SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Nasal Nitric Oxide Sampling

All sampling occurred by placing a small, occlusive nasal olive at the entrance of the naris. For patients mature enough to cooperate, nasal nitric oxide (nNO) was measured by having the patient exhale against an oral resistor to raise the soft palate and isolate the nasal passages from the rest of the lower airway during sampling. For patients mature enough to cooperate but not able to adequately execute exhalation through the resistor, an alternative forced exhalation technique, such as blowing bubbles or blowing a pinwheel, was implemented. Each naris was sampled twice for 15 seconds. For small children or those unable to cooperate, sampling was performed using the tidal breathing method or immediately upon being intubated for their procedure. Each naris was sampled once for 50 seconds. Three seconds of plateau nNO concentrations (in parts per billion) from each resistor sampling, or five peak inflections from each tidal breathing nNO concentration-sampling curve were analyzed against the sampling flow (0.3 L/min) and averaged to yield a final nNO value in nl/min. Each of these methods have been validated in the literature[1-3]. Subjects with multiple measurements had their most recent value used in the primary analysis. Patients who underwent heart transplant (HTx) and had both pre-HTx and post-HTx measurements had each of those values used in the respective analyses.

Physiologic Variables

Patients were categorized as oxygen desaturated if their SpO2 was <90%. Because quantitative ventricular function measures are not always possible for all congenital heart disease (CHD) subtypes, systemic ventricular systolic function was categorized as either normal or dysfunctional based on the qualitative assessment by the reading pediatric cardiologist. Older, clinically stable, biventricular (BV) CHD patients who did not have oxygen saturation documented or a transthoracic echocardiogram performed on the day of their nNO assessment had their most immediate preceding values used. Data was omitted for patients if no oxygen saturation or ventricular function values were documented within one year of the nNO measurement. Patients with CHD that only had nNO values after their HTx were not included in the primary CHD analysis, since their transplanted physiology precluded them from experiencing some of the effects of certain CHD physiologies (i.e. single ventricle (SV) and systemic right ventricle [SysRV]). Because nNO increases dynamically with age until it plateaus at approximately 10 years old[4], patients were separated into older (≥10-years-old) and younger groups (<10-years-old) and continuous nNO data was analyzed only for the older group. Intergroup continuous nNO data comparisons were analyzed using one way analysis of variance (ANOVA) and Student's t-test.

Composite Endpoint Analysis

To examine the relationship between low nNO and the composite endpoint of HTx or death, four CHD subjects were matched to each HTx/deceased CHD subject based on the underlying CHD phenotype and the age that the nNO was measured (to eliminate age-related effects on nNO). Kaplan-Meier plots were generated to display time-to-event based on nNO status. The date of nNO sampling was used as the initiating event. The

terminating event was HTx/death or last documented clinical visit for censored subjects. Time-to-event distributions between low and normal nNO groups were analyzed using log-rank test. In addition to the nNO log-rank univariate analysis, Cox proportional-hazards model was used to investigate time-to-event risk based on nNO status with the five physiologic variables from the primary analysis serving as covariates.

Supplemental Results

Analysis of Subjects with nNO Measurements ≥ 10 Years of Age

Because nNO rises with age and stabilizes to adult levels at 10 years of age, we also conducted a sub-analysis of the 402 (65%) subjects ≥10-years-old, comprising 318 (79%) CHD patients, 5 (1%) post-HTx only, and 79 (20%) control subjects (Table S3). This analysis showed nNO levels that were significantly different between controls (284+/-89 nl/min), BV-CHD (255+/-94 nl/min), and SV-CHD (218+/-90 nl/min) (ANOVA P<0.001). Pairwise comparisons showed significant differences between control vs. BV-CHD (P=0.045), control vs. SV-CHD (P<0.001), and BV-CHD vs. SV-CHD (P=0.013). When analyzing the CHD subjects based on the five variables from the categorical analysis, nNO levels were significantly lower in those with SV-CHD, sysRV, and SpO2 <90% (Table S3). Similar to the categorical analysis incorporating the entire cohort, those with ventricular dysfunction had lower nNO levels, however this did not reach significance after Bonferroni adjustment of the P-value (Table S3). No difference in nNO levels were observed between heterotaxy and non-heterotaxy CHD patients. Compared to the non-transplanted CHD patients ≥10-years-old, the post-HTx patients ≥10-years-old had lower nNO, 194±67 vs. 248±95 nl/min, but this did not reach statistical significance (*P*=0.133) (Table S3).

Table S1. Prevalence of low nNO between various patient characteristics for both CHD and control subjects

N = 606	CHD (N = 471)			Control (N = 135)		
	Low nNO	**Normal nNO**	***P*-value**	**Low nNO**	**Normal nNO**	***P*-value**
Male	84 (28%)	214 (72%)	0.109	13 (18%)	58 (82%)	0.505
Female	61 (35%)	112 (65%)		9 (14%)	55 (86%)	
White	131 (30%)	308 (70%)	0.1	21 (18%)	97 (82%)	0.306
Non-white	14 (44%)	18 (56%)		1 (6%)	16 (94%)	
Resistor	107 (31%)	237 (69%)	0.805	12 (15%)	69 (85%)	0.568
Tidal breathing	38 (30%)	89 (70%)		10 (19%)	44 (81%)	
Age < 10 yrs*	48 (31%)	105 (69%)	0.848	10 (18%)	46 (82%)	0.679
Age ≥ 10 yrs	97 (30%)	221 (70%)		12 (15%)	67 (85%)	

*There is a normal, physiologic increase in nNO levels until approximately 10 years of age[4]
CHD = congenital heart disease, nNO = nasal nitric oxide

Table S2. Spectrum of CHD subtypes and proportions with low nNO (nNO), SV-CHD, and sysRV

CHD	Total	Low nNO	% Low nNO	SV-CHD	% SV-CHD	sysRV	% sysRV
HLHS	30	17	57%	30	100%	30	100%
ccTGA	24	10	42%	2	8%	23	96%
d-TGA	49	18	37%	0	0%	28	57%
DORV	45	16	36%	23	51%	23	51%
Truncus	11	4	36%	0	0%	0	0%
TOF	70	22	31%	0	0%	0	0%
PA	20	6	30%	10	50%	1	5%
ASD/VSD	46	14	30%	0	0%	0	0%
AVSD	24	7	29%	6	25%	5	21%
TA	19	5	26%	19	100%	0	0%
Arch	34	8	24%	1	3%	1	3%
DILV	13	3	23%	13	100%	0	0%
Ebstein	19	4	21%	0	0%	0	0%
AV	61	8	13%	0	0%	0	0%
Transplant*	16	11	69%	0	0%	0	0%
Other	6	3	50%	2	33%	1	17%
Total	487*	156*		106	21%	112	23%

*Totals higher than described in main manuscript figures and tables because four transplant patients had both pre- and post-HTx measurements, all of which were low.
HLHS = hypoplastic left heart syndrome, ccTGA = congenitally corrected transposition of the great arteries, d-TGA = dextro-transposition of the great arteries, DORV = double outlet right ventricle, Truncus = truncus arteriosus, TOF = tetralogy of Fallot, PA = pulmonary atresia (with and without intact ventricular septum), ASD/VSD = either isolated atrial septal defect, ventricular septal defect, or both atrial and ventricular septal defects, AVSD = atrioventricular septal defect (inclusive of complete/partial and balanced/unbalanced), TA = tricuspid atresia, Arch = hypoplastic aortic arch, Shone complex, DILV = double inlet left ventricle, Ebstein = Ebstein anomaly, AV = aortic valve disease including congenital aortic stenosis and bicuspid aortic valve with or without discrete coarctation, Other = one each of isolated total anomalous pulmonary venous return, anomalous left coronary artery arising from the pulmonary artery, and two each of double outlet left ventricle, and partial anomalous pulmonary venous return.

Table S3. Comparison of nNO values for physiologic variables in CHD subjects ≥10 years-old

N=318*	Count	nNO (nl/min)	SD	CI	*P*-value
Single ventricle CHD	65	218	90	196-240	0.005
Biventricular CHD	253	255	94	243-267	
Systemic RV	65	209	75	190-228	<0.001
Systemic LV	253	257	97	245-269	
Ventricular dysfunction	67	224	94	201-246	0.022
Normal ventricular function	242	253	94	241-265	
SpO_2 < 90%	21	184	87	144-223	0.002
SpO_2 ≥ 90%	244	247	89	236-258	
Heterotaxy	29	233	116	189-277	0.391
Non-heterotaxy	289	249	92	238-260	
N=323	**Count**	**nNO (nl/min)**	**SD**	**CI**	***P*-value**
Transplant	7	194	67	131-256	0.133
No transplant	316	248	95	238-258	

*Bonferroni adjustment for five CHD characteristics (0.05 / 5 = 0.01)
CHD=congenital heart disease, RV=right ventricle, LV=left ventricle, nNO=nasal nitric oxide

Table S4. Congenital heart disease (CHD) patients used for the survival analysis. Censored patients were matched based on CHD type and age at nNO measurements

ID#	CHD*	nNO (nl/min)	Age at nNO (years)	Low nNO	SV-CHD	sysRV	Time-to-event (months)
Transplant							
7351	HLHS	5	infant	+	+	+	8
7382	HLHS	188	21	+	+	+	12
7453	HLHS	5	infant	+	+	+	8
7522	ccTGA	143	8	+		+	17
7538	HLHS	155	15	+	+	+	1
Deceased							
7005	DORV	204	15		+	+	3
7006	DOLV†	125	41	+	+		82
7011	DORV	85	11	+	+	+	26
7194	TOF	161	26	+			42
7283	AVSD	166	51	+	+	+	3
7289	HLHS	32	infant		+	+	1
7328	DORV	16	Infant		+		22
7332	TOF	202	56				37
7428	Arch	19	Infant				2
7446	PA/IVS	160	32	+	+		12
7462	d-TGA	257	32			+	23
7489	ccTGA	11	Infant			+	1
7537	Arch	6	Infant	+			13
7596	HLHS	117	6	+	+	+	3
7669	d-TGA	7	Infant	+		+	4
Matched CHD subjects							
7018	DORV	307	13		+	+	66
7077	TGA	281	44			+	58
7078	HLHS	161	18	+	+	+	32
7090	HLHS	107	8	+	+	+	64
7092	PA/IVS	352	28				2
7120	ccTGA	193	23	+		+	58
7150	TOF	222	25				58
7169	HLHS	179	23	+	+	+	63
7171	AVSD	208	36				63
7173	PA/IVS	77	25	+	+		63
7188	d-TGA	259	34			+	55
7197	PA/IVS	304	28		+		12
7199	HLHS	10	infant		+	+	63
7216	ccTGA	294	18			+	25
7218	TA	161	36	+	+		63
7224	TA	240	40		+		24
7225	HLHS	19	infant		+	+	63
7226	TOF	168	32	+			62
7228	DORV	178	9	+	+	+	63
7233	ccTGA	229	25			+	61
7236	TOF	150	26	+			24
7249	TOF	200	53				61
7254	ccTGA	200	23			+	60

7272	DORV	239	14		+		55
7278	HLHS	122	14	+	+	+	22
7286	ccTGA	309	14			+	19
7287	d-TGA	149	29	+		+	48
7295	d-TGA	11	infant			+	41
7296	TOF	259	48				51
7302	HLHS	6	infant	+	+	+	55
7303	TOF	209	54				50
7310	HLHS	151	18	+	+	+	53
7312	DORV	153	8	+	+	+	55
7325	AVSD	300	33		+	+	17
7329	TA	362	40		+		51
7336	d-TGA	20	infant			+	48
7350	DORV	198	21	+	+	+	23
7364	ccTGA	239	24			+	46
7372	HLHS	245	12		+	+	46
7375	HLHS	191	16	+	+	+	43
7381	d-TGA	12	infant			+	44
7386	HLHS	3	infant	+	+	+	43
7389	HLHS	52	2	+	+	+	18
7390	d-TGA	272	36			+	44
7400	HLHS	9	infant		+	+	42
7403	ccTGA	273	22			+	20
7411	Arch	15	infant				18
7422	d-TGA	2	infant	+		+	40
7425	Arch	17	infant				40
7430	DORV	15	infant		+		37
7434	HLHS	264	17		+	+	34
7438	HLHS	12	infant		+	+	36
7464	HLHS	40	1		+	+	23
7472	ccTGA	172	23	+		+	11
7479	AVSD	250	23		+	+	27
7504	AVSD	181	52	+			20
7507	HLHS	342	20		+	+	7
7513	DORV	133	19	+	+	+	25
7523	HLHS	13	infant	+	+	+	25
7526	PA/IVS	235	29		+		19
7532	HLHS	233	8		+	+	23
7547	TA	315	39		+		23
7559	DORV	127	12	+	+	+	23
7567	TOF	307	26				5
7578	Arch	10	infant				19
7583	HLHS	17	infant		+	+	18
7585	Arch	6	infant	+			15
7597	Arch	18	infant				12
7605	HLHS	18	5	+	+		10
7611	HLHS	147	11	+	+	+	5
7617	DORV	10	infant				12
7620	Arch	6	infant	+			12
7627	Arch	26	infant				11
7630	HLHS	17	infant		+	+	10

7633	DORV	1	infant	+	+	+	11
7635	DORV	226	13		+	+	9
7644	DORV	8	infant	+			7
7647	HLHS	250	28		+	+	8
7651	TOF	194	53	+			4
7673	Arch	20	infant				5

* Primary CHD diagnosis listed

† Only DOLV patient in the study. Matched with four tricuspid atresia patients of the same age, all single ventricle and all with systemic left ventricles.

CHD = congenital heart disease, nNO = nasal nitric oxide, SV = single ventricle, RV = right ventricle, HLHS = hypoplastic left heart syndrome, ccTGA = congenitally corrected transposition of the great arteries, DORV = double outlet right ventricle, DOLV = double outlet left ventricle, TOF = tetralogy of Fallot, AVSD = atrioventricular septal defect, Arch = hypoplastic aortic arch, Shone complex, PA/IVS = pulmonary atresia with intact ventricular septum, d-TGA = dextro-transposition of the great arteries, TA = tricuspid atresia

Table S5. Variable comparison between the low nNO vs. normal nNO groups used in the composite endpoint analysis

Variable	Low nNO (N = 44)	Normal nNO (N = 56)	*P*-value
Single ventricle	29 (66%)	26 (46%)	0.052
Systemic right ventricle	30 (68%)	32 (57%)	0.259
Heterotaxy	4 (9%)	9 (16%)	0.303
Ventricular dysfunction	16 (36%)	15 (27%)	0.332
$SpO_2 \leq 90\%$	17 (40%)	19 (35%)	0.595
Experienced endpoint	13 (30%)	7 (13%)	0.034

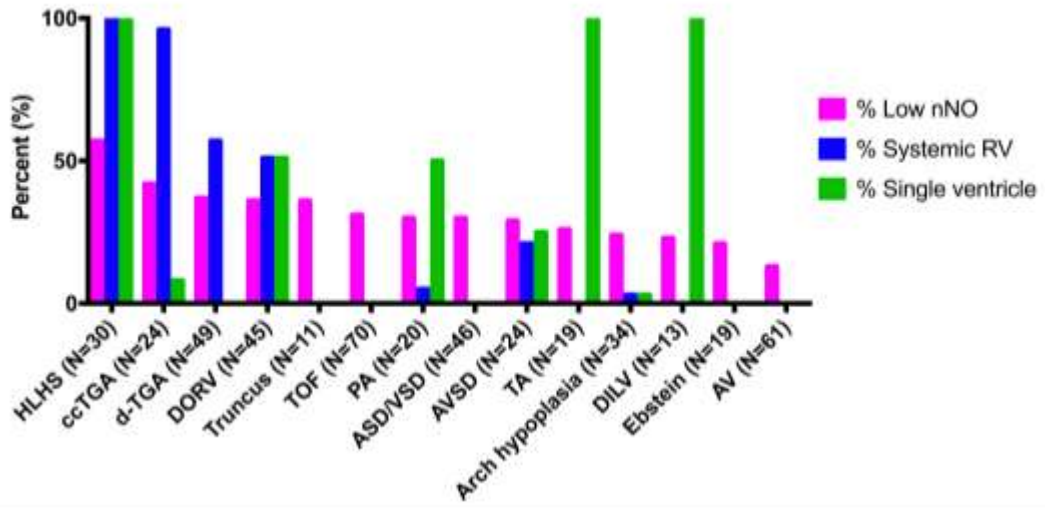
Table S6. List of patients who underwent HTx with pre- and/or post-HTx nNO measurements

ID#	Original CHD*	SV	sysRV	Age Pre-HTx nNO (years)	Pre-HTx nNO (nl/min)	Low nNO Pre-Htx	Age at HTx (years)	Age at Post-HTx nNO (years)	Post-HTx nNO (nl/min)	Low nNO Post-Htx
7013	TA	+					15	18	260	
7014	DORV	+	+				infant	6	145	+
7042	HLHS	+	+				infant	5	170	
7076	DORV	+	+				12	17	70	+
7113	DORV	+	+				2	5	119	
7351	HLHS	+	+	infant	5	+	infant	2	52	+
7382	HLHS	+	+	21	188	+	22	23	178	+
7413	AVSD	+	+				1	3	4	+
7418	PA/IVS	+					infant	3	7	+
7432	HLHS	+	+				infant	10	182	+
7435	TGA						3	10	271	
7442	TGA						14	14	221	
7453	HLHS	+	+	infant	5	+	infant	1	37	+
7522	ccTGA		+	8	143	+				
7538	HLHS	+	+	15	155	+				
7596	HLHS	+	+	5	41	+	5	6	117	+
7632	TGA						infant	8	152	+
7668	HLHS	+	+				infant	14	174	+

* Primary CHD diagnosis listed

HTx = heart transplant, nNO = nasal nitric oxide, CHD = congenital heart disease, SV = single ventricle, SysRV = systemic right ventricle, TA = tricuspid atresia, DORV = double outlet right ventricle, HLHS = hypoplastic left heart syndrome, AVSD = atrioventricular septal defect, PA/IVS = pulmonary atresia with intact ventricular septum, TGA = d-transposition of the great arteries, ccTGA = congenitally corrected transposition of the great arteries

Figure S1. Bar graph illustrating the prevalence of low nNO by specific CHD phenotypes (magenta bars).



Co-illustrated are the percentages of systemic right ventricle (blue bars) and single ventricle phenotypes (green bars). Note the four CHD types with the highest prevalence of low nNO also have the highest prevalence of systemic right ventricle. TA and DILV are 100% single ventricle but 0% systemic right ventricle and as such, have a relatively low prevalence of low nNO.
HLHS = hypoplastic left heart syndrome, ccTGA = congenitally corrected transposition of the great arteries, d-TGA = dextro-transposition of the great arteries, DORV = double outlet right ventricle, Truncus = truncus arteriosus, TOF = tetralogy of Fallot, PA = pulmonary atresia (with and without intact ventricular septum), ASD/VSD = either isolated atrial septal defect, ventricular septal defect, or both atrial and ventricular septal defects, AVSD = atrioventricular septal defect (inclusive of complete/partial and balanced/unbalanced), TA = tricuspid atresia, Arch = hypoplastic aortic arch, Shone complex, DILV = double inlet left ventricle, Ebstein = Ebstein anomaly, AV = aortic valve disease including congenital aortic stenosis and bicuspid aortic valve with or without discrete coarctation

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