Low Nasal NO in Congenital Heart Disease With Systemic Right Ventricle and Postcardiac Transplantation

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Background—NO bioavailability has not been systematically examined in congenital heart disease (CHD). To assess NO in patients with CHD, we measured nasal NO (nNO) generated by the nasal epithelia, given blood NO is difficult to measure (half-life, <2 ms). Given NO's role in hemodynamic regulation and the association of NO bioavailability with heart failure risk, we hypothesized NO levels may differ with varying severity of CHD physiologic characteristics.

Methods and Results—Six-hundred eighteen subjects, 483 with CHD and 135 controls, had nNO measured noninvasively via the nares using American Thoracic Society/European Respiratory Society guidelines. Subjects were dichotomized as having low or normal nNO based on age-specific cutoff values. Prevalence of low nNO was examined by various CHD physiologic feature types. Low nNO was more prevalent with CHD than controls (odds ratio, 2.28; P=0.001). A logistic regression model showed overall significance (P=0.035) for single ventricle, systemic right ventricle, ventricular dysfunction, oxygen desaturation, and heterotaxy predicting low nNO, with systemic right ventricle independently having twice the odds of low nNO (odds ratio, 2.04; P=0.014). Patients with low nNO had a higher risk of experiencing heart transplant or death (hazard ratio, 2.75; P=0.048), and heart transplant recipients (N=16) exhibited 5 times the odds of low nNO (69% versus 30%; odds ratio, 5.1; P=0.001).

Conclusions—Patients with CHD have increased prevalence of low nNO, with highest odds seen with systemic right ventricle and heart transplant. Further studies are needed to investigate heart failure risks in patients with CHD with left versus right systemic ventricle physiologic characteristics and utility of low nNO for predicting heart failure risk. (*J Am Heart Assoc.* 2017;6:e007447. DOI: 10.1161/JAHA.117.007447.)

Key Words: congenital heart disease • nasal NO • NO • right ventricle • single ventricle • transplant

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Congenital heart disease (CHD) is the most common birth defect, with an estimated 2.4 million individuals living with CHD in the United States.[1,2] The leading CHD-related cause of death after having congenital cardiac surgery is heart failure.[3] CHD is the cause of heart failure in 55% of infants, 41% of 1- to 5-year-old children, 35% of 6- to 10-year-old children, and 23% of adolescents who received heart

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Accompanying Data S1, Tables S1 through S6, and Figure S1 are available at http://jaha.ahajournals.org/content/6/12/e007447/DC1/embed/inline-supplementary-material-1.pdf

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transplants (HTx) (from 2009 to 2015).[4] Norozi et al observed an overall heart failure rate of 26% in patients with a heterogeneous mix of CHD, but 47% among those with single-ventricle (SV) CHD.[5] It is perhaps not surprising that SV CHD has the highest incidence of heart failure among all CHD types,[6] because SV physiologic features necessitate that a single functioning ventricle assume the workload typically shared by 2 ventricles. In some instances, the right ventricle (RV) is the systemic ventricle (sysRV), which has been associated with worse outcomes,[6] indicating the RV may not be able to meet the demands of a systemic ventricle. Consistent with this, patients with CHD most commonly listed for HTx are those with SV CHD (36%), followed by sysRV (20%), suggesting these unfavorable CHD physiologic features place patients at high risk for heart failure.[7]

Recent studies revealed a novel paradigm for heart failure, one in which vascular endothelial health and coronary microvascular function are of pivotal importance.[8] Because NO is an important mediator of coronary microcirculatory function,[9] and its bioavailability may be crucial to the development of heart failure,[10,11] this suggests NO measurements may have clinical utility as a biomarker for assessing

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Clinical Perspective

What Is New?

- Patients with congenital heart disease with a systemic right ventricle have twice the odds of having low nasal NO (nNO).
- Patients with congenital heart disease with low nNO may be at higher risk of requiring heart transplant or death.
- The highest prevalence of low nNO was in patients with congenital heart disease who had received a heart transplant.

What Are the Clinical Implications?

- nNO measurements can easily be trended over time, because they are simple and noninvasive.
- Deviation from one's nNO curve may help identify patients with congenital heart disease at risk for poor outcomes.

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heart failure risks. However, NO measurement in blood is difficult because of NO's extremely short half-life (<2 ms).[12] Given NO is also continuously produced by the airway epithelium,[13,14] this suggests the possibility that nasal NO (nNO) measurements, easily obtained noninvasively via the nares,[15] might be suitable as a proxy for blood NO. Measurement of nNO is commonly used in assessments of various respiratory diseases,[16] and nNO measurements have also been obtained in prior studies of patients with CHD. One such study demonstrated a 42% (18/43) prevalence of low nNO in a small cohort of patients with heterotaxy CHD.[17] A subsequent study of a larger cohort of patients with CHD (with and without heterotaxy) found 36% (64/180) had low nNO.[18]

Whether varying physiologic characteristics among patients with CHD differentially affect nNO levels remains unknown. In the current study, we investigated nNO levels in the context of different CHD physiologic features to assess the hypothesis that patients with unfavorable CHD physiologic features may have lower nNO and that this may portend heart failure risk.

Methods

Study Design

This was a prospective observational study of patients with CHD, with exposure being favorable versus unfavorable CHD physiologic features and the outcome being normal or low nNO status. Secondarily, the relationship between low nNO and the composite end point of HTx or death was investigated. A third and final analysis was performed to compare nNO measurements after HTx with those of patients with CHD who had not undergone HTx.

Patient Recruitment

Patients with CHD of all ages and all structural CHD phenotypes presenting to the Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center (Pittsburgh, PA) were recruited into the study from January 25, 2010 through January 17, 2017. Written parental informed consent was obtained for all minors, and adults gave their own written informed consent. This study was performed in compliance with our full institutional review board–approved protocol (PRO 09090021).

Subjects were recruited from the cardiology clinic for patients being seen for routine appointments and from the surgical preoperative suite for those being seen for cardiac catheterization or surgical intervention. Neonates who were born and admitted directly to the cardiac intensive care unit were recruited and had testing only if clinically stable. Acutely ill or clinically decompensating inpatients were excluded. Patients with isolated dilated or restrictive cardiomyopathies were excluded.

Controls consisted of healthy volunteers, healthy parents and siblings of subjects with CHD, and infants and children without CHD who were being seen for simple outpatient surgical procedures (eg, hernia repair, blepharoplasty, or small lesion/cyst removals). Subjects with nasal/airway pathological features, any trauma, or acutely ill, urgent, or emergent patient status were excluded.

nNO Assessment

nNO was measured as per the American Thoracic Society/ European Respiratory Society guidelines (Data S1).[19] nNO values (nl/min) were acquired using a CLD88sp chemiluminescence NO analyzer (EcoPhysics, Ann Arbor, MI). Patients were categorized as having normal or low nNO based on established cutoff values from the literature.[17,18,20]

Demographic and Clinical Variables

Each subject's sex, race, and age at nNO measurement were recorded. In addition, for the primary analysis, we chose 5 physiologic variables to examine for correlation with nNO levels. These variables were chosen on the basis of their predilection for heart failure and potential influence on oxygen content/delivery (SV status, systemic ventricle status, ventricular function, and blood oxygenation) or on previous published findings showing correlation with nNO level (heterotaxy status).[17,18]

Each subject's surgical stage of correction at nNO measurement and whether the subject had a systemic right or left ventricle were documented. Heterotaxy status was abstracted from the medical record, clinic notes, or imaging studies stating atrial isomerism and/or thoracoabdominal

situs abnormalities. Oxygen saturation and systemic ventricular systolic function, as determined by transthoracic echocardiography, were abstracted from the medical record from the same day that subjects had their nNO measured (Data S1). These tests were done as part of their routine care and not specifically as part of the study design. All clinicians were blinded to each patient's nNO status.

Statistical Analysis

All patients sampled were included in the analysis. Summary statistics are presented as the count with percentage and as the mean with SD. Comparisons of the previously mentioned categorical variables with nNO status were analyzed using χ^2 testing and the Fisher exact test when appropriate. All subjects were used in the categorical analysis. Logistic regression with Pearson goodness-of-fit postestimation testing was used to examine the effect of CHD physiologic features on nNO status. Time-to-event distributions for the composite end point of transplant or death, on the basis of nNO status, were analyzed using the log-rank test and Cox proportional-hazards modeling (Data S1). $P=0.05$ was considered statistically significant, with Bonferroni adjustment for correction of the α level for multiple comparisons. Statistical analysis was completed using STATA 14.1 (StataCorp, College Station, TX) and GraphPad Prism 7.0a (GraphPad Software, La Jolla, CA).

Results

Patient Characteristics

For the primary analysis examining physiologic variables and nNO, measurements were available for 606 subjects, composed of 471 patients with CHD (78%), of whom 365 (77%) had biventricular CHD and 106 (23%) had SV CHD; also, 135 (22%) were control subjects (Figure 1A). Along with heterotaxy status, the physiologic variables examined included status for SV and systemic ventricle, ventricular systolic function (458 patients [97%]), and oxygen saturation (415 patients [88%]). From this cohort, 6 patients with CHD (1%) progressed to requiring HTx and 16 (3%) died within the study period, providing 22 patients for composite end point analysis (Figure 1B). Measurement of nNO was repeated on 4 of the 6 patients requiring HTx after HTx, and an additional 12 patients were recruited after their HTx. Together, this yielded 16 patients for the post-HTx nNO analysis (Figure 1C).

General nNO Assessment

We observed an age-related increase in nNO measurements, which appeared to plateau at ≈10 years old, consistent with
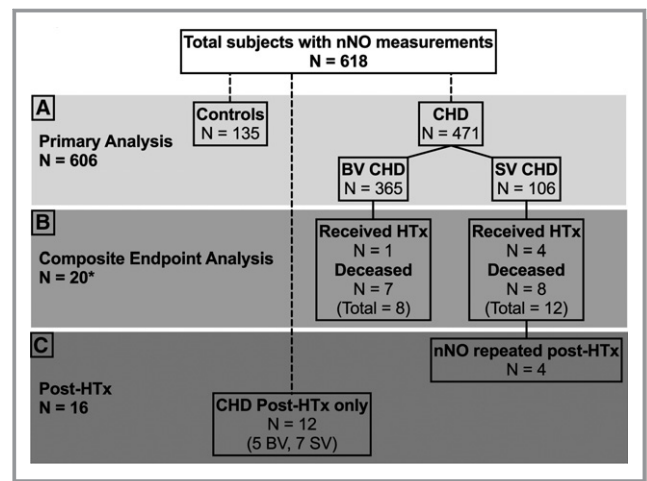
Figure 1. Flow chart showing subject numbers used for analyses. A, A total of 606 subjects with nasal NO (nNO) measurements were available for the primary analysis. B, There were 6 subjects who underwent heart transplant (HTx) and 16 who died. Of the 22 patients who experienced these end points, only 20 (asterisk) were appropriate for the outcome analysis (see Results). C, Of the 6 patients with HTx, 4 had repeated nNO values after HTx in addition to 12 patients who only had post-HTx nNO measurements, thus providing a total 16 patients after HTx. CHD indicates congenital heart disease; and SV, single ventricle.

prior literature (Figure 2).[21] Although nNO increased with age (as expected), the prevalence of low nNO in patients <10 years old versus those ≥10 years old was not different (Table S1). We subdivided the 606 subjects into controls, biventricular CHD, and SV CHD. Both a 1-phase association and quadratic nonlinear curves were generated, with 1-phase association being the preferred model in all 3 instances (Figure 2A through 2C, respectively). The nNO regression curves with 95% prediction intervals appear to progressively decrease from controls, to biventricular CHD, to SV CHD.

Patients with CHD in the primary analysis (Figure 1A) had a significantly higher prevalence of low nNO than control subjects (31% versus 16%; $P=0.001$) (Table 1). Group comparison showed a significant difference for the prevalence of low nNO between controls (16%), SV CHD (42%), and biventricular CHD (27%) ($P<0.001$), with additional subgroup analysis showing patients with SV CHD having nearly 4 times the odds of low nNO measurements compared with control subjects (odds ratio, 3.79; $P<0.001$) (Table 1). There was no significant difference in the prevalence of low nNO by sex, race, or sampling method within either the CHD or control groups (Table S1). The CHD type with the highest prevalence of low nNO was hypoplastic left heart syndrome (17/30 [57%]), followed by congenitally corrected transposition of the great arteries (10/24 [42%]), D-transposition of the great arteries (18/49 [37%]), and double-outlet RV (16/45 [36%]) (Figure S1, Table S2). Interestingly, these 4 CHD types were
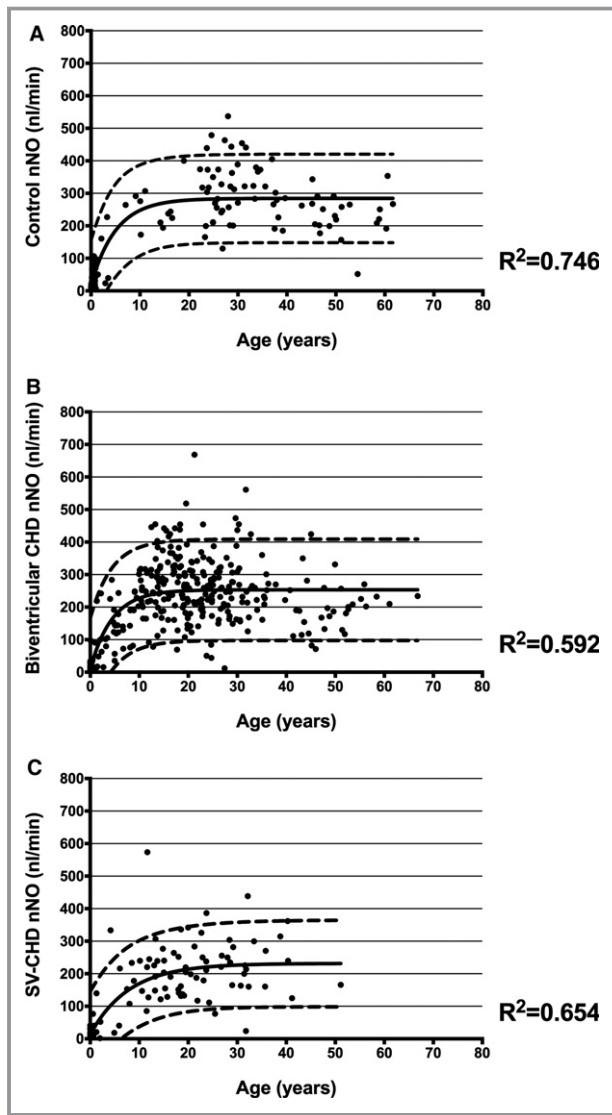
Figure 2. Age-related changes in nasal NO (nNO) measurements (nl/min). Our study sample demonstrated an age-related increase in nNO measurements until the age of ≈10 years. Fitted nNO regression curves and 95% prediction intervals appear to decrease from controls (A), to biventricular congenital heart disease (CHD) (B), to single-ventricle (SV) CHD (C).

also observed to have the highest percentage of a sysRV (Figure S1). Analysis of the stage of surgical palliation showed patients with both Fontan SV CHD and pre-Fontan SV CHD had a significantly higher prevalence of low nNO than controls (Figure 3). In addition, Fontan SV CHD also had a higher prevalence of low nNO when compared with biventricular CHD (Figure 3).

nNO by Various CHD Physiologic Features

The prevalence of low nNO was significantly higher in SV CHD versus biventricular CHD (42% versus 27%; $P=0.003$) and sysRV versus systemic left ventricle (46% versus 26%; $P<0.001$) (Table 2). Patients with more systemic ventricular dysfunction had a higher prevalence of low nNO than those with better ventricular function; however, differences between the groups were not significant (Table 2). There were no significant differences in the prevalence of low nNO when comparing patients by oxygenation or heterotaxy status (Table 2). Logistic regression of these 5 variables for predicting low nNO produced an overall significant model ($P=0.035$) with a postestimation goodness-of-fit test that supports the model ($P=0.592$) (Table 3). On the basis of this model, having a sysRV was an independent predictor of low nNO, with odds twice as high for low nNO in sysRV patients (odds ratio, 2.04; $P=0.014$) (Table 3). Because nNO increases dynamically with age until it plateaus at ≈10 years old,[21] patients were separated into older (≥10 years old) and younger (<10 years old) groups, and continuous nNO data were analyzed only for the older group. nNO measurements were significantly lower in those with SV CHD, sysRV, and oxygen saturation <90% (Table S3).

Composite End Point Analysis

There were 16 deaths and 6 patients with pre–HTx nNO values who progressed to requiring HTx, thus providing 22 possible subjects for end point analysis comprising either HTx or death. One deceased patient (a 2-month-old patient who was the only patient with isolated total anomalous pulmonary venous return) had no suitable CHD match and was excluded from the analysis. One patient experienced both HTx and death, but because the pre–HTx nNO was measured the day of HTx, only the death event and post–HTx/predeath nNO measurement were used. This resulted in 20 patients for

Table 1. Low nNO Prevalence Between Control, Biventricular CHD, and SV CHD Groups

Group	Total	Low nNO	% Low nNO	P Value	OR	95% CI
Control*	135	22	16	...	...	...
CHD (biventricular+SV)	471	145	31	0.001	2.28	1.37–3.94
SV CHD	106	45	42	<0.001	3.79	2.01–7.24
Biventricular CHD	365	100	27	0.010	1.94	1.14–3.40

N=606. CHD indicates congenital heart disease; CI, confidence interval; nNO, nasal NO; OR, odds ratio; and SV, single ventricle.
*P values and ORs reflect comparison to control.
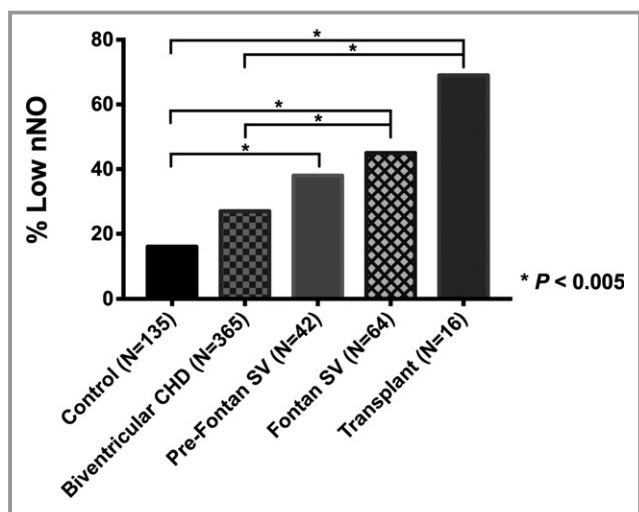
Figure 3. Bar graph illustrating the prevalence of low nasal NO (nNO) by stage of congenital heart disease (CHD) repair. Both patients with Fontan single-ventricle (SV) and transplanted CHD had a significantly higher prevalence of low nNO than both subjects with biventricular CHD and control subjects. Patients with pre-Fontan SV CHD had a higher prevalence of low nNO than controls. The asterisk indicates Bonferroni adjustment for 10 comparisons (0.05/10=0.005).

the final composite end point analysis (Figure 1B). For comparison, 80 patients with CHD were matched to these 20 on the basis of CHD type and age at nNO measurement (Table S4).

When these 100 subjects were divided into low nNO (N=44) versus normal nNO (N=56) groups, there were no significant differences in SV status, systemic ventricle, heterotaxy status, ventricular dysfunction, or oxygenation level, although there was a trend for more SV CHD in the low nNO group (66% versus 44%; P=0.052) (Table S5). The composite end point comprising death or transplant was observed in a higher percentage of the low nNO group (13/44 [30%]) than those with normal nNO (7/56 [12%]) (P=0.034) (Table S5). Survival analysis for end point comprising either HTx or death showed a lower percentage of event-free subjects through the 7-year study period for those with low nNO compared with those with normal nNO (Figure 4), but this difference did not reach statistical significance (log-rank P=0.074). We performed a Cox proportional-hazards regression analysis for the composite end point, with low nNO as a predictor and ventricular function and oxygenation status as covariates (anatomic covariates were controlled by matching). Low nNO was found to be associated with increased risk for HTx/death (hazard ratio, 2.75; P=0.048) (Table 4).

Post-HTx Analysis

Comprising the 11 of 16 post-HTx patients with low nNO measurements were 10 patients with SV CHD (91%), 9 (83%)

of whom had sysRV (Table S6). Of the 5 patients with normal post-HTx nNO, 3 (60%) were prior patients with SV CHD, 2 (40%) of whom had a sysRV. When compared against patients with nontransplanted CHD, significantly more post-HTx patients had a low nNO (69% versus 30%; P=0.001) (Table 2). Overall, the post-HTx group showed a significantly higher prevalence of low nNO compared with the biventricular CHD group and control subjects (Figure 3).

Discussion

To our knowledge, this is the largest study of nNO measurements in patients with CHD. Our study included a wide spectrum of CHD phenotypes and varying physiologic features. Each group, control, biventricular CHD, and SV CHD, appreciated the same age-related increase in nNO levels as described in the literature[21]; however, there was a progressive decline in the best-fit line and 95% prediction intervals from controls, to patients with biventricular CHD, to patients with SV CHD. Overall, we observed patients with CHD had twice the odds of having low nNO compared with non-CHD control subjects. We further showed having a sysRV was an independent predictor for having low nNO. An association was also observed between low nNO and having SV CHD, ventricular dysfunction, and low oxygen saturation. Analysis of outcomes showed the highest prevalence of low nNO at 69% (11/16) in patients with CHD who had undergone HTx. More important, we observed fewer event-free patients in the low nNO group using the composite end point of HTx or death (log-rank P=0.074), and our proportional-hazards model was significant for low nNO having a hazard ratio of 2.75 (P=0.048).

In contrast to the study of Garrod et al,[18] we did not see a significant difference in the prevalence of low nNO in patients with heterotaxy. Because the population with heterotaxy-CHD in the study by Nakhleh et al included a large proportion of subjects with double-outlet RV and congenitally corrected transposition of the great arteries, the high prevalence of low nNO (42%) in this cohort of patients with heterotaxy may, in fact, reflect the high proportion of patients with sysRV physiologic features.[17] Similarly, in the study of Garrod et al, the proportion of patients with heterotaxy CHD was 18% compared with only 9% in our current CHD cohort and, thus, those results may have been skewed by a similar trend.[18]

Physiologic Variables Associated With Low nNO

Our finding of a high prevalence of low nNO in patients with CHD with SV and sysRV amalgamates prior publications showing a central role for NO in the development of heart failure[8] and the known increased risk for heart failure among patients with SV/sysRV-CHD.[6] We showed an association of

Table 2. Prevalence of Low nNO by Various CHD Physiologic Variables

Variables	Total	Low nNO	% Low nNO	P Value	OR	95% CI
N=471*						
Single-ventricle CHD	106	45	42	0.003	1.95	1.21–3.13
Biventricular CHD	365	100	27	...	...	...
Systemic RV	112	52	46	<0.001	2.48	1.56–3.94
Systemic LV	359	93	26	...	...	...
Ventricular dysfunction				0.135		
None	320	93	29		...	...
Low-normal	61	19	31		...	...
Mild	52	19	37		...	...
Moderate	22	11	50		...	...
Severe	3	2	67		...	...
Sp_{O_2} <90%	77	32	42	0.069	...	...
Sp_{O_2} ≥90%	338	104	31		...	...
Heterotaxy	42	14	33	0.708	...	...
Nonheterotaxy	429	131	31		...	...
N=483						
Transplanted	16	11	69	0.001	5.09	1.59–18.96
Not transplanted	467	141	30		...	...

CHD indicates congenital heart disease; CI, confidence interval; LV, left ventricle; nNO, nasal NO; OR, odds ratio; RV, right ventricle; and Sp_{O_2}, oxygen saturation.
*Bonferroni adjustment for 5 CHD characteristics (0.05/5=0.01).

low nNO with ventricular dysfunction and with oxygen desaturation, begging the question as to whether low nNO is the cause or effect of these undesirable physiologic states. Lundberg and Weitzberg suggested low nNO may result from impaired substrate delivery.[21] A lack of substrate (hypoxemia) or inadequate substrate delivery (ventricular dysfunction) to

Table 3. Logistic Regression Model for Low nNO in the Population With CHD

Variable	OR	95% CI	P Value
Single-ventricle CHD	1.57	0.88–2.79	0.124
Systemic RV	2.04	1.16–3.59	0.014
Ventricular dysfunction			
Low-normal	0.67	0.33–1.33	0.25
Mild	0.97	0.49–1.94	0.934
Moderate	1.68	0.65–4.33	0.286
Severe	2.25	0.19–26.72	0.52
Sp_{O_2} <90%	1.02	0.55–1.88	0.961
Heterotaxy	0.69	0.33–1.44	0.319

N=413. Overall model: P=0.035. Pearson goodness of fit: P=0.592. CHD indicates congenital heart disease; CI, confidence interval; nNO, nasal NO; OR, odds ratio; RV, right ventricle; and Sp_{O_2}, oxygen saturation.
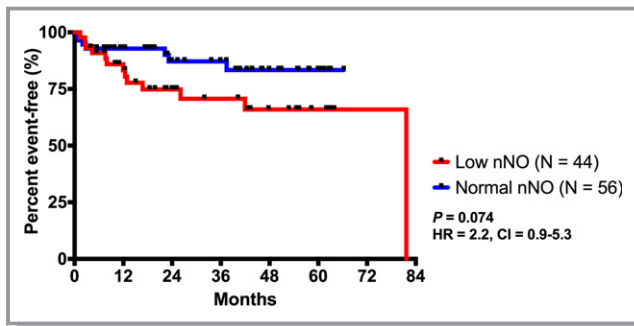
the nasal epithelia may manifest outwardly as low nNO, and this could be accompanied by a parallel reduction in substrate availability for coronary and myocardial NO production. Because NO is an essential effector of myocardial microvascular function,[22] insufficient NO can cause cardiac dysfunction and increased tissue ischemia/acidosis. This can lead to oxidative stress, resulting in NO synthase (NOS) uncoupling,[23] further impairing NO production and leading to even more NO/nitrite consumption and further reduction in substrate availability. This spiraling effect may lead to heart failure and could contribute to the increased heart failure risks in patients with CHD with low nNO. In addition, patients with complex CHD are at higher risk of NO scavenging, because some of the palliative shunts and valve replacements used in CHD surgery can result in high shear stress that may injure blood cells, causing decompartmentalized hemoglobin, which can scavenge endothelial-derived NO 600-fold faster than hemoglobin within intact erythrocytes.[24] This may be exacerbated by the fact that at the low oxygen concentrations seen in patients with cyanotic CHD, the NO binding affinity for various globin species is even higher.[25] Thus, an NO scavenging mechanism may play an important role in NO bioavailability in patients with complex CHD.

Interestingly, the patients with pre-Fontan SV CHD had a higher prevalence of low nNO compared with controls, but

Figure 4. Kaplan-Meier plot showing time from nasal NO (nNO) measurement to either heart transplant or death. There was a lower proportion of event-free patients in the low nNO group within the 7-year study period, although this did not reach statistical significance. CI indicates confidence interval; and HR, hazard ratio.

those who had undergone the Fontan operation showed an even higher prevalence of low nNO. This was unexpected because the Fontan procedure should resolve the hypoxemia experienced by the patients with pre-Fontan SV CHD. However, it is possible that these older patients may have been in more advanced states of heart failure at their nNO measurement, so although adequate substrate (oxygen) may have been available in the patients with Fontan SV CHD physiologic features, the delivery may have been impaired. This may suggest that NO-producing capacity is an intrinsic individual trait, under the theory that the functionality of the vascular endothelium (and other NO-producing tissues) may play a larger role than appreciated in this population. Furthermore, because mutations in endothelial NOS can result in CHD,[26,27] it is possible that genetic causes of impaired NO production capacity may both drive abnormal cardiac development, causing CHD, and result in low NO bioavailability in postnatal and adult life. Whether it is vascular endothelium, myocardial cells, or nasal epithelium, NO is

Table 4. Proportional-Hazards Model for the Composite End Point Analysis

Variable	Hazard Ratio	95% CI	P Value
Low nNO	2.75	1.01–7.47	0.048
Ventricular dysfunction			
Low-normal	0.67	0.2–2.27	0.522
Mild	0.33	0.07–1.6	0.169
Moderate	2.13	0.55–8.19	0.273
Severe	2.01	0.25–16.29	0.514
Sp_{O_2} <90%	1.63	0.63–4.22	0.317

N=95. Overall model: *P*=0.098. Harrell C: 0.677. CI indicates confidence interval; nNO, nasal NO; and Sp_{O_2}, oxygen saturation.

produced by either the oxygen-dependent NOS pathway or nitrite reduction, the latter predominating with ischemia and/or acidosis. nNO can be informative of NO bioavailability given all 3 NOS isoforms have been identified in both the nasal epithelia[13,14] and the myocardium.[28]

Low nNO and Outcomes

Our analysis of the 20 patients with preevent nNO measurements who progressed to the end point of either HTx or death and 80 matched, censored subjects showed no significant difference in the prevalence of SV or sysRV, ventricular dysfunction, or oxygen desaturation between those with low or normal nNO. Without any significant difference in their underlying CHD physiologic features, those who experienced the composite end point had a higher prevalence of low nNO than their CHD- and age-matched controls. Although our univariate survival analysis did not reach statistical significance, our proportional-hazards model showed low nNO to be a significant factor. These analyses demonstrate the potential utility of using nNO as a biomarker for monitoring patients longitudinally to identify those at risk for heart failure. Thus, by trending a patient's nNO over time, it might be possible to predict impending heart failure in patients showing a decrease on their nNO curve, similar to that of a failure-to-thrive patient deviating from his or her curve on a height and weight growth chart. This would allow earlier intervention for at-risk patients to improve outcome.[29]

HTx and Low nNO

The single parameter associated with the highest prevalence of low nNO was being post-HTx (11/16 [69%] with low nNO). Interestingly, of the 6 patients with pre-HTx nNO, all 6 (100%) had low nNO. Of these 6 patients, 4 had both pre- and post-HTx nNO measurements, with all 4 (100%) showing the same low nNO after HTx. There are several potential causes for NO bioavailability remaining low in patients after HTx. First, all common immunosuppressant agents can either directly or indirectly inhibit NOS.[30,31] An additional untoward effect of immunosuppression is the vulnerability for opportunistic infections and the use of prophylactic antibiotics. Prophylactic antibiotic use can negatively affect the enterosalivary circulation of nitrate through the unintended alteration of commensal bacteria in the oral cavity, which are responsible for reduction of nitrate to nitrite and, thus, contributing to a reduced NO bioavailability.[32]

However, if immunosuppression and antibiotics were the sole causes for low nNO after HTx, all patients with HTx would be predicted to have low nNO, not the 69% observed. On the contrary, one could expect the prevalence of low nNO to be

much lower after HTx, because HTx should remedy low nNO that may arise from the abnormal physiologic features associated with the SV CHD or sysRV, systemic ventricular dysfunction, and arterial hypoxemia. Instead, our observations suggest other underlying mechanisms causing low nNO in these patients. We hypothesize this may involve intrinsic endothelial dysfunction with resultant poor NO production and this may contribute to impaired cardiovascular development and continuing pathologic features because of the intrinsic limited bioavailability of NO that cannot be corrected by the palliative surgical procedures.

Indeed, if low nNO is reflective of systemic NO bioavailability and, therefore, myocardial NO bioavailability, this would contribute prominently to the progression towards end-stage heart failure and the subsequent need for HTx. Cardiac allograft vasculopathy continues to be a leading cause of death after HTx for CHD, and it may be that NO bioavailability plays an important role in this critical outcome.

Airway NO and Heart Failure

There is mounting interest in NO research with regard to cardiovascular function and heart failure. We should point out that, to our knowledge, all heart failure studies use exhaled NO (eNO) measurements, whereas our study used nNO measurements. Exhaled NO largely measures NO generated in the lower airway, whereas nNO measures NO produced in the nasal sinuses. The dilution, fluctuations, and confounders that can affect lower airway eNO measurements are important reasons why we believe nNO may be a superior measurement compared with eNO.

In contrast to our nNO results, a study by Hare et al concluded that adult patients with heart failure have increased eNO.[33] Conversely, a few years after the publication by Hare et al,[33] Katz et al showed results similar to ours, where adults with low eNO were more likely to experience the same end point we examined, that of HTx or death.[34] Indeed, Hare et al[33] point out, in their discussion, that there are several conflicting studies on eNO and heart failure. Again, we believe nNO is likely more sensitive as a marker for systemic NO bioavailability compared with lower airway NO (eNO), because the eNO would be more diluted by the larger lung volume compared with nNO measurements. Also of great importance in studying patients with CHD is the fact that nNO measurement does not require patient cooperation, which allows for data to be more easily obtained from the pediatric population, including infants. Overall, we agree with both Hare et al[33] and Katz et al[34] that airway NO, at least to some degree, does, in fact, reflect endothelial NO production capacity, and this is supported by the fact that all NOS isoforms, including endothelial NOS, have been identified in nasal/airway epithelium.[13]

Although some heart failure studies have yielded evidence of pathophysiologic features arising from increased peroxynitrite production attributable to elevated NO production, we note at the same time that there is also extensive evidence of decreased NO bioavailability playing a role in heart failure. Relevant to this is a point emphasized in the Pall review, where it is described that a basic mechanism of the NO cycle in disease must involve local effects, given the limited half-lives of NO metabolites in biological tissues.[10] This would suggest, in the context of CHD, given the likely presence of oxidative stress, there may be NOS uncoupling and alterations of numerous bioenergetic mechanisms (mitochondrial dysfunction, intracellular calcium abnormalities, and tetrahydrobiopterin depletion) that could affect NO levels. Thus, the role of NO in the pathophysiologic features of heart failure in patients with CHD will require further analysis of the complex interplay between different NOS isoforms in regulating myocardial versus vascular NO homeostasis and its effects on systemic NO.

Limitations of the Study

Despite having >600 subjects in this study, we recognize the sample sizes for the composite end point and post-HTx analyses were relatively small; hence, these were included only as secondary analyses. We acknowledge that 7 years may not be sufficient time to experience HTx/death attributable to CHD, especially for those enrolled in the later years of the study.

Another limitation is the fact that we do not have oxygen saturation values for 12% and transthoracic echocardiograms for 2% of the CHD cohort. The standard practice of our pediatric cardiologists does not include routine measurement of oxygen saturation on predominantly healthy patients with biventricular CHD being seen at the outpatient clinic for routine assessment.

In addition, we chose to apply the Bonferroni adjustment to our *P* values for analyses with multiple comparisons/hypotheses. This highly conservative adjustment, while accurately identifying statistical insignificance, may also result in false negatives by rejecting clinically significant associations of low nNO with various physiologic parameters. We chose to err on the conservative side to ensure our study does not overstate the evidence supporting the potential use of nNO as a clinical biomarker for assessing heart failure risk.

Conclusion

Low nNO observed in some patients with CHD is especially prevalent in those with sysRV, suggesting a subpopulation of patients with CHD may have a defect in NO generation and/or overuse. Low nNO was associated with increased risk of HTx

and/or mortality, with low nNO continuing to be highly prevalent in the patients after HTx. This would suggest the low nNO results from intrinsic patient factors and is not solely attributable to the physiologic characteristics of the congenitally malformed heart.

Sources of Funding

Disclosures

None.

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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).
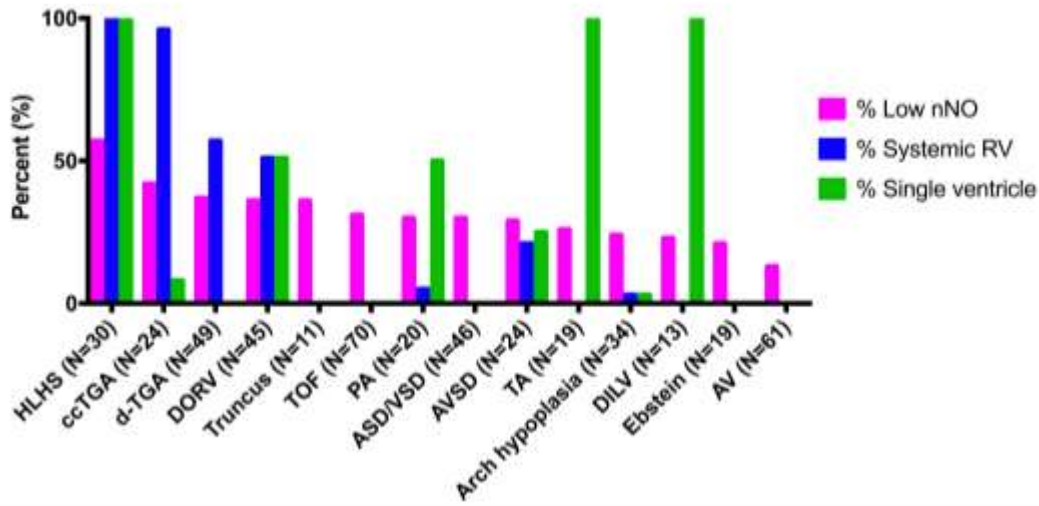
Co-illustrated are the percentages of systemic right ventricle (blue bars) and single ventricle phenotypes (green bars). Note the four CHD types with the highest prevalence of low nNO also have the highest prevalence of systemic right ventricle. TA and DILV are 100% single ventricle but 0% systemic right 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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Low Nasal NO in Congenital Heart Disease With Systemic Right Ventricle and Postcardiac Transplantation

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