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# Enhanced Mitochondrial Calcium Uptake Suppresses Atrial Fibrillation Associated With Metabolic Syndrome

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The authors attest they are in compliance with human studies committees and animal welfare regulations of the authors' institutions and Food and Drug Administration guidelines, including patient consent where appropriate.

## ABBREVIATIONS AND ACRONYMS

AF = atrial fibrillation, AP = action potential, ATP = adenosine triphosphate, ECG = electrocardiogram, HFS = high-fat sucrose diet, IP-GTT = intraperitoneal glucose tolerance, IP-ITT = intraperitoneal insulin tolerance, k<sub>pf</sub> = kaempferol, MCUC = mitochondrial Ca<sup>2+</sup> uniporter complex, MetS = metabolic syndrome, Ob-T2D = obese with type 2 diabetes Ow-InsR = overweight with insulin resistance, PDH = pyruvate dehydrogenase, PDK = pyruvate dehydrogenase kinase, pSer = phosphorylated serine residue, ROS = reactive oxygen species, SR = sarcoplasmic reticulum, T2D = type 2 diabetes

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## KEY WORDS

atrial arrhythmias, calcium, diabetic cardiomyopathy, metabolism

# ABSTRACT

## BACKGROUND

Patients with metabolic syndrome (MetS) have an increased risk of atrial fibrillation (AF). Impaired Ca<sup>2+</sup> homeostasis and mitochondrial dysfunction have emerged as an arrhythmogenic substrate in both patients and animal models of MetS. Whether impaired mitochondrial Ca<sup>2+</sup> handling underlies AF associated with MetS remains poorly explored.

## OBJECTIVES

The aim of this study was to determine the initial mechanisms related to AF susceptibility and mitochondrial dysfunction encountered in metabolic cardiomyopathy.

## METHODS

A total of 161 mice and 34 patients were studied. Mitochondrial Ca<sup>2+</sup> and mitochondrial Ca<sup>2+</sup> uniporter complex (MCUC) were investigated in right atrial tissue of patients with (n = 18) or without (n = 16) MetS and of C57Bl/6J mice fed with a high-fat sucrose diet (HFS) for 2 (n = 42) or 12 (n = 39) weeks. Susceptibility to AF was evaluated in isolated sinoatrial tissue and in vivo in mice.

## RESULTS

Increased expression of the MICUs subunits of the MCUC (1.00 ± 0.33 AU vs 1.29 ± 0.23 AU; P = 0.034) was associated with impaired mitochondrial Ca<sup>2+</sup> uptake in patients (168.7 ± 31.3 nmol/min/mg vs 127.3 ± 18.4 nmol/min/mg; P = 0.026) and HFS mice (0.10 ± 0.04 DF/F0 · ms<sup>-1</sup> vs 0.06 ± 0.03 DF/F0 · ms<sup>-1</sup>; P = 0.0086, and 0.15 ± 0.07 DF/F0 · ms<sup>-1</sup> vs 0.046 ± 0.03 DF/F0 · ms<sup>-1</sup>; P = 0.0076 in 2- and 12-week HFS mice, respectively). HFS mice elicited a 70% increased susceptibility to AF. The MCUC agonist kaempferol restored MCUC activity in vitro and abolished the occurrence of AF in HFS mice.

## CONCLUSIONS

Impaired MCUC activity and mitochondrial Ca<sup>2+</sup> homeostasis from the early stage of metabolic cardiomyopathy in mice lead to AF. Given that similar defects in cardiac mitochondrial Ca<sup>2+</sup> handling are present in MetS patients, the modulation of the MCUC activity represents an attractive antiarrhythmic strategy.

## INTRODUCTION

Sedentary and dietary habits have led to an explosion of overweight and obesity over the past decade, especially among children and teenagers. During obesity, type 2 diabetes (T2D) is preceded by a silent phase of metabolic dysregulation of variable duration leading to the metabolic syndrome (MetS). More importantly, the onset of MetS during adolescence is accompanied by metabolic cardiomyopathy, which progresses with age and is characterized by an increased risk for atrial fibrillation (AF).<sup>1</sup> According to population-based cohort studies, obesity increases the risk of AF by 49% in comparison with nonobese people.<sup>2</sup> The underlying mechanisms initiating and promoting AF associated with the MetS are

multifactorial and still poorly understood. At the cellular level, shorter action potential (AP) and also altered sarcoplasmic reticulum (SR) Ca<sup>2+</sup> cycling predispose to ectopic beats and triggered activity.<sup>3</sup> In addition, recent evidence suggests that impaired metabolic supply and mitochondrial function are arrhythmogenic substrates especially in the context of diabetes.<sup>4</sup>

Mitochondria play major roles in cellular biological processes by: 1) providing the cell with a constant amount of energy through the synthesis of adenosine triphosphate (ATP); 2) dealing with reactive oxygen species (ROS); and 3) participating in Ca<sup>2+</sup> homeostasis through cross-talk with the SR.<sup>5</sup> To dynamically adapt the ATP production to the needs imposed by the contractile demand, mitochondria can transiently take up part of the Ca<sup>2+</sup> released by the SR via the mitochondrial Ca<sup>2+</sup> uniporter complex (MCUC). The MCUC is composed of the MCU pore-forming subunits and its regulatory subunits MICU1, MICU2, EMRE, and MCUb.<sup>5</sup> The MICU1:MCU confers its tissue specificity and Ca<sup>2+</sup> sensitivity.<sup>6,7</sup> Within mitochondria, Ca<sup>2+</sup> modulates activities of enzymes involved in the metabolic fluxes, the RO production, the Krebs cycle, and the electron transport chain.<sup>5</sup> Therefore, mitochondrial Ca<sup>2+</sup> is the masterpiece in the dynamic control of the cellular respiration and ATP production.<sup>8</sup>

The mitochondria involvement in cardiac excitability has emerged from preclinical studies showing that modulation of mitochondrial respiration, membrane potential, and ion channels alter AP genesis and myocardial conduction properties.<sup>9</sup> Our group demonstrated that preoperative mitochondrial dysfunction of the atrial myocardium is associated with increased incidence of AF after cardiac surgery, identifying the mitochondrion as a potential player in clinically relevant arrhythmias.<sup>4</sup> These translational data warrant further investigation to explore mitochondria as potential therapeutic targets in clinical arrhythmias, particularly in MetS patients who have cardiac mitochondrial dysfunction.<sup>10</sup>

Therefore, we aimed to establish whether mitochondrial Ca<sup>2+</sup> homeostasis underlines the development of AF associated with the MetS. Using a translational and multidisciplinary approach, we demonstrated that MCUC-supported mitochondrial Ca<sup>2+</sup> uptake is impaired in the atria of both MetS patients and mice fed a high-fat sucrose (HFS) diet for 3 or 12 weeks. Pharmacologic modulation of MCUC function suppresses atrial arrhythmias, providing a new paradigm in the pathogenesis of AF.

## METHODS

### PATIENTS.

Thirty-four patients (16 without MetS and 18 with MetS) undergoing coronary artery bypass graft surgery in the heart surgery ward at the Lille University Hospital were included in the POMI-AF (Post-Operative Myocardial Incident & Atrial Fibrillation; [NCT03376165](#)) cohort. The study was approved by the local Patient Protection Committee (Lille, France), and patients gave informed consent. The metabolic syndrome was diagnosed according to the criteria of Alberti et al.<sup>11</sup>

### ANIMALS.

A total of 161 8-week-old C57Bl/6J male mice (Janvier, France) were fed ad libitum an HFS diet (SAFE 260HF: 20% proteins, 36% lipids, 37% sucrose, 7% other) or a standard diet (SAFE A04, Augy, France: 16% proteins, 3% lipids, 60% starch, 21% other) for 2 or 12 weeks.

## **SINGLE-CHANNEL RECORDINGS.**

Murine atrial submitochondrial particles (SMP) were prepared and spontaneously fused into a planar lipid bilayer. After incorporation, MCUC channel activity was measured in the presence of 1 mM Ca<sup>2+</sup> at fixed potential (-40 mV).<sup>12</sup>

## **KAEMPFEROL ADMINISTRATION.**

In vivo, Alzet 2004 osmotic mini pumps (Durect Corp) were used for the continuous administration of kaempferol (kpf) (EtOH, Carl Roth) or dimethyl sulfoxide (DMSO, Sigma-Aldrich, Merck KGaA) at a rate of 1 mL/h or 0.15 min/24 h for 7 days before transesophageal stimulation as previously described.<sup>13</sup> For voltage optical mapping, sinoatrial tissues were continuously perfused during 20 minutes with 10 mM kpf.

## **STATISTICAL ANALYSIS.**

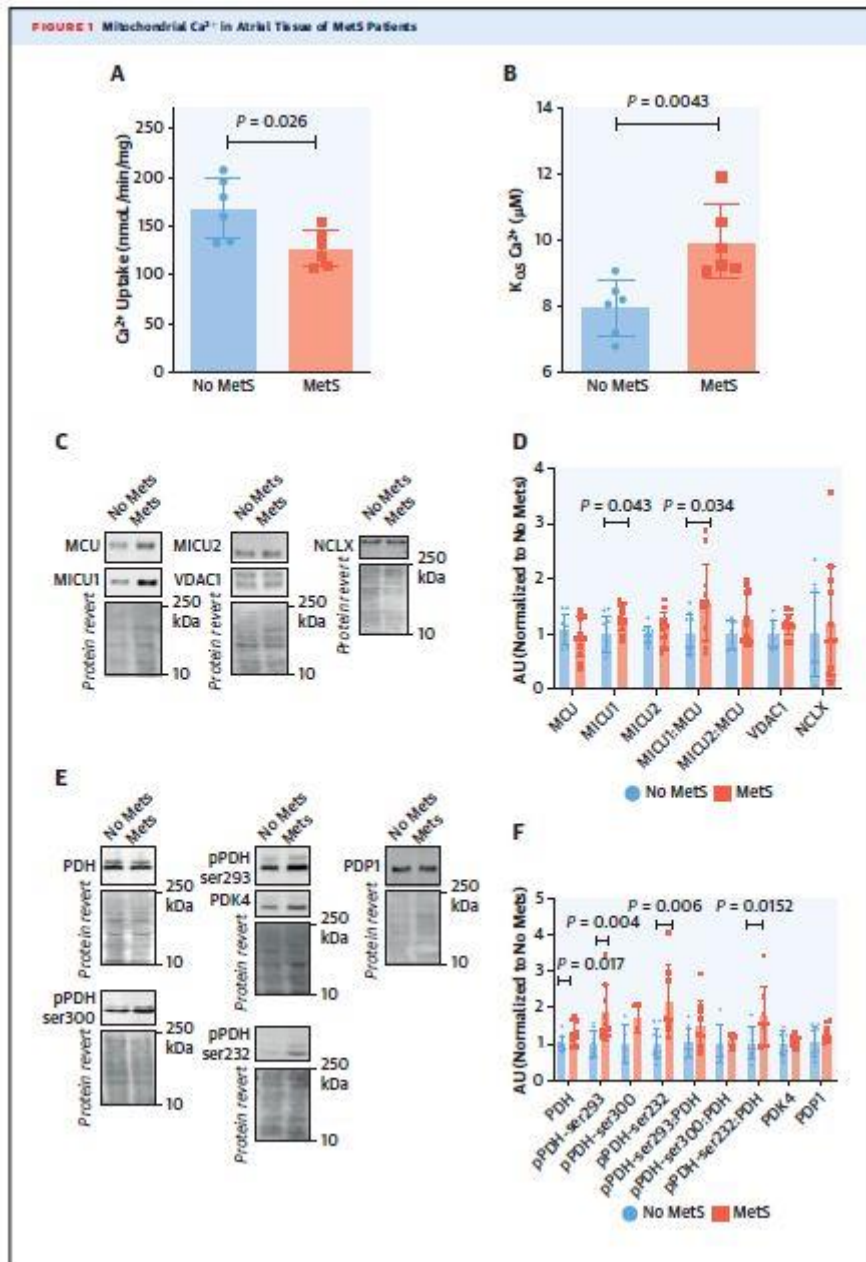
Statistical analyses were performed with Prism 7.0 (GraphPad) software and Origin 8.5 (OriginLab). Data are presented as mean  $\pm$  SD. The normal distribution of the samples was tested by the Shapiro-Wilk normality test. The Student's t-test was used for normally distributed samples, and the Mann-Whitney U test if the normality test failed. For transesophageal stimulation experiments, a 2-way analysis of variance followed by a Tukey's test was performed. The P values and 95% CIs presented in this report have not been adjusted for multiplicity, and therefore inferences drawn from these statistics may not be reproducible. The significance level was set at  $P < 0.05$ .

# **RESULTS**

## **MetS WAS ASSOCIATED WITH ALTERED ATRIAL MITOCHONDRIAL Ca<sup>2+</sup> UPTAKE IN PATIENTS AND RODENTS.**

Isolated cardiac mitochondria from MetS patients revealed abnormal mitochondrial Ca<sup>2+</sup> uptake with decreased maximal rates of Ca<sup>2+</sup> uptake (168.7  $\pm$  31.3 nmol/min/mg vs 127.3  $\pm$  18.4 nmol/min/mg;  $P = 0.026$ ) (Figure 1A) and higher K<sub>0.5</sub> (7.98  $\pm$  0.8 mM vs 9.968  $\pm$  1.1 mM;  $P = 0.0043$ ) (Figure 1B), whereas glutamate/malate mediated mitochondrial respiration remained unchanged. The respiratory control ratio and mitochondrial membrane potential were preserved. Therefore, decreased mitochondrial Ca<sup>2+</sup> uptake was not related to impaired mitochondrial respiration. The expression of the Ca<sup>2+</sup> sensitive subunit MICU1 was increased in MetS patients (1.00  $\pm$  0.33 AU vs 1.29  $\pm$  0.23 AU;  $P = 0.043$ ), whereas MCU expression was unchanged, leading to an increased MICU1:MCU (1.00  $\pm$  0.37 AU vs 1.57  $\pm$  0.69 AU;  $P = 0.034$ ) (Figures 1C and 1D). No difference was observed in the expression of MICU2, voltage-dependent anion channel 1 (VDAC1), or mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger. To explore changes in mitochondrial Ca<sup>2+</sup> homeostasis, the phosphorylation status of the pyruvate dehydrogenase (PDH) was evaluated. The PDH is the entry point for the pyruvate into oxidative metabolism. PDH kinase (PDK) phosphorylates and inhibits PDH activity, whereas the Ca<sup>2+</sup>-dependent PDH phosphatase (PDP) dephosphorylates and increases its activity. In MetS patients, the PDH phosphorylation at the phosphorylated serine residues, phosphorylated serine residue (pSer)<sup>293</sup> and pSer<sup>232</sup>, as well as the PDH expression, were significantly increased (1.00  $\pm$  0.35 AU vs 1.86  $\pm$  0.76 AU;  $P = 0.004$  and 1.00  $\pm$  0.43 AU vs 2.15  $\pm$  0.99 AU;  $P = 0.006$ , respectively) (Figure 1E), although the expression of PDK

isozyme 4 (PDK4) and the PDP1 were unchanged (Figure 1F). When normalized to PDH, the pSer232 level remained significantly increased (1.0 ± 0.43 AU vs 1.75 ± 0.8 AU; P = 0.0152) (Figure 1F) in line with the decreased mitochondrial Ca<sup>2+</sup> uptake (Figure 1A).<sup>14</sup>

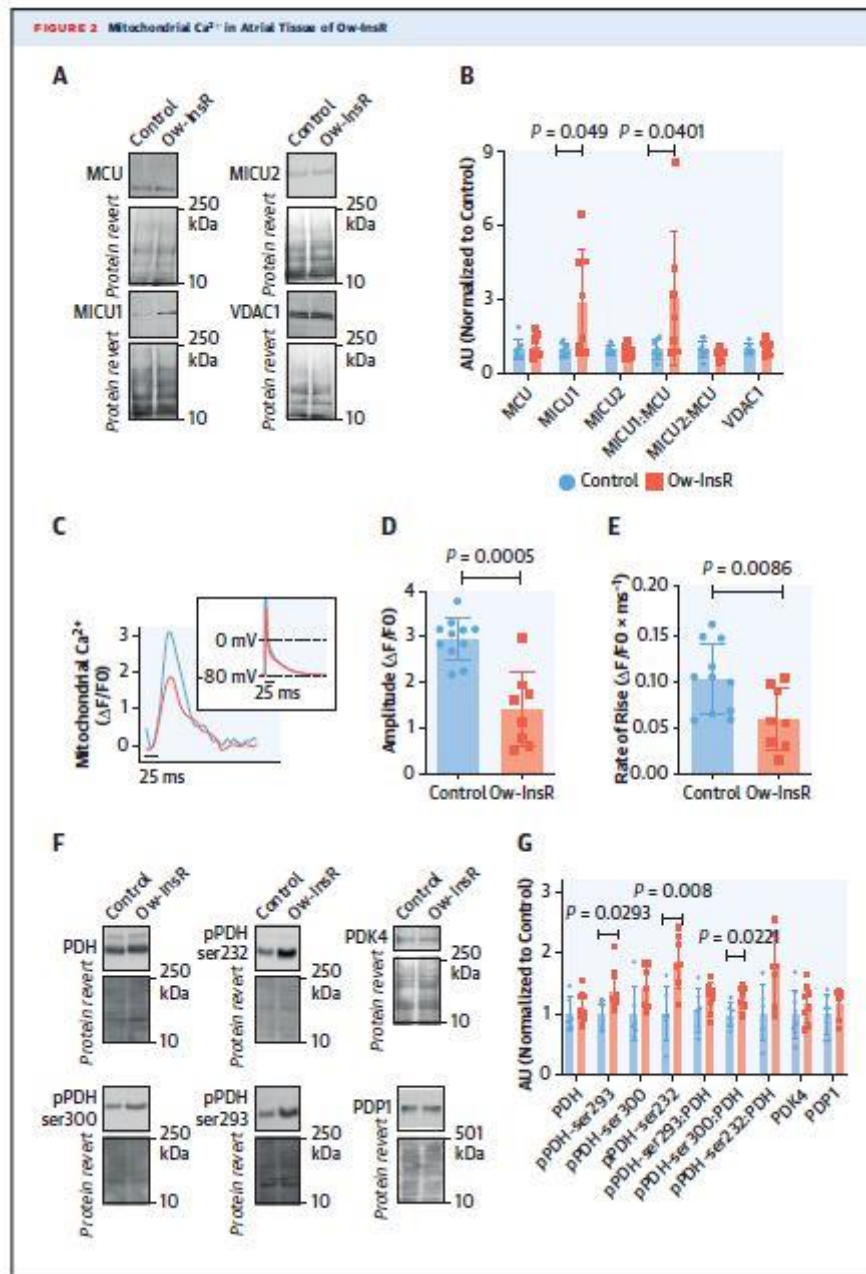


**FIGURE 1 Continued**

(A) Mitochondrial Ca<sup>2+</sup> uptake from atria of patients without MetS and (B) Ca<sup>2+</sup> concentration at 50% of the maximum uptake rate (K<sub>0.5</sub>) (n = 6 per group). (C) Representative Western blot and (D) relative expressions of MCU, MICU1, MICU2, VDAC1, and NCLX and MICU1:MCU, MICU2:MCU in patients without MetS. (E) Representative Western blot and (F) relative expressions of PDH (n = 10 no MetS vs n = 9 MetS), pSer293 (n = 10 no MetS vs n = 9 MetS), pSer300 (n = 5 no MetS vs n = 4 MetS), pSer232 (n = 9 no MetS vs n = 8 MetS), PDK4 (n = 10 no MetS vs n = 11 MetS), and PDP1 (n = 10 no MetS vs n = 10 MetS). Data are mean ± SD. No corrections for multiple testing were applied. MetS = metabolic syndrome; MCU = mitochondrial Ca<sup>2+</sup> uniporter complex; NCLX = mitochondrial Na<sup>+</sup>/Ca<sup>2+</sup> exchanger; PDH = pyruvate dehydrogenase; PDK = pyruvate dehydrogenase kinase; PDP = pyruvate dehydrogenase phosphatase; pSer = phosphorylated serine residue; VDAC1 = voltage-dependent anion channel 1.

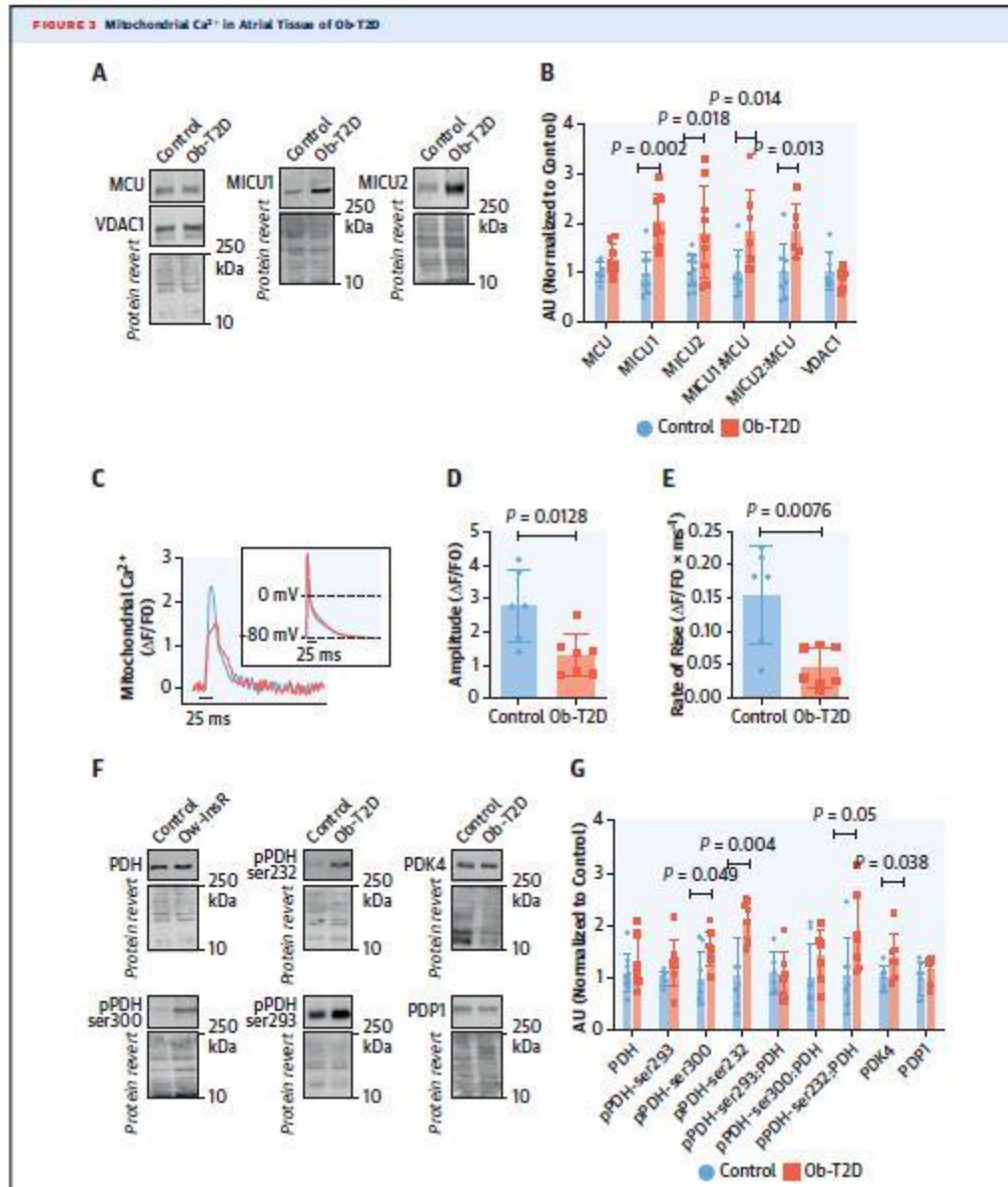
To determine whether impaired mitochondrial Ca<sup>2+</sup> uptake is an early feature of MetS, we established a mouse model of MetS by feeding mice an HFS diet for 2 or 12 weeks. Mice fed with a short-term HFS diet already showed an increase in body weight and an intermediate

metabolic impairment with a decrease in insulin sensitivity but maintained a normal glucose tolerance, normoglycemia, with increased insulinemia, and circulating nonesterified fatty acids and cholesterol. In addition to weight gain, mice fed with a long-term HFS diet exhibited an established T2D state, characterized by insulin resistance, glucose intolerance, and hyperglycemia, associated with dyslipidemia. Therefore, after 2 weeks of HFS, mice were overweight with insulin resistance (Ow-InsR), whereas 12 weeks of HFS diet induced an obese T2D mouse model (Ob-T2D).



**FIGURE 2 Continued**

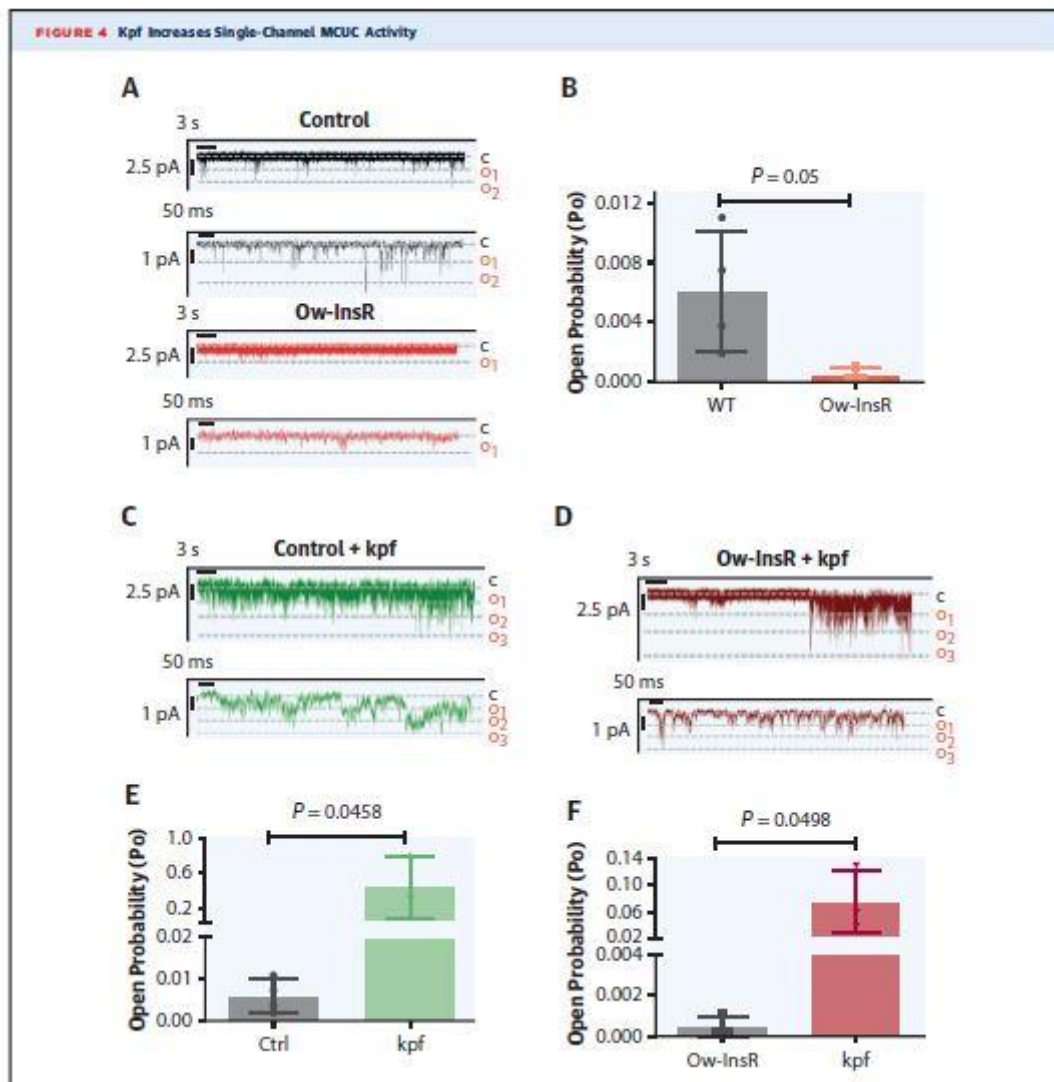
(A) Representative Western blot and (B) relative expressions of MCU, MICU1, MICU2, VDAC1, and MICU1:MCU, MICU2:MCU ( $n = 8$  per group). (C) Representative traces of mitochondrial Rhod-2  $\text{Ca}^{2+}$  transients recorded in adult atrial cardiomyocytes during an AP (inset). Mitochondrial  $\text{Ca}^{2+}$  transient parameters: (D) amplitude, (E) rate of rise;  $n = 8$ -11 cardiomyocytes;  $n = 4$  per group. (F) Representative Western blot and (B) relative expressions of PDH, pSer293, pSer300, pSer232, PDK4, and PDP1;  $n = 6$ -8 per group. Data are mean  $\pm$  SD. No corrections for multiple testing were applied. AP = action potential; other abbreviations as in Figure 1.



Representative of single-channel recording of the MCUC incorporated in planar lipid bilayer from (A) control or Ow-InsR, recorded in Ca-gluconate (1 μM free Ca<sup>2+</sup>) at -40 mV. (Above) 40-s recording. (Below) 1.5-s subset of recording. (B) Single-channel open probability of the MCUC from control and Ow-InsR; n = 4 channels from n = 2 hearts per group. Representative of (C) control and Ow-InsR (D) single-channel recording of the MCUC in presence of 10 μM kpf at -40 mV. Mean MCUC open probability from (E) control and (F) Ow-InsR; n = 3 channels from n = 2 hearts per group. Data are mean ± SD. No corrections for multiple testing were applied. kpf = kaempferol; MCUC = mitochondrial Ca<sup>2+</sup> uniporter complex; Ob-T2D = obese with type 2 diabetes; Ow-InsR = overweight with insulin resistance.

Similarly to MetS patients, both mouse models displayed an atrial remodeling of the MCUC. Ow-InsR mice showed an increase in MICU1 expression (1.00 ± 0.26 AU vs 2.89 ± 2.14 AU; P = 0.049), resulting in a higher MICU1:MCU (1.00 ± 0.39 AU vs 3.10 ± 2.75 AU; P = 0.0401) (Figures 2A and 2B). The amplitude (2.94 ± 0.46 DF/F0 vs 1.41 ± 0.83 DF/F0; P = 0.0005) (Figures 2C and 2D) and the rate of rise of the mitochondrial Ca<sup>2+</sup> transients recorded during ventricular AP was significantly reduced (0.10 ± 0.04 DF/F0 × ms<sup>-1</sup> vs 0.06 ± 0.03 DF/F0 × ms<sup>-1</sup>; P = 0.0086) (Figure 2E), whereas the decay time was unchanged. This impaired mitochondrial Ca<sup>2+</sup> uptake was not related to changes in AP duration or the resting membrane potential.

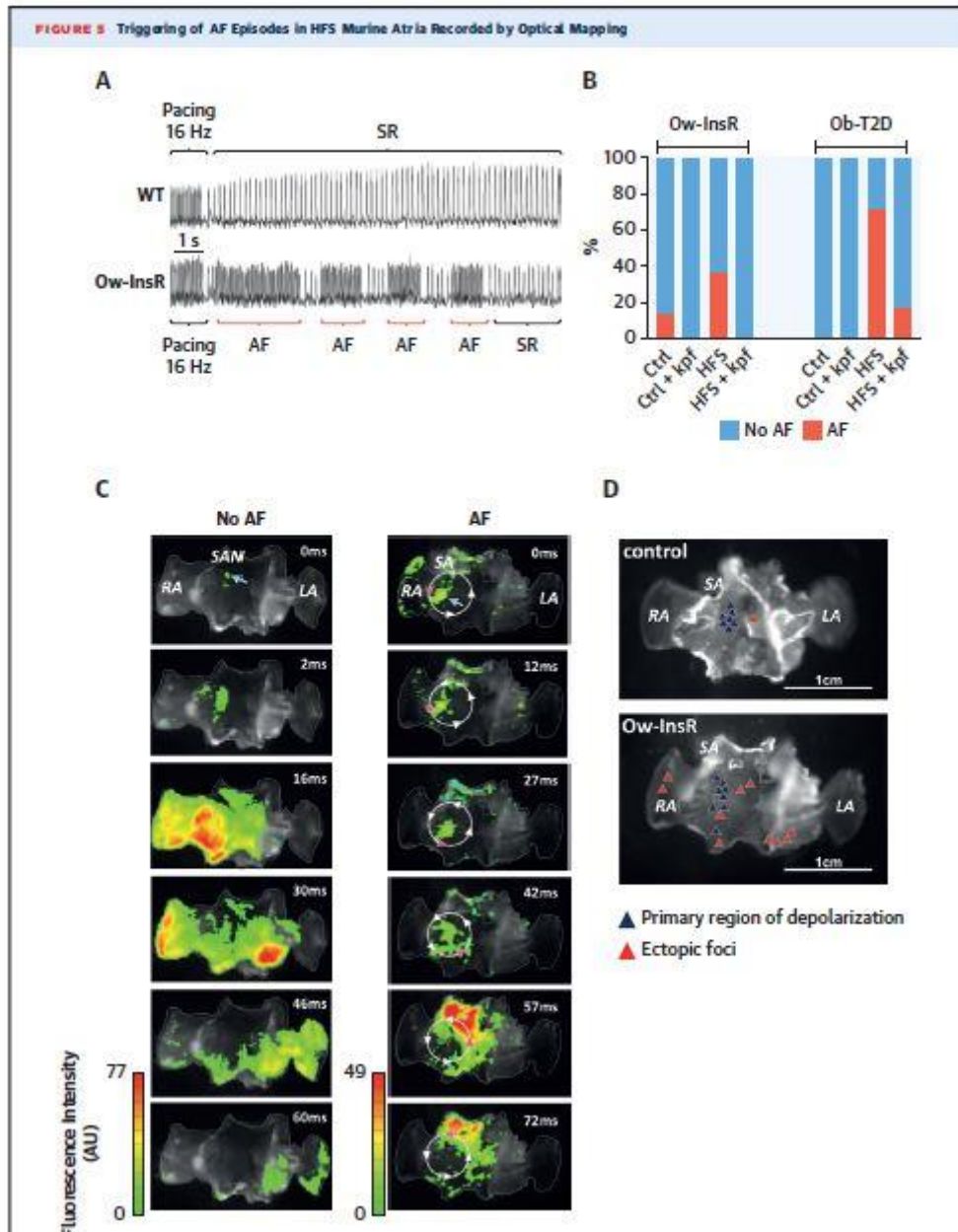




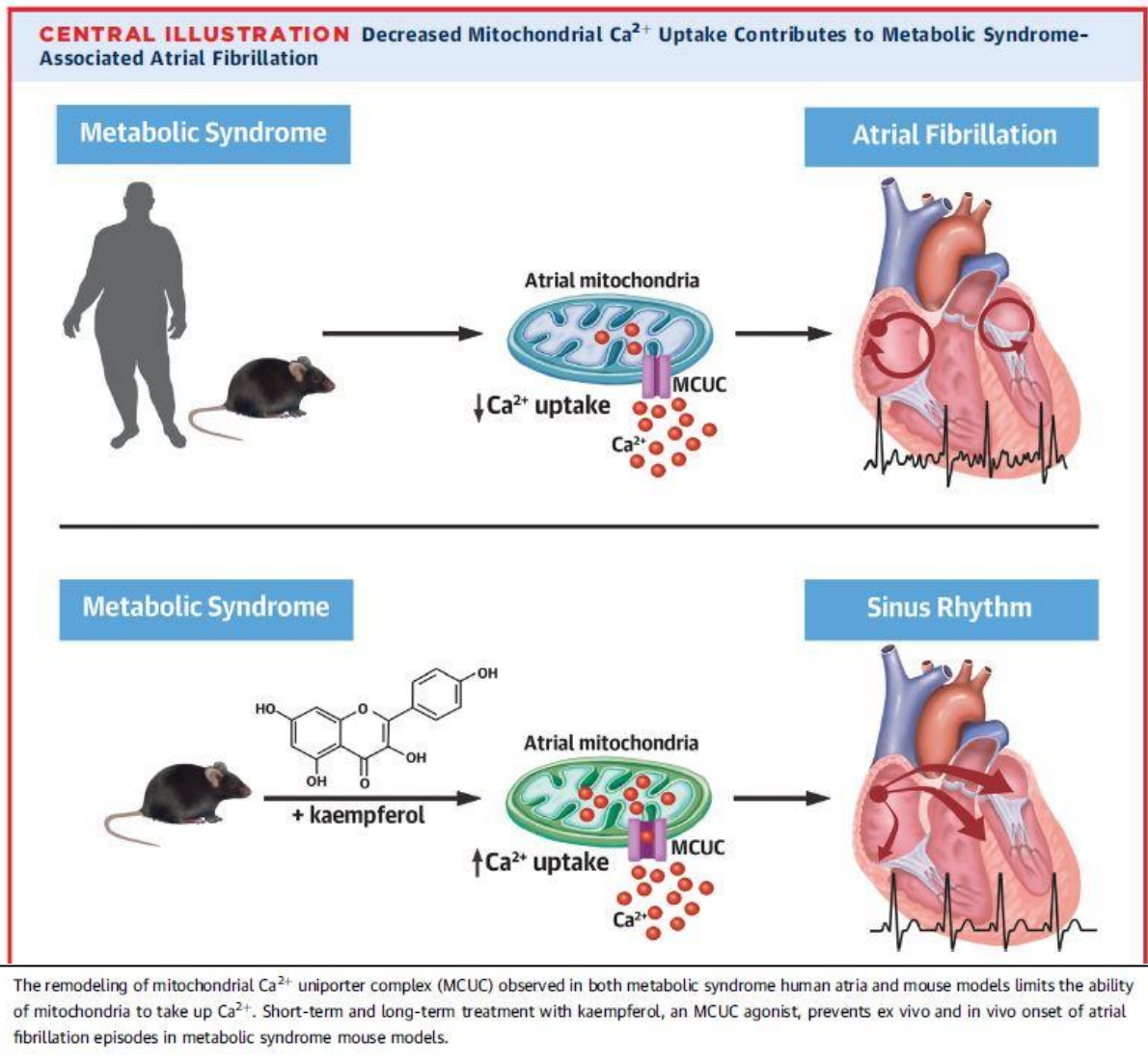
Representative of single-channel recording of the MCUC incorporated in planar lipid bilayer from (A) control or Ow-InsR, recorded in Ca-gluconate (1  $\mu$ M free  $\text{Ca}^{2+}$ ) at  $-40$  mV. (Above) 40-s recording. (Below) 1.5-s subset of recording. (B) Single-channel open probability of the MCUC from control and Ow-InsR;  $n = 4$  channels from  $n = 2$  hearts per group. Representative of (C) control and Ow-InsR (D) single-channel recording of the MCUC in presence of 10  $\mu$ M kpf at  $-40$  mV. Mean MCUC open probability from (E) control and (F) Ow-InsR;  $n = 3$  channels from  $n = 2$  hearts per group. Data are mean  $\pm$  SD. No corrections for multiple testing were applied. kpf = kaempferol; MCUC = mitochondrial  $\text{Ca}^{2+}$  uniporter complex; Ob-T2D = obese with type 2 diabetes; Ow-InsR = overweight with insulin resistance.

This decreased mitochondrial  $\text{Ca}^{2+}$  uptake was associated with an absolute increased PDH phosphorylation at the Ser293 and Ser232 (1.00  $\pm$  0.21 AU vs 1.37  $\pm$  0.34 AU;  $P = 0.0293$ , and 1.00  $\pm$  0.45 AU vs 1.87  $\pm$  0.44 AU;  $P = 0.008$ , respectively) (Figures 2F and 2G) and an increased PDH phosphorylation at the Ser300 when normalized to the PDH expression (1.00  $\pm$  0.20 AU vs 1.27  $\pm$  0.18 AU;  $P = 0.0221$ ) (Figures 2F and 2G). In Ob-T2D mice, the increases in MICU1 expression (1.00  $\pm$  0.44 AU vs 2.04  $\pm$  0.52 AU;  $P = 0.002$ ) and the MICU1:MCU (1.00  $\pm$  0.46 AU vs 1.84  $\pm$  0.83 AU,  $P = 0.014$ ) were conserved. We additionally observed a significant increase in MICU2 expression (1.00  $\pm$  0.33 AU vs 1.82  $\pm$  0.92 AU;  $P = 0.018$ ) and an elevation of the MICU2:MCU (1.00  $\pm$  0.56 AU vs 1.84  $\pm$  0.53 AU;  $P = 0.013$ ) (Figures 3A and 3B). The amplitude and the rate of rise of the mitochondrial  $\text{Ca}^{2+}$  uptake were also significantly decreased (2.8  $\pm$  1.09 DF/F0 vs 1.3  $\pm$  0.65 DF/F0;  $P = 0.0128$ , and 0.15  $\pm$  0.07 DF/F0  $\cdot$  ms $^{-1}$  vs 0.046  $\pm$  0.03 DF/F0  $\cdot$  ms $^{-1}$ ;  $P = 0.0076$ , respectively) (Figures 3C to 3E). The decay time constant remained comparable with that in

control animals, confirming the specific defects in mitochondrial Ca<sup>2+</sup> uptake. The AP duration and the resting membrane potential recorded alongside the mitochondrial Ca<sup>2+</sup> transients did not show any significant difference in both groups. Similarly, Ser300 and Ser232 phosphorylation levels of the PDH increased (1.00 ± 0.52 AU vs 1.57 ± 0.33 AU; P = 0.049, and 1.00 ± 0.68 AU vs 2.14 ± 0.38 AU; P = 0.004, respectively) as well as the pSer232:PDH (1.00 ± 0.7 vs 1.9 ± 0.75 AU); however, the PDK4 expression also increased (1.00 ± 0.22 AU vs 1.56 ± 0.59 AU; P = 0.038) (Figures 3F and 3G). Taken together, these results demonstrate that cardiac MCUC remodeling and the associated reduction in mitochondrial Ca<sup>2+</sup> uptake constitute a pathogenic feature that appears from the early stage of the MetS.



(A) Representative traces of AF episodes after a 16-Hz pacing in Ow-InsR. Spontaneous APs corresponding to the sinus rhythm of sinoatrial tissues from control and HFS animals. (B) Incidence of AF in Ow-InsR and Ob-T2D animals. Sinoatrial tissues were continuously perfused with or without kpf (10 μM) (n = 8 per group). (C) Normal (left) and rotor propagation during AF episode (right) visualized by optical voltage mapping in the SA of Ow-InsR animals. Green to red colors indicate the propagation of the depolarization within the SA toward the atria. (D) Origin of depolarization (blue triangle) and ectopic foci (red triangle) in control or Ow-InsR SA tissues. AF = atrial fibrillation; HFS = high-fat sucrose diet; other abbreviations as in Figures 2 and 4.



## HFS DIET ALTERED ATRIAL MCUC SINGLE-CHANNEL BIOPHYSICAL PROPERTIES.

To establish the specific functional remodeling of the atrial MCUC, we characterized the MCUC's single-channel biophysical properties in artificial planar lipid bilayers. The results showed a drastic decrease in MCUC open probability, with only 1 open state in Ow-InsR atria ( $6.10^{-3} \text{ } _{4.10^{-3}}$  vs  $4.10^{-4} \text{ } _{4.10^{-4}}$ ;  $P \text{ } \frac{1}{4} 0.05$ ) (Figures 4A and 4B). MCUC conductance was also significantly decreased ( $50.4 \text{ } _{4.2}$  pS vs  $25.5 \text{ } _{3.3}$  pS;  $P \text{ } \frac{1}{4} 0.034$ ). Next, we evaluated whether kpf (10 mM), a polyphenol known to increase MCUC-mediated  $\text{Ca}^{2+}$  uptake<sup>15</sup>, enhances MCUC activity. As shown in Figures 4C and 4D, and occurrence of high-amplitude opening events in both control and Ow-InsR animals ( $6.10^{-3} \text{ } _{4.10^{-3}}$  vs  $0.44 \text{ } _{0.34}$ ;  $P \text{ } \frac{1}{4} 0.0458$ , and  $4.10^{-4} \text{ } _{4.10^{-4}}$  vs  $0.080.05$ ;  $P \text{ } \frac{1}{4} 0.0498$ ) (Figures 4C to 4F). Therefore, kpf showed a direct effect on MCUC biophysical behavior.

## KPF SUPPRESSED AF TRIGGERING EX VIVO.

Given that MetS is a risk factor for AF, we next determined whether the genesis and propagation of the electrical impulse from the SA node was affected after an HFS diet. We therefore performed optical voltage mapping in an isolated atrial preparation. After a pacing

burst (16 Hz), 40% Ow-InsR and 70% Ob-T2D atria triggered AF episodes (Figures 5A and 5B). In HFS, the electrical impulse was generated in alternate depolarization regions and propagated in a rotor-shaped direction (Figures 5C and 5D). Furthermore, control tissues exhibited leading regions of depolarization in the upper area of the SA tissue, near the superior vena cava and the crista terminalis (Figure 5D). Conversely, in HFS tissues, alternative leading regions appeared in the SA peripheral region, in the right atrium and near the inferior vena cava but also in the left atrium (Figure 5D). The emergence of extranodal depolarizations in HFS was associated with AF occurrence (Figure 5C). Finally, the short-term application of kpf (10 mM) prevented AF in atrial preparations from Ow-InsR mice and reduced to 17% the AF episodes in Ob-T2D (Figure 5B), supporting the hypothesis of a key role of MCUC in the genesis of AF associated with MetS.

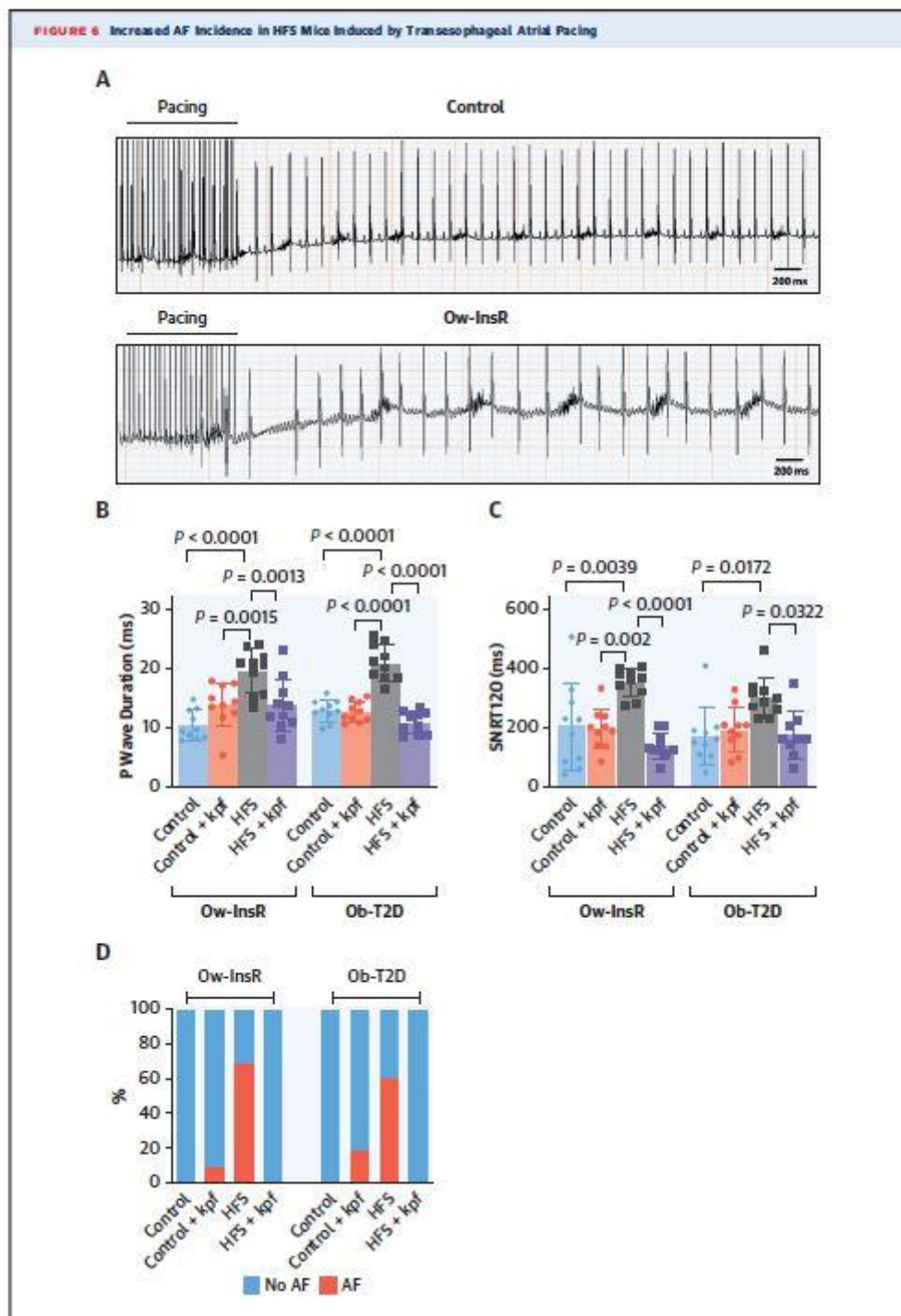
### **KPF PREVENTED AF TRIGGERING IN VIVO.**

To further confirm the protective effects of kpf against AF in vivo, kpf was administered for 1 week before transesophageal stimulation. P-wave duration was increased in both Ow-InsR and Ob-T2D animals compared with control groups (10.5 ± 2.5 ms vs 19.6 ± 3.7 ms;  $P < 0.00001$ ;  $n = 10$ , and 13.9 ± 3.7 ms vs 21.1 ± 3 ms;  $P < 0.0001$ ;  $n = 10$ ) (Figures 6A and 6B). By contrast, SCL duration, PR interval, QRS duration, and both QT and QTc intervals were unchanged. Kpf restored P-wave duration in both groups (Figure 6B). Furthermore, transesophageal stimulation revealed that sinus node recovery time increased in HFS mice (205.8 ± 144.6 ms vs 355.2 ± 46.8 ms;  $P = 0.0039$ ;  $n = 10$ , and 172.6 ± 95.8 ms;  $P = 0.0172$ ;  $n = 10$ ) (Figure 6C), suggesting an impairment of sinus node function. Kpf treatment restored the sinus node recovery time in both HFS groups (Figure 6C). Finally, none of the control animals triggered AF, whereas 70% of the Ow-InsR animals and 60% of the Ob-T2D animals exhibited episodes of AF, characterized by baseline tremulation, loss of the P-wave, and irregular RR intervals (Figures 6A and 6D). Kpf abolished the occurrence of arrhythmia in almost all animals and therefore exerted a protective antiarrhythmic effect (Figure 6D). Of note, in control animals, the kpf treatment increased AF occurrence, suggesting that enhancing mitochondrial Ca<sup>2+</sup> uptake in normal conditions may be deleterious (Figure 6D).

## **DISCUSSION**

Over the past decades, the cardiac consequences of obesity and diabetes have been widely documented. Among the well-established mechanisms, mitochondrial dysfunction plays a central role in the development of the structural and functional cardiac changes associated with metabolic disorders.<sup>4,10,16</sup> Here, AF associated with MetS can be attributed to a very early remodeling of the structure and function of atrial MCUC. Particularly, remodeling of MCUC stoichiometry and function leads to a decrease in the beat-to-beat mitochondrial Ca<sup>2+</sup> uptake and mitochondrial Ca<sup>2+</sup> dynamics in the atria of MetS patients and mice (Central Illustration). This decrease affected PDH phosphorylation, one of the first changes responsible for decreased oxidation of carbohydrates.<sup>17</sup> Indeed, the metabolic switch associated with MetS was attributed to a switch in substrate utilization, where energy production relies almost exclusively on fatty acid oxidation, despite hyperglycemia.<sup>18</sup> PDK4 inhibits PDH activity, whereas its dephosphorylation by Ca<sup>2+</sup>-dependent PDP increases its activity.<sup>19</sup> Although the increased PDK4 is reported in a severe model of obesity-induced T2DM,<sup>20</sup> the increased

phosphorylation of PDH at the early stage of the pathologic condition is most likely related to the impaired mitochondrial Ca<sup>2+</sup> uptake.<sup>14</sup>



**FIGURE 6 Continued**

(A) Representative recording of AF triggered by a 25-Hz pacing stimulation rate in Ow-InsR. (B) Mean P-wave duration in control and Ow-InsR or Ob-T2D mice. (C) Mean sinus recovery time (SNRT) after 120 ms pacing and (D) incidence of AF in Ow-InsR and Ob-T2D mice. Kpf was administered for 1 week using implantable osmotic pumps before transesophageal stimulation ( $n = 10$  per group). Data are mean  $\pm$  SD. \* $P < 0.05$ ; 2-way analysis of variance. Abbreviations as in Figures 1, 4, and 5.

Prolonged P-wave is associated with diabetes and is strongly correlated with the incidence of AF.<sup>21,22</sup> Most importantly, such an ECG abnormality has also been reported in metabolically healthy obese patients<sup>2</sup> and is positively correlated with insulin resistance in obese adolescents.<sup>23</sup> Accordingly, epidemiologic studies have established that obesity and

elevated body mass index independently increase the risk of AF.<sup>2</sup> This is also true for patients with impaired glycemic control who see their risk for the development of AF increased by >30%.<sup>24</sup> From a pathophysiological point of view, dilation of the atria, fibrosis, pericardial adipose tissue, myocardial steatosis, infiltration of inflammatory cells, and/or an alteration of the autonomic nervous system constitute anatomopathological substrates of AF.<sup>25</sup> Furthermore, the triggering of ectopic electrophysiological activities is required and is attributed to cellular early and delayed after the occurrence of depolarization.<sup>3</sup> The conjunction of both the arrhythmogenic substrate and ectopic activity increases the incidence of the reentry phenomenon that characterizes episodes of AF. Although baseline ECG parameters showed no difference between control and HFS animals, the latter have prolonged P-wave duration, indicating decreased atrial conduction velocity.<sup>22</sup> In addition, HFS sinoatrial tissues exhibited extranodal ectopic foci, resulting in the appearance of re-entry phenomena in the right atrium, and transesophageal recordings also showed increased susceptibility to triggering AF.

Several experimental studies and clinical evidence support the fact that mitochondrial impairment contributes to ventricular arrhythmogenesis, primarily by modulating redox and/or ATP-dependent signaling pathways.<sup>26,27</sup> Although the contribution of mitochondrial dysfunction to the onset and progression of AF is still puzzling, we demonstrated in a prospective study that in the context of MetS, preoperative atrial mitochondrial dysfunction is associated with postoperative AF.<sup>4</sup> Here, we further establish a reduced capacity of mitochondria to take up Ca<sup>2+</sup> associated with increased MICU1:MCU in these patients. In mice, an increase in MICU1:MCU is observed at the very early stage of the pathologic changes, and for more severe metabolic phenotypes, when the T2D is established, the MICU2 expression also increases. In both cases this MCUC remodeling results in a reduction in the mitochondrial Ca<sup>2+</sup> uptake rate, which agrees with the impaired mitochondrial Ca<sup>2+</sup> uptake currently reported in preclinical models of metabolic cardiomyopathy.<sup>28</sup> MICUs subunits are key regulators of the MCU gating functions and confer the tissue specificity of the mitochondrial Ca<sup>2+</sup> uptake. Indeed, MICUs settle the Ca<sup>2+</sup>-dependent threshold for Ca<sup>2+</sup> uptake at approximately 400 nM in control conditions, and the removal of MICU1 lowers this threshold and increases MCU conductance for physiological Ca<sup>2+</sup> cytosolic concentration.<sup>7</sup> Conversely, increased MICU1 expression and MICU1:MCU decrease the capacity of the mitochondria to take up Ca<sup>2+</sup> at submicromolar physiological concentrations.<sup>6</sup>

Although the biophysical properties of the MCUC from atrial tissue have not been characterized so far, here the higher MICU:MCU accordingly reduced the open probability and conductance of the channel in the presence of a physiologically relevant Ca<sup>2+</sup> concentration. Moreover, the MCUC enhancer, kpf, increased open probability and high-amplitude events in both control and HFS SMP, pharmacologic activation of mitochondrial Ca<sup>2+</sup> uptake in pathophysiological contexts has been the subject of much interest for years.<sup>29</sup> For instance, Montero et al<sup>15</sup> first showed a direct activation of mitochondrial Ca<sup>2+</sup> uptake by several flavonoids. Among them, kpf was reported to enhance the Ca<sup>2+</sup> affinity of the MCUC. Although kpf has no or little effect on Ca<sup>2+</sup> uptake in the absence of MICUs subunits,<sup>30</sup> the exact mechanisms whereby kpf increases the MCUC open probability remain unclear and would require further investigation.

Under basal conditions, a limitation in mitochondrial Ca<sup>2+</sup> has little impact on myocardial ATP synthesis; however, when the cardiac workload increases, a rise in mitochondrial Ca<sup>2+</sup> uptake is required to match the energetic demand.<sup>5</sup> Decreased dynamic mitochondrial Ca<sup>2+</sup> uptake produces fluctuations in ATP synthesis under an increased cardiac workload. Concomitantly, activation of the ATP-dependent potassium channel shortens the AP and decreases refractory

periods promoting re-entrant arrhythmia.<sup>3</sup> Moreover, oscillation in ATP synthesis affects the SERCA2a activity and SR Ca<sup>2+</sup> load. The beat-to-beat fluctuations in SR Ca<sup>2+</sup> content induce alternans in SR Ca<sup>2+</sup> release, which affect AP duration.<sup>3,16,31</sup> Finally, a lower mitochondrial Ca<sup>2+</sup> uptake is also prone to directly affect the RyR2 activities by increasing the Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release process and the propagation of the local Ca<sup>2+</sup> release.<sup>32</sup> Fluctuations in the mitochondrial respiration rate also increase ROS production and subsequent RyR2 oxidation, thereby increasing its open probability.<sup>33</sup> Consequently, the Ca<sup>2+</sup> leak from the SR predisposes to spontaneous diastolic SR Ca<sup>2+</sup> release events and triggers after depolarization.<sup>31</sup> Therefore, positive modulation of mitochondrial Ca<sup>2+</sup> uptake has demonstrated beneficial impacts in several pathophysiological contexts. It regulates cardiac rhythmicity,<sup>34</sup> suppresses Ca<sup>2+</sup>-dependent arrhythmia in preclinical models of catecholaminergic polymorphic ventricular tachycardia,<sup>13</sup> improves energy metabolism and ROS production in heart failure, <sup>35,36</sup> and protects against alternans in atrial myocytes.<sup>37</sup> Here, the pharmacologic activation of MCUC prevented the triggering of AF in the context of MetS. Although stimulating mitochondrial Ca<sup>2+</sup> uptake is an attractive therapeutic strategy, it also should be considered a double-edged sword. Indeed, mitochondrial Ca<sup>2+</sup> overload is a prerequisite for mPTP opening and is a major trigger of mitochondrial ROS production. Thus, enhancement of mitochondrial Ca<sup>2+</sup> uptake in a context where impairment of mitochondrial Ca<sup>2+</sup> homeostasis is not established may have some negative consequences on myocardial function.<sup>5</sup>

## STUDY LIMITATIONS.

Postoperative AF in patients with MetS is positively correlated with mitochondrial dysfunction with an increased ROS production. Here the MCUC remodeling was associated with decreased mitochondrial Ca<sup>2+</sup> uptake in MetS patients. However, the link between changes in mitochondrial Ca<sup>2+</sup> handling and the previously described mitochondrial dysfunction in MetS patients remains to be established. Additionally, further studies will be needed to elucidate the underlying mechanisms whereby mitochondrial Ca<sup>2+</sup> triggers ectopic activity and AF. Finally, the amount of tissue obtained from patient biopsy specimens was not sufficient to enable extraction of SMP for single-channel MCUC recordings and for analysis of the biophysical properties of human atrial MCUC.

## CONCLUSIONS

In this study, we provide a new paradigm in which mitochondrial Ca<sup>2+</sup> dynamics play a central role in the early phases of AF pathogenesis. Indeed: 1) obesity and the associated metabolic changes are closely related to an alteration in the expression and function of MCUC in both patients and mice; and 2) impaired mitochondrial Ca<sup>2+</sup> homeostasis in a preclinical model of MetS contributes to the AF triggering and can be reversed by pharmacologic improvement of MCUC function, reducing AF vulnerability. Therefore, our data open new opportunities to optimize therapies aimed at restoring electrical function in metabolic disorders.

## **PERSPECTIVES**

### **COMPETENCY IN MEDICAL KNOWLEDGE:**

Defects in mitochondrial calcium handling in patients with metabolic syndrome play a key role in their propensity to atrial fibrillation.

### **TRANSLATIONAL OUTLOOK:**

Further research is needed to understand the regulation of mitochondrial Ca<sup>2+</sup> signaling in the context of human disease and to identify potential therapeutic avenues to reduce the incidence of atrial arrhythmias.

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### **COMPETENCY IN MEDICAL KNOWLEDGE:**

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