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Citation for published version (APA):
Rana, M. S. (2014). Molecular and genetic basis of congenital conotruncal heart defects

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Chapter 3

Development of the Human Aortic Arch System Captured in an Interactive Three-Dimensional Reference Model

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American Journal of Medical Genetics Part A 2013

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Abstract

Variations and mutations in the human genome, such as 22q11.2 microdeletion, can increase the risk for congenital defects, including aortic arch malformations. Animal models are increasingly expanding our molecular and genetic insights into aortic arch development. However, in order to justify animal-to-human extrapolations, a human morphological and molecular reference model would be of great value, but is currently lacking. Here, we present interactive three-dimensional reconstructions of the developing human aortic arch system, supplemented with the protein distribution of developmental markers for patterning and growth, including T-box transcription factor TBX1, a major candidate for the phenotypes found in patients with the 22q11.2 microdeletion. These reconstructions and expression data facilitate unbiased interpretations, and reveal previously unappreciated aspects of human aortic arch development. Based on our reconstructions and on reported congenital anomalies of the pulmonary trunk and tributaries, we postulate that the pulmonary arteries originate from the aortic sac, rather than from the sixth pharyngeal arch arteries. Similar to mouse, TBX1 is expressed in pharyngeal mesenchyme and epithelia. The endothelium of the pharyngeal arch arteries is largely negative for TBX1 and family member TBX2 but expresses neural crest marker AP2α, which gradually decreases with ongoing development of vascular smooth muscle. At early stages, the pharyngeal arch arteries, aortic sac, and the dorsal aortae in particular were largely negative for proliferation marker Ki67, potentially an important parameter during aortic arch system remodelling. Together, our data support current animal-to-human extrapolations and future genetic and molecular analyses using animal models of congenital heart disease.
Introduction

Anomalous patterning and remodelling of the embryonic arterial pole can result in anomalies of the aortic arch and great vessels, representing approximately one-third of all congenital cardiovascular defects\(^1\). Moreover, congenital aortic arch malformations occur in approximately 80% of all 22q11.2 deletion syndrome (22q11.2DS) patients\(^2\), indicating that 22q11.2DS is an important risk factor for aortic arch anomalies. Within the 22q11.2 locus, the gene encoding the transcription factor TBX1 is the major candidate in the etiology of the clinical features of 22q11.2DS and has been shown to be crucial for the correct development of the aortic arch system in mouse\(^3\)\(^-\)\(^5\).

Theorizing the origin of common clinical phenotypes such as aortic arch anomalies has proven to be challenging. The characterization of the developmental disturbances underlying congenital vascular defects necessitates a thorough knowledge of proper aortic arch system development. Following the description of the developing pharyngeal arch arteries (PAAs) by Rathke (1843)\(^6\), many researchers have debated and complemented his analysis\(^7\)\(^-\)\(^10\). The long-standing tradition of projecting the rapidly transforming components of the embryonic aortic arch system upon the postnatal situation as the method of deducing the origins of the definitive vascular structures is highly prone to mistakes and controversies. The most accurate approach to study the transformation of PAAs is by means of physical or genetic labelling within the living embryo, which is impossible in human. Because of these limitations, morphological methods, such as three-dimensional reconstructions from serial sections and preparation of vascular casts, became the most widely used alternatives. Yet, an accurate and unbiased presentation of the findings is of crucial importance. In contrast to recent mouse and chicken studies with exquisite morphological detail\(^11\),\(^12\), the most complete description of human aortic arch metamorphosis is almost 100 years old\(^9\). Although Congdon’s report has contributed significantly to our understanding of human PAA transformation, it unfortunately has been incorrectly interpreted and mixed up with the configuration found in lower vertebrates\(^13\)\(^-\)\(^16\).

Proper remodelling of the PAAs into the aortic arch system is a complex process, requiring intercellular signalling events and correct spatiotemporal gene expression in multiple cell types. Migration and differentiation of neural crest-derived cells as well as growth and apoptosis of the developing vessels within a biomechanically active environment is a prerequisite of normal PAA transformation\(^17\),\(^18\). Errors in the regression of distinct PAAs may result in several life-threatening aortic arch malformations. Although the transformation of the PAAs has been studied extensively in animal models and has provided fundamental insights, molecular data relevant for the development of the human aortic arch system are lacking. In order to test whether current animal-derived insights are also applicable to the human situation, we generated interactive three-dimensional reconstructions of the PAAs and related structures in human embryos to facilitate unbiased interpretations and comparisons with previously generated data. In addition, we studied the expression patterns of developmental regulators relevant during PAA development. The interactive presentation of the remodelling process of the PAAs is of educational and clinical value. In addition, our molecular analysis indicates that the genetic programs underlying aortic arch system development are comparable and evolutionarily conserved.
Materials and methods

Human embryos

Collection of human embryonic material and preparation for histology was as described previously\textsuperscript{19}. Human embryos were collected from medically induced abortions performed for socio-economic reasons at the Gynecology Department of the Tartu University Hospital, Estonia. Collection and use of human material was approved by the Medical Ethical Committees of the University of Tartu, Estonia, and the University of Amsterdam, The Netherlands. The aborted human embryos were fixed in 4\% paraformaldehyde, examined for gross morphological anomalies under a stereomicroscope, photographed, staged using the Carnegie series of developmental stages and included in our study only when considered normal when compared to the Carnegie series\textsuperscript{20}. We included seven embryos at stage 12-13, five embryos at stage 16, four embryos at stage 18, and three embryos at stage 23 allowing us to compare and confirm the obtained results. Additionally, the photomicrographs of complete series of hematoxylin-azophloxin stained sections of human embryos at four different stages were used, one of which obtained from the Carnegie collection (Human Developmental Anatomy Center of the Armed Forces Institute of Pathology, Washington, USA), and others from the collection of the Department of Anatomy, Embryology & Physiology of the University of Amsterdam, The Netherlands.

Immunohistochemistry and three-dimensional (3D) reconstruction

Immunofluorescent staining and 3D reconstructions were essentially performed as described previously\textsuperscript{19,21}. The 3D-PDF file was generated as described\textsuperscript{22}. Immunofluorescent staining was performed with the following primary antibodies: goat polyclonal for connexin40 (Cx40, diluted 1:250, Santa Cruz Biotechnology), mouse monoclonal for α-smooth muscle actin (αSMA, 1:500, Sigma-Aldrich), rabbit polyclonal for proliferation marker Ki67 (1:500, Monosan), mouse monoclonal for neural crest marker Activating enhancer binding Protein 2 alpha (AP2α, 1:25, Developmental Studies Hybridoma Bank), goat polyclonal T-box transcription factor TBX1 (1:250, Santa Cruz Biotechnology), mouse monoclonal T-box transcription factor TBX2 (1:100, gift from Colin Godin\textsuperscript{23}).

Limitations of our study

The limited numbers of available embryos did not permit complete optimization of the staining protocol for some antibodies and the assessment of biological variation between specimens at similar developmental stages was therefore challenging. Nevertheless, immunohistochemical stainings were found to be reproducible. The reconstructions had to be corrected for surface distortions due to uneven stretching and occasional tissue damage, which could have affected some details of structures in the 3D models. However, no spurious structures were introduced, nor important details lost, during this manual correction.
Results

The reader is encouraged to read the results along with the interactive 3D-PDF file in the online-only Data Supplement.

Stage 13: Formation of the aortic sac and third, fourth and sixth pharyngeal arch arteries

In stage 13 human embryos, corresponding to 28-32 days of development and equivalent to mouse embryonic day 9.5, blood exits the heart via the outflow tract and enters the extrapericardial aortic sac. The aortic sac connects with the bilateral dorsal aortae via the bilateral and symmetrical third, fourth and sixth PAAs (Figure 1A, B), which run in corresponding pharyngeal arches and diverge dorsally to meet the paired symmetrical dorsal aortae (Figure 1A). The fifth pair of PAAs was not detected in the embryos analyzed. The fourth PAAs are thicker and more prominent than both the third and sixth PAAs (Figure 1A, 2A). In agreement with previous reports26,27, only remnants of the first and second PAAs were observed and were already detached from the aortic sac at this stage (Figure 1A). The third PAAs connect with the cranial part of the dorsal aorta in an “end-to-side” fashion, which in turn continue cranially as the developing internal carotid arteries (Figure 2A, B, arrowheads). The segment of the dorsal aortae between the third and fourth PAAs, known as the carotid duct8, is narrow (Figure 2B, arrow) in comparison with the more caudally located dorsal aortae. At the caudal surface of the sixth PAAs, sprouting primordia of the pulmonary arteries are already visible (Figure 2A). At these early stages, the pulmonary arteries are situated far from each other with the lumen of the aortic sac interposed between them. The right pulmonary primordium was more laterally located with respect to the midline of the aortic sac compared to the left primordium (Figure 2A, double-arrowed bars). At the dorsal aspect of the dorsal aortae, small intersegmental arteries are sprouting at regular intervals and are running toward the developing spinal cord (not reconstructed). Caudally to the confluence of the dorsal aortae the seventh pair of intersegmental arteries gives rise to the future left and right subclavian arteries running into the respective upper limb buds (Figure 1B).

The endothelium of the aortic sac, PAAs, dorsal aortae and all other arteries express gap junction protein connexin40 (Cx40, Figure 3A), similar to the endothelium of postnatal arteries29. Both the pharyngeal arch mesenchyme and endothelium of the developing aortic arch vasculature express the proliferation marker Ki67, without robust differences between left- and right-sided structures (Figure 3A). AP2α, a transcription factor expressed in migrating and differentiating neural crest-derived cells, is required during PAA development26,27, and was found to be expressed in the fourth and sixth PAA endothelium, and in the mesenchyme surrounding these arteries (Figure 3D). The endothelium of the dorsal aortae and the neighboring pharyngeal mesenchyme does not express AP2α (Figure 3D). αSMA is one of the earliest markers of differentiating smooth muscle cells28, and is known to be expressed in neural crest-derived smooth muscle cells of PAAs27,29. In contrast to AP2α, αSMA is robustly expressed in the developing wall of the dorsal aortae, but only weakly in cells surrounding the aortic sac and fourth and sixth PAAs (Figure 3G). T-box transcription factor and 22q11.2DS candidate TBX1 is expressed in pharyngeal epithelia, where it is has been shown to regulate fourth PAA development30. TBX1 is also expressed in
pharyngeal mesenchymal cells adjacent to the fourth PAAs, but not in AP2α-positive neural crest cells (Figure 3J). Interestingly, cells surrounding the dorsal aortae express TBX1 (Figure 3J, asterisk), but are virtually devoid of proliferation marker Ki67 (Figure 3A, asterisk). The endothelium of the PAAs, aortic sac and dorsal aortae are also negative for Ki67 (Figure 3A, arrow in enlarged box). T-box family member TBX2, a transcriptional repressor involved in arterial pole development, is known to genetically interact with TBX1 and is expressed in neural crest-derived cells flanking the proximal portions of the fourth and sixth PAAs (Figure 3M). The dorsal aortae are negative for AP2α and TBX2, in agreement with the observation that neural crest cells do not contribute to these vessels (Figure 3D, M).

**Stage 16: Remodelling of the aortic sac and third, fourth and sixth pharyngeal arch arteries**

At stage 16 (37-42 days of human development, embryonic day 10.5 in mouse), the characteristic aortic sac present in the previous stage is no longer recognizable as a cavity, as it has been transformed into two separate arterial channels (Figure 1C). Protrusion of pharyngeal mesenchyme into the distal outflow tract has already separated the developing ascending aorta from the future pulmonary trunk (Figure 1C, D, 2C). The spatial orientation of the future intrapericardial aortic and pulmonary arterial channels, as seen in the adult heart, is already clearly recognizable at this stage with the future ascending aorta and pulmonary trunk running perpendicular to one another (large arrows in Figure 1C). The more cranially located channel, representing the developing ascending aorta, remains connected to the remnant of the remodeled aortic sac, which connects to the bilateral third and fourth PAAs (Figure 2C, arrows). This remnant of the aortic sac and the future ascending aorta together form a ‘T’-like figure, because the distance between the pericardial reflection and the origins of the third and fourth PAAs is increasing (brackets in Figure 2A, D). The more caudally located developing pulmonary trunk continues extrapericardially as the paired sixth PAAs, the lumina of which are increasing in size and are now comparable to the fourth PAAs (Figure 1C). Nevertheless, the lumen of right sixth PAA is substantially narrower than its left counterpart (Figure 2C, arrowhead). Shortly after branching from the dorsal aortae, the bilateral seventh intersegmental arteries give rise to the future subclavian and forming vertebral arteries. The future subclavian arteries, running into the upper limb buds, are now located more cranially relative to the confluence of the dorsal aortae (CDAo, Figure 1C, D). The more distal parts of the future vertebral arteries arise as the dorsal ends of the consecutive intersegmental arteries and form anastomoses with one another (not shown). The pulmonary arteries are growing toward the developing lung buds and have become clearly recognizable (Figure 1C). The part of the dorsal aortae between the third and fourth PAAs, the so-called carotid duct, has not yet fully regressed at this stage, but has become considerably narrower (Figure 1C, small arrow). Upon regression of this segment, the third PAAs will only be connected to the developing internal carotid arteries (Figure 2C).
Figure 1. Morphological overview of the developing human pharyngeal arch arteries. At stage 13, the third, fourth, and sixth bilateral and symmetrical pharyngeal arch arteries are connected to the aortic sac, which is the direct continuation of the undivided lumen of the outflow tract (A and B). The pulmonary arteries are already present as sprouting primordia. At stage 16, the aortic sac has remodeled into two arterial channels (the future systemic and pulmonary circulations) and the arch arteries are more prominent (C and D). In addition, the right and left pulmonary arteries are growing toward the developing right and left lung buds. At stage 18, the left subclavian artery is in the process of attaining its final position close to where the arterial duct connects to the dorsal aorta (E and F), and at stage 23 distal to the left common carotid arteries (G and H). The innominate artery grows from stage 16 onward and bifurcates into the right subclavian artery and the right common carotid artery (F and H). The right and left vertebral arteries arise from the subclavian arteries (F and H). The innominate artery, ascending aorta and the aortic arch are remnants of the initial aortic sac (F and H). For abbreviations, see Box 1. Scale bar is 2 mm.
Proliferation of the tissues surrounding the aortic arch arteries at these stages does not differ overtly between the right and left sides (Figure 3B). Similar to stage 13 embryos, Ki67 and AP2α were not detected in the endothelial cells of the dorsal aortae or in TBX1-positive mesenchyme surrounding the dorsal aortae (Figure 3B,E, K). The lack of proliferation could play a role in the positional change of the future subclavian arteries relative to the confluence of the dorsal aortae (Figure 1D). AP2α-positive cells surrounding the fourth and sixth PAAs (Figure 3E) are also positive for αSMA (Figure 3H), reflecting a neural crest-derived origin of the smooth muscular vasculature. TBX2 is expressed in both mesodermal and neural crest-derived cells, including the developing pharyngeal arches (Figure 3N) and both TBX2 and AP2α are expressed in the mesenchyme of the developing larynx (Figure 3E, N). TBX1 and TBX2 are not expressed around the fourth PAAs at this stage (Figure 3K, N).

**Figure 2.** Remodelling of the human pharyngeal arch arteries. 3D reconstructions of the pharyngeal arch arteries in stage 13 embryos (A and B). The pulmonary arteries sprout as primordia, but are not arranged symmetrically (A, double-arrowed bars). The third PAAs make end-to-side anastomoses (arrowhead) with the most cranial part of the dorsal aortae, which continues as the future internal carotid arteries. The distance between the third and fourth PAAs increases (C, arrows; compare brackets in C with A). The left sixth PAA is narrower (arrowhead in C) than the right sixth PAA. The disappearance of the (distal) right sixth PAA occurs between stages 16 and 18 (compare D and F). At stage 18 (E and F), the sixth PAA is present as the arterial duct and is connected to the dorsal aorta next to the left subclavian artery. The future innominate artery (C, left arrow) gives rise to the right subclavian artery and right common carotid artery (E and F). The definitive aortic arch is the part of the aortic sac that connects to the innominate artery, left common carotid artery and left subclavian artery. The right and left pulmonary arteries acquire their final configuration relative to the pulmonary trunk from stage 18 onward (F and H). The configuration as observed at stage 23 highly resembles the postnatal situation (G and H). See Figure 1 for additional descriptions. For abbreviations, see Box 1. Scale bar is 200 μm.
Figure 3. Molecular analysis of the developing human pharyngeal arch arteries. Transverse sections of the pharyngeal region at stages 13, 16 and 18, showing Cx40-positive endothelium surrounded by proliferating Ki67-positive pharyngeal mesenchyme (A–C) and AP2α-positive neural crest-derived cells (D–F). At stage 13, the aortic sac (AS) and PAAs are virtually negative for αSMA (G). Ki67 is only weakly expressed in the endothelium of the dorsal aorta (A, arrow in enlarged box), aortic sac and PAAs (A–C). During subsequent development, the third, fourth and sixth PAAs (3rd, 4th, 6th) start expressing αSMA, reflecting neural crest-derived smooth muscle cell formation (H and I). The 22q11.2DS candidate TBX1 is expressed in the pharyngeal mesoderm surrounding the dorsal aortae, pharyngeal epithelia and but not in PAA endothelium (J–L). Importantly, pharyngeal TBX1-positive cells are largely negative for Ki67 (compare A, B with J, asterisks). At stage 18, TBX1-positive mesenchymal cells were only found adjacent to the dorsal aorta (L). T-box family member TBX2 is expressed in pharyngeal mesoderm and pharyngeal neural crest-derived cells surrounding the PAAs (M). Endothelial cells are negative for TBX2 at stage 16 (N), but TBX2-positive cells were found in the fourth PAA or future aortic arch from stage 18 onward (O). For abbreviations, see Box 1. Scale bar is 200 μm.
Stages 18 through 23: Formation of the definitive aortic arch and subclavian, carotid and pulmonary arteries

At stages 18 through 23, corresponding to 44-56 days of development and equivalent to embryonic day 12.5-15.5 in mouse, the asymmetrical configuration of the aortic arch system gradually becomes identical to the postnatal situation. From stage 18 onward, the arterial channel, which connects the lumen of the OFT to the third and fourth PAAs, is almost completely transformed into the ascending aorta. The elongation process of the right lateral part of the most cranial remnant of the remodeled aortic sac, which at the previous stage formed the upper part of the ‘T’-like figure, contributes to the appearance of the so-called innominate or brachiocephalic artery. This vascular segment bifurcates into the right common carotid artery and the right subclavian artery, the derivatives of the right third and fourth PAAs, respectively (Figure 1F, H and 2E, G). The part of the right-sided dorsal aorta distal to the origin of the seventh intersegmental artery has involuted at stage 18. Thus, remodelling of the remnant of the aortic sac together with the involution of the right-sided dorsal aorta leads to a “translocation” of the initially distant origins of the right-sided subclavian and common carotid arteries to form the bifurcation of the innominate artery. The bilateral carotid ducts have now disappeared, and, as a consequence, the arteries previously recognizable as the third PAAs, now become the common carotid arteries (Figure 1F, H and 2F, H). Both common carotid arteries give rise to the internal and external carotid arteries (not shown). Because the left lateral part of the remnant of the remodeled aortic sac does not elongate to the same extent as the right part, the left common carotid artery arises directly from the future definitive aortic arch (Figure 1H, 2G, H).

The right subclavian artery, initially originating from the right dorsal aorta via the seventh intersegmental artery, becomes distinguishable as a branch of the innominate artery (Figure 1F, H and 2G). The left subclavian artery, originally recognizable as the branch of the left seventh intersegmental artery at stages 13 through 16, is connected to the distal part of the future definitive aortic arch at stage 18, close to where the left sixth PAA connects to the dorsal aorta (Figure 2F). At stage 23, the left subclavian artery has attained a distal position at the definitive aortic arch close to the origin of the left common carotid artery, similar to the adult situation (Figure 2H). Vertebral arteries branching from the subclavian arteries were observed to divert cranially to form the basilar artery in the vertebro-basilar system. The right sixth PAA distal to the origin of the right pulmonary artery has regressed at stage 18 (Figure 1F, 2F), while the distal left sixth PAA is now recognizable as the arterial duct (Figure 1E). The pulmonary trunk has fully developed at stage 23, bifurcating into the right and left pulmonary arteries (Figure 1G, 2G). No other obvious differences were observed at stages 18 through 23.

The tissues around the fourth PAAs, or future definitive aortic arch and innominate artery, express the neural crest marker AP2α (Figure 3F) at stage 18, but TBX1 and TBX2 expression was weak in these cells (Figure 3L,O). No AP2α-positive cells were detected at stage 23 (not shown), reflecting a decrease in migrating neural crest-derived cells toward the transforming PAA system. The expression of Ki67 was highly similar to stage 16, but was slightly increased in the Cx40-positive endothelium of the fourth PAA (Figure 3C). Similar to stages 13 and 16, the dorsal aortae are negative for Ki67, which could explain why the left and right subclavian arteries are now found closer to the common carotids (Figure 2E-H).
The thickness of the αSMA-positive arteries increases from stage 18 onward (Figure 3H,I), indicating the formation of a smooth muscular media layer.

Discussion

Animal models have considerably expanded our insight into the genetics of aortic arch system development and concomitant congenital heart defects\. A reference model of the developing human aortic arch system is essential in order to fully infer novel animal model-based insights into human congenital heart defects. Currently used schematic representations of the transforming human aortic arch system are largely based on Congdon’s work\(^8\) (1922), but, unfortunately, have incorporated data from lower vertebrates\(^{13-16}\). The complex morphology and rapidly changing environment in which the human PAAs transform into the adult aortic arch system, is difficult to grasp, even for expert morphologists. PAA remodelling should therefore be presented within its proper 3D context. In this study, we present such a reference model by generating interactive 3D reconstructions of the human aortic arch system at consecutive stages of development, supplemented with gene expression data. Our findings, as summarized in Figure 4, indicate that Congdon’s (1922) original description is the most accurate one, but that schematic representations based on his description have gradually changed along the years, resulting in erroneous descriptions in some medical textbooks. The significance of our reconstructions and expression analyses is 2-fold. First, our interactive reconstructions of the remodelling of the human PAAs provide crucial morphological information and facilitate unbiased interpretation, which is of educational and diagnostic value. Second, our expression analyses reveal that the genetic programs underlying aortic arch system development are similar to those found in mouse, supporting hypotheses of the conservation of developmental and genetic programs underlying PAA remodelling. In the next sections, we will elaborate on the development of regions that are prone to be affected during the process of PAA transformation.

Developmental defects of the ascending aorta, the aortic arch and its tributaries: linking embryology to postnatal anatomy

At stage 13, the aortic sac connects with the bilateral PAAs, which run toward the dorsal aortae. Our morphological analyses revealed that, at stages 16 and 18, remodelling of the aortic sac results in a T-shaped structure formed by the extra pericardial ascending aorta and the most cranial remnant of the aortic sac. Furthermore, it is clear that the right lateral part of this aortic sac remnant subsequently elongates, which is required to form the innominate artery, the first vessel branching off from the aortic arch (Figure 2C, E, G). Although the contribution of the aortic sac to the innominate artery and the developmental mechanisms underlying the localized growth and subsequent configuration is poorly understood, we suggest that aberrations in the development of this segment of the aortic sac is likely to result in abnormalities of the innominate artery. The right-sided part of the dorsal aorta regresses between stages 16 and 18 (Figure 4C). In case of a double aortic arch, the right-sided dorsal aorta persists, and the cranial aortic sac remnant does not elongate, hindering
the formation of the innominate artery and stressing the importance of these remodelling processes. The innominate artery, in theory, could contain parts of the third and fourth PAAs, however, the contribution of the right third PAA to the innominate artery seems to be modest, as mice lacking the Hoxa3 gene fail to form the third PAAs, but do not display signs of an abnormally developed innominate artery\textsuperscript{34}. Genetic and lineage data in mouse will be crucial in determining whether or to what the extent remnants of the different PAAs are still present in the adult innominate artery.

The contribution of the third PAAs in the formation of the common carotids has also been demonstrated in Hoxa3-deficient mice, which lack the third PAAs and fail to develop the common carotid arteries\textsuperscript{34}. Our morphological analyses support that the third PAAs form a substantial portion of common carotid arteries\textsuperscript{3} (Figure 2C-H). Remodelling defects of the third PAAs could therefore result in carotid artery malformations, such as unilateral or bilateral separate origins of the internal and external arteries with absent common carotid arteries\textsuperscript{35}. Theoretically, absence of common carotid arteries should result from agenesis or involution of the third PAAs, and such a configuration, in turn, would require persistence of the carotid duct in order to maintain a cranial circulation.

In the postnatal situation, the extrapericardial tributaries of the arterial pole possess considerable asymmetry. This transformation requires involution of the right-sided dorsal aorta distal to the origin of the seventh intersegmental artery, which occurs between stages 16 and 18 (Figure 1D, F and 2E, G). The future subclavian arteries initially arise as the bilateral seventh intersegmental arteries of the single dorsal aorta just below the confluence of the bilateral dorsal aortae (Fig 1B). However, from stage 18 onward the left subclavian artery originates from the distal part of the definitive aortic arch, whereas the right subclavian connects to the innominate artery and not directly to the aortic arch. Virtually nothing is known about the mechanism, by which the subclavian arteries change their distant aortic origins and acquire their final positions. It is tempting to hypothesize that this results from the lack of proliferation in the bilateral dorsal aortae between the fourth PAAs and the seventh intersegmental arteries (Figure 3A), a fact that has not been previously reported. An increase in size of the left dorsal aorta caudally to the origin of the seventh intersegmental artery may therefore underlie the observation that the distance between the future left subclavian artery and the dorsal aorta and remnant of the left fourth PAA remains constant throughout development, being around 1.5 mm (Figure 4).

Our immunohistochemical analyses revealed that the endothelial and developing tunica media of the dorsal aortae are negative for AP2\alpha and TBX1 (Figure 3D-F, J-L), but that the mesenchymal cells in between the dorsal aortae robustly express TBX1 in a symmetrical fashion. These cells furthermore hardly express the proliferation marker Ki67, indicating differences in the proliferation rate of cells within and outside the TBX1-positive domain (Figure 3A, B, J, K). Whether the expression of TBX1 in pharyngeal mesoderm underlies the asymmetrical development of the aortic arch is not fully clear, but TBX1 is known to impact on neural crest cell migration during mouse PAA development and therefore could be involved\textsuperscript{36}. Interestingly, 22q11.2DS patients frequently suffer from tetralogy of Fallot, of which some possess right-sided definitive aortic arch\textsuperscript{37}. During subsequent development, the TBX1-negative endothelium of the right and left fourth PAAs mature and transform into the
proximal right subclavian artery and definitive aortic arch, respectively, and start expressing AP2α, αSMA and TBX2 (Figure 3).

Numerous reports have drawn attention to TBX1 in aortic arch morphogenesis, since Tbx1-deficiency in mice has been associated with abnormal development of the aortic arch and its branches\(^3\)\(^5\). In addition, mice deficient for Raldh2, Chd7, Fgf8, Crkl, Gbx2 or Eya1/Six1 have been shown to display developmental defects of the arterial outlets and are known to interact with or require Tbx1, highlighting the relevance of these pathways in aortic arch remodelling\(^38\)\(^44\). Future studies using combinations of these and additional genetic mouse models will be pivotal to unravel normal and abnormal development of the aortic arch and its branches, with a central focus on Tbx1.

**Anomalies of the pulmonary trunk and its tributaries: role of the remodelling aortic sac and sixth pharyngeal arch arteries**

The distal part of the right sixth PAA is known to disappear early in development and the distal left sixth PAA is known to form the arterial duct. The distal right sixth PAA was found to regress between stage 16 and 18 (Figure 2D-F; Figure 4C). The pulmonary arteries are suggested to sprout from the sixth PAAs from the moment of their first appearance (Figure 2A). However, the central pulmonary arteries have been reported to originate from the ascending aorta, which is one of the aortic sac derivatives\(^35\). Furthermore, there are forms of pulmonary atresia associated with the absence of the pulmonary trunk and its bifurcation, in which the distal pulmonary artery branches are still present\(^46\), or an isolated right subclavian artery connected to the bifurcation of the pulmonary trunk\(^47\). These observations suggest that the region encompassing the pulmonary trunk and its bifurcation represents another derivative of the aortic sac, rather than being part of the sixth PAAs. Therefore, we propose a developmental view in which the distal pulmonary artery branches, from the moment of their first appearance, arise directly from the caudal part of the remodelling aortic sac, which persists at later stages to form the bifurcation of the pulmonary trunk and central pulmonary arteries (Figure 2C, D). The observation that the distal sixth PAAs eventually disappear further underscores the differences in morphogenesis, and could be linked to different developmental origins of proximal sixth PAAs\(^9\)\(^12\). From stage 16 onward, the extent of the right and left pulmonary arteries toward the developing lungs and bronchial capillary network was clearly identifiable. Although it remains challenging to fully elucidate the relations between the different components of the sixth PAAs and central pulmonary arteries, reports on congenital aberrations of these arteries are of great value in determining their origins.

Disturbances in the outgrowth of the primordia of the pulmonary arteries from the aortic sac can result in a congenital absence of the right or left pulmonary artery, a relatively rare anomaly\(^48\)\(^50\). The distal left sixth PAA will become converted into the arterial duct between stages 18 through 23, as confirmed by our reconstructions (Figure 2F, H). Our expression data show that both αSMA and AP2α are co-expressed in vascular smooth muscle cells in the sixth PAAs (Figure 3E,H), in agreement with earlier reports describing smooth muscle cells as important targets during remodelling of the arterial duct\(^26\)\(^51\).
Figure 4. Schematic representation of the presumed contribution of the human pharyngeal arch arteries (PAAs) to the adult aortic arch system. At CS13 (A), the centrally located aortic sac (orange) and paired third (dark-blue), fourth (green) and sixth (light blue and yellow) PAAs. Note that the origins of the sixth PAAs are included as part of aortic sac (see Discussion). The third PAAs continue cranially as developing internal carotid arteries (light violet) and caudally as the bilateral carotid ducts (light pink), whereas the fourth and sixth PAAs directly connect to the paired dorsal aortae (red). Appreciate the distance (~1.5mm) between the origin of the future left subclavian artery (brown) and the connection of the left fourth PAA with the dorsal aorta. At CS16 (B), the aortic sac has remodeled to form the future extra-pericardial (thus, distal to the pericardial reflection, pr) ascending aorta and pulmonary trunk (orange). The most cranial aortic sac remnant has already acquired a configuration that will facilitate the formation of the innominate artery and the definitive aortic arch. The most caudal aortic sac remnant resembles the bifurcation of the developing pulmonary trunk and gives off the pulmonary arteries (purple), which are increasing in size. The bilateral carotid ducts and the left sixth PAA become substantially narrower. At CS18 (C), the most cranial remnant of the remodeled aortic sac (orange) forms the proximal part of the definitive aortic arch and the innominate artery, both continuing as asymmetric left- and right-sided third and fourth PAAs. The enlarged left-sided sixth PAA fulfills the function of the arterial duct (yellow), enabling the shunting of the blood from pulmonary trunk to the descending aorta. The right-sided sixth PAA and dorsal aorta distal to origin of the subclavian artery are disappearing, whereas the carotid ducts are no longer recognizable. These changes allow the recognition of an almost definitive configuration of the aortic arch and pulmonary artery system. Disturbances in the correct disappearance or persistence of the embryonic vessels near this stage will produce different types of vascular rings. After birth (D), differential growth of the left- and right-sided dorsal aortae and PAAs contributes to the final appearance of the aortic arch and pulmonary artery system, where right subclavian and common carotid arteries originate from innominate artery and left common carotid and subclavian arteries arise directly from the definitive aortic arch. Note that the distance (~1.5mm) between aortic origin of the developing subclavian artery and the junction between the left fourth PAA (later transverse part of the definitive aortic arch) and left dorsal (later descending) aorta hardly changes at consecutive stages. The asterisks in panel D indicate the yet experimentally to be confirmed contributions of the initial aortic sac, fourth PAAs and dorsal aortae to the formation of the definitive aortic arch and innominate and right-sided proximal subclavian arteries. For abbreviations, see Box 1.

CONCLUDING REMARKS

A thorough knowledge of the development of PAAs and their derivatives is imperative in order to expand our understanding of the embryological basis of congenital arterial pole defects and to improve diagnostic accuracy when looking at congenital aortic arch defects. Here, we have summarized our observations regarding the contribution of the embryonic aortic sac and PAAs to the definitive aortic arch and pulmonary artery system (Figure 4). However, some of these suggested contributions still need to be elucidated. For instance, the innominate artery could primarily be an aortic sac-derivative, but could also contain parts of the right third and fourth PAAs. Similarly, the right fourth PAA and proximal right dorsal aorta are likely to contribute to the right subclavian artery, but whether this contribution is modest or extensive remains unclear. The 3D-reconstructions presented in this study lend support to the currently used morphological concepts, but also highlight that for some regions, little is known about the relative size and final contribution in the adult. Lineage and cell-labelling experiments will be pivotal to shed light on the developmental origin of the adult aortic arch and pulmonary artery system.

Aortic arch anomalies can result in serious clinical problems due to tracheobronchial or esophageal compression, and abnormal blood flow patterns. The accurate description of the continuously changing and complex 3D morphology of the transforming PAAs, accompanied by gene expression data in human embryos presented here, attempt to provide a better understanding of human aortic arch morphogenesis. Our findings support animal-to-human extrapolations. The interactive reference model presented here may
further increase our understanding of the etiology of a variety of aortic arch abnormalities. Finally, future genetic and molecular analyses of the developing vasculature using animal model systems will aid in unravelling the underlying pathogenesis of common aortic arch malformations.

Acknowledgements

We are indebted to the personnel of the Gynaecology Department of the Tartu University Hospital, to Dr. M. Aunapuu and Prof. A. Arend from the Anatomy Institute of the University of Tartu, Estonia, for their help with the collection of the human embryos. We thank J. Hagoort for his continuous support and invaluable help with the preparation of the interactive 3D-PDF file. This work was supported by the European Community’s Framework Programmes’ contracts LSHM-CT-2005-018630 (‘HeartRepair’), Health-F2-2008-223040 (‘CHeartED’), Health-2007-B-223463 (‘CardioGeNet’), and by the Netherlands Organization for Scientific Research (‘Mosaic’ grant 017.004.040 to M.S.R.). Collection of human embryonic material was supported by grant 7301 from the Estonian Science Foundation.

Box 1. Abbreviations:

AA aortic arch
AAo ascending aorta
AS aortic sac
Ca caudal
Cr cranial
D dorsal
DAo dorsal aorta
DsAo descending aorta
duct arterial duct
E esophagus
E/ICAs external/internal carotid arteries
IA innominate artery (brachiocephalic trunk)
Lb lungbud
lig ligamentum arteriosum
L/RCCA left/right common carotid arteries
L/RLb left/right lungbud
L/RPA left/right pulmonary artery
L/RSA left/right subclavian artery
L/RVA left/right vertebral artery
L/RVOT left/right ventricular outflow tract
NC neural crest
OFT outflow tract
PA pulmonary artery
PAAs pharyngeal arch arteries
PE pharyngeal ectoderm
Ph pharynx
Pr pericardial reflection
PT pulmonary trunk
Tr trachea
V ventral

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