Circulating Factors in Single-Ventricle Congenital Heart Disease Promote Pathological Metabolic Remodeling in Cardiac Myocytes, Which is Abrogated with Phosphodiesterase-5 Inhibitor Therapy

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Background

Single ventricle (SV) congenital heart disease (CHD) is the leading indication for heart transplantation in infancy and is the most common cause of cardiovascular death in infants.

While remarkable advancements in surgical and post-operative care have increased the pre-transplant survival of SV patients, eventual heart failure (HF) remains a leading cause of death and indication for transplant in infants.

The molecular mechanisms associated with the progression to HF in SV are poorly understood, and it remains a challenge to predict which patients will develop clinically significant HF, and when.

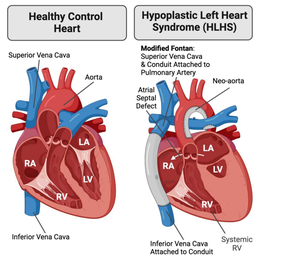

Figure 1. Anatomy of the Single Ventricle (SV) Heart
As compared to the normal heart, patients born with Hypoplastic Left Heart Syndrome (HLHS), the most common SV defect, have severe underdevelopment of the left ventricle (LV) and rely on a single systemic right ventricle (RV).

Healthy Control	Single Ventricle Heart Failure SVHF
Control – Donors with normal heart structure (biventricular) and function	SV patient explants with Heart Failure (identified by echocardiography)

Table 1. Patients (male and female) included in the study; Healthy Ctrl Median Age: 8.7 yrs and SVHF Median Age 3.9 yrs

While there are no proven medical therapies for the treatment or prevention of HF in the SV population, selective and competitive Phosphodiesterase-5 Inhibitors (PDE5i), such as Sildenafil, are increasingly utilized.

In addition to lowering pulmonary vascular resistance, there is increased evidence that **PDE5i improves exercise tolerance and hemodynamics in patients with SVHF (PMID: 31736357).**

Although the pulmonary vasculature is thought to be the primary target, the ***direct* effects of PDE5i on the SV myocardium are unknown.**

However, our previous data, suggests mitochondrial metabolism may act as a target for PDE5i therapy in the failing SV population.

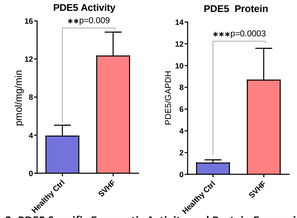

Figure 2. PDE5 Specific Enzymatic Activity and Protein Expression are Increased in Explanted Human SVHF RV Myocardium (PMID: 30354365) (A) PDE5 specific enzymatic activity in healthy control and SVHF RV myocardium Bar equals mean±SEM, n=7 Healthy Control and n=13 SVHF RV samples. (B) Quantification of PDE5 protein relative to GAPDH protein in healthy control and SVHF RV myocardium. Quantitative data were $\log_2$ transformed, bar equals $\log_2$(mean±SEM), n=9 Healthy Control and n=8 SVHF RV samples.

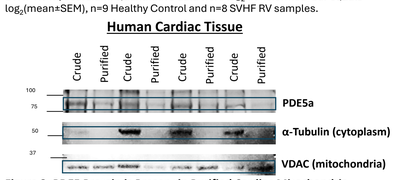

Figure 3. PDE5 Protein is Present in Purified Cardiac Mitochondria
Representative blot of (A) PDE5a (B) cytoplasmic protein expression: α-Tubulin. (C) mitochondrial protein expression: Voltage-Dependent Anion Channel (VDAC). For all groups, n=4 matched, crude and purified freshly isolated human cardiac mitochondrial samples.

Hypothesis

We hypothesize SVHF serum circulating factors are distinct from healthy controls and contribute to cardiomyocyte metabolic dysfunction, thereby promoting heart failure progression, and that PDE5i abrogates these pathologic metabolic alterations via improving cardiomyocyte mitochondrial bioenergetic function.

Methods

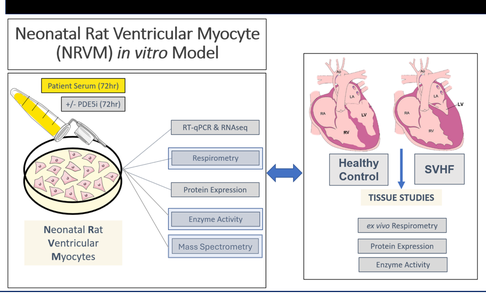

Figure 4. Summary of Methods to Assess Circulating Proteins and Metabolites (Mass Spectrometry), Mitochondrial Bioenergetics (High-Resolution Respirometry), and Enzymatic Activity (Stable Isotopes)
In order to investigate the specific mechanisms by which PDE5 inhibitors are protective in SVHF, we utilized an in vitro model of primary cardiomyocytes called Neonatal Rat Ventricular Myocytes or NRVMs, which were treated with 2% human serum from SVHF patients or Healthy Controls +/-PDE5i for 72 hours prior to harvest or assay. We have previously shown this model recapitulates pathological cardiomyocyte remodeling that is associated with SV heart failure (PMID: 34383712).

Using this model, we can assess the impact of patient serum circulating factors +/- PDE5 inhibitors on cardiomyocyte metabolism, (including both oxidative phosphorylation and mitochondrial fatty acid β-oxidation), along with Mass spectrometry-based lipidomics and metabolomic analysis.

Results

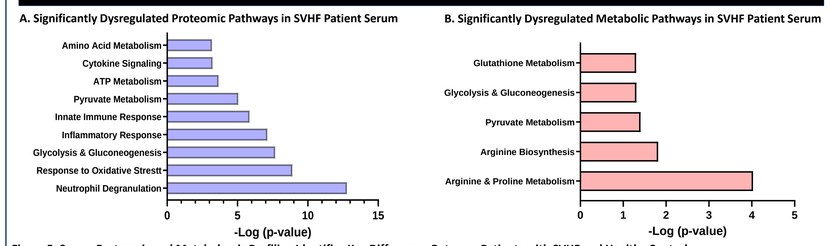

Figure 5. Serum Proteomic and Metabolomic Profiling Identifies Key Differences Between Patients with SVHF and Healthy Controls
Unbiased proteomic and metabolomic analysis suggests the serum circulating peptide and metabolite milieu in SVHF is significantly altered, including significant dysregulation of canonical pathways involved in mitochondrial metabolism, glycolysis, amino acid metabolism, oxidative stress, and inflammation. Pathways were identified in Metascape using significantly dysregulated peptides (A, n=4 healthy control and n=10 SVHF) or metabolites (B, n=5 healthy control and n=5 SVHF); Fisher's exact test, –log10 [P value] >1.3 or P <0.05.

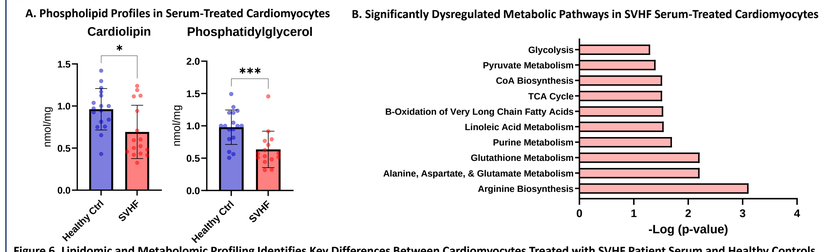

Figure 6. Lipidomic and Metabolomic Profiling Identifies Key Differences Between Cardiomyocytes Treated with SVHF Patient Serum and Healthy Controls
SVHF serum circulating factors pathologically remodel cardiomyocytes, including decreasing cardiolipin and phosphatidylglycerol lipid species (A), and shifted metabolite profiles (B) suggesting altered glycolysis, CoA biosynthesis, oxidative phosphorylation, and fatty acid beta-oxidation in response to SVHF sera. For all groups, asterisk denotes significant difference among groups; ∗P <0.05 and ∗∗∗P<0.001, analysis using a Mann-Whitney Test; Bar equals mean ± SEM; : n=6 healthy controls, n=7 SVHF serum-treated NRVM samples. Pathways were identified with Metaboanalyst using significantly dysregulated metabolites (n=3 Healthy Controls and n=3 SVHF serum-treated NRVMs; Fisher's exact test, -log10 (P-value) >1.3 or P<0.05

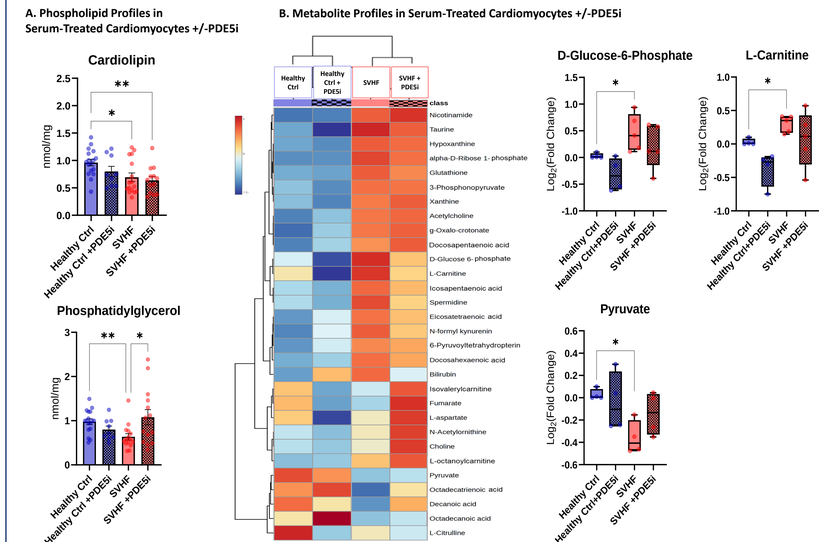

Figure 7. PDE5i Attenuates SVHF Serum-Induced Lipidomic and Metabolite Changes in Cardiomyocytes *in vitro*
A: PDE5i treatment abrogates the SVHF serum-induced changes in phosphatidylglycerol. Quantification (nmol/mg) of each detected phospholipids species; cardiolipin (CL) and phosphatidylglycerol (PG) with and without PDE5i. Bar equals mean ± SEM; each dot represents an individual patient serum-treated NRVM: n=13 (6 patient replicates) Healthy Controls and n=6 (2 patient replicates) failing SV serum-treated NRVMs +/- PDE5i across a total of 10 NRVM preparations; asterisks denote significant differences among groups; ∗P <0.05 and ∗∗P<0.01; analysis using Kruskal-Wallis test. B: Heatmap representation of the top 30 differentially expressed metabolites in serum-treated NRVMs +/- PDE5i and Log2 (Fold Change) representation of including D-Glucose-6-Phosphate, L-Carnitine, and Pyruvate which are attenuated with PDE5i; asterisks denote significant differences among groups; ∗P <0.05 and ∗∗∗∗P <0.0001; analysis using Brown-Forsythe and Welch ANOVA tests. For all groups, average of n=3 (1 patient replicate) Healthy Controls and n=3 (2 patient replicates) failing SV-serum treated NRVMs across a total of 5 NRVM preparations.

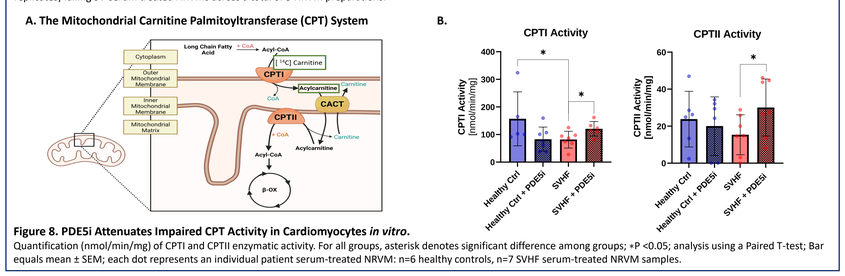

Figure 8. PDE5i Attenuates Impaired CPT Activity in Cardiomyocytes *in vitro*.
Quantification (nmol/min/mg) of CPTI and CPTII enzymatic activity. For all groups, asterisk denotes significant difference among groups; ∗P <0.05; analysis using a Paired T-test; Bar equals mean ± SEM; each dot represents an individual patient serum-treated NRVM: n=6 healthy controls, n=7 SVHF serum-treated NRVM samples.

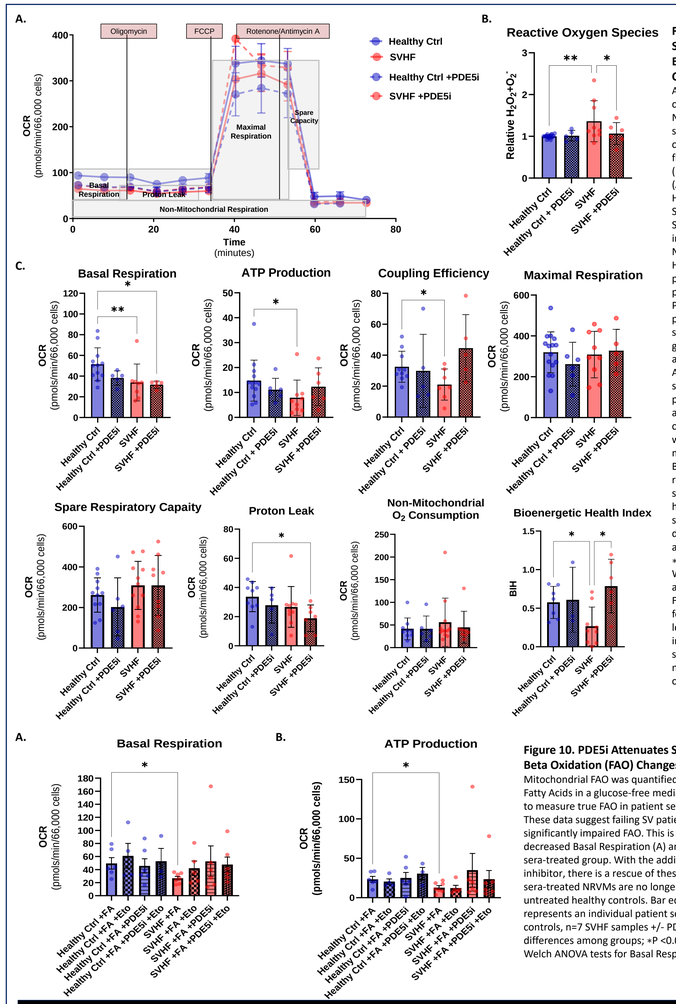

Figure 9. PDE5i Attenuates SV Serum-Induced Mitochondrial Bioenergetic Changes in Cardiomyocytes *in vitro*
A: Representative trace of oxygen consumption rate (OCR) curve in NRVMs. Cells were exposed sequentially to oligomycin, carbonyl cyanide p-(tri-fluromethoxy)phenyl-hydrazone (FCCP), and rotenone/antimycin (AA). B: Quantification (relative H_2O_2+O_2^-) of Reactive Oxygen Species (ROS). Bar equals mean ± SEM; each dot represents an individual patient serum-treated NRVM: n=13 (6 patient replicates) Healthy Controls and n=6 (3 patient replicates) failing SV patient-serum-treated NRVMs +/- PDE5i across a total of 10 NRVM preparations; asterisks denote significant differences among groups; ∗P <0.05 and ∗∗P<0.01; analysis using Ordinary one-way ANOVA. C: Respiratory parameters such as basal respiration, ATP production, coupling efficiency, and maximal respiration were calculated from OCR data with and without PDE5i (B) according to mitochondrial stress test protocol. Bar equals mean ± SEM; each dot represents an individual patient serum-treated NRVM: n=12 healthy controls, n=8 SVHF samples +/- PDE5i; asterisks denote significant differences among groups; ∗P <0.05 and ∗∗P<0.01; analysis using Kruskal-Wallis test for basal respiration and ATP production, Brown-Forsythe and Welch ANOVA tests for coupling efficiency, proton leak, and bioenergetic health index, Mann-Whitney test for spare respiratory capacity and non-mitochondrial oxygen consumption.

Figure 10. PDE5i Attenuates SV Serum-Induced Fatty-Acid Beta Oxidation (FAO) Changes in Cardiomyocytes *in vitro*
Mitochondrial FAO was quantified using the addition of Long-Chain Fatty Acids in a glucose-free media +/- Etomoxir, a CPT1 inhibitor, to measure true FAO in patient sera-treated NRVMs +/- PDE5i. These data suggest failing SV patient sera-treated NRVMs have significantly impaired FAO. This is illustrated by significantly decreased Basal Respiration (A) and ATP Production (B) in SVHF sera-treated group. With the additional treatment of PDE5 inhibitor, there is a rescue of these parameters such that the SVHF sera-treated NRVMs are no longer significantly decreased from the untreated healthy controls. Bar equals mean ± SEM; each dot represents an individual patient serum-treated NRVM: n=6 healthy controls, n=7 SVHF samples +/- PDE5i; asterisks denote significant differences among groups; ∗P <0.05; analysis Brown-Forsythe and Welch ANOVA tests for Basal Respiration and ATP Production.

Conclusions

While there are no proven medical therapies for SV heart failure, PDE5i therapy is increasingly used. However, the specific direct effects of PDE5i on the SV myocardium are remain incompletely understood.

Here we show: (1) The presence of PDE5 in purified mitochondria suggests a potential direct role for PDE5i therapy in modulating mitochondrial bioenergetics in this population. (2) Proteomic and metabolomic analysis of SVHF sera implicates altered signaling and metabolism as hallmarks of SV failure. (3) Using an established *in vitro* model whereby primary cardiomyocytes are treated with patient sera +/- PDE5i, we identified that serum circulating factors may potentiate progressive cardiac dysfunction via inducing alterations in cardiomyocyte phospholipids, metabolites, mitochondrial long-chain fatty acid transport, mitochondrial function, and substrate utilization. (4) Many of these pathological metabolic changes are abrogated with PDE5i, suggesting PDE5i therapy has direct myocardial effects, and likely contributes to beneficial cardiomyocyte metabolic remodeling in SVHF.

Acknowledgments

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