliott and coworkers, the plasma membrane buckles prior the shedding of MPs, as a consequence of cell shrinkage. At the apex of the blebs, the packing of PC in the outward leaflet is relatively loose, reducing the energetic barrier of the outward movement of PS and PE

("flop"). Conversely, at the base of the blebs, PS and PE are tightly packed in the inner leaflet, increasing the energetic probability of the inverse transport of PC ("flip"). The swift randomization of the membrane phospholipids independently of specific transporters would be ultimately resolved into the shedding of MPs. This hypothesis gives to ion channels a central role in membrane remodeling and raises the question of their ability to sensor different types of stress [29].

By triggering endogenous sphingomyelinase in the inner monolayer of the membrane, the raise of cytosolic [Ca^{2+}] may also lead to the formation of temporary pores. As a result of swift sphingomyelin hydrolysis by sphingomyelinase, ceramide concentrations reach a threshold enabling a mismatch between the two membrane leaflets and the creation of an invagination. The unstability between leaflets is eventually solved through the formation of transient pores. During the lifetime of pores, lipids have free access to both sides of the membrane [30, 31]. Ceramide-induced pore formation which fosters cell membrane scrambling and PS exposure was found determinant in erythrocyte programmed cell death (eryptosis), in sepsis, haemolytic uremic syndrome or malaria [32].

Transmembrane ion transport

Cell shrinkage results from cell dehydration involving a Ca^{2+}-activated efflux of K^+ and Cl^- ions [29, 33]. While numerous experimental data point at cytosolic [Ca^{2+}] as the trigger of PS exposure, investigations of other ion fluxes provide further insights. For instance, the α-hemolysin from *Escherichia coli* triggers erythrocyte shrinkage and PS exposure through the dual Ca^{2+}activation of the K^+ Gardos channel and of the TMEM16A Cl^- channel [34]. The relevance of K^+ Gardos channels as other possible contributors to PS exposure was also illustrated in Scott syndrome [35]. The lack of procoagulant platelet response observed in one patient, was partially restored by pre-treatment with valinomycin, a selective K^+ ionophore that favors K^+ efflux. The prime importance of Ca^{2+}-activated efflux of Cl^- in the regulation of PS exposure was very recently illustrated in a subline of a mouse B cell line, Ba/F3,that strongly exposed PS. A cDNA that caused PS spontaneous exposure and encoded a constitutively active mutant of TMEM16F was identified by expression cloning (Fig. 1). Wild-type TMEM16F was localized on the plasma membrane and conferred Ca^{2+}-dependent scrambling of phospholipids. In two patients with Scott syndrome 3 identified mutations in TMEM16F predicted the truncation of the protein and premature termination of the protein devoid of its calcium sensing domain [36, 37]. Interestingly, other authors showed that in platelets stimulated by thrombin/collagen, Na^+ influx is able to trigger phospholipid scrambling via a Na^+/H^+ exchanger [38]. Hence, intracellular Ca^{2+} may well not be the sole sensor of the collapse of membrane asymmetry.

Mitochondria and tubular dense system in the regulation of membrane remodeling

Initially known as the power factory of the cells, mitochondria are now considered a check point in the apoptotic machinery. Recently, their incidence in the procoagulant functions of vascular cells and platelets has been investigated. Mitochondrial permeability appears an important event for MP shedding. Various agonists promote the opening of the mitochondrial permeability transition pores (mPTP) leading to the release in cytosol of cytochrome *c* and apoptosis-inducing factor that enable the activation of effector caspases [39]. Consitutive proteins of the mPTP include the adenine nucleotide translocator, the voltage-dependent anion channel, and cyclophylin D. mPTP opening is blocked by CsA that binds to cyclophylin D and dissociates it from the translocator [40]. Mitochondrial membrane depolarization appears an integral event leading to the outward transport of PS in response to physiological agonist such as thrombin and collagen [41, 42]. The contribution of mPTP to plasma membrane remodeling is suggested by the observation that CsA prevents the loss of the mitochondrial transmembrane potential together with MP release and PS exposure in thrombin/convulxin stimulated platelets. This link was confirmed in platelets from mice in which cyclophilin D was invalidated. They were characterized by a defective PS externalization and prothrombinase activity in response to thrombin and convulxin [43]. Interestingly, in the canine Scott syndrome [44], the trait locus includes several genes coding for mitochondria-associated proteins and apoptosis regulators. It is thus likely, that multiple actors govern PS exposure and MP release at different steps of the cell response machinery among which (1) specific caspase-dependent cytoskeleton degradation (2) enhancement of mitochondria permeability (3) sustained increase in intracellular calcium.

Role of MPS in transcellular communication

Whatever the type of cell stimulation responsible for membrane shedding, MPs do not behave as inert cellular debris but should be considered as potent mediators of a new cellular communication network enabling information exchanges in body fluids. It should however be emphasized that the mode of cellular activation, the type of stress (i.e., stimulation vs. apoptosis...) together with the micro-

environnement where MPs were generated are probably determinant in the nature and composition of MPs. For instance, past studies have suggested that different MP protein expression patterns are generated under conditions of rapid stimulation or of apoptosis [45]. Even the type of stimulus is able to elicit a private response characterized by a different MP composition. By comparison to MPs shed by LPS-stimulated leukocytes those shed after P-selectin stimulation are enriched in lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18). Importantly, the presence of LFA-1 at MP surface suggests a possible role in immune responses. In addition, LFA-1 may promote MP interaction with the endothelium, causing endothelial activation or dysfunction [46].

Because they harbor or contain ligands, receptors, counter-receptors, active lipids, mRNA, MPs are able to modulate biological properties of neighboring cells through several mechanisms including (1) transfer of membrane-associated receptors (2) release of proteins or active biolipids (3) exchange of genetic information by transfer of RNA (4) induction of adaptative immune response. This MP-mediated intercellular delivery system allows the establishment of an integrated communication network in which specific properties and information among cells can be swiftly shared enabling the coordination of complex responses such as immunoregulation and the maintenance of the homeostasis equation [47]. However, under pathophysiological conditions, the spreading of MPs can amplify noxious responses such as inflammation, cell invasion, thrombosis and vascular damage.

MPs as a source prothrombotic vascular effectors

A dual procoagulant character: blood-borne tissue factor

In the vessel, rapid phospholipid membrane remodeling and PS exposure characterize a physiologically relevant procoagulant response. Owing to their high membrane plasticity ensuring a rapid procoagulant response at site of vascular injury, platelets are the main provider of circulating MPs in healthy individuals. In other vascular cell types, scrambling is less effective, with the lowest ability being found in red blood cells. Beside platelets, leukocytes and endothelial cells, MPs are key determinants of the hemostasis equation. Furthermore, MPs constitute the main reservoir of blood-borne tissue factor (TF) released from [48] cells with inducible (monocytes, macrophages, endothelial cells, etc.) or constitutive TF expression (vascular smooth cells, fibroblasts, and tumor cells). TF is the major cellular initiator of the coagulation cascade that acts synergistically with PS when expressed by the membrane of stimulated cells (Fig. 2). In this context, shed MPs could be viewed as protective in case of bleeding, whereas their dramatic release could favor thrombotic complications in the arterial or venous bed. In the vasculature, various agonists such as cytokines, microorganisms, thrombin, low shear stress, oxidative stress, hyperglycemia, smoking, dylipidemia, complement, immune reaction induce procoagulant phospholipid exposure, TF induction and its release in an active MP-borne form [49, 50]. For instance, in heart transplant recipients, acute rejection triggers the release of a specific MP pattern. Significant elevation of MPs bearing TF, Fas, or E-selectin was associated with acute allograft rejection and suggested endothelial cell activation and Fas-mediated apoptosis [51]. Of note, human leukocyte antigen class I antibodies was demonstrated a potent inducer of TF expression by endothelial cells.

At the onset of thrombosis, it is likely that the accumulation of leukocyte-derived MPs harboring TF activities is mandatory whereas the level of platelet-derived MPs mostly testify to the extent of platelet stimulation. In an experimental mouse model of venous thrombosis, circulating leukocyte-derived MP levels were negatively correlated with the thrombus weight, suggesting their early incorporation within the growing thrombus [52]. Conversely, platelet-derived MPs were positively correlated with the thrombus growth [52]. In acute myocardial infarction, sequestered inflammatory cells together with the shedding of leukocyte MPs harboring TF activity are crucial to thrombus formation. Independent of the eventual role played by platelet-derived MPs, we have shown that leukocyte-derived MPs are the main contributor of TF activity at the vicinity of the arterial thrombus (see below). Altogether, these data underline the prime importance of leukocyte-derived MPs in the concentration of TF activity at site of endothelium injury [53]. However, besides the central role of the leukocyte-platelet-endothelium triad in thrombus generation, it is worth mentioning that other cellular lineages may contribute to MP-driven blood coagulation. In traumatic brain injury, we have demonstrated an increased generation of procoagulant MPs in the cerebrospinal fluid that may reflect neuronal apoptosis. MPs were also detected in the peripheral blood, a mechanism possibly involved in trauma-induced coagulopathy [54]. Accordingly, recent studies raised the possibility that central nervous system-derived MPs may enter the bloodstream as the result of blood brain-barrier disruption and interact with endothelial cells [55]. In others clinical settings (malaria, sickle-cell disease, etc.), erythrocyte-derived MPs appeared important contributors to the circulating storage pool of MPs, levels being correlated with the extent of blood coagulation activation [56, 57].

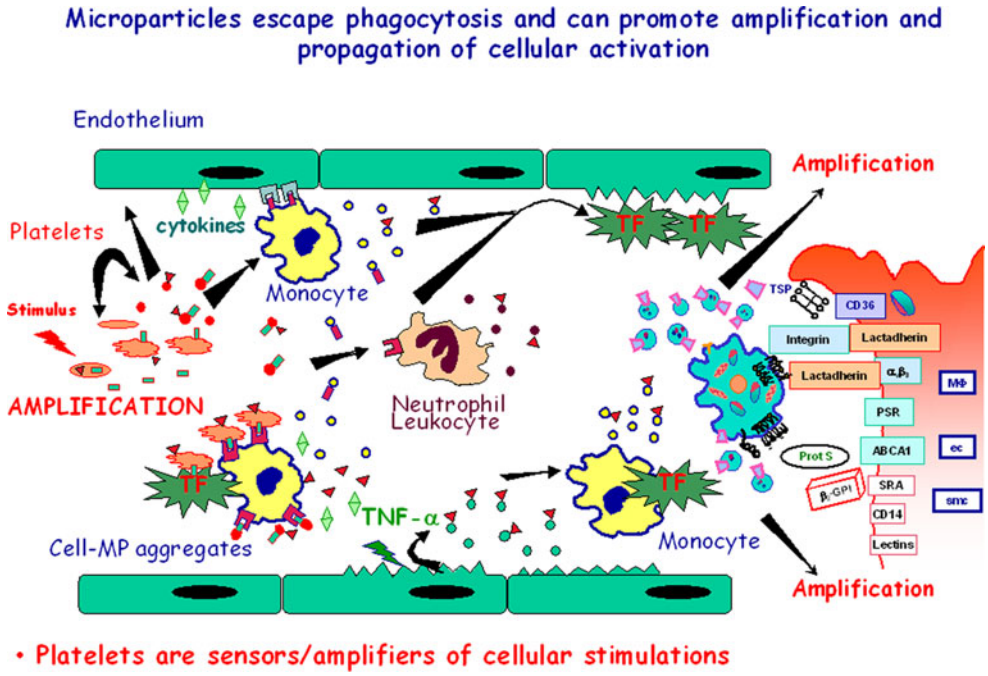

Fig. 2 Role of microparticles (MPs) in the vasculature. Microparticles escape phagocytosis and can promote amplification and propagation of cellular activation. Owing to their ability to cargo bioactive signals, MPs could be viewed as an integrated communication network enabling the coordination of complex cellular responses in biological fluids. Under pathophysiological conditions, the spreading of MPs can amplify noxious responses such as inflammation, cell invasion, thrombosis and vascular damage. Owing to their high membrane plasticity ensuring a rapid procoagulant response at site of vascular injury, platelets are the main provider of circulating MPs in healthy individuals. In other vascular cell types, scrambling is less effective, with the lowest ability being found in red blood cells. Beside platelets, leukocytes and endothelial cells MPs are key determinants of the hemostasis equation. Furthermore, MPs constitute the main reservoir of blood-borne tissue factor (*TF*) released from cells with inducible (monocytes, macrophages, endothelial cells, etc.) or constitutive TF expression (vascular smooth cells, fibroblasts, and tumor cells). TF is the major cellular initiator of the coagulation cascade that acts synergistically with PS when expressed by the membrane of stimulated cells

Intertwined proinflammatory and prothrombotic MP-mediated responses

The first hint suggesting a prothrombotic pathway mediated by MPs in inflammatory conditions was brought by Mesri and Altieri that evidenced the up-regulation of circulating leukocyte MPs in vivo and a stress signaling pathway in endothelial cells, leading to increased TF activity [58]. Similarly, monocyte-derived-MPs up-regulated the expression of active TF by endothelial cells and a rapid expression of von Willebrand factor at the cell surface favoring a transient attachment of non-activated platelets to the endothelium. In vitro experiments demonstrated that these two major cell responses are under the control of reactive oxygen species (ROS) delivered by MPs [59]. Under conditions of oxidative stress, endothelial-derived MPs contain oxidized phospholipids that promote monocyte–endothelial interactions. Importantly, platelet-derived MPs (PMPs) are able to up-regulate cytoadhesin expression in monocytes and endothelial cells through the delivery of arachidonic acid, thus reinforcing cell adhesiveness. At high shear stress, platelet-derived MPs (PMPs) GPIb and P-selectin-dependent rolling would enable the delivery of RANTES, a CC chemokine, to the inflamed endothelium and favor monocyte adhesion [60]. MPs also contribute to endothelial inflammation as providers of proinflammatory interleukin (IL)-1β. The relevance of these findings is also suggested in human pathological settings. For instance, in patients with pulmonary arterial hypertension, we have previously shown that RANTES was associated with high levels of procoagulant MPs of leukocyte, endothelial, and platelet origin in the pulmonary vascular bed, possibly reflecting multiple pathways in which MPs contribute to inflammation and thrombus formation [61]. In the same clinical setting, increased platelet-, leukocyte- and endothelium-derived MPs were recently found to predict enhanced coagulation and vascular inflammation [62].

P-selectin: soluble and membranous

During the past decade, selectins, leukocytes-derived MPs, and TF have merged into a determinant triad in thrombosis [63]. The first demonstration of the importance of leukocyte shedding in the hemostatic equation came from the group

of Denisa Wagner. Using a mice model of hemophilia A, Hrachovinova et al. demonstrated that the infusion of soluble P-selectin prompts the generation of leukocyte-derived MPs harboring TF and able to correct a bleeding phenotype [64]. Other mechanisms triggered by P-selectin could contribute to increased thrombotic propensity. P-selectin was shown to favor the transfer of TF sorted from raft into monocyte-derived MPs and delivered as a functional entity to platelets [65]. P-selectin also promotes PS exposure by monocytes and TF expression [66, 67]. Finally, P-selectin stimulation of monocytes generated MPs enriched in lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) that enabled interactions between leukocytes and endothelial cells [46]. The prime importance of this pathway was later emphasized in experimental models of thrombosis. Using *intravital* real time microscopy several groups have established that the swift recruitment at the edge of the thrombus of leukocytes and leukocyte-derived MPs through P-selection/P-selectin glycoprotein ligand-1 interactions promotes thrombus growth [68–70].

Atherothrombosis and MP storages pools: inflammation, thrombosis, and plaque vulnerability

Beside the circulating pool of MPs, another reservoir worth considering in the setting of atherothrombosis is the pool of MPs sequestrated in the plaque. Exposed upon plaque disruption, the acellular lipid-rich core of an atherosclerotic plaque represents its most thrombogenic part, with enhanced TF activity being directly supported by TF^+-MPs exposing PS [71]. Apoptotic macrophages together with smooth muscle cells (SMCs) may also contribute to TF^+-MP accumulation in the lipid core. In vitro, under settings of minimal apoptosis, human SMC release TF^+-MPs whereas TF expression is up-regulated by both native or aggregated low-density lipoprotein (LDL), the engagement of the latter with LDL receptor-related protein leading to the expression of cellular TF activity and TF^+-MP release [72]. A recent report has demonstrated that plaque MPs induce T cell proliferation, and that immunoglobulins are trapped within macrophage-derived MPs. Plaque sequestered MPs express major histocompatibility complexes on their surface and activate $CD4^+$ T lymphocytes, possibly cooperating locally with the scarce B lymphocytes to produce immunoglobulins directed against plaque antigens. Besides being an important determinant of plaque thrombogenicity, MPs might thus play a previously unrecognized role in modulating tissue inflammation as supported by their proliferative effect on $CD4^+$ T lymphocytes [73].

In the kinetics of arterial thrombus formation, the respective contributions of blood-borne TF spread by MPs and the trapped pool of TF^+-MPs are still on debate [74]. To decipher the role of circulating or sequestered TF^+-MPs, reciprocal bone marrow transplants were performed between wild-type and engineered mice expressing minimal TF [75, 76]. Results suggest that whereas arterial vessel-wall TF is involved in the initiation of platelet activation, blood-borne TF spread by MPs mediates thrombus propagation. Once vessel-wall TF is covered by a layer of fibrin and platelets, the plaque might become impermeable to circulating clotting factors. Thus, blood-borne TF would prevail in puncture wounds or venous thrombosis, its contribution being less effective in the presence of high amounts of plaque TF.

In addition to their prothrombotic and proinflammatory potential within the atheromatous plaque, MPs are also likely to contribute to plaque neoangiogenesis and proteolysis, 2 major determinants of plaque vulnerability. MPs isolated from plaques enhance VEGF production endothelial proliferation and neovessel formation in a CD40L dependent pathway [77]. Increased vasa vasorum neovascularization can be associated with a higher risk of intraplaque hemorrhage and subsequent rupture or thrombosis [78]. The recent recognition of erythrocyte-derived MPs as another important contributor to the pool of trapped MPs is in line with the latter paradigm [73]. However, although deleterious within the plaque, MPs contribute to vascular repair. For instance, platelet-derived MPs (PMPs) augment the adhesion of early angiogenic cell outgrowth to extracellular matrix components resulting in accelerated re-endothelialization after arterial denudation injury [79]. Because MPs harbored functional metalloproteinase, one could not exclude that they promote matrix proteolysis, a main factor in plaque destabilization [80]. Recently, monocyte- and endothelial-derived MPs were shown to harbor functional urokinase able to promote the transformation of plasminogen into plasmin. In situ, this uPA-driven cross talk generates plasmin with a high efficiency underlining its potential physiological relevance in fibronolysis and matrix proteolysis induced by inflammatory cells or derived-MPs. Indeed, plasmin formed on these cellular membranes is implicated in the proteolytic processing of extracellular matrix components, cell migration, angiogenesis through endothelial progenitor cell homing, and in cell blebbing and shedding [81–83]. TACE/ADAM-17 is another metalloprotease evidenced in human atherosclerotic plaques that may contribute to plaque vulnerability and cleaves tumor necrosis factor (TNF) and its receptors, TNFR-1 and TNFR-2. $TACE^+$-MPs from the plaque were shown to promote the shedding of the endothelial protein C receptor (EPCR) from endothelial cells, providing another mechanism by which they contribute to enhanced in situ thrombogenicity [84, 85]. Indeed, the loss of EPCR from the endothelial cell surface would impede the binding of anticoagulant activated protein C and the limitation of

thrombin auto-amplification. Finally, endothelial MPs themselves are part of the amplification loop leading to the [85] enhanced in situ thrombogenicity. Released upon various types of aggression, they induce monocyte TF expression and procoagulant activity [86].

Microparticles in venous thrombosis

By contrast to the mechanisms underlying arterial thrombosis, several lines of evidence have suggest that blood-borne TF could be a determinant factor in venous thrombosis, especially in the setting of neoplasia, the contribution of tissular MPs trapped within the vascular wall being probably marginal [87–89]. Another crucial difference between arterial and venous thrombotic process rely on the differential impact of blood flow, venous stasis being a crucial determinant of venous thrombosis whereas arterial thrombosis likely occurs at site of high shear stress. It should also be emphasized that venous stasis promotes cell-cell and MP-cell interactions reinforcing thrombotic and proinflammatory loops. The careful dissection of the sequence linking endothelial damage and thrombus growth in animal models has allow a better understanding of the role of MP and TF in venous thrombosis. In a rat model of inferior vena cava ligation, TF staining was observed in both leukocytes and endothelial cells associated with the clot [90]. Likewise, one study showed that inhibition of the TF factor VIIa complex reduced thrombosis in an inferior vena cava ligation model in primates [91]. As depicted in animal models using *intravital* real time microscopy to monitor the thrombus growth, it is likely that under stasis, leukocyte- and tumor-derived MPs expressing P-selectin glycoprotein-1 bind to endothelial and platelet P-selectin. In this setting, it is worth mentioning that high plasma levels of soluble P-selectin were found predictive of venous thromboembolism in cancer patients [92]. Several studies demonstrated that levels of procoagulant MPs, eventually harboring enhanced circulating TF activity are significantly elevated in cancer patients with respect to healthy individuals or non cancer patients [89]. A recent retrospective analysis using impedance-based flow cytometry to detect TF-positive MPs reported that tumor-derived TF-positive MPs are elevated in cancer patients with venous thromboembolism [93, 94]. The putative role of TF^{+}-MPs as a marker of venous thrombotic risk was recently assessed by measurement of MP TF activity. Among 11 pancreatic cancer patients, MP TF activity increased over time (30 weeks) in the two patients who developed VTE whereas such activity remained at baseline in patients with no sign of thrombosis [88, 89]. Although the limited size of the cohort did not allow to raise definite conclusion, this result suggested that MP TF activity may be predictive of cancer-associated VTE and that TF^{+}-MPs may be the vehicle that transports the procoagulant activity from the tumor site to the deep veins to trigger thrombosis [73].

The deleterious role of MPs in the regulation of vascular tone

Apart their role in the direct modulation of prothrombotic, proadhesive and proinflammatory properties of vascular cells, circulating MPs could also be an important regulator of the vascular tone, a key component of the Virchow's triad. Indeed, circulating endothelial-derived MPs (EMPs) correlate with endothelial dysfunction in patients with ischemic left ventricular dysfunction [95] or within the coronary circulation [96]. Platelet-derived MPs are a source of thromboxane A2, a potent regulator of vascular tone, as shown in rabbit aorta [97]. In addition, MPs from apoptotic T lymphocytes impair endothelium-dependent relaxation through eNOS down-regulation and caveolin-1 overexpression [98]. Endothelial dysfunction was also targeted by MPs derived from apoptotic SMCs that diminished NO production in mouse aorta [99].

Microparticles in immune-mediated acquired thrombophilic disorders

In "immune-mediated acquired thrombophilic disorders" such as the antiphospholipid syndrome and heparin-induced thrombocytopenia, auto-antibodies could contribute to the release of procoagulant MPs. Elevated levels of EMPs are a common characteristic of antiphospholipid syndrome, solely detected in patients with systemic lupus erythematosus presenting antiphospholipid antibodies and correlated with the lupus anticoagulant moiety that is strongly associated with thrombotic propensity. Plasma samples from antiphospholipid syndrome patients promote EMP release, pointing at a possible antiphospholipid antibody-driven effect. Heparin-induced thrombocytopenia is a common cause of drug-related immune-mediated thrombocytopenia known to favor thrombotic diathesis and the production of antibodies against circulating heparin platelet factor-4 complexes, involved in platelet crosslinking and activation. In patients, circulating MPs expose GPIb, GPIIbIIIa, P-selectin, and thrombospondin and were found highly thrombogenic with the potency to trigger the activation. Likewise, in other drug-induced thrombocytopenia, elevated levels of procoagulant MPs could be detectable as the consequence of drastic platelet stimulation. In the general context of cytopenia, because shed MPs provide an additional phospholipid procoagulant catalytic surface one should keep in mind that a careful evaluation of the thrombotic and bleeding risk should take into account (1) the extent of thrombocytopenia and (2) the extent of procoagulant MP shedding. For instance, in patients with

paroxysmal nocturnal hemoglobinura (PNH) patients with pancytopenia and high levels of circulating MPs should be at low risk of bleeding, whereas PNH patients with pancytopenia but low levels of circulating MPs might probably be [100]. Likewise, elevated levels of circulating platelet MPs were precisely found protective against bleeding in patients with auto-immune thrombocytopenia but were associated with the occurrence of small cerebral vessel infarcts when very high [101].

Microparticles in the restoration of hemostasis: role in transfusion and in hemophilia

If the drastic shedding of procoagulant MPs appears clearly noxious in thrombotic disorder, this does not necessarily endorse their invariably harmful nature, the activation of blood coagulation being considered beneficial in bleeding disorders (see above) [64]. In humans, part of the beneficial effect of rVIIa in hemophilic patients could rely on the shedding of procoagulant MPs, some of them expressing TF [102]. Because MPs are also present, and accumulate, in blood products such as erythrocyte and platelet concentrates during storage, one could not exclude that they act as submicron clotting bumbs. Recently, the use of warm fresh whole blood (WFWB) transfusion has gained interest in patients with severe life-threatening hemorrhagic traumatic injuries, retrospective analysis of American casualties in Iraq and Afghanistan showing lower mortality rates associated with this approach [103]. One of the singularities of the WFWB is the transfusion of leukocytes and derived MPs. Because cell stimulation leads to functional TF exposure on both moieties, it is likely that they might act as potent effectors of the coagulation process in transfused patients, explaining the benefit of WFWB in the restoration of efficient vascular homeostasis [104].

MPs as a source of proinflammatory effectors

Proinflammatory effects of MPs in the vessel

In the settings of inflammatory disorders, various agonists such as cytokines, LPS, microorganisms, toxins, autoantibodies, heatstroke [105] were described as potent inducers of membrane remodeling, PS exposure and MP release. In the early 1990s, we have demonstrated that following LPS stimulation, monocyte disseminate procoagulant and proadhesives activities in the form of circulating MPs enriched in TF, PS, and cytoadhesins [106]. The importance of this pathway in inflammatory induced coagulopathy was underlined by several experimental models. In endotoxemic mice, levels of TF^+-MPs activity correlate with coagulation [107]. Likewise, in enterohemorrhagic *E. coli* infection associated with the release of Shiga toxin, the formation of aggregates between platelets and leukocytes led to the generation of TF^+-MPs that could contribute to microthrombi, tissue damage, and the onset of hemolytic and uremic syndrome [108]. Similarly, in Ebola hemorrhagic fever, overexpression of TF in monocyte/macrophages and derived-MPs is a key event triggering the onset of intravascular disseminated intravascular coagulopathy [109]. The link between sepsis and increased coagulopathy was nicely illustrated by the work of Aras and coworkers in human endotoxemia [110]. Volunteers receiving an infusion of endotoxin exhibited an early increase in TF^+-MPs and to a lesser extent of EMPs [110].

Numerous studies have pointed at the proinflammatory effects of MPs on the endothelium and its phenotype alteration. It is likely that MPs, by releasing a broad panel of inflammatory mediators are key features in the regulation of inflammatory process and cellular infiltration within tissues. The first demonstration that MPs may affect the endothelial phenotype came from studies depicting a transcellular PMP-mediated delivery of arachidonic acid to endothelial cells and the concomitant expression of cyclooxygenase type 2 [111]. In addition, MPs are able to stimulate the release of proinflammatory endothelial cytokines, including IL-6 or MCP-1 and to induce the expression of ICAM-1, VCAM-1 and E-selectin cytoadhesins [58, 111–113]. Amplification loops are probably maintained through the MP-driven expression of appropriate counter-receptors at leukocyte membrane surface. More recently, the transcellular delivery of the chemokines RANTES by platelet-derived MPs was demonstrated to promote monocyte arrest at the inflamed endothelial surface and diapedesis [60].

Beyond their effect on vascular cells, circulating MPs also contribute to an enhanced inflammatory vascular response. MPs are lipid substrate for secretory phospholipase A2 enabling the production of lysophosphatidic acid, a potent proinflammatory mediator and platelet agonist. In addition, the release by activated platelets of inorganic polyphosphates, a polymer that directly binds to and activates the plasma protease factor XII, triggers the secretion of the inflammatory mediator bradykinin and induces fluid extravasation in microvessels [114]. Amplification loops may also result from MP-driven cell crosstalk in aggregates, as suggested in vitro by PMP-driven neutrophil aggregation and activation [115]. Monocyte-derived MPs constitute a secretion pathway for IL-1β and have chemotactism abilities [116]. Another reservoir of proinflammatory MPs is $TACE^+$-MPs from the atherosclerotic plaque that are able to stimulate the release of TNF-α by the endothelial cell (see above).

Proinflammatory effects of MPs in other biological fluids: insights from clinical issues

Inflammatory diseases

Apart from their putative role in the regulation of inflammatory status within the vasculature, a growing literature reporting the proinflammatory character of MPs prompted the clinical investigation in other storage compartments and fluids. In immunoinflammatory diseases and in the absence of an infectious agent (i.e., in non-septic conditions), the nature of the factors triggering the production of proinflammatory TNF and IL-1β cytokines, mainly produced upon activation of monocytes, is still elusive. Imbalance in cytokine homeostasis plays an important role in the pathogenesis of chronic inflammatory diseases such as multiple sclerosis and rheumatoid arthritis (RA). In such settings, mechanisms ruling the balance between TNF and IL-1β and their inhibitors (soluble receptors and sIL-1Ra secreted IL-1 receptor antagonist), escape normal controls. It was previously demonstrated that direct cellular contact with stimulated T cells induces the massive up-regulation of IL-1β and TNF-α in monocytes. Interestingly, MPs shed by stimulated T cells mimic the cellular contact achieved by their parental cell [117]. At the opposite, HDL were demonstrated to interfere in the binding of T lymphoyte-derived MPs to monocytes, thereby reducing the production of proinflammatory cytokines [118].

In the synovial fluid of patients with RA, MPs from monocytes and granulocytes have been identified as procoagulant entities bearing TF and as modulators of chemokine and cytokine production by Fibroblast like synoviocytes (FLS) [119, 120]. Using genetically deficient mice and pharmacological blockade, PMPs could recently be identified in joint synovial fluids as a possible marker of platelet adhesion on the collagen of the FLS extracellular matrix via platelet glycoprotein GPVI. In this particular setting, it is likely that the contact between the extracellular matrix enriched in collagen IV and the platelets contributed to MP release. Moreover, released MPs were identified as potent effectors contributing to joint inflammation through the up-regulation of IL-6 and IL-8 in FLS [121].

Cerebral malaria

Cerebral malaria is another example of the MP proinflammatory potential. During the final stages, alteration of the endothelial blood–brain barrier and the sequestration of *Plasmodium falciparum*-parasitized red blood cells (PRBCs), platelets, and leukocytes within the brain microvasculature occur. MP generation appears a critical step in this noxious loop. Indeed, circulating EMPs in Malawian children were found significantly elevated during crisis and were correlated with plasma TNF concentration. At follow-up, both EMP and TNF levels returned to control values [122]. Interestingly in deficient mice for the ABCA-1 gene, an occasional floppase, protection against neuropaludism could be evidenced, possibly related to the reduced shedding of vascular cells, including platelets and macrophages. Importantly, MPs from infected mice displayed enhanced proinflammatory and procoagulant properties with respect to normal mice [123]. Furthermore, they induced macrophage activation as mirrored by CD40 up-regulation and TNF production.

Experimental data have established erythrocytes-derived MPs as the central inducer of systemic inflammation during malaria infection. Evidences were provided that the binding of PMPs to PRBCs enables the transfer of cytoadhesins to PRBCs leading to a major enhancement in PRBC adherence to the endothelium. It is thus likely that PMPs participate in the pathogenesis of cerebral malaria while interacting with both PRBCs and endothelium [124].

Acute lung injury

Elevated levels of TF^{+}-MPs could be evidenced in the broncho-alveolar fluid of patients with adult respiratory distress syndrome. In murine models, EMPs contribute by themselves to acute lung injury. This conclusion was initially based on multiple measures of histological parameters, lung permeability indexes and dysregulation of endothelial nitric oxide formation and release [125]. Moreover, recent data point at EMPs as inducers of inflammation. They significantly increase pulmonary and systemic IL-1β and TNF-α levels which correlate with increased neutrophil recruitment to the lung. Within the lung, the release of myeloperoxidase, a large source of oxidative injury, is thought to reinforce the noxious action of MPs release [126].

Sepsis

In sepsis, NO and bacterial elements are responsible for the generation of platelet-derived exosome that have an active role in vascular signaling. They behave as redox-active particles that can activate endothelial cell caspase-3 and induce apoptosis through the generation of superoxide, NO and peroxynitrite [127]. Inoculation of MPs isolated from septic rats in healthy counterparts reproduced the hemodynamic septic inflammatory patterns associated with oxidative and nitrosative stresses. The increase in superoxide ion production and NF-KB activity, the overexpression of inducible NO synthase and the overproduction of NO together with decreased endothelial NO synthase activation were evidenced in MP-treated rats [128].

Protective effects of MPs

Altogether, it is likely that the respective importance of each cell lineage and stress in the development of a MP-driven response varies with respect to the location of the thrombotic process and according to the size and nature of the vessel (macroangiopathy/microangiopathy and arterial/venous). MPs could substantially interfere in the three aspects of the Virchow's triad namely vascular inflammation and thrombogenicity, blood thrombogenicity and alteration of vascular reactivity. At the opposite, the shedding of endothelial-derived MPs could be viewed as beneficial since they contribute to the "sorting" of caspase-3, thereby preventing endothelial cell apoptosis and detachment [129].

Anti-inflammatory effects of MPs

Another complexity was brought to our current understanding of MP-mediated inflammatory responses by Gasser and Schifferli [130] who suggested that MPs could promote anti-inflammatory properties. It is likely that neutrophils are able to release potent anti-inflammatory effectors, in the form of membrane vesicles, at the earliest stage of inflammation, already providing a drive to its resolution [130]. Indeed, neutrophil MPs have no proinflammatory activity on human macrophages assessed by the release of IL-8 and TNF-α but increase the secretion of TGF-β, a potent inhibitor of macrophage activation. As a proof of evidence, such vesicles are able to inhibit the macrophage anti-inflammatory response to LPS.

Similarly, erythrocytes-derived ectosomes, another name for MPs, may react with and down-regulate cells of the immune system. MPs are taken up by macrophages, and they significantly reduce their activation by zymosan A and LPS, with a drastic drop in TNF-α and IL-8 release, an effect that lasted for at least 24 h [131]. Other mechanisms were recently proposed to depict the link between MPs and the resolution of the inflammatory response. When shed from adherent neutrophils to endothelial cells, MPs bear annexin A1, an endogenous anti-inflammatory protein able to inhibit the adhesion of stimulated neutrophils adhesion to the endothelium [132]. AnxA1 acts in a paracrine/autocrine fashion and triggers signaling pathways that down-regulate PMN activation while it up-regulates the anti-inflammatory IL-10, leading to the inhibition of inducible NO synthase mRNA expression. The relevance of this anti-inflammatory pathway was nicely illustrated in cystic fibrosis (CF), a disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Typical chronic bacterial infections and inflammation in the lung in CF was associated with lower expression of AnxA1. In vitro assays with human PMN demonstrated that a CFTR inhibitor prompted the sorting of AnxA1 in MPs. A defective counter-regulatory property of AnxA1 in CF cells, owing to its sorting in MPs, might thus contribute to the inflammatory background of the disease, at least in situ [133].

Anticoagulant potential of MPs: a possible role in cytoprotection and in the limitation of the inflammatory response

Anionic phospholipids exposed by activated vascular cells or derived MPs promote the assembly of both procoagulant and protein C anticoagulant enzyme complexes, the latter probably requiring ~10-fold higher PS concentrations. Depending on their cell origin, the presence of anticoagulant thrombomodulin, TFPI, EPCR or protein S at MP surface is another indication of an eventual MP-driven anticoagulant regulatory pathway [85, 134]. Strikingly, although MPs from stimulated endothelial cells are appropriately recognized as potent procoagulant mediators, especially when they harbor active TF, EMPs induced by APC were recently demonstrated to support anticoagulant activities enabling efficient FVa and FVIIIa inactivation at membrane surface. Of note, the MP anticoagulant effect was borne by MPs that exposed EPCR and could be also observed on monocyte-derived MPs. Both APC and PAR-1 active sites were mandatory in anticoagulant MP release [135]. Treatment by anticoagulant rhAPC known to reduce disseminated coagulation and mortality in human severe sepsis is also neuroprotective in ischemic stroke. In a recent report, Pérez-Casal and coworkers provide evidences that part of the cytoprotective effects of APC is mediated by MPs and that APC^{+}-MPs levels are raised in patients with sepsis after treatment by rhAPC [136]. A variety of APC cytoprotective effects like the modulation of cytokine or cytoadhesin transcription levels, antiapoptotic activity and endothelial barrier stabilization, could be induced in vitro by APC^{+}-MPs [137]. In a baboon, heatstroke model and despite a lack of demonstrable antithrombotic effect, rhAPC cytoprotective and anti-inflammatory properties were confirmed by a decrease in thrombomodulin, IL-6, and procoagulant MP levels presumably reflecting diminished cell apoptosis. [138]. This observation also suggests that MP-conveyed APC displays cytoprotective effects at lower doses than those necessary to blunt the activation of coagulation. In humans, heatstroke is characterized by substantial leukocyte activation and MP release that could mediate intravascular coagulopathy in severe clinical settings [105]. Altogether, it is likely that during sepsis or heatstroke, the infusion of APC induces the release of anticoagulant MPs harboring EPCR and APC whilst it diminishes the generation of procoagulant MPs shed upon vascular cell apoptosis.

Of particular importance for pharmacological development, the report by Griffin demonstrating that the APC cytoprotective effects could at least in part be dissociated from its anticoagulant activity, supports the concept that EMPs do not convey an univoque prothrombotic potential, and that anticoagulant and cytoprotective properties may override thrombotic ones upon APC stimulation [137]. Altogether, these observations shed light on the physiopathological relevance of MPs as modulators of cell death and survival eventually associated to that of the inflammatory balance.

Protective effect of MPs on vascular hyporeactivity

In critical disease conditions such as septic shock or trauma, MPs were first considered as deleterious conveyers prompting endothelial dysfunction, blood coagulation, intravascular disseminated coagulopathy and death. Elevated MP levels of platelet, granulocyte and endothelial origin were reported in patients with septic shock, meningococcemia or traumatic brain injury [54, 139, 140]. A beneficial effect conveyed by MPs in patients with septic shock was more recently suggested by the observation that higher mortality rates and organ dysfunction were associated with lower levels of endothelial, platelet, and leukocyte-derived MPs [141]. Because MPs from patients with septic shock enhance contraction of aorta in lipopolyssacharide-treated mice and possibly through the delivery of thromboxane A2, a challenging hypothesis would be that MPs protect septic patients from vascular hyporeactivity by maintaining a tonic pressure response [139]. In line with the observation, ex vivo application of rhAPC, that increases MP generation, also improves arterial contractility and endothelial dysfunction in a murine endotoxemia model [136, 142].

Microparticles and immunity

If the immune response is able to trigger the release of procoagulant MPs as demonstrated in acute allograft rejection following heart transplantation [51], shed MPs are also known to harbor major histocompatibility complex molecules, making them responsible for the stimulation of T cells in an antigen-specific manner, eventually promoting an immune response in vivo. Exosomes, that are other small vesicles, released as a consequence of the fusion of intraluminal membrane-bound multivesicular bodies with the plasma membrane are important mediators in immunity. When released by antigen-presenting cells, one of the major properties of exosomes is to induce antigen-specific T cell activation [143]. Because MPs display autoantigens, such as RNA and DNA, in a highly immunostimulatory manner, they may also act as potent autoadjuvants. In the bone marrow, nucleic-acid-containing MP autoadjuvants might induce B cell tolerance, whereas in the periphery, they might stimulate mature B cells that have escaped central tolerance. Indeed, because MP autoadjuvants can trigger several receptors, they could effectively provide apoptotic or activating signals to B cells [144]. Conversely, MPs could also support a sort of physiological immune escape as that described in pregnancy, a situation in which the invading trophoblast represents a semi-allograft. In this particular setting, the shedding of Fas ligand (CD95 ligand)-exposing MPs may constitute a mechanism by which trophoblast cells promote a state of immune privilege and, therefore protect themselves from maternal immune recognition. Indeed, Abrahams et al. have demonstrated that first-trimester trophoblast cells lack membrane-associated FasL but constitutively secrete active FasL through the release of MPs, and that such MPs are able to induce Fas-presenting T cell death by apoptosis [145]. Cancer represents another situation in which MPs constitute a way to escape from the immune system. Epithelial ovarian cancer cells were shown to secrete functional FasL via the release of MPs [146]. In contrast, normal ovarian epithelial cells express but do not secrete FasL. Tumor-derived MPs may serve as shuttle for FasL, which is known to induce apoptosis of sensitive human lymphoid target via interaction with the Fas receptor. Together, these two studies suggest a mechanism by which tumors might neutralize Fas-bearing immune cells, thus facilitating escape and promoting survival [146]. Of note, tumor-derived vesicles were proposed as actors in the establishment of tumoral niche through the degradation of the extracellular matrix, the activation of fibroblasts, of their resistance to apoptosis and the promotion of an increased motility [147].

Conclusions

MPs serve as biological shuttles enabling cell interactions in various pathological settings and may represent one of the elements coupling thrombosis and inflammation. If the drastic shedding of procoagulant MPs appears clearly noxious in thrombotic disorders or in some models of inflammation-induced coagulopathy, this does not necessarily endorse their invariably harmful nature. In the vessel, endothelial cytoprotection reported in the early regulation of inflammation-induced coagulopathy is emblematic of the beneficial effects other than the prevention of blood leakage. Because of their multiple properties that are characteristic of a private response of the parental cell, MPs prompt target cells through different ways: they are able to cargo active enzymes or anti-inflammatory proteins and to deliver effectors of cell signaling. MP favor the establishment of an integrated communication network in

biological fluids. Further investigations of the mechanisms tuning MP generation and of their mode of action should help our current understanding of the significance of MPs in physiopathological issues and appears a prerequisite in the development of a pharmacological regulation.

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Cellular Mechanisms Underlying the Formation of Circulating Microparticles

Olivier Morel, Laurence Jesel, Jean-Marie Freyssinet, Florence Toti

Abstract—Microparticles (MPs) derived from platelets, monocytes, endothelial cells, red blood cells, and granulocytes may be detected in low concentrations in normal plasma and at increased levels in atherothrombotic cardiovascular diseases. The elucidation of the cellular mechanisms underlying the generation of circulating MPs is crucial for improving our understanding of their pathophysiological role in health and disease. The flopping of phosphatidylserine (PS) to the outer leaflet of the plasma membrane is the key event that will ultimately lead to the shedding of procoagulant MPs from activated or apoptotic cells. Research over the last few years has revealed important roles for calcium-, mitochondrial-, and caspase-dependent mechanisms leading to PS exposure. The study of Scott cells has unraveled different molecular mechanisms that may contribute to fine-tuning of PS exposure and MP release in response to a variety of specific stimuli. The pharmacological modulation of MP release may have a substantial therapeutic impact in the management of atherothrombotic vascular disorders. Because PS exposure is a key feature in pathological processes different from hemostasis and thrombosis, the most important obstacle in the field of MP-modulating drugs seems to be carefully targeting MP release to relevant cell types at an optimal level, so as to achieve a beneficial action and limit possible adverse effects. **(*Arterioscler Thromb Vasc Biol*. 2011;31:15-26.)**

Key Words: calcium ■ cell physiology ■ coagulation ■ pharmacology ■ platelets ■ vascular biology ■ flip-flop ■ plasma membrane remodeling

Microparticles (MPs) are a heterogeneous population of small plasma membrane structures that serve as important signaling structures between cells.[1,2] MPs are composed of a phospholipid bilayer that exposes transmembrane proteins and receptors and encloses cytosolic components such as enzymes, transcription factors, and mRNA derived from their parent cells.[3] Growing evidence suggests that MPs regulate inflammation, stimulate coagulation, affect vascular functions and apoptosis, and can also play a role in cell proliferation or differentiation.[1,4] MPs circulate in the bloodstream, can be detected in the peripheral blood, and may originate from different vascular cell types (eg, platelets, monocytes, endothelial cells, red blood cells, and granulocytes). In health, it has been reported than >80% of circulating MPs express membrane antigens that suggest a platelet origin.[5] Typically, increased MP levels have been associated with thrombotic disorders and systemic inflammatory conditions, including atherothrombosis.[6,7] A prolonged generation of MPs can be deleterious, as it may exert significant procoagulant and inflammatory effects favoring adverse clinical outcomes in a variety of cardiovascular diseases.[1,7] Importantly, circulating MPs can mediate the communication between vascular cells in that they allow membrane interactions between cells at distance.[8] Therefore, the elucidation of the molecular mechanisms underlying the generation of circulating MPs is crucial for improving our understanding of their role in health and disease.[9,10]

It is generally assumed that MPs form when the asymmetrical distribution of lipids between the inner and outer leaflets of a plasma membrane is lost.[11] Under resting conditions, phosphatidylserine (PS) is located almost exclusively in the inner monolayer.[9] When cells undergo activation or apoptosis, PS externalization is one of the earliest observable indicators of the process. Its translocation to the outer leaflet is the initial event that will ultimately lead to the shedding of procoagulant MPs that are therefore regarded as reliable markers of cell stress.[11] The dynamic balance of cell stimulation, cell proliferation, and death within the vessels is reflected by the formation and release of MPs that may thus represent a vascular storage pool of bioeffectors.[9]

MPs shed from activated, necrotic, or apoptotic cells provide a catalytic phospholipid surface for the assembly of blood coagulation factors, thereby promoting the coag-

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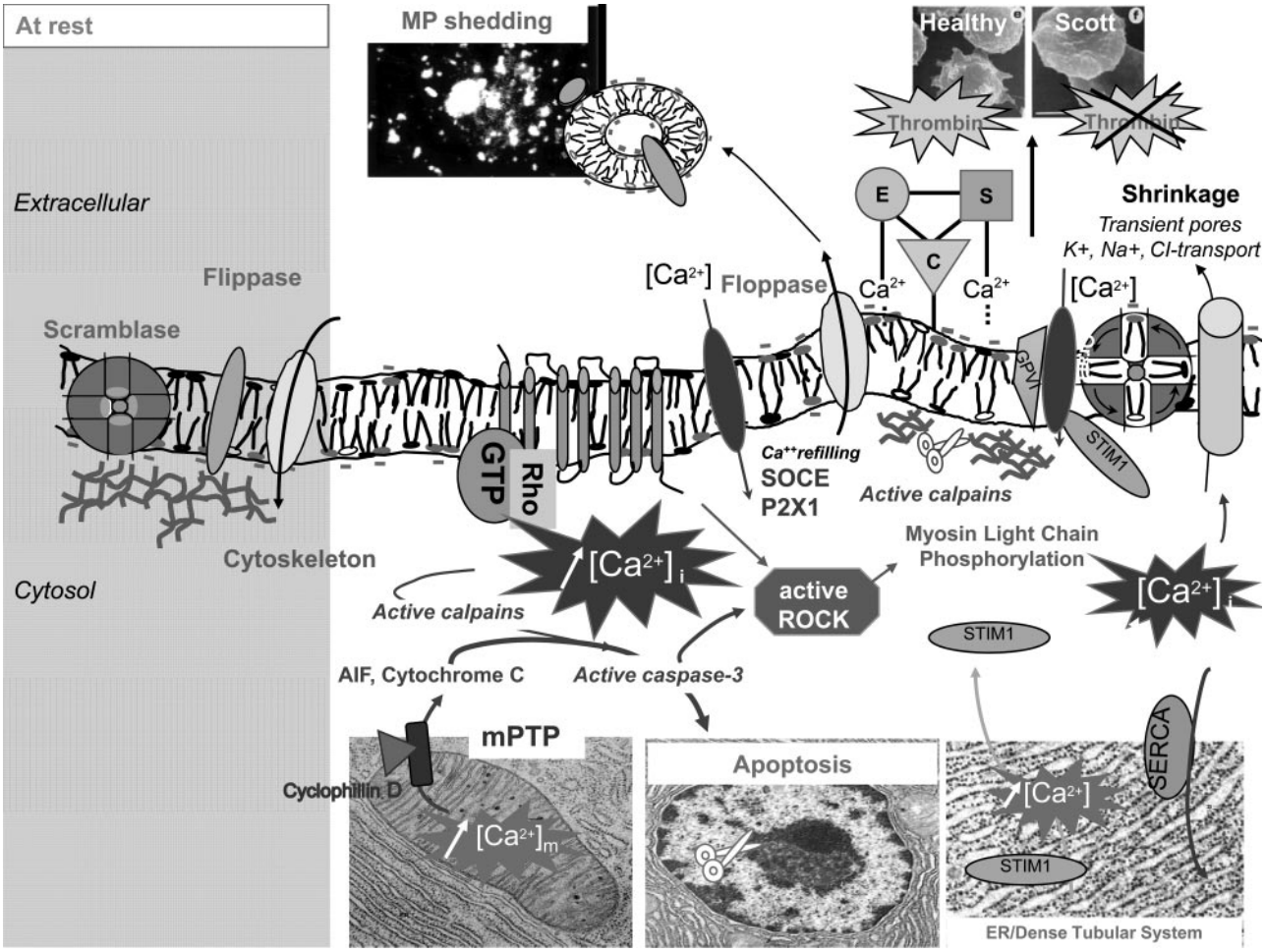

Figure. Key players in membrane remodeling and shedding of procoagulant MPs. Phospholipids in the plasma membranes of resting cells (left) show an asymmetrical distribution. In particular, aminophospholipids (red headgroups), PS (negatively charged) and phosphatidylethanolamine (PE) are located in the inner leaflet. Membrane phospholipid randomization requires cytoskeleton reorganization involving calcium-activated proteases such as caspases and calpains. A sustained increase in intracellular calcium ions is mandatory to PS exposure, eventually associated with changes in mitochondria permeability. The cleavage and reorganization of the cytoskeleton during apoptosis is dependent on the activation of ROCK-I, ultimately resulting in the release of MPs into the extracellular fluid. The exact role of transmembrane ion transport, transient transmembrane pores, and SOCE in PS exposure deserves further investigations to gain insight into a potential pharmacological approach for thrombotic disorders. Recent studies indicate that mitochondrial membrane depolarization is an integral event leading to the outward transport of PS. Membrane budding ultimately leads to the shedding of MPs that expose PS and hijack membrane antigens and cytoplasm components that promote remote cell cross-talk. At the outer leaflet of activated or apoptotic cells and MPs released in the bloodstream, PS enables the assembly of enzymatic complexes involved in the coagulation cascade.

ulation cascade and thrombin generation.[6] MPs can harbor active tissue factor (TF), the cellular initiator of blood coagulation in vivo.[12–14] Because PS and TF are known to act synergistically as potent triggers of blood coagulation, it has been suggested that TF-bearing MPs represent the so-called blood-borne TF.[15–17] These observations suggest that MPs can be viewed as a major therapeutic target, not only in the inhibition of arterial and venous thrombosis but also in the containment of the systemic inflammatory response and atherosclerosis.[18]

In this report, we review the cellular mechanisms governing the formation and the dynamics of circulating MPs. The significance of MPs as potential therapeutic and diagnostic targets for a number of atherothrombotic diseases with unmet medical needs will be discussed.

Loss of Membrane Phospholipid Asymmetry and MP Formation

Under resting conditions, phospholipids are asymmetrically distributed in the membrane of eukaryotic cells.[19] The outer leaflet is enriched in phosphatidylcholine and sphingomyelin, whereas the inner leaflet contains the aminophospholipids PS and phosphatidylethanolamine.[20] The distribution of the membrane lipids is the result of an active process under the dependence of complementary phospholipid transporters governing either the inward (flip) or the outward (flop) translocation. The appearance of PS or phosphatidylethanolamine on the outer leaflet results in a back-transportation to the inner leaflet by an aminophospholipid translocase with "flippase" activity that maintains the normal resting phospholipid distribution (Figure).[21] Because uncatalyzed transbilayer

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transport is slow, lipid asymmetry is stable in quiescent cells. It has been suggested that a "floppase" (ie, an ATP-dependent protein that specifically transfers PS from the interior to exterior leaflet) mediates the rapid outward translocation that is directly responsible for the disruption of asymmetry when cells are activated.[22] In addition, a "scramblase" bidirectional nonspecific lipid transporter—which is activated by an influx of calcium ions—enables all phospholipids to flow down their concentration gradients, thereby favoring randomization.[23] Exposure of PS is the consequence of the rapid overwhelming translocation exerted by floppase(s)[24] and the inhibition of flippase activity by calcium influx.[21] The phospholipid transient mass imbalance between the 2 leaflets due to membrane randomization and the proteolysis of the cytoskeleton promoted by calcium-activated calpains promote the shedding of MPs.[11] Importantly, PS exposure appears to be a nearly universal feature of cells undergoing activation or apoptosis and a common underlying feature of MP release by these cells.[25]

Platelets have the highest scrambling rate known, which ensures a rapid and localized blood coagulation at sites of vascular injury.[23] In other vascular cell types, the scrambling is less effective, with the lowest ability being found in red blood cells.[23]

It is important to emphasize that rapid phospholipid membrane remodeling and PS exposure characterize a physiologically relevant procoagulant response. Accordingly, PS borne by MPs provides additional accessible sites for the assembly of the prothrombinase and tenase complexes, leading to thrombin generation and clotting.[6] In this context, shed MPs could protect from bleeding during idiopathic thrombocytopenic purpura, whereas their dramatic release could favor neurological complications of thrombotic origin.[26] Importantly, platelet-derived MP surfaces have 50- to 100-fold higher procoagulant activity compared with the normal platelet surface.[27]

Microparticles Without Procoagulant Activity: Formation, Detection, and Significance

According to the widely accepted principle that MPs are released by cells on the collapse of the plasma membrane phospholipids asymmetry, the existence of MPs that do not expose PS remains a matter of debate. Preliminary evidence suggests that some vesicles derived from endothelial cells are PS-negative by annexin-V labeling.[28] In the clinical setting, however, the accurate detection of platelet-derived procoagulant MPs remains of paramount importance for the accurate assessment of prothrombotic risk.[29] Both in platelet-rich plasma[30] and isolated platelet suspensions,[31] the efficacy of platelet agonists to trigger PS exposure and the release of procoagulant MPs may display a significant degree of variability, suggesting that concentrations of MPs can mirror the extent of platelet stimulation. For example, physiological agonists (eg, collagen) are able to induce the release of a fewer number of procoagulant MPs compared with non physiological agonists (eg, calcium ionophores). In addition, procoagulant MPs are enriched in P-selectin, CD63,[30] GPIIb-IIIa, or calpains.[32,33] These observations suggest that an efficient generation of procoagulant MPs is accompanied by a major remodeling of the platelet membrane, as well as by platelet granule secretion (see below). Connor et al[30] have reported that up to 80% of platelet-derived CD41-positive MPs circulating in the platelet poor plasma (PPP) of healthy donors cannot be labeled by annexin V. In addition, the proportion of procoagulant MPs generated in the platelet-rich plasma following exposure to collagen was less than 40%. Taken together, these data question our current understanding of the nature and significance of such circulating nonprocoagulant platelet-derived vesicles in plasma.[30] Recently, Flaumenhaft et al[5] have reported that megakaryocytes constitute a physiological source of circulating procoagulant MPs characterized by the exposure of PS, CD42, and CD41, as well as the presence of full-length filamin.

As the methodological approach is crucial for the assessment of MP shedding, it is difficult to reconcile the large amount of data currently available in the literature. For example, it is possible that experimental conditions can impair the use of annexin V as a probe for PS or immunologic labeling, particularly when using blood samples in the absence of washing steps. As recently confirmed by Connor et al using a functional coagulation assay and flow cytometry,[30] there is no doubt that annexin V is appropriate and specific for the detection of procoagulant MPs, at least at tightly regulated calcium concentrations allowing a specific binding to PS.[33,34] Notably, cell activation and treatment can further complicate the analytic methodology, thus making the preanalytical sample treatment of crucial importance. In this regard, annexin V is known to inhibit platelet MP release, whereas calcium-induced calpain activity is considered mandatory for platelet shedding following exposure to agonists.[31] The potential contribution of the exosomes present in platelet-rich plasma during platelet activation and shedding remains a matter of debate. Indeed, these exosomes expose very low (if any) PS and cannot be detected by flow cytometry because of their small size.[35]

The nature of the annexin V–negative vesicles detected by flow cytometry in platelets or endothelial cells[28,30]—as well as the mechanisms enabling their release—is poorly understood. One potential explanation is that they can reflect basal tissue homeostasis. Alternatively, they can result from multiple fusion events between cell debris or small endosomal-secreted vesicles with the medium or plasma components, possibly through interactions with lipophilic proteins. Another putative mechanism may rely on the release from the plasma membrane due to a particular cytoskeleton cleavage. This would allow the maintenance of the phospholipid asymmetrical distribution, as observed in megakaryocytes (see below).[36] Nevertheless, the generation of MPs from resting platelets cannot be visualized by real-time imaging.[5]

Although the functional differences between activated cells and cell-derived MPs are not entirely understood in vivo, these findings suggest that cell-derived MPs are not only potent procoagulant effectors in atherothrombosis but can also convey a variety of different molecules, such as adhesion receptors, cytokines, bioactive lipids, mRNA, caspases, and calpains, to the neighboring cells (see below).[37]

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Membrane Remodeling and Microparticle Formation: Insights From Scott Syndrome

The physiological relevance of membrane remodeling and the shedding of MPs in hemostasis is well exemplified by Scott syndrome. This rare bleeding disorder is characterized by provoked hemorrhages caused by the reduced floppase activity of vascular cells, the diminished externalization of negatively charged phospholipids, and reduced MP shedding.[38,39] As a consequence, the binding of coagulation factors and their assembly to the platelet surface is decreased and thrombin generation limited.[40–42] Low prothrombin consumption in the serum is the sole abnormal hemostasis parameter. Although initial studies could not clearly distinguish between a hereditary and an acquired bleeding disorder, recent evidence unequivocally indicates that Scott syndrome has a genetic etiology.[39,43] The hereditary nature of the disorder has been corroborated by the discovery of a single, inbred colony of German shepherd dogs with virtually identical symptoms.[43] In both human patients and affected dogs, a defective floppase activity is considered the core pathophysiological defect of Scott syndrome.[43,44] Pedigree studies have shown that this defect equally affects males and females, consistent with an autosomal recessive inheritance pattern of the disorder.[42]

To date, only 3 documented human cases of Scott syndrome have been reported.[42] The paucity of human cases has hindered genetic analyses of the trait. As the underlying defect seems to be the deficiency of phospholipid floppase, the initial candidate gene for Scott syndrome was the canonical phospholipid scramblase (*PLSCR1*).[45] PLSCR1 mediates accelerated transbilayer unspecific migration of phospholipids, facilitating rapid translocation of PS to the cell surface on elevation of intracellular calcium concentrations. Although expression levels of PLSCR1 mRNA have a good degree of correlation with scrambling efficiency in different cell types,[45] PLSCR1-null mice show normal hemostasis and PS externalization.[46] In addition, the product of *PLSCR1* gene is normally expressed in Scott cells.[47,48] The fact that occasional and specific PS transporters probably coexist in the membrane represent the main obstacle in the identification of the PS-floppase necessary for the hemostatic response. Like most occasional PS translocators, multidrug resistance protein-1 (MDR1) or ABCA1 are members of the ATP-binding cassette protein family[49,50] that have been investigated in Scott cells.[51] In humans, the loss of function of ATP-binding cassette transporter ABCA1 leads to the dyslipidemic phenotype of Tangier disease, an autosomal recessive disorder of lipid metabolism characterized, at the cellular level, by the defective translocation of membrane lipids to specific apolipoprotein acceptors. In mice, ABCA1 promotes Ca^{2+}-induced exposure of PS at the outer leaflet of the plasma membrane of Ca^{2+} ionophore-stimulated red blood cells, as determined by a prothrombinase assay, membrane shedding, and measurement of transbilayer redistribution of spin-labeled phospholipids. In addition, the rate of PS translocation has been found sensitive to the altered expression of ABCA1 in knock-out mice. In a patient with Scott syndrome, Albrecht et al[49] identified a heterozygous missense mutation (Arg1925Gln) in the ATP-binding cassette transporter *ABCA1* gene, which was not found in unaffected family members or controls. However, the authors found no causative mutation for this phenomenon in the *ABCA1* gene or its proximal promoter and suggested that a putative second mutation in a transacting regulatory gene might be involved.[49] In the canine hereditary bleeding disorder, *ABCA1* has been firmly excluded as the causative genetic defect.[52] Recently, a genome-wide linkage scan in German shepherd dogs has localized canine platelet procoagulant deficiency (Scott syndrome) to canine chromosome 27.[53] Of note, this region does not contain the canine homologues of human *PLSCR1* or *ABCA1* genes.[53] Another rare bleeding disorder reported by Castaman and coworkers shares clinical and laboratory findings with Scott syndrome.[42] This defect was found to be associated with an inability to form platelet-derived MPs on cell activation, despite normal PS expression on the platelet surface.[42] Although the reason for this defective shedding remains elusive, both disorders highlight the importance of platelet-derived MPs for efficient hemostasis.

Previous studies have shown that the abnormal membrane remodeling in Scott syndrome can also affect red blood cells and lymphocytic cells, indicating that it occurs in multiple hematopoietic lineages.[41,42] Importantly, Scott lymphoblasts show normal exposure of PS and MPs shedding when subjected to apoptotic stress.[54] It is clear that the elucidation of molecular pathways active in Scott syndrome—different from those operating in the process of membrane remodeling typical of hemostatic responses—holds great promise for our understanding of the mechanisms governing MPs formation in health and disease.

Calcium Channels, Ions, and Microparticle Formation

The exposure of procoagulant phospholipids and the shedding of MPs are cellular responses that depend on activating conditions. Notably, membrane remodeling and PS externalization is dependent on an increase in cytosolic calcium.[55] Activation of human platelets by a Ca^{2+} ionophore results in the surface exposure of PS. Conversely, the inhibition of Ca^{2+} influx abolishes agonist-induced PS externalization and the procoagulant response in activated platelets.[56] For more than 20 years, it has been known that calcium ion influx and activation of the Ca^{2+}-dependent protease calpain stimulate platelets to shed MPs. In a landmark study, Chang et al[57] have reported that MP formation and the surface exposure of PS show a similar requirement for Ca^{2+}. The Ca^{2+}-driven mechanisms underlying rapid phospholipid redistribution include a number of different events, including the activation of transmembrane outward PS transporters, the inhibition of the inward aminophospholipid translocase, the transmembrane redistribution of PS, and even cell shrinkage with a decrease in lipid packing of both leaflets. As small increases in Ca^{2+} lead to platelet activation, the maintenance of a stable Ca^{2+} is essential to keep platelets in a resting state. The mechanisms limiting platelet activation by counteracting Ca^{2+} leakage from the intracellular stores rely on sarcoplasmic/endoplasmic Ca^{2+} ATPases that pump the calcium ions back to the stores (targeted by the inhibitor thapsigargin) and on a plasma membrane Ca^{2+} ATPase that pumps Ca^{2+} out of the cell.

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Calcium entry in the platelet occurs via at least 2 different mechanisms. The first consists in the agonist-induced release of Ca^{2+} from cytosolic stores, followed by an influx of calcium ions through the plasma membrane. This process is commonly referred to as store-operated calcium entry (SOCE). The second mechanism depends on the purinergic calcium channel P2X1.[58] SOCE may act as a modulator of aminophospholipid exposure (Figure). In platelets, the release of Ca^{2+} from the endoplasmic reticulum induces SOCE and a rapid PS exposure without the need for ligand-receptor interactions.[54,59] Importantly, this mechanism of refilling empty internal stores[59] has been found to be reduced in Scott B-lymphoblasts of one Scott patient,[54] although it was normal in others.[60,61] This clearly suggests that defective SOCE cannot be considered a typical feature of Scott syndrome. Nonetheless, this apparent discrepancy might be explained by the heterogeneity of transformation by Epstein-Barr virus.

In human erythroleukemic cell line (HEL) cells, SOCE and the exposure of PS are regulated through the reorganization of actin cytoskeleton by Rho A (a small GTPase), and not by ROCK, highlight the presence of intricate control pathways (Figure and section below).[62] More recently, STIM1 (stromal-interacting molecule 1), a transmembrane protein located in the tubular dense system, has emerged as the key regulatory protein of SOCE in platelet with Orai1 as principal Ca^{2+} entry channel after depletion of the Ca^{2+} stores.[63] The interaction between oligomerized Orai1 and STIM1 leads to channel opening. Research has suggested that mice expressing a mutated form of Orai1 (Arg93Try) show a marked inhibition of platelet SOCE together with impaired agonist-induced cytosolic Ca^{2+} elevation.[64] However, Orai1 (Arg93Try) platelets normally aggregate and adhere to collagen under arterial flow conditions ex vivo. Nonetheless, adherent Orai1 (Arg93Try) platelets are defective in PS exposure, suggesting that Orai1 plays a pivotal role in the platelet procoagulant response rather than in other Ca^{2+}-dependent cellular responses.[64]

The relevance of the SOCE pathway to thrombosis is suggested by the protection against collagen-dependent arterial thrombus formation and brain infarction associated with Orai1 or STIM1 deficiency.[65,66] Nevertheless, platelets possess 2 distinct mechanisms of Ca^{2+} entry and PS exposure. The first relies on STIM1-Orai1 interaction, whereas the second most likely acts in a thrombin receptor-operator fashion.[63] Accordingly, the reduction of PS externalization and thrombus formation observed in STIM1- and Orai1-deficient platelets is evident at high shear flow over collagen and is associated with impaired GPVI-dependent Ca^{2+}. In contrast, the lacking of STIM1 and Orai1 does not abolish PS exposure and thrombus formation in the presence of thrombin. This observation is in good agreement with the finding of an unchanged intraplatelet Ca^{2+} signaling following collagen-thrombin activation in one Scott patient.[60] STIM1 and Orai1 jointly contribute to glycoprotein VI-induced SOCE and procoagulant activity, but a different Ca^{2+} entry mechanism provides a compensatory pathway when thrombin acts as a coagonist.

Interestingly, platelets from STIM1- and Orai1-null mice correctly exert most of their physiological functions despite a lack of SOCE-mediated calcium entry. These results clearly suggest that Ca^{2+} store release is sufficient to trigger important basic platelet functions such as shape change, integrin activation, and granule release in a SOCE-independent fashion. It is thus feasible that SOCE may be of special relevance in the processes following initial platelet adhesion and primary activation (PS exposure, coagulant activity, and thrombus stabilization).

It should be kept in mind that Ca^{2+} signaling pathways may be cell type specific. For example, raft integrity, SOCE involving transient receptor potential channel 1, and cGMP have been shown to play an important role in the completion of PS transmembrane redistribution in megakaryocytic HEL cells.[62,67,68]

However, the actual role of SOCE in cells activated by nonphysiological agonists is still a matter of debate. Munnix et al have shown that elevated SOCE-driven Ca^{2+} elevation is not sufficient to trigger PS exposure.[60] In addition, Ca^{2+} ionophores may trigger membrane remodeling without a need for store-operated Ca^{2+} entry.[69] Similarly, the increase in cytosolic Ca^{2+} elicited by the reticular Ca^{2+}-ATPase inhibitor thapsigargin in lymphocyte cells is not sufficient to induce scrambling.[60,69] Importantly, evidence suggests that modulation of intracellular free calcium in human platelets affects the formation of MPs in clinical settings. Labiós et al demonstrated that treatment of hypertensive patients with doxazosin leads to normalization of the kinetics of cytoplasmic cytoplasmic-free calcium, leading to a reduced release of MPs.[70] Further study is needed to confirm whether the pharmacological modulation of procoagulant MP release may improve the clinical outcomes of patients with atherothrombotic disorders.

Although PS externalization is largely believed to be calcium ion–dependent, regardless of the cell lineage, this pathway alone may not be sufficient to promote the release of MPs. Indeed, evidence suggests that flux of ions other than calcium may play a role in the exposure of anionic phospholipids at the cell surface (Figure). Following activation, shrinkage is the result of cell dehydration due to a Ca^{2+}-activated efflux of potassium and chloride ions.[71] In addition, the activation of Ca^{2+}-sensitive K^+ channels (Gardos channels) has recently been implicated in the mechanisms regulating PS exposure in platelets activated by collagen and thrombin.[72] In accordance with this, the lack of procoagulant platelet response observed in one patient with Scott syndrome was partially restored by pretreatment with valinomycin, a selective K^+ ionophore. Another study has suggested that Na^+ loading via Na^+/H^+ exchange is involved in the regulation of PS scrambling in activated platelets.[73] These results clearly indicate that cytosolic calcium ions levels are not a unique player leading to the collapse of the membrane asymmetry. Whether these mechanisms may have a role in modulating the release of MPs in vivo deserves further scrutiny.

Cytoskeleton Reorganization and Microparticle Formation

Cytoskeleton integrity is believed to participate in the maintenance of membrane asymmetry and cell shape, its reorganization could therefore favor membrane budding in stimulated cells (Figure). Cauwenberghs et al[36] have shown that

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MP formation by resting platelets results from αIIbβ3 signaling to destabilization of the actin cytoskeleton in the absence of calpain activation. Unlike agonist-induced MP generation, this mechanism does not require either Ca^{2+} elevation or the activity of calpain, a Ca^{2+}-dependent thiol-protease known to modify platelet cytoskeleton and some signal transduction enzymes. Although baseline calpain activity seems insufficient to degrade cytoskeleton components, agonist-induced Ca^{2+} influx is necessary to reach Ca^{2+} concentrations sufficiently high to induce maximum protease activity.[36] Calpains have been known for more than a decade to play a crucial role in platelet shedding. The inhibition of calpain reduces the degree of MP release from collagen- and thrombin-stimulated platelets.[74] A similar decrease has been also observed after treatment with cytochalasin D, an actin depolymerizer.[31] Recently, Flaumenhaft et al[5] have reported that megakaryocyte MP generation is resistant to inhibition of microtubule assembly—which is critical to platelet formation—and is increased by the inhibition of actin polymerization, an observation that questions the incidence of the fate of the cell on its ability to shed MPs. Caspases are cytoplasm proteases from the apoptotic cascade involved in the reorganization of the cytoskeleton, possibly related to either membrane remodeling or cell differentiation. Caspases or calpain is responsible for the proteolytic cleavage of prominent cytoskeletal proteins such as filamin-1 (a marker of megakaryocyte-derived MPs),[5] gelosin, talin, and myosin.[75] Of note, the induction of the platelet apoptotic machinery by the BH3 mimetic compound ABT-737 has been shown to result in a time-dependent cleavage of both gelsolin and filamin that paralleled exposure of PS and platelet membrane blebbing.[76] The release of MPs can be also triggered by activation of caspases in other vascular lineages. In the Jurkat T-lymphocyte cell line, caspase-3 mediates the cleavage of ROCK I, a Rho-kinase acting by myosin light chain phosphorylation that induces cell membrane contraction and MP release during apoptosis (Figure).[77] In the absence of cell death and in the presence of thrombin (ie, in a condition more closely related to hemostasis), Sapet et al[78] identified by microarray a group of genes linked to the cytoskeleton reorganization and involved in MP release from endothelial cells. Of these, the Rho-kinase ROCK-II activated by caspase-2 showed a high transcription rate. ROCK-I, the Rho-kinase isoform that is activated by caspase-3, was not modulated by thrombin. These results are in keeping with previous findings by Tramontano et al[79] that suggested that the Rho activation is the key element involved in endothelial shedding under inflammatory conditions. These authors showed that fluvastatin decreased the generation of MPs from tumor necrosis factor-α stimulated human coronary artery endothelial cells, as well as targeted Rho activation.[79] In addition, the rho-kinase inhibitor Y26732 produced a similar effect. Together, these results point to a critical role for Rho kinases, which was also confirmed by granzyme-triggered caspase-3-independent apoptosis.[80] It should be emphasized that the contribution of different signal transduction pathways involved in cytoskeleton reorganization to MP release is a function of cell stress or cell lineages, and their physiopathological relevance in the vascular compartment deserves further studies.

Signal Transduction Pathways and Microparticle Formation

Numerous signal transduction pathways (activation-induced protein tyrosine dephosphorylation, protein phosphorylation, and calmodulin activation) have been implicated in the generation of MPs from activated cells. In a seminal study, Wiedmer and Sims[81] demonstrated that the phosphatase inhibitor vanadate increased the formation of platelet-derived MPs, Interestingly, MP formation was partially inhibited in platelets treated with the protein kinase inhibitor sphingosine, the myosin light chain kinase inhibitor ML-7, and the calmodulin-antagonist W-7 and under conditions of elevated cytosolic concentration of cAMP.[81] Pasquet et al[82] have reported that microvesicle release during platelet activation is specifically associated with μ-calpain activation, increased protein tyrosine phosphatase activity, and decreased tyrosine phosphorylation. Other studies have shown that adhesion-induced procoagulant activity of platelets is regulated by protein tyrosine kinase-dependent Ca^{2+} responses.[83,84] Altogether, these results clearly indicated that platelet MP formation is influenced by the state of phosphorylation of the platelet cellular compartments and by calmodulin, myosin light chain kinase, and other calmodulin-regulated effectors. Experimental data in human HEL cells have shown that extracellular signal-regulated kinase (ERK) phosphorylation acts as a cornerstone in response to a variety of external stimuli.[67] In HEL cells, the phosphorylation of the 2 isoforms ERK1 and ERK2 is increased during PS exposure induced by the Ca^{2+} ionophore A23187. The reduction of procoagulant activity induced by ERK inhibitors suggests that these proteins are involved in the signal transduction pathways initiated by the increase of Ca^{2+}.[67] Nevertheless, ERK phosphorylation induced by Ca^{2+} occurs in a cell-specific manner, requiring, for example, an external Ca^{2+} influx in Jurkat cells but not in platelets. It is difficult to reconcile current data in the context of a procoagulant cell response, as ERK phosphorylation and membrane scrambling leading to PS exposure are independent mechanisms occurring in platelets.[85]

Yan et al[86] have recently reported that inhibition of basal cAMP-dependent protein kinase activity resulted in an increased platelet MP formation and PS exposure. Pretreatment of platelets with the cAMP-elevating agent forskolin abolished thrombin plus collagen-induced MPs formation and PS exposure and obviously decreased calcium ionophore-evoked MPs shedding. These results imply that protein kinase plays key roles in the regulation of platelet MPs formation and PS exposure.[86] Signal transduction pathways and MP formation have been studied in other cell lineages and under various stress conditions. Curtis et al[87] have shown that the pharmacological inhibition of p38 mitogen-activated protein kinase in human aortic endothelial cells, resulted in a 50% reduction of tumor necrosis factor α–induced endothelial MPs. These findings implicate p38 mitogen-activated protein kinase signaling as significant and selective in the formation and maturation of endothelial MPs.

Interestingly, in a subproteomics analysis of B-lymphoblasts from a patient experiencing Scott syndrome, Imam-Sghiouar et al[88] failed to find any differential expression of tyrosine-phos-

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phorylated proteins that can account for the lack of exposure of procoagulant PS at the exoplasmic leaflet of plasma membrane.

Hashimoto et al[89] have recently investigated whether the platelet expression of toll-like receptor 4, the receptor for Gram-negative bacterial lipopolysaccharide, may influence the release of MPs. Acute direct activation of toll-like receptor 4 increased phosphorylation of p38 mitogen-activated protein kinase and decreased production of prothrombotic PS and P-selectin-positive MPs in response to TRAP, the thrombin receptor agonist peptide. Therefore, reduced platelet reactivity and procoagulant potency could result in vitro from toll-like receptor 4–driven prestimulation.[89] Another line of evidence questioning the role of initial platelet conditions in the promotion of membrane remodeling comes from transgenic mice overexpressing the platelet purinoreceptor receptor-operated calcium channel $P2X_1$. Indeed, the perfusion of whole blood from $P2X_1$-overexpressing mice over collagen fibers under shear results in increased $P2X_1$-dependent aggregation and PS exposure.[90]

Whatever the initial mechanism leading to PS exposure and MP release may be, the generation of circulating thrombin contributes to the autoamplification loops by targeting resting platelets and endothelial cells, ultimately leading to an enhanced release of deleterious MPs in the vessel. It has recently been demonstrated that thrombin can also increase the expression of cell-associated and soluble form of TRAIL, a cytokine belonging to the tumor necrosis factor family. The TRAIL/Apo2L complex has been shown to mediate the release of endothelial-derived MPs by initiating the recruitment of adaptor proteins and the activation of nuclear factor-κB.[91] Inflammatory mediators secreted after tumor necrosis factor treatment (intercellular adhesion molecule-1) or upregulated by thrombin (intercellular adhesion molecule-1, interleukin-8) can also contribute to the autocrine pathways that amplify endothelial vesiculation.[92] Indirect evidence also suggests that interleukin-1 and its receptor may play a role in the amplified generation of MPs from thrombin-treated human microvascular endothelial cells. Therefore, additional complexity is brought by the cross-talk of elements of the coagulation and inflammation processes in the release of MPs.[91,92] Together, these findings indicate that the relative contribution of the pathways involved in the tuning of membrane remodeling is dependent on both the cell lineage and the type of stress, some of them being linked to the mechanisms of acute cell response.

Blebs, Pores, and Microparticle Formation

Traditionally, the transbilayer movement (flip-flop) of PS has been suggested to be the key mechanism in the induction of membrane lipid asymmetry. However, a temporary, localized disordering of the lipid bilayer structure with the formation of transient membrane pores may serve as another pathway for movement of lipid molecules from one leaflet of the membrane to the other (Figure), ultimately leading to membrane disruption.[93] Following the formation of hydrophilic pores, PS has access to both leaflets of the membrane.[94,95] The formation of membrane blebs is another mechanism putatively involved in PS externalization.[96] In this regard, Elliott et al[96] have shown that Ca^{2+}-stimulated PS exposure in B cells is strain variable, ABCA1 independent, and both preceded by and dependent on a decrease in lipid packing. Following stimulation with Ca^{2+} ionophore, K^+ and Cl^- ions leave the cells that shrinks, shedding appearing as the consequent membrane distortion. The importance of this work lies in the fact that changes in the plasma membrane organization may occur by a translocase-independent mechanism.[96] Whether this MP-forming mechanism may serve as a novel target that can be moved toward the clinic deserves further study.

Platelets, Apoptosis, and Microparticle Formation

Before cells undergo nuclear apoptotic DNA fragmentation, they show early cell-membrane asymmetry and expose PS.[97] Importantly, PS exposure on the cell surface during apoptosis occurs on a different time scale than platelet activation. In cells that show inducible tissue-factor expression, apoptosis promotes a high-dependent procoagulant activity because of PS expression.[6] There is convincing evidence that the membrane of platelet-derived MPs arising from megakaryocyte fragmentation through an apoptotic process also retain procoagulant properties.[5] Of note, platelets contain several key regulators of apoptotic cell death, including caspases and different members of the Bcl-2 protein family. Previous studies have shown that the apoptotic machinery may contribute to the agonist-driven PS exposure by platelets.[74] For example, one prosurvival member of the Bcl-2 protein family, termed Bcl-xl, plays a crucial role in the maintenance of platelet viability.[98] Interestingly, a decline in Bcl-xl levels occurs in stored platelets and is associated with an increase in PS exposure and platelet procoagulant activity.[99] The activation of apoptotic signaling in platelet can include both death receptors and mitochondrial pathways. These cytotoxic signaling pathways result in activation of effector caspases.[99] Another recent study has shown that the platelet apoptosis machinery and the classical calcium-driven rapid agonist pathways, although both able to promote PS exposure, are distinct.[76] The coexistence of different signaling pathways triggered either by activation or apoptotic stimuli is not restricted to platelets. The differences related to PS exposure in MPs generated from apoptotic or activated endothelial cells may reflect distinct mechanisms leading to the release of MPs (see above).[28]

Membrane Remodeling, Calcium, and Tuning of Different Signaling Pathways

During apoptosis, the exposure of PS is a relatively early event, taking place before DNA degradation and the loss of membrane integrity.[23] Although PS exposure is a downstream event during the apoptotic cascade, evidence suggests that caspases do not exert a direct effect on the floppase activity. Accordingly, floppase activation in certain cell types proceeds efficiently even in the presence of broad spectrum caspases inhibitors that block downstream event such as DNA degradation.[100] It is noteworthy that the kinetics of PS exposure and MP release differ significantly in cells entering apoptosis compared with in those undergoing activation. Although shedding of MPs occurs on the hour time scale in apoptosis,[101] platelet activation

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results in a rapid exposure of PS and the release of MPs is observed within a few minutes.[102]

The question of whether apoptosis and procoagulant PS exposure share the same molecular pathways remains a matter of debate. The exact role played by calcium ions in the mechanisms of PS exposure occurring in apoptotic cells is still incompletely understood. Interestingly, the removal of extracellular Ca^{2+} has been shown to prevent PS exposure in Jurkat cells undergoing apoptosis, whereas chelation of intracellular Ca^{2+} did not.[103] Another study has shown that Ca^{2+} flux—and not a net change in intracellular Ca^{2+} levels—is essential for the activation of the scramblase activity in apoptotic cells.[104] It is worth noting that the loss of PS asymmetry does not always result in the activation of the apoptotic machinery.[105] In light of these findings, it has recently been suggested that PS exposure can occur through at least 2 different pathways.[76] The first is strictly calcium ion dependent and is amplified by the mitochondrial depolarization, whereas the second (the apoptotic pathway) occurs independently of intracellular Ca^{2+} concentration changes.[76] This possibility fits well with the observation that PS exposure occurs in Scott lymphoblasts during apoptosis.[100] A previous study has shown that inhibition of caspase-3 leads to a blockade of PS exposure and MP shedding in platelets. Conversely, other platelet responses (α-granule secretion, shape changes, and aggregation) remained unaffected.[74]

Mitochondrial Permeability Transition Pore and PS Exposure

In recent years, studies of murine platelets lacking key mitochondria-associated proteins have demonstrated the role played by mitochondrial permeability transition pore (mPTP) in the regulation of PS exposure. For example, the cyclophilin D knockout mice (lacking a key component of the mPTP) show marked defects in PS externalization and prothrombinase activity after platelet stimulation.[101] Conversely, platelets from mice lacking Bax and Bad (2 critical sensitizers of the mPTP) have a normal rate of PS exposure following stimulation.[76] Therefore, mitochondrial membrane depolarization appears to be an integral event leading to exposure of PS in platelets exposed to thrombin and collagen.[102,103] The mPTP is a channel that causes the loss of the mitochondrial at high Ca^{2+} mitochondrial concentration.[104] When opened, mPTP allows the release of proapoptotic into the cytosol (Figure).[105] Importantly, the mitochondrial permeability transition appears an important checkpoint for the shedding of platelet-derived MPs.[106] The engagement of the mitochondrial-dependent pathway is characterized by cytochrome c release, a prolonged increase in cytosolic Ca^{2+} concentrations, the processing of caspases 0 and 3 by calpains and is accompanied by the externalization of PS, even in the absence of extracellular calcium ions. The reciprocal influences between the signaling pathways regulating membrane remodeling deserve further investigation. However, a large body of evidence points to a role for mitochondria as the key regulators in PS exposure and MP shedding. Very intriguingly, the Scott syndrome trait locus recently identified in dogs includes the canine homologues of several mitochondria-associated proteins and apoptosis regulators.[53]

In line with our findings in one human Scott patient,[107] tetracaine (a local anesthetic agent that triggers mitochondrial depolarization) induces PS exposure in the canine Scott syndrome to an extent similar to that in canine control platelets.[43] Although platelets from Scott syndrome dogs show a loss of mitochondrial potential following exposure to thrombin and collagen, PS externalization does not occur.

A recent study has focused on the reciprocal importance of the increase of cytosolic calcium ions and the mitochondrial membrane depolarization in the induction of PS exposure.[108] The results demonstrated that—regardless of the loss of membrane mitochondrial permeability—PS exposure is triggered by a marked increase in cytosolic calcium ions concentrations. Challenging previous observations, these findings clearly indicate that PS exposure can occur even without loss of membrane mitochondrial permeability. In turn, evidence in rabbits suggests that the loss of membrane mitochondrial permeability in aged platelets can take place without PS exposure.[109] Taken together, these results indicate that the increase of cytosolic calcium ions concentration rather than mitochondrial membrane depolarization is the critical event leading to PS exposure.

Toward a Pharmacological Control of PS Exposure and Microparticle Shedding?

The pharmacological control of flip-flop and MP shedding remains a challenging issue because of the high number of intricate pathways leading to membrane remodeling. Some of these pathways are crucial to tissue homeostasis, whereas others are involved in the cellular response to acute stress (eg, blood leakage). Additional lines of evidence in support of this complexity are derived from the understanding of the effects of cyclosporin A both in vitro and in vivo. Cyclosporin A, a ligand for cyclophilin D, is known to block mPTP opening.[104] When cyclophilin D–null platelets are exposed to Ca^{2+} ionophores, cyclosporin A does not inhibit PS externalization.[99] Accordingly, PS exposure is triggered by a marked increase in cytosolic Ca^{2+} concentrations in platelets regardless of a loss of membrane mitochondrial permeability.[108] In any case, cyclosporin A holds promise for the pharmacological modulation of MP shedding in the clinical setting of kidney transplantation because treatment with cyclosporin A results in an earlier reduction of endothelial-derived MPs levels than in patients treated with tacrolimus/mycophenolate.[110] The above evidence indicates that different molecular pathways (caspase-dependent mechanisms, increased mitochondria permeability, sustained increase in intracellular calcium ions) can mediate membrane lipid scrambling and ultimately lead to PS exposure. Importantly, these mechanisms might have different sensitivity to pharmacological agents. The exact role played by transmembrane ion transport or transient transmembrane pores in phospholipid scrambling remains to be established. Notably, PS exposure may also represent a signal for platelets clearance at the steady state. This possibility is supported by experimental data showing a reduced adhesive function and an enhanced clearance of senescent platelets from the bloodstream that may actually lead to a reduced prothrombotic potential.[76]

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Besides their procoagulant activity, MPs may play a regulatory role in vascular remodeling and fibrinolysis, which may ultimately have therapeutic implications. In this regard, Podor et al[111] have demonstrated that the vimentin-dependent localization of active plasminogen activator inhibitor-1 on the surface of activated platelets and platelet-derived MPs is suggestive of an intricate role played by MPs in the context of atherothrombosis. Of note, endothelial MPs also support plasmin generation by expressing the urokinase-type plasminogen activator and its receptor, enabling urokinase-type plasminogen activator–driven fibrinolytic cross-talk in situ.[112] In addition, they are able to convey metalloproteinases that could contribute to vascular remodeling by influencing the properties of endothelial progenitor cells.[113,114]

Given the noxious role of procoagulant MPs in a variety of inflammation-induced coagulopathy disorders (malaria, sepsis, heat stroke, and others), the control of MP release may be a relevant pharmacological target.[115] Another attractive pharmacological target is represented by Orai1 inhibitors. It is likely that specific targeting of Orai1 may reduce platelet procoagulant activity and thrombus formation when the pathophysiological role of TF is limited. In contrast, the blockade of Orai1 may not affect the bleeding risk following wounds given the predominance of the TF pathway. It should, however, be emphasized that the possible existence of 2 pathways that lead to PS translocation and exposure in platelets—which is certainly protective from a phylogenic point of view—could hamper the development of potent pharmacological modulators.

Conclusions and Perspectives

Although circulating MPs are increased in patients with atherothrombotic and inflammatory disorders, this does not necessarily endorse their invariably harmful nature. MPs are ubiquitously shed from cell membranes, and the intercellular exchange of protein and RNA-containing MPs is an important mode of cell-cell communication.[116] Despite theoretical advantages of novel MP-targeted treatment strategies in atherothrombotic disorders, some can potentially be harmful to the patient. However, the absence of bleeding symptoms in heterozygous Scott subjects[39] clearly indicates that the pharmacological modulation of the release of procoagulant MPs may have an important therapeutic impact in the management of atherothrombotic vascular disorders. At present, the most important obstacle in the field of MP-modulating drugs seems to be carefully targeting MP release to particular cell types at an optimal level, so as to achieve a beneficial action and to limit possible adverse effects. Unfortunately, the genetic cause of Scott syndrome remains elusive. Pharmacological manipulation of the biochemical defect of Scott syndrome will provide an alternative or complementary approach to treatment of atherothrombosis through the modulation of procoagulant activity of MPs.[114] In addition, the contribution of the recently discovered calcium entry pathways to the regulation of platelet procoagulant activity[117] represents another attractive target for pharmacological modulation because platelets are the main providers of circulating procoagulant MPs in thrombotic disorders.

Disclosures

None.

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LISTE DES ANNEXES

1. L. Jesel, M. Abbas, F. Toti, A. Cohen, T. Arentz, O. Morel. Microparticles in atrial fibrillation: A link between cell activation or apoptosis, tissue remodelling and thrombogenicity. *International Journal of Cardiology*.2013;168:660-669

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UNIVERSITÉ DE STRASBOURG

Laurence JESEL

Sénescence, remodelage tissulaire et membranaire,risque thrombotique au cours de la Fibrillation Auriculaire

Résumé en français

Nos travaux montrent qu'au cours de la fibrillation atriale (FA), les microparticules (MP) reflètent et contribuent à un état d'hypercoagulabilité et pro-inflammatoire. Leurs concentrations similaires dans les deux oreillettes de patients en FA témoignent d'une absence de différence de statut pro-thrombotique entre ces deux cavités cardiaques. Au cours des procédures d'ablation de FA, les concentrations de MP évoluent parallèlement à l'augmentation de l'activation cellulaire et plaquettaire. Nous avons également montré dans l'altération tissulaire des oreillettes en FA, l'importance de la sénescence qui évolue avec la progression du trouble du rythme. Nous avons caractérisé un modèle cellulaire de sénescence réplicative de cellules endothéliales auriculaires de porc permettant d'identifier l'apparition d'un phénotype pro-thrombotique, pro-inflammatoire, pro-adhésif et de mieux comprendre la physiologie de la cellule endothéliale atriale sénescente et le rôle majeur du système rénine-angiotensine dans ces mécanismes.

Fibrillation atriale ; Microparticules ; Thrombogénicité ; Sénescence ; Cellule endothéliale ; Remodelage

Résumé en anglais

Our data evidence that during atrial fibrillation (AF), microparticles (MP) contribute to an enhanced hypercoagulable and pro-inflammatory state. Similar concentrations of MP measured in left and right atria of AF patients highlight the absence of chamber-specific enhanced thrombogenic status. During AF ablation procedures, MP concentrations progress in parallel with cell and platelet activation. We also showed that AF progression is strongly related to human atrial senescence burden pointing toward a possible network that links in human atrium, senescence burden, endothelial dysfunction, thrombogenicity and atrial remodeling. We also developed a model of left atrium endothelial cell replicative senescence providing compelling evidences indicating that atrial endothelial senescence promotes thrombogenicity, inflammation and proteolysis. These data underline the major role of renin-angiotensin system in endothelial atrial cell senescence.

Atrial fibrillation ; Microparticles ; Thrombogenicity ; Senescence ; Endothelial cell ; Remodeling