Aus der Medizinischen Klinik und Poliklinik I
des Klinikums der Ludwig-Maximilians-Universität München
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Proarrhythmic atrial remodeling mechanisms leading to arrhythmias

Mechanismen des zu Rhythmusstörungen führenden proarrhythmogenen atrialen Remodelings

Kumulative Habilitationsschrift
zur Erlangung der Venia Legendi
für das Fach Innere Medizin und Kardiologie
der Medizinischen Fakultät
der Ludwig-Maximilians-Universität München

vorgelegt von
Dr. med. Sebastian Thomas Wolfgang Clauß

2020

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Publications summarized within this work

1. Dawson K., Wakili R., Ordög B., **Clauss S.**, Chen Y., Iwasaki Y., Voigt N., Qi XY., Sinner MF., Dobrev D., Kääb S., Nattel S. MicroRNA29: a mechanistic contributor and potential biomarker in atrial fibrillation. Circulation. 2013 Apr 9;127(14):1466-75, 1475e1-28.

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Introduction

Epidemiology of Arrhythmias

Arrhythmias are one of the major healthcare challenges since they are very common affecting millions of Europeans[1-4] and are associated with significant morbidity and mortality since they are the main cause for sudden cardiac death[5, 6].

Various arrhythmias exist, ranging from less harmful premature extra beats to life-threatening heart block, from inherited preexcitation syndromes in children to acquired post-infarction ventricular fibrillation in aged patients, and from rare familial arrhythmia syndromes to the most common arrhythmia, atrial fibrillation (AF). Epidemiology data demonstrate an overall prevalence of arrhythmias of 3,4% (ranging from 1,4% in patients younger than 18 years to 10,1% in patients older than 75 years)[5]. Thus, arrhythmias affect many patients at all ages with or without concomitant heart disease and represent a major public health burden. Arrhythmias are the main reason for sudden cardiac death that account for 25% of all deaths[7-10] and are associated with significant morbidity such as a five-fold increased stroke risk, a doubling in dementia risk, and a tripling in heart failure risk[2, 6]. Improved treatment options in cardiology such as coronary interventions in patients with myocardial infarction or implantable defibrillators in patients with heart failure have led to survival of previously fatal diseases but have also led to increased numbers of acquired arrhythmias. Thus, the overall incidence of arrhythmias is rising and accounts for an increasing proportion of hospitalizations and healthcare costs[11-13].

Current treatment options

Therapy depends on the specific arrhythmia and includes drug therapy, catheter ablation, device implantation as well as treatment of concomitant diseases[5, 14]. Pharmacotherapy using betablockers or antiarrhythmic drugs such as ion channel blockers are used to control heart rate, to suppress premature extra beats, to slow down electrical conduction through the AV node in case of atrial tachycardias, to convert arrhythmias to sinus rhythm, or to stabilize sinus rhythm[15-19]. Oral anticoagulation is further necessary to prevent thromboembolic events such as strokes in AF[20, 21]. In recent years, interventional catheter-based therapies were developed[22, 23]. Ablation of accessory muscle bundles can cure several forms of re-entrant tachycardias[24, 25], pulmonary vein isolation (PVI) [22, 23] became a standard treatment for AF, and ablation of ventricular tachycardia is performed to prevent life-threatening arrhythmias[26, 27]. More than 50 years ago cardiac pacemakers were developed that allowed to treat life-threatening bradycardia such as sinus arrest. Furthermore, implantable cardioverter-defibrillators (ICD) have been developed to terminate ventricular tachyarrhythmias. This is of special interest for patients with increased risk for lethal arrhythmias, e.g. patients with arrhythmia syndromes such as Long QT Syndrome and for patients with reduced left ventricular function after myocardial infarction. In those patients ICD implantation has been demonstrated to allow prevention of sudden cardiac death[28, 29].

Those innovative treatment options have substantially improved survival but remain ineffective in a number of patients, remain largely symptomatic therapies without targeting the causal factors and are associated with numerous complications[30, 31] and side effects[32]. Preventing tachyarrhythmias with betablockers might result in bradycardia and syncope, oral anticoagulants for stroke prevention might result in major bleedings[32], and implanted devices might cause lead infection, heart failure due to permanent ventricular stimulation, or

repetitive hospitalisations due to inadequate ICD therapies[33, 34]. Therefore, current treatment options have to be improved by innovative therapies that are ideally targeting causal proarrhythmic mechanisms.

Arrhythmia Mechanisms

The pathophysiologic basis of arrhythmias is complex (Fig.1) and still incompletely understood but similarities of fundamental mechanisms between different forms of arrhythmias have been identified[35-37]. In general, arrhythmias occur either due to impaired impulse formation and/or impulse propagation. Physiologically, generation and propagation of electrical impulses in the heart is achieved by a characteristic sequence of voltage changes implemented by depolarizing and repolarizing ion currents. The normal resting membrane potential of cardiomyocytes is around -80 mV. An electrical impulse causes a depolarization by rapidly activated sodium channels with following influx of sodium ions. Subsequently, potassium channels open initiating the repolarization of the cell (re-establishing the resting membrane potential again). Simultaneously, calcium ions enter the cell leading to cell contraction (excitation-contraction-coupling) and slowing of the repolarization.

In the healthy heart, the sinus node expresses specific ion channels (funny channels) that are responsible for the so-called automaticity (i.e. the progressive diastolic depolarization) and dominates the impulse generation. An upregulation of these ion channels, as seen in heart failure, could be a possible mechanism leading to enhanced automaticity[38]. Furthermore, it is possible that a cell outside the sinus node reaches the threshold potential earlier resulting in ectopic firing at a more rapid rate potentially leading to atrial tachycardia. In this regard, alterations of the cellular calcium homeostasis may play an important role as it has been demonstrated in animal models and patients[37]. Physiologically, cellular calcium is removed by the Ca^{2+}-ATPase (SERCA) and the Na^{+}-Ca^{2+}-exchanger (NCX) during diastole to re-establish ionic homeostasis at the end of the cardiac cycle. During atrial tachycardia, however, calcium is progressively accumulated in the cell because of repetitive activation of the L-Type Ca^{2+} channel resulting in a calcium overload that in turn leads to multiple maladaptive changes such as impairment of the ryanodine receptor (calcium release channel in the sarcoplasmatic reticulum) with subsequent calcium leak from the sarcoplasmatic reticulum and activation of NCX causing a depolarizing current leading to so-called delayed afterdepolarizations (DAD). This leads to a progressive depolarization of the cell and ectopic firing (when the threshold potential is reached; so-called triggered activity)[36, 39, 40]. Early afterdepolarizations (EAD) occur when the action potential duration (APD) is excessively prolonged, for example in the context of arrhythmia syndromes such as Long QT syndrome[41, 42].

Another hallmark in arrhythmogenesis is reentry that can occur when altered electrical properties cause a shortening of the refractory period or conduction slowing and when structural changes (e.g. fibrosis) establish an anatomical substrate[37]. In the healthy heart, reentry cannot occur because cardiomyocytes display a certain refractory period that prevents premature stimuli to conduct. When the refractory period is shortened, however, the cell is excitable earlier, ectopic activity can be conducted, and reentry can be initiated. Additionally, slowed conduction velocity can also allow reentry because cells are excitable again when the impulse arrives. Structural changes as dilatation and fibrosis extend conduction pathways, slow conduction, and create conduction barriers favoring initiation and maintenance of reentry circuits[43].

Besides these fundamental mechanisms several other factors may lead to arrhythmias, all of them are summarized under the term "**proarrhythmic atrial remodeling**" with mechanisms leading to altered ion currents (e.g. enhanced automaticity) categorized as "electrical remodeling" and mechanisms leading to anatomic changes (e.g. atrial fibrosis) categorized as "structural remodeling" (Fig.1).

Interestingly, some of the electro-anatomical changes implicated in arrhythmia pathophysiology initially act as a protective mechanism of the cell, but finally result in a fixed substrate for arrhythmia maintenance. For example, atrial tachycardia (as seen in AF) causes a cellular calcium overload. In order to reduce the calcium influx and to antagonize the calcium overload the calcium current is reduced (by inactivation of calcium channels (short-term effect) and reduced gene expression of the calcium channels (long-term effects)) leading to a shortening of the action potential. This favors reentry and therefore contributes to AF maintenance ("AF begets AF")[44].

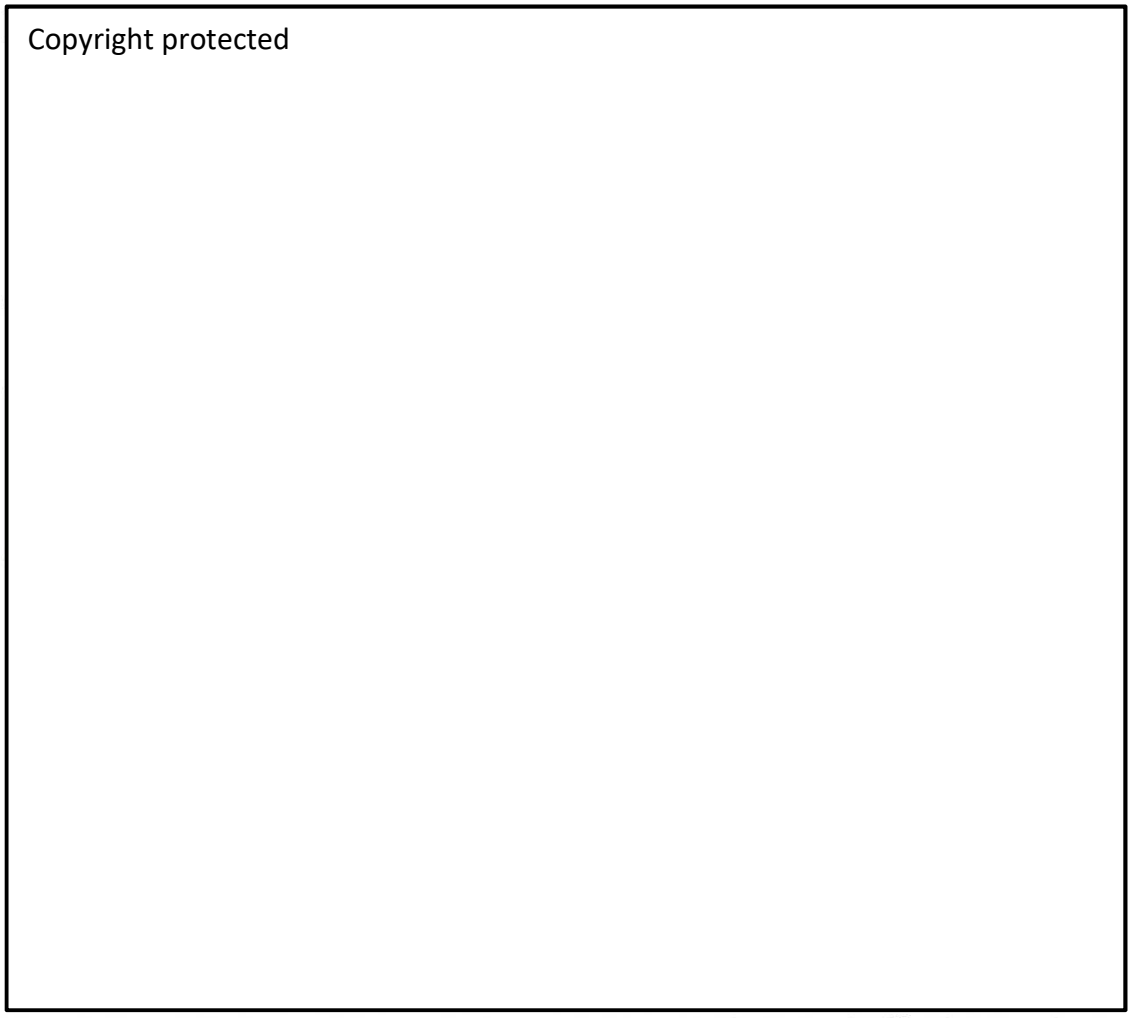

Figure 1. Proarrhythmic remodeling mechanisms underlying atrial fibrillation (AF)[51]. *TGFb*, Transforming Growth Factor-b, *CTGF*, Connective Tissue Growth Factor; *PTEN*, Phosphatase and Tensin homolog; *MMP2*, Matrix Metalloproteinase 2; *TRPC3*, Transient Receptor Potential Channel 3; *ECM*, Extracellular Matrix; *Cx*, Connexin; *RYR2*, Ryanodine Receptor Type 2; *SR*, Sarcoplasmatic Reticulum; *DAD*, Delayed Afterdepolarisation; *APD*, Action Potential Duration; *SK3*, small-conductance calcium-activated potassium channel.

MicroRNA mediated atrial remodeling

In 1993 microRNAs (miRNAs) have been discovered as important developmental mediators in Caenorhabditis elegans as they regulate gene expression[45, 46]. Since then, thousands of miRNAs, which are short (i.e. 20-22 base pairs), single-stranded, non-coding RNA fragments, have been identified in other animals and plants playing key roles in various physiologic and pathophysiologic conditions. MiRNA binding to the 3‘ untranslated region (3’ UTR) of a target mRNA results in post-transcriptional inhibition of gene expression by repression of the translation or the degradation of the target mRNA[47, 48]. Thus, miRNAs can both cause downregulation of genes (by direct binding to target mRNAs) but also (indirect) upregulation of genes (by binding and downregulation of intrinsic inhibitors of these genes). Since miRNAs can bind to various target genes and one gene can be targeted by various miRNAs a complex regulatory network with up- and downregulation of agonists and antagonists at the same time is established leading to the concept of miRNAs as „fine tuners“ of gene expression[49]. Over the last years, several miRNAs have been demonstrated as key regulators in cardiac development and cardiovascular disease leading to a growing number of studies evaluating the role of miRNAs in proarrhythmic remodeling as well[37, 50]. By targeting genes encoding ion channels, connexins, or calcium handling-related proteins miRNAs such as miR-1, -26, -106b-25, -133, -208, -328, or -499 are mediating electrical remodeling whereas miR-21, -26, -29b, -30, -499, -590 are mediating structural remodeling by regulating extracellular matrix turnover and profibrotic signaling[51].

In a canine ventricular tachypacing model (VTP, 240 bpm) that has previously established as model for AF in the context of congestive heart failure[52, 53], we studied the role of miR-29b in regulating proarrhythmic remodeling[54]. Already 24 hours after initiation of VTP miR-29b was significantly downregulated (Fig.2A) abolishing its inhibitory effects on the expression of several extracellular matrix genes such as collagens (COL1A1, COL3A1) and fibrillin (FBN1) that were upregulated 2 weeks later (Fig.2D-F) when significant atrial fibrosis and increased vulnerability for AF were also present (Fig.2B-C). To confirm the causal effects of miR-29b on matrix gene expression we isolated fibroblasts from the left atrium and infected these cells with a lentivirus containing the miR-29b precursor sequence or a complementary target sequence (a so-called miR-sponge) to induce a miR-29b overexpression or knockdown, respectively. Upregulation of miR-29b in these cells resulted in significantly reduced expression of COL1A1, COL3A1, and FBN1 whereas a downregulation of miR-29b showed the opposite effect (Fig.2G-H). In sum, these data suggest a causal role for miR-29b in regulating extracellular matrix expression and structural remodeling in the atrium.

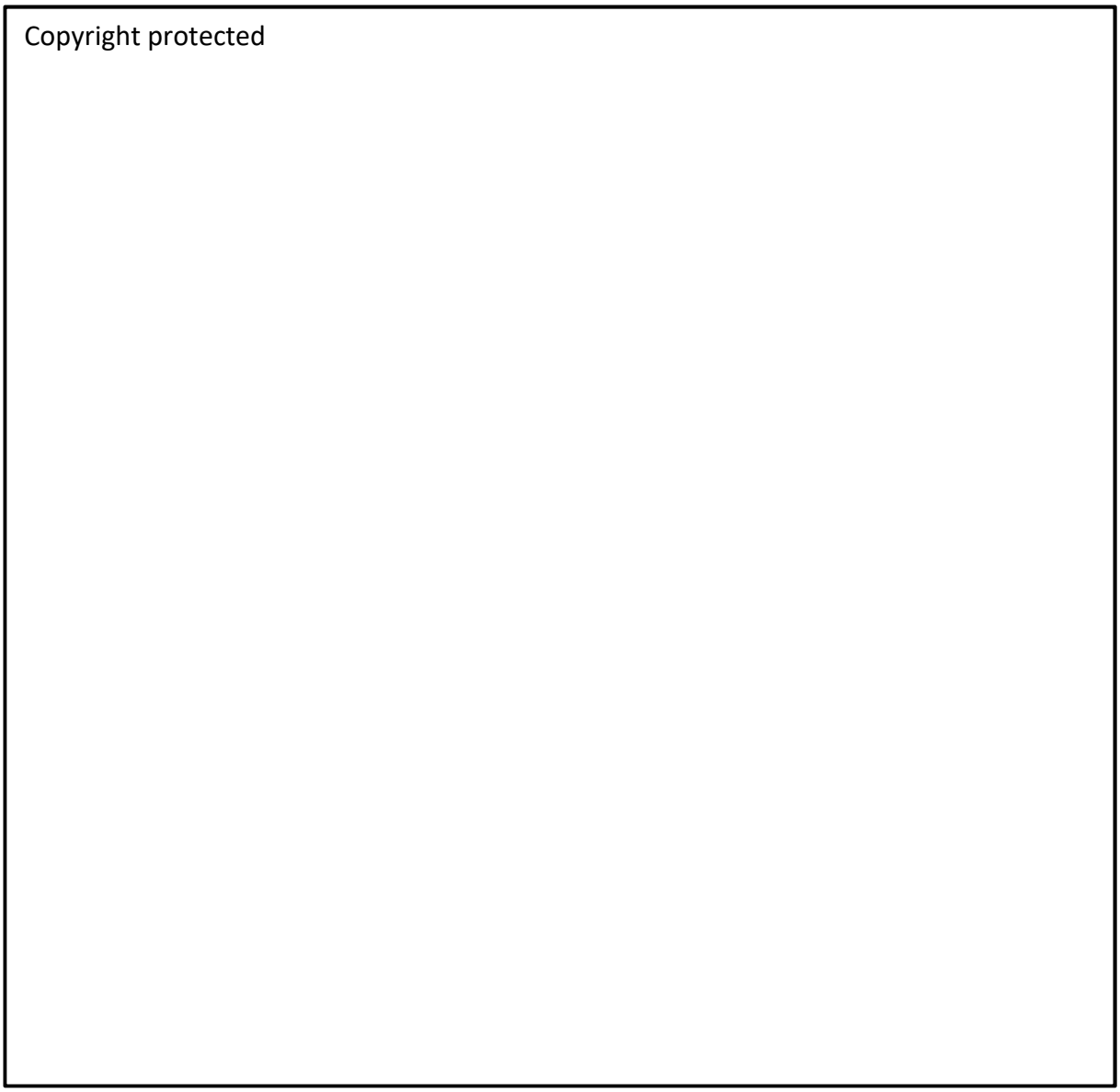

Figure 2. Evaluation of miR-29b as mediator of proarrhythmic remodeling[54]. A. Atrial expression of miR-29b in a dog model for AF. **B**. Duration of induced AF. **C**. Interstitial fibrosis. **D**. Expression of collagen 1 alpha 1 (COL1A1) in fibroblasts isolated from canine atria. **E**. Expression of collagen 3 alpha 1 (COL3A1) in fibroblasts isolated from canine atria. **F**. Expression of fibrillin 1 (FBN1) in fibroblasts isolated from canine atria. **G**. Expression of miR-29b and its target genes in cells infected with a control virus (white bars) and a miR-29b sponge (blue bars). **H**. Expression of miR-29b and its target genes in cells infected with a control (scrambled) virus (white bars) and a miR-29b over-expressing virus (red bars). Data are presented as MEAN±SEM. * $p<0.05$, ** $p<0.01$, *** $p < 0.001$. *AF*, atrial fibrillation, *a.u.*, arbitrary units, *CTL*, control, *COL1A1*, collagen 1 alpha 1, *COL3A1*, collagen 3 alpha 1, *FBN1*, fibrillin 1, *hr*, hour, *miR*, microRNA, *VTP*, ventricular tachypacing.

In a next step we studied the expression of miR-29b in human heart tissue obtained from patients who underwent cardiac surgery. We could show that the expression of miR-29b in the atrium of patients with AF was significantly reduced compared to patients without AF (Fig.3A)[54].

Figure 3. miR-29b expression in human atrial tissue and plasma[54]. A. Atrial expression of miR-29b in human patients without arrhythmia, with paroxysmal AF, with chronic AF. B. Plasma expression of circulating miR-29b in healthy controls without AF or heart failure, in patients with AF, in patients with heart failure, and in patients with both AF and heart failure. Data are presented as MEAN±SEM. * p<0.05, ** p<0.01, *** p < 0.001. *AF*, atrial fibrillation, *a.u.*, arbitrary units, *CTL*, control, *CHF*, congestive heart failure, *miR*, microRNA, *w*, with, *w/o*, without.

The extent of atrial fibrosis is associated with the outcome after AF therapy, but measurement of atrial fibrosis *in vivo* remains challenging since the gold standard for fibrosis quantification is still histology which requires the staining of tissue samples obtained from the individual patient[55, 56]. Innovative attempts using MRI scans are promising but still experimental and not available in clinical routine. Since miRNAs such as miR-29b regulate structural remodeling and are expressed in the human heart as well (Fig.3A), we studied the expression of miRNAs in human blood samples to evaluate their role as potential biomarkers for atrial fibrosis. However, it was not known how blood samples have to be processed to allow reliable measurement of miRNAs. To test this and to establish preanalytical quality criteria we measured miR-21 and miR-1 expression in blood samples under various conditions[57]. We collected blood samples in different collection tubes containing ETDA, heparin or serum separator, processed samples immediately to plasma/serum, isolated RNA and measured miRNA levels. We could show that miRNA measurement can be achieved from blood samples collected both in EDTA plasma and serum tubes whereas miRNAs were undetectable upon contact to heparin (Fig.4A). We then applied repetitive freeze-thaw cycles to plasma and serum samples and observed a decrease in miR-21 both in plasma (ΔCT values: 5.59±0.19 vs. 6.38±0.21, p<0.05) and serum (ΔCT values: 4.45±0.75 vs. 8.74±0.88, p<0.01) and a decrease in miR-1 levels in plasma (ΔCT values: 15.57±0.69 vs. 16.31±0.62, p<0.05) after 4 freeze-thaw cycles (Fig.4B-C). In daily practice it might not be possible to immediately process blood samples, thus, we mimicked this situation by incubating the samples at room temperature for 24 hours and 4 days before (full blood samples) and after processing (plasma/serum samples). In full blood samples collected into EDTA containing tubes miRNAs were stable for after 4 days whereas in serum separator containing tubes miR-21 levels were significantly reduced after 4 days (ΔCT values: 4.5±0.75 vs. 8.5±0.64, p<0.01, Fig.4D-E). In processed plasma/serum samples, however, both miR-21 (ΔCT values in plasma: 5.60±0.19 vs. 7.65±0.68, p<0.05; ΔCT values in serum: 4.45±0.75 vs. 7.97±0.65, p<0.01) and miR-1 (ΔCT values in plasma: 15.6±0.69 vs. 18.7±0.83, p<0.001; ΔCT values in serum: 15.72±1.02 vs. 18.29±0.40, p<0.05) were significantly reduced already after 24 hours (Fig.4F-G). To evaluate the effect of physical disturbance that may occur during sample transport we placed samples before (full blood)

and after processing (plasma/serum) on a shaker for one and eight hours. We showed that one hour of shaking did not affect miRNA levels in any of the sample types, after eight hours of shaking, however, miR-1 levels in full blood collected in serum tubes (15.72±1.02 vs. 17.50±0.31, $p<0.05$) and in plasma (15.57±0.69 vs. 18.77±0.81, $p<0.01$) as well as miR-21 levels in plasma (5.59±0.19 vs. 7.20±0.40, $p<0.01$) were significantly increased (Fig.4H-K).

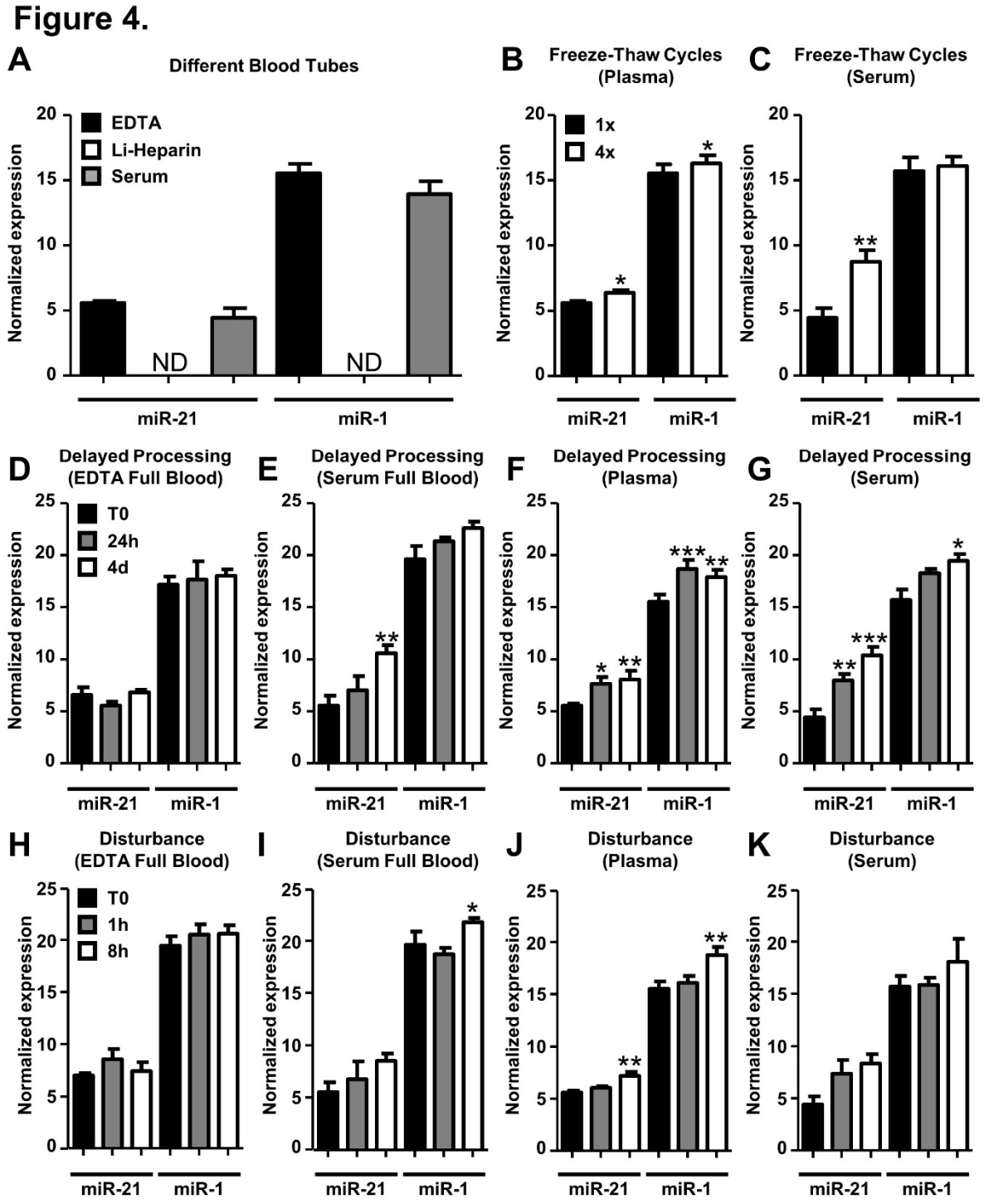

Figure 4. Measurement of miRNAs in patient blood under various conditions[57]. A. Impact of different blood collection tubes. Impact of repetitive freeze-thaw cycles using plasma (B) and serum samples (C). Impact of delayed processing on full blood collected in EDTA plasma tubes (D), full blood collected in serum tubes (E), processed plasma samples (F), processed serum samples (G). Impact of physical disturbance on full blood collected in EDTA plasma tubes (H), full blood collected in serum tubes (I), processed plasma samples (FJ), processed serum samples (K). ΔCT values presented as MEAN±SEM. * $p<0.05$, ** $p<0.01$, *** $p < 0.001$. *d*, days, *h*, hour(s), *miR*, microRNA, *ND*, not detected.

According to these preanalytical considerations we measured the expression of miR-29b in human plasma samples (Fig.3B)[54]. We could show that miR-29b is significantly reduced in plasma obtained from patients with AF (8.96±2.67 vs. 19.54±1.97, p<0.001), from patients with heart failure without AF (5.82±0.88 vs. 19.54±1.97, p<0.001) and from patients with AF and concomitant heart failure (10.03±1.25 vs. 19.54±1.97, p<0.001) compared to patients without heart disease. A multivariate regression analysis showed that miR-29b plasma levels remained significantly reduced in all groups after adjusting for age, sex, cardiovascular risk factors (hypertension, diabetes mellitus, hypercholesterolemia), concomitant coronary artery disease, or drug treatment (treatment with ACE inhibitors/AT1 blockers, aldosterone antagonists, statins) suggesting a potential role for circulating miR-29b as biomarker for proarrhythmic remodeling.

Heart diseases such as congestive heart failure are an important predisposing factor for arrhythmias as they induce proarrhythmic remodeling[58]. However, even without underlying heart diseases arrhythmias can occur[59-61]. In recent years, a growing body of evidence suggests that genetics may be an important factor responsible for developing arrhythmias[62-65]. Genome-wide association studies (GWAS) revealed significant associations between genetic variants and an increased risk for AF[66, 67] and other arrhythmias[68-71]. However, the underlying mechanisms how these variants may cause arrhythmias are unknown in most cases especially since these risk variants are often located in „genomic deserts“ without any known gene. To further evaluate if altered miRNA signaling could be the mechanistic link to genetically increased AF risk we studied the gene SHOX2, a transcription factor with an essential role in cardiac development, especially of the sinus node[72]. Since sinus node dysfunction is associated with AF[73], SHOX2 is a plausible candidate gene to study genetic susceptibility. We measured expression of SHOX2 in right atrial tissue and could show that SHOX2 expression is significantly reduced in patients with AF compared to patients in sinus rhythm (Fig.5A). We then performed SHOX2 sequencing in 378 patients with early-onset AF (i.e. before the age of 60 years) and identified a genetic variant within the 3‘ untranslated region of the SHOX2 gene (c.*28T>C) that was significantly associated with AF (p<0.01). AF Patients with this genetic variant show a prolonged PR interval during sinus rhythm which is a surrogate parameter for altered atrial conduction properties indicating a functional effect of this genetic variant (Fig.5B). Since miRNAs regulate gene expression by binding to target sequences in non-coding regions we hypothesized that this variant generates a novel miRNA binding site and thus affects arrhythmogenesis. Indeed, we could demonstrate that this genetic variant cause a novel binding site for miR-92b-5p which may explain the reduced SHOX2 expression in the atria of AF patients. This is further supported by the finding that miR-92b-5p plasma levels were similar in patients with or without AF (Fig.5C) but were significantly reduced in patients carrying the variant (Fig.5D) suggesting a miR-92b-5p enrichment in the heart in patients carrying the genetic variant. In sum, our data suggest that miRNA-mediated remodeling may be the functional link between an increased genetic risk and arrhythmias.

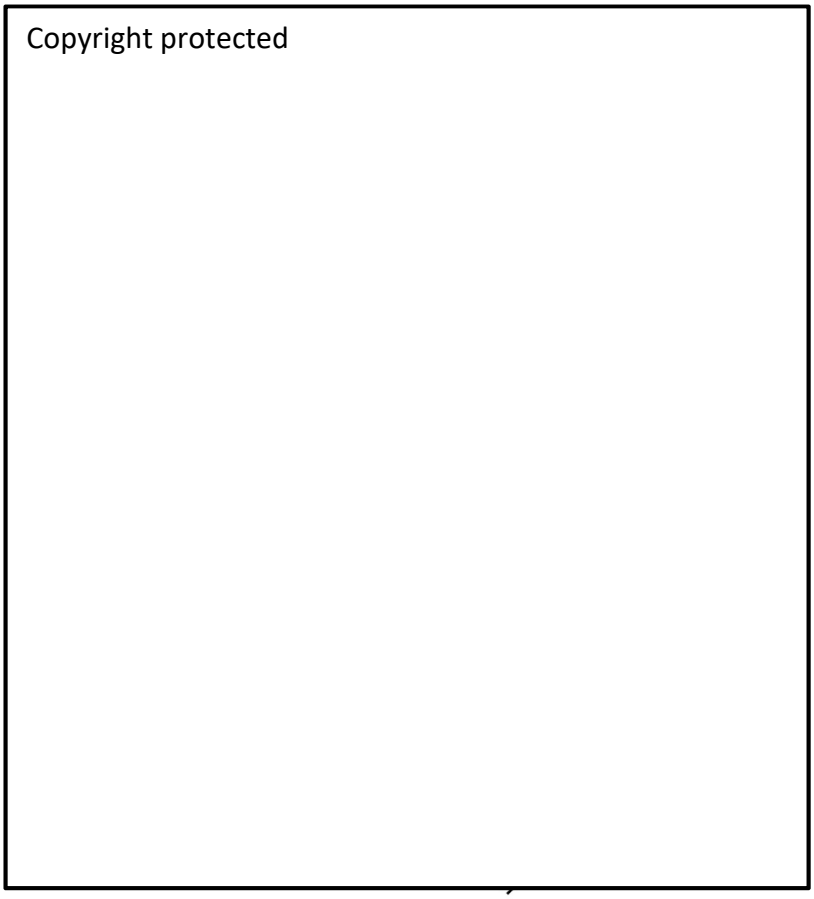

Figure 5. Investigating a genetic variant creating a novel miRNA binding site[72]. A. Atrial expression of Shox2 in patients with and without AF. **B**. PR interval during sinus rhythm in patients with and without the genetic variant. **C**. Plasma expression of circulating miR-92b-5p in healthy controls without AF and in patients with AF. **D**. Plasma expression of circulating miR-92b-5p in AF patients with and without the genetic variant. Data are presented as MEAN±SEM. * p<0.05, *** p < 0.001. *AF*, atrial fibrillation, *miRNA*, microRNA, *SNP*, single nucleotide polymorphism, *w*, with, *w/o*, without.

An individual‘s lifestyle is – besides genetic factors – an important risk factor for the development of arrhythmias, especially in otherwise healthy people such as athletes[74-76]. The underlying mechanisms however, are incompletely understood but miRNA-mediated remodeling may play an important role[77]. To study the potential effects of miRNAs in athletes we measured circulating miRNAs in healthy people participating in a marathon[78]. We recruited 15 runners who performed intense physical training throughout the year (“elite runners”) and 15 runners who performed only seasonal training (“non-elite runners”). We collected blood at different time points (before and after a 10 week training period before the marathon, immediately and 24 hours after the marathon) and correlated miRNA expression levels with echocardiographic parameters for remodeling. We focused on 5 miRNAs that have previously been demonstrated as mediators of cardiac remodeling[51]: miR-1, miR-133a, miR-30a, miR-26a, and miR-29b. In both groups, expression of all 5 miRNAs was similar over time (Fig.6A-E). After the marathon miR-1, miR-133a and miR-30a increased (significant increases of miR-30a in both groups and miR-1/-133a in elite runners) whereas miR-26a and miR-29b decreased (significant decrease of miR-26a in elite runners). In non-elite runners we could not identify correlations between miRNA levels and echocardiographic parameters, while in elite runners miR-1 (r=-0.6897, p<0.01) and miR-133a r=-0.7090, p<0.01) plasma levels significantly

correlated with LA diameter, a surrogate parameter for structural remodeling (Fig. 6F-G). In our study, alterations in miRNA plasma levels are pronounced and correlate with LA diameter only in elite runners with regular and more intense training suggesting that i) miRNAs might be involved in proarrhythmic remodeling in athletes, and ii) that circulating miRNAs could serve as potential biomarkers for atrial remodeling.

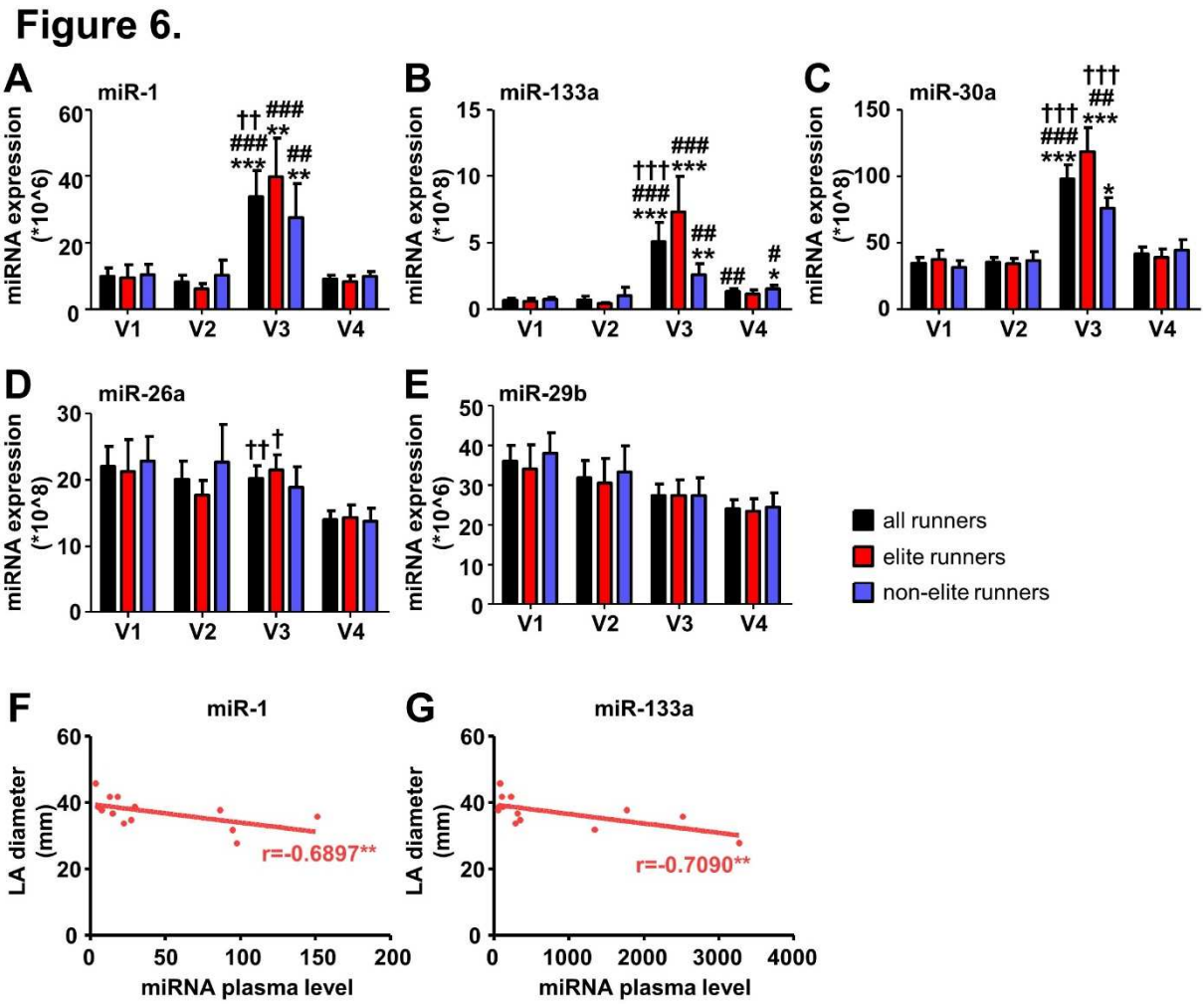

Figure 6. MicroRNAs in marathon runners[78]. Plasma expression of miR-1 (**A**), miR-133a (**B**), miR-30a (**C**), miR-26a (**D**), miR-29b (**E**) at 4 time points. V1, at the beginning of a 10 week training program before the marathan, V2, at the end of the training program, V3, immediately after the marathon, V4, 24 hours after the marathon. Correlation between LA diameter and miRNA plasma levels for miR-1 (**F**) and miR-133a (**G**) at V3. Data are presented as MEAN±SEM. * p<0.05, ** p<0.01, *** p<0.001 vs. V1, # p<0.05, ## p<0.01, ### p<0.001 vs. V2, † p<0.05, †† p<0.01, ††† p<0.001 vs. V4. *LA*, left atrium, *miRNA*, microRNA.

Macrophage mediated atrial remodeling

Macrophages are important cells of the innate immune system which identify and eliminate pathogens by phagocytosis and release cytokines and chemokines to recruit further immune cells to the site of inflammation[79]. Besides their role in host defense against infectious pathogens macrophages have also been demonstrated to play important regulatory roles in the context of sterile inflammation as seen in many chronic diseases such as diabetes mellitus[80-83].

Diabetes mellitus is associated with an increased risk for arrhythmias, but the underlying mechanisms are unknown so far[84-87]. Recently, a study using a mouse model of diabetes suggests an inflammatory mechanism and a role for macrophages[88]. In this model macrophages are recruited to the heart where they release the cytokine IL-1β which mediates an electrical remodeling resulting in reduced potassium currents, altered calcium homeostasis, prolonged QT interval and action potential duration, and increased susceptibility for arrhythmias.

Recent studies suggest that inflammatory mechanisms are also involved in the pathophysiology of arrhythmias such as AF even in the absence of overt chronic inflammatory diseases[89, 90]. We hypothesized that macrophages are involved in the pathogenesis of AF but due to the very limited availability of human heart tissue samples it was not possible to directly study macrophages in the heart of patients with AF. Thus, we studied the monocyte chemoattractant protein-1 (MCP-1) that has been demonstrated to attract macrophages to the site of an inflammation[82, 91]. Thus, we measured MCP-1 in the blood of patients with AF as an indirect marker for macrophage recruitment[92]. We measured MCP-1 plasma levels in 96 patients with AF and in 40 patients without AF and observed significantly elevated levels of MCP-1 in AF patients (268 pg/ml vs. 227 pg/ml; p=0.03; Fig.7A). Since the patient cohort was adjusted for age, sex, type of AF, cardiovascular risk factors, echocardiographic parameters, as well as concomitant coronary artery disease, renal failure or antiarrhythmic drug therapy, an elevated MCP-1 plasma level indicates a pro-inflammatory condition in AF. To study whether MCP-1 plasma levels are directly associated with the heart rhythm or indirectly with the degree of atrial remodeling we studied MCP-1 plasma levels before and three months after pulmonary vein isolation (PVI). We revealed that MCP-1 levels significantly increased after PVI both in patients with stable sinus rhythm (268 pg/ml vs. 349 pg/ml, baseline vs. 3 months after PVI; p<0.01; Fig.7B-C) and in patients with AF recurrence (281 pg/ml vs. 355 pg/ml, baseline vs. 3 months after PVI; p=0.03; Fig.7B-C). These data suggest that MCP-1 levels i) are not associated with the current heart rhythm (no difference between sinus rhythm vs. AF post PVI), ii) might rather indicate a preexistent atrial remodeling and does therefore not allow to predict short-term ablation success (no difference at baseline), and iii) might be associated with a PVI-induced pro-inflammatory and pro-fibrotic response potentially indicating scar formation after ablation. In sum, these data provide evidence that MCP-1 is altered in patients with AF indicating that a macrophage-related inflammatory mechanism could be involved in proarrhythmic atrial remodeling.

Figure 7.

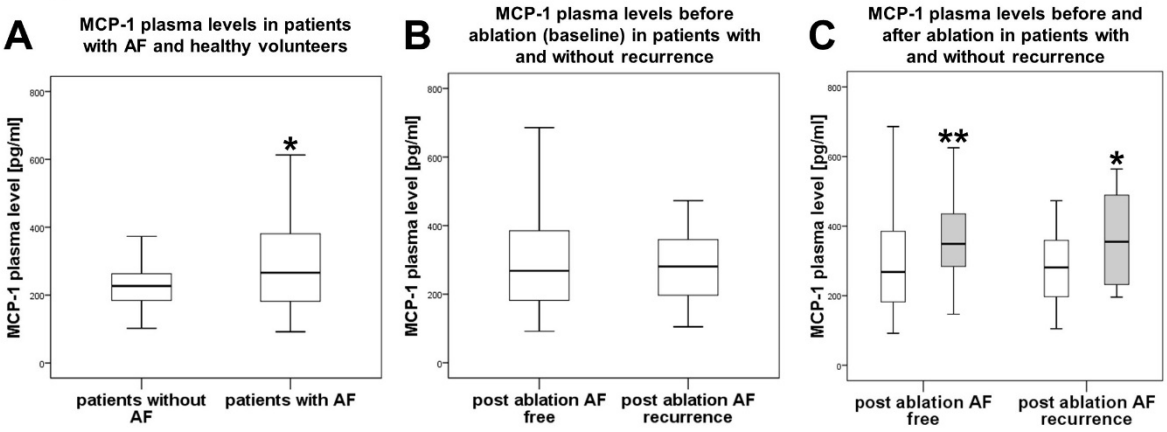

Figure 7. MCP-1 in patients[92]. A. Plasma levels of MCP-1 in patients with and without AF. **B**. MCP-1 plasma levels before ablation in AF patients with and without AF recurrence after ablation. **C**. MCP-1 plasma levels before and after ablation in AF patients with and without AF recurrence after ablation. Data are presented as median with interquartile ranges. * p<0.05, ** p<0.01, *AF*, atrial fibrillation, *MCP-1*, monocyte chemoattractant protein-1.

Over the recent years several macrophage subsets have been demonstrated, most importantly "inflammatory macrophages" originating from circulating monocytes that are recruited to sites of inflammation and "resident macrophages" which are located within different organs during steady-state, i.e. without any inflammation mediating tissue homeostasis[79, 93, 94]. Although resident macrophages have been described in the healthy heart as well, their spatial distribution and their specific function was unknown.

In a genetically modified mouse model where macrophages are fluorescently labeled ($Cx_3cr1^{GFP/+}$) we could demonstrate that resident cardiac macrophages are enriched within the AV node by flow cytometry and histology (Fig.8)[95]. We then performed optical clearing and confocal microscopy imaging of AV nodes and revealed that resident macrophages frequently intersperse with cardiomyocytes especially in the lower AV node.

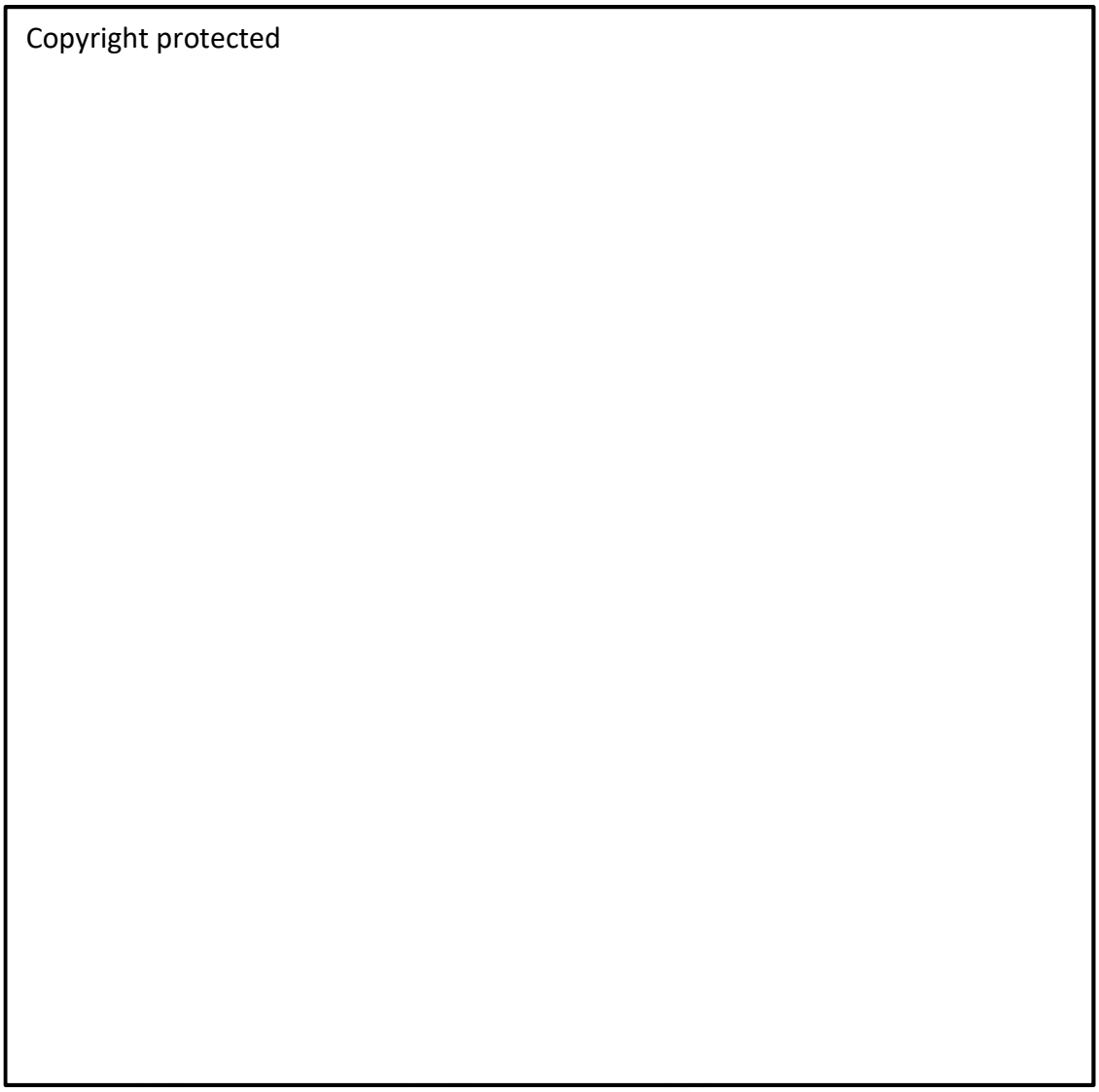

Figure 8. Resident cardiac macrophages in the AV node[95]. A. Volumetric reconstruction of confocal microscopy after optical clearing in $Cx_3Cr1^{GFP/+}$ mouse stained with HCN4 (red). **B**. Higher magnification of dashed square in A. **C**. Flow cytometric macrophage quantification in microdissected AV node and left ventricular free wall of C57BL/6 mice. Data presented as MEAN±SEM. ** $p<0.01$. AV, atrioventricular, CD, cluster of differentiation, CFB, central fibrous body, CN, compact node, IAS, interatrial septum, IVS, interventricular septum, LV, left ventricle, mg, milligram, µm, micrometer.

Since cardiomyocytes are functionally connected with adjacent cells by gap junctions formed by connexins we isolated AV node macrophages by fluorescent-activated cell sorting (FACS) and determined expression of connexins (Fig.9A). We found that AV node macrophages mainly express Connexin-43 (Cx43) establishing on average three contact points between macrophages and cardiomyocytes indicating gap junctions (Fig.9B). Since gap junctions allow electrical conduction between neighboring cardiomyocytes, we therefore hypothesized that macrophages are electrotonically coupled to cardiomyocytes via gap junctions as well.

To test this we co-cultured FACS-purified AV nodal macrophages and neonatal cardiomyocytes and confirmed Cx43-positive contact points indicating gap junctions between macrophages and cardiomyocytes (Fig.9C). Whole-cell patch-clamp experiments (Fig.9D-E) revealed typical resting membrane potentials and action potentials in cardiomyocytes (Fig.9F). In isolated macrophages the resting membrane potential was more depolarized (compared to

cardiomyocytes) and we did not observe spontaneous electrical activity (Fig.9G). After three days of co-culture of neonatal cardiomyocytes with isolated macrophages, however, we found rhythmic depolarizations in 23% and irregular electrical activity in another 23% of the macrophages connected to cardiomyocytes (Fig.9H). The resting membrane potential of these macrophages was hyperpolarized compared to solitary macrophages indicating electrical coupling. To further study the interaction between both cell types we measured resting membrane potentials in cardiomyocytes coupled to macrophages and could demonstrate that these cardiomyocytes are more depolarized (compared to solitary cardiomyocytes) and that treatment of co-cultured cells with a Cx34 inhibitor could reverse this effect while treatment of solitary cardiomyocytes with the Cx34 inhibitor did not affect the resting membrane potential (Fig.9I-J).

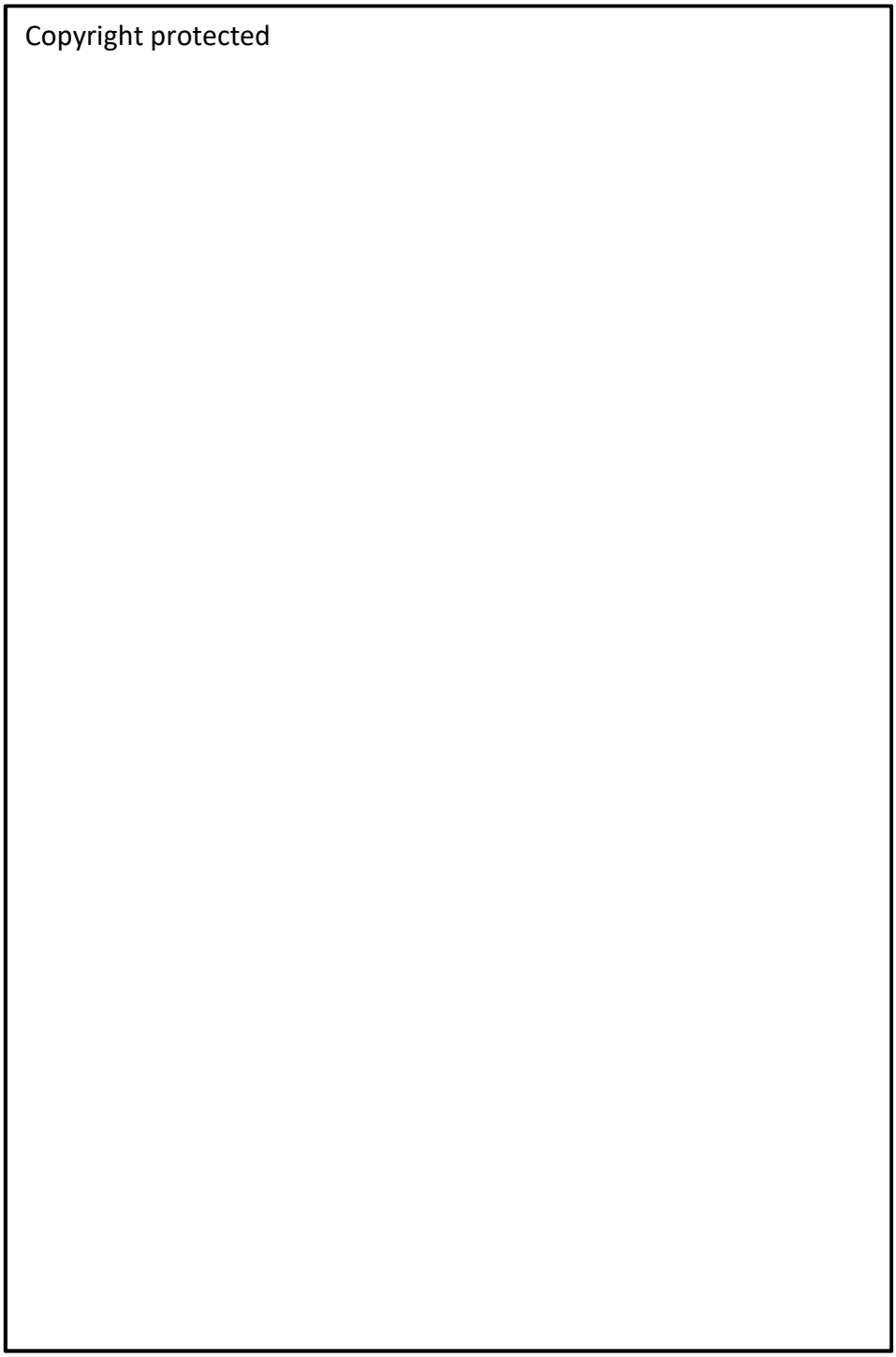

Figure 9. Resident cardiac macrophages couple to cardiomyocytes[95]. A. Expression of connexins in FACS-purified AV node macrophages. **B**. Whole-mount immunofluorescence (IF) microscopy of AV node from $Cx_3cr1^{GFP/+}$ mouse stained with Cx43 (red) and HCN4 (white). Arrowheads indicate Cx43 colocalisation with GFP^+ macrophages (green). **C**. IF image of a co-cultured $desmin^+$ neonatal mouse cardiomyocyte (white) and GFP^+ cardiac macrophage (green) stained with Cx43 (red, arrow). **D-E**. IF images of dextran diffusion during whole-cell patch-clamp with a dextran loaded pipette. Arrowhead indicates GFP^+ cardiac macrophage (green). **F.-H.** Spontaneous recordings and **I-J.** resting membrane potential of solitary cardiac macrophages and macrophages attached to cardiomyocytes. Data presented as MEAN±SEM. * $p<0.05$, ** $p<0.01$. AV, atrioventricular, Cx, connexin,mV, millivolts, µm, micrometer.

Next, we used the data from patch clamp experiments to perform a mathematical modeling of electrical interactions between macrophages and cardiomyocytes (Fig.10). According to this modeling macrophage coupling depolarizes the resting membrane potential and shortens the action potential duration of the coupled cardiomyocyte, effects that are further enhanced if more macrophages are coupled to the cardiomyocyte. These data indicate that macrophage coupling may enhance electrical conduction since an increased resting membrane potential allows to easier/faster reach the depolarization threshold and a shortened action potential duration, i.e. a shortened refractory period allows a higher depolarization frequency.

Figure 10. Mathematical modeling of single-sided coupling between one AV bundle cardiomyocyte and an increasing number of cardiac macrophages[95]. The graph shows the AV bundle cardiomyocyte membrane potential uncoupled or coupled to one, two, or four cardiac macrophages. *APD*, Action potential duration, *RMP*, resting membrane potential, *mV*, millivolts.

To test this directly in the AV node we generated a transgenic mouse model expressing a photoactivatable channelrhodopsin 2 (ChR2) specifically in macrophages. ChR2 is a cation channel that opens upon stimulation by light and allows influx of sodium ions, i.e. a depolarization of the cell. Hearts from these mice (Cx_3cr1 ChR2) were then perfused using a Langendorff apparatus and the RV free wall was removed to expose the AV node allowing to specifically illuminate the AV node (Fig.11A-D). To study the electrical conduction through the AV node we performed atrial pacing to assess the Wenckebach point, i.e. the cycle length at which an electrical impulse from the atrium is no longer conducted to the ventricles. When we turned on the light illuminating the AV node at the Wenckebach cycle length we observed a significantly increased number of stimuli that were conducted indicating an enhanced electrical conduction when AV node macrophages were depolarized (Fig.11E-G).

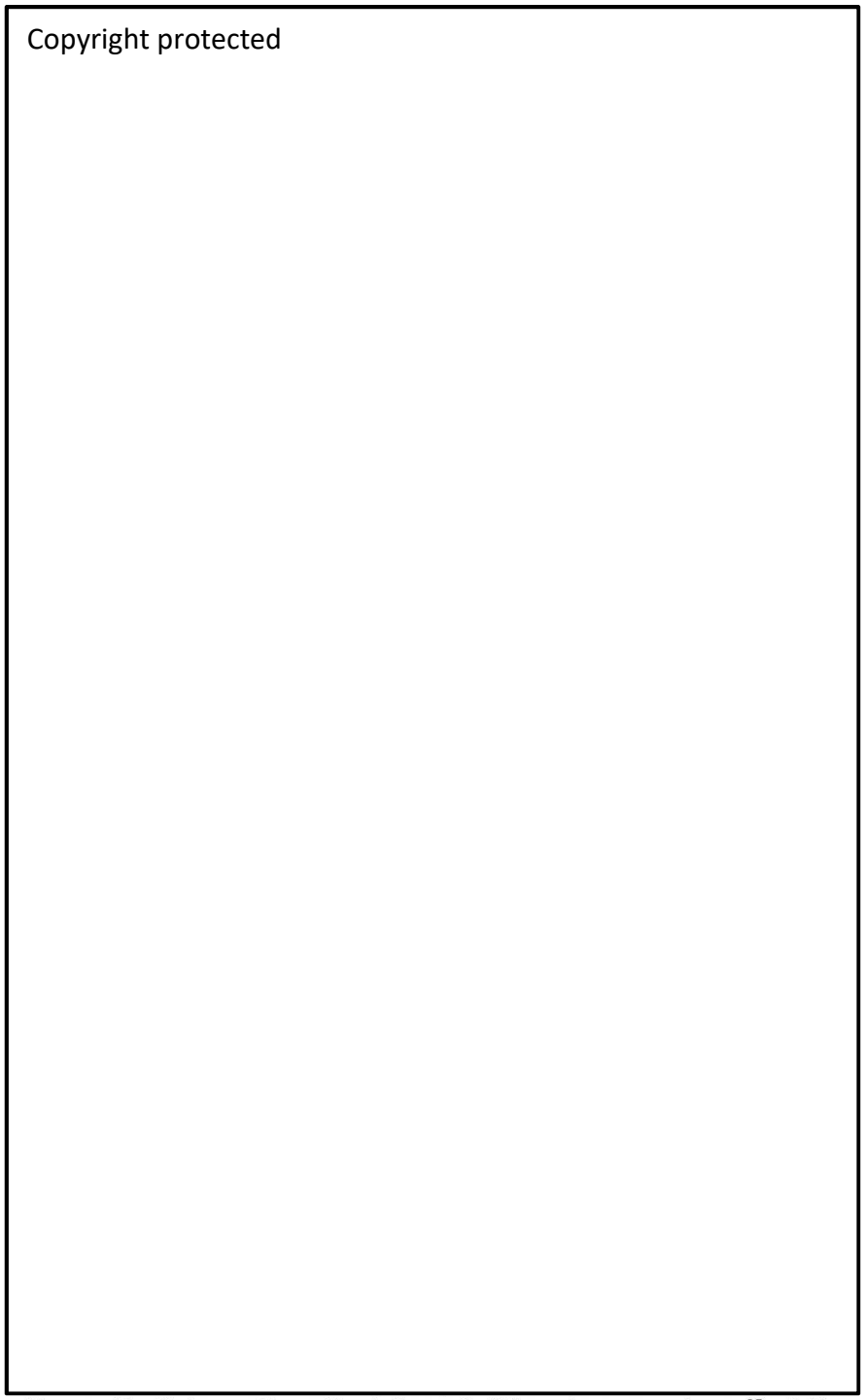

Figure 11. Optogenetics stimulation of AV node macrophages[95]. A-B. Experimental Outline. **C-D.** Images illustrating the optogenetics experimental setup during a light off and on cycle. **E-F.** Representative ECG recordings from a Cx_3cr1 ChR2 heart, illustrating the number of conducted atrial stimuli between two non-conducted impulses of a Wenckebach period during light off (**E**) and on (**F**) cycles. Arrows indicate failure of conduction leading to missing QRS complexes. **G**. Number of conducted atrial stimuli between two non-conducted impulses of a Wenckebach period during light off and on cycles. Data presented as MEAN±SEM. ** p<0.01. *AV*, atrioventricular, *ECG*, electrocardiography.

To further confirm the electrophysiology-modulating role of cardiac macrophages we studied several mouse models. First, we generated a transgenic mouse model with macrophage-specific Cx43 downregulation after tamoxifen treatment (Cx_3cr1 $Cx43^{-/-}$; Fig.12A). *In vivo*

electrophysiology (EP) studies in these mice revealed prolongation of the PR and AH intervals, the AV node refractory periods, the Wenckebach cycle length and the retrograde VA conduction cycle length demonstrating an impaired AV conduction (Fig.12B-E). Since the number of AV node macrophages was not altered in $Cx_3cr1\ Cx43^{-/-}$ mice these findings indicate that macrophages facilitate electrical conduction in the AV node via Cx43. Next, we performed *in vivo* EP studies in $Csf1^{op}$ mice that congenitally lack macrophages and could confirm the findings mentioned above: absence of macrophages in these mice resulted in prolongation of the PR and AV intervals, the AV refractory periods, and the Wenckebach cycle length (Fig.12F-I).

Figure 12. *In vivo* EP studies in mice with Cx43 knockdown and congenital lack of macrophages[95]. A. Experimental Outline. **B-C**. AV node effective refractory period at 120 ms pacing frequency (**B**) and pacing cycle lengths at which Wenckebach conduction (**C**) occurred. **D-E.** Surface ECG from control and $Cx_3cr1\ Cx43^{-/-}$ mice illustrating the Wenckebach cycle length. Arrows indicate missing QRS complexes. **F-G**. Quantification of AV node macrophages in control and $Csf1^{op}$ mice by flow cytometry. **H-I.** AV node effective refractory period at 120 ms pacing frequency (**H**) and pacing cycle lengths at which Wenckebach conduction occurred (**I**) in control and $Csf1^{op}$ mice. Data presented as MEAN±SEM. * $p<0.05$, ** $p<0.01$. *AV*, atrioventricular, *ECG*, electrocardiography, *EP*, electrophysiology, *ms*, milliseconds.

Finally, we studied $CD11b^{DTR}$ mice that allow depletion of CD11b-positive cells (including resident cardiac macrophages) upon treatment with diphtheria toxin (DT; Fig.13). We implanted telemetry transmitters to continuously monitor the ECG in these mice. Within one

day after DT injection all mice developed first degree AV block (Fig.13C, E) that progressively evolved to second- and finally to third-degree AV block (Fig.13F-G) at the time of maximal macrophage depletion about three days after DT injection (Fig.13B). DT injection into C57BL6 control mice did not alter the ECG excluding toxic effects of DT, parabiosis experiments with $Cx_3cr1^{GFP/+}$ mice joined to $CD11b^{DTR}$ mice excluded circulating factors since only in $CD11b^{DTR}$ and not in $Cx_3cr1^{GFP/+}$ mice DT injection resulted in AV block, histologic assessment excluded apoptosis and fibrosis, blood chemistry tests excluded electrolyte disturbances, and treatment with isoproterenol, epinephrine or atropine excluded autonomic regulation as causal factors for altered AV conduction. In sum, these *in vivo* studies suggest that resident cardiac macrophages facilitate electrical conduction in the AV node.

Figure 13. Telemetry monitoring in $CD11b^{DTR}$ mice[95]. A. Experimental Outline. **B.** Flow cytometric quantification of AV node macrophages three days after intraperitoneal injection of DT (25 ng/g) into C57BL/6 and $CD11b^{DTR}$ mice. **C.** Onset of first degree AV block in $CD11b^{DTR}$ and C57BL/6 mice after DT injection. **D-G.** Telemetric ECG recordings before and after DT injection (25 ng/g) in $CD11b^{DTR}$ mice illustrating a normal ECG (**D**), first degree AV block (**E**), second-degree AV block (**F**) and third-degree AV block (**G**). Arrows indicate non-conducted P waves in second degree AV block. **** $p<0.0001$. *AV*, atrioventricular, *DT*, diphteria toxin, *ms*, milliseconds.

Summary and future perspectives

Arrhythmias are among the most common diseases and are clinically highly relevant since they account for significant morbidity and mortality. Over the last decades arrhythmia management has been improved, but preventive strategies, diagnostic approaches and therapeutic options are still limited and require the development of innovative concepts in the future.

The main reason for this currently still insufficient clinical management of arrhythmias is the complex underlying pathophysiology that is still not fully understood despite numerous advances that have been made in recent years. In this *Habilitation* some novel insights into cellular and molecular mechanisms leading to proarrhythmic remodeling and arrhythmogenesis have been gained. Studies on microRNAs revealed an important role for miR-29b in mediating atrial fibrosis in a dog model of heart failure induced AF and altered expression in human cardiac tissue suggested a role for miR-29b in humans as well. Furthermore, miRNAs circulating in the blood have been evaluated as potential biomarkers suggesting a role for miR-29b in profibrotic remodeling in AF patients and for miR-1 and miR-133a in exercise-induced remodeling in athletes. Additionally, our study on SHOX2 provided evidence for novel miRNA binding sites generated by genetic variants, thus, suggesting a potential mechanistic link how genetic variants might cause an increased risk for arrhythmias.

In addition to miRNA mediated mechanisms the role of immune cells, especially macrophages in arrhythmogenesis has been investigated. Our study on circulating MCP-1 revealed that MCP-1 levels are increased in AF patients and are further increasing after pulmonary vein isolation pointing towards immunologic mechanisms. Most importantly, cardiac resident macrophages have been identified as key regulators of electrical conduction in the AV node. It has been demonstrated that resident macrophages are enriched in the AV node, are electrically coupled to cardiomyocytes via connexin-43, and alter the electrophysiologic properties of coupled cardiomyocytes by increasing the resting membrane potential and shortening the action potential duration which facilitates electrical conduction.

The results of this *Habilitation* helped to increase our knowledge on proarrhythmic remodeling but several challenges remain. Some of the investigations were carried out on human samples and are thus highly valuable but provide rather indirect results and remain descriptive in most cases. Furthermore, human biosamples, especially heart tissue is very limited and even if available only some experiments are possible (e.g. expression analysis) while others are technically highly challenging (e.g. isolation of cells for patch clamp studies) or even impossible (e.g. genetic manipulation). To study causal arrhythmia mechanisms animal models are necessary and some of the findings presented here have been acquired in animals. The majority of basic science findings have been obtained in mouse models, these findings, however, cannot be directly applied to patients but require validation in animal models that closely resemble the human disease. This so-called translation of basic science findings into clinical practice will be the biggest challenge for researchers in the future.

Every animal species used in arrhythmia research has some specific advantages and disadvantages[96, 97]. However, the pig seems to be the most suitable species for most studies for several reasons: i) the porcine cardiac anatomy, hemodynamic properties, and electrophysiology closely resembles the human situation (Fig.14); ii) instrumentation in pigs is relatively simple as human equipment can be used; iii) pigs are widely available and their use for research purposes is socially more accepted compared to other species such as dogs; and

iv) genetic engineering is available resulting in a growing number of genetically modified pigs (e.g. pigs with neonatal diabetes mellitus). These characteristics will allow to generate tailored, close-to-human pig models facilitating translational research.

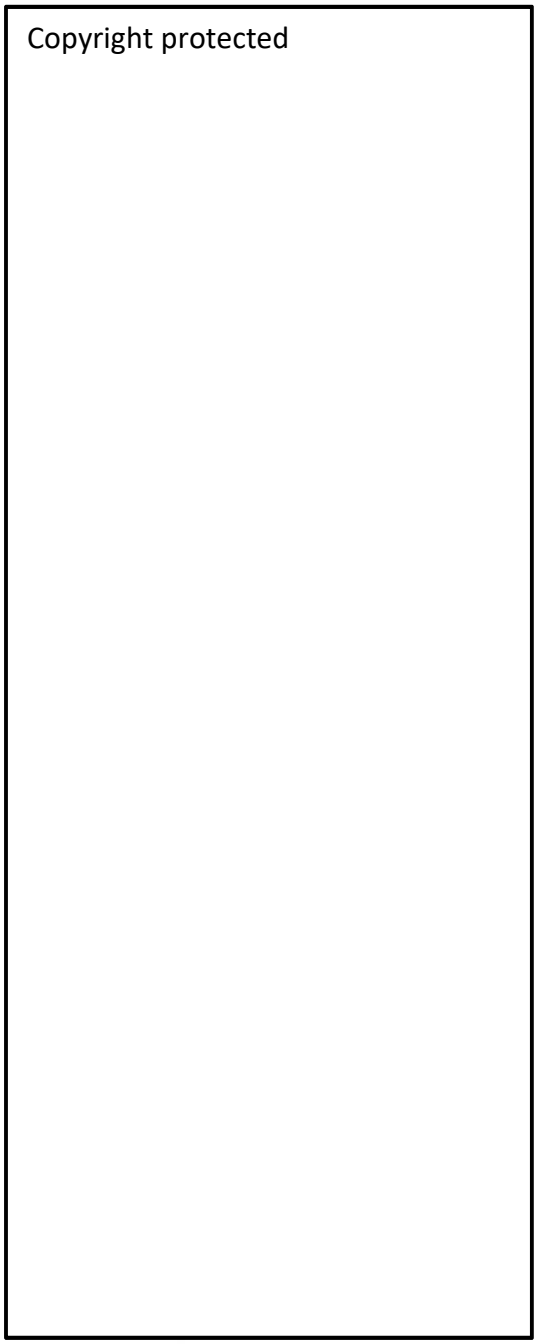

Figure 14. Comparison of atrial and ventricular action potentials in different species[96]. Dashed lines depict the action potential duration in humans.

Recently, we have demonstrated the translational potential of pigs for arrhythmia research[98]. In clinical practice, the most common trigger for AF is myocardial ischemia. Thus, we induced a myocardial infarction by ballon occlusion of the LAD for 90 minutes inducing an ischemic heart failure that closely resembles the situation in patients after myocardial infarction.

Ejection fraction was significantly reduced and the left ventricular end-diastolic pressure was significantly elevated which caused an increase in both left and right atrial pressure and finally the development of atrial fibrosis (Fig.15E-F). This structural remodeling finally resulted in an enhanced susceptibility for induced AF, longer AF episodes, and an increased AF burden in pigs with ischemic heart failure (Fig.15A-D). In sum, we could demonstrate that myocardial ischemia induces a proarrhythmic atrial remodeling leading to AF and could confirm the pig as a highly suitable species for translational studies in arrhythmia research.

Figure 15.

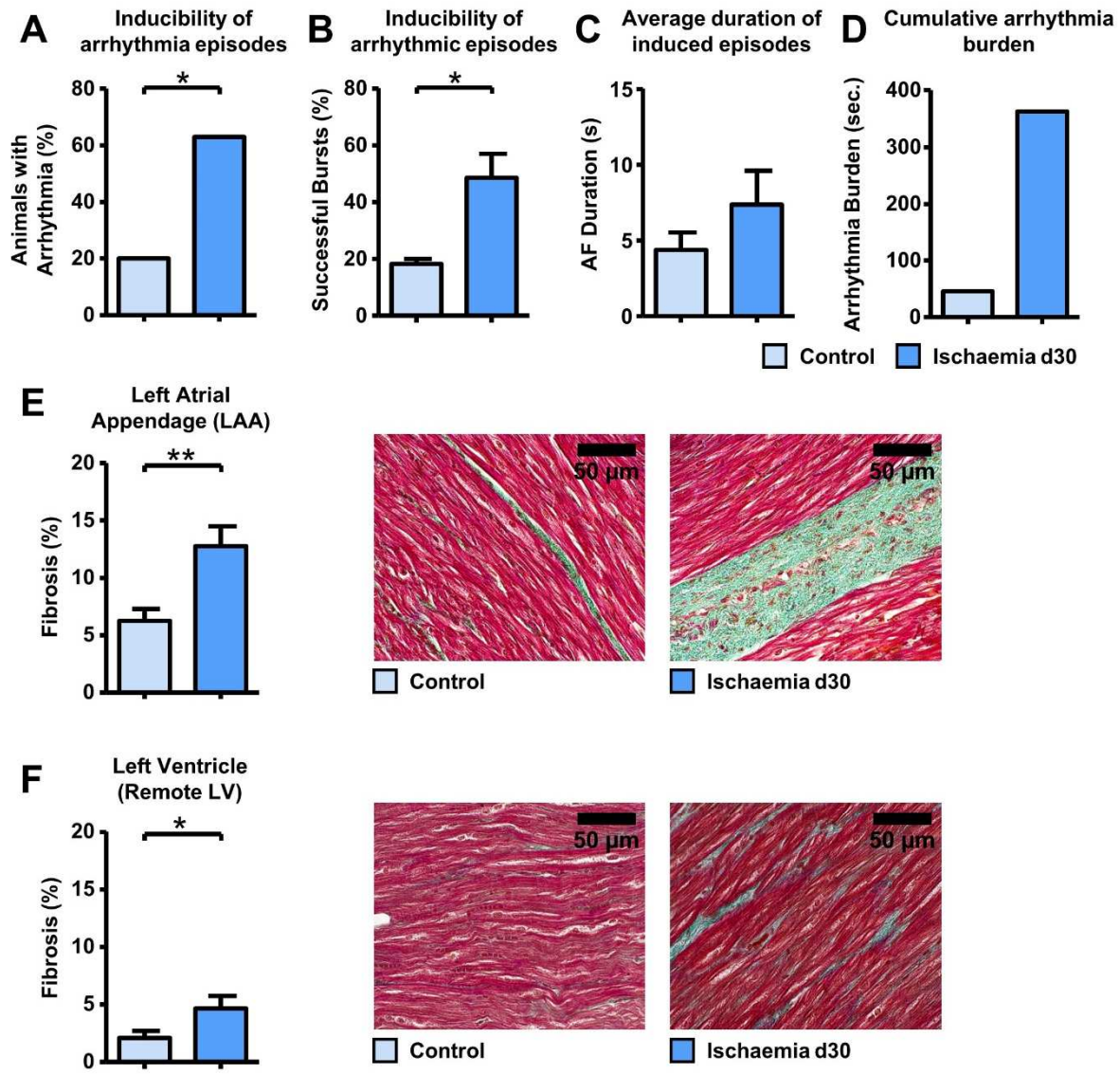

Figure 15. Porcine ischemic heart failure model[98]. A-D. Electrophysiology studies. Inducibility of atrial arrhythmia episodes per animal (**A**), percentage of successful bursts (**B**), average duration of induced arrhythmia episodes (**C**), and cumulative arrhythmia burden (**D**) in control and ischaemic pigs at day 30. **E-F**. Histologic assessment. Quantification of fibrosis in left atrial appendages (**E**) and remote left ventricle (**F**) between control pigs and ischaemic pigs at day 30 after infarction. * p<0.05, ** p<0.01. *AF*, atrial fibrillation, *d*, day, *LAA*, left atrial appendage, *LV*, left ventricle, *µm*, micrometer.

List of Abbreviations

µm	micrometer
a.u.	arbitrary units
ACE	Angiotensin converting enzyme
AF	Atrial fibrillation
APD	Action potential duration
AT1	angiotensin receptor type 1
AV	Atrioventricular
bpm	Beats per minute
CD	Cluster of differentiation
CD11b	Cluster of differentiation molecule 11B
CFB	central fibrous body
CHF	congestive heart failure
ChR2	Channelrhodopsin 2
CN	Compact node
COL1A1	Collagen 1 alpha 1
COL3A1	Collagen 3 alpha 1
Csf1	Colony stimulating factor 1
CT	Cycle threshold
CTGF	Connective Tissue Growth Factor
CTL	Control
Cx	Connexin
Cx3cr1	CX3C chemokine receptor 1
Cx43	Connexin-43
d	day(s)
DAD	Delayed afterdepolarisations
DT	Diphteria toxin
DTR	Diphteria toxin receptor
EAD	Early afterdepolarizations
ECG	Electrocardiography
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EP	Electrophysiology
FACS	Fluorescent-activated cell sorting

FBN1	Fibrillin 1
GFP	Green fluorescent protein
GWAS	Genome-wide association study
h	hour(s)
hr	hour(s)
IAS	Interatrial septum
ICD	Implantable cardioverter defibrillator
IL-1β	Interleukin-1β
IVS	Interventricular septum
LA	Left atrium
LAA	Left atrial appendage
LAD	Left anterior descending artery
LV	Left ventricle
MCP-1	Monocyte chemoattractant protein-1
mg	Milligram
miR	microRNA
miRNA	microRNA
ml	milliliter
MMP2	Matrix Metalloproteinase 2
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mV	millivolts
NCX	Na^{+}-Ca^{2+}-exchanger
ND	not detected
pg	picogram
PTEN	Phosphatase and Tensin homolog
PVI	Pulmonary vein isolation
RMP	Resting membrane potential
RNA	Ribonucleid acid
RV	Right ventricle
RYR2	Ryanodine Receptor Type 2
SEM	Standard error of the mean
SERCA	sarco/endoplasmic reticulum Ca2+-ATPase
SHOX2	Short-stature homeobox 2

SK3	Small-conductance calcium-activated potassium channel
SNP	Single nucleotide polymorphism
SR	Sarcoplasmatic Reticulum
TGFb	Transforming Growth Factor-β
TRPC3	Transient Receptor Potential Channel 3
UTR	Untranslated region
VA	Ventriculo-atrial
VTP	Ventricular tachypacing
w	With
w/o	Without

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Acknowledgments

I would like to thank Professor Steffen Massberg, Director of the Department of Medicine I at the University Hospital Munich for providing an excellent research environment and for supporting my scientific work.

I also want to express my particular gratitude to Professor Stefan Kääb who invited me into his research group, became my most influential clinical and scientific teacher, supported me whenever needed in professional and personal situations, helped me to build my own research group and to become an independent researcher, and remained a true mentor.

Furthermore, I am very thankful to know Professor Daphne Merkus who treated me as a researcher at eye level from the very beginning, established an inspiring atmosphere, and simply supported me in all my activities.

I would also like to thank Dr. Philipp Tomsits and Dr. Dominik Schüttler who were the first members of my research team, believed in me and stayed with me even during challenging times, and became my dear friends.

Science is pure team work, therefore I want to thank all the members of my group for all their hard work and the familiar atmosphere in our laboratory. Thank you, Julia V., Christina, Sarah, Christine, Jan, Ruibing, Florian, Kavi, Julia B., Aparna, Li, Simone, Valerie, Nora, Lina.

I want to particularly thank my friend Dr. Ling Xiao who I strongly admire for her profound knowledge, her widespread technical expertise and her unbelievable work ethic being the role model of a 'perfect scientist'.

Many thanks to Professor Patrick Ellinor for inviting me to his laboratory as a postdoctoral fellow and for supervising me during these terrific years.

I also thank all the other collegues, friends and family members who supported me during all these years.

Finally, I would like to thank my wife Dr. Ina Klier for the wonderful time I have since I met her and our son Anton for showing me what is really important in life.

Curriculum vitae

Not available online

Not available online

Not available online

Publication List

IF Impact Factor (Clarivate Analytics, InCites Journal Citation Reports)

* shared authorship/equal contribution

1. Original publications as first or senior author

1. **Clauss S.***, Schüttler D.*, Bleyer C., Vlcek J., Shakarami M., Tomsits P., Schneider S., Maderspacher F., Chataut K., Trebo A., Wang C., Kleeberger J., Xia R., Baloch E., Hildebrand B., Massberg S., Wakili R., Kääb S. Characterization of a porcine model of atrial arrhythmogenicity in the context of ischaemic heart failure. PLoS One. 2020 May 4;15(5):e0232374. **(IF 2.776)**
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3. Case Reports

None.

4. Reviews

1. Schüttler D., Bapat A., Kääb S., Lee K., Tomsits P., **Clauss S.***, Hucker W.* Animal Models of Atrial Fibrillation. Circ Res. 2020 Jun 19;127(1):91-110. doi: 10.1161/CIRCRESAHA.120.316366. **(IF (2018) 15.862)**

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6. Other Publications (Editorials)

1. Tomsits P., **Clauss S.**, Kääb S. Genetic Burden of Birthweight on Atrial Fibrillation: Translational Challenges in Genetic Atrial Fibrillation Studies. Circ Genom Precis Med. 2020 Jun;13(3):e002987. doi: 10.1161/CIRCGEN.120.002987. (**IF (2019) 4,063**)
2. **Clauss S.**, Sinner MF., Kääb S. Genome-Wide Association Studies Revealing the Heritability of Common Atrial Fibrillation: Is Bigger Always Better? Circ Cardiovasc Genet. 2017 Dec;10(6). (**IF 5.664**)
3. **Clauss S.**, Ellinor P. Does AF Follow Function? Ion Channel Mutations and Lone AF. CircArrhythmElectrophysiol. 2015 Oct;8(5):1005-6. (**IF 4.428**)
4. **Clauss S.**, Kääb S., Is pitx2 growing up? Circ Cardiovasc Genet, 4(2):105-7, 2011.(**IF 6.105**)

Teaching activities

Winter semester 2016/2017	7M1293	Seminar
	7M1292	Bedside Teaching
	7M1294	Tutorial (Problem-based learning)
Summer semester 2017	7M1293	Seminar
	7M1294	Tutorial (Problem-based learning)
Winter semester 2017/2018	7M1293	Seminar
	7M1292	Bedside Teaching
Summer semester 2018	7M1292	Bedside Teaching
	7M1298	Techniques in Cardiology (“Kardiologische Funktion”)
Winter semester 2018/2019	7M1293	Seminar
	7M1292	Bedside Teaching
	7M1298	Techniques in Cardiology (“Kardiologische Funktion”)
Summer semester 2019	7M1293	Seminar
	7M1292	Bedside Teaching
	7M1298	Techniques in Cardiology (“Kardiologische Funktion”)
Winter semester 2019/2020	7M1290	Lecture ECG
	7M1292	Bedside Teaching
	7M1298	Techniques in Cardiology (“Kardiologische Funktion”)
Summer semester 2020	7M1294	Tutorial (Problem-based learning)

Appendix