Targeting proteostasis in atrial fibrillation

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Chapter 1

General introduction and Aim of the thesis

GENERAL INTRODUCTION

1. Atrial fibrillation, clinical aspects

Atrial fibrillation (AF) is the most common clinical tachyarrhythmia accounting for approximately one-third of hospitalizations for cardiac rhythm disturbances with an annual cost of 13 billion euro in the European Union.[1] Its incidence is age-related and growing alarmingly in the ageing population. With the present trend, more than 30 million North Americans and Europeans will be affected with AF by 2050.[1] AF can be caused by underlying cardiovascular conditions, including hypertension, cardiac surgery, pericarditis, congestive heart failure, coronary heart disease, congenital heart disease, pneumonia or other acute pulmonary diseases.[2, 3] In about 30% of AF patients, clinical signs of underlying heart diseases are absent, and these patients are referred to as having 'lone' AF. Recent studies showed that additional risk factors, such as alcohol abuse, obesity, metabolic syndrome, psychological stress or genetic factors, are linked to this group of 'lone' AF patients.[4-6]

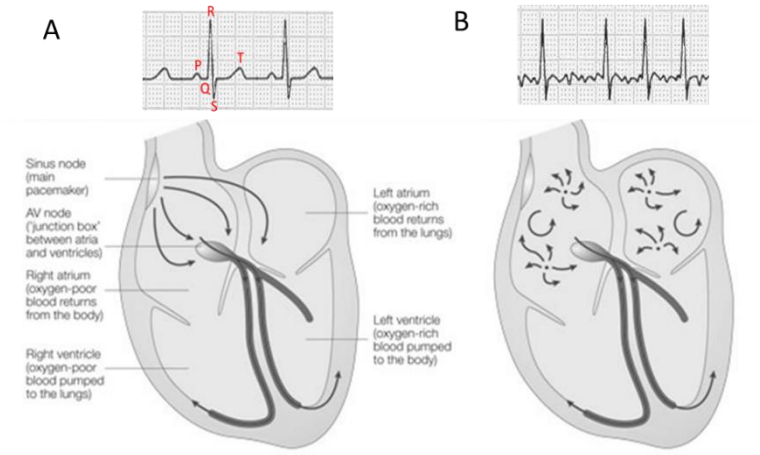

Figure 1: Electrical cardiac activation in sinus rhythm and atrial fibrillation. A) In sinus rhythm (SR), the electrical impulse emanates from the sinus node, causing stimulation (P wave on electrocardiogram (ECG)) and contraction in the atria. This impulse slows as it passes through the atrioventricular (AV) node (generating the PR interval) before reaching the ventricles via rapidly conducting specialized tissue. Ventricular activation provides for coordinated contraction of the ventricles and is visible on the ECG as the QRS complex. B) During AF, coordinated electrical and mechanical activity is replaced by multiple reentrant electrical wavelets and absence of effective contraction of the atria. On the ECG, organized P waves are absent, and the QRS complexes are irregularly spaced. Figures adapted from Page et al. Nat Rev Drug Discov. 2005.[7]

In the healthy heart, the normal electrical impulses are generated by auto-rhythmic cells of the sinus node and travel through the two upper chambers (atria) of the heart to reach the ventricles via the purkinje fibers and the atrio-ventricular node (AV node). At rest, the normal impulses lead to a typical heartbeat of about 60-70 beats per minute (bpm),

which can reach up to 180-200 bpm during heavy exercise. During an episode of AF, the normal electrical impulses are overwhelmed by disorganized and much faster impulses (400-600bpm), derived from the atria and/or pulmonary veins, resulting the atria quivering or fibrillating (Figure 1). By virtue of the limited conductive capacity of the AV node, only about a third of these irregular impulses are transferred, generating a ventricular rate of about 100-200 bpm.[8] When AF persists, the likelihood to develop chronic heart failure, stroke, thromboembolism or infarction increases.[2, 8] Therefore, an important therapeutic aim is to prevent or attenuate AF induction and progression.

In AF, the irregular heartbeats can occur in episodes lasting from several minutes to weeks, till months to years. In many cases, AF is asymptomatic and the arrhythmia is only discovered during a routine physical examination. In other patients, AF is diagnosed by the clinician because of symptoms related to a rapid heartbeat, like light-headedness, palpitations or chest discomfort. Occasionally, rapid and irregular heartbeats may also be perceived as angina, shortness of breath or edema.

According the 2014 AHA/ACC/HRS guidelines for the management of patients with AF, AF can be divided into 4 categories:

- **paroxysmal AF:** AF that terminates spontaneously or with intervention within 7 d of onset. Also, episodes may recur with variable frequency.
- **persistent AF:** continuous AF that is sustained >7 days.
- **long-standing persistent AF:** continuous AF >12 months.
- **permanent AF:** this term is used when the patient and clinician make a joint decision to stop further attempts to restore and/or maintain sinus rhythm. Acceptance of AF represents a therapeutic attitude on the part of the patient and clinician rather than an inherent pathophysiological attribute of AF. Also, the acceptance of AF may change as symptoms, efficacy of therapeutic interventions, and patient and clinician preferences evolve.

An important feature of AF is its natural tendency to progress towards longer and more frequent attacks. The consequence of AF progression is that many patients with paroxysmal AF develop the persistent form of the disease. AF persistence is rooted in the progressive changes in cardiomyocytes and/or their connections with each other or other cell types, which make the atria more vulnerable for the arrhythmia ('AF begets AF').[9] This progressive changes in cardiomyocytes are also known as atrial arrythmogenic remodeling.[10]

2. State of the art: molecular mechanisms underlying AF progression

Atrial arrythmogenic remodeling, defined as any change in atrial structure or function that promotes atrial arrhythmia, is central to AF progression.[9] During the past decades, various mechanisms have been identified which promote the occurrence or maintenance of AF (Figure 2).

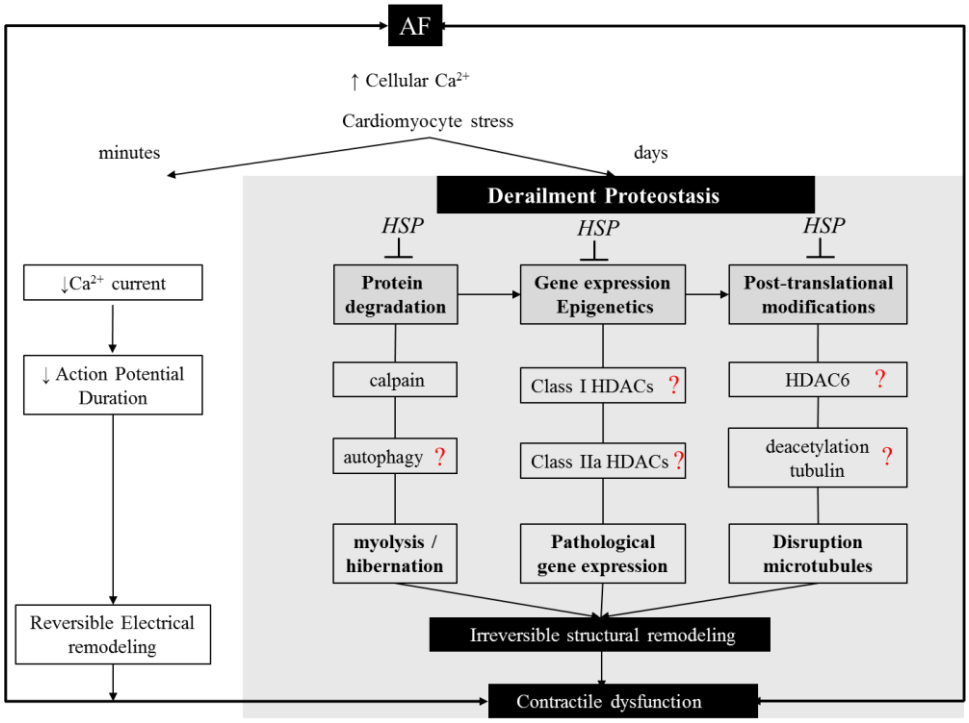

Figure 2: **Overview of AF-induced cardiomyocyte remodeling.** AF induces time-related progressive remodeling. First, AF causes a stressful cellular Ca^{2+} overload, which results in a direct inhibition of the L-type Ca^{2+} channel, shortening of action potential duration and contractile dysfunction. These changes have an early onset and are reversible.[9, 11] The early processes protect the cardiomyocyte against Ca^{2+} overload but at the expense of creating a substrate for persistent AF. When AF persists derailment of proteostasis occurs, including induction of protein degradation,[12, 13] changes in gene expression (epigenetics), post-translational modifications, and exhaustion of protective HSPs.[14-16] The key modulators also activate each other.[17] Derailment of proteostasis results in sustainable structural remodeling, myolysis/ hibernation, and consequently impaired contractile function and AF persistence.[17] Autophagy, class I/ IIa HDACs and HDAC6 may represent key modulators of structural remodeling and consequently contractile dysfunction due to their role in protein degradation[18] , regulation of pathological gene expression [19] and posttranslational modification of cytoskeletal and contractile proteins.[19]

Reversible Electrical remodeling

An important concept in AF research originated from the notion that AF, once initiated, alters atrial electrophysiological properties in a manner that favours the induction and persistence of AF.[9] During AF, atrial cardiomyocytes are subjected to very rapid (400-600 times per min) and irregular firing, causing Ca^{2+} overload, which triggers a stress

response in affected cardiomyocytes, resulting in functional down-regulation of the L-type Ca^{2+} channel.[20] In turn, this leads to shortening of the action potential duration and contractile dysfunction (hypo-contractility), thus providing a further substrate for AF (Figure 2).[11, 20] These changes have an early onset and are reversible.[11]

Innovative AF research: derailed proteostasis underlies sustainable structural remodeling

In addition to the reversible electrical remodeling, various research findings point to a key role of structural remodeling in AF progression.[12, 13, 21] Since structural changes are progressive and sustained over time they may explain why patients with longstanding persistent and permanent AF are difficult to treat.

The first study describing AF-induced alterations in the ultrastructure of atrial cardiomyocytes was the study of Morillo et al. in 1995. They utilized dogs subjected to prolonged periods of rapid atrial pacing (6 weeks).[22] Several additional studies confirmed their observations including studies in experimental dog and goat models for AF and patients with persistent AF.[21, 23, 24] The structural changes observed in atrial cardiomyocytes after sustained AF closely resemble the changes in ventricular myocytes due to chronic low flow ischemia (cardiac hibernation).[21, 23, 24] Cardiac hibernation is a form of tissue adaptation defined by the ability of cardiomyocytes to transform into a non-functional phenotype through irreversible degradation of the myofibril structure (myolysis), which leads to contractile dysfunction (Figure 3).[25] Both in chronic hibernating ventricular myocardium and in fibrillating atria, a phenotypic adaptation occurs towards a more fetal stage of development (dedifferentiation).[26] Other AF-induced structural changes include: (1) fibrosis, (2) cell hypertrophy and (3) cell death. In addition, structural changes at the subcellular level include: (1) perinuclear accumulation of glycogen, (3) changes in mitochondrial shape, (4) fragmentation of sarcoplasmic reticulum, (5) homogeneous distribution of nuclear chromatin, and (6) changes in quantity and localization of structural cellular proteins.[26] Most prominent is an increase in atrial cell size associated with myolysis (Figure 3).[26] While the early electrical remodeling is reversible[27], the structural changes are sustained and impair electrical coupling and the functional recovery to sinus rhythm by pharmacological and electrical cardioversion. In addition, it was found that alterations of the myocardial structure underlie changes in electrophysiology as observed in AF.[9, 21, 28] This phenomenon is commonly defined as electropathology. It should be noted that electropathology is already present when a patient enters the clinic for the first time with an AF episode.

Currently available pharmacological therapies are mostly directed at alleviation of electrical activity (rhythm control), but have limited effect on patient outcome.[29]

Therapeutic approaches that halt the mechanisms conveying the AF-induced structural remodeling, may be more effective and improve clinical outcomes.

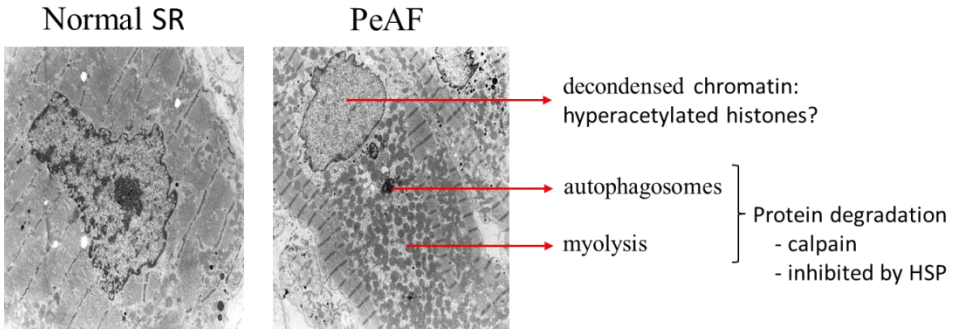

Figure 3: Electron microscopic picture revealing hibernation in patients with longstanding, permanent AF. Patients with longstanding, permanent AF (PeAF) show signs of sustained structural remodeling, including 1) pale nucleus, suggesting that the chromatin is decondensed via hyperacetylation of histones 2) autophagosomes, indicating modulation of autophagy, 3) myolysis, implying the activation of protein degradation pathways, such as calpain[12, 13] and autophagy.

Proteostasis is defined as the homeostasis of protein production, breakdown and function.[30] Proteostasis is therefore involved in controlling the concentration, conformation, binding interaction, kinetics and location of individual proteins via diverse signaling pathways, called the proteostasis network. Our research group has strong indications that a derailment of proteostasis underlies AF-induced structural remodeling.[17] By studying the structural remodeling in patients with longstanding, persistent and permanent AF, we identified four key factors that may modulate the derailment of proteostasis and therefore underlie AF progression (Figure 1, 3). These key factors include: heat shock proteins, epigenetic regulation, post-translational modifications and protein degradation by autophagy.

3. Cardioprotective heat shock proteins

Our research group was the first to demonstrate that conservation of a healthy proteostasis through overexpression of heat shock proteins (HSPs) attenuates structural remodeling in AF. We previously disclosed that some small HSPs, particularly HSPB1 (HSP27), bind to myofibrils and protect against myofibril degradation in tachypaced atrial cardiomyocytes and human AF.[14-16] Also, HSPB1, prevents structural remodeling by attenuating the conversion of G-actin to F-actin stress fibers and consequently protects against contractile dysfunction.[14] In addition, we showed that the HSP response is temporarily activated in patients with short duration of AF, but exhausts when AF persists.[15] Consequently, cardiomyocytes lose defense against structural remodeling, such as hibernation, consequently resulting in the progression of AF.

In addition to the elucidation of the cardio-protective role of HSPs in AF, we previously showed that treatment with a non-toxic HSP-inducing compound geranylgeranylacetone (GGA), protects cardiomyocytes against structural remodeling and AF progression.[14-16, 31] Based on these findings it is envisioned that boosting of specific HSP expression attenuates structural cardiomyocyte remodeling and as a result prevents AF progression.

4. HDACs: epigenetic regulation and post-translational modifications

In addition to the role of exhaustion of HSP in AF progression, also experimental and clinical studies revealed AF to induce changes in gene expression, related to dedifferentiation of the cardiomyocytes.[32-34] This suggests a role for epigenetic regulation in AF-induced structural remodeling. Epigenetic regulation affects the packaging of the chromatin of the nuclear DNA and thereby influences the on/off states of multiple genes.[19, 35] Importantly, the packaging of chromatin is largely dependent on acetylation of histones[35] representing a key switch in the regulation of gene expression. Acetylation is controlled by two separate families of enzymes: histone acetyltransferases (HATs) and deacetylases (HDACs).[35] Class II HDACs (especially HDAC4 and HDAC5) are highly expressed in the heart and are phosphorylated by CaMKII,[36, 37] resulting in the translocation of HDAC from the nucleus to the cytoplasm, thereby promoting histone acetylation and activation of transcription factors including NFAT, MEF2, GATA4 and SRF[35, 38], generally causing the activation of stress-responsive genes and structural remodeling.[19, 35]

HDACs catalyze the removal of acetyl-groups from lysine residues within nucleosomal histone tails and various non-histone proteins (Table 1). The 18 mammalian HDACs are encoded by distinct genes and are grouped into four classes on the basis of similarity to yeast transcriptional repressors. Class I HDACs (HDAC1, HDAC2, HDAC3 and HDAC8) are related to yeast RPD3, class II HDACs (HDAC4, HDAC5, HDAC6, HDAC9 and HDAC10) to yeast HDAC1, and class III HDACs (sirtuin 1–7), usually named as sirtuins, to yeast Sir2. Class II HDACs are further divided into two subclasses, IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and IIb (HDAC6 and HDAC10). HDAC11 belongs to a fourth class. Classes I, II and IV HDACs have a highly conserved zinc-dependent deacetylase domain and are referred as classical HDACs (Figure 4), which differ in structure, enzymatic function, subcellular localization and expression patterns (Figure 4, Table 1). In contrast, class III HDACs (sirtuins) utilize nicotinamide adenine dinucleotide (NAD^+) as a co-factor for catalytic activity.

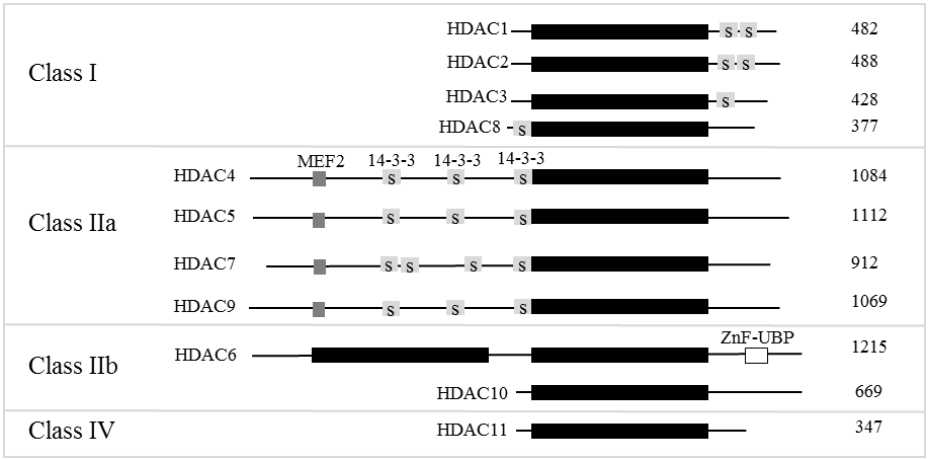

Figure 4: Overview of the classical zinc dependent histone deacetylase (HDAC) superfamily. Black rectangles indicate the conserved HDAC catalytic domain; Numbers following the HDAC domain indicate the number of amino acids in *Homo sapiens*. Myocyte enhancer factor 2 (MEF2)-binding sites and binding sites for the 14-3-3 chaperone protein sites are marked by grey squares. S represents the serine residue which can be phosphorylated by kinases. HDAC6 has a zinc-finger ubiquitin-specific protease (ZnF-UBP) domain, which can bind to ubiquitin and plays an important role in autophagy. Modified from Haberland M et al, Nat Rev Genet. 2009.[19]

Class I HDACs are expressed ubiquitously, localize predominantly to the nucleus and display high enzymatic activity towards histone substrates.[19] Cardiac deletion of all HDAC1 and HDAC2 alleles in mice results in neonatal lethality, accompanied by cardiac arrhythmias, dilated cardiomyopathy and upregulation of genes encoding skeletal muscle-specific contractile proteins and calcium channels in heart.[39] Cardiac deletion of HDAC3 in mice results in a metabolic catastrophe in the heart, with massive cardiac hypertrophy and excessive myocardial lipid accumulation.[40]

Class IIa HDACs (HDACs 4, 5, 7, 9) have several unique features. First, they have long (~ 500 amino acids) amino-terminal extensions that harbor binding sites for transcription factors and cofactors, such as myocyte enhancer factor 2 (MEF2). Secondly, they undergo signal-dependent nuclear export upon phosphorylation of two serine residues within their amino-terminal extensions.[41] Thirdly, in contrast to what the name suggests and despite having conserved catalytic domains, these proteins are unable to deacetylate histones *in vivo*.[42] Class IIa HDACs normally repress pathological cardiac gene expression. In response to stress signals, class IIa HDACs become phosphorylated and undergo nuclear export, resulting in the derepression of pathologic genes.[43] The transcription factors regulated by class IIa include NFAT, MEF2, GATA4 and SRF, stress-responsive genes such as atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), contractile protein genes (β-MHC), and ion channel genes including sodium-calcium exchanger (NCX1).[36, 44-48] All of these genes contribute to cardiomyocyte remodeling.

Table 1: HDAC characteristics

Class	Member	Subcellular localization	Tissue distribution	Substrates (partial)	Binding partners	Knockout phenotype	Ref.
I	HDAC1	nucleus	ubiquitous	Androgen receptor, p53, MyoD, STAT3		Embryonic lethal, proliferation defects	39, 54, 55
	HDAC2	nucleus	ubiquitous	YY1, BCL6, STAT3		Postnatal lethal, cardiac defect	39, 55, 56
	HDAC3	nucleus	ubiquitous	YY1, GATA1, STAT3, MEF2D		Embryonic lethal, gastrulation defects	55, 57, 58
	HDAC8	nucleus/cytoplasm	ubiquitous	–	EST1B	Postnatal lethal, Cranial defects	55, 59
IIa	HDAC4	nucleus / cytoplasm	heart, skeletal muscle, brain	GATA1, HP1,NFAT	MEF2, RUNX2	Defects in chondrocyte differentiation	55, 60, 61
	HDAC5	nucleus / cytoplasm	heart, skeletal muscle, brain	SMAD7, HP1, SRF,NKX2.5	MEF2,myocardin	Cardiac defect	55, 61-63
	HDAC7	nucleus / cytoplasm	pancreas, placenta, heart, skeletal muscle	PLAG1, PLAG2	MEF2, HIF1A, BCL6	Endothelial dysfunction	55, 61, 64
	HDAC9	Nucleus / cytoplasm	heart, skeletal muscle, brain	–	MEF2, FOXP3	Cardiac defect	55, 61, 65, 66
IIb	HDAC6	cytoplasm	heart, liver, kidney, placenta	α-Tubulin, HSP90, SMAD7	RUNX2, mDia2 P97/VCP	Increased tubulin acetylation	55, 66-68
	HDAC10	cytoplasm	liver, spleen, kidney	–		–	55
V	HDAC11	nucleus / cytoplasm	brain, heart, skeletal muscle, kidney	–			55

HDAC6 and HDAC10 form the class IIb family. HDAC6 is the main cytoplasmic deacetylase in mammalian cells, whereas little is known about the functions of HDAC10.[49] Among the targets which are directly deacetylated by HDAC6, reside cytoskeletal proteins such as α-tubulin and cortactin [49], but also chaperones[50] which play an important role in AF protection.[16, 51-53]

Small molecule inhibitors of HDACs are efficacious in multiple pre-clinical models of pressure overload and ischemic cardiomyopathy, reducing pathological hypertrophy and fibrosis, and improving contractile function.[43] Emerging data have revealed various mechanisms by which HDAC inhibitors benefit the heart, including the suppression of oxidative stress and inflammation, inhibition of MAP kinase signaling, enhancement of cardiac protein aggregate clearance and increased autophagic flux.[43] Recently, Class I HDAC inhibition was found to suppress angiotensin II-dependent cardiac fibrosis by

blocking cardiac fibroblasts in the G0/G1 phase of the cell cycle.[77] However, the involvement of HDACs in AF is not clear.

5. Protein degradation by autophagy

Our recent research described a role for persistent activation of proteases, especially calpain, which results in degradation of contractile and structural proteins.[12, 13, 69] In addition to protease activation, activation of auxiliary cellular protein degradation pathways, such as autophagy may play an important role in AF progression. Autophagy is an evolutionarily conserved cellular degradation pathway to maintain cell proteostasis by removing damaged or long-lived proteins and organelles. Controlled autophagy during (mild) cardiac stress conditions, such as nutrient deprivation, hypoxia, and oxidative stress, supports cardiomyocyte survival. In contrast, stress-induced excessive activation of autophagy causes derailment of cell proteostasis by degradation of essential proteins and organelles and thereby triggers autophagic cell damage and death as found in mitral valve regurgitation [18, 70] and cardiac hypertrophy.[71] The contribution of autophagy to the initiation and progression of AF has not yet been investigated.

6. Novel experimental models to study AF

A versatile model system is crucial to study the mechanisms underlying AF-related cardiomyocyte remodeling. Recently, we developed an *in vitro* tachypaced HL-1 atrial cardiomyocyte model, to obtain insights into the mechanisms underlying AF progression and development.[51] Over the past decades, several animal models such as dog, goat, rabbit, and sheep have been extensively used to explore underlying mechanisms and potential treatment of AF.[72,73] Due to the low speed, high costs, and limited availability of approaches to genetically manipulate the animals, these (large) *in vivo* models are not convenient for the dissection of molecular pathways underlying AF progression and compound screening.

Drosophila melanogaster is one of the most popular invertebrate model organisms and has been used extensively in many areas of biological research, especially genetics and development. The use of this model is supported by the existence of functionally conserved features between *Drosophila* and humans, including cardiac aging and development of heart failure.[74,75] These features, combined with the short life-cycle, its cost efficiency and the powerful techniques for genetic and molecular manipulations, make the *Drosophila* system highly suitable for elucidation of molecular mechanisms involved in heart diseases and (high-throughput) compound testing.[76] Based on these considerations, we hypothesized that *Drosophila* can be exploited to study tachycardia remodeling related to AF.

In summary, our previous research findings indicate that the derailment of proteostasis causes sustainable structural remodeling. Various pathways of cardiomyocyte proteostasis are involved, including HSP exhaustion, post-translational modifications of proteins, changes in gene expression by epigenetics and protein degradation via autophagy. By exploiting experimental model systems of AF, including the tachypaced HL-1 cardiomyocyte and *Drosophila* model, we may identify whether key modulators of these pathways represent druggable targets to prevent AF progression.

AIM OF THIS THESIS

The main goal of this thesis is to elucidate if key modulators of proteostasis are derailed in AF and whether these key modulators represent druggable targets to attenuate AF initiation and progression. In **chapter 2**, we present an overview of studies indicating that upregulation of the heat shock protein expression represents a novel therapeutic target to prevent derailment of proteostasis and consequently progression and recurrence of AF. In **chapter 3**, we describe a tachypaced *Drosophila* model to study AF-related remodeling. We verified this model by investigating the efficacy of HSP-inducing compounds, and specific overexpression of individual small HSPs in the heart, to restore AF-induced alterations in contractile function. To explore the role of HDACs in AF, we first screened broad HDAC inhibitors (TSA, sodium butyrate, nicotinamide) and a specific HDAC6 inhibitor (tubacin) in tachypaced HL-1 cardiomyocytes and *Drosophila* and found tubacin and nicotinamide to protect against AF progression in both models, as described in **chapter 4**. We continued to elucidate the protective mechanisms of HDAC6 inhibition in AF by examining the impact of HDAC6 inhibition on the microtubule network. Since we observed in **chapter 4** that the protective effect of nicotinamide is independent of sirtuin inhibition, we studied in **chapter 5** the molecular mechanisms underlying the protective effect of nicotinamide, by examining its role in PARP inhibition in tachypaced HL-1 cardiomyocytes and *Drosophila*. In **chapter 6**, we explored the involvement of class I HDACs and class IIa HDACs in AF by using retroviral transfection of HL-1 cardiomyocytes with constructs of class I HDACs (HDAC1, HDAC3) and class IIa HDACs (HDAC4, HDAC5, HDAC7 and HDAC9). The involvement of autophagy in AF progression is described in **chapter 7** by testing various inhibitors of the autophagy pathway in tachypaced HL-1 cardiomyocytes and *Drosophila*, as well as autophagy markers in AF patients. Lastly, in **chapter 8**, we summarize and discuss the data obtained in our experimental chapters and provide future perspectives.

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