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# Atrial fibrosis heterogeneity is a risk for atrial fibrillation in pigs with ischemic heart failure

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# Table of content

# List of abbreviations

| | |
|---|---|
| AF | atrial fibrillation |
| APD | action potential duration |
| AP | action potential |
| ABC | Atrial fibrillation Better Care |
| AADs | Anti arrhythmic drugs |
| AV WCL | atrioventricular Wenkebach cycle length |
| AVERP | atrioventricular node effective refractory period |
| AERP | atrial effective refractory period |
| CMR | cardiac magnetic resonance |
| DADs | delayed afterdepolarizations |
| ECG | electrocardiogram |
| ESC | European Society of Cardiology |
| EADs | early afterdepolarizations |
| ECM | extracellular matrix |
| FHS | Framingham Heart Study |
| HF | heart failure |
| ID | intercalated disk |
| LTCCs | L-type $Ca^{2+}$ channels |
| LV | left ventricle/ventricular |
| LAA | left atrial appendage |
| LAD | left anterior descending artery |
| LVEDP | left ventricular enddiastolic pressure |
| MRI | magnetic resonance imaging |
| NCX | $Na^{+}/Ca^{2+}$ exchanger |
| PVI | pulmonary vein isolation |
| RyR2s | ryanodine receptors |
| RAAS | renin-angiotensin-aldosterone system |
| SR | sarcoplasmic reticulum |
| SERCA2a | sarcoplasmic reticulum $Ca^{2+}$-adenosine triphosphatase |

# List of publications

1. **Zhang, Z.**, Vlcek, J., Pauly, V., Hesse, N., Bauer, J., Chataut, K. R., Maderspacher, F., Volz, L. S., Buchberger, K., Xia, R., Hildebrand, B., Kääb, S., Schüttler, D., Tomsits, P., & Clauss, S. (2024). Atrial fibrosis heterogeneity is a risk for atrial fibrillation in pigs with ischaemic heart failure. *European journal of clinical investigation*, *54*(4), e14137. https://doi.org/10.1111/eci.14137 **(Part of cumulative dissertation, JCR Q1)**
2. Pauly, V., Vlcek, J., **Zhang, Z.**, Hesse, N., Xia, R., Bauer, J., Loy, S., Schneider, S., Renner, S., Wolf, E., Kääb, S., Schüttler, D., Tomsits, P., & Clauss, S. (2023). Effects of Sex on the Susceptibility for Atrial Fibrillation in Pigs with Ischemic Heart Failure. *Cells*, *12*(7), 973. https://doi.org/10.3390/cells12070973
3. Xia, R., Tomsits, P., Loy, S., **Zhang, Z.**, Pauly, V., Schüttler, D., & Clauss, S. (2022). Cardiac Macrophages and Their Effects on Arrhythmogenesis. *Frontiers in physiology*, *13*, 900094. https://doi.org/10.3389/fphys.2022.900094
4. **Zhang Z.**, Clauss, S. Electrophysiological Repair, in: Heart and Vascular System: An Encyclopedia. (Book Chapter), in press.

# 1. Contribution to the publications

## 1.1 Atrial fibrosis heterogeneity is a risk for atrial fibrillation in pigs with ischaemic heart failure

In this project, I assisted in pig *in vivo* experiments, including preparation of the experiments, support during the operation, and sample preparation after the operation. I analyzed all *in vivo* results. I performed histological experiments, including staining, microscopy and analysis. I performed statistics and prepared all figures. I discussed all results with Sebastian Clauss. We conceived the manuscript. I drafted the manuscript and performed all revisions needed during the review process.

## 1.2 Effects of Sex on the Susceptibility for Atrial Fibrillation in Pigs with Ischemic Heart Failure

In this project, I contributed to formal analysis, investigation and funding acquisition.

## 1.3 Cardiac Macrophages and Their Effects on Arrhythmogenesis

For this paper, I performed literature research, and provided parts of the manuscript.

## 1.4 Electrophysiological Repair, in: Heart and Vascular System: An Encyclopedia

In this book chapter, I performed literature research, conceived the manuscript, designed figures and drafted the manuscript with Sebastian Clauss.

# 2. Introduction

Atrial fibrillation (AF) stands as the most common cardiac arrhythmia, associated with considerable morbidity and mortality (G. Y. Lip et al., 2016). Ischemic heart failure is regarded as one of the leading causes for AF (Belkouche et al., 2021). Patients with ischemic heart failure and AF often have poor clinical outcome with high all-cause mortality rates (Benjamin et al., 1998). Current treatments for AF remain limited due to our incomplete understanding of the underlying mechanisms, especially in ischemia-mediated AF (Brundel et al., 2022). Therefore, we designed this project to improve our understanding by investigating a porcine ischemic heart failure model, which is associated with an increased susceptibility to AF.

## 2.1 Atrial fibrillation in ischemic heart failure – The scientific context

### 2.1.1 Definition of atrial fibrillation

AF is an irregular rhythm of the atria, accompanied by rapid and chaotic atrial contractions (Baman & Passman, 2021). Typical electrocardiography (ECG) findings of AF include the absence of distinctly visible P waves, the presence of rapid fibrillatory waves, and irregular RR intervals (Cai et al., 2020). Diagnosis of AF typically requires the documentation of an episode lasting at least 30 seconds by surface ECG (Kotalczyk, Lip, & Calkins, 2021) and is categorized into five types (**Table 1**) (Hindricks et al., 2021).

**Table 1. Classification of AF**

| **AF type** | **Definition** |
|---|---|
| First diagnosed | AF not diagnosed before |
| Paroxysmal | AF that terminates spontaneously or with intervention within 7 days of onset |
| Persistent | AF that lasts longer than 7 days, including episodes terminated by cardioversion after $\geq$ 7 days |

| | |
|---|---|
| Long-standing persistent | Continuous AF that lasts more than 1 year when decided to adopt a rhythm control strategy |
| Permanent | AF that is accepted by the clinician and patient and no further attempts to restore and/or maintain sinus rhythm will be undertaken |

### 2.1.2 Epidemiology of atrial fibrillation

AF is the most common cardiac arrhythmia in adults affecting approximately 69 million people (Elliott, Middeldorp, Van Gelder, Albert, & Sanders, 2023; G. Y. Lip et al., 2016). The overall prevalence of AF in adults is currently estimated between 2% to 4% (Benjamin et al., 2019) with a substantial increase worldwide being observed in recent decades (Elliott et al., 2023). According to data from the Framingham Heart Study (FHS), AF prevalence had tripled over the past 50 years (Schnabel et al., 2015). Similarly, a clinical study from the United Kingdom noted a continued rise in AF prevalence from 1998 to 2010 (Lane, Skjoth, Lip, Larsen, & Kotecha, 2017). With the improved detection of AF, the prevalence of AF is estimated to keep rising to a global epidemic in the coming decades (Kornej, Borschel, Benjamin, & Schnabel, 2020) which will impose a tremendous economic burden on healthcare systems. In Europe, the annual costs of AF were reported to range from 660 to 3280 million Euros, accounting for 0.28% to 2.60% of the total healthcare expenditure (Ball, Carrington, McMurray, & Stewart, 2013; Cotte et al., 2016). Due to the increasing prevalence of AF, the economic burden of AF is expected to rise even more in the coming decades (Wolowacz, Samuel, Brennan, Jasso-Mosqueda, & Van Gelder, 2011).

### 2.1.3 Ischemic heart failure as a leading cardiovascular risk factor for atrial fibrillation

The prevalence of AF is fueled by the increasing incidence of cardiovascular diseases, including hypertension, coronary heart disease, heart failure (HF) and other cardiovascular risk factors (Chung, Eckhardt, et al., 2020). In approximately 70% of the patients, AF is secondary to other cardiovascular conditions, most importantly ischemic heart disease or (ischemic) heart failure (Hindricks et al., 2021). The epidemiological profile of ischemic heart disease draws parallels with that of AF. Approximately 1.7% of the global population is affected by ischemic heart disease, making it the most prevalent cardiovascular disease (Roth et al., 2017). Furthermore, it

stands as the top cause of death worldwide, accounting for 16% of total deaths (Zhang et al., 2023). Ischemic heart disease can clinically manifest as acute or chronic coronary syndrome and ischemic cardiomyopathy (Khan et al., 2020). A growing body of evidence indicates that AF commonly occurs in the setting of myocardial ischemia (Violi, Soliman, Pignatelli, & Pastori, 2016). The cumulative AF incidence in patients within 5 years after myocardial infarction was noted to range from 6% to 21%, which is higher than the incidence of 3% in the general population (Belkouche et al., 2021). Myocardial ischemia can progress to ischemic heart failure (Elgendy, Mahtta, & Pepine, 2019), which further exacerbates the development of AF (Kornej et al., 2020; Luo et al., 2020). The occurrence of AF in ischemic heart failure is highly associated with the level of ejection fraction and Killip class (Jabre et al., 2011). In ischemic heart failure patients with a low left ventricular ejection fraction (< 40%), the one-year incidence of AF was found to be 32% (Jons et al., 2011). Vice versa, AF also precipitates the progression of ischemic heart failure, which increases the risk of heart failure by 20% to 51% after myocardial infarction (Jenca et al., 2021). This bidirectional pathological relationship between AF and ischemic heart failure exerts a 'fire and fury' effect complicating each other (Carlisle, Fudim, DeVore, & Piccini, 2019).

### 2.1.4 Clinical outcome and management of atrial fibrillation

The uncoordinated contraction of the heart during AF leads to various symptoms including palpitations, angina, dizziness, fatigue, dyspnea, and weakness (Brundel et al., 2022). Various complications and related comorbidities, such as stroke, heart failure, myocardial infarction, and other cardiovascular diseases, occur in AF patients leading to adverse clinical outcomes (Staerk, Sherer, Ko, Benjamin, & Helm, 2017). A 10-year follow-up study in AF patients reported an all-cause mortality rate at 10 years following the initial AF diagnosis of 30.3% (Padfield et al., 2017). Approximately half of the deaths in AF patients are due to cardiovascular events (Gomez-Outes, Suarez-Gea, & Garcia-Pinilla, 2017), and the most common cause during hospitalization was heart failure (Fauchier et al., 2015). In a large cohort study from 47 countries, heart failure as the top cause of death accounted for 30% of all deaths within one year of AF diagnosis (Healey et al., 2016). Furthermore, when AF complicates pre-existing ischemic heart disease, it confers to a joint influence resulting in higher risks of stroke, cardiogenic shock, and heart failure which doubles the overall cardiovascular mortality compared to each condition alone (Batta, Hatwal, Batta, Verma, & Sharma, 2023; Prabhu, Voskoboinik, Kaye, & Kistler, 2017).

Aiming to improve quality of life and the outcomes of severe complications, the 2020 European Society of Cardiology (ESC) guidelines for AF management proposed a holistic approach known as the Atrial fibrillation Better Care (ABC) pathway (Hindricks et al., 2021), focusing on three key aspects: 'A' (Avoid stroke), 'B' (Better symptom management), and 'C' (Cardiovascular and comorbidities risk reduction) (G. Y. H. Lip, 2017). Although the management strategy of AF has been optimized over the years, the limited efficiency of current therapeutic approaches with high AF recurrence rates has to be admitted (Chung, Refaat, et al., 2020). The principal reason for the moderate efficiency lies in an incomplete understanding of the mechanisms driving AF (Brundel et al., 2022). Thus, current therapies can be considered as 'symptomatic' rather than 'causal' treatments. With this background, more efforts to study AF mechanisms are needed to better understand the pathophysiology of AF and thus, to improve current AF prevention and treatment.

### 2.1.5 Mechanisms of atrial fibrillation

The most widely accepted underlying hallmarks of AF center to ectopic activity and reentry, which promote AF onset and maintenance (Wijesurendra & Casadei, 2019). Cardiovascular diseases can cause electrical and structural remodeling in the atria, which contribute to the development of ectopic beats and reentry mechanisms leading to AF (Jansen, Bohne, Gillis, & Rose, 2020). AF itself also aggravates electrical and structural remodeling processes, i.e. "AF begets AF" (Nattel & Harada, 2014; Wijffels, Kirchhof, Dorland, & Allessie, 1995).

Electrical remodeling of cardiac ion channels, pumps and exchangers alters atrial electrophysiological characteristics, which enhances ectopic activity and creates a reentry prone substrate (Heijman et al., 2016). The principal components of electrical remodeling include abnormal $Ca^{2+}$ handling, altered ion currents and changes in gap junction expression/distribution (Nattel & Harada, 2014):

**i) $Ca^{2+}$ handling abnormalities:** $Ca^{2+}$ handling plays an important role to maintain normal cardiac electrophysiological properties (Smith & Eisner, 2019). Under physiological conditions, $Ca^{2+}$ enters the cytosol via L-type $Ca^{2+}$ channels (LTCCs) during depolarization, triggering $Ca^{2+}$ release from the sarcoplasmic reticulum (SR) through ryanodine receptors (RyR2s), which drives cardiomyocyte contraction in systole (Bers, 2014). During diastole, RyR2s are closed and

cytosolic $Ca^{2+}$ is cleared either by $Ca^{2+}$ uptake into the SR via the SR $Ca^{2+}$-adenosine triphosphatase (SERCA2a) or by transmembrane extrusion via the $Na^{+}/Ca^{2+}$ exchanger (NCX) (Nattel & Dobrev, 2012). $Ca^{2+}$ handling abnormalities cause early and delayed afterdepolarizations (EADs and DADs) underlying ectopic firing (Denham et al., 2018). EADs are generally favored by prolonged action potential durations (APD) due to decreased repolarizing $K^{+}$ currents (Zellerhoff et al., 2009) or/and increased $Na^{+}$ currents (Lemoine et al., 2011). APD prolongation allows the recovery of L-type $Ca^{2+}$ channels which augments inward currents resulting in membrane depolarization during action potential (AP) phase 2 or 3 (Heijman, Voigt, Nattel, & Dobrev, 2014). DADs predominately occur in the context of abnormal SR $Ca^{2+}$ release events and SR $Ca^{2+}$ leak due to increased SR $Ca^{2+}$ overload or/and RyR2 dysfunction (Heijman et al., 2014). Diastolic $Ca^{2+}$ release activates NCX, producing a depolarizing transient inward current during AP phase 4 (Nattel & Dobrev, 2012).

**ii) Abnormal ion currents:** The cardiac AP is determined by the opening and closing of various transmembrane ion channels, i.e. $Na^{+}$, $K^{+}$ and $Ca^{2+}$ channels (Varro et al., 2021). Altered expression, distribution, or function of ion channels allows abnormal ion currents, which disturbs impulse generation and conduction, underlying an important contributor to AF (Nattel, Maguy, Le Bouter, & Yeh, 2007). Several studies demonstrated that gain-of-function mutations in *SCNA5* increased persistent $Na^{+}$ current and prolonged atrial APD heterogeneously, which might promote EADs and initiate AF (Avula et al., 2019; Remme, 2013). The principal and acetylcholine-dependent inward rectifier $K^{+}$ currents ($I_{K1}$ and $I_{K,Ach}$) play a pivotal role in AP repolarization and determine the resting potential (Varro et al., 2021). Increased $I_{K1}$ and $I_{K,Ach}$ are partly regulated by intracellular $Ca^{2+}$, which abbreviates the APD creating a substrate prone to reentry activity (Heijman et al., 2016). In addition, reduced L-type $Ca^{2+}$ current caused by the downregulation of Cav1.2 $\alpha$-subunit also decreases the APD, which favors AF maintenance (Yue, Melnyk, Gaspo, Wang, & Nattel, 1999).

**iii) Abnormal gap junction expression/distribution:** Electrical propagation in the working myocardium is conducted from cell to cell via connexin-based gap junctions (Leybaert et al., 2023). The gap junctions located at the intercalated disk (ID) in the atrium formed by connexin 40 and/or connexin 43, establish electrical coupling between adjacent cells by passing ions, e.g. $Na^{+}$, $K^{+}$, and $Ca^{2+}$ (Dhillon et al., 2014; Leybaert et al., 2023). Myocardial injury can affect connexin

expression or/and location (distribution from the ID to the lateral sides), disrupting electrical coupling and resulting in reentry-favoring slow conduction zones (Kato, Iwasaki, & Nattel, 2012). A brief overview of electrical remodeling leading to AF is summarized in **Figure 1**.

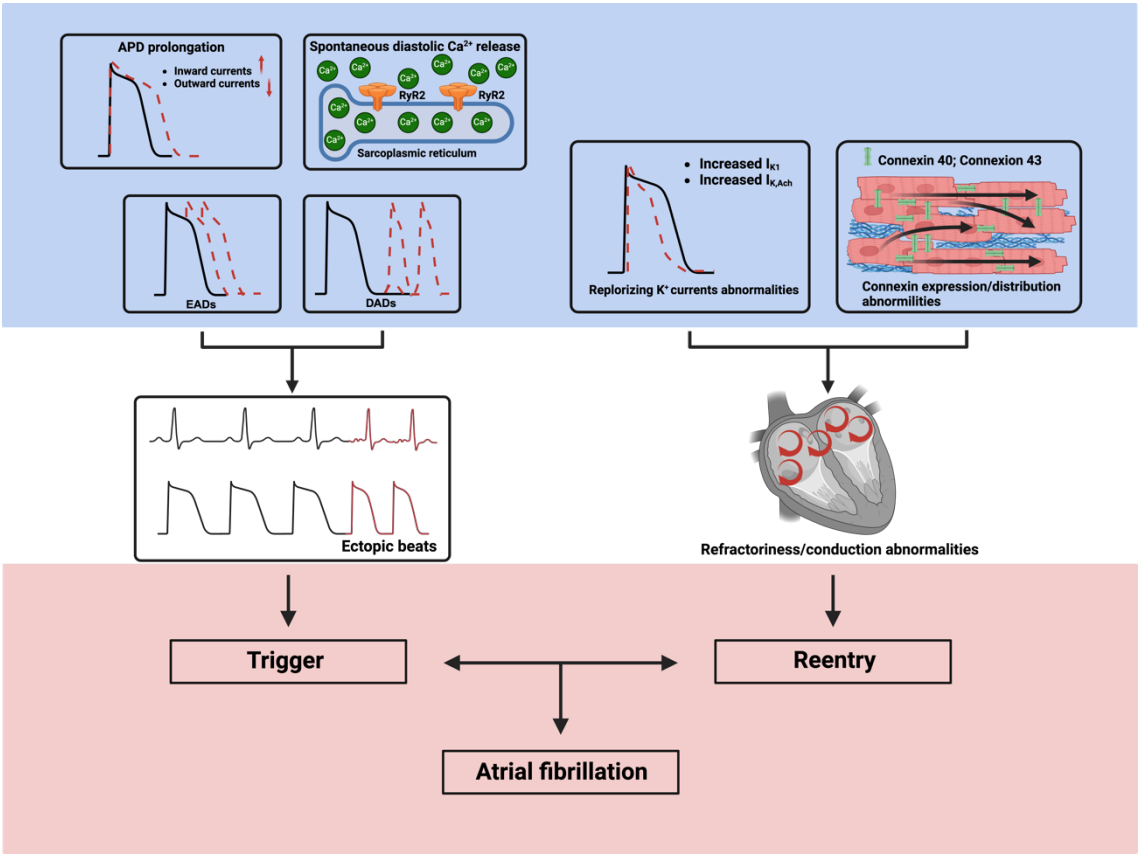


**Figure 1. Electrical remodeling leading to AF.** $Ca^{2+}$ handling abnormalities play a crucial role for AF initiation. APD prolongation and diastolic $Ca^{2+}$ release result in EADs and DADs causing ectopic firing as a trigger for AF onset. Increased repolarization $K^+$ currents lead to abbreviated refractoriness, increasing the likelihood of reentry. Additionally, the altered expression and lateralization of atrial connexin 40/ connexin 43 impair electrical conduction, thereby facilitating the generation of reentry circuits. Both trigger reentry and promote AF. Created with BioRender.com

Structural remodeling is another important contributor to the substrate for AF (Li, Fareh, Leung, & Nattel, 1999; Yue, Xie, & Nattel, 2011). The heart functions as an electrically continuous syncytium and relies on the proper architecture of muscle bundles (Heijman et al., 2014). Excessive deposition of extracellular matrix (ECM) in the interstitium interrupts cardiac muscle bundles, thus impairing normal continuity. The interposition of such fibrotic barriers in the normal myocardium facilitates reentry by slowing conduction and causing unidirectional conduction block

(Nattel, 2016; Zlochiver et al., 2008). Additionally, interposed fiber strands disrupt side-to-side cardiomyocyte connections, leading to electrical decoupling that slows transverse wavefront propagation, thereby enhancing anisotropic reentry activity (Spach, Dolber, & Heidlage, 1988). Interestingly, fibrosis-related cellular changes can also exert a direct arrhythmogenic effect. Cardiac fibroblasts play an important role in the generation of fibrosis. They are numerous in the myocardium, accounting for 75% of cardiac cell numbers but only 10% to 15% by mass (Yue et al., 2011). *In vitro*, cardiomyocytes and fibroblasts develop low-resistance electrical junctions and interact with each other electrically (Nattel, 2017). The coupling between cardiomyocytes and fibroblasts can enhance phase 4 depolarization, promoting ectopic impulse activities (Nattel, 2018). Fibroblasts activated by profibrotic stimulation can proliferate and differentiate into myofibroblasts (Nattel, 2017). It has been demonstrated that myofibroblasts interact with cardiomyocytes via heterocellular gap junctions, triggering myocyte automaticity in a density-dependent way. Higher myofibroblast density induces more intense proarrhythmic intercellular interaction (Miragoli, Salvarani, & Rohr, 2007). Furthermore, myofibroblasts can secret paracrine factors, such as TGF-β1, which was demonstrated to increase $I_{Na}$ and reduce $I_{to}$ in cultured rat myocytes (in addition to its profibrotic effects) (Kaur et al., 2013). These effects likely induce APD prolongation, exerting an “at-a-distance” proarrhythmic effect (Kaur et al., 2013). In summary, structural remodeling can contribute to AF development by influencing cardiomyocyte electrophysiology through intercellular interaction or paracrine factors (enhancing ectopic impulse generation) and by interrupting myocardium architecture (allowing reentry) **(Figure 2)**.

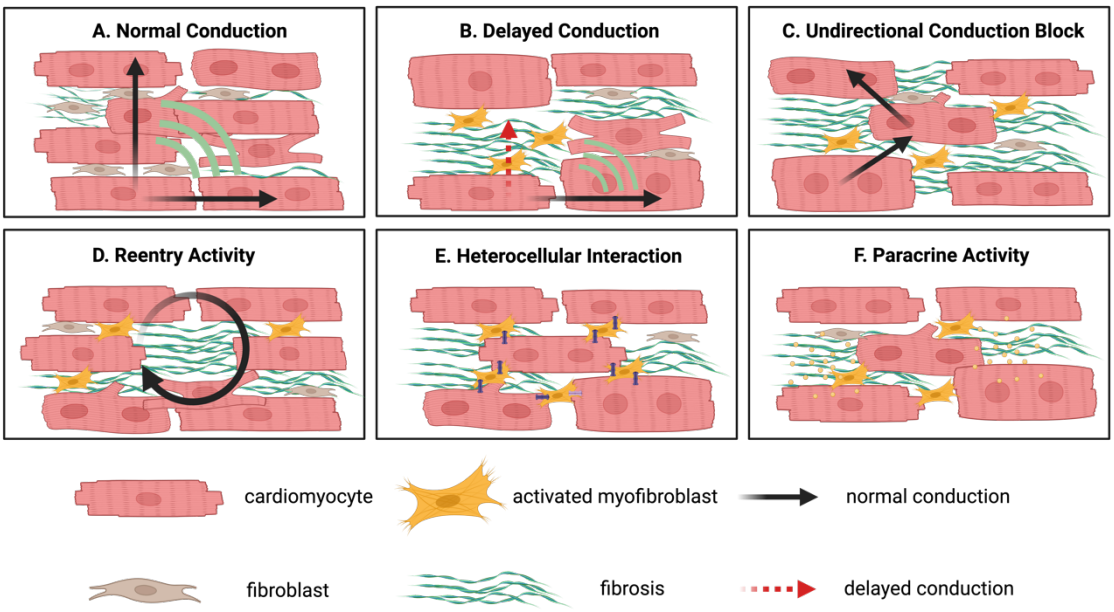


**Figure 2. Atrial structural remodeling.** (A) The normal atrium allows regular electrical conduction. (B) Increased interstitial fibrosis disrupts cardiomyocyte connections, leading to delayed electrical conduction. (C) The interposition of fibrotic barriers facilitates slow zig-zag conduction. (D) Interposed fiber strands promote reentry activity. (E) Activated myofibroblasts interact with cardiomyocytes through gap junctions, enhancing cardiomyocyte automaticity. (F) Activated myofibroblasts secret paracrine factors exerting a "at-a-distance" proarrhythmic effect. Created with BioRender.com

### 2.1.6 Shared pathological mechanisms between AF and ischemic heart failure: structural remodeling leads to a vicious cycle

The frequent co-existence of AF and ischemic heart failure results from shared pathological mechanisms (Prabhu et al., 2017), and one of the most recognized mechanisms is structural remodeling (Frederiksen et al., 2023; Kotecha et al., 2016). In ischemic heart failure, significantly increased left ventricular (LV) pressure results in elevated atrial filling pressure, in turn leading to structural remodeling of the atrial wall (Verhaert, Brunner-La Rocca, van Veldhuisen, & Vernooy, 2021). A common observation of structural remodeling in the atria during heart failure is enhanced fibrosis, which creates an arrhythmic substrate for AF initiation and maintenance (Sridhar et al., 2009). Conversely, AF resulting in rapid ventricular response can further trigger more ventricular fibrosis (Avitall, Bi, Mykytsey, & Chicos, 2008). The increased ventricular fibrosis contributes to ventricular stiffness, which leads to abnormalities in cardiac relaxation and contractility, thereby

fostering the development of ischemic heart failure (Dzeshka, Lip, Snezhitskiy, & Shantsila, 2015). In this regard, structural remodeling in ischemic heart failure facilitates the occurrence of AF. In turn, AF exacerbates structural remodeling that impairs cardiac function, creating a vicious cycle to promote the progression of both AF and heart failure (Burstein & Nattel, 2008). Given the central role of structural remodeling in this deleterious feedback cycle, targeting structural remodeling as a 'causal' treatment strategy might break the vicious cycle, thereby potentially optimizing the benefits for AF patients.

### 2.1.7 Advancements in understanding structural remodeling leading to AF

Considering fibrosis as the key component of the structural remodeling in AF patients, recent advancements in late gadolinium enhancement cardiac magnetic resonance (CMR)-guided fibrosis assessment provides novel insights into structural remodeling in AF (Sohns & Marrouche, 2020). In this context, recent studies suggest that AF might be more a state of structural remodeling linked to electrical alterations, leading to increased AF susceptibility (Cochet et al., 2018; Goette et al., 2017). This theory was also supported by the multicenter DECAAF trial, which demonstrated that the degree of atrial fibrosis was a strong predictor of AF recurrence post ablation (Akoum et al., 2015). Furthermore, a follow-up CMR study showed a significant reduction in left atrial fibrosis in patients free of AF recurrence after catheter ablation (Gal & Marrouche, 2017). Given that these conclusions suggest improved outcomes in AF patients with low atrial fibrosis, the subsequent DECAAF II trial further investigated the feasibility of a fibrosis-guided therapeutic strategy: a MRI-guided fibrosis ablation. In this randomized controlled trial, 843 patients with persistent AF were randomly treated by pulmonary vein isolation (PVI) plus MRI-guided atrial fibrosis ablation (421 patients) or PVI alone (422 patients). However, no significant differences in AF recurrence were found between the groups (43% in the fibrosis-guided ablation group vs. 46.1% in the PVI only group) (Marrouche et al., 2022). This lack of benefit from fibrosis-guided ablation strategy conflicted somewhat with the existing conclusions drawn from available data, which strongly suggested a link between fibrosis and AF. DECAAF II authors suggested that the failure to improve AF outcome by this strategy might be due to the complex arrhythmogenic propensity of fibrosis in AF, which depends not only on its extent but also its spatial distribution (Marrouche et al., 2022). Furthermore, a following *post-hoc* analysis of patients in the DECAAF II trial confirmed this notion and indicated that regional fibrosis distribution in the

left atrial appendage (LAA) linked to AF recurrence after ablation in patients who underwent fibrosis modification (Assaf et al., 2023). There is accumulating evidence indicating that structural remodeling in AF is not a homogenous process, and the individual distribution of fibrosis rather than its mere presence may determine arrhythmic risk (Caixal et al., 2021; Roy et al., 2020). These findings provide a new insight: regional substrate analysis may provide better predictive value than global analyses which do not consider spatial distribution (Althoff & Porta-Sanchez, 2023). Therefore, in the future, a more comprehensive approach should be followed to consider multiple dimensions of fibrosis characteristics, including both extent and distribution, to better understand the pathology of structural remodeling leading to AF.

## 2.2 Investigating the effect of structural remodeling on AF risk in ischemic heart failure using a porcine model

To summarize the scientific context previously described, AF is the most common cardiac arrhythmia worldwide, which has emerged as a critical public health concern with high morbidity and mortality (Elliott et al., 2023). Myocardial ischemia or (ischemic) heart failure represents the most prevalent trigger for AF in patients, with an incidence of AF after myocardial infarction reaching up to 21% (Schmitt, Duray, Gersh, & Hohnloser, 2009). AF and ischemic heart failure exhibit a strong bidirectional association by promoting the progression of each other in a vicious cycle (Anter, Jessup, & Callans, 2009). AF patients with pre-existing ischemic heart failure normally carry a worse prognosis than patients suffering from each condition alone (Chamberlain, Redfield, Alonso, Weston, & Roger, 2011). However, current AF treatment approaches, whether using antiarrhythmic drugs (AADs) or AF ablation, mainly aim to alleviate symptoms rather than address causal mechanisms, which have limited efficacy and raise safety concerns (Buist, Zipes, & Elvan, 2021; Woods & Olgin, 2014). A better understanding of AF disease mechanisms is necessary to develop novel ‘causal’ treatments with higher efficacy for treating AF in the future. Structural remodeling has been recognized as an important mechanism in AF pathogenesis (Dzeshka et al., 2015), which is also a primary factor in ischemic heart failure (Frangogiannis & Kovacic, 2020). Recent advancements in understanding structural remodeling have indicated that the spatial distribution of fibrosis rather than its mere extent determines the risk of AF recurrence in patients after AF ablation (Althoff & Porta-Sanchez, 2023). However, in ischemic heart failure,

it remains unclear whether its structural remodeling is homogeneous and whether the pattern of fibrosis distribution affects the incidence of AF. Therefore, we designed this project to investigate structural remodeling leading to AF in ischemic heart failure.

Since pigs are considered one of the most suitable animal species for electrophysiological research due to their cardiac anatomy, hemodynamics and electrophysical properties which closely resemble those of humans (Clauss et al., 2019), the project was conducted using a porcine ischemic heart failure model. In pigs, a myocardial infarction was induced by the occlusion of the left anterior descending artery (LAD) for 90 minutes. After 30 days, hemodynamic parameters were measured to evaluate cardiac function. Electrophysiological studies were conducted to measure conduction properties and to perform burst pacing to assess AF inducibility. Masson-Trichrome and immunofluorescence staining were performed to assess structural remodeling in different heart regions including atrial appendages, atrial free walls, and ventricles.

# 3. Summary

## 3.1 Results of the project

In this project, we studied structural remodeling in a translational pig model. In this model, the LAD is occluded for 90 minutes, mimicking the typical situation in patients with ST elevation myocardial infarction undergoing emergency revascularization. In this pig model, we observed a significant heart failure phenotype similar to that observed in humans with ischemic cardiomyopathy, characterized by a reduced ejection fraction and an elevated left ventricular diastolic pressure (LVEDP). Furthermore, we found a significantly enhanced AF susceptibility in pigs with ischemic heart failure. These findings justified using this model to investigate proarrhythmic structural remodeling in ischemic heart failure.

To explore potential mechanisms underlying ischemia-mediated AF susceptibility, we further performed electrophysiological studies *in vivo* and histologic analyses to assess electrical and structural remodeling. We found no significant alterations in electrophysiological properties including atrioventricular conduction (Wenkebach cycle length (AV WCL), atrioventricular node effective refractory periods (AVERP)) or atrial conduction properties (atrial effective refractory periods (AERP)) in pigs with ischemic heart failure. This finding suggested that electrical remodeling might not be a major driver for increased AF susceptibility in ischemic heart failure. Additionally, histologic experiments showed a significant increase in interstitial fibrosis in both left and right atrial appendages rather than in the atrial free walls and ventricles in pigs with ischemic heart failure, which was also paralleled by an increase in the number of activated myofibroblasts. To further investigate whether the overall content of increased fibrosis has an impact on AF susceptibility, we assessed the correlation between fibrosis amount and the number of AF episodes but found no significant correlation. This result suggested that overall fibrosis levels might not determine AF vulnerability in this model. However, given that the marked regional differences in fibrosis distribution were also observed in the left and right atrium, we further investigated whether cross-regional difference in fibrosis was associated with AF vulnerability. Interestingly, we found a significant correlation between left atrial fibrosis difference and the number of AF episodes. This finding implied that left atrial fibrosis heterogeneity rather than the overall atrial fibrosis level may determine AF susceptibility in ischemic heart failure.

In conclusion, we demonstrate heterogeneous cross-regional fibrosis in the left atrium as a potential mechanism for AF in a pig model of ischemic heart failure. These results confirm previous studies that similarly underscore the importance of localized fibrosis heterogeneity in arrhythmogenesis. For example, in a rat myocardial infarction model, heterogeneous fibrosis in the infarction border zone was found to link to the generation of reentry through 3-dimensional topology and image-based computer modeling (Rutherford, Trew, Sands, LeGrice, & Smaill, 2012). Moreover, a larger degree of fibrosis heterogeneity has been shown to more likely result in arrhythmias *in silico* (Kazbanov, ten Tusscher, & Panfilov, 2016). Our results demonstrate that in a pig model of ischemic heart failure, increased susceptibility to AF is associated with the heterogeneity of fibrosis distribution across the left atrium rather than the overall extent of fibrosis. This finding suggests that not fibrosis extent but rather its heterogenous distribution in the atrium, determines increased AF susceptibility in ischemic heart failure. It emphasizes the important role of atrial fibrosis distribution in AF generation, which is in line with the most recent finding from a *post hoc* analysis of patients in the DECAAF II clinical trial. This study demonstrated that regional variation of fibrosis in the left atrium was associated with AF recurrence in patients after AF ablation (Assaf et al., 2023). Thus, our study provides an experimental insight that the detection of fibrosis heterogeneity across the atrium *in vivo* by cardiac MRI or voltage mapping is important and may offer benefits for the preventive and therapeutic strategies in patients at risk of developing AF.

## 3.2 My contribution to the project

In the project, I participated in the pig *in vivo* experiments as an assistant, including preparatory work for the procedures and support for investigations during the operation. I performed sample preparation after surgeries including tissue sampling, fixation, and cutting. Additionally, I performed histological experiments including staining and microscopy. Furthermore, I collected and analyzed all results obtained from the investigations *in vivo* and the histological experiments. Subsequently, I discussed these results with my supervisor. We conceived the manuscript together. I prepared all figures and wrote the manuscript.

# 4. Zusammenfassung

## 4.1 Ergebnisse des Projekts

In diesem Projekt haben wir in einem translationalen Schweinemodell strukturelles Remodeling untersucht. In unserem Modell wird die linke vordere absteigende Koronararterie (engl. left anterior descending artery, LAD) für 90 Minuten verschlossen, um die typische Situation bei Patienten mit ST-Hebungs-Myokardinfarkt mit Wiedereröffnung des akuten Gefäßverschlusses nachzuahmen. Ähnlich wie bei Menschen mit ischämischer Kardiomyopathie, beobachteten wir einen signifikanten Herzinsuffizienz-Phänotyp, gekennzeichnet durch eine reduzierte Ejektionsfraktion und einen erhöhten linksventrikulären enddiastolischen Druck (LVEDP). Darüber hinaus stellten wir eine signifikant erhöhte Induzierbarkeit von Vorhofflimmern (engl. atrial fibrillation, AF) bei Schweinen mit ischämischer Herzinsuffizienz fest. Diese Befunde rechtfertigten die Verwendung dieses Modells zur Untersuchung des proarrhythmischen strukturellen Remodelings bei ischämischer Herzinsuffizienz.

Um potenzielle Mechanismen zu erforschen, die der durch Ischämie vermittelten AF-Anfälligkeit zugrunde liegen, führten wir elektrophysiologische Studien *in vivo* und histologische Analysen durch, um elektrische und strukturelle Veränderungen zu bewerten. Wir fanden keine signifikanten Veränderungen der elektrophysiologischen Eigenschaften. Unter anderem waren die atrioventrikuläre (AV) Überleitung (Wenckebach-Zykluslänge (AV WCL), effektive Refraktärzeit des AV-Knotens (AVERP)) oder die atrialen Überleitungseigenschaften (atriale effektive Refraktärzeit (AERP)) bei Schweinen mit ischämischer Herzinsuffizienz unverändert. Diese Befunde deuteten darauf hin, dass elektrisches Remodeling möglicherweise nicht zu den Haupttreibern für die erhöhte AF-Anfälligkeit bei ischämischer Herzinsuffizienz zählt. Allerdings zeigten histologische Untersuchungen eine signifikante Zunahme der interstitiellen Fibrose sowohl im linken als auch im rechten Vorhofohr bei Schweinen mit ischämischer Herzinsuffizienz, nicht jedoch in den freien Vorhofwänden und Ventrikeln, was auch mit einer Zunahme der Anzahl aktivierter Myofibroblasten einherging. Um weiter zu untersuchen, ob die Gesamtmenge der interstitiellen Fibrose einen Einfluss auf die AF-Anfälligkeit hat, versuchten wir die Korrelation zwischen der Menge an Fibrose und der Anzahl der AF-Episoden zu beurteilen, fanden jedoch keine signifikante Korrelation. Dieses Ergebnis deutete darauf hin, dass die Gesamtfibrose

möglicherweise nicht die AF-Anfälligkeit in diesem Modell bestimmt. Da jedoch auch ausgeprägte regionale Unterschiede in der Verteilung der Fibrose im linken und rechten Vorhof beobachtet wurden, untersuchten wir weiter, ob regionale Unterschiede der Fibrosierung mit der AF-Anfälligkeit in Zusammenhang stehen. Interessanterweise fanden wir eine signifikante Korrelation zwischen der linksatrialen Fibroseunterschiede und der Anzahl der AF-Episoden. Dieser Befund deutete darauf hin, dass die Heterogenität der linksatrialen Fibrose und nicht die Gesamtmenge der atrialen Fibrose die AF-Anfälligkeit bei ischämischer Herzinsuffizienz bestimmen könnte.

Zusammenfassend konnten wir zeigen, dass heterogene regionale Fibrose im linken Vorhof einen potenziellen Mechanismus für AF in einem Schweinemodell der ischämischen Herzinsuffizienz darstellt. Diese Ergebnisse bestätigen frühere Studien, die ebenfalls die Bedeutung der lokalen Heterogenität der Fibrose bei der Arrhythmogenese hervorheben. Beispielsweise wurde in einem Myokardinfarkt-Modell in der Ratte durch 3D-Topologie und bildbasierte Computermodellierung festgestellt, dass heterogene Fibrose in der Infarktgrenzzone mit der Entstehung von Reentry verbunden ist (Rutherford, Trew, Sands, LeGrice, & Smaill, 2012). Darüber hinaus wurde gezeigt, dass eine größere Fibrose-Heterogenität *in silico* eher zu Arrhythmien führt (Kazbanov, ten Tusscher, & Panfilov, 2016). Unsere Ergebnisse zeigen, dass in einem Schweinemodell der ischämischen Herzinsuffizienz die erhöhte AF-Anfälligkeit mit der Heterogenität der Fibrose im linken Vorhof und nicht mit der Gesamtmenge der Fibrose verbunden ist. Dieser Befund legt nahe, dass nicht das Ausmaß der Fibrose, sondern deren heterogene Verteilung im Vorhof die erhöhte AF-Anfälligkeit bei ischämischer Herzinsuffizienz bestimmt. Er unterstreicht die wichtige Rolle der Verteilung der atrialen Fibrose bei der AF-Entstehung, was im Einklang mit den neuesten Erkenntnissen einer *post hoc* Analyse von Patienten in der DECAAF II-Studie steht. Diese Studie zeigte, dass regionale Unterschiede der Fibrose im linken Vorhof mit einem wiederholten Auftreten von Vorhofflimmern bei Patienten nach Ablation assoziiert waren (Assaf et al., 2023). Somit liefert unsere Studie einen experimentellen Einblick, dass die Erkennung der Heterogenität der Fibroseverteilung im Vorhof *in vivo* mittels kardialer MRT oder sog. Voltage-Mapping wichtig sein könnte und Vorteile für präventive und therapeutische Strategien bei Patienten mit einem erhöhten Vorhofflimmerrisiko bieten könnte.

## 4.2 Mein Beitrag zum Projekt

In diesem Projekt habe ich als Assistent an den *in vivo* Eingriffen mit Schweinen teilgenommen und leistete Unterstützung während der Untersuchungen. Auch beteiligte ich mich an den Vorbereitungsarbeiten für die Experimente und an der Probenverarbeitung nach den Operationen. Dazu zählten Gewebeentnahme, Fixierung und Schneiden. Zusätzlich führte ich histologische Experimente, einschließlich des Schneidens, der Färbung und der Mikroskopie durch. Darüber hinaus sammelte und analysierte ich alle Ergebnisse aus den *in vivo* Untersuchungen und den histologischen Experimenten. Anschließend besprach ich diese Ergebnisse mit meinem Betreuer. Wir konzipierten gemeinsam das Manuskript. Ich bereitete alle Abbildungen vor und schrieb das Manuskript.

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# Atrial fibrosis heterogeneity is a risk for atrial fibrillation in pigs with ischaemic heart failure

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## Abstract

**Background:** Atrial fibrillation (AF) is the most common arrhythmia and is associated with considerable morbidity and mortality. Ischaemic heart failure (IHF) remains one of the most common causes of AF in clinical practice. However, ischaemia-mediated mechanisms leading to AF are still incompletely understood, and thus, current treatment approaches are limited. To improve our understanding of the pathophysiology, we studied a porcine IHF model.

**Methods:** In pigs, IHF was induced by balloon occlusion of the left anterior descending artery for 90 min. After 30 days of reperfusion, invasive haemodynamic measurements and electrophysiological studies were performed. Masson trichrome and immunofluorescence staining were conducted to assess interstitial fibrosis and myofibroblast activation in different heart regions.

**Results:** After 30 days of reperfusion, heart failure with significantly reduced ejection fraction (left anterior obique 30°, 34.78 ± 3.29% [IHF] vs. 62.03 ± 2.36% [control], $p < .001$; anterior–posterior 0°, 29.16 ± 3.61% vs. 59.54 ± 1.09%, $p < .01$) was observed. These pigs showed a significantly higher susceptibility to AF (33.90% [IHF] vs. 12.98% [control], $p < .05$). Histological assessment revealed aggravated fibrosis in atrial appendages but not in atrial free walls in IHF pigs (11.13 ± 1.44% vs. 5.99 ± .86%, $p < .01$ [LAA], 8.28 ± .56% vs. 6.01 ± .35%, $p < .01$ [RAA]), which was paralleled by enhanced myofibroblast activation (12.09 ± .65% vs. 9.00 ± .94%, $p < .05$ [LAA], 14.37 ± .60% vs. 10.30 ± 1.41%, $p < .05$ [RAA]). Correlation analysis indicated that not fibrosis per se but its cross-regional heterogeneous distribution across the left atrium was associated with AF susceptibility ($r = .6344$, $p < .01$).

**Conclusion:** Our results suggest that left atrial cross-regional fibrosis difference rather than overall fibrosis level is associated with IHF-related AF susceptibility, presumably by establishing local conduction disturbances and heterogeneity.

Philipp Tomsits and Sebastian Clauss contributed equally to the work and share senior authorship.

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**KEYWORDS**

atrial fibrillation, fibrosis, ischaemic heart failure, left atrial fibrosis, myocardial infarction, pig model, structural remodelling

## 1 | INTRODUCTION

More than 33 million people worldwide are suffering from atrial fibrillation (AF), the most common sustained cardiac arrhythmia and this number is still increasing by around 5 million new cases per year.[1] AF is associated with numerous complications, most importantly with a fivefold increased risk for stroke,[1] a doubling in dementia risk, a tripling in heart failure risk as well as a doubling in mortality.[1] Current treatment options are limited due to insufficient effectivity or relevant side effects, including proarrhythmic effects, most probably because therapies are not targeting causal mechanisms underlying AF.[1–4] Since AF pathophysiology is complex and still not fully understood, an improved understanding of AF disease mechanisms is the key to identifying novel targets and developing improved treatment approaches.[2–4]

Different mechanistic paradigms summarize our understanding of AF pathophysiology, including the establishment of a vulnerable substrate and enhanced triggered activity as a result of various proarrhythmic remodelling processes.[2–4] Enhanced automaticity or altered calcium homeostasis may lead to focal ectopic firing, whereas action potential shortening or the establishment of conduction barriers by atrial fibrosis result in a vulnerable substrate allowing reentry.[2–4] In around 70% of the patients, AF is thought to be secondary to the underlying heart diseases, most importantly myocardial ischaemia or (ischaemic) heart failure.[1] According to several clinical trials, 6.8%–21% of the patients presenting with an acute myocardial infarction will develop AF.[5,6]

Previously, we have demonstrated significantly increased left atrial fibrosis in pigs with ischaemic heart failure (IHF) and AF.[7] However, it remains unclear, whether fibrosis is homogeneously distributed across the atrium and whether the distribution pattern has an impact on AF susceptibility in IHF.

Since pigs are among the most valuable species in electrophysiology research due to their porcine anatomy, haemodynamics and conduction properties, which resemble the human situation quite well, especially when compared to rodent models,[8,9] we studied the above-mentioned porcine model of IHF to further investigate the ischaemia-mediated proarrhythmic atrial remodelling leading to AF.

## 2 | MATERIAL AND METHODS

### 2.1 | Animals

German landrace pigs (age range from 3 to 4 months, average body weight 38.3 ± 2.8 kg) were obtained from *Landwirtschaftliche Forschungsstation Thalhausen*, Technical University of Munich, Kranzberg, Germany, *Moorversuchsgut*, Ludwig-Maximilians-University Munich, Oberschleissheim, Germany and *Lehr- und Versuchsgut der LMU*, Ludwig-Maximilians-University, Munich, Oberschleissheim, Germany. Instrumentation of pigs was conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals' and was approved by the Regierung von Oberbayern (ROB-55.2-2631.Vet_02–10-130 and ROB-55.2-2532.Vet_02–15-209). Twenty-two pigs with IHF and 18 age- and weight-matched control pigs without IHF were included in the study. As animal guidelines evolve and tend to get stricter, it has never been as important to respect the 3R (replacement, reduction and refinement) principle when designing a study. Thus, the current manuscript uses in vivo tracings as well as tissue samples from a previous study,[7] in addition to new animals. The entire tissue workup is done from first-use tissue samples, and all analyses shown are new and original; none of the data has been published elsewhere.

### 2.2 | Pig model

Model induction was previously described in detail.[7,10] In brief, pigs were sedated (by intramuscular ketamine [20 mg/kg], azaperone [10 mg/kg] and atropine [.05 mg/kg]), anaesthetized (induced by intravenous midazolam [.5 mg/kg] and maintained by intravenous fentanyl [.05 mg/kg], propofol [.5 mg/kg/min]) and mechanically ventilated (initial parameters: peak pressure 18–25 mmHg, peep 5 mmHg, tidal volume 6–8 mL/kg, FiO2 21%; further adjustments according to regular blood gas test results). The right external jugular vein and right carotid artery were surgically prepared, and sheaths were inserted (9F and 8F, respectively). Myocardial infarction was induced by occlusion of the left anterior descending coronary artery distal to the first diagonal branch for 90 min by a PTCA balloon. The

correct localization of the occlusion was confirmed by angiography. Afterwards, sheaths were removed, the wound was closed and pigs were transferred to the stalls, where they were kept and monitored until the final experiment 30 days later.

## 2.3 | Invasive haemodynamic measurements

Thirty days after myocardial infarction, invasive haemodynamic measurements were performed as previously described,[7,10] including left ventricular laevocardiography in two planes (30° left anterior obique [LAO] and 0° anterior–posterior [AP] projection) as well as measurement of left ventricular systolic and enddiastolic pressure, pulmonary capillary wedge pressure, pulmonary artery pressure, right ventricular pressure and right atrial pressure. All measurements were performed under high right atrial electrical stimulation at 130 bpm (with 1:1 atrioventricular [AV] conduction). Control pigs were assessed in the same fashion but without prior myocardial infarction.

## 2.4 | Electrocardiography and electrophysiological studies

Electrophysiological studies were performed following a standardized protocol as reported previously.[7,10] Briefly, a multipolar electrophysiology catheter was placed at the high right atrium to allow atrial stimulation (at 2× pacing threshold). The Wenckebach point was assessed by a progressively decreased pacing cycle length until the atrial signal was no longer conducted 1:1 via the AV node. The effective refractory periods of the atrium and the AV node (AERP and AVERP) were determined by a train of eight fixed stimulations followed by a single premature stimulation at a stepwise decreased pacing cycle length until the premature stimulation failed to induce an atrial signal (AERP) or to propagate to the ventricle via the AV node (AVERP). Six fixed pacing cycle lengths, including 500, 450, 400, 350, 300 and 250 ms, were used to perform ERP measurements. Finally, arrhythmias were induced by rapid burst pacing (at the 2× pacing threshold) at 1200 bpm for 6 s. Per pig, 10 burst pacings were performed. AF was defined as an atrial arrhythmic episode with irregular RR intervals longer than 10 s.

## 2.5 | Histology

After the electrophysiological study, pigs were euthanized in deep sedation and hearts were removed. Tissue samples from left and right atrial appendages, atrial free walls and ventricular free walls (remote to the infarct zone) were harvested, fixed in 4% formaldehyde and embedded in paraffin for the following histological staining.

Masson's trichrome staining was performed in paraffin-embedded sections (5 μm) using a Masson-Goldner Trichrome staining kit (Carl Roth GmbH + Co. KG, Germany). After the staining, pictures were acquired at a high-resolution microscope (DM6 B, Leica, Germany) with a 40-fold objective. Ten nonoverlapping pictures per region were analysed by three blinded observers using Adobe Photoshop software. Interstitial fibrosis was assessed by pixel counting using Adobe Photoshop software (the percentage of fibrosis was calculated as the number of interstitial fibrosis pixels [excluding perivascular fibrosis] divided by the total number of pixels per picture). Per pig, 10 nonoverlapping pictures per region were analysed.

Immunofluorescence staining was performed in paraffin-embedded sections (5 μm) by the following steps: The tissue was rehydrated by immersing in xylene, 100% ethanol, 95% ethanol, 70% ethanol and 1× phosphate-buffered saline for 5 min per step. Antigen retrieval was performed at 95°C for 20 min. Then .1% Triton X-100 solution was used for tissue permeabilization, and block buffer (1% goat serum in phosphate-buffered saline) was used to block nonspecific staining for 1 h at room temperature. After blocking, tissue was incubated with anti-α-SMA antibody (Cat#ab150301, Abcam, UK, 1:500) and anti-α-actinin antibody (Cat#A7811, Sigmal-Aldrich, USA, 1:200) overnight at 4°C. After washing three times in washing buffer (5% bovine serum albumin +.1% Tween 20 in phosphate-buffered saline) for 5 min, tissue was subsequently incubated with Alexa Fluor™ 488 anti-mouse secondary antibody (Cat#4408S, Cell Signaling Technology, USA, 1:100) and Alexa Fluor™ 647 anti-rabbit secondary antibody (Cat#A21245, Thermo Fisher Scientific, USA, 1:100) for 1 h each. The diluted DAPI solution (Cat#H3570, Thermo Fisher Scientific, USA, 1:1000 in phosphate-buffered saline) was applied to incubate tissue for 10 min following the secondary antibody incubation. After washing three times in phosphate-buffered saline for 5 min, slides were covered by fluorescence mounting medium (Cat#S3023, Dako, Denmark) and sealed by glass coverslips. Immunofluorescence staining pictures were acquired at a high-resolution microscope (DM6 B, Leica, Germany) with a 40-fold objective. Five nonoverlapping pictures per region were taken and analysed by using ImageJ software (National Institutes of Health). The number of α-SMA-positive cells and the total cell number were quantified using the ImageJ counting tool. The percentage of α-SMA-positive cells was calculated by the number of positive cells divided by the total number of cells. Per pig, five nonoverlapping pictures per region were analysed.

### 2.6 | Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed using GraphPad Prism 9. Differences between two groups were calculated by unpaired *t*-tests. Categorical variables were compared by Fisher's exact test. A significant correlation was identified by the Pearson correlation. A *p*-value of <.05 was considered statistically significant.

## 3 | RESULTS

### 3.1 | Left anterior descending coronary artery occlusion leads to significant IHF

Myocardial infarction in pigs resulted in a significantly reduced ejection fraction compared to control pigs (LAO 30°, 34.78 ± 3.29% vs. 62.03 ± 2.36%, ***$p < .001$; Figure 1A and AP 0°, 29.16 ± 3.61% vs. 59.54 ± 1.09%, **$p < .01$; Figure 1B). Consistently, left ventricular enddiastolic pressure (LVEDP, 18.02 ± 1.16 mmHg vs. 14.11 ± 1.31 mmHg, *$p < .05$; Figure 1D), pulmonary capillary wedge pressure (PCWP, 21.34 ± .97 mmHg vs. 16.40 ± 1.33 mmHg, **$p < .01$; Figure 1E) and right atrial pressure (16.30 ± .85 mmHg vs. 13.40 ± .79 mmHg, *$p < .05$; Figure 1H) were significantly increased in IHF pigs compared to control pigs.

There were no differences in left ventricular systolic pressure, pulmonary artery pressure and right ventricular pressure between IHF and control pigs (Figure 1C,F,G, respectively).

### 3.2 | IHF results in an increased susceptibility for AF without affecting electrical conduction

Pigs with IHF showed a clear arrhythmic phenotype. Compared to control pigs, IHF pigs demonstrated a significantly higher inducibility of AF (33.90% of IHF pigs vs. 12.98% of control pigs, *$p < .05$; Figure 2A) and a significantly higher percentage of burst pacing attempts resulting in AF (31.72% vs. 15.06%, ***$p < .001$; Figure 2B). To further investigate electrical conduction properties,

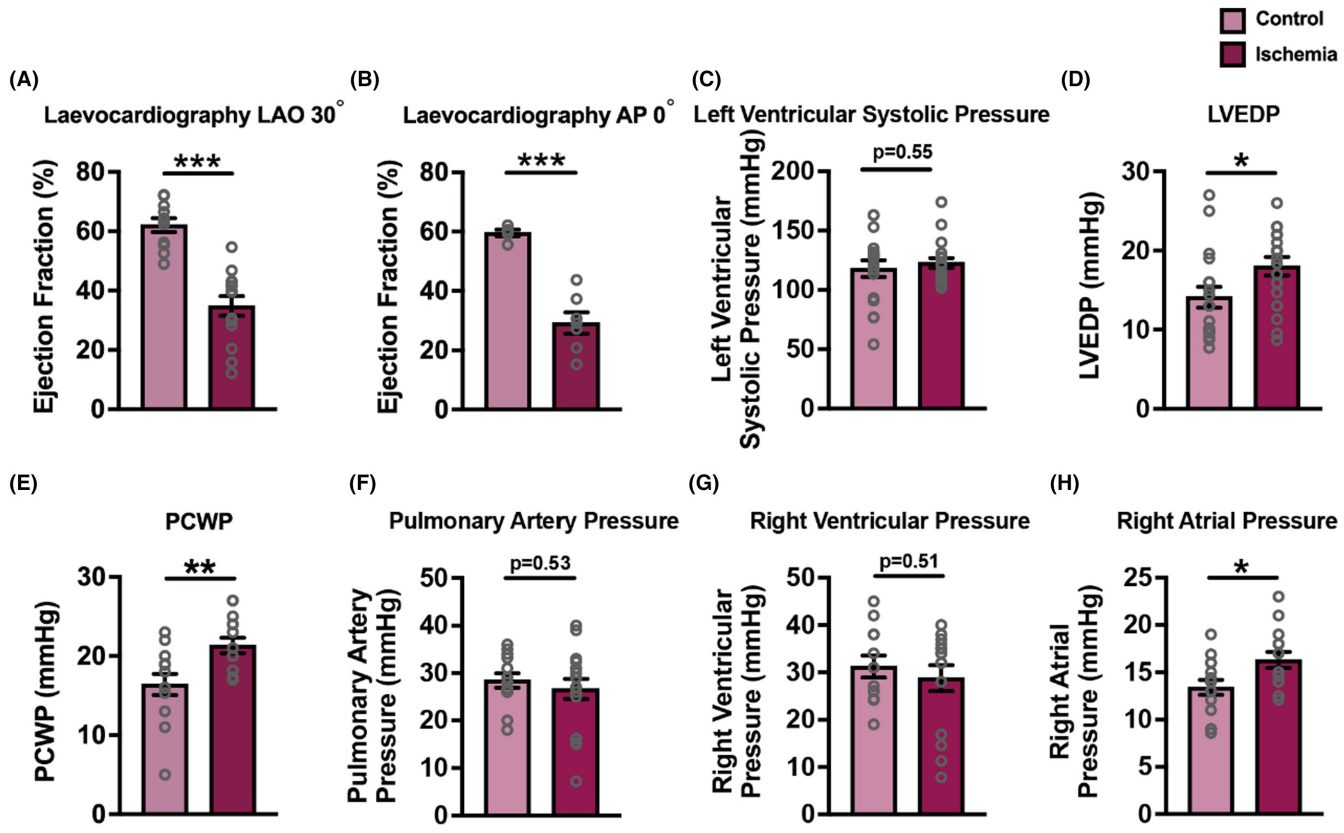


FIGURE 1 Myocardial infarction resulted in significant ischaemic heart failure as observed by haemodynamic measurements. (A) Left ventricular ejection fraction assessed by laevocardiography at left anterior obique 30° and (B) anterior–posterior 0°; (C) left ventricular systolic pressure; (D) left ventricular enddiastolic pressure (LVEDP); (E) pulmonary capillary wedge pressure (PCWP); (F) pulmonary artery pressure; (G) right ventricular pressure; and (H) right atrial pressure. Bar graphs represent the mean ± SEM, and grey circles represent the data of individual pigs. Unpaired *t*-tests were applied. *$p < .05$, **$p < .01$, ***$p < .001$.

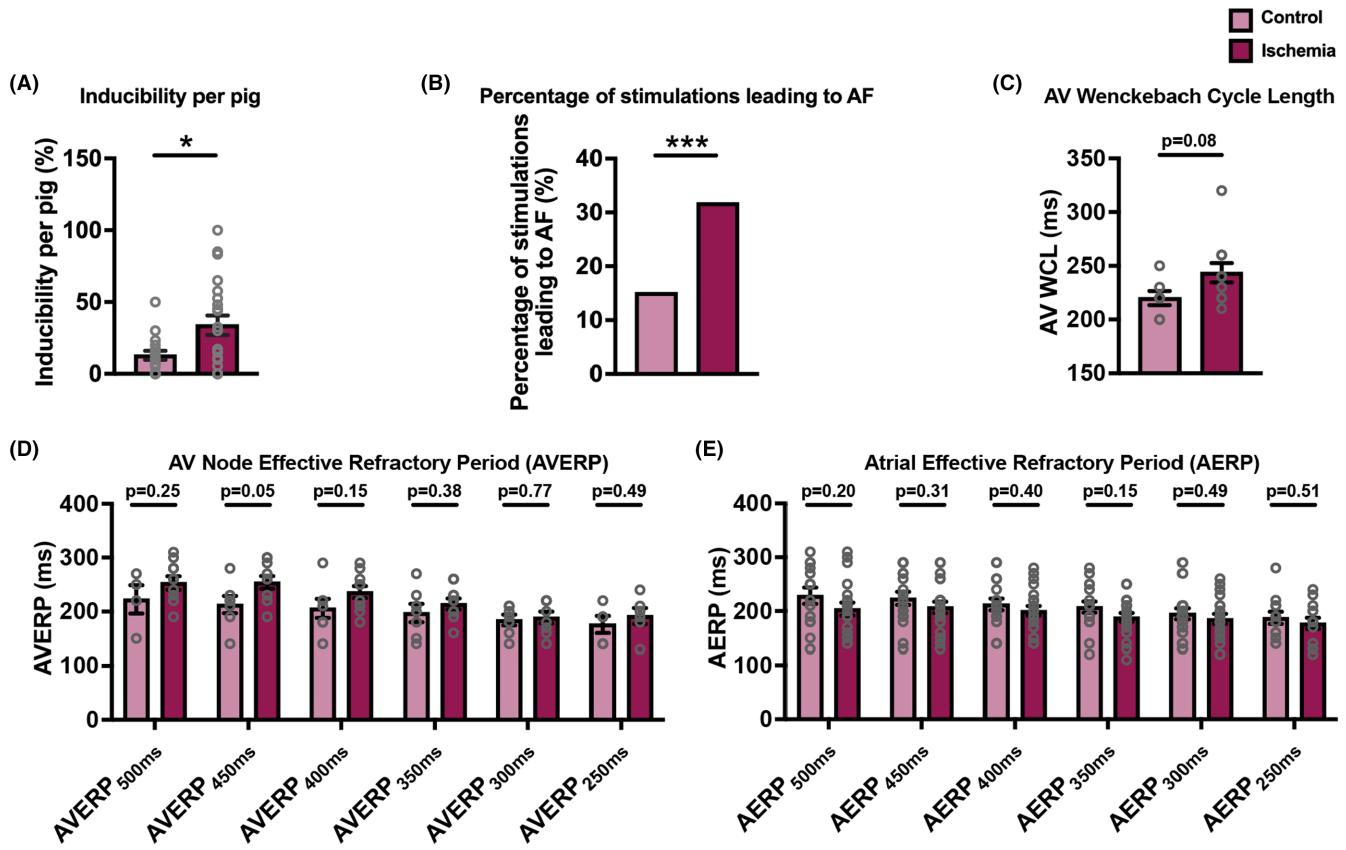


FIGURE 2 Arrhythmia phenotype and electrophysiological properties in control and ischaemic heart failure pigs. (A) Inducibility of atrial fibrillation (AF) per pig; (B) percentage of bursts inducing AF; (C) atrioventricular (AV) Wenckebach cycle length; (D) effective refractory period of the AV node (AVERP); (E) effective refractory period of the atrium (AERP). Bar graphs represent the mean ± SEM where applicable, and grey circles represent the data of individual pigs. An unpaired *t*-test was applied in (A), (C), (D) and (E), and Fisher's exact test was applied in (B). $*p < .05$, $***p < .001$.

invasive electrophysiological studies were performed 30 days after myocardial infarction (IHF pigs) and in age- and weight-matched pigs without myocardial infarction (control pigs). AV Wenckebach cycle length (AV WCL) was prolonged in IHF pigs without reaching statistical significance (243.6 ± 9.0 ms vs. 220.0 ± 6.5 ms, $p = .08$; Figure 2C). Effective refractory periods of the AV node (AVERP, Figure 2D) and the atrium (AERP, Figure 2E) did not differ between groups.

## 3.3 | IHF results in heterogeneous distribution of interstitial atrial fibrosis

Structural remodelling, especially the development of fibrosis, has been identified as one of the hallmarks of AF pathophysiology. Thus, we quantified interstitial fibrosis in different regions of the heart separately, both in control and IHF pigs. We observed a significant increase of fibrosis in left atrial appendage and right atrial appendage in IHF pigs (11.13 ± 1.44% vs. 5.99 ± .86%, $**p < .01$, Figure 3A,B and 8.28 ± .56% vs. 6.01 ± .35%, $**p < .01$, Figure 3A,E). In the left atrial free wall (Figure 3A,C), right atrial free wall (Figure 3A,F), left ventricular free wall (Figure 3A,D) and right ventricular free wall (Figure 3A,G), however, the overall level of fibrosis did not significantly differ between control and IHF pigs, demonstrating a heterogeneous distribution of fibrosis in IHF.

## 3.4 | Aggravated interstitial fibrosis in IHF is paralleled by increased numbers of myofibroblasts in atrial appendages

Activated myofibroblasts are the main source of fibrosis.[11] Thus, we quantified myofibroblasts by counting α-SMA-positive cells[11] in different regions of the heart, both in control and IHF pigs. We observed a significant increase of myofibroblast numbers only in left atrial appendage and right atrial appendage of IHF pigs (12.09 ± .65% vs. 9.00 ± .94%, $*p < .05$, Figure 4A,B and 14.37 ± .60% vs. 10.30 ± 1.41%, $*p < .05$, Figure 4A,E), whereas myofibroblast numbers in left atrial free wall (Figure 4A,C), right atrial free wall (Figure 4A,F), left ventricular free wall

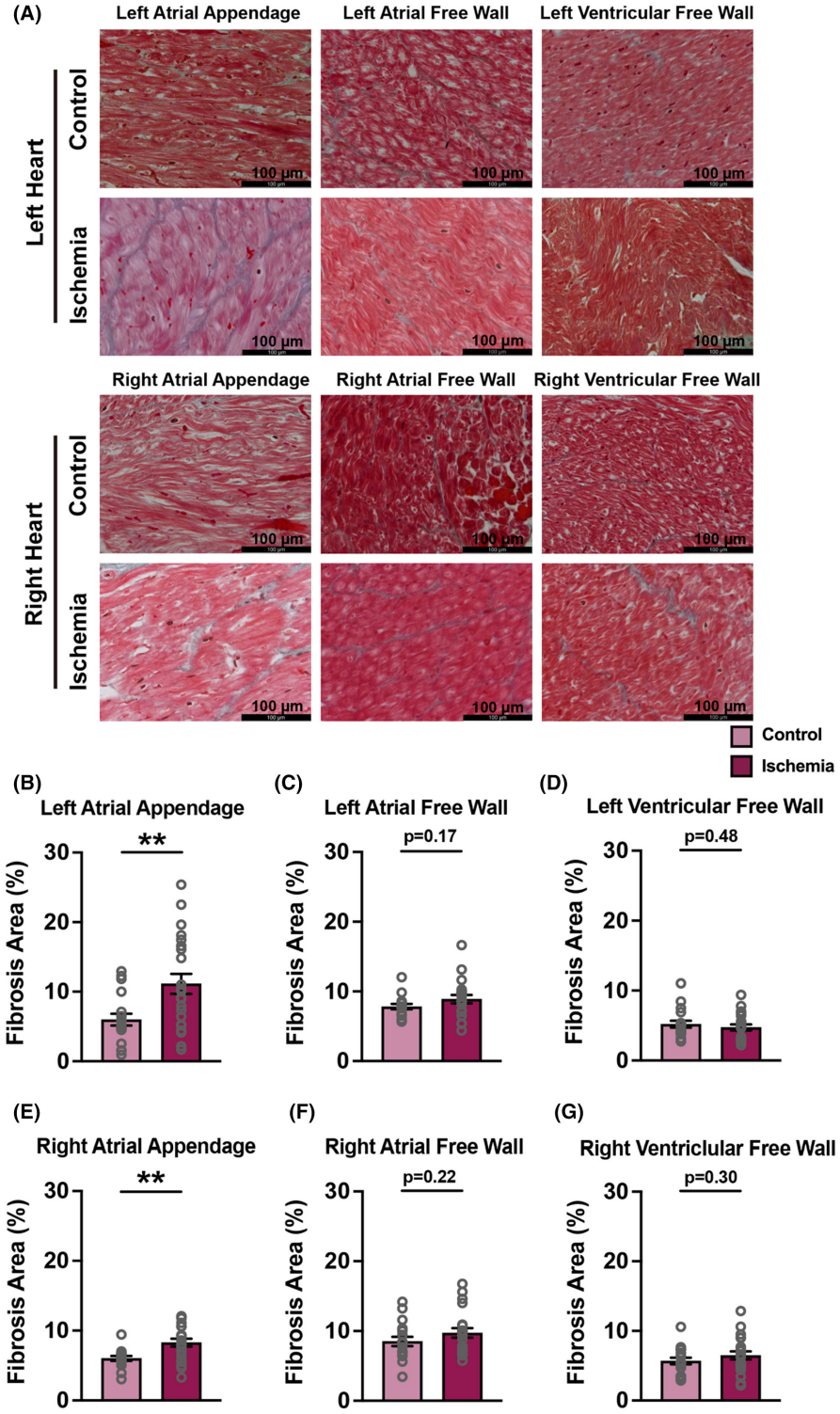


**FIGURE 3** Quantification of interstitial fibrosis. (A) Representative images of Masson trichrome-stained tissue slides in control and ischaemic heart failure pigs, quantification of interstitial fibrosis in (B) left atrial appendage, (C) left atrial free wall, (D) left ventricular free wall, (E) right atrial appendage, (F) right atrial free wall and (G) right ventricular free wall. Bar graphs represent the mean ± SEM, and grey circles represent the data of individual pigs. Unpaired *t*-tests were applied. $^{**}p < .01$.

(Figure 4A,D) and right ventricular free wall (Figure 4A,G) did not differ between control and IHF pigs.

## 3.5 | Left atrial cross-regional fibrosis difference rather than the overall level of fibrosis is associated with AF

Ischaemia resulted in significantly enhanced fibrosis in atrial appendages. However, in pigs with IHF, the overall level of fibrosis did not correlate with the number of AF episodes in these pigs, neither in atrial appendages (Figure 5A,D) nor in the other regions (Figure 5B,C,E,F). Since we observed that fibrosis was not homogenously distributed across the atria, we calculated the fibrosis difference between appendages and free walls in both the left and right atriums and investigated a potential correlation between fibrosis differences and AF episode numbers. This analysis revealed a significant correlation between fibrosis differences and AF episode numbers in the left

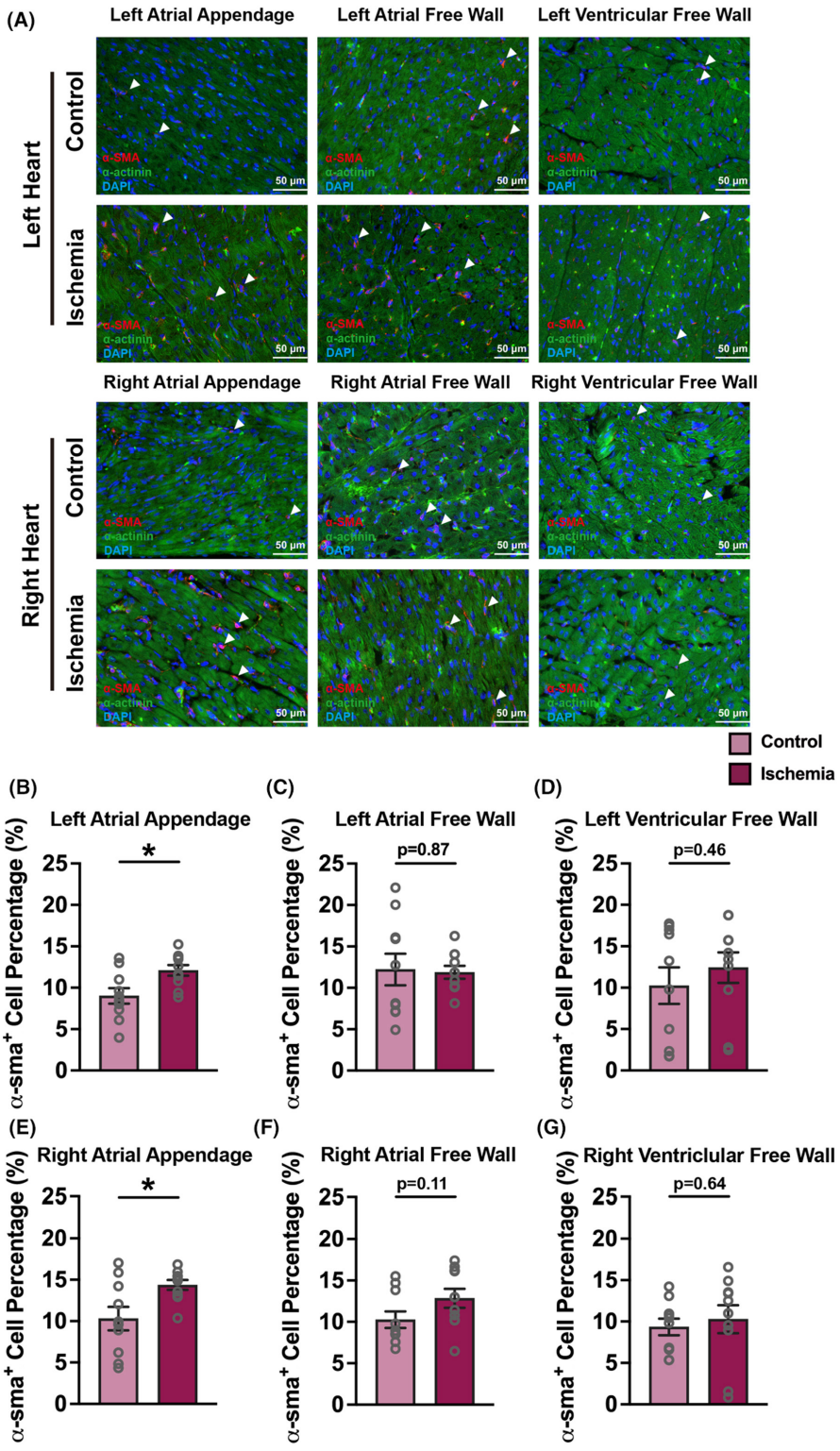


**FIGURE 4** Quantification of myofibroblasts. (A) Representative images of immunofluorescence staining in control and ischaemic heart failure pigs; arrowheads indicate myofibroblasts. Quantification of myofibroblasts (α-SMA-positive cell) in (B) left atrial appendage, (C) left atrial free wall, (D) left ventricular free wall, (E) right atrial appendage, (F) right atrial free wall and (G) right ventricular free wall. Bar graphs represent the mean ± SEM, and grey circles represent the data of individual pigs. Unpaired *t*-tests were applied. $*p < .05$.

atrium ($r = .6344$, $**p < .01$, Figure 5G), but not in the right atrium of IHF pigs (Figure 5H).

## 4 | DISCUSSION

Various animal models for AF mimicking different aspects of the disease exist and are highly valuable to investigate specific underlying mechanisms and to improve our understanding of AF.[12] From a clinical perspective, however, models closely resembling the situation in patients are more relevant, as they may allow direct transfer of novel findings into clinical application, improving patients' health. Thus, we studied an ischaemia model in pigs as (i) myocardial ischaemia is the most common trigger for AF in patients with an incidence of AF

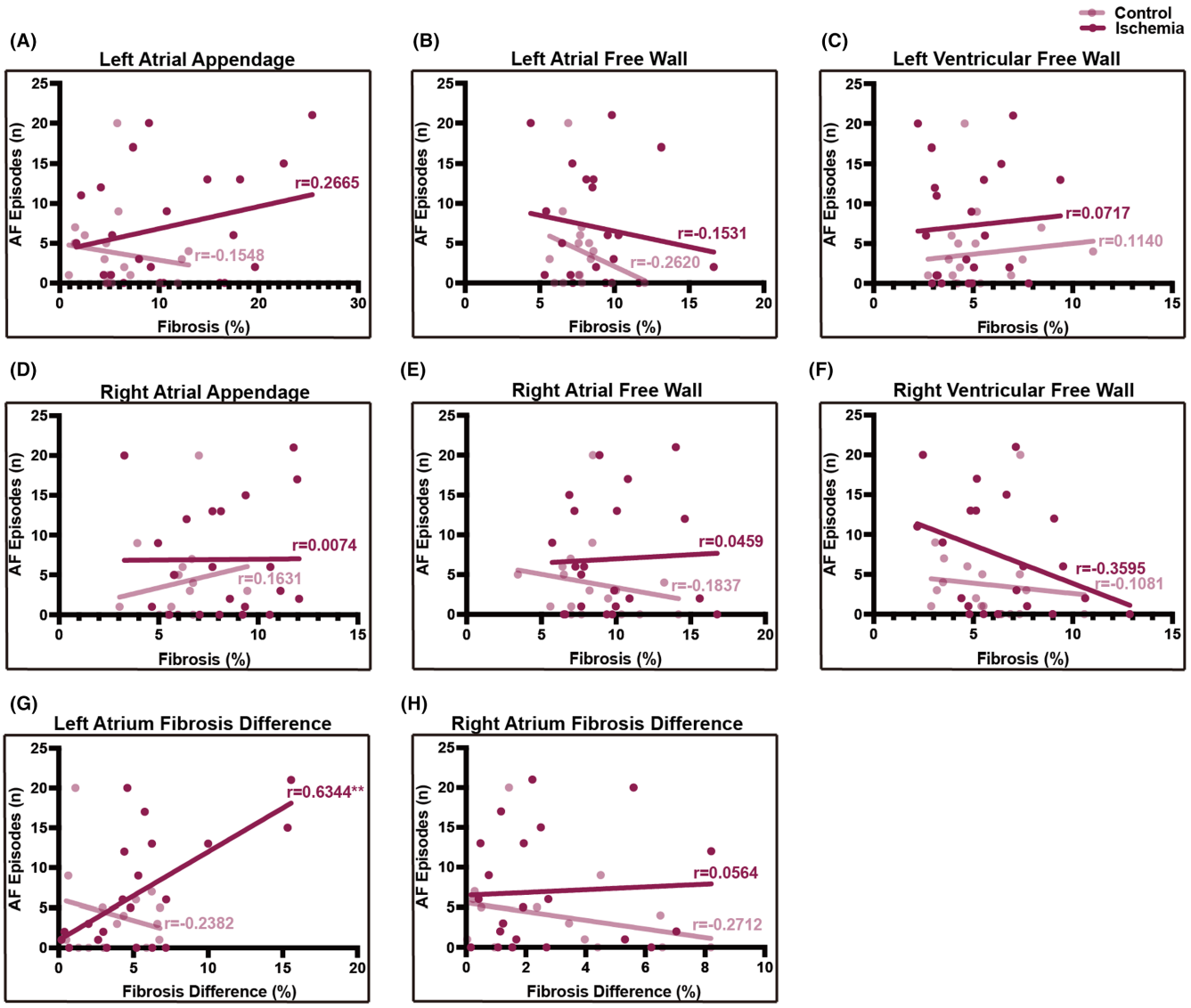


FIGURE 5 Correlation analysis. Correlation of the number of atrial fibrillation episodes with (A) left atrial appendage fibrosis, (B) left atrial free wall fibrosis, (C) left ventricular free wall fibrosis, (D) right atrial appendage fibrosis, (E) right atrial free wall fibrosis, (F) right ventricular free wall fibrosis, (G) left atrium fibrosis difference and (H) right atrium fibrosis difference. Dots represent data of individual pigs. Pearson correlation coefficients were applied. $^{**}p < .01$.

after myocardial infarction of up to 21%[1,5,13] and (ii) pigs closely resemble the human anatomy and (electro-)physiology, making pigs an ideal model species for translational AF research.[8,9] Specifically, the model we used mimics a common clinical situation in patients with ST elevation myocardial infarction that is revascularized 90 min after coronary occlusion, but nevertheless, this short-term ischaemia triggers the development of IHF with subsequent proarrhythmic remodelling, resulting in enhanced vulnerability for AF.[7,10] As we wanted to investigate ischaemia-mediated proarrhythmic structural remodelling, this preclinical pig model seemed to be well suited.

In our study, we observed a significant and similar to human heart failure phenotype demonstrated by reduced ejection fraction and altered haemodynamics of both the ventricles (elevated LVEDP) and atria (elevated right atrial and postcapillary wedge pressure, a surrogate for left atrial pressure). We also observed a significantly increased vulnerability to AF in pigs with IHF, which confirms our previous findings in an additional cohort of pigs and justifies using this model to further investigate ischaemia-induced proarrhythmic remodelling.[7]

Next, we explored potential mechanisms underlying this ischaemia-mediated AF susceptibility by in vivo electrophysiology studies and histologic analysis to assess electrical and structural remodelling as two of the major hallmarks of AF pathophysiology.[2–4,14] Atrial electrophysiological properties, for example AERP, vary in different heart failure models and different animal species.[8,9] In our IHF pigs, we found no significant alterations

in sinus node function, AV node conduction or atrial effective refractory periods, which is in line with insignificant atrial conduction alterations observed in ischaemic canine hearts previously,[15] suggesting that, 30 days after myocardial infarction, electrical remodelling is not the major driver for enhanced arrhythmogenicity in our model. Myocardial infarction induces necrosis or apoptosis of cardiomyocytes, which are then replaced by reparative fibrosis, establishing a stabilizing scar in the infarct zone. Additionally, ischaemia with progressive heart failure also triggers profibrotic signalling, leading to interstitial fibrosis in other (nonischaemic) areas of the heart including the atria.[16] This results in local conduction heterogeneity, which in turn favours re-entry.[2–4,14]

In our study, we observed marked regional differences in fibrosis distribution, with significantly increased fibrosis in atrial appendages rather than in atrial free walls and ventricles in IHF pigs. Myofibroblasts have been demonstrated as the main contributor to structural remodelling after heart injury.[11] We observed a significantly increased myofibroblast number in atrial appendages but not in atrial free walls and ventricles in IHF pigs. To explore the differences between IHF pigs developing AF and those without AF, we further performed subgroup analyses regarding haemodynamics, electrophysiological measurements, fibrosis amounts and myofibroblast numbers. However, we found no significant differences between groups (data not shown).

Several studies have demonstrated that atrial appendages are an important source of AF.[17] In patients with AF recurrence after ablation, the prevalence of left atrial appendage firing was 27%, with 8.7% of cases being the only source of AF.[17] The BELIFE study also showed that additional electrical left atrial appendage isolation resulted in lower AF recurrence rates compared to extensive ablation of the left atrium alone.[18] In sum, these studies indicate that not only the atria itself but also atrial appendages play an important role in AF.

To further investigate whether the overall degree of fibrosis is important, we evaluated the correlation between fibrosis and the number of AF episodes and could not detect a significant correlation, indicating that the overall level of fibrosis may not be the major determinant of AF, susceptibility in this model. Recently, Ramos and colleagues could similarly demonstrate that in patients with AF the absolute degree of fibrosis is not the key driver for AF.[19] Furthermore, they could show that the heterogeneous distribution of fibrosis within the right atrial appendage does not correlate with altered electrical conduction properties.[19]

As interstitial fibrosis has been demonstrated to establish local conduction heterogeneities leading to AF,[2–4,14] we hypothesized that not only localized but also cross-regional fibrosis heterogeneity contributes to AF. To address this, we assessed atrial cross-regional differences in fibrosis distribution by subtracting values from appendages and free walls. We could demonstrate that the left atrial fibrosis difference significantly correlates with the number of AF episodes, indicating that not the overall level of atrial fibrosis but rather the left atrial cross-regional fibrosis difference may determine the vulnerability for AF in IHF. Previous studies have indicated that the gross structure and myoarchitecture between the left atrium and the right atrium are markedly different, resulting in differences in electrical conduction as well.[20] Thus, left and right atrial remodelling might exert variable effects on AF susceptibility. Compared to the right atrium, the left atrium has been well explored in most previous studies,[21] and left atrium size has been demonstrated as the strongest independent predictor of new-onset AF in the Framingham study.[22] However, we cannot exclude an important role of right atrial remodelling in AF, although we did not find a correlation between right atrial fibrosis differences and AF episode numbers.

Our results demonstrate left atrial cross-regional fibrotic differences in IHF as a potential mechanism for AF in a pig model for the first time. This is in line with previous studies similarly showing the importance of localized fibrosis heterogeneity in AF, for example in the rapid ventricular pacing model in sheep or in the rapid atrial pacing model in dogs.[8,9] In human end-stage heart failure, nonuniform patchy fibrosis and long fibrotic strands were associated with progressive activation delay, potentially leading to arrhythmias and sudden cardiac death.[23] In a recent study on patients with persistent AF, endomysial fibrosis rather than the overall fibrosis has been shown to determine AF complexity.[24]

As fibrosis is believed to slow conduction and, for example Kazbanov et al. recently demonstrated in silico that a larger degree of fibrosis heterogeneity induced more arrhythmias,[25] further investigation into this phenomenon could involve visualization of electrical conduction in vivo, for example by 3D mapping. Cardiac MRI can be used to anatomically correlate the voltage mapping with cardiac fibrosis and provide further insights into the underlying mechanisms.[26,27] We believe that a better understanding of the interplay between anatomy/fibrosis and electrical conduction will benefit catheter ablation strategies for AF. Numerous studies indicated that the low effectiveness of AF catheter ablation and impaired atrial function recovery could be linked with interstitial fibrosis across the atrium.[28] As pigs share many anatomic and physiological characteristics with humans,[9] pig models are ideal translational models and can be used to investigate new ablation strategies on the translational road from bench to bedside.

Despite our findings linking left atrial cross-regional fibrosis heterogeneity to AF susceptibility, conclusions with clinical impact should be drawn only with caution. In our pig model, AF episodes were short, self-limiting and induced by burst pacing, probably best representing patients at risk of developing paroxysmal AF. Whether spontaneous AF occurs in this model, that is fully mimicking patients with paroxysmal or persistent AF, needs to be studied, for example by implantable loop recorders in the future.

In sum, we demonstrate that increased AF susceptibility in a porcine IHF model is associated with a heterogeneous distribution of fibrosis across left atrial regions rather than with the overall level of fibrosis. This finding suggests that not fibrosis quantity per se but rather its distribution across the atrium seems to determine AF susceptibility in IHF. Thus, in vivo detection of atrial fibrosis in human patients (e.g. by cardiac MRI or voltage mapping) is important and may help to guide preventive or therapeutic strategies (e.g. specific ablation of the left atrial appendage) in patients at risk to develop AF or patients already suffering from AF, respectively. However, further studies, both in suitable animal models and in human patients, are warranted.

## AUTHOR CONTRIBUTIONS

Sebastian Clauss: Supervised the study and designed experiments. Zhihao Zhang, Julia Vlcek, Valerie Pauly, Nora Hesse, Julia Bauer, Kavi Raj Chataut, Florian Maderspacher, Lina Sophie Volz, Katharina Buchberger, Ruibing Xia, Bianca Hildebrand, Dominik Schüttler, Philipp Tomsits and Sebastian Clauss: Performed experiments and analysed results. Sebastian Clauss and Zhihao Zhang: Discussed data and conceived the manuscript. Zhihao Zhang: Prepared the figures. Zhihao Zhang, Philipp Tomsits and Sebastian Clauss: Wrote the manuscript. Sebastian Clauss: Supported and gave suggestions to the study. All authors carefully read and revised the manuscript and agreed to the published version of the manuscript including the authors' names.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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# Effects of Sex on the Susceptibility for Atrial Fibrillation in Pigs with Ischemic Heart Failure

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**Abstract:** Atrial fibrillation (AF) is the most prevalent arrhythmia, often caused by myocardial ischemia/infarction (MI). Men have a 1.5× higher prevalence of AF, whereas women show a higher risk for new onset AF after MI. However, the underlying mechanisms of how sex affects AF pathophysiology are largely unknown. In 72 pigs with/without ischemic heart failure (IHF) we investigated the impact of sex on ischemia-induced proarrhythmic atrial remodeling and the susceptibility for AF. Electrocardiogram (ECG) and electrophysiological studies were conducted to assess electrical remodeling; histological analyses were performed to assess atrial fibrosis in male and female pigs. IHF pigs of both sexes showed a significantly increased vulnerability for AF, but in male pigs more and longer episodes were observed. Unchanged conduction properties but enhanced left atrial fibrosis indicated structural rather than electrical remodeling underlying AF susceptibility. Sex differences were only observed in controls with female pigs showing an increased intrinsic heart rate, a prolonged QRS interval and a prolonged sinus node recovery time. In sum, susceptibility for AF is significantly increased both in male and female pigs with ischemic heart failure. Differences between males and females are moderate, including more and longer AF episodes in male pigs and sinus node dysfunction in female pigs.

**Keywords:** atrial fibrillation; atrial arrhythmias; electrical remodeling; structural remodeling; sex differences; large animal model; pig model; translational medicine



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## 1. Introduction

Atrial fibrillation (AF) is the most prevalent cardiac arrhythmia worldwide: over 37.5 million people were diagnosed with AF and nearly 287,000 deaths were associated with AF in 2017, with predictions estimating that both prevalence and incidence will continue to increase drastically in the next years [1].

Myocardial ischemia is an independent risk factor for AF and both diseases often co-exist [2]. Although the overall prevalence of AF is estimated to be 1.5 times higher in

men, women seem to be at higher risk of suffering from new onset AF after myocardial infarction [2]. However, despite these known differences, the impact of sex on AF is still incompletely understood, especially regarding the clinical presentation and evaluation of AF in women [3]. Multiple studies report that women have been underrepresented in clinical trials investigating AF in the past [4,5]. In particular, an analysis of all studies cited by the "2020 Canadian Cardiovascular Society/Canadian Heart Rhythm Society Comprehensive Guidelines for the Management of Atrial Fibrillation" showed that only 39.1% of the study population were women [3,6]. In recent years, research began setting a greater focus on investigating differences between men and women concerning pathophysiology, treatment, and prevention [7]. It has been observed that treatment strategies and outcome of cardiovascular diseases are influenced by the gender of patients: women less often receive electrical cardioversion or catheter ablation upon the diagnosis of AF [8] and a nationwide study in Germany showed that the in-hospital mortality of patients with AF, who underwent atrial ablation or atrial appendage occlusion therapy, is also associated with the female sex [9].

Given that, it is clearly necessary to consider the impact of sex on electrophysiology and arrhythmogenesis in basic, translational, and clinical research.

Rodent models have been used to investigate sex-based differences in cardiovascular disease development [10], but there is still a lack of inclusion of sex as a biological variable in animal models in cardiovascular research as most studies use exclusively male or female animals or did not specify the sex at all [11,12]. Although the necessity of reporting sex differences in clinical trials has increased, these requirements have not been converted to preclinical research [12]. Therefore, we wanted to investigate possible biological sex-related differences in heart function, electrophysiology and arrhythmogenicity in a translational porcine model of atrial fibrillation (AF) in the context of ischemic heart failure (IHF), which we have previously characterized [13].

## 2. Materials and Methods

### 2.1. Animals

All animal experiments were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" and were approved by the *Regierung von Oberbayern* (ROB-55.2-2631.Vet_02-10-130, ROB-55.2-2532.Vet_02-15-209 and ROB-55.2-2532.Vet_02-18-69) at the Institute of Surgical Research at the Walter-Brendel-Centre of Experimental Medicine, Munich, Germany. The animals included in this work were bred in research facilities of the *Landwirtschaftliche Forschungsstation Thalhausen*, Technical University of Munich (TUM), Kranzberg, Germany, the *Moorversuchsgut* and the Center for Innovative Medical Models (CiMM), Ludwig-Maximilians-University (LMU), Oberschleissheim, Germany, and *Lehr-und Versuchsgut der LMU*, Oberschleissheim, Germany.

In total, 72 domestic swine aged 3–6 months with an average weight of 66.1 ± 2.9 kg were included in this analysis. We studied four groups: (1) Male control pigs (63.3 ± 5.1 kg, $n$ = 22), (2) Female control pigs (69.7 ± 5.2 kg, $n$ = 22), (3) Male IHF pigs (65.6 ± 6.5 kg, $n$ = 15) and (4) Female IHF pigs (65.1 ± 7.9 kg, $n$ = 13). Inclusion in either the CTL or the IHF group was random. Ischemic heart failure was induced by occlusion of the left anterior descending artery (LAD) for 90 min as previously described [13,14]. After 30 days, IHF pigs underwent the same experimental procedures as age- and weight-matched controls. The pigs were sacrificed after in vivo measurements and hearts were removed for further histological experiments.

### 2.2. Anesthesia and Surgical Preparation

Experimental procedures were recently described in detail [13,14]. In short, all pigs were given seven days of acclimatization in the facility to avoid any stress-induced alterations of experimental results. On the day of surgery, the pigs were sedated by intramuscular (IM) injection of ketamine (20 mg/kg, Zoetis, Berlin, Germany) and azaperone (10 mg/kg, Elanco, Bad Homburg, Germany) in the lateral cervical musculature behind the

ear in combination with atropine (0.05 mg/kg, Braun, Melsungen, Germany) to reduce salivation during the endotracheal intubation. Once sedated, an intravenous (IV) access was placed in the outer auricular vein followed by IV injection of midazolam (0.5 mg/kg, Braun, Melsungen, Germany), propofol (0.5 mg/kg/min, Fresenius, Bad Homburg, Germany), and fentanyl (0.05 mg/kg, Dechra, Aulendorf, Germany) to maintain general anesthesia and analgesia at a level of surgical tolerance throughout the experimental procedure.

After intubation and preparation of the surgical site in dorsal recumbency, 8F and 9F sheaths (Cordis, Norderstedt, Germany) were surgically introduced into the right external jugular vein and the right carotid artery. The LAD was occluded by an angioplasty balloon (Merit Medical, South Jordan, UT, USA) placed distal of the first diagonal branch. To prevent ventricular tachyarrhythmias, 150 mg/kg amiodarone (Hikma Pharma, Martinsried, Germany) were administered upon LAD occlusion. The location of the inflated balloon was intermittently confirmed by X-ray (Ziehm Imaging, Nuremberg, Germany). Furthermore, all vital parameters were closely monitored during 90 min of occlusion and following reperfusion. If the pigs remained stable after the reperfusion period, all catheters and sheaths were removed, and blood vessels were ligated. The surgical site was then thoroughly closed and bandaged, anesthesia was stopped, and pigs were transferred to the housing facility where they were monitored for 30 days.

### *2.3. Assessment of the Ejection Fraction*

Using a 6F or 7F Pigtail catheter (Cordis, Norderstedt, Germany), laevocardiography of the left ventricle (LV) was performed to assess the ejection fraction in the left anterior-oblique (LAO) angulation at 30° and anterior-posterior (AP) angulation at 0° at a paced heart rate of 130 bpm.

### *2.4. 12-Lead Electrocardiogram and Electrophysiological Study*

12-lead surface electrocardiograms (ECGs) were recorded to measure heart rate (bpm), P wave duration (ms), PR interval (ms), QRS interval (ms) and QT interval (ms). For electrophysiological (EP) studies, a multipolar electrophysiology catheter (Abbott, Eschborn, Germany) was placed at the high right atrium to allow atrial stimulation (at 2× pacing threshold) to assess sinus node recovery time (SNRT), atrioventricular conduction properties, as well as atrial and atrioventricular refractory periods.

The sinus node recovery time (SNRT) is defined as the interval between the last atrial stimulus and the first intrinsic P wave following 30 s of atrial stimulation. SNRT was evaluated at paced cycle lengths of 500 ms, 450 ms, and 400 ms, and was corrected for the intrinsic basic cycle length (SNRT/BCL). The SNRT/BCL is calculated by multiplying the quotient of the SNRT and the BCL by 100. Atrioventricular conduction properties were assessed by measuring the Wenckebach cycle length (WB), the 2:1 conduction cycle length (2:1 CL), the atrioventricular effective refractory period (AVERP) and the atrial effective refractory period (AERP). We determined the Wenckebach point by progressively shortening the atrial pacing cycle length from 500 ms in decrements of 10 ms until there was no longer a 1:1 AV conduction of the atrial signal. The 2:1 conduction cycle length was assessed in the same manner. The effective refractory periods of the atrium and the AV node were assessed by series of seven fixed stimulations at each basic cycle length (500 ms, 450 ms, 400 ms, 350 ms, 300 ms, and 250 ms, respectively) followed by a decrementally coupled, premature, eighth stimulus. AERP and AVERP were defined as the cycle lengths of the premature stimulus at which the atrial signal did not induce a P wave (AERP) or was not conducted to the ventricles (AVERP).

Finally, arrhythmias were induced by burst pacing in the high right atrium for 6 s. Atrial Fibrillation (AF) was defined as an atrial arrhythmia with irregular RR intervals lasting at least 10 s.

### 2.5. Assessment of Structural Remodeling

Structural remodeling was assessed by quantifying interstitial fibrosis in the left atrium and left ventricle (remote of infarcted area). Paraffin-embedded tissue samples were cut at a thickness of 5 µm and stained according to the Masson's trichrome protocol (Masson-Goldner-Trichrome Staining Kit 3459.1, Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Ten random, non-overlapping images per region were taken using a high-resolution microscope (DM6 B, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) at a 40× magnification. Interstitial fibrosis was quantified by a blinded observer.

### 2.6. Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8.0.1. Data are presented as Mean ± SEM. The standard error of the mean (SEM) was calculated dividing the standard deviation by the square root of the sample size ($n$) of each data set. Differences between two cohorts were calculated by applying the non-parametric Mann-Whitney-U Test. Fisher's-Exact Test was used to compare categorical values (presented as percentages). Figures were designed using GraphPad Prism 8.0.1 Individual values of each pig are shown as grey circles. Results were considered statistically significant with $p$-values less than 0.05.

## 3. Results

### 3.1. Induction of Ischemic Heart Failure

In IHF a significant reduction of the ventricular ejection fraction (EF) was observed (Figures 1A and 2A). In male IHF pigs the EF was significantly lower than in male control pigs (EF 0° AP CTL vs. IHF: 59.6 ± 2.5% vs. 22.9 ± 4.1%, *** $p < 0.001$; Figure 1A; 30° LAO: 59.0 ± 2.2% vs. 35.4 ± 3.2%, *** $p < 0.001$; Figure 2A). Consistently, in female pigs the EF was significantly reduced in IHF animals as well (EF 0° AP CTL vs. IHF: 57.2 ± 2.8% vs. 26.8 ± 4.2%, *** $p < 0.001$; Figure 1A; 30° LAO: 58.6 ± 3.0% vs. 31.8 ± 3.9%, *** $p < 0.001$; Figure 2A). No differences were observed between male and female pigs. Hemodynamic measurements obtained from right heart catheterization using a Swan-Ganz catheter (Edwards Lifesciences, Irvine, CA, USA) indicated an increase in pulmonary arterial pressure (PAP), pulmonary capillary wedge pressure (PCWP) and right arterial pressure (RAP) in both IHF groups compared to their respective control groups (Supplemental Figure S1).

### 3.2. ECG

ECG analysis revealed two significant differences between male and female control pigs, which disappeared in IHF: female control pigs showed a significantly lower heart rate compared to male control pigs (male vs. female: 95.1 ± 6.1 bpm vs. 77.7 ± 5.4 bpm, * $p < 0.05$; Figure 3A) and a significantly prolonged QRS interval (male vs. female: 68.4 ± 2.1 ms vs. 75.3 ± 2.8 ms, * $p < 0.05$; Figure 3D). Other ECG parameters such as P wave duration (Figure 3B), PR interval (Figure 3C), and $QT_C$ interval (Figure 3E) did not differ between male and female control pigs. In pigs with ischemic heart failure, no differences were observed in these ECG parameters between males and females or compared to their respective controls (Figure 3).

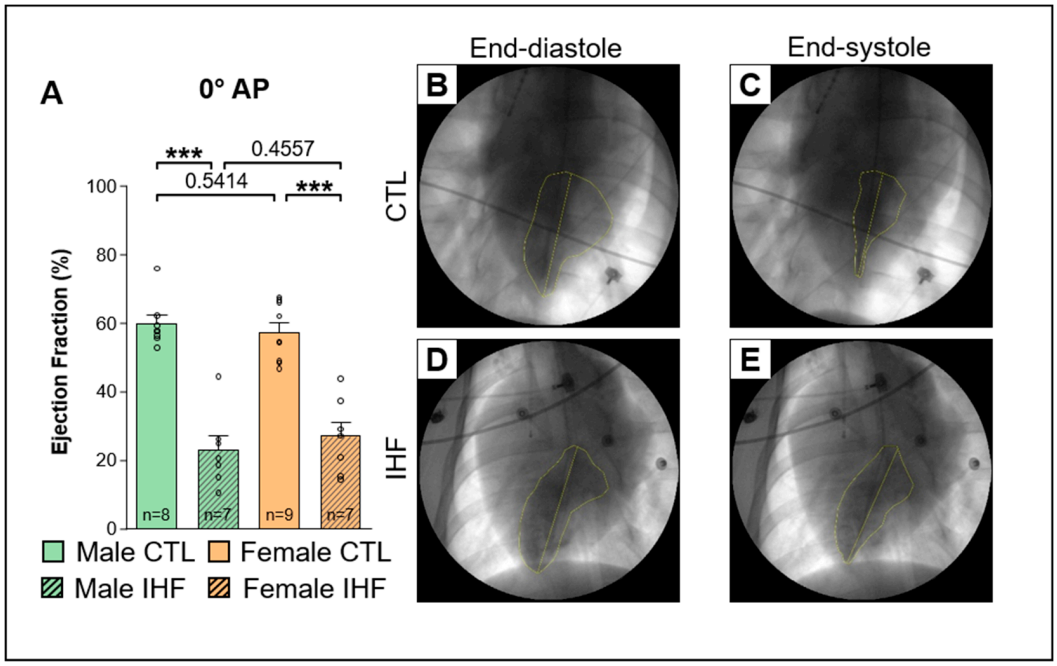


**Figure 1.** Ejection fraction (EF) assessed by laevocardiography at 130 bpm at 0° AP (anterior posterior). (**A**), Ejection Fraction. (**B**,**C**), Representative images of a control pig at end-diastole (**B**) and end-systole (**C**). (**D**,**E**) Representative images of an IHF pig at end-diastole (**D**) and end-systole (**E**). *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. *** $p < 0.001$.

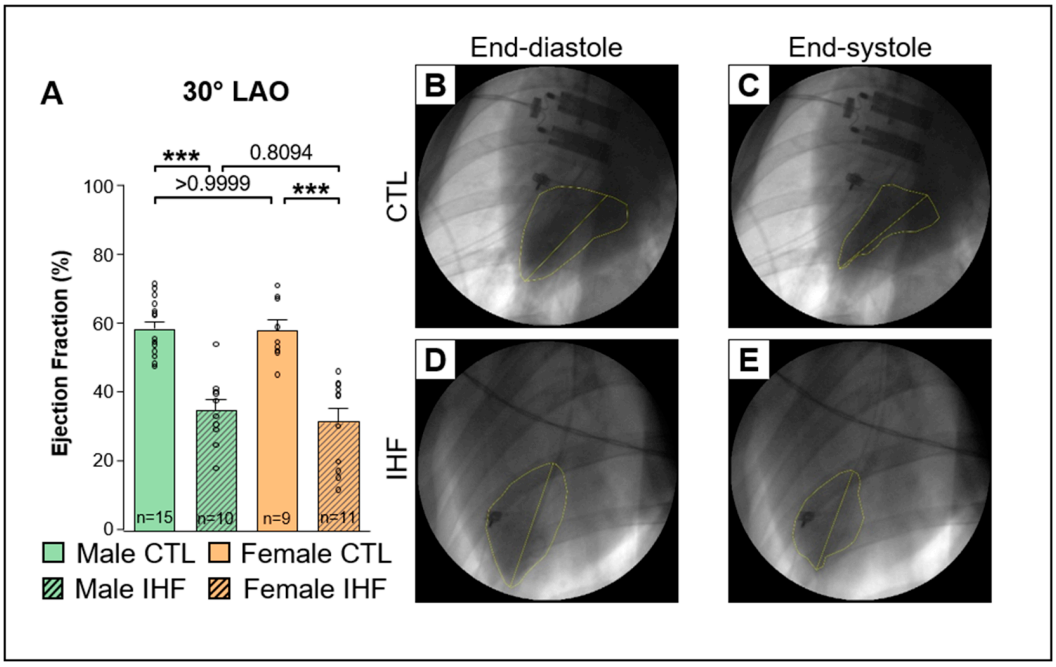


**Figure 2.** Ejection Fraction (EF) assessed by laevocardiography at 130 bpm at 30° LAO (left anterior oblique). (**A**), Ejection Fraction. (**B**,**C**) Representative images of a control pig at end-diastole (**B**) and end-systole (**C**). (**D**,**E**) Representative images of an IHF pig at end-diastole (**D**) and end-systole (**E**). *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. *** $p < 0.001$.

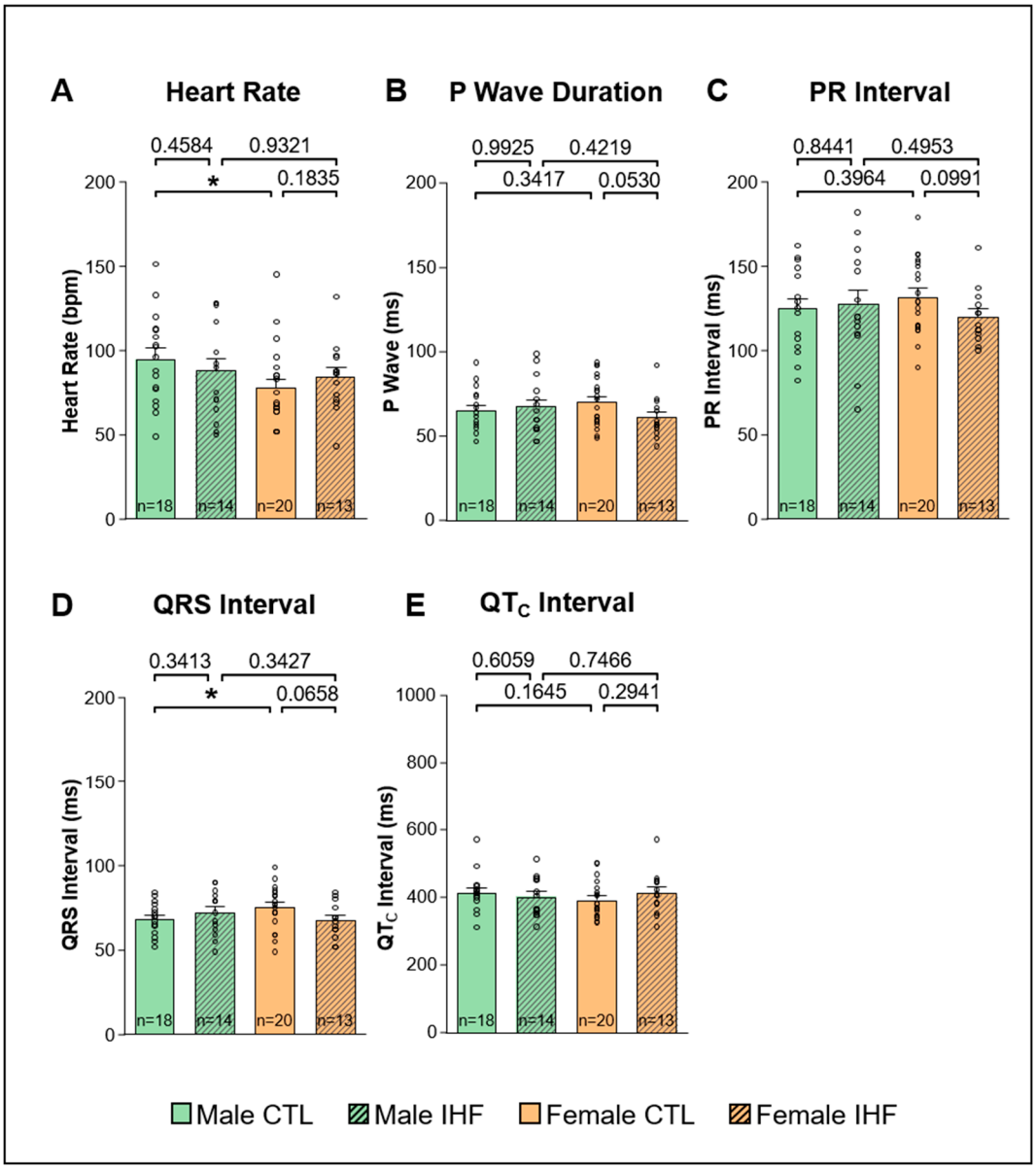


**Figure 3.** ECG parameters. (**A**), Heart Rate. (**B**), P Wave Duration. (**C**), PR Interval. (**D**), QRS Interval. (**E**), Corrected QT Interval (calculated according to Bazett). *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. * $p < 0.05$.

### *3.3. Arrhythmia Inducibility*

The overall inducibility of AF was assessed as the percentage of pigs with AF episodes longer than 10 s among all pigs of each group (Figure 4A). IHF significantly increased the susceptibility for AF in both sexes with 42.9% of the male pigs and 46.2% of the female pigs showing AF (** $p < 0.01$, compared to sex-matched control pigs, respectively; Figure 4A). Furthermore, in both sexes with IHF, a significantly higher number of burst stimulations resulted in AF compared to control pigs (CTL vs. IHF: 2.2% vs. 12.1% in males, *** $p < 0.001$; and 0.4% vs. 4.9% in females, ** $p < 0.01$; Figure 4B). As a result, the average AF burden was significantly increased in IHF pigs compared to control pigs in both sexes (CTL vs. IHF: 6.2 ± 6.2 s vs. 40.6 ± 22.9 s in males, * $p < 0.05$; and 0.9 ± 0.9 s vs. 8.9 ± 3.3 s in females, ** $p < 0.01$; Figure 4C). A similar number of male and female pigs both in control and IHF groups developed AF; however, in male pigs a higher number of stimulations resulted in AF with a statistically significant difference in IHF animals (male vs. female: 12.1% vs.

4.9% in IHF pigs, * $p < 0.05$; and 2.2% vs. 0.4% in control pigs, $p = 0.1116$; Figure 4B). We observed a trend towards a lower AF burden in female pigs, however, these differences were not statistically significant (male vs. female: 40.6 ± 22.9 s vs. 8.9 ± 3.3 s in IHF pigs, $p = 0.5453$; and 6.2 ± 6.2 s vs. 0.9 ± 0.9 s in control pigs, $p = 0.7445$; Figure 4C).

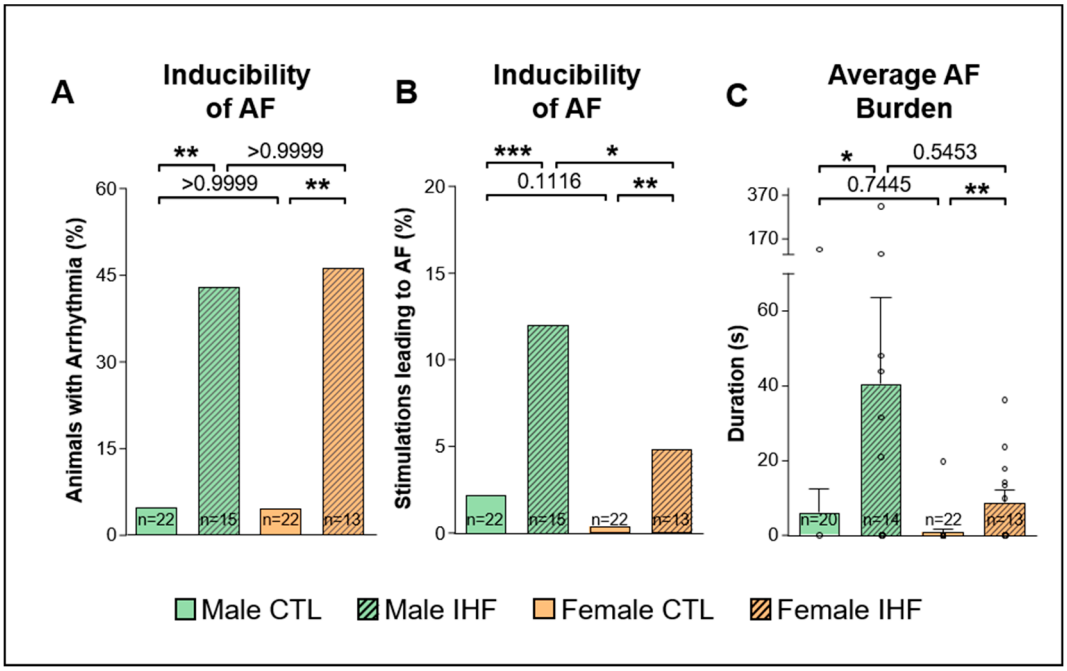


**Figure 4.** Inducibility of Atrial Fibrillation (AF) (**A**), Percentage of pigs with AF episodes longer than 10 s. (**B**), Percentage of burst stimulations leading to AF episodes longer than l0 s. (**C**), Average AF burden. *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Fisher's Exact Test (**A**,**B**) and Mann-Whitney-U Test (**C**). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

### *3.4. Electrophysiological Studies (EPS)*

The uncorrected sinus node recovery times measured at pacing cycle lengths of 500 ms, 450 ms, and 400 ms showed significant differences between male and female control pigs (Supplemental Figure S2). Female control pigs showed a significantly longer sinus node recovery time to basic cycle length quotient (SNRT/BCL) at all stimulated cycle lengths compared to male control animals (male vs. female: at 500 ms: 119.6 ± 4.3% vs. 152.6 ± 9.7%, * $p < 0.05$; at 450 ms: 120.5 ± 4.3% vs. 159.1 ± 10.7%, ** $p < 0.01$; at 400 ms: 119.9 ± 4.5% vs. 157.9 ± 10.0%, *** $p < 0.001$; Figure 5A–C). A similar trend between the two sexes could be observed in IHF pigs, but only at a stimulated cycle length of 400 ms the difference was statistically significant (male vs. female: 123.6 ± 8.8% vs. 172.5 ± 22.1%, * $p < 0.05$; Figure 5C). Between control and IHF animals of each respective sex, however, no difference was observed.

To determine atrioventricular (AV) conduction properties we measured the Wenckebach cycle length and the 2:1 AV conduction cycle length, but could not observe any statistically significant differences, either between males and females or between control and IHF pigs (Figure 6).

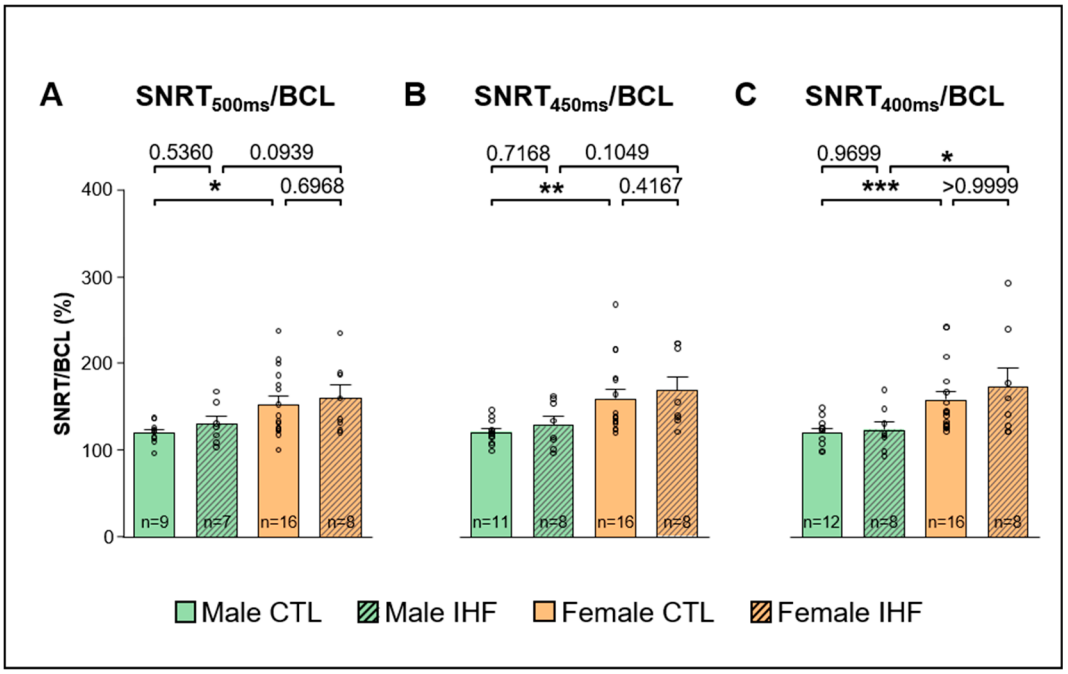


**Figure 5.** Corrected Sinus Node Recovery Time (SNRT/BCL). (**A**), SNRT/BCL at a pacing cycle length of 500 ms. (**B**), SNRT/BCL at a pacing cycle length of 450 ms. (**C**), SNRT/BCL at a pacing cycle length of 400 ms. *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

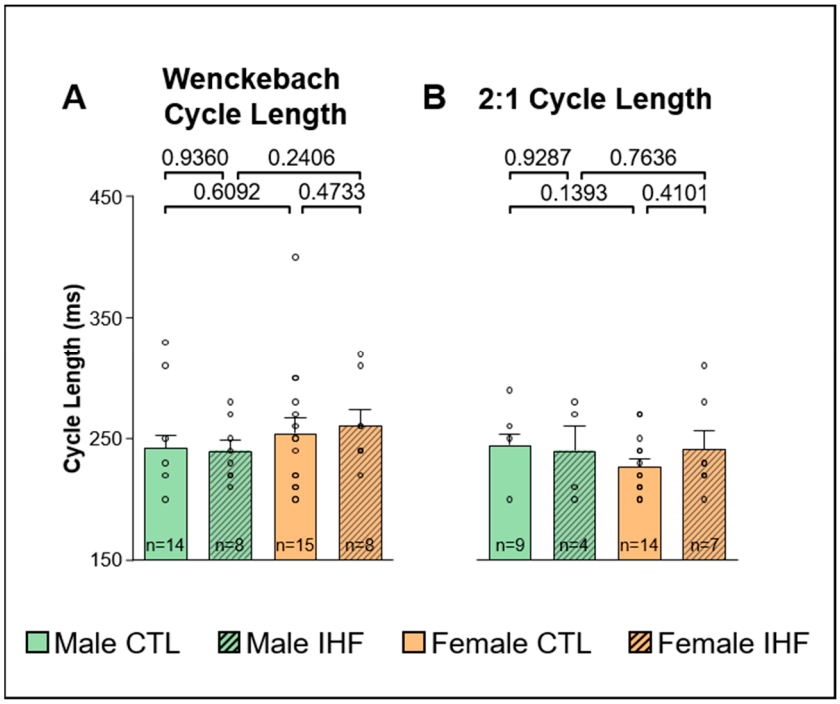


**Figure 6.** Atrioventricular conduction properties. (**A**), Wenckebach cycle length. (**B**), 2:1 atrioventricular conduction cycle length. *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test.

The effective refractory period of the AV node (AVERP) was significantly prolonged in female controls compared to male controls at a basic cycle length of 450 ms (male vs. female: 241.8 ± 9.1 ms vs. 268.7 ± 10.5 ms, * $p < 0.05$; Figure 7B), whereas the other stimulated basic cycle lengths did not show any differences (Figure 7A,C–F). In pigs with IHF, no difference was observed between males and females nor compared to their respective controls (Figure 7). Similarly, we did not see any differences between sexes or between controls and IHF pigs regarding AERP (Figure 8).

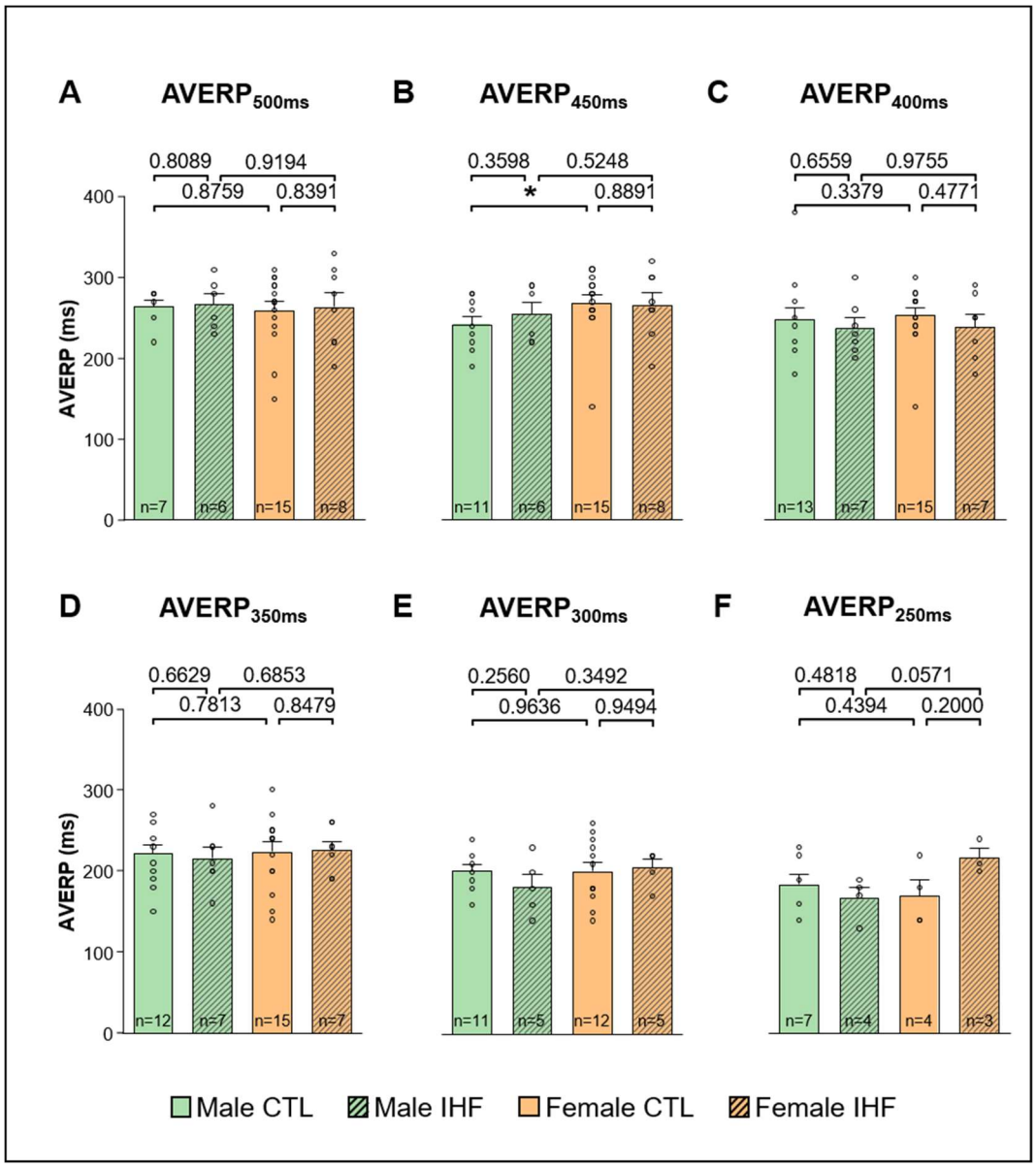


**Figure 7.** Atrioventricular Effective Refractory Period (AVERP). (**A**), AVERP at a basic cycle length of 500 ms. (**B**), AVERP at a basic cycle length of 450 ms. (**C**), AVERP at a basic cycle length of 400 ms. (**D**), AVERP at a basic cycle length of 350 ms. (**E**), AVERP at a basic cycle length of 300 ms. (**F**), AVERP at a basic cycle length of 250 ms. *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. * $p < 0.05$.

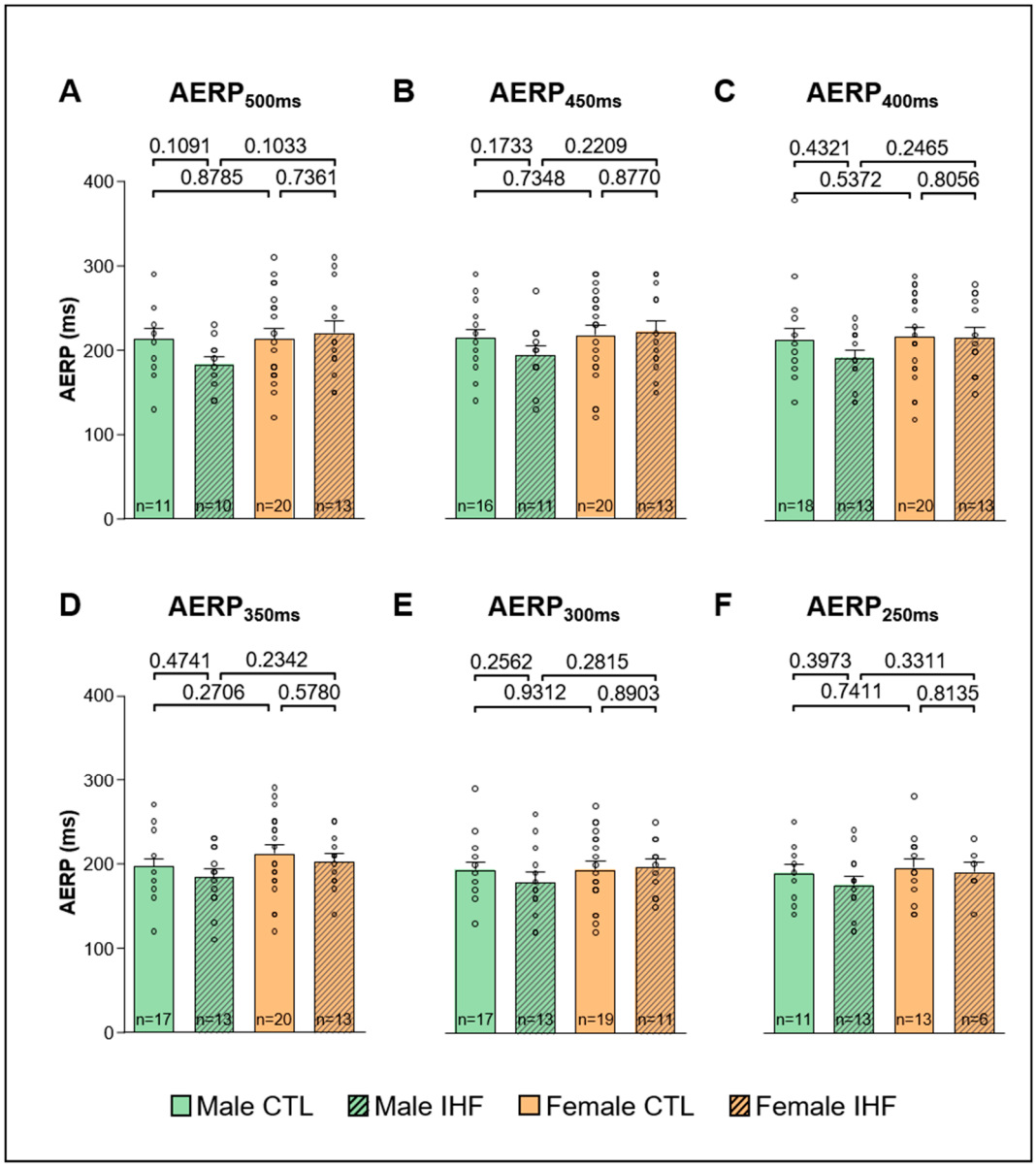


**Figure 8.** Atrial Effective Refractory Period (AERP). (**A**), AERP at a basic cycle length of 500 ms. (**B**), AERP at a basic cycle length of 450 ms. (**C**), AERP at a basic cycle length of 400 ms. (**D**), AERP at a basic cycle length of 350 ms. (**E**), AERP at a basic cycle length of 300 ms. (**F**), AERP at a basic cycle length of 250 ms. *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test.

### *3.5. Assessment of Structural Remodeling*

Histologic analysis revealed a significant increase in left atrial (LA) interstitial fibrosis in both female and male IHF pigs compared to controls (CTL vs. IHF: 5.0 ± 1.1% vs. 11.3 ± 1.8% in males, * $p < 0.05$; and 3.7 ± 0.7% vs. 11.0 ± 2.2% in females, ** $p < 0.01$; Figure 9A). However, there were no significant differences between males and females of CTL or IHF animals. In the left ventricle (LV), IHF resulted in significantly increased fibrosis only in females (CTL vs. IHF: 2.6 ± 0.7% vs. 4.5 ± 0.4%, * $p < 0.05$; Figure 10A), but not in males (CTL vs. IHF: 4.0 ± 0.7% vs. 5.0 ± 0.8%, $p = 0.3864$; Figure 10A). Within each sex, there were no statistical differences between the control and IHF group (male vs. female: $p = 0.1423$ in CTL; $p = 0.7544$ in IHF).

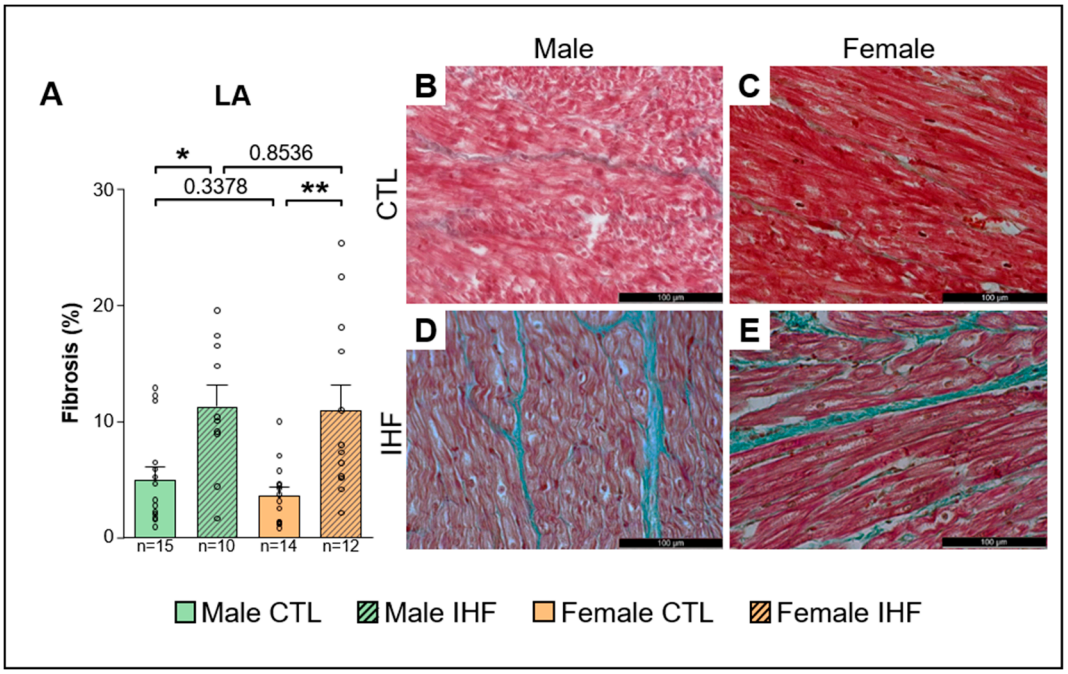


**Figure 9.** Interstitial fibrosis assessed by Masson's trichrome staining in the Left Atrium (LA). (**A**), LA Fibrosis. (**B**,**C**), Representative images of control pigs in the LA of males (**B**) and females (**C**). (**D**,**E**), Representative images of IHF pigs in the LA of males (**D**) and females (**E**). *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. * $p < 0.05$; ** $p < 0.01$.

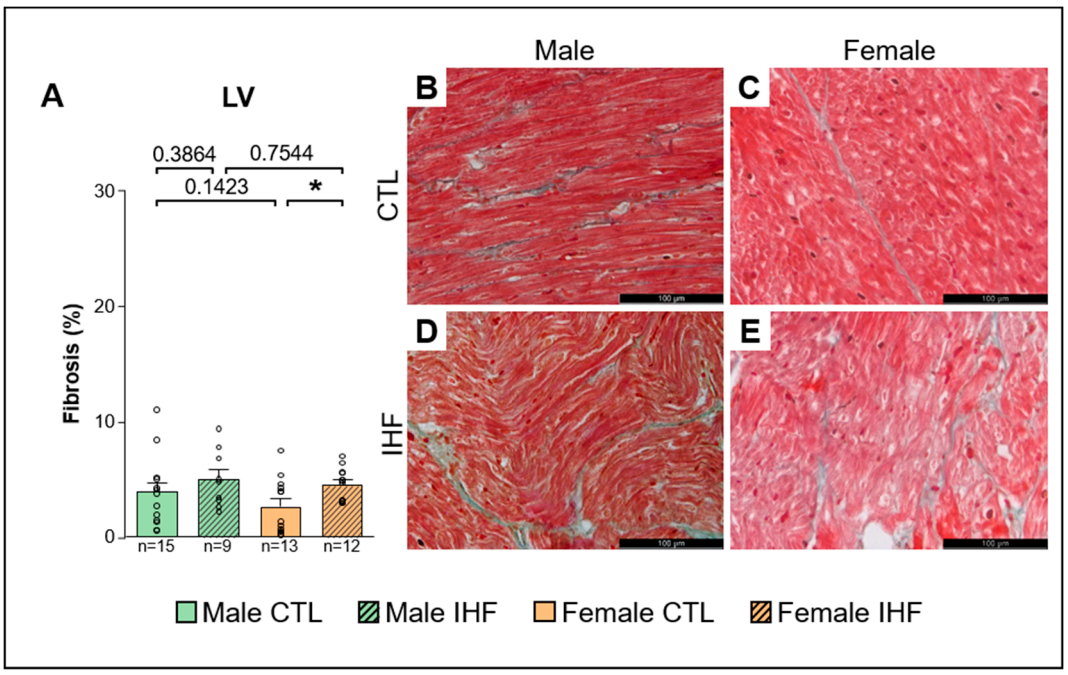


**Figure 10.** Interstitial fibrosis assessed by Masson's trichrome staining in the Left Ventricle (LV). (**A**), LV Fibrosis. (**B**,**C**), Representative images of control pigs in the LV of males (**B**) and females (**C**). (**D**,**E**), Representative images of IHF pigs in the LV of males (**D**) and females (**E**). *CTL*, control animals without ischemic heart failure; *IHF*, animals with ischemic heart failure. Bar graphs represent Mean + SEM, grey circles represent data of individual pigs. Mann-Whitney-U Test. * $p < 0.05$.

## 4. Discussion

In the presented study, we investigated the potential impact of sex on susceptibility for atrial fibrillation (AF) using a pig model of ischemic heart failure (IHF), and observed a significantly increased vulnerability for AF in IHF pigs of both sexes.

Although sex-related differences in patients with AF are well known, it has not been fully elucidated *how* sex affects AF pathophysiology [8,15]. There is a strong body of evidence suggesting that pre-menopausal women have a lower risk of cardiovascular diseases compared to men, indicating a strong effect of sex hormones on cardiac pathophysiology [16]. This is further supported by data showing that in post-menopausal women the concentration of estrogens, especially estradiol, decrease to the same basal level as in men [17] which is paralleled by an increase in cardiovascular risk compared to age-matched men [18]. Nevertheless, it has been shown that even prepubertal boys and girls have different blood concentrations of estrone (E1) and estradiol (E2) [19], indicating that sex hormones may already play a role in the development of children and contribute to a biological dimorphism even in the young. Mechanistically, sex hormones act via specific receptors for estrogen (ERα and ERβ) and androgen isoforms [16,20], which are phosphorylated in a sex-specific manner in cardiomyocytes of men and women [21]. Myocardial calcium handling and, thus, cardiac electrophysiology are also directly affected by estrogens and androgens [21–23]. Furthermore, some studies have shown modulatory effects of sex-steroids on mRNA levels of ion channels [20,23]. One study has recently shown that there is a significant difference in the resting membrane potential (RMP) between men and women, possibly due to fewer inward rectifier channels in women, thus resulting in a slower conduction in the atria of women [24]. This could also be a potential mechanistic explanation for the lower incidence of AF in women [24].

Numerous animal models have been used to evaluate sex differences in cardiac electrophysiology and arrhythmogenesis [8,15,20,22,25]. However, most studies so far have mainly focused on the ventricles, but not on the atria [8,15,20,22,26]. In mice—although they are commonly used in arrhythmia research in general [27]—only a few studies evaluated sex effects on atrial electrophysiology or arrhythmogenesis and revealed inconsistent results. In regard to ECG parameters, some studies have shown longer QRS durations in female mice compared to male mice [28,29]. In other studies, however, no difference was observed [30]. One study also demonstrated a prolonged PR interval in female mice [30], whereas others did not report such a difference [28,29]. Male mice are more susceptible to develop AF induced by programmed electrical stimulation, an effect that is abolished by orchiectomy, which further emphasizes the role of sex hormones [31,32]. Potential mechanisms described include altered calcium homeostasis (increased calcium transient amplitude, more frequent spontaneous calcium releases, faster decay time, higher $Na^{+}Ca^{2+}$ exchanger current density and a lower L-type calcium current density in male mice) resulting in enhanced triggered activity in male mice [31] and connexin lateralization [32]. Regarding atrial action potential shape/duration or potassium currents, however, no differences were observed [32]. In contrast, other studies demonstrated no differences between male and female mice in regard to intracardiac conduction properties, or susceptibility to arrhythmias [28,29]. These inconsistencies, which also include contradictory reports on ion channel distribution, are most likely due to different mouse strains or ages studied, as these aspects have been demonstrated as key determinants in murine electrophysiology [25,33–35].

Similarly, in other species sex differences have been predominantly studied in regard to ventricular electrophysiology [22,25,36]. In rabbits, only a few studies have evaluated atrial electrophysiology and showed a higher incidence of delayed afterdepolarizations, larger late sodium current, larger calcium transients and larger sarcoplasmic reticulum calcium contents in atrial cardiomyocytes isolated from male left atria [37]. Interestingly, no differences were observed in cardiomyocytes isolated from right atria [37]. In tissue preparations from male rabbits' pulmonary veins or left atrium a higher spontaneous beating rate and incidence of burst firing as well as longer action potentials have been

observed [38]; however, it has not been studied whether these alterations result in an increased AF susceptibility in male rabbits.

Despite numerous advantages of small animal species and their usefulness, especially in studying fundamental proarrhythmic pathways, the translatability into clinical application remains challenging, mainly because of substantial differences in size, anatomy, and electrophysiology, especially in mice [27,39]. Thus, findings from small animal models need to be confirmed in large animal models prior to clinical application in human patients [27,39]. In the presented study, we used a pig model to investigate sex-related differences in AF susceptibility, as pigs are a well-established species for translational cardiovascular research with great advantages over other commonly used large animal species, such as dogs and sheep [40,41]. Pigs share a vast number of physiological and anatomical similarities with humans. More importantly, humans and pigs express the same major ion channels in cardiac myocytes, resulting in similar action potential length and morphology, in both the atria and ventricles [27,39,42], making the pig an ideal model for the study of electrophysiology [27]. We investigated a porcine ischemic heart failure model, since one of the main triggers leading to AF is acute myocardial infarction (AMI) [26,43] with up to 25% of patients developing AF during or after AMI [43]. AMI commonly causes ischemic heart failure with reduced ejection fraction (HFrEF) [44], an effect that was seen in both sexes in our study. Our model therefore closely resembles the clinical situation of patients with AMI and allows an in-depth analysis of sex effects on ischemia-mediated atrial arrhythmogenicity.

In our study, IHF resulted in an enhanced susceptibility for AF in both sexes with male pigs showing significantly more frequent and a trend towards longer AF episodes compared to female animals. These findings are consistent with studies in mice demonstrating an enhanced AF susceptibility in male mice [31,32,45]. Furthermore, these data are in line with a clinical study including 27,512 patients (42% women and 58% men) that demonstrated a similar AF incidence in female and male patients during the 30-day monitoring period (50.2% vs. 49.8%) with a significantly increased AF episode duration and overall AF burden in men [46].

We revealed differences in several quantitative ECG traits between male and female pigs. Female control pigs showed a significantly lower resting heart rate and longer QRS intervals compared to male pigs, possibly due to a correlation between body weight/size with heart rate. It has been demonstrated in humans that a higher BMI positively correlates to a longer QRS interval [47]. Similarly, the higher QRS interval in the female control pigs might be attributed to their higher average body weight compared to the age-matched male control animals. In their book, *Swine in the Laboratory,* M. Swindle and A. Smith published ECG parameters of several minipig breeds such as the Göttingen or Hanford Minipig. Minipigs show higher heart rates, shorter QRS and QTc intervals compared to large domestic swine, but no significant differences between males and females were observed [48]. Overall, the higher resting heart rates and shorter QRS/QTc intervals correlated with a lower mature body weight, ranging from 12–45 kg in miniature breeds compared to large domestic swine breeds. In humans, women have a higher resting heart rate than men, which may be due to their smaller heart size and lower body weight [49]. Also, a longer QTc interval is seen in women [47], however, we did not observe this in our female pigs. Nevertheless, as we studied juvenile pigs, of which only some had already reached sexual maturity, our results are in line with studies in humans, which suggest that the QTc interval changes throughout development, probably due to different testosterone levels: in neonates and young children, no differences in QTc interval between sexes have been observed [19,50]. During puberty it is shortened in males, and in late adulthood (from 50 years) the QTc interval slowly prolongs and reaches a similar duration as in women [20], therefore we cannot rule out that the QTc interval may still change throughout the pigs' lifetime.

Both in control and IHF pigs, the SNRT/BCL was longer in female pigs. IHF even caused a prolongation above 160%, which is considered pathologic in humans [51], in-

dicating relevant sinus node dysfunction (SND) whereas in male IHF pigs, SNRT/BCL remained within a physiologic range. The SNRT/BCL is directly dependent on the heart rate, therefore it remains debatable whether the prolonged SNRT/BCL measured in females is true or false-positive due to the lower heart rate seen in female pigs. However, even the uncorrected SNRT is elevated in female control pigs (Supplementary Figure S2), indicating a true finding in females. Separating animals according to those with and without AF reveals that in female animals with AF SNRT/BCL was slightly elevated compared to females without AF, both in the control and IHF group (Supplementary Figure S3). Consistently, male pigs with and without AF had lower SNRT/BCL values than the female cohorts and differences between the male cohorts were not visible. However, the sample size per subgroup was low and did not allow reliable statistical analysis as to whether SND and AF correlate. In humans, SNRT is shorter in women but does not significantly differ from that in men when corrected to the intrinsic heart rate [52,53]. However, when affected, women more frequently suffer from SND such as sick sinus syndrome (SSS) compared to men [15,53]. SND and AF are closely linked as they often coexist (40–70% of patients diagnosed with SND already have AF), and SND markedly increases the risk to develop AF [54]. One study even suggests that a prolonged corrected SNRT (cSNRT) can be used as a predictor for paroxysmal AF recurrence following radiofrequency catheter ablation [55]. However, despite showing SND, female IHF pigs did not show more AF than male IHF pigs. To our knowledge, sex-related differences regarding sinus node function have not been investigated in any other animal model so far, therefore it remains unclear, whether a prolonged SNRT in females is physiologic in pigs. However current evidence demonstrates that pigs in general have a shorter SNRT than humans [52,56,57], supporting the interpretation of prolonged SNRT in our female IHF pigs. This suggests, female IHF pigs showing both SND (indicated by prolonged SNRT/BCL) and enhanced susceptibility for AF compared to controls closely resemble the situation in human patients and may establish a valuable model to investigate the mechanistic links between both diseases, which are still largely unknown so far.

As structural remodeling is one of the major hallmarks of AF pathophysiology, we investigated whether myocardial ischemia induced the development of interstitial fibrosis. We found significantly increased levels of LA fibrosis in both male and female IHF pigs as well as LV fibrosis in female IHF pigs indicating structural remodeling following myocardial injury. A recent study using late gadolinium-enhanced magnetic resonance imaging (LGE-MRI) for quantification of atrial fibrosis has shown increased atrial fibrosis in women with AF [58]. However, this may be an effect of age rather than sex since the women included in the AF group were significantly older than the male patients and since it has been demonstrated before that fibrosis gradually increases with advanced age [51,58]. In another study, Li et al. showed enhanced fibrosis in pulmonary vein sleeves from women but not men with long-standing persistent AF (LSP-AF) [59]. Gene and protein expression studies from these patients further indicated differences between men and women, specifically, an up-regulation of the TGFβ/Smad3 pathway that was observed in women [59]. However, in the studies conducted by Akoum et al. and Li et al., the average age of participants with AF was above 50 years, suggesting that age is an important contributor to the AF substrate [58,59], an effect that is probably not yet seen in our young, still growing pigs. Additionally, the left atrial tissue samples analyzed in our study do not depict the whole atria, as was shown in the above-mentioned LGE-MRI study. We therefore cannot rule out sex-related differences in regard to the distribution of fibrosis throughout the atrium including the pulmonary vein sleeves.

## 5. Limitations and Outlook

Although porcine models can generally serve as close-to-human models for cardiac research, there are, in contrast to small animal models such as mice, only a few genetically modified swine models available, as genetic engineering has proven to be more difficult in large animals [60]. This limits the spectrum of human diseases for which the pig as an

experimental species can be used to dietary-, medically- or instrumentally-induced disease models. However, with ongoing advancement of genetic engineering and CRISPR-Cas, more and more genetically modified swine models have been developed recently [27,60,61].

In humans, substantial differences in electrophysiology and arrhythmogenesis between men and women can be observed. In our study, however, we observed only moderate differences between male and female pigs, which can potentially be attributed to the age of our pigs. We included young, still growing pigs whereas sex differences in human patients are mostly shown for adults or even in an aged population. As the influence of the biological sex on cardiac electrophysiology is probably mainly driven by sex hormones with varying blood levels over time, it remains unclear whether the differences found in our pigs can be explained by fluctuations of hormone levels, since there are only limited data available on sex hormone levels or receptor distribution in pigs over time. Therefore, studies on older pigs should be performed in the future as well. This can be challenging in our selected swine breed, as mature domestic wild type swine can weigh up to 350 kg and would require specialized animal facilities and well-trained staff. An alternative could be to use minipig breeds, such as the Göttingen Minipig, for long-term studies.

Reports on physiological ranges of many parameters in pigs are inconsistent, as animals of various breeds and ages are used, and often do not differ between males and females due to low sample sizes. Although our overall sample size is small on a clinical scale, the group sizes in our study exceed most study sizes in large animal research, thus showing robust results. Yet, to fully exploit the potential of our pig model, more extensive study protocols could verify the differences between the male and female ECG parameters and EP properties, such as sinus node function.

## 6. Conclusions

Our study demonstrates that ischemic heart failure increases the vulnerability for atrial fibrillation (AF) in pigs and thus confirms this model as a clinically highly relevant close-to-human model for atrial arrhythmogenesis. In this model, we observed biological differences between male and female swine, most importantly a significantly prolonged sinus node recovery time in female IHF pigs, mirroring the situation in female patients who suffer more frequently from sinus node disease. In sum, this model seems to be ideally suited to further investigate IHF-related atrial arrhythmogenicity in a close-to-human environment, even mimicking sex-related effects on electrophysiology.

**Supplementary Materials:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/cells12070973/s1, Figure S1: Hemodynamic Parameters; Figure S2: Sinus Node Recovery Time (SNRT); Figure S3: Corrected Sinus Node Recovery Time (SNRT/BCL) in animals with and without Atrial Fibrillation (AF).

**Author Contributions:** Conceptualization, S.C.; methodology, J.V., S.R., E.W., D.S., P.T. and S.C.; software, not applicable, validation, V.P., P.T. and S.C.; formal analysis, V.P., J.V., Z.Z., D.S., P.T. and S.C.; investigation, V.P., J.V., Z.Z., N.H., R.X., J.B., S.L., S.S., D.S., P.T. and S.C.; resources, S.R., E.W., S.K.; data curation, V.P., P.T. and S.C.; writing—original draft preparation, V.P.; writing—review and editing, V.P., P.T. and S.C.; visualization, V.P.; supervision, S.C.; project administration, S.C.; funding acquisition, Z.Z., R.X., S.L., S.S., D.S., P.T., J.B., S.K. and S.C. All authors have read and agreed to the published version of the manuscript.

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**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data is available upon reasonable request to the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

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REVIEW
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# Cardiac Macrophages and Their Effects on Arrhythmogenesis

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Cardiac electrophysiology is a complex system established by a plethora of inward and outward ion currents in cardiomyocytes generating and conducting electrical signals in the heart. However, not only cardiomyocytes but also other cell types can modulate the heart rhythm. Recently, cardiac macrophages were demonstrated as important players in both electrophysiology and arrhythmogenesis. Cardiac macrophages are a heterogeneous group of immune cells including resident macrophages derived from embryonic and fetal precursors and recruited macrophages derived from circulating monocytes from the bone marrow. Recent studies suggest antiarrhythmic as well as proarrhythmic effects of cardiac macrophages. The proposed mechanisms of how cardiac macrophages affect electrophysiology vary and include both direct and indirect interactions with other cardiac cells. In this review, we provide an overview of the different subsets of macrophages in the heart and their possible interactions with cardiomyocytes under both physiologic conditions and heart disease. Furthermore, we elucidate similarities and differences between human, murine and porcine cardiac macrophages, thus providing detailed information for researchers investigating cardiac macrophages in important animal species for electrophysiologic research. Finally, we discuss the pros and cons of mice and pigs to investigate the role of cardiac macrophages in arrhythmogenesis from a translational perspective.

**Keywords: Cardiac electrophysiogy, macrophages, arrhythmia, inflammation, animal models, translational medicine**

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## 1 INTRODUCTION

Cardiac arrhythmias affect millions of individuals worldwide and are a major cause of morbidity and mortality, thereby causing a significant medical but also socioeconomic burden (Boriani et al., 2006; Bloch Thomsen et al., 2010; Gorenek et al., 2014). The pathophysiology of arrhythmias is complex and includes alterations of electrical, structural, and contractile properties of the heart as well as changes of autonomic cardiac innervation (Nattel et al., 2020). Cardiac inflammatory responses e.g., in the context of myocardial infarction (MI) or heart failure (HF), are thought to crucially contribute to the proarrhythmic remodeling resulting in the development of vulnerable substrates in the heart (Engelmann and Svendsen, 2005; Francis Stuart et al., 2016). Recently, studies have revealed the various roles played by immune cells, especially macrophages, in the maintenance of cardiac homeostasis (Hulsmans et al., 2017; Sugita et al., 2021; Simon-Chica et al., 2022) and—if disrupted—the development of cardiac arrhythmias (Monnerat et al., 2016; Sun et al., 2016; Yin et al., 2016; Fei et al., 2019; Hu et al., 2019; Liu et al., 2019; Lubos et al., 2020; Lyu et al., 2020; Miyosawa et al., 2020; Zhang et al., 2020; Hiram et al., 2021; Zhang et al., 2021).

Being a key component of the innate immunity, cardiac macrophages reside in the heart from the embryonic stage and unfold significant roles during heart development (Epelman et al., 2014). In these early stages, macrophages from the embryonic yolk-sac or the fetal liver migrate into the heart where they continue to proliferate locally, thus establishing the pool of "resident cardiac macrophages," which can be identified by a characteristic combination of surface markers such as $CD45^{+}CX_3CR1^{+}F4/80^{high}$ (Schulz et al., 2012; Molawi et al., 2014). After birth, bone marrow (BM)-derived monocytes circulate in the blood and—especially under pathologic conditions—migrate into the heart where they develop to macrophages (Epelman et al., 2014; Heidt et al., 2014). Due to their different origin these can be called "recruited cardiac macrophages" and can be identified as $CD11b^{high}F4/80^{low}$.

The majority of research focused on these BM-derived "recruited macrophages", which can be grouped according to their functional phenotype into classic inflammatory and noncanonical reparative macrophages (Mills, 2012; Lavine et al., 2014). The exact identities of these macrophages remain undefined. Pro-inflammatory macrophages release cytokines and chemokines and have been demonstrated to be involved in electrical (Monnerat et al., 2016; Sun et al., 2016; Fei et al., 2019; Liu et al., 2019; Zhang et al., 2020) as well as autonomic remodeling of the heart (Yin et al., 2016; Hu et al., 2019; Lyu et al., 2020; Zhang et al., 2021). Noncanonical reparative (or alternatively-activated) macrophages release anti-inflammatory cytokines, participate in post-inflammatory tissue repair, and contribute to cardiac structural remodeling by promoting cardiac fibrosis (Wynn and Barron, 2010; Dobaczewski et al., 2011; Mills, 2012; Liao et al., 2018; Miyosawa et al., 2020; Watson et al., 2020; Hiram et al., 2021; Revelo et al., 2021). Furthermore, it has been shown that cardiac resident macrophages are functionally connected to cardiomyocytes by gap junctions (Simon-Chica et al., 2022), thereby facilitating the physiologic electrical conduction in the atrioventricular (AV) node (Hulsmans et al., 2017) or preserving electrical conduction in the context of disease (Sugita et al., 2021).

In sum, a growing body of evidence suggests a key role of cardiac macrophage populations in regulating both physiologic electrical conduction in the healthy heart and proarrhythmic remodeling processes in the diseased heart. With this review, we provide a comprehensive overview on cardiac macrophages and their effects on cardiac electrophysiology. We describe different macrophage populations in the heart and we summarize recent findings on how they affect regular electrophysiology and proarrhythmic electrical, structural, and autonomic remodeling. Since translating basic scientific findings into clinical practice is one of the major challenges in modern medicine, we summarize and discuss the current knowledge on cardiac macrophage populations in mice, pigs, and humans to support translational research on cardiac macrophages in the future.

## 2 CARDIAC MACROPHAGE SUBPOPULATIONS

Macrophages are the most abundant leukocytes in the heart (Pinto et al., 2016; Skelly et al., 2018). They are highly plastic, play significant roles both in cardiovascular homeostasis and pathophysiology (Gomez et al., 2018) and can be identified by certain markers expressed on the cell surface.

Most knowledge about cardiac macrophage subpopulations is derived from studies in mice: during development, macrophages from the embryonic yolk-sac (at embryonic day (E) 7.25) and the fetal liver (at E8.25) migrate into the heart where they persist as "resident cardiac macrophages" by *in situ* proliferation (Schulz et al., 2012; Hashimoto et al., 2013). In the healthy heart, these resident macrophages are the predominant macrophage population, while in the diseased heart so called "recruited macrophages" derived from infiltrating bone-marrow monocytes (from E17.5 to adulthood) are the dominant population (Ingersoll et al., 2011; Epelman et al., 2014; Dick et al., 2019). Moreover, a recent report indicated the endocardium as an additional source of resident cardiac macrophages, called endocardial-derived macrophages, which emerge at E9.5, exhibit an intensive phagocytic activity, then proliferate *in situ*, and are indispensable for development and remodeling of extracellular matrix (ECM) and heart valves (Zhang et al., 2018; Shigeta et al., 2019).

In steady-state, resident macrophages are exclusively ($CCR2^{-}MHC\text{-}II^{low}$) or partly ($CCR2^{-}MHC\text{-}II^{high}$) replenished through *in situ* proliferation with negligible monocyte input (Dick et al., 2019). *In situ* proliferation also contributes to the macrophage proliferation driven by the interleukin (IL)-4 triggered inflammatory response (Jenkins et al., 2011). However, if resident macrophages are depleted (e.g. in transgenic mouse models), monocytes from the bone marrow and splenic reservoirs repopulate the heart and differentiate to macrophages (Heidt et al., 2014). Murine monocytes can be grouped into two major functionally distinct subsets: inflammatory $Ly6C^{high}$ ($CCR2^{+}CX_3CR1^{low}Gr1^{+}$) and noninflammatory $Ly6C^{low}$ ($CCR2^{-}CX_3CR1^{high}Gr1^{-}$) monocytes (Geissmann et al., 2003). All these different blood monocyte subsets differentiate into macrophages when stimulated with macrophage colony stimulating factor (M-CSF) *in vitro* (Sunderkotter et al., 2004), but in response to inflammatory stimuli bone marrow-derived $Ly6C^{high}$ monocytes preferentially infiltrate inflammatory sites and differentiate into $Ly6C^{high}F4/80^{high}$ macrophages as demonstrated in a myocardial infarction (MI) mouse model (Nahrendorf et al., 2007).

Recruited and resident macrophages have different functions in the context of cardiac inflammation (Bajpai et al., 2019). Monocyte-derived macrophage subsets ($CCR2^{+}MHC\text{-}II^{high}$, $CCR2^{+}MHC\text{-}II^{low}$ and $CCR2^{+}Ly6C^{high}$) are preferentially recruited to sites of injury, where they enhance inflammation by promoting further monocyte and neutrophil recruitment and cause cardiomyocyte hypertrophy, interstitial fibrosis and adverse cardiac remodeling resulting in poor outcome, e.g. after myocardial infarction (Lavine et al., 2014; Li et al., 2016; Lorchner et al., 2018). In contrast, resident macrophage subsets ($CCR2^{-}MHC\text{-}II^{low}Ly6C^{low}$ and $CCR2^{-}MHC\text{-}II^{high}Ly6C^{low}$) demonstrate robust proangiogenic properties, suppress inflammation by inhibiting monocyte recruitment and enhance cardiac repair (Kaikita et al., 2004; Lavine et al., 2014; Bajpai et al., 2019). Profibrotic monocyte-derived MHC-

II$^{high}$ macrophages accumulate in mouse hearts exposed to aldosterone, activate fibroblasts by releasing IL-10 and transforming growth factor-β1 (TGF-β1), thereby causing collagen deposition and diastolic dysfunction (Hulsmans et al., 2018). In contrast, MHC-II$^{low}$ macrophages favor the clearance of excess collagen by activating *in vivo* protease sensors and matrix metalloproteinases (MMPs) activity (Hulsmans et al., 2018).

Single-cell RNA sequencing (scRNA-seq) allows precise identification of macrophage subpopulations in the heart. In heart failure mice, two CCR2$^{-}$ populations in the heart showed diverse functions: The MHC-II$^{high}$CD163$^{+}$Mrc1$^{+}$ population is associated with antigen processing and shows a repair-mediating identity, while the other MHC-II$^{low}$ population mediates phagocytosis and proinflammatory changes. The latter CCR2$^{-}$MHC-II$^{low}$ population can further be subdivided into two subclusters based on the cells' different expresses level of the repair-associated genes *Mmp9* and *Arg2* and the neutrophil-associated gene *Csf3r*. On the other hand, two CCR2$^{+}$ populations highly express the proinflammatory cytokines oncostatin M (Osm) and IL-1β. Intriguingly, overexpression of Osm was also observed in cardiac patient hearts suggesting a role in heart disease (Gautier et al., 2012; Skelly et al., 2018; Martini et al., 2019). scRNA-seq data indicates that human and murine TLF$^{+}$ (FOLR2$^{+}$ and/or TIMD4$^{+}$ and/or LYVE1$^{+}$) macrophages originate from both yolk sac and fetal monocyte precursors, and are the most transcriptionally conserved subset (Dick et al., 2022). In the adult human heart, LYVE1$^{+}$TIMD4$^{-}$ macrophages appear to be related to resident macrophages and are associated with cardiovascular remodeling. LYVE1$^{+}$FOLR2$^{+}$ macrophages are monocyte-derived and express the chemoattractant cytokine genes *CCL13* and *CCL18*. Patients with dilated cardiomyopathy showed reduced numbers of LYVE1$^{+}$ resident macrophages (subsets expressed *FOLR2* and *HSPH1*) and an increased number of inflammatory macrophages (subsets expressed *TREM2*, *CCL3* and *KLF2*) (Koenig et al., 2022). In contrast, LYVE1$^{-}$FOLR2$^{-}$MERTK$^{-}$ macrophages are associated with antigen presentation (Litvinukova et al., 2020).

In addition to the classification based on their origin, macrophages can be categorized depending on their cytokine secretion profile studied *in vitro* (Murray and Wynn, 2011). Commonly, macrophages producing proinflammatory cytokines such as IL-1β and tumor necrosis factor-α (TNF-α) which mediate pathogen clearance and tissue destruction, are defined as M1 macrophages. In contrast, macrophages releasing anti-inflammatory cytokines such as IL-10 and TGF-β1 contribute to healing and tissue repair and are commonly referred to as M2 macrophages (Chavez-Galan et al., 2015). Of note, the M1/M2 classification, while useful, is an oversimplification and applies to *in vitro* polarized macrophages, and should therefore be used with caution, especially when discussing *in vivo* macrophage phenotypes.

Generally, all kinds of inflammation contain a mixture of M1 and M2 responses (Atri et al., 2018). For example, in an infectious myocarditis mouse model, M1 macrophages which are activated by cytokines such as interferon-γ produced by T helper cells (Th1), support polarization of CD4$^{+}$ T cells to produce IL-12, IL-23 and nitric oxide (NO). In addition, T helper cell (Th2)-produced cytokines such as IL-4 and IL-13, activate M2 macrophages, which control the proliferation of T cells and attenuate the immune response demonstrating that pro- and anti-inflammatory processes of cardiac macrophages happen simultaneously and disrupt homeostasis (Mills et al., 2000; Gutierrez et al., 2014). Furthermore, within this M1/M2 classification, different macrophage phenotypes partly express similar surface markers, and the exact lineage relationship of Ly6C$^{high}$ and Ly6C$^{low}$ monocytes to M1 and M2 macrophages has yet to be determined. In the acute phase of a myocardial infarction, Ly6C$^{high}$ monocyte-derived macrophages are activated and generate proinflammatory cytokines, remove debris and apoptotic neutrophils, and contribute to tissue-rebuilding post-MI, a process to which Ly6C$^{low}$ macrophages also contribute by regulating wound healing, angiogenesis and myofibroblast activation (Nahrendorf et al., 2007; Frantz and Nahrendorf, 2014).

In sum, there are several macrophage subpopulations in the heart that can be grouped by their origin, their functional impact, or the cytokines produced. However, there are significant overlaps between groups and several surface markers are not exclusively expressed by only one specific macrophage subset. In this review, we try to follow an origin-oriented classification of macrophages into embryonic/fetal precursor-derived "resident cardiac macrophages" and bone marrow monocyte-derived "recruited cardiac macrophages" wherever possible.

# 3 CARDIAC ELECTROPHYSIOLOGY AND POTENTIAL ARRHYTHMIA MECHANISMS

Studying the effects of immune cells on arrhythmogenesis requires profound knowledge about normal electrophysiology, especially regarding cardiac ion currents, calcium homeostasis, and cell-to-cell contacts. For an in-depth overview on cardiac ion channels and electrophysiology, the interested reader may be referred to specific reviews (Bosch, 2002; Bartos et al., 2015; Landstrom et al., 2017; Kistamas et al., 2020). For context, we provide a brief overview on fundamental concepts in the following paragraphs.

## 3.1 Cardiac Ion Channels and Action Potentials

Myocardial electrical activity is generated by sequential opening and closing of ion channels and transporters establishing a transmembrane action potential (AP) in individual cardiomyocytes (**Figure 1**) (Nerbonne and Kass, 2005). The duration of the action potential (APD) reflects the interplay of inward and outward currents (Grant, 2009). In general, the cardiac action potential is divided into five phases: phase 0 reflects a rapid upstroke, which is generated by the activation of a fast inward $Na^{+}$ current ($I_{Na}$). Immediately following the AP upstroke, a transient repolarization produced by an outward potassium current ($I_{to}$) follows (phase 1). Phase 2 represents a slowly decaying plateau, resulting from an equilibrium of the delayed-rectifier potassium current with slow, rapid, and

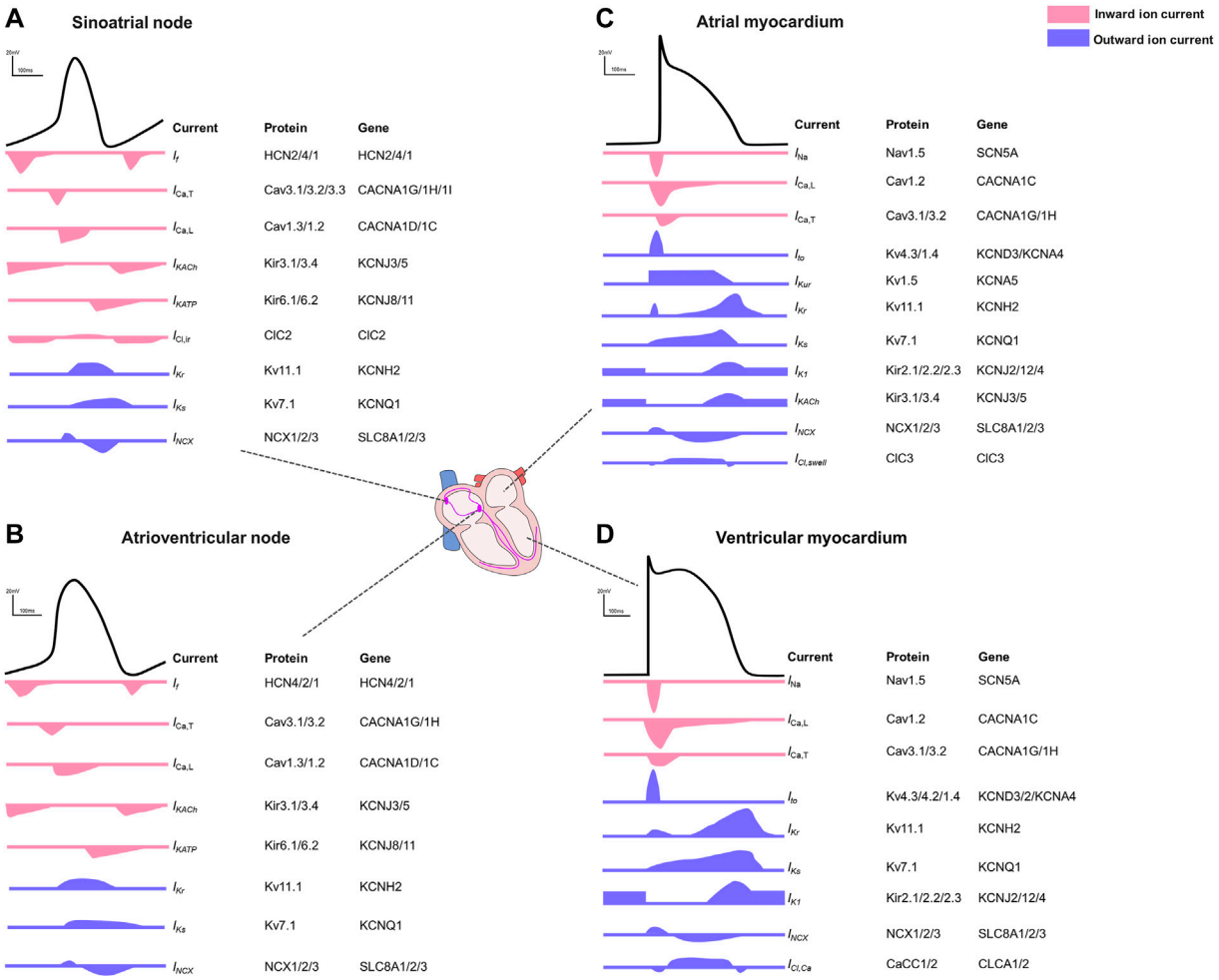


**FIGURE 1 |** Action potentials and the contributing ion currents in different regions of the human heart. **(A)**. Sinoatrial node. **(B)**. Atrioventricular node. **(C)**. Atrial myocardium. **(D)**. Ventricular myocardium.

ultrarapid activation kinetics ($I_{Ks}$, $I_{Kr}$, and $I_{Kur}$, respectively), the $Na^+$-$K^+$-ATPase current ($I_{NKA}$), the late sodium current ($I_{Na,Late}$) and a simultaneous prominent calcium influx ($I_{Ca,L}$, $I_{Ca,T}$). Phase 3 is characterized by a rapid repolarization, resulting from the inactivation of calcium channels and the activation of an inward rectifier potassium current ($I_{K1}$). The resting state (phase 4), which is also mainly driven by the potassium current $I_{K1}$ establishes the resting membrane potential (RMP). Under physiologic conditions, the APD determines the effective refractory period (ERP), which is defined as the shortest time interval needed before a new stimulus can depolarize the cell again causing another AP (Nerbonne and Kass, 2005; Grant, 2009; Varro et al., 2021).

The electrophysiological properties illustrated by different AP shapes and durations differ between the different regions within the heart (Bartos et al., 2015) and between species (Clauss et al., 2019). In pacemaker cells of the sinoatrial node (SAN) or the atrioventricular node (AVN), the pacemaker current ($I_f$) is responsible for the membrane hyperpolarization (Aziz et al., 2018). Inward $Ca^{2+}$ currents, regulated mainly by the L-type calcium channel ($I_{Ca,L}$, $Ca_v1.2$), play a fundamental role in both depolarization of SAN and AVN cells and in counteracting repolarization despite lacking a clear plateau phase (Bers, 2008). Acetylcholine-activated $K^+$ channels ($I_{K,Ach}$ current) are most abundantly expressed in SAN and AVN where this current contributes to the diastolic depolarization and in the atria where it hyperpolarizes the cell contributing to the phase 3 repolarization (Nerbonne, 2016). In general, atrial action potentials are shorter than those in ventricles with a less clear plateau phase, mainly because of potassium channels with faster activation kinetics and larger conductance. In phase 4, the ventricular resting membrane potential is more negative because ventricular myocytes have higher inward rectifier currents than atrial myocytes (Ng et al., 2010). The regional differences driven by the diversity of ion channels (Joukar, 2021; Varro et al., 2021) are summarized in **Figure 1**.

## 3.2 Arrhythmia Mechanisms

Current paradigms in arrhythmogenesis include electrical ectopy (acting as a trigger) and reentry (acting as substrate) which are

reviewed in detail elsewhere (Nattel et al., 2007; Michael et al., 2009; Wakili et al., 2011; Sanchez-Quintana et al., 2012; Clauss et al., 2015). Here, we briefly summarize some of the main aspects to allow a better understanding of macrophage effects on arrhythmogenesis which are provided in **section 4**.

### 3.2.1 Ectopy/Arrhythmia Triggers

Any change to the subtle equilibrium of currents underlying cell type-specific action potentials (**Figure 1**) resulting from altered channel conduction or kinetics (e.g., by mutation, ligand binding or posttranslational modification) can potentially lead to focal ectopic activity which may in turn act as arrhythmia "trigger". The main mechanisms underlying ectopic impulse generation include early (EADs) and delayed afterdepolarizations (DADs) which are voltage oscillations known to cause cardiac arrhythmias (Song et al., 2015). EADs appear at phase 2 (in the context of APD prolongation due to e.g., activation of L-type $Ca^{2+}$ channels ($I_{Ca,L}$ current)) or phase 3 (in the context of $Ca^{2+}$ overload and activated $Na^+/Ca^{2+}$ exchange current (NCX) resulting in $Na^+$ influx) (Tse, 2016). DADs usually occur following AP repolarization and are associated with an intracellular $Ca^{2+}$ accumulation (Pogwizd et al., 2001).

### 3.2.2 Reentry/Arrhythmia Substrate

Aside from altered impulse formation, abnormal impulse conduction represents another main mechanism of arrhythmogenesis (Nguyen et al., 2017). This can be caused by anatomic changes (such as hypertrophy or fibrosis) indirectly influencing conduction, by altered electrical conduction properties itself (functional reentry, e.g., by modified gap junction expression/function) (Kamjoo et al., 1997) as well as by changes in autonomic tone (Verheule and Schotten, 2021).

The major hallmark of structural remodeling and subsequently maintenance of arrhythmia is cardiac fibrosis, characterized by an imbalance between the generation and degradation of extracellular matrix (ECM) (Merchant and Armoundas, 2012). Under myocardial injury, activated fibroblasts differentiate into myofibroblasts, which release profibrotic factors such as transforming growth factor-β1 (TGF-β1) and stimulate expression of ECM, thereby promoting fibrogenesis (Khalil et al., 2017). Interstitial fibrosis forms electrical barriers between myocardial bundles, causing the electrical impulse to travel a longer distance, resulting in conduction delay and potential asynchronous activation of different areas of the myocardium (de Jong et al., 2011). Such a delayed impulse conduction, which also happens after myocardial infarction where a myocardial scar acts as a central obstacle, allows the myocardium to recover from a previous excitation. The following depolarizing front can continuously encounter excitable myocardium, thereby promoting circular excitation, so-called reentry (Verheule and Schotten, 2021). A murine TGF-β1 overexpression model for example shows that atrial fibrosis itself is sufficient to cause AF (Verheule et al., 2004). In ventricular myocardium, the slow conduction zone formed by fibrosis in post-MI and advanced HF patients creates a substrate for reentry of sustained monomorphic ventricular tachycardia (VT) (St John Sutton et al., 2003; Yokokawa et al., 2009).

The AP propagation depends on gap junctions, transmembrane proteins that mediate cell-to-cell coupling (Kanno and Saffitz, 2001). Gap junctions are composed of connexin (Cx) subunits. Cx43 is the most abundant connexin expressed in both atrial and ventricular cardiomyocytes of mammalian species (Severs et al., 2004). In healthy hearts, gap junctions are predominantly expressed in the intercalated disc regions between cardiomyocytes and facilitate the longitudinal flow of electrical currents (Rohr, 2004). Downregulated expression and lateralization of Cx43 increase arrhythmia susceptibility, as slowed and multidirectional conduction occurs (Kostin et al., 2004). Ischemia induced dephosphorylation and progressive reduction of Cx43 can lead to electrical uncoupling of ventricular myocytes, potentially playing an important role in arrhythmogenesis after MI (Beardslee et al., 2000).

Cardiac electrophysiology is modulated by the autonomic nervous system (ANS) consisting of sympathetic and parasympathetic nerves (Herring et al., 2019). An imbalance of the autonomic innervation may induce an inhomogeneous conduction and distribution of refractoriness promoting susceptibility to arrhythmia (Shen and Zipes, 2014). Autonomic stimulation (bilateral either sympathetic or parasympathetic) of isolated rabbit hearts for example shows spatial dispersion of repolarization (nonuniformity of repolarization) and heterogenous APD, which contributes to arrhythmia vulnerability (Mantravadi et al., 2007). In a diabetic rat model, increased heterogeneity of sympathetic nerves and defected parasympathetic nerves leads to decreased atrial ERP and increased incidence of AF under sympathetic nerve stimulation (Otake et al., 2009).

## 4 CARDIAC MACROPHAGES MEDIATE ARRHYTHMOGENESIS BY PROMOTING CARDIAC REMODELING

It has been suggested that macrophages play an important role in arrhythmogenesis since they can produce a number of cytokines which have been linked to cardiac remodeling. However, only recently a few studies have been published that clearly demonstrate specific macrophage-dependent mechanisms regulating physiologic conduction (Hulsmans et al., 2017; Sugita et al., 2021; Simon-Chica et al., 2022) as well as electrical, structural, or autonomic remodeling leading to arrhythmias (Monnerat et al., 2016; Sun et al., 2016; Yin et al., 2016; Fei et al., 2019; Hu et al., 2019; Liu et al., 2019; Lubos et al., 2020; Lyu et al., 2020; Miyosawa et al., 2020; Zhang et al., 2020; Hiram et al., 2021; Zhang et al., 2021) (**Figure 2**).

## 4.1 Cardiac Macrophages Modulate Cell-To-Cell Coupling *via* Gap Junctions

Cardiomyocytes are electrically connected *via* gap junction channels (established by connexins) which is an essential prerequisite for electrical impulse propagation. Modifications of connexins such as phosphorylation or altered distribution

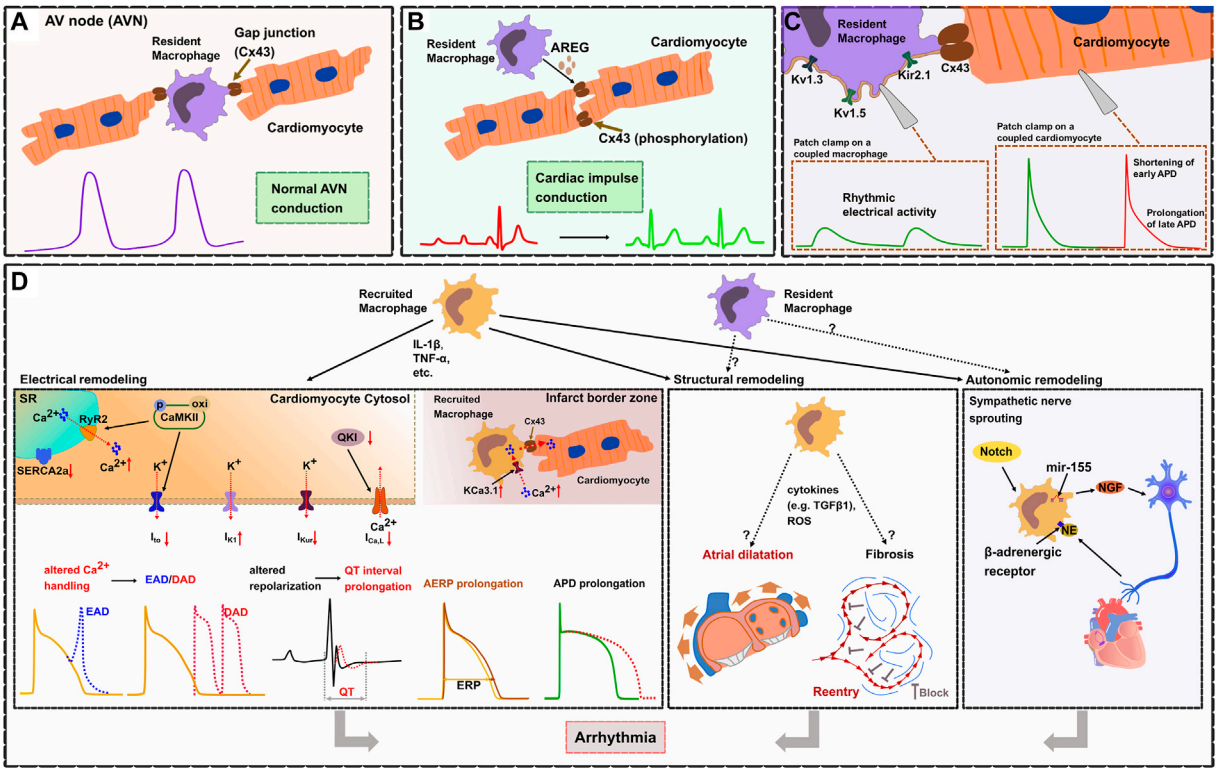


**FIGURE 2 |** Cardiac macrophages in electrophysiology and arrhythmogenesis. **(A)**. In the healthy heart, cardiac resident macrophages are functionally linked to cardiomyocytes through gap junctions (Cx43), thereby facilitating electrical conduction in the atrioventricular node (AVN). **(B)**. Macrophages prevent arrhythmias by regulating the phosphorylation of Cx43 *via* AREG (Amphiregulin). **(C)**. Resident cardiac macrophages express potassium channels (including Kv1.3, Kv1.5, and Kir2.1), Patch clamp experiments show that resident macrophages can depolarize coupled cardiomyocytes, shorten early APD and prolong late APD. **(D)**. Electrical remodeling (left frame). During inflammation, recruited macrophages produce cytokines (IL-1β, TNF-α) which affect ion currents and calcium homeostasis resulting in increased electrical vulnerability to arrhythmias. TNF-α causes abnormal SR $Ca^{2+}$-ATPase (SERCA2a) function which reduces the SR $Ca^{2+}$ uptake. IL-1β induces AP prolongation through a decrease in $I_{to}$ current and an increased diastolic sarcoplasmic reticulum (SR) $Ca^{2+}$ leak *via* ryanodine receptors (RyR2). This is promoted through CaMKII oxidation/phosphorylation causing cytosol $Ca^{2+}$ overload leading to delayed afterdepolarizations (DAD). CaMKII inactivates $I_{to}$ which contributes to the prolongation of APD and predisposes to early or delayed afterdepolarizations (EAD). IL-1β reduces $I_{Ca,L}$ by inhibiting the expression of atrial quaking protein (QKI) facilitating atrial fibrillation. Cytokines (IL-1β, TNF-α, TGF-β, IL-6) lead to APD prolongation by altering potassium current densities (increased $I_{K1}$ and reduced $I_{Kur}$). Reduced atrial conduction velocity and AERP prolongation, also results in enhanced susceptibility for atrial fibrillation (AF). Within infarct border zones, upregulated potassium channel KCa3.1 in recruited macrophages facilitate $Ca^{2+}$ influx into the macrophages. Elevated intracellular $Ca^{2+}$ then flows from recruited macrophages to adjacent cardiomyocytes via Cx43, which causes APD prolongation of cardiomyocytes. Structural remodeling (middle frame). Recruited macrophages cause atrial dilatation and fibrosis probably by releasing cytokines (e.g., TGF-β1) or reactive oxygen species (ROS), but the exact mechanisms have not been fully elucidated. Atrial dilatation and formation of reentry subsequently results in enhanced susceptibility for AF. Autonomic remodeling (right frame). Recruited macrophages induce autonomic nerve sprouting by synthesizing nerve growth factor (NGF). Norepinephrine (NE) from sympathetic nerve endings can in turn activate *β*-adrenergic receptors on macrophages, which enhances the expression of NGF. Activation of Notch signaling and microRNA-155 expression in recruited macrophages also promotes sympathetic outgrowth.

of gap junctions result in reduced conduction velocity or altered pathways of conduction and anisotropy leading to a proarrhythmic substrate predisposing to reentry (Jongsma and Wilders, 2000; Dhein and Salameh, 2021).

Recently, Hulsmans et al. (2017) demonstrated that cardiac resident macrophages are enriched in the healthy AV node both in mouse ($CD11b^{+}CD64^{+}CX_3CR_1{}^{+}F4/80^{+}Ly6C^{low}$) and human ($CD68^{+}CD163^{+}$) and that they are functionally coupled to cardiomyocytes *via* Cx43. They revealed that macrophages coupled to cardiomyocytes show spontaneous rhythmic depolarizations whereas cardiomyocytes coupled to AV nodal macrophages show an elevated resting membrane potential and a shortened action potential, which facilitates electrical conduction in the AV node. These findings were further supported by several *in vivo* models: 1) using an optogenetics approach they were able to specifically depolarize AV nodal macrophages *in situ* (transgenic expression of the fluorescently activated channel rhodopsin-2 specifically in macrophages, $Cx_3cr1^{wt/CreER}$ $ChR2^{wt/fl}$; focused light on the exposed AV node region) which resulted in enhanced electrical conduction through the AV node illustrated by a higher number of conducted stimuli at the Wenckebach point. 2) In a transgenic mouse model with macrophage-specific knockdown of Cx43 ($Cx_3cr1^{wt/CreER}$ $Cx43^{fl/fl}$) they demonstrated an increased Wenckebach cycle length and AV node refractory period indicating that disruption of the macrophage-cardiomyocyte interaction

results in impaired electrical conduction in the AV node. These findings were further confirmed by 3) a transgenic mouse model that congenitally lacks macrophages ($Csf1^{op}$). 4) Inducible macrophage depletion in $CD11b^{DTR}$ mice finally showed progressive AV block within 1–2 days. All these findings illustrate that cardiac resident macrophages facilitate electrical conduction in the AV node by electrical coupling to cardiomyocytes *via* Cx43 (Hulsmans et al., 2017) (**Figure 2A**).

In a mouse model of right ventricular (RV) stress (pulmonary artery banding leading to RV pressure overload), cardiac macrophages protect the heart from arrhythmia and sudden cardiac death (SCD) by maintaining proper electrical conduction through gap junctions (Sugita et al., 2021). Depletion of macrophages in this model resulted in advanced heart block and lethal cardiac arrest indicating that macrophages play an essential role for survival. Further studies identified macrophage-derived amphiregulin (AREG) as key regulator of Cx43 phosphorylation and translocation in cardiomyocytes. AREG knockout mice showed reduced Cx43 phosphorylation and a pronounced Cx43 lateralization leading to arrhythmias and increased mortality after pulmonary artery banding, while bone marrow transplantation (wildtype mice serving as donors) or treatment with recombinant AREG restored Cx43 phosphorylation/localization and prevented SCD (Sugita et al., 2021) (**Figure 2B**).

A recent study by Simon-Chica and colleagues comprehensively characterized the electrophysiologic properties of murine cardiac resident ($Cx_3cr_1^{eYFP/+}$) macrophages and reported a membrane resistance of 2.2 ± 0.1 GΩ, a capacitance of 18.3 ± 0.1 pF, and a resting membrane potential of -39.6 ± 0.3 mV (Simon-Chica et al., 2022) (**Figure 2C**). They revealed that resident macrophages express potassium channels including Kv1.3, Kv1.5, and Kir2.1 which establish several inward and outward rectifying currents. Furthermore, resident macrophages have been shown to express Cx43. Computer modeling demonstrates that resident macrophages can depolarize coupled cardiomyocytes and shorten APD. Besides a number of novel findings, the authors could independently confirm several observations such as the macrophages' resting membrane potential, their Cx43 expression and their ability to shorten the APD of coupled cardiomyocytes which have been previously reported by Hulsmans et al. (Hulsmans et al., 2017).

In patients with arrhythmias after myocardial infarction (but not in patients without post-MI arrhythmias) an elevated number of recruited macrophages have been detected in the infarct border zone where they form gap junctions with adjacent cardiomyocytes, a finding that was confirmed in a mouse model of MI (Fei et al., 2019). Recruited macrophages couple to cardiomyocytes *via* Cx43 in border zones of murine infarcted hearts and show an upregulation of the potassium channel KCa3.1 (Fei et al., 2019). This facilitates $Ca^{2+}$ influx and causes APD prolongation in connected cardiomyocytes which results from the intracellular $Ca^{2+}$ flow from macrophages to cardiomyocytes through Cx43, demonstrating that recruited macrophages can be functionally linked to cardiomyocytes modulating cardiac conduction (Fei et al., 2019) (**Figure 2D**).

In mice with myocardial infarction macrophage-produced proinflammatory cytokine MMP-7 was shown to be able to process Cx43 at a C-terminus cleavage site. The degradation of ventricular Cx43 is linked with decreased conduction velocity and increased incidence of arrhythmia and SCD (Gutstein et al., 2001; Lindsey et al., 2006). IL-1β has also been shown to lead to defective excitation-contraction coupling and arrhythmogenesis by Cx43 degradation in the post-MI heart (De Jesus et al., 2017). In the border zone of a MI, myofibroblasts emerge from fibroblasts under the stimulation of IL-1β, which then further produce more IL-1β, and cause Cx43 downregulation and thereby abnormal conduction of cardiac impulse and formation of arrhythmogenesis substrate (Baum et al., 2012).

Altogether, there is strong evidence that cardiac macrophages can be functionally linked to cardiomyocytes, modulate the cardiomyocytes' electrophysiologic properties and may thus be key players in arrhythmogenesis.

## 4.2 Cardiac Macrophages Induce Cardiac Electrical Remodeling

A growing body of evidence suggests that inflammatory processes are key factors in the pathophysiology of atrial fibrillation (AF) with several remodeling mechanisms being regulated by macrophages (Engelmann and Svendsen, 2005; Yamashita et al., 2010; Hu et al., 2015; Mitrofanova et al., 2016; Watson et al., 2020). In patients with AF increased levels of inflammatory markers (e.g., CRP, IL-6, IL-8, TNF-α etc.) and an elevated number of recruited macrophages (identified as $CD68^+$ and $CD14^{++}CD16^-$ macrophages) have been described in left and right atria (Patel et al., 2010; Yamashita et al., 2010; He et al., 2016; Sun et al., 2016; Aguiar et al., 2019).

Further mechanistic studies in lipopolysaccharide (LPS)-treated mouse and canine models revealed that recruited macrophages induce electrical remodeling by secreting pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (Sun et al., 2016) (**Figure 2D**). In these models, IL-1β has been shown to inhibit the expression of atrial quaking protein (QKI), a RNA-binding protein that regulates RNA splicing and maintains RNA stability and has been shown to reduce CACNA1C (L-type calcium channel subunit) expression and $I_{Ca,L}$ in atrial myocytes leading to AF (Tili et al., 2015; Sun et al., 2016).

Liu and colleagues induced myocardial infarction in rats and treated these rats with Fisetin, a flavonoid with proposed anti-inflammatory effects (Liu et al., 2019). After 4 weeks post-MI rats showed significant LA fibrosis, prolonged interatrial conduction time and atrial refractory periods as well as an increased inducibility of AF, accompanied by elevated numbers of recruited $CD68^+$ macrophages and increased expression of IL-1β and TNF-α in LA indicating proarrhythmic structural and electrical remodeling together with an inflammatory response (**Figure 2D**). In rats treated with Fisetin, macrophage recruitment to the heart, proarrhythmic remodeling and susceptibility for AF was significantly reduced suggesting a causal role for recruited cardiac macrophages in ischemia-related arrhythmogenesis (Liu et al., 2019).

Zhang and colleagues studied arrhythmogenesis in spontaneously hypertensive rats and could demonstrate that mechanisms mediated by recruited macrophages play an important role (Zhang et al., 2020). Hypertensive rats showed electrical and structural remodeling including APD prolongation, altered potassium current densities (increased $I_{K1}$ and reduced $I_{Kur}$), reduced expression of Cx43 as well as enlarged atria, enhanced atrial fibrosis and increased levels of reactive oxygen species, ultimately leading to an increased susceptibility for AF (**Figure 2D**). The authors proposed a macrophage-mediated mechanism due to elevated numbers of $Mac2^+$ recruited macrophages in the atria of hypertensive rats and increased levels of common macrophage-derived cytokines such as TGF-β, IL-1β, IL-6, and TNF-α. CXCR2 inhibition blocked recruitment of macrophages, reversed all these effects and could finally prevent AF inducibility suggesting a macrophage-dependent mechanism (Zhang et al., 2020).

Inflammatory and macrophage-mediated arrhythmia mechanisms have also been shown for ventricular arrhythmias. Recent studies revealed that expression of *KCNN4* is upregulated in macrophages recruited to the heart after myocardial infarction. *KCNN4* encodes the intermediate conductance $Ca^{2+}$-activated $K^+$ channel KCa3.1, which can preserve the negative membrane potential required for sustained $Ca^{2+}$ influx (Xu et al., 2017). As already mentioned in **section 4.1**, in the mouse MI model KCa3.1 activation in recruited macrophages facilitates $Ca^{2+}$ influx and causes APD prolongation of cardiomyocytes which are connected to these macrophages *via* gap junctions, ultimately resulting in prolonged QTc duration and enhanced susceptibility to ventricular arrhythmias (**Figure 2D**) (Fei et al., 2019).

Diabetes mellitus is associated with an increased risk for arrhythmias (Kahn et al., 1987; El-Atat et al., 2004; Renner et al., 2020). For a long time, inflammation was proposed to play an important role in diabetic arrhythmogenesis, but only recently Monnerat and colleagues could demonstrate a specific macrophage-dependent mechanism (**Figure 2D**) (Monnerat et al., 2016). In a mouse model of diabetes mellitus, they showed that hyperglycemia activates the toll-like receptor 2 (TLR2) and the NLRP3 inflammasome in recruited cardiac MHC-$II^{high}$ macrophages which in turn causes release of IL-1β. Elevated IL-1β leads to ventricular arrhythmias by prolongation of the ventricular action potential, reduction of the potassium current $I_{to}$ and enhancement of a diastolic SR calcium leak which is mediated by increasing CaMKII oxidation/phosphorylation. Targeting the IL-1β axis by either inhibiting the IL-1β receptor or inhibiting the NLRP3 inflammasome was further shown as potential therapeutic approach protecting against arrhythmias (Monnerat et al., 2016).

## 4.3 Cardiac Macrophages Mediate Structural Remodeling

Studies on cardiac remodeling after tissue injury (e.g. myocardial infarction) revealed that macrophages play a pivotal role in cardiac remodeling as they may have both profibrotic and antifibrotic functions (Wynn and Barron, 2010; Liao et al., 2018; Revelo et al., 2021; Dobaczewski et al., 2011). $CD68^+$ or $CD11c^+$ macrophages infiltrate the epicardial adipose tissue of AF patients and are associated with atrial fibrotic remodeling leading to a proarrhythmic substrate for AF (Abe et al., 2018). As fibrosis is one of the hallmarks of arrhythmogenesis, it seems obvious that macrophages may play an important role in proarrhythmic structural remodeling, but specific studies are rare (**Figure 2D**).

The crosstalk between macrophages and cardiac fibroblasts regulates the balance of cardiac fibrosis (Van Linthout et al., 2014). Ly6C$^{high}$ macrophages release anti-fibrotic cytokines like Osm to inhibit the conversion of fibroblasts to myofibroblasts. Ly6C$^{low}$ macrophages in contrast produce profibrotic cytokines such as TGF-β1 and IL-10 to promote fibrosis (Weber et al., 2013; Abe et al., 2019). In turn, cardiac fibroblasts, as a major source of cytokines, can instruct resident macrophages to recruit other immune cells (monocytes, neutrophils) (Van Linthout et al., 2014). The cytokine secretion (IL-6, TGF-β1) of cardiac fibroblasts relies on the presence of macrophages in an *in vitro* co-culture model (Ma et al., 2012). Alvarez et al. (2011) explored the relationship between macrophages and fibroblasts in association with congenital heart block (CHB). They found that human fetal cardiac fibroblasts secreted TGF-β under the stimulation of macrophage-produced endothelin-1 in an *in vitro* experiment, and endothelin-1 presented in the septal region in areas of calcification and fibrosis in two fetal hearts of CHB. Furthermore, cardiac fibroblasts can influence cardiac function through direct (through gap junctions or membrane nanotubes) and indirect (paracrine signaling) effects on cardiomyocytes (Camelliti et al., 2004; Miragoli et al., 2006; Quinn et al., 2016).

In AF patients without concomitant heart failure, elevated numbers of macrophages ($CD163^+$) were detected in right atrial appendages which was associated with increased atrial gene expression of procollagen and B-type natriuretic peptide (BNP) and atrial fibrosis (Watson et al., 2020). Although sample size was small (RA tissue from 10 patients with AF and 27 patients without AF) this study indicates a direct link between cardiac macrophages, structural remodeling and AF.

In another small study, elevated numbers of recruited $CCR2^+$ macrophages have been observed in left atrial appendages obtained from AF patients (Miyosawa et al., 2020). Macrophage numbers were higher in patients with left atrial dilatation compared to patients without LA dilatation, indicating an association between increased macrophage numbers and atrial structural remodeling. In a larger cohort (83 patients with AF and normal LA diameter, 78 patients with AF and LA dilatation, 22 patients without AF) the authors further studied circulating monocytes. In AF patients the numbers of monocytes were significantly lower compared to patients without AF but among AF groups total numbers or proportion of monocyte subsets were not different. However, in patients with LA dilatation monocytes expressed higher levels of CCR2 and exhibited an enhanced migratory activity *in vitro*. Although this study is also purely descriptive, investigated a small number of patients and lacks a comprehensive analysis of structural remodeling (e.g. atrial fibrosis) it is another hint towards a direct role of recruited cardiac macrophages in proarrhythmic structural remodeling (Miyosawa et al., 2020).

In rats, monocrotaline-induced pulmonary hypertension causes proarrhythmic atrial remodeling including reduced atrial conduction velocity, atrial dilatation and fibrosis which subsequently results in enhanced susceptibility for AF (Hiram et al., 2021) (**Figure 2D**). The authors proposed a macrophage-related mechanism since an elevated number of recruited $CD68^+$ macrophages was observed in the right atrium. Treatment with resolvin-D1 significantly attenuated proarrhythmic remodeling and reduced the inducibility of AF which was accompanied by significant reduction of $CD68^+$ macrophages and an increase of $CD206^+$ macrophages in the heart (Hiram et al., 2021).

Several independent studies performed in rat models provide some more mechanistic data which support a clear link between cardiac macrophages and proarrhythmic remodeling (**Figure 2D**). As these studies by Yun-Long Zhang et al. (2020), Liang Liu et al. (2019) indicated macrophage-related effects both on structural and electrical remodeling, they are already discussed in **section 4.2**.

Arrhythmogenic cardiomyopathy is an inherited disease characterized by progressive structural remodeling ultimately leading to arrhythmias and SCD (Elliott et al., 2019). In mice, inflammation has been identified as a major mechanism of macrophage recruitment to the heart (Lubos et al., 2020). Histologic analyses revealed that $CD11b^+CD206^+F4/80^+$ macrophages accumulate and persist in fibrotic regions/scars over several weeks and that the macrophage populations include both proinflammatory (expressing MMP12) and reparative (expressing osteopontin) macrophages. Although this work illustrates the association of macrophages with fibrotic remodeling, potential conclusions on arrhythmogenesis must be drawn with caution as 1) there was no direct evidence for arrhythmias in these mice, 2) direct macrophage function/effects were not investigated, and 3) a direct causal role for macrophages remains elusive.

All these studies mentioned above demonstrate a potential role for recruited, i.e., monocyte-derived cardiac macrophages in proarrhythmic remodeling. In heart failure induced by aortic constriction in mice (pressure overload TAC model) it has been indicated that cardiac resident ($CCR2^-$) macrophages and recruited monocyte-derived ($CCR2^+Ly6C^{high}$) macrophages may have distinct and at least partly antagonistic effects (Liao et al., 2018; Revelo et al., 2021) suggesting that different macrophage populations in the heart may also differentially affect proarrhythmic remodeling.

## 4.4 Macrophages Mediate Autonomic Nerve Remodeling

The heart is innervated both by sympathetic and parasympathetic nerves and alterations in this innervation—the so-called autonomic remodeling—have been demonstrated to play important roles in arrhythmogenesis (Cao et al., 2000; Stavrakis et al., 2020). Macrophages are able to synthesize nerve growth factor (NGF), an essential protein for promoting sympathetic nerve sprouting (Brown et al., 1991; Wernli et al., 2009) indicating a potential role for macrophages in autonomic remodeling.

This is further supported by the presence of $CD68^+$ macrophages in stellate ganglia from patients with Long QT syndrome (LQTS) or catecholaminergic polymorphic VT (CPVT) where the autonomic innervation has been identified as key factor in arrhythmogenesis (Rizzo et al., 2014). Studies in a rat heart failure model revealed that local depletion of macrophages in stellate ganglia attenuate cardiac sympathetic overactivation and susceptibility to ventricular arrhythmias (Zhang et al., 2021). This is due to reduced levels of proinflammatory cytokines (TNF-α and IL-1β) and reduced N-type $Ca^{2+}$ currents as well as excitability of cardiac sympathetic neurons (Zhang et al., 2021). Norepinephrine (NE) from sympathetic nerve endings can in turn activate $\beta$-adrenergic receptors on macrophages, which enhances the expression of NGF and establishes a vicious circle of sympathetic nerve remodeling, thereby aggravating electrophysiological heterogeneity and increasing the risk for ventricular arrhythmias (Lyu et al., 2020).

Notch signaling is an important pattern receptor for myeloid cell differentiation and macrophage activation (Monsalve et al., 2006). In post-MI rat hearts, the activation of Notch signaling predominantly promotes polarization of recruited macrophages toward the proinflammatory phenotype in the infarcted border zone as well as NGF expression, which then stimulates sympathetic outgrowth (Yin et al., 2016). Inhibition of Notch in contrast induces a phenotype switch from proinflammatory to reparative macrophages and decreases NGF expression, consequently ameliorating sympathetic nerve sprouting (Yin et al., 2016). The macrophage-derived microRNA-155 has been shown to suppress genes related to neural stem cell self-renewal by downregulation of cytokine signaling 1 (Scos1) (Zingale et al., 2021). Inhibition of microRNA-155 expression in recruited macrophages in a MI mouse model displays decreased density of tyrosine hydroxylase and GAP43 (neuromodulin) positive nerve fibers in myocardial tissue, which was associated with reduced ventricular arrhythmias (Hu et al., 2019) (**Figure 2D**).

In sum, a number of studies indicate that recruited macrophages may play an important role in arrhythmogenesis by regulating autonomic remodeling after myocardial injury.

# 5 FUTURE CHALLENGES: STUDYING MACROPHAGES IN ARRHYTHMOGENESIS FOLLOWING THE TRANSLATIONAL ROAD FROM RODENTS TO HUMANS

To investigate the complex role of macrophages in electrophysiology and arrhythmogenesis, delicately designed *in vivo* experiments are required. Model organisms vary in regard to anatomy, physiology, electrophysiological properties, immunology, genetic background, availability, costs, practical, and ethical considerations (Clauss et al., 2019; Schuttler et al., 2020). All species used in research have distinct advantages and disadvantages and every species might be the ideal one for a specific research question. Since mouse models are widely

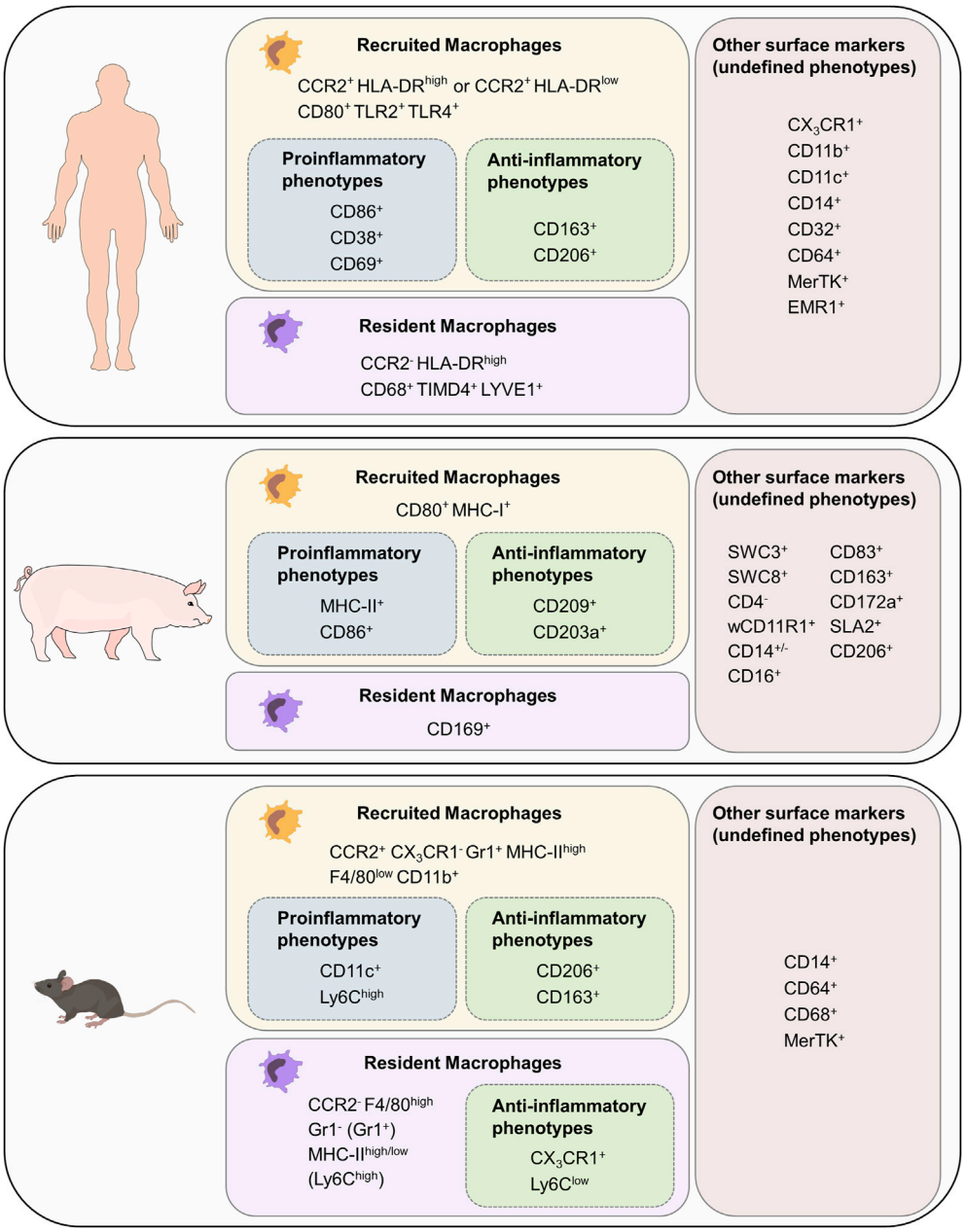


**FIGURE 3 |** Cardiac macrophage surface markers in mouse, pig and human. List of surface markers for recruited and resident macrophages as well as markers for proinflammatory and anti-inflammatory phenotypes. Surface markers with unknown association to function have been grouped as "other surface markers (undefined phenotypes). F4/80 in mouse is the homolog for EMR in humans.

available at different genetic backgrounds with various genetic modifications and manifold options for disease modeling they are highly suitable and widely used for initial investigations both in electrophysiology and immunology research. However, compared to humans mice have notable differences regarding both electrophysiology and immunology, which limits the transferability of findings obtained in mouse models to a clinical setting in humans. Thus, suitable so-called preclinical models in large animals are necessary to validate findings obtained in mouse models prior to clinical application. The most widely used large animal species include sheep, dogs and pigs. In regard to electrophysiology research we have proposed a "practical trio" including mice, rabbits and pigs as these species are most suitable for the majority of researchers in the field (Clauss et al., 2019). Furthermore, such a "practical trio" establishes a translational concept paving the way from early *in vitro* studies for screening, over initial *in vivo* validation in mice and further mechanistic studies in rabbits to confirmation and

preclinical testing in close-to-human pig models prior to clinical application in human patients (Clauss et al., 2019). Continuing our proposed concept, we will discuss how cardiac macrophage populations differ between mice, pigs, and humans, by which surface markers they can be identified (**Figure 3**), and finally demonstrate that the pig is a suitable large animal species to study cardiac macrophages in the context of arrhythmias.

As described in **section 1**, murine resident macrophages derived from embryonic/fetal precursors and recruited macrophages derived from bone-marrow monocytes can be distinguished based on their CCR2 and $CX_3CR_1$ expression (Pinto et al., 2012; Bajpai et al., 2019). Resident macrophages ($CCR2^-$ $CX_3CR_1^+$) can be further grouped into two numerically dominant $Ly6C^{low}Gr1^-MHC\text{-}II^{high}$ and $Ly6C^{low}Gr1^-MHC\text{-}II^{low}$ subsets, and the $Ly6C^{high}Gr1^+$ subset. Furthermore, resident macrophages can be identified as $F4/80^{high}$, whereas recruited macrophages ($CCR2^+CX_3CR_1^-$) are $F4/80^{low}CD11b^{high}$. Recruited macrophages with a proinflammatory phenotype also express CD11c, whereas those with a reparative phenotype can be identified as $CD163^+CD206^+$ (Zhu et al., 2017; Ma et al., 2018). Other classic murine macrophage markers include CD14, CD64, CD68, and MerTK (Gautier et al., 2012).

Though rabbits are a valued model in electrophysiology research, cardiac macrophages in rabbits have been poorly characterized so far. Rabbit macrophages can be polarized into proinflammatory ($MHC\text{-}II^{high}$) and reparative ($CD206^+$) phenotypes using human recombinant GM-CSF and M-CSF, respectively (Yamane and Leung, 2016). Rabbit macrophages do not express the tissue macrophage marker CD68, but express RAM11, a widely used macrophage marker in rabbits (Lis et al., 2010). CD14, CD163, CD200 and Arginase-1 can also be used to further group rabbit macrophages (Sanz et al., 2007; Hoemann et al., 2010; Akkaya et al., 2016; Desando et al., 2019). Rabbits, as they are still easy to house, can be used to fill the gap between rodents and large animals.

Human macrophages are grouped into three major subpopulations depending on the cell surface markers CCR2 and HLA-DR. Since attraction of monocytes into tissue depends on CCR2 signaling, recruited macrophage populations can be identified as $CCR2^+HLA\text{-}DR^{low}$ and $CCR2^+HLA\text{-}DR^{high}$ (Svedberg and Guilliams, 2018). Resident cardiac macrophages, however, are $CCR2^-HLA\text{-}DR^{high}$ (Bajpai et al., 2018; Sansonetti et al., 2020). Both $CCR2^+$ and $CCR2^-$ macrophages express common markers of monocytes and macrophages including $CX_3CR_1$, CD11b, CD11c, CD14, CD32, CD64, CD86, MerTK, and EMR1 (Bajpai et al., 2018). The majority of $CD68^+$ cells represent $CCR2^-$ resident macrophages which also express TIMD4 and LYVE1 (Dick et al., 2019). To distinguish functional phenotypes of recruited macrophages many additional markers can be used: human proinflammatory macrophage markers include CD38, CD69, CD80, TLR2, and TLR4, whereas human anti-inflammatory macrophage markers include CD163 and CD206 (Singleton et al., 2016; Yunna et al., 2020; Kim et al., 2021).

Porcine macrophages have not been characterized to the same extent, making it challenging to identify monocyte/macrophage lineage cells based on their surface markers (Piriou-Guzylack and Salmon, 2008; Ezquerra et al., 2009; Mair et al., 2014; Dawson and Lunney, 2018; Nikovics and Favier, 2021). In blood cell maturation, two cell lineages can be distinguished from each other: The lymphoid lineage leads to T-, B- and natural killer (NK) cells. Granulocytes, monocytes, macrophages and polymorphonuclear cells (PMCs) are part of the myeloid cell lineage (Kondo, 2010). Porcine memory helper T cells present as $CD4^+CD8^+$cells. B cells can be identified with the marker CD21, T cells can be furthermore distinguished with being $CD3e^+CD56^+$. NK cells express the marker CD56 (Piriou-Guzylack and Salmon, 2008). As these markers are not present in the myeloid lineage, spotting and excluding cells of the lymphoid lineage eases identification of macrophages. All cells with origin from the myeloid progenitor line express high levels of the swine workshop cluster SWC3, which makes it an ideal marker for myeloid cells (Summerfield and McCullough, 1997). Porcine PMCs are described as $SCW1^+SWC3^+SWC8^+SWC9^-CD14^{low}CD163^-$. Dendritic cells can be identified as $SWC3^+WC1^+wCD11R1^-CD16^+CD163^-$ cells (Piriou-Guzylack and Salmon, 2008). CD203a and SWC8 function as potential identification markers for myeloid differentiation: when monocytes develop into macrophages, they express more CD203a. SWC8 can be found on pig PMCs in the blood and on macrophages (Piriou-Guzylack and Salmon, 2008). CD11b is a commonly used marker for human monocytes and macrophages. The anti-human CD11b antibody reacts with the porcine wCD11R1 but in contrast wCD11R1 is not expressed on porcine monocytes or alveolar macrophages. CD14, like in humans, is highly expressed on porcine monocytes, is also present on a low level on tissue macrophages and has a low expression level on porcine granulocytes (Piriou-Guzylack and Salmon, 2008; Ezquerra et al., 2009). CD172a serves as a marker for bone marrow cells, which origin in the myeloid/monocyte/macrophage lineage, and is therefore present on macrophages, neutrophils and dendritic cells. CD16 is expressed on porcine NK cells, monocytes and macrophages (Ezquerra et al., 2009). In sum, porcine monocytes can be described as $SWC1^+SWC3^{high}CD11a^{high}wCD11r1^{low/-}CD14^{high}MHC\text{-}II^{+/-}SWC8^-SWC9^-CD163^{+/-}CD49e^{high}CD49d^{high}$ cells (Piriou-Guzylack and Salmon, 2008). F4/80 encoded by EMR1, which was later renamed to Adgre1, is a commonly used marker for tissue-resident macrophages in mice. Anti-porcine Adgre1 monoclonal antibodies detect monocytes and granulocytes in bone marrow and blood as well as porcine tissue macrophages (Waddell et al., 2018). CD163 has an especially high expression on porcine monocytes and macrophages which makes it an ideal marker for these cells (Mair et al., 2014; Dawson and Lunney, 2018), whereas CD169 is only expressed on tissue macrophages. MHC-II can be used as a marker for porcine recruited macrophages but is more present on B lymphocytes, microglia and dendritic cells. SLA2 is expressed on dendritic cells, B cells, monocytes, macrophages and also on some T cell subpopulations (Dawson and Lunney, 2018). CD80 is an established porcine macrophage marker, but cannot distinguish between classic proinflammatory and alternatively activated anti-inflammatory macrophages as it is expressed on both cell types (Nikovics and

Favier, 2021). To further distinguish different porcine macrophage subsets, proinflammatory macrophages can be identified as $CD86^+CD203a^+$ (Singleton et al., 2016) whereas anti-inflammatory macrophages can present as $MHC\text{-}I^+CD86^+$ cells (Carta et al., 2021).

When choosing a suitable model for arrythmia studies, many aspects have to be taken into consideration. There are notable differences between species in regard to electrophysiology, including different expression and distribution of ion channel subunits in the heart, or action potential morphology and duration (Kaese and Verheule, 2012; Kaese et al., 2013; Clauss et al., 2019; Schuttler et al., 2020). Compared to the human AP, the murine AP is characterized by a faster repolarization phase, no prominent plateau phase, and an overall more negative membrane potential (Kaese et al., 2013; Boukens et al., 2014; Clauss et al., 2019; Schuttler et al., 2020). In the mouse, $I_{to}$ and $I_{Kur}$ play important roles in ventricular repolarization, whereas in humans $I_{Kr}$ and $I_{Ks}$ are the main repolarization currents (Joukar, 2021). Further differences include NCX which contributes only around 7% to $Ca^{2+}$ clearance (in humans: around 28%–29%) (Edwards and Louch, 2017), the $I_{Ca,L}$ current causing the lack of an obvious plateau phase (Kaese and Verheule, 2012), and $I_{K1}$ which is less important for repolarization in mice (Boukens et al., 2014).

Pigs have been increasingly used in cardiovascular research as they closely resemble the human cardiac anatomy and (electro) physiology (Verma et al., 2011). Furthermore, the porcine immune system is very similar to the human immune system, especially compared to mice (Mair et al., 2014; Pabst, 2020). Clinical routine techniques and equipment used for humans can easily be applied to pigs, a large number of disease models have been established and well characterized (Schuttler et al., 2022), and targeted genetic modification has become feasible with recent technological advances (Park et al., 2015; Langin et al., 2018; Renner et al., 2020; Riedel et al., 2020; Stirm et al., 2021). Moreover, pigs present action potentials and electrophysiological properties similar to humans as they express the same major ion currents (except $I_{to}$) (Kaese et al., 2013; Piktel and Wilson, 2019). Major differences exist in regard to the phase 1 notch in ventricular cardiomyocytes resulting from $I_{to2}$ ($Ca^{2+}$-activated $Cl^-$, also called $I_{Ca,Cl}$) current in pigs and from $I_{to}$ current in humans (Li et al., 2003).

Although more research on specific porcine macrophage subsets is warranted to allow a more precise classification, pigs have several obvious advantages over other species especially regarding the resemblance to human electrophysiology and immunology, which make them an ideal choice as large animal species for studying cardiac macrophages in arrhythmogenesis.

## 6 CONCLUSION

Inflammation is thought to crucially contribute to the development of an arrhythmogenic substrate. Emerging data underlines vital roles of distinct cardiac macrophage subsets for regulating proarrhythmic electrical, structural, or autonomic remodeling. Given their remarkable plasticity, multiple origins and phenotypes, further analysis of the functionality of macrophage subsets in arrhythmogenesis is clearly necessary. A better understanding of arrhythmia mechanisms will reveal new potential therapeutic targets allowing the development of innovative therapeutic strategies for patients suffering from arrhythmias. To achieve this goal, initial findings on macrophage-mediated arrhythmia mechanisms obtained in rodent models need to be validated in preclinical close-to-human large animal models prior to clinical application. As they share close similarities with humans regarding cardiovascular anatomy, electrophysiology and immunology we propose pigs as suitable large animal species for translational research on cardiac macrophages and their role in arrhythmias.

## AUTHOR CONTRIBUTIONS

RX and PT performed literature research and provided a manuscript draft and drafted illustrations. SL and ZZ performed literature research and provided parts of a manuscript draft. VP corrected academic writing and reviewed the manuscript. DS reviewed, edited, and finalized the manuscript and illustrations for publication. SC conceptualized the manuscript's aim, scope and content, reviewed, edited, and finalized the manuscript and illustrations for publication. All authors contributed to the article and approved the submitted version.

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