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Original Research

Atrial Fibrillation Is Associated With Impaired Atrial Mitochondrial Energetics and Supercomplex Formation in Adults With Type 2 Diabetes

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Key Messages

- Mitochondrial respiration is impaired in adults with diabetes and atrial fibrillation.
- Diabetes and atrial fibrillation lead to impaired supercomplex assembly in adults.

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ABSTRACT

Objectives: Type 2 diabetes mellitus is a chronic progressive disease that is associated with increased risk for cardiovascular diseases and with impaired mitochondrial metabolism in cardiac and skeletal muscles. Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia and is associated with significant morbidity and mortality. Type 2 diabetes is also one of the prevalent concomitant diseases in patients with AF. During AF, myocardial energy demand is high due to electrical activity. To date, however, very little is known about the effects of AF on atrial muscle mitochondrial energetics. We hypothesized that preexisting fibrillation or type 2 diabetes impacts atrial mitochondrial energetics and electron transport chain supercomplexes.
Methods: Atrial appendages were collected from patients who had consented and who had and did not have preexisting AF and were undergoing coronary artery bypass graft surgery. Mitochondrial functional analyses were conducted in permeabilized myofibers using high-resolution respirometry.
Results: Results show impaired complex I and II function in addition to impaired electron transport chain supercomplex assembly in patients with diabetes and AF compared to patients with diabetes but without AF. There were no differences in mitochondrial content in atrial muscle between the groups. There was a strong trend for increased oxidative damage (protein carbonyls) in patients with diabetes and AF compared to patients with diabetes but without AF.
Conclusions: Overall, findings suggest impaired mitochondrial function in AF and type 2 diabetes.

Mots clés :
fibrillation auriculaire
métabolisme cardiaque
diabète
cœur
mitochondrie
stress oxydatif

RÉSUMÉ

Objectifs : Le diabète sucré de type 2 est une maladie chronique évolutive qui est associée à l'augmentation du risque de maladies cardiovasculaires et à une altération du métabolisme des mitochondries dans le muscle cardiaque et le muscle squelettique. La fibrillation auriculaire (FA) qui est l'arythmie cardiaque soutenue la plus fréquente est associée à une morbidité et à une mortalité significatives. Le diabète de type 2 est également l'une des maladies concomitantes prévalentes chez les patients atteints de FA. Au cours de la FA, la demande énergétique du myocarde est élevée en raison de l'activité électrique. Toutefois, on en connaît jusqu'à maintenant très peu sur les effets de la FA sur l'énergétique des mitochondries du muscle auriculaire. Nous posons l'hypothèse que la préexistence de la fibrillation ou du diabète de type 2 a des répercussions sur l'énergétique des mitochondries du muscle auriculaire et les supercomplexes de la chaîne de transport d'électrons.

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Méthodes : Les appendices auriculaires provenaient de patients qui avaient donné leur consentement, qui étaient atteints ou non d'une FA préexistante, et qui avaient subi un pontage aortocoronarien. Nous avons effectué les analyses fonctionnelles des mitochondries dans les myofibres perméabilisées au moyen de la respirométrie à haute résolution.
Résultats : Comparativement aux résultats des patients diabétiques non atteints de FA, les résultats des patients diabétiques atteints de FA montrent une altération du fonctionnement des complexes I et II en plus de la dégradation de l'assemblage des supercomplexes de la chaîne de transport d'électrons. Nous n'avons observé aucune différence dans la teneur en mitochondries du muscle auriculaire entre les groupes. Nous avons observé une forte tendance à l'augmentation des dommages oxydatifs (protéines carbonylées) chez les patients diabétiques atteints de FA par rapport aux patients diabétiques non atteints de FA.
Conclusions : Dans l'ensemble, les résultats montrent une altération du fonctionnement des mitochondries en présence de diabète de type 2 et de FA.

Introduction

Insulin resistance and abnormal or impaired glucose metabolism are early signs of type 2 diabetes mellitus, a chronic progressive disease that represents 90% of all diabetes cases and is on the rise internationally.

Type 2 diabetes commonly coexists (≤20%) in patients with atrial fibrillation (AF) and is considered by some to promote the development of AF independently (1,2). However, a causal relationship between type 2 diabetes and AF has not yet been confirmed. It has been established that type 2 diabetes-associated metabolic defects cause endothelial dysfunction, acceleration of atherogenesis and activation of the renin-angiotensin-aldosterone system which, alone or in combination with other factors, may promote the development of AF (3). AF is the most common sustained cardiac arrhythmia in humans and is increasing in prevalence (4). Currently, approximately 350,000 Canadians experience AF, which confers significantly increased morbidity and mortality (4,5). The molecular origins of AF are complex and, despite recent progress, little if anything is known about the fundamental mechanisms linking type 2 diabetes and AF (6,7). It is intuitively clear that AF must increase the energy demands on the atrial muscle; however, our understanding of the metabolic mechanisms and overall impact of fibrillation is still preliminary (8).

During AF, rapid electrical activity increases adenosine triphosphate (ATP) demands that need to be met through increases in the nutrient and oxygen supply that support mitochondrial oxidative phosphorylation, which comprises electron transport chain complexes I through IV and ATP synthase (complex V) (8). It is unknown whether these increased ATP demands place an excessive demand on atrial mitochondria, leading to collateral negative effects due to prolonged stress responses. Recent research has demonstrated that high ATP demands in muscle are, in part, met by the formation of mitochondrial supercomplexes, which are higher-order protein structures that improve oxidative phosphorylation (OXPHOS) capacity (9,10). Impairments in the formation of these supercomplexes can affect ATP supply, leading to increased risk for heart failure. Moreover, we have previously demonstrated that type 2 diabetes is associated with impaired mitochondrial supercomplex formation in skeletal muscle (9). Thus, the overall aim of this study was to assess atrial muscle mitochondrial energetics and supercomplexes in patients with AF in the presence and absence of type 2 diabetes. Here we show that atrial mitochondrial respiration and supercomplex assembly are impaired in patients with AF and type 2 diabetes.

Methods

Atrial appendage collection

Atrial appendages were collected from patients who had consented and were undergoing coronary artery bypass graft surgery. This study was approved by the Ottawa Hospital Research Ethics Board, and patients provided informed written consent. Tissue was collected, placed immediately in a respiratory buffer (0.5 mM ethylene glycol tetraacetic acid, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 110 mM D-sucrose, 0.1% bovine serum albumin and 60 mM lactobionic acid; pH 7.1) and analyzed less than 3 h after coronary artery bypass graft surgery.

High-resolution respirometry of permeabilized atrial myofibers

A small fraction (roughly 15 mg) of atrial tissue was used to prepare atrial fibres for high-resolution respirometry analyses of mitochondrial energetics. The remainder of the atrial appendage was frozen for storage at –80°C. Fibres were mechanically teased apart using fine forceps under a dissection microscope and were then permeabilized with 50 µg/mL of saponin. High-resolution respirometry was conducted at 37°C and in duplicate (0.5 mM ethylene glycol tetraacetic acid, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid, 110 mM D-sucrose, 0.1% bovine serum albumin and 60 mM lactobionic acid; pH 7.1) using an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Malate (2 mM), pyruvate (5 mM) and glutamate (10 mM) were added to the incubation medium, followed by the addition of adenosine diphosphate and Mg^{2+} (5 mM) to assess adenylate-free proton leak respiration and complex I-driven respiration, respectively. Succinate (10 mM) and adenosine diphosphate (5 mM) were then added to determine maximum oxidative phosphorylation capacity fueled by complex I and II energy substrates. Proton leak respiration was assessed again in the presence of oligomycin (2 µg/mL), which inhibits ATP synthase. The complex III inhibitor antimycin A (2.5 µM) was added to inhibit mitochondrial respiration. Finally N,N,N′,N′-tetramethyl-p-phenylenediamine (0.5 mM), ascorbate (2 mM) and sodium azide (15 mM) were subsequently added to assess complex IV activity. All values were corrected for residual nonmitochondrial oxygen consumption.

Blue native gel electrophoresis

Blue native gel electrophoresis was conducted as described previously (9,11). Briefly, atrial tissues were homogenized in sucrose buffer (250 mM sucrose, 20 mM imidazole/HCl, pH 7). The membrane fraction containing mitochondria was pelleted at 10,000×*g* for 10 min and resuspended in 50 mM imidiazole/HCl pH 7.0, 50 mM NaCl, 5 mM 6-aminohexanoic acid, 1 mM EDTA with 1% digitonin (experimentally determined; digitonin/tissue ratio [w/w] was 1:10) for 30 min. Then, samples were centrifuged for 30 min at 14,000×g; 5% glycerol and a 1:10 dye:digiton in a ratio of Coomassie blue G-250 were added to the proteins. Proteins were loaded onto 3% to 13% gradient gels. The gels were run in high Coomassie blue cathode buffer for 2 h at 150V and switched to low Coomassie blue cathode

buffer overnight at 200V. After transferring to a nitrocellulose membrane, the following proteins were probed: complex I (NDUFA9) (459100; Invitrogen, Carlsbad, California, United States); complex II (Fp) (459200; Invitrogen); complex III (UQCRC2) (Ab14745; MitoSciences [Abcam], Cambridge, United Kingdom); complex IV (subunit I) (459600; Invitrogen); and complex V (ATP5A) (Ab14748; MitoSciences). Bands were visualized using the Pierce ECL Western Blotting Substrate (32106; Thermo Fisher Scientific, Waltham, Massachusetts, United States) and quantified using the software ImageJ.

Western blot analysis

Atrial tissues were weighed and homogenized in 10 mM Tris HCl, 150 mM NaCl, 1 mM EDTA with 0.5% Triton-X100, pH 7.4, using homogenization bead tubes CK14 (Precellys, Bertin Technologies, Montigny-le-Bretonneux, France) and the MagNa Lyser instrument (Roche, Basel, Switzerland). Protein content was measured using a Bradford assay, and samples were stored at –80°C. Samples were prepared, subjected to reducing sodium dodecyl sulfate-gel electrophoresis (4% to 12%) and transferred to a nitrocellulose membrane. Membranes were blocked for 1 h at room temperature using either 5% milk or 5% bis(trimethylsilyl)acetamide in Tris-buffered saline containing 0.1% Tween-20. All primary antibodies were incubated overnight at 4°C followed by a 2-h incubation at room temperature with the appropriate horseradish peroxidase-conjugated secondary antibody. The following primary antibodies were used at the indicated dilutions: MitoProfile Total OXPHOS Human WB Antibody Cocktail (ab110411; Abcam) 1:3000 and COL1A antibody (sc-59772; Santa Cruz Biotechnology, Santa Cruz, California, United States) 1:1000. Blots were developed with the Pierce ECL Western Blotting Substrate (32106; Thermo) according to the manufacturer's protocols. Bands were quantified using ImageJ software.

OxyBlot analysis

To determine levels of oxidative stress, we measured protein carbonyls (OxyBlot, S7510; EMD Millipore, Burlington, Massachusetts, United States). Briefly, after homogenization, 15 µg of protein in each sample were derivatized, separated on 12% sodium dodecyl sulfate gels and transferred onto nitrocellulose membranes. After the transfer, membranes were blocked with 5% bis(trimethylsilyl)acetamide in phosphate buffered saline solution with Tween for 1 h at room temperature, then incubated with the provided primary rabbit anti-DNPH protein antibody (1:200, overnight at 4°C). Secondary goat antirabbit diluted in the blocking solution was used in a dilution of 1:5,000 for 2 h at room temperature. Blots were developed with the Pierce ECL Western Blotting Substrate (32106; Thermo). Bands were quantified using the software ImageJ.

Statistical analyses

All data are represented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 7 (GraphPad Prism, La Jolla, California, United States). Data were analyzed either by Student t tests or by 2-way repeated measures analysis of variance (ANOVA) with Tukey post hoc tests, as indicated; $p<0.05$ was considered significant.

Results

Patients' characteristics

Our study investigated patients with and without type 2 diabetes and with and without preoperative AF. The clinical characteristics of these patients are shown in Supplementary Table 1. There were no significant differences in age, weight, height or body mass index. Glycated hemoglobin percentages were higher in patients with diabetes than in those without diabetes, but were not different within the groups without and with diabetes.

AF impairs mitochondrial respiration in atrial myofibers

High-resolution respirometry of the atrial myofibers showed no differences in complex I (nonphosphorylating), maximal- or leak-dependent respiration, or in complex IV activity when we compared results between patients without AF with patients with AF (Figure 1A–D). On the other hand, when we compared results from patients with type 2 diabetes (D) and with and without AF (D^+AF^+ vs. D^+AF), we observed significantly lower complex I-driven phosphorylating respiration (Figure 1E). This impaired oxygen consumption was still observed when succinate was subsequently added to determine complex I- and II-driven phosphorylating respiration (Figure 1F). The latter respiration rate is also referred to as maximal phosphorylating respiration because these respiratory chain substrates in the presence of adenosine diphosphate support the highest rates of adenosine diphosphate-coupled respiration. There were no differences in leak (nonphosphorylating) respiration or in complex IV activity (Figure 1G and 1H).

Unchanged levels of mitochondrial OXPHOS proteins

In order to determine whether mitochondrial dysfunction is due to decreases in levels of OXPHOS proteins, we next used western blotting of atrial muscle homogenate to assess levels of proteins representing complexes I, II, III, IV and V. There were no changes in protein levels, whether bands were normalized to glyceraldehyde 3-phosphate dehydrogenase or vinculin (Figure 2A and 2B).

AF impairs mitochondrial supercomplex assembly

Recently, we reported that skeletal muscle from obese individuals with type 2 diabetes had dysfunctional mitochondrial bioenergetics compared to obese controls, without concomitant decreases in mitochondrial content (9). We were surprised to discover a significant decrease in electron transport chain (ETC) supercomplexes, which have been shown to affect respiration (12). Here, we similarly rationalized that decreased complexes I, II and IV activities in atrial appendages from D^+AF^+ vs. D^+AF^- could be affected by altered ETC supercomplex assembly. Because complex II does not participate in the formation of supercomplexes (13,14), we normalized our densitometry data to complex II monomer, as others have done (9,15). Results show significantly lower complex I- and complex IV-related supercomplex formation, with no changes in complex III supercomplexes or in complex IV or V monomers in D^+AF^+ vs. D^+AF^- (Figure 3A–E). Furthermore, in D^+AF^+ vs. D^+AF^-, there was a decrease in the high oligomeric supercomplexes. When these high oligomeric supercomplexes were normalized to complex II, and traditional supercomplexes were normalized to complex II, it was clear that the reductions in supercomplex formation were due to drastic reductions in these high-oligomeric supercomplexes in D^+AF^+ vs. D^+AF^- (Figure 3F and 3G).

AF tends to increase oxidative damage in the absence of fibrosis

Oxyblot determinations of protein carbonyl adducts in atrial tissue demonstrated a strong trend for increased protein oxidation in patients with D^+AF^+ vs. D^+AF^- (Figure 4B). In order to check for inflammation and fibrosis, we then measured collagen protein levels. There were no significant differences between groups (Figure 2C and 2D).

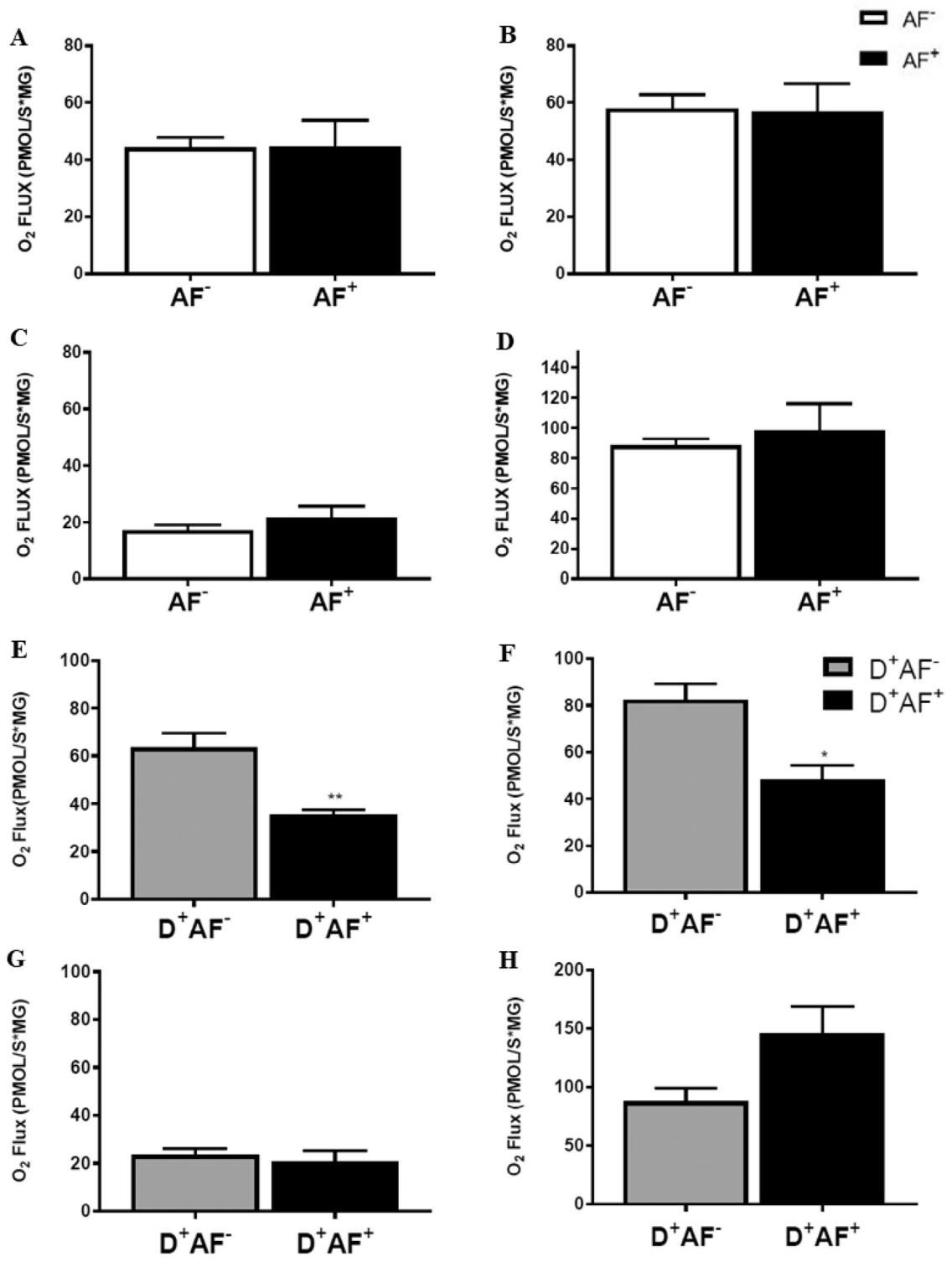

Figure 1. Atrial mitochondrial respiration analyses in adult patients. Oxygen consumption in atrial appendages was measured using high-resolution respirometry. In patients with and without AF, complex I respiration, complex I+II respiration, leak respiration and complex IV activity were determined (A through D, respectively). The same determinations were conducted in patients with type 2 diabetes, with and without AF (E through H, respectively). Data are represented as mean ± SEM. Unpaired Student t test: *p<0.05; **p<0.01. *AF,* atrial fibrillation; *D,* diabtees.

Discussion

Clinical risk factors for AF are well recognized (e.g. aging, obesity, type 2 diabetes), and recent research has elucidated aspects of AF-induced remodeling (5,16,17). However, our understanding of metabolic factors in the atrium remains limited (8). Indeed, atrial metabolism is not well studied, although some important advances have been made in recent years. In 2003, Lai et al showed that in older patients with AF there is an accumulation of mtDNA mutations in atrial tissue (18). Furthermore, there is an association between the fibrillating atrium and mitochondrial dysfunction and morphology (19–23). Thus, to understand the role of mitochondrial

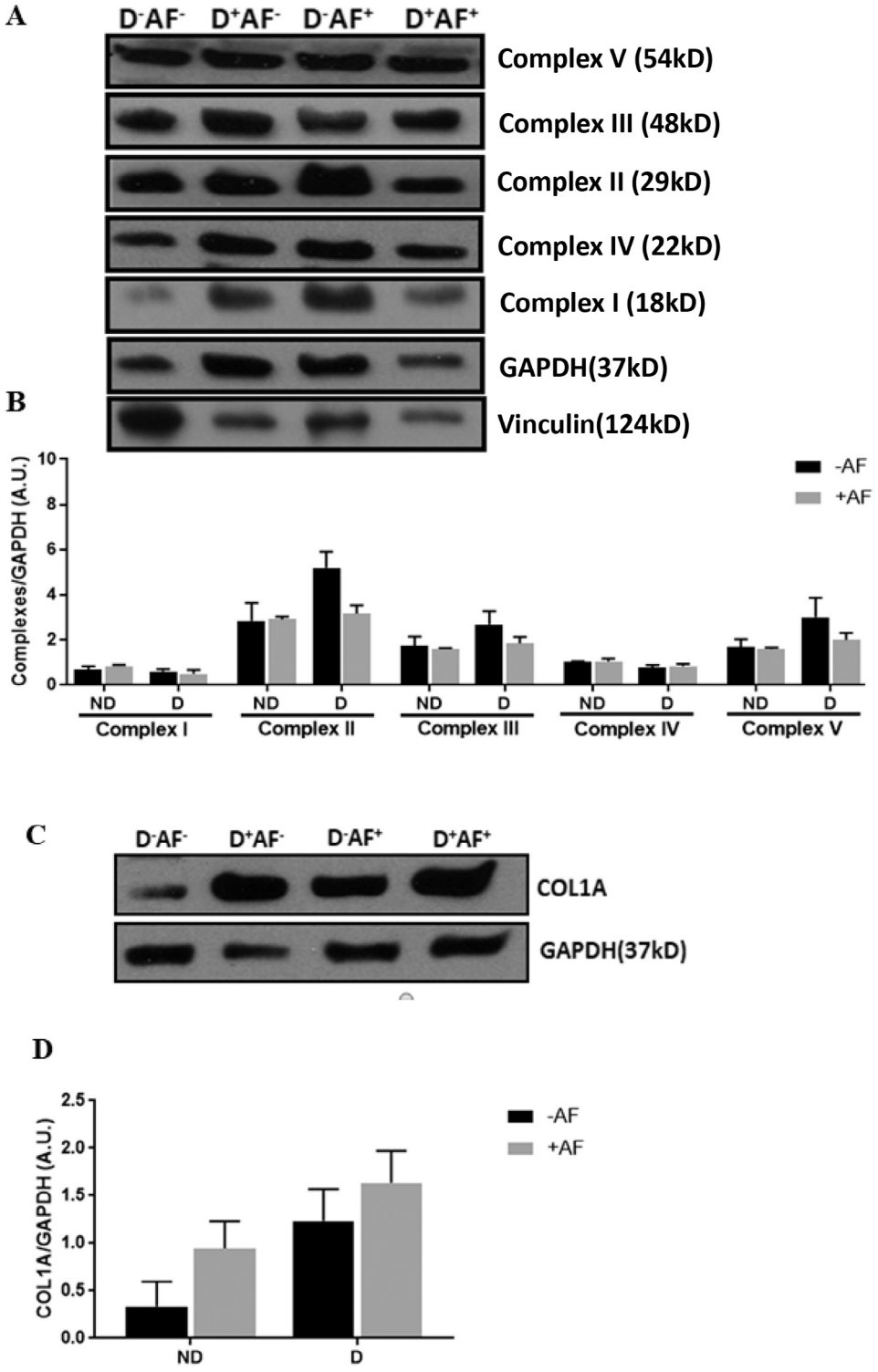

Figure 2. Mitochondrial content is not changed. Protein expression of mitochondrial OXPHOS complexes (I through V), normalized to GAPDH (A and B) and protein expression of collagen (C and D). Quantification was done by ImageJ, and data are represented as mean ± SEM. A 2-way ANOVA with Tukey post hoc test: *p<0.05. *AF,* atrial fibrillation; *COL1A,* antibody; *D,* diabetes; *GADPH,* glyceraldehyde 3-phosphate dehydrogenase; *ND*, no diabetes; *OXPHOS*, oxidative phosphorylation.

oxidative phosphorylation in AF, we examined characteristics of mitochondrial structure and function in human cardiac myofibers. Our study is the first to address such factors in human patients with type 2 diabetes and is the first, to our knowledge, to analyze mitochondrial bioenergetics or supercomplexes in the human atrium.

Mitochondrial OXPHOS is responsible for the production of more than 90% of myocardial cell ATP production (24). Myocardial ATP demand is constant due to its vital role in maintaining cardiac output (25), and such demand is met largely, in the healthy heart, through fatty acid oxidation (60% to 90%) and, to a lesser extent, through glycolysis (10% to 40%) (26). In patients with type 2 diabetes, the heart relies on lipid oxidation more than on glucose oxidation, even during hyperglycemia (27,28). The implications of sustained high-frequency activation on cardiac tissue, as occurs in AF, are unknown.

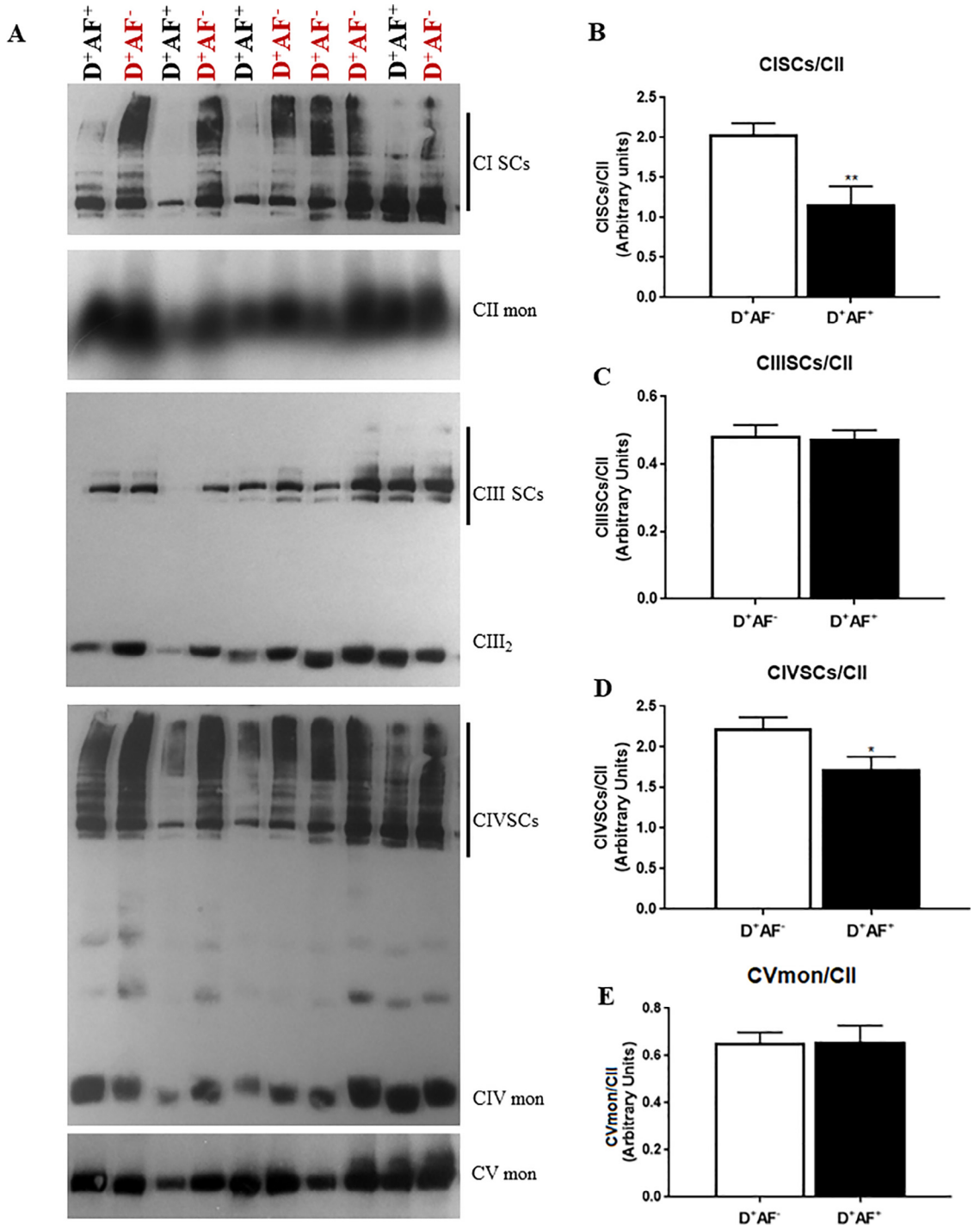

Figure 3. Supercomplex assembly is altered in patients with type 2 diabetes and AF. A, Representative blue native gel electrophoresis blot of the indicated respiratory complexes (C I through V), supercomplexes (SCs) and monomers (mon) using anti-NDUFA9 (complex I), antiflavoprotein (complex II), antiUQCRC2 (complex III), anticomplex IV and antiATP5a (complex V) antibodies. B through E, Quantification of expression of the indicated respiratory supercomplex normalized to complex II monomer levels. Higher order oligomeric C I- and C IV-containing ETC supercomplexes were separated from traditional ETC supercomplexes and quantified (F through K). Quantification was done by ImageJ, and data are represented as mean ± SEM. Unpaired Student t test: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. *AF*, atrial fibrillation; *ETC*, electron transport chain.

Thus, the application of novel approaches to the study of mitochondrial structure and function is important for improved understanding of atrial function. We used high-resolution respirometry of atrial muscle fibers, which were studied immediately (<3 h) after coronary artery bypass graft surgery. Results from these experiments showed impaired complex I and maximal (I+II) phosphorylating respiration in patients with diabetes and AF compared to patients with diabetes but without AF. This was associated with an increase in maximal complex IV activity. In a recent study, homogenates of atrial tissues from patients with and without AF (without diabetes) were studied, and enzyme activities of the complexes were determined spectrophotometrically. Lower enzymatic activities were recorded for complex I and complex II, but an increase in complex V (ATP synthase) activity was observed,

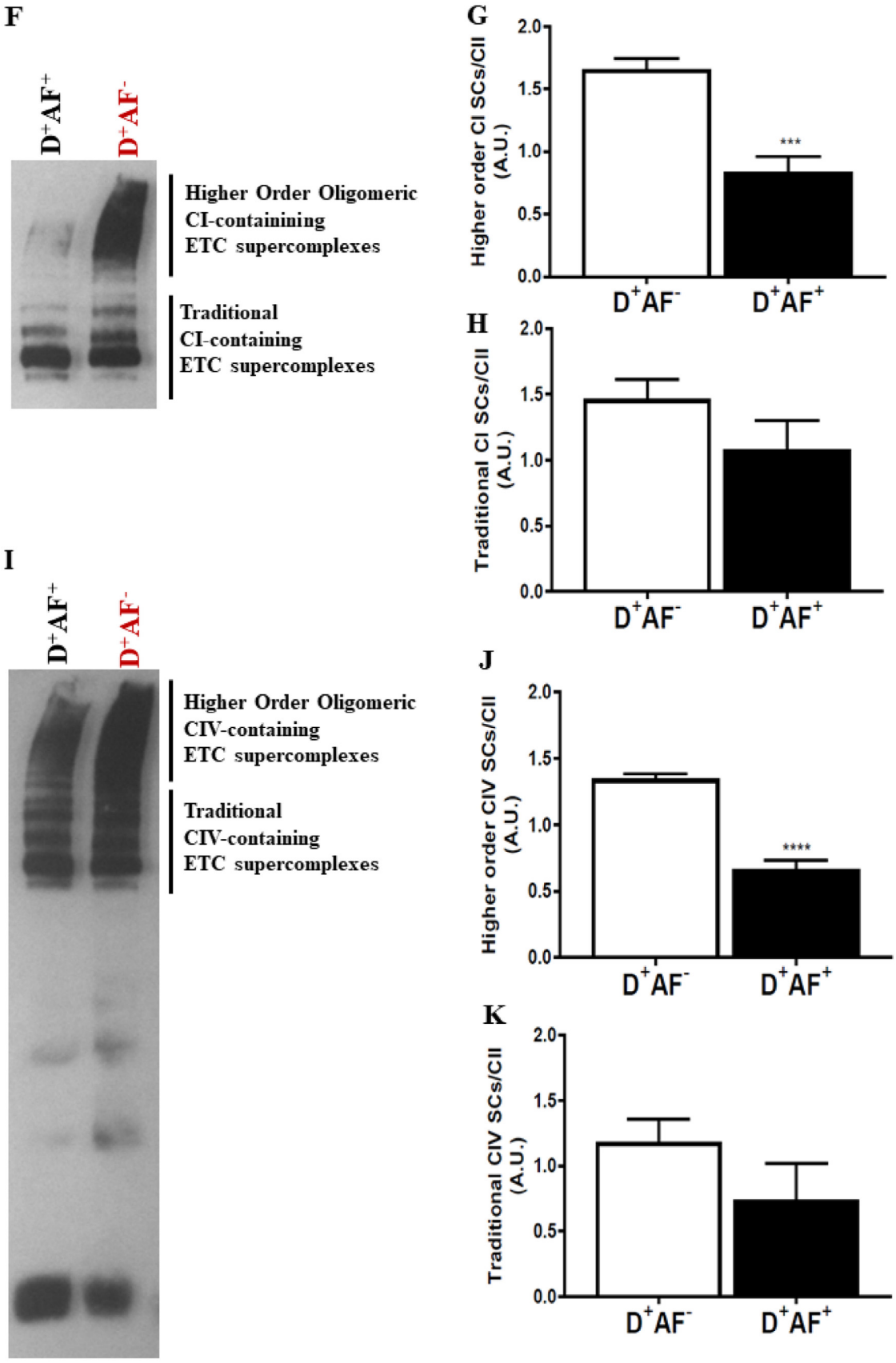

Fig. 3. (*continued*)

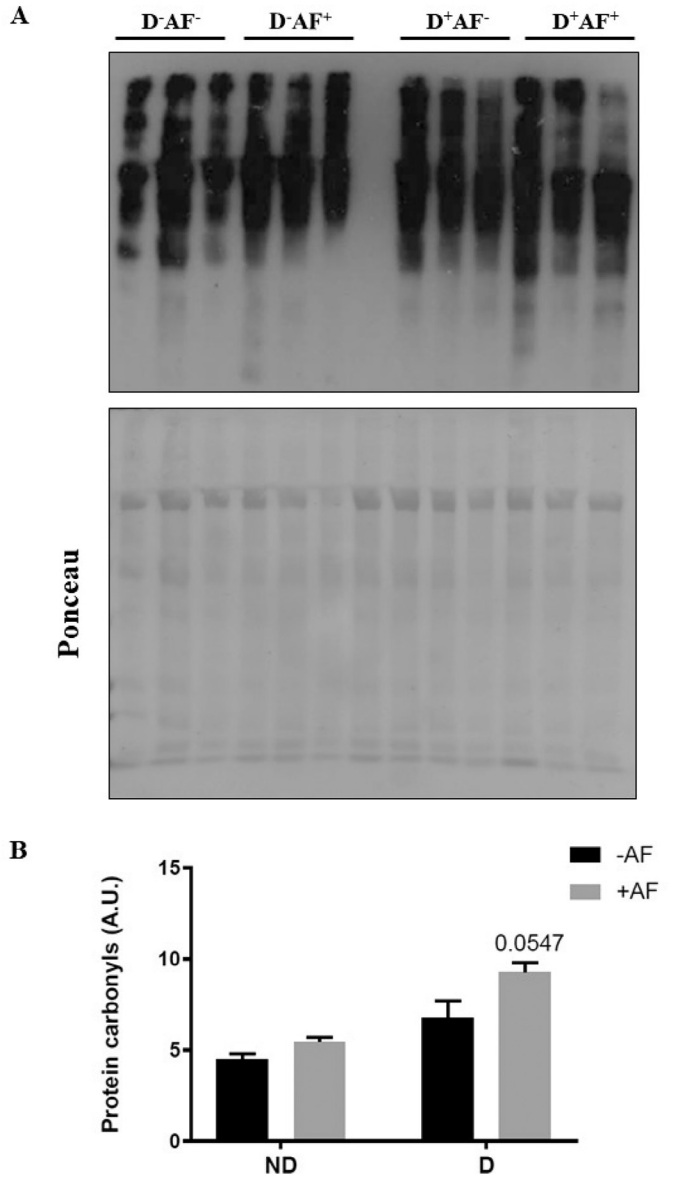

Figure 4. Protein carbonyls in atrial tissue of patients with type 2 diabetes and AF. A, Representative blot of an Oxyblot and Ponceau stain. B, Quantification was done by ImageJ, and data are represented as mean ± SEM. A 2-way ANOVA with Tukey post hoc test: *p<0.05. *AF,* atrial fibrillation; *D,* diabetes.

consistent with the possibility of impaired mitochondrial respiration in patients with AF (29). However, these determinations were made in homogenates of frozen tissues by using enzymatic assays. Our study addressed these questions by measuring the OXPHOS system in fresh atrial tissue. Using this approach, mitochondria remain in their reticular structures in situ in the muscle fibres, and the activities of the complexes are assessed in the intact OXPHOS system by examining the coordinated activities of electron flow and proton efflux.

The idea that OXPHOS complexes can bind together to form supercomplexes was first proposed by Chance and Williams more than 60 years ago (30), but our understanding of these structures remains very limited. It was only recently discovered that supercomplex assembly factors can affect mitochondrial respiration and production of reactive oxygen species (12,31). Here, we investigated supercomplex formation and showed impaired supercomplex assembly in patients with AF. Supercomplexes can be divided into 4 main groups according to their assembly of ETC complexes: $I+III_2$ (abundant in plants); III_2+IV_{1-2} (abundant in fungi); and $I+III_2+IV_{1-4}$ (abundant in mammals) in addition to complex V dimer formation (14) (Supplementary Figure 1A–D).

In addition to traditional $I+III_2+IV_{1-4}$ supercomplexes, we found much larger higher-order supercomplexes than had previously been documented for other human tissues (9,10) (Supplementary Figure 1E). These high-order supercomplexes are consistent with the respiratory string theory (32), which contends that supercomplex formation is not the highest level of OXPHOS organization. The string form is likely to be due to complex IV interaction with a neighbouring complex IV, aligning 2 traditional $I+III_2+IV_4$ supercomplexes side by side (32), although little is known about the species and tissue specificity of respiratory strings. It has been hypothesized that strings of complexes enhance electron flow as a result of reduced distances between the complexes and reduced production of reactive oxygen species, especially at complexes I and III (33,34). Further experimentation is required to clarify why higher-order respiratory strings were detectable when immunoblotting for complex I and complex IV but not for complex III antibodies. Speculatively, the complex III epitope could be masked in the higher-order string confirmation and is, thus, not detectable. Nevertheless, our findings are consistent with the notion that the reduction in respiratory strings in the atrial myocardium of patients with AF could impair electron flow and cause an increase in the production of reactive oxygen species, which could, in turn, result in oxidative damage. There were no changes in the levels of atrial collagen, so it appears that the mitochondrial dysfunction and atrial protein oxidation were not associated with concomitant fibrosis.

Conclusions

In summary, our findings show that AF is associated with impaired mitochondrial OXPHOS, decreased supercomplex assembly into respiratory strings, and increased protein oxidative damage in atrial myocardial tissue of patients with type 2 diabetes. Despite our small sample size, our findings represent important proof-of-concept discoveries. This is the first study, to our knowledge, that has examined mitochondrial energetics in intact atrial myofibers in patients with AF. Future research should address the factors underlying the impaired mitochondrial energetics and supercomplex formation.

Supplementary Material

To access the supplementary material accompanying this article, visit the online version of *Canadian Journal of Diabetes* at https://www.canadianjournalofdiabetes.com.

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Author Disclosures

Conflicts of interest: None.

Author Contributions

GNK, CJR and MEH conceived the idea for the project; GNK conducted most of the experiments and analyzed most of the results; DAP conducted and analyzed blue native gel electrophoresis determinations; GNK, CJR and MEH wrote the paper; all authors reviewed and approved the article.

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Supplementary Material

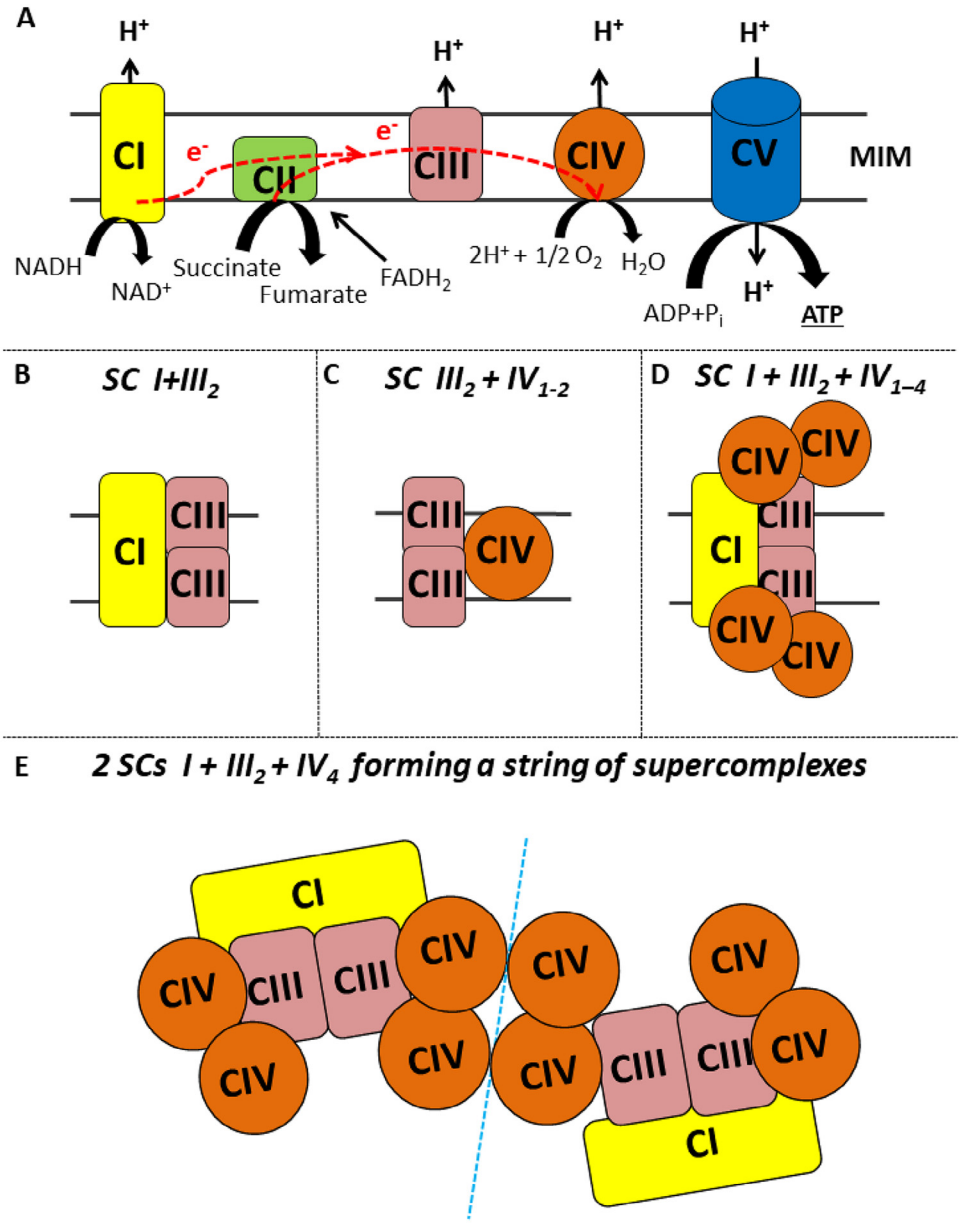

Figure S1. Various known forms of mitochondrial supercomplexes. A, Representation of the complexes of the electron transport chain, which traditionally were thought to be distinct but fluidly interacting in the inner membrane. B through D, Different types of supercomplexes shown to exist in mammals: B, Complex I and III2 supercomplex; C, Complex III2 and IV supercomplex; D, Complex I, III2 and IV1 through 4 supercomplex, also called a respirasome. E, Formation of a string of supercomplexes by the linkage of I+III2+IV1 through 4 supercomplexes.

Table S1
Patients' characteristics

	Patient characteristics				p value
	No diabetes, no AF	No diabetes, AF	Diabetes, no AF	Diabetes, AF	
N	12	2	16	4	
Age (y)	69	76	65	68	0.6471
Weight (kg)	79.33	83.50	84.44	111.25	0.0849
Height (cm)	168.00	169.00	172.56	171.50	0.6541
BMI (kg/m^2)	28.17	29.16	28.42	36.81	0.1191
Glycated hemoglobin (%)	5.55	5.40	7.97	6.85	**0.001**

Notes. Age, weight, height, body mass index (BMI) and glycated hemoglobin levels of people with or without type 2 diabetes and with or without preoperative atrial fibrillation (AF). Data are presented as mean, 1-way ANOVA. Bold text represents statistical significance.