Nitric oxide synthase expression by pulmonary arteries: A predictive marker of Fontan procedure outcome?

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Objectives: We retrospectively analyzed lung biopsy specimens from patients who underwent the Fontan procedure to identify predictive markers of outcome.

Methods: We studied the intra-acinar pulmonary arteries present in lung biopsy specimens from 17 patients undergoing the Fontan procedure. We evaluated both their morphology and their expression of endothelial nitric oxide synthase and endothelin 1. We compared these data with those of 6 patients who died of no pulmonary cause (control group).

Results: Eight patients had a good surgical outcome (group 1). Their distal arteries were thin and weakly expressed endothelin 1 and endothelial nitric oxide synthase. The procedure failed in 9 patients (group 2). Their distal arteries displayed muscle extension with an increased wall thickness ($P < .01$ vs group 1). Their endothelin 1 expression remained low (not significant vs group 1). By contrast, endothelial nitric oxide synthase was markedly overexpressed ($P < .001$ vs group 1).

Conclusion: Distal pulmonary arteries of patients in whom the Fontan procedure failed exhibited a markedly increased wall thickness and a clear endothelial nitric oxide synthase overexpression. In addition to giving clues to the pathogenesis of the procedure's failure, our study might help to define reliable predictive markers of its outcome.

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The Fontan operation has been shown to benefit patients with a single functioning ventricle and low pulmonary resistance.^{1,2} The Fontan operation is proposed to patients to separate the systemic venous and arterial circulations by means of direct anastomosis between the vena cava and the pulmonary arteries. Its outcome is highly dependent on several risk factors, chief of which is pulmonary vascular resistance. Some patients have a poor outcome even when clinical and hemodynamic parameters indicate that conditions for the Fontan procedure are ideal.³ Increased medial thickness of the intra-acinar pulmonary arteries is found in patients with normal pulmonary pressure and might increase pulmonary vascular resistance after cardiopulmonary bypass, leading to failure of the Fontan procedure.⁴ Predicting the status of pulmonary vascular structure from hemodynamic data is very difficult.⁵ Endothelial dysfunction could be involved in these patients.

The vascular endothelium is known to play a crucial role in the local regulation of pulmonary vascular tone and in smooth muscle cell function.⁶⁻⁸ The vascular endothelium produces and releases a variety of substances. Among them, nitric oxide is a potent endothelium-derived vasorelaxant that prevents the proliferation of smooth muscle cells.⁷ Endothelin 1 (ET-1) is also a potent endothelium-derived peptide with vasoconstrictive and mitogenic properties.^{9,10} In adult patients with

TABLE 1. Characteristics of patients with single ventricle

Patient no.	Sex	Additional diagnosis	Previous operation	Age (y)	Saturation	Catheterization results				Outcome/sat	Biopsy grade	NOS grade
						PAP	SAP	QP/QS	VEDP			
1	M	TA, PA	APS	9	78%	13	110/60	1.3	8	Good result	1	0.9
2	F	MA, TGA, PS	APS	7	80%	17	115/70	2	10	Good result	N	0.9
3	M	TA, PS	None	16	62%	9	120/65	0.5	6	Good result, fen	1	0.3
4	F	TGA	PAB, PCPC	6	85%	13	115/70	1.4	9	Good result	1	0.9
5	M	TA, PS	APS	7	80%	14	100/55	1.5	8	Good result	N	0.9
6	F	TA, PA	APS	11	78%	15	110/65	2	6	Good result, fen	1	0.5
7	F	TA, PS	None	14	74%	14	110/65	0.8	7	Good result	1	0.4
8	F	PS	None	13	80%	16	110/65	1.5	7	Pleural effusion, fen	2	1.1
9	F	TA, TGA, PS	None	5	75%	18	110/60	1.5	10	85%, fen	N	1.4
10	M	TGA	PAB, PCPC	3.5	70%	11	100/50	0.8	10	60% then 83%, fen	2	2.5
11	F	TA, PS	None	2	74%	13	100/60	0.9	7	Take-down, fen	3	1.9
12	M	TA, PS	None	3	76%	13	110/65	1.5	6	Take-down, 85%	1	1.4
13	M	TA	PAB, PCPC	3	65%	14	95/60	0.8	8	Take-down, died	3	2
14	F	TGA, PS	APSx2	4	70%	18	100/65	1.7	7	Take-down, died	3	2
15	F	PS	APS	23	82%	10	130/70	NA	10	Take-down, died	4	2.2
16	M	PS	APS	6	84%	18	95/60	NA	6	Died	3	1.3
17	M	None	PAB, PCPC	2	80%	15	100/65	NA	10	Take-down, died	3	1.5

PAP, Pulmonary artery pressure; SAP, systemic artery pressure; QP/QS, pulmonary to systemic flow ratio; TA, tricuspid atresia; PA, pulmonary atresia; APS, aortopulmonary shunt; MA, mitral atresia; TGA, transposition of great arteries; PS, pulmonary stenosis; N, normal; PAB, pulmonary artery banding; PCPC, partial cavopulmonary connection; Fen, fenestrated Fontan operation; NA, not available.

pulmonary hypertension, the expression of endothelial nitric oxide synthase (eNOS) has been shown to decrease, and that of ET-1 has been shown to increase.^{11,12}

On the basis of these considerations, we postulated that the pulmonary circulation was involved in failure of the Fontan procedure, despite the satisfactory hemodynamic criteria of the patients concerned. To support this hypothesis, we attempted to determine whether the structure of the pulmonary arteries or endothelial expression of vasoactive substances were altered in these patients.

Methods

Study Population

The Fontan procedure is indicated for patients with a single functioning ventricle or with complex congenital heart disease causing similar impairment. It consists of anastomosing first the superior vena cava and right pulmonary artery and then the inferior vena cava and pulmonary artery with either an extracardiac tunnel or an intracardiac conduit. Thus, the systemic venous circulation is drained directly through the lungs and is totally separated from the arterial circulation.

Of the 66 patients who underwent the Fontan procedure at the Laennec and Necker-Enfants Malades hospitals between 1993 and 1999, 17 patients aged 2 to 23 years (median, 6 years) had a lung biopsy analysis. The aim of the study was explained to only 30 patients and their families, depending on the impact of the surgeon on the study, and the biopsy specimen was taken with their agreement for 14 patients. We also analyzed 3 postmortem specimens from patients who died as a result of the Fontan procedure before the study period. The other patients underwent the Fontan procedure without lung biopsy. No difference could be found between the patients who underwent the Fontan procedure with or

without lung biopsy according to sex, age, and hemodynamic parameters.

Of the 17 patients, the last 5 had a fenestrated Fontan operation. The fenestration was chosen arbitrarily to facilitate the postoperative course. We performed a histomorphometric analysis of these specimens and used immunohistochemistry to assess eNOS and ET-1 expression.

The clinical and hemodynamic data for our 17 patients, including previous surgical procedures, are shown in Table 1. All patients underwent cardiac catheterization before the Fontan procedure with oxygen and nitric oxide challenge. The time period between catheterization and lung biopsy was less than 1 month. Because most children were referred from institutions in which the techniques for measuring oxygen consumption in small children were not always available, we reported their pulmonary pressures and pulmonary/systemic flow ratios (Table 1). Patients were considered as good candidates for the Fontan procedure on the basis of good ventricular function, absence of atrioventricular valve regurgitation, and indirect factors assuming low pulmonary resistance. Because increased eNOS levels could be related to liver dysfunction, we evaluated liver function, which was normal in all patients. Patients were retrospectively divided into 2 groups on the basis of successful or unsuccessful surgical results. Group 1 comprised 8 patients aged 6 to 16 years (median, 10 years) with satisfactory results, and group 2 comprised 9 patients aged 2 to 23 years (median, 3.5 years) with poor results defined as takedown, prolonged pleural effusion requiring chest tube drainage for more than 14 days and hospitalization for more than 21 days, or death. With the exception of age (10.4 ± 3.7 vs 5.7 ± 6.6 years, $P = .04$), other factors, such as the hemoglobin level, saturation, and pulmonary pressure (17.3 ± 1.6 vs 17.4 ± 1.3 g/dL [$P = .76$], $77.1\% \pm 6.8\%$ vs $75.1\% \pm 6.2\%$ [$P = .15$], and 13.8 ± 2.4 vs 14.4 ± 3 mm Hg [$P = .8$], respectively), were comparable in both groups.

Figure 1. Light microscopy patterns of lung biopsies performed during Fontan procedures. (Original magnification for all panels 400 $\times$.) **A**, Normal nonmuscularized alveolar duct artery (arrowheads). **B**, Extension of muscle into the distal alveolar duct artery. Note the presence of muscle between the 2 laminae (arrowheads).

We did not find any difference in the previous surgical management, the degree of pulmonary vascular bed protection, or the age at the first operation that could explain the difference in outcome of the Fontan procedure. In addition, no difference was observed in the patients who had a fenestrated Fontan procedure (3 in group 1 and 2 in group 2). The myocardial protection and aortic crossclamp times were comparable in all cases, and the postoperative management included pulmonary vasodilators, such as inhaled nitric oxide and spontaneous breathing, if possible. All the patients had postoperative measurement of the pressure in the Fontan circuit and measurement of the transpulmonary gradient. The transpulmonary gradient was significantly higher in group 2 patients than in group 1 patients ($P = .01$). The takedown was decided on the basis of hemodynamic deterioration with increased transpulmonary gradient or increased left pulmonary pressure caused by ventricular failure confirmed with the echocardiogram. Surgical biopsy specimens were also analyzed from 6 control subjects (aged 3 months to 9 years; median, 0.5 years) who died of no pulmonary cause (3 of sudden infant death syndrome and 3 of cerebral tumors). Although patients who died of sudden infant death syndrome are younger than those in our patient population, we considered them as relevant control subjects because histomorphometric studies have shown that by the age of 4 to 6 months, arterial structure is similar to that observed in older children.¹³

The lung biopsy specimens were taken from the lower lobes when the lung was inflated, and tissues were fixed in 10% buffered formalin solution and embedded in paraffin for routine pathologic examination and immunohistochemical analysis.

Histomorphometric Study

Serial paraffin-embedded 4- μ m-thick sections were stained with hematoxylin and eosin, Perls stain for iron, and modified orcein for elastic fibers. In each biopsy specimen the pulmonary vascular structure was analyzed by using quantitative morphometric techniques, as previously described.¹³⁻¹⁵ Pulmonary arterial muscularity was assessed by determining the mean percentage arterial medial thickness of at least 40 arteries in different size ranges and was compared with the normal values for age. The proportion of

muscular, partially muscular, and nonmuscular arteries accompanying terminal and respiratory bronchioles and alveolar ducts was also assessed to evaluate potential muscle extension to more peripheral arteries than is normal for age. In each biopsy specimen the size of the intra-acinar arteries was determined by measuring the external diameter of all intra-acinar arteries cut in a perpendicular fashion. We calculated the mean external diameter at each airway level. The measurements were performed by 2 blinded observers. Because the Heath-Edwards grades are inappropriate for assessing pulmonary hypertension in congenital heart disease and because the Rabinovitch classification concerns more severe lesions than that observed in our population, we scored the morphometric grades of lung biopsy specimens as follows: normal, normal arterial medial thickness of proximal and distal pulmonary arteries with no intimal damage (Figure 1, A); grade 1, medial hypertrophy of proximal arteries less than 2 $\times$ normal but normal medial thickness of distal arteries accompanying alveolar ducts and alveolar walls; grade 2, medial hypertrophy of proximal and distal pulmonary arteries less than twice the normal wall thickness; grade 3, medial hypertrophy of proximal and distal arteries more than twice the normal wall thickness (Figure 1, B); and grade 4, intimal thickening.

Grades 1 and 2 are not described in the Rabinovitch classification, and grade 3 would correspond to its grade A.¹⁶

Immunohistochemical Analysis

Serial paraffin sections of lung tissue were immunostained with antiserum to human eNOS (Transduction Laboratory) and to ET-1 (affinity bioreagent, Golden). All the slides were stained and developed at the same time to avoid variability. Tissue sections were deparaffinized in toluene, rehydrated through graded concentrations of ethanol to water, and heated for 40 minutes in buffered citrate at pH 6. Slides were incubated in hydrogen peroxide to block endogenous peroxidase activity, washed in Tris-buffered saline solution, and incubated for 1 hour with anti-NOS (diluted 1:100) or anti-ET-1 (diluted 1:200) primary antibodies or with normal serum used as a negative control. Sections were then incubated for 15 minutes with a biotinylated secondary antibody

Figure 2. Relationship between the percentage wall thickness of the distal pulmonary arteries (external diameter <50 μm , 50-100 μm , or 100-200 μm) and outcome of the Fontan procedure. *NS*, Not significant. * $P < .01$, + $P < .05$.

and stained with streptavidine labeled with peroxidase, according to the manufacturer's instructions (Dako Corp). Slides were counterstained with Harris hematoxylin. Three investigators blinded to all clinical information examined all the slides to assess interobserver variations. They used a semiquantitative system to grade the staining intensity (0, no staining; 4, maximal staining). No significant difference between observers was noted. The staining was evaluated for both the proximal ($>100 \mu\text{m}$) and distal (50-100 μm) intra-acinar arteries and the capillaries. Because the immunostaining was similar in different-sized arteries, we pooled these results. We retrospectively assigned the data to the patients and divided them into 3 groups (group 1, group 2, and the control group). We expressed the results as a score (mean $\pm$ SD of determinations performed on 20 arteries by all investigators).

Statistical Analysis

Results are expressed as means $\pm$ SD. The significance of differences between groups was assessed by the Student *t* test or Fisher exact test, as appropriate. The Pearson coefficient was used to calculate correlations.

Results

Histomorphometric and immunohistochemical studies were performed on 14 lung biopsy specimens taken during the Fontan procedure and on 3 postmortem specimens (patients 15-17).

Histomorphometric Study

Histomorphometric study data are shown in Figures 1 and 2. Seven of the 8 patients who had good outcomes (group 1) exhibited normal distal intra-acinar pulmonary arteries (external diameter, $<50 \mu\text{m}$), and 1 patient exhibited muscle extension into the distal arteries. This patient had postoperative pleural effusion for 12 days. In this group the percentage wall thickness of the distal arteries was low (9.6% $\pm$

2.5%) and was comparable with normal values for age.¹³ All patients exhibited a small increase in proximal intra-acinar artery thickness, but the difference versus values in the normal control subjects was not significant.

In contrast, 7 of the 9 patients with poor results (group 2) exhibited muscle extension of grade 2 or 3 in distal intra-acinar pulmonary arteries, whereas only 2 of them had normal distal pulmonary arteries. As a whole, the mean percentage wall thickness of the distal pulmonary arteries was markedly increased in group 2 (20.45% $\pm$ 7.1%, $P < .01$ compared with group 1; Figure 2).

It is noteworthy that none of the patients with normal criteria for the distal intra-acinar pulmonary artery died of the Fontan procedure, whereas 5 of the 8 with grade 2 or greater muscle extension died shortly thereafter ($P = .001$).

Immunohistochemical Study

NOS immunostaining. In the control group eNOS reactivity was low in the pulmonary arteries (score, 0.88 ± 0.32 ; Figure 3, A) and mainly observed in endothelial cells of the proximal and distal muscular pulmonary arteries and capillaries. Endothelial NOS was also detected in the bronchiolar epithelium to a lesser extent. No difference was noted between the proximal and distal pulmonary arteries regarding the staining intensity (Figure 4).

In group 1 patients immunostaining for eNOS was very weak (score, 0.86 ± 0.35 ; Figure 3, B) and similar to that of the control group, with no difference between the proximal and distal pulmonary arteries (Figure 4).

In contrast, group 2 patients exhibited a strong eNOS immunoreactivity in the pulmonary vascular endothelium of their proximal and distal intrapulmonary arteries (score, 1.86 ± 0.88 ; $P < .01$ vs group 1; Figure 3, C).

Figure 3. Immunohistochemical studies showing eNOS expression in the pulmonary arteries: **A**, healthy control patients; **B**, group 1 patients (successful Fontan procedures); **C**, group 2 patients (failed Fontan procedures).

There was a good correlation between the histomorphometric and immunohistochemical results, except for patients 8 and 9 ($r = 0.76$, $P = .001$; Figure 5).

Endothelin immunostaining. Endothelin immunostaining data are shown in Figure 6.

In the control group ET-1 immunoreactivity was weak in both the proximal and distal muscular pulmonary arteries and mainly observed in endothelial and smooth muscle cells of muscular pulmonary arteries, capillaries, inflammatory cells, and type II pneumocytes. In epithelial cells immunoreactivity was strong in all patients.

In group 1 ET-1 expression remained low (score, 0.9 ± 0.3) and comparable with that of the control subjects (0.7 ± 0.2), whereas group 2 patients exhibited a more intense ET-1 immunoreactivity (score, 1.3 ± 0.3) than the control subjects ($P < .01$). The difference between immunoreactivity observed in the proximal pulmonary arteries of groups 1 and 2 was not significant (Figure 6).

Discussion

The Fontan procedure has been proposed for the correction of complex cardiac lesions for which biventricular repair is impossible. In the absence of a pulmonary ventricle, even a

slight increase in pulmonary vascular resistance can result in failure of the procedure. In our study, as reported by others, we observed that the Fontan procedure failed in some patients despite fulfilment of the usual hemodynamic criteria. Predictive factors of outcome are presently lacking. Failure of the Fontan procedure is frequently due to increased pulmonary resistance after cardiopulmonary bypass.

We postulated that endothelial dysfunction might be at least partly responsible for this situation.

To find out whether markers of such dysfunction could be evidenced in patients with a failed procedure, we retrospectively evaluated the pulmonary vascular structure and the expression of endothelial vasoactive factors in lung biopsy specimens obtained during surgical intervention from patients undergoing the Fontan procedure. For this purpose, we used both histomorphometric criteria and immunohistochemical markers of eNOS and ET-1 expression on endothelium.

We could not find any differences between group 1 and 2 patients that were predictive of these histologic and immunohistochemical changes. Indeed, saturation, degree of pulmonary protection, surgical technique, aortic cross-

Figure 4. NOS immunostaining score in proximal (*prox*) and distal (*dist*) intra-acinar pulmonary arteries and capillaries (*cap*) in group 1 patients (successful Fontan procedures) and group 2 patients (failed Fontan procedures) versus that in normal control subjects. NOS expression is stronger in group 2 patients than in group 1 patients and normal control subjects. * $P < .01$.

Figure 5. Correlation between histomorphometric and immunohistochemical data.

clamping time, and postoperative management were comparable in both groups. The only difference between groups is age. However, we would expect the opposite because the younger age of group 2 is usually recommended to avoid alteration of pulmonary vascular resistance or ventricular failure.

Our results showed abnormal muscle extension into the distal pulmonary arteries of the majority of patients in whom surgical intervention failed. Indeed, although group 1 patients exhibited normal distal pulmonary artery muscularity in all but one patient, 7 of the 9 patients in group 2 had

muscle extension in the distal pulmonary arteries of grade 2 or greater. It should be underlined that most of these samples would have been considered as normal by using standard pathologic criteria because the changes in the muscularity of the distal pulmonary arteries were minor. However, these arteries contribute greatly to pulmonary vascular resistance and to failure of the Fontan procedure because of an impaired vascular reactivity after a stimulus such as cardio-pulmonary bypass.

We also postulated that expression of endothelial vasoactive factors might be altered in patients in whom the procedure failed, as observed in patients with pulmonary hypertension.^{11,12} Immunoreactivity was indeed weak for both eNOS and ET-1 in group 1 patients and similar to that of the control group. By contrast, eNOS was intensely expressed in the vascular endothelium of group 2. Endothelial NOS expression was comparable all along the arterial axis. It was significantly stronger in group 2 patients than in group 1 patients and control subjects. It should be underlined that this criterion might appear as more sensitive than histomorphometry. Indeed, patients 9 and 12 had normal distal wall thickness but increased eNOS reactivity, and their operations failed. Patient 8 had thick-walled distal pulmonary arteries with only a slight increase in NOS expression. This patient had an overall good outcome, despite a pleural effusion for 12 days.

Altogether, all 9 patients whose Fontan procedures failed exhibited a marked expression of NOS and ET-1 compared

Figure 6. ET-1 immunostaining score in proximal and distal intra-acinar pulmonary arteries and capillaries in group 1 patients (successful Fontan procedures) and group 2 patients (failed Fontan procedures) versus that in normal control subjects. * $P < .01$. *Prox*, Proximal intra-acinar pulmonary arteries; *dist*, distal intra-acinar pulmonary arteries; *cap*, capillaries.

with that seen in the control subjects, despite a low pulmonary pressure.

Overexpression of endothelial factors in patients with failure of the procedure might indicate an endothelial dysfunction. Increased shear stress caused by abnormal pulmonary blood flow, and polycythemia might cause endothelial dysfunction with overstimulation of the nitric oxide pathway to maintain low pulmonary pressure. Endothelial NOS is indeed upregulated in shunt-induced pulmonary hypertension in lambs, in rats exposed to hypoxia, and in polycythemic rats.¹⁷⁻²⁰ By contrast, in adult patients with pulmonary hypertension, eNOS expression is reduced.^{11,12} The mechanism underlying pulmonary hypertension might be involved in the differences between endothelial factor synthesis because eNOS is upregulated in shunt-induced pulmonary hypertension, whereas it is reduced in ductus arteriosus ligation.^{17,21} However, 4 patients with successful operations had increased pulmonary blood flow without the eNOS overexpression observed in all patients in whom surgical intervention failed. Therefore, increased pulmonary blood flow does not necessarily impair endothelial functions. Unlike adult patients with primary pulmonary hypertension, the eNOS is upregulated in our patients with failed Fontan procedures. This could be due to an attempt to improve the pulmonary vascular resistance and facilitate the Fontan circulation. Indeed, adult patients with primary pulmonary hypertension could have an incapacity to produce eNOS, which is not the case in our population. Whether this

upregulation of eNOS is to balance the endothelin production or the lack of other vasodilative substances remains to be elucidated.

Of interest is the fact that histomorphometric and immunohistochemical data were correlated ($P = .001$). The former data appear to have a good predictive value, but the latter might be more sensitive. Bearing in mind that immunohistochemistry only allows for a semiquantitative assessment of protein expression, we are currently investigating the possibility of more accurate procedures, Western blotting, and enzyme-linked immunosorbent assay to quantify eNOS activity and ET-1 on frozen tissues.

The results of this study warrant further investigation of these markers by a prospective study of patients considered good candidates for the Fontan procedure. Our results might support the idea that this procedure should be performed in 2 steps, with a lung biopsy performed during the first step (ie, the partial cavopulmonary connection). This sample would undergo histomorphometric studies, immunohistochemical studies, and quantification of eNOS and ET-1, and the decision concerning whether to complete the procedure would be based on the absence of endothelial dysfunction.

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