Chapter 7

Cardiomyocyte mitochondrial stress and dysfunction in experimental Atrial Fibrillation is blocked by inhibition of the mitochondrial calcium uniporter

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ABSTRACT
Atrial fibrillation (AF), the most common sustained clinical tachyarrhythmia, is characterized by electrical and structural remodeling. Derailment of proteostasis, the homeostasis of protein production, function and degradation, contributes significantly to AF-induced remodeling. Endoplasmic reticulum (ER) stress was previously found present in AF, which may lead to myolysis. The ER is closely linked to mitochondria and ER stress can result in mitochondrial stress. Importantly, mitochondrial stress is observed in AF pathogenesis. Here, we report that in vitro tachypacing of HL-1 cardiomyocytes induces mitochondrial dysfunction and fragmentation of the mitochondrial network, which was verified in AF patients. Partial blocking or downregulating the mitochondrial calcium uniporter (MCU) precludes mitochondrial dysfunction and mitochondrial fragmentation in tachypaced cardiomyocytes, suggesting that augmented mitochondrial Ca\textsuperscript{2+} influx constitutes a key pathophysiological mechanism. Blocking the MCU pharmacologically, by Ru360, also protects against contractile dysfunction in an in vivo Drosophila AF model. In addition, AF patients display increased levels of circulating mitochondrial DNA, indicating mitochondrial DNA may act as a biomarker for AF. Together, these results suggest that inhibition of the MCU may represent a novel therapeutic target to counteract AF-induced mitochondrial dysfunction, and that circulating mitochondrial DNA in serum may represent a biomarker for AF.

INTRODUCTION
Atrial fibrillation (AF) is the most common sustained clinical tachyarrhythmia and is associated with increased mortality and morbidity.\textsuperscript{1,2} Its incidence is age-related and expected to rise due to the aging population, which will increase hospitalization and medical costs, contributing significantly to the socioeconomic burden.\textsuperscript{3} Due to its progressive nature, patients undergo transition from paroxysmal AF to persistent, longstanding persistent and, ultimately, permanent AF.\textsuperscript{2} All AF stages are characterized by electrical and structural remodeling, which increases the chance of re-entry and, thereby, AF progression.\textsuperscript{1,2} Electrical remodeling is accomplished by reduced effective refractory period and action potential duration, due to aberrant function of cardiac ion channels, increased cytosolic calcium levels and atrial hypocontractility, but is often reversible.\textsuperscript{1,2} Structural remodeling, however, is irreversible and includes atrial hypertrophy, fibrosis and myolysis.\textsuperscript{1,4} Current therapies have limited efficacy, especially in the longstanding persistent and permanent AF stages, likely due to the lack of knowledge about the underlying molecular mechanisms of AF-related cardiac structural...
remodeling.\textsuperscript{2} Therefore, recent research is directed at revealing the pathways leading to AF-induced cardiac structural remodeling in order to develop more mechanism-related AF therapies.

One of the mechanisms underlying AF-induced structural remodeling is derailment of proteostasis, i.e. the homeostasis of protein synthesis, folding, assembly, trafficking, function and degradation.\textsuperscript{5} Activation of proteases\textsuperscript{6-8} and histone deacetylases\textsuperscript{9} contributes to degradation of contractile and structural proteins, resulting in proteostasis derailment and structural remodeling. In addition, \textit{in vitro} AF initiation leads to increased RhoA activation, changes in structural proteins and failure to mount the heat shock response.\textsuperscript{10,11} In accord, induction of the heat shock response, a primary defense mechanism against derailment of proteostasis, attenuates cardiomyocyte remodeling and preserves the cardiomyocyte contractile function.\textsuperscript{10,12-14} In addition, we recently identified activation of macroautophagy, in response to endoplasmic reticulum (ER) stress, to constitute an important route involved in degradation of structural proteins in AF.\textsuperscript{15} As the ER is in close contact with mitochondria through so called mitochondria-associated membranes, which promote the exchange of metabolites, including lipids and Ca\textsuperscript{2+}, mitochondria respond with stress to ER stress.\textsuperscript{16} Interestingly, there are indications that mitochondrial stress contributes to AF pathogenesis. One of the most direct indications comprises a study demonstrating the association between decreased mitochondrial respiration and expression of respiratory chain proteins and the incidence of post-operative AF in obese patients.\textsuperscript{17} Likewise, protein expression profiling in a small cohort of valvular disease patients demonstrated differential expression of important energy metabolism-related proteins between sinus rhythm and AF patients.\textsuperscript{18} Moreover, the presence of oxidative stress in \textit{in vivo} models of AF\textsuperscript{19} and in AF patients\textsuperscript{20-22} indicates disruption of normal respiration. Despite these indications of aberrant mitochondrial function in AF, characterization of these changes, putative mechanism and contribution to AF pathogenesis has not been studied. This is striking, as mitochondrial ATP production is vital for cardiac contraction. Moreover, on a theoretical level, the relationship between mitochondrial ATP production and Ca\textsuperscript{2+} influx from the ER\textsuperscript{23} immediately links cellular Ca\textsuperscript{2+} overload in AF to mitochondrial dysfunction. Following cellular Ca\textsuperscript{2+} overload, which is toxic for the cardiomyocytes, excessive Ca\textsuperscript{2+} is stored in the ER and mitochondria, thereby causing an ER stress response and swelling and dysfunction of mitochondria, respectively.\textsuperscript{23-27} Here, we examined mitochondrial function in experimental models of AF remodeling and in AF patients. Furthermore, we determined the role of the mitochondrial Ca\textsuperscript{2+} uniporter
Cardiomyocyte mitochondrial stress and dysfunction in experimental Atrial Fibrillation is blocked by inhibition of the mitochondrial calcium uniporter (MCU), which is responsible for mitochondrial Ca\(^{2+}\) uptake, in AF. As dysfunction of mitochondria induces release of its components from the cardiomyocytes,\(^{28-30}\) we also assessed whether mitochondrial DNA represents a biomarker for AF in patients.

**MATERIALS AND METHODS**

**HL-1 cardiomyocyte cell culture and tachypacing**

HL-1 atrial cardiomyocytes, derived from adult mouse atria, were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, USA)\(^{31}\) and were maintained in complete Claycomb Medium (Sigma, The Netherlands) supplemented with 10% FBS (PAA Laboratories GmbH, Austria), 100 U/ml penicillin (Gibco, The Netherlands), 100 µg/ml streptomycin (Gibco, The Netherlands), 4 mM L-glutamine (Gibco, The Netherlands), 0.3 mM L-ascorbic acid (Sigma, The Netherlands) and 100 µM norepinephrine (Sigma, The Netherlands). The cardiomyocytes were cultured on cell culture plastics or on glass coverslips coated with 0.02% gelatin (Sigma, The Netherlands) and were grown at 37°C in 5% CO\(_2\).

HL-1 atrial cardiomyocytes were subjected to 6Hz (tachypacing), 40V and 20ms pulses, for maximal 8 hours via the C-Pace100 TM-culture pacer (IonOptix Corporation, The Netherlands).

**Transfection, drug treatment and mitochondrial calcium transient measurement**

HL-1 cardiomyocytes were transiently transfected with pDEST40-MCU-V5-HIS (Addgene, USA) by the use of Lipofectamin 2000 (Life Technologies, The Netherlands). MCU knockdown was accomplished by transiently transfecting the cardiomyocytes with Mission MCU esiRNA (EMU213891, Sigma, The Netherlands) by the use of Lipofectamin RNAiMAX (Life Technologies, The Netherlands). Ru360 was purchased from Millipore (USA) and dissolved in deoxygenated water, according to manufacturer's instructions. Ru360 treatment (5 µM) was started 30 minutes prior to and was continued during tachypacing. Mdivi-1 (Sigma, The Netherlands) and mitoTEMPO (Santa Cruz Biotechnology, USA) were dissolved according to manufacturer's instructions and added 40 minutes and 1 hour prior to and during tachypacing, respectively.

To measure mitochondrial calcium transients (Ca\(_{\text{mito}}\)T), HL-1 cardiomyocytes were incubated for 30 minutes with 5 µM of the mitochondrial Ca\(^{2+}\)-sensitive dye Rhod-2 AM\(^{32}\) (Abcam, UK) at 37°C in DMEM (Gibco, The Netherlands), followed by three times washing with DMEM (Gibco, The Netherlands). Rhod-2 AM-loaded cardiomyocytes were
excited by a 600 nm laser with emission at 605 nm and CaT$_{mito}$ were recorded with the Myocyte Calcium and Contractility System (IonOptix Corporation, The Netherlands). The live recording of the CaT$_{mito}$ was performed at 1Hz stimulation (normal pacing) at 37°C. The relative value of fluorescent signals was determined utilizing the following calculation: Fcal=F1/F0, where F1 is the fluorescent dye signal at any given time and F0 is the fluorescent signal at rest. Mean values and SEM from each experimental condition were based on 7 consecutive CaT$_{mito}$ in at least 25 cardiomyocytes.

**ATP, mitochondrial membrane potential and morphology analysis**

Cellular ATP levels were measured according to the manufacturer’s instructions of the ATP Bioluminescence Assay Kit CLS II (Roche, The Netherlands). In short, HL-1 atrial cardiomyocytes were lysed in 50 µl 1.5% TCA and 1 ml Tris-buffer (pH 8.0), supplemented with 1 mM NaF (Sigma, The Netherlands), according to the following protocol: ENLITEN ATP assay system bioluminescence detection for ATP measurement – instructions for the use of product FF2000 (Promega). The protein concentration was determined (Bio-Rad, The Netherlands). Dog and human atrial tissue were lysed in TE-saturated phenol, after which chloroform and demineralized water were added. 33 50 µl of each extracted protein samples was transferred into a 96-well plate and mixed with 50 µl luciferase reagent (supplied) and ATP levels were bioluminescently measured using the SynergyH4 Hybrid Reader (BioTek, USA).

Mitochondrial membrane potential was determined by incubating the HL-1 atrial cardiomyocytes with 100 nM tetramethyl rhodamine methylester (TMRM, Sigma, The Netherlands) in DMEM (Gibco, The Netherlands) for 30 minutes at 37°C, followed by washing with DMEM (Gibco, The Netherlands) and PBS. The cardiomyocytes were resuspended in 1% BSA in PBS and mean fluorescence intensity of 10.000 cardiomyocytes was analyzed by the LSR-II flow cytometer (BD Biosciences, USA).

To measure mitochondrial morphology, HL-1 cardiomyocytes were incubated with 100 nM Mitotracker Deep Red (Life Technologies, The Netherlands) in DMEM (Gibco, The Netherlands) for 30 minutes at 37°C, followed by three times washing with DMEM (Gibco, The Netherlands). Mitotracker Deep Red-loaded cardiomyocytes were excited by a 647 nm laser with emission at 665 nm and were visually recorded with a 63x-objective, using a Solamere-Nipkow-Confocal-Live-Cell-Imaging system (based on a Leica DM IRE2 inverted microscope). The live recording was performed at 37°C. Ten random fields containing at least 15 cardiomyocytes were recorded and the mitochondrial
morphology per cardiomyocyte was scored as tubular, intermediate or fragmented\textsuperscript{34} by an investigator blinded for the treatment conditions.

**Protein extraction and Western blot analysis**

HL-1 atrial cardiomyocytes or human tissue samples were lysed in radioimmunoprecipitation assay buffer and Western blot analysis was performed as described before.\textsuperscript{9} In short, equal amounts of protein homogenates in SDS-PAGE sample buffer were separated on SDS-PAGE 4-20% Precise\textsuperscript{TM} Protein gels (Thermo Scientific, The Netherlands) and transferred to nitrocellulose membranes (Bio-Rad, The Netherlands). Subsequently, membranes were incubated with primary antibodies, followed by incubation with secondary antibodies. Signals were detected by the Western Lightning Ultra (PerkinElmer, USA) method and quantified by densitometry (Syngene, UK). The following primary antibodies were used: anti-HSP60 (ADI-SPA-805, Enzo Life Sciences, USA), anti-TOM20 (MCA4300Z, Bio-Rad, The Netherlands), anti-MCU (14997S, Cell Signaling Technology, The Netherlands), OXPHOS Antibody Cocktail (MS604, Abcam, UK) and anti-GAPDH (10R-G109a, Fitzgerald, USA). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit (Dako, Denmark) were used as secondary antibodies, depending on the species origin of the primary antibody.

**Quantitative RT-PCR**

Total RNA was isolated from HL-1 atrial cardiomyocytes using the Nucleospin RNA isolation kit (Machery-nagel, The Netherlands). First strand cDNA was generated by M-MLV reverse transcriptase (Promega, The Netherlands) and random hexamers primers (Promega, The Netherlands). Subsequently, the cDNA was used as a template for quantitative real-time PCR. Relative changes in transcription level were determined utilizing the CFX384 Real-time system C1000 Thermocycler (Bio-Rad, The Netherlands) in combination with SYBR green ROX-mix (Westburg, The Netherlands). mRNA levels were expressed in relative units on the basis of a standard curve (serial dilutions of a calibrator cDNA mixture). Fold inductions were adjusted for GAPDH and the PCR efficiencies for all primers were between 90-110%. Primer pairs used are the following:


Total DNA was isolated from HL-1 cardiomyocytes, Claycomb medium from cultured
HL-1 cardiomyocyte plates or patient serum utilizing the Nucleospin Tissue kit (Machernagel, The Netherlands), according to manufacturer’s instructions. Isolated DNA was used to determine mitochondrial DNA levels utilizing the CFX384 Real-time system C1000 Thermocycler (Bio-Rad, The Netherlands) in combination with SYBR green ROX-mix (Westburg, The Netherlands). Mitochondrial DNA levels were adjusted for nuclear DNA levels,\(^{35}\) and analyzed using the ΔC\(_{t}\) method. Primer pairs used are the following: mouse ND1 fw: AAACATATGTTTCGCCACAA and rv: TGGAGTCAGTGACATTTGGC, mouse COX1 fw: GCCCCAGATACTAGCCATC and rv: GTTCATCTGTTCC-TGCTCC, mouse 18S rRNA fw: TAGAGGACAAGTGG-CGTTTC and rv: CGCTGAGCCAGTCAGTGT, human ND1 fw: ATACCATGGCCAACCTCCT and rv: GGGGCTTGGCGTAGTTGTAT, human COX3 fw: ATGACCCACCAA-TACATGC and rv: ATCACATGCTAGGGCCGAG and human 18S rRNA fw: AGAAACCGCTACCACATCCA and rv: CCCTCCAATGGATCCCGT.

**Measurement of mitochondrial oxygen consumption rate (OCR)**

Mitochondrial OCR was measured utilizing the XF24 extracellular flux analyzer (Seahorse Bioscience, USA). After tachypacing, HL-1 cardiomyocytes were trypsinized and 8 x 10\(^4\) cardiomyocytes were replated in triplicate in 0.02% gelatin-coated XF 24-well cell culture microplates (Seahorse Bioscience, USA) and incubated at 37°C and 5% CO\(_2\) for 24 hours. Full Claycomb medium was removed and replaced by assay medium (Seahorse Bioscience, USA) supplemented with 10 mM D(+)-glucose monohydrate (Sigma, The Netherlands), 1 mM sodium pyruvate (Sigma, The Netherlands) and 2 mM glutamine (Gibco, The Netherlands), adjusted to pH 7.4 and incubated 1 hour at 37°C in a CO\(_2\)-free incubator. The calibration cartridge was loaded with 1 μM oligomycin (port A), 300 nM carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, port B) and 1 μM rotenone plus 1 μM antimycin A (port C) in assay medium. After the assay, cardiomyocytes in each well were lysed in radioimmunoprecipitation assay buffer and protein concentration was determined (Bio-Rad, The Netherlands), which was used to calibrate the OCR data.

**Drosophila heart wall contraction measurement**

*Drosophila melanogaster* heart wall contraction measurements were performed with the w\(^{1118}\) strains (Genetic Services Inc, USA), which were maintained at 25°C on standard medium. Adult *Drosophilas* were removed after fertilization and the medium, containing the fly embryos, was supplied with Ru360 (20, 50 and 100 μM), freshly dissolved in demineralized water. Controls were subjected to demineralized water only. After at least
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48 hours, prepupae were selected as described before. In short, groups of at least 5 transparent prepupae were selected at entry of the immobile phase and were placed on a 1% agarose gel in PBS. The prepupae were subjected to tachypacing (5Hz for 20 minutes, 20V and 5ms pulses) with a C-Pace100TM-culture pacer (IonOptix Corporation, The Netherlands). Before and after tachypacing, the heart wall contractions in whole prepupae were measured for a period of 30 seconds and analyzed with the Myocyte Calcium and Contractility System (IonOptix Corporation, The Netherlands).

Table 1 Demographic and clinical characteristics of patients with AF and control patients in SR, used for Western blot analysis of atrial appendages.

<table>
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<th>SR (N=8)</th>
<th>AF (N=11)</th>
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<tbody>
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<td>Gender</td>
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<tr>
<td>Male (N, %)</td>
<td>5 (63)</td>
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<tr>
<td>Aorta</td>
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<td>Paroxysmal + AT</td>
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<tr>
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<tr>
<td>LS Persistent</td>
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<tr>
<td>Permanent</td>
<td>1 (9)</td>
<td></td>
</tr>
<tr>
<td>Months since diagnosis (median (range))</td>
<td>39 (33-44) (n=3)</td>
<td>69 (1-150)</td>
</tr>
<tr>
<td>LA diameter, mm (median (range))</td>
<td>39 (33-44) (n=3)</td>
<td>42 (35-74) (n=7)</td>
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<tr>
<td>LVF (N, %)</td>
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<td>Normal</td>
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<td>Mild impairment</td>
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<td>NYHA classification (N, %)</td>
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<td></td>
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<tr>
<td>I</td>
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<tr>
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<td>2 (18)</td>
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<tr>
<td>IV</td>
<td>1 (13)</td>
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Patient material

Before surgery, one investigator assessed patient characteristics (Table 1 and 2) as described before. Right and left atrial appendages (RAA and LAA, respectively) were obtained from patients with AF and control patients in sinus rhythm (SR) as described in the Halt and Reverse study (MEC 2014-393). Both AF and SR patients groups suffered from underlying heart disease. After excision, the atrial appendages were immediately snap-frozen in liquid nitrogen and stored at -80 ºC. The study conforms to the principles of the Declaration of Helsinki. The institutional review board approved the study and patient gave written informed consent.

Canine in vivo AF model

The dog left atrial tissue used for ATP measurement was obtained from experiments performed at the Montreal Heart Institute according to the guidelines for animal

<table>
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<tr>
<th>Table 2 Demographic and clinical characteristics of patients with AF and control patients in SR, used for mitochondrial DNA analysis in serum.</th>
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<tbody>
<tr>
<td>Gender</td>
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<td>LA dilatation (&gt;45mm, %)</td>
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<td>II</td>
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</table>

CAD: coronary artery disease, VHD: valvular heart disease, CHD: congenital heart defect, LS: longstanding, LA: left atrium, LVF: left ventricular function, NYHA: New York Health Association for exercise tolerance
handling of the National Institutes of Health and approved by the Animal Research Ethics Committee of the Montreal Heart Institute. Two dog groups were included from a previous conducted study,\textsuperscript{15} non-paced control (C) and 7 days atrial tachypaced (TP) dogs, and consisted of 7 mongrel dogs (28 to 38 kg) per group. For ATP analysis, left atrial tissue was snap-frozen and stored at -80°C.

Statistical analysis
Results are expressed as mean ± SEM of at least two independent experiments. Multiple-group comparisons were obtained by a one-way ANOVA with a Bonferroni correction. Individual group-mean differences were evaluated with a Student’s t-test. All $P$-values were two-sided. A value of $P \leq 0.05$ was considered statistically significant. SPSS version 22 was used for all statistical evaluations.

RESULTS
Tachypacing induces mitochondrial stress and dysfunction
To examine the influence of tachypacing on mitochondrial function, we first determined cellular ATP levels in tachypaced HL-1 cardiomyocytes. Tachypacing for 2h induced a large decrease in cellular ATP levels, which gradually reduced further upon extended pacing (Figure 1A). As the decrease in cellular ATP may have been caused by excessive consumption and/or impaired production, we next examined mitochondrial respiration. To that end, oxygen consumption was measured in whole cardiomyocytes under basal conditions and after addition of oligomycin to inhibit ATP synthesis, FCCP to determine the maximal respiratory capacity and rotenone/antimycin A to determine non-mitochondrial respiration (Figure 1B; 1, 33, 56 and 96 minutes, respectively). Tachypacing for 2h initially increased basal oxygen consumption rate and spare respiratory capacity. However, tachypacing beyond 2h progressively decreased the basal oxygen consumption rate and very markedly inhibited spare respiratory capacity (Figure 1B), which accounts for a less adequate response to cellular stress or increased cardiac workload.\textsuperscript{37} However, protein expression of complex I, II, III and V of the respiratory chain did not change during tachypacing (Figure 1C), indicating that the decreased respiration during tachypacing is not due to diminished respiratory chain protein expression. Together, these results demonstrate that tachypacing induces mitochondrial dysfunction, likely leading to a progressive impairment of ATP synthesis. Cytosolic Ca\textsuperscript{2+} overload constitutes the most obvious mechanism underlying the tachypaced-induced mitochondrial dysfunction. Ca\textsuperscript{2+} overload in both cytosol and ER,
Figure 1 Tachypacing induces mitochondrial dysfunction. A) Quantified data showing reduced cellular ATP levels during tachypacing. B) The oxygen consumption rate (OCR) showing the mitochondrial respiration. 1-33 minutes: basal respiration, 33-56 minutes: addition of oligomycin to inhibit ATP synthesis, 56-96 minutes: addition of FCCP for the maximal respiratory capacity, 96-119 minutes: addition of rotenone and antimycin A for the non-mitochondrial respiration. C) Top panel represent Western blot of respiratory chain complexes I, II, III and V. Lower panels reveal quantified data of the respiratory chain complexes normalized for basal GAPDH protein level. D) Quantified data showing mitochondrial calcium transients (CaT_{mito}) during tachypacing. E) Representative CaT_{mito} of HL-1 cardiomyocytes after normal pacing (NP) or tachypacing (TP). F) Quantified data showing mitochondrial membrane potential during tachypacing. *P≤0.05, **P≤0.01, ***P≤0.001 versus NP, #P≤0.05, ##P≤0.01 versus 2hTP.
as encountered in tachypacing and AF, will result in Ca\textsuperscript{2+} buffering by mitochondria\textsuperscript{38,39}. The subsequent excessive mitochondrial Ca\textsuperscript{2+} buffering leads to mitochondrial Ca\textsuperscript{2+} overload, and consequently mitochondrial swelling, dysfunction\textsuperscript{24} and reduced mitochondrial Ca\textsuperscript{2+} uptake. Reduced mitochondrial Ca\textsuperscript{2+} uptake may be a trigger for AF, as it encounters for enhanced cytosolic Ca\textsuperscript{2+} levels. Thus, we examined mitochondrial calcium transients (Ca\textsubscript{T_mito}) during normal pacing at 1Hz and during a time course of tachypacing. Tachypacing beyond 2h significantly reduced Ca\textsubscript{T_mito}, mainly characterized by the reduction in amplitude (Figure 1D and E). Both the mitochondrial respiration and Ca\textsuperscript{2+} influx are dependent on the mitochondrial membrane potential (ΔΨ\textsubscript{mito}),\textsuperscript{38} therefore we measured ΔΨ\textsubscript{mito} by the fluorescent probe TMRM, which is readily sequestered in polarized mitochondria. Tachypacing strongly and progressively reduced ΔΨ\textsubscript{mito} as from 2h onwards (Figure 1F). Next, we examined the morphology of the mitochondrial network, as dysfunction of respiratory chain complexes is accompanied by network fragmentation, either as a cause\textsuperscript{40,41} or as a consequence.\textsuperscript{42} Indeed, tachypacing as early as 2h induced a transition from a tubular to a fragmented mitochondrial network (Figure 2A and B). Fragmentation of the network was progressive over time, as evidenced by the time-dependent decrease and the time-dependent increase in a tubular and fragmented mitochondrial network, respectively.

In addition, we examined whether tachypacing also induces mitochondrial stress by measuring levels of mitochondrial chaperones upregulated upon mitochondrial stress. Tachypacing resulted in a significant and progressive upregulation of mRNA of both HSP60 and HSP10 (Figure 2C and D). Finally, we examined whether tachypacing affected the number of mitochondria by measuring the amount of mitochondrial DNA (mtDNA) and TOM20 levels. Both cellular mtDNA and TOM20 levels showed no changes upon tachypacing (Figure 2E and F), suggesting that the number of mitochondria did not change upon tachypacing. Therefore, the mitochondrial dysfunction upon tachypacing is not due to a decreased number of mitochondria. Together, these data demonstrate tachypacing to progressively impair mitochondrial function of cultured cardiomyocytes, characterized by a very early reduction in cellular ATP, loss of ΔΨ\textsubscript{mito} and fragmentation of the mitochondrial network, followed by dysfunction of the respiratory chain, impaired mitochondrial Ca\textsuperscript{2+} handling and induction of mitochondrial stress chaperones.

**A mitochondrial Ca\textsuperscript{2+} uniporter inhibitor protects from tachypacing-induced mitochondrial stress and dysfunction**

The findings reveal that tachypacing induces impaired mitochondrial function. To uncover by which mechanism mitochondria are impaired, the effects of three
compounds targeting mitochondrial function on a different level, i.e. mdivi-1, a chemical mitochondrial fragmentation inhibitor,\textsuperscript{43} mitoTEMPO, a mitochondrial antioxidant\textsuperscript{44} and Ru360, an inhibitor of the MCU,\textsuperscript{45} were explored. The effect of these compounds to counteract tachypacing-induced loss of CaT\textsubscript{mito} was examined after 6 hours of pacing, when the CaT\textsubscript{mito} are significantly reduced (Figure 1D and E). Both mdivi-1 and mitoTEMPO did not protect against tachypacing-induced CaT\textsubscript{mito} reduction (Figure 3A and B) at any concentration applied (Figure S1A and B). Interestingly, mdivi-1 treatment showed opposite effects as reported previously,\textsuperscript{43,46} as it did not induce mitochondrial

Figure 2 Tachypacing induces mitochondrial network fragmentation and stress. A) Quantified data showing the transition of the mitochondrial network from tubular to fragmented during tachypacing. B) Representative confocal images of tachypaced HL-1 cardiomyocytes of the mitochondrial network morphology, for the period as indicated. Quantitative real-time PCR of mitochondrial stress markers C) HSP60 and D) HSP10 in response to tachypacing for the indicated duration. E) Quantified data showing no changes in mitochondrial DNA during tachypacing. F) Top panel represent Western blot and lower panel reveal quantified data of TOM20 normalized for basal GAPDH protein level. *P≤0.05, ***P≤0.001 versus NP.
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network formation, but rather concentration-dependently promoted mitochondrial fragmentation (Figure 3C and D). However, Ru360 significantly and concentration-dependently protected against CaT_mito reduction, induced by tachypacing (Figure 3A and S1C). Next, we explored whether Ru360 treatment also ameliorated mitochondrial stress and dysfunction. Ru360 treatment normalized transcription levels of HSP60 and HSP10, cellular ATP levels and CaT_mito to non-treated control levels (Figure 4A-E). Interestingly, Ru360 treatment even enhanced cellular ATP levels and CaT_mito significantly in normal-paced cardiomyocytes (Figure 4C-E). Furthermore, Ru360 treatment protected the mitochondrial network from tachypacing-induced fragmentation (Figure 4F and S2). These results strongly suggest that inhibition of the MCU-mediated Ca^{2+} influx into the mitochondria protects against mitochondrial stress and dysfunction and preserves cellular function in tachypaced cardiomyocytes.

The MCU mediates tachypacing-induced mitochondrial changes

To determine whether tachypacing-induced mitochondrial changes are specifically mediated by the MCU, we first examined its protein and mRNA levels. Tachypacing did not affect protein expression of MCU, but significantly reduced MCU mRNA (Figure 3 Ru360, but not mdivi-1 or mitoTEMPO protects against tachypacing-induced CaT_mito loss. A) Representative CaT_mito of HL-1 cardiomyocytes after normal paced (NP) and tachypaced (TP), each from groups as indicated. B) Quantified CaT_mito amplitude of NP and TP HL-1 cardiomyocytes either non-treated or treated with 50 µM mdivi-1, 10 µM mitoTEMPO or 5 µM Ru360. C) Quantified data showing the transition of the mitochondrial network from tubular to fragmented after NP or 6 hours tachypacing (TP) either non-treated or treated with 10 µM, 50 µM or 100 µM mdivi-1. D) Representative confocal images of tachypaced HL-1 cardiomyocytes of the mitochondrial network morphology, each from groups as indicated. ***P<0.001 versus NP, ###P<0.001 versus 6hTP C.
5A and B). Next, we manipulated MCU levels by overexpression and siRNA treatment. Overexpression of MCU did not affect tachypacing-induced loss of CaT\textsubscript{mito} (Figure 5C and D, S3A). In contrast, reducing MCU expression by 20% protected against tachypacing-induced CaT\textsubscript{mito} loss, without affecting CaT\textsubscript{mito} in normal-paced cardiomyocytes (MCU

Figure 4 Inhibition of the MCU protects against mitochondrial stress and dysfunction. Quantified data showing protection of Ru360 treatment on A) HSP60 and B) HSP10 transcription, C) cellular ATP levels and D) CaT\textsubscript{mito} after normal pacing (NP) or tachypacing (TP). Black bars represent non-treated HL-1 cardiomyocytes; white bars represent Ru360-treated cardiomyocytes. E) Representative CaT\textsubscript{mito} of HL-1 cardiomyocytes after NP or TP with (Ru360) or without (Control) treatment. F) Representative confocal images of HL-1 cardiomyocytes of the mitochondrial network morphology, showing preservation of the mitochondrial network formation with Ru360 treatment during TP compared to TP without treatment (Control). *P≤0.05, **P≤0.01, ***P≤0.001 versus NP C no treatment (black bar), #P≤0.05, ##P≤0.01, ###P≤0.001 versus NP C Ru360 (white bar).
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siRNA low, Figure 5E and F, S3B and C). Interestingly, an approximate 60% reduction of MCU expression lowered CaT_mito in normal-paced cardiomyocytes and conferred no protection in tachypaced cardiomyocytes (MCU siRNA high, Figure 5E and F, S3B and C). These results suggest that a small reduction in MCU, not affecting normal

Figure 5 Mitochondrial changes are due to the MCU. A) Top panel represent Western blot of MCU and GAPDH and lower panel reveals quantified date of MCU normalized for basal GAPDH levels. B) Quantitative real-time PCR of MCU in response to tachypacing (TP) for the indicated duration relative to normal pacing (NP). C) Representative CaT_mito of NP or TP HL-1 cardiomyocytes either non-transfected or transiently transfected with MCU, generating MCU overexpression (OE). D) Quantified CaT_mito amplitude of NP and 6 hour TP HL-1 cardiomyocytes either non-transfected (C) or transfected with MCU. E) Quantified CaT_mito amplitude of NP and 6 hour TP HL-1 cardiomyocytes either non-transfected (C) or transfected with MCU siRNA with 60% or 20% reduced MCU expression (high and low, respectively). F) Representative CaT_mito of NP or TP HL-1 cardiomyocytes either non-transfected or transiently transfected with MCU siRNA. G) Quantified data showing heart wall contraction rates each from groups as indicated. White bars represent normal paced (NP in HL-1 cardiomyocytes) or spontaneous heart rate (SR in Drosophila) and black bars represent tachypaced HL-1 cardiomyocytes or Drosophila. *P≤0.05, **P≤0.01, ***P≤0.001 versus control NP or SR, #P≤0.05, ###P≤0.001 versus control TP.
mitochondrial Ca\(^{2+}\) handling, is beneficial to counteract tachypacing effects. However, a larger reduction of MCU levels seems detrimental, likely due to an impairment of physiological mitochondrial Ca\(^{2+}\) influx, which is already observed under baseline conditions. To confirm the importance of the MCU, the effect of Ru360 was explored in tachypaced *Drosophila melanogaster*.\(^9,14\) Comparable to findings in tachypaced HL-1 cardiomyocytes, Ru360 conferred a dose-dependent protection against tachypacing-induced decrease of heart wall contraction in *Drosophila* (Figure 5G and S4). The optimal concentration of Ru360 needed, 50 µM in *Drosophila* as opposed to 5 µM in HL-1 cardiomyocytes, corresponds well with previous experiments in which concentrations needed to confer protection in *Drosophila* are generally 10x higher than in HL-1 cardiomyocytes.\(^14\)

**Markers of mitochondrial dysfunction are present in AF patients and a dog model of AF**

To extend our findings to human AF, we examined mitochondrial dysfunction in left and right atrial appendages (LAA and RAA, respectively) in patients with AF and control
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patients in sinus rhythm (SR). Upon electron microscopic examination, patients in SR show mitochondrial localization along the entire length of the sarcomeres. In contrast, in AF patients the mitochondria are fragmented and dispersed and sarcomeres are degraded (myolysis), the latter being absent in SR patients (Figure 6A and B). Besides divergent distribution of mitochondria, cellular ATP levels are also significantly lower in AF patients (Figure 6C). Mitochondrial dysfunction is further evidenced in AF patients by increased protein expression of HSP60 and MCU in LAA, while there is no change in expression of TOM20 (Figure 6D-G). Changes in expression were only present in LAA, as observed before,\textsuperscript{9,47,48} as the expression of HSP60 and MCU in RAA is similar to SR. To extend these findings, we measured cellular ATP levels in a canine model for AF, in which dogs were subjected to 7 days of tachypacing to induce AF-associated atrial remodeling. Similar to tachypaced cardiomyocytes and AF patients, cellular ATP levels in tachypaced dogs were significantly reduced (Figure S5). These results suggest that mitochondrial dysfunction is not only found in an \textit{in vitro} model of AF, but is also present in an \textit{in vivo} model of AF and in AF patients.

**Mitochondrial DNA as a possible biomarker for AF**

Mitochondrial dysfunction can lead to the release of mtDNA into the medium of cultured cardiomyocytes or in the circulation of patients, where it acts as a damage associated molecular pattern.\textsuperscript{28,29} Thus, circulating mtDNA in serum may be used as a biomarker in AF, as already shown for several diseases, including cancer,\textsuperscript{49,50} acetaminophen hepatotoxicity\textsuperscript{51} and myocardial infarction.\textsuperscript{52} In addition, cancer progression is associated with the level of circulating mtDNA in serum.\textsuperscript{50} Thus, we examined the potential of circulating mtDNA in serum as a biomarker for AF. First we measured DNA of 2 mitochondrially encoded genes, cytochrome c oxidase subunit 1 (COX1) and NADH dehydrogenase subunit 1 (ND1), in the medium of tachypaced cardiomyocytes. Tachypacing increased the levels of both COX1 and ND1 in the medium, albeit with a large variation (Figure 7A and B). Next, levels of COX3 (cytochrome c oxidase subunit 3) and ND1 DNA were determined in serum of patients in SR or AF. Expression of both COX3 and ND1 showed a trend towards increase in AF patients (\(P<0.06\), Figure 7C), which probably originates from the limited number of AF patients included (n=10 persons). Although circulating mtDNA in serum increases with age,\textsuperscript{53} the age of patients in SR or AF was similar (Table 2). Thus, measuring circulating mtDNA in serum may be a promising biomarker for AF, which can be elucidated by testing a larger cohort.
DISCUSSION

In the current study, we show tachypacing to induce substantial mitochondrial dysfunction, including failure of respiration, most likely resulting from enhanced Ca$^{2+}$ influx through the MCU, consequently impairing mitochondrial calcium transients. Our data also demonstrate that tachypacing induces mitochondrial stress, as exemplified by increased transcription of the mitochondrial stress chaperones HSP60 and HSP10 and fragmentation of the mitochondrial network (Figure 8). Moreover, mitochondrial changes, including decreased cellular ATP levels and increased HSP60 expression, are present in AF patients, which also show myolysis and fragmented and dispersed mitochondrial localization. Likewise, cellular ATP levels are decreased in a canine \textit{in vivo} model of AF. Treatment with Ru360, an inhibitor of the MCU, or modest MCU downregulation restored these detrimental mitochondrial changes upon tachypacing. Furthermore, Ru360 treatment protected against contractile dysfunction in a \textit{Drosophila} model for AF. In addition, our data indicates that circulating mitochondrial DNA in serum may be a potential biomarker of AF. Together, these results suggest inhibition of the MCU as a novel therapeutic target in AF-induced mitochondrial dysfunction and the potential of circulating mitochondrial DNA in serum as a biomarker for AF.

\textbf{MCU-mediated mitochondrial stress and dysfunction in AF}

Our data indicate that mitochondrial stress and dysfunction in an \textit{in vitro} model of AF is due to MCU-mediated Ca$^{2+}$ influx. The MCU regulates the Ca$^{2+}$ influx through the inner mitochondrial membrane and is important for maintenance of the mitochondrial Ca$^{2+}$ homeostasis, which, in turn, is essential for cellular physiology.\textsuperscript{54} The MCU is part of the mitochondrial calcium uniporter complex, consisting of MCU, which is the

\begin{figure}
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\caption{Mitochondrial DNA is a potential biomarker for AF. Quantitative real-time PCR of the mitochondrial-transcribed A) cytochrome c oxidase subunit 1 (COX1) and B) NADH dehydrogenase subunit 1 (ND1) genes in HL-1 cardiomyocyte culture medium in response to tachypacing (TP) for the indicated duration relative to normal pacing (NP). C) Quantitative real-time PCR of the mitochondrial-transcribed cytochrome c oxidase subunit 3 (COX3) and ND1 genes in serum of SR and AF patients.}
\end{figure}
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pore-forming unit, MCUb, a negative regulator, MICU1, MICU2 and MCUR1, all required for MCU-mediated Ca\(^{2+}\) uptake and EMRE, a mediator between MCU and MICU.\(^{54}\) The MCU is specifically blocked by Ru360,\(^{45}\) which protected against tachypacing-induced mitochondrial stress and dysfunction in the current study, most likely by attenuation of mitochondrial Ca\(^{2+}\) overload. Such view is consistent with observations on the key role of MCU in heart failure\(^{55}\) and findings that Ru360 protects against ischemia-reperfusion injury \textit{in vitro}\(^{55}\) and \textit{in vivo}.\(^{57}\) Moreover, in addition to inhibition of Ca\(^{2+}\) influx, Ru360 may also stimulate mitochondrial Ca\(^{2+}\) efflux, as observed for ruthenium red.\(^{58,59}\)

The optimal treatment dose of Ru360 used in this study, 5 \(\mu\)M, does not confer a complete block of the MCU. A complete block is accomplished by treatment with 10 \(\mu\)M Ru360, a concentration that was without protective effects in the current study. Similarly, we found MCU siRNA treatment reducing MCU expression by 20% protected cardiomyocytes against tachypacing-induced Ca\(T_{\text{mito}}\) loss, in contrast to a reduction of 60%, which showed no protection. The latter is in agreement with other studies,\(^{60}\) in which a large reduction in MCU expression to 20% of normal revealed no protective effects. Together, these data suggest that modestly decreasing MCU functionality inhibits mitochondrial Ca\(^{2+}\) overload and its detrimental effects, but preserves adequate MCU functioning during cellular stress, such as tachycardia.

Indeed, experiments in MCU\(^{-/-}\) mice support that complete or almost complete downregulation of MCU has detrimental effects. Although MCU\(^{-/-}\) mice were protected...
against ischemia-reperfusion injury, they were unable to respond to an increased cardiac workload and showed elevated cytosolic Ca\(^{2+}\) levels, a phenomena also observed in mice expressing a dominant-negative MCU. This is in accordance with a study showing increased cytosolic Ca\(^{2+}\) oscillations in MCU siRNA-transfected cardiomyocytes. Cytosolic Ca\(^{2+}\) levels influence mitochondrial morphology and cytosolic Ca\(^{2+}\) overload leads to mitochondrial fragmentation. Mitochondrial fragmentation leads to dysfunction of the respiratory chain complexes and, thus, results in decreased ATP production. This decrease may result in opening of the sarcolemmal K\(_{ATP}\) channels, which reduces the length of the effective refractory period and shortens action potential duration, thereby promoting development of arrhythmias, such as AF.

**Mitochondrial dysfunction and its implication in cardiac diseases**

Multiple cardiac diseases, including heart failure, myocardial infarction, ischemic heart disease, dilated cardiomyopathy, diabetic cardiomyopathy and hypertension-induced cardiomyopathy, are associated with mitochondrial dysfunction, such as reduced mitochondrial respiration, membrane potential and cellular ATP levels and aberrant mitochondrial morphology. Aberrant mitochondrial morphology includes mitochondrial fragmentation and swelling, disorganized and/or dismantled cristae, smaller mitochondria and/or reduced amount of mitochondria. Both the mitochondrial fragmentation and destroyed cristae result in impairment of respiration, which compromise ATP production and, consequently, cardiac contraction. In addition, MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes) patients with the m.3243A>G mutation in MT-TL1, a mitochondrial leucine transfer RNA gene, have a high risk of cardiac death.

As mitochondria comprise approximately 30% of the cardiomyocyte volume and account for 90% of the provided cardiac contraction energy, mitochondrial dysfunction is detrimental for the heart. This is exemplified by the vast amount of cardiac diseases caused or worsened by mitochondrial dysfunction. In this study, we show that mitochondrial dysfunction also underlies AF progression.

**Mitochondrial DNA as a potential biomarker in AF**

Our study suggests a potential role of circulating mitochondrial DNA in serum as a biomarker for AF as the expression of two measured mitochondrial genes, COX3 and ND1, is induced in serum of AF patients compared to age-related SR patients. Although the upregulation is borderline significant, it is based on only 10 patients and inclusion
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of more patients in various stages of AF, both before and after treatment and/or surgery, will give a better appreciation of the potency of serum mitochondrial DNA as a biomarker for AF.

Interestingly, the potential of circulating mitochondrial DNA in serum as a biomarker has been evaluated in a variety of diseases, including cancer, viral infections, neurodegenerative and cardiac diseases. In several cancers, including bladder, lung and breast cancer, circulating mitochondrial DNA in serum is elevated and can, in some cases, be associated with disease stage or progression. Mitochondrial DNA in serum in HIV-infected persons was elevated and could predict the presence of lipodystrophy. Reduced circulating mitochondrial DNA in cerebral spinal fluid was found in patients with Parkinson’s disease. Cardiac disorders, such as acute myocardial infarction and diabetes-induced coronary heart disease, were also associated with elevated mitochondrial DNA in serum. Moreover, increased plasma mitochondrial DNA levels associates with increased mortality risk in patients in the intensive care unit.

As mitochondrial DNA acts as a damage-associated molecular pattern, increased levels might lead to inflammatory responses, organ injury and increased mortality. Importantly, release of mitochondrial DNA is primarily used to restore homeostasis, but prolonged exposure leads to detrimental changes. As circulating mitochondrial DNA increases with age, the increased risk of inflammation might lead to (age-related) diseases, including cardiac diseases such as heart failure and AF. This makes circulating mitochondrial DNA in serum an interesting biomarker.

Future directions

As this manuscript is still in preparation, multiple experiments are yet to be implemented. The effect of Ru360 on mitochondrial dysfunction has mostly been elucidated. However, the effect of Ru360 treatment on cellular cardiomyocytes stress and cardiomyocyte contractile function is still to be determined. Furthermore, although MCU siRNA treatment attenuates tachypacing-induced CaT_mito loss, the effect on other parameters of mitochondrial dysfunction, including cellular ATP levels, respiration, mitochondrial network morphology and mitochondrial stress should be determined.

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SUPPLEMENTAL FIGURES

Figure S1 Concentration-dependent effects of mdivi-1, mitoTEMPO and Ru360 on CaTmito. Quantified CaTmito amplitude of normal paced (NP C) and 6 hour tachypaced (TP) HL-1 cardiomyocytes either non-treated (C) or treated with different concentrations of A) mdivi-1, B) mitoTEMPO and C) Ru360. **P≤0.01, ***P≤0.001 versus NP C, #P≤0.05, ##P≤.01 versus 6hTP C.

Figure S2 Ru360 preserves the mitochondrial morphology. Quantified data showing the preservation of the mitochondrial network during tachypacing with Ru360 treatment.

Figure S3 Overexpression of MCU in normal-paced HL-1 cardiomyocytes does not change CaTmito, while downregulating MCU does. A) Quantified CaTmito of HL-1 cardiomyocytes transfected with MCU and subjected to normal pacing. B) Quantitative real-time PCR of MCU expression showing a high and a low MCU mRNA reduction (40% and 20%, respectively). C) Quantified CaTmito of HL-1 cardiomyocytes transfected with MCU siRNA and subjected to normal pacing. ***P≤0.001 versus C, ###P≤0.001 versus MCU siRNA high.
Figure S4 Ru360 protects against *Drosophila* heart wall contractile dysfunction. A) Representative heart wall contractions of *Drosophila* monitored before TP (SR) and after TP with demineralized water (Control) or Ru360 pretreatment (20 µM, 50 µM or 100 µM). B) Quantified heart wall contractions of *Drosophila* monitored before TP (SR) with demineralized water (Control) or Ru360 pretreatment (20 µM, 50 µM or 100 µM).

Figure S5 Reduced ATP levels in an AF in vivo model. 7 days atrial tachypaced dogs (TP) show significantly reduced ATP levels compared to control, non-paced (C) dogs. **P≤0.01 versus C.
Part III: Summary, General discussion, Addendum