Dissertation

THE EFFECTS OF CHOLESTASIS ON THE HEART

submitted by

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‘‘Declaration I hereby declare that this dissertation is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this dissertation. Due acknowledgement has been made in the text to all other material used. Throughout this dissertation and in all related publications I followed the guidelines of “Good Scientific Practice”.

Dr. med. univ. Peter P. Rainer

Graz, July 7, 2014

I. Dedication

This work is dedicated to my wife and to my mother. Always there. Always encouraging. Always loving. I could not have done it without you.

II. Acknowledgements

These times it is virtually impossible to work on a scientific project without help. I understand this piece of work and the resulting publication as a result of many hands and brains. Hence I am indebted to the people who worked with me in the last couple of years. My thanks go to all co-authors on the resulting publication (see appendix). I would like to especially stress the contribution of Dr. Uwe Primessnig and Dr. Sandra Harenkamp who conducted their diploma thesis on the topic of bile acid effects on the heart under my supervision. Further, I would like to acknowledge my colleagues Dr. Bernhard Doleschal and Dr. Markus Wallner who conducted important experiments, and Dr. Marie-Sophie Huber who was working in the lab in the very first hours of this project and conducted preliminary experiments with me.

Of note, the current thesis was the foundation for a peer reviewed and published manuscript, which was accompanied by an editorial article; significant parts of the thesis and published manuscript are similar.

I am also indebted to my teachers Dr. Dirk von Lewinski, Dr. Michael Trauner, and Dr. Burkert Pieske who supervised all aspects of this thesis and had open ears for my questions and requests.

Lastly, I would like to acknowledge Dr. David Kass, all the members of his lab, and the basic science folks of Johns Hopkins Cardiology, Baltimore MD, USA, where I spent the last four years. While the focus of my work there was a different one, they had most

profound influence on my views and understanding of science. I am immensely grateful for the time and the privilege to work with them.

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IV. Abbreviations and Definitions

ACE	Angiotensin converting enzyme
AEC	Arrhythmic extra contraction
AF	Atrial fibrillation
AI	Aortic regurgitation
AKT	Protein kinase B
Aldo	Aldosterone
ANOVA	Analysis of Variance
ARB	Angiotensin II receptor blocker
AS	Aortic stenosis
AVR	Aortic valve replacement
BA	Bile acid
BB	Beta blocker
BDM	2,3-butanedione-monoxime
BMI	Body mass index
BSEP	Bile salt export pump
$[Ca^{2+}]_i$	Intracellular calcium concentration
CA	Cholic acid
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
cAMP	3'-5' cyclic adenosine mono phosphate
CDCA	Chenodeoxycholic acid
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CNHF	Competence Network Heart Failure
CO_2	Carbon dioxide
DAD	Delayed afterdepolarization
DCA	Deoxycholic acid

DCB	3’,4’-dichlorobenzamil hydrochloride
dF/dt_{max}, dF/dt_{min}	Maximal velocity of force generation/relaxation
DIG	Digitalis glycosides
DIU	Diuretics
EAD	Early afterdepolarization
ECG	Electrocardiogram
EF	Ejection fraction
EGTA	Ethylene glycol tetra acetic acid (calcium chelator)
FIC1	Familial intrahepatic cholestasis 1
FVB	Friend virus B
FXR	Bile acid farnesoid receptor X
GCA	Glycine-conjugated cholic acid
GCDCA	Glycine-conjugated chenodeoxycholic acid
GSK3beta	Glycogen synthase kinase 3 beta
Hz	Hertz
I / V	Current / Voltage
I_{CaL}	L-type inward calcium current
ICP	Intrahepatic cholestasis of pregnancy
ICV	Inferior cave vein
I_{si}	Slow inward current
kDA	Kilodalton
LCA	Lithocholic acid
L_{max}	Length with maximum twitch force generation
LPL	Lipoprotein lipase
LV	Left ventricle
LXRalpha, LXRbeta	Liver X receptor alpha/beta
MDR1	Multidrug resistance 1
MEM	Modified Eagle’s medium

MI	Mitral regurgitation
mN/mm^2	Millinewton per square millimeter
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MRP2-4	Multidrug resistance-associated protein 2, 3, and 4
ms	Milliseconds
MS	Mitral stenosis
mV	Millivolt
n.s.	Not significant at $p<0.05$
NCX	$Na+/Ca^{2+}$ exchanger
NRVM	Neonatal rat ventricular myocyte
NTCP	Na^+/Bile co-transporter
NTproBNP	N-terminal pro brain natriuretic peptide
O_2	Oxygen
OR	Odds Ratio
OSTalpha, OSTbeta	Organic solute transporter alpha / beta
PCR	Polymerase chain reaction
pH	Pondus hydrogeni
pPLB, tPLB	Phospho/Total phospholamban
PQ	PQ time (ECG parameter)
QRS	QRS complex duration (ECG parameter)
QTc	Frequency corrected QT interval duration
RNA	Ribonucleic acid
RT50, TT90	Time to 50/90% relaxation
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SHP	Small heterodimer partner
SR	Sinus rhythm

TCA	Taurine-conjugated cholic acid
TCDCA	Taurine-conjugated chenodeoxycholic acid
TGR5	G protein-coupled bile acid receptor 1
TUDCA	Taurine-conjugated ursodeoxycholic acid
UDCA	Ursodeoxycholic acid

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create a vicious circle. Additionally, other groups found that bile acids may exert effects via their cognate bile acid or other receptors (7). 60

VI. List of Tables

1. Zusammenfassung

Einleitung: Der Zusammenhang zwischen Herz- und Lebererkrankungen ist vor allem für die hämodynamischen Effekte einer fortgeschrittenen Herzinsuffizienz auf die Leber etabliert (Cirrhose cardiaque). Weniger gut sind die Auswirkungen von Lebererkrankungen auf die Herzfunktion charakterisiert, insbesondere die Effekte von hohen systemischen Gallensäure-Spiegeln bei Cholestase.

Das Ziel dieser Dissertation war zu untersuchen, ob (1) hohe Gallensäure Konzentrationen am menschlichen Herzmuskelgewebe Arrhythmien auslösen, (2) der vermutete arrhythmogene Effekt von der Konjugierung der Gallensäuren abhängig ist, (3) die Gallensäure Serum Konzentration oder Zusammensetzung bei Patienten mit Arrhythmien verändert ist, und 4) verantwortliche Mechanismen zu identifizieren.

Methoden und Ergebnisse: Taurocholsäure induzierte konzentrationsabhängig arrhythmische Extrakontraktionen in humanen multizellulären atrialen Präparaten (14 von 28 bei 300µM TCA, $p<0.01$). Ursodeoxycholsäure hingegen war nicht arrhythmogen. Patienten mit Vorhofflimmerarrhythmie zeigten reduzierte Serumkonzentrationen von Ursodeoxycholsäure Konjugaten, während nicht-Ursodeoxchol Gallensäuren erhöht waren. Taurocholsäure depolarisierte das Ruhemembranpotential in isolierten Kardiomyozyten, stimulierte den Na^+/Ca^{2+} Austauscher (NCX), und induzierte Nachdepolarisationen. Pharmakologische NCX Inhibition verhinderte das Auftreten von arrhythmischen Extrakontraktionen in atrialen multizellulären Präparaten.

Schlussfolgerung: Taurocholsäure induziert akut und konzentrationsabhängig Arrhythmien in menschlichen Vorhöfen. Der Effekt von Gallensäuren ist von ihrer Konjugation abhängig und fehlt bei der hydrophileren Ursodeoxycholsäure. Patienten mit Vorhofflimmerarrhythmie weisen ein verändertes Serum Gallensäureprofil auf, das sich durch eine Verringerung von Ursodeoxycholsäure-Konjugaten auszeichnet. Mechanistisch konnte eine Beeinflussung des sarkolemmalen Na^+/Ca^{2+} Transport durch hydrophobe Gallensäuren mit Ruhemembranpotential Depolarisation und vermehrtem Auftreten von Nachdepolarisationen nachgewiesen werden.

2. Abstract

Objective: High bile acid serum concentrations have been implicated in cardiac disease, particularly in arrhythmias. Most data originate from *in vitro* studies and animal models. We tested the hypotheses that (1) high bile acid concentrations are arrhythmogenic in adult human myocardium, (2) serum bile acid concentrations and composition are altered in patients with atrial fibrillation, and (3) the therapeutically used ursodeoxycholic acid has different effects than other potentially toxic bile acids.

Methods and Results: Multicellular human atrial preparations ('trabeculae') were exposed to primary bile acids and the incidence of arrhythmic events was assessed. Bile acid concentrations were measured in serum samples from 250 patients and their association with atrial fibrillation and ECG parameters analyzed. Additionally, we conducted electrophysiological studies in murine myocytes and assessed expression of bile acid transporters and receptors in human hearts.

Taurocholic acid concentration-dependently induced arrhythmias in atrial trabeculae (14/28 at 300µM TCA, $p<0.01$) while ursodeoxycholic acid did not. Patients with atrial fibrillation had significantly decreased serum levels of ursodeoxycholic acid conjugates and increased levels of non-ursodeoxycholic bile acids. In isolated myocytes, taurocholic acid depolarized the resting membrane potential, enhanced Na^+/Ca^{2+}

exchanger (NCX) tail current density, and induced afterdepolarizations. Inhibition of NCX prevented arrhythmias in atrial trabeculae.

Conclusion: High taurocholic acid concentrations induce arrhythmia in adult human atria while ursodeoxycholic acid does not. Atrial fibrillation is associated with higher serum levels of non-ursodeoxycholic bile acid conjugates and low levels of ursodeoxycholic acid conjugates. These data suggest that higher levels of toxic (arrhythmogenic) and low levels of protective bile acids create a milieu with a decreased arrhythmic threshold and thus may facilitate arrhythmic events.

3. Introduction

Cholestasis is encountered in various clinical scenarios including large bile duct obstruction, vanishing (small) bile duct syndromes, and hepatocellular dysfunction of bile acid secretion due to drugs, hormones, or genetic defects[1]. Studies in rodent models of liver injury and cholestasis[2-4], as well as clinical observations in patients with end-stage liver failure and cholestasis[5, 6] found alterations in cardiac structure, function, and electrical properties ('cirrhotic cardiomyopathy'). One of the earliest reports dates from 1863, from the dissertation conducted by Armin Röhrig at the Universities of Würzburg and Leipzig[7]. Röhrig started from the clinical observation that icteric patients often exhibit bradycardia. He then tested the effects of injecting bile or its constituents intravenously in rabbits or dogs and found that bile as well as cholate and its taurine and glycine conjugates consistently produced bradycardia (and in high concentrations cardiac arrest and death) while other bile components (e.g. bilirubin or cholesterol) did not. In addition, he demonstrated that the bradycardic effects are preserved when the heart is denervated, either by cutting the vagal nerve or exposing explanted frog hearts to diluted bile. Later reports confirmed the negative chronotropic properties of cholic acid [8, 9], and either found negative, positive, or no inotropic effects. Electrophysiological studies demonstrated shortening of the action potential duration in ventricular myocytes linked to a depressed slow inward current (I_{si}) [10], as well as reduced spontaneous discharge of sino-atrial preparations linked to depressed slow inward calcium currents and time-dependent potassium outward currents[11].

More recently, Desai and co-workers found reduced exercise capacity, bradycardia, QT interval prolongation, and cardiac hypertrophy coupled to activation of AKT signalling and inhibition of glycogen synthase kinase 3 beta (GSK3beta) in a murine model of biliary fibrosis with high levels of circulating bile acids[2]. Stimulation of myocytes with the TGR5 agonist taurochenodeoxycholic acid (TCDCA) *in vitro* recapitulated the changes observed *in vivo* and induced AKT signalling. Epidemiologic studies in patients with advanced liver disease/cirrhosis have also demonstrated QTc interval prolongation, which correlates with disease severity and prognosis[12].

Moreover, evidence concerning a particular form of cholestasis, intrahepatic cholestasis of pregnancy (ICP), which is a substantial cause for morbidity and mortality of the fetus[13], suggests that fetal arrhythmias may be responsible for adverse outcomes[14-16]. The incidence of fetal complications in ICP increases with serum bile acid concentrations, particularly at levels higher than 40μM[17].

Clinical evidence has recently been supported by *in vitro* studies that demonstrate clear effects of bile acids on neonatal cardiomyocytes[18-21]. Gorelik and Williamson have contributed several papers to the literature in the field. They demonstrated that taurocholic acid induces decreased rate of contraction and reduced the proportion of beating cells in cultured neonatal rat ventricular myocytes (NRVM)[21] linked to decreased amplitude of contraction and calcium transients[19]. Further, they demonstrated that dexamethasone and ursodeoxycholic acid (UDCA) protect against TCA induced arrhythmias[22, 23] and that genes encoding bile acid transporters are expressed

in cultured human fetal cardiomyocytes[24]. They propose that taurocholate acts as a partial agonist on the M2 muscarinic receptor and reduces intracellular 3'-5' cyclic adenosine mono phosphate (cAMP) levels and hence reduces amplitude of contraction, calcium transients, and beating frequency. The nuclear and trans membrane bile acid receptors FXR and TGR5 were expressed in neonatal cultured rat cardiomyocytes, however at low levels and they and did not appear to play a functional role[20].

Despite these studies in isolated cell or animal models, and anecdotal clinical reports, the effects of high serum bile acid concentrations on the adult human heart have scarcely been studied systematically yet.

Bile acids are amphiphilic molecules and consist of a lipophilic steroid ring with a side chain carrying a carboxyl group that can be differentially conjugated by liver cells (**Figure i**). Primary bile acids are synthetized in liver cells from cholesterol and are cholic acid (CA) and chenodeoxycholic acid (CDCA), which in turn can be conjugated with taurine or glycine to increase their solubility. After secretion into the duodenum gut bacteria may dehydroxylate primary bile acids and form the secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA). Large parts of the enteric bile acid pool are reabsorbed in the ileum into the mesenteric venous blood flow and recycled in the enterohepatic circulation.

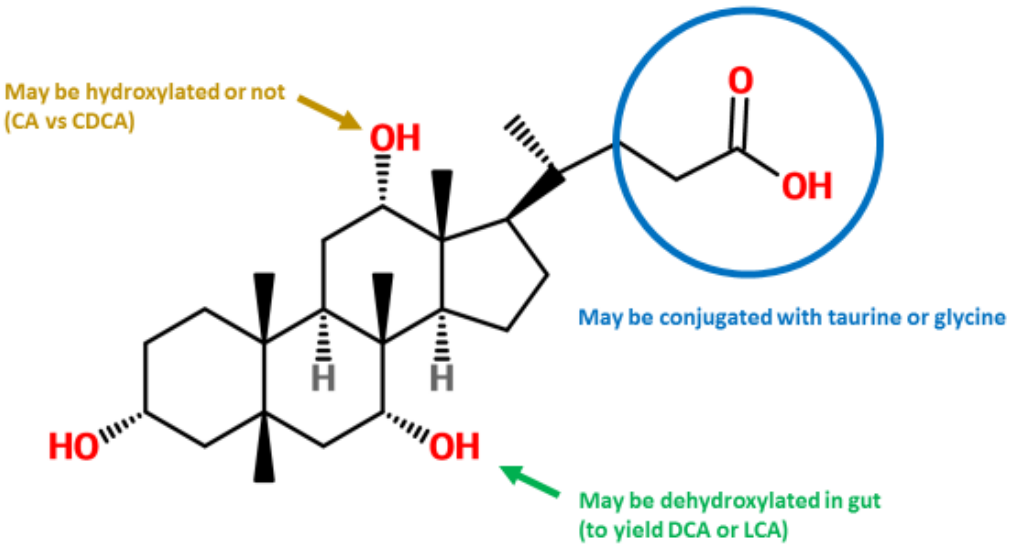


Figure i Molecular structure of bile acids (here cholic acid). A lipophilic steroid skeleton can be differentially conjugated and hydroxylated to yield bile acid sub species.

Endogenously, most bile acids are glycine- or taurine conjugated in humans (**Figure ii**). Ursodeoxycholic acid, a bile acid that occurs in minor quantities in humans, differs from most other bile acids as it is more hydrophilic (**Figure iii**) and can stimulate bile acid excretion by hepatocytes[25].

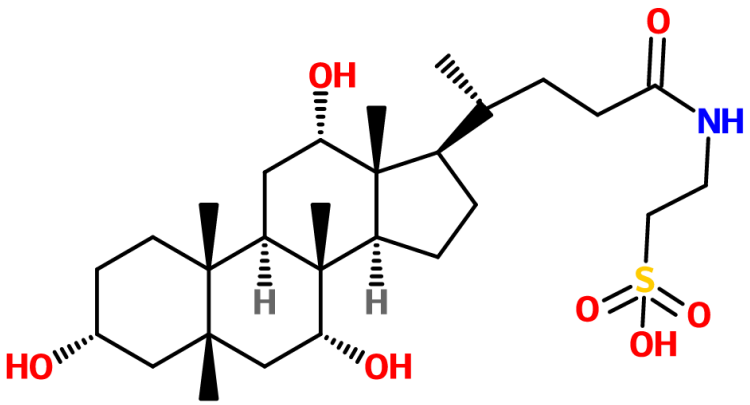


Figure ii Molecular structure of taurocholic acid (TCA)

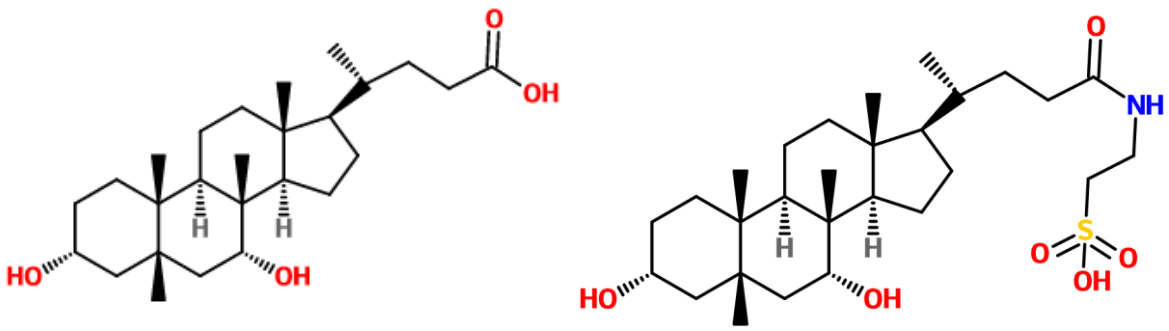


Figure iii Molecular structures of ursodeoxycholic acid (UDCA, **left**) and tauro ursodeoxycholic acid (TUDCA, **right**). Note the absence of a hydroxyl group at carbon 12 when compared to cholic acid or taurocholic acid.

In fact, ursodeoxycholic acid is used therapeutically for various cholestatic disorders and has recently been demonstrated to be beneficial in heart failure in humans[26], in a murine heart transplantation model[27], and in a cell culture model of the fetal heart [23]. The rationale of these studies is that ursodeoxycholic acid exerts pleiotropic effects such as anti-inflammatory and anti-apoptotic effects beside its classical role as bile acid.

In contrast to the well-established arrhythmogenic effects of bile acids in models of the fetal heart little is known about potential implications for the adult heart. We sought to investigate if and how human adult myocardium reacts to elevations in serum bile acid levels and whether the therapeutically used ursodeoxycholic acid has differential effects. To accomplish this we used an *in vitro* model of adult human atrial myocardium. Additionally, we measured bile acid levels in a precisely characterized human patient collective and tested if bile acid levels and composition differ between patients with or

without atrial fibrillation (AF). To obtain mechanistic insights, we tested the electrophysiological effects of bile acids on isolated cardiomyocytes.

The hypotheses underlying this thesis were:

1) Bile acids are arrhythmogenic in adult human atrial myocardium.

2) The arrhythmogenic potency of bile acids depends on their conjugation.

3) Serum bile acid concentrations and the constitution of the bile acid pool differ in patients with atrial fibrillation.

4) Mechanistically, the arrhythmogenic properties of bile acids may be due to specific interactions with bile acid receptors on myocytes or unspecific (detergent-like) actions of the sarcolemma.

4. Material and Methods

Human myocardium and muscle strip preparation

A small (about 5-8 mm x 8-16 mm) piece of heart was excised from the right atrium of patients undergoing heart surgery to cannulate the heart for extracorporeal circulation (**Figure iv**).

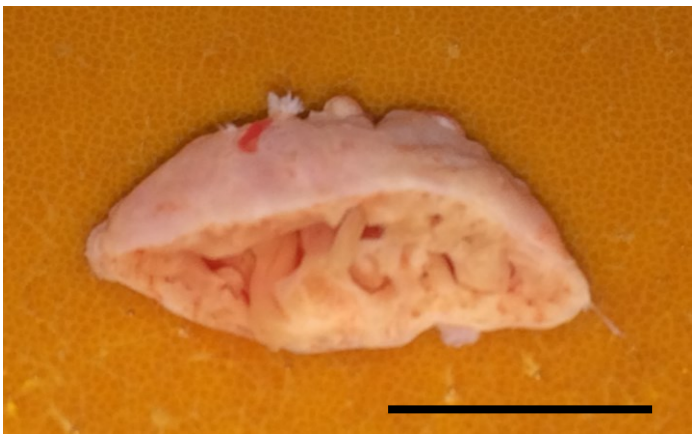


Figure iv Human right atrial appendage with trabeculations. Scale bar: 5mm (*Photograph by Markus Wallner, used with permission*).

The tissue was immediately transported to the laboratory in ice-cold cardioplegic solution that contained the following: 152 mM Na+, 3.6 mM K+, 135 mM Cl-, 25 mM HCO3-, 0.6 mM Mg2+, 1.3 mM H2PO4-, 0.6 mM SO42-, 0.2 mM Ca2+, 11.2 mM glucose and 30 mM 2,3-butanedione-monoxime (BDM) and was oxygenated with 95% O2 and 5% CO2, pH 7.4. Small endocardial trabeculae ("muscle strips" with a cross-sectional area of <0.5 mm2) were dissected with the help of a stereo-microscope. The muscle strips were

then transferred to an organ bath, mounted on miniature hooks connected to a force-transducer (Scientific Instruments, Germany) and a length-controller, and superfused with modified Tyrode´s solution (37°C) of the same composition as the cardioplegic solution described above, except that BDM was omitted, and Ca2+ was stepwise increased to 2.5 mM (**Figures v, vi**).

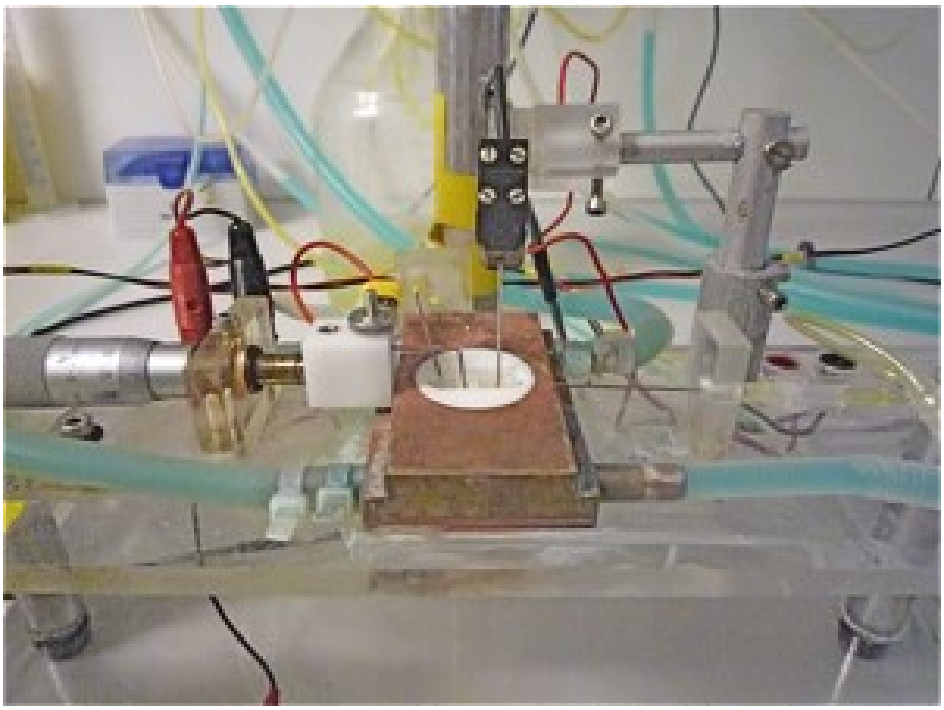


Figure v Organ bath with length controller (left) and force transducer (right) connected to miniature hooks, which are also connected to a pacer.

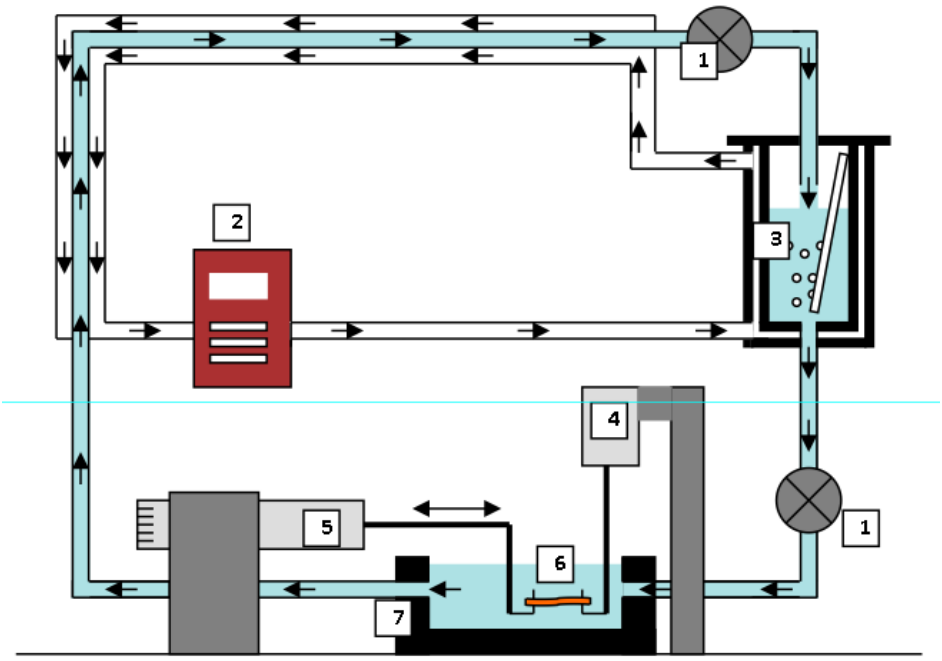


Figure vi Muscle strip setup (1 roller pump, 2 heater, 3 reservoir, 4 force transducer, 5 micrometer adjusting length, 6 muscle strip, 7 organ bath (*cartoon by Mounir Khafaga, used with permission*).

Muscles were paced at 0.5 and 1 Hertz (Hz, voltage 25% over threshold) and gradually stretched to optimum preload (Lmax, i.e., the length with maximum twitch force generation) until steady state conditions were reached. Bile acids (purchased from Sigma-Aldrich, Vienna, Austria) were added to the perfusate in increasing concentrations (10, 30, 100, 300, 1000 µM) for at least 15 minutes per concentration step. These concentrations match concentrations that have been used in previous studies[2, 11, 19-21]. Developed force and twitch kinetics (developed systolic and diastolic force (mN/mm2), time to maximum tension (ms), velocity of force generation (dF/dt max) and relaxation (dF/dt min), time to 50% and 90% relaxation (RT50, TT90), and the appearance of arrhythmic extra contractions (AECs) were analyzed. The contractile refractory period

was determined by reducing stimulation intervals (constant voltage) until the muscle strip was unresponsive to the electric impulse.

In total, tissue from 84 patients was used **(Supplementary Table 1)**: 49 patients required coronary artery bypass grafting (CABG), 20 patients aortic valve replacement, and 15 patients both. 25 patients were female; the mean age was 66±13 years, and the mean ejection fraction (EF) 56±9%; medications being taken included beta blockers in 60% and angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers in 66%. When analysing comorbidities and premedication we found a non-significant trend towards higher AEC induction in patients undergoing CABG surgery (p=0.077) and with lower EF. We routinely use matched trabeculae from the same hearts for different treatment groups. Tissue from patients with atrial fibrillation was not used for functional studies. The local ethics committee approved the study, all patients gave written informed consent to participate, and the study conformed to the principles of the declaration of Helsinki.

Measurement of serum bile acid levels and logistic regression analysis

Serum samples of 250 patients were obtained from the German Competence Network Heart Failure (CNHF, subproject 7). All trials within the CNHF comply with the declaration of Helsinki; the protocols were approved by the responsible ethics committees and all patients gave written informed consent[28].

Bile acids were determined as unconjugated acids and as taurine and glycine conjugates using a tandem mass spectrometry method. The free acids and the corresponding conjugates were measured by 3 different multiple reaction monitoring (MRM) experiments within one high-performance liquid chromatography run. Quantitation was performed by the use of deuterated internal standards and correlation of peak area ratios in linear regression[29].

Electrophysiology

Two to four month-old FVB mice (Animal Services, Medical University of Vienna, Austria) were injected with heparin (400U/kg) anesthetized with isoflurane 5% and euthanized by cervical dislocation. Hearts were quickly removed and washed in Ca2+- free modified Eagle's medium (MEM, Sigma Aldrich, Vienna, Austria) followed by coronary perfusion with digestion buffer containing Liberase TH (Roche, Austria) at 37°C for 10min. The myocardium was then minced and further digested with gentle agitation. After digestion, myocytes were stored in MEM 0.1 mM Ca2+ at room temperature until use.

Myocytes were superfused at a flow rate of 3-5ml/min with modified Tyrode's solution containing (mM): 137NaCl, 5KCl 10D-Glc, 1MgCl, 1CaCl2, 10Hepes, pH 7.4 and 35°C and clamped using the whole cell patch clamp technique (ruptured patch). Current was measured using an Axopatch 200B (Axon Instruments, Molecular Devices, CA, USA) amplifier controlled by a personal computer using a Digidata 1322A analog to digital converter (Axon Instruments) driven by pCLAMP 8 software (Axon Instruments). For

action potential recording patch pipettes made from glass capillaries (Harvard Instruments, MA, USA) were pulled on a P-97 puller (Sutter, CA, USA) up to a resistance of 2-4MOhm and filled with internal solution containing (mM): 20KCl, 110K-Asp, 0.5MgCl, 10Hepes, 5MgATP, 5NaCl, 0.1Li-GTP, 5Na-PCrea; pH7.2.

Action potentials were evoked by a depolarizing intracellular current injection (1-2nA amplitude, 2-4ms duration) at a pacing cycle length of 2sec; at steady-state conditions myocytes were exposed to taurocholic acid. Only rod-shaped, cross-striated myocytes with a resting membrane potential lower than -70mV at baseline were included in studies.

For studying L-Typ Ca2+ current KCl in the extracellular solution was replaced by equimolar CsCl. The intracellular solution was composed of [mM]: 110 CsCl, 10 TEACl, 0.5 MgCl, 10 Hepes, 5 MgATP, 5 NaCl, 11 EGTA, 1 CaCl2, 0.1Li-GTP, 5Na-PCrea, pH 7.2. After capacitance and series resistance (up to 60%) were compensated I_{Ca} was elicited by 400ms voltage steps in 10mV increments from -40 to +80mV, every 5 seconds. To allow steady-state conditions a voltage protocol including voltage steps (150ms) from -40 to 0mV was applied before and voltage-gated Na+-channels were inactivated by a 50ms ramp from -80 to 40mV. $I_{Ca,L}$ was determined as the difference between the maximum inward current and the current at the end of the pulse and we constructed the mean current–density/voltage relationship by normalizing $I_{Ca,L}$ to cell capacitance, which was calculated as the time-integral of the capacitive surge measured in response to 10mV hyperpolarizing steps from a holding potential of -50mV divided by the voltage change.

For selective determination of NCX inward current function cells were superfused with a Cs based Tyrode and dialyzed with a Cs based intracellular solution (see above). $[Ca2+]_i$ was buffered constantly to diastolic values and NCX tail currents were measured as peak inward currents 20ms after repolarization (and thus after the capacitive spike) to -80mV following 400ms depolarization steps from +40 to +80mV. Steady-state holding currents were subtracted and values normalized to cell capacitance were plotted against test potentials (40 to 80mV).

The protocol was approved by the local animal use and care committee and followed U.S. National Institutes of Health guidelines.

Sarcoplasmic reticulum Ca^{2+} content

Isolated myocytes were loaded with 3µM Fura2-AM (Invitrogen) for 10 minutes at room temperature. After de-esterification cells were placed in a perfusion chamber and superfused with Tyrode's solution containing 1mM Ca2+ and electrically field stimulated at 0.5Hz. Whole cell Ca2+ transients (excitation 340/380nM, emission 510nM) were recorded with an inverted fluorescence microscope (Nikon, TE2000) and IonOptix (Myocam) software. Sarcoplasmic reticulum (SR) Ca2+ content was assessed as the amplitude of the Ca2+ transient induced by rapid application of 20mM caffeine 5s after turning of field stimulation **(Figure vii)**.

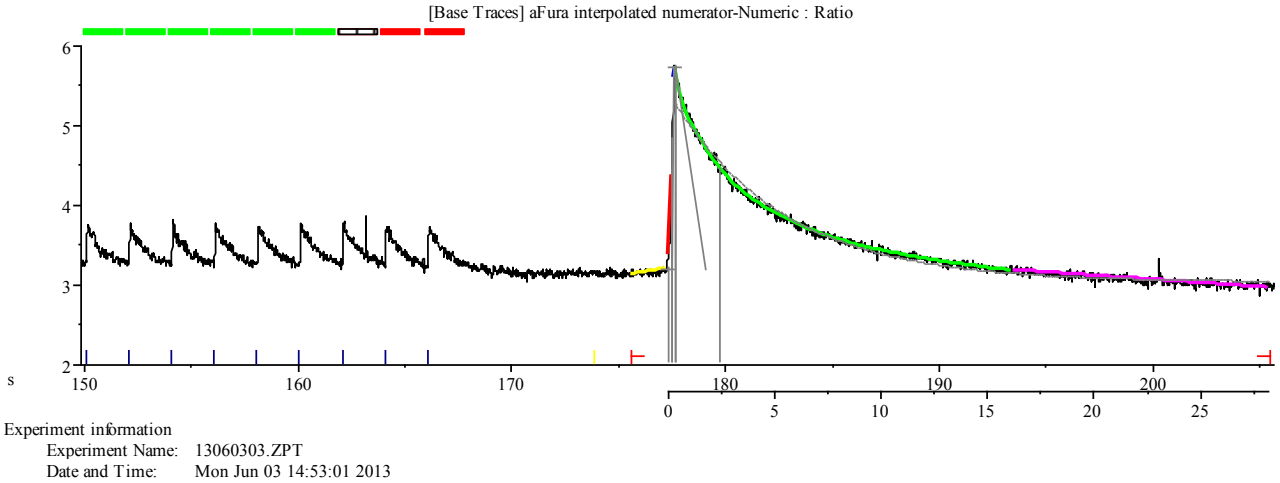


Figure vii Calcium transients of an isolated murine ventricular cardiomyocyte. The smaller transients to the left are paced transients (pacing spikes visible at the bottom). Once the pacing is stopped caffeine (20mM) is rapidly applied to the perfusion chamber. The amplitude of the caffeine-induced calcium transient is used as a measure of the SR calcium content. In this depiction the curve fitted to the calcium transient is overlaid with color.

RNA Isolation and quantitative real-time PCR

Total myocardial ribonucleic acid (RNA) was isolated with TRIzol reagent (Invitrogen, Austria) and reverse-transcribed using the GeneAmp Gold RNA PCR Core Kit (Applied Biosystems, Austria) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was performed on a GeneAmp 7900 Sequence Detection System with GeneAmp 7900 SDS software (Applied Biosystems, CA), using a 20 µL reaction mixture containing TaqMan or SYBR Universal PCR Master Mix (Applied Biosystems, CA). The following PCR primers were used (MRP2-4, MDR1, MDR3, BSEP, NTCP, OSTalpha, OSTbeta, TGR5, FXRalpha, SHP, LXRalpha (control), LXRbeta (control) as previously published[30]. Liver complementary deoxyribonucleic acid (cDNA) was used as positive control and to generate a standard-curve for each gene per run.

Western Blot

Myocytes were incubated for 15 minutes with 300µM TCA or TUDCA at room temperature with gentle agitation. After centrifugation and snap freezing protein was isolated using cell lysis buffer (Cell Signaling Technologies, MA, USA) according to manufacturer’s protocol, separated by SDS-PAGE on Tris-Glycine gels (Nupage, Invitrogen, NY, USA), and transferred to nitrocellulose membranes. Primary antibodies to pPLB (Ser16) and tPLB were purchased from Millipore (pPLB) and Pierce (tPLB) and incubated at 4°C overnight. Fluorescence-labeled secondary antibodies were obtained from Licor (NE, USA), and membranes were scanned on an infrared imaging system (Odyssey, Licor).

Statistics

Data are given as mean±SEM. Means were compared by two-tailed Student's t-test or one way Analysis of variance (ANOVA) with Bonferroni corrected t-tests. Non parametric data were compared using Whitney Mann U-test or Kruskal-Wallis analysis. Proportions were compared using the Chi-squared distribution or Fisher's exact test. Multiple logistic regression was performed using SPSS 20 statistical software with atrial fibrillation as dependent variable and bile acid levels, N-terminal pro brain natriuretic peptide (NTproBNP) concentration, age, and presence of inferior cave vein (ICV) dilation as covariates.

5. Results

Bile acids induce arrhythmic extra contractions in human atrial myocardium

We exposed human atrial myocardium to taurocholic acid (TCA) and analyzed the occurrence of arrhythmic extra contractions (AECs). At baseline AECs occurred in 11% of muscle strips (3/28); by increasing TCA concentrations in the perfusate to 10, 30, 100, 300, and 1000µM, AEC occurrence increased to 32, 48, 46, 50 and 54%, respectively ($p=0.09$ for 10µM TCA and $p<0.01$ for TCA concentrations ≥30µM. **Figure 1a, 1b, 1d, Table 1**).

Table 1 AEC incidence and functional characteristics of atrial trabeculae with TCA or UDCA incubation

	TCA (n=28)			**UDCA (n=9-11)**		
	Baseline	**300 µM TCA**	**p-value**	**Baseline**	**300 µM UDCA**	**p-value**
AEC Incidence [%]	11% (3 of 28)	50% (14 of 28)	0.003	0% (0 of 11)	0% (0 of 11)	1.000
Developed Force [mN/mm²]	9.88±1.37	8.79±1.44	0.586	9.97±1.43	10.98±1.70	0.672
Diastolic Tension [mN/mm²]	2.71±1.12	1.21±0.83	0.290	2.15±0.52	0.85±0.45	0.010
dF/dt max [mN/mm²/s]	178.96±24.14	155.97±27.83	0.056	186.71±27.99	213.30±37.46	0.340
dF/dt min [mN/mm²/s]	-86.08±10.87	-79.81±13.16	0.296	-82.53±13.10	-79.27±14.78	0.569
RT50 [ms]	86.56±5.91	84.06±6.02	0.464	111.71±8.46	131.71±8.03	0.164

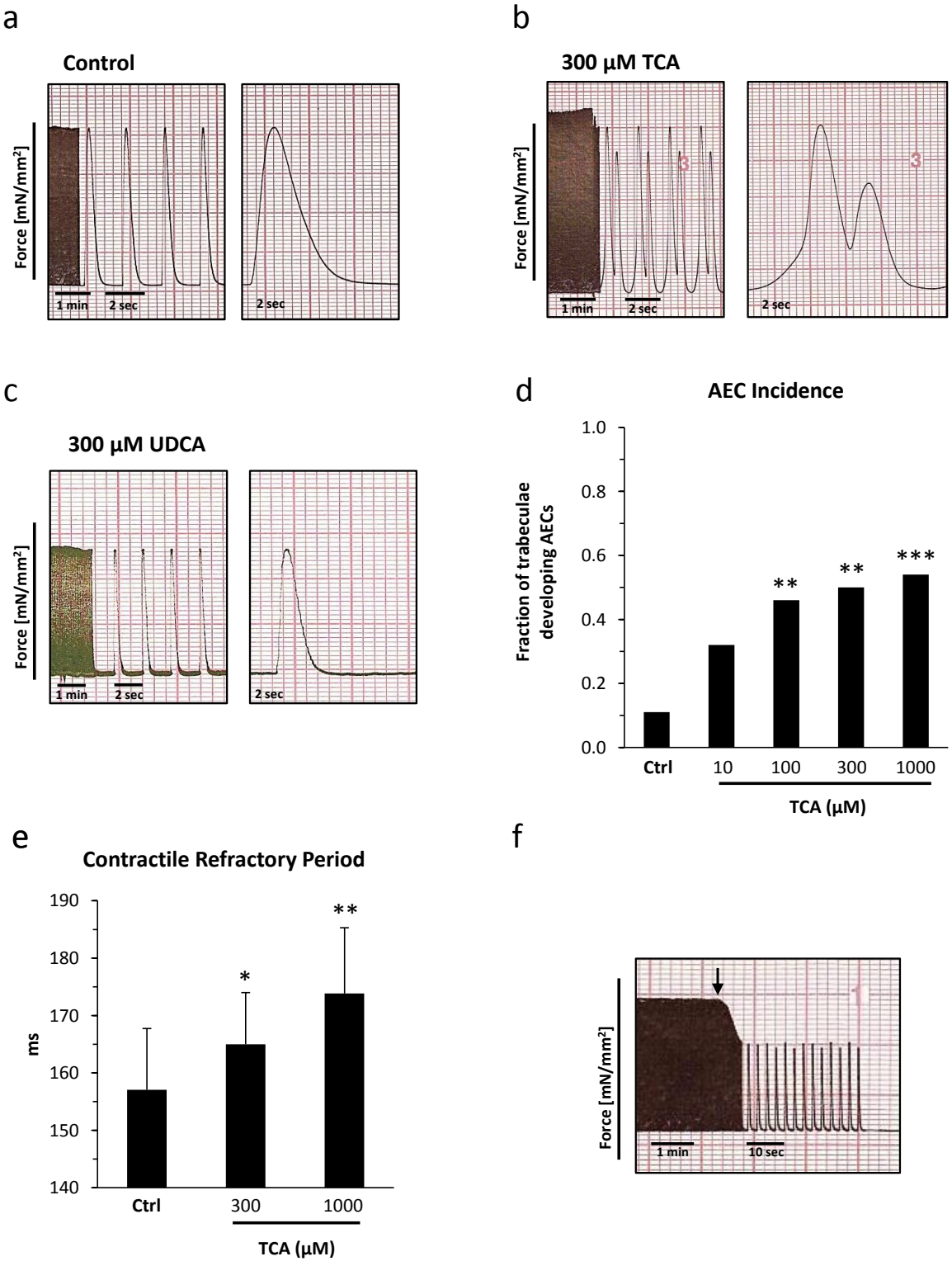


Figure 1 Taurocholic acid concentration-dependently induces AECs in atrial trabeculae. Representative recordings **(a, b)** and summary data **(d)** [n=24-28, pacing at 0.5 Hz]. Ursodeoxycholic acid was not effective in inducing AECs **(c)** [n=9-11]. Taurocholic acid prolonged the contractile refractory period of trabeculae **(e)** [n=13-17; * $p<0.05$, ** $p<0.01$]. At the highest concentration tested (1000 µM) taurocholic acid elicited an immediate negative inotropic effect **(f)**.

Functional parameters of myocardial inotropy and kinetics (developed force, diastolic tension, velocity of force generation and relaxation parameters) were not acutely altered at pathophysiologic relevant bile salt concentrations (**Table 1**). Only at the highest concentration tested (1000µM) developed force decreased significantly (**Figure 1f**).

We also tested the other main conjugated primary bile acids: taurine-conjugated chenodeoxycholic acid (TCDCA), glycine-conjugated cholic- (GCA), and chenodeoxycholic acid (GCDCA) and found AEC-induction to be present with GCA and GCDCA (**Table 2,** p-value for TCDCA n.s.). In contrast, ursodeoxycholic acid (UDCA) and taurine-conjugated ursodeoxycholic acid (TUDCA), were not effective in inducing any AECs at any concentration tested (**Figure 1c, Table 1, Figure 2**).

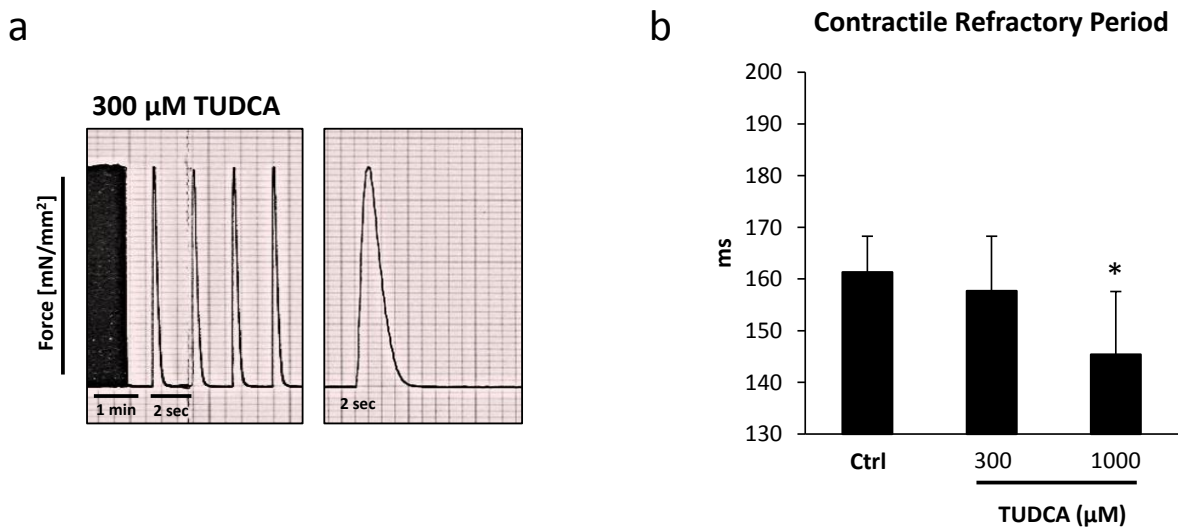


Figure 2 Tauroursodeoxycholic acid (TUDCA, 300 µM) is not effective in inducing arrhythmias in trabeculae **(a)** [n=12], and rather shortens than prolongs contractile refractory period **(b)** [*p<0.05 vs. ctrl, n=13].

Table 2 AEC incidence and functional characteristics of atrial trabeculae with GCA, TCDCA, or GCDCA incubation.

	GCA (n=7)			**TCDCA (n=7)**			**GCDCA (n=4)**		
	Baseline	**300 µM GCA**	**p-value**	**Baseline**	**300 µM TCDCA**	**p-value**	**Baseline**	**300 µM GCDCA**	**p-value**
AEC Incidence [%]	0% (0 of 7)	67% (4 of 6)	0.021	0% (0 of 7)	43% (3 of 7)	0.192	0% (0 of 5)	75% (3 of 4)	0.048
Developed Force [mN/mm²]	8.33 ±1.54	6.97 ±1.41	0.245	8.31 ±1.58	7.73 ±1.83	0.363	14.34 ±2.49	13.59 ±2.71	0.131
Diastolic Tension [mN/mm²]	1.53 ±0.08	1.57 ±0.17	0.176	2.58 ±0.27	1.96 ±0.24	0.001	2.27 ±0.38	1.77 ±0.26	0.138
dF/dt max [mN/mm²/s]	110.24 ±15.07	87.85 ±14.32	0.110	122.98 ±22.28	92.93 ±19.57	0.094	165.06 ±30.42	161.19 ±31.54	0.397
dF/dt min [mN/mm²/s]	-60.40 ±10.76	-49.54 ±9.56	0.106	-60.48 ±9.51	-53.57 ±11.75	0.102	-98.16 ±18.66	-93.59 ±2099	0.446
RT50 [ms]	97.5 ±6.35	125.83 ±15.65	0.302	93.00 ±5.39	110.17 ±8.34	0.224	111.33 ±11.57	114 ±14.17	0.642

To further characterize functional effects of bile acids on atrial myocardium we determined the contractile refractory period by reducing stimulation intervals until the muscle strip was unresponsive to the electric impulse (absolute refractory) and tested higher stimulation frequencies (1Hz). As the arrhythmogenic capacity of different bile acid conjugates had not differed in the previous experiments we used TCA in ensuing experiments.

At baseline the contractile refractory period was 157.1±10.7ms. Exposing the muscle strips to increasing TCA concentrations prolonged the refractory period to 165.0±9 and 173.8±11.5ms at 300 and 1000µM TCA ($p<0.05$ for both, $n\geq13$, **Figure 1e**). In contrast, TUDCA rather shortened the contractile refractory period **(Figure 2b)**.

When stimulating the muscle strips with 1Hz the occurrence of AECs was markedly reduced. We did not observe any AECs at baseline and only a slight increase in AEC occurrence at concentrations between 10 and 300µM TCA (6% for 300µM, p=n.s.); only at the highest concentration tested (1000µM) TCA induced AECs in 37% of cases (10/27, p=0.001). Taken together, the studies in trabeculae demonstrate concentration dependent induction of arrhythmic extra contractions in human atrial myocardium by the taurine- and glycine-conjugated primary bile acids cholic and chenodeoxycholic acid. Notably, ursodeoxycholic acid did not induce any AECs at any concentration tested.

Concentration and composition of serum bile acids in patients with atrial fibrillation

We measured the serum bile acid concentration in 250 patients of the CNHF network. Patients without pre-existing liver disease and an unambiguous diagnosis of atrial fibrillation (as evidenced by ECG recordings) were included in the analysis (n=195). Patient characteristics are given in **Table 3**.

Table 3 Patient characteristics

Parameter	**Sinus Rhythm (n=144)**	**Atrial Fibrillation (n=51)**	**p-value**
Male Sex [%]	68.8%	76.5%	0.298
Age [years]	68.17±0.66	71.12±1.05	**0.022**
BMI [kg/m^2]	30.15±0.50	29.89±0.63	0.951
Smoker [%]	34%	41.2%	0.360
LV Ejection Fraction [%]	49.27±0.85	51.87±1.70	0.173
NTproBNP [ng/l]	4820.67±216.45	8541.61±730.67	**<0.001**
IVC Dilation [%]	2.8%	30.6%	**<0.001**
Non-UDCA BA [µmol/l]	2.72±.014	3.55±0.35	***0.055***
UDCA BA [µmol/l]	1.03±0.07	0.62±0.14	**0.001**

The total bile acid concentration was 3.75±0.17µM (95% CI 3.41-4.09µM) in patients with sinus rhythm and 4.17±0.42µM (95% CI 3.32-5.02µM) in patients with atrial fibrillation. The difference in the mean values between groups was not significant (p=0.985; 95% CI for difference: -1.33, 0.49).

Ursodeoxycholic acid-conjugate levels, however, contrasted the behaviour of total bile acid concentrations. When analyzing the concentration of total bile acids without ursodeoxycholic acid (non-UDCA bile acids) and ursodeoxycholic acid independently, we found that the total concentration of non-UDCA bile acids was increased from 2.72±0.14µM (95% CI 2.45-3.00µM) in patients with sinus rhythm to 3.55±0.35µM (95% CI 2.85-4.25µM) in patients with atrial fibrillation (p=0.055; 95% CI for difference: 0.08, 1.58 µM). On contrast, ursodeoxycholic acid levels decreased from 1.03±0.07µM (95% CI: 0.88, 1.17µM) to 0.62±0.14µM (95% CI: 0.34, 0.89µM) in patients with atrial fibrillation (p=0.001; 95% CI for difference: -0.72, -0.10; **Figure 3**).

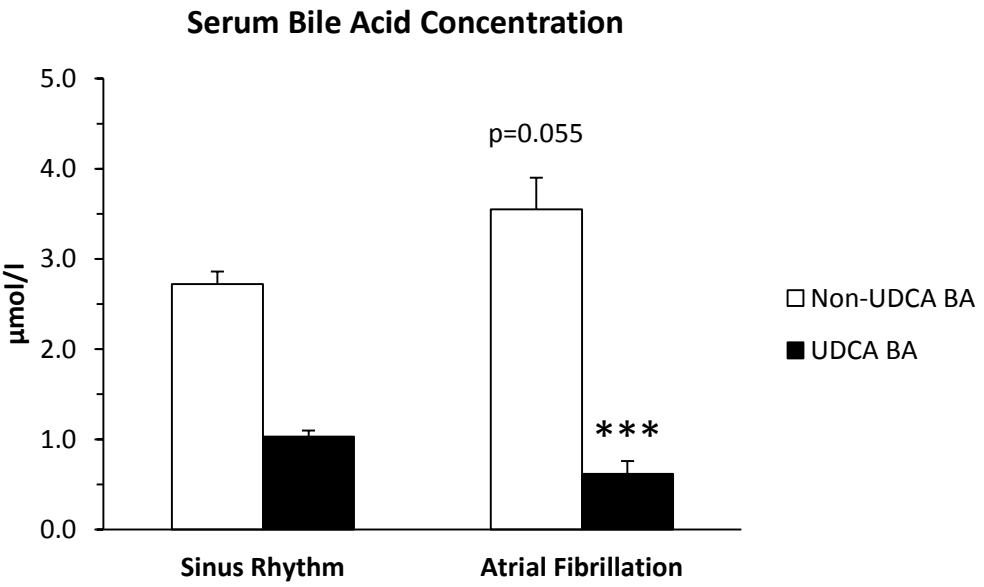


Figure 3 Non-ursodeoxycholic bile acid-conjugates were increased in patients with atrial fibrillation while ursodeoxycholic acid-conjugate levels were decreased [3.55±0.35 µM vs. 2.72±0.14 µM for non-UDCA bile acids, p=0.055, 95% CI for difference: 0.08, 1.58; and 0.62±0.14 µM vs. 1.03±0.07 µM for UDCA, *** p<0.001; 95% CI for difference: -0.72, -0.10, respectively; n=144 for SR, n=51 for AF].

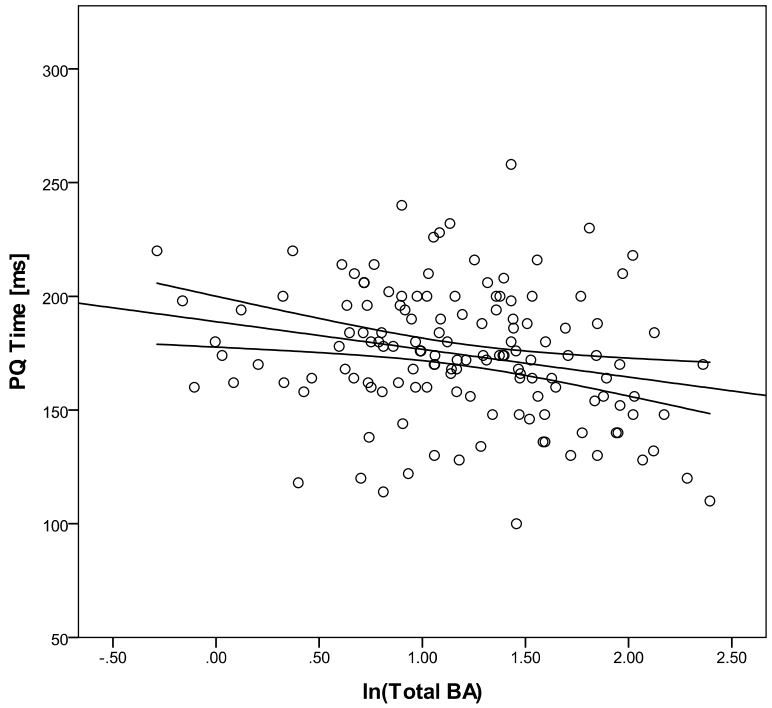
To adjust for covariates we performed multiple logistic regression analysis with atrial fibrillation status as the dependent variable. Both the total bile acid concentration without ursodeoxycholic acid and ursodeoxycholic acid were significant predictors of the occurrence of atrial fibrillation in the logistic regression analysis when controlling for age, NTproBNP values, and the presence of inferior cave vein (ICV) dilation (adjusted odds ratio (OR) for atrial fibrillation for one unit increase in non-UDCA bile acid levels: 1.46; 95% CI: 1.12, 1.90; OR for UDCA: 0.34; 95% CI: 0.18, 0.64; **Table 4**).

Table 4 Multiple Logistic Regression Analysis

Model Summary	
Nagelkerke R^2	0.699
Hosmer Lemeshow Goodness of Fit	p=0.413

Covariate	**Coefficient**	**Standard Error**	**p-value**	**Odds Ratio**	**95% CI for OR**	
					Lower	**Upper**
Non-UDCA BA	0.377	0.134	**0.005**	1.458	1.121	1.897
UDCA BA	-1.092	0.325	**0.001**	0.336	0.177	0.635
NTproBNP	0.003	0.001	**<0.001**	1.003	1.002	1.004
Age	-0.045	0.038	0.234	0.956	0.888	1.029
ICV Dilation	3.628	0.984	**<0.001**	37.623	5.473	258.615
Constant	-0.904	2.428	0.710	0.405 (Odds)		

We also investigated the correlation of serum bile acid levels with ECG parameters and found a significant, albeit weak negative correlation of total bile acid levels with PQ time in patients with sinus rhythm (Pearson's r=-0.234, p=0.005, **Figure 4**). Bile acids levels did not correlate with heart rate, QRS time, or QT interval duration.



r= -0.234 (Pearson)
p=0.005
n=140

No significant linear correlation between total BA levels and:

Heart Rate
QRS Time
QT Interval

Figure 4 PQ time is weakly correlated with total bile acid levels in patients with sinus rhythm [r=-0.234 (Pearson), p=0.005, n=140]. We did not find significant correlations between other ECG parameters and total bile acid levels.

Myocardial expression patterns of bile acid transporters and receptors

Different cell membrane- and nuclear receptors have been demonstrated to be bile acid targets and to mediate specific effects of bile acids in various tissues. Particularly the G-protein coupled bile acid receptor TGR5 has been implicated in the cardiovascular effects of bile acids[2], though other studies found no involvement of TGR5 or the nuclear bile acid receptor FXR[20]. Another nuclear (bile acid) receptor, liver X receptor beta (LXRbeta), has been shown to mediate anti-hypertrophic effects in a pressure-overload model of heart failure[31] and to be protective in a cardiac ischemia-reperfusion model [32]. Thus we tested the presence and regulation of mRNA transcripts of bile acid receptors and transporters in human atria (12 patients with sinus rhythm, 9 with atrial fibrillation).

We could not detect transcripts for the classic bile acid farnesoid receptor X (FXR), Na+/Bile co-transporter (NTCP), bile salt export pump (BSEP), organic solute transporter alpha (OSTalpha), multidrug resistance-associated protein 2, 3, and 4 (MRP2, MRP3, MRP4) in human atria. Transcripts for the liver X receptor (LXRbeta), the G-protein coupled bile salt receptor (TGR5), lipoprotein lipase (LPL), small heterodimer partner (SHP), and familial intrahepatic cholestasis 1 (FIC1) were expressed in human atria and thus may principally mediate bile acid effects. However, of these only SHP was differentially expressed in patients with or without atrial fibrillation (0.42-fold±0.08, $p<0.05$, **Figure 5**) LXRbeta transcript levels demonstrated a non-significant trend towards lower expression levels in patients with AF (0.4-fold±0.17, $p=0.076$).

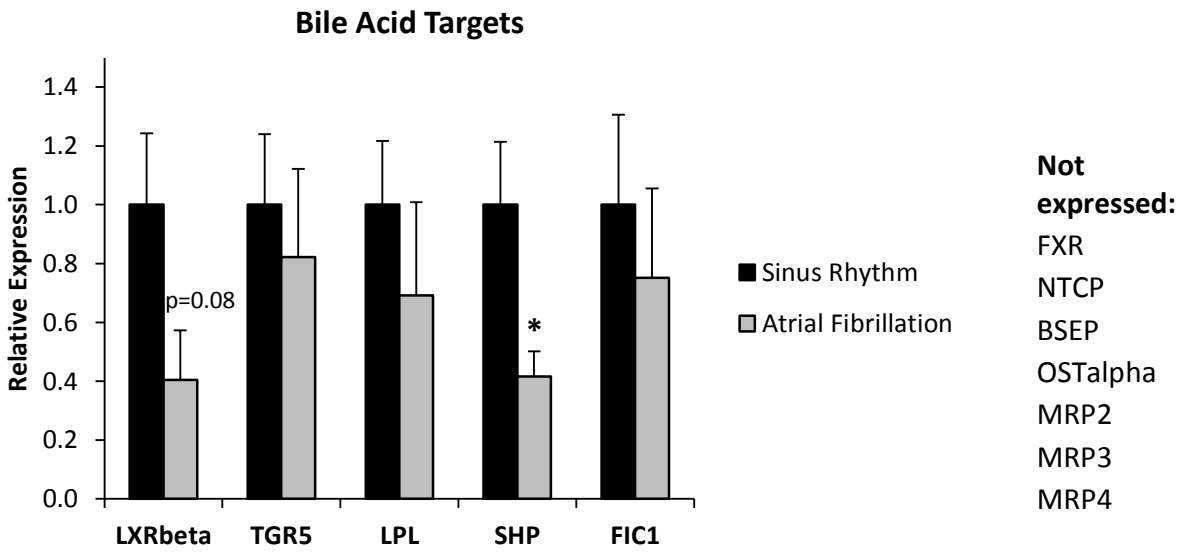


Figure 5 Myocardial expression patterns of bile acid targets. LXRbeta, TGR5, LPL, SHP, and FIC1 are expressed in human atrial myocardium. LXRbeta (trend, p=0.076) and SHP (* p<0.05) were down-regulated in patients with atrial fibrillation (n=9-12).

Taurocholic acid depolarizes the resting membrane potential and induces afterdepolarizations in isolated cardiomyocytes

TCA significantly depolarized the resting membrane potential of isolated cardiomyocytes, from -80±1.4mV at baseline to -72±4.0mV and -56±5.4mV at 5 minutes after administration of 300 or 1000µM TCA, respectively (n=6-11 per group, $p<0.05$, **Figure 6a**).

The occurrence of early- and delayed afterdepolarizations (EADs and DADs), which give rise to triggered activity and thus arrhythmic events was increased to 1.2±0.5, 4.3±1.9 and 10.1±3.5 afterdepolarizations/minute at 100, 200 and 300 seconds after exposure to 300µM TCA ($p<0.05$, n=21, **Figure 6b-d**). Delayed afterdepolarizations (DADs) predominated, however, we did not observe a statistically significant difference between early- or delayed afterdepolarizations.

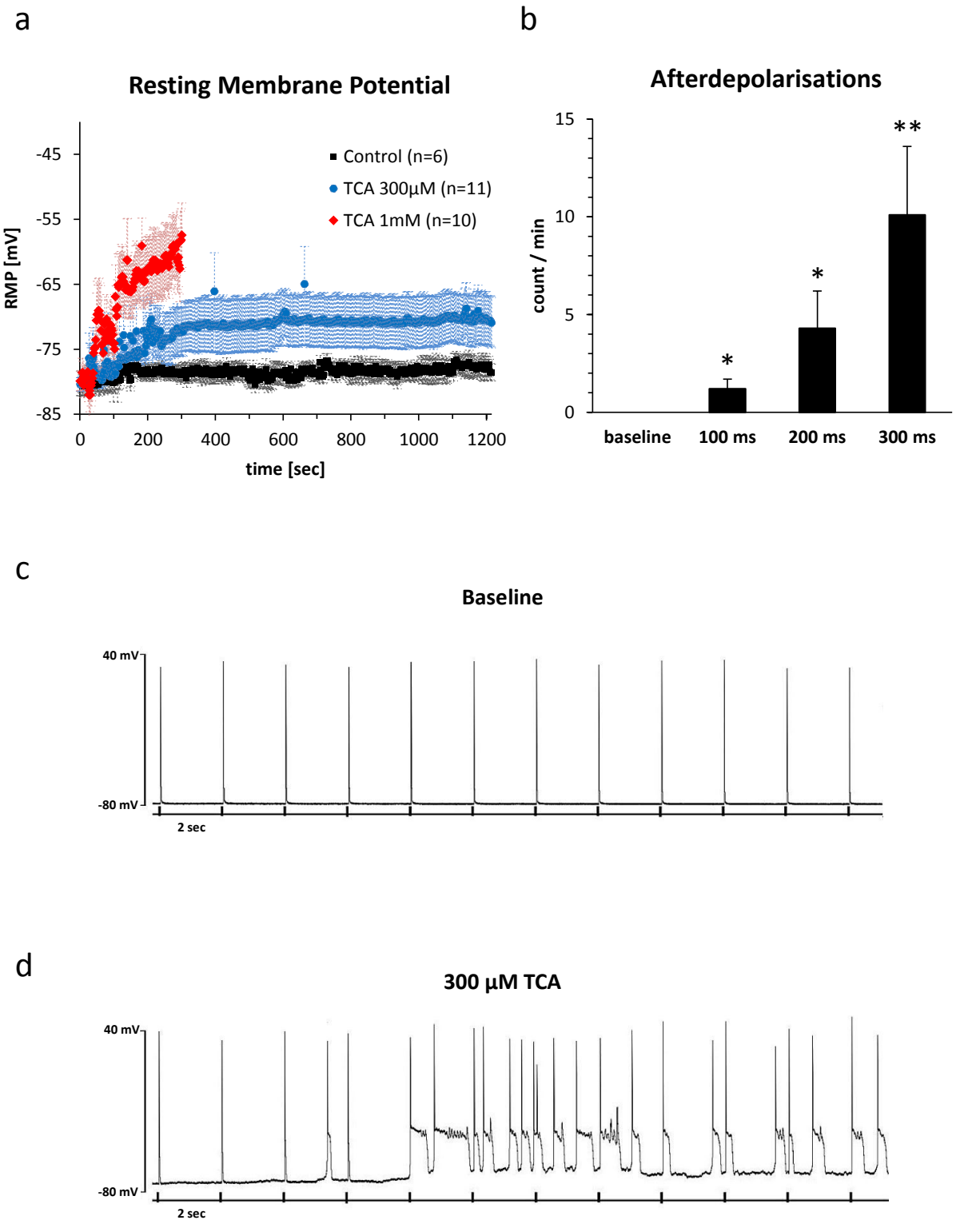


Figure 6 Taurocholic acid depolarized the resting membrane potential **(a)** and induced the appearance of afterdepolarizations [original traces **(c, d)**, and summary data **(b)**]. At 1000 µM TCA myocytes became unstable after incubation periods longer than 300 seconds (* $p<0.05$, ** $p<0.01$ vs. baseline).

Taurocholic acid increases Na+/Ca2+ exchanger (NCX) inward current density. Inhibition of NCX abolishes TCA induced arrhythmias

The sarcolemmal Na+/Ca2+ exchanger (NCX) is prone to perturbations of lipid bilayer properties by charged amphiphiles[33, 34]. We assessed the effect of TCA on NCX current density in isolated myocytes and found enhanced NCX inward current after exposure to 300µM TCA **(Figure 7a, b)**. Inhibition of NCX by 3',4'-dichlorobenzamil hydrochloride (DCB, 10µM) decreased the occurrence of TCA induced afterdepolarizations in isolated myocytes (trend, p=0.071, **Figure 7c**) and prevented depolarization of the resting membrane potential (-84±1.9mV 5 minutes after TCA administration, p=n.s. vs. ctrl, **Figure 7d**). In atrial trabeculae, DCB completely abolished TCA induced AECs (**Figure 7e**, $p<0.05$ vs. TCA) and blunted prolongation of the contractile refractory period (**Figure 7f**, p=n.s.).

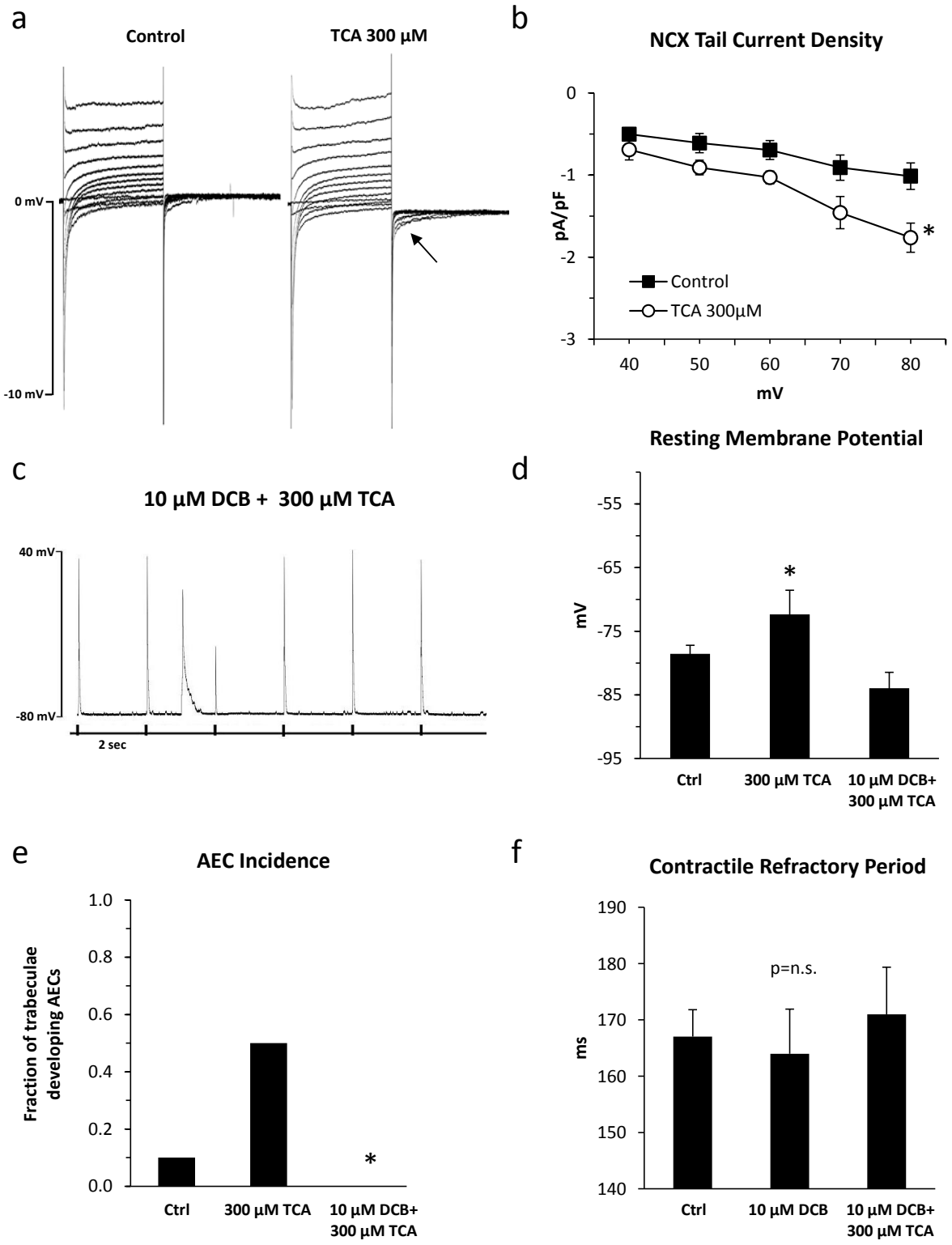


Figure 7 TCA (300µM) increases NCX tail current density (arrow) at -40mV, representative traces **(a)** and summary data **(b)** [*p<0.05, n=8). NCX inhibition (10µM DCB) reduced afterdepolarizations in isolated myocytes **(c)** [trend, p=0.071, n=7], prevented depolarization of the resting membrane potential **(d)** [*p<0.05 vs. ctrl, n=7], completely abolished AECs in trabeculae **(e)**, [*p<0.05 vs. TCA, n=8-12] and prevented prolongation of the contractile refractory period by TCA **(f)** [p=n.s., n=10]

L-type Ca2+ current-voltage relationships **(Figure 8a)**, sarcoplasmic reticulum (SR) Ca2+ content **(Figure 8b)**, and phospholamban phosphorylation **(Figure 8c)** were not altered by TCA.

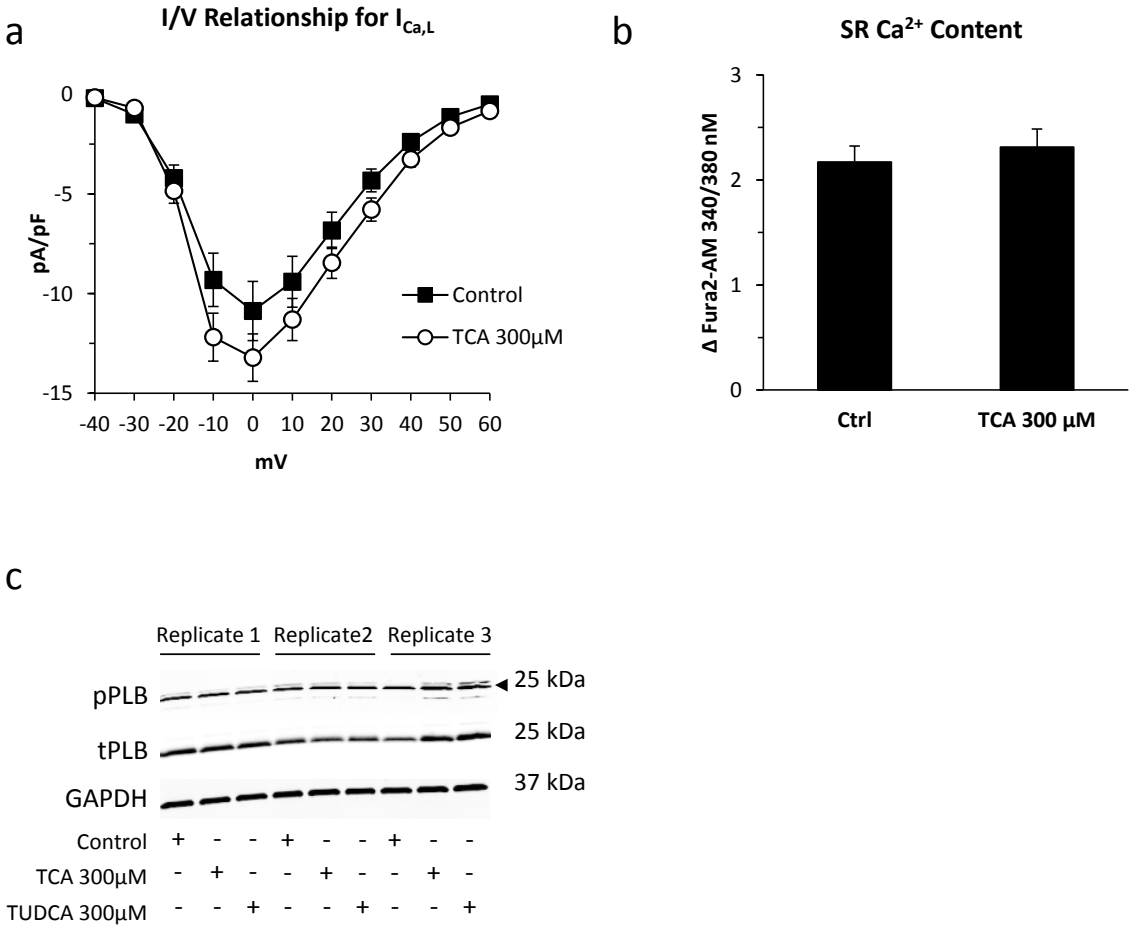


Figure 8 TCA (300µM) does not significantly shift L-Type Ca^{2+} current density-voltage relationships **(a)** [n=8-10], sarcoplasmic reticulum Ca^{2+} content **(b)** [n=18-21], or phospholamban phosphorylation **(c)** [n=3].

6. Discussion

We demonstrate for the first time that primary bile acids concentration-dependently induce arrhythmia in adult human atrial tissue at concentrations that are seen in patients with cholestasis. Previous work mainly investigated the effects of cholestasis on fetal complication rates in intrahepatic cholestasis of pregnancy (ICP)[17], and animal or cell culture models of cholestasis[2, 18, 21]. Our results suggest that not only fetal hearts but also adult myocardium is susceptible to bile acid-induced arrhythmia.

Moreover, we show that ursodeoxycholic acid, a bile acid that is therapeutically used to treat cholestatic disease, does not elicit arrhythmias in isolated myocardium. The most appreciated mechanism of action of UDCA is an increase in hepatocellular bile acid secretion and a lowering of endogenous serum bile acid levels[25], thus making a direct interpretation of UDCA effects on the heart difficult. In our *in vitro* studies, where we set bile acid levels and preclude hepatic effects, UDCA did not induce arrhythmias while the other primary bile acids we tested did, suggesting that in addition to positive effects on hepatocyte bile acid secretion UDCA lacks the direct arrhythmogenic capacity exerted by other primary bile acids.

To test the potential clinical relevance of these findings, we measured bile acid levels in a well-defined patient collective and investigated if serum levels and composition differ between patients with or without atrial fibrillation. We chose AF because it is the

most prevalent cardiac rhythm disorder, responsible for significant morbidity and mortality[35], and we had demonstrated in our *in vitro* studies that bile acids are potent inducers of arrhythmic extra contractions in atrial tissue. The common denominator of patients in this collective was at least one risk factor for the development of heart failure, meaning that the majority of patients that we studied were rather healthy individuals in terms of bile acid levels. Additionally, we excluded patients with pre-existing liver disease and adjusted for NTproBNP levels and the presence of ICV dilation to minimize possible confounding by elevated central venous pressures or cardiac hepatopathy. Thus these patients did not have cholestasis and had low serum bile acid levels.

We found that total bile acid levels did not differ between the two groups, while the contribution of ursodeoxycholic acid to the total bile acid pool differed significantly. Ursodeoxycholic acid levels were lower in patients with AF while non-UDCA bile acid levels were higher in this group. In the multiple logistic regression analysis both ursodeoxycholic and non-UDCA bile acid levels were significant predictors of atrial fibrillation. This finding is in line with our *in vitro* data, where non-UDCA bile acids were arrhythmogenic.

As mentioned, serum bile acid levels were in the normal range in our patient collective. At these concentrations we do not expect to see direct induction of atrial fibrillation by bile acids (as we have demonstrated for the acute induction of arrhythmic contractions in isolated atria), however, the observed difference suggests that bile acid composition and particularly the ratio of UDCA to non-UDCA bile acids may create a milieu with a lower arrhythmic threshold.

Bile acid targets that have been suggested to account for arrhythmogenesis include the nuclear receptor FXR and the G-protein coupled receptor TGR5[2], and partial agonism at muscarinic M2 receptors[20]. In our samples we did not find expression of FXR. TGR5 was expressed, however, levels did not differ between patients with or without atrial fibrillation. This is consistent with work from Gorelik and co-workers that demonstrated a lack of functional effects of both receptors in cultured neonatal cardiomyocytes[20]. LXRbeta and SHP were differentially expressed between groups (trend for LXRbeta), with lower transcript levels in patients with atrial fibrillation. LXRbeta is a transcription factor, hence it is unlikely to play an immediate role in the induction of arrhythmias that we observed, however, it may still be involved in the chronic structural and electrophysiological remodeling seen in atrial fibrillation. Indeed, LXRbeta has been demonstrated to be a regulator of cardiac hypertrophy and ischemia-reperfusion injury[31, 32]. SHP is also a member of the nuclear receptor family; it is unusual as it lacks the DNA-binding domain but it can associate with other nuclear receptors and inhibit their transcriptional activity[36]. SHP has been demonstrated to be expressed in in the heart[37], its role, however, remains unclear. In fact, to our knowledge, we are the first to describe differential expression in cardiac pathology.

Another possible mechanism for bile acid-induced arrhythmogenesis is interaction of bile acids with the cell membrane and cell membrane ion channels or transporters[8, 33, 38]. Bile acids are amphiphilic molecules and are negatively charged at physiologic conditions[39]. The hydrophobic steroid structure enables bile acids to get embedded in

the lipid bilayer of the cell membrane, and it has been shown that anionic amphiphiles can stimulate the cardiac sodium-calcium exchanger (NCX)[33]. The accepted stoichiometry of NCX is three Na+ ions for one Ca2+ ion, thus it is electrogenic and one net positive charge is moved into the cell[40]. We observed enhanced NCX inward current density and resting membrane potential depolarization after TCA incubation, and that TCA gives rise to afterdepolarizations in adult cardiomyocytes. Inhibition of NCX prevented resting membrane potential depolarization and TCA induced arrhythmias (for working model see **Figure 9**). These findings are consistent with previous findings describing resting membrane potential depolarization and generation of afterdepolarizations[23]. Importantly, afterdepolarizations and triggered activity are one of the mechanisms believed to initiate atrial fibrillation[41]. We also found a prolongation of the contractile refractory period upon bile acid incubation and that arrhythmogenesis is prevented by shorter stimulation intervals. A previous study demonstrated that taurocholic acid slows spontaneous diastolic depolarization and hence heart rate in rabbit sino-atrial node preparations[11]; moreover, sinus bradycardia is a frequent clinical feature of patients with end-stage liver disease[6]. We did not assess sino-atrial node properties in our experiments but it is conceivable that a negative chronotropic effect on the sino-atrial node together with facilitated triggered activity in the atrial myocardium may promote AF initiation.

The fact that ursodeoxycholic acid was not effective in inducing arrhythmias suggests that in addition to enhanced hepatic excretion of bile acids with ursodeoxycholic acid treatment, UDCA also has differential effects on the heart. Earlier studies found that

ursodeoxycholic acid conjugates protect cholesterol-rich plasma membranes from toxic effects of more hydrophobic bile acids[42] and that UDCA can hyperpolarise and thus protect cells from arrhythmias[23]. Intriguingly, the cholesterol content of the cardiomyocyte sarcolemma is increased in cholestatic conditions[4].

In conclusion, we demonstrate that

1) high concentrations of primary bile acids acutely induce arrhythmia in adult human atria,

2) the more hydrophilic ursodeoxycholic acid is not effective in inducing arrhythmia at any concentration tested,

3) the primary bile acid taurocholic acid depolarizes the resting membrane potential and facilitates afterdepolarizations in isolated myocytes,

4) NCX inward current density is increased in myocytes exposed to TCA, and NCX inhibition prevents arrhythmia,

5) atrial fibrillation is associated with an altered composition of the serum bile acid pool with low UDCA and higher non-UDCA bile acid levels.

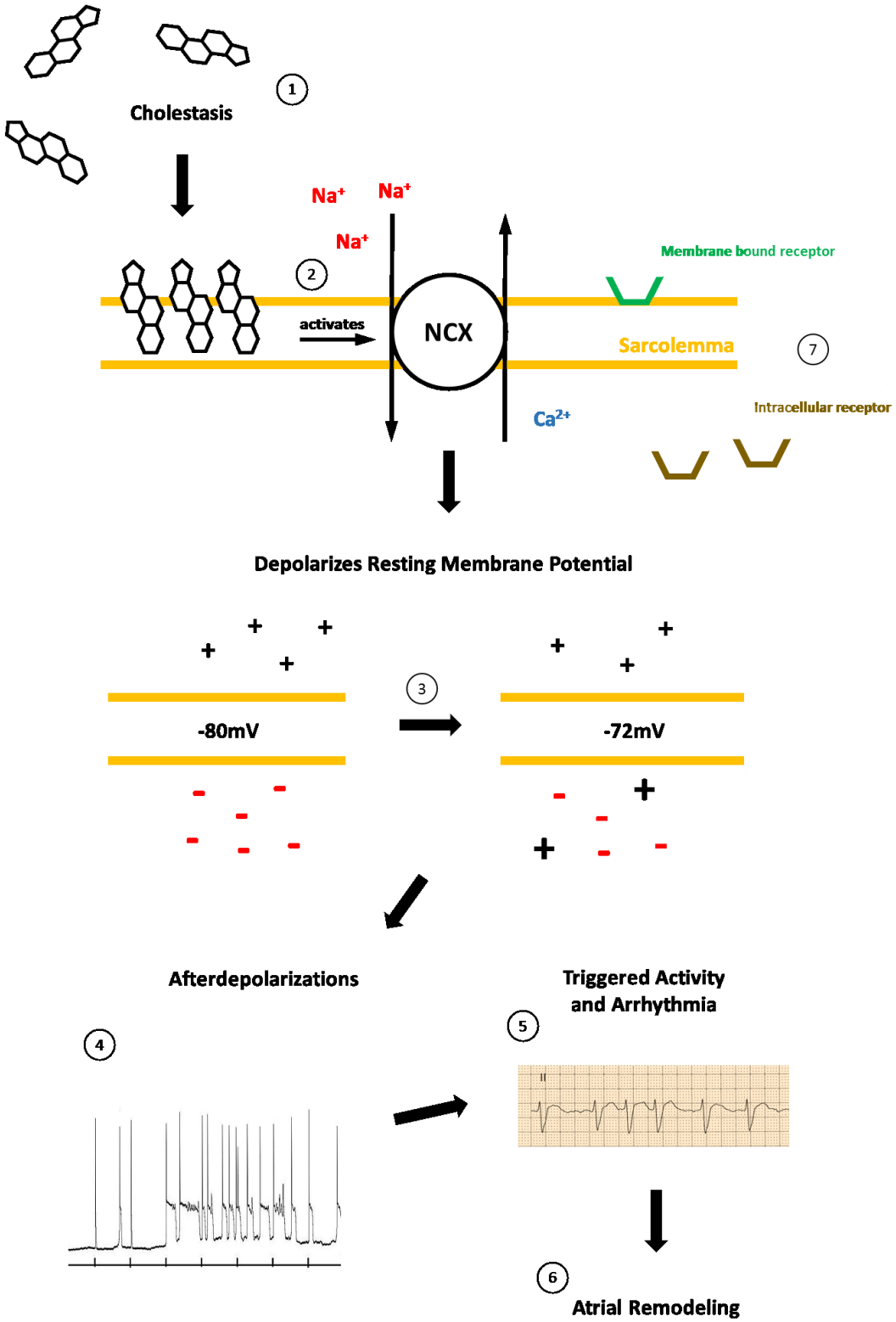


Figure 9 Working model. In conditions of elevated bile acid serum concentrations **(1)** bile acids may associate with the lipid bilayer of the cell membrane (particularly less hydrophilic bile acid subspecies) and alter membrane properties. This activates the

Na^+/Ca^{2+} exchanger (NCX) **(2)** and depolarizes the resting membrane potential **(3)**, which induces the occurrence of afterdepolarizations **(4)**, triggered activity, and cardiac arrhythmia **(5)**. Ultimately, cardiac arrhythmias, such as atrial fibrillation, induce electric and structural remodeling **(6)** which perpetuate the arrhythmia, alter cardiac function, and may increase venous filling pressure. This in turn may affect hepatic function and bile acid excretion and augment the described process to create a vicious circle. Additionally, other groups found that bile acids may exert effects via their cognate bile acid or other receptors **(7)**. *For details see text. Depictions are schematic and do not represent factual proportions or stoichiometry.*

7. Limitations

Tissue and cells used in this study originate from different sources. While atrial multicellular preparations were obtained from patients, murine myocytes have been isolated from healthy hearts. The key finding of bile acid-induced arrhythmia is present in both experimental set-ups. Further, unspecific interactions of bile acids with the lipid bilayer may not only affect NCX but also other membrane proteins and hence trigger additional changes. This may reconcile our findings with previous findings that have shown the importance of muscarinic and AKT/GSK3beta signalling in bile acid-related cardiac disease[2, 20]. Additionally, chronic low-dose effects of bile acids on myocardium may differ from the acute effects observed in our study[2]. Lastly, while DCB is routinely used as NCX inhibitor it may affect other ion channels and receptors, particularly at higher concentrations. These limitation is common to pharmacological inhibition of Na+/Ca2+ exchange[34].

8. References

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9. Appendix

Supplementary Table 1

A. Curriculum Vitae

B. Publication List

C. Published Manuscript

Supplementary Table 1 Patient characteristics of right atrial appendage donors

Patient (coded)	Date of surgery (month and year)	Age	Sex	Height	Weight	BMI	EF	Surgery type	CAD	AS	AI	MI	MS	DIG	DIU	BB	Aldo	Statins	ACE	ARB	ACE or ARB
1	06.2009	74	m	172	78	26	55	CABG	1	1	1	1	0	0	1	1	0	1	1	0	1
2	06.2009	80	f	159	53	23	55	CABG	1	0	0	0	0	0	1	1	0	0	1	0	1
3	07.2009	72	f	157	91	37	55	AVR	0	1	0	0	0	0	1	0	0	0	1	0	1
4	07.2009	79	f	168	88	31	40	CABG+AVR	1	1	0	1	0	0	1	1	0	1	1	0	1
5	08.2009	84	m	165	69	25		CABG+AVR	1	1	0	0	0	0	0	1	0	1	0	0	0
6	08.2009	75	m				60	CABG	1	0	1	0	0	0	0	1	0	1	1	0	1
7	08.2009	67	m	173	82	27	50	CABG+AVR	1	1	0	1	0	0	0	0	0	1	0	1	1
8	08.2009	87	f				50	CABG+AVR	1	1	0	1	0	0	0	0	0	1	0	1	1
9	08.2009	85	f	162	48	18	50	CABG+AVR	1	1	1	1	0	0	1	1	0	0	1	0	1
10	09.2009	85	m	170	70	24	45	CABG+AVR	1	1	1	1	0	0	1	1	0	1	1	0	1
11	09.2009	71	f	160	73	29	50	CABG+AVR	1	1	0	0	0	0	0	0	0	1	0	1	1
12	09.2009	80	m	179	90	28	55	CABG	1	0	0	1	0	0	0	1	0	1	1	0	1
13	09.2009	79	f	170	80	28	50	CABG+AVR	1	1	0	1	0	0	0	0	0	1	0	0	0
14	09.2009	79	m	174	91	30	55	CABG	1	0	0	1	0	0	1	1	0	0	1	0	1
15	10.2009	72	m	152	70	30	55	CABG	1	0	0	0	0	1	0	1	0	1	1	0	1
16	10.2009	71	f	159	60	24	60	CABG	1	0	0	0	0	0	0	1	0	1	0	0	0
17	10.2009	63	m	178	94	30	43	AVR	0	1	0	0	0	0	0	0	0	1	1	0	1
18	10.2009	60	m	175	98	32	55	AVR	0	1	0	0	0	0	1	1	0	0	0	1	1
19	10.2009	64	m	178	78	25	45	CABG	1	0	0	0	1	1	0	1	0	1	0	0	0
20	10.2009	59	m	176	86	28	40	CABG	1	0	0	0	0	0	1	1	0	1	0	1	1
21	10.2009	54	f	157	71	29	68	CABG	1	1	0	0	0	0	0	1	0	0	0	0	0
22	11.2009	77	m	168	78	28		CABG	1	0	0	1	0	0	0	1	0	1	1	0	1
23	11.2009	69	m	174	88	29	40	CABG	1	0	0	0	0	0	1	1	0	1	1	0	1
24	12.2009	68	m	178	96	30	50	CABG	1	0	0	0	0	0	0	1	0	1	0	0	0
25	12.2009	81	f	157	82	33	50	CABG	1	0	0	1	0	0	0	1	0	1	1	0	1
26	12.2009	72	m	164	79	29	65	CABG+AVR	1	1	0	0	0	0	1	1	0	1	0	1	1
27	12.2009	56	m	180	76	24	60	AVR	0	1	0	1	0	0	0	0	0	0	1	0	1
28	01.2010	64	f				65	AVR	0	1	0	0	0	0	0	1	0	1	1	0	1
29	01.2010	69	m	165	92	34	74	CABG	1	0	0	0	0	0	1	0	0	0	1	0	1
30	01.2010	79	m	167	85	30		AVR	0	1	0	0	0	0	1	0	0	0	0	0	0
31	02.2010	49	m	173	87	29		CABG	1	0	0	0	0	0	0	1	0	1	0	0	0
32	02.2010	53	m	185	90	26	45	AVR	0	1	0	1	0	0	0	0	0	0	0	0	0
33	02.2010	63	m	176	80	26	60	CABG	1	0	0	0	0	0	0	1	0	1	1	0	1
34	02.2010	55	m	174	72	24	65	CABG	1	0	0	0	0	0	0	0	0	1	1	0	1
35	02.2010	65	m	178	97	32	58	CABG	1	0	0	0	0	0	0	0	0	1	0	0	0
36	02.2010	53	m	175	86	28	60	CABG	1	0	0	0	0	0	0	1	0	1	1	0	1
37	02.2010	69	m	176	93	30	65	CABG	1	0	0	1	0	0	0	0	0	1	1	0	1
38	02.2010	50	m	186	83	24	70	CABG	1	0	0	0	0	0	0	0	0	1	1	0	1
39	02.2010	71	m	173	80	27	45	CABG	1	0	0	0	0	0	0	0	0	1	1	0	1
40	02.2010	57	m	175	119	39	50	CABG+AVR	1	0	0	1	0	0	1	1	1	1	1	0	1
41	02.2010	52	m	183	87	26	65	CABG	1	0	0	0	0	0	0	1	0	1	1	0	1
42	02.2010	81	f	160	60	23	50	CABG+AVR	1	1	0	1	0	0	1	1	1	1	0	0	0
43	02.2010	60	f	164	60	22	60	CABG	1	0	0	0	0	0	0	0	0	1	0	1	1
44	02.2010	45	m	176	90	29	60	AVR	0	1	0	0	0	0	0	0	0	0	1	0	1
45	03.2010	64	m	172	81	27	60	CABG	1	0	0	0	0	1	1	1	0	1	1	1	1
46	03.2010	72	f	159	65	26	60	CABG	1	0	0	1	0	0	0	1	0	1	1	0	1
47	03.2010	64	m	170	72	25		AVR	1	1	0	1	0	0	1	1	0	0	0	0	0
48	03.2010	59	f	164	80	30	60	CABG	1	0	0	0	0	0	1	1	0	1	0	0	0
49	03.2010	72	m				65	CABG	1	0	0	0	0	0	1	1	0	1	0	0	0
50	03.2010	95	m	164	84	31	64	CABG	1	0	0	0	0	0	0	1	0	1	1	0	1

Supplementary Table 1 continued

Patient (coded)	Date of surgery (month and year)	Age	Sex	Height	Weight	BMI	EF	Surgery type	CAD	AS	AI	MI	MS	DIG	DIU	BB	Aldo	Statins	ACE	ARB
51	03.2010	78	m	178	92	29	75	CABG	1	1	0	0	0	0	0	0	0	1	1	0
52	03.2010	42	m	178	107	34	60	AVR	0	1	0	0	0	0	0	0	0	1	0	0
53	03.2010	54	m	163	80	30	55	CABG	1	0	0	0	0	0	0	0	0	0	1	1
54	03.2010	66	m	170	71	25	60	CABG	1	0	0	0	0	0	0	1	0	1	1	0
55	04.2010	78	f	150	58	26	65	CABG	1	1	1	1	0	0	1	1	0	0	1	0
56	04.2010	70	m	182	79	24	65	CABG+AVR	1	1	0	0	0	0	0	0	0	1	0	0
57	04.2010	61	f	170	70	24	83	CABG	1	0	1	0	0	0	0	1	0	1	1	0
58	05.2010	73	m				50	CABG	1	0	1	1	0	0	0	1	0	0	0	0
59	05.2010	71	m	172	104	35	48	CABG	1	0	0	0	0	0	0	1	0	1	0	1
60	05.2010	70	m	182	85	26		CABG	1	0	0	0	0	0	0	0	0	1	1	0
61	05.2010	79	m	182	88	27	65	AVR	1	1	0	0	0	0	0	0	0	0	0	0
62	05.2010	55	m	179	90	28	65	AVR	0	1	0	0	0	0	0	0	0	1	0	0
63	05.2010	87	m	174	79	26	60	CABG	1	1	0	1	0	0	1	0	0	1	1	0
64	05.2010	71	f	167	88	32		CABG	1	0	0	0	0	0	0	1	0	1	0	1
65	05.2010	69	m	176	85	27	50	CABG	1	0	0	0	0	0	0	1	0	0	0	0
66	05.2010	80	m	165	74	27	45	CABG	1	0	1	1	0	0	1	1	1	1	1	0
67	05.2010	66	f	166	84	31		CABG	1	0	0	0	0	0	1	1	0	1	1	0
68	12.2010	75	m	160	75	29	50	CABG+AVR	1	1	0	0	0	0	0	0	0	0	1	0
69	12.2010	57	f	163	79	30	45	AVR	0	1	1	0	0	0	1	0	0	0	0	0
70	12.2010	72	f	170	68	24	55	AVR	0	1	0	0	0	0	1	1	0	1	1	0
71	12.2010	84	f	156	52	21	60	AVR	1	1	1	1	0	0	1	1	0	1	1	0
72	12.2010	57	m	177	99	32	40	CABG+AVR	1	1	1	0	0	0	1	1	0	1	1	0
73	01.2011	40	m	188	105	30	50	AVR	0	0	1	0	0	0	0	0	0	1	0	0
74	01.2011	26	m	174	81	26	50	CABG	1	1	0	0	0	0	0	0	0	1	0	0
75	01.2011	38	f	178	80	25	55	AVR	0	1	0	0	0	0	0	1	0	0	0	0
76	05.2013	52	m	163	68	26	55	CABG	1	0	0	0	0	0	0	1	0	1	0	0
77	05.2013	39	m	172	104	35	55	CABG	1	0	0	0	0	0	0	1	0	1	0	0
78	05.2013	79	m	181	77	24	55	CABG+AVR	1	1	0	0	0	1	0	1	0	1	0	0
79	05.2013	73	m	168	67	24	60	CABG	1	0	0	0	0	0	0	0	0	0	1	0
80	05.2013	62	m	178	96	30	55	AVR	1	1	1	0	0	0	0	0	0	1	1	0
81	05.2013	59	f	172	66	22	60	AVR	0	1	0	0	0	0	0	0	0	0	0	0
82	05.2013	48	m	174	101	33	55	CABG	1	0	0	0	0	0	1	1	0	1	1	0
83	06.2013	55	f	157	68	28		AVR	0	1	0	0	0	0	1	0	0	0	1	0
84	06.2013	57	m					CABG	1											

Mean	66		171	81	28	56	Yes (n)	68	39	13	23	1	4	29	50	3	60	46	11
SD	13,0		8,4	13,4	3,8	8,6	No (n)	16	44	70	60	82	79	54	33	80	23	37	72
SEM	1,50		1,0	1,6	0,4	1,1	Total (n)	85	84	84	84	84	84	84	84	84	84	84	84
Min	26		150	48	18	40													
Max	95		188	119	39	83	Yes (%)	80	46	15	27	1	5	35	60	4	71	55	13
n	84		78	78	78	74	No (%)	19	52	83	71	98	94	64	39	95	27	44	86
							Total (%)	99	99	99	99	99	99	99	99	99	99	99	99

Male	59
Female	25
Total	85

CABG	49
AVR	20
Both	15
Total	85

A. Curriculum Vitae

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2008-2010	Clinical and Research Fellow, Division of Cardiology, Department of Medicine, Laboratory of Burkert Pieske, Medical University of Graz, Austria
2006-2008	Fellow, Division of Rheumatology, Department of Medicine, Medical University of Graz, Austria; Resident, Department of Cardiology and Intensive Care Medicine, Private Medical University of Salzburg; Resident and Research Assistant, Department of Internal Medicine A.o. KH Oberndorf b. Salzburg

Education

2009 – present	Graduate student in the MD DSc - program (*Dr. scient. med.,* curriculum O 790) at the Medical University of Graz, Doctoral School for Cardiovascular Research; Research Topic: *Pathophysiological effects of liver failure and cholestasis on myocardial function*.
2012-2013	Graduate Courses at the Bloomberg School of Public Health, Baltimore
2000-2006	Graduate program for Medicine, Medical University of Graz, Austria and University of Florence, Italy (curriculum O 201) Internships in Austria, Italy and Viet Nam Graduation summa cum laude October 18, 2006
1995-1999	High School/College (BORG Bad Hofgastein), Major in Natural Sciences, Graduation with distinction, June 17, 1999

Grants and Awards

2013	Sanofi-Aventis Prize for manuscript 'Cardiac fibrosis in human transplanted hearts is mainly driven by cells of intracardiac origin, Pichler M., Rainer PP, et. al, J Am Coll Cardiol. 2012 Mar 13;59(11):1008-16.
2013	Start Grant by the Medical University of Graz (€61 000)
2013	Stanley L. Blumenthal Award at the Annual Johns Hopkins Cardiovascular Research Retreat; Baltimore, MD
2013	Johns Hopkins Post-doctoral Association Poster Prize; Baltimore, MD
2013	Finalist ISHR World Congress Poster Competition, San Diego, CA
2012	Featured Speaker at the Basic and Translational Science Hot Line, ESC Meeting, Munich, Germany
2012	Travel Grant of the ESC Council for Basic Cardiovascular Science to the ESC Meeting, Munich, Germany
2011- 2012	Max Kade Fellowship by the Max Kade Foundation and the Austrian Academy of Sciences ($77 000)
2010	BA-CA Visiting scientists scholarship (€2 000)
2010	Travel Grant to the FCVB (Frontiers of Cardiovascular Biology meeting, ESC), Berlin, Germany
2009	ESC Grant for the CBCS Summer School on Basic Cardiovascular Science, Nice, France
2006	Award for outstanding Achievement (Federal Ministry of Education, Science and Culture)
2006	Graduation sub auspiciis praesidentis rei publicae
2003, 2004, 2006	Scholarship Merit, Medical University of Graz

2004	ASEA-Uninet Travel Grant/Internship at the Wellcome Trust-Oxford University Clinical Research Unit for Tropical Diseases, Ho Chi Minh City, Vietnam
2004	Erasmus Grant to the Universita' degli Studi di Firenze, Italy

Committees and Memberships

American Heart Association – Council on Basic Cardiovascular Sciences, European Society of Cardiology – Working Group on Myocardial Function and Heart Failure Association, International Society for Heart Research – Interest Group for Signaling in Hypertrophy and Failure, Austrian Society of Cardiology, Austrian Society of Internal Medicine, Austrian Society for Alpine and High Altitude Medicine, Member of the Organizing Committee of the international heart failure with preserved ejection fraction meeting (Pichlarn, Austria, October 2009)

Teaching

Supervision of undergraduate summer and research elective students (Johns Hopkins University, Baltimore, USA, 2011-2013) and supervision of graduate students (M.D. programme, Medical University of Graz, Austria) for their diploma thesis (2008-2011). Lectures and labs for M.D. and nursing students (coronary artery disease, risk factors, ECG, echocardiography) at the Medical University of Graz (2007-2010).

Current Research

Cell specific transforming growth factor beta signaling in maladaptive remodeling following ischemia or pressure overload.

Molecular mechanisms of heart failure and abnormal mechano-force/calcium transduction in dystrophic muscle.

Interaction of TRPC channels in the hypertrophic response to pressure-overload and Gαq stimulation.

B. Publications

Rainer PP, Primessnig U, Harenkamp S, Doleschal B, Wallner M, Fauler G, Stojakovic T, Wachter R, Yates A, Groschner K, Trauner M, Pieske BM, von Lewinski D Bile Acids Induce Arrhythmias in Human Atrial Myocardium - Implications for altered Serum Bile Acid Composition in Patients with Atrial Fibrillation. Heart. 2013 Nov;99(22):1685-92.

Rainer PP, Hao S, Vanhoutte D, Lee DI, Koitabashi N, Molkentin JD, Kass DA Cardiomyocyte-selective TGFb Inhibition Prevents Neutrophil Infiltration and Early Mortality after Myocardial Infarction by Inducing Protective Endoplasmic Reticulum Stress and Anti-Inflammatory and Cytoprotective Cytokines. Circ Res. 2014 Apr 11;114(8):1246-57].

Seo K*, Rainer PP*, Lee DI, Hao S, Cingolani OH, Kass DA Adverse Mechanical-Stress Response in Dystrophic Heart Is Blocked by cGMP-PKG Modulation of TRPC6. Circ Res. 2014 Feb 28;114(5):823-32. ** These authors contributed equally.*

Seo K*, Rainer PP*, Shalkey-Hahn* V, Jo SH, Lee DI, Andersen, A, Liu T, Xu X, Willette RN, Lepore JJ, Marino JP, Schnackenberg CG, Kass, DA TRPC3/6 Blockade by Selective Small Molecule or Genetic Deletion inhibits Pathological Cardiac Hypertrophy. Proc Natl Acad Sci U S A. 2014 Jan 28;111(4):1551-6. ** These authors contributed equally.*

Shenje L, Uosaki H, Fernandez L, Andersen P, Rainer PP, Kass DA, Kwon C Numb and Numb-like Are Required for Renewing Cardiac Progenitor Fate. eLife 2014;10.7554/eLife.02164.

Sivakumaran V, Stanley B, Tocchetti CG, Ballin JD, Caceres V, Zhou L, Keceli G, Rainer PP, Lee DI, Huke S, Ziolo MT, Kranias EG, Toscano JP, Wilson GM, O'Rourke B, Kass DA, Mahaney JA, Paolocci, N HNO enhances SERCA2a activity and cardiomyocyte function by promoting redox-dependent phospholamban oligomerization. Antioxid Redox Signal. 2013 Oct 10;19(11):1185-97.

Pichler M, Rainer PP, Schauer S, Hoefler G Cardiac fibrosis in human transplanted hearts is mainly driven by cells of intracardiac origin. J Am Coll Cardiol. 2012 Mar 13;59(11):1008-16.

Rainer PP, Doleschal B, Kirk JA, Sivakumaran V, Saad Z, Groschner K, Maechler H, Hoefler G, Bauernhofer T, Samonigg H, Hutterer G, Kass DA, Pieske BM, vonLewinski D, Pichler M Sunitinib causes dose-dependent negative functional effects on myocardium and cardiomyocytes. BJU Int. 2012 Nov;110(10):1455-62.

Rainer PP, Schmidt A; Anelli-Monti M, Kleinert, R, Pieske BM, Maier RM A Swinging Pacemaker Lead Promoting Endocarditis and Severe Tricuspid Regurgitation. J Am Coll Cardiol. 2012 Jun 5; 59(23):e45

Larson JE, Rainer PP, Watts VL, Yang R, Miller KL, Phan A, Barouch LA Dependence of β3-adrenergic signaling on the adipokine leptin in cardiac myocytes. Int J Obes (Lond). 2012 Jun;36(6):876-9.

von Lewinski D, Gasser R, Rainer PP, Huber MS, Wilhelm B, Roessl U, Haas T, Wasler A, Grimm M, Bisping E, Pieske B. Functional effects of glucose transporters in human ventricular myocardium. Eur J Heart Fail. 2010 Feb;12(2):106-13.

von Lewinski D, Rainer PP, Gasser R, Huber MS, Khafaga M, Wilhelm B, Haas T, Mächler H, Rössl U, Pieske B. Glucose-transporter-mediated positive inotropic effects in human myocardium of diabetic and nondiabetic patients. Metabolism. 2010 Jul;59(7):1020-8.

Rainer, PP; Kaufmann, P; Smolle-Juettner, FM; Krejs, GJ Hyperbaric oxygen in the treatment of puff adder (Bitis arietans) bite. Undersea and Hyperbaric Medicine Journal. 2010 Nov-Dec;37(6):395-8

Rainer PP, Eherer A, Langner,C, Graninger WB, Spreizer C, Weber K Esophageal ulceration mimicking malignancy in a patient with severe kyphoscoliosis. Endoscopy. 2010; 42(S 02): E119-E120.

C. Published Manuscript

The published manuscript can be downloaded at

(Accessed on July 7, 2014)

http://heart.bmj.com/content/99/22/1685.full?sid=bcbd0809-54d6-445d-8a6f-61b6c14fbf12

Rainer PP, Primessnig U, Harenkamp S, Doleschal B, Wallner M, Fauler G, Stojakovic T, Wachter R, Yates A, Groschner K, Trauner M, Pieske BM, von Lewinski D Bile Acids Induce Arrhythmias in Human Atrial Myocardium - Implications for altered Serum Bile Acid Composition in Patients with Atrial Fibrillation. Heart. 2013 Nov;99(22):1685-92. doi:10.1136/heartjnl-2013-304163

The accompanying editorial article can be downloaded at

(Accessed on July 7, 2014)

http://heart.bmj.com/content/99/22/1629.full?sid=bcbd0809-54d6-445d-8a6f-61b6c14fbf12

Desai MS, Penny DJ Bile acids induce arrhythmias: old metabolite, new tricks Heart 2013;99:1629-1630 doi:10.1136/heartjnl-2013-304546