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High Serum Levels of Procollagen Type III-N-terminal Amino Peptide in Patients with Congenital Heart Disease

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Abstract

Objective: The serum concentration of amino-terminal procollagen type III (PIIIP) is considered a useful marker of tissue fibrogenesis. The present study tested the hypothesis that: 1) serum PIIIP levels are elevated in patients with congenital heart disease (CHD) and abnormal hemodynamic loading and/or hypoxemia, 2) PIIIP levels are associated with severity of hemodynamic load or hypoxemia, both of which enhance myocardial fibrosis.

Methods and Results: Serum PIIIP levels were measured in 5 groups of CHD patients [42 patients with ventricular septal defect (VSD), 26 with coarctation of the aorta (COA, n=19) or aortic stenosis (AS, n=7), 36 with atrial septal defect (ASD), 39 with pulmonary stenosis (PS) and 20 with tetralogy of Fallot (TOF)]. PIIIP levels of CHD patients were significantly higher than those of 42 control subjects ($p<0.05$, each). Serum PIIIP levels increased in parallel with increased ventricular volume load in VSD and ASD, and with severity of PS. In TOF patients, PIIIP levels correlated negatively with arterial oxygen saturation. Treatment with angiotensin converting enzyme inhibitor (ACEI) was associated with low levels of PIIIP in COA/AS patients despite existing hemodynamic load.

Conclusion: The increased serum PIIIP levels in proportion with the severity of ventricular load or cyanosis suggest enhanced myocardial synthesis of collagen type III in patients with CHD. Suppression of PIIIP level by ACEI suggests the involvement of the renin-angiotensin-aldosterone system in myocardial fibrosis. These data provide the basis for the development of new diagnostic and therapeutic strategies in patients with CHD.

Key words: congenital heart defects, hemodynamic load, ventricular fibrosis

Introduction

Collagen deposition in myocardial interstitial fibrosis is a key feature of ventricular remodelling, and associated with ventricular dysfunction and arrhythmogenicity,[1] thus potentially contributing to the progression of heart failure and sudden cardiac death.[2] Accumulating evidence indicates that serum levels of procollagen peptide fragments are useful markers of cardiac collagen turnover. Amino-terminal procollagen type III (PIIIP), which is the most frequently and extensively studied marker, is cleaved off during conversion from type III procollagen to type III collagen and is released into the blood stream. The serum levels of PIIIP correlate significantly with myocardial collagen type III contents,[3, 4] and are significantly elevated in pathophysiological conditions associated with enhanced myocardial collagen deposition, such as myocardial infarction,[5] hypertension,[6] and idiopathic or ischemic cardiomyopathy.[3, 7] Furthermore, the levels of PIIIP are decreased in patients on therapies that potentially inhibit cardiac fibrosis.[7-10] Serum PIIIP levels can also predict progression to ventricular dysfunction and prognosis of patients with dilated cardiomyopathy or those with myocardial infarction.[3, 11, 12]

In congenital heart disease (CHD), the anatomical abnormalities of the heart are generally associated with abnormal hemodynamic loading[13] and neurohumoral activation,[14] both of which are important stimuli that promote cardiac fibrosis.[15-18] Hypoxemia, generally accompanied with so-called cyanotic type of CHD, is also known to stimulate collagen synthesis, thereby promotes myocardial fibrosis.[17, 19] Thus, it is possible that collagen synthesis and fibril formation are also increased in CHD patients and serum concentration of PIIIP may provide useful biomarker for monitoring cardiac tissue repair and fibrosis in this condition.

To delineate the value of serum PIIIP as a potential marker of ventricular fibrosis in patients with CHD, the present study tested our hypotheses that *1)* serum levels of PIIIP are elevated in patients with CHD who present with abnormal hemodynamic loading and/or hypoxemia and that *2)* PIIIP levels correlate with the magnitude of hemodynamic load or hypoxemia, which are both known to enhance cardiac fibrosis.

Methods

Patients

The study subjects were 163 children with various types of CHD and 41 control subjects under the age of 10 years. We selected this age group because a previous report that examined age-associated serum levels of PIIIP in children indicated that PIIIP decreases linearly with age after birth but increases significantly later at about 10 years of age (possibly reflecting changes in collagen type III synthesis rate in healthy tissues during growth) and then decreases thereafter, reaching adult levels by 16 years of age.[20] All subjects studied had undergone appropriate clinical and laboratory evaluation to exclude conditions associated with elevated serum concentrations of PIIIP (e.g., chronic liver disease, renal failure, pulmonary fibrosis, extensive wounds). CHD patients were subdivided into five groups that are hemodynamically representative of CHD. These included 42 patients with ventricular

septal defect (VSD), representing left ventricular (LV) volume overload (VSD group), 26 patients with coarctation of the aorta (COA, n=19) or aortic stenosis (AS, n=7), representative of LV pressure overload (COA/AS group), 36 patients with atrial septal defect (ASD), representative of right ventricular (RV) volume overload (ASD group), and 39 patients with pulmonary stenosis (PS), representative of RV pressure overload (PS group). In addition, to elucidate the effects of cyanosis (hypoxemia) on tissue collagen turnover, 20 patients with tetralogy of Fallot (TOF) and nonrestrictive VSD, in whom the degree of RV pressure load could be considered similar within the group, were also examined (TOF group). In the TOF group, to eliminate the confounding effect of LV volume overload, only those with pulmonary-to-systemic shunt ratio (Qp/Qs) no greater than 1 were examined. The control group consisted of patients with post-Kawasaki disease who had no evidence of cardiovascular lesions. Written informed consent was obtained from the parents of all patients, and the procedures were approved by the Committee on Clinical Investigation of each institution.

Laboratory analysis

Blood samples were obtained from the vena cava during cardiac catheterization, centrifuged immediately after sampling, and the separated serum was stored at –40°C for subsequent analysis. Serum PIIIP levels were measured by immunoradiometry using a mouse monoclonal antibody and an IRMA kit for PIIIP measurement (Riagnost P-III-P, CIS Bio International, Ceze, France).[21] The interassay and intra-assay variations were 7% and 3%, respectively.

Hemodynamic analysis

Cardiac catheterization was performed using the same anaesthesia protocol for all subjects (pre-medication with intramuscular pethidine and atropine, and sedation with continuous infusion of sodium thiamylal during catheterization). Blood samples were obtained as necessary for measurement of oxygen saturation and angio/ventriculography. For stenotic lesions, the pressure gradient was measured with a catheter drawn back across the stenotic site. The Qp/Qs was calculated using the Fick method.

Statistical analysis

All data were expressed as mean±SD. Serum PIIIP levels were compared among the CHD groups and the control group by analysis of variance (ANOVA), followed by post hoc Dunnett multiple comparison test. Multivariate regression analysis was performed to test the effects of age, hemodynamic load, and cyanosis on serum PIIIP levels. The correlation between continuously distributed variables was tested by linear regression analysis. A p value <0.05 was considered to indicate statistical significance. All statistical analyses were performed using Systat® ver 6.0 (Hearne Scientific Software, Chicago IL).

Results

Table 1 summarizes the demographic and hemodynamic characteristics of the six groups. With regard to the medications prescribed by the attending physicians, 31% of

VSD patients were on diuretics, and 38 and 15% of COA/AS patients were on angiotensin converting enzyme inhibitor (ACEI) and β-blocker, respectively. Consistent with previous reports of age-associated changes in serum PIIIP levels,[20] PIIIP levels in control subjects decreased linearly with advancing age (regression equation: PIIIP = -0.075 × age + 1.4 ($r=0.59$, $p<0.001$). As shown in Table 2, serum levels of PIIIP were significantly elevated in all patient groups compared to those in controls. This was also true when age as well as group were included as independent variables in multivariate regression analysis ($p<0.05$ for both age and group).

In VSD patients, Qp/Qs correlated with PIIIP levels (Figure 1A) (PIIIP = 0.92 × Qp/Qs + 0.5, $r=0.45$, $p<0.01$). Because increased pulmonary flow in VSD is generally coupled with a rise in pulmonary and thus RV pressure, therefore, to discriminate the effects of RV pressure (RVP) overload from those of LV volume overload on elevated PIIIP levels, multiple regression analysis was performed with RVP as well as Qp/Qs and age included as independent variables. The results demonstrated that Qp/Qs ($p=0.008$) and age, but not RVP ($P=0.311$), significantly correlated with PIIIP level (Table 3).

In COA/AS patients, neither pressure gradient nor LVP correlated with PIIIP levels. Because some of these patients received medications (ACEI) that could potentially inhibit ventricular fibrosis and thus alter serum PIIIP levels, the effect of medication on serum PIIIP concentration was evaluated by multivariate regression analysis. The mean pressure gradient across the stenotic site (ΔP) was significantly higher in the medication COA/AS group than in the medication-free COA/AS group (50.6±31.0 vs. 19.8±9.8 mmHg, $p<0.01$). Under this background condition, multivariate regression analysis identified the use of ACEI as significantly associated with lower PIIIP levels (Table 3).

Significant positive correlations were identified between Qp/Qs and serum PIIIP in the ASD group (Figure 1B; PIIIP = 0.73 × Qp/Qs + 0.28, $r=0.40$, $p<0.05$) and between ΔP and serum PIIIP in the PS group (Figure 1C; PIIIP = 0.08 × ΔP + 0.42, $r=0.51$, $p<0.01$). This was also the case in multivariate regression analysis after taking into account the age of the patients (Table 3).

In TOF patients, the severity of cyanosis correlated significantly with serum PIIIP level as demonstrated by the inverse correlation between arterial oxygen saturation (SaO_2) and serum PIIIP (Figure 1D and Table 3, PIIIP = -0.55 × SaO_2 + 10.9, $r=0.31$, $p<0.05$).

To further elucidate the relative contribution of pressure (ΔP across the stenotic site), volume (Qp/Qs) overload and hypoxemia (SaO_2) to the level of PIIIP, we performed multivariate regression analysis using data of all patients, and calculated the standardized coefficient (beta value). The results indicated that each of the above three covariates correlated significantly with PIIIP level (PIIIP = 14.1 + 0.03 × ΔP + 0.99 × Qp/Qs – 0.14 × SaO_2 – 0.12 × age, $p<0.001$ for all coefficients) and almost equally contributed to the levels of PIIIP (beta values; 0.22 for ΔP, 0.30 for QpQs, -0.34 for SaO_2 and –0.25 for age).

Discussion

The present study demonstrated for the first time that serum concentrations of PIIIP, a marker of tissue fibrosis, are significantly elevated in patients with various types of CHD. In addition, PIIIP levels correlated significantly with associated abnormalities in hemodynamic load or severity of hypoxemia (cyanosis), which are well-known factors that trigger myocardial fibrosis. Furthermore, in a subset of patients who received ACEI, which reportedly inhibits collagen synthesis, low serum levels of PIIIP correlated with ACEI treatment despite an existing hemodynamic load. These results add to the mounting evidence that serum PIIIP levels are elevated in pathophysiological conditions associated with enhanced collagen deposition, and suggest that serum PIIIP may provide indirectly important diagnostic information on myocardial fibrosis associated with CHD.

The results of the present study indicating augmented fibrosis in CHD patients are consistent with several previously reported data demonstrating ventricular myocardial fibrosis in patients with CHD [21-24]. Using cardiac magnetic resonance imaging (MRI), Babu-Narayan et al.[22] reported that ventricular fibrosis was evident in both the left and right ventricles after TOF repair and this pathological state correlated with adverse clinical markers, including ventricular dysfunction, exercise intolerance, neurohormonal activation, and clinical arrhythmia. Studies in CHD patients with right heart lesions also demonstrated that the combination of right ventricular pressure load and systemic hypoxemia was a strong predictor of right ventricular fibrosis indicated by late gadolinium enhancement of MRI.[23] Histopathological studies using postmortem hearts of tricuspid atresia[24] or hypoplastic left heart syndrome,[25] which respectively represent LV volume overload and RV volume overload under cyanotic conditions, have demonstrated that the LV of tricuspid atresia and RV of hypoplastic left heart syndrome were more fibrotic than age-matched normal hearts. It is well documented that myocardial mechanical stimuli[11, 15-18, 26] and/or hypoxia[17, 19] can induce myocardial collagen synthesis. Cardiac fibroblasts play a key role in this process by altering cell proliferation, growth factor release or gene expression.[27] Importantly, serum PIIIP levels are reported to increase in such mechanical load- or ischemia-induced increased myocardial fibrotic conditions.[3, 5, 6, 12, 28] Taken together, our data demonstrating not only increased serum levels of the myocardial fibrosis marker but also its association with ventricular hemodynamic load or hypoxemia point to the possibility of increased ventricular fibrosis in our CHD patients. Given the importance of myocardial fibrosis in leading to myocardial failure and poor prognosis in cardiovascular diseases, non-invasive monitoring by using a serological marker, PIIIP, should greatly help improve the management of patients with CHD.

It is interesting that although PIIIP levels were significantly elevated in CHD patients, the magnitude of the change was different among the groups. In particular, patients with VSD had a similar degree of left-to-right shunt but much higher levels of PIIIP compared to patients with ASD. This may be due to the difference in the age of the two groups (Table 1). However, multivariate regression analysis that included age as a covariate also showed significantly higher PIIIP levels in VSD patients than in ASD patients ($PIIIP = -0.072 \times age + 0.496 \times Qp/Qs + 0.376 \times Group$, $p<0.03$ for each coefficient), suggesting that PIIIP was higher in VSD patients than in ASD patients independent of age and Qp/Qs and that some other factor(s) associated with the difference in conditions of VSD and ASD contributed to the difference in PIIIP levels.

The higher levels of PIIIP in VSD than in ASD may also be due to the elevated RVP in association with increased left-to-right shunt, which was seen in VSD patients but not in ASD patients. In addition, the fibrogenic response to volume loads may be different between LV and RV. The results of multivariate regression noted above add support to the latter mechanism, but this needs to be confirmed in future studies.

In the present study, we also observed that the use of ACEI was associated with lower levels of serum PIIIP despite increased LV afterload by COA or AS. These results are consistent with several basic and clinical studies that have emphasized the important role of the renin-angiotensin-aldosterone (RAA) system in the development of myocardial fibrosis. For example, *in vivo* studies have shown that the presence of chronically high circulating angiotensin II and aldosterone levels provoke accumulation of fibrillar collagen in the myocardium.[29] *In vitro*, the effector hormones of the RAA system directly enhance collagen synthesis in rat fibroblasts.[30, 31] In addition, a significant association between plasma renin activity and serum PIIIP levels and reductions in serum PIIIP levels after blockade of the RAA system with ACEI (lisinopril) have been reported in patients with essential hypertension.[6, 8] It has been demonstrated also that blockade of aldosterone receptor by spironolactone prevents ventricular remodelling after myocardial infarction[9] and improves survival of patients with congestive heart failure.[32] Importantly, such beneficial effects of aldosterone blockade are associated with suppression of myocardial collagen synthesis as represented by a decrease in serum PIIIP level.[9, 32] Thus, our finding of low PIIIP levels in COA/AS patients treated with ACEI may add support to the involvement of angiotensin II and/or aldosterone in collagen overproduction in CHD patients. Interestingly, our observation is, in some sense, consistent with a previous report by Timonen et al.[33] who showed low serum PIIIP levels in patients with dilated cardiomyopathies (DCM). Compared to other studies that consistently showed high serum concentrations of PIIIP in DCM patients,[3, 7, 9, 32, 34] their patients were on antifibrotic medication (ACEI), which commenced well before study entry, suggesting pharmacological modulation of fibrogenesis by ACEI. Because ACE inhibitors were not administered in a controlled manner in the present study, but were more frequently used by patients with more severe hemodynamic impairment, future prospective randomized study examining serial changes in serum PIIIP before and after the treatment would help delineate the involvement of the RAA system in the development of myocardial fibrosis in CHD population. Furthermore, such study may provide evidence that ACEI is a potentially valuable therapy and that PIIIP is a useful tool for monitoring the effects of therapy.

Activation of reactive oxygen species (ROS) is a major step in the process of cardiac fibrogenesis, and ROS activation through NADPH oxidases is the downstream mechanism through which the RAA system can induce ventricular fibrosis.[35] In addition to the RAA system, recent studies have highlighted the importance of nitric oxide synthase (NOS) as a source of ROS, and have suggested that NOS uncoupling may play a pivotal role in the development of cardiac fibrosis.[36] Future studies exploring the effects of modulation of these pathways on cardiac fibrogenesis and resultant changes in PIIIP levels in CHD population are warranted.

In the same context, the relationships between various serological markers of fibrogenesis and ventricular diastolic dysfunction (stiffening) are of great clinical

interest because myocardial fibrosis is an important pathophysiological process underlying ventricular stiffening[37, 38]. Indeed, several studies have described a close correlation between serum levels of PIIIP and diastolic dysfunction in adults.[34, 39] Future studies designed to examine the correlation between serum PIIIP levels and diastolic dysfunction in CHD population should be also of potential importance.

Study limitations

There are several limitations to this study that need to be taken into consideration. First, although the group mean value of PIIIP of patients with CHD was significantly higher than the mean value of patients with post-Kawasaki disease (control group), overlap of the data was noted in the two groups. This observation may be because patients with post-Kawasaki disease do not truly represent healthy children and thus the PIIIP values of our control group could be higher than those of normal healthy subjects. However, PIIIP levels of our patients with post-Kawasaki disease were close to those reported previously for healthy children.[20] Therefore, the data overlap does not appear to be related to the inclusion of patients with post-Kawasaki disease as the control group. Instead, we believe the data overlap relates to the fact that CHD patients with mild degree of volume/pressure overloads had PIIIP levels within the normal range. Second, the present study relied on peripheral markers of collagen turnover without confirmatory endomyocardial biopsy data or coronary sinus sampling. Although the exclusion of patients with chronic liver/renal disease, pulmonary fibrosis, or extensive wounds, which potentially increase serum concentrations of PIIIP, supports the likelihood of cardiac source of PIIIP in the present study, comparative study with cardiac MRI or endomyocardial biopsy is needed to directly answer this question. Third, serum PIIIP is eliminated from the blood by the liver and kidney, and thus, hepatic and/or renal dysfunction can affect serum PIIIP levels. However, because such effect appears to become evident only in the advanced stages of hepatic/renal dysfunction,[3] and because the enrolled patients were free of liver and renal dysfunction, we believe that the high serum levels of PIIIP in CHD patients represent overproduction of the peptide rather than reduced excretion. Fourth, in the present study, PIIIP was measured only once and the potential dynamics of PIIIP in various pressure and volume overload conditions should be investigated to further delineate the value of serum PIIIP. Lastly, the process of cardiac fibrosis does not only involve collagen type III but also several other compounds, such as collagen type I, fibronectin, and laminin.[40] In addition, fibrosis by collagen deposition is determined by a balance between its synthesis and degradation. Matrix metalloproteinases and their endogenous inhibitors play important roles in the fibrolytic process.[41] Therefore, future studies incorporating assessment of the fibrolytic system together with assessment of the entire fibrogenetic system should help further clarify the pathophysiological significance of serum concentrations of PIIIP in patients with CHD.

Summary

Serum PIIIP levels are increased and related to the severity of ventricular load or cyanosis of patients with CHD, suggesting abnormally high myocardial synthesis of collagen type III in this population. In addition, the suppression of PIIIP level by

ACEI suggests possible involvement of the RAA system in myocardial fibrosis. The results provide scientific basis for the development of new diagnostic and therapeutic strategies in patients with CHD.

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Figure legends

Figure 1. Relationship between serum PIIIP level and pulmonary to systemic flow ratio (Qp/Qs) in patients with ventricular septal defect.

Figure 2. Relationship between serum PIIIP level and pulmonary to systemic flow ratio (Qp/Qs) in patients with atrial septal defect (A) and with pulmonary stenosis (ΔP) (B).

Figure 3. Relationship between serum PIIIP level and arterial oxygen saturation (SaO_2) in patients with tetralogy of Fallot.

Table 1. Characteristics of study populations

	Control	VSD	COA/AS	ASD	PS	TOF
Age (years)	4.7±2.2	2.1±2.7*	4.4±3.9	6.9±3.9*	4.9±3.9*	1.1±1.4*
Range (years)	0.7-8.8	0.3-9.6	0.2-9.7	0.9-9.0	0.2-9.4	0.2-6.5
Qp/Qs	1	2.3±0.8*	1	2.1±0.8*	1	0.8±0.1
RVP (mmHg)	24±5	54±25*	32±18*	34±13*	69±16*	74±13*
LVP (mmHg)	94±11	88±12*	138±30*	93±12	94±17	74±13*
ΔP	0	0	41±30*	0	33±17*	0
SaO_2	97±1	96±3	96±2	98±2	97±1	86±8*
Medication (n)	none			none		
Diuretics		13	3			
ACEI			11			
β-blocker			4		2	5

Data are mean±SD or number of patients.
*$p<0.05$ vs. control
Qp/Qs: pulmonary to systemic flow ratio; RVP, right ventricular pressure; LVP, left ventricular pressure; ΔP, pressure gradient across the stenotic site; SaO_2, arterial oxygen saturation; ACEI, angiotensin converting enzyme inhibitor.

Table 2. Serum PIIIP levels in each group.

	Control	VSD	COA/AS	ASD	PS	TOF
Mean±SD (U/ml)	1.09±0.29	2.60±1.66	1.60±0.84	1.34±0.6	2.33±2.82	2.83±1.36
p value vs. control		<0.001	0.007	0.021	0.005	<0.001
age-included analysis by multiple regression		<0.001	<0.001	0.001	0.002	<0.001

Age-included analysis shows the results of multiple regression analysis with age as well as group included as independent variables.

Table 3. Results of multiple regression analysis.

	VSD		COA/AS		ASD		PS		TOF	
	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*
Age	-0.123	0.063	-0.05	0.036	-0.045	0.01	-0.027	<0.001	-0.066	0.015
Qp/Qs	0.67	0.008			0.20	0.08				
RVP		0.311								
ΔP			0.01	0.039			0.005	0.012		
Medication (ACEI)			-0.05	0.002						
SaO_2									-0.011	0.023

In COA/AS group, the medication status was coded as 1 (medication-free patients) and 2 (medicated patients).
Abbreviations as in Table 1. R, correlation coefficient.

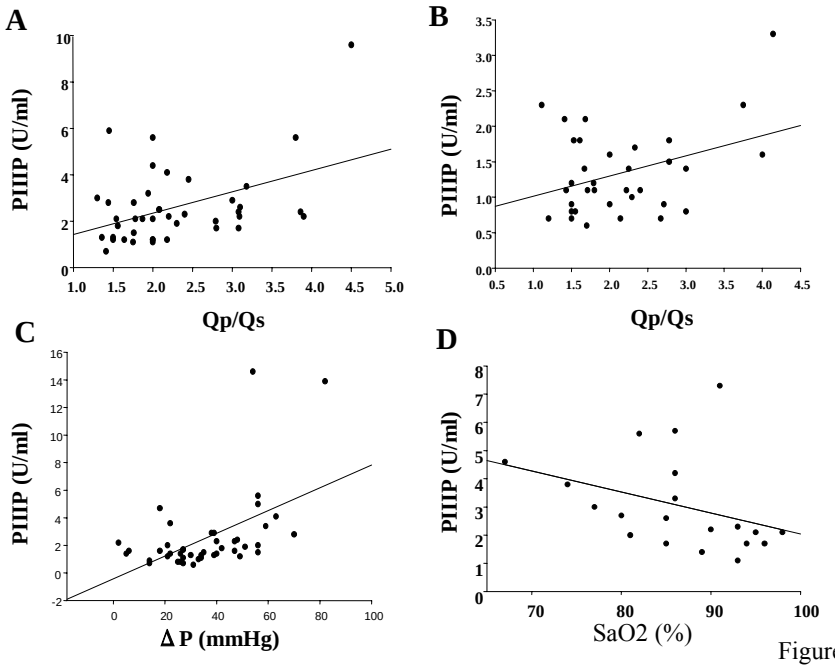

Figure 1

Heart

High Serum Levels of Procollagen Type III-N-terminal Amino Peptide in Patients with Congenital Heart Disease

Masaya Sugimoto, Satoshi Masutani, Mitsuru Seki, Hiroki Kajino, Kenji Fujieda and Hideaki Senzaki

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Institute for Health and Clinical Excellence (NICE).[3] Bellmunt and colleagues focus upon the limitations of the meta-analysis upon which the NICE guidelines were based,[4] the uncertainty surrounding which also having been highlighted in our article.[2]

We agree that a large randomised trial, with appropriate end points and long-term follow-up, directly comparing cliostazol, naftidrofuryl oxalate and placebo would be valuable in contributing to the evidence base in this context.

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Contributors The response was written by both authors listed.

Competing interests None.

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CORRECTION

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H Senzaki, Y Iwamoto, H Ishido, T Matsunaga, M Taketazu, T Kobayashi, H Asano, T Katogi, S Kyo. Arterial haemodynamics in patients after repair of tetralogy of Fallot: influence on left ventricular after load and aortic dilatation *Heart* 2008;**94**:70–4 Published Online First: 31 May 2007 doi:10.1136/hrt.2006.114306

M Sugimoto, S Masutani, M Seki, H Kajino, K Fujieda, H Senzaki. High serum levels of procollagen type III N-terminal amino peptide in patients with congenital heart disease *Heart* 2009;**95**:2023–28 Published Online First: 6 August 2009 doi:10.1136/hrt.2009.170241

The author Hideaki Senzaki was the chief investigator and was responsible for the mis-statement: "the procedures were approved by the Committee on Clinical Investigation of Saitama Medical University" which was mentioned in each of these publications. The statement was inaccurate because he did not obtain approval for the procedures from the ethics review committee of the hospital. The procedures were approved for clinical use and all patients provided informed consent, but a Clinical Investigation Committee had not reviewed this specifically, and the studies themselves involved retrospective analysis of previously obtained data.

The author apologises but would also like to add these points:

1.There were no ethical guidelines for clinical research issued by the Japanese Government until July 2003. In fact, the institutional review board (IRB) for clinical research was established in the author's hospital in 2004. Referring to IRB before performing and/or publishing clinical studies of a retrospective nature was not a routine process in Japan in those days.

2. As mentioned, the studies described in the above publications relate to analysis of data collected from routine medical care and catheterization, which had been initiated before 2003. In other words, the study protocol was retrospective in nature.

3. In each study, a signed informed consent form was obtained from each patient or parents/guardians.