# Metabolic aspects of cardiac arrhythmias

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*"If we knew what we were doing it wouldn't be research"*

Albert Einstein

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# ABSTRACT

Cardiac arrhythmias are an important cause of mortality and morbidity in patients with cardiac diseases. Sudden death due to ventricular tachycardia and fibrillation (VT/VF) in the setting of acute myocardial infarction (AMI) and heart failure (HF) is a frequent cause of premature death. Another recognized cause for sudden cardiac death is acquired complete heart block (CHB), a condition where the heart must quickly adapt to volume overload and increased wall stress to maintain normal hemodynamics. In the pre-pacemaker era this was a condition with a very high mortality mostly due to sudden death and progressive HF. The most common sustained arrhythmia, atrial fibrillation causes significant morbidity and is associated with stroke, HF and risk of premature death. The heart is an electro-mechanic pump that metabolizes mostly fatty acids for energy generation. Most of the energy is fuelling contractile work but approximately one-third is designated for ion pumps that maintain the electrochemical homeostasis of the cardiomyocyte, and give rise to the cell depolarization and repolarization. As the ATP pool is completely turned over every 10s, effective metabolism is imperative for the maintenance of electrical stability in the cell. In different pathophysiological states, i.e. obesity and diabetes, there is a mismatch between uptake and utilization of fatty acids leading to intracellular lipid accumulation. This may lead to the production of toxic lipid metabolites (e.g. lysophosphatidylcholine (LPC), diacylglycerol (DAG) and ceramides) and is referred to as lipotoxicity. LPC is also generated during myocardial ischemia and has been proposed as a contributor to the generation of ventricular arrhythmias during AMI. An import metabolic regulatory hormone, growth hormone (GH), has been shown to exert various positive effects in post-infarction HF.

**Aims**

I.      To evaluate the short- and long-term effects of CHB on cardiac function, morphology and energy metabolism in the rat

II.     To investigate the effects of GH on ischemic and non-ischemic arrhythmogenesis in the rat

III.    To set up and validate an *in vitro* experimental system for studies of the effects of LPC on electrophysiological parameters in beating cardiomyocytes.

IV.     To evaluate associated risk factors for having AF in patients admitted to cardiac care units and to evaluate the role of lipid metabolism in the pathophysiology of AF.

**Results and conclusions**

I.      Rats with CHB compensate for the reduction in heart rate by doubling the stroke volume and thereby maintaining cardiac output. Increases in wall tension leads to eccentric left ventricular hypertrophy. After long-term CHB there were no hemodynamic or metabolic signs of HF.

II.     GH reduced the occurrence of spontaneous VT/VF in rats with induced AMI as well as reducing induced VT/VF in anesthetized rats. This adds to previously described beneficial effects of GH in HF and AMI, and we suggest that the effect is partly mediated via decreased sympathetic stimulation.

III.    LPC induced prompt and pronounced electrophysiological alterations that may underlie its observed pro-arrhythmic properties. Our model may be a useful tool for preclinical studies of electrophysiological effects of various pathophysiological concepts.

IV.     In a multivariate analysis we found that obesity was associated with AF, but diabetes was not. AF was associated with quantitative and qualitative alterations in atrial lipid content but not with signs of lipotoxicity. Polyunsaturated DAG was increased in AF patients and may play a role in the pathophysiology of AF.

# SAMMANFATTNING

Hjärtarytmi är en viktig orsak till död och lidande hos patienter med underliggande hjärtsjukdomar. Kammartakykardi och kammarflimmer vid akut hjärtinfarkt och hjärtsvikt är en vanlig orsak till förtidig död. Totalt hjärtblock, där förmak och kammare inte kommunicerar på ordinärt sätt, är en annan känd orsak till plötslig hjärtdöd. Vid hjärtblock måste hjärtat anpassa sig till volymsbelastning och ökat väggtryck för att bibehålla normal hjärtfunktion. Före pacemakerns tillkomst hade detta tillstånd en mycket dålig prognos, på grund av försämring av hjärtfunktionen och plötslig hjärtdöd. Den vanligaste ihållande hjärtarytmin, förmaksflimmer orsakar i tillägg till besvärliga symptom även ökad risk för stroke, hjärtsvikt och dubblerar risken att dö i förtid.

Hjärtat är en elektromekanisk pump som metaboliserar i huvudsak fettsyror för att alstra energi. Huvuddelen av energin går till att generera mekanisk kontraktion men cirka en tredjedel utnyttjas för driften av jonpumpar, som upprätthåller elektrisk stabilitet i cellen samt ger upphov till signaler som styr över när hjärtat skall kontrahera och relaxera. Dessa signaler är så pass tydliga att de kan registreras på kroppens yta, då i form av s.k. elektrokardiogram (EKG). Hjärtats ämnesomsättning är mycket snabb och är därför helt beroende av en effektiv produktion av energirika molekyler för att kunna upprätthålla elektrisk stabilitet. Vid vissa sjukdomstillstånd, t.ex. diabetes och fetma, finns en obalans i upptag och utnyttjandet av fettsyror som leder till att dessa ansamlas inne i hjärtcellen. Hjärtat, till skillnad från fettceller, är inte anpassat till att lagra stora mängder fett, vilket kan leda till en produktion av för cellen giftiga fettämnen, vilket ger upphov till s.k. lipotoxicitet. Dessa fettämnen kan i vissa fall även bildas vid hjärtinfarkt och tros vara en av förklaringarna till varför alvarliga hjärtrytmrubbningar uppkommer vid akut hjärtinfarkt. Tillväxthormon har en viktig roll i styrningen av ämnesomsättningen i kroppens celler och har visat sig ha positiva effekter på hjärtfunktionen vid bland annat hjärtsvikt efter hjärtinfarkt.

**Syfte**

I. Att utvärdera kort- och långtidseffekterna av totalt hjärtblock på hjärtats form, funktion och ämnesomsättning i en råttmodell.

II. Att undersöka tillväxthormonets effekter på uppkomst av alvarliga rytmstörningar vid experimentell hjärtinfarkt hos råtta.

III. Att sätta upp och validera ett experimentellt system för bedömning av effekterna av giftiga fettämnen på elektrofysiologiska parametrar, i en kultur av slående hjärtceller.

IV. Att utvärdera risk faktorer för förekomsten av förmaksflimmer hos patienter som blir inlagda på hjärtintensivvårdsavdelningar i Västra Götaland, samt att undersöka rollen av olika kardiella fettämnen vid förmaksflimmer.

**Resultat och slutsatser**

I. Råttor med totalt hjärtblock kompenserar för en kraftig reduktion i hjärtfrekvensen med en dubblering av slagvolymen (dvs. den mängd blod som hjärtat pumpar vid varje kontraktion) och bibehåller därmed normal hjärtminutvolym. Ökat väggtryck orsakar hypertrofi i vänster kammaren. Efter en längre tid av hjärtblock ses inga hemodynamiska eller metabola tecken till hjärtsvikt.

II. Tillväxthormon minskar förekomsten av alvarliga rytmstörningar vid akut hjärtinfarkt, liksom minskar inducerbarheten av kammararytmier hos råttor. Detta är i tillägg till tidigare beskrivna positiva effekter av GH vid hjärtsvikt, och vi föreslår att effekten medieras delvis via sympatiska nervsystemet.

III. Det toxiska fettämnet lysophophatidylcholine orsakar tydliga elektrofysiologiska förändringar som kan ligga till grund för dess observerade negativa effekter på hjärtrytmen. Vår modell kan utgöra ett användbart verktyg för fortsatta pre-kliniska elektrofysiologiska studier av olika patofysiologiska koncept.

IV. I en multivariat analys fann vi att fetma, men inte diabetes var associerat med förekomst av förmaksflimmer. Flimmer var associerat med kvantitativa och kvalitativa förändringar i fettsammansättningen i förmaken men vi fann inga hållpunkter för s.k. lipotoxicitet. Fleromättat diacylglycerol kan spela en roll i patofysiologin bakom förmaksflimmer.

# LIST OF ORIGINAL PAPERS

This thesis is based on the following studies, which will be referred to in the text by their Roman numerals.

I.  Sigfus Gizurarson, Malin Lorentzon, Truls Råmunddal, Finn Waagstein, Lennart Bergfeldt, Elmir Omerovic. **Effects of complete heart block on myocardial function, morphology, and energy metabolism in the rat.** Europace. 2007 Jun;9(6):411-6.

II.  Truls Råmunddal*, Sigfus Gizurarson**, Malin Lorentzon, Elmir Omerovic.**Antiarrhythmic effects of growth hormone—in vivo evidence from small-animal models of acute myocardial infarction and invasive electrophysiology.** J Electrocardiol. 2008 Mar-Apr;41(2):144-51.

III.  Sigfus Gizurarson*, Yangzhen Shao*, Azra Miljanovic, Truls Råmunddal, Jan Borén, Lennart Bergfeldt, Elmir Omerovic. **Electrophysiological effects of lysophosphatidylcholine on HL-1 cardiomyocytes assessed with a microelectrode array system.** Submitted

IV.  Sigfus Gizurarson, Marcus Ståhlman, Anders Jeppsson, Yangzhen Shao, Björn Redfors, Lennart Bergfeldt, Jan Borén, Elmir Omerovic. **Atrial Fibrillation in Patients Admitted to Coronary Care Units in Western Sweden - The Role of Obesity and Lipotoxicity.** In manuscript

 * indicates that these authors contributed equally to these publications

# ABBREVIATIONS

| | |
|---|---|
| ADP | Adenosine di-phosphate |
| AF | Atrial fibrillation |
| AMI | Acute myocardial infarction |
| AP | Action potential |
| APD | Action potential duration |
| ATP | Adenosine tri-phosphate |
| AV | Atrioventricular |
| BMI | Body mass index ($kg/m^2$) |
| BR | Beating rate |
| CAC | Citric acid cycle |
| CAD | Coronary artery disease |
| CCU | Cardiac care unit |
| CHB | Complete heart block |
| CK | Creatine kinase |
| Cr | Creatine |
| CrP | Creatine phosphate=Phosphocreatine |
| DAG | Diacylglycerol |
| ECG | Electrocardiogram |
| FA | Fatty acid |
| FFA | Free fatty acid |
| FP | Field potential |
| FS | Fraction of shortening |
| GH | Growth hormone |
| GHD | Growth hormone deficiency |
| HF | Heart failure |
| HPLC | High performance liquid chromatography |
| IGF-1 | Insulin-like growth factor 1 |
| IHD | Ischemic heart disease |
| LAD | Left anterior descending artery |
| LPC | Lysophosphatidylcholine |
| MEA | Multielectrode array |
| NA | Noradrenaline |
| NYHA | New York Heart Association |
| PC | Phosphatidylcholine |
| PKC | Protein kinase C |
| RVOT | Right ventricular outflow tract |
| SR | Sinus rhythm |
| TAG | Triacylglycerol |
| TAN | Total adenine nucleotides |
| VA | Ventricular arrhythmia |
| VERP | Ventricular effective refractory period |
| VF | Ventricular fibrillation |
| VPB | Ventricular premature beat |
| VT | Ventricular tachycardia |
| VTI | Velocity-time integral |

# INTRODUCTION

Cardiac arrhythmias are an important cause of mortality and morbidity in western societies. Ventricular tachycardia/fibrillation *(Figure 1)* is the most common cause of death in the setting of acute myocardial infarction (AMI) and heart failure (HF)[1]. Severe bradycardia due to complete heart block also contributes substantially to sudden cardiac death[2]. At a time when the Western societies experience lower incidences of coronary heart disease[3], atrial fibrillation (AF) is increasing in prevalence, and is expected to reach epidemic proportions during this century[4]. AF is the most common sustained arrhythmia and affects quality of life and increases the risk of stroke, HF and death[5]. The latter half of the 20th century has seen new important treatment options for different types of arrhythmias, both in pharmacological treatments and non-pharmacological treatments, including the pacemaker, arrhythmia surgery, percutaneous ablation procedures, implantable defibrillators and cardiac resynchronization therapy. Two notable milestones are the introduction of the internal pacemaker in the late fifties for treatment of severe bradyarrhythmia and percutaneous ablation procedures for paroxysmal supraventricular tachycardia. Yet, in the last 20 years there has been a scarcity of major advances in new pharmacological treatments and devices. Besides issues of costs there are several important restrictions to the current treatment options, namely safety issues, shortage of experienced physicians performing complex procedures, and increasing volumes of patients seeking treatment.
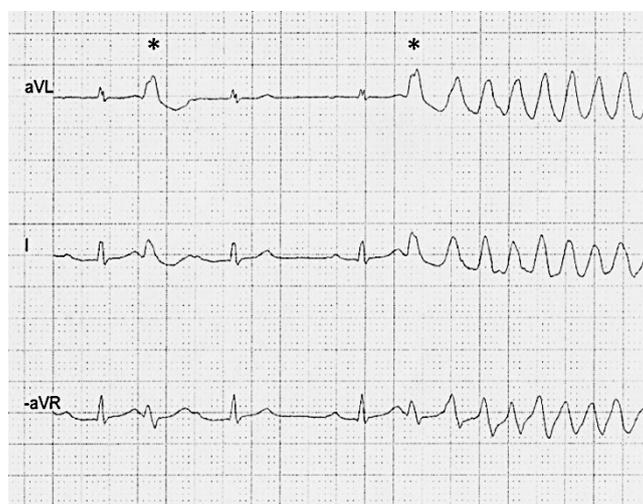


*Figure 1. An example of ventricular premature beats (\*) eventually triggering polymorphic ventricular tachycardia in a patient with idiopathic ventricular fibrillation.*

The heart is an electromechanical pump, and energy is spent on fuelling contractile work and maintaining the electrochemical gradients by means of the ion channels and pumps that uphold appropriate gradients of calcium, potassium and sodium across the cellular membrane. The ionic channels are also the fundament in the electric propagation by which the chambers of the heart work in synergy thus giving rise to the contractile pump function and the characteristic electrocardiogram. Historically, the attention has focused on characterizing and

understanding the ionic pumps and channels and this has led to important discoveries and introduction of anti-arrhythmic drugs and principles. One major drawback of drugs that affect the ion channels is the risk of introducing unwanted pro-arrhythmias, which has impeded the widespread use these agents, especially in patients with significant cardiac disease. Better understanding of the cardiac metabolism and how changes in the delivery and utilization of substrate may be involved in the risk developing arrhythmias holds the promise of improving the treatment of patients suffering from cardiac arrhythmias. This is especially the case when there is significant cardiac comorbidity, and this applies to most arrhythmias occurring in this setting ranging from AF to ischemic ventricular fibrillation (VF).

## *Basic cellular electrophysiology and the action potential*

Electrical signaling in the cardiomyocyte is based on the passage of ions in accordance to their electrochemical gradient through an intricate system of ion channels and on the pumping of ions against their electrochemical gradients by means of energy dependent ion pumps. The major charge carriers are sodium, potassium and calcium. Ion channels are pores that span the lipid bilayer of the cell membrane and are usually named after the most permeant ion, e.g. $Na^+$-channels. The main cardiac ion channels and pumps are depicted in *Figure 2*.
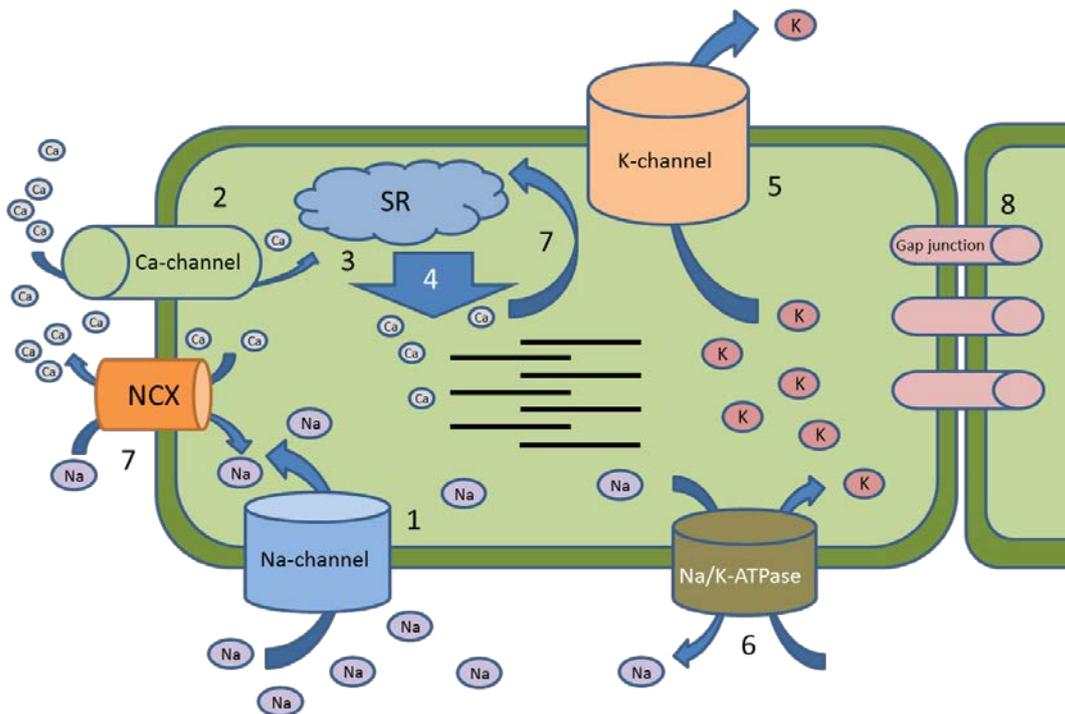


***Figure 2.*** *Simplified overview of the main ion channels.*
*Depolarization starts with rapid inward flow of sodium (1). This causes the membrane voltage to increase and this in turn opens voltage sensitive L-type $Ca^{++}$-channels (2). The inwardly flowing calcium ions react with the ryanodine receptor on the sarcoplasmic reticulum (SR) and trigger a massive calcium release from the SR (3). Calcium binds to the myofibrils in order to induce contraction (4). During these depolarizing currents the potassium channels have opened to allow potassium to flow outward (5). This eventually repolarizes the cell back to resting voltage. The $Na^+/K^+$-ATPase then pumps sodium back to the extracellular space and potassium into the cytoplasm to restore ionic homeostasis (6). Calcium is pumped back into the SR and out of the cell via a $Na^+/Ca^{++}$ exchanger (NCX)(7). Gap junctions are non-selective channels that interconnect the cytoplasm of neighboring cells (8).*

The cell has a high intracellular concentration of potassium, but low concentrations of sodium and calcium. The opposite is true for the extracellular space. The depolarization of the cell starts by an inward sodium currents that is intense but short-lived. This depolarizes the cell from the normal transmembrane voltage of -90mV to around +20 mV, which is followed by opening of voltage dependent calcium channels. This produces a calcium current that prolongs the action potential and induces calcium-induced calcium release from the sarcoplasmic reticulum that acts like a chain reaction to release large quantities of calcium that in turn trigger the contraction of the actin-myosin complex *(Figure 2)*. During the depolarization, potassium channels open to release potassium from the cell, thus restoring the negative transmembrane voltage in a process called repolarization. Calcium is pumped back into the sarcoplasmatic reticulum and the $Na^+/K^+$-ATPase pumps potassium back inside the cell and sodium to the extracellular space, as the cell prepares for yet another cycle of depolarization and repolarization. In order to have homogenous contraction of the muscle there are ion channels linking the cytoplasm of one cell to that of other neighboring cells. These channels are called gap junctions and they make the depolarization currents move faster over a large area of myocardium, hence promoting almost simultaneous contraction of a large area of myocardium. The changes in voltage over the cell membrane, caused by the elaborate flow of ions over the membrane, give rise to the cardiac action potential (AP). The cardiomyocyte action potential differs from that of e.g. neurons in that it has a prolonged depolarization phase (plateau phase), during which the cell is unexcitable. In the repolarization phase the cell regains excitability in a successive manner. An example of an action potential acquired from one cell is depicted in *Figure 3*. The vectorial sum of all unique action potentials from every cell in the heart gives rise to the characteristic surface electrocardiogram (ECG) used in everyday clinical medicine. An intermediary form is the electrocardiogram recorded from a syncytium of cells, e.g. cells in a culture. An example of this is the field potential (FP), here *(Figure 3)* in the form of a unipolar registration that is closely related to the cardiomyocyte AP, given that the syncytium studied is homogenous. Actually the FP can be considered to be the negative first derivative of the AP[6], and this gives the possibility to estimate the AP characteristics in a non-invasive fashion.
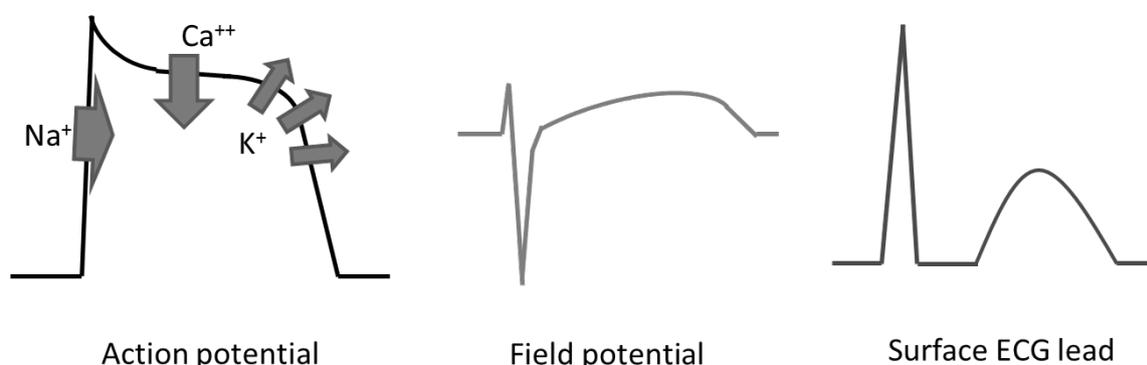


*Figure 3. Different types of electrical registrations from cardiac tissue.*
*The action potential registers what is happening in a single cell (on the picture the main ion currents and directions are shown). The field potentials are registrations from a syncytium of cells and the surface ECG represents a registration from the whole organ.*

## Ischemic ventricular arrhythmias

In the previous decades, there has been dramatic progress in the treatment of patients with AMI, leading to great improvements in the survival of these patients[7]. The in-hospital mortality has now reached such levels, that the margin for further improvements is rather small. However, this has not been the case for the substantial portion of patients that die out of hospital, i.e. before coming to medical attention. This group constitutes the largest portion of patients that die from AMI[8]. The out-of-hospital cause of death from AMI is largely due to ventricular arrhythmias (VA), usually VF[9]. Adding to the complexity is the fact that VF may be the first manifestation of ischemic heart disease (IHD) in about one quarter of the patients[10]. Although VF is most often effectively treated by direct-current cardioversion in the hospital or ambulance, there is a short window of time from the onset of VF until the patient will suffer irreversible brain damage from cerebral ischemia. This poses logistical problems for the timely and effective treatment of these patients and translates directly into a poor prognosis for sufferers of out-of-hospital cardiac arrest[11]. This problem is further complicated by the fact that many cardiac arrests are not witnessed. Ischemia and VAs are closely related phenomena. An AMI caused by interruption of blood flow in a coronary artery leads to profound changes in cellular metabolism and electrophysiology within minutes and is highly related to the occurrence of VF. VF in itself causes cessation of effective cardiac pump function, leading to global ischemia, further aggravating the metabolic and electrical derangement. In addition, the reperfusion of ischemic myocardial tissue also predisposes to VA. Three different temporal patterns of VA have been identified from pioneering work in dogs[12]. The early phase of arrhythmia is usually called phase I and this is usually limited to the first 30-45 min of ischemia. The late phase (II) is usually considered to last between 5-72 hours after the onset of ischemia and the arrhythmias occurring during the chronic phase (weeks to years later) are referred to as phase III arrhythmias. In humans and some animal models the first phase consists of two peaks in VA incidence, one that occurs after a few minutes and then another after about 15-40 min, subsequently termed Ia and Ib. There are however some important differences in species, and for instance rats have only one peak in incidence during phase I[13]. During phase I, the injury to the myocardium is reversible upon restoration of the blood supply, but during phase II the infarction is starting to evolve and the injury can at best be regarded as partly reversible. In humans it has been estimated that about half of all sudden deaths occurring during an AMI, take place during phase I.

The pathogenesis of VA during acute ischemia is similar to that of other arrhythmias in the sense that it depends on triggers, substrate and modulating factors. The substrate consists of regional ischemic myocardium that is surrounded by a non-ischemic myocardium. In the area between the two there is a zone, often referred to as the border zone, of myocardium that has varying degree of ischemia. The trigger is most often a critically timed ventricular premature beat (VPB) and the relative frequency of VPBs mirrors that of VF during the different acute ischemic phases. In the ischemic myocardium there is an extracellular accumulation of potassium that gives rise to a current (injury current) that can cause depolarization of myocytes (VPBs) on the normal side of the border zone. An important modulating factor is the autonomic system. Noradrenaline (NA) increases 100-1000 times its normal plasma concentration in the extracellular space of the ischemic tissue during the first 30 minutes after onset of ischemia, and this phenomenon seems to be related to reversal of neuronal reuptake

of NA[14, 15]. An example of the importance of the autonomic nervous system is exemplified by the almost total absence of VF in an ex-vivo ischemic porcine heart, even after the introduction of mechanical work[16]

In patients, acute coronary ischemia is most often caused by acute thrombosis in a coronary artery with previous signs of atherosclerosis[17]. In experimental settings, there is a higher incidence of ischemic VA when an artery is thrombotically occluded in contrast to mechanically occluded arteries[18]. One reason for this is the release of thrombin, which apart from being a key factor in the coagulation cascade, activates phospholipase $A_2$ ($PLA_2$)[19]. $PLA_2$ cleaves membrane phosphatidylcholine (PC) to lysophosphatidylcholine (LPC) and a free fatty acid (FFA). $PLA_2$ can also be activated by β-adrenergic receptor stimulation[20]. Prior studies suggest that early-phase arrhythmic events are caused by accumulating metabolites, such as LPC, in the ischemic myocardium[21]. Under normal physiological conditions, accumulation of LPC is precluded by reacylation and hydrolysis[22], but in the setting of myocardial ischemia, $PLA_2$ activity increases while reacylation and washout of LPC decreases *(Figure 4)*. Within the first few minutes after the onset of ischemia, LPC levels increase by 50-60% with a protracted normalization[23, 24]. LPC exerts various electrophysiological effects, which may be considered pro-arrhythmic. It inhibits fast $Na^+$ currents and inwardly rectifying $K^+$ currents[25, 26] and causes intracellular $Ca^{++}$ accumulation[27, 28]. LPC also decreases cell-to-cell coupling[29]. The consequences of these alterations are abnormal rhythmic activity, triggered activity, delayed after-depolarization and slowing of conduction, all of which facilitate intramyocardial re-entry.
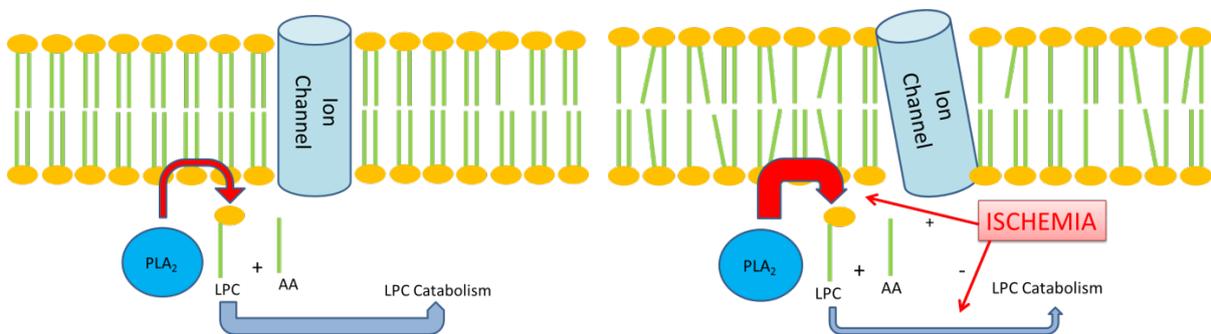


*Figure 4. Effects of ischemia on the production and catabolism of lysophosphatidylcholine (LPC). To the left is a highly schematic overview of LPC production and catabolism under normal condition. Normally the cell membrane phospholipids consist of less than 2% LPC. To the right the condition of ischemia is depicted. During ischemia there is an increased $PLA_2$ activity which generates LPC (which has only one acyl chain) and one fatty acid chain. The ischemia reduces the catabolism of LPC at the same time leading to accumulation of LPC. The LPC is incorporated into the cell membrane and having just one fatty acid chain it causes significant perturbation of the orderly packed phospholipids, ultimately influencing membrane proteins, such as ion channels. $PLA_2$=phospholipase $A_2$, AA=free fatty acid, e.g. arachidonic acid.*

The effects of LPC and other metabolites released during myocardial ischemia and metabolic stress can be studied by several established methods, including patch clamping and optical mapping. The study of electrical currents in a living, intact myocardial syncytium has several

advantages. This technology offers the possibility to estimate electrophysiological variables such as action potential duration in addition to simultaneous analysis of impulse-generation, impulse-propagation, wave front velocity, and arrhythmic activity [30].


## *The cardiac conduction system*

The sinus node is located at the border between the superior vena cava and the right atrium (*sulcus terminalis*). The sinus node is made up of densely packed cells that discharge synchronously and make up the working pacemaker of the heart. The electrical impulse is then propagated by atrial myocardial tissue anteriorly to the Bachman´s bundle, which conducts the impulse to the left atrium anteriorly. The impulse is at the same time propagated to the atrioventricular (AV) node.

The AV node in the formed heart is a superficial atrial structure with its boundaries being the triangle of Koch, which is made up by the septal leaflet of the tricuspid valve, the orifice of the coronary sinus and the tendon of Todaro. Its arterial supply is the dominant artery at the crux of the heart, usually the right coronary artery. In direct continuation from the compact AV node is the atrioventricular bundle of His, that traverses the fibrous body and branches in the ventricular septum. The main function of the AV node is to modulate the transmission of the electrical impulse to the ventricles in order for the atria and ventricles to contract in a coordinated manner. The bundle of His then branches into the bundle branches finally forming a network of Purkinje fibers that cover the endocardial surface and penetrate the ventricular myocardium facilitating fast and synchronized ventricular depolarization.

Both the sinus node and the AV node are densely innervated by the autonomic system with a much lesser supply to the atrial and ventricular myocardium. Ganglion cells, that are vagal postganglionic fibers, are confined to the atrium with preponderance for the sinus node.


## *Complete heart block*

Complete heart block (CHB) is as the name implies a total electrical dissociation between the atria and ventricles. It is frequently called total AV block or third degree AV block. Often there is a gradual worsening of AV conduction with progressively longer AV conduction times (first degree AV block), then progressing to non-conducted ventricular beats (second degree AV block) and finally develops into the fulminant form as complete heart block.  In CHB the ventricles are not activated by the sinus rhythm (SR) activity, but rather from foci located just below the level of block, giving rise to a so called escape rhythm. These foci may be located close to the AV node or more distally in the fascicles or ventricles.  Pacemaker activity in or close to the bundle of His is faster and seemingly more stable than that located more distally. In humans the escape rhythm is usually less than 40/min in acquired CHB, but can be faster for example in congenital heart block. CHB can be either temporary or chronic. CHB in children is usually congenital and most often the site of block is the AV node with a relatively rapid escape rhythm and stable hemodynamics. Acquired CHB on the other hand is located more distally in the conduction system, and therefore has a slower escape rhythm. Therefore it is usually poorly tolerated and accompanied by signs and symptoms of palpitations and reduced cardiac output, i.e. pre-syncope, syncope, dizziness and hypotension.

The etiology of heart block is usually idiopathic bundle branch fibrosis, IHD, infectious disease, rheumatic disease, calcific valve disease, cardiomyopathies and surgery[31]. In a study in the fifties and sixties, Johansson et al described 204 patients with CHB from the Malmö region in southern Sweden. In these years they calculated a yearly incidence of 63 patients/million. The mean age of these patients was 68.3 years and men were in majority (63%)[32]. The prognosis of CHB in the pre-pacemaker era was poor, one third of the patients died within 1 month and half were dead by one year. Sudden death was the most common mode of death, followed by progressive HF. The mortality and mode of death has changed dramatically after the introduction and widespread use of the implantable pacemaker. Animal models for complete heart block (CHB) have a long history. The earliest and most commonly used model is the canine model that has been in use for more than one century and has been extensively characterized[33]. There are however important societal, economical and institutional impediments for the routine use of large animal models in medical research. This has led to the development of small animal models of CHB, e.g. rodents such as rabbits[34], rats[35] and recently mice[36]. Rabbits are larger and easier to instrument, but tolerate CHB very poorly without the use of pacing. Mice are much smaller than rats and there are inherent methodological difficulties in creating CHB. Recently a novel method for induction of CHB by using a transvenous radiofrequency ablation in mice has been presented[36].

Preclinical research of CHB has wide potential implications, including studies of bradycardia related torsade de pointes[37], stem cell regenerative therapies[38] and physiological hypertrophy.


## *Myocardial metabolism*

The heart performs an extraordinary workload, by pumping almost ten tons of blood through the body every day. The energy needed comes mostly from the metabolism of fatty acids and to a lesser extent from carbohydrates. Metabolisms of ketones play a limited role in cardiac energy metabolism, except during prolonged starvation. Acetyl-CoA is produced by β-oxidation of fatty acids (FA) and from pyruvate (mostly from glucose through glycolysis). Acetyl-CoA fuels the citric acid cycle (CAC) that provides electrons for the respiratory chain. This results in ATP production by the use of oxygen. During normal conditions almost all ATP is produced in the mitochondria by oxidative phosphorylation, with a small contribution from glycolysis. The ATP content of the heart is relatively low with a high rate of hydrolysis, and all cellular ATP content is turned over every 10 seconds. Most of the produced ATP is fuelling contractile work but approximately 30-40% is spent on ion pumps in the sarcoplasmic reticulum and sarcolemma[39]. Maintenance of $Na^+$ and $K^+$ gradients across the membrane is among the most energy demanding cellular processes in the myocardium.

For the transport of high energy phosphates (such as ATP) from the mitochondria to the sites of usage, the cell utilizes a small molecule, creatine (Cr). Mitochondrial creatine kinase (CK) transfers one phosphate from ATP to Cr to yield phosphocreatine (CrP). CrP is a smaller and less charged molecule than ATP, and can thus move freely through the cytosol to the place of utilization, where the cytosolic CK can restore ATP from ADP via the reverse reaction *(Figure 5)*. This provides adequate levels of ATP at the myofibrils and ion pumps[40]. CrP is an important storage of high energy phosphates in the cell and depletion of Cr leads to HF[41].
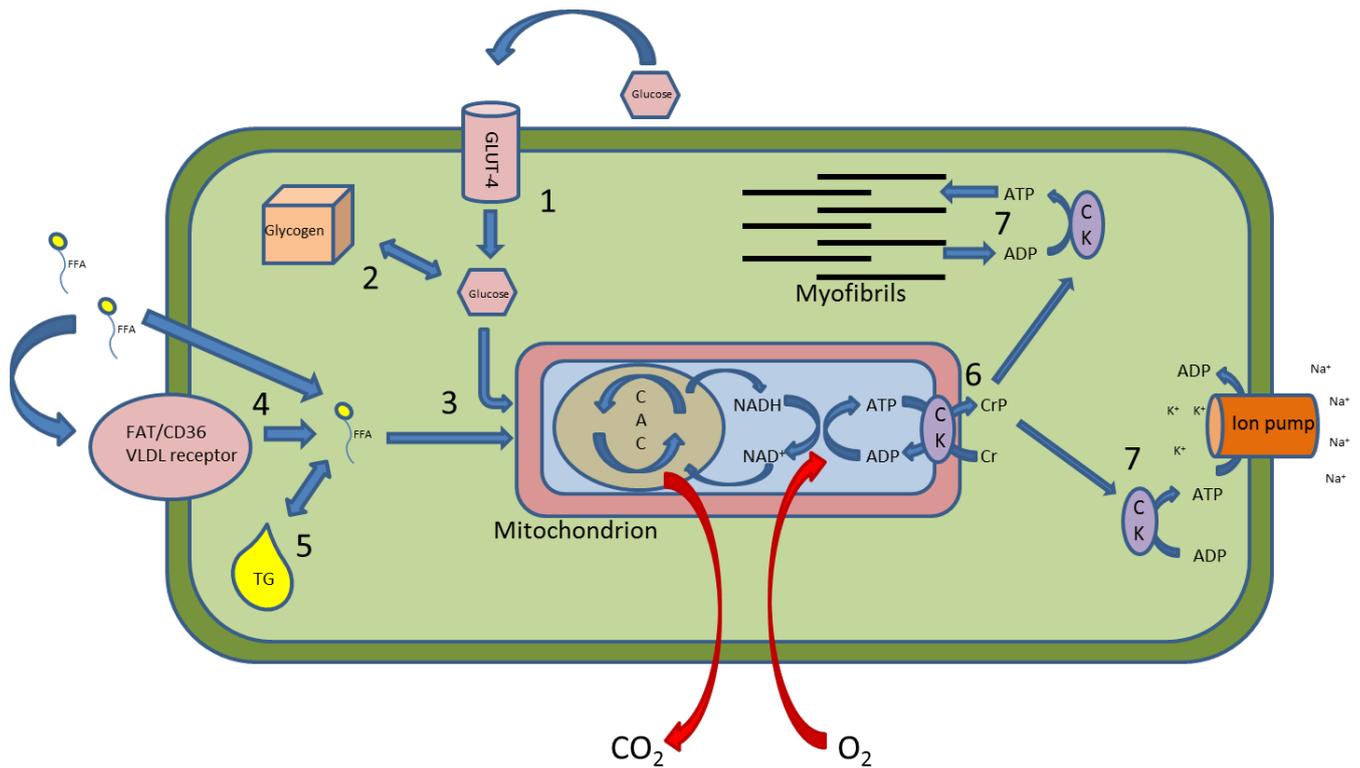
*Figure 5. Simplified overview of substrate import and energy metabolism in the cardiomyocyte. Glucose is transported into the cell via the GLUT-4 glucose transporter (1). Once in the cell, it can be stored as glycogen (2) or utilized for energy production in the mitochondria (3). Fatty acids can either be imported via receptor uptake or diffuse via the cell membrane (4). Once in the cell they can be stored as triacylglycerides (TG) (5) or utilized for energy production (3). The ATP is produced in the mitochondrion and can be used to produce CrP (6) which then diffuses to the site of usage, here demonstrated as ion pumps or myofibrils (7). Abbreviations: FAT/CD36=fatty acid transporter protein, VLDL=very low density lipoprotein, CAC=citric acid cycle, NAD=Nicotinamide Adenine Dinucleotide, Cr=Creatine, CrP=phosphocreatine, CK=creatine kinase*

There are some studies that particularly focus on the atrial metabolism during AF. These have suggested important changes in substrate utilization[42, 43]. AF imposes severe strain on atrial metabolism, and oxygen consumption is increased three-fold during AF[44]. In a goat model, AF causes initial depletion of high energy phosphates but subsequently these levels return to baseline, indicating that the cells are capable of adaptive metabolic remodeling[45]. One possible negative effect of this remodeling is that AF causes severe oxidative stress that is partly due to decreased CAC function. This leaves less energy available for the respiratory chain which causes ATP depletion and promotes free radical formation. During AF there is up regulation of several genes involved in the production of free radicals and concomitant down regulation of genes that inhibit oxidative stress[46]. It has been demonstrated that glucose utilization is increased early during AF and this has been linked to an increase in intracellular $Ca^{++}$. Intracellular calcium is an activator of glycogenolytic pathways and is known to be increased early during AF[47]. However, during prolonged AF there is normalization in intracellular calcium leading to lower glucose levels[42] and accumulation of glycogen in the cell[48] indicating that glucose utilization decreases over time. Another study even demonstrated an increased role of ketone body metabolism during long-standing AF[42]. This is

in contrast to the very limited ketone metabolism in normal myocardium. Taken together, these findings imply a change in substrate utilization occurring over time during AF which may play an important role in the perpetuation of the arrhythmia.

## *Fatty acid metabolism and lipotoxicity*

The heart metabolizes mostly fatty acids (FA) and has relatively small glycogen storage compared to skeletal muscle[49]. FA uptake is primarily determined by the concentration of non-esterified FAs in plasma[50] which can vary over a fourfold range during normal condition and even more, during metabolic stress (e.g. ischemia, diabetes and starvation) [51]. FAs can diffuse across the lipid bilayer of the cell by means of its hydrophobic nature but most of the uptake is protein-mediated. The major fatty acid transporter (FAT/CD36) has been shown to be a key factor in upregulating FA oxidation in diabetic mice[52, 53]. Recently, the role of the VLDL-receptor has been emphasized as an import mode of lipid import in hypoxia and ischemia[54], highlighting the importance of several systems for cellular uptake of lipids. Once the FAs are across the sarcolemma they are bound to fatty acid binding proteins that bind with high affinity to long chain FAs and a large portion is rapidly esterified to fatty acyl-CoA by acyl-CoA synthetase which keeps it from diffusing to the extracellular matrix. Fatty acyl-CoA is then shuttled to the target sites of β-oxidation or for further esterification[55]. Fatty acid β-oxidation takes place in the mitochondria, which are not permeable to long chain acyl-CoA and they have to be transported from the cytosol to the mitochondrial matrix by a carnitine-dependent transport system[56]. Carnitine palmitoyltransferase I (CPT-I) has been identified as a key regulator of fatty acid oxidation by its involvement in mitochondrial translocation of fatty acids for β-oxidation[57].

As the metabolism of the heart depends largely on long chain FAs for energy production[58], the balance of cellular fatty acid uptake and metabolism is tightly regulated. There are three major metabolic fates of fatty acids in the heart cells. They can be converted to glycerolipids (e.g. phospholipids, triglycerides), they can be converted to sphingolipids (e.g. sphingomyelin or ceramide) or they can be oxidized for energy. If the amount of FAs taken up by the cardiomyocyte exceeds the metabolic demand for FAs a state of lipid excess (steatosis) occurs. The cells can store small amounts as triacylglycerides (TAG) but eventually the excess lipids may be shunted into non-oxidative pathways resulting in accumulation of toxic lipid species[59], such as ceramides (CER) and diacylglycerol (DAG). There is experimental evidence showing that cardiac TAG amount correlates both with left ventricular (LV) dysfunction and hypertrophy[60, 61]. However, the exact mechanism for how the excess lipids harm the cells is only partly understood. Even though TAGs are at the center of the concept of lipotoxicity, there is good evidence that they are not harmful by themselves. This is substantiated by several experimental studies, for instance the overexpression of the enzyme DGAT-1 (that converts DAG to TAG) in mice skeletal muscle decreases the levels of CER and DAG and promotes inert TAG accumulation[62] that seems beneficial to most cells. So TAGs appear to be markers of lipotoxicity but may not be responsible for the observed toxic effects. Adding to the complexity is that certain metabolic adaptions can be harmful during non-stressed conditions but may be beneficial under certain stress (e.g. hypertension or

ischemia)[63]. This phenomenon has also been observed in human epidemiological studies and has been the basis of the "obesity paradox"[64].

The proposed mechanisms of lipotoxicity include increased stress on the endoplasmic reticulum (ER), mitochondrial dysfunction and altered gene expression [59] ultimately leading to increased amounts of reactive oxygen species and CER production causing cellular dysfunction and apoptosis[65]. The amphiphilic lipid molecule LPC is known to cause several electrophysiological alterations and is also considered to be a lipotoxic intermediate[66].

## *Growth hormone and the heart*

Growth hormone (GH) is a peptide hormone that is secreted from the anterior pituary gland in a pulsatile manner. GH is principally involved in somatic growth regulation and exerts its effects either directly or via the stimulation of insulin-like growth factor-1 (IGF-1). GH has attracted public attention from its use in the food industry and its illegal use among body-builders and athletes. The relationship between GH and the heart has been the focus of many experimental and clinical studies and some of the proposed mechanisms are summarized in *Figure 6*. Cardiomyocytes have a relatively high expression of GH receptors and they also express the IGF-1 receptor[67].
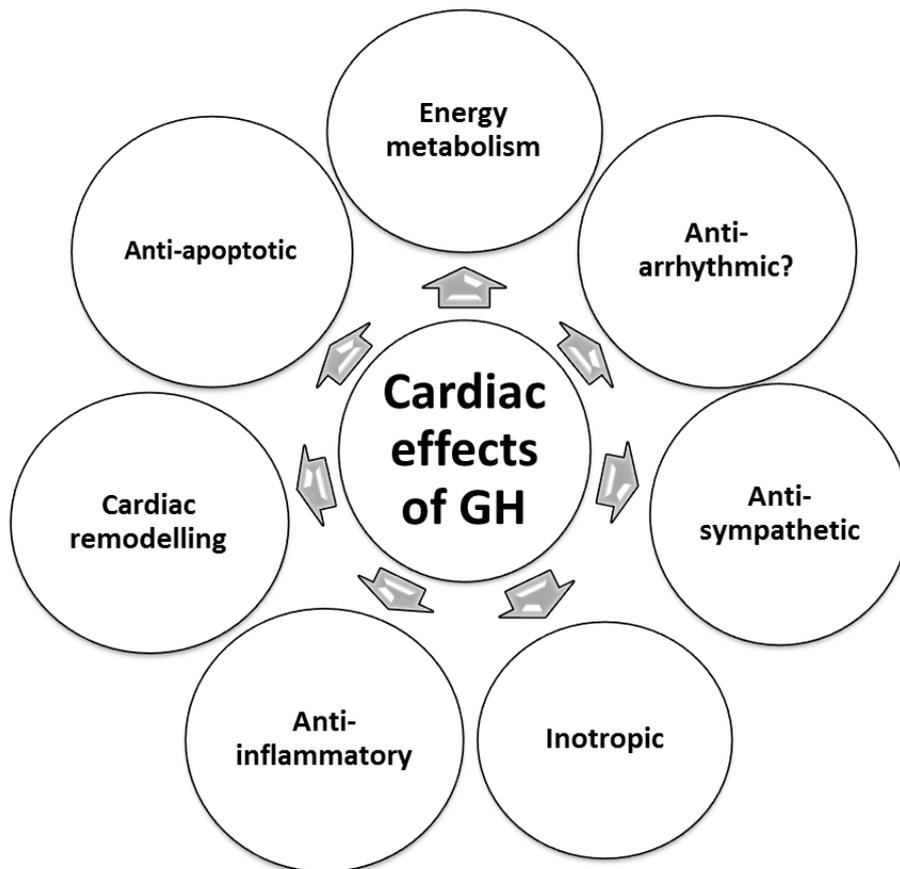


*Figure 6.* Postulated cardiac effects of growth hormone.

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The important role of GH for the human heart is strengthened by observations from patients with GH deficiency (GHD). GHD patients have an increased risk for cardiovascular disease and a Swedish retrospective study found that cardiovascular mortality was doubled in these patients compared to healthy controls[68]. Patients with GHD have reduced left ventricular mass and performance, which is more pronounced if the deficiency develops at a young age[69] and there is also evidence for increased sympathetic nervous activity in GHD patients[70]. Additional important effects of GHD on the cardiovascular system include central obesity[71] and an adverse lipid profile[72]. Interestingly there is a relationship between low IGF-1 levels and risk of IHD and HF in population studies[73, 74]. Supplementation of GH ameliorates many of the adverse cardiovascular effects seen during states of GHD[75]. Studies on GH excess have focused on observations from patients with acromegaly. Since acromegaly is related to hypertension, diabetes and coronary artery disease (CAD), the direct role of GH can be difficult to elucidate, since all of the aforementioned conditions have important negative cardiovascular effects. One consistent finding in uncomplicated acromegaly is increased LV mass and a hyperkinetic circulation[76]. Long standing acromegaly however induces interstitial fibrosis and myocardial necrosis[77]. As for GHD, correction of the GH imbalance in acromegaly ameliorates the cardiac abnormalities seen[78]. At least in the short term, GH seems to exert various positive effects on cardiac morphology and function. It has anti-remodeling effects after myocardial infarction and experimental HF[79, 80]. Previous research from our group has revealed positive effects of GH on myocardial energy status after experimental AMI that is associated with the upregulation of the creatine translocator in the heart[81, 82] in addition to decreased sympathetic activity [81, 83]. Clinical studies have shown that GH therapy can generate more mechanical work despite a lower oxygen consumption[84], which is substantiated by experimental work where GH seems to act as a calcium sensitizer[85]. GH may therefore be able to improve myocardial energy metabolism, a fact that may explain its positive effects in various experimental myocardial diseases. These promising experimental data and other clinical and experimental data showing positive effects on apoptosis[86] and inflammation[87] have prompted clinical trials of GH treatment (with or without concomitant IGF-1) in HF. The results have been inconsistent, however a metaanalysis of a total of 195 patients with HF treated with GH revealed positive effects on cardiac morphology and function in addition to improvement in NYHA function class[88].

Based on available preclinical and clinical data, there is strong evidence linking GH with the sympathetic nervous system in addition to other beneficial effects, including metabolic, structural and functional effects. However, there is limited knowledge on whether these effects may translate to positive GH effects on the electrical stability of the heart, i.e. being antiarrhythmic. As sudden death due to VA is a common mode of death in these patients, prevention of VA would be of great importance for improving long-term prognosis.


## *Atrial fibrillation*

The western world is experiencing an increase in the prevalence of AF, partly due to the aging of the population. AF is the most common sustained arrhythmia in humans and has a prevalence of 2-3%, translating to about 250.000 people in Sweden[89]. In addition to

sometimes very bothersome symptoms, patients with AF have a doubled mortality compared to their matched controls in SR[5] and the risk of ischemic stroke is almost five times higher in patients with AF[90]. As a consequence, the Swedish health care cost for the treatment of AF and its consequences has been estimated to be a staggering 7 billion Swedish krona (€700 million)[91].

The pathophysiology of AF is complex and involves electrical, structural, metabolic, contractile, neurohormonal and inflammatory remodeling of the atria[92-94]. Established risk factors for AF include age, sex, HF, valvular heart disease, hypertension and myocardial infarction[95]. More recently suggested risk factors include sleep apnea[96], chronic kidney disease[97], diabetes and obesity. Obesity has been identified in several recent studies as a risk factor for new onset AF, with an increased risk of approximately 50%[98-101], even though some early studies did not support this finding.[95, 102]. The contribution of diabetes to the risk of AF has been examined in a few studies with contradictory results[95, 103-105]. Interestingly, insulin resistance was not found to predispose to AF in a recent study from the Framingham research group[106], but another study reported that the levels of $HbA_1C$ were associated with the occurrence of AF[107]. Hence the debate regarding whether diabetes plays a role in the pathophysiology of AF is yet to be settled.

Studies in animals, supported by clinical findings, have shown that the arrhythmia is self-perpetuating[108]. This means that AF itself produces changes in the atrium that increase the susceptibility for the arrhythmia to become more persistent. Some of the remodeling processes are reversible upon the cessation of the arrhythmia, for example the electrical and contractile remodeling, related to down regulation of the L-type $Ca^{++}$ currents are rapidly reversible and are almost completely reversed within a few days upon restoration of SR[109]. Some changes are however more or less permanent, for instance the structural remodelling that is characterized by the loss of cardiomyocytes, increased fibrosis and loss of tissue integrity[110-113].

AF is often divided into paroxysmal, persistent and chronic forms. *Paroxysmal AF* consists of short episodes that spontaneously revert to SR. They often last a few hours, but can per definition last up to one week at a time. *Persistent AF* lasts more than one week at a time and frequently needs pharmacological or electrical cardioversion for conversion to SR. Finally, *chronic AF* is when the treating physician has accepted the arrhythmia as chronic, or when it is no longer possible to convert the patient to sustained SR.

The main symptoms of AF are palpitations, dizziness, tiredness, dyspnea and chest pain. *Figure 7* demonstrates an example of the irregularity and faster heart rate during AF, which causes the palpitations. Patients with symptomatic AF have a poor quality of life which may in part be due to the randomness of attacks, and that bouts of AF often start at times of minimal physical exertion such as during rest or sleep. This makes the attacks hard to avoid. The arrhythmia is frequently asymptomatic[114], but it has recently been demonstrated that patients with asymptomatic AF have similar risk of complications such as stroke[115], and may be at a higher risk for development of heart failure.

The treatment of AF is focused on prevention of thromboembolic sequelae and controlling the rate or rhythm. The indication for anticoagulation is based on risk factors such as age, HF, hypertension and previous thromboembolic history, rather than on the pattern of the arrhythmia or symptoms in a given patient. This has greatly improved the selection of patients

suitable for anticoagulation therapy. The choice on whether to pursue rate or rhythm control is also in largely based on underlying patient characteristics, such as age and co-morbidity in addition to symptoms.

Even though great advances have been made in the last decades there are still large gaps in the knowledge of the pathophysiology of AF, and increased understanding will be imperative for effective treatment strategies in the future.
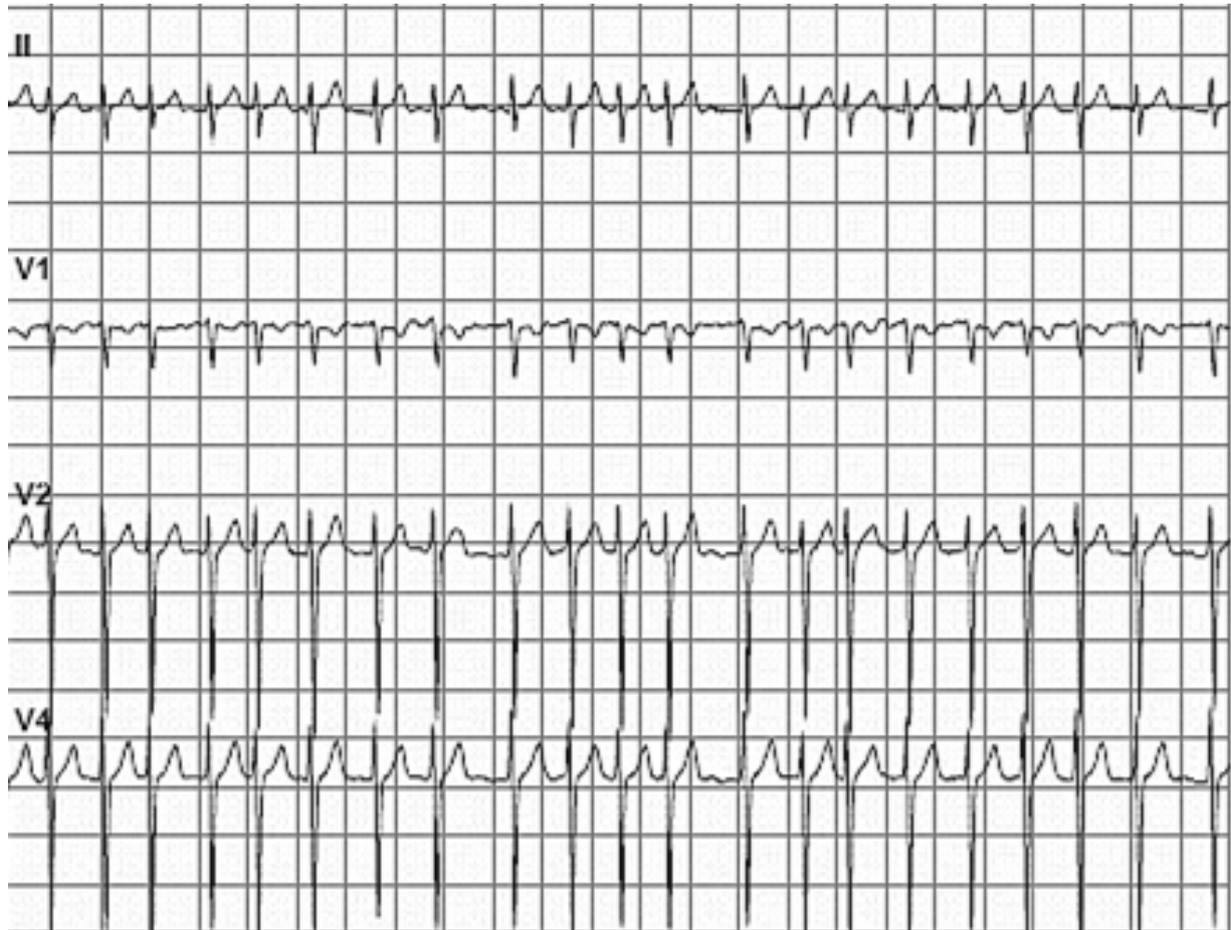


*Figure 7.* *An electrocardiogram (ECG) from a patient with persistent AF. Note the irregularity of the QRS complexes and the accelerated rate (approx. 125/min). In addition there are note discernible P waves seen, but rather an undulating baseline between the QRS complexes. The sweep of the ECG is 12,5 mm/s.*

# AIMS OF THE THESIS

## I.

To investigate the acute and chronic effects of complete heart block on cardiac function, morphology and energy metabolism.

## II.

To investigate the effects of growth hormone on cardiac arrhythmogenesis.

## III.

To investigate the electrophysiological effects of lysophosphatidylcholine on atrial cardiomyocytes.

## IV.

To investigate whether atrial fibrillation is associated with development of lipotoxicity in human atria.

# MATERIALS & METHODS

Detailed descriptions of the materials and methods are given in each individual paper. In this chapter there will be a more focused description of certain methods.

## *The rat model (Paper I and II)*

Male Sprague-Dawley rats were used for the experiments in both these papers. In *Paper I* slightly younger animals were used, weighing 220–240 g, compared to *Paper II* where they were slightly larger, weighing approximately 350 g. All animals were fed with standard rat pellets and tap water *ad libitum* and housed in cages in groups of two-five animals, at 26°C with 60% humidity and a 05.00–19.00-h light regimen. During the experiments the animals were anesthetized with isoflurane and orally intubated. They were connected to a mechanical respiratory support (Carlsson ventilator, Astra Zeneca, Mölndal, Sweden) and to a three lead ECG by connecting standard ECG electrodes to three paws, visualized on-line by an oscilloscope and downloaded to a computer for later off-line analysis by Biopac® acquisition software. The chest was shaved and the heart was accessed via a mid-line *(Paper I)* or a left lateral *(Paper II)* thoracotomy. After a successful operation, the thoracotomy was closed by interrupted sutures and the lungs were hyperinflated by applying positive end-expiratory pressure. Before awakening, all animals received post-operative opioid analgesia in addition to atropine.

In *Paper II*, the animals were divided into three groups. The first group was pre-treated with amiodarone, the second group was pre-treated with human recombinant GH and finally one group received an injection of saline and served as the control group. For all animals the administration of the drugs was performed 6 hours prior to the induction of AMI or invasive programmed stimulation.

## *Induction of complete heart block (Paper I)*

After accessing the pericardium, we retracted the right atrial appendage and exposed the aortic root. There is a fat pad between the aortic root and right atrium that serves as a landmark for access to the AV node. The AV node is an endocardial structure and has to be approached by puncturing the right atrial wall. We initially followed the description given by Lee et al[35], where 30µL of 70% alcohol was injected via a thin needle to the area of the AV node. However, we found this method to be unsuccessful for the induction of permanent CHB. Only 1/20 animals developed chronic CHB even though far more alcohol than described in their paper was used. For some animals only transient block was seen. We therefore abandoned this method and employed a diathermy in a similar manner. With this approach, an instant and permanent CHB was accomplished in all animals *(Figure 8)*. This method however has the drawback that serious hemorrhage may occur when retracting the diathermy needle from the right atrial wall, and some animals did not survive the operation due to extensive bleeding. All the animals with CHB after the operation maintained this for

the remainder of the study. No animal died during follow up, but one animal had to be re-operated due to a wound infection in the thoracic wall.
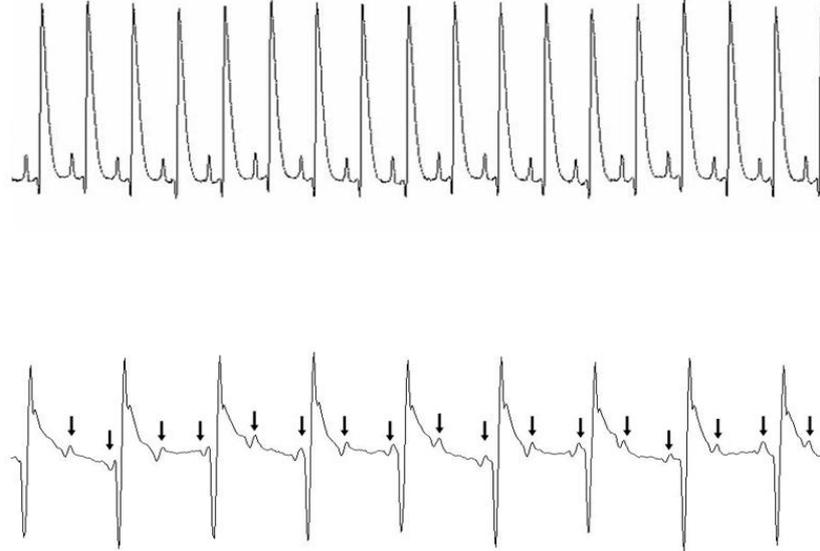


*Figure 8. Example of electrocardiogram recordings.*
*The above recording is an example of sinus rhythm. In the rat there is no clearly discernible ST segment as the QRS komplex and T wave are fused in a triangular shape. In the lower recording there is atrioventricular dissociation (complete heart block) with the P-waves (arrows)dissociated from the broad complex ventricular escape rhythm.*

## Induction of myocardial infarction (Paper II)
After gaining access to the anterolateral heart wall, the trunk of the pulmonary artery and left atrial appendage are easily identified. A suture was placed between the two structures, where the proximal left anterior descending artery (LAD) is located while looking for characteristic changes confirming ischemia, such as typical ECG changes and visual signs of wall motion disturbance in the anterolateral LV free wall. This procedure typically results in acute ischemia of approximately 40% of the left ventricle. All animals were sacrificed after the duration of the study protocol, and were not allowed to wake up.

## Ischemic arrhythmia analysis (Paper II)
A continuous recording of the limb ECG was presented, recorded and stored on Biopac® acquisition software for later off-line analysis. The animals were monitored before occluding the LAD and up to 60 min after induction of AMI. The arrhythmic events were classified according to the Lambeth Convention[116]. Ventricular tachycardia (VT) was defined as >3 consecutive broad complex beats. VF was defined as rapid ventricular activity with changing

ECG configuration and the absence of recordable pressure measurements *(Figure 9)*. A previously validated arrhythmia score was used[117], that takes into account not only the seriousness of the arrhythmia, but also the timing and consequence of it. This means that a VF that leads to death after a few minutes will get a high score contrary to a VF occurring later or spontaneously converting which will be attributed a lower score, although the arrhythmia itself is the same (VF).
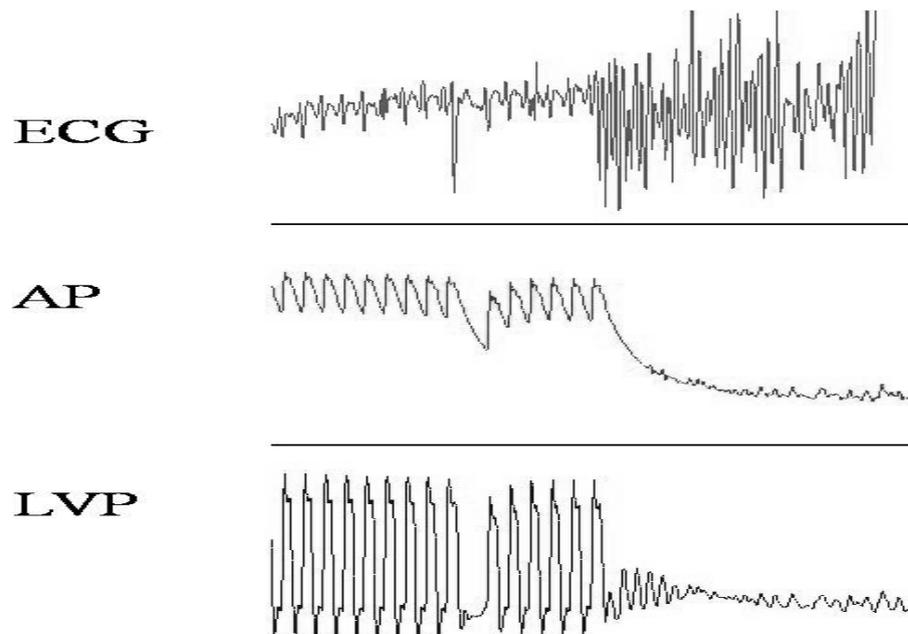


*Figure 9. An example of an ischemic VF with subsequent loss of ventricular and arterial pressure. ECG=electrocardiogram, AP=arterial pressure, LVP=left ventricular pressure.*

### *Invasive electrophysiological stimulation protocol (Paper II)*

An 8 polar electrophysiology catheter (Millar instruments) 1.1 French ($\varnothing$=0.36 mm) was introduced via the right internal jugular vein into the right atrium and RV. The catheter was slightly bent at the end for easier access to the RV *(Figure 10)*. The correct placement was verified by the intracardiac ECG recording. Stimulation and recording was performed on a custom made hardware/software developed by Astra Zeneca (Mölndal, Sweden).

After placement of the catheter in the chamber of interest, the threshold potential for stable pacing was assessed at a cycle length of 100ms. The programmed stimulation study was conducted at two times the threshold. Ventricular effective refractory period ($VERP_{100}$) was assessed by introducing a single extrastimulus (S2) after a train of 8 paced beats (S1) with a S1-S1 interval of 100ms. S2 was initially set at 60ms and successively lowered by 2ms at a time until loss of capture, which equals $VERP_{100}$.

For double extrastimuli, S3 is then introduced after S2. The coupling interval of S2 and S3 were set at $VERP_{100}$+20ms, and at first S3 was lowered by 2ms at a time until loss of capture and then set at the shortest coupling interval with maintained ventricular capture. S2 was then lowered at 2ms at a time until loss of capture, taking care of not losing capture of S3.

Similarly, we introduced the third extrastimulus; S4 at the same coupling interval as above, i.e. VERP$_{100}$+20ms and so S2, S3 and S4 were all set at this level. The procedure was then repeated as for the double extrastimuli study.
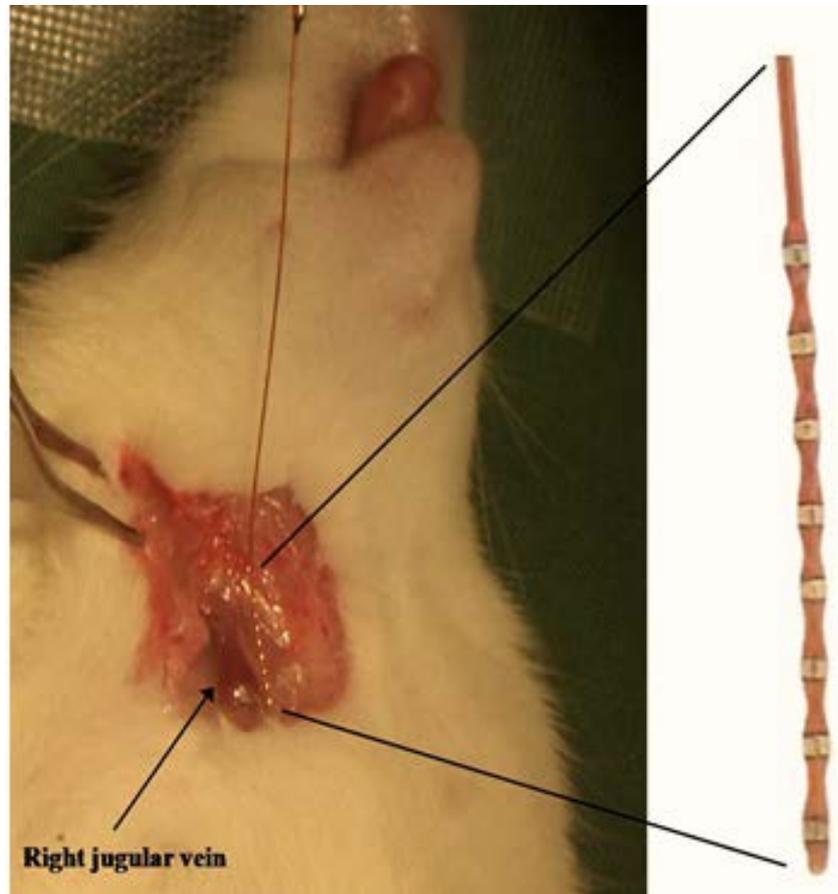


***Figure 10.** Demonstration of the electrophysiology catheter.*
*The right jugular vein has been accessed surgically just above the clavicle. The 1.1Fr electrophysiology catheter is seen beside the vein and can be seen magnified to the right, were the 8 poles are readily visualized. Adapted from Råmunddal,T. 2008. Myocardial metabolism in experimental infarction and heart failure. Doctoral Thesis. Sahlgrenska Academy, Gothenburg University.*

The arrhythmia score for the induced arrhythmia was different from that of the AMI sub-study. An arrhythmia score was given using a binary system (VT/no VT), where a significant VT was defined as more or equal to 6 beats *(Figure 11)*. A VT of less than 6 beats was considered non-significant. After the first stimulation protocol, isoprenaline was then given subcutaneously (0.02 mg/kg) to increase the heart rate 10-20%, and the stimulation protocol was then repeated.

After ventricular stimulation, the catheter was placed in the right atrium and a new threshold was determined. There we performed burst pacing at 5x threshold, 1ms pulse width and S1-S1 of 10ms (100 Hz) for a duration of 10s. After burst pacing was stopped we used the intracardiac recording to determine the supraventricular rhythm and the total time of

arrhythmia duration *(Figure 12)*. Similarly, the observed arrhythmia was scored using a binary system, where an atrial tachycardia/fibrillation lasting more than 10s was considered significant. All atrial stimulation was performed under constant isoprenaline infusion (0.02 mg/kg).
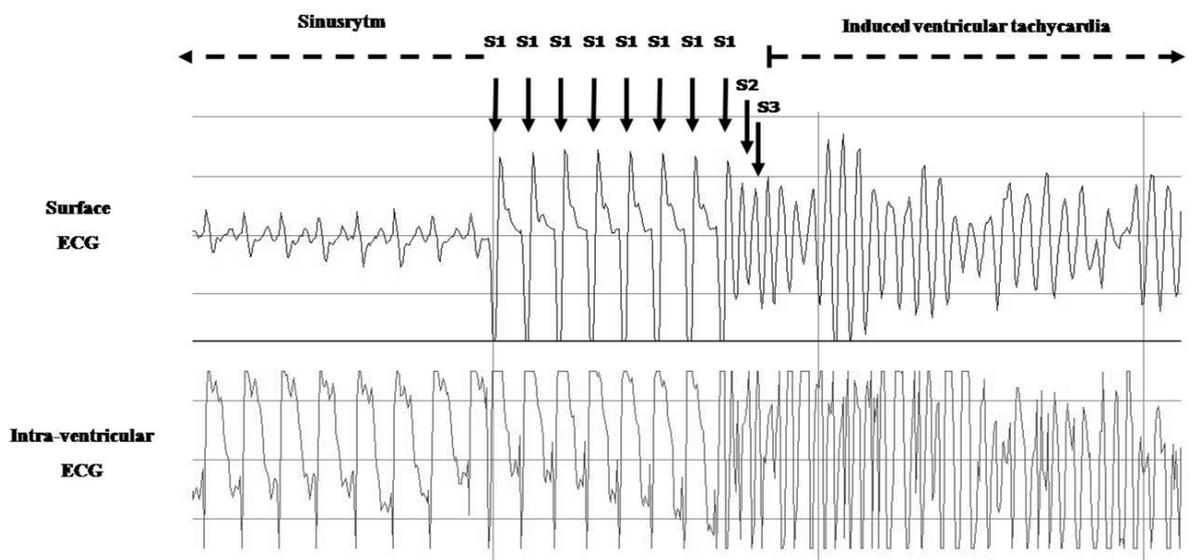


***Figure 11**. Example of the induction of ventricular tachycardia (VT).*
*To the left there is initially sinus rhythm, with the corresponding intracardiac recording from the right ventricle.*
*Then there is a train of 8 paced beats (S1), followed by double extrastimuli (S2 and S3) with subsequent*
*induction of polymorphic VT.*
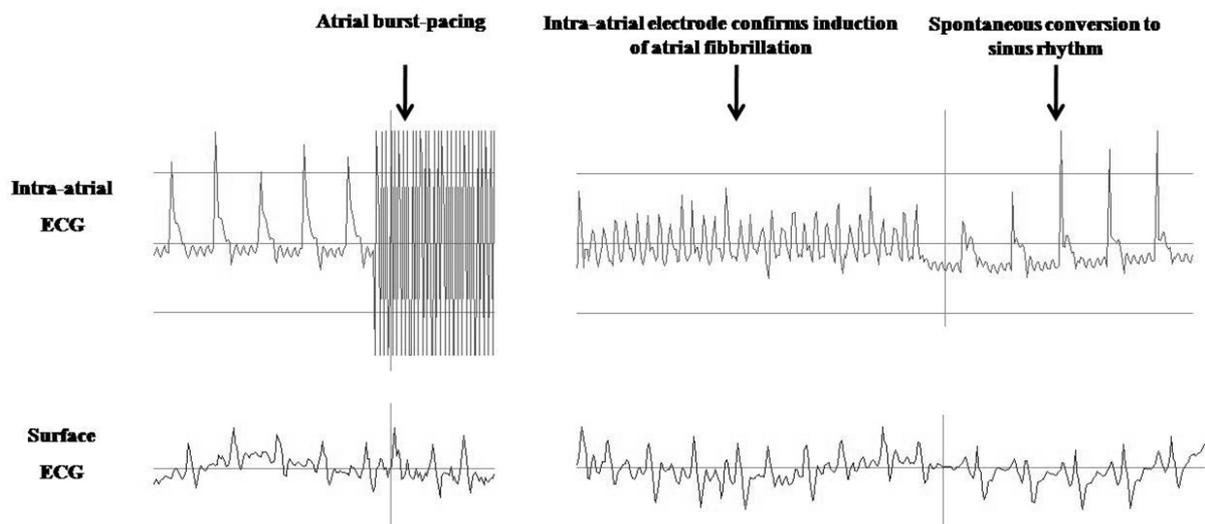


***Figure 12.** Example of the induction of atrial fibrillation.*
*The left panel shows a surface ECG with clearly demonstrable p-waves and above the corresponding*
*intracardiac recording from the right atrium showing large atrial signals, followed by 100Hz burst pace. The*
*right panel demonstrates an irregular ventricular rhythm on the surface ECG without discernible p-waves and*
*the intracardiac recording confirms a very rapid atrial rhythm (atrial fibrillation) with a subsequent*
*spontaneous conversion to sinus rhythm.*

## Echocardiography (Paper I)

Prior to the echocardiography study, the rats were anesthetized with isoflurane and kept asleep for the procedure by spontaneously breathing a mixture of isoflurane (0.4-0.6%) and 70% $O_2$. The echocardiogram was performed with a standard pediatric 15 MHz linear transducer and the images were recorded on a HDI 5000 (Philips Medical Systems) and analyzed off-line with EchoPac 5.4 (General Electric, Vingmed Ultrasound, Horten, Norway). Standard projections included 2D parasternal short axis views of the LV at the level of the papillary muscles, where M-mode recordings of the LV were obtained. The M-mode was used for calculating the left ventricular systolic and diastolic dimensions in addition to anterior and posterior wall thickness *(Figure 13)*. From these measurements, ejection fraction and left ventricular mass was derived, using previously described standard methods[118].
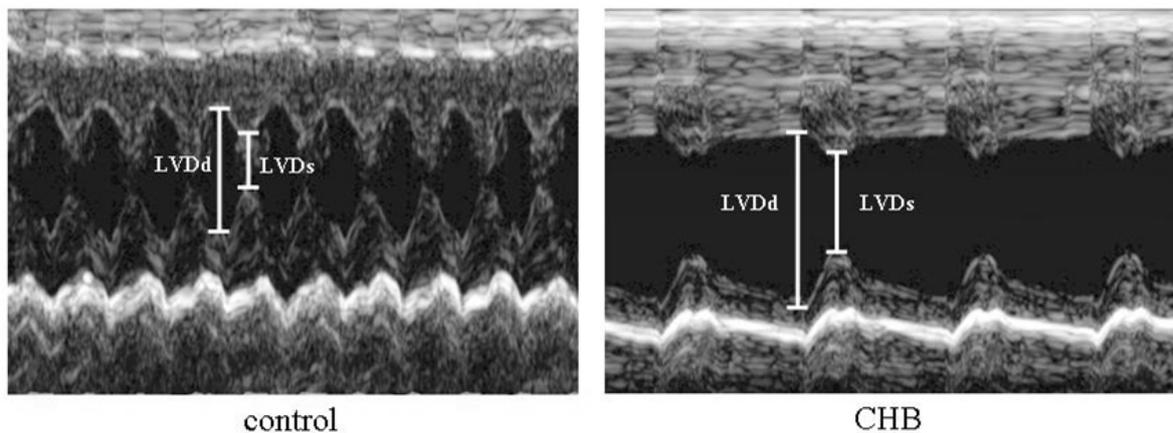


**Figure 13.** *Parasternal M-mode from the mid-basal left ventricle.*
*On the left side there is a recording from a control animal in sinus rhythm, and on the right side from an animal with complete heart block (CHB). The sweep of the recording is the same and the difference in rate and internal dimensions is clearly seen. LVDd=Left ventricular dimension in diastole, LVDs=Left ventricular dimension in systole.*
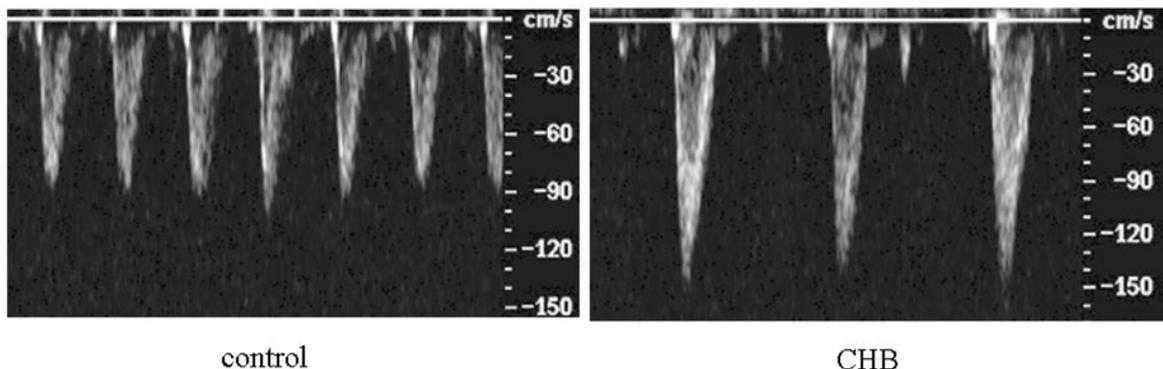


**Figure 14.** *Pulsed-wave Doppler from the right ventricular outflow tract*
*On the left is an example from an animal in sinus rhythm (control) and to the right from an animal in complete heart block (CHB). The sweep of the recording is identical. Note the slower rate and larger velocity/time integral in the CHB animal, indicative of a larger stroke volume.*

The right ventricular outflow tract (RVOT) was targeted for measurements with pulsed Doppler, for estimations of stroke volume and cardiac output. This was done by measuring the velocity time integral (VTI) and the diameter of RVOT and applying standard clinical formulas *(Figure 14)*. The rationale for using the RVOT as opposed to the left ventricular outflow tract is that the angle for Doppler registrations in the rat is far better for the RVOT. Similarly the measurement of the diameter is easy to perform.

### *Invasive hemodynamics (Paper I and II)*
In order to obtain hemodynamic data from the RV and LV, the chamber of interest was punctured with a 21G needle connected to a standard clinical fluid filled pressure transducer system. In *Paper II* the LV was cannulated for the whole duration of the protocol, but in *Paper I* it was only used for the terminal phase of the study. For arterial pressure measurements, the carotid artery was surgically identified and instrumented *(Paper II)*.

### *Quantifications of creatine and energy rich phosphometabolites (Paper I)*
After the pressure measurement we excised the apical part of the LV and this was freeze clamped, put in liquid nitrogen and stored at -80°C pending analysis. The myocardial samples were homogenized on ice and extracted with 0.5 M perchloric acid. An aliquot of the homogenate was neutralized with 1M potassium hydroxide (KOH), centrifuged for 6 min at 5500 rpm at 4°C. After filtrating the supernatant it was injected into the high performance liquid chromatograph (HPLC) (SMART system Pharmacia, Uppsala, Sweden) as previously described[119]. The column used for analysis was a Luna 5u C18(2) column (Phenomenex). The high energy metabolite content was related to total protein content of each sample, which was measured from the homogenate by means of a BCA protein assay reagent kit. The measured metabolites were CrP, Cr, AMP, ADP, ATP and total adenine purine pool (TAN).

### *Murine cardiomyocytes and multielectrode array recordings (Paper III)*
For this paper we used an immortalized murine atrial cardiomyocyte cell line, initially isolated from an atrial tumor (HL-1). The cells were donated to us by Dr. Claycomb (Louisiana State University Medical Centre, New Orleans, LA). The cells have been extensively characterized and express receptors and ion channels similarly to normal murine atrial cardiomyocytes[120], This includes $Na^+$, $K^+$ and $Ca^{++}$ channels and the $Na^+/Ca^{++}$ - exchanger. The cells were seeded on sterile gelatin coated multielectrode array (MEA) plates and were allowed to grow for three days until confluent and homogenous growth was observed in addition to spontaneous beating activity. Plates with overgrowth, insufficient growth or lack of beating activity were discarded. The standard substrate-integrated MEA culture dish contains 60 Titanium Nitride coated gold electrodes (30µm diameter) arranged in an 8x8 electrode grid with an inter-electrode distance of 200 µm. The system offers a unique possibility to non-invasively study extracellular recordings from 60 sites simultaneously at a sampling rate of 10 kHz *(Figure 15)*. The recording system has an integral heating device that maintains the temperature stable.
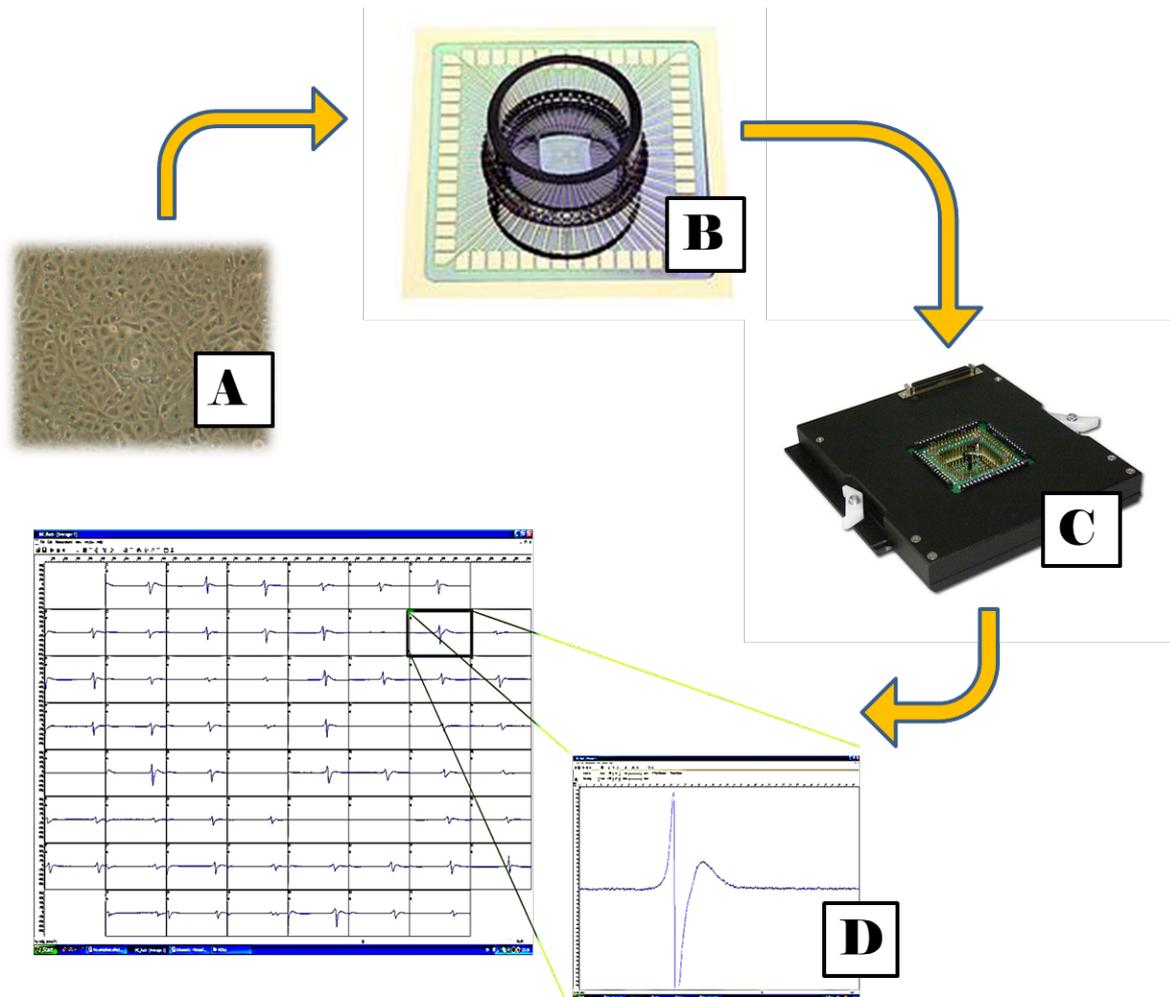
*Figure 15. Experimental setup with the multielectrode array (MEA)*
*The HL-1 cells (A) are cultured on the MEA (B) for several days until continuous growth and spontaneous beating is observed. During the experiments the MEA plate is connected to an amplified unit with integrated heating device (C) and the signal is exported to a computer and presented as shown in (D).*

All data were analyzed off-line using dedicated software, MC Rack version 3.9 (Multi Channel Systems, Reutlingen, Germany) or a customized toolbox programmed with MATLAB (The Mathworks, Natick, MA, USA). The following parameters were analyzed as previously described[121] : beating rate (BR), FP duration ($FP_{dur}$), rise time of FP upstroke ($FP_{rise}$) and peak-peak FP amplitude ($FP_{AMP}$). Propagation velocity was evaluated in accordance to standard methods[122], and we included the MEA plates that had initial signals (pacemaker zones) lying at one of the four edges of the cubic 8x8 electrode matrix. The total propagation distance was a constant of around 1400 µm. Propagation velocity was then evaluated in the form of propagation time, i.e. the time required for signals propagating from the "pacemaker" edge to the opposing edge of the electrode matrix.

The cell culture medium was mixed with increasing concentrations of lysophosphatidylcholine (LPC) and heptanol. A flowchart for the LPC experiments is shown below in *Figure 16*, where a washout of medium containing LPC was performed between

neighboring concentrations. The experimental setup also included pre-treatment with isoprenaline and atropine, prior to the LPC challenge.
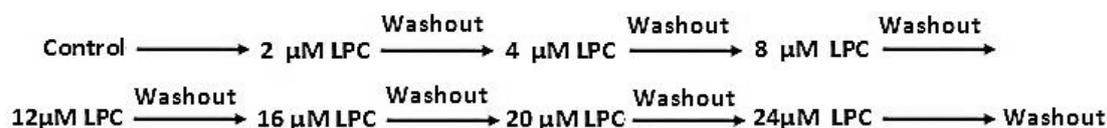


**Figure 16.** *Flowchart of experiments with lysophosphatidylcholine (LPC).*

## *Human atrial biopsies and lipidomics (Paper IV)*

Biopsies were obtained from 54 patients scheduled for cardiac surgery for coronary artery disease, valvular disease, thoracic aortic aneurysm or AF. Two biopsies were lost, leaving 52 biopsies available for analysis. The patients were divided into three groups: A) *no history of AF*, B) *history of paroxysmal AF* and C) *history of persistent AF*, but for some analyses we used only dichotomized values (AF/no AF). The biopsies were harvested from the right atrial appendage while the surgeon was cannulating the right atrium as a standard procedure before commencing extracorporeal circulation. The biopsies were immediately put in liquid nitrogen and stored at -80°C pending analysis. Heart tissue (20-50 mg) was homogenized in methanol. Lipids from homogenized heart tissue were extracted using the Folch procedure[123]. Heptadecanoyl (C17:0) -containing internal standards of phosphatidylcholine (PC), phosphatidylethanolamine (PE), DAG, sphingomyelin (SM), lysophosphatidylcholine (LPC) and CER were added during the extraction. The extracts were evaporated using nitrogen, reconstituted in chloroform:methanol [2:1] and stored at -20ºC. Cholesteryl esters (CE), triacylglycerols (TAG) and free cholesterol (FC) were quantified using straight-phase HPLC with evaporative light scattering detection according to previous work[124].
Phospholipids, SM and LPC were quantified as described by Ekroos et al[125] using direct infusion on a  QTRAP 5500® mass spectrometer (AB Sciex, Concord, Canada, ) equipped with a robotic nanoflow ion source NanoMate HD (Advion Biosciences, Ithaca, NY, USA). Cholesteryl ester profiles were also attained using direct infusion as previously described[126]. DAGs and CER were purified using straight-phase HPLC prior to quantification. DAGs were quantified using precursor ion scanning[127] and CER using HPLC/MS/MS[128]. Mass spectrometry data files were processed using Lipid Profiler™[129] (*Figure 17*). The amount of cholesterol ester (CE), triacylglycerides (TAG), free cholesterol (FC), DAG, CER, phosphatidylethanolamine (PE), phosphaditylcholine (PC), sphingomyelin (SM) and lysophosphatidylcholine (LPC) was measured in nmol/mg tissue. Analysis of composition of different lipid subspecies was presented as mol%.
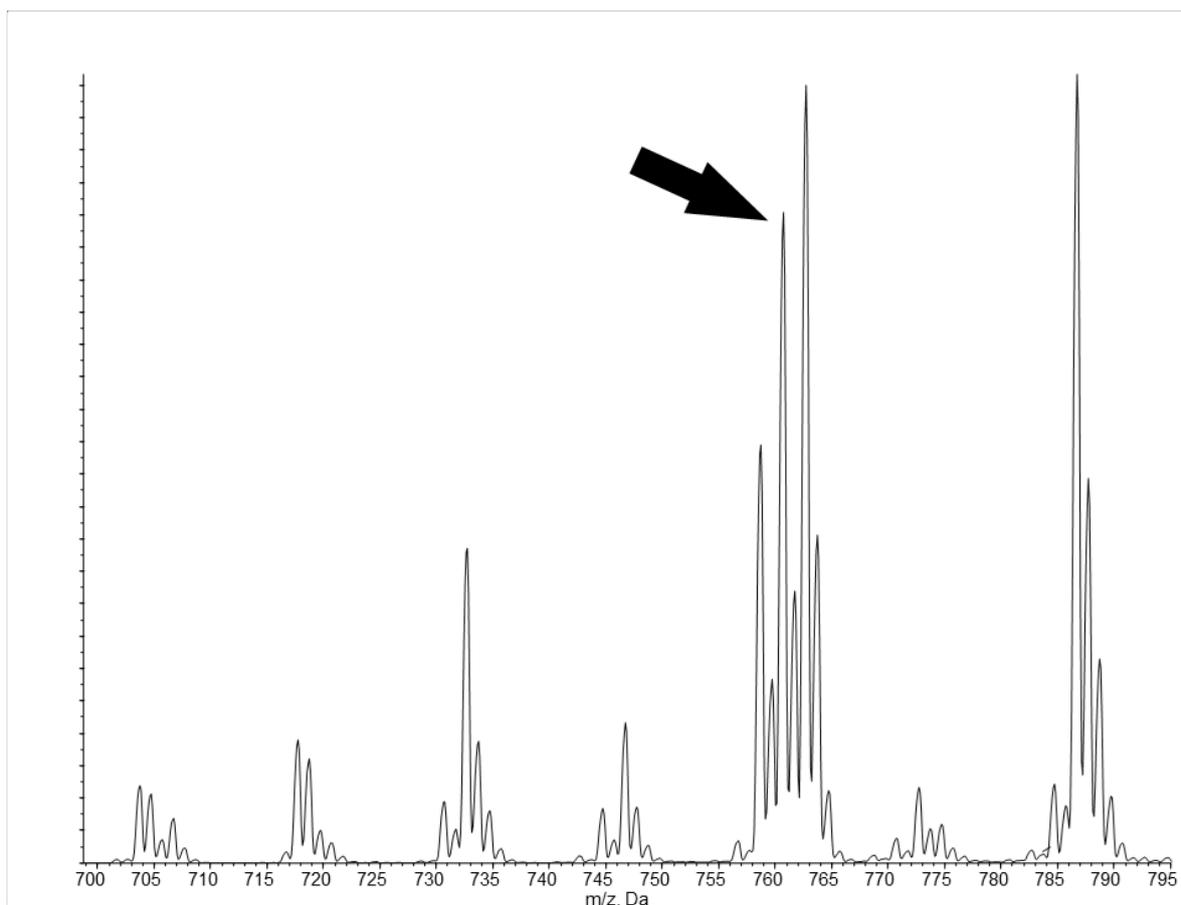
*Figure 17. Example of spectral profiles obtained by lipid class specific precursor ion scanning. The X-axis represents the mass-to-charge ratio (m/z) in Dalton (Da) and the Y-axis correlates to the relative abundance of the particulate species. In this example demonstrates the composition of phosphatidylcholine (PC) species. For example the peak m/z 760 (arrow) is PC 36:1, which is relatively abundant in cardiac tissue. This is a PC that has a total of 36 carbons with 1 unsaturated bond. Further analysis is then needed to see whether the PC is 18:0/18:1 or 16:0/20:1, etc.*
*Courtesy of Marcus Ståhlman, Wallenberg laboratory, Sahlgrenska University Hospital, Gothenburg.*

## *The RIKS-HIA register (Paper IV)*

The Register of Information and Knowledge About Swedish Heart Intensive Care Admissions (RIKS-HIA) includes all patients admitted to hospitals with coronary care units (CCUs) in Sweden. The register started in 1991 and became a national quality register in 1995. After that, all hospitals that admit cardiac patients report to the register. The current study was conducted on a population from western Sweden (Västra Götaland), which has a population of approximately 1,5 million inhabitants and is served by ten hospitals that report to the register. A number of patient variables are reported at admission, including height, weight, diabetes, smoking history, previous cardiovascular disease, current medication, blood pressure, pulse and ECG rhythm. The full protocol has been previously published[130] and is available on-line (http://www.ucr.uu.se/rikshia). In the register, AF is a combined variable including both AF and atrial flutter with no distinction made between the two. All data is entered in a prospective manner and all diagnoses at discharge are registered.

Due to limitation in data quality (missing data) in the early years of the register, we chose to limit the study to the years 2006-2011, were there was far less missing data, and the treatment of acute coronary syndromes and AF is similar to present standards of care.

### *Ethics*

All investigations concerning humans and laboratory animals (*Papers I,II and IV*) were approved by the local ethics committee at the University of Gothenburg. Care was taken to limit the number of animals used and terminal experiments were preferred when possible.

# RESULTS

This chapter summarizes the main results from the papers. The full results are given in each individual paper.

## *Paper I*

This paper describes the effects of CHB on cardiac morphology, function, hemodynamics and energy metabolism over a relatively long-term period of three months. In addition we have described a novel method for the induction of CHB.

After induction of CHB, the atrial rate in both groups was similar, but there was an approximately halved ventricular rate in the CHB animals. The animals adapted to this by a large increase in left ventricular diameter (and volume) and after 3 weeks, this was followed by a profound increase in left ventricular mass *(Figure 18)*. The hypertrophy observed was eccentric and there was no difference in wall thickness between the groups. The stroke volume of the CHB animals was twice that of the controls over the duration of the study *(Figure 19)*, thus compensating for the lower ventricular rate thereby maintaining cardiac output. Parallel to the observed remodeling we noticed a gradual decrease in the fraction of shortening, a measure of the contractility. The animals grew normally and showed no signs of distress when observed in the cages.
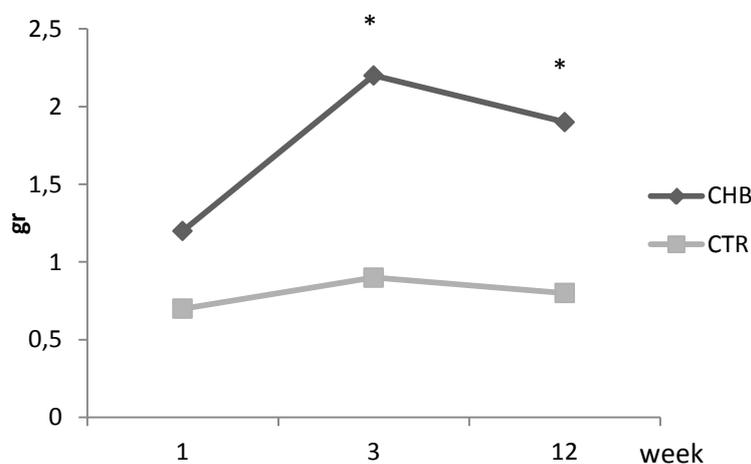


*Figure 18. Myocardial mass calculated from M-mode echocardiogram data was significantly increased at 3 and 12 weeks. CHB=complete heart block, CTR= controls, *= P<0.05 vs. controls.*

After 12 weeks the animals were examined by invasive pressure measurement in both ventricles. There were no observed differences in the systolic and diastolic intracardiac pressures between the groups. We found no evidence of altered ventricular metabolism based on measurements of the levels of Cr and high-energy phosphometabolites *(Figure 20)*.

## Conclusion

CHB in rats leads to early, pronounced and sustained remodeling with development of eccentric hypertrophy not associated with LV dysfunction or disturbed energy metabolism.
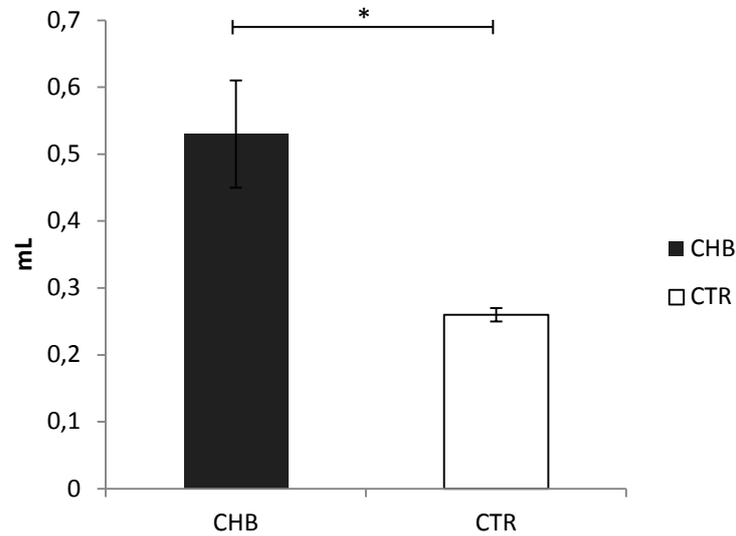


*Figure 19. Stroke volume estimated by echocardiography at 12 weeks. CHB=complete heart block, CTR= controls, *= P<0.05.*
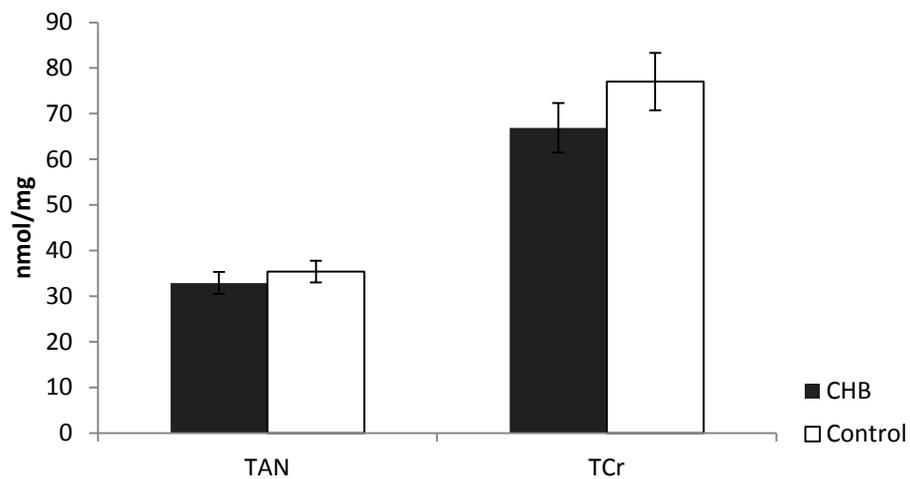


*Figure 20. Myocardial content of total adenine nucleotides (TAN) and total creatine (TCr) did not differ significantly at 12 weeks between the controls and the animals with complete heart block (CHB).*

## *Paper II*

This paper evaluated the effects of growth hormone (GH) on the occurrence of VAs in the setting of experimental AMI and in a separate model we investigated the inducibility of atrial and ventricular arrhythmias.

### *AMI substudy*

The heart rate prior to induction of AMI was lower in both the GH and amiodarone pre-treated rats as compared to the control group. There were no differences in the hemodynamic parameters, such as left ventricular systolic and diastolic pressure at baseline and post AMI induction. The animals pretreated by amiodarone and GH had significantly lower arrhythmia scores after AMI induction as compared to controls *(Figure 21)*. For the control group, all deaths occurring during the study were due to VAs while for the amiodarone and GH group approximately half of the deaths were due to progressive left ventricular pump failure.
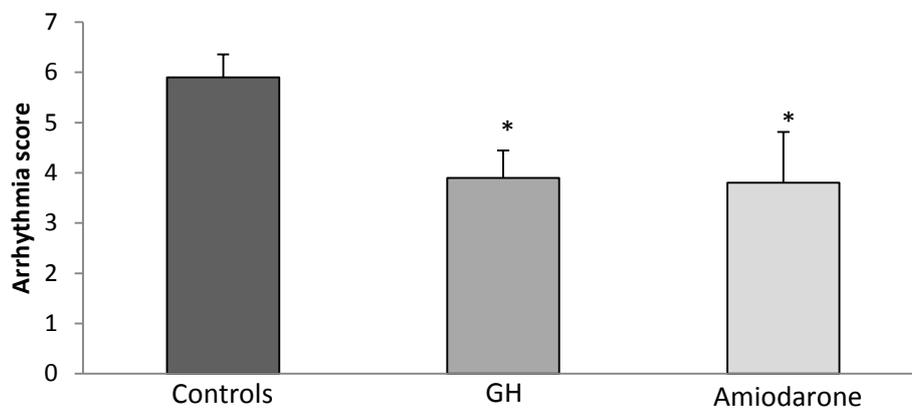


*Figure 21. The incidence and seriousness of ventricular arrhythmias was decreased by the pretreatment with growth hormone (GH) and amiodarone in the setting of acute myocardial infarction. \*= P<0.05.*

### *Programmed stimulation substudy*

The inducibility of VT was lower in both amiodarone and GH pre-treated animals as compared to controls *(Figure 22)*. One-third of the animals in the GH and amiodarone groups developed VT in contrast to 81% of the control animals. Amiodarone, but not GH decreased the inducibility of AF as compared to controls *(Figure 23)*. There were no significant differences in the electrocardiographic variables, such as PR interval, QRS duration and corrected QT interval, across the three groups.

### *Conclusion*

GH demonstrates antiarrhythmic properties in the setting of AMI and prevents induced VA, but not AF. This further adds to the concept of GH being a promising treatment in myocardial infarction and HF.
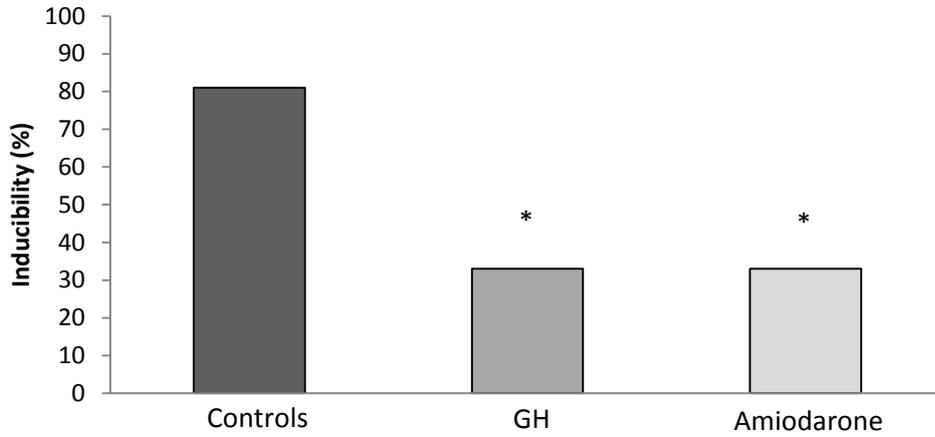
***Figure 22.*** *The inducibility of ventricular arrhythmia was decreased in the amiodarone and growth hormone (GH) pretreated animals. \*= P<0.05.*
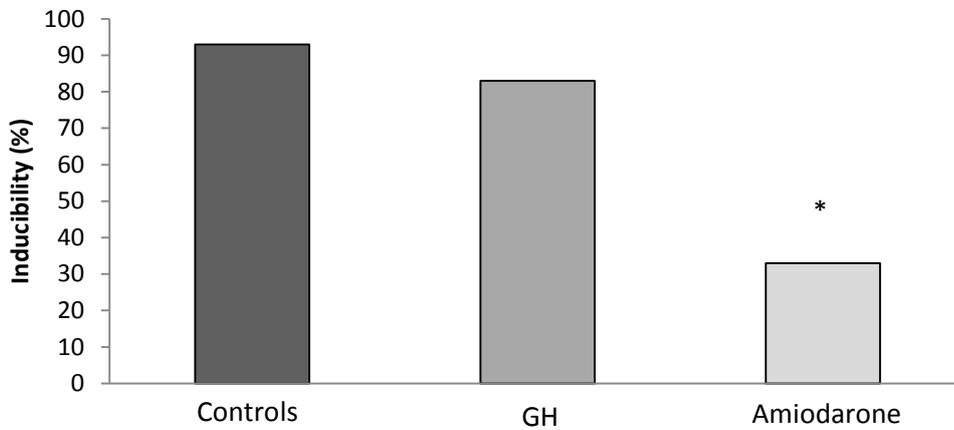


***Figure 23.*** *The inducibility of atrial fibrillation was decreased in the amiodarone, but not the growth hormone (GH) pretreated animals. \*= P<0.05.*

## Paper III

Lysophosphatidylcholine (LPC) exerted pronounced electrophysiological effects on the HL-1 cardiomyocytes. The spontaneous beating rate (BR), amplitude of the field potentials (FP) and propagation velocity all decreased significantly. An example is the concentration-effect of LPC on the amplitude of the FPs as shown in *Figure 24*. The duration of the FPs *(Figure 25)* and FP rise time was significantly prolonged. In all aforementioned parameters, we noted an initial trend in the opposite direction to the main findings at concentrations of LPC less than 8 µM. We also demonstrated slowed signal propagation by LPC, which was similar to that observed by heptanol, which is a known blocker of gap junctions *(Figure 26)*.
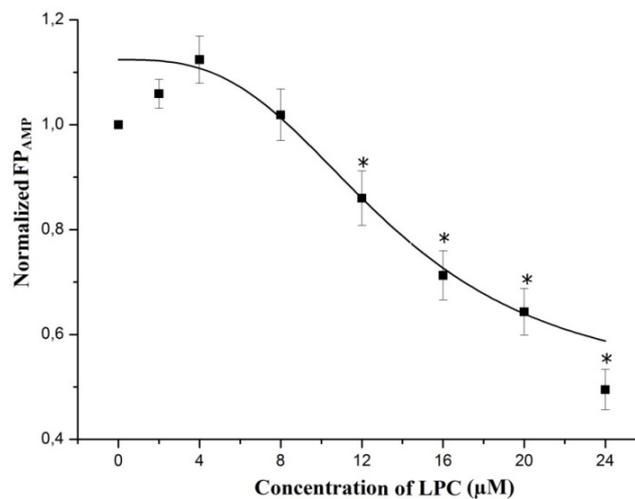


*Figure 24. Effects of lysophosphatidylcholine (LPC) on field potential amplitude (FP$_{AMP}$). Depicted is a concentration-effect curve fitted with a Boltzmann function. * P<0.05 compared with baseline.*
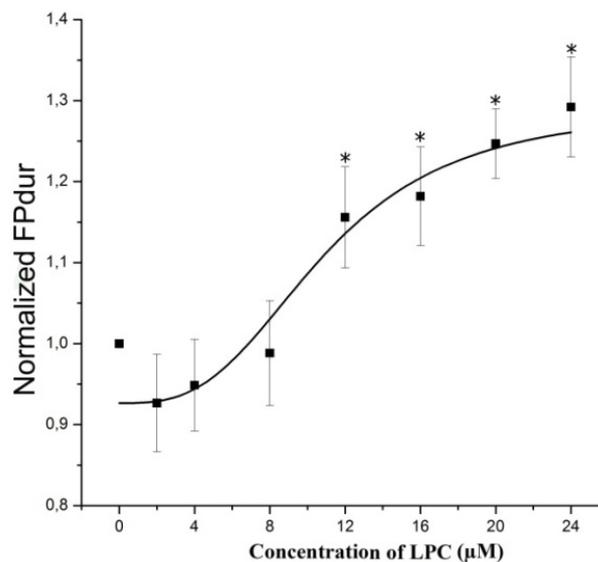


*Figure 25. Effects of lysophosphatidylcholine (LPC) on field potential duration (FP$_{dur}$).*
*Depicted is a concentration-effect curve fitted with a Boltzmann function. * P<0.05 compared with baseline.*

In order to examine whether LPC might exert some of its effects by modulation of the β- or muscarinic receptors, we performed additional experiments by pre-treating the cells with the β-adrenoceptor agonist *isoprenaline* and the muscarinic antagonist *atropine*. We noticed that both these substances attenuated the LPC-induced delay in signal propagation, while no apparent effects were observed on LPC-induced changes of the FP parameters.

*Conclusion*

LPC induces several electrophysiological alterations in murine atrial cardiomyocytes. An important advantage of the method is the possibility to analyze the net-effects of all cellular events elicited by experimental perturbation (e.g. caused by addition of an exogenous substance) on electrical properties, frequency, and rhythm. This provides the possibility to perform high-throughput screening of different pro-arrhythmic and anti-arrhythmic substances. The information gathered from this model can be complementary to that obtained from the existing *in vitro* cellular models and *in vivo* animal models.
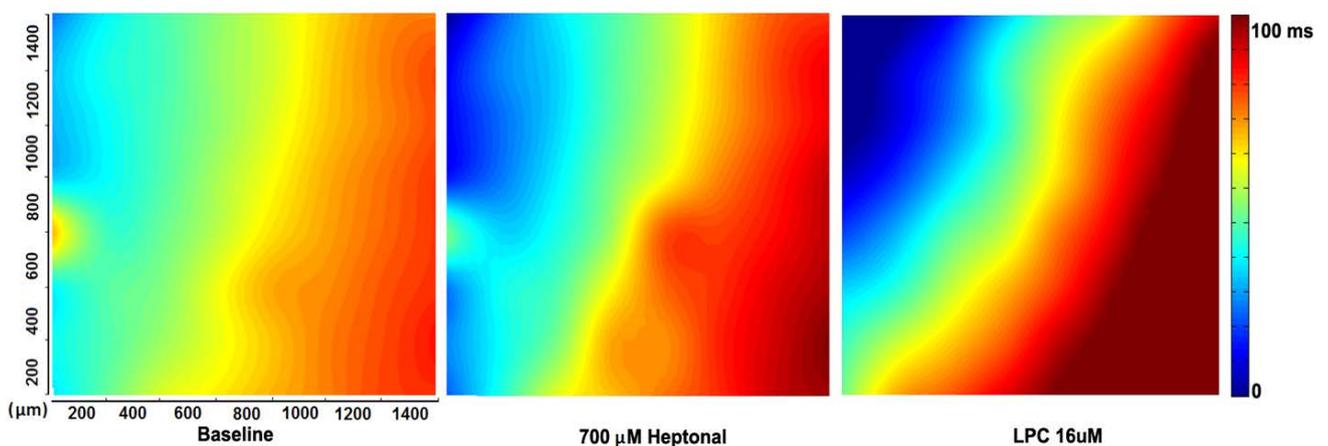


*Figure 26. Representative colour maps demonstrating the electrical signal propagation in the HL-1 cell culture at baseline (left) and slowed propagation induced by heptanol (centre) and lysophosphatidylcholine (LPC), giving rise to a larger area of late propagation (deep red).*

## *Paper IV*

In this paper our hypothesis was that obesity and diabetes confer a higher risk to develop AF and that this predisposition is mediated by lipotoxicity. The paper consists of two parts.

### *RIKS-HIA registry substudy*

During the period of 2006-2011 there were 36,682 consecutive patients admitted to cardiac care units in Västra Götaland. There were 5,396 patients with AF and 29,836 had SR. More than 80 per cent of the patients were admitted because of IHD or unspecific chest pain. The AF patients were older and were more likely to be male compared to the SR patients. In addition they had more hypertension, diabetes, HF and previous AMI, whereas the SR patients had more previous percutaneous interventions and were more likely to be smokers. In a multivariate logistic regression model we found that obesity, but not diabetes, was an independent predictor for having AF at admission. In addition, we found that age was strongly predictive of having AF at admission but hypertension was not *(Table 1)*.

| | Range | Odds ratio | 95% CI | P-value |
|---|---|---|---|---|
| **Sex** | Male | 1.36 | 1.23-1.50 | <0.001 |
| **Age** | Per decade | 1.54 | 1.50-1.59 | <0.001 |
| **Hypertension** | Yes | 1.05 | 0.95-1.15 | 0.24 |
| **Diabetes** | Yes | 0.92 | 0.82-1.04 | 0.19 |
| **Previous MI** | Yes | 1.08 | 0.96-1.22 | 0.20 |
| **History of CHF** | Yes | 1.72 | 1.54-1.92 | <0.001 |
| **History of stroke** | Yes | 1.49 | 1.29-1.73 | <0.001 |
| **Active smoker** | Yes | 1.05 | 0.96-0.1.16 | 0.38 |
| **BMI category:** | | | | |
|   Underweight | | 1.37 | 0.95-1.90 | 0.05 |
|   Normal weight | Reference | 1 | - | - |
|   Overweight | | 0.99 | 0.90-1.10 | 0.92 |
|   Obese | | 1.35 | 1.17-1.55 | <0.001 |
|   Very obese | | 1.61 | 1.29-1.98 | <0.001 |
| **Indication** | | | | |
|   IHD | Reference | 1 | - | - |
|   CHF | | 4.71 | 4.04-5.49 | <0.001 |
|   Arrhythmia | | 15.83 | 13.95-17.96 | <0.001 |
|   Chest pain | | 0.43 | 0.29- 0.65 | <0.001 |
|   Other | | 0.12 | 0.08-0.15 | <0.001 |

*Table 1. Multivariable logistic regression analysis for the likelihood of atrial fibrillation at admission. MI=myocardial infarction, CHF=congestive heart failure, BMI=body mass index, IHD= disease*

### *Atrial biopsy substudy*

The patients undergoing surgery were categorized based on prior history of AF. There were more patients with IHD, statin treatment and diabetes in the group without a previous history of AF, but otherwise the groups were similar. The main finding was that the patients with AF had significantly less atrial TAG compared to the patients without AF history *(Figure 27)*. We found no significant differences in the levels of other lipid species. Analysis of DAG

subspecies revealed a qualitative difference where the AF patients had higher relative levels of polyunsaturated DAG *(Figure 28)*

## Conclusion

Obesity but not diabetes was found to be an independent predictor of AF. AF is associated with quantitative and qualitative alterations in atrial lipid content but not with lipotoxicity. Polyunsaturated DAG may be involved in the pathophysiology of AF.
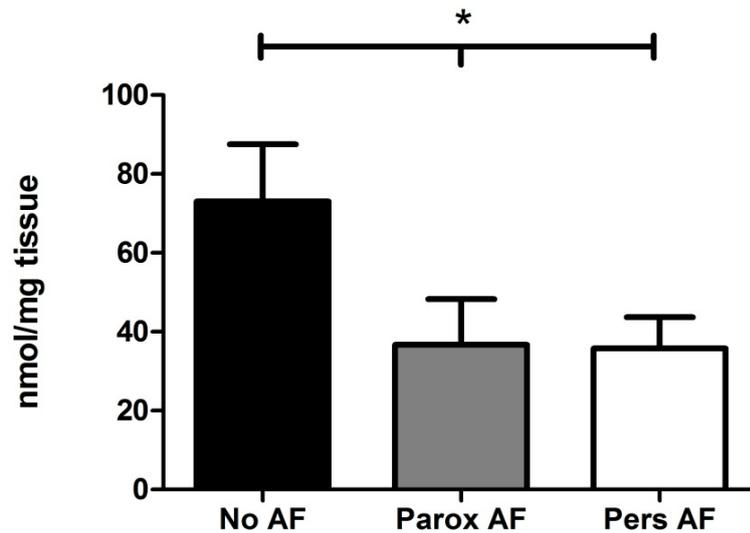


*Figure 27. Levels of atrial triacylglycerides (TAG) from patients with no history of atrial fibrillation (No AF), history of paroxysmal (Parox AF) and persistent (Pers AF) atrial fibrillation. at the time of surgery. There is significantly less TAG in the atria of patients with AF. (\* P for trend <0.05)*
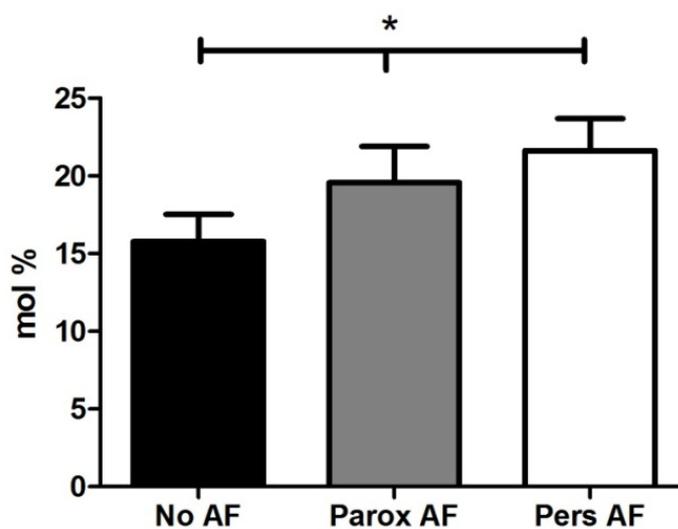


*Figure 28. Proportion of polyunsaturated diacylglycerol (DAG) from patients with no history of atrial fibrillation (No AF), history of paroxysmal (Parox AF) and persistent (Pers AF) atrial fibrillation. Proportion of polyunsaturated DAGs was higher in patients with AF (\* P for trend <0.05).*

# DISCUSSION

## *Paper I*

### *Development of the rat model*

In a previous publication, Lee et al described the induction of CHB in rats by means of ethanol injection[35]. We have demonstrated a further development of their method, by applying electrical diathermic energy for AV nodal destruction, with a good success rate. Prior to our study, research regarding the long term consequences of chronic CHB has been limited to larger animal models[131]. In order to characterize this model for further studies, we performed serial long-term follow-up by echocardiography and further characterization of intra-cardiac hemodynamics and energy metabolism at the end of the study.

### *Morphological remodelling and function*

By longitudinal follow-up by echocardiograms we could reveal important information on post-CHB remodelling in the rat. Already by one week we noticed an increase in left ventricular diameter and mass. Although we were only able to measure the function of the left ventricle by echocardiogram, there was an equally dramatic increase in RV mass when the hearts were examined macroscopically at the end of the study (results not shown). Previous studies have demonstrated that the biventricular hypertrophy seen in CHB is more pronounced in the right ventricle[131]. The biventricular hypertrophy seen in dogs is evident as early as 6 days post CHB and is more pronounced than in other models of volume overload[132, 133]. Even though the reason for this is unclear it may involve a higher wall stress in the CHB model and thus a more pronounced neurohormonal response and that the longer diastole provides more time for growth. The intense neurohormonal activation seen in CHB models normalizes over time as the remodelling process plateaus[134].

An interesting finding in our study is that the contractility decreases over time in the CHB animals. This finding is not previously reported in similar models, and in the canine model there is actually an increase in the contractility over a similar time frame[131, 135]. While this observed decrease in fraction of shortening (FS) could be related to incipient HF, we found that the filling pressures in both ventricles were normal which would argue against this explanation. We rather propose an alternative explanation for this finding, i.e. the force-frequency relationship, sometimes referred to as the "Bodwitch phenomenon". Most mammals including humans have positive force-frequency relationships (i.e. the contractile force increases with increased frequency and vice-versa) while some species have negative or blunted responses[136, 137]. The rat has a positive force-frequency relationship at physiological rates[138]. This effect is believed to be mediated by $Ca^{++}$ ions entering the cell during the plateau phase of the action potential[139]. The uptake of $Ca^{++}$ into the sarcoplasmic reticulum (SR) increases relative to $Ca^{++}$ extrusion via the $Na^+/Ca^{++}$-exchanger at higher rates, making more $Ca^{++}$ available for release from the SR during the contraction phase[140]. In addition there is an increase in the L-type $Ca^{++}$ current at higher rates, making it possible for more $Ca^{++}$ to enter the cell and thus "loading" the cell with $Ca^{++}$[141]. Decreased FS and decreased

contractility is a fairly common and well described finding in elite athletes and is accompanied by physiological hypertrophy and increased LV dimensions[142-144]. This decrease in contractility is believed to be caused to the Bodwitch phenomenon. The phenotype of elite athletes thus has several similarities to that seen in the CHB animals and the mechanisms may be similar.

*Energy metabolism*

After 3 months of CHB we did not find significant biochemical signs of disturbed myocardial energy metabolism. Previous reports in other animal models of HF and pathological hypertrophy have been associated with a decreased total adenine nucleotide pool (TAN) and decrease in Cr[145, 146]. Especially Cr depletion seems to be a ubiquitous finding in these states. The metabolism of high energy phosphates (CrP and ATP in particular) is extremely rapid, hence reliable biochemical measurements may be difficult[147]. Since TAN and total Cr are the sum of ATP, ADP,AMP and Cr and CrP respectively, these values are less sensitive to the degradation of the high energy phosphates[148]. We found the levels of TAN and total Cr to be similar in both groups, strongly arguing against impaired metabolism in the CHB animals. During early HF, signs of metabolic disturbance are usually first seen in the levels of CrP and Cr, whilst changes in ATP levels and TAN are normally later changes indicative of more advanced energy depletion[149]. We found a trend for lower levels of CrP in the CHB animals, but as approximately 70% of all myocardial Cr is phosphorylated it is highly unlikely that the CrP levels are abnormal when the total Cr levels are normal.  Other models of volume overload lead to diminished Cr and TAN levels and our findings imply that the CHB model causes less biochemical metabolic alterations. The reason for this is not completely understood but CHB induced remodelling may be more physiological and thus better tolerated. In support of this previous studies have found a normal capillary to myocyte ratio in the hypertrophic CHB dog heart and absence of fibrosis[150]. The explanation for this may be that bradycardia per se, stimulates angiogenesis in the heart by production of angiogenic factors[151].

*Future studies*

The CHB model provides important insights into an adaptive and more physiological remodelling as opposed to pathological remodelling occurring in various heart diseases, ultimately causing HF[152]. This might assist in development of strategies to facilitate physiological remodelling in the face of cardiac compromise. The age of the animals when they develop the CHB is likely to play a role and in our study they were young animals, which could explain the adaptive remodeling. Whether older animals also have this capacity has to be subject to future studies. With the introduction of genetically modified "knock-out" models in rats[153], these could play a greater role in future studies.

## Paper II

### The model

We used a previously validated model for the induction of AMI in rats[154], as well as an *in vivo* electrophysiology study model previously described for small animal studies[155]. We found no previous studies on the rat model, and we chose to use a more specific protocol than the one previously reported for mice. We kept a longer basic driving cycle (S1) of 100ms (compared to 50ms) and the definition of VT was stricter (6 versus 3 beats). As the rat heart in contrast to humans has a predominance of α-receptors[156], we chose to use amiodarone as a positive control in light of its known antiarrhythmic properties in rats[157].

### Postulated mechanisms for GH antiarrhythmic effects

The sympathetic nervous system (SNS) is known to be intensely activated during and after myocardial infarction[158], and NA is known to exert pathological effects on the myocardium in high concentrations[159]. The activation of the SNS in HF has also been recognized as a negative prognostic marker in these patients[160]. A frequent mode of death in AMI and HF is that of sudden arrhythmic death. Several studies in HF show that β-blocking therapy can effectively reduce sudden death by up to 40%[161-163], further adding evidence to the deleterious effects of SNS activation on ventricular arrhythmogenesis. There is good evidence that GH interacts with the SNS. Firstly, patients with GH deficiency have increased sympathetic nerve activity in muscle that decreases with the exogenous administration of GH[70, 164]. Secondly, GH administration in patients with dilated cardiomyopathy reduces myocardial release of NA[165]. Thirdly, in previous work from our laboratory it has been demonstrated that both myocardial and plasma NA is markedly lowered in rats treated with GH early after AMI[81] and similar findings were made in transgenic mice with cerebral overexpression of GH[83].

There are other possible mechanisms that can contribute to the effects seen with GH. GH and insulin-like growth factor-1 (IGF-1) have effects on both calcium[166] and potassium[167] currents in cultured ventricular myocytes as well as increasing $Na^+/K^+$-ATPase activity in muscle[168]. This suggests that GH has direct electrophysiological effects on cardiomyocytes that could partly explain our observed antiarrhythmic properties. In an AMI model in rats pretreated with GH, the monophasic action potentials from the infarction region were preserved and action potential duration was not prolonged compared to non-infarcted controls[169]. Whether these findings are due to direct electrophysiological effects or indirectly by means of improved metabolic status of the cells is not clear.

Previous reports have indicated that GH has important cytoprotective effects on the heart, resulting in decreased ischemia and infarct size[170]. This is likely to translate into better arrhythmic survival in the longer term, but it is uncertain whether this explains our results of decreased arrhythmias <60 min after coronary occlusion (phase I arrhythmias). In a similar study of AMI induction, Elaiopoulos et al could demonstrate a modest non-significant reduction in phase I arrhythmias, but saw a large decrease in phase II arrhythmias[169]. This finding was interpreted as possibly mediated by the reduction in infarct size, although this finding failed to reach statistical significance in their study. The reason that we were able to show a difference in the early arrhythmias and they were not, may be due to a different timing in the administration of GH.

Finally, GH has important effects on the energetic balance and metabolic activity in the cardiomyocyte. GH given to rats with HF at a dose of 1 mg/kg enhanced antioxidant defenses, reduced oxidative stress and improved energy generation[171]. In post-infarction rats GH administration improved bioenergetics as demonstrated by a higher CrP/ATP ratio[81]. GH mediated improvement in bioenergetics status in the face of cardiac compromise might therefore be an important mechanism for the maintenance of electrical stability in the cardiomyocyte, possibly contributing to the positive findings observed with GH in our study.

*Future studies*

The major finding of this paper was the demonstration of the antiarrhythmic effects of growth hormone (GH) in rats with experimental AMI. These properties were further substantiated in a different *in vivo* model of VAs, and the effects of GH were similar to those of amiodarone, which is a known and effective antiarrhythmic agent in humans and rats[157, 172]. No effect on the inducibility of AF could be shown, indicating that GH antiarrhythmic effects may be specific to the ventricles.

GH treatment in the setting of chronic HF has received some attention in the previous years, following an important pilot study by Fazio et al[84], that has prompted further clinical research. A metaanalysis has suggested improvement in several important parameters such as ejection fraction and NYHA function class[88]. Our and others findings imply that VA, an important mode of death in cardiac patients, can be reduced by GH treatment. Further characterization of the cellular electrophysiological effects of GH in the setting of HF and myocardial ischemia may reveal important targets for treatment of these patients. The observed ventricular specificity of GH effects is also an intriguing observation that may reveal important differences in the generation of arrhythmias on the atrial and ventricular level.

## Paper III

In this paper we set up and validated a model for studies of *in vitro* electrophysiological effects of LPC on HL-1 cardiomyocytes. We have demonstrated that LPC has profound effects on field potential attributes, probably due to multiple ion channel effects of LPC. Furthermore we have demonstrated an attenuation of LPC-induced signal propagation delay with the pre-treatment of ISO and atropine.

### *LPC and ischemia*

LPC is an amphiphilic metabolite that is promptly released during myocardial ischemia in mammalian hearts, including humans. During ischemia, the production of LPC is increased by the action of phospholipase-$A_2$ while the catabolism of LPC is markedly reduced, resulting in rapid LPC accumulation [24]. In addition, the frequency and seriousness of VAs in the settings of myocardial ischemia correlate well to the LPC concentration[173, 174]. The concentration of LPC in ischemic myocardium is typically in the range of 100-300 µM[175]. The major part is protein-bound (~90%) while free LPC is likely to be in the range of 10-30 µM. This corresponds well to the measured extracellular concentrations during cardiac ischemia (5-20 µM)[176], and is similar to the concentration-range used in this study. Our results are therefore likely to be suggestive of the cellular effects of LPC at clinically relevant concentrations.

### *The sodium channel*

The effect of LPC on ion-channels has been described in previous studies. For the sodium channel, LPC causes complex changes in $Na^+$-channel gating. At relatively high concentrations (20 µM), it reduces the upstroke of the action potential - an effect linked to the inhibition of peak $Na^+$ currents[26]. This causes slowing of conduction, slowing of membrane depolarization and post-repolarization refractoriness. The effects on peak $Na^+$ currents are combined with a marked slowing of $I_{Na}$ decay giving rise to increased late $Na^+$ currents[177]. Recently the mechanism of the increased late sodium current was found to be mediated by the formation of peroxynitrite[178]. In this study, a low concentration of LPC (5µM) increased the late inward $Na^+$ current. This action prolongs action potential duration (APD), slows conduction and increases APD dispersion, thereby increasing the propensity for early and delayed after-depolarization. In addition, augmented late sodium currents can increase automaticity. LPC inhibits the $Na^+/K^+$ ATP-ase which may cause intracellular $Na^+$ accumulation and consequently $Ca^{++}$ accumulation via the $Na^+/Ca^{++}$ exchanger working in reverse mode[179]. Similarly, the LPC-caused prolongation of late inward $Na^+$ current can aggravate cellular $Ca^{++}$ overload which eventually might trigger after-depolarisations. Sodium currents along with pacemaker currents ($I_f$) play an important role in the pacemaker activity in the HL-1 cells[180]. In our model, the inhibitory effects of LPC on the fast inward $Na^+$ currents are reflected by decreased $FP_{AMP}$, decrease in BR, slower propagation velocity, and prolonged FP rise time. However, our model cannot discern the contribution of the increased late $Na^+$ currents from decreased $K^+$ currents, as the FP changes are expected to be similar, i.e. prolonged $FP_{dur}$.

*The potassium channels*

LPC also has important effects on outward $K^+$-currents. The findings have been variable, depending on the type of channel and concentration of LPC. A study by Kyosue et al, demonstrated inhibition of the inwardly rectifying $K^+$ current ($I_{K1}$) at relatively high LPC concentrations (50 µM)[25] which would be expected to cause AP prolongation and depolarization of resting membrane potentials. Even though there are no reports of LPC effects on the transient outward potassium current ($I_{to}$), there are data in rodents showing suppression of $I_{to}$ by other amphiphilic fatty acid metabolites such as palmitoylcarnitine[181]. Studies on the delayed rectifier $K^+$-current ($I_{Kr}$), a major determinant of resting membrane potential, has shown the opposite effect. Increased $K^+$-currents caused by $I_{Kr}$ were demonstrated at low levels of LPC[182]. Recent evidence reveals that the slow delayed rectifier $K^+$-current ($I_{Ks}$) can also be reversibly increased, at low concentration of LPC. Combined, these increased $K^+$ currents would cause shortening of APD and increased concentrations of extracellular $K^+$, a common finding in experimental myocardial ischemia[183].

*The calcium channels*

There has been some controversy regarding the effects of LPC on L-type $Ca^{++}$-currents. In a recent study, LPC in a moderate concentration of 10 µM, did not affect L-type $Ca^{++}$-channel currents[28]. In the same publication the authors could demonstrate augmentation of T-type $Ca^{++}$-channel currents, but noticed no effects on other pacemaker currents, such as $I_f$, over a wide range of LPC concentrations (5-50 µM). The increase in T-type $Ca^{++}$-channel currents could contribute to $Ca^{++}$ loading and increased automaticity seen during LPC challenge (premature beats).

*Electrophysiological effects*

The APD has been shown to correlate well with $FP_{dur}$ in other cell models[6]. In our study $FP_{dur}$ prolongation with the successively higher doses of LPC is likely to be due partly to inhibition of potassium currents ($I_{K1}$, $I_{to}$) with a contribution from enhanced late sodium currents. There is however a non-significant trend at lower concentration of LPC for a shortened $FP_{dur}$ and this could possibly be explained by a bimodal effect on potassium channels (activation at lower concentrations and inhibition at higher concentrations as described above).

The observed electrophysiological effects of LPC in our model are reversible by washout. The time during which the cells are incubated with LPC seems to be important. In our experiments we had short incubations with LPC which might explain the observed reversibility in our study. However, when incubated for longer periods, we saw only partial reversibility (data not shown). This phenomenon has been observed in studies using similar concentrations of LPC (10-20 µM)[26, 29, 177] but some studies have also reported a total reversibility of the electrophysiological effects induced by LPC[184-186]. The latter experiments generally used a lower concentration of LPC (2-5 µM) and this discrepancy might explain the different findings during washout. A reasonable explanation for this may be found in the chemical properties of amphiphilic lipid substances such as LPC, i.e. the ability to form micelles. At low concentrations, LPC molecules are absorbed in the membrane as monomers. However, at higher concentrations the monomers tend to form micelles[184]. This is reflected by the critical micelle concentration (CMC). For LPC-16 (used in the present study), the CMC is

approximately 5-7 µM [187]. Above the CMC, LPC forms micelle-membrane interactions which alter the permeability of the sarcolemma and may cause cell membrane poration. These pores are so large that they could allow passage of among other $Ca^{++}$ -ions, eliciting many of the observed changes with higher doses of LPC[188]. These changes are likely to be irreversible as the membrane is permanently damaged, while the reversible effects of LPC (similar to those seen in our study) are likely to be mediated via direct interaction with the sarcolemma and ion channels.

### *The gap junctions*
LPC is a potent inhibitor of gap junctional communication between cardiac cells[29]. We found significantly retarded signal propagation by LPC. Compared with the delayed signal propagation induced by heptanol (a gap junction blocker), LPC seems to effect signal propagation to a similar extent. The effect of LPC on gap junctions is further aggravated by a decreased inward $Na^+$ current and increased intracellular $Ca^{++}$ load induced by LPC.

### *Future studies*
This is the first study to examine the effects of LPC on HL-1 cardiomyocytes using a multichannel MEA system. We have verified that LPC induces several electrophysiological alterations in the myocardial syncytium. An important advantage of the method is the possibility to non-invasively analyze the net effect of all cellular events elicited by experimental changes (e.g. by addition of an exogenous substance) on the FP properties, frequency, and rhythm. Another possibility is to perform high-throughput electrophysiological screening of different pro-arrhythmic or anti-arrhythmic substances. The information gathered from this model can be complementary to the information obtained from the existing *in vivo* animal models employing invasive cardiac mapping and from the *in vitro* patch-clamp techniques. Future studies should address the variation seen in effects of low contra high concentrations of LPC, the breakpoint in our studies is likely found around 8µM. At higher concentrations, surface active substances could be evaluated to determine if electroporation is contributing to the effects seen. To further understand the participation of individual ion channels in the observed electrophysiological alterations, various blockers and enhancers of specific ion channels may be utilized.

## Paper IV

The major finding of this study was that obesity but not diabetes was independently associated with AF in a population with a high prevalence of concomitant heart disease. AF was associated with quantitative and qualitative changes in atrial lipid content, but not with lipotoxicity.

### The RIKS-HIA registry data

This study addresses the prevalence of AF and correlates in a prospective registry of Swedish individuals that were admitted for a cardiac condition. The validity of the study is supported by the large sample size and consistency in multiple analyses adjusting for known confounders. In addition, all cases were ECG verified AF. Our study largely confirms earlier studies demonstrating that obesity is associated with AF and our finding extend to a previously unstudied group of patients. The RIKS-HIA is a prospective cohort consisting mostly of patients admitted for acute coronary syndromes and unspecific chest pains, but also includes admissions for HF, palpitations, syncope etc. We found a high prevalence (15%) of AF in this population that is likely due to the high degree of cardiac comorbidity and the relatively advanced age of the population studied.

Our results point to obesity being associated with a 32% increase in risk of AF and that severe obesity carries a 58% increase in risk of having AF at admission. This corresponds well with the findings of a recent meta-analysis on the risk of AF due to obesity, which demonstrated an OR of 1.49 in the obese[98]. Obesity has also been found to predict recurrence of AF after ablation treatment[189] as well as increasing the risk of post-operative AF[190]. However the outcome for patients with established AF is not worse for overweight or obese[191] and may in fact be better[192]. This has been related to the obesity paradox, with better outcomes observed among overweight in several cardiovascular diseases such as HF, coronary heart disease and hypertension[193].

Earlier reports have been inconsistent regarding whether diabetes increases the risk of AF. Given the association between obesity, diabetes and the metabolic syndrome with the incident of AF, it has been speculated that insulin resistance could be a possible common mechanism[105], but this could not be substantiated in a recent publication from the Framingham group[106]. Our study included a substantial amount of diabetics (15.8%) and we did not find any contribution from diabetes to the risk of AF in a multinomial logistic regression analysis. This adds to the understanding of how diabetes may be involved in the pathophysiology of AF and questions whether diabetes in fact is a risk factor in itself, implying that the observed risk may be mediated by other common factors, e.g. obesity.

The potential mechanisms by which obesity might contribute to the pathophysiology of AF are numerous. Obesity is related to an elevated plasma volume[194], diastolic dysfunction[195] and increased neurohormonal activation[196], all condition that increase left atrial volume and stretch . An increase in left atrial stretch may lead to atrial extra-systoles and over time to fibrosis and structural changes in the atria[197]. One study speculated that that obesity mediates its effects by dilatation of the left atrium[101], but others have found the relationship between BMI and AF to be significant even after adjustments for left atrial size[198]. Increased BMI has been linked to increased P-wave duration[199] and poorer atrial mechanical function[200] which

indicate structural remodelling (scarring and dilatation) of the atria that in turn predisposes to AF. This is supported by recent work in which there were signs of DNA fragmentation, as a marker of apoptosis, in atrial biopsies from overweight individuals[201]. Obesity has also been associated with inflammation[202] even though a recent study could not find a major contribution from inflammation on the observed obesity associated risk[100]. The autonomic nervous system has been implicated in the initiation and maintenance of AF[203], and obesity is known to cause cardiac autonomic disturbance[204]. Finally, obesity has been linked to sleep apnea[96], where the mechanism might involve hypoxemia or increased pressure in the pulmonary circulation.

*Lipotoxicity*

In our hypothesis we proposed a novel mechanism by which obesity and diabetes could predispose to AF. In recent years there has been accumulating knowledge on heart steatosis occurring in obese and diabetic individuals reflecting an altered metabolism in the cardiomyocytes[205] which might contribute to increased metabolic stress[206]. However, the exact mechanism for how the excess lipids harm the cells is only partly understood. TAG themselves seem to be an inert bystander and are unlikely to be responsible for the observed toxic effects and are therefore often seen as markers of lipotoxicity[62]. The proposed mechanisms of lipotoxicity include increased stress on the endoplasmic reticulum (ER), mitochondrial dysfunction and altered gene expression [59] ultimately leading to increased amounts of reactive oxygen species in addition to DAG and CER production causing cellular dysfunction and apoptosis[65]. We looked at several different lipid subspecies and found no signs of atrial lipid accumulation; on the contrary we found lower levels of TAG in the atria of AF patients compared to controls and similar levels of the lipotoxic species DAG, LPC and CER. Normal levels of CER and sphingoid bases were also found in the atria of overweight and diabetic patients in a recent study by Baranowski et al[201].

Polyunsaturated DAG has been proposed to have lipotoxic properties by its actions as a second messenger; binding to and activating certain isoforms of protein kinase C (PKC). PKC can exert various potentially cardiotoxic effects; including impairment of beta receptor function[207], uncoupling effects on gap junctional communication[208], reduction of cardiac contractility[209], increased apoptosis[210], activation of transient receptor potential channels[211] and cause intracellular $Ca^{++}$ loading via activation of L-type $Ca^{++}$ channels[212] (summarized in *Figure 29*). Activation of PKC can also inhibit the delayed rectifier potassium currents ($I_{Kr}$), causing prolonged repolarization further aggravating the $Ca^{++}$ loading[213]. Hence, PKC activation may expose the atrial myocardium to arrhythmia via $Ca^{++}$ loading, decreased cell-cell communication and by substrate modification.

Diacylglycerol kinase (DAGK) is the enzyme that catalyzes the conversion of DAG to phophatidic acid utilizing ATP. In animals with increased activity of transient receptor potential channels, increased expression of DAGK was able to inhibit structural remodelling and arrhythmias both in the ventricles[214] and in the atria[215]. This further substantiates the role of polyunsaturated DAG in arrhythmogenesis.

Based on these findings we explored if the qualitative distribution of DAG subspecies was associated with AF. We found a higher proportion of polyunsaturated DAG in the AF patients

as compared to the non-AF controls. Although this may be a chance finding, it is possible that in these patients polyunsaturated DAG may play a role in the pathophysiology of AF.

## *Future studies*

Although we found no evidence of lipotoxicity in the pathogenesis of AF, the possible role of polyunsaturated DAG in the pathogenesis of AF is an interesting and previously unexplored mechanism. This hypothesis may be addressed in cellular models such as the MEA cell model. Further studies are needed on the effects of weight reduction to reduce AF risk or burden in order to further substantiate the obesity related risks and propose effective treatment strategies.
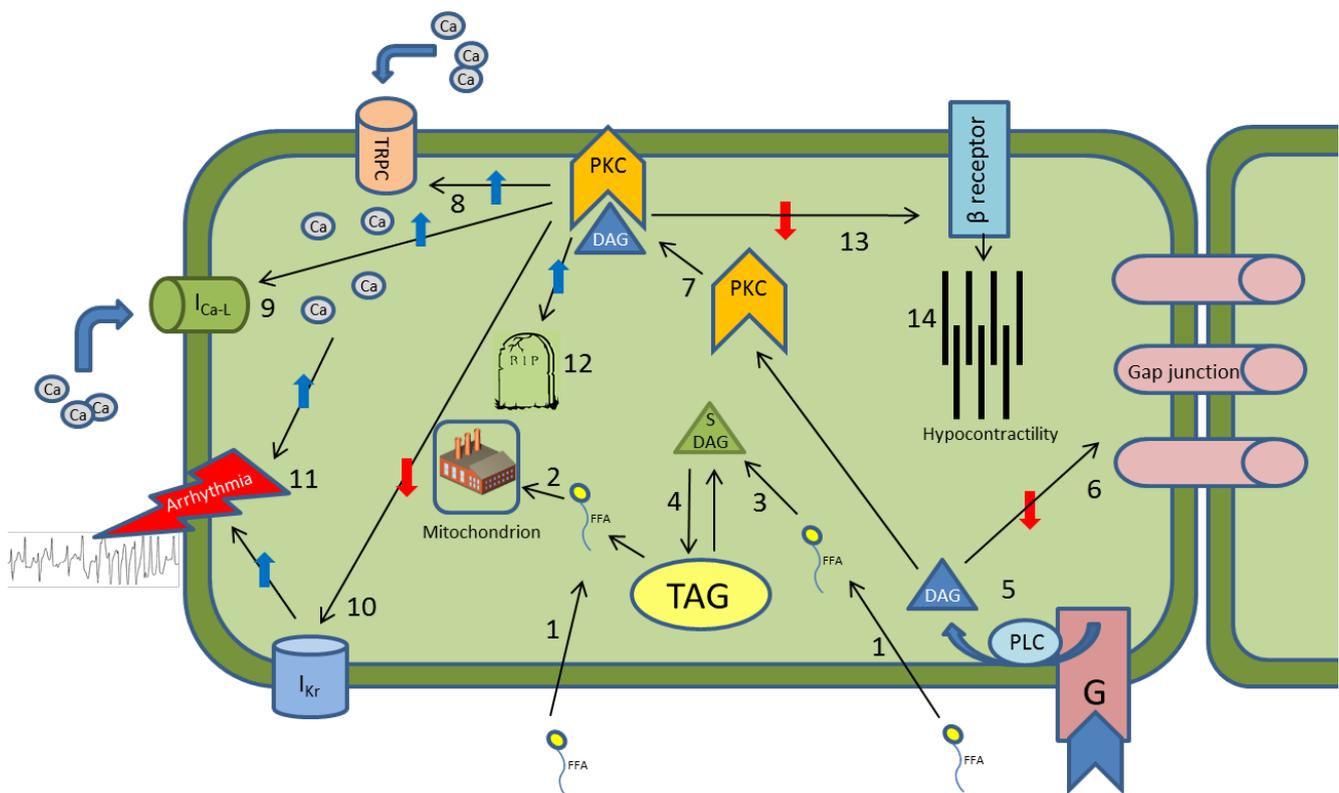


*Figure 29. Schematic overview of some of diacylglycerol (DAG) effects in the cardiomyocyte. Free fatty acids (FFA) are taken up by the cell by diffusion and transporters (1) and in the cell they are metabolized for energy production (2) or converted to saturated diacylglycerol (s-DAG) (3). DAG can be further acylated by means of DGAT1 to triacylglycerol (TAG) (4), which is the preferred means to store lipids. Polyunsaturated DAG is produced via a G-protein coupled (G) activation of phospholipase C (PLC) (5). DAG can via direct action uncouple the gap junctional mechanisms (6) but most of the observed effects are through its action as a secondary messenger, activating protein kinase C (PKC) (7). PKC exert various effects, including activation of transient receptor potential channels (TRCP) (8) and L-type calcium channels ($I_{Ca-L}$) (9) that both are permeable to $Ca^{++}$. Additionally PKC inhibits delayed rectifier potassium channels ($I_{Kr}$), further aggravating the calcium depolarizing currents (10). The Ca-loading can lead to after-depolarisations and arrhythmias (11). PKC activation stimulates apoptotic signaling in the cell, causing programmed cell death (12) Finally, PKC may impair β-receptor function (13) which can lead to hypocontractility (14). DGAT1=Diglyceride acyltransferase 1.*

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