Impaired dynamics and function of mitochondria caused by mtDNA toxicity leads to heart failure

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mtDNA damage exclusively in cardiomyocytes results in fatal heart failure in 8 wk.

CARDIOVASCULAR DISEASE is an increasing health concern worldwide and a leading cause of morbidity and mortality in industrialized countries (40). The incidence and prevalence of heart failure, the clinical syndrome and common terminal end-point of cardiovascular disease of diverse etiologies, are also rising due to altered demographics, with a higher proportion of elderly, as well as implementation of new treatment modalities during recent years that delay onset of heart failure. Even though persistent exposure to cardiovascular risk factors increases the incidence of cardiovascular disease with age, cardiovascular senescence itself may also be an important factor precipitating the onset of heart failure. In this perspective, mitochondrial dysfunction of the aging heart may contribute in the pathophysiological mechanisms of heart failure (36).

The heart is the organ with the greatest oxygen consumption in the body (47) and consumes 100 times its weight in ATP, constituting 30 kg/day, to fuel ~100,000 contractions each day (1). Thus the heart cannot rely on stored reserves, but must constantly generate new ATP, and this is mostly done by heart mitochondria, which comprise ~35% of the cardiomyocyte volume in a healthy heart. The mitochondrial ATP production is firmly and effectively regulated to meet varying demands resulting from minute-by-minute changes in cardiac work (19), and mitochondria use Ca2+ as a key regulator for controlling their metabolic activities (37). This is illustrated by the strategic mitochondrial positioning, where mitochondria in association with sarcoplasmic reticulum (SR) are able to contribute to balancing localized cellular Ca2+ homeostasis and ATP generation during excitation-contraction coupling, thereby connecting energy metabolism and myocardial contraction (19). Mitochondria are also an endogenous source of reactive oxygen species (ROS), and increased generation of ROS may directly harm proteins involved in contractile function of the heart or in mitochondrial function (53). Paradoxically, mitochondria, the major producers of ROS in the heart, are extra prone to oxidative stress-induced damage because they contain the source of ROS generation (the electron transport chain). Furthermore, the intronless mitochondrial DNA

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(mtDNA) is not protected by histones like nuclear DNA, increasing the susceptibility to oxidative damage and to introduction of deleterious mutations (21). Dysfunctional mitochondria are not only bioenergetically less efficient but may also trigger cardiac myocyte apoptosis, myocardial dysfunction, and heart failure (5).

The importance of mitochondrial homeostasis in cardiac physiology is demonstrated by the fact that primary mitochondrial diseases often present as syndromes that include deranged cardiac function (for review, see Ref. 38). Interestingly, high levels of oxidative damage to mtDNA with acquired mutations and deletions have been detected in the aging heart (25). However, it is still not known how such instability of mtDNA affects mitochondrial dynamics and function. The topic is difficult to address, since alterations of mtDNA are often associated with other pathological states that separately may influence the mitochondrial homeostasis. To overcome this difficulty, we have generated transgenic mice that enable us to inflict damage to mtDNA in the form of introduction of apyrimidinic sites in vivo through inducible expression of a mutated version of the mitochondrial uracil-DNA glycosylase 1 (mutUNG1) specifically in cardiomycocytes. UNG (EC 3.2.2.27, HGNC 12572) is a central part of the DNA-repair machinery as it initiates the DNA base-excision repair pathway through cleaving the γ-glycosidic bond between uracil and deoxyribose, preventing mutagenesis by removing misincorporated uracil from DNA. The isoform UNG1 is targeted specifically to mitochondria. An amino acid substitution mutation (Y147A) in the catalytic domain of UNG1 changes the substrate properties of this mtDNA-specific enzyme, causing it to remove naturally occurring thymine in addition to uracil from mitochondria (mtDNA) (23). Removing bases from DNA in this manner leaves excess apyrimidinic (AP) sites, which are highly cytotoxic (13, 31). By placing the Tet-on system under control of the heart-specific α-myosin heavy chain (αMHC) promoter, the lesions are induced specifically in the cardiomycocytes (54). Here, we present evidence that accumulating instability of mtDNA in the heart leads to severe heart failure and death via mitochondrial dysfunction, entailing impaired mitochondrial respiration, signatures of increased load of ROS with elevated antioxidant defenses in the heart, increased mitochondrial mass but perturbed mitochondrial dynamics, and ultimately reduced myocardial contractility.

MATERIALS AND METHODS

Transgenic constructs and mice. Transgenic mice with cardiac-specific expression (due to the α-MHC promoter) of mutant uracil-DNA glycosylase 1 (mutUNG1) were developed in this study. The previously described (28) mutUNG1 founder mice with reporter luciferase (mouse I; Fig. 1A) were bred with transgenic mice carrying the rtTA Tet-on system (mouse II; Fig. 1A), under the control of the cardiac muscle-specific α-MHC promoter (54), obtained from the Mutant Mouse Regional Resource Center (MMRRC); strain name: FVB/N-Tg(Myh6-rTta)8585Jam/Mmmh; stock no., 010478-MU. Of the offspring, mutUNG1-luciferase/α-MHC-rtTA (25%) mice were used for experiments with wild-type/wild-type littermates (25%) as controls, whereas mutUNG1-luciferase/wild-type (25%) and α-MHC-rtTA/wild-type (25%) mice were used for the next generation breeding. The mice used for the described experiments were of the fifth or higher generations of cross-breeding. Expression of mutUNG1 was induced by adding doxycycline to the Chow at 6 mg/g (in wet food, corresponding to ~3.5 mg doxycycline·g body wt·day⁻¹) (39). All mice were used at 8 wk after start of doxycycline, except for luciferase (Fig. 1C; 4 wk), and echocardiography (see Fig. 4; 7.5 wk).

In agreement with our previous studies on mutUNG1 expression in brain (26-28), we found that α-MHC-rtTA mice have no phenotype, and neither have wild-type mice fed doxycycline. Importantly, when not induced by doxycycline, mutUNG1 mice (α-MHC-rtTA × Luciferase-TetOn-mutUNG1 mice) showed no phenotype. We therefore used wild-type mice of the same litter as controls.

All experimental procedures were approved by the Section for Comparative Medicine at the University Hospital of Oslo and by the Norwegian Animal Research Authority, and complied with national laws and institutional regulations governing the use of animals in research.

Immunohistochemistry. Whole hearts were immersion fixed in 10% neutral-buffered formalin (Richard-Allan Scientific), paraffin embedded, and cut into 4-μm-thick sections using a rotary microtome (Microm 355 S). The procedure for fluorescence immunohistochemistry was performed as described (26), with the exception of UNG1 + complex II (70-kDa Fp subunit) double labeling and WGA, for which pictures were taken using AxiocamHR camera and Zeiss LSM 510 Meta confocal laser scanning microscope. To image UNG1 + complex II, ×40 and ×63 (oil immersion) objectives were used. For images used to calculate the size of cardiomycocytes (data not shown) and their nuclei at 8 wk of induction, ×20 objective was used. The Alexa Fluor 594 WGA labeled heart sections from wild-type (same age as mice with 4- to 8-wk induction of mutUNG1) and 2-, 4-, 6-, and 8-wk induction of mutUNG1 expression were imaged using a Leica DFC450 microscope, ×10 objective, LAS version 4.5 program. Also, in the case of UNG1 + complex II double staining, the protocol was slightly different in the points mentioned; the antigen retrieval was performed in a PT-link at 97°C for 20 min, the secondary antibodies to rabbit anti-UNG1 or mouse anti-complex II, 70-kDa Fp subunit were Alexa goat anti rabbit 568 (Invitrogen, All 029), and Alexa goat anti mouse 488 (Invitrogen, All 029), respectively. The following antibodies were used: anti-UNG1 (ProSci, dilution 1:1,000), anti-Sod2 (AbCam, ab13533; dilution, 1:1,000), anti-complex II, 70-kDa Fp subunit (Invitrogen, 459200; dilution, 1:1,000), and anti-Pgc-1α (AbCam, ab54481; dilution 1:1,000). Lectin was used for membrane labeling of heart cells, 5 μg/ml anti-wheat germ agglutinin (WGA) conjugated to Alexa fluor 594 (Life Technologies). There were 4 animals in each group, except for the group of 2 mice expressing mutUNG1 for 4 wk, and the wild-type group with 10 animals. Cross-sectional areas of cardiomyocytes (data not shown) and cardiomycocyte nuclei from mice with 8 wk of mutUNG1 expression were measured manually in Image J (http://imagej.nih.gov/ij/). The sizes of WGA-594-labeled cardiomycocytes from mice expressing mutUNG1 for 2, 4, 6, and 8 wk and from wild-types were measured in images from transverse sections of the left-ventricular wall. An automated color thresholding algorithm, Percentile, and an automatic particle analysis (with input range of size and circularity) was included in the macro used to measure cell size in the ImageJ software. Further data analysis of cell size was performed in Microsoft Excel. The mean individual cardiomycocyte transsectional area from mice for the different mutUNG1 expression times were presented relative to the mean of wild-type values (set as 1). There were in total 5–14 images from 2–3 sections per animal, and 2–4 animals per age-matched group.

Fibrosis and apoptosis analysis. Paraffin sections (4 μm) from wild-type and mutUNG1-expressing mouse hearts were stained with Masson’s trichrome (Sigma, HT15-1KT), according to the kit protocol, including refixation of the formalin fixed tissue in Bouin’s solution (Sigma, HT10-1), and finally mounted with Eukitt, a resinous mounting medium. A Pascal confocal microscope was used to image the Trichrome-stained sections, looking for blue-stained areas indicative of fibrosis/collagen (n = 3 wild-type and n = 4 mutUNG1-expressing trichrome-stained heart sections).
Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL) staining was performed on paraffin-embedded tissue to analyze for apoptosis. In brief, 4-H9262 heart sections were deparaffinized and rehydrated, and then permeabilized, either with proteinase K (20 g/ml in PBS) or by high-power microwave irradiation for 1 min in preheated 0.1 M citrate buffer, pH 6.0. An in situ apoptotic cell death detection kit (in situ cell death detection kit, TMR red; Roche Applied Biosystems) was used to detect myocardium apoptotic nuclei. Nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI; 1 g/ml in PBS) staining. Heart sections were thereafter mounted with Mowiol 4-88 mounting medium. Sections were observed under AxioCamHR camera and Zeiss LSM 510 Meta confocal microscope (several heart regions from n/4 animals/group).

Electron microscopy. For electron microscopy, small blocks from the heart apex of wild-type and mutUNG1 mice, perfusion-fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in phosphate buffer, were embedded in plastic, as described (6). Ultrathin sections (70–100 nm) were cut with a Reichert ultramicrotome, mounted on nickel mesh grids, and processed for immunogold cytochemistry as described previously (6). There were three animals in each group.

Luciferase measurements. Doxycycline was administered as previously described (28). Transgenic founders were selected by investigating luciferase expression in hearts and other organs utilizing in vivo and ex vivo imaging. In vivo imaging was performed by administering D-luciferin intraperitoneally (120–150 mg/kg) to mice anesthetized with 2.5% isoflurane using 1 min as exposure time. Bioluminescence was recorded by the IVIS100 imaging system (Caliper/Perkin Elmer). Images were analyzed by Living Image software 2.5 (Xenogen/Caliper). Immediately after in vivo imaging, mice were killed and organs were dissected out to perform ex vivo imaging.

Respiration and citrate synthase assay. Mitochondrial complex II-driven respiratory capacity and citrate synthase activity were measured in heart homogenates from transgenic and wild-type mice. Respiratory capacity was analyzed by high-resolution respirometry.

Fig. 1. Verification of the α-myosin heavy chain (α-MHC)-inducible mutUNG1 mouse model. A: working model of the transgenic mouse. B: genotyping reveals the presence of mutUNG1, luciferase, and rtTA. C: luciferase activity of heart in an induced mutUNG1 mouse measured with a CCD camera, in vivo and ex vivo (B, brain; I, intestine; L, liver; S, spleen; K, kidney; Lu, lung; H, heart; M, muscle). D: survival of wild-type (black) and mutUNG1-expressing mice (red); 20 mice in each group. E: quantitative rt-PCR proves expression of mutUNG1 transcript in the induced mutUNG1 mice, whereas in wild-type controls no specific rt-PCR product is formed. Values are means ± SD (n = 3 mice). *Significant difference (P < 0.05). F: Western blot analysis showing UNG1 expression in heart whole-cell extracts from wild-type and mutUNG1-expressing mice. Actb was used as a loading control. G: quantification of an ~31-kDa protein band, assumed to be mitochondrial UNG1, detected by anti-UNG1. Values are means ± SD (n = 3 mice). *Significant difference (P < 0.05).
T-3 -GAC TCC TAC CAC CAT CAT TTC TCC

GTT CAA TGG TGG GG-

-GCT TGA TAG CCT CCA

GAT GGT TTG GGA GAT TGG TTG ATG-3

Biosystems). The following primers were used: Sod2: 5'-GCC TCA CAA TCA ACT TAT CCC-3' and 5'-GGGG TTG GCA GGT GGC TTT TT-3; Ung: 5'-CCA CCA CAA GTA GGT CTA TCC G-3' and 5'-GCC CAT GCC GGC TTT TTT TT-3; OGG1: 5'-ATG AGG ACC AAG-3' and 5'-GCC TCA CAA TCA ACT TAT CCC-3; OPA1: 5'-TCC TGG TGA AGA GCT-3' and 5'-GAT GGT TTG GCA GGT GAA CTG GTC TTT GG-5'; Drp1: 5'-CCA TGA CAC CAC CGA-3' and 5'-CCAATTTTGCAACC-3; Mfn2: 5'-TCA ATG-3' and 5'-GCC TCA CAA TCA ACT TAT CCC-3; Cat: 5'-GCC TCA CAC CAT CAT CTA AGT-3' and 5'-GCC TCC GAC GGA

values of <0.99 were rejected. There were three animals in each group. The results were expressed relative to the mean of wild-type values (set as 1, except for in Fig. 1E, where the mutUNG1 gene is not expressed in wild-type and the mean of relative gene expression from mutUNG1-expressing mice is set to 1).

Protein. Dissected heart tissue (50–100 mg, snap frozen in liquid nitrogen and stored at -80°C until use) from wild-type and mutUNG1-expressing mice, was homogenized in 0.8 ml of modified RIPA lysis buffer [20 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP-40/0.5% deoxy 630, 0.1% SDS, 0.5 mM PMSF and 1× complete protease inhibitor cocktail (Roche) using Lysing Matrix D on the Fastprep-24 Instrument (MP Biomedical)]. The homogenates were then subjected to total protein quantification using the BCA protein assay (Pierce, Thermo Fisher Scientific). The protein lysates were used for Western blots, OxyBlot, and measurement of free thiol.

Western blotting. Total protein (25 μg) was subjected to SDS PAGE electrophoresis (12.5% Criterion gel) and Western blotting using the Criterion gel and blotting system (Bio-Rad Laboratories). After incubation of the nitrocellulose membranes with the primary antibody and secondary antibody (GE Healthcare anti-rabbit IgG from donkey, HRP linked, NA934V, 1:20,000), the immunoreactive bands were detected using the chemiluminescence kit ECL Super-Signal (Thermo Scientific) with a Fujiﬁlm LAS3000 imager (Fujiﬁlm). From the following primary antibodies were used: anti-UNG1 (Pro Sci, catalog no. 3863; dilution 1:1,000); anti-Pgc-1α (Abcam, ab54481; dilution 1:1,000) and anti-VDAC1/Prinomitocondrial Loading Control (Abcam, ab15895; dilution 1:1,000). Restore TM stripping buffer (Thermo Scientiﬁc) was used followed by PBS washes to remove bound antibodies before incubation with the HRP-conjugated anti-Acb [Abcam, Anti-Beta Actin antibody (mAbcam 8226) Loading Control (HRP), ab20272; dilution 1:5,000]. Image J software was used for protein quantiﬁcation.

OxyBlot, protein carbonyl ﬂuorometric assay, and measurement of free thiolis. OxyBlots were performed according to the manufacturer’s instructions with 20 μg of proteins (OxyBlot Protein Oxidation Detection Kit, Millipore). Brieﬂy, 40 μg of protein lysate from each sample were reduced by adding DTT to 50 mM, denatured in SDS, at a ﬁnal concentration of 6%, and then the sample was split into two tubes, 20 μg of protein in each. 2,4-Dinitrophenylhydrazine (DNPH) was added (one tube to derivatize the carbonyl groups, the other served as a control). Derivatization solution was added to the other tube of each sample as a negative control. Following derivatization and neutralization, the proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane (0.45 μm), blocked with 1% BSA in PBS-T for 1 h, incubated with primary antibody rabbit anti-DNP at 1:150 dilution in blocking buffer for 1–2 h at room temperature, washed in PBS-T, incubated with goat anti-rabbit IgG HRP-labeled secondary antibody at 1:300 in blocking buffer, and washed in PBS-T before ECL (Super Signal West Dura Extended Duration Substrate, Thermo Scientiﬁc) was added and the blot was scanned in a LAS 3000 Image Analyzer to visualize the oxidized protein carbonyl groups of the samples.

For the OxiSelect Protein Carboxyl Fluorometric Assay (Cell Biosabs, 90–145 μg of protein lysates from three wild-type and three mutUNG1-expressing mice were used and treated according to the protocol manual of the kit. Dissected heart tissue (50–100 mg, snap frozen in liquid nitrogen and stored at -80°C until use) from wild-type and mutUNG1-expressing mice was homogenized in 1 ml of 1× sample diluent from the kit, using a 2-ml Dounce homogenizer (Kontes glass, Vineland, NJ). The homogenate was centrifuged at 10,000 g for 5 min at 4°C, and the supernatant was kept for Bio-Rad protein concentration measurement on a biophotometer before downstream applications. Briefly, protein carboxyls in the protein samples were first derivatized with the protein carbonyl fluorophore. Proteins were then TCA precipitated, and free fluorophore was removed by washing the protein pellet with acetone. The protein pellet was dissolved in guanidine hydrochloride, the absorbance of protein-fluorophore product was measured fluorometrically with a 485/
535-nm filter set in a Wallac plate reader, and the protein carbonyl content was calculated in Excel.

Free thiols/sulfhydryls in 95 μg of heart protein lysates (homogenates with modified RIPA or sample diluent, as described elsewhere for use in Western blot and carbonyl assay) from six wild-type and six mutUNG1-expressing mice were quantified using 5,5'-dithio-bis (2-nitrobenzoic acid), DTNB/Ellman's reagent (0.1 mM in 0.1 M Tris, pH 8) in a colorometric assay, and absorbance in samples and standard containing
serial dilution of DTT was measured at 412 nm in an Ultrospec 2000 spectrophotometer, and results were calculated in Excel.

Statistical analysis. All quantitative data are represented as means ± SD or SE, as indicated in the figure legends. When relevant, the results are expressed in relative units, setting the average in wild-type mice = 1. Unpaired, two-tailed \( t \)-tests were performed. The null hypothesis was rejected at the \( P < 0.05 \) level (asterisks in figures).

RESULTS

Characterization of the αMHC-inducible mutUNG1 mouse model. To investigate the impact of mtDNA damage on myocardial structure and function in adult mice, we chose a similar strategy as reported previously for forebrain neurons (26–28). The Tet-on system in combination with the αMHC-promoter was used to restrict mutUNG1 expression to cardiomyocytes in adult mice (2, 14, 54). This was achieved by breeding mice carrying a bi-directional Tet-on responsive promoter, which controls mutUNG1 in addition to luciferase (mouse II in Fig. 1A) (26–28) with mice carrying a Tet-on transactivator [reverse tetracycline transactivator (rtTA)] controlled by the cardiomyocyte-specific αMHC-promoter (mouse I in Fig. 1A) (54). The activity of the mutUNG1 enzyme has already been thoroughly investigated and confirmed in vivo and has been shown not to

Fig. 2. Hypertrophic hearts of mutUNG1-expressing mice after induction. A: dissected hearts of wild-type (left) and induced mutUNG1-expressing (right) mice. Scale bar = 2 mm. B: mean heart weight/body weight of wild-type (black) and induced mutUNG1 (gray) mice. Values are means ± SE (n = 6 mice). C: size of cardiomyocytes from the free left ventricle wall of induced (for the time indicated) mutUNG1 mice (n = 2–4) relative to wild-type mice (n = 10). Mean wild-type cardiomyocyte size is set to 1. Values are means ± SD. D: representative images of cardiomyocyte nuclear size in paraffin sections in left ventricle from wild-type (left) and mutUNG1 (right) mouse heart stained Alexa fluor 594-conjugated WGA (red) and DAPI (blue). Representative cardiomyocyte nuclei are encircled for each genotype. Scale bar = 25 μm. E: quantification of the average cardiomyocyte nuclear size. Values are means ± SE (n = 4 mice). *Significant difference (\( P < 0.05 \)). F: representative immunofluorescence image after double-labeling with anti-UNG1 and anti-Complex II in heart paraffin sections from wild-type and mutUNG1-expressing mice (n = 3 in each group). Nuclei are encircled, and nuclear UNG1-expression is indicated by arrow heads; mitochondrial UNG1-expression is indicated by arrows. Scale bar = 10 μm. G: quantitative rt-PCR shows expression of mouse Ung1 transcript in wild-type and induced mutUNG1 mice. Values are means ± SD (n = 3 mice). *Significant difference (\( P < 0.05 \)). **Significant difference (\( P < 0.01 \)).
damage nuclear DNA (28). The Tet-on system, and transgene expression, was induced by adding doxycycline in the chow when the mice were 8 wk of age. The presence of the transgenes rtTA, mutUNG1, and luciferase in the hybrid mouse genome was verified by genotyping (Fig. 1B). To test the inducible Tet-on system and the specificity of transgene expression in the mice, luciferase activity was measured using a charge-coupled device (CCD) camera and showed high and specific activity in the mouse heart both in vivo and ex vivo (Fig. 1C). A first sign of illness observed in mutUNG1-expressing mice was a rapid weight loss, which was typically followed by death. Close to 100% mortality was observed ~2

**Fig. 4. Echocardiographic evaluation of left ventricular dimensions and function after mutUNG1 induction.** A and B: M-mode of left ventricle in wild-type (A) and induced mutUNG1 (B) mice demonstrates increased left ventricular wall thickness and smaller contractions in induced mutUNG1 mice. C and D: raw data of left ventricular tissue velocities determined by tissue Doppler-ultrasound in wild-type (C) and induced mutUNG1 (D) mice demonstrates reduced peak systolic and peak early diastolic tissue velocities, reflecting heart failure in induced mutUNG1. E: increased left ventricular end-diastolic wall thickness of mutUNG1 mice indicates cardiac hypertrophy. F: there is unaltered left ventricular end-diastolic diameter among all mice investigated. G: left ventricular fractional shortening in induced mutUNG1 mice compared with wild-type mice, demonstrating reduced cardiac contractility of mutUNG1 expressing mice. H: peak left ventricular contraction velocities determined from tissue Doppler recording illustrated in C and D demonstrates reduced contractility in mutUNG1expressing mice. I: reduced peak diastolic velocities of the left ventricle (determined from tissue Doppler ultrasound recording), demonstrating slower left ventricular relaxation in mutUNG1-expressing mice. J: hearts of mutUNG1 mice have increased left atrial diameter, demonstrating congestive heart failure. All quantitative data: means ± SE (n = 6 mice). *Significant difference (P < 0.05).
mo after induction of expression of mutUNG1 (Fig. 1D). In agreement with our previous studies on mutUNG1 expression in brain (26–28), uninduced mutUNG1 mice (i.e., fed on a normal diet and thereby not expressing mutUNG1) as well as wild-type littersmates that were on a doxycycline diet did not exhibit the phenotype of mutUNG1-expressing mice and sustained a normal lifespan (data not shown).

As expected, the transgene mutUNG1 was highly expressed as mRNA in myocardial tissue of mutUNG1-induced mice but not in that of wild-type mice (Fig. 1E). No antibody exists that specifically detects mutUNG1, but the Ung1 antibody detects both the mutated human and the native mouse protein. Western blotting with antibody to Ung1 (Fig. 1F) showed two bands at ~37 kDa and two at ~30 kDa, likely representing the unprocessed protein and the result of processing in the mitochondria, respectively (7). In the wild-type mice, the bands represent endogenous unprocessed mouse Ung1 and its mitochondrial product. In the mutUNG1-induced mice, the bands represent the sum of the endogenous mouse Ung1 and the transgenic mutUNG1, the unprocessed forms of which have the same expected molecular mass. The mitochondrialy processed form at 31 kDa increased by more than five times after mutUNG1 induction (Fig. 1G). At the same time, the unprocessed form at ~39 kDa was reduced, indicating that any mutUNG1 not going to the mitochondria is only a small proportion and that the mitochondrial import of mutUNG1 after translation is effective. Immunofluorescence showed that Ung1 immunoreactivity was strong in mitochondria and low in nuclei (Fig. 2F). The immunoblots analysis also suggested that Ung1 unprocessed protein is reduced after induction of mutUNG1, whereas Ung1/mutUNG1 processed in mitochondria is increased. This was confirmed by rt-PCR, which showed a 60% reduction in total mRNA transcript from the mouse Ung-gene (Fig. 2G). The primers for the rt-PCR-assay recognize sequences in the catalytic domain common for both Ung1 and Ung2, both of which are encoded by the same gene (40, 41, 50), indicating a reactive downregulation of the Ung-gene expression during induced expression of the transgene.

**mutUNG1-expressing mice acquired pathological cardiac hypertrophy.** After ~2 mo of induced mutUNG1 expression in the cardiomyocytes, i.e., when the mutUNG1 mice displayed signs of illness and succumbed to sudden death, substantially enlarged hearts were observed compared with wild-type littersmates (Fig. 2, A and B) or uninduced mutUNG1 mice. Increased cardiac mass as well as increased transsectional area of the cardiac myocytes revealed significant cardiac myocyte hypertrophy in induced mutUNG1 mice (Fig. 2C), the transsectional area less than the weight, possibly reflecting the stretching of myocardiocytes on cardiac dilatation. In addition, the nuclei of cardiomyocytes of mutUNG1-expressing mice were significantly enlarged compared with wild-type littersmates (Fig. 2, D and E), which is a characteristic of pathological myocardial hypertrophy (51). Among mutUNG1-expressing mice, but not in the wild-type controls, pulmonary edema with dyspnea, pleural exudate and/or increased lung weight were often observed (Fig. 3A), i.e., signs of congestive heart failure (24). Some of the mutUNG1-expressing mice had extensive pleural effusion and minimal increase of lung weight. Thus, as a group, mutUNG1 mice did not display significant increase of lung weight compared with wild-type control mice (Fig. 3A). Masson’s Trichrome staining of myocardi-tissue of mutUNG1-expressing mice compared with wild-type littersmates. As observed before in heart failure, cardiomyocytes as well as the more numerous other cell types showed apoptosis (43).

**Transthoracic echocardiographic analyses of cardiac structure and function revealed left ventricular hypertrophy, reduced systolic function, and signs of congestive heart failure in mutUNG1-expressing mice.** The internal left ventricular end-diastolic diameter of mutUNG1-expressing mice was similar to that of wild-type mice (Fig. 4F). However, the end-diastolic left-ventricular wall thickness (posterior wall thickness) was significantly increased in mutUNG1-expressing mice com-

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**Fig. 5. Mitochondrial mass increases in cardiomyocytes, while mitochondrial antioxidant systems are induced.** A: immunostaining of mitochondrial outer-membrane protein Vdac1 (green) in paraffin-embedded sections from mouse heart of wild-type (left) and induced mutUNG1 mice (right). Sections were counterstained with nuclear dye DAPI. Scale bar = 100 μm. F: Western blot analysis and quantification of Vdac1 (31 kDa), showing increased expression of Vdac1 in mutUNG1-expressing mice compared with wild-type control mice. Ponceau staining was used as a loading control. Values are means ± SD (n = 3 mice). *Significant difference (P < 0.05). C: citrate synthase assay reveals increased citrate synthase activity in induced mutUNG1 mice (gray) compared with wild-type controls (black), indicating increased mitochondrial mass. Values are means ± SE (n = 4 mice).
pared with that in wild-type mice, demonstrating robust myocardial hypertrophy in mutUNG1 expressing mice (Fig. 4, A, B, and E). Left-ventricular contractility was reduced in mutUNG1-expressing mice (Fig. 4, B and G) compared with wild-type mice (Fig. 4, A and G), as assessed by determination of fractional shortening. Reduced left-ventricular systolic and diastolic function in induced mutUNG1-mice (Fig. 4, D, H, and I) compared with wild-type mice (Fig. 4, C, H, and I) were also confirmed by lower peak systolic and early diastolic left ventricular tissue velocities determined by tissue Doppler ultrasound. The mutUNG1-expressing mice also displayed increased left atrial diameter, indicating congestive heart failure (Fig. 4J).

Fig. 6. Mitochondrial antioxidant systems are induced in mutUNG1-expressing mice. A: immunostaining of Sod2 (green) and mitochondrial complex II (Mito CII; red) of paraffin-embedded sections from mouse hearts of wild-type and mice induced for 2–8 wk to express mutUNG1, showing colocalization of antioxidant system marker Sod2 with mitochondrial marker CII, which increase after 6 wk of induction of mutUNG1. Sections were counterstained with nuclear dye DAPI. Scale bar = 100 μm. Wild-type mice are the same age as the mice induced for mutUNG1 for 8 wk. At each time point, four mice were examined (2 at 4 wk), 4–6 sections from each, one of which is shown. In all of the sections, Sod2 staining at 8 wk > 6 wk > 4 wk > 2 wk — wild-type control, based on visual evaluation. The same ranking applied for mitochondrial complex II. B–D: quantitative rt-PCR shows significantly increased expression of transcripts for antioxidant enzymes Sod2 (B), glutathione peroxidase 1 (C), and catalase (D) in hearts from mutUNG1-expressing mice compared with wild-type mice. Values are means ± SD (n = 3 mice). *Significant difference (P < 0.05).
Mitochondrial mass increased, and mitochondria accumulated centrally in cardiomyocytes of mutUNG1-expressing mice, and mitochondrial antioxidant systems were induced. Fluorescence microscopy (Fig. 5A) and Western blotting (Fig. 5B) with mitochondrial markers (here: outer membrane mitochondrial protein Vdac1, voltage-dependent anion channel 1) showed that mitochondrial mass was increased in mutUNG1-expressing mice compared with wild-type littermates, which is a common finding in heart disease (47, 49). In addition, the mitochondria appeared to aggregate centrally in the cell, especially around the nucleus, whereas they tended to be more evenly distributed in wild-type mice (Fig. 5A). The increase in mitochondrial mass was substantiated by the significantly higher citrate synthase activity in mutUNG1-expressing mice compared with wild-type mice (Fig. 5B). In addition, there was induction of the endogenous antioxidant systems, with upregulation of the gene encoding the mitochondrial antioxidant enzyme, superoxide dismutase 2 (Sod2), in mutUNG1-expressing mice, at both the mRNA-level (Fig. 6A) and the protein-level (Fig. 6A). The induction of Sod2 was visible after 6 wk of mutUNG1 expression and increased progressively (Fig. 6A). The expression of two further antioxidant defense enzymes, glutathione peroxidase 1 (Gpx1; Fig. 6C) and catalase (Cat; Fig. 6D) was also upregulated. These changes are indicative of elevated ROS production, which is evidence of a malfunctioning electron transport chain.

To demonstrate signs of a general influence of ROS activity on tissue macromolecules, we examined carbonylation of proteins and the levels of free-SH groups. Surprisingly, OxyBlot results indicated a lower rather than a higher oxidative protein damage in mutUNG1 mice than in controls (Fig. 7, A and B). However, the results varied considerably, and no statistically significant effect was demonstrated (Fig. 7B). Similar results were obtained by analyzing three mutUNG1 and three controls by OxiSelect Protein Carbonyl Fluorometric Assay, without statistical significance (not shown). Free thiol groups likewise showed no statistically significant change in six mutUNG1-induced compared with six control mice (Fig. 7C). A plausible interpretation of these findings is that the demonstrated increase in oxidative defense enzymes (Sod2, Gpx1, catalase; Fig. 6, B–D) suffices to overcome protein and thiol oxidation in the mutUNG1 model mice.

The mitochondria in the hearts of induced mutUNG1 mice showed dysfunction with lowered respiration activity and reduced mtDNA copy number and mtDNA expression. Respiration analyses showed a severe and significant reduction in mitochondrial respiration in hearts of mutUNG1-expressing mice (Fig. 8A), which indicates reduced function in energy conversion/ATP production. Succinate was used as substrate because it does not depend on the nuclear-encoded complex II, but on the mtDNA-encoded complex III/IV. Since the heart has a high energy demand, an energy deficit will be harmful and is most likely contributing to the fatal phenotype seen in the induced mutUNG1 mice. Rt-PCR studies also showed a significant 30% decrease in mtDNA copy number in the total mitochondrial population of hearts of mutUNG1-expressing mice (Fig. 8B). Since there is an increase in mitochondrial biogenesis in the mutUNG1-expressing mice (Fig. 4, A and B), mitochondrial DNA copy number is most likely even lower in each individual mitochondrion. In addition, mtDNA expression was severely affected and was almost absent in genes on both heavy-strand (e.g., mt-Cox1, mitochondrially encoded cytochrome c oxidase 1) and light-strand (e.g., mt-Nd6, mitochondrially encoded NADH dehydrogenase 6) mtDNA (Fig. 8, C and D), indicating malfunction of the extant mtDNA copies.

Mitochondria of cardiomyocytes in mutUNG1-expressing mice showed a breakdown in mitochondrial dynamics. Mitochondria in heart tissue are highly organized to provide energy and also calcium buffering in an efficient manner (44). The normal appearance of myocardial mitochondria in transmission electron microscopy is shown in images of wild-type mice (Fig. 9, A–C). Mitochondria are known to be highly dynamic organelles that move around in the cell to satisfy local cellular demands, and also fuse and divide (57). The fusion/fission mechanisms are important for upholding a healthy mitochondrial pool in the heart (12). By studying the cardiomyocytes, using transmission electron microscopy, we found the mitochondria to be disorganized and abnormal in the mutUNG1-expressing mice. The normally highly organized cardiac tissue
also seemed to be unstructured with myofibril displacement (Fig. 9, D–L). In addition to general disorganization of the anatomy of the tissue, there was an unusual accumulation of mitochondria (Fig. 9, D–F), as indicated by increased mitochondrial marker immunofluorescence (Figs. 5A and 6A). The accumulated mitochondria showed structures reminiscent of mitophagic processes (Fig. 9G). Also, some mitochondria were atypically large with irregular shapes, and small round mito-

chondria were numerous (Fig. 9, H–J). This indicates failure of the fusion and fission mechanisms of mitochondria in cardiomyocytes in the mutUNG1-expressing mice. In addition, there were mitochondria with amorphic internal structures, where the cristae arrangement looked as if it were collapsing (Fig. 9K) or falling apart (Fig. 9L). These observations, of accumulating mitochondria with abnormal structure, indicate failure of the fusion-fission and mitophagy processes designed to uphold a healthy mitochondrial population.

Expression of genes controlling mitochondrial fusion and fission were upregulated in mutUNG1-expressing mice. Due to the apparent breakdown in mitochondrial dynamics shown by electron microscopy, we examined the expression of genes important for mitochondrial fission and fusion using rt-PCR. Genes associated with fission (Drp1 and Fis1) (Fig. 10, A and B), fusion (Mfn1 and Mfn2) (Fig. 10, C and D), and cristae-organization (Opal1) (Fig. 10E) showed significant upregulation in mutUNG1-expressing mice compared with wild-type littermates. There was an approximately threefold upregulation in all genes directly involved in mitochondrial fusion and fission, except Mfn2, which was upregulated approximately twofold. The expression of the gene encoding Fis1, which functions as a scaffolding protein for Drp1 during fission, was upregulated close to twofold. The activation of both fusion and fission genes indicates that both processes are needed in the efforts to counteract the serious mitochondrial malfunction caused by mutUNG1.

The multifaceted Pgc-1α, key regulator of energy metabolism, ROS scavenging enzymes, and mitochondrial biogenesis, was upregulated in mutUNG1-expressing mice. Finally, we determined the mRNA and protein level of peroxisome proliferator-activated receptor gamma coactivator-1α (Pgc-1α), which is a chief regulator of mitochondrial biogenesis as well as of fusion/ fission dynamics and mitochondrial energy production (35), and is upregulated by various forms of stress, including ROS (55). Although there was no significant change in Pgc-1α mRNA (Fig. 10F), an upregulation of Pgc-1α protein was visible after 6 wk of mutUNG1 expression and increased progressively (Fig. 11A). The Pgc-1α content was doubled after 8 wk of induction of mutUNG1 (Fig. 11, B and C), suggesting that a central regulatory mechanism is recruited to cope with the stress inflicted on the myocardium by mutUNG1 expression.

DISCUSSION

Here, we demonstrate that selective introduction of mtDNA damage in cardiomyocytes results in mitochondrial dysfunction and ensuing congestive heart failure and death. Indeed, exposure to the transgene for no more than 2 mo reveals that mutUNG1 is able to inflict serious, and eventually fatal, derangement of cardiac function. A previous report of mutUNG1 expression under control of Ca2+/calmodulin-dependent protein kinase II (CamKIIα) promoter revealed a dramatic increase of cytotoxic AP sites in mtDNA in forebrain neurons, causing neurodegeneration and illness ~6 mo after induction of mutUNG1 (28). Thus αMHC-driven expression of mutUNG1 would be likely to inflict similar increase of AP sites in mtDNA of cardiac myocytes. Although AP sites are common intermediates of the base-excision-repair pathway, accumulating AP sites pose a serious threat to the mitochondrial genome by blocking mtDNA replication and transcription, leading to mitochondrial dysfunction and subsequent increase of free-oxygen radicals. The fact that mutUNG1 not only removes uracil from mtDNA but more importantly also thymine accounts for the dramatic increase of AP sites in the mtDNA.

The model described here provides rapid and specific induction of mtDNA damage in cardiomyocytes, suitable for exploring mechanisms of mtDNA-based cardiomyopathy that are relevant to aging-dependent disease without having to wait for the animals to age. A remaining question is to what extent lower levels of mutUNG1 activities may inflict moderate damage of mtDNA that leads to overt heart disease only in aging or after imposing other forms of increased cardiac stress, for example, chronic blood pressure overload. Lower levels of accumulating mtDNA perturbation would mimic more closely the conditions
in human disease and may in future studies be obtained at lower mutUNG1-inducing dosage of doxycycline, since the approach implies the possibility of doxycycline dose-dependent gene expression (39).

As seen with mitochondrial immunohistochemical staining and citrate synthase-assays, there is an increase in mitochondrial mass in mutUNG1-expressing mice compared with wild-type littermates. Upregulation of mitochondrial biogenesis is often seen in mitochondrial disorders, where it serves as a compensatory mechanism to uphold the required ATP production (4, 15). Studies have shown that excessive mitochondrial biogenesis in the myocardium can cause mechanical damage by disturbing the tight cellular arrangement and lead to myofibril displacement, interfering with sarcomeric function and contributing to severe cardiac remodeling (49). This is also observed in the mutUNG1-expressing mice, where myofibril displacement and a higher degree of cellular disorganization are seen in electron micrographs.

The state of the mitochondria in mutUNG1-expressing mice indicates imbalance between mtDNA-related factors and nuclear DNA-related factors. The mutUNG1-expressing mice show a decrease in mtDNA copy number and a heavy reduction in expression from the mitochondrial genome. This is probably due to the high levels of mtDNA damage in the form of AP sites,
Significant difference (P expressing mice compared with wild-type controls (mitochondrial biogenesis and function, shows no significant change in mutUNG1-coactivator (regulating genes involved in energy metabolism) and a regulator of mitochondrial transcription. Ectopic expression of a mutant mitochondrial polymerase γ with a mutation in the catalytic domain, mirroring the human syndrome chronic progressive external ophthalmoplegia (CPEO) in murine heart, is associated with a decrease in mtDNA copy number and increased levels of oxidative mtDNA damage. These mice also have abnormally shaped mitochondria, enlarged hearts, and a shortened lifespan (29). In the mtDNA mutator mouse, where the proof-reading domain of polymerase γ has been deleted (Polgmut), the animals had hypertrophic hearts with accumulation of abnormal cardiomyocyte mitochondria (52). This was confirmed in another study, where the Polgmut protein was specifically expressed in the heart, causing cardiomyopathy with severely dilated hearts and death from congestive heart failure (58).

Interestingly, cardiomyocyte-specific overexpression of the DNA repair enzyme 8-oxoguanine DNA glycosylase 1 (OGG1) isoform 2a selectively protected mtDNA from damage and counteracted the hypertrophic response and myocardial fibrosis (ascribed to signaling from stressed cardiomyocytes) in a heart failure model (56).

Sod2 is essential for dismutating the superoxide anion to hydrogen peroxide in mitochondria, and Sod2−/− knockout animals die early postnatally of cardiomyopathy and neuropathy. Sod2+/− heterozygous mice eventually develop cardiomyopathy and heart hypertrophy with accompanying fibrosis and necrosis, but importantly not apoptosis (30, 32).

High levels of ROS are toxic not only to the mitochondria themselves but to all components of the cell (5). Several experimental and clinical studies have shown that ROS is commonly increased in cardiomyopathy and heart failure (17, 18, 34), and dysfunctional mitochondria have been recognized as the main source of excess ROS causing oxidative stress (21, 48). ROS can directly harm proteins essential to excitation-contraction coupling, thereby impairing the contractile function of the heart. In addition, ROS can set in motion a number of prohypertrophy signaling and apoptosis pathways (53). We found a strong induction of the mitochondrial antioxidant enzyme Sod2 in mutUNG1-expressing mice, as well as of two other antioxidant enzymes, Gpx1 and catalase, indicating increased levels of ROS of mitochondrial origin. Given the abundant mitochondrial mass in the heart, the potential for toxic oxidative stress is particularly high in mutUNG1-expressing mice, where all cardiomyocyte mitochondria are subject to the mtDNA damage-inducing protein mutUNG1 causing mitochondrial dysfunction. The importance of keeping the ROS levels low by cellular antioxidant enzymes has been demonstrated in experiments using animal models where overexpression of Sod2 proved to protect against heart failure (42).

Surprisingly, we found no evidence of general ROS action on tissue protein, neither as protein carbonylation nor as reduced tissue free thiol groups. Nevertheless, three different oxidation defense enzymes (Sod2, Gpx1, Cat) showed upregulation in mutUNG1-expressing mice. Likewise, the “master regulator” of metabolism and mitochondrial biogenesis, Pgc-1α, was increased. Coactivators such as Pgc-1α are required for high-level expression of nuclear- and mitochondrial-encoded genes involved in mitochondrial dynamics and energy transduction in the heart (35). Since Pgc-1α expression is increased by many stress factors, including ROS (55), and is known to upregulate oxidative defense mechanisms such as

Fig. 10. All genes investigated that are involved in mitochondrial fusion and fission are upregulated in mutUNG1-expressing mice. Quantitative r-PCR shows increased expression of genes governing mitochondrial fusion Drp1 (A) and Fis1 (B) as well as of genes implicated in mitochondrial fusion Mfn1 (C), Mfn2 (D), and Opal (E) in mutUNG1-expressing mice compared with wild-type littermates (n = 3 mice). Quantitative r-PCR of Pgc-1α (F), which encodes a protein that is both a transcriptional coactivator (regulating genes involved in energy metabolism) and a regulator of mitochondrial biogenesis and function, shows no significant change in mutUNG1-expressing mice compared with wild-type controls (n = 5). Values are means ± SD. *Significant difference (P < 0.05).

which slow down or inhibit the polymerases and prevent them from acting on their substrate. Interestingly, although mtDNA copy number was reduced by “only” 30% (with an associated 50% reduction in respiration capacity), there was a much more pronounced reduction of the mitochondrial transcripts. Our data therefore suggest that the compensatory increase in mitochondrial biogenesis to reverse the mtDNA toxicity in the mutUNG1-expressing mice is responsible for the relatively high functional capacity of the mitochondria despite the dramatic reduction in mtDNA transcription. It is of interest to note that mtDNA synthesis is less sensitive to AP site accumulation than mitochondrial transcription.
Sod2 (33), its observed upregulation may therefore link the findings made in the present study. Together, these factors may possibly have caused enough upregulation of oxidation defense to prevent ROS from causing lasting evidence of oxidative stress in the form of protein carbonylation or consumption of thiols.

Alternatively, the observed alterations in gene expression could possibly be mediated by Pgc-1α in the absence of excessive ROS production: in cardiomyocytes, Pgc-1α decreases in hypoxia but increases in fuel shortage (45); both of these opposing effects might occur in fatal heart failure.

There was upregulation of all genes involving fusion and fission mechanisms in mutUNG1-expressing mice, which might be an attempt by the failing myocardium to rescue the dysfunctional mitochondrial population. But an overcompensation of these systems leads to a further collapse of mitochon-

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**Fig. 11.** Pgc-1α, key regulator of energy metabolism, ROS scavenging enzymes, and mitochondrial biogenesis, was upregulated at the protein level in mutUNG1-expressing mice. A: immunostaining of Pgc-1α (green) and mitochondrial complex II (Mito CII, red) of paraffin-embedded sections from hearts of wild-type mice and mice induced for mutUNG1 expression at different time intervals show a progressive increase of Pgc-1α after 6 wk of induction of mutUNG1. At each time point, four mice were examined (two at 4 wk), 4–6 sections from each, one of which is shown. In all of the sections, Pgc-1α staining at 8 wk > 6 wk > 4 wk ~ 2 wk ~ wild-type control, based on visual evaluation. The same ranking applied for mitochondrial complex II. Sections were counterstained with nuclear dye DAPI. Scale bar = 100 μm. Wild-type mice are the same age as the mice induced for mutUNG1 for 8 wk. Western blot (B) and concomitant quantification (C) show upregulation of Pgc-1α in mice expressing mutUNG1 for 8 wk compared with wild-type controls; Actb was used as a reference loading control. Values are means ± SD (n = 3 mice). *Significant difference (P < 0.05).
drial organization and ultimately to dysfunction. Unbalanced cycles of fusion and fission of mitochondria, in addition to increased levels of ROS and loss of energy due to lowered mitochondrial respiration, can contribute to the progressive breakdown of mitochondrial dynamics and subsequent heart failure in these mice. Since chronic heart failure is commonly associated with anomalies in mitochondrial morphology, regarding number, organelle size, and structural integrity, there has recently been a rising notion that the mechanisms controlling mitochondrial shape may contribute to the progression of several cardiac pathologies. There is also an association between mitochondrial dynamics and mtDNA (44). Studies with S. cerevisiae and mouse embryonic fibroblast (MEF) cells have shown that the activities of Opal (10, 22) and Mfn2 (9, 10, 16) are essential for the integrity of mtDNA nucleoids (44), and Opal1−/− mice suffer from cardiomyopathy (11). The imbalanced mitochondrial dynamics observed in mutUNG1-expressing mice might contribute to the diminished levels of mtDNA, exacerbating the process of mitochondrial dysfunction. An increase in mitochondrial biogenesis and overexpression of genes involved in fusion and fission can be a compensatory attempt to rescue the progressively pathological heart tissue in mutUNG1-expressing mice. Given the reduced respiratory capacity of the mitochondria of these mice, and thereby lessened ATP production and impaired calcium handling, the generation of more mitochondria seems to be a rational strategy. However, since damage in mtDNA is continuously being generated, the elevated mass of mitochondria is ROS-producing and dysfunctional, ending up being harmful instead of beneficial and causing cellular toxicity. The excessive mass of mitochondria, with unbalanced mitochondrial dynamics, also causes disruption of the highly organized cardiac architecture.

It has recently been shown that mitochondrial dysfunction triggers a p53-mediated response that regulates mitophagy and that this response is crucial to prevent heart failure in mice (20). Along these lines, we show that mtDNA instability in the heart tissue decreases mitochondrial function and impairs mitochondrial fission and fusion. We have recently proposed a model according to which intermittent enhancement of mitochondrial biogenesis by stressful episodes with subsequent recovery promotes adaptive changes supporting mitochondrial health (46). Here, we expand this model to include biogenesis of heart mitochondria and explain how quality control of mtDNA can influence cardiac function. We propose that amplification of defective mitochondria in combination with continuous attempts at enhancement of mitochondrial biogenesis without efficient recovery promotes mitochondrial dysfunction and cardiac failure. In conclusion, our data underline the importance of balanced and well functioning mitochondrial dynamics and the relevance of mitochondrial biogenesis in determining mitochondrial and cardiac failure. In conclusion, our data underline the importance of balanced and well functioning mitochondrial dynamics and the relevance of mitochondrial biogenesis in determining mitochondrial and cardiac failure.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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