



# Cardiac Left Ventricle Mitochondrial Dysfunction After Neonatal Exposure to Hyperoxia: Relevance for Cardiomyopathy After Preterm Birth

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**BACKGROUND:** Individuals born preterm present left ventricle changes and increased risk of cardiac diseases and heart failure. The pathophysiology of heart disease after preterm birth is incompletely understood. Mitochondria dysfunction is a hallmark of cardiomyopathy resulting in heart failure. We hypothesized that neonatal hyperoxia in rats, a recognized model simulating preterm birth conditions and resulting in oxygen-induced cardiomyopathy, induce left ventricle mitochondrial changes in juvenile rats. We also hypothesized that humanin, a mitochondrial-derived peptide, would be reduced in young adults born preterm.

**METHODS:** Sprague-Dawley pups were exposed to room air (controls) or 80% O<sub>2</sub> at postnatal days 3 to 10 (oxygen-induced cardiomyopathy). We studied left ventricle mitochondrial changes in 4 weeks old males. In a cohort of young adults born preterm (n=55) and age-matched term (n=54), we compared circulating levels of humanin.

**RESULTS:** Compared with controls, oxygen-exposed rats showed smaller left ventricle mitochondria with disrupted integrity on electron microscopy, decreased oxidative phosphorylation, increased glycolysis markers, and reduced mitochondrial biogenesis and abundance. In oxygen-exposed rats, we observed lipid deposits, increased superoxide production (isolated cardiomyocytes), and reduced *Nrf2* gene expression. In the cohort, left ventricle ejection fraction and peak global longitudinal strain were similar between groups however humanin levels were lower in preterm and associated with left ventricle ejection fraction and peak global longitudinal strain.

**CONCLUSIONS:** In conclusion, neonatal hyperoxia impaired left ventricle mitochondrial structure and function in juvenile animals. Serum humanin level was reduced in preterm adults. This study suggests that preterm birth-related conditions entail left ventricle mitochondrial alterations that may underlie cardiac changes perpetuated into adulthood.

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**Key Words:** cardiomyopathies ■ humanin ■ hyperoxia ■ mitochondria ■ premature birth

Nearly 10% of all infants worldwide are born preterm (before 37 weeks' gestation).<sup>1</sup> Although preterm birth remains one of the most important determinants of perinatal mortality and morbidity,<sup>2</sup> improvements in perinatal care over the last decades have enabled the survival of infants born preterm at rates approaching

those of infants born full-term. Accordingly, the prevalence of adults born preterm is increasing.<sup>1</sup> Despite their relatively young age, they are at greater risk of cardiovascular disease.<sup>3</sup> Young adults born preterm display altered shape and mass of the left ventricle of the heart and slightly impaired systolic and diastolic function.<sup>4,5</sup>

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## NOVELTY AND RELEVANCE

### What Is New?

The current study shows that neonatal hyperoxia in rats leads to abnormal mitochondria structure and function in the cardiac left ventricle later in life but before overt cardiac dysfunction.

The current study is the first clinical report correlating circulating levels of the mitochondria-derived peptide humanin with left ventricle function and reveals lower humanin levels in adults born preterm compared with full-term.

### What Is Relevant?

The current study provides novel elements in the understanding of the pathophysiological pathway to left

ventricle cardiac disease after preterm birth and supports the rationale to examine the circulating mitochondria-derived peptide humanin as an early biomarker for cardiac disease in individuals born preterm.

### Clinical/Pathophysiological Implications?

In this study, we revealed that left ventricle mitochondrial alterations may underlie the cardiac changes perpetuated into adulthood following transient neonatal hyperoxia, a model of preterm birth-related conditions. We also show that serum humanin levels, a mitochondria-derived peptide, are lower in adults born preterm versus term.

## Nonstandard Abbreviations and Acronyms

<b>HAPI</b>	Health of Adults Born Preterm Investigation
<b>mtDNA</b>	mitochondrial DNA
<b>Nrf2</b>	nuclear factor erythroid 2-related factor 2
<b>OIC</b>	oxygen-induced cardiomyopathy
<b>SOD2</b>	superoxide dismutase 2

Preterm birth is associated with increased risk of heart failure from childhood into adulthood.<sup>6,7</sup> Overall, the mechanisms underlying increased susceptibility of the preterm-born to heart disease, as observed in young adults, remain unknown.

A well-recognized rodent model simulating preterm birth conditions is that of rats exposed to neonatal hyperoxia. In previous work with this model, we demonstrated the development of cardiac dysfunction, with impaired contractile response to stressors, which we termed oxygen-induced cardiomyopathy (OIC).<sup>8–10</sup> Exposure of an immature myocardium to increased tissue oxygen, as happens at preterm birth, might impair cardiac mitochondria; normal perinatal adaptation to postnatal life is coupled with mitochondrial maturation.<sup>11–14</sup> We, therefore, postulated that neonatal exposure to hyperoxia could lead to mitochondrial changes in the left ventricle, preceding the rise in blood pressure and significant cardiac dysfunction that we observed in the model.<sup>8,15</sup>

To evaluate myocardial mitochondrial dysfunction in the clinical setting remains a challenge, as it demands fresh biological specimens, a procedure not inherently feasible in otherwise healthy individuals or biobank samples. Humanin is a mitochondria-derived peptide increasingly recognized for its cytoprotective and mitoprotective properties, including in cardiac tissue.<sup>16–18</sup> Data suggest that

circulating levels of humanin could constitute a marker of mitochondrial health and function.<sup>19</sup> We, therefore, conducted a 2-pronged investigation to test our hypothesis. We examined cardiac tissue samples from rats with OIC for mitochondrial changes and compared them to those of normal rats. To translate to the clinical context, we compared serum humanin levels in our cohort of young adults born very preterm to those of age-matched term-born adults,<sup>20,21</sup> and assessed the association of humanin with left ventricle ejection fraction.

## MATERIALS AND METHODS

Materials and methods are presented in further detail in the [Supplemental Material](#). The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Animal Model

Sprague-Dawley litters obtained from Charles River laboratories (Saint-Constant, QC, Ca; culled to n=12 pups/litter, 6/sex) were kept in either: (1) room air (control group); or (2) an oxycycler (ProOx P110, BioSpherix, Lacona, NY) calibrated at 80% O<sub>2</sub> from postnatal day 3 to day 10 (OIC group), as previously reported.<sup>8–10,15</sup> Male rats were studied at 4 weeks of age when OIC rats do not yet present hemodynamic alterations (blood pressure levels) that could be a confounder for cardiac phenotype.<sup>8,15</sup> Litters were kept co-ed to preserve normal group dynamics that might otherwise affect stress response. Only males were used in the experiments as in previous studies.<sup>8–10</sup> We included no more than 2 animals per litter per experiment to avoid experimental bias or genetic predisposition. To confirm the cardiac phenotype of OIC following neonatal exposure to hyperoxia, we assessed cardiac structure and function in random littermates of the pups allocated in the mitochondria assessments study using the VEVO 3100 ultrasound imaging system (Visual Sonics) equipped with a 510 MHz scan head, as previously described.<sup>8–10</sup>

The Animal Ethics Committee of the Centre Hospitalier Universitaire Sainte-Justine Research Centre approved the experimental procedure, according to the guidelines of the Canadian Council on Animal Care and the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Researchers performed analyses while blinded to the experimental group of the animals.

### Cardiac Tissue Sampling

For all tissue samples (except as stated below), used for molecular and bioenergetics evaluations, rats were placed in a plastic chamber connected to a vaporizer machine (Dispomed, Joliette, QC, Canada) to be first anesthetized with inhaled Isoflurane USP (Batch number: N0G120H20A; Fresenius Kabi, Toronto, ON, Canada; 4 vol% in O<sub>2</sub>; ratio 4:1).<sup>8–10</sup> Sedation was monitored through the absence of painful response to pedal and toe pinch. Rats were then rapidly killed by decapitation.

For electron microscopy studies, rats were anesthetized with the intraperitoneal injection of a mixture of ketamine (Vetalar, 90 mg/kg; Bioniche Animal Health Canada, Inc, Belleville, Ontario) and xylazine (Rompun, 10 mg/kg; Bayer Healthcare LCC, Whippany). Plane anesthesia was confirmed by the loss of pedal and toe pinch reflex. Animals were then placed in dorsal position near the peristaltic pump for transcatheter perfusion, which consists in an end-point procedure. All animals were killed following the guidelines of the Canadian Council on Animal Care and the US National Institutes of Health Guide for the Care and Use of Laboratory Animals to ensure minimum pain, suffering, and distress.

### Mitochondrial Ultrastructure

Quantitative transmission electron microscopy (Facility for Electron Microscopy Research, McGill University, Montreal, Canada) at  $\times 1400$ ,  $\times 2900$ , and  $\times 7000$  magnification was used to image cardiomyocytes in sections of left ventricle papillary muscle ( $n=3$  rats/group, 10 random visual fields/rat/magnification). Morphological characteristics of mitochondria were quantified, including individual surface area, perimeter, and frequency of area distribution, as well as mitochondria/cardiomyocyte ratio (total mitochondrial area/cardiomyocyte surface area). Mitochondrial integrity scores on a 4-point scale were determined according to Mofarrah et al,<sup>22</sup> taking into account intact matrix, cristae definition, outer mitochondrial membrane damage, and homogeneous mitochondrial density (electron density). All images were analyzed with ImageJ (National Institutes of Health).

### Mitochondrial Respiration

We isolated mitochondria from  $\approx 300$  mg of fresh left ventricle tissue, using differential centrifugation.<sup>23,24</sup> The rate of oxygen consumption in isolated mitochondria, representing the mitochondrial oxidative phosphorylation, was measured with a Clark electrode (Oxytherm+ System, Hansatech Instruments, Ltd, Norfolk, England) in response to the sequential addition of mitochondrial respiratory chain complex II substrate (succinate) and complex I blocker (rotenone), ADP to induce maximal rate of oxygen consumption (state 3), followed by the ATP synthase inhibitor oligomycin (state 4), and the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone. The respiratory control ratio was calculated as the ratio of state 3 to

state 4. Results were expressed as oxygen consumption rate/ min per milligram protein.<sup>25,26</sup>

### Cardiac Lipid Deposits

Lipid depositions were evaluated in left ventricle cryosections (10  $\mu$ m) using 2 methods: LipidTox<sup>27</sup> (Invitrogen Thermo Fisher, Toronto, Canada, H34475) to quantify intracellular lipid deposition under confocal microscopy and with Oil Red O<sup>28,29</sup> (ab150678; Abcam, Toronto, Canada) to visualize cardiac lipid deposits under both bright-field and confocal microscopy. These were quantified automatically in Image J (National Institutes of Health; see [Supplemental Material](#)).

### Mitochondrial Copy Number

Mitochondrial DNA copy number, determined by the ratio of mitochondrial DNA (mtDNA) to genomic DNA, was measured by quantitative polymerase chain reaction, as a marker of relative abundance of mtDNA in cells.<sup>30</sup>

### Mitochondrial Superoxide Production in Isolated Cardiomyocytes

To isolate left ventricle cardiomyocytes, the hearts were rapidly excised and manually perfused using a Langendorff-free method.<sup>31,32</sup> The dissected left ventricle was then processed in collagenase type II solution to isolate individual cardiomyocytes. Mitochondrial superoxide (O<sub>2</sub><sup>-</sup>) production was assessed using the Invitrogen MitoSOX Red Mitochondrial Superoxide Indicator (Thermo Fischer Scientific, Waltham, MA).<sup>33</sup>

### Human Study Population

Data were obtained from the HAPI (Health of Adults Born Preterm Investigation) cohort, which has been previously described.<sup>20,21</sup> We evaluated young adults aged 18 to 29 years, born in 1987 to 1997 at  $<30$  weeks' gestation, and compared them to peers matched for age but born full-term at  $\geq 37$  weeks' gestation. Echocardiography was performed with a Vivid E9 ultrasound machine (GE Healthcare, Milwaukee). Standard 2-dimensional echocardiographic measurements were extracted and analyzed according to the American Society of Echocardiography guidelines.<sup>34,35</sup> Peak global longitudinal strain of the left ventricle by speckle-tracking echocardiography, a marker of systolic deformation, was measured using Tomtec Arena (Munich, Germany). Ethics approval was obtained from the Research Ethics Boards of Sainte-Justine Hospital (Centre Hospitalier Universitaire Sainte-Justine; number 3901), McGill University Health Centre, and the Sir Mortimer B. Davis Jewish General Hospital (number 2139). Participants gave written informed consent.

### Serum Humanin Levels

Blood was collected from cohort participants on the morning of the study visit, after overnight fasting, and was immediately centrifuged. Serum samples were kept frozen at  $-80^{\circ}\text{C}$  for subsequent analysis. Humanin levels were determined by ELISA (CSB-EL015084HU- MT-RNR2, Cusabio Technology LLC, Wuhan, China), as per manufacturer's instructions. This kit has an intraassay coefficient of variation  $<8\%$ , interassay coefficient of variation  $<10\%$ , detection range 28 to 1800 pg/mL, and

sensitivity of 7 pg/mL. All measurements were within detection range and were performed in duplicate. Measurements with a coefficient of variation >8% were repeated if possible or excluded from analysis if no additional sample was available. In rats, a humanin equivalent remains to be identified with certainty and was therefore not measured in the current study.

## Statistical Analysis

Data are presented as mean±SEM unless otherwise indicated. We used the Shapiro-Wilk test for normality and Student *t* test for analysis. We used  $\chi^2$  tests to compare mitochondrial surface area frequency distribution and mitochondrial integrity between groups. Because the serum humanin distribution was not normal (Shapiro-Wilk test <0.05), humanin levels are shown as median (interquartile range). We used the nonparametric Mann-Whitney *U* test and Fisher exact test for between-group comparisons of human results. Significance level was set at  $P<0.05$ . All statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA) and R version 3.6.0 (open-source collaborative).

## RESULTS

### Animal Model

There were no differences between control and OIC groups in terms of survival, growth, or other parameters such as body weight, heart weight, and heart/body weight ratio at the time of euthanization;  $P>0.05$ , Student *t* test (Table S1 in the [Supplemental Material](#)). Echocardiography studies (Table S2) confirmed OIC similar to what we previously reported<sup>8</sup> and showed mild alteration in left ventricular structural parameters characterized by increased posterior wall thickness (indexed), smaller cavity, and reduced end-diastolic volume and cardiac output in OIC versus control. Mitral E-wave to A-wave ratio was also significantly decreased in OIC. The expression of profibrotic factor TGF (transforming growth factor)  $1\beta$  was significantly increased in the left ventricle of OIC when compared to the control group, whereas TNF (tumor necrosis factor)- $\alpha$ , associated with molecular pathways of established cardiac disease, was not different between groups (Figure S1).

### Mitochondrial Ultrastructure

Left ventricle mitochondrial ultrastructure is shown under transmission electron microscopy (Figure 1A). In the OIC group, mitochondrial surface area (Figure 1B) and perimeter (Figure 1C) were smaller than in the control group. Overall, the cardiomyocyte area occupied by mitochondria was smaller in OIC animals as compared to controls (Figure 1D). The frequency distribution of mitochondrial size was shifted left toward the smaller sizes (Figure 1E). In terms of integrity, whereas nearly 90% of mitochondria in the control group had integrity scores of 3 or 4, over 70% in the OIC had scores of  $\leq 2$ : organelles were fragmented, swollen, with loss in cristae structure and disrupted inner and outer membranes (Figure 1F).

Mitochondrial morphology and integrity also result from the dynamic process of mitochondrial fusion and fission. We found an overall downregulation in gene expression of fusion genes (mitofusins [*Mfn1*] and *Mfn2*) and fission genes (mitochondrial dynamin-related protein [*Drp1*] and mitochondrial fission 1 protein [*Fis1*]) in OIC rats (Figure S2).

### Mitochondrial Respiration and Bioenergetics

Mitochondrial oxidative phosphorylation capacity was evaluated by measuring oxygen consumption rates at different states, after sequential incubation with rotenone and succinate (state 2), ADP (state 3), oligomycin as inhibitor of ATP synthase (state 4), and carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone as an uncoupler. OIC mitochondria showed lower rates of oxygen consumption than controls at baseline (state 2; Figure 2A). When stimulated with ADP, the oxygen consumption rate decreases in OIC group during state 3 (Figure 2B). No between-group differences were observed at state 4 with oligomycin (Figure 2C) or with carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone-stimulated uncoupling (Figure 2D). Respiratory control ratios were similar in both groups (Figure 2E), indicating that both mitochondria integrity were intact.

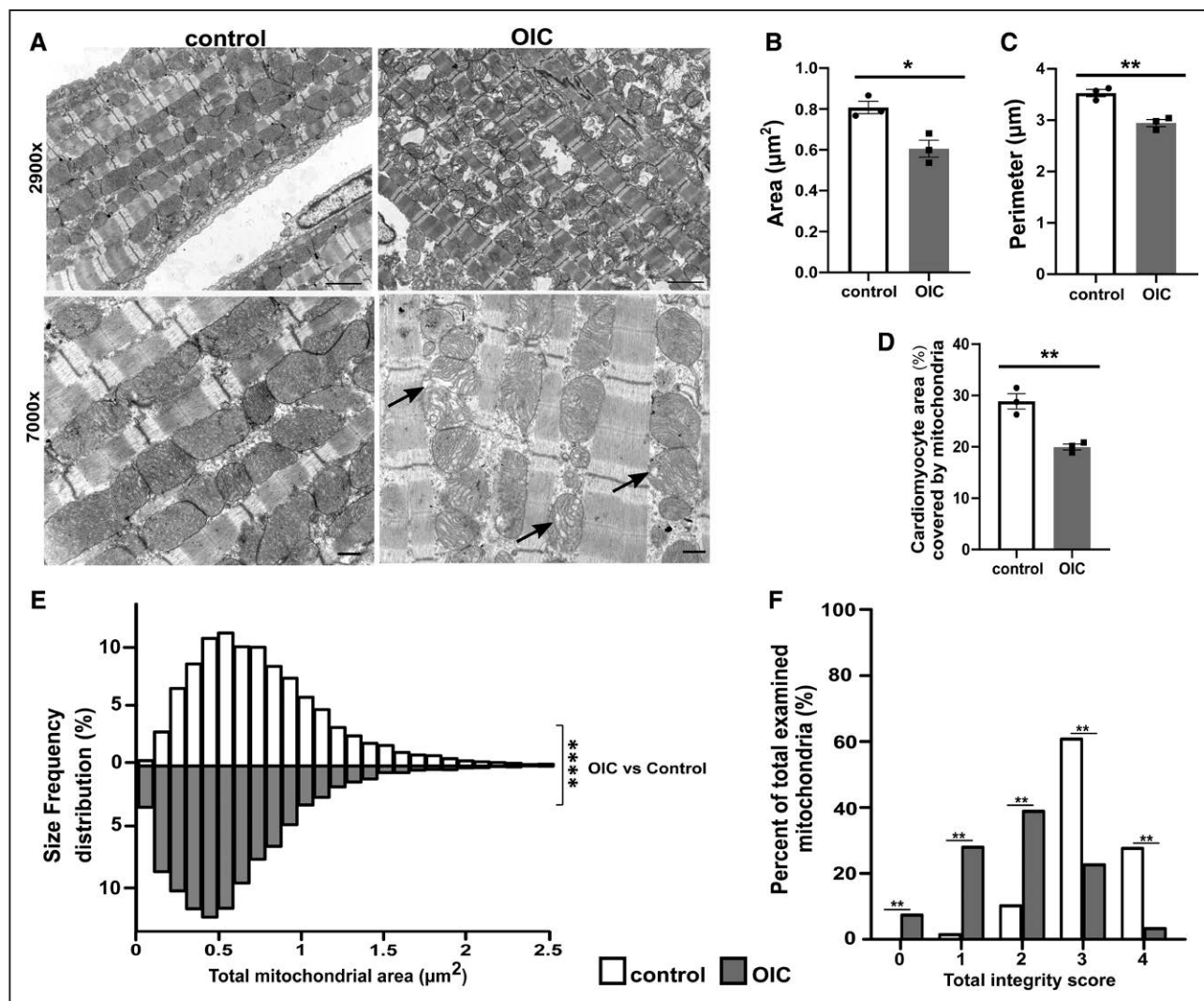
Protein content of all electron transport chain complexes, except II, was significantly reduced in left ventricle isolated mitochondria from OIC group (Figure S3), corroborating the defect in mitochondrial oxidative phosphorylation observed in Figure 2. Complexes I, III, and IV are integral proteins of the inner mitochondrial membrane that generate the proton gradient that drives ATP synthesis; Complex 5 is ATP synthase. HIF (hypoxia-inducible transcription factor)- $1\alpha$  protein (Figure 3A) and hexokinase 1 mRNA (Figure 3B), primary drivers of glycolysis, were increased in OIC, as compared to controls. There was no between-group difference in hexokinase 2 mRNA expression (Figure 3C). We observed significantly increased intracardiac lipid deposits in left ventricle from OIC but not in control rats (Figure 3D and 3E).

### Mitochondrial Biogenesis and Abundance

As compared to the control group, mitochondrial biogenesis and abundance in left ventricle muscle tissue was reduced in the OIC group. Markers such as gene expression of PGC (peroxisome proliferator-activated receptor gamma coactivator)- $1\alpha$  (Figure 4A) and of citrate synthase (Figure 4B) were decreased, as was the ratio mtDNA/genomic DNA (Figure 4C).

### Left Ventricle Superoxide Production

Mitochondrial superoxide production was increased in isolated left ventricle cardiomyocytes (Figure 4D) from OIC versus control rats. Whereas expression of mitochondrial SOD2 (superoxide dismutase 2) did not differ in left ventricle tissue between groups (Figure 4E), *Nfr2* (nuclear factor erythroid 2-related factor 2), involved in



**Figure 1. Mitochondrial ultrastructure, morphology, and integrity in left ventricle cardiac muscle.**

Electron microscope images of left ventricular fibers (A) from rats exposed only to room air (control) and rats exposed to hyperoxia as newborns (oxygen-induced cardiomyopathy [OIC]). **Top** views were magnified  $\times 2900$  and **bottom** views  $\times 7000$ . Individual mitochondria are indicated by arrows. Mitochondrial average surface area (B), perimeter (C), proportion (%) of cardiomyocyte area covered by mitochondria (D), and frequency distribution of mitochondrial size (E) all showed lower values for the OIC group than for controls. F presents the distribution (%) of mitochondria according to integrity score (0–4). Data shown are mean  $\pm$  SEM (SEM);  $n=3$ /group. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\*\* $P<0.0001$  comparing control vs OIC with unpaired  $t$  test.

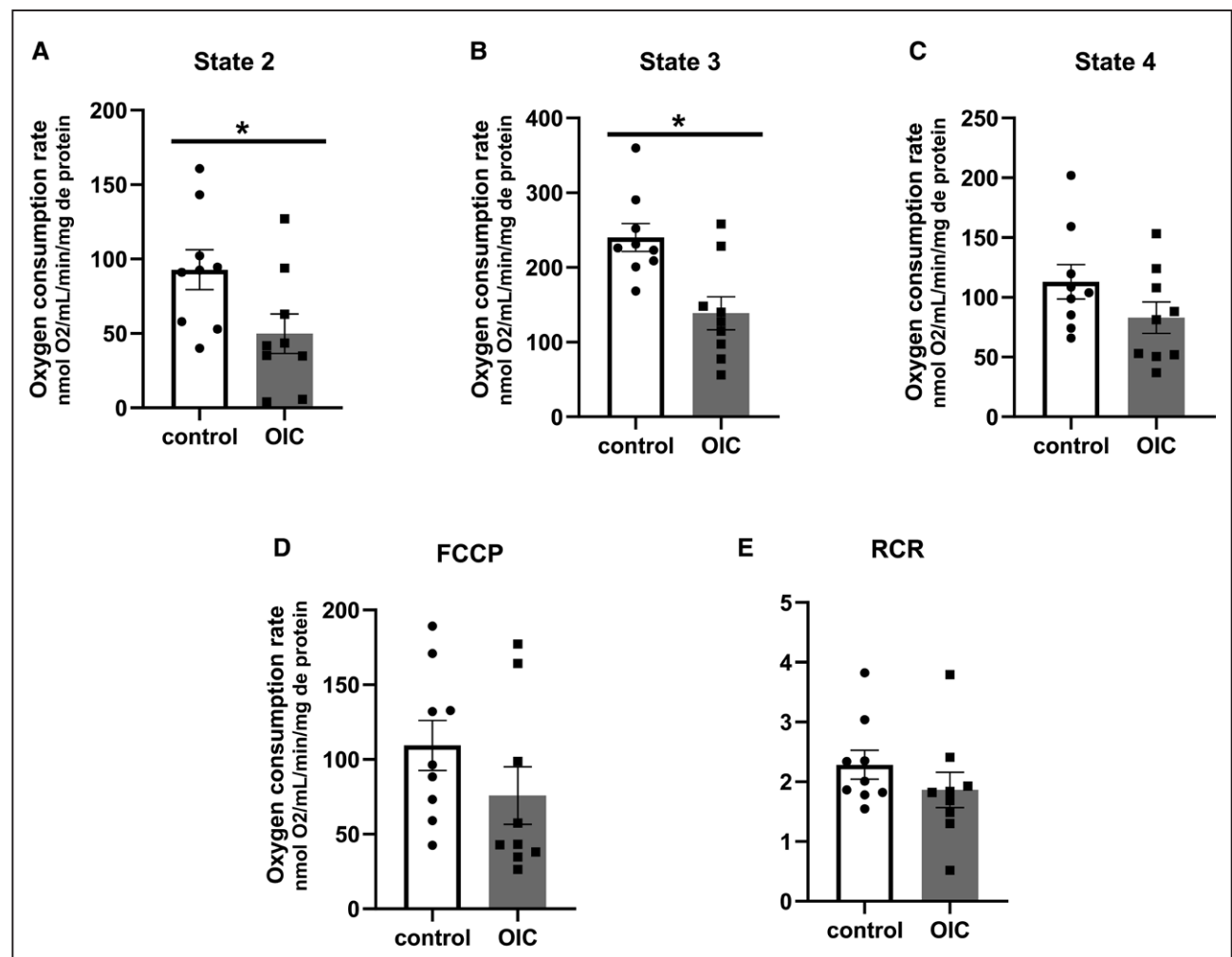
antioxidant capacity stimulating antioxidant response elements and in the promotion of mitogenesis, was reduced in OIC rats (Figure 4F).

### Mitochondria-Derived Peptide Humanin and Left Ventricle Function in Young Adults Born Preterm Versus Term

There were 55 participants in the preterm group, 54 in the full-term group, with 53% and 59% women, respectively. Median age on study day was 23 years (range,  $\approx 21$ –25). There were no significant differences between groups in baseline characteristics (Table), except for a slight elevation in median diastolic blood pressure (about 3 mmHg) in the preterm group. Left

ventricle ejection fraction and systolic blood pressure were within normal range.

Serum humanin levels (Figure 5A) were lower in the preterm group than in the full-term group (median [interquartile range], 133 [105–189] pg/mL versus 161 [124–252] pg/mL, respectively;  $P=0.041$ ). Left ventricle ejection fraction was similar and within the normal range in the term and the preterm groups. However, lower serum humanin levels were associated with lower left ventricular ejection fraction, both in the preterm group (Figure 5B) and in the term group (Figure 5C). Lower humanin levels were also associated with a less negative peak global longitudinal strain (less myocardial shortening during contraction) both in preterm (Figure 5D) and in term group (Figure 5E). When stratified by sex, similar



**Figure 2. Mitochondrial respiration in isolated mitochondria from left ventricle cardiac muscle.**

Rates of oxygen consumption in isolated left ventricular mitochondria from rats exposed only to room air (control) and rats exposed to hyperoxia as newborns (oxygen-induced cardiomyopathy [OIC]). Values are shown at metabolic state 2 (A); metabolic state 3 (B) metabolic state 4 (C); and uncoupled respiration, using carbonyl cyanide-4- (trifluoromethoxy)phenylhydrazone (FCCP; D). Respiratory control ratio (RCR) indicates functional integrity of mitochondria (E). Data shown are mean $\pm$ SEM; n=9/group. \* $P$ <0.05 comparing control vs OIC with unpaired  $t$  test.

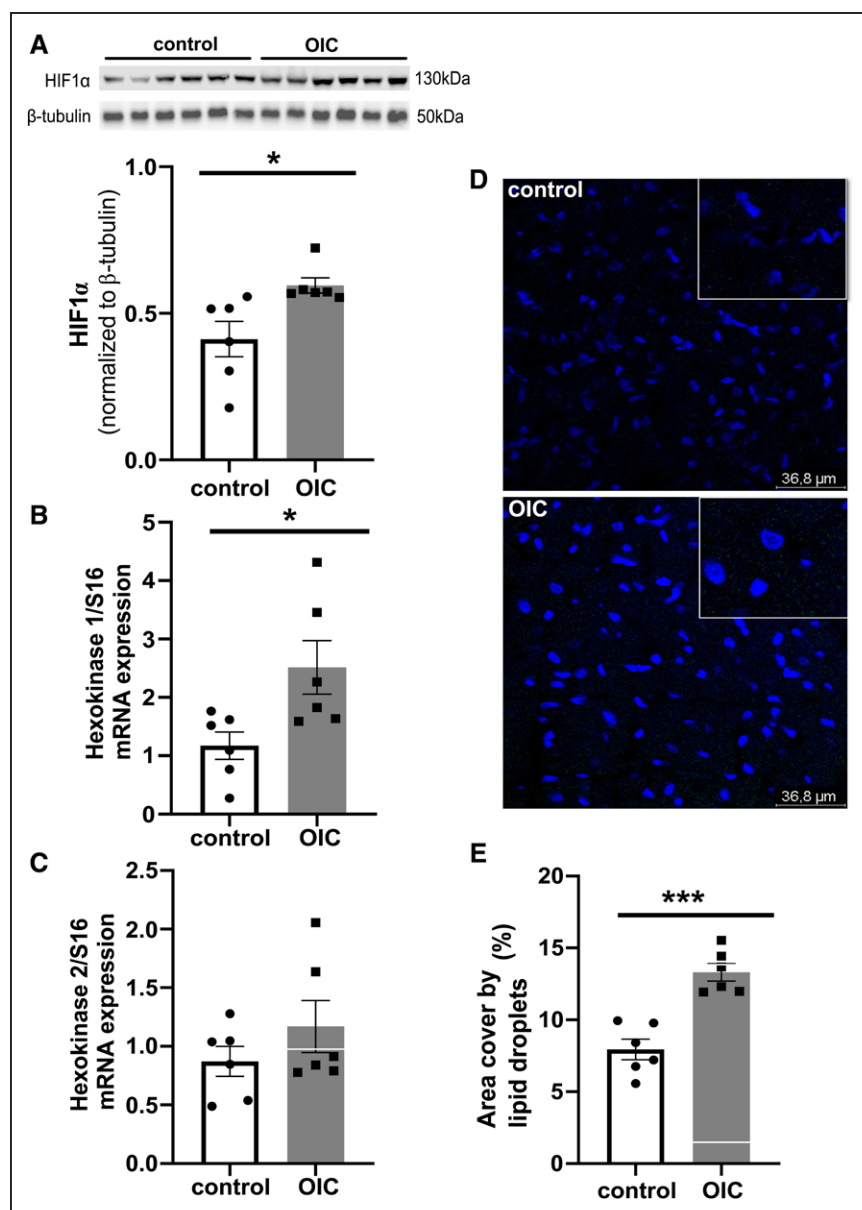
pattern in humanin serum levels was present between preterm and full-term. In males, humanin level in full-term (n=22) group were 208 (155–264) pg/mL versus 151 (103–193) pg/mL in preterm (n=25). In females, humanin serum levels in full-term (n=32) group were 147 (120–196) pg/mL versus 130 (109–184) pg/mL in preterm (n=29).

## DISCUSSION

The current study showed that transient neonatal exposure to hyperoxia in rats (a model of preterm birth) leads to alterations in cardiac left ventricle mitochondrial structure and function later in life. Disruptions included reduction of mitochondrial size and integrity, decreased oxidative phosphorylation capacity, reduced mitochondrial biogenesis, and abundance. Glycolysis markers were upregulated, as was intracardiac lipid deposition and production of mitochondrial superoxide, whereas gene expression of

antioxidant response element inducer Nrf2 was reduced. In the investigation of young adults born preterm versus full-term, we showed lower serum levels of humanin, a mitochondrial-derived peptide, and biomarker of mitochondrial function.<sup>19</sup> Humanin levels were associated with left ventricle ejection fraction and peak global longitudinal strain. Taken together, these results suggest that preterm birth-related neonatal conditions result in significant cardiac mitochondrial dysfunction beyond the neonatal period.

At birth, the myocardium must shift from normal hypoxic conditions of fetal life to ex utero oxygen levels.<sup>36</sup> This process is intricately tied to mitochondrial maturation and function.<sup>11,12,14</sup> When birth is preterm, an immature myocardium must undertake the transition. Clinical<sup>3–7</sup> and experimental<sup>8,9,15,37</sup> studies have demonstrated morphological and functional cardiac alterations in adults born preterm, as well as in animal models<sup>37,38</sup> simulating conditions associated with preterm birth.



**Figure 3. Glycolysis activation and lipid deposition in left ventricle cardiac tissue.**

**A**, Representative blot and histogram of compiled data of the protein HIF (hypoxia-inducible factor)-1 $\alpha$  expression in rats exposed only to room air (control) and rats exposed to hyperoxia as newborns (oxygen-induced cardiomyopathy [OIC]);  $\beta$ -tubulin was used as internal standard. Hexokinase 1 mRNA (**B**) and hexokinase 2 mRNA (**C**) are expressed relative to the 40S ribosomal protein S16 (S16). Representative image (**D**) and quantification (**E**) of intracardiac lipid deposition in left ventricle sections. Scale bar: 36.8  $\mu$ m. Data shown are mean  $\pm$  SEM;  $n=6$ /group. Lipid droplets appear as green fluorescent dots in the representative images. \* $P<0.05$ ; \*\*\* $P<0.001$  comparing control vs OIC with unpaired  $t$  test.

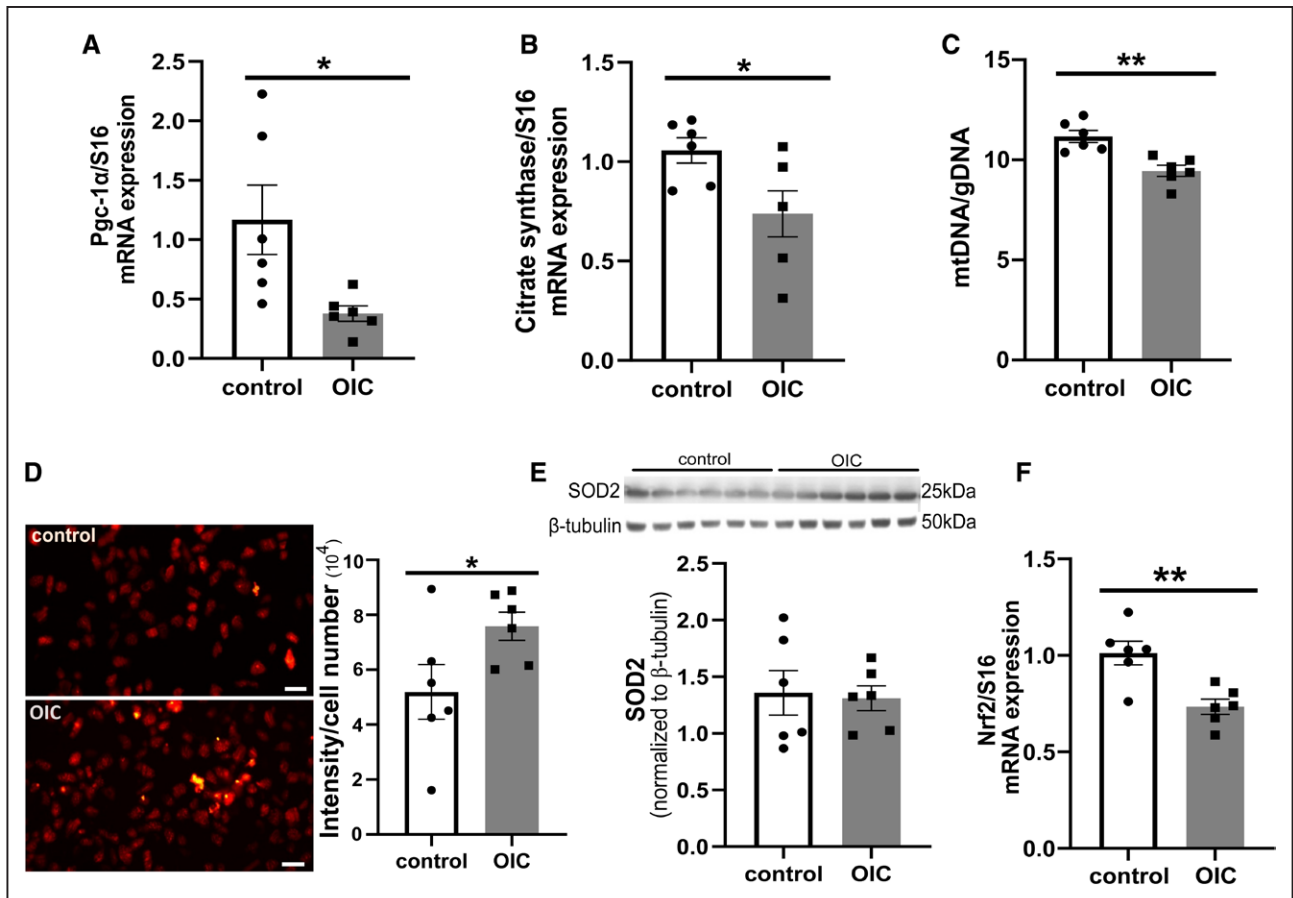
However, mechanisms of cardiac alterations after preterm birth are not well known.

The present study demonstrated substantial changes in left ventricle mitochondrial ultrastructure and function. It is in the perinatal period that important morphological changes of cardiac mitochondria occur, with increasing level of structural complexity and spatial organization that were found clearly disrupted in the left ventricle of OIC rats. These changes were observed in 4-week-old animals, in which we had previously shown normal blood pressure and minimal changes in left ventricle function.<sup>8</sup> Along with increased profibrotic signaling pathway (increased expression of TGF1 $\beta$ ) but normal TNF $\alpha$ , these mild alterations in the left ventricle echocardiography parameters in OIC are in line with cardiac functional and structural changes observed in young adults born preterm.<sup>4,39,40</sup> Taken together, these studies show the

clinical relevance of the experimental model and that, in OIC, mitochondrial dysfunction is present before significant cardiac dysfunction.

Left ventricle mitochondria from OIC rats showed reduced oxidative phosphorylation capacity and mitochondrial respiration compared with controls as well as lower protein expression of the electron transport chain subunits encoded by mtDNA (complex I, III, IV, and V). Reduced oxidative phosphorylation was associated with increased expression of HIF-1 $\alpha$  and hexokinase 1 in OIC hearts, both known to be involved in glycolysis upregulation.<sup>14,41</sup> The mechanism by which neonatal hyperoxia results in these changes is unknown and could imply a reprogramming in mitochondrial function.

In adult humans and animal models for cardiac diseases, left ventricle hypertrophy and heart failure are characterized by a metabolic reprogramming



**Figure 4. Mitochondrial biogenesis and abundance, mitochondrial superoxide production, SOD2 (superoxide dismutase 2), and Nrf2 (nuclear factor erythroid 2–related factor) expression in cardiac left ventricle.**

Markers of mitochondrial biogenesis and abundance in left ventricle tissue from rats exposed only to room air (control) and rats exposed to hyperoxia as newborns (oxygen-induced cardiomyopathy [OIC]): Pgc (peroxisome proliferator-activated receptor gamma coactivator)-1 $\alpha$  gene expression (A), citrate synthase (B), the ratio of mitochondrial DNA (mtDNA) to genomic DNA (gDNA; C) were all reduced in rats with OIC compared with controls. Mitochondrial superoxide production was increased in isolated left ventricular cardiomyocytes from OIC rats as compared to room air (control); representative images and quantification (D). SOD2 protein expression (representative blot with  $\beta$ -tubulin as internal standard and histograms of compiled data) is shown in (E) and Nrf2 gene expression in (F) in left ventricle. Gene mRNA expression are expressed relative to the 40S ribosomal protein S16 (S16). Data shown are mean $\pm$ SEM; n=6/group. \* $P$ <0.05, \*\* $P$ <0.01 comparing control vs OIC with unpaired  $t$  test.

with increased dependency on glycolysis to generate ATP.<sup>42,43</sup> A reduction in HIF-1 $\alpha$  signaling is also required for mitochondrial maturation and adaptation during the transition from late gestation to postnatal life.<sup>14,44</sup> HIF-1 $\alpha$  is a key determinant of metabolic regulation and the glycolytic metabolic program; its (over)activation is associated with lipid droplet accumulation<sup>41</sup> as we found in the OIC left ventricle. A major role for metabolic alterations in the cardiac consequences following neonatal hyperoxia exposure has recently been suggested at the level of the atria.<sup>45</sup> Cohen et al<sup>45</sup> showed in mice that neonatal hyperoxia exposure impairs the proliferation of left atria cardiomyocytes by repressing major components of fatty acid synthesis.

A reduction in oxidative phosphorylation capacity can also be partially compensated by increased mitochondrial mass.<sup>46</sup> We observed rather the opposite, with a marked downregulation of major regulators of mitochondrial

biogenesis, and of total mitochondrial area/cardiomyocyte.<sup>47</sup> Expression of Pgc-1 $\alpha$ , a major coordinator of mitochondrial biogenesis, was reduced which in turn can contribute to the decrease in oxidative phosphorylation capacity in the OIC rats. Current results are consistent with observations by Goss et al,<sup>38</sup> who reported decreased PGC-1 $\alpha$  in the right ventricle of 1-year old rats exposed to neonatal hyperoxia; the left ventricle was not evaluated in that study.

Supraphysiological levels of oxygen decrease mitochondrial respiration and promote mitochondrial production of reactive oxygen species.<sup>48</sup> Mitochondrial superoxide production was increased in left ventricle myocytes from OIC. Furthermore, whereas SOD2 expression was similar between groups, expression of Nrf2, a transcription factor involved in the induction of a battery of antioxidant defenses and mitochondrial biogenesis, was reduced in OIC, suggesting a lesser



**Table. Clinical Characteristics of Study Participants**

Characteristics	Full-term birth (n=54)	Preterm birth (n=55)	P value*
Male sex, n (%)	22 (41)	26 (47)	0.56
Gestational age, wk, median (range)	39.8 (39 to 40.4)	27.5 (26.2 to 28.4)	...
Birth weight, g, median (range)	3358 (3131 to 3641)	940 (855 to 1135)	...
Maternal preeclampsia, n (%)	4 (13)	6 (11)	>0.99
Bronchopulmonary dysplasia,† n (%)	...	17 (31)	...
Age on study day, y, median (range)	23.2 (21.9 to 24.8)	23.5 (21.2 to 25.4)	0.69
Systolic blood pressure, mmHg, median (range)	114 (105 to 122)	116 (108 to 131)	0.084
Diastolic blood pressure, mmHg, median (range)	67 (62 to 74)	70 (60 to 75)	0.027
Left ventricular ejection fraction, %, median (range)	57.6 (52.1 to 60.3)	57.0 (52.3 to 61.2)	0.71
Peak global longitudinal strain, %, median (range)	-21.8 (-23.9 to 0.86 -17.9)	-21.3 (-23.7 to -18.8)	0.86

\*P values were calculated using Fisher's exact test or the Mann-Whitney U test.

†Bronchopulmonary dysplasia defined as oxygen or respiratory support at 36 wk postmenstrual age.

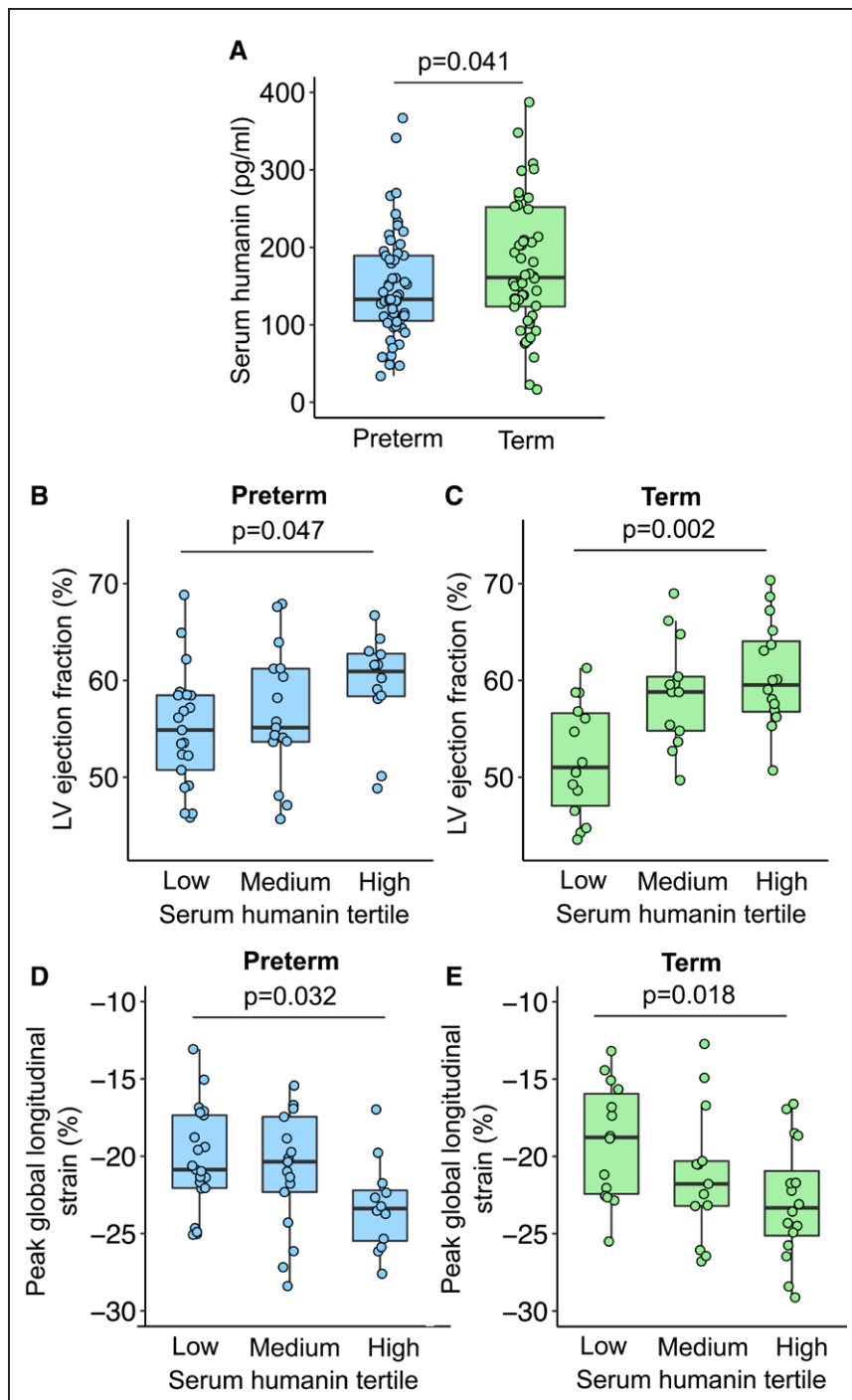
capacity to regulate oxidative stress.<sup>49</sup> Electron transport chain and oxidative phosphorylation deficits are associated with increased reactive oxygen species production that can be both cause and/or effect of mitochondrial dysfunction.<sup>50</sup>

Under physiological conditions, fatty acid oxidation at the mitochondrial matrix increases permanently in the perinatal period. Although we did not specifically assess substrate oxidation at mitochondrial level and considering that cardiac metabolism regulation includes components beyond the mitochondrial step itself, the evidence of oxidative phosphorylation machinery rarefaction (demonstrated by decreased oxygen consumption rate and reduced protein expression of electron transport chain complexes), along with accumulation of intracardiac lipid, support the presence of mitochondrial energetic dysfunction in the left ventricle of OIC rats.

A role for altered mitochondrial oxidative phosphorylation capacity in preterm birth-related complications in humans has been suggested by other reports. Endothelial cells taken from the umbilical cord vein of extremely preterm neonates who later died or developed bronchopulmonary dysplasia showed lower oxygen consumption rates and increased superoxide and hydrogen peroxide production as compared to others.<sup>51</sup> In adults born preterm, mitochondrial oxygen consumption patterns in circulating peripheral blood mononuclear cells were similar to those reported for individuals diagnosed with metabolic diseases, such as type II diabetes.<sup>52</sup> These studies support the concept that preterm birth impacts cellular energetic metabolism in several tissues. To translate our experimental findings to a clinical context, we measured circulating levels of humanin, a peptide encoded by mtDNA and considered mitoprotective and cytoprotective. We found lower humanin levels in young adults born preterm versus full-term. Lower humanin levels were associated with decreased left ventricular ejection fraction and decreased left ventricle performance by strain analysis (less negative peak global longitudinal strain) in both preterm and full-term participants. To our

knowledge, there are no previous studies reporting a correlation between circulating humanin levels and left ventricle function in humans, nor the measure of humanin in preterm-born subjects. As per current knowledge, higher humanin levels are associated with preserved coronary endothelial function in adults<sup>16</sup> and the administration of humanin analogs improves ventricular function in rodent<sup>18,53</sup> and pig<sup>17</sup> models of heart failure. In vitro treatment with humanin analogs improved cardiac myoblast function and survival, protecting mitochondria from oxidative stress induced by hydrogen peroxide.<sup>54</sup> In newborns, cord blood humanin correlated with mitochondrial function measured in cord blood peripheral blood mononuclear cells<sup>19</sup>; whether cord blood humanin levels are modulated by perinatal factors such as prematurity is unknown. Our results suggest that mitochondrial dysfunction is present in preterm-born adults and that these alterations may be related to left ventricular systolic function. Because left ventricle ejection fraction and left ventricle systolic deformation by speckle-tracking echocardiography were within normal range for most participants,<sup>20</sup> we cannot extrapolate current results to diseased states. How left ventricle systolic function in preterm individuals will evolve with aging and associated age-related risk factors, and whether serum humanin could be used to predict risk of heart disease, remain to be determined.

Limitations apply to our study. The OIC model does not fully capture the spectrum of conditions experienced by infants born preterm. Nonetheless, the critical role of high oxygen in the causal pathway of the most important short-term complications of preterm birth, as well as our previous reports showing how accurately this model mimics clinical cardiovascular findings, support its relevance. Only males were studied and accordingly we cannot extrapolate the findings to female rats which would need to be included in future assessments. We expect to find similar results between males and females as we previously reported that male and female rats showed similar blood pressure, vascular, renal, and hemoglobin



**Figure 5. Clinical study: humanin levels and left ventricle (LV) ejection fraction in young adults born preterm vs full-term.**

Serum humanin levels in young adults born preterm (<30 wk' gestation) and full-term ( $\geq 37$  wk' gestation; **A**). LV ejection fraction in preterm (**B**) and full-term groups (**C**) and peak global longitudinal strain (%) in preterm (**D**) and full-term groups (**E**) by humanin tertile. Tertiles: low ( $\leq 130$  pg/mL), medium (131–193 pg/mL), high ( $> 193$  pg/mL). Statistical tests: Mann-Whitney *U* (**A**) and Kendall (**B** and **C**).

changes after neonatal hyperoxia.<sup>15,55,56</sup> Pups survival and growth are also similar between sex and groups.<sup>15</sup> In clinical studies, increased neonatal risk related to males born preterm has been documented in large cohorts.<sup>57</sup> It remains unclear, however, if sex differences between preterm males and females persist similarly when examining adult cardiovascular disease: overall only a few studies to this date have the power to compare outcomes in males versus females and the vast majority of these studies are derived from one population. These reports showed either no difference, increased risk of disease for males,

or increased risk for females depending on the outcome examined.<sup>58,59</sup> Clinical papers from our group previously showed no sex-based difference between preterm and term individuals on overall cardiovascular risk factors, on the left ventricle<sup>20</sup> and the right ventricle<sup>60</sup> and on hemoglobin levels.<sup>61</sup> Nevertheless, in the clinical part of our study, both men and women were evaluated and although the lack of power prevent us to perform statistical analyses of sex-stratified results, humanin serum levels were similarly lower in preterm versus term for both males and females.

In conclusion, this 2-pronged study shows that transient neonatal hyperoxic exposure results in significant structural and functional mitochondrial alterations in cardiac muscle cells of the left ventricle in juvenile animals. In a cohort of young adults born extremely preterm (average 27 weeks' gestation), we report lower levels of the mitochondrial-derived peptide humanin; humanin levels correlated with left ventricular ejection fraction.

## PERSPECTIVES

Our results combining experimental and clinical studies indicate that preterm birth can result in left ventricle mitochondrial alterations that are perpetuated into adulthood. The role of mitochondrial dysfunction in the development of cardiac disease after preterm birth, and whether humanin can be used as a biomarker of disease and to monitor the benefits of intervention, will be important to examine.

## ARTICLE INFORMATION

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### Disclosures

None.

### Supplemental Materials

Expanded Methods

Table S1 and S2

Figures S1–S4

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