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The role of the second heart field in pulmonary vein development : new insights in the origin of clinical abnormalities

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The Role of the Second Heart Field in Pulmonary Vein Development

New insights in the origin of clinical abnormalities

Yvonne Louise Douglas

Colofon

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This thesis was prepared at the Department of Anatomy and Embryology of the Leiden University Medical Center, Leiden, The Netherlands.

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The Role of the Second Heart Field in Pulmonary Vein Development

New insights in the origin of clinical abnormalities

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Aan mijn ouders

Voor Jos, Frits, Pien en Jur

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1

General Introduction

The interest in pulmonary vein development has rapidly increased since the role of the pulmonary venous myocardial sleeve in the foundation of potential atrial arrhythmias[1,2] and the vulnerability for potential pathogenic processes[3] has become more and more clear. A more detailed understanding of normal and abnormal pulmonary vein development requires comprehension of general cardiac development, since the so-called heart fields, that play a very important role in the formation of the complete heart, play an essential role in the composition and differentiation of the pulmonary venous wall as well[4]. Abnormalities in this (vessel) wall composition as well as the presence and re-activation of embryonic cardiac conduction system cells can be responsible for the onset of arrhythmias[1]. Besides that, abnormal pulmonary vein development, leading to abnormal pulmonary vein anatomy, may be held responsible for a lot of clinical entities.

Cardiac development

The primary heart tube develops from two parallel cardiogenic plates located in the anterior splanchnic mesoderm. After fusion of these crescent shaped cardiogenic plates in the midline, a primary myocardial heart tube is formed, cranially connected to the pharyngeal arches and caudally to the omphalomesenteric veins. The myocardial tube is lined on the inside by cardiac jelly (later on replaced by the endocardial cushions) and endocardial cells that are continuous with the endothelium of the embryonic vascular plexus[5,6]. This primary heart tube has an asymmetric shape as a consequence of predetermined left-right patterning and cardiac looping[6-8]. Cardiac differentiation into future atrial and ventricular components already takes place in the cardiogenic plate stage, based on expression of cardiac-specific genes like Nkx2.5[8] and GATA4-6[9].

In normal development, the heart tube loops to the right, resulting in a position of the outflow tract frontal to the atria and wedging of the aorta in between the atrioventricular orifices. During heart development, cardiac chambers and transitional zones are distinguished, the latter being involved in cardiac septation, valve formation and development of the cardiac conduction system. There has been much debate about which heart segments are discernable in the primitive heart tube, but most data point out that it is initially composed of a small atrial segment,

an atrio-ventricular canal and a primitive left ventricle[5,6]. The splanchnic mesoderm forming the primary heart tube is referred to as the **first heart field** or first lineage, and has the potential to generate differentiation into myocardial cells[10,11]. To this primary myocardium, new myocardium is secondarily contributed (second lineage), recruited from the so-called **second heart field,** located in the splanchnic mesoderm dorsal to the primary tube (Fig.1)[12]. Islet 1 (Isl 1), a marker of undifferentiated cardiac progenitor cells, and inhibitor of DNA binding 2 (Id2) have shown that major parts of the arterial as well as the venous pole of the heart are derived from this second heart field[13,14]. Therefore, to simplify understanding of terms, processes at the arterial pole (outflow tract) and venous pole (inflow tract) will be described separately.

Myocardial recruitment at the arterial pole

Several studies have been performed using different lineage markers such as *fibroblast growth factor*(*Fgf*)*8* and *10*[15], *Islet* (*Isl*)*1*[13] and *Tbx1*[16] to trace these cells into their cardiac destination. As a result of this, we know that undifferentiated mesoderm, recruited from the **anterior heart field,** is responsible for addition of myocardium of the complete right ventricle and the proximal arterial outflow tract[12,17], whereas the **secondary heart field**, being the distal part of the anterior heart field, forms the distal outflow tract[18]. Both the anterior heart field and the secondary heart field are part of the second heart field (Fig.1).

An extracardiac cell type contributive to the formation of the definitive heart at the arterial pole is the neural crest cell, derived from the crest of the neural tube[18,19]. At the arterial pole, these cells have an active role in the formation of the pharyngeal arch arteries and, to a lesser extent, in the septation of the outflow tract (Fig.1), since neural crest ablation has lead to cardiac outflow tract malformations[19]. Therefore, it is not surprising that a good interaction of both second heart field and neural crest cells is a prerequisite for the normal outflow tract development.

Finally, besides genetic factors being the cause of cardiac anomalies, also epigenetic (environmental) factors can play a role in remodeling of the outflow tract, as seen in diabetes[20,21], hyperhomocysteinemia[22] and shear stress[23].

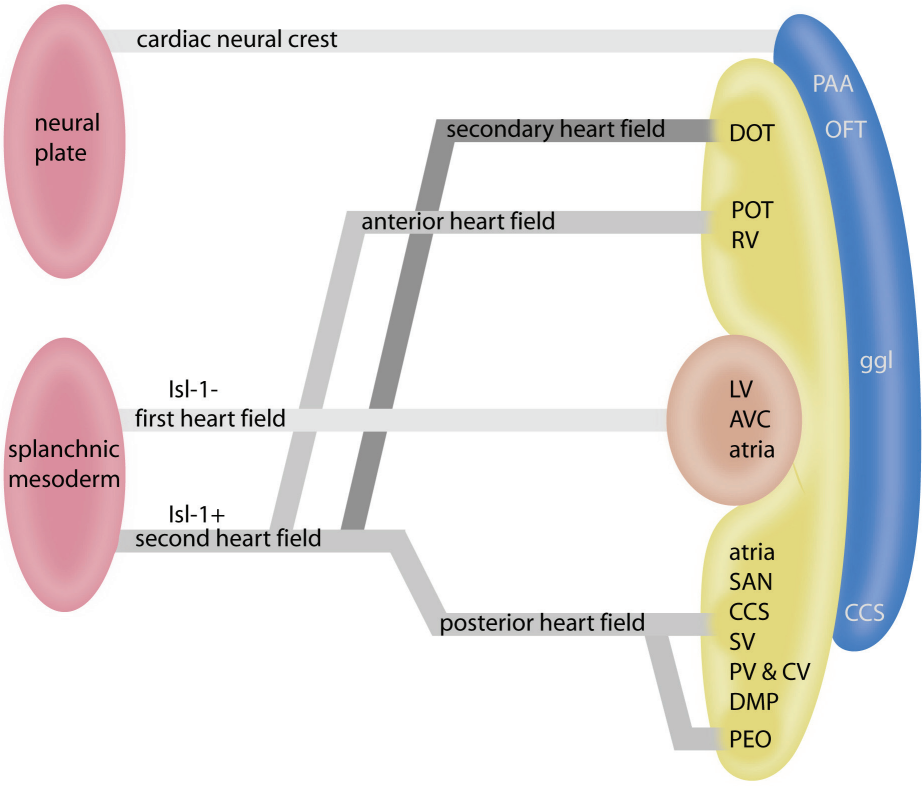


Figure 1. Schematic representation of the first and second heart field contribution to cardiac development. The primary heart tube, which contains the left ventricle (**LV**), the atrioventricular canal (**AVC**) and parts of the atria, is derived from the Isl-1 negative precursors of the first heart field and is depicted in **brown**. The secondary added myocardium, derived from Isl-1 positive precursors of the second heart field, is depicted in **yellow**. The second heart field is divided into a *secondary heart field*, that contributes to the distal outflow tract (**DOT**) and an *anterior heart field* that contributes to the right ventricle (**RV**) and the proximal outflow tract (**POT**) at the arterial pole of the heart. At the venous pole of the heart, myocardium is added from the *posterior heart field*, which contributes to the posterior wall of the atria and the interatrial septum, the dorsal mesenchymal protrusion (**DMP**), the sinoatrial node (**SAN**), the myocardium of the sinus venosus (**SV**), the pulmonary veins (**PV**), and the cardinal veins (**CV**), including the coronary sinus (**CS**), and probably part of the central conduction system (**CCS**). The posterior heart field also contributes to the proepicardial organ (**PEO**), which is the source of the epicardium and epicardium derived cells. Cardiac neural crest cells (**dark blue**) migrate to the heart and enter the heart both at the arterial and venous pole. Inflow tract (**IFT**); ganglia (**ggL**); outflow tract (**OFT**); pharyngeal arch arteries (**PAA**). Adapted from Gittenberger-de Groot et al.[6].

Myocardial recruitment at the venous pole

Analogous to the arterial pole, there has been special interest for markers specific for recruitment of myocardium from the second heart field to the venous pole of the heart, such as *Pitx2c*[24], *Nkx2.5*[24,25], *Tbx18*[26], *Shox2*[27] and podoplanin[25,28]. This region of the second heart field, located posteriorly to the primary heart tube, is involved in the addition of myocardium to the sinus venosus area, including the sino-atrial node, the pulmonary veins, the cardinal veins and the coronary sinus, as well as the main body of the atria, and is referred to as the **posterior heart field** (Fig.1).

Moreover, at the venous pole, two other extracardiac cell types play a role in the formation of the definitive heart structures. In the first place, neural crest cells that migrate into the inflow tract[29], are important for the development of the sympathic and parasypmpathic ganglia[30], and possibly are the indication of the atrioventricular conduction system[31]. Secondly, epicardial cells that are derived from the coelomic epithelial lining (coelomic mesothelium) of the posterior heart field, forming the proepicardial organ (PEO)[32-34] (Fig.1). Cells derived from this PEO grow out over the myocardial heart tube[35] and undergo epithelial-to-mesenchymal transformation (EMT). The so-called epicardium-derived cells (EPDC's)[36-38] migrate into the atrial and ventricular myocardium and are essential for the formation of compact myocardium[39], the atrioventricular valves[36], the main coronary arteries[40,41] and differentiation of the Purkinje network[42]. Moreover, they might play a role in the pathogenesis of endocardial fibroelastosis.

General pulmonary vein development

During the fourth week of embryonic development, two primary lung buds can be discriminated at the caudal end of the respiratory diverticulum, that develop in the surrounding splanchnic mesenchyme[43]. Subsequently, these lung buds differentiate into the bronchi and their bifurcations in the future lungs. The cartilaginous plates, the bronchial smooth muscle and connective tissue as well as the pulmonary connective tissue and capillaries are derived from the splanchnic mesenchyme, that forms a plexus of veins, by which the primitive lungs drain to the systemic circulation. This splanchnic plexus is also connected to the endocardium of the primitive heart tube by means of a strand of endothelial cells, situated in the mesocardium behind the heart, which is the anlage of the future common pulmonary vein[44] (Fig.2). When this strand lumenizes, initially, the lungs can drain centrally, to the atrial part of the heart, as well as peripherally, to the systemic veins. After atrial septation, the common pulmonary vein drains to the left atrium and starts to grow. The pulmonary-to-systemic venous connections are not necessary anymore and regress[45]. Abnormal development of the common pulmonary vein might be related to persistence of the primitive pulmonary-to-systemic connections which is the likely substrate for anomalous pulmonary venous connection[45].

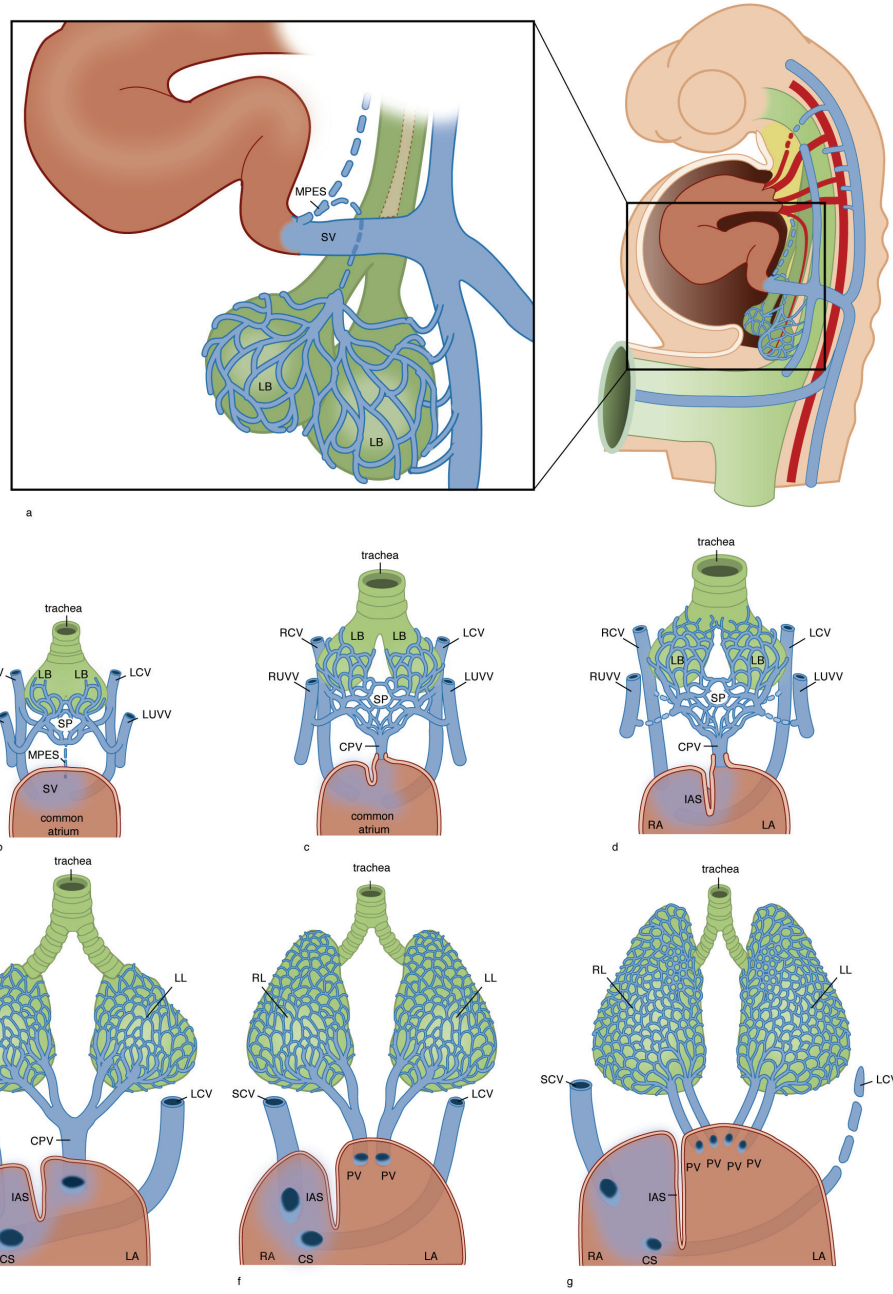

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Figure 2. Schematic depiction of pulmonary vein development.

a,b. Lateral (a) and frontal (b) view. The splanchnic vascular network surrounds the two lung buds (**LB**) and drains to the systemic circulation by means of primitive pulmonary-to-systemic connections to the right and left cardinal veins (**RCV/LCV**) or the right and left umbilical and vitelline veins (**RUVV/LUVV**). In the region of the heart, this splanchnic plexus (**SP**) is connected to the sinus venosus segment of the heart (**SV,** blue) by means of the midpharyngeal endothelial strand (**MPES**), which is the remaining part of a strand of endothelial cells that initially runs from the arterial to the venous pole in the dorsal mesocardium.

c. When the endothelial strand, which is the anlage of the future common pulmonary vein (**CPV**) lumenizes, the splanchnic plexus can drain its blood to the systemic circulation as well as to the heart. Although atrial septation has started, the CPV still drains to the sinus venosus segment that is connected to a common atrium.

d. The CPV grows and dilates, becoming the main route for drainage of pulmonary venous blood. The primitive pulmonary-to-systemic connections are not necessary anymore and regress. After atrial septation has completed (**IAS**), the part of the sinus venosus containing the CPV is placed to the left, becoming part of the left atrium (**LA**).

e. The CPV has bifurcated, so that the right and left lung (**RL/LL**) drain to the heart by means of two left and two right pulmonary veins. The right cardinal vein becomes the superior caval vein (**SCV**) and the left cardinal vein gives rise to the coronary sinus (**CS**), both connecting to the sinus venosus part of the right atrium (**RA**).

f,g. By incorporation of the CPV, initially, one right and one left CPV drain to the LA (f). After incorporation has completed, four separate pulmonary vein (**PV**) ostia can be identified on the inner side of the LA (g). The extracardiac part of the LCV regresses, becoming the so-called ligament of Marshall.

Development of the cardiac conduction system

In human embryos peristaltic contractions of the heart tube can be observed from the age of 3 weeks of development. After the heart has developed into its definitive segments, the myocardium differentiates either into working myocardium or in conducting myocardium, in which process neural crest cells as wells as EDPC's play an inductive role[31,46]. According to the ballooning model[47], the working myocardium of the atria and the ventricles differentiates from the outer curvatures of the looping heart tube, whereas the conducting myocardium might originate from the inner heart curvature.

The conduction system can be divided into a central conduction system and a peripheral Purkinje network. The central conduction system has a sino-atrial and an atrioventricular component connected by the atrioventricular node. Studies using the antibody against HNK-1 and the transgenic CCS-LacZ mouse strain have described the developing cardiac conduction system in mice and men[48-50] and show that there are two main transitional zones contributing to the central conduction system: the sinus venosus myocardium including the sino-atrial transition (or ring) and the primary fold (or ring) between the ventricular segments. The sino-atrial ring includes the left and right venous valves, the septum spurium, the myocardium surrounding the coronary sinus and the pulmonary veins and the interatrial Bachmann's bundle[51]. Three internodal pathways, derived from the sino-atrial ring, connect the sino-atrial (SA) node with the atrioventricular (AV) node (Fig.3). The first pathway, represented by the fused venous valves or septum spurium, runs anterosuperiorly and connects to the transient anterior AV node, located to the anterior side of the right atrioventricular ring (rAVR), posterior to the aortic root. The second pathway runs laterally in the base of the right venous valve (the later crista terminalis of the right atrium) and connects together with the third pathway, running within the left venous valve through the base of the atrial septum, to the posterior AV node. Whether these anterior and posterior AV node anlagen fuse or whether the regular AV node solely consists of the embryonic posterior AV node is not completely clear[6], but the functional AV node connects to the His bundle and the bundle branches. The atrioventricular conduction system is mainly derived from the primary ring, which encircles the aortic orifice and the right atrioventricular canal, thus forming the rAVR. (Fig.3). It was shown that the right ventricular moderator band is also continuous with the rAVR[51].

In the normal heart, only the SA node, the AV node, the His bundle and both bundle branches remain functional, while the internodal pathways, the embryonic conduction tissue surrounding the coronary sinus and the pulmonary veins and the rAVR disappear[6]. Therefore, discrimination between the *developmental* conduction system and the *functional* conduction system is essential since rhythm disturbances can be explained from the embryonic disposition of the conduction system as a result of dedifferentiation of embryonic cells and reinitiation of conduction system capacities[51].

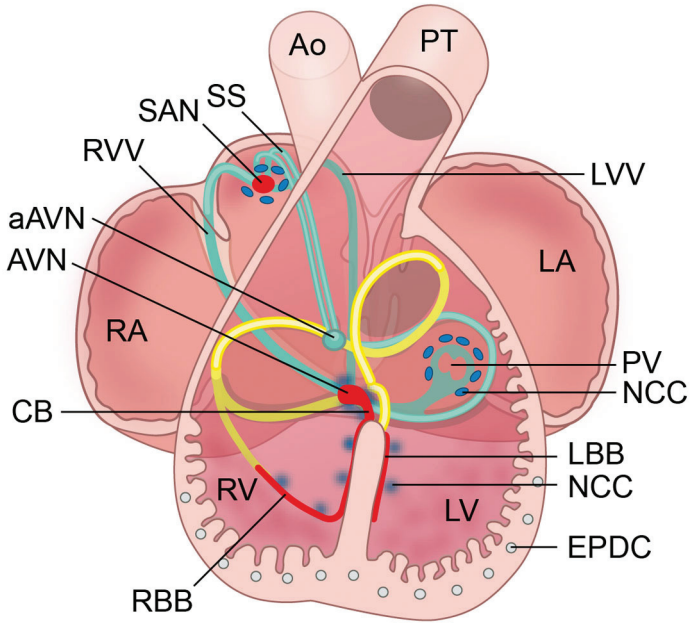


Figure 3. Embryonic parts of the conduction system derived from the sinoatrial ring (**green**) and the primary fold (**yellow**). The sinoatrial ring includes the right venous valve (**RVV**), the left venous valve (**LVV**), the septum spurium (**SS**), the myocardium surrounding the coronary sinus and the pulmonary veins (**PV**) and Bachmann's bundle, being part of the left atrioventricular ring, running posterior to the aorta, in the roof of the left atrium (**LA**) to the right atrium (**RA**). The tissue of the primary fold or ring encircles the right atrioventricular canal (forming the right atrioventricular ring), and the aortic orifice. The definitive conduction system (**depicted in red**) consists of the sinoatrial node (**SAN**), the atrioventricular node (**AVN**), the common atrioventricular bundle (**CB**), the left bundle branch (**LBB**), and the right bundle branch (**RBB**). The parts of the conduction system depicted in yellow and green are supposed to disappear. It remains to be investigated if the anterior atrioventricular node (**aAVN**), which is connected to the SAN by the anterior internodal pathway running in the SS, becomes part of the regular AVN or solely features as an embryonic structure in addition to persisting in some cardiac malformations. Neural crest cells (**NCC**) can be found in proximity to structures of the cardiac conduction system, both as precursors of the autonomic neuronal network (**depicted in dark blue**) and after apoptosis as possible inducing cells (**depicted in shaded blue**). Aorta (**Ao)**; epicardial derived cell (**EPDC)**; left ventricle (**LV)**; pulmonary trunk (**PT)**; right ventricle (**RV)**. Modified after Gittenberger-de Groot et al.[6].

Although molecular biology, by the use of transgenic animal models, has attributed to the knowledge of normal and abnormal heart development, one has to realize that congenital heart malformations mostly are the result of highly complex cascades of gene interactions combined with epigenetic factors that cannot be deduced to isolated entities.

Aim of this thesis

In this thesis we describe normal and abnormal pulmonary vein development in human and mouse hearts, and focus on the histo(patho)logy of the pulmonary venous and left atrial dorsal wall, in order to elucidate the role of the posterior heart field in the formation and differentiation of the pulmonary venous vessel wall and its possible consequences for the onset of arrhythmias and susceptibility for pulmonary vein stenosis. Another aim of this thesis is that the understanding of normal pulmonary vein development will contribute to the understanding of the development of abnormal pulmonary venous connections and its clinical consequences.

Chapter outline

In **Chapter 2** the normal development of the pulmonary veins and the left atrial dorsal wall is described in embryonic, neonatal and adult human hearts, with special focus on the histological consequences of the incorporation process of the pulmonary veins into the left atrium. The findings are discussed in relation to the pathogenesis of atrial arrhythmias.

In **Chapter 3** the development and differentiation of the myocardium and vascular wall of the pulmonary veins, left atrial dorsal wall and atrial septum in wild type and *podoplanin* knockout mouse embryos of embryonic stages (E) 10.5-E18.5 is described, using 3-D reconstruction and immunohistochemistry. The role of the posterior heart field myocardial marker podoplanin in this developmental process is discussed.

In **Chapter 4** the histopathological findings of the pulmonary venous and left atrial dorsal wall in human neonatal hearts with total anomalous pulmonary venous connection are attributed to abnormal posterior heart field contribution to the development of the venous pole of the heart and related to the findings in Chapter three. A clinical link to the origin of pulmonary vein stenosis is made.

Chapter 5 presents a clinical report on unilateral pulmonary vein stenosis with a contralateral pulmonary varix as an example of abnormal pulmonary vein development. A pulmonary varix is a dilated, tortuous pulmonary vein, usually resulting from obstructed pulmonary vein drainage. In this report, we try to link the clinical condition to an embryologic explanatory hypothesis which may or may not be combined with environmental factors (acquired disease).

Chapter 6 is an extended general discussion which reviews normal and abnormal pulmonary vein development, including the content of the chapters two to six. It outlines the importance of the posterior heart field in the etiology of congenital heart disease and emphasizes the need for improvement of knowledge on its (epi) genetic background in the future.

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2

Histology of Vascular-Myocardial Wall of Left Atrial Body after Pulmonary Venous Incorporation

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Abstract

During embryonic development the common pulmonary vein (PV) becomes incorporated into the left atrium (LA), giving rise to separate PV ostia. We describe the consequences of this incorporation process for the histology of the LA and the possible clinical implications. The histology of the LA wall in relation to PV incorporation was studied immunohistochemically in 16 human embryos and fetuses, one neonate and 5 adults. The PV wall, surrounded by extrapericardially differentiated myocardial cells, incorporated into the LA body. After incorporation, the composition of PVs and smooth-walled LA body wall was histologically identical. The LA appendage however, consisted of an endocardial and myocardial layer without a vessel wall component. In 2 adults, the myocardium in the LA posterior wall was absent. At the transition of the LA body and the LA appendage, a smooth-walled myocardial zone lacking the venous wall was observed. This zone was histologically identical to the sinus venarum of the right atrium. In conclusion, the LA body arises by incorporation and growth of the PVs, presenting with a histologically identical structure of vessel wall covered by extrapericardially differentiated myocardium of the PVs. Discontinuity of myocardium may be the substrate for arrhythmias, whereas absence of myocardium in some individuals makes this area potentially vulnerable to damage inflicted by ablation strategies. A borderzone between the LA body and the LA appendage is hypothesized to be the left part of the embryonic sinus venosus.

Introduction

During development, the common PV becomes myocardialized whereafter it is incorporated into the posterior LA wall giving rise to separate PV orifices[1]. The degree of incorporation is variable between individuals and only about 75% to 80% of individuals possess 4 discrete PV orifices[2,3]. Though the development and incorporation of the PVs has been studied and several histological studies of the LA and the PVs have been performed, until now, there are no extensive reports that emphasize the histological result of PV incorporation in the LA in subsequent stages in the human. It seems logical to expect characteristic venous wall tissue, consisting of an intima, media with elastin and smooth muscle cells (SMCs) in the LA after the incorporation process, but no histological veno-atrial border has been described in the literature. In the cardiology cathlab this atriovenous border and the structure of the LA and the PV have gained interest in recent years, in relation to the treatment of atrial fibrillation originating from the PV[4]. Ablation strategies, aimed at electrical isolation of the PV from the LA are often targeted at the left atrial tissue just outside the atriovenous junction to avoid the occurrence of PV stenosis by damaging the vulnerable PV vessel wall[5]. The present study investigates the histological outcome of the incorporation process of the PVs in the LA during subsequent stages of development.

Methods

Normal hearts of 16 human embryos and fetuses, 1 neonate and 5 adults were studied. The embryos and fetuses, obtained by legal or spontaneous abortion, ranged from 7 to 22 weeks (crown-rump lengths from 19 to 170 mm). The studies were approved by the local medical-ethical committee. The neonate had died during delivery as a result of asphyxia. Adult hearts were obtained from human bodies used in anatomy teaching lessons. The cause of death was unknown due to privacy regulations. Preservation of the bodies was performed by injection of embalming fluid in the femoral artery, consisting of 36% formaldehyde with a mixture of ethanol, glycerine,

phenol, K_2SO_4, Na_2SO_4, $NaHCO_3$ and Na_2SO_3. The fetal and neonatal specimens were fixed in a 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.2).

Samples/sectioning of material. Embryonic hearts were embedded in paraffin and serially sectioned transversely (5-10 µm). From the neonatal and adult hearts, cross-sectional samples were taken from all PVs, the posterior and anterior LA wall, and the LA appendage. Samples taken from the posterior and anterior right atrial wall and the right atrial appendage served as reference. Samples were embedded in paraffin and sectioned (5 µm) using a Leica microtome. Sections were mounted onto slides using chicken albumine dissolved in glycerine (1:1) and dried at 37º C for at least 24 hours.

Staining. Standard histological stainings were performed with Haematoxilin-Eosin (HE), Elastic Van Gieson (EvG) or elastic Resorcin-Fuchsin (RF) to detect elastic filaments, and Sirius Red to demonstrate collagen fibers [6]. Furthermore, sections were stained immunohistochemically with the pan-muscle actin antibody HHF35 (1:500, Dako, M0635), to study myocardial structure, 1A4 (1:3000, Sigma Aldrich, Product No. A2547) against alpha-smooth muscle (SM) actin, and an antibody against atrial myosin light chain (MLC2a, 1:2000, a generous gift of Steve Kubalak), specific for atrial myocardium. Staining procedures were performed as described previously[7,8].

Results

Embryonic stage: *Seven to 7.5 weeks of gestation, crown-rump length 19 to 24 mm:* Proximal to the heart, 4 PVs drained via a common PV into the smooth-walled segment of the LA (Fig.1a), also called the LA body, that could already be distinguished from the trabeculated LA appendage. The wall of both the intra- and extrapulmonary parts of the veins consisted of 1-3 layers of alpha-SM actin expressing SMCs around the endothelium (Fig.1b,e). The extrapulmonary part of the PVs being continuous with the dorsal wall of the LA were encircled by an additional layer of MLC-positive myocardial cells (Fig.1c,d). In the wall of the intrapulmonary part of the veins myocardial cells were absent (Fig.1f). The LA wall does not express smooth muscle cell actin at this stage.

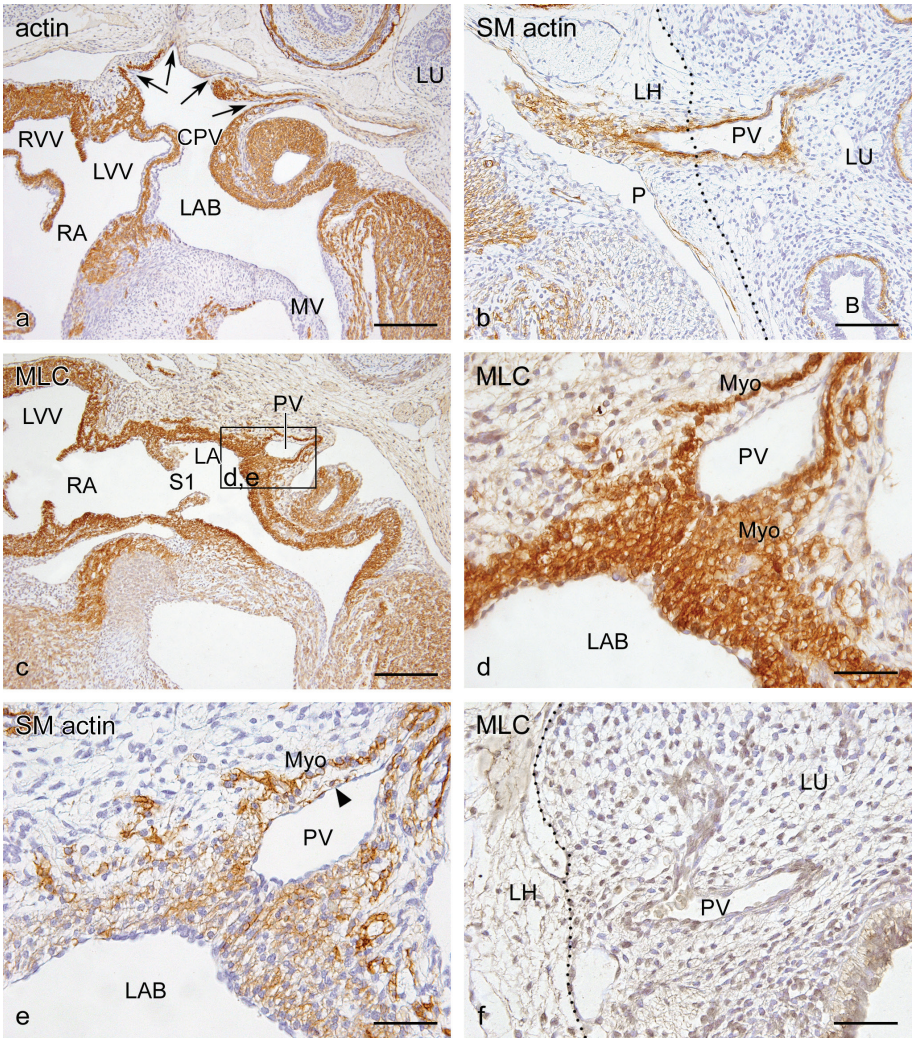
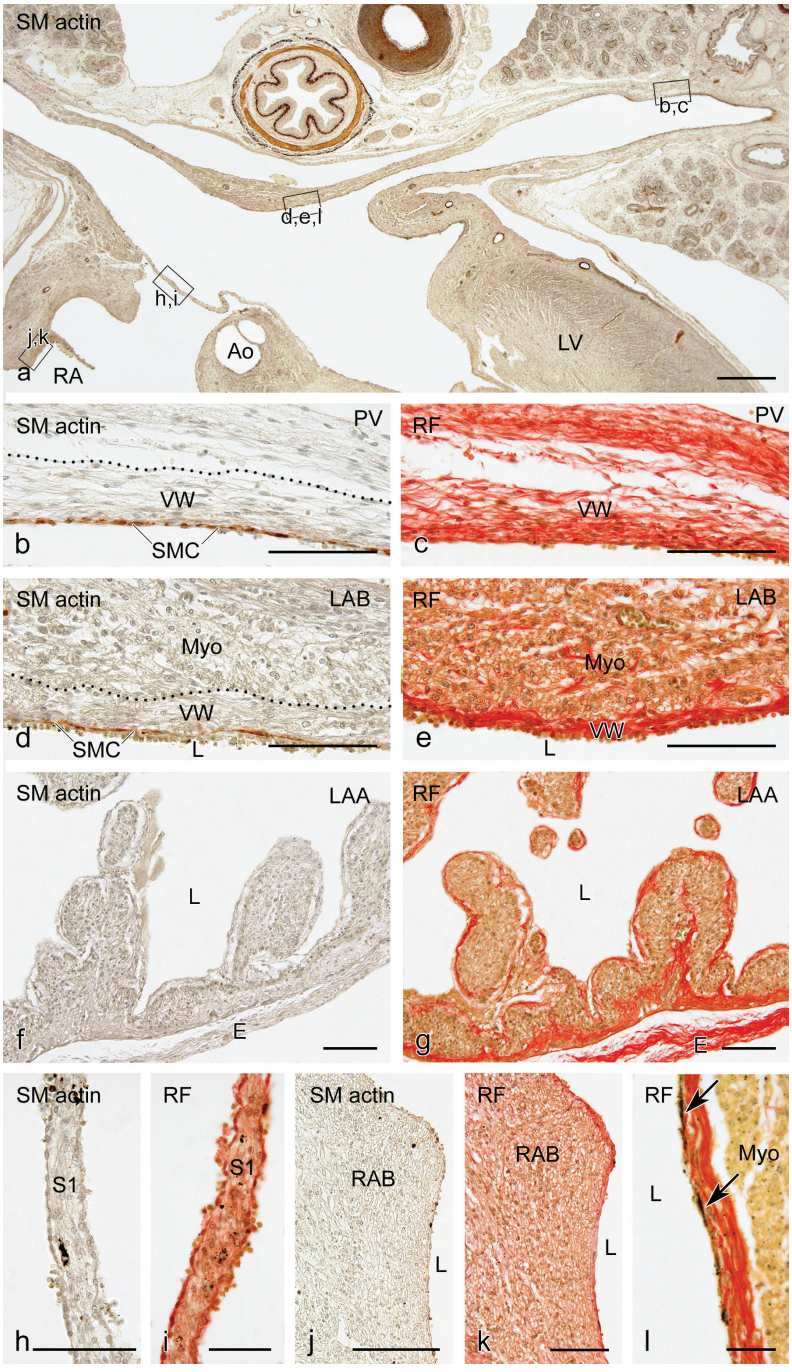


Figure 1a-f

Sections of a 54 days old human embryo stained with HHF35 for muscle actins (a; **actin**), alpha-smooth muscle actin (b,e; **SM actin**), atrial myosin light chain (c,d,f; **MLC**), showing the differentiation of the pulmonary veins (PVs) and their entrance in the left atrial body (**LAB**). a. Four PVs (arrows) drain via one common pulmonary vein (**CPV**) into the LAB. The wall of both intra- and extrapulmonary PVs consists of 1-3 layers of smooth muscle cells (SMC) (b), while an additional layer of actin and MLC positive myocardium (**Myo**) only encircles the extrapulmonary part of the vessel wall (c-f). Consecutive sections in c, d and e demonstrate that part of the MLC-positive myocardial cells surrounding the smooth muscle cells of the PVs (**Myo**) are also 1A4-positive, which is related to early differentiating myocardial cells[21]. Beneath these myocardial cells a real SMC layer develops (e; arrow head). Bronchus (**B**), Intra/extrapulmonary borderline (----), lung (**Lu**), left venous valve (**LVV**), lung hilum (**LH**), mitral valve (**MV**), pericardial cavity (**P**), right atrium (**RA**), right venous valve (**RVV**), septum primum (**S1**). Scale bar = 200 µm in a,c; 100 µm in b,d-f.

Fetal stages: *Ten to 22 weeks' gestation, crown-rump length 42 to 170 mm:* As incorporation of the common PV had progressed, in all but 2 fetuses there were 2 separate right pulmonary venous orifices, while 2 left PVs drained via one common left PV into the LA. At 16 weeks, in 2 fetuses (out of 5) four separate PV ostia were observed. From the lung hilum to the heart, the PVs were completely covered by myocardium. Subendothelially, the wall of the PVs consisted of 1-3 layers of SMCs embedded within a collagenous matrix (Fig.2a-c). This layer extended from the intrapulmonary veins to the smooth-walled LA body (Fig.2d,e). A thin layer of collagen was also found in the LA appendage, atrial septum and in the complete right atrium, but no SMCs were observed (Fig.2f-k). The myocardial layer around the PVs had thickened as well as the subendothelial collagenous vessel wall with SMCs in the PVs and the LA body. The presence of vascular SMCs in the heart was restricted to the LA body. At 21 weeks, the first indication of elastic fiber formation was observed within this layer (Fig.2l).

Figure 2a-l
Sections of a 15.5 week old human embryo. Boxes in Fig.a indicate the position of the enlargements in b-k. RF-sirius red stained sections in c, e, g, i, k and l are adjacent sections of Fig.a, b, d, f, h and j, that are stained for alpha-SM actin. Collagen fibers are red and elastic fibers are black in the RF stained sections.
a-c. Smooth muscle cells (SMCs) have differentiated within the subendothelial collagenous matrix of the pulmonary vessel wall (**VW**). d,e. This differentiating vessel wall extends into the smooth-walled left atrial body (**LAB**). Although the left atrial appendage (**LAA**), the septum primum (**S1**), and the right atrial body (**RAB**) have a thin subendothelial collagen matrix (g, i, k) no SMCs are found (f, h, j). l. At 21 weeks elastin is deposited (arrows) in the differentiating venous vessel wall in the LAB. Aorta (**Ao**), epicardium (**E**), external borderline (------) of the VW, left ventricle (**LV**), lumen (**L**), myocardium (**Myo**). Scale bar = 500 μm in a; 100 μm in b-h,k,l; 50μm in i; 200 μm in j.

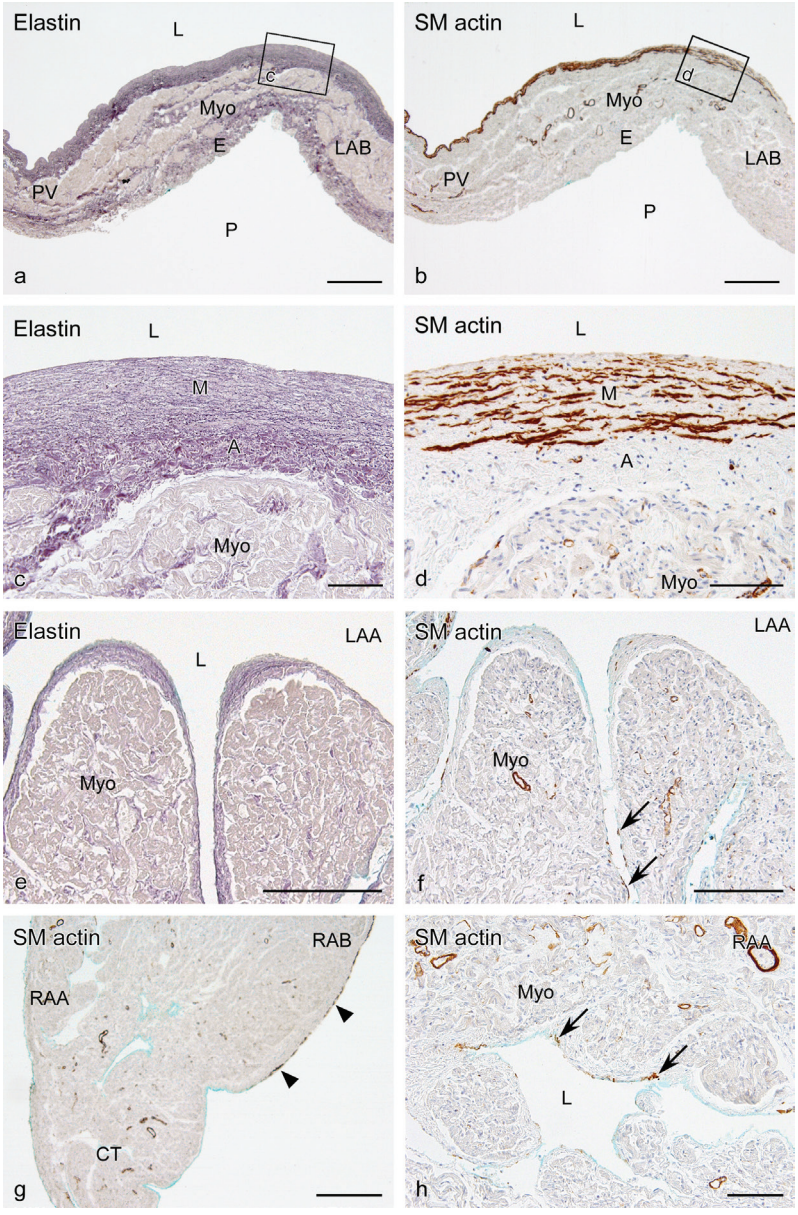

SM actin
b,c
d,e,l
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Ao
LV
a
RA
SM actin
PV
VW
SMC
b
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PV
VW
c
SM actin
LAB
Myo
VW
SMC
L
d
RF
LAB
Myo
VW
L
e
SM actin
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Neonatal stage: *Pulmonary veins and left atrial body:* As in previous stages there were 2 separate right PV ostia and one common left PV ostium. At the transition of the PV ostia and LA body no histological veno-atrial demarcation was found (Fig.3a,b). In both the PVs and the LA body the following layers could be distinguished: an inner venous vessel wall with an endothelium, several medial layers of SMCs with longitudinally oriented elastic fibers, and an adventitial layer with randomly distributed elastic fibers with fibroblasts and vasa vasorum. These vessel wall structures were covered by a myocardial layer and an epicardium on the outside (Fig.3a-d).

Left atrial appendage: In the trabeculated LA appendage the inner surface consisted of an endocardium with a thin subendocardial sheet consisting of well-organized collagen and elastic fibers, but without well-organized layers of alpha-SM actin positive cells. Only a few scattered actin positive cells were found. Characteristic vessel wall layers as seen in the body of the atrium, were lacking (Fig.3e,f). At the outer side, a thick layer of myocardium covered by an epicardium was present.

Right atrium: The right atrial body or sinus venarum consisted of smooth-walled myocardium. The inner side of the wall near the systemic veins consisted of one layer of SMCs. This layer did not represent a proper tunica media as seen in the systemic veins which had a well organized vessel wall with five layers of SMCs. At the junction of the terminal crest with the right atrial body, this smooth muscle layer disappeared (Fig.3g). Within the trabeculated right atrial appendage, only scattered subendocardial SMCs were observed (Fig.3h).

Figure 3a-h
Sections of various segments of a human neonatal heart. a-d At the transition of the PV and LA body no histological veno-atrial demarcation was found. The wall consisted of an endothelium/endocardium, a medial layer (**M**) with smooth muscle cells (SMCs) and elastic fibers, an adventitial layer (**A**) with fibroblasts and elastic fibers, a sheet of myocardium (**Myo**) and an outer epicardium (**E**). e,f In the left atrial appendage (**LAA**) no organized vessel wall was present. Only a subendothelial layer of elastin (e) and isolated SMCs (arrows in f) was present. In the right atrium only one layer of SMCs, not characteristic for a tunica media, could be detected in the right atrial body (arrow heads in g) near the entrance of the caval veins. The histology of the right atrial appendage (**RAA**) is comparable with the LAA with only isolated SMCs (arrows; h). Terminal crest (**CT**), Lumen (**L**), pericardial cavity (**P**). Scale bar = 500 µm in a,b,g; 100 µm in c-f; 200 µm in h.



Adult stage: *Pulmonary veins and left atrial body:* The PVs of 4 adult hearts had discrete PV orifices. In one heart a common left PV orifice was still present. In 2 hearts, an early branching pattern of the right inferior PV was observed. The outer side of

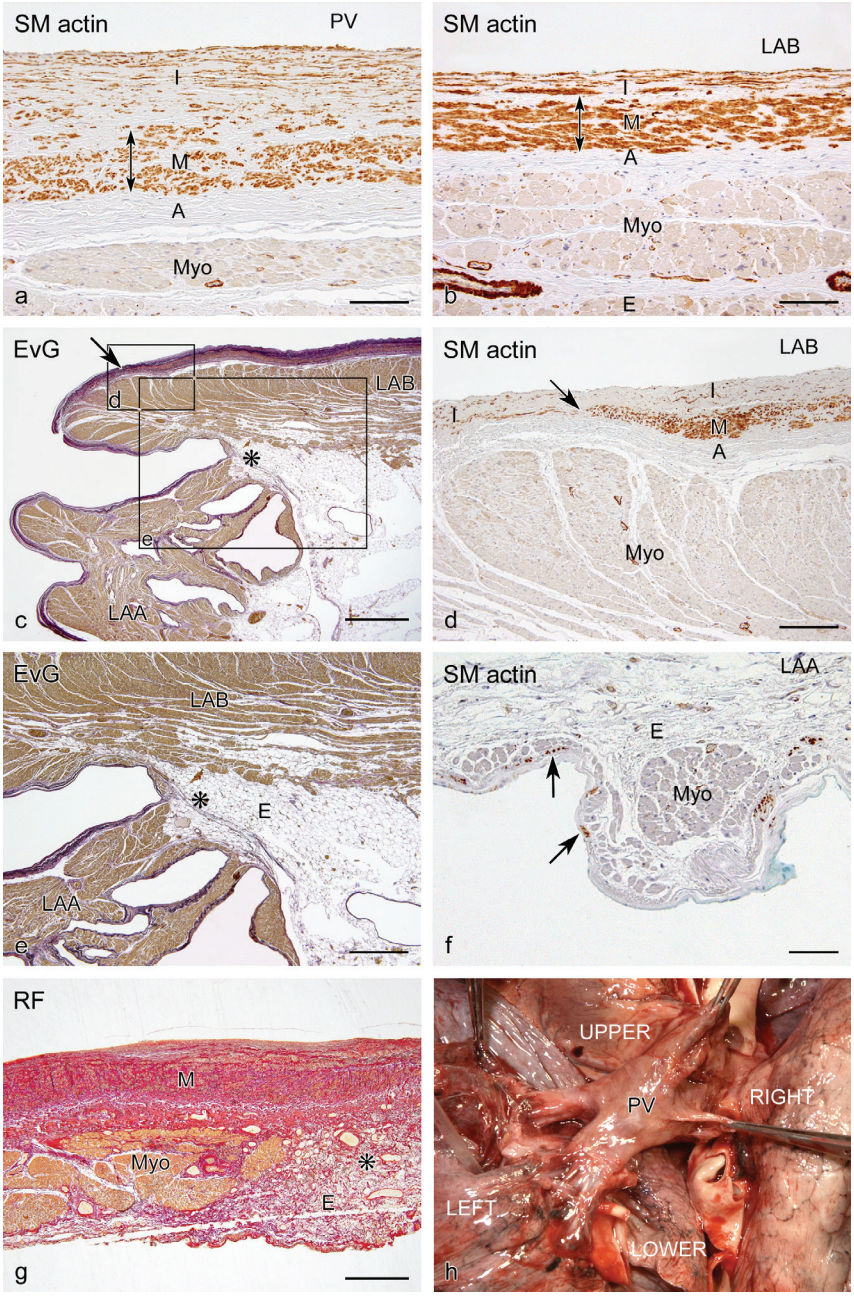
the wall of the LA body consisted of a non-trabeculated myocardial layer. Around the outside of the PVs this myocardium formed a circular or spirally arranged muscular sleeve. The sleeves tended to be thicker and more complete near the junction with the posterior LA body, but tapered more distally. In the PVs, a characteristic vessel wall was found consisting of an intimal layer and a medial layer with SMCs (Fig.4a). In the wall of the entire LA body, in addition to the outer myocardial layer, a prominent vessel wall layer being continuous with the PV vessel wall was present (Fig.4b). In between the media and myocardium an adventitial layer with fibroblasts (not expressing SM actin) was found (Fig.4a,b). In contrast to the neonatal stage, intimal thickening was observed in the PVs and the LA body, which was most profound in the PVs (Fig.4a,b). At the entry of the PVs into the LA, no histological demarcation was found. Interestingly, at the junction of the LA body with the LA appendage, a borderzone with distinctive tissue was identified (Fig.4c-e). The inner surface of this area lacked trabeculation. In contrast to the remaining part of the LA body and the PVs, the characteristic tunica media was lacking in this borderzone (Fig.4d,e). Another finding was that the myocardium at the transition of the LA body and the trabeculated LA appendage was either very thin or absent (Fig.4c,e). Moreover, in 2 of the adult hearts examined, the myocardium covering the LA body in between the confluence of the 4 PVs was discontinuous thus allowing the epicardium to line the vessel wall of the PVs (Fig.4g,h). These areas of discontinuous myocardium were only several millimeters wide. It should, however, be noted that in these areas (partly) isolated bundles of myocytes were observed.

Figure 4a-h

Adult stage sections stained for alpha-smooth muscle actin (a,b,d,f; **SM actin**), Elastic Van Gieson (c,e; **EvG**) and Resorcin-Fuchsin (g; **RF**). (h) is a macroscopical picture of the area in between the confluence of the PVs where discontinuous myocardium is found as shown in (g). Boxes in (c) indicate the position of the enlargements in (d,e). In EvG stained sections collagen fibers are purple and elastic fibers are black, in RF stained sections collagen fibers are red and elastic fibers are black.

a,b. In the pulmonary veins (**PV**) as well as in the left atrial body (**LAB**) an identical and characteristic vessel wall is found consisting of an intimal layer (**I**), a medial layer (**M;** double arrow) with smooth muscle cells (SMCs) and elastic lamellae, and an adventitial layer (**A**) in between the media and myocardium (**Myo**). In contrast to the neonatal stage, a profound intimal thickening is observed, most profound in the PVs. At the junction of the LAB with the left atrial appendage (**LAA**) a borderzone with distinctive tissue is identified (c,d; arrow). The inner surface of this area lacks trabeculation and vessel wall tissue (d). The myocardium at the transition between LAB and LAA is very thin or absent, facing the epicardium (c,e: asterisk). f. On the inner surface of the LAA occasional SMCs are found (arrows) but a vessel wall component is lacking.

g,h. In between the confluence of the four PVs, in two adults, the myocardium (**Myo**) is discontinuous or absent (asterisk in g). Epicardium (**E**). Scale bar = 100 μm in a,b,f; 1mm in c; 200 μm in d; 500 μm in e,g.


SM actin
PV
I
M
A
Myo
a
SM actin
LAB
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M
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Myo
E
b
EvG
d
LAB
e
LAA
c
SM actin
LAB
I
I
M
A
Myo
d
EvG
LAB
E
LAA
e
SM actin
LAA
E
Myo
f
RF
M
Myo
E
g
UPPER
PV
RIGHT
LEFT
LOWER
h

Atrial appendages: The wall of the trabeculated LA appendage differed from the PVs and LA body in that it did not contain a vessel wall component. The inner surface consisted of a thin subendocardial collagenous layer with some elastic fibers and an occasional SMC (Fig.4f). The histology of the LA appendage was similar to that of the right atrial appendage (Fig.5a,b).

Right atrial body: The non-trabeculated sinus venarum of the right atrium consisted at the inside of a thin subendocardial collagenous layer covered by myocardium. No characteristics of vessel wall structures were present at this site (Fig.5c,d).

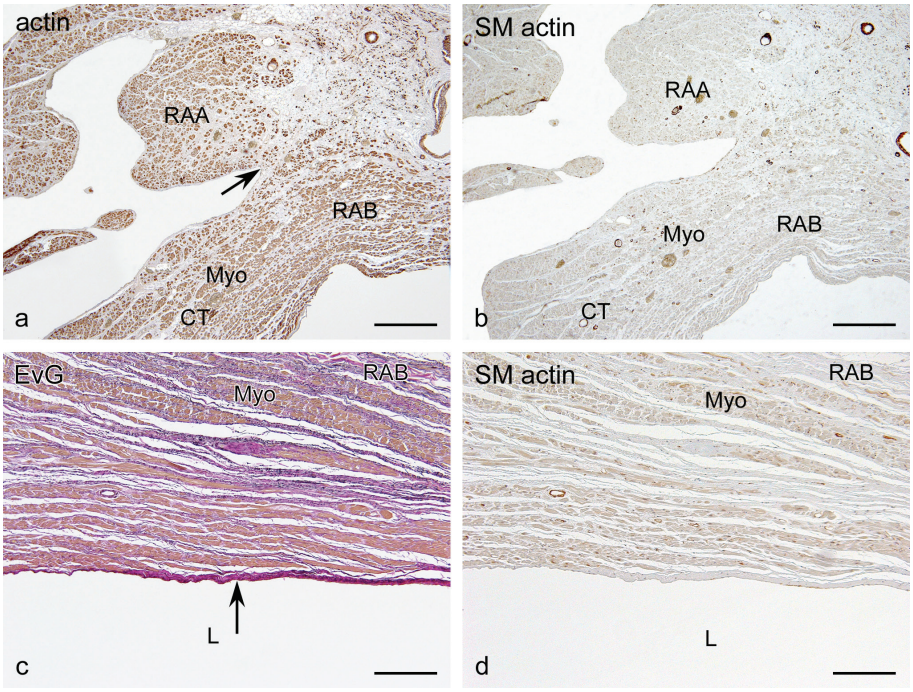


Figure 5a-d
Adult stage sections stained with HHF35 for muscle actins (a; **actin**), alpha-smooth muscle actin (b,d-f; **SM actin**) and Elastic Van Gieson (c; **EvG**). (b) and (d) are adjacent sections of (a) and (c).
a,b. At the transition of the smooth-walled right atrial body (**RAB**) to the trabeculated right atrial appendage (**RAA**), the myocardial layer (**Myo**) is very thin (a; arrow) comparable to the left side. In the RAA, vessel wall tissue is absent (b) which is comparable to the left atrial appendage. c,d. The right atrial body (**RAB**) consists at the inner side of a thin subendocardial layer (c; arrow), covered by myocardium (**Myo**). No characteristics of vessel wall tissue were present at this site (d). Terminal crest (**CT**), lumen (**L**). Scale bar = 500 µm in a,b; 200 µm in c-d.

Discussion

In this study, the histological outcome of the incorporation of the PVs into the LA was described in subsequent developmental stages. Our study demonstrates that, based on histological criteria, 3 different compartments can be distinguished in the LA (schematically demonstrated in Fig.6): the smooth-walled LA body with vessel wall tissue (i.e. incorporated PV tissue); the trabeculated LA appendage, without vessel wall tissue; and a transitional zone in between the LA appendage and the LA body, which is smooth-walled and lacks vessel wall tissue. The right atrial appendage, the body of the right atrium and the atrial septum are not lined by vessel wall tissue. The borderzone between the LA appendage and the LA body histologically resembles the smooth-walled sinus venarum (body) of the right atrium.

The origin of the PVs is an issue of controversy. Based on findings using the marker HNK-1, several authors have described that the myocardium surrounding the primitive PV in the LA is continuous with the sinus venosus in the right atrium[9-11]. However, other authors have disputed this relation[12]. These discrepancies depend largely on different application of definitions (especially how to define the sinu-atrial transition and the sinus venosus), and on different interpretation of possibly similar observations. Regardless of these differences, most authors seem to agree that the myocardium surrounding the primitive PV possesses different characteristics and can be differentiated from the working myocardium of the LA appendage. The aim of the current study was not to provide the answer for the origin of the primitive PV. However, based on the observation of a zone of myocardium at the transition of the LA appendage with the LA body that histologically resembles the sinus venosus in the right atrium, we hypothesize that during incorporation of the PV and the surrounding myocardium into the LA, the contribution of vessel wall to the body of the LA increases, which may reduce the area of sinus venosus myocardium to a small zone encircling the entrance to the LA appendage. An interesting finding was the very thin or even absent myocardium at this junction zone between the LA body and the LA appendage; within this zone slow conduction or conduction block may occur that are prerequisites for the induction of reentry[13].

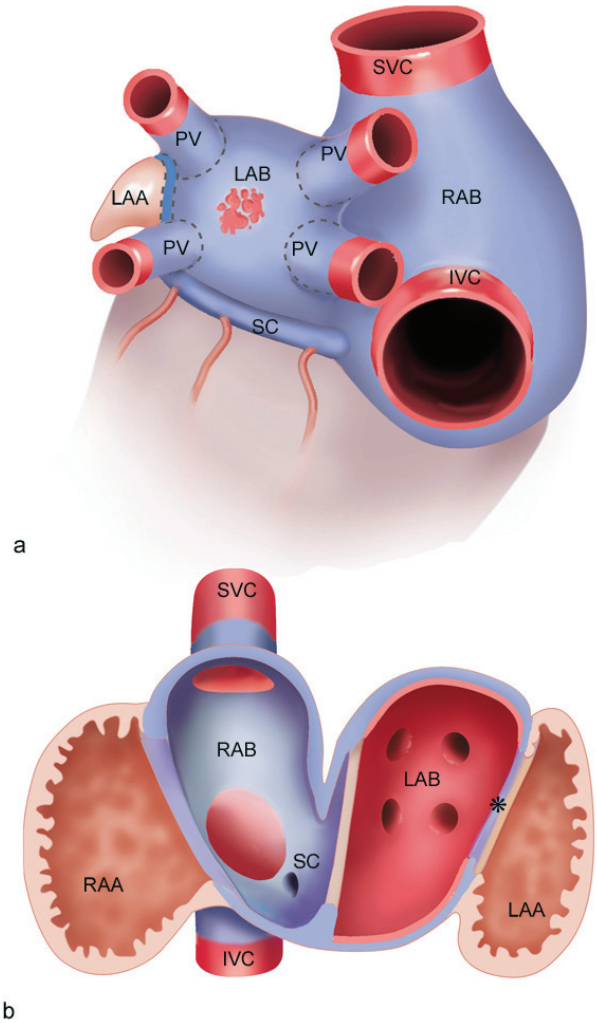


Figure 6a,b

a. Schematic depiction of the outer side of the atrial chambers with the pulmonary veins (**PV**) and systemic veins. The left atrial body (**LAB**) and right atrial body (**RAB**) are covered by myocardium with a smooth-walled inner aspect (**blue**), which stretches out over the extracardiac segments of the PV and over a small peripheral part of the systemic veins (**blue area above and below dotted line**). The left atrial appendage (**LAA**) and right atrial appendage (**RAA**) consist of trabeculated myocardium (**brown**). A zone of smooth-walled myocardium that lacks characteristics of vessel wall tissue is found at the junction of the LAB to the LAA (**blue between dotted lines**). b. Schematic depiction of the tissue types found in the left and right atria seen from the inside of the atria. Vessel wall tissue (**red**), myocardial tissue with a smooth-walled inner aspect (**blue**), primary atrial segment tissue (**brown**), transitional zone (**asterisk**) which is smooth-walled, lacking vessel wall tissue (hypothesized to be left-sided sinus venosus tissue). Coronary sinus (**SC**), inferior caval vein (**IVC**), superior caval vein (**SVC**).

At early stages, the wall of the PVs consisted of a mixture of extrapericardially differentiated myocardial cells and SMCs. These SMCs proliferated and differentiated into an inner venous vessel wall. In the neonate and the adult, due to the incorporation process of the PVs, vessel wall tissue and extrapericardially differentiated myocardium were not only present in the PVs, but also in the LA body. It is currently unclear whether the outer circular or spirally arranged myocardial sleeves are formed due to extracardial mesenchymal triggers as is strongly supported by studies of Kruithof et al.[14,15], or are a direct continuation of the LA myocardium as supposed by Millino in the mouse[16]. Our study demonstrates that the myocardium and venous wall differentiate simultaneously, but does not support either a cardiac or an extracardiac origin.

In the adult PVs and the LA body, beneath the endothelium/endocardium a prominent fibroelastic subendothelial layer was observed, which extended to a lesser extent in the right and left appendages and the atrial septum. Interestingly, in the neonatal stage, only a very thin subendothelial layer was observed, raising the possibility of an aging reaction to different stimuli e.g. continuous shear stress. In congenital heart diseases causing persistent and increased wall tension on the ventricles e.g. aortic stenosis or atresia and hypoplastic left heart, a comparable intimal thickening, so-called fibroelastosis, can be found[17]. However, as all our adult hearts showed this intimal thickening, it is debatable whether these findings represent a true form of fibroelastosis or are part of normal aging. Reactive intimal thickening in the PVs, leading to PV stenosis, can be seen after ablative therapy for arrhythmias, which suggests that the intimal layer is not only susceptible to internal but also to external stimuli[18]. Especially in this context, the results of this study can be of importance since it demonstrates that the LA body contains areas of discontinuous myocardium and areas without myocardium, e.g. in between the PV orifices, that might be the electrophysiological substrate of arrhythmias[13]. An increased susceptibility for arrhythmias may be explained by a difference in the composition of the extracellular matrix, the cellular junctions or the phenotype of these extrapericardially differentiated myocardial cells with regard to the intrapericardially differentiated myocardial cells of the sinus venosus and the atrial segment. These arrhythmias are amenable to catheter ablation but one must be alert that the vessel wall can be easily damaged due to a lack of myocardium. These considerations are specifically

important when performing ablation at the LA dorsal wall, that is in close contact to the esophagus. Recent studies have emphasized the risk of atrio-esophageal fistula due to the application of radiofrequency current at this site[19,20]. Lower dosage of radiofrequency current is therefore recommended when ablating in this area.

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3

Pulmonary Vein, Dorsal Atrial Wall and Atrial Septum Abnormalities in *Podoplanin* Knockout Mice with Disturbed Posterior Heart Field Contribution

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Abstract

The developing sinus venosus myocardium, derived from the posterior heart field, contributes to the atrial septum, the posterior atrial wall, the sino-atrial node and myocardium lining the pulmonary and cardinal veins, all expressing podoplanin, a coelomic and myocardial marker. We compared development and differentiation of the myocardium and vascular wall of the pulmonary veins, left atrial dorsal wall and atrial septum in wild type with *podoplanin* knockout mouse embryos (E10.5-E18.5) by 3-D reconstruction and immunohistochemistry. Expression of Nkx2.5 in the pulmonary venous myocardium changes from mosaic to positive during development pointing out a high proliferative rate compared to Nkx2.5 negative myocardium of the sino-atrial node and cardinal veins. In mutants, myocardium of the pulmonary veins, dorsal atrial wall and atrial septum was hypoplastic. The atrial septum and right sided wall of the pulmonary vein almost lacked interposed mesenchyme. Extension of smooth muscle cells into the left atrial body was diminished. We conclude that myocardium of the pulmonary veins, dorsal atrial wall and atrial septum as well as the smooth muscle cells are derived from the posterior heart field regulated by *podoplanin*.

Introduction

The developmental origin and molecular mechanisms controlling the formation of the myocardium of the pulmonary veins (PV) and the smooth muscle cells (SMCs) of the PV media are a matter of debate. The PV myocardial sleeve develops by either migration of myocardial cells from the left atrium (LA)[1] or by recruitment of extracardiac mesenchymal cells which differentiate into myocardial cells[2]. Based on expression patterns of *Nkx2.5*, *Islet1* and *Pitx2c* the LA and PV myocardium was suggested to be formed by the addition of myocardial cells from the second heart field at the venous pole[3].

The second heart field, part of the splanchnic mesoderm, is involved in the addition of (myocardial) cells to the primary heart tube at both the arterial (the *secondary* and *anterior* heart field) and the venous pole (the *posterior* heart field)[4]. *Islet1*, a marker of undifferentiated cardiac progenitor cells, is expressed throughout the second heart field[5]. Myocardial cells can differentiate from second heart field derived mesenchymal cells, and SMCs may also differentiate from mesenchyme[6].

The transmembrane glycoprotein podoplanin, a coelomic and myocardial marker expressed in the posterior heart field[7], has gained interest for its role in epithelial-to-mesenchymal transformation (EMT) and in formation of the myocardium and coronary SMCs at the venous pole of the heart[8]. It is found in the proepicardial organ, epicardium, sinus venosus myocardium including the sino-atrial node, the PVs and cardinal veins, the dorsal atrial wall and the base of the atrial septum extending into the developing atrial and ventricular cardiac conduction system. *Podoplanin* promotes EMT by binding ezrin, radixin, moesin (ERM) proteins that activate RhoA, and by downregulation of the cell-to-cell adhesion molecule E-cadherin[9]. Lack of *podoplanin* leads to altered EMT and e.g., to abnormal formation of sinus venosus myocardium and decreased numbers of SMCs of the coronary artery media[8]. In extra cardiac tissues *podoplanin* functions as a possible signalling molecule and is involved in the development of osteoblasts[10] intestinal and alveolar epithelium[11] podocytes and mesothelium of the visceral peritoneum[12] and lymphatic endothelium[13]. During cardiac development, podoplanin is expressed specifically in the second heart field derived myocardium and mesenchyme at the venous pole of the heart[7,8].

Our previous study in humans[14] demonstrated that during incorporation of the PVs in the LA body, the inner lining of the LA body presents vessel wall tissue. The outer layer of the LA is formed by myocardium covered by epicardium. The origin of the SMCs of the LA dorsal wall is still a topic of discussion.

In the present study, we have compared the morphology and development of the wall of the PVs, LA dorsal wall and atrial septum between wild type and *podoplanin* knockout mouse embryos of embryonic stages (E) 10.5-18.5. We hypothesize that the posterior heart field is involved in the formation and differentiation of both myocardial cells and SMCs of the PVs and LA dorsal wall regulated by *podoplanin*.

Material and methods

Generation of the embryonic and neonatal mice

This study was performed at the department of Anatomy & Embryology of the Leiden University Medical Center and was approved by the Animal Research Committee. *Podoplanin* knockout mice were generated by homologous recombination in embryonic stem cells from the 129S/v mouse line as reported previously[8].

General description

We investigated the morphology and development of the sinus venosus region of the heart, especially the PVs, LA dorsal wall and atrial septum in 32 wild type mouse embryos of E10.5 (n=4), E11.5 (n=3), E12.5 (n=4), E13.5 (n=5), E14.5 (n=4), E15.5 (n=3), E16.5 (n=3), E17.5 (n=3) and E18.5 (n=3), and compared these with 28 *podoplanin* knockout mouse embryos of E10.5 (n=4), E11.5 (n=5), E12.5 (n=5), E13.5 (n=3), E14.5 (n=4), E15.5 (n=4) and E18.5 (n=3).

Immunohistochemistry

Immunohistochemistry was performed with antibodies against alpha-smooth-muscle actin (1A4, 1:2000, Sigma Aldrich, Product No.A2547, USA) to detect differentiated SMCs and developing myocardium, atrial myosin light chain 2 (MLC2a, 1/6000, kindly provided by S.W. Kubalak, Charleston, SC, USA) specific for (atrial) myocardium, NK2

transcription factor related locus 5 (Nkx2.5, 1/4000, Santa Cruz Biotechnology, Inc., CA, USA, SC-8697) as an early marker of undifferentiated cardiac progenitor cells, and podoplanin (clone 8.1.1., 1/500, Hybridomabank, Iowa, USA) as a marker of the posterior heart field myocardium. Fixation, preparation and staining procedures were completed according to standard protocols[8].

3-D reconstructions

We made 3-D reconstructions, as described previously[8], of the composition of PV, LA dorsal wall and atrial septum of MLC-2a and 1A4 stained sections of *podoplanin* wild type and knockout embryos (E15.5).

Morphometry

Sinus venosus myocardial volume estimation was performed of 12 wildtype mouse hearts of E11.5 (n=3), E12.5 (n=3), E15.5 (n=3) and E18.5 (n=3) and 12 *podoplanin* knockout hearts of E11.5 (n=3), E12.5 (n=3), E15.5 (n=4) and E18.5 (n=2). Statistical analysis was performed with an independent sample-*t*-test ($P<0.05$) using SPSS 11.0 software (SPSS Inc, Chicago, III) as described previously[8].

Results

Podoplanin mutants demonstrate marked mesenchymal and myocardial abnormalities, including hypoplasia of the pro-epicardial organ, abnormal epicardium, hypoplastic chamber myocardium, atrial, ventricular and atrioventricular septal defects, and an abnormal coronary arterial vascular wall[7,8]. These abnormalities are related to diminished EMT which was reported in a previous paper[8]. Below, the morphological development of the wall of the PV, LA and atrial septum will be described in subsequent stages. Abnormal PV connections were not observed. In Table 1a sinus venosus myocardial volumes between wild type and knockout mice are compared. Table 1b provides an overview of qualitative expression of myocardial and SMCs markers used.

Myocardial Morphometry

a

E	WT		*Podoplanin*$^{-/-}$		t-test p value
	Myocardial volume (mm^3)	SD	Myocardial volume (mm^3)	SD	
11.5	0.0094 (n=3)	0.0029	0.0050 (n=3)	0.0017	0.0457
12.5	0.0120 (n=3)	0.0025	0.0071 (n=3)	0.0032	0.0348
15.5	0.0424 (n=3)	0.0052	0.0300 (n=4)	0.0047	0.0108
18.5	0.2871 (n=3)	0.0307	0.1688 (n=2)	0.0287	0.0115

Expression of Markers

b

E	Markers	PV wall		LA dorsal wall		Atrial septum		Media PV	
		WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
10.5	MLC2a	++	+	++	+	Not Developed Yet			
	Nkx2.5	++	+	++	+				
	Actin	++	+	++	+				
12.5	MLC2a	++	+	+++	++	+++	++	++	++
	Nkx2.5	++	+	++	++	++	++	++	++
	Actin	+	+	+	+	+	+	-	-
15.5	MLC2a	+++	+++	+++	+++	+++	+++	+	+
	Nkx2.5	+++	+++	+++	+++	+++	+++	+	+
	Actin	-	-	-	-	-	-	++	+
18.5	MLC2a	+++	+++	+++	+++	+++	+++	-	-
	Nkx2.5	+++	+++	+++	+++	+++	+++	-	-
	Actin	-	-	-	-	-	-	+++	+

Table 1. a: Sinus venosus myocardial volume estimation of 12 wild type (**WT**) and 12 *podoplanin*$^{-/-}$ mouse hearts. In all stages, mutants have a significant smaller myocardial volume ($P<0.05$) compared to WT embryos. E: embryonic day, SD: standard deviation. b: A qualitative overview of the expression of markers at several locations in *podoplanin* WT and knockout (**mutant**) embryos. At early stages, the expression of all markers is diminished in mutants compared to the WT embryos, whereas in advanced stages these differences are no longer apparent, indicating delayed differentiation in mutants. Left atrium (**LA**); pulmonary vein (**PV**). +++strong, ++medium, +weak, -absent expression.

E10.5

In the right part of the common atrium, the right and left venous valves delineated the ostia of the cardinal veins entering the sinus venosus. In both wild type and knockout

embryos, MLC2a was expressed in the sinus venosus and chamber myocardium (Fig.1a,c-e). In the mesenchyme of the dorsal mesocardium in wild type embryos, the lumen of the primitive PV was surrounded by a mainly left sided concentration of MLC2a positive myocardium that stained, however, less markedly for MLC2a compared to the common atrium (Fig.1a,d). In knockouts, this myocardium was thinner with clearly diminished MLC2a expression in a larger part in the dorsal mesocardium and left part of the atrial dorsal wall (compare Fig.1d with e). This diminished expression was seen in the region normally expressing podoplanin (Fig.1b, Table 1b). In wild types the primitive PV myocardium in the region of the dorsal mesocardium had mosaic Nkx2.5 expression, defined as a mixture of Nkx2.5 positive and negative cells. Similar to MLC2a expression, the Nkx2.5 mosaic myocardial cells were mainly situated on the left side of the dorsal mesocardium (Fig.1f). Moreover, in knockouts, this area was hypoplastic showing less Nkx2.5 expressing cells (compare Fig.1f with g).

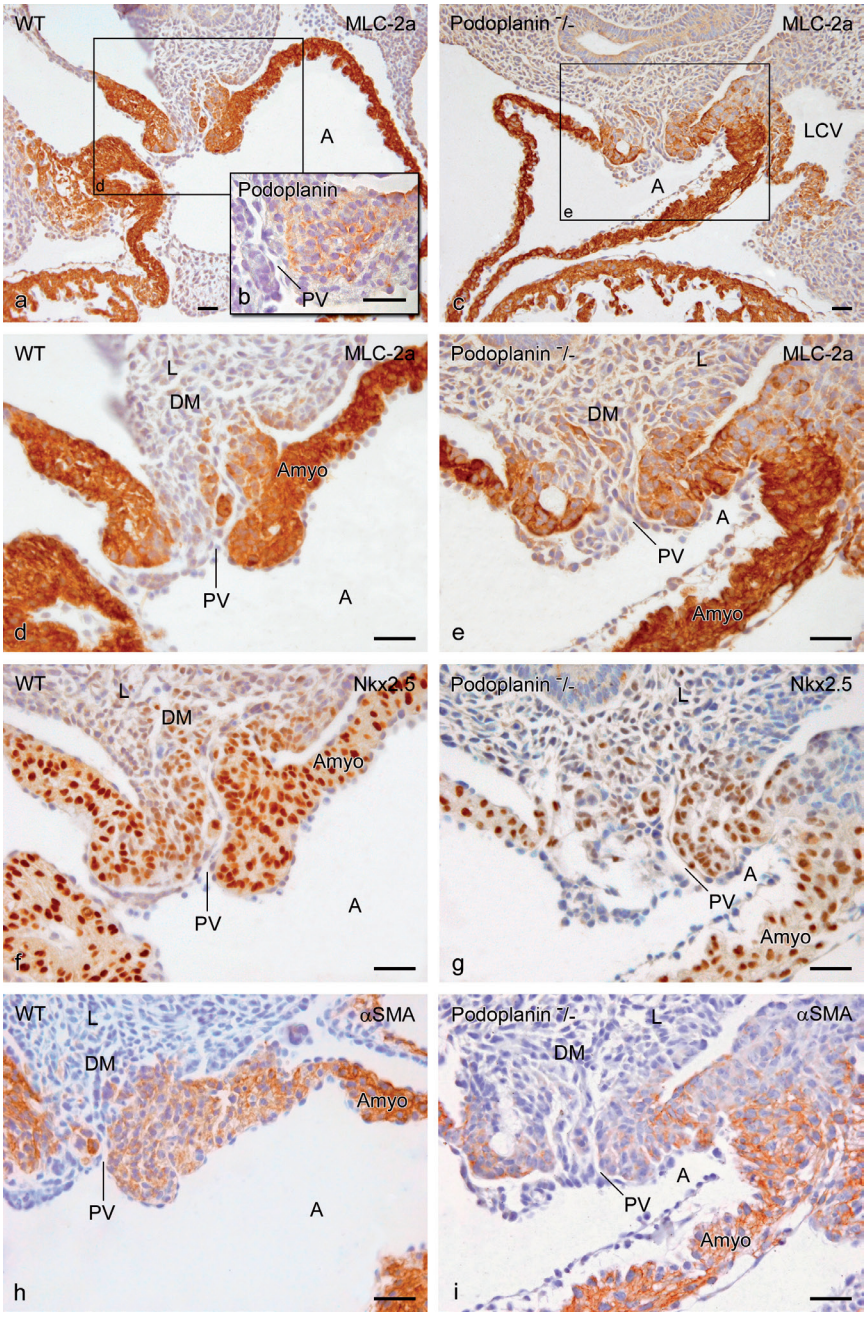
The alpha-smooth-muscle actin antibody 1A4 was co-expressed in the MLC2a-stained myocardium of the common atrium and ventricle of the wild type embryos. Similar to MLC2a, actin expression around the primitive PV and left part of the atrial dorsal wall was weaker (Fig.1h). In mutants, actin expression was diminished in a larger region compared to the wild type (Fig.1i).

E12.5

The common PV bifurcated into tiny left and right PVs, covered by a MLC2a positive myocardial layer which, in contrast to E10.5, could now be observed in equal density on both sides of the PV (Fig.2a,c). MLC2a expression around the PV was weaker than the expression in the myocardium of the atria (Fig.2a,c). In the dorsal mesocardium and around the PV Nkx2.5 expression was mosaic, while in the wall of the cardinal veins Nkx2.5 staining was negative (Fig.2d). In the atrial myocardium of the wild type embryos MLC2a as well as Nkx2.5 were positive, while smooth muscle actin had almost disappeared, particularly in the LA dorsal wall (Fig.2e). In the myocardium of the ventricles (not shown) and the wall of the cardinal veins (Fig.2e) smooth muscle actin was still present. Actin expression in the wall of the PV was more extensive than the MLC2a expression (Fig.2e).

In the *podoplanin* knockout embryos, MLC2a (Fig.2f,g) and actin positive (Fig.2i) as well as Nkx2.5 mosaic (Fig.2h) myocardium around the PV was thin and locally even absent (compare Fig.2a,c with f,g) in a region where podoplanin expression was seen in wild type embryos (Fig.2b). The LA dorsal wall and the atrial septum were thin and the myocardium was hypoplastic (compare Fig.2j,k with l,m). The atrial septum showed a large secondary foramen and myocardialization at the base of the atrial septum was absent (Fig.2k,m). Additionally, the atrioventricular cushion was not fused properly to the top of the ventricular septum resulting in a interventricular communication (Fig.2j,l).

Figure 1. Transverse sections of *podoplanin* wild type (**WT**,a,b,d,f,h) and knockout (Podoplanin-/-,c,e,g,i) mouse embryos of stage (E)10.5 comparing the development of the pulmonary vein (**PV**). Boxed area in a and c are magnified in d and e. MLC2a expression is seen around the primitive PV on the left side of the dorsal mesocardium (**DM**) (a,d) where also podoplanin (b) and mosaic Nkx2.5 expression (f) is seen. Compared to the atrial myocardium (**Amyo**) the MLC2a expression at the DM and atrial (**A**) dorsal wall is weaker corresponding with the expression pattern of alpha-smooth-muscle actin (α**SMA**, h), which marks the developing myocardium. In knockouts, a larger region shows less expression of MLC2a (c,e), Nkx2.5 (g) and αSMA (i), corresponding with altered differentiation rate of myocardium at these regions. Left cardinal vein (**LCV**), Lung (**L**). Scale bars 30μm.


WT
MLC-2a
A
d
Podoplanin
a
b
PV
Podoplanin ¯/-
MLC-2a
LCV
A
e
c
WT
L
MLC-2a
DM
Amyo
PV
A
d
Podoplanin ¯/-
L
MLC-2a
DM
A
PV
Amyo
e
WT
L
Nkx2.5
DM
Amyo
PV
A
f
Podoplanin ¯/-
L
Nkx2.5
A
PV
Amyo
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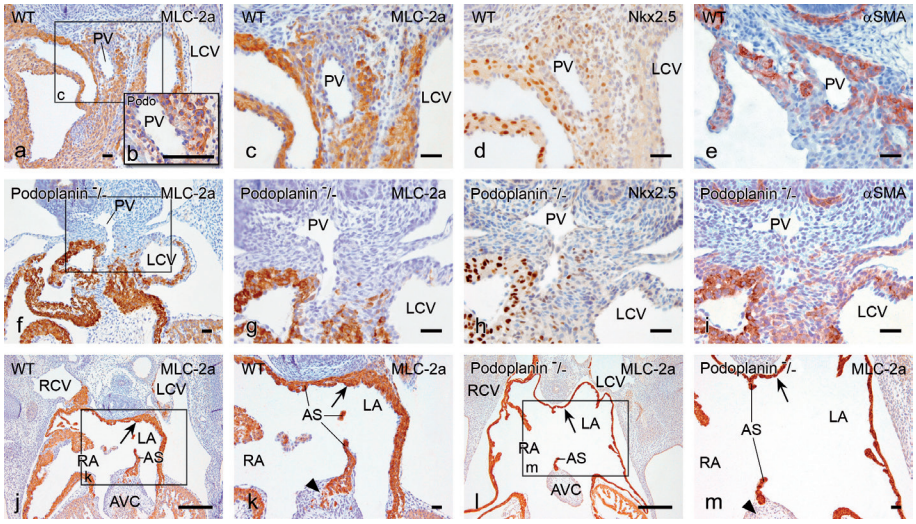


Figure 2. Transverse sections of *podoplanin* wild type (**WT**,a-e,j,k) and knockout (Podoplanin-/-,f-i,l,m) mouse embryos of stage (E)12.5 comparing the development of the pulmonary vein (**PV**,a-i), atrial septum (**AS**,j-m) and left atrial (**LA**) dorsal wall (j-m). In WT embryos PV is surrounded by myocardium expressing MLC2a (a,c), podoplanin (b) and mosaic Nkx2.5 (d) and completely positive for alpha-smooth-muscle actin (α**SMA**,e). In mutants the expression of these markers around the PV is almost absent (compare a,c-e with f-i) and myocardium of the LA dorsal wall is hypoplastic (compare arrow in j,k with l,m). The AS in the knockouts is thin and deficient with a large secondary foramen (k,m). Moreover, myocardialization process of the AS with the deficient atrioventricular cushion (**AVC**, compare j with l) is absent (see arrowhead in k and m), which might cause an atrioventricular septal defect. Boxed area in a,f,j and l are magnified in c,g,k and m. Left cardinal vein (**LCV**), Right atrium (**RA**), Right cardinal vein (**RCV**). Scale bars a-i,k,m 30µm, j,l 200 µm.

E15.5

In both wild types and knockouts, the myocardium of the atria, the atrial septum and of the wall of the cardinal and PVs expressed MLC2a (Fig.3a-f). In mutants, the myocardium of the atrial septum and the PVs was hypoplastic (compare Fig.3c,d with e,f). Moreover, compared to wild type, the PVs in knockout embryos seemed to be dilated (compare Fig.3c with e). In both wild type and knockout embryos, the Nkx2.5 mosaic expression in the wall of the PV became overall positive (Fig.3g,i).

In wild type embryos 1A4 co-staining was specifically observed in the MLC2a positive sub-endothelial layer of the PVs and LA dorsal wall (Fig.3h). In the outer MLC2a positive myocardial layer of the wall of the PVs, cardinal veins, dorsal atrial wall and the entire atrial myocardium (not shown), 1A4 staining had disappeared (Fig.3h). In

knockout embryos, smooth muscle actin was almost absent in the sub-endothelial layer of the PVs and LA dorsal wall, which still showed MLC2a staining (compare Fig.3a,h with b,j). In areas with 1A4 expression, distribution of positive cells was discontinuous compared to the wild type embryos (compare Fig.3h with j).

E18.5

The common part of the PVs was incorporated into the LA. In both wild type and knockout embryos MLC2a expressing myocardium was seen in the atria and the wall of the cardinal and PVs, while the myocardium now extended into the lungs (Fig.4a,b). Similar to previous stages, the myocardium was hypoplastic in knockouts, with dilation of the atria, the cardinal and PVs (compare Fig.4a,b with c,d). In the dorsal mesocardium less mesenchymal cells were observed compared to the wild type (compare asterisk in Fig.4 a with b).

In the PV and LA dorsal wall of the wild type embryos, smooth muscle actin (1A4) was present between endothelium and MLC2a positive myocardium (Fig.4e). Hence, the 1A4 positive actin layer was MLC2a negative in contrast to the previous stages (compare Fig.3d,h with Fig.4c,e). The 1A4 staining at other parts of the heart was absent (not shown), except for the wall of the cardinal veins and ventricular myocardium. Again, hardly any 1A4 staining was seen in the sub-endothelial layer of the PV and LA dorsal wall of the *podoplanin* knockout embryos (compare Fig.4e with f).

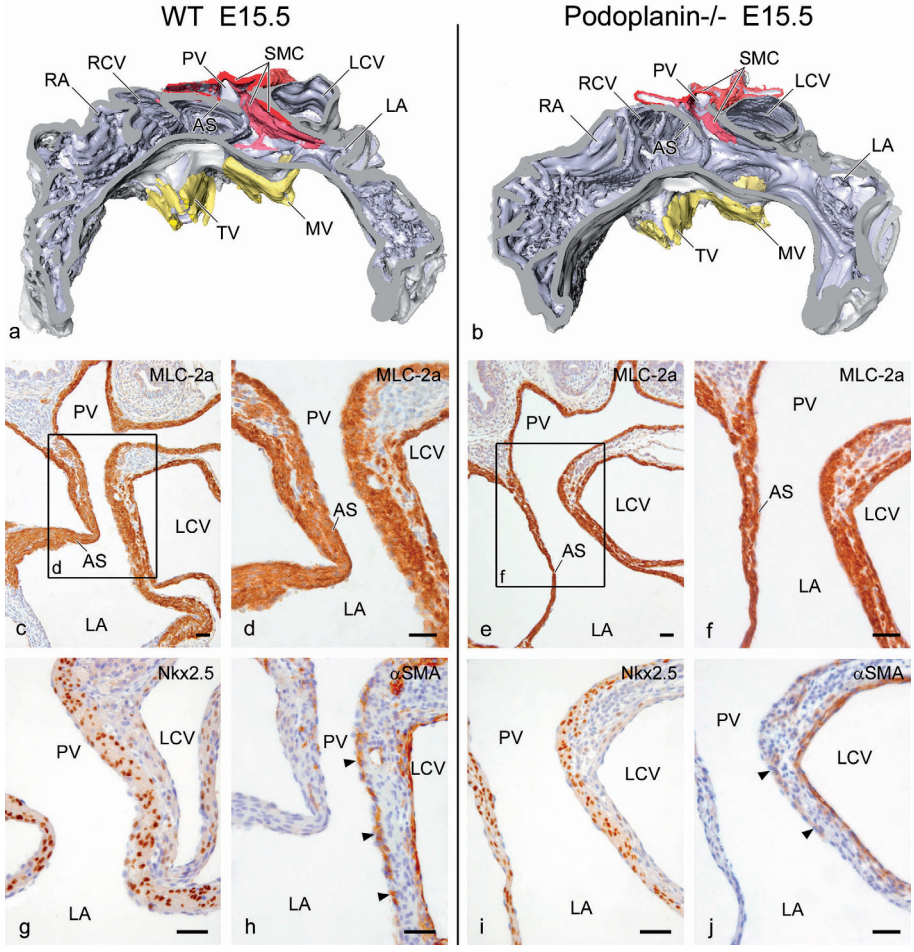


Figure 3. 3-D reconstructions (a-b) and transverse sections (c-j) of *podoplanin* wild type (**WT**,a,c,d,g,h) and knockout (Podoplanin -/-,b,e,f,i,j) mouse embryos of stage (E)15.5 showing hypoplasia of atrial septum (**AS**) and altered smooth muscle cells (**SMC**,red) formation in the wall of the common pulmonary vein (**PV**) and the posterior wall of left atrium (**LA**) of the mutants. a and b are a cranial view of the PV orifice into the LA showing the diminished amount of SMCs in the PV and LA dorsal wall in knockouts. Boxes in c and e are the positions of the enlargements in d and f. Compared to WT embryos, the MLC2a positive myocardium of the AS, the left cardinal vein (**LCV**) and the somewhat dilated PVs in knockouts is hypoplastic with interposition of less mesenchymal tissue (compare c,d with e,f). The myocardium of the wall of the PV is differentiated and is seen as a myocardial cuff around the PV, which expresses Nkx2.5 (g,i). In WT embryos SMCs (arrowheads in h) are incorporated into the LA, whereas in knockouts SMCs are almost absent (arrowheads in j). Mitral valve (**MV**,yellow), Right atrium lumen (**RA**,light gray), Right cardinal vein lumen (**RCV**,light gray), tricuspid valve (**TV**,yellow). Color codes: light gray (lumen), dark gray (MLC2a positive myocardium). Scale bars 30µm.

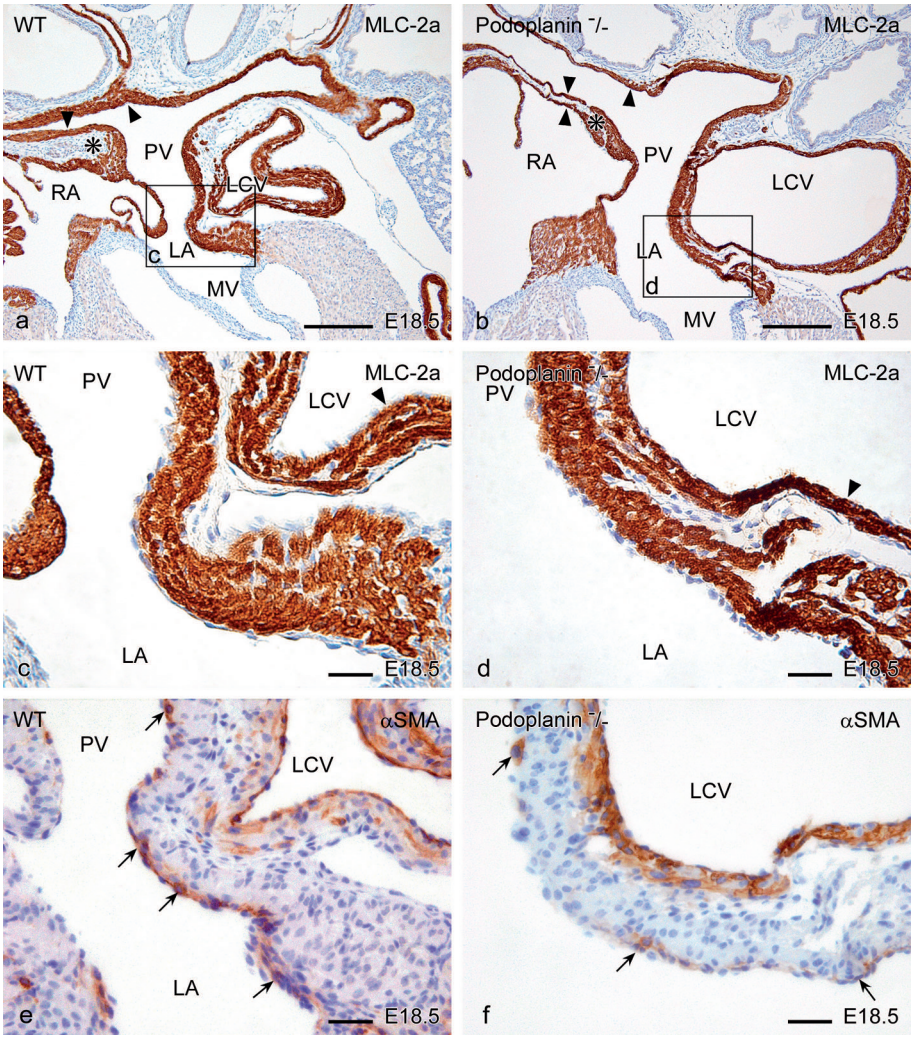


Figure 4. Transverse sections of *podoplanin* wild type (**WT**,a,c,e) and knockout (Podoplanin-/-,b,d,f) mouse embryos of stage (E) 18.5 comparing the development of pulmonary vein (**PV**). Sections a-d are stained with MLC2a as myocardial marker and sections e and f are stained with alpha-smooth-muscle cell actin (**αSMA**). Boxes in a,b are the positions of the enlargements in c,d. a,b. MLC2a expression is seen in WT and mutants in the myocardium of left (**LA**) and right atrium (**RA**), left cardinal vein (**LCV**) and pulmonary veins (**PV**) (a,b). In knockouts, these structures are dilated and have hypoplastic myocardium (arrowheads in a-d) with interposition of less mesenchymal tissue (a,b; asterisks). Smooth muscle cells (SMC) are seen in the sub-endothelial layer of the PV and LA dorsal wall (arrows in e). In the mutants, SMCs are almost absent, which might be caused by either impaired SMC formation or differentiation due to the lack of *podoplanin* (compare arrows in e with f). Mitral valve (**MV**). Scale bars a,b 200μm; c-f 30μm.

Discussion

During human development, a vascular lining comprising SMCs and an adventitia will evolve at the inside of the LA body, that is lined on the outside by atrial myocardium[14]. Findings in the current study show a similar phenomenon in the LA of wild type mouse embryos. A difference between mouse and human specimen concerns the extension of the myocardial tissue surrounding the PV into the lung. The merits of our current study are that the posterior heart field plays a role in the addition of not only PV myocardium, but also in the formation and differentiation of SMCs that line the LA body. We demonstrated hypoplasia of the myocardium of the PV, LA dorsal wall and the atrial septum in *podoplanin* knockout mice. The vessel wall of the PVs and its extension into the LA was underdeveloped in knockout embryos.

Myocardial development

It has been reported earlier that the myocardial sleeve around the PV is formed by either migration of existing atrial cardiomyocytes[1], or by recruitment and differentiation of mesenchymal cells of the splanchnic mesoderm[2]. The latter supports earlier advances in the study of cardiac development that underline the relevance of addition of myocardium to the primary heart tube[15]. The second heart field[5] or second lineage[4,5] concerns an anteroposterior extension of splanchnic mesenchyme from where cells are recruited for addition to both the arterial pole and the venous pole of the heart. Several studies have been performed using different lineage markers such as *fibroblast growth factor* (*Fgf*)*8* and *10*[16], *Islet* (*Isl*)*1*[5] and *Tbx1* and *18*[17,18] to trace these cells into their cardiac destination. Special interest was raised in markers specific for recruitment of myocardium from the second heart field to the venous pole of the heart such as *Pitx2c*[3], *Nkx2.5*[3,7], *Shox2*[19] and podoplanin[7,8]. *Podoplanin* promotes EMT by binding ERM proteins that activate RhoA and by downregulation of the cell-to-cell adhesion molecule E-cadherin[8,9]. In *podoplanin* knockout mice E-cadherin is upregulated causing abnormal EMT which may lead to abnormal formation of myocardium at the venous pole of the heart[8]. Thus, the hypoplasia of the myocardium of the wall of the PVs, atrial septum and LA dorsal wall observed in the current study in *podoplanin* knockout mice could be explained

by impaired addition of myocardium from the posterior heart field due to abnormal EMT by lack of *podoplanin*.

Another explanation for the myocardial hypoplasia in the *podoplanin* knockouts could be abnormal epicardial-myocardial interaction. Previously we demonstrated that altered epicardial-myocardial interaction leads to deficient ventricular myocardial formation in *SP3* mutants[20] as well as in *podoplanin* mutant embryos[8]. In *SP3* mutants, WT-1 expression, a transcription factor involved in development of epicardium derived cells (EPDCs)[21], was downregulated and the mutant hearts showed EPDC-related cardiac abnormalities comparable to *podoplanin* mutants. The impaired formation of EPDCs and altered epicardial-myocardial interaction, resulting in hypoplastic atrial and ventricular myocardium in *podoplanin* knockouts, has already been described and related to the deficient contribution of the posterior heart field[8,22]. Therefore, the hypoplasia of the PVs, atrial septum and LA dorsal wall reported in the current study could be related to the altered contribution of epicardium and myocardium from the posterior heart field.

In early stages, Nkx2.5 and alpha-smooth-muscle actin (1A4) are expressed in undifferentiated myocardium. At these stages, Nkx2.5 is mosaic around the PVs and absent in the wall of the cardinal veins[7], whereas actin expression is present in the wall of the pulmonary and cardinal veins. The Nkx2.5 mosaic expression in the wall of the PVs rapidly becomes completely positive concomitant with a higher proliferation rate of the PV myocardium compared to the cardinal veins[3]. Consequently, 1A4 expression from E15.5 on is confined to the medial layer of the vascular wall of the pulmonary and cardinal veins. These findings suggest that the pulmonary and cardinal veins have a common precursor derived from the posterior heart field[7], but a distinct proliferation rate[3] accounting for a distinct differentiation based on Nkx2.5 expression.

Expression of markers in putative PV myocardium starts at the left side of the dorsal mesocardium, indicating that PV myocardium is preferentially added from the left side of the posterior heart field, regulated by progenitor cells that play a role in left-right patterning, as was reported for Pitx2c[3,23,24]. Next to hypoplasia, mutants showed diminished expression, that was predominantly observed in the earlier stages (Table 1a,b). As in later stages these differences were no longer apparent, this suggests a delayed differentiation of the myocardium and SMCs in mutants.

Smooth muscle cell development

The origin of the SMCs at the venous pole is as yet not well understood. SMCs may differentiate from mesenchymal cells[6]. At the arterial pole formation and differentiation of SMCs has been reported from the splanchnic mesoderm or from the neural crest[25]. The latter process requires cross-talk between the endothelial and the muscular component[26]. DeRuiter and colleagues have described the formation of the SMCs of the dorsal aorta by transdifferentiation from endothelial cells[27].

Research in this field is complicated as alpha-smooth-muscle actin also stains the primitive myocardium. We demonstrated that this staining was less extensive in the developing myocardium of the *podoplanin* mutant mice, supporting delayed or defective myocardial differentiation. At the stage that normally alpha-smooth-muscle actin disappears from the myocardium, only the SMCs retain their expression of this marker. In *podoplanin* knockout mice we observed a diminished extension of SMCs in the LA dorsal wall as compared to normal. This phenomenon may be caused by impaired formation of SMCs from the posterior heart field derived dorsal mesocardium or abnormal or delayed differentiation of the SMCs in the PV and LA body in absence of *podoplanin*. The disturbed EMT in *podoplanin* knockouts probably leads to abnormal formation of SMCs as was shown for the coronary artery SMCs development in mutant mice[8].

In conclusion, as *podoplanin* is a marker of the myocardial and mesenchymal cells derived from the second heart field at the venous pole of the heart, this study supports evidence that the myocardium of the PVs, LA dorsal wall and atrial septum is derived from the posterior heart field. Moreover, we can state that *podoplanin* not only plays a role in the development of myocardium, but also in the formation of the SMCs.

The clinical relevance of our findings needs further research. In the mutants, complex atrial defects were observed. In the majority, there was fusion of the primary atrial septum with the AV cushions, but the secondary foramen was enlarged and the AV cushions did not fuse properly with the ventricular septum resulting in an AVSD with shunting at the ventricular level. These findings were described in a previous study[8].

The study of human and mouse models with isomerism, atrial arrhythmias and cases with abnormal pulmonary venous return are on their way. More insight into the variation in myocardial cuffing of the PVs in the human population might enlighten us on the variability of occurrence of ectopic automaticity in the PV myocardial sleeve[28], which is suggested by the finding that the length of the PV sleeve corresponds to the frequency of occurrence of ectopic PV beats as observed in electrophysiological studies[28,29]. In podoplanin knockout embryos, we have observed deficient sinus venosus myocardium with myocardial discontinuities. Areas lacking myocardium can be regarded as low voltage areas (comparable to scar tissue) that may form the substrate of reentry circuits. Electrophysiological testing in mutant mice is necessary to further investigate this hypothesis.

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4

Pulmonary Vein and Atrial Wall Pathology in Human Total Anomalous Pulmonary Venous Connection

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Abstract

Background. Normally, the inside of the left atrial (LA) body and pulmonary veins (PVs) is lined by vessel wall tissue covered by myocardium. In total anomalous pulmonary venous connection (TAPVC), no connection of the PVs with the LA body exists. These veins have an increased incidence of PV stenosis. We describe the consequences of the absent connection for the histopathology of the wall of the LA body and the PVs, and hypothesize on a mechanism predisposing to PV stenosis.

Methods and results. In 10 human neonates with TAPVC, the wall of the LA body and PVs were studied using histological and immunohistochemical techniques. As controls, 2 normal neonatal and adult hearts and 5 neonatal hearts with partial anomalous venous connection (PAPVC) or situs inversus were studied. In hearts with TAPVC, no vessel wall tissue was found in the LA body and its myocardial layer was hypoplastic. No myocardial sleeve was found around the abnormally draining PVs. In hearts with PAPVC, only the non-LA draining PV lacked myocardial covering, whereas in situs inversus, PVs connecting to the right-sided LA, were normally myocardialized.

Conclusion. An open connection of the PVs with the morphological LA is necessary for the presence of vessel wall tissue in the LA and myocardialization of the PVs. Absence of myocardium covering the PVs is hypothesized to enhance susceptibility to PV stenosis and prevent onset of PV originating arrhythmias. The embryonic posterior heart field may be responsible for the abnormal myocardialization and smooth muscle cell formation in TAPVC.

Introduction

In human cardiac development, at the end of the fifth week, in the dorsal mesocardium the common pulmonary vein (PV) lumenizes, thus forming an open connection between the pulmonary vascular network and the left atrium (LA)[1-4]. Therefore, connections to the systemic circulation[3,5] have become non-functional and regress. Thereafter, a myocardial sleeve, which is continuous with the LA myocardium, is formed around the common PV. The origin of this myocardial layer lining the PVs is a topic of debate. Either LA cardiomyocytes extend into the extracardiac mesenchyme and cover the PVs[6-8], or non-cardiac cells are recruited from the splanchnic mesenchyme and differentiate into myocardial cells which line the PVs[9-11]. Recently, in mice a biphasic process of formation of PV myocardium was described, favouring both points of view[12].

From the tenth week onwards, the PVs grow and are incorporated into the also expanding dorsal wall of the LA. This process results in a smooth-walled LA body consisting of vessel wall tissue on the inner side covered by myocardium on the outside[13]. In hearts with total anomalous pulmonary venous connection (TAPVC), the common PV forms no open connection between the splanchnic plexus and the LA, thus resulting in persistence of (one of) the cardinal veins draining the pulmonary venous blood to the systemic venous circulation[3] which can be divided in a supracardiac, a cardiac and a infracardiac type of drainage. At presentation and especially after repair of anomalous pulmonary venous connections, increased rates of PV stenosis are reported, suggesting susceptibility of these veins to PV stenosis[14-16]. A remarkably low incidence of significant arrhythmias in these patients is reported in literature[17].

Our previous findings[13] have shown that the common PV connects to and incorporates into the expanding LA dorsal wall resulting in vessel wall tissue lining the smooth wall of the LA and a covering of the PVs by a myocardial layer. We hypothesize that, if this process does not take place, as in hearts with TAPVC, there will be an abnormal histology of the structures involved, which may also have clinical consequences. We also postulate on the basis of mouse model studies[12] that PV myocardialization is a genetically left-sided regulated process.

Methods

We studied 5 neonatal heart-lung specimens from the Leiden collection with supracardiac (n=4) or infracardiac (n=1) TAPVC as well as biopsy material of another 5 neonates, taken intra-operatively prior to surgical correction, of either the supracardiac (n=3) or infracardiac (n=2) type of TAPVC. These patients were operated in the University Medical Center of Groningen or Rotterdam. The local Medical Ethical Committee approved the studies. The age of the neonates ranged from 1 day to 2 years. Hearts with atrial isomerism were excluded.

Controls.

Normal neonatal (n=2) and adult hearts (n=2) were used as controls. Three neonatal heart-lung specimens from the Leiden collection with cardiac (n=1) and supracardiac (n=2) partial abnormal pulmonary venous connection (PAPVC) were used to study the myocardialization process of PVs when normal and abnormal pulmonary venous drainage coexist. We additionally examined two neonatal heart-lung specimens with situs inversus to study the influence of laterality, in this case of a right-sided position of the LA, on the myocardialization of the PVs.

The adult hearts were obtained from human bodies used in anatomy teaching lessons. The cause of death was unknown due to privacy regulations.

Samples/sectioning of material:

Study material. From the neonatal heart-lung specimens with TAPVC, tissue blocks were taken from the dorsal wall of the smooth-walled LA body, the LA appendage and the PVs. The surgical biopsies obtained from patients of the University Medical Center of Groningen and Erasmus Medical Center Rotterdam were derived from the LA dorsal wall and the pulmonary venous confluence (exactly taken at the site of the incisions necessary for the correction) and, in some cases, the vertical vein.

Controls.

Normal neonatal and adult hearts. Tissue blocks were taken from the LA dorsal wall and the PVs. In adults, tissue blocks were also taken from the smooth-walled

posterior and anterior right atrial (RA) body, the RA appendage, both caval veins, the smooth-walled RA body above the tricuspid valve and the coronary sinus to compare the presence of vessel wall in the LA and RA.
*Neonatal hearts with abnormalities***.** From the specimens with PAPVC, samples of the normally as well as abnormally draining PVs were taken. In hearts with situs inversus, tissue blocks were taken from the PV-LA transition.

The samples were embedded in paraffin and sectioned (5 µm) using a Leica microtome. The sections were mounted onto slides using chicken albumin dissolved in glycerine (1:1) and dried at 37 °C for ≥ 24 h.

Staining: Standard histological staining procedures were performed with Hematoxylin-Eosin, Verhoeff-Van Gieson or elastic Resorcin-Fuchsin to detect elastic filaments. Additional sections were stained immunohistochemically with a mouse monoclonal against alpha smooth muscle (SM) actin (1A4, 1:10.000, Sigma Aldrich, Product No. A2547, USA), indicating the presence of a vascular wall, and a rabbit polyclonal antibody against atrial myosin light chain (MLC2a, 1:2000, a generous gift of Steve Kubalak), specific for atrial myocardium. After incubation with the primary antibodies overnight, antibody binding for 1A4 was demonstrated with rabbit anti-mouse peroxidase (RAM-PO, 1:250, Dako, Product No. P0260, Denmark) and for MLC2a with goat anti-rabbit biotin (GAR-biotin, 1:200, Vector labs, Product No. BA-1000, USA). The latter procedure was followed by a rabbit biotinylated reagent (ABC, 1:100, Vector Labs, Product No. PK-6100). After sections were rinsed with phosphate buffered saline and tris/maleate (pH 7.6), 3-3' diaminobenzidine tetrahydrochloride (DAB) was used as chromogen and counterstaining was performed with Mayer's haematoxylin. The remaining part of the staining procedures was performed as described previously[1].

Results

Normal neonatal and adult hearts.

In the PVs, a characteristic venous vessel wall was found, consisting of an intimal layer, a medial layer with smooth muscle cells, embedded in a matrix of collagen and elastic fibers and an adventitial layer with fibroblasts and vasa vasorum. On the outside, the PVs were covered by a myocardial sleeve which tapered off towards the lung hilum. In the entire smooth-walled LA body, a prominent vessel wall layer, continuous with that of the PVs, was found, covered on the outside by myocardium. At the transition of the PV ostia and the LA body no histologic veno-atrial demarcation was found. In contrast to the neonatal hearts, in the PVs and LA body of adult hearts intimal thickening was observed, most profound in the PVs (Fig.1a-f).

The RA dorsal wall of the adult hearts was smooth-walled and consisted of myocardium. The inner side of the RA body wall near the superior caval vein consisted of a layer of endocardium overlying a discontinuous layer of elastic fibers, generally indicated as the lamina elastica interna. Between these layers and the myocardium, a vascular tunica media was observed, continuous with that of the superior caval vein (Fig.2a,b). This smooth muscle cell layer was also seen in the region of the orifice of the inferior caval vein. In the circumference of the orifice of the coronary sinus no smooth muscle cells were found. The coronary sinus itself contained a proper tunica media as seen in the superior caval vein, covered on the outside by a myocardial layer (Fig.2c,e). In the remaining part of the smooth-walled RA body no vessel wall tissue was observed (Fig.2c,d).

The RA and LA appendages were trabeculated and lined by endocardium with a subendocardial presence of elastin and collagen fibers and an occasional smooth muscle cell, and a myocardial layer on the outside. No vessel wall layer was present (Fig.2f,g).

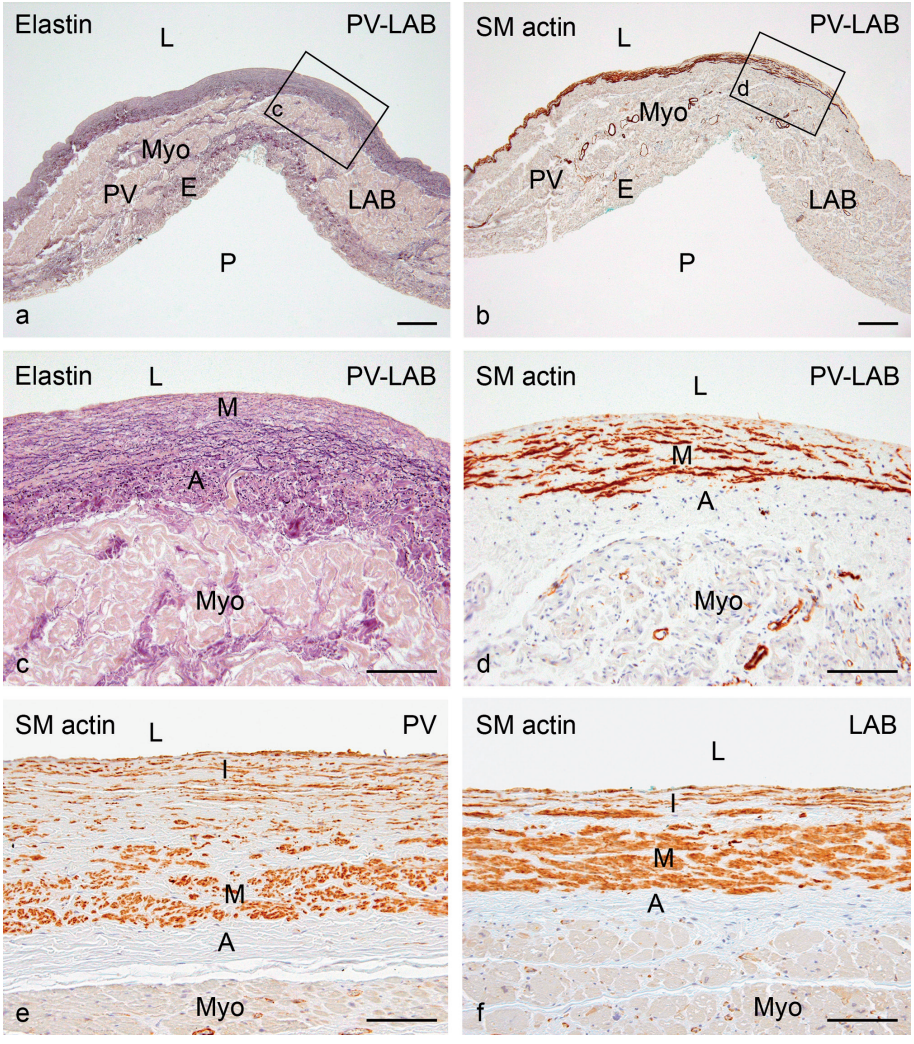


Figure 1a-f

Sections of a normal neonatal heart (a-d**)** and adult heart (e,f**)** stained with elastic Resorcin-Fuchsin (a,c; **Elastin**) and alpha smooth muscle actin (b,d-f; **SM actin**), showing the histological structure of pulmonary venous and left atrial wall. Boxes in a,b indicate the positions of the enlargements in c,d.

a-d. At the anatomical transition of pulmonary veins (**PV**) and LA body (**LAB**), no histologic veno-atrial demarcation is found. The wall consists of endothelium/endocardium, a medial layer (**M**), with smooth muscle cells and elastic fibers, an adventitial layer (**A**), myocardium (**Myo**), and an outer epicardium (**E**).

e,f**.** In the PV as well as in the LAB, conforming to the sections in a-d, an identical and characteristic vessel wall is found. In contrast to the neonatal stage profound intimal thickening (**I**) is observed most obviously in the PV. Lumen (**L),** pericardial cavity (**P**). Scale bar= 300µm in a,b; 100µm in c-f.

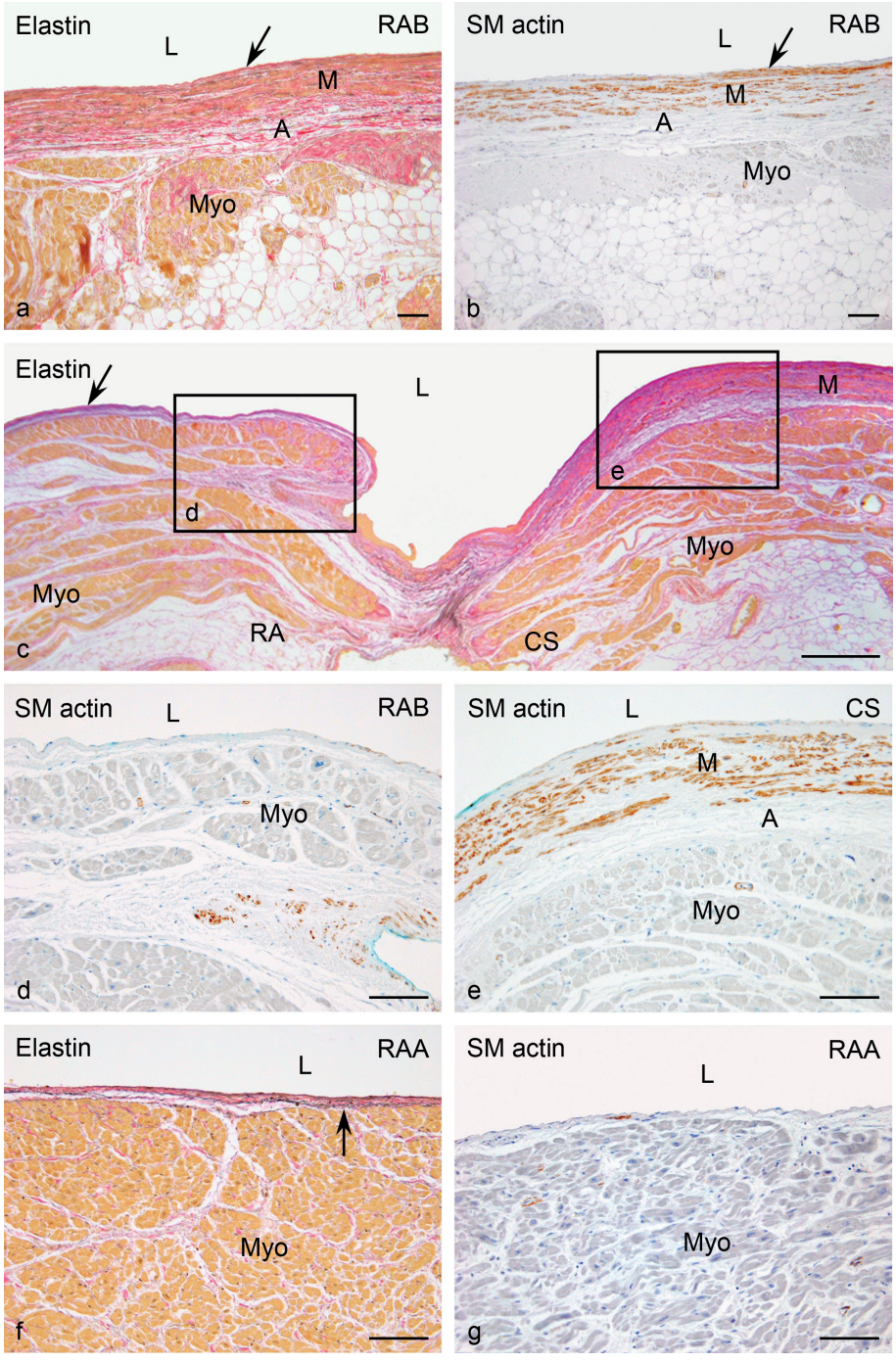

Elastin
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RAB
M
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Myo
a
SM actin
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RAB
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Myo
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Myo
RA
CS
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SM actin
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RAB
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d
SM actin
L
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SM actin
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RAA
Myo
g

Figure 2a-g
Sections of the right atrial body of normal adult hearts, stained with Verhoeff-Van Gieson (a,c,f; **Elastin**) and alpha smooth muscle actin (b,d,e,g; **SM actin**). Boxes in c indicate the positions of the enlargements in d,e.
a,b**.** Right atrial body (**RAB**) near the superior caval vein showing organized vessel wall tissue with multiple layers of smooth muscle cells. Intima (arrow**).**
c-e. In the region next to the coronary sinus (**CS**), no vessel wall tissue is found, which is representative for the major part of the smooth-walled RAB. The CS is covered by myocardium. Endothelium (arrow).
f,g. Trabeculated RA appendage (**RAA**). On the inner surface a thin endocardium (arrow) is present and a subendocardial layer with elastic fibers, collagen and occasional smooth muscle cells. There is no proper tunica media. These thin layers are covered by myocardium.
adventitia (**A**)**,** Lumen (**L**), media (**M**), myocardium (**Myo**). Scale bar = 100 µm in a,b,d-g; 300µm in c.

Heart-lung specimens with abnormal PV connections.

Macroscopic findings. Two of the four hearts with the supracardiac type of TAPVC had a situs solitus of the atria, with concordant atrioventricular and ventriculo-arterial connections, a small mitral valve and a hypoplastic left ventricle. Both had a secundum type of atrial septal defect (ASD), one of which had been surgically closed. The PV confluence originally drained via a vertical vein into a systemic vein, in this case the brachiocephalic vein. In the surgically corrected heart, the vertical vein was ligated and the PV confluence was connected to the roof of the LA body, thus establishing a continuous blood flow from the PVs to the LA. One of the other two hearts had a discordant ventriculo-arterial connection, with pulmonary atresia. The other heart had a double outlet right ventricle with infundibular pulmonary stenosis. Both of these hearts had a secundum type of ASD as well as an atrioventricular septal defect (AVSD).

The heart with the infracardiac type of TAPVC also presented with a situs solitus of the atria. There was a concordant atrioventricular connection with mitral atresia and a discordant ventriculo-arterial connection. The LA body and the enlarged RA body were smooth-walled. A secundum type of ASD was present. The PV confluence connected to a vertical vein, which descended through the diaphragm to a systemic vein, in this case the right gastro-epiploic vein that drained into the portal vein.

The three neonatal heart-lung specimens with PAPVC had a situs solitus, with concordant atrioventricular connections. In two specimens, the ventriculo-arterial connections were discordant. One of the four PVs drained either directly or via the brachiocephalic vein into the right superior caval vein in two of the three specimens, and in one the right PVs originally drained directly into the RA which was operatively corrected. Besides a ventricular septal defect (VSD) in two, there were no other serious cardiac abnormalities.

The hearts with situs inversus both had concordant atrioventricular and ventriculo-arterial connections with a right-sided aortic arch. In one a subaortic stenosis, dysplastic aortic valve, secundum type of ASD and an AVSD were present, the other had a perimembranous subaortic VSD.

Microscopic findings. In both the supra- and infracardiac type of TAPVC, the wall of the PV confluence had an intimal, medial and an adventitial layer. The intima consisted of an endothelial layer, a subendothelial layer of collagen and a clear lamina elastica interna with well-oriented elastic fibers. The media consisted of smooth muscle cells embedded in collagen and some elastic fibers. The adventitial layer had randomly distributed collagen fibers, some elastic fibers and scattered smooth muscle cells. Interestingly, in contrast to normally connecting PVs, no myocardial layer was observed around the anomalous connecting PVs (Fig.3a,b).

The posterior wall of the smooth-walled LA body consisted of a slightly thickened subendocardial layer of collagen, elastic fibers and sporadic smooth muscle cells, covered by myocardium. In contrast to the normal LA body, no distinct vessel wall tissue was observed (Fig.3c,d). Moreover, compared to normal, the size of the LA body was significantly diminished and its myocardial layer was translucent and hypoplastic (Fig.4a-d). In one case, even spongious (non-compact) LA myocardium was seen (not shown). Similar to the RA appendage in normal hearts (Fig.2f,g), the LA appendage was trabeculated and consisted of an endocardial layer covered by myocardium.

In the heart-lung specimens with either cardiac or supracardiac PAPVC, the PVs that connected to the LA were normally myocardialized (not shown), whereas the abnormally draining veins (i.e. the veins that did not connect to the LA) had no myocardial covering (Fig.5a-d). Moreover, similar to normal hearts, in cardiac PAPVC no vessel wall tissue was found in the RA body (not shown). The heart-lung specimens with situs inversus showed normally myocardialized PVs connecting to the right-sided morphological LA (Fig.5e,f).

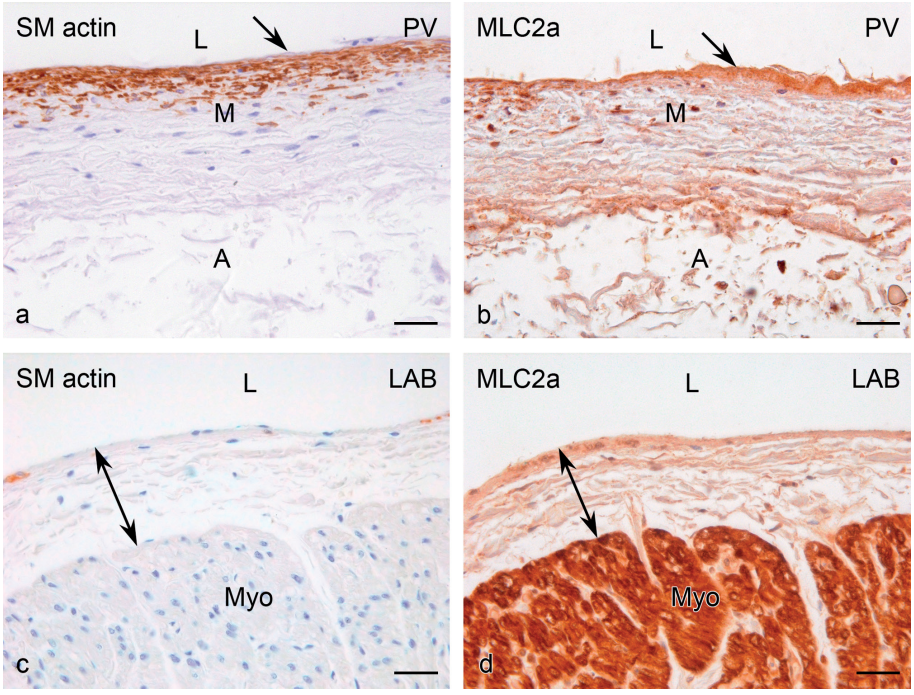


Figure 3a-d
Sections of neonatal heart-lung specimens stained with 1A4 against alpha smooth muscle actin (a,c; **SM actin**) and atrial myosin light chain (b,d; **MLC2a**), showing the histological structure of pulmonary venous and left atrial wall in hearts with total anomalous pulmonary venous connection (TAPVC).
a,b. Pulmonary venous (**PV**) confluence representative for any kind of TAPVC showing vessel wall consisting of a tunica intima (arrow), media (**M**) and a tunica adventitia (**A**) without myocardial covering.
c,d. Smooth-walled left atrial body (**LAB**) with a slightly thickened (sub)endocardial layer (arrow, double head) of collagen, elastic fibers and occasional smooth muscle cells covered by myocardium (**Myo**). No vessel wall tissue was found.
Lumen (**L**). Scale bar = 30µm in a-d.

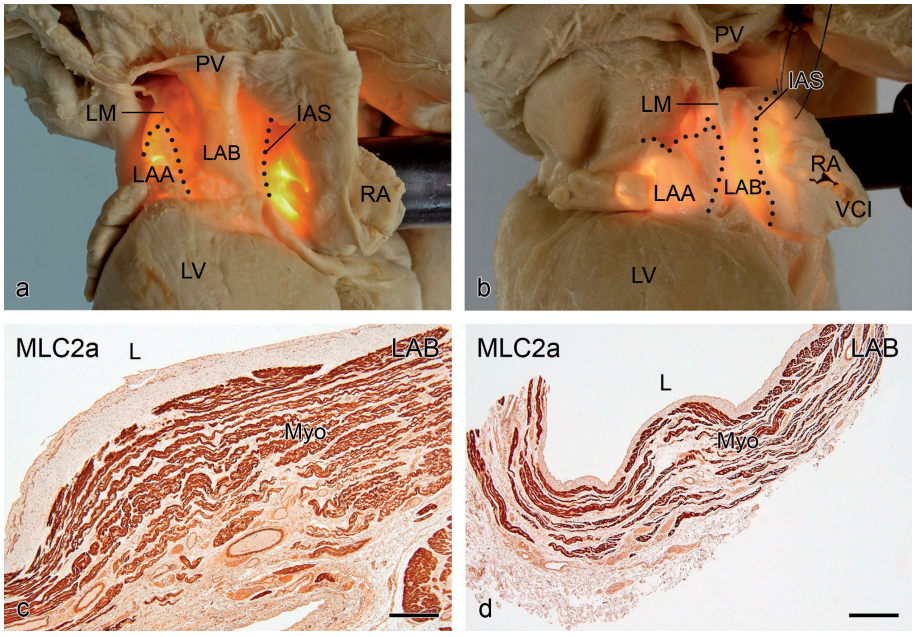


Figure 4a-d

Pictures of a normal neonatal heart (a) and a neonatal heart with the supracardiac type of TAPVC (b) correlated to sections of their LA dorsal wall stained with atrial myosin light chain (c,d; **MLC2a**).

a. Normal heart. The left atrial body (**LAB,** between dotted lines) receives the PVs and is demarcated by the ligament of Marshall (**LM**), at the base of the left atrial appendage (**LAA**), and the atrial septum (**IAS**).

b**.** Supracardiac type of TAPVC. The pulmonary veins (**PV**) are not connected to the LAB but drain via a confluence into the systemic venous circulation. In this case, the LM connects the PV confluence to the left atrium. Note that in TAPVC, the size of the LAB (between dotted lines) is remarkably diminished and that there is more translucency compared to the normal heart.

c. Normal heart. The myocardium (**Myo**) of the LAB has a normal, compact structure.

d. TAPVC. Compared to the normal heart, the myocardium is hypoplastic, causing translucency.

Lumen (**L**), left ventricle (**LV**), right atrium (**RA**), inferior caval vein (**VCI**). Scale bar = 300 µm in c,d.

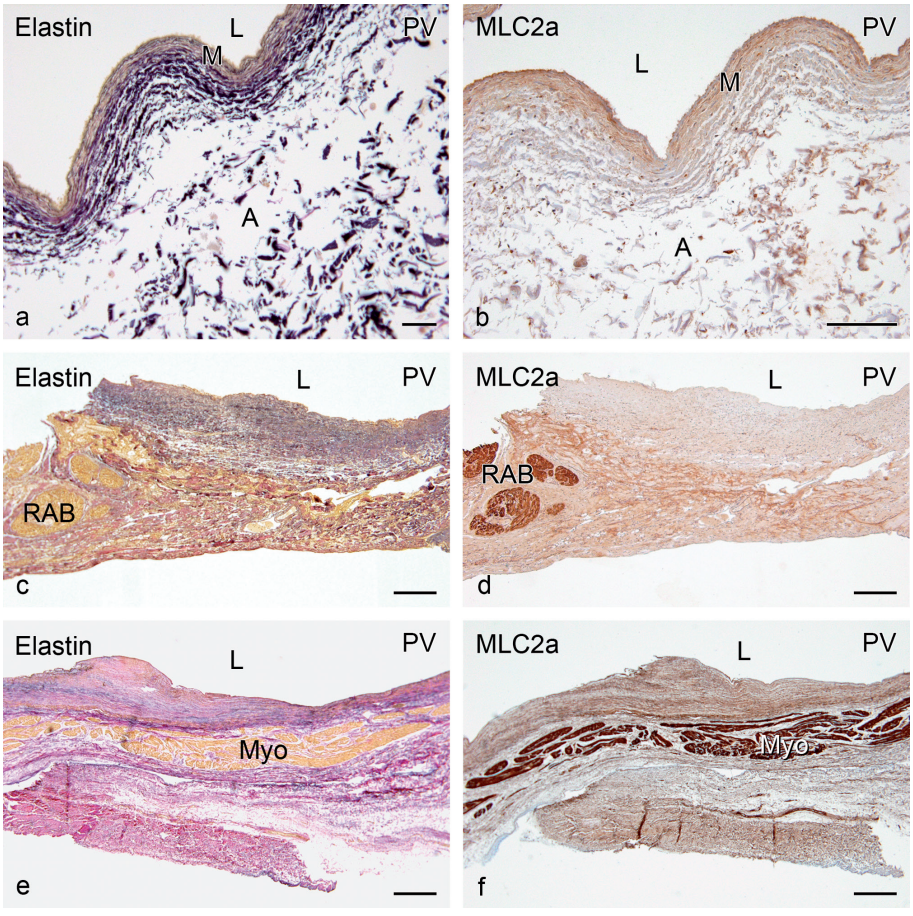


Figure 5a-f
Sections of neonatal heart-lung specimens stained with Verhoeff-Van Gieson for elastic filaments (a,c,e; **Elastin**) and atrial myosin light chain (b,d,f; **MLC2a**), showing the histological structure of the abnormally draining pulmonary vein (**PV**) in partial anomalous pulmonary venous connection (PAPVC; a-d) as well as the histological structure of the PV in case of situs inversus (e,f).
a,b**.** Extracardiac PAPVC. Section of the abnormally draining PV showing a normal vessel wall structure (a), and absence of myocardium (b).
c,d. Cardiac PAPVC. The right atrial body (**RAB**) receives one of the PVs. This PV is not myocardialized.
e,f**.** Situs inversus. The PVs, though right-sided, do have a myocardial layer.
Adventitia (**A**). lumen (**L**), media (**M**). Scale bar = 100µm in a,b and 300 µm in c-f.

Biopsies of patients during surgical correction for TAPVC

Macroscopic findings. In all hearts with the supracardiac type, the PV confluence drained by means of a vertical vein into the brachiocephalic vein. Naturally, in hearts with the infracardiac type, only a vertical vein descending downwards could be identified during operation. In none of these 5 hearts other cardiac anomalies were found.

Microscopic findings. Within the samples taken from the smooth-walled LA body, no vessel wall tissue was detected. The smooth-walled LA body consisted of endocardium and a subendocardial layer, the latter consisting of randomly scattered collagen and elastic fibers, covered by myocardium (Fig.6a-c). The PV confluence consisted of a well organized subendothelial layer of collagen and elastic fibers. The medial layer resembled a proper tunica media and consisted of multiple layers of smooth muscle cells embedded in collagen and some elastic fibers (Fig.6d,e). On the outside of the confluence a proper tunica adventitia was observed. Again, in these veins, no myocardial layer surrounding the vein was present (Fig.6f). Samples of the vertical vein could be taken in two hearts with the supracardiac type and in two hearts with the infracardiac type. In both types the intimal layer was locally thickened (not shown). As in the sections of the anomalous connecting PVs, no myocardial layer was observed (Fig.6g-i).

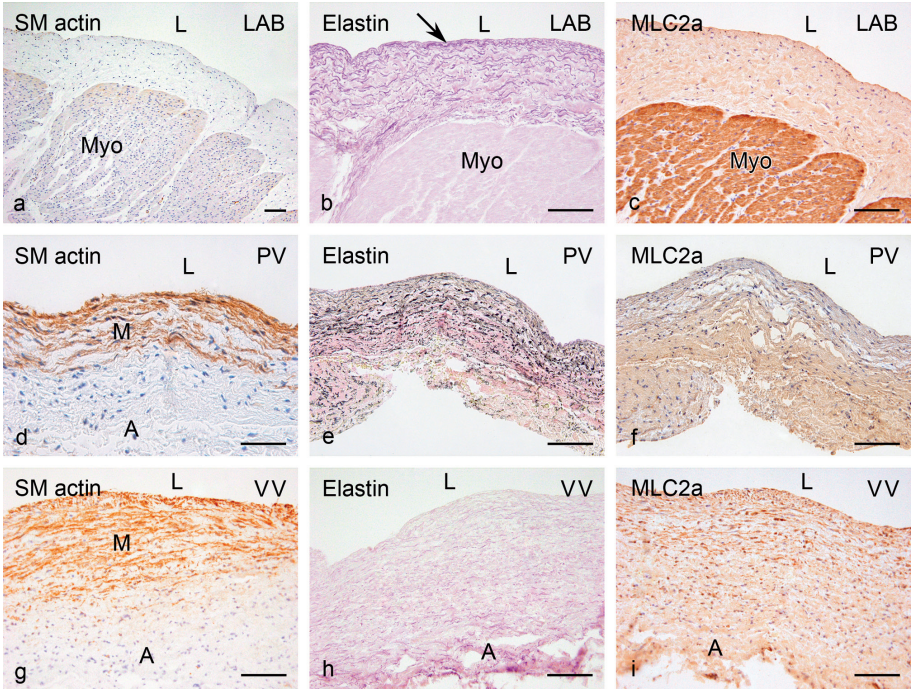
Figure 6a-i

Sections of peroperatively taken samples of left atrial body (**LAB**), pulmonary veins (**PV**), and the vertical vein (**VV**) from neonates operated on for total anomalous pulmonary venous connection. Staining with elastic Resorcin-Fuchsin (b,h; **Elastin**) and Verhoeff-Van Gieson (e; **Elastin**) for elastic filaments; alpha smooth muscle actin (a,d,g; **SM actin**), and atrial myosin light chain (c,f,i; **MLC2a**).

a-c. Smooth-walled LAB, showing absence of vessel wall tissue (a). The thin endocardium (b,arrow**)** and subendocardial layer with collagen and elastic fibers (b), is covered by myocardium (c, **Myo**).

d-f. PV confluence consisting of proper vessel wall components (d,e) but lacking a myocardial covering (f).

g-i**.** Vertical vein histologically resembling the PV confluence with absence of myocardial covering (i**).** Adventitia (**A**), lumen (**L**), media (**M**). Scale bar = 100μm in a-c,f-i; 50μm in d,e.



Discussion

In this study we examined the morphology and tissue characteristics of hearts from patients with anomalous pulmonary venous connections, and compared the results with findings in normal controls. Key findings of our study in patients with TAPVC are: 1. a significantly diminished size of the LA body and reduced thickness of the myocardium; 2. the lack of a vessel wall in the LA body; and 3. the lack of formation of a myocardial muscular sleeve surrounding the PVs.

The origin of the myocardium surrounding the common PV in normal hearts remains an issue of debate. On the basis of morphology and expression patterns of the marker HNK-1 in avian[18], rat[19] and human[20] embryos and Nkx2.5 expression in human embryos[9], our group showed that the myocardium around the common PV belongs to the sinus venosus segment. Based on morphology[4,21] and expression patterns of connexin40 and atrial natriuretic peptide[22], the myocardium around the primitive

PV can be discriminated from the myocardium of the primary heart tube. There are different theories about the (secondary) addition of myocardium lining the common PV.

Recent research using the novel coelomic and myocardial marker podoplanin, has shown that the development of sinus venosus myocardium, derived by recruitment of progenitor cells at the venous pole (the so-called posterior heart field), and surrounding the common PV, is demarcated by podoplanin expression and the absence of Nkx2.5 expression.

Compared to the rest of the sinus venosus myocardium, the PV myocardium undergoes a rapid differentiation and proliferation phase[12], during which the Nkx2.5 expression changes from a mosaic to positive expression pattern[9]. Diminished Nkx2.5 expression is accompanied by expression of the sinus venosus marker HCN4 in the PV myocardium[12], suggesting early transient expression of HCN4 in the PVs during the mosaic Nkx2.5 expression phase. Eventually, HCN4 becomes confined to the sino-atrial node[12]. The myocardium surrounding the common PV is linked to the development of parts of the cardiac conduction system[9,12]. These findings are supported by the (transient) expression of several molecular markers in the myocardial sleeve surrounding the PV, including HNK-1 and CCS-lacZ[20,23]. Therefore, second heart field derived myocardial cells surrounding the PVs may have conduction properties responsible, together with areas of myocardial discontinuity, for the increased susceptibility for arrhythmias arising from the PV area and the LA dorsal wall[10,13,24]. We postulate that the lack of a myocardial sleeve surrounding the PVs in patients with TAPVC, may explain the low incidence of arrhythmias observed in this patient group[17].

Since in TAPVC hearts addition of myocardium at the venous pole is impaired, also sino-atrial node dysfunction can be expected. This hypothesis is supported by several clinical studies[17,25] as well as by the observation that in podoplanin knockout mice, the sino-atrial node is hypoplastic[26].

The findings of our current study that in human hearts with supra- or infracardiac TAPVC, myocardium around the PVs is absent, the LA myocardium is hypoplastic and no vessel wall tissue is found in the LA body, suggest that impaired formation and differentiation of posterior heart field myocardial and smooth muscle cells contributes to the development of TAPVC.

As we have shown, in hearts with TAPVC, as a consequence of absent PV connection and incorporation, the complete LA body is small and smooth-walled, and the myocardium is hypoplastic. Additionally, reduced fetal hemodynamic flow may contribute to the observed small size of the LA in these hearts. Therefore, it is not surprising that surgeons have tried to optimize results of TAPVC correction by augmentation of the LA[15,27].

In this study, in one heart with TAPVC, non-compaction of LA myocardium was seen. Since the posterior heart field seems to contribute to TAPVC and the proepicardial organ is derived from the posterior heart field, an explanation for the spongious LA myocardium can be impaired formation of epicardium derived cells (EPDC's) and altered epicardial-myocardial interaction, which are known to be responsible for abnormal atrial and ventricular myocardium[28,29].

In our study in 60% of TAPVC heart-lung specimens also outflow tract abnormalities were found. Because the posterior heart field is part of the second heart field which contributes to myocardial addition at the arterial as well as the venous pole[30], co-existence of outflow tract and inflow tract abnormalities can be explained.

We hypothesize that an open connection of the common PV into the morphological LA is necessary to trigger recruitment of mediastinal mesenchymal cells, that subsequently differentiate into cardiomyocytes and smooth muscle cells. Based on our findings in hearts with (extra)cardiac PAPVC and in situs inversus, we now know that only PVs connecting to the LA body are myocardialized (Fig.7a-h). This is an important finding which confirms that for PVs, addition of myocardial cells recruited from the posterior heart field, mainly takes place from the morphological left side[9].

In this context, an interesting finding in hearts with cardiac TAPVC and isomeric left atrial appendages was that myocardialization was only found in one "LA", irrespective of the side of PV incorporation. This myocardialization is probably based on left-right patterning as was shown in studies using the left-sided transcription factor Pitx2c[12,31,32]. Pitx2c deficient mice exhibit right atrial isomerism and lack the development of a myocardial sleeve surrounding the PVs[12].

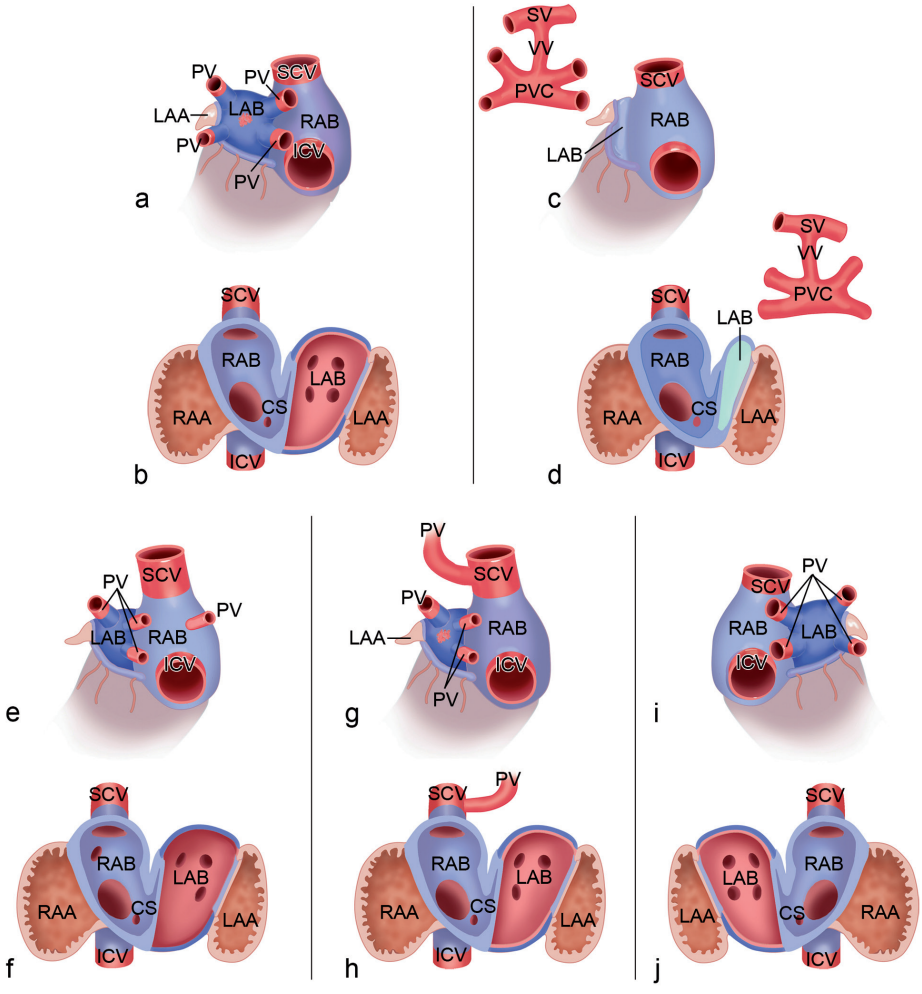

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Figure 7a-j
Schematic depiction of outer (a,c,e,g,i) and inner (b,d,f,h,j) side of atrial chambers and pulmonary veins (**PV**) in normal hearts (a,b), hearts with total anomalous pulmonary venous connection (TAPVC;c,d**),** hearts with (extra)cardiac partial anomalous pulmonary venous connection (PAPVC; e-h) and hearts with situs inversus (i,j).
a,b**.** Normal heart. a. Left atrial body (**LAB**) and pulmonary veins (**PV)** covered by myocardium derived from the posterior heart field (***dark blue***). In the area between the PVs the myocardium can be discontinuous or absent. Right atrial body (**RAB**) covered by sinus venosus myocardium (***light blue***). b. The inner aspect of both LAB and RAB is smooth-walled. In the LAB vessel wall tissue (***red***) is found parallel to PV incorporation. The left and right atrial appendages (**LAA, RAA**) consist of trabeculated myocardium (***brown***).
c,d. TAPVC, extracardiac type. The PVs drain via a vertical vein (**VV)** into a systemic vein (**SV**) and are not covered by myocardium. The LAB consists of smooth-walled hypoplastic myocardium, derived from the posterior heart field. Because of the absence of PV connection and incorporation into the LA, the LAB is small. Moreover the LAB does not contain vessel wall tissue, probably because of impaired differentiation of smooth muscle cells.
e,f. PAPVC, cardiac type. The abnormally connected PV drains directly into the RAB, but no vessel wall tissue is found in the RAB. In contrast to the PVs that are connected to the LAB, the abnormal PV is not covered by myocardium.
g,h. PAPVC, extracardiac type. The abnormally connected PV drains (in)directly into the superior caval vein and is, similar to the cardiac type of PAPVC, not covered by myocardium. The LAB and its incorporated PVs are myocardialized and comparable to the normal heart, vessel wall tissue is present in the LAB.
i,j. Situs inversus. The LAB is located on the right side of the heart and receives the four PVs, which are covered by myocardium. In the LAB vessel wall is found parallel to normal PV incorporation.
Coronary sinus (**CS**), inferior caval vein (**IVC**), pulmonary venous confluence (**PVC),** superior caval vein (**SCV**).

After surgical correction of TAPVC, the pulmonary venous confluence is connected with the LA body. Because of an increased incidence of PV stenosis at the site of the anastomosis, so-called sutureless techniques have been developed and successfully applied[33,34]. We hypothesize that this PV stenosis might be enhanced in TAPVC by differences in wall thickness and contractile properties of cells at the transition of PVs to LA, where vessel wall is directly sutured to myocardium, without an intermediate layer of myocardium protecting the PV. Also, primary PV stenosis is observed frequently in this patient group[35]. With respect to this, absence of the protective layer of myocardium surrounding the PVs may be responsible for the enhanced susceptibility of the PV wall in response to internal or environmental influences, thus making it prone to stenosis. The interaction between the lack of PV myocardial layer and the development of PV stenosis requires further investigation.

In conclusion, in hearts with TAPVC no vessel wall was found in the smooth-walled LA body and its myocardial layer was structurally abnormal, demonstrating marked hypoplasia. No myocardial layer was formed around the PVs. Absence of this

myocardium may prevent the onset of arrhythmias in the PVs but may enhance PV stenosis. Based on our findings in PAPVC and situs inversus, we hypothesize that recruitment of cells, for addition of myocardium and smooth muscle cells at the venous pole, mainly takes place from the morphological left side. An open connection to the LA is mandatory for proper development of the LA vascular wall and myocardium and the pulmonary venous muscular sleeve. The posterior heart field is suggested to be responsible for the abnormal myocardialization and smooth muscle cell formation of the LA dorsal wall and PVs in TAPVC hearts. This knowledge about the PV and atrial morphology in TAPVC hearts may help to understand the mechanisms related to the high occurrence of primary PV stenosis and PV stenosis after surgery for TAPVC.

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5

Unilateral Pulmonary Vein Stenosis with a Contralateral Pulmonary Varix

Modified after
Journal of Thoracic and Cardiovascular Surgery 2007;134:496-501

Case Presentation

A 41-year-old woman was admitted by the pulmonologist in July 2004 because of progressive complaints of dyspnea on exertion. There was no remarkable medical history except for mild dyspnea existing from about 1996. Physical examination revealed no dyspnea at rest, with normal breath sounds. There were no signs of cyanosis. Laboratory data demonstrated a normal hematologic profile and there was no serologic evidence for a systemic disease. The chest film showed bilateral interstitial abnormalities and a blurred left hemidiaphragm (Fig.1).



Figure 1. Chest film showing bilateral interstitial abnormalities and a left-sided blurry vascular structure above the diaphragm.

Bronchoscopic findings were within normal limits. Pulmonary function tests showed mild bronchial obstruction with normal static volumes (Table 1). Ventilation and perfusion lung scintigraphy revealed nearly symmetrical ventilation (R 58%, L 42%) but very asymmetrical perfusion to the detriment of the left lung (R 83%, L 17%) (Fig.2a,b).

Table 1. Preoperative and postoperative pulmonary function

	Pre Op	Post Op	Predicted
FEV_1 (L)	3.0	3.0	3.0
VC (L)	4.1	3.8	3.6
FEV_1/VC	74	78	81
TLCO/VA, % pred.	68	72	100

Forced expiratory volume in 1 second (**FEV1**) in liters (**L**); diffusion capacity corrected for alveolar volume (**TLCO/VA**); vital capacity (**VC**).

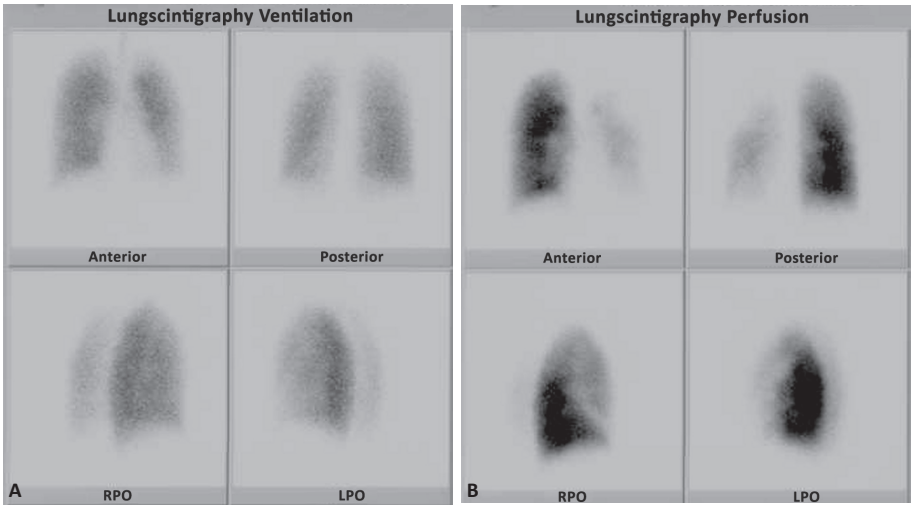


Figure 2. Ventilation (a**)** and perfusion (b) lung scintiscans showing symmetrical ventilation (right 58% , left 42%) but asymmetrical perfusion to the detriment of the left lung (right 83%, left 17%). Right posterior oblique (**RPO**), left posterior oblique (**LPO**).

Cardiac auscultation was completely normal. The electrocardiogram showed signs of right ventricular overload and transesophageal echocardiography showed turbulent flow at the level of the right pulmonary veins (PVs), together with mild mitral valve regurgitation. Cardiac catheterisation showed a pulmonary arterial hypertension (40/15-28 mm Hg), discrepant high left pulmonary artery wedge pressure (30 mmHg) compared to the left ventricular end-diastolic pressure (LVEDP, 14 mmHg) together with a high pulmonary vascular resistance (PVR): 9,5 Woodunits (left); 5 Woodunits (right)). There were no signs of coronary artery sclerosis.

Cardiac triggered multidetector computed tomography during intravenous contrast administration demonstrated bilateral pulmonary arteriovenous malformations most pronounced on the left side (Fig.3a,b). Magnetic resonance (MR) angiography was performed to clarify flow directions in the arteriovenous malformations, but it left the origin of the tortuous PVs unclear. Stenosis of the PVs at the level of the entrance in the left atrium (LA) was most pronounced on the right side. A digital substraction angiogram of the pulmonary vessels confirmed a much smaller caliber of the left pulmonary artery compared to the right, together with a corresponding difference in lung perfusion. The left PVs seemed to be aberrant and varicous but not stenotic.

Based on these findings, surgical resection of the stenotic parts of the right PVs was suggested without intervention on the left side because of its underdeveloped pulmonary vasculature and its aberrant but uninterrupted inflow of the PVs into the LA. Besides that, inspection and possible repair of the mitral valve was indicated.

By means of a median sternotomy, both lungs could be inspected. They had an abnormally pale appearance with multiple small vessel injections, more pronounced on the left side, so that a lingular as well as a middle lobe biopsy was done. Intrapericardially, the right superior and inferior PVs were macroscopically stenotic at the entrance to the LA. On the left side, a small vein resembling a left superior caval vein was seen together with a vein to the lower lobe. The origin and course of these veins were not visible. By inspection inside the right atrium, the possibility of a left superior caval vein could be rejected. After atrial septotomy, the entrance of the left-sided veins in the LA could be passed and appeared not to be stenotic. However, on the right side the diameters of the superior and inferior PVs at their entrance into the LA were only 4 and 7 mm, respectively. The mitral valve was sufficient and structurally normal. Both right PVs were incised longitudinally into the LA. The circumscript stenotic parts were resected, which resulted in an increase in diameter to 12 and 13 mm, respectively. A so-called sutureless pulmonary venoplasty[1,2] was performed by suturing the LA wall to the pericardium posteriorly and using an autologous pericardial patch anteriorly. The postoperative course was uncomplicated.

The lung biopsies were sent for pathologic analysis in Groningen and the resected stenotic parts of the right-sided PVs were analyzed in the Department of Anatomy and Embryology of Leiden.

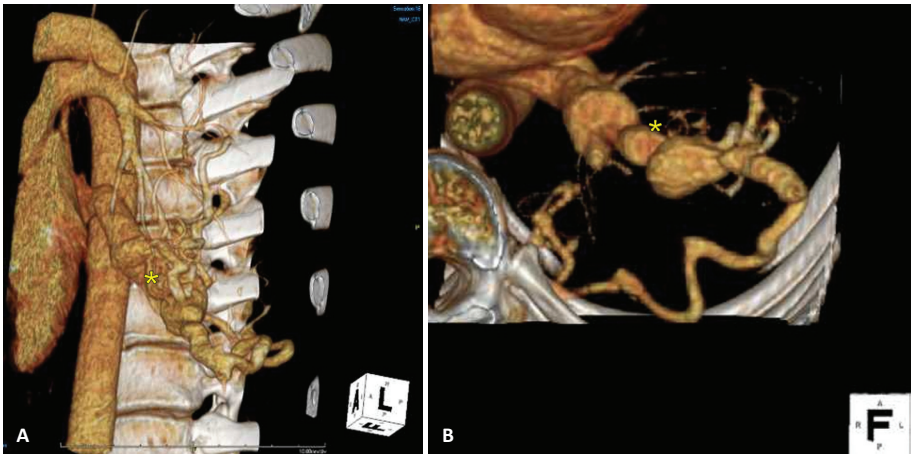


Figure 3. Multidetector computed tomography. a. Left lateral view and b. Caudal view. A varicous pulmonary vein (asterisk) connects to the original pulmonary vein ostium.

Histological examination of the pulmonary biopsies revealed serious vascular pathology with asymmetric and eccentric obliteration of venous as well as arterial branches, most pronounced on the left side, to be interpreted as post-thrombotic vasculopathy. Thereby, extensive hemosiderosis was seen on the right side.

Staining of the stenotic rim revealed a large mass of collagen (Fig.4a,b).

Two weeks after the operation the patient was discharged from the hospital. On echocardiography, no turbulence was seen in the right PVs.

After one year's follow-up, the ventilation-perfusion mismatch between the right and left lungs was exactly the same as preoperatively. Exercise capacity, assessed by cycle-ergometry, had not improved. Maximal oxygen uptake was 71% predicted at a maximal workload of 90 Watts (78% of predicted). Large disturbances in gas exchange were shown during the test compatible with large ventilation-perfusion mismatch. Also, these findings were comparable with the preoperative situation (Table 1). It was decided to be expectative about probable future effects of dead space ventilation on the left side.

High-resolution CT demonstrated non-stenotic veno-atrial transitions on the right side (Fig.5). In relation to the right inferior PV, the right superior PV had a smaller caliber and a more tortuous course.

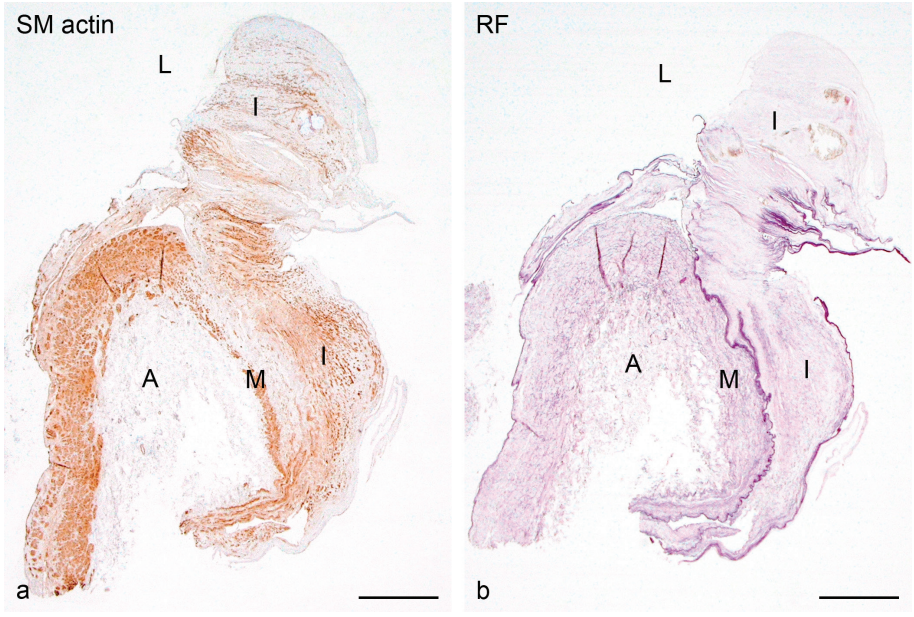


Figure 4. Sections of the excised circumferential stenosis of the right superior pulmonary vein stained for α-smooth muscle (**SM**) actin (a), and Resorcin-Fuchsin (**RF**) (b)**,** showing severe intimal proliferation (**I**) by means of a stenotic rim of collagen without elastic fibers. Lumen (**L**), media (**M**), adventitia (**A**). Scale bars are 600μm.

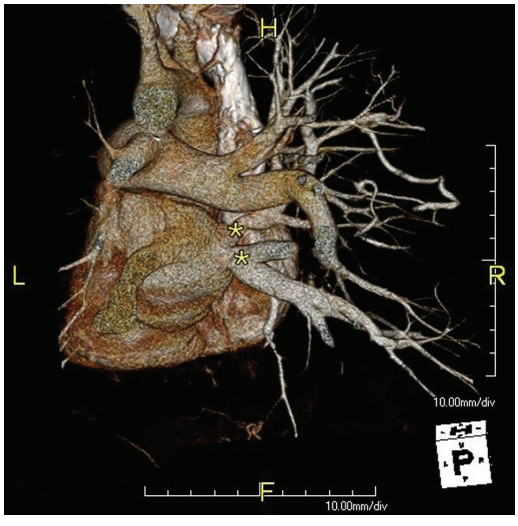


Figure 5. Multidetector computed tomography. Posterior view. The repaired right pulmonary veins (asterisks) are nonstenotic. Compared to the right inferior pulmonary vein, the right superior pulmonary vein is relatively small and tortuous**.**

Discussion

PV stenosis is part of the spectrum of abnormal PV drainage. Usually congenital in origin, the site of the stenosis depends on the embryologic stage at which it develops[3,4]. The stenosis can also be acquired, called pulmonary veno-occlusive disease (PVOD), a condition for which various agents can be responsible[5,6]. Especially in adults, it can be very difficult to determine whether the origin of the anomaly in a particular patient is congenital, acquired, or a combination of both[7]. Extremely rare is a PV varix[8], which can be associated with a diseased mitral valve[9].

When PV stenosis is congenital, two different types can be distinguished[10]. In the first, the so-called hypoplasia of the PVs, narrowing involves the intrapulmonary and extrapulmonary part of the PVs for a variable distance. The second type is characterized by narrowing at the veno-atrial junction, commonly called localized PV stenosis.

In normal development, the embryonic heart is connected to the mediastinum by means of the dorsal mesocardium. The major part of this structure regresses, after which, at the end of the fifth embryonal week, the lumen of the common PV develops in the remaining part of the dorsal mesocardium, and the common PV connects with the peripheral PV system[11-13]. By this point, the lung buds, which drained via the splanchnic plexus into the cardinal and umbilical veins, drain directly via the common PV into the LA. The connections with the splanchnic plexus and the umbilical and cardinal venous system become of less importance and regress. The common PV is then incorporated into the posterior LA wall, finally resulting in four individual PVs entering the LA. On the left side, also two PVs forming one common PV ostium that enters the LA are seen[14,15].

If atresia of the common PV occurs very early in development, total anomalous PV drainage develops and the embryonic systemic venous connections do not regress but establish a supra- or infracardiac drainage unless the PVs drain into the right atrium. If stenosis of the common PV occurs later, incorporation is disturbed which may result in cor triatriatum[3,4]. Atresia or stenosis of the left or right common PV leads to partial anomalous PV drainage so that the ipsilateral systemic venous drainage persists. If stenosis occurs very late, after PV incorporation has been completed, stenosis or atresia of individual PVs is the result. During fetal life, the hemodynamic effect of

partial obstruction of one or more PVs may not be enough to preserve embryologic (systemic) venous drainage[16,17]. After delivery, an increasing amount of blood volume reaches the lungs by which the hemodynamic effect of individual PV stenosis can be more pronounced.

If necessary, therapeutic options are percutaneous transluminal balloon angioplasty, stenting or surgical resection.

Acquired PV stenosis or PVOD is characterized by extensive and diffuse occlusion of PVs by fibrous tissue. Usually the (eccentric) intimal thickening involves venules and small veins but, occasionally, also larger veins and, in later stages, arterioles. The media of the veins may become arterialized with an increase in elastic fibers. The most consistent early parenchymal change is interstitial edema, which may progress to deposition of collagen fibers in the lobular septa and alveolar walls, as often observed when histologic features are obtained[18]. Lymphatic vessels are dilated. A more obvious finding, in particular in later stages, is hemosiderosis, found commonly in alveolar macrophages or less commonly also in the interstitium.

Clinically, cases present with pulmonary arterial hypertension without elevation of the pulmonary arterial wedge pressure[7,18]. Many agents may be responsible for the etiology of PVOD. Infectious, genetic, and toxic factors have been described as well as a thrombotic diathesis and autoimmune disorders[18]. Because PVOD is a rare condition, no randomized therapeutic trials have been undertaken to compare the effect of current treatment modalities, that is, vasodilators, immunosuppressives, anticoagulants and oxygen, on outcome. Because the prognosis is usually bad within 2 years after diagnosis, lung transplantation seems the only option for significantly prolonging life expectancy.

A PV varix is also a very uncommon anomaly that can be congenital or acquired. The congenital form usually is asymptomatic. The acquired form develops as a result of obstructed PV drainage and presents with symptoms of the underlying disease causing PV hypertension, for instance mitral valve disease or PV stenosis. Treatment consists of observation. If size increases, or in case of hemoptysis, surgical treatment is indicated[8].

In our patient, the etiology of the right-sided focal PV stenosis at the veno-atrial junction is likely to be congenital in origin in view of the combination with a diseased (nonfunctional) left lung. Acquired central PV stenosis in combination with a nonfunctional left lung would be very unlikely because in hemodynamically comparable situations of normal PVs in patients who have had pneumonectomy, subsequent development of contralateral pinpoint central PV stenosis is very uncommon. However, an additional acquired component in this region must have been responsible for the progression of the dyspnea; the function of the left lung decreased and flow to the right lung subsequently increased, thereby inducing intimal proliferation at the right veno-atrial junction to a critical point by means of altered flow profiles as turbulence[19]. Nonetheless, the significant ipsilateral hemosiderosis with moderate obliteration of small veins and arteries in the biopsy specimen, together with a normal right-sided pulmonary arterial wedge pressure, suggests a possible superposed PVOD. On the left side, the small caliber pulmonary artery, the elevated pulmonary artery wedge pressure, and the decreased perfusion of the left lung because of a very high PVR in combination with histologic fibrotic changes mimicking micro-infarction suggests left-sided PV atresia. Considering the two apparently normal left PV ostia entering the LA and the nonhypoplastic left lung, this atresia must have been developed during the late fetal period. After this, and under pressure, the varicous vein must have developed and connected with both left PVs, after which left-sided PV drainage was delayed but uninterrupted. If we deal in this case with some degree of PVOD in the right lung, careful follow-up is necessary because of its aggressive behaviour. Now the venous flow of the right lung to the LA is unobstructed, theoretically, dead space ventilation on the left side will increase. So long as this patient is doing well, an expectant policy is warranted. Thus for ethical reasons, we have not yet performed a postoperative recatheterization. If her situation deteriorates, cardiac recatheterization should be performed. In case of increased dead space ventilation on the left side, combined with acceptable PA pressures and PVRs on the right, a left pneumonectomy would be an option. In case of an irreversible increased right-sided PVR and a very bad clinical condition, this patient should be screened for lung transplantation.

In conclusion, differentiation between congenital or acquired stenosis of PVs or a combination of both can be very difficult. However, cardiac catheterization and a lung biopsy can bring more clarity. This is necessary inasmuch as the acquired form behaves more aggressively than the congenital version and needs a closer follow-up because of its tendency to relapse and its worse prognosis on the long term.

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6

Discussion

Normal and Abnormal Development of Pulmonary Veins: State of the Art and Correlation with Clinical Entities

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Review article

Abstract

Interest for the pulmonary veins has increased in the past decade after the potential arrhythmogenicity of the myocardial sleeve surrounding these structures has been recognized. Furthermore, there are several clinical entities, such as anomalous connection pattern and pulmonary vein stenosis, that are related to abnormal pulmonary vein development.

In this review, we will describe current literature and aim to elucidate and reorganize current opinions on normal and abnormal pulmonary vein development in relation to clinical (management of) diseases. Several unresolved questions will be addressed, as well as current conceptual controversies. First, a general overview of development of structures at the venous pole of the heart, including normal development of the pulmonary vein from a primitive Anlage, will be provided. Recent insights indicate an important contributory role of the mesoderm behind the heart, the so-called second heart field, to this area. Subsequently, the formation of a myocardial and smooth muscle vascular wall of the pulmonary veins and the left atrium is described, as well as current insights in the mechanisms involved in the differentiation of these different cell types in this area. Next, developmental concepts of normal pulmonary venous drainage patterns are reviewed, and an overview is provided of clinical entities related to abnormal development at several anatomical levels. Lastly, attention is paid to arrhythmogenesis in relation to pulmonary vein development, as well the consequences for clinical management.

Introduction

In recent years, the involvement of the pulmonary veins (PVs) in several pathogenetic processes, has increasingly been recognized. The PVs play a role in the onset of atrial arrhythmias, such as atrial fibrillation[1]. Furthermore, congenital malformations, including abnormal pulmonary venous drainage patterns and PV stenosis, may warrant surgical or percutaneous interventions. Also the structure of specific components of the PVs may be involved in pathogenesis, e.g. vulnerability of the smooth muscle and myocardial wall for acquired PV stenosis.

In the light of these clinical considerations, understanding of both normal and abnormal PV development is mandatory. PV development is narrowly related to development of the venous pole of the heart, that includes the sinus venosus and atrial segment. Recent new insights have demonstrated that during cardiac development large compartments of the heart are developed in and subsequently incorporated from the mesocardium behind the heart, the so-called second heart field[2,3].

Current concepts of PV development result in several questions that seem to recirculate and ask for clarification. We should in this regard also take notice of the fact that differences in semantics, definitions and interpretations of findings might be of great influence. After giving a general overview of development, in this review we will focus on the following questions:

1. How are the different components of the venous pole defined and what are current controversies?
2. Which role does the second heart field play in the development of the venous pole, myocardialization and smooth muscle cell (SMC) formation of the PVs?
3. What, in the view of previous points, are the current developmental concepts on the origin of normal and abnormal PV connection?
4. How is PV development related to arrhythmogenesis?
5. What are the consequences of the various concepts of PV development on clinical management?

We will try to clarify these topics by describing normal and abnormal PV development, as well as the relation to clinical entities, based on our own research and current literature.

With regard to abnormal PV development, in literature different terms are used to describe the same condition. While “anomalous PV drainage or return” mainly focuses on the physiological condition, “anomalous PV connection”, in our opinion, describes most accurately the anatomic condition.

General overview of early cardiac development.

The embryo starts as an embryonic plate, in which a primitive streak area develops[4]. The primitive streak is formed as an invagination in the midline of the embryonic plate, at the initiation of gastrulation (the formation of the three embryonic germ layers). From the primitive streak, two large lateral plate mesodermal compartments are derived on both sides of the embryonic axis[4] (Fig.1a, upper panel). In each compartment a coelomic cavity develops that splits the mesoderm into a somatic layer, lining the ectoderm, and a splanchnic layer, lining the endoderm[5]. The bilateral splanchnic mesoderm contains the cardiac precursor cells. Anterior to the embryonic axis (notochord), these mesodermal compartments fuse to form a primitive myocardial heart tube (primary heart tube) that is lined on the inside with endocardium and separated from the myocardial layer by cardiac jelly[4,5]. After a complicated bending of the head region of the embryo, this heart tube obtains an arterial (anterior/cranial) pole and a venous (posterior/caudal) pole (Fig.1a, lower panel).

During further development of the heart, splanchnic mesodermal cells from the so-called second heart field will continue to be added to the heart[2,3]. The second heart field is defined as an anteroposterior extension of mesoderm, located behind the heart, from which myocardium is incorporated to the heart during further development. The contribution to the venous pole of the heart is referred to as derived from the posterior heart field[6,7], whereas the contribution at the arterial pole is referred to as derived from the anterior or secondary heart field[8,9] (Fig.1b-d). The second heart field in relation to PV development is discussed in detail in section 2.

The splanchnic mesoderm also differentiates into a vascular plexus of endothelial cells. This plexus is the source of the lining of the vitelline veins, the pharyngeal arch arteries and the initially bilateral dorsal aortae[4,5]. The splanchnic plexus is connected to the sinus venosus, being itself in connection with the common atrium, by means of a small strand of endothelial cells, the so-called midpharyngeal endothelial strand, which runs from the arterial to the venous pole of the heart[10]. This structure is the Anlage of the primitive PV (see section 3), that is situated in the mesocardium behind the heart (the dorsal mesocardium). We will focus on structures that will develop at the venous pole of the heart, being the area in which the PV develops.

1. How are the different components of the venous pole defined?

The inflow part or venous pole of the heart includes the embryological sinus venosus segment and the atrial segment. These compartments constitute the most caudal parts of the primary heart tube (Fig.1a). In this paragraph we will clarify the nomenclature of embryological structures at this site, as well as their counterparts in the adult. As will become clear, definitions and descriptions of some structures have been the subject of vivid discussions over the past decades. We will also address these controversies where appropriate.

After development from the second heart field (see section 2), the sinus venosus is connected to the common atrium and consists during the 4th week of development of a left and right part, each receiving blood from the vitelline/omphalomesenteric vein, the umbilical vein and the common cardinal vein. During early stages of development, the border between the sinus venosus and the still unseptated common atrium, can be distinguished as a fold of myocardium, the sino-atrial fold. Asymmetrical growth of the right part of the common atrium causes a rightward shift of the site of connection of the sinus venosus to the common atrium. The right part of the sinus venosus and corresponding veins also enlarge and form the caval veins that are incorporated into the future right atrium. The left part of the sinus venosus obliterates to a large extent in human development. The left common cardinal vein will form the coronary sinus. In mice, the left anterior cardinal vein (the cranial branch of the common cardinal vein) will persist as the left superior caval vein, while in human the left caval vein usually obliterates, forming the ligament of Marshall

(oblique vein of Marshall in case of incomplete obliteration). After septation, the right atrium consists of 2 parts: the smooth-walled main body derived from the sinus venosus and the right trabeculated atrial appendage. The sinus venosus part of the right atrium, receiving the caval veins and the coronary sinus (in the adult called sinus venarum) contains during development 2 muscular valves: the right and left venous valves. The inferior part of the right venous valve will form in the mature heart both the Eustachian valve, covering the inferior caval vein, and the Thebesian valve, covering the coronary sinus. The left venous valve in human fetuses fuses with the atrial septum that develops at the end of the 4th week[11], whereas in mice it can still be distinguished as a separate structure. The superior part of the right venous valve will develop into the terminal crest (or crista terminalis), that forms a border structure between the right atrial appendage and the right atrial body or sinus venarum in the adult.

Between the 4th and 5th week of development a lumenized PV can be discriminated in the dorsal mesocardium, derived from the midpharyngeal endothelial strand[10]. The PV is at this stage a single structure, that is, at the entrance into the sinus venosus (connecting to the common atrium), bordered by 2 muscular ridges, the left and right pulmonary ridges. These ridges are part of the so-called dorsal mesenchymal protrusion (DMP)[12,13], an accumulation of mesenchymal cells that encompasses the primitive PV, and that contributes to the atrial septum[14]. During atrial septation, the DMP is responsible for the shift of the PV towards the left side[13]. Thus, after atrial septation, the common PV drains to that part of the sinus venosus that is incorporated in the posterior wall of the left atrium (LA) after which the right pulmonary ridge fuses with the left side of the atrial septum[14]. Subsequently, the PV bifurcates, dilates and incorporates into the LA, contributing to the size and consistency of the smooth-walled LA body, which has histological identical characteristics to the PV, with an inner vascular layer covered on the outside by myocardium[15]. Usually, by the end of this process, two right and two left PVs enter the LA, although variations in anatomy are very common[16]. Similar to the situation on the right side, the trabeculated LA appendage can be distinguished from the smooth-walled LA body. Histologically, a transitional zone between these parts can be recognized that is similar to the sinus venosus segment of the right atrium[15].

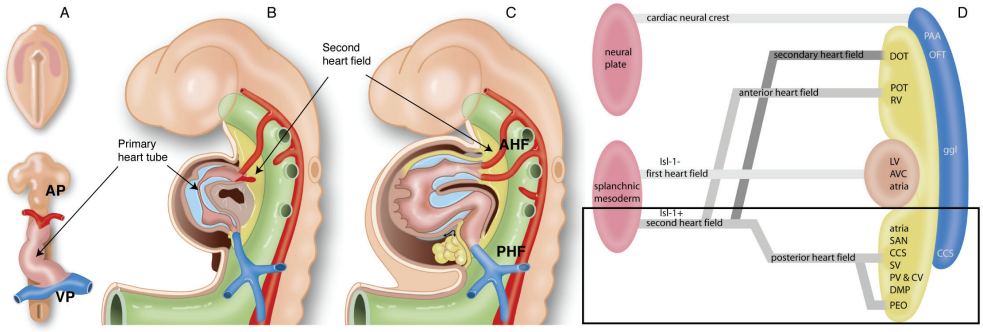


Figure 1.

Schematic representation of sequential stages of cardiac development and the contribution from first and second heart field. The primary heart tube is depicted in brown and the myocardium that is derived from the second heart field (and incorporated later in the heart) in yellow. A. The primary heart tube is formed after fusion of bilateral plates of splanchnic mesoderm in the primitive plate. This tube already has a venous pole (**VP**), and an arterial pole (**AP**). B. Lateral view of embryo (approximately 23 days in human), showing the primary heart tube, surrounded by cardiac jelly (blue), and the second heart field situated dorsally to the heart. C. Human embryo at 25 days. The primary heart tube has expanded both at the venous and at the arterial pole with myocardium derived from the second heart field (depicted in yellow). D. Scheme of the nomenclature the primary and second heart field (see also Chapter 1, Fig.1). At the venous pole of the heart, myocardium is derived from the *posterior heart field*, that contributes to the posterior wall of the atria and the interatrial septum, the sino-atrial node (**SAN**), the myocardium of the sinus venosus (**SV**), pulmonary veins (**PV**) and cardinal veins (**CV**) including the coronary sinus, part of the central conduction system (**CCS**) and the dorsal mesenchymal protrusion **(DMP)**. The second heart field contribution to the venous pole is discussed in more detail later on in this review. Modified after: Crawford and DiMarco, third edition 2009[125].

1.1 Current controversies regarding definitions

1.1.1. Drainage of the primitive PV to the sinus venosus or to the LA segment?

The question whether the common PV originally is connected to the sinus venosus segment or to the LA segment has been a topic of vivid discussion over the past decades. In literature there have been advocates of an original connection of the PV to the sinus venosus[14,17-23] as well as to the LA[24-29]. A probable explanation for

these divergent opinions in the past might be the absence of adequate techniques for a distinct demarcation of the sino-atrial fold in the region of the PV, making it difficult to decide whether the PV connected to a sinus venosus or LA structure. Other limitations were the lack of availability of human embryos in good condition, (resulting in some cases in incorrect interpretations[25] that were revised later on[30]) and the comparability of stages between different species. Although recent studies using novel molecular techniques have contributed new data, this discussion is still alive and seems at least partly to be based on different interpretations of similar findings.

On the basis of scanning electron microscopic studies it was reported by some authors that the PV is connected to the LA, cranial to the systemic venous tributaries at gestational day 10 and 11 in mouse[27], at HH 15-30 in chick[28] and at Carnegie stage 12 in human embryos[29]. In these studies, the sinus venosus segment was defined as the part of the atrium that was encompassed by the venous valves. Indeed, when the left and right venous valves are taken as border structures for the sinus venosus, then the PV does not drain in this area. A limitation of this definition is however, that in very early stages, venous valves can not be distinguished yet. Furthermore, on the basis of expression of the myocardial markers Cx40 and ANF, in combination with 3-dimensional reconstructions, it has been concluded that the venous valves themselves are not completely part of the sinus venosus[26]. According to these studies, from the outset, the myocardium surrounding the PV is distinct from that lining the systemic veno-atrial junctions based on expression patterns of molecular markers[26,31]. It should be noted however in this regard, that, there is no evidence that the PV is surrounded by myocardium from the outset, as it has been demonstrated that the PV myocardial sleeve only develops from stage E11.5 and further on[32-34].

In contrast to these studies, other studies propagate an original connection of the primitive PV to the sinus venosus segment of the heart. In the mouse, at day 9.5 (Carnegie stages 11-12), the primitive PV was recognized posterior to the sino-atrial fold, its endothelium being continuous with that of the sinus venosus[22]. Also in quail embryos, from stage HH15 onwards, expression patterns of the marker HNK-1, which is expressed in the sinus venosus myocardium, suggested that the orifice of the common PV drains to the left part of the sinus venosus[18] (Fig.2). Transient

HNK-1 expression of the embryonic myocardium surrounding the PV was also seen in rat (E 13.5)[23] and human (CRL14-23mm)[35] embryos. Consistent with these findings are more recent data published by our group on CCS-LacZ expression[32] and expression of podoplanin in the developing myocardium surrounding the PVs in mice[6]. The marker CCS-LacZ is expressed in the myocardium surrounding the PV, that becomes continuous with the CCS-LacZ positive sinus venosus area of the right atrium. The posterior heart field marker podoplanin is expressed in major parts of the cardiac conduction system and also found in the myocardium surrounding the PV. It's expression is linked to the Nkx2.5 negative sinus venosus myocardium of the posterior heart field[6,36] (see section 2).

Based on the results of our studies, we favour an original connection of the primitive PV to the sinus venosus segment of the heart.

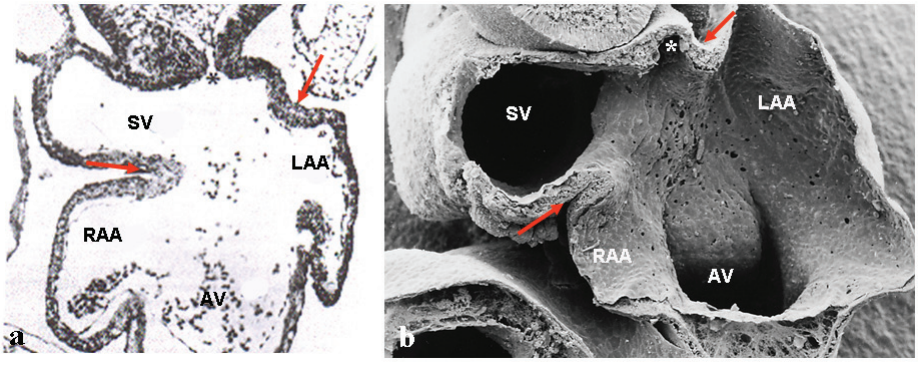


Figure 2.

a. Transverse section through the sinus venosus and left and right atrial appendage of a chick embryo (HH17).

b. Electron microscopic picture at the same level. Arrows point at the sino-atrial fold, which separates the sinus venosus from the atrial segment. The asterisk indicates the common pulmonary vein, that appears to drain in the sinus venosus. Atrio-ventricular canal (**AV),** left atrial appendage (**LAA**), right atrial appendage (**RAA**), sinus venosus (**SV**). Modified after DeRuiter et al., Anat Rec 1995[18].

1.1.2. Is there a direction of growth of the primitive PV in relation to the heart?

Already since the middle of the nineteenth century there have been different opinions about the direction of growth of the PV in relation to the heart, in other words: does the primitive PV grow out from the heart towards the lungs, or vice versa? In former reports by Buell in chick[17], Schornstein in pig[37], Auër in rat and rabbit embryos[38], and Los in human embryos[25], the stem of the PV was considered to be an outgrowth of the dorsal wall of the atrium, whereas by Brown in domestic cats[39], and by Davies in a human neonate[40], the primordium of the PV was considered to grow out of the splanchnic plexus of veins towards the heart. The splanchnic plexus is not species-specific[17,39,41]. In quail embryos, this vascular plexus arises at HH8 by differentiation of splanchnic mesodermal cells into endothelial precursor cells (vasculogenesis). The midpart of this splanchnic plexus is the earlier mentioned midpharyngeal endothelial strand. Immunohistochemical studies performed in avian embryos, using antibodies against quail endothelial cells and precursors, showed that this midpharyngeal endothelial strand from early stages on connects the endocardium of the primitive heart tube to the splanchnic vascular plexus around the foregut and the lung buds and contains the Anlage of the non-lumenized common PV[10]. This is supported by studies in mouse, chick and human embryos by Webb et al.[27-29]. This implies that, in contrast to the concepts of PV ingrowth[39,40] and outgrowth[17,25,37-39], the endothelium of the PVs has been connected to the endocardium of the heart right from the beginning[10,18].

2. Which role does the second heart field play in the development of the venous pole?

As mentioned earlier, there is increasing attention for the role of the second heart field in cardiac development. Different lineage markers have been used to study the final destination of cells in the heart[2,7,42-44]. Islet (Isl)1 is a marker for cells recruited from the second heart field and is expressed at the arterial as well as the venous pole of the heart during differentiation[2] (Fig.1d). There are several markers expressed in the posterior heart field, including, Shox2[45], Tbx18[42], Pitx2[46], podoplanin[6,7], but excluding (lack of expression of) Nkx2.5[6,46]. The posterior heart field, as a part of the second heart field, contributes to the sinus venosus, the cardiac conduction system and the

atrial septum[6,7] (Fig.1d). A subset of cardiac progenitor cells in the posterior heart field receives Hedgehog (Hh) signals, likely from pulmonary endoderm, resulting in migration of these progenitor cells through the dorsal mesocardium to form the atrial septum as well as the DMP[47]. Similar to Hh signaling, also, PDGF-signaling is known to play a role in the development of second heart field derived structures of the venous pole[48].

Next to a contribution to these aforementioned structures, in recent years, second heart field contribution to the vascular wall and myocardium surrounding the PVs[36,49] has been established.

2.1 Second heart field contribution to formation of a PV myocardial sleeve.

Several theories exist about the origin of the myocardium surrounding the PVs. The three most important processes described are: First the process of myocardialization which refers to *migration* of atrial cardiomyocytes into the extracardiac mesenchyme and extending towards the PV[34,50-52]. Between species, differences exist in the extension of the myocardial sleeve. In mice, this sleeve extends intrapulmonary[49,53], while in the chick this intrapulmonary (i.e.peripheral) myocardial extension is very limited[54]. The second process is *recruitment*, which refers to differentiation of splanchnic mesenchymal cells into cardiomyocytes[6,33,55,56] and the third process is a *combination* of the two aforementioned[46]. The recruitment of mesenchymal cells fits smoothly with earlier research in cardiac development underlining the addition of myocardium to the primary heart tube[57], from the second heart field[2,3].

In the context of the origin of myocardium surrounding the PV, as aforementioned, the myocardial markers Nkx2.5[6,46], Pitx2[46], Shox2[45], Tbx18[42] and podoplanin[6,7] are of interest for their expression in cells that contribute to the venous pole of the heart. Here, a distinction must be made between the *mesoderm* that surrounds the primitive PV in early stages (when formation of a myocardial sleeve around the PV has not taken place yet), and that is Nkx2.5 positive already in early stages; and the newly added *myocardial sleeve* that develops around the PV at stage 12.5 and further on[32-34]. Nkx2.5 expression in this newly forming myocardium around the PV initially is absent during a brief time period[6] (like in the sinus venosus myocardium), rapidly turns into a mosaic pattern with positive and negative cells, and finally is completely Nkx2.5

positive, indicating newly added myocardium that has differentiated. Compared to the cardinal veins that become Nkx2.5 positive at a later stage as well, this was considered as the result of a higher proliferation rate of myocardium of the PVs[7,49], which is in line with previous findings by other groups of a high proliferation rate of the PV myocardium as compared to the proliferation rate of the atrial myocardium[46]. Several markers expressed in putative PV myocardium, including the myocardial marker MLC2a and the posterior heart field marker podoplanin[6], are initially expressed at the left side of the dorsal mesocardium, indicating that PV myocardium is preferentially added from the left side of the posterior heart field, regulated by progenitor cells that play a role in left-right patterning, as was reported for Pitx2c[46,58,59]. Podoplanin is a transmembrane protein that was originally described in the kidney and in later studies was found to be expressed in the heart. Podoplanin knockout embryos show marked hypoplasia of the posterior heart field derived myocardium of the sinus venosus, including the sinus node and the myocardium surrounding the PVs and covering the posterior atrial wall[36]. Interestingly, in this model also a reduced number of SMCs was observed, indicating that the posterior heart field has a role in myocardial and SMC contribution at the venous pole of the heart[36,49]. This will be further addressed in section 2.2. Analogous to podoplanin mutants, chick, mouse and human embryos with DMP abnormalities caused by defective Hh or PGDF-signaling, also show hypoplastic PV myocardium, indicating a role of the DMP in proper PV development[48].

2.2 Second heart field contribution to smooth muscle cell (SMC) formation in the PVs and LA.

SMCs are another important source of cells that contribute to the vascular layer of the PVs and the inner layer of the LA[15]. It has been described that SMCs can differentiate from mesenchymal cells[60]. At the arterial pole SMCs have been described to originate from the splanchnic mesoderm, the neural crest[61] or endothelial cells[62]. At the venous pole however, not much is known about the origin of SMCs. As was implied above, several hypotheses consist about the origin of the SMCs in the LA body. Initially, SMCs were considered to migrate from the PVs towards the heart and into the LA[34]. SMCs in the LA body have also been considered to be the result of PV incorporation in the

LA[15]. The above mentioned data[60-62] have suggested differentiation as a mechanism by which the presence of SMCs in the LA can be explained, which has been supported by studies using the posterior heart field marker podoplanin[49]. In *podoplanin* knock-out embryos there is a significantly reduced myocardial cuff around the PVs as well as a reduced number of SMCs. Similar findings were reported by Bleyl et al. in embryos with DMP abnormalities[48]. These SMCs do not extend in the complete smooth-walled LA body. These findings indicate that a process of differentiation rather than incorporation is the mechanism responsible for SMC formation in the LA (Fig.3; compare with Chapter 1, Fig.2, adapted to new insights). These findings are in line with observations that have been made in the inhibitor of differentiation (Id2) mouse model. Id2 knockout mice seem to have small-sized LA bodies -probably caused by impaired or delayed PV incorporation-, while the amount of differentiated smooth muscle cells in the LA did not differ from wild type mice (Jongbloed et al., unpublished results).
These data suggest that *incorporation* and *differentiation* are two autonomous independent processes.

3. What are the current concepts on the origin of normal and abnormal PV drainage?
In the paragraphs above, we have described the origin of the PV Anlage from the midpharyngeal endothelial strand, that is connected to the heart from the beginning. During development different drainage patterns of the primitive PV can be distinguished (Fig.3a-g). We will describe here the drainage patterns according to Rammos[30], who discriminated three periods with different drainage patterns in **normal** human PV development: 1. A *peripheral period* (CRL 4.4-7mm) during which there is no open connection yet between the primitive atrium and the developing lung vessels. The mid-pharyngeal endothelial strand has not lumenized yet. The primitive lung drains via the splanchnic venous plexus into the systemic venous circulation (cardinal and umbilical veins). 2. An *intermediate period* (CRL 7-11mm) during which the lumenized common PV connects to the LA as well as to the lung plexus. The lungs can drain their blood either centrally to the LA, or peripherally to the systemic venous system. 3. A *central period* during which the only possible drainage is via the common PV to the heart (Fig.4a).

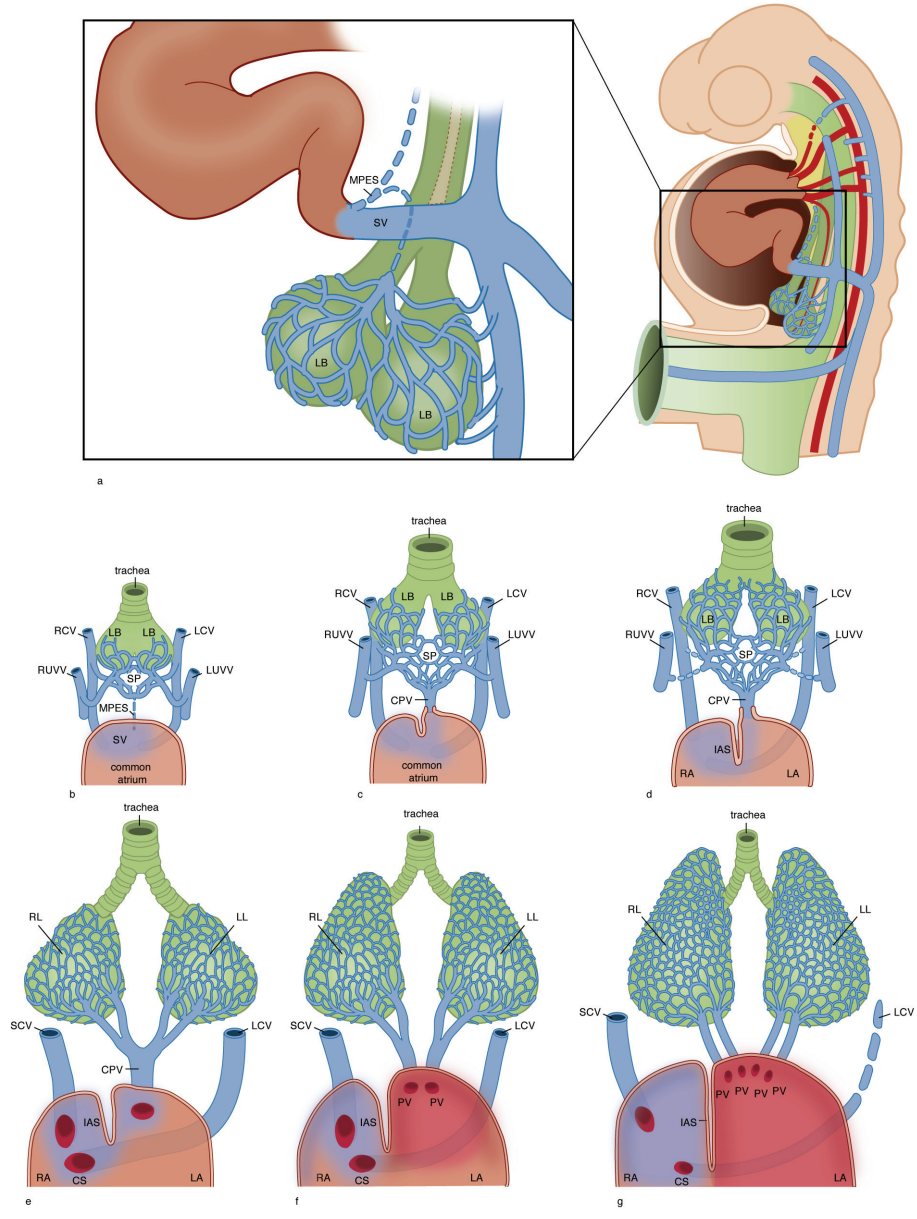

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Figure 3a-g. Schematic depiction of pulmonary vein development, adapted to new insights.

a,b. Peripheral period, lateral (a) and frontal (b) view. The splanchnic vascular network surrounds the two lung buds (**LB**) and drains to the systemic circulation by means of primitive pulmonary-to- systemic connections to the right and left cardinal veins (**RCV/LCV**) or the right and left umbilical and vitelline veins (**RUVV/LUVV**). In the region of the heart, this splanchnic plexus (**SP**) is connected to the sinus venosus segment of the heart (**SV**, blue) by means of the midpharyngeal endothelial strand (**MPES**), which is the remaining part of a strand of endothelial cells that initially runs from the arterial to the venous pole in the dorsal mesocardium.

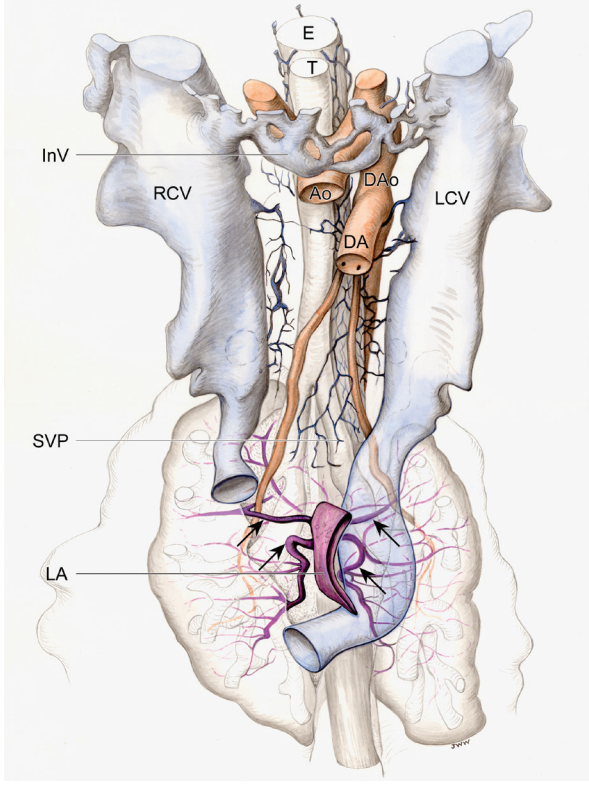
c. Intermediate period. When the endothelial strand, which is the anlage of the future common pulmonary vein (**CPV**) lumenizes, the splanchnic plexus can drain its blood to the systemic circulation as well as to the heart. Although atrial septation has started, the CPV still drains to the sinus venosus segment that is connected to a common atrium.

d. Central period. The CPV grows and dilates, becoming the main route for drainage of pulmonary venous blood. The primitive pulmonary-to-systemic connections are not necessary anymore and regress. After atrial septation has completed (**IAS**), the part of the sinus venosus containing the CPV is placed to the left, becoming part of the left atrium (**LA**).

e. The CPV has bifurcated, so that the right and left lung (**RL/LL**) drain to the heart by means of two left and two right pulmonary veins. The right cardinal vein becomes the superior caval vein (**SCV**) and the left cardinal vein gives rise to the coronary sinus (**CS**), both connecting to the sinus venosus part of the right atrium (**RA**).

f,g. By incorporation of the CPV, initially, one right and one left CPV drain to the LA (f). After incorporation has completed, four separate pulmonary vein (**PV**) ostia can be identified on the inner side of the LA (g). Independent on PV incorporation, smooth muscle cells (red) can be identified in the LA as a result of differentiation. The extracardiac part of the LCV regresses, becoming the so-called ligament of Marshall.

A



B

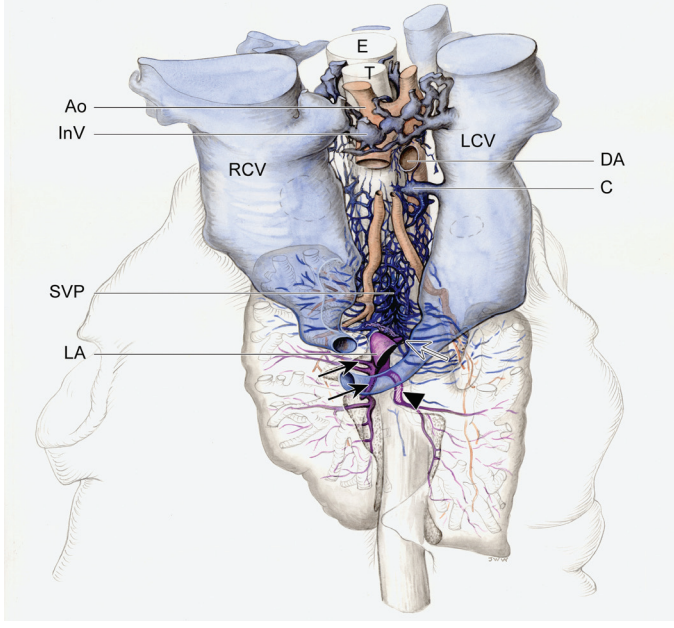


Figure 4a,b.

a. 3-D Reconstruction of the thoracic contents of a normal human embryo of 18 mm crown-rump length (approximate gestational age 8 weeks; Carnegie stage 14). Frontal view, after resection of the heart including part of the left atrial dorsal wall. Four pulmonary veins (arrows**)** are connected to the lung region and to a short common pulmonary vein that has an open connection with the left atrial (**LA**) dorsal wall. At this stage there is no connection anymore of the splanchnic plexus (**SVP**) to the lungs, but still very small communications of the SVP with the right (**RCV**) and left cardinal veins (**LCV**) exist caudal to the innominate vein (**InV**). Aorta (**Ao**), descending aorta (**DAo**), ductus arteriosus (**DA**), esophagus (**E**), trachea (**T**).

b. 3-D Reconstruction of an abnormal human embryo of 18mm crown-rump length (approximate gestational age 8 weeks; Carnegie stage 14). Frontal view, after resection of the heart, including part of the left atrial dorsal wall. Posteriorly to the left atrial (**LA**) dorsal wall three individual pulmonary veins, originating from a common pulmonary vein run towards the lungs. On the left side the left upper PV is lacking (open arrow), the left lower PV does not contain blood (black arrowhead). The two right PVs (black arrows) are normal. The splanchnic vessels (**SVP**) extend a great deal further than in the normal embryo and with numerous connections to both lungs. A large communication analogous to the vertical vein (**C**) exists between the splanchnic plexus and the left cardinal vein (**LCV**). Aorta (**Ao**), ductus arteriosus (**DA**), esophagus (**E**), innominate vein (**InV**), right cardinal vein (**RCV**), trachea (**T**).

The primitive connections of the lung to the systemic veins are not necessary anymore at this stage and regress[30,63] by a process called remodeling, analogous to the developmental processes at the arterial pole[64]. Persistence of pulmonary-to-systemic communications is the likely substrate of *extracardiac* anomalous PV connections, probably related to abnormal development of the common PV (TAPVC) or one of its branches (PAPVC)[30,63,65]. On the other hand, *cardiac* anomalous PV connections seem to result from absent or impaired mesenchymal contribution to atrial septation[14,48].

Insights in **abnormal** PV development historically have mainly been based on post-mortem studies leading to classifications and tentative embryological explanations of the encountered anomalies[63,66]. Total anomalous PV connection was explained by a shift of the common PV with regard to the atrial septum, leading to a complete connection to the right atrium, or, when the PV connection with the atrium was lost, to the use of the nearest systemic vein for definitive drainage[66].

Abnormal PV development can be based on various processes that take place at different developmental stages and at different anatomical levels. These are summarized in Table 1. These processes lead to different congenital heart anomalies (clinical entities), that we will subsequently describe in relation to embryonic development.

Noteworthy is that, regularly, inflow and outflow tract abnormalities are found to co-exist[67,68]. Since the second heart field contributes to incorporation of mesenchymal cells at both the venous as well as the arterial pole of the heart[2], second heart field impairment may be the underlying cause for this co-existence.

3.1. Clinical entities (also see Table 1).

3.1.1 Total anomalous pulmonary venous connection (TAPVC).

In this condition there is no open connection to the LA because of non-lumenization of the midpharyngeal endothelial strand (PV Anlage) or atresia of an initially lumenized common PV. If the latter takes place at a late embryonic stage, the condition is lethal, with 4 PVs draining to a blind ending confluence (Table 1). Usually, all the PVs drain to the systemic venous circulation (extracardially), but rarely, they have a cardiac connection as a consequence of impaired mesenchymal contribution to atrial septation[14,48]. Four types of TAPVC can be discriminated: A *supracardiac* type accounting for 45% of cases; an *infracardiac* type for 25%; a *cardiac* type for 25%; and a *mixed* type for 5%[69].

The supra- and infracardiac type both belong to the extracardiac group of TAPVC which means that, in absence of an open connection to the LA, the nearest extracardiac systemic vein is used for PV drainage. The preserved primitive pulmonary-to-systemic connection is represented by the so-called vertical vein. In *supracardiac* TAPVC the (left) vertical vein usually connects to the innominate (brachiocephalic) vein, less often to the superior caval vein -at the cavo-atrial junction-, and rarely to the azygos vein. These systemic veins are representatives of the peripheral part of the former cardinal veins. The commonest sites of connection in patients with *infracardiac* TAPVC are the portal vein (65%) and the embryonic ductus venosus, that has persisted in these patients. Less common are connections to the gastric vein, the hepatic veins and the inferior caval vein. These systemic veins originate from the former umbilicovitelline veins[11].

Tabel 1. Abnormal pulmonary vein development in relation to anatomical level, time and clinical entities. On the left side, the developmental impairments and different anatomical levels are depicted, and on the right the clinical entities that result from this abnormal development. These clinical entities are described in the text in section 3.1. Abbreviations: atrio(ventricular) septal defect (**A(V)SD**); mid-pharyngeal endothelial strand (**MPES**); partial anomalous PV connection (**PAPVC**); pulmonary vein(s) (**PV(s)**); total anomalous PV connection (**TAPVC**); second heart field (**SHF**).

Anatomical level	Anomaly	Developmental impairment	Time relation (related to atrial septation)	Pulmonary-to-systemic connections	Clinical entity
common PV/ MPES	absence	absent connection PV-LA (absent MPES)	before	will persist	TAPVC (extracardiac type)
	atresia	non-lumenization of the MPES (PV Anlage)	before	will persist	TAPVC (extracardiac type)
	stenosis	secondary stenosis of an initially lumenized common PV	during/after	will disappear	cor triatriatum
	atresia	secondary obliteration of an initially lumenized PV	after	have disappeared	TAPVC (lethal)
first or second PV bifurcation	atresia	non-lumenization of 1 or more individual PV(s)/tributaries	during	will persist	PAPVC (extracardiac type)
	stenosis	secondary stenosis of an initially lumenized individual PV	during/after after	will disappear have disappeared	congenital PV stenosis acquired PV stenosis
	atresia	obliteration of 1 or more initially lumenized PVs	after	have disappeared	(solitary) PV atresia
veno-atrial junction	variant number of PVs	abnormal PV incorporation	after	have disappeared	
	<4	incomplete			unilateral common PV ostium*
	>4	extreme			>4 PV ostia*
dorsal mesenchymal protrusion	hypoplasia	abnormal mesenchymal contribution from SHF	during	will disappear	sinus venosus defect with PAPVC (cardiac type) A(V)SD
	absence	absent mesenchymal contribution from SHF	during	will mostly disappear	TAPVC (cardiac type) A(V)SD

* Note: not regarded as pathological but rather as anatomical variations

In the *cardiac* type of TAPVC, the PV confluence drains directly to the right atrium, or more often, directly to the coronary sinus. Drainage to the coronary sinus is the result of persistence of pulmonary-to-systemic venous connections to the left common cardinal vein (which becomes the coronary sinus). The etiology of the direct connection to the right(-sided) atrium is a topic of discussion since this condition is very rare in case of situs solitus (i.e. in normal atrial arrangement), and almost always associated with (right) atrial isomerism[70]. Leftward displacement of the atrial septum has been proposed[71], although this was also considered a secondary phenomenon to the abnormal expansion of the developing right atrium due to incorporation of the PVs[72]. The fact that this condition is more often seen in case of right atrial isomerism suggests that inhibition of left-sided structures is a more probable explanation. This would fit in the concept of a bilateral Anlage (left and right pulmonary pit) with development of the right pulmonary pit and inhibition of the left part as was suggested by Männer[72]. Such a bilateral Anlage was never identified by our group. We hypothesize that in case of right atrial isomerism, the unilateral Anlage or common PV connects to one of the "right" atria or maintains a midline position, connecting to both atria, since atrial septation is also disturbed in this condition[73]. Another component that might play a role in the onset of cardiac TAPVC to the right atrium is the dorsal mesenchymal protrusion, which role in atrial septation and leftward displacement of the PV has already been established[12,13]. Defective development of this dorsal mesenchymal protrusion might lead to a rightward shift of the PV, resulting in PV drainage to the sinus venosus part of the right atrium[14,48].

The *mixed* type usually is a combination of the cardiac and supracardiac type of TAPVC, in which the left PVs drain to a vertical vein and the right PVs to the coronary sinus, as already explained. Also this type finds its origin in an absent or atretic PV Anlage.

3.1.1.1. TAPVC and immunohistochemical findings.

On the basis of comparative immunohistochemical studies of the LA dorsal wall and the PVs between normal human hearts and hearts with TAPVC, we know that, in contrast to normal hearts, in hearts with TAPVC a. PVs have no myocardial sleeve b. the LA has no inner smooth muscle cell layer and c. the LA body is very small and thin-walled because of non-compact hypoplastic myocardium[15,49,67].

a.: *Lack of a PV myocardial sleeve in TAPVC*. It is noteworthy that in this condition, also the vertical vein, i.e. the persisted pulmonary-to-systemic connection, lacks myocardial covering. In both normal hearts and hearts with situs inversus, the PVs are normally myocardialized which means that myocardialization is not dependent on left-sidedness in the body but probably on left second heart field signaling. In hearts with partial anomalous pulmonary venous connection (PAPVC), only the normally connecting PVs are myocardialized, from which we hypothesized that a connection of a PV to the LA is necessary for myocardialization to occur[67] (Fig.5).

The fact that left-right patterning seems to play a role in myocardialization of the PVs is supported by studies using the differentiation marker Pitx2[46,58,59,74-76]. Pitx2 is a homeodomain transcriptional protein, which Pitx2c isoform is exclusively expressed in the heart, driving asymmetrical cardiac morphogenesis. Initially, Pitx2c is asymmetrically expressed in the left lateral plate mesoderm and subsequently in the cardiogenic precursors of the left second heart field, supplying both the venous[46] and the arterial pole[77]. At the venous pole, Pitx2c is present in the posterior heart field in mesenchymal cells, that will later on differentiate into myocardial cells. PVs of Pitx2c mutants have no myocardial sleeve[46]. Pitx2 represses the transcriptional pathway of right atrial identity, also suppressing the SA node transcriptional program on the left side[75]. So, Pitx2c mutants show right atrial isomerism[46]. Since both Pitx2c mutants and TAPVC patients lack a PV myocardial sleeve, there might be a connection between Pitx2c and TAPVC. This possibility is supported by the fact that right atrial isomerism as well as TAPVC frequently go together with asplenia[78]. Until now, a relation between Pitx2c and asplenia, was not reported in literature.

b.: *Lack of LA inner smooth muscle cell layer in TAPVC*. As mentioned above, *differentiation* rather than *incorporation* is the mechanism responsible for SMC formation in the LA[49]. The podoplanin knockout mouse model, shows an impaired formation of SMCs in the LA body, similar to human TAPVC[67], suggesting that lack of a SMC layer in TAPVC is also based on a differentiation impairment.

c.: *Small LA size*. The small size of the LA in TAPVC is the consequence of non-incorporation of the PVs. In fact, the LA body has not fully developed and the main part of the LA is formed by the LA appendage. Additionally, reduced hemodynamic fetal flow may contribute to the small size of the LA.

Non-compact, hypoplastic LA myocardium in TAPVC. Impaired formation and differentiation of posterior heart field derived myocardial and smooth muscle cells in the PVs and the LA dorsal wall are findings both observed in knockout models of PHF markers and in TAPVC, suggesting that impairment in the posterior heart field contributes to TAPVC. A developmental explanation for the spongious LA myocardium in TAPVC may be the impaired formation of epicardium derived cells (EPDC's) and altered epicardial-myocardial interaction, which are known to be responsible for abnormal atrial and ventricular myocardium[7,79].

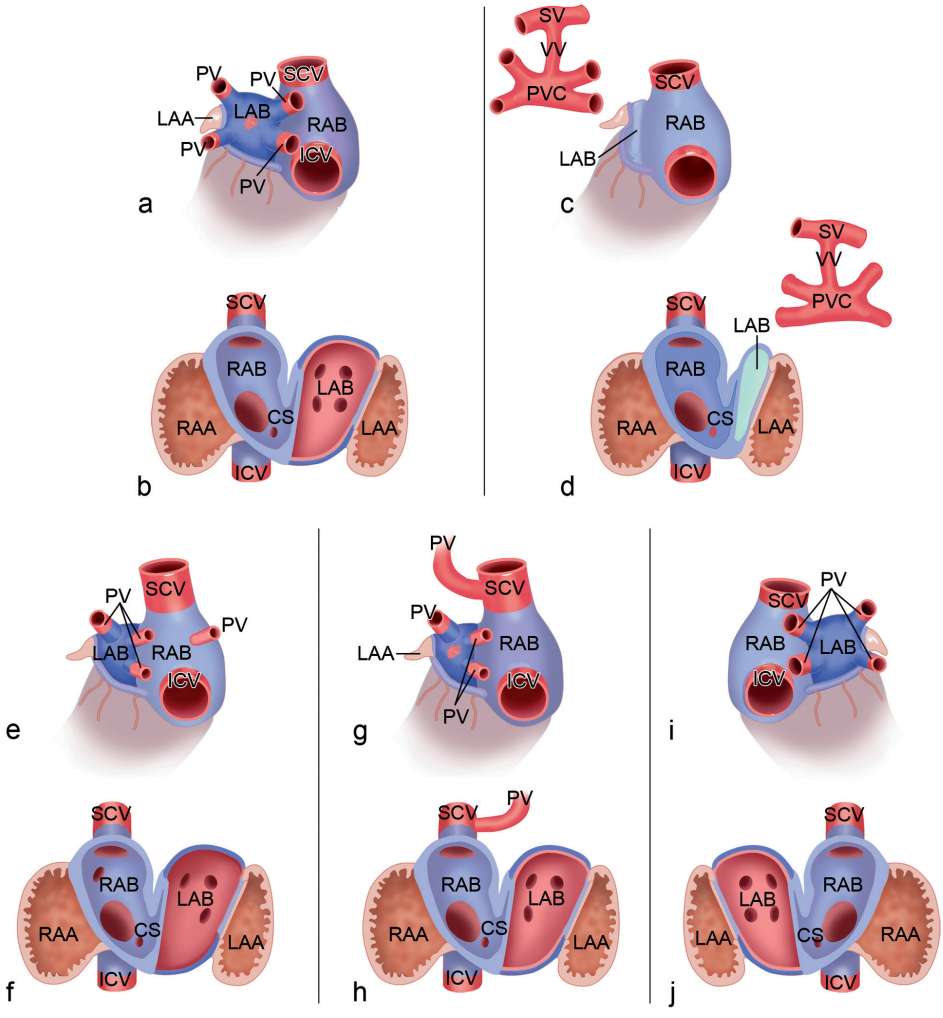


Figure 5a-j.

Schematic depiction of outer (a,c,e,g,i) and inner (b,d,f,h,j) side of the atrial chambers and the pulmonary veins (**PV**) in normal hearts (a,b), hearts with total anomalous pulmonary venous connection (TAPVC;c,d**),** hearts with (extra)cardiac partial anomalous pulmonary venous connection (PAPVC; e-h) and hearts with situs inversus (i,j).

a,b. Normal heart. a. The left atrial body (**LAB**) and pulmonary veins (**PV)** are covered by myocardium derived from the posterior heart field (***dark blue****).* In the area between the PVs the myocardium can be discontinuous or absent. The right atrial body (**RAB**) is covered by sinus venosus myocardium (***light blue****).* b. The inner aspect of both LAB and RAB is smooth-walled. In the LAB vessel wall tissue (***red***) is found. The left and right atrial appendages (**LAA, RAA**) consist of trabeculated myocardium (***brown****)*.

c,d. TAPVC, extracardiac type. The PVs drain via a vertical vein (**VV)** into a systemic vein (**SV**) and are not covered by myocardium. The LAB consists of smooth-walled hypoplastic myocardium, derived from the posterior heart field. Because of the absence of a PV connection and incorporation into the LA, the LAB is small. Moreover, the LAB does not contain vessel wall tissue, probably because of impaired differentiation of smooth muscle cells.

e,f. PAPVC, cardiac type. The abnormally connected PV drains directly into the right atrial body (**RAB**), but (as in the normal situation) no vessel wall tissue is found in the RAB. In contrast to the PVs that are connected to the LAB, the abnormal PV is not covered by myocardium.

g,h. PAPVC, extracardiac type. The abnormally connected PV drains (in)directly into the superior caval vein **(SCV)** and is, similar to the cardiac type of PAPVC, not covered by myocardium. The LAB and its incorporated PVs are myocardialized and, also comparable to the normal heart, vessel wall tissue is present in the LAB.

i,j. Situs inversus. The LAB is located on the right side of the heart and receives the four PVs, which are covered by myocardium. In the LAB vessel wall is found parallel to normal PV incorporation.

Coronary sinus (**CS**), inferior caval vein (**IVC**), pulmonary venous confluence (**PVC).** Modified after Douglas et al., Int J Cardiol 2009[67].

3.1.1.2.

TAPVC and genetics. Maternal exposure to environmental teratogens as lead, paint or pesticides have been described to cause a familial susceptibility for TAPVC, mostly in the presence of a positive family history of cardiac and non-cardiac malformations[80,81]. Until now, familial incidence of TAPVC appeared not to be related to ethnic background and was seen in siblings, twins and cousins of the same family with a male preponderance[80,82]. Moreover, TAPVC has been described in association with genetic syndromes, such as the Holt-Oram syndrome, cat eye syndrome and craniofacial and skeletal dysmorphias[80,83], so that the question has raised whether there is a heritable component. Since then, various genetic hypotheses have been proposed. Multifactorial inheritance[84] seemed unlikely in families with two or more positive members, as well as autosomal recessive inheritance in the absence of consanguinity in positive families[85]. More likely is an X-linked inheritance[86] and autosomal dominance with variable expressivity and incomplete penetrance[83,87]. As beforementioned, in 70-80% of patients with the asplenia syndrome TAPVC is the most characteristic heart defect[78]. Both of these laterality disorders are suggested to be different expressions of a single genetic disorder[78]. Because of scarcity of multigeneration TAPVC families, identification of candidate genes for TAPVC is difficult. Until now, two candidate genes have been proposed. The *TAPVR1* gene, playing a role in vasculogenesis, maps to chromosome 4q12, which centromeric region contains receptor tyrosine kinase genes as kinase domain receptor (KDR)[88]. Recently, the *TAPVR1* susceptibility locus was specified to the PDGFRA-KIT intergenic interval of chromosome 4q12[48]. The *ANKRD1/CARP* gene, encoding a cardiac transcriptional regulator, was localized proximally to the breakpoint of a previously found translocation site on chromosome 10[89,90]. Investigations to limit the *TAPVR1* gene interval and specify the role of the *CARP* gene in the pathogenesis of TAPVC are on their way.

Another unsolved issue is the etiology of primary PV stenosis. Until now, in literature, a genetic basis for this entity has not been reported.

3.1.2. PAPVC.

In this condition, two or more PVs connect normally to the LA and two or less PVs do not lumenize or become atretic after initial lumenization. The corresponding lung(segment) drains its blood to the systemic venous circulation, usually extracardially by means of persistence of connections to the superior caval vein (Fig.4b) or to the inferior caval vein (Scimitar syndrome[91]). However, two or more PVs can also connect cardially, i.e. to the sinus venosus part of the right atrium, which is the consequence of defective mesenchymal contribution to atrial septation, obligatory coexisting with a sinus venosus defect[14] (section 3.1.5).

3.1.3. Cor triatriatum.

This condition is based on stenosis of the common PV, leading to non-incorporation (Table 1). The PVs connect to a separate chamber that either drains to the left or (rarely) to the right atrium, dependent on initially normal or abnormal connection of the common PV[92]. The LA body is very small due to absence of PV incorporation. Moreover, cor triatriatum in combination with left or right atrial isomerism can be found[70].

3.1.4. PV stenosis.

This condition can be congenital or acquired[93].

3.1.4.1 Primary PV stenosis.

This is the consequence of abnormal remodeling of individual PVs. It can be found in hearts with normally[94] as well as abnormally connected PVs[95]. The stenosis can be present in either all the PVs, or in individual PVs, localized either at the veno-atrial transition or more peripherally towards the lung. In hearts with normal PV connection, the PV return to the LA will be obstructed, whereas in case of anomalous PV connection, pulmonary venous return to the right atrium is obstructed. Both situations can lead to uni- or bilateral pulmonary hypertension on the long-term.

3.1.4.2. Acquired PV stenosis.

Whereas the exact etiology of congenital PV stenosis is unknown, acquired PV stenosis can be the consequence of radiofrequency ablation[96], surgery[97,98] or by a so-called pulmonary veno-occlusive disease[99,100]. Pulmonary veno-occlusive disease is characterized by extensive and diffuse occlusion of PVs by fibrous tissue. Usually the (eccentric) intimal thickening involves venules and small veins but, occasionally, also larger veins are involved. Clinically, patients have pulmonary arterial hypertension without elevation of the pulmonary arterial wedge pressure. Infectious, genetic, and toxic agents as well as a thrombotic diathesis and autoimmune disorders may be responsible for the etiology of pulmonary veno-occlusive disease[99,100].

3.1.5. Sinus venosus superior or inferior defect.

Normally, during atrial septation, mesenchyme of the dorsal mesocardium protrudes in the dorsal wall of the primary atrial segment (dorsal mesenchymal protrusion[12] or spina vestibuli[13]) being responsible for leftward displacement of the common PV. When this mesenchymal contribution to atrial septation from the posterior heart field is impaired, a so-called sinus venosus defect can develop, often resulting in anomalous connection of one or two right PVs (PAPVC) to the right-sided sinus venosus: the sinus venarum of the right atrium, one of the atrio-caval junctions or the superior or inferior caval vein[14]. Similarly, severe hypoplasia of this DMP (may) lead to TAPVC to the sinus venosus part of the right atrium (cardiac type)[48].

3.1.6. Variant number of PVs.

This condition is based on variations in PV incorporation which can be caused by PV stenosis at the veno-atrial junction. Incomplete PV incorporation can result in a unilateral common PV ostium, mostly seen on the left side[15,16,101]. Extreme PV incorporation can lead to more than four PV ostia, more frequently seen on the right side as an additional right PV draining the right middle lung lobe[16] (Table 1). In our opinion, both common ostia and additional PVs should not be regarded as an abnormality but rather as normal anatomical variations.

4. How is PV development related to arrhythmogenesis?

4.1. Normal PV development.

The majority of atrial paroxysms of arrhythmia start with earliest activation at the PV ostium or within the PVs[1]. Characteristics of the PV myocardial sleeves are held responsible for this potential to generate arrhythmias. These myocardial sleeves are thickest at the veno-atrial junction and become thinner peripherally, towards the lung[101,102]. The myocardial architecture in normal PVs is highly variable[102]. Usually, the sleeves are better developed in the superior than in the inferior PVs, specifically at the inferior side of the superior PVs and at the superior side of the inferior PVs[53,102]. At the endocardial (inner) side of the vessel a circumferentially oriented myocardial fibre arrangement is described, while at the epicardial (outer) side this arrangement is longitudinally[103]. These transitions in fibre arrangement in combination with interpositions of fibrous tissue, separating myocardial fibres, can lead to activation delay and conduction block, so creating a milieu for reentry[102,104]. Another predisposing factor to reentry in normal development may be the myocardial discontinuity in the area between the PVs, as well as in the transitional zone between the LA body and the LA appendage[15].

Next to these architectural characteristics of the PVs, several other mechanisms may contribute to the arrhythmogenic capacities of the PVs. Morphologic studies have described cells resembling pacemaker cells in the myocardium surrounding the PVs[105,106]. These observations would explain the possible independent pacemaker activity that was observed in the PVs in otherwise mechanical silent hearts of rabbits and cats, described as early as 1874. It is tempting to speculate that the presence of node-like cells would also be responsible for the initiations of paroxysms of atrial arrhythmias described later on[1].

In this light, it is interesting to note that several immunohistochemical and molecular markers related to the developing cardiac conduction system are (transiently) expressed in the myocardium surrounding the developing PV, including HNK-1, CCS-LacZ, and podoplanin, suggesting that areas related to the occurrence of arrhythmias correspond to areas derived from the embryonic cardiac conduction system[32,35]. It was hypothesized that embryonic remnants or re-expression of embryonic genes could be related to clinical arrhythmias in neonates and adults, respectively.

These findings are strengthened by several electrophysiological studies that have demonstrated specific electrophysiological capacities of the PVs as compared to the atria[107-109], with a reduced resting membrane potential, action potential amplitude, a smaller phase 0 upstroke velocity and a shorter duration of the action potential resulting in shorter refractory periods and slowed conduction. These cellular characteristics in combination with the anisotropic arrangement of the PV myocardial fibres favour the occurrence of reentry and probably the maintenance of initiated arrhythmias[103,104].

Furthermore, spontaneous or enhanced pacemaker activity (abnormal automaticity) in the PVs has been reported[110,111].

Recently, human genetics studies identified two sequence variants on chromosome 4q25 that were strongly associated with increased risk for atrial fibrillation[112]. The gene closest to these sequence variants is Pitx2, which is critical for the development of PV myocardium, making Pitx2 a likely candidate locus for atrial fibrillation[112,113]. Moreover, the presence of a sequence variant increased the risk for both early and late recurrence of atrial fibrillation[114].

4.2. Abnormal PV development.

In patients with TAPVC, conduction abnormalities, including sinus node dysfunction (sick sinus syndrome, tachy- and bradyarrhythmia) are reported[115-117]. In hearts with TAPVC, the PVs lack a myocardial sleeve and the myocardium of the LA dorsal wall is hypoplastic, with discontinuities[67]. The combination of hypoplastic myocardium and sinus node dysfunction could be related to an abnormal contribution from the posterior heart field, as studies in podoplanin knockout mice demonstrate both hypoplastic and discontinuous myocardium surrounding the PVs, as well as an hypoplastic sino-atrial node[36,49]. Hypoplasia of the sinus node in TAPVC would provide a plausible explanation to the observed conduction disorders in TAPVC. However, further research is needed to reveal a possible co-existence of pulmonary venous myocardial hypoplasia and hypoplasia of the sinus node in TAPVC[67]. Although myocardial discontinuities of the LA dorsal wall could form a substrate for generation of arrhythmias in TAPVC hearts, total absence of a PV myocardial sleeve may explain the low incidence of atrial arrhythmias originating from the PV observed in this patient group[117].

After surgical repair of TAPVC, compared to other atrial surgery, significant atrial arrhthymias as atrial flutter and reentry tachycardias are uncommon, although sinus node dysfunction (sick sinus syndrome, tachy- and bradyarrhythmia) has been reported[115-117].

In general, in this patient group, arrhythmias are asymptomatic and not related to the type of surgery[118] (and Douglas et al. unpublished results). Therefore, it is arguable whether the reported arrhythmias are related to the operation or to the pathological findings of TAPVC itself. Given the possible relation with sinus node dysfunction, for this patient category a careful follow-up including Holter registration is warranted.

5. What are consequences of the various concepts of PV development on clinical management?

For surgical correction of TAPVC, the PV confluence has to be connected to the LA. This can be performed by an anteroposterior right-to-left atrial incision after median sternotomy, or by a single LA incision after a left lateral thoracotomy. Subsequently, an anastomosis is made between the PV confluence and the LA after ligation of the former connection to the systemic circulation, the vertical vein. Since the LA in TAPVC is very small[67], in the past, surgeons have tried to augment the LA with a patch, however this appeared to be the substrate for arrhythmias[95,98]. In hearts with TAPVC, the PVs have no myocardial layer resulting in an enhanced susceptibility for internal and environmental influences making them more prone to congenital and acquired PV stenosis[93,96], which is still one of the main unravelled problems. For postoperative PV stenosis at the site of the anastomosis surgeons successfully developed and applied so-called sutureless techniques, leaving the PVs untouched[119,120]. Primary PV stenosis is still a major cause of death in this patient group[97,98].

In PAPVC, frequently presenting as part of a sinus venosus defect, drainage of the abnormally connecting PVs has to be “rerouted” to the LA which usually is performed by an atrial patch that is fixed around the abnormally connecting PV ostia, so directing drainage to the LA. If this is anatomically not possible, the PVs have to be reimplanted in the LA. Surgery of one abnormally connecting PV usually will not be performed because of absence of hemodynamic complaints and consequences.

In hearts with normal as well as abnormal PV connections, myocardial discontinuities are found in the LA dorsal wall, which can form a substrate for the onset of arrhythmias[15,67]. Interventional cardiologists performing ablational techniques for these arrhythmias should be aware of the increased vulnerability to damage of these areas in the LA where a myocardial wall is lacking, specifically in the proximity of the esophagus. Recent studies have emphasized the risk of atrio-esophageal fistula due to the application of radiofrequency current at this site[121,122]. Lower dosage of radiofrequency current is therefore recommended when ablating in this area.

Future perspectives and conclusions.

In this review normal and abnormal PV development is described and updated based on the current literature. We have described how normal PV development starts with lumenization of a midpharyngeal endothelial strand, that provides a connection to the endocardium of the heart right from early development on. Although discussion on whether the primitive PV after lumenization drains into the atrial segment or the sinus venosus segment of the heart is still ongoing, based on the results of our studies, we favour an original connection of the primitive PV to the sinus venosus segment of the heart. From the posterior heart field, as part of the second heart field, SMCs and myocardial cells are contributed to the PV and LA dorsal wall. Normally, pulmonary-to-systemic connections disappear during development. Abnormal PV development may lead to persistence of pulmonary-to-systemic connections which is a substrate of anomalous PV connection(s), related to different clinical entities. In normal PV development the myocardium surrounding the PVs has specific characteristics creating a milieu for arrhythmias and reentry. In abnormal PV development, we hypothesize that rhythm- or conduction disorders might be explained in some patients by an insufficient contribution of posterior heart field myocardium to the sinus node and LA dorsal wall.

Surgical procedures have been developed to correct anomalous PV connections and to manage surgery-related complications.

There are still a number of topics that remain unresolved and have to be addressed by future research. Additional cell tracing studies are necessary to unequivocally determine the origin of SMCs in the PVs and LA.

With regard to arrhythmia treatment, currently mainly conventional anti-arrhythmic therapies and ablational strategies have been applied to control atrial arrhythmias. Development of new ablation techniques, and the expanding possibilities to integrate imaging techniques with electrophysiological mapping data and genetic data will improve patient selection and selectivity of application sites in order to decrease the recurrence rate of the arrhythmias[114,123]. Acquired PV stenosis as a consequence of surgical correction or resulting from ablation therapy needs further improvement of surgical techniques and refinement of useful energy sources. Pre-interventional imaging by echocardiography or computed tomography may also helpful in localizing areas at risk and preventing this potentially serious disorder.

The underlying mechanisms leading to primary PV stenosis are still unknown. Future investigation of a potential genetic basis is an important next step in understanding and manipulating this serious disease.

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S

Summary

Samenvatting

In **Chapter 1** the necessity of knowledge of general cardiac development for a proper understanding of normal and abnormal pulmonary vein development is emphasized. A general overview of cardiac and pulmonary vein development is provided as well as an overview of the development of the cardiac conduction system, since (ab)normal pulmonary vein development and the potency for development of atrial arrythmias are embryonically related. Under certain circumstances, initially non-functional embryonic deposited cardiac conduction cells are supposed to be reactivated and to contribute to the onset of arrhythmias.
Different heart fields are discriminated that contribute to the formation of the final heart. The *first heart field* forms the primitive heart tube that consists of a small atrial segment, an atrioventricular canal and a primitive left ventricle. The *second heart field* consists of an *anterior heart field* that contributes cells for the formation of the right ventricle and the proximal outflow tract, a *secondary heart field,* that contributes cells for the distal outflow tract, and a *posterior heart field* from which cells are derived for the development of the atria, the cardiac conduction system, and the sinus venosus, including the sino-atrial node, the pulmonary veins, the cardinal veins and the coronary sinus. Since this thesis deals with pulmonary vein development this *posterior heart field* is our main region of interest.
With regard to abnormal pulmonary vein development in relation to clinical entities it is emphasized that heart malformations are mostly the result of highly complex cascades of gene interactions combined with epigenetic factors that cannot be simply deduced to isolated clinical entities.

In **Chapter 2** the histology of the left atrial wall in relation to the incorporation of the pulmonary veins is studied immunohistochemically in sixteen human embryos and fetuses, one neonate and five adults. Based on histological criteria, three different compartments can be distinguished in the left atrium: 1. the smooth-walled left atrial body with vessel wall tissue, which is histologically identical to the vessel wall of the incorporated pulmonary veins, 2. the trabeculated left atrial appendage without vessel wall tissue and 3. a transitional zone in between the left atrial body and the left atrial appendage which is smooth-walled, lacks vessel wall tissue and histologically resembles the smooth-walled sinus venarum (body) of the right atrium. This border

zone is hypothesized to be the left part of the embryonic sinus venosus, that has shifted to the left due to incorporation of the pulmonary veins. Furthermore, areas of discontinuous or even absent myocardium are demonstrated in between the pulmonary veins and in the transitional zone. These areas are probably the substrate for arrhythmias. Besides that, the left atrial wall is supposed to be vulnerable to damage because of this myocardial discontinuity and the fact that in case of ablation strategies the histological border between the left atrium and the pulmonary veins cannot be found.

In **Chapter 3** we compare the development and differentiation of the myocardium and vascular wall of the pulmonary veins, the left atrial dorsal wall and the atrial septum in wild type with *podoplanin* knockout embryos (E10-E18.5) by 3D reconstruction and immunohistochemistry and demonstrate that in mutants, the myocardium of the pulmonary veins, the left atrial dorsal wall and the atrial septum is hypoplastic and that extension of smooth muscle cells into the left atrial body is diminished. Since *podoplanin* is a marker of myocardial and mesenchymal cells derived from the posterior heart field, we conclude that the myocardium of the pulmonary veins, the dorsal atrial wall and the atrial septum, as well as the smooth muscle cells are derived from the posterior heart field, regulated by *podoplanin*. We state that more insight in the variation in myocardial cuffing of the pulmonary veins might enlighten us on the variability of the occurrence of ectopic automaticity in the pulmonary vein myocardial sleeve.

In **Chapter 4** we investigate the wall of the pulmonary veins and the left atrial body in ten human neonates with total anomalous pulmonary venous connection (TAPVC), using histological and immunohistochemical techniques. As controls, two normal neonatal and adult hearts and five neonatal hearts with partial anomalous pulmonary venous connection (PAPVC) or situs inversus are studied. We demonstrate that in hearts with TAPVC, the left atrial body is small, lacks vessel wall tissue and has a hypoplastic myocardial layer. No myocardial layer is formed around the pulmonary veins. In hearts with PAPVC, only the non-left atrial draining pulmonary vein lacks a myocardial covering, whereas in hearts with situs inversus, pulmonary

veins connecting to the right-sided left atrium are normally myocardialized. Based on these results, we conclude that recruitment of cells, for addition of myocardium and smooth muscle cells at the venous pole, mainly takes place from the morphological left side. Moreover, we state that an open connection of the pulmonary veins with the morphological left atrium is mandatory for a normal left atrial size, the presence of vessel wall tissue and myocardialization of the left atrial body and pulmonary veins. Absence of myocardium covering the pulmonary veins is hypothesized to enhance susceptibility to pulmonary vein stenosis and to prevent the onset of pulmonary vein originating arrhythmias. As a link to chapter 3, we postulate that the embryonic posterior heart field might be responsible for the abnormal myocardialization and smooth muscle cell formation in TAPVC.

In **Chapter 5** a clinical case with right-sided pulmonary vein stenosis together with a left-sided pulmonary varix is embryologically and hemodynamically unravelled. A pulmonary varix is a dilated and tortuous pulmonary vein resulting from congenital or acquired obstruction of pulmonary vein drainage. In this case, we deal with a combination of congenital and acquired pulmonary vein stenosis on the right side and a congenitally formed pulmonary varix on the left. The difficulty and importance to determine whether the pulmonary vein stenosis is congenital or acquired, is emphasized.

In conclusion in **Chapter 6** current opinions on normal and abnormal pulmonary vein development in relation to clinical (management of) diseases are presented, based on the literature. This review serves as a general discussion of the contents of this thesis. Several questions are addressed as well as conceptual controversies. A general overview of cardiac development is provided, followed by definitions of the different components of the venous pole, the role of the second heart field at the venous pole, developmental concepts on the origin of normal and abnormal pulmonary vein drainage, the relation of pulmonary vein development to arrhythmogenesis and the consequences of the various concepts of pulmonary vein development on clinical management. Finally, conclusions and future perspectives are expressed.

In **Hoofdstuk 1** wordt het belang van het vergaren van kennis over de algemene hart ontwikkeling voor een goed begrip van normale en abnormale pulmonaalvenen (longader) ontwikkeling benadrukt. Er wordt een algemeen overzicht gegeven van de hart en pulmonaalvenen ontwikkeling. Aangezien (ab)normale pulmonaalvenen ontwikkeling en de potentie om atriale ritmestoornissen te ontwikkelen embryonaal aan elkaar zijn gerelateerd, wordt eveneens een overzicht gegeven van de ontwikkeling van het geleidingssysteem van het hart. Onder bepaalde omstandigheden, kunnen embryonaal ontstane geleidingssysteem eigenschappen in tot dan toe niet-functionele geleidingssysteem cellen gereactiveerd worden en bijdragen aan het ontstaan van ritmestoornissen.
Er kunnen verschillende “heart fields” worden onderscheiden die bijdragen aan de vorming van het volgroeide hart. Het zogenaamde *first of primary heart field* vormt de primitieve hartbuis, die bestaat uit een klein atriaal (boezem) segment, een atrioventriculair kanaal en een primitieve linker ventrikel (kamer). Het zogenaamde *second heart field* bestaat uit een *anterior heart field* dat bijdraagt aan de vorming van de rechter ventrikel en het aansluitende eerste deel van het uitstroomsegment, een *secondary heart field,* dat cellen aanlevert voor het laatste deel van het uitstroomsegment, en een *posterior heart field* van waaruit cellen verkregen worden voor de ontwikkeling van de atria, het geleidingssysteem van het hart en de sinus venosus, waaronder de sinusknoop, de pulmonaalvenen, de cardinaalvenen en de sinus coronarius vallen. Aangezien dit proefschrift gaat over de ontwikkeling van de pulmonaalvenen, is dit laatstgenoemde *posterior heart field* het gebied van focus van dit proefschrift.
Wat betreft abnormale pulmonaalvenen ontwikkeling in relatie tot specifieke klinische aandoeningen, moet benadrukt worden dat hartafwijkingen meestal de resultante zijn van zeer complexe cascades van geninteracties gecombineerd met omgevingsfactoren van waaruit deze specifieke klinische aandoeningen niet eenvoudigweg herleidbaar zijn.

In **Hoofdstuk 2** wordt de histologie van de linker atriumwand in relatie tot de incorporatie van de pulmonaalvenen immunohistochemisch bestudeerd in zestien humane embryo’s en foetussen, een neonaat en vijf volwassenenen. Gebaseerd

op histologische criteria, kunnen drie verschillende compartimenten worden onderscheiden in het linker atrium: 1. het gladwandige corpus van het linker atrium bekleed met vaatwand weefsel, histologisch identiek aan de vaatwand van de geincorporeerde pulmonaalvenen, 2. het getrabeculariseerde linker hartoor zonder vaatwand weefsel, en 3. een gladwandige overgangszone tussen het corpus van het linker atrium en het linker hartoor, zonder vaatwand en histologisch erg lijkend op het weefel van de sinus venarum (het corpus) van het rechter atrium. Verondersteld wordt dat deze overgangszone het linkszijdige deel van de embryonale sinus venosus is, die ten gevolge van de incorporatie van de pulmonaalvenen is "opgeschoven" naar de overgang met het linker hartoor. Daarnaast wordt aangetoond, dat zowel in het gebied tussen de pulmonaalvenen als in de genoemde overgangszone, het myocard plaatselijk discontinu of zelfs afwezig is. Deze plekken kunnen het substraat voor atriale ritmestoornissen zijn en kunnen bovendien de linker atriumwand kwetsbaar maken voor beschadiging. Het feit dat, tijdens ablatie technieken, de histologische grens tussen het linker atrium en de pulmonaalvenen niet zichtbaar is, maakt de linker atriumwand extra kwetsbaar.

In **Hoofstuk 3** vergelijken we, met behulp van 3D reconstructies en immunohistochemische technieken, de ontwikkeling en differentiatie van het myocard en de vaatwand van de pulmonaalvenen, de linker atrium achterwand en het atrium septum in wild type muizen embryo's met die van *podoplanine* knockout muizen embryo's (E10-E18.5). We tonen aan dat in mutanten, het myocard van de pulmonaalvenen, de linker atrium achterwand en het atrium septum hypoplastisch is en dat bovendien de aanwezigheid van gladde spiercellen in het corpus van het linker atrium verminderd is. Aangezien *podoplanine* een marker is voor myocardiale en mesenchymale cellen afkomstig uit het posterior heart field, concluderen we dat zowel het myocard van de pulmonaalvenen, de linker atrium achterwand en het atrium septum als de gladde spiercellen, afkomstig zijn uit dit posterior heart field, gereguleerd door *podoplanine*. We stellen vast dat meer inzicht in variatie in myocardiale bekleding van de pulmonaalvenen ons opheldering zou kunnen geven over de variabiliteit in het ontstaan van ectopische automaticiteit in deze myocardiale laag rond de pulmonaalvenen.

In **Hoofdstuk 4** onderzoeken we de wand van de pulmonaalvenen en het linker atrium in tien humane neonaten met totaal abnormale pulmonaalvenen connectie (TAPVC), gebruik makend van histologische en immunohistochemische technieken. Ter controle worden twee normale humane neonatale en volwassen harten en vijf humane neonatale harten met partieel abnormale pulmonaalvenen connectie (PAPVC) of situs inversus bestudeerd. We stellen vast dat in harten met TAPVC, het corpus van het linker atrium onderontwikkeld is, geen vaatwand bevat en uit hypoplastisch myocard bestaat. Een myocardiale laag rond de pulmonaalvenen wordt niet aangelegd. In harten met PAPVC, ontbreekt de myocardiale laag alleen rond de pulmonaalvene die niet naar het linker atrium draineert, terwijl in harten met situs inversus, pulmonaalvenen die verbonden zijn met het rechtsgelegen linker atrium normaal gemyocardialiseerd zijn. Gebaseerd op deze resultaten, concluderen we dat de rekrutering van cellen, voor aanbouw van myocard en gladde spiercellen ter plaatse van de veneuze pool, hoofdzakelijk plaats vindt vanuit de morfologisch linker kant van het posterior heart field. We stellen bovendien vast, dat een open connectie van de pulmonaalvenen met het morfologische linker atrium een voorwaarde is voor het ontwikkelen van een normale linker atrium grootte, de aanwezigheid van vaatwand weefsel in het linker atrium en een normale myocardialisatie van het corpus van het linker atrium en de pulmonaalvenen. We veronderstellen, dat de afwezigheid van myocard rond de pulmonaalvenen de kwetsbaarheid van de pulmonaalvenen voor het ontstaan van pulmonaalvenen stenose verhoogt, en tegelijkertijd het ontstaan van ritmestoornissen, afkomstig uit de pulmonaalvenen, voorkomt. In combinatie met de opgedane kennis in hoofdstuk 3, constateren we, dat het embryonale posterior heart field verantwoordelijk zou kunnen zijn voor de abnormale myocardialisatie en de abnormale vorming van gladde spiercellen in TAPVC harten.

In **Hoofdstuk 5** wordt een klinische casus met rechtszijdige pulmonaalvenen stenose gepaard gaande met een linkszijdige pulmonale varix embryologisch en hemodynamisch onder de loep genomen. Een pulmonale varix is een aangeboren of verworven spatader in de long die kan ontstaan ten gevolge van een belemmerde pulmonaalvenen drainage. In deze casus is er sprake van een combinatie van

congenitale en verworven pulmonaalvenen stenose rechts en een congenitaal ontstane pulmonale varix links. De complexiteit en het belang van het vaststellen of de pulmonaalvenen stenose congenitaal danwel verworven is, wordt benadrukt.

We eindigen in **Hoofdstuk 6** met de huidige opinie over normale en abnormale pulmonaalvenen ontwikkeling in relatie tot klinische (behandeling van) ziekten, gebaseerd op de literatuur. Dit review dient als algemene discussie voor de inhoud van dit proefschrift. Verscheidene vragen worden behandeld en beantwoord evenals conceptuele controverses. Er wordt een algemeen overzicht van de hart ontwikkeling gegeven, gevolgd door definities van de verschillende componenten van de veneuze pool, de rol van het second heart field ter plaatse van de veneuze pool, concepten over de ontwikkeling van normale en abnormale pulmonaalvenen drainage, de relatie van pulmonaalvenen ontwikkeling tot arrhythmogenese en de consequenties die de verscheidene concepten over pulmonaalvenen ontwikkeling hebben voor klinisch management. Tot slot worden conclusies en toekomstperspectieven weergegeven.

P

List of Publications

Atresia of a solitary pulmonary vein.
Douglas YL, van der Werf TS, Bink-Boelkens MThE, Nikkels PGJ, Ebels T.
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Osteosarcoma of the sternum.
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C

Curriculum Vitae

De auteur van dit proefschrift is geboren op 16 maart 1964 te Amsterdam. In 1982 deed zij eindexamen Atheneum-B aan de Openbare Scholengemeenschap Erasmus te Almelo. In 1982 startte zij met de studie Geneeskunde aan de Rijksuniversiteit van Groningen. In 1986 behaalde zij haar doctoraal examen en in 1989 het artsexamen. Vervolgens werkte zij van 1989 tot 1992 als AGNIO Interne Geneeskunde, Neurologie, Chirurgie en Plastische Chirurgie. Na tevens ruim een jaar ervaring te hebben opgedaan als AGNIO Cardio-thoracale chirurgie in Groningen startte zij aldaar in 1993 met haar opleiding tot Cardio-thoracaal chirurg (opleiders Prof. Dr. R. van Schilfgaarde en Prof. Dr. T. Ebels), welke op 1 maart 2000 werd voltooid. Tijdens haar opleiding werden haar zoon Frits (1996) en dochter Pien (1998) geboren en na haar opleiding haar jongste zoon Jur (2001). Sinds maart 2000 is zij werkzaam als staflid Cardio-thoracale chirurgie te Groningen met een speciale belangstelling voor de Congenitale hartchirurgie en Anatomie. Dit resulteerde in 2003 in het starten van basaal onderzoek naar de ontwikkeling van de pulmonaalvenen en de linker atrium achterwand en naar abnormale longvenendrainage onder leiding van Prof. Dr. A.C. Gittenberger-de Groot in het laboratorium voor Anatomie en Embryologie te Leiden. De resultaten hiervan staan beschreven in dit proefschrift.

Als Cardio-thoracaal chirurg houdt zij zich momenteel bezig met de volwassen Cardio-thoracale chirurgie met als aandachtsgebieden de mitraalklep- en aorta(wortel) chirurgie en de transapicale aortaklep chirurgie. In de toekomst wil zij daarnaast meer organisatorische taken op zich nemen en hoopt zij haar wetenschappelijke activiteiten te verleggen naar Groningen. Daarnaast zou zij ook graag betrokken blijven bij de huidige onderzoekslijn op pulmonaalvenen gebied in Leiden.