Biomechanical and Molecular Aspects of Pulmonary Vascular Disease in Children with Congenital Heart Disease

Biomechanische en moleculaire aspecten van pulmonale vaatziekte bij kinderen met een aangeboren hartafwijking

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CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

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Biomechanical and molecular aspects of pulmonary vascular disease in children with congenital heart disease / Rudolphus Maria Franciscus Berger.
Thesis Rotterdam. - With ref. - With summary in Dutch
ISBN 90-9011731-8

Subject headings: pulmonary hypertension / pulmonary plexogenic arteriopathy / congenital heart disease / intravascular ultrasound / vascular endothelial growth factor / nitric oxide synthase

Lay-out: Joop van Dijk

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PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. dr P.W.C. Akkerman M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 24 juni 1998 om 15.45 uur

door

Rudolphus Maria Franciscus Berger
geboren te Venlo

Promotiecommissie:

Promotores:	Prof. dr J. Hess
	Prof. dr W.J. Mooi
Overige leden:	Prof. dr A.J.J.C. Bogers
	Prof. dr A.C. Gittenberger-de Groot
	Prof. dr Ir N. Bom

The Netherlands Heart Foundation is gratefully acknowledged for the financial support of the work presented in this thesis (research grant 94.046).
The Austrian Science Foundation, Vienna, Austria is gratefully acknowledged for the financial support of the work presented in the chapters 9 and 10 of this thesis (research grant J 1282).
Financial support by The Netherlands Heart Foundation for the publication of this thesis is gratefully acknowledged.
Printing of this thesis was possible with generous suppoprt from:
Hoek Loos B.V. Schiedam, Boston Scientific, Medtronic B.V.,
Hewlett Packard Nederland B.V., Medicor Nederland B.V., Kardia/Acuson B.V.,
Pfizer B.V., Nitinol Medical Technologies International B.V.,
Arrow Holland Medical Products B.V., Jaeger Nederland B.V.,
Glaxo Wellcom B.V., Cook Nederland B.V. and Ellerbrock & Nauta.

The search for truth is in one way hard and in another way easy
for no one can master it fully nor miss it fully...
but each adds a little to our knowledge of nature
and from all things assembled there arises a certain grandeur

Aristotle (384-322 B.C.)

Aan Anita
Aan Wouter, Danique, Florine en Lotje

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General introduction

Pulmonary hypertension and increased pulmonary blood flow both lead to functional and structural changes in the pulmonary vasculature. Pulmonary vascular disease constitutes an ongoing threat to children with congenital heart disease. Without early surgical repair, an estimated 30% of patients with congenital heart disease will develop significant pulmonary vascular disease. Pulmonary hypertension and pulmonary vascular disease are important causes of morbidity and mortality in patients with congenital heart disease. Pulmonary plexogenic arteriopathy is a characteristic form of pulmonary vascular disease and is most frequently associated with congenital heart defects. The combination of increased pulmonary blood flow with elevated pulmonary artery pressure, causes a rapid progression of the pulmonary vascular remodeling process that may progress to irreversibility and, then, precludes curative therapy of both the heart- and the vascular disease. Although the time course in which pulmonary arteriopathy progresses towards this stage is highly variable in different patients and different heart defects, our current knowledge, mainly based on empiricism, has lead to management strategies in pediatric cardiology that aims at surgical interventions early in life. On the other hand, at present, surgical corrections of complex congenital heart diseases may be delayed because of a staged approach. In addition, earlier and potentially reversible stages of plexogenic arteriopathy can jeopardize the outcome of surgical procedures because of acute pulmonary hypertensive crises perioperatively. This aspect is of special importance in the management of patients with a univentricular heart, who will undergo Norwood- or Fontan procedures. These procedures are being performed in a rapidly increasing frequency in the current era and create a circulation, in which no ventricular force faces the pulmonary vasculature. In such a condition, already early stages of pulmonary vascular disease may be detrimental to surgical outcome and prognosis.

An accurate assessment of the progression and functional consequences of pulmonary vascular disease is crucial in the management and prognosis of children with congenital heart defects. The management of univentricular hearts makes new demands on the evaluation of the pulmonary vasculature. The possibilities of the currently available

techniques to assess the progression of the vascular disease and its functional consequences for the integrated pulmonary circulation and, thus, the work load of the heart, are restricted by conceptual and practical limitations.
To evaluate the progression and consequences of a disease, it is important to understand its pathophysiology. Research in vascular biology provides rapidly expanding knowledge on humoral and cellular mechanisms that are probably involved in vascular injury and vascular remodeling. However, the specific role of the various substances and mechanisms, their interactions and the sequence of the various events remains to be unravelled. Endothelial dysfunction is believed to play a key role in vascular remodeling and various endothelial-derived vasoactive substances have been suggested to be involved in pulmonary vascular disease.
Pulmonary plexogenic arteriopathy is a specific form of pulmonary vascular disease, with a characteristic clinical course and unique morphological vascular lesions. Investigation of its pathophysiology is hampered by the lack of an adequate experimental model of this disease.

The aims of the studies described in this thesis were to determine the feasibility of a new technique, pulmonary intravascular ultrasound imaging, in children and infants and to investigate its potential to assess, *in vivo,* morphological and functional changes of pulmonary arteries in the course of pulmonary vascular disease in children with congenital heart disease. Furthermore, in our search for specific cellular events and biologic modulators that are involved in the emergence of advanced lesions of plexogenic arteriopathy, and thus must be characteristic for this typical disease, we studied the vascular expression of two potential candidates, vascular endothelial growth factor and nitric oxide synthase, at different stages of the disease process.

The pulmonary circulation: Development, postnatal adaptation and disease

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Introduction

To define pulmonary hypertension and pulmonary vascular disease, whether related to congenital heart disease, persistent pulmonary hypertension of the newborn or other conditions, insight into the normal pulmonary developmental changes in utero, at birth and during the postnatal period is necessary. Many mechanisms involved in pulmonary vascular remodeling in disease, are probably closely related, or may be even similar to mechanisms involved in the normal development, growth and adaptation of the pulmonary vasculature. Therefore the pulmonary vascular anatomy, physiology and humoral and cellular regulation in characteristic conditions will be successively discussed. These conditions are (1) the normal mature lung, (2) lung development, (3) postnatal transition and, finally, (4) pulmonary vascular disease.

The mature lung

Anatomy

The human lung can be divided into two functionally different components: *the conducting system* (1), consisting of airways and blood vessels, which function is the distribution of air and blood to the basic unit of the lung, formed by *the acinus* (2), in which the gas exchange takes place.

The conducting airways consist of the trachea, which originates at the larynx and after descending in the mediastinum branches into the left and right main bronchi. Within the lung the bronchi branche dichotomously, giving rise to progressively smaller airways. The number of airway generations from the main bronchus to the acini varies from 8 to 25, depending on the region of the lung. The branching pattern is asymmetric. Conducting airways 1 mm or more in diameter are reinforced by cartilage and are referred to as bronchi. Conducting airways without cartilage are named bronchioli. The terminal bronchiole is the most peripheral bronchiole, free of alveoli and not involved in gas exchange. The intra acinar airways of the adult lung consist of respiratory bronchioli, alveolar ducts and alveoli. The normal anatomy of the airways have been described in detail[1-3].

The pulmonary arteries accompany the bronchi, following the same branching pattern, until they reach the acini. The structure of these preacinar arteries can be predicted from its size[4, 5]: arteries with a diameter of approximately 1 mm or more are *elastic arteries*. In contrast to the pulmonary trunk and main arteries, in which the elastic laminae are irregular, disrupted and fragmented, in peripheral elastic arteries these laminae are regular, intact and parallel to one another. At the level of diameters between approximately 1 and 0.5 mm there is a gradual transition to a muscular type of artery. The preacinar arteries between 0.5 mm and 150 micrometer are most *muscular arteries*. The characteristic part of these arteries is its media or muscular coat. It is formed by vascular smooth muscle cells (VSMC's) with collagen and elastic fibers in between. The media is bounded by distinct internal and external elastic laminae[5].
In addition to the dichotomous branching pattern, following the airways, there are numerous additional branches, which do not follow the airways, but arise perpendicular or obliquely from elastic as well as from muscular arteries. These additional branches are known as *supernumerary arteries* and are muscular arteries[6].
Along the intra acinar arterial pathway the fully muscular media gives way to a partially muscular region, where muscle is present only as a spiral, before it disappears. This results in a non-muscular artery, still larger than a capillary, often controversially referred to as *arteriole*[4, 5]. In contrast to preacinar arteries in which the structure of the artery can be predicted from its size, in intra acinar arteries the transition from one type of artery to another does not always occur in arteries from the same size[4]. As described later, the proportion of arteries of a given size that shows a specific type of structure changes with growth, but is also modified by disease[4]. The non-muscular arterioles taper in calibre and give rise to dense alveolar capillary networks. The walls of *alveolar capillaries* consist of a single endothelial layer resting upon a basal continuous layer. At the site of gas exchange the alveolar wall also consists of a single layer of alveolar epithelium resting upon a common basal lamina[5].
Small *pulmonary veins*, with a very thin wall consisting of an endothelial layer resting upon a basement membrane, collect the blood from the alveolar capillaries and merge with each other to form larger veins with a wall containing collagen and elastic fibers and some VSMC's. These veins enter, independently from the bronchi, the mesenchyme of the interlobular septa and drain into the left atrium of the heart[5, 7].

Bronchial arteries provide nutrient blood supply to the bronchi, major vessels and part of the pleura and originate from the systemic circulation. As in systemic vessels, the media of bronchial arteries is considerably thicker than that of pulmonary arteries. Their caliber, however, is much smaller than that of the pulmonary artery accompanying the same bronchus. They show a distinct internal elastic lamina but the outer elastic lamina is usually interrupted or even absent. In general, the bronchial arteries accompany the bronchi as long as the latter contain cartilage[5].

Physiology

In the human circulation, the pulmonary vasculature is unique in receiving the total ventricular output, having a very active metabolism and producing, metabolizing and inactivating a variety of active substances[8]. The primary function of the pulmonary circulation is not the nutrition of an organ, but the exchange of oxygen and carbon dioxide. The pulmonary circulation is a high flow, low pressure and high capacitance, low resistance circuit and, thus, has unique functional and structural properties.

The physiologic regulation of the pulmonary circulation, which involves many mechanisms, is very complex and far from elucidated completely[8]. One of the most important mechanisms is the modulation of pulmonary vascular tone. Many interacting factors are responsible for this physiologic process, however, the final common pathway by which the pulmonary VSMC constricts apparently is the Ca^{2+}-mediated stimulation of excitation-contraction coupling; whereas relaxation occurs mainly through either a cGMP- or cAMP-mediated mechanism[8]. In the normal pulmonary circulation the basal vascular tone is low and is the result of a subtle balance between vasoconstrictor and vasodilator activity[9-11]. The normal pulmonary vasculature has the capacity to adapt to large fluctuations in pulmonary blood flow. During exercise, or in congenital heart disease with left-to-right shunt, pulmonary blood flow may increase enormously, without a rise in pulmonary artery pressure[12]. This can be achieved due to the great range of vascular tone modulation, high vascular compliance and the possibility to recruit "unopened" vessels. For studying the physiology of the pulmonary circulation, the use of hemodynamic variables and a simple formula for pulmonary vascular resistance (PVR), analogous to Ohm's law of electricity, has proved to be extremely useful from a clinical point of view[13, 14]:

$$PVR = \frac{mean\ PAP - mean\ LAP}{Qp} \qquad \textit{(equation 1)}$$

in which:	PVR	=	pulmonary vascular resistance	($WU.m^2$)
	PAP	=	pulmonary artery pressure	(mmHg)
	LAP	=	left atrial pressure	(mmHg)
	Qp	=	pulmonary blood flow	($l/min/m^2$)

Pulmonary vascular resistance, corrected for body surface, is expressed as Woods Units times m^2. Although there is a wide range of normality, depending on various conditions at the time of the study, the normal pressure- and flow values in the young human are the following: mean PAP is around 15mmHg, about one-sixth of that in the systemic circulation, mean LAP approximately 8mmHg. Qp around 4 $l/min/m^2$ and, consequently, PVR approximately 1.5 Woods Units.m^2 [15, 16].
Poiseuille and Hagen, who both studied the flow of liquids along narrow tubes, discovered independently that, once steady flow has started, the pressure drop (ΔP), between two points L cm apart could be related to the flow rate (Q), the radius of the tube (r) and the viscosity of the liquid (η), by the expression[13, 17].

$$\frac{\Delta P}{Q} = \frac{8}{\pi} \cdot \frac{L}{r^4} \cdot \eta \qquad \textit{(equation 2)}$$

Focusing on PVR, several aspects have to be taken into consideration. Although the comparison of the complex pulmonary circulation to a simple rigid tube is a gross simplification, the relationship does indicate the importance of vessel radius. Because it is a fourth power function, a small change in radius alters resistance markedly in an individual vessel. The overall cross-sectional area of the pulmonary vascular bed undergoes dramatic changes in growth and disease[13]. Another feature of this formula that applies to clinical conditions is the factor viscosity. Even without pulmonary vascular changes, PVR will increase in conditions with polycythemia, associated with hyperviscosity. In children with cyanotic heart disease or pulmonary vascular disease this factor may not be neglected. Another factor, that have to be taken into account

when interpreting PVR, is the critical closing pressure[16, 18]. This is the pressure which results from the forces which tend to collapse a vessel. In the lung these may originate from the alveoli or from the vessel itself, in the latter case due to its elastic or muscular recoil. In the physiologic condition, LAP exceeds the critical closing pressure and is the relevant downstream pressure. However, when critical closure pressure exceeds LAP, as may occur in pathologic conditions such as emphysema or pulmonary hypertension, the usual calculation of PVR becomes misleading because LAP is no longer the appropriate downstream pressure[18].

PVR as primary measure of the state of the pulmonary vasculature has conceptual limitations[15, 18]. PVR as calculated above, represents the resistance to continuous flow from the main pulmonary arteries to the pulmonary veins, but does not take into account the dynamic nature of pulsatile flow[15, 18, 19]. The pulmonary vascular system distends during systole and acts as a reservoir for blood, which will continue to flow during diastole. In this way, pulsatile flow is converted to virtually continuous flow in the pulmonary capillaries. In the pulmonary capillaries pulsatile flow can still be recognized, although the pulsations are relatively small and superimposed upon a strong mean flow[15, 16, 19, 20].The vascular reservoir capacity is called capacitance, and is determined by the distensibility or compliance of the pulmonary arteries. The pulmonary artery distensibility is determined by elastic properties of the arterial wall, the tone of its smooth muscle and environmental forces like, for instance, alveolar surface tension or excessive interstitial fluid[15, 19]. An additional characteristic of pulsatile flow is inertia, due to viscous forces in the blood, which will oppose the action of the heart. Inertia varies directly with the heart rate[15, 19]. A third important component of pulsatile flow is the effect of reflected pulse waves[15, 18, 19]. In principle, pressure wave reflections originate from the pulmonary microcirculation, but also from the frequent branching of large vessels. In the normal pulmonary vasculature, wave reflections are of small amplitude because of excellent matching of impedances in the distal pulmonary vessels[18]. However, pulmonary hypertension may increase the amplitude of the reflected waves and if pulmonary arterial compliance is decreased, their velocity will also be increased. These early reflection waves will enhance systolic pressure and the load on the right ventricle[18]. Approximately 70% of the total right ventricular work load is formed by the steady load and 30% by the pulsatile load[18].

The pulsatile power is dissipated mainly in the large pulmonary arterial branches, while the steady work is lost in the small vessels[19, 21].
Finally, the resistance to pulmonary blood flow in the normal lung occurs not only upstream to the gas exchange vessels (30-50%), but also in the microcirculation itself (approximately 30%) as well as downstream in the venous component of the pulmonary vasculature (20-30%)[15]

Humoral and cellular mechanisms in the mature lung
Only recently the pivotal role of the vascular endothelium in the homeostasis of pulmonary circulation has been recognized (4, 5, 11). The functions of the pulmonary endothelium include biosynthesis and metabolism of circulating hormones and vasoactive substances, modulation of vascular tone, regulation of hemostasis and thrombosis and influencing inflammatory processes. In recent years, in which molecular and cellular biology techniques have provided new insights in vascular biology, various mediators and growth factors have been shown to have vasoactive and growth modulating properties. The endothelial cell has been shown to be able to produce both vasoconstrictor (e.g. endothelin-1, platelet derived growth factor) and vasodilator (e.g. nitric oxide, prostacycline) substances. Many of these substances have been suggested to play a role in the maintenance of basal pulmonary vascular tone[9-11, 22, 23]. Although subject of extensive investigations, the roles of the various substances in the physiology of the pulmonary circulation has not been fully elucidated yet.
The vascular endothelium produces a short lived dilator substance, which has been identified as nitric oxide (NO)[24-26]. NO is synthesized from L-arginine, by the calcium-dependent, endothelial-specific isoform of nitric oxide synthase (ecNOS). A calcium-independent isoform of NOS can be induced by cytokines and lipopolysaccharides in endothelial cells and VSMC's[27-30]. This latter isoform is called inducible NOS (iNOS). After release by the endothelial cell, NO diffuses to the VSMC and stimulates soluble guanylate cyclase, increasing cGMP levels and lowering intracellular calcium. cGMP mediates vasodilatation, probably through GMP-kinase mediated activation of calcium-gated K^+-channels[31]. In addition to its vasodilator properties, NO modulates vascular growth and structure, decreases platelet aggregation, neutrophil

adherence and vascular permeability[30, 32]. Physiologic stimuli, including increased pO2 and hemodynamic forces, such as pulsatile flow or shear stress, are known to induce ecNOS activity. Basal NO formation has been demonstrated to contribute to the maintenance of basal pulmonary vascular tone and appear to augment the decline in PVR during exercise[11, 22, 33].

Development

Anatomy

The pulmonary vasculature does not have only special features, but it also develops and mature in a special way[4]. The embryonic development of the lungs starts in the 4th week after conception, but is not yet completed at birth. Therefore, the lung of the newborn cannot be regarded as a miniature adult lung[4]. The characteristics of airway development and differentiation have been extensively described[34].

At 4 wk of human development, an endodermal pair of lung buds originates from the esophagus and differentiates into bronchi[35-37]. These bronchi multiply by branching until at 16 wk all non-respiratory airways are present[4, 38]. Respiratory airways develop between 16 wk of gestation and birth. The airspaces present at birth are referred to as primitive sacculi. At birth, approximately 20 million sacculi are present in both lungs[4, 38]. Alveoli, as in the adult lung, are not present at birth. In mature infants they first appear approximately 2 months after birth, then multiply fast. This multiplying is most prominent in the first years of life, but continues until the age of 8 years, when approximately 300 million alveoli have developed. The alveoli keep growing in size until adulthood[4].

Pulmonary vascular development also starts early in the embryogenesis, probably simultaneously with the development of the airways. The mesenchyme surrounding the pair of lung buds contains a vascular network derived from the foregut[36]. Vessels following the branching airways differentiate into arteries and later join the pulmonary arteries, which have grown toward the lung buds from the sixth branchial arch[35, 36]. Two morphogenic processes contribute to the development of lung vasculature: vasculogenesis and angiogenesis[36].

In the process of vasculogenesis, preexisting endothelial cell precursors or angioblasts assemble in situ to form primitive vascular channels. These channels remodel, giving rise to arteries, veins and lymphatics, depending upon apparently local stimuli and information within the surrounding mesenchyme[36].

In the process of angiogenesis, new vessels form by budding or sprouting from preexisting vascular channels, which have groups of migrating cells at their tip. Differences in endothelial cell properties between the proximal and distal lung vasculature have been contributed to both the differences in origin (i.e., sixth branchial arch and mesenchyme) and the different processes involved (vasculogenesis and angiogenesis) in lung vascular development[36, 39].

A close association of pulmonary arteries and airways starts early in development. During development preacinar arteries and airways show a synchronized branching pattern[40]. Remaining separate, however, are the veins that arise within the loose mesenchyme of the lung septa. All preacinar vessels are present at the 16 wk of intra uterine life[4, 38]. The intra acinar blood vessels appear when the alveoli develop. They multiply and keep pace with the alveolar multiplication. The presence of muscularisation of the arterial wall lags behind the appearance of new vessels[4, 38]. The proportion of arteries of a given size that show a muscular media changes with growth and development, but is also modified with disease[4, 41]. Not only size, but also the position in the arterial tree determines wall structure in the intra acinar arteries. The extension of muscular arteries into the acinus progresses from fetus to adult. In the fetus, muscular arteries are not found with the respiratory bronchiole or beyond. Virtually all muscular arteries are found in the preacinar region. During childhood and adolescence, there is extension of muscle, so that it appears in the arterial wall progressively further into the periphery of the respiratory unit. Respiratory bronchioli develop a muscular coat during the first year of life. At the age of four years approximately all arteries accompanying alveolar ducts are muscular. At that time the majority of these arteries is partially muscularized and becomes completely muscularized by 7 years of age (figure 1)[4].

Pulmonary arterial muscularity, defined as percentage medial thickness, is higher in the fetus than in the adult. After birth, percentage medial thicknes decreases at first rapidly, and then more gradually, to reach the low mature level at three months of age

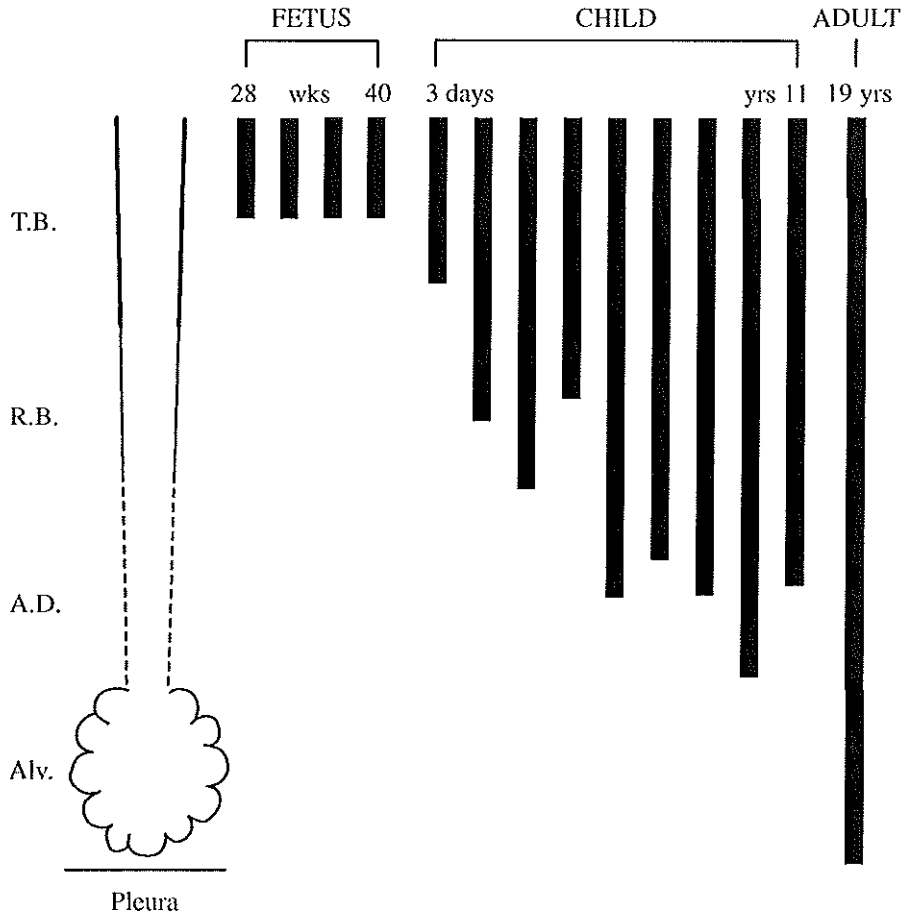

Figure 1. *The distance or 'extension' within the acinus of muscular arteries. In the fetus, muscular arteries are not found with the respiratory bronchiolus (R.B.) or beyond. During childhood, they extend further until, in the adult, they reach the pleural level. The acinus is the respiratory unit supplied by a terminal bronchiolus (T.B.). A.D. = alveolar duct; Alv. = alveolar region. Adapted from Reid, LM. The pulmonary circulation: remodeling in growth and disease. The 1978 J. Burns Amberson lecture. Am. Rev Respir Dis 1979; 119:531-46.*

in most normal infants (figure 2)[4, 42, 43]. As explained later, this first postnatal drop in muscularity is associated with changes in cell shape and position, without reduction of the amount of pulmonary vascular smooth muscle[44, 45].

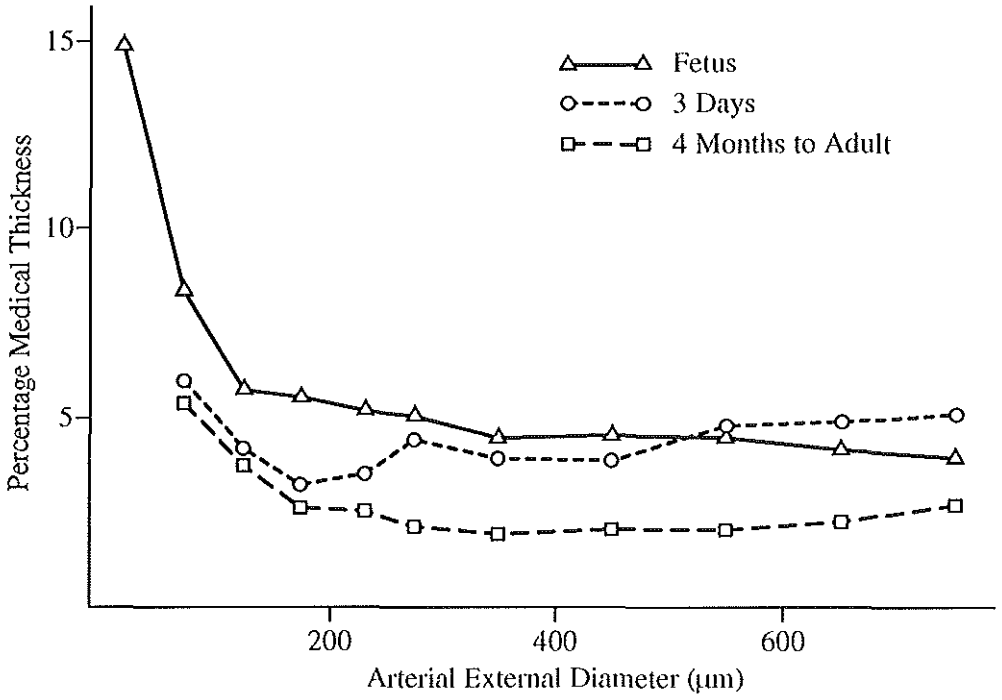

Figure 2. *Medial thickness as a per cent of external diameter related to arterial diameter. Arteries of all sizes are thicker in the fetus than the adult. By 3 days, the increase in compliance of small arteries is apparent by their decrease in wall thickness to adult levels.*
Adapted from Reid, LM. The pulmonary circulation: remodeling in growth and disease. The 1978 J. Burns Amberson lecture. Am. Rev Respir Dis 1979; 119:531-46.

If judged by the percentage medial thickness of a given artery, pulmonary arterial muscularity is larger in the fetal lung compared to the adult lung. However, judged by the level to which muscular arteries are found, muscularity in the fetus is strikingly less than in the adult lung.

During maturation of the pulmonary vasculature, contractile, cytoskeletal and extracellular matrix protein production is subject to important changes, which will be discussed later[36].

Physiology

Most of our knowledge of the fetal circulation is derived from studies in fetal lambs and most of the data reviewed here refer to the fetal lamb. In utero, the pulmonary blood flow is very low. During 2/3 of gestation the lungs receive approximately 4% of the combined ventricular output, rising to 8-10% at the end of gestation[46-48]. Most of the right ventricular output is diverted away from the lungs through the widely patent ductus arteriosus to the descending aorta and the placenta for oxygenation.

Although the number of small blood vessels per unit of lung increases by 10-fold in the second half of gestation (i.e. the number of small vessels increases 40-fold, while the wet weight of the lung increases 4 fold), the blood flow per unit of lung does not change in this period of gestation[46]. As many vasodilators can increase pulmonary blood flow dramatically in the near-term lamb, fetal blood flow apparently is limited by vasoconstriction[49, 50]. Fetal pulmonary circulation probably is modulated through a balance between vasoconstrictor and vasodilator stimuli, including endogenous mediators and mechanical factors (e.g. lung volume).

Sympathic chain stimulation, norepinephrine, arachidonic acid, prostaglandin F2, thromboxane A2 and endothelin-1 (ET-1), an endothelium-derived vasoactive peptide, all are demonstrated to cause vasoconstriction in the fetal pulmonary circulation[36, 51]. However, neither experimental sympathectomy nor alpha-adrenergic blockade decreased fetal PVR, indicating that the high PVR of the normal fetus is not caused by sympatic tone[36]. Similarly, blocking prostaglandin and thromboxane A2 synthesis does not decrease resistance, so prostaglandins or thromboxane do not appear to cause the high vascular tone in the fetal lung[36].

In the late gestational ovine fetus, prolonged infusion of ET-1 causes increase of PVR, whereas intrapulmonary blocking of the ET_A receptor, localized at the VSMC, results in a decrease of vascular resistance. In contrast, selective stimulation of the ET_B receptor causes pulmonary vasodilation through endothelial cell NO release. It is suggested that endogenous ET-1 production primarily contributes to the high basal pulmonary vascular tone in the fetus by its action on the A receptor of the VSMC[52, 53]. Vasoconstriction in response to the low fetal oxygen tension (around 19 mmHg) also does contribute to fetal pulmonary vasoconstriction. Decreasing oxygen tension in near-term fetal lambs doubled PVR, whereas increasing oxygen tension markedly

decreased resistance and increased pulmonary blood flow to normal newborn levels[46, 54].

Prostaglandins, in particular prostaglandin-E2 (PGE_2) and prostacyclin (PGI_2), have shown to be potent vasodilators in the fetal lung[55, 56]. PGI2 is produced by endothelial cells and has its smooth muscle relaxing effect via cAMP. Acetylcholine, bradykinin and histamine are fetal pulmonary vasodilators, which act by stimulating the formation of NO[26, 57-60]. The presence of NO has been reported from in the first half of gestation in the fetal lamb. NO is formed in the endothelial cell during the conversion of L-arginine to L-citrulline by NOS. Next, NO diffuses rapidly into the VSMC and stimulates soluble guanylate cyclase to produce cGMP[32]. Despite high fetal PVR, NO modulates basal pulmonary vascular tone in the late-gestation fetus and postnatal adaptation (see later)[61]. Recent studies suggest that high fetal cGMP-specific phosphodiesterase (PDE5) activity may limit NO activity[62, 63].

In the late gestational fetal circulation vasoconstrictor stimuli evidently dominate over the vasodilator stimuli, resulting in a high fetal pulmonary vascular tone and resistance.

Humoral and cellular mechanisms in pulmonary vascular development

The close association of arteries and airways begins early in development, and as development proceeds, there is a synchronization of vessel and airway branching, implying that they respond to common mediators[40]. The exact mechanisms that control vascular cell proliferation during lung development *in vivo* are not known. However, circumstantial evidence from *in vitro* studies of vascular cells from the lung or other organs suggest that the local production and action of growth factors are involved[36]. Growth factors who are likely to play a role in pulmonary vascular development are the fibroblast growth factor (FGF), both the basic and acidic type, the transforming growth factors ß (TGFß), platelet derived growth factor (PDGF), the insulin-like growth factors (IGF) type 1 and 2 and the vascular endothelial growth factor (VEGF)[64].

Both acidic and basic FGFs have been shown to be mitogenic and chemotactic for vascular wall cells as well as a potent angiogenic factors[64, 65]. Antibodies to FGF have been demonstrated to retard vascular development[66]. TGFß increases production of collagens I and III and fibronectin by lung fibroblasts and is associated with angiogenic

activity[36, 67]. It has been shown to be expressed in the developing lung in a specific spatial and temporal pattern and to have potent effects on cell proliferation[36]. PDGF is also expressed at different stages in the developing lung and is shown to be mitogenic for VSMC's and fibroblasts, especially in combination with cyclic mechanical load, and to influence cell differentiation[36, 68-70]. The IGFs, both type 1 and 2, stimulate proliferation of VSMC's and fibroblasts and also increase elastin and collagen synthesis in neonatal pulmonary vascular wall cells. IGF-1 levels within the vascular walls are shown to vary with hemodynamic load and medial IGF-1 expression parallels the expression of elastin[36, 70].

Mediators involved in the modulation of pulmonary vascular tone, also have effects on cell growth: ET-1, a potent vasoconstrictive peptide, has a mitogenic effect on pulmonary VSMC's. In contrast, the pulmonary vasodilators NO and prostacyclin inhibit proliferation of VSMC's[8, 71]. Hemodynamic factors are also of great importance in directing vascular growth. It has been suggested that the mechanical stress acting on vascular endothelial cells after the onset of circulation is the major force responsible for the development of large vessels[72]. A "shear stress responsive element" has been identified in a progressive number of growth factors, suggesting that its production and activity can be altered by hemodynamic forces[73, 74].

In the fetus and newborn, VSMC's are immature: synthetic and reproductive rather than contractile properties predominate[75-78]. In this stage, production of growth factors and deposition of connective tissue seems more important than contractility. During development, fetal VSMC's mature to an adult phenotype, characterized by increased concentration of myofilaments and predominantly contractile properties[79]. This is accompanied by the acquisition of growth restrictive characteristics. However in contrast to skeletal muscle, VSMC's do not show terminal differentiation when they loose the capacity to replicate[36, 79]. In case of *in vivo* vascular injury, apparently fully differentiated VSMC's are able to "redifferentiate" to the synthetic phenotype with great potential to replicate[36].

At all ages, the medial VSMC's form a phenotypically heterogeneous, highly organized cell population. The cells close to the adventitia contain a significantly greater concentration of myofilaments than those close to the intima[76, 77, 79]. This gradient in VSMC differentiation is present at all age despite all the cells' showing an increase in

myofilament concentration with age. The predominantly secretory properties of the VSMC's in the inner media may indicate a greater potential for growth and adaptation, which helps to explain the phenomena of intima proliferation, containing migrated VSMC's, in pulmonary vascular disease. Phenotypically different VSMC's also express genes for different growth factors[45].

Production of extracellular matrix by cells of the developing vessel is also essential for vascular integrity and continued cell differentiation. A major function of the VSMC in the developing vessel is the production of connective tissue, particularly elastin and collagen, which ultimately constitute some 70% of the mass of the large pulmonary arterial walls[36]. The amount of connective tissue increases after birth in both media and adventitia. The proportion of the different types of interstitial connective tissue also changes with age, which directly affects the mechanical properties of the vessel wall[80, 81]. Elastin is largely produced during fetal development, with production essentially concluded by the first decade of life. *In vitro* experiments have demonstrated that TGFß, IGF-1 and -2 and glucocorticoids increase tropoelastin-mRNA and elastin synthesis, whereas epidermal growth factor, interleukin-1B, phorbolesters and 1,25-dihydroxyvitamin D3 suppress tropoelastin synthesis[36, 82]. Control of elastogenesis in vivo, however, is poorly understood.

At birth, immunolocalization shows an abundance of collagen types III and V and a relative paucity of type I[77, 81]. With age the proportion of type I collagen increases, mainly in the outer media and adventitia. Because collagen type I is the collagen of high tensile strength, this suggests that the newborn pulmonary vasculature is more plastic than that of the adult, facilitating rapid changes in cell shape and orientation[45, 75, 77]. Factors assumed to be involved in collagen deposition include IGF-1, TGFß, leukotriene C4 and PDGF, which stimulate collagen synthesis, and PGE2, epidermal growth factor and gamma-interferon, inhibiting collagen synthesis[36, 83, 84].

Postnatal adaptation

Anatomy

Instantly after birth the lungs have to take care of the newborns gas exchange and

start immediately to adapt to extra-uterine life. This process, called neonatal transition, aims at an abrupt increase in pulmonary blood flow. It is accompanied by a rapid remodeling of the pulmonary vasculature and can be divided in two stages[77]. Stage 1, the direct adaptation, that lasts from birth until approximately 4 days of age[75, 77]. Stage 2, the structural stabilization, that begins at birth and lasts until 3-4 weeks of age[76, 77]. As outlined before, postnatal pulmonary arterial development also comprise vascular growth, continuing until adulthood.

In stage 1, adaptation involves both the elastic conducting arteries and the resistance arteries, as the pulmonary vasculature behaves in a closely integrated manner[75, 76]. The earliest and most prominent changes, however, are seen in the precapillary arteries. At birth the endothelial cells are squat and have a low surface/volume ratio. Already 5 minutes after birth, these cells show stretching and spreading, leading to a thinning of the vascular wall and an increase in luminal diameter, while the external diameter is constant[75, 77]. Similar structural changes occur in the small muscular arteries, although slower. Many small, "unopened", muscular arteries are recruited into the pulmonary circulation during the first four days of life. Thus, direct structural adaptation to extra-uterine life consists of changes in cell shape and position, without reduction of the amount of pulmonary vascular smooth muscle[44, 45]. These changes may be facilitated by the lack of fixed interstitial connective tissue in the peripheral vascular wall at birth.

In stage 2, after the cells have taken their new position within the vessel wall, structural stabilization is accomplished by deposition of connective tissue[77]. The formation of the definitive lamina elastica interna, consisting at birth of tiny amounts of amorphous elastin in the small muscular arteries, takes place in the first 3 weeks. In the larger arteries all elastic laminae increase in length and thickness and occasional collagen fibrils becomes substantial collagen fibers during the first 3 weeks of life[76]. Also the type of collagen changes: at birth there is mainly type III and V collagen, while postnatal deposition is mainly type I[81]. The increase in medial elastin is relatively greater than that of collagen.

Physiology

At birth there must be a sudden and complete transition from gas exchange by the

placenta to gas exchange by the lungs. Instantaneously, the newborn depends on pulmonary ventilation and perfusion. After the umbilical artery has been clamped, systemic resistance rises, partly caused by the removal of the low resistance vascular bed of the placenta. Immediately after birth a 8 to 10-fold increase in pulmonary blood flow occurs, from 30-35 ml/kg/min to 350-400 ml/kg/min and is associated with a dramatic fall in PVR[47]. At 24 hours after birth the mean pulmonary artery pressure is only half systemic [85]. After the initial, rapid fall in PVR and pulmonary artery pressure there is a gradual, progressive fall, with adult levels reached in 2-6 weeks[86, 87]. The rapid fall is mainly due to lowering vascular tone and modulations in cellular shape (see above), the gradual fall to vascular remodeling, muscular involution and rheologic changes.

The decrease in resistance is initiated by the rhythmic distension of the lungs by ventilation, increase of oxygen tension (pO_2) and the decrease of carbon dioxide tension (pCO_2)[88, 89]. Experiments in lambs have shown that each of this stimuli separately lowers PVR, however, it has been demonstrated that the changes are predominantly due to the local vasodilatatory effects of increased pO_2[46, 88]. The mechanism through which O_2 causes pulmonary vasodilation is not known. The rise in O_2 could directly affect the pulmonary VSMC or it could stimulate the release of a substance or substances that, directly or indirectly, dilate the pulmonary circulation.

Increasing oxygen tensions have been demonstrated to increase basal and stimulated NO release as well as bradykinin, a stimulator of NO synthesis[90, 91]. Another mechanism could be that the increase in O_2 inhibits vasoconstriction, direct or via vasoactive substances. Finally and most likely, a combination of these may be responsible for the effect of pO_2[92].

Humoral and cellular mechanisms in the transition process

Various potential mediators of transition have been investigated, however, the complex mechanisms of neonatal transition are not fully understood.

Prostaglandins. One of the most extensively studied mediators have been the prostaglandins. The decrease in PVR caused by mechanical distension of the lung, without changing gas tensions, could be blocked completely by indomethacin, a prostaglandin synthesis inhibitor[93, 94]. The particular prostaglandin involved, appears

to be prostacyclin (PGI2)[95]. However, the effect of pO_2 is not associated with an increase in PGI2 and cannot be blocked by indomethacin[96, 97]. Since in the intact lamb indomethacin only modestly disrupts the decline in PVR, but does not disrupt the transition to gas exchange by the lungs, the role of prostaglandins in transition is assumed to be modest[36].

Nitric oxide. Increasing experimental data exist suggesting a central role of endogenous NO in the perinatal period. Both basal and stimulated release of NO appear to peak in the immediate postnatal period[98]. L-NA, a blocker of NO production, blocks the increase in fetal pulmonary blood flow caused by hyperbaric oxygenation without ventilation[99]. Infusion of a low dose L-NA for 10 days before delivery, did not increase basal PVR, but it markedly blunted the decrease in resistance and increase in flow at birth, mimicking the physiology of persistent pulmonary hypertension of the newborn (PPHN)[100]. Shear stress increases the synthesis of NO in the fetal pulmonary circulation, providing a positive feedback mechanism for increases in pulmonary blood flow at birth[101].

Endothelin. In the late gestation ovine fetal lung, brief infusions of ET-1 cause transient vasodilatation; however, with prolonged infusion, PVR increases[102]. The effects of ET-1 on PVR in the normal fetus are mediated through distinct receptor subtypes[103]. The ET_A receptor has been identified to the VSMC[104]. The ET_B receptor is present on the vascular endothelial cell[105]. As summarized earlier, ET-1 is suggested to contribute to high fetal basal pulmonary vascular tone. In human neonates with PPHN circulating ET-1 levels are markedly elevated, correlate with disease severity and decline with the resolution of PPHN[106]. These findings suggest that ET-1 activity decreases during normal transition.

Thus, summarizing, multiple mechanisms appear to independently and synergistically cause the crucial increase in pulmonary blood flow at birth.

Pulmonary vascular disease in congenital heart disease

Pulmonary plexogenic arteriopathy

Pulmonary hypertension is not a disease in itself, but rather a symptom of underlying

disease. The underlying disease may have its origin in the heart, the pulmonary vasculature or both. Secondarily, pulmonary hypertension will lead to pulmonary vascular disease, including functional and structural changes of the pulmonary vessels. Pulmonary hypertension caused by heart defects is associated with increased pulmonary blood flow and/or pulmonary venous congestion. Various types of pulmonary vascular disease (PVD) have been identified with different etiologic factors[107, 108].

1. Alveolar hypoxia, resulting from a variety of disorders, including exposure to high altitude, chronic obstructive airway disease and cystic fibrosis, will lead to hypoxic arteriopathy, characterized by pulmonary vasoconstriction and an increase in muscularization of all levels of the pulmonary arborization, except the pulmonary veins.
2. Left ventricular dysfunction, mitral or pulmonary venous stenosis, all may lead to pulmonary congestive vasculopathy, characterized by muscularization of the pulmonary veins and secondarily of the pulmonary arteries and hemosiderin-containing macrophages in the alveoli.
3. Congenital heart disease associated with increased pulmonary blood flow will lead to pulmonary plexogenic arteriopathy (PPA). This is a characteristic type of pulmonary vascular disease, most frequently found in patients with congenital heart disease, but also occurring under various, apparently unrelated conditions, including hepatic disease, schistosomiasis, HIV and ingestion of anorexigenic drugs. It also occurs without a recognized etiological factor, where it constitutes one of the histological subtypes of the clinical entity called 'unexplained' or primary pulmonary hypertension .
4. Other forms of pulmonary vascular disease include thromboembolic arteriopathy, pulmonary fibrosis, pulmonary vasculitis and pulmonary veno-occlusive disease. These types of pulmonary vascular disease are out the scope of this study and will not be discussed here.

When discussing pulmonary vascular disease, it is of eminent importance to distinguish the different types of pulmonary arteriopathy. Crucial differences in etiology, pathophysiology, morphology of vascular changes and, most important, in clinical

course distinguish plexogenic arteriopathy from other vasculopathies, such as hypoxic or congestive vasculopathy[109]. In the early stage of PPA, morphologic vascular changes may be similar to that in other arteriopathies. However, when PPA advances, characteristic lesions emerge, such as concentric laminar intimal fibrosis and plexiform lesions, that are never seen in other types of pulmonary arteriopathy. Also the clinical course distinguishes PPA from other pulmonary vasculopathies. Hypoxic and congestive pulmonary vasculopathies are reversible if the causing factor, the hypoxia or the venous congestion, can be removed. In contrast, PPA, if untreated, advances to a certain 'point of no return' at which the disease process has not only become irreversible, but even progresses when the underlying heart defect is corrected[110, 111]. This review will further focus on plexogenic arteriopathy in congenital heart disease.

Congenital heart diseases that are associated with increased pulmonary blood flow, especially if combined with increased pulmonary artery pressure, will lead to PPA. The time course in which PPA develops seems related to hemodynamic variables. In heart defects with left-to-right shunts and increased pulmonary artery pressure persisting after birth, e.g. a large unrestrictive ventricular septal defect (VSD) or a complete atrioventricular septal defect, PPA commonly progresses to the advanced stage within the first years of life . In contrast, in cardiac anomalies with substantially increased pulmonary blood flow but normal pulmonary artery pressure, e.g an atrial septal defect, PPA generally advances slowly and, untreated, may lead to severe, irreversible disease associated with pulmonary hypertension and death, after the third decade of life in more than 50% of the cases[112]. Although advanced PPA in the latter type of defects has been reported in the pediatric age group, this is rare[108, 113]. Besides the hemodynamic characteristics, the anatomical defect has been suggested to affect the time course in which advanced PPA develops[77, 114]. For example, both an isolated VSD and one associated with a transposition of the great arteries (TGA) cause a left-to-right shunt and pulmonary hypertension, but, if untreated, the former rarely causes advanced PPA during the first year, whilst the latter usually has developed irreversible PPA before patients are one year old[77, 115].

Anatomy

Pulmonary plexogenic arteriopathy is named after its most characteristic lesion, the so called plexiform lesion, which occurs, however, only in the final stage of the disease. Plexogenic arteriopathy, i.e. potentially leading to plexiform lesions, is thus a vascular disease with a sequence of vascular changes, that may eventually include plexiform lesions. From a histological point of view, the most prominent vascular changes are localized in the small, muscular pulmonary arteries.

ENDOTHELIAL CELL ALTERATIONS

Ultrastructural studies, by scanning and transmission electron microscopy (EM), of lung biopsy material of patients with congenital heart disease revealed alterations in endothelial cell appearance as the vessel becomes thick-walled[116]. By scanning EM, the normal "corduroy-appearance" of the arterial inner surface texture, consisting of cells in neat, even, aligned ridges, changed to "cable-like" with twisted hulls and gulleys. On transmission EM, compared to control pulmonary arteries, the cytoplasm of endothelial cells showed an increased concentration of rough endoplasmatic reticulum and microfilament bundles. Further it was observed that the internal elastic lamina of the muscular arteries appeared fragmented in patients with early PVD.

MEDIAL HYPERTROPHY AND MUSCULARIZATION OF ARTERIOLES

If elevated pulmonary artery pressure persists after birth the pulmonary vasculature fails to remodel normally[77, 117, 118]. In the presence of an increased pulmonary blood flow, pericytes and intermediate cells in precapillary vessels differentiate to VSMC, giving rise to extension of muscle into more peripheral arteries than normal[119, 120]. This "muscularization of arterioles" occurs almost always in association with thickening of the media of the muscular arteries[77, 108, 120]. This thickening is due to an increase in number and size of the VSMC's. Although it is clear that both hypertrophy and hyperplasia are involved, this is usually referred to as medial hypertrophy. In congenital heart disease medial hypertrophy already occurs during the first two months of life, probably when the VSMC's are in their least differentiated state and consequently highly responsive to injury[36, 77, 118]. The VSMC's, which are relatively undifferentiated at birth, show an accelerated maturation in the presence of elevated

pulmonary artery pressure, associated with an increase in myofilament concentration and an increase in contractile capability[79]. This is further enhanced by a premature innervation of these thick-walled arteries by sympathic, vasoconstrictive nerves[77]. This stage of increased muscularity of the pulmonary arterial tree is accompanied by an extensive modulation of the extracellular matrix composition, including increased deposition of collagen and glycoproteins[36, 77, 120].

INTIMAL THICKENING

Intimal thickening is the formation of a de novo layer in a normally non-cellular stratum between endothelium and internal elastic lamina[108]. Although various types of intimal thickening may occur in plexogenic arteriopathy, two forms, representing two successive stages of disease, are the most common and characteristic for this arteriopathy:

CELLULAR INTIMAL PROLIFERATION

This constitutes the first stage in the development of intimal thickening and is most striking in muscular arteries smaller than 100 μm in diameter[108]. Ultrastucturally, the proliferated cells within the intima have the appearance of "myofibroblasts" and are suggested to be VSMC's that have migrated from the media, through gaps in the internal elastic lamina to the subendothelium[121]. The VSMC's of the inner media have a predominance of secretory over contractile organelles indicating a great potential to growth, adaptation and migration[36, 76, 79]. Within the layer of proliferating cells, there is accumulation of mucopolysaccharides, but collagen fibers are scarce initially[107]. Cellular proliferation of the intima may cause considerable narrowing of muscular pulmonary arteries.

CONCENTRIC-LAMINAR INTIMAL FIBROSIS

Deposition of collagen and, usually to a lesser extent, elastin within the layers of proliferated intimal cell leads to the stage of intimal fibrosis. This process starts within the layer of cellular proliferation close to the internal elastic lamina. In the course of time the intimal thickening becomes more and more fibrotic and lacking in cells. Concentric layers are formed by which the intimal thickening acquires a peculiar

onion-skin appearance. This lesion may be considered pathognomic for plexogenic arteriopathy[108]. As with cellular proliferation, concentric-laminar intimal fibrosis is most severe in the proximal segments of supernumerary branches near their origin from larger arteries and eventually may completely occlude the arterial lumen and may protrude into the parent artery as a thick collar around the orifice[108]. Severe intimal fibrosis may cause secondary medial atrophy by lack of medial nutrition, normally supplied by blood flowing through the lumen, or by lack of persistence of the high blood flow and/or pressure in the more peripheral artery segments, resolving the stimuli for VSMC proliferation[107, 122].

DILATATION LESIONS

Sustained elevation of pulmonary arterial transmural pressure may lead to localized dilatation of pulmonary arteries, so that the lumen becomes wider and the wall thinner. Consequently, preexisting medial hypertrophy seems to diminish or to disappear in some arteries, in spite of sustained pulmonary hypertension. Usually, dilatation leads to extreme thinning of the wall of muscular arteries and their branches, in combination with pronounced widening of lumina, producing the so called "vein-like branches of arteries", that arise from thick-walled arteries[110]. These branches may form clusters of wide, thin-walled vessels, that occasionally become so large and compact as to resemble small angiomas, referred to as "angiomatoid lesions"[110]. Vein-like branches, clusters of vessels and angiomatoid lesions are collectively known as dilatation lesions. The place of dilatation lesions in the sequence of changes of plexogenic arteriopathy is not beyond doubt[108-110]. It is certainly one of the advanced lesions and most likely the development of dilatation lesions generally precedes that of fibrinoïd necrosis and plexiform lesions[108, 109].

FIBRINOÏD NECROSIS AND ARTERITIS

In fibrinoïd necrosis, the wall of muscular pulmonary arteries, and particularly the medial smooth muscle cells, degenerate and become necrotic, probably preceded by imbibition of the wall with fibrin[107, 108]. This process usually occurs over short stretches of small arterial branches, preferably shortly after their origin from a larger artery. In such a segment the media is swollen with an eosinophilic, groundglass appearance,

while the VSMC's become unrecognizable due to karyolysis. In some cases fibrinoïd necrosis is complicated by an inflammatory response, leading to necrotizing arteritis. Experimental evidence suggests that the injury to the arterial wall leading to fibrinoïd necrosis is related to severe, spastic contraction of the media[123].

PLEXIFORM LESIONS

Plexiform lesions are very characteristic arterial alterations that develop in the final stage of plexogenic arteriopathy. The predilection place is, again, in the small arterial branches, the supernumerary arteries, directly after their origin from larger arteries. A plexiform lesion consist, in its most typical form, of a circumscript dilatation of a branche with destruction of the arterial thinning of the media, loss of VSMC's and rupture or even disappearance of elastic laminae. Within the dilated lumen a plexus of narrow slit-like channels develops. These channels communicate with each other and with the proximal and distal lumen of the branch, the latter being very dilated and thin-walled over some distance[108, 110]. The proliferated cells within a plexiform lesion are numerous and show characteristic hyperchromatic nuclei. Active proliferation of endothelial cells and active collagen synthesis have been demonstrated within plexiform lesions[124, 125].

Summarizing, in the early stages of PPA, increased muscularization of the pulmonary arterial tree is found, constituted by medial thickening of the pulmonary muscular arteries and muscularization of normally non-muscularized arterioles. This is followed by cellular intimal proliferation. As plexogenic arteriopathy advances, characteristic lesions emerge, such as concentric laminar intimal fibrosis, angiomatoid dilation lesions, fibrinoid necrosis and plexiform lesions. These advanced lesions are associated with irreversible disease[111].

In the larger, elastic pulmonary arteries, structural changes are less prominent. However, these arteries are not exluded from the disease process. The media of the elastic arteries increases in thickness, due to an increase in size of the solitary VSMC's and changes in the extracellular matrix, including an increase in collagen and glycoprotein deposition[126]. Intimal fibrosis occurs in the larger elastic arteries, but is not characteristic for plexogenic arteriopathy and, in pathological studies, did not correlate with the histological changes of the vascular bed[108, 126].

Physiology

The equation for PVR (*equation 1*) can be rearranged to give:

$$PAP = LAP + PVR \times Qp$$

It can be derived that pulmonary hypertension may be due to three basic physiologic phenomena. Firstly, if pulmonary blood flow is increased, whereas the PVR is unchanged, this will result in an increased pressure gradient over the pulmonary vascular bed and thus an elevated pulmonary arterie pressure. This may be the case in young children with congenital heart disease with left-to-right shunting, before advanced PVD has developed. Secondly, if the downstream pressure (LAP or PWP) is elevated and pulmonary blood flow and PVR are unchanged, pulmonary artery pressure has to rise to maintain the required pressure gradient over the vascular bed. This may be the case in mitral stenosis or left ventricular dysfunction. And thirdly, PVR is increased, and consequently more pressure is needed to maintain equal pulmonary blood flow. This will be the case in PVD. Obviously, various combinations of the described conditions are possible.

It is important to realize that flow per se is not a cause of pulmonary hypertension. Large increases in pulmonary blood flow due to excersise or atrial septal defect cause almost no increase in mean pulmonary artery pressure because, as outlined earlier, previously closed arteries are recruited and existing open vessels dilate[13]. Consequently, PVR is below normal in these conditions. Therefore the finding of pulmonary hypertension, when there is a large pulmonary blood flow implies that PVR has not fallen appropriately and indicates some PVD[13, 127, 128].

PVR rises in pulmonary vascular disease. Evaluated from the Poiseuille equation (*equation 2*), this may be caused by changes in vessel diameter, length of the vessels or blood viscosity. In PVD, the pulmonary arterial lumen will narrow because of vasoconstriction, due to increased muscularization, and because of cellular intimal proliferation; in advanced stages the arterial lumen may even obliterate completely. This will result in a decrease in the overall cross-sectional area of the vascular bed. To differentiate between the vasoconstrictive and structural component of vessel narrowing, PVR can be determined, subsequently, during administration of

-pulmonary- vasodilators. Although not based on adequate follow-up data, the response of the vascular bed to vasodilators, such as inhalation of 100% oxygen and/or NO, has been assumed to indicate the reversibility of the disease process[14, 129-131].
Independent from vessel narrowing, the number of pulmonary vessels affects the total cross-sectional area of the pulmonary vascular bed. In certain conditions it is known that the pulmonary vascular bed may be hypoplastic, such as congenital diaphragmatic hernia and anomalies with decreased fetal bloodflow, like pulmonary atresia. This may increase PVR, however independent from vascular hindrance and thus from the degree of vascular disease[13, 128]. The number of pulmonary vessels is a variable that is difficult to evaluate.
An increase in blood viscosity will increase PVR by increasing the pressure gradient across the vascular bed for a given flow[13, 132]. Since hematocrit may be increased in patients with cyanotic heart disease or decreased in children with large shunts, this variable should be taken into account. For example, a hematocrit of about 70% can roughly double PVR[133]. The exact increase will depend on the hematocrit, the flow rate and the LAP. Therefore, with a high hematocrit, an increased PVR should not be interpreted only in terms of narrowed resistance vessels.
The length of the vessel seems a relatively unimportant variable in PVD because it seems unlikely that the small resistance vessels will changes much in length during the disease process[13].
In the presence of pulmonary hypertension the distensibility of the elastic pulmonary arteries decreases, probably due both to structural and functional changes in the arterial wall and to the artery operating on a steeper part of its pressure-volume relationship[18, 134]. Reflected pressure waves will be increased in pulmonary hypertension. The decreased distensibility, and thus decreased capacitance of the pulmonary arteries may hamper the smooth conversion of pulsatile flow to virtually continuous perfusion of the pulmonary capillaries, and will lead to an accelerated return of the reflect waves [18, 134]. Both these physical phenomena will increase the mechanical, potentially damaging forces on the vasculature.
Consequently, in the course of PVD both the steady load and the pulsatile load of the subpulmonary ventricle will increase dramatically, eventually resulting in ventricular failure.

Humoral and cellular mechanisms in pulmonary vascular disease

Recent research in vascular biology has demonstrated that a large variety of growth factors and mediators are likely to be involved in vascular remodeling. One has to realize, however, that our current knowledge has largely evolved from experiments using cultured vascular cells *in vitro*. Although this simplified experimental approach is necessary to determine capabilities of cells and to elucidate the mechanisms responsible for regulation of cell to cell interactions, these methods carry unavoidable limitations. Cells in culture may dedifferentiate and be removed of a myriad of blood-borne and cell-derived controlling elements that are present *in vivo*, with unknown consequences. A second indispensible source of our knowledge are experiments using animal models of pulmonary vascular disease. However, these experiments have demonstrated that the effects and actions of growth factors and their receptors show important inter-species differences[135]. And, last but not least, these experimental models often use hypoxia, air-emboli or chemicals, such as monocrotaline, to induce pulmonary vascular disease[136]. These factors, however, do not lead to pulmonary plexogenic arteriopathy. Unfortunately, no well-functioning animal model for this characteristic arteriopathy is currently in use. So, the results of these experiments must be interpreted cautiously and can not simply be translated to humans with pulmonary vascular disease associated with congenital heart defects.

The pulmonary vascular remodeling process is associated with excessive cell proliferation and extracellular matrix modulation. Marked changes in cellular phenotypes occur during the remodeling process. Precursor cells in precapillary vessels differentiate into VSMC's resulting in muscularization of arterioles[77]. In the presence of neonatal pulmonary hypertension, accelerated maturation of VSMC from the immature, synthetic phenotype to the adult-like, predominantly contractile phenotype has been demonstrated[36, 79]. On the other hand, a subset of VSMC's increase their replicating and matrix protein synthetizing properties[137-139]. Immature VSMC's migrate from the confines of the media into the subendothelium where they produce a new extracellular matrix. Further, apparently fully differentiated VSMC's may "redifferentiate" to a secretory phenotypy in the course of pulmonary vascular disease[36, 79]. The pulmonary vascular endothelium, with its unique location, lining the inner vessel

wall, seems pre-eminently suited to detect changes in mechanical forces, such as stretch and shear stress, and is assumed to play an important role in the initiation of PVD associated with increased pulmonary blood flow and/or pulmonary hypertension[8]. A shear stress responsive element (SSRE) has recently been identified in the promotor region of genes, encoding for various vascular modulators, including ecNOS, PDGF and endothelin[29, 140]. Endothelial cell release of vasoactive substances, including PGI_2, ET-1 and NO, was increased by cyclic strain on cultured human endothelial cell layers[74]. Shear-stress has been demonstrated to induce the endothelial release of substances, including PDGF, NO and PGI_2[32, 141].

Endothelial injury appears to be an important mechanism of enhanced release of ET-1 in animal models[142], lung ET-1 production is increased in rats with idiopathic pulmonary hypertension[143] and the pulmonary arterial-to-venous ratio of immunoreactive ET-1 was increased in patients with pulmonary hypertension compared to controls[144]. Finally, increased expression of ET-1 in pulmonary endothelial cells has been demonstrated in adults with pulmonary hypertension[145].

NOS activity is stimulated by hemodynamic forces, such as pulsatile flow and shear stress[32]. However, in patients with plexogenic arteriopathy, expression of ecNOS appeared to be diminished in the course of the disease[146]. In adolescents with advanced PVD, but also in babies with potentially reversible PVD, the excretion of prostacyclin metabolites was reduced, whereas that of thromboxane metabolites was increased[147]. Already in an early stage of pulmonary vascular disease in children with congenital heart disease and increased pulmonary blood flow, impaired endothelium-dependent pulmonary vasorelaxation has been demonstrated[148].

In short, numerous observations suggest that PVD is the consequence of imbalance between substances with vasoconstrictor/growth promoting properties on the one hand, and vasodilator/growth inhibiting properties on the other; either by an increased release of the former or by an inhibited release of the latter. The net result will be pulmonary vasoconstriction and proliferation of pulmonary vascular cells.

During the process of vascular remodeling there is extensive extracellular matrix modulation, associated with changes in proteolytic, chemotactic and synthetic activity. Alteration of extracellular matrix can change cell phenotype[120], so it has been suggested that release or activation of proteolytic enzymes may be responsible for initiating the

series of switches in cell phenotypes[149, 150]. Increased activity of an endogenous vascular elastase (EVE) has been observed in VSMC's of rats with both hypoxia and monocrotaline induced pulmonary hypertension[151-153]. Elastase-inhibitor studies strongly suggest that increased activity of EVE leads to proliferation and migration of VSMC's and increased production of extracellular matrix[154]. Other growth factors and mediators that have been suggested to be involved in the pulmonary vascular remodeling process include TGFß, bFGF, IGF-1, IGF-2, VEGF, protoglycans and glycoproteins[36, 71, 120, 149]. TGFß and IGF-1 and -2, induce the synthesis of collagen and other extracellular matrix proteins, such as fibronectin[64, 67]. The expression of isoforms TGFß-2 and TGFß-3 apeared to be increased in the media and intima of pulmonary arteries in patients with PPA[155]. Increases in IGF-1 and IGF-2 mRNA expression have been demonstrated in experimental pulmonary hypertension[156]. bFGF not only induces proliferation of vascular cells, but also the synthesis of proteases. These activities, in combination with its chemotactic properties, make bFGF suitable to play a role in the migration of VSMC's[64]. Finally, bFGF is directly upregulated by mechanical forces.
In advanced PPA, exuberant endothelial cell proliferation has been demonstrated in plexiform lesions[124]. VEGF, an important mitogen for endothelial cells and mediator of angiogenesis, is induced by TGFß and has been suggested to play a role in pulmonary vascular disease. In an *ex vivo* rat model, hypoxia enhanced VEGF-synthesis and upregulated VEGF-receptor mRNA expression in alveolar cells and pulmonary macrophages[157, 158].

The growth factors under study in PVD, are similar to the factors involved in pulmonary vascular development and growth. It is unclear whether vascular remodeling in disease results from a recapitulation of mechanisms associated with normal vascular development or from entirely different pathological mecha-nisms[36]. Up to now, the pathophysiology of pulmonary plexogenic arteriopathy is poorly understood. The sequence of activation or inhibition of the various factors in the time course of the disease, so crucial to obtain insight in pathophysiology, remains to be elucidated.

Aims of the studies

Pulmonary vascular disease is an important determinant in the management and prognosis of children with congenital heart disease. The assessment of the progression of plexogenic arteriopathy in these children is hampered by the limited knowledge of the pathophysiology of the disease.
In the early stage of pulmonary vascular disease, the functional consequences of the alterations in contractile properties and the proliferation of VSMC's and the modulation of extracellular matrix, for the integrated, pulsatile pulmonary circulation are only partly understood. This is due to the difficulties in studying the intrinsic, biomechanical arterial wall properties *in vivo*.
We sought for new diagnostic variables that include both structural and functional properties of a pulsatile circulation. Intravascular ultrasound has the potential to evaluate both the structural appearance of the pulmonary artery *in vivo* and functional properties of the arterial wall itself.

The aims of the described intravascular ultrasound studies in patients with congenital heart diseases were:

1. To assess the feasibility of pulmonary intravascular ultrasound in infants and children and the ability to measure vascular dynamics accurately.
2. To assess the ability of pulmonary intravascular ultrasound to detect changes in the appearance of elastic arteries in pulmonary vascular disease.
3. To study vascular pulsatility and arterial wall distensibility, which may be new functional variables of the pulmonary vasculature, in patients with and without pulmonary vascular disease.
4. To assess how these variables are associated with hemodynamic data, with structural changes of the pulmonary vascular bed and with the progression of the vascular disease.

In plexogenic pulmonary arteriopathy, characteristic lesions emerge in the course of the disease process, that are associated with severe, irreversible disease, and will progress even when the underlying heart defect is corrected. Determination of this so

called "point of no return" is hazardous: the techniques that are currently in use to evaluate the pulmonary vasculature leave a grey zone between reversible and irreversible disease. This is due to our lack of knowledge on how the pathognomic lesions of plexogenic arteriopathy develop and at what point in the time course of the disease, plexogenic arteriopathy distinghuish itself from other pulmonary arteriopathies.
Characteristic features of the advanced lesions of plexogenic arteriopathy include endothelial cell proliferation, abnormal angiogenesis and dilatation. We sought for factors that could be involved in these specific processes and therefore may play a role in the development of the characteristic lesions of plexogenic pulmonary arteriopathy. VEGF has been demonstrated to induce endothelial proliferation and angiogenesis, including abnormal angiogenesis in malignancies. The properties of NO include vasodilatatory activity, inhibition of VSMC proliferation and stimulation of endothelial cell proliferation. Large amounts of NO, as produced by the inducible isoform of NOS, may lead to oxidative vascular injury.

The immunohistochemical studies, using lung biopsy tissue of children with congenital heart diseases, were aimed to answer the following questions:

1. Is Vascular Endothelial Growth Factor involved in the development of the characteristic lesions of PPA and if so, at which stage of the disease?
2. Is increased nitric oxide production likely to play a role in the development of the characteristic lesions of PPA?
3. Is the inducible isoform of nitric oxide synthase involved in pulmonary vascular remodeling and is there a differentiated expression of the different isoforms?
4. Is the expression of vascular endothelial growth factor and nitric oxide synthase in the pulmonary vasculature associated with mechanical forces, reflected by pulmonary hemodynamic data?

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Comparison of hemodynamic and histologic evaluation of the pulmonary vasculature and the relationship with clinical outcome in children with congenital heart disease: Is there a gold standard?

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Abstract

To determine the correlation between hemodynamic and histological evaluation of the pulmonary vasculature and clinical outcome, we retrospectively studied contemporaneous hemodynamic and histological data and outcome in a selected group of 47 children with complex congenital heart disease.

Pulmonary artery pressures correlated weakly with increased muscularity of the pulmonary arterial tree ($r = .46$ to $.67$; $p < .05$) and with the presence of advanced plexogenic arteriopathy ($p < .01$). Diastolic pulmonary artery pressure $\leq$ 25mmHg or pulmonary vascular resistance ≤ 6 WU.m^2 excluded features of advanced plexogenic arteriopathy. Perioperative mortality, associated with acute pulmonary hypertensive crises, was correlated with increased arterial muscularity ($p = .01$). Pulmonary hypertensive crises were not correlated with preoperative hemodynamics. Of 15 survivors, 5 showed persistent pulmonary hypertension at follow up (69 ± 39 months; mean $\pm$ SD). No correlation existed between preoperative hemodynamics or the histologic presence of advanced plexogenic arteriopathy and persistent pulmonary hypertension after surgery. All 3 patients with plexiform lesions showed persistent pulmonary hypertension ($p = .02$).

Conclusions: We found merely limited correlations between hemodynamic and histological assessment of pulmonary plexogenic arteriopathy. Perioperative mortality due to acute pulmonary hypertensive crises was associated with increased muscularity of the pulmonary arterial tree, but could not be predicted from preoperative hemodynamics. Both hemodynamic and histologic assessment of plexogenic arteriopathy were of limited value in predicting persistent pulmonary hypertension after corrective surgery. Only plexiform lesions were inevitably predictive for persisting pulmonary hypertension.

Introduction

Pulmonary plexogenic arteriopathy constitutes a serious threat to children with congenital heart disease and increased pulmonary blood flow or increased pulmonary artery pressures. The state of the pulmonary vasculature is an important determinator of the management and prognosis of these children[1,2]. As long as increased pulmonary blood flow or elevated pressure in the pulmonary arteries persists, the process of plexogenic arteriopathy may progress towards a "point of no return", at which the process becomes irreversible and precludes a curative effect of surgical repair of the cardiac disease[3,4]. In addition, earlier and potentially reversible stages of plexogenic arteriopathy can jeopardize the outcome of surgical procedures because of acute pulmonary hypertensive crises direct postoperatively[5]. In Fontan or Norwood-like procedures, even a slight postoperative increase in the resistance of the pulmonary vascular bed will cause pulmonary hypoperfusion, which may easily result in clinical detoriation. The techniques most commonly used to assess the state of the pulmonary vasculature and to determine operability in children with congenital heart disease are hemodynamic evaluation by cardiac catheterization[1,2] and histological evaluation of an open lung biopsy[3-7]. However, important discrepancies between the results of both techniques have been reported in individual patients with pulmonary vascular disease[7-11], hampering the interpretation of such findings. Furthermore, although widely used for over thirty years, data on the predictive value of both techniques with regards to outcome are scarce[4,8,12,13]. The purpose of the present study is to correlate pulmonary vascular morphology and hemodynamic data, and relate the findings to clinical outcome in infants and children with complex congenital heart disease.

Methods

Patients

Patients with congenital heart disease, of whom contemporaneous hemodynamic and histological data, obtained between 1983 and 1994, were available, formed the population of this retrospective study.

Lung tissue specimens

Biopsy specimens were taken as an isolated procedure or during cardiac surgery in children judged to be at risk for pulmonary vascular disease (biopsy group). In addition, specimens taken at autopsy were included in the study (autopsy group). The indication for taking these latter specimens was not necessarily based on the suspicion of pulmonary vascular disease. Specimens were formalin fixed under vacuum, cut in 2 mm thick blocks and embedded totally in paraffin. Five μm sections were cut at various levels and stained with hematoxylin-eosin. Elastic van Gieson stain and Perls' iron stain were included in all cases. All specimens were re-examined independently by two pathologists (CAW, RJS), who were unaware of the diagnosis and hemodynamic data. Only the age of the patient was revealed. Pulmonary vascular lesions were described according to Wagenvoort and Mooi[3]. Morphologic features such as medial hypertrophy, muscularisation of arterioles, cellular intimal proliferation and concentric-laminar intimal fibrosis were scored semi-quantitatively with regard to severity and frequency (0 = absent, 1 = mild, 2 = moderate, 3 = severe), as described earlier[3]. In evaluating the muscularity of the pulmonary arterial tree, the scores for medial hypertrophy and muscularisation of arterioles were adjusted to the age of the patient[14-16]. Subsequently, a concensus session produced the definite scores. For the overall degree of muscularity a single composite score, the total muscularity score (TMS, 0 = normal, 1 = mildly increased, 2 = moderately increased, 3 = severely increased), was obtained on the basis of the scores for medial hypertrophy and arteriolar muscularisation. The presence or absence of arterial tortuosity, longitudinal smooth muscle cells in arterioles and sphincters at the origin of branches were used as secondary indices to turn the balance in cases where the results were borderline. Dilatation lesions, fibrinoid necrosis and plexiform lesions were documented as present or absent. The presence of plexiform lesions, fibrinoid necrosis, dilatation lesions or concentric-laminar intimal fibrosis was considered as "advanced pulmonary plexogenic arteriopathy".

Hemodynamic data

Cardiac catheterization was performed in all patients under general anesthesia. Data on pulmonary and systemic arterial and venous pressures, pulmonary blood flow,

shunt size and pulmonary and systemic vascular resistance were collected. Pulmonary vascular resistance was determined using pulmonary blood flow, calculated with the Fick principle using assumed oxygen consumption. Pulse pressure and the ratios of pulmonary-to-systemic pressure and pulmonary-to-systemic vascular resistance were calculated. Pulmonary vasodilator capacity was studied in 11 cases as the response of pulmonary vascular resistance to 100% oxygen inhalation.

Follow up data

Outcome was assigned to short-term and mid-term outcome. Perioperative death associated with pulmonary hypertensive crises was studied as short-term outcome variable. Perioperative death was defined as death during cardiac surgery or within 21 days after the procedure. Acute pulmonary hypertensive crises were defined as events of progressive and profound pulmonary hypertension and/or systemic desaturation, in the absence of obstruction of the pulmonary artery, combined with a decreased systemic circulation. In patients who underwent a univentricular repair, perioperative death associated with pulmonary hypoperfusion was studied as outcome variable. As mid-term outcome variable was studied the persistence of pulmonary hypertension after corrective surgery. Persistent pulmonary hypertension was defined as a loud second heart sound at physical examination, electrocardiographical evidence of right ventricular hypertrophy and echocardiographical features suggestive for elevated pulmonary artery pressure, in the absence of residual cardiac lesions.

Statistical analysis

Patients in the autopsy and biopsy groups were analysed seperately because the indications to obtain lung tissue were dissimilar in both groups. Hemodynamic data were correlated with histologic findings using Pearson's correlation coëfficiënt. Categorical data were compared by Fisher's exact test. Statistical comparisons of hemodynamic and histologic data between groups of patients were made using the Student's *t* test or Mann-Whitney U test when distribution was not normal. In the biopsy group comparisons of data were made between patients with and without clinical features of pulmonary hypertension during follow up, using the same tests. A value of $p < .05$ was considered significant.

Table 1 *Anatomical Diagnosis*

		n
VSD	• isolated	6
	• with leftsided obstructive lesions	5
cAVSD	• isolated	6
	• complex	3
TGA	• complex	9
Truncus arteriosus		1
ASD with/without PAPVC		5
TAPVC		1
Tricuspid atresia	• complex	3
Fallot		6
Others		2
		47

ASD indicates atrial septal defect; cAVSD, complete atrioventricular septal defect; PAPVC, partial abnormal pulmonary venous connection; TAPVC, total abnormal pulmonary venous connection; TGA, transposition of the great arteries; VSD, ventricular septal defect; complex indicates with associated lesions; n, number of patients.

Results

In the study period, 47 patients underwent concomitant histologic and hemodynamic evaluation of the pulmonary vasculature. The total study group consisted of 29 males and 18 females, with a variety of complex congenital heart diseases (table 1).
The median age at lung biopsy was 6.5 months with a range from 1 day to 16.8 years. The age distribution and the distribution of histological features in relation to age are shown in fig. 1.

One patient had two lung biopsies with concomitant cardiac catheterizations. Three biopsies were taken as an isolated procedure and 14 during cardiac surgery (biopsy group, n = 17). 31 Specimens were taken at autopsy (autopsy group, n = 31). The median time period between collection of lung specimen and hemodynamic data was 1,0 month, ranging from 3 days - 24 months. In 4 patients this period exceeded 6 months.

number of patients

MH	8	15	2	4	1	3
MA	7	15	2	4	1	4
ICP	0	3	0	0	0	2
CLIF	0	2	0	0	1	1
DL	0	0	0	0	1	1
FN	0	2	0	0	1	0
PL	0	1	0	0	1	1

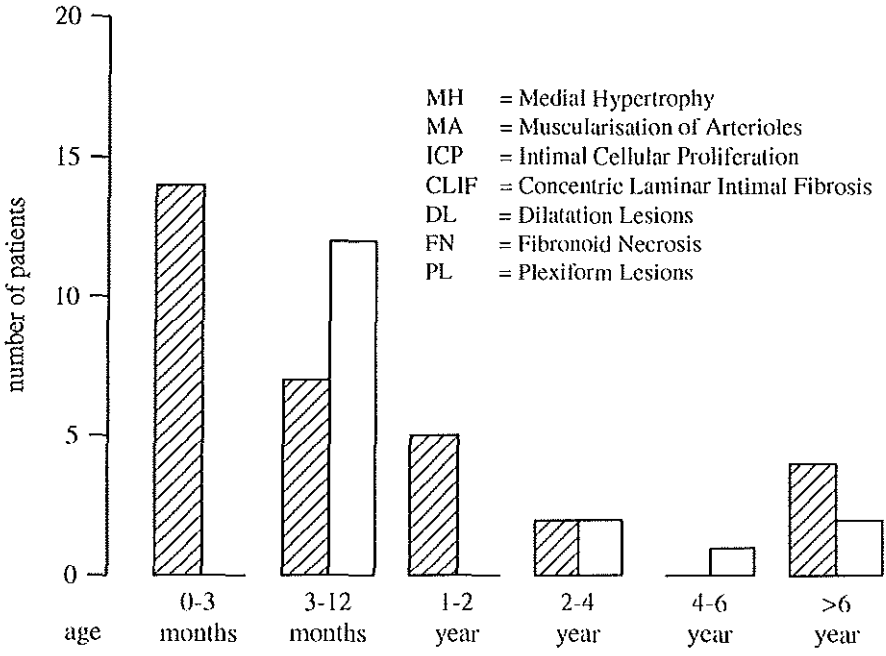

Figure 1 *Age distribution and the distribution of histological features in relation to age. ☑: Autopsy patients; ☐: Biopsy patients*

Table 2 *Patient characteristics of the autopsy and biopsy groups*

group	autopsy	biopsy	
n*	31	17	
median age (mo) (range)	4.5 (0.1-192)	8.5 (3.1-201)	ns
M : F	2.8 : 1	0.9 : 1	ns
hemodynamics			
	(mean ± SD)	(mean ± SD)	
syst PAP	43 ± 22	60 ± 20	p = 0.016
diast PAP	21 ± 11	26 ± 10	ns
mean PAP	30 ± 15	41 ± 15	p = 0.02
pulse pressure	22 ± 14	34 ± 12	p = 0.006
PAP/SAP ratio	0.56 ± 0.34	0.64 ± 0.27	ns
PVR	4.6 ± 4.5	8.0 ± 3.5	p = 0.01
PVR/SVR ratio	0.49 ± 0.99	0.32 ± 0.14	ns
histology			
MH	1.3 ± 1.1	1.5 ± 1.1	ns
MA	1.2 ± 1.1	1.3 ± 1.0	ns
TMS	1.3 ± 1.2	1.6 ± 1.1	ns
APA	0/31	5/17	p = 0.003

APA indicates features of advanced plexogenic arteriopathy; MA, arteriolar muscularisation; M : F, sex ratio; MH, medial hypertrophy; n, number of specimens; PAP, pulmonary artery pressure (mmHg); syst, systolic; diast, diastolic; PVR, pulmonary vascular resistance (WU.m^2); SAP, systemic arterial pressure; TMS, total muscularity score;

* one patient had two lung tissue specimens with concomitant hemodynamic data, see text.

Table 3 *Pulmonary hemodynamic characteristics of the total study population*

		1	2	3	4	Total
n		10	28	4	6	48†
Qp (shuntsize)	↑	–	28	–	3	31
	↓	–	–	4	1	5
	=	10	–	–	2	12
	mean ± SD	1.1 ± 0.1	4.0 ± 4.3*	0.6 ± 0.2*	1.5 ± 0.8	2.8 ± 3.6
mPAP (mmHg)	↑	5	25	2	5	37
	=	5	3	2	1	11
	mean ± SD	19 ± 6	38 ± 14*	31 ± 22	48 ± 15*	35 ± 16
PVR (WU.m2)						
	< 4	4	9	–	2	15
	4-7	4	8	2	–	14
	> 7	1	9	1	4	15
	mean ± SD	3.9 ± 2.6	6.4 ± 5.1	8.1 ± 5.8	9.2 ± 7.1	6.3 ± 5.1
mPVP (mmHg)	↑	–	–	–	6	6
	=	10	28	4	–	42
	mean ± SD	8 ± 3	9 ± 2	6 ± 1	21 ± 3*	9 ± 5

1, indicates normal pulmonary blood flow (Qp/Qs ratio: 0.8-1.2); 2, increased pulmonary blood flow (Qp/Qs ratio > 1.2); 3, decreased pulmonary blood flow (Qp/Qs ratio < 0.8); 4, pulmonary venous congestion (pulmonary venous pressure > 17 mmHg). mPAP indicates mean pulmonary artery pressure (↑ > 20 mmHg); mPVP, mean pulmonary venous pressure (↑ > 17 mmHg); n, number of patients; PVR, pulmonary vascular resistance; Qp, pulmonary blood flow;

* significant difference with group I ($p < .05$).

† one patient had two lung tissue specimens with concomitant hemodynamic data, see text.

By reanalysis, excluding the latter 4 patients, it was verified that the findings were not essentially affected by these cases. Baseline characteristics of the autopsy biopsy goups are shown in table 2. Pulmonary hemodynamics of the total study population are summarized in table 3. Medial hypertrophy and arteriolar muscularisation were present in patients with increased, decreased as well as normal pulmonary blood flow, but were significantly more frequent in patients with increased pulmonary blood flow and/or pulmonary venous congestion ($p = .03$). Intimal cellular proliferation, concentric laminar intimal fibrosis, dilatation lesions, fibrinoid necrosis and plexiform lesions were present only in patients with increased pulmonary blood flow, with or without pulmonary venous congestion.

Hemodynamic-histologic correlation

In the autopsy group, systolic, diastolic and mean pulmonary artery pressure, pulmonary pulse pressure and the pulmonary-to-systemic pressure ratio correlated significantly with the severity of medial hypertrophy ($r = .55$, $r = .46$, $r = .51$, $r = .60$ and $r = .60$ respectively; all $p < .05$), arteriolar muscularisation ($r = .53$, $r = .46$, $r = .50$,

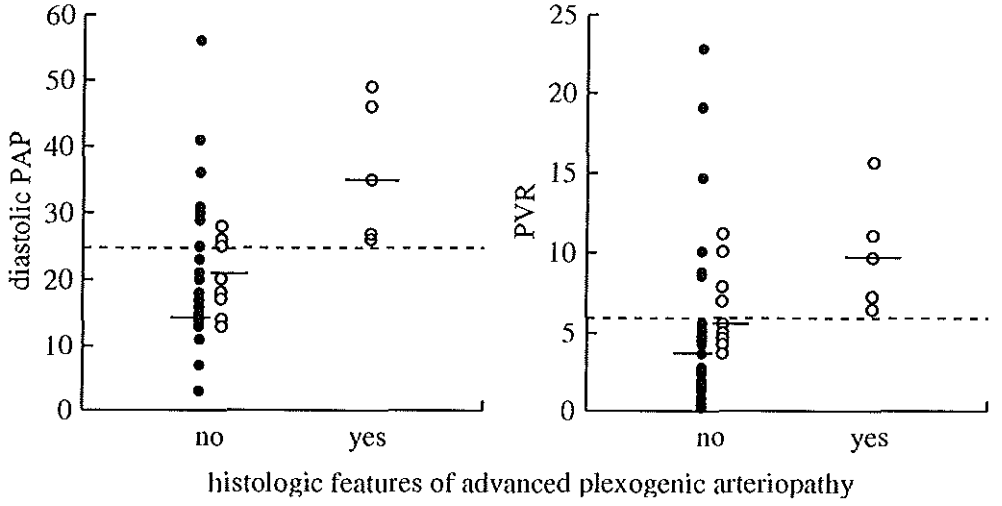

Figure 2 *Relation between the presence of histologic features of advanced plexogenic arteriopathy and diastolic PAP (pulmonary artery pressure, mmHg) and PVR (pulmonary vascular resistance, WU.m²).*
●: autopsy patients; ○: biopsy patients.

r = .57 and r = .56 respectively; all $p < .05$) and total muscularisation score (r = .59, r = .46, r = .54, r = .67 and r = .64 respectively; all $p < .05$). Pulmonary vascular resistance was correlated significantly only with medial hypertrophy ($r = .39$, $p < .05$). Pulmonary blood flow, shunt size, pulmonary-to-systemic resistance ratio and pulmonary venous pressures showed no correlation with the features of arterial muscularity. Cellular intimal proliferation or features of advanced plexogenic arteriopathy were not present in this group. In the biopsy group, none of the hemodynamic parameters correlated significantly with the features of arterial muscularisation or the presence of cellular intimal proliferation. Pulmonary artery pressures and pulmonary-to-systemic pressure ratio were significantly higher in patients with histologic lesions of "advanced plexogenic arteriopathy" compared to those without these features: systolic pulmonary artery pressure, 75 ± 18 vs. 53 ± 18 mmHg ($p = .03$); diastolic pulmonary artery pressure, 36 ± 11 vs. 21 ± 5 mmHg ($p = .002$); mean pulmonary artery pressure, 53 ± 14 vs 36 ± 12 mmHg ($p = .02$); pulmonary-to-systemic pressure ratio, 0.94 ± 0.19 vs. 0.51 ± 0.18 ($p = .01$, mean ± SD). Pulmonary vascular resistance, pulmonary-to-systemic resistance ratio, pulmonary blood flow or shunt size did not differ significantly in patients with or without features of advanced plexogenic arteriopathy.

None out of 7 patients with pulmonary vascular resistance ≤ 6WU.m2 and none out of 9 patients with diastolic pulmonary artery pressure ≤ 25mmHg, cut-off values reported in earlier studies[8, 11, 17] showed features of advanced plexogenic arteriopathy in the lung biopsy, whereas 5 out of 10 patients with pulmonary vascular resistance > 6WU.m2 and 5 out of 8 patients with a diastolic pulmonary artery pressure > 25mmHg did show these features ($p = .04$ and $p = .009$ respectively; analysed for biopsy and autopsy groups together: $p = .003$ and $p = .002$ respectively; fig. 2). The respons of pulmonary vascular resistance to inhalation of 100% oxygen showed no significant correlation with any of the histological variables in the 11 studied patients.

Perioperative mortality

In the autopsy group, 26 of 31 patients had undergone cardiac surgery. In 5 this concerned a univentricular repair. Sixteen patients had died perioperatively. Ten patients had died after the perioperative period without clinical involvement of pulmonary

vascular disease. In 9 out of the 21 patients who had undergone a biventricular repair, perioperative death was associated with pulmonary hypertensive crises. Preoperative hemodynamic parameters of these 9 patients did not differ significantly with that from patients who died perioperatively without pulmonary hypertensive crises (n = 4) or patients who died after the perioperative period (n = 8). Of the 5 patients who had a univentricular repair, 3 died perioperatively due to pulmonary hypoperfusion, whereas 2 died after the perioperative period. Preoperative hemodynamic data did not differ between the former and the latter patients. Patients who died perioperatively associated with pulmonary hypertensive crises, showed significantly increased medial hypertrophy, arteriolar muscularisation and a higher muscularisation score compared to those who died perioperatively without such crises (medial hypertrophy: 2.1 ± 0.9 vs. 0.5 ± 1.0 (p = .01); arteriolar muscularisation: 1.8 ± 1.1 vs. 0.5 ± 1.0 (p = .04); muscularisation score: 2.2 ± 1.0 vs. 0.5 ± 1.0 (p = .01, mean ± SD). In patients with a univentricular repair, no difference in arterial muscularity could be demonstrated between patients who died with and patients who died without pulmonary hypoperfusion. The features of pulmonary arterial muscularisation tended to be lower in patients who underwent a univentricular repair compared to those who underwent biventricular repair, however, these differences did not reach statistical significance. In the biopsy group, no patient had undergone a univentricular repair and no perioperative mortality had occurred.

Mid term outcome

In the biopsy group, 2 of the 17 patients died of causes not related to pulmonary vascular disease (1 infectious disease, 1 during a later surgical procedure) after 55 and 11 months respectively. The remaining 15 patients are still alive with a mean follow up of 69 ± 39 months (mean ± SD), ranging from 14 months to 12.3 years. Five of these patients showed clinical features of persistent pulmonary hypertension in the absence of residual cardiac lesions. No significant differences in age at biopsy, time period between collection of lung specimen and hemodynamic data and follow up period could be demonstrated between children with and without features of pulmonary hypertension at follow up. Preoperative hemodynamic data from children with persistent pulmonary hypertension did not differ significantly from those with-

out persistent pulmonary hypertension: mean pulmonary artery pressure: 50 ± 18 vs. 41 ± 9 mmHg (p = .22), diastolic pulmonary artery pressure 32 ± 15 vs. 24 ± 6 mmHg (p = .38), pulmonary-to-systemic pressure ratio 0.81 ± 0.32 vs. 0.58 ± 0.15 (p = .17), pulmonary vascular resistance 10.1 ± 4.7 vs. 7.7 ± 2.2 WU.m^2 (p = .20) and pulmonary-to-systemic resistance ratio 0.31 ± 0.18 vs. 0.34 ± 0.13 (p = .82, mean ± SD). Of the histological features studied, only the presence of plexiform lesions was significantly associated with signs of persistent pulmonary hypertension: all 3 patients with plexiform lesions showed persistent pulmonary hypertension (p = .02, positive predictive value 100%). The histological classification "features of advanced plexogenic arteriopathy" did not correlate significantly with signs of pulmonary hypertension at follow up (p = .26, positive predictive value 60%). Two out of 10 patients without features of advanced plexogenic arteriopathy in their original lung biopsy showed persistent pulmonary hypertension at follow-up which could not be explained by residual anatomical lesions. However, 2 out of 5 patients with histological features of advanced plexogenic arteriopathy showed no evidence of persistent pulmonary hypertension after 69 and 77 months, respectively. At the time of the lung biopsy, one of these 2 patients showed severe concentric laminar intimal fibrosis with some fibrinoid necrosis, the other also showed fibrinoid necrosis next to severe cellular intimal proliferation. The remaining 3 patients who did develop persistent pulmonary hypertension all showed concentric laminar intimal fibrosis and plexiform lesions, 2 of them also showed dilatation lesions and 1 additionally showed fibrinoid necrosis.

Discussion

The present study demonstrates merely limited correlations between hemodynamic and histologic assessment of the pulmonary vascular bed in a highly selected group of children with complex congenital heart disease. In this patient group, both techniques appeared of limited value in predicting persistent pulmonary hypertension after corrective surgery at midterm follow up. Only plexiform lesions in the lung biopsy were inevitably predictive for persisting pulmonary hypertension.

Lung specimens were obtained from biopsies or autopsies. Pulmonary artery pressures

and pulmonary vascular resistance were significantly higher and histological advanced vascular lesions significantly more frequent in biopsy compared to autopsy patients (table 2). This may be the result of the fact that patients in the biopsy group were selected on the suspicion of pulmonary vascular disease. In the autopsy group, the hemodynamic variables pulmonary artery pressure, pulmonary-to-systemic arterial pressure ratio and pulmonary vascular resistance showed significant correlations only with the muscularity of the pulmonary arterial tree. Correlation coefficients, however, were not high, indicating that, in the individual patient, muscularity could not be predicted accurately from hemodynamic data, or vice versa. In contrast to the findings in the autopsy group, no significant correlation between pulmonary artery pressures and muscularity could be demonstrated in the biopsy group. The grades of medial hypertrophy, arteriolar muscularisation and total muscularity score, however, did not differ significantly in both groups. One could speculate that, in the biopsy group, containing patients with more advanced vascular disease, this lack of correlation could be caused by the effect of pre-acinar obstructive intimal proliferation, resulting in a decreased muscularity of the intra-acinar arteries, as suggested by Haworth[11]. However in our material we found no support for this explanation.

Patients with features of advanced plexogenic arteriopathy had significantly higher pulmonary artery pressures. However, again the scatter was wide, so no accurate predictions could be made in the individual patient. In our study, a diastolic pulmonary artery pressure $\leq$ 25 mmHg or a pulmonary vascular resistance $\leq$ 6 WU.m^2 was never associated with histological features of advanced plexogenic arteriopathy in the lung biopsy. A high diastolic pulmonary artery pressure or pulmonary vascular resistance, however, were not predictive for the presence of advanced lesions.

Discrepancies between the interpretation of hemodynamic and histological data in the individual patient, as frequently found in our patients, are not surprising if one realises that the information obtained by both techniques is far from identical. Histologic examination of lung biopsy is a static, *in vitro*, description of morphologic vascular changes, which does not take into account the functional activity of the endothelium and smooth muscle cells[16, 18, 19]. Although a lung biopsy of adequate size is thought to be representative of the entire pulmonary vascular bed[20], the vascular changes may be distributed heterogenously in the lung. On the other hand, hemo-

dynamic data are derived data, which reflect merely indirect the functional consequences of changes in the vascular bed. Moreover, various assumptions have to be made in calculating flows and resistances, resulting in potential errors[1]. Realising that both diagnostic techniques reveal different aspects of the state of the pulmonary vasculature, the question is which of both methods reveals the most accurate information on clinical outcome in the individual patient. Do we have a gold standard? The value of the information has to be weighed against the risks of both diagnostic procedures, which are not equal. While the risks of pediatric cardiac catheterisation are relatively low[21], the mortality rate of a lung biopsy as an isolated procedure has been reported to reach 20%[17]. Although both diagnostic tools are in clinical use for over 30 years, reports on mid and long term follow up are few[4, 8, 12, 13].

Perioperative mortality

Patients with early, potentially reversible, stages of pulmonary plexogenic arteriopathy may develop pulmonary hypertensive crises in perioperative periods. Although treatment results have improved with the introduction of vasodilators like epoprostenol and, more recently, inhaled nitric oxide, these life-threatening events continue to lead to significant perioperative mortality. In our study, preoperative hemodynamic data did not allow the prediction of perioperative mortality associated with pulmonary hypertensive crises. From the histologic features, increased muscularity of the pulmonary tree, expressed as medial hypertrophy, muscularisation of arterioles or the combined total muscularity score, was associated significantly with perioperative pulmonary hypertensive crises. In univentricular heart repairs, which create a circulation without a ventricle facing the pulmonary vasculature, already a mildly elevated pulmonary resistance can cause pulmonary hypoperfusion and clinical detoriation. In these patients neither preoperative hemodynamics nor muscularity could predict perioperative death with pulmonary hypoperfusion. This is of major concern now that Fontan- and Norwood-procedures are performed with increasing frequency[5, 10-12]. How then, if hemodynamic data do not allow an accurate prediction for the risk of postoperative pulmonary hypoperfusion after univentricular repair or for the risk of perioperative death by pulmonary hypertensive crises after biventricular repair, can we determine the optimal time for surgery, other than just operate at young age?

Furthermore, if this optimal time has passed, can benificial effects still be expected from postoperative supportive therapy, like nitric oxide or other vasodilators? Our current diagnostic tools do not provide answers to these questions.

Mid term outcome

The main aim of evaluating the state of the pulmonary vasculature is the assessment of reversibility of the disease process. Although the irreversible stage of plexogenic arteriopathy most commonly occurs in older patients, with sustained pulmonary hypertension, it may also occur in infants younger than one year of age, hampering surgical decision-making[9, 17, 22]. Data on the predictive value, however, of both preoperative histologic and hemodynamic evaluation, with respect to the progression of plexogenic arteriopathy after corrective surgery, are scarce. Severe concentric laminar intimal fibrosis, fibrinoid necrosis, dilatation lesions and plexiform lesions are features of advanced plexogenic arteriopathy and are generally regarded as signs of irreversible disease[3, 4, 6]. We found morphologic features of advanced plexogenic arteriopathy under the age of one year (fig. 1). No significant correlation between age and any of the hemodynamic or histologic variables could be demonstrated. At follow up, 2 of our patients showed to have persisting pulmonary hypertension after corrective surgery, despite the absence of advanced vascular lesions in their preoperative biopsy. On the other hand, the presence of these features did not predict accurately the persistence of pulmonary hypertension at a mean follow up of almost 6 years. In our series, only plexiform lesions were inevitably predictive of pulmonary hypertension at follow up. Although one should realize that patients with advanced lesions of plexogenic arteriopathy in their lung biopsy may develop pulmonary hypertension at longer follow up, these findings lead to hesitation in accepting histologic assessment as the gold standard with respect to long term prognosis. Especially since recent reports suggest that chronic therapy with vasodilatory and antiproliferative agents, such as continuous intravenous epoprostenol, may result in a remodeling of vascular changes in primary pulmonary hypertension, including reversal of plexogenic lesions[23]. These suggestions challenge the currently used concept of irreversibility of advanced plexogenic arteriopathy. Similarly, hemodynamic evaluation did not allow an accurate prediction of persistent pulmonary hypertension at follow up. Reactivity of the

pulmonary vascular bed to vasodilators is a commonly used tool to assess reversibility of plexogenic arteriopathy[24-26]. However, in our study, response of pulmonary vascular resistance to 100% oxygen inhalation, a very potent pulmonary vasodilator, was assessed in 9 of the 15 survivors and showed no significant correlation with pulmonary hypertension at follow up. Response to other vasodilators was not studied in our series.

Study limitations

A retrospective study has inevitably shortcomings. Lung biopsies were not performed routinely in our institution, but in patients judged to be at risk for pulmonary vascular disease. Thirty-one samples were obtained at autopsy. As a result, the patients in this study represent a highly selected group with a variety of complex cardiac anomalies. However, it is the subset of patients with complex congenital heart disease, like the study population, in which the assessment of the pulmonary vascular bed becomes especially crucial, when correction is delayed because of a staged approach or when Fontan-like procedures will be involved. In this retrospective study hemodynamic evaluation of the pulmonary vascular bed included acute vasodilator testing in only 11 patients. Although inhaled oxygen is one of the most powerful pulmonary vasodilators, additional vasodilator testing with epoprostenol or inhaled nitric oxide in a larger number of patients might have provided additional information[26]. The follow up data on the presence of pulmonary hypertension in our study are based on clinical, electro- and echocardiographical findings suggestive for pulmonary hypertension. Obviously, these findings are not synonymous with the actual presence of plexogenic arteriopathy. However, pulmonary hypertension as a longterm sequel after cardiac repair, is the main, clinically important consequence of plexogenic arteriopathy. Cases were not classified on the basis of anatomical diagnosis because we feel that mechanical forces, acting on the pulmonary vasculature and resulting from abnormal pulmonary hemodynamics caused by the anomaly, rather than the anatomical diagnosis itself, are responsible for the initiation of this specific vascular disease process in children with congenital heart disease.

Both hemodynamic and histologic assessment of the pulmonary vasculature have proved to be of great value in the management of children with congenital heart disease.

However, our study indicates the limitations of both techniques. In our opinion, an effort has to be made to develop additional tools to assess *in vivo* the functional status of the pulmonary vascular bed. The intravascular ultrasound imaging technique may contribute to this ultimate goal. It has the potential to assess directly and simultaneously, *in vivo*, functional and morphological features of the pulmonary arteries. Intravascular ultrasound imaging has shown to be feasible in infants and children and has shown to be able to assess "real time" pulmonary vascular dynamics *in vivo*[27]. In addition, current research on modulators and growth factors involved in pulmonary vascular disease will extend our insight in the pathophysiology of the process of vascular remodeling[19, 28, 29]. Molecular biology may hand new clues with respect to the progression and functional consequences of the pulmonary vascular disease process in children with congenital heart disease, and may thus aid the clinician in choosing the optimal therapy and its timing.

We conclude that, in the selected patients of the present study, correlation between hemodynamic and histological evaluation of the pulmonary vascular bed was merely limited. Patients with a diastolic pulmonary artery pressure $\leq$ 25mmHg or a pulmonary vascular resistance $\leq$ 6WU.m^2 did not show concentric laminar intimal fibrosis, dilatation lesions, fibrinoid necrosis or plexiform lesions in their lung tissue. However, in patients with a diastolic pulmonary artery pressure > 25mmHg and a pulmonary vascular resistance > 6WU.m^2 histology of the lung biopsy could not be predicted from hemodynamic variables. No hemodynamic parameter was predictive for perioperative death associated with pulmonary hypertensive crises. Increased pulmonary arteterial muscularisation was associated with an increased risk of perioperative death associated with pulmonary hypertensive crises. This was not the case in patients who had undergone a univentricular repair. In our study both hemodynamic and histologic assessment of the pulmonary vascular bed were of limited value in predicting persistent pulmonary hypertension after corrective surgery. Only plexiform lesions were inevitably predictive for persisting pulmonary hypertension.

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Possibilities and impossibilities in the evaluation of the pulmonary vasculature in congenital heart disease

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Introduction

Increased pulmonary blood flow and pulmonary hypertension in congenital heart disease will lead to structural and functional changes in the pulmonary vasculature[1-3]. These changes include an increased muscularisation, altered vasoconstrictive and impaired vasodilatatory responses of the arterial tree, extensive extracellular matrix modulation, including increased collagen deposition, and gradual occlusion of the small pulmonary arteries by intima proliferation and fibrosis[4-6]. These alterations will result in a decreased compliance of the pulmonary vessels and, thus, a stiffening of the arterial wall[7, 8]. In plexogenic pulmonary arteriopathy, characteristic lesions emerge in the course of the disease process, including concentric laminar intimal fibrosis, fibrinoid necrosis and plexiform lesions. The combined vascular changes will lead to an increase in both steady and pulsatile hydraulic load of the subpulmonary ventricle. Depending on the progression of the process and the type of surgical procedure, they may jeopardize surgical correction of the heart defect or seriously affect the patients' prognosis. In plexogenic pulmonary arteriopathy, the early vascular changes are reversible after the underlying heart defect has been corrected[9]. However, if untreated, plexogenic pulmonary arteriopathy advances to a certain 'point of no return' at which the disease process has not only become irreversible, but progresses even when the underlying heart defect is corrected[9].

In patients with congenital heart disease, the state of the pulmonary vasculature is, thus, an important determinant of management, clinical course and prognosis. Various diagnostic techniques are available to evaluate the progression of pulmonary vascular disease (PVD) in the clinical setting. However, although all these techniques have undisputable value, they all have specific drawbacks and intrinsic limitations[10, 11]. The purpose of this paper is to review these currently available diagnostic techniques.

Non-invasive techniques to evaluate the pulmonary vasculature

Clinical examination, chest X-ray and electrocardiographic evaluation may suggest the presence of pulmonary hypertension, however sensitivity is very low[12, 13].

Echocardiography with pulsed doppler is more reliable in determining the presence of pulmonary hypertension, using tricuspid- or pulmonary regurgitation velocity measurements[14-17]. However, in congenital heart disease the presence of pulmonary hypertension provides limited information on the state of the pulmonary vascular bed and, in general, the progression of PVD cannot be assessed with these diagnostic tools.
Estimation of left-to-right intracardiac shunting and measurement of pulmonary blood flow using velocity-encoded, phase-difference magnetic resonance imaging have been reported[18-20]. However, its value in the evaluation of PVD remains to be investigated.

Hemodynamic evaluation of the pulmonary vasculature

The technique most frequently used to evaluate the pulmonary vascular bed in congenital heart disease is cardiac catheterization[21-23]. In this procedure the pulmonary artery pressures and pulmonary wedge pressure or left atrial pressure are measured, shunt size and pulmonary blood flow are determined and, subsequently, pulmonary vascular resistance is calculated by dividing the pressure gradient across the lungs by the pulmonary blood flow. The pulmonary vascular resistance constitutes a key parameter in the preoperative evaluation of the pulmonary vasculature[22]. It is regarded as a measure for the functional state of the vascular bed, and its reaction to vasodilators as a measure for reversibility of the disease process. Although hemodynamic evaluation of the pulmonary vascular bed has proven to be extremely valuable in clinical practice, it has both conceptual and practical limitation[24].

Practical considerations. Like any other invasive technique, cardiac catheterization has certain risks, especially in patients with PVD[25, 26]. However, with the currently available techniques and materials, including nonionic contrast media, the risks of pediatric cardiac catheterization are relatively low[27, 28]. The conditions under which the hemodynamic measurements are performed, such as position of the patient and level of agitation, may influence the results of the measurements[29]. Standardized conditions during the complete procedure are imperative in order to obtain reliable

and comparable hemodynamic data. A serious drawback of the hemodynamic evaluation, however, is the difficulty to determine accurately blood flow in the clinical setting. Various methods exist to quantitate cardiac output and intracardiac shunting. These methods are based on the Fick principle: a known amount of a specific indicator is added to a volume of fluid. If the concentrations of the indicator in the blood before and after this addition is known, the volume of fluid can be calculated[26, 30, 31]. The Fick method uses the physiologic uptake of oxygen as indicator. Pulmonary blood flow can be calculated by dividing oxygen consumption through the difference in oxygen content between the pulmonary artery and the veins. The oxygen content of the blood is calculated using measured oxygen saturation, hemoglobin concentration, oxygen binding capacity and dissolved oxygen. The advantages of oximetry are that it is easy to perform, results are available immediately and it allows one to ascertain the site and magnitude of the shunt. However, in patients without intracardiac shunts, the oxygen content has been demonstrated to gradually increase from the caval veins, right atrium (RA), right ventricle (RV) to the pulmonary artery[32-35]. Dexter et al. defined criteria for normal right-sided oxygen step-ups, which resulted in a normal oxygen content difference of maximal 1.9 ml/dl between superior caval vein and RA, 0.9 ml/dl between RA and RV and 0.5 mg/dl between RV and pulmonary artery[32]. This means that the minimal ratio of pulmonary-to-systemic flow (Qp/Qs) that can be reliable detected by oximetry is 1.5-1.9 at the atrial level, 1.3-1.5 at the ventricular level and 1.3 at the level of the great arteries[26, 36]. Measuring oxygen saturations nowadays can be performed relatively easy using paramagnetic oxygen sensors, within an accuracy of approximately 2%[37]. However, in heart defects with large left to right shunts, pulmonary arterial oxygen saturation is high, and variations in oxygen saturations of 2% may result in a change in calculated blood flow of 25% or more[26]. Measurement of the patient's oxygen consumption is another potential source of error. In the past, rather cumbersome methods, like the douglas bag method, have been used for this purpose. However, procedural complexity prohibited its wide use in routine catheterization laboratories. In recent years more practical instrumentation has become available, that can be used routinely[38]. However, measuring oxygen consumption is based on measuring the difference in oxygen content between inspired and expired air. In contrast to metabolic studies, determination of cardiac output

requires an absolute value for oxygen consumption. This, in turn requires precise, absolute measurements of respiratory gas volumes, so that leakage of respiratory gasses has to be precluded. This is cumbersome in most pediatric patients, but is virtually impossible in small, intubated children. For this reason, many routine catheterization laboratories continue to use assumed oxygen consumption, based on body weight or surface area, derived from predictive tables, like that of LaFarge and Miettinen[39]. This is a serious limitation since these figures may not be reliable,

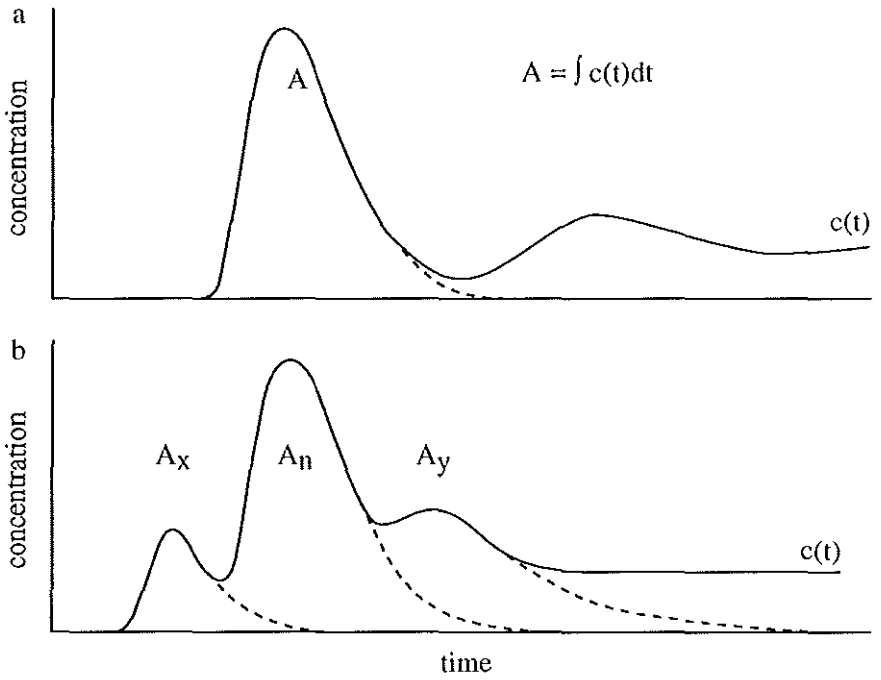


Figure 1. *a. Relationship between time and dye concentration at the femoral artery of a normal subject, after injection of a bolus of dye into the pulmonary artery. The dotted line is the replotting from the linear extrapolation of the descending limb on a semilogarithmic scale.*
b. Relationship between time and dye concentration at the femoral artery after injection of dye into the right heart. A pre-normal so-called x-peak is due to a right-to-left shunt. A post-normal y-peak is due to a left-to-right shunt. The dotted lines are the replottings from the linear extrapolation of the descending limbs on a semilogarithmic scale.

especially in congenital heart disease, where oxygen consumption may be influenced by intracardiac shunts[26, 40, 41]. Finally, when 100% oxygen inhalation is used to test pulmonary vasoreactivity, measuring oxygen consumption as described is not possible because the Haldane equation cannot be applied[42].

The dye dilution technique, first described by Stewart, is often regarded as the gold standard in measuring blood flow in patients[30]. A bolus of dye, most frequently indocyanine green, is injected into one part of the circulation (e.g. pulmonary artery) and sampled at another site (e.g. femoral artery) by continuous withdrawal through a densitometer cuvette, leading to a concentration-time curve, characterized by a primary peak and a recirculation peak (figure 1a)[30, 31]. In left-to-right shunts the curve demonstrates a prominent early recirculation (figure 1b)[43]. The area of the primary curve is proportional to Qp, whereas the area of the early recirculation curve is proportional to shunt flow. Both can be calculated by different mathematical techniques[44-47]. These techniques require a complex analysis of the inscribed curve, a process that may be especially difficult in the setting of very large or small shunts. A method developed by Carter et al. is much simpler but restricts itself to quantitating the intracardiac shunt[48]. To detect a left-to-right shunt using the direct mathematical analysis of the indocyanine green curve, the Qp/Qs ratio has to be at least 1.15[49], whereas the method of Carter can reliably detect left-to-right shunts only when $Qp/Qs \geq 1.35$ [50, 51]. Limitations of the indocyanine green dilution technique include further: 1) the complexity and time-consuming nature of the procedure, 2) the need for -temporary- withdrawal of fairly large volumes of blood from the systemic circulation, which may constitute a potential hazard in infants, 3) problems in cuvette sterilisation and 4) very important in patients with congenital heart disease: the rapid recirculation in large left-to-right shunts may hamper the description of the primary circulation curve, influencing the accuracy of the calculation[26, 36, 52].

The thermodilution technique, that uses temperature difference after injection of a bolus of a cold solution as indicator, is found to be quite reliable in patients without shunts, but is not accurate in the presence of left to right shunts[21, 23]. The difficulties in quantitative determination of blood flows are illustrated by the limited correlations between the different methods[53, 54]. When reviewing the intrinsic limitations of the various techniques, it has to be acknowledged that the determination of shunt size

and pulmonary blood flow, and thus pulmonary vascular resistance, are at best estimations.

Conceptual considerations. The equation used to calculate pulmonary vascular resistance, analogous to Ohm's law in electrical resistance, assumes a constant, steady flow. The Poiseuill-Hagen equation, derived from a rigid tube, indicates the importance of the vessel radius, or cross sectional area of the vascular bed, to steady flow resistance[55, 56]:

$$PVR = \frac{mean\ PAP - mean\ LAP}{Qp} = \frac{8}{\pi} \cdot \frac{L}{r^4} \cdot \eta$$

in which:	PVR	=	pulmonary vascular resistance	(WU.m^2)
	PAP	=	pulmonary artery pressure	(mmHg)
	LAP	=	left atrial pressure	(mmHg)
	Qp	=	pulmonary blood flow	(l/min/m^2)
	L	=	length of the vessel(s)	
	r	=	radius	
	η	=	viscosity of the fluid	

The differences between the steady flow of water throuh a long straight glass tube on the one hand and the pulmonary circulation on the other, are very clearly lined out by Hoffman in 1972[56]. Several considerations have to be taken into account, when interpreting pulmonary vascular resistance in children with congenital heart disease: 1) The total cross-sectional area of the pulmonary vascular bed may be diminished due to other reasons than vasoconstriction or structural vascular changes, occurring in PVD. For instance, vascular underdevelopment in congenital diaphragmatic hernia, or pulmonary thrombi may cause a decrease in total cross-sectional area. The increased pulmonary vascular resistance in these conditions does not represent a decreased vascular hindrance due to vascular disease, but a reduced number of vessels. Narrowing of arteries may be due to extrinsic pressure, for instance in case of perivascular edema fluid. 2) Blood viscosity, a factor in the equation that applies to clinical conditions, may be increased due to polycythemia in children with cyanotic heart disease or PVD, and may raise pulmonary vascular resistance, independent from

pulmonary vascular changes. 3) A third factor to take into account is the critical closing pressure, resulting from the forces which tend to collapse a vessel. In the lung these may originate from the alveoli or from the vessel itself, in the latter case due to its elastic or muscular recoil[57]. In physiologic conditions, left atrial pressure exceeds the critical closing pressure. However, when critical closure pressure exceeds left atrial pressure, as may occur in pathologic conditions such as emphysema or pulmonary hypertension, the usual calculation of pulmonary vascular resistance becomes misleading because left atrial pressure is no longer the appropriate downstream pressure[57]. Finally, a conceptual limitation of pulmonary vascular resistance, representing the resistance of the pulmonary vasculature to steady flow, is that it neglects the pulsatile nature of the pulmonary circulation. In this context one has to realize that in normal conditions approximately 30% of the work load of the right ventricle consists of pulsatile load[57]. In pulmonary hypertension this load increases, because of decreased distensibility of the pulmonary arteries and increased amplitude and velocity of reflected pulse waves[57]. However, this cannot be evaluated using pulmonary vascular resistance.

The response of the vascular bed to vasodilators is regarded as a measure for reversibility of the disease process[58-60]. This is based on the concept that changes in PVD consist of a vasoconstrictive component and structural component[26]. Many studies focus on the question which pharmacological substances produce the most powerfull and selective pulmonary vasodilation[10]. However, it has to be acknowledged that the predictive value of pulmonary vasoreactivity with regards to operability of the cardiac defect, the likelihood for perioperative pulmonary hypertensive crises, and the likelihood of persistent pulmonary hypertension following repair, has never been convincingly demonstrated in children with congenital heart disease. Furthermore, recent observations in patients with advanced primary pulmonary hypertension, who did not respond to acute vasodilator testing, showed that long-term continuous prostacyclin therapy improved the clinical status and hemodynamic data[61, 62]. These observations seriously challenge the concept of reversibility and irreversibility of PVD[63].

Histologic evaluation of lung biopsy

Although the value of this method is also defined by its ability to determine the type of pulmonary arteriopathy and the morphological substrate for clinically unexplained pulmonary hypertension, we will focus here on its use in assessing plexogenic pulmonary arteriopathy in congenital heart defects. From a histological point of view, the most prominent vascular changes are localized in the small, muscular pulmonary arteries (figure 2, see page 180).

In 1958, Heath an Edwards described six grades of progressive structural changes in the small pulmonary arteries in congenital cardiac defects[64]: In *grade I* there was medial hypertrophy of arteries and arterioles, but no intimal changes; In *grade II*, additional cellular intima proliferation was present; In *grade III*, intimal fibrosis was present, in addition to medial hypertrophy. *Grade IV* showed progressive generalized vascular dilatation and plexiform lesions. In *grade V*, other dilatation lesions were present, including vein-like branches and angiomatoid lesions. Finally, *grade VI*, was the stage in which necrotizing arteritis occurred. This grading system has been widely used for assessment of the severity of hypertensive PVD. However, with the increasing awareness of the complexity of the vascular lesions, the sequence of the vascular changes in the course of the disease process and their biological meaning, have been debated[65]. Wagenvoort advocated to abandon the grading principle, because the degree and extent of the various lesions, the different types of intimal fibrosis and additional features should all be assessed, not only for arteries but also for other vessels[65-67]. Careful consideration and weighing of all these features are necessary in order to form an opinion on reversibility and prognosis of PVD. In general, severe concentric laminar intimal fibrosis, angiomatoid dilatation lesion, fibrinoid necrosis and plexiform lesions are considered advanced vascular lesions, associated with irreversible disease[9, 68]. Finally, Rabinovitch and Haworth suggested a morphometric approach, in which they judged the extension of muscle into peripherial arteries which are normally non-muscularized, quantified medial thickening and assessed the number of small arteries by the alveolar-artery ratio[69].

The natural history of the various vascular changes has been assessed in patients with shunts at the ventricular level, who had first undergone a pulmonary artery banding

and subsequent surgical repair of their cardiac anomalies. However, systematic studies of long-term follow-up of patients evaluated by any method are scarce[9, 58, 70, 71].

Practical considerations. To assess PVD, a lung biopsy of adequate size requires an open lung biopsy and, thus, a thoracotomy. The amount of lung tissue obtained by transbronchial biopsies or transthoracic needle biopsies is too small and the vessels are often severely damaged, precluding adequate evaluation[67]. Inadequate lung biopsy size, that may be caused by the concern to take away too much tissue or by pleural thickening, means that the patient is subjected to the risks of the procedure without benefit[67]. An open lung biopsy as isolated procedure carries a certain risk, that substantially increases in patients with PVD[72]. The processing of biopsy specimens is important for a reliable evaluation. For example, handling by the surgeon or the pathologist may easily result in collapse of tissue, whereas intravascular injection of fixative may lead to unpredictable dilatation of vessels, both leading to qualitative and quantitative misinterpretation of the pulmonary blood vessels. Fixation of lung tissue under vacuum has been recommended[67].

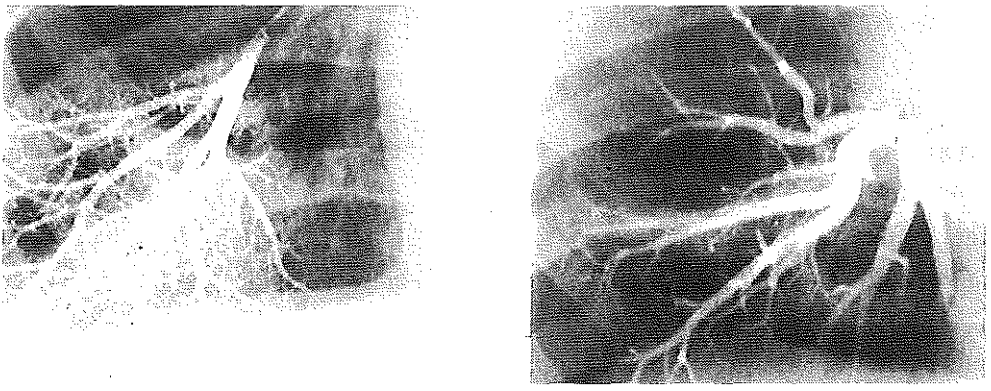
Conceptual considerations. Histologic examination of lung biopsy yields a static description of morphologic changes in the vascular bed and does not take into account the functional activity of the vascular endothelium and smooth muscle cells[73-75]. Controversy exists on the predictive value of evaluation of lung biopsy, since it has been suggested that the vascular changes in PVD, especially the advanced lesions, may be distributed unequally in the lung and thus may be missed in a lung biopsy[69, 76] Also, the presence of these advanced lesions is unusual in the first two years of life, even in the presence of severely, elevated, non-responsive pulmonary vascular resistance[69]. The classification of Rabinovitch et al., studying features that are distributed uniformely through the lung, is based on the concept that small arteries become reduced in size and number in the course of PVD[69, 77, 78]. However, this concept has been seriously challenged[79, 80].

Pulmonary wedge angiography

The pulmonary wedge angiogram provides an angiographic visualisation of pulmonary arteries as small as 100 μm and allows definition of tapering and tortuosity of muscular arteries, supernumerary arteries and of the background capillary blush, representing the perfusion of intraacinar vessels (figure 3)[81-84]. These variables can be described and quantitated and have been demonstrated to correlate with both hemodynamic data and structural findings in lung biopsies[82-84]. It has been suggested that the presence of obstructive, irreversible PVD can be determined by wedge angiography[82]. Two techniques of pulmonary wedge angiography have been described. In the first, an end-hole catheter is placed in wedge position and 0.5-3 ml contrast medium is injected slowly by hand until most of the small arteries and some of the interlobular veins are seen. Then 5-10ml flush solution is flushed through the catheter until the major pulmonary veins fill and the lobular capillary blush disappears[82]. In the second method, a balloon-tipped, end-hole catheter is used. The catheter is placed in a segment artery and after the artery is occluded by inflation of the balloon, 0.3ml/kg contrast medium (with a minimum of 2ml) is injected at a flow rate of approximately 5 ml/s. After the injection, the balloon is deflated and the venophase can be examined separately[83].

Practical considerations. The risks of the procedure consist of the risk of lung parenchymal damage, pulmonary infarction and hemoptysis after injection of contrast medium at a such peripheral localisation, and of the general risks of pulmonary angiography in patients with PVD[25,81]. However, with the currently available materials, such as nonionic contrast fluids, and with the described techniques, these risks are low[82-84]. A limitation of the method is that features such as abrupt termination of vessels, tortuosity, arborization and background flush are largely qualitative and rather subjective. This has been accommodated partly by Rabinovitch et al., who provided quantitative variables, like tapering rate and circulation time[83]. High resolution angiography facilities are required in the catheterization laboratory.

Conceptual considerations. The tapering rate of a vessel may be decreased by dilatation of the proximal part of the artery, as is the case in high flow states, or by narrowing of



a b

Figure 3 *Pulmonary wedge angiography in a 6 years old child with normal pulmonary circulation (a) and in a 5 years old child with a patent duct and advanced pulmonary plexogenic arteriopathy (b). Note the dilated arteries with increased tapering rate, the reduced number of monopedial branches and a markedly reduced background haze.*

the distal part of the artery as occurs in obstructive disease, or by vasoconstriction of the distal part of the artery or, finally, by a combination of these phenomena. These cannot be distinguished by angiography. Circulation time may reflect a structurally reduced pulmonary vascular bed, due to partially or completely obstructed vessels. However, circulation time may be also directly related to flow, and consequently may reflect just pulmonary blood flow. Indeed, circulation time appeared to be decreased in patients with pulmonary stenosis[83].

Evaluation of pulmonary vascular compliance

In the concept of pulsatile flow, the total forces opposing the blood flow consist of the vascular resistance component, the vascular elasticity component and the blood volume related inertia component[85]. Although blood flow through the pulmonary vasculature is equal to that through the systemic vasculature, the pulmonary pressure wave is more rounded and of lower amplitude. This means that the compliance of the pulmonary arteries is substantially higher than that of its systemic counterparts[85].

In the normal pulmonary vasculature elastic arteries extend to arteries of approximately 1 mm in size and have much thinner walls than systemic arteries[67]. In PVD, the compliance of the elastic pulmonary arteries decreases, probably due both to structural changes in the arterial wall and, when pulmonary hypertension is present, to the artery operating on a steeper part of its pressure-volume relationship. Consequently, forces opposing the pulsatile flow, such as arterial compliance and reflected pulse waves, which have little importance in the normal pulmonary circulation, may become major components of the right ventricular load in pulmonary vascular disease[57].
Using harmonic analysis of pressure and flow waves, pulmonary vascular impedance can be studied. However, until now its use has been restricted to animals and experimental models[86-88]. In systemic arteries, vascular compliance has been studied using pulse wave velocity, however this has not been feasible in pulmonary arteries. Recently, pulsatility and distensibility of pulmonary arteries have been studied, using ultrasound or magnetic resonance techniques[89, 90]. These data, however, remain to be validated and interpreted in the clinical context.

Discussion

The techniques described, all evaluate different properties of the pulmonary vasculature. As lined out, the hemodynamic evaluation describes functional aspects of the pulmonary vascular bed associated with the total cross-sectional area of that bed, whereas lung biopsy describes morphological, structural changes of the small pulmonary arteries. Pulmonary wedge angiography describes changes in the course, the luminal diameter and the perfusion of peripheral pulmonary arteries. Pulmonary arterial distensibility describes the functional behaviour and properties of the larger pulmonary arteries and its consequences for the pulsatile circulation. Although, all these different aspects of the pulmonary vasculature are subject to changes in the course of PVD, the information obtained by the various techniques is far from identical. And therefore it may not be surprising, that although the results of the different techniques are correlated, these correlations are limited and results may appear conflicting in the individual patient[56, 58, 69, 79, 82, 83, 91].

No doubt, hemodynamic evaluation, lung biopsy and pulmonary wedge angiography have contributed greatly to our knowledge of PVD and to the management of children with congenital heart disease at risk for this disease. However, realizing the different aspects that are evaluated by each technique and its intrinsic limitations, we are still not confident on how to explain and how to handle discrepancies between the different technique results in individual patients. Clinicians each have developed their own preferences and strategies in evaluating the pulmonary vasculature in children with congenital heart disease, frequently dictated by institutional experience and facilities. A three-pronged approach has been suggested to determine surgical risk and outcome[11]. However, although in use for over 30 years now, reliable data on the predictive values of the different techniques, with regard to reversibility and long term prognosis are scarce[9, 58, 70, 71, 92]. This is especially the case when the results indicate that the disease process is in the grey zone between reversible and irreversible disease. Moreover, in the current era, in which univentricular heart repairs, such as Fontan- and Norwood procedures, are performed in an increasing frequency, the evaluation of the pulmonary vasculature faces higher demands. For instance, on the pathologic functional activity of the pulmonary arteries in early stages of PVD. Efforts should be made to develop new clinical, diagnostic tools, that provide additional information on the state of the pulmonary vasculature, with special attention for aspects of the pulsatile nature of the pulmonary circulation.

Our limited capability of a refined assessment of the pulmonary vasculature in PVD, may be explained by the acknowledgement that the pathophysiology of this characteristic vascular disease process is still largely unknown. In the last decade, due to the emerging research in vascular cell biology, a significant progress has been made in our insights in mechanisms on cellular and molecular level that appear to be involved in PVD[5, 6, 74, 75, 93]. The vascular disease process is characterized by excessive cell proliferation, changes in cellular phenotype and extracellular matrix modulation, directed by various vasoactive modulators and growth factors. The unraveling of these mechanisms may not only provide insight in the progression and functional consequences of the disease process, but may also lead to novel therapeutic strategies directed to prevent or reverse the vascular disease[6, 63, 74].

In conclusion, each of the diagnostic techniques to evaluate PVD in patients with congenital heart disease, describes specific aspects of the pulmonary vasculature and each has its specific practical and conceptual limitations. Data on the predictive value of the techniques regarding reversibility and long term outcom are scarce. The value of the obtained information has to be weighed against the risks of the diagnostic procedure. The results of the different techniques may provide discrepancies in individual patients, hampering the interpretation of the findings. Research focused on the pathophysiology of PVD in congenital heart disease will increase our understanding of these patients and may aid the clinician to ascertain optimal management and its timing.

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Evaluation of the pulmonary vasculature and dynamics with intravascular ultrasound imaging in children and infants

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Pediatr Res 1995;38:36-41

Abstract

The ability to assess the pulmonary vasculature in pulmonary vascular disease by hemodynamic or histological evaluation is limited.

We sought to determine the feasibility of intravascular ultrasound techniques in infants and children and to assess simultaneously the morphology and dynamics of pulmonary arteries.

Patients were seen in the department of Pediatrics, division of Pediatric Cardiology, Sophia Children's Hospital, Rotterdam, The Netherlands.

We performed intravascular ultrasound imaging in 11 pediatric patients with congenital heart disease undergoing cardiac catheterization. Luminal diameter, area and pulsatility were determined at two to five sites in the pulmonary branches. Pulmonary vascular reaction to 100% oxygen inhalation was studied.

Patients weighed 4.1 to 51.0 kg (21.8 ± 16.3 kg, mean ± SD). Luminal diameters, areas and pulsatilities could be determined reproducibly in arteries with diameters from 1.6 to 9.3 mm. In total 39 sites were studied in 11 patients. Pulsatility was related to vessel size ($r = 0.81$), although a substantial interindividual variation was present. After 100% oxygen inhalation pulsatility increased in all arteries (from 20.0 ± 3.3% to 25.9 ± 2.9%, $p < 0.05$) and a vasodilatation could be directly visualised, most prominently in the smallest arteries (percentage change in diameter mean 7.4 ± 2.8% vs. -2.8 ± 3.1% in the largest arteries, $p < 0.001$). Measurement of wall thickness was not feasible, but specific changes in the appearance of the wall structure could be recognized in a patient with severely elevated pulmonary vascular resistance.

The specific advantages of intravascular ultrasound in assessing pulmonary vascular disease are discussed.

Conclusions: Intravascular ultrasound imaging of the pulmonary vasculature is feasible in infants and children and provides a unique opportunity to assess directly pulmonary dynamics *in vivo*. Therefore it may be a valuable tool in evaluating the pulmonary vasculature and its responses to normal and pathological conditions.

Introduction

The state of the pulmonary vasculature plays a crucial role in the management and prognosis of children with congenital heart disease[1, 2]. To assess the pulmonary vasculature, mainly two diagnostic tools are currently in use: haemodynamic evaluation by cardiac catheterization and examination of lung histology. Although both techniques provide valuable information, both also have important limitations. One must realise that, on the one hand, hemodynamic data, like pulmonary artery pressure, blood flow, and resistance, reflect merely indirect consequences of the vascular process; moreover, they are based on various assumptions and, thus, inevitably are accompanied by potential errors[2]. On the other hand, histologic examination of lung biopsy tissue provides a qualitative and quantitative description of morphologic changes in the vascular wall, but remains a static, *in vitro* examination without functional validation[3, 4]. Furthermore, it requires a thoracotomy. These limitations are illustrated by reports of the correlation of the two techniques, showing important discrepancies in individual patients with pulmonary vascular disease (PVD)[5-8].

It has been shown that by means of intravascular ultrasound (IVUS) imaging vascular luminal diameter, area and pulsatility can be determined accurately, as well as qualitative and quantitative aspects of the vascular wall[9-12]. We hypothesized that with this technique it is possible to assess directly and simultaneously vessel wall appearance and "real time" vessel wall dynamics, *in vivo*, in peripheral pulmonary arteries of children with congenital heart disease. This would give direct, unique information on the appearance and functional state of the pulmonary vascular bed, which is directly available during cardiac catheterization and might obviate the need for a thoracotomy.

We therefore performed a study to determine the feasibility of IVUS techniques in infants and children and to assess the appearance and the dynamics of the pulmonary vasculature *in vivo*.

Methods

Eleven patients, requiring diagnostic or therapeutic cardiac catheterization because of congenital heart disease in the Sophia Children's Hospital, Rotterdam, the Netherlands, underwent simultaneous IVUS imaging of the pulmonary arteries. IVUS was performed using disposable ultrasound catheters, 4.2F CVIS (Cardiovascular Imaging Systems Inc., Sunnyvale, CA) or 3.5F Sonicath (Boston Scientific Corp., Watertown, MA), both having a 30MHz transducer at its tip. These were connected to the CVIS Insight system or the HP Sonos Intravascular Imaging System (Hewlett Packard, Andover, MA) respectively. All patients underwent complete right heart catheterization, under general anesthesia, including determination of pulmonary artery pressure, capillary wedge pressure, pulmonary blood flow calculated by the Fick principle, and pulmonary vascular resistance. A 6F, 70cm long sheath (Cordis, Miami, FL) was advanced from the femoral vein into the proximal pulmonary artery. Through this sheath the ultrasound catheter was directed along the pulmonary branch, towards a wedged position. At consecutive sites, from the proximal artery, via the segment arteries to the peripheral arteries, identified and defined by fluoroscopy[13], ultrasound images were recorded, simultaneously with the ECG, and analyzed off-line. Pulmonary artery diameter and area were measured at end-diastolic (minimal) and peak-systolic (maximal) dimensions. Vascular pulsatility was defined as the difference between planimetered peak-systolic and end-diastolic areas divided by end-diastolic area x 100%. The average value of 3 cardiac cycles was used for analysis. In 6 of 11 patients all measurements were repeated at identical sites after inhalation of 100% oxygen as pulmonary vasodilator. Written informed consent was obtained from the parents of all children. The study protocol was approved by the Medical Ethical Comittee of the University Hospital Rotterdam.

Statistical analysis

Data are expressed as mean values and standard deviations. Correlations were calculated using linear regression and correlation coefficient. Student's t-test and Wilcoxon rank sum test were used to evaluate the effect of oxygen inhalation. A p value < 0.05 was considered significant.

Results

IVUS imaging of the pulmonary arteries was performed in 11 children. Patients characteristics are shown in table 1. Pulmonary vascular resistance was normal in eight patients, slightly to moderately elevated in two and severely elevated in one patient. In all patients the positioning of the long sheath in the proximal pulmonary artery and guiding of the ultrasound catheter through this sheath along the pulmonary branch could be achieved easily. Once the catheter was placed in a stable, non-branching position, the obtained images were of good quality in all patients (fig 1). There was a clear delineation between blood and the inner vessel wall, allowing reproducible measurements of systolic and diastolic vessel dimensions in all recordings. Measurements sites are shown in figure 2. End-diastolic diameter of the vessels

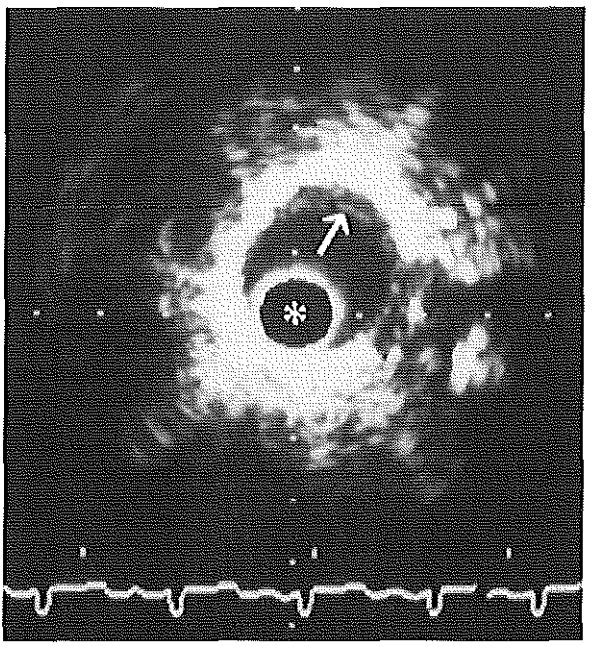

Figure 1 *Intravascular ultrasound image of a peripheral pulmonary artery of patient 11. *: ultrasound catheter. Note the gray layer at the inner border of the vessel wall (arrow). Distance between two white points is 1 mm.*

Table 1 *Patients characteristics*

patient	age	weight	diagnosis	mPAP	Qp	PVR 30%	PVR 100%
	yrs	kg		mmHg	l/min/m^2	WU. m^2	WU. m^2
1	3.1	15.6	PDA	12	4.3	1.0	0.2
2	11.4	51.0	PDA	17	6.0	1.2	*
3	3.3	15.4	PDA	11	6.7	0.2	-
4	3.0	15.8	PDA	11	5.3	0.8	-
5	6.5	24.7	PDA	13	4.4	0.9	-
6	0.7	5.3	VSD	24	8.6	1.6	1.7
7	11.5	48.0	ASDII	13	4.4	1.6	-
8	5.0	12.0	ASDII	12	7.3	1.0	-
9	16.0	36.5	ASDI	24	4.3	4.2	2.3
10	0.4	4.1	AVSD	33	4.2	5.5	2.8
11	4.4	11.7	AVSD	72	4.4	15.0	19.0
mean	6.0	21.8		22	5.5	3.0	
(SD)	5.1	16.3		18	1.5	4.3	

*ASDII indicates atrial septal defect of the secundum type; AVSD, atrioventricular septal defect; PDA, patent ductus arteriosus; VSD, ventricular septal defect; mPAP, mean pulmonary artery pressure; Qp, pulmonary blood flow; PVR 30%, pulmonary vascular resistance with 30% oxygen inhalation; PVR 100%, idem with 100% oxygen inhalation. WU, Wood's units; *, IVUS study, but no hemodynamic data during 100% oxygen inhalation.*

reached, varied from 1.6 to 9.3 mm. Larger arteries could not be visualised adequately because of limited penetration power of the 30 MHz transducer. The pulsatility of the pulmonary artery varied at different sites in the arterial branch and showed a relation with the size of the vessel, expressed as end-diastolic area. In all patients but one (patient 11) the pulsatility increased in the more proximal vessels. The relation for

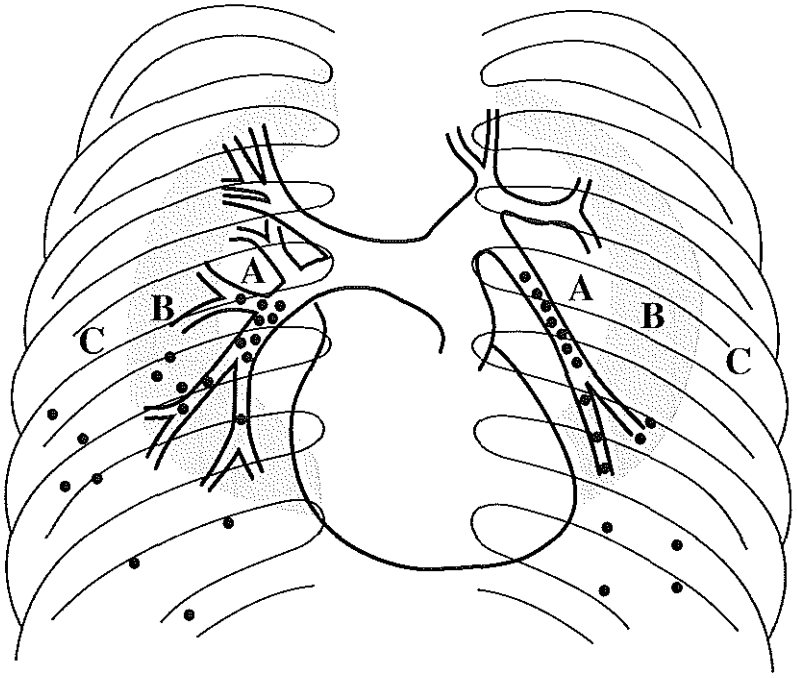

Figure 2 *Measurement sites in the pulmonary vascular tree. A: proximal arteries, luminal diameter 4.2 to 9.3 mm (6.4 ± 1.3 mm, mean ± SD), n = 15; B: segment arteries, luminal diameters 2.3-5.9 mm (3.9 ± 1.1 mm), n = 12; C: peripheral arteries, luminal diameters 1.6-2.9 mm (2.3 ± 0.4 mm), n = 12. n: number of measurement sites.*

the total patient group ($r = 0.81$; $p < 0.05$) is shown in fig. 3a. Between the individual patients there was a substantial variation in pulsatility per vessel diameter and in the extent of pulsatility increase (fig. 3b). In 6 patients the reaction to 100% oxygen inhalation was studied. Pulsatility increased in all vessels. The larger vessels tended to show the largest increase in pulsatility (fig. 4). Only patient 11 showed a different reaction pattern. The mean pulsatility in this patient was smaller than the mean pulsatility in the other patients (9.5 ± 0.7%, n = 2 vs. 21.5 ± 13.6%, n = 14). Furthermore, the increase in pulsatility after oxygen inhalation tended to be diminished (13.5 ± 0.7% vs. 27.6 ± 13.5%). End-diastolic diameters increased after oxygen inhalation. This reaction was most prominent in the smallest vessels (percentage change in diameter 4-11%, mean 7.4 ± 2.8%, $p = 0.001$) and was less prominent in the medium

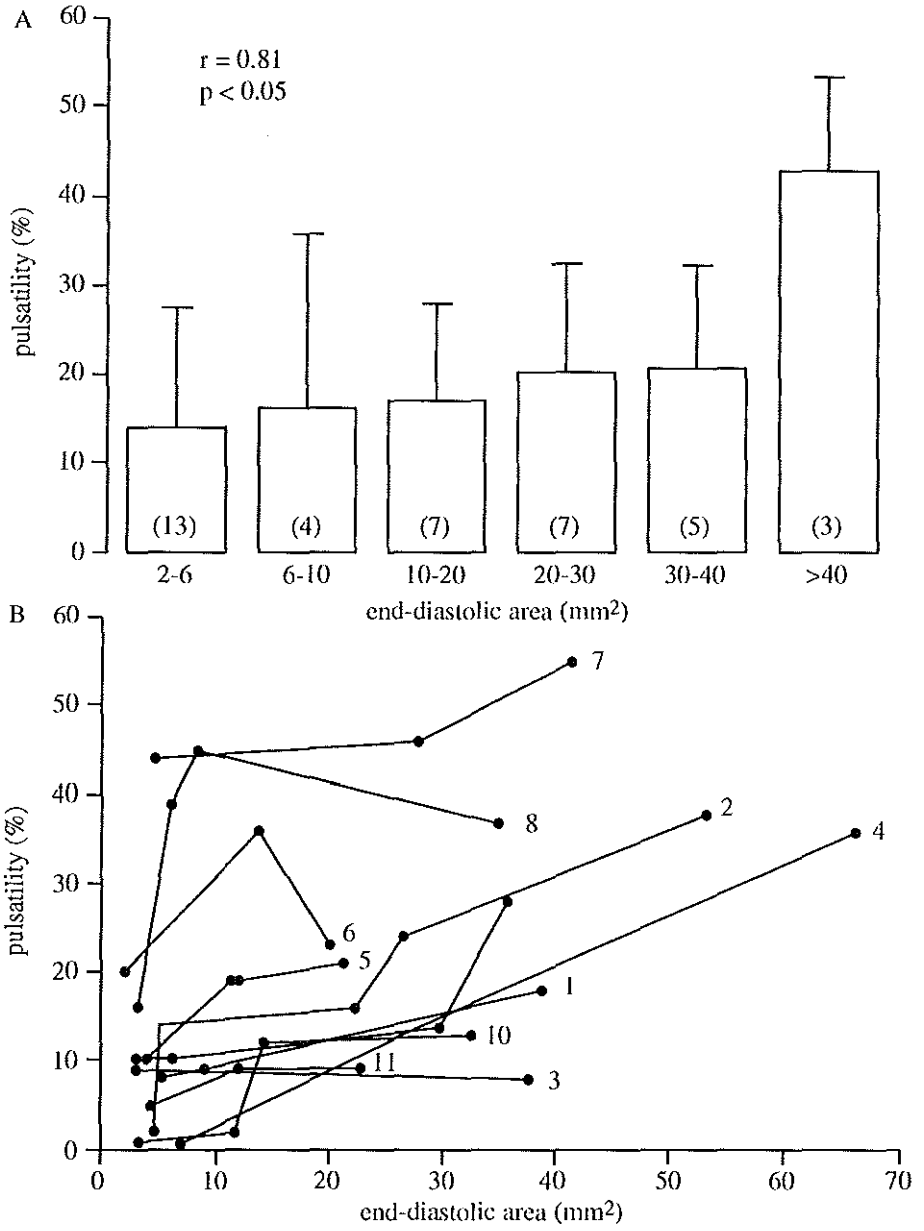

Figure 3 *Pulsatility in relation to vessel size in 11 patients. A: measurements of the total patient group, expressed as mean ± SD. (n) = number of measurements. B: measurements of the individual patients. (n) = patient number as shown in table 1.*

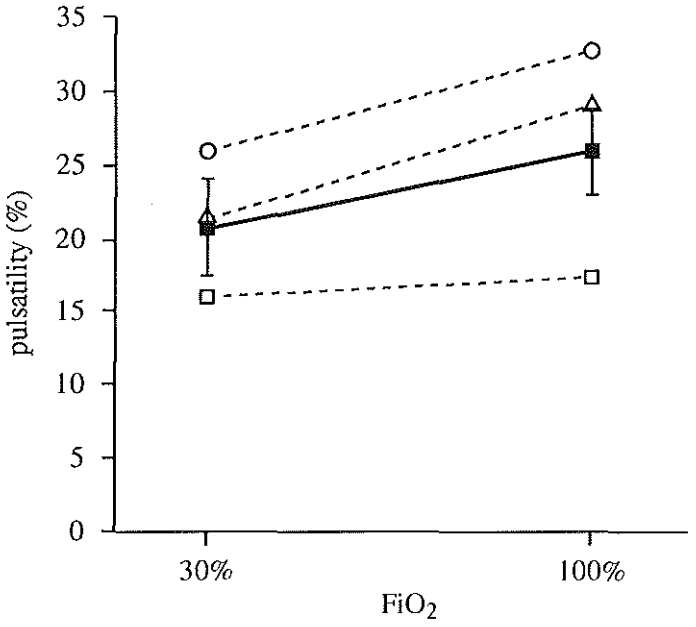

Figure 4 *Effect of 100% oxygen inhalation on pulsatility in 6 patients. FiO_2, percentage of inspired oxygen. Vessel end-diastolic diameter: 1-3 mm, n=7 (□); 3-6 mm, n=6 (△); 6-9 mm, n=3 (○); total group, n=16 (■). n, number of measurements. Pulsatility is expressed as mean ± SEM for the total group. The increase in mean pulsatility at 100% inspired oxygen concentration was statistically significant ($p < 0.05$, Wilcoxon signed rank sum test for total group).*

sized vessels (2-6%, mean 4.2 ± 1.8%, p = 0.002). There was a slight decrease in diameter in the largest vessels (–6 to –2%, mean –2.8 ± 3.1%, p = 0.047) (fig. 5). Again, this reaction pattern was similar in all patients except patient 11. In this patient the end-diastolic diameter of the largest vessels increased 2%.

The adventitial border could not be delineated adequately. Consequently, wall thickness could not be measured. However, there was a specific appearance of the wall aspect in patient 11, in whom a grey layer could be distinguished at the inner border of the vessel wall (fig. 1). Such layer was not seen in the other patients. There were no procedure related complications in any of the patients.

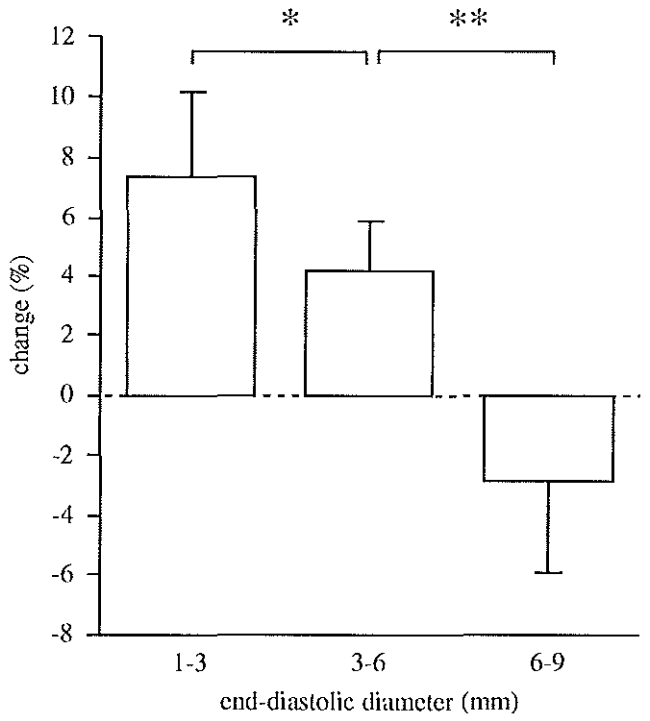

Figure 5 *Effect of 100% oxygen inhalation on end-diastolic vessel diameter. Percentage change in diameter expressed as means ± SD. Vessels of various diameters showed a statistically significant different effect of 100% oxygen inhalation (t-test: * $p < 0.05$; ** $p = 0.001$).*

Discussion

Our data show that IVUS imaging of the pulmonary arteries in infants and children is feasible. There was a clear delineation between blood and the inner vessel wall, allowing reproducible measurements of systolic and diastolic vessel dimensions. Accuracy of vascular luminal diameter and area measurements with IVUS has been demonstrated[9-12]. Measurements of luminal diameters and areas during the cardiac cycle provide the possibility of studying pulmonary vascular dynamics *in vivo* in a unique, direct way.

The pulsatility of the pulmonary artery is one expression of this dynamics. We found a significant relationship between vessel size and pulsatility. Although the pattern of increasing pulsatility towards the proximal arteries was present in all patients, the variation between the individual patients was considerable. One might speculate that the diminished pulsatility in the smaller vessels is caused by a progressive obstruction by the ultrasound catheter in these vessels, thus affecting their responses. However, the interindividual variation, specifically in vessels at similar sites, with similar area in patients with similar weights, makes this hypothesis unlikely. This far more suggests that, as expected, there are more factors than size that affect pulsatility. Pulsatility will be determined by two major components: (1) mechanical forces working on the vessel wall and (2) intrinsic viscomechanical properties of the vessel wall.

Mechanical forces

In vivo, various mechanical forces have their effects on the vascular wall. Radially directed forces, such as pulmonary artery or pulse pressure and the contradirected intrapulmonary pressure, will affect wall dynamics. Also pressure wave reflections, originating from the pulmonary microcirculation and branching of larger vessels, will contribute to the pulsatility, varying with propagation down the pulmonary branches[14]. Next, tangential forces, in both longitudinal and circumferential directions, such as shear stress, will affect the mechanics of the vascular wall[15, 16]. In pulmonary vascular disease these mechanical forces and their effect on wall dynamics will alter. For instance, in the normal pulmonary circulation wave reflections are of small amplitude because of excellent matching of impedances in the distal pulmonary vessels. However, in pulmonary vascular disease these reflection waves will increase in amplitude as well as in velocity with which they return, resulting in an increasing effect on vessel wall dynamics[14, 17]. It has been demonstrated that mechanical forces have their effects on the metabolic activity in the vascular wall, resulting, on the short term, in adaptation of vascular tone and, on the longer term, changes in the elastic properties of the wall itself[18-20].

Properties of the vessel wall

Referring to the vessel wall itself, this exhibits an anisotropic, nonlinear, viscoelastic

are using indirect parameters to estimate dilatatory vascular effects or less reliable methods like angiografic diameter determination[2, 20].

In our experience, the adventitial border could not be delineated well, probably because the pulmonary vessels are embedded in air, frustrating quantitative assessment of the vessel wall thickness. This confirms the limited experience reported in adult patients [22]. However, qualitative aspects of the vascular wall could be studied. The inability to distinguish the three layer structure of the wall corresponds with the fact that we studied elastic arteries[12, 23]. However, a grey inner layer was identified at the inner border of the vessel wall of the one patient with irreversible PVD, which might reflect intima thickening. A congruency may exist with IVUS aspects of coronary intima proliferation in adult patients[24, 25]. However, histological validation was not performed in this patient. Few earlier reports claim to distinguish qualitative changes in the vessel wall in pulmonary hypertension[22, 26, 27]. We think larger series, including normal pulmonary arteries, are needed to evaluate the value of this qualitative vessel wall assessment.

The smallest vessels we reached with the ultrasound catheter had a diameter of 1.6 mm. In PVD the most striking changes, from a morphological point of view, occur in the muscular arteries and smaller arterioles, which are vessels with a diameter of less than 1 mm[23]. Although new ultrasound catheters, with a diameter less than 1 mm, are about to become available, the vessels accessible for ultrasound imaging will not be the "resistance vessels". However, as stated before, PVD is a process of the whole pulmonary vasculature, which is not restricted to the small peripheral vessels, but also affects the larger pulmonary arteries[19, 21]. Every functional change in the pulmonary microcirculation will directly affect the dynamic behaviour of the more proximal vessels. The large and medium sized pulmonary arteries are not just passive conduit vessels, but represent an important determinant of integrated pulmonary circulation[14, 17]. So, their mechanical behaviour in PVD is of major interest. In contrast to both hemodynamic and histological evaluation, IVUS imaging has the possibility to assess vessels of different sizes separately.

In this feasibility study, the patient number was too small, with regard to the number of hemodynamic parameters, to perform adequate analysis of possible relationships between hemodynamic variables and ultrasound data. However, there was a tendency

of less pulsatile arteries in the patients with elevated pulmonary vascular resistance. Furthermore, in all previously described relations and responses, patient 11, with severe, irreversible PVD, showed aberrant patterns. This suggests the ability of intravascular ultrasound to assess altered vascular dynamic behaviour in pulmonary vascular disease.

At the moment we are performing a prospective study in children with congenital heart disease, including not only the various stages of PVD but also normal pulmonary vasculature, in which we will correlate IVUS data with complete hemodynamic, angiographical and histological evaluation. This study will demonstrate whether the described advantages of IVUS imaging will have additional value to the currently available diagnostic techniques, in assessing the state of the pulmonary vasculature for clinical as well as research purposes.

We conclude that IVUS imaging of the proximal and peripheral pulmonary arteries is feasible in children and infants using routine catheter techniques. This technique provides a unique opportunity to assess the state of the pulmonary vasculature and its dynamics *in vivo* directly. Therefore it may be a valuable tool in evaluating the pulmonary vasculature and its changes and responses in normal and pathological conditions. The additional value with regard to conventional techniques, such as hemodynamic and histologic evaluation, remains to be determined. A prospective, comparative study to answer this question is in progress at our department.

Acknowledgements

The authors would like to thank TD Medical b.v., Eindhoven and Hewlett Packard, Amstelveen, the Netherlands, for their support by providing the equipments used in the described study.

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Abstract

Assessment of the progression of pulmonary vascular disease (PVD) associated with congenital heart disease is limited with current techniques.This study aimed to assess the ability of intravascular ultrasound (IVUS) to visualize changes in the pulmonary arterial wall associated with PVD in children.

Pulmonary IVUS was performed in 35 patients with moderate PVD, 12 with advanced PVD and in 14 controls. The findings were compared with hemodynamic variables and, in 31 patients, with lung biopsy findings. In controls, the arterial lumen was most frequently (71%) surrounded by an echodense ring with a clearly defined inner border, but without a clearly defined outer border. This pattern was found less frequently in patients with moderate ($p < .01$) and advanced ($p < .05$) PVD. In patients with moderate PVD an inner layer of soft echo reflections was recognized more frequently than in controls ($p < .01$). The thickness of this layer correlated with shunt size ($r = .39$; $p < .01$), pulmonary artery pressure ($r = .31$; $p < .05$) and percent medial thickness as determined in lung biopsies ($r = .44$; $p < .05$). When adjacent structures allowed the outer border of the arterial wall to be defined, wall thickness correlated with pulmonary artery pressure ($r = .52$; $p < .05$) and pulmonary vascular resistance ($r = .65$; $p < .01$). The ultrasound appearance of the arterial wall did not correlate with advanced lesions in lung biopsies.

Conclusions: IVUS is able to visualize changes in the wall of elastic pulmonary arteries in PVD. Quantitative assessment of these changes correlated with hemodynamics and quantitative data on medial hypertrophy in lung biopsy. The ultrasound changes of the arterial wall did not correlate with the presence of advanced lesions in lung biopsy.

Introduction

Congenital heart disease associated with pulmonary hypertension or pulmonary overflow leads to functional and morphological changes in the pulmonary vascular bed[1-5]. This pulmonary vascular disease (PVD), referred to as plexogenic arteriopathy, is an important determinant in the management and prognosis of children with this type of heart disease[2, 3, 6-8]. As long as plexogenic arteriopathy has not progressed beyond a certain stage, surgical correction of the heart defect will result in regression of vascular changes. However, when the arteriopathy has progressed beyond a "point of no return," the disease process becomes irreversible and will progress despite corrective surgery[7]. Current diagnostic techniques, used to assess the state of the pulmonary vasculature, i.e. cardiac catheterization and lung biopsy, have intrinsic limitations[1, 5-7, 9-12]. Interpretation of the results of both techniques leaves a "grey" zone between reversible and irreversible disease[7].

It has been suggested that intravascular ultrasound imaging (IVUS) of the pulmonary arteries may complement the assessment of pulmonary vascular disease[13, 14]. IVUS has been shown to provide accurate measurements of vascular area, wall characteristics and pulsatility *in vivo*[13, 15-17]. The currently available ultrasound catheters have diameters down to 3.0 French and can reach vessels of 1.4 mm diameter. In the pulmonary vasculature, where the transition to a muscular type of artery occurs at a diameter of approximately 1 mm, this means that only elastic arteries are accessible for investigation by IVUS. This may be a potential limitation, since histologically the most prominent vascular changes are localized in the smaller, muscular pulmonary arteries. However, anecdotal data, suggesting changes of the echographic appearance of the vascular wall in patients with pulmonary hypertension, have been reported [14, 18-20]. To assess, systematically, the ability of IVUS to visualize changes in the pulmonary arterial wall associated with PVD, we prospectively studied the echographic appearance of the pulmonary arterial wall, *in vivo*, in control children and in patients with different stages of pulmonary vascular disease and correlated the findings with hemodynamic and histologic evaluation of the pulmonary vasculature.

Materials and Methods

Patients

This study was approved by the Medical Ethical Committee of the University Hospital Rotterdam. After written informed consent of the parents, patients who required cardiac catheterization and had increased pulmonary blood flow (pulmonary-to-systemic blood flow ratio > 1.2) and/or pulmonary hypertension (mean pulmonary artery pressure > 20 mmHg), were prospectively enrolled in the study. Children with a normal pulmonary circulation, who underwent cardiac catheterization to exclude cardiac disease or with mild left-sided obstructive lesions and a normal left ventricular end-diastolic pressure and pulmonary wedge pressures, served as controls.

Catheterization and IVUS procedure

All procedures were performed under standardized general anaesthesia[21]. Hemodynamic evaluation and IVUS imaging of the pulmonary arteries were performed in the same procedure according to a protocol previously reported[13]. Briefly, all patients underwent complete hemodynamic evaluation of the pulmonary and systemic vascular beds, including vasodilator response to inhalation of 100% oxygen, as a powerful pulmonary vasodilator. An HP Sonos Intravascular Imaging System (Hewlett Packard, Andover, MA) was used. Sonicath 3.5 F or Spy 3.0 F ultrasound catheters (Boston Scientific Corp., Watertown, MA) were used, both having a 30 MHz transducer at its tip. The ultrasound catheter was directed through a 6 F long sheath along the pulmonary branch. At consecutive sites in the proximal artery, the segment arteries and the peripheral arteries, identified and defined by fluoroscopy[13, 22], ultrasound images were obtained and recorded on S-VHS videotape simultaneously with the ECG.

Histologic evaluation

In patients who underwent subsequent cardiac surgery, an open lung biopsy was taken during the surgical procedure. In one patient an open lung biopsy was taken as isolated procedure for diagnostic purposes. Tissue was formalin fixed under vacuum. After slicing and routine paraffin embedding, histological sections were cut at 3 different levels of each slice. Sections from each level were stained by hematoxylin-

eosin, elastic- van Gieson stain and iron stain. Vascular changes were described according to Wagenvoort[5, 7]. The medial thickness of the muscular arteries was calculated as a percentage of the total artery diameter, excluding the adventitial layer. The presence or absence of intimal proliferation, concentric laminar intimal fibrosis, angiomatoid dilatation lesions, fibrinoid necrosis or plexiform lesions was recorded.

Patient classification

Patients were classified on the basis of hemodynamic data and, separately, on the basis of histologic data.

HEMODYNAMIC CLASSIFICATION: patients with normal pulmonary vascular resistance (PVR baseline < 4 WU.m^2) or responsive PVR (PVR at 100% oxygen inhalation < 4 WU.m^2 or vasodilator response ≥ 50%) were classified as having mild-to-moderate PVD. Patients with an increased PVR, not responsive to 100% oxygen (PVR at 100% oxygen inhalation ≥ 4 WU.m^2 and vasodilator response < 50%) were classified as having advanced PVD. Vasodilator response was defined as 100 x (PVR baseline - PVR at 100% oxygen inhalation) / PVR baseline.

HISTOLOGIC CLASSIFICATION: patients with a lung biopsy showing one ore more of the advanced lesions of plexogenic arteriopathy: concentric laminar intimal fibrosis, angiomatoid dilatation lesions, fibrinoid necrosis or plexiform lesions, were classified as having irreversible PVD[7]. In the absence of any of these lesions, the patients were classified as having reversible PVD.

Intravascular ultrasound analysis

The ultrasound cross-sections were analyzed at end-diastole, independently by two observers blinded for diagnosis, hemodynamic and histological data. For qualitative and quantitative analysis the cross-sections were divided in four quadrants (each 90°). Based on ultrasound appearance the pulmonary arterial wall was categorized in five patterns: A, an echodense ring with a clearly defined inner border, but without a clearly defined outer border (Fig. 1A); B, an inner layer of soft echo reflections within the echodense outer layer (Fig. 1B); C, two layers separated by a thin echolucent layer with minimal reflection (Fig. 1C); D, a well-defined outer border of the arterial wall (Fig. 1D); E, a three-layered arterial wall, as described in systemic muscular

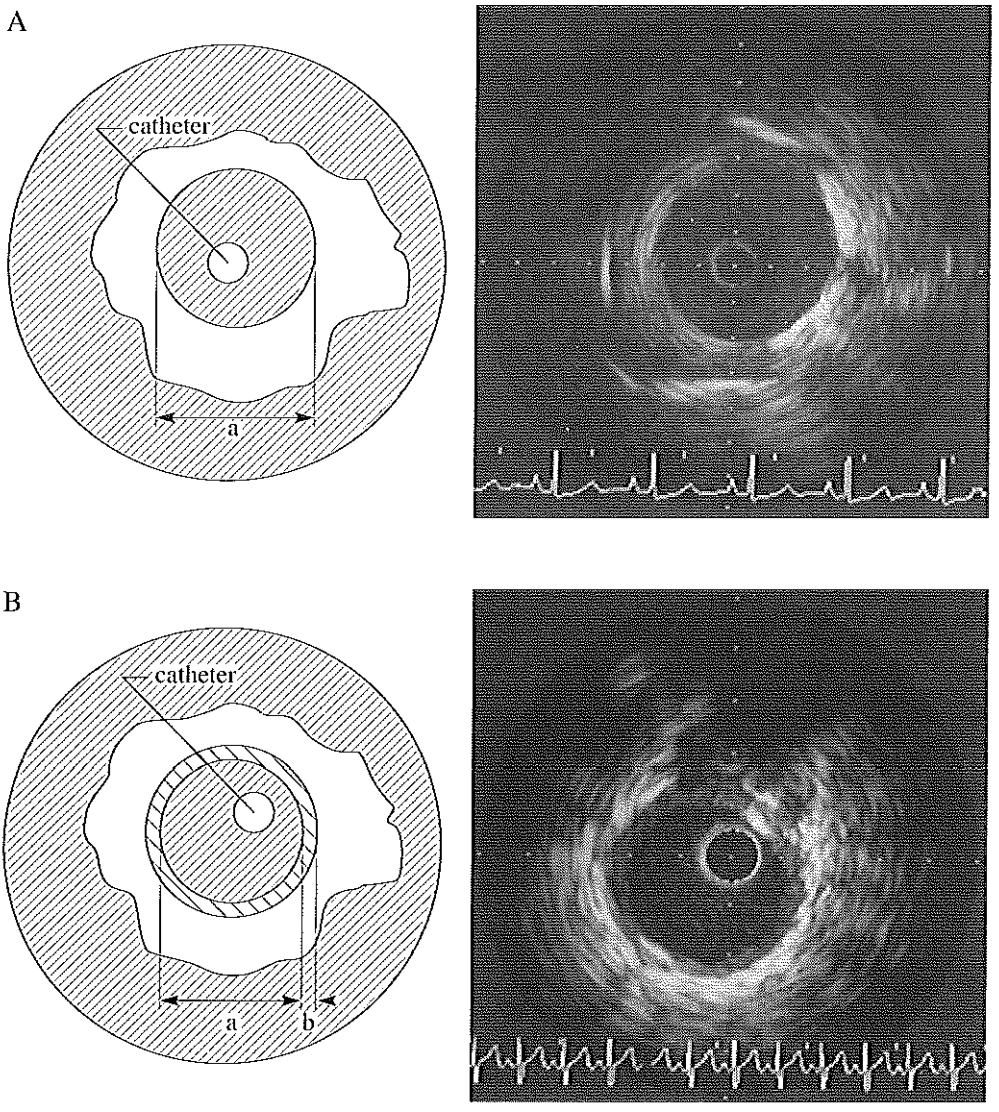
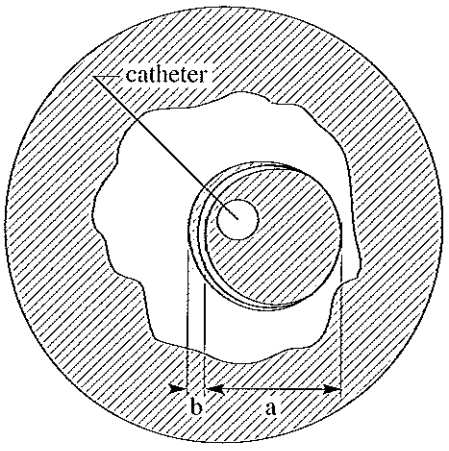


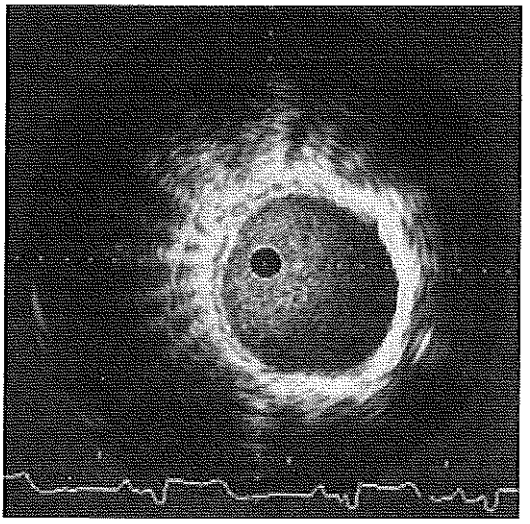
Figure 1 *Pulmonary arterial wall characteristics as defined by intravascular ultrasound.*

A. Pattern A*: an echodense ring with a clearly defined inner border, but without a clearly defined outer border.*

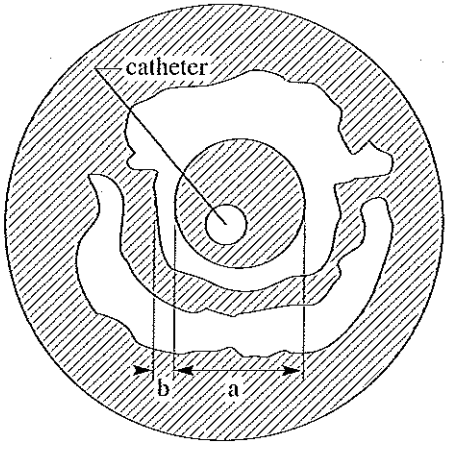
B. Pattern B*: an inner layer of soft echo reflections within the echodense outer layer.*

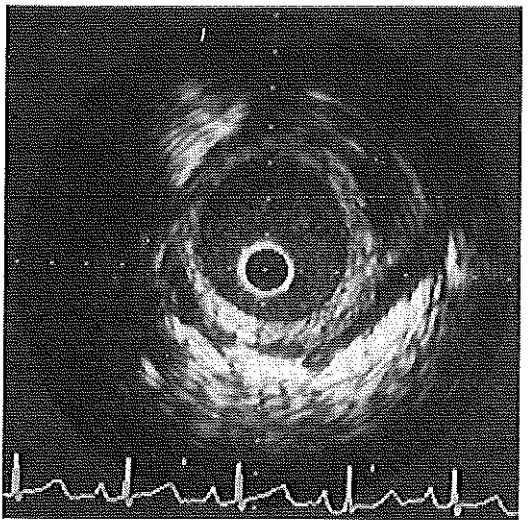
C





D





C. Pattern C*: 2 layers separated by a thin echolucent layer with minimal reflection.*

D. Pattern D*: a well-defined outer border of the arterial wall, due to adjacent structures.*

a = arterial lumen diameter; b = layer thickness; The distance between two white dots is 1 mm

arteries [16] and *in vitro* in pulmonary arteries[14]. The wall characteristics were described for each quadrant. The mean number of quadrants with a specific pattern per vessel was calculated for each patient. Quantitative analysis was performed using a protractor. The vessel diameter, bounded by the leading edge of the outer echodense ring, and the maximum layer thickness per cross-section were measured for each artery. The layer thickness was determined as the perpendicular distance between the leading edge of the inner layer and the leading edge of the outer layer (Fig. 1b-d). A layer was measured only if the distance was ≥ 0.1 mm. In the absence of a distinct layer (pattern A) no layer thickness was measured. The percentage of layer thickness of a vessel was calculated as (maximum layer thickness / vessel diameter) x 100. The mean percentage of layer thickness was calculated for each pattern in each patient. Because the resolution of the ultrasound catheter may influence the findings in arteries of different size, the IVUS cross-sections were divided in small (diameter < 2.5 mm), medium-sized (diameter 2.5-6.5 mm) and large (diameter > 6.5 mm) arteries.

Statistical analysis

The interobserver variability for the presence or absence of each qualitative parameter was assessed using the unweighted Cohen's Kappa coefficient [23, 24]. Kappa values range from −1 to +1. Kappa equals +1 when there is complete agreement between two observers. Kappa equals 0 when observed agreement is equal to that expected by chance alone. The number of quadrants with each pattern per vessel, as determined by the two observers, was compared using the Wilcoxon matched-pairs signed-ranks test. The interobserver agreement for the quantitative parameters was determined by calculating correlation coefficients. The Student's t-test for paired observations was used to test whether there were systematic differences between the observers.

The incidence of the different wall characteristics was compared between patient groups using Chi-square test, followed by Fisher's Exact test. The correlations between continuous variables were calculated using linear regression. A p-value < 0.05 was considered statistically significant.

Table 1 *Diagnoses of the study patients*

Diagnosis	n
Left-to-right shunt	
VSD with or without ASD or PDA	23
ASD	6
PDA	3
cAVSD	2
Truncus arteriosus	1
No left-to-right shunt	
Persistent PH after corrected heart defect	1
Unexplained PH	2
Left sided obstructive lesions with PVC	9
Controls	14
Total	61

ASD indicates atrial septal defect; cAVSD, complete atrioventricular septal defect; n, number of patients; PDA, patent ductus arteriosus; PH, pulmonary hypertension; PVC, pulmonary venous congestion; VSD, ventricular septal defect.

Results

Patients

In total 61 children were enrolled in the study. These included 47 patients with PVD and 14 controls. Diagnoses are shown in table 1. Based on hemodynamic evaluation 35 patients had mild-to-moderate PVD and 12 patients had advanced PVD. Lung biopsies were obtained in 31 children. Based on histological evaluation 23 patients had reversible PVD and 4 patients had irreversible PVD. In 4 controls, biopsies were

Table 2 *Patients classification based on hemodynamics and histology.*

		Hemodynamics		
		moderate PVD	advanced PVD	control
n		35	12	14
age	(years)	3.6	2.1	6.3
		(0.2-30.7)	(0.2-15.8)	(0.5-16.9)
mean PAP	(mmHg)	22 ± 9	50 ± 12	12 ± 3
shunt ratio	(Qp/Qs)	2.6 ± 1.5	1.2 ± 0.3	1.0 ± 0.0
$PVR_{BASELINE}$	(WU.m2)	2.0 ± 1.2	9.7 ± 3.5	1.2 ± 0.5
$PVR_{AT\ 100\%\ O_2}$	(WU.m2)	1.6 ± 1.1	9.5 ± 4.1	0.7 ± 0.3
		Histology		
		reversible PVD	irreversible PVD	control
n		23	4	4
age	(years)	1.2	2.0	3.3
		(0.3-15.2)	(0.3-30.7)	(1.8-4.8)
medial thickness	(%)	11.0 ± 5.1	13.0 ± 1.4	9.8 ± 2.9

PAP indicates pulmonary artery pressure; PVD, pulmonary vascular disease; PVR, pulmonary vascular resistance; Qp/Qs, pulmonary-to-systemic blood flow ratio; WU.m2, Woods units times square meter. Age is expressed as median (range), other data are expressed as mean ± standard deviation.

available. Patient characteristics, hemodynamic and histological data are summarized in table 2.

Intravascular ultrasound analysis

A total of 229 pulmonary arterial IVUS cross-sections was analyzed. Image quality

Table 3 *Echographic wall characteristics related to vessel diameter*

	vessel diameter					
	small (< 2.5 mm)		medium-sized (2.5-6.5 mm)		large (> 6.5 mm)	
	n	(%)	n	(%)	n	(%)
pattern A	143	(71)	116*†	(54)	43	(73)
pattern B	38	(19)	59*†	(28)	6	(10)
pattern C	7	(4)	21*	(10)	6	(10)
pattern D	12	(6)	18	(8)	4	(7)
	200	(100)	214	(100)	59	(100)

n indicates the sum of the mean numbers of quadrants per vessel per patient;
*$p < .05$ compared to small arteries; † $p < .01$ compared to large arteries.
For the description of patterns see text.

was moderate or good in 220 cross-sections and poor in 9 cross-sections. The latter 9 cross-sections were excluded for analysis. Of the cross-sections selected, all 4 quadrants could be analyzed in 84.5%, 3 quadrants in 10%, 2 quadrants in 5% and only 1 quadrant could be analyzed in 0.5%. In 62% of the analyzed quadrants pattern A was identified, in 22% pattern B, in 8% pattern C and in 8% pattern D. Pattern E could not be identified in any of the cross-sections.

Vessel diameter ranged from 1.4 to 10.4 mm. As shown in table 3, the frequency of the described patterns differed between small, medium-sized and large arteries ($p < .001$). Pattern A presented less frequently in medium-sized arteries compared to both small ($p < .001$) and large arteries ($p < .05$). In contrast, pattern B occurred more frequently in the medium-sized arteries compared to the other diameter categories ($p < .05$). No differences in echographic wall characteristics were demonstrated between the small and large pulmonary arteries.

Table 4 *Echographic wall patterns in the patient groups based on hemodynamics*

	pattern	moderate PVD		advanced PVD		control	
		n	(%)	n	(%)	n	(%)
diameter	A	78.1	(69)	30.7	(76)	34.5	(73)
< 2.5 mm	B	25.3	(22)	5.5	(14)	7.5	(16)
	C	5.0	(4)	0.8	(2)	1.0	(2)
	D	5.3	(5)	3.0	(8)	4.0	(9)
	Total	113.7	(100)	40.0	(100)	47.0	(100)
diameter	A	56.4**	(48)	21.0*	(49)	38.3	(69)
2.5-6.5 mm	B	41.2**	(36)	10.5	(25)	7.0	(13)
	C	11.6	(10)	5.0	(12)	4.8	(9)
	D	7.3	(6)	6.0	(14)	5.0	(9)
	Total	116.5	(100)	42.5	(100)	55.1	(100)
diameter	A	32.5	(75)	2.0	(67)	8.0	(67)
> 6.5 mm	B	4.0	(9)	1.0	(33)	1.0	(8)
	C	4.0	(9)	–	–	2.0	(17)
	D	3.0	(7)	–	–	1.0	(8)
	Total	43.5	(100)	3.0	(100)	12.0	(100)

n indicates the sum of the mean numbers of quadrants per vessel per patient;
PVD, pulmonary vascular disease. *p < .05 and **p < .01 compared to controls.

The interobserver agreement for the qualitative ultrasound parameters revealed kappa values of .82 for the described pattern in quadrant 1, .79 in quadrant 2, .88 in quadrant 3 and .78 in quadrant 4. No systematic differences were demonstrated between the number of quadrants with each pattern per vessel, as determined by the two observers. Correlation coefficients for the quantitative ultrasound parameters, as determined by both observers, were .99 for vessel diameter, .95 for percentage wall thickness in

Table 5 *Echographic wall patterns in the patient groups based on histology*

	pattern	reversible PVD		irreversible PVD		control	
		n	(%)	n	(%)	n	(%)
diameter	A	51.6	(61)	11.2	(83)	10.5	(70)
< 2.5 mm	B	24.3	(28)	1.0	(7)	2.5	(17)
	C	3.5	(4)	1.3	(10)	–	(–)
	D	6.3	(7)	–	(–)	2.0	(13)
	Total	85.7	(100)	13.5	(100)	15.0	(100)
diameter	A	44.0	(54)	8.0	(53)	7.0	(44)
2.5-6.5 mm	B	28.0	(34)	3.0	(20)	5.0	(31)
	C	6.0	(7)	1.0	(7)	1.0	(6)
	D	4.0	(5)	3.0	(20)	3.0	(19)
	Total	82.0	(100)	15.0	(100)	26.0	(100)

n indicates the sum of the mean numbers of quadrants per vessel per patient;
PVD, pulmonary vascular disease.

pattern B, .94 for percentage wall thickness in pattern C and .89 for percentage wall thickness in pattern D. No systematic differences between the observers for these quantitative parameters were demonstrated.

Correlations between IVUS and hemodynamic evaluation

In the control group, predominantly pattern A was found in small (73%), medium-sized (69%) and large (67%) arteries. The incidence of the ultrasound wall patterns in the "hemodynamics-based" patient groups is given in table 4.

In the small (< 2.5 mm) and large (> 6.5 mm) arteries no differences in echographic wall patterns could be demonstrated between patients with mild-to-moderate PVD, patients with advanced PVD and controls. No significant correlations between the mean percent layer thickness and hemodynamic data could be demonstrated in the

small and large arteries. In the medium-sized arteries, pattern A was found less frequently in patients with mild-to-moderate and advanced PVD compared to controls ($p < .01$ and $p < .05$, respectively), whereas pattern B was seen more frequently in patients with mild-to-moderate PVD compared to controls ($p < .01$). The mean percent layer thickness in pattern B correlated with the mean pulmonary artery pressure ($r = .31$; $p < .05$) and with the size of the left-to-right shunt ($r = .39$; $p < .01$). In the medium-sized arteries, pattern D was found in similar frequencies in all patient groups. However, the mean percent layer thickness in this pattern correlated with mean pulmonary artery pressure ($r = .52$; $p < .05$) and with pulmonary vascular resistance ($r = .65$; $p < .01$).

Correlations between IVUS and histologic evaluation

In the patients with irreversible PVD and in the controls, arteries with a diameter > 6.5 mm were not studied. In the small and medium-sized arteries, no differences in the incidence of echographic wall patterns nor in layer thickness could be demonstrated between the "histology-based" patient groups (table 5). The percentage of medial thickness of muscular arteries, as determined in lung biopsy, correlated with the layer thickness in pattern B only in the medium-sized arteries ($r = 0.44$; $p = 0.02$). The echographically discernible layers in elastic arteries were not correlated with the presence of intima hyperplasia or fibrosis in lung biopsy.

Discussion

The present study demonstrates differences in the echographic appearance of the elastic pulmonary arteries between children with and without PVD. Quantitative measurement of echographic layers in the arterial wall correlated with both hemodynamic data and the media thickness of muscular arteries in lung biopsy. The interobserver agreement for qualitative and quantitative assessment of IVUS images was good. As far as we know, this is the first study that correlates IVUS imaging of the pulmonary arteries *in vivo*, both in controls and in patients with PVD, with contemporaneous hemodynamic and histologic evaluation.

Although IVUS imaging has been extensively described in systemic and coronary arterial disease, it has not been studied systematically in PVD[25-30]. In PVD, the muscular arteries and arterioles, with a diameter smaller than 0.5-1 mm, may show medial thickening, muscularisation of arterioles and intima proliferation. In plexogenic arteriopathy, characteristic lesions may emerge, such as concentric laminar intimal fibrosis, angiomatoid dilation lesions, fibrinoid necrosis and plexiform lesions. These advanced lesions are associated with irreversible disease. However, such small arteries can be assessed only in lung biopsy tissue[4, 7]. Structural changes in the larger, elastic pulmonary arteries are less prominent, but increased medial thickness, with an altered elastin arrangement and changes in extracellular matrix composition, such as an increased collagen content and deposition of glycoproteins, have been demonstrated in PVD[31].

In this study, IVUS in children with a normal pulmonary circulation, predominantly showed an arterial lumen surrounded by a single, echodense ring, with no discernible separate layers. This is in concordance with previous findings[13, 15, 18]. The media of elastic arteries mainly consists of densely packed, concentrically arranged elastin fibers and some sole smooth muscle cells. The acoustic impedance of elastin fibers is more or less comparable to that of intima and adventitia. Therefore, in normal elastic arteries it is expected that no separate layers can be discerned echographically[16]. However, in autopsy specimens of patients with pulmonary hypertension, Ishii et al., using a 20 MHz ultrasound catheter, described a three-layer appearance, with a thick echodense inner layer, an echolucent zone and an echodense outer layer[14]. Scott et al., also using a 20 MHz ultrasound catheter, reported another type of three-layer-appearance in three adults with systemic pulmonary hypertension and described a thin echodense layer visible at the intima media interface[18]. Although we used 30 MHz ultrasound catheters, allowing for greater delineation of structural anatomy, we found no three-layer appearance as reported by Ishii, in any of our patients *in vivo*.

When evaluating the arterial wall with IVUS, one has to acknowledge that acoustic factors, gain setting and catheter position may cause visualization of an echodense layer at the intima-media interface, that provide no anatomic information per se[32]. In our experience, increasing the gain setting sometimes created an echodense line at the luminal border of the pulmonary arterial wall, leading to an artificial three-layer

appearance. The wall characteristics, in the present study described as pattern C and seen in a similar frequency in all patient groups, is comparable to the description of Scott et al. and may be such a non-anatomic layer.

In our series, IVUS showed differences in the appearance of the pulmonary arterial wall in medium-sized arteries between controls, patients with hemodynamically mild-to-moderate PVD and those with severe PVD. Pattern A, most frequently found in controls, occurred significantly less frequent in patients with mild-to-moderate and advanced PVD. An inner layer with soft echo reflections (pattern B, Fig. 1b) was seen more frequently in patients with mild-to-moderate PVD compared to controls. The thickness of this layer correlated positively with the size of the left-to-right shunt and with mean PAP. It also correlated with the medial thickness of muscular arteries, as assessed in lung biopsy. The morphologic substrate of this "soft" layer is not clear. In elastic vessels of this size the solitary vascular smooth muscle cells, located between the elastic laminae, will increase in size in the course of PVD, but it is unlikely that this will lead to this echographic layer[31]. One may speculate that the acoustic properties of the intima-media complex change because of modulation of the extracellular matrix or intima proliferation. Then the layer could reflect medial thickening or intima proliferation, as suggested earlier[14, 18]. The finding that the occurrence of this pattern appears to diminish in patients with advanced PVD, might be explained by the increase in collagen deposition and fibrosis in media and intima at this stage of the disease, increasing again the echodensity of the layer.

In most IVUS cross-sections the outer border of the arterial wall could not be defined, presumably because of air in adjacent tissue, hampering echo transmission. In cross-sections with pattern D (Fig. 1d) it was possible to define the outer border of the arterial wall. This characteristic pattern was recognized in similar frequencies in the different patient groups.The possibility to delineate the outer border of the arterial wall may be due to the presence of adjacent structures, such as arterial branches or bronchial vessels, taking the place of air-filled lung parenchyma. The thickness of the wall, as determined in this pattern, was correlated with mean pulmonary artery pressure and PVR.

No differences in the appearance of the arterial wall could be demonstrated in the small and large arteries. This may be explained by the limited penetration of the

30 MHz catheters in the large arteries and the limited image resolution in the small arteries. The use of lower frequency ultrasound catheters in the large arteries and higher frequency catheters in the small ones, may demonstrate whether structural changes can be identified by IVUS in these arteries.
In contrast to the in vitro findings of Ishii et al.[14], we found no significant correlation between qualitative echographic wall characteristics and the histological features of the pulmonary vascular bed. Of the quantitative ultrasound findings, only the percent layer thickness of pattern B correlated with medial thickness in muscular arteries. Pulmonary IVUS and lung biopsy evaluate the pulmonary vasculature at different levels. In early pathologic studies, medial thickness of elastic arteries did not correlate with hypertensive changes in the vascular bed, nor did non-specific intimal changes, such as atheroma[31].

Limitations of the study.
The design of this *in vivo* study allowed no corresponding histologic sections of the pulmonary arteries studied with IVUS. Therefore, the echographic appearance of the arterial wall could not be correlated directly with anatomic structures. The number of patients with irreversible PVD in whom a lung biopsy was available, was relatively small. Functional vascular changes have been reported in both small and large pulmonary arteries in the course of pulmonary vascular disease[33, 34]. IVUS of the pulmonary arteries also has the potential to assess functional properties of the pulmonary vasculature *in vivo* by investigating pulsatility and arterial wall distensibility[13, 35]. However, this was not the focus of the present study.

We conclude that IVUS has the ability to visualize changes in the wall of elastic pulmonary arteries in the course of PVD. Quantitative assessment of these echographic changes correlated with hemodynamic data, pulmonary artery pressure, shunt size and PVR, and also with the quantitative assessment of medial hypertrophy in lung biopsy. The echographic changes in the pulmonary arterial wall did not correlate with the histologic presence of advanced lesions in lung biopsy. Therefore, we demonstrated that IVUS of the pulmonary vasculature provides additional information in the assessment of progression of pulmonary vascular disease. Long-term outcome analysis

is needed to determine the predictive value and, with that, the clinical utility of echographic assessment of the pulmonary arterial wall in children with congenital heart disease associated PVD.

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Pulmonary arterial wall distensibility assessed by intravascular ultrasound in children with congenital heart disease: An indicator for pulmonary vascular disease?

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Abstract

Vascular remodeling in pulmonary hypertension leads to functional and structural changes in the pulmonary vasculature. The available techniques to evaluate the pulmonary vasculature in congenital heart disease have practical and conceptual limitations.

We investigated whether pulmonary arterial wall properties, assessed with intravascular ultrasound, are altered in children with congenital heart disease and pulmonary vascular disease and whether these are associated with the progression of the disease.

Pulmonary arterial pulsatility and distensibility were determined, using intravascular ultrasound, in 43 children with congenital heart disease, associated with mild to moderate (n = 31) or advanced (n = 12) pulmonary vascular disease, and in 12 children with normal pulmonary circulation. Vascular pulsatility correlated independently with pulmonary pulse pressure, pulmonary-to-systemic vascular resistance ratio and hemoglobin concentration. Adjusted for these variables, pulsatility did not differ between patient groups. In contrast, arterial wall distensibility decreased with the severity of vascular disease and correlated with the ratios of pulmonary-to-systemic pressure and resistance and with hemoglobin concentration. Adjusted for hemodynamic variables, distensibility was still decreased in patients with pulmonary vascular disease compared to controls. This difference was most pronounced after inhalation of 100% oxygen.

Conclusions: These data demonstrate that distensibility is progressively decreased in pulmonary vascular disease and suggest that this decrease is, in part, related to an increased distending pressure as a result of pulmonary hypertension and, in part, to a stiffening of the arterial wall in the disease process. Arterial wall distensibility may be a variable of additional value in the evaluation of the pulmonary vasculature.

Introduction

Pulmonary hypertension, with or without increased pulmonary blood flow, in children with congenital heart disease, will lead to functional and structural changes of the pulmonary vasculature[1-4]. The progression and reversibility of these vascular changes, referred to as pulmonary vascular disease (PVD), are important determinants of management and prognosis of these patients[1, 2]. In the clinical setting, the state of the pulmonary vasculature is currently evaluated at cardiac catheterization by calculating pulmonary vascular resistance and its response to vasodilators. Although these hemodynamic data provide valuable information, they are conceptual restricted to the resistance of the pulmonary vasculature to continuous flow and, thus, neglect the essentials of a pulsatile circulation [5]. However, with the increasing recognition of the importance of the pulsatile component of blood pressure in the pathogenesis of various cardiovascular diseases, this aspect of pulmonary blood flow has become of major interest[6-8]. In normal conditions, approximately 30% of the work load of the right ventricle is formed by the pulsatile load and this will increase in pulmonary hypertension[8, 9]. A major determinant of this load is the capacitance of the pulmonary vasculature. That is, the reservoir capacity of the pulmonary vasculature that distends during systole, absorbing the energy in the elastic components of the arterial walls, and releases this energy during diastole to continue blood flow[9, 10]. The capacitance of the pulmonary vasculature depends of the compliance or distensibility of the vessel wall. The functional and structural changes of the large pulmonary arteries in pulmonary hypertension may lead to stiffening of the arterial wall and may affect arterial wall dynamics[11-13].

Intravascular ultrasound (IVUS) of the pulmonary vasculature can visualize vascular dynamics *in vivo*, is feasible in infants and children and has been suggested to document changes in arterial wall properties in pulmonary hypertension[14-16]. The appearance of the arterial wall, as determined *in vitro* by IVUS in patients with congenital heart disease and PVD, has been reported to correlate with histopathologic grade[17]. However, vascular dynamics and pulmonary arterial wall properties in children with congenital heart disease and different stages of PVD have not been reported earlier. The purpose of this study was to assess pulmonary arterial wall dynamics in

children with a normal and abnormal pulmonary circulation. We sought for the correlation of these findings with hemodynamic data and the progression of the vascular disease.

Methods

Patients

This study was approved by the institutional Medical Ethical Committee. After written informed consent of the parents, patients who required cardiac catheterization and had increased pulmonary blood flow (pulmonary-to-systemic blood flow ratio > 1.2) and/or pulmonary hypertension (mean pulmonary artery pressure > 20mmHg), were prospectively enrolled in the study. Children with a normal pulmonary circulation, who underwent cardiac catheterization to exclude cardiac disease or with mild left-sided obstructive lesions and a normal left ventricular end-diastolic pressure and pulmonary wedge pressures, served as controls. Patients with hemoglobinopathies, interstitial pulmonary disease, liver or renal dysfunction or unstable condition were excluded.

Catheterization and IVUS procedure

All procedures were performed under standardized general anaesthesia[18]. Hemodynamic evaluation and IVUS imaging of the pulmonary arteries were performed in the same procedure according to a protocol previously reported[14]. Briefly, all patients underwent complete hemodynamic evaluation of the pulmonary and systemic vascular beds, including vasodilator response to inhalation of 100% oxygen, as a powerful pulmonary vasodilator. Pulmonary blood flow was determined using dye dilution technique, the shunt size was determined by oximetry. Before, during and after the procedure blood gas analyses were performed to maintain metabolic stability. An HP Sonos Intravascular Imaging System (Hewlett Packard, Andover, MA) was used. Sonicath 3.5 F or Spy 3.0 F ultrasound catheters (Boston Scientific Corp., Watertown, MA) were used, both having a 30 MHz transducer at its tip. The ultrasound catheter was directed through a 6 F long sheath along the pulmonary branch. At consecutive

sites in the proximal artery, the segment arteries and the peripheral arteries, identified and defined by fluoroscopy[14, 19], ultrasound images were obtained and recorded on S-VHS videotape, simultaneously with the ECG. IVUS imaging of the pulmonary arteries was performed, during 30% and 100% oxygen inhalation. The images were analyzed off-line independently by two observers, blinded for clinical and hemodynamic data (AC,RB). Pulmonary arterial luminal diameters and areas were measured at end-diastolic (minimal) and peak-systolic (maximal) dimensions. The values of three cardiac cycles were averaged for each observer and the mean of both averages was used for further analyses. Vascular pulsatility was defined as the difference between planimetered peak-systolic (ESA) and end-diastolic area (EDA), divided by EDA x 100%[15]. Arterial wall distensibility, the inverse of the stress/strain elastic modulus and a measure of arterial wall compliance, was calculated by dividing pulsatility by pulmonary pulse pressure (pulsatility / systolic minus diastolic pulmonary artery pressure)[7, 20]. Because adequate measurement of wall dynamics was not feasible in arteries with an end-diastolic diameter < 2.0 mm, these arteries were excluded from pulsatility and distensibility analyses.

Patient classification

The enrolled children were classified into three groups. Children with a normal pulmonary circulation formed the control group (group 1). Patients with normal pulmonary vascular resistance ($PVR_{baseline} < 4$ WU.m^2) or responsive PVR ($PVR_{at\ 100\%\ oxygen\ inhalation} < 4$ WU.m^2 or vasodilator response $\geq 50\%$) were classified as having mild-to-moderate PVD (group 2). Patients with an increased PVR, not responsive to 100% oxygen ($PVR_{at\ 100\%\ oxygen\ inhalation} \geq 4$ WU.m^2 and vasodilator response < 50%) were classified as having advanced PVD (group 3). Vasodilator response was defined as 100 x ($PVR_{baseline}$ - $PVR_{at\ 100\%\ oxygen\ inhalation}$) / $PVR_{baseline}$.

Statistical analysis

Interobserver agreement was determined by calculating intraclass correlation coefficients (R_i). Pulsatility and distensibility outcomes, taking account of differences between as well within patients, were evaluated using regression analysis for repeated

measurements using the BMDP-statistical software package [21]. Both outcomes were logarythmically transformed in order to obtain approximate normal distributions. Univariate comparison of IVUS data between the patient groups was done using mixed-model analysis of variance. Using backward elimination, the clinical and hemodynamic variables, most predictive for pulsatility and distensibility, respectively, were determined. To allow a comparison between results at baseline and at 100% oxygen inhalation, however, regression coefficients are given for both conditions if one of them proved to be significant. In the multivariate analysis we have chosen pulmonary-to-systemic pressure ratio and pulmonary-to-systemic vascular resistance ratio to represent measures for pulmonary pressure and resistance respectively. The limit of significance was set at $p = 0.05$ (two-sided).

Results

Clinical and hemodynamic data

In total 55 patients were enrolled in the study. These included 12 controls, 31 patients with mild to moderate PVD and 12 patients with advanced PVD. Patient characteristics are shown in table 1.

Intravascular ultrasound data

IVUS studies were performed in 2-7 consecutive sites along the the pulmonary arterial tree per patient. In total, 199 artery segments were studied. There was an adequate delineation between blood and the inner vessel wall, allowing reproducible measurements of vessel dimensions during the cardiac cycle in 196 recordings. Three recordings were excluded from the analysis because of poor image quality. End-diastolic diameter of the studied vessels varied from 1.4 mm to 10.4 mm. 148 Vessels were $\geq$ 2 mm in diameter. There was a high degree of agreement between both observers. The intraclass correlation coefficients for the measurements of EDD, ESD, EDA, and ESA were 0.997, 0.998, 0.998 and 0.998 respectively. The sizes of the studied arteries, determined by end-diastolic diameter, were similar in the three patient groups ($p = .20$).

Table 1 *Patient characteristics*

		control	mild to moderate PVD	advanced PVD
n		12	31	12
age	(yrs)	5.6 (0.5-16.9)	3.7 (0.2-30.7)	2.6 (0.2-15.8)
weight	(kg)	21.0 (8.2-51.9)	13.0 (3.0-76)	11.4 (4.3-44)
Hemodynamics				
meanPAP	(mmHg)	13 ± 3	22 ± 9	50 ± 12
PWP	(mmHg)	8 ± 3	9 ± 4	11 ± 7
shunt size	(Qp:Qs)	1.0 ± 0.0	2.6 ± 1.5	1.2 ± 0.4
PVR	(WU.m2)	1.2 ± 0.6	1.9 ± 1.2	10.2 ± 3.5

PAP indicates pulmonary artery pressure; PVD, pulmonary vascular disease; PWP, pulmonary wedge pressure; PVR, pulmonary vascular resistance; Qp:Qs, pulmonary-to-systemic blood flow ratio; Age and weight are expressed as median and range; hemodynamic data are expressed as mean ± standard deviation.

Vascular pulsatility did not differ significantly ($p = .29$) between patients with mild to moderate PVD (geometric mean; 95% confidence interval: 23%; 20-26), patients with advanced PVD (18%; 14-24) and controls (21%; 16-27) (fig 1a). In contrast, arterial wall distensibility was decreased ($p < .001$) in patients with mild-to-moderate PVD compared to controls (1.22, 1.04-1.42 vs 2.15, 1.67-2.77). Wall distensibility in patients with advanced PVD (0.55, 0.42-0.71) was decreased compared to patients with mild to moderate PVD and to controls (both $p < 0.001$) (fig.1b).
Univariate correlations between age, hemoglobin concentration and hemodynamic data and the measurements of pulsatility respectively distensibility are shown in table 2. Results of the multivariate regression analyses are shown in table 3.

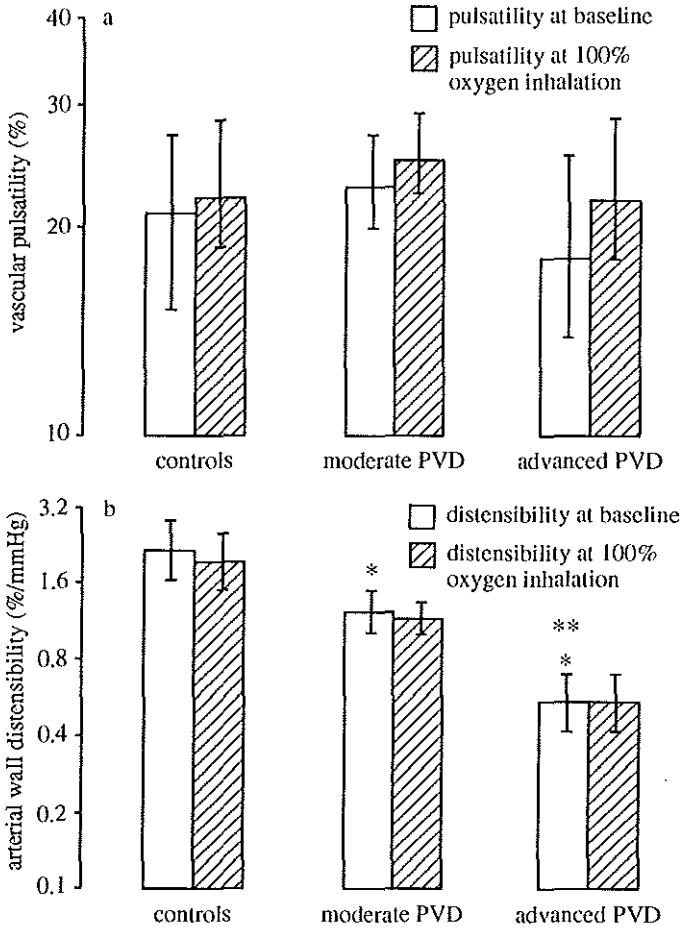

Figure 1 *Vascular pulsatility (a) and arterial wall distensibility (b) of the pulmonary arteries at baseline and at 100% oxygen inhalation in the three patient groups. Data are expressed as geometric means and 95% confidence intervals. * $p < 0.001$ compared to controls, ** $p < 0.001$ compared to moderate PVD.*

At baseline conditions and adjusted for patient group, the hemodynamic parameters pulmonary pulse pressure and pulmonary-to-systemic vascular resistance ratio (PVR/SVR) and, in addition, hemoglobin concentration were independently associated with vascular pulsatility. Adjusted for these variables, no difference in pulsatility between

Table 2a *Univariate correlations between the various variables and vascular pulsatility. Data were analyzed separately for controls (n = 12) and patients (n = 43)*

	Pulsatility at 30%				Pulsatility at 100%			
	controls	p	patients	p	controls	p	patients	p
age (years)	-.010 ± .009	.27	.004 ± .005	.37	-.005 ± .009	.57	.001 ± .004	.88
Hb (mmol/l)	-.04 ± .06	.50	-.06 ± .02	.006	-.03 ± .06	.65	-.05 ± .02	.03
meanPAP	.02 ± .02	.27	-.002 ± .002	.36	.016 ± .011	.15	-.000 ± .002	.99
diastPAP	.006 ± .014	.66	-.005 ± .002	.03	.005 ± .011	.67	-.002 ± .002	.22
systPAP	.009 ± .013	.50	-.000 ± .001	.83	.01 ± .01	.29	.001 ± .001	.40
pulm PP	.01 ± .02	.67	.004 ± .002	.08	.03 ± .02	.19	.005 ± .002	.002
shunt size	.51 ± 1.30	.69	.04 ± .02	.08	-.41 ± 1.58	.80	.016 ± .015	.30
PVR	.15 ± .08	.07	-.011 ± .007	.12	-.18 ± .19	.35	-.005 ± .006	.42
PVR/SVR	3.19 ± 1.80	.08	-.18 ± .11	.10	.10 ± 1.21	.94	-.05 ± .08	.50
PAP/SAP	1.53 ± 1.23	.21	-.06 ± .11	.55	.58 ± .73	.43	.05 ± .08	.56
EDA	.002 ± .003	.64	.001 ± .001	.48	.004 ± .003	.21	.002 ± .001	.05

Data given are regression coefficients ± standard error, with p-values. Pulsatility data are $\log_{10}$ transformed.
EDA indicates end-diastolic area (mm^2); Hb, hemoglobin concentration (mmol/l); mPAP, mean pulmonary artery pressure (mmHg); diast, diastolic; syst, systolic; pulm. PP, pulmonary pulse pressure (mmHg); PAP/SAP, pulmonary-to-systemic arterial pressure ratio; PVR, pulmonary vascular resistance (Woods' units.m^2); PVR/SVR, pulmonary-to-systemic vascular resistance ratio; shunt size, pulmonary-to-systemic blood flow ratio.

Table 2b *Univariate correlations between the various variables and arterial wall distensibility. Data were analyzed separately for controls (n=12) and patients (n=43)*

	Distensibility at 30%				Distensibility at 100%			
	controls	p	patients	p	controls	p	patients	p
age (years)	-.019 ± .008	.02	.012 ± .006	.05	-.001 ± .009	.96	.007 ± .005	.22
Hb (mmol/l)	-.12 ± .06	.06	-.050 ± .001	.08	.02 ± .06	.70	-.02 ± .03	.41
mPAP	.01 ± .02	.43	-.013 ± .001	<.001	.02 ± .01	.10	-.010 ± .001	<.001
shunt size	-.67 ± 1.44	.65	.05 ± .03	.06	-.26 ± 1.54	.87	.01 ± .02	.48
PVR	.07 ± .10	.48	-.042 ± .006	<.001	-.24 ± .18	.18	-.030 ± .006	<.001
PVR/SVR	2.00 ± 2.10	.33	-.72 ± .10	<.001	-.47 ± 1.15	.69	-.38 ± .08	<.001
PAP/SAP	1.98 ± 1.32	.14	-.74 ± .08	<.001	.24 ± .71	.74	-.53 ± .06	<.001
EDA	.001 ± .003	.75	.001 ± .001	.44	.004 ± .003	.25	.003 ± .001	.02

Data are given as regression coefficients ± standard error, with p-values. Distensibility data are $\log_{10}$ transformed. EDA indicates end-diastolic area (mm^2); Hb, hemoglobin concentration (mmol/l); mPAP, mean pulmonary artery pressure (mmHg); PAP/SAP, pulmonary-to-systemic arterial pressure ratio; PVR, pulmonary vascular resistance (Woods' units.m^2); PVR/SVR, pulmonary-to-systemic vascular resistance ratio; shunt size, pulmonary-to-systemic blood flow ratio.

Table 3a *Multivariate analysis of pulsatility at baseline and at 100% oxygen inhalation. Data given are regression coefficients with ^{10}log pulsatility as dependent variable.*

	Pulsatility at baseline			Pulsatility at 100% oxygen		
		change	p		change	p
Pulse pressure	.010 ± .003	+2%	<.001	.009 ± .002	+2%	<.001
PVR/SVR	-.05 ± .02	-11%	.001	-.01 ± .01	-2%	.20
Hb	-.05 ± .02	-11%	.01	-.02 ± .02	-4%	.41
EDA	.001 ± .001	+.2%	.30	.003 ± .001	+.7%	.01

EDA indicates end-diastolic area (mm^2); Hb, hemoglobin concentration (mmol/l); PVR/SVR, pulmonary-to-systemic vascular resistance ratio. Change indicates the percentage change in pulsatility per mmHg change in pulse pressure, per 0.1 unit change in PVR/SVR, per mmol/l change in Hb and per mm^2 change in EDA.
Data given are adjusted for patient group. Adjusted for the described variables, no differences between patient groups could be demonstrated for pulsatility (at baseline: p = .58; at oxygen inhalation: p = .23).

patient groups was demonstrated (p = .58). Distensibility was independently associated with the pulmonary-to-systemic mean arterial pressure ratio (PAP/SAP), PVR/SVR and Hb. At baseline conditions, differences between patient groups, adjusted for the described variables, just failed to reach statistical significance (p = .06). At 100% oxygen inhalation, pulsatility was independently correlated with pulse pressure and the end-diastolic area of the vessel, without significant differences between groups. At 100% oxygen inhalation, PAP/SAP and the end-diastolic area of the vessel were identified as variables independently correlated with distensibility.
Adjusted for these variables, distensibility at 100% oxygen inhalation differed between the patient groups (p = .03). Pulmonary arterial wall distensibility in a vessel with a given end-diastolic area, at a given PAP/SAP was decreased with 26% in patients with mild-to-moderate PVD compared to controls (p = .012). In patients with advanced PVD, adjusted wall distensibility was decreased with 39% compared to controls

Table 3b *Multivariate analysis of distensibility at baseline and at 100% oxygen inhalation. Data given are regression coefficients with ^{10}log distensibility as dependent variable.*

	Distensibility at baseline			Distensibility at 100% oxygen		
		change	p		change	p
PAP/SAP	-.06 ± .01	-13%	<. 001	-.05 ± .01	-11%	<. 001
PVR/SVR	-.04 ± .02	-9%	.03	.004 ± .011	-1%	.71
Hb	-.05 ± .02	-11%	.008	-.007 ± .018	-2%	.70
EDA	.001 ± .001	+.2%	.22	.003 ± .001	+.7%	.02

EDA indicates end-diastolic area (mm^2); Hb, hemoglobin concentration (mmol/l); PAP/SAP, ratio of pulmonary-to-systemic pressure; PVR/SVR, pulmonary-to-systemic vascular resistance ratio. Change indicates the percentage change in distensibility per 0.1 unit change in PAP/SAP and PVR/SVR, per mmol/l change in Hb and per mm^2 change in EDA.
Differences between patient groups for distensibility, adjusted for the described variables, just failed to reach statistical significance at baseline (p = .06). Distensibility at oxygen inhalation, adjusted for the described variables, differed between patient groups (p = .03): see text.

(p = .024). The difference in adjusted distensibility between groups 2 and 3 (13%) was not statistically significant (p = .27) (fig. 2).

After 100% oxygen inhalation, as pulmonary vasodilator, the mean increase in end-diastolic vessel area for the combined group of all patients was 2.7% (0.9-4.5, 95%-CI; p = .003). No significant correlations could be demonstrated between the increase in vessel area and vessel size, patient group, PVR/SVR or the vasodilator response, defined as percentage change in PVR.

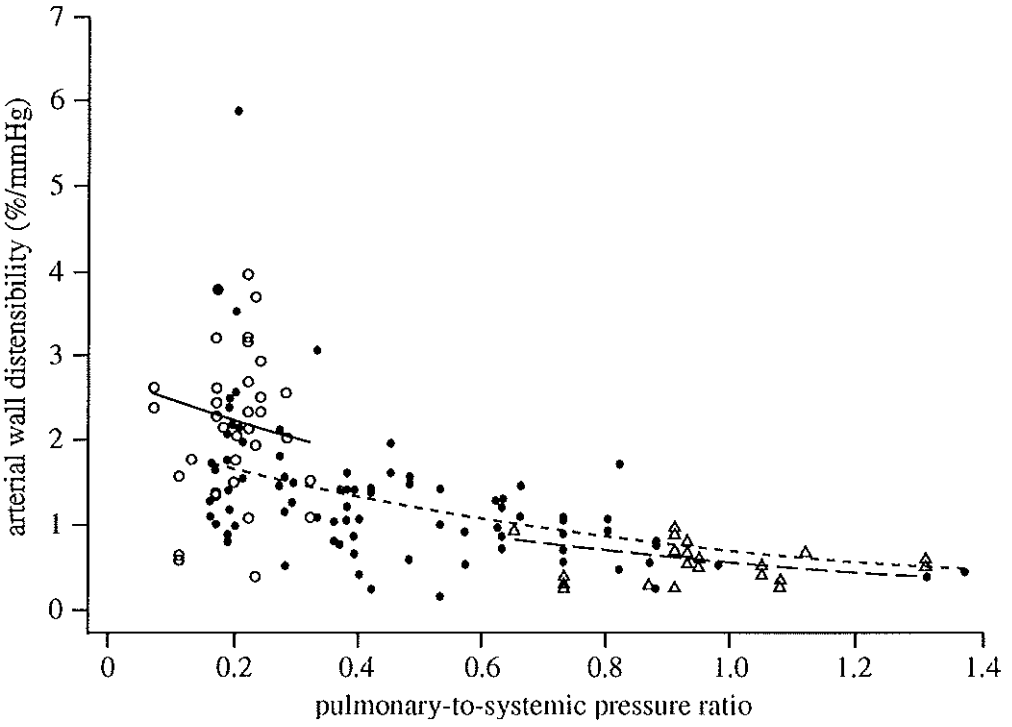

Figure 2 *Relation between arterial wall distensibility at 100% oxygen inhalation, adjusted for an end-diastolic vessel area of 15 mm², and the pulmonary-to-systemic arterial pressure ratio. The solid regression line represents the relation in controls (open circles), the dotted regression line represents the relation in patients with mild-to-moderate pulmonary vascular disease (closed circles) and the dashed regression line represents the relation in patients with advanced pulmonary vascular disease (triangles)*

Discussion

Our data demonstrate that pulmonary arterial wall distensibility is progressively decreased in patients with mild-to-moderate PVD and advanced PVD compared to controls. This in contrast to arterial pulsatility, which did not differ between the patient groups. Arterial wall distensibility correlated with distending pressure, represented

by PAP/SAP, but also, independently, with PVR/SVR and hemoglobin concentration at baseline conditions, and with vessel area at 100% oxygen inhalation. Also taking account of these variables, arterial wall distensibility appeared substantially decreased in patients with PVD compared to controls. This difference was accentuated by inhalation of 100% oxygen. These findings suggest a stiffening of the pulmonary arterial wall in PVD that can be measured *in vivo* with IVUS.

In patients with congenital heart diseases, associated with pulmonary hypertension or increased pulmonary blood flow, the pulmonary vasculature is subject to structural and functional changes[1-4]. Although the most prominent vascular changes, from a morphological point of view, occur in the small muscular pulmonary arteries, not accessible for the ultrasound catheter, also in the larger, elastic pulmonary arteries structural and functional alterations have been demonstrated[4, 11]. Structural alterations consist of increased medial thickness, intima fibrosis and extracellular matrix modulation, including an increased deposition of collagen[13, 22, 23]. Functionally, impaired endothelial-dependent vasorelaxation of both the small and large arteries has been demonstrated already early in PVD[3]. Both the structural and functional changes will lead to a loss of elastic properties of the arterial wall[12, 24]. The stiffening of the pulmonary arteries is likely to be reflected in their dynamic behavior.

Vascular pulsatility

Vascular dynamics can be described with the use of pulsatility, defined in this study as the relative increase in vessel area during the cardiac cycle. Vascular pulsatility, the net result of a complex interaction between mechanical forces, acting on the vessel wall, on the one hand, and intrinsic properties of the wall itself on the other, did not differ between patients with PVD and controls. Pulsatility was most strongly associated with pulmonary pulse pressure. Pulse pressure forms a direct radial mechanical force acting on the arterial wall and is expected to be a major determinant of vascular pulsatility. In addition to pulse pressure, PVR/SVR and Hb were independent predictors of pulsatility, at baseline conditions, however not at vasodilation.

Arterial wall distensibility

The elastic properties of a vessel wall can be characterized *in vivo* by arterial

distensibility, the inverse of the stress/strain elastic modulus. Arterial distensibility reflects the relative increase in arterial area (strain) per mmHg increase in arterial blood pressure, i.e. pulse pressure (stress), and represents a measure for arterial compliance[7, 20, 24]. A decreased distensibility of systemic arteries has been demonstrated in essential hypertension, coronary artery disease, diabetes mellitus and aging[7]. However, the arterial walls exhibits a nonlinear, viscoelastic behavior and therefore, distensibility is directly affected by the distending pressure. An ongoing debate exists about whether, in these conditions, the decrease in distensibility is a consequence of elevated distending pressure and, thus, of the artery operating on a steeper part of its pressure-volume relationship, or a result of changes in the vessel wall itself[7]. In our series, pulmonary arterial wall distensibility decreased progressively with the severity of PVD. The decrease in distensibility correlated strongly with an increase in pulmonary artery pressure expressed as PAP/SAP, reflecting an elevated distending pressure. However, independent from this distending pressure, distensibility at baseline conditions, similar to pulsatility, correlated with PVR/SVR and Hb.

These findings suggest that changes in the pulmonary vascular bed at the level of the resistance vessels, reflected by PVR/SVR, do correlate with pulsatility and distensibility of the larger pulmonary arteries, independent from a possible rise in pulmonary artery pressure. Hemoglobin concentration, regarded a measure for blood viscosity, will affect inertia, pulse wave propagation and the shear stress on the arterial wall and, consequently, may influence directly vascular dynamics[9, 10].

During 100% oxygen inhalation, the independent correlation of both pulsatility and distensibility with PVR/SVR and hemoglobin concentration was lost, whereas the difference in distensibility between patient groups, after allowance for the impact of other variables, became more prominent. How can the effects of oxygen inhalation on these relations be explained? Inhaled oxygen is a powerful pulmonary vasodilator. In PVD, the effect of pulmonary vasodilation, if any, will be most prominent in the small muscular arteries and muscularized arterioles and is reflected in a decrease in PVR and PVR/SVR[5]. Apparently, oxygen did not affect the distensibility of the studied larger pulmonary arteries, suggesting that muscular tone is not an important contributing factor in the distensibility of these elastic arteries. This is in concordance with experimental data in the rat lung, suggesting that decreased distensibility in

monocrotaline-induced pulmonary hypertension is due rather to structural changes in the vascular wall than to increased muscular tonus[25, 26]. The discrepancy in vasodilator respons between muscular and elastic arteries may have led to the loss of the independent correlation between pulsatility and PVR/SVR after pulmonary vasodilation. The effect of viscosity on vascular dynamics depends on the area of the vessel: the larger the vessel, the lesser the effect of viscosity[10]. After 100% oxygen inhalation, the end- diastolic areas of the studied vessels increased. One could speculate that this vasodilation diminished, or eliminated, the measurable effect of Hb on vascular wall dynamics.

Vascular pulsatility, determined by IVUS, has been reported to correlate with vessel diameter[14]. In the present study we demonstrated end-diastolic vessel area to be correlated with pulsatility and distensibility only during oxygen inhalation. However, the effect of end-diastolic area on both pulsatility and distensibility was limited to approximately 0.7% decrease per mm^2 decrease in vessel area. Whether this relation is caused by differences in arterial wall properties in arteries of different sizes or by the effect of the ultrasound catheter obstructing the arterial lumen relatively more in smaller vessels, can not be deduced from our data.

An important finding of our study is that, when taking account of all the described variables, pulmonary arterial wall distensibility still appeared to be decreased in patients with PVD compared to controls. An adjusted, mean decrease of approximately 40% was found in patients with advanced PVD. In our opinion, this decrease indicates a stiffening of the pulmonary arterial wall caused by intrinsic properties of the arterial wall itself, that could not be assessed with the studied hemodynamic variables. Arterial wall stiffness is believed to be important in both the pathogenesis and the clinical course of pulmonary vascular disease[7, 8]. This means that pulmonary arterial wall distensibility is a new feature in the assessment of the pulmonary vasculature, that may provide additional information on pulmonary vascular disease and its effect on the right ventricular load.

Oxygen is a powerful vasodilator of the pulmonary vascular bed and was associated in our series with a modest vasodilation of the arteries studied by IVUS. In contrast to our previous findings, the increase in vessel diameter could not be not associated with

vessel size[14]. Neither was it associated with PVR/SVR, the vasodilator response as determined by percentage change in PVR or with patient group. This means that determination of pulmonary artery dilatation with IVUS is not interchangeable with the assessment of vasodilator response by currently used techniques, such as response of PVR[27, 28].

In conclusion, we demonstrated that pulmonary arterial wall distensibility is progressively decreased in PVD. Our data suggest that this decrease is, in part, related to an increased distending pressure as a result of pulmonary hypertension and, in part, to a stiffening of the arterial wall in the disease process. These changes in arterial wall properties appear independent from hemodynamic data, obtained at cardiac catheterization, but can be assessed with IVUS of the pulmonary arteries, on line, in the same procedure. Pulmonary arterial wall distensibility may be a new variable in the *in vivo* evaluation of the pulmonary vasculature. This variable takes into account the pulsatile nature of the pulmonary circulation and thereby provides additional information on the progression of PVD and its consequences for the integrated pulmonary circulation and right ventricular load.

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Pulmonary arterial wall distensibility, assessed by intravascular ultrasound, correlates with structural changes of the pulmonary vascular bed in children with congenital heart disease

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Introduction

Children with congenital heart disease associated with pulmonary hypertension and/or increased pulmonary blood flow, develop functional and structural alterations of the pulmonary vasculature[1-4]. These vascular changes are a major determinant in morbidity and mortality in these patients[2, 5, 6]. Pulmonary vascular disease may lead to elevated pulmonary vascular resistance, which may hamper surgical intervention or limit long-time survival. The functional state of the pulmonary vascular bed is currently assessed by hemodynamic evaluation, including respons to vasodilators, whereas structural alterations are evaluated by pulmonary wedge angiography and histological examination of lung biopsy. However, these current techniques are limited by practical and conceptual restrictions[7-13]. Vascular changes in pulmonary vascular disease have been demonstrated to affect pulmonary vascular impedance and stiffness[9, 14, 15]. These latter properties of the pulmonary vasculature cannot be assessed in the clinical setting, using the available techniques. Intravascular ultrasound (IVUS), using high-frequency imaging, enables examination of pulmonary vascular dynamics and biomechanical arterial wall properties *in vivo* in infants and children[16]. Recently, children with congenital heart disease and associated pulmonary vascular disease were demonstrated to have a decreased wall distensibility of the elastic pulmonary arteries (see chapter 7). That study suggested that the decreased distensibility was due not only to elevated distending pressure in case of pulmonary hypertension, but also to altered intrinsic wall properties in pulmonary vascular disease[17]. Furthermore, changes of the arterial wall, observed with intravascular ultrasound, have been reported to be correlated with structural changes in the pulmonary vascular bed[18, 19]. A part of the patient population described in this study, has been reported earlier with respect to qualitative IVUS parameters and the correlation between arterial wall distensibility and hemodynamic data[17, 19].

The purpose of the present study was to determine whether pulmonary arterial wall distensibility in children with pulmonary vascular disease, assessed with IVUS, is correlated with structural changes of the pulmonary vascular bed. We therefore compared pulmonary intravascular ultrasound findings with the results of pulmonary wedge angiography and histological examination of lung biopsy in children with

congenital heart disease associated with pulmonary vascular disease and in children with a normal pulmonary circulation.

Methods

Patients. Patients who required cardiac catheterization and had increased pulmonary blood flow (pulmonary-to-systemic blood flow ratio > 1.2) and/or pulmonary hypertension (mean pulmonary artery pressure > 20mmHg), were prospectively enrolled in the study. Children with a normal pulmonary circulation, who underwent cardiac catheterization to exclude cardiac disease or with mild left-sided obstructive lesions and a normal left ventricular end-diastolic pressure and pulmonary wedge pressures, served as controls. Patients with hemoglobinopathies, interstitial pulmonary disease, liver or renal dysfunction or unstable condition were excluded. The study protocol was approved by the institutional Medical Ethical Committee and informed written consent was obtained from the parents of all patients before enrollment.

Catheterization and IVUS procedure

All procedures were performed under standardized general anesthesia[20]. Hemodynamic evaluation and IVUS imaging of the pulmonary arteries were performed in the same procedure, according to a protocol previously reported[16]. Briefly, all patients underwent complete hemodynamic evaluation of the pulmonary and systemic vascular beds, including vasodilator response to inhalation of 100% oxygen, is a powerful pulmonary vasodilator. Pulmonary blood flow was determined using dye dilution technique, the shunt size was determined by oximetry. Before, during and after the procedure blood gas analyses were performed to maintain metabolic stability. An HP Sonos Intravascular Imaging System (Hewlett Packard, Andover, MA) was used. Sonicath 3.5 F or Spy 3.0 F ultrasound catheters (Boston Scientific Corp., Watertown, MA) were used, both having a 30 MHz transducer at its tip. The ultrasound catheter was directed through a 6 F long sheath along the pulmonary branch. At consecutive sites in the proximal artery, the segment arteries and the peripheral arteries, identified and defined by fluoroscopy[10, 16], ultrasound images were obtained and recorded on S-VHS video-

tape, simultaneously with the ECG. The images were analyzed off-line independently by two observers, blinded for clinical and hemodynamic data (AC,RB). Pulmonary arterial luminal diameters and areas were measured at end-diastolic (minimal) and peak-systolic (maximal) dimensions. The values of three cardiac cycles were averaged for each observer and the mean of both averages was used for further analyses. Arterial wall distensibility, the inverse of the stress/strain elastic modulus and a measure of arterial wall compliance, was calculated by dividing the relative increase in planimetered vessel area during the cardiac cycle by pulmonary pulse pressure (100 x peak-systolic (ESA) minus end-diastolic area (EDA), divided by EDA x (systolic minus diastolic pulmonary artery pressure)[21, 22].

Wedge angiography

Pulmonary wedge angiography was performed in the same procedure and in the same lung segments as IVUS, before the conventional angiographies. The balloon occlusion technique, described by Rabinovitch, was used[10]. Tapering rate was measured, after allowance for magnification, as the distance over which vessels tapered from 2.5 to 1.5 mm luminal diameter. This was measured for as many vessels as possible and a mean tapering rate was then calculated[10]. Pulmonary circulation time was defined as the time between deflation of the balloon and visualization of contrast in the pulmonary veins at the site of their entrance into the left atrium, and was determined by counting the number of cine frames [10]. Circulation time could not be accurately assessed in 9 cases, because of incomplete balloon occlusion. The background haze, determined by the degree of filling of small peripheral arteries was assessed as being moderately or markedly reduced by comparing the angiograms with standards, established by the control group[10].

Histologic evaluation

In patients who underwent subsequent cardiac surgery an open lung biopsy was taken during the surgical procedure. In one patient an open lung biopsy was taken as isolated procedure for diagnostic purposes. Tissue was formalin fixed under vacuum and paraffin embedded. Five μm sections were cut at various levels and stained with hematoxylin-eosin (HE) and Elastic-van Gieson (EvG) and iron stain to allow for

proper identification of morphological structures. Vascular changes were described according to Wagenvoort[1, 23]. The medial thickness of the muscular arteries was calculated as a percentage of the total artery diameter, excluding the adventitial layer. The presence or absence of the following vascular changes was recorded: intimal longitudinal smooth muscle, intimal proliferation, concentric laminar intimal fibrosis, angiomatoid dilatation lesions, fibrinoid necrosis, plexiform lesions[23]. The latter four vascular changes were regarded as advanced lesions. Further the presence or absence of eccentric intimal fibrosis, recent thrombi and arterialisation of veins was recorded[23].

Statistical analysis

Arterial wall distensibility, taking account of differences between as well within patients, were evaluated by regression analysis for repeated measurements using the BMDP-statistical software package[24]. Using backward elimination, the angiographical and histological variables, most predictive for arterial wall distensibility were determined. In this analysis distensibility data were logarithmically transformed in order to obtain approximate normal distributions. In first instance, the angiographical and histological variables were analysed separately. In a following analysis, the resulting most important variables from each technique were evaluated simultaneously. Finally, the pulmonary-to-systemic pressure ratio, reflecting arterial distending pressure, was included in the multivariate analysis. A univariate comparison of geometric mean distensibility between patients and controls was done using mixed model ANOVA. The limit of significance was set at $p = 0.05$ (two-sided).

Results

In total, 51 patients with an increased pulmonary blood flow and/or pulmonary hypertension were enrolled in the study. Additionally, 13 patients with a normal pulmonary circulation were studied (table 1). All patients underwent hemodynamic evaluation, a pulmonary wedge angiogram and intravascular ultrasound study. In 32 patients, including 4 controls, a lung biopsy was taken during subsequent cardiac surgery (n=31) or by means of a diagnostic open lung biopsy procedure (n=1). The

Table 1 *Diagnoses of the study patients*

Diagnosis	n
Left-to-right shunt:	
VSD with or without ASD or PDA	23
with pulmonary arterial banding	2
ASD	6
PDA	3
cAVSD	2
Truncus arteriosus	1
No left-to-right shunt:	
Persistent PH after corrected heart defect	2
Unexplained PH	3
Left sided obstructive lesions with PVC	9
Controls	13
Total	64

ASD indicates atrial septal defect; cAVSD, complete atrioventricular septal defect; n, number; PDA, persistent arterial duct; PH, pulmonary hypertension; PVC, pulmonary venous congestion; VSD, ventricular septal defect.

interval between catheterization procedure and biopsy ranged from 2 days to 12 months, median interval 2.1 months. Age, hemodynamic and angiographical characteristics of the patients are summarized in table 2.

Examination of lung biopsies revealed essentially normal pulmonary arteries in the control group and the complete spectrum of morphologic vascular changes, ranging from muscular hypertrophy and intima hyperplasia to the characteristic lesions of advanced plexogenic arteriopathy in the patient group. Medial thickness ranged from 6 to 13% (mean 10%) in the control group and from 3 to 30% (mean 12%) in the patient group. Six patients had intimal hyperplasia as most severe vascular change, 1 had concentric laminar intimal fibrosis, without other advanced lesions and three

Table 2 *Hemodynamic and angiographic characteristics of the study patients*

	control		patients	
n	13		51	
age (years)	4.8	(0.5 - 16.9)	2.8	(0.2 - 30.7)
Hemodynamics				
mean PAP (mmHg)	13	(7 - 18)	26	(8 - 72)
PAP/SAP	0.19	(0.13 - 0.25)	0.47	(0.12 - 1.02)
shunt size (Qp/Qs)	1.0	(0.95 - 1.1)	2.1	(1.0 - 7.2)
PVR (WU.m2)	1.3	(0.4 - 2.6)	2.4	(0.4 - 15.0)
Wedge angiography				
tapering rate (mm)	14	(9.2 - 23.8)	13	(6.6 - 24.5)
circulation time (sec)	0.89	(0.2 - 1.7)	0.74	(0.0 - 1.9)
background haze (n)				
normal	13		28	
moderately reduced	0		16	
markedly reduced	0		6	

PAP indicates pulmonary artery pressure; PAP/SAP, pulmonary-to-systemic arterial pressure ratio; PVR, pulmonary vascular resistance; Qp/Qs, pulmonary-to-systemic blood flow ratio; WU, Woods' units; n, number of patients. Data are presented as median value and range.

patients showed combinations of concentric laminar intimal fibrosis, dilatation lesions and plexiform lesions.

Intravascular ultrasound data

IVUS studies were performed in 2-7 consecutive sites along the the pulmonary arterial tree per patient. In total, 186 artery segments were studied. End-diastolic diameters of the studied vessels ranged from 2.0 mm to 10.4 mm.

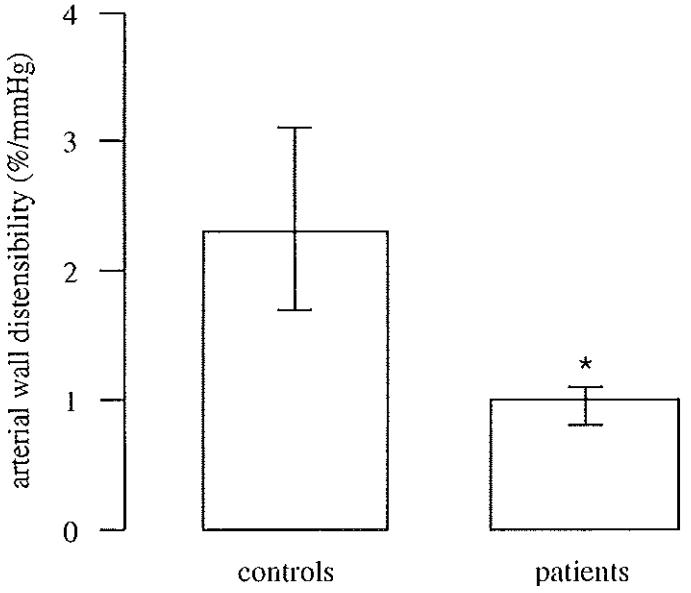

Figure 1 *Arterial wall distensibility of the elastic pulmonary arteries in patients with a normal pulmonary circulation (controls) and patients with pulmonary vascular disease. Data are expressed as geometric means and 95% confidence intervals. * $p < .001$ compared to controls.*

Arterial wall distensibility was decreased in patients with an abnormal pulmonary circulation compared to controls (geometric mean 1.0 %/mmHg; 95% confidence interval: 0.8-1.1 vs. 2.3 %/mmHg: 1.7-3.1; $p < .001$) (figure 1). Univariate correlations in the patient group between arterial wall distensibility and the variables of angiographic respectively histological evaluation of the pulmonary vascular bed are shown in table 3. The results of the multiple regression analysis for the angiographic variables are summarized in table 4a and for the histologic variables in table 4b. Subsequently, these angiographic and histologic variables were included simultaneously in a regression analysis, which identified the tapering rate and a markedly decreased background haze on the wedge angiogram, the pulmonary arterial medial thickness and the finding of recent thrombi in the lung biopsy as independent predictors for arterial wall distensibility (table 5a). Moreover, adjusted for these variables, arterial wall distensibility was still decreased in patients with pulmonary hypertension and/or

Table 3 *Univariate correlations between the studied variables and arterial wall distensibility. Data given are regression coefficients with ^{10}log distensibility as dependent variable. Data were analyzed for the patient group (angiography: n = 51; biopsy: n = 28)*

		patients	p
Pulmonary wedge angiography			
Tapering rate	(mm)	.018 ± .007	.016
Circulation time	(sec)	–.074 ± .084	.38
Background haze	normal	0	-
	moderately reduced	–.034 ± .060	.58
	severely reduced	–.420 ± .087	< .001
End-diastolic area (mm^2)		.001 ± .001	.24
Lung biopsy			
Medial thickness (%)		–.019 ± .007	.01
Longitudinal smooth muscle		–.024 ± .108	.83
Recent luminal thrombi		–.395 ± .141	.005
Eccentric intimal fibrosis		–.046 ± .099	.64
Intimal thickening		–.196 ± .071	.006
Dilatation lesions		.116 ± .122	.34
Advanced lesions		–.316 ± .122	.001
Arterialisation of veins		.072 ± .095	.45
End-diastolic area (mm^2)		.0003 ± .001	.79

The effects of the variables longitudinal smooth muscle, recent luminal thrombi, eccentric intimal fibrosis, dilatation lesions, advanced lesions and arterialisation of veins were all described as present versus absent. Intimal thickening was tested for trend in the sequence of severity in pulmonary plexogenic arteriopathy: absent (0), intimal proliferation (1) or concentric laminar intimal fibrosis (2). Advanced lesions are concentric laminar intimal fibrosis, dilatation lesions and plexiform lesions. End-diastolic area indicates the arterial luminal area measured by intravascular ultrasound.

Table 4 *Multivariate analysis of the angiographic variables (A) and the histologic variables (B). Data given are regression coefficients with ^{10}log distensibility as dependent variable.*

A			
angiographic variables		change	p
background haze:	moderately reduced	–.020 ± .057	–4%	.73
	markedly reduced	–.421 ± .083	–62%	< .001
tapering rate	(mm)	.014 ± .006	3%	.02
pulmonary vascular disease		–.303 ± .064	–50%	< .001
end-diastolic area	(mm2)	.002 ± .001	0.5%	.10

B			
histological variables		change	p
medial thickness	(%)	–.020 ± .007	–4%	.005
recent luminal thrombi		–.274 ± .117	–47%	.020
advanced lesions		–.240 ± .095	–42%	.01
pulmonary vascular disease		–.238 ± .095	–42%	.012
end-diastolic area	(mm2)	.002 ± .001	0.5%	.16

Change indicates the percentage change in distensibility per: mm tapering rate, per % increase of medial wall thickness, per mm^2 end-diastolic vessel area. It further indicates the change of distensibility in case of a markedly or moderately reduced compared to a normal background haze, in the presence compared to the absence of recent thrombi respectively advanced lesions, and in the presence compared to the absence of pulmonary vascular disease.

increased pulmonary blood flow compared to patients with a normal pulmonary circulation ($p < 0.001$; fig. 2).

To evaluate the effect of arterial distending pressure on these correlations, the analysis was repeated with the introduction of the pulmonary-to-systemic arterial pressure

Table 5. *Multivariate analysis of the angiographic and histological variables (A), and including the pulmonary-to-systemic arterial pressure ratio (B). Data given are regression coefficients with ^{10}log distensibility as dependent variable.*

A

			change	p
tapering rate	(mm)	.016 ± .006	4%	.01
background haze:	markedly reduced	–.447 ± .116	–64%	< .001
medial thickness	(%)	–.017 ± .006	–4%	.002
recent luminal thrombi		–.226 ± .099	–41%	.02
pulmonary vascular disease		–.240 ± .072	–42%	<. 001
end-diastolic area	(mm^2)	.002 ± .001	0.5%	.18

B

			change	p
pulmonary-to-systemic arterial pressure ratio		–.054 ± .180	–12%	.76
tapering rate	(mm)	.016 ± .007	4%	.02
background haze:	markedly reduced	–.434 ± .124	–63%	<. 001
medial thickness	(%)	–.015 ± .008	–3%	.07
recent luminal thrombi		–.210 ± .112	–38%	.06
pulmonary vascular disease		–.229 ± .081	–41%	.005
end-diastolic area	(mm^2)	.002 ± .001	0.5%	.18

Change indicates the percentage change of distensibility: per mm tapering rate, per % increase of medial wall thickness, per 0.1 unit change in the ratio of pulmonary-to-systemic arterial pressure, per mm^2 end-diastolic vessel area, in case of a markedly reduced compared to a normal or moderately reduced background haze, in the presence compared to the absence of recent thrombi, and finally, in the presence compared to the absence of pulmonary vascular disease.

ratio (PAP/SAP) as a variable into the model. The variable PAP/SAP provided no additional value in predicting wall distensibility, while the correlations with the angiographical and histologicle variables appeared to be essentially unchanged (table 5b).

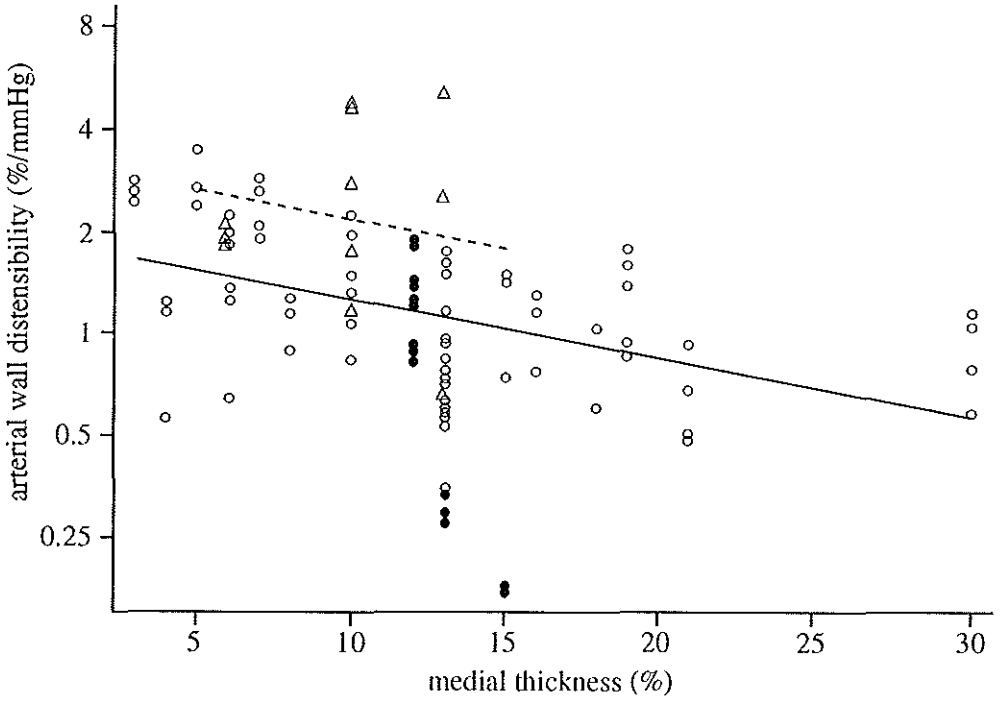

Figure 2 *Relation between arterial wall distensibility (^{10}log)and medial wall thickness in the lung biopsy. Arterial wall distensibility is adjusted for a tapering rate of 13 mm and a normal background haze on pulmonary wedge angiography, for the absence of recent thrombi in the lung biopsy and for an end-diastolic vessel area of 15 mm^2. The dashed regression line represents the relation in controls (triangles), the solid regression line represents the relation in patients with pulmonary vascular disease (circles). The solid circles represent values of patients with advanced lesions in their lung biopsy.*

Discussion

Our study demonstrates that, in children with pulmonary vascular disease associated with congenital heart disease, pulmonary arterial wall distensibility, assessed with IVUS, is decreased. This decrease was correlated with structural properties of the pulmonary vascular bed, as determined with pulmonary wedge angiography and lung biopsy. This correlation appears to be independent from pulmonary artery pressure, expressed as the ratio of pulmonary-to-systemic arterial pressure. Moreover, after adjustment for the various angiographic and histologic variables, arterial wall distensibility still appeared to be decreased in patients with pulmonary vascular disease compared to that in controls.

The IVUS technique has been recently proposed as a tool in the assessment of pulmonary vascular disease[16, 18, 25-27]. Accuracy and a high degree of interobserver agreement of ultrasound measurements of luminal area have been reported earlier[16, 17, 28]. Changes in the appearance of elastic pulmonary arteries, assessed with IVUS both *in vitro* and *in vivo,* have been correlated with histologic assessment of pulmonary vascular disease[18, 19]. However, the *in vivo* investigation of functional arterial wall properties of proximal pulmonary arteries in children with pulmonary vascular disease have not been correlated before with structural changes of the pulmonary vascular bed.

Pulmonary arteriopathy in congenital heart disease involves the complete pulmonary vasculature[1]. Although the morphological changes are most impressive in the small muscular arteries, both functional and structural alterations of the proximal, elastic pulmonary arteries have been demonstrated in pulmonary vascular disease. These alterations include impaired endothelial-dependent relaxation and medial thickening by extensive extracellular matrix modulation[3, 29-31]. These vascular changes will lead to a decreased compliance of the pulmonary arteries[9, 14, 15]. In a previous study, described in chapter 7, we demonstrated that pulmonary arterial wall distensibility was decreased in the course of pulmonary vascular disease[17]. This decrease in distensibility could be partly related to an elevated arterial distending pressure in pulmonary hypertension, and additionally to the presence of pulmonary vascular disease itself, suggesting altered intrinsic wall properties in that condition. The pre-

sent study demonstrates the decrease in distensibility to be correlated with structural changes in the vascular bed, assessed with pulmonary wedge angiography and lung biopsy.

Pulmonary wedge angiography enables the visualisation of peripheral pulmonary arteries as small as 100-300 μm in diameter [32]. A rapid tapering of the pulmonary arteries have been reported to correlate with both hemodynamic parameters and structural vascular changes in lung biopsies, although the predictive value for a given taper length was low[10, 32, 33]. A reduced background haze, due to diminished or absent filling of the small, so-called supernumerary branches, thin-walled arteries that branche perpendicular from muscular and elastic arteries, has been shown to correlate with the number of occluded small vessels in lung biopsy and to predict advanced plexogenic arteriopathy[32, 33]. From the angiographic variables, both a rapid tapering of peripheral pulmonary arteries and a markedly decreased background haze were independently predictive for a decreased distensibility.

From the histologic variables, an increased medial thickness of muscular arteries, the presence of recent thrombi and the presence of advanced vascular lesions were independently predictive for a decreased arterial wall distensibility. Increased muscularity of the pulmonary arterial tree, judged by extension of muscle to peripheral, normally non-muscularised arteries and increased medial thickness of muscular arteries, is an early feature of pulmonary arteriopathy[1, 11]. The proliferation of vascular smooth muscle cells is associated with an extensive modulation of the extracellular matrix, including increased connective tissue deposition[31, 34]. This results in a stiffening of the arterial wall[14, 35-37].

Both the rapid tapering rate and the increased muscularization of pulmonary arteries are features of early stage of pulmonary vascular disease, whereas a markedly decreased background haze and the presence of advanced lesions are characterics of severe pulmonary arteriopathy[1, 32, 33]. The significance of the presence of recent thrombi in lung biopsy is not clear, and in plexogenic arteriopathy it is probably an accompanying phenomena. In our series recent thrombi were found in lung biopsy of only three patients, all of who had a severely elevated pulmonary artery pressure and pulmonary vascular resistance.

When the information obtained by wedge angiography and lung biopsy was analysed

simultaneously, the tapering rate, markedly reduced background haze, medial thickness and the presence of recent thrombi, all appeared to be independent predictors for wall distensibility. However, in combination with the information from the wedge angiogram, the presence of advanced lesions in the lung biopsy was not of significant predictive value for distensibility anymore. This may be due to the fact that a markedly reduced background haze and the presence of advanced lesions in lung biopsy were highly related in our series: 2 out of the 4 patients with histologically advanced lesions, and none out of the 28 patients without advanced lesions, had a markedly reduced background haze on the wedge angiography ($p = 0.01$).
Because of the viscoelastic properties of the arterial wall, the stress-strain relationship is not linear[22]. Therefore, arterial distending pressure affects the functional distensibility of the arterial wall in such a way that an elevated distending pressure will decrease distensibility. We demonstrated earlier, that the arterial distending pressure, reflected by PAP/SAP, was correlated inversely with arterial wall distensi-bility[17]. However, the introduction of the hemodynamic variable PAP/SAP in the analysis showed that the correlations between arterial wall distensibility and angiographic and histological parameters were independent of this arterial pressure variable.

A second important finding of the present study is that, after allowance has been made for the studied variables, arterial wall distensibility appeared to be still significantly decreased in patients with pulmonary vascular disease. Apparently, in patients with an abnormal pulmonary circulation functional alterations of elastic pulmonary arteries occur already early in the course of vascular disease. These alterations constitute an increased functional arterial stiffening, and may be due to changes in the extracellular matrix in the elastic arteries that paralel the muscularisation and matrix modulation of the muscular arteries. These changes in functional properties cannot be assessed with the commonly used techniques, but undoubtly will have unfavourable effects on the pulmonary circulation and the work load of the right ventricle[9, 15].

Limitations of the study
The number of control patients in whom a lung biopsy was taken was small. Also the

number of patients with advanced lesions in their lung biopsy was relatively small. Therefore, the finding that some variables did not show independent correlations with arterial wall distensibility, could have been due to the numbers studied. The design of this *in vivo* study allowed no corresponding histologic sections of the pulmonary arteries studied with IVUS. Therefore, the measured arterial wall distensibility could not be correlated directly with the histopathological and chemical composition of the vascular wall.

Future applications

The finding that arterial wall distensibility of the elastic pulmonary arteries is correlated with structural changes of both early and advanced arteriopathy at the level of the vascular bed, independent from arterial distending pressure, may provide new opportunities in the clinical assessment of pulmonary vascular disease. The possibility to measure functional changes early in the course of the disease process may be of special interest in the preoperative evaluation of patients who will need univentricular heart repair. Furthermore, it will increase our insight into the functional consequences of the various aspects of pulmonary vascular remodeling.

In conclusion, intravascular ultrasound of the pulmonary arteries allows *in vivo* measurement of pulmonary arterial wall distensibility. Pulmonary arterial wall distensibility was decreased in patients with pulmonary vascular disease and this decrease was correlated with structural changes of the pulmonary vascular bed assessed with pulmonary wedge angiography and histologic examination of lung biopsy. These correlations were independent from pulmonary-to-systemic arterial pressure ratio, as a measure for arterial distending pressure. Moreover, adjusted for the angiographic and histological parameters, arterial wall distensibility appeared to be still decreased in patients with pulmonary vascular disease. Pulmonary arterial wall distensibility may constitute a new parameter in the assessment of pulmonary vascular disease, that provides information on both functional and structural properties of the pulmonary vasculature.

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Immunohistochemical localization of Vascular Endothelial Growth Factor in pulmonary plexogenic arteriopathy

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Abstract

Congenital heart disease (CHD) associated with increased pulmonary blood flow leads to pulmonary plexogenic arteriopathy (PPA). The pathogenesis of the characteristic lesions in PPA is not well understood. Active endothelial cell proliferation has been demonstrated in these lesions and therefore VEGF, a key mediator of angionesis, might play a role in their pathogenesis.

The aims of the study were to assess the pulmonary vascular expression of vascular endothelial growth factor (VEGF) in patients with pulmonary plexogenic arteriopathy, and to compare the findings to the expression in patients with pulmonary venous congestion and in controls. We further sought for correlations between the immunolocalization of VEGF and hemodynamic and histological data.

Thirty-four patients, undergoing cardiac catheterization and subsequent cardiac surgery were studied prospectively. Patients were grouped in moderate PPA (n = 18), advanced PPA (n = 7), pulmonary congestive vasculopathy (PCV) (n = 5) and controls (n = 4). ImmunoreactiveVEGF was detected in pulmonary arterial smooth muscle and endothelial cells in 13 out 34 cases. The staining was more frequent in patients with advanced PPA as compared to patients with moderate PPA ($p = 0.006$) and controls ($p = 0.08$). No differences in the frequency of VEGF expression could be demonstrated between controls, patients with PVC and moderate PPA. Measured hemodynamic parameters did not differ significantly between VEGF-positive and VEGF-negative patients. Marked VEGF positivity was detected in the characteristic lesions of advanced PPA.

Conclusion: The pulmonary arterial expression pattern suggests an role for VEGF in the pathogenesis of the proliferative lesions of advanced PPA.

Introduction

Pulmonary hypertension in congenital heart disease (CHD) may be associated with different types of pulmonary arteriopathy. CHD with left-to-right shunts and increased pulmonary blood flow leads to plexogenic arteriopathy (PPA)[1, 2]. The morphologic vascular changes of PPA have been documented extensively[3]. Advanced lesions of PPA include concentric-laminar intimal fibrosis (CLIF), fibrinoid necrosis, dilatation lesions and plexiform lesions. These lesions of advanced PPA distinguish it from other pulmonary vasculopathies, resulting from, e.g., hypoxia or pulmonary congestion. The latter, also termed pulmonary congestive vasculopathy (PVC) develops in cases of mitral stenosis or left ventriclular dysfunction. The recognition of the type of pulmonary arteriopathy is of paramount clinical importance[2, 4]: PCV is reversible if the cause of pulmonary venous congestion can be removed[5]. In contrast, untreated PPA advances to a certain "point of no return", hallmarked histologically by the emergence of the characteristic advanced lesions of PPA, mentioned above. When such lesions are found, the disease process has not only become irreversible, but also progresses even when the underlying heart defect is corrected[5]. PPA may also occur in the absence of CHD and even without a known etiological factor, where it constitutes one of the histological subtypes of so-called clinically unexplained pulmonary hypertension[6].

Interestingly, fibrinoid necrosis, CLIF and the plexiform lesion, three of the main histological hallmarks of advanced PPA, occur at a very specific site of the pulmonary arterial tree, namely at the origin of so-called supernumerary arteries (SNA's)[7, 8]. These are small arterial branches which arise perpendicularly from larger parent arteries and run straight into the pulmonary alveolar parenchyma. Apparently, this site is uniquely vulnerable to injury in case of high flow/high pressure pulmonary hypertension. At present, the exact cause of the vascular injury, and the vascular proliferative remodelling process presumably resulting from it and leading to intimal cellular proliferation and the formation of the plexiform lesion, have not yet been elucidated at the molecular level.

Vascular endothelial growth factor (VEGF), a potent mitogen apparently acting exclusively on vascular endothelial cells, is known to play a role in angiogenesis in

widely divergent circumstances, such as embryonic development, wound healing, tumor growth, rheumatoid arthritis and ischemic retinopathy[9, 10]. VEGF has been demonstrated to induce and facilitate angiogenesis, to promote endothelial cell growth and migration and to influence capillary permeability[10]. It has been shown that endothelial proliferation and angiogenesis occur in advanced plexogenic lesions[11]. In this study we tested the hypothesis that VEGF plays a role in the remodeling process occuring at the origin of SNAs in PPA. Therefore, we examined the expression patterns of VEGF in pulmonary arteries in PPA and compared it to the expression in normal pulmonary arteries and in PCV. Furthermore, we correlated the pattern of VEGF expression with hemodynamic and morphologic characteristics.

Methods

Clinical parameters

After approval of the Medical Ethical Committee of the University of Rotterdam and after parental informed consent, patients with PPA, associated with congenital heart disease or idiopathic pulmonary hypertension, and patients with PCV, who underwent diagnostic cardiac catheterization, were prospectively enrolled in the study. Pulmonary venous congestion was defined as a pulmonary capillary wedge pressure (PCW) >15 mmHg in the absence of a left to right shunt. Children with a normal pulmonary circulation and mild left-sided obstructive lesions (PCW < 15 mmHg), who underwent diagnostic cardiac catheterization, served as controls. Complete hemodynamic evaluation was performed, including measurement of pulmonary arterial pressure, calculation of shunt size, pulmonary and systemic vascular resistance. Shunt size was determined using oxymetry. Pulmonary blood flow was determined using the dye dilution technique.

Tissue specimens

Lung biopsies were taken subsequently during cardiac surgery for CHD or as an open lung biopsy procedure for diagnostic purposes. Tissue was formalin fixed under vacuum and paraffin embedded[1].

Routine histochemical staining and immunohistochemical staining was performed on serial sections to allow for proper identification of morphological structures, and a precise correlation of morphology with patterns of immunoreactivity.

Immunohistochemistry

Sections (5 μm) of paraffin-embedded lung tissues were deparaffinized in xylene and graded ethanols and hydrated in 5% phosphate-buffered saline (PBS). Routine histology was performed by staining sections with hematoxylin-eosin and Elastic-van Gieson, using standard protocols. For immunohistochemistry the sections were incubated with 10% normal goat serum to block nonspecific binding. Thereafter, the sections were incubated with affinity purified rabbit polyclonal antibody against human VEGF (Santa Cruz Biotechnology, Santa Cruz, USA) in dilution 1:200 for 30 minutes. This antibody reacts with the human 121, 165, and 189 amino acid splice variants of VEGF by immunohistochemistry on formalin-fixed, paraffin-embedded tissues[12]. After rinsing twice with PBS-tween 0.2%, the sections were incubated for 30 minutes with biotinylated antiimmunoglobulins (Multilink, Biogenex, San Ramon, CA) in a dilution of 1:50, with 2% normal goat serum and 2% normal human serum in PBS. After rinsing twice with PBS, sections were incubated with preformed avidin-biotin-enzyme complex (ABC, Biogenix, San Ramon, CA) 1: 50 in PBS for another 30 minutes. After rinsing with PBS, and washing in tris-HCL at pH 8.0, chromogen was added for the enzyme detection with the alkaline-phosphatase method using freshly prepared solutions of Naphtol AS-MX -phosphate, New Fuchsin, Sodium nitrite and Levamisole in tris-HCL at pH 8.0. The chromogene reaction was allowed to take place over 30 minutes in the dark. Sections were rinsed with tris-HCL at pH 8.0, slightly counterstained with Meyer's hematoxylin and mounted in an aqueous-base mounting medium. Negative controls consisted of replacement of the primary antibody by 1:10 diluted normal rabbit serum (Dako, Glostrup, Denmark). Placental blood vessels where used as positive controls for VEGF immunostaining. The presence and the micro-anatomical pattern of expression of VEGF in the lung biopsies was investigated in detail. VEGF expression was defined as negative or positive staining. The entire series of sectios was processed and evaluated twice, in order to document consistency of immunostaining patterns.

Vascular lesions, present in the lung biopsies were classified histologically as described by Wagenvoort and Mooi[3, 13]. Patients with PPA were regarded as having mild to moderate PPA if concentric laminar intimal fibrosis, angiomatoid dilatation lesions, fibrinoid necrosis and plexiform lesions were absent from the biopsy. The presence of one or more of the above mentioned lesions in the lung biopsy was considered to indicate advanced PPA[3].

Statistical analysis

Values are given in means ± standard deviation, if not indicated otherwise. For comparison of continous variables between groups analysis of variance (ANOVA) or Kruskal-Wallis tests were used, where apropriate. For comparison of categorical variables between groups the chi square test and subsequently the Fisher exact test was used. P-values < 0.05 were considered significant.

Results

Thirty-nine patients were included in the study. In 5 patients, interpretation of VEGF immunostaining was not possible because of insufficient sample size or suboptimal tissue quality, leaving 34 evaluable cases. There were 18 patients with mild to moderate PPA and 7 patients with advanced PPA. The group with PCV consisted of 5 patients and the control group included 4 children (tables 1 and 2). Staining for VEGF was found in 13 out of the 34 lung biopsies. In these cases VEGF was localized in pulmonary arteries, as described in detail below. In addition a faint to moderate positivity was seen, especially in bronchial and bronchiolar epithelium and smooth muscle.

In 13 cases, VEGF positivity was detected in vascular smooth muscle cells (VSMC's) of the media (figure 1, page 177). In addition, arterial endothelial cells, including those of non-muscularized branches, were positive. VEGF immunostaining was often more prominent in supernumerary arteries then in the adjacent much larger parent artery (figure 1). In the characteristic lesions of advanced PPA we detected increased VEGF positivity. In lesions of concentric laminar intimal fibrosis VEGF was expressed in the intimal cells forming the characteristic onion skin-like layer (figure 1).

Table 1 *Anatomical and histological diagnosis*

Diagnosis	number of patients			
	controls	PVC	moderate PPA	advanced PPA
Left-sided obstructive lesions	4	5	-	-
VSD				
- isolated ± ASD II	-	-	11	1
- with left-sided obstr. lesion	-	-	2	1
ASD	-	-	3	-
cAVSD	-	-	2	2
PDA	-	-	-	1
ToF with left pulmonary artery from aorta	-	-	-	1
Idiopathic PPA	-	-	-	1
Total	4	5	18	7

ASD indicates atrial septal defect; cAVSD complete atrioventricular septal defect; PDA, persistent ductus arteriosus; PPA, pulmonary plexogenic arteriopathy; ToF, Tetralogie of Fallot, VSD, ventricular septal defect.

Table 2 *Baseline characteristics*

		controls (n = 4)	PCV (n = 5)	moderate PPA (n = 18)	advanced PPA (n = 7)*
Age	(yr)	3.3 ± 1.3	3.9 ± 3.9	4.8 ± 7.6	9.9 ± 12.3
mean PAP	(mmHg)	13 ± 2	31 ± 12	24 ± 12	51 ± 16
Qp/Qs		1.0 ± 0.0	1.0 ± 0.0	2.9 ± 1.6	2.3 ± 1.6
PVR	($WU.m^2$)	0.9 ± 0.3	5.0 ± 4.0	2.3 ± 1.6	8.2 ± 4.2

* n = 6 for hemodynamic data. PAP = pulmonary artery pressure; PCV = pulmonary congestive vasculopathy; PPA = pulmonary plexogenic arteriopathy; Qp/Qs = pulmonary-to-sytemic blood flow ratio; PVR = indexed pulmonary vascular resistance. Data given are mean ± SD.

In plexiform lesions, pronounced staining of VEGF was seen in the cells forming the plexus as well as in the cells lining dilated, angiomatoid vessels (figure 1).
The number of patients who showed detectable expression of VEGF in VSMC's and endothelial cells of the muscularized and non-muscularized pulmonary arteries varied between the patient groups ($p = 0.03$). No differences in the frequency of VEGF expression could be demonstrated between controls, patients with PCV and mild-to-moderate PPA. However, VEGF expression occurred more frequently in patients with advanced PPA (table 3). When compared to patients with reversible arteriopathy (moderate PPA and PCV), VEGF positivity in VSMCs was significantly more frequent in patients with advanced PPA ($p = 0.007$). The age of the patients with VEGF expression was higher than that of those negative for VEGF (median 6.0 vs 1.1 years, $p = 0.006$). However, when stratified for patient group, age was not independently related to VEGF expression. The hemodynamic parameters mean, systolic and diastolic pulmonary artery pressure did not differ between VEGF positive and VEGF negative patients (32 mmHg ± 20 vs 27 mmHg ± 14; 49 mmHg ± 28 vs 39 mmHg ± 20, and 20 mmHg ± 15 vs 16 mmHg ± 9, respectively), neither did pulmonary arterial pulse pressure (28 ± 16 vs 24 ± 13). The pulmonary-to-systemic blood flow ratio (2.7 ± 2.1 vs 2.0 ± 1.3) and the pulmonary vascular resistance (4.6 WUm^2 ± 4.4 vs 3.2 WUm^2 ± 3.2) did not differ between the groups (for all $p > 0.05$).

Discussion

To our knowledge, this is the first study documenting VEGF positivity in the arterial lesions characteristic of advanced PPA. On the basis of its known biological activities, it is quite possible that VEGF is involved in the pathogenesis of the advanced lesions of PPA. VEGF is a direct angiogenic factor, that promotes endothelial cell proliferation[9], an important histological feature of advanced PPA. A potential role for VEGF in the process of structural vascular changes in pulmonary hypertension was firstly suggested by Tuder et al., who briefly reported on increased levels of mRNAs encoding VEGF in the lung of a patient with unexplained pulmonary hypertension[14]. In an *ex vivo* rat lung model of acute and chronic hypoxia, the same investigators

Table 3 *VEGF expression in pulmonary artery smooth muscle cells*

	VEGF + (n)	VEGF – (n)	Significance (p-value) compared to:	
Controls	1	3	controls	mod.PPA
PCV	2	3	0.6	0.4
Moderate PPA	4	14	0.7	–
Advanced PPA	6	1	0.08	0.006

PCV indicates pulmonary congestive vasculopathy; PPA, pulmonary plexogenic arteriopathy.

found increased VEGF gene transcripts, VEGF protein content and also increased levels of mRNA for its receptors Flk-1 and Flt-1. They localized the increased VEGF mRNA expression in alveolar cells and, to a lesser degree, in alveolar macrophages and vascular smooth muscle cells[15, 16]. In this respect, however, it should be noticed that PPA differs essentially from hypoxia-induced pulmonary hypertension, which does not associate with the characteristic lesions of advanced PPA, even when it is pronounced and has been present for a long period of time[17]. We immunolocalized VEGF protein predominantly in vascular structures and found a positive correlation between the staining for VEGF in the smooth muscle cells of the pulmonary arterial wall and the presence of histological lesions indicative for advanced PPA.

Responsiveness of VEGF expression to hypoxia has been related to its gene promoter and 3'-untranslated region, which contain sequence motifs that share high homology with hypoxia-responsive enhancer elements of the human erythropoietin gene[18]. In PPA, mechanisms other than those operating in hypoxic pulmonary hypertension may be responsible for the vascular VEGF expression. If exposed to increased pulsatile flow, vascular endothelial cells are altered in their surface characteristics and, together with smooth muscle cells, in their functional state by fluid mechanical forces[19]. A shear stress responsive element (SSRE) has recently been identified in the promoter region of genes, encoding for various vascular modulators[20], including the VEGF receptors Flk-1and Flt-1[16]. VEGF-inducing growth factors, like TGF-ß, PDGF-BB and bFGF, which show restricted sequence homology with VEGF, are now known to

be directly upregulated at the transcriptional level by mechanically transduced mechanisms[20]. VEGF expression may be upregulated by similar mechanisms. In our study we were unable to demonstrate a correlation between pulmonary hemodynamic features and the expression of VEGF protein. However, the subtle, local fluid mechanical forces, regulating vascular tone and homeostasis at the cellular level are not necessarily reflected directly by hemodynamic data. As indicated earlier, plexiform lesions occur immediately distal to ramification sites and especially at the origin of SNA's[7,8]. At these sites, complex hemodynamic flow profiles with areas of recirculation may result in steep gradients of shear stress, that parallel those in systemic arterial branching points[21,22], which have recently been demonstrated to have a great impact on endothelial adaptive functions[23]. Whether VEGF expression is upregulated directly by altered fluid mechanical forces or indirectly by induction through other factors remains to be elucidated.

The occurence of VEGF in cells of the characterisistic lesions of advanced PPA suggests a role of VEGF in the development of the irreversible pulmonary vascular changes. Wether this role is causal to the development of these lesions cannot be deduced from our data. Unfortunately, no well-functioning animal model exists, to study, in an experimental setting, the effects of pulmonary overflow on the pulmonary vasculature leading to PPA, which would allow to evaluate the effects of VEGF-blocking therapy. Such anti-VEGF therapy is expected to have therapeutic potential for a variety of neoplastic and angiogenic disorders[10].

Recently, a soluble VEGF-receptor has been identified, which blocks the mitogenic effects of VEGF in vitro and in vivo[10].

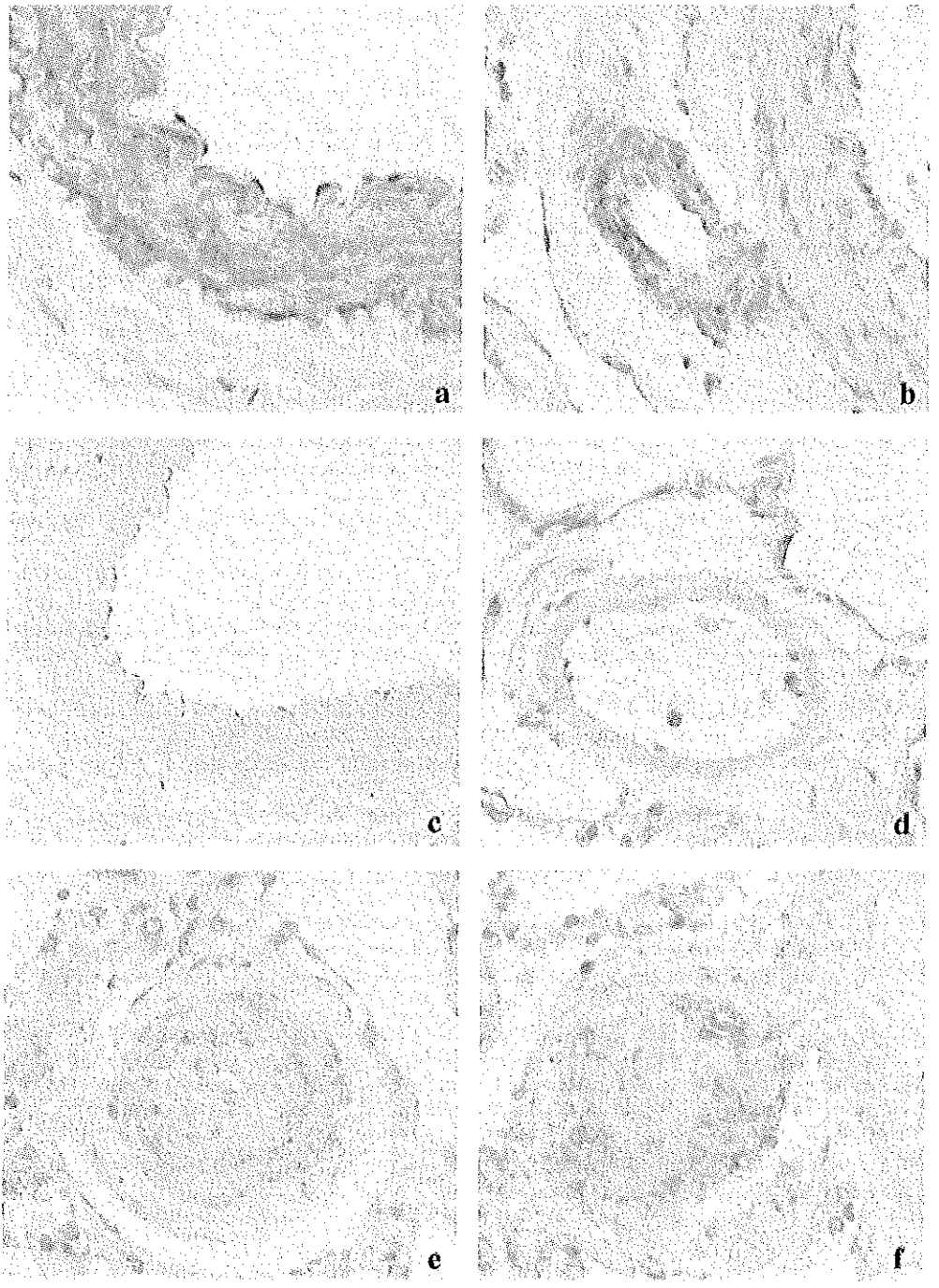
In conlusion, we demonstrated that VEGF protein is more frequently expressed in smooth muscle cells and endothelial cells of pulmonary arteries of patients with advanced PPA compared to patients with moderate PPA, PCV and controls. We further demonstrated that immunoreactive VEGF is localized in the characteristic lesions of advanced PPA. The localization of VEGF in the wall of small pulmonary arteries and at the sites where the characteristic lesions originate is in accordance with our hypothesis, that local, mechano-transducive mechanisms are responsible for the expression of VEGF during progression of the disease.

Acknowledgements

The authors gratefully acknowledge the experience and technical assistance of Johan van Lier and Frieda van der Ham in processing the immunohistochemical staining.

Figure 1

a. *Positive VEGF expression in vascular smooth muscle and endothelial cells of a small pulmonary artery in a patient with multiple VSD's (moderate PPA), biopsy taken at the time of pulmonary artery banding; magnification 20x.*
b. *Pronounced positive staining for VEGF in endothelial cells of a supernumerary artery in a patient with isolated ventricular septal defect, who had moderate PPA; magnification 20x.*
c. *No detectableVEGF-positivity in the medial layer of pulmonary arteries and supernumerary arteries in a control patient; magnification 20x.*
d. *Normal arteriole of comparable size to that shown in 1e in a control patient; magnification 40x.*
e. *Positive staining for VEGF in a lesion of concentric laminar intimal fibrosis in a patient with ventricular and atrial septal defect, who had advanced PPA; magnification 40x.*
f. *Distinct positivity for VEGF in the cell convolute of a plexiform lesion, immediately distal to the origin of a supernumerary artery in a patient with advanced idiopathic PPA. Note also positivity for VEGF in some of the surrounding pneumocytes and alveolar macrophages; magnification 20x.*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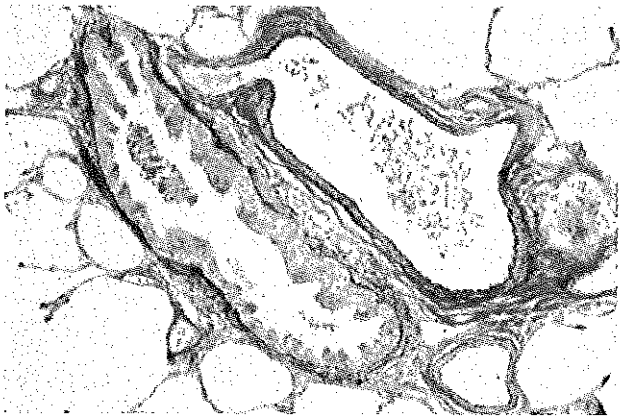
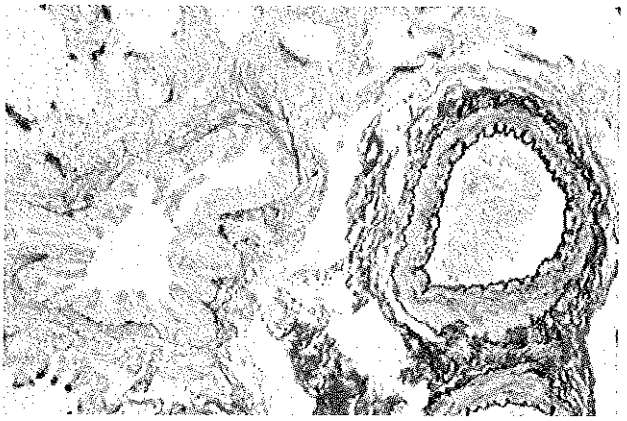
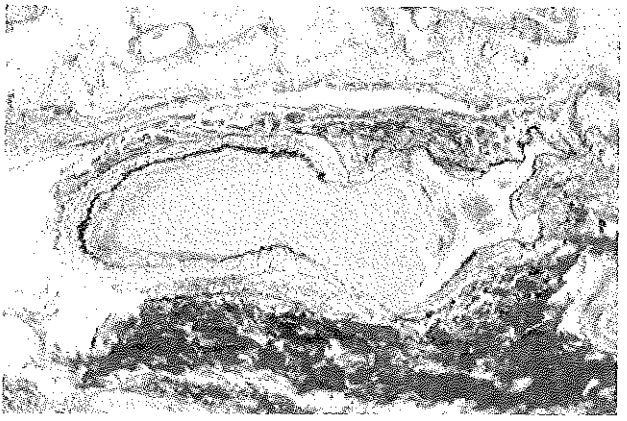


a. normal muscular pulmonary artery with a thin media, bounded by internal and external lamina elastica, accompanying a bronchus (Elastica-van Gieson stain, x 40).



b. medial hypertrophy of a muscular pulmonary artery in the presence of pulmonary hypertension (Elastica-van Gieson stain, x 40).



c. plexiform lesion at the origin of a supernumerary artery, branching from a muscular pulmonary artery. The distal part of the supernumerary artery is very dilated and thin-walled. Intimal fibrosis is present in the parent artery (Elastica-van Gieson, x 100).

Figure 2 (chapter 4) *Histologic evaluation of lung biopsy*

Immunohistochemical localization of inducible and endothelial Nitric Oxide Synthase in lung biopsies of children with congenital heart disease and pulmonary vascular disease.

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Abstract

The vascular lesions in advanced pulmonary plexogenic arteriopathy (PPA) are characterized by local abnormal vasodilatation, arterial wall injury and endothelial cell proliferation. The pathogenesis of these typical lesions is poorly understood.

The aim of the study was to investigate whether endothelial and inducible nitric oxide synthase may be involved in the development of these specific arteriopathy.

We investigated the expression of endothelial and inducible nitric oxide synthase by immunohistochemical analysis in lung biopsies of 29 patients with early and advanced plexogenic arteriopathy, 6 children with congestive vasculopathy and 4 patients with a normal pulmonary circulation. Both endothelial and inducible nitric oxide were weakly expressed in the endothelium of muscular pulmonary arteries in control patients. The level of expression of endothelial nitric oxide synthase did not differ significantly between the patient groups and was not correlated with histological vascular changes. In contrast, the immunoreactivity to inducible nitric oxide was increased in patients with pulmonary hypertension associated with increased pulmonary blood flow, compared to controls ($p = .012$) and to patients with increased pulmonary blood flow and normal pulmonary artery pressure ($p = .013$). The level of immunoreactivity to both isoforms of nitric oxide synthase was positively correlated with pulmonary pulse pressure and pulmonary blood flow. Marked immunoreactivity to both endothelial and inducible nitric oxide was observed in various cells forming the plexus in plexiform lesions.

Conclusions: The expression of inducible nitric oxide synthase appears to be increased in patients at risk for pulmonary plexogenic arteriopathy. Both endothelial and inducible nitric oxide synthase are markedly expressed in advanced lesions of PPA. These findings suggest that both isoforms of nitric oxide synthase are involved in the pathogenesis of pulmonary plexiform arteriopathy.

Introduction

Pulmonary hypertension leads to structural and functional changes of the pulmonary arteries, which, in turn, sustain or even aggrevate the pulmonary hypertension[1-3]. Structural changes include medial hypertrophy of the arterial wall and narrowing of the vascular lumen by intimal thickening, leading to an increase in pulmonary vascular resistance[4]. Pulmonary plexogenic arteriopathy (PPA) is a specific form of pulmonary vascular disease, in which histologically characteristic vascular alterations, such as dilatation lesions and plexiform lesions, may emerge[4,5]. PPA may develop in patients with congenital heart disease, where an increased pulmonary blood flow appears to be an obligatory factor and elevated pulmonary artery pressure seems to accelerate disease progression[6]. The mechanism through which these characteristic lesions develop is unknown. It has become clear that the endothelium plays an important role in the pulmonary vascular remodeling process[6]. The vascular endothelium, pre-eminently suited to detect changes in mechanical forces, such as cyclic strain and shear stress, produces substances with both vasodilator or vasoconstrictor properties[7,8]. Endothelial alterations early in pulmonary vascular disease, may cause an imbalance between vasodilatory and vasoconstrictive activity, resulting in pulmonary vasoconstriction and vascular cell proliferation[6,9-13]. Whether or not it plays a causative role in vascular remodeling, the endothelial dysfunction in itself may promote disease progression[14]. An important role in the maintenance of basal pulmonary vascular tone and in pulmonary hypertension is attributed to the endothelial-derived relaxing factor nitric oxide (NO), synthesized by the enzyme nitric oxide synthase (NOS) converting L-arginine to L-citrulline[6,15-18]. Experimental and clinical evidence exist that endothelium-dependent relaxation is impaired in pulmonary vascular disease, although data on the involvement of NOS appear to be controversial[19,20]. Giaid et al. showed that expression of the endothelial isoform of NOS (ecNOS) was decreased in patients with pulmonary hypertension and that it was absent in plexiform lesions[13], whereas other experimental studies showed the expression of ecNOS to be enhanced in pulmonary hypertension[21,22]. To date, three isoforms of NOS have been identified. The original classification in the endothelial and neuronal "constitutive" forms (ecNOS and nNOS) and an "inducible" form (iNOS) is misleading, because it has become

clear that gene expression of both ecNOS and nNOS is upregulated under different conditions (e.g. shear stress, nerve injury) and that, conversely, iNOS functions as a constitutive enzyme under physiologic conditions in a variety of cells, including vascular endothelial cells[23]. All three isoforms of NOS have been demonstrated in endothelial cells of the normal human pulmonary vasculature[13, 24, 25].
Advanced vascular lesions in plexogenic arteriopathy, such as dilatation lesions and plexiform lesions, are characterized by local abnormal vasodilatation and endothelial cell proliferation[26]. The effects of NO on blood vessels include vasodilation and stimulation of angiogenesis by migration and proliferation of endothelial cells[18, 27]. Therefore, we considered that NO might be involved in the abnormal local vasodilation and angiogenesis in advanced plexogenic arteriopathy. Inflammatory and immunologic stimuli, such as cytokines, are suggested to be involved in the pathogenesis of plexiform lesions. Since the same stimuli have been demonstrated to induce iNOS expression, leading to a more prolonged and higher output of NO than the other two isoforms, the inducible isoform of NOS is of special interest in this respect[14, 23, 26, 28]. Therefore, we investigated immunohistochemically the presence of iNOS and ecNOS protein in the pulmonary arteries of patients with congenital heart disease associated with pulmonary plexogenic arteriopathy and congestive vasculopathy and compared the findings with those in controls.

Patients and Methods

Patients

Thirty five patients with congenital heart disease, who underwent cardiac catheterization and had increased pulmonary blood flow (pulmonary-to-sytemic blood flow ratio > 1.2) and/or pulmonary hypertension (mean pulmonary artery pressure > 20 mmHg) and in whom subsequently a lung tissue specimen was obtained, were enrolled in the study, after written informed parental consent. Four patients with a normal pulmonary circulation, who underwent cardiac catheterization and cardiac surgery because of mild left sided obstructive lesions (PCW < 15 mmHg) served as controls. In all patients a complete hemodynamic evaluation was performed, including

measurements of pulmonary artery pressure, determination of shunt size by oximetry and calculation of pulmonary and systemic vascular resistance. Blood flow was determined using the dye dilution technique. In addition to the control subjects (group 1), 10 patients had a congenital heart defect associated with increased pulmonary blood flow and normal pulmonary artery pressure (group 2), 6 patients without intracardiac shunts had pulmonary hypertension due to pulmonary venous congestion (group 3). Eighteen patients had heart defects associated with both increased pulmonary blood flow and pulmonary hypertension (group 4). One patient with clinically unexplained pulmonary hypertension, showed histologically advanced PPA. We have chosen to assign this patient to group 4, because those were the patients at risk for advanced PPA.

Tissue specimens

Lung tissue was obtained as a biopsy during cardiac surgery (n = 36), as an open lung biopsy procedure for diagnostic purpose (n = 2) or at autopsy (n = 1). Tissue was formalin fixed under vacuum and paraffin embedded. Five μm sections were cut at various levels and stained with hematoxylin-eosin (HE) and Elastic-van Gieson (EvG) to allow for proper identification of morphological structures and precise correlation of morphology with patterns of immunoreactivity. Vascular changes were described according to Wagenvoort[2, 29].

Immunohistochemistry

The peroxidase-antiperoxidase (PAP) technique was used for staining ecNOS and the avidin-biotin complex (ABC) method was used for staining iNOS. Sections (5 μm) of paraffin-embedded lung tissues were deparaffinized in xylene, degraded in ethanol and rinsed in water. For the PAP method, sections were put in methanol/ hydrogen peroxide 3% for 20 minutes to quench of endogenous peroxidase. For antigen-retrieval sections were cooked in citric acid buffer at 100°C for 10 minutes. Slides were allowed to return to room temperature over several hours to minimize tissue destruction. Subsequently, sections were rinsed in 5% phosphate-buffered saline (PBS) and incubated with 10% normal rabbit serum (PAP) or normal goat serum (ABC) for 15 minutes to block nonspecific binding. The sections were then incubated overnight

with mouse monoclonal antibody either against ecNOS in dilution 1:40 or against iNOS in dilution 1:50. The antibody reacting with the endothelial NOS was raised against a 140 kDa immunogen, corresponding to amino acids 1030-1209 of the carboxy terminal reductase domain of human ecNOS and has specifity for human, rat and mouse ecNOS. The antibody reacting with the inducible NOS was raised against a 130 kDa immunogen of mouse iNOS, corresponding to amino acids 961-1144 of the carboxy terminal half, that has a specifity for human, dog, rat and mouse iNOS (Transduction Laboratories, Lexington, KY, USA). Both antibodies are monospecific for each their NOS isoform and showed no cross reactivity using Western blotting techniques. Specifity on paraffine material was tested by using aortic endothelium for ecNOS and activated macrophages in rheumathoid arthritis for iNOS as positive controls (data not shown). After rinsing once with PBS-tween 0.2%, and once with PBS 5% the sections were incubated for 30 minutes with rabbit-anti-mouse serum in a dilution of 1:25 (DAKO, Glostrup, Denmark), rinsed with PBS 5%, incubated with mouse PAP-complex 1:300 (Sigma Chemical CO, St. Louis, MO, USA) for another 30 minutes. Chromogen consisted of 3,3' diaminobenzidine tetrahydrochloride (DAB)/ hydrogenperoxide in PBS 5%. The chromogen reaction was allowed to take place over 7 minutes in the dark. Sections were rinsed with water, slightly counterstained with Mayer's hematoxylin and regraded to xylene by increasing alcohol concentrations. For iNOS detection sections were incubated with biotinylated anti-immunoglobulins (Multilink, Biogenex, San Ramon, CA, USA) in dilution 1:50 with 2% normal goat serum and 2% normal human serum for 30 minutes, followed by incubation with preformed avidin-biotin-alkaline phospatase complex (ABC, Biogenex, San Ramon, CA, USA) 1:50 for another 30 minutes. Chromogen consisted of Naphtol AS-MX-phosphate, New Fuchsin, Sodium Nitrite and Levamisole in tris-HCL at pH 8.0, which was added for 30 minutes. Sections were slightly counterstained with Mayer's hematoxiylin and mounted in an aqueous-base mounting medium. Negative controls consisted of replacement of the primary antibody by non-immunogen serum (Dako, Glostrup, Denmark).

Immunohistochemical analysis

Large and small muscular arteries, identified on the basis of accompanying airways,

were studied. The intensity of the immunostaining was graded semiquantitatively from 1 to 3, with 1 corresponding to the weak staining in controls and 3 representing strong staining. The grading was performed by two of the investigators, who were unaware of the clinical or hemodynamic data.

Statistical analysis

For comparison of continuous variables between groups analysis of variance (ANOVA) or Kruskal-Wallis tests were used, where appropriate. For comparison of categorical variables between groups, chi square tests were used. Correlation coefficients were calculated using linear regression technique. A p-value $< .05$ was considered significant. Values are given as means ± standard deviation, unless indicated otherwise.

Results

In total, 39 patients were enrolled in the study: Anatomical diagnoses of the congenital heart disease and the clinical and hemodynamic characteristics of the patients are shown in table 1 and 2. Lung tissue specimens, stained with HE and EvG revealed essentially normal pulmonary arteries in the control group and the complete spectrum of morphologic vascular changes, ranging from muscular hypertrophy and intima hyperplasia to the characteristic lesions of advanced plexogenic arteriopathy in the other patient groups. Histological characteristics of the patients are shown in table 2.

Endothelial nitric oxide synthase

Immunohistochemical analysis revealed weak staining for ecNOS in the pulmonary vascular endothelium in the control group. No differences in staining pattern were observed between muscular arteries of different sizes. Also in the patients, a positive staining of vascular endothelium was present in muscular arteries, both with or without morphologic changes. In the advanced lesions of plexogenic arteriopathy positive immunoreactivity was observed. In concentric laminar intimal fibrosis, positivity was seen in endothelial cells but not in the subintimal cells (fig 1). In plexiform lesions, distinct immunostaining for ecNOS was present in several cells forming the plexus

Table 1 *Clinical diagnoses of the study patients*

Diagnosis		group 1 (n)	group 2 (n)	group 3 (n)	group 4 (n)
VSD	isolated		7		7
	+ PDA				2
	+ ASD				3
cAVSD					4
PDA					1
Left pulmonary artery from aorta					1
ASD			3		
(sub)valv. AoS ± CoAo ± MS				6	
Unexplained PH					1
Controls		4			
Total		4	10	6	19

ASD indicates atrial septal defect; cAVSD, complete atrioventricular septal defect; CoAo, coarctation of the aorta; MS, mitral stenosis; PDA, persistent ductus arteriosus; PH, pulmonary hypertension; VSD, ventricular sepal defect.
Group 1: controls; **group 2**: patients with increased pulmonary blood flow and normal pulmonary artery pressure; **group 3**: patients with pulmonary hypertension not associated with increased pulmonary blood flow; **group 4**: patients with pulmonary hypertension associated with increased pulmonary blood flow, including one patient with unexplained plexogenic arteriopathy.

(fig 1). Immunoreactivity was not found in the cells lining the dilated angiomatoid vessels. The intensity of the immunoreaction in vascular endothelium, as graded semiquantitatively, was comparable to the control subjects in 26 patients. In 9 patients the level of immunostaining of the vascular endothelium of muscular arteries of all sizes, was increased. Semiquantitative grading of the immunoreaction did not correlate with the severity of the morphologic changes (fig 2). Seven of the 9 patients with

Table 2 *Characteristics of the study patients*

	controls normal Qp normal PAP	**group 2** increased Qp normal PAP	**group 3** normal Qp increased PAP	**group 4** increased Qp increased PAP
n	4	10	6	19
median age (years) (range)	3.3 (1.8-4.8)	6.6 (0.6-32.1)	2.4 (1.1-10.9)	0.7 (0.3-30.7)
Hemodynamics				
meanPAP (mmHg)	13 ± 2	13 ± 4	31 ± 12	41 ± 14
PCW (mmHg)	9 ± 2	7 ± 2	16 ± 4	10 ± 2
shunt size (Qp/Qs)	1.0 ± 0.0	2.3 ± 1.8	1.0 ± 0.0	3.1 ± 1.5
PVR (WU.m2)	0.9 ± 0.4	1.4 ± 0.8	5.0 ± 4.0	5.1 ± 3.8
Histology				
medial thickness (%)	9 ± 3	7 ± 3	12 ± 4	16 ± 5
patients with as most advanced lesions:				
intima proliferation	0	2	1	3
CLIF	0	0	0	1
advanced lesions	0	0	0	7
Immunohistochemistry				
endothelial NOS	1.0 ± 0.0	1.1 ± 0.3	1.2 ± 0.3	1.4 ± 0.5
inducible NOS	1.2 ± 0.5	1.6 ± 0.7	1.8 ± 1.0	2.4 ± 0.7

Advanced lesions indicate dilatation lesions and/or plexiform lesions; CLIF, concentric laminar intimal fibrosis; n, number of patients; NOS, nitric oxide synthase; PAP, pulmonary artery pressure; PCW, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance; Qp, pulmonary blood flow; Qs, systemic blood flow; WU, Woods' units. Increased Qp is defined as Qp/Qs > 1.2; increased PAP is defined as meanPAP > 20mmHg. Age is given as median and range.

increased intensity of staining were from group 4. Difference in staining intensity between the groups did not reach statistical significance ($p = 0.16$) (fig 3). Patients with an increased level of immunoreactivity had a larger pulmonary-to-systemic blood flow ratio (3.2 ± 1.3 vs. 2.0 ± 1.6; $p = 0.018$) and a larger pulmonary pulse pressure (40 ± 13 mmHg vs. 25 ± 14 mmHg; $p = 0.008$) compared to that in the other patients. No differences in mean pulmonary artery pressure or pulmonary vascular resistance could be demonstrated between patients with different staining level.

Inducible nitric oxide synthase

Immunohistochemical analysis revealed weak staining for iNOS in the pulmonary vascular endothelial cells in controls (fig 1). No differences in staining pattern were observed between muscular arteries of different sizes. Positive staining for iNOS was present in all patients. However, the intensity of the immunoreaction differed remarkably between patients (fig 1). Intense immunostaining was present in the endothelium of arteries affected by concentric laminar intimal fibrosis, as long as the vessel was not completely occluded (fig 1). Also in the cellular plexus of plexiform lesions a marked immunoreaction for iNOS was present (fig 1f). In addition, endothelial cells lining dilated, angiomatoid vessels showed iNOS positivity. Semiquantitative analysis of the immunohistochemical data revealed no significant correlation with the severity of morphologic vascular changes ($p = 0.25$) (fig 2). The immunoreactivity to iNOS differed significantly between the patient groups ($p = 0.012$) (fig 3). The level of immunostaining in patients with both increased pulmonary blood flow and pulmonary hypertension was higher than in controls ($p = 0.012$) and in patients with increased pulmonary blood flow and normal pulmonary artery pressure ($p = 0.013$). The level of immunostaining did not differ significantly between patients with pulmonary hypertension with or without increased pulmonary blood flow ($p = 0.19$). The level of immunostaining was higher in patients with pulmonary hypertension than in those with normal pulmonary artery pressure (2.2 ± 0.8 vs. 1.5 ± 0.7, $p = 0.006$). The level of immunoreactivity showed a positive correlation with the size of the left-to-right shunt ($r = 0.39$; $p = 0.019$) and the pulse pressure ($r = 0.35$; $p = 0.034$), but not with mean pulmonary artery pressure or pulmonary vascular resistance.

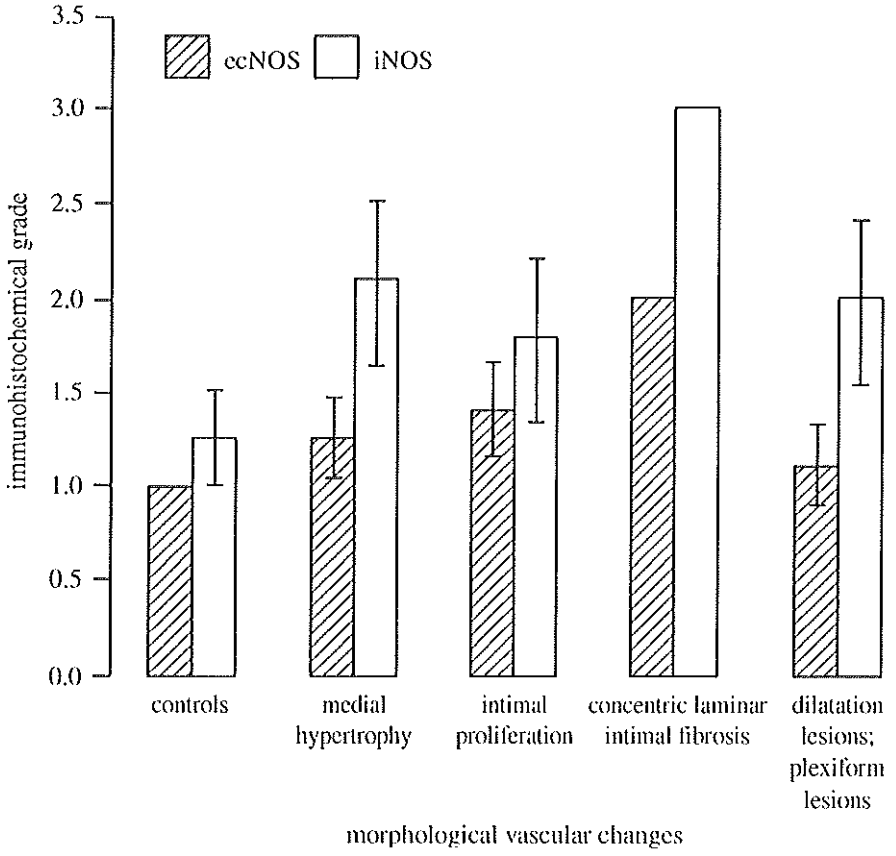
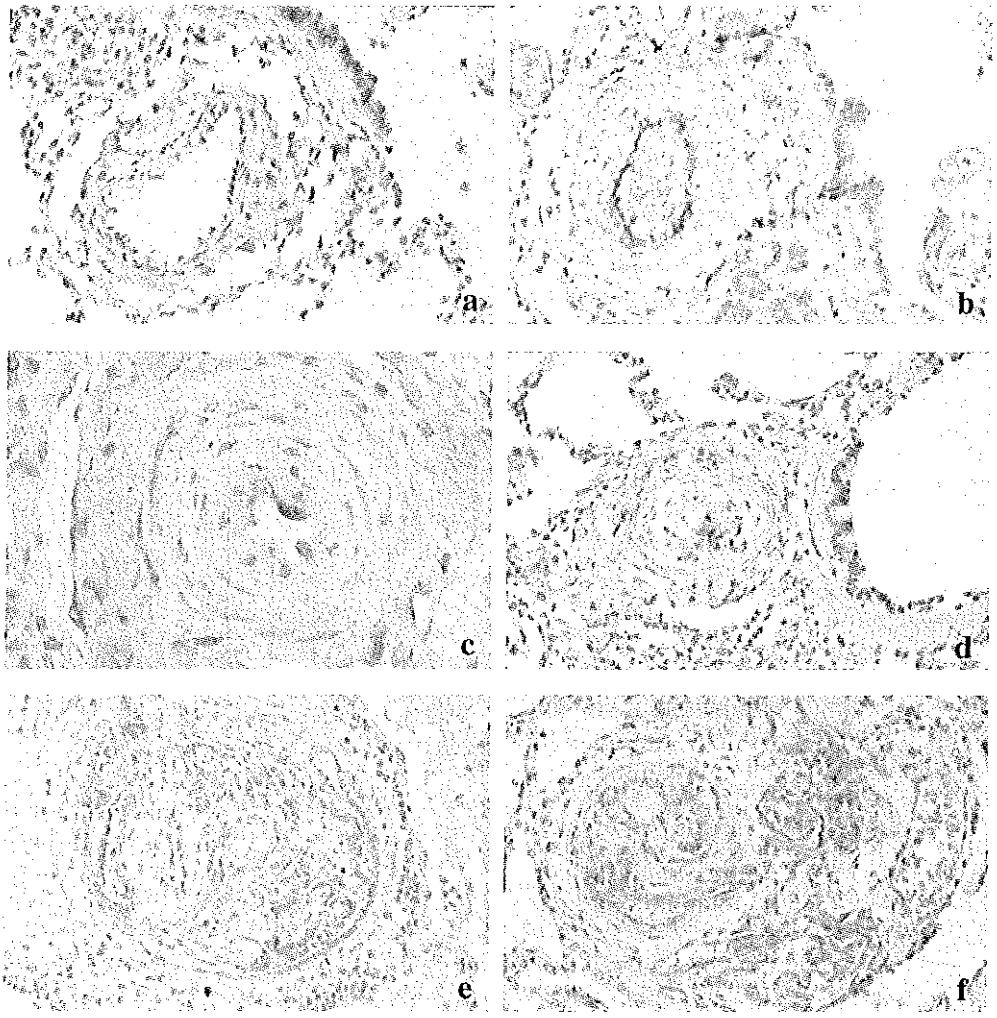


Figure 2 *Relation between the semiquantative grading of immunoreactivity to the endothelial respectively inducible isoform of nitric oxide synthase in muscular arteries and the morphologic vascular changes. No significant differences in immunoreactivity between the various vascular changes were demonstrated. Data are expressed as mean ± standard deviation.*

Discussion

We found that both the endothelial and inducible isoforms of nitric oxide synthase protein are expressed in the pulmonary vascular endothelium in children with a normal pulmonary circulation, as well as in patients with congenital heart disease



associated with increased pulmonary blood flow and/or pulmonary hypertension. In plexogenic arteriopathy, marked immunoreactivity for ecNOS and iNOS was identified in advanced vascular lesions, including concentric laminar intimal fibrosis, dilatation lesions and plexiform lesions. Semiquantitative analyses of the immunohistochemical data revealed that a higher intensity of the immunostaining for both isoforms was associated with an increased pulmonary arterial pulse pressure and an increased pulmonary blood flow. No correlation, however, was found with the severity of the

Figure 1 *Immunostaining for ecNOS (immunoperoxidase; brown) and iNOS (alkaline phosphatase; red) in lung biopsy sections of patients with different morphological vascular changes.*

a. weak immunostaining for iNOS in endothelial cells of a muscular artery in a control patient (x 40);
b. pronounced immunostaining for iNOS in the endothelium of an artery with medial hypertrophy in a patient with pulmonary hypertension and increased pulmonary blood flow (x 40);
c. immunostaining for ecNOS in endothelial cells of a pulmonary artery affected by severe concentric laminar intimal fibrosis (x 100);
d. distinct immunostaining for ecNOS in cells within a plexiform lesion (x 40);
e. immunostaining for iNOS in endothelial cells of a pulmonary artery affected by severe concentric laminar intimal fibrosis (x 40);
f. distinct immunostaining for iNOS in cells within a plexiform lesion (x 40).

histological vascular changes. The level of immunoreactivity for iNOS was significantly increased in patients with pulmonary hypertension compared to those without pulmonary hypertension and was most prominent in patients in which pulmonary hypertension was associated with increased pulmonary blood flow.

The finding of increased NOS protein in pulmonary endothelium in patients with pulmonary hypertension, seems in conflict with the impaired endothelium-dependent vasorelaxation that has been demonstrated in both clinical and experimental conditions of pulmonary hypertension[19, 20]. However, several mechanisms other than decreased NOS expression, including decreased respons to NO or dominant vasoconstrictive activity, have been suggested to be involved in this impairment[17, 30-33]. To date, it remains unclear whether impaired endothelium-dependent vasorelaxation in flow-related pulmonary hypertension is associated with decreased NO activity and, if so, at which level of the NO pathway this occurs. Studies on the expression of NOS in pulmonary arteriopathy have yielded conflicting results. In various experiments, for example, hypoxia has been shown to up regulate cNOS, whereas other experiments suggest down regulation of cNOS in similar conditions[21, 22, 34]. In contrast to the findings of Giaid et al., our data showed that the expression of ecNOS protein was not decreased in pulmonary vascular endothelium of children with congenital heart disease

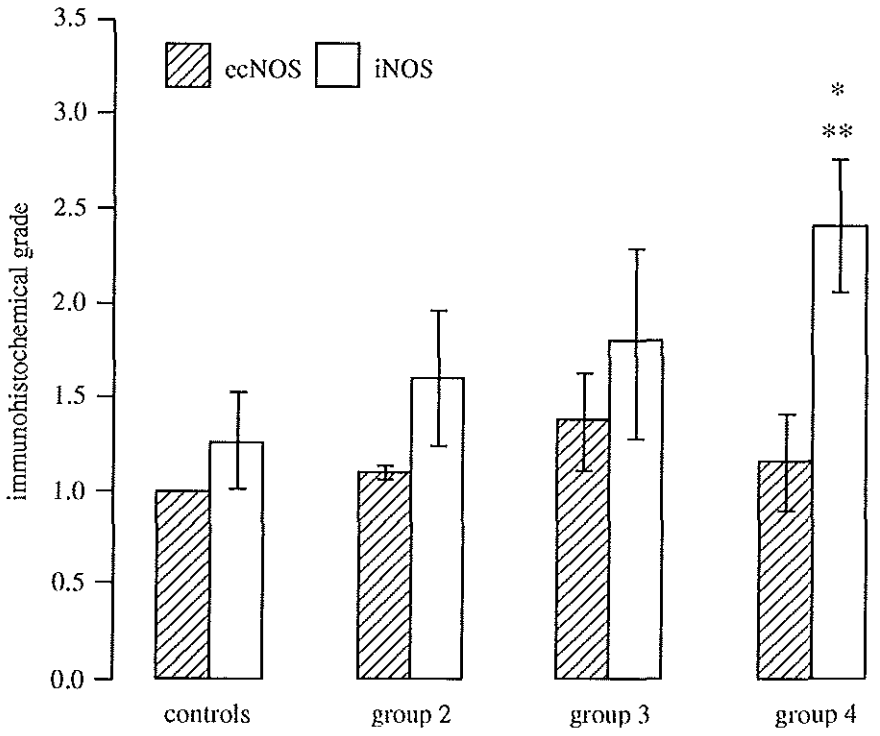


Figure 3. *The immunoreactivity to endothelial and inducible nitric oxide synthase, as graded semiquantitatively, in the different patient groups. Patients with pulmonary hypertension, associated with increased pulmonary blood flow (group 4), had significantly higher immunoreactivity than controls and than patients with increased pulmonary blood flow, but normal pulmonary artery pressure (group 2). * indicates p = .012 compared to controls; **, p = .013 compared to group 2. Data are expressed as mean ± standard deviation.*

and did not correlate with the severity of morphological vascular lesions. The expression of iNOS in pulmonary endothelial cells, to our knowledge not previously studied in patients with pulmonary hypertension, was increased in pulmonary hypertension. The observation that staining for both ecNOS and iNOS was present in pulmonary arteries with severe concentric laminar intimal fibrosis, until complete occlusion of the vessel, suggests prolonged NO production until late in plexogenic arteriopathy. Semiquantitative analysis suggested the expression of both isoforms of

NOS to be associated with pulmonary flow and pulse pressure. Although this is obviously no evidence for a causal relation, it is in concordance with experimental data showing pulmonary endothelial ecNOS expression to be upregulated by shear stress and cyclic strain[18, 35]. This is further supported by the presence of a "shear stress-responsive element", in both the human ecNOS and iNOS gene, similar to that found in the promotor of several shear stress inducible endothelial genes[36].

An important finding in our study is that iNOS expression was most prominent in patients in whom pulmonary hypertension was associated with increased pulmonary blood flow, which is exactly the subset of patients at risk for developing advanced PPA. Plexiform lesions and dilatation lesion are pathognomic for this type of pulmonary arteriopathy. The predilection place for these lesions is close to the origin of supernumerary arteries. These are small, additional arterial branches, which arise perpendicular or obliquely from elastic as well as from muscular arteries.

Morphologically, dilatation lesions develop as a circumscript dilatation of the muscular part of a supernumerary artery, close to its orifice of the parent artery, leading to extreme thinning of the wall. Proximal to this dilated segment there is destruction of the arterial wall, referred to as fibrinoid necrosis, with thinning of the media. In the plexiform lesion, a plexus of narrow slit-like vascular channels develops within the dilated lumen.The distal portion of the artery is very dilated and thin-walled[2]. The local wall injury has been assumed to be associated with increased exposition to mechanical stress at the origin of supernumerary arteries[2], whereas the morphological changes are regarded as the result of abnormal dilation and angiogenesis.

The known biological effects of NO, include vasodilation, inhibition of smooth muscle cell proliferation and stimulation of angiogenesis by migration and proliferation of endothelial cells[18, 27]. The constitutive forms of NOS synthesize small amounts of NO on demand. Stimuli, including shear stress and cyclic strain, lead to an increase in cytosolic calcium, which activates the NOS to give of a short burst of NO[18]. In contrast, iNOS is functionally calcium independent and releases large amounts of NO continuously[18]. The large amounts of NO, that are characteristically produced by iNOS, may lead to oxidative injury[18, 25]. Although this may be of benefit in infection-control reactions, in the vascular system increased iNOS expression has been associated with the development of vasculitis[37, 38].

Our findings of increased iNOS expression in patients at risk for the development of fibrinoid necrosis, dilatation lesions and plexiform lesions, as well as our findings of marked expression of ecNOS and iNOS in these specific lesions, are suggestive for the involvement of iNOS in the development of arterial wall injury occurring in PPA and for the involvement of both ecNOS and iNOS in the pathogenesis of dilatation lesions and plexiform lesions.
The expression of iNOS is known to be induced by inflammatory and immulogic stimuli. In this respect, the findings of Tuder et al, who demonstrated perivascular T- and B-cells and macrophages in plexogenic arteriopathy, infiltrating plexiform lesions, and those of Humbert et al., who found elevated serum levels of cytokines in patients with PPH, are of special interest[26, 28, 39].

Limitations of the study
The use of immunohistochemistry rather allows conclusions on the presence of the protein under study than on its activity or the actual protein production. An advantage of the immunohistochemistry technique, important to this study, is that it demonstrates directly the microanatomical localization of the protein under study. Our data does not allow conclusions on a causal or accompanying nature of the associations found in our study. Semiquantitative analysis of immunohistochemical data cannot be more than an estimation of relative differences in staining intensity, and may be hampered by numerous confounding factors. However, the influence of these factors was minimized by standardized processing of biopsy material, staining the entire series of sections simultaneously and relate staining intensity to a standard staining.

In conclusion, the present study demonstrates characteristic patterns of the expression of ecNOS and iNOS in the pulmonary vascular remodeling process in human pulmonary hypertension. The increased presence of iNOS in the pulmonary vascular endothelium of patients at risk for PPA and the positive correlation of this increased presence with pulmonary blood flow and pulse pressure, suggests that iNOS is involved in the pulmonary vascular remodeling process in this characteristic disease. By showing the presence of both ecNOS and iNOS in dilatation lesions and plexiform lesions the present study emphasizes the potential importance of these enzymes in the development of the advanced vascular lesions of plexogenic arteriopathy.

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General discussion

In the described studies we investigated biomechanical and molecular aspects of pulmonary vascular remodeling in children with congenital heart disease, in order to increase insight in the pathophysiology of this still unraveled process. Pulmonary vascular disease forms an ongoing threat for children with congenital heart disease and pulmonary hypertension or increased pulmonary blood flow. The pulmonary vascular remodeling process in plexogenic arteriopathy may progress to irreversibility and thereby precludes curative therapy of both heart and vascular disease[1]. On the other hand, early stages of pulmonary arteriopathy, associated with increased contractile vascular responses, thickening and stiffening of the pulmonary arteries, may seriously complicate clinical course and corrective surgery[2, 3]. A new challenge in the current era is formed by the surgical procedures in the management of univentricular hearts, such as Norwood- and Fontan procedures, that are performed in a rapidly increasing frequency and require higher demands on the evaluation and understanding of the pulmonary vasculature. In these procedures, in which no ventricular force faces the pulmonary vasculature, already early stages of pulmonary vascular disease may jeopardize surgical outcome and prognosis.

In the past 40 years, the clinical assessment of pulmonary vascular disease in children with congenital heart disease has been refined by hemodynamic criteria, lung biopsy, including morphometry, and wedge angiography[4]. Pathological studies have revealed insights in the sequence of morphologic changes of the pulmonary vasculature in development and disease[5-7]. However, it became clear that comprehension of the pathophysiology of pulmonary vascular disease, would require fundamental studies of the processes of cellular and molecular dysregulation.

In the recent years, basic research in vascular biology has rapidly expanded our knowledge on an impressive list of cells, modulators, mediators, growth factors, molecules and mechanisms that may be involved in vascular remodeling (for reviews see[8-11]).

Pulmonary vascular remodeling in pulmonary hypertension includes a series of switches in vascular smooth muscle cell phenotype. Differentiation from precursor cells to VSMC's causes muscularisation of normally non-muscular peripheral arteries.

Hypertrophy and hyperplasia of vascular smooth muscle cells and extracellular matrix modulation lead to thickening of the arterial media, already early in pulmonary vascular disease[4, 10], whereas migration of VSMC's into the subendothelial space is the basis of intimal proliferation. The modulation of the extracellular matrix includes increased deposition of glycoproteins and connective tissue, such as collagen. These changes in the arterial wall, combined with an increased vasoconstrictive respons, will lead to a stiffening of the wall and, thus, loss of arterial compliance[10, 12, 13]. Although predominantly in the small muscular arteries, these changes also occur in elastic arteries[14, 15]. One of the major characteristics of the pulmonary vasculature is its low arterial compliance, crucial for the unique property of the pulmonary circulation to be a low resistance, low pressure and high flow circulation. Since the normal postnatal pulmonary remodeling is disturbed in congenital heart defects and since the proliferative and matrix-producing potential of the neonatal pulmonary circulation in respons to stress is high compared to adults, arterial wall compliance may progressively decrease already early in the course of vascular disease[10, 16]. The physiologic and clinical consequences of these pathologic vascular changes for the integrated pulmonary circulation and ventricular-vascular coupling are not well understood. Our studies have shown that intravascular ultrasound of the pulmonary arteries provides a new and direct measure for arterial wall distensibility, that is correlated with altered functional wall properties early in pulmonary vascular disease and with structural properties of the vascular bed, both independent from hemodynamic parameters. Although the predictive value of this new variable in the individual patient, with respect to progression and consequences of the disease, remains to be determined, the opportunity to measure directly intrinsic arterial wall properties *in vivo*, creates various possibilities. In an experimental setting, it allows the investigation of the functional consequences of different aspects of vascular remodeling and the effects of intervention. In the clinical setting it may allow a more functional assessment of the pulmonary vasculature early in PVD, which is of special interest in the context of univentricular heart repairs. Finally, direct assessment of pulmonary vascular dynamics and functional wall properties will increase our insight in the vascular and physiological consequences of the so-called Fontan circulation, in which the pulsatile pulmonary flow has been converted to a nearly continuous flow.

Endothelial dysfunction has been recognized to play a vital role in pulmonary hypertension. The pulmonary vascular endothelium is a metabolically active and dynamic organ with many functions, including biosynthesis and metabolism of circulating hormones and vasoactive substances, modulation in vascular tone, regulation of hemostasis and thrombosis, and participation in inflammation and local vessel wall function[17]. The vascular endothelial cells, lining the inner pulmonary arterial wall, are pre-eminently suited to signal a variety of stimuli, including changes in mechanical forces such as shear stress and cyclic strain, and to respond by producing vasoactive substances, cytokines and growth factors. This has been suggested to initiate a cascade of cellular and humoral interactions and responses, leading to vasoconstriction and cellular proliferation and migration. These processes are being studied intensively in a variety of models, both animal or isolated cell systems, of pulmonary vascular disease, which represent predominantly hypoxia or monocrotaline induced pulmonary hypertension. Pulmonary plexogenic arteriopathy, however, cannot be studied in these models. Although the early stages of PPA may show striking similarities with the mentioned arteriopathies, and even may share a common pathway, the characteristic advanced lesions do not occur in any of the experimental models that are currently in use. Due to this restriction, knowledge on the pathogenesis of this specific entity is scarce. What factors, mechanical, chemical or humoral, are involved in the development of the pathognomic lesions of PPA and at what point in the time course of the disease, does PPA distinghuish itself from other pulmonary arteriopathies? To date, these questions cannot be answered.

The purpose of our immunohistochemical studies was to find clues on what humoral factors might be involved in the development of the characteristic lesions of PPA. Because of their known biological activities, both VEGF and NO were, in our opinion, canditates in this respect. Advanced vascular lesions in plexogenic arteriopathy, such as dilatation lesions and plexiform lesions, are characterized by local abnormal vasodilatation and angiogenesis. Endothelial cell proliferation has been demonstrated to be an important feature of plexiform lesions[18, 19]. VEGF is known to be a direct angiogenic factor, that promotes endothelial cell proliferation[20]. Further, it has been demonstrated to play a role in the embryonic development of the pulmonary vasculature and different types of pathological angiogenesis[20, 21]. Our finding that a pronounced

immunoreactivity to VEGF protein was present in the characteristic lesions and the finding that VEGF was expressed more frequently in pulmonary VSMC's and endothelial cells of patients with advanced PPA compared to patients with reversible arteriopathies, strongly support a role for VEGF in the development of advanced PPA.

In addition to vasodilation and inhibition of smooth muscle cell proliferation, the properties of NO include stimulation of angiogenesis by migration and proliferation of endothelial cells[22, 23]. Our hypothesis holded that increased NO release may induce dilatation lesions and plexiform lesions. Indeed, we found a marked immunoreaction to both ecNOS and iNOS localized in cells forming the plexus in these lesion. Further, semiquantitative analysis suggested that iNOS expression was increased predominantly in patients with both increased pulmonary blood flow and pulmonary hypertension, which is exactly the subset of patients at risk for advanced PPA. Oxidative injury may play a role in the mechanism of fibrinoid necrosis, a typical form of arterial wall injury that occurs at the origin of supernumerary arteries, the predilection place for the plexiform lesion.

Obviously, the presence of protein, as demonstrated with immunohistochemistry, is not synonymous with biological activity of the protein. Neither do these findings allow conclusions on a causal or accompanying relationship between the presence of these proteins and the development of vascular lesions. As described earlier, the process of vascular remodeling involves a myriad of factors and interactions and it would be ingenuous to suggest that one or two factors were responsible for the characteristic morphological and clinical patterns of PPA. On the other hand, the presence and the specific micro-anatomical localisation of the protein under study can be reliable demonstrated with immunohistochemical techniques. And, as history has proven many times, the key to resolve apparently inconceivable phenomena rests in starting with close observation of the event. Our observations direct, as far as we know for the first time, at two humoral factors that may be involved specifically in the development of advanced PPA. These should form the starting point for further research on the exact functional role of VEGF and both isoforms of NOS in this context. This, however, will require longitudinal and interventional studies, which for obvious reasons are not possible in human plexogenic arteriopathy and will also not be possible in the

commonly used experimental models of pulmonary hypertension. To unravel the pathogenetic mechanisms of pulmonary plexogenic arteriopathy, an experimental model of this characteristic entity is mandatory. In our opinion, a major effort has to be made to develop a model of flow- and pressure related pulmonary plexogenic arteriopathy, leading to the characteristic lesions of this mysterious disease. Such a model would allow functional studies, time-related studies and interventional studies.

The comprehension of the specific activities of VEGF and iNOS as well as those of many other factors, would help to understand the pathophysiology of pulmonary vascular remodeling and would provide opportunities for novel therapeutic strategies, directed at patients with secondary, as well as unexplained pulmonary hypertension[11, 24].

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Summary

Children with congenital heart disease and increased pulmonary blood flow, with or without pulmonary hypertension are subject to pulmonary vascular disease, called plexogenic arteriopathy. This disease constitutes a pulmonary vascular remodeling process, in which functional and structural changes of the pulmonary vasculature will lead to an increase in pulmonary vascular resistance and, thus, in workload of the right ventricle. In pulmonary plexogenic arteriopathy, if untreated, the vascular remodeling proces may become irreversible, especially when a combination of high pulmonary blood flow and high pressure is present. Pulmonary vascular disease (PVD) is an important cause of morbidity and mortality in children with congenital heart disease. The assessment of the progression and the functional consequences of PVD is rough and hampered by the lack of knowledge on the pathogenesis and pathophysiology of the process.

In the described studies, biomechanical and molecular aspects of pulmonary vascular remodeling in children with congenital heart disease were investigated in order to increase insight in the pathophysiology of this process and its consequences for the pulmonary circulation.

To understand the mechanisms used by the pulmonary vasculature to adapt to pathological conditions, it is of eminent importance to have insight into the normal pulmonary developmental changes in utero, at birth and during the postnatal period. The pulmonary vasculature has a unique position in the human circulation and is capable of enormous structural and functional adaptations, for instance during intrauterine life and in the transition period. In *chapter 2*, a concise state of the art is given on pulmonary vascular anatomy, physiologic events and humoral and cellular mechanisms in the vasculature during these characteristic time periods. Subsequently, the same aspects are discussed in relation to PVD. One should acknowledge that the pathophysiology of plexogenic arteriopathy is practically unknown.

This lack of knowledge, hampers the clinical assessment of the progression and functional consequences of the disease process. The study in *chapter 3* compares the results of hemodynamic and histologic evaluation of the pulmonary vasculature in a selected group of patients with complex congenital heart disease, and studied the

relationship with clinical outcome. Discrepancies in the results of both techniques occurred especially in patients with unfavourable hemodynamic characteristics, who frequently showed morphological vascular changes in their lung biopsy indicating only early stages of PVD. Preoperative hemodynamic data did not allow prediction of perioperative pulmonary hypertensive crises. In the 15 surviving patients both techniques did not accurately predict persisting pulmonary hypertension after surgical correction of the heart defect at a mean follow up of 6 years. This study illustrates the limitations of hemodynamic and histological evaluation of the pulmonary vascular bed and indicates the urge to develop additional diagnostic tools. In *chapter 4*, the currently available diagnostic techniques are discussed. All techniques have their indisputable value, but they also have their practical and conceptual limitations. The functional consequences of pulmonary vascular disease for the integrated pulmonary circulation and, thus, for the work load of the heart can be evaluated only partly. Aspects of the integrated pulmonary circulation include the pulmonary vascular bed with its specific functional and structural properties, and also the vascular capacitance function, the reflection of pulse waves and the resistance to inertia. Pulmonary vascular disease affects all these aspects.

The study in *chapter 5* demonstrates the feasibility of pulmonary intravascular ultrasound imaging in infants and children and the unique potential of the technique to study simultaneously the appearance of the pulmonary arterial wall and its dynamics *in vivo*. The specific potential advantages of intravascular ultrasound in assessing pulmonary vascular disease are discussed.

Chapter 6 describes the study that investigated the *in vivo* appearance of the pulmonary arterial wall, as assessed by pulmonary intravascular ultrasound, in 47 children with congenital heart disease and different stages of pulmonary vasculopathy and in 14 children with a normal pulmonary circulation. The findings were compared with hemodynamic and histological evaluation. A normal ultrasound aspect of pulmonary arteries was defined. IVUS was able to detect differences in arterial wall appearance between controls, patients with mild-to-moderate PVD and advanced PVD. The normal aspect was found less frequently in patients with PVD, whereas an inner layer of soft echo reflections was found more frequently in patients with moderate PVD. The thickness of this specific layer correlated with pulmonary blood flow and pulmonary

artery pressure and, interestingly, with the medial thickness of muscular arteries in the lung biopsy. When adjacent structures allowed the outer border of the arterial wall to be defined, wall thickness could be measured and correlated with pulmonary artery pressure and pulmonary vascular resistance. The echographic changes of the arterial wall did not correlate with the presence of advanced lesions in lung biopsy.
In nearly the same patient groups, the effects of pulmonary vascular remodeling on vascular wall dynamics, assessed *in vivo* with IVUS, were studied. In the *chapters 7 and 8* the dynamic variable pulmonary arterial wall distensibility is introduced. Associations between this new pulmonary variable and functional aspects of the pulmonary vasculature, as determined by hemodynamic evaluation are studied and described in *chapter 7*. Pulmonary arterial wall distensibility was progressively decreased in pulmonary vascular disease, and multivariate analysis suggests that this decrease is, in part, related to an increased distending pressure as a result of pulmonary hypertension and, in part, to a stiffening of the arterial wall in the disease process.
On the other hand, associations between arterial wall distensibility and structural aspects of the pulmonary vasculature, as determined by pulmonary wedge angiography and lung biopsy are described in *chapter 8*. The decrease in arterial wall distensibility, found in patients with pulmonary vascular disease, was correlated with structural changes at the level of the pulmonary vascular bed. These correlations were independent from pulmonary-to-systemic arterial pressure ratio, as a measure for arterial distending pressure. Moreover, taking account of the distending pressure and the angiographic and histological parameters, arterial wall distensibility appeared to be still decreased in patients with pulmonary vascular disease.
Chapters 9 and 10 describe two of the studies in our search for specific cellular events and biologic modulators that are involved in the emergence of the advanced lesions of plexogenic arteriopathy, and that thus must be characteristic for this typical disease. Because of its specific biological properties, we thought vascular endothelial growth factor (VEGF) a candidate to be involved in the development of plexiform lesions.
In *chapter 9* we report on the pulmonary arterial expression of VEGF protein, assessed with immunohistochemistry, in lung biopsy tissue of patients with different types of PVD and controls. Vascular immunoreactivity to VEGF was detected in 13 out of 34 cases and was localized in pulmonary arterial smooth muscle and endothelial cells.

Immunoreactivity was present more frequently in patients with advanced pulmonary plexogenic arteriopathy (PPA) compared to controls and patients with reversible pulmonary vascular disease. Moreover, marked immunoreactivity for VEGF was detected in the characteristic lesions of PPA. Although the presence of VEGF does not allow conclusions on causal relationships, these data suggest an active role for VEGF in the pathogenesis of the characteristic lesions of PPA.
In *chapter 10*, data are presented on the endothelial expression of the endothelial constitutive and the inducible isoforms of nitric oxide synthase (ecNOS and iNOS resp.) in lung biopsy tissue of patients with different types of PVD and controls. Because of the various biological activities of NO, local overproduction of this molecule could explain specific features of the characteristic dilatation lesions, fibrinoid necrosis and plexiform lesions. We found the immunoreactivity to ecNOS, compared to controls, unchanged in children with congenital heart disease and pulmonary hypertension, independent of the type of arteriopathy and independent of the severity of morphological vascular lesions. Semiquantitative analysis of the immunohistochemical data suggested the amount of ecNOS to be associated with pulmonary flow and pulse pressure. The level of immunoreactivity to iNOS was increased in patients with compared to those without pulmonary hypertension, and correlated with pulmonary blood flow and pulmonary pulse pressure. Moreover, expression appeared to be highest in patients with the combination of increased pulmonary blood flow and pulmonary hypertension. Marked immunoreactivity to both ecNOS and iNOS protein was present in the endothelium in dilatation lesions and in a subset of endothelial cells in plexiform lesions. These data suggest a pulmonary arterial upregulation of iNOS in children with congenital heart disease and pulmonary hypertension and a local role for NO in the pathogenesis of advanced lesions of PPA.

The studies in chapters 6, 7 and 8 demonstrate that the pulmonary vascular remodeling process in children with congenital heart disease not only affects the pulmonary vascular bed, but also the larger elastic arteries. It further demonstrate that this remodeling is associated with changes in arterial wall properties of the elastic arteries and does lead to functional consequences, such as an increase in the pulsatile load of the right heart. These vascular changes can be assessed *in vivo* with pulmonary

intravascular ultrasound during a cardiac catheterization procedure. Qualitative aspects of the pulmonary arterial wall and arterial wall distensibility both provide additional information on the pulmonary vasculature and allow the investigation of the functional consequences of different aspects of vascular remodeling and the effects of intervention. Long-term outcome analyses will be needed to determine if these variables are of value in the clinical evaluation of pulmonary vascular disease.

The studies in chapters 9 and 10 identify three proteins, with potentially very strong biological activities, that show characteristic expression patterns in the course of pulmonary plexogenic arteriopathy, suggestive for a role in the pathogenesis of the advanced lesions in this arteriopathy. Further research will be needed to find out if these proteins really represent increased activity and have a causal effect in pathogenesis.

In order to answer these and many more questions, in our opinion, a strong effort has to be made to develop an adequate animal model of flow- and pressure-related pulmonary plexogenic arteriopathy. Basic studies of mechanisms to altered vessel growth and remodeling will lead to further therapeutic strategies for pulmonary vascular disease.

Samenvatting

Kinderen met een aangeboren hartafwijking, welke gepaard gaat met een toegenomen longdoorstroming, al dan niet met een verhoogde druk in de longvaten, ontwikkelen een pulmonale vaatziekte. Deze vaatziekte wordt pulmonale plexogene arteriopathie (PPA) genoemd en bestaat uit een proces van vaatwand remodellering. Functionele en structurele vaatafwijkingen leiden hierbij uiteindelijk tot een verhoging van de longvaatweerstand en dus tot een toegenomen belasting van het hart. Indien geen behandeling plaatsvindt, kan dit ziekteproces op een bepaald moment onomkeerbaar worden. Dit gebeurt met name wanneer de toegenomen longdoorstroming gepaard gaat met een verhoogde druk in de longvaten. Pulmonale vaatziekte vormt een belangrijke oorzaak van morbiditeit en mortaliteit bij kinderen met aangeboren hartafwijkingen.

De progressie van deze vaatziekte kan slechts grofweg vastgesteld worden met de huidige diagnostische technieken. Dit wordt mede veroorzaakt door een gebrek aan inzicht in de pathogenese en pathofysiologie van het ziekteproces.

De studies, beschreven in dit proefschrift, bestuderen biomechanische en moleculaire aspecten van pulmonale vaatwand remodellering bij kinderen met aangeboren hartafwijkingen, met als doel het inzicht te vergroten in de pathofysiologie van dit proces en de consequenties voor de pulmonale circulatie.

Om de mechanismen te begrijpen, die een rol spelen bij de aanpassing van de pulmonale vasculatuur aan pathologische omstandigheden, is het van groot belang om inzicht te hebben in de veranderingen die optreden in de longvaten tijdens de normale ontwikkeling in utero, tijdens de geboorte en tijdens de postnatale periode. De pulmonale vasculatuur bezet een unieke plaats in de menselijke circulatie en is in staat tot indrukwekkende structurele en functionele aanpassingen, bijvoorbeeld tijdens de intrauterine ontwikkeling en tijdens de transitie periode. In *hoofdstuk 2* wordt de huidige stand van zaken samengevat betreffende de kennis over de anatomische en fysiologische gebeurtenissen en de humorale en cellulaire regulatie van de pulmonale vasculatuur tijdens deze kenmerkende perioden. Vervolgens worden diezelfde aspecten beschreven in relatie tot pulmonale vaatziekte. Men dient zich te realiseren dat de pathofysiologie van PPA vrijwel onopgehelderd is.

Dit gebrek aan inzicht bemoeilijkt in de kliniek het vaststellen van zowel de progressie als de functionele gevolgen van het ziekteproces. In *hoofdstuk 3* worden de resultaten vergeleken van hemodynamische en histologische evaluatie van het longvaatbed in een geselecteerde groep patiënten met complexe hartafwijkingen. Tevens wordt de relatie van deze resultaten met het klinisch beloop bestudeerd. Er bleek sprake van discrepanties tussen de resultaten van beide technieken, met name bij patiënten met ongunstige hemodynamische kenmerken, bij wie de histologie frequent vaatafwijkingen liet zien passend bij nog vroege stadia van de vaatziekte. Pre-operatieve hemodynamische data bleken geen voorspelling toe te laten over het optreden van postoperatief optredende, zogenaamde pulmonale hypertensieve crisen. Bij de 15 overlevende patiënten in dit onderzoek, bleken beide diagnostische technieken, bij een gemiddelde follow-up duur van 6 jaar, niet in staat het persisteren van pulmonale hypertensie, na chirurgische correctie van de hartafwijking, accuraat te voorspellen. Deze studie illustreert de beperkingen van hemodynamische en histologische evaluatie van het longvaatbed en benadrukt de behoefte aan aanvullende diagnostische mogelijkheden.

In *hoofdstuk 4* worden de diagnostische technieken besproken, welke tegenwoordig in gebruik zijn om de pulmonale vasculatuur te beoordelen. Al deze technieken hebben onbetwist hun waarde, echter allen hebben zij ook hun praktische en conceptuele beperkingen. De functionele gevolgen van pulmonale vaatziekte voor de geïntegreerde pulmonale circulatie, en dus voor de werkbelasting van het hart, kunnen slechts gedeeltelijk geëvalueerd worden De geïntegreerde pulmonale circulatie bevat namelijk niet alleen het longvaatvaatbed met zijn specifieke functionele en structurele eigenschappen, maar ook de vaatwand compliantie, de reflectie van polsgolven en de weerstand tegen inertie. Pulmonale vaatziekte beïnvloedt al deze aspecten.

De studie in *hoofdstuk 5* toont de uitvoerbaarheid aan van intravasculaire echografie in de longvaten van kinderen en zuigelingen en, bovendien, de unieke mogelijkheid van deze techniek om tegelijkertijd zowel het aspect als de dynamiek van de pulmonale vaatwand *in vivo* te bestuderen. De specifieke, potentiële voordelen van intravasculaire echografie in de beoordeling van pulmonale vaatziekte worden besproken.

Hoofdstuk 6 beschrijft het aspect van de pulmonale vaatwand *in vivo*, beoordeeld met intravasculaire echografie, bij 47 kinderen met een aangeboren hartafwijking en ver-

schillende stadia van pulmonale vaatziekte, en in 14 kinderen met een normale pulmonale circulatie. De bevindingen werden vergeleken met hemodynamische en histologische evaluatie. Het normale echografische aspect van een longslagader werd gedefinieerd. Met behulp van intravasculaire echografie was het mogelijk om verschillen in het aspect van de vaatwand te detecteren tussen controle kinderen, kinderen met milde tot matige pulmonale vaatziekte en kinderen met ernstige vaatziekte. Het normale patroon kwam minder vaak voor bij de kinderen met pulmonale vaatziekte, terwijl bij de kinderen met milde tot matige pulmonale vaatziekte aan lumen zijde, een band van zachte echoreflecties werd waargenomen. De dikte van deze band correleerde met de bloedstroom door de longen, de druk in de longvaten en met de mediadikte van musculaire arteriën in de long biopsie. Wanneer de buitengrens van de vaatwand goed herkend kon worden, als gevolg van aangrenzende structuren, kon de totale vaatwand-dikte gemeten worden. Deze correleerde eveneens met de druk in de longvaten, maar ook met de longvaatweerstand. De veranderingen in het echografisch aspect van de vaatwand correleerden niet met de aanwezigheid van gevorderde vaatafwijkingen in de long biopsie.

In de, voor een belangrijk deel, zelfde patiënten groep, werd het effect van het remodellerings proces op de dynamiek van de vaatwand bestudeerd met behulp van intravasculaire echografie. In de *hoofdstukken 7 en 8* wordt de nieuwe variabele "distensibiliteit van de longslagader" geïntroduceerd.

De associatie tussen deze nieuwe variabele en de functionele aspecten van de pulmonale vasculatuur, bepaald door middel van hemodynamische evaluatie, wordt beschreven in *hoofdstuk 7*. De arteriële distensibiliteit was progressief verlaagd bij pulmonale vaatziekte. De resultaten van multvariate analyse suggereren dat dit deels samenhangt met een toegenomen rek van de vaatwand door verhoogde druk in de longvaten, en deels met het stugger worden van de vaatwand zelf tijdens het ziekte proces.

In *hoofdstuk 8* worden de associaties bestudeerd tussen arteriële distensibiliteit en structurele aspecten van de pulmonale vasculatuur, vastgesteld door middel van pulmonale wedge angiografie en long biopsie. De verminderde arteriële distensibiliteit in patiënten met pulmonale vaatziekte bleek gecorreleerd met structurele vaatafwijkingen op het niveau van het vaatbed. Deze correlaties waren onafhankelijk van de druk in de longvaten. Bovendien bleek dat wanneer behalve met de druk in de

longvaten, ook rekening werd gehouden met de angiografische en histologische variabelen, de arteriële distensibiliteit nog steeds verlaagd was in kinderen met pulmonale vaatziekte.

De *hoofdstukken 9 en 10* beschrijven twee van de studies in onze zoektocht naar specifieke cellulaire gebeurtenissen en biologisch actieve stoffen, die betrokken zijn bij de ontwikkeling van de karakteristieke vaatafwijkingen van PPA en die dus kenmerkend moeten zijn voor deze typische ziekte.

Op grond van zijn specifieke biologische eigenschappen, was ons inziens "vascular endothelial growth factor"(VEGF) een potentiele kandidaat om betrokken te zijn bij het ontstaan van een zogenaamde plexiforme laesie. In *hoofdstuk 9* wordt de arteriële expressie beschreven van het VEGF eiwit, bepaald met immunohistochemische technieken, in long biopsieën van patiënten met verschillende typen pulmonale vaatziekte en van controle patiënten. Immunoreactiviteit gericht tegen VEGF werd gevonden in 13 van de 34 patiënten en was gelokaliseerd in de arteriële gladde spiercellen en in de endotheelcellen. Immunoreactiviteit werd vaker gevonden bij patiënten met gevorderde, onomkeerbare PPA dan in patiënten met een normale pulmonale circulatie en patiënten met wel omkeerbare vormen van pulmonale vaatziekte. Bovendien werd een duidelijke immunoreactiviteit tegen VEGF aangetoond in de karakteristieke vaatafwijkingen van PPA. Hoewel de aanwezigheid van VEGF geen conclusies toelaat over een oorzakelijke relatie, suggereren deze bevindingen een actieve rol voor VEGF in de pathogenese van de karakteristieke vaatafwijkingen van PPA.

In *hoofdstuk 10* wordt de arteriële expressie van de "endothelial constitutive" en de "inducible" vorm van nitric oxide synthase (ecNOS en iNOS) beschreven in long biopsie materiaal van nagenoeg dezelfde studie populatie. Op grond van de diverse bekende biologische eigenschappen van stikstofmonoxide, zou een locale overproduktie van dit molecuul enkele specifieke aspecten kunnen verklaren van de karakteristieke dilatatie laesies, fibrinoïde necrose en plexiforme lesies in PPA. De immunoreactiviteit tegen ecNOS was vergelijkbaar in patiënten met een normale pulmonale circulatie en die met pulmonale vaatziekte, ongeacht het type vaatziekte of de ernst van de histologische vaatafwijkingen. Semiquantitatieve analyse van de immunohistochemische gegevens liet een correlatie zien tussen de intensiteit van de immunoreactie, tegen zowel ecNOS als iNOS, en de bloed doorstroming van de long

en de polsdruk in de longvaten. De intensiteit van de immunoreactie tegen iNOS was toegenomen in patiënten met een verhoogde druk in de longvaten, vergeleken met diegenen met een normale druk, en was het hoogst wanneer de verhoogde druk was gecombineerd met een toegenomen longdoorstroming. Duidelijke immunoreactiviteit tegen ecNOS en iNOS werd aangetoond in endotheelcellen in dilatatielaesies en in diverse endotheelcellen in plexiforme laesies. Deze bevindingen suggereren een toegenomen productie van iNOS in de longvaten van kinderen met een aangeboren hartafwijking en verhoogde druk in de longvaten, en een locale rol voor NO in the pathogenese van de geavanceerde laesies van PPA.

De studies in de *hoofdstukken 6.7 en 8* tonen aan dat het proces van pulmonale vaatwand remodellering in kinderen met een aangeboren hartafwijking zich niet beperkt tot het longvaatbed, maar dat ook de grotere elastische longslagaders zijn betrokken in het ziekteproces. Zij tonen verder aan dat deze remodellering is geassocieerd met veranderingen in de dynamische eigenschappen van de vaatwand van elastische arteriën, welke functionele gevolgen hebben, zoals een toename van de pulsatiele werkbelasting van het hart. Deze vasculaire veranderingen kunnen *in vivo* beoordeeld worden met pulmonale intravasculaire echografie tijdens een hartcatheterisatie procedure. Kwalitatieve aspecten van de pulmonale vaatwand en arteriële distensibiliteit leveren nieuwe informatie betreffende de pulmonale vasculatuur en maken het mogelijk de functionele consequenties van de verschillende aspecten van vaatwand remodellering te onderzoeken, evenals het effect van interventies.
Lange termijn analyse zal moeten uitwijzen of deze nieuwe variabelen van waarde zijn in de klinische evaluatie van pulmonale vaatziekte.
De studies in de *hoofdstukken 9 en 10* identificeren drie eiwitten, met krachtige biologische activiteit, met karakteristieke expressie patronen in het beloop van PPA, welke een rol suggereren in de pathogenese van de karakteristieke laesies in deze vaatziekte. Verder onderzoek zal nodig zijn om het oorzakelijke effect van deze drie proteïnen vast te stellen.
Om deze en nog vele andere vragen te kunnen beantwoorden, dient een krachtige inspanning ondernomen te worden om een adequaat diermodel te ontwikkelen voor deze bloedstroom- en druk gerelateerde pulmonale plexogene arteriopathie. Basale

studies naar mechanismen betrokken bij afwijkende vasculaire groei en remodellering, zullen uiteindelijk leiden tot nieuwe preventieve en therapeutische mogelijkheden voor patiënten met pulmonale vaatziekte.

List of abbreviations

ABC	avidin-biotin-enzyme complex
cAMP	cyclic adenosine monophospate
cGMP	cyclic guanosine monophosphate
CHD	congenital heart disease
CLIF	concentric laminar intimal fibrosis
EM	electron microscopy
ECG	electrocardiography
EDA	end-diastolic area
ESA	end-systolic area
ET-1	endothelin-1
EVE	endogenous vascular elastase
EvG	elastic-van Gieson
FGF	fibroblast growth factor
Hb	hemoglobin concentration
HE	hematoxylin-eosin
IGF	insulin-like growth factor
IVUS	intravascular ultrasound
LAP	left atrial pressure
L-NA	N^G-nitro-L-arginine
NO	nitric oxide
ecNOS	endothelial constitutive nitric oxide synthase
iNOS	inducible nitric oxide synthase
pO2	oxygen tension
pCO2	carbon dioxide tension
PAP	pulmonary artery pressure
PBS	phosphate-buffered saline
PCV	pulmonary congestive vasculopathy
PDE5	phosphodiesterase type V
PDGF	platelet derived growth factor
PGE2	prostaglandin E2

PGI2	prostacyclin
PPA	pulmonary plexogenic arteriopathy
PPHN	persistent pulmonary hypertension of the newborn
PVD	pulmonary vascular disease
PVR	pulmonary vascular resistance
PWP	pulmonary wedge pressure
Qp	pulmonary blood flow
Qs	systemic blood flow
RA	right atrium
RV	right ventricle
SAP	systemic arterial pressure
SNA	supernumerary artery
SSRE	shear stress responsive element
SVR	systemic vascular resistance
TGA	transposition of the great arteries
TGFß	transforming growth factor ß
VSD	ventricular septal defect
VSMC	vascular smooth muscle cell
VEGF	vascular endothelial growth factor
WU	Woods' units

Tot slot

Een verkeerd gekozen dessert kan een geslaagd diner bederven. Een dankwoord in een proefschrift mondt helaas nogal eens uit in al te zoet proza. Echter, voor diverse gastronomen is een diner niet af zonder een toetje ...
Een aantal mensen wil ik welgemeend bedanken voor hun hulp, steun en bijdragen aan het tot stand komen van dit proefschrift:

Allereerst bedank ik de kinderen en hun ouders voor hun bereidheid om te participeren in het onderzoek.
John Hess, of je nu in Rotterdam zat of in München, het maakte niet uit. Van begin tot einde stond (en sta) jij vierkant achter het hele project. Met je enthousiasmerende manier van "gedoseerde begeleiding" heb je een enorme invloed gehad op al het werk, waarbij je je behalve als promotor als vriend hebt opgesteld.
Wolter Mooi, ik wil jou bijzonder bedanken voor de manier waarop jij, na het ongelukkige overlijden van Prof. dr C.A. Wagenvoort, welhaast geruisloos ons onderzoek binnengleed, om vervolgens zeer nadrukkelijk en met veel elan je stempel op het werk te drukken.
Ralf Geiger, was gibt es in Insbruck, das wir nicht haben in Rotterdam? Bedankt voor de anderhalf jaar dat je onze ploeg versterkt hebt en voor alles wat je in die tijd gebracht hebt.

Met name bedank ik de mensen die vele uren noeste arbeid gestoken hebben in diverse onderdelen van de studies:
Adri Cromme-Dijkhuis, voor het tot bloedens toe analyseren van al die intravasculaire echoballen.
Wim Hop, voor de uitputtende, complexe maar uiteindelijk verhelderende statistische analyses.
Marco Kruit en Arno van Vliet, respectievelijk mister Dye and mister Wedge, voor al jullie hulp.
Daphne van Zuuren, Mohammed Ouhlous en Jaap van Oeveren, voor jullie zeer bijzondere inzet tijdens de respectievelijke onderzoekstages.

Freek van den Heuvel, voor het opzetten van de database.
Anton Timmermans, Frieda van der Ham en Johan van Lier, voor de ondersteuning in het laboratorium.
Ad Bogers, Robert-Jan van Suylen en Elma Gussenhoven, voor ieders gespecialiseerde hulp.
Anja Ellerbrock, voor je steun op zeer uiteenlopend gebied.

De huidige en voormalige hartfunctie-assistentes, Marian, Carola, Laura, Marjo, Cari, Anja, Louise, Mieke, Ineke en Miranda bedank ik voor de hulp bij de IVUS-studies en jullie uitgebreide interresse in vele terreinen buiten dit proefschrift

Tot slot bedank ik zeer bijzonder mijn collega's van de afdeling Kindercardiologie, Adri Cromme-Dijkhuis, Michiel Dalinghaus, Ingrid Frohn-Mulder, Folkert Meijboom en Maarten Witsenburg, en in de laatste drie intensieve maanden natuurlijk ook Leo Torn, voor het overnemen van vele klinische werkzaamheden gedurende een belangrijke periode van het onderzoek.

Lot, Floor, Daan, Wouter en Anita, bedankt voor alles!

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