



Atrial fibrillation : insights concerning the arrhythmogenic substrate

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Résumé en français

La Fibrillation Atriale – Aperçus Concernant le Substrat Arythmogène

Introduction et objectifs de l'étude

La fibrillation atriale est l'arythmie cardiaque la plus fréquente, elle touche environ 1% de la population générale et jusqu'à 8% des sujets âgés de plus de 80 ans.¹ La présence de l'arythmie est associée à une altération significative de la qualité de vie et à une importante morbi-mortalité par accident vasculaire cérébral, thromboembolisme, ou encore insuffisance cardiaque.² Selon les estimations, l'incidence de la fibrillation atriale est en augmentation dans le monde entier et sa prévalence doublerait au cours des 50 prochaines années, ce qui entraîne un important problème de santé publique.¹ Ceci est principalement la conséquence du nombre croissant de patients atteints de maladies cardiaques, d'une amélioration dans la gestion de l'infarctus du myocarde et de l'insuffisance cardiaque, ainsi que l'augmentation du nombre de patients âgés et l'amélioration des outils diagnostiques.

Ces raisons épidémiologiques ont incité à de nombreuses études, afin de développer des traitements plus efficaces pour la fibrillation atriale et ses complications. Jusqu'à présent, aucun traitement n'a donné les résultats escomptés. Les stratégies pharmacologiques actuellement disponibles sont souvent peu efficaces, et leurs effets secondaires désagréables expliquent la fréquente absence d'adhérence au traitement. Cela est dû largement à une compréhension incomplète des mécanismes physiopathologiques de ces arythmies.

Il est généralement admis qu'il n'existe pas de mécanisme unique de la fibrillation atriale et qu'un mécanisme peut prédominer sur un autre chez certains patients, généralement avec la participation de plusieurs mécanismes chez le même patient. La physiopathologie de la fibrillation atriale semble être influencée par des mécanismes multiples, qui agissent à des degrés divers dans différentes situations cliniques et qui interagissent les uns avec les autres d'une manière dynamique à différents stades de l'histoire naturelle de l'arythmie.

Un déséquilibre autonome, des anomalies structurelles de l'oreillette gauche, ou encore diverses anomalies moléculaires ont été récemment incriminés dans la survenue de la fibrillation atriale et des complications liées à la fibrillation atriale telles que les accidents vasculaires cérébraux,³⁻⁵ mais un certain nombre de questions restent toujours sans réponse. À ce jour, il est difficile de savoir si 1) il existe une relation directe entre le déséquilibre autonome et la survenue des arythmies atriales et quel est le sens de ce déséquilibre, 2) la

complexité du remodelage structurel de l'oreillette gauche est similaire dans la population générale des patients avec fibrillation atriale à celle rapportée dans les études autopsiques précédentes et quelles sont les principales composantes de ce remodelage structurel, ou 3) la diminution de l'expression du gène *Pitx2* est présente seulement dans l'oreillette gauche de patients atteints de fibrillation atriale ou cette anomalie moléculaire précède et favorise l'apparition de ces arythmies. De même, les anomalies d'expression des gènes codant pour des courants ioniques, des protéines de la matrice extracellulaire, ou encore des protéines impliquées dans le couplage intercellulaire ont donné des résultats contradictoires et d'autres marqueurs moléculaires de la fibrillation atriale restent certainement à identifier. La question probablement la plus importante est de savoir si ces anomalies accompagnent les arythmies auriculaires ou si elles précèdent et favorisent la survenue de ces arythmies. Répondre à ces questions devrait permettre d'identifier de nouvelles cibles thérapeutiques pour le traitement et la prévention des arythmies atriales. Par conséquent, nous avons cherché à identifier la présence, les composants et la sévérité des anomalies autonomes, structurales et moléculaires dans un nouveau modèle animal d'arythmies atriales spontanées en effectuant une étude multifacettes.

La participation de l'inflammation dans la survenue d'arythmies auriculaires a également été signalée dans plusieurs études cliniques et expérimentales.⁶ Cependant, l'origine et la sévérité du syndrome inflammatoire chez les patients atteints de fibrillation atriale restent incomplètement comprises. En outre, l'existence d'un tel syndrome dans la population entière des patients atteints de fibrillation atriale est un sujet de débat, étant donné que plusieurs études n'ont pas réussi à démontrer des niveaux élevés de marqueurs d'inflammation chez les patients atteints de fibrillation atriale.⁷⁻⁹ Ainsi, la sévérité d'un syndrome inflammatoire chez les patients atteints de fibrillation atriale et la source de niveaux élevés de marqueurs inflammatoires chez les patients atteints de fibrillation atriale restent à établir. Nous avons émis l'hypothèse que des dosages étagés, intracardiaques et extracardiaques, de plusieurs protéines inflammatoires, nous permettront d'évaluer la sévérité d'un syndrome inflammatoire chez les patients avec fibrillation atriale et d'établir l'origine des taux élevés de marqueurs inflammatoires chez ces patients.

Des données récentes suggèrent la participation d'une dysfonction endothéliale dans la physiopathologie du thromboembolisme chez les patients atteints de fibrillation atriale.^{10,11} Des niveaux élevés de différents marqueurs pro-thrombotiques d'origine endothéliale, y compris le facteur von Willebrand (vWF), ont déjà été documentés chez les patients atteints

de fibrillation atriale.^{12,13} Toutefois, la source de vWF dans cette population reste inconnue. Des études expérimentales ont suggéré que la sécrétion du vWF pourrait provenir de l'endocarde atrial,¹⁴ ou qu'elle pourrait être la conséquence d'un dysfonctionnement endothélial généralisé,¹⁵ mais, à ce jour, l'origine précise du vWF reste à identifier. Nous avons émis l'hypothèse que des dosages multiples du vWF, intracardiaques et extracardiaques, aideront à identifier l'origine du vWF chez les patients avec fibrillation atriale.

La complexité des mécanismes physiopathologiques de la fibrillation atriale souligne la nécessité d'une approche pluridisciplinaire. L'objectif principal de cette thèse a été de développer le premier modèle expérimental d'arythmies atriales spontanées chez le rat et d'évaluer les différents mécanismes physiopathologiques impliqués dans la fibrillation atriale et ses complications, à l'aide d'une approche translationnelle, multidisciplinaire.

Un nouveau modèle d'arythmies atriales spontanées chez le rat

Les mécanismes classiques, qui continuent de former la base de notre compréhension de la physiopathologie de la fibrillation atriale, ont été établis à l'aide d'études animales. Ces dernières années ont vu une accélération considérable des travaux dans ce domaine, y compris le développement d'une large gamme de modèles de fibrillation atriale dans des substrats pathologiques cliniquement pertinents et d'un nombre croissant de modèles de souris transgéniques.

Cependant, aucun de ces modèles n'a réussi à reproduire fidèlement le cadre clinique de l'arythmie. Des modèles expérimentaux de tachyarythmies atriales spontanées manquent toujours. En effet, des études antérieures ont rapporté des « tachycardie ou fibrillation atriale spontanées » chez les animaux.^{16,17} Cependant, dans tous ces modèles, l'arythmie s'est développée seulement après que différents protocoles de stimulation ou des interventions pharmacologiques aient été appliqués, ou en réalisant les expériences sur des cœurs isolés. Plusieurs caractéristiques essentielles pour l'apparition des arythmies, comme le remodelage structurel, électrique, ou autonome, pourraient avoir été provoquées artificiellement dans ces modèles. Ainsi, « spontané » pourrait ne pas être le terme le plus approprié pour décrire le type d'arythmies rapporté dans ces études. La principale question est de savoir si les observations formulées sur de tels modèles peuvent être extrapolées chez l'Homme et si des mécanismes similaires participent dans la survenue de l'arythmie dans des milieux aussi différents, étant donné le manque de points communs évidents entre ces modèles animaux et l'arythmie auriculaire observée chez l'Homme.

En conséquence, le but principal de cette étude a été de développer un modèle expérimental qui pourrait présenter de manière reproductible des tachyarythmies atriales spontanées chez le rat et de décrire ses caractéristiques phénotypiques. Étant donné que le vieillissement et l'hypertension artérielle sont les deux facteurs de risque les plus fréquemment associés à une arythmogénicité auriculaire accrue,^{10,11} nous avons émis l'hypothèse que les rats âgés spontanément hypertendus (SHR) pourraient fournir un modèle approprié d'arythmie atriale spontanée.

Pour cette étude, des émetteurs ECG de radiotélémétrie ont été implantés chez 8 SHR âgés (≥ 48 semaines), 4 rats Wistar-Kyoto (WKY) âgés, et aussi chez 8 SHR jeunes (14 semaines) et 4 rats WKY jeunes. Pour la quantification de base d'événements arythmiques atriaux, une surveillance ECG de 24 h a été effectuée sur des rats conscients, non-restreints. Les données ECG ont été analysées par deux cardiologues indépendants en utilisant un programme récemment développé dans notre laboratoire. Le nombre total d'épisodes tachyarythmiques atriaux, d'extrasystoles auriculaires et de couplets et bigeminismes atriaux a été déterminé sur les enregistrements de 24 h. La reproductibilité à court terme (d'un jour à l'autre) et à moyen terme (d'une semaine à l'autre) du nombre d'événements arythmiques a également été testée.

La surveillance ECG de 24h a clairement démontré la présence d'épisodes de tachyarythmie atriale spontanés (Fig. 3.2, page 99), non-soutenus, chez tous les SHR âgés (médiane 91,5 ; étendue 4 - 444 épisodes/24 h), alors que seulement deux des huit SHR jeunes ont présenté un seul épisode de tachyarythmie atriale, et aucun épisode n'a été enregistré chez les rats WKY, jeunes ou âgés. Des différences similaires ont été observées dans le nombre d'extrasystoles auriculaires, de couplets et bigeminismes (Fig. 3.3, page 100). Chez les SHR âgés la durée des épisodes a varié entre 0,43 s et 10,90 s, alors que chez les 2 SHR jeunes qui ont présenté des tachyarythmies atriales, les durées des épisodes ont été de 0,4 s et 0,52 s, respectivement. L'aspect ECG était évocateur d'un diagnostic de fibrillation atriale dans 30,6% des cas. Dans les autres cas, la morphologie ECG a suggéré un diagnostic de tachycardie atriale multifocale. Les SHR âgés ont montré une grande reproductibilité à court terme (variation moyenne du nombre d'épisodes d'arythmie $18,1 \% \pm 4,7 \%$) et à moyen terme (Spearman $r = 0,83$; $P = 0,01$) du nombre d'épisodes d'arythmie (Fig. 3.4, page 101).

Ainsi, la surveillance ECG de 24h a clairement montré la présence de tachyarythmies atriales non-soutenues, spontanées, chez tous les SHR âgés. En outre, le nombre élevé d'extrasystoles atriales isolées, couplées et bigéminées dans ce groupe, aide également à

confirmer la présence d'une importante instabilité électrique auriculaire chez ces rats. Ces résultats sont en accord avec les observations cliniques et confirment la ressemblance entre ce modèle expérimental et la présentation clinique de l'arythmie. L'apparition spontanée de tachyarythmies atriales chez les rats hypertendus et âgés correspond aux observations cliniques qui relient les arythmies auriculaires avec le vieillissement et l'hypertension artérielle.^{8,9,18} La courte durée des épisodes tachyarythmiques est une caractéristique de ce modèle. Cela est dû aux particularités anatomiques des petits animaux (masse cardiaque réduite).¹⁹ En conséquence, chez les SHR, la plupart des épisodes de tachyarythmie atriale se sont spontanément convertis au rythme sinusal, généralement après un nombre réduit de battements arythmiques, ce qui confirme que le rôle de la masse myocardique critique ne peut pas être sous-estimé.

A notre connaissance, cette étude est la première à rapporter la survenue d'épisodes spontanés d'arythmie atriale chez le rat génétiquement hypertendu, en absence de tout facteur interventionnel qui pourrait avoir favorisé l'apparition de l'arythmie, et l'augmentation de l'incidence de ces épisodes d'arythmie avec le vieillissement. Ce travail caractérise un nouveau modèle expérimental d'arythmies auriculaires chez le rat. Ce modèle pourrait faciliter l'étude des mécanismes physiopathologiques de la fibrillation atriale et l'étude de l'efficacité et de la toxicité de divers agents thérapeutiques dans un cadre qui reproduit fidèlement la présentation clinique de l'arythmie.

Compte tenu des possibilités offertes par ce modèle nous avons ensuite cherché à évaluer l'implication de différents mécanismes physiopathologiques autonomes, structurels et moléculaires dans la survenue d'arythmies atriales dans ce nouveau modèle animal d'arythmies atriales spontanées en effectuant une étude multi-approche.

Étude de la balance sympatho-vagale chez les rats arythmiques

Le rôle d'un tonus autonome anormal dans le déclenchement et la persistance de la fibrillation atriale est bien reconnu.^{20,21} Des travaux antérieurs ont démontré qu'un déséquilibre dans l'activité du système nerveux autonome peut entraîner des changements importants des propriétés électrophysiologiques du myocarde auriculaire et peut faciliter la survenue de la fibrillation atriale.²²⁻²⁴ La stimulation parasympathique par stimulation électrique continue bilatérale des troncs vago-sympathiques cervicaux, par perfusion d'acétylcholine, ou par dénervation sympathique, a été efficace dans l'induction de la fibrillation atriale dans divers modèles animaux.²²⁻²⁴ D'un point de vue électrophysiologique,

la stimulation vagale raccourcit les périodes réfractaires des oreillettes et des veines pulmonaires et augmente la dispersion des périodes réfractaires.²⁵ La stimulation sympathique avec de l'isoprotérénol ou de l'adrénaline peut également faciliter le développement de la fibrillation atriale en réduisant les périodes réfractaires auriculaires. Toutefois, la stimulation sympathique ne favorise pas la fibrillation atriale autant que la stimulation vagale. Ceci est probablement dû à une réduction beaucoup plus homogène de périodes réfractaires lors de la stimulation sympathique.²³ Cependant, la plupart des études portant sur le rôle du système nerveux autonome dans la survenue d'arythmies auriculaires suggère une participation combinée des deux branches, avec un déséquilibre sympatho-vagal comme principal élément déclencheur de ces arythmies, plutôt qu'une anomalie d'une seule branche.^{26,27} À ce jour, la direction du déséquilibre autonome facilitant la survenue d'arythmies auriculaires est encore un sujet de débat.

En conséquence, nous avons cherché à évaluer la présence et les caractéristiques d'un déséquilibre autonome dans notre modèle d'arythmies auriculaires spontanées chez le rat, en utilisant l'analyse de la variabilité sinusale et deux protocoles expérimentaux qui ciblent les deux branches du système nerveux autonome (un protocole de stress émotionnel et l'administration de carbamylcholine).

Des enregistrements ECG de 24 h ont été obtenus chez 8 SHR âgés, 4 rats WKY âgés, 8 SHR jeunes, et 4 rats WKY jeunes. La variabilité du rythme cardiaque pendant le rythme sinusal a été évaluée sur les enregistrements de base de 24 h pour chaque animal. En outre, afin d'identifier d'éventuelles modifications du tonus autonome précédant les événements arythmiques, nous avons sélectionné plusieurs intervalles d'activité arythmique intense et analysé les périodes de 5 min précédant ces intervalles. Les valeurs des paramètres fréquents ont été comparées avec les valeurs mesurées sur les enregistrements de base.

Compte tenu de sa capacité à induire une stimulation sympathique pure tout en réduisant la sensibilité du baroréflexe cardiaque, un protocole de stress émotionnel a été appliqué à chaque animal par envoi d'un jet d'air dans la cage.²⁸ L'activité arythmique et la variabilité du rythme cardiaque ont été évaluées sur des périodes de 10 minutes, juste avant et pendant le stress, puis comparées. Afin d'évaluer l'arythmogénicité auriculaire en réponse à un tonus vagal augmenté chez les SHR, des injections sous-cutanées de carbamylcholine, un agoniste parasympathique, ont également été réalisées chez ces rats. L'activité arythmique et la variabilité du rythme cardiaque ont été évaluées dans les intervalles de 10 min avant et après administration de carbamylcholine puis comparées.

La surveillance ECG de 24h a indiqué que les SHR âgés étaient plus bradycardes que les rats WKY âgés, les SHR jeunes et les rats WKY jeunes. L'analyse de la variabilité sinusale a démontré une diminution significative des composantes de basse fréquence chez les SHR âgés par rapport aux rats WKY âgés et une diminution des rapports des composantes de basse et de haute fréquence chez les SHR, quel que soit leur âge, par rapport aux rats WKY du même âge (tous $P < 0,05$; Tableau 4.1, page 111). Chez les SHR âgés, les enregistrements effectués à une semaine d'intervalle ont montré une bonne reproductibilité de tous les paramètres de variabilité sinusale (tous $P > 0,05$). Chez les SHR âgés, l'analyse de la variabilité sinusale n'a montré aucun changement significatif des composantes fréquentielles dans les intervalles de 5 min précédant le début des périodes d'activité arythmique intense par rapport aux valeurs de base (tous $P > 0,05$). La plupart des épisodes d'arythmie ont eu lieu pendant des périodes d'activité arythmique intense (extrasystoles auriculaires fréquentes), mais dans 28% des cas les épisodes de tachyarythmie atriale sont survenus après une bradycardie sinusale ou des pauses sinusales.

Chez les SHR âgés le stress émotionnel a supprimé tous les épisodes tachyarythmiques (médiane 0,5 ; étendue 0 - 5 épisodes dans les 10 min précédant le début du protocole par rapport à aucun épisode pendant l'exposition au stress) et a induit une diminution significative du nombre d'extrasystoles auriculaires (médiane 13,5 ; étendue 1 - 33 épisodes avant le protocole de stress par rapport à une médiane de 1, range 0 - 14 épisodes pendant l'exposition au stress; $P = 0,04$) (Fig. 4.1, page 112). Aucun épisode de tachyarythmie atriale et aucune extrasystole auriculaire n'ont été observés chez les rats WKY, quel que soit leur âge, avant ou après le début du protocole de stress. Aucun épisode de tachyarythmie atriale et seulement quelques extrasystoles auriculaires ont été observés chez les SHR jeunes, avant ou pendant l'exposition au stress. Chez les SHR âgés, l'analyse de la variabilité sinusale a montré une augmentation significative des composantes de haute fréquence au cours du stress par rapport aux valeurs de base ($P = 0,01$). Des résultats comparables ont été obtenus chez les SHR jeunes.

Avant l'administration de carbamylcholine, les SHR âgés ont présenté une médiane de 15 extrasystoles auriculaires (étendue 6 - 29), et aucun épisode de tachyarythmie atriale n'a été enregistré. Dans l'intervalle de 10 min suivant l'injection de carbamylcholine, une médiane de 70 (étendue 40 - 271) extrasystoles atriales et une médiane de 3 (étendue 1 - 29) épisodes de tachyarythmie atriale ont été observées (Fig. 4.2, page 113). En outre, les épisodes de tachyarythmie atriale enregistrés après l'administration de carbamylcholine (durée

médiane 1,89 s, étendue 0,90 - 10,13 s), ont été significativement plus longs que les épisodes de tachyarythmie atriale observés sur les enregistrements de base de 24h (durée médiane 0,71 s, étendue 0,53 - 1,63 s; $P < 0,001$). Aucun épisode de tachyarythmie atriale et seulement quelques extrasystoles auriculaires ont été observés chez les SHR jeunes, avant ou après administration de carbamylcholine. Chez les SHR âgés, l'instabilité électrique élevée observée après administration de carbamylcholine, avec un nombre important d'extrasystoles auriculaires et d'épisodes de tachyarythmie atriale et avec très peu de périodes de rythme sinusal stable, n'a pas permis une analyse correcte de la variabilité de la fréquence cardiaque.

Dans cette étude, la variabilité de haute fréquence du rythme cardiaque, un indice de modulation parasympathique, n'est pas différente entre les SHR âgés, qui ont présenté de nombreux épisodes de tachyarythmie atriale, et les rats WKY du même âge, qui n'ont pas présenté d'arythmies auriculaires. Cependant, la variabilité de basse fréquence a été significativement plus diminuée chez les SHR âgés par rapport à celle des rats WKY âgés, et, par conséquent, les rapports des composantes de basse et de haute fréquence ont été significativement plus diminuée chez les SHR âgés que chez les rats WKY âgés. Par conséquent, l'analyse de la variabilité sinusale a révélé la présence d'un important déséquilibre sympatho-vagal, avec un tonus sympathique diminué et une hyperactivité vagale relative chez les SHR âgés qui ont présenté des arythmies atriales. Les changements aigus du tonus autonome qui ont précédé l'apparition des épisodes de tachyarythmie atriale ont été difficiles à évaluer, en raison du fait que les épisodes de tachyarythmie atriale se sont produits pendant des périodes d'activité arythmique intense (extrasystoles auriculaires fréquentes). Par conséquent, la plupart de l'analyse de la variabilité sinusale a été effectivement réalisée sur des intervalles précédant immédiatement les périodes d'extrasystoles auriculaires fréquentes plutôt qu'avant de réels épisodes de tachyarythmie atriale, ce qui pourrait avoir influencé nos résultats.

Dans ce modèle, le stress émotionnel a rétabli l'équilibre autonome en augmentant le tonus sympathique et a eu d'importants effets antiarythmiques, en supprimant tous les épisodes de tachyarythmie atriale et en réduisant considérablement le nombre d'extrasystoles auriculaires. D'autre part, la stimulation parasympathique avec carbamylcholine a eu d'importants effets pro-arythmiques, en déclenchant des tachyarythmies atriales et en augmentant le nombre d'extrasystoles auriculaires.

Une observation intéressante est la présence d'un déséquilibre sympatho-vagal comparable chez les SHR jeunes et âgés, alors que seuls les SHR âgés présentent des

événements arythmiques. Cette observation suggère que le déséquilibre sympatho-vagal est déjà présent chez le rat hypertendu bien avant la survenue d'arythmies, mais aussi que cette anomalie autonome ne semble pas être suffisante pour induire les arythmies dans ce modèle. En effet, les rapports des composantes de basse et de haute fréquence de la variabilité du rythme cardiaque ont été plus altérés chez les SHR âgés que chez les SHR jeunes, ce qui suggère que le déséquilibre autonome est devenu plus sévère avec l'âge. Toutefois, d'autres éléments, tels que le remodelage anatomique avec une fibrose auriculaire extensive, la distribution anormale ou la densité anormale des nerfs autonomes dans l'oreillette, ou diverses anomalies moléculaires, pourraient également agir en tant que facilitateurs de l'arythmie dans ce modèle.

Étude de l'histologie atriale gauche chez les rats arythmiques

Il a été démontré que les anomalies structurelles auriculaires gauches précèdent l'apparition d'arythmies auriculaires, mais elles évoluent également avec la progression de ces arythmies.²⁹ Les anomalies histologiques sont constamment observées dans les échantillons auriculaires gauches des patients qui présentent des arythmies auriculaires et comprennent un large éventail d'anomalies cellulaires et ultrastructurales.⁴ Ensemble, ces altérations créent un substrat arythmogène, essentiel pour l'apparition et surtout pour la persistance de ces arythmies.

Une augmentation de la taille des cellules, une myolyse, une accumulation de glycogène périnucléaire, des altérations de l'expression des connexines, une fragmentation du réticulum sarcoplasmique et des changements dans la forme mitochondriale sont fréquemment observés dans les oreillettes gauches de divers modèles animaux d'arythmie auriculaire.³⁰ Au niveau tissulaire, le remodelage structurel est caractérisé par la perte des myocytes et des changements dans la composition de la matrice extracellulaire, associés à une fibrose interstitielle diffuse.⁴ Des anomalies structurelles et ultrastructurales comparables à celles observées dans divers modèles animaux de fibrillation atriale ont été décrites chez des patients atteints de fibrillation atriale. Néanmoins, des lésions plus sévères ont été signalées chez les patients atteints de fibrillation atriale par rapport à celles observées dans des modèles animaux de fibrillation atriale.^{4,14} Cependant, les anomalies structurelles de l'oreillette gauche ont seulement été étudiées dans des modèles où l'arythmie a été déclenchée par des moyens interventionnels et n'ont jamais été évaluées dans un modèle animal d'arythmies auriculaires spontanées.

En conséquence, nous avons cherché à évaluer la présence, les composants et la sévérité des anomalies histologiques de l'oreillette gauche dans notre modèle d'arythmies atriales spontanées chez le rat, en utilisant un examen histologique standard. Pour cette étude, des échantillons d'oreillette gauche ont été obtenus à partir de 8 SHR âgés et 4 rats WKY âgés. Seule une évaluation visuelle a été réalisée, étant donné que le but de cet examen était de décrire les caractéristiques structurelles de nos populations et non pas de quantifier précisément ces résultats. Un système de notation a été utilisé pour quantifier la sévérité des lésions. Selon ce système, 0 correspond à l'absence d'anomalies, 1 correspond à des anomalies légères, 2 à des anomalies modérées et 3 à des anomalies graves.

Dans 6 des 8 SHR âgés, une fibrose auriculaire endocardique et interstitielle, des infiltrats inflammatoires, et une nécrose myocytaire de divers degrés de sévérité ont été observés. La caractéristique la plus fréquente a été la fibrose endocardique (Fig. 5.2, page 123), qui a été observée chez 6 SHR âgés (score moyen $1,5 \pm 0,4$). Aucun des rats WKY âgés n'a présenté de fibrose endocardique. Chez 4 SHR âgés, plusieurs aspects d'une fibrose endocardique précoce, évolutive, ont été identifiés sur le même échantillon. Aucune des autres anomalies histologiques n'a présenté un score significativement différent entre les SHR et les rats WKY âgés (tous $P > 0,05$). En outre, chez 3 SHR âgés, l'examen histologique a montré la présence d'une thrombose intra-auriculaire (Fig. 5.3, page 124), alors qu'aucun des rats WKY n'a présenté de thrombus intracardiaque.

Nos résultats renforcent le rôle du vieillissement et de l'hypertension artérielle dans le remodelage auriculaire gauche et dans l'arythmogénèse auriculaire. Toutefois, dans l'ensemble, les anomalies histologiques auriculaires gauches observées chez les SHR âgés qui ont présenté des arythmies auriculaires ont été significativement moins sévères que celles précédemment rapportées chez des patients atteints de fibrillation atriale. En outre, seulement une fibrose auriculaire interstitielle légère à modérée a été retrouvée chez seulement trois SHR âgés, alors que cette anomalie est constante dans les échantillons auriculaires gauches de patients atteints de fibrillation atriale.^{4,31}

De manière intéressante, certains SHR âgés qui ont présenté des arythmies auriculaires n'ont pas présenté de fibrose endocardique, ou toute autre anomalie structurelle de l'oreillette gauche. Cette constatation suggère que la relation entre le remodelage auriculaire gauche, la fibrose auriculaire et la tachyarythmie atriale pourrait être en fait non-linéaire et que la fibrose auriculaire est plus probablement un facilitateur de l'arythmogénicité auriculaire dans ce modèle, plutôt qu'une condition *sine qua non*.

Chez 3 des 8 SHR âgés qui ont présenté des arythmies auriculaires l'examen histologique a révélé la présence de thrombus intra-auriculaires, alors qu'aucun des rats WKY âgés n'a présenté de thrombose intra-auriculaire. Ces résultats sont en accord avec les données cliniques qui associent la fibrillation atriale avec un risque élevé de morbidité et de mortalité par thromboembolies et accidents vasculaires cérébraux.³² D'autres études devront être menées pour élucider les mécanismes responsables de la thrombose intra-auriculaire dans ce modèle.

Étude des mécanismes moléculaires d'arythmies atriales chez les rats arythmiques

Plusieurs observations cliniques ont suggéré que, bien que la plupart des cas de fibrillation atriale survient dans un environnement cardiaque favorable, dans 15% à 30% des cas, une étiologie est absente.^{33,34} En outre, certains de ces patients ont des antécédents familiaux de fibrillation atriale.³⁵ Ces observations suggèrent que des facteurs génétiques pourraient également contribuer au développement de l'arythmie. Les gènes qui pourraient être responsables du déclenchement et du maintien de la fibrillation atriale sont des gènes qui jouent sur l'automaticité, la durée de la période réfractaire auriculaire ou encore la conduction. Toutefois, les mutations reconnues ne représentent probablement qu'une petite fraction de tous les cas de fibrillation atriale, tandis que les polymorphismes de nucléotides simples sont beaucoup plus fréquents, mais elles agissent avec un effet plus faible et un phénotype moins évident.³⁶

Les polymorphismes les plus fréquemment associés à la fibrillation atriale sont situés dans la proximité du gène *Pitx2*, qui joue un rôle important dans la morphogénèse.^{37,38} Plusieurs études récentes ont suggéré que la diminution de l'expression du gène *Pitx2* pourrait être un facteur causal de l'apparition de la fibrillation atriale.^{5,39}

Alors que la diminution de l'expression du gène *Pitx2* est une constatation courante dans les échantillons auriculaires gauches des patients atteints de fibrillation atriale,⁵ à ce jour, aucune étude n'a fourni de données concernant les changements d'expression de ce gène qui précèdent le début de l'arythmie. Étant donné que, selon nos résultats, tous les SHR présentent des arythmies auriculaires une fois qu'ils ont atteint un âge avancé, nous avons cherché à évaluer, pour la première fois, les changements d'expression du gène *Pitx2* qui précèdent le début de l'arythmie. En conséquence, nous avons cherché à étudier l'expression du gène *Pitx2* dans les oreillettes gauches de SHR âgés qui ont présenté des arythmies auriculaires, mais

aussi chez des SHR plus jeunes (SHR jeunes et adultes(24 semaines)) qui n'ont pas encore présenté des arythmies auriculaires, en comparaison à des rats WKY du même âge.

Les gènes expriment généralement leur fonction par le biais d'une protéine. Sous l'influence de stimuli appropriés, une première phase de transcription est déclenchée dans le noyau, à travers laquelle les ARN messagers sont produits à partir de la séquence ADN du gène. La totalité des ARN messagers présents dans une cellule à un moment donné définit le transcriptome de cette cellule. Contrairement au génome, la « composition » du transcriptome est en permanente évolution. Le transcriptome est une structure très dynamique qui reflète l'adaptation de la cellule à son environnement.

Chez les patients atteints de fibrillation atriale, l'analyse du transcriptome a montré un profil cohérent de changements, incluant des modifications dans les gènes codant pour des canaux ioniques, pour des protéines impliquées dans la dynamique du calcium, le stress oxydatif et une signature caractéristique aux ventricules.⁴⁰⁻⁴² Cependant, les mécanismes moléculaires précis impliqués dans les processus d'adaptation au cours de la fibrillation atriale sont loin d'être clairs. L'identification des voies de signalisation et de leurs gènes cibles pourrait conduire à de nouvelles options thérapeutiques pour les patients atteints de fibrillation atriale.

Ainsi, nous avons cherché à identifier les anomalies transcriptomiques dans les oreillettes gauches de rats arythmiques, en évaluant l'expression des ARN messagers de 89 gènes codant pour des protéines impliquées dans le métabolisme calcique, les canaux ioniques, la matrice extracellulaire et le couplage intercellulaire, dans des échantillons auriculaires gauches de SHR âgés par rapport à des rats WKY âgés. Pour analyser les données, nous avons appliqué un fold-change de 1,5 pour l'ensemble des données.

À l'exception des SHR âgés, tous les autres groupes (SHR jeunes et adultes, rats WKY jeunes, adultes, et âgés) ont présenté une expression significativement plus élevée de *Pitx2* dans l'oreillette gauche que dans l'oreillette droite (tous $P < 0,05$). Chez les SHR âgés il n'y a pas eu de différence significative entre l'oreillette gauche et l'oreillette droite concernant les niveaux de *Pitx2* ($P = 0,45$). Aucune différence significative n'a été retrouvée entre les niveaux de *Pitx2* dans l'oreillette gauche des SHR jeunes par rapport aux rats WKY jeunes ($P = 0,09$), ni chez les SHR adultes par rapport aux rats WKY adultes ($P = 0,49$). D'autre part, l'expression du gène *Pitx2* a été significativement inférieure au niveau des oreillettes gauches des SHR âgés par rapport aux rats WKY âgés ($P = 0,02$; Fig. 6.1, page 134). En outre, chez les SHR âgés, l'expression de *Pitx2* a montré un déclin progressif, dépendant de l'âge, et cette

diminution a été corrélée négativement avec l'âge de façon significative (Spearman $r = -0,86$; $P < 0,01$).

Sur le total de 89 gènes étudiés, 6 gènes ont été différemment exprimés entre les SHR âgés et les rats WKY âgés (Fig. 6.2, page 135). Parmi ces 6 gènes, 4 étaient sous-exprimés et 2 étaient surexprimés chez les SHR âgés par rapport aux rats WKY du même âge. La sous-expression a été particulièrement observée pour les gènes codant pour le courant calcique de type L, *Cacng6* ($P = 0,03$) et *Cacnb4* ($P = 0,02$). Conformément à la présence d'une fibrose et d'un remodelage structurel important dans l'oreillette gauche des patients avec fibrillation atriale, nous avons noté des expressions plus fortes du gène codant pour le collagène de type VI, *Col6a1* ($P = 0,006$) chez les rats arythmiques. Une sous-expression des gènes codant pour des protéines impliquées dans la communication intercellulaire, comme la connexine-43 (*Gjal* ; $P = 0,04$) et la desmocolline 2 (*Dsc2* ; $P = 0,03$) a également été notée. En outre, nous avons trouvé une augmentation de l'expression du gène codant pour un composant *bêta* du courant potassique rectificateur retardé ultra-rapide (*Kcnab1* ; $P = 0,02$).

L'analyse de l'expression du gène *Pitx2* a mis en évidence des niveaux significativement plus faibles de *Pitx2* dans les oreillettes gauches des SHR âgés par rapport aux rats WKY du même âge. Par ailleurs, les SHR âgés présentent des épisodes spontanés, non-provoqués, de tachyarythmie auriculaire. Ces observations suggèrent un lien entre la diminution de l'expression du gène *Pitx2* et l'apparition de tachyarythmies auriculaires chez les SHR âgés. En effet, ces résultats sont en accord avec les données rapportées précédemment chez l'homme et chez des souris transgéniques qui ont apporté les preuves d'un lien moléculaire entre une perte de fonction du gène *Pitx2* et la survenue de la fibrillation atriale.⁵

En plus de la diminution de l'expression du gène *Pitx2* chez les rats arythmiques, nous avons également montré que, parmi les SHR, l'expression du *Pitx2* présente une diminution graduelle, dépendante de l'âge. Ces résultats suggèrent que chez les SHR, la diminution de l'expression du gène *Pitx2* commence avant la survenue d'arythmies, malgré le fait que chez les SHR plus jeunes l'expression du *Pitx2* n'était pas significativement différente de celle des rats WKY. Cette observation suggère qu'une thérapie appropriée précoce chez les patients hypertendus pourrait prévenir l'apparition de la fibrillation atriale.

L'analyse du transcriptome a permis d'identifier des altérations d'ARN messagers dans plusieurs gènes codant pour des protéines impliquées dans des processus électriques, structurels et de couplage intercellulaire.

Une réduction significative de 63% de la densité du courant calcique de type L a été observée dans les myocytes auriculaires des patients atteints de fibrillation atriale chronique. Une diminution significative du courant calcique de type L est aussi susceptible de contribuer à l'altération de la fonction mécanique auriculaire chez ces patients.⁴³ La réduction de la densité du courant calcique de type L semble être principalement due à une sous-expression transcriptionnelle des sous-unités *alpha* de ce courant.⁴⁴ Dans la présente étude, nous n'avons pas noté de changement significatif dans la transcription des sous-unités *alpha*, mais celles des sous-unités *beta* 4 et *gamma* 6 du courant calcique de type L ont été significativement diminuées chez les rats qui ont présenté des arythmies auriculaires par rapport aux rats témoins. La réduction de ces sous-unités contribue à une réduction de la densité du courant calcique de type L, induisant un raccourcissement du potentiel d'action et de la période réfractaire auriculaire, caractéristiques de la fibrillation atriale.⁴⁵

Le gène *Kcnab1* code pour la sous-unité *bêta* du courant potassique rectificateur retardé ultra-rapide et altère l'activité du courant.⁴⁶ Ainsi, une surexpression du gène *Kcnab1* provoquerait une diminution de ce courant, ce qui induirait à son tour un allongement de la durée du potentiel d'action et de la période réfractaire auriculaire. Cette observation est tout à fait surprenante, étant donné que le courant potassique rectificateur retardé ultra-rapide a été considéré comme une cible prometteuse pour la thérapie de la fibrillation atriale.⁴⁷ Toutefois, la diminution de ce courant a déjà été signalée dans plusieurs études sur la fibrillation atriale⁴⁸ et il a été démontré que le blocage de ce courant induit le raccourcissement du potentiel d'action chez les patients atteints de fibrillation atriale.⁴⁹

Des changements substantiels dans la composition de la matrice extracellulaire, comme un dépôt anormal de collagène dans les oreillettes, augmentent l'hétérogénéité de la conduction auriculaire et fournissent les bases pour le déclenchement et la persistance de l'arythmie.⁵⁰ Alors que les collagènes de type I et type III ont généralement été considérés comme les sous-types principaux de collagène dans la matrice myocardique, des études récentes suggèrent que le dépôt de collagène non fibrillaire participe également au processus de remodelage.⁵¹ Dans cette étude, l'expression du gène *Col6a1*, codant pour le collagène non fibrillaire de type VI, a été significativement plus élevée chez les rats hypertendus arythmiques par rapport aux contrôles normotendus du même âge, en accord avec des données précédemment rapportées chez des patients avec une fibrillation atriale permanente⁵² et chez des rats atteints d'une hypertension rénovasculaire.⁵³

Les données obtenues suggèrent une relation étroite entre l'expression anormale de gènes codant pour des protéines qui présentent une activité de jonctions gap et la fibrillation atriale.⁵⁴ L'analyse des gènes codant pour des protéines impliquées dans la communication intercellulaire a apporté d'importantes informations sur les mécanismes d'arythmies auriculaires. Les SHR âgés qui ont présenté des arythmies auriculaires ont également présenté une expression diminuée du gène *Gjal*, codant pour la connexine-43, par rapport aux rats témoins non-arythmiques. Cette observation est en accord avec les données rapportées chez les patients atteints d'une cardiopathie rhumatismale, qui ont démontré que la réduction et la redistribution de la connexine-43 contribuent à la fois à l'initiation et à la persistance de la fibrillation atriale.⁵⁵

Nous avons également montré que l'expression du gène *Dsc2*, qui code pour la desmocolline 2, est significativement plus faible chez les rats hypertendus âgés qui ont présenté des arythmies auriculaires que chez les rats normotendus du même âge. Cette anomalie transcriptomique n'a jamais été rapportée chez des patients atteints de fibrillation atriale. Des mutations dans le gène codant pour la desmocolline 2 ont été identifiées chez les patients atteints de cardiomyopathie arythmogène du ventricule droit, une canalopathie héréditaire associée à un risque accru d'arythmies ventriculaires.⁵⁶ Plusieurs études cliniques ont rapporté une incidence accrue de troubles du rythme supra-ventriculaires chez les patients atteints de cardiomyopathie arythmogène du ventricule droit et une prévalence élevée de tachyarythmies auriculaires soutenues induites à l'étude électrophysiologique par rapport aux patients sans maladie cardiaque.⁵⁷ Toutefois, à ce jour, les mécanismes sous-jacents de tachyarythmies auriculaires dans la cardiomyopathie arythmogène du ventricule droit sont inconnus. Étant donné que, selon nos résultats, une perte de fonction du gène *Dsc2* semble être liée à une propension accrue aux arythmies auriculaires, cette anomalie transcriptomique pourrait être responsable de l'arythmogénicité auriculaire importante observée chez les patients atteints de cardiomyopathie arythmogène du ventricule droit. D'autres études sur les conséquences d'une sous-expression du gène *Dsc2* sur l'arythmogénicité auriculaire seront donc d'un grand intérêt.

Étude de l'arythmogénicité ventriculaire et du remodelage moléculaire du ventricule gauche chez les rats hypertendus

L'hypertrophie ventriculaire gauche se développe généralement comme un processus adaptatif bénin, servant à compenser la surcharge hémodynamique imposée par une pression

artérielle élevée persistante.⁵⁸ Cependant, l'évolution de l'hypertrophie cardiaque a été associée à des conséquences néfastes. L'hypertrophie ventriculaire gauche a été identifiée comme un facteur de risque important de mort subite, d'insuffisance cardiaque et d'infarctus aigu du myocarde.⁵⁹ Plusieurs études ont rapporté une incidence accrue d'arythmies ventriculaires en présence d'une hypertrophie ventriculaire gauche par rapport aux patients hypertendus ne présentant aucun signe d'hypertrophie ventriculaire gauche et ont trouvé une relation linéaire entre la charge arythmique et la masse ventriculaire gauche.⁶⁰ Cependant, la question de la mort subite chez les patients hypertendus est encore un sujet de débat et les formes malignes d'arythmie ventriculaire (tachycardie ventriculaire et fibrillation ventriculaire) sont encore assez rares dans ce groupe de patients. En outre, James et al. ont suggéré que les patients hypertendus présentant une hypertrophie ventriculaire gauche pourraient en fait présenter moins d'arythmies.⁶¹ Ainsi, les éléments reliant l'hypertrophie ventriculaire gauche, les arythmies ventriculaires et la mort subite sont, au mieux, indirects, et il reste à déterminer si ces arythmies sont d'une importance cruciale.

En conséquence, nous avons cherché à évaluer, dans des conditions de base, la survenue d'arythmies ventriculaires spontanées (fibrillation ventriculaire et tachycardie ventriculaire) et plusieurs autres marqueurs d'arythmogénicité ventriculaire accrue (extrasystoles ventriculaires, couplets, bigeminismes et salves d'extrasystoles ventriculaires) chez des SHR jeunes, adultes et âgés. Ces paramètres ont été comparés à ceux observés chez des rats normotendus du même âge.

Comme résultat d'une hypertension artérielle persistante et de l'hypertrophie ventriculaire gauche secondaire, le cœur subit un processus complexe de remodelage électrique, structurel et du couplage intercellulaire. Le large éventail de gènes impliqués dans la croissance cardiaque physiologique et pathologique soutient l'hypothèse d'une contribution génétique à l'hypertrophie ventriculaire gauche.⁶² Toutefois, les données concernant les anomalies transcriptomiques dans l'hypertension et l'hypertrophie ventriculaire gauche sont très discordantes. Différentes espèces animales ou différents degrés et étapes du développement de l'hypertrophie pourraient contribuer à la discordance des données disponibles. En outre, la progression avec l'âge du remodelage moléculaire ventriculaire gauche induite par l'hypertension persistante n'a jamais été évaluée auparavant.

En conséquence, nous avons cherché à fournir des informations sur le remodelage moléculaire ventriculaire gauche dans les cœurs hypertrophiés. Pour cette étude, nous avons

effectué une analyse de l'expression de 89 gènes dans des échantillons de ventricule gauche de SHR jeunes, adultes, et âgés en comparaison aux rats WKY du même âge.

Des émetteurs ECG de radiotélémétrie ont été implantés chez 8 SHR jeunes, et chez 8 rats WKY jeunes. Pour la quantification de base d'événements arythmiques ventriculaires, trois enregistrements ECG individuels de 24h ont été effectués chez des SHR et des rats WKY conscients, non-restreints, à l'âge jeune, à l'âge adulte et âgé. Les données ECG ont été analysées par deux cardiologues indépendants en utilisant un programme récemment développé dans notre laboratoire. Le nombre total d'épisodes de tachycardie ventriculaire et de fibrillation ventriculaire, d'extrasystoles ventriculaires, de couplets et de bigeminismes ventriculaires et aussi de salves d'extrasystoles ventriculaires a été quantifié sur les enregistrements de 24h.

Afin de procéder à l'analyse du transcriptome, trois groupes supplémentaires de SHR ($n = 4$ chacun) et de rats WKY ($n = 4$ chacun) jeunes, adultes et âgés ont été euthanasiés, les cœurs ont été excisés et des échantillons de ventricule gauche ont été obtenus et analysés. L'expression de 89 gènes codant pour des canaux ioniques, des protéines de la matrice extracellulaire et des protéines impliquées dans le couplage intercellulaire a été évaluée dans des échantillons de ventricule gauche de SHR jeunes, adultes et âgés par rapport aux rats WKY du même âge. Pour analyser les données, nous avons appliqué un fold-change de 1,5 pour l'ensemble des données.

Indépendamment de leur âge, les SHR ont présenté des extrasystoles ventriculaires significativement plus fréquentes par rapport aux rats WKY du même âge (tous $P = 0,004$; Fig. 7.2, page 147). Des différences similaires ont été observées dans le nombre de couplets ventriculaires, bigeminismes et salves d'extrasystoles ventriculaires. En fait, aucun des rats WKY, quel que soit l'âge, n'a présenté de couplets ventriculaires ou de salves d'extrasystoles ventriculaires et un seul rat WKY jeune a présenté un seul épisode de bigeminisme ventriculaire. Parmi les SHR, le plus grand nombre d'extrasystoles ventriculaires a été enregistré à l'âge adulte (médiane 99, étendue 58 - 309 extrasystoles ventriculaires / 24 h), par rapport à l'âge jeune (médiane 57, étendue 23 - 88 extrasystoles ventriculaires / 24 h, $P = 0,008$) et aux SHR âgés (médiane 43, étendue 23 - 69 extrasystoles ventriculaires / 24 h, $P = 0,008$). De même, le nombre de couplets ventriculaires ($P = 0,02$), de bigeminismes ($P = 0,01$), et de salves d'extrasystoles ventriculaires ($P = 0,04$) a été significativement plus élevé à l'âge adulte que chez les SHR âgés ou de jeune âge. Un SHR adulte a présenté 3 épisodes de tachycardie ventriculaire non-soutenue lors de l'enregistrement de 24 h, tandis que 2 SHR

adultes ont présenté un seul épisode de tachycardie ventriculaire non-soutenue / 24 h. Aucun épisode de tachycardie ventriculaire n'a été enregistré chez les SHR jeunes ou âgés, ou les rats WKY, quel que soit leur âge. Aucun épisode de fibrillation ventriculaire n'a été enregistré dans les conditions de base chez aucun des SHR et des rats WKY.

Chez les SHR, quel que soit leur âge, les anomalies transcriptomiques ont présenté un profil de gain de fonction par rapport aux rats WKY du même âge. Le nombre de gènes dérégulés chez les SHR par rapport aux rats WKY du même âge a augmenté avec l'âge : 9 gènes ont été dérégulés chez les SHR jeunes (Fig. 7.3, page 148), par rapport à 17 gènes dérégulés chez les SHR adultes (Fig. 7.4, page 149), et enfin 22 gènes dérégulés chez les SHR âgés (Fig. 7.5, page 150). Lorsque le profil moléculaire a été superposé pour les trois groupes de SHR, 6 gènes sont apparus comme étant dérégulés chez tous les groupes de SHR par rapport aux rats WKY: *Kcne1*, *Scn3b*, *Myh7*, *Npr3*, *Kcnj5* et *Cacna1h* (Fig. 7.6, page 151). Les 5 premiers gènes étaient surexprimés chez tous les groupes de SHR par rapport aux rats WKY du même âge, alors que le dernier gène a été significativement sous-exprimé chez les SHR par rapport aux rats WKY. Chez les SHR, l'expression des gènes *Kcne1* (Spearman $r = 0,56$; $P = 0,05$), *Scn3b* (Spearman $r = 0,62$; $P = 0,03$), *Npr3* (Spearman $r = 0,92$; $P < 0,001$) et *Cacna1h* (Spearman $r = 0,68$; $P = 0,02$) a été significativement corrélée avec l'âge.

Nos résultats ont confirmé la présence d'une ectopie ventriculaire plus importante chez les rats hypertendus, indépendamment de leur âge, par rapport aux contrôles normotendus du même âge. Néanmoins, le nombre d'événements arythmiques à la fois isolés (extrasystoles ventriculaires) et plus complexes (couplets ventriculaires, bigeminismes et salves d'extrasystoles ventriculaires) a été assez modeste, avec une médiane de moins de 100 extrasystoles ventriculaires / 24h chez tous les groupes de SHR, indépendamment de leur âge.

De façon intéressante, les SHR adultes ont été les seuls à présenter des épisodes de tachycardie ventriculaire non-soutenue, alors qu'aucun épisode n'a été enregistré chez les SHR jeunes ou âgés. Ces résultats suggèrent que l'hypertrophie ventriculaire gauche précoce pourrait en fait être plus arythmogène que l'hypertrophie ventriculaire gauche chronique, évoluée. Des résultats comparables ont été signalés dans plusieurs études cliniques, qui ont également échoué à trouver une corrélation positive entre la sévérité de l'hypertrophie ventriculaire gauche et le nombre d'arythmies ventriculaires complexes.⁶³ En fait, plusieurs études ont suggéré que, dans ce cadre, ce ne serait pas l'hypertrophie ventriculaire elle-même, mais la combinaison de l'hypertrophie et d'autres conditions assez fréquentes chez les patients hypertendus, telles que les déséquilibres électrolytiques induits par un traitement diurétique,

qui induiraient la survenue d'arythmies ventriculaires chez les patients atteints d'une hypertrophie ventriculaire gauche.⁶⁴ Des études supplémentaires doivent être menées pour élucider les mécanismes qui sous-tendent l'arythmogénicité ventriculaire accrue observée chez les SHR adultes par rapport aux SHR jeunes et âgés.

L'utilisation de rats hypertendus d'âges différents nous a permis d'évaluer la progression du remodelage moléculaire ventriculaire gauche lié à l'hypertension artérielle. Chez les SHR, les anomalies transcriptomiques ont présenté un profil de gain de fonction, par rapport aux rats WKY. Ce fut particulièrement le cas pour les gènes codant pour des canaux ioniques, mais aussi pour des protéines de la matrice extracellulaire, ou des gènes du cytosquelette. Une tendance similaire, avec les gènes surexprimés prédominant sur les gènes sous-exprimés, a été démontrée chez le rat avec surcharge chronique de pression. Le nombre de gènes dérégulés chez les SHR par rapport aux rats WKY a augmenté avec l'âge, suggérant un remodelage moléculaire progressif dans les ventricules gauches de rats hypertendus avec l'âge.

Les données concernant les densités des canaux potassiques dans l'hypertension et l'hypertrophie ventriculaire gauche sont très discordantes. Dans la présente étude, les SHR ont présenté des expressions significativement plus élevées de *Kcnc3*, *Kcnd1* et *Kcnd2*, codant pour le courant potassique transitoire sortant, par rapport aux rats WKY de même âge. Ces résultats sont en accord avec des études antérieures qui ont démontré une augmentation de la densité du courant potassique transitoire sortant dans les cœurs hypertrophiés.⁶⁵ Plusieurs autres gènes, codant pour le courant potassique rectificateur retardé, ont été également significativement surexprimés chez tous les groupes de SHR par rapport aux rats WKY du même âge. Nos résultats ont particulièrement mis en évidence le gène *Kcne1*, codant pour la composante activante lente du courant potassique rectificateur retardé, qui a été considérablement surexprimé chez tous les groupes de SHR, indépendamment de leur âge. Des résultats similaires ont déjà été signalés chez des rats atteints d'une hypertension rénovasculaire et d'une hypertrophie ventriculaire gauche, ainsi que post-infarctus du myocarde.⁶⁶

La surexpression classique des gènes codant pour le courant calcique de type L⁶⁵ n'a pas été détectée dans tous les groupes de SHR. Une surexpression du gène *Cacnb3* a été observée uniquement chez les SHR âgés, mais pas chez les SHR jeunes ou adultes. Cette observation confirme l'existence d'un lien entre les densités des courants calciques de type L actuelles et l'hypertrophie ventriculaire gauche. Cette hypothèse est également étayée par des données montrant une forte corrélation entre la densité du courant calcique de type L et la sévérité de

l'hypertension.⁶⁷ Nos résultats indiquent également une surexpression de gènes codant pour le courant calcique de type T. L'expression du gène *Cacnalg*, codant pour ce courant, a été significativement plus importante chez les SHR adultes par rapport aux rats WKY adultes, et des expressions encore plus élevées de *Cacnalg* ont été observées chez les SHR âgés. Ces résultats suggèrent que dans les cœurs de SHR, le courant calcique de type T pourrait être influencé par la durée de l'hypertension et par la sévérité de l'hypertrophie ventriculaire gauche. Cependant, le courant calcique de type T ne semble pas être exprimé dans les myocytes ventriculaires humains, qu'ils soient normaux ou hypertrophiés.⁶⁸ Par conséquent, le rôle de ce courant dans l'hypertrophie ventriculaire gauche de l'homme est peu probable.

Le fait que 6 gènes aient été dérégulés chez tous les SHR, indépendamment de leur âge, pointe vers une implication potentielle de ces gènes dans l'hypertension. Ces 6 gènes ont tous présenté le même profil de changement d'expression (soit surexprimé, soit sous-exprimé) chez tous les groupes de SHR. En outre, les niveaux d'expression de 4 de ces 6 gènes ont été significativement corrélés avec l'âge, suggérant une implication potentielle de ces gènes dans les mécanismes physiopathologiques adaptatifs liés à l'hypertension et dans le développement de l'hypertrophie ventriculaire gauche. Ainsi, *Kcne1*, *Scnb3*, *Npr3* et *Cacnalh* pourraient représenter des marqueurs spécifiques de l'hypertrophie ventriculaire gauche.

Étude du syndrome inflammatoire chez les patients atteints de fibrillation atriale

Au cours de la dernière décennie, de plus en plus de preuves se sont accumulées impliquant l'inflammation dans la pathogenèse de la fibrillation atriale et des complications liées à cette arythmie.⁶⁹ L'observation d'infiltrats inflammatoires, d'une nécrose des myocytes et de fibrose dans les biopsies auriculaires de patients atteints de fibrillation atriale isolée réfractaire à la thérapie médicamenteuse antiarythmique soutient l'hypothèse que la fibrillation atriale est étroitement liée à un processus inflammatoire.⁴ La fréquence de la fibrillation atriale chez les patients atteints de maladies inflammatoires telles que la myocardite, la péricardite, ou après une chirurgie cardiaque, suggère également une contribution de l'inflammation dans la survenue de la fibrillation atriale.⁶⁹

Des cytokines inflammatoires telles que la protéine C réactive ou l'interleukine-6, ont été associées avec le développement futur, la récurrence de la fibrillation atriale et la probabilité d'une cardioversion réussie.⁷⁰ En outre, une plus longue durée de fibrillation atriale a été associée à des niveaux plus élevés de protéine C réactive, ce qui suggère un lien entre la charge arythmique et la présence d'une inflammation systémique.⁷¹

Cependant, l'origine et la sévérité du syndrome inflammatoire chez les patients atteints de fibrillation atriale restent incomplètement comprises. En outre, l'existence d'un syndrome inflammatoire dans toute la population de patients atteints de fibrillation atriale est un sujet de débat, étant donné que plusieurs études n'ont pas réussi à démontrer des niveaux élevés de protéines inflammatoires chez les patients atteints de fibrillation atriale.⁷⁻⁹

Par conséquent, nous avons cherché à évaluer la présence, la sévérité et l'origine d'un syndrome inflammatoire chez les patients atteints de différentes formes cliniques de fibrillation atriale en comparaison à des contrôles en rythme sinusal.

Un total de 81 patients programmés pour une étude électrophysiologique ou des procédures d'ablation par cathéter ont participé à cette étude. Le groupe témoin a compris 9 patients atteints d'un syndrome de Wolff-Parkinson-White avec une voie accessoire gauche et sans antécédents de fibrillation atriale. Trente-neuf patients atteints de fibrillation atriale paroxystique et 33 patients avec fibrillation atriale persistante ont été inclus dans cette étude.

Les patients ayant des antécédents récents d'un événement cardiovasculaire ou cérébrovasculaire aigu, traumatisme majeur ou intervention chirurgicale, cardiopathie ischémique apparente, cancer, maladie inflammatoire ou du tissu conjonctif, infection aiguë ou chronique, maladie pulmonaire, hépatique ou rénale n'étaient pas éligibles pour cette étude. Les patients sous traitement anti-inflammatoire stéroïdien ou non-stéroïdien en cours ont également été exclus. Une histoire médicale détaillée a été obtenue pour chaque patient participant à l'étude. Tous les patients ont subi un examen physique et des tests de laboratoire de routine. La surface auriculaire gauche a été évaluée par échocardiographie transthoracique avant l'étude électrophysiologique ou les procédures d'ablation par cathéter.

Les prises de sang ont été effectuées pendant le cathétérisme cardiaque de routine, avant toute ablation ou administration d'héparine. Des échantillons de sang de la veine fémorale, du sinus coronaire, de l'oreillette gauche et des veines pulmonaires ont été obtenus pour chaque patient. Les concentrations sériques de la molécule soluble d'adhésion intercellulaire-1, du facteur de croissance de transformation *bêta*-1, d'interleukine-8 et de facteur de croissance vasculaire endothélial (VEGF) ont été déterminées dans les 4 échantillons.

Il n'y a pas eu de différences significatives entre les trois groupes en ce qui concerne les niveaux de molécule soluble d'adhésion intercellulaire-1, du facteur de croissance de transformation *bêta*-1 et d'interleukine-8 (tous $P > 0,05$; Tableau 8.2, page 162). De même, les taux de protéine C réactive (mesurée dans le sang périphérique uniquement) n'ont pas été différents entre les trois groupes ($P = 0,60$).

Les niveaux périphériques de VEGF ont été plus élevés chez les patients atteints de fibrillation atriale paroxystique ou persistante par rapport aux témoins ($P = 0,03$ et $P = 0,02$, respectivement; Fig. 8.1, page 163). Dans l'oreillette gauche, les taux de VEGF ont été plus élevés dans le groupe avec fibrillation atriale paroxystique par rapport au groupe témoin ($P = 0,05$), mais il n'y a pas eu de différence significative entre les niveaux auriculaires gauches de VEGF des patients atteints de fibrillation atriale persistante et ceux des témoins ($P = 0,32$). Ni les niveaux de VEGF du sinus coronaire, ni ceux des veines pulmonaires n'ont été significativement différents entre les trois groupes (les deux $P > 0,05$).

Même si beaucoup d'études suggèrent un lien entre l'inflammation et la présence de la fibrillation atriale, plusieurs autres n'ont pas réussi à montrer des niveaux élevés de protéine C réactive, d'interleukine-6, ou encore d'interleukine-8 chez les patients atteints de fibrillation atriale.⁷⁻⁹ En outre, les études évaluant l'efficacité de la thérapie anti-inflammatoire dans la prévention de la fibrillation atriale ont donné des résultats contradictoires,⁷² ce qui suggère que le rôle des processus inflammatoires dans la physiopathologie de la fibrillation atriale pourrait avoir été surestimé. Notre étude n'a pas démontré de différence significative dans les niveaux de protéine C réactive, d'interleukine -8 ou de molécule soluble d'adhésion intercellulaire-1 entre les trois groupes de patients. Ces résultats suggèrent que les niveaux élevés de marqueurs inflammatoires observés chez les patients atteints de fibrillation atriale pourraient être dus à des comorbidités associées plutôt qu'à la fibrillation atriale elle-même.

Chez les patients atteints de fibrillation auriculaire paroxystique la combinaison des niveaux normaux de VEGF dans les veines pulmonaires et des niveaux élevés dans l'oreillette gauche suggère le cœur lui-même comme la source la plus probable de VEGF dans ce groupe. Un certain nombre de conditions pathologiques, tels que l'hypoxie, l'inflammation, ou, selon des rapports récents, l'étirement mécanique pulsatile,⁷³ sont connus pour induire la sécrétion de VEGF. Les faibles niveaux de marqueurs d'inflammation chez les patients atteints de fibrillation atriale paroxystique ou persistante suggèrent que, dans les populations étudiées, le processus inflammatoire est de faible intensité, si présent. Par conséquent, il semble peu probable que ce processus puisse expliquer les taux élevés de VEGF observés chez ces patients. Malgré l'absence de preuves d'une angiogénèse accélérée chez les patients atteints de fibrillation auriculaire paroxystique, nous avons trouvé des niveaux élevés de VEGF dans l'oreillette gauche de ces patients. Une augmentation transitoire de l'expression du gène codant pour le facteur inductible par l'hypoxie *alpha*, un déclencheur connu de la sécrétion de VEGF, a été rapportée dans les myocytes cardiaques des patients atteints de fibrillation

atriale.⁷⁴ D'autre part, la relation inverse est également possible, avec une ischémie favorisant la survenue de la fibrillation atriale. L'oreillette en fibrillation conduisant à un débit sanguin irrégulier pourrait induire un étirement vasculaire pulsatile et une rhéologie sanguine anormale. Ces deux phénomènes pourraient ensuite déclencher la sécrétion de VEGF, ainsi que sa libération de cellules musculaires lisses et de cellules sanguines circulantes chez les patients atteints de fibrillation atriale, quelle que soit la forme clinique de l'arythmie.⁷⁵ L'étirement mécanique pulsatile a récemment été proposé comme un déclencheur puissant de la sécrétion de VEGF dans les myocytes cardiaques.⁷⁶ Ce phénomène pourrait être responsable des niveaux intracardiaques élevés de VEGF observés chez les patients atteints de fibrillation atriale paroxystique.

Cependant, nos résultats suggèrent que la sécrétion auriculaire gauche de VEGF est un événement transitoire dans l'histoire naturelle de la fibrillation atriale. Le VEGF est déjà connu pour stimuler le processus fibrotique dans le tissu auriculaire, probablement par induction de l'angiogenèse.⁷⁷ Plusieurs études ont souligné que les patients atteints de fibrillation atriale persistante présentent des changements fibrotiques plus importants par rapport aux patients atteints de fibrillation atriale paroxystique.⁷⁸ La fibrose cardiaque importante pourrait expliquer le raidissement cardiaque progressif dans ce contexte, réduisant ainsi le degré d'étirement pulsatile et par la suite diminuant la sécrétion de VEGF.

Étude de la dysfonction endothéliale chez les patients atteints de fibrillation atriale

La prédisposition d'accident vasculaire cérébral chez les patients atteints de fibrillation atriale est estimée à environ 5% par an.⁷⁹ En outre, les patients atteints de fibrillation atriale et qui présentent des accidents vasculaires cérébraux développent des déficits plus graves par rapport aux patients ayant subi un accident vasculaire cérébral qui n'est pas associé à la fibrillation atriale.⁸⁰

La triade de coagulation de Virchow demande que des anomalies du flux sanguin, des parois vasculaires et des facteurs de coagulation soient présentes pour que la formation d'un thrombus puisse se produire.⁸¹ Dans la fibrillation atriale, il est largement accepté que la stagnation du sang dans l'oreillette gauche est un facteur thrombogène important.⁸²

Cependant, la coexistence d'autres conditions, telles que l'hypertension artérielle, le vieillissement, ou encore le diabète augmente le risque d'accident vasculaire cérébral encore plus.⁸² Cette observation suggère que, bien que la stagnation du sang dans l'oreillette gauche

puisse être une source de thromboembolisme chez les patients atteints de fibrillation atriale, ceci ne fournit pas une explication complète.

Des constituants hémostatiques anormaux indicatifs de thrombogénèse ont été démontrés chez des patients atteints de fibrillation atriale et des données récentes soutiennent la participation d'une dysfonction endothéliale dans la physiopathologie de la fibrillation atriale.^{10,11} Des niveaux élevés de différents marqueurs pro-thrombotiques d'origine endothéliale, y compris le vWF, un marqueur accepté de dysfonction endothéliale, ont déjà été documentés dans ce groupe de patients.^{12,13} Comme marqueur pronostique, des niveaux élevés de vWF ont été associés à un risque accru d'accident vasculaire cérébral et à un pronostic plus réservé chez les patients atteints de fibrillation atriale,⁸³ mais la source de vWF chez ces patients reste inconnue. L'observation que les cellules endocardiques auriculaires sont capables de sécréter du vWF pourrait suggérer une origine cardiaque.¹⁴ D'autre part, Conway et al. ont démontré que les patients atteints de fibrillation atriale et chez lesquels on retrouve des taux plasmatiques élevés de vWF présentent le plus souvent des accidents non-cardioemboliques et ont suggéré un dysfonctionnement endothélial généralisé comme source de vWF chez ces patients.¹⁵

Par conséquent, nous avons cherché à évaluer la sévérité de la dysfonction endothéliale chez les patients atteints de différentes formes cliniques de fibrillation atriale et à identifier la source du vWF chez les patients atteints de fibrillation atriale.

Un total de 110 patients programmés pour une étude électrophysiologique ou des procédures d'ablation par cathéter ont participé à cette étude. Le groupe témoin a compris 9 patients atteints d'un syndrome de Wolff-Parkinson-White avec une voie accessoire gauche et sans antécédents de fibrillation atriale. Soixante-trois patients atteints de fibrillation atriale paroxystique et 38 patients avec fibrillation atriale persistante ont été inclus dans cette étude.

Les patients ayant des antécédents récents d'événement cardiovasculaire ou cérébrovasculaire aigu, traumatisme majeur ou intervention chirurgicale, cardiopathie ischémique apparente, cancer, maladie inflammatoire ou du tissu conjonctif, infection aiguë ou chronique, et maladie pulmonaire, hépatique ou rénale, n'étaient pas éligibles pour cette étude. Les patients sous traitement anti-inflammatoire stéroïdien ou non-stéroïdien en cours ont également été exclus. Une histoire médicale détaillée a été obtenue pour chaque patient. Tous les patients ont subi un examen physique et des tests de laboratoire de routine. La surface de l'oreillette gauche a été évaluée par échocardiographie transthoracique avant l'étude électrophysiologique ou les procédures d'ablation par cathéter. Pour chaque patient, le

risque d'accident vasculaire cérébral a été évalué selon les recommandations actuelles en utilisant le score CHA₂DS₂VASc.

Les prises de sang ont été effectuées pendant le cathétérisme cardiaque de routine, avant toute ablation ou administration d'héparine. Des échantillons de sang de la veine fémorale, du sinus coronaire et de l'oreillette gauche ont été obtenus pour chaque patient. Le vWF a été dosé en utilisant des particules de polystyrène recouvertes d'anticorps polyclonaux de lapin anti-vWF humain.

Les niveaux périphériques de vWF ont été significativement plus élevés chez les patients atteints de fibrillation atriale persistante par rapport aux témoins ($P = 0,04$), mais il n'y a pas eu de différence significative entre les patients atteints de fibrillation atriale paroxystique et les contrôles ($P = 0,29$; Tableau 9.2, page 174).

Les patients atteints de fibrillation atriale persistante ont présenté des niveaux auriculaires gauches de vWF significativement plus élevés par rapport aux patients du groupe contrôle ($P = 0,004$) et aussi par rapport aux patients atteints de fibrillation atriale paroxystiques ($P = 0,02$). De même, les niveaux auriculaires gauches de vWF ont été significativement plus élevés chez les patients atteints de fibrillation atriale paroxystique par rapport aux témoins ($P = 0,02$). Des résultats comparables ont été obtenus pour les échantillons du sinus coronaire.

Les patients atteints de fibrillation atriale paroxystique et persistante ont ensuite été répartis en fonction du risque d'accidents vasculaires cérébraux évalué par le score CHA₂DS₂VASc en groupe à risque faible-intermédiaire (CHA₂DS₂VASc = 0 - 1) et groupe à haut risque (CHA₂DS₂VASc ≥ 2). Les patients à haut risque selon le score CHA₂DS₂VASc ont présenté des niveaux périphériques de vWF significativement plus élevés par rapport aux patients à risque faible-intermédiaire ($P = 0,04$; Fig. 9.1, page 175). Les niveaux auriculaires gauches de vWF ont eu aussi tendance à être plus élevés chez les patients à haut risque par rapport au groupe à risque faible-intermédiaire ($P = 0,08$).

En plus de son rôle de transporteur du facteur de coagulation VIII, le vWF est également le médiateur de différentes étapes clés dans la formation d'un thrombus, tels que l'adhésion des plaquettes à la paroi vasculaire de-endothélialisée et l'agrégation plaquettaire consécutive.⁸⁴ Son rôle d'élément clé dans la thrombose fait du vWF un marqueur fiable du risque prothrombotique.

La présente étude a démontré une augmentation des taux plasmatiques de vWF chez les patients atteints de fibrillation atriale par rapport à des contrôles en rythme sinusal. En outre,

nous avons trouvé des niveaux significativement plus élevés de vWF chez les patients atteints de fibrillation atriale considérés à haut risque d'accident vasculaire cérébral selon le score CHA₂DS₂VASc par rapport aux patients atteints de fibrillation atriale considérés à risque faible-intermédiaire. Ces résultats suggèrent un lien entre la sévérité de la dysfonction endothéliale chez les patients atteints de fibrillation atriale et le risque d'accident vasculaire cérébral de ces patients.

Les niveaux élevés de vWF dans l'oreillette gauche des patients atteints de fibrillation atriale paroxystique et persistante par rapport aux témoins en rythme sinusal suggèrent une dysfonction endothéliale localisée, limitée à l'endocarde auriculaire, en tant que source de vWF chez ces patients. Nos résultats suggèrent également que, avec l'évolution de la fibrillation atriale paroxystique vers la forme persistante, cette dysfonction endothéliale progresse également d'une dysfonction localisée à une anomalie généralisée. Les niveaux élevés de vWF dans le sinus coronaire et le sang périphérique des patients atteints de fibrillation atriale persistante, mais pas dans celui des patients atteints de fibrillation atriale paroxystique, suggèrent la généralisation de la dysfonction endothéliale au-delà de l'endocarde auriculaire et le développement d'un dysfonctionnement endothélial généralisé en parallèle avec la perpétuation de l'arythmie. En outre, les niveaux auriculaires gauches de vWF ont été significativement plus élevés chez les patients atteints de fibrillation atriale persistante par rapport aux patients atteints de fibrillation atriale paroxystique. Ces résultats suggèrent aussi une dysfonction endocardique auriculaire progressive en parallèle avec la perpétuation de l'arythmie.

Les données cliniques ont démontré que les patients atteints de fibrillation atriale paroxystique ont un risque d'accident vasculaire cérébral similaire à celui des patients atteints des formes persistantes ou permanentes.⁸¹ La dysfonction endothéliale est un marqueur de risque accru de thrombose intra-auriculaire locale et, selon une étude réalisée par Heppel et al., les niveaux de vWF sont un facteur prédictif indépendant de la présence de thrombus auriculaire gauche en échocardiographie transœsophagienne chez les patients atteints de fibrillation atriale.⁸⁵ La présente étude a montré que les niveaux intracardiaques de vWF ont été plus élevés chez les patients atteints de fibrillation atriale paroxystique et persistante que chez les patients sans antécédents de fibrillation atriale. Ainsi, le risque d'accident vasculaire cérébral similaire chez les patients atteints de fibrillation atriale paroxystique et persistante pourrait être expliqué par l'important dysfonctionnement endothélial et le risque consécutif de

thrombose auriculaire locale chez les patients atteints de fibrillation atriale, quelle que soit la forme clinique de l'arythmie.

Nous avons également apporté la preuve que chez les patients atteints de fibrillation atriale paroxystique, les niveaux périphériques normaux de vWF n'excluent pas des niveaux auriculaires gauches élevés. Selon cette observation, les niveaux de vWF ne semblent pas être un marqueur approprié de risque d'accident vasculaire cérébral, étant donné que les niveaux périphériques normaux n'excluent pas des niveaux intracardiaques élevés.

En outre, nos résultats suggèrent que, selon la forme clinique de l'arythmie, les patients atteints de fibrillation atriale pourraient présenter des risques thromboemboliques spécifiques. Ainsi, les patients atteints de fibrillation atriale paroxystique, où seule une dysfonction auriculaire localisée a été identifiée, devraient présenter des accidents cardioemboliques, tandis que les patients atteints de fibrillation atriale persistante, qui ont présenté des niveaux de vWF prédictifs à la fois d'un dysfonctionnement endocardique auriculaire et d'une atteinte endothéliale vasculaire généralisée, pourraient présenter à la fois des accidents cardioemboliques et non-cardioemboliques. Cette hypothèse est soutenue par les résultats de Conway et al., qui ont démontré que les patients atteints de fibrillation atriale ayant de faibles niveaux périphériques de vWF, qui correspondent au profil des patients atteints de fibrillation atriale paroxystique dans notre étude, présentent uniquement des accidents cardioemboliques, alors que les patients atteints de fibrillation atriale et qui ont des niveaux périphériques élevés de vWF, qui correspondent au profil des patients atteints de fibrillation atriale persistante, présentent à la fois des accidents cardioemboliques et non-cardioemboliques.¹⁵

Ainsi, les résultats de cette étude mettent en évidence les relations complexes et dynamiques qui s'établissent entre l'endocarde auriculaire, l'endothélium vasculaire et la progression de la fibrillation atriale et suggèrent que l'endothélium est un « organe » clé dans la physiopathologie de la fibrillation atriale et du risque thromboembolique associé.

En conclusion, notre étude chez l'animal est la première à signaler la survenue d'épisodes spontanés d'arythmie atriale au cours du vieillissement chez les rats hypertendus, en absence de tout facteur interventionnel qui aurait pu favoriser l'apparition de l'arythmie. L'approche multi-facettes utilisée dans cette étude a permis d'apporter des informations nouvelles et importantes concernant les mécanismes des arythmies auriculaires. Les résultats de cette étude soulignent la nature multicausale de la fibrillation atriale et la complexité des mécanismes physiopathologiques de cette arythmie.

Nos résultats suggèrent qu'un déséquilibre autonome avec hyperactivité vagale relative pourrait être responsable de l'arythmogénicité atriale accrue observée dans ce modèle. Cette anomalie autonome était présente chez le rat hypertendu bien avant la survenue d'arythmies, mais elle ne semble pas être suffisante pour provoquer seule ces arythmies.

Nous avons également montré que des anomalies structurelles et notamment une fibrose auriculaire endocardique, sont présentes dans l'oreillette gauche des rats arythmiques. Cependant, l'absence d'anomalies histologiques chez certains de ces rats suggère que la fibrose auriculaire est plus probablement un facilitateur d'arythmogénicité dans ce modèle, plutôt qu'une condition *sine qua non*.

En accord avec les données rapportées chez les patients atteints de fibrillation atriale, les rats arythmiques ont présenté une diminution de l'expression du *Pitx2* dans l'oreillette gauche et des anomalies transcriptomiques évocatrices d'une diminution du courant calcique de type L, du courant potassique rectificateur retardé ultra-rapide et de la connexine-43, ainsi qu'une augmentation du collagène de type VI. En outre, nous montrons que chez les SHR, la diminution de l'expression du gène *Pitx2* est un processus évolutif, dépendant de l'âge.

Chez les patients atteints de fibrillation atriale, de faibles niveaux de marqueurs inflammatoires suggèrent que dans les populations étudiées, le processus inflammatoire est de faible intensité, si présent. Les niveaux élevés de VEGF dans les oreillettes gauches, mais pas dans les veines pulmonaires de patients atteints de fibrillation atriale paroxystique, suggèrent que le cœur lui-même est la source la plus probable de VEGF dans ce groupe, mais ce trouble semble être un événement transitoire dans l'histoire naturelle de la fibrillation atriale. En outre, les dosages étagés du vWF soutiennent l'existence d'une évolution parallèle de la fibrillation atriale et de la dysfonction endothéliale, avec une atteinte auriculaire endocardique précédant la dysfonction endothéliale généralisée.

Enfin, la surveillance ECG a confirmé la présence d'une ectopie ventriculaire plus importante chez les rats hypertendus, indépendamment de leur âge, par rapport aux contrôles normotendus. Cependant, l'hypertrophie ventriculaire gauche précoce semble être plus arythmogène que l'hypertrophie ventriculaire gauche chronique, évoluée. Nos résultats suggèrent également que le remodelage moléculaire ventriculaire gauche progresse avec l'âge.

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List of Abbreviations

ACEI	angiotensin converting enzyme inhibitor
AF	atrial fibrillation
ANS	autonomic nervous system
APD	action potential duration
ARB	angiotensin receptor blocker
AT	atrial tachyarrhythmia
ATP	adenosine triphosphate
cDNA	complementary DNA
CRP	C-reactive protein
C _x 40	connexin-40
C _x 43	connexin-43
DNA	deoxyribonucleic acid
ECG	electrocardiogram
ESC	European Society of Cardiology
HF	high-frequency
HRV	heart rate variability
hs-CRP	high sensitivity C-reactive protein
I _{Ca, L}	L-type calcium current
I _{Ca, T}	T-type calcium current
I _{KAch}	acetylcholine-activated potassium current
I _{K, ATP}	ATP-dependent potassium current
I _{Ks}	slowly activating delayed rectifier potassium current
I _{Kur}	ultra-rapid delayed rectifier potassium current
IL-6	interleukin-6
IL-8	interleukin-8
I _{Na}	sodium current
INR	International Normalized Ratio
IQR	interquartile range
Ito	transient outward potassium current
LA	left atrium
LF	low-frequency
LVH	left ventricular hypertrophy

mRNA	messenger ribonucleic acid
N/App	not applicable
PAC	premature atrial contraction
PCR	polymerase chain reaction
pNN5	percentage of adjacent RR intervals that differed by >5 ms
PV	pulmonary vein
PVC	premature ventricular contraction
RMSSD	root-mean-square of successive RR-interval differences
SDNN	standard deviation of normal RR intervals
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
sICAM-1	soluble intercellular adhesion molecule-1
TBP	TATA box binding protein
TGF- β 1	transforming growth factor- β 1
TLDA	TaqMan Low-Density Array
VEGF	vascular endothelial growth factor
VF	ventricular fibrillation
VT	ventricular tachycardia
vWF	von Willebrand factor
WKY	Wistar-Kyoto

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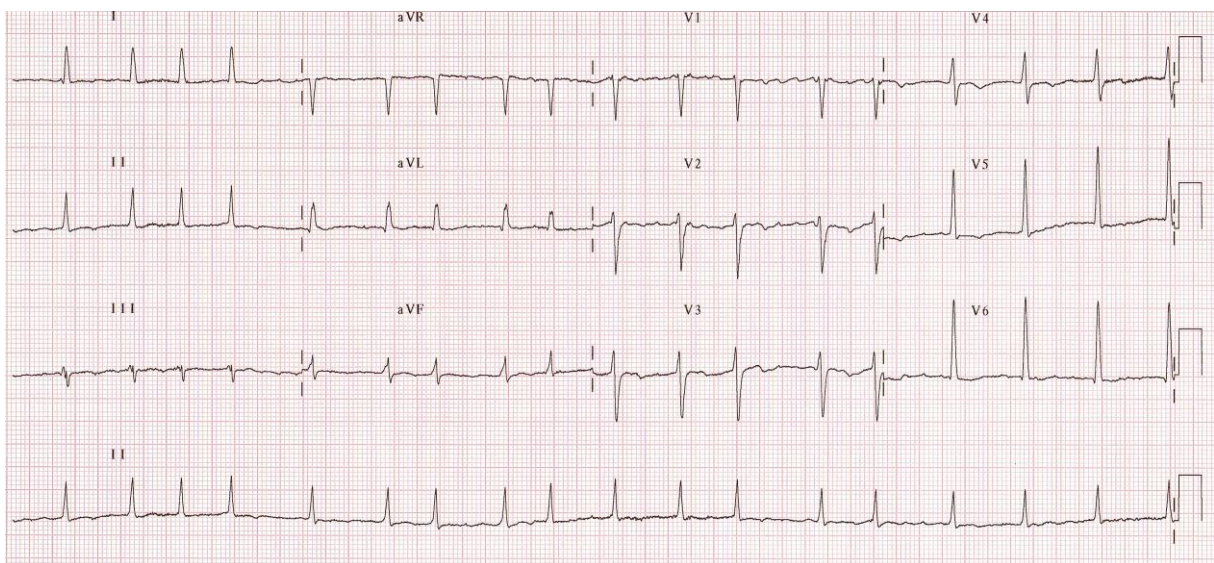
CHAPTER 1

Atrial Fibrillation – The Context

1.1. What is atrial fibrillation?

Atrial fibrillation (AF) is an abnormality of the heart's rhythm that is characterized by rapid and irregular electrical activation of the atria, leading to chaotic and uncoordinated atrial contraction.⁸⁹ On the electrocardiogram (ECG), sinus P-waves are replaced by rapid oscillations, with varying amplitude, morphology, and cycle length (Fig. 1.1).⁸⁹ Consequently, ventricular activation is also irregular, and usually rapid, as long as atrioventricular conduction pathways are functional, but autonomic tone and concomitant medication can also influence ventricular rate.^{89,252}

Figure 1.1. Electrocardiogram showing atrial fibrillation

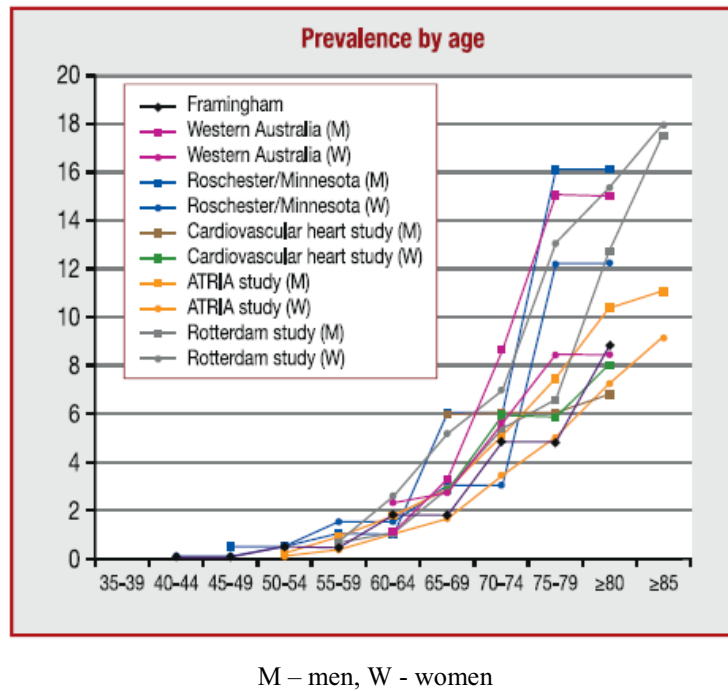


1.2. Atrial fibrillation – a worldwide public health issue

Atrial fibrillation is the most prevalent sustained form of cardiac arrhythmia, accounting for approximately one third of all patient discharge diagnoses where the arrhythmia is the principal diagnosis.¹⁰¹ The arrhythmia affects approximately 1% of the general population and up to 8% of subjects over the age of 80 years.¹⁶⁹ According to a meta-analysis of four large population-based surveys,^{100,168,246,321} the prevalence of AF doubles with each decade of life after the age of 50 years⁹³ (Fig. 1.2).

According to estimates, the incidence of AF is on increase worldwide and its prevalence is estimated to at least double in the next 50 years. The arrhythmia is 1.5 times more frequent in men than in women, but the difference between genders diminishes with age.

Figure 1.2. Prevalence of atrial fibrillation according to age in different studies⁴³



In the past 20 years, the percentage of AF-related hospitalizations has increased by 66%, which represents a significant impact for health care providers, with a total cost burden of approximately 13.5 billion Euros in Europe.¹⁶⁹ This is mainly the consequence of increasing number of patients with cardiac diseases, coupled with the expanding segment of elderly patients and improved diagnostic tools.¹⁸⁵ Maglio et al. reported that over a 2-year period, an AF patient requires an average of 14 office visits, 12 outpatient visits, 2 admissions to hospital and one emergency department visit.¹⁹⁵

The presence of the arrhythmia is associated with significant impairment of quality of life, making an important contribution to general morbidity and mortality.^{24,292} In 2001, AF was the primary and / or contributing cause of more than 70,000 deaths. Atrial fibrillation is associated with a 5-fold increase in the risk of ischemic stroke and it is estimated that one of every 6 strokes occurs in AF patients. Even in the absence of severe complications, patients that present AF-related symptoms such as exercise intolerance, palpitations, fatigue, congestive heart failure, angina, hypotension or presyncope, have impaired quality of life and lower functional status.

1.3. Clinical presentation of atrial fibrillation

Clinical presentation of patients with AF varies from asymptomatic arrhythmia (silent AF) to cardiogenic shock or devastating stroke. Although many patients experience various symptoms, such as palpitations, dizziness, dyspnoea, angina, or heart failure symptoms, up to 90% of AF episodes may not cause any symptoms.²³⁸ When present, AF-related symptoms result from the irregular and often rapid ventricular response, as well as from ensuing autonomic reflex changes and loss of atrial systole.

1.4. Atrial fibrillation classification

Given the wide range of clinical settings, several criteria have been proposed in order to classify AF. Eventually, one classification has been withheld because of the simplicity of its use in clinical practice. Thus, according to the 2010 European Society of Cardiology (ESC) Guidelines for the Management of AF,⁸⁵ several clinical forms of AF have been defined:

- **first diagnosed AF** defines the first episode of AF ever detected, symptomatic or not, irrespective of its duration;
- if the arrhythmia is spontaneously reversible to sinus rhythm within 7 days, it will be classified as **paroxysmal**;
- an arrhythmia lasting for more than 7 days or requiring cardioversion will be considered **persistent**;
- **long-standing persistent AF** has lasted for at least one year when it is decided to adopt a rhythm control strategy;
- **permanent AF** defines the forms of arrhythmia in which rhythm control strategies are no longer considered as therapeutic option.

Paroxysmal and persistent AF may both be recurrent and are often progressive. It is generally accepted that AF usually progresses from short, rare episodes, to longer and more frequent attacks, and that, over time, many patients will develop sustained forms of AF. It has been reported that 8% to 9% of patients with paroxysmal AF progress to permanent AF within one year, and 25% within 5 years.¹⁵⁰ However, it is still a matter of debate whether paroxysmal, persistent and permanent AF represent different stages of the same pathophysiological process or whether they represent distinct disorders.

Additionally, based on various other criteria, several other forms of AF have been defined. **Secondary AF** defines the occurrence of the arrhythmia in a favorable clinical context, such as myocardial infarction or cardiac surgery. In such cases, resolution of the

initial condition usually solves the arrhythmia. **Non-valvular AF** is defined as AF occurring in the absence of post-rheumatic valvular disease, valvular prosthesis or surgical repair of the mitral valve. **Lone AF** should be considered when the arrhythmia occurs in a patient 60 years or younger, in the absence of any clinical or echocardiographic sign of cardiac structural abnormality. **Silent AF** defines the presence of the arrhythmia in the absence of any related symptoms.

1.5. Atrial fibrillation – associated comorbidities, risk factors

With the exception of relatively rare cases of lone AF, the arrhythmia is commonly associated with other cardiac conditions. In fact, an underlying heart disease is present in 70% of patients with AF. The presence of various comorbidities creates a substrate for AF and complicates therapy for these patients.

In its most common form, AF is a degenerative disease occurring in the elderly patient. A number of specific predictors for AF occurrence have been identified, including hypertension, valvular (mitral) disease, coronary artery disease, cardiomyopathy, heart failure, supraventricular and ventricular rhythm disturbances, lung disease or diabetes. Multivariate analysis identified advancing age, male sex, smoking, diabetes, hypertension, left ventricular hypertrophy (LVH), myocardial infarction, valvular heart disease, and heart failure, as risk factors for AF onset.²³

The prevalence of AF has been found to increase in a linear manner with the severity of heart disease (NYHA I prevalence 4% *versus* NYHA IV prevalence 50%).¹⁰² Patients with asymptomatic AF are more likely to have less severe heart disease and a lower incidence of ischemic heart disease, heart failure or peripheral vascular disease, but this precise category is more prone to experience strokes or transient ischemic attacks compared to symptomatic patients.⁹⁵ On the other hand, the prevalence of symptomatic AF increases with the severity of coexisting cardiovascular diseases.

Obesity was associated with a 0.5-fold higher risk of AF in both men and women, probably mediated by left atrial (LA) dilation.³⁰⁹ A number of other cardiovascular and non-cardiovascular factors have been reported to trigger acute, temporary AF, including alcohol intoxication, cardiac or thoracic surgery, electrocution, myocardial infarction, pericarditis, myocarditis, pulmonary embolism or other pulmonary disorders, hyperthyroidism or other metabolic or endocrine disorders, as well as severe infections.¹⁰² Risk factors such as genetic

or socioeconomic factors, chronic physical training and various biomarkers are currently under evaluation as potential novel risk factors for AF.

Aging and high blood pressure are the two risk factors most frequently associated with high atrial arrhythmogenicity^{5,24,58} and the coexistence of both conditions in AF patients is associated with increased morbidity and mortality.¹⁸⁹

1.5.1. The role of aging

The incidence of AF increases with age. The adjusted incidence and prevalence of AF roughly double for each advancing decade of life. At 40 years of age, the remaining lifetime risk for developing AF is 1 in 4 for both white men and women, and it remains as high at older ages because of the steeply increasing risk for AF with advancing age. The comparable lifetime risk in men and women is due to the greater longevity of women.

Data from Europe and North America suggest that the age-adjusted incidence and prevalence of AF are increasing. Based solely on the aging of the population, the prevalence of AF in the United States has been projected to increase from 2 to 5 million in 2000 to 6 to 12 million in 2050, with estimates reaching almost 16 million if the increase in age-adjusted AF incidence continues. The pathophysiology underpinning the greater age-adjusted likelihood of AF in men and the increased risk with advancing age is incompletely understood.

1.5.2. The role of hypertension

In the Manitoba study, the prevalence of hypertension among patients with AF was 53% and the estimated risk of AF was 1.4 times higher in hypertensive patients than in normotensive subjects.¹⁶³ The Framingham Heart Study reported a 1.9-fold higher risk of AF in hypertensive than in normotensive individuals.¹⁴⁵ In fact, because of its high prevalence, hypertension is responsible for more AF in the general population (14%) than any other risk factor.¹⁴⁶

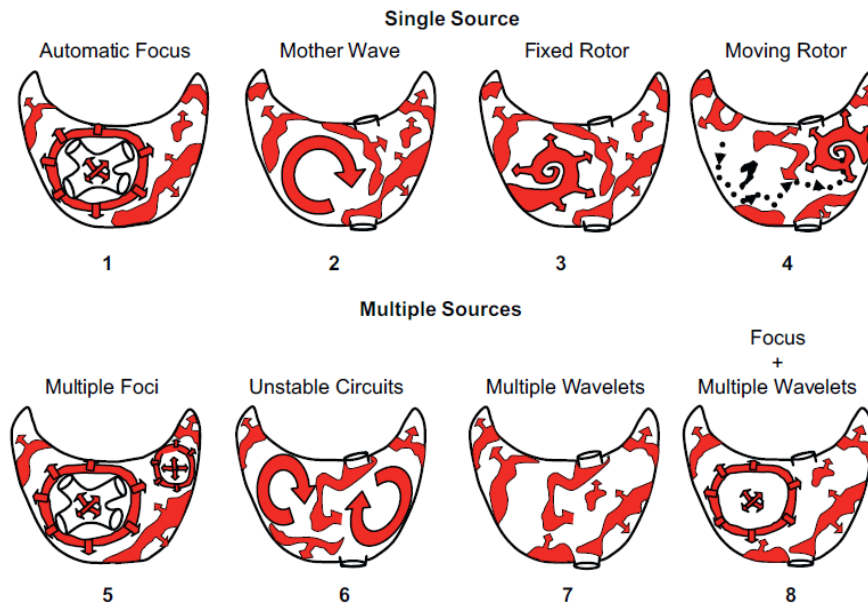
Several pathophysiological mechanisms, including increased aortic stiffness, extensive LA fibrosis induced by elevated blood pressure, significant LVH and dilated LA, or a combination of several of these factors, could contribute to hypertension-related AF.

1.6. Pathophysiology of atrial fibrillation - Coumel's triangle of arrhythmogenesis

Multiple mechanisms have been proposed in order to explain AF occurrence and maintenance, including automatic focus, multiple wavelets, mother waves, fixed or moving

rotors, or macro-re-entrant circuits (Fig. 1.3).⁸⁰ Several mechanisms may coexist in one patient.

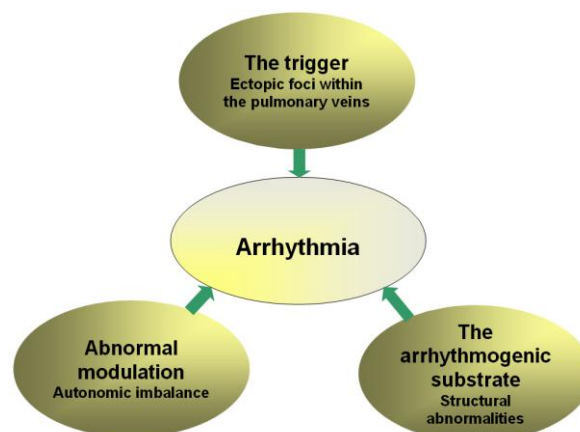
Figure 1.3. Cartoons of left and right atria presenting potential mechanisms of atrial fibrillation⁸⁰



Fibrillation waves are displayed in red with arrows indicating the direction of propagation.

According to Coumel's triangle of arrhythmogenesis,⁹¹ three elements are required in order to start cardiac arrhythmias – a trigger, an arrhythmogenic substrate, and abnormal modulating factors (Fig. 1.4). These three precise elements have been associated with AF onset and maintenance: rapidly discharging triggers or foci, substrate abnormalities that permit and promote wavelet re-entry, and autonomic imbalance, which may promote trigger activity and modify the substrate to facilitate the arrhythmia.

Figure 1.4. Coumel's triangle of arrhythmogenesis



The complex interactions established between these three components, which are not mutually exclusive and are likely to co-exist, determine the presentation of the arrhythmia. In the absence of significant substrate, the ectopic activity can only induce self-terminating episodes of AF (paroxysmal AF). Instead, in the presence of extensively remodeled atria, even limited ectopy can start persistent or permanent AF.

1.6.1. Automatic focus – the role of pulmonary veins

Already in the 1940's, Scherf advanced the hypothesis of 'focal AF', based on an animal model in which local administration of aconitine induced focal atrial tachycardia rapidly degenerating into AF.²⁶⁰ Termination of the arrhythmia could be achieved by isolating the focus of the arrhythmia from the rest of the atrium. However, these data have been overlooked until Haïssaguerre et al. identified ectopic activity originating in the pulmonary veins (PVs) of AF patients.¹²³ Experiences with AF ablation showed that radiofrequency elimination of PV foci could terminate the arrhythmia. Several hypotheses, including abnormal automaticity, triggered activity, and re-entry mechanisms, have been proposed in order to explain 'focal AF' originating in the PVs.

Accumulating evidence suggests that PVs are capable of automaticity and that PV myocardial cells themselves contribute to arrhythmogenic triggers. These specialized myocytes are highly excitable in the presence of altered autonomic tone. It has been suggested that the unique electrophysiological properties of PV cardiomyocytes, coupled with the dense autonomic innervation of PVs, produce a highly arrhythmogenic region within the posterior LA around the PVs. However, while PV cardiomyocytes probably do contribute to atrial arrhythmias, these cells are not likely to be the only ones involved in atrial arrhythmias' initiation. To date, three populations of non-myocardial cells have been incriminated in the trigger activity of PVs: melanocytes, pale cells, and interstitial Cajal-like cells.^{174,199,212} These cells might be residua of the embryonic myocardium.

In addition, foci outside the PVs are known to initiate atrial arrhythmias, and the unique electrophysiologic properties of PV cardiomyocytes cannot account for the origin of triggers outside the PVs. Such foci have been described throughout the atria, including the LA posterior wall, the right atrium, superior vena cava, the ligament of Marshall, crista terminalis, or the coronary sinus.

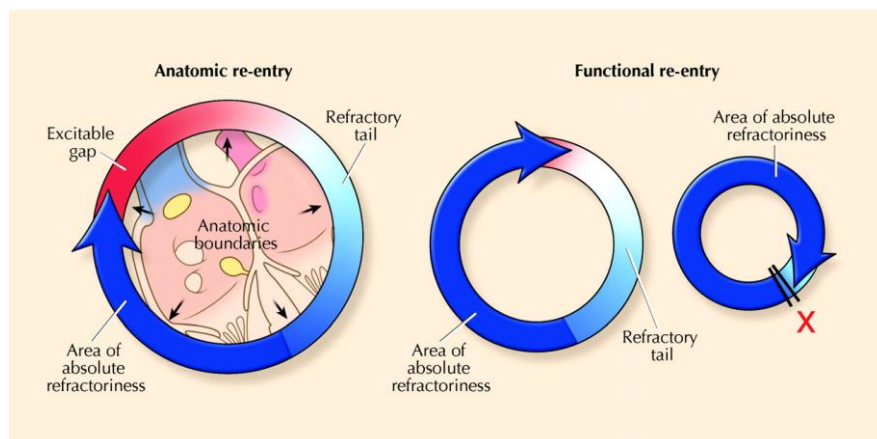
Although focal activity seems to play a crucial role in AF occurrence, PVs appear to be also implicated in the development of re-entry circuits. The myocardial architecture in PVs is

highly variable. The transition from atrial to venous walls is gradual as the LA myocardial sleeves overlap with smooth muscle of the venous myocardium.¹³³ The muscle fibers in PVs are usually orientated perpendicularly to the blood flow. Such an arrangement, together with increased anisotropy due to aging-induced fibrosis, may facilitate re-entry within the PVs. The cellular properties of PV cardiomyocytes, including shorter action potential duration (APD) and smaller 0-phase upstroke velocities than in the LA are also in favor of re-entry.⁸¹

1.6.2. Re-entry circuits

In order to assure AF perpetuation, multiple re-entry circuits must coexist within the atria. A circuit arises when an ectopic beat encounters refractory tissue when propagating in one direction, but it is still able to conduct in the other direction, where the tissue has already recovered, generating a so-called ‘unidirectional block’. For the re-entry circuit to be maintained, the impulse must traverse the circuit slow enough for all points of the circuit to regain their excitability. Thus, the conduction time through the circuit must be longer than its refractory period. The main determinants of the conduction time within the circuit are circuit path length and conduction velocity. The longer the path length and the slower the conduction velocity, the more likely is for all points of the circuit to recover fast enough their excitability to be reactivated by the re-circulating impulse (Fig. 1.5).

Figure 1.5. Anatomical *versus* functional re-entry²⁹⁹

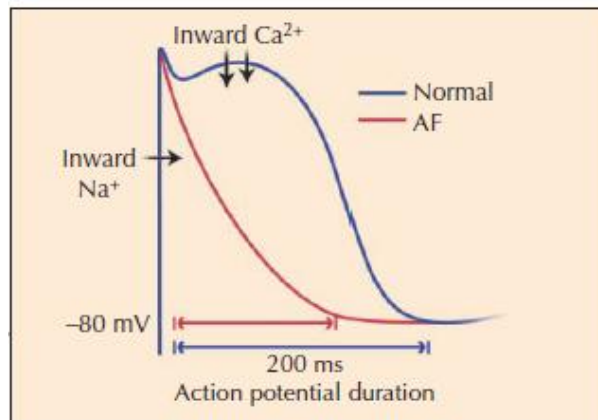


In anatomical re-entry, circuit size is determined by fixed anatomic obstacles (left). In functional re-entry (middle), circuit size depends on conduction velocity and refractory period (length of the refractory tail). If the wavefront travels too quickly or its refractory period is too long, its leading end would ‘bite its tail’ and extinguish itself (right).

Short refractory periods increase the likelihood for circuit points to be available for reactivation when the impulse passes through. The ‘wavelength’, or the distance traveled by

an impulse in one refractory period, represents the product of refractory period and conduction speed. Atrial refractoriness depends on cardiac APD, which is significantly reduced in the presence of AF (Fig. 1.6).

Figure 1.6. Action potential duration: normal *versus* atrial fibrillation²⁹⁹



Atrial conduction slowing can result from changes in sarcolemal Na^+ channels, gap junctions, or tissue structure. The wavelength determines the size of functional re-entry circuits. Factors that reduce wavelength decrease re-entry circuit dimensions, which increase the potential number of simultaneous circuits and augment the probability of AF maintenance.

According to the multiple wavelet hypothesis, fractionation of wavefronts propagating through the atria results in self-perpetuating wavelets in a seemingly chaotic manner. The wavefront-waveback interactions will generate new wavefronts. The number of resulting wavelets is parallel with increased atrial mass, intra-atrial conduction abnormalities, and shortened atrial refractory periods. Parasympathetic stimulation reduces atrial refractory period, thus reducing the size of re-entry circuits and allowing more circuits to coexist in the same space, thereby favoring arrhythmia perpetuation.

1.6.3. *Abnormal autonomic tone*

It is generally accepted that changes in the autonomic tone are present before, during, and after episodes of AF. However, the role of autonomic variations in the initiation, maintenance, and termination of AF remains controversial.

It has been recently suggested that the distribution of autonomic nerves throughout the atria and abnormal autonomic tone could represent a substrate for AF,²⁶⁸ particularly in settings where little or no anatomic abnormalities are present. Clinical and experimental observations suggest that the onset of AF is often related to variations in autonomic tone,^{268,312} but the direction of the autonomic imbalance facilitating AF occurrence is still a matter of

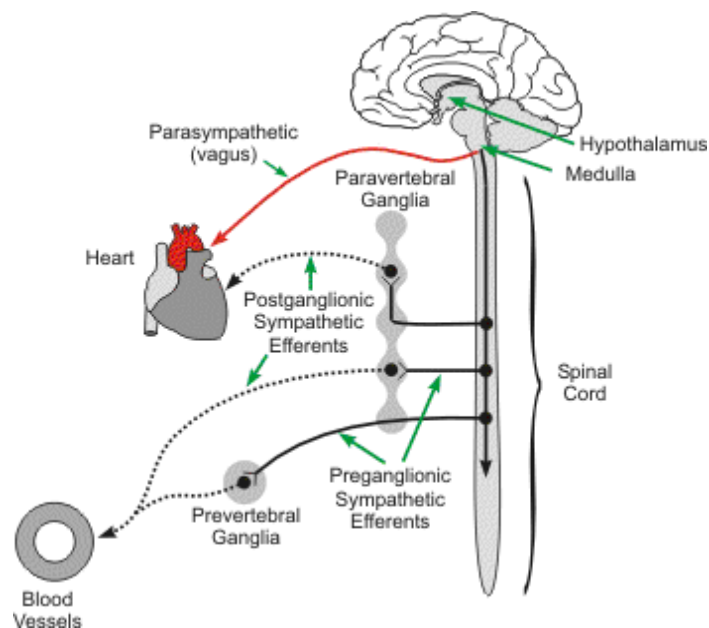
debate. Several different patterns of sympatho-vagal imbalance have been identified as AF facilitators in both clinical and experimental conditions. Various factors, such as heterogeneous autonomic innervation of the atria¹⁷⁵ or a complex and dynamic inter-relationship between the two branches of the autonomic nervous system (ANS),^{268,312} could be involved in arrhythmia occurrence.

1.6.3.1. *The cardiac nervous system*

The cardiac ANS can be divided into extrinsic and intrinsic components. The extrinsic part consists of brain nuclei and chains of ganglia along the spinal cord with axons terminating in the heart, while the intrinsic component incorporates a network of axons and autonomic ganglia embedded within epicardial fat pads located above both atria and ventricles.

The autonomic innervation of the heart consists of both parasympathetic and sympathetic fibers. The medulla is the primary site in the brain for regulating sympathetic and parasympathetic outflow to the heart and blood vessels, while the hypothalamus and higher centers modify the activity of medullary centers (Fig. 1.7).

Figure 1.7. Autonomic nerves and their innervation of heart and blood vessels¹⁵⁶



The autonomic innervation of the heart is very rich, yet asymmetric. Postganglionic parasympathetic neurons are primarily located in three fat pads - one adjacent to the right PV-atrial junction which sends postganglionic fibers to the sinus node, one at the junction of the inferior vena cava and the right atrium that sends vagal postganglionic neurons to the

atrioventricular node, and a third one referred to as the ‘head station’, located between the superior vena cava and the aortic root through which the majority of these efferent fibers travel before reaching their destinations. However, there can be significant overlap in the anatomical distribution of autonomic nerves.

The atria are innervated by both parasympathetic and sympathetic nerves, whereas the ventricles are only sparsely innervated by vagal efferents. Sympathetic efferent nerves are present throughout the atria and the ventricles, including the conduction system of the heart. Additionally, a population of mechanosensitive and chemosensitive receptors sends afferent signals back to the central nervous system and influence cardiac function *via* local intrathoracic circuits.

The complex interactions between the sympathetic and parasympathetic outflows to the heart have been shown not only to control normal cardiac function, but also to modulate the susceptibility to cardiac arrhythmias. The ANS can facilitate arrhythmia occurrence by triggering arrhythmogenic foci on the one hand, and modifying the heterogeneity of refractory periods on the other hand.

1.6.3.2. Atrial fibrillation onset – vagal, sympathetic, or both?

Philippe Coumel was the first to differentiate vagal and sympathetic AF.⁶⁴ He reported that vagal AF usually occurs in men, around the age of 30 to 50 years. It can usually be found in patients without concomitant heart conditions, in a context of progressive bradycardia. The onset of the arrhythmia is frequently nocturnal, postprandial or after alcohol intake. In contrast, adrenergic AF frequently occurs after exercise, during stress, or strong emotions. An underlying heart disease is often observed. Excessive adrenergic stimulation is also believed to be the dominant mechanism in post-operative AF and prophylactic use of *beta*-blockers efficiently reduces the incidence of post-operative AF.¹⁹⁷ In the large cohort of patients who participated in the Euro Heart Survey, an autonomic trigger, vagal or adrenergic, was found in almost one-third of patients. Vagal AF was found in 6% of patients, adrenergic AF in 15% of patients and mixed AF in 12% of patients.⁷⁰

The parasympathetic nervous system has long been recognized to play an important role in AF onset. Parasympathetic stimulation with either carbamylcholine, continuous bilateral electrical stimulation of the cervical vago-sympathetic trunks, acetylcholine infusion, or sympathetic denervation, was efficient in inducing AF in various animal models.^{112,230,265} From an electrophysiologic point of view, vagal stimulation shortens atrial and PVs refractory

periods due to activation of the acetylcholine-activated potassium current (I_{KACh}), while increasing the dispersion of refractory periods and causing cell hyperpolarization. Vagal AF promotion seems to be related to this spatially heterogeneous reduction in atrial effective refractory periods.³⁰⁸ On the contrary, parasympathetic denervation prevents the occurrence of AF.

On the other hand, AF can also be induced by adrenergic stimulation with isoproterenol or adrenaline.²⁶⁵ Sympathetic stimulation also produces reductions in atrial effective refractory periods, but does not promote AF as much as vagal stimulation. This could be due to a much more homogeneous reduction of atrial effective refractory periods with sympathetic stimulation.¹⁸⁶ Increased slowly activating delayed rectifier potassium current (I_{Ks}) activity is the likely source for action potential alterations with adrenergic stimulation.

However, most of the studies addressing the role of the ANS in atrial arrhythmia occurrence suggest a combined participation of both branches, with sympatho-vagal imbalance as main trigger of these arrhythmias, rather than an anomaly of one branch alone. Autonomic fluctuations before the onset of paroxysmal AF, measured primarily by using heart rate variability (HRV) analysis, have been assessed in several studies, but with conflicting results.

Holter ECG monitoring and subsequent HRV analysis in 13 patients diagnosed with 'focal AF' showed an increase in sympathetic activity, followed by a vagal predominance immediately before the onset of AF.²⁵ In a canine model of AF, it has been shown that the sympathetic nervous system modulates and maintains vagally induced AF.²⁶⁵ Tan et al. implanted a pacemaker and a radiotransmitter in dogs to simultaneously record nerve activities of the left stellate ganglion and left vagal nerve over a period of several weeks.²⁷⁹ Intermittent rapid atrial pacing was performed and ANS activity was monitored when the pacemaker was turned off. Simultaneous sympatho-vagal discharge was the most frequent trigger of paroxysmal atrial tachycardias and AF, and a common trigger for premature atrial contractions (PACs). On the other hand, stellate ganglion and vagal ablation helped to prevent AF.⁵⁵

Tomita et al. performed HRV analysis in two groups of patients according to the mode of occurrence of the arrhythmia.²⁸⁸ In patients with nighttime paroxysmal AF, despite a progressive increase in low-frequency (LF) and high-frequency (HF) components before AF onset, the authors found no change in the LF/HF ratio. On the other hand, patients with daytime paroxysmal AF presented an increase in LF/HF ratio that preceded the onset of AF

episodes. Based on these results, the authors suggested that vagal tone is more dominant in nighttime paroxysmal AF, whereas adrenergic tone is more dominant in daytime paroxysmal AF.²⁸⁸

These conflicting results could be due to the differences in patient population, the multitude of clinical forms of arrhythmia, the time frame during which HRV was measured, or the intrinsic limitations of this technique. While HRV analysis provides a general picture on the autonomic balance, it has serious limitations when assessing individual branches of the ANS and it provides no insight into the autonomic background during AF. Furthermore, the mechanisms responsible of AF onset and maintenance are most certainly not limited to autonomic abnormalities, and the role of the ANS in patients with focal AF and structurally normal hearts is not likely to be the same to that in patients who have structural heart disease.

Indeed, the study by Tai et al. suggested that in certain cases, vagal stimulation could actually interrupt AF.²⁷⁸ In patients undergoing radiofrequency ablation procedures, administration of phenylephrine, which is known to increase blood pressure and trigger reflex vagal activation, suppressed the activity of PV ectopic foci. Chen et al. suggested that these conflicting results could originate from the differential effect of vagal stimulation on the trigger *versus* the substrate.⁴⁸ While vagal stimulation might actually suppress the ectopy originating from the PVs and thus the trigger for AF, its effect on the substrate is far from being antiarrhythmic.

1.6.3.3. The role of autonomic nervous system and autonomic reflexes in atrial fibrillation maintenance

As previously mentioned, increased vagal tone shortens the effective refractory period and increases the dispersion of refractoriness,³⁰⁸ leading to an increased number of functional obstacles and hence wavebreaks, and resulting in new wavelet formation and perpetuation of AF. Experimentally, it has been demonstrated that an increase in atrial pressure during tachycardia could activate cardiac mechanoreceptors leading to an increase in vagal activity.²³⁷ Thus, these results suggest that besides acting as arrhythmia onset facilitator, vagal hyperactivity also promotes AF persistence and that parasympathetic nervous system plays an important role in the electrical remodeling associated with AF.

Sympathetic hyperactivity has also been proposed as facilitator for AF maintenance. Studies using rapid atrial pacing have shown nerve sprouting and sympathetic hyperinnervation in the stimulated atria, probably as response to electrical pacing.^{42,141} The

authors suggested that heterogeneous sympathetic innervation of the atria could lead to an increase in dispersion of refractoriness and thus to increased susceptibility to persistent arrhythmias.

It seems that during AF, increased cardiac filling pressure and decreased arterial blood pressure send conflicting messages to the spinal cord, confusing the ANS. While the decrease in cardiac output leads to unloading of the arterial baroreceptors and reflex sympatho-excitation, the increase in filling pressure leads to activation of the cardiopulmonary baroreceptors and sympatho-inhibition.²⁷³ The importance of each of these mechanisms seems to vary from one individual to another. While Wasmund et al. showed that sympathetic nerve activity increased following AF onset, suggesting that the arterial baroreflex plays a major role in sympatho-excitation mediation with minimal contribution from the cardiopulmonary baroreflex, in other patients the cardiopulmonary baroreflexes could be playing a greater role, resulting in a greater degree of sympatho-inhibition and vagotonia.³² The question remains if impaired baroreflex is only the cause of persistent AF or whether it contributes itself to the perpetuation of AF.

1.6.3.4. Autonomic nervous system and atrial fibrillation termination

If recent data brought much information about AF onset and maintenance, little is known about the spontaneous termination of AF episodes. One could hypothesize that reversing autonomic abnormalities that triggered the arrhythmia will be efficient in restoring normal sinus rhythm. Future studies will have to provide an answer. Meanwhile, radiofrequency or surgical parasympathetic denervation emerged as efficient techniques for AF resolution. Recently, Pappone et al. showed that complete vagal denervation during circumferential PV ablation significantly reduces AF recurrence.²⁴² In the study of Tan et al., cryoablation of bilateral stellate ganglia and of the superior cardiac branches of the left vagus nerve in dogs eliminated paroxysmal AF and atrial tachycardia.²⁷⁹ Similarly, in a canine model of pacing-induced heart failure, atrial tachyarrhythmias (ATs) could be prevented by cryoablation of the stellate ganglion and of the T₂₋₄ thoracic sympathetic ganglia.²²⁷ Results of a study by Scherlag et al. showed that ablation of ganglionated plexi in addition to PV-antrum isolation increase ablation success from 70% to 91% among patients with paroxysmal or persistent AF after 12 months of follow-up.²⁶¹

In fact, because linear lesions from standard PV-directed ablation run through areas with high concentrations of ganglionated plexi, autonomic modification or denervation could be one of the mechanisms that contribute to the effectiveness of standard ablation procedures.

1.6.4. Inflammation – part of the triangle of arrhythmogenesis

Both clinical and experimental studies highlight inflammation as a modulating factor predisposing for AF and its complications.¹⁰¹ The first observation linking atrial remodeling to inflammation was made by Frustaci et al., who demonstrated a high prevalence of inflammatory infiltrates, myocyte necrosis, and fibrosis in atrial biopsies from twelve patients with lone AF, whereas biopsies from control patients were normal.⁹⁸ Similar findings have been reported by Nakamura et al.²¹⁸ and confirmed in a dog model of sustained AF where the authors demonstrated active atrial perimyocarditis, with inflammatory infiltrates, lipid degeneration, and fibrosis in arrhythmic hearts.¹⁶⁵

Observations of inflammatory infiltrates, myocyte necrosis and fibrosis in atrial biopsies from patients with lone AF refractory to antiarrhythmic drug therapy also support the hypothesis that AF is closely related to an inflammatory process.^{162,169} Liao et al. reported mast cells infiltrates in the atria of mice with atrial burst stimulation-induced AF in pressure-overloaded hearts, and showed that stabilizing these cells with cromolyn, a mast cell stabilizer, attenuates AF inducibility and duration. The authors concluded that mast cells participated actively to AF in this model.¹⁸²

The contribution of inflammation to AF is also suggested by the high incidence of the arrhythmia after cardiac surgery.^{24,185,292} Atrial fibrillation occurs in up to 40% of patients undergoing cardiac bypass surgery and in up to 50% of patients undergoing cardiac valvular surgery.^{6,178} The inflammatory cascade and catecholamine surge associated with surgery could play a prominent role in initiating ATs after cardiac surgery. Bruins et al. found that interleukin-6 (IL-6) rises initially and peaks at 6h after surgery. Then, a second phase occurs in which C-reactive protein (CRP) levels peak on post-operative day 2, with complement-CRP complexes peaking on post-operative day 2 or 3.³⁶ The incidence of atrial arrhythmias follows a similar pattern and peaks on post-operative day 2 or 3.

Inflammatory cytokines have been recently described as facilitators for AF development and recurrence.^{5,24,58} Several reports showed a strong correlation between inflammatory markers (CRP, tumor necrosis factor, interleukins, cytokines) and the presence or the future development of AF. In a population study of 5,806 subjects 65 years or older, CRP levels at

baseline predicted future development of AF.¹⁶ Dernellis and Panaretou reported that for every 1 mg/dL increase in serum CRP, the risk for recurrent AF is increased 7 times and the risk for permanent AF is 12 times higher than in control subjects.⁷² Moreover, lower high sensitivity-CRP (hs-CRP) at baseline was associated with maintenance of sinus rhythm after planned electrical cardioversion for persistent AF.²⁹¹

The exact mechanisms that link inflammation to tissue remodeling in AF patients are unclear and deserve further research. CRP could be not just a marker of ongoing cardiac inflammation, but it could also contribute to cardiac damage, by activating serum complement system. However, whether complement-mediated inflammation plays a role in human AF remains to be demonstrated. Several studies have suggested that inflammation exerts its remodeling effects through reactive oxygen species. It has been shown that CRP promotes the generation of reactive oxygen species by altering the homeostatic balance of antioxidative enzymes in endothelial progenitor cells. Oxygen-free radicals can also activate matrix metalloproteinase resulting in an imbalance between accumulation and breakdown of extracellular matrix, enhancing LA fibrosis with consequent dilatation and loss of function of the atria. In fact, LA dysfunction has been described in patients with increased CRP levels even in the absence of AF, suggesting that inflammation *per se* affects LA function.²³ Thus, the presence of systemic inflammation with increased circulating CRP levels may predispose patients with sensitive atrial foci to develop AF.

Alternatively, rapid atrial activation has been shown to induce calcium accumulation within atrial myocytes leading to overload, and in some cases to initiation of apoptotic loss of atrial myocytes.²²² CRP might then act as an opsonin that binds to atrial myocytes, inducing a local low-grade inflammatory response and complement activation. Tissue damage then ensues and fibrosis sets in.²²⁹ Specifically, in the presence of Ca^{2+} ions, CRP binds to phosphatidylcholine. Long-chain acylcarnitines and lysophosphatidylcholines are generated from phosphatidylcholine and can further contribute to membrane dysfunction by inhibiting the exchange of Na^+ and Ca^{2+} ions in sarcomeres. This can eventually lead to AF maintenance.

Whether initiation of AF activates inflammation pathways or whether the presence of a pre-existing systemic inflammatory status promotes further persistence of AF remains unclear. Increased incidence of AF during inflammatory states such as cardiac surgery, and the fact that baseline levels of CRP predict AF occurrence support the hypothesis that inflammation could be a causative factor of AF. On the other hand, the fact that CRP levels

decrease after restoration of sinus rhythm leans towards the idea that inflammation could be a consequence of AF.

Although numerous studies support the existence of a link between inflammation and AF, several issues remain unsolved and require further investigation. A major limitation is the lack of data regarding inflammatory markers in patients before the onset of AF. Moreover, several studies failed to demonstrate elevated levels of CRP, IL-6, and interleukin 8 (IL-8) in AF patients compared to sinus rhythm controls.^{83,188,200,295}

1.6.5. Structural basis of an electrical disease

Cardiac remodeling often precedes the onset of AF, since it emanates from cardiac damage due to coronary artery disease, hemodynamic overload from valve disease or hypertension. The resulting atrial remodeling involves changes in structure and function of the atria, modifications of atrial electrical and contractile functions, changes in atrial extracellular matrix. Together, these alterations create an arrhythmogenic substrate, essential for the onset, and particularly for the persistence of AF. Structural alterations comprise a wide range of cellular and ultrastructural abnormalities, with components varying depending particularly on coexisting cardiac conditions and specie.⁹⁸

The severity of structural abnormalities seems to be related to the degree of LA enlargement. In the 1980s Boyden et al. reported dilated atria, associated with increased amounts of connective tissue between enlarged myocytes, as well as signs of degeneration, myofilaments loss and thickened basement membranes in a model of mitral valve disease and ventricular cardiomyopathy in dogs and cats.³⁰ These dilated atria presented a high susceptibility to atrial arrhythmias. Small, diffuse areas of interstitial and patchy fibrosis with unaltered interstitial space, homogeneous redistribution of nuclear chromatin resembling embryonic cardiomyocytes and changes in localization and quantity of structural cellular proteins seem to participate to this remodeling process.

Regardless of the model, a consistent finding is that of the major heterogeneity of lesions, not only within different sites, but also regionally, with severely injured myocytes next to virtually unaltered cells.

Comparable structural and ultrastructural abnormalities with those encountered in various animal models of AF have been described in AF patients. Nevertheless, more severe and extensive lesions have been reported in humans with both long-term lone AF and in AF associated with other cardiac diseases than in animal models of AF. In contrast with the data

reported in animals, in humans, atrial fibrosis is a common feature of both lone AF and AF associated with an underlying organic disease.^{98,165,218} In light microscopy, both intercellular and inter-bundle interstitial fibrosis appear more pronounced compared to experimental observations in animals. Concomitant heart conditions such as coronary artery disease, valve disease or congenital heart defects could be at least partially responsible for the more extensive structural changes observed in humans.

Several animal models proposed conduction abnormalities favoring unidirectional conduction block and macro-re-entry, conduction heterogeneities secondary to regional fibrotic differences, or focal atrial activity as potential mechanisms for atrial fibrosis-related arrhythmias. On the other hand, pathophysiological and genetic factors that promote atrial fibrosis remain incompletely understood. Three main pathways seem to be involved - renin-angiotensin system, transforming growth factor- β 1 (TGF- β 1), and inflammation/oxidative stress.

The renin-angiotensin system is known to be involved in structural cardiac remodeling and in the development of cardiac fibrosis in various disease states, including heart failure and myocardial infarction. Patients with primary hyperaldosteronism have an increased incidence of AF,²⁰⁷ and locally produced angiotensin II is associated with cardiomyocyte apoptosis and reactive interstitial fibrosis.³¹⁵ Increased angiotensin II production in transgenic mice with cardiac-restricted angiotensin converting enzyme overexpression causes marked atrial dilation, focal fibrosis and AF.³²⁴ On the other hand, AF susceptibility and atrial fibrosis were decreased by candesartan or enalapril, but not by hydralazine or isosorbide mononitrate, despite similar hemodynamic effects.^{178,229} These results suggested a key antiarrhythmic role of blockade of the renin-angiotensin system, rather than of improving hemodynamics. A meta-analysis of all trials using angiotensin converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) showed that these drugs possess quite potent antiarrhythmic effects.⁶ In the overall population, these drugs reduced new-onset AF by 18%, and among heart failure patients as much as 43%.⁶

Transforming growth factor-*beta*1 overexpression has been shown to induce myocardial fibrosis. The critical role of the TGF- β 1 pathway in AF occurrence has been demonstrated in transgenic mice overexpressing constitutively active forms of TGF- β 1, which led to selective atrial fibrosis, increased conduction heterogeneity and enhanced AF susceptibility, despite normal atrial APD and normal ventricular structure and function.³⁰⁰ In fact, TGF- β 1 effects seem to be limited to the atrial level, despite equal overexpression of TGF- β 1 in the atria and

the ventricles.³⁰⁰ On the other hand, TGF- β 1 inhibition with pirfenidone appears to reduce TGF- β 1 expression, the severity of atrial fibrosis and AF vulnerability.¹⁷⁰ The mechanism of inflammation leading to tissue remodeling and atrial fibrosis in AF patients has been already discussed (see section 1.6.4).

It remains to be established if targeting prevention or reversal of structural atrial remodeling will result in reduction of the major impact that AF represents for the public health and to determine the best time to intervene to lower the risk of occurrence and/or recurrence of atrial arrhythmias.

1.7. Atrial fibrillation begets atrial fibrillation

Clinical experience shows that paroxysmal AF frequently progresses towards persistent AF and that the longer the duration of AF the harder it becomes to establish and maintain sinus rhythm. Once established, AF itself participates to further alterations in electrical and structural properties of the atrial tissue, which will consequently lead to perpetuation of the arrhythmia.¹⁶⁹ Thus, from a therapeutic point of view, it is essential to interrupt this cycle as quickly as possible, to prevent remodeling and its deleterious consequences.

Two distinct types of atrial remodeling have been described, electrical and structural, which differ in etiology, involved mechanisms, and potential reversibility.

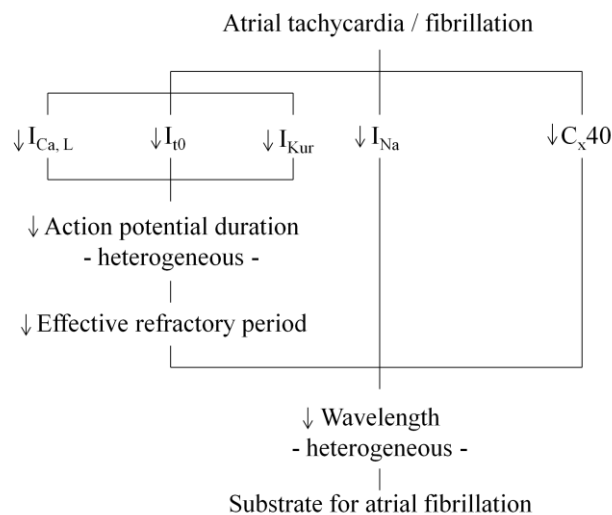
1.7.1. Atrial fibrillation-induced electrical remodeling of the atria

The concept of atrial electrical remodeling due to chronic high atrial rates or ‘tachycardia-induced electrical remodeling’ was first described in experimental studies.^{213,319} Based on a goat model in which AF was induced by atrial tachypacing, it has been shown that more frequent induction of AF was associated with more sustained episodes of arrhythmia, and that this phenomenon was accompanied by shortening and maladaptation of atrial refractory periods.³¹⁹ In this model, electrical remodeling has been shown to occur primarily by virtue of a very rapid atrial rate and included changes in ionic properties of atrial myocytes, particularly shortening of the atrial refractory period and abnormal rate-adaptation of refractoriness, alterations in sarcolemmal ion channel gene expression, cellular size and content, in addition to changes in connexins that couple cells electrically.³¹⁹

Shortly after the demonstration of tachycardia-induced electrical remodeling, the ionic mechanisms underlying this arrhythmogenic process have been elucidated by a number of convincing studies.^{29,105,111,297,327} Action potential recordings and patch clamp experiments in isolated atrial cells from animal models and patients with chronic AF showed a consistent

pattern. The reported abnormalities are probably largely due to impaired Ca^{2+} homeostasis, with reduction of L-type calcium currents ($I_{\text{Ca,L}}$). This explains the shortening of the atrial action potential and the loss of the physiological rate adaptation of the APD.³²⁷ A significant reduction in the sodium current (I_{Na}) density was also reported in a dog model.¹⁰⁵ The decrease in $I_{\text{Ca,L}}$ leads to a reduction in APD, whereas the reduction in I_{Na} may contribute to the decrease in the conduction velocity. These changes concurrently facilitate re-entry mechanisms. In several other studies, the transient outward potassium current (I_{to}) and the ultra-rapid delayed rectifier potassium current (I_{Kur}) were also reduced (Fig. 1.8).^{29,327}

Figure 1.8. Schema of mechanisms involved in atrial fibrillation promotion by atrial tachycardia-induced remodeling (adapted after Nattel S, 2001²²¹)



$I_{\text{Ca,L}}$ - L-type calcium current, I_{to} - transient outward potassium current, I_{Kur} - ultra-rapid delayed rectifier potassium current, I_{Na} - sodium current, C_{x40} - connexin-40

The onset of electrical remodeling has been established as early as few minutes after AF onset, and complete remodeling was achieved in 24h to 48h after the onset of the arrhythmia. However, electrical remodeling appears to be completely reversible after restoration of sinus rhythm. In fact, even after prolonged periods of AF, atrial refractoriness was shown to return to normal within a few days after sinus rhythm restoration.²⁴⁰

1.7.2. Atrial fibrillation-induced contractile and structural remodeling of the atria: a chicken-egg relationship

Contractile remodeling of the atria has been shown to begin shortly after the onset of AF. Similarly to electrical remodeling, this mechanism seems to be also related to Ca^{2+} -handling impairment.

Ausma et al. documented in goats the time-dependency of structural remodeling, with first cellular and ultrastructural changes developing after one week, and a maximum of lesions of myolysis reached after about 8 weeks of AF.¹⁴ Progressive morphological changes in mitochondria and sarcoplasmic reticulum and homogeneous chromatin distribution peaked one week after AF induction. From a functional point of view, atrial paralysis associated with increased atrial compliance will lead to LA enlargement within a few weeks after AF onset. Increased wall stress is likely to play a crucial role in this structural remodeling.

Experimental studies demonstrated that prolonged rapid atrial pacing induced changes in atrial myocytes including increase in cell-size, myolysis, perinuclear accumulation of glycogen, alterations in connexin expression, fragmentation of sarcoplasmic reticulum and changes in mitochondrial shape.¹⁵ At the tissue level, structural remodeling in various animal models is characterized by myocyte loss, and by changes in extracellular matrix composition, with both diffuse interstitial and patchy fibrosis. Similar alterations were observed in the atrial tissue of patients with AF.⁹⁸ Structural remodeling results in electrical tissue inhomogeneity, slowed conduction and electrical uncoupling, which facilitate AF continuation without inducing changes in atrial action potential properties.

Evidence thus suggests that atrial structural abnormalities are not only the cause, but also the consequence of AF, participating in the maintenance of a vicious circle, with AF-induced atrial structural remodeling promoting AF persistence. In contrast to electrical remodeling, structural changes are far less reversible and they tend to persist even after reestablishment of sinus rhythm.¹⁴

1.7.3. Atrial fibrillation-induced autonomic remodeling of the atria

While structural and electrical remodeling are recognized as key pieces in the vicious circle by which AF favors AF, recent data suggest that autonomic remodeling also participates to the auto-perpetuation of AF.^{42,141}

Using positron emission tomography in dogs with AF induced by chronic rapid right atrial pacing, Jayachandran et al. showed that the paced atrium had a greater heterogeneity of

sympathetic innervation than the non-paced atrium and demonstrated a greater magnitude of sympathetic nerve sprouting in the right atrium than in the LA.¹⁴¹ In a heart failure model induced by rapid ventricular pacing in dogs, Ng et al. showed that both parasympathetic and sympathetic remodeling occur in the LA, with an increase in both parasympathetic and sympathetic innervation.²²⁴ Evidence of sympathetic remodeling was an increase in sympathetic nerve fiber density and a concomitant increase in *beta1*-adrenoreceptors. Parasympathetic remodeling was more complex, with an increase in nerve bundle size, number of parasympathetic fibers, cardiac ganglia density, and number of cell bodies in the ganglia. In contrast, in a canine model of sustained AF produced by prolonged LA pacing, Yu et al. reported sympathetic hyperinnervation and vagal denervation.³²⁶ Further studies in this area would definitely be of interest.

1.8. Atrial fibrillation – a genetic disease?

As previously mentioned, most of AF cases develop in patients with significant cardiac comorbidities. However, in 15% to 30% of patients, an etiology is absent.^{176,216} Moreover, some of these patients have a positive family history of AF.^{96,320} Heritability of AF appears even stronger in individuals with lone AF. These observations suggested that genetic factors could also contribute to the development of the arrhythmia. A study on the heritability of AF in an Icelandic population showed that first degree relatives of patients with AF diagnosed before the age of 60 years are almost 5 times more likely to develop AF compared to the general population.⁸

Possible genes responsible for AF onset and persistence include genes that affect automaticity, atrial refractory period duration and conduction.

In 1997, Brugada et al. were the first to identify linkage to a locus on chromosome 10q22-24 for familial AF in three different Spanish families.³⁵ In these families, AF segregated as an autosomal dominant disease with high penetrance. In a three-generation family, 10 of the 26 living family members were affected. The causative gene has not been identified yet. In 2003, Chen et al. published data of a large Chinese family with autosomal dominantly inherited lone AF.⁵¹ Atrial fibrillation was permanent in all patients. The causative mutation was located in the *Kcnq1* gene on chromosome 11p15.5. Functional analysis of this mutation revealed a gain-of-function effect on the *Kcnq1/Kcne1* and the *Kcnq1/Kcne2* gene complexes. These mutations induced shortening of the APD and of the effective refractory period in atrial myocytes, which in turn could set the stage for initiation

and maintenance of AF. The same group identified another mutation, in the *Kcne2* gene, in two Chinese families with lone AF.³²⁵ Functional analyses also revealed a gain-of-function effect in both inward and outward *Kcnq1/Kcne2* potassium current genes leading to a shortening of the APD, which again could trigger and perpetuate AF. Recently, a locus on chromosome 5p13 has been identified in autosomal recessively inherited AF.²²⁶ The affected children showed a rapidly progressive disease, including AF with early onset, at fetal stage. The implication of genetic abnormalities in certain cases of AF that develop in the presence of associated comorbidities cannot be excluded.

However, resequencing of AF patient cohorts suggests that recognized ion channel mutations account for only a small fraction of all AF cases. On the other hand, common genetic variants, single-nucleotide polymorphisms, are much more frequent, though they act with a weaker effect and a less-clear phenotype.²⁷¹ Genome-wide association studies identified 3 genetic loci on chromosomes 4q25, 16q22, and 1q21 associated with AF.^{84,117,118}

1.8.1. Pitx2 in cardiac development

The single-nucleotide polymorphisms most significantly associated with AF are located on chromosome 4q25, in what is commonly called a ‘genomic desert’, a large, intergenic region, without any known genes.¹⁹⁴ The closest gene in the region is *Pitx2*, a member of the pituitary homeobox family of transcription factors that plays an important role in morphogenesis in early development.²⁴⁸

Pitx2 is a transcription factor known to play a critical role in the left-right asymmetry of the heart, differentially regulating LA identity and ventricular asymmetrical remodeling programs. This functional role is supported by reports showing that *Pitx2*^{-/-} knockout mice die before birth and present right atrial isomerism and both atrial and ventricular septal defects.²⁸³ Additionally, *Pitx2* plays essential roles in regulating the development of the cardiac conduction system, particularly the sinus node, but also the LA and the pulmonary myocardium.

All cardiomyocytes of the developing heart initially possess pacemaker properties, but only a small proportion of cells differentiate into pacemaker cells that form the cardiac conduction system, including the sinus node. *Pitx2* plays a crucial role in this setting. *Pitx2* represses *Shox2*, a transcription factor expressed in sinus node precursors. Repression of *Shox2* by *Pitx2* results in down-regulation of a nodal gene program and up-regulation of a gene program characteristic for a working myocardium phenotype. Because *Pitx2* expression

is restricted to LA *Shox2* expression, development of the sinus node is only repressed in the LA. In the right atrium, missing *Pitx2* results in *Shox2* up-regulation, which will assure sinus node development.^{53,191}

The importance of *Pitx2* during cardiogenesis has been illustrated by the complex and robust cardiac defects observed in systemic deletion of *Pitx2* in mice. Lack of *Pitx2* expression leads to embryonic lethality at mid-term, and *Pitx2* deficient embryos display isomeric hearts and incomplete closure of the body wall.^{53,191} In humans, *Pitx2* mutations are known to induce the Axenfeld-Rieger syndrome, which includes umbilical, ocular and facial malformations. In addition to this syndrome, mutations in the *Pitx2* gene have been described in cardiac malformations like double-outlet right ventricle or arterial transposition.²⁴⁸

1.8.2. *Pitx2* in atrial fibrillation

Chinchilla et al. were the first to demonstrate that *Pitx2* is significantly decreased in patients with sustained AF, thus providing a molecular link between *Pitx2* loss-of-function and AF.⁵³ However, it seems that *Pitx2* expression is not altered in the developmental period, but its impairment occurs in the adult heart. Indeed, an early defect in *Pitx2* function would be expected to have severe consequences for cardiac embryogenesis, and thus it is more likely for an adult dysfunction to underlie *Pitx2* down-regulation-related predisposition to AF.

Wang et al. investigated mice that were heterozygous for a *Pitx2* null allele that removed all isoform function and could show that these mice were more susceptible to pacing-induced atrial arrhythmias. The authors hypothesized that *Pitx2* is an inhibitor of a pacemaker gene program in the LA and that down-regulated *Pitx2* expression may underlie the propensity of atrial arrhythmias in their model.³⁰⁷

Pitx2 mis-expression impairs C_x40 expression, both in embryonic stem cell-derived cardiomyocytes and in fetal and adult atrial and ventricular chamber-specific *Pitx2* loss-of-function mice.^{53,191} These data provide a putative link to AF, since mutations in C_x40 gene are associated with AF and further implicate impaired *Pitx2* function at adult stages in predisposition to AF. In addition, *Pitx2* might be modulating ion channel expression, and therein provide molecular substrates for atrial arrhythmogenesis. In the study of Kirchoff et al., *Pitx2*^{-/+} hearts were susceptible to AF elicited by programmed stimulation with a single atrial premature beat.¹⁵⁴ The authors also showed that reduced expression of *Pitx2* in mice causes shortening of atrial APD and susceptibility to induced AF. Gene expression patterns and functional analyses in *Pitx2* deficient atria suggest that modified Ca²⁺-handling, cell-cell

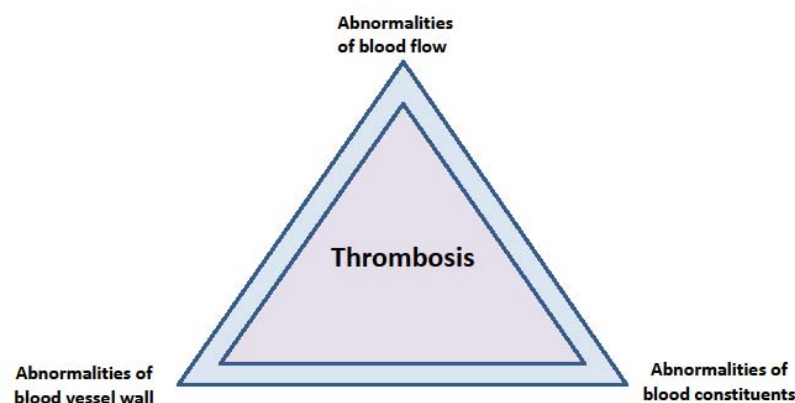
communication, or altered function of melanocytes could also participate to AF predisposition in this setting.^{46,50,82,116,257,262,302} Finally, *Pitx2* could also trigger AF by altering the conduction properties of the cardiac conduction system.^{209,210}

1.9. Thromboembolic risk – the hidden face of atrial fibrillation

Atrial fibrillation is associated with a high risk of mortality and morbidity from stroke and thromboembolism. The predisposition for stroke among patients with AF is estimated at around 5% per year.²⁰⁵ Furthermore, patients with AF and stroke have worse outcomes and develop more serious deficits compared to patients with strokes that are not related to AF.²

Over 150 years ago, Virchow stated that for thrombus formation there should be abnormalities in blood flow, vessel wall and blood constituents (Fig. 1.9).¹⁸⁴

Figure 1.9. Virchow's triad of coagulation



In AF, it is largely accepted that blood stagnation in the atrium is an important thrombogenic factor.²⁵¹ However, the coexistence of other conditions, such as hypertension, aging, or diabetes increases the risk of stroke even more.²⁵¹ This observation suggests that although LA stasis and subsequent thrombus formation may be one source of thromboembolism in AF patients, this does not provide a complete explanation.

It is well-established that AF is associated with abnormal haemostatic constituents indicative of thrombogenesis.⁵⁷ Gustaffsson et al. demonstrated the presence of haemostatic abnormalities in the plasma of patients with non-valvular AF compared to sinus rhythm controls, and associated these abnormalities with stroke.¹²⁰ Increased thrombogenesis has been further demonstrated by increased prothrombin fragments 1 and 2 and thrombin-antithrombin III complex levels as indicators of increased thrombin generation in AF patients, and it has

been shown that prothrombin fragments are particularly raised in AF patients with stroke.¹² However, although thrombotic markers confirm the association of AF with an intravascular environment in which there is an increased tendency to clot, the origins of this coagulation activation are yet to be elucidated.

Recent data support the participation of endothelial dysfunction in the pathophysiology of thromboembolic risk in AF patients,^{5,58,145,163,189} as part of a vicious circle, with atrial endocardial impairment favoring arrhythmia occurrence and AF aggravating endothelial dysfunction. Several hypotheses have been advanced to explain endothelial dysfunction in AF. The endothelium could be rendered dysfunctional by a localized mechanism involving blood flow stasis in the LA, or systemically due to turbulent flow and reduction in shear stresses on the vessel wall, or simply by associated cardiovascular risk factors.⁵⁴ Several of these mechanisms might coexist in the same patient.

1.10. Atrial fibrillation management

The main therapeutic goals in AF management are to prevent severe complications, particularly thromboembolic events, and to control symptoms. Both rate and rhythm control strategies have been shown to improve symptoms, but neither appears to be superior to the other when comparing survival rates.¹⁵⁹ Thus, the cornerstones of AF management are rate control and anticoagulation, and rhythm control for those symptomatically limited by the presence of the arrhythmia.

1.10.1. Prevention of thromboembolic events

Interestingly, permanent and persistent AF are associated with similar thromboembolic risk to that of paroxysmal AF.^{184,274} Thus, both rhythm and rate control strategies must be accompanied by strategies aiming to prevent thromboembolic events in high-risk patients.

A rapid and easy-to-remember scheme, the CHA₂DS₂VASc score, has been proposed by the 2010 ESC Guidelines for the Management of AF to facilitate the assessment of thromboembolic risk in AF patients.⁸⁵ This score assigns one point for each of the following risk factors: congestive heart failure, hypertension, age 65 to 74 years, diabetes, female sex, and vascular disease. Two points are assigned for a history of stroke or transient ischemic attack and age ≥ 75 years.

A meta-analysis performed by Hart et al. in AF patients, reported a 64% reduction in the relative risk of stroke with vitamin K antagonists compared to placebo,¹²⁵ while antiplatelet therapy showed much less impressive results. Compared to antiplatelet therapy, vitamin K

antagonists showed clear superiority in reducing thromboembolic risk, without significant difference in the risk of major haemorrhage.¹⁹⁸ The combination of anticoagulants and antiplatelet agents does not seem to bring any benefit compared to vitamin K antagonists alone.

Based on the results of these large clinical trials, chronic oral anticoagulation is recommended in high risk patients (CHA₂DS₂VASc score ≥ 2).⁸⁵ Patients with a CHA₂DS₂VASc score of 1 are candidates for oral anticoagulation or antiplatelet therapy, but anticoagulants are preferred in the absence of contraindications, while patients with a CHA₂DS₂VASc score of 0 could be candidates for antiplatelet therapy, but no antithrombotic therapy is considered a better option.⁸⁵

Currently, the International Normalized Ratio (INR), which is derived from the ratio between the actual prothrombin time and that of a standardized control serum, is the most frequently used parameter to assess the efficacy of anticoagulation. However, despite an INR within the target range of 2.0 to 3.0, some patients continue to experience thromboembolic events.^{59,267} Gatt et al. suggested that INR monitoring may actually not be the most appropriate approach to assess adequate anticoagulation, given that this parameter does not take into account the participation of important pro-thrombotic factors, such as von Willebrand factor (vWF).¹⁰⁶

New oral anticoagulants that may not need monitoring are on the horizon, namely oral direct thrombin inhibitor dabigatran etexilate and oral factor Xa inhibitors.⁵⁹

1.10.2. Rhythm control versus rate control in atrial fibrillation

1.10.2.1. Acute situations

In acute situations, therapeutic strategy is mostly driven by the severity of AF-related symptoms and hemodynamic stability. Since many episodes of AF terminate spontaneously within the first hours or days, adequate rate control of symptomatic patients with *beta*-blocking agents, calcium antagonists, or digoxin is recommended.⁸⁵

However, in the presence of hypotension with signs of hypoperfusion, severe heart failure or intractable ischemic chest pain, or if long-term rhythm control is considered, pharmacologic or electrical cardioversion should be considered as therapeutic options.⁸⁵ Several pharmacologic agents have proven their efficacy in acute rhythm control, including flecainide, propafenone, amiodarone, and ibutilide. Other agents, including *beta*-blockers, verapamil, sotalol, or digoxin seem to have no or negligible effect when administered for AF

termination. Highly symptomatic patients with infrequent recurrences of AF may benefit from the ‘pill-in-the-pocket’ strategy with flecainide or propafenone.

One new antiarrhythmic agent, vernakalant, appears to be efficient and safe for conversion of short-duration AF to sinus rhythm.²⁶ This drug is a relatively atrial-selective compound, blocking early-activating potassium currents and frequency-dependent sodium current, while not significantly affecting the length of the QT interval.²⁰ However, definitive recommendations cannot be made without additional data.

1.10.2.2. Long-term management

Large clinical trials found no significant difference between rate control and rhythm control strategies regarding all-cause or cardiovascular mortality, stroke rate, quality of life, or heart failure.¹⁵⁹ The decision of one long-term therapeutic strategy over another should be based on integrated data including patient-specific factors and the likelihood of success of the chosen strategy.

1.10.2.2.1. Rate control

The main goals of rate control strategy in AF patients are relief of symptoms and prevention of tachycardiomyopathy. The drugs most frequently used to control heart rate are *beta*-blockers, non-dihydropyridine calcium channel antagonists, and digoxin, or various combinations of these agents.⁸⁵ Both amiodarone and dronedarone have proved their efficacy as rate-control drugs in AF patients.^{68,76}

Data regarding the optimal heart rate in AF patients with respect to morbidity, mortality, quality of life, and symptoms are still lacking. Based on the results of the AFFIRM trial, a resting heart rate between 60 beats/min and 80 beats/min, and 90 beats/min and 115 beats/min during moderate exercise should be the target values in this population.²⁸⁵ However, RACE II did not find any benefit in patients with strict rate control (resting heart rate < 80 beats/min), compared to patients with lenient rate control (resting heart rate < 110 beats/min).²⁹⁴ Current recommendations suggest ventricular rate reduction to the point where the patient becomes asymptomatic or symptoms become tolerable.

When pharmacological approach does not seem to be efficient, complete heart block by atrioventricular node ablation can be considered as therapeutic option.⁴⁴ Since this approach is irreversible, it should only be considered in patients in whom conservative rate control and/or rhythm control strategies have failed.

1.10.2.2.2. Rhythm control

According to the 2010 ESC Guidelines for the Management of AF,⁸⁵ rhythm control is the best alternative whenever the patient remains symptomatic despite efficient rate control, in young symptomatic subjects, patients with AF-related heart failure, or AF secondary to a trigger that has been removed.

Drugs such as sodium-channel blockers (class I antiarrhythmic agents), potassium-channel blockers with *beta*-blocker effects (sotalol) and mixed ion channel blockers with *beta*-blocker effects (amiodarone) have all proved their efficacy in the prevention of AF recurrences.

One new antiarrhythmic agent, with properties belonging to all four antiarrhythmic classes, dronedarone, has achieved class I recommendations in the 2010 ESC Guidelines as first-line treatment in patients who do not present concomitant heart failure.⁸⁵ Unfortunately, its use has quickly been encumbered by serious adverse events (several cases of severe liver injury and hepatic failure, including two that required liver transplants).¹³⁷

The main concern associated with the use of antiarrhythmic drugs remains their pro-arrhythmic potential, despite the relatively low risk reported by a multivariate analysis of the AFFIRM data (7% cumulative incidence at 6 years).¹⁴⁹

In contrast to other arrhythmias, for which effective non-pharmacological therapies are currently available, the best therapeutic strategy in AF patients remains difficult to establish. Catheter-based ablation of the PVs has the advantage of addressing all three elements of arrhythmia triangle – isolates the trigger, modifies the substrate and affects local vagal innervations. Given the high efficacy and low-risk of severe complications when performed by experienced operators, radiofrequency ablation may be considered as first-line strategy in patients with paroxysmal AF and no/little structural heart disease.⁸⁵

Available studies show higher efficacy of ablation procedures over antiarrhythmic drugs. On one year follow-up, Wazni et al. showed 87% lack of AF recurrences after ablation, compared to 37% in the medical treatment group.³¹⁴ However, most of the studies that address this issue were performed on younger patients, with paroxysmal AF and no structural heart disease, with usually short-term follow-up, in specialized institutions, by highly trained physicians. These results might not be completely applicable in all centers.

New insights into the mechanisms of AF suggest that a number of non-antiarrhythmic drugs could be useful in reducing AF recurrences. Angiotensin-converting-enzyme inhibitors and ARBs seem to bring some benefits when used in addition to antiarrhythmic drugs, but

their use for preventing AF recurrences does not seem justified in the absence of other conditions demanding their use.⁷³ Experimental studies suggest that aldosterone antagonists could be good candidates for the prevention of AF recurrences.⁶⁶ Statins have shown a benefit for the primary prevention of AF, but they were less efficient for preventing AF recurrences after cardioversion or ablation.⁹⁰ Regarding polyunsaturated fatty acids, current data are scarce and contradictory.¹⁵¹

1.11. Information learned from animal models of atrial fibrillation

Studies in animal models have provided extremely important insights into AF mechanisms. In fact, the classic mechanisms that still form the basis for our understanding of the pathophysiology of AF were established based on animal studies. The past years have witnessed a tremendous acceleration of animal work in this area, including the development of a wide range of AF models in clinically relevant pathological substrates and of an increasing number of transgenic mouse models.

1.11.1. Autonomic stimulation

Some of the earliest models of AF manipulated autonomic tone in the atria of dogs. The simplest model involved applying a drop of carbamylcholine to the atrial appendage, which caused sustained AF lasting for the duration of carbamylcholine exposure.⁹⁴ Clamping the appendage separated the fibrillatory source from the substrate, causing cardioversion of the main atrial body, while allowing the affected appendage to continue fibrillating. This observation supported the idea that fibrillation comes from a rapidly firing point source with fibrillatory conduction away from the stimulus.

Until the mid-1990s, virtually all experimental studies of AF were performed in animals with normal hearts, with autonomic manipulation often used when sustained AF was needed.

Although AF can obviously occur in patients without evident heart disease, an organic heart disease is involved in about half of the cases of paroxysmal AF and in more than 80% of persistent or permanent AF.⁵ The development of numerous animal models of AF with clinically relevant diseases has been a major advance of these later years.

1.11.2. Atrial burst pacing

The first reported method for achieving sustained AF in an animal model was atrial tachypacing.³¹⁹ Using this protocol, the episodes of non-sustained AF progressively lengthened over time until sustained AF was achieved. The concept that ‘AF begets AF’ was

first substantiated in this model.⁷⁴ Within 24h of burst pacing, atrial effective refractory periods decreased by 35% in a spatially heterogeneous fashion, without significant change in conduction velocity.⁷⁴ This electrical response favors the persistence of the arrhythmia by shortening the wavelength necessary to sustain re-entry. In addition to this electrical remodeling, structural remodeling was also noted. After 9 to 23 wks of sustained AF, Ausma et al. demonstrated structural changes in atrial myocytes similar to those observed in ventricular myocytes from chronic hibernating myocardium.¹⁵

Atrial tachycardia-induced remodeling has also been demonstrated in dogs,^{327,124} sheep,⁷ and pigs.^{47,78} This process is believed to contribute to a variety of clinically important phenomena, including the tendency of paroxysmal AF to become persistent, the tendency of AF to recur soon after electrical cardioversion, and the tendency for longer-lasting AF to become refractory to pharmacologic cardioversion.²²⁰

1.11.3. Heart failure models

Congestive heart failure, one of the most common clinical causes of AF, has been investigated in experimental dog and sheep models. In these models, heart failure was generally induced by right ventricular pacing for several weeks, generating tachycardiomyopathy.¹⁷⁸ While electrical remodeling appears to be the main determinant for AF promotion in the atrial tachypacing model, structural remodeling seems to play the major role in heart failure models of AF. Atrial effective refractory period was unchanged or increased by congestive heart failure, but localized regions of conduction slowing occurred in association with marked atrial fibrosis.¹⁷⁸ Cessation of ventricular tachypacing allowed ventricular function and atrial dilation to reverse completely, along with the disappearance of hemodynamic and clinical signs of congestive heart failure, but fibrosis remained and the ability to induce prolonged AF was maintained.²⁶⁹

1.11.4. Sterile pericarditis

Atrial fibrillation frequently occurs in post-thoracotomy patients and inflammatory pericarditis is felt to be a significant contributor. The dog model of sterile pericarditis was developed in Dr. Waldo's laboratory. The authors created pericarditis by irritating the pericardium with talcum, after sterile mediastinotomy and pericardiotomy. The predominant arrhythmia in the model was atrial flutter, but AF was also induced.²³⁶ Kumagai et al. suggested that unstable and migratory re-entrant circuits of very short cycle length involving

the inter-atrial septum were responsible for arrhythmia maintenance.¹⁶⁴ Heterogeneous reductions in conduction velocity have also been described in this model.

1.11.5. Mitral regurgitation

Mitral regurgitation is a common cause of clinical AF. Cox et al. found that chronic mitral regurgitation in dogs permits the induction of sustained AF, apparently by the means of macro-re-entry.⁸⁸ Altered gap-junction-connexin distribution and inflammatory response contributed to abnormal atrial conduction and AF vulnerability, and agents with anti-inflammatory effects such as atorvastatin or prednisone prevented AF in this model.¹⁶⁵

An AF model using combined mitral regurgitation and atrial tachypacing showed typical atrial tachycardia remodeling, including effective refractory period abbreviation and decreased AF cycle length, as well as marked ultrastructural remodeling.⁸⁸

1.11.6. Aging

Anyukhovskiy et al. assessed various parameters in older dogs in comparison to younger adult dogs. The authors found significant morphological differences of the action potential, including a decrease in peak and plateau action potential voltage, a decrease in the rate of cellular depolarization, a slight decrease in resting membrane potential, and an increased dispersion of APD across the tissue.⁹ The authors also reported significant fibrosis in older animals. From these data, the authors speculated that fibrosis, slowed conduction of premature beats, and increased heterogeneity of repolarization could be important determinants of both initiation and maintenance of AF in the elderly.⁹

1.11.7. Hypertension

Sheep with pre-natal corticosteroid-induced high blood pressure exhibited enlarged LA and increased AF durations.¹⁵⁵ Widespread conduction abnormalities and atrial wavelength shortening were observed; however, refractoriness did not change significantly. Atrial tissue presented significant structural remodeling, including central myofibrillarolysis, myocyte hypertrophy, mitochondrial and nuclear enlargement and fibrosis, as well as evidence of apoptosis.¹⁵⁵

1.11.8. Small animal models of atrial fibrillation

Re-entrant arrhythmias have generally been considered to be restricted to large animals, because the generation and maintenance of re-entrant arrhythmias require a minimum tissue mass.¹⁰⁴ Consequently, to date, re-entrant arrhythmias are often considered impossible in

small rodents, given that the myocardial mass of rats and mice is too small to sustain multiple re-entrant wavelets. However, experimental models of atrial arrhythmias in small animals started to emerge.

1.11.8.1. Rat models

1.11.8.1.1. Spontaneously hypertensive rats

Increased susceptibility to atrial arrhythmias was demonstrated in excised perfused hearts from aging spontaneously hypertensive rats (SHRs), compared to hearts from both young SHRs and normotensive controls.⁵⁶ Aging SHRs seemed to develop a substrate for AF that included increased LA interstitial fibrosis, in the absence of significant changes in effective refractory period.^{56,201} Hypertension-induced activation of the renin–angiotensin system could explain these findings, given that ARBs reduced fibrosis in this model.²⁰¹

1.11.8.1.2. Glycolytic inhibition

Ono et al. reported spontaneous episodes of AF, related to triggered activity arising in the vicinity of the LA-PV junction, in old, but not in young rats, after using a modified Tyrode solution to perfuse isolated hearts.²³³ Sarcoplasmic reticulum uptake of intracellular Ca^{2+} is adenosine triphosphate (ATP)-dependent, and addition of sodium pyruvate to glucose-free perfusate selectively inhibited glycolysis in those hearts.²³³ In this model, AF was probably mediated by Ca^{2+} -handling abnormalities. In old rats, early after depolarizations originating in the vicinity of the LA-PV junction were associated with prolonged APD and elevated diastolic Ca^{2+} .²³³

1.11.8.2. Transgenic mice

Transgenic knockout mice models are fast becoming an effective tool in the exploration of mechanisms responsible for various disease states. In the context of atrial arrhythmias, reports are scarce in mouse models, though not surprising since setting up a model in an animal with such a small mass of atrial tissue could be somewhat prohibitive.

In a report by Hagendorff et al., a series of mouse mutants that possessed a deficiency in the hexameric assembly of connexins was used to assess conduction abnormalities.¹²¹ The authors subjected $\text{Cx}40$ heterozygous and homozygous knockout mice to rapid atrial transesophageal stimulation. In mice that were either $\text{Cx}40$ intact or heterozygous there was no evidence of inducible arrhythmias. In contrast, in $\text{Cx}40$ deficient mice burst pacing generated atrial arrhythmias in 63% of animals.¹²¹

The hypothesis that inflammation and fibrosis affect AF vulnerability was highlighted in transgenic mice overexpressing tumor necrosis factor-*alpha*,²⁵⁵ TGF- β 1,³⁰⁰ Rac1 guanine triphosphate (GTP)-ase² and angiotensin converting enzyme.³²⁴ Each of these proteins favors inflammation and/or fibrosis of the atria, and each animal presented increased propensity to AF.

1.11.9. Limitations of currently available atrial tachyarrhythmia models

Numerous experimental models have been developed in which AF pathophysiology can be studied. However, many important questions remain unanswered. To date, there is no such thing as a ‘perfect’ animal model of AF.

With the wide array of pathophysiological findings in varying animal models, it is difficult to see any commonalities between these models and human AF. Human AF is likely an endpoint of numerous disease states, structural alterations and inherited defects.

The most important question is if observations made upon models of artificially induced atrial arrhythmias can be extrapolated in humans, particularly when it comes to therapeutic strategies. From this point a view, one more animal model of atrial arrhythmia is lacking – a model of spontaneous atrial arrhythmias, in which no interventional means are used to favor the occurrence of arrhythmias. It is true that a number of previous studies have reported ‘spontaneous AT or AF’ in animals. However, in those models, arrhythmia occurred only after programmed stimulation protocols or pharmacologic interventions were applied, or experiments were performed on isolated hearts. A number of essential features for AT occurrence, such as structural, electrical, or autonomic remodeling could have been artificially induced in those models. Therefore, ‘spontaneous’ might not be the proper term for describing the type of arrhythmias reported in those papers. The term ‘spontaneous’ should probably be reserved for situations that have not used interventional means to trigger the arrhythmia. Results obtained on such a model should be easier to extrapolate in humans.

CHAPTER 2

Aims and Hypotheses

Growth in the size of the AF population and increased recognition of the morbidity, mortality, and diminished quality of life associated with AF, have spurred numerous investigations to develop more effective treatments for AF and its complications. So far none has provided the expected results. Currently available pharmacological strategies are often inefficient, with enough unpleasant side effects explaining the frequent lack of adherence to treatment. This is highly due to the fact that the precise mechanisms underlying atrial arrhythmias are poorly understood. The complexity of the pathophysiological mechanisms of AF emphasizes the need for a multidisciplinary approach.

It is generally accepted that there is no unifying mechanism of AF, and that a mechanism may predominate over another in certain patients, generally with the participation of several mechanisms in the same patient.

The aim of this thesis was to assess various pathophysiological mechanisms involved in AF and AF-related complications by using a translational, multidisciplinary approach.

In order to perform a comprehensive approach of atrial arrhythmias we performed both experimental and clinical studies.

2.1. Hypotheses

2.1.1. *Experimental study in rats*

To date, we do not dispose of any animal model of spontaneous atrial arrhythmias. Since aging and high blood pressure are the two risk factors most frequently associated with atrial arrhythmias, we hypothesized that aging hypertensive rats could provide a suitable model of spontaneous, unprovoked atrial arrhythmias.

Given that no interventional means are used to favor the occurrence of arrhythmias, this model would offer the possibility to study pathophysiological mechanisms of atrial arrhythmias in a setting that faithfully reproduces the human clinical condition. Thus, results obtained in this model should be easier to extrapolate in humans. Furthermore, in human studies, obtaining cardiac tissue samples is restricted to subjects undergoing cardiac surgery or necropsy, and thus to subjects with severely impaired cardiac function and severely remodeled atria. In contrast, a model of spontaneous atrial arrhythmias in rats has the advantage that it can provide large amounts of tissue samples and allows us to perform various studies in a setting similar to that observed in the majority of AF patients.

Autonomic imbalance, LA structural abnormalities, and various molecular changes have been recently incriminated in AF occurrence and AF-related complications such as stroke, but

a number of questions remain to be answered. To date, it is unclear whether there is a direct relationship between autonomic imbalance and atrial arrhythmias and what is the direction of this imbalance, whether the complexity of LA structural remodeling is similar in the general AF population to that reported in previous autopsy studies and what are the main components of this structural remodeling, or whether *Pitx2* is only down-regulated in the LA of AF patients or if this molecular abnormality precedes and favors the occurrence of these arrhythmias. Similarly, expression changes in genes encoding for ion currents, proteins of the extracellular matrix, or cell-cell coupling, offered conflicting results and additional molecular markers of AF remain to be identified. Probably the most important question is if these abnormalities only accompany atrial arrhythmias or whether they precede and favor the occurrence of these arrhythmias. Answering these questions is expected to identify new therapeutic targets for the treatment and/or the prevention of atrial arrhythmias. Accordingly, we aimed to identify the presence, the components and the severity of autonomic, structural and molecular abnormalities in our animal model of spontaneous atrial arrhythmias by performing a multifaceted study.

Both benign and malignant ventricular arrhythmias have been reported in aging hypertensive subjects, and LVH has been identified as a strong risk factor for sudden death. However, the question of sudden death in hypertensive patients is still a matter of debate, and malignant forms of ventricular arrhythmia are still quite rare in this setting. In fact, several studies reported more frequent premature ventricular contractions (PVCs) in untreated hypertensive patients with normal left ventricular mass than in the presence of LVH. These results suggested that hypertensive patients with LVH might actually experience fewer arrhythmias. Thus, the evidence linking LVH, ventricular arrhythmias and sudden death is, at best, circumstantial, and it remains to be determined whether these arrhythmias are of crucial significance. Accordingly, we aimed to assess the frequency and the complexity of ventricular ectopy in hypertensive rats with different stages of LVH and to provide molecular insights into the LV remodeling of hypertrophied hearts.

2.1.2. *Clinical studies in atrial fibrillation patients and control subjects*

The participation of inflammation in atrial arrhythmia occurrence has been reported in several clinical and experimental studies. However, the origin and the extent of the inflammatory syndrome in AF patients remain incompletely understood. Moreover, the existence of such a syndrome in the entire population of AF patients is a matter of debate,

given that several studies failed to demonstrate elevated levels of inflammatory markers in AF patients. Thus, we hypothesized that multilevel intracardiac and extracardiac measurements of several inflammatory proteins will allow us to assess the severity of an inflammatory syndrome in AF patients, and to establish the source of high inflammatory markers levels in AF patients.

Recent data support the participation of endothelial dysfunction in the pathophysiology of thromboembolism in AF patients. High levels of different pro-thrombotic markers of endothelial origin, including von Willebrand factor (vWF), have already been documented in AF patients. However, the source of vWF in this population remains unknown. Experimental studies suggested that the secretion of vWF could originate either in endocardial atrial cells, or in a generalized endothelial dysfunction, but, to date, the precise origin of vWF remains to be identified. We hypothesized that multiple intracardiac and extracardiac dosages of vWF, at different levels of the circulatory tree, would help to identify the origin of vWF in atrial AF patients.

2.2. Aims

The aims of the experimental study were:

1. To develop the first animal model of spontaneous, unprovoked atrial arrhythmias in rats;
2. To assess the presence and the pattern of an autonomic imbalance in this new animal model of atrial arrhythmias;
3. To assess the presence, the components and the severity of LA histological abnormalities in this new animal model of atrial arrhythmias;
4. To identify the molecular abnormalities associated with this new animal model of atrial arrhythmias; and
5. To assess the frequency and the complexity of ventricular ectopy in hypertensive rats and to provide molecular insights into the LV remodeling of hypertrophied hearts.

The aims of the clinical study were:

1. To assess the extent and the source of inflammation in AF patients; and
2. To confirm the presence of high vWF levels in patients with different clinical forms of AF and to identify the source of high vWF levels in these populations.

2.3. Outline of the studies contained within this thesis

Seven principle studies were performed and are detailed in Chapters 3 - 9:

- Chapter 3: A new model of spontaneous atrial arrhythmias in rats
- Chapter 4: A study of autonomic balance in arrhythmic rats
- Chapter 5: A study of left atrial histology in arrhythmic rats
- Chapter 6: A study of molecular mechanisms of atrial arrhythmias in arrhythmic rats
- Chapter 7: A study of ventricular arrhythmogenicity and molecular left ventricular remodeling in hypertensive rats
- Chapter 8: A study of inflammatory syndrome in atrial fibrillation patients
- Chapter 9: A study of endothelial dysfunction in atrial fibrillation patients

CHAPTER 3

A New Model of Spontaneous Atrial Arrhythmias in Rats

3.1. Introduction

Currently available antiarrhythmic strategies for the treatment and prevention of atrial arrhythmia's recurrences are often inefficient.²² This is highly due to incomplete understanding of the pathophysiological mechanisms of these arrhythmias.

Numerous experimental models have been developed in which atrial arrhythmias' pathophysiology may be studied.^{127,244} Yet, none of these models manage to faithfully reproduce the human clinical condition. Experimental models of spontaneous ATs are still lacking. Indeed, previous studies have reported 'spontaneous atrial tachycardia or AF' in animals.^{55,233} However, in those models, arrhythmia occurred only after programmed stimulation protocols or pharmacologic interventions were applied, or experiments were performed on isolated hearts. A number of essential features for AT occurrence, such as structural, electrical, or autonomic remodeling could have been artificially induced in these models. Thus, 'spontaneous' might not be the most appropriate term for describing the type of arrhythmias reported in those papers; we believe that the term 'spontaneous' should be reserved for situations that have not used interventional means to trigger the arrhythmia. The main question is if observations made upon such models can be extrapolated in humans and if similar mechanisms participate in arrhythmia occurrence in both human and experimental settings, given the lack of obvious commonalities between those models and human atrial arrhythmias. In contrast, results obtained on an animal model of spontaneous arrhythmias should be easier to extrapolate in humans.

The purpose of the present study was to develop a model that could reproducibly exhibit spontaneous ATs in rats and to describe its phenotypic features. Frequent PACs and several other markers of increased atrial electrical instability (i.e., atrial couplets and bigeminisms) are common findings in patients who present atrial arrhythmias.³⁰⁶ Furthermore, frequent PACs in 24-h ECG monitoring can be used as marker to identify patients who are at increased risk to present or to develop paroxysmal AF.³⁰⁸ In some patients, it is likely that these ectopic beats represent the trigger for 'focal AF', while in others they probably represent the initiator of multiple wavelet re-entry AF.

Aging⁵ and arterial hypertension^{5,58} are the two risk factors most frequently associated with high atrial arrhythmogenicity. Therefore, we hypothesized that aging rats of the SHR strain might provide a suitable model of spontaneous atrial arrhythmia.

Accordingly, we aimed to assess, in baseline conditions, the occurrence of spontaneous ATs and several other markers of increased atrial arrhythmogenicity (i.e., PACs, couplets and

bigeminisms) in young and aging SHR. These parameters were compared to those observed in young and aging normotensive controls. For this study, we used 24-h continuous ECG recordings, acquired with radiotelemetry.

3.2. Materials and Methods

3.2.1. Animals

Male SHR and normotensive Wistar-Kyoto (WKY) rats were purchased from Elevage Janvier (Le Genest Saint Isle, France).

All animals were housed in a climate-controlled room (21 to 22°C), with a 12-h light/dark cycle (on 7 AM/off 7 PM), in an accredited animal facility. The rats were housed individually in polycarbonate cages and fed standard rat pellets and tap water *ad libitum*.

All experiments were performed in compliance with the French Ministry of Agriculture guidelines for animal experimentation and were approved by the local Animal Ethics Committee.

3.2.2. Transmitter implantation procedure

Radiotelemetry ECG transmitters (TA11 CA-F40; Data Sciences International, St. Paul, MN) were implanted under isoflurane anesthesia. Each animal received subcutaneous prophylactic injections of penicillin G (50,000 IU) and ketoprofen (2 mg/kg).

The body of each transmitter was placed in a dorsal, subcutaneous pocket, and sutured to the underlying tissue under aseptic conditions. The two ECG leads were tunneled subcutaneously and secured in a lead II configuration (Fig. 3.1).

Figure 3.1. Device and lead placement



After the implantation procedure, animals were allowed at least one week of recovery prior to recording.

3.2.3. ECG recording

For baseline quantification of atrial arrhythmic events, 24-h continuous ECG monitoring was performed on unrestrained, conscious rats. Recordings were conducted on eight 55-wk-old SHR (471 \pm 5 g), 4 age-matched WKY rats (576 \pm 11 g), 8 young (14-wk-old) SHR (342 \pm 3 g), and 4 young (14-wk-old) WKY rats (397 \pm 5 g). ECG signal capture was accomplished with receivers (RPC-1; Data Sciences International) placed under each experimental cage. Telemetry ECGs were converted to analog signal (Analog ECG Output Adapter R08; Data Sciences International) and routed to a personal computer equipped with a signal acquisition card (NI PCIe-6251; National Instruments, Austin, TX). An acquisition program developed in our laboratory using LabVIEW 2009 software (National Instruments) allowed the signal to be continuously recorded with a 2000 Hz sampling frequency and stored on hard disk.

3.2.4. ECG analysis

ECG data were analyzed using a program recently developed in our laboratory using LabVIEW 2010 software (National Instruments) to automatically detect R waves and measure RR intervals, as previously described.²³⁹ All ECG tracings were visually assessed by two independent cardiologists. In the vast majority of time ECG quality allowed easy recognition of all atrial arrhythmic events. Infrequently, intense animal somatomotor activity was associated with altered ECG signal and artifacts; this impaired proper assessment of the atrial electrical activity. Therefore, all artifactual periods were discarded prior to analysis.

Atrial tachyarrhythmia was defined as rapid, irregular, supraventricular rhythm (irregular ventricular response with narrow QRS complexes, exceeding by at least 50 beats/min the heart rate measured during sinus rhythm), of at least 3 beats. The diagnosis also required that P waves were absent or replaced by atrial waves with morphology significantly different compared to sinus rhythm P waves. Premature atrial contractions were defined as early atrial activations, with morphology different compared to sinus rhythm P waves. Atrial couplets were defined as a pair of atrial premature activations in a row, whereas the regular succession of a normal sinus beat and a premature atrial activation was defined as atrial bigeminism.

The total numbers of AT episodes, PACs, atrial couplets, and bigeminisms were quantified on the 24-h recordings. The duration of an AT episode was measured from the first arrhythmic beat of the episode (top of the R wave) to the first sinus rhythm beat that followed the episode.

To test for day-to-day reproducibility, 4 aging SHR_s underwent 48-h continuous recording, instead of the standard 24-h recording. Data used for between-group comparisons corresponded to the first 24-h period.

To test for medium-term reproducibility, comparisons were also performed, for all 8 aging SHR_s, between the 24-h recording used in between-group comparisons and a second recording, performed one week earlier.

3.2.5. Invasive blood pressure measurement

To evaluate the degree of hypertension, intra-arterial blood pressure was measured in conscious, unrestrained rats. The catheterization procedure was performed under isoflurane anesthesia in 5 SHR_s at the age of 64 ± 2 wk and in all 4 WKY rats at the age of 68 wk. None of the young SHR_s or WKY rats underwent this procedure. However, blood pressure values of young SHR_s and WKY rats were expected to conform to the extensive reports in the literature; in contrast, to the best of our knowledge, no reports had previously measured blood pressure in aging rats.

Skin was opened over the femoral vessels. The femoral artery was carefully isolated and a sterile polyethylene PE-10 fused PE-50 catheter was inserted into the vessel and advanced beyond the iliac bifurcation until the pressure-sensing tip has reached the abdominal aorta. A second skin incision was performed at the back of the neck and the catheter was tunneled subcutaneously from the femoral region to the dorsal neck and exteriorized for approximately 3 cm.

Animals were allowed at least 24h of recovery before blood pressure measurement. Arterial pressure was measured by connecting the arterial catheter via PE-50 tubing to a pressure transducer (TNF-R; Becton Dickinson, Sandy, UT) coupled to the amplifier (13-4615-52; Gould, Cleveland, OH) and chart recorder (8802; Gould). Analog outputs of pulsatile arterial pressure were converted into digital signal with an analog-to-digital converter (AT-MIO-16; National Instruments) sampled at 500 Hz, continuously recorded on a personal computer using LabVIEW 5.1 software (National Instruments) and stored on hard

disk. Off-line processing was performed with a program developed in our laboratory under LabVIEW 2009 software (National Instruments). All artifacts were manually removed.

For each cardiac cycle, mean arterial pressure and pulse pressure were automatically calculated. Systolic, diastolic and mean blood pressure values, as well as pulse pressure values were obtained over 1-h interval recordings, once blood pressure was stable.

3.2.6. Statistics

Data are expressed as mean \pm standard error of the mean (SEM) or median and range, as appropriate. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test using Holm's method for multiple comparisons. Medium-term reproducibility in the number of AT episodes was analyzed using Spearman correlation. To assess day-to-day reproducibility, the mean percent variation of the number of AT episodes was calculated.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software (GraphPad Software; San Diego, CA).

3.3. Results

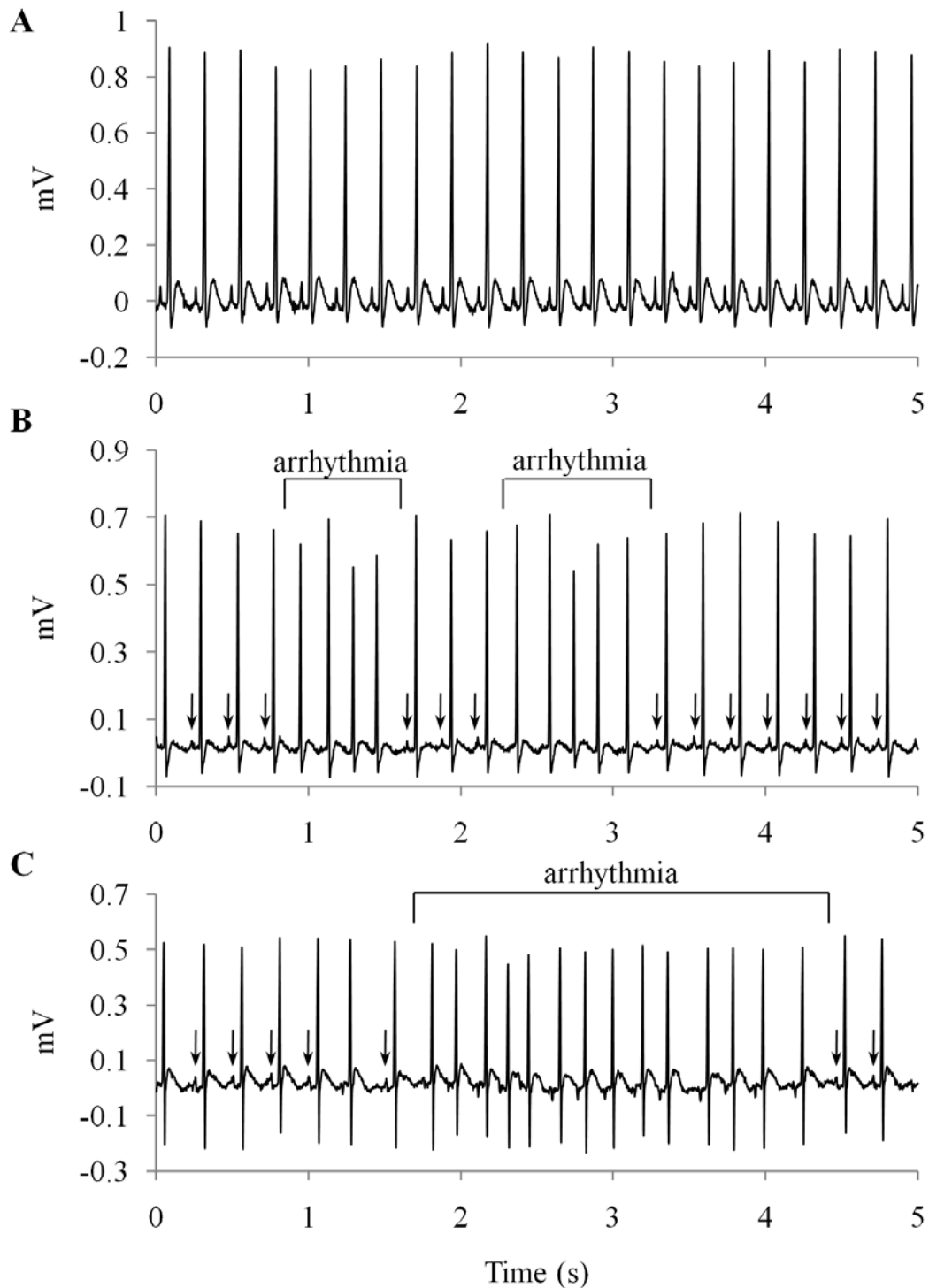
3.3.1. Atrial arrhythmic activity at baseline

Aging SHR^s presented spontaneous, unprovoked episodes of AT (Fig. 3.2).

The number of tachyarrhythmic episodes in aging SHR^s was significantly greater than in young SHR^s ($P < 0.001$), while no episodes were recorded in normotensive rats, regardless of their age (Fig. 3.3, A). Two of the 8 young SHR^s presented a single episode of AT, while all 8 aging SHR^s had tachyarrhythmic episodes (median 91.5, range 4 – 444 episodes / 24h). Similar differences were observed in the number of PACs (Fig. 3.3, B), atrial couplets (Fig. 3.3, C), and bigeminisms (Fig. 3.3, D).

In aging SHR^s, over 24h, the median total duration of episodes was 60 s / 24h (range 2 – 372 s). The median duration of tachyarrhythmic episodes was 0.71 s (range 0.53 – 1.63 s). In this group, episode duration ranged from 0.43 s to 10.90 s; in the 2 young SHR^s that presented ATs, the episode durations were 0.4 s and 0.52 s, respectively.

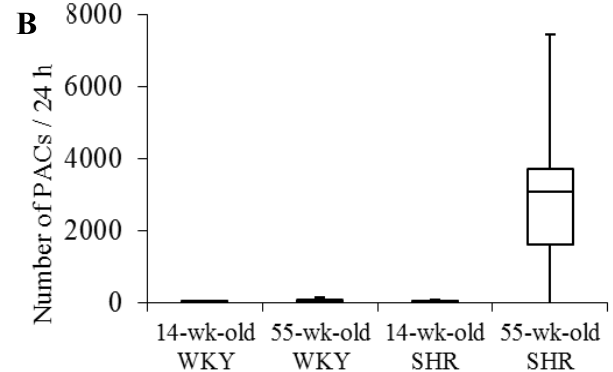
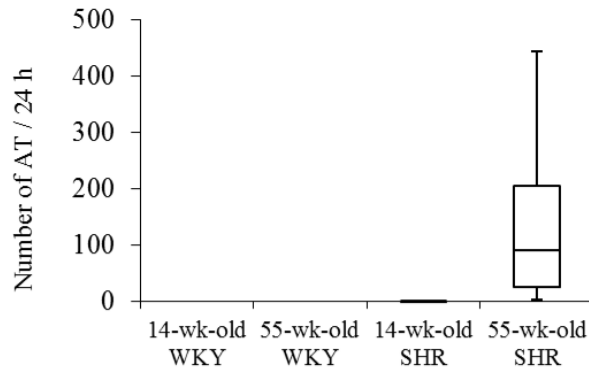
Figure 3.2. ECG tracings in 55-wk-old SHR showing - (A) sinus rhythm; (B) two arrhythmic episodes suggesting a diagnosis of AF (rapid, irregular rhythm, with persistence of narrow QRS complexes, in the absence of visible P waves) and (C) arrhythmic episode suggesting a diagnosis of multifocal AT (rapid, irregular rhythm, with persistence of narrow QRS complexes and wide, inverted atrial waves, with continually changing morphology)



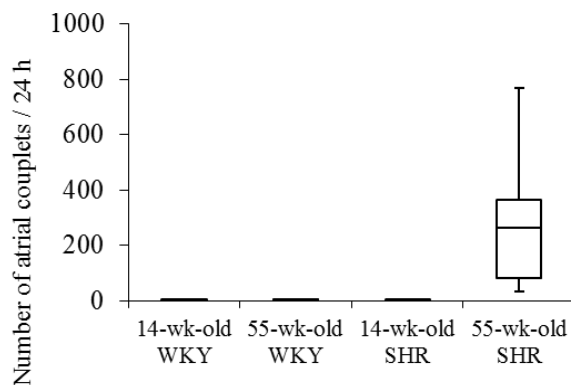
Arrows on panels (B) and (C) indicate sinus rhythm beats.

Figure 3.3. Number of supraventricular arrhythmic events - (A) episodes of AT; (B) PACs; (C) atrial couplets; and (D) atrial bigeminisms, during 24-h ECG recordings in 14-wk-old (n = 4) and 55-wk-old (n = 4) WKY rats, and in 14-wk-old (n = 8) and 55-wk-old (n = 8) SHRs

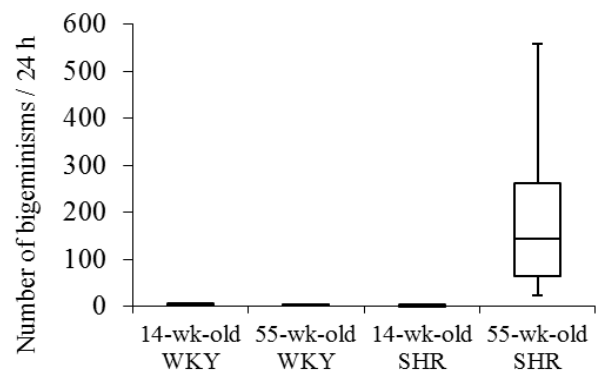
A



C



D

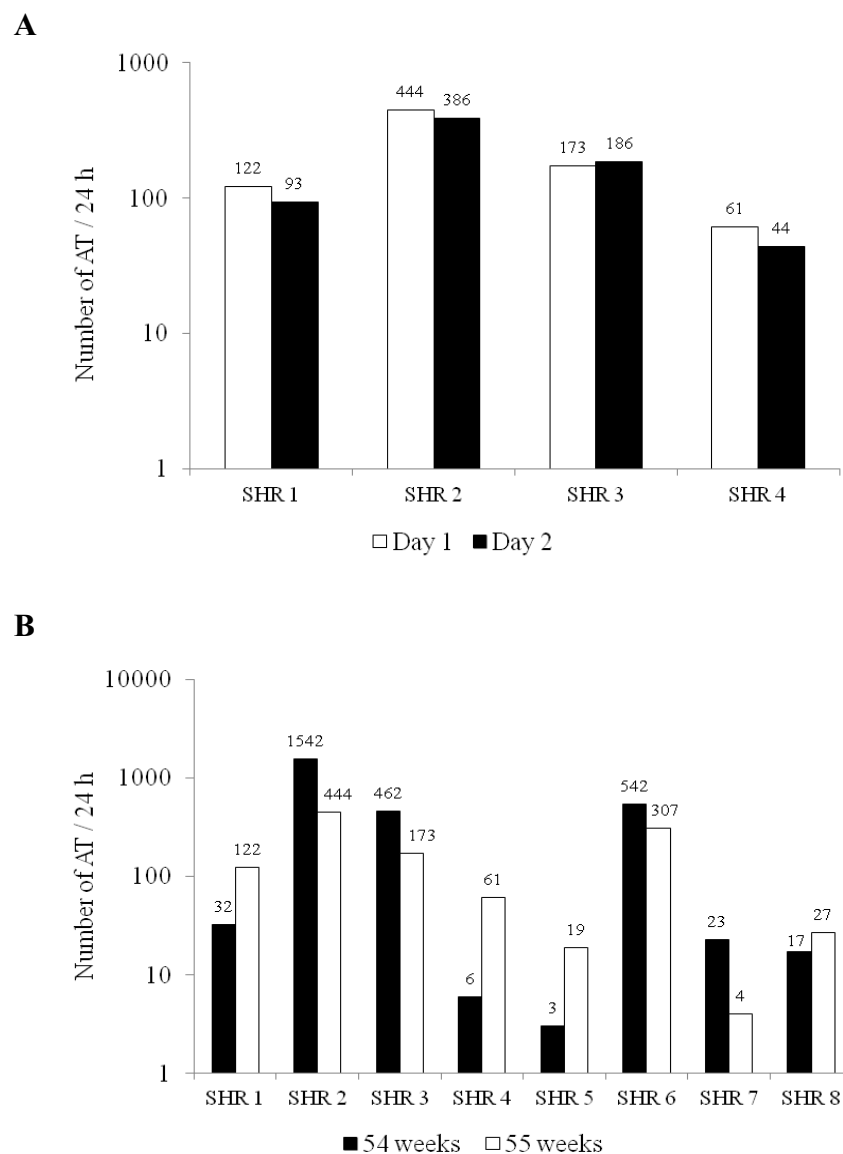


AT – atrial tachyarrhythmia, PACs – premature atrial contractions. Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile.

In aging SHRs, the RR intervals during AT episodes were 69 ± 1 % shorter than the RR intervals during sinus rhythm ($P < 0.001$). The ECG aspect was consistent with the diagnosis of AF (rapid, irregular, supraventricular rhythm, in the absence of visible P waves) in 30.6% of cases. In the remaining cases, the ECG morphology suggested a diagnosis of atrial tachycardia (rapid, irregular, supraventricular rhythm, with atrial waves with morphology significantly different compared to sinus rhythm P waves).

Aging SHRs showed high day-to-day (mean percent variation of the number of AT episodes $18.1\% \pm 4.7\%$) and medium-term (Spearman $r = 0.83$, $P = 0.01$) reproducibility of the number of arrhythmic episodes (Fig. 3.4, A and B).

Figure 3.4. Day-to-day (A) and medium-term (B) reproducibility of arrhythmias in individual aging SHRs - (A) Comparison between the total number of arrhythmic episodes during the first (*day 1*) and second (*day 2*) 24-h period in 4 individual 55-wk-old SHRs that underwent 48-h continuous ECG monitoring; (B) Comparison between the total number of arrhythmic episodes in 8 individual SHRs at 54 wks of age and 55 wks of age, respectively



AT – atrial tachyarrhythmia. Results are shown in log scale.

3.3.2. Blood pressure measurement

Systolic, diastolic and mean blood pressure, and pulse pressure values in aging SHR and WKY rats are presented in Table 3.1. As expected, SHRs had significantly higher systolic, diastolic and mean blood pressure than WKY rats (all $P = 0.02$). Similarly, pulse pressure was significantly higher in SHRs than in normotensive controls ($P = 0.03$).

Table 3.1. Blood pressure values in aging WKY rats and aging SHRs

Variables	WKY rats (n = 4)	SHRs (n = 5)	<i>P</i> -value
Systolic blood pressure (mmHg)	135 ± 2.0	236 ± 7.0	0.02
Diastolic blood pressure (mmHg)	90 ± 0.4	171 ± 8.0	0.02
Mean blood pressure (mmHg)	107 ± 0.5	199 ± 8.0	0.02
Pulse pressure (mmHg)	46 ± 2.0	65 ± 5.0	0.03

Data are expressed as the mean ± SEM. *P*-values refer to comparisons between groups based on the Mann-Whitney *U* test.

3.4. Discussion

The main findings of the present study were (i) all aging SHRs presented spontaneous, unsustained ATs, while young SHRs and both young and aging WKY rats did not; and (ii) in aging SHRs, the arrhythmic burden showed high day-to-day and medium-term reproducibility.

3.4.1. Limitations of currently available atrial tachyarrhythmia models

In humans, atrial arrhythmias are likely the endpoint of numerous disease states, structural alterations and inherited defects.^{75,92,126} The large diversity of clinical forms of atrial arrhythmias explains the difficulties in obtaining an experimental model that faithfully reproduces the clinical presentation of these arrhythmias.

As previously mentioned, the majority of the currently available experimental models of atrial arrhythmias use programmed stimulation to achieve the arrhythmic state.^{263,305} However, it is likely that aggressive stimulation will eventually induce atrial arrhythmias, even in normal hearts,¹⁴⁷ and atrial arrhythmias triggered by spontaneous initiators could be quite different from pacing-induced arrhythmias. Models using anesthetized animals can be imputed with the fact that anesthetic agents might influence the duration and the inducibility of the arrhythmia in *in vivo* models,²³¹ while experiments performed on isolated hearts avoid the participation of extrinsic mechanisms of cardiac regulation in response to artificially

imposed changes. Therefore, the question remains if results provided by such models can be extrapolated to spontaneous arrhythmias observed in humans.

Both aging and hypertensive animals have been shown to present increased susceptibility to ATs.^{56,155} However, to the best of our knowledge, this is the first study to report the occurrence of unprovoked episodes of atrial arrhythmia in hypertensive rats, in the absence of any interventional means that could have favored the onset of arrhythmia, and the increased incidence of these arrhythmic episodes with aging.

3.4.2. Spontaneous, unsustained atrial tachyarrhythmias in aging spontaneously hypertensive rats

Twenty-four hour ECG monitoring clearly documented the presence of unsustained, spontaneous ATs, in all aging SHR. In addition, the high number of isolated, coupled and bigeminal PACs in this group further confirmed the presence of a significant atrial electrical instability in these rats. These results are in accordance with clinical observations and sustain the close resemblance between our model and the human presentation of the arrhythmia. The spontaneous occurrence of ATs in aging, hypertensive rats, corresponded to clinical observations that linked atrial arrhythmias with aging and high blood pressure.^{5,145,146,163} Frequent PACs have also been reported in hypertensive patients and in elder subjects.²⁸¹

The inter-individual variability in the number of AT episodes in the 24-h recordings further supported the close resemblance between this model and the human presentation of atrial arrhythmias.¹³⁴

Even though small animals are usually preferred when it comes to experimental models of disease states, reports of small animal models of atrial arrhythmia are scarce. Re-entrant arrhythmias have generally been considered to be restricted to large animals. This is far from being surprising, considering that generation and maintenance of re-entrant arrhythmias require a minimum tissue mass ($> 100 - 200 \text{ mm}^3$).¹⁰⁴ Consequently, to date, re-entrant arrhythmias are often considered impossible in small rodents, since the myocardial mass of rats and mice is $< 35 \text{ mm}^3$, too small to sustain multiple re-entrant wavelets.²⁰⁸

The present study provides evidence that atrial arrhythmias are possible in rat hearts and that spontaneous arrhythmic episodes can develop in rats despite their reduced cardiac mass. However, the short duration of AT episodes was a characteristic of our model. This was due to these characteristic anatomical features of small animals. Accordingly, in SHR, most episodes of AT spontaneously reversed to sinus rhythm, usually after a small number of

arrhythmic beats; this confirmed that the role of a critical myocardial mass cannot be underestimated.

3.4.3. *Potential limitations*

Based solely on the results from a surface ECG recording, a junctional origin of arrhythmias cannot be excluded. However, rapid ectopic activity arising from the atrioventricular node or His bundle has only been reported in special circumstances, such as digitalis toxicity, recent cardiac surgery, acute myocardial infarction, or isoproterenol infusion,^{17,235} but not in patients with hypertension alone, and, to the best of our knowledge, has never been reported in rats. Additionally, the presence of relatively long PQ intervals during arrhythmic episodes that presented atrial waves, and the continual changes in the morphology of atrial waves during those episodes, strongly supported the diagnosis of multifocal AT and not of a tachycardia originating from a single subsidiary pacemaker.

3.4.4. *Conclusions*

This study reports the occurrence of unprovoked episodes of atrial arrhythmia in hypertensive rats, and their increased incidence with aging. This work represents a potentially useful, new experimental model of atrial arrhythmias. This model could facilitate studies of pathophysiological mechanisms involved in atrial arrhythmias such as AF, and allow assessment of efficacy and toxicity of various therapeutic agents in a setting that faithfully reproduces the clinical presentation of the arrhythmia.

CHAPTER 4

A Study of Autonomic Balance in Arrhythmic Rats

4.1. Introduction

Recent data suggest that abnormal autonomic tone could represent a substrate for atrial arrhythmias, particularly in settings where little or no anatomic abnormalities are present.^{52,122,286,313} Several factors, such as heterogeneous autonomic innervation of the atria¹⁷⁵ or a complex and dynamic inter-relationship between the two branches of the ANS,^{268,312} could be involved in arrhythmia occurrence.

Coumel et al. were the first to describe different clinical types of AF, resulting from increased vagal or adrenergic tone.⁶⁴ Clinical and experimental observations suggest that the onset of atrial arrhythmias is often related to variations in autonomic tone.^{25,49} Both sympathetic and parasympathetic stimulation were able to induce atrial arrhythmias in various experimental models.²⁶⁵ However, several studies that addressed the role of the ANS in arrhythmia occurrence suggested a combined participation of both branches of the ANS, with sympatho-vagal imbalance as main trigger of these arrhythmias, rather than anomalies of one branch alone.^{48,278,288} Thus, the direction of the autonomic imbalance facilitating atrial arrhythmia occurrence is still a matter of debate.

Accordingly, we aimed to assess the presence and the pattern of an autonomic imbalance in our rat model of spontaneous atrial arrhythmias, using HRV analysis and two experimental protocols that targeted the two branches of the ANS. A mild emotional stress protocol elicited by blowing a jet of air into the cage was chosen because of its ability to induce pure sympathetic stimulation, while lowering the sensitivity of the cardiac baroreceptor reflex.^{39,144} In order to assess atrial arrhythmogenicity in response to increased vagal tone, injections of carbamylcholine, a parasympathetic agonist, were also performed.

4.2. Materials and Methods

All animals used to assess the autonomic tone in this study coincided with the animals used in the previous study to assess the presence of arrhythmic events (Chapter 3). Heart rate variability analysis in baseline conditions was also performed based on the 24-h continuous ECG recordings previously used to assess the occurrence of arrhythmic episodes. All details regarding provenience and care of animals, transmitter implantation procedure, ECG recordings and analysis have already been described in the previous chapter (Chapter 3).

All experiments were performed in accordance with the guidelines of the French Ministry of Agriculture for animal experimentation and were approved by the local Animal Ethics Committee.

4.2.1. Heart rate variability analysis

All arrhythmic events and compensatory pauses were excluded manually prior to analysis. The mean heart rate was automatically calculated over 24-h intervals. The HRV during sinus rhythm was assessed in the 24-h baseline recordings for each animal.

Heart rate variability was assessed by analyzing beat-to-beat variations in RR intervals in the time and frequency domains, as previously described.¹³ For the time domain analysis, standard deviation of normal RR intervals (SDNN), root-mean-square of successive RR-interval differences (RMSSD), and percentage of adjacent RR intervals that differed by > 5 ms (pNN5) were calculated. For the frequency domain analysis, time series were over-sampled at 20 Hz using a cubic spline, then spectral power in the LF band (0.3 - 0.6 Hz), HF band (0.6 - 2.5 Hz), and the LF/HF ratio were estimated on 2048-point (102.4 s) segments windowed by the Hanning function and overlapping by 50% using a fast Fourier transform.

Additionally, in order to identify potential significant changes in autonomic tone preceding arrhythmic events, we analyzed several intervals of intense arrhythmic activity (frequent PACs and AT episodes) and the 5-min periods preceding these intervals. The resulting frequency-domain parameter values were compared to baseline values.

4.2.2. Emotional stress protocol in conscious rats

A mild emotional stress protocol elicited by blowing a jet of air into the cage for 20 min was applied to each animal: at the age of 16 wks for young SHRs and WKY rats, 47 wks for aging WKY rats, and 64 wks for 6 aging SHRs. Air was delivered through a tube (6 mm internal diameter) inserted in a hole at the bottom of the cage, as previously described.³²⁸ The pressure in the relief valve of the air tank was set at 1 bar. The stress test was applied when rats were in a quiet resting state.

A baseline recording was acquired for each animal before the onset of the protocol. Arrhythmic events and HRV analyses were assessed over 10-min intervals. The stress-related values were compared to baseline values.

4.2.3. Subcutaneous injection of carbamylcholine in conscious rats

A baseline recording was obtained for 3 aging SHRs at the age of 60 wks and for all 8 young SHRs at the age of 16 wks. Then, a 0.4 mg/kg dose of carbamylcholine was injected subcutaneously. A 10-min period initiated 5 min after carbamylcholine injection was analyzed, and the number of arrhythmic events was compared with that recorded during the 10-min pre-injection period. None of the WKY rats underwent this protocol.

4.2.4. *Statistics*

Data are expressed as the mean \pm SEM or median and range, as appropriate. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test using Holm's method for multiple comparisons. Differences within the same group were tested for significance with the Wilcoxon signed-rank test. Nonparametric repeated-measures ANOVA (Friedman test) were used for repeatability data. Frequency domain parameters were also analyzed by a nonparametric (using a rank transform) two-way analysis of variance (ANOVA), factoring for the effects of both blood pressure status (normotensive *versus* hypertensive) and age (young *versus* aging).

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software.

4.3. *Results*

4.3.1. *Assessment of the autonomic tone*

The 24-h ECG monitoring indicated that aging SHR rats were more bradycardic (278 ± 4 beats/min) than age-matched WKY rats (335 ± 2 beats/min, $P = 0.01$), young SHR rats (326 ± 6 beats/min, $P < 0.01$) or young WKY rats (355 ± 5 beats/min, $P < 0.01$).

Heart rate variability analysis demonstrated significantly decreased LF components in aging SHR rats compared to age-matched WKY rats, and decreased LF/HF ratios in both young and aging SHR rats compared to age-matched WKY rats (Table 4.1). A two-factor ANOVA demonstrated that LF components were significantly affected by blood pressure status ($P = 0.002$), but they were not significantly affected by age ($P > 0.05$), and there was no interaction between blood pressure status and age ($P > 0.05$). The same analysis demonstrated that the LF/HF ratio was significantly affected by both age and blood pressure status (both $P < 0.05$); again, we found no interaction between age and blood pressure status ($P > 0.05$).

In aging SHR rats, recordings performed one week apart showed significant reproducibility of all HRV parameters (all $P > 0.05$).

In aging SHR rats, the HRV analysis showed no significant changes in LF, HF, or LF/HF components in the 5-min intervals that preceded the onset of periods of intense arrhythmic activity compared to baseline values (all $P > 0.05$). Most of the arrhythmic episodes occurred during periods of intense arrhythmic activity (frequent PACs), but in 28% of cases AT episodes occurred after sinus bradycardia or sinus pauses.

Table 4.1. Baseline heart rate variability parameters in young and aging groups of WKY rats and SHRs

Parameter	14-wk-old WKY rats (n = 4)	55-wk-old WKY rats (n = 4)	14-wk-old SHRs (n = 8)	55-wk-old SHRs (n = 8)	<i>P</i> -value
<i>Time-domain</i>					
SDNN (ms)	28.33 ± 1.21	25.14 ± 1.82	21.42 ± 0.85*	27.09 ± 3.16 [†]	0.01
RMSSD (ms)	4.05 ± 0.45	4.85 ± 0.54	3.58 ± 0.32	3.80 ± 0.32	0.28
pNN5 (%)	0.17 ± 0.03	0.26 ± 0.04	0.14 ± 0.03	0.16 ± 0.03	0.21
<i>Frequency-domain</i>					
LF (ms ²)	1.51 ± 0.30	1.96 ± 0.31	1.00 ± 0.17	0.86 ± 0.10*	0.03
HF (ms ²)	5.99 ± 1.33	7.70 ± 1.56	5.53 ± 1.11	6.13 ± 1.37	0.62
LF/HF	0.25 ± 0.01	0.27 ± 0.04	0.18 ± 0.01*	0.14 ± 0.01* [†]	0.001

Data are expressed as the mean ± SEM. *P*-values refer to comparisons between groups based on multiple comparisons ANOVA. * - *P* < 0.05 for SHRs *versus* age-matched WKY rats. [†] - *P* < 0.05 for 55-wk-old SHRs *versus* 14-wk-old SHRs

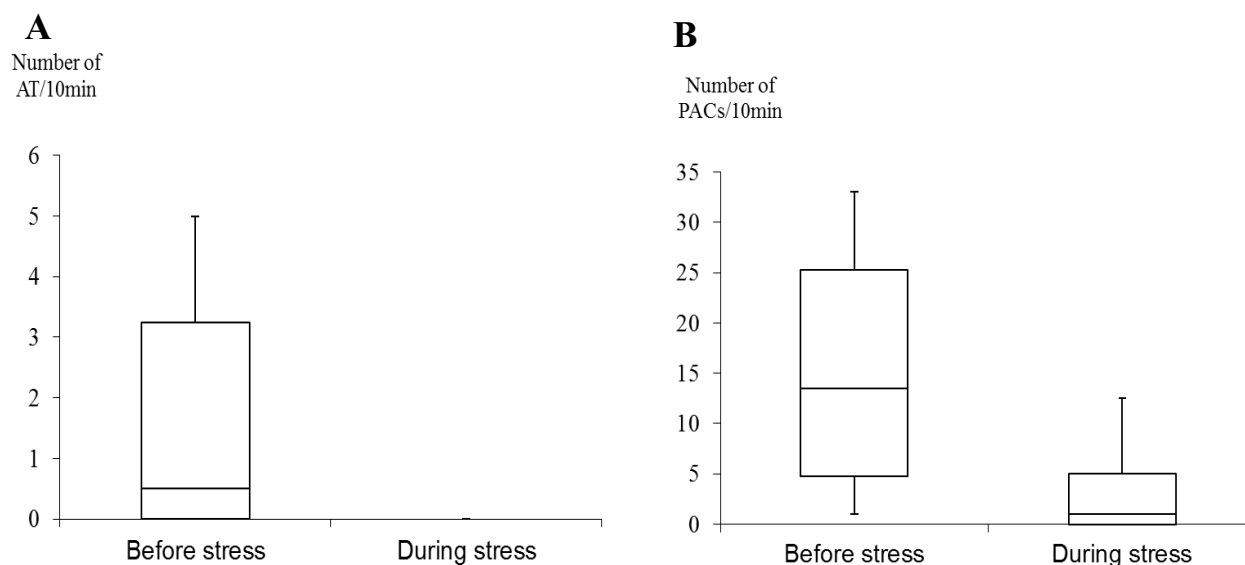
4.3.2. *The effect of stress on atrial arrhythmogenicity*

In the 6 aging SHRs, a median of 0.5 (range 0 – 5) tachyarrhythmic episodes were observed during the 10-min interval preceding the onset of the stress protocol, while no episode was recorded during stress exposure (Figure 4.2, A). Similarly, the number of PACs decreased from a median of 13.5 (range 1 – 33) before stress to a median of 1 (range 0 – 14) during stress exposure (*P* = 0.04) (Fig. 4.2, B).

No ATs or PACs were observed in WKY rats, regardless of their age, before or after the onset of the stress protocol. No ATs and few PACs were observed in young SHRs, before or during stress exposure.

The emotional stress induced an increase in heart rate: heart rates increased in young WKY rats from 288 ± 9 beats/min to 313 ± 18 beats/min, *P* = 0.25; in aging WKY rats from 315 ± 10 beats/min to 341 ± 12 beats/min, *P* = 0.38; in young SHRs from 282 ± 6 beats/min to 412 ± 10 beats/min, *P* = 0.007; and in aging SHRs from 273 ± 10 beats/min to 363 ± 9 beats/min, *P* = 0.03. Since only SHRs presented spontaneous episodes of AT, HRV analysis before and during stress was only performed in these groups. In aging SHRs, the HRV analysis showed significant increases in the LF/HF ratio during stress compared to pre-stress values (Table 4.2).

Figure 4.1. Number of (A) AT episodes and (B) PACs before and during stress protocol in aging SHR



AT – atrial tachyarrhythmia, PACs – premature atrial contractions. Data represent the total numbers of AT episodes and PACs recorded over a 10-min pre-stress period (*before stress*) and a 10-min intra-stress period (*during stress*). Data are expressed as median and IQR. The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile.

Table 4.2. Heart rate variability parameters before and during stress in aging SHR (n = 6)

Parameter	Before stress	During stress	P-value
RMSSD (ms)	3.58 ± 0.66	2.22 ± 0.53	0.01
pNN5 (%)	0.14 ± 0.05	0.04 ± 0.02	0.06
LF (ms ²)	0.67 ± 0.15	1.70 ± 0.69	0.20
HF (ms ²)	4.33 ± 1.35	2.57 ± 0.86	0.27
LF/HF	0.19 ± 0.03	0.56 ± 0.09	0.01

Data are expressed as the mean ± SEM. P-values refer to comparisons between conditions based on the Wilcoxon signed-rank test.

Comparable results were obtained in young SHR: LF increased during stress from $0.60 \pm 0.14 \text{ ms}^2$ to $1.11 \pm 0.23 \text{ ms}^2$ ($P = 0.007$), HF decreased from $5.62 \pm 0.10 \text{ ms}^2$ to $3.19 \pm 0.39 \text{ ms}^2$ ($P = 0.03$), and the LF/HF ratio increased from 0.11 ± 0.01 to 0.35 ± 0.05 ($P = 0.007$).

4.3.3. Carbamylcholine administration and atrial arrhythmias

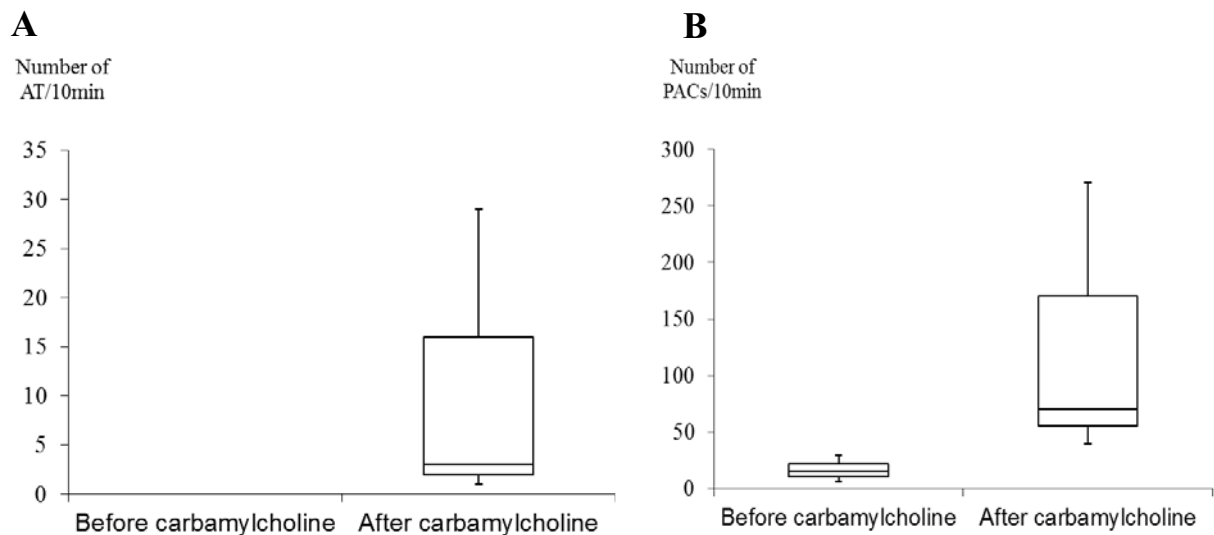
Prior to carbamylcholine administration, aging SHR presented a median of 15 PACs (range 6 - 29), and no episodes of AT were recorded. In the 10-min interval following carbamylcholine injection, a median of 70 PACs (range 40 - 271) and a median of 3 (range 1

– 29) episodes of AT were observed (Fig. 4.3, A and B). The AT episodes that occurred after carbamylcholine administration had a median duration of 1.89 s (range 0.90 – 10.13 s), significantly longer than the duration of AT episodes in the 24-h baseline recordings (0.71 s, range 0.53 – 1.63 s; $P < 0.001$).

No ATs and few PACs were observed in young SHR, before or after carbamylcholine administration.

In aging SHRs, the high electrical instability observed after carbamylcholine administration, with an elevated number of PACs and AT episodes and very few periods of stable sinus rhythm, did not allow HRV analysis to be properly performed.

Figure 4.2. Number of (A) AT episodes and (B) PACs before and after carbamylcholine administration in aging SHRs



AT – atrial tachyarrhythmia, PACs – premature atrial contractions. Data represent the total numbers of AT episodes and PACs recorded over a 10-min pre-injection period (*before carbamylcholine*) and a 10-min period initiated 5 min after carbamylcholine injection (*after carbamylcholine*). Data are expressed as median and IQR.

The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile.

4.4. Discussion

The main findings of the present study were (i) in aging SHRs, a sympatho-vagal imbalance, with reduced sympathetic tone, was present; (ii) in aging SHRs, sympathetic activation by emotional stress removed all ATs and significantly reduced the number of PACs, while parasympathetic stimulation with carbamylcholine had marked pro-arrhythmic effects; and (iii) this sympatho-vagal imbalance was already present in young SHRs and preceded the occurrence of arrhythmias.

4.4.1. *The role of the autonomic nervous system*

It was previously proposed that sympatho-vagal imbalance could facilitate atrial arrhythmias.²⁶⁶ In a study by Patterson et al., application of acetylcholine shortened the APD in PVs and induced early-after-depolarizations, while simultaneous infusion of acetylcholine and norepinephrine triggered tachycardia-pause rapid firing in the same preparations.²⁴³ The authors also demonstrated that combined parasympathetic and sympathetic nerve stimulation triggered firing in canine PVs.²⁴⁴ Chen and colleagues used extrinsic and intrinsic cardiac nerve activity recordings in ambulatory dogs and applied intermittent rapid LA pacing to induce paroxysmal AF or atrial tachycardia. Their results suggested that intrinsic cardiac nerve activation, alone or associated with extrinsic cardiac nerve activation, could invariably trigger paroxysmal ATs.⁵⁵ Along the same lines, Tan et al. reported that simultaneous sympatho-vagal discharges were common triggers for spontaneous atrial arrhythmias, and cryoablation of extrinsic sympatho-vagal nerves eliminated arrhythmic episodes; these results suggested a causal relationship between the discharges and the occurrence of the arrhythmia.²⁷⁹

In the present study, HF components, an accepted index of parasympathetic modulation,¹²⁹ did not differ between aging SHR, which presented numerous episodes of AT, and age-matched WKY rats, which did not experience atrial arrhythmias. However, LF components were significantly reduced in aging SHR compared to those in WKY rats, and, consequently, the LF/HF ratio was significantly smaller in aging SHR than in WKY rats. Given that the LF/HF ratio is a convenient index of parasympathetic and sympathetic interactions in the rat heart,¹⁶⁶ our results highlighted the presence of a significant sympatho-vagal imbalance in animals that experienced ATs. The reduction of sympathetic tone, and therefore, the relative vagal hyperactivity, appeared to facilitate the occurrence of arrhythmias. In this model, reduced sympathetic tone appeared to result from persistent hypertension, while both aging and high blood pressure appeared to affect the sympatho-vagal balance.

Acute changes in autonomic tone that preceded the onset of AT episodes were difficult to assess, due to the fact that the AT episodes occurred during periods of intense arrhythmic activity (frequent PACs). Consequently, most of the HRV analysis was actually performed on intervals immediately preceding periods of frequent PACs rather than actual AT episodes. This could have influenced our results. Therefore, although frequency-domain parameters showed no acute changes before the onset of arrhythmic episodes, these results should be

interpreted with caution; the role of the ANS should not be oversimplified based on these results.

The high frequency of PACs before AT onset is in line with previous papers. A progressive increase in atrial ectopy over the last minutes prior to AF onset was previously reported.^{18,103,152} The higher density of ectopic beats in this setting may reflect enhanced automaticity *per se*, or may be mediated by fluctuations in autonomic balance^{62,63} or increased atrial stretch.²⁷⁵

In our model, emotional stress restored the autonomic balance by increasing sympathetic tone and had marked antiarrhythmic effects. Indeed, in aging SHR, the LF/HF ratio increased during stress compared to the baseline value, despite unchanged LF values. However, given the reduction in total heart rate variance in aging SHR during stress (decreased RMSSD and pNN5), it is not surprising that the absolute values of LF did not significantly change.

In aging SHR, carbamylcholine administration had marked pro-arrhythmic effects. This was shown by the increased number of PACs and AT episodes and the significantly longer duration of AT episodes following carbamylcholine administration compared to baseline recordings. Unfortunately, HRV analysis after carbamylcholine administration could not be achieved, due to the increased number of arrhythmic events. However, based on the well-known effects of the stress protocol (pure sympathetic stimulation),^{39,144} the known parasympathetic effect of carbamylcholine, and the opposite effects induced by stress and carbamylcholine on atrial arrhythmogenicity, it appears that accentuating the autonomic imbalance by further increasing vagal tone enhances even more atrial arrhythmogenicity in this setting.

As suggested by the absence of significant AT events in young SHR, despite the decreased LF/HF ratio, the presence of an autonomic imbalance precedes the occurrence of arrhythmias in hypertensive rats, but does not seem to be sufficient to induce ATs. Indeed, the LF/HF ratio was altered more in aging SHR than in young SHR; this suggested that autonomic imbalance became more severe with advancing age. However, other features, such as anatomical remodeling with extensive atrial fibrosis, abnormal distribution and/or density of autonomic nerves within the atria, or various molecular abnormalities, may also act as arrhythmia facilitators in this model. In this vein, the role of atrial fibrosis in atrial arrhythmogenicity was previously reported in senescent normotensive rats.²³³

4.4.2. *Potential limitations*

The protocols used in this study provided only indirect evidence of autonomic imbalance in SHR that presented atrial arrhythmias. Additional functional studies are required to quantify the extent of abnormal sympatho-vagal imbalance and its precise role in atrial arrhythmogenicity.

4.4.3. *Conclusions*

Our findings reinforce the role of abnormal autonomic tone in atrial electrical instability. Our results suggest that autonomic imbalance with relative vagal hyperactivity may be responsible for the increased atrial arrhythmogenicity observed in this model. We also provide evidence that the sympatho-vagal imbalance was present in hypertensive rats long before the occurrence of arrhythmias. However, this autonomic abnormality does not seem to be sufficient to induce arrhythmias.

CHAPTER 5

A Study of Left Atrial Histology in Arrhythmic Rats

5.1. Introduction

Cardiac remodeling with LA structural abnormalities has been shown to precede the onset of atrial arrhythmias, but also to evolve along with the progression of these arrhythmias.¹⁴ Histological abnormalities are a common finding in LA samples of patients that present atrial arrhythmias and particularly AF, and include a wide range of cellular and ultrastructural abnormalities.⁹⁸ In this setting, atrial remodeling involves changes in structure and function of the atria, modifications of atrial electrical and contractile functions, changes in atrial extracellular matrix.³⁰ Together, these alterations create an arrhythmogenic substrate, essential for the onset and particularly for the persistence of atrial arrhythmias.

Experimental studies suggest that the components and the severity of atrial structural remodeling vary depending particularly on coexisting cardiac conditions and specie.^{15,213,293} Comparable structural and ultrastructural abnormalities with those encountered in patients with AF have been described in various animal models of atrial arrhythmia.^{15,30} Nevertheless, more severe and extensive lesions have been reported in humans with both long-term lone AF and in AF associated with other cardiac diseases than in animal models of atrial arrhythmia. Particularly, atrial interstitial fibrosis is a common feature of AF in humans,⁹⁸ while both intercellular and inter-bundle interstitial fibrosis seem to be less important in animal models of atrial arrhythmia.¹⁴ However, LA structural abnormalities have only been studied in models in which arrhythmia was triggered by interventional means, and have never been assessed in an animal model of spontaneous atrial arrhythmias.

Accordingly, we aimed to assess the presence, the components and the severity of LA histological abnormalities in our rat model of spontaneous atrial arrhythmias, using standard histological examination.

5.2. Material and Methods

All samples used for the histological examination originated from the animals previously used to assess the presence of arrhythmic events (Chapter 3) and the role of autonomic imbalance in arrhythmia occurrence (Chapter 4). All details regarding provenience and care of animals have already been described in Chapter 3.

All experiments were performed in accordance with the guidelines of the French Ministry of Agriculture for animal experimentation and were approved by the local Animal Ethics Committee.

5.2.1. Heart sampling and histological examination

Once experimental protocols were completed (i.e., 24-h continuous ECG recordings, emotional stress, and carbamylcholine injections), aging SHR_s (n = 8) and WKY rats (n = 4) were euthanatized with an intraperitoneal injection of a terminal dose of sodium pentobarbital (> 100 mg/kg).

Necropsy, heart sampling and histological examination were performed by an independent anatomopathologist, who was unaware of the rat's blood pressure status (hypertensive or normotensive), age (aging or young) or cardiac rhythm (presence or absence of atrial arrhythmias).

The hearts were excised and underwent immediate (within 2 min after removal) fixation in 10% buffered formalin and were processed for paraffin histology. Deparaffined tissue sections 5 µm thick were stained with Masson's trichrome and haematoxylin-eosin-safran and examined with an Axiolab (Carl Zeiss MicroImaging GmbH, Germany) light microscope. The region examined was the lateral wall of the LA. Characteristic areas were photographed using the Zeiss AxioCam ICc1 digital camera system (Carl Zeiss MicroImaging GmbH) and AxioVision Release 4.8.2. software (Carl Zeiss MicroImaging GmbH) was used for image processing.

Only visual evaluation was performed, as the purpose of this examination was to describe characteristic structural features in our populations and not to precisely quantify these findings.

A scoring system was used to quantify the degree of fibrosis, myocyte necrosis, and inflammatory cell infiltration in LA samples. According to this scoring system 0 corresponded to the absence of abnormalities, 1 defined mild abnormalities, 2 moderate abnormalities, and 3 severe abnormalities. Left ventricular wall thickness was measured for each heart sample at 2.5 magnification.

5.2.2. Statistics

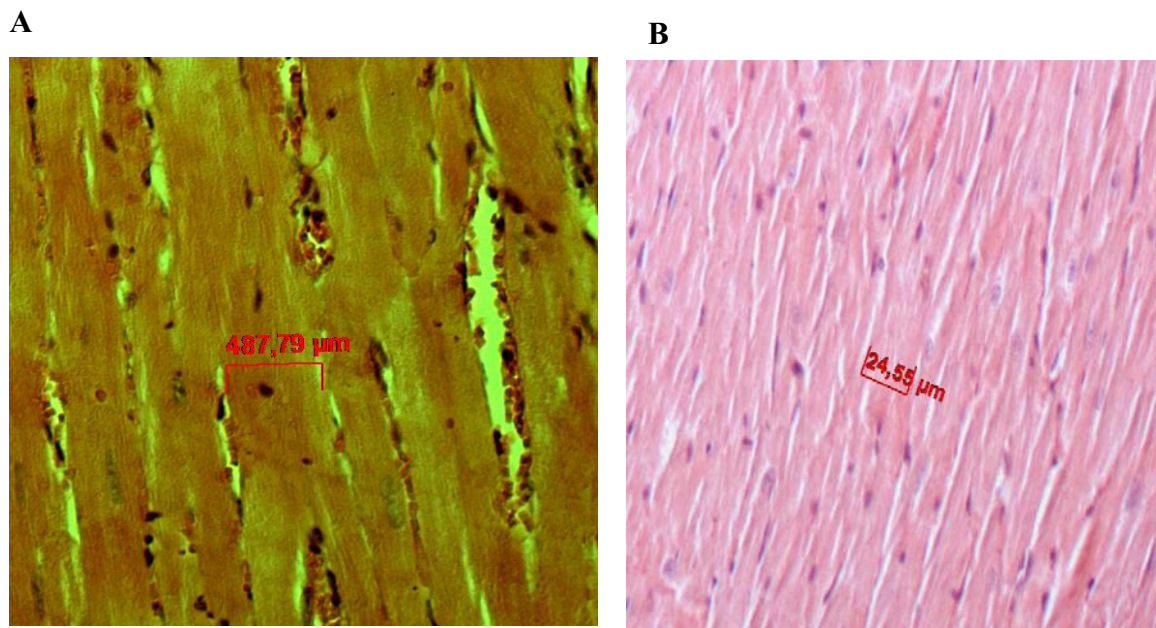
Data are expressed as the mean ± SEM. Between-group comparisons were performed using the Mann-Whitney *U* test. A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software.

5.3. Results

5.3.1. Left ventricular hypertrophy indexes

Left ventricular wall thickness was 67% greater ($P < 0.01$) in aging SHR (5513 \pm 201 μ m) than in aging WKY rats (3300 \pm 238 μ m). Similarly, myocyte diameter was significantly greater in aging SHR compared to age-matched WKY rats (Fig. 5.1, A and B). These results confirmed the presence of significant LVH in hypertensive rats.

Figure 5.1. Left ventricular samples (haematoxylin-eosin-safran staining) from aging SHR (A) and WKY rats (B) showing increased myocyte diameter in aging SHR compared to WKY rats



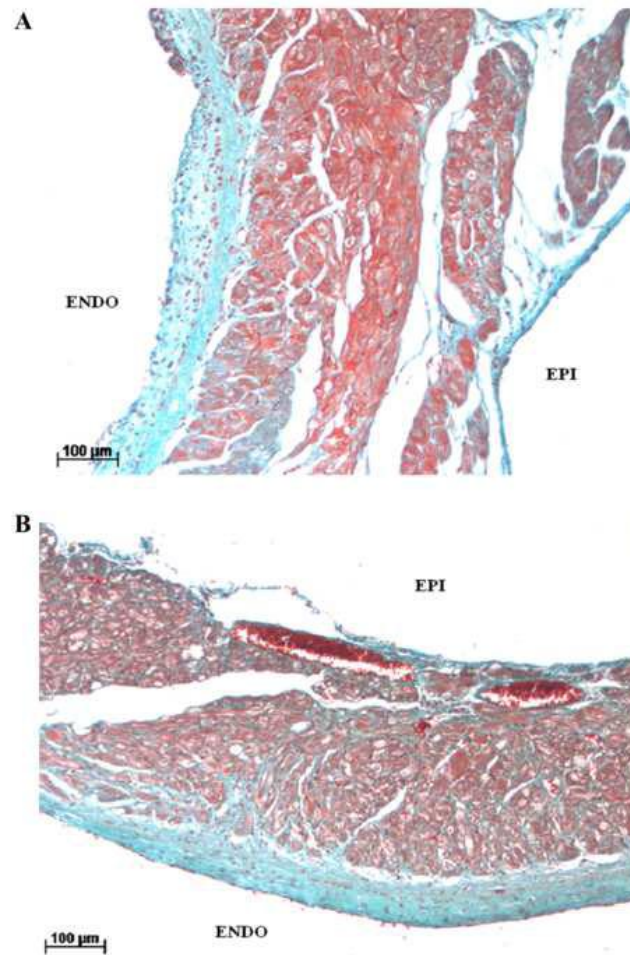
5.3.2. Left atrial structural remodeling

In 6 of the 8 aging SHR, LA endocardial and interstitial fibrosis, inflammatory infiltrates, and myocyte necrosis of varying grades of severity were observed. The most frequent feature was endocardial fibrosis (Fig. 5.2, A and B), which was observed in 6 aging SHR (mean score 1.5 \pm 0.4). None of the aging WKY rats presented endocardial fibrosis. In 4 aging SHR, several aspects of early-stage, progressive endocardial fibrosis were identified on the same sample (Fig. 5.2, B).

None of the other histological abnormalities presented a significantly different score between aging SHR and aging WKY rats (interstitial fibrosis: 0.6 \pm 0.3 in aging SHR *versus* 0.25 \pm 0.25 in WKY rats, $P = 0.10$; myocyte necrosis: 1.0 \pm 0.4 in aging SHR, *versus* 0.25 \pm 0.25 in WKY rats, $P = 0.39$; inflammatory infiltrates: 0.6 \pm 0.4 in aging SHR *versus* 0.25 \pm 0.25 in WKY rats, $P = 0.39$).

0.25 in WKY rats, $P = 0.93$). In aging SHR, the severity of structural lesions was highly variable among individuals and heterogeneous within the same sample.

Figure 5.2. Left atrial samples (Masson's trichrome staining) from aging SHR showing – (A) progressive, stratified endocardial fibrosis, and (B) stable, evolved endocardial fibrosis

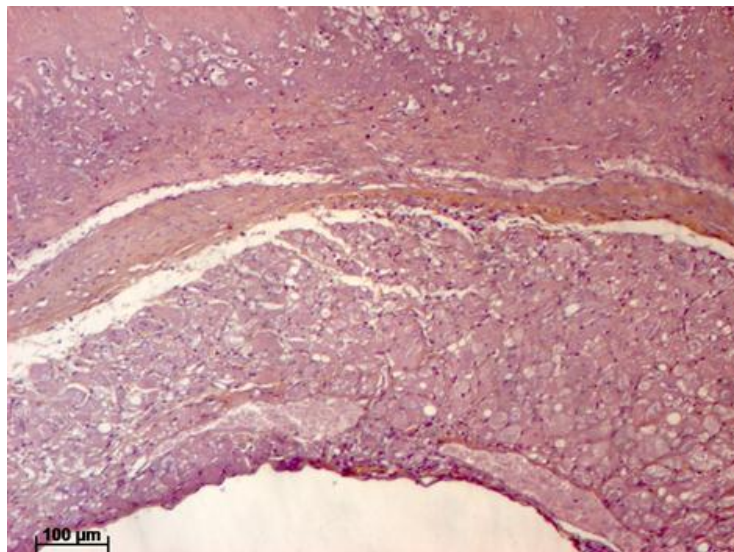


ENDO (endocardial) and EPI (epicardial) borders of the left atrium.

In 3 aging SHR, histological examination identified the presence of intra-atrial thrombosis (Fig. 5.3). None of the WKY rats presented intracardiac thrombi.

There was no significant difference regarding any of the histological abnormalities (i.e., endocardial or interstitial fibrosis, myocyte necrosis, or inflammatory infiltrates) between SHR that presented intra-atrial thrombosis and SHR that did not. Also, we found no correlation between the presence of intra-atrial thrombosis and any of the histological abnormalities.

Figure 5.3. Intra-atrial thrombosis in an aging SHR (haematoxylin-eosin-safran staining)



5.4. Discussion

The main findings of the present study were (i) LA histological abnormalities were present in 6 of the 8 aging SHRs; the severity of structural lesions was highly variable among individuals and heterogeneous within the same sample; (ii) in aging SHRs, LA endocardial fibrosis was the most frequent feature, while in aging WKY rats endocardial fibrosis was completely absent; (iii) three of the 8 aging SHRs presented intra-atrial thrombosis, while none of the WKY rats did so; and (iv) in 2 of the 8 aging SHRs histological examination did not reveal any LA structural abnormality.

5.4.1. Atrial fibrosis as arrhythmia substrate

Atrial remodeling can occur as response to cardiac damage due to coronary artery disease, aging, hemodynamic overload from valve disease or hypertension.²⁷ Changes in structure and function of the atria, modifications of atrial electrical and contractile functions, and changes in atrial extracellular matrix, accompany cardiac remodeling and create an arrhythmogenic substrate essential for the occurrence of atrial arrhythmias.³⁰ Several experimental studies have provided evidence of an association between altered atrial structure and increased inducibility of AT.^{56,98,128,177,301} In dogs, ventricular tachypacing induced heart failure¹⁰ and produced atrial interstitial fibrosis comparable to that previously reported in AF patients.¹⁷⁷ In that model, atrial fibrosis was associated with localized regions of conduction slowing, and increased conduction heterogeneity.¹⁷⁷ In a rat model, Hayashi et al. demonstrated increased susceptibility to AF in aged rats and suggested that heterogeneous

atrial interstitial fibrosis and atrial cell hypertrophy could contribute to aging-related atrial conduction slowing, conduction block, and inducible AF.¹²⁸ Atrial fibrosis has been shown to increase AF vulnerability in several other animal models^{119,170,177} and in a transgenic mouse model for selective atrial fibrosis.³⁰⁰

Recent studies in both humans¹⁶² and in animal models of aging^{8,128} demonstrated a strong correlation between age and atrial fibrosis. Furthermore, in the presence of hypertension, the remodeling process seems to be accelerated,²⁹⁰ probably due to a combination of factors such as earlier development and more severe diastolic dysfunction, impaired plasma volume control, intensified neurohormonal activation, and atrial myopathy secondary to oxidative stress and lipoapoptosis.²⁹⁰

Our results reinforce the role of aging and hypertension in LA remodeling and atrial arrhythmogenesis. Indeed, 6 of the 8 aging SHR rats presented varying degrees of endocardial fibrosis, while no such abnormality was observed in WKY rats. However, LA histological abnormalities observed in aging SHR rats that presented atrial arrhythmias were significantly less severe than those previously reported in AF patients. Moreover, only mild to moderate LA interstitial fibrosis was present in no more than 3 aging SHR rats, whilst this feature is a constant finding in LA samples of patients with AF.^{98,4} However, studies in humans are limited to LA samples obtained from patients that undergo cardiac surgery or explantation. Concomitant serious heart conditions such as coronary artery disease, valve disease or congenital heart defects in these patients could be at least partially responsible for the more extensive structural changes observed in human LA samples.

Interestingly, some of the rats that presented atrial arrhythmias did not present endocardial fibrosis, or any other LA structural abnormality. This finding suggested that the LA remodeling / fibrosis-AT relationship could be in fact highly nonlinear, and that atrial fibrosis is more likely to be a facilitator of atrial arrhythmogenicity in our model, rather than a prerequisite.

5.4.2. Increased atrial thrombogenicity in arrhythmic rats

In 3 of the 8 aging SHR rats that presented atrial arrhythmias, histological examination revealed the presence of intra-atrial thrombi, while no such abnormality was observed in age-matched WKY rats. These results are in accordance with previous clinical data that link AF with a high risk of morbidity and mortality from thromboembolism and stroke.¹²⁰

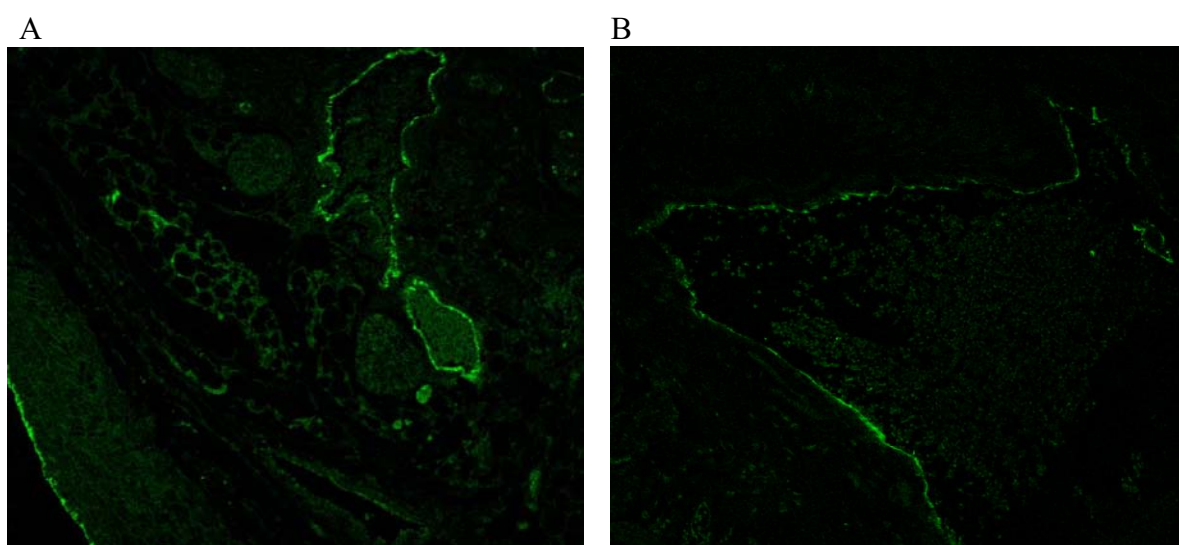
The pathophysiology of thrombosis relies on Virchow's triad of coagulation, which includes abnormalities in blood flow, vessel wall and blood constituents.¹⁸⁴ While LA stasis and subsequent thrombus formation may be one source of thromboembolism in AF patients,²⁵¹ recent data suggest that additional factors could contribute to the thromboembolic risk of this patient group. Abnormal haemostatic constituents indicative of thrombogenesis have been demonstrated in AF patients.⁵⁷ Recent data support a critical role of endothelial dysfunction in the AF-related thromboembolic risk.⁵⁴

Accordingly, we thought to assess the presence and the extent of atrial endocardial impairment in aging SHR rats compared to young and normotensive rats. Therefore, we performed immunohistochemical staining of vWF, a marker of both increased thrombogenicity and endothelial dysfunction, in LA samples of young, adult and aging SHR rats in comparison to age-matched WKY rats.

Preliminary results suggest that atrial endocardial impairment is significantly more severe in aging SHR rats (Fig. 5.4, A) compared to both young SHR rats and age-matched WKY rats (Fig. 5.4, B). These results suggest that both aging and high blood pressure participate to atrial endocardial impairment in this model.

Indeed, previous data demonstrated that endothelial functional and morphological alterations are favored by both aging and hypertension.^{202,214} In several species, relaxation in response to various endothelium-dependent stimuli decreased with age in isolated or perfused arteries.^{77,135,158,217} According to Taddei et al., endothelial dysfunction that occurs in hypertension could be an accelerated form of the dysfunction that occurs with aging.^{277S}

Figure 5.4. Immunostaining for vWF (fluorescent green) reveals higher intensity of staining in aging SHR rats (A) compared to aging WKY rats (B)



Additional analysis, with precise quantification of the intensity of vWF immunostaining is ongoing and is expected to bring additional information on the pathophysiology of thromboembolic risk in aging, hypertensive rats.

5.4.3. *Potential limitations*

Histological examination performed in this study was only visual and thus only semiquantitative results were available. Also, the protocol used in this study cannot exclude the presence of microfibrosis in the LA samples of studied animals. Additional studies are required to precisely quantify LA structural abnormalities and particularly LA fibrosis in this model. Additionally, studies to directly address the mechanisms responsible of intra-atrial thrombosis in this model will be of interest.

5.4.4. *Conclusions*

Our findings reinforce the role of LA structural abnormalities and particularly LA fibrosis in atrial electrical instability. However, the fact that 2 of the 8 aging SHR rats did not present LA histological abnormalities despite the presence of arrhythmias suggests that the LA remodeling / fibrosis-AT relationship could be in fact highly nonlinear, and that atrial fibrosis is more likely to be a facilitator of atrial arrhythmogenicity in our model, rather than a prerequisite. We also provide evidence that intra-atrial thrombosis accompanies LA structural remodeling in arrhythmic rats.

CHAPTER 6

A Study of Molecular Mechanisms of Atrial Arrhythmias in Arrhythmic Rats

6.1. Introduction

Although most AF patients have associated cardiac diseases, in 15% to 30% of patients, an etiology is absent.^{176,216} Moreover, some of these patients have a positive family history for AF.^{96,320} These observations suggested that genetic factors could also contribute to the development of the arrhythmia. Possible genes responsible for triggering and maintaining AF include genes that affect automaticity, atrial refractory period duration and conduction. However, recognized mutations probably account for only a small fraction of all AF cases, while common genetic variants, single-nucleotide polymorphisms, are much more frequent, though they act with a weaker effect and a less-clear phenotype.²⁷¹

The single-nucleotide polymorphisms most significantly associated with AF are located on chromosome 4q25.¹¹⁸ The closest gene in the region is *Pitx2*, a member of the pituitary homeobox family of transcription factors that plays an important role in morphogenesis.²⁴⁸ Several recent studies postulated that *Pitx2* insufficiency might be a causative factor for the onset of AF.^{53,154} Chinchilla et al. were the first to demonstrate that *Pitx2* expression is significantly decreased in AF patients, providing a link between *Pitx2* loss-of-function and AF.⁵³ In transgenic mice with heterozygous deletion of the *Pitx2* gene, reduction of *Pitx2* expression to about 60% of normal was associated with increased susceptibility to AF.¹⁵⁴ While down-regulation of *Pitx2* expression is a common finding in LA samples of AF patients, to date, no study has provided any data concerning *Pitx2* expression changes that precede the onset of the arrhythmia. In fact, it would be difficult to imagine such a large-scale study in humans. Given that, according to our results, all SHRs present atrial arrhythmias once they have reached advanced age, this study aimed to assess, for the first time, expression changes in the *Pitx2* gene that preceded the onset of the arrhythmia. Accordingly, we studied *Pitx2* expression in the LA of aging SHRs that presented atrial arrhythmias, but also in younger SHRs (i.e., young and adult SHRs) that did not yet experience atrial arrhythmias, in comparison to age-matched WKY rats.

Molecular research in AF has focused on two main fields: identification of genes that play a role in AF onset and identification of altered gene expression during AF. For most genes, there is a close relationship between the level of expression and the associated function. Genes generally express their function by the means of a protein. The two main steps in the transition from a gene to the resulting protein are transcription and translation. Under the influence of appropriate stimuli, a first phase of transcription is triggered in the nucleus. Messenger ribonucleic acids (mRNAs) are thus produced from the deoxyribonucleic

acid (DNA) sequence of the gene. The totality of mRNAs present in a cell at a given time defines the transcriptome of that cell. Unlike for the genome, the ‘composition’ of the transcriptome is constantly changing as a result of newly generated mRNAs and enzymatic degradation of other mRNAs. The transcriptome is a highly dynamic structure that reflects cell’s adaptation to its environment. In AF patients, transcriptome analysis showed a consistent pattern of expression changes, including alterations in genes encoding for ion channels and Ca^{2+} -handling proteins,³⁷ oxidative stress,¹⁵³ and a ventricular-like expression signature.¹⁹ However, the exact molecular mechanisms involved in the adaptation processes during AF are far from clear. Identification of the signaling pathways and their target genes could lead to new therapeutic options for AF patients. In order to elucidate the molecular profile of arrhythmic rats, we investigated alterations in mRNA expression of 89 genes encoding for proteins involved in Ca^{2+} -handling, ion channels, extracellular matrix, and cell-cell communication, in LA samples of aging SHR rats in comparison to age-matched WKY rats.

6.2. Materials and Methods

6.2.1. Animals

Three groups of male SHR rats ($n = 4$ each) of 14 wks (further mentioned as young), 24 wks (further mentioned as adult), and 48 wks (further mentioned as aging) of age, respectively, and three groups of age-matched WKY rats ($n = 4$ each) were purchased from Elevage Janvier (Le Genest Saint Isle).

All experiments were performed in compliance with the French Ministry of Agriculture guidelines for animal experimentation and were approved by the local Animal Ethics Committee.

6.2.2. Heart sampling

All animals were euthanatized with an intraperitoneal injection of a terminal dose of sodium pentobarbital ($> 100 \text{ mg/kg}$).

Heart sampling was performed by a cardiac surgeon, who excised the hearts. The LA was then isolated and was immediately (within 2 min after removal) placed in a sterile dry tube and was stored at -80°C until processing and analysis.

6.2.3. *Pitx2* expression analysis

Total RNA was isolated from frozen biopsies using TRIzol[®] Reagent (Life Technologies, Villebon sur Yvette, France) following manufacturer's instructions. Ribonucleic acid concentration was measured using Nanodrop ND1000 (Thermo scientific, Illkirch, France) and quality was verified using the Agilent 2100 BioAnalyser (Agilent Technologies, Massy, France).

One µg of total RNA was reverse-transcribed to complementary DNA (cDNA) by using 100 U Superscript II (Life Technologies) and a mixture of random hexamers and oligo(dT) primers (Promega, Charbonnières-les-Bains, France). Real-time polymerase chain reaction (PCR) assays were performed as previously described,²⁰⁶ using a Rotor-Gene 6000 (QIAGEN). Values were normalized using TATA box binding protein (TBP). Primers used to detect *Pitx2* were GTG-TGG-ACC-AAC-CTT-ACG-G (sense primer) and AGT-TGA-AGA-AGG-GGA-AGC-TC (antisense primer).

6.2.4. *Molecular analysis of 89 selected genes*

Total RNA was isolated from frozen biopsies using TRIzol[®] Reagent (Life Technologies) following manufacturer's instructions. Ribonucleic acid concentration was measured using Nanodrop ND1000 (Thermo Scientific) and quality was verified using the Agilent 2100 BioAnalyser (Agilent Technologies).

One µg of total RNA was reverse-transcribed to cDNA by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following manufacturer's instructions.

To identify altered gene expression, we used a TaqMan Low-Density Array (TLDA) to examine the expression of 89 human genes, encoding for ion channels, proteins of the extracellular matrix, and cell-cell coupling. For the present study, the TLDA card was configured into four identical 89 genes set. For each set of genes, we used 6 endogenous control genes, *Ppia*, *β-actin*, *Gapdh*, *Hprt1*, *Tbp* and *18s RNA*. The cDNA (2 µL) was mixed with 48 µL of H₂O and 50 µL of 2X TaqMan Universal PCR Mix (Applied Biosystems). Each sample (100 µL) was loaded into a port of the micro-fluid card and run on an ABI 7900HT System (Applied Biosystems) for 2 min at 50°C, 10 min at 94.5°C, followed by 40 cycles for 30 s at 97°C and 1 min at 59.7°C.

The TLDA data were analyzed by SDS 2.3 software (Applied Biosystems). Threshold cycle data for all target and control gene were transformed into molecule number (attomoles)

considering PCR efficiency of 100%. Gene expression levels were normalized using TBP. A detailed list of the 89 examined genes is provided in Appendix 1.

6.2.5. Statistics

Data are expressed as the mean \pm SEM. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test. Correlations were ascertained with Spearman's rank correlation method.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software.

6.3. Results

6.3.1. *Pitx2* expression in the left atria of hypertensive rats

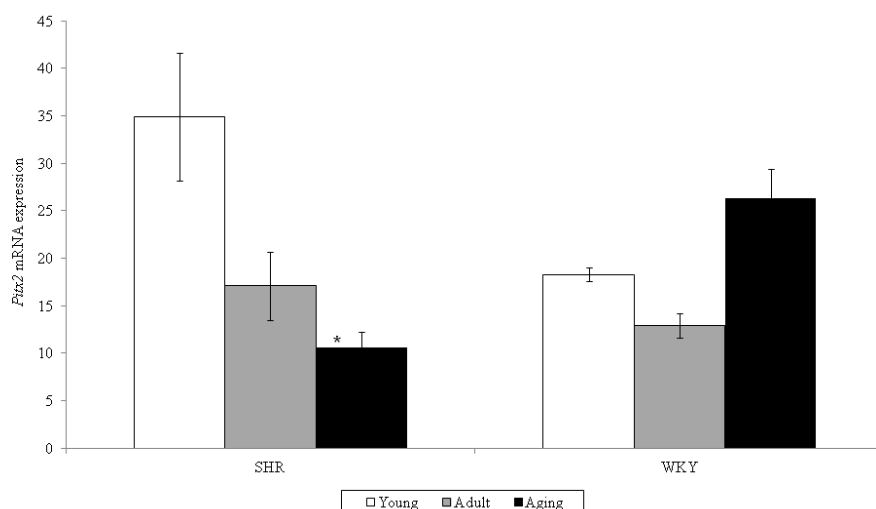
With the exception of aging SHR, all other groups (i.e., young and adult SHR, and young, adult, and aging WKY rats) presented significantly higher expression of *Pitx2* in the LA than in the right atrium (all $P < 0.05$). In aging SHR there was no significant difference between LA and right atrial levels of *Pitx2* ($P = 0.45$). When right atrial expression of *Pitx2* was compared between hypertensive and normotensive rats no significant difference was observed between groups, regardless of the age of animals (all $P > 0.05$).

Similarly, no significant difference was found between LA levels of *Pitx2* in young SHR *versus* age-matched WKY rats ($P = 0.09$), nor in adult SHR *versus* age-matched WKY rats ($P = 0.49$) (Fig. 6.1).

On the other hand, aging SHR had significantly lower LA level of expression of the *Pitx2* gene compared to age-matched WKY rats ($P = 0.02$) (Fig. 6.1).

As the Fig. 6.1 clearly highlights, among hypertensive rats, *Pitx2* expression showed a gradual, age-dependent, decrease (34.9 ± 6.7 in young SHR *versus* 17.1 ± 3.6 in adult SHR *versus* 10.7 ± 1.7 in aging SHR, $P = 0.04$). In SHR, *Pitx2* expression was significantly negatively correlated with age (Spearman $r = -0.86$, $P < 0.01$).

Figure 6.1. Left atrial expression of *Pitx2* in young (n = 4), adult (n = 4) and aging (n = 4) SHRs and age-matched WKY rats (n = 4 each)



Data are expressed as the mean \pm SEM. * - $P = 0.02$ for aging SHRs versus aging WKY rats using the Mann-Whitney U test

6.3.2. Molecular profile of aging hypertensive rats versus normotensive controls

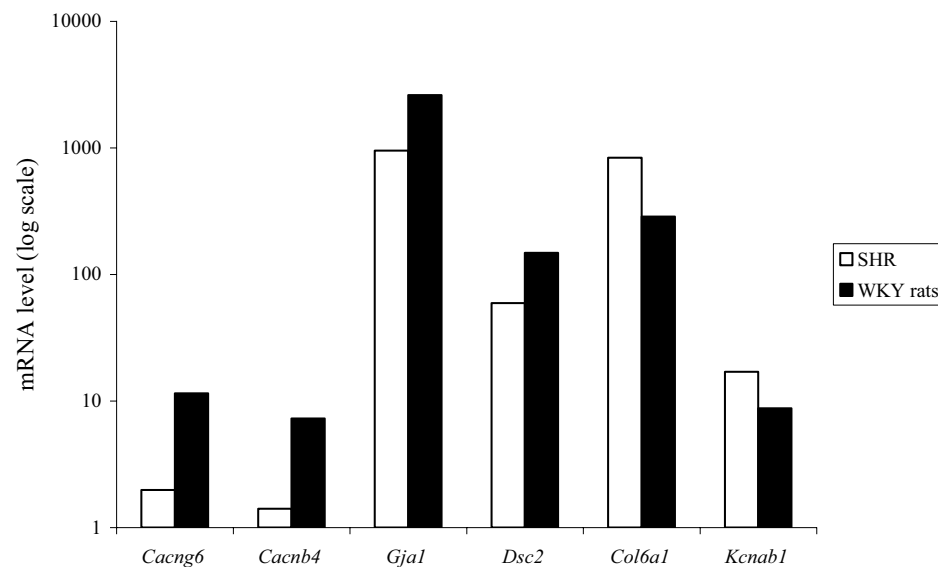
Left atrial gene expression levels of aging SHRs were compared to those of age-matched WKY rats. To analyze the data, we applied a 1.5 fold-change cut-off point to the entire data set.

Of the total of 89 studied genes, 6 genes were differentially expressed between aging SHRs and WKY rats (Fig. 6.2). Among these 6 genes, 4 were down-regulated and 2 were up-regulated in aging SHRs compared to age-matched WKY rats. The absolute fold-change values of underexpressed genes were higher than those of overexpressed genes (absolute fold-change for down-regulated genes 16.2 versus 4.9 for up-regulated genes).

Down-regulation was particularly observed for genes encoding for the $I_{Ca, L}$ current, *Cacng6* and *Cacnb4*. *Cacng6* expression was 5.8 times lower in aging SHRs compared to age-matched WKY rats ($P = 0.03$). Similarly, aging SHRs presented a 5.2-fold lower *Cacnb4* expression than aging WKY rats ($P = 0.02$). Consistent with prominent fibrosis and structural remodeling in the LA of AF patients, we noted increased expressions of the gene encoding for type VI collagen, *Col6a1*. *Col6a1* expression was 2.9 times higher in aging SHRs than in age-matched WKY rats ($P = 0.006$). Down-regulation of the genes encoding for proteins involved in cell-cell communication, connexin-43 (C_{x43} ; *Gjal*) and desmocollin 2 (*Dsc2*) was also noted. *Gjal* expression was 2.8 times lower in aging SHRs compared to age-matched WKY rats ($P = 0.04$). Similarly, aging SHRs presented a 2.5-fold lower expression of the *Dsc2* gene

compared to age-matched WKY rats ($P = 0.03$). Additionally, we found a 1.9-fold increase ($P = 0.02$) in the expression of the gene encoding for a *beta* component of the I_{Kur} current (*Kcnab1*) (Fig. 6.2).

Figure 6.2. Expression levels of genes differentially expressed between aging SHR and age-matched WKY rats



$P < 0.05$ for aging SHR versus age-matched WKY rats for all displayed genes using the Mann-Whitney U test

6.4. Discussion

The main findings of the present study were (i) in aging SHR, LA *Pitx2* expression was significantly down-regulated compared to age-matched WKY rats; (ii) among SHR, *Pitx2* expression was significantly negatively correlated with age; (iii) aging SHR presented significantly lower expressions of genes encoding for $I_{Ca,L}$ current, C_{x43} and desmocollin 2; (iv) and higher expressions of genes encoding for type VI collagen and for the *beta* component of the I_{Kur} current compared to age-matched WKY rats.

6.4.1. *Pitx2* insufficiency and atrial arrhythmias in spontaneously hypertensive rats

Pitx2 expression analysis demonstrated significantly lower levels of *Pitx2* in the LA of aging SHR compared to age-matched WKY rats. The exact same category of rats (i.e., aging SHR) was previously shown to present spontaneous, unprovoked episodes of AT, while aging WKY rats did not (Chapter 3). These results suggested a link between *Pitx2* insufficiency and the occurrence of ATs in aging SHR. Indeed, these results are in line with

previous data reported in humans and transgenic mice that provide evidence of a molecular link between *Pitx2* loss-of-function and AF.^{53,154}

While homozygous *Pitx2*^{-/-} mice exhibited embryonic lethality, heterozygous deletion of the *Pitx2* gene isoform permitted viable mice, without obviously altered cardiac morphology and function.¹⁵⁴ Mice that were heterozygous for a *Pitx2* null allele that removed all isoform function had increased susceptibility to pacing-induced atrial arrhythmias.³⁰⁷ In humans, decreased expression of the *Pitx2* gene was demonstrated in the LA of AF patients and, according to Chinchilla et al., down-regulation of the *Pitx2* gene provides the cellular, molecular, and electrophysiological substrates for atrial arrhythmogenesis.⁵³

Additionally to the decreased expression of *Pitx2* in the LA of arrhythmic rats, we also provide evidence that among SHRs, *Pitx2* expression presents a gradual, age-dependent decrease. These results suggest that in SHRs, *Pitx2* down-regulation starts before the occurrence of arrhythmias, despite the fact that at younger ages *Pitx2* expression was not significantly different from that of WKY rats. This observation suggests that timely appropriate therapy in hypertensive patients might prevent AF onset.

However, to date, no study has identified the mechanisms responsible of *Pitx2* insufficiency in AF patients. Preliminary analyses performed in our laboratory suggested that, similarly to what has been reported in patients with breast¹⁹⁶ or prostate cancer,³¹⁷ decreased expression of *Pitx2* in arrhythmic rats could also be due to a methylation process. Further studies will have to confirm these preliminary results. If *Pitx2* down-regulation is indeed due to a methylation process, new therapeutic agents that target this precise mechanism, such as demethylating agents, could open new ways in AF prophylaxis.

6.4.2. Molecular insights into the left atrial arrhythmogenicity of spontaneously hypertensive rats

6.4.2.1. Molecular changes underlying atrial electrical remodeling

Significant shortening of the atrial effective refractory period and of the APD are constant findings in both paroxysmal and persistent AF.⁶⁷ Atrial repolarization and refractoriness are the result of a balance between inward Ca^{2+} and outward K^{+} currents. Thus, decreased APD could result from either decreased inward Ca^{2+} current or increased outward K^{+} current densities.

A significant 63% reduction in the density of $\text{I}_{\text{Ca,L}}$ was observed in atrial myocytes of patients with chronic AF; significantly decreased $\text{I}_{\text{Ca,L}}$ is likely to contribute to the

impairment of atrial mechanical function in these patients.²⁹⁶ Reduced $I_{Ca, L}$ density seems to be mainly due to transcriptional down-regulation of *alpha* subunits.¹⁵⁷ In the present study we did not note any significant transcriptional change in *alpha* subunits, but *beta* 4 and *gamma* 6 subunits of the $I_{Ca, L}$ current were significantly down-regulated in rats that presented atrial arrhythmias compared to control rats. Down-regulation of both *beta* and *gamma* accessory subunits has been previously reported in AF patients.²⁸ Reduction in either of these subunits contribute to a reduction in $I_{Ca, L}$ current density. Bosch et al. demonstrated that depressed transcription of the auxiliary channel subunit *beta* 2 precedes alterations in *alpha* subunits, and in fact any changes in $I_{Ca, L}$ current densities.²⁸

Kcnab1 encodes for the *beta* subunit of the I_{Kur} current and alters the activity of the current.¹⁶⁷ Thus, up-regulation in the *Kcnab1* gene will result in decreased I_{Kur} activity, which would be expected to induce prolonged atrial APD and refractoriness. This observation is quite surprising, given that I_{Kur} blockade was thought to be a promising approach for AF therapy.¹⁷⁹ However, reduced activity of I_{Kur} has already been reported in several AF studies.^{29,297,327} Moreover, Burashnikov et al. demonstrated that I_{Kur} blockade abbreviates APD in AF patients.³⁸ Comparable results were reported by Olson et al., who demonstrated that deficit in *Kcna5* expression, the main gene encoding for the I_{Kur} current, increased susceptibility to arrhythmogenic after-depolarizations and facilitated stress-induced atrial arrhythmias.²³² *Kcna5* loss-of-function mutations were recently reported in familial AF.²³²

6.4.2.2. *Molecular correlates to atrial structural remodeling*

The extracellular matrix provides the skeleton for cardiomyocyte alignment, the basis for force transmission, and assures the geometry of the atria. Substantial changes in the composition of the extracellular matrix, such as abnormal collagen deposition in the atria, increase the heterogeneity of atrial conduction and provide the basis for arrhythmia occurrence and persistence.¹¹⁴

While collagen I and III have generally been considered as the main collagen subtypes of the myocardial matrix, recent studies suggest that non-fibrillar collagen deposition also participates to pathological healing processes.²⁶⁴

In our study, *Col6a1* gene expression, encoding for the non-fibrillar type VI collagen, was significantly higher in arrhythmic, hypertensive rats, compared to age-matched normotensive controls. Similar results were reported by Polyakova et al., who found significantly increased type VI collagen in permanent AF patients compared to sinus rhythm

controls.²⁴⁹ High collagen VI levels were also noted in the hearts of rats with renovascular hypertension.³⁰⁴

6.4.2.3. *Molecular insights into the cell-cell coupling impairment*

Accumulating data suggest a close association between abnormal expression of genes encoding for proteins that exhibit gap junction activity and AF.¹¹³ In our study, aging SHR that presented atrial arrhythmias showed decreased expression of the *Gjal* gene, encoding for C_x43, compared to non-arrhythmic control rats. In patients with rheumatic heart disease, reduction and redistribution of C_x43 was shown to contribute to both initiation and persistence of AF.¹⁸⁰

Dsc2 down-regulation has never been reported in AF patients. In our study, *Dsc2* expression, which encodes for desmocollin 2, was 2.5 times lower in aging, hypertensive rats that presented atrial arrhythmias than in age-matched, normotensive, non-arrhythmic controls.

Mutations in the gene encoding for desmocollin 2 are known to cause arrhythmogenic right ventricular cardiomyopathy, an inherited channelopathy associated with increased risk of ventricular arrhythmias.¹³² Several clinical studies reported increased incidence of supraventricular arrhythmias in patients with arrhythmogenic right ventricular cardiomyopathy and high prevalence of sustained ATs induced at electrophysiological study compared to patients without cardiac disease.³¹ To date, the underlying mechanisms of ATs in arrhythmogenic right ventricular cardiomyopathy are unknown. Long-term follow-up demonstrated that right ventricular abnormalities are frequently progressive, and that the condition can also affect the LV.¹⁷² It has been suggested that supraventricular arrhythmias could be the consequence of right and LV impairment. However, Brembilla-Perrot et al. found no correlation between the presence of spontaneous or inducible atrial arrhythmias and the degree of right ventricular or LV impairment.³¹ These results suggested that the risk of atrial arrhythmias could be directly related to atrial involvement.

This study provides evidence of decreased *Dsc2* expression in rats that presented atrial arrhythmias compared to sinus rhythm controls. These results suggest that *Dsc2* loss-of-function could underlie the increased atrial arrhythmogenicity observed in patients with arrhythmogenic right ventricular cardiomyopathy. Further studies to directly address the consequences of decreased *Dsc2* expression on atrial arrhythmogenicity will be of interest.

6.4.3. Potential limitations

The changes in Ca^{2+} -handling proteins, ion channels, proteins of the extracellular matrix, or cell-cell communication, were only assessed at the mRNA level. There was no investigation about changes at protein or ion current level. Although a change in gene expression would be expected to result into proportional protein levels, other regulatory mechanisms at posttranscriptional or posttranslational levels could also influence protein levels. Also, the small number of animals could have mitigated the differences between groups, particularly for genes for which the fold-change was just below the cut-off point of 1.5.

6.4.4. Conclusions

Similarly to the data reported in AF patients, rats that presented atrial arrhythmias also presented reduced *Pitx2* LA expression and transcriptional levels suggestive of reduced $\text{I}_{\text{Ca, L}}$, I_{Kur} , and C_{x43} densities, and increased type VI collagen. Furthermore, we also provide evidence that in SHRs, *Pitx2* gene expression follows a progressive, age-dependent decrease. This observation suggests that timely appropriate therapy in hypertensive patients might prevent AF onset. We also identified, for the first time, decreased expression of the gene encoding for desmocollin 2 in rats that presented atrial arrhythmias. This finding could explain the increased propensity to atrial arrhythmias of patients with arrhythmogenic right ventricular cardiomyopathy.

CHAPTER 7

A Study of Ventricular Arrhythmogenicity and Molecular Left Ventricular Remodeling in Hypertensive Rats

7.1. Introduction

Left ventricular hypertrophy generally develops as a benign adaptive process, serving to compensate increased hemodynamic burden imposed by persistent high blood pressure. In turn, its evolution has been associated with deleterious outcomes. Left ventricular hypertrophy has been identified as a strong risk factor for sudden death, heart failure and acute myocardial infarction.¹⁶¹ Several studies reported more likelihood of ventricular arrhythmias in the presence of LVH than in hypertensive patients with no signs of LVH,^{110,234} and found a linear relationship between arrhythmia burden and left ventricular mass.¹¹⁰ However, the question of sudden death in hypertensive patients is still a matter of debate, and malignant forms of ventricular arrhythmia (ventricular tachycardia (VT) and ventricular fibrillation (VF)) are still quite rare in this group of patients. James et al. found more frequent PVCs in untreated hypertensive patients with normal left ventricular mass than in the presence of LVH, and suggested that hypertensive patients with LVH might actually experience fewer arrhythmias.¹⁴⁰ Thus, the evidence linking LVH, ventricular arrhythmias and sudden death is, at best, circumstantial, and it remains to be determined whether these arrhythmias are of crucial significance.

In this study, we aimed to assess, in baseline conditions, the occurrence of spontaneous ventricular arrhythmias (i.e., VF and VT) and several other markers of increased ventricular arrhythmogenicity (i.e., PVCs, couplets, bigeminisms, and salvos of PVCs) in young, adult and aging SHR. These parameters were compared to those observed in age-matched normotensive controls. For this study, we used 24-h continuous ECG recordings, acquired with radiotelemetry. We also aimed to provide molecular insights into the LV remodeling of hypertrophied hearts. Accordingly, we performed gene expression analysis in LV samples of young, adult, and aging SHR in comparison to age-matched WKY rats.

7.2. Material and Methods

7.2.1. Animals

Male SHR and normotensive WKY rats were purchased from Elevage Janvier (Le Genest Saint Isle). All animals were housed in a climate-controlled room (21 to 22°C), with a 12-hour light/dark cycle (on 7 AM/off 7 PM), in an accredited animal facility. Rats undergoing ECG recordings (SHR, n = 8; WKY rats, n = 4) were housed individually in polycarbonate cages. All other animals (three groups of SHR, n = 4 each - 14 wks of age, 24 wks of age, and 48 wks of age, respectively, and three groups of age-matched WKY rats, n =

4 each), were housed in groups of 2 to 3 rats per cage under the same controlled conditions. These latter animals, used to assess the alterations in mRNA expression of the 89 selected genes, coincided with the animals used in the previous study to assess atrial expression of *Pitx2* (Chapter 6). All rats were fed standard rat pellets and tap water *ad libitum*.

All experiments were performed in compliance with the French Ministry of Agriculture guidelines for animal experimentation and were approved by the local Animal Ethics Committee.

7.2.2. Transmitter implantation procedure

Radiotelemetry ECG transmitters (TA11 CA-F40; Data Sciences International) were implanted under isoflurane anesthesia in 8 young (14-wk-old) SHR (342 ± 3 g) and 4 young (14-wk-old) WKY rats (397 ± 5 g). Each animal received subcutaneous prophylactic injections of penicillin G (50,000 IU) and ketoprofen (2 mg/kg).

The body of each transmitter was placed in a dorsal, subcutaneous pocket, and sutured to the underlying tissue under aseptic conditions. The two ECG leads were tunneled subcutaneously and secured in a lead II configuration. After the implantation procedure, animals were allowed at least one week of recovery prior to recording.

7.2.3. ECG recording

For baseline quantification of ventricular arrhythmic events, 24-h continuous ECG monitoring was performed on unrestrained, conscious rats. For all SHR and WKY rats, three 24-h continuous ECG recordings were obtained at different ages. A first 24-h recording was performed for each animal at 14 wks of age (further mentioned as young), a second 24-h continuous ECG monitoring was performed at 24 wks of age (further mentioned as adult), followed by a third recording at 48 wks of age (further mentioned as aging). ECG signal capture was accomplished with receivers (RPC-1; Data Sciences International) placed under each experimental cage. Telemetry ECGs were converted to analog signal (Analog ECG Output Adapter R08; Data Sciences International) and routed to a personal computer equipped with a signal acquisition card (NI PCIe-6251; National Instruments). An acquisition program developed in our laboratory using LabVIEW 2009 software (National Instruments) allowed the signal to be continuously recorded with a 2000 Hz sampling frequency and stored on hard disk.

7.2.4. ECG analysis

Electrocardiographic data were analyzed using a program recently developed in our laboratory using LabVIEW 2010 software (National Instruments) to automatically detect R waves and measure RR intervals, as previously described.²³⁹ All ECG tracings were visually assessed and artifactual periods were discarded prior to analysis. Rhythm analysis was visually performed by two independent cardiologists.

Premature ventricular contractions were defined as enlarged QRS complexes, with morphology significantly different compared to sinus rhythm beats. Ventricular couplets were defined as a pair of ventricular premature activations in a row, while the regular succession of a normal sinus beat and a ventricular premature activation was defined as ventricular bigeminism. The succession of 3 PVCs was defined as salvo of PVCs. Ventricular tachycardia was defined as rapid ventricular rhythm (enlarged and distorted QRS complexes) of at least 4 beats. Ventricular fibrillation was defined as wandering, bizarre, irregular baseline, with no clearly identifiable QRS complexes or P waves.

The total numbers of VT and VF episodes, PVCs, ventricular couplets and bigeminisms, and salvos of PVCs, were quantified on the 24-h recordings.

7.2.5. Heart sampling

The three groups of young, adult and aging SHR (n = 4 each) and the three groups of age-matched WKY rats (n = 4 each) were weighted and euthanatized with an intraperitoneal injection of a terminal dose of sodium pentobarbital (> 100 mg/kg).

Heart sampling was performed by a cardiac surgeon, who excised and weighted the hearts, and immediately isolated the LV. A sample originating from the LV lateral wall was obtained for each animal and was immediately (within 2 min after removal) placed in a sterile dry tube and stored at -80°C until processing and analysis.

7.2.6. Ribonucleic acid extraction

Total RNA was isolated from frozen biopsies using TRIzol[®] Reagent (Life Technologies) following manufacturer's instructions. Ribonucleic acid concentration was measured using Nanodrop ND1000 (Thermo Scientific) and quality was verified using the Agilent 2100 BioAnalyser (Agilent Technologies).

7.2.7. Complementary deoxyribonucleic acid synthesis

One µg of total RNA was reverse-transcribed to cDNA by using High-Capacity cDNA

Reverse Transcription Kits (Applied Biosystems) following manufacturer's instructions.

7.2.8. *TaqMan Low-Density Array*

To identify altered gene expression, we used a TaqMan Low-Density Array (TLDA) to examine the expression of 89 human genes encoding for ion channels, proteins of the extracellular matrix, and cell-cell coupling. For the present study, the TLDA card was configured into four identical 89 genes set. For each set of genes we used 6 endogenous control genes, *Ppia*, *beta-actin*, *Gapdh*, *Hprt1*, *Tbp* and *18s RNA*. The cDNA (2 μ L) was mixed with 48 μ L of H₂O and 50 μ L of 2X TaqMan Universal PCR Mix (Applied Biosystems). Each sample (100 μ L) was loaded into a port of the micro-fluid card and run on an ABI 7900HT System (Applied Biosystems) for 2 min at 50°C, 10 min at 94.5°C, followed by 40 cycles for 30 s at 97°C and 1 min at 59.7°C.

7.2.9. *Data Analysis*

The TLDA data were analyzed by SDS 2.3 software (Applied Biosystems). Threshold cycle data for all target genes and control genes were transformed into molecule number (attomoles) considering PCR efficiency of 100%. Gene expression levels were normalized using TBP. The 89 studied genes coincided with those studied in LA samples of SHR and WKY rats (Chapter 6). A detailed list of these 89 genes is provided in Appendix 1.

7.2.10. *Statistics*

Data are expressed as the mean \pm SEM or median and range, as appropriate. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test with Holm's method for multiple comparisons. Differences within the same group were tested for significance with the Wilcoxon signed-rank test. Nonparametric repeated-measures ANOVA (Friedman test) were used for repeatability data.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software.

7.3. Results

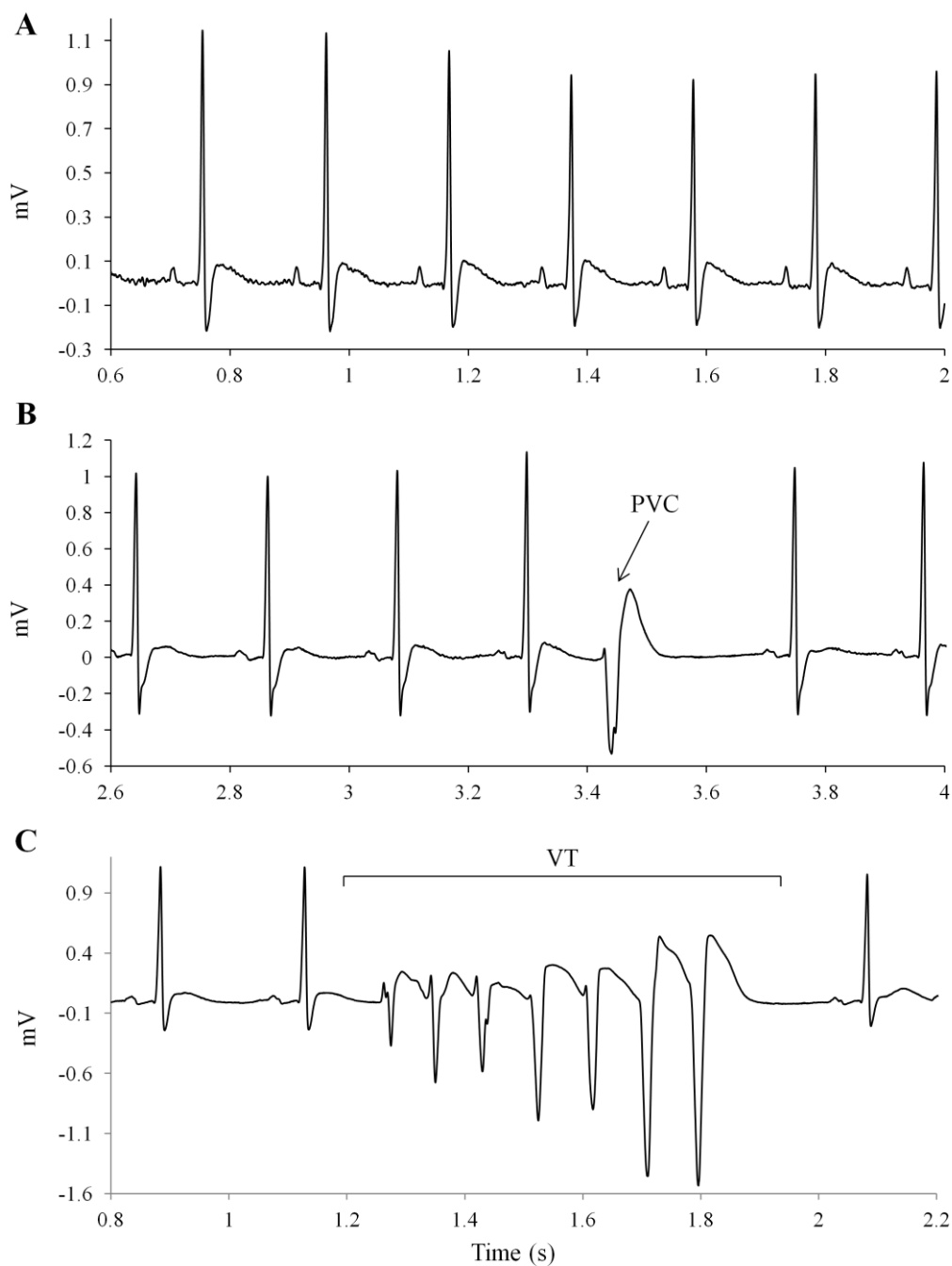
Heart weight-to-body weight ratios were calculated in order to assess the presence and the severity of LVH in the three groups of SHRs. In young rats, heart weight-to-body weight ratios were not significantly different between SHRs and WKY rats (*P* = 0.11). In adult SHRs, heart weight-to-body weight ratios were 30 ± 2 % higher compared to those of age-

matched WKY rats ($P = 0.02$), while in aging SHR heart weight-to-body weight ratios were $56 \pm 3 \%$ higher than in age-matched WKY rats ($P = 0.01$).

7.3.1. Ventricular arrhythmic activity at baseline

Figure 7.1 depicts typical ECG tracings recorded in adult SHRs.

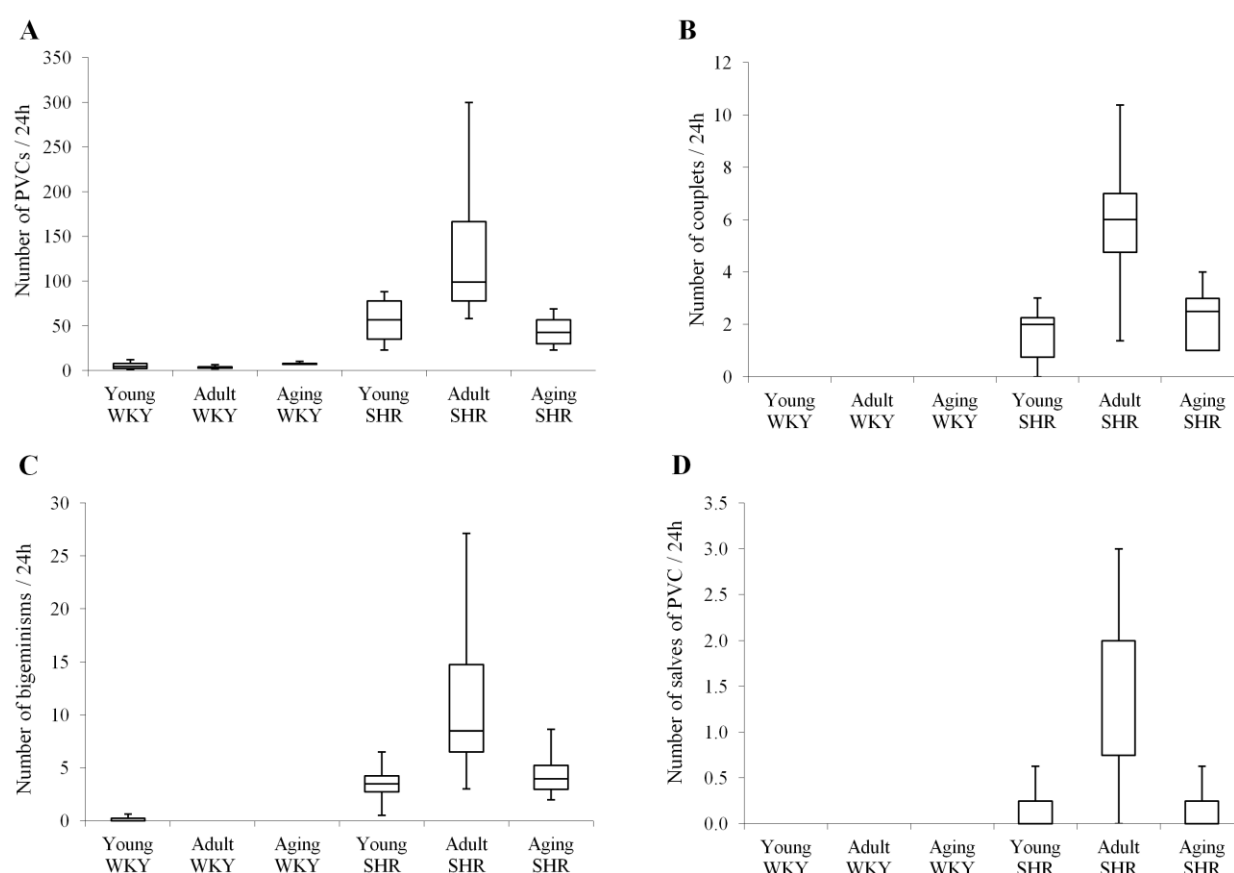
Figure 7.1. ECG tracings in adult SHRs showing – (A) sinus rhythm; (B) PVC; (C) unsustained VT



PVC – premature ventricular contraction, VT – ventricular tachycardia

Regardless of their age, SHR rats presented significantly more frequent PVCs (Fig. 7.2, A) compared to age-matched WKY rats (all $P = 0.004$). Similar differences were observed in the numbers of ventricular couplets (Fig. 7.2, B), bigeminisms (Fig. 7.2, C) and salves of PVCs (Fig. 7.2, D). In fact, none of the WKY rats, regardless of their age, presented ventricular couplets or salves of PVCs, and only one young WKY rat presented a single ventricular bigeminism.

Figure 7.2. Number of ventricular arrhythmic events - (A) PVCs; (B) ventricular couplets; (C) ventricular bigeminisms; and (D) salves of PVCs, during 24-h ECG recordings in young, adult and aging WKY rats ($n = 4$ each) and age-matched SHR rats ($n = 8$ each)



PVCs – premature ventricular contractions. Data are expressed as median and IQR. The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile.

Among SHR rats, the highest number of PVCs was recorded at adult age (median 99, range 58 – 309 PVCs / 24h), compared to both young age (median 57, range 23 – 88 PVCs / 24h, $P = 0.008$) and aging SHR rats (median 43, range 23 – 69 PVCs / 24h, $P = 0.008$), while no difference was observed between young and aging SHR rats ($P = 0.46$). Similarly, the numbers

of ventricular couplets ($P = 0.02$), bigeminisms ($P = 0.01$), and salves of PVCs ($P = 0.04$) were significantly greater at adult age than at aging or young age SHR. In multiple comparison, the differences between groups remained significant ($P = 0.01$ for PVCs, $P = 0.02$ for ventricular couplets, $P = 0.04$ for ventricular bigeminisms, and $P = 0.02$ for salves of PVCs).

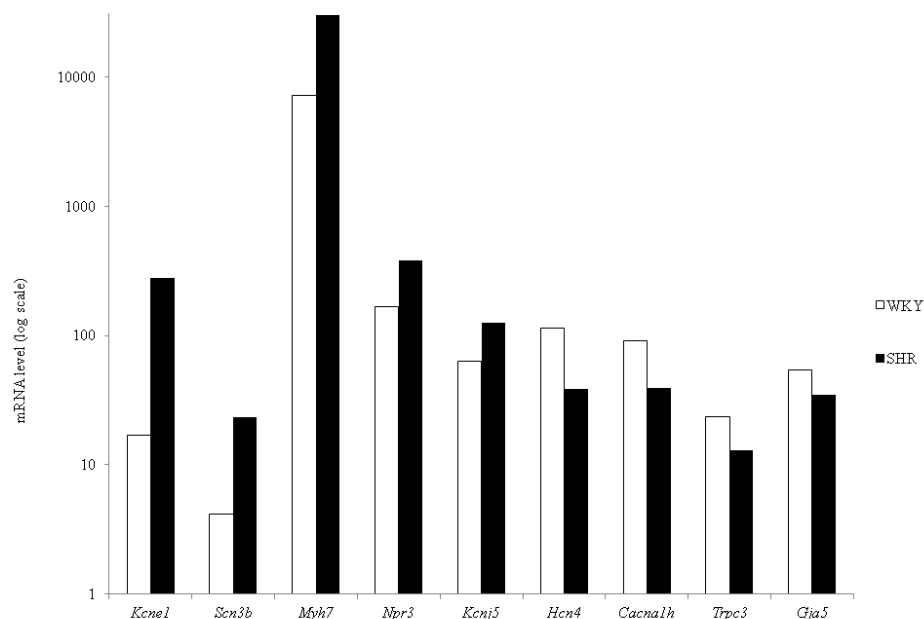
One adult SHR presented 3 episodes of unsustained VT during the 24-h recording, while 2 adult SHRs presented a single episode of unsustained VT / 24h. No episode of VT was recorded in aging SHRs. Similarly, no VTs were recorded in young SHRs, or in WKY rats, regardless of their age. No episode of VF was recorded in baseline conditions in neither of the SHRs or WKY rats.

7.3.2. Molecular profile of hypertensive rats versus normotensive controls

For this analysis, we compared LV gene expression levels of SHRs with those of age-matched WKY rats. To analyze the data we applied a 1.5 fold change cut-off point to the entire data set.

Nine genes (10%) of the total of 89 studied genes were differentially expressed between young SHRs and age-matched WKY rats (Fig. 7.3). Among these 9 genes, 5 were up-regulated and 4 were down-regulated.

Figure 7.3. Expression of genes differentially expressed between young SHRs and age-matched WKY rats

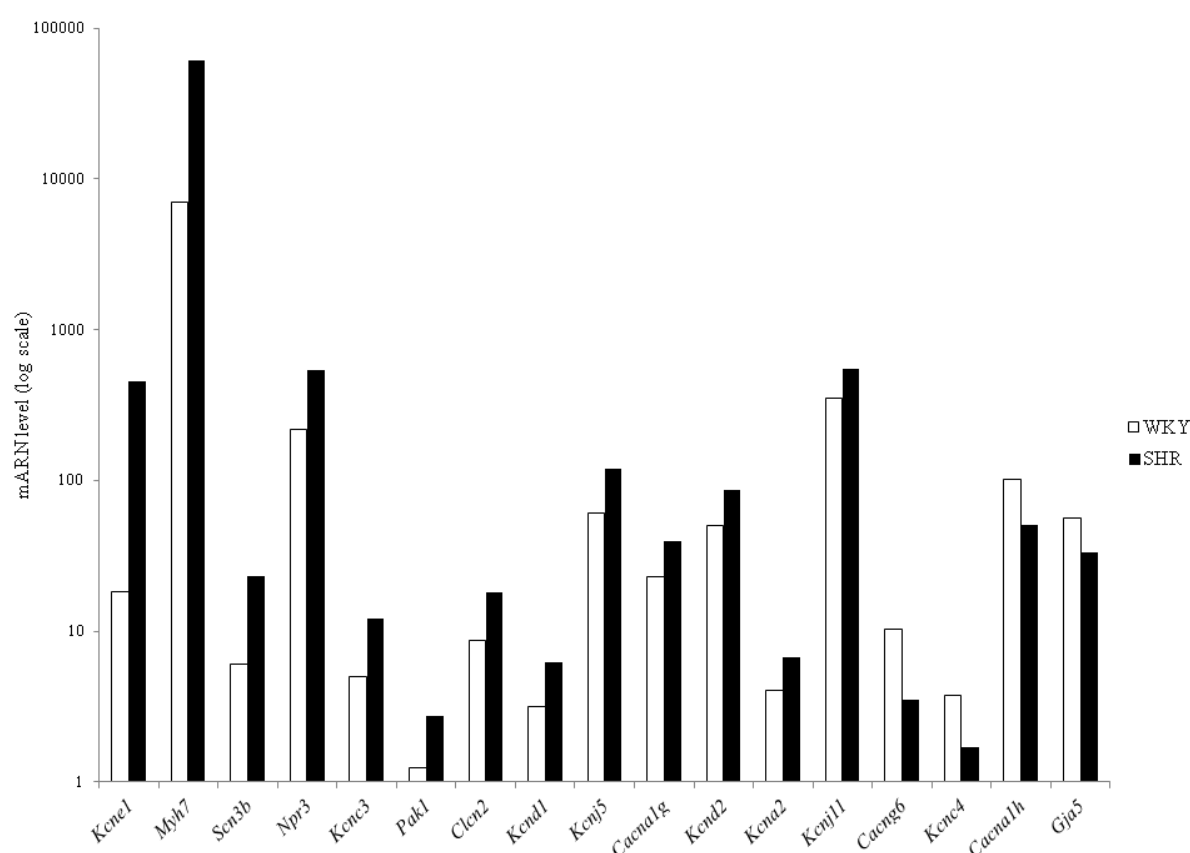


$P < 0.05$ for young SHRs versus age-matched WKY rats for all displayed genes using the Mann-Whitney U test

The main genes identified in this group had a tendency to be rather up-regulated. The absolute fold-change values of overexpressed genes were higher than those of underexpressed genes (30.6 total fold-change for overexpressed genes *versus* 8.7 total fold-change for underexpressed genes).

In adult rats, we identified 17 genes (19%) of the total of 89 studied genes to be differentially expressed between SHRs and WKY rats (Fig. 7.4). Among these 17 genes, 13 genes were up-regulated and 4 genes were down-regulated.

Figure 7.4. Expression of genes differentially expressed between adult SHRs and age-matched WKY rats



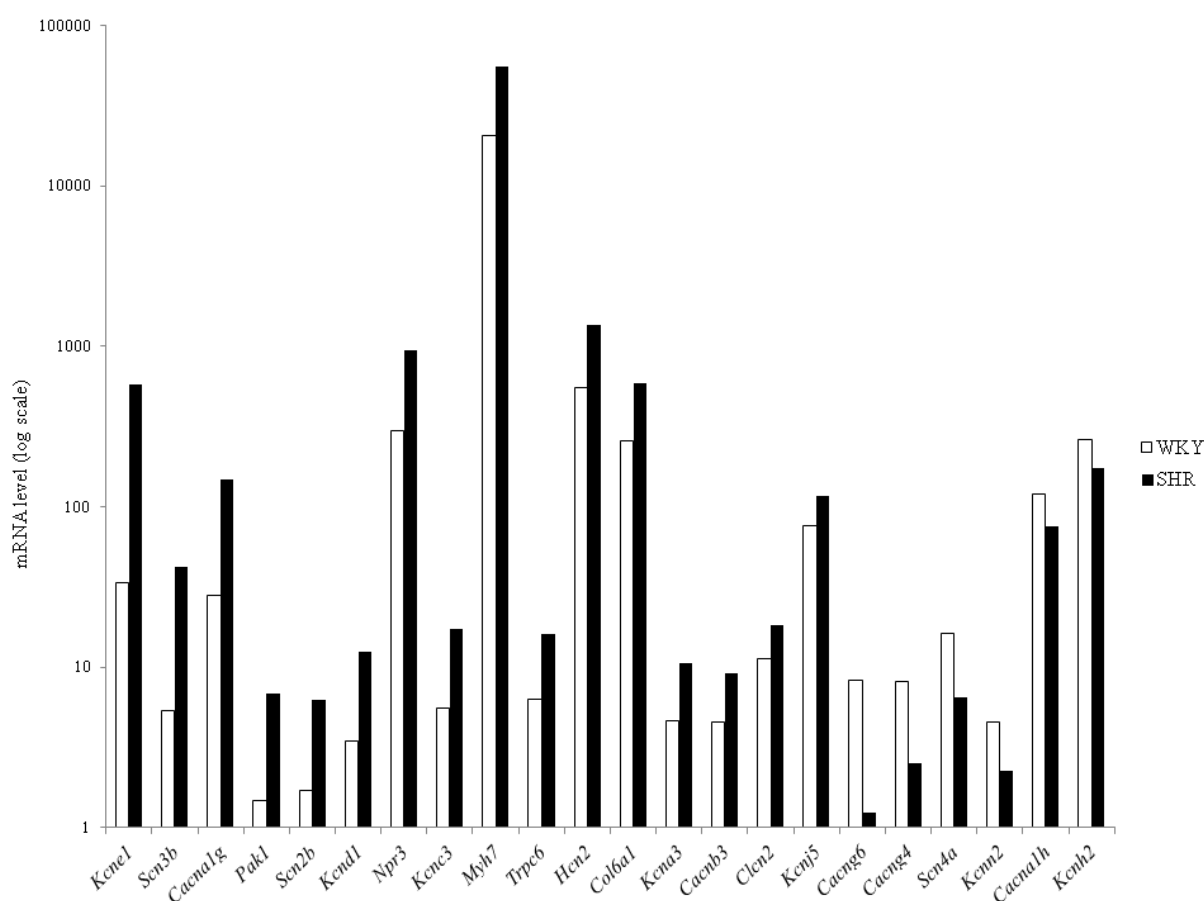
$P < 0.05$ for adult SHRs *versus* age-matched WKY rats for all displayed genes using the Mann-Whitney U test

Similarly to what we observed in young rats, the main genes identified in this group had a tendency to be rather up-regulated. The absolute fold-change values of overexpressed genes were higher than those of underexpressed genes (57.3 total fold-change for overexpressed genes *versus* 8.9 total fold-change for underexpressed genes). Seven of the 9 genes that were deregulated in young SHRs presented the same pattern in adult SHRs. *Kcne1* (gene

expression 24.8 times higher in adult SHR compared to adult WKY rats *versus* 16.5 times higher in young SHR compared to young WKY rats), and *Myh7* (gene expression 8.8 times higher in adult SHR compared to adult WKY rats *versus* 4.2 times higher in young SHR compared to young WKY rats) were significantly more up-regulated in adult SHR than in young SHR compared to age-matched WKY rats. All other genes were equally deregulated in young and adult SHR age compared to age-matched WKY rats.

In aging rats, 22 genes (25%) of the total of 89 studied genes were differentially expressed between SHR and WKY rats (Fig. 7.5). Among these 22 genes, 16 genes were up-regulated and 6 genes were down-regulated.

Figure 7.5. Expression of genes differentially expressed between aging SHR and age-matched WKY rats

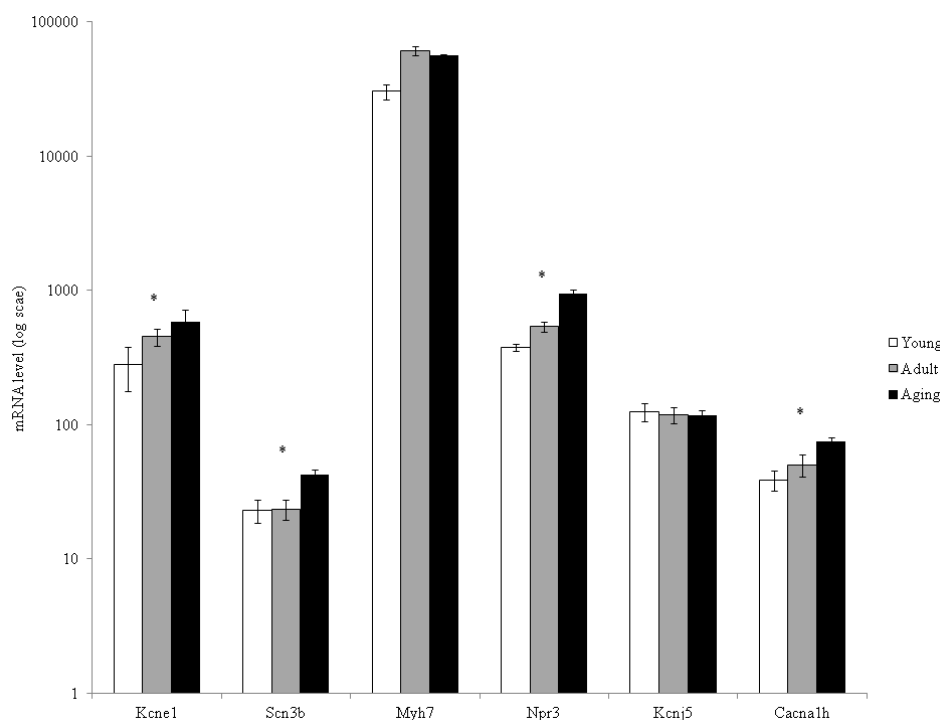


$P < 0.05$ for aging SHR *versus* age-matched WKY rats for all displayed genes using the Mann-Whitney U test.

Similarly to what we observed in young and adult rats, the main genes identified in this group also had a tendency to be up-regulated. Eleven of the 17 genes that were deregulated in adult SHR presented the same pattern in aging rats. *Kcne1* (gene expression 24.8 times higher in adult SHR compared to adult WKY rats *versus* 17.4 times higher in aging SHR

compared to aging WKY rats) and *Myh7* (gene expression 8.8 times higher in adult SHR compared to adult WKY rats *versus* 2.7 times higher in aging SHR compared to aging WKY rats) were significantly more up-regulated in adult SHR than in aging SHR compared to age-matched WKY rats. *Scn3b* (gene expression 3.9 times higher in adult SHR compared to adult WKY rats *versus* 7.9 times higher in aging SHR compared to aging WKY rats), *Pak1* (gene expression 2.2 times higher in adult SHR compared to adult WKY rats *versus* 4.7 times higher in aging SHR compared to aging WKY rats), *Kcnd1* (gene expression 2.0 times higher in adult SHR compared to adult WKY rats *versus* 3.6 times higher in aging SHR compared to aging WKY rats), *Cacnalg* (gene expression 1.7 times higher in adult SHR compared to adult WKY rats *versus* 5.2 times higher in aging SHR compared to aging WKY rats), and *Cacng6* (gene expression 3.0 times lower in adult SHR compared to adult WKY rats *versus* 6.7 times lower in aging SHR compared to aging WKY rats) were significantly more deregulated in aging SHR than in adult SHR compared to age-matched WKY rats. The rest of the genes were equally deregulated in adult and aging SHR compared to age-matched WKY rats.

Figure 7.6. Expression levels of genes deregulated in all SHR groups according to age



Data are expressed as the mean \pm SEM. * - gene expressions were significantly correlated with age

When molecular profile was superimposed for the 3 groups of SHRs, 6 genes appeared deregulated in all age-groups compared to age-matched WKY rats: *Kcne1*, *Scn3b*, *Myh7*,

Npr3, *Kcnj5*, and *Cacna1h* (Fig. 7.6). The first 5 genes were up-regulated in all SHR groups compared to age-matched WKY rats, while the last gene was significantly down-regulated in SHRs of all ages compared to age-matched WKY rats.

In SHRs, *Kcne1* (Spearman $r = 0.56$, $P = 0.05$), *Scn3b* (Spearman $r = 0.62$, $P = 0.03$), *Npr3* (Spearman $r = 0.92$, $P < 0.001$), and *Cacna1h* (Spearman $r = 0.68$, $P = 0.02$) expressions were significantly correlated with age (Fig. 7.6). Neither *Kcnj5* nor *Myh7* expressions were significantly correlated with age (both $P > 0.05$).

7.4. Discussion

The main findings of the present study were (i) regardless of their age, hypertensive rats presented more frequent ventricular arrhythmic events compared to age-matched normotensive rats; (ii) adult SHRs presented spontaneous, unsustained episodes of VT, while neither young or aging SHRs did so; (iii) hypertensive rats displayed a gain-of-function molecular pattern compared to age-matched WKY rats; (iv) in SHRs, the number of deregulated genes increased with age; and (v) 6 genes were significantly deregulated in all SHRs, regardless of their age, and the expression of 4 of these genes was significantly correlated with age.

7.4.1. Evolved versus early-stage left ventricular hypertrophy – which of them is really arrhythmogenic?

A number of studies have amassed evidence that within the same age group the incidence of arrhythmias becomes higher in the presence of LVH. However, most of these studies did not find sustained episodes of VT or VF, but rather isolated PVCs, sometimes bigeminal or multi-form.^{204,303}

Our results confirmed the presence of higher ventricular ectopy in hypertensive rats, regardless of their age, compared to age-matched normotensive controls. Nevertheless, the number of both isolated (PVCs) and more complex (i.e., ventricular couplets, bigeminisms, and salves of PVCs) ventricular arrhythmic events was still quite small, with a median of less than 100 PVCs / 24h in all SHR groups, regardless of their age.

According to heart weight-to-body weight ratios, as expected, LVH was not yet present in young SHRs; early-stage LVH was present in adult SHRs, while LVH was much more severe in aging SHRs. Interestingly, adult SHRs were the only ones who presented episodes of unsustained VT, while no such episodes were recorded in young or aging SHRs. These results suggested that early-stage LVH could in fact be more arrhythmogenic than evolved,

chronic LVH. Comparable results were reported in several clinical studies, who also failed to prove a correlation between the severity of LVH and the number of complex ventricular arrhythmias.^{107,190,298} In a rat model of LVH, James et al. tested the effect of sudden blood pressure changes on normal and hypertrophied isolated hearts and succeeded to induce ventricular arrhythmias in normal, but not in hypertrophied hearts.¹³⁹ When experiments were conducted in cation-depleted environment, hypertrophied hearts were particularly sensitive to sudden blood pressure changes and presented significantly more frequent and more severe ventricular arrhythmias than normal hearts. The authors concluded that not ventricular hypertrophy itself, but the combination of hypertrophy and other conditions rather frequent in hypertensive patients, such as cation depletion induced by diuretic therapy, could explain the occurrence of ventricular arrhythmias in patients with LVH.¹³⁹ In the same vein, Evans et al. demonstrated that combined low magnesium and low potassium perfusion increase the complexity of ventricular arrhythmias in hypertrophied hearts, VT occurring in 9 of 10 SHR but in only 2 of 6 normotensive controls.⁸⁶ The potential role of antihypertensive therapy in ventricular arrhythmia occurrence was also suggested by the study of Mayet et al., who demonstrated significantly more frequent ventricular ectopy in hypertensive patients who had received long-term antihypertensive therapy compared to untreated hypertensive patients.²⁰³

7.4.2. Left ventricular molecular remodeling in hypertrophied hearts

As result of persistently increased blood pressure and secondary LVH, the heart undergoes a complex process of electrical, structural, and cell-cell coupling remodeling. The wide array of genes involved in physiological and pathological cardiac growth supports the hypothesis of a genetic contribution to LVH.^{11,148} The use of hypertensive rats of different ages allowed us to assess the progression of LV molecular remodeling according to age.

In SHRs, transcriptional changes presented a gain-of-function pattern, compared to age-matched WKY rats. This was the case for genes encoding particularly ion channels, but also proteins of the extracellular matrix, or cytoskeletal genes. A similar pattern, with up-regulated genes predominating over down-regulated genes, was demonstrated in rats with chronic pressure overload.³¹⁶

The number of genes deregulated in SHRs compared to age-matched WKY rats increased with age, from 9 deregulated genes in young SHRs, to 17 deregulated genes in adult SHRs, and finally to 22 deregulated genes in aging SHRs. Overall, these results suggest a progressive molecular remodeling in the LV of hypertensive rats with advancing age.

Myh7 gene expression was significantly up-regulated in all SHR groups compared to normotensive controls. This gene encodes for the cardiac *beta*-myosin heavy chain, which is a marker for cardiac hypertrophy.²⁴¹ *Col6a1* mRNA levels, encoding for type VI collagen, were more than 2.2 times higher in aging SHRs compared to age-matched WKY rats, but no difference was observed in *Col6a1* between young or adult SHRs and age-matched WKY rats. The age-dependency of genes encoding for collagen *alpha* subunits in SHRs has already been reported.²⁵⁴

Data regarding K^+ channels densities in hypertension and LVH are highly discordant.^{34,250,282} The variability of heart transcriptome is highly dependent on the model, suggesting that each model of hypertension develops its own transcriptional adaptations.⁴¹ Different animal specie or different degree and time-course of development of hypertrophy could also contribute to the discordance of available data. In the present study, SHRs presented significantly higher expressions of *Kcnc3*, *Kcnd1* and *Kcnd2* genes, encoding for the I_{to} current, compared to age-matched WKY rats. These results are in accordance with previous studies that reported increased I_{to} densities in hypertrophied hearts.²⁸² Several other genes, encoding for the I_K current, were also significantly up-regulated in all SHR groups compared to age-matched WKY rats. Our results pointed out particularly one gene, *Kcne1*, encoding for the I_{Ks} current, which was significantly overexpressed in all SHR groups, regardless of their age. Similar results have already been reported in rats with renovascular hypertension and LVH,¹⁹² as well as post-myocardial infarction.³²³

The classical up-regulation of genes encoding for the $I_{Ca,L}$ current²⁵⁹ was not detected in all SHR groups. *Cacnb3* overexpression was only observed in aging SHRs, but not in young or adult SHRs. This observation supports a link between $I_{Ca,L}$ current densities and the grade of LVH. This hypothesis is also supported by previous data showing a strong correlation between $I_{Ca,L}$ density and the severity of hypertension.²⁸⁹ Moreover, our results indicated an overexpression of genes encoding for the T-type Ca^{2+} current ($I_{Ca,T}$). *Cacnalg* expression, encoding for the $I_{Ca,T}$ current, was significantly up-regulated in adult SHRs compared to age-matched WKY rats, and even higher expressions of *Cacnalg* were observed in aging SHRs. These results suggested that in rat hearts, $I_{Ca,T}$ could be influenced by the duration of hypertension and the severity of LVH. However, $I_{Ca,T}$ does not seem to be expressed in cells isolated from either normal, hypertrophied, or failing human ventricles.²²⁵ Therefore, a role for this current in human LVH is unlikely.

The fact that 6 genes were deregulated in all SHRs, regardless of their age, point to a

potential implication of these genes in hypertension. All these 6 genes exhibited the same directional change (either up-regulation or down-regulation) in all SHR groups. Moreover, expression levels of 4 of these 6 genes were significantly correlated with age, supporting a potential implication of these genes in adaptive pathophysiological mechanisms related to hypertension and in LVH development. Thus, *Kcne1*, *Scnb3*, *Npr3*, and *Cacna1h* could be potential specific markers of hypertension-induced LVH.

7.4.3. Potential limitations

The changes in ion channels, proteins of the extracellular matrix, or cell-cell coupling, were only assessed at the mRNA level. There was no investigation about changes at protein or ion current level. Although a change in gene expression would be expected to result into proportional protein levels, other regulatory mechanisms at posttranscriptional or posttranslational levels could also influence protein levels. Also, the small number of animals could have mitigated differences between groups, particularly for genes for which the fold-change was just below the cut-off point of 1.5.

7.4.4. Conclusions

Our study confirmed the presence of higher ventricular ectopy in hypertensive rats, regardless of their age, compared to age-matched normotensive controls. We also provide evidence that in this model, early-stage LVH is more arrhythmogenic than evolved, chronic LVH. A gain-of-function pattern of the transcriptional changes was present in SHRs compared to age-matched WKY rats. Our results suggest that in this model the extent of LV molecular remodeling progresses with age.

CHAPTER 8

A Study of Inflammatory Syndrome in Atrial Fibrillation Patients

8.1. Introduction

Over the past decade there has been accumulating evidence implicating inflammation in the pathogenesis of AF and AF-related complications.²⁷ Observation of inflammatory infiltrates, myocyte necrosis, and fibrosis in atrial biopsies from patients with lone AF refractory to antiarrhythmic drug therapy supports the hypothesis that AF is closely related to an inflammatory process.⁹⁸ The frequent occurrence of AF in patients with inflammatory conditions such as myocarditis, pericarditis, or after cardiac surgery, also suggests a contribution of inflammation to AF onset.^{27,61}

Inflammatory cytokines such as CRP or IL-6, have been shown to be associated with the future development and recurrence of AF, and the likelihood of successful cardioversion.^{16,193} A multivariate analysis of the Cardiovascular Health Study, including 5,806 patients, found CRP levels to be predictive of both the presence and the future development of AF, even after adjustment for potential confounders.¹⁶ Furthermore, longer duration of AF has been found to be associated with higher levels of hs-CRP, suggesting a link between AF burden and systemic inflammation.³¹⁰

However, the origin and the extent of the inflammatory syndrome in AF patients remain incompletely understood. Moreover, the existence of an inflammatory syndrome in the entire population of AF patients is a matter of debate, given that several studies failed to demonstrate elevated levels of inflammatory proteins in AF patients.^{83,188,200,295}

Therefore, we aimed to assess the presence, the extent and the origin of an inflammatory syndrome in patients with different clinical forms of AF in comparison to sinus rhythm controls. For this study, we performed multilevel intracardiac and extracardiac measurements of several inflammatory proteins.

8.2. Material and Methods

8.2.1. Patient selection

A total of 81 patients scheduled for electrophysiological study or catheter ablation procedures at the Rhythmology Department of the Louis Pradel Cardiologic Hospital in Lyon, France, between August 2008 and June 2010 were screened for participation in this study. The control group included 9 patients with left-sided accessory pathway Wolff-Parkinson-White syndrome and without any history of AF.

A total of 39 patients with paroxysmal AF and 33 patients with persistent AF were enrolled. According to the 2010 ESC Guidelines for the Management of AF,⁸⁵ AF was defined

as ‘paroxysmal’ when the arrhythmia was spontaneously reversible to sinus rhythm within 7 days and ‘persistent’ when the AF episode persisted for more than 7 days or pharmacological or electrical cardioversion were required to terminate the arrhythmia.

Exclusion criteria included recent history of an acute cardiovascular or cerebrovascular event, major trauma or surgery, apparent ischemic heart disease, malignancy, connective tissue or inflammatory disease, acute or chronic infection, and pulmonary, hepatic, or renal impairment. Patients under ongoing anti-inflammatory or steroid treatment were also excluded.

A detailed medical and drug history was obtained for each patient enrolled. All patients underwent physical examination and routine laboratory tests. The LA area was assessed by transthoracic echocardiography prior to electrophysiological study or catheter ablation procedures. For each patient, the stroke risk was evaluated according to current recommendations using the CHA₂DS₂VASc score.⁸⁵ This score assigns one point for each of the following risk factors for patients with AF: congestive heart failure, hypertension, age 65 to 74 years, diabetes, female sex, and vascular disease. Two points are assigned for a history of stroke or transient ischemic attack and age ≥ 75 years. For AF patients, the moment of the first arrhythmic episode was documented based on ECG documentation or physician-confirmed diagnosis. The presence or absence of AF at the moment of blood drawn was assessed electrocardiographically.

All participants provided written informed consent in accordance with the requirements of the local ethics committee. The study protocol complied with the Declaration of Helsinki and was approved by the South-East II People’s Protection Committee and the Advisory Committee on Information Processing in Research in the Field of Health.

8.2.2. *Blood sampling and laboratory analysis*

Blood sampling was performed during routine cardiac catheterization, prior to any ablations or administration of heparin. Blood samples were obtained serially from the femoral vein and coronary sinus. The LA was accessed through trans-septal puncture and a third blood sample was taken from the LA *via* the trans-septal sheath. Blood samples from the left superior PV were collected *via* the trans-septal sheath advanced into the proximal segment of the sampled vein.

Vacutainer (BD Plymouth, UK) tubes (6.0 mL) containing ethylenediaminetetraacetic acid were used. The tubes were stored and shipped on ice within 30 min after sampling.

Serum concentrations of soluble intercellular adhesion molecule-1 (sICAM-1) and TGF- β 1 were measured using commercially available quantitative enzyme linked immunosorbent assay kits (Human TGF- β 1 and Human sICAM-1/CD54 Quantikine ELISA kits; R&D Systems, Inc., Minneapolis, MN). Luminex methodology was used to measure serum levels of vascular endothelial growth factor (VEGF) and IL-8, with R&D System kits (Human VEGF and Human CXCL8/IL-8 Fluorokine Multi-analyte Profiling ELISA; R&D Systems, Inc.). The technician performing the analysis was not aware of the patient's diagnosis or rhythm. The lower limits of detection were 4 pg/mL for VEGF, IL-8, and TGF- β 1, and 30 ng/mL for sICAM-1.

8.2.3. Statistics

Values are expressed as the mean \pm SEM. Comparisons between control and AF groups were made using the Mann-Whitney *U* test. Categorical data were compared using the chi-square test. Correlations were ascertained with Spearman's rank correlation method. Stepwise multiple regression analysis was performed to identify independent predictors of VEGF levels.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software.

8.3. Results

8.3.1. Patient clinical characteristics

Demographic and clinical data regarding AF patients and control subjects are summarized in Table 8.1.

There was no significant difference among the three groups in terms of sex ratio, smoking status, history of hypertension or diabetes, or statin treatment (all *P* > 0.10). Atrial fibrillation patients were more likely to be treated with antialdosteronics (*P* = 0.02) and ACEIs or ARBs (*P* < 0.01). They were also older (*P* < 0.01) and had higher body mass index (*P* < 0.01). As expected, LA area as assessed by echocardiography in the parasternal long axis was higher in paroxysmal AF patients than in controls, and even higher in persistent AF patients (*P* = 0.001). Persistent AF patients were more frequently in arrhythmia at the moment of blood draw compared to paroxysmal AF patients (*P* < 0.001).

Table 8.1. Clinical characteristics of study groups

Variables	Control (n=9)	Paroxysmal AF (n=39)	Persistent AF (n=33)	<i>P</i> -value
Age (years)	43 ± 18	56 ± 9	58 ± 8	< 0.01
Men (n, %)	6 (67%)	32 (82%)	28 (85%)	0.46
Body mass index (kg/m ²)	22.2 ± 3.5	27.1 ± 3.4	29.2 ± 5.2	< 0.01
Hypertension (n, %)	2 (22%)	16 (41%)	19 (58%)	0.12
Diabetes (n, %)	0 (0%)	5 (13%)	3 (9%)	0.50
Smokers (n, %)	1 (11%)	12 (31%)	11 (33%)	0.42
History of AF (months)	N/App	95 ± 86	77 ± 58	0.29
Antialdosteronics (n, %)	0 (0%)	2 (5%)	7 (21%)	0.02
Statins (n, %)	0 (0%)	9 (23%)	10 (30%)	0.16
ACEIs / ARBs (n, %)	0 (0%)	8 (21%)	17 (52%)	< 0.01
Left atrial area (cm ²)	15.7 ± 2.5	20.6 ± 3.7	25.2 ± 6.1	< 0.01
AF during blood sampling (n, %)	N/App	8 (21%)	24 (73%)	< 0.001
Prior ablation procedure (n, %)	N/App	12 (31%)	13 (39%)	0.26
CHA ₂ DS ₂ VASc score	0.63 ± 0.26	1.33 ± 0.21	1.21 ± 0.21	0.34

ACEIs - angiotensin converting enzyme inhibitors, AF - atrial fibrillation, ARBs - angiotensin receptor blockers, N/App - not applicable. Quantitative data are expressed as the mean ± SEM. Categorical data are expressed as number (percentage). *P*-values refer to comparisons between groups based on multiple comparisons ANOVA.

Twelve patients in the paroxysmal AF group (31%) and 13 patients in the persistent AF group (39%) had single or multiple prior ablation procedures (*P* = 0.26). Mean duration of AF history was 95 ± 86 months in paroxysmal AF patients and 77 ± 58 months in persistent AF patients (*P* = 0.29). None of the control patients presented AF during electrophysiological study or ablation procedure.

8.3.2. *Inflammatory marker levels*

Mean levels of inflammatory markers in the three studied groups are presented in Table 8.2.

There were no significant differences among the three groups regarding IL-8, sICAM-1, or TGF-β1 levels at any of the sampling sites (all *P* > 0.05). Similarly, CRP levels (measured in peripheral blood only) did not differ among the three groups (*P* = 0.60).

Table 8.2. Inflammatory marker levels in the three study groups

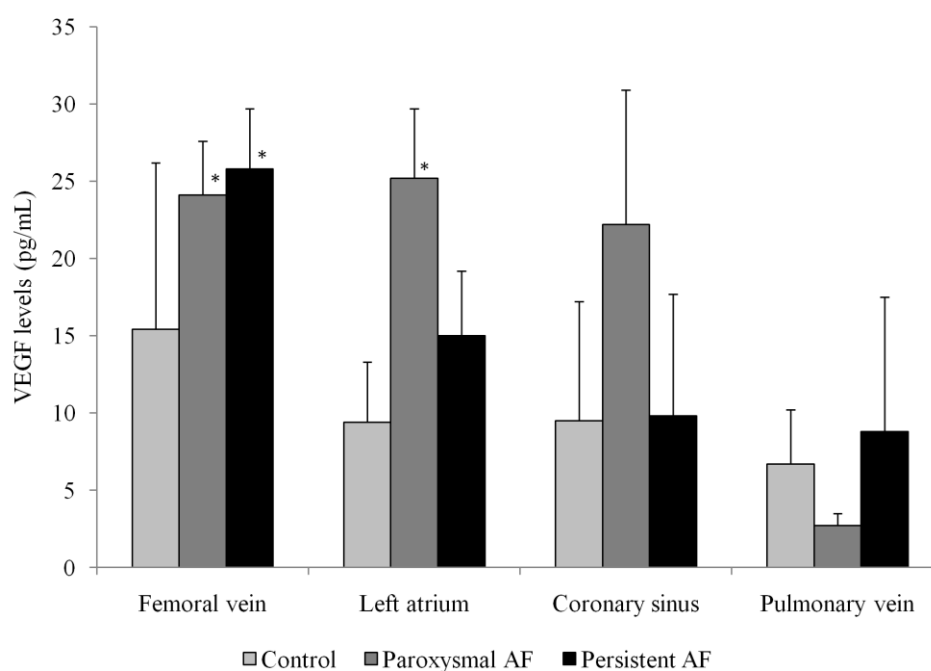
	Control (n=9)	Paroxysmal AF (n=39)	<i>P</i> -value ^a	Persistent AF (n=33)	<i>P</i> -value ^b
<i>VEGF (pg/mL)</i>					
Femoral vein	15.4 ± 10.8	24.1 ± 3.5	0.03	25.8 ± 3.9	0.02
Left atrium	9.4 ± 3.9	25.2 ± 4.5	0.05	15.0 ± 4.2	0.32
Coronary sinus	9.5 ± 7.7	22.2 ± 8.7	0.77	9.8 ± 7.9	0.81
Pulmonary vein	6.7 ± 3.5	2.7 ± 0.8	0.15	8.8 ± 8.7	0.72
<i>IL8 (pg/mL)</i>					
Femoral vein	7.0 ± 4.9	6.9 ± 0.6	0.21	8.0 ± 1.4	0.67
Left atrium	7.7 ± 3.4	7.8 ± 0.7	0.17	8.0 ± 1.7	0.37
<i>sICAM-1 (ng/mL)</i>					
Femoral vein	161.4 ± 15.8	178.5 ± 8.3	0.36	179.7 ± 7.0	0.25
Left atrium	160.1 ± 15.5	174.4 ± 8.1	0.43	173.2 ± 7.1	0.46
<i>TGF-β1 (ng/mL)</i>					
Femoral vein	25.0 ± 10.0	15.9 ± 1.8	0.63	22.3 ± 3.2	0.87
Left atrium	13.1 ± 3.3	15.7 ± 2.5	0.88	15.5 ± 2.0	0.41
<i>CRP (μg/mL)</i>					
Peripheral blood	9.6 ± 7.0	3.6 ± 0.9	0.10	6.6 ± 2.4	0.60

AF - atrial fibrillation. ^a - paroxysmal AF *versus* control. ^b - persistent AF *versus* control. Data are expressed as the mean ± SEM. *P*-values refer to comparisons between groups based on the Mann-Whitney *U* test.

Peripheral levels of VEGF were higher in both paroxysmal and persistent AF patients compared to controls ($P = 0.03$ and $P = 0.02$, respectively) (Fig. 8.1). In the LA, VEGF levels were higher in the paroxysmal AF group compared to control group ($P = 0.05$); however, there was no significant difference between LA VEGF levels in persistent AF patients *versus* controls ($P = 0.32$) (Fig. 8.1).

Although not statistically significant ($P > 0.10$), coronary sinus levels of VEGF were higher in paroxysmal AF patients compared to both controls and persistent AF patients (22.2 ± 8.7 pg/mL in paroxysmal AF patients *versus* 9.5 ± 7.7 pg/mL in control patients and 9.8 ± 7.9 pg/mL in persistent AF patients) (Fig. 8.1). Pulmonary vein levels of VEGF did not differ significantly among the three groups ($P = 0.73$).

Figure 8.1. Mean levels of vascular endothelial growth factor in the femoral vein, left atrium, coronary sinus, and pulmonary vein in the three study groups



AF – atrial fibrillation, VEGF – vascular endothelial growth factor. Data are expressed as the mean \pm SEM.

* - $P < 0.05$ versus control using the Mann-Whitney U test.

8.3.3. Inter-site vascular endothelial growth factor gradients

In patients with paroxysmal AF, no significant difference was observed between peripheral and LA or coronary sinus and LA levels of VEGF (both $P > 0.05$); however, both LA and peripheral levels of VEGF were significantly higher than PV levels (both $P < 0.01$). In patients with persistent AF, peripheral levels of VEGF were higher than LA levels ($P = 0.05$); however, no significant difference was observed among LA, coronary sinus, and PV levels of VEGF ($P > 0.05$).

8.3.4. Vascular endothelial growth factor levels predictors

Neither gender, the presence of hypertension or diabetes, current smoking status, antialdosteronics, statins, treatment with ACEIs or ARBs, previous ablation procedures, nor rhythm during blood sampling (i.e., sinus rhythm or AF) were significantly correlated with VEGF levels at any of the sampled sites (all $P > 0.10$). Body mass index, LA area, and duration of AF history were also not significantly correlated with VEGF levels at any of the sampled sites (all $P > 0.10$). There was no correlation between age and VEGF levels in neither

of the sampled sites and in neither of the studied groups (for paroxysmal AF patients: Spearman $r = 0.17$, $P = 0.28$ for peripheral VEGF levels and Spearman $r = 0.03$, $P = 0.82$ for LA VEGF levels; for persistent AF patients: Spearman $r = -0.1$, $P = 0.10$ for peripheral VEGF levels and Spearman $r = 0.11$, $P = 0.57$ for LA VEGF levels).

When paroxysmal AF patients and persistent AF patients were considered as a single group, LA levels of VEGF were significantly negatively correlated with ACEI or ARB therapy (Spearman $r = -0.25$, $P = 0.03$). In multivariate analysis, the presence of AF independently predicted elevated peripheral levels of VEGF (Spearman $r = 0.31$, $P = 0.04$), whereas there was no significant correlation between the presence of AF and LA, coronary sinus, or PV levels of VEGF (all $P > 0.05$).

Patients with both paroxysmal and persistent AF were then divided according to stroke risk as assessed by the CHA₂DS₂VASc score into low–intermediate (CHA₂DS₂VASc = 0 – 1, n = 46) and high (CHA₂DS₂VASc ≥ 2 , n = 26) stroke risk groups. We did not find any significant differences between peripheral or LA VEGF levels between patients considered at low-intermediate and high stroke risk according to the CHA₂DS₂VASc score (peripheral VEGF levels were 21.31 pg/mL in the low-intermediate stroke risk group and 27.73 pg/mL in the high stroke risk group; LA levels of VEGF were 16.42 pg/mL in the low-intermediate stroke risk group and 23.19 pg/mL in the high stroke risk group, both $P > 0.10$).

8.4. Discussion

The main findings of the present study were (i) patients with paroxysmal or persistent AF and no concomitant inflammatory disease did not have high CRP, IL-8, TGF- β 1, or sICAM-1 levels; (ii) patients with paroxysmal AF had high peripheral and LA, but similar PV levels of VEGF compared to non-AF controls; (iii) patients with persistent AF had high peripheral levels of VEGF, but similar LA, coronary sinus, and PV levels of VEGF compared to non-AF controls.

8.4.1. *Could inflammation in atrial fibrillation patients have been overestimated?*

Although a large amount of data suggest a link between inflammation and the presence of AF, several studies failed to show elevated levels of CRP, IL-6, or IL-8 in AF patients.^{83,188,200,295} Furthermore, studies assessing the efficacy of anti-inflammatory therapy in AF prevention have yielded conflicting results,^{160,187} suggesting that the role of inflammatory processes in the pathophysiology of AF might have been overestimated. Our study did not demonstrate any significant difference in CRP, IL-8, or sICAM-1 levels among

the three patient groups. These results suggested that the high inflammatory marker levels reported in AF patients could be due to associated comorbidities rather than to AF *per se*.

Inflammation and pro-thrombotic status seem to be related in AF patients.²⁴⁷ However, we did not find any significant correlation between age, smoking, hypertension, diabetes, or CHA₂DS₂VASc score and any of the inflammatory markers. The low risk profile of our cohort could explain the absence of high inflammatory marker levels and the relatively low risk of stroke (mean CHA₂DS₂VASc score 1.33 ± 0.21 in paroxysmal AF patients and 1.21 ± 0.21 in persistent AF patients). This observation further supports the hypothesis that associated comorbidities and not AF itself induces the inflammatory and pro-thrombotic states reported in AF patients, as previously suggested Lip and colleagues.³¹¹

8.4.2. The source of vascular endothelial growth factor in atrial fibrillation patients

In paroxysmal AF patients, the combination of normal PV and high LA levels of VEGF suggests the heart itself as the most likely source of high LA VEGF levels in this patients group. Accumulating evidence indicates that VEGF and VEGF receptor mRNA are expressed in heart tissue.^{173,258} Ogi et al. recently reported high VEGF levels in the atrial tissue of AF patients compared with non-AF controls.²²⁸

A number of pathological conditions, such as oxygen deficiency, inflammation, or, according to recent reports, pulsatile mechanical stretch,¹⁸¹ are known to induce VEGF secretion. Low levels of inflammatory markers in both paroxysmal and persistent AF patients suggest that, in the studied populations, the inflammatory process is of low grade, if present. Therefore, it seems unlikely that this process could account for the high VEGF levels observed in these patients. High VEGF levels have been reported in the myocardium and peripheral blood following myocardial infarction and unstable angina.¹⁷¹ Despite the absence of evidence for accelerated angiogenesis in paroxysmal AF patients, we found high LA levels of VEGF in this population. Histopathological studies demonstrate a high incidence of atrial ischemia, in up to 17%, and isolated atrial infarctions in more than 20% of autopsied patients with a history of AF.³²² A transient increase in the hypoxia inducible factor *alpha* gene expression, a known trigger for VEGF secretion, has been reported in cardiac myocytes in the early onset of AF.²⁸⁷ On the other hand, the inverse relationship is also possible, with ischemia favoring AF occurrence.²⁷²

The fibrillating atrium leading to an irregular blood flow could induce pulsatile vascular stretch and impaired blood rheology. Both phenomena could trigger VEGF secretion, as well

as its release from smooth muscle cells and circulating blood cells in AF patients, regardless of the clinical form of the arrhythmia.¹⁰⁸ Pulsatile mechanical stretch, recently postulated as a potent trigger for VEGF secretion from cardiac myocytes,^{253,258} could be responsible for the high intracardiac VEGF levels observed in paroxysmal AF patients.

8.4.3. *Progressive left atrial remodeling affects cardiac secretion of vascular endothelial growth factor (and the inverse)*

Our results suggest that LA secretion of VEGF is a transient event in the natural history of AF. Vascular endothelial growth factor has been previously shown to stimulate the fibrotic process within the atrial tissue, probably through induction of angiogenesis.⁴⁵ Several studies have emphasized that patients with persistent AF present more important fibrotic changes compared to patients with paroxysmal AF.^{115,143} The spreading of cardiac fibrosis could account for progressive cardiac stiffening in this setting, thereby reducing the degree of pulsatile stretch and subsequently diminishing VEGF secretion.

Despite the important role that TGF- β 1 plays in the genesis of fibrosis in patients with AF, we did not find a significant difference in TGF- β 1 levels between patients with and without AF. The larger use of ACEIs or ARBs in AF patients (35% in AF patients *versus* 0% in control patients) could have mitigated the differences between groups. Transforming growth factor-*beta*1 expression is known to be induced by angiotensin II and blockade of the angiotensin II type 1 receptor is known to suppress TGF- β 1 up-regulation.⁸⁷ Furthermore, the larger use of ACEIs and ARBs in AF patients could also have mitigated even higher VEGF levels in this population.

Our results suggest that in the context of persistent AF the damaged and remodeled atrial tissue becomes less able to secrete and release VEGF. Meanwhile, VEGF inhibition has been associated with extensive apoptotic changes in the vasculature of neonatal mice.¹⁰⁹ The low intracardiac VEGF levels observed in persistent AF patients could signify the loss of this protective mechanism of endothelial cells, subsequently leading to progressive endothelial and atrial endocardial damage. High VEGF levels observed in paroxysmal AF patients could participate in the progressive spread of atrial fibrosis, which, in turn, could diminish VEGF secretion by reducing atrial responsiveness to pulsatile mechanical stretch. Later, the resulting low intracardiac VEGF levels could contribute to progressive endothelial dysfunction.

8.4.4. *Potential limitations*

This study is limited by its cross-sectional design, which only permitted associations to be observed; cause-effect relationships could not be established. The small number of patients in the control group could have lowered the study's statistical power. Control patients were also significantly younger than AF patients. However, there was no correlation between age and VEGF levels whatever the sampled site or the studied group. Additionally, one could argue that high VEGF levels might be due to coexisting cardiovascular conditions; however, there was no significant difference among the three groups regarding the proportion of hypertensive or diabetic patients.

8.4.5. *Conclusions*

Low levels of several inflammatory markers in both paroxysmal and persistent AF patients suggest that in the studied populations, the inflammatory process is of low grade, if present. In the context of normal PV levels of VEGF, the heart itself is the most likely source of high LA levels of VEGF in paroxysmal AF patients; however, this disorder appears to be a transient event in the natural history of AF.

CHAPTER 9

A Study of Endothelial Dysfunction in Atrial Fibrillation Patients

9.1. Introduction

The predisposition for stroke among patients with AF is estimated at around 5% per year.²⁰⁵ Moreover, patients with AF and stroke have worse outcomes and develop more serious deficits compared to patients with strokes that are not related to AF.¹⁸³

Virchow's triad of coagulation demands that anomalies of blood flow, vessel walls, and coagulation factors be present so that thrombus formation can occur.¹⁸⁴ In AF, it is largely accepted that blood stagnation in the LA is an important thrombogenic factor.²⁵¹ However, the existence of other conditions, such as arterial hypertension, aging, or diabetes, increases the risk of stroke even more.²⁵¹ This observation suggests that although LA stasis may be one source of thromboembolism in AF patients, this does not provide a complete explanation.

Abnormal haemostatic constituents indicative of thrombogenesis have been demonstrated in AF patients,^{12,57,120} and recent data support the participation of endothelial dysfunction in the pathophysiology of AF.⁵⁴ High levels of different pro-thrombotic markers of endothelial origin, including vWF, an accepted marker of endothelial damage/dysfunction, have already been documented in this patient group.^{120,211} As prognostic marker, high vWF levels have been associated with increased risk of stroke and poorer prognosis in AF patients,³¹⁸ but the source of vWF in these patients remains unknown. The observation that endocardial atrial cells produce vWF could suggest a cardiac origin.²¹⁸ On the other hand, Conway et al. demonstrated that patients with AF and elevated plasma levels of vWF most often experience non-cardioembolic strokes, and suggested a generalized endothelial dysfunction as source of vWF in these patients.⁶⁰

Therefore, we aimed to assess the extent of endothelial impairment in patients with different clinical forms of AF in comparison to sinus rhythm controls, and to identify the source of vWF in AF patients. For this study, we performed multilevel intracardiac and extracardiac measurements of vWF.

9.2. Material and Methods

9.2.1. Patient selection

The study enrolled a total of 110 patients, scheduled for electrophysiological study or catheter ablation procedures at the Rhythmology Department of the Louis Pradel Cardiology Hospital in Lyon, France, between August 2008 and January 2011. The control group included 9 patients with left-sided accessory pathway Wolff-Parkinson-White syndrome and without any history of AF.

A total of 63 patients with paroxysmal AF and 38 patients with persistent AF were enrolled. According to the 2010 ESC Guidelines for the Management of AF,⁸⁵ AF was defined as ‘paroxysmal’ when the arrhythmia was spontaneously reversible to sinus rhythm within 7 days and ‘persistent’ when the AF episode persisted for more than 7 days or pharmacological or electrical cardioversion were required to terminate the arrhythmia.

Exclusion criteria included recent history of acute cardiovascular or cerebrovascular event, major trauma or surgery, apparent ischemic heart disease, malignancy, connective tissue or inflammatory disease, acute or chronic infection, and pulmonary, hepatic, or renal impairment. Patients under ongoing anti-inflammatory or steroid treatment were also excluded.

Detailed medical and drug history was obtained for each patient enrolled. All patients underwent physical examination and routine laboratory tests. The LA area was assessed by transthoracic echocardiography prior to electrophysiological study or catheter ablation procedures. For each patient, the stroke risk was evaluated according to current recommendations using the CHA₂DS₂VASc score.⁸⁵ This score assigns one point for each of the following risk factors for patients with AF: congestive heart failure, hypertension, age 65 to 74 years, diabetes, female sex, and vascular disease. Two points are assigned for a history of stroke or transient ischemic attack and age ≥ 75 years. For AF patients, the moment of the first arrhythmic episode was documented based on ECG documentation or physician-confirmed diagnosis. The presence or absence of AF at the moment of blood drawn was assessed electrocardiographically.

All participants provided written informed consent in accordance with the requirements of the local ethics committee. The study protocol complied with the Declaration of Helsinki and was approved by the South-East II People’s Protection Committee and the Advisory Committee on Information Processing in Research in the Field of Health.

9.2.2. Blood sampling and laboratory analysis

Blood sampling was performed during routine cardiac catheterization, prior to any ablations or administration of heparin. Blood samples were obtained serially from the femoral vein and the coronary sinus. The LA was accessed through trans-septal puncture and a third blood sample was taken from the LA *via* the trans-septal sheath.

Vacutainer (BD Plymouth) tubes (6.0 mL) containing ethylenediaminetetraacetic acid were used. The tubes were stored and shipped on ice within 30 min after sampling. Plasma

was extracted from each blood sample and divided into four aliquots that were stored at -80°C until analysis. Plasma vWF was dosed on a BCS analyzer (Siemens Healthcare Diagnostics, Inc., Deerfield, IL) with a Samsung Kit test (Marburg, Germany) by immunoturbidimetric assay, using polystyrene particles coated with polyclonal rabbit antihuman vWF antibodies.

9.2.3. Statistics

Values are expressed as the mean \pm SEM. Comparisons between control and AF groups were made using the Mann-Whitney U test. Categorical data were compared using the chi-square test. Correlations were ascertained with Spearman's rank correlation method. Stepwise multiple regression analysis was performed to identify independent predictors of vWF levels.

A P value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism[®] software.

9.3. Results

9.3.1. Patient clinical characteristics

Demographic and clinical data regarding AF patients and control subjects are summarized in Table 9.1.

There was no significant difference among the three groups in terms of sex ratio, smoking status, history of diabetes, statin or antiplatelet treatment (all $P > 0.10$). Atrial fibrillation patients were more likely to be treated with ACEIs or ARBs ($P = 0.01$), antialdosteronics ($P = 0.02$) or anticoagulants ($P < 0.001$). They were also older ($P = 0.05$), had higher body mass index ($P < 0.001$) and were more frequently hypertensive ($P = 0.04$).

As expected, LA area as assessed by echocardiography in the parasternal long axis was higher in paroxysmal AF patients than in controls, and even higher in persistent AF patients ($P < 0.001$).

Mean duration of AF history was 79 ± 81 months in the paroxysmal AF group and 81 ± 59 months in the persistent AF group ($P = 0.85$). Six patients (10%) in the paroxysmal AF group and 31 patients (82%) in the persistent AF group were in arrhythmia at the moment of blood sampling ($P < 0.001$), while the rest were in sinus rhythm. None of the control patients presented AF during electrophysiological study or ablation procedure.

Table 9.1. Clinical characteristics of study groups

Variables	Control (n = 9)	Paroxysmal AF (n = 63)	Persistent AF (n = 38)	<i>P</i> -value
Age (years)	42 ± 19	57 ± 10	58 ± 8	0.05
Men (n, %)	6 (67%)	50 (79%)	35 (92%)	0.10
Body mass index (kg/m ²)	22.06 ± 3.49	27.02 ± 3.87	29.71 ± 5.09	<0.001
Hypertension (n, %)	1 (11%)	25 (40%)	21 (55%)	0.04
Diabetes (n, %)	0 (0%)	6 (10%)	7 (18%)	0.21
Smokers (n, %)	2 (22%)	6 (10%)	2 (5%)	0.20
History of AF (months)	N/App	79 ± 81	81 ± 59	0.85
Antialdosteronics (n, %)	0 (0%)	5 (8%)	4 (26%)	0.02
Antiplatelets (n, %)	2 (22%)	3 (5%)	3 (8%)	0.17
Anticoagulants (n, %)	0 (0%)	62 (98%)	29 (97%)	<0.001
Statins (n, %)	0 (0%)	16 (25%)	8 (32%)	0.15
ACEIs/ARBs (n, %)	0 (0%)	18 (29%)	10 (47%)	0.01
Left atrial area (cm ²)	17.0 ± 0.6	20.9 ± 4.8	25.0 ± 6.8	<0.001
AF during sampling (n, %)	N/App	6 (10%)	31 (82%)	<0.001
CHA ₂ DS ₂ VASc score	0.7 ± 0.7	1.5 ± 1.4	1.3 ± 1.1	0.23

ACEIs - angiotensin converting enzyme inhibitors, AF - atrial fibrillation, ARBs - angiotensin receptor blockers, N/App - not applicable. Quantitative data are expressed as the mean ± SEM. Categorical data are expressed as number (percentage). *P*-values refer to comparisons between groups based on multiple comparisons ANOVA.

9.3.2. von Willebrand factor levels

Mean levels of vWF in the femoral vein, LA, and coronary sinus in the three studied groups are presented in Table 9.2.

Peripheral levels of vWF were significantly higher in patients with persistent AF compared to controls (*P* = 0.04); however, there was no significant difference between paroxysmal AF patients and controls (*P* = 0.29), or between paroxysmal AF and persistent AF patients (*P* = 0.21).

Patients with persistent AF had significantly higher LA levels of vWF compared to both control patients (*P* = 0.004) and paroxysmal AF patients (*P* = 0.02). Similarly, LA levels of vWF were significantly higher in patients with paroxysmal AF compared to controls (*P* = 0.02).

Table 9.2. Mean levels of von Willebrand factor in the femoral vein, left atrium, and coronary sinus in the three study groups

vWF levels (IU/dL)	Control (n = 9)	Paroxysmal AF (n = 63)	Persistent AF (n = 38)
Femoral vein	88.9 ± 37.4	108.6 ± 54.1	122.1 ± 51.3*
Left atrium	81.1 ± 29.7	112.9 ± 50.5*	137.6 ± 49.9*†
Coronary sinus	82.0 ± 23.5	86.9 ± 45.9	132.7 ± 48.9*†

AF – atrial fibrillation, vWF – von Willebrand factor. Data are expressed as the mean ± SEM. * - $P < 0.05$ versus control, † - $P < 0.05$ versus paroxysmal atrial fibrillation using the Mann-Whitney U test.

Comparable results were obtained for the coronary sinus samples. Patients with persistent AF had significantly higher coronary sinus levels of vWF compared to both control patients ($P = 0.003$) and paroxysmal AF patients ($P < 0.001$). However, there was no significant difference between patients with paroxysmal AF and controls ($P = 0.89$).

When paroxysmal AF patients and persistent AF patients were considered as a single group and compared to controls, the former combined groups had significantly higher LA vWF levels ($P = 0.02$). Peripheral vWF levels also tended to be higher in the AF group compared to controls ($P = 0.09$), but no significant difference was found in coronary sinus vWF levels between these two groups ($P = 0.48$).

9.3.3. von Willebrand factor levels predictors

Neither gender, the presence of diabetes, current smoking status, antialdosteronics, antiplatelets, anticoagulants, statins, treatment with ACEIs or ARBs, nor age, nor body mass index were significantly correlated with peripheral, LA, or coronary sinus levels of vWF in any of the three groups (all $P > 0.10$).

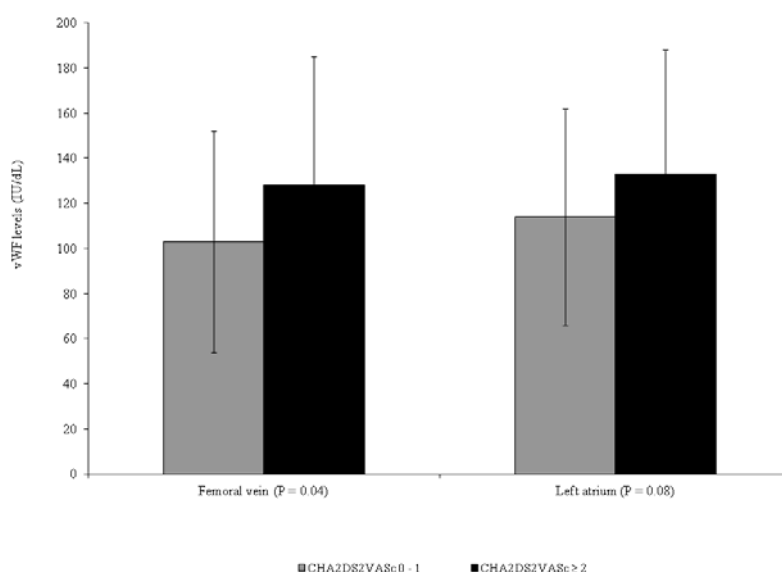
In patients with persistent AF, LA levels of vWF were significantly correlated with the duration of AF history (Spearman $r = 0.36$, $P = 0.03$).

When paroxysmal AF patients and persistent AF patients were considered as a single group, peripheral levels of vWF were significantly correlated with age (Spearman $r = 0.29$, $P = 0.002$), LA area (Spearman $r = 0.29$, $P < 0.001$), and the duration of AF history (Spearman $r = 0.19$, $P = 0.04$). Similarly, LA levels of vWF were significantly correlated with age (Spearman $r = 0.27$, $P < 0.001$), LA area (Spearman $r = 0.30$, $P = 0.004$), and the duration of AF history (Spearman $r = 0.20$, $P = 0.05$). However, in multivariate analysis, neither of the variables was significantly correlated with vWF levels (all $P > 0.05$).

9.3.4. von Willebrand factor levels and the CHA₂DS₂VASc score

Patients with both paroxysmal and persistent AF were divided according to stroke risk as assessed by the CHA₂DS₂VASc score into low–intermediate (CHA₂DS₂VASc = 0 – 1, n = 67) and high (CHA₂DS₂VASc ≥ 2, n = 34) stroke risk groups.

Figure 9.1. von Willebrand factor levels according to the CHA₂DS₂VASc score in atrial fibrillation patients



vWF – von Willebrand factor. Data are expressed as the mean ± SEM. *P*-values refer to comparisons between groups based on the Mann-Whitney *U* test.

Patients at high risk according to the CHA₂DS₂VASc score had significantly higher peripheral vWF levels compared to patients at low–intermediate risk (*P* = 0.04). Left atrial levels of vWF also tended to be higher in high-risk patients compared to the low–intermediate risk group (*P* = 0.08) (Fig. 9.1).

9.4. Discussion

The main findings of this study were (i) overall, patients with AF presented higher levels of vWF compared to sinus rhythm controls; (ii) patients with paroxysmal AF presented higher LA, but similar peripheral and coronary sinus levels of vWF compared to controls; (iii) patients with persistent AF presented higher vWF levels compared to controls in all sampled sites; (iv) patients with persistent AF presented significantly higher LA levels of vWF compared to paroxysmal AF patients; and (iv) in AF patients, peripheral levels of vWF were

significantly higher in patients with increased risk of stroke according to the CHA₂DS₂VASc score.

In addition to its role in transporting coagulation factor VIII, vWF also mediates key steps in thrombus formation, such as platelet adhesion to the de-endothelialized wall and subsequent platelet aggregation.²⁵⁶ Its role as a key element in the thrombotic process makes vWF a reliable marker of pro-thrombotic risk.

The present study demonstrated increased plasma levels of vWF in patients with AF compared to sinus rhythm controls. Moreover, we found significantly higher levels of vWF in patients with AF and high stroke risk according to the CHA₂DS₂VASc score compared to AF patients considered at low-intermediate risk of stroke. These results support the close relationship established in AF patients between the severity of endothelial damage/dysfunction and the risk of stroke.

9.4.1. Atrial fibrillation and endothelial dysfunction progression

In vitro studies have shown that endothelial cells do not bind or take up exogenous vWF.¹⁴² Thus, high vWF levels in the atria of patients with both paroxysmal and persistent AF compared to sinus rhythm controls suggest localized endothelial injury, limited to the atrial endocardium, as source of vWF in these patients. Several previous findings, such as a positive linear correlation between LA volume and plasma vWF levels reported by Mondillo et al.,²¹¹ or increased endocardial expression of vWF in the overloaded human atrial appendage demonstrated by Fukuchi et al.,⁹⁹ also suggest that the atrial endocardium is the most likely source of vWF in AF patients. In paroxysmal AF patients, the lack of increased levels of vWF in the venous blood that were as high as in the LA could be due to its sequestration in peripheral capillaries, a consequence of the extremely large dimensions of vWF multimers, exceeding 20,000 kDa.

Our results also suggest that along with paroxysmal AF evolution towards the persistent form, endothelial dysfunction also progresses from a localized feature to a widespread abnormality. High peripheral and coronary sinus levels of vWF in patients with persistent AF, but not in patients with paroxysmal AF, suggest the development of a widespread endothelial dysfunction beyond the atrial endocardium, that parallels the clinical perpetuation of the arrhythmia. Moreover, LA levels of vWF were significantly higher in patients with persistent AF compared to paroxysmal AF patients. These results suggest that the atrial endocardial impairment also progresses alongside with the perpetuation of the arrhythmia.

The hypothesis that atrial endocardium impairment precedes and favors a generalized vascular endothelial dysfunction has been demonstrated by Cai et al. and appears to be due to abnormal nitroso compound levels.⁴⁰ Given that endocardial expression of nitric oxide is decreased in patients with AF as a result of loss of organized contraction, the authors suggested that abnormal nitroso compound levels originating in the atrial endocardium could induce a generalized impairment of the vascular endothelium in this population.⁴⁰

Thus, the results of this study highlight the complex and dynamic relationships established between atrial endocardium, vascular endothelium, and AF progression and suggest endothelium as a key ‘organ’ in the pathophysiology of AF.

9.4.2. High intracardiac von Willebrand factor levels and the risk of thromboembolic stroke in different clinical forms of atrial fibrillation

Clinical data demonstrated that patients with paroxysmal AF have a risk of stroke similar to that of patients with the persistent or permanent forms.¹⁸⁴ Endothelial dysfunction/damage is a marker of increased risk of local intra-atrial thrombosis and according to a study by Heppel et al., vWF levels were an independent predictor of LA thrombus presence in transesophageal echocardiography in patients with AF.¹³¹ The present study showed that intra-cardiac levels of vWF were high in patients with both paroxysmal and persistent AF compared to patients without any history of AF. Thus, the similar risk of thromboembolic stroke in patients with paroxysmal and persistent AF could be explained by the substantial atrial endothelial damage/dysfunction and the subsequent risk of local atrial thrombosis in AF patients, regardless of the clinical form of the arrhythmia.

We also provide evidence that in patients with paroxysmal AF, normal peripheral levels of vWF do not exclude high LA levels. Thus, vWF levels do not seem to be a convenient marker of increased risk of stroke, given that normal peripheral vWF levels do not exclude high intra-cardiac levels.

Our results also suggest specific thromboembolic risk patterns in patients with different clinical forms of AF. Thus, paroxysmal AF may be more frequently associated with cardioembolic strokes, while persistent AF patients would experience both cardioembolic and non-cardioembolic strokes. Indeed, Conway et al. found that patients with low peripheral vWF levels, which match the profile of paroxysmal AF patients in our study, suffered only cardioembolic strokes, while patients with high peripheral vWF levels, which match the

profile of persistent AF patients, experienced both cardioembolic and non-cardioembolic strokes.⁶⁰

9.4.3. *Potential limitations*

This study is limited by its cross-sectional design, which only permitted associations to be observed; cause-effect relationships could not be established. The small number of patients in the control group could have lowered the study's statistical power. However, the levels of vWF in this group were comparable to the data reported in the literature.⁹⁷

9.4.4. *Conclusions*

Our results suggest a parallel between the evolution of AF and the progression of endothelial dysfunction, with atrial endocardial impairment preceding a widespread endothelial dysfunction. These findings bring new insights into the thromboembolic risk of AF patients and highlight the complex and dynamic relationship established between the progression of the arrhythmia and the evolution of endothelial impairment. Our results also suggest specific thromboembolic risk patterns in patients with paroxysmal and persistent AF, as assessed by intracardiac vWF dosage.

CHAPTER 10

General Discussion and Conclusions

10.1. General discussion

Although AF is the most prevalent sustained form of cardiac arrhythmia,¹⁰¹ the precise mechanisms that lead to the onset and persistence of AF are far from being completely elucidated. It is obvious that there is no unifying mechanism of AF, and that a mechanism may predominate over another in certain patients, generally with the participation of several mechanisms in the same patient. The pathophysiology of AF appears to be influenced by multiple mechanisms, which act to a different extent in different clinical settings and interact with each other in a dynamic way at different stages of the natural history of the arrhythmia. The complexity of the pathophysiological mechanisms of AF prompted us to develop a multifaceted approach.

Studies in animal models have provided extremely important insights into the mechanisms of AF. In fact, the classic mechanisms that form the basis for our understanding of the pathophysiology of AF were elucidated based on animal studies. This is not surprising, since several objectives are achievable in animal models but difficult, if not impossible, in clinical studies. Animals offer, first of all, wide accessibility to tissues, cells, and highly invasive measurements. Secondly, whilst in AF patients it is impossible to separate the effect of confounding factors such as underlying diseases, age, or ongoing therapy on AF mechanisms, in animal models variables can be carefully controlled.

However, with the wide array of pathophysiological findings in varying animal models, it is difficult to see any commonalities between these models and human AF. Moreover, in all those models, arrhythmia occurred only after interventional means, such as programmed stimulation protocols or pharmacologic interventions, were applied.^{56,319} Thus, a number of essential features for AF occurrence, such as structural, electrical, or autonomic remodeling could have been artificially induced in those models. The most important question is if observations made upon such models of artificially induced atrial arrhythmias can be extrapolated in humans, particularly when it comes to therapeutic strategies. To date, we do not dispose of any animal model of spontaneous AF. Results obtained on such a model should be easier to extrapolate in humans. Therefore, the main objective of the present study was to develop a model that could reproducibly exhibit spontaneous atrial arrhythmias in rats and to describe its phenotypic features.

Aging and hypertension account for more AF than any other predisposing factors.^{5,24,58} Moreover, studies in various experimental models have provided evidence of increased inducibility of AT and AF in both aging and hypertensive animals.^{9,155} Choisy et al.

demonstrated increased susceptibility to atrial arrhythmias in excised perfused hearts from aging SHR, compared to hearts from both young SHR and normotensive controls.⁵⁶ Using a modified Tyrode solution to perfuse isolated hearts from young and old rats, Ono et al. reported episodes of AF in old, but not in young rats.²³³ However, none of these studies has ever reported the occurrence of spontaneous atrial arrhythmias in these settings. Given the important synergistic association between aging and hypertension as risk factors for AF, we hypothesized that aging SHR could represent a reliable model of spontaneous atrial arrhythmias. The next step was the choice of the most appropriate monitoring technique, in order to avoid interference of external confounders.

With the exception of studies of anesthetic drugs, special interest is granted to achieve data from conscious, unrestrained animals, because such conditions mimic the best physiological or pathophysiological conditions and the results of such measurements have the highest chances to be correctly extrapolated to humans. Radiotelemetry ECG monitoring corresponds to all these requirements and was therefore the natural choice to assess cardiac rhythm in our study. This technique allowed us to monitor rats' heart rhythm without interference of external stressors such as handling of animals before or during measurements, which could lead to inconsistencies and affect the reproducibility of the method.

Thus, using 24-h continuous ECG recordings obtained by radiotelemetry, we have successfully demonstrated, for the first time, the occurrence of unprovoked atrial arrhythmias in aging hypertensive rats, but not in young hypertensive rats, nor in young or aging normotensive rats. This new model offers tremendous opportunities to study the pathophysiological mechanisms of atrial arrhythmias and to assess the efficacy and toxicity of various therapeutic agents in a setting that faithfully reproduces the clinical presentation of the arrhythmia.

Given the wide array of advantages that this model provides, particularly the similarities with the clinical form of the arrhythmia by the fact that it uses no interventional means to favor the occurrence of these arrhythmias and the wide availability of tissue samples, we decided to study various pathophysiological mechanisms of atrial arrhythmias in this model. Therefore, we aimed to assess several mechanisms recognized to play key roles in the pathophysiology of AF, particularly autonomic imbalance, structural and electrical remodeling, and molecular abnormalities, related to this arrhythmia. In addition, the roles of inflammation and endothelial dysfunction in AF occurrence were also addressed in two clinical studies.

These studies brought new and important insights into the mechanisms of atrial arrhythmias, starting with the demonstration of an autonomic imbalance in rats that presented atrial arrhythmias, with decreased sympathetic tone and relative vagal hyperactivity. Furthermore, the antiarrhythmic effect of sympathetic stimulation by emotional stress and the pro-arrhythmic effect of parasympathetic stimulation by carbamylcholine suggested a putative link between the autonomic imbalance and arrhythmia onset. Overall, aging SHR rats seem to reflect Coumel's vagal AF.⁶⁴ Indeed, vagal stimulation can alter atrial electrophysiology, can affect automaticity, re-entry, fibrillatory conduction, and triggered automaticity.^{112,230,265} More importantly, we demonstrated that this autonomic imbalance was present in young SHR rats that did not yet present arrhythmic events. Thus, it appears that impaired sympatho-vagal balance precedes and probably favors the occurrence of arrhythmias, but does not seem to be sufficient to induce atrial arrhythmias on its own. These findings suggest that changes in autonomic tone are likely to have different effects depending on the timing of these changes and the coexistence of other predisposing factors.

Accumulating evidence suggests that the longer the duration of AF the harder it becomes to establish and maintain sinus rhythm.²¹⁹ In fact, once established, AF itself participates to further alterations in electrical and structural properties of the atrial tissue, inducing several changes in atrial myocardium at electrophysiologic and structural levels, summarized as atrial remodeling.⁷⁴ Shortly after the onset of AF, electrical remodeling consisting in changes in ionic properties of atrial myocytes occurs due to high atrial rates.^{213,319} The most consistent findings in both paroxysmal and persistent AF are significant shortening of the atrial effective refractory period and of the APD.⁶⁷ This is probably largely due to impaired Ca^{2+} homeostasis.³²⁷ In arrhythmic rats, transcriptome analysis revealed decreased mRNA levels of genes encoding for various subunits of the $\text{I}_{\text{Ca,L}}$ current. Indeed, this ionic change could explain the shortening of the atrial action potential and the loss of the physiological rate adaptation of the APD reported in AF patients.³²⁷ Increased atrial rate increase atrial myocyte Ca^{2+} loading.²⁷⁶ Given that Ca^{2+} overload exhibits cytotoxic effects on atrial myocytes,²⁷⁶ decreased $\text{I}_{\text{Ca,L}}$ could occur as an adaptive, protective mechanism. Decreased $\text{I}_{\text{Ca,L}}$ would decrease indeed Ca^{2+} loading, but would simultaneously lead to shortening of the APD, decreasing atrial refractory periods and wavelength, thus favoring multiple re-entry circuits and perpetuation of the arrhythmia.

The second component of AF-related remodeling is structural remodeling of the atria. While electrical remodeling seems to be the consequence of AF, LA structural abnormalities

can be both precursors and consequences of AF.^{14,98} Cardiac remodeling often precedes the onset of AF, since it emanates from cardiac damage due to coronary artery disease, hemodynamic overload from valve disease or hypertension.⁹⁸ On the other hand, AF itself promotes and aggravates LA structural abnormalities,¹⁴ promoting the development of a vicious circle where AF-induced atrial structural remodeling promotes AF persistence. Histological examination of LA samples of aging SHR^s revealed LA endocardial and interstitial fibrosis, inflammatory infiltrates, and myocyte necrosis of varying grades of severity in the majority of aging SHR^s, in accordance with previously published data.^{98,162,182} Consistent with the presence of fibrosis in the LA of aging SHR^s, molecular analysis of LA samples revealed increased expressions of the gene encoding for type VI collagen in this population. Furthermore, transcriptome analysis also demonstrated decreased expression of genes encoding for C_x43 and desmocollin 2. Disruption of cell-cell coupling has already been demonstrated in atrial tissue prone to AF.¹¹³ Altered cell-cell communications could result in abnormal atrial conduction and contribute to AF vulnerability.^{113,180} However, the detailed mechanism by which cell-cell coupling impairment favors atrial arrhythmogenicity is probably much more complex. Subtle changes in the relative expression or the degree of membrane incorporation of proteins involved in cell-cell communication could modulate the coupling of these proteins. In conjunction with metabolic modulators such as pH, Ca²⁺ concentration, oxygenation, or phosphorylation status,²⁷⁶ it could represent modulating mechanisms that act in concert and over different time scales, and, when specific circumstances are met, it could promote arrhythmogenesis. Left atrial histological abnormalities observed in arrhythmic rats were significantly less severe than what has been previously reported in AF patients.^{98,162} However, human LA sampling is limited to patients that undergo cardiac surgery or cardiac explantation. Thus, concomitant serious heart conditions such as coronary artery disease, valve disease or congenital heart defects in these patients could be at least partially responsible for the more extensive structural changes observed in human LA samples. One could conclude that LA structural abnormalities of most AF patients could actually be much less severe than those reported so far. Interestingly, some of the aging SHR^s that presented atrial arrhythmias did not present LA structural abnormalities. This finding suggested that the LA remodeling / fibrosis-AT relationship could be in fact highly nonlinear, and that atrial fibrosis is more likely to be a facilitator of atrial arrhythmogenicity in this model, rather than a prerequisite. Abnormal autonomic tone or

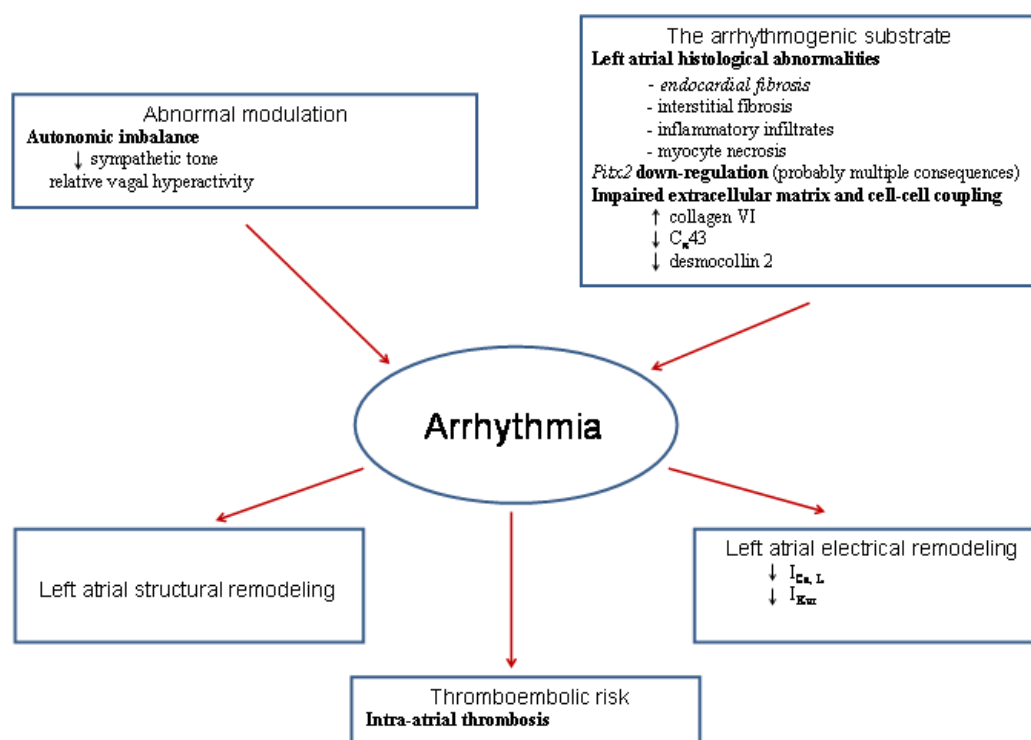
molecular abnormalities could play critical roles in such settings were little or no anatomic abnormalities are present.^{52,122,286,313}

Finally, *Pitx2* analysis brought new insights into the mechanisms underlying atrial arrhythmias. Recent clinical and experimental studies provided conclusive data regarding the role of *Pitx2* in AF,^{53,154} and demonstrated a molecular link between *Pitx2* loss-of-function and the presence of the arrhythmia.⁵³ In our study, *Pitx2* expression analysis demonstrated significantly lower levels of *Pitx2* in the LA of aging SHR rats compared to age-matched WKY rats. These results support a link between *Pitx2* insufficiency and the occurrence of ATs in aging SHR rats. More importantly, we provided evidence that among hypertensive rats, *Pitx2* expression presents a gradual, age-dependent decrease. These results provide for the first time the proof of a temporal relationship between *Pitx2* down-regulation and AF onset. However, whether AF activates *Pitx2* down-regulation or whether the presence of a pre-existing *Pitx2* down-regulation promotes AF remains unclear. The fact that an age-dependant *Pitx2* diminution precedes the onset of the arrhythmia supports the hypothesis that this molecular abnormality could be a causative factor of AF. On the other hand, *Pitx2* was more down-regulated in the presence of the arrhythmia than in its absence, suggesting that AF itself could play a role in *Pitx2* down-regulation in this setting.

The multifaceted approach utilized in this study brought new and important insights into the mechanisms of atrial arrhythmias. The results of this study highlight the multicausal nature of AF and the complexity of pathophysiological mechanisms of this arrhythmia.

A multitude of factors, including autonomic imbalance, LA structural abnormalities, *Pitx2* down-regulation, expression changes in genes encoding for ion channels, proteins of the extracellular matrix, and proteins involved in cell-cell coupling, seem to act synergistically to promote arrhythmia occurrence in aging hypertensive rats. While expression changes in genes encoding for ion channels are most probably a consequence of AF, *Pitx2* down-regulation and autonomic imbalance seem to be precursors of AF, and LA structural abnormalities could be both causative factors and consequences of AF (Fig. 10.1).

Figure 10.1. Schematic representation of pathophysiological mechanisms involved in atrial arrhythmia occurrence in aging hypertensive rats according to the results of the present study



C_x43 – connexin-43, I_{Ca,L} - L-type calcium current, I_{Kur} - ultra-rapid delayed rectifier potassium current

It remains to be established if targeting prevention or reversal of electrical, structural, and/or autonomic atrial remodeling will result in the reduction of the major impact that AF represents for the public health and to determine the best time to intervene to lower the risk of occurrence and/or recurrence of atrial arrhythmias. Whereas electrical remodeling appears to be completely reversible within a few days after sinus rhythm restoration,²⁴⁰ the reversibility of other types of atrial remodeling is far from clear.

Since structural remodeling of the atria often emanates from cardiac damage due to coronary artery disease, hemodynamic overload from valve disease or hypertension,⁹⁸ it seems unlikely that antiarrhythmic strategies could modify these alterations. In fact, experimental studies have demonstrated that, in contrast to electrical remodeling, structural changes are far less reversible and they tend to persist even after reestablishment of sinus rhythm.¹⁴ However, a number of non-antiarrhythmic drugs with antifibrotic effects have shown some benefits in reducing AF recurrences.^{66,73,90,151} On the other hand, reversing autonomic abnormalities seems to be efficient in restoring and maintaining sinus rhythm.

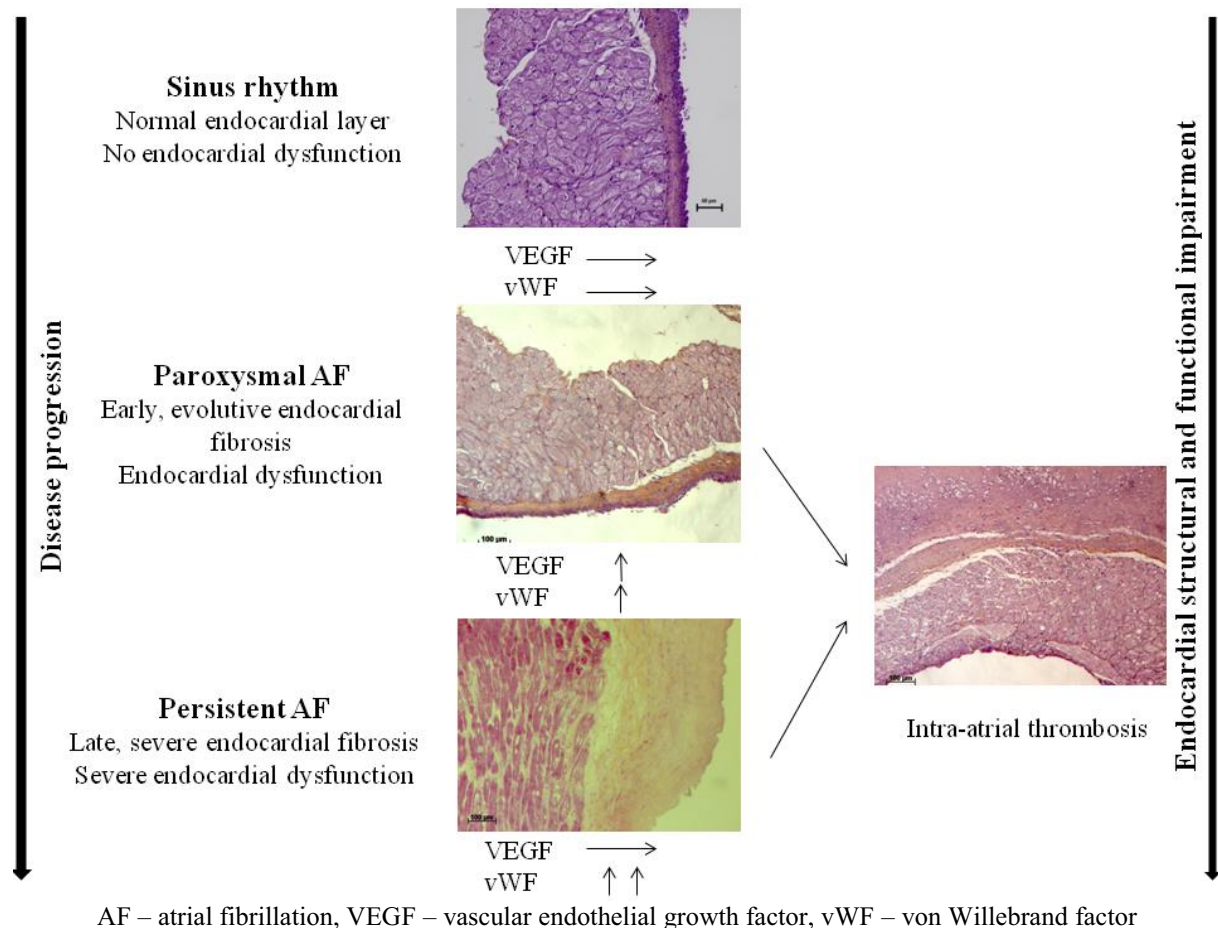
Radiofrequency or surgical parasympathetic denervation emerged as efficient techniques for AF resolution. According to Scherlag et al., ablation of ganglionated plexi in addition to PV-antrum isolation increases ablation success among patients with paroxysmal or persistent AF.²⁶¹ In fact, because linear lesions from standard PV-directed ablation run through areas with high concentrations of ganglionated plexi, autonomic modification or denervation could be one of the mechanisms that contribute to the effectiveness of standard ablation procedures.

Multilevel VEGF dosages in patients with different clinical forms of AF provided interesting data regarding the relationship between LA remodeling and AF. Both paroxysmal and persistent AF patients presented high peripheral levels of VEGF, probably due to pulsatile vascular stretch and impaired blood rheology,¹⁰⁸ while, interestingly, high LA levels of VEGF were only observed in paroxysmal, but not in persistent AF patients. Given that PV levels of VEGF were normal, the heart itself is the most likely source of high LA levels of VEGF in paroxysmal AF patients, probably as a consequence of VEGF secretion from cardiac myocytes in response to pulsatile mechanical stretch.^{181,253,258} The lack of similarly high levels of VEGF in the LA of patients with persistent AF suggest that this disorder is a transient event in the natural history of AF. Indeed, abnormal VEGF levels could promote atrial fibrosis through the induction of angiogenesis.⁴⁵ Then, extensive atrial fibrosis could generate progressive cardiac stiffening, reducing the degree of pulsatile stretch, and subsequently diminishing VEGF secretion. Thus, progressive LA remodeling, in which VEGF itself appears to play a crucial role, seems to affect cardiac secretion of VEGF (Fig. 10.2).

The data obtained using multilevel intracardiac and extracardiac measurements of vWF reinforced the progressive nature of AF-related LA remodeling (Fig. 10.2). This study demonstrated increased LA levels of vWF in patients with both paroxysmal and persistent AF. However, LA levels of vWF were significantly higher in patients with persistent AF compared to those with paroxysmal AF. These results suggest a progressive atrial endocardial impairment, in parallel with clinical evolution of the arrhythmia.

Furthermore, peripheral vWF levels were only elevated in persistent, but not in paroxysmal AF patients. Thus, it appears that a parallel also exists between the evolution of AF and the progression of endothelial dysfunction, with atrial endocardial impairment preceding widespread vascular endothelial dysfunction. This relationship between atrial endocardial impairment and generalized vascular endothelial dysfunction seems to be due to abnormal nitroso compound levels originating in the atrial endocardium.⁴⁰

Figure 10.2. The parallel between the clinical evolution of atrial fibrillation, the progression of endocardial dysfunction, as suggested by VEGF and vWF levels, and the progression of atrial endocardial fibrosis, as suggested by histological analysis



10.2. Clinical implications and further work

There are a number of clinical implications from the findings and observations in this thesis. Probably the most important achievement of the present study was the development of the first animal model of spontaneous, unprovoked atrial arrhythmias in rats. This model offers tremendous opportunities to study the pathophysiological mechanisms of atrial arrhythmias and to assess the efficacy and toxicity of various therapeutic agents in a setting that manages, for the first time, to faithfully reproduce the clinical presentation of the arrhythmia.

Using this model, we have succeeded to bring additional information regarding the complex mechanisms that participate in atrial arrhythmia occurrence and to identify potentially new targets for atrial arrhythmia prevention. A better understanding of the mechanisms underlying atrial arrhythmias is expected to result in more efficient

antiarrhythmic strategies and to have a positive impact on the social and economic burdens that AF represents.

10.2.1. New targets for atrial fibrillation prophylaxis

Clinical experience has demonstrated that antiarrhythmic strategies in AF are often inefficient.²² Data concerning AF prophylaxis are even more elusive and so far no prophylactic strategy has provided the expected results. This study revealed potentially new targets for AF prevention in hypertensive patients. We demonstrated that in hypertensive rats, both sympatho-vagal imbalance and *Pitx2* down-regulation preceded and favored the occurrence of atrial arrhythmias. Thus, autonomic imbalance could possibly serve as predictor of future development of AF in hypertensive patients. Furthermore, targeting this sympatho-vagal imbalance could represent a new approach in AF prophylaxis in this population. Indeed, ablation of ganglionated plexi in addition to PV-antrum isolation has been shown to increase ablation success among patients with paroxysmal or persistent AF,²⁶¹ and experimental data in dogs suggested that stellate ganglion and vagal ablation also helps to prevent AF inducibility by intermittent rapid atrial pacing.⁵⁵ To date, no study has focused on the potential role of sympatho-vagal imbalance correction in AF prevention. Further work will need to be done to assess this issue.

The relationships between *Pitx2* and AF are important, given the increasing evidence indicating a link between *Pitx2* expression and the occurrence of the arrhythmia.^{53,307} Whilst decreased expression of *Pitx2* in the LA of AF patients has been previously reported,⁵³ *Pitx2* changes before the occurrence of arrhythmia have never been studied in this population. This study provided evidence that among hypertensive rats, *Pitx2* expression presents a gradual, age-dependent decrease. These results suggest that in hypertensive rats, *Pitx2* down-regulation starts before the occurrence of arrhythmias and that timely appropriate therapy in hypertensive patients might prevent AF onset. However, to date, no study has identified the mechanisms responsible of *Pitx2* insufficiency in AF patients. Preliminary analyses performed in our laboratory suggest that decreased expression of *Pitx2* in arrhythmic rats could be due to a methylation process. If this is indeed the case, new therapeutic agents that target this precise mechanism, such as demethylating agents, could open new ways in AF prophylaxis.

10.2.2. A new look at old problems

This thesis has provided a series of results that might help understanding a number of clinical observations.

Atrial fibrillation confers a 5-fold increase in the risk of stroke, and one in every five strokes is attributed to this arrhythmia.²⁷⁰ In agreement with these data, we also found increased incidence of atrial thrombosis in arrhythmic rats. Moreover, AF is associated with worse deficits and worse outcomes than for stroke patients in sinus rhythm.²⁵² Blood stagnation in the LA is an important thrombogenic factor in AF patients.²⁵¹ However, the coexistence of other conditions, such as hypertension, aging, or diabetes increases the risk of stroke even more.²⁵¹ Moreover, the LA, particularly the LA appendage, is the most frequent location of embolic thrombi, even when the right atrium undergoes the same pathological process,²⁸⁴ and despite the fact that the blood stasis in the left and the right atrial appendage is similar.⁶⁹ Taken together, these observations suggest that although LA stasis may be one source of thromboembolism in AF patients, this does not provide a complete explanation. Recently, attention has focused on vWF, a large plasma glycoprotein that mediates platelet adhesion and aggregation.³³ This protein has been associated with increased stroke risk in AF patients.^{136,215} The present study demonstrated increased plasma levels of vWF in patients with AF compared to sinus rhythm controls. Moreover, we found significantly higher levels of vWF in patients with AF and high stroke risk according to the CHA₂DS₂VASc score compared to AF patients considered at low-intermediate risk of stroke. These results reinforce the link between endothelial damage/dysfunction and the risk of stroke. Additionally, preliminary results on LA vWF immunostaining showed higher intensity of staining in aging hypertensive rats compared to both young hypertensive rats and aging normotensive rats. These findings support the role of aging and hypertension, both important risk factors for stroke in AF patients,⁸⁵ in endothelial dysfunction.

An interesting clinical observation is that AF patients have similar risk of stroke, regardless of the clinical form of the arrhythmia.¹⁸⁴ Our results could help to explain this predisposition. Endothelial dysfunction/damage confers an increased risk of local intra-atrial thrombosis and according to a study of Heppel et al., vWF levels were an independent predictor of LA thrombus presence in transesophageal echocardiography in patients with AF.¹³¹ The present study demonstrated high intra-cardiac levels of vWF in patients with both paroxysmal and persistent AF, indicating the presence of atrial endocardial impairment in both forms of arrhythmia. Thus, the similar risk of thromboembolic stroke in patients with

paroxysmal and persistent AF could be explained by the substantial atrial endothelial damage/dysfunction and the subsequent risk of local atrial thrombosis in AF patients, regardless of the clinical form of the arrhythmia.

Furthermore, our results suggest that according to the clinical form of arrhythmia, AF patients could present specific thromboembolic risk patterns. Thus, paroxysmal AF patients, in which only localized LA endocardial dysfunction was identified, should present cardioembolic strokes, while persistent AF patients, which presented vWF levels suggestive for both atrial endocardial and widespread vascular endothelial impairment, would experience both cardioembolic and non-cardioembolic strokes. This hypothesis is supported by the results obtained by Conway et al., who found that AF patients with low peripheral vWF levels, which match the profile of paroxysmal AF patients in our study, suffered only cardioembolic strokes, while AF patients with high peripheral vWF levels, which match the profile of persistent AF patients, experienced both cardioembolic and non-cardioembolic strokes.⁶⁰ However, given that in paroxysmal AF patients normal peripheral levels of vWF did not exclude high LA levels, peripheral vWF levels do not seem to be a convenient marker of increased risk of stroke in this population.

Both clinical and experimental studies postulated inflammation as a predisposing factor for AF.^{27,61} However, several studies failed to show elevated levels of inflammatory proteins in this population,^{83,188,200,295} suggesting that the role of inflammatory processes in the pathophysiology of AF might have been overestimated. In our study, we found no difference in the levels of several inflammatory markers (i.e., CRP, IL-8, sICAM-1, and TGF- β 1) between paroxysmal or persistent AF patients and sinus rhythm controls. These results suggested that, at least in the studied populations, the inflammatory process was of low grade, if present. Given that this study enrolled patients with a relatively low-risk profile, it seems reasonable to conclude that associated comorbidities might play a more important role in the inflammatory state reported in AF patients than AF itself, as previously suggested by Lip and colleagues.³¹¹ Furthermore, the participation of inflammation in AF occurrence is probably highly depending on the substrate of the arrhythmia. While inflammation has probably a minor or no role in lone AF, it is probably a key element in other settings such as post-operative AF. This could explain the deceiving results reported by several studies assessing the efficacy of anti-inflammatory therapy in AF.²⁸⁰ On the other hand, anti-inflammatory agents might be useful in patients in which inflammation is a main determinant of AF onset.

Further studies will need to be carried out to assess the efficacy of anti-inflammatory agents in specific AF sub-groups.

Molecular analysis in the LA of arrhythmic rats revealed decreased expression of the *Dcs2* gene, encoding for desmocollin 2. This transcriptional change has not been previously reported in AF patients. Mutations in *Dcs2* are known to cause arrhythmogenic right ventricular cardiomyopathy, an inherited channelopathy associated with increased risk of ventricular arrhythmias.¹³² Several clinical studies reported increased incidence of supraventricular arrhythmias in patients with arrhythmogenic right ventricular cardiomyopathy and high prevalence of sustained ATs induced at electrophysiological study compared to patients without cardiac disease.³¹ To date, the underlying mechanisms of ATs in arrhythmogenic right ventricular cardiomyopathy are unknown. Our results suggest a putative link between *Dsc2* insufficiency and atrial arrhythmias. Thus, *Dsc2* loss-of-function could underlie the increased atrial arrhythmogenicity observed in patients with arrhythmogenic right ventricular cardiomyopathy. Further studies to directly address the consequences of decreased *Dsc2* expression on atrial arrhythmogenicity will be of interest.

Left ventricular hypertrophy has been identified as a strong risk factor for sudden death, heart failure and acute myocardial infarction.¹⁶¹ Several studies reported more likelihood of ventricular arrhythmias in the presence of LVH than in hypertensive patients with no signs of LVH,^{110,234} and found a linear relationship between arrhythmia burden and left ventricular mass.¹¹⁰ Quite surprisingly, 24-h continuous ECG monitoring in hypertensive rats showed more frequent and more complex ventricular arrhythmic events in adult SHR than in aging SHR. These results suggested that in hypertensive rats, early-stage LVH could in fact be more arrhythmogenic than evolved, chronic LVH. Further studies need to be carried out to elucidate the mechanisms underlying the increased ventricular arrhythmogenicity observed in adult SHR compared to young and aging SHR.

10.2.3. Biomarkers of atrial fibrillation progression

Paroxysmal AF frequently progresses towards persistent AF and the longer the duration of AF the harder it becomes to establish and maintain sinus rhythm.²¹⁹ Moreover, once established, AF itself participates to further alterations in electrical and structural properties of the atrial tissue, which will promote the perpetuation of the arrhythmia.⁴ Thus, from a

therapeutic point of view, it seems essential to interrupt this circle as quickly as possible, to prevent remodeling and its deleterious consequences.

A number of clinical variables, such as duration of the arrhythmia, the presence and the severity of LV dysfunction, or prolonged filtered P-wave duration,¹ have been proposed as predictors of AF progression. In a retrospective study, Jahangir et al. identified older age at diagnosis and the presence of QRS abnormalities on ECG as predictors of progression towards permanent AF.¹³⁸ On the other hand, a faster heart rate during AF at baseline was associated with a lower risk of progression towards permanent AF.²²³ Recently, de Vos et al. proposed the HATCH score to predict the risk of paroxysmal AF to evolve towards more persistent arrhythmia.⁷¹ This score assigns one point for hypertension, age > 75 years, and chronic obstructive pulmonary disease. Two points are assigned for a history of stroke or transient ischemic attack and heart failure. In their study, nearly 50% of the patients with a HATCH score > 5 progressed to persistent AF compared to only 6% of the patients with a HATCH score of 0.⁷¹ In addition to clinical variables, a series of biological markers have been associated with increased risk of AF progression. Higher levels of CRP and YKL-40, a new biomarker of inflammation, in persistent and permanent AF patients compared to paroxysmal AF patients suggested a link between AF progression and the inflammatory burden.¹³⁰

In our study, both VEGF and vWF levels presented specific patterns, according to the clinical form of arrhythmia. Intracardiac VEGF levels were high in paroxysmal AF patients, but the capacity of secretion of atrial myocytes seemed to be lost once the arrhythmia progressed to persistent AF. On the other hand, vWF levels were high in paroxysmal AF patients, but even higher levels were observed in persistent AF. We can therefore conclude that VEGF and vWF levels vary according to the clinical form of arrhythmia and that VEGF, vWF, or a combination of both, could serve as markers of AF progression.

Identifying biomarkers of AF progression would be of most interest in patients presenting silent, asymptomatic AF. Furthermore, these biomarkers may be used for early selection of patients for rhythm control therapy in an effort to prevent disease progression.

10.2.4. Ongoing studies

Histological analysis brought additional information regarding LA remodeling in aging hypertensive rats. However, only visual examination was performed and this limited the power of our study. Precise quantification of LA structural abnormalities, and particularly LA

fibrosis, is ongoing and is expected to bring additional data concerning LA remodeling in this model.

An important finding of this study was the presence of intra-atrial thrombosis in arrhythmic rats, but the precise mechanisms predisposing these rats to thrombosis remain to be elucidated. Preliminary results from immunohistochemical staining of vWF showed more severe atrial endocardial impairment in aging SHR rats compared to both young SHR rats and age-matched WKY rats. This endocardial dysfunction could contribute to the increased atrial thrombogenicity observed in this setting. Additional analysis, with precise quantification of the intensity of vWF immunostaining is ongoing and is expected to bring additional information on the pathophysiology of thromboembolic risk in aging, hypertensive rats.

Additionally, the mechanisms responsible of *Pitx2* down-regulation in arrhythmic rats are unknown. Analyses performed in our laboratory suggest that *Pitx2* down-regulation could be due to a methylation process. Further studies will have to confirm these preliminary results.

10.3. General conclusion

This is the first study to report the occurrence of unprovoked episodes of atrial arrhythmia in aging hypertensive rats, in the absence of any interventional means that could have favored the onset of arrhythmia. With this thesis we confirmed the multicausal nature of AF and the complexity of the pathophysiological mechanisms involved in atrial arrhythmia occurrence.

Our results suggest that autonomic imbalance with relative vagal hyperactivity may be responsible for the increased atrial arrhythmogenicity observed in this model. This autonomic abnormality was present in hypertensive rats long before the occurrence of arrhythmias, although it did not seem to be sufficient to induce arrhythmias.

We also provide evidence that structural abnormalities and particularly atrial endocardial fibrosis are present in the LA of arrhythmic rats; however, the absence of histological abnormalities in certain rats that presented arrhythmias suggests that atrial fibrosis is more likely a facilitator of atrial arrhythmogenicity in our model, rather than a prerequisite.

Similarly to the data reported in AF patients, rats that presented atrial arrhythmias also presented reduced *Pitx2* LA expression and transcriptional changes suggestive of reduced I_{Ca} ,

I_{L} , I_{Kur} , and C_{x43} densities, as well as increased type VI collagen. Furthermore, we provide evidence that in SHRs, *Pitx2* gene expression follows a progressive, age-dependent decrease.

In AF patients, low levels of inflammatory markers suggest that in the studied populations, the inflammatory process is of low grade, if present. High VEGF levels in the LA, but not in the PVs of paroxysmal AF patients, suggest that the heart itself is the most likely source of high LA levels of VEGF in this group; however, this disorder appears to be a transient event in the natural history of AF.

Multilevel vWF dosages further supported a parallel between the evolution of AF and the progression of endothelial dysfunction, with atrial endocardial impairment preceding widespread endothelial dysfunction. Our results also suggest that according to the clinical form of arrhythmia, AF patients could present specific thromboembolic risk patterns.

Finally, ECG monitoring confirmed the presence of higher ventricular ectopy in hypertensive rats, regardless of their age, compared to age-matched normotensive controls. However, early-stage LVH appeared to be more arrhythmogenic than evolved, chronic LVH. A gain-of-function pattern of transcriptional changes was present in SHRs compared to age-matched WKY rats. Our results also suggest that in this model the extent of LV molecular remodeling progresses with age.

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Appendixes

Appendix 1. List of the 89 genes for which mRNA expressions were studied in left atrial and left ventricular samples

Symbol	Name
<i>Cacna1c</i>	calcium channel, voltage-dependent, L type, <i>alpha</i> 1C subunit
<i>Cacna1d</i>	calcium channel, voltage-dependent, L type, <i>alpha</i> 1D subunit
<i>Cacna1g</i>	calcium channel, voltage-dependent, T type, <i>alpha</i> 1G subunit
<i>Cacna1h</i>	calcium channel, voltage-dependent, T type, <i>alpha</i> 1H subunit
<i>Cacna2d1</i>	calcium channel, voltage-dependent, <i>alpha</i> 2/delta subunit 1
<i>Cacna2d2</i>	calcium channel, voltage-dependent, <i>alpha</i> 2/delta subunit 2
<i>Cacnb1</i>	calcium channel, voltage-dependent, <i>beta</i> 1 subunit
<i>Cacnb2</i>	calcium channel, voltage-dependent, <i>beta</i> 2 subunit
<i>Cacnb3</i>	calcium channel, voltage-dependent, <i>beta</i> 3 subunit
<i>Cacnb4</i>	calcium channel, voltage-dependent, <i>beta</i> 4 subunit
<i>Cacng4</i>	calcium channel, voltage-dependent, <i>gamma</i> subunit 4
<i>Cacng6</i>	calcium channel, voltage-dependent, <i>gamma</i> subunit 6
<i>Casq1</i>	calsequestrin 1 (fast-twitch, skeletal muscle)
<i>Casq2</i>	calsequestrin 2 (cardiac muscle)
<i>Clcn2</i>	chloride channel 2
<i>Clcn3</i>	chloride channel 3
<i>Col6a1</i>	collagen, type VI, <i>alpha</i> 1
<i>Dsc2</i>	desmocollin 2
<i>Dsp</i>	desmoplakin
<i>Gja1</i>	gap junction protein, <i>alpha</i> 1
<i>Gja5</i>	gap junction protein, <i>alpha</i> 5
<i>Gja7</i>	gap junction membrane channel protein <i>alpha</i> 7
<i>Hcn1</i>	hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
<i>Hcn2</i>	hyperpolarization activated cyclic nucleotide-gated potassium channel 2
<i>Hcn3</i>	hyperpolarization-activated cyclic nucleotide-gated potassium channel 3
<i>Hcn4</i>	hyperpolarization activated cyclic nucleotide-gated potassium channel 4
<i>Kcna1</i>	potassium voltage-gated channel, shaker-related subfamily, member 1
<i>Kcna2</i>	potassium voltage-gated channel, shaker-related subfamily, member 2
<i>Kcna3</i>	potassium voltage-gated channel, shaker-related subfamily, member 3
<i>Kcna4</i>	potassium voltage-gated channel, shaker-related subfamily, member 4
<i>Kcna5</i>	potassium voltage-gated channel, shaker-related subfamily, member 5
<i>Kcna6</i>	potassium voltage gated channel, shaker related subfamily, member 6
<i>Kcnab1</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 1
<i>Kcnab2</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 2
<i>Kcnab3</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 3
<i>Kcnb1</i>	potassium voltage gated channel, Shab-related subfamily, member 1
<i>Kcnc3</i>	potassium voltage gated channel, Shaw-related subfamily, member 3
<i>Kcnc4</i>	potassium voltage gated channel, Shaw-related subfamily, member 4
<i>Kcnd1</i>	potassium voltage-gated channel, Shal-related subfamily, member 1
<i>Kcnd2</i>	potassium voltage-gated channel, Shal-related subfamily, member 2
<i>Kcnd3</i>	potassium voltage-gated channel, Shal-related subfamily, member 3
<i>Kcne1</i>	potassium voltage-gated channel, Isk-related family, member 1
<i>Kcne1l</i>	KCNE1-like

<i>Kcne2</i>	potassium voltage-gated channel, Isk-related family, member 2
<i>Kcne3</i>	potassium voltage-gated channel, Isk-related family, member 3
<i>Kcne4</i>	potassium voltage-gated channel, Isk-related family, member 4
<i>Kcnh2</i>	potassium voltage-gated channel, subfamily H (eag-related), member 2
<i>Kcnh7</i>	potassium voltage-gated channel, subfamily H (eag-related), member 7
<i>Kcnp2</i>	Kv channel-interacting protein 2
<i>Kcnj11</i>	potassium inwardly rectifying channel, subfamily J, member 11
<i>Kcnj12</i>	potassium inwardly-rectifying channel, subfamily J, member 12
<i>Kcnj2</i>	potassium inwardly-rectifying channel, subfamily J, member 2
<i>Kcnj3</i>	potassium inwardly-rectifying channel, subfamily J, member 3
<i>Kcnj4</i>	potassium inwardly-rectifying channel, subfamily J, member 4
<i>Kcnj5</i>	potassium inwardly-rectifying channel, subfamily J, member 5
<i>Kcnj8</i>	potassium inwardly-rectifying channel, subfamily J, member 8
<i>Kcnk1</i>	potassium channel, subfamily K, member 1
<i>Kcnk3</i>	potassium channel, subfamily K, member 3
<i>Kcnk5</i>	potassium channel, subfamily K, member 5
<i>Kcnn2</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
<i>Kcnq1</i>	potassium voltage-gated channel, KQT-like subfamily, member 1
<i>Npr3</i>	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
<i>Pak1</i>	p21 protein (Cdc42/Rac)-activated kinase 1
<i>Myh7</i>	myosin, heavy chain 7, cardiac muscle, <i>beta</i>
<i>Pkp2</i>	plakophilin 2
<i>Ryr2</i>	ryanodine receptor 2, cardiac
<i>Scn1a</i>	sodium channel, voltage-gated, type I, <i>alpha</i>
<i>Scn1b</i>	sodium channel, voltage-gated, type I, <i>beta</i>
<i>Scn2b</i>	sodium channel, voltage-gated, type II, <i>beta</i>
<i>Scn3a</i>	sodium channel, voltage-gated, type III, <i>alpha</i>
<i>Scn3b</i>	sodium channel, voltage-gated, type III, <i>beta</i>
<i>Scn4a</i>	sodium channel, voltage-gated, type IV, <i>alpha</i> subunit
<i>Scn5a</i>	sodium channel, voltage-gated, type V, <i>alpha</i> subunit
<i>Scn7a</i>	sodium channel, voltage-gated, type VII, <i>alpha</i>
<i>Scn9a</i>	sodium channel, voltage-gated, type IX, <i>alpha</i>
<i>Smad6</i>	SMAD family member 6
<i>Smad9</i>	SMAD family member 9
<i>Smyd2</i>	SET and MYND domain containing 2
<i>Trpc1</i>	transient receptor potential cation channel, subfamily C, member 1
<i>Trpc3</i>	transient receptor potential cation channel, subfamily C, member 3
<i>Trpc4</i>	transient receptor potential cation channel, subfamily C, member 4
<i>Trpc5</i>	transient receptor potential cation channel, subfamily C, member 5
<i>Trpc6</i>	transient receptor potential cation channel, subfamily C, member 6
<i>Trpm4</i>	transient receptor potential cation channel, subfamily M, member 4
<i>Trpm5</i>	transient receptor potential cation channel, subfamily M, member 5
<i>Trpv1</i>	transient receptor potential cation channel, subfamily V, member 1
<i>Trpv6</i>	transient receptor potential cation channel, subfamily V, member 6
<i>Tgfb1</i>	transforming growth factor, <i>beta</i> 1
<i>Camk2d</i>	calcium/calmodulin-dependent protein kinase II <i>delta</i>

Appendix 2. People's Protection Committee agreement for the study on human subjects

COMITE DES PROTECTION DES PERSONNES SUD-EST II

Hôpital HOTEL-DIEU
Porte 16
Place de l'Hôpital
69288 LYON CEDEX 02
Tél : 04.78.42.94.48
Fax : 04.78.42.94.69
E-mail : francoise.leclet@chu-lyon.fr

Lyon le 4 octobre 2007

Monsieur le Professeur CHEVALIER
Service de Rythmologie
HOPITAL CARDIOLOGIQUE

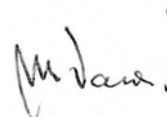
Monsieur,

Le Comité de Protection des Personnes SUD-EST II, lors de sa séance du 3 octobre 2007, a pris connaissance du projet d'étude que vous envisagez de mener et relatif à la mesure des paramètres inflammatoires chez les patients avec fibrillation atriale.

A l'issue de la délibération, le Comité a considéré que *cette étude n'entraîne pas dans le champ d'application de la Loi du 9 août 2004* dans la mesure où il s'agit d'une étude, « non interventionnelle ».

La procédure est habituelle, sans aucun geste invasif ou positionnement d'un cathéter autres que ceux faits dans la pratique courante. Il n'y a aucun risque lié à l'étude proprement dite.

Recevez, Monsieur, mes salutations les meilleures.



Pour le CPP SUD-EST-II
Le Président – Professeur Michel David

Membres ayant participé à la séance :

AMIET Nicole (Domaine Juridique) - BIENVENU Jacques (Recherche Biomédicale) - BOISRIVEAUD Christine (Psychologue) - BONIFAIT Christian (Domaine Juridique) - CHEVAUX Raphaël (Représentant Association Malades et Usagers) - DAVID Michel (Recherche Biomédicale) - GIMENEZ-GEAY Isabelle (Infirmière) - GUEYFFIER François (Recherche Biomédicale – méthodologie) - HODGKINSON Isabelle (Recherche Biomédicale) - PAULIGNAN Yves (Recherche Biomédicale) - PELEGRIN Serge (Représentant Association Malades et Usagers) - PHILIPPE Noël (Recherche Biomédicale) - PHILIPPE-JANON Chantal (Domaine Social) -

Appendix 3. Local Ethics Committee agreements for studies on rats

Université Claude Bernard  Lyon 1

DEPARTEMENT DE FORMATION
ET CENTRE DE RECHERCHE
EN BIOLOGIE HUMAINE

Adresse : Domaine Rockefeller
8, avenue Rockefeller
69373 Lyon cedex 08
Tel : 04.78.77.70.38

Lyon, le 05 Juillet 2010

M. Claude JULIEN

Objet : Votre protocole BH2010-28

Cher collègue,

Votre protocole BH 2010-28 intitulé « Etude du protéome urinaire et mesure de l'électrocardiogramme chez des rats SHR âgés et leurs contrôles WKY » a reçu un avis favorable du Comité d'Expérimentation Animale de l'Université Claude Bernard Lyon I lors de sa séance du 05 Juillet 2010.

Je vous prie d'agréer, cher collègue, l'assurance de mes sentiments les meilleurs.




Pr. Pierre FARGE

Université Claude Bernard  Lyon 1

DEPARTEMENT DE FORMATION
ET CENTRE DE RECHERCHE
EN BIOLOGIE HUMAINE

Adresse : Domaine Rockefeller
8, avenue Rockefeller
69373 Lyon cedex 08
Tél : 04.78.77.70.38

Lyon, le 04 Avril 2011

M. le Dr Claude JULIEN

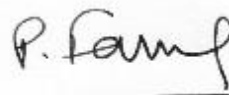
Objet : Votre protocole BH2011-17

Cher collègue,

Votre protocole BH 2010-17 « Etude du protéome urinaire et mesure de l'électrocardiogramme chez des rats SHR adultes (protocole complémentaire au BH2010-28 et BH2010-08) » a reçu un avis favorable du Comité d'Expérimentation Animale de l'Université Claude Bernard Lyon I lors de sa séance du 04 Avril 2011 mais le Comité recommande une analgésie au kétoprofène 30 minutes avant l'injection au penthobarbital, celui-ci n'étant pas analgésique.

Je vous prie d'agréer, cher collègue, l'assurance de mes sentiments les meilleurs.





Pr. Pierre FARGE



Increased intracardiac vascular endothelial growth factor levels in patients with paroxysmal, but not persistent atrial fibrillation

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Aims	Although inflammation appears to play a pivotal role in the pathophysiology of atrial fibrillation (AF), the source of inflammation is unknown. We hypothesized that multilevel measurement of several inflammatory proteins in AF patients would help assess the extent and the source of inflammation.
Methods and results	Thirty-nine patients with paroxysmal AF, 33 with persistent AF, and 9 control patients with Wolff–Parkinson–White syndrome were enrolled. Peripheral, left atrial, coronary sinus, and pulmonary vein blood samples were obtained during catheterization. Serum levels of vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), soluble intercellular adhesion molecule 1 (sICAM-1), and transforming growth factor-β1 (TGF-β1) were measured at the four sampled sites. Interleukin-8, sICAM-1, and TGF-β1 levels did not differ among groups at any of the sampled sites. Peripheral VEGF levels were higher in both paroxysmal and persistent AF patients than in controls ($P \leq 0.03$). Left atrial VEGF levels were higher in paroxysmal AF ($P = 0.05$), but not in persistent AF ($P = 0.32$), compared with controls. Coronary sinus and pulmonary vein VEGF levels did not differ significantly among groups.
Conclusions	Low levels of several inflammatory markers in both paroxysmal and persistent AF patients suggest that the inflammatory process is of low grade, if present. In the context of normal pulmonary vein VEGF levels, the heart itself is the most likely source of high left atrial VEGF levels in paroxysmal AF patients; however, this disorder appears to be a transient event in the natural history of AF.
Keywords	Atrial fibrillation • Inflammation • Vascular endothelial growth factor

Introduction

Atrial fibrillation (AF), the most common clinical arrhythmia, is associated with significant impairment of quality of life, and makes an important contribution to general morbidity and mortality.^{1,2} Both clinical and experimental studies postulate inflammation as a predisposing factor for AF, as well as for its complications.³ Observation of inflammatory infiltrates, myocyte necrosis, and fibrosis in atrial biopsies from patients with lone AF refractory to anti-arrhythmic drug therapy supports the hypothesis that AF is

closely related to the inflammatory process.⁴ The contribution of inflammation to AF is also suggested by the high incidence of AF after cardiac surgery.^{5,6} Inflammatory cytokines have been recently described as facilitators of AF development and recurrence.^{7,8} However, several studies have failed to demonstrate elevated levels of C-reactive protein (CRP), interleukin-6 (IL-6), and interleukin-8 (IL-8) in AF patients.^{1,2,9–11}

We hypothesized that multilevel intracardiac and extracardiac measurements of several inflammatory proteins would help assess the extent and source of inflammation in AF patients.

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Methods

Patient selection

A total of 81 patients scheduled for electrophysiological study or catheter ablation procedures at the Rhythmology Department of the Louis Pradel Cardiology Hospital in Lyon, France, between August 2008 and June 2010 were screened for participation in this study. The control group included nine patients with left-sided accessory pathway Wolff–Parkinson–White syndrome and without any history of AF.

A total of 39 patients with paroxysmal AF and 33 patients with persistent AF were enrolled. Atrial fibrillation was defined as ‘paroxysmal’ when the arrhythmia was self-terminating within 7 days and ‘persistent’ when the AF episode persisted for >7 days or pharmacological or electrical cardioversion were required to terminate the arrhythmia.

Exclusion criteria included recent history of an acute cardiovascular or cerebrovascular event, major trauma or surgery, apparent ischaemic heart disease, malignancy, connective tissue or inflammatory disease, acute or chronic infection, and pulmonary, hepatic, or renal impairment. Patients under ongoing anti-inflammatory or steroid treatment were also excluded. A detailed medical and drug history was obtained for each patient enrolled. All patients underwent physical examination and routine laboratory tests. The left atrial area was assessed by trans-thoracic echocardiography prior to electrophysiological study or catheter ablation procedures. For each patient stroke risk was evaluated according to current recommendations (CHA₂DS₂-VASc score).¹² This score assigns one point for each of the following risk factors for patients with AF: congestive heart failure, hypertension, age 65–74 years, diabetes, female sex, and vascular disease. Two points are assigned for a history of stroke or transient ischaemic attack and age ≥75 years.

All participants provided written informed consent in accordance with the local ethics committee. The study protocol complies with the Declaration of Helsinki and was approved by the South-East II People’s Protection Committee and the Advisory Committee on Information Processing in Research in the Field of Health.

Blood sampling and laboratory analysis

Blood sampling was performed during routine cardiac catheterization, prior to any ablations or administration of heparin. Blood samples were obtained serially from the femoral vein and coronary sinus. The left atrium was accessed through trans-septal puncture and a third blood sample was taken from the left atrium via the trans-septal sheath. Blood samples from the left superior pulmonary vein were collected via the trans-septal sheath advanced into the proximal segment of the sampled vein.

Vacutainer (BD, Plymouth, UK) tubes (6.0 mL) containing EDTA were used. The tubes were stored and shipped on ice within 30 min after sampling.

Serum concentrations of soluble intercellular adhesion molecule-1 (sICAM-1) and transforming growth factor-β1 (TGF-β1) were measured using commercially available quantitative enzyme-linked immunosorbent assay kits (Human TGF-β1 and Human sICAM-1/CD54 Quantikine ELISA kits, R&D Systems, Inc., Minneapolis, MN, USA). Luminex methodology was used to measure serum levels of vascular endothelial growth factor (VEGF) and IL-8, with R&D System kits (Human VEGF and Human CXCL8/IL-8 Fluorokine Multi-analyte Profiling ELISA, R&D Systems, Inc.). The technician performing the analysis was not aware of the patient’s diagnosis or rhythm. The lower limits of detection were 4 pg/mL for VEGF, IL-8, and TGF-β1, and 30 ng/mL for sICAM-1.

Statistics

Values are expressed as mean ± SEM. Comparisons between control and AF groups were made using Student’s *t*-test or the Mann–Whitney *U* test, as appropriate. Categorical data were compared using the χ^2 test. Correlations were ascertained with Pearson’s correlation or Spearman’s rank correlation method, as appropriate. Stepwise multiple regression analysis was performed to identify independent predictors of VEGF levels. Two-tailed *P* < 0.05 was considered statistically significant. All data were computed using GraphPad Prism® (GraphPad Software, San Diego, CA, USA).

Results

Patient clinical characteristics

Demographic and clinical data regarding patients and control subjects are summarized in Table 1.

There was no significant difference among the three groups in terms of sex ratio, smoking status, history of hypertension or diabetes, or statin treatment (all *P* > 0.10). Atrial fibrillation patients were more likely to be treated with anti-aldosteronics and angiotensin converting enzyme inhibitors (ACEIs) or angiotensin II receptor blockers (ARBs) (*P* < 0.01). They were also older (*P* < 0.01) and had a higher body mass index (*P* < 0.01). As expected, left atrial area as assessed by echocardiography in the parasternal long axis was higher in paroxysmal AF patients than in controls and in persistent AF than in paroxysmal AF patients (*P* = 0.001). Persistent AF patients were more frequently in arrhythmia at the moment of blood draw compared with paroxysmal AF patients (*P* < 0.001).

Twelve patients in the paroxysmal AF group (31%) and 13 (39%) in the persistent AF group had single or multiple prior ablation procedures (*P* = 0.26). Mean duration of AF history was 95 ± 86 months in paroxysmal AF patients and 77 ± 58 months in persistent AF patients (*P* = 0.29). None of the control patients presented AF during electrophysiological study or ablation procedure.

Inflammatory marker levels

Mean levels of inflammatory markers are presented in Table 2.

There were no significant differences among the three groups regarding IL-8, sICAM-1, or TGF-β1 levels at any of the sampling sites (all *P* > 0.05). Similarly, CRP levels (measured in peripheral blood only) did not differ among the three groups (*P* = 0.60).

Peripheral levels of VEGF were higher in both paroxysmal and persistent AF patients than in controls (*P* = 0.03 and 0.02, respectively) (Figure 1). In the left atrium, VEGF levels were higher in the paroxysmal AF group than the control group (*P* = 0.05); however, there was no significant difference between persistent AF patients and controls (*P* = 0.32) (Figure 1). Although not statistically significant (*P* > 0.10), coronary sinus levels of VEGF were higher in paroxysmal AF patients compared with both controls and persistent AF patients (22.2 ± 8.7 pg/mL in paroxysmal AF patients vs. 9.5 ± 7.7 pg/mL in control patients and 9.8 ± 7.9 pg/mL in persistent AF patients) (Figure 1).

Pulmonary vein levels of VEGF did not differ significantly among the three groups (*P* = 0.73).

Table 1 Clinical characteristic of study groups

	Control (n = 9)	Paroxysmal atrial fibrillation (n = 39)	Persistent atrial fibrillation (n = 33)	P value
Age (years)	43 ± 18	56 ± 9	58 ± 8	<0.01
Men (n, %)	6 (67%)	32 (82%)	28 (85%)	0.46
Body mass index (kg/m ²)	22.2 ± 3.5	27.1 ± 3.4	29.2 ± 5.2	<0.01
Hypertension (n, %)	2 (22%)	16 (41%)	19 (58%)	0.12
Diabetes (n, %)	0 (0%)	5 (13%)	3 (9%)	0.50
Smokers (n, %)	1 (11%)	12 (31%)	11 (33%)	0.42
History of AF (months)	N/App	95 ± 86	77 ± 58	0.29
Anti-aldosteronics (n, %)	0 (0%)	2 (5%)	7 (21%)	0.02
Statins (n, %)	0 (0%)	9 (23%)	10 (30%)	0.16
ACEI/ARB (n, %)	0 (0%)	8 (21%)	17 (52%)	<0.01
Left atrial surface (cm ²)	15.7 ± 2.5	20.6 ± 3.7	25.2 ± 6.1	0.001
AF during blood sampling (n, %)	N/App	8 (21%)	24 (73%)	<0.001
Prior ablation procedure (n, %)	N/App	12 (31%)	13 (39%)	0.26
CHA ₂ DS ₂ VASc score	0.63 ± 0.26	1.33 ± 0.21	1.21 ± 0.21	0.34

AF, atrial fibrillation; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; N/App, not applicable.

Table 2 Inflammatory marker levels in the three study groups

	Control (n = 9)	Paroxysmal atrial fibrillation (n = 39)	P value ^a	Persistent atrial fibrillation (n = 33)	P value ^b
VEGF (pg/mL)					
Femoral vein	15.4 ± 10.8	24.1 ± 3.5	0.03	25.8 ± 3.9	0.02
Left atrium	9.4 ± 3.9	25.2 ± 4.5	0.05	15.0 ± 4.2	0.32
Coronary sinus	9.5 ± 7.7	22.2 ± 8.7	0.77	9.8 ± 7.9	0.81
Pulmonary vein	6.7 ± 3.5	2.7 ± 0.8	0.15	8.8 ± 8.7	0.72
IL-8 (pg/mL)					
Femoral vein	7.0 ± 4.9	6.9 ± 0.6	0.21	8.0 ± 1.4	0.67
Left atrium	7.7 ± 3.4	7.8 ± 0.7	0.17	8.0 ± 1.7	0.37
sICAM-1 (ng/mL)					
Femoral vein	161.4 ± 15.8	178.5 ± 8.3	0.36	179.7 ± 7.0	0.25
Left atrium	160.1 ± 15.5	174.4 ± 8.1	0.43	173.2 ± 7.1	0.46
TGF-β1 (ng/mL)					
Femoral vein	25.0 ± 10.0	15.9 ± 1.8	0.63	22.3 ± 3.2	0.87
Left atrium	13.1 ± 3.3	15.7 ± 2.5	0.88	15.5 ± 2.0	0.41
CRP (μg/mL)					
Peripheral blood	9.6 ± 7.0	3.6 ± 0.9	0.10	6.6 ± 2.4	0.60

^aParoxysmal atrial fibrillation vs. control.

^bPersistent atrial fibrillation vs. control.

Intersite vascular endothelial growth factor gradients

In patients with paroxysmal AF, no significant difference was observed between peripheral and left atrial or coronary sinus and left atrial levels of VEGF (both $P > 0.05$); however, both left atrial and peripheral levels of VEGF were significantly higher than pulmonary vein levels (both $P < 0.01$). In patients with persistent AF, peripheral levels of VEGF were higher than left atrial levels

($P = 0.05$); however, no significant difference was observed among left atrial, coronary sinus, and pulmonary vein levels of VEGF ($P > 0.05$).

Vascular endothelial growth factor predictors

Neither gender, the presence of hypertension or diabetes, current smoking status, anti-aldosteronics, statins, treatment with ACEI or

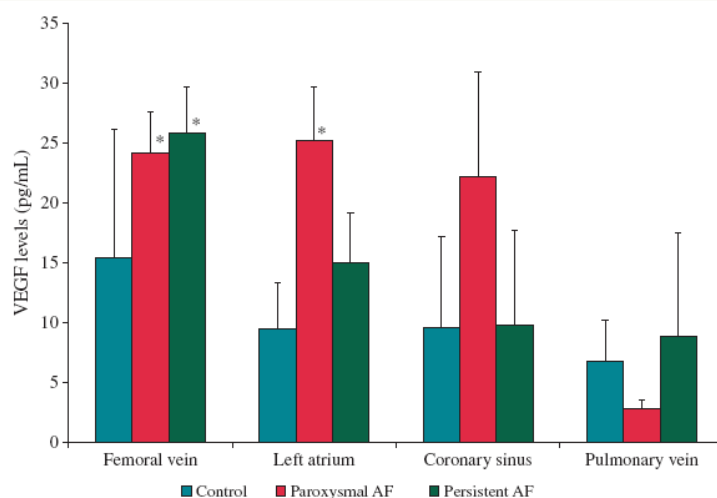


Figure 1 Mean levels of vascular endothelial growth factor in the femoral vein, left atrium, coronary sinus, and pulmonary vein in the three groups. * $P < 0.05$ vs. control.

ARB, previous ablation procedures, nor rhythm during blood sampling (i.e. sinus rhythm or AF) were significantly correlated with VEGF levels at any of the sampled sites (all $P > 0.10$). Body mass index, left atrial area, and duration of AF history were also not significantly correlated with VEGF levels at any of the sampled sites (all $P > 0.10$). There was no correlation between age and VEGF levels in neither of the sampled sites nor of the studied groups (for paroxysmal AF patients $r = 0.17$, $P = 0.28$ for peripheral VEGF levels and $r = 0.03$, $P = 0.82$ for left atrial VEGF levels, for persistent AF patients $r = -0.1$, $P = 0.10$ for peripheral VEGF levels and $r = 0.11$, $P = 0.57$ for left atrial VEGF levels).

When paroxysmal AF patients and persistent AF patients were considered as a single group, left atrial levels of VEGF were significantly negatively correlated with ACEI or ARB therapy (Spearman's rank correlation coefficient $r = -0.25$, $P = 0.03$). In multivariate analysis, the presence of AF independently predicted elevated peripheral levels of VEGF ($r = 0.31$, $P = 0.04$), whereas there was no significant association between AF and left atrial, coronary sinus, or pulmonary vein levels of VEGF (all $P > 0.05$).

Patients with both paroxysmal and persistent AF were then divided according to stroke risk as assessed by CHA₂DS₂VASc score into low-intermediate (CHA₂DS₂VASc = 0–1, $n = 46$) and high (CHA₂DS₂VASc ≥ 2 , $n = 26$) stroke risk groups. We did not find any significant differences between peripheral or left atrial VEGF levels between patients considered at low-intermediate and high stroke risk according to CHA₂DS₂VASc score (peripheral VEGF levels were 21.31 pg/mL in low-intermediate stroke risk group and 27.73 pg/mL in high stroke risk group, left atrial levels of VEGF were 16.42 pg/mL in low-intermediate stroke risk group and 23.19 pg/mL in high stroke risk group, both $P > 0.1$).

Discussion

The main findings of the present study are that (i) patients with paroxysmal or persistent AF and no concomitant inflammatory disease did not have high CRP, IL-8, TGF- β 1, or sICAM-1 levels; (ii) patients with paroxysmal AF had high peripheral and left atrial, but similar pulmonary vein levels of VEGF compared with non-AF controls; (iii) although patients with persistent AF had high peripheral levels of VEGF, their left atrial, coronary sinus, and pulmonary vein levels of VEGF were not significantly elevated compared with non-AF controls.

Could inflammation in atrial fibrillation have been overestimated?

Although a large amount of data suggest a link between inflammation and AF, several studies failed to show elevated levels of CRP, IL-6, or IL-8 in AF patients.^{1,2,9–11} Furthermore, studies assessing the efficacy of anti-inflammatory therapy in AF prevention have yielded conflicting results,^{13,14} suggesting that the role of inflammatory processes in the pathophysiology of AF might be overestimated. Our study did not demonstrate any significant difference in CRP, IL-8, or sICAM-1 levels among the three patient groups. These results suggest that the high inflammatory marker levels reported in AF patients could be due to associated co-morbidities rather than to AF *per se*.

Inflammation and pro-thrombotic state seem to be related in AF patients. However, we did not find any significant correlation between age, smoking, hypertension, diabetes, or CHA₂DS₂VASc score and any of the inflammatory markers. The low-risk profile of our cohort could explain the absence of high inflammatory marker levels and the relatively low risk of stroke (mean CHA₂DS₂VASc score 1.33 ± 0.21 in paroxysmal AF and 1.21 ± 0.21 in

persistent AF). This further supports the hypothesis that associated co-morbidities and not AF itself induces the inflammatory and pro-thrombotic states reported in AF patients, as previously stated by Lip and colleagues.¹⁵

The source of vascular endothelial growth factor in atrial fibrillation patients

In paroxysmal AF patients, the combination of normal pulmonary vein and high left atrial VEGF levels suggests the heart itself as the most likely source of high left atrial VEGF levels. Accumulated evidence indicates that VEGF and VEGF receptor mRNA are expressed in heart tissue.^{16,17} Ogi et al.¹⁸ recently reported high VEGF levels in the atrial tissue of AF patients compared with non-AF controls.

A number of pathological conditions, such as oxygen deficiency, inflammation, or, according to recent reports, pulsatile mechanical stretch,¹⁹ are known to induce VEGF secretion. Low levels of several inflammatory markers in both paroxysmal and persistent AF patients suggest that the inflammatory process is of low grade, if present. Therefore, it seems unlikely that this process could account for the high peripheral VEGF levels observed in these patients. High VEGF levels have been reported in the myocardium and peripheral blood following myocardial infarction and unstable angina.^{20,21} Despite the absence of evidence for accelerated angiogenesis in paroxysmal AF patients, we found high left atrial levels of VEGF in this population. Histopathological studies report a high incidence of atrial ischaemia, in up to 17%, and isolated atrial infarctions in >20% of autopsied patients with a history of AF.²² A transient increase in hypoxia inducible factor α gene expression, a known trigger for VEGF secretion, has been reported in cardiac myocytes in the early onset of AF.²³ On the other hand, the inverse relationship is also possible, with ischaemia favouring AF occurrence.

The fibrillating atrium leading to an irregular blood flow may induce pulsatile vascular stretch and impaired blood rheology. Both phenomena could trigger VEGF secretion, as well as its release from smooth muscle and circulating blood cells^{24–26} in AF patients, regardless of the clinical form of the arrhythmia. Pulsatile mechanical stretch, recently postulated as a potent trigger for VEGF secretion from cardiac myocytes,^{17,19} could be responsible for the high intracardiac VEGF levels observed in paroxysmal AF patients.

Progressive left atrial remodelling affects cardiac secretion of vascular endothelial growth factor (and the inverse)

Our results suggest that left atrial secretion of VEGF is a transient event in the natural history of AF. Vascular endothelial growth factor has been previously shown to stimulate the fibrotic process within the atrial tissue, probably through the induction of angiogenesis.^{27,28} Several studies have emphasized that patients with persistent AF present more important fibrotic changes compared with patients with paroxysmal AF.^{29,30} The spreading of cardiac fibrosis could account for progressive cardiac stiffening in this setting, thereby reducing the degree of pulsatile stretch and subsequently diminishing VEGF levels.

Despite the important role that TGF- β 1 plays in the genesis of fibrosis in patients with AF, we did not find a significant difference in TGF- β 1 levels between patients with and without AF. The larger use of ACEI or ARB in AF patients (35% in AF patients vs. 0% in control patients) might have mitigated the differences between the groups. TGF- β 1 expression is known to be induced by angiotensin II³¹ and blockade of the angiotensin II type 1 receptor is known to suppress TGF- β 1 upregulation.³² Furthermore, the larger use of ACEI or ARB could also have mitigated even higher VEGF levels in AF patients.

Our results suggest that in the context of persistent AF the damaged and remodelled atrial tissue becomes less able to secrete and release VEGF. Meanwhile, VEGF inhibition has been associated with extensive apoptotic changes in the vasculature of neonatal mice.³³ The low intracardiac VEGF levels observed in persistent AF patients might signify the loss of this protective mechanism of endothelial cells, subsequently leading to progressive endothelial and atrial endocardial damage. High VEGF levels observed in paroxysmal AF patients may participate in the progressive spread of atrial fibrosis, which, in turn, would diminish VEGF secretion by reducing atrial responsiveness to pulsatile mechanical stretch. Later, the resulting low intracardiac VEGF levels could contribute to progressive endothelial dysfunction.

Limitations

This study is limited by its cross-sectional design, which only permitted associations to be observed; cause–effect relationships could not be established. The small number of patients in the control group may have lowered the study's statistical power. Control patients were also significantly younger than AF patients. However, there was no correlation between age and VEGF levels whatever the sampled site or the studied group. Additionally, one could argue that high VEGF levels might be due to coexisting cardiovascular conditions; however, there was no significant difference among the three groups regarding the proportion of hypertensive or diabetic patients.

Conclusions

Low levels of several inflammatory markers in both paroxysmal and persistent AF patients suggest that the inflammatory process is of low grade, if present. In the context of normal pulmonary vein VEGF levels, the heart itself is the most likely source of high left atrial VEGF levels in paroxysmal AF patients; however, this disorder appears to be a transient event in the natural history of AF.

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Unprovoked atrial tachyarrhythmias in aging spontaneously hypertensive rats: the role of the autonomic nervous system

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Scridon A, Gallet C, Arisha MM, Oréa V, Chapuis B, Li N, Tabib A, Christé G, Barrès C, Julien C, Chevalier P. Unprovoked atrial tachyarrhythmias in aging spontaneously hypertensive rats: the role of the autonomic nervous system. *Am J Physiol Heart Circ Physiol* 303: H000–H000, 2012. First published June 1, 2012; doi:10.1152/ajpheart.00004.2012.—Experimental models of unprovoked atrial tachyarrhythmias (AT) in conscious, ambulatory animals are lacking. We hypothesized that the aging, spontaneously hypertensive rat (SHR) may provide such a model. Baseline ECG recordings were acquired with radiotelemetry in eight young (14-wk-old) and eight aging (55-wk-old) SHRs and in two groups of four age-matched Wistar-Kyoto (WKY) rats. Quantification of AT and heart rate variability (HRV) analysis were performed based on 24-h ECG recordings in unrestrained rats. All animals were submitted to an emotional stress protocol (air-jet). In SHRs, carbamylcholine injections were also performed. Spontaneous AT episodes were observed in all eight aging SHRs (median, 91.5; range, 4–444 episodes/24 h), but not in young SHRs or WKY rats. HRV analysis demonstrated significantly decreased low frequency components in aging SHRs compared with age-matched WKY rats ($P < 0.01$) and decreased low/high frequency ratios in both young ($P < 0.01$) and aging ($P = 0.01$) SHRs compared with normotensive controls. In aging SHRs, emotional stress significantly reduced the number of arrhythmic events, whereas carbamylcholine triggered AT and significantly increased atrial electrical instability. This study reports the occurrence of unprovoked episodes of atrial arrhythmia in hypertensive rats, and their increased incidence with aging. Our results suggest that autonomic imbalance with relative vagal hyperactivity may be responsible for the increased atrial arrhythmogenicity observed in this model. We also provide evidence that, in this model, the sympatho-vagal imbalance preceded the occurrence of arrhythmia. These results indicate that aging SHRs may provide valuable insight into the understanding of atrial arrhythmias.

spontaneous atrial arrhythmias; experimental model; autonomic imbalance

ATRIAL FIBRILLATION (AF) AFFECTS ~1% of the general population and up to 8% of subjects over the age of 80 (9). Incomplete understanding of the pathophysiological mechanisms of arrhythmia explains the relative failure of available antiarrhythmic strategies to effectively treat atrial electrical instability (3, 21).

Numerous animal models have been developed to explore the mechanisms that initiate and maintain atrial tachyarrhythmias (AT), including AF, and to evaluate different therapeutic strategies (6, 12, 30). Yet, none of these models manage to faithfully reproduce the human clinical condition. Experimen-

tal models of spontaneous AT in conscious, ambulatory animals are still lacking. Previous studies have reported “spontaneous AT or AF” in animals. However, in those models, arrhythmia occurred only after applying programmed stimulation protocols (6). A number of essential features for AT occurrence, such as structural, electrical, or autonomic remodeling, could have been artificially induced in these models. Therefore, in our opinion, “spontaneous” is not the proper term for describing the type of arrhythmias reported in those articles; we believe that the term “spontaneous” should be reserved for situations that have not used artificial means to trigger the arrhythmia.

The purpose of the present study was to develop a model that could reproducibly exhibit spontaneous AT in rats and to describe its phenotypic features. Because aging (1) and arterial hypertension (1, 7) are the most frequent risk factors associated with atrial arrhythmogenicity, we hypothesized that aging rats of the spontaneously hypertensive rat (SHR) strain might provide a reliable model of spontaneous atrial arrhythmia. Accordingly, we aimed to assess, in baseline conditions, the occurrence of spontaneous AT and several other markers of increased atrial arrhythmogenicity (i.e., atrial premature contractions, couplets, and bigeminisms) in young and aging SHRs. These parameters were compared with those observed in normotensive controls. For this study, we used 24-h continuous ECG recordings, acquired with radiotelemetry.

Autonomic imbalance has been implicated as a potential facilitator in the occurrence and maintenance of AT in humans and various animal models. Several factors, such as heterogeneous autonomic innervation of the atria (19) or a complex and dynamic inter-relationship between the two branches of the autonomic nervous system (26, 31), may be involved in arrhythmia occurrence. Accordingly, the presence and the pattern of an autonomic imbalance were searched using heart rate (HR) variability (HRV) analysis and two experimental protocols that targeted the autonomic nervous system (i.e., emotional stress and carbamylcholine administration).

MATERIALS AND METHODS

Animals. Male SHRs and normotensive Wistar-Kyoto (WKY) rats were purchased from Elevage Janvier (Le Genest Saint Isle, France).

All animals were housed in a climate-controlled room (21° to 22°C), with a 12-h:12-h light/dark cycle, in an accredited animal facility. The rats were housed individually in polycarbonate cages and fed standard rat pellets and tap water ad libitum.

All experiments were performed in compliance with the French Ministry of Agriculture guidelines for animal experimentation and adhered to the American Physiological Society Guiding Principles in

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the Care and Use of Vertebrate Animals in Research and Training, and they were approved by the local Animal Ethics Committee.

Transmitter implantation procedure. Radiotelemetry ECG transmitters (TA11 CA-F40; Data Sciences International, St. Paul, MN) were implanted under isoflurane anesthesia. Each animal received subcutaneous prophylactic injections of penicillin G (50,000 IU) and ketoprofen (2 mg/kg). The body of each transmitter was placed in a dorsal, subcutaneous pocket, and the two ECG leads were tunneled subcutaneously and secured in a lead II configuration. After the implantation procedure, animals were allowed at least 1 wk of recovery before recording.

ECG recording. For baseline quantification of atrial arrhythmic events, 24-h continuous ECG monitoring was performed on unrestrained, conscious rats. Recordings were conducted on eight 55-wk-old SHR (471 \pm 5 g), four age-matched WKY rats (576 \pm 11 g), eight young (14-wk-old) SHR (342 \pm 3 g), and four young (14-wk-old) WKY rats (397 \pm 5 g). ECG signal capture was accomplished with receivers (RPC-1; Data Sciences International) placed under each experimental cage. Telemetry ECGs were converted to analog signal (Analog ECG Output Adapter R08; Data Sciences International) and routed to a personal computer equipped with a signal acquisition card (NI PCIe-6251; National Instruments, Austin, TX). An acquisition program developed in our laboratory using LabVIEW 2009 software (National Instruments) allowed the signal to be continuously recorded with a 2,000-Hz sampling frequency and stored on hard disk.

ECG analysis. ECG data were analyzed using a program recently developed in our laboratory using LabVIEW 2010 software (National Instruments) to automatically detect R waves and measure RR intervals, as previously described (23). All ECG tracings were visually assessed by two independent cardiologists. In the vast majority of time ECG quality allowed easy recognition of all atrial arrhythmic events. Infrequently, intense animal somatomotor activity was associated with altered ECG signal and artifacts; this impaired proper assessment of the atrial electrical activity. Therefore, all artifactual periods were discarded before analysis.

AT was defined as rapid, irregular, supraventricular rhythm [irregular ventricular response with narrow QRS complexes, exceeding by at least 50 beats/min the HR measured during sinus rhythm], of at least 3 beats. The diagnosis also required that P waves were absent or replaced by atrial waves with morphology significantly different compared with sinus rhythm P waves. Premature atrial contractions (PACs) were defined as early atrial activations, with morphology different compared with sinus rhythm P waves. Atrial couplets were defined as a pair of atrial premature activations in a row, whereas the regular succession of a normal sinus beat and an atrial premature activation was defined as atrial bigeminism.

The total numbers of AT episodes, PACs, atrial couplets, and bigeminisms were quantified on the 24-h recording. The duration of an AT episode was measured from the first arrhythmic beat of the episode (top of the R wave) to the first sinus rhythm beat that followed the episode.

To test for day-to-day reproducibility, four aging SHRs underwent 48-h continuous recording, instead of the standard 24-h recording. Data used for between-group comparisons corresponded to the first 24-h period. To test for medium-term reproducibility, comparisons were also performed, for all eight aging SHRs, between the 24-h recording used in between-group comparisons and a second recording, performed 1 wk earlier.

HRV analysis. All arrhythmic events and compensatory pauses were excluded manually before analysis. The mean HR was automatically calculated over 24-h intervals. The HRV during sinus rhythm was assessed in the 24-h recordings for each animal.

HRV was assessed by analyzing beat-to-beat variations in RR intervals in the time and frequency domains, as previously described (2). For the time domain analysis, SD of normal RR intervals, root-mean-square of successive RR-interval differences, and percent-

age of adjacent RR intervals that differed by >5 ms (pNN5) were calculated. For the frequency domain analysis, time series were resampled at 20 Hz using a cubic spline, then spectral power in the low-frequency (LF) band (0.3–0.6 Hz), high-frequency (HF) band (0.6–2.5 Hz), the LF-to-HF ratio were estimated on 2,048-point (102.4 s) segments windowed by the Hanning function and overlapping by 50% using a fast Fourier transform.

Additionally, to identify potential significant changes in autonomic tone preceding arrhythmic events, we analyzed several intervals of intense arrhythmic activity (frequent PACs and AT episodes) and the 5-min periods preceding these intervals. The resulting frequency-domain parameter values were compared with baseline values.

Emotional stress protocol in conscious rats. Because of its ability to induce pure sympathetic stimulation, while lowering the sensitivity of the cardiac baroreceptor reflex (4, 14), a mild emotional stress protocol elicited by blowing a jet of air into the cage for 20 min was applied to each of the following animals: at the age of 16 wk for young SHRs and WKY rats, 47 wk for aging WKY rats, and 64 wk for six aging SHRs. Air was delivered through a tube (6 mm i.d.) inserted in a hole at the bottom of the cage, as previously described (33). The pressure in the relief valve of the air tank was set at 1 bar. The stress test was applied when rats were in a quiet resting state.

A baseline recording was acquired for each animal before the onset of the protocol. Arrhythmic events and HRV analyses were assessed over 10-min intervals. The stress-related values were compared with baseline values.

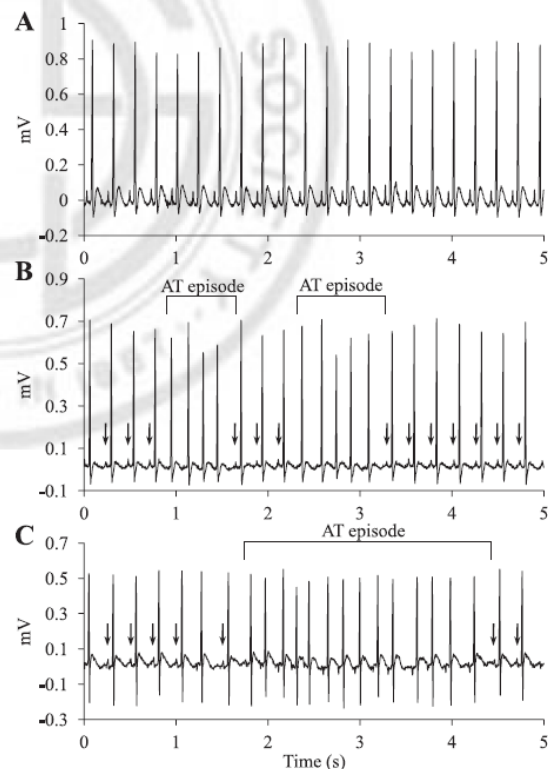


Fig. 1. ECG tracings in 55-wk-old spontaneously hypertensive rats (SHRs) showing sinus rhythm (A); 2 atrial tachyarrhythmias (AT) episodes suggesting a diagnosis of atrial fibrillation (AF; rapid, irregular rhythm, with persistence of narrow QRS complexes, in the absence of visible P waves) (B); and AT episode suggesting a diagnosis of multifocal AT (rapid, irregular rhythm, with persistence of narrow QRS complexes and wide, inverted atrial waves, with continually changing morphology) (C). B and C, arrows: sinus rhythm beats.

Subcutaneous injection of carbamylcholine in conscious rats. A baseline recording was obtained for three aging SHR rats at the age of 60 wk and for all eight young SHR rats at the age of 16 wk. A 0.4 mg/kg dose of carbamylcholine was then injected subcutaneously. A 10-min period initiated 5 min after carbamylcholine injection was analyzed, and the number of arrhythmic events was compared with that recorded during the 10-min preinjection period. None of the WKY rats underwent this protocol.

Invasive blood pressure measurement. To evaluate the degree of hypertension, intra-arterial blood pressure was measured in conscious, unrestrained rats, as previously described (13). The catheterization procedure was performed in five SHR rats at the age of 64 ± 2 wk and in all four WKY rats at the age of 68 wk. One rat in the aging SHR group died during arterial catheterization. None of the young SHR rats or WKY rats underwent this procedure.

Statistics. Data are expressed as means \pm SE or median and range, as appropriate. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test using Holm's method for multiple comparisons. Differences within the same group were tested for significance with the Wilcoxon signed-rank test. Nonparametric repeated-measures ANOVA (Friedman test) were used for repeatability data. Frequency domain parameters were also analyzed by a nonparametric (using a rank transform) two-way ANOVA, factoring for the effects of both blood pressure status (normotensive vs. hypertensive) and age (young vs. aging). Medium-term reproducibility in the number of AT episodes was analyzed using Spearman correlation. To assess day-to-day reproducibility, the mean percent variation of the number of AT episodes was calculated.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism software (GraphPad Software; San Diego, CA).

RESULTS

Atrial arrhythmic activity at baseline. Figure 1 depicts typical ECG tracings recorded in 55-wk-old SHR rats.

The number of tachyarrhythmic episodes in aging SHR rats was significantly greater than that in young SHR rats ($P < 0.001$), whereas no episodes were recorded in normotensive rats, regardless of their age (Fig. 2A). Two of the eight young SHR rats

presented a single episode of AT, while all eight aging SHR rats had tachyarrhythmic episodes (median, 91.5; range, 4–444 episodes/24 h). Similar differences were observed in the number of PACs (Fig. 2B), atrial couplets (Fig. 2C), and bigemini (Fig. 2D).

In aging SHR rats, over 24 h, the median total duration of episodes was 60 s/24 h (range, 2–372 s). The median duration of tachyarrhythmic episodes was 0.71 s (range, 0.53–1.63 s). In this group, episode duration ranged from 0.43 s to 10.90 s; in the two young SHR rats that presented AT, the episode durations were 0.4 s and 0.52 s.

In aging SHR rats, the RR intervals during AT episodes were $69 \pm 1\%$ shorter than the RR intervals during sinus rhythm ($P < 0.001$). The ECG aspect was consistent with the diagnosis of AF (rapid, irregular, supraventricular rhythm, in the absence of visible P waves) in 30.6% of cases. In the remaining cases, the ECG morphology suggested a diagnosis of atrial tachycardia (rapid, irregular, supraventricular rhythm, with atrial waves with significantly different morphology compared with sinus rhythm P waves).

Aging SHR rats showed high day-to-day (mean percent variation of the number of AT episodes $18.1\% \pm 4.7\%$) and medium-term ($P = 0.01$) reproducibility of the number of arrhythmic episodes (Fig. 3, A and B).

Occasionally, aging SHR rats showed isolated, monomorphic, premature ventricular contractions.

Assessment of the autonomic tone. The 24-h ECG monitoring indicated that aging SHR rats were more bradycardic (278 ± 4 beats/min) than age-matched WKY rats (335 ± 2 beats/min, $P = 0.01$), young SHR rats (326 ± 6 beats/min, $P < 0.01$), or young WKY rats (355 ± 5 beats/min, $P < 0.01$).

HRV analysis demonstrated significantly decreased LF components in aging SHR rats compared with age-matched WKY rats, and decreased LF-to-HF ratios in both young and aging SHR rats compared with age-matched WKY rats (Table 1). A two-factor ANOVA demonstrated that LF components were significantly affected by blood pressure status ($P = 0.002$), but they were

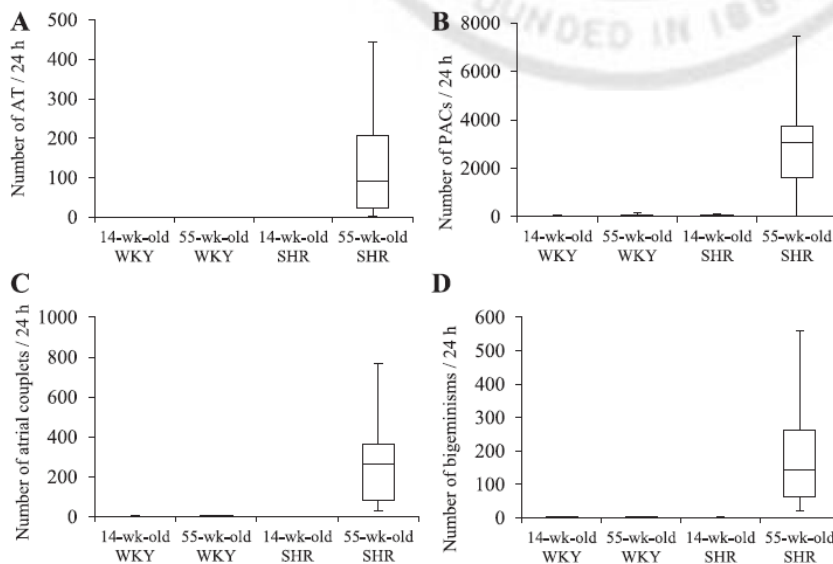


Fig. 2. Number of supraventricular arrhythmic events: episodes of AT (A); premature atrial contractions (PACs; B); atrial couplets (C); and atrial bigemini (D), during 24-h ECG recordings in 14-wk-old ($n = 4$) and 55-wk-old ($n = 4$) Wistar-Kyoto (WKY) rats, and in 14-wk-old ($n = 8$) and 55-wk-old ($n = 8$) SHR rats. Data are expressed as median and interquartile range (IQR). The ends of the whiskers represent the lowest value within 1.5 IQR of the first quartile and the highest value within 1.5 IQR of the third quartile.

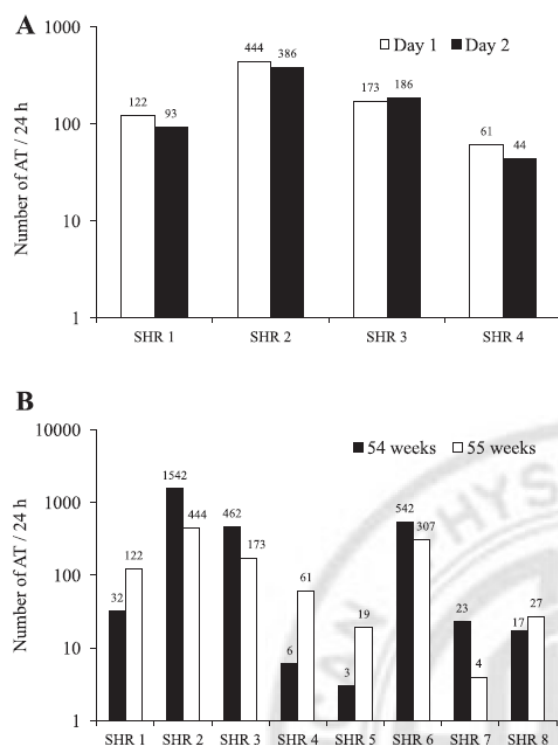


Fig. 3. Day-to-day (A) and medium-term (B) reproducibility of arrhythmias in individual aging SHR rats. A: comparison between the total number of arrhythmic episodes during the first (day 1) and second (day 2) 24-h period in 4 individual, 55-wk-old SHR rats that underwent 48-h continuous ECG monitoring. The mean percent variation in the number of AT episodes was $18.1\% \pm 4.7\%$. B: comparison between the total number of arrhythmic episodes in 8 individual SHR rats at 54 and 55 wk of age, respectively (Spearman correlation coefficient, 0.83; $P = 0.01$). Results are shown in log scale.

not significantly affected by age ($P > 0.05$), and there was no interaction between blood pressure status and age ($P > 0.05$). The same analysis demonstrated that the LF-to-HF ratio was significantly affected by both age and blood pressure status (both $P < 0.05$); again, we found no interaction between age and blood pressure status ($P > 0.05$).

In aging SHR rats, recordings performed 1 wk apart showed significant reproducibility of all HRV parameters (all $P > 0.05$).

In aging SHR rats, the HRV analysis showed no significant changes in LF, HF, or LF-to-HF components in the 5-min intervals that preceded the onset of periods of intense arrhythmic activity compared with baseline values (all $P > 0.05$). Most of the arrhythmic episodes occurred during periods of intense arrhythmic activity (frequent PACs), but in 28% of cases AT episodes occurred after sinus bradycardia or sinus pauses.

The effect of stress on atrial arrhythmogenicity. In the six aging SHR rats, a median of 0.5 (range, 0–5) tachyarrhythmic episodes was observed during the 10-min interval preceding the onset of the stress protocol, whereas no episode was recorded during stress exposure. Similarly, the number of PACs decreased from a median of 13.5 (range, 1–33) before stress to a median of 1 (range, 0–14) during stress exposure ($P = 0.04$).

No AT or PACs were present in WKY rats, regardless of their age, before or after the onset of the stress protocol. No AT and few PACs were observed in young SHR rats, before or during stress exposure.

The emotional stress induced an increase in HR; HR increased in young WKY rats from 288 ± 9 to 313 ± 18 beats/min, $P = 0.25$; in aging WKY rats from 315 ± 10 to 341 ± 12 beats/min, $P = 0.38$; in young SHR rats from 282 ± 6 to 412 ± 10 beats/min, $P = 0.007$; and in aging SHR rats from 273 ± 10 to 363 ± 9 beats/min, $P = 0.03$. Since only SHR rats presented spontaneous episodes of AT, HRV analysis before and during stress was only performed in these groups. In aging SHR rats, the HRV analysis showed significant increases in the LF-to-HF ratio during stress compared with prestress values (Table 2). Comparable results were obtained in young SHR rats: LF increased during stress from 0.60 ± 0.14 ms² to 1.11 ± 0.23 ms² ($P = 0.007$), HF decreased from 5.62 ± 0.10 ms² to 3.19 ± 0.39 ms² ($P = 0.03$), and the LF-to-HF ratio increased from 0.11 ± 0.01 to 0.35 ± 0.05 ($P = 0.007$).

Carbamylcholine administration and atrial arrhythmias. Before carbamylcholine administration, aging SHR rats presented a median of 15 PACs (range 6–29), and no episodes of AT were recorded. In the 10-min interval following carbamylcholine injection, a median of 70 PACs (range 40–271) and a

Table 1. Baseline heart rate variability parameters in young and aging groups of WKY rats and SHR

Parameter	WKY rats, weeks		SHRs, weeks		P Value
	14	55	14	55	
n	4	4	8	8	
Time domain					
SDNN, ms	28.33 ± 1.21	25.14 ± 1.82	$21.42 \pm 0.85^*$	$27.09 \pm 3.16^\dagger$	0.01
RMSSD, ms	4.05 ± 0.45	4.85 ± 0.54	3.58 ± 0.32	3.80 ± 0.32	0.28
pNN5, %	0.17 ± 0.03	0.26 ± 0.04	0.14 ± 0.03	0.16 ± 0.03	0.21
Frequency domain					
LF, ms ²	1.57 ± 0.35	1.96 ± 0.31	1.00 ± 0.17	$0.86 \pm 0.16^*$	0.03
HF, ms ²	5.99 ± 1.33	7.70 ± 1.56	5.53 ± 1.11	6.13 ± 1.37	0.62
LF/HF	0.26 ± 0.01	0.27 ± 0.04	$0.19 \pm 0.01^*$	$0.14 \pm 0.01^{*\dagger}$	0.001

Values are means \pm SE. Recordings were characterized in the time domain: SDNN, SD of normal RR intervals; RMSSD, root-mean-square of successive RR-interval differences; pNN5, percentage of adjacent RR intervals that differed by >5 ms. Recordings were characterized in the frequency domain: LF and HF, low-frequency (0.3–0.6 Hz) and high-frequency (0.6–2.5 Hz) signals, respectively; LF/HF, the ratio of low to high frequency components. P values refer to comparisons between groups based on multiple comparisons ANOVA. * $P < 0.05$ for spontaneously hypertensive rats (SHRs) vs. age-matched Wistar-Kyoto (WKY) rats; $^\dagger P < 0.05$ for 55-wk-old SHRs vs. 14-wk-old SHRs.

Table 2. Heart rate variability parameters before and during stress in aging SHR

Parameter	Stress		P Value
	Before	During	
Time domain			
RMSSD, ms	3.58 ± 0.66	2.22 ± 0.53	0.01
pNNS, %	0.14 ± 0.05	0.04 ± 0.02	0.06
Frequency domain			
LF, ms ²	0.67 ± 0.15	1.70 ± 0.69	0.20
HF, ms ²	4.33 ± 1.35	2.57 ± 0.86	0.27
LF/HF	0.19 ± 0.03	0.56 ± 0.09	0.01

Values are means ± SE; *n* = 6 aging SHRs. Recordings were characterized in the time and frequency domains. *P* values refer to comparisons between conditions based on the Wilcoxon signed-rank test.

median of 3 (range 1–29) episodes of AT were observed. The AT episodes that occurred after carbamylcholine administration had a median duration of 1.89 s (range 0.90–10.13 s), significantly longer than the duration of AT episodes in the 24-h baseline recordings (*P* < 0.001).

No AT and few PACs were observed in young SHRs, before or after carbamylcholine administration.

In aging SHRs, the high electrical instability observed after carbamylcholine administration, with an elevated number of PACs and AT episodes and very few periods of stable sinus rhythm, did not allow HRV analysis to be properly performed.

Blood pressure measurement. Systolic, diastolic, and mean blood pressure, and pulse pressure values in aging SHRs and WKY rats are presented in Table 3. As expected, SHRs had significantly higher systolic, diastolic, and mean blood pressure than WKY rats (all *P* = 0.02). Similarly, pulse pressure was significantly higher in SHRs than in normotensive controls (*P* = 0.03).

DISCUSSION

The main findings of the present study were 1) all aging SHRs developed spontaneous, unsustained AT, whereas young SHRs and both young and aging WKY rats did not; 2) in aging SHRs, the arrhythmic burden showed high day-to-day and medium-term reproducibility; 3) in aging SHRs, sympatho-vagal imbalance, with reduced sympathetic tone, was observed; 4) in aging SHRs, sympathetic activation by emotional stress removed all AT and significantly reduced the number of PACs, whereas parasympathetic stimulation with carbamylcholine had marked pro-arrhythmic effects; and 5) sympatho-vagal imbalance was already present in young SHRs and preceded the occurrence of arrhythmia.

To the best of our knowledge, this is the first study to report the occurrence of spontaneous AT in animals, in the absence of any artificial means that could have favored the onset of arrhythmia. The short duration of AT episodes in our model was due to characteristic anatomical features of small animals. Reentrant arrhythmias have generally been considered to be restricted to large animals, because the generation and maintenance of reentrant arrhythmias require a minimum tissue mass (>100–200 mm³) (10). Consequently, to date, reentrant arrhythmias are often considered impossible in small rodents, since the myocardial mass of rats and mice is <35 mm³, too small to sustain multiple reentrant wavelets. Accordingly, in SHRs, most episodes of AT spontaneously reversed to sinus

rhythm, usually after a small number of arrhythmic beats; this confirmed that the role of a critical myocardial mass cannot be underestimated.

Despite this relative limitation, our model closely resembled the human presentation of arrhythmia. The spontaneous occurrence of AT in aging, hypertensive subjects corresponded to clinical observations that linked AT with aging and high blood pressure (1, 16, 17). In the Manitoba study, the prevalence of hypertension among patients with AF was 53% and the estimated risk of AF was 1.42 times higher in hypertensive than in normotensive subjects (17). The Framingham Heart Study reported a 1.9-fold higher risk of AF in hypertensive than in normotensive individuals (15). Frequent PACs have also been reported in hypertensive patients and in elder subjects (28). The inter-individual variability in the number of AT episodes in the 24-h recordings further supported the close resemblance between this model and the human presentation of AT.

The autonomic nervous system. It was previously proposed that sympatho-vagal imbalance could facilitate atrial arrhythmias. A number of clinical and experimental studies have provided evidence that the autonomic nervous system plays a crucial role in the pathophysiology of AF. Coumel (8) described different types of AF resulting from increased vagal or adrenergic tone. A number of studies have amassed evidence for an enhanced vagal tone in AT (5, 11, 29, 32). In a study by Patterson et al. (24), application of acetylcholine shortened the action potential duration in pulmonary veins and induced early-after-depolarizations, whereas simultaneous infusion of acetylcholine and norepinephrine triggered tachycardia-pause rapid firing in the same preparations. Those authors also demonstrated that combined parasympathetic and sympathetic nerve stimulation triggered firing in canine pulmonary veins (25). Chen and colleagues (6) used extrinsic and intrinsic cardiac nerve activity recordings in ambulatory dogs and applied intermittent rapid left atrial pacing to induce paroxysmal AF or AT. Their results suggested that intrinsic cardiac nerve activation, alone or associated with extrinsic cardiac nerve activation, could invariably trigger paroxysmal AT. Along the same lines, Tan et al. (27) reported that simultaneous sympatho-vagal discharges were common triggers for spontaneous atrial arrhythmias, and cryoablation of extrinsic sympatho-vagal nerves eliminated these episodes; those results suggested a causal relationship between the discharges and the occurrence of arrhythmia (27).

In the present study, HF components, an index of parasympathetic modulation, did not differ between aging SHRs, which presented numerous episodes of AT, and age-matched WKY rats, which did not experience AT. However, LF components

Table 3. Blood pressure values in aging WKY rats and aging SHRs

Variables	WKY rats	SHRs	P Value
<i>n</i>	4	5	
Blood pressure, mmHg			
Systolic	135 ± 2	236 ± 7	0.02
Diastolic	90 ± 0.4	171 ± 8	0.02
Mean	107 ± 0.5	199 ± 8	0.02
Pulse pressure, mmHg	46 ± 2	65 ± 5	0.03

Values are means ± SE. *P* values refer to comparisons between groups based on the Mann-Whitney U test.

were significantly reduced in aging SHR rats compared with those in WKY rats, and the LF-to-HF ratio was significantly smaller in aging SHR rats than in WKY rats. Given that the LF-to-HF ratio is an index of parasympathetic and sympathetic interactions in the rat heart (18), our results highlighted a significant sympatho-vagal imbalance in animals that experienced AT. The reduction of sympathetic tone, and therefore, the relative vagal hyperactivity, appeared to facilitate the occurrence of arrhythmias. In our model, reduced sympathetic tone appeared to result from persistent hypertension, but both aging and high blood pressure appeared to affect the sympatho-vagal balance.

Acute changes in autonomic tone that preceded the onset of AT episodes were difficult to assess, due to the fact that the AT episodes occurred during periods of intense arrhythmic activity (frequent PACs). Consequently, most of the HRV analysis was actually performed on intervals immediately preceding periods of frequent PACs rather than actual AT episodes. This could have influenced our results. Therefore, although frequency-domain parameters showed no acute changes before the onset of arrhythmic episodes, these results should be interpreted with caution; the role of the autonomic nervous system should not be oversimplified based on these results.

Emotional stress restored the autonomic balance by increasing sympathetic tone and had marked antiarrhythmic effects. Indeed, in aging SHR rats, the LF-to-HF ratio increased during stress compared with the baseline value, despite unchanged LF values. However, given the reduction in total HR variance in aging SHR rats during stress (decreased root-mean-square of successive RR-interval differences and pNN5), it is not surprising that the absolute values of LF did not significantly change.

In aging SHR rats, carbamylcholine had marked pro-arrhythmic effects. This was shown by the increased number of PACs and AT episodes and the significantly longer duration of AT episodes following carbamylcholine administration compared with baseline recordings. Unfortunately, HRV analysis after carbamylcholine administration could not be achieved, due to the increased number of arrhythmic events. However, based on the well-known effects of the stress protocol (pure sympathetic stimulation), the known parasympathetic effect of carbamylcholine, and the opposite effects induced by stress and carbamylcholine on atrial arrhythmogenicity, it appears that accentuating the autonomic imbalance by further increasing vagal tone enhances even more atrial arrhythmogenicity in this setting.

As suggested by the absence of AT episodes in young SHR rats, despite the decreased LF-to-HF ratio, the presence of an autonomic imbalance did not appear to be sufficient for inducing AT. Indeed, LF-to-HF ratio was altered more in aging SHR rats than in young SHR rats; this suggested that autonomic imbalance became more severe with advancing age. However, other features, such as anatomical remodeling with extensive atrial fibrosis, abnormal distribution and/or density of autonomic nerves within the atria, or molecular abnormalities, may also act as arrhythmia facilitators in this model. The role of atrial fibrosis in atrial arrhythmogenicity was previously reported in senescent normotensive rats (22).

Potential limitations. Based solely on the results from a surface ECG recording, a junctional origin of arrhythmias cannot be excluded. However, rapid ectopic activity arising from the atrio-ventricular node or His bundle has only been reported in special circumstances, including recent cardiac surgery or acute myocardial infarction, but not in patients with

hypertension alone, and, to the best of our knowledge, has never been reported in rats. Additionally, the presence of relatively long PQ intervals during arrhythmic episodes that presented atrial waves and the continual changes in the morphology of atrial waves during those episodes strongly support the diagnosis of multifocal AT and not of a tachycardia originating from a single subsidiary pacemaker.

The protocols used in this study provide only indirect evidence of autonomic imbalance in SHR rats that presented atrial arrhythmias. Additional functional studies are required to quantify the extent of abnormal sympatho-vagal imbalance and its precise role in atrial arrhythmogenicity.

Conclusions

This study reports the occurrence of unprovoked episodes of atrial arrhythmia in hypertensive rats, and their increased incidence with aging. Our results suggest that autonomic imbalance with relative vagal hyperactivity may be responsible for the increased atrial arrhythmogenicity observed in this model. We also provide evidence that, in this model, the sympatho-vagal imbalance preceded the occurrence of arrhythmia. This work represents a potentially useful, new experimental model of atrial arrhythmias that may facilitate studies of pathophysiological mechanisms involved in atrial arrhythmias, including AF. Additional functional, anatomic-histological, and molecular studies in this setting will be of interest.

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GRANTS

Alina Scridon held a doctoral fellowship from la Région Rhône-Alpes.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.S., C.J., and P.C. conception and design of research; A.S., V.O., N.L., and A.T. performed experiments; A.S., C.G., M.M.A., V.O., B.C., N.L., and A.T. analyzed data; A.S., C.G., B.C., A.T., G.C., C.B., C.J., and P.C. interpreted results of experiments; A.S. and B.C. prepared figures; A.S. drafted manuscript; A.S., C.G., M.M.A., V.O., B.C., N.L., A.T., G.C., C.B., C.J., and P.C. approved final version of manuscript; C.G., M.M.A., V.O., B.C., N.L., A.T., G.C., C.B., C.J., and P.C. edited and revised manuscript.

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Appendix 5. Submitted papers

Age-dependency of Pitx2 down-regulation in rats with spontaneous atrial tachyarrhythmias

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Structured abstract

Objectives To assess atrial transcriptomic profile associated with atrial electrical instability in an experimental model of spontaneous hypertension and to evaluate Pitx2 changes that precede the onset of atrial arrhythmias.

Background The time-course of Pitx2 down-regulation and the associated molecular pro-arrhythmic changes in the left atria (LA) of atrial fibrillation (AF) patients are unknown.

Methods LA sampling was performed in 3 groups of young, adult, and aging spontaneously hypertensive rats (SHR; n=4 each), and 3 groups of age-matched Wistar-Kyoto (WKY) rats (n=4 each). Atrial mRNA expressions of Pitx2 and 89 additional genes were studied using TaqMan Low-Density Array.

Results No difference was found between LA Pitx2 levels in young or adult SHRs compared to age-matched WKY rats (both $P>0.05$). Aging SHRs had significantly lower LA Pitx2 levels compared to age-matched WKY rats ($P=0.02$). Among SHRs, Pitx2 expression showed a gradual, age-dependent, decrease (34.9 ± 6.7 in young SHRs, 17.1 ± 3.6 in adult SHRs, 10.7 ± 1.7 in aging SHRs, $P=0.04$) and was significantly negatively correlated with age (Spearman $r=-0.86$, $P<0.01$). Of the 89 studied genes, 6 were differentially expressed between aging SHRs and WKY rats. Down-regulation was observed for genes encoding for the I_{CaL} current, connexin-43 and desmocollin 2.

Conclusion Similarly to the data reported in AF patients, we found reduced LA Pitx2 expression in aging SHRs with spontaneous atrial arrhythmias. This was associated with reduced I_{CaL} , desmocollin 2 and connexin-43 densities and increased collagen VI. The age-dependency of Pitx2 suggests that a timely appropriate therapy in hypertensive patients may prevent AF onset.

Keywords Atrial fibrillation, Experimental model, Transcriptomics, Pitx2

Abbreviations List

AF = atrial fibrillation

cDNA = complementary DNA

C_x43 = connexin-43

I_{CaL} = L-type calcium current

I_{Kur} = ultra-rapid delayed rectifier potassium current

LA = left atria

SHR = spontaneously hypertensive rat

TBP = TATA box binding protein

TLDA = TaqMan Low-Density Array

WKY = Wistar-Kyoto

INTRODUCTION

Atrial fibrillation (AF) is the most common sustained arrhythmia. Molecular substrate of this arrhythmia, which is frequently associated with hypertension, remains elusive (1-3). According to recent genome-wide association studies in humans, the single-nucleotide polymorphisms most significantly associated with AF are located on chromosome 4q25 (4). The closest gene in the region is *Pitx2*, a member of the pituitary homeobox family of transcription factors that plays an important role in morphogenesis (5). Chinchilla et al. were the first to demonstrate that *Pitx2c* expression is significantly decreased in AF patients, providing a link between *Pitx2* loss-of-function and AF (6). In transgenic mice with heterozygous deletion of the *Pitx2* gene, reduction of *Pitx2* expression to about 60% of normal was associated with increased susceptibility to AF (7). Whereas down-regulation of *Pitx2* expression is a common finding in left atrial (LA) samples of AF patients, we do not know whether *Pitx2* expression changes precede the onset of the arrhythmia.

We recently demonstrated the age-dependency of unprovoked atrial tachyarrhythmias in spontaneously hypertensive rats (SHR) (8). In order to document the molecular changes associated with this increased atrial arrhythmogenicity in SHRs, we assessed the time-course of transcriptomic profile modifications in the atrial tissue of these rats. For this study, left and right atrial mRNA expression of *Pitx2*, as well as LA mRNA expression of 89 additional genes encoding for proteins involved in Ca^{2+} -handling, ion channels, extracellular matrix, and cell-cell communication, were investigated in SHRs in comparison to age-matched Wistar-Kyoto (WKY) rats. The choice of the 89 genes selected for mRNA expression analysis was based on their conclusive role in cardiac arrhythmogenicity (9).

METHODS

Animals

Three groups of male SHR_s (n = 4 each) of 14 weeks (further mentioned as young), 24 weeks (further mentioned as adult), and 48 weeks (further mentioned as aging) of age, and three groups of age-matched WKY rats (n = 4 each) were purchased from Elevage Janvier (Le Genest Saint Isle, France).

All experiments were performed in compliance with the French Ministry of Agriculture and Food and Drug Administration guidelines for animal experimentation and were approved by the local Animal Ethics Committee. These regulations are in accordance with the “Position of the American Heart Association on Research Animal Use”.

Heart sampling

All animals were euthanized with an intraperitoneal injection of a terminal dose of sodium pentobarbital (> 100 mg/kg). Left and right atrial samples were isolated, placed in a sterile dry tube and snap frozen in liquid nitrogen then stored at -80°C until processing.

Gene expression analysis

RNA extraction

Total RNA was isolated from frozen biopsies using TRIzol[®] Reagent (Life Technologies, Villebon sur Yvette, France) following manufacturer’s instructions. RNA concentration was measured using Nanodrop ND1000 (Thermo scientific, Illkirch, France) and quality was verified using the Agilent 2100 BioAnalyser (Agilent Technologies, Massy, France).

Pitx2 expression analysis

One µg of total RNA was reverse-transcribed to complementary DNA (cDNA) by using 100 U Superscript II (Life Technologies) and a mixture of random hexamers and oligo(dT)

primers (Promega, Charbonnières-les-Bains, France). Real-time polymerase chain reaction assays were performed as previously described (10), using a Rotor-Gene 6000 (QIAGEN). Values were normalized using TATA box binding protein (TBP). Primers used to detect Pitx2 were GTG-TGG-ACC-AAC-CTT-ACG-G (sense primer) and AGT-TGA-AGA-AGG-GGA-AGC-TC (antisense primer).

TaqMan Low Density Array Analysis

To identify altered gene expression, we used a TaqMan Low-Density Array (TLDA) to examine the expression of 89 human genes, encoding for ion channels, proteins of the extracellular matrix, and proteins involved in cell-cell coupling. For the present study, the TLDA card was configured into four identical 89 genes set. For each set of genes, we used 6 endogenous control genes: Ppia, β -actin, Gapdh, Hprt1, Tbp and 18s RNA.

Briefly, one μ g of total RNA was reverse-transcribed to cDNA by using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems) following manufacturer's instructions. The cDNA (2 μ L) was mixed with 48 μ L of H₂O and 50 μ L of 2X TaqMan Universal PCR Mix (Applied Biosystems). Each sample (100 μ L) was loaded into a port of the micro-fluid card and run on an ABI 7900HT System (Applied Biosystems) for 2 min at 50°C, 10 min at 94.5°C, followed by 40 cycles for 30 s at 97°C and 1 min at 59.7°C.

The TLDA data were analyzed using SDS 2.3 software (Applied Biosystems). Threshold cycle data for all target and control gene were transformed into molecule number (attomoles) considering polymerase chain reaction efficiency of 100%. Gene expression levels were normalized using TBP. A detailed list of the 89 examined genes is provided in Table 1.

Statistics

Data are expressed as the mean \pm SEM. Nonparametric ANOVA (Kruskal-Wallis test) was used for multiple comparisons. Between-group comparisons were performed using the Mann-Whitney *U* test. Correlations were ascertained with Spearman's rank correlation method.

A *P* value of less than 0.05 was considered statistically significant. Statistical analyses were undertaken using GraphPad Prism® software (GraphPad Software; San Diego, CA).

RESULTS

Pitx2 expression in the left atria of hypertensive rats

With the exception of aging SHR, all other groups (i.e., young and adult SHR, and young, adult, and aging WKY rats) presented significantly higher expression of Pitx2 in the LA than in the right atrium (all $P < 0.05$). In aging SHR there was no significant difference between LA and right atrial levels of *Pitx2* ($P = 0.45$). When right atrial expression of *Pitx2* was compared between hypertensive and normotensive rats no significant difference was observed between groups, regardless of the age of animals (all $P > 0.05$).

Similarly, no significant difference was found between LA levels of Pitx2 in young SHR versus age-matched WKY rats ($P = 0.09$), nor in adult SHR versus age-matched WKY rats ($P = 0.49$) (Figure 1).

On the other hand, aging SHR had significantly lower LA level of expression of the Pitx2 gene compared to age-matched WKY rats ($P = 0.02$) (Figure 1).

As Figure 1 clearly highlights, among hypertensive rats, Pitx2 expression showed a gradual, age-dependent, decrease (34.9 ± 6.7 in young SHR versus 17.1 ± 3.6 in adult SHR versus 10.7 ± 1.7 in aging SHR, $P = 0.04$). Moreover, in SHR, Pitx2 expression levels were significantly negatively correlated with age (Spearman $r = -0.86$, $P < 0.01$).

Gene expression changes in aging hypertensive rats versus normotensive controls

Left atrial gene expression levels in aging SHR rats were compared to those obtained in age-matched WKY rats. Significantly altered transcripts were selected by applying a fold change-cutoff of 1.5 and a *P*-value cutoff of 0.05.

Of the total of 89 studied genes, 6 genes were differentially expressed between aging SHR rats and WKY rats (Figure 2). Among these 6 genes, 4 were down-regulated and 2 were up-regulated in aging SHR rats compared to age-matched WKY rats.

Down-regulation was particularly observed for genes encoding for the L-type calcium current (I_{CaL}), *Cacng6* and *Cacnb4*. *Cacng6* expression was 5.8 times lower in aging SHR rats compared to age-matched WKY rats ($P = 0.03$). Similarly, aging SHR rats presented a 5.2-fold lower *Cacnb4* expression than aging WKY rats ($P = 0.02$). We also noted increased expression of the gene encoding for type VI collagen, *Col6a1*. *Col6a1* expression was 2.9 times higher in aging SHR rats than in age-matched WKY rats ($P = 0.006$). Down-regulation of the genes encoding for proteins involved in cell-cell communication, connexin-43 (*Cx43*; *Gja1*) and desmocollin 2 (*Dsc2*) was also noted. *Gja1* expression was 2.8 times lower in aging SHR rats compared to age-matched WKY rats ($P = 0.04$). Similarly, aging SHR rats presented a 2.5-fold lower expression of the *Dsc2* gene compared to age-matched WKY rats ($P = 0.03$). Additionally, we found a 1.9-fold increase ($P = 0.02$) in the expression of the gene encoding for a *beta* subunit of the ultra-rapid delayed rectifier potassium current (I_{Kur} ; *Kcnab1*) (Figure 2).

DISCUSSION

The main findings of the present study were (i) in aging SHR rats, LA *Pitx2* expression was significantly down-regulated compared to age-matched WKY rats; (ii) among SHR rats, *Pitx2* expression was significantly negatively correlated with age; (iii) aging SHR rats presented significantly lower expressions of genes encoding for I_{CaL} current, *Cx43* and desmocollin 2; (iv)

and higher expressions of genes encoding for type VI collagen and for a *beta* subunit of the I_{Kur} current compared to age-matched WKY rats.

Pitx2 insufficiency and atrial arrhythmias in spontaneously hypertensive rats

Similarly to what is found in AF patients, rats that were previously shown to present unprovoked episodes of atrial tachyarrhythmia (8) had reduced Pitx2 LA expression. Studies in humans and transgenic mice suggested a relationship between Pitx2 loss-of-function and AF (6, 7). While homozygous Pitx2^{-/-} mice exhibited embryonic lethality, mice with heterozygous deletion of the Pitx2 gene are viable and do not present altered cardiac morphology and function (7). Mice that are heterozygous for a Pitx2 null allele have increased susceptibility to pacing-induced atrial arrhythmias (11). In humans, decreased LA expression of the Pitx2 gene was demonstrated in AF patients (6). According to Chinchilla et al., this down-regulation provides the cellular, molecular, and electrophysiological substrates for atrial arrhythmogenesis (6).

In addition to the decreased expression of Pitx2 in the LA of arrhythmic rats, we also found that among SHR, Pitx2 expression presents a gradual, age-dependent decrease. These results suggest that Pitx2 down-regulation starts before the occurrence of atrial arrhythmias. We may thus speculate that appropriate therapy early in life in hypertensive patients may prevent AF onset. Further studies will need to address this issue.

Molecular changes underlying atrial electrical remodeling

Significant shortening of the atrial effective refractory period and of the action potential duration are constant findings in both paroxysmal and persistent AF (12). Decreased action potential duration has also been demonstrated in mice that are heterozygous for a Pitx2 null allele (11). This decreased action potential duration could result from either decreased inward Ca^{2+} current or increased outward K^{+} current densities.

A significant 63% reduction in the density of $I_{Ca,L}$ was observed in atrial myocytes of patients with chronic AF (13). Reduced $I_{Ca,L}$ density seems to be mainly due to transcriptional down-regulation of *alpha* subunits (14). In the present study we did not note significant transcriptional changes in *alpha* subunits, but *beta* 4 and *gamma* 6 subunits of the $I_{Ca,L}$ current were significantly down-regulated in rats that presented atrial arrhythmias compared to control rats. Down-regulation of both *beta* and *gamma* accessory subunits has been previously reported in AF patients (15) and reduction in either of these subunits contributes to a reduction in $I_{Ca,L}$ current density. Bosch et al. demonstrated that depressed transcription of the auxiliary channel subunit *beta* 2 precedes alterations in *alpha* subunits, and any other changes in $I_{Ca,L}$ current densities (15).

Kcnab1 encodes for a *beta* subunit of I_{Kur} and alters the activity of the current (16). Thus, Kcnab1 up-regulation will result in decreased I_{Kur} activity, which would be expected to induce prolonged atrial action potential duration and refractoriness. This observation is quite surprising, given that I_{Kur} blockade was thought to be a promising approach for AF therapy (17). However, reduced activity of I_{Kur} has already been reported in several AF studies (18-20). Moreover, Burashnikov et al. demonstrated that I_{Kur} blockade abbreviates action potential duration in AF patients (21). Comparable results were reported by Olson et al., who demonstrated that deficit in Kcna5 expression, the main gene encoding for the I_{Kur} current, increased susceptibility to arrhythmogenic after-depolarizations and facilitated stress-induced atrial arrhythmias (22). Kcna5 loss-of-function mutations were recently reported in familial AF (22).

Molecular correlates to atrial structural remodeling

Substantial changes in the composition of the extracellular matrix, such as abnormal collagen deposition in the atria, increase the heterogeneity of atrial conduction and provide the basis for arrhythmia occurrence and persistence (23).

While type I and III collagen have generally been considered as the main collagen subtypes of the myocardial matrix, recent studies suggest that non-fibrillar collagen deposition also participates to pathological healing processes (24). In the present study, Col6a1 gene expression, encoding for the non-fibrillar type VI collagen, was significantly higher in arrhythmic, hypertensive rats, compared to age-matched normotensive controls. Similar results were reported by Polyakova et al., who found significantly increased type VI collagen in permanent AF patients compared to sinus rhythm controls (25).

Cell-cell coupling impairment

Accumulating data suggest a close association between abnormal expression of genes encoding for proteins that exhibit gap junction activity and AF (26). In the present study, aging SHRs that presented atrial arrhythmias showed decreased expression of the Gja1 gene, encoding for Cx43, compared to non-arrhythmic control rats. In patients with rheumatic heart disease, reduction and redistribution of Cx43 has been shown to contribute to both initiation and persistence of AF (27).

Dsc2 down-regulation has never been reported in AF patients. We found that Dsc2 expression, which encodes for desmocollin 2, was 2.5 times lower in aging, hypertensive rats that presented atrial arrhythmias than in age-matched, normotensive, non-arrhythmic controls.

Mutations in the gene encoding for desmocollin 2 are known to cause arrhythmogenic right ventricular dysplasia (28). Although several clinical studies reported an increased incidence of

atrial arrhythmias in patients with arrhythmogenic right ventricular dysplasia (29), the underlying arrhythmogenic mechanisms remain unknown.

The present study provides evidence of decreased Dsc2 expression in rats that presented atrial arrhythmias. These results suggest that Dsc2 loss-of-function may explain the increased atrial arrhythmogenicity observed in some patients with arrhythmogenic right ventricular dysplasia and Dsc2 mutations. Polymerase chain reaction analysis of atrial tissue in an arrhythmogenic right ventricular dysplasia patient that presented AF and a Dsc2 variant (p.Arg132Cys) has demonstrated the absence of Dsc2 and the presence of Dsc3, a protein usually absent in the myocardium (30).

Given that Pitx2 is a transcription factor, we could hypothesize a potential participation of impaired Pitx2 expression in altered mRNA levels of genes differentially expressed in the LA of aging SHR. Kirchhof et al. have previously demonstrated significantly different expression levels of genes involved in calcium ion binding and in cell junctions and adhesion and shorter atrial action potential duration in *Pitx2c*^{+/-} heterozygous knockout mice compared to wild-type littermates (7). The most consistently affected pathway was gap junction regulation pathway. Similar findings were reported by Chinchilla et al. (6).

Potential limitations

Gene expression changes were only assessed by mRNA quantification. There was no investigation about changes at protein or ion current levels. Although altered gene expression would probably result in abnormal protein variations, other posttranscriptional or posttranslational regulatory mechanisms are known to influence protein level and function.

Conclusion

Similarly to what is found in AF patients, rats with atrial arrhythmias had reduced Pitx2 LA expression. Transcriptomic profiles of SHRs also demonstrated reduced I_{CaL} , I_{Kur} , desmocoline 2 and Cx43 densities, associated with increased type VI collagen. Finally, in the present model, Pitx2 gene expression follows a progressive, age-dependent decrease, suggesting that a timely appropriate therapy in hypertensive patients may prevent AF onset.

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FIGURE LEGENDS

Figure 1: Left atrial Pitx2 / TBP mRNA expression in young (n = 4), adult (n = 4) and aging (n = 4) SHR rats and age-matched WKY rats (n = 4 each).

Data are expressed as the mean \pm SEM. * - $P = 0.02$ for aging SHR rats *versus* aging WKY rats using the Mann-Whitney U test.

Figure 2: mRNA / TBP ratios for genes differentially expressed between aging SHR rats (n = 4) and age-matched WKY rats (n = 4).

$P < 0.05$ for aging SHR rats *versus* age-matched WKY rats for all displayed genes using the Mann-Whitney U test.

Table 1. List of the 89 genes for which mRNA expressions were studied in left atrial samples of aging SHR and WKY rats

Symbol	Name
<i>Cacna1c</i>	calcium channel, voltage-dependent, L type, <i>alpha</i> 1C subunit
<i>Cacna1d</i>	calcium channel, voltage-dependent, L type, <i>alpha</i> 1D subunit
<i>Cacna1g</i>	calcium channel, voltage-dependent, T type, <i>alpha</i> 1G subunit
<i>Cacna1h</i>	calcium channel, voltage-dependent, T type, <i>alpha</i> 1H subunit
<i>Cacna2d1</i>	calcium channel, voltage-dependent, <i>alpha</i> 2/delta subunit 1
<i>Cacna2d2</i>	calcium channel, voltage-dependent, <i>alpha</i> 2/delta subunit 2
<i>Cacnb1</i>	calcium channel, voltage-dependent, <i>beta</i> 1 subunit
<i>Cacnb2</i>	calcium channel, voltage-dependent, <i>beta</i> 2 subunit
<i>Cacnb3</i>	calcium channel, voltage-dependent, <i>beta</i> 3 subunit
<i>Cacnb4</i>	calcium channel, voltage-dependent, <i>beta</i> 4 subunit
<i>Cacng4</i>	calcium channel, voltage-dependent, <i>gamma</i> subunit 4
<i>Cacng6</i>	calcium channel, voltage-dependent, <i>gamma</i> subunit 6
<i>Casq1</i>	calsequestrin 1 (fast-twitch, skeletal muscle)
<i>Casq2</i>	calsequestrin 2 (cardiac muscle)
<i>Clcn2</i>	chloride channel 2
<i>Clcn3</i>	chloride channel 3
<i>Col6a1</i>	collagen, type VI, <i>alpha</i> 1
<i>Dsc2</i>	desmocollin 2
<i>Dsp</i>	desmoplakin
<i>Gja1</i>	gap junction protein, <i>alpha</i> 1
<i>Gja5</i>	gap junction protein, <i>alpha</i> 5
<i>Gja7</i>	gap junction membrane channel protein <i>alpha</i> 7
<i>Hcn1</i>	hyperpolarization-activated cyclic nucleotide-gated potassium channel 1
<i>Hcn2</i>	hyperpolarization activated cyclic nucleotide-gated potassium channel 2
<i>Hcn3</i>	hyperpolarization-activated cyclic nucleotide-gated potassium channel 3
<i>Hcn4</i>	hyperpolarization activated cyclic nucleotide-gated potassium channel 4
<i>Kcna1</i>	potassium voltage-gated channel, shaker-related subfamily, member 1
<i>Kcna2</i>	potassium voltage-gated channel, shaker-related subfamily, member 2
<i>Kcna3</i>	potassium voltage-gated channel, shaker-related subfamily, member 3
<i>Kcna4</i>	potassium voltage-gated channel, shaker-related subfamily, member 4
<i>Kcna5</i>	potassium voltage-gated channel, shaker-related subfamily, member 5
<i>Kcna6</i>	potassium voltage gated channel, shaker related subfamily, member 6
<i>Kenab1</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 1
<i>Kenab2</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 2
<i>Kenab3</i>	potassium voltage-gated channel, shaker-related subfamily, beta member 3
<i>Kcnb1</i>	potassium voltage gated channel, Shab-related subfamily, member 1
<i>Kcnc3</i>	potassium voltage gated channel, Shaw-related subfamily, member 3

<i>Kcne1</i>	potassium voltage-gated channel, Isk-related family, member 1
<i>Kcne11</i>	KCNE1-like
<i>Kcne2</i>	potassium voltage-gated channel, Isk-related family, member 2
<i>Kcne3</i>	potassium voltage-gated channel, Isk-related family, member 3
<i>Kcne4</i>	potassium voltage-gated channel, Isk-related family, member 4
<i>Kcnh2</i>	potassium voltage-gated channel, subfamily H (eag-related), member 2
<i>Kcnh7</i>	potassium voltage-gated channel, subfamily H (eag-related), member 7
<i>Kcnip2</i>	Kv channel-interacting protein 2
<i>Kcnj11</i>	potassium inwardly rectifying channel, subfamily J, member 11
<i>Kcnj12</i>	potassium inwardly-rectifying channel, subfamily J, member 12
<i>Kcnj2</i>	potassium inwardly-rectifying channel, subfamily J, member 2
<i>Kcnj3</i>	potassium inwardly-rectifying channel, subfamily J, member 3
<i>Kcnj4</i>	potassium inwardly-rectifying channel, subfamily J, member 4
<i>Kcnj5</i>	potassium inwardly-rectifying channel, subfamily J, member 5
<i>Kcnj8</i>	potassium inwardly-rectifying channel, subfamily J, member 8
<i>Kcnk1</i>	potassium channel, subfamily K, member 1
<i>Kcnk3</i>	potassium channel, subfamily K, member 3
<i>Kcnk5</i>	potassium channel, subfamily K, member 5
<i>Kcm2</i>	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 2
<i>Kcnq1</i>	potassium voltage-gated channel, KQT-like subfamily, member 1
<i>Npr3</i>	natriuretic peptide receptor C/guanylate cyclase C (atrionatriuretic peptide receptor C)
<i>Pak1</i>	p21 protein (Cdc42/Rac)-activated kinase 1
<i>Myh7</i>	myosin, heavy chain 7, cardiac muscle, <i>beta</i>
<i>Pkpx2</i>	plakophilin 2
<i>Ryr2</i>	ryanodine receptor 2, cardiac
<i>Scn1a</i>	sodium channel, voltage-gated, type I, <i>alpha</i>
<i>Scn1b</i>	sodium channel, voltage-gated, type I, <i>beta</i>
<i>Scn2b</i>	sodium channel, voltage-gated, type II, <i>beta</i>
<i>Scn3a</i>	sodium channel, voltage-gated, type III, <i>alpha</i>
<i>Scn3b</i>	sodium channel, voltage-gated, type III, <i>beta</i>
<i>Scn4a</i>	sodium channel, voltage-gated, type IV, <i>alpha</i> subunit
<i>Scn5a</i>	sodium channel, voltage-gated, type V, <i>alpha</i> subunit
<i>Scn7a</i>	sodium channel, voltage-gated, type VII, <i>alpha</i>
<i>Scn9a</i>	sodium channel, voltage-gated, type IX, <i>alpha</i>
<i>Smad6</i>	SMAD family member 6
<i>Smad9</i>	SMAD family member 9
<i>Smad2</i>	SET and MYND domain containing 2
<i>Trpc1</i>	transient receptor potential cation channel, subfamily C, member 1
<i>Trpc3</i>	transient receptor potential cation channel, subfamily C, member 3
<i>Trpc4</i>	transient receptor potential cation channel, subfamily C, member 4

<i>Trpc5</i>	transient receptor potential cation channel, subfamily C, member 5
<i>Trpc6</i>	transient receptor potential cation channel, subfamily C, member 6
<i>Trpm4</i>	transient receptor potential cation channel, subfamily M, member 4
<i>Trpm5</i>	transient receptor potential cation channel, subfamily M, member 5
<i>Trpv1</i>	transient receptor potential cation channel, subfamily V, member 1
<i>Trpv6</i>	transient receptor potential cation channel, subfamily V, member 6
<i>Tgfb1</i>	transforming growth factor, <i>beta</i> 1
<i>Camk2d</i>	calcium/calmodulin-dependent protein kinase II <i>delta</i>

Figure 1.

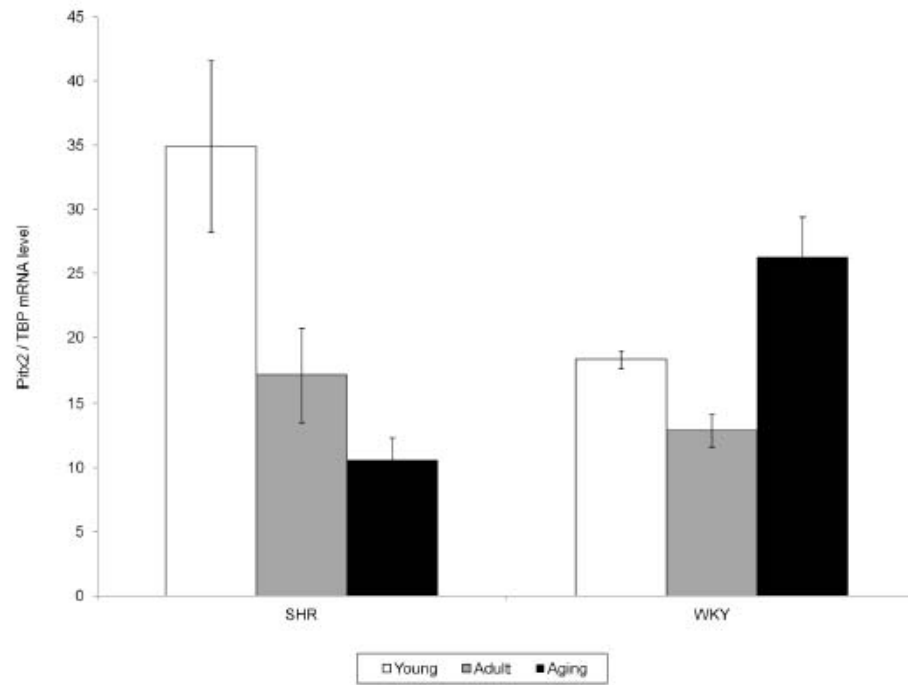
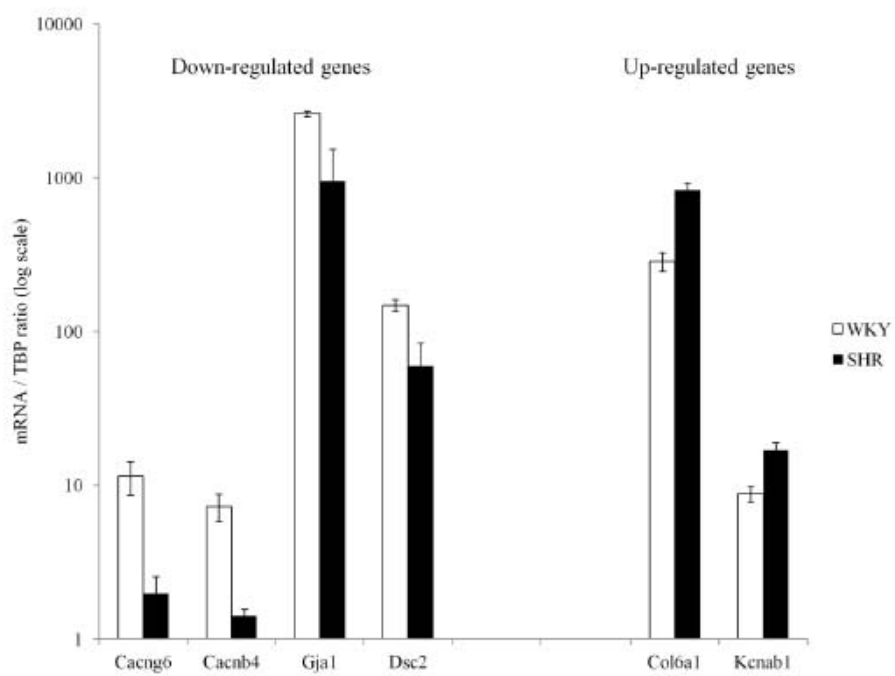


Figure 2.



TITRE en français

La fibrillation atriale – Aperçus concernant le substrat arythmogène

RESUME en français

La fibrillation atriale est l'arythmie cardiaque la plus fréquente. Les études sur des modèles animaux ont fourni beaucoup de renseignements sur les mécanismes de cette arythmie, mais, à ce jour, nous ne disposons pas de modèle animal d'arythmie atriale spontanée.

Nous avons cherché à développer un modèle d'arythmie atriale spontanée chez le rat et à identifier les mécanismes physiopathologiques de ces arythmies. Nous avons également cherché à évaluer la présence et la sévérité de l'inflammation et de la dysfonction endothéliale, impliquées dans la survenue des complications de la fibrillation atriale comme les accidents vasculaires cérébraux, chez les patients avec fibrillation atriale.

Cette étude décrit le premier modèle animal d'arythmies atriales spontanées. De multiples mécanismes semblent participer à la survenue d'arythmies dans ce modèle, particulièrement un déséquilibre autonome avec hyperactivité vagale relative, une fibrose endocardique atriale et une diminution de l'expression atriale gauche du gène *Pitx2*. Nous avons également constaté des niveaux élevés de facteur de croissance endothélial vasculaire et de facteur von Willebrand chez les patients avec fibrillation atriale par rapport aux contrôles. Ces résultats suggèrent un profil spécifique du risque thromboembolique en fonction de la forme clinique de l'arythmie et mettent en évidence une évolution parallèle de la fibrillation atriale et de la dysfonction endothéliale.

Ce nouveau modèle animal permettra d'étudier les mécanismes physiopathologiques des arythmies atriales et d'évaluer de nouveaux agents thérapeutiques dans un cadre qui reproduit fidèlement la présentation clinique de l'arythmie.

TITRE en anglais

Atrial fibrillation – insights concerning the arrhythmogenic substrate

RESUME en anglais

Atrial fibrillation is the most prevalent form of cardiac arrhythmia. Studies in animal models have provided important insights into arrhythmia mechanisms. However, to date, we do not dispose of animal models of spontaneous atrial arrhythmia.

Thus, we aimed to develop a model of spontaneous atrial arrhythmia in rats and to assess pathophysiological mechanisms of these arrhythmias by using a multidisciplinary approach. We also aimed to assess the presence and the extent of inflammation and endothelial dysfunction, incriminated in atrial fibrillation-related complications such as stroke, in atrial fibrillation patients.

The animal study describes the first animal model of spontaneous atrial arrhythmias. We also provide evidence that multiple mechanisms participate in arrhythmia occurrence in this model, particularly autonomic imbalance with relative vagal hyperactivity, left atrial endocardial fibrosis, and decreased left atrial expression of the *Pitx2* gene. In our clinical study, we found high levels of vascular endothelial growth factor and von Willebrand factor in atrial fibrillation patients compared to sinus rhythm controls. These results suggest specific thromboembolic risk patterns according to the clinical form of arrhythmia and highlight a parallel evolution of atrial fibrillation and endothelial dysfunction.

These results add new insights into the understanding of atrial arrhythmias. This new animal model could facilitate studies of pathophysiological mechanisms involved in atrial arrhythmias and allow assessment of efficacy and toxicity of therapeutic agents in a setting that faithfully reproduces the clinical presentation of the arrhythmia.

DISCIPLINE : Cardiologie

MOTS-CLES : Fibrillation atriale, Modèle expérimental, Système nerveux autonome, Anomalies moléculaires, Inflammation, Dysfonction endothéliale

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