

# Prenatal development of coronary arteries in the rat: morphometric patterns

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*The aim of this work was to address morphometric patterns of coronary artery (c.a.) development in the rat based on serial section analysis of hearts at different stages of prenatal development. Studies were performed on foetal hearts 15–21 days (ED) post-conception. Paraffin sections were stained with haematoxylin-eosin (H&E) and frozen sections were labelled with Griffonia simplicifolia I (GSI) lectin (endothelial cell marker). Coronary arteries' luminal diameters were measured at different distances from the aortic roots and the main c.a. branch lengths were calculated from serial sections. All measured values were compared to heart length and to foetal stages. On ED15 precursors of c.a. were distinguished as tubes running on both sides of the outflow tract. Below the aortic valves the tubes had the largest diameter. Formation and development of c.a. proceeded by elongation of vascular tubes distally, ramification and formation of the media and the adventitia. During the prenatal period the c.a. length increased approximately 14-fold, while heart length increased about 4-fold, and crown-rump length about 2.5-fold. The lumen of the proximal part of c.a. increased 4-fold during ED18–21. An increase in c.a. length is the fastest compared to the heart growth, and crown-rump growth during the foetal life.*

**key words:** coronary artery development, angiogenesis, coronary artery, rat, embryonic heart, morphometry

## INTRODUCTION

The earliest morphological signs of coronary artery formation can be distinguished as vascular tubes running along the ED15 rat hearts. These tubes consist of endothelial cells lining their lumen and mesenchymal cells attached to them. The blood is not flowing within the lumen yet, because the vascular tubes do not form a patent connection with the aorta at this stage [18]. It seems that the development of larger vessels which are predestined to contain the media and the adventitia starts at preferred areas of the myocardium [16]. The vasculogenesis, which leads to the larger vessel development, includes endothelial cell assembly and a recruitment of other cell types followed by differentiation to

smooth muscle cell phenotype of the media, fibroblasts of the adventitia and connective tissue deposition [12,14,23]. The formation of the coronary ostia occurs as a consequence of the ingrowth of vascular tubes into the aorta. Although many of these activities have been addressed recently by numerous studies [2–5,7,8,13,17,20–23,26] some questions regarding heart vascularisation remain unresolved.

There is no data on morphometrical parameters of coronary artery formation during foetal life. Such data would help to understand developmental dynamics of coronary arteries and would shed some light on the mechanism(s) of larger vessel formation during foetal development.

The present work was designed to: 1) address the spatio-temporal and selected morphometrical parameters of coronary artery development in rats; 2) characterise growth rates of coronary arteries in relation to the foetal age, crown-rump and heart length.

## METHODS

### Animals and tissues

The investigation conforms to the requirements for the care and use of Laboratory Animals of the Medical University of Warsaw, Poland and of the European Communities Council Directive of November 24, 1986 (National Institutes of Health Publications No. 80-23, Revised 1978).

Hearts were obtained from pregnant rat dams (Wistar WAG) at various times of gestation, between ED14 and ED21 (ED21 is the last day of gestation). Foetuses were removed from uteri, under ether and chloral hydrate anaesthesia and decapitated. Then their hearts were excised and placed in Tris-Tyrode solution (pH 7.4). The foetal crown-rump and heart lengths were obtained under a dissecting microscope by the use of a calibrated micrometer scale. The hearts from the same litter were either: 1) mounted onto a chuck with OCT compound and frozen for immunohistochemical staining or 2) paraffin embedded after formalin fixation. While embedding all hearts were oriented with the longitudinal axis perpendicular to the plane of section so that cross-sections of coronary arteries could be obtained.

### Immunohistochemical staining

Frozen serial sections were placed on sialinised slides, air-dried and stained with Griffonia simplicifolia I (GSI) lectin conjugated with rhodamine (Sigma)(1:100 dilution in PBS containing 1% BSA). This lectin has an affinity to alpha-methyl-D-galactopyranosyl group specific for endothelial cells of microvasculature in rodents [1,9]. It also has an affinity for some basement membrane components, but to a lesser extent. The following numbers of hearts were used for serial sectioning: ED14 — four, ED15 — six, ED16 — six, ED17, ED18, ED20 and ED21 — three of each. Larger hearts (ED18-21) were cut serially at the base, but towards the apex sections were cut every 50 or 100  $\mu\text{m}$ .

### Light microscopy and morphometry

Paraffin sections (6  $\mu\text{m}$  thick) were serially cut, and stained with haematoxylin-eosin. These sections were

used for the morphometric analysis using Multiscan computer system connected with a light microscope. The hearts from paraffin blocks of ED15, ED16, ED17, ED18 and ED21 specimens (three of each) were taken for morphometry. A vessel having fully developed aortic orifice and covered with the primitive media (and the adventitia) was considered a nascent coronary artery. A structure having an endothelial cell lining without a lumenised connection with the aorta was considered a coronary artery precursor. This structure was recognised by its spatial position within the embryonic heart. From each heart the longest branches of both coronary arteries were analysed, provided they were recognisable on sections and had a proper orientation (perpendicular to the plane of sections); otherwise they were not taken for the measurement. Lumen diameters of the left and the right c.a. or their precursors were measured at various section levels. The cross section of the lumen was in the shape of a circle or an ellipse. The shortest axes of ellipses were taken as the vessel's diameter, as explained in Figure 1. The large hearts (ED18-21) were measured serially at the base but towards the apex the distal sections were measured every 50 or 100  $\mu\text{m}$ . This was because the course and dimensions of distal c.a. did not change at these stages of development (based on the pilot study results).

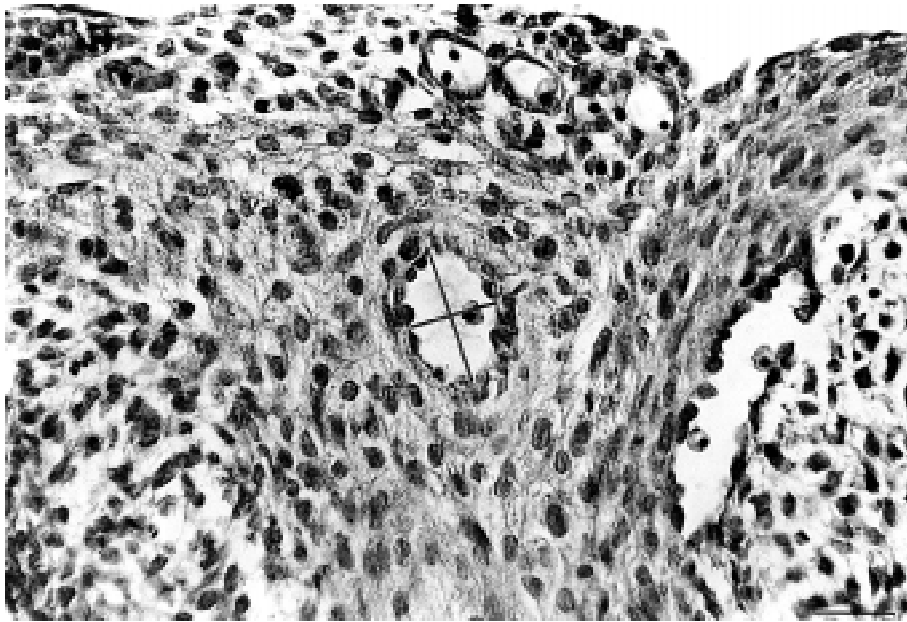
Heart cranio-caudal dimension was measured and expressed as heart length.

### Statistical analysis

Three types of measurement for each foetal heart were obtained. The length of the c.a. was estimated based on the thickness of sections and the number of sections in which the vessel was visible. The mean luminal diameter of the proximal part of c.a. was calculated from measurements performed on the first 15 sections nearest to the aortic roots. Finally the hearts' lengths were obtained. Mean values were estimated from normal distribution and expressed as mean  $\pm$  SEM.

The method of the least squares was used to fit the straight line and/or power function between mean luminal diameter and the foetal age. Based on the course of these lines a growth coefficient of the mean luminal diameter was estimated.

Luminal reconstruction of a coronary artery at representative foetal stages was presented graphically as a two-dimensional model in which the length of the vessel was shown on the abscissa (X-axis) and the lumen diameter was shown on the ordinate (Y-axis). Each value of the luminal diameter was divided into two



**Figure 1.** H&E stained section of ED15.5 heart. C.a. wall consists of the endothelial cells and the primitive tunica media visible on the cross section. The short and the long lines represent the lumen dimensions; the short line from each measurement has been taken as lumen diameter. Bar = 50  $\mu\text{m}$ .

radii of identical length and was shown on both sides of "0 level" on the ordinate. The long axis of the vessel was represented as a straight line (abscissa). This approximation neglected curvatures resulting from the natural course of the vessel within the myocardium.

In order to exclude the influence of possible artefacts caused by the heart shrinkage upon the measurements, some hearts were immersed in 1M KCl solution inducing heart relaxation before fixation. Then, c.a. morphometry was performed on those hearts, as described above.

## RESULTS

### Immunohistochemical and histological identification of coronary artery constituents and precursor(s)

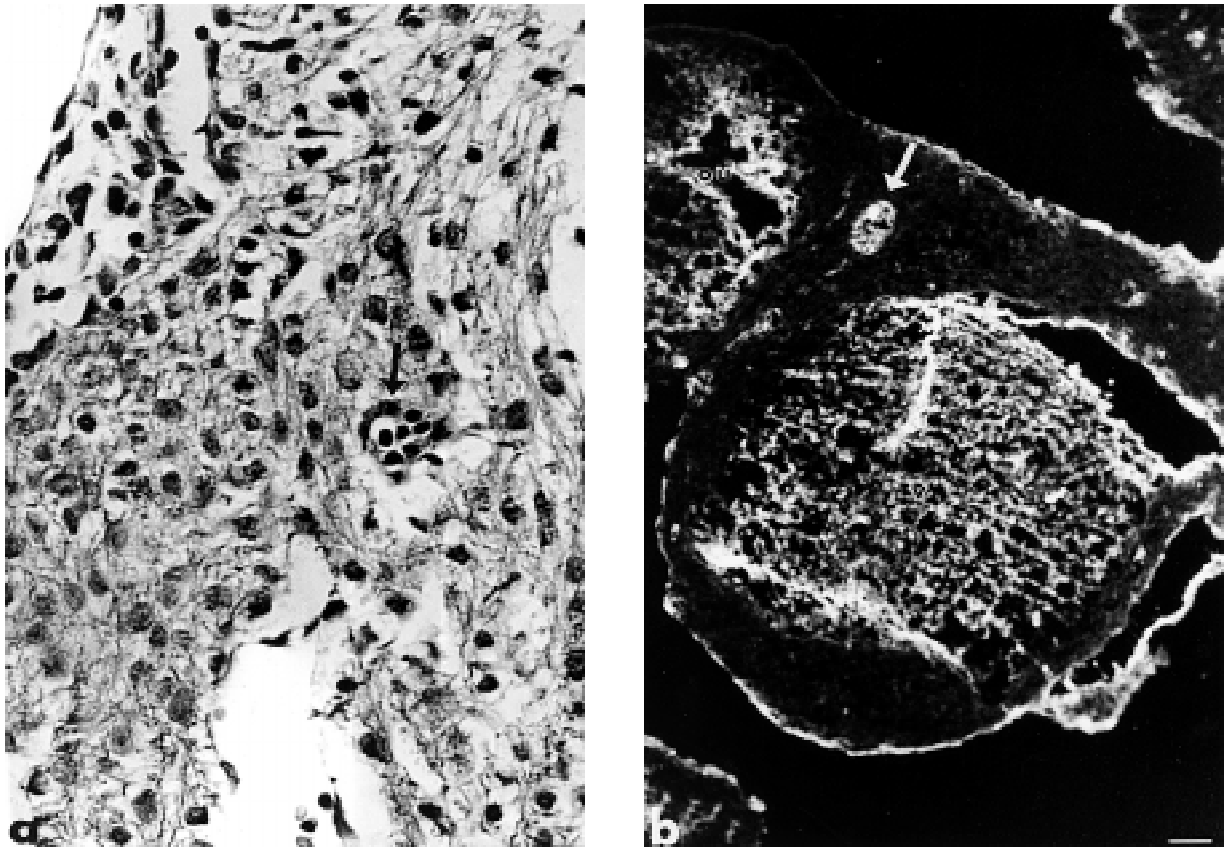
The labelling of tissue sections with Griffonia simplicifolia I lectin made it possible to identify all vascular structures within the myocardium. Among these were structures that had an appearance of typical blood islands (Fig. 2). These structures were c.a. precursors. On ED14 GSI-positive cells were seen forming clusters within the outflow tract (= conotruncus and truncus arteriosus) and the proximal ventricles of the heart on ED14 (Fig. 2B). On the following day these structures transformed into vascular tubes that were forerunners of the proximal coronary arteries. The vascular tubes were distinguished as coronary

artery precursors based on their localisation within the myocardium and their lumen diameters that were the largest compared to the lumen of other vascular structures (capillary plexuses).

The main tubes were running along both sides of the outflow tract and the ventricles with their distal end still not far away from the base of the heart. The tube corresponding to the main branch of the left coronary artery occurred earlier in development than the tube corresponding to the future right coronary artery.

The arborisations of the coronary arteries became visible within the precursor vessels at the stage when their walls consisted of one layer of cells (in ED15 hearts).

While analysing ED15 hearts we had seen in most cases one single tube as the c.a. precursor. Apart from this two different tendencies have been observed in the course of the c.a. precursors. Some of those precursors seemed to have a discontinuous course: they appeared in basal sections, were absent in consecutive sections, and were visible again towards the apex. Other c.a. precursors were initially distinguished as a single tube (while analysing the serial sections), subsequently as arborised capillaries, and finally (on consecutive sections) a single vascular tube again was seen. We could not prove, using our methods, whether vascular tubes separated by capillary plexuses were continuous with each other along the myocardial wall.



**Figure 2.** ED14 heart. A) H&E stained section with blood island (arrow). Blood island is localised within the myocardium in the area of future coronary artery; ou — outflow tract; v — primitive ventricle; B) GSI — stained sections of the same stage heart. Blood island (arrow) represents the structure that on ED15 would form vascular tube (the c.a. precursor). Bar = 50  $\mu$ m.

In ED16 hearts the endothelium-lined tubes were seen to be continuous in every section, i.e. two tubes (on each side of the aorta) were continuous with the peritruncal plexus, which at this time developed a lumenised connection with the aortic sinus. On ED17 two predominant vascular tubes with a patent lumen and arborisations towards the apex were found in the serial sections. These tubes became major branches of the coronary arteries and contained the multi-layered media and the adventitia. The distal end of the artery appeared on H&E stained sections as a cellular clusters with no recognisable lumen.

In older hearts (ED18–21) distal ends of coronary arteries extended further towards the apex of the heart and their branches developed the media and the adventitia.

#### Morphometry and graphical models

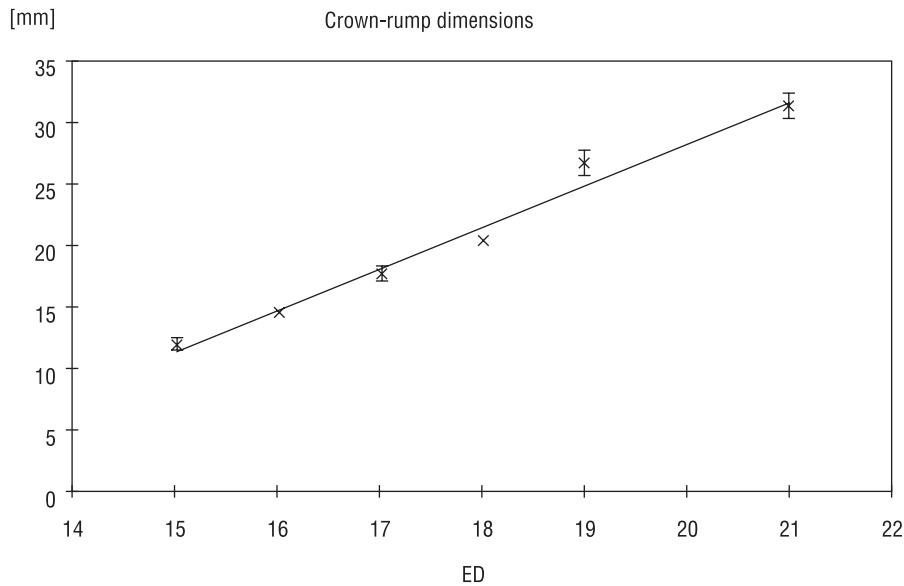
Figure 3 represents the crown-rump dimensions of the rat foetuses used for the experiment. Between ED15 and ED21 the foetuses grew 2.5 times in length.

The growth of the foetus was slow during the period of our interest and had a linear character.

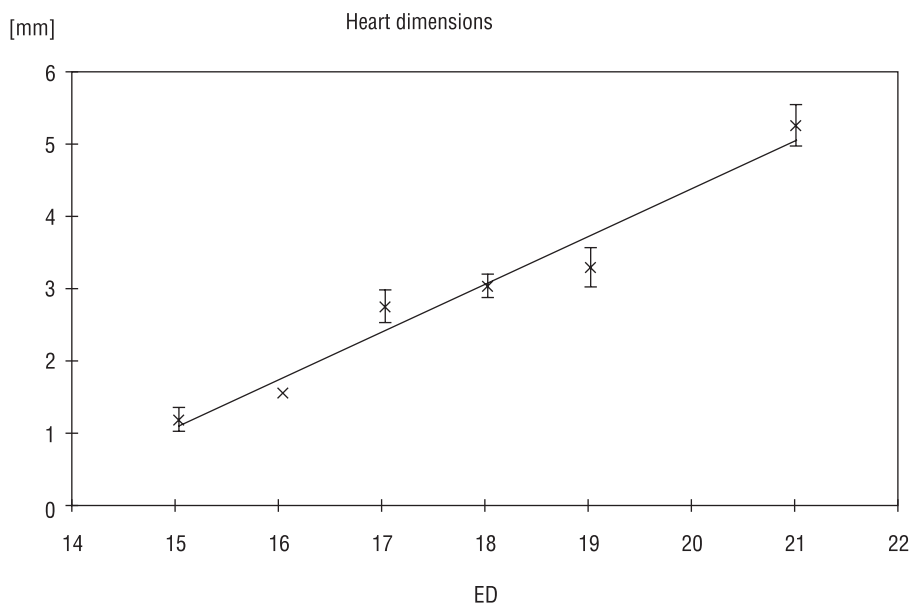
The heart length increased 4 times during the period studied. The growth was approximately linear, with a tendency to be faster by the end of the prenatal life (Fig. 4).

On ED15 the vascular tube corresponding to the main branch of a coronary artery was aligned approximately with the long axis of the heart; the total length of its main branch was about 150  $\mu$ m, which is 1/8 to 1/10 of the heart length at this stage. One day later the length of the coronary artery on ED16 doubled, and equalled 1/4 to 1/6 of the heart's length (Fig. 5). On the last day of the prenatal life the length of the c.a. was estimated to be about 14 times greater compared to the length of the c.a. on ED15. The c.a. growth in length proceeded in a linear fashion and its rate was greater than the growth rate of both the foetus itself and its heart.

Since the internal diameter of the c.a. during the development could also characterise the growth of this vessel, we wanted to see how the width of the



**Figure 3.** Development of foetuses during the prenatal period of c.a. formation represented as linear growth of the body length. The abscissa: embryonic day (ED) post-conception; the ordinate: crown-rump length (in mm).

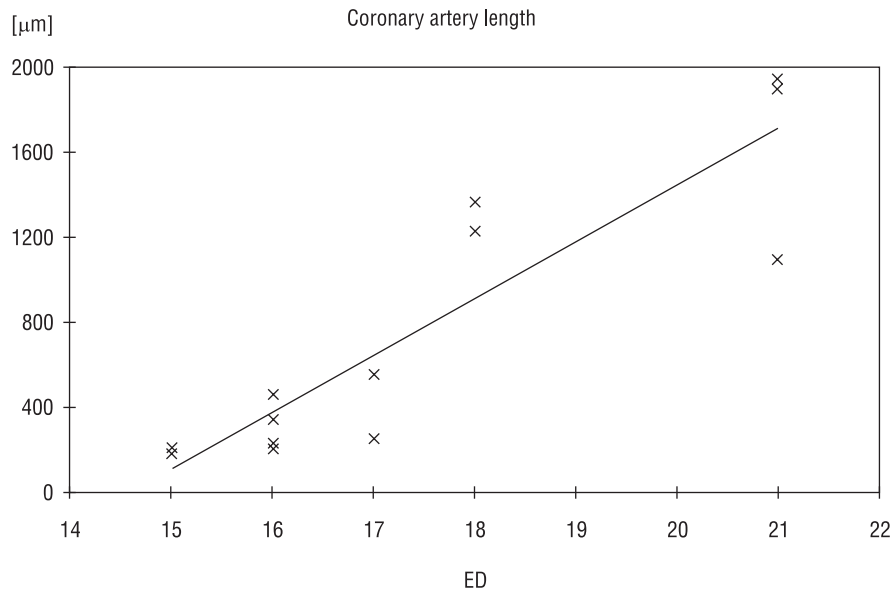


**Figure 4.** Growth of the heart during the foetal life represented as linear dimension of the heart between ED15 and ED21.

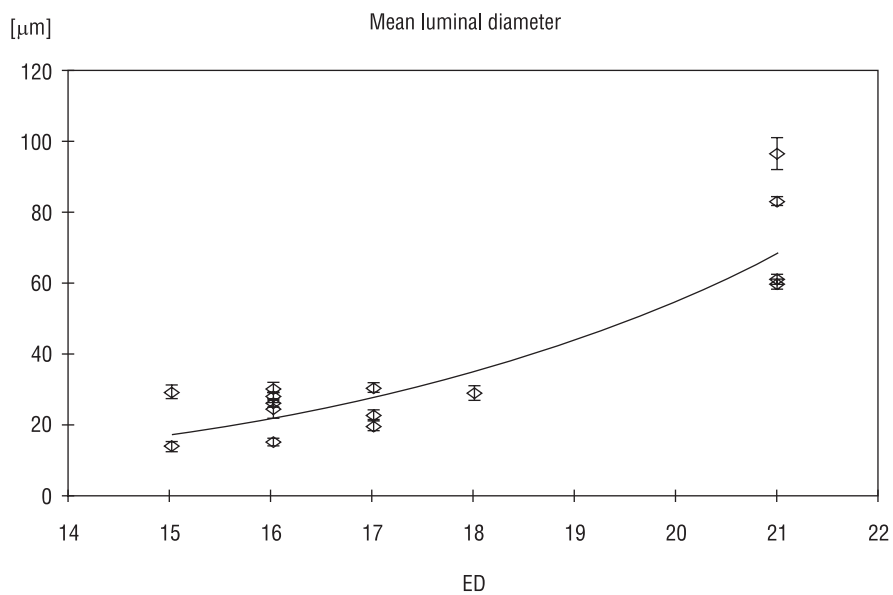
proximal c.a. had changed during the prenatal period studied. We found that the internal diameter of the proximal c.a. did not change significantly between E15 and ED18 and grew fast after ED18 (Fig. 6).

Figure 7 presents graphical models of coronary artery luminal reconstruction at various distances from the orifice at different foetal stages. Since the lumen diameter of the large vessels increased during development, we wanted to study how the c.a. lumen diameter had changed depending on foetal stage and

the distance from the orifice. As has been shown on the graphs the lumen diameter of c.a. differed depending on the distance from the aortic sinus (Fig. 7). On ED15 a connection with the aorta was not developed yet, so the lumen was absent within the proximal part (Fig. 7A) at this stage. On ED16 the coronary ostium became evident but the c.a. still had a narrow lumen in the proximal part. This lumen became wider at a certain distance from the aortic orifice (Fig. 7B). In the rostral direction the lumen of the coronary artery



**Figure 5.** The c.a. growth during the foetal life (between ED15 and 21) represented as linear growth of c.a. length. The line forms a large angle with the abscissa, which is indicative of a rapid growth.



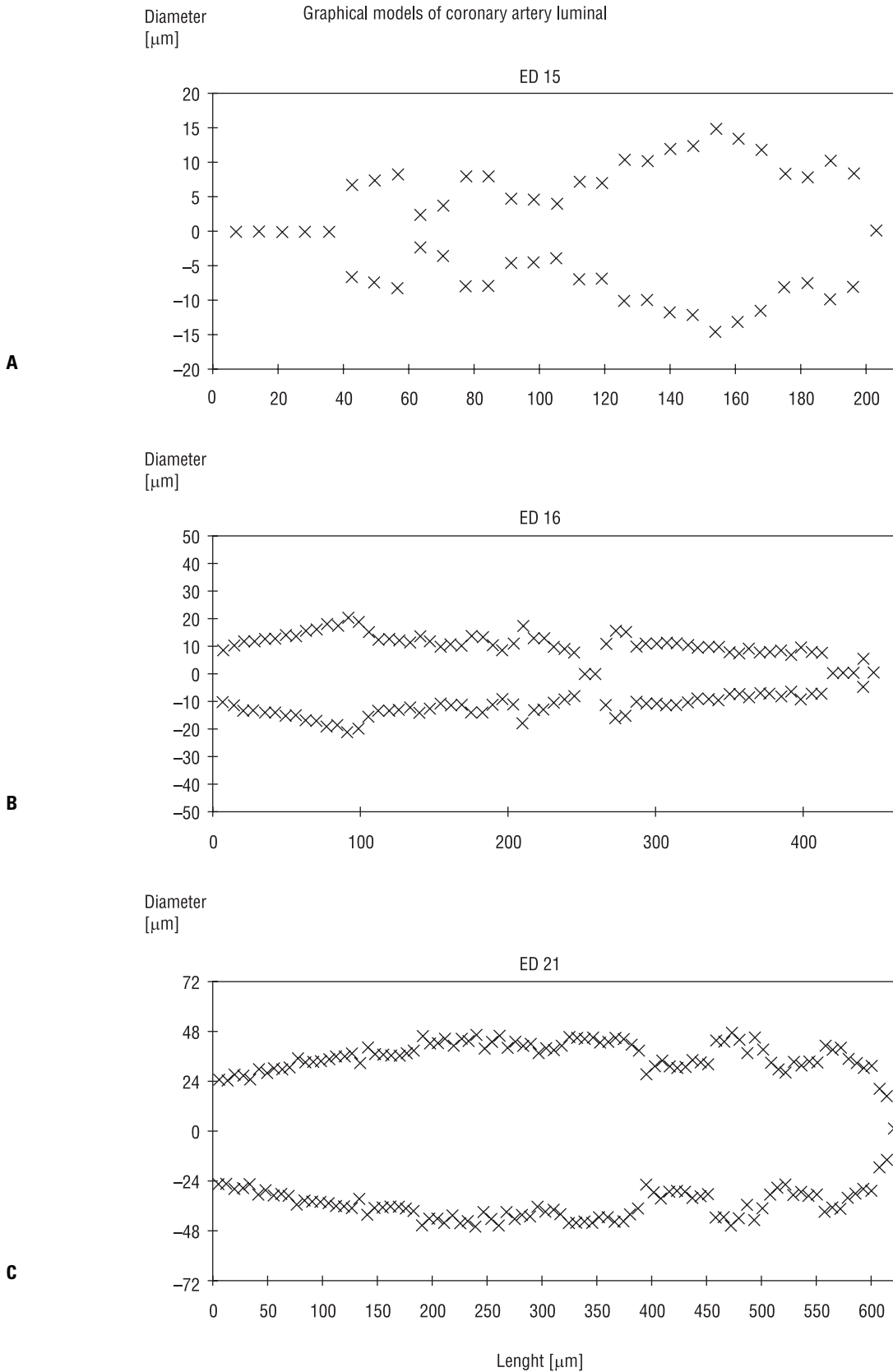
**Figure 6.** Graphical presentation of growth of the proximal c.a. luminal diameter during the foetal life (ED15–21).

widened and narrowed alternatively. By the end of the prenatal life (ED21) the length of the c.a. was equal to the length of the ventricles (Fig. 7C and Table 1).

We wanted to find out whether in areas of the largest lumen diameter the c.a. ramified to form two sister vessels, which would become separate branches of the c.a. The analysis of serially cut sections allowed us to ascertain that there was no relationship between the lumen diameter and the sites of c.a. ramification. The same pattern of c.a. luminal shape was also found in KCl-dilated hearts.

**Table 1.** Foetal dimensions and heart dimensions represented as crown-rump length and heart length, respectively, between ED15 and ED21

ED	Crown rump length [mm] (mean $\pm$ SEM)	Heart length [mm] (mean $\pm$ SEM)
15	11.8 $\pm$ 0.5	1.17 $\pm$ 0.16
16	14.3 $\pm$ 0.25	1.52 $\pm$ 0.04
17	17.4 $\pm$ 0.6	2.72 $\pm$ 0.23
18	20.0 $\pm$ 0.2	3.0 $\pm$ 0.16
19	26.3 $\pm$ 1.03	3.25 $\pm$ 0.27
21	31.0 $\pm$ 1.0	5.20 $\pm$ 0.3



**Figure 7.** Graphical model of coronary artery luminal shape is expressed as a diameter of its lumen (Y-axis) measured at different distances from the orifice (X-axis). Point "0" on the ordinate represents the level of aortic orifice. "Length" is the length of the c.a. (X-axis). Three stages of prenatal life are detailed: ED15, ED16, and ED21. On ED15 the coronary orifice is usually not seen, thus the c.a. lumen is marked with XXX at point "0". Note, that on ED15, and 16 the lumen diameter of c.a. is largest at a certain distance from the orifice.

## DISCUSSION

Recently numerous studies have addressed the embryonic c.a. formation from vascular precursors in different species [3–6,24,26]. Our paper further defines the precursor of c.a. development and is the first documentation of the histomorphometric characteristics of coronary artery formation. Since the GSI-positive cells were recognised in ED14 hearts as separate clusters at areas corresponding to the future c.a. development, we could suggest that the first steps of the c.a. formation proceed by vasculogenesis. Vasculogenesis and angiogenesis have been documented to occur during the embryonic coronary vessel development [19], however, it has not been certain whether coronary arteries developed by vasculogenesis [22]. Our data indicate further that the forerunners of the c.a. in rats are recognised on ED15 as the vascular tubes aligned with the long axis of the heart. These tubes are very short compared to the heart's length: they run along the outflow tract and reach only the proximal part of ventricles. Between ED16 and 21 the tubes reorganise to form the main branch of the coronary artery, whose length increases markedly in this period of time. Compared to the heart growth and crown-rump dimensions the c.a. increase in length is much faster (14-fold compared to 2.5-fold foetal growth increase). In one of the previous studies which utilised india ink injections on staged quail hearts it has been found that the distal part of the coronary artery is situated initially not far away from the base of the heart and later (as development proceeds) it "moves" towards the heart's apex. This may indicate that with time the c.a. increases in length and its distal part is approaching the heart apex [22].

The mechanism of c.a. increase in length is unknown, but based on our graphical patterns at different developmental stages we can speculate that the elongation of the coronary artery seems to proceed by adding the tube-like or blood-island-like structures to the distal part of the forming vessel. Considering our statistical results of a rapid increase in the length of the vessel we can exclude the notion that the growth of the c.a. proceeds by a simple elongation of the distal end. We suggest the mechanism of end-to-end tube coalescing for coronary artery formation in embryogenesis. This mechanism based on studies utilising retroviral tagging was also proposed by Mikawa & Fischman [15], and Mikawa & Gourdie [16].

On ED15 a coronary artery precursor consists of a single layer of GSI-positive cells, while the proximal part is not yet connected to the aorta. At some

point along the course of the outflow tract the lumen of the c.a. attains its maximal diameter and in some cases at this stage the vessel wall consists of two cell layers. The largest lumen diameter is found below the level of the aortic valves. Observations made by Waldo et al. [26] using india ink injections into the chicken heart have suggested that the proximal part of the c.a. close to the aortic sinus has a narrow lumen. These authors, however, have not presented any measurement data on this topic.

According to our previous data [18], the second layer of cells (the embryonic media) appears at the time when primordial endothelial cells are approaching the aorta. These results would indicate a discontinuous way of embryonic media formation during the first stages of the c.a. development. Once a wide lumenised connection with the aortic sinus has been established, the decreasing proximo-distal gradient of smooth muscle media coating is clearly distinguished [11,18,22]. Thus, the largest lumen diameter and the start of the media formation occur at a certain distance from the aortic orifice.

Based on the mathematical criteria and morpho-histochemical analysis we can state that the increase in luminal diameter of the proximal c.a. is initially slow (ED15–18), and subsequently becomes rapid (ED18–21), in other words it proceeds according to a power function. The time course for the differentiation of the proximal part occurs from ED15 to ED18. Our observations and literature reports indicate that this period is characterised by a marked remodelling of the proximal c.a. in order to form a single lumen and develop an aortic orifice. The process of remodelling also involves the peritruncal connections: some branches (slit-like channels) from the peritruncal plexus disappear and eventually only two develop into the coronary artery stems [17,23]. Thus, while the remodelling takes place, the diameter of the proximal c.a. does not increase.

At the time of the c.a. formation the crown-rump length, the heart length, and the c.a. length increase linearly. The body weight of the embryos during the prenatal life proceeds according to a power function [25], whereas during the stages of coronary artery formation (the second half of the embryonic life) the crown-rump length increases linearly.

As the analysis of the graphical models and the serial sections immunostained with the GSI lectin indicates, the stages of c.a. maturation differ along certain points of the forming vessel. In early stages (ED15–16) the lumenisation of the cellular clusters and/or cords and the widening of the tube diameter



as well as the addition of a second layer of cells begin at some distance from the aortic sinuses. After this time (ED17) maturation proceeds distally (towards the apex). Further elongation (after ED16) appears to result from the addition of vascular structures to the distal end. We cannot exclude the possibility that there are also other patterns of ramification within the coronary system. The c.a. starting from one vessel ramifies to sister vessels towards the apex, which then coalesce into one tube again. This pattern of arborisation may persist to the end of the prenatal life [20].

Our data indicate that the shape of the c.a. lumen is not changed at areas where ramifications occur and is not the result of a fixation artefact. Thus, the presented shape of the c.a. lumen may reflect the pattern of its embryonic development. Since the c.a. consists of bulgy tubes, we can speculate that these tubes are added to the distal end of c.a. while development proceeds.

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