RESEARCH ARTICLE

Quantified growth of the human embryonic heart
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ABSTRACT
The size and growth patterns of the components of the human embryonic heart have remained largely undefined. To provide these data, three-dimensional heart models were generated from immunohistochemically stained sections of ten human embryonic hearts ranging from Carnegie stage 10 to 23. Fifty-eight key structures were annotated and volumetrically assessed. Sizes of the sepal foramina and atrioventricular canal opening were also measured. The heart grows exponentially throughout embryonic development. There was consistently less left than right atrial myocardium, and less right than left ventricular myocardium. We observed a later onset of trabeculation in the left atrium compared to the right. Morphometry showed that the rightward expansion of the atrioventricular canal starts in week 5. The sepal foramina are less than 0.1 mm² and are, therefore, much smaller than postnatal septal defects. This chronological, graphical atlas of the growth patterns of cardiac components in the human embryo provides quantified references for normal heart development. Thereby, this atlas may support early detection of cardiac malformations in the foetus.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: Human heart development, Embryology, Growth, Morphogenesis

INTRODUCTION
The study of normal and abnormal growth of individual organs such as the brain, kidney, liver and spleen in the developing human can inform us on disease processes that might become of importance later in life (Latini et al., 2004; Wahab Abdel Latif et al., 2017). For the heart, early diagnoses of malformations allow for better adjusted postpartum care (Thakur et al., 2016) and lead to better survival (Holland et al., 2015). Heart development is a highly complex multistep process. Most comprehensive descriptions of human heart development are qualitative in nature and are summarised in textbooks (Filipoiu, 2014; Oostra et al., 2007; Sadler, 2006; Schoenwolf et al., 2009). These textbooks make use of the many detailed qualitative studies on the emergence and appearance of structures (e.g. Lockhart et al., 2011 and Sylva et al., 2013) or processes like septation (e.g. Anderson et al., 2002; Patten, 1938 and Van Mierop et al., 1963). However, what constitutes normal and abnormal growth of the structures of the human embryonic heart is poorly understood. Nevertheless, in recent years, immunohistochemical studies, regarding for example the development of the cardiac conduction system, have clarified some of the controversies emerging from purely anatomical descriptions (Sizarov et al., 2011a). But, for the single cell transcriptomic data of the developing heart, which is becoming available (e.g. Cui et al., 2019), more detailed knowledge regarding cardiac structures and their sizes may help to validate the assignment of cell identities and their anatomical positions.

The few publications that do address quantitative descriptions of embryonic cardiac growth mostly focus on a detailed point such as ventricular wall thickness (e.g. Goor et al., 1970) or ventricular myocardial volume (e.g. Wenink, 1992). But, most of what is known about quantitative morphology stems from non-human heart development, such as mouse (de Boer et al., 2012; Ishiwata et al., 2003) and chicken (Rana et al., 2007; Rychterová, 1971). No quantitative reference work exists on the growth of key structures of the human heart that can indicate whether cardiac development might be deviating from normal.

Cardiac malformations are usually diagnosed after a (first trimester) ultrasound and/or with an abotion after (spontaneous) abortion (Huggon et al., 2002; Rasiah et al., 2006). With the advancement of visualisation technology, the threshold for detecting abnormalities is shifting to younger stages of development. Since many cardiac malformations are defined by a disproportion and, therefore, by a change in differential growth, a reference work on cardiac growth could be useful. For example, hypoplastic left heart and obstructed foramen ovale as well as septal defects and absence of valvular structures like in tricuspid atresia, result from one structure growing faster or slower than others (Maeno et al., 1999). Additionally, more subtle phenotypes are defined by changes in relative size of myocardial structures, like, for example, left ventricular non-compaction (Menon et al., 2007) in which the proportion of trabecular to compact ventricular myocardium is greater than normal.

Here we present an atlas of normal human embryonic heart development. Given the number of structures investigated, numerous analyses can be made. For the sake of brevity, we have focused on a few processes to exemplify the utility of such quantifications.

RESULTS
We segmented all major components of ten embryonic hearts of subsequent stages of development (Fig. 1) resulting in three-dimensional (3D) models in which all individual structures can be rotated and inspected (Figshare Supplement). The chronological appearance of the morphologically distinct structures of the embryonic heart have been represented in a graph (Fig. 2). This indicates that most myocardial structures become recognisable during the period of CS12-15, in the fifth week of gestation, which suggests this is a highly critical period for heart development.

Volumetry
Reconstruction of the myocardium, cardiac jelly/cushion tissue, and lumen of the embryonic hearts revealed differences in their relative growth patterns (Fig. 3). The myocardium grows exponentially while
the cardiac jelly/cushions slow their growth after CS16. There is no indication that the total amount of cushion mesenchyme decreases in the embryonic period, instead it plateaus after initial growth (logistic regression curve fit $R^2=0.91$), which fits with previous reports (Wenink, 1992). The luminal volume increased exponentially over time, although the variance there is larger than for the myocardium, likely because luminal volume is dependent on the state of contraction, which varies among the specimens, whereas the myocardial volume is not.

**Chambers**
The myocardial growth of the cardiac chambers is outlined in Fig. 4. The inflow tract myocardium, which surrounds the two superior caval veins and may act as a chamber in early human development (Faber et al., 2019), initially grows exponentially, but its growth levels off halfway through embryonic development. It remains to be shown whether this change in growth rate coincides with the so-called atrialisation of this myocardium, whereby the sinus venosus loses its identity as a separate chamber. It can be seen that the amount of right atrial myocardium is always larger than that of the left atrium [non-linear regression, exponential curve fit ($R^2$ of 0.98 for both curves), $P<0.0001$]. Conversely, the left ventricular myocardial volume is greater than the right in the entire embryonic period [non-linear regression, exponential curve fit ($R^2$ of 0.98 for right and 0.96 for left ventricular myocardial volume), $P=0.002$].

![Fig. 1. Methodology.](image)
The right ventricular myocardial volume only approaches that of the left ventricle towards the end of the embryonic period. During the first stages of development, the right ventricular growth is driven by the addition of outflow tract myocardium (Kelly et al., 2001; Rana et al., 2007), which is reflected in the drop of outflow tract myocardial volume (Fig. 4B). The atrioventricular canal myocardium initially consists of primary myocardium. This is known to grow very little (Hoogaars et al., 2004; Sizarov et al., 2011b). Therefore, the amount of atrioventricular canal myocardium decreases relatively to the rest of the heart (Fig. 4C).

**Trabeculae**

Both atria and ventricles show development of trabecular structures (see Fig. 1D for examples of the ventricular trabecular labels). In the atria they give rise to the pectinate muscles and in the ventricles they become the trabeculae carneae, papillary muscles and Purkinje network. The distinction between the different trabecular muscle types is more pronounced in adult hearts, where the pectinate muscles also show a lot of individual variation in morphology (Loukas et al., 2008). It can be seen that in the right atrium the trabeculae develop earlier than in the left atrium (Fig. 5A), this is corroborated by data found in several publications (Anderson et al., 2003; Kim et al., 2001; Mandarim-de-Lacerda and Sampaio, 1987; Picazo-Angelin et al., 2018; Wessels et al., 1992, 2000). The left atrium in the embryo is, therefore, smoother than the right atrium, a characteristic that will persist into adulthood (Ho et al., 2002). In contrast to the right ventricle, the left ventricle contains more compact than trabecular myocardium (Fig. 5B,C). The volume of ventricular trabecular myocardium increased for every stage analysed. This contrasts the expectation that a substantial amount of trabecular muscle is added to the compact wall in the process of compaction (Oechslin and Jenni, 2011).
Morphometry

The 3D reconstructions also allow for morphometric investigations (Fig. 6). Around CS16, in the sixth week of development, the primary foramen of the atrial septum closes by approximation of the mesenchymal cap mesenchyme at the leading edge of the primary atrial septum to the atrioventricular cushions (Fig. 6A). The primary foramen has never been larger than 0.1 mm² (Fig. 6A). Simultaneous with the closure of the primary foramen of the atrial septum, the secondary foramen of the atrial septum opens (Fig. 6B). The volume of primary atrial septum myocardium stabilises over time, while the area of the secondary atrial foramen shows only a trend towards narrowing from 0.15 mm² to 0.04 mm² towards the end of the embryonic development (quadratic curve over straight line fit \( P = 0.183 \)). It is unlikely that the secondary atrial foramen narrows in development since it has to grow to approximately 45 mm² perinatally (Kiserud and Rasmussen, 2001).

The opening of the atrioventricular canal in the transverse plane changes from a circular lumen to a more oval lumen with its long-axis from right to left. The canal widens linearly. Additionally, the canal shifts to the right relative to the crest of the interventricular septum (Fig. 6C). This shift of approximately 0.5 mm causes the atrioventricular canal to keep overriding the right ventricle.

The interventricular foramen is tiny from its first appearance, less than 0.1 mm² (Fig. 6D). It is closed by mesenchymal tissue towards the end of the embryonic period, which, from then on constitutes the still very small membranous septum. The atrioventricular cushions play a key role in the division of the interventricular foramen in the right ventricular inlet and the left ventricular outlet. It is noteworthy that the cushions that will later form the membranous septum, at this stage are still thicker than the myocardial septum, leaving only small slits for the right and left atrioventricular channels.

DISCUSSION

We have labelled 58 structures in ten embryonic human hearts ranging from gestational weeks 4 to 8. All structures have their own developmental stage of origin and growth rate (Supplemental materials). The observed developmental appearance of morphologically recognisable structures in the heart corresponds well with previous accounts focusing on fewer structures (Arráez-Aybar et al., 2008; Dhanantwari et al., 2009; O’Rahilly, 1971). Some detailed differences do occur, however. We distinguished the interventricular septum at CS14, slightly earlier than some (CS16) (Dhanantwari et al., 2009), though later than others (CS12) (O’Rahilly, 1971). Additionally, we observed that in our specimens the primary atrial septum and the sinuatrial valves appeared slightly later (CS14 versus CS12 and 13) (O’Rahilly, 1971). The precise staging of the human embryos is difficult and may be imprecise and may, therefore, slightly differ between studies, which could account for the reported differences in the first appearance of structures. However, at CS14 we did see the first connection of the pulmonary vein to the left atrium, which is similar to previous reports (Blom et al., 2001). Overall, most structures visibly appear around CS12-15. This period, which corresponds to the fifth week of gestation, can be considered of particular importance for the formation of the 4-chambered heart. For example, the univentricular heart, where the right ventricle is absent or only rudimentary visible (Anderson et al., 1976; Khairy et al., 2007), as well as septal defects may find their origin in this particular period of development. Growth of the heart is rapid and exponential, because it is synchronised with the exponential growth of the whole embryo (de Bakker et al., 2016), both before, during and after the morphologically important fifth week of gestation.

The fast-growing cardiac chambers show a gradual development towards adult proportions, whereas the slow-
Fig. 4. Growth of the cardiac chambers and myocardial structures. (A) Absolute growth of the inflow tract (IFT; including IFT_myocardium, R_sinus_horn_myocardium and L_sinus_horn_myocardium), common atrium (A; Atrial_myocardium), right atrium (RA; including RA_myocardium, RA_trabecular_myocardium, R_sinuatrial_valve, L_sinuatrial_valve, Septum_spurium_myocardium, Inferior_rim_oval_fossa_myocardium and Secondary_atrial_septum_sulcus), left atrium (LA; including LA_myocardium, LA_trabecular_myocardium and Pulmonary_vein_myocardium), common ventricle (V; including Ventricular_myocardium and Ventricular_trabecular_myocardium), left ventricle (LV; including LV_compact_myocardium and LV_trabecular_myocardium), and right ventricle (RV; including RV_compact_myocardium and RV_trabecular_myocardium). There is always significantly less left atrial than right atrial myocardium (P<0.0001) and less right ventricular than left ventricular myocardium (P=0.002). (B) Absolute growth of the septa (S; including Primary_atrial_septum_myocardium and Interventricular_septum_myocardium), atrioventricular canal (AVC; AV_canal_myocardium), and the outflow tract (OFT; OFT_myocardium). (C) Relative growth of chamber myocardium. Rest includes Primary_atrial_septum_myocardium, Interventricular_septum_myocardium and Myocardialised_AV_cushion. (D) Four chamber view cross-sections of hearts of CS12, 13 and 14 illustrating the transition from common atrium (dark blue) and common ventricle (light green) to recognisable right and left atria (intermediate and light blue) and ventricles (orange and yellow). N=1 for each time point.
growing components of the primary heart tube become proportionally smaller (Fig. 4) (van den Berg et al., 2009). In the adult, the left and right ventricle, and the interventricular septum encompass approximately $37\pm6\%$, $19\pm4\%$ and $24\pm4\%$ of total myocardial mass (Hayes and Lovell, 1966). When we look at the end of the embryonic period, the chamber proportions are $32\%$, $30\%$ and $7\%$, indicating that the right ventricle is relatively large, which is supported by previous foetal dissections and ultrasounds (Alvarez et al., 1987; Keen, 1955; Kim et al., 1992; Rowlatt et al., 1963; St John Sutton et al., 1984), and the interventricular septum lags behind in growth as can be expected at this stage (Rolo et al., 2011, 2015).

We report in this study that the formation of the trabeculae in the left atrium starts after that of the right atrium. This is in contrast to...
Fig. 6. Morphometric quantifications. (A) Closure of the primary atrial foramen. In purple, Primary_atrial_septum_myocardium; in grey, all other myocardium; in yellow, cardiac cushions. The angle of cross-section was determined by the primary atrial septum. Slices represent 5% of the total model thickness. Models are of CS14, 15, 16, 18, 20 and 23. (B) Growth of the primary atrial septum (purple) and the secondary atrial foramen. The secondary atrial foramen only shows a trend towards narrowing (Strait line fit $P=0.183$). In grey, all myocardium belonging to the left atrium; in purple, Primary_atrial_septum_myocardium. Models are of CS14, 15, 16, 18, 20 and 23. (C) Widening of the atrioventricular canal and the distance between the interventricular septal wall and the right wall of the atrioventricular canal. In grey, AV_canal_myocardium, in orange Interventricular_septum_myocardium. Models are of CS10, 12, 14, 16, 18 and 20. (D) Closure of the interventricular foramen. In purple, Primary_atrial_septum_myocardium; in orange, the Interventricular_septum_myocardium; in grey, all other myocardium. The angle of cross-section was determined by the interventricular septum. Slices represent 10% of the total model thickness. Models are of CS 14, 15, 16, 18 and 20. X-axes of the graphs are in average gestational age (days). L, left; R, right; Dm, dorsal; V, ventral. Foramina are indicated with a red arrow. N=1 for each time point.
observations in mouse, where the left atrium acquires trabeculae simultaneously with the right atrium (Savolainen et al., 2009). At a comparable developmental stage (Sylva et al., 2013), both atria in the mouse are trabeculated (embryonic day 11.5-12.5) (Hooogaars et al., 2004; Savolainen et al., 2009), whereas this is not the case in human (CS14-16) (Anderson et al., 2018; Wessels et al., 2000). This may indicate that the developmental stage of onset of trabeculation is decisive for the degree of trabeculation of the adult atrial wall. There is the notion that the left atrial wall owns its smooth appearance to the incorporation of pulmonary venous myocardium (e.g. Sadler, 2006; Schoenwolf et al., 2009). Our observations, instead, suggest an important role of the late onset of trabeculation and this is entirely compatible with the previous observation that the left atrium is smooth even if no pulmonary veins connect to it (Douglas et al., 2009).

We have shown in this study that the ventricular trabecular myocardial volume increases during the entire embryonic period, which is consistent with the only other observations on trabecular volume in human embryos (Blausen et al., 1990). In our specimens, the amount of right ventricular trabeculae approached that of the left ventricle slightly later (CS23 versus CS19), possibly because the previous study included the entire ventricular septum in the measurements of trabeculae (Blausen et al., 1990). Importantly, our data together with those of Blausen et al. do not support a process of myocardial compaction, that is a decrease in trabecular myocardium by its addition to the compact wall, to occur in the embryo, as has been suggested previously (Almeida and Pinto, 2013; Sedmra, 2000). Although compaction appears to be well documented in chicken (Rychterová, 1971; Sedmra et al., 2000) and other animals (Hanemaaijer et al., 2019), we know of no measurements that support compaction in human. Therefore, if left ventricular non-compaction cardiomyopathy occurs, where compaction is assumed to fail (Finsterer et al., 2017; Towbin and Jeferies, 2017), it does not originate in the embryo.

We found the interventricular foramen closing between CS20 and CS23, similar to previous reports (Arráez-Aybar et al., 2008; Goor et al., 1970). The largest size of the interventricular foramen in the embryo was found to be 0.07 mm², which corresponds with a foramen diameter of 0.3 mm, reported previously (Goor et al., 1970). Membranous septal defects in hearts of newborns can have diameters of 3 to 25 mm (Sharif et al., 1989), equalling 7 to 490 mm². Such orders of magnitude differences in size of the foramen between the embryo and the newborn indicate that these defects should not be considered the persistence of an embryonic defect only. Similarly, the secondary atrial foramen or foramen ovale did not exceed the 0.1 mm² at the end of embryonic development. It is known from ultrasounds that it increases in foetal development till an average of 0.45 cm² (Kiserud and Rasmussen, 2001). This, too, indicates that the foramen itself has temporary regression, such as the primary atrial septum, which serves as its control. For smaller structures that undergo some level of temporary regression, such as the primary atrial septum, which develops secondary perforations, addition of more samples could substantially improve the assessment of growth. In particular, there may be substantial variation in the development of the secondary perforations as suggested by the heterogeneous appearance of primary septal defects (Patten, 1938). Because we plotted our findings on an average gestational age scale rather than a CS scale, there is some space for variation in age as well. The heart of CS15, which is slightly larger than the heart of CS16, illustrates this problem.

The embryos we used will have shrunken due to the fixation process. Previous reports have measured the shrinkage associated with 10% formalin fixation to range between 10 and 26% (Mandarim-de-Lacerda, 1991; Patten et al., 1929) and we, therefore, consider it likely that our measurements are slight underestimations. We do not know of studies that show that shrinkage is greater in some embryonic stages than in others, therefore, we expect that direction of growth and the relative growth will be correct even if the absolute values are underestimated.

CONCLUSION
This study provides a quantitative description of the growth of the different cardiac structures recognisable in the embryonic heart. We observed exponential growth of the heart and measured the development of the sepal foramina. By comparing our data with pathological reports and ultrasound investigations, we show that our quantitative description of heart development can serve as a supportive document to several fields of research and can give new insights in complex processes.

MATERIALS AND METHODS

Embryos
This study makes use of a human embryonic section series that was published previously (Sizarov et al., 2011a,b, 2012) so no informed consent was obtained for this investigation. The embryos were leftover material from induced abortions on social indication performed at the Gynaecology Department of the Tartu University Hospital, Estonia. They had been collected with permission of the Medical Ethics committees of the University of Tartu, Estonia, and of the University of Amsterdam, the Netherlands. The investigation conformed to the principles outlined in the Declaration of Helsinki. The embryos were fixed in 4% paraformaldehyde after collection.
After exclusion of outward abnormalities, embryos had been staged on the basis of outward appearances before they were dehydrated, immersed in butanol, and embedded in paraffin (Sizarov et al., 2011a). We included embryos of Carnegie Stage (CS) 10, 11, 12, 13, 14, 15, 16, 18, 20 and 23. Since CS does not correspond linearly to time, average ages were calculated (Sylva et al., 2013). This study did not use animal models or tissues.

**Immunofluorescent staining**

The embryos had previously been sectioned at 7 (CS10, 11, 12, 13, 14, 15, 16, 18, 23) or 10 μm (CS20) thickness. Thereafter, sections that contained heart had been immunofluorescently stained with Troponin I [1:250; MAB1691, Chemicon or 1:250; Hytest, 4T21/2 (only on CS11, 12 and 13)] or, on alternating sections, Troponin I, SERCA2a [1:250; ab2817, Abcam] (Sizarov et al., 2010) and MF20 (produced in house after Bader et al., 1982).

**Cardiac reconstruction**

With a fluorescence microscope Leica DM6000, driven by ImagePro Plus 6.2 software (Media Cybernetics), each stained section had been photographed (Sizarov et al., 2010) and was imported in Amina 6.5.0 (Konrad-Zuse-Zentrum Berlin; FEI SAS, Thermo Fisher Scientific) (Fig. 1A). The x and y values of the voxel size were set to correspond with the actual tissue size on a section. The z value corresponded to section thickness. If a section was severely damaged, it was substituted with a copy of an adjacent section.

The AlignSlices module of Amina software allows for automatic alignment of the sections without deformation correction, while permitting manual adjustments of transformation and rotation. This was done for all hearts. Next, all recognised cardiac structures were manually segmented on each individual section (Fig. 1B). The description of all label definitions can be found in the Supplemental materials. Hereafter, with the Material Statistics tool, volumes of each labelled structure were exported. The absolute volumes and the volume growth curves of all cardiac structures can be found in the Supplemental materials. Hereafter, with the Material Statistics tool, volumes of each labelled structure were exported. The absolute volumes and the volume growth curves of all cardiac structures can be found in the Supplemental materials. Hereafter, with the Material Statistics tool, volumes of each labelled structure were exported.

The authors declare no competing or financial interests.

**Competing interests**

**Author contributions**


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**Data availability**

The data underlying this article, including the 3D-pdfs, is available on Figshare https://doi.org/10.6084/m9.figshare.13378742.

**Supplementary information**

Supplementary information available online at https://bio.biologists.org/lookup doi:10.1242/bio.057059.supplemental

**References**


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