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ERBB4 Stimulation as a Therapeutic Strategy for Atrial Myopathy and Atrial Fibrillation

Proefschrift voorgelegd tot het behalen van de graad van doctor in de medische wetenschappen aan de Universiteit Antwerpen te verdedigen door Michiel Tubeeckx

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Non-standard abbreviations and acronyms

AAD	Anti-arrhythmic Drug
ACEi	Angiotensin Converting Enzyme inhibitor
ADMA	Asymmetric Dimethylarginine
AERP	Atrial Effective Refractory Period
AF	Atrial Fibrillation
AHA	American Heart Association
AHT	Arterial Hypertension
Akt	Protein Kinase B
AMP	Adenosine Monophosphate
AMPK	Adenosine Monophosphate activated Protein Kinase
Ang-II	Angiotensin-II
ANOVA	Analysis Of the Variance
ANP	Atrial Natriuretic Peptide
ANS	Autonomic Nervous System
Ao	Max/mean gradient over the Aortic valve
APD	Action Potential Duration
ARB	Angiotensin Receptor Blocker
ASC	Apoptosis-associated Speck-like protein containing a CARD
AT1R	Ang-II Type 1 Receptors
ATP	Adenosine Triphosphate
AV	Atrioventricular
AVR	Aortic Valve Replacement
BMI	Body Mass Index
Brpm	Breath Rate Per Minute
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Grafting
CAD	Coronary Artery Disease
CALM1	Calcium Regulator
CaMKII	Calcium-Calmodulin Kinase II
CARD	Caspase Activation and Recruitment Domain
CLCF1	Chloride Channel
CM	Cardiomyocyte
CMR	Cardiac Magnetic Resonance
CREM	cAMP Responsive Element Modulator
CRI	Continuous Rate Infusion
CRP	C-Reactive Protein
CT	Computed Tomography
CTGF	Connective Tissue Growth Factor

CTRL	Control
CV	Conduction Velocity
CVC	Central Venous Catheter
Cx_{43}	Connexin-43
DAD	Delayed AfterDepolarizations
DAPI	4',6-diamidino-2-phenylindole
DEG	Differentially Expressed Gene
DHA	Docosaheaenoic Acid
DOAC	Direct Oral Anticoagulant
DOCA	Deoxycorticosterone Acetate
DTT	Dithiothreitol
EAD	Early Afterdepolarizations
ECG	Electrocardiogram
ECM	Extra Cellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGM	Electrogram
EHRAS	EHRA-HRS-APHRS-SOLAECE
ELISA	Enzyme-Linked Immuno Sorbent Assay
EndMT	Endothelial-Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
EP	Electrophysiology
EPA	Eicosapentaenoic Acid
EpiEG	Epicardial Electrogram
ERBB	V-erb-b avian erythroblastic viral oncogene homolog
ERK	Extracellular Signal Regulated Kinases
ERP	Effective Refractory Period
ESC	European Society of Cardiology
$ETCO_2$	End-Tidal CO_2
ETT	Endotracheal Tube
Etv1	ETS Variant Transcription Factor 1
FGF23	Fibroblast Growth Factor-23
Fxa	Factor Xa
GABA	Gamma-Amino-Butyric Acid
GDF-15	Growth Differentiation Factor-15
Gja5	Gap Junction 5
GLUT-4	Glucose Transporter-4
GO	Gene Ontology
GP	Ganglionated Plexi
GWAS	Genome-Wide Association Studies

HER	Human Epidermal growth factor Receptor
HF	Heart Failure
HF patients	Heart Failure patients
HFpEF	Heart Failure with preserved Ejection Fraction
HFrEF/HFpEF	Heart Failure with reduced and preserved Ejection Fraction
IAA	Isolated Atrial Amyloidosis
ICTP	Type I Carboxy-Terminal Peptide
IGF1	Insulin-like Growth Factor-1
IGFBP1	IGF-Binding Protein-1
IL-1ß	Interleukin-1 beta
IL-6	InterLeukin-6
IVSd	InterVentricular Septum thickness in diastole
IVSs	InterVentricular Septum thickness in systole
JAK/STAT	Janus Kinase / Signal Transducer and Activator of Transcription proteins
JNK2	Jun N-terminal Kinase-2
Kca3	Ca^{2+}-sensitive K^+ channel
KCNJ3	K+ Channel J3
LA	Left Atrium
LAA	Left Atrial Appendage
LAA	Left Atrial Appendage
LAT	Local Activation Times
LAVI	Left Atrial Volume Index
LGE	Late Gadolinium Enhancement
LGE-CMR	Late Gadolinium EnhancementCardiac Magnetic Resonance
LKB1	Liver Kinase B1
LV	Left Ventricular
LV	Left Ventricle
LVEF	Left Ventricular Ejection Fraction
LVIDd	Left Ventricular Internal Diameter in diastole
LVIDs	Left Ventricular Internal Diameter in systole
LVPWd	Left Ventricular Posterior Wall thickness in diastole
LVPWs	Left Ventricular Posterior Wall thickness in systole
MAPK	Mitogen-Activated Protein Kinase
MEA	Muti-electrode Array
MHC	Myosin Heavy Chain
MI	Mitral Insufficiency
miRNA	Micro-RiboNucleic Acid
MMP	Matrix MetalloProteinases
MR	Mineralocorticoid Receptors
MRA	Mineralocorticoid Receptor Antagonist
NADPH	Nicotinamide Adenine Dinucleotide Phosphate

NCX	Na+/Ca2+ Exchanger
NFAT	Nuclear Factor of Activated T-cells
NLRP3	Nucleotide-binding Domain, Leucine-rich Repeat-Family Pyrin-domain containing
NO	Nitric Oxide
NO	Nitric Oxide
NRG-1	Neuregulin-1
NT-pro-BNP	N-Terminal pro-B-type Natriuretic Peptide
OT	Omnipolar Technology
P-SAECG	P-wave duration on Signal-Avereged ECG
PAC	Premature Atrial Complex
PAR	Protease Activated Receptor
PBS	Phosphate-buffered Saline
PCM1	PeriCentriolar Material 1
PCR	Polymerase Chain Reaction
PCWP	Pulmonary Capillary Wedge Pressure
PDGF	Platelet Derived Growth Factor
PDK	Pyruvate Dehydrogenase Kinase
PEEP	Positive EndExpiratory Pressure
PES	Programmed Electrical Stimulation
PET	Positron Emission Tomography
PGI_2	Prostacyclin
PIIINP	Procollagen type III N-terminal Peptide
PIP	Peak Inspiratory Pressure
PKCζ	Protein Kinase C-Zeta
PLN	Phospholamban
PTFV1	P-wave Terminal Force in lead V1
PVI	Pulmonary Vein Isolation
PVs	Pulmonary Veins
RA	Right Atrium
RAA	Right Atrial Appendage
RAAS	Renin-Angiotensin-Aldosterone System
RAP	Rapid Atrial Pacing
RMS	Root Main Square
ROS	Reactive Oxygen Species
RyR	Ryanodine Receptor
RyR2	Ryanodine Receptor type 2
SAC	Stretch-Activated Channel
SAC_K	K^+-selective SAC
SACNS	Non-Specific Cation SAC
Scn5a	Sodium Channel 5A
SD	Standard Deviation

SEM	Standard Error of the Mean
SERCA	Saroplasmic/endoplasmic reticulum Ca2+ ATPase
SGLT2i	Sodium-Glucose Linked Transporter-2 Inhibitor
SNP	Single Nucleotide Polymorphism
SP	Sterile Pericarditis
SPECT	Single Photon Emission Computed Tomography
SR	Sarcoplasmic Reticulum
sST2	soluble Suppressor of Tumorigenicity 2
TEE	Transesophageal Echocardiography
TF	Tissue Factor
TGF-b_1	Transforming Growth Factor -ß1
TGF-α	Transforming Growth Factor Alpha
TGF-β	Transforming growth Factor-ß
Tgfbr1	Transforming growth Factor-ß receptor 1
Tgfbr2	Transforming growth Factor-ß receptor 2
TNF-α	Tumor Necrosis Factor-α
tPA	Tissue Plasminogen Activator
TRAM-34	Triarylmethane-34
TRP	Transient Receptor Potential
TRPC3	Transient Receptor Potential Canonical-3
TRPM7	Transient Receptor Potential Melastin related 7
VCV	Volume Controlled Ventilation
VEH	Vehicle
VKF	Voorkamerfibrillatie
vWF	von Willebrand Factor
WGA	Wheat Germ Agglutinin
WOV	Window Of Vulnerability
α-SMA	Alpha-Smooth Muscle Actin
χ^2	Chi-squared

Chapter 1:

General introduction on atrial myopathy

ADAPTED FROM:

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Atrial fibrillation

In the healthy human heart, the sinoatrial node generates electrical impulses that are then conducted throughout the atria leading to a well-organised contraction. These impulses are subsequently transmitted via the atrioventricular (AV) node to the His-Purkinje system, which propagates the depolarization wave throughout the ventricles. This process gives rise to a regular heart rhythm known as sinus rhythm.

On the other hand, atrial fibrillation (AF) is a supraventricular tachyarrhythmia characterized by disorganized atrial electrical activation and ineffective atrial contraction. Typically, rapid and irregular conduction through the AV node result in 'irregularly irregular' ventricular contractions (R-R intervals) without distinct P waves on the electrocardiogram (ECG; see figure 1). A diagnosis of AF is made if this irregular pattern persists for at least 30 seconds. [1]

The clinical presentation of AF can vary widely and may include palpitations, dizziness, syncope, dyspnea, or chest pain. In about one third of patients, AF may be entirely asymptomatic.

AF is a highly prevalent arrhythmia, with individuals of European ancestry having a lifetime risk of 37% by the age of 55 years. Predictions indicate a 2.3-fold increase in prevalence over the coming decades due to factors such as longer life expectancy, improved detection of subclinical AF, and an increasing burden of risk factors (hypertension, obesity, diabetes, heart failure, coronary artery disease, etc.). [2] The significant morbidity and mortality associated with AF make it a substantial societal burden, impacting not only individual patients but also healthcare economics. Consequently, there is an urgent need for further research to develop more effective and affordable therapeutic strategies to address this impending epidemic.

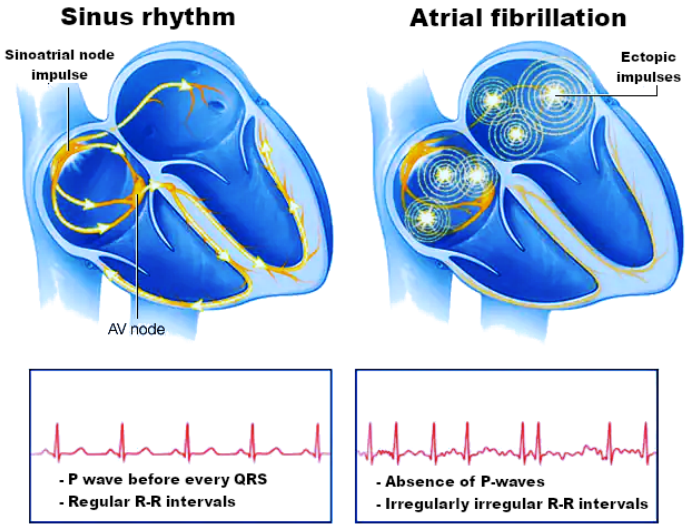

Figure 1: In sinus rhythm (left), the sinoatrial node generates electrical impulses that are then conducted throughout the atria. These impulses are subsequently transmitted via the atrioventricular node to the His-Purkinje system, which propagates the depolarization wave throughout the ventricles. In atrial fibrillation, rapid and irregular activations result in irregularly irregular R-R intervals without distinct repeating P waves on the electrocardiogram. Image adapted from MayoClinic.org.

From a pathophysiological standpoint, it is now recognized that AF should not be viewed as a disease in itself, but rather as an electrical manifestation of an underlying atrial myopathy. This myopathy can also contribute to thromboembolism (stroke) and heart failure symptoms. Current anti-arrhythmic drugs and ablation strategies primarily focus on suppressing the arrhythmia by blocking ion channels and isolating triggering ectopic foci, typically located in the pulmonary veins. While these approaches may alleviate AF and its symptoms, they do not address the underlying atrial myopathy, which worsens over time and contributes to treatment resistance. [3] The concept of atrial myopathy is complex and will be thoroughly reviewed in the following sections, that provide a state of the art on the pathophysiology, diagnosis and management of this condition. The sections are based on a review article written during my Ph.D. trajectory and published in *Basic Research in Cardiology*.

Atrial myopathy is a condition that consists of electrical, structural, contractile, metabolic, and autonomic remodeling of the atria and is the substrate for development of atrial fibrillation, the most common arrhythmia. Pathophysiologic mechanisms driving atrial myopathy are inflammation, oxidative stress, atrial stretch, and neurohormonal signals, e.g., angiotensin-II and aldosterone. These mechanisms initiate the structural and functional remodeling of the atrial myocardium. Novel therapeutic strategies are being developed that target the pathophysiologic mechanisms of atrial myopathy. In this review, we will discuss the pathophysiology of atrial myopathy, as well as diagnostic and therapeutic strategies.

Introduction

Atrial fibrillation (AF) is caused by underlying alterations in the atrium that often take decades to culminate in the arrhythmia. Various risk factors, such as aging, hypertension, and obesity, contribute to atrial wall stretch, activation of the renin-angiotensin-aldosterone (RAAS) system, oxidative stress and inflammation. These detrimental stimuli induce phenotypic alterations in different cardiac cell types, collectively referred to as atrial remodeling. Atrial remodeling encompasses structural, electrical, contractile, autonomic, metabolic, and endothelial remodeling. Consequently, atrial myopathy can induce clinically relevant manifestations, including AF, stroke and atrial stiffening causing dyspnea. [3,4] (Fig. 1)

The complex interplay between AF and atrial myopathy involves electric remodeling, which leads to ectopic firing, decreased refractoriness and conduction slowing, thereby increasing susceptibility to AF initiation and sustainability.[5] Structural remodeling, particularly atrial fibrosis, creates conduction heterogeneity, stabilizes re-entrant circuits and perpetuates AF. [6] Additionally, the rapid atrial depolarizations during AF perpetuate electric remodeling, establishing a vicious cycle known as "AF begets AF". [7,8] However, AF persistence cannot solely be attributed to this phenomenon, as atrial myopathy can progress even with adequate anti-arrhythmic drug or ablation strategies, contributing to therapy refractoriness.

Atrial myopathy is not only implicated in AF but also plays a significant role in thromboembolism. [3,9] Endothelial remodeling in atrial myopathy causes a shift towards a procoagulant phenotype of the endothelium, elevating the risk of thrombo-embolism independent of AF. Stroke can be the first symptom of atrial myopathy, preceding the first episode of AF. [10-13] Although atrial myopathy develops before AF, mechanistic data from patients with atrial myopathy, but before development of AF are scarce. For this reason, most clinical papers included here concern patients with atrial myopathy associated with AF.

Understanding the broader concept of atrial myopathy is instrumental in elucidating the pathophysiology of AF and may inspire novel therapeutic approaches. Intensive research has been conducted on atrial myopathy in recent years. This paper reviews the latest insights in the pathophysiology of atrial myopathy and its clinical implications.

Pathophysiology

Atrial myopathy is a complex disorder characterized by diverse pathophysiologic mechanisms, resulting in distinct phenotypes observed among individual patients. Various subtypes of atrial remodeling, including structural, electrical, contractile, autonomic, and endothelial remodeling, are consistently present in all patients with atrial myopathy, albeit in varying proportions. Figure 2 provides an overview of the key pathways implicated in this condition.

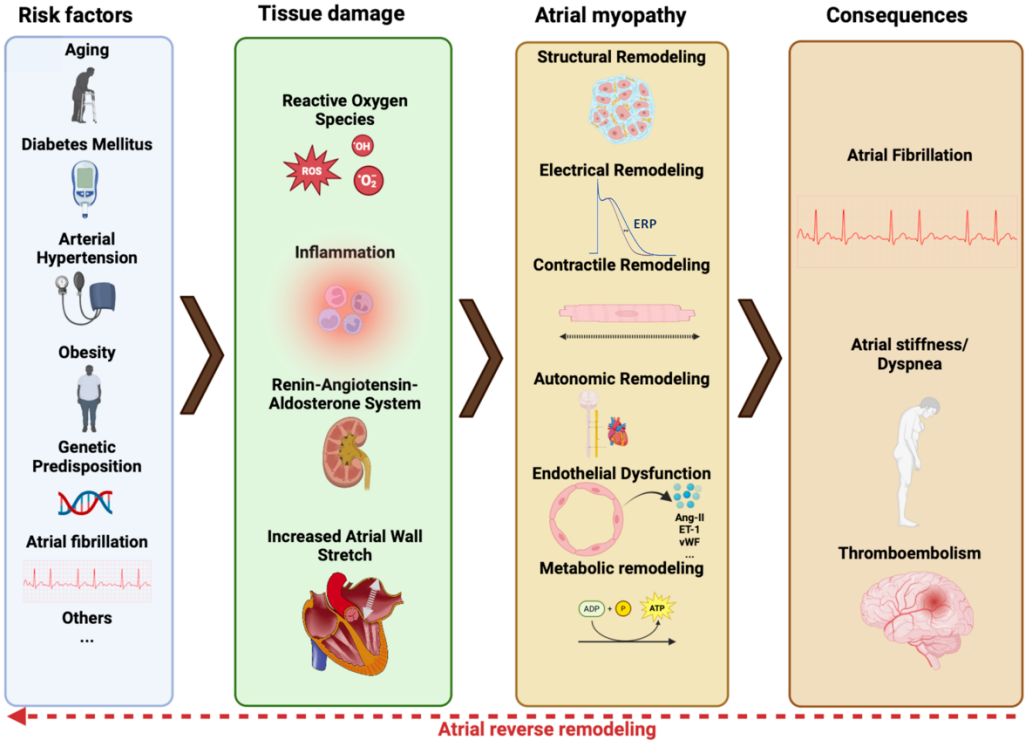

Fig. 2 Central illustration *Overview of the different pathophysiological aspects of atrial myopathy. Different risk factors induce tissue damage to the atrium through various mechanisms. All cardiac cell types react by phenotypic alterations, leading to a state of atrial myopathy, characterized by structural, electrical, contractile, autonomous, endothelial, and metabolic remodeling. These changes lead to atrial fibrillation, exertional dyspnea ('stiff left atrium syndrome', a form of heart failure) and thromboembolism.*

Initiating factors

Genetic predisposition

Despite the classical paradigm that AF and atrial myopathy are acquired, heritability studies with monozygotic twins reported an estimated heritability of AF of 62%.[14] Linkage analysis identified genes in selected families with Mendelian inheritance patterns of early-onset lone AF phenotypes. The first to be discovered this way was a mutation in the KCNQ1 gene, encoding the α-subunit of the K^+-channel responsible for the I_{Ks} current. [15] Later, mutations were identified in the NPPA gene encoding for atrial natriuretic peptide (ANP), TBX5 encoding for T-box transcription factor 5 which is associated with Holt-Oram syndrome, and MYL4 encoding for myosin light chain. [16] The fact that genes like MYL4 unrelated to electrophysiology are involved in AF pathogenesis formed a clue to the importance of structural remodeling in AF pathophysiology and the role of the myopathic substrate. Another surprising finding was the observation of frequent early-onset AF in patients with SCN5A mutations leading to long QT syndrome by *prolongation* of atrial refractoriness, while classically ERP shortening is seen as a causative factor for the development of AF. [17]

Genome-wide association studies (GWAS) have identified more than 140 AF loci of interest (reviewed in [18]). The advantage of GWAS is that AF cases can be compared to controls in a more general population to identify regions of interest. [16,18,19] From these data, polygenic risk scores have been derived, which can potentially guide personalized medicine in the future. [20-22] Furthermore, GWAS data were used to inform pathway analysis to identify transcriptional networks underlying AF. [23]

A limitation of GWAS is the fact that only SNPs are analyzed, focusing on loss-of-function mutations, potentially overlooking numerous mutations in other parts of the genome. Technological advancements reduced the price of whole exome and whole genome sequencing, which made it possible to perform sequencing-based studies, as was done in an Icelandic study that included 14255 AF and 374939 control patients, identifying missense mutation in the PLEC, MYH6 and TTN genes. [24]

Table 1 gives an overview of the most common genetic mutations associated with AF and atrial myopathy, classified according to the function of the proteins they encode. However, this is not a complete overview of every single genetic mutation associated with AF in humans. For further reading we advise review articles focusing on AF genetics. [16,18,19,25,26]

CLASS	GENE	PROTEIN	CARDIAC PHENOTYPE	REF.
ION CHANNELS	SCN5A	Na^+ channel	AF, Atrial standstill	[4]
	GJA5	Connexin-43	AF, Atrial standstill	[4]
	KCNQ1, KCNH2, KCND3, KCNE5, KCN5A, KCNN2, KCNN3, KCNJ5, KCNH2	K^+ channel	Repolarization disturbances, AF	[4,18,19]
	HCN4	HCN-channel	AF, conduction defects, sick sinus syndrome	[18,27]
PARACRINE SIGNALING	NPPA	Atrial natriuretic peptide	Massive atrial enlargement, AF, atrial standstill, ERP shortening	[4,28,29]
CONTRACTILE APPARATUS	DMD, BMD	Dystrophin	Diffuse atrial fibrosis, AF, atrial standstill	[4,30,31]
	LMNA	Lamin A/C	Diffuse atrial fibrosis, AF, atrial standstill	[4,30,31]
	SGCG	Sarcoglycan	Diffuse atrial fibrosis, AF, atrial standstill	[4,30,31]
	DMPK	Dystrophia myotonica protein kinase	Diffuse atrial fibrosis, AF, atrial standstill	[4,30,31]
	MYL4	Myosin light chain 4	Atrial dilation, sarcomere disruption, atrial standstill, AF	[19,32]
	MYH6	α-Myosin heavy chain	Sick sinus syndrome, AF	[16]
	TTN	Titin	Atrial dilation, prolonged PR, AF	[19,33]
CELL STRUCTURE	AKAP9	A-kinase anchoring protein 9	Atrial dilation, AF	[25]
	PRRX1	Paired related homeobox 1	Patent ductus arteriosus and AF	[18,34,35]
	SYNE2	Nesprin-2	Emery-Dreifuss muscular dystrophy, increased nuclear stiffness, AF	[36]
	SYNPO2L	Synaptopodin 2-like protein	Disorganized sarcomeres, contractile dysfunction, AF	[37]
	CAV1	Caveolin-1	Atrial fibrosis, AF	[38]
	SLC35F1	Solute carrier family 35 member F1	Associated with AF	[39]
	NEBL	Nebulette	Various cardiomyopathies: dilated, hypertrophic, non-compaction	[40]
	PLEC	Plectin	Associated with AF	[16,24]
ATRIAL DEVELOPMENT AND	PITX2	Paired-like homeodomain transcription factor 2	Impaired expression of Shox2, Cx-40 and ion channels, leading to AF	[19,41,42]

TRANSCRIPTION FACTORS				
	TBX5	T-box transcription factor 5	Holt-Oram syndrome AF, atrial septal defect, conduction defects	[18]
	ZFHX3	Zinc fingers and homeoboxes protein 3	Atrial (and ventricular) dilation and fibrosis, conduction delay, shortened refractoriness, atrial thrombus, AF	[19,43]
	PRRX1	Paired related homeobox 1	Associated with AF	[34]
	NEURL	Neuralized E3 ubiquitin protein ligase 1	APD prolongation, AF	[44]
	CAND2	Cullin associated neddylation dissociated 2	APD prolongation, AF	[45]
	GATA6	GATA-binding factor 6	Conduction defects, sick sinus syndrome, AF	[46]
	NKX2-5	Homeobox protein Nkx-2.5	Atrial septal defect, AF	[47]
	HAND2	Hand and Neural crest Derivatives expressed 2	Associated with AF	[48]

Table 1: Common genetic mutations leading to atrial fibrillation and atrial myopathy.

Aging

Increasing age is one of the most important risk factors and affects various types of remodeling: structural, electrical, contractile, autonomic, metabolic, and endothelial. Aging indirectly affects the atria by increasing the prevalence of hypertension, diabetes, heart failure, ischemic or valvular heart disease, among others. [49] With age, cumulative atrial damage by hypertension, diabetes, inflammation, etc. increases. Aging also directly induces deleterious effects on the atrial tissue: senescence and apoptosis of atrial cells lead to cell loss with fibrotic replacement, which creates conduction heterogeneity and a substrate for AF. [50-52] Aging also increases leakiness of the ryanodine receptor (RyR) and impairs SERCA function, leading to calcium dysregulation and delayed afterdepolarizations (DAD). [50-52] Aging leads to decreased expression and activity of muscarinic and beta-adrenergic receptors, leading to autonomic dysregulation. [53] Furthermore, elderly patients have elevated levels of the inflammatory cytokines C-reactive protein (CRP), Interleukin-6 (IL-6) and Tumor Necrosis Factor-α (TNF-α), as well as reactive oxygen species (ROS). [50-52] Finally, aging is associated with endothelial dysfunction, which is reflected by capillary rarefaction, reduced nitric oxide (NO) bioavailability, and increased thromboembolic risk. [54-57]

The quantity of DNA methylation, as a measure of cumulative life insults, can be seen as an epigenetic clock, enabling the prediction of chronological age with high accuracy. [58] However, the epigenetic age can also differ from the chronological age, termed epigenetic age acceleration (EAA). Positive EAA, indicating an older epigenetic age, is strongly linked to AF. [59] This in contrast to earlier research that showed that telomere length did not correlate with AF. [60] Additionally, other forms of epigenetic alterations, such as histone modifications and non-coding RNA's, are currently being explored in relation to AF. [61]

Arterial hypertension (AHT)

Hypertension correlates strongly with AF and is the most frequent underlying condition, present in 80% of AF patients. [62,63] Various pathophysiologic mechanisms link AF to AHT. First, AHT increases LA pressure, which leads to atrial dilation, but also to activation of stretch-activated channels (see below) which increase cytosolic Ca^{2+}, a trigger for AF. Second, atrial stretch induces Angiotensin-II (Ang-II), TGF-β1 and platelet derived growth factor (PDGF) release, stimulating profibrotic pathways. Third, pathophysiologic mechanisms of hypertension, including autonomic dysfunction, neurohormonal activation, chronic inflammation, and renin-angiotensin-aldosterone system (RAAS) activation, are important drivers of AF pathogenesis. [63]

Obesity

Obesity is strongly correlated with the prevalence of AF. [64] Not only is obesity often accompanied by other risk factors, e.g., AHT or sleep apnea, but factors secreted by the epicardial adipose tissue—e.g., adipokines—have a direct effect on atrial inflammation and fibrosis. [65,66] It has been shown that the extent of epicardial adipose tissue measured with CT or MRI is a strong predictor of AF occurrence, severity and recurrence. [67] A recent study demonstrated upregulation of the NLRP3 inflammasome (see below) in obese patients. The NLRP3 inflammasome upregulates profibrotic pathways, abnormal sarcoplasmic reticulum (SR) Ca^{2+}-release, and ultra-rapid delayed-rectifier K^+ channels (Kv1.5) that shorten the effective refractory period (ERP). Genetic ablation of *Nlrp3* in mice prevented these obesity-induced alterations. [68] Interestingly, limited data reported ERP-*prolongation* in cardiomyocytes in response to adipokines. [69]

Diabetes mellitus

Diabetes is associated with increased levels of Ang-II and TGF-β as well as thickened epicardial adipose tissue, leading to inflammation, fibrosis and atrial enlargement, all of which lead to higher risk of AF, faster progression and recurrence after ablation. [70-72]

Furthermore, insulin resistance is also present in cardiomyocytes of AF patients, leading to metabolic inflexibility (see 'metabolic remodeling'). [73]

Heart disease

Atrial myopathy can exist solitary but is often observed in the presence of other cardiovascular diseases, including heart failure, ischemic and valvular heart disease. [4] An observational study by *Molina et al.* illustrates the electrophysiological changes in right atrial cardiomyocytes of heart failure patients, where proarrhythmogenic alterations were observed, including increased expression of pro-fibrotic markers and abnormalities in Ca^{2+}-handling. [74] The mechanism through which heart disease leads to atrial tissue damage is not only due to overlap of risk factors of these syndromes, but atrial volume overload leads to oxidative stress, inflammation, wall stress and RAAS activation. [3,4,10,75-78] Finally, AF itself can further induce progression of atrial myopathy. [7] How these mechanisms lead to atrial myopathy will be described in the following section.

Mechanisms of tissue damage

Oxidative stress

Signs of increased oxidative stress have been widely reported in atrial tissue of patients with AF. [51,79-82] ROS originate from various sources: exogenous—e.g., alcohol or tobacco—and intracellular through activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. [50,51,79,83-85] Oxidative stress induces activation (phosphorylation) of the Ca^{2+}/calmodulin kinase II (CaMKII) through c-Jun N-terminal Kinase-2 (JNK2) and directly through oxidation of CaMKII at Met281/282. CaMKII activation increases the open-probability of the RyR, increasing cytosolic Ca^{2+}.[5] Interestingly, a study in old rats described how oxidative stress can acutely activate CaMKII, leading to triggered activity by *early* afterdepolarizations (EAD) in presence of a *prolonged* action potential duration, in contrast with the classical theorem of DAD-mediated triggered activity. [86] In two different transgenic mouse models that increased resistance of CaMKII to oxidative stress (overexpression of reductive enzymes and ablation of critical oxidation sites), AF was not inducible after Ang-II infusion. [87]

In the fibroblast, ROS activates mitogen-activated protein kinase (MAPK), leading to myofibroblast differentiation and tissue fibrosis. [6] Finally, oxidative stress induces a phenotypic switch in the endothelial cell, stimulating the secretion of inflammatory cytokines. [5,88]

Inflammatory signaling

Inflammation is a central mechanism in the development and progression of atrial myopathy, which is reflected by the link between increased levels of the inflammatory markers IL-1ß, IL-6 and TNF-α and the development and persistence of AF. [89] An interesting mechanism is the nucleotide-binding domain, leucine-rich repeat (NLR)-family pyrin-domain containing protein 3 (NLRP3) inflammasome. The NLRP3 inflammasome is a multimeric protein complex present in various cell types, and consists of three molecules: NLRP3, ASC (apoptosis-associated speck-like protein containing a CARD) and procaspase-1. Activation of toll-like receptors or IL-1ß receptors induces priming of the NLRP3 inflammasome by nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB) mediated transcription of NLRP3, pro-IL-1ß and pro-IL-18. When specific stimuli are present—e.g., K^+ efflux, Ca^{2+} influx, ROS, mitochondrial DNA or cathepsin-B—they trigger the oligomerization of NLRP3, ASC and procaspase-1. In this process, procaspase-1 is cleaved into mature caspase-1, which cleaves pro-IL-1ß and pro-IL-18 into mature IL-1ß and IL-18, which both contribute to atrial fibrosis and electrical remodeling. Caspase-1 also cleaves gasdermin-D, creating pores in the sarcolemma of the cardiomyocyte, which can lead to myocytolysis through a specific type of cell death, called *pyroptosis*. [5,89,90] Cardiomyocyte-specific constitutive overexpression of NLRP3 in mice induced atrial dilation, shortened refractoriness, frequent premature atrial complexes (PAC) and increased AF inducibility; all of these are typical characteristics of atrial myopathy that can be abrogated by pharmacological or virally transduced short hairpin RNA targeted NLRP3 inhibition. In a CREM-transgenic mouse model of spontaneous AF, genetic ablation of NLRP3 prevented the occurrence of spontaneous AF. [91] The NLRP3 inflammasome has proven to be a central and necessary pathway in the development of atrial myopathy as a result of various stimuli: obesity, chronic kidney disease, hypercoagulation and post-operative AF. [68,92-95] Because of its central role in atrial myopathy, the NLRP3 inflammasome could be an interesting therapeutic target.

Recent insights showed that not only the initiation of inflammation but also inflammation-resolution is important in the pathogenesis of atrial myopathy. In the acute setting of inflammation, eicosapentaenoic acid (EPA) and docosaheaenoic acid (DHA) are metabolized to resolvins (series D and E), which halt the inflammatory reaction by inhibiting chemotaxis of polymorphonuclear cells, by activating phagocytosis and efferocytosis by M2 macrophages and by clearing inflammatory cells. [95-99] Defective reversal of the inflammatory cascade can lead to chronic inflammation, which is proarrhythmic. [99] For an excellent review on this topic, we refer the reader to [95]

Atrial stretch

Increased atrial stretch is an important driver of atrial myopathy, especially in heart failure and valvular disease. Multiple studies have proven atrial dilation to be an independent risk factor for AF. [100,101] Several mechanisms have been proposed to explain how atrial stretch induces heterogenous conduction slowing, shortened refractoriness and triggered activity—all of them making the atria vulnerable to AF. Increased atrial wall strain activates stretch-activated channels (SAC) on cardiomyocytes. These can be divided into non-specific cation SAC (SAC_{NS}), which produce outward currents when activated during early systole (shortening ERP) or DADs when activated during late systole. Specific cation SAC include the K^+-selective SAC (SAC_K), which mediates a K^+ efflux, shortening the ERP and decreasing excitability, resulting in heterogeneous conduction block and favoring reentry. The best studied SAC_K is the TREK-1 channel, which is downregulated in AF patients. [102] TREK-1 is a mechanically gated K^+ channel, that is activated by thinning of the cell membrane, which is sensed by an amphiphatic helix that extends from within the inner leaflet of the membrane. [103] Activation of TREK-1 occurs independently from the cytoskeleton.

Cardiomyocyte stretch also stimulates cytoskeletal proteins connected to integrins, activating RyR, increasing diastolic Ca^{2+} concentration and hence the probability of DADs. [102,104] Fibroblasts can also activated upon stretch, resulting in the secretion of collagens and matrix metalloproteases, and thus contributing to interstitial fibrosis. Increased stretch over longer periods induces differentiation into myofibroblasts, which can electrically couple to cardiomyocytes (see “fibroblast remodeling”). [6,102,105,106]

Finally, atrial stretch also results in natriuretic peptide secretion. ANP not only induces natriuresis, but also cGMP-mediated decrease of the cytosolic Ca^{2+} concentration in cardiomyocytes, and thus can be antiarrhythmic. [107]

RAAS activation

Ang-II type 1 receptors (AT1R) and mineralocorticoid (MR) receptors are present on cardiomyocytes and fibroblasts. When activated, they are proarrhythmic by activating several pathways. AT1R activates NADPH oxidase, CaMKII and JNK, leading to Cx40/43 function impairment, downregulation and lateralization to transverse borders. AT1R also increases intracellular Ca^{2+} through MAPK and through JAK/STAT, resulting in transcriptional activation of pathways involved in remodeling. [5,108] Furthermore, AT1R activates Arkadia, which results in ubiquitination of Smad7 and disinhibition of TGF-ß signaling. Combined with connective tissue growth factor (CTGF), NLRP3 and IL-1ß

secretion, TGF-ß signaling leads to increased atrial-selective inflammation. [108] Activation of AT1R and MR on fibroblasts induces a profibrotic phenotype by upregulation of collagen production and differentiation into myofibroblasts. [5,108,109]

Clinical data also support the crucial role of RAAS activation in atrial myopathy. For instance, patients with hyperaldosteronism have a 12-fold increase in AF incidence compared to primary hypertension, despite similar blood pressures. [102,110] Administration of deoxycorticosterone acetate (DOCA) in pigs results in increased atrial fibrosis and inducibility of AF without changes in left atrial pressures. [111] In patients, lowering of serum aldosterone after cardioversion predicts maintenance of sinus rhythm. [112] Thus, direct action of aldosterone and Ang-II on the atrial myocardium appears to be an important contributor to atrial myopathy. [72,102]

Clinical manifestations of atrial myopathy

Atrial fibrillation

Atrial myopathy increases the vulnerability to AF through various abnormalities in impulse generation, impulse conduction and electrical refractoriness (Figure 2). [5] In the early stages of atrial myopathy, abnormal impulses typically originate from within the pulmonary veins (PVs) due to triggered activity or micro-reentry. As the disease progresses, AF-induced remodeling and the advancement of atrial myopathy lead to the emergence of ectopic sources and reentry outside the PVs. [5] Clinically, this is characterized by frequent PACs. [113] Impaired conduction arises from dysregulation of sodium channels or connexins, and from tissue fibrosis (or other interstitial depositions). [5] The rapid depolarizations of cardiomyocytes result in an increase in Ca^{2+} loading, leading to a reduction in $I_{Ca,L}$ and an upregulation of I_{K1}, $I_{K,ACh}$, I_{Kur}, I_{K2P} and I_{SK}. [5,114,115] Consequently, this shortens the action potential duration (APD) and subsequently, the effective refractory period (ERP), thus promoting micro-reentry in the PVs and the atrium. [5]

Increased atrial stiffness

The "stiff left atrium syndrome" was initially used to describe a heart failure syndrome based on a diastolic deficit, decreased LA compliance and pulmonary venous hypertension with its onset after excessive catheter ablation of the LA. [116-118] However, decreased LA compliance caused by atrial myopathy can lead to left atrial (i.e. pulmonary venous) hypertension leading to dyspnea, even in absence of prior ablation. [119-122]

Atrial contractile remodeling and dilation have mainly been investigated in the setting of heart failure and valvular disease. They are the result of Ca^{2+} dysregulation, elevated filling pressures, autonomic and neuroendocrine dysfunction, and interstitial fibrosis. Even in the absence of AF and heart failure, atrial myopathy can cause exertional dyspnea. Stiffening of the atria impedes LA filling and passive emptying, while Ca^{2+} dysregulation and conduction slowing–induced dyssynchronous atrial contractions impair LA active emptying. The LA is in direct communication with the pulmonary vasculature, hence a rise in LA pressure (measured as the pulmonary capillary wedge pressure, PCWP) can result in hydrostatic pulmonary edema, leading to dyspnea. [120-122] However, the link between stiff atria and dyspnea is dubious since many patients with atrial myopathy or AF are asymptomatic.

Regarding LA hypertension, an important overlap exists between atrial myopathy and heart failure with preserved ejection fraction (HFpEF). Not only do both conditions have common risk factors and common pathophysiologic traits (Ca^{2+} dysregulation, fibrosis, inflammation, oxidative stress, mitochondrial dysfunction). But AF can exacerbate HFpEF and vice versa: on the one hand, stiffening and decreased contractility of the LA lead to worsening of restrictive LV filling. On the other hand, increased left ventricular (LV) pressures lead to LA hypertension and congestion through atrioventricular coupling. [120,121] Finally, AF can induce an exacerbation of HFpEF complaints due to LA hypertension caused by ineffective atrial drainage and fast and irregular ventricular contractions.

Thromboembolism

Atrial myopathy disrupts the physiological regulation of prothrombotic and antithrombotic factors. There is a dysregulation of endothelial function, resulting in impaired release of endothelial nitric oxide (NO), prostacyclin (PGI_2), and tissue plasminogen activator (tPA), which are critical for maintaining an antithrombotic state. Concurrently, there is an upregulation of procoagulant factors such as tissue factor (TF) and von Willebrand factor (vWF), promoting a prothrombotic environment. [9,123-125]

Furthermore, structural changes such as fibrosis and atrial dilation contribute to blood stasis and altered blood flow dynamics. Turbulent blood flow patterns, particularly in enlarged and dysfunctional atria, create zones of low flow and stasis, providing a fertile environment for thrombus formation. Additionally, the loss of atrial contractility and impaired mechanical function diminish the efficacy of blood clearance, further contributing to stasis. The latter is exacerbated during an episode of AF. [9,126]

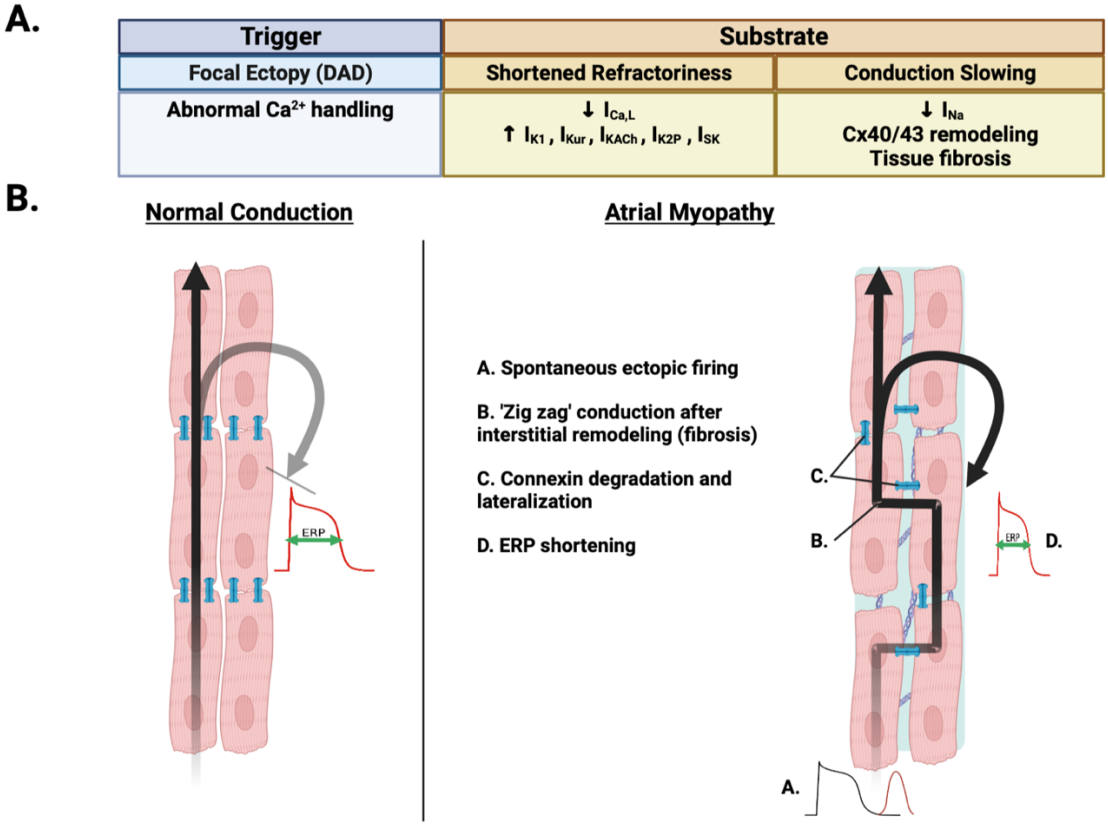

Fig. 3 *The link between atrial remodeling and AF vulnerability. A: schematic overview of the most important cellular mechanisms leading to AF vulnerability. B (left): in normal conduction, there is homogenous impulse conduction throughout the atria. B (right): In atrial myopathy, spontaneous ectopic firing triggers AF, while fibrosis, connexin remodeling and ERP shortening create a substrate for reentry, which in turn may lead to initiation and/or maintenance of AF.*

Molecular and cellular remodeling in atrial myopathy

Remodeling of the atrial interstitium

The EHRAS classification of atrial myopathy[4] distinguishes several types of atrial myopathy based on histology. Class I represents atrial myopathy characterized by myocytolysis and hypertrophy of cardiomyocytes, without fibrotic or interstitial changes. Class II represents atrial myopathy characterized by fibrotic alterations, without changes in cardiomyocytes. Class III represents atrial myopathy characterized by a combination of cardiomyocytes and interstitial changes. Class IV represents atrial myopathy with additional interstitial matrix changes, such as the accumulation of amyloid (class IV_a), fat deposits (class IV_f), inflammatory cells (class IV_i), or other interstitial changes (class IV_o), including granulomatosis or glycosphingolipids. The accumulation of these infiltrates contributes to

the development of electrical conduction heterogeneity, manifesting as "zig zag" conduction patterns. Such aberrant conduction dynamics facilitate the formation of reentry circuits and stabilize rotors at the tissue level, thereby rendering the atria susceptible to the initiation and perpetuation of AF. [4,6,72,108]

Atrial fibrosis is the most common interstitial deposition. These consist of collagen (mostly type I), secreted by fibroblasts. Therefore, we refer to the paragraph "remodeling of fibroblasts".

Isolated atrial amyloidosis (IAA) represents the atrial-specific deposition of insoluble misfolded proteins, for instance ANP. [127] Like fibrotic depositions, these induce local conduction block and prolong P-wave duration but are also directly toxic to atrial cardiomyocytes. The prevalence of IAA strongly increases with age: up to 8.2% of people older than 80 years in one post-mortem study. In people undergoing cardiac surgery, a prevalence of 16% has been reported. In both studies, IAA strongly correlated with AF, independent of age or sex. These data indicate that IAA is a common cause of intra-atrial conduction block and AF in older people. [128-130]

Fatty infiltration of the atrium arises from the epicardial adipose tissue and causes local conduction block. Adipocytes secrete adipokines—e.g., Activin A—that decrease Ca^{2+} influx and contractility in cardiomyocytes [131] and induce fibrosis. [66,124] Over time, fatty infiltrates can evolve into subepicardial fibro-fatty infiltrations, observed in more advanced stages of atrial myopathy with persistent AF. [78,132]

Rare forms of interstitial infiltration in the atria are inflammatory infiltrates in myocarditis, [54,133] granulomatosis in cardiac sarcoidosis[134] or glycosphingolipids in Anderson-Fabry disease. [135]

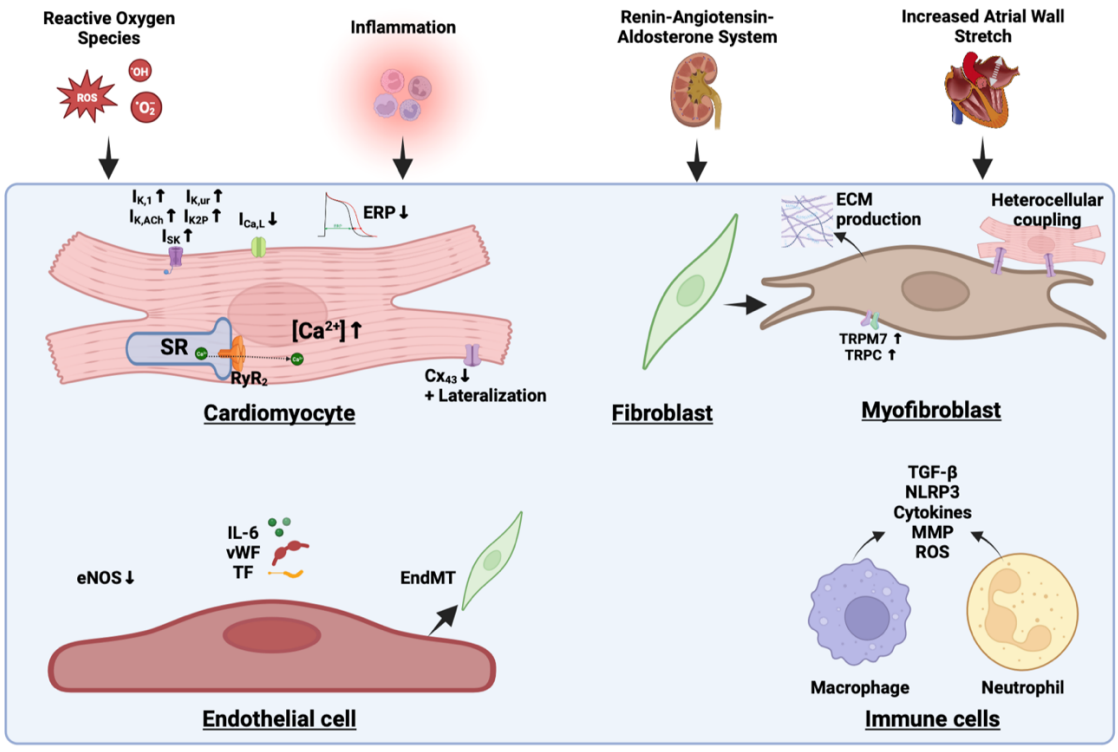

Fig. 4 *Overview of cellular remodeling in atrial myopathy*

Cx_{43} = connexin-43, ECM = extracellular matrix, EndMT = endothelial-mesenchymal transition, eNOS = endothelial nitric oxide synthase, ERP = effective refractory period, IL-6 = interleukin-6, MMP = matrix metalloprotease, NLRP3 = nucleotide-binding domain, leucine-rich repeat (NLR)-family pyrin-domain containing protein 3, ROS = reactive oxygen species, RyR2 = ryanodine receptor type 2, SR = sarcoplasmic reticulum, TGF-ß = transforming growth factor-ß, TF = tissue factor, TRP = transient receptor potential, vWF = von Willebrand Factor.

Remodeling of cardiomyocytes

Cardiomyocyte remodeling is a key process in atrial myopathy and AF, as reviewed previously [5,108,136]. Here, we provide a brief overview of the main mechanisms involved in this process and illustrate these in Fig. 3 and Fig. 4. One of these mechanisms is the activation of the NLRP3 inflammasome and ROS, which increase the RyR_2 channel open-probability and cause sarcoplasmic reticulum (SR) Ca^{2+}-overload. This leads to spontaneous SR Ca^{2+}-release and triggered activity due to enhanced Na^+/Ca^{2+} exchanger (NCX) activity, resulting in DADs. [137] Cytosolic Ca^{2+} overload leads to ion channel remodeling due to NFAT-regulated miR-26 reduction and altered expression of $I_{Ca,L}$, $I_{K,1}$, $I_{K,ur}$ and $I_{K,ACh}$. This causes a shortening of the effective refractory period. Another mechanism is the impairment of impulse conduction by Connexin-43 dysregulation and

lateralization, which are influenced by aging, IL-6, Ang-II and ROS. Moreover, fibrosis causes "zig zag" conduction, which further disrupts normal impulse propagation. [5,108,136]

Remodeling of fibroblasts, myofibroblasts

Myofibroblasts are fibroblasts that have been activated by various stimuli (atrial stretch, TGF-ß, PDGF, CTGF and Ang-II) to differentiate and produce more procollagen and matrix metalloproteinases (MMP), which are mediated through various intracellular profibrotic signaling pathways (MAPK, SMAD, NLRP3, ROS…).[6] During fibroblast differentiation, expression of the transient receptor potential (TRP) melastatin related 7 (TRPM7) and TRP canonical-3 (TRPC3) receptors is increased. TRPM7 and TRPC3 are inward non-selective Ca^{2+}-channels, that stimulate myofibroblast differentiation and proliferation. [138,139] Myofibroblasts express contractile proteins, such as alpha-smooth muscle actin (α-SMA), a distinctive marker. [6] They also express connexin-43, which allows them to form electrical connections with cardiomyocytes; these connections affect the conductivity and rotor susceptibility of cardiomyocytes in vitro. [140-142] However, it is not clear whether myofibroblasts can couple with cardiomyocytes in vivo.

Recently, *Moreira et al.* discovered that cardiomyocyte-secreted calcitonin inhibits fibroblast proliferation and differentiation. In right atrial appendages of AF patients, calcitonin secretion was decreased and calcitonin receptors were internalized. In a LKB1-knockout mouse model of spontaneous AF, additional atrium-specific knockout of calcitonin increased cardiac fibrosis 2.5-fold and mean duration of spontaneous AF episodes 16-fold compared to the single-knockout LKB1 mice, while calcitonin overexpression led to reduced fibrosis and no spontaneous AF. [143]

Remodeling of immune cells

Important cells in atrial appendages from AF patients are macrophages, which contribute to inflammation and fibrosis. A recent single-nucleus RNA-sequencing study demonstrated that expansion of the macrophage population was higher than of any other cell type and that macrophage-specific genetic ablation of the inflammatory mediators *Spp1* and *Ccr2* resulted in decreased arrhythmogenicity. [144] Macrophages respond to inflammatory signals and produce TGF-ß, which induces the differentiation of fibroblasts into myofibroblasts. Through NLRP3 activation, inflammatory cytokines released by the macrophage affect electrical properties of cardiomyocytes by reducing $I_{Ca,L}$ and altering Ca^{2+} handling. [124,145] In explanted human hearts from end-stage heart failure patients undergoing transplant, a marked tendency was observed for increased atrial NLRP3 inflammasome activation in AF patients compared to sinus rhythm. [146] In a canine rapid

atrial pacing model, TRAM-34—an inhibitor of the Ca^{2+}-sensitive K^+ channel KCa3.1 which is expressed on macrophages—was able to prevent macrophage switching into a proinflammatory phenotype, decreasing AF vulnerability. [147]

Neutrophils are involved in atrial remodeling after surgery or ablation for AF. They release inflammatory mediators, matrix metalloproteases and ROS, which cause changes in the structure and function of the atria. [124,145] Mast cells and lymphocytes may also play a role in some cases of atrial myopathy. [145]

Remodeling of endothelial cells

Inflammation and oxidative stress induce endothelial dysfunction, endothelial activation and endothelial-to mesenchymal transition (EndMT). These processes increase the risk of thromboembolic complications. Studies have shown that patients with AF have endothelial dysfunction. For example, they have decreased flow-mediated dilation and lower levels of endothelial NO synthase (eNOS). [123-125] They also have higher levels of inflammatory and prothrombotic factors, such has IL-6, vWF and TF, all markers of endothelial activation. [124,125] Moreover, they have more EndMT in their atrial appendages. [148] These fibroblast-like mesenchymal cells are derived from endothelial cells through the action of TGF-ß, which reduces expression of E-cadherin and enables them to migrate and secrete extracellular matrix components, leading to fibrosis. [149,150]

Remodeling of the autonomic nervous system (ANS)

Auto-antibodies that target muscarinic receptors and $ß_1$-adrenergic receptors have been implicated in some cases of AF. [145] These receptors mediate the effect of vagal and sympathetic stimulation on cardiomyocytes and increase the risk of arrhythmia: ß-adrenergic receptor stimulation induces hyperphosphorylation of RyR_2 channels and increased intracellular Ca^{2+} concentrations, while vagal activation enhances $I_{K,ACh}$. [76] Zhou et al. showed that microinjections with Neuregulin-1 into the ganglionated plexi (GP) of dogs prevented rapid atrial pacing (RAP)-induced upregulation of GP neural activity; decreased GP neural activity suggests that it could eventually decrease AF vulnerability. [151] As a therapeutic approach, targeted ablation of the ganglionated plexi in humans on top of conventional PVI did not deliver significant increase in AF-free survival. [152,153] However, in another attempt to target autonomic remodeling in AF patients, intermittent low-level tragus stimulation to increase vagal tone reduced AF burden in patients with paroxysmal AF. [154]

AF-related atrial remodeling

When atrial myopathy is established, it can predispose individuals to atrial fibrillation (AF). However, the presence of AF itself exerts detrimental effects on various cardiac cell types, contributing to the progression of atrial myopathy and creating a self-perpetuating cycle known as “AF begets AF”. [7]

At the electrophysiological level, the rapid depolarizations observed during AF induce Ca^{2+} loading within the cardiomyocytes. Consequently, there is downregulation of $I_{Ca,L}$ and upregulation of I_{K1}, I_{Kur}, , $I_{K,Ach}$, I_{K2P} and I_{SK}. [114,115] These changes result in a shortened ERP, which promotes the occurrence of reentrant circuits. Additionally, the increased diastolic Ca^{2+} levels activate NCX, enhancing the likelihood of triggered activity. [5,108,136]

Beyond electrical remodeling, the intracellular Ca^{2+} overload in the cardiomyocytes triggers the activation of NADPH oxidase, leading to the generation of reactive oxygen species (ROS) and the activation of the NLRP3 inflammasome. These signaling pathways extend their influence to neighboring fibroblasts, promoting their differentiation into myofibroblasts and ultimately contributing to the development of atrial fibrosis, which in turn stabilizes reentrant circuits and promotes AF. [5,6,108]

Metabolic remodeling

Cardiomyocytes oxidate fatty acids (FA) and glucose in the mitochondria to produce acetyl-coA that is consumed in the oxygen-dependent Krebs cycle to generate nicotinamide adenine dinucleotide and flavin adenine dinucleotide, the latter only in FA oxidation. These molecules are transported towards the electron transporter chain, where they undergo redox reactions eventually resulting in adenosine triphosphate (ATP), the universal energy source of the cell. [155] FA oxidation produces more ATP than glucose, but also requires more oxygen. During stress situations, e.g., during high atrial rates observed in AF, glucose metabolism is favored. In patients with AF, there is also increased expression of the less energy consuming, but slower contracting myofilament fetal-phenotype beta-myosin heavy chain (MHC) instead of the adult myofilament alpha-MHC. [155] In response to energy depletion, adenosine monophosphate (AMP)-activated protein kinase (AMPK) is activated (phosphorylated), increasing glucose and FA uptake in the mitochondrion. AMPK phosphorylation is increased in paroxysmal AF, but decreased in permanent AF, indicating failure of this adaptive mechanism as atrial myopathy progresses. [155]

In patients with AF, atrial myocytes have decreased ability to switch to glucose, either favoring glycolysis or FA oxidation. [73] Pyruvate dehydrogenase kinase (PDK) is upregulated in AF and inactivates pyruvate dehydrogenase, leading to uncoupling of glycolysis and glucose oxygenation, which is called the Warburg effect, originally described in tumor cells. [156] PDK is upregulated in AF patients, and its inactivation by dichloroacetic acid was able to counteract electrical and structural remodeling in a mouse model of paroxysmal AF. [157] Similar to the Warburg effect, FA transportation and oxidation are uncoupled from FA uptake, leading to FA overload and lipotoxicity, resulting in fibrosis and connexin-43 lateralization in a mouse model. [73,158]

Finally, during AF, an imbalance can arise between energy supply and demand. On the one hand, high rates of atrial excitation and increased cardiac work (due to LA dilation) lead to increased atrial ATP consumption. On the other hand, coronary flow reserve decreases, leading to metabolic stress. This results in mitochondrial dysfunction due to redox imbalance, resulting in increased ROS production and mitochondrial damage, a characteristic histological change in atrial tissue of AF patients. [54,155]

Diagnosis of atrial myopathy

Atrial myopathy is a scientific concept that has not been translated to daily clinical practice yet, in part because of the lack of diagnostic criteria. However, many clinical findings indicate the presence of atrial myopathy; some of them are listed below. Future research should develop a scoring or classification system, or perhaps serum biomarkers, that reflects the degree and reversibility of atrial tissue damage and the patient's prognosis. Furthermore, a quantitative assessment of various components contributing to atrial myopathy could give insight into a hierarchical classification of these factors. Potential avenues include investigating the association between biomarkers reflecting underlying types of remodeling, and their correlation with quantitative proxies for atrial myopathy, e.g., atrial arrhythmia burden, imaging proxies like atrial size or strain, and stroke risk. Addressing these aspects could enhance our understanding of atrial myopathy, guide future therapeutic interventions, and help clustering different types of atrial myopathy.

History

A detailed history can reveal the patient's risk profile and possible mechanisms or triggers for atrial remodeling. The $CHADS_2$-VA_2Sc score is part of this risk profile. Personal and family history, lifestyle habits—such as drug use (alcohol, nicotine, caffeine, cocaine…), medication use, endurance sports and sleeping apnea—should also be investigated. These factors can increase the risk of atrial myopathy and can be modified to reduce it (see management).

Electrocardiogram (ECG)

The ECG is a widely used tool in cardiology that can reveal signs of atrial myopathy. The most obvious sign of atrial myopathy on the ECG is the presence of AF, which is a strong argument for an underlying atrial myopathy and requires treatment according to current guidelines.[1] Several studies have shown a correlation between the amplitude of fibrillatory f-waves and the degree of LA enlargement, AF chronicity, and response to therapy. [78,159-162] However, ECG findings during sinus rhythm can also provide valuable insights into the presence of atrial myopathy. Specifically, the P-wave terminal force in lead V1 (PTFV1) and P-wave duration on signal-averaged ECG (P-SAECG) serve as indicators of left atrial enlargement and conduction abnormalities. Furthermore, the frequency of PACs reflects atrial electrical remodeling. Increased PTFV1 and PAC frequencies have been associated with a higher risk of AF incidence, stroke, AF recurrence after PVI, and mortality. Although less data is available for P-SAECG, it has shown promise

as a predictor of AF and AF recurrence following PVI. Notably, one small study[163] demonstrated the predictive value of P-SAECG in stroke recurrence among patients with cryptogenic stroke. [78,113,163-174]

A recent advancement in ECG analysis is the use of artificial intelligence (AI) to predict AF occurrence from sinus rhythm recordings. [175] *Attia et al.* used a deep learning system trained on a dataset of 454 789 ECGs in sinus rhythm to identify AF patients in a separate testing dataset of 130 802 ECGs with a sensitivity of 79.0% and specificity of 79.5%.[176] They also showed that AI-based screening of ECGs was more sensitive than conventional care to detect AF. [177] The deep learning system was able to extract subtle—predominantly P-wave—features invisible to the human eye to predict AF. [178] Despite promising results, implementing AI into clinical practice requires cautiousness because the outcome is strongly dependent on the learning database. [175]

Cardiac imaging

Echocardiography is the most common and accessible technique for cardiac imaging in clinical practice. It can measure several parameters related to the LA. The most typical parameter is the left atrial volume (index), but it does not correlate seamlessly with stroke risk or AF occurrence and recurrence after treatment. [75,78] LA strain, which is the deformation of the LA wall during the cardiac cycle using speckle tracking echocardiography, has been studied extensively in recent years. Reduced LA strain reflects LA stiffness and fibrosis. [179] It also predicts stroke, and AF occurrence and recurrence after ablation. [180-182] Transesophageal echocardiography (TEE) can evaluate blood flow velocities in the LAA and detect spontaneous echo contrast, both of which indicate a higher risk of thrombo-embolism. [126,183]

Late gadolinium enhancement *cardiac magnetic resonance* (LGE-CMR) allows the detection of fibrotic areas in the heart, where gadolinium remains longer than in normal tissue after 10-15 minutes. LGE-CMR can quantify the extent of fibrosis according to the Utah classification, which is a strong predictor for AF recurrency after catheter ablation, as shown in the DECAAF-trial. [184,185] Moreover, increased fibrosis assessed with LGE-CMR correlates with AF occurrence, chronicity and stroke. [186-188] Another parameter that can be measured with CMR is the amount of epicardial fat, which is associated with AF occurrence, ablation outcome and stroke. [67,189]

Besides gadolinium, new *molecular imaging* probes are being developed to target specific markers of atrial myopathy, for instance collagen, inflammatory cells, coagulation factors

and ganglionated plexi. These probes can be visualized by CMR, positron emission tomography (PET) or single photon emission computed tomography (SPECT), depending on the probe. [190]

In patients undergoing an electrophysiology study, three-dimensional *electroanatomical voltage maps* can be created to visualize the substrate of the arrhythmia. On these maps, low voltage areas, electrical silence, fractionation or double potentials indicate fibrotic areas that can cause reentry. Additionally, activation maps can show zones of conduction slowing. [4,78,191] These abnormal areas inside the atria can be ablated to improve outcome. [192,193] Electroanatomic mapping is probably the most accurate technique for estimating atrial substrate, but it is invasive and not suitable for patients in early stages of atrial remodeling.

Circulating biomarkers

Numerous studies aim to identify blood biomarkers signaling the presence of atrial myopathy, indicating an elevated risk of atrial fibrillation (AF), stroke, and AF recurrence post-catheter ablation. Atrial myopathy, a common endpoint for various remodeling types, has diverse biomarkers measuring specific remodeling aspects: myocyte stress, damage, inflammation, fibrosis, endothelial, metabolic, and electrical remodeling.

Micro-ribonucleic acids (miRNAs) are emerging biomarkers. These short non-coding RNA strands can bind to messenger RNA, epigenetically altering gene transcription. Some miRNAs released into serum/plasma (focused on in this article) provide clinicians with a means to assess remodeling-related pathways with targeted precision. [194-199]

While no single biomarker directly links to myopathy, simultaneous use of multiple biomarkers offers insights into its underlying mechanisms, as highlighted in Table 2.

PARAMETER	MECHANISM	PREDICTS AF	PREDICTS STROKE	PREDICTS RESPONSE TO PVI	REF.
ECG					
- $PTFV_1$	Intra-atrial conduction delay	Yes	Yes	Yes	73,160-162
- P-SAECG	Intra-atrial conduction delay	Yes	?	Yes	159,169,170
- FREQUENT PAC	Triggered activity	Yes	Yes	Yes	108,164-168
- DEEP LEARNING	Unknown	Yes	?	?	171-174
ULTRASOUND					
- ATRIAL DIMENSIONS	Struct./contractile remodeling	(Yes)	(Yes)	No	70,196
- LA STRAIN	Contractile remodeling, Î stiffness	Yes	Yes	Yes	175-178,197
- LA EMPTYING FRACTION	Contractile remodeling	Yes	Yes	Yes	72,114,198-200
- A' WAVE VELOCITY	Contractile remodeling	Yes	?	?	72,201-203
- LA FUNCTIONAL INDEX	Structural / contractile remodeling	Yes	Yes	Yes	204,205
- INTEGRATED BACKSCATTER	Structural remodeling	Yes	?	Yes	206-209
- SPONTANEOUS ECHO CONTRAST	Endothelial / contractile remodeling	Yes	Yes	Yes	210-212
CT					
- LA ASYMMETRY INDEX	Structural / contractile remodeling	?	?	Yes	213
- LAA MORPHOLOGY	Blood stasis	?	Yes	?	214
CMR					
- ATRIAL LGE	Fibrosis	Yes	Yes	Yes	180-184
- EPICARDIAL FAT	Adipokines	Yes	Yes	Yes	63,185
- LA STRAIN	Contractile remodeling, Î stiffness	Yes	Yes	Yes	215-217
- LA EMPTYING FRACTION	Contractile remodeling	Yes	Yes	Yes	215-217

BIOCHEMICAL MARKERS					
- TROPONIN	Myocardial injury	Yes	Yes	?	63,73,218-220
- NT-PRO-BNP	Myocardial stretch	Yes	Yes	Controversial	63,73,218-220
- CRP	Inflammation	Yes	Yes	Yes	221-223
- IL-6	Inflammation	Yes	Yes	Yes	221-223
- GDF-15	Inflammation / oxidative stress	Yes	Yes	Yes	224-227
- MIRNA-150	Inflammation (IL-6, IL-8, TNF-α)	Yes	?	(Yes)	228
- SST2	Fibrosis	Controversial	Yes	Yes	229-234
- GALECTIN-3	Fibrosis	Yes	Yes	Yes	235-237
- PIIINP/ICTP	Fibrosis	Yes	No/Yes	Yes	9,50,74,120,238
- FGF-23	Fibrosis	Yes	No	No	239-242
- TGF-β_1	Fibrosis	Yes	?	Yes	191,243-245
- MIRNA-29	Fibrosis (COL1A1, COL3A1)	Yes	Yes	?	219,246-248
- VWF	Endothelial remodeling	Yes	Yes	Yes	9,120,249-251
- ADMA	Endothelial remodeling	Yes	Yes	Yes	252-255
- IGF-1	Metabolic remodeling	Yes	Controversial	?	255-257
- IGFBP-1	Metabolic remodeling	Yes	No	?	255,256
- MIRNA-1	Electrical (KCNE1, KCNB2)	Yes	No	?	190,194,258,259
- MIRNA-328	Electrical (CACNA1C, CACNB1)	Yes	Yes	No	195,260,261
- MIRNA-106	Electrical (RYR2)	Yes	(Yes)	?	262
- MIRNA-208	Electrical (SERCA2A)	Yes	No	?	228,263
- MIRNA-21	Electrical / Fibrotic / inflammatory remodeling (CACNA1C, CACNB2, MAPK/ERK, Smad7)	Yes	Yes	Yes	193,228,243,264-267

ELECTRO-ANATOMIC MAPPING					
- LOW VOLTAGE AREAS	Fibrotic / Electrical remodeling	Yes	Yes	Yes	268-270

Table 2: Diagnostic tools to detect atrial myopathy and their predictive values for AF incidence, stroke and response to ablation therapy.

Abbreviations (alphabetically): ADMA = asymmetric dimethylarginine, CMR = cardiac magnetic resonance imaging, CRP = c-reactive protein, CT = computed tomography, ECG = electrocardiogram, FGF23 = fibroblast growth factor-23, GDF-15 = growth differentiation factor-15, ICTP = type I carboxy-terminal peptide, IGF1 = insulin-like growth factor-1, IGFBP1 = IGF-binding protein-1, IL-6 = interleukin-6, LA = left atrium, LAA = left atrial appendage, LGE = late gadolinium enhancement, miRNA = micro-ribonucleic acid, NT-pro-BNP = N-terminal pro-B-type natriuretic peptide, PAC = premature atrial contraction, PIIINP = procollagen type III N-terminal peptide, P-SAECG = P-wave duration in signal-averaged electrocardiography, PTFV1 = pre-terminal force in lead V1, PVI = pulmonal vein isolation, sST2 = soluble suppressor of tumorigenicity 2, TGF-β_1= transforming growth factor-β_1, vWF = von Willebrand factor.

Management of atrial myopathy

The ESC and AHA provide guidelines for the management of AF, [1,275] but they do not address interventions that target the underlying atrial myopathy. This section summarizes some of the main therapeutic strategies that target the atrial substrate.

Atrial reverse remodeling

As can be appreciated in Figure 2, atrial myopathy is a heterogenous disorder that is a common endpoint of (a combination of) various pathogenic driving mechanisms. *Shen et al*. proposed 4 stages of atrial myopathy, similar to the 4 stages of AF in the recent ACC/AHA/ACCP/HRS guidelines on AF: [276] stage A (at risk of developing atrial myopathy), stage B (asymptomatic but detectable atrial myopathy), stage C (manifest disease but reversable), and stage D (irreversible). [3] Without intervention, patients progress towards stage D, but therapy directed at the driving mechanism of atrial myopathy can induce reverse remodeling, which is often objectivated by reduction in LA volume, increase in LA strain, or decrease in symptom burden. [277]

One of the most important strategies is to modify the risk factors that contribute to atrial myopathy, such as obesity, alcohol consumption, and high blood pressure. For example, weight loss can reduce epicardial fat, improve symptoms and quality of life, and lower the recurrence of AF after ablation and even reverse persistent AF to paroxysmal AF. [278-280] Quitting alcohol can improve the oxidative balance and reduce both AF recurrence and stroke risk. [281,282]

Successful early rhythm control can lead to atrial reverse remodeling via inhibition of AF-induced atrial myopathy. [283] Successful maintenance of sinus rhythm after catheter ablation leads to a decrease in LA volume and an increase in LA strain. [284] Successful DC cardioversion also leads to a prolongation of the ERP, and sustained ERP prolongation post-cardioversion predicts longer-term rhythm control. [285] On a molecular level, sinus rhythm maintenance is accompanied by a reduction in oxidative stress[51] and inflammation parameters. [286] Thus, ample evidence exists that early rhythm control with existing therapies is effective to halt and even reverse atrial myopathy. To widen the therapeutic arsenal of anti-arrhythmic drugs, highly specific ion-channel blocking agents inhibiting I_{SK} [115] or K_{Ca}-2 [287] are currently being developed.

Combining several targets increases success rates: aggressive risk factor reduction after AF ablation not only increased arrhythmia-free survival compared to standard care, but also significantly reduces left atrial volumes, suggesting a reversing effect on atrial myopathy. [279,288]

Therapies targeting the atrial substrate

Treating atrial myopathy in patients presenting in later stages, e.g. stage C, is challenging, because they often progress to persistent and permanent AF (stage D) despite rhythm control efforts. Current strategies targeting the atrial substrate include ACE inhibitors, Ang-II receptor blockers, and mineralocorticoid receptor antagonists. However, large clinical trials have not been able to confirm their efficacy in atrial myopathy. [72,289-297] Diuretics may alleviate LA hypertension during early atrial myopathy stages, [298] but their impact on later stages of atrial myopathy is limited. [289,299] Statins show promise in reducing inflammation and fibrosis during early stages, but their effectiveness in persistent AF is unclear. [300-302] Novel therapies targeting atrial fibrosis, such as pirfenidone, show potential but lack convincing clinical data. [303,304]

Sodium-glucose linked transporter-2 inhibitors (SGLT2i) may reduce AF incidence in diabetic patients by targeting several mechanisms [305-309] with ongoing trials like the BEYOND trial to validate their efficacy in humans. [310] In the trials investigating glucagon-like peptide-1 receptor agonists like semaglutide, an increase in heart rate was observed, and albiglutide induced significant increase in AF risk. However, a recent meta-analysis showed a trend towards a protective effect of GLP1-RA towards AF development, however more data is required. [311] The NLRP3 inflammasome is a promising target; inhibitors like glibenclamide [312]and colchicine [313,314] exhibit (weak) anti-fibrotic effects. Ongoing developments include selective NLRP3 inhibitors (oridonin, MCC950), that show promising data, [315-318] but are still being clinically tested. [95] Resolvins, a novel class promoting inflammation-resolution. A member of this family, Resolvin-D1, has proven anti-inflammatory, anti-fibrotic, and anti-arrhythmic effects in a rat model. [319] A recent drug repurposing study showed that ruxolitinib, a drug used to treat myelofibrosis, is a potent CaMKII inhibitor and is able to inhibit AF in vitro and in mouse models [320,321] Future research should further identify possible therapies targeting and ideally reversing atrial remodeling, in order to get patients from stage C or D back to earlier stages of the disease in order to enhance rhythm control and patient wellbeing.

Anticoagulation

Another challenge in managing atrial myopathy is the initiation of anticoagulation therapy to prevent thrombo-embolic complications, particularly in the absence of AF. Some studies suggest that atrial remodeling, rather than rhythm, is a significant stroke risk factor, [3,9] and therefore should guide initiation of anticoagulant therapy. [322,323] The ARCADIA trial[324] is currently investigating apixaban with aspirin in stroke patients with atrial myopathy without AF. While awaiting trial results, it may be more practical to use markers for atrial myopathy to identify patients at risk for developing AF and stroke. [325,326] However, there is a need for standardized diagnostic tools to asses and stage atrial myopathy in individual patients.

The bidirectional relationship between coagulation and AF is gaining attention. AF promotes a hypercoagulant state, contributing to atrial myopathy progression. Thrombin and Factor Xa (FXa) are serine proteases and activate protease activated receptors (PAR 1-4), which induce inflammatory signaling, oxidative stress, and inositol triphosphate (IP_3)-mediated SR Ca^{2+} release in cardiomyocytes. They also induce differentiation of fibroblasts into myofibroblasts. Furthermore, thrombin and FXa activate MMP9 and the NLRP3 inflammasome through PAR4 activation, triggering fibrosis and inflammation. [93] These detrimental effects can potentially be prevented by direct oral anticoagulants (DOACs). Additionally, DOACs exhibit direct antiarrhythmic properties by prolonging ERP through upregulation of $I_{Ca,L}$ in isolated cardiomyocytes. [327,328] More research is needed to elucidate the clinical importance of these findings. Finally, selective PAR4-inhibitors are under investigation in clinical trials. [329]

Conclusion

Rhythm control therapy proves highly effective in many patients with AF, even those exhibiting early signs of atrial myopathy. Addressing underlying factors contributing to myopathy is a prudent approach, amplifying the impact on reverse remodeling. However, the inefficacy of long-term rhythm control in some subjects displaying signs of more advanced atrial myopathy suggests the need for interventions targeting the underlying substrate to treat this large subgroup of patients. Improved criteria for diagnosing and staging atrial myopathy are needed for the clinical implementation of this concept.

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Chapter 2:

Rationale and hypothesis

The Neuregulin-1/ErbB system

Neuregulin-1 (NRG1) is a paracrine peptide that is released by the endothelium and that plays a vital role in embryonic cardiac development and trabeculation but that is also important in normal adult homeostasis. NRG1 is a member of the superfamily of epidermal growth factors, in the subfamily of the four structurally related transmembrane polypeptides encoded by four different genes: NRG1, NRG2, NRG3 and NRG4. In this subfamily, NRG1 is the most abundant in the cardiovascular system and the most broadly studied in cardiovascular physiology. [1]

NRG1 binds on the V-erb-b avian erythroblastic viral oncogene homolog (ERBB) receptors ERBB3 and ERBB4. ERBB receptors—also called human epidermal growth factor receptors (HER)— are a subfamily of tyrosine kinase receptors that regulate differentiation, proliferation and migration. The ERBB receptor subfamily consists of four members: ERBB1 or synonym epidermal growth factor receptor (EGFR), ERBB2, ERBB3 and ERBB4, but NRG1 only binds ERBB3 and ERBB4. [2-5]

Ligands for EGFR include epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) but not neuregulins, and therefore fall outside the scope of this dissertation. ERBB2 has no known ligands but can be activated and phosphorylated by heterodimerization with other ERBB receptors. The extracellular domain of ERBB3 does bind neuregulins, but lacks a functional intracellular tyrosine kinase subdomain, and therefore its effects are mainly exerted by heterodimerization with other ERBB receptors, mainly ERBB2. [2-5]

ERBB4 is activated by all neuregulins and ERBB4 signaling pathways are involved in the regulation of cell proliferation, survival, differentiation, and other cellular processes. [2-5]

NRG1/ERBB4 signaling in the heart

The neuregulin-1 (NRG-1) and ERBB4 system plays an important role in cardiac development but also as a cardiac stress-response mitigating factor during adulthood. Genetic ablation of *Nrg1*, *Erbb2* or *Erbb4* in mice induces a similar phenotype of fetal death at mid-gestation (10.5 days) due to defective heart development, including lack of ventricular trabeculation. Postnatal heterozygous deletion of *Nrg1* in mice exacerbates doxorubicin-induced heart failure (HF) and endothelial-specific knock-out of *Nrg1* decreases cardiac tolerance to ischemia. [1,2,6-9]

These preclinical data are supported by the fact that in humans, trastuzumab —an ERBB2-antagonizing antibody used to treat breast cancer— exerts cardiotoxic effects, resulting in heart failure, especially when combined with anthracyclins. [10] The fact that inhibition of the NRG1/ERBB pathway induced adverse remodeling and heart failure raised the question whether stimulation of this pathway could bear therapeutic potential, especially in HF patients.

Previous data from our lab have shown that treatment with NRG1 was able to prevent myocardial fibrosis and heart failure in an angiotensin-II mouse model. [11] In humans, phase I and II clinical trials have been conducted to test recombinant NRG1 in heart failure patients, where early data showed a dose-dependent improvement in left ventricular ejection fraction (LVEF). [12-14] An ongoing clinical trial will recruit 1600 HF patients to evaluate the efficacy of recombinant NRG1 treatment (NCT03388593).

NRG1/ERBB signaling in atrial fibrillation

Little is known on the role of ERBB4 in AF. Recent work by *Yamaguchi et al.* showed that pressure overload in the LA of heart failure patients and in transverse aortic constriction and angiotensin-II mouse models led to downregulation of *Erbb4* transcription, which through *Etv1* downregulation induced atrial conduction slowing by downregulation of *Scn5a* and *Gja5* and upregulation of profibrotic pathways by *Tgfbr1* and *Tgfbr2* as well as collagen genes. These data suggest a pivotal role of the NRG1/ERBB4/ETV1 pathway in atrial remodeling, because of inhibiting effects on both electrical and structural remodeling. [15] The role of NRG1 activation as a negative feedback mechanism is also highlighted by an increase in NRG1 serum levels in paroxysmal AF patients compared to controls. [16]

One study demonstrated in a canine rapid atrial pacing model that NRG1 injection into the ganglionated plexi counteracted shortening of the effective refractory period (ERP), decreased activity of the ganglionated plexi, and decreased the window of vulnerability (WOV), i.e. the stimulation frequency range where it is possible to induce AF. [17] The authors claimed that WOV is a proxy of AF inducibility but no AF inducibility data were shown. The effects of NRG1 on ERP and WOV were abolished after injection of PD158780, which is described in the article as an ERBB4-antagonist, but PD158780 inhibits the whole ERBB family of receptors and is not ERBB4-specific.[18] In conclusion, the study demonstrated an effect of NRG1 on secondary endpoints for AF vulnerability, but the data did not provide proof to claim a therapeutic role for ERBB4 stimulation in atrial fibrillation.

In summary, the NRG1/ERBB4 system plays a critical role in cardiac development and maintenance of cardiac function as a stress-response mitigating factor. Current data show promising results for ERBB4 agonists to treat heart failure. Limited data on its role in atrial fibrillation and atrial myopathy suggest that ERBB4 stimulation might also be a potential therapeutic target in this area.

Effects of NRG1 on cardiac cells

NRG1 is secreted by the endothelium throughout various tissues and cells throughout the body, including the heart. The heart is a pluricellular organ, that consists of cardiomyocytes, endothelial cells, fibroblasts, and inflammatory cells (most importantly macrophages). The ERBB4 receptor is expressed in all cardiac cell types, more than ERBB2 and ERBB3, [19] and the cardioprotective effects of NRG1 are mediated through ERBB4. [1,2,20,21] The different effects of NRG1 in each cell type are summarized in Figure 1.

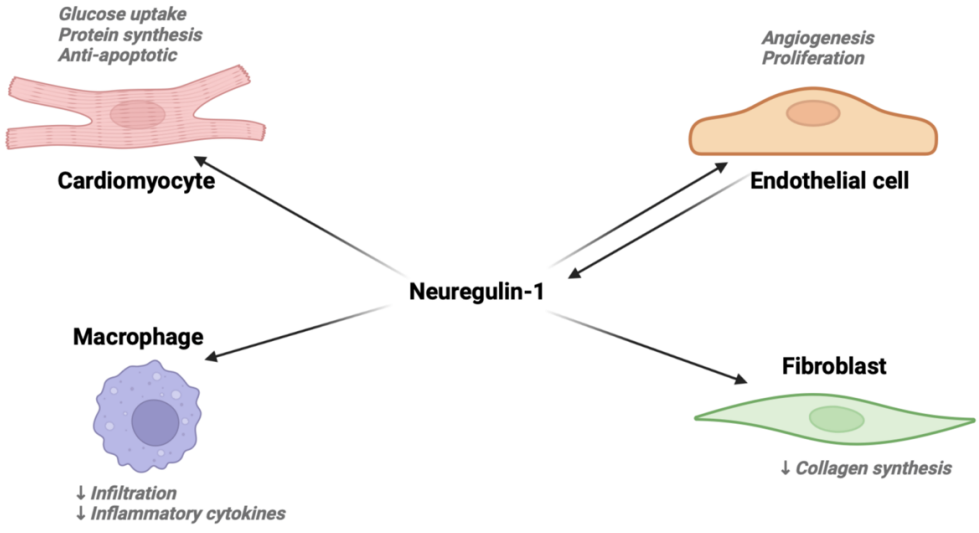

Figure 1: Effects of NRG1 in cardiac cells. *Details: see text.*

<u>Cardiomyocytes</u>

Cardiomyocytes exhibit various responses to NRG-1, both structurally and functionally. In vitro studies highlight NRG-1's role in promoting cardiomyocyte survival, growth, and proliferation. Interestingly, in 3-dimensional cardiac tissue engineered from human embryonic stem cells, NRG-1 stimulates metabolic and contractile maturation including increased glucose uptake and protein synthesis. [22] Moreover, NRG-1 negatively impacts inotropy in isolated papillary muscles and increases lusitropy by enhancing calcium reuptake. [23] Notably, in rodent models of diabetes, NRG-1 reverses diabetes-induced myocyte stiffening and titin hypophosphorylation, implicating translational potential in e.g. diabetic cardiomyopathy. [1,24] However, it should be noted that these data stem from ventricular cardiomyocytes and no data is available about atrial cardiomyocytes.

<u>Endothelial Cells</u>

The endothelium is the main source of NRG-1 in the body, but endothelial cells also express ERBB2, ERBB3 and ERBB4 receptors, creating an autocrine loop. Genetic ablation of NRG-1 or its receptors leads deficient ventricular trabecularisation and endocardial cushion formation resulting in intra-uterine death.[7,25] Cellular responses of the endothelium include induction of rapid calcium fluxes and proliferation, which is implicated in angiogenesis and blood-brain barrier preservation.[26] Additionally, exogenous NRG-1 administration restores myocardial angiogenesis in diabetic cardiomyopathy, suggesting a regenerative role. [27] However, nothing is known yet on the

impact of NRG-1 on the endo- and paracrine function of the endothelium, warranting further studies.

Macrophages

Macrophages are the main inflammatory cells expressing ERBB4 and respond to pro-inflammatory stimuli by upregulation of ERBB4.[1] Macrophages respond to NRG-1 administration by reduced proliferation and cytokine release, and even apoptosis.[28] Additionally, ErbB4 activation attenuates proinflammatory cytokine release from macrophages, underscoring its regulatory role in inflammation and fibrosis.[28] These findings suggest a potential therapeutic avenue for NRG-1 in managing inflammatory conditions.

Fibroblasts

NRG-1 signaling in fibroblasts influences various cellular processes, including cell growth, survival, and cytokine secretion. Activation of ERBB receptors by NRG-1 in isolated fibroblasts suppresses fibrotic responses induced by transforming growth factor-β and decreases the secretion of profibrotic cytokines. Furthermore, NRG-1 inhibits α-smooth muscle actin expression, and hence myofibroblast differentiation.[29] In conclusion, current knowledge converges to potential therapeutic implications in mitigating fibrosis and inflammation.[1]

JK07

The downside of recombinant NRG1 is its short half-life, which necessitates 6-8 h infusion times, for 10 consecutive days. [12] This limits clinical applicability and efficacy in chronic diseases, like HF and AF. Furthermore, NRG1 binds non-selectively to both the ERBB3 and ERBB4 receptors. Activation of ERBB3 by NRG1 in cancer cells that overexpress ERBB2 has been related to enhanced cancer cell proliferation in various tissues and induces therapeutic resistance against anti-ERBB2 therapy. These are regarded as serious potential adverse events in the clinical use of NRG1. Therefore, selective ERBB4 activation would be preferred to attenuate the risk of inducing or accelerating cancer.

JK07 is a fusion protein developed by Salubris Biotherapeutics, Inc. (Gaithersburg, MD USA). It consists of a fully human immunoglobulin IgG1 monoclonal antagonistic ERBB3 antibody fused to two active polypeptide fragments of NRG1 (Figure 2). JK07 has improved pharmacokinetic properties: The half-life in vivo varies from one to three days, depending on dose and species, compared to approximately 2 hours for recombinant NRG1 (Table 1). Moreover, by inhibiting ERBB3 activation, JK07 is a more selective agonist of ERBB4 than NRG1.

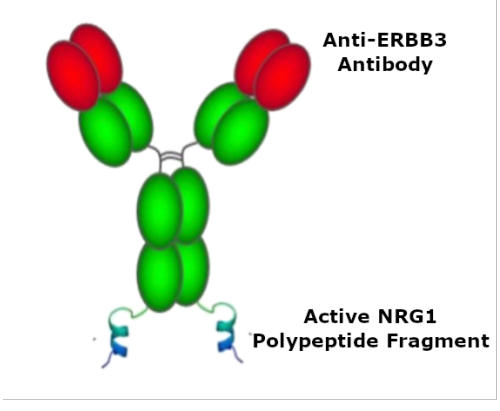

Figure 2: Structure of JK07*. An anti-ERBB3 antibody is fused to two active polypeptide fragments of NRG1.*

	Recombinant NRG1	***JK07***
Receptor activation	ERBB3 and ERBB4	ERBB4 selective
Molecular weight	40 kDa	165 kDa
Half-life in vivo	< 2 hours	1-3 days
Current clinical phase	Phase III	Starting Phase II

Table 1: Comparison between cimaglermin— a form of recombinant NRG1— and JK07.

Hypothesis

Given the existing data on the cardioprotective effects of ERBB4 stimulation on ventricles in the setting of heart failure, we hypothesized that its effects in the atria in the setting of atrial myopathy and AF could also counteract these entities. Therefore, the main hypothesis that was tested in this dissertation was the following:

GENERAL HYPOTHESIS

JK07 counteracts inflammation and fibrosis present in atrial myopathy and therefore decreases vulnerability of the atria towards induction of atrial fibrillation.

To test this hypothesis, we developed two different pig models of atrial myopathy, which will be discussed in the next chapters.

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Chapter 3:

Characterization of a porcine sterile pericarditis model

ADAPTED FROM:

Tubeeckx M, Laga S, Jacobs C, Stroe M, Van Cruchten S, Goovaerts B, Van Fraeyenhove J, Miljoen H, De Meyer GRY, De Keulenaer GW, Heidbuchel H, Segers VFM. Sterile Pericarditis in Aachener Minipigs As a Model for Atrial Myopathy and Atrial Fibrillation. J Vis Exp. 2021 Sep 24;(175). doi: 10.3791/63094. PMID: 34633365.

Introduction

In many cases, AF is merely the electrical symptom of underlying atrial myopathy. Most therapies target the electrical remodeling but few target the underlying structural changes in the atria (inflammation and fibrosis). [1-4] This is probably one of the reasons why current therapies are only marginally effective, especially in more advanced atrial myopathy. [5]

A reproducible animal model is crucial to test novel therapies that target the inflammation and fibrosis present in atrial myopathy. Atrial tachypacing models have been typically used in several large animal species. [6-9] In these models, the atrium is paced continuously or intermittently for long periods to induce electrical and eventually structural changes that lead to AF. The major disadvantages of tachypacing models are the long duration before structural signs of atrial myopathy appear and their relevance only for clinical syndromes in which electrical abnormalities develop before the atrial myopathy. A theoretical risk is pacing-lead failure due to fibrosis during long follow-up[6].

In models of sterile pericarditis, sterile talcum is sprayed over the epicardial surface of the atria to induce an acute inflammatory and fibrotic reaction, resulting in atrial myopathy. [10,11] Pigs have cardiac anatomy and physiology similar to that of humans and therefore, porcine models have high translational relevance. [7] The advantages of using minipigs are that these are easier to handle due to their smaller size compared to conventional pig strains and can be maintained for a long period without any significant increase in body weight. [7] All these reasons make sterile pericarditis in minipigs an appealing model for the investigation of atrial myopathy and fibrillation.

Because there was no previous experience at the University of Antwerp with the use of non-terminal large animal models, the initial step was to set up and characterize this animal model. To facilitate the set-up of this model in different research facilities and standardize protocols to study the inducibility of AF, the protocol was also published in the Journal of Visualized Experiments[12] and elaborately described here for the purpose of increasing know-how and expertise for a future generation of researchers that will be conducting large animal experiments.

Methods

This protocol has been approved by the University of Antwerp Ethical Committee for Animal Testing (case number 2019-29) and follows the animal care guidelines of the University of Antwerp. Ten 6-month-old Aachener minipigs (male, castrated) weighing approximately 20 kg were selected for this study and were randomly divided between the sterile pericarditis (SP) group and the control (CTRL) group. They underwent electrophysiologic testing twice weekly and were sacrificed after 31 days.

Medication and anesthesia

Premedication and installing

Pigs were fasted for 12 hours, but with unlimited access to water. For sedation, a single intramuscular injection of the following medications was administered: atropine (0.05 mg/kg), ketamine (10 mg/kg), and midazolam (0.5 mg/kg). After the pig lost consciousness —approximately 10 minutes post-dose— its weight was determined, and it was transported to the operating theater. The animal was placed on a heating pad to maintain body temperature. ECG electrodes, pulse oximetry, and initial thermometry were attached to ensure proper monitoring. An over-the-needle catheter (22 G) was inserted into the marginal ear vein or external saphenous vein.

Anesthesia

To induce anesthesia, a bolus of propofol (1-4 mg/kg IV) was administered prior to intubation. If superficial anesthesia was observed, an additional bolus of midazolam (0.2 mg/kg IV) was given, and intubation was performed after approximately 5 minutes. The pig was placed in a prone position and an assistant held the animal's mouth open using gauze slings and/or a mouth spreader. Lidocaine (10 mg) was sprayed into the larynx to desensitize it before continuing with intubation. Then, an endotracheal tube (ETT) with an internal diameter of 6.5 mm was placed using a laryngoscope. The ETT was visualized, and a stylet was inserted for better manipulation.* When connecting the ventilator,

* Due to limited visualization of the rima glottis in pigs, the ETT and stylet aided in intubation.

supplementary medication (midazolam 0.5 mg/kg IV and/or alfentanil 30 µg/kg IV) was given if needed.

Ventilator settings included volume control ventilation (VCV) with a pre-set tidal volume of 10 mL/kg, resulting in a peak inspiratory pressure (PIP) of 11-15 cmH_20, a positive end-expiratory pressure (PEEP) of 2-5 cmH_20, a respiratory rate of 12-16 breaths per minute (Brpm) to maintain end-tidal CO_2 ($ETCO_2$) between 35-45 mmHg, FiO_2 of 50% (to be reduced when saturation is 100%), and sevoflurane at 2.5%. For analgesia, alfentanil was administered as a continuous rate infusion (CRI) at a dose of 0.5-1 $\mu g/(kg \cdot min)^{-1}$. A bolus of plasmalyte (10 mL/kg) was given over 10-20 minutes at a rate of 3-5 $mL/(kg \cdot h)^{-1}$ to correct hypotension resulting from hypovolemia. Cefazolin (1 g IV) was administered, and an additional 500 mg IV was given for every 2 hours of surgery.[†] The thoracic and neck regions of the animal were shaved. Vet ointment was applied to the eyes to prevent dryness and eye irritation during anesthesia. Vital parameters were continuously monitored. The depth of anesthesia was assessed at least every 10 minutes by evaluating relaxed jaw tonus, absence of palpebral reflex, rotated eyes, and no signs of excitation. Mucosa color and capillary refill time were checked to evaluate tissue perfusion. All data, along with administered medication, were recorded in an individual anesthetic chart.

An arterial line was placed as follows: the pressure conducting system was prepared by adding 5000 IU of heparin to a 500 mL intravenous bag of 0.9% NaCl. The animal was returned to a supine position, and the femoral artery was located using ultrasound with the vascular probe in the carotid setting. The inguinal zone was disinfected with chlorhexidine.[‡] Under ultrasound guidance, the femoral artery was punctured. The Seldinger technique was used to insert a 3 Fr sheath.[§] The sheath was secured with a

[†] Male pigs may have difficulty with urinary bladder catheterization, which is generally unnecessary for this procedure.
[‡] To ensure an antiseptic technique, the ultrasound probe was sterilized with umonium and the investigator wore sterile gloves.
[§] To prevent dislocation of the needle tip, it was helpful to have an assistant insert the guide wire through the needle, as the act of lifting the ultrasound probe can cause displacement.

suture and connected to the transducer. After flushing, arterial blood pressure was monitored in real time.

Surgery

Preparation for surgery

To prepare for the procedure, the animal was positioned supine in a stable position, with prewarmed IV bags placed in a paraspinal position for additional support. The earthing plate of the electrocautery was placed underneath the animal, ensuring proper contact with the skin using ultrasound gel. The skin was shaved in specific regions, including the neck, upper limbs, anterior thorax, upper part of the abdomen, and inguinal area. Thorough disinfection of the skin was performed using alternating scrubs with alcohol 70% and iodine 2%. Sterile drapes were applied, and the claws of the animal were wrapped in sterile sheets or gloves, with sterile gauze used for retraction. The surgical area was draped with sterile covers, and sterile instruments were used throughout the procedure. Surgeons wore a hair cap, mouth mask, surgical gown, and sterile gloves.

Surgical Placement of a Permanent Central Venous Catheter (CVC)

A 5 cm incision was made in the groove at the medial border of the sternocleidomastoid muscle. Blunt dissection was performed until the internal jugular vein was reached. Fibrous tissue around the vein was removed, and a squared suture (consisting of 3 to 4 stitches forming a circle) with Prolene 6-0 was placed around the desired catheterization site for vessel control. The internal jugular vein was cannulated using a 3 French triple-lumen CVC, employing the Seldinger technique. The Prolene suture was tightened around the catheter, and the handle of the catheter was fixated to the sternocleidomastoid muscle. The three catheter lumina were tunneled separately using blunt dissecting scissors to create the tunnel, and an atraumatic clamp was used to pull the catheter lumina through. The ends of the catheter were securely attached to the skin, and a needle-free injection port was applied. The exit sites of the catheter lumina were positioned behind the ear, maximizing the trajectory length under the skin, and located away from the incision site. The incision site was closed in two layers.

Sternotomy

A median incision was made from the manubrium of the sternum to 3 cm below the xiphoid process, exposing the sternum. Blunt dissection was performed caudally from the xiphoid process, with a finger placed on the visceral side of the sternum to remove

connective tissue along the visceral sternal surface. This step was crucial to prevent myocardial injury during sternotomy.

The sternum was cleaved using a sternum saw, ensuring control of all bleeding sites. The sternum spreader was then utilized to enlarge the access to the thoracic cavity, while taking care to avoid damaging the pleura. The pericardium was carefully opened, and suspension sutures were employed to keep it away from the surgical field.

Pacemaker Lead Placement

Before implantation, the extension and retraction mechanism of the lead's fixation screw were tested to ensure proper functioning. Then, the tip was placed on a (curved) forceps. If necessary, the stylet was curved by 60° to facilitate implantation. A compress was placed on the left ventricle and gently pulled aside to obtain a view of the left atrium.[**] Once the left atrium was visualized, the lead tip was firmly placed on the left atrial free wall, as close as possible to the pulmonary veins and as far as possible from the ventricle. The helix was extended into the atrial tissue by screwing it in, preferably with a slight inclination. This step was performed quickly, and the pressure on the left ventricle was released immediately. The sensing and pacing threshold,[††] and lead impedance were measured using a programmable electrical stimulator or pacemaker programmer. An important criterium for lead placement was the absence of ventricular overcapture (broad QRS on ECG) when pacing at high voltages (10 V). If criteria were unmet, the helix of the lead was retracted, and reimplanted in a slightly different position until satisfactory parameters were obtained.

The placement of a pacemaker lead on the right atrium was performed in an analogous manner to the left atrial lead placement.

Both leads left the thorax at the midline. The left atrial lead was tunneled through the abdominal subcutaneous fat from the xiphoid process to the back of the animal, where

[**] Pressure on the ventricle could lead to hypotension. The anesthesiologist was advised to administer low-dose norepinephrine through the CVC to anticipate this. The ventricle was released when the mean blood pressure dropped below 40 mmHg for more than 20 seconds. The procedure was only resumed after normalization of the animal's blood pressure.

[††] The normal pacing threshold should be less than 1 V with a pulse width of 0.5 ms (normally around 0.5 V @ 0.5 ms).

the tip of the lead was externalized, secured to the skin with sutures and covered with sterile bandages. The right atrial lead was tunneled to the right flank where a pocket was made for a pacemaker to which the lead was connected. A summary of this setup can be seen in Figure 1.

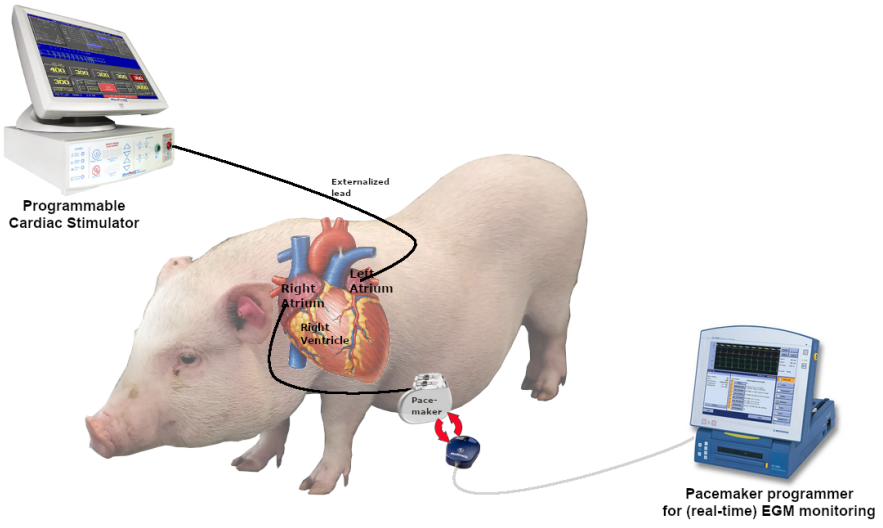

***Figure 1: Experimental setup of the pacing leads**. A pacemaker lead is screwed into the left atrium and externalized. When connected to a programmable cardiac stimulator, atrial tachypacing could be performed. A pacemaker for sensing of the right atrial electromyogram is connected to a lead screwed into the right atrium of the pig. EGM = electrogram.*

Induction of Sterile Pericarditis:

The atria were exposed again by gently pulling aside the ventricles. Gauze was used to cover the ventricles from accidentally being talcated, which was later removed. Sterile talcum was sprayed over the epicardial surface of both atria using the dispenser included in the pack. As bradycardia and hypotension could follow this manipulation, sufficient time was given for the heart to recover spontaneously after approximately one minute. If necessary, a norepinephrine drip was started or increased in infusion rate. One layer of sterile gauze (5 cm x 5 cm) was left on the epicardial surface of both atria, with one piece on the left and one on the right. Afterwards, the position of the pacemaker leads was checked one last time before starting closure.

For the sham operated animals, this step was skipped. In these animals, the same protocol was performed without spraying talcum over the atrial epicardium or leaving a layer of sterile gauze.

Closing the Chest:

A drain was left in the mediastinum and tunneled to the skin surface. The drain was connected to a sterile vacuum jar, and the connection was opened when the first layer of the skin was closed to avoid air leakage. The drain was removed when the animal was brought back to a stable condition.

The pericardium was closed with Prolene 6-0. The sternum was closed using a classical cerclage technique with stainless steel wire. The subcutis was closed in two layers with resorbable thread. A sternal block was performed by infiltrating 5 mL of 0.5% bupivacaine into the skin, ensuring bone contact with the sternum to infiltrate the periosteum.[‡‡] The skin was closed with a continuous intradermal suture using resorbable thread.

Postoperative care

At the end of the surgery, all sedatives were gradually turned off while closing the skin. The pig was kept in the surgery room with close monitoring of body temperature, ventilation, airway patency, oxygenation, and hemodynamic parameters. Due to the frequent drop in body temperature during the procedure, the animal was kept warm using blankets, a heating pad, and hot packs. Oxygen was provided during recovery, especially when shivering was noted. A fentanyl patch of 50 µg/h was applied for postoperative analgesia. To bridge the period before the fentanyl patch became effective, subcutaneous administration of 0.05-0.1 mg/kg of morphine was administered.

The animal could be transported back to the stable if the following conditions were met[§§]: if the animal was hemodynamically stable, showed an increase in body temperature, could lift its head, swallow, exhibited normal ocular reflexes, and breathed spontaneously, freely, and deeply without an ETT in place, without signs of upper airway obstruction.

[‡‡] Alternatively, pre-emptive analgesia could be achieved by performing the sternal block before incision of the sternum.

[§§] The animal was not returned to the stable too soon, as respiratory arrest is possible even hours after the cessation of narcotics.

Means of heating were also provided during the recovery phase in the stable, such as an infrared lamp, heating mat, and blankets.

Regular check-ups were performed on the animal, with assessments made every 15 minutes during the first hour postoperatively, then hourly for the first 4-6 hours, or more frequently if the animal was not comfortable. Supplementary morphine was administered subcutaneously at a dose of 0.025-0.05 mg/kg every 2 hours until the animal was comfortable.[***]

Cefazolin was administered at a dose of 1 g, 8 and 16 hours after surgery. All animals returned to their normal status and behavior within 24 hours. The fentanyl patch was removed on day 3 post-operation.

Atrial tachypacing for induction of AF

After intramuscular injection of ketamine (10 mg/kg) and midazolam (0.5 mg/kg) (without atropine), the pig was observed in the stable until a sufficient level of sedation was reached. The pig was weighed again for follow-up, placed in a restraining sling, and brought to the operating theater. ECG and oxygen saturation monitoring were attached, and the programmer head was placed over their pacemaker for interrogation. The arrhythmia logs were checked for the occurrence of spontaneous AF — which never occurred in any of our subjects. The externalized left atrial lead was connected to the programmable cardiac stimulator for electrophysiology (EP) testing. Pacing thresholds were determined. During EP testing, pacing was always performed at twice the threshold voltage.

[***] Pain assessment consisted of subjective elements such as attitude, behavior (standing, eating, drinking), and grimace. Objective signs of pain included elevated heart rate, elevated respiratory rate, and superficial respiration.

$S_1S_{1,min}$ was defined as the shortest cycle length at which 1:1 capture was maintained during burst pacing and should correlate with the atrial effective refractory period (AERP).[†††]

AF inducibility testing consisted of 10 burst pacing episodes. During one burst pacing episode, atrial electrical stimulation was applied for 60 seconds, starting with a cycle length of $S_1S_{1,min}$ + 20 ms to ensure atrial capture, to quickly decrease the cycle length until $S_1S_{1,min}$. After cessation of pacing, the EGM was observed for the presence of AF, and the duration of the episode was measured. There was a pause of at least 5 seconds between each pacing episode, and the sinus rhythm heart rate was allowed to recover to baseline. When an AF episode lasted longer than 120 seconds, electrical cardioversion was performed. AF inducibility was defined as a percentage—the proportion of "successful" attempts to induce AF for at least 5 seconds out of the total attempts.

Tissue sampling

Euthanasia

After the experiment, which lasted one month, the animals were euthanized with an overdose of IV pentobarbital (50 mg/kg, IV). Humane endpoints for euthanasia were assessed clinically on a daily basis and included persisting signs of severe pain or discomfort despite adequate treatment. Alarming signs included hypertension, tachycardia, increased respiratory rate, behavioral changes (restlessness, immobilization, vocalization), and jaw clenching.

Sampling

Immediately after euthanasia, sternotomy was performed as described in the previous section. After obtaining visualization on the thoracic cavity, vascular clamps were placed on the superior and inferior vena cava, and on the ascending aorta. The whole heart was excised and samples were taken from the left and right atrium.

[†††] This method differed from clinical AERP determination but was more relevant to this protocol because to induce AF, sustained capture on tachypacing is needed. Furthermore, this method is independent of basic cycle length.

Histology

Samples were fixated in a formaldehyde 4% solution and after 24 h transferred to a isopropanol 60% solution. Next, the samples were embedded in paraffin, to be stained with a Masson's trichrome staining. Overview images were taken with a BX43 microscope (Olympus) and analyzed after blinding of the investigator. After manual correction for epicardial fibrotic tissue, the percentage of blue pixels out of the total amount of non-white pixels was determined using ImageJ software. This is a measure of combined interstitial and perivascular fibrosis.

Statistics

Groups were compared using the Mann-Whitney U test. All graphs and analyses were made using GraphPad Prism software (version 9.5.1).

Results

Morbidity and mortality

Data are presented in Figure 2. A perioperative mortality was observed of 3 out of 10 pigs (30%). All deaths occurred in the first 6 surgeries, indicating a "learning curve effect". The causes of death were the following: 2 pigs died because of postoperative respiratory arrest; this problem was later solved by reducing the dose of alfentanil. One pig died because of ventricular fibrillation during testing of the pacing lead: this was due to ventricular overcapture because the left atrial lead was placed too close to the ventricle. Since no defibrillator was at hand (at that time), the animal unfortunately died. During the follow-up period, all animals survived until sacrifice (Figure 2A). During these weeks, however, dislocation or dysfunction of the externalized LA lead was noted because often the pigs pulled out their own or their neighbor's externalized lead. (Figure 2B). In the first 10 days after implantation, an increase in voltage threshold was also observed (Figure 2C). All signs of discomfort disappeared within 24 h postoperatively and a gradual weight gain was observed in both groups (Figure 2D).

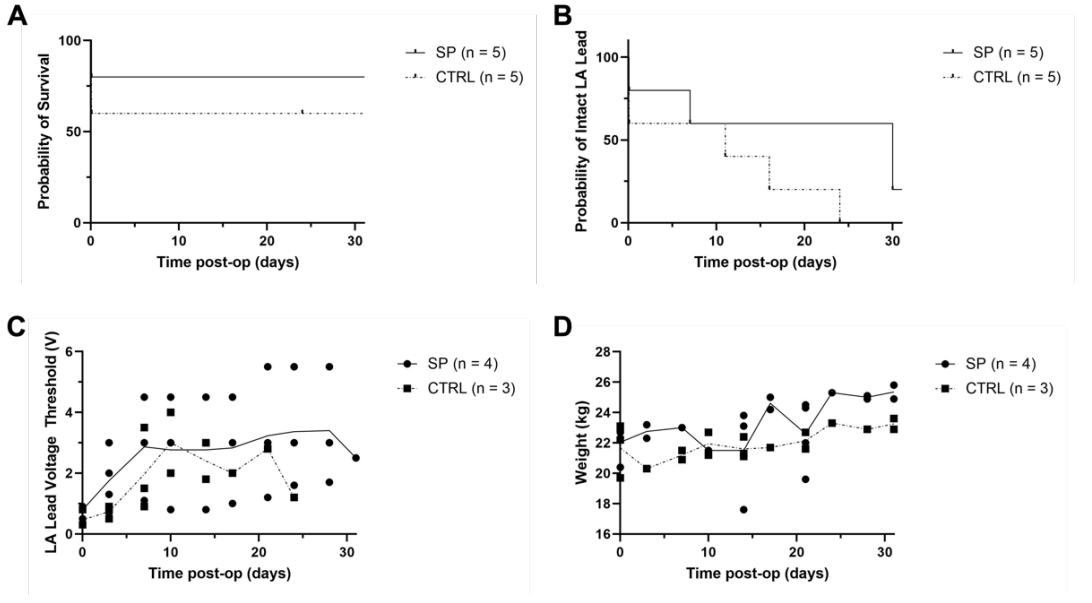

***Figure 2: General parameters of success**. **A)** Peri-operative mortality was 30%, but surviving pigs always reached the end of the experiment. **B)** During the experiments, the occurrence of lead failure was highly prevalent. **C)** After implantation, pacing thresholds tended to rise in the first 10 days and then reach a plateau. **D)** Pigs in both groups gradually gained weight. SP = sterile pericarditis, CTRL = control, LA = left atrium.*

EP testing

Data are represented in Figure 3. AF inducibility began to increase two weeks after surgery (Figure 3A) with a total average of 26% in the SP group compared to 3% in CTRL (p=0.0001; Fig 3B). Similarly, AF duration was higher in the SP group compared to CTRL (37 ± 51 s vs. 8 ± 21 s, p = 0.0073; Figure 3C + D), but it should be noted that this parameter has much higher variability because it is more prone to outliers. Finally, a slight decrease in $S_1S_{1,min}$ (a surrogate for atrial refractory period) was observed in the SP group compared to CTRL, although variability was high (123 ± 12 ms vs. 137 ± 22 ms; p = 0.019; Figure 3E + F).

As a general remark, these data should be interpreted with caution, since due to pacer lead failure, there is a great loss of data towards the end of the experiment.

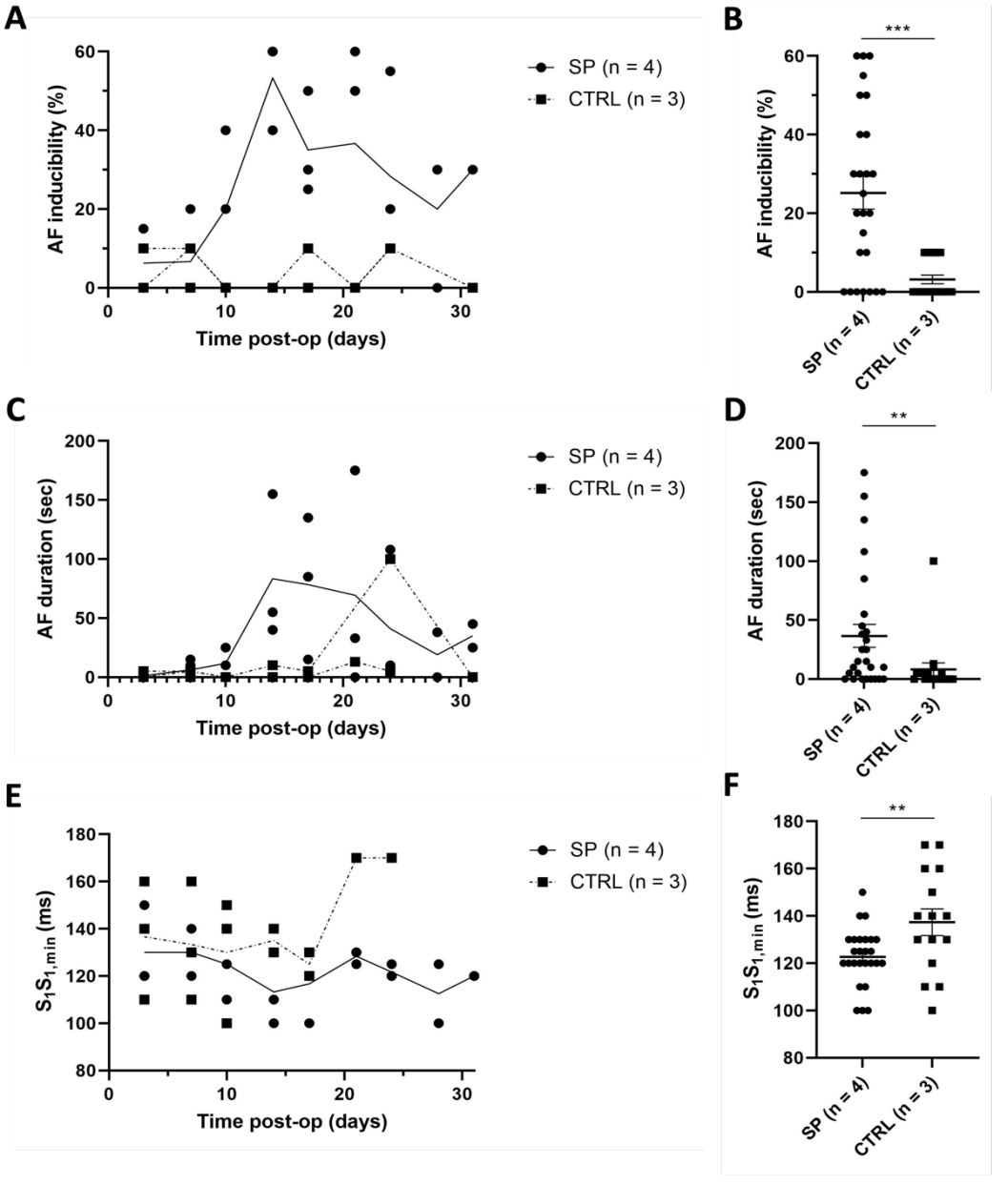

Figure 3: Evolution of EP parameters. A) *In the SP group, AF inducibility increases after two weeks and then reaches a plateau, while in the CTRL group, AF inducibility remains low.* ***B)*** *AF inducibility is significantly higher in the SP group.* ***C)*** *Similarly, AF duration increased after two weeks in the SP group, but remained low in the CTRL group.* ***D)*** *Although variability is high, AF duration is increased in the SP group compared to CTRL.* ***E+F)*** $S_1S_{1,min}$ *also shows high variability, where a slight decrease is observed in the SP group. AF = atrial fibrillation, CTRL = control, EP = electrophysiology, SP = sterile pericarditis. Data displayed as mean ± SEM. **: p < 0.01; ***: p < 0.0001*

Histology

Data and representative images are displayed in Figure 4. The last two pigs were excluded because they were used for the JK07 dosing study (see next chapter). Because the n-values are low, no statistical significance was reached, but a trend was observed for increased levels of interstitial/perivascular fibrosis in the SP group compared to the CTRL group in the left atrium (12.5 ± 2.4% vs. 9.7 ± 1.7%, p = 0.23), right atrium (13.9 ± 4.4%vs. 8.0 ± 3.3%, p = 0.11) and mean of both (13.2 ± 1.0% vs. 8.8 ± 0.9%, p = 0.057).

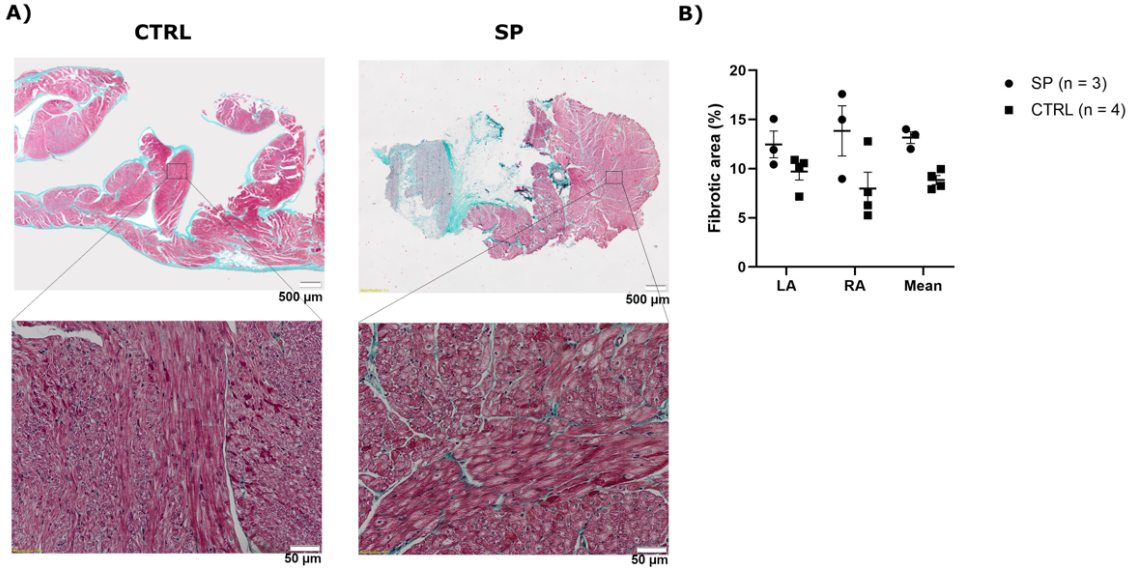

Figure 4: Atrial fibrosis in the sterile pericarditis animals compared to shams. A) *Masson's trichrome staining of left atrial tissue. Blue color = fibrotic tissue. Sterile pericarditis induces more perivascular and interstitial fibrosis in atrial tissue than sham surgery. Upper: 4x magnification; scale bars = 500 µm. Lower: 20x magnification; scale bars = 50 µm.* ***B)*** *Blinded quantification of the fibrotic area shows increased fibrosis for the SP animals compared to CTRL, in the LA, RA and mean with a trend towards statistical significance. Data displayed as mean ± SEM.*

Discussion

In this chapter, we described the development of a sterile pericarditis model in minipigs to induce atrial fibrosis and AF. A reliable large animal model is a major asset for the study of atrial myopathy and AF and the development of novel therapies for AF. In this model, implantation of pacemaker leads on the atrial epicardium allowed a longitudinal follow-up and repetitive electrophysiologic testing, which is difficult in small animals. Minipigs are easy to handle, and their hearts are structurally and physiologically similar to the human heart. [7]

The sterile pericarditis model is relatively straightforward compared to continuous atrial tachypacing because no customized programmed pacemakers are needed. The pathophysiology induced in this model also more closely resembles the pathophysiology often observed in humans, as inflammation and fibrosis precede the induction of AF, which makes it valuable from a translational perspective. [13] Other models, where AF is secondary to ventricular dysfunction or mitral valve regurgitation, tend to be more complicated to develop, and the presence of a non-atrial primary disease confounds the interpretation of effects induced by therapeutic interventions.

A limitation is the arbitrary cut-off of 5 seconds to define inducibility of AF. However, the definition of induced AF is very variable between different papers, and in pig models there is no consensus about the standard cut-off. So, this cut-off was selected at our discretion, based on practical aspects of AF induction since it seemed the shortest duration of AF where no doubt could remain that AF was present with a good “signal-to-noise ratio” since sometimes very short runs (< 3 sec) of atrial arrhythmia follow the burst pacing. To increase uniformity in different animal experiments, was one of the reasons why we published the methods paper in JoVE.

However, some drawbacks were noted with this large animal model. First, there was a high mortality rate, which decreased during the experiment, and likely reflects a learning effect. In the last 4 minipigs we did not encounter early death anymore. The main complication was respiratory arrest, which could later be solved by decreasing the alfentanil dose. Follow-up data were compromised because 5 out of 7 animals that survived the surgery experienced dislocation of the externalized LA lead. This problem was later solved by the implantation of two pacemakers (see Chapter 5). A possible methodological flaw was that —due to stock shortages of the used brand of sterile talcum

spray— two different spraying applicators (insufflation bulb vs. spray) were used interchangeably in the experiment, which could have theoretically affected particle velocity with whom talcum was delivered on the myocardium, and hence penetration depth and myocardial dissolution. Compared to small-animal models, the present protocol is expensive, time-consuming, and labor-intensive, limiting the overall N-value that can be achieved. However, the extra effort of setting up a large animal model is compensated by the high translational relevance and opportunity to perform investigations that are technically impossible in small laboratory animals.

Currently, Schwartzman et al. [11] were the only other investigators who induced sterile pericarditis in pigs. In that study, AF inducibility was higher (10%) immediately after surgery and rose to 80% after 1 week postoperatively. In contrast, our experiments only showed AF inducibility after 2 weeks, and it did not exceed 40%. A possible explanation is the older age and larger body weight of their pigs as well as the higher talcum dose that they used, which makes their model a more acute and aggressive model.

Conclusion

We have shown the feasibility of setting up an animal model of AF with high translational value. Although no statistical significance was reached, it showed a clear increase in atrial fibrosis and AF inducibility in the sterile pericarditis group compared to sham surgery. Therefore, we decided to continue with the model to test the efficacy of JK07 after a limited dosage study (see next chapters). We modified the model only to reduce issues with pacemaker lead dislocation by implanting a second pacemaker connected to the LA lead (since no problems were observed with the RA lead that was already connected to a pacemaker). Furthermore, because significant experience and know-how were gained on surgical, anesthetic, and pacing techniques, the expectation was to have a significant decrease in mortality.

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Chapter 4:

Optimization of JK07 Dosing in minipigs

Background

Since we were the first to assess the efficacy of JK07 in swine, there were no data available on ideal dosage. Therefore, we conducted a small series of experiments to find the maximal tolerable dose, that will be described here. Previously conducted unpublished pharmacodynamic studies by the manufacturer evaluating JK07 in rhesus macaques with heart failure showed increasing activity at dose levels of 0.1 mg/kg, 0.3 mg/kg and 1.0 mg/kg. A study with a full-length recombinant form of NRG-1, cimaglermin, in Yorkshire pigs showed unacceptable tolerability (deep hypoglycemia with transient lethargy and convulsions, occurring within 15 minutes of treatment and persisting 60-90 minutes after treatment) at a dose level of 2 mg/kg administered twice weekly, and acceptable tolerability with the same molecule at a dose level of 0.67 mg/kg administered twice weekly. [1] In NRG-1 molar equivalents, 0.67 mg/kg cimaglermin is equal to approximately 1.34 mg/kg JK07. Cimaglermin has a short half-life of approximately 2 hours in rodents, whereas JK07 has been shown to have a half-life of 24-48 hours in animal studies. [2,*] Finally, the extent to which ERBB3 antagonism of JK07 will reduce the tolerability issues observed in swine following administration of cimaglermin are unknown. Collectively, these data supported a starting dose of 0.1 mg/kg JK07 in this study, with escalation to a maximum dose of 0.6 mg/kg.

Anticipated side effects included bradycardia, hypoglycemia, nausea and sialorrhea. Bradycardia is a known ERBB4-mediated effect, since ERBB4 stimulation of parvalbumin-positive interneurons in the cardiac ganglionated plexi leads to increased secretion of gamma-amino-butyric acid (GABA), which inhibits sympathetic stimulation. [3] Similarly, hypoglycemia is ERBB4/ERBB2-mediated through protein kinase C-zeta (PKCζ)-induced translocation of glucose transporter-4 (GLUT-4) to the cell membrane of skeletal muscle cells. [4,5] Nausea has been described before as well with administration of neuregulins. [6,7]

[*] Based on patent and unpublished experimental data from manufacturer

Methods

Preparation of the JK07 solution

Polysorbate-80 from a 1% stock solution (m/v, "formulation buffer") was added to a NaCl 0.9% solution to reach a concentration of 0.005% (m/v). JK07 was added to this solution in different concentrations and calculated for the pig's body weight as in Table 1.

Dose Level (mg/kg)	Total Dose (estimated per 25 kg)	JK07 Volume (estimated, based on 25kg & 20mg/mL)	Dose Volume (mL/kg)	Formulation buffer (mL)	Final volume for infusion (mL)	Dose Concentration (mg/mL)
0.1	2.5 mg	.125 mL	2	7.375 mL	50 mL	**0.05**
0.3	7.5 mg	.375 mL	2	7.125 mL	50 mL	**0.15**
0.6	15.0 mg	.75 mL	2	6.75 mL	50 mL	**0.3**

Table 1: *Solution preparation for ascending doses of JK07.*

Administration of JK07

Two male castrated Aachener minipigs, 7 months old, underwent treatment with ascending doses of 0.1 mg/kg, 0.3 mg/kg and 0.6 mg/kg JK07 respectively at day 1, 8 and 15. Before treatment, animals were fasted for at least 12h, and sedated with an intramuscular injection of ketamine 10 mg/kg and midazolam 0.5 mg/kg. They were placed in a restraining sling and JK07 in varying doses was administered in 50 ml NaCl 0.9% with polysorbate-80 (see Table 1) over 30 minutes via a syringe pump driver for continuous rate infusion. Blood was drawn from the central venous catheter for measurement of glycemia with Verio OneTouch Ultra glucose test strips. Glycemia measurement was performed before, 1 h after, 4 h after, 24 h after and 72 h after the initiation of each JK07 administration. If neuroglycopenic symptoms would be observed (lethargy, decreased consciousness) with glycemia under 50 mg/dl, an IV bolus of 5g glucose would be administered. Measurements were performed *in duplo* and the mean value was used. Measurements never differed more than 4 mg/dl. Animals were fed after glucose measurements of 4 h post dose. At least twice daily, the animals were evaluated for the presence of side effects, including signs of pain, anorexia, vomiting, diarrhea, behavioral changes, skin or fur changes.

On day 22, an additional dose was administered of 0.3 mg/kg to rule out accumulation of JK07. After this dose, the animals were observed for 24 h, after which they were euthanized.

Results

The evolution of glycemia in the JK07 treated animals is displayed in Figure 1. No side effects were observed during or after the 0.1 mg/kg dose or 0.3 mg/kg dose. During the 0.6 mg/kg dose however, both animals showed slowing of the heart rate from 120 to 80 bpm and 115 to 75 bpm during administration combined with sialorrhea. Furthermore, both animals showed neuroglycopenic symptoms with very low glycemia (23 and 35 mg/dl) 1 h after administration of JK07, which necessitated an IV bolus of 5 g glucose. Within one minute after the glucose administration, both animals regained consciousness and started to walk around, indicative of neuroglycopenia. After regaining consciousness, both animals were gagging without vomitus, which was alleviated with administration of alizapride 10 mg IV. Four hours after administration, there were no signs of anorexia. A low glycemia (37 mg/dl) was also noted in one pig 1 h after the 0.1 mg/kg dose, but neuroglycopenic or other symptoms were absent and therefore no IV glucose bolus was necessary. (Note that normal glycemia in pigs is substantially lower compared to humans with normal values of 50-100 mg/dl. [8]) In this animal, a spontaneous recovery of the glycemia was noted 4 h after the dose.

Glycemia 1 h after the second dose of 0.3 mg/kg on day 22 to rule out accumulation was 71 and 64 mg/dl in the pigs and no adverse effects were observed.

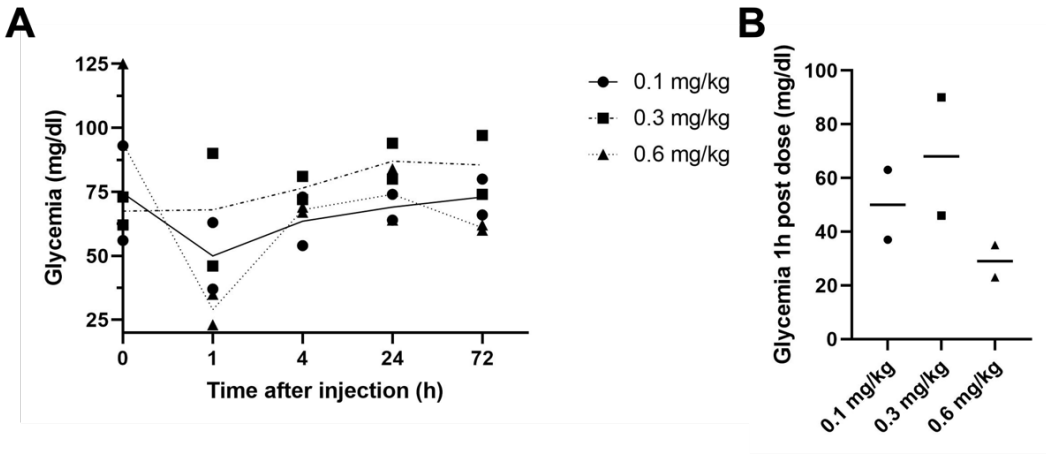

***Figure 1: Evolution of glycemia after JK07 injection**. **A)** Hypoglycemia manifests as an acute effect most pronounced 1h after administration and only in the highest dose of 0.6 mg/kg. B) Detail of the glycemia 1h after JK07 injection.*

Discussion and conclusion

Adverse effects were only observed acutely after the 0.6 mg/kg dose and were absent during and after the 0.1 and 0.3 mg/kg doses. The adverse effects were the anticipated bradycardia, sialorrhea, nausea and hypoglycemia. Adverse effects were easily reversible, did not occur frequently and showed a clear correlation with the administered dose.

In conclusion, this study showed that the highest tolerable dose in pigs was 0.3 mg/kg, which will be the dose we have chosen to proceed with in further experiments. Since the adverse effects are typical and specific to ERBB4-agonists, this is also a reassuring argument that JK07 stimulates the ERBB4 receptor in pigs.

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Chapter 5:

JK07 as a therapeutic strategy for atrial myopathy in a porcine sterile pericarditis model

Background

In Chapter 1 was discussed that inflammation and fibrosis are key mechanisms in the pathogenesis of atrial myopathy. Therefore, we set up and characterized the sterile pericarditis model at the University of Antwerp, where an increase in AF inducibility and duration was observed 2 weeks after surgery, as well as increased fibrosis in the atrial myocardium (Chapter 3).

Neuregulin-1, through activation of the ERBB4 receptor, exerts anti-inflammatory and antifibrotic effects. Administration of recombinant NRG1 improves cardiac contractility in heart failure patients. JK07 was developed to increase ERBB4 selectivity (Chapter 2). Therefore, we hypothesized that JK07, through ERBB4 activation, will also exert antifibrotic and cardioprotective effects in the atria, which will result in decreased AF inducibility.

Methods

Twenty-one male castrated Aachener minpigs, 6 months old, were randomly divided into two groups. A JK07 group received weekly treatment of JK07 at a dose of 0.3 mg/kg, while the VEH group only received vehicle to serve as a control. This happened in a blinded setting, where a second researcher labeled the medication for each animal. This researcher also performed glycemia measurements 1 h after administration.

On day 0, all animals underwent sterile pericarditis surgery as elaborately described in Chapter 3. Because lead dislodgement of the externalized left atrial (LA) lead was a frequent problem in the previous protocol, the LA lead in these experiments was connected to a second pacemaker capable of performing 50 Hz burst pacing. Twice weekly, animals were sedated for electrophysiology (EP) testing, during which a pacing protocol was used (see below). On day 3, 10, 17, 24 and 31, animals also underwent transthoracic echocardiography.

Weekly treatment with JK07 or vehicle started at day 3 after surgery until day 24. Animals were sacrificed on day 31 and tissues were sampled as described in Chapter 3.

Surgery

All animals underwent sternotomy for induction of sterile pericarditis as described in Chapter 3. In short, animals were sedated and intubated. A central venous catheter was placed in the internal jugular vein for intravenous access throughout the experiment. Sternotomy was performed to perform epicardial implantation of pacing leads on the LA and right atrium (RA). Both leads leave the thorax under the xiphoid process. The LA lead is tunneled subcutaneously to the left flank and connected to a (Medtronic) pacemaker capable of performing 50 Hz burst pacing. Similarly, the RA lead is connected to a (Biotronik) pacemaker for real-time electrogram (EGM) monitoring (see Figure 1). All animals underwent induction of sterile pericarditis by spraying 3 g of sterile talcum on the atrial epicardial surface and placement of a layer of sterile gauze. After closure of the sternum, the animals were allowed to wake up and recover from the surgery.

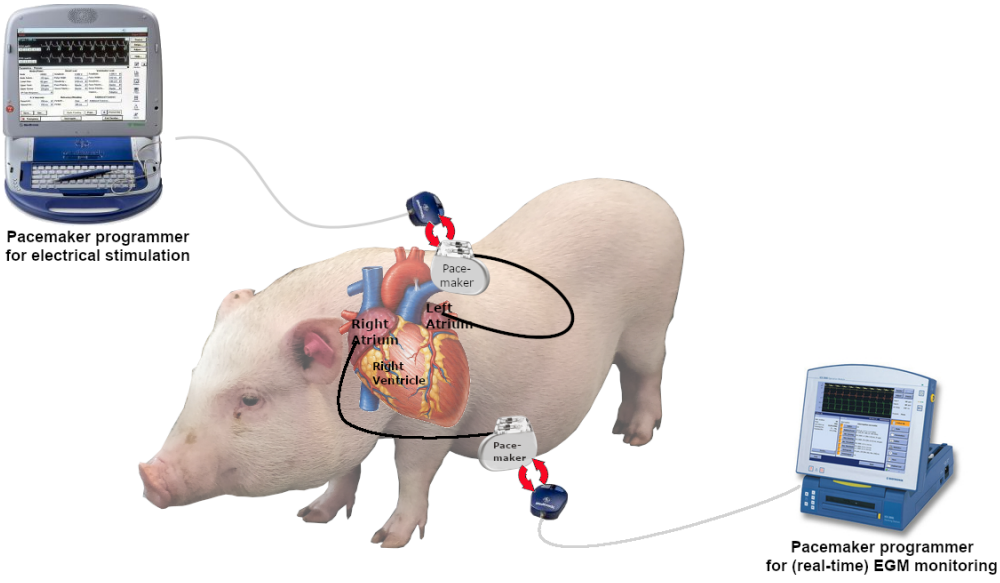


Figure 1: Experimental setup of the pacing leads. *A pacemaker for atrial tachypacing is connected to a lead screwed into the left atrium. Similarly, a pacemaker for sensing of the right atrial electrogram is connected to a lead screwed into the right atrium of the pig. EGM = electrogram.*

Atrial tachypacing for induction of AF

The previous pacing protocol was rather limited, and therefore, a novel more extensive protocol was employed, consisting of three different tachypacing techniques in ascending aggressiveness to test for AF inducibility.

After intramuscular injection of ketamine (10 mg/kg) and midazolam (0.5 mg/kg) (without atropine), the pig was observed in the stable until a sufficient level of sedation was reached. The pig was weighed, placed in a restraining sling, and brought to the operating theater. ECG and oxygen saturation monitoring were attached, and the programmer heads were placed over their corresponding pacemakers for interrogation. The arrhythmia logs were checked for the occurrence of spontaneous AF, which never occurred in any of the pigs. Impedance and pacing thresholds were determined on the LA lead. During electrophysiology (EP) studies, pacing was always performed at twice the threshold voltage.

$S_1S_{1,min}$ was defined as the shortest cycle length at which 1:1 capture was maintained during burst pacing. Conduction time between the left and right atrial leads was

determined by measuring the time between the initiation of the pacing spike on the LA lead and the atrial depolarization on the right atrial lead.

Three different burst pacing protocols were performed, all consisting of 10 burst pacing episodes (so a total of 30 bursts). After cessation of pacing, the EGM was observed for the presence of AF, and the duration of the episode was measured. There was a pause of at least 5 seconds between each pacing episode, and the sinus rhythm heart rate was allowed to recover to baseline. AF inducibility was defined as a percentage, i.e. the proportion of "successful" attempts to induce AF with a duration of more than 5 seconds, out of the total attempts.

For the first protocol, a burst pacing train was applied for 20 seconds with a cycle length of $S_1S_{1,min}$ + 30 ms. For the second protocol, a burst was applied for 20 seconds, starting with a cycle length of $S_1S_{1,min}$ + 20 ms. During the following bursts, the cycle length was decreased until $S_1S_{1,min}$. For the third protocol, a burst was applied for 5 seconds at 50 Hz. After the pacing session, other procedures (e.g., echocardiography, treatment, blood draw) were performed, and the pig waken up from anesthesia.

Transthoracic Echocardiography

On treatment days, echocardiography was performed in the sedated pig, after EP testing and before blood draw and drug administration. Ultrasound was performed with a Epiq7c ultrasound device (Philips Medical). Because the apex of the porcine heart is located behind the sternum, it was technically impossible to acquire apical four-chamber views, limiting the number of measurements. However, when the probe was placed in the right axilla of the pig, images could be made similar to parasternal long and short axis views. Images were stored and analyzed at the end of the experiment to minimize differences in measurements between animals and timepoints. Analysis was conducted before unblinding. The following parameters were measured in long axis view: left ventricular (LV) global longitudinal strain, LV ejection fraction (LVEF), LA area in diastole and systole, LA volume in diastole and systole. The following parameters were measured in short axis view: interventricular septum thickness in diastole (IVSd) and in systole (IVSS), LV internal diameter in diastole (LVIDd) and in systole (LVIDs), LV posterior wall thickness in diastole (LVPWd) and in systole (LVPWs).

Statistical analysis

Crosstabs were examined using a chi-squared (χ^2) test. Statistical significance for comparison between groups is examined using independent samples T-tests. For comparison of groups for measurement on different time points, repeated measures ANOVA was used. When sphericity wasn't present, a Greenhouse-Geisser correction was applied. In case other tests were used, this was specified in the text. A p-value <0.05 was considered significant. Data in text are displayed as mean ± SD, unless specified otherwise. The analyses were performed using SPSS software. Graphs were made using GraphPad Prism.

Results

Two pigs died during the experiment: one pig in the early post-operative period due to respiratory arrest, and one on day 3, probably due to an accidental epidural/intrathecal injection of ketamine during intramuscular administration of sedation for pacing and treatment. Two pigs (one JK07 and one VEH treated) were excluded because of purulent pericarditis (as evidenced by pus in the pacing lead trajectory). Cultures revealed *Actinomyces hyovaginalis* in one pig and *Trueperella pyogenes* in the other, which are typical porcine opportunist infections. Seventeen animals were analysed: 9 from the VEH group and 8 from the JK07 group.

No adverse events were observed in either group during or after JK07 or vehicle administration. Glycemia 1 hour after initiation of JK07 administration was never below 40 mg/dl and did never cause symptoms (Figure 2A) and did not differ between JK07 and Vehicle (77 ± 17 mg/dl vs 72 ± 18 mg/dl, p=0.321). Therefore, after 11 animals, we stopped measuring post-treatment glycemia. Gradual weight gain was observed in all animals post-surgery with no difference between JK07 and Vehicle (28.8 ± 2.7 kg vs. 28.2 ± 3.4 kg p=0.708; Figure 2B). Finally, no significant bradycardia was observed, although overall heart rate was lower in the JK07 group compared to VEH (120 ± 19 bpm vs. 129 ± 20 bpm, p=0.003; Figure 2C).

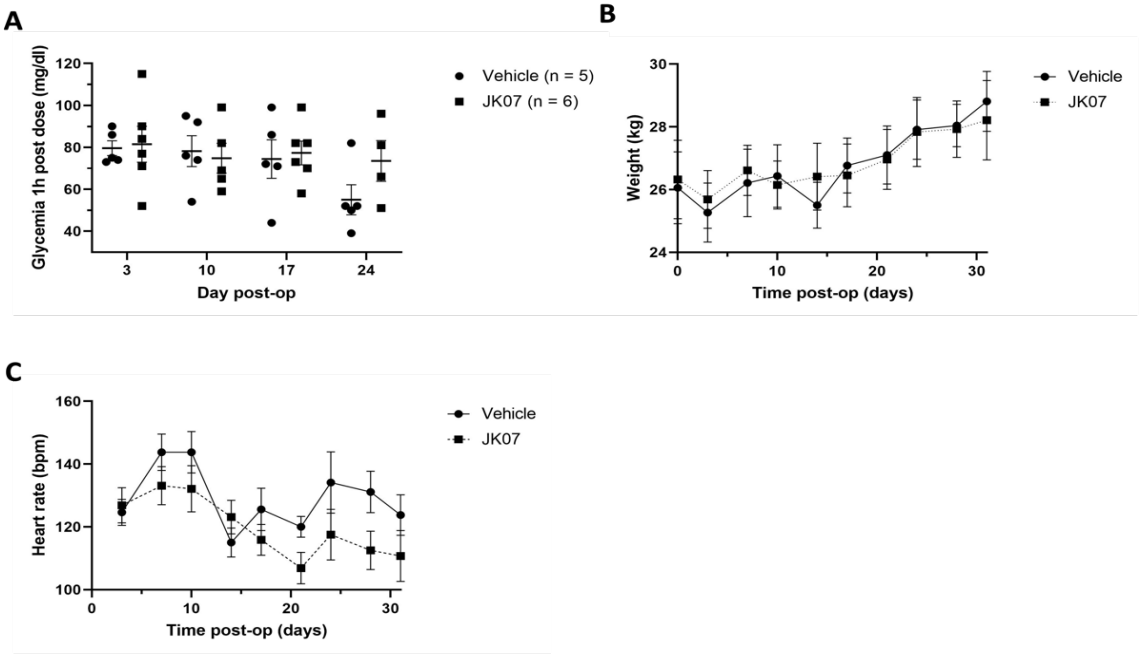


Figure 2: Anticipated adverse effects were absent. A) *Glycemia 1 hour after initiation of JK07 administration did not differ between VEH (n=5) and JK07 (n=6) groups.* ***B)*** *Gradual weight gain was observed in both groups post-surgery.* ***C)*** *Heart rate slightly varied between examination sessions with no significant difference between groups. Mean ± SEM.*

No lead dislodgement or pacemaker malfunction was observed. Data regarding lead function are shown in Figure 3. A gradual increase was observed in the impedance of the LA lead on day 31 compared to day 3 in the JK07 group (540 ± 196 Ohm vs. 398 ± 151 Ohm, p=0.156) and Vehicle group (594 ± 181 Ohm vs. 397 ± 82 Ohm, p=0.014), with no difference on day 31 between the JK07 group and Vehicle group (540 ± 196 Ohm vs. 594 ± 181 Ohm, p=0.591). Similarly, a gradual increase was observed in the impedance of the RA lead on day 31 compared to day 3 in the JK07 group (710 ± 108 Ohm vs. 476 ± 156 Ohm, p=0.007) and Vehicle group (624 ± 145 Ohm vs. 435 ± 84 Ohm, p=0.004), with no difference on day 31 between the JK07 group and Vehicle group (710 ± 108 Ohm vs. 624 ± 145 Ohm, p=0.219). Finally, a gradual increase was observed in the pacing threshold of the LA lead on day 31 compared to day 3 in the JK07 group (2.61 ± 1.42 V vs. 1.94 ± 1.41 V, p=0.376) and Vehicle group (1.72 ± 0.86 V vs. 1.28 ± 0.45 V, p=0.224), with no difference on day 31 between the JK07 group and Vehicle group (2.61 ± 1.42 V vs. 1.72 ± 0.86 V, p=0.160).

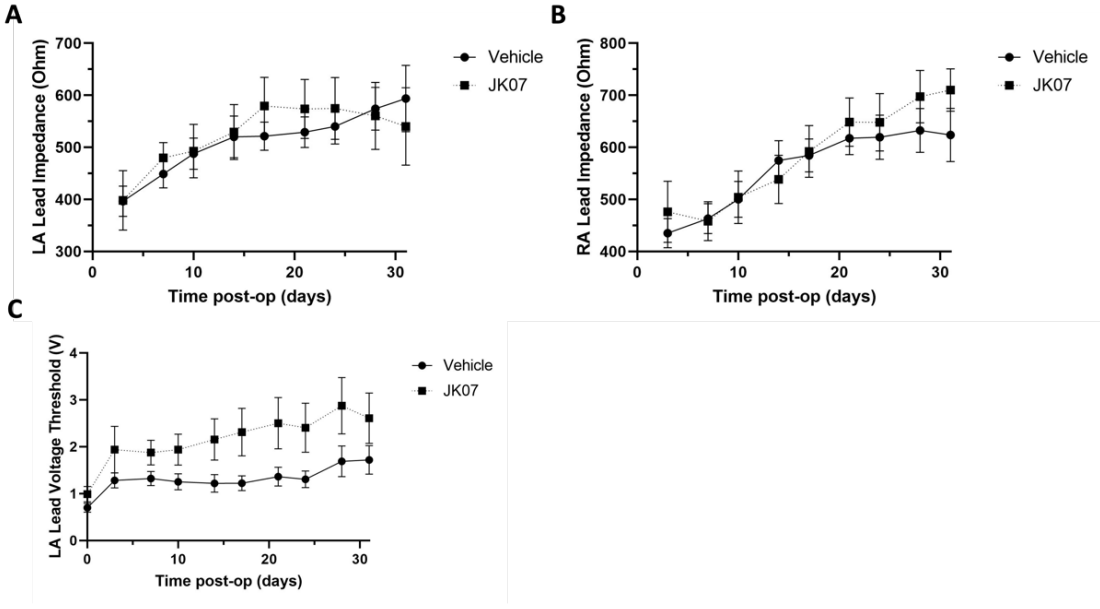


Figure 3: Evolution of pacing lead properties. A+B) *An increase in lead impedance of the RA and LA is observed during the experiment.* ***C)*** *LA voltage threshold increases throughout the experiment, more pronounced in the JK07 group. Mean ± SEM.*

Electrophysiology testing

Effectiveness of the novel pacing protocol

Data on the effectiveness of the extended pacing protocol are displayed in Figure 4. Conform the previous pacing protocol described in Chapter 3, AF inducibility and AF duration increased two weeks after the induction of sterile pericarditis. To test the effectiveness of the protocols, we merely looked at data in the Vehicle group to characterize the sterile pericarditis model without any further intervention (JK07 administration). The $S_1S_{1,min}$ + 30 ms protocol showed significantly lower AF inducibility compared to the decremental pacing protocol (2 ± 7% vs. 9 ± 14%, p<0.001, paired T-test). Similarly, induced AF duration was shorter (2 ± 8 s vs. 9 ± 16 s, p<0.001, paired T-test). The 50 Hz burst protocol showed the highest AF inducibility (16 ± 23%, p=0.004 vs. decremental pacing protocol) as well as longer AF duration (22 ± 53 s, p=0.026 vs. decremental).

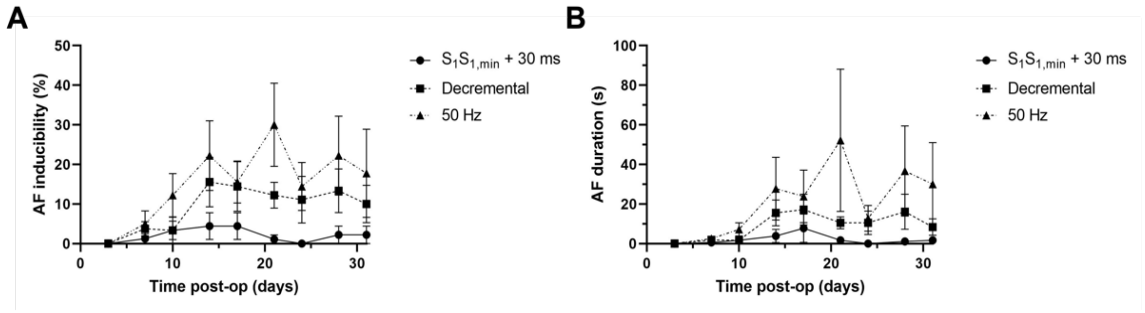


Figure 4: Evolution of AF inducibility (A) and AF duration (B) in the various pacing modalities in the Vehicle group (n=9)*. Mean ± SEM.*

EP data for JK07 vs. Vehicle

The evolution of EP parameters is displayed in Figure 5.

An increase in AF inducibility over time was observed (within-subject effect, p = 0.002) which was not affected by treatment (between-subject effect, p = 0.321). Similarly, a clear trend towards an increase in AF duration was present, but not statistically significant (p = 0.055) without an effect of treatment (p = 0.302). $S_1S_{1,min}$ decreased significantly over time in both groups (p < 0.001), which was also unaffected by treatment (p = 0.765). The corrected conduction time increased over time in both groups (p < 0.001) without an effect of treatment (p = 0.280).

When comparing the separate pacing modalities, there was no statistically significant difference in AF inducibility or AF duration between the treatment groups, except for $S_1S_{1,min}$ +30 ms where the between-subject difference was not clinically significant (Supplementary Figure 1).

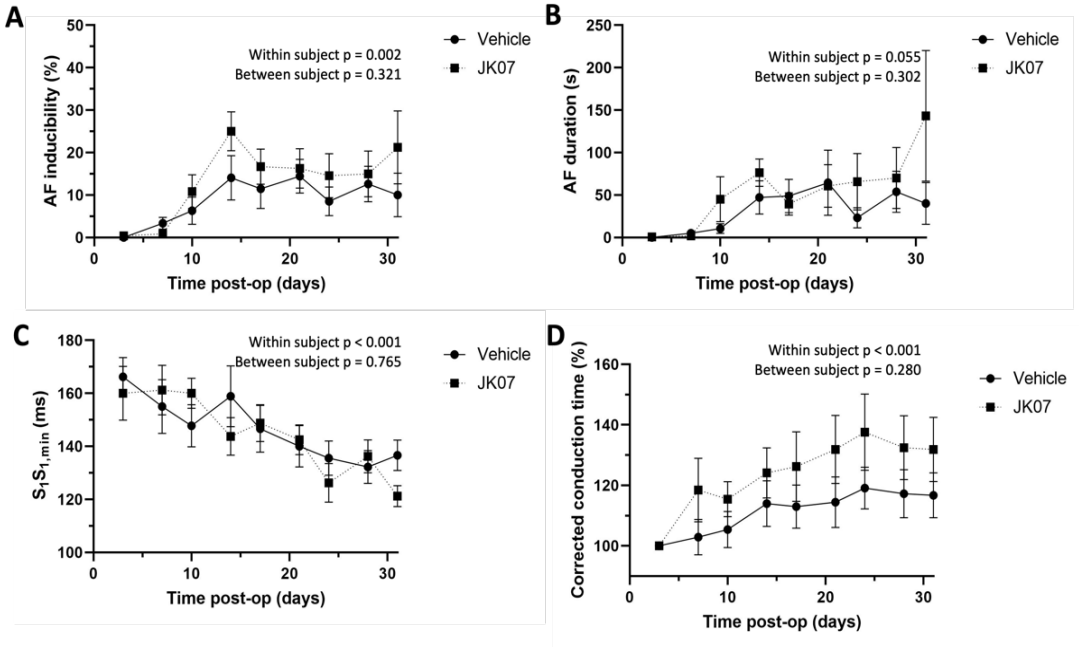


Figure 5: Evolution of EP parameters. A) *No differences in total AF inducibility or AF duration* ***(B)*** *are observed between the Vehicle (n=9) and JK07 (n=8) groups.* ***C)*** *$S_1S_{1,min}$ does not differ between groups.* ***D)*** *Corrected conduction time does not differ between groups.*

Echocardiography

Representative images obtained with cardiac echocardiography are shown in Figure 6 and the data are represented in Figure 7.

Overall, no important echocardiographic differences were observed over time or between both treatment groups.

LVEF did not change over time in both groups ($p = 0.226$) and was unaffected by treatment ($p = 0.089$). Similarly, left ventricular strain showed no significant difference over time ($p = 0.602$), but had slightly lower negative values (increased deformation) in the JK07 group compared to vehicle ($p = 0.030$).

The interventricular septal thickness in diastole (IVSd) did not significantly differ over time ($p = 0.056$) without an effect of JK07 ($p = 0.339$). Concordantly, the left ventricular posterior wall thickness in diastole (LVPWd) did not evolve over time ($p = 0.785$) and was also unaffected by treatment ($p = 0.092$).

Finally, weight-corrected left atrial volumes increased over time ($p < 0.001$) without an effect of JK07 ($p = 0.608$).

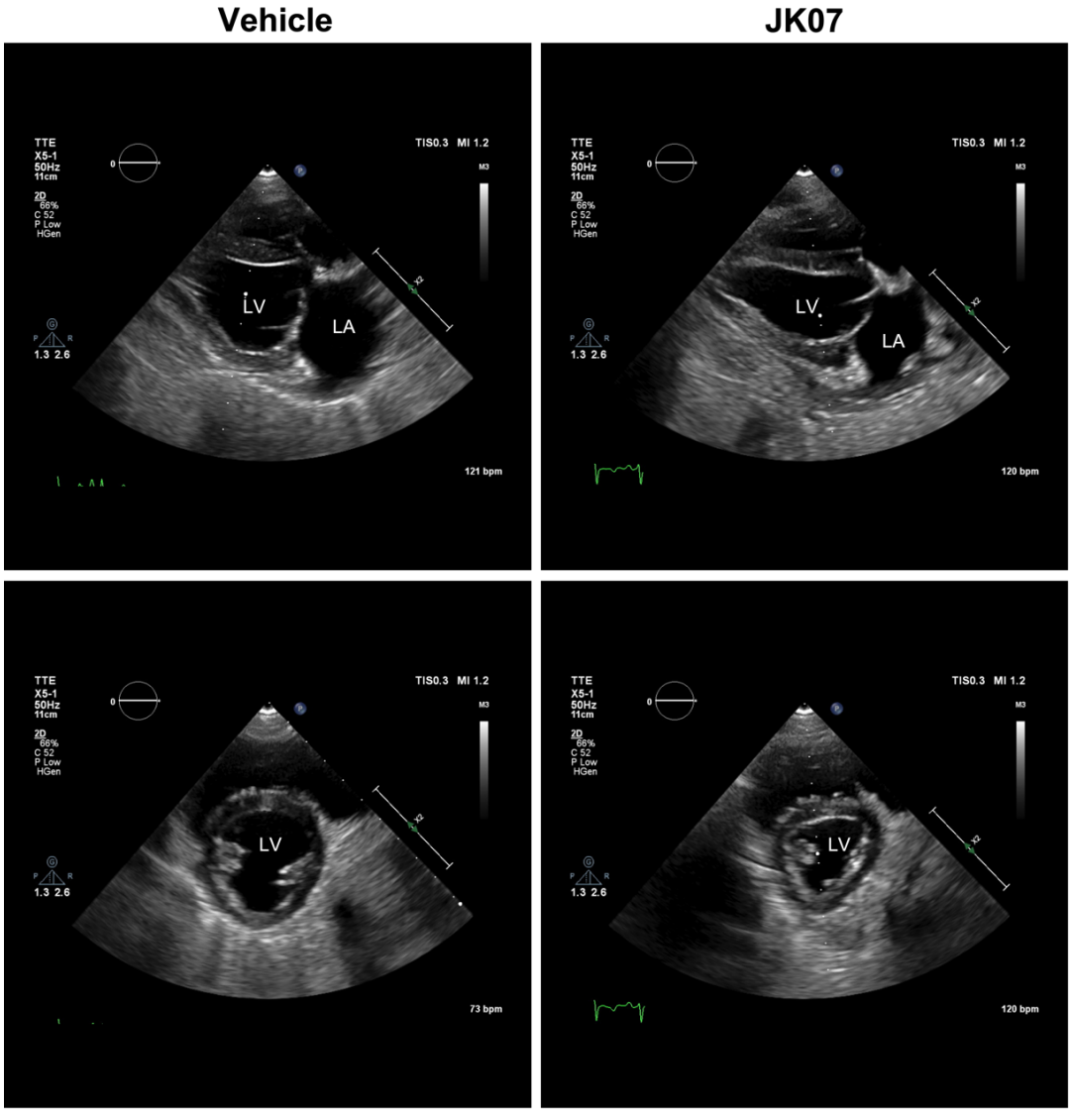


Figure 6: Representative echocardiographic images*. Upper: images taken in a view similar to parasternal long axis. Lower: images taken in a view similar to parasternal short axis. All images displayed were taken on day 31 of the experiment and pictures were taken in diastole. LA = left atrium, LV = left ventricle.*

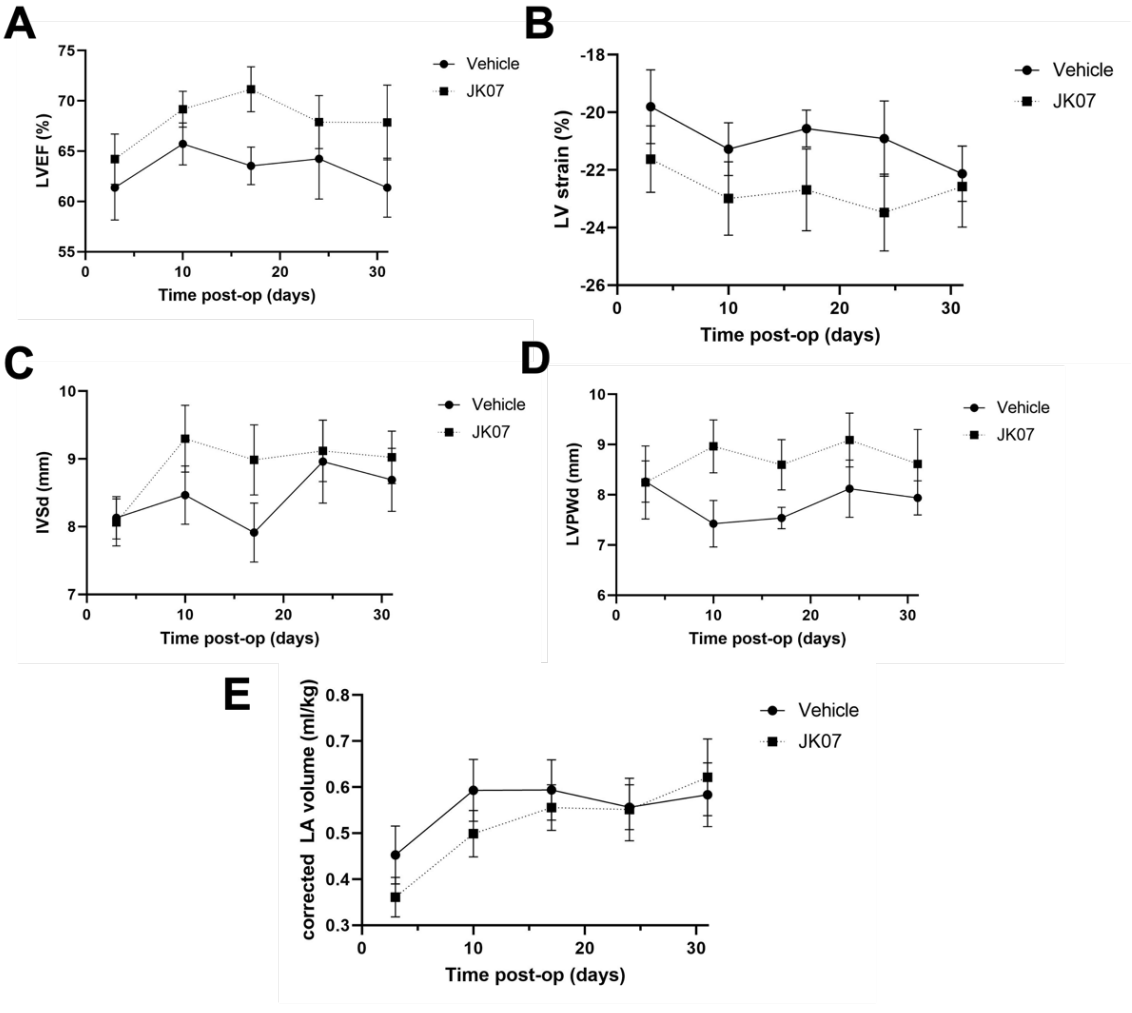


Figure 7: Evolution of echocardiographic parameters. *The parameters for contractility LVEF* ***(A)*** *and LV strain* ***(B)*** *remained constant during the timeframe of the experiment. The parameters for hypertrophy IVSd* ***(C)*** *and LVPWd* ***(D)*** *also remained constant.* ***E)*** *There was a gradual dilation of the LA over the first 2 weeks, that stabilized afterwards. Mean ± SEM.*

IVSd = interventricular septal thickness in diastole, LA = left atrium, LV = left ventricle, LVEF = LV ejection fraction, LVPWd = LV posterior wall thickness in diastole.

Histology

Data are presented in Figure 8. Five animals were excluded from histologic examination because after day 31, they were kept alive conduct testing of different AF models (additional atrial tachypacing was performed after day 31 to increase atrial electrical remodeling). There was no statistically significant difference observed in the LA fibrotic area of the Vehicle vs. JK07 group (13.0 ± 3.4% vs 12.1 ± 5.5%, p=0.726) as well as the RA fibrotic area (14.3 ± 6.7% vs. 11.5 ± 3.4%, p=0.381).

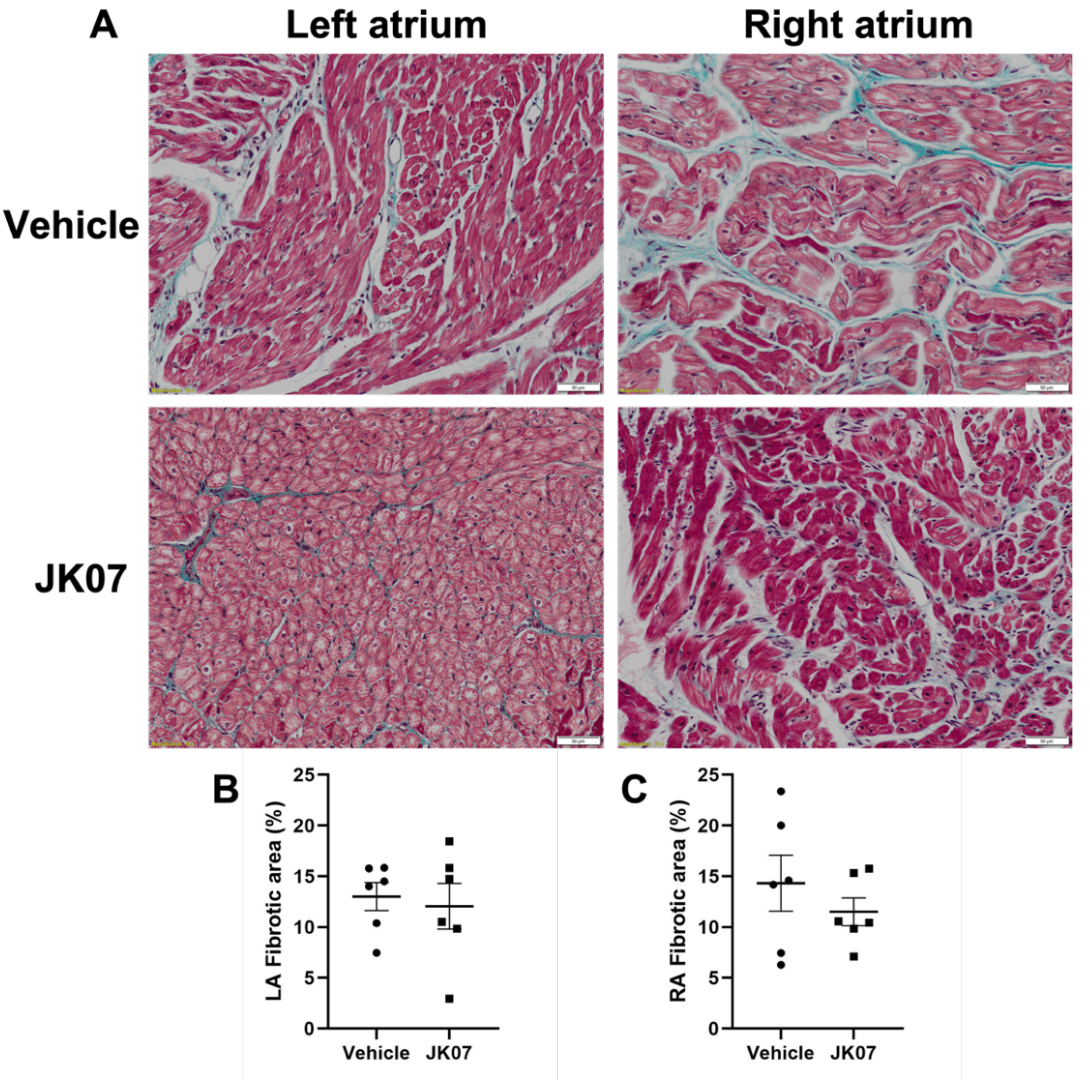


Figure 8: Fibrosis in the left and right atrium*. A) representative images of Masson's trichrome staining at 20x enlargement, scale bar = 30 mcm. B+C) Quantification of fibrosis shows no difference in fibrosis between the Vehicle and JK07 groups in the left and right atrium.*

Discussion

This study was the first ever to test the efficacy of JK07 for the treatment of atrial myopathy and atrial fibrillation in a large animal model. Overall, JK07 was well tolerated as no side effects were observed with the previously determined dose of 0.3 mg/kg (Chapter 4).

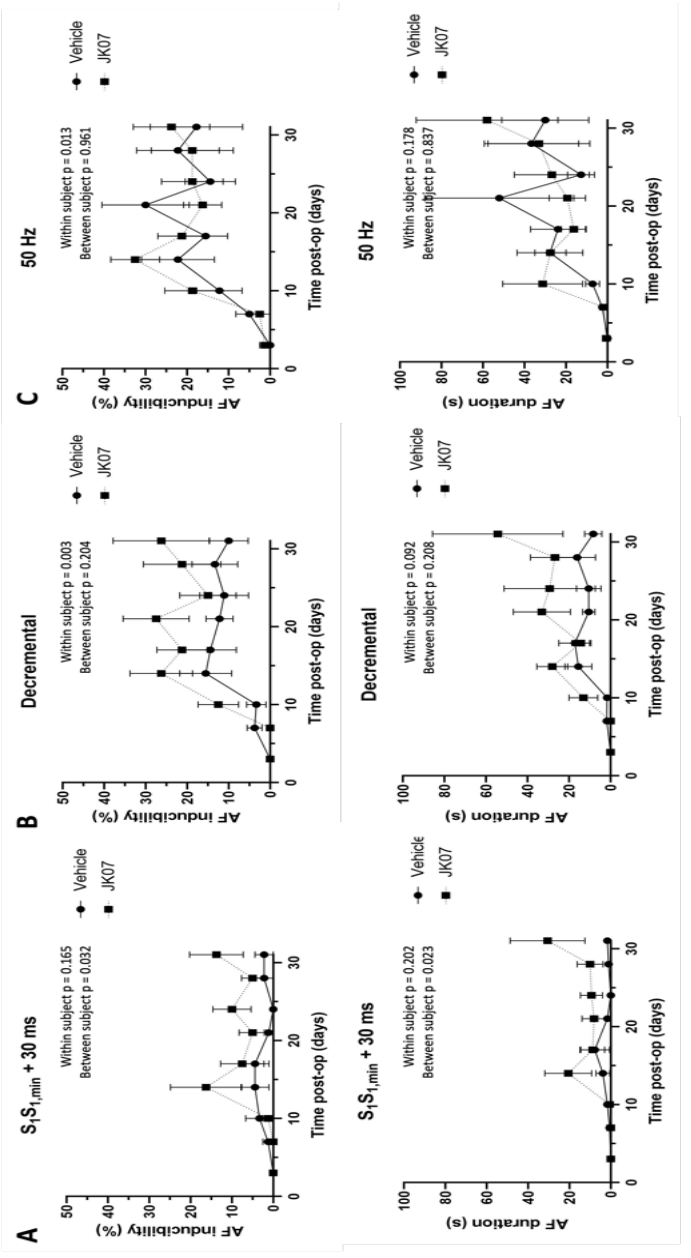
By using the novel technique with two pacemakers, we encountered no more spontaneous lead externalization, and all leads could be used until the end of the experiment. However, over the duration of the experiment, a gradual increase was seen in impedance and voltage threshold (Figure 3). These have been described before as a species-specific property for pigs, forming fibrotic encapsulations around foreign material. This could be a limiting factor for longer experiments but no animal in our experiments showed loss-of-capture. [1]

The new pacing protocol showed a similar temporal evolution of AF inducibility and duration compared to the previous protocol. Pacing episodes in this protocol were much shorter in duration (20 or 5 s, in contrast to 60 s previously), which allowed for more bursts in each animal. Furthermore, since three different kinds of bursts were applied with increasing "aggressiveness" for induction of AF, this created the opportunity to better differentiate between different groups.

Surprisingly, animals treated with JK07 showed decreased $S_1S_{1,min}$ compared with Vehicle, as well as slightly lower heart rate and possibly lower excitability, as measured by a trend towards higher LA voltage thresholds with equal impedance and a trend towards slower impulse conduction. These effects are contradictory to the anticipated results and remain enigmatic.

No difference was observed between the JK07 and Vehicle group regarding AF inducibility or AF duration. JK07 was unable in this model to reverse inflammation-induced ERP shortening (as measured by $S_1S_{1,min}$) and fibrosis. A possible explanation for this is that the inflammatory stimulus in this model (sterile talcum and gauze on the atria) was so strong and irreversible that the endpoint of atrial myopathy was deterministic and no drug could interfere with the inflammation caused by the gauze. Since it was thought that the model was not the right model for pharmacologic testing after all, we decided to test the therapeutic effect of JK07 in a different model with a more reversible stimulus.

Supplement



Supplementary Figure 1: AF inducibility and duration among various burst pacing techniques. A) *AF inducibility (upper) and AF duration (lower) in the $S_1S_{1,min}$ + 30 ms protocol;* ***B)*** *AF inducibility (upper) and duration (lower) in the decremental pacing protocol;* ***C)*** *AF inducibility (upper) and duration (lower) in the 50 Hz burst protocol. Mean ± SEM.*

References

1. Dosdall DJ, Ranjan R, Higuchi K, Kholmovski E, Angel N, Li L, Macleod R, Norlund L, Olsen A, Davies CJ, et al. Chronic atrial fibrillation causes left ventricular dysfunction in dogs but not goats: experience with dogs, goats, and pigs. *Am J Physiol Heart Circ Physiol*. 2013;305:H725-731. doi: 10.1152/ajpheart.00440.2013

Chapter 6:

JK07 as a therapeutic strategy for atrial myopathy in a porcine DOCA model

Background

With the experiment described in Chapter 5, it was not possible to either prove or reject our hypothesis that by counteracting atrial fibrosis, vulnerability to AF induction would be reduced. JK07 was unable to reduce fibrosis as well as AF inducibility. We speculated that this was most likely related to the specific characteristics of the disease model, in which atrial fibrosis was induced in an almost irreversible fashion, or at least too aggressively for a drug to interfere with. Therefore, we opted for an alternative large animal model.

It has been previously described that deoxycorticosterone acetate (DOCA) slow-release pellets, implanted in pigs, induce hypertension (DOCA is an aldosterone precursor and analog), atrial fibrosis, atrial dilation, and increased vulnerability towards AF induction. [1-5] The DOCA model is clinically relevant since arterial hypertension is a comorbidity in 80% of all patients with AF. [6,7] We found no publications where the DOCA model was used to test therapeutic strategies, e.g to treat AF, but we estimated that this model was less aggressive and more reversible as compared to the sterile pericarditis model.

Therefore, we tested the efficacy of JK07 to prevent atrial fibrosis and to decrease vulnerability to AF induction. Furthermore, we set up a multi-electrode array (MEA) to measure conduction velocity, and cardiomyocyte nuclei were isolated from the left atrial tissue and analyzed with RNA sequencing, to investigate changes in the expression profile with emphasis on ion channels.

Materials and Methods

All experiments were approved by University of Antwerp Ethical Committee for Animal Testing (file number 2019-29) and were conform with the ARRIVE guidelines, with the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, with the Belgian Royal Decree of 2013, and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animals were kept on a standard chow and water *ad libitum* at a constant temperature of 22°C and humidity of 50% in a 12 h controlled light/dark cycle.

Minipig model of atrial myopathy

Eighteen castrated male Aachener minipigs, six months old, (Carfil, Oud-Turnhout, Belgium) were randomly divided into three groups. A first group (CTRL) did not receive therapeutic intervention. In the other minipigs, pellets with sustained release of deoxycorticosterone acetate (DOCA, 100 mg/kg over 60 days, Innovative Research of America) were implanted. Pigs with DOCA pellets were randomized to receive either vehicle (once a week; DOCA group) or JK07 (0.3 mg/kg, IV infusion over 30 minutes, once a week; DOCA + JK07 group) treatment.

Before each treatment, minipigs were sedated[8] and weighed, and a cardiac ultrasound (EPIQ 7C, Philips Medical) was performed as described in Chapter 5. At the end of the study period, invasive measurement of arterial blood pressure and electrophysiology testing were performed. Afterwards, minipigs were euthanized with pentobarbital 1 g (IV). See Figure 1 for the treatment timeline.

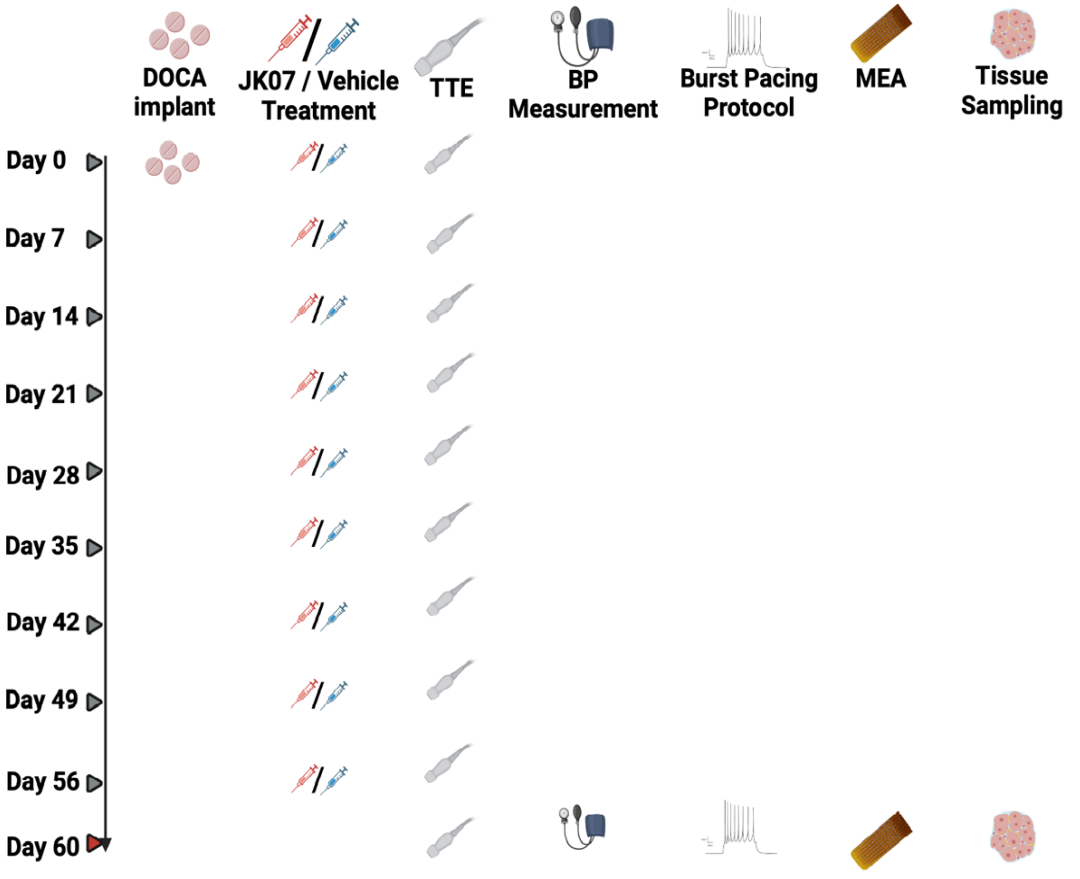


Figure 1: Timeline of treatment for DOCA and DOCA+JK07 groups. *On day 0, The animals received DOCA pellets (100 mg/kg) with continuous release over 60 days. Weekly treatment with either JK07 or its vehicle started on day 0. CTRL pigs did not receive DOCA or any treatment. All animals were sacrificed at day 60 after examinations including BP measurement, electrophysiology testing with a burst pacing protocol, placement of a MEA on the atria to make conduction velocity maps and sampling of atrial tissue for histology and RNA sequencing. BP = blood pressure, DOCA = deoxycorticosterone acetate, MEA = multi-electrode array.*

Electrophysiology testing

An 8 French sheath was inserted into the femoral vein. Next, a decapolar catheter (Biosense Webster) was placed on the right atrial free wall under fluoroscopic guidance. Epicardial electrograms (EpiEGs) were measured with an Axiom Sensis XP electrophysiology system (Siemens, Germany), and cardiac pacing was performed with a programmable electrical stimulator (Micropace). Pacing was performed at twice the voltage threshold. $S_1S_{1,min}$ was measured as previously described. [8] Five different programmed electrical stimulation (PES) protocols were sequentially executed, all

consisting of 10 episodes (50 episodes total). In the first protocol, pacing was performed during 20 s at a fixed cycle length (CL) of $S_1S_{1,min}$ + 30 ms. In the second protocol, pacing was performed during 20 seconds, starting at a CL of 20 msec above $S_1S_{1,min}$, with a gradual decrement until $S_1S_{1,min}$. In the third, fourth, and fifth protocol, pacing was performed at 33 Hz during respectively 5 s, 10 s and 15 s. After each PES protocol, the duration of the AF episode was recorded. The threshold duration for AF was defined as an episode lasting ≥ 5 s.

Multi-electrode array and activation maps

A multi-electrode array (MEA) was designed in-house and comprised of 8 rows of 16 (total of 128) electrodes in a rectangular configuration and with an inter-electrode distance of 2.5 mm. The MEA was connected to a Sensis Axiom XP electrophysiology system. This allows to simultaneously record 64 unipolar unipolar epicardial electrograms (EpiEGs) in a checkered pattern to cover maximal area (the software was limited to 64 simultaneous recordings).

In sedated state, pigs were anesthetized with propofol IV (2 mg/kg, bolus) and alfentanil (continuous infusion rate of 1.4 mcg/kg/min) and intubated. After sternotomy, the pericardium was opened, and the MEA was placed on the left atrial epicardial surface to record EpiEGs for 5 s during sinus rhythm. A schematic overview of the experimental setup is presented in Figure 2. EpiEG tracings were interpreted semi-automatically using customized software, [9,10] that determined local activation times (LAT) using omnipolar technology (OT). [11] From the temporal differences in LAT, conduction velocity (CV) vectors and maps were derived. CVs > 4 m/s were excluded because not physiologically plausible. In a similar way, voltage maps were derived from the root main square (RMS) of the electrograms.*

* We want to kindly thank Arthur Santos Bezerra, Eike Wülfers and Nele Vandersickel from the University of Ghent for the analysis of the data.

Figure 2: MEA setup. *From left to right: in an open-chest state, the MEA was placed on the left atrial epicardium. The MEA was able to simultaneously record 64 unipolar electrograms. From differences in local activation times, conduction velocity maps were derived. MEA = multi-electrode array*

Histology

Immediately after euthanasia, a vascular clamp was placed on the superior and inferior vena cava and the aorta. The heart was excised, and tissue samples were taken from left and right atrial appendages, and atrial free walls. Tissue samples were first fixed in 4% buffered formalin and transferred to isopropanol 60% after 24 h until embedded in paraffin. Samples were stained with Masson's trichrome and tissue fibrosis was quantified on five random high-power field (40x magnification) images. Fibrosis was quantified using AFAT software as a percentage of blue pixels divided by the total amount of non-white pixels. [12]

Plasma samples

Blood samples were taken on EDTA tubes and centrifuged at 800 x g for 10 minutes. Plasma was collected and stored in cryogenic tubes at -80°C. These can be used for further research. However, no analyses were done on these samples yet.

RNA sequencing on isolated cardiomyocytes[†]

Cardiomyocyte sorting

Cardiomyocyte nuclei were isolated from the left atrial free wall as described previously with modifications. [13,14] Frozen heart tissue (~100 mg) was suspended in Dissociation

[†] We want to kindly thank Yile Fu and Llewelyn Roderick from the University of Leuven for the execution of these experiments and the analysis of the data.

Buffer supplemented with Protease Inhibitor Cocktail and homogenized using a gentleMACS dissociator (Myltenyi). The suspension was filtered, and the homogenate was centrifuged to recover the nuclei, which were then resuspended in Sucrose Buffer. After centrifugation, the pellet was washed with Wash Buffer and incubated in Staining Buffer containing specific antibodies and fluorescent markers. Subsequently, 200,000 DAPI+, PLN+, PCM1+ nuclei were purified by flow cytometry and collected in a buffer solution. The sorted nuclei were harvested by centrifugation and the supernatant was removed.

Nuclear RNA isolating and sequencing

Nuclear RNA extraction from sorted nuclei was performed using Trizol LS reagent (InVitrogen). Following phase separation by adding a solution of Chloroform in Isoamyl Alcohol, the upper aqueous phase containing RNA was collected and mixed with Sodium Acetate, Linear Acrylamide, and Isopropanol, and then incubated overnight. After centrifugation, the RNA pellet was washed with ethanol and air-dried before being resuspended in DNase/RNase-free water. Genomic DNA was removed using the TURBO DNA-free Kit. RNA concentration was quantified using a Qubit fluorometer. Subsequently, sequencing libraries were prepared and subjected to paired-end sequencing using a NovaSeq 6000.

Statistical analysis

Statistical parameters including descriptive statistics (mean and standard error of the mean), significance (p) are reported in the text and figures. Groups were compared using either Student's independent samples t-test or analysis of the variance (ANOVA) followed by Tamhane's *post-hoc* test. For group comparison with datasets consisting of different timepoints, repeated measures ANOVA was used, and non-sphericity was corrected with Greenhouse-Geisser correction. Crosstabulation (chi-squared (χ^2) test) was used to examine the relationship between categorical variables. All analyses were performed using SPSS software (version 28.0.1.1). Graphs were made using GraphPad Prism (version 9.5.1).

RNAseq data analysis

Quality and adapter trimming of sequencing reads was performed prior to mapping to remove low-quality reads and adapter contaminations. RNA-seq data were mapped to the pig genome (Sscrofa11.1) using HISAT2. [15] PCR duplicates were removed using SAMtools. [16] Owing to the high number of intronic reads arising from unspliced RNAs in nuclear RNA-seq data, [17,18] we used intronic and exonic regions of coding genes for gene expression analysis. Mapped RNA fragments were further processed using StringTie[19] to calculate CPM values as an estimate of transcript expression. edgeR[20] was used for differential gene expression. A q-value of <0.05 was considered as significant. Genes with expression values of <3 CPM in all the compared groups were excluded from the differential gene expression analysis. To identify enriched GO terms in the category "biological process", a hypergeometric test in the clusterProfiler package was used[21] with the Benjamini and Hochberg procedure. Terms enriched with an adjusted p-value < 0.05 were considered significant.

Results

One minipig (DOCA group) died because of technical issues during catheterization (accidental coronary air embolus during a non-related additional experiment). Mean arterial pressure was significantly higher in minipigs treated with DOCA (142 ± 5 mmHg) compared to CTRL (105 ± 3 mmHg, $p < 0.0001$). JK07 did not influence arterial pressure in minipigs implanted with DOCA pellets (132 ± 6 mmHg, DOCA + JK07; Figure 3A). Animals showed no adverse signs or discomfort. Body weight of DOCA (28.2 ± 1.9 kg) and DOCA + JK07 (28.4 ± 2.8 kg) animals did not differ ($p = 0.906$; Figure 3B).

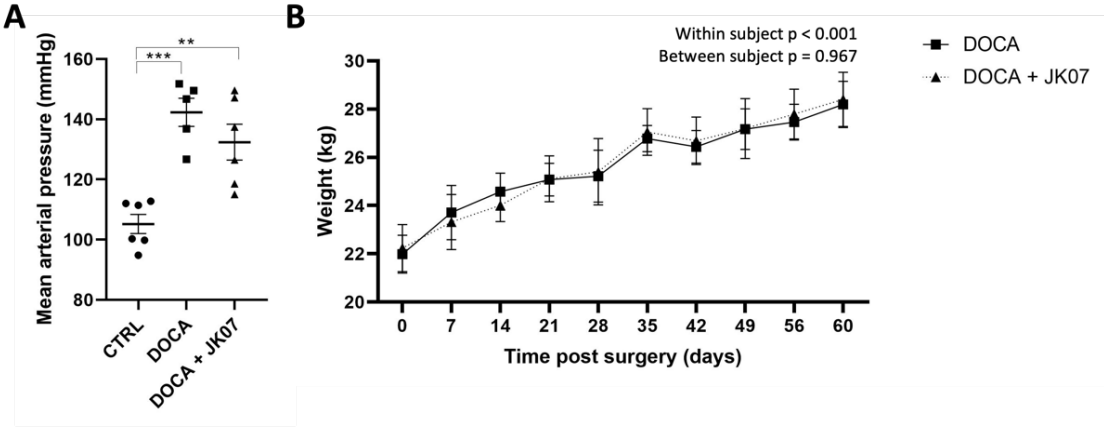


Figure 3: General characteristics. A) *DOCA induces significant hypertension, which is unaffected by JK07. This validates the model.* ***B)*** *No difference in weight was observed between both DOCA treated groups. Data presented as mean ± SEM.*

Electrophysiology

There was no significant difference between the groups in heart rate (92 ± 5 bpm for CTRL; 89 ± 4 bpm for DOCA and 84 ± 4 bpm for DOCA + JK07; Figure 4A) as well as $S_1S_{1,min}$ (163 ± 10 ms for CTRL; 156 ± 7 ms for DOCA and 162 ± 10 for DOCA + JK07; Figure 4B). AF inducibility per animal showed a trend towards increase in DOCA compared to CTRL (26 ± 11% vs. 3 ± 3%; p = 0.069) and likewise, a trend was also observed for decrease in DOCA + JK07 compared to DOCA (3 ± 1% vs. 26 ± 11%; p = 0.13). Analyzed per group, AF inducibility in the DOCA group was significantly higher than CTRL (66/250 vs. 9/300 PES trains; p < 0.0001), and JK07-treated DOCA pigs (66/250 vs. 8/300 PES trains; p < 0.0001; Figure 4C). AF duration in the DOCA group (11.5 ± 1.9 s) was significantly higher than in the CTRL (0.8 ± 0.3 s; p < 0.0001) and the DOCA + JK07 group (1.0 ± 0.4 s; p < 0.0001; Figure 4D). Waterfall plots (Figure 4E and 4F) indicate consistency of the effect throughout all animals.

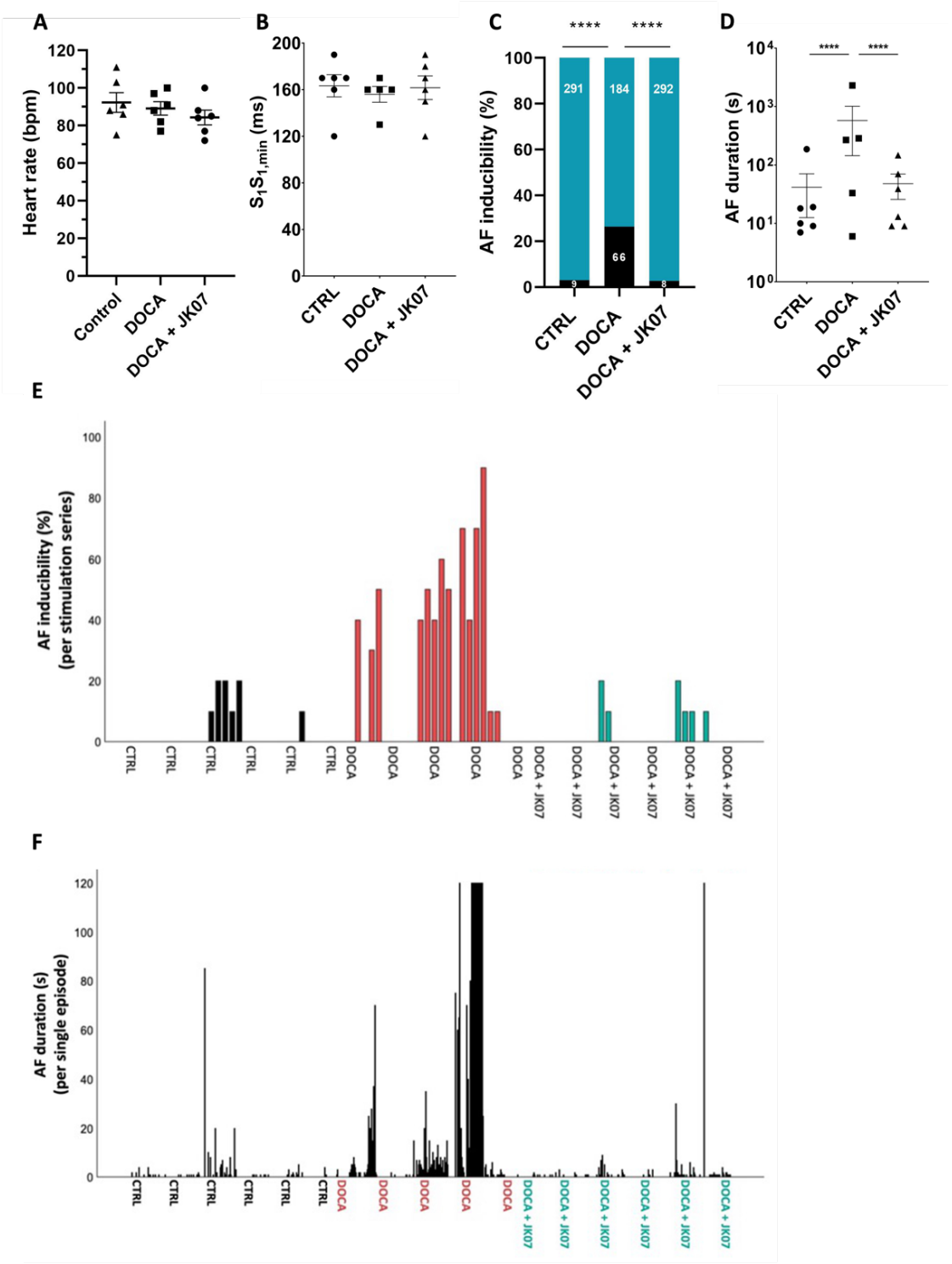

A
Heart rate (bpm)
120
100
80
60
40
20
0
Control
DOCA
DOCA + JK07
B
$S_1S_{1,min}$ (ms)
200
160
120
80
40
0
CTRL
DOCA
DOCA + JK07
C
****
****
AF inducibility (%)
100
80
60
40
20
0
291
184
292
66
9
8
CTRL
DOCA
DOCA + JK07
D
****
****
AF duration (s)
10^4
10^3
10^2
10^1
10^0
CTRL
DOCA
DOCA + JK07
E
AF inducibility (%)
(per stimulation series)
100
80
60
40
20
0
CTRL
DOCA
DOCA + JK07
F
AF duration (s)
(per single episode)
120
100
80
60
40
20
0
CTRL
DOCA
DOCA + JK07

Figure 4: Electrophysiology results. A+B) *No difference was observed in basic heart rate* ***(A)****, as well as in* $S_1S_{1,min}$ ***(B)*** *between the three groups.* ***C)*** *AF inducibility is significantly increased in the DOCA group compared to CTRL and DOCA + JK07. (Black = AF inducible for ≥ 5 s; Blue = AF not inducible or inducible for < 5 s, numbers in bars indicate number of episodes)* ***D)*** *AF duration per animal was significantly increased by DOCA, which could be prevented by JK07.* ***E)*** *Plot of AF inducibility per stimulation series.* ***F)*** *Plot of AF duration per pacing episode per animal. Error bars indicate Standard Error of the Mean. **** = p < 0.0001; AF = atrial fibrillation, DOCA = deoxycorticosterone acetate.*

The inducibility by each different pacing modality is compared in Figure 5, where AF duration and inducibility are increased in the DOCA group compared to the CTRL and DOCA + JK07 in every pacing modality.

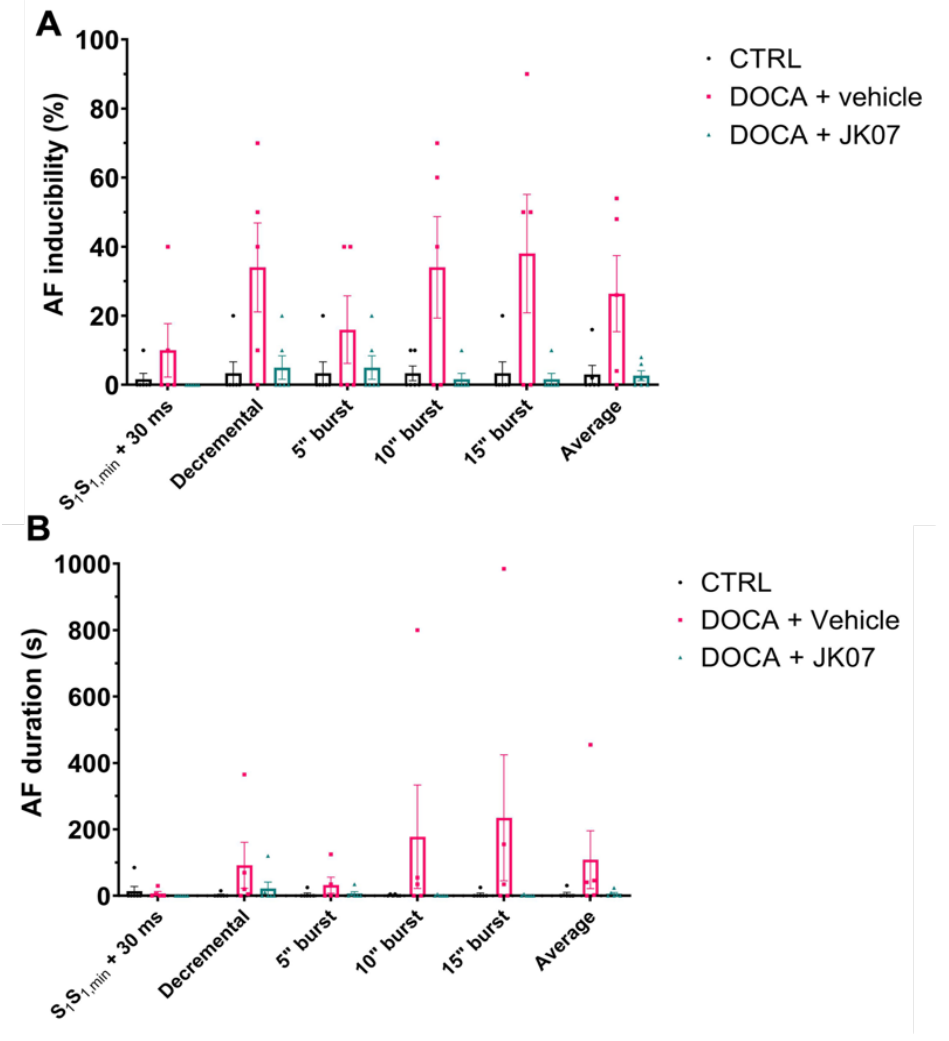


Figure 5: Comparison of pacing modalities. *Regardless of the pacing modality, AF inducibility and duration are highest in the DOCA group. The pacing protocols increase in % of inducible attempts, except for decremental pacing, which has higher inducibility and duration than the 5" burst.*

Compared to the CTRL group, a reduction in conduction velocity was observed in the DOCA group (1.20 ± 0.03 m/s vs. 1.55 ± 0.03 m/s, $p < 0.0001$) and the DOCA + JK07 group (1.20 ± 0.03 m/s vs. 1.36 ± 0.03 m/s, $p < 0.0001$; Figure 6A). Compared to CTRL, an average increase in voltage was observed in the DOCA group (2.45 ± 0.09 mV vs. 1.30 ± 0.05 mV, $p < 0.0001$) which was attenuated in the DOCA + JK07 group (2.45 ± 0.09 mV vs. 1.95 ± 0.05, $p < 0.0001$; Figure 6B).

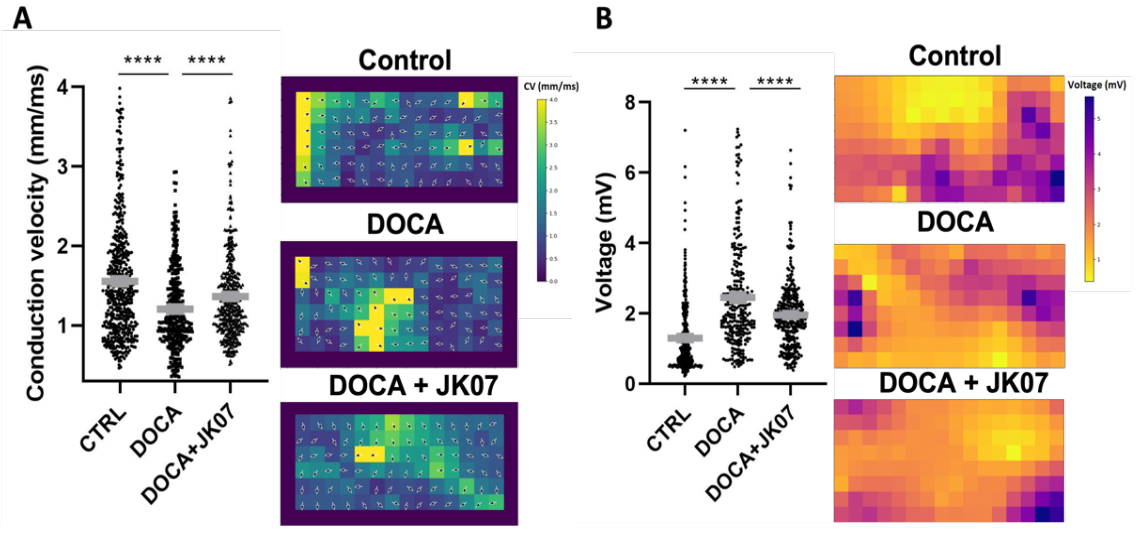


Figure 6: Results from the multi-electrode array (MEA). A) *Left: JK07 reversed the DOCA-induced conduction slowing. Right: representative conduction velocity maps.* ***B)*** *Left: JK07 partially reversed the DOCA-induced voltage increase. Right: representative voltage maps. Data presented as mean ± SEM.*

Echocardiography

Echocardiographic parameters at day 60, normalized to body weight, are displayed in Table 1. Representative images are displayed in Figure 7. Diastolic volume of the left atrium was significantly increased with DOCA group, while JK07 showed a trend to partially reverse this effect (Table 1). DOCA-induced hypertrophy was unaffected by JK07. The LV contractile parameters LVEF and LV strain also did not differ between groups. Figure 8 shows the evolution of the echocardiographic parameters over time.

	CTRL	**DOCA**	**DOCA + JK07**
LA diastolic volume, corrected (ml/kg)	0.51 ± 0.09	0.78 ± 0.15 **	0.64 ± 0.15
IVSd, corrected (mm/kg)	0.26 ± 0.03	0.31 ± 0.03 *	0.29 ± 0.04
LVPWd, corrected (mm/kg)	0.25 ± 0.03	0.35 ± 0.03 ***	0.33 ± 0.08
LVEF (%)	76.0 ± 5.4	76.2 ± 2.6	77.3 ± 3.4
LV strain (%)	-31.1 ± 4.0	-32.6 ± 1.7	-33.5 ± 4.5

Table 1: Echocardiographic measurements of minipigs on day 60. *Data are presented as mean ± standard deviation. LV = left ventricle, IVSd = interventricular septum thickness in diastole, LVPWd = left ventricular posterior wall thickness in diastole, LA = left atrium. * : p < 0.05; ** : p < 0.01; *** : p < 0,0001. DOCA was compared to CTRL and DOCA + JK07 to DOCA.*

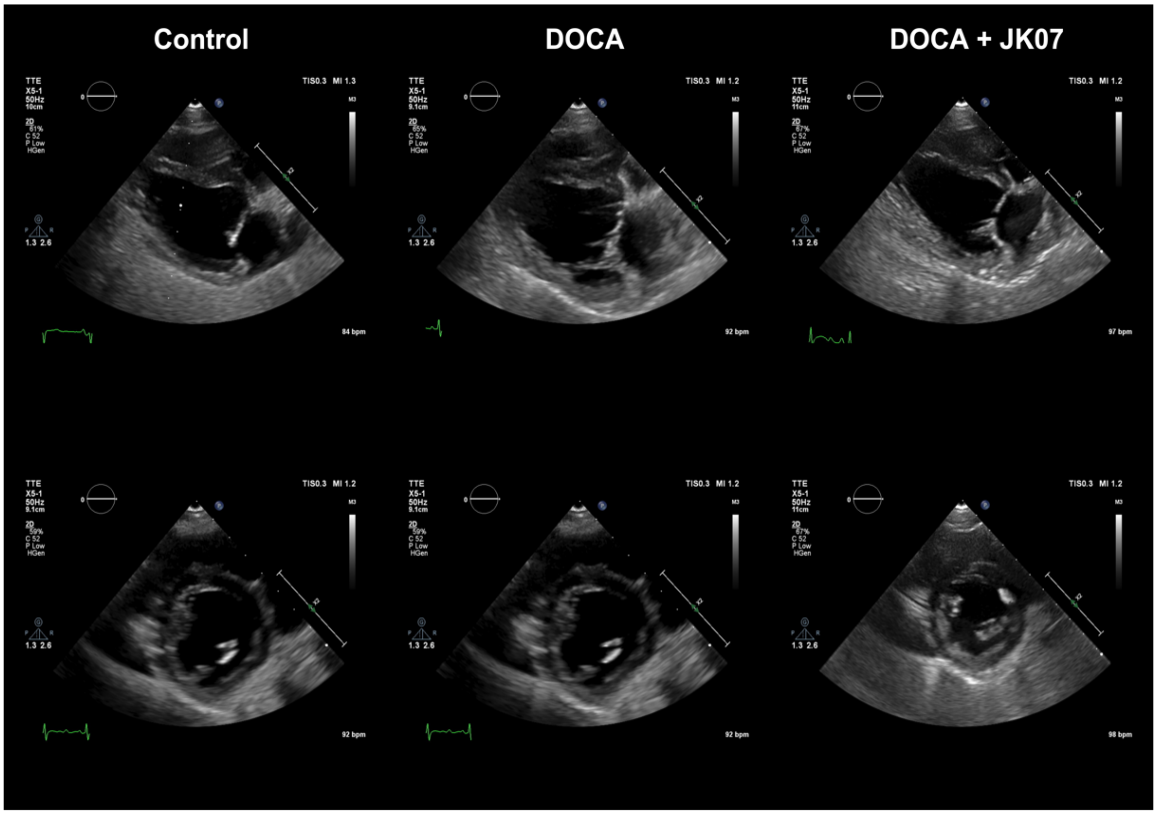


Figure 7: Representative ultrasound images *of each group in parasternal long axis-like (upper) and parasternal short axis-like (lower) views.*

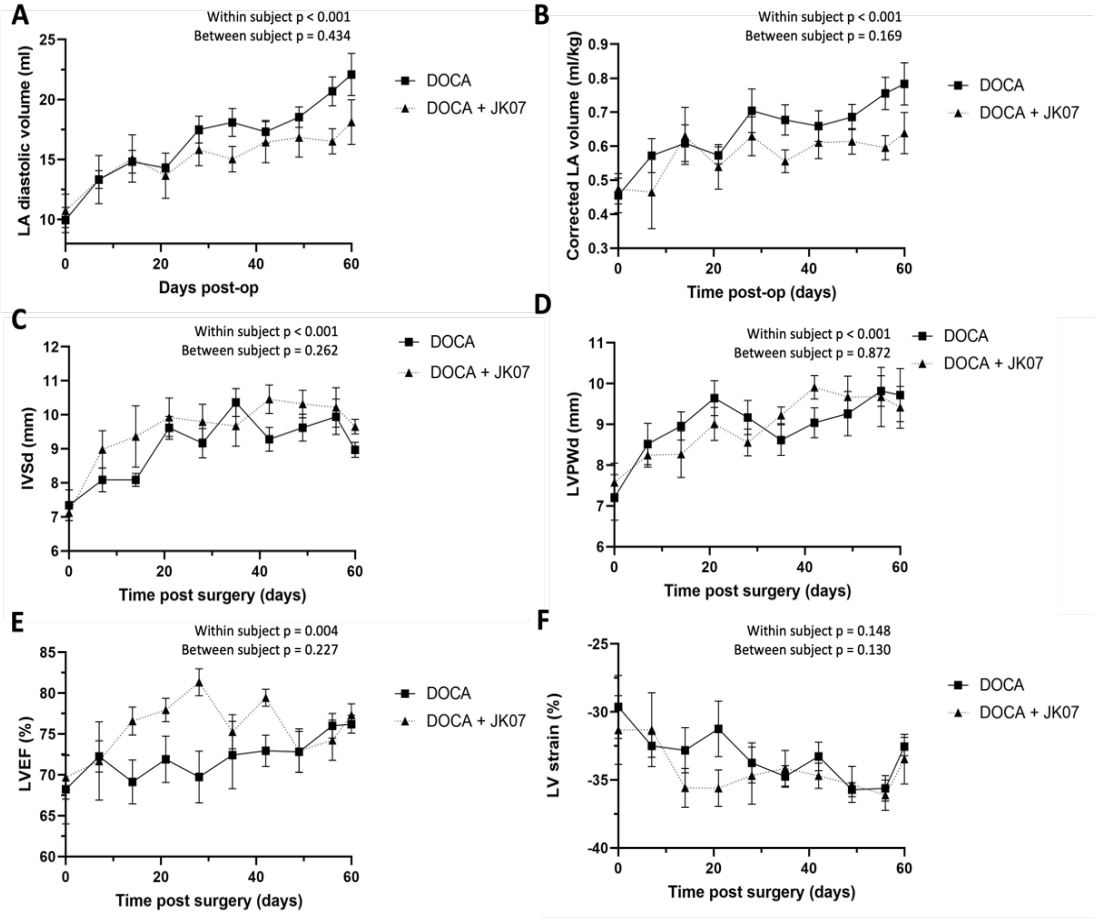


Figure 8: Evolution of echocardiographic parameters.

Effect of JK07 on atrial fibrosis

Atrial interstitial fibrosis in the left atrial free wall was significantly higher in the DOCA group compared to CTRL (12.2 ± 2.1% fibrotic area vs. 5.1 ± 0.6% fibrotic area p = 0.009); this increase was completely prevented by JK07 (12.2 ± 2.1% fibrotic area vs. 5.5 ± 1.5%; p = 0.03). Similar results were found at all sampling sites, i.e., in the left atrial appendage, right atrial free wall, and right atrial appendage (Figure 9A).

In contrast to interstitial fibrosis, there was no difference in perivascular fibrosis between the groups (Figure 10).

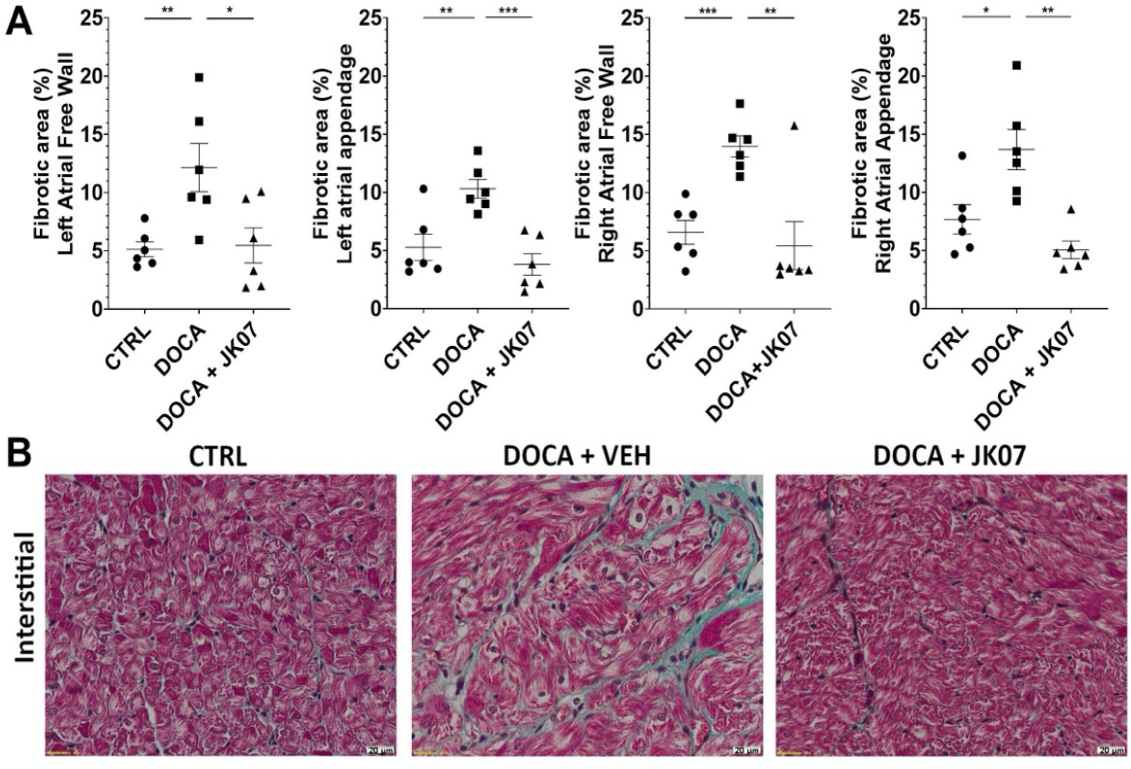


Figure 9: *Atrial interstitial fibrosis. A) Fibrotic area is significantly increased at all sampling sites in the DOCA group compared to CTRL and DOCA + JK07. B) Representative images, Masson's trichrome staining. Data presented as mean ± SEM.*

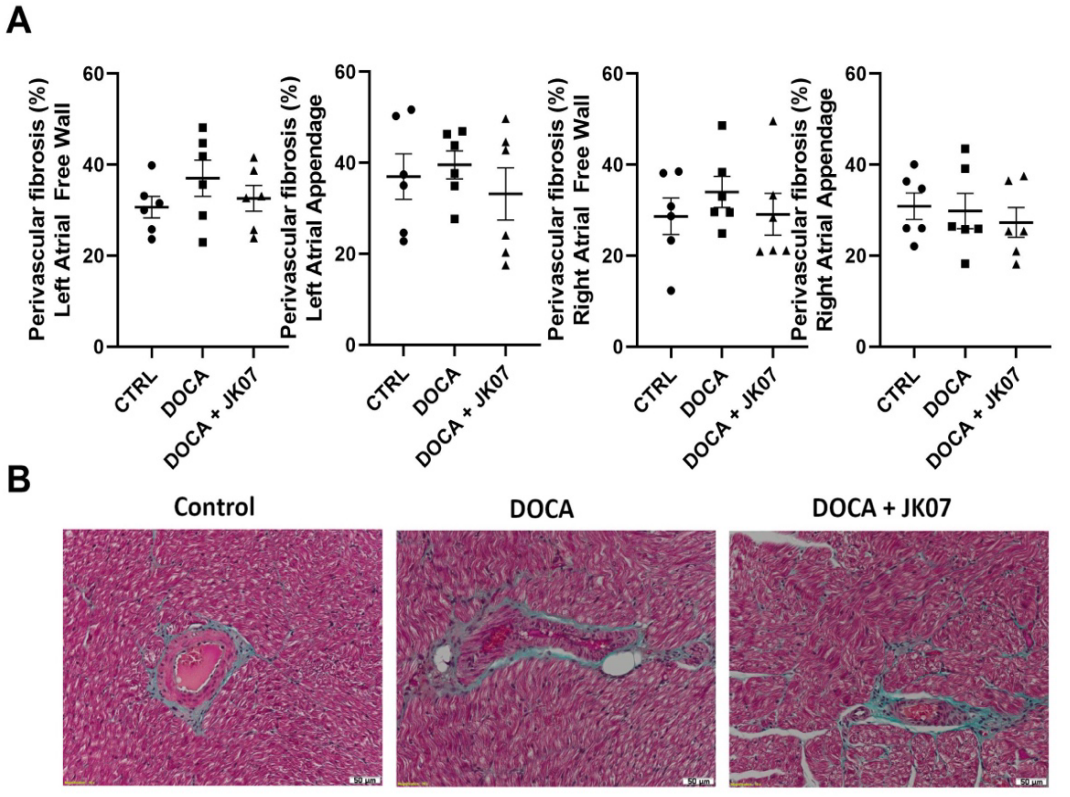


***Figure 10: Atrial perivascular fibrosis**. **A)** No difference in perivascular fibrosis was observed between the groups at any atrial sample site. **B)** Representative images, Masson's trichrome staining. Data presented as mean ± SEM.*

Transcriptomic changes

To gain insight into the remodeling of CM of the pig atria during AF and the effects thereon of JK07, RNA-Seq analysis was performed on CM isolated from the free wall of the pig left atria. For these experiments, three groups were analyzed (control group, DOCA group, DOCA + JK07 group). CM nuclei were purified from the atrial tissue by fluorescence-activated sorting using antibodies against pericentriolar material 1 (PCM1) and phospholamban (PLN) to ensure CM specificity and the absence of other cardiac cell types (Figure 12). [22-24] Differentially expressed gene (DEG) analysis identified 697 significantly upregulated and 919 downregulated genes in CM from the DOCA-induced AF samples in comparison to non-treated controls (log2-fold change ≥ 1, p adj < 0.05) (Figure 11A). Given their importance in atrial remodeling during AF, we specifically examined the expression of ion channel genes. Notably, genes encoding ion channels including potassium channels (KCNJ3, KCNJ5) and calcium regulators (CALM1) were found to be differentially expressed in DOCA (Figure 1A). A comparison of gene expression between DOCA and JK07 group revealed 473 genes to be significantly upregulated, whereas 403 genes were downregulated (log2-fold change ≥ 1, p adj < 0.05) (Figure 11B). Significantly altered ion-channel genes including chloride channel (CLCF1) and calcium regulators (PLN) were observed (Figure 11B). Gene ontology (GO) analysis of genes differentially expressed between control and DOCA groups were involved in p38MAPK cascade (Figure 11C), whereas genes significantly altered between the DOCA and the JK07 group were associated with ERBB signaling and the stressed-activated MAPK cascade (Figure 11D). To determine whether JK07 was reversing the remodeling of the atrial transcriptome associated with AF induced by DOCA, we examined the overlap between genes upregulated by DOCA and downregulated by JKO7 as well as those genes downregulated by DOCA and increased by JK07. This analysis revealed an overlap of 328 of the up-regulated DOCA DEGs with the down-regulated DEGs of JK07 and 456 of the down-regulated DOCA DEGs with those up-regulated DEGs by JK07 (Figure 11 E-F). Change in ion channel gene expression in response to DOCA and treatment with JK07 is shown in the hierarchical gene clustering (Figure 12).

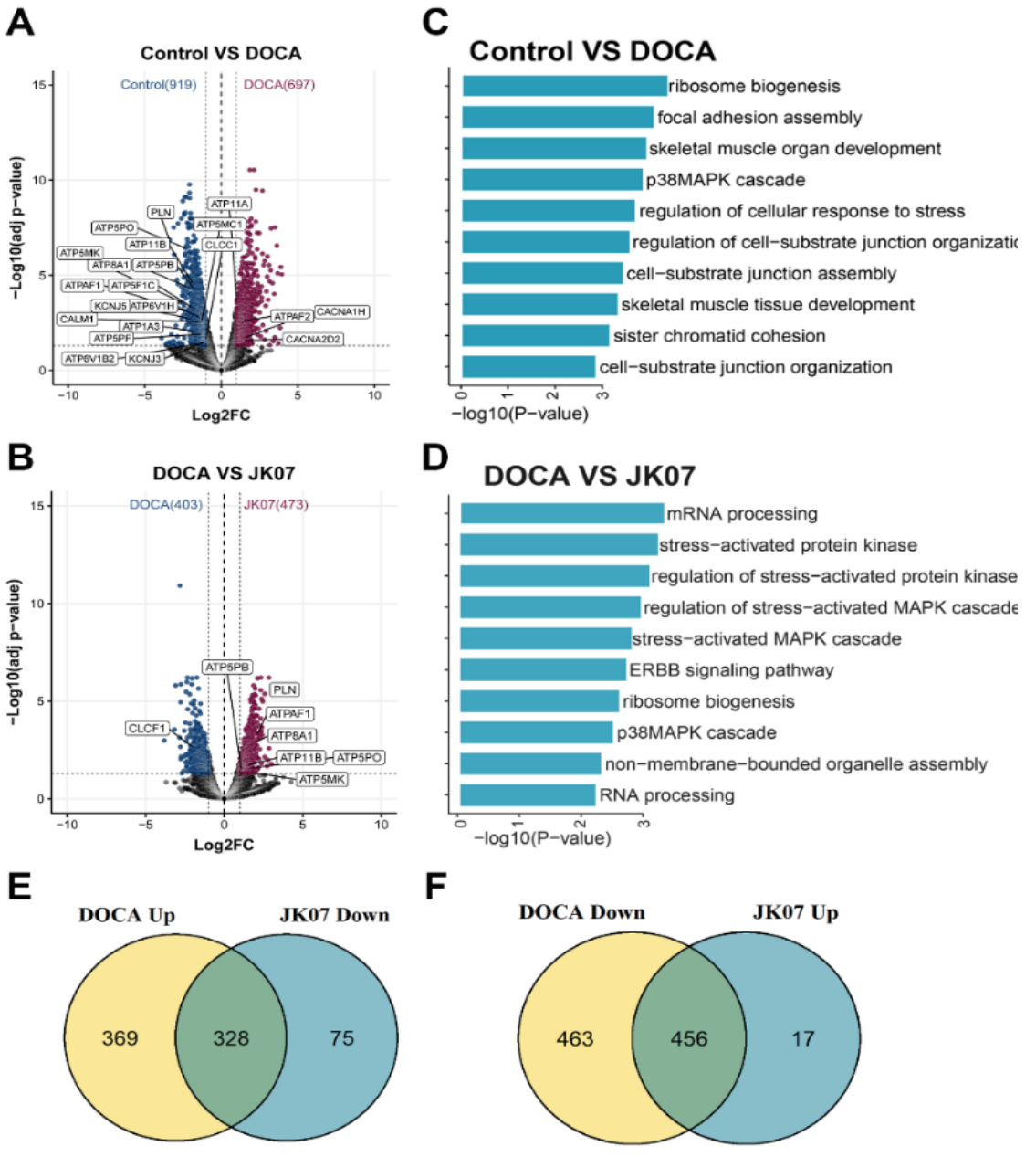


Figure 11: Transcriptomic changes in CM during induction of AF with DOCA and response to treatment with JK07. (A-B) *Volcano plot showing gene expression changes (edgeR, p adj < 0.05) in response to DOCA (n = 2-5) and to JK07 (2-3), respectively. Vertical dashed lines indicate an absolute fold change (log2) ≥ 1. Horizontal dashed lines indicate a significance cut-off of adj-p value < 0.05. Significantly altered ion channel genes are highlighted.* ***(C-D)*** *Gene ontology (GO) biological process terms between DOCA and JK07, respectively.* ***(E-F)*** *Venn diagram showing overlaps of the up- or down-regulated genes between the response to DOCA and JK07.*

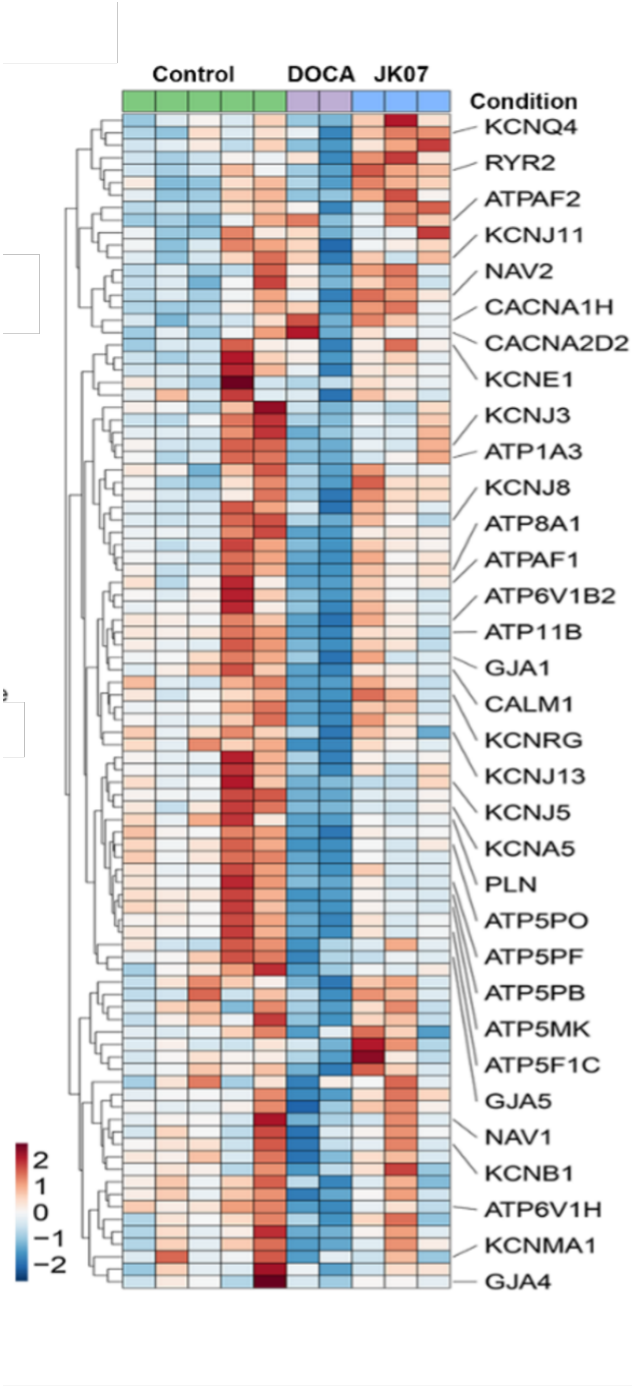


Figure 12: *Heatmap showing log fold-change estimates for ion channel genes in the control, DOCA, and JK07 groups.*

Discussion

This study demonstrates the antiarrhythmic effects of JK07, an agonist of the ERBB4 receptor. More specifically, we showed that JK07 reduces the inducibility of AF in a porcine DOCA model. The mechanism of antiarrhythmic action is — unlike any other antiarrhythmic drug currently available — not due to prolongation of the effective refractory period (ERP), as demonstrated by the lack of effect of JK07 on $S_1S_{1,min}$. A possible antiarrhythmic mechanism is the JK07-mediated attenuation of DOCA-induced conduction slowing, as shown in Figure 6A. This effect is likely related to the simultaneous effects on atrial fibrosis and dilation.

In addition to its antifibrotic effects, JK07 reverses electrical remodeling of cardiomyocytes, as shown in Figure 11. Transcriptomic changes induced by DOCA align with those observed in AF, including downregulation of connexins, Ca^{2+} regulators, Na^{+} and Ca^{2+} channels[25,26] (see Chapter 1). These alterations are hierarchically clustered through the activation of the p38-MAPK cascade, which has previously been associated with electrical and structural remodeling in atrial myopathy. [27,28] A potential antiarrhythmic mechanism of ERBB4 stimulation involves inhibition of p38-MAPK, [29] offering a plausible explanation for the reversal of DOCA-induced electrical remodeling. Furthermore, given MAPK's established role in atrial fibrosis, [30] this pathway could connect JK07's antifibrotic properties and its capacity to reverse electrical remodeling.

Another striking detail about the RNAseq analysis is the ability of JK07 to reverse the downregulation of multiple ATP-sensitive channels. This may indicate increased cellular availability of ATP, and therefore enhanced glucose metabolism. Previous studies have already demonstrated insulin-independent increase in GLUT-4 expression in skeletal muscle secondary to ERBB4 stimulation, [31] which may be a possible mechanism in the cardiomyocyte for mitigation of metabolic remodeling.

These findings are clinically relevant because cardiac anatomy and physiology of pigs is very similar to humans. [32] In addition, a large gap in the therapeutic options for AF exists because the current antiarrhythmic drugs rely on prolonging ERP (with increased risk of Torsades de pointes) or slowing of conduction (with increased risk of reentrant proarrhythmia), are marginally effective, and do not target the underlying atrial myopathy. Pulmonary vein isolation (PVI) is a useful tool to help maintain rhythm control, but once atrial fibrosis —as a proxy for atrial myopathy— is present, the success rate of

PVI decreases, [33] and this cannot be attenuated by additional targeted ablation of fibrotic regions. [34] Pirfenidone has been shown to slightly modify atrial fibrosis, but effects on arrhythmogenesis remain unknown. [35] Accordingly, there is no therapy available that targets both electrical and structural atrial remodeling. A recent publication of *Yamaguchi et al.* [36] concluded that the NRG1/ERBB4/ETV1 pathway is correlated with both electrical and structural remodeling. Our study supports that the ERBB4 pathway is involved in pressure overload-related atrial myopathy, is a suitable therapeutic target for treatment of atrial myopathy, and is able to modify both structural and electrical remodeling. In contrast to existing therapies, JK07 does not target a single ion channel, but its effects are more pleiotropic, which appears essential to address the complex and multifactorial atrial remodeling in AF patients. As illustrated in Figure 11, JK07 mitigates approximately half of the transcriptomic alterations induced by DOCA, implying a multifaceted and targeted approach to tackle cardiomyocyte remodeling in atrial myopathy.

A limitation of the current study is that treatment was initiated together with DOCA implantation, implying a more preventive setting. Future research is warranted to prove the ability of JK07 to successfully reverse established atrial remodeling in a curative setting, which is a more frequent clinical scenario. Furthermore, the RNA sequencing analysis zoomed in on isolated cardiomyocytes, which leaves open questions for the effects of respectively DOCA and JK07 on different cardiac cell types. Two paradoxical phenomena remain unexplained: while atrial dilation was present in the DOCA model, this did not affect ERP/ $S_1S_{1,min}$: this points to other factors than atrial stretch-activated ion channels as explanation. [37] Furthermore, the DOCA-treated pigs showed higher voltages on the MEA, which was again attenuated by concomitant JK07 treatment. The anticipated effect was to see on average more low voltage (fibrotic) areas. Currently, we cannot explain these paradoxical effects and more research is warranted to elucidate the exact electrophysiological effects of ERBB4 stimulation.

Conclusion

JK07, a NRG1 fusion protein with increased ERBB4 selectivity, prevents atrial fibrosis, AF inducibility and AF duration *in vivo* in a minipig model of DOCA (hypertension) induced atrial myopathy.

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Chapter 7:

Atrial fibrosis in patients undergoing cardiac surgery

Introduction

The EHRAS classification of atrial myopathy is mainly based on the presence or absence of atrial fibrosis: fibrosis is the main characteristic of EHRAS class II and III. [1] Fibrosis plays a crucial role in the development and maintenance of atrial fibrillation (AF) and is therefore linked to increased AF chronicity and therapy resistance. [2] However, precise assessment of atrial fibrosis requires tissue samples. This section focuses on atrial fibrosis and its correlation with clinical and electrocardiographic parameters.

We assessed atrial fibrosis in samples from both the right and left atrial appendage, obtained during open-chest on-pump cardiac surgery.

Methods

All experimental protocols were approved by the Ethics Committee of the University Hospital Antwerp (file no. B3002021000041). Fifty-four patients who were scheduled for coronary artery bypass grafting (CABG) or surgical aortic valve replacement (AVR) at the University Hospital Antwerp participated in this study after providing written informed consent. Patient selection was conducted randomly and occurred between September 2019 and March 2023: every patient planned for an open-chest, on-pump CABG or AVR procedure was considered, after consent of the patient and the surgeon. History of AF and its subtype were obtained from the patient's medical record. Echocardiographic parameters were obtained from the standard pre-operative ultrasound. During the surgical procedure, a small tissue sample of approximately 2 cm^2 from the right atrial appendage (RAA) was obtained when the incision was made for canulation. This sample was divided into four cryotubes and immediately frozen in liquid nitrogen. The frozen samples were then stored at -80 degrees Celsius. The left atrial appendage (LAA) was sampled at the discretion of the surgeon.

For histological analysis, samples were thawed in a formaldehyde 4% solution and 24 hours later transferred to an isopropanol 60% solution. Next, samples were embedded in paraffin and stained with Masson's trichrome. High power field images with a magnification of 40x were made with an Olympus BX43 microscope. Representative images of patients are displayed in Figure 1. Fibrosis was quantified using AFAT software. [3] Statistical analysis was performed with SPSS software. Data are displayed as mean $\pm$ standard deviation unless otherwise specified. P-values less than 0.05 were considered statistically significant.

Results

Study population

Patient characteristics are summarized in Table 1. The group of patients without AF (n=33) was larger than the group with AF (n=21). A significantly higher proportion of patients in the AF group underwent surgical AVR compared to those without AF. Moreover, a significantly higher percentage of male patients was observed in the AF group compared to the non-AF group. However, patient age, length, or weight did not differ between the two groups. A trend towards a higher prevalence of heart failure was observed in the AF group. No significant differences were detected in the cardiovascular risk profiles of both groups. Significantly more patients in the group without AF used antiplatelet therapy. Conversely, significantly more patients in the group with AF used direct oral anticoagulants (DOACs) and class III antiarrhythmic drugs.

The AF group showed more pre-operative valvular heart disease compared to the non-AF group, with significantly more mitral insufficiency and higher aortic pressure gradients. Additionally, the AF group showed increased left ventricular septum and posterior wall thickness, and a higher left atrial volume index. However, the left ventricular ejection fraction (LVEF) was the same between both groups. Finally, there were no differences in fibrotic area, neither in the RAA nor in the LAA (Figure 1).

Parameter	***No AF group***	***AF group***	***p-value***
No. of patients	33	21 (Par = 14; Pers = 1; Perm = 6)	
Patient characteristics			
Male (%)	67	90	0.046*
CABG (%)	97	57	2×10^{-4} ***
AVR (%)	15	48	0.009 *
Heart failure (%)	24	43	0.151
Age (years)	68 ± 9	69 ± 9	0.833
Length (cm)	170 ± 9	175 ± 11	0.600
Weight (kg)	79 ± 10	88 ± 24	0.055
Risk profile			
BMI (kg/m^2)	27 ± 3	29 ± 8	0.311
Smoker (%)	39	43	0.801
Arterial hypertension (%)	82	86	0.708
Hypercholesterolemia (%)	82	81	0.936

Familial cardiac disease (%)	24	43	0.151
Diabetes (%)	27	19	0.491
Medication use			
Anti-aggregant (%)	76	29	6×10^{-4} ***
DOAC (%)	6	76	1×10^{-7} *
Class I AAD	0	10	0.071
Beta-blocker	58	71	0.304
Class III AAD	0	19	0.009**
Calcium channel blocker	33	43	0.480
Digoxin	0	5	0.206
ACEi/ARB	58	62	0.752
MRA (%)	3	10	0.310
Diuretics	21	48	0.042
Nitrate	24	5	0.061
Statin	73	76	0.777
Echocardiography			
MI (out of 4)	0.6 ± 0.7	1.2 ± 0.8	0.009**
Ao max (mmHg)	16 ± 23	33 ± 31	0.029*
Ao mean (mmHg)	10 ± 15	20 ± 19	0.038*
LVEF Simpson (%)	58 ± 10	57 ± 13	0.733
IVSd (mm)	13 ± 3	17 ± 3	5×10^{-6} ***
LVPWd (mm)	11 ± 2	13 ± 2	7×10^{-4} ***
LAVI (ml/m^2)	32.8 ± 10.1	48.2 ± 15.9	0.001**
Fibrosis			
RAA fibrosis (%)	31.9 ± 7.0	30.5 ± 8.7	0.518
LAA fibrosis (%)	26.8 ± 4.5	28.2 ± 8.8	0.572

Table 1: Patient characteristics in the AF group and non-AF group. *Mean $\pm$ standard deviation. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.0001$.*

ACEi = angiotensin converting enzyme inhibitor, AAD = antiarrhythmic drug, AF= atrial fibrillation, Ao max/mean = max/mean gradient over the aortic valve, ARB = angiotensin receptor blocker, AVR = aortic valve replacement, BMI = body mass index, CABG = coronary artery bypass graft, DOAC = direct oral anticoagulant, IVSd = interventricular septum thickness in diastole, LAA = left atrial appendage, LAVI = left atrial volume index, LVEF = left ventricular ejection fraction, LVPWd = left ventricular posterior wall thickness in diastole, MI = mitral insufficiency, MRA = mineralocorticoid receptor antagonist, RAA = right atrial appendage.

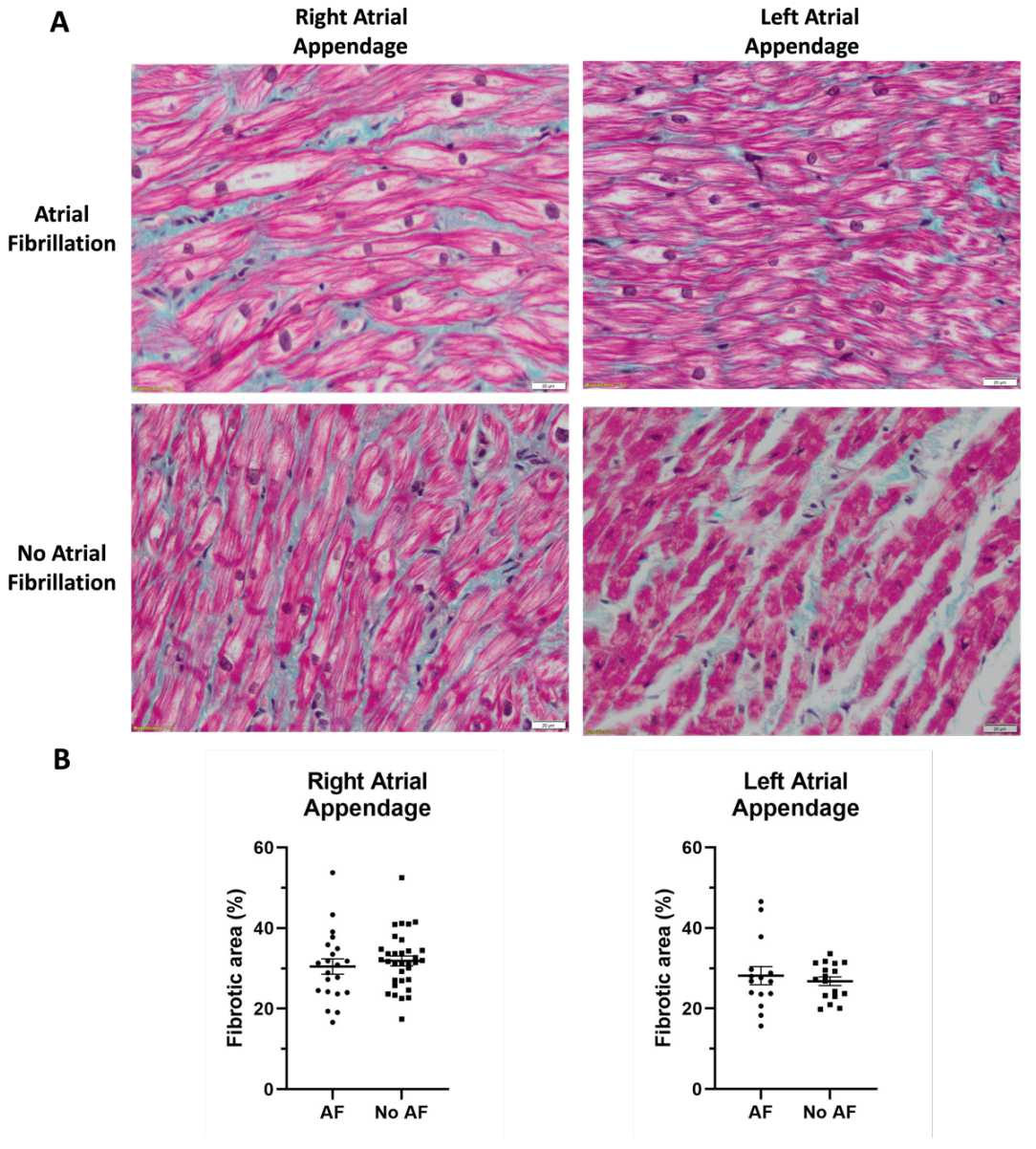

Figure 1: A) *Representative images of left and right atrial appendages in AF and non-AF patients. Scale bar = 20 µm;* ***B)*** *No difference is observed in quantity of fibrosis between the AF and no AF group in the right (30.5 ± 8.7% vs. 31.9 ± 7.0%; p=0.518) and left (28.2 ± 8.8% vs.26.8 ± 4.5%; p=0.572) atrial appendage. AF = atrial fibrillation. Data presented as mean ±SEM.*

Correlations

Correlations between clinical and structural parameters are displayed in Table 2. A robust correlation was observed between fibrotic area in the LAA and RAA. The fibrotic area was higher in the RAA than in the LAA (Y = 0.727X + 5.28, Figure 2). A weak inverse correlation was found between RAA fibrosis and LAVI, whereas no significant correlation was observed between LAA fibrosis and LAVI. Stratification into different subtypes of atrial fibrillation (AF) did not reveal any correlation with LAA or RAA fibrosis, although increasing AF chronicity did correlate with LAVI. Neither age nor the duration since the first AF episode have an impact on fibrosis or atrial dilation. A weak correlation is noted between the extent of coronary artery disease (CAD) and LAA fibrosis, with a weak negative correlation to LAVI, while no correlation is observed with RAA fibrosis.

Patients with a history of heart failure (HF) exhibited increased fibrosis in the LAA compared to those without HF. Similarly, LAVI was increased in the HF group, but no effect of HF was observed on RAA fibrosis. Left ventricular ejection fraction (LVEF) exhibited a negative correlation with LAVI, but no correlation was found with LAA or RAA fibrosis.

Additionally, echocardiographic parameters of interventricular septal thickness in diastole (IVSd) and left ventricular posterior wall thickness in diastole (LVPWd), exhibited weak correlations with LAVI, but no significant correlations are found with RAA or LAA fibrosis.

	Fibrosis RAA			*Fibrosis LAA*			*LAVI*		
	Pearson R	**P-value**	**N**	**Pearson R**	**P-value**	**N**	**Pearson R**	**P-value**	**N**
Fibrosis RAA (%)				**0.784****	**<0.001**	32	**-0.319***	**0.027**	48
Fibrosis LAA (%)	**0.784****	**<0.001**	32				-0.136	0.474	30
AF subtype+	-0.034	0.807	54	0.242	0.182	32	**0.524****	**<0.001**	48
Age	0.128	0.357	54	0.119	0.516	32	0.040	0.789	48
Years since first AF episode	-0.011	0.963	21	-0.009	0.974	15	-0.215	0.407	17
Coronary status°	0.175	0.204	54	**0.379***	**0.032**	32	**-0.353***	**0.014**	48
Heart failure	-0.032	0.816	54	**0.350***	**0.050**	32	**0.524****	**<0.001**	48
LVEF (%)	0.082	0.565	52	0.011	0.951	32	**-0.403****	**0.005**	47
Mitral regurgitaton	-0.022	0.875	52	-0.073	0.689	32	**0.425****	**0.003**	47
Ao grad max	**-0.329***	**0.018**	51	-0.329	0.082	32	**0.317***	**0.034**	45
Ao grad mean	**-0.320***	**0.022**	51	-0.315	0.096	32	**0.322***	**0.031**	45
IVSd	-0.225	0.116	50	-0.182	0.336	30	**0.444****	**0.002**	46
LVPWd	-0.208	0.160	47	-0.125	0.534	27	**0.470****	**0.001**	43

Table 2: *Correlations between clinical and structural remodeling parameters.*

+: AF subtype expressed as an ordinal variable where 0 = no AF, 1 = paroxysmal AF, 2 = persistent AF, 3 = permanent AF.

°: Coronary status expressed as an ordinal variable where 0 = no CAD, 1 = single vessel disease, 2 = two vessel disease, 3 = three vessel disease.

AF= atrial fibrillation, Ao max/mean = max/mean gradient over the aortic valve, IVSd = interventricular septum thickness in diastole, LAA = left atrial appendage, LAVI = left atrial volume index, LVEF = left ventricular ejection fraction, LVPWd = left ventricular posterior wall thickness in diastole, RAA = right atrial appendage.

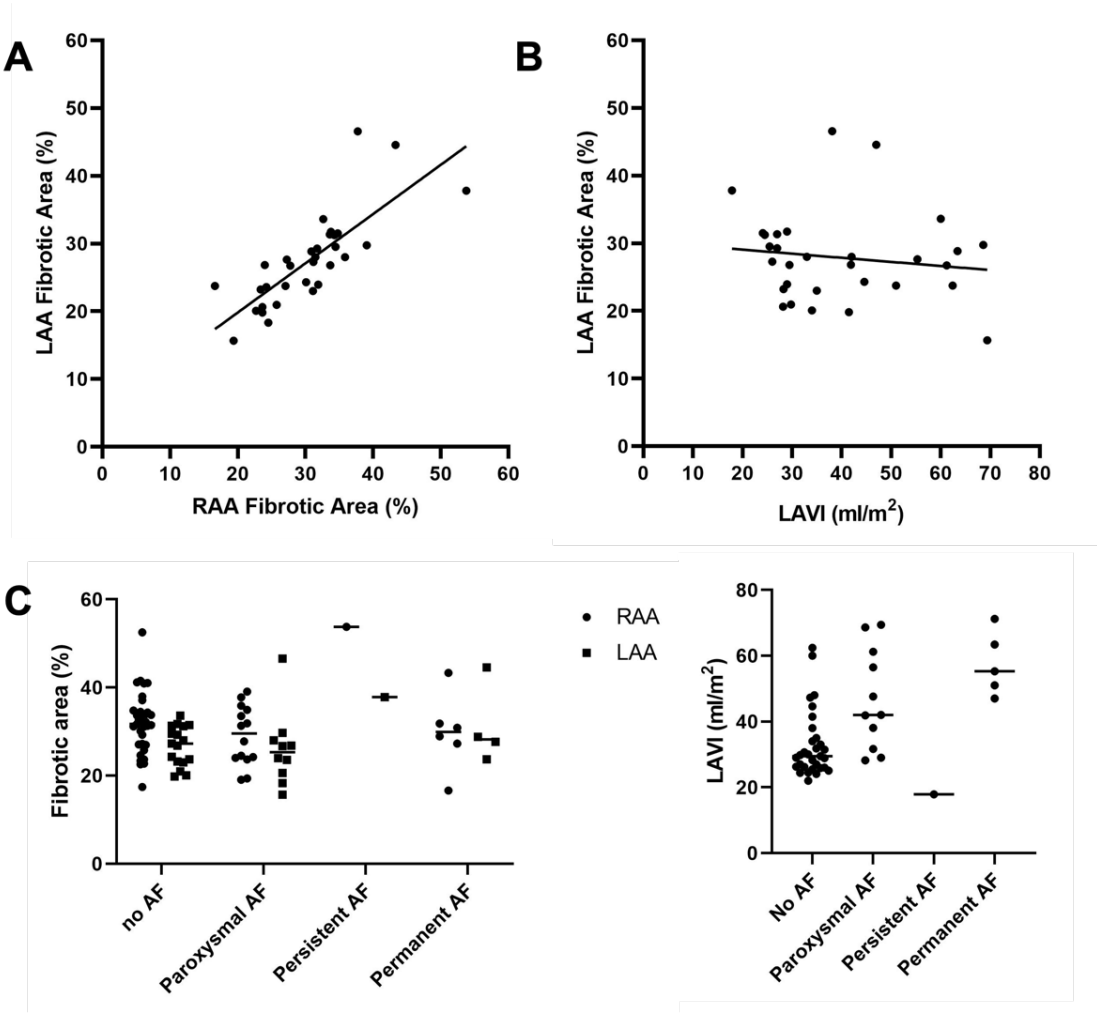

Figure 2: *Correlations between atrial structural remodeling parameters.* ***A)*** *Fibrotic area in the RAA and LAA correlate well. (slope=0.727±0.105; R=0.784; p<0.0001)* ***B)*** *LAVI does not correlate to LAA fibrotic area. (slope=-0.061±0.083; R=-0.136; p=0.474)* ***C)*** *AF subtype does not influence RAA and LAA fibrotic area (left; slope=-0.266±1.086; R=-0.034; p=0.807 for RAA and slope=1.607±1.176; R=0.242; p=0.182 for LAA), but AF subtype influences LAVI (right; slope=7.831±1.877; R=0.524; p<0.0001). AF = atrial fibrillation, LAA = left atrial appendage, LAVI = left atrial volume index, RAA = right atrial appendage.*

Discussion

Atrial fibrosis

This study aimed to investigate the relationship between intersitial fibrosis in the atrial appendages and clinical characteristics, particularly history of AF. We observed a significant correlation between fibrosis in the LAA and RAA, validating the quantification method. Interestingly, it was observed that RAA fibrosis was higher than LAA fibrosis, despite the fact that the right heart is normally seen as the lower-pressure system. Similar findings have been observed in our own pig model (Chapter 6) and in another DOCA pig model[4]. Other clinical studies have also found a higher degree fibrosis in the RAA than the LAA, [5-7] while others have found more fibrosis in the LAA, [8] or no difference between RAA and LAA. [9] Geuzebroek et al. — who also found more fibrosis in the RAA — speculated a developmental explanation for this pnenomenon, since the right and left heart have different embryogenic origin and in the fetal circulation, pressures are higher in the right-sided circulation.[6] Another explanation could be different expression of matrix mealloproteases.[10] Earlier studies ruled out neurohumoral explanations, since no difference could be observed in expression of mineralocorticoid or angiotensin-II receptors in RAA vs. LAA. [11,12]

Surprisingly, no significant impact of AF or AF subtypes (paroxysmal, persistent, or permanent) on fibrosis was found. These findings are consistent with a study by *Ramos et al.*, [13] which reported no difference in RAA fibrosis among patients with different AF subtypes. Another study by *Eckstein et al*. [14] also found no difference in LAA fibrosis between AF and sinus rhythm patients, and LAA fibrosis did not predict postoperative AF, stroke, or mortality. However, in their study, a slight difference in LAA fibrosis was observed when stratifying for mitral regurgitation, but in our dataset, this difference was not present, also after this stratification. However, it should be noted that n-values were small.

On the contrary, an autopsy study[9] demonstrated significantly more fibrosis in AF patients compared to those without AF, with higher levels in persistent AF than in paroxysmal AF. Animal models have provided mixed correlations with fibrosis, with a goat model of long-standing AF showing no increase in atrial fibrosis, [15] while in pig models atrial fibrosis correlates well with AF inducibility. [16-19] Up until now, the link between atrial fibrosis and AF remains enigmatic.

Several limitations should be noted in this study. The study population is rather small and predominantly male. Furthermore, the cardiac surgery population has per definition a very high burden of heart disease, which interferes with normal atrial physiology. A control group with no cardiovascular disease is missing. Several clinical differences between the AF and non-AF groups raise questions about their comparability. The AF group consisted of significantly more male subjects, more patients that underwent AVR, and on average a higher degree of mitral insufficiency and ventricular hypertrophy.

For ethical reasons, only RAA and LAA samples were taken. The relevance of the RAA and LAA as representative sites for atrial fibrosis remains uncertain, although a recent MRI study revealed a strong correlation between fibrosis in the left atrium and LAA. [20]

Finally, a significant limitation is that interstitial fibrosis is the only histological parameter. As the data show, interstitial fibrosis might not be the main hallmark of AF and atrial myopathy. Therefore, examination of more localized types of fibrosis (pericellular, perivascular) as well as other analyses, e.g., hypertrophy and gap junctions would be interesting additions to the current dataset.

Atrial dilation

Although no significant correlations between fibrosis and clinical parameters were observed, there was a better correlation between LAVI and clinical parameters, including AF and its subtypes. This raises the question of whether LAVI might be a more informative parameter in assessing atrial pathology. Despite the modest sample size of this cohort, weak correlations were detected between LAVI and parameters known to contribute to the development of left atrial hypertension —such as heart failure, left-sided valvular disease, etc. These findings align with existing literature and are physiologically plausible.

Conclusion

Among patients undergoing cardiac surgery, the presence or subtype of AF did not show an association with increased fibrosis in either the left or right atrial appendage. Moreover, the correlation between the degree of atrial fibrosis and other clinical and electrocardiographic parameters was found to be non-significant to weak. In contrast, left atrial volume index (LAVI) demonstrated superior predictive ability for AF and displayed stronger correlations with clinical parameters.

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Chapter 8

Summary, General discussion and future perspectives

Summary

Background and hypothesis

Atrial myopathy is an important contributor to the pathophysiologic substrate that underlies AF development, recurrence, and refractoriness to rhythm control therapy. [1] AF is a condition with increasing prevalence, and is associated with significant morbidity, mortality, and healthcare costs. [2] Current antiarrhythmic drugs inhibit ion channels to prolong the effective refractory period and/or slow conduction velocity, but they do not target the underlying atrial myopathy, likely explaining why these drugs become ineffective in the long term. Similarly, pulmonary vein isolation (PVI) merely abolishes the triggers in early phases of atrial myopathy, but once fibrosis manifests in the atria, PVI loses its effectiveness. [3] These observations highlight the urgent need for novel therapies to target atrial myopathy.

In Chapter 2, the cardioprotective effects of Neuregulin-1 were described, based on its anti-fibrotic, anti-apoptotic and stress-response mitigating properties through the ERBB4 receptor. JK07 is a NRG1 fusion protein that has increased ERBB4 selectiveness and a longer half-life, enhancing its pharmacological usability. Recombinant NRG1 and JK07 have therapeutic effects in preclinical models of heart failure as well as in early phase clinical trials. For this dissertation, we aimed to focus on its potential role in modifying atrial myopathy and AF. More specifically, **we hypothesized that JK07 counteracts atrial fibrosis present in atrial myopathy and therefore decreases vulnerability of the atria towards induction of AF.** (Figure 1)

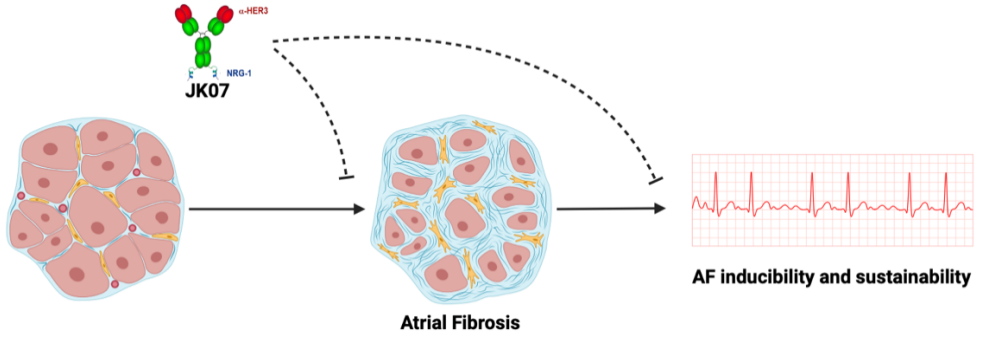

Figure 1: General hypothesis: *JK07 counteracts atrial fibrosis present in atrial myopathy and therefore decreases vulnerability of the atria towards induction of AF.*

Overview of key findings

In Chapter 1, we discussed the pathophysiology of atrial myopathy, possible diagnostic tools to detect it, and highlighted the lack of effective therapies targeting atrial myopathy.

In Chapter 3, we described that spontaneous AF was not observed in any of the pigs with sterile pericarditis (nor with sham surgery), but there was an increase in AF inducibility and atrial fibrosis upon two weeks after sterile pericarditis. This seemed to make it a suitable model to study fibrotic atrial myopathy. Before testing the efficacy of JK07 for treatment of atrial myopathy, we determined that the highest tolerated dose of JK07 in minipigs was 0.3 mg/kg, as described in Chapter 4. Chapter 5, however, could not discern efficacy of JK07 in the sterile pericarditis model. with both AF inducibility and atrial fibrosis unaffected in JK07 treated animals. We speculated that the sterile pericarditis model was likely too aggressive for a drug to interfere with its pathophysiology.

Therefore, we tested the efficacy of JK07 in another, more gentle model of hypertension-induced atrial myopathy (DOCA model), which was described in Chapter 6. In this model, we observed that JK07, without lowering blood pressure, counteracted atrial fibrosis and decreased AF inducibility and sustainability without an effect on $S_1S_{1,min}$ —as a proxy for atrial effective refractory period (AERP). JK07 attenuated DOCA-induced conduction slowing, which might be attributable to the prevention of interstitial fibrosis. In addition, echocardiographic observations showed that JK07 induced a trend towards attenuation of atrial dilation.

Tissue samples were collected from the atrial appendages of cardiac surgery patients, and Chapter 7 described how the degree of fibrosis in the atrial appendages was linked to clinical characteristics of the patients, including a history of AF. Surprisingly, the degree of fibrosis in the left and right atrial appendage was not higher in patients with prior AF, nor was there a correlation with AF chronicity. In contrast, echocardiographic measurements of left atrial volume index showed a better correlation with AF.

Taken together, this study provides the first scientific evidence for a therapeutic role for ERBB4 agonism in the treatment of atrial myopathy and atrial fibrillation by affecting electrical and structural remodeling without prolongation of the refractory period.

General discussion

Models matter.

It may appear contradictory that JK07 showed no effect on AF vulnerability in the sterile pericarditis model, while exerting antiarrhythmic effects in the DOCA model. However, this observation supports our earlier speculation that the profibrotic and proarrhythmogenic stimulus in the sterile pericarditis model was too strong, providing no therapeutic window for a pharmacologic treatment. Another potential reason for decreased efficacy of JK07 in the sterile pericarditis model is decreased penetrance of the drug into the epicardial space, that is occupied by foreign material. Obviously, the DOCA model is more gentle, allowing JK07 to fill the therapeutic window in this model. From a clinical point of view, the DOCA model is more relevant as it is closer to the clinical reality of hypertension-induced AF in many patients, [4] compared to placement of sterile talcum and gauze on the atrial epicardium, which was developed as an exaggerated model of post-operative AF. [5]

JK07 shows therapeutic potential for the treatment of atrial myopathy.

The data from Chapter 6 support our hypothesis that ERBB4 activation decreases vulnerability towards AF induction and sustainability by counteracting atrial fibrosis. An interesting aspect was that JK07, unlike other antiarrhythmic drugs, did not affect AERP. Neither did DOCA, as described elsewhere. [6] By contrast, JK07 attenuated DOCA-induced electrical conduction slowing, which can be explained by a decrease of interstitial fibrosis. Presumably, this effect is caused by a direct effect of ERBB4 activation on the cardiac fibroblast, inhibiting myofibroblast differentiation and extracellular matrix production. [7,8] In cardiomyocytes, there was an inhibiting effect through the p38-MAPK pathway, that is also related to fibrosis [9] and could be a possible mechanism in the fibroblast as well, although the classic paradigm states that ERBB4 stimulation mainly works through Akt and ERK, although effects on MAPK have been described. [10] Another possible mechanism is the ERBB4-dependent inhibition of macrophages releasing inflammatory cytokines by inhibition of the PI3K/Akt/STAT3 pathway. [11]

Besides anti-inflammatory and anti-fibrotic effects, we found that typical characteristics of electrical remodeling as seen in AF – e.g. downregulation of Na^+ and Ca^{2+} channels, downregulation of Ca^{2+}-regulators, like phospholamban - were present in the DOCA treated pigs, and inhibited by JK07, resulting in lower AF inducibility. Those findings indicate prevention of decreasing cardiomyocyte excitability, which maintains conduction

velocity. Another effect of JK07 was the prevention of downregulation of *Gja1*, *Gja4* and *Gja5*, genes respectively encoding Connexin-43, -37 and -40, all of which form important gap junctions for atrial impulse propagation, and that are classically downregulated in AF patients. [12] These are novel findings since no literature is available on the atrial-specific effects of cardiomyocyte ERBB4 stimulation. Other antiarrhythmic mechanisms have been described in ventricular cardiomyocytes, e.g., NRG1 increases contractile and metabolic maturity in ventricular cardiomyocytes, desensitizes to β_1-adrenergic stimulation, and enhances diastolic Ca^{2+} reuptake by the sarcoplasmic reticulum. [8,13-15]

With this, some possible effects have been described, all of which could theoretically be antiarrhythmic. Further studies are warranted to elucidate the exact antiarrhythmic mechanism of JK07.

The relation between atrial fibrosis and AF: do we need to change the paradigm?

We observed no difference in fibrotic area in human atrial appendages between AF patients and patients without AF. One possible hypothesis could be that all surgical patients have an equally high risk of developing AF. However, the permanent AF patients that were included have per definition a 100% risk of developing AF and did not show increased fibrosis. Similar observations were recently made by *Ramos et al*., [16] suggesting that the degree of atrial fibrosis is not the sole key factor to predict development of AF. A lot of recent research on atrial substrate for AF is directed at detecting and quantifying atrial fibrosis, but the reduction of atrial myopathy to mere atrial fibrosis is an oversimplification. Different mechanisms are at play, including different interstitial depositions (amyloid, fat, inflammatory cells,…) and phenotypic remodeling of different cell types, as described in Chapter 1. However, when only looking at the pig model, atrial fibrosis is a good predictor of AF inducibility and sustainability, which might be species-specific.

Remarkable is that the amount of interstitial fibrosis in both pig models (around 10-15% in both models) was lower compared to cardiac surgery patients (around 20-40%). This might suggest that the atrial myopathy in these pig models still is in an early stage compared to human atrial myopathy, that is probably present in every cardiac surgery patient.

One remaining question is whether atrial fibrosis causes AF or that it is merely an epiphenomenon in the process of atrial myopathy. On the one hand, atrial fibrosis is consistently reported to be present in the atria of AF patients and has proven to increase

anisotropy in electrical conduction, but on the other hand, there are many patients with high amounts of atrial fibrosis, that remain arrhythmia-free. [17] This is also reflected in the findings in this thesis: on the one hand, fibrosis is not increased in AF patients in an at-risk patient group (cardiac surgery), but on the other hand, prevention of fibrosis led to decreased conduction delay and lower AF inducibility in the pig model. These findings altogether made us suspect that the relationship between atrial fibrosis and AF is complex and non-linear and, in order to develop (vulnerability towards) AF, other factors must be present, in the form of other types of remodeling, e.g. electric remodeling. This is why RNA sequencing was performed on isolated cardiomyocytes at the end of the Ph.D. trajectory, to examine the effect of JK07 on electrical remodeling in cardiomyocytes.

In humans and pigs, more fibrosis was observed in the RAA compared to the LAA.

In the two pig models as well as in the human atrial appendages, right atrial fibrosis was higher compared to left atrial fibrosis. This seems paradoxical, since blood pressure is higher in the left atrium and therefore, higher collagen content is expected in the left atrium to withstand increased wall tension. Clinical studies also report variable findings, with some showing more fibrosis in the RAA, [18-20] while others indicate higher fibrosis in the LAA[21] or no significant difference. [22] However, in rodent studies, no difference was observed between RA and LA fibrotic content. [6] Geuzebroek et al. proposed a developmental explanation, considering the distinct embryogenic origin of the right and left heart, and fetal circulation pressures being higher on the right side. [19] Another plausible explanation could involve differential expression of matrix metalloproteases. [23] In conclusion, this finding as well as its possible cause still remains controversial; more research is needed to explore the reasons behind this phenomenon, since pursuing this could give insights into the hierarchy of factors that lead to atrial fibrosis.

Future perspectives

We demonstrated the capacity of JK07 to prevent atrial electrical and structural remodeling in one specific pig model (the DOCA model, not the pericarditis model), resulting in decreased vulnerability towards AF induction. However, many questions remain currently unanswered.

For translation into clinical use, merely preventive effects of JK07 on atrial fibrosis and electrical remodeling might prevent proarrhythmic remodeling, but might not be able to revert (i.e. treat) it. Therefore, a useful next step would be to repeat the experiments but start JK07 treatment on day 60 instead of day 0, when electrical and structural remodeling have been established. To maintain hypertensive cardiac remodeling, additional implantation of new DOCA pellets on day 60 would be necessary.

Neither in the sterile pericarditis nor in the DOCA model did we observe spontaneous AF. Therefore, it might be that we tested in an early stage of the atrial myopathy long before clinical manifestations become apparent. Since (spontaneous) AF is often the first symptom of atrial myopathy, many patients will probably already be in a later stage of atrial myopathy at the time of diagnosis. So, another question that needs to be answered is whether JK07 exerts an antiarrhythmic effect in a model of paroxysmal or persistent AF. Modeling spontaneous AF in large animals is difficult and in order to avoid very long treatment with DOCA, which is very costly, an additional stimulus will be necessary, which has been previously done with atrial tachypacing. [24,25] In this model, we will need to test the effect of JK07 on AF burden.

Further exploration of the exact antiarrhythmic mechanism of JK07 is necessary. The data that came from the bulk RNA sequencing of the isolated cardiomyocytes have been helpful in gaining insights in predominantly electrical remodeling, as described above and in Chapter 6. However, these genomic data also need to be correlated with phenotypic alterations. For this, it would be ideal to perform patch clamping and optical voltage mapping on freshly isolated cardiomyocytes from pigs of the three groups as well as perform ELISA or Western blotting of the most important up- or downregulated proteins.

Furthermore, besides cardiomyocyte effects, the effects of JK07 on different cell types should be investigated. For this, single-cell RNA sequencing would be an ideal first step. To investigate the effect of JK07 on endothelial function, it would be interesting to analyze

plasma samples for molecular signs of endothelial damage and activation, including von Willebrand Factor, soluble P-selectin, and Platelet Activation Factor. To investigate immune cell activation, analysis of the plasma samples for interleukins and inflammatory cytokines would be interesting. To investigate (myo)fibroblast activation, an additional staining of the atrial tissue with alpha-smooth muscle actin would be a feasible option.

The ERBB4 selectivity of JK07 has been demonstrated by the manufacturer in various cells and species. [26] However, for definite proof that the antiarrhythmic effect is ERBB4-mediated, ideally a knock-out model should be made. Despite the fact that it is not common to create a knock-out large animal model, this has been done before with different knock-outs.[27] A knock-out model of ERBB4 could also provide information about secondary up-and downregulated genes and hence, elaborate the electrophysiological effects of ERBB4 activation. However, more realistic would be to perform this knock-out in a small animal, where unfortunately very little atrial tissue is available for molecular biology.

Recently, two Phase 1b clinical trials of JK07 in the setting of heart failure with reduced and preserved ejection fraction (HFrEF/HFpEF) were successfully completed (NCT04210375 and NCT05322616) and will move on towards Phase 2. Largely based on our preclinical data, the manufacturer will also initiate a Phase 2 trial for patients with paroxysmal and persistent AF, with specific targeting of the combined AF + HFpEF phenotype, since they have substantial overlap in pathophysiology.

JK07 has the advantage of a long half-life compared to recombinant NRG1, that needs daily intravenous administration for 6-8 hours, [28,29] where JK07 is administered in single dose over 60 minutes in humans. However, since JK07 is a protein, it needs to be administered parenterally. Combined with the fact that antibodies are expensive, this decreases clinical applicability. For this reason, it would be interesting to develop small molecule ERBB4 agonists, that are suitable for oral administration and with the potential to selectively activate the ERBB4 receptor.

Finally, the examination of human atrial appendage samples with quantification of fibrosis did not deliver an explanation for increasing AF chronicity. It is possible that, next to fibrosis, other factors are at play. Possible explanations include the presence of other types of extracellular matrix remodeling, e.g. amyloid or fat deposition. These could easily be identified using classic tissue stainings (Congo Red, Hematoxylin & Eosin). Next to that, cellular types of remodeling would also be interesting, especially hypertrophy, connexin

remodeling, calcium handling, ion channel remodeling, as well as markers for metabolic remodeling (AMPK expression), autonomic remodeling and contractile remodeling. One of these factors or a combination of different factors could expose the mechanisms of increasing AF chronicity and hence the advancement of atrial myopathy.

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Hoofdstuk 8 bis:

Samenvatting en conclusies

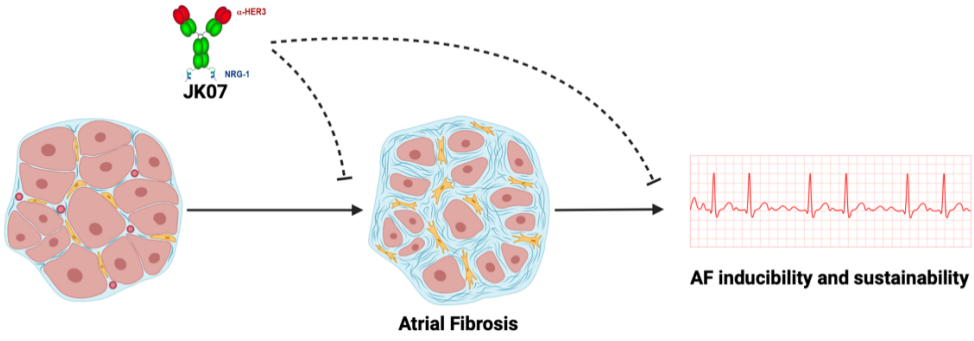
Samenvatting

Introductie en hypothese

Atriale myopathie is het geheel van pathofysiologische veranderingen in de voorkamers dat zorgt voor het ontstaan, terugkeren en therapieresistent worden van voorkamerfibrillatie (VKF). [1] VKF is een aandoening met een stijgende prevalentie en is geassocieerd met significante morbiditeit, mortaliteit en kosten aan het gezondheidszorgsysteem. [2] Anti-aritmica die worden gebruikt ter behandeling van VKF blokkeren ionenkanalen en verlengen de effectief refractaire periode, maar grijpen niet in op de onderliggende myopathie, waardoor ze op lange termijn hun effect verliezen. Pulmonaalvene isolatie (PVI) is een andere behandeloptie die triggers wegneemt in vroege fases van atriale myopathie, maar PVI verliest zijn effect eens er fibrose aanwezig is in de atria. [3] Om deze redenen zijn nieuwe therapieën nodig gericht tegen atriale myopathie.

In Hoofdstuk 3 werd besproken hoe Neureguline-1 (NRG1) het hart beschermt door activatie van de ERBB4 receptor, wat leidt tot onderdrukking van fibrose en apoptose. JK07 is een fusie-eiwit van NRG1 met een veel langere halfwaardetijd dan recombinant NRG1, dat ook selectiever bindt aan de ERBB4 receptor. Hierdoor bezit deze molecule een hoog translationeel potentieel (d.w.z. het zal vermoedelijk goed kunnen worden toegepast in patiënten).

Zowel JK07 als recombinant NRG1 hebben hun effect bewezen in diermodellen van hartfalen en in vroege fases van klinische trials voor hartfalen. In deze thesis testen we of de effecten van ERBB4-stimulatie ook van toepassing zijn op atriale myopathie en VKF. **Onze hypothese is dat JK07 fibrose in de voorkamers zal tegengaan die aanwezig is in atriale myopathie, en daardoor de kwetsbaarheid voor VKF zal afnemen.** (Figuur 1)



Figuur 1: Algemene hypothese: *JK07 remt atriale fibrose, die aanwezig is in atriale myopathie, en vermindert daardoor de kwetsbaarheid voor VKF.*

Overzicht van de belangrijkste bevindingen en conclusies

In Hoofdstuk 1 werd dieper ingegaan op de pathofysiologie van atriale myopathie, de mogelijke diagnostische technieken om dit vast te stellen, en het gebrek aan effectieve behandelings-mogelijkheden.

In Hoofdstuk 3 werd beschreven dat in het steriele pericarditis model geen spontane VKF werd waargenomen. Wel was er een duidelijke toename in induceerbaarheid van VKF en fibrose van de voorkamers, vanaf twee weken na steriele pericarditis. Dit model kan worden gebruikt om een vorm atriale myopathie te bestuderen waar fibrose centraal staat. Echter, vooraleer we het effect van JK07 op induceerbaarheid van VKF konden testen, hebben we eerst een beperkte doseerstudie uitgevoerd, waarin werd vastgesteld dat de hoogst getolereerde dosis 0.3 mg/kg bedroeg, zoals beschreven in Hoofdstuk 4. In Hoofdstuk 5 werd beschreven hoe het effect van JK07 werd getest in het steriele pericarditis varkensmodel. Hier zagen we echter geen therapeutisch effect van JK07: induceerbaarheid en duur van VKF zowel als fibrose van de voorkamers verschilden niet tussen de dieren behandeld met JK07 enerzijds en vehikel anderzijds. We speculeerden dat het steriele pericarditis model vermoedelijk te agressief was voor een medicijn om iets te kunnen veranderen aan de pathofysiologie.

In hoofdstuk 6 werd beschreven hoe een tweede, milder varkensmodel werd opgezet: het DOCA model. In dit model werden pellets geïmplanteerd die langzaam DOCA (een aldosterone-analoog) vrijstelden in de varkens, wat zorgde voor hoge bloeddruk en fibrose van de voorkamers. In dit model zagen we dat JK07 fibrose tegenging in de voorkamers, en de induceerbaarheid en duur van VKF-episodes verminderde, zonder enig

effect te hebben op de effectief refractaire periode of op de bloeddruk. JK07 ging ook geleidingsvertraging van elektrische prikkels tegen in de voorkamers; deze was het gevolg van “zig zag conductie”, die dan weer het gevolg was van fibrose door DOCA. Daarenboven toonden echocardiografische metingen een trend dat JK07 atriale dilatatie zou tegengaan.

We kunnen besluiten dat de data uit Hoofdstuk 7 het eerste wetenschappelijk bewijs vormen dat stimulatie van de ERBB4-receptor nuttig kan zijn om atriale myopathie en VKF te behandelen door in te grijpen op elektrische en structurele veranderingen in de voorkamers, en dit zonder de refractaire periode te verlengen.

Tot slot werden weefselstalen verzameld van hartoren afkomstig van cardiochirurgische patiënten, en in Hoofdstuk 7 wordt beschreven hoe de hoeveelheid fibrose in de hartoortjes correleerde met klinische eigenschappen van de patiënten, zoals het optreden van VKF in de voorgeschiedenis. Onverwacht was dat de hoeveelheid fibrose in zowel linker als rechter hartoor niet verhoogd was bij patiënten met voorafgaande geschiedenis van VKF, en dat er geen verband was met de hoeveelheid van de tijd de patiënt in VKF was. Meting van de linker atriale volume index (LAVI) met echografie daarentegen toonde echter een veel duidelijkere relatie met de aanwezigheid van VKF. De relatie tussen atriale fibrose en VKF blijft een enigma.

Algemene discussie

Het ene model is het andere niet.

Op het eerste zicht lijkt het tegenstrijdig dat JK07 geen effect toont op kwetsbaarheid voor VKF in het steriele pericarditis model, terwijl JK07 in het DOCA model dan weer duidelijk ritmestoornissen lijkt tegen te gaan. Echter ondersteunt dit juist onze eerdere veronderstelling dat de ziekmakende stimulus in het steriele pericarditis model zo fors is dat een geneesmiddel niets kan veranderen in het verloop van dit ziekteproces. Een andere mogelijke verklaring waarom JK07 geen effect heeft in het steriele pericarditis model is een verminderde penetratie van het geneesmiddel tot in het epicard, waar vreemd materiaal aanwezig is. Logischerwijs is het DOCA model milder, waardoor JK07 in dat model wel iets kan veranderen aan het verloop van het ziekteproces. Vanuit klinisch standpunt is het DOCA model ook relevanter, gezien hoge bloeddruk aanwezig is in de meerderheid van de patiënten met VKF, [4] terwijl steriele pericarditis werd ontwikkeld als een uitvergroot model van post-operatieve VKF na cardiochirurgie, wat een zeer specifieke subpopulatie is. [5]

JK07 toont potentieel als behandeling voor atriale myopathie.

De gegevens van Hoofdstuk 6 ondersteunen onze initiële hypothese, namelijk dat ERBB4-activatie de kwetsbaarheid voor VKF tegengaat door fibrose in de voorkamers tegen te gaan. Een interessante bevinding was dat JK07 geen effect had op de ERP, in tegenstelling tot andere anti-aritmica. Evenmin beïnvloedde DOCA de ERP, zoals eerder beschreven. [6] Daarentegen werd een attenuatie gezien van de DOCA-geïnduceerde conductievertraging. Dit is een mogelijk gevolg van verminderde “zig zag conductie”, die dan weer het gevolg is van interstitiële fibrose in de voorkamers. Dit effect komt vermoedelijk voort uit een direct effect van ERBB4-activatie van de cardiale fibroblast, wat de differentiatie tot myofibroblast remt, net als de productie van extracellulaire matrix. [7,8] In de cardiomyocyt zagen we een remmend effect op de p38-MAPK pathway, die gerelateerd is aan fibrose, [9] hetgeen mogelijks ook een mechanism in de fibroblast kan zijn, al medieert ERBB4 zijn effecten klassiek volgens Akt en ERK, al zijn effecten op MAPK beschreven. [10] Een ander mogelijk mechanisme is de ERBB4-afhankelijke remming van macrofagen door remming van de PI3K/Akt/STAT3 pathway, waardoor minder ontstekingsmediatoren worden vrijgesteld. [11]

Naast remmende effecten op ontsteking en fibrose vonden we ook transcriptomische handtekens van elektrische remodelering in de DOCA-varkens, die typisch gezien worden

bij VKF-patiënten, zoals downregulering van Na^+ en Ca^{2+}-kanalen en Ca^{2+}-regulatoren zoals fosfolamban. Deze veranderingen konden worden voorkomen door behandeling met JK07, wat mee leidde tot een verminderde induceerbaarheid van VKF. Deze bevindingen wijzen vermoedelijk op een preventie van verlies aan exciteerbaarheid van de cardiomyocyt, waardoor de conductiesnelheid bewaart blijft. Een ander mechanisme is de JK07-gemedieerde preventie van downregulering van connexine (Cx)-43, Cx-40 en Cx-37, die belangrijke gap junctions vormen voor impulsvoortgeleiding in de voorkamers, en dewelke klassiek zijn downgereguleerd in VKF-patiënten. [12] Deze bevindingen zijn allemaal nieuw, gezien er geen literatuur bestond over de voorkamerspecifieke effecten van ERBB4 stimulatie in hartspiercellen, al zijn effecten van NRG1 op hartspiercellen van de kamers wel beschreven. Zo is het geweten dat NRG1 zorgt voor contractiele en metabole maturiteit in hartspiercellen, deze minder gevoelig maakt voor het effect van adrenaline op β_1-receptoren en de Ca^{2+}-reuptake door het sarcoplasmatisch reticulum tijdens de diastole verhoogt. [8,13-15]

Al deze effecten kunnen theoretisch gezien beschermen tegen ritmestoornissen. Desalniettemin zullen verdere studies nodig zijn om het anti-aritmische mechanisme van JK07 te ontsluieren.

Het verband tussen atriale fibrose en VKF: moeten we het paradigma veranderen?

We zagen geen verschil in de hoeveelheid fibrose in de hartoortjes van VKF-patiënten en patiënten die nog nooit VKF hadden. Een mogelijke verklaring is dat alle chirurgische patiënten een gelijkaardig (verhoogd) risico hebben op het ontwikkelen van VKF. Echter, de patiënten met permanente VKF hebben per definitie een 100% risico op ontwikkelen van VKF en bij deze groep werd geen toename in fibrose gezien. Soortgelijke bevindingen werden recent beschreven door *Ramos et al.,* [16] waarbij zij suggereerden dat fibrose niet de enige belangrijke factor is om de ontwikkeling van VKF te voorspellen. Veel recent onderzoek is gericht op het vaststellen van de hoeveelheid fibrose in de voorkamers als maat voor atriale myopathie, maar de reductie van atriale myopathie tot louter voorkamerfibrose is een oversimplificatie. Verschillende mechanismen spelen een rol, waaronder verschillende deposities tussen de weefsels (eiwit, vet, ontstekingsinfiltraten,…) en reacties van verschillende celtypes op veranderende omstandigheden, zoals beschreven in Hoofdstuk 1. Als we echter uitsluitend naar de varkensmodellen kijken, is fibrose in de voorkamers wel een goede predictor van kwetsbaarheid voor VKF, hoewel dit mogelijk eerder diersoort-afhankelijk zou kunnen zijn.

Opmerkelijk is dat de hoeveelheid interstitiële fibrose in beide varkensmodellen (bij allebei ongeveer 10-15%) lager is in vergelijking met die in chirurgische patiënten (ongeveer 20-40%). Dit kan betekenen dat de atriale myopathie in deze varkensmodellen nog in een vroeg stadium zit vergeleken met humane atriale myopathie, welke vermoedelijk aanwezig is in elke cardio-chirurgische patiënt. Ook omdat er nooit spontane VKF werd gezien en de pacing protocols relatief agressief dienden te zijn om korte runs VKF uit te lokken.

Een resterende vraag is of atriale fibrose VKF veroorzaakt, of dat dit slechts een bijkomstigheid is in het proces van atriale myopathie. Enerzijds wordt consistent gerapporteerd dat atriale fibrose aanwezig is in de atria van patiënten met VKF en de anisotropie in elektrische geleiding verhoogt. Anderzijds zijn er veel patiënten met aanzienlijke atriale fibrose die geen hartritmestoornissen ontwikkelen. [17] Deze dubbelzinnigheid komt ook naar voren in de bevindingen van dit proefschrift: enerzijds is fibrose niet verhoogd bij VKF-patiënten in een risicogroep (hartchirurgie), maar anderzijds leidde preventie van fibrose door JK07 tot verminderde geleidingsvertraging en lagere indcuceerbaarheid voor VKF in het varkensmodel. Deze bevindingen doen ons vermoeden dat de verhouding tussen atriale fibrose en VKF niet-lineair en complex is, en dat om (kwetsbaarheid voor) VKF te ontwikkelen andere factoren nodig zijn, zoals elektrische remodelering. Dit is de reden waarom RNA-sequencing werd uitgevoerd op geïsoleerde hartspiercellen, om het effect van JK07 op elektrische remodeling in cardiomyocyten te onderzoeken.

In varkens en mensen zagen we meer fibrose in het rechter hartoor vergeleken met links.

In beide varkensmodellen en in de humane hartoren was fibrose in de rechter hartoor meer uitgesproken in vergelijking met de fibrose in de linker voorkamer. Dit lijkt paradoxaal, aangezien de bloeddruk hoger is in het linker atrium en daarom wordt verwacht dat er meer collageen aanwezig is in het linker atrium om de verhoogde wandspanning te weerstaan. Klinische studies melden ook wisselende resultaten, waarbij sommige meer fibrose laten zien in de rechter hartoor, [18-20] terwijl anderen meer fibrose aangeven in het linker hartoor, [21] en anderen dan weer geen significant verschil zien. [22] Bij knaagdierstudies werd dan weer geen verschil waargenomen in het de hoeveelheid fibrose tussen het rechter en het linker atrium. [6] *Geuzebroek et al.* suggereerden dat dit effect zou berusten op het verschil in embryonale oorsprong van het rechter- en linkerventrikel en de hogere druk in de rechterventrikel tijdens de foetale circulatie. [19] Een andere plausibele verklaring zou kunnen liggen in andere expressie van matrixmetalloproteasen. [23] Kortom, deze bevinding en de mogelijke oorzaak blijven

controversieel; meer onderzoek is nodig om de redenen achter dit fenomeen te verkennen, aangezien dit inzicht kan bieden in de (hiërarchie van) factoren die leiden tot fibrose van de voorkamer.

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Scientific curriculum vitae

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Scientific activities

Theses

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Result: *Summa cum laude*

Patent applications

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Title: Methods of treating fibrosis and arrhythmia with a Neuregulin-1 fusion protein. Application filed by Salubris Biotherapeutics, Inc. on December 1, 2022.

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Tubeeckx M, Goovaerts B, Van fraeyenhove J, De Meyer G, De Keulenaer G, Heidbuchel H, Segers V; JK07 prevents atrial fibrosis and atrial fibrillation in a porcine DOCA model. EHRA, Barcelonoa, 2023 April.

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Tubeeckx M, Van Fraeyenhove J, Laga S, De Meyer G, De Keulenaer G, Heidbuchel H, Segers V; Over varkentjes en muizen met VKF die niet 'knr' of 'piep', maar 'nrg' en 'Erb' zeggen: de rol van het NRG1/ERBB4 systeem in voorkamerfibrillatie en atriale myopathie. Evening seminar Cardiology dept. UZA, Antwerp, 2021 May.

Feyen E, Cools J, Van fraeyenhove J, Tubeeckx M, De Winter H, Audenaert D, De Keulenaer G, Segers V; Small-molecule ERBB4 agonists as a treatment for heart failure. Heart Failure, online congress, 2022 May.

Feyen E, Cools J, Van fraeyenhove J, Tubeeckx M, Bruyns T, De Winter H, Audenaert D, De Keulenaer G, Segers V; Identification of small-molecule ERBB4 agonists to treat heart failure. American Heart Association, Chicago, 2021 November.

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