Modulators of proteostasis: therapeutic targets and diagnostic markers to halt and reverse atrial fibrillation

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Chapter 1

General introduction and scope of the thesis

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The most common tachyarrhythmia: atrial fibrillation

Atrial fibrillation (AF) is the most common age-related cardiac arrhythmia accounting for one-third of hospitalizations related to cardiac rhythm disturbances [1]. AF is present in 3% of the total population, with a higher prevalence in the elderly, and is associated with serious complications such as stroke, heart failure, impaired cognitive function, and increased mortality [2-4]. To understand the pathophysiology underlying AF, we first need to understand the physiology of the normal heart.

The physiology of the heart

In a healthy heart, electrical impulses are generated by pacemaker cells in the sinus / sinoatrial node (SA node), near the vena cava superior (Figure 1A). Although other cardiac cells have pacemaker capacities, the pacemaker cells in the sinus node are the fastest to be depolarized, setting the pace for both the atria and the ventricles of the heart. At rest, the SA node provides a regular rhythm of approximately 60-70 beats per minute (bpm) [5].

Pacemaker cells are auto-rhythmic cells, having an instable membrane potential. Pacemaker cells are smaller than the contractile cells and contain a limited amount of contractile fibers. As their function is to electrically stimulate neighboring contractile cells, i.e. cardiomyocytes, they have no contractile function [5]. Cardiomyocytes are muscle cells with contractile fibers organized into sarcomeres (Figure 1B). As cardiomyocyte contraction is an energy consuming process, cardiomyocytes contain for about one-third of their volume mitochondria for energy production [6]. Cardiomyocytes are connected to their neighboring cells trough intercalated disks which consists of desmosomes, to tie the cardiomyocytes together, and gap junctions, which electrically connect the cardiomyocytes to one another. As such, electrical waves can spread rapidly from cell to cell [5].

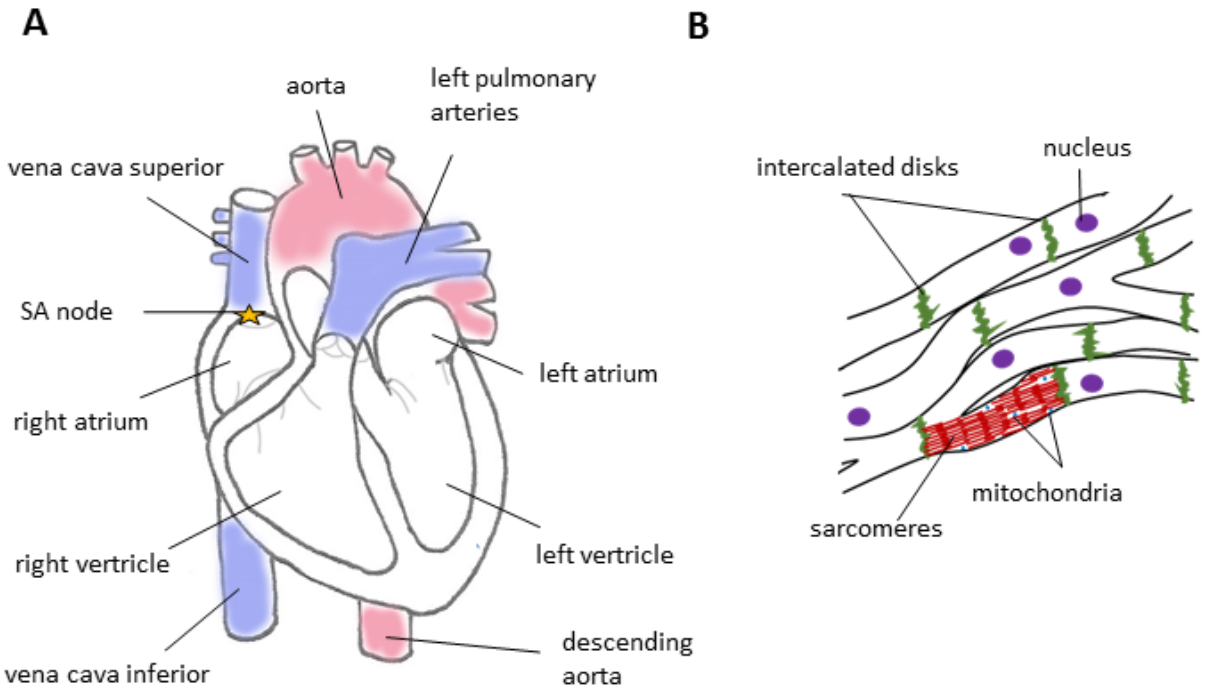

Figure 1. (**A**) Schematic structure of the heart. (**B**) Schematic structure of cardiomyocytes. (Drawn after example of Figure 14-7 from [5])

Excitation of pacemaker cells and cardiomyocytes

The instable membrane potential of pacemaker cells (Figure 2, green line) is about -60 mV [5]. At -60 mV, special channels, called I_f channels, open (0), allowing Na^+ influx and K^+ efflux. As Na^+ influx exceeds K^+ efflux, a net positive charge depolarizes the auto-rhythmic cells. With the membrane potential becoming more positive, the I_f channels gradually close and some Ca^{2+} channels open (1). The subsequent influx of Ca^{2+} continues during the depolarization and the membrane potential moves steadily towards threshold (2). When the membrane potential reaches threshold, additional Ca^{2+} channels open causing additional Ca^{2+} influx, resulting in a steep depolarization phase. When Ca^{2+} channels close at the peak of the action potential (3), slow K^+ channels open and cells repolarize due to K^+ efflux. Later K^+ channels close (4) and the cycle starts again (0).

The depolarization of cardiomyocytes is different. Cardiomyocytes have a stable resting potential of about -90 mV (Figure 2, red line). When a depolarization wave (from a pacemaker cell) moves into a

cardiomyocyte through gap junctions, the membrane potential becomes more positive. Voltage gated Na^+ channels open (1) for Na^+ influx, causing a rapid depolarization. Than Na^+ channels close (2), and initial repolarization starts with K^+ leaving the cell through fast K^+ channels, which readily close, followed by the plateau phase where voltage gated Ca^{2+} channels open for Ca^{2+} influx (3). Ca^{2+} channels subsequently close and slow K^+ channels open for additional K^+ efflux (4): the repolarization phase, followed by the resting potential (0) [5].

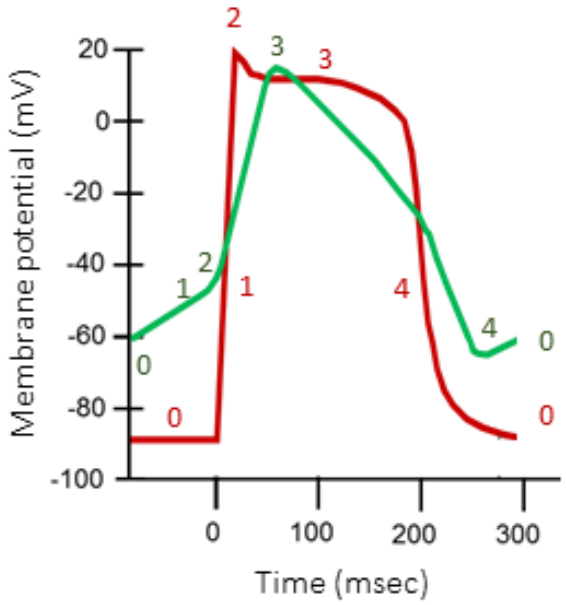

Figure 2. The pacemaker potential (green line) and the action potential of a cardiomyocyte (red line).

Excitation-to-contraction coupling in a cardiomyocyte

A change in voltage, induced by pacemaker cells that enters a (ventricle) cardiomyocyte, moves across the sarcolemma and into the t-tubules, where it opens voltage-gated Ca^{2+} channels in the cell membrane (Figure 3). Ca^{2+} enters the cell and opens ryanodine receptor channels in the sarcoplasmic reticulum (SR). The ryanodine receptor channels are Ca^{2+} channels and opening causes Ca^{2+}-induced-Ca^{2+} release, creating a Ca^{2+} spark. Free Ca^{2+} diffuses through the cytosol to the contractile elements, where it binds to troponin and initiates the cycle of cross bridge formation between myosin and actin filaments for contraction.

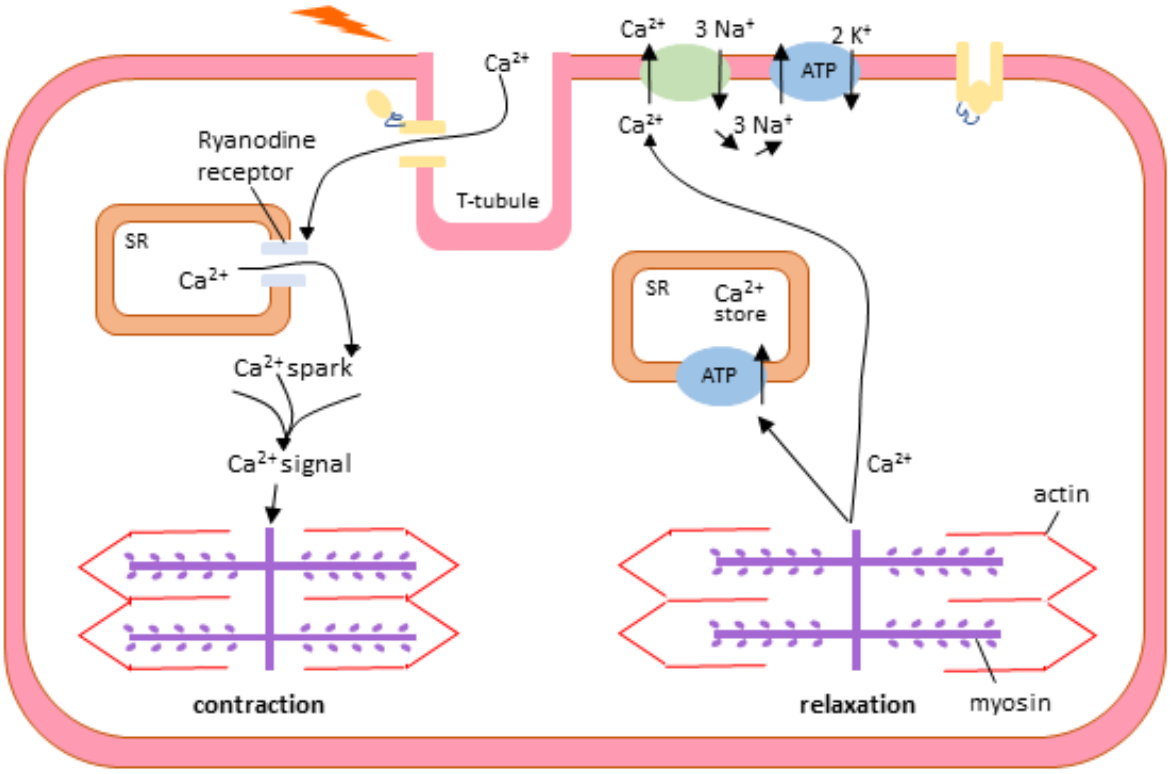

Figure 3. Excitation-to-contraction coupling and relaxation in a cardiomyocyte. (Drawn after example of Figure 14-11 from [5])

When Ca^{2+} concentrations decrease, via reuptake into the SR (via Ca^{2+}-ATPase) and across the cell membrane via Na^{+}-Ca^{2+} exchange, Ca^{2+} unbinds from troponin, myosin releases actin and the contractile filaments slide back into their relaxed position. The Na^{+}, exchanged for Ca^{2+}, is subsequently removed from the cell via a Na^{+}-K^{+}-ATPase [5].

Contraction of the heart

After a contraction, the ventricles are relaxed and the atrioventricular (AV) valves open. Blood flows from the veins into the atria - from the vena cavae (superior and inferior) into the right atrium and from the pulmonary veins into the left atrium - and subsequently by gravity from the atria into the ventricles.

The depolarization of the pacemaker cells in the SA node spreads to adjacent cardiomyocytes through gap junctions in the intercalated disks (slower conduction) and also through a branched internodal pathway (fast conduction) between the SA node and the AV node, enabling the atria to contract from top to bottom (Figure 4). At the AV node the electrical signals are slightly delayed before continuing to the ventricles to ensure that atrial contraction fills the ventricles with a remaining 20% of blood from the atria.

As the fibrous skeleton between the atria and ventricles prevent the transfer of electrical signals from the atria to the ventricles, transfer of electrical signals are conducted from the AV node to the bundle of His and Purkinje fibers in the septum between the ventricles, after which they branch into right and left bundle branches towards the apex. Smaller branches spread outward among the contractile cells in the ventricles, allowing the ventricles to contract from the apex to the base, squeezing the blood upwards after which the AV valves are forced to close (tricuspid valve on the right and mitral valve on the left, first heart sound). Meanwhile during ventricular contraction, the atria are repolarizing and relaxing and when atrial pressure falls below that in the veins, blood flows from the veins into the atria again. Isovolumic ventricular contraction continues, forcing the opening of a second set of valves, pumping blood into the pulmonary (right) arteries and the aorta (left, which branches into the coronary arteries) for supply to the rest of the body. As the ventricles repolarize and relax, ventricular pressure decreases. Once ventricular pressure falls below the pressure in the arteries, blood starts to flow back to the heart from the arteries and the artery valves close (second heart sound). During isovolumic ventricular relaxation, the ventricular pressure will eventually fall below atrial pressure and the AV valves will open again for a new cycle [5].

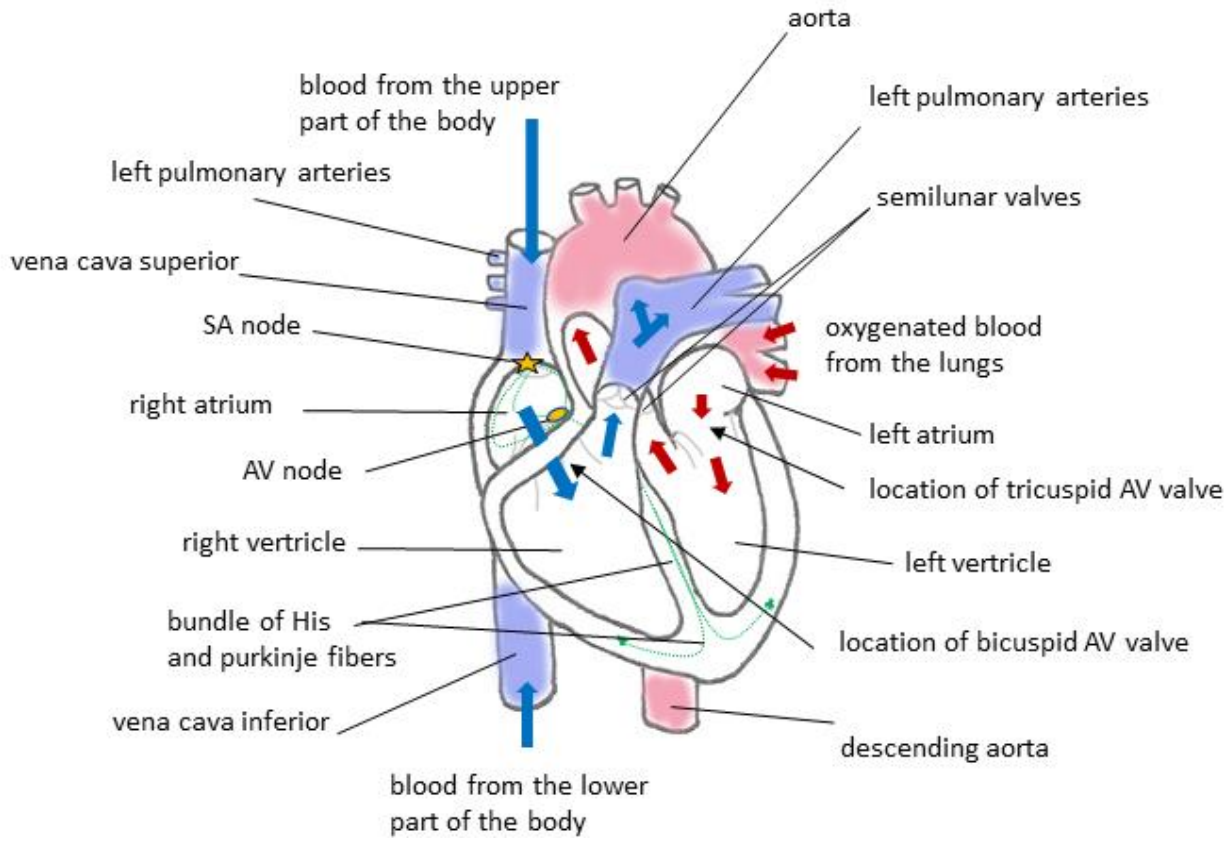

Figure 4. Structure of the heart, indicating the deoxygenated blood flow in blue and the oxygenated blood flow in red. The electrical signals, starting from the sinus (SA) node follow the green dotted line via the AV node via the bundle of His and the purkinje fibers to the apex. (Drawn after example of Figure 14-7 from [5])

Atrial fibrillation

Electrical chaos

AF is a disease where the atria contract in a disorganized manner. During a normal cardiac rhythm, the atrial electrical conduction wave dies out after it has activated the AV node, because each atrial cardiomyocyte is surrounded by refractory tissue. Anatomical anomalies, or, more common, structural (cardiomyocyte) damage, including fibrosis and myolysis, can deflect electrical impulses, and re-excite regions of myocardium after the refractory period has subsided (and before the pacemaker cells can depolarize these cells again) [5] giving rise to uncoordinated, chaotic electrical signals and disorganized contraction, leading to an increased and irregular heart rate [1]. Cardiac output is diminished, as the

blood is not pumped efficiently from the atria to ventricles and overall heart contraction is less effective. Instead, part of the blood may stay in the atria leading to atrial enlargement and form blood cloths (thromboembolism), which may cause a stroke. Atrial enlargement can conceivably drive development of other severe cardiovascular problems, such as chronic heart failure [3, 7].

Clinical characteristics

Approximately 30% of the AF population has 'lone AF'. These patients are generally <60 years of age and their AF is found to be unrelated to underlying lung or cardiovascular conditions [8]. In the remainder of the AF population, AF has its origin in (a combination) underlying conditions, such as common risk factors for AF, including advanced age, hypertension, obesity, diabetes mellitus, hyperthyroidism, heart failure, valvular heart disease, coronary artery disease, congenital heart disease and recent cardiothoracic surgery [1, 3, 8].

Some patients have asymptomatic AF, which was previously hard to diagnose. However, with recent developed optical sensors on wearable devices, irregular pulses can be detected which may ease the detection of asymptomatic AF [9]. More often, AF is related to rapid-heartbeat-related symptoms like palpitations and chest discomfort, and symptoms related to improper blood pumping, like lightheadedness, dizziness, shortness of breath, chest pain and fatigue occur [10].

Patients presenting to the hospital with symptomatic AF are currently classified into paroxysmal AF (PAF, <7 days), persistent AF (PeAF, >7 days <1 year) and longstanding persistent AF (LSPeAF, >1 year of AF). Either AF is left untreated or patients with AF are treated.

Treatment of AF patients

Treatment of AF patients may occur using medication to control:

- thrombosis with anti-coagulants,
- hypertension with diuretics; angiotensin-converting enzyme (ACE)-inhibitors or angiotensin II receptor blockers,
- cholesterol with cholesterol synthase inhibitors; statins,
- the arrhythmia with:
    - **Class I** anti-arrhythmic drugs: Na^+ channel blockers, which reduce the maximum rate of depolarization (such as flecainide),
    - **Class II** anti-arrhythmic drugs: β-adrenoreceptor antagonists, which antagonize sympathetic stimulation (such as propranolol),
    - **Class III** anti-arrhythmic drugs: drugs that prolong the action potential by delaying slow outward K^+ current, increasing the refractory period (such as amiodarone and sotalol),
    - **Class IV** anti-arrhythmic drugs: calcium antagonists which slow down conduction in the SA and AV nodes where action potential propagation depends on slow inward Ca^{2+} current (such as verapamil and diltiazem)
    - prevention of heart failure with **digoxin**: inhibits Na^+/K^+ pump, slowing AV conduction, and subsequently slowing the ventricular rate to improve ventricular filling (rate control) [11].

Besides pharmacological treatment, rhythm control can also be accomplished by electrical cardioversion or by physically interrupting specific electrical pathways (electrical isolation of regions which are the origin of the aberrant disorganized electrical impulses, often originating from the pulmonary veins) by creating scar tissue (ablation), for example with a Maze procedure or pulmonary vein isolation (PVI) [3].

Atrial fibrillation treatment: the complication

The current treatment alleviates the electrical refractoriness and therefore the symptoms, however does not halt or cure the disease. Already at the first onset of AF, structural damage is present in the atria: fibrosis may impair electrical conduction and provoke chaotic electrical signaling and, in the cardiomyocytes, loss of sarcomeres (myolysis) impairs excitation-to-contraction coupling [12-21]. As structural damage is persistent and accumulates over time, it also becomes increasingly difficult to reverse AF to sinus rhythm with the current treatment strategies [13, 14]. The deterioration of the cardiomyocytes explains the progression of the disease and the high recurrence rates after treatment. To illustrate, patients with AF episodes limited to less than 24 continuous hours had a significantly lower rate of recurrence (approximately 40% within 1 year) following an ablation procedure than patients with longer than 24 hours of AF episodes, in which approximately 60% recurred within one year after ablation [22]. Accordingly, it is essential to investigate the underlying mechanisms driving cardiomyocyte damage and test novel drugs to halt or even reverse structural damage.

Derailment of proteostasis underlies structural damage

In clinical AF, structural changes in the atrial cardiomyocytes, including myolysis, correlate with electrical remodeling and AF progression [12, 16, 19]. Recent research findings indicate that derailment of protein homeostasis (proteostasis), underlies structural changes and consequently AF progression [23]. Proteostasis is the homeostasis of protein production, folding, function and degradation. A balanced proteostasis is the basis for a healthy cell. Proteins are made at the ribosomes and chaperones assist correct folding and function after translation. Damage to proteins, incorrect folding, or aggregation is toxic for the cardiomyocytes and if not reversed, this may lead to cardiomyocyte damage, changes in gene expression and changes in phenotype. Each cardiomyocyte expresses

chaperones, mainly heat shock proteins, which assist in correct (re-)folding and function of proteins and as such conserve proteostasis.

Under stressful conditions, such as during paroxysmal AF, HSPs are upregulated to reverse damage in the cardiomyocytes. However, during more persistent forms of AF, HSP levels become exhausted, resulting in proteostasis derailment and progression of the arrhythmia [15, 18]. In **chapter 2**, the protective role of small HSPs in cardiac diseases, and their key role in AF is reviewed. In **chapter 3,** state of the art knowledge on electrical and molecular mechanisms underlying AF is summarized, shortcomings of present diagnostic instruments and therapeutic options are discussed and potential novel diagnostic tools and therapeutic targets directed at restoration of proteostasis are presented. In **chapter 4,** the rationale behind the Halt & Reverse study is described, starting with explaining the term electropathology and how HSPs may halt and reverse electropathology. In **chapter 5**, the trial protocol for the Halt & Reverse study is described. The Halt & Reverse study aims to elucidate the correlation between electropathology and HSP levels and AF following pulmonary vein isolation, electrical cardioversion or cardiothoracic surgery. This knowledge may help to stage AF patients and identify patients at risk for an AF recurrence after AF treatment.

The experimental part of this thesis starts with **chapter 6,** in which the role of RhoA on HSP expression is investigated. We found that RhoA activation suppresses the heat shock response (HSR) in HL-1 atrial cardiomyocytes by suppressing the binding of heat shock factor 1 (HSF1) to the heat shock element (HSE) in the promotor sequence of the *hsp* genes, thereby inhibiting HSP expression. Inversely, RhoA inhibition boosts the proteotoxic stress-induced HSR. This study reveals that active RhoA negatively regulates the HSR via attenuation of the HSF1-HSE binding by which HSP expression is suppressed. Since HSPs are important in safeguarding a healthy proteostasis and HSP levels are exhausted in atrial tissue of patients with persistent AF, pharmacological induction of HSP expression is an interesting potential therapy in clinical AF. Hereto, 81 new compounds, all derivatives of the well-known HSP-inducer geranylgeranylacetone (GGA), were tested for their HSP-inducing capacity and protective and

reversal effects against tachypacing-induced contractile dysfunction in experimental models for AF. Protective effects of the derivatives seems mainly dependent on the effects of increased HSPB1 levels. The mode of action of the derivatives to induce HSP levels was also investigated and we observed that GGA-derivatives enhance the activation of HSF1. The results are presented in **chapter 7**. Out of the 81 derivatives, the most potent compound, GGA*-59, was further investigated for its properties to reverse tachypacing-induced contractile dysfunction and structural damage in **chapter 8**. GGA*-59, as well as recombinant HSPB1, accelerate recovery from tachypacing (TP)-induced calcium transient loss, microtubule network disruption and sarcomeric protein loss.

Although GGA is known to induce HSPs in various tissues in experimental settings, including cardiomyocytes, the HSP-inducing properties of GGA has not been demonstrated in human heart tissue. In **chapter 9**, we investigated whether oral GGA treatment increases HSF1-mediated HSP expression in atrial appendages of patients undergoing cardiac surgery. We observed that 3 days of oral GGA treatment increases the expression levels of HSPB1 and HSPA1 in these patients.

Staging of AF is essential to select an appropriate treatment strategy and to prevent disease progression and the accompanied increase in therapy failure. In **chapter 10,** we investigate whether serum HSP levels determine AF stage and identify patients with a higher risk for AF recurrence after treatment. Hereto, we evaluated the relation between blood-based (i.e. serum) HSPB1, HSPA1, HSPB7 and HSPD1 levels, the presence and stage of AF and AF recurrence following electrocardioversion (ECV) or pulmonary vein isolation (PVI). None of the HSP levels in baseline serum samples correlate with the presence, stage or recurrence of AF, however HSPB1 levels in serum samples taken at 3, 6 and 12 months after PVI were higher in patients with AF recurrence compared to patients with no AF recurrence. Additional to **chapter 10**, in **chapter 11,** we evaluated the relation between atrial tissue and serum levels of HSPs, and AF presence, AF stage, the occurrence of post-operative AF (PoAF) and AF recurrence. Serum HSP levels do not associate with the presence or stage of AF compared to control, development of PoAF or AF recurrence. Atrial tissue (right and left atrial appendages, RAA and

LAA, respectively) levels of HSPB1, HSPA1, HSPB5 and pHSF1 were similar between control and PAF, PeAF and LSPeAF. RAA HSPA5 levels were significantly lower in LSPeAF and HSPD1 levels higher in PeAF and in the total AF group compared to control. Both HSPA1 and HSPA5 RAA levels were higher in control patients who developed PoAF, compared to patients without PoAF. In AF patients treated with Maze surgery, HSPB1 RAA levels were significantly lower and HSPA5 LAA levels higher in patients with AF recurrence, compared to no AF recurrence.

Next to HSP levels, in the cardiomyocytes also the energy producing entities, the mitochondria, are affected in AF. Mitochondrial dysfunction is demonstrated in experimental models for AF as well as in atrial tissue samples of patients with AF, as described in **chapter 12**. Elaborating on this, in **chapter 13,** it is evaluated whether cell-free circulating mitochondrial DNA (cfc-mtDNA) represents a potential serum marker for AF stage, sex differences, and recurrence of AF after treatment, in a population of patients undergoing AF treatment (ECV or PVI) or cardiac surgery compared to controls with no history of AF. It was observed that the level of cfc-mtDNA (COX3 and ND1) in serum is associated with AF stage and with recurrence of AF, especially in patients with paroxysmal AF undergoing ECV and PVI treatment. In addition, increased levels of cfc-mtDNA in the medium of *in vitro* tachypaced HL-1 cardiomyocytes were associated with enhanced mitochondrial damage and stress in these cardiomyocytes, suggesting that during AF mtDNA is released from the cardiomyocytes into the circulation.

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