

Diploma thesis

**Atrial Fibrillation and Hypertension –  
Structural Remodelling in a Porcine Model  
of Rapid Atrial Pacing**

submitted by  
**Ursula Rohrer**

attaining the academic degree  
**Doktor der gesamten Heilkunde  
(Dr. med. univ.)**

at the

**Medical University Graz**

conducted by the  
**University Clinic for Internal Medicine  
Department of Cardiology**

supervised by  
**Assoz. Prof. Priv.-Doz. Dr.med.univ. Daniel Scherr  
Dr.med.univ. Martin Manninger-Wünscher**

Graz, 30<sup>th</sup> of July 2016

## **Eidesstattliche Erklärung**

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 30.Juli 2016

Ursula Rohrer eh.

## **Affidavit**

I hereby declare that I did write the following diploma thesis by myself and without any assistance from third parties. Furthermore, I confirm that no sources have been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, 30<sup>th</sup> of July 2016

Ursula Rohrer eh.

## **Acknowledgement**

First of all, I would like to express my sincere gratitude to Assoz. Prof. Priv.-Doz. Dr.med.univ. Daniel Scherr that he gave me the honour to be a member of his research group and for encouraging me in conducting this thesis.

I am very grateful to Dr.med.univ. Martin Manninger-Wünscher for his continuous support, for his patience and his motivation.

Thanks to both of my supervisors for their guidance and their constructive criticism that helped me all the time of research and writing of this thesis.

Finally, I would like to express my gratitude and appreciation to my parents, Marianne and Harald, my sister Katharina, Philipp and my friends for their never-ending support and encouragement throughout my studies.

## Table of contents

<b>Eidesstattliche Erklärung</b> .....	<b>2</b>
<b>Affidavit</b> .....	<b>2</b>
<b>Acknowledgement</b> .....	<b>3</b>
<b>Table of contents</b> .....	<b>4</b>
<b>List of abbreviations</b> .....	<b>7</b>
<b>Table of figures</b> .....	<b>9</b>
<b>List of tables</b> .....	<b>10</b>
<b>Abstract in English</b> .....	<b>11</b>
<b>Zusammenfassung in Deutsch</b> .....	<b>13</b>
<b>Introduction</b> .....	<b>15</b>
<b>1 Atrial anatomy and electrophysiology</b> .....	<b>17</b>
1.1 Atrial anatomy .....	17
1.1.1 Left atrium.....	17
1.1.2 Right atrium .....	18
1.2 Action potential and ion channels .....	19
<b>2 Atrial fibrillation</b> .....	<b>20</b>
2.1 Definition .....	20
2.2 Epidemiology .....	20
2.3 Classification .....	21
2.4 Symptoms .....	21
2.5 ECG characteristics .....	22
2.6 Complications and morbidity.....	22
2.7 Therapy.....	23
2.7.1 Anticoagulation .....	23
2.7.2 Rate or rhythm control .....	24
2.7.3 Catheter ablation and surgical procedures .....	24
<b>3 Pathophysiology of AF</b> .....	<b>24</b>

3.1	Anarchical organization.....	25
3.1.1	Multiple wavelet theory .....	25
3.2	Hierarchical organization .....	25
3.2.1	Focal sources .....	26
3.2.2	Enhanced automaticity .....	27
3.2.3	Triggered activity.....	27
3.2.4	Reentries and rotors .....	29
<b>4</b>	<b>Risk factors .....</b>	<b>31</b>
4.1	Hypertension.....	31
4.1.1	Definition.....	31
4.1.2	Pathophysiology .....	32
4.1.3	Aetiology .....	32
4.1.4	Complications .....	34
4.1.5	Therapy.....	34
4.1.6	Adverse effects of HT on AF .....	36
<b>5</b>	<b>Structural remodelling.....</b>	<b>37</b>
5.1.1	Atrial dilatation .....	37
5.1.2	Atrial architecture .....	38
5.1.3	Myocyte hypertrophy .....	38
5.1.4	Profibrotic pathways .....	39
5.1.5	Gap junctions.....	39
<b>6</b>	<b>Materials and methods .....</b>	<b>40</b>
6.1	Porcine model .....	40
6.1.1	Topography.....	41
6.1.2	Conduction system .....	41
6.2	Rapid atrial pacing .....	42
6.2.1	Pacemakers.....	42
6.3	Desoxycorticosterone .....	43
6.4	Processing and staining.....	43

6.4.1	HE staining .....	44
6.4.2	Picrosirius-Red staining .....	44
6.4.3	Immunofluorescence for confocal microscopic analysis .....	44
6.5	Experimental setup .....	44
<b>7</b>	<b>Results .....</b>	<b>46</b>
7.1	Macroscopic changes .....	46
7.2	Cardiomyocyte hypertrophy .....	48
7.3	Interstitial fibrosis .....	49
7.4	Distribution of connexin 43.....	50
<b>8</b>	<b>Discussion.....</b>	<b>51</b>
	<b>Bibliography.....</b>	<b>53</b>
	<b>Annex.....</b>	<b>57</b>
	Cutting with the rotation-microtome .....	57
	Staining solutions.....	57
	HE staining .....	58
	Configuration of the solutions.....	58
	Picrosirius-Red staining .....	59
	Cx43 Immunfluorescence .....	60

## List of abbreviations

<b>ACE inhibitors</b>	angiotensin-converting-enzyme inhibitors
<b>AERP</b>	atrial effective refractory period
<b>AF</b>	atrial fibrillation
<b>APD</b>	action potential duration
<b>AV</b>	atrioventricular
<b>BP</b>	blood pressure
<b>CAD</b>	coronary artery disease
<b>CHD</b>	coronary heart disease
<b>CHF</b>	congestive heart failure
<b>CKD</b>	chronic kidney disease
<b>CO</b>	cardiac output
<b>CS</b>	coronary sinus
<b>CV</b>	cardiovascular
<b>DBP</b>	diastolic blood pressure
<b>DM</b>	diabetes mellitus
<b>DOCA</b>	desoxycorticosterone acetate
<b>EF</b>	ejection fraction
<b>EP study</b>	electrophysiological study
<b>ERP</b>	effective refractory period
<b>HRA</b>	high right atrial location
<b>HT</b>	hypertension
<b>HV – interval</b>	interval from his bundle to chamber
<b>IVC</b>	inferior vena cava
<b>IVS</b>	interventricular septum
<b>LA</b>	left atrium
<b>LAA</b>	left atrial appendage
<b>LIPV</b>	left inferior pulmonary vein
<b>LSPV</b>	left superior pulmonary vein
<b>LV</b>	left ventricle
<b>LVEDD</b>	left ventricular enddiastolic diameter
<b>LVESD</b>	left ventricular endsystolic diameter
<b>NYHA</b>	New York Heart Association
<b>OAC</b>	oral anticoagulation
<b>OD</b>	organ damage
<b>PR - interval</b>	interval from the p to the r wave
<b>PV</b>	pulmonary vein
<b>PW</b>	posterior wall
<b>RA</b>	right atrium

<b>RAA</b>	right atrial appendage
<b>RAAS</b>	renin-angiotensin-aldosterone-system
<b>RAP</b>	rapid atrial pacing
<b>RCT</b>	randomized controlled trial
<b>RF</b>	risk factor
<b>RIPV</b>	right inferior pulmonary vein
<b>RMP</b>	resting membrane potential
<b>RSPV</b>	right superior pulmonary vein
<b>SBP</b>	systolic blood pressure
<b>SVC</b>	superior vena cava
<b>TIA</b>	transitory ischaemic attack
<b>TPR</b>	total peripheral resistance

## Table of figures

Figure 1: Schematic anatomy of the left atrium (1) .....	17
Figure 2: Schematic anatomy of the right atrium (4).....	18
Figure 3: Atrial AP and ventricular AP (5).....	19
Figure 4: 48 year old, female patient with de-novo AF .....	22
Figure 5: Early after depolarizations .....	28
Figure 6: Delayed after depolarizations .....	28
Figure 7: Circus movement reentry (25) .....	29
Figure 8: Leading circle without excitable gap (25).....	29
Figure 9: Rotors (25).....	30
Figure 10: Fluoroscopy of the porcine heart - catheter positioning: HRA, CS, LAA .....	41
Figure 11: The porcine heart during epicardial mapping .....	42
Figure 12: Custom-made pacemakers .....	43
Figure 13: RAP vs. RAP + DOCA .....	44
Figure 14: Experimental set-up in a time frame.....	45
Figure 15: Parasternal long axis.....	46
Figure 16: Parameters of the macroscopic changes .....	47
Figure 17: HE stained samples of the left and right atria .....	48
Figure 18: Picrosirius-Red stained left and right atria .....	49
Figure 19: Distribution of connexin visualized with immunofluorescence.....	50

## List of tables

Table 1: Prevalence of AF linked to the age (8).....	20
Table 2: CHA <sub>2</sub> DS <sub>2</sub> VASC- Score .....	23
Table 3: Overview of abnormal cell findings .....	27
Table 4: Classification of arterial hypertension regarding the BP values (30).....	32
Table 5: Major causes of secondary hypertension (31).....	33
Table 6: Risk stratification chart including the BP and the prevalence of RFs (31).....	35
Table 7: Therapeutical approach adjusted to BP grades and risk factors (31) .....	35
Table 8: Summary of macroscopic changes.....	46
Table 9: Summary of findings .....	51

## Abstract in English

### Introduction:

Atrial fibrillation (AF) is the most common sustained arrhythmia in humans and is associated with an increased risk of stroke, morbidity and death. Arterial hypertension (HT) is found in 60-80% of AF patients, is an independent predictor of new-onset AF and triggers hypertrophic and profibrotic pathways resulting in structural remodelling potentially favouring the progression of the arrhythmia. Due to overlap of multiple possible risk factors and large heterogeneity in patients, risk-factor dependent structural remodelling remains incompletely understood. In a large animal model we combined rapid atrial pacing (RAP) generating atrial fibrillation with DOCA-(desoxycorticosterone acetate)-induced arterial hypertension giving the opportunity to elucidate risk-factor dependent mechanisms favouring the progression of atrial fibrillation. In this study, we aimed to investigate the impact of arterial hypertension on structural remodelling during atrial fibrillation.

### Material and Methods:

17 landrace pigs were implanted with custom-made, telemetrically controllable pacemakers to induce AF. DOCA pellets were subcutaneously implanted in a subgroup of 9 animals (RAP+DOCA), the other 8 animals served as controls (RAP). Pacemakers were activated at a rate of 600/min two weeks before histological samples of both atria were obtained for analysis of cardiomyocyte area (HE staining), atrial fibrosis (Picrosirius-Red staining) and distribution of connexin 43 (Cx43 immunofluorescence for confocal microscopic analysis).

### Results:

Animals in the RAP+DOCA group had significant arterial hypertension and concentric left ventricular hypertrophy. HT was associated with severe structural remodelling. Histologic evaluation showed biatrial cardiomyocyte hypertrophy (cross-section cardiomyocyte area: LA:  $243.7 \pm 41.8$  vs.  $174.4 \pm 36.0$   $\mu\text{m}^2$ ,  $p < 0.01$ , RA:  $271.6$  (232,326) vs.  $186.8$ (169,202)  $\mu\text{m}^2$ ,  $p < 0.01$ ) as well as interstitial fibrosis (LA:  $14.0 \pm 2.2$  vs.  $8.5 \pm 1.6$  %,  $p < 0.001$ ; RA:  $14.4 \pm 3.4$  vs.  $8.3 \pm 1.5$  %,  $p < 0.001$ ) while distribution of

Cx43 remained unchanged ( $0.37\pm 0.1$  vs.  $0.39\pm 0.1$  ratio between Cx43 located on longitudinal sides and at intercalated disks, n.s.).

**Conclusion:**

In this model of secondary hypertension, HT triggers hypertrophic and profibrotic pathways revealing a distinct form of risk factor dependent structural remodelling in atrial fibrillation. These findings will help to understand mechanisms favouring the progression of atrial fibrillation in patients with arterial hypertension.

## Zusammenfassung in Deutsch

### **Einleitung:**

Vorhofflimmern ist die häufigste anhaltende Herzrhythmusstörung des Menschen und geht mit einem erhöhten Risiko für Schlaganfälle, mit erhöhter Morbidität und Mortalität einher. 60-80% aller Patienten mit Vorhofflimmern leiden an arterieller Hypertonie, welche ein unabhängiger Prädiktor für neu aufgetretenes Vorhofflimmern ist. Außerdem fördert dieser Risikofaktor strukturelle Veränderungen wie Hypertrophie und Fibrose, die als "Remodelling" bezeichnet werden und schlussendlich das Voranschreiten der Arrhythmie fördern.

Aufgrund der Überlappung mehrerer möglicher Risikofaktoren und der großen Heterogenität der Patienten, ist das von Risikofaktoren abhängige strukturelle "Remodelling" noch nicht vollständig geklärt. Wir haben Vorhofflimmern, durch schnelle Vorhofstimulation ("rapid atrial pacing" - RAP) ausgelöst, mit dem sogenannten "DOCA" (Desoxycorticosteronacetat), das arterielle Hypertonie induziert, in einem Großtiermodell kombiniert. Dadurch können die Mechanismen, die von Risikofaktoren abhängig sind und die Progression von Vorhofflimmern begünstigen, näher aufgeklärt werden.

Das Ziel dieser Studie war es, den Einfluss von arterieller Hypertonie auf das strukturelle Remodelling beim Vorhofflimmern näher zu erforschen.

### **Material und Methoden:**

17 Hausschweinen wurden eigens angefertigte, telemetrisch steuerbare Schrittmacher implantiert, um Vorhofflimmern auszulösen. In einer Kohorte von 9 Tieren (RAP+DOCA) wurden zusätzlich subkutan DOCA Pellets implantiert um einen arteriellen Hypertonus auszulösen. Die anderen 8 Tiere (RAP) dienten als Kontrollgruppe. Die Schrittmacher wurden auf eine Frequenz von 600/Minute eingestellt und zwei Wochen später wurden histologische Proben beider Atrien entnommen um die Kardiomyozytengröße (mittels HE Färbung), die Fibrosierung der Vorhöfe (Picosirius-Rot Färbung) und die Verteilung des Connexins 43 (mittels konfokaler Immunfluoreszenzmikroskopie) zu analysieren.

## **Ergebnisse:**

Die Tiere in der RAP+DOCA Gruppe zeigten einen signifikanten arteriellen Hypertonus und eine konzentrische linksventrikuläre Hypertrophie. Arterielle Hypertonie war mit erheblichem strukturellem Umbau assoziiert. Bei der histologischen Evaluierung zeigte sich biatrial eine Hypertrophie der Kardiomyozyten (Kardiomyozyten Querschnittsfläche: LA:  $243.7 \pm 41.8$  vs.  $174.4 \pm 36.0$   $\mu\text{m}^2$ ,  $p < 0.01$ , RA:  $271.6$  ( $232,326$ ) vs.  $186.8$  ( $169,202$ )  $\mu\text{m}^2$ ,  $p < 0.01$ ) und interstitielle Fibrose (LA:  $14.0 \pm 2.2$  vs.  $8.5 \pm 1.6$  %,  $p < 0.001$ ; RA:  $14.4 \pm 3.4$  vs.  $8.3 \pm 1.5$  %,  $p < 0.001$ ). Es zeigte sich jedoch kein Unterschied in der Verteilung von Cx43 ( $0.37 \pm 0.1$  vs.  $0.39 \pm 0.1$  Verhältnis von Cx43 an der Längsseite zu Cx43 an Glanzstreifen, n.s.).

## **Conclusio:**

In diesem Modell von sekundärem Hypertonus konnte nachgewiesen werden, dass der arterielle Hypertonus eine Hypertrophie der Myokardiozyten und vermehrte Fibrose verursacht. Es ergab sich eine ausgeprägte Form von Risikofaktor-abhängigem Strukturumbau beim Vorhofflimmern. Diese Ergebnisse können dazu beitragen, zugrundeliegende Mechanismen, die den Fortschritt von Vorhofflimmern bei Patienten mit arteriellem Hypertonus fördern, zu verstehen.

## Introduction

Atrial Fibrillation is the most common sustained cardiac arrhythmia – the prevalence rises especially in the elderly part of the population. Not just the number of hospitalizations increases but also the patients' morbidity and mortality multiplies significantly.

The risk of ischaemic strokes enhances notably and the outcome after a thromboembolic event cause by atrial fibrillation (AF) is poor compared to strokes due to other reasons. Therefore, AF is an important economic factor in the modern health system as well as an impairment of every single patient's health and life quality.

Hypertension (HT) counts to the scientifically proven risk factors but still not much is known on the specific pathways promoting the stability of AF and precipitating the progression of structural and electrical remodelling.

The goal of this study was to explore the pathways and mechanisms of AF and its progression in several ways. The setup compared two groups of pigs – one group with AF and HT and the control group with AF only. To put this idea into practice we used a well-established porcine model. We combined rapid atrial pacing (RAP) with custom-made telemetrically controllable pacemakers to induce AF with implanted DOCA-pellets (desoxycorticosterone acetate) to generate hypertension.

After a macroscopic exploration of the induced changes, this diploma thesis will focus on histological samples obtained of both atria. The main focus of attention were the size of the cardiomyocytes, the degree of fibrosis and the distribution of connexin 43.

The first chapter gives a brief insight of the atrial anatomy and physiology, while chapter two will focus on the pathology of atrial fibrillation.

Chapter three discusses the mechanisms leading to AF – the development of theories from the multiple wavelet theory as representative for anarchical organization to reentries and rotors to explain the idea of hierarchical organized AF:

The fourth chapter will discuss risk factors – more specifically arterial hypertension and its influence on arrhythmias.

The fifth chapter is a review of the current knowledge about structural remodelling and chapter six explains the materials and methods used in the experiments. The porcine specific anatomy and physiology compared to the human one, especially the topography and conduction system will be discussed as well as rapid atrial pacing, DOCA pellets and the processing and staining of the samples.

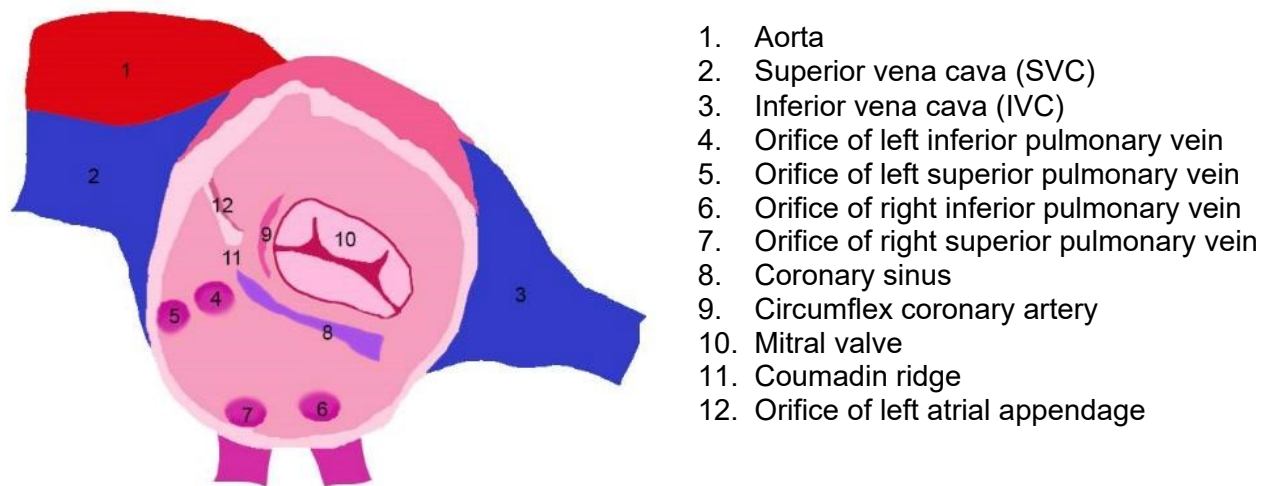
Finally, the practical part of this thesis covers the results and the obtained conclusions.

# 1 Atrial anatomy and electrophysiology

## 1.1 Atrial anatomy

The left and right atrium are fundamentally different to each other and this chapter will explain briefly the most important anatomical landmarks and their contribution to arrhythmogenesis.

### 1.1.1 Left atrium



*Figure 1: Schematic anatomy of the left atrium (1)*

The left atrium (LA) consists of different components: the left atrial appendage (LAA), the vestibule, the venous part and the septum.

The left atrial appendage is the only part with trabecular structure formed by pectinate muscles in the left atrium except of a small rim between the orifice of the LAA and the left superior pulmonary vein.

The so-called “vestibule” is the name for the smooth, muscular area around the mitral valve.

The four pulmonary veins (left and right superior and inferior pulmonary vein) continue in myocardial sleeves and end in the venous part. Fibro fatty tissue surrounds the muscle cells of the veins. The fibres are directed in different directions described as longitudinal, circular and oblique. Whereas fibres between the left and right pulmonary veins are superiorly-inferiorly orientated. (2,3)

This part together with the posterior wall of the left atrium contribute to the genesis of AF and play an important role in ablation strategies.

The mentioned posterior wall is composed of complex interlocked myocardial fibres orientated in various directions. This elaborate architecture helps maintaining AF and may be hypothetically a substrate for rotors (see chapter 3.2.4).

Generally, the left atrium shows less anatomical barriers than the right one and supports multiple wavelet reentries (see chapter 3.1.1) possibly leading to induce AF.(2)

### 1.1.2 Right atrium

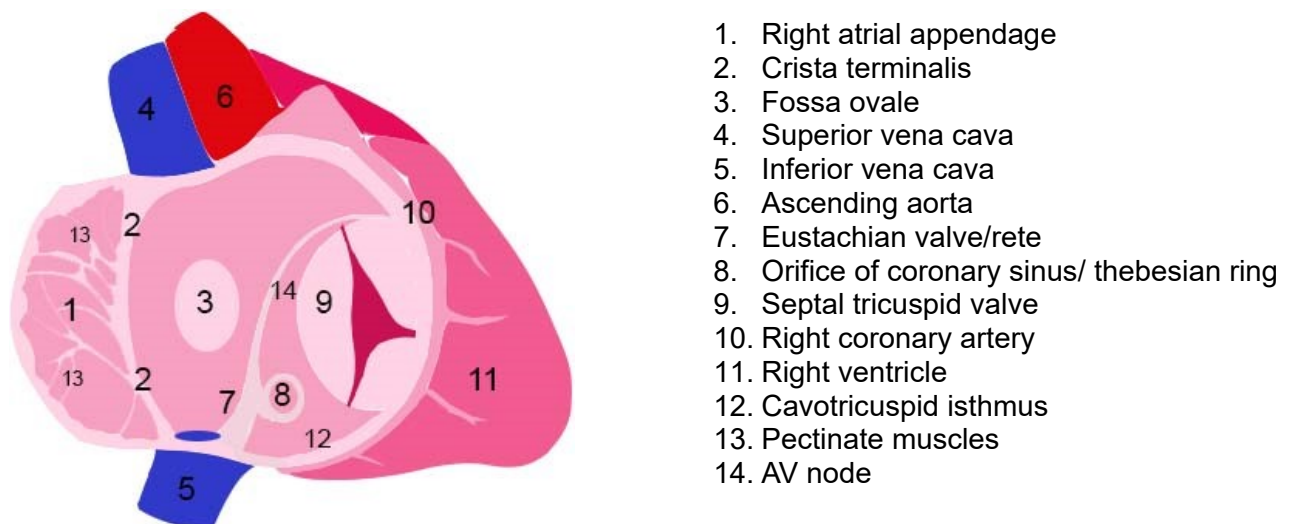


Figure 2: Schematic anatomy of the right atrium (4)

The right atrium (RA) contains important parts of the cardiac conduction system: the sinus node, the AV-node and the His bundle.

Anatomical structures and histological patterns divide this atrium into the appendage, the vestibule and the venous part.

The pectinate muscles again characterize the right atrial appendage. Its trabeculae originate from and are confined by the crista terminalis.

The vestibule is a smooth muscular part around the atrioventricular valve – in this case the tricuspid valve.

The right atrial venous part receives SVC and IVC and goes from the terminal groove to the interatrial groove.

An important structure in electrophysiology is the crista terminalis. It is a remarkable u-shaped, muscular structure eventually joining the Bachmann's bundle. This bundle is the electrophysiological interatrial pathway and consists of parallel-orientated muscle fibres. The terminal crest is known to cause anisotropic conduction with fast velocity in longitudinal direction and slow conduction in transversal direction due to a disperse distribution of gap junction density. This may reflect as well an arrhythmogenic substrate for atrial tachycardias – predominantly atrial flutter in contrast to the left atrium.(2)

## 1.2 Action potential and ion channels

The atrial action potential (AP) has a triangular shape and the plateau is either not present or less pronounced compared to the ventricular AP. Furthermore the variety of the AP duration and morphology within the atria is striking. These facts are due to the different concentration of ion channel currents and kinetic of repolarizing currents.

For example the  $I_{K1}$  is 5-10 times less denser represented than in the ventricles which leads to a high resting membrane potential of atrial myocytes and so the threshold to a new AP can be easily reached, i.e. myocytes are more easily excitable. Consequently, atrial foci need a smaller size and less depolarizing current to activate the surrounding cells. Some other ion channels like the ultrarapid delayed-rectifier ( $I_{Kur}$ ) and the acetylcholine-regulated current ( $I_{KACh}$ ) are predominantly expressed in the atria.(3)

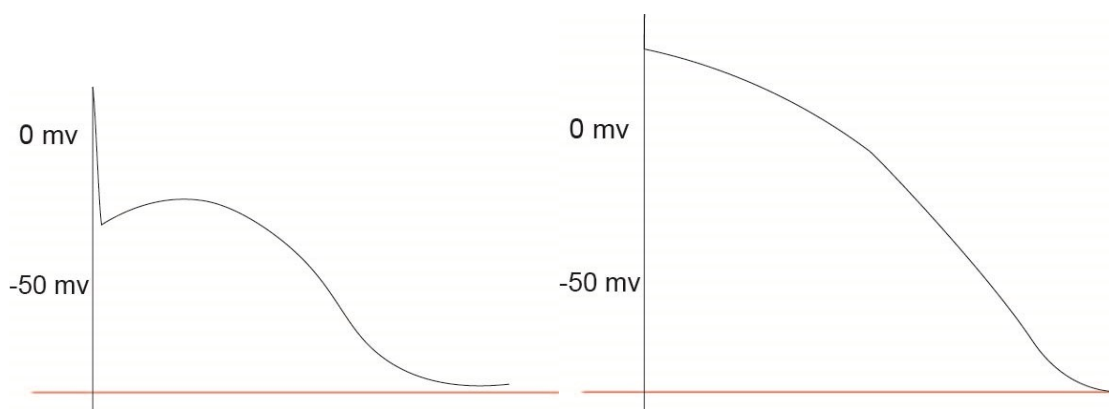


Figure 3: Atrial AP and ventricular AP (5)

## 2 Atrial fibrillation

### 2.1 Definition

Atrial fibrillation is the most common cardiac arrhythmia. Its origin is in the atrial myocardium and evokes unorganized electrical potentials with a very high rate (300-600 bpm), which overlaps the physiological pacemaker – the sinoatrial node. These waves vary in their amplitude, shape and timing. They produce “low amplitude baseline oscillations” (6) and are sometimes not perceptible in the ECG. This electrical potential does not lead to any effective contraction in the atrium and comes along with an absolute arrhythmic (=“irregularly irregular”) contraction in the chambers. This effect also is called “Arrhythmia absoluta”.

The effective heart rate is usually increased (100-160 bpm) and the end-diastolic filling is consequently decreased due to the shortened R-R intervals. As a result, the chambers adapt insufficiently during physical exertion because this fact can result in a low stroke volume and a pulse deficit. The distinctive abnormalities in a physical examination would be an irregular jugular venous pulse, a variation of the qualities of the first heart sound and the striking irregular pulse.(6)

### 2.2 Epidemiology

As already mentioned, atrial fibrillation is the most common disturbance of the cardiac rhythm particularly in the elderly part of our population. Around 1-2% of the European population suffer from AF and these numbers are still rising. The prevalence of atrial fibrillation in Europe is approximately 9% in octogenarians.(7) Obviously, the increasing age correlates directly with the incidence.(3)

A large epidemiological study in Massachusetts/ USA called the “Framingham study”, shows that the lifetime risk for developing AF after 40 years of age is 26% for men and 23 % for women.(8)

Prevalence of AF	Age
1,00%	<60 years old
~12%	75-84 years old
> 1/3	80 years old(9)

Table 1: Prevalence of AF linked to the age (8)

## 2.3 Classification

*Paroxysmal AF* – This type is lasting less than 7 days (seconds to hours) and usually converts back to a sinus rhythm spontaneously. Typically starting with atrial runs or ectopic atrial beats, the paroxysmal type always shows an initiating trigger. Consequently, there is a substrate for ablation. If there is a proof of a focal monomorphic trigger – it is called “focal AF”.(2)

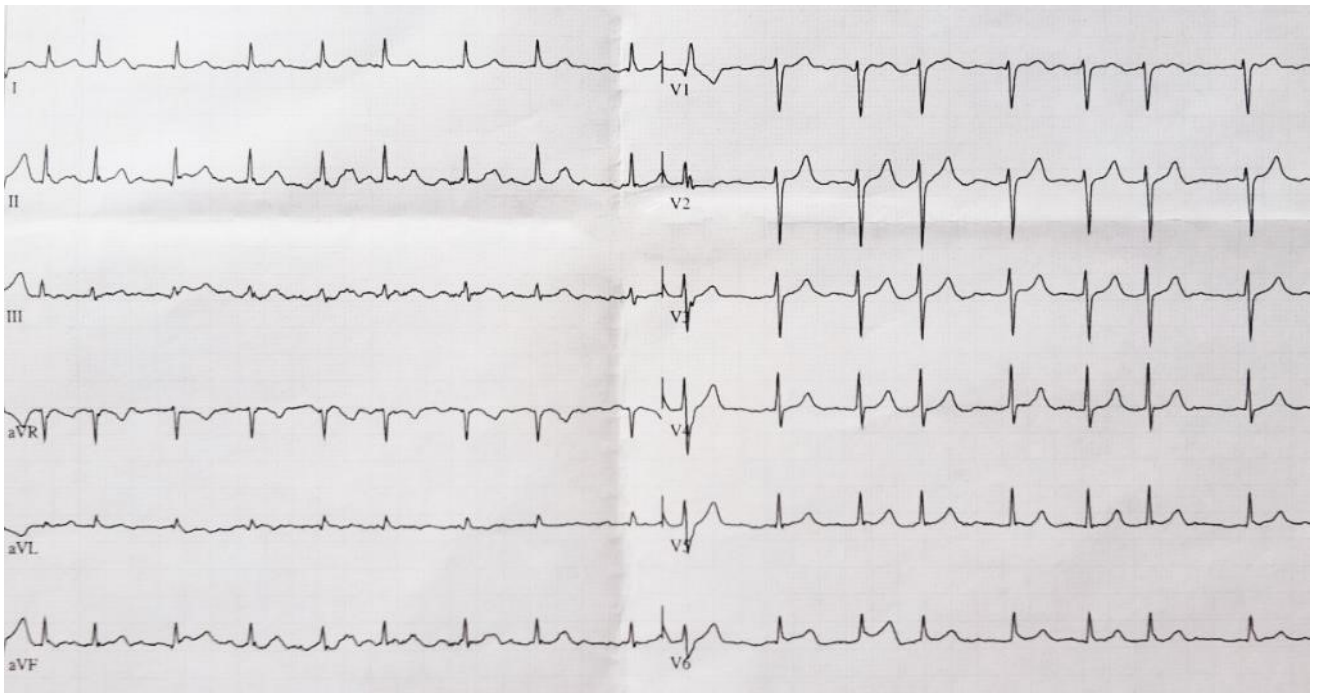
*Persistent AF* – No spontaneous conversion, but pharmacological/electrical cardioversion is still possible. It is lasting 7 days to several months and can potentially reoccur after a successful cardioversion after weeks to months. If it is lasting longer than 1 year, it is considered “longstanding”. (6)

*Permanent (chronic) AF* – Typically associated with unsuccessful cardioversion, neither electrical nor pharmacological. The duration can be several months up to several years.(7)

## 2.4 Symptoms

Symptoms and signs can vary. Patients with self-terminating episodes of AF sometimes are asymptomatic. With a progression to longer episodes – which can happen over decades – patients can feel palpitations, an irregular heartbeat, fatigue, effort intolerance, angina pectoris, shortness of breath, dizziness and eventually presyncope or syncope.(3) In more severe cases, the first symptoms can be an embolic event or right heart failure.

## 2.5 ECG characteristics



*Figure 4: 48 year old, female patient with de-novo AF*

Figure 4: 48 year old, female patient with de-novo AF. Typical ECG features from this patient with new onset atrial fibrillation are: No p-waves, arrhythmia absoluta, narrow QRS complexes, 100-110 bpm and fibrillation of the baseline.

## 2.6 Complications and morbidity

AF leads to dilatation and fibrotic remodelling in the atria. Due to that fact, the laminar flow converts into a turbulent flow, which can subsequently lead to thromboembolic complications like an ischaemic stroke. Eventually this remodelling process will lead to chronic heart failure.

AF is the proven cause of 20 - 25% of all strokes and the overall risk for a stroke is 5 times higher with a history of AF. Compared to an atherosclerotic aetiology the neurologic impairment is incomparably higher. The risk for a cardio embolic stroke decreases significantly when patients are under antithrombotic treatment when risk factors can be assessed (see chapter 2.7.1). Clinical studies comparing the left ventricular function for the cardiac prognosis in two populations (rate versus rhythm control) did not show any advantage in maintaining sinus rhythm. In terms of heart failure, AF increases the risk three times and the total mortality two times.(3)

## 2.7 Therapy

This chapter summarizes briefly the up-to-date therapeutical options and approaches according to the current guidelines. (For further information see: 2014 AHA/ACC/HRS guideline for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on practice guidelines and the Heart Rhythm Society (9), ESC Clinical Practice Guidelines: Management of Atrial Fibrillation 2010 (10) and Focused Update 2012 (11))

### 2.7.1 Anticoagulation

After the first clinical presentation of AF an assessment of the risk factors for ischaemic strokes or the recurrence of AF should be made to determine the future therapy. If risk factors can be stated, the therapy should be continued with either oral anticoagulants (OAC) or new oral anticoagulants (NOAC) lifelong. The present rhythm – whether sinus rhythm or AF – has no impact on the therapy.(10) CHA<sub>2</sub>DS<sub>2</sub> VASC is a score developed for the evaluation of the stroke risk in patients with non-valvular AF in Birmingham in 2009 and is an advancement of the previously used CHADS<sub>2</sub> risk score. Non-valvular AF indicates the absence of any valve disease and / or intervention like synthetic heart valves as patients with these risk factors need an antithrombotic therapy anyways. CHA<sub>2</sub>DS<sub>2</sub> VASC is an acronym and stands for the comorbidities listed in Table 2.(12)

Risk factor/ comorbidity	Points
<b>Congestive heart failure/ Left ventricular dysfunction (LV-Ejection fraction ≤40%)</b>	1
<b>Hypertension</b>	1
<b>Age (&gt; 75)</b>	2
<b>Diabetes mellitus</b>	1
<b>Stroke/Transient ischaemic attack/thromboembolism</b>	2
<b>Vascular disease</b>	1
<b>Age: 65-74</b>	1
<b>Sex Category: Female</b>	1

Table 2: CHA<sub>2</sub>DS<sub>2</sub> VASC- Score

### 2.7.2 Rate or rhythm control

There are two approaches to manage AF: either to control the rhythm with antiarrhythmic drugs or direct-current cardioversion or to keep the ventricular rate under control with oral drugs in a chronic setting or i.v. drugs in an acute setting.

Rhythm control can be established through direct-current cardioversion, antiarrhythmic drugs and/or catheter ablation, and is indicated amongst others in younger patients and/or whenever the rate control is unsuccessful, causes symptoms and/or leads to end-organ damage like myocardial ischaemia or heart failure. The preferable antiarrhythmic agents for rhythm control are amiodarone, flecainide, propafenone, ibutilide or vernakalant after assessing comorbidities and excluding contraindications.(10)

The medical approach to control the ventricular rate recommends beta-blockers or non-dihydropyridine calcium channel antagonist, digitalis glycosides or others like amiodarone after assessing possible contraindications or interaction with other medications.(10)

### 2.7.3 Catheter ablation and surgical procedures

Different strategies using radio-frequency ablation like AV node ablation (limited to patients with implanted pacemakers), PV isolation or ablation of complex fractionated atrial electrograms can be considered after failure of all current conservative treatments and in patients with remaining symptoms. The benefit and the risks need a very close evaluation as all these procedures have their adverse effects and are irreversible. Similar procedures exist using cryoablation, high-intensity focused ultrasound or surgical techniques.(10)

## 3 Pathophysiology of AF

The idea of AF is a chaotic circle excitement, which is triggered and maintained through atrial fibrosis and dilatation. In case it lasts for a long time, atrial remodelling takes place – electrically and structurally. The most important electrical parameters that are changing are the action potential and the refractory period. The structural remodelling will be discussed in Chapter 5: *Structural Remodelling*.

These processes support the stability and chronicity of AF, lessens the chance of a successful cardioversion and subsequently promotes a relapse. So the longer the

episodes of AF persist, the more profound these pathways establish a morphological substrate that again helps to sustain the arrhythmia. A study published by Wijffels et al. in 1995 describes the electrical remodelling that leads to this “vicious circle”: “Atrial fibrillation begets atrial fibrillation”.(13)

We partially understood the accurate mechanisms, which differ between each patient and offer different therapeutic options. In this chapter, a few pathways leading to arrhythmias and especially to AF will be described.

### **3.1 Anarchical organization**

Multiple non-organized sources inducing AF are called “anarchical AF”.(3)

#### **3.1.1 Multiple wavelet theory**

After many different theories on the initiation of AF Moe et al. established the multiple wavelet hypothesis in 1959 (14). This hypothesis proposed several simultaneous atrial reentries and self-perpetuating wavelets as a way to maintain AF. Nevertheless, it requires a sufficient number of sources to sustain AF as well as a certain atrial mass. A defined conduction velocity and refractory period may be necessary for a long and self-sustaining arrhythmia.(14) The therapeutic approach is to restrict the propagation of the wavelets by a surgical procedure called “Cox Maze”: multiple incisions in the right and left atrium – like a maze - should induce scar tissue that prevent the conduction of these multiple wavelets and should help to cure AF.(15)

### **3.2 Hierarchical organization**

This theory on pathomechanisms in AF states that there are rapid sources of activation which discharge focally. These sources have a higher rate than the physiological pacemaker and can be reentry circuits as well as local sources. The atrial myocardium cannot follow at the same frequency and so the conduction is irregular at a lower frequency and consequently leads to re-entrant circuits.(3)

Concerning the therapy, the focal source can be ablated with radio frequency to terminate the AF. These local sources can be anywhere in the left or right atria but usually a high percentage is located in/around the pulmonary veins. The term for (idiopathic) AF without any detectable cause is “lone atrial fibrillation”. (7)

### 3.2.1 Focal sources

In 1998, Haissaguerre et al. discovered a focal source of ectopic atrial beats in the confluence of the PV or the crista terminalis in a significant percentage of patients via mapping with a multielectrode catheter. The location of earliest activity in these electrophysiological studies was located in the myocardial sleeves of the pulmonary veins in 94%. These cells showed a more depolarized resting membrane potential, the upstroke velocity was decreased as well as the action potential duration.(16) These paroxysms originating from rapid discharges from the PVs could be terminated by radio-frequency ablation. In a follow-up several months later 69% of the patients were free of AF after ablation.(17) More elaborate techniques of radio-frequency ablation are established by now.

Microscopic studies proved a similarity between atrial myocytes and myocytes in the pulmonary veins. On the other hand different studies showed abnormal myocytes, like for example cells similar to the ones of the nodal tissues, as well as striking changes in the fibre orientation in PVs with different methods. Even interstitial cajal-like cells (ICLC) that show pacemaking activity in the gastrointestinal tract have been found in human atria and PVs. These findings may explain the triggered activity in this tissue and may have an influence on the conduction although the exact relevance of these changes remains unclear.(3) Table 3 shows an overview of these studies.

Authors	Method	Tissue	Findings
<b>Masani F.(18)</b>	Electron microscopy	Normal rat PV	Node-like cells with probable pacemaking activity
<b>Hocini M. et al.(19)</b>	Mapping	Normal dog PV	No evidence for abnormal myocytes. Abrupt changes in fibre orientation
<b>Verheule S. et al.(20)</b>	Electron and light microscopy	Normal dog PV	Normal myocytes, circumferential orientation of myocytes
<b>Chou CC. et al.(21)</b>	Electron and light microscopy	Normal dog PV	PAS-positive node-like cells, abrupt changes in fibre orientation
<b>Perez-Lugones et al.(22)</b>	Electron microscopy	Human PV	Presence of nodal like P cells, transitional cells, large Purkinje-like myocytes when history of AF → not in patients without history of AF
<b>Morel et al.(23)</b>	Electron microscopy and immunostaining	Human PV	Small ICLC (interstitial cajal-like cells)
<b>Hinescu ME. Et al. (24)</b>	Non conventional light microscopy	Human atrial myocardium	ICLC

*Table 3: Overview of abnormal cell findings*

### 3.2.2 Enhanced automaticity

The myocytes with pacemaking activity show a higher rate of spontaneous discharge. A lower threshold of the action potential (AP) upstroke can cause this in phase 0, a less negative maximal diastolic potential or an increase in the slope of spontaneous diastolic repolarization in phase 4.(3) This mechanism could be shown in human myocytes but a causative correlation with cardiac arrhythmias may need further investigation. (6)

### 3.2.3 Triggered activity

Cells can be activated by membrane oscillations of prior action potentials through reaching the individual threshold and end up in depolarization of the cells and a new AP. There are two different points in time when these triggers can activate the cells: before or after full repolarization.

- **EADs** – early afterdepolarizations occur in bradycardic conditions with normal calcium levels. Usually the EADs can be induced in phase 2 or phase 3 when the membrane potential of the AP is less negative.

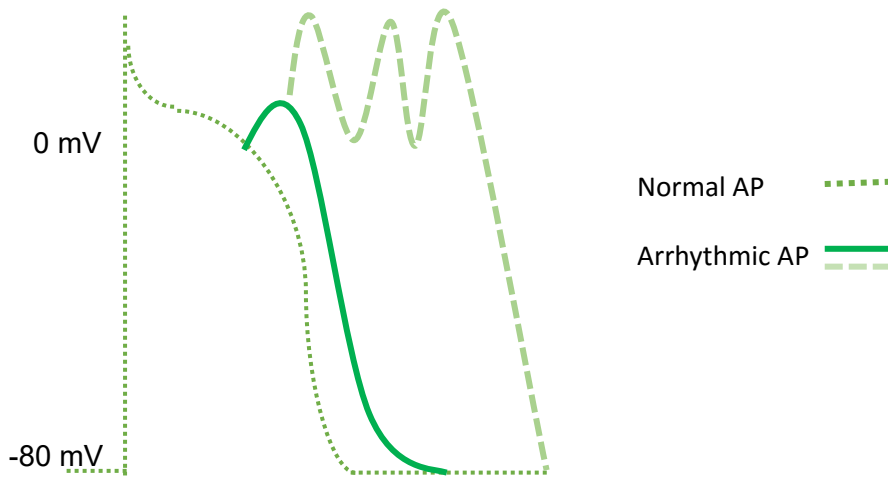


Figure 5: Early after depolarizations

- **DADs** – delayed after depolarizations occur after full repolarization in late phase 3/4 when the AP is more negative compared to the level in EADs and mainly during rapid pacing. Furthermore, this condition is associated with abnormal intracellular calcium levels typically observed in situations like digitalis intoxication, excessive catecholaminergic stimulation, ischaemia, tachycardia and/or low extracellular potassium levels.(3,6)

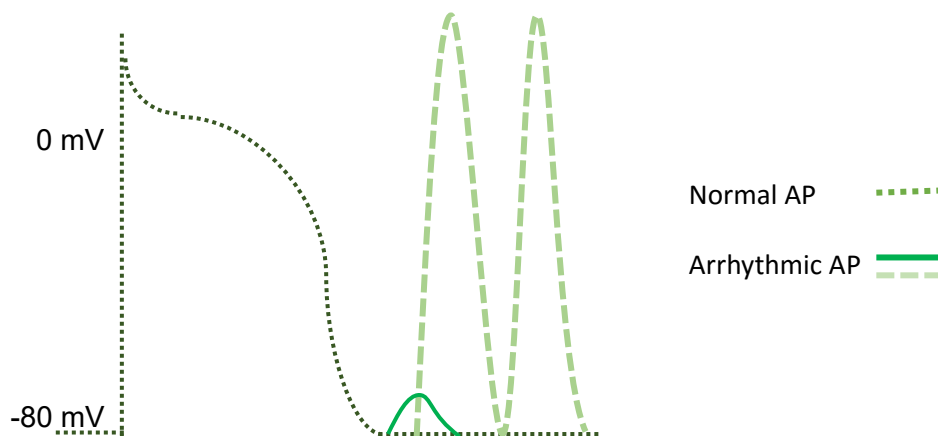


Figure 6: Delayed after depolarizations

### 3.2.4 Reentries and rotors

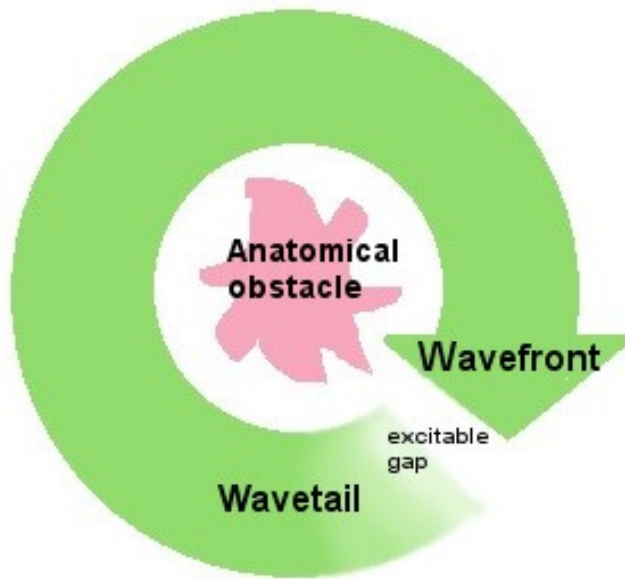


Figure 7: Circus movement reentry (25)

**Circus movement reentry:** Reentries can either arise around an anatomical obstacle, so that the excitation wave front reactivates tissue that's already excitable again shortly after its previous excitation. This part of the reentry is called "excitable gap" and develops because the anatomical barrier defines the size of the circus movement.(25)

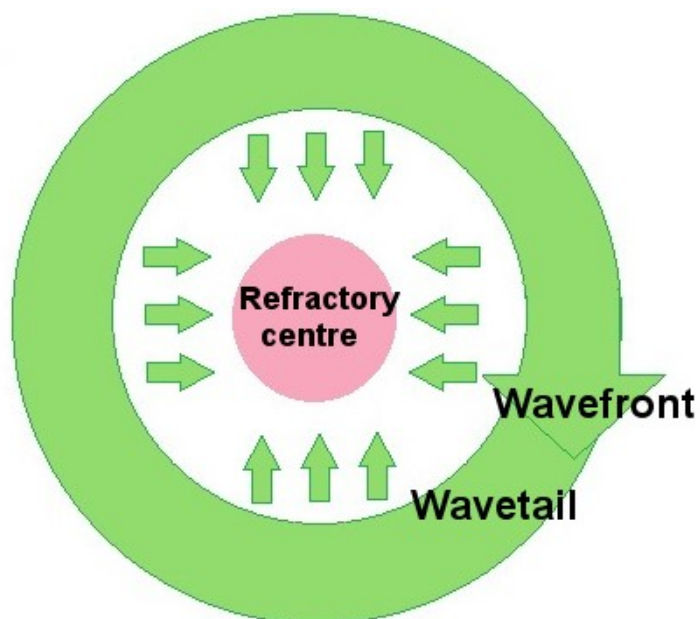


Figure 8: Leading circle without excitable gap (25)

**Leading circle reentry:** This hypothesis advances the circus movement reentry model. Allesie et al. found that in absence of an anatomical barrier the minimal necessary size of a self-sustaining reentry is determined by the fibre's attributes. This means that the length of the reentry circuit is equivalent to the wavelength of the electrical impulse – the product of the conduction velocity and the refractory period - and they called it “leading circle”.(26) This form of reentry is missing out on an excitable gap, so the wavefront always follows the wavetail and closes the re-entrant circuit. In contrast to a “normal” circus movement reentry the centre is continuously activated by small centripetal orientated waves coming from the leading circle. This produces a centre of refractory tissue and makes an anatomical obstacle unnecessary.(25,26)

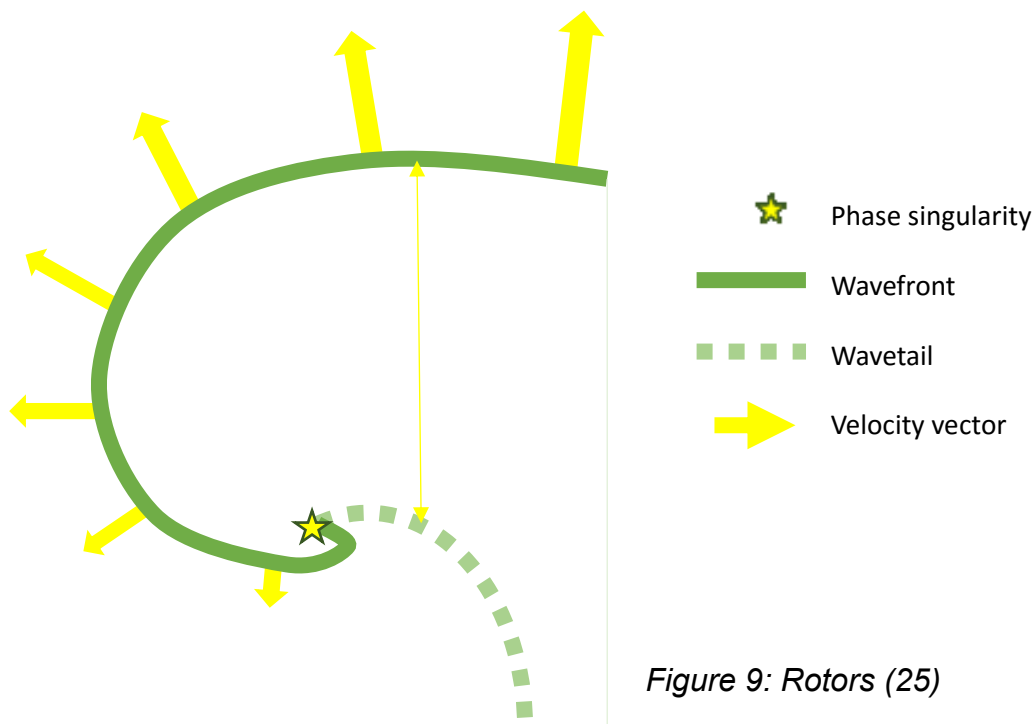


Figure 9: Rotors (25)

**Rotors:** The idea of a rotor means that an excitation “rotates”/moves around an unexcited core while the different points of the rotor show different conduction velocities (different APD) along its curvature. The wavefront and wavetail meet at a point called “phase singularity” and the conduction velocity diminishes to this point as well as the wavelength of the rotor. Basically the unexcited core is excitable but due to the attributes of the “phase singularity”, the core cannot be excited. Rotors seem to be more flexible in size and shape and do not seem to be fixed on a certain place. Furthermore, they might be able to break and bend around anatomical obstacles and build multiple daughter rotors.(25)

## 4 Risk factors

Several chronic diseases support the development of a substrate for AF. These include:(27)

- **Hypertensive heart disease (60-80%)**
- **Cardiovascular disease (25-30%)**

This includes cardiomyopathies, valvular and coronary artery diseases.

- **Heart failure**

Heart failure and AF are interrelated: AF will eventually compromise the LV function and this LV dysfunction will cause atrial dilatation and pressure overload, which promotes the development of a substrate for AF

- **Diabetes mellitus (20%)**
- **Age**
- **Secondary AF**

There are potentially reversible causes such as conditions after cardiac and noncardiac surgery and hyperthyroidism.(28)

These risk factors enhance the susceptibility in additive manner -the more risk factors, the more AF.

### 4.1 Hypertension

Arterial hypertension has a prevalence of 50% (29) in the general population in Europe and 60-80% (3) in AF patients – the prevalence correlates directly with increasing age. Hypertension itself has a causal connection with stroke and stroke mortality.

#### 4.1.1 Definition

“Hypertension” is the medical term for an elevated blood pressure and the cut-off value is either  $\geq 140$  mmHg systolic blood pressure (SBP) and/ or  $\geq 90$  mmHg diastolic blood pressure (DBP). The generally approved way to define it more accurately is shown in Table 4.

Category	SBP		DBP
<b>Optimal</b>	<120	And	<80
<b>Normal</b>	120-129	And/or	80-84
<b>High Normal</b>	130-139	And/or	85-89
<b>Grade 1 hypertension</b>	140-159	And/or	90-99
<b>Grade 2 hypertension</b>	160-179	And/or	100-109
<b>Grade 3 hypertension</b>	≥180	And/or	≥110
<b>Isolated systolic hypertension</b>	≥140	and	<90

*Table 4: Classification of arterial hypertension regarding the BP values (30)*

These values result from large RCT studies and provide help and recommendations for the therapeutical approach (see chapter 4.1.5 *Therapy*). Epidemiological studies showed that the SBP and additionally the pulse pressure (= difference between SBP and DBP) might be more accurate than the DBP to assess the cardiovascular (CV) risk in patients older than 50 years of age.(30)

#### 4.1.2 Pathophysiology

Generally, the blood pressure is defined as the product of the cardiac output (CO) and the total peripheral resistance (TPR).

$$BP = CO \times TPR$$

Consequently, a high blood pressure results from a high cardiac output, an increased TPR or a combination of both. The sympathetic nervous system, the RAAS (Renin-Angiotensin-Aldosterone-System) and the plasma volume as a surrogate for the renal function can influence these factors.(31)

#### 4.1.3 Aetiology

Arterial Hypertension can be classified in primary and secondary hypertension.

#### 4.1.3.1 Primary hypertension

Primary hypertension can also be called “essential hypertension” and its aetiology is not fully proven by science. We know little about the exact mechanism leading to increased blood pressure values in about 90% of all hypertensive patients.(29) Nevertheless there is a strong association with several risk factors like age, obesity, family history, diabetes and dyslipidaemia as well as life style factors, such as high sodium diet, alcohol consumption or physical inactivity.(31) As known so far the pathophysiological mechanism is an elevated CO in early stages but the TPR will increase consequently due to enhanced sympathetic activity and structural remodelling of the vessel walls.

#### 4.1.3.2 Secondary hypertension

The other 10 percent of the hypertensive patients suffer from some kind of secondary hypertension. There are several conditions secondarily leading to hypertension as listed in Table 5.

<b>Conditions leading to hypertension</b>	
<b>Legal drugs/medications</b>	
	oral contraceptives, NSAIDs, antidepressants (SSRIs, TCAs), glucocorticoids, decongestants (like pseudoephedrine), weight loss medication, cyclosporine, stimulants (methylphenidate and amphetamines)
<b>Illegal drugs</b>	
	methamphetamines and cocaine which are acting sympathomimetic
<b>Renal diseases</b>	
	primary renovascular (associated with fibromuscular dysplasia in younger or atherosclerosis in older patients) or renoparenchymal diseases (any kind of acute or chronic kidney disease – glomerular or vascular)
<b>Endocrine disorders</b>	
	primary aldosteronism, pheochromocytoma, Cushing’s syndrome, endocrine disorders of the thyroid or the parathyroid glands
<b>Obstructive sleep apnea</b>	
<b>Coarctation of the aorta</b>	

Table 5: Major causes of secondary hypertension (31)

The current recommendation is to look for secondary hypertension in the regular assessment but not to start specific blood tests or imaging procedures unless the patient is very young and cannot be controlled with a combination of three antihypertensive drugs or there are clues to it in the anamnesis and physical examination. These specific tests can show positive negative results so it is important to have a high pre-test probability to rule out the non-diagnostic findings.(29)

#### 4.1.4 Complications

HT may eventually lead to various severe disorders if the therapy is not ideally adjusted to the patient's needs and comorbidities. Early findings in these patients are a left ventricular hypertrophy and consequently a higher risk for heart failure, ischaemic strokes as well as intracerebral haemorrhage, ischaemic heart disease and chronic kidney disease (CKD) as far as end-stage renal disease.(31)

#### 4.1.5 Therapy

According to current guidelines, it is essential to assess the cardiovascular risk factors (RF) to initiate a therapy because these factors can potentiate each other and the overall cardiovascular mortality.

The ESH/ESC guidelines provide a chart considering the blood pressure level as well as the number of risk factors or already present organ damage (OD). This chart as seen in Table 6 *Table 6: Risk stratification chart including the BP and the prevalence of RFs (31)*

stratifies the risk from low to very high and serves as a device to assess the indication of a therapy.

Other asymptomatic or disease	RFs, OD	Blood Pressure (mmHg)			
		High normal	Grade I HT	Grade 2 HT	Grade 3 HT
No other RF			Low risk	Moderate risk	High risk
1-2 RFs		Low risk	Moderate risk	Moderate - high risk	High risk
≥3 RFs		Low - moderate risk	Moderate - high risk	High risk	High risk
OD, CKD stage 3, diabetes		Moderate - high risk	High risk	High risk	High - very high risk
Symptomatic CV disease, CKD stage ≥4 or diabetes with OD/RFs		Very high risk			

Table 6: Risk stratification chart including the BP and the prevalence of RFs (31)

Stratification of the CV risk in addition to grading the blood pressure helps to evaluate the need for a medical approach. The ESH/ESH “Practice guideline for the management of arterial hypertension” from 2013 (31) recommends the therapeutical approach as reproduced in Table 7.

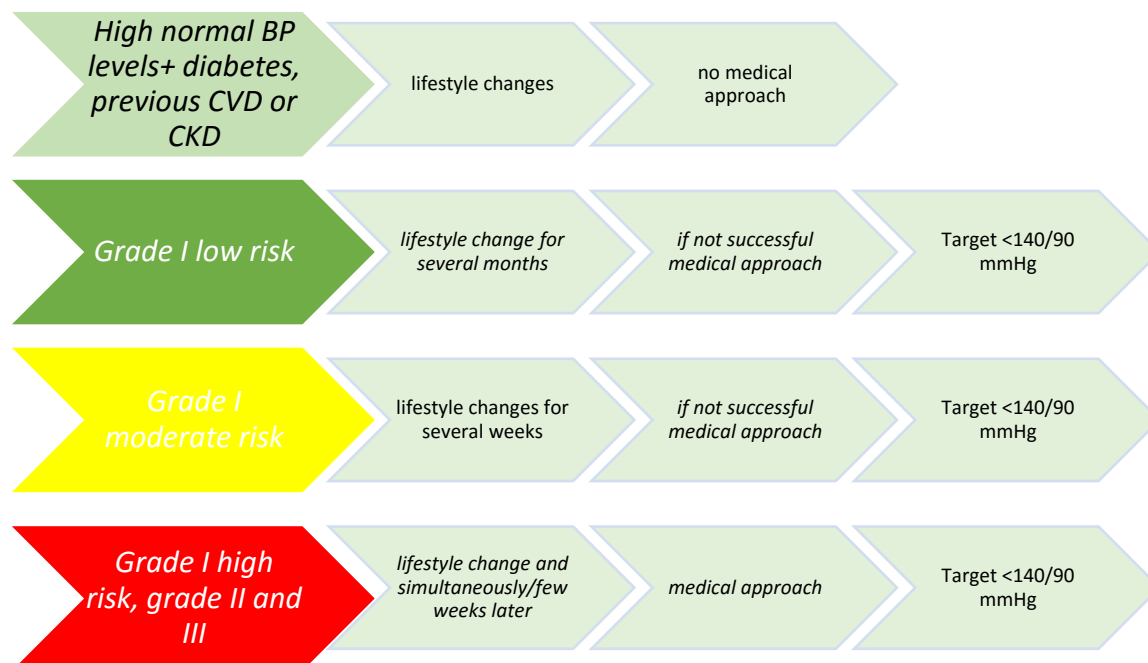


Table 7: Therapeutical approach adjusted to BP grades and risk factors (31)

Lifestyle changes like the reduction of the body mass index, salt restriction, introduction of a mediterranean diet, reduction/ total abstinence of tobacco, alcohol, caffeine and the start of daily physical activity are highly recommended. Most of

these recommendations are proven to have a positive effect on morbidity and mortality in multiple randomized clinical trials or meta-analyses.

After a certain time of lifestyle change the BP should be reassessed and if the levels are still not satisfying a medical therapy should be initiated. The first line medications are thiazide diuretics, ACE inhibitors, angiotensin II- receptor- antagonists, long-acting calcium channel blocker or beta-blockers. It is the individual choice of the treating physician to initiate whether a monotherapy or start with a combination straight away adjusted to risk factors or comorbidities like heart failure or coronary artery disease.(31) HT should be controlled to prevent AF and its progression to avoid structural changes as discussed below.(10)

#### 4.1.6 Adverse effects of HT on AF

There are practically no clinical studies investigating the direct effects of hypertension as an independent risk factor for the progression of AF as most of the patients are having more than one risk factor. Therefore, it is difficult to differentiate between the effects of the different risk factors as their effects seem to be overlapping and may be synergistic. However, various animal models can show direct effects of hypertension on the electrophysiology and structure of fibrillating hearts.

An Australian study in an ovine hypertension model proved the correlation of short-time hypertension and atrial remodelling. In detail, the research group found enlarged LA, reduced LA ejection fraction, higher mean ERP, slower mean conduction velocity, higher conduction velocity index, greater AF inducibility, increased AF duration, increased interstitial fibrosis and increased inflammatory cell infiltrates.(32)

Other electrophysiological studies show decreased conduction velocities but no alteration in the atrial effective refractory period resulting in increased susceptibility and stability of AF in spontaneously hypertensive rats. Furthermore, changes in the  $Ca^{2+}$  current density in the left atria could be examined.(33)

Structural studies show profibrotic changes and myocyte hypertrophy as well as myolysis.(3,33,34)

These effects may be explained by the higher vulnerability of the atrial myocardium to pressure and volume overload. These profibrotic pathways seem to be mediated by angiotensin II-dependent and angiotensin II-independent mechanism and show increased apoptosis and fibrotic remodelling following chronic atrial stretch. Although

this mechanism was demonstrated in a canine model of congestive heart failure (CHF), it may explain the structural remodelling in hypertension as well.(35)

## **5 Structural remodelling**

The following chapters give an overview of the current scientific knowledge about structural changes developing through AF. Structural remodelling occurs in the course of the time and its role in contributing to the progression of AF is still under investigation. Usually the structural alteration needs more time (weeks-months) compared to the electrical alterations.(3) The latter will not be discussed in this thesis.

### **5.1.1 Atrial dilatation**

Atrial dilatation occurs in AF and has a strong correlation with different risk factors. Most notably the left atrium is well studied and clinical data suggest a causative relation between its size and the presence of AF in patients with a structural heart disease (isolated mitral or aortic valve disease, asymmetric septal hypertrophy). Furthermore, the atrial size acts inversely proportional to the long lasting (>6 months) success of cardioversion.(36)

Only very few clinical studies focus on structural remodelling, but regarding the factor atrial dilatation a small prospective study examined the atrial size of human patients with AF. The echocardiographic studies in patient with initial normal atrial sizes showed a significant increase in size after 12-28 months of AF (LA mean volume 45.2 to 64.1 cm<sup>3</sup>, RA mean volume 49.2 to 66.2 cm<sup>3</sup>). The exclusion criteria were strict regarding comorbidities supporting atrial dilatation (i.e. significant mitral or tricuspid valve disease, left ventricular pathologies and pre-existing abnormal left atrial sizes). Therefore, these data support that sustained AF induces atrial dilatation while the extent of the influence of synergistic risk factors remains unclear.(37)

Another prospective clinical study published in 2005 supports this hypothesis. The aim of this study was to quantify the influence of the heart rhythm (i.e. sinus rhythm versus AF) on the echocardiographically determined atrial sizes. After initial echocardiographic studies, 81 patients with persistent AF and an underlying hypertensive heart disease underwent electrical cardioversion. They were then divided into two cohorts, one consisted of patients who remained in sinus rhythm

after a successful cardioversion (25%) while the other included patients who relapsed to AF. The echocardiographic studies have been redone in both groups in the 5-year-follow-up and showed an increase in the left atrial volume by 7.3 to 21.4% in the group with persisting AF depending on the patients' NYHA class. On the other hand the group maintaining sinus rhythm showed even a decrease of left atrial parameters compared to the initial studies.(38)

### 5.1.2 Atrial architecture

The various fibre orientation at certain landmarks of the atria contributes to conduction anisotropy (see chapter 1.1 *Atrial Anatomy*). These fibres may run in a parallel pattern like in the Bachmann's bundle or at the terminal crest and between/around the pulmonary veins. These specifically orientated running fibres may induce dissociated conduction patterns and can lead to complex fractionated electrograms when the atria undergo structural remodelling in the course of ongoing AF.(3)

Another anatomical fact showing a substrate for AF is the combination of endocardial trabecular structures over epicardial layers in both atria. In experimental animal models the activation patterns in these different layers were very different and complex which may make AF less organized and more stable.(3)

### 5.1.3 Myocyte hypertrophy

An elaborate animal model in a study from Maastricht examined goats under sustained atrial fibrillation. This established model of chronic atrial fibrillation induced by rapid atrial pacing (RAP) described by Wijffels et al. in long-term instrumented goats focused on structural changes in both atria. Already one week after continuous application of the automatic AF pacemaker the animals showed sustained AF (defined as AF>24 hours).(13) After 20 weeks of sustained AF the samples of both atria could be obtained and showed a hypertrophy of atrial myocytes up to 195% compared to the atrial myocytes of the control group.(39,40) Nevertheless the influence of these structural changes on the electrical conduction is not yet fully investigated. Myocytal hypertrophy influences scientifically proven the anisotropy of the conduction while its effect on the conduction velocity may need further research on the other hand.(3)

#### 5.1.4 Profibrotic pathways

Many studies (most of them using animal models with RAP) have been conducted to study the role of atrial fibrosis in the development and maintenance of AF. Some of these studies could not prove a higher percentage of fibrosis between the RAP and the control group. Nevertheless, the extracellular matrix of the myocytes increased suggesting a higher percentage of interstitial fibrosis.(3,41) These changes could affect the conduction and induce a higher complexity of AF.

Contrary to these results, other RAP studies opposing cohorts with and without controlled ventricular rate found increased fibrosis either in both groups (42) or in the group without controlled ventricular rate.(43) The latter study indicates a significantly elevated percentage of fibrosis particularly in the group with a high ventricular rate only after 15 weeks of RAP. The examinations (electrophysiological studies, epicardial mapping and histological studies) showed evidence for conduction heterogeneity, atrial fibrosis and the rapid progression to persistent AF in this group. This results may have a consequence on the treatment and support the importance of rate control in AF.(3)

#### 5.1.5 Gap junctions

Cardiac muscles are stretched, intertwining skeletal muscles connected via intercalated discs. These discs contain desmosomes on one hand and gap junctions on the other hand. These gap junctions are called “connexins”.

These connexins serve as intercellular communication media - chemically and electrically whereas desmosomes are responsible for the mechanic stabilization of the cells.

Especially the cells of the conduction system below the AV node show a high density of gap junctions resulting in high conduction velocities.(44) In a regular working myocardium the electrical excitation is conducted faster in longitudinal direction compared to conduction in transverse direction. Because the gap junctions at side-to-side connection of myocytes are smaller and their density is lower, they represent a bigger hurdle than the larger and plentiful connexins of the intercalated discs at the end-to-end connection.

Connexin 40 (Cx40) and Cx43 are the predominant atrial gap junction channels. Cx45 is also expressed in the myocardium. Cx40 is expressed in most part of the

conduction system downwards the AV node and endothelial cells. Cx43 is expressed in the atria as well as in the ventricles. Cx45 occurs mainly in the sinus and AV node and is co-expressed with Cx43 downstream of the His bundle.(45,46)

There is evidence that an alteration of connexins in models of sterile pericarditis leads to an alteration of conduction velocities and may lead to atrial arrhythmias.(3)

Different studies in animals as well as in humans show inconsistent results. Connexins may play a different role at every stage of the development and chronification of atrial arrhythmias. Especially in AF a possible lateralization or heterogeneity of distribution is discussed. Furthermore, the density of the particular connexin types has been examined. Until now there are no significant alteration found that might support a specific pathway to disturb electrical coupling.(3,45)

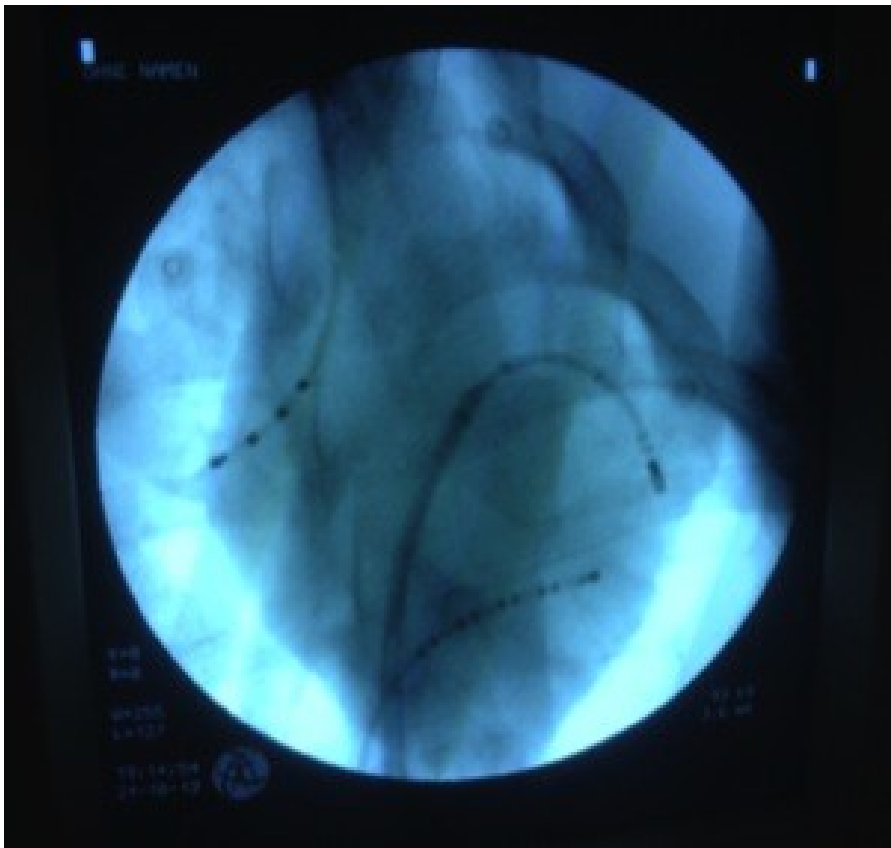
## **6 Materials and methods**

### **6.1 Porcine model**

The porcine anatomy and physiology in general are very similar to the human one, especially concerning the cardiac conduction system. Additionally, the size of the animals and their cardiovascular system facilitate accurate haemodynamic measurements.(47) This fact makes the porcine model very representative and effective for electrophysiological studies.

Nevertheless, there are some important differences regarding the topography and the conduction system that need to be considered while using fluoroscopy or electrophysiological mapping systems.

### 6.1.1 Topography



*Figure 10: Fluoroscopy of the porcine heart - catheter positioning: HRA, CS, LAA*

At first, the heart's orientation in the thorax in fluoroscopy is 60° rotated compared to humans, so the posterior surface is adjacent to diaphragm while the anterior surface is next to the sternum.

Secondly, considering the heart itself: The appendages are equally sized but the atria are smaller. Contrarily to human hearts, the apex is entirely formed by the left ventricle.

The ventricular myocardium is up to two centimetres thick, which consequently leads to smaller volumes. The aorta leaves the heart more posterior than in human hearts and there are only 2 pulmonary veins which split early in multiple larger branches.

The dimensions can vary hugely with race, weight and size.

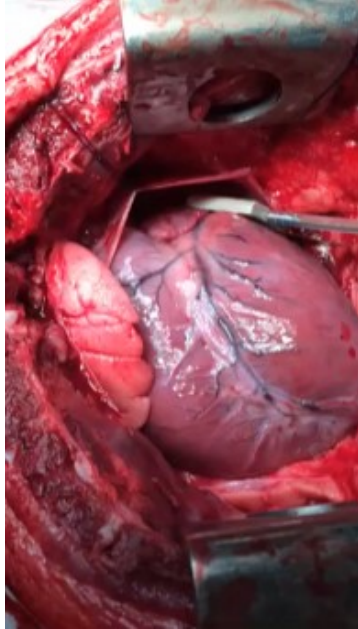
### 6.1.2 Conduction system

The sinus node is situated at the junction between SVC and RAA but its position can be at different heights at the upper to the middle third of the crista terminalis.

The intrinsic heart rate is usually higher than the human one and highly variable.

PR and HV intervals are shorter, so subsequently, the sinoatrial conduction time is shorter.

The AV - node is located more to the right side of the septum, the bundle is short and the bifurcation is more proximal compared to the human one.(48)



*Figure 11: The porcine heart during epicardial mapping*

## **6.2 Rapid atrial pacing**

RAP is a well-established method to induce AF in different animal models.(49) In this model, the right atrium was paced with a rate of 600bpm.

### **6.2.1 Pacemakers**

Commercially available pacemakers cannot stimulate with very high rates so our study group developed custom-made pacemakers to reach the desired stimulation rate of 600 bpm. These pacemakers were constructed consisting of a telemetry unit and a freely programmable pacing unit casted in biocompatible epoxy resin. The telemetry unit could be connected via a radio link to a computer to activate the stimulation unit and to do regular rhythm checks. The pacemaker weighed 78 grams and its size was 19x48x10 mm. The pacemaker probe was a commercially available model for human use. These custom-made pacemakers were implanted in all 17 pigs by heart surgeons in a sterile setting. After 2 weeks of healing they could be started at a rate of 600bpm to induce AF.



Figure 12: Custom-made pacemakers

### 6.3 Desoxycorticosterone

DOCA (desoxycorticosterone acetate) is used to induce secondary hypertension. The chemical slightly different and physiological variant DOC is a precursor to aldosterone. In this model, DOCA-pellets are implanted subcutaneously into the pig's groin. Also, salt is added to their regular nutriment.

The mechanism to induce hypertension is via fluid retention and a higher circulating blood volume. Desoxycorticosterone acetate is a steroid hormone and acts like a mineralocorticoid by retention of sodium and excretion of potassium.(50)

So this mechanism increases the blood volume by retaining the sodium. Therefore, it is important to monitor potassium levels and replace the excreted potassium either orally or intravenously. The advantage of this model is the reproducibility and the rapid raise of the blood pressure, which can be observed within few days. (47)

### 6.4 Processing and staining

The final experiment consisted of echocardiographic studies, invasive haemodynamic measurements, electrophysiological studies and epicardial mapping while the animals were under general anaesthesia. Afterwards the heart was removed to obtain samples of both atria. These samples were conserved in 4% paraformaldehyde in phosphate buffer solution. Later, tissues were embedded in paraffin to be cut into slices of 3  $\mu\text{m}$  by a microtome. The samples underwent different staining for assessment of cardiomyocyte size, collagen content and connexion distribution. Detailed staining protocols can be found in the annex.

### 6.4.1 HE staining

Haematoxylin and Eosin are a widely used routine staining to differentiate cells. Haematoxylin stains basophile structures in deep blue to purple and is responsible for the nuclear detail and definition. It stains nucleic acids by a complex. Eosin, on the other hand, serves as the contrasting counterstain and stains all eosinophil structures such as collagen, proteins of the cytoplasm and mitochondria in a pink colour. Here, it was used for the analysis of the cardiomyocyte area.(51)

### 6.4.2 Picrosirius-Red staining

This staining is mainly used to visualize the collagen contained in muscular tissue. Collagen is stained in red which helps analysing area fractions of extracellular matrix in a histological sample.

### 6.4.3 Immunofluorescence for confocal microscopic analysis

Immunofluorescence is used to visualize connexin 43 and to evaluate its distribution with the help of confocal microscopy.

## 6.5 Experimental setup

An earlier experiment showed that after two weeks 80% of the animals develop more sustained AF (free of AF for more than 60 minutes) if they are treated with DOCA pellets in addition to RAP as seen in Figure 13.

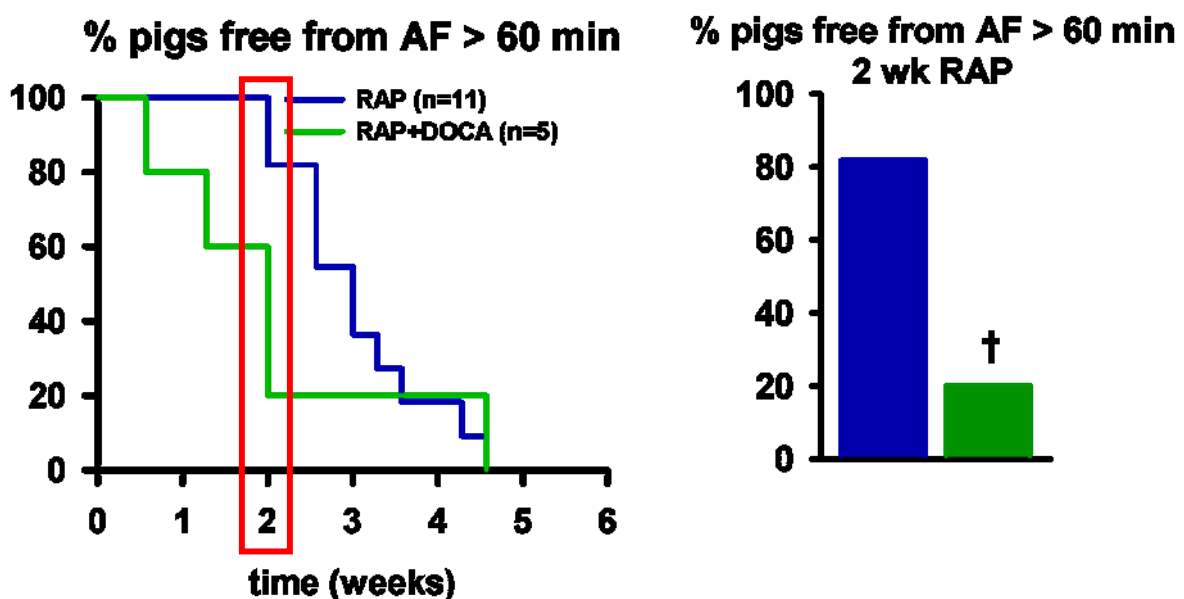


Figure 13: RAP vs. RAP + DOCA

The experiment consisted of 2 groups: While both were implanted with pacemakers and probes in the right atria, one cohort additionally received DOCA pellets one to two weeks later. After two more weeks all pacemakers were activated at a rate of 600 bpm. We added Digoxin to the food to slow down AV conduction and subsequently ventricular heart rate and potassium to balance the DOCA induced loss.

As soon as the RAP started, pigs were under regular telemetric control to check the rhythm and to detect malfunctions like a damage or dislocation of the probe every second day. In addition, echocardiography was performed and blood samples were taken. After a total of 4 weeks, the final experiment took place and the macro- and microscopic samples were obtained. A detailed overview of the study protocol is illustrated in figure 16.

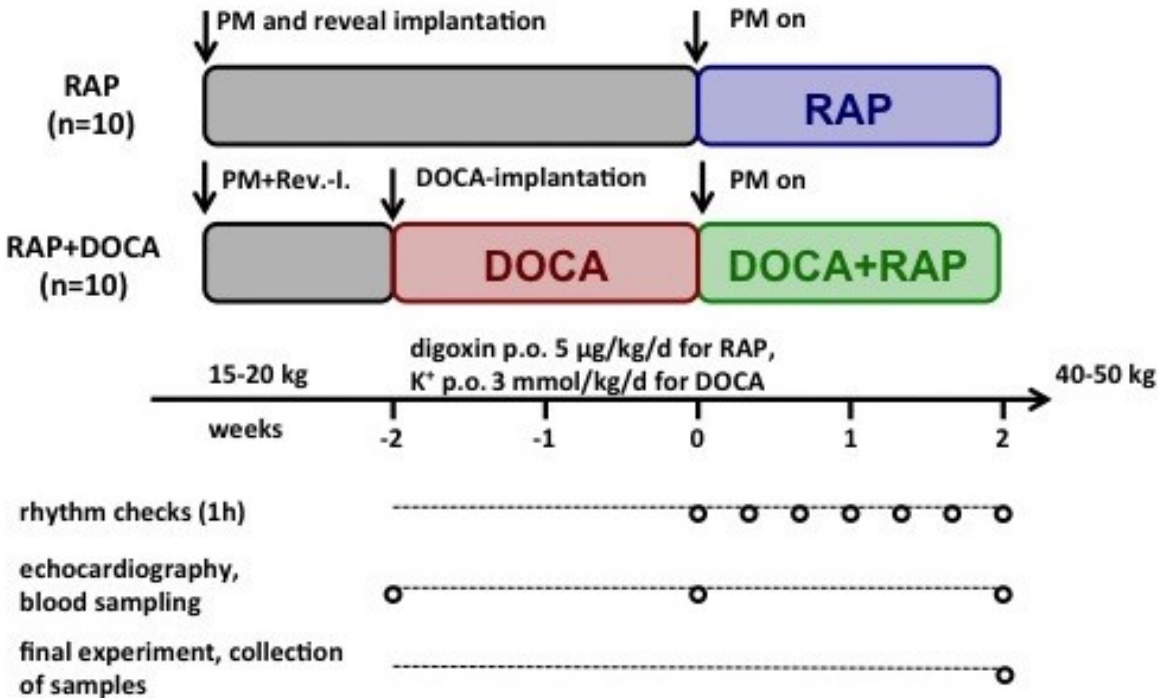


Figure 14: Experimental set-up in a time frame

## 7 Results

### 7.1 Macroscopic changes

The atrial mass was determined after the final experiment and the LA weighed  $24,859 \pm 5,625$ g in the RAP group compared to  $33,513 \pm 8,428$ g in the RAP+DOCA group. The right atrial weight was  $19,376 \pm 3,143$ g in the RAP group and  $23,7 \pm 2,914$ g in the combined group.

The left atrial diameter were assessed in the parasternal long axis and the other diameters in the parasternal short axis at the height of the papillary muscles. The diastolic septa, the posterior walls and the left atria were compared and showed a significant increase in all the above mentioned diameters. Results are summarized in detail in Table 8.



Figure 15: Parasternal long axis

The echocardiographic measured values of the left ventricle (LV) and the LA suggest increased hypertrophy and additional dilatation in the LA. The left ventricular end diastolic diameter (LVEDD) together with the EF are important surrogates for the left ventricular function. The obtained values as summarized in Table 8 do not show a significant difference between both groups. Therefore, there is no sign for a left ventricular impairment.

	RAP	RAP+DOCA	p
LA weight (g)	$24,859 \pm 5,625$	$33,513 \pm 8,428$	0,0268
RA weight (g)	$19,376 \pm 3,143$	$23,7 \pm 2,914$	0,0101
LA diameter (cm <sup>2</sup> )	$77,875 \pm 23,388$	$119,111 \pm 31,279$	0,0082
IVS syst (mm)	13,5(13;14)	18(16;20)	0,002
IVS diast. (mm)	11,5(10;12,75)	17(14,5;17,5)	0,005
PW syst. (mm)	9(8,25;9,75)	14(11;16)	<0,001
PW diast. (mm)	8(8;8)	10(9;11,5)	0,008
LVEDD (mm)	$43 \pm 4$	$40,667 \pm 5,148$	0,318
LVESD (mm)	$33,375 \pm 3,543$	$29,333 \pm 5,979$	0,116

Table 8: Summary of macroscopic changes

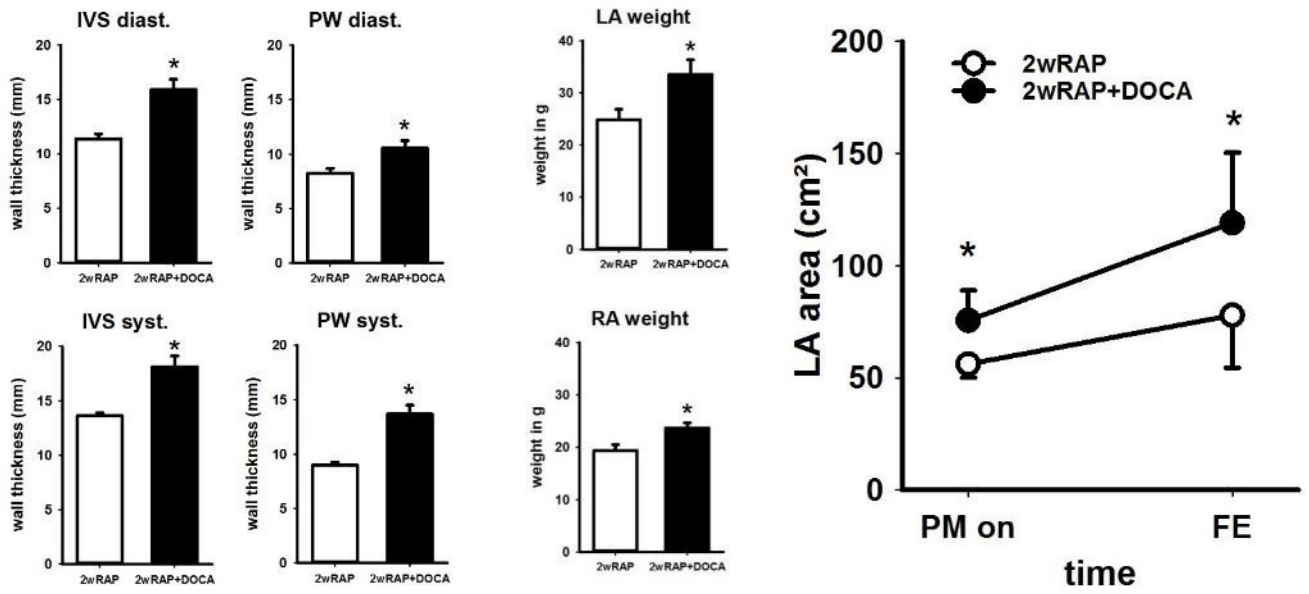


Figure 16: Parameters of the macroscopic changes

## 7.2 Cardiomyocyte hypertrophy

Samples of both atria were obtained throughout the final experiment and processed as described in Chapter 6.4 *Processing and Staining*. We took random snapshots and selected transversely cut myocytes to measure equal numbers of both cohorts. We compared the cross-sectional areas of these myocytes.

As visualized in Figure 17 the cross-sectional area of the myocytes in the left and right atria of the combined (RAP+DOCA) model show a statistically significant hypertrophy, i.e. the p-value is  $<0.01$ . The cross-sectional cardiomyocyte area of the left atria in the combined model was  $243.7 \pm 41.8 \mu\text{m}^2$  compared to  $174.4 \pm 36.0 \mu\text{m}^2$  of the RAP only model. The right atria of the combined group were  $271.6 \mu\text{m}^2$  (232,326) compared to  $186.8 \mu\text{m}^2$  (169,202) of the other group.

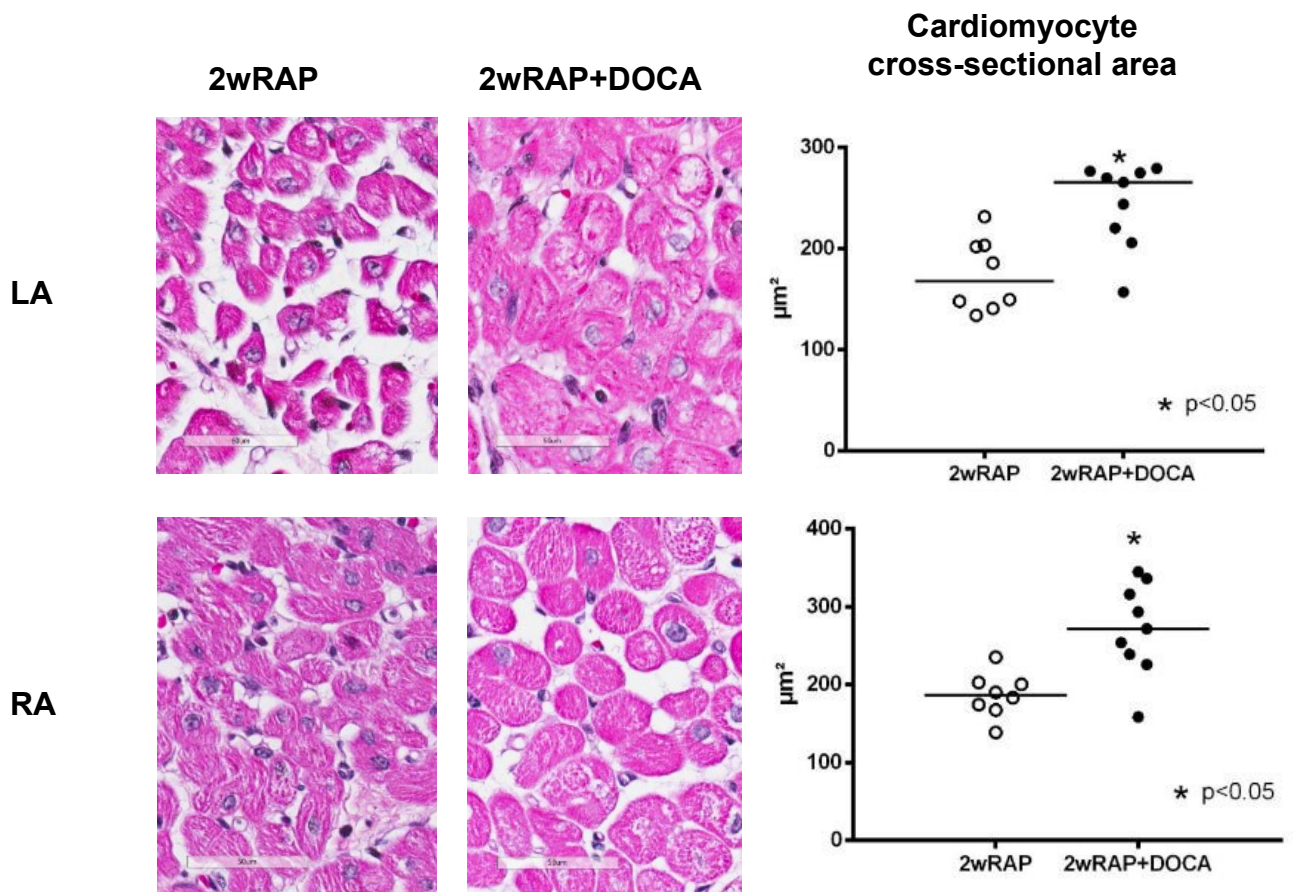


Figure 17: HE stained samples of the left and right atria

### 7.3 Interstitial fibrosis

Only after 2 weeks of RAP and implantation of DOCA pellets the cohort with the combined treatment showed impressively increased collagen contents. We could visualize the interstitial fibrosis with Picosirius-Red staining and quantify it afterwards. The collagen content in the combined model was  $14.0 \pm 2.2\%$  in the LA compared to  $8.5 \pm 1.6\%$  and  $14.4 \pm 3.4$  vs.  $8.3 \pm 1.5\%$  in the right atria as illustrated in Figure 18 below. The p-value was  $<0.001$  in the comparison of the left as well as the right atria of the different groups and indicates a statistically significant difference.

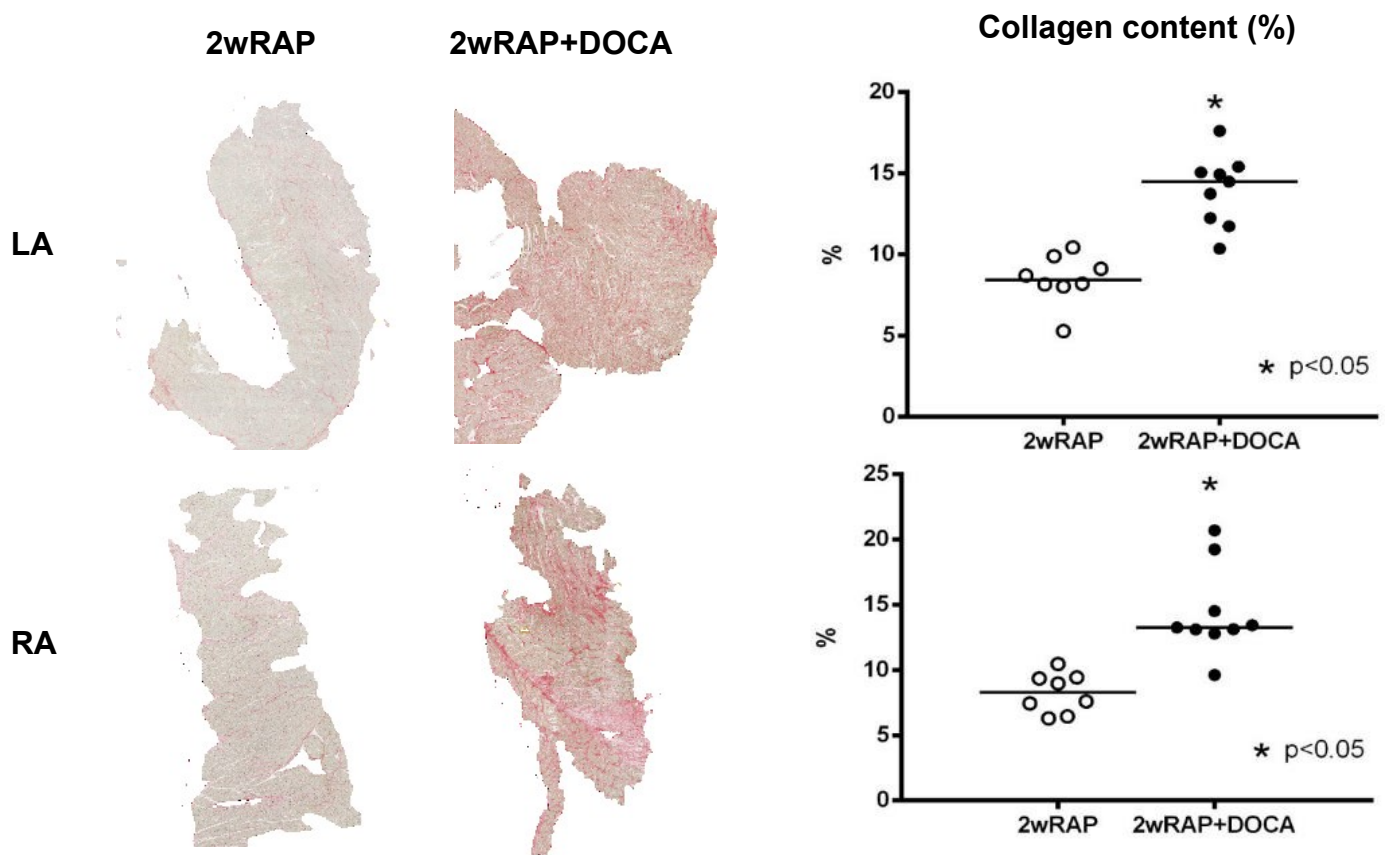


Figure 18: Picosirius-Red stained left and right atria

## 7.4 Distribution of connexin 43

We compared the distribution of Cx43 with immunofluorescence under confocal electron microscopy. The ratio between Cx43 located on longitudinal sides and at intercalated disks in the left atria (RAP  $0,3734 \pm 0,03779$  vs RAP+DOCA  $0,3854 \pm 0,01757$ ) and the right atria (RAP  $0,4569 \pm 0,04119$  vs RAP+DOCA  $0,3730 \pm 0,01883$ ) remained unchanged as shown in Figure 19 below. The p-values are 0,76 comparing the LA of both groups and 0,0733 for the RA and so these results do not show any significant difference between the groups.

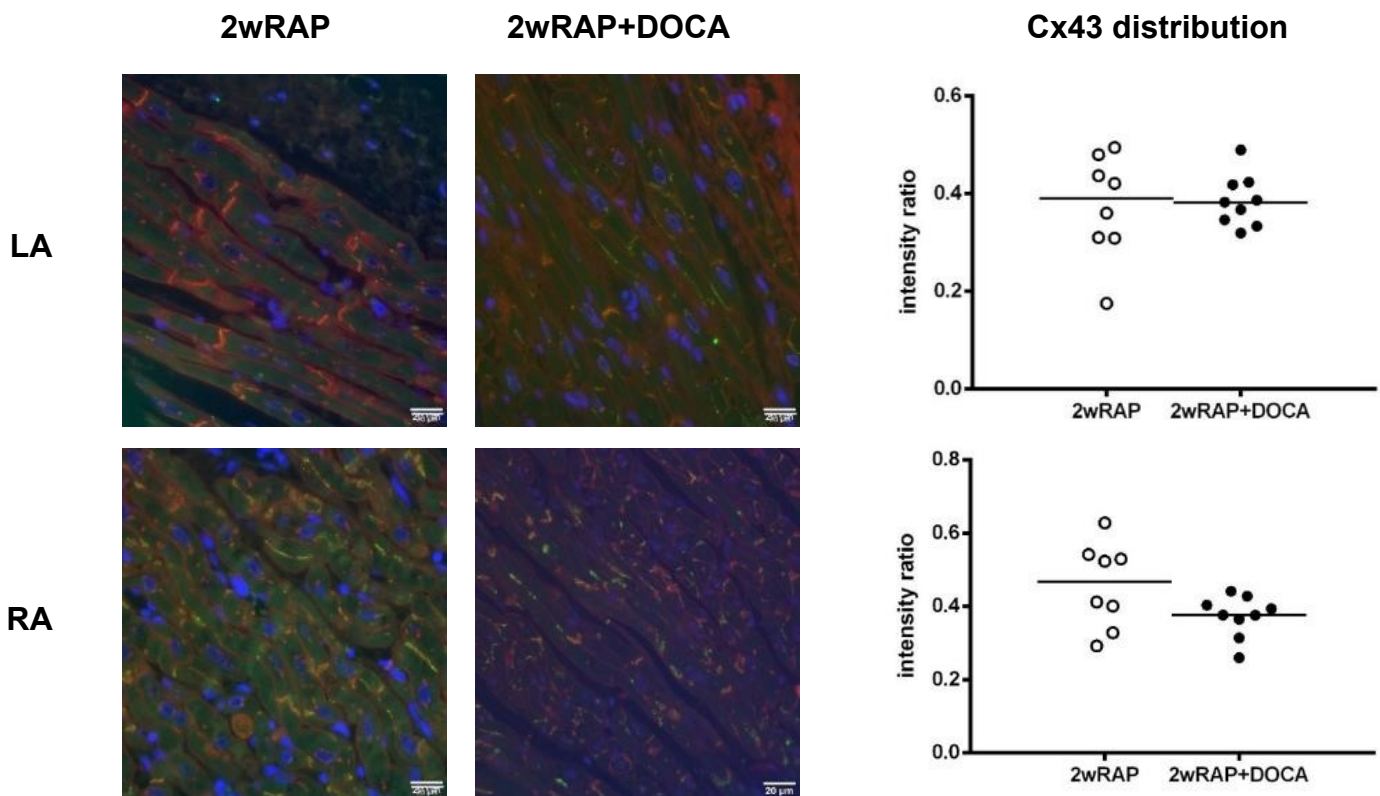


Figure 19: Distribution of connexin visualized with immunofluorescence

## 8 Discussion

The aim of this experiment was to reveal risk-factor dependent pathways helping to develop a structural substrate for arrhythmias and especially atrial fibrillation in hypertensive pigs. These pathways seemed to support increased stability and chronicity of AF.

The results as described in chapter 7 show a massive structural remodelling of the combined group compared to the group with RAP only after only two weeks of atrial fibrillation.

	RAP	RAP+DOCA	p
<b>LA weight (g)</b>	24,859±5,625	33,513±8,428	0,0268
<b>RA weight (g)</b>	19,376±3,143	23,7±2,914	0,0101
<b>LA diameter (cm<sup>2</sup>)</b>	77,875±23,388	119,111±31,279	0,0082
<b>LVEDD (mm)</b>	43±4	40,667±5,148	0,318
<b>LVESD (mm)</b>	33,375±3,543	29,333±5,979	0,116
<b>IVS syst (mm)</b>	13,5(13;14)	18(16;20)	0,002
<b>IVS diast. (mm)</b>	11,5(10;12,75)	17(14,5;17,5)	0,005
<b>PW syst. (mm)</b>	9(8,25;9,75)	14(11;16)	<0,001
<b>PW diast. (mm)</b>	8(8;8)	10(9;11,5)	0,008
<b>cardiomyocyte cross-sectional area LA (µm<sup>2</sup>)</b>	174,4±36,0	243,7±41,8	<0,01
<b>cardiomyocyte cross-sectional area RA (µm<sup>2</sup>)</b>	186,8(169;202)	271,6(232;326)	<0,01
<b>collagen content LA (%)</b>	8,5±1,6	14,0±2,2	<0,001
<b>collagen content RA (%)</b>	8,3±1,5	14,4±3,4	<0,001
<b>Cx43 distribution LA</b>	0,3734 ± 0,03779	0,3854 ± 0,01757	0,76
<b>Cx43 distribution RA</b>	0,4569 ± 0,04119	0,3730 ± 0,01883	0,0733

*Table 9: Summary of findings*

The high blood pressure levels have a synergistic effect on these changes as the DOCA group shows a much faster development of LV hypertrophy as well as left atrial hypertrophy and dilatation that could be already seen during the echocardiographic examinations. In this model, we could recreate specific forms of remodelling like left ventricular hypertrophy and left atrial size, which were linked to AF progression in earlier studies.(37)

The histological samples taken after the final experiment underline these macroscopic changes: we could observe cardiomyocyte hypertrophy and interstitial

fibrosis. Again, in the DOCA these structural alteration occurred earlier than in the RAP only group.

The distribution of connexin 43 shows very diverse results in different papers as discussed in chapter 5.1.5 and in this experiment, there is no change between both groups that may demonstrate a significant effect as shown in Table 9.

This experiment excludes all other diseases and conditions leading to specific changes having proven adverse effects on the findings such as any kind of valve disease that accelerate changes of the atrial size. The combination of different risk factors and their interacting influences on structural changes may need further investigations to study their impact.

Moreover, it is difficult to prove these results in large random controlled clinical studies, as there are few patients with AF and HT as an isolated risk factor. It is difficult to quantify adverse effects and interactions of other comorbidities and it is almost impossible to exclude them in clinical settings. The porcine model is very precise and highly reproducible but the results obtained in an animal model may not be 100 % transferable to the human species and its pathologies. Moreover, the induced hypertension is a secondary hypertension and the influence may differ compared to primary hypertension.

Nevertheless, these findings will help to understand mechanisms favouring the progression of atrial fibrillation in patients with arterial hypertension and emphasize the importance of blood pressure control in patients with AF as well as in patients without AF.

All these results show a risk-factor dependent form of structural remodelling that can be set in correlation with an arrhythmogenic substrate. The strict and early BP control could help to prevent the development of a substrate for this arrhythmia.

## Bibliography

- (1) Robertson JO, Saint LL, Leidenfrost JE, Damiano RJ, Jr. Illustrated techniques for performing the Cox-Maze IV procedure through a right mini-thoracotomy. *Ann Cardiothorac Surg* 2014 Jan;3(1):105-116.
- (2) Schmitt C, Deisenhofer I, Zrenner B. *Catheter Ablation of Cardiac Arrhythmias*. 2006.
- (3) Schotten U, Verheule S, Kirchhof P, Goette A. Pathophysiological mechanisms of atrial fibrillation: a translational appraisal. *Physiol Rev* 2011 Jan;91(1):265-325.
- (4) Anatomy and physiology of the right atrium. Available at: <https://123sonography.com/node/871>. Accessed 06/13/2016, 2016.
- (5) Schmitt N, Grunnet M, Olesen SP. Cardiac potassium channel subtypes: new roles in repolarization and arrhythmia. *Physiol Rev* 2014 Apr;94(2):609-653.
- (6) Robert I. Simon MD, Douglas L. Mann MD FACC, Douglas S. Fenton MD, MD PR. *Braunwald's Heart Disease: A Textbook of Cardiovascular Medicine, Single Volume: Expert Consult Premium Edition - Enhanced Online Features and Print*. : Saunders.
- (7) Erdmann E. *Klinische Kardiologie: Krankheiten des Herzens, des Kreislaufs und der herznahen Gefäße; mit 185 Tabellen; [Therapie evidenz-basiert]*. 8., vollst.überarb. und aktualisierte Aufl. ed. Heidelberg: Springer Medizin; 2011.
- (8) Lloyd-Jones DM, Wang TJ, Leip EP, Larson MG, Levy D, Vasan RS, et al. Lifetime risk for development of atrial fibrillation: the Framingham Heart Study. *Circulation* 2004 Aug 31;110(9):1042-1046.
- (9) January CT, Wann LS, Alpert JS, Calkins H, Cigarroa JE, Cleveland JC, Jr, et al. 2014 AHA/ACC/HRS guideline for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the Heart Rhythm Society. *J Am Coll Cardiol* 2014 Dec 2;64(21):e1-76.
- (10) European Heart Rhythm Association, European Association for Cardio-Thoracic Surgery, Camm AJ, Kirchhof P, Lip GY, Schotten U, et al. Guidelines for the management of atrial fibrillation: the Task Force for the Management of Atrial Fibrillation of the European Society of Cardiology (ESC). *Eur Heart J* 2010 Oct;31(19):2369-2429.
- (11) Camm AJ, Lip GY, De Caterina R, Savelieva I, Atar D, Hohnloser SH, et al. 2012 focused update of the ESC Guidelines for the management of atrial fibrillation: an update of the 2010 ESC Guidelines for the management of atrial fibrillation. Developed with the special contribution of the European Heart Rhythm Association. *Eur Heart J* 2012 Nov;33(21):2719-2747.
- (12) Pamukcu B, Lip GY, Lane DA. Simplifying stroke risk stratification in atrial fibrillation patients: implications of the CHA2DS2-VASc risk stratification scores. *Age Ageing* 2010 Sep;39(5):533-535.
- (13) Wijffels MC, Kirchhof CJ, Dorland R, Allessie MA. Atrial fibrillation begets atrial fibrillation. A study in awake chronically instrumented goats. *Circulation* 1995 Oct 1;92(7):1954-1968.

- (14) Moe GK, Abildskov JA. Atrial fibrillation as a self-sustaining arrhythmia independent of focal discharge. *Am Heart J* 1959 Jul;58(1):59-70.
- (15) Cox JL, Boineau JP, Schuessler RB, Kater KM, Lappas DG. Five-year experience with the maze procedure for atrial fibrillation. *Ann Thorac Surg* 1993 Oct;56(4):814-823; discussion 823-4.
- (16) Haissaguerre M, Jais P, Shah DC, Takahashi A, Hocini M, Quiniou G, et al. Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. *N Engl J Med* 1998 Sep 3;339(10):659-666.
- (17) Shah DC, Haissaguerre M, Jais P. Catheter ablation of pulmonary vein foci for atrial fibrillation: PV foci ablation for atrial fibrillation. *Thorac Cardiovasc Surg* 1999 Aug;47 Suppl 3:352-356.
- (18) Masani F. Node-like cells in the myocardial layer of the pulmonary vein of rats: an ultrastructural study. *J Anat* 1986 Apr;145:133-142.
- (19) Hocini M, Ho SY, Kawara T, Linnenbank AC, Potse M, Shah D, et al. Electrical conduction in canine pulmonary veins: electrophysiological and anatomic correlation. *Circulation* 2002 May 21;105(20):2442-2448.
- (20) Verheule S, Wilson EE, Arora R, Engle SK, Scott LR, Olgin JE. Tissue structure and connexin expression of canine pulmonary veins. *Cardiovasc Res* 2002 Sep;55(4):727-738.
- (21) Chou CC, Nihei M, Zhou S, Tan A, Kawase A, Macias ES, et al. Intracellular calcium dynamics and anisotropic reentry in isolated canine pulmonary veins and left atrium. *Circulation* 2005 Jun 7;111(22):2889-2897.
- (22) Perez-Lugones A, McMahon JT, Ratliff NB, Saliba WI, Schweikert RA, Marrouche NF, et al. Evidence of specialized conduction cells in human pulmonary veins of patients with atrial fibrillation. *J Cardiovasc Electrophysiol* 2003 Aug;14(8):803-809.
- (23) Morel E, Meyronet D, Thivolet-Bejuy F, Chevalier P. Identification and distribution of interstitial Cajal cells in human pulmonary veins. *Heart Rhythm* 2008 Jul;5(7):1063-1067.
- (24) Hinescu ME, Gherghiceanu M, Mandache E, Ciontea SM, Popescu LM. Interstitial Cajal-like cells (ICLC) in atrial myocardium: ultrastructural and immunohistochemical characterization. *J Cell Mol Med* 2006 Jan-Mar;10(1):243-257.
- (25) Waks JW, Josephson ME. Mechanisms of Atrial Fibrillation - Reentry, Rotors and Reality. *Arrhythm Electrophysiol Rev* 2014 Aug;3(2):90-100.
- (26) Allesie MA, Bonke FI, Schopman FJ. Circus movement in rabbit atrial muscle as a mechanism of tachycardia. III. The "leading circle" concept: a new model of circus movement in cardiac tissue without the involvement of an anatomical obstacle. *Circ Res* 1977 Jul;41(1):9-18.
- (27) Mechanisms of atrial fibrillation. Available at:  
<http://www.uptodate.com/contents/mechanisms-of-atrial-fibrillation?topicKey=CARD/16402&elapsedTimeMs=0&source=machineLearning&searchTerm=rapid+atrial+pacing&selectedTitle=9~150&view=print&displayedView=full&anchor=H11423999>. Accessed 5/4/2016, 2016.

- (28) Epidemiology of and risk factors for atrial fibrillation. Available at: [http://www.uptodate.com/contents/epidemiology-of-and-risk-factors-for-atrial-fibrillation?source=see\\_link#H23733690](http://www.uptodate.com/contents/epidemiology-of-and-risk-factors-for-atrial-fibrillation?source=see_link#H23733690). Accessed 4/28/2016, 2016.
- (29) Herold G. Innere Medizin 2016 - eine vorlesungsorientierte Darstellung. Köln: Gerd Herold und seine Mitarbeiter; 2016.
- (30) ESH/ESC Task Force for the Management of Arterial Hypertension. 2013 Practice guidelines for the management of arterial hypertension of the European Society of Hypertension (ESH) and the European Society of Cardiology (ESC): ESH/ESC Task Force for the Management of Arterial Hypertension. *J Hypertens* 2013 Oct;31(10):1925-1938.
- (31) Overview of Hypertension in Adults. Available at: <http://www.uptodate.com/contents/overview-of-hypertension-in-adults>. Accessed 09/18/2015, 2015.
- (32) Lau DH, Mackenzie L, Kelly DJ, Psaltis PJ, Worthington M, Rajendram A, et al. Short-term hypertension is associated with the development of atrial fibrillation substrate: a study in an ovine hypertensive model. *Heart Rhythm* 2010 Mar;7(3):396-404.
- (33) Choisy SC, Arberry LA, Hancox JC, James AF. Increased susceptibility to atrial tachyarrhythmia in spontaneously hypertensive rat hearts. *Hypertension* 2007 Mar;49(3):498-505.
- (34) Kirchhof P, Eckardt L, Franz MR, Monnig G, Loh P, Wedekind H, et al. Prolonged atrial action potential durations and polymorphic atrial tachyarrhythmias in patients with long QT syndrome. *J Cardiovasc Electrophysiol* 2003 Oct;14(10):1027-1033.
- (35) Hanna N, Cardin S, Leung TK, Nattel S. Differences in atrial versus ventricular remodeling in dogs with ventricular tachypacing-induced congestive heart failure. *Cardiovasc Res* 2004 Aug 1;63(2):236-244.
- (36) Henry WL, Morganroth J, Pearlman AS, Clark CE, Redwood DR, Itscoitz SB, et al. Relation between echocardiographically determined left atrial size and atrial fibrillation. *Circulation* 1976 Feb;53(2):273-279.
- (37) Sanfilippo AJ, Abascal VM, Sheehan M, Oertel LB, Harrigan P, Hughes RA, et al. Atrial enlargement as a consequence of atrial fibrillation. A prospective echocardiographic study. *Circulation* 1990;82(3):792-797.
- (38) Wozakowska-Kaplon B. Changes in left atrial size in patients with persistent atrial fibrillation: a prospective echocardiographic study with a 5-year follow-up period. *Int J Cardiol* 2005 May 11;101(1):47-52.
- (39) Thijssen VL, Ausma J, Liu GS, Allessie MA, van Eys GJ, Borgers M. Structural changes of atrial myocardium during chronic atrial fibrillation. *Cardiovasc Pathol* 2000 Jan-Feb;9(1):17-28.
- (40) Ausma J, Wijffels M, Thone F, Wouters L, Allessie M, Borgers M. Structural changes of atrial myocardium due to sustained atrial fibrillation in the goat. *Circulation* 1997 Nov 4;96(9):3157-3163.

- (41) Ausma J, van der Velden HM, Lenders MH, van Ankeren EP, Jongsma HJ, Ramaekers FC, et al. Reverse structural and gap-junctional remodeling after prolonged atrial fibrillation in the goat. *Circulation* 2003 Apr 22;107(15):2051-2058.
- (42) Avitall B, Bi J, Mykitysey A, Chicos A. Atrial and ventricular fibrosis induced by atrial fibrillation: evidence to support early rhythm control. *Heart Rhythm* 2008 Jun;5(6):839-845.
- (43) Anne W, Willems R, Holemans P, Beckers F, Roskams T, Lenaerts I, et al. Self-terminating AF depends on electrical remodeling while persistent AF depends on additional structural changes in a rapid atrially paced sheep model. *J Mol Cell Cardiol* 2007 Aug;43(2):148-158.
- (44) Bischofberger J, Behrends JC, Ehmke H, et al. *Duale Reihe Physiologie. 2.aktualisierte Auflage* ed.: Georg Thieme Verlag KG; 2012.
- (45) Severs NJ, Bruce AF, Dupont E, Rothery S. Remodelling of gap junctions and connexin expression in diseased myocardium. *Cardiovasc Res* 2008;80(1):9 19.
- (46) Gros D. Genetically modified mice: tools to decode the functions of connexins in the heart—new models for cardiovascular research. *Cardiovasc Res* 2004;62(2):299 308.
- (47) Grekin RJ, Terris JM, Bohr DF. Electrolyte and hormonal effects of deoxycorticosterone acetate in young pigs. *Hypertension* 1980;2(3):326 332.
- (48) Hilbert S, Kosiuk J, John S, Hindricks G, Bollmann A. A guide to the porcine anatomy for the interventional electrophysiologist. Fluoroscopy and high density electroanatomical mapping. *J Cardiovasc Transl Res* 2015 Feb;8(1):67-75.
- (49) Morillo CA, Klein GJ, Jones DL, Guiraudon CM. Chronic rapid atrial pacing. Structural, functional, and electrophysiological characteristics of a new model of sustained atrial fibrillation. 1995;91:1588-1595.
- (50) Gross D. *Animal Models in Cardiovascular Research*. 3rd ed.: Springer-Verlag New York; 2009.
- (51) Fischer AH, Jacobson KA, Rose J, Zeller R. Hematoxylin and eosin staining of tissue and cell sections. *CSH Protoc* 2008 May 1;2008:pdb.prot4986.

## Annex

### Cutting with the rotation-microtome

- Set the cooling plate to -14°C
- Put the paraffin embedded tissue on the cooling plate for ~ 30 minutes
- Set the heating plate and the water bath to 45°C (optionally increase the temperature to 50 °C)
- Cool the holder of the paraffin embedded tissue
- Cut slices with a thickness of ~ 3 µm
- Take the slices with a brush and put them first in the cold water bath and then in the hot water bath
- Take them out of the water bath and put them on the slides
- Put the slides on the heating plate for drying
- Prior to staining put the slides in the incubator (70°C) for 30 minutes

### Staining solutions

<b>0,2% Phosphomolybdic Acid</b>	10 ml 20 % Phosphomolybdic Acid in EOH + 990 ml Aqua Dest.
<b>0,01 N HCL</b>	2 ml 5 M HCL + 998 ml Aqua Dest.
<b>Sirius Red, 0,1 % Picric Acid</b>	100 ml Sirius Red Solution 900 ml Picric Acid + add some (2-3 small spoons) Picric Acid Powder for the saturation
<b>1 % Sirius Red Solution Stock</b>	1g Sirius Red Powder 100 ml Aqua Dest.

## HE staining

Färbeautomat DRS2000 - Programm: H&E (Oridis)

Step	Station	Lösung	Genauigkeit	Zeit	Mix	Spülen
1	S*	Start Station				
2	25	Xylol	∞	2 min	+	
3	24	Xylol	∞	2 min	+	
4	23	EtOH abs/Xylol	∞	2 min	+	
5	5	EtOH abs	Δ	2 min	+	
6	6	EtOH abs	Δ	2 min	+	
7	3	Alk:70%	Δ	2 min		
8	2	Alk:50%	Δ	2 min		
9	7	A.d.	●	2 min	+	
10	9	Hämalaun	●	1 min	+	
11	Wash	Wasser	●	1 min	+	
12	Wash	Wasser	●	1 min	+	
13	20	Eosin-Phloxin	●	2 min	+	
14	7	A.d.	●	2 min		
15	2	Alk:50%	Δ	1 min		
16	3	Alk:70%	Δ	1 min		
17	19	EtOH abs	Δ	2 min	+	
18	18	EtOH abs	Δ	2 min	+	
19	17	EtOH abs/Xylol	Δ	2 min	+	
20	16	Xylol	∞	2 min	+	
21	E*	End				

## Configuration of the solutions

1	2	3	4	5	6	7	8	9	10	11	12	13		
14	15	16	17	18	19	20	21	22	23	24	25	26	27	
E1	E2	Xylol	Alk/Xylol	Alk 100%	Alk 100%	Aqua dest Eosin-Phloxin	Gill II W	Hämalaun W	Alk/Xylol	Xylol I	Xylol	S1	S2	

## Picrosirius-Red staining

Staining protocol for paraffin embedded slices:

- Deparaffinize and hydrate to distilled water:
  - Xylol: 2x (6 min)
  - 100 % Ethanol: 2x (3 min)
  - 75 % Ethanol: 1x (3 min)
- Running distilled water (10 min)
- Rinse in distilled water
- Treat in Phosphomolybdic Acid 0.2 % Aqueous for 4 minutes (1-5 min)
- Stain in Sirius Red (Direct Red 80, Sigma Cat. No. 365548 or 43665), 0.1 % Picric Acid for 70 minutes (60-90 min)
- Prepare 0,5 g Sirius Red in 500 ml saturated aqueous solution of Picric Acid (add some Picric Acid powder to assure full saturation-crystals)
- Wash for 2 min in Acetic Acid (5 ml in 1 liter of distilled water) for 3 min or alternatively for 2 min in 0.01 N HCl
- Rinse in:
  - 100 % Ethanol: 3x (3 min)
  - Xylol 1x (3 min)
- Mount slides

## Cx43 Immunfluorescence

- **Deparaffinize:**

Dry 15 to 30 min in incubator (60°C)

Xylol	10 min
100% EtOH	3 min
90% EtOH	3 min
70% EtOH	3 min
50% EtOH	3 min
Aqua dest	3 min

- **Antigen retrieval**

Fill cuvette with citrat-Na Pf. pH6

Fill pressure cooker with 500 ml Aqua Dest.

Put cuvette in the middle and close the cooker

Press display set button

**SP 1** 120°C

**SP 2** 15 min

**SP 3** 85°C                    cooling down to this temperature

**SP 4** 14 sec                how long it holds the lower temperature- signal sounds

**SP 5** 5°C                    shows max. temperature deviation

**SP 6** zb. 23°C            shows current temperature

Press start/stop

Wait until the signal sounds – press Start/Stop

It cools down – will take ~30 min. – then the signal sounds again – press

Start/Stop – switch off

Cool down slices for 30 min at room temperature, rinse thoroughly with PBS

- **Block with 1% BSA**

ca. 100 µl 1% BSA for a minimum of 10 min

3 x 5 min PBS

- **1. antibodies Cx43**

ca. 100 µl incubate overnight/ 4°C

Cx43 1/100 in 1% BSA

3 x 5 min PBS

- **2. Antibodies**

1/1000 (dilute in PBS) work protected from light

ca. 100 µl incubate 45 min / RT

3 x 5 min PBS

- **Staining of the cell membrane**

Ca. 100µl WGA 1:3000/10 min (in PBS)

3 x 5 min PBS

- **Nuclear staining**

ca. 100 µl DAPI 1/200; 5 min /RT work protected from light (in PBS)

wash with PBS

- **Embedding**

Embed with fluorescence mounting medium

Let dry and close the edges of the cover glass with transparent nail polish