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## Sex differences in metabolic adaptation in infants with cyanotic congenital heart disease

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### Abstract

**BACKGROUND:** Female infants with congenital heart disease (CHD) face significantly higher postoperative mortality rates after adjusting for cardiac complexity. Sex differences in metabolic adaptation to cardiac stressors may be an early contributor to cardiac dysfunction. In adult diseases, hypoxic/ischemic cardiomyocytes undergo a cardioprotective metabolic shift from oxidative phosphorylation to glycolysis which appears to be regulated in a sexually dimorphic

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#### AUTHOR CONTRIBUTIONS

T.O.F. and L.M. conceived and planned the experiments, T.O.F. carried out the experiments. A.C.P., G.M., and J.S. provided the samples. Z.Z., K.S.C., and C.S. analyzed and interpreted the data and produced the figures. T.O.F. drafted the manuscript and all authors contributed to the final manuscript.

#### COMPETING INTERESTS

The authors declare no competing interests

#### INFORMED CONSENT

Parental permission was obtained for patient participation for each respective institutional biorepository.

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manner. We hypothesize sex differences in cardiac metabolism are present in cyanotic CHD and detectable as early as the infant period.

**METHODS:** RNA sequencing was performed on blood samples (cyanotic CHD cases,  $n = 11$ ; controls,  $n = 11$ ) and analyzed using gene set enrichment analysis (GSEA). Global plasma metabolite profiling (UPLC-MS/MS) was performed using a larger representative cohort (cyanotic CHD,  $n = 27$ ; non-cyanotic CHD,  $n = 11$ ; unaffected controls,  $n = 12$ ).

**RESULTS:** Hallmark gene sets in glycolysis, fatty acid metabolism, and oxidative phosphorylation were significantly enriched in cyanotic CHD females compared to male counterparts, which was consistent with metabolomic differences between sexes. Minimal sex differences in metabolic pathways were observed in normoxic patients (both controls and non-cyanotic CHD cases).

**CONCLUSION:** These observations suggest underlying differences in metabolic adaptation to chronic hypoxia between males and females with cyanotic CHD.

## INTRODUCTION

Congenital heart diseases (CHD) are the most common type of birth defect worldwide, with the highest rates of birth defect-related morbidity and mortality.<sup>1</sup> Over the past forty years, medical and surgical advancements have resulted in steady improvements in survival rates and long-term outcomes.<sup>2</sup> And yet, postoperative morbidity and mortality for the most severe forms of CHD remains high with a one-year survival rate of ~75%.<sup>3</sup> Overall, infant deaths make up half of all CHD mortality.<sup>4</sup> In this high-risk age group, females with cyanotic CHDs have a 30–50% higher odds of mortality following cardiac surgery compared to their male counterparts after risk adjustment for CHD complexity and other attributable factors.<sup>5–7</sup> Considering the high energy demands of the heart, metabolic response to chronic hypoxia may be different between males and females contributing to sex differences in mortality rates.

During normal fetal development, the placental blood supply to the fetus is relatively hypoxic. Hypoxia upregulates transcription factor hypoxia-inducible factor 1 subunit alpha (HIF-1 $\alpha$ ), driving anaerobic glycolysis to provide the main source of energy for the heart and promoting cardiomyocyte proliferation.<sup>8–11</sup> At the birth of unaffected newborns, delivery into an oxygen-rich environment inactivates HIF-1 $\alpha$  and activates transcription factor peroxisome proliferator activated receptor alpha (PPAR $\alpha$ ), triggering a gradual metabolic shift from glycolysis to oxygen-dependent oxidative phosphorylation.<sup>8,12–14</sup> In contrast, neonates with cyanotic CHD remain chronically hypoxic after birth, but the newborn heart is tolerant to oxygen deprivation likely due to an increased capacity for glycolysis.<sup>14,15</sup> Adult studies have shown the counterbalancing roles of HIF-1 $\alpha$  and PPAR $\alpha$  also regulate metabolic shifts triggered by cardiac stressors such as hypoxia/ischemic injury, ventricular hypertrophy, and heart failure, reverting back to glycolysis as a form of cardioprotection.<sup>12,16–19</sup> Impaired or inappropriate metabolic regulation results in cardiac dysfunction and appears to be a key driver in sex-dependent heart failure.<sup>18,20–24</sup>

Compared to acquired adult diseases, less is known on the early metabolic impact of hypoxia exposure in CHD. Prior studies involving infants with CHD have sought to detect

global differences in metabolite levels related to cardiopulmonary bypass during surgery, identifying biomarkers associated with clinical outcomes.<sup>25–29</sup> However, dedicated pediatric studies remain limited and sex differences remain relatively unexplored. Our study objective is to investigate sex differences in gene expression and metabolic profiles of infants with cyanotic CHD along key metabolic pathways utilized by cardiomyocytes. We hypothesize that there is a sexually dimorphic metabolic adaptation to chronic hypoxia in cyanotic CHD cases, providing insight into the biological mechanisms that may contribute to early infant mortality in females.

## METHODS

### Patient sample collection

Patients were enrolled into biobank programs at two separate institutions. In Houston, Texas, newborns admitted to the neonatal intensive care unit (NICU) were enrolled into the UTHealth Houston BioRepository for NICU Diseases with parental consent from January 2020 to December 2021. A small volume of blood (1 mL) was collected in EDTA-coated tubes and tubes containing RNA stabilizer in the first month of the NICU hospitalization and prior to any cardiac surgery for CHD cases. EDTA tubes were centrifuged within six hours of collection at 1850 g for 10 min at 4 °C. Approximately 100 to 200 µL of plasma was extracted without disturbing the buffy coat and stored at –80 °C. Blood samples in tubes containing RNA stabilizer were stored unprocessed at –80 °C for subsequent use. In Jackson, Mississippi, pediatric patients undergoing cardiac surgery at the University of Mississippi Medical Center (UMMC) were enrolled into the UMMC Biobank with parental consent from January 2013 to December 2017. Blood samples were collected in the operating room prior to surgical incision; no blood samples were stored in tubes containing RNA stabilizer. Similar processing and storage procedures for plasma were used at UMMC. The age of the patients at the time of collection was influenced by the biobank protocol at each participating institution. The timing and frequency of blood collection was limited to minimize extraneous needle sticks and iatrogenic blood loss. Samples at UMMC were collected prior to cardiac surgery and so were influenced by the timing of surgery. See other patient characteristics and cardiac phenotypes in Supplementary Table 1. Informed patient consent was obtained for study participants, and the study received approval from the Institutional Review Board at the University of Texas Health Science Center at Houston.

Patients from both institutions were selected and divided into three groups based on cardiac phenotype: cyanotic CHD, non-cyanotic (normoxic) CHD, and normoxic controls without CHD (Fig. 1). Inclusion criteria for control patients were the absence of cardiopulmonary anomalies, gestational age greater than 32 weeks, and absent or minimal respiratory support, as defined as non-invasive respiratory support with fractional inspired oxygen (FiO<sub>2</sub>) of 21% (room air) with normal oxygen saturations. Exclusion criteria were mechanical ventilation or requirement of supplementary oxygen (FiO<sub>2</sub> > 21%).

### Bulk RNA sequencing and data analysis

Cyanotic CHD cases ( $n = 11$ ) and controls without CHD ( $n = 11$ ) were chosen as comparative cohorts for the blood transcriptomics study as they were the most

phenotypically distinct and similar in age. RNA-stabilized samples were thawed and incubated for a minimum of 2 h at room temperature to allow for complete lysis of blood cells. Total RNA extraction was performed using RNeasy® blood kits in accordance with the manufacturer's protocol (Qiagen, Germantown, Maryland). RNA samples were submitted to the Cancer Genomics Center at The University of Texas Health Science Center at Houston. Total RNA was quality-checked using Agilent RNA 6000 Pico kit (#5067–1513) by Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California). RNA with an integrity number of greater than 7 was used for library preparation. Libraries were prepared with New England Biolabs's NEBNext Ploy(A) mRNA Magnetic Isolation Module (E7490L), NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (E7760L), and NEBNext Multiplex Oligos for Illumina (E6609S) by following the manufacturer's instructions. The quality of the final libraries was examined using Agilent High Sensitive DNA Kit (#5067–4626) by Agilent Bioanalyzer 2100, and the library concentrations were determined by qPCR using Colibri Library Quantification kit (#A38524500, Thermo Fisher Scientific, Waltham, Massachusetts). The libraries were pooled evenly and underwent paired-end 75-cycle sequencing on an Illumina NextSeq 550 System using High Output Kit v2.5 (#20024907, Illumina, San Diego, California).

Raw mRNA sequence reads were processed using Cutadapt and bases with quality scores less than 20 were removed.<sup>30</sup> Filtered RNA-sequence reads were aligned to the human reference genome (GRCh38.102) and gene read counts were generated by STAR.<sup>31</sup> Genes with less than 5 reads among the samples were removed. The differential gene expression analysis was conducted by DESeq2, which models the read counts to follow the negative binomial distribution.<sup>32</sup> Differentially expressed genes (DEG) were defined as genes with a fold change greater than 1.2 ( $\log_2$  fold change  $> 0.26$ ) and  $p < 0.05$  after adjusting for multiple comparisons. Functional enrichment was analyzed by gene set enrichment analysis (GSEA) to compare gene expression levels to an a priori established gene set.<sup>33,34</sup> GSEA was utilized to compare gene expression between females and males (cyanotic CHD patients analyzed separately from controls). The magnitude of enrichment was quantified by normalized enrichment score (NES) with significance determined by  $|\text{NES}| > 1$ , nominal  $p < 0.05$ , and  $q < 0.25$ . The comparison was performed using the Molecular Signatures Database (MSigDB) Hallmark gene set<sup>35</sup> as a reference, which highlights well-characterized biological states and processes by summarizing multiple gene sets and eliminating overlapping genes.

### Global plasma metabolite profiling and data analysis

Cyanotic CHD patients ( $n = 27$ ), non-cyanotic CHD ( $n = 11$ ), and normoxic controls without CHD ( $n = 12$ ) were included in the plasma metabolomics study. Plasma samples were thawed on ice, aliquoted, and shipped on dry ice to Metabolon (Metabolon, Morrisville, North Carolina). Following receipt, all samples were maintained at  $-80^\circ\text{C}$  until processed per standardized protocols and quality control measures using the automated MicroLab STAR® system (Hamilton Company, Reno, Nevada). The extract was divided into five fractions: two for analysis by two separate reverse phase (RP)/ultra-performance liquid chromatography (UPLC)- mass spectrometry (MS)/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode

ESI, one for analysis by hydrophilic interaction chromatography (HILIC)/UPLC-MS/MS with negative ion mode ESI, and one sample was reserved for backup. Instrument variability was determined by calculating the median relative standard deviation for the standards added to each sample. Process variability was determined by calculating the median relative standard deviation for all endogenous metabolites present in 100% of the pooled matrix samples.

Raw data was extracted, peak-identified, and processed for quality control. Compounds were identified by comparison to library entries of purified standards or recurrent unknown entities. A total of 1012 compounds of known identity were included in the analysis. Biochemical identifications were based on three criteria: retention index within a narrow window of the proposed biochemical identification, accurate mass match to the library  $\pm$  10 ppm, and the MS/MS forward and reverse scores between the experimental data and standards. Peaks were quantified using area-under-the-curve.

In the statistical analysis, Welch's two-sample t-test was used to compare metabolite levels between the study groups. Threshold of statistical significance was  $p < 0.05$ . An estimate of the false discovery rate ( $q$  value) for multiple comparisons was set at  $q < 0.10$  for statistical significance. Sparse partial least squares discriminant analysis (sPLS-DA) was performed to provide a comparative overview of the metabolomics data by study groups.

## RESULTS

### Gene expression in metabolic pathways

Gene expression was compared between sexes within each study cohort of cyanotic CHD cases and controls without CHD. This study design allowed for more precise examination of sex differences in hypoxic and normoxic conditions. Among cyanotic CHD cases, there were 655 differentially expressed genes between females and males (see volcano plot, Fig. 2a). As expected, the expression of genes located on sex chromosomes (e.g., *RPS4Y1*, *EIF1AY*, *KDM5D*, *UTY*, *XIST*) were differentially expressed between sexes in both study groups, confirming that expression of those genes is sex-determined in neonates.<sup>36</sup> The top differentially-expressed genes in both study groups were present on sex chromosomes (Table 1). Eight other genes were differentially expressed only in cyanotic CHD patients, and one gene was differentially expressed exclusively in normoxic controls. Differential expression of target genes of *HIF-1 $\alpha$*  were explored but none were statistically significant after adjusting for multiple comparisons (data not shown).

Utilizing GSEA allows incorporation of enriched transcriptional events along signaling pathways as opposed to single gene events.<sup>33</sup> Critical hypoxia-regulated transcriptional events involving *HIF-1 $\alpha$*  and *PPAR* regulate glycolysis and fatty acid oxidation.<sup>37</sup> In the comparison of cyanotic CHD females and cyanotic CHD males, GSEA identified 19 Hallmark gene sets that were significantly enriched (Fig. 2b). Key metabolic pathways were significantly overrepresented in cyanotic females including Hallmark\_glycolysis (NES = 1.298,  $p = 0.031$ ,  $q = 0.109$ ); Hallmark\_oxidative phosphorylation (NES = 1.543,  $p < 0.002$ ,  $q = 0.024$ ); Hallmark\_fatty acid metabolism (NES = 1.543,  $p = 0.002$ ,  $q = 0.022$ ) (see enrichment plots in Fig. 2d–g). Additionally, expression of hypoxic-related genes was

sexually dimorphic with Hallmark\_hypoxia gene set overrepresented in cyanotic females (NES = 1.387,  $p = 0.007$ ,  $q$  value 0.076). No metabolism gene sets were enriched by sex among normoxic controls without CHD (Fig. 2c).

### Metabolic adaptation to hypoxia

A larger representative cohort was used for global plasma metabolomic profiling including cyanotic CHD, non-cyanotic CHD, and controls without CHD. To illustrate overall group effects, comprehensive sPLS-DA was performed involving the three study groups. The results of sPLS-DA revealed that non-cyanotic CHD patients exhibited the highest degree of clustering for normoxic groups and therefore served as the reference group for subsequent analysis (Fig. 3a). When cyanotic CHD cases were analyzed separately, there was distinct clustering of males and females (Fig. 3b). Random forest analysis was performed to identify top-ranking discriminating metabolites between study groups. A predictive accuracy of 75% was obtained compared to 33% expected by random chance alone. The top discriminating metabolites included markers of amino acid, carbohydrate, lipid, and nucleic acid metabolism (Fig. 3c). Among these, 5-oxoproline (or pyroglutamic acid), the end-product of glutathione metabolism,<sup>38</sup> was the most discriminating metabolite between study groups. Aggregated cyanotic CHD cases had a 1.44-fold increase in 5-oxoproline when compared to non-cyanotic CHD cases ( $p = 0.0011$ ,  $q = 0.0010$ ). When disaggregated by sex, heightened levels of 5-oxoproline were predominantly observed in female cyanotic CHD cases by a 1.55-fold increase compared to female non-cyanotic CHD cases ( $p = 0.051$ ,  $q = 0.0157$ ). Conversely, no significant elevation in 5-oxoproline levels was noted in the male comparison groups ( $p = 0.1111$ ,  $q = 0.0480$ ).

Results of GSEA were used to inform the approach for comparative metabolic analysis between cyanotic CHD cases, non-cyanotic CHD cases, and controls without CHD. Each study group underwent separate analysis to pinpoint sex differences while addressing potential confounding variables such as differences in age and the presence/absence of CHD across groups. In normoxic patients (non-cyanotic CHD cases and controls without CHD), the metabolic profiles were similar between females and males. However, in cyanotic CHD cases, hypoxic females demonstrated nominally significant elevations in glycolytic intermediates (e.g., 2,3-diphosphoglycerate and 3-phosphoglycerate) and long-chain acylcarnitines compared to hypoxic males (Supplementary Table 2A). These differences suggested increased activity in glycolysis and fatty acid oxidation in female cyanotic CHD cases compared to males.

To further test our hypothesis, hypoxic patients were directly compared against normoxic patients with non-cyanotic CHD cases serving as the reference group. In the aggregated data, cyanotic CHD cases exhibited increased levels of lactate (fold change 1.32,  $p = 0.0113$ ,  $q = 0.0073$ ), the final by-product of glycolysis,<sup>39</sup> suggesting increased glycolytic activity in the presence of hypoxia which was an expected result. Along the citric acid cycle, early intermediates (e.g., citrate, isocitrate) were decreased but downstream intermediates were significantly increased, likely through a compensatory pathway.<sup>40</sup> Levels of long-chain acylcarnitines, markers of fatty acid oxidation activity,<sup>41,42</sup> were significantly reduced in cyanotic CHD cases (e.g., stearyl carnitine, fold change 0.77,  $p = 0.0045$ ,  $q = 0.0034$ ).

Overall, a metabolic shift away from fatty acid oxidation towards glycolysis was observed in cyanotic CHD cases compared to the reference group but the data was confounded by age differences limiting our interpretation (Supplementary Table 2B). However, these findings are consistent with the anticipated metabolic responses attributed to varying levels of oxygen. As a result, they provide a solid basis for deeper exploration of sex differences between these two groups.

In the sex disaggregated comparative analysis, male cyanotic CHD cases were compared with male non-cyanotic CHD cases as the reference group. Similarly, female cyanotic CHD cases were compared with female non-cyanotic CHD cases. If findings in the sex disaggregated data were similar to the aggregated data, this would suggest our observations were due to age differences alone and sex was unlikely a contributing factor. However, intermediates in glycolysis, citric acid cycle, and fatty acid oxidation were more disparate when analyzed by sex. In the aggregated dataset, glycolytic intermediates were mostly similar between cyanotic CHD cases and the reference group. Upon closer examination by sex, heightened levels of glycolytic intermediates were predominantly observed in female cyanotic CHD cases compared to female non-cyanotic CHD cases, including a significant 1.5-fold increase in lactate compared to female non-cyanotic CHD cases ( $p = 0.0005$ ,  $q = 0.0075$ ). In contrast, no significant elevation in lactate was seen in the male comparison groups (Fig. 4c). Evaluation of mitochondrial oxidation revealed that suppression of early intermediates in the citric acid cycle was only observed in male cyanotic CHD cases ( $p = 0.0188$ ,  $q = 0.0128$ ); there was no difference in the female comparison groups ( $p = 0.5077$ ,  $q = 0.3074$ ; Fig. 4d). Downstream citric cycle intermediates were significantly increased in both sexes, likely from a downstream compensatory pathway. Similarly for fatty acid oxidation, significant reductions in long-chain acylcarnitines were observed in the male comparison groups with no difference in the female comparison groups (Fig. 4h). Overall, these findings further confirm a sexually dimorphic response in glycolysis and mitochondrial oxidation in neonates exposed to chronic hypoxia (Fig. 5).

## DISCUSSION

Chronic hypoxia triggers a cascade of cellular mechanisms leading to a metabolic shift towards alternative sources of energy for cardioprotection.<sup>12,43,44</sup> Sex differences in metabolic responses to both acute and chronic hypoxia have been observed in animal models and adult diseases.<sup>18,20,45–48</sup> However, this remains understudied in children with CHD even though female sex is an independent risk factor for mortality after adjusting for age and surgical complexity.<sup>5</sup> Further investigation is warranted in this fragile patient population. Our study demonstrates that a sexually dimorphic metabolic change occurs in neonates with cyanotic CHD, likely attributable to chronic hypoxia. Females with cyanotic CHD exhibit augmented transcriptomic and metabolomic fatty acid and oxidative phosphorylation activity compared to their male counterparts. Suppression of mitochondrial oxidative activity is a known cardioprotective response in the setting of oxygen deprivation.<sup>12,17,18</sup> Somewhat unexpectedly, hypoxic females also showed increased expression of hypoxia-related genes and glycolytic activity.<sup>45</sup> Although differential expression of individual target genes of *HIF-1 $\alpha$*  were similar between sexes, expression of the Hallmark\_hypoxia gene set representing 200 genes targeted by *HIF-1 $\alpha$*  was upregulated in females with cyanotic

CHD.<sup>35</sup> Analyzing the collective expression pattern of gene sets can amplify coordinated gene changes compared to more subtle changes that may be missed when analyzing genes separately. While HIF and PPAR may appear to be in opposition, in postnatal cardiac mitochondria, PPAR gamma co-activator 1 (*PGC1 $\alpha$* ) expression does not depend on downregulation of *HIF*, but instead on oxygen levels.<sup>49</sup> We speculate that females with cyanotic CHD may have inappropriate responses to hypoxia that underlie these observed differences. Differences in energy capacity and utilization in the neonatal myocardium continues to be explored, such as whether increased glycolytic activity in neonatal females indicates early exhaustion of glycogen stores.<sup>45,50,12,17,18</sup> Overall, these findings are consistent with animal studies indicating females may be at a metabolic disadvantage to ischemic/hypoxic injury with higher rates of cardiac dysfunction and ultimately, ventricular failure.<sup>22,45,51</sup> Possible mechanisms include the PPAR $\alpha$ /estrogen signaling pathway which has been shown to be differentially regulated by sex and implicated in the pathophysiology of heart failure.<sup>20,23,24,52–54</sup>

Pediatric studies on metabolic effects of CHD have been more limited and will be reviewed here. Mitochondrial dysfunction and oxidative stress were found to be important contributors to severe heart failure in iPSC-derived cardiomyocytes in hypoplastic left heart syndrome, a severe form of cyanotic CHD.<sup>55</sup> In this study by Xu et al., groups were defined by severity of heart failure. However, females were only represented in the group of the most severe phenotype and excluded in the single-cell transcriptome profiling of iPSC-derived cardiomyocytes, limiting analysis of sex-related factors. Other metabolic effects of hypoxia on pediatric cardiomyocytes have been examined from discarded tissue excised during cardiac repair.<sup>56–58</sup> Of note, these studies lack unaffected controls as tissue from normal, healthy hearts is extremely difficult to obtain due to ethical and practical constraints. One study involving patients with cyanotic CHD ( $n = 25$ ) demonstrated that at the onset of puberty, suppression of *HIF-1 $\alpha$*  in cardiac tissue co-occurred with metabolic maladaptation and impaired cardiac function.<sup>57</sup> Others studies involving infants with CHD have utilized serum or plasma metabolites as a more feasible, non-invasive alternative.<sup>25,28,29,59</sup> Differentially expressed genes in oxidative phosphorylation, citric acid cycle, and fatty acid metabolism, in blood obtained from HLHS neonates were associated with post-operative low cardiac output syndrome ( $n = 13$ ), but the effect of sex was not explored.<sup>60</sup> Differences in metabolite panels (e.g., targeted vs. untargeted LC-MS panels) hinders grouped interpretation of multiple studies, nonetheless, glutathione metabolism was identified as a top discriminating pathways in another study comparing cyanotic and non-cyanotic CHD infants, consistent with our findings.<sup>28</sup> In these studies including ours, total metabolite concentrations do not accurately represent metabolite flux, limiting the interpretation of metabolic activity along interrelated pathways, including intracellular redox homeostasis. However, our findings indicate oxidative stress is handled differently between cyanotic males and females, potentially bearing implications for cell survival mechanisms.

Our study is limited to the use of blood samples which may not directly reflect the metabolic activity of the heart. However, plasma shares a similar profile with cardiac biochemistry while being minimally invasive,<sup>42,61</sup> permitting the inclusion of unaffected controls in our study. Another study limitation was that age differences were not balanced across the study groups introducing a potential confounder as the duration of hypoxia could influence results.



This is a common issue in studies involving blood and tissue collected at the time of cardiac surgery since children with non-cyanotic CHD often have cardiac surgery performed at an older age.<sup>28</sup> Our approach to first compare transcriptomic data between sexes confined to each study group was performed to abate potential age-related confounders. As a result, non-cyanotic CHD patients were not included in the transcriptomic analysis. While this is a notable study limitation, our primary objective was to elucidate potential pathways of interest specific to hypoxia and cyanotic CHD. Focusing the scope to two relatively homogeneous groups (cyanotic CHD and unaffected controls) avoided potential confounders related to the wider phenotypic variability in non-cyanotic CHD. Lastly, the small sample size posed a limitation to the robustness and generalizability of our findings. To mitigate the risk of spurious results from multiple comparisons, stringent correction methods using FDR adjustment was used, ultimately influencing the significance of the results.

This study presents evidence that genetic expression and metabolic activity in circulating blood undergo alterations in the setting of chronic hypoxia from cyanotic CHD. Furthermore, these alterations occur in a sexually dimorphic manner. Based on the known cardioprotective and diverse cellular functions driven by hypoxia, glycolysis and mitochondrial oxidation appear to be key distinguishing metabolic pathways between males and females with cyanotic CHD. Larger-scale metabolomic and transcriptomic sex-disaggregated studies involving pediatric patients with CHD are needed to confirm findings. Importantly, our findings suggest alternative metabolic pathways are utilized in neonates with chronically hypoxic conditions.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## DATA AVAILABILITY

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE269353 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE269353>).

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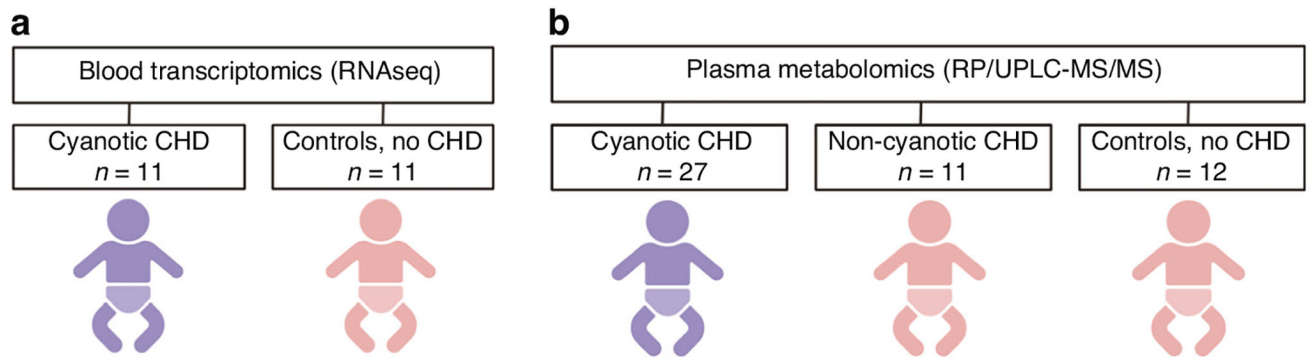
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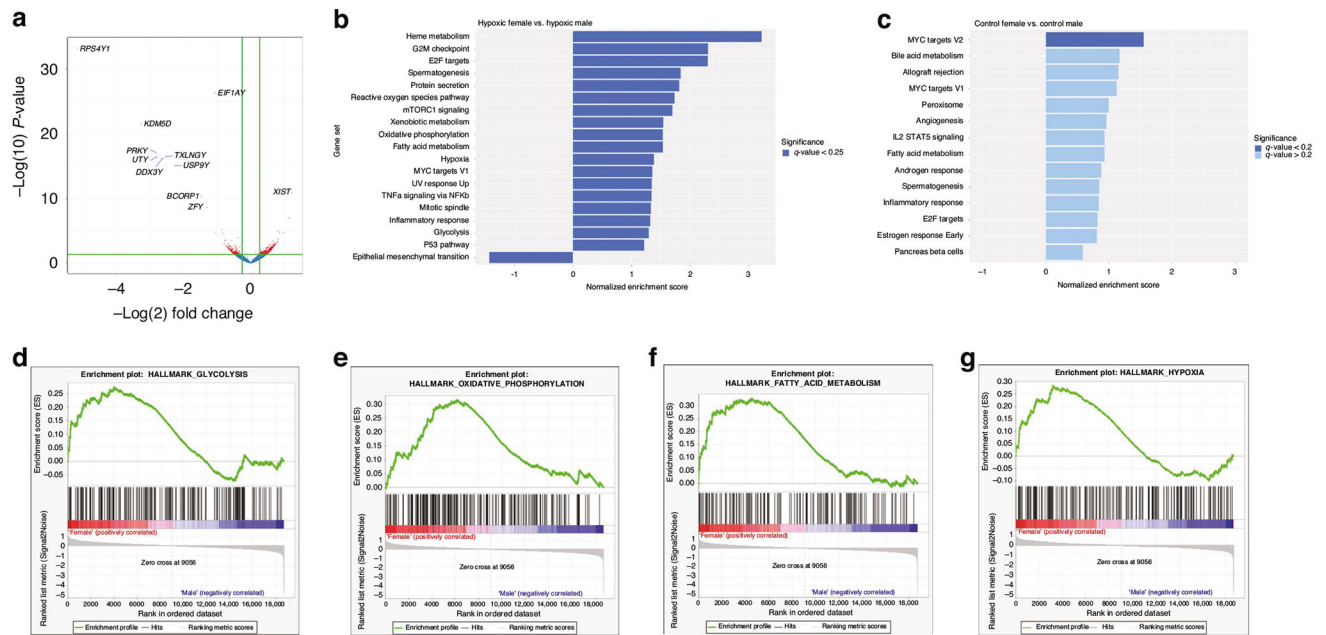
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**IMPACT:**

- Children with cyanotic CHD exhibit sex differences in utilization of glycolysis vs. fatty acid oxidation pathways to meet the high-energy demands of the heart in the neonatal period.
- Transcriptomic and metabolomic results suggest that under hypoxic conditions, males and females undergo metabolic shifts that are sexually dimorphic. These sex differences were not observed in neonates in normoxic conditions (i.e., non-cyanotic CHD and unaffected controls).
- The involved metabolic pathways are similar to those observed in advanced heart failure, suggesting metabolic adaptations beginning in the neonatal period may contribute to sex differences in infant survival.



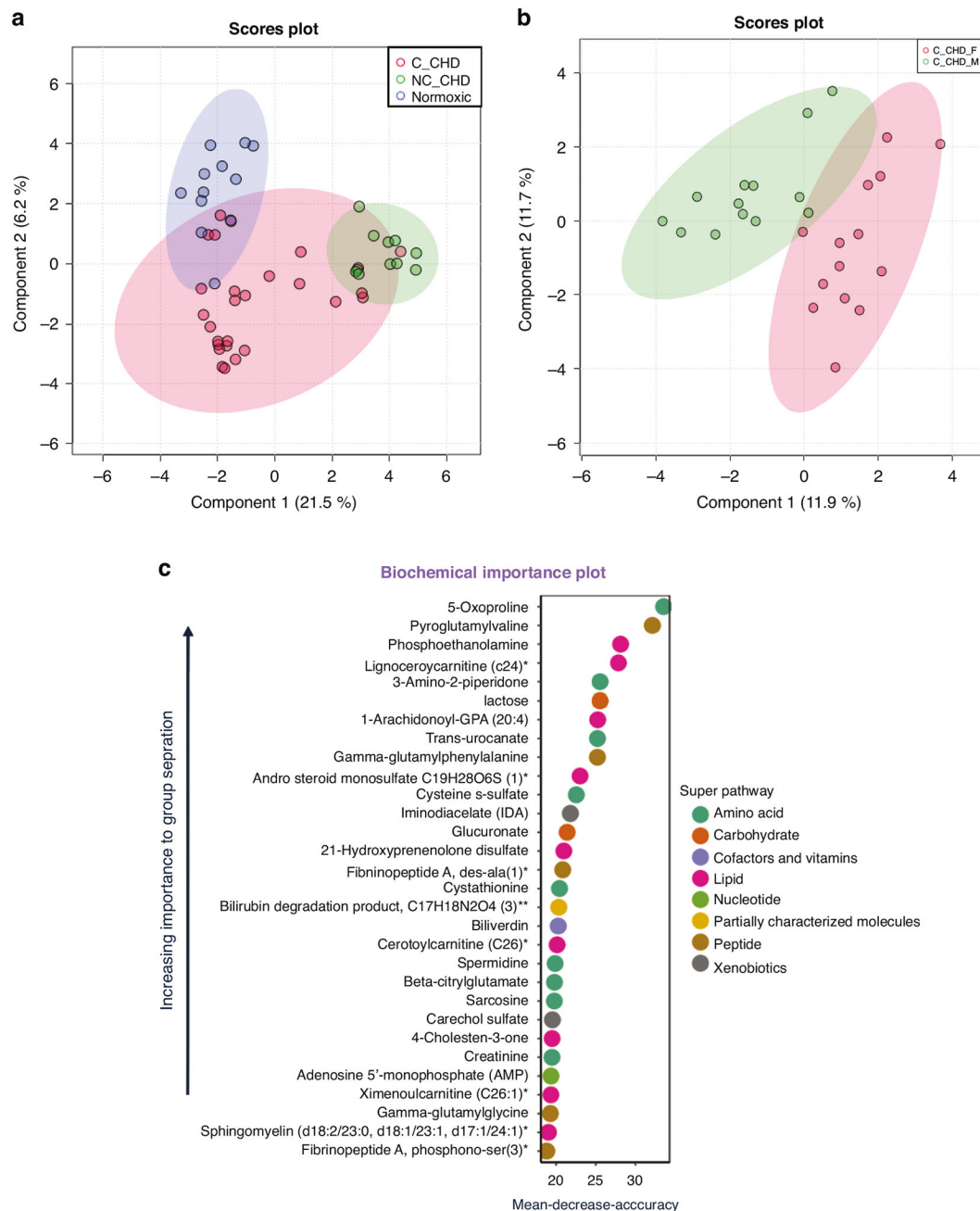
**Fig. 1. Comparison of differential expression of genes between cyanotic CHD females vs. males.** Cohort groups for (a) blood transcriptomics and (b) plasma metabolomics studies. Created with [BioRender.com](https://BioRender.com).



**Fig. 2. Comparison of differential expression of genes between sexes.**

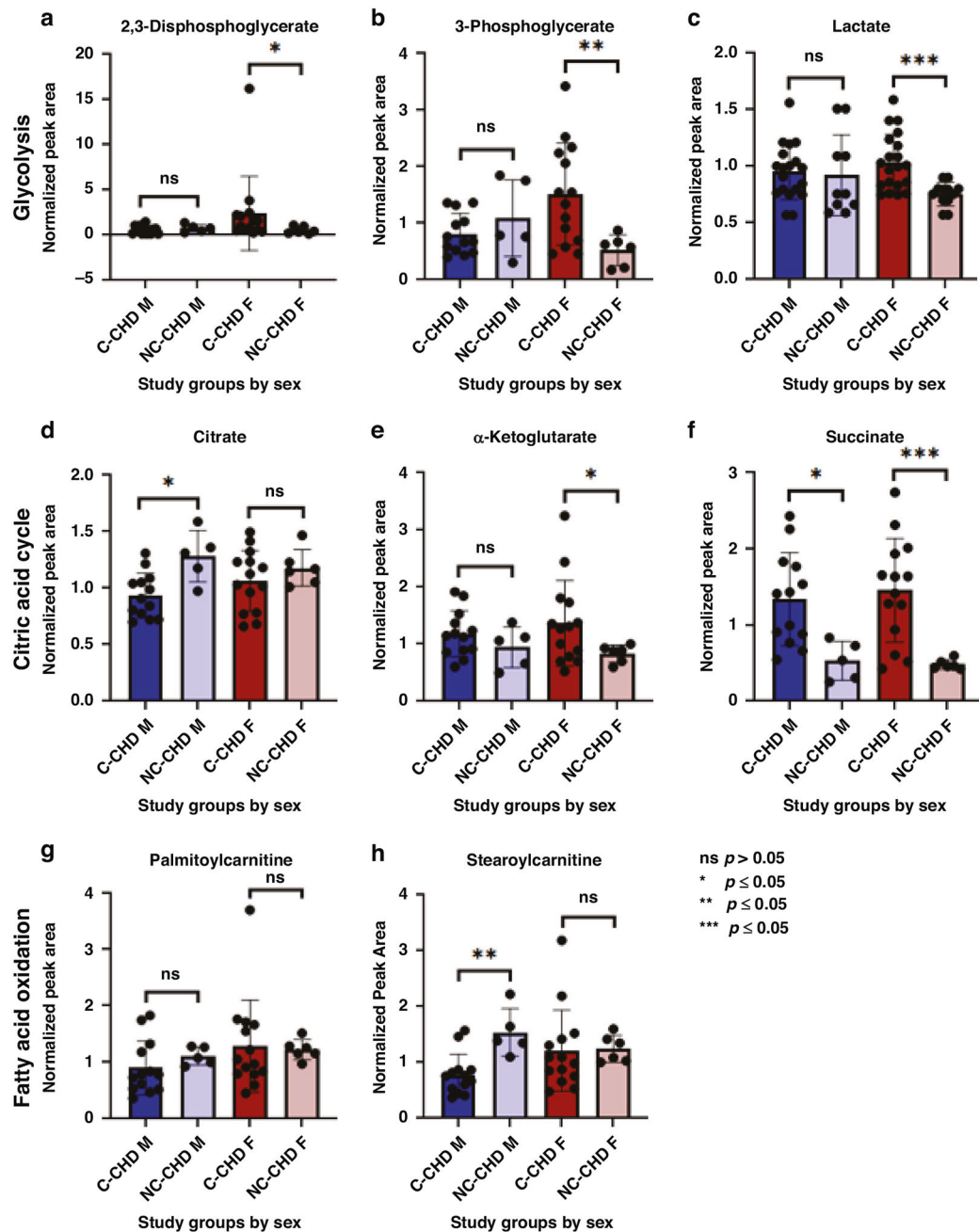
**a** Volcano plot comparing expression of genes between cyanotic CHD females vs. males. Threshold for significance (in red) of fold change  $>1.2$ ,  $p < 0.05$ . **b** GSEA between cyanotic females vs. cyanotic males and **(c)** normoxic control females vs. normoxic control males using MSigDB Hallmark gene set ranked by normalized enrichment score (NES) with significance threshold of  $|NES| > 1$ , nominal  $p < 0.05$ . Enrichment plots between cyanotic females vs. cyanotic males of **(d)** Hallmark\_glycolysis, **(e)** Hallmark\_oxidative\_phosphorylation, **(f)** Hallmark\_fatty\_acid\_metabolism, and **(g)** Hallmark\_hypoxia.





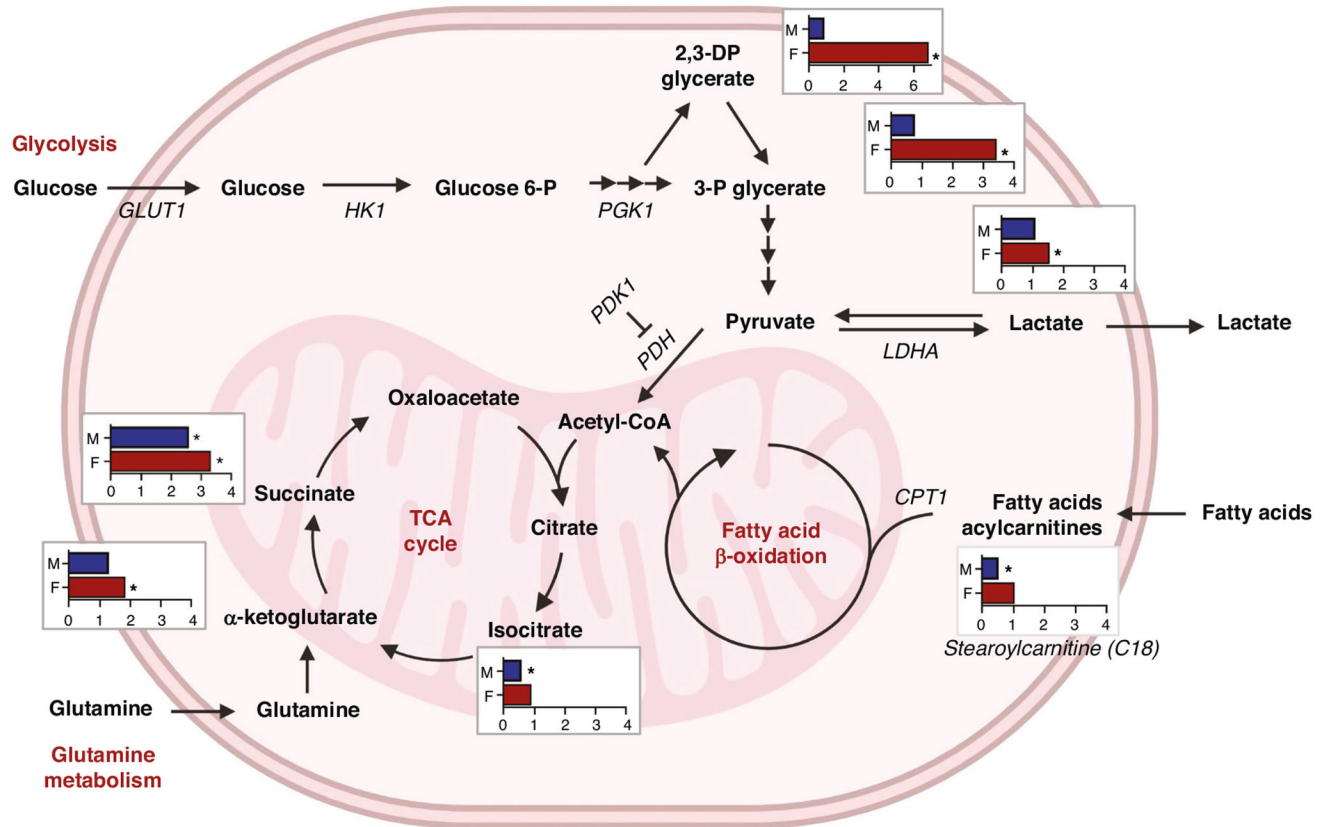
**Fig. 3. Overall group effects in plasma metabolic profiling between study groups.**

**a** Sparse partial least squares discriminant analysis plot of plasma metabolites grouped by study groups (normoxic control, non-cyanotic congenital heart defect (NC\_CHD), and cyanotic CHD (C\_CHD) patients). **b** Sparse partial least squares discriminant analysis plot of plasma metabolites grouped by cyanotic congenital heart defect (CDH) females and cyanotic CDH males. **c** Random forest results generated with normoxic control, non-cyanotic congenital heart defect (NC\_CHD), and cyanotic CHD (C\_CHD) patients.



**Fig. 4.** Normalized metabolite peak area levels among study groups of cyanotic congenital heart defect (C-CHD) and non-cyanotic CHD (NC-CHD) by sex (F, female; M, male).

Intermediates in glycolysis are represented by (a) 2,3-diphosphoglycerate, (b) 3-phosphoglycerate, and (c) lactate; citric acid cycle are represented by (d) citrate, (e)  $\alpha$ -ketoglutarate, and (f) succinate; fatty acid oxidation are represented by (g) palmitoylcarnitine (C16) and (h) stearoylcarnitine (C18).



**Fig. 5. Comparison of metabolic levels in fold of change (FC) between cyanotic and non-cyanotic congenital heart defect patients by sex (F, female; M, male) in interrelated metabolic pathways (glycolysis, citric acid or TCA cycle, fatty acid oxidation, and glutamine metabolism).**

FC of 1 indicates no difference; \* indicates  $p < 0.05$ . Metabolites: glucose 6-P, glucose 6-phosphate; 2,3-DP glycerate, 2,3-diphosphoglycerate; 3-P glycerate, 3-phosphoglycerate. Gene names: *GLUT1*, glucose transporter 1; *HK1*, hexokinase 1; *PGK1*, phosphoglycerate kinase; *LDHA*, lactate dehydrogenase A; *PDK1*, pyruvate dehydrogenase kinase 1; *PDH*, pyruvate dehydrogenase complex; *CPT1*, carnitine palmitoyltransferase. Created with [BioRender.com](https://www.biorender.com).

Table 1.

Differential expression of genes reported in log<sub>2</sub> fold change (LFC) between sexes (F, females; M, males) within study groups of cyanotic congenital heart defects (CHD) and normoxic control patients.

Symbol	Chromosome	Gene name	Cyanotic F:M		Control F:M	
			LFC	<i>P</i> <sub>adj</sub> value	LFC	<i>P</i> <sub>adj</sub> value
<i>RPS4Y1</i>	chrY	ribosomal protein S4, Y-linked 1	-5.15	<b>1.14E-29</b>	-12.51	<b>5.81E-44</b>
<i>EIF1AY</i>	chrY	eukaryotic translation initiation factor 1A, Y-linked	-1.06	<b>4.03E-23</b>	-12.19	<b>2.65E-44</b>
<i>KDM5D</i>	chrY	lysine demethylase 5D	-3.25	<b>1.75E-18</b>	-11.58	<b>6.68E-23</b>
<i>PRKY</i>	chrY	protein kinase, Y-linked, pseudogene	-2.81	<b>4.64E-14</b>	-11.00	<b>5.46E-19</b>
<i>UTY</i>	chrY	ubiquitously transcribed tetranucleotide repeat containing, Y-linked	-2.78	<b>1.15E-13</b>	-10.98	<b>1.33E-19</b>
<i>TXLNGY</i>	chrY	taxilin gamma pseudogene, Y-linked	-2.52	<b>1.15E-13</b>	-11.07	<b>5.46E-19</b>
<i>DDX3Y</i>	chrY	DEAD-box helicase 3, Y-linked	-2.60	<b>1.48E-13</b>	-9.60	<b>2.08E-22</b>
<i>USP9Y</i>	chrY	ubiquitin specific peptidase 9, Y-linked	-2.26	<b>1.95E-12</b>	-8.14	<b>3.60E-16</b>
<i>XIST</i>	chrX	<i>X inactive specific transcript (non-protein coding)</i>	1.23	<b>2.72E-08</b>	8.31	<b>3.52E-09</b>
<i>ZFY</i>	chrY	zinc finger protein, Y-linked	-1.31	<b>3.59E-06</b>	-9.64	<b>1.24E-12</b>
<i>BCORP1</i>	chrY	BCL6 corepressor pseudogene 1	-1.46	<b>7.94E-08</b>	-8.28E-06	3.14E-07
<i>AC100801.1</i>	chr8	<i>long non-protein coding RNA</i>	1.14	<b>0.0001</b>	-3.83E-07	0.9999
<i>TTY14</i>	chrY	<i>long non-protein coding RNA</i>	-0.87	<b>0.0092</b>	-2.37E-06	0.5363
<i>AC021713.1</i>	chr11	<i>long non-protein coding RNA</i>	0.81	<b>0.0146</b>	9.26E-08	0.9999
<i>IGHV4-34</i>	chr14	immunoglobulin variable gene	-1.05	<b>0.0188</b>	-1.20E-06	0.9999
<i>CA2</i>	chr8	carbonic anhydrase 2	0.86	<b>0.0198</b>	7.91E-07	0.9999
<i>MICAL2</i>	chr7	MICAL like 2	0.98	<b>0.0213</b>	1.02E-06	0.9999
<i>LINC00278</i>	chrY	<i>long non-protein coding RNA</i>	-0.83	<b>0.0246</b>	-5.08E-06	1.28E-04
<i>OLRI</i>	chr12	oxidized low density lipoprotein receptor 1	0.29	0.9998	2.56	<b>0.0447</b>

Threshold of statistical significance of fold change  $> 1.2$  (or  $|LFC| > 0.26$ ) and  $p < 0.05$  after adjusting for multiple comparisons.

Bold indicates statistical significant of  $p < 0.05$  adjusted for multiple comparisons.