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Research article

# Retrograde dye perfusion of the proximal aorta – A postmortem technical study

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## HIGHLIGHTS

- Retrograde dye perfusion facilitates functional analysis of aortic valve competency.
- Retrograde dye perfusion visualizes vasa vasorum of the aorta and pulmonary artery.
- Vasa vasorum of the pulmonary artery derive from conal coronary branches in humans.

## ARTICLE INFO

*Keywords:*
Methylene blue perfusion
Aortic valve competency
Coronary perfusion
Vasa vasorum
Optical clearing
Sudden cardiac death

## ABSTRACT

*Introduction:* Multiple cardiovascular conditions can lead to unexpected fatality, which is defined as sudden cardiac death. One of these potentially underlying conditions is aortic regurgitation, which can be caused by discrete changes of the geometry of the proximal aorta. To analyze aortic valve competency and furthermore to elucidate underlying pathological alterations of the coronary arteries and the vasa vasorum a perfusion method to simulate a diastolic state was designed.

*Material and methods:* A postmortem approach with retrograde perfusion of the ascending aorta with methylene blue was applied to three bodies. The procedure comprised cannulation of the brachiocephalic trunk, clamping of the aortic arch between brachiocephalic trunk and left carotid artery, infusion of 250 ml of methylene blue, and optical clearing of the superficial tissue layers after perfusion. Organs were examined directly following perfusion and after optical clearing.

*Results:* Assessment and visualization of aortic valve competency and the vasa vasorum were possible in all three instances. Visualization of the coronary perfusion was impaired by postmortem thrombus formation. Optical clearing did not provide additional information.

*Discussion:* The method presented here is a time- and cost-efficient way of visualizing aortic valve competency and the vasa vasorum. The visualization of the vasa vasorum highlights the potential of this method in basic research on diseases of the great arteries and coronaries. However, for a time-efficient functional analysis of the coronaries, other methods must be applied.

## 1. Introduction

One of the central tasks in legal medicine is to elucidate the cause of unexplained and sudden deaths [1]. When evaluating such cases, investigators must bear in mind that, under its current definition, the term *sudden cardiac death* is a rather broad category (see recent clinical guidelines on this topic [2, 3]). Indeed, various pathologies can lead to sudden cardiac death. In this context, clarification of the underlying condition leading to fatality is crucial as it provides exact epidemiological data and might even have some "preventive" benefit, if a genetic condition is involved and next-of-kin can be informed of this. Additionally, identifying the underlying cardiac condition can confirm the diagnosis of sudden cardiac death, ensuring diagnostic accuracy as required in the medico-legal field. This underlines why recent guidelines recommend

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autopsy in cases suspected of suffering sudden cardiac death [3]. An autopsy can be beneficial because some cardiovascular conditions are accompanied by clear gross sectional findings (e.g., hypertrophic cardiomyopathy [4]), while other conditions can hardly be recognized without further analysis (e.g., acute myocardial infarction without occlusion of the coronaries [5]).

### 1.1. Aortic regurgitation

One of the entities potentially associated with minor gross sectional changes is aortic regurgitation. Only some causes of aortic regurgitation can be identified macroscopically (e.g., perforation [6]). Aortic regurgitation appears to be a common condition, being found in up to 13% of males and 8.5% of females included in the Framingham Heart Studys [7]. Conservatively treated aortic regurgitation is associated with a 10-year mortality rate of up to 34% [8], making it a considerable cause of sudden cardiac death. To diagnose aortic regurgitation in a clinical setting, functional analysis, for example, by echocardiography, is recommended [9]. Such functional analysis is necessary given the complex functional anatomy and geometry of the aortic root and valve, as they significantly contribute to valve competency [10, 11, 12]. For example, changes in the sino-tubular junction [13, 14], basal ring [15], or aorto-ventricular junction [15] can lead to aortic regurgitation. Additionally, the aortic root undergoes geometric changes during the cardiac cycle [11, 12, 14]. Therefore, aortic regurgitation caused by changes of root and/or valve geometry can hardly be seen during routine autopsy, especially if the aortic root is longitudinally opened. In addition, the scarcity of clinical data concerning aortic regurgitation poses an additional challenge on the postmortem examination in medico-legal casework. According to a systematic literature search (for details, see S.1), no technical study on how to diagnose aortic regurgitation caused by geometric changes of the aortic root and valve has yet been reported. According to the linkage between valve function and the sino-tubular junction [13, 14], the ascending aorta must be included in such a technical study. Given the background of aortic valve function, a perfusion method should be appropriate to examine aortic valve competency. To incorporate the ascending aorta, the brachiocephalic trunk appears to be a suitable cannulation site for such an approach barely affecting the ascending aorta.

### 1.2. Vasa vasorum

Vasa vasorum are small nutritive vessels that are crucial for maintaining the vessel wall structure [16], with potential involvement in pathologies, e. g. in atherosclerosis [16]. Both structural changes [17] and hypoperfusion [18] of vasa vasorum have been linked to vascular disease. For example, spasms of the vasa vasorum are associated with degeneration of the arterial wall following vasospasm consecutive to a subarachnoid hemorrhage [19]. These vessels are the basis of the integrity of structures relevant to the preservation of aortic valve competency. The vasa vasorum of the proximal aorta originate from the brachiocephalic trunk and the coronary arteries [20]. Studies on the vasa vasorum have been performed either on animal models (e.g., [21, 22]) or in human studies with a very limited case number (e.g., [17]), with few exceptions (e.g., [23]). A method applied to analyze the aortic valve and root that additionally allows visualization of the vasa vasorum would facilitate evaluation of the geometric changes of the aortic valve and root in conjunction with their nutritive supply.

### 1.3. Coronary arteries

In the field of cardiac death, (forensic) pathologists are regularly confronted with cases of ischemic heart disease, with it being one of the most common causes of death globally [24]. The coronary arteries' vasa vasorum have been linked to coronary heart disease. For example, rupture of these small vessels has been identified as a potential cause of acute myocardial infarction [25]. Some authors have also asserted that the vasa vasorum are pivotal in atherosclerosis [26]. The coronary arteries' vasa vasorum directly originate from their lumen [22]. It has also been demonstrated in an animal model that the coronaries give rise to the vasa vasorum supplying the pulmonary trunk [21] as well. In the past, the vasculature of the heart was investigated using dye perfusion (e.g., [27]). With the coronaries originating from the aortic root [10], dye perfusion applied to assess aortic root and valve would lead to run-off into the coronaries. Therefore, such an approach based on dye perfusion would also have the potential to provide further information about the coronaries and the vasa vasorum originating from these.

### 1.4. Dye perfusion

Retrograde dye perfusion of the ascending aorta would have the potential to (1) analyze the aortic valve by approximating the diastolic state of the proximal aorta, (2) evaluate coronary perfusion, and (3) visualize the vasa vasorum.

### 1.5. Optical clearing

Regarding the microcirculation (i.e., smaller branches of the vasa vasorum and small coronary branches), dye perfusion is unlikely to

**Table 1.** Donor characteristics and gross sectional findings.

| | Case 1 | Case 2 | Case 3 |
|---|---|---|---|
| Information on death certificate | Sepsis, pneumonia, acute myelogenous leukemia, pancytopenia | Suspected pulmonary embolism | Stroke, arterial hypertension, peripheral artery disease |
| *Gross sectional findings* | | | |
| Aorta | Pale unstained area at the cranial anterior and concave aspect of the ascending aorta visible after organ excision. | Transmural aortic blue staining. | Stain partially removed by clearing solution over time. Initially, transmural blue-stained aortic wall. |
| Aortic valve and left-sided cavities | Scattered blue staining of the left ventricular endocardium. Native color of the left atrium; ring and cusp calcifications as well as sclerosis. | Extensive blue staining of the left ventricular and atrial endocardium; restricted and ridge leaflets. | Native left ventricular and atrial endocardium. Aortic valve inconspicuous. |
| Coronaries and myocardium | Sclerotic, mild stenosis. | Proximal stent in the left anterior descending artery. | Sclerosis with scattered calcifications and repeated stenoses (3-vessel disease). |
| | Not wall-adhesive, crumbly, and loose thrombi in the proximal sections of all 3 main coronary arteries. | Not wall-adhesive, crumbly, and loose thrombi in the proximal sections of all 3 main coronary arteries. | Not wall-adhesive, crumbly, and loose thrombi in the proximal sections of all 3 main coronary arteries. |
| | By perfusion, areas that were blue-stained were reached, otherwise native color of the myocardium. | By perfusion, areas that were blue-stained were reached, otherwise native color of the myocardium. | Minimal extravasation of the methylene blue into the myocardium. |

provide additional information by itself. Optical clearing is a method that can be used to improve the visibility of small structures, usually in small samples (e. g. [28]). The use of xylene for optical clearing is well established [29], but its toxicity has led to alternatives being deployed [30]. For larger samples, such as heart and ascending aorta, little is known about the benefits of partial, superficial translucence. Besides xylene, a clearing solution made by Saarland University Medical Center's hospital pharmacy is already established at our campus. Thus, the present study aimed to compare these two established clearing solutions. Details on the clearing solutions can be found in supplement S.2.

### 1.6. Study objective

This technical study performed during the autopsy of three body donors aimed to analyze the diagnostic potential of retrograde dye perfusion (methylene blue) of the ascending aorta followed by optical clearing. This work was intended to answer the following questions: Does retrograde dye perfusion of the ascending aorta (A) allow functional analysis of the aortic valve and root, (B) provide additional findings regarding the coronaries, and (C) visualize the vasa vasorum? (D) Does optical clearing add further information following retrograde dye perfusion? (E) Are there differences between different available clearing agents?

## 2. Materials and methods

Further background information on study conceptualization and design is summarized in supplement S.3.

### 2.1. Ethical statement

The presented study is in compliance with the local rules of professional conduct (i.e., §15 "Berufsordnung für die Ärztinnen und Ärzte des Saarlandes in der Fassung des Beschlusses der *Vertreterversammlung vom* 10. April 2019"). Additionally, the presented study was approved by the local ethical committee (approval number 162/20; "Ständige Ethikkommission der Ärztekammer des Saarlandes", Faktoreistraße 4, 66111 Saarbrücken, Saarland, Germany). When alive, the body donors gave written informed consent to the postmortem utilization of their bodies for research and education.

### 2.2. Body donors

Experiments were performed on the organs of three body donors (median age 84 years; case 1: 79-year-old male; case 2: 84-year-old male; case 3: 92-year-old female). The time from death to fixation ranged between 48 and 96 h (case 1: 96 h, case 2: 48 h, case 3: 96 h). Information on the death certificates is summarized in Table 1. Before the experiment, the bodies were fixed using a nitrate-ethanol-pickling salt solution (for details, see supplement S.4).

### 2.3. Perfusion and obtaining organ samples

For the retrograde dye perfusion, a cannula (16 FR; DLP® Medtronic®, Dublin, Ireland United Kingdom) was placed in the brachiocephalic trunk (Figure 1 A and B). For details on the preparation of the ascending aorta and the supra-aortic branches, see supplement S.5. After fixation of the cannula with double ligation, 250 ml of 2% methylene blue solution was injected rapidly using a syringe. The injection was performed as rapidly as possible and took approximately 15–25 s per cadaver. A visual check was performed to confirm that ballooning of the proximal aorta was achieved, resulting in an upright position for at least the duration of the perfusion (Figure 1 B). As the aorta started collapsing, clamps, ligation, and cannula were removed followed by excision of the heart. The time from skin incision to extraction of the organ was measured.

The extracted organs were inspected and longitudinally incised to determine how far the perfusion reached (Figure 2 A to G) and to assess the perfusion of the trans- (Figure 2 A and C) and endomural (Figure 2 B)

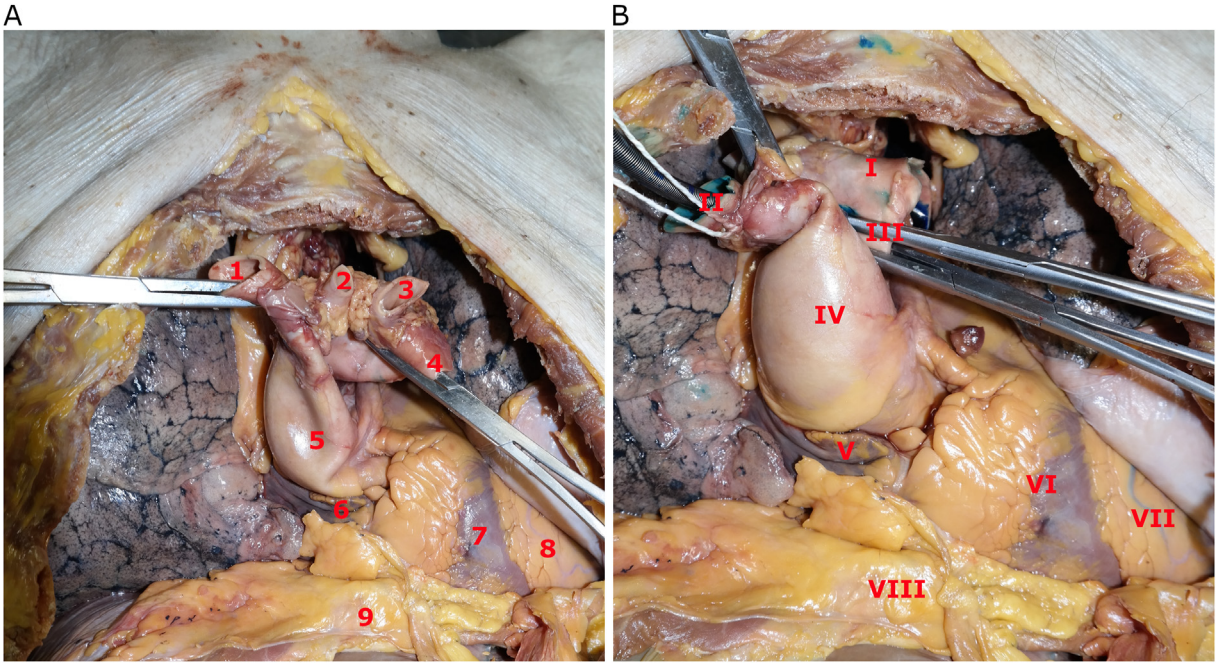


**Figure 1.** Cannulation and retrograde perfusion *(A):* Thoracic situs after preparation of the ascending aorta and the supra-aortic branches before perfusion. (1) Clamp placed at the brachiocephalic trunk. (2) Left carotid artery. (3) Left subclavian artery. (4) Clamp in the thoracic and descending aorta and the distal aortic arch. (5) Collapsed ascending aorta. (6) Right atrial appendage. (7) Right ventricle. (8) Left anterior descending. (9) Diaphragm. *(B):* Thoracic situs during retrograde dye perfusion of the ascending aorta. (I) Thoracic descending aorta. (II) Cannula placed in the brachiocephalic trunk fixed with double ligation. (III) Two clamps were placed between the brachiocephalic trunk and the left carotid artery to ensure appropriate occlusion. (IV) Ballooned ascending aorta in an upright position in an approximated "diastolic state". (V) Right atrial appendage. (VI) Right ventricle. (VII) Left anterior descending. (VIII) Diaphragm. (Image editing: Gimp Version 2.10.32).

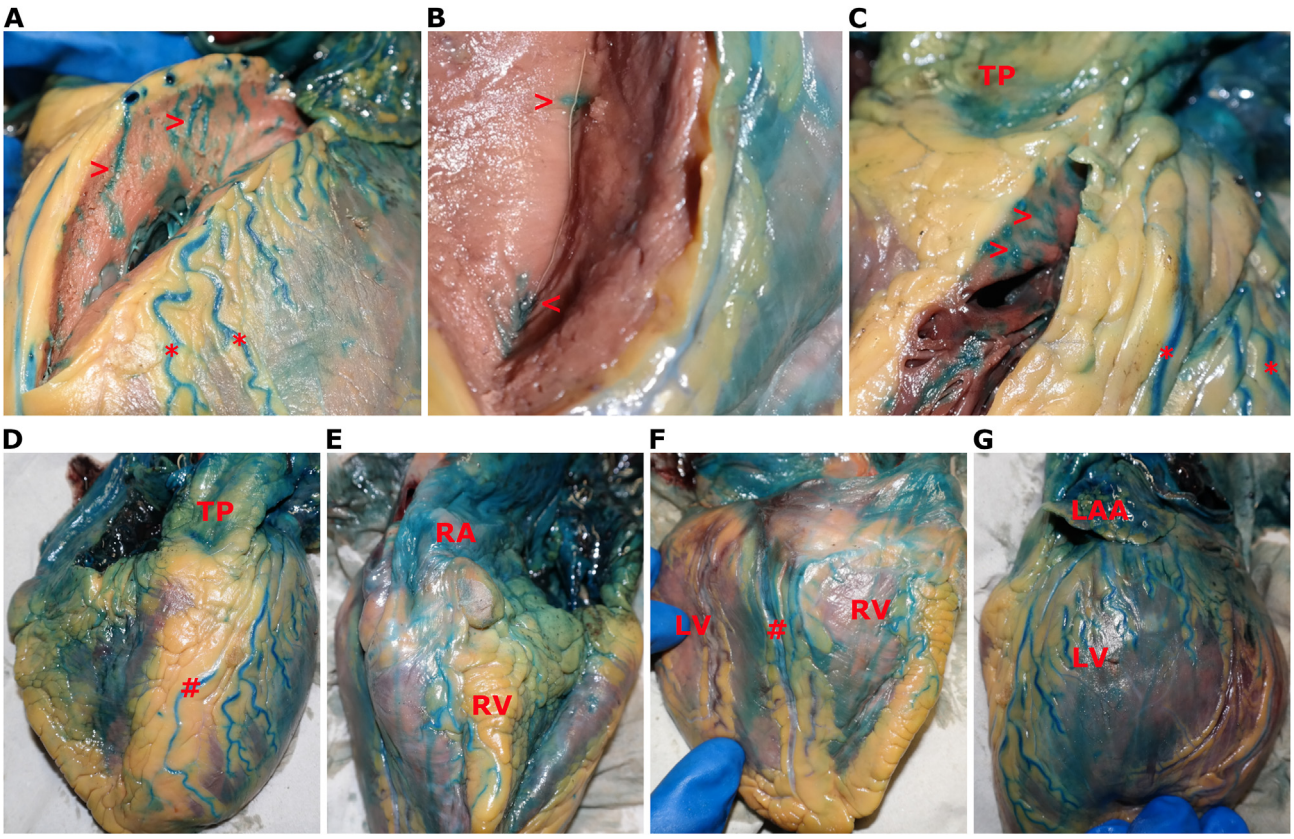


**Figure 2.** Coronary arteries after perfusion (A) Longitudinal incision into the anterior left ventricular wall with stained transmural coronary branches (>). Also, smaller epicardial branches exhibit blue staining (*). (B) Longitudinal incision into the posterior wall of the left ventricle with displayed endomural vessels (>). (C) Longitudinal incision into the anterior aspect of the right ventricle adjacent to the truncus pulmonalis (TP) showing stained transmural coronary branches (>). Additionally, epicardial coronary branches present blue stained (*). (D) Anterior aspect of the heart (TP – truncus pulmonalis, # - left anterior descending). (E) Right aspect of the heart (RA – right atrium, RV – right ventricle). (F) Posterior aspect of the heart (LV – left ventricle, RV – right ventricle, # - posterior descending artery). (G) Left aspect of the heart (LAA – left atrial appendage, LV – left ventricle). (Image editing: Gimp Version 2.10.32).

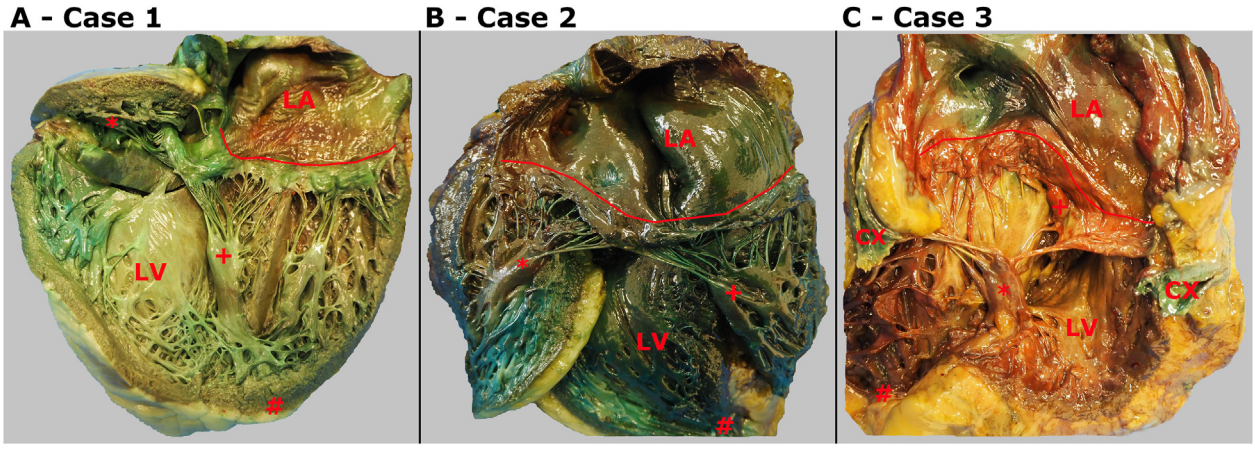


**Figure 3.** Blue staining of left-sided endocardium.
Displayed is the endocardium of the left atrium (LA) and the left ventricle (LV) for case 1 (A), case 2 (B) and case 3 (C). The insertion line of the mitral valve is highlighted by the red line. Marked are the anterior papillary muscle (*), the posterior papillary muscle (+) and the apex (#). In case 3 (C) additionally the blue stained circumflex artery (CX) is highlighted. Case 1 (A): Scattered blue-stained endocardium of the left ventricle following methylene blue perfusion without staining of the left atrium as an expression of aortic regurgitation. Case 2 (B): Extensive blue stain of left ventricular and left atrial endocardium following the perfusion as an expression of aortic regurgitation and potentially secondary mitral regurgitation. Case 3 (C): Unstained left ventricular endocardium following perfusion. (Image editing: Gimp Version 2.10.32).

vascular systems. Additionally, the heart size was compared to the respective body donors' fist to get an idea of the heart size in relation to the body. Although heart weight is usually used for this purpose [31, 32], this measure is not appropriate for the present study for three reasons. First, the organ samples comprised the complete ascending aorta, parts of the aortic arch, and the brachiocephalic trunk. Thus, the weight of this organ sample hardly resembles the “heart weight” as used in (forensic) pathologists’ routine casework. Second, owing to the pending further

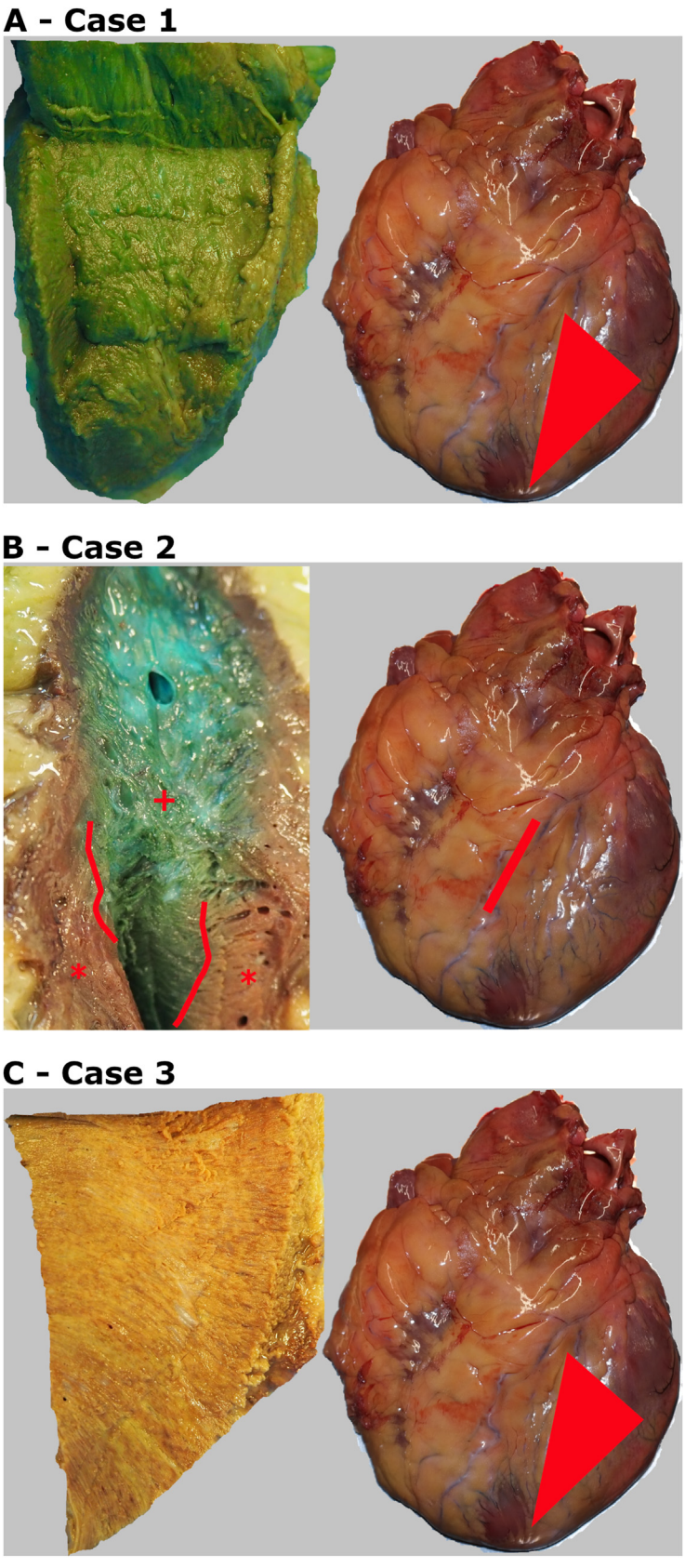


**Figure 4.** Myocardium after optical clearing.
For each case, a section through the myocardium (left) and the respective section plane (right) are displayed. Case 1 (A): Transmural blue-stained left ventricular anterior wall displayed in a laminating section following xylene treatment. The section plane is approximated by the right triangle in the scheme. Case 2 (B): Longitudinal section through the muscular interventricular septum (top – basal, bottom – apical). The section plane is shown in the scheme. Displayed is the border line (right line) between blue stained (+) and unstained myocardium (*). Case 3 (C): Unstained myocardium of the left ventricular anterior wall shown in a laminating section following treatment with the clearing solution. The section plane is approximated by the right triangle in the scheme. (Image editing: Gimp Version 2.10.32).

processing of the organ samples, clots within the heart could not be removed. As a result, the weight of these samples can hardly be assumed to be reliable. Third, it is not known how the different clearing solutions affect organ weight, for example, by accumulating in the tissue or dissolving epicardial fat. Therefore, no organ weights were measured after the optical clearing procedure.

### 2.4. Optical clearing

Two organs were placed in xylene (cases 1 and 2; isomeric mixture, concentration at least 98%; VWR Chemicals, Radnor, PA, USA) and one organ was placed in the clearing solution provided by the hospital pharmacy (case 3; for details, see supplement S.2). Solutions were changed at 9-day intervals. The organs were placed in the solutions until translucence of the superficial epicardial layers was observed in repeated checks at 3-day intervals. These checks comprised both visual and palpable inspections.

### 2.5. Further processing

As superficial translucence was achieved, organ autopsy was performed. This involved all four cavities, the three main branches of the coronary arteries, and myocardial structure being examined. The hearts and coronaries were opened following the bloodstream. Subsequently, blood clots were removed, and the surfaces were cleaned using water. In this study, coronary stenosis was defined as severe if the constriction could no longer be transited by scissors while the lumen was still visible. Mild stenosis was defined if there was narrowing of the lumen visible but with the vessel still being easily transited by scissors at the same time. A status between these two was defined as moderate stenosis. The term sclerosis was used if the tissue was markedly hardened and appeared thickened. Calcification was defined if the tissue was hardened due to the deposition of calcium salts.

Samples for histological work-up (see supplement S.6 for details) were obtained. Owing to the focus of this study being on the technical aspects of retrograde dye perfusion, histological analysis of unstained and hematoxylin-eosin-stained tissues was performed. For this, tissue was dehydrated and then embedded in paraffin. Subsequently, 5-µm-thick sections were obtained by microtomy. The unstained tissue was screened for remnant blue stain following the tissue processing. Upon examining the hematoxylin-eosin-stained slides, orienting and abridged histological assessment was performed. With regard to the great arteries, the analysis was simply aimed at differentiating whether severe and excessive degeneration was present. The analysis was performed with reference to the consensus statements on the surgical pathology of the aorta [33, 34]. The pulmonary trunk was similarly assessed. For the myocardium, the analysis was aimed at determining whether signs of cardiomyocyte death or damage were present. Here, nucleus loss, hypereosinophilia, contraction band necrosis, and infiltrates of granulocytes [5] were rated as confirmatory findings. Likewise, the presence of extensive fibrosis (i.e., affecting more than 50% of the examined area [35], with no further differentiation of interstitial and replacement fibrosis) was assessed. Further details regarding tissue processing and microscopy are provided in supplement S.7.

## 3. Results

The time from skin incision to excision of the heart ranged between 20 and 30 min. A learning curve was seen, with the procedure being performed faster from one donor to the next (case 1: 30 min, case 2: 25 min, case 3: 20 min).

Immediately after perfusion, the vasa vasorum were assessed. In all three instances, the vasa vasorum on the aorta and truncus pulmonalis showed blue staining. The vasa vasorum on the pulmonary trunk originated from the conal coronary arteries. Vasa vasorum of the coronaries were not visible.

In two of the examined cases, the heart size was approximately the same size as the fist of the respective body donor (cases 1 and 3). In case 2, the heart was approximately 50% larger than the body donor's fist.

During excision of the organs in cases 1 and 3, no further pathological findings were obtained. In case 2, a ventriculo-peritoneal shunt was found.

Translucence of the superficial layer was achieved in all three instances on day 27 after two changes of the clearing agent. In all three cases, small and branching non-perfused epicardial vessels became visible. The effect was particularly remarkable in areas where the clearing agents removed the epicardial fat.

The results of the macroscopic analysis after optical clearing are summarized in Table 1. The results are presented in Figure 2 for the coronaries (Figure 2 A and C: transmural system, B: endomural system, D to G: larger subepicardial branches), in Figure 3 (Figure 3 A: case 1, B: case 2, C: case 3) for the aortic valve competency, in Figure 4 (Figure 4 A: case 1, B: case 2, C: case 3) for the myocardium, and in Figure 5 for the vasa vasorum (Figure 5 A: truncus pulmonalis, B and C: ascending aorta).

Figure 6 shows representative images of the histological analysis (Figure 6 A: hematoxylin-eosin stain, B to D native tissue). In all three cases, no blue staining was retained by the tissue following the processing for microtomy. None of the cases exhibited signs of acute cardiomyocyte death or damage, excessive fibrosis, or excessive degeneration of the arterial wall.

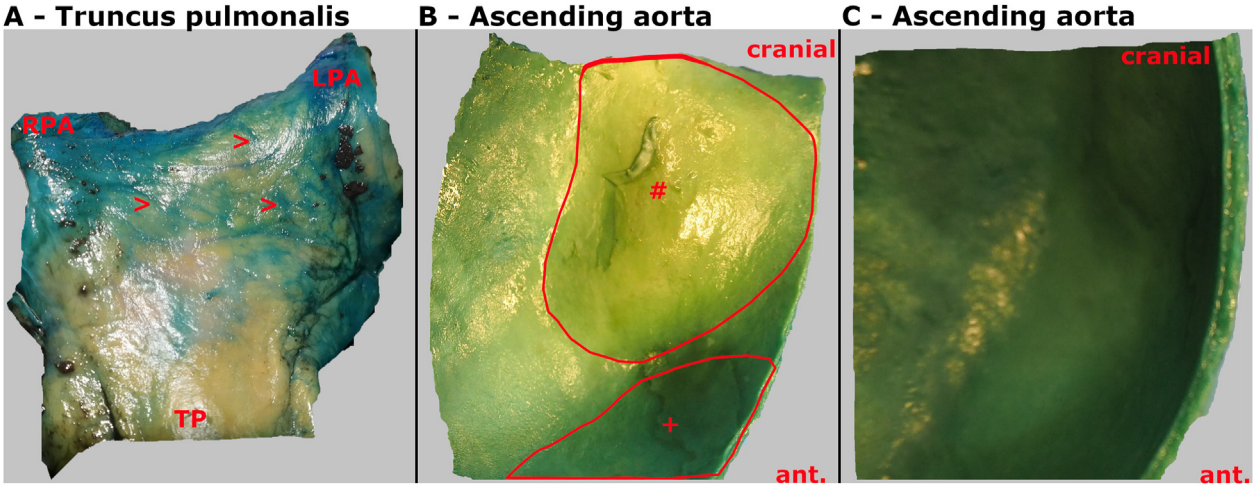


**Figure 5.** Vasa vasorum and great arteries. (A) *Truncus pulmonalis* (TP) with its bifurcation into the right pulmonary artery (RPA) and the left pulmonary artery (LPA) with blue-stained vasa vasorum (>). (B) Longitudinally opened ascending aorta with an unstained area at the anterior (ant.) and cranial aspect adjacent to the brachiocephalic trunk (#). Adjacent blue strained aortic wall (+). (C) Detailed picture of the longitudinally opened and transmural stained aortic wall in the anterior mid-ascending aortic part (right – anterior, top – cranial).

The clearing solution removed staining from the aortic intima and decreased its intensity in other parts of the organs. Xylene preserved the staining. Over time, xylene induced extravasation of the methylene blue into the perfused areas of the myocardium. The clearing solution caused minimal extravasation of the dye into the myocardium (Figure 4 C). During the repeated checks, the tissues placed in the clearing solution – especially the walls of the large arteries – lost their elasticity and became somewhat rigid. At the time of the final inspection, the tissue placed in the clearing solution appeared partially brittle for example, folding of the ascending aorta resulted in small crack-like lesions of the tunica adventitia. The coronaries did exhibit less change than the aorta and pulmonary trunk. Xylene treatment softened the tissue without causing marked organ vulnerability during preparation.

## 4. Discussion

Autopsy is a well-standardized and -structured procedure [36]. Nevertheless, reliable detection of discrete but potentially important pathologies requires vast experience and may even then be difficult. Autopsy also does not allow functional analyses of the different structures. An "augmented" autopsy (in the sense of an improved methodological approach) may facilitate the detection of discrete changes and functional impairment of structures. Thus, the aim of this study was to analyze structures depending on or influencing the flow in the proximal aorta, namely, the aortic valve competency, the coronary perfusion, and the vasa vasorum. Furthermore, the study was intended to examine postmortem retrograde dye perfusion of the ascending aorta as a method. Additionally, the value of optical clearing by different agents as subsequent treatment was assessed. The method presented here using methylene blue was fast (Table 1) and cost-efficient. Regarding its practical performance, "spill--over" of the chromogen during the extraction of the heart and aorta occurred. Hence, we would recommend performing the perfusion after preparation of the upper abdomen and lungs has been completed.

### 4.1. Left-sided heart valves

Left ventricular dilatation without any other discernible cause may suggest preexisting aortic regurgitation due to changes of the aortic root. However, pathognomonic findings supporting this diagnosis can hardly be found during routine autopsy. As such, to date, the diagnosis has had to be supported by antemortem data of functional testing, such as echocardiography and analogous examinations. Such background information is scarce in daily routine casework. The available records on the medical history of the decedent sometimes do not provide much information. It must thus be considered that the information provided on the death certificate is likely to be at least partially incorrect, as has been reported in Germany [37]. In the current study, only limited information on the cardiovascular conditions of the analyzed individuals was given on the death certificate (arterial hypertension in case 3, see Table 1 for comparison). The information on the stent in the left anterior descending in case 2 was not yielded by the death certificate. Therefore, the perfusion scenario aims to simulate the "diastolic state" of the aortic valve, root, and the ascending aorta, although it only approximates the "real diastole" in a living individual. To achieve this, perfusion-caused ballooning inducing an upright position of the ascending aorta is crucial.

In case 2, aortic regurgitation with a dilated left ventricle was identified. Additionally, in this case, the endocardium of the left atrium showed blue staining, while the mitral valve apparatus showed no gross sectionally perceptible hint of mitral valve disease, such as rupture of the tendon chords [38]. Therefore, secondary mitral valve regurgitation might be a potential explanation. This would mean that the retrograde dye perfusion worked somewhat like a water test and was established as a tool to estimate mitral valve competence during mitral valve surgery [39]. Here, water would be injected across the mitral valve into the left ventricle. However, in our case, methylene blue was injected in the proximal aorta and reached the left ventricular cavity, crossing the aortic valve instead the mitral valve. Regarding the linkage between ventricular geometry and mitral valve competence [40, 41], it is unclear whether the regurgitant

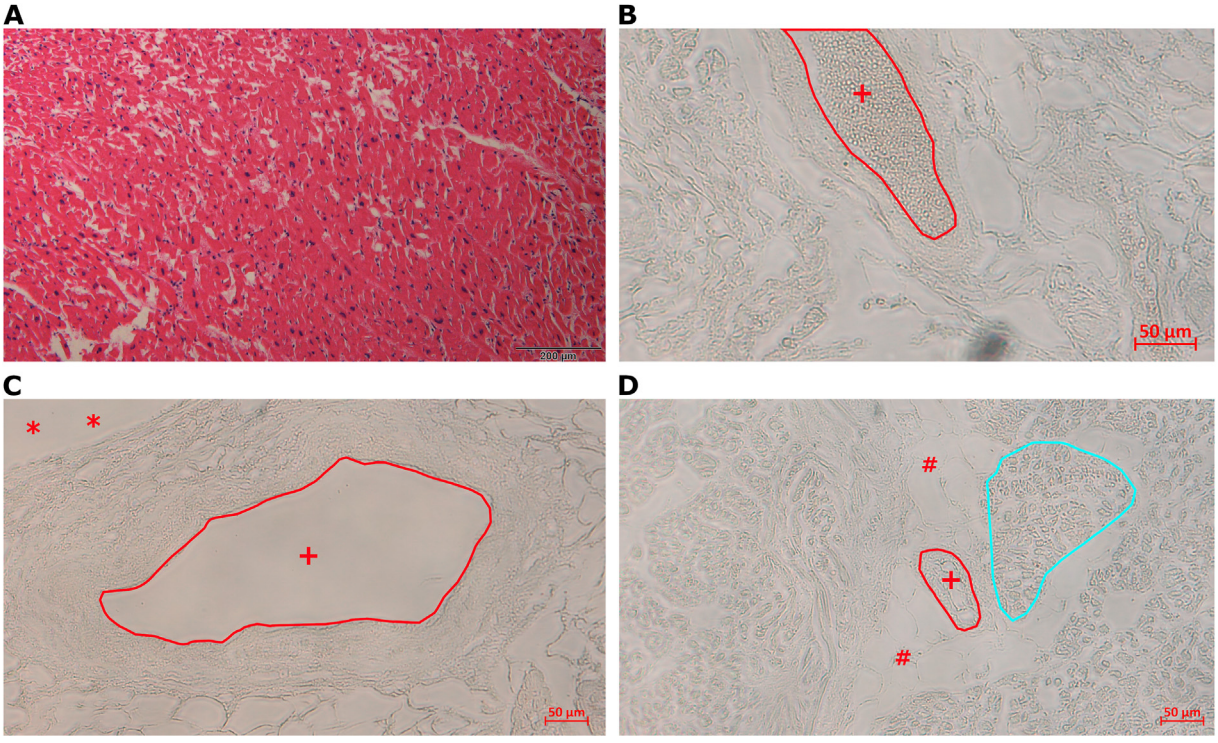


**Figure 6.** Histology (A) Representative area of the myocardium in all three hearts in hematoxylin-eosin staining. (B) Native tunica adventitia of the ascending aorta with unstained vas vasorum. The tunica intima of the vessel is marked with a red line (+- lumen). (C) Native section through a subepicardial branch of the coronary arteries without staining (red line – tunica intima, + - lumen, * - outside). (D) Native myocardium with no blue staining (red line – small vessel, + - lumen of the intramural vessel, # - fatty tissue, blue line – cardiomyocyte bundle).

volume of the dye fully resembles a water test. Besides, mitral regurgitation physiological shunts would be a possible explanation for the observed left atrial blue stain. In this context, the Thebesian veins have to be taken into consideration [42, 43, 44], as it has been shown that they can cause a shunt to the left ventricle [43]. This physiological shunt has been shown to range between 0.12% and 0.43% of aortic flow [43]. Therefore, for the present study with an overall aortic flow volume of 250 ml, this would result in a total shunt volume of approximately 1 ml. Thus, physiological shunts are a potential explanation of the left atrial blue staining, although the observed stain appears unlikely to be explained by 1 ml of the dye. This would again favor mitral regurgitation as an explanation of the left atrial stain. In summary, the methylene blue perfusion supports the autoptically motivated assumption of aortic regurgitation derived from the ventricular dilatation observed. Additionally, hints of a likely secondary mitral valve regurgitation are provided. However, the method does not allow verification of the mitral regurgitation.

### 4.2. Coronary arteries

It can be challenging to identify myocardial infarction by autopsy alone [5]. Therefore, extensive sampling for histological analysis is sometimes crucial to correctly identify the pathology [5]. In the three cases presented here, the proximal parts of all main branches of the coronary arteries were sufficiently reached by the applied methylene blue. More distal parts of the arteries were occluded by non-adhesive and crumbly thrombi, which were potentially an artifact of the retrograde perfusion of the body for fixation. Nevertheless, the transmural (left and right ventricle) and endomural systems (left ventricle) were displayed (Figure 4). Over time, the perfused parts of the myocardium developed blue staining during treatment with xylene (Figure 4 A and B), whereas non-perfused parts remained unstained (Figure 4 C). These unstained areas corresponded to the encountered thrombi.

The hematoxylin-eosin staining of the specimens obtained in these unstained areas did not yield signs of acute cardiomyocyte death or damage. Thus, the impaired perfusion is likely to be an artifact of likely postmortem clots abducted to more distal parts of the coronary arteries. This is opposite to the finding that the coronaries were passable by scissors.

Regarding the practicability of this method to search for hypoperfused areas in a common autoptic setting, it should be noted that the time until the myocardial staining procedure was completed is rather long. Thus, faster techniques such as fluorescence angiography [45] appear to be more promising for functional analysis of the coronary arteries and cardiac microcirculation.

Besides such perfusion-based techniques, imaging techniques to assess the heart and coronaries are available. Among others, magnetic resonance imaging [46], postmortem computed tomography-angiography [47], and optical coherence tomography [48] are available. The literature suggests that these methods [46, 47, 48], especially in combination [49, 50], allow much better visualization and assessment of the coronaries than retrograde dye perfusion. Additionally, optical coherence tomography appears to be a powerful tool for analyzing the vasa vasorum [51].

At the end of life, agonal [52] and postmortem thrombi [53] form. This can impede the diagnosis of acute coronary occlusion during autopsy. Like the method presented here, postmortem computed tomography and angiography also struggle with these phenomena [54]. They are difficult to address prior to both investigation types, namely, retrograde dye perfusion and postmortem computed tomography, as there is usually a delay between agony, death, and autopsy in routine casework. Therefore, at the time of autopsy, postmortem computed tomography, or retrograde dye perfusion, these thrombi have already formed. It has been shown that fibrinolytic activity persists for an unknown time postmortem [55]. Nevertheless, it remains controversial how to weigh the odds of the two processes of postmortem blood clotting and fibrinolysis [50]. However, it has been demonstrated that, in anatomical organ preparations a mixture of heparin and water can be used to wash the capillary bed [56]. Although this is described elsewhere, in the present study agents like heparin were not added to the dye. This decision was made considering the postmortem interval, the duration of the fixation procedure (see supplement S.4 for comparison), and the fixation itself inducing protein cross-linking [57]. Nonetheless, it appears very unlikely that, at the time of the retrograde dye perfusion, heparin could somehow positively affect the thrombi in the vessels and heart.

### 4.3. Vasa vasorum

To the best of our knowledge, the origin of the vasa vasorum of the truncus pulmonalis has so far only been described for rabbit [21], but not for human. Using methylene blue perfusion, we were able to identify the origin of the vasa vasorum of the truncus pulmonalis and the proximal pulmonary arteries in the human body (Figure 5), namely, the conal branches of the coronary arteries. Additionally, the method presented here visualized the vasa vasorum of the aorta, providing information on the vascularization of the aortic wall (Figure 5). However, visualization of the vasa vasorum supplying the coronaries was not possible. Thus, retrograde dye perfusion could be used for research on the vascular supply of the great arteries (i.e., the proximal aorta and the truncus pulmonalis), but not for studies on the vascular supply of the coronaries.

### 4.4. Optical clearing

Some diseases affect small vessels [58] or can be caused by alterations of small vessels [18]. Therefore, we included optical clearing procedures to obtain deeper insights into the respective small vessel systems of the great arteries and myocardium. Here, xylene performed better regarding the preservation of dye perfusion results and tissue properties. Additionally, xylene facilitated extravasation of the methylene blue into the perfused parts of the myocardium (Figure 4). The use of the clearing solution did not provide this additional information (Figure 4).

Given the sample size and thickness, complete translucence of the whole human heart is unlikely to be achieved. Nevertheless, translucence affected superficial layers in our samples. As pertains to the coronaries, a growing number of small, superficial, and branching vessels became visible over time, which were not reached by methylene blue. In synopsis, the application of clearing agents to induce tissue translucence only provided additional information after a rather long time and only on superficial vessels. Therefore, optical clearing like that performed in this study is not a useful adjunct to a routine autopsy. Additionally, it has to be pointed out that the small vessels of particular relevance in cardiac disease are the small vessels within the myocardium (e.g., shown in hypertrophic cardiomyopathy [35]). Therefore, optical clearing like that applied in this study is not only time-consuming but also does not reach the structures of interest for both agents applied.

The clearing solution did cause tissue hardening and fragility, while xylene smoothed the tissue more. This effect of the clearing solution was particularly pronounced at the great arteries' walls. The finding that this effect was stronger than at other tissues might have been due to its large contact surface in relation to its volume and thickness.

We were unable to identify reports providing a clearing protocol for such large organ samples. Regarding the large volumes required to fully cover the samples, as well as the size and thickness of the samples, we simply decided to inspect the organs every 3 days. In addition, to test the clearing, we decided to change the solutions at every third inspection of the organs. Therefore, to develop proper protocols for at least partial superficial optical clearing of such large samples, systematic studies that methodically vary the clearing solution and its exposure time are needed. Moreover, different organ processing should be assessed in such studies. For example, systematically puncturing the organ could allow for better action of the clearing solution. For smaller organ samples, it has been demonstrated that perfusion with clearing solution is a useful application mode [59]. Besides the clearing solutions assessed, a broad variety of other clearing agents are available [30, 59, 60, 61].

Comparing our results to the literature, it seems like the optical clearing would be a much better adjunct to the histological analyses than to the gross sectional analysis. If a chunk of tissue of a gross sectional suspected area is obtained, it could be cut in half. One-half of the specimen could be allocated to routine histology, while the other half could undergo optical clearing in order to prepare the tissue for three-dimensional analysis (e.g., as shown by Kim et al. [59]). This would allow both structural analysis and assessment of the three-dimensional architecture, facilitating more exact descriptions of the localization and extent of a lesion.

### 4.5. Limitations

#### 4.5.1. Study design and technical issues

The present manuscript summarizes the results of a technical study exploring a simple and cost-efficient method. Focus was applied to aortic valve competency. However, given the nature of this study, a larger series is required to verify the value of the applied techniques and to determine its significance. To assess the presented method, there is a particular need to analyze individuals with known and antemortem-assessed and quantified aortic valve regurgitation.

The assessment of aortic valve competency is based on the staining of the left ventricle caused by "regurgitation" of the dye through the aortic valve. As such, the study design does not allow quantification of the aortic regurgitation.

Although the heart remained in situ, the aortic arch was transected, and a cannula was placed in the brachiocephalic trunk. These manipulations of the arch increase the mobility of the proximal aorta during the procedure. However, owing to the course of the aorta ballooning of the aortic root not being associated with elevation of the aortic arch, geometric changes might be induced, which could again be associated with impairment of the aortic valve competency.

For this orienting study, the retrograde perfusion was performed using a syringe. This technique typically results in retrograde flow into the aortic root of approximately 750 ml per min. The method aimed to simulate the diastolic state. Diastolic backward flow, that is, from the ascending aorta in the direction of the aortic valve, has previously been measured in magnetic resonance imaging studies (e.g., [62]). This study reported diastolic backward flow volumes between 24 and 208 ml [62]. Although the presented study could not provide data on the pressure applied to the aortic valve and root, the technique appears to be quite a tough test in terms of "volume load".

Instead of this very basic technique using a syringe, other techniques for cadaver perfusion are available, as summarized by Bellier et al. [63]. Apart from using a pump, even postmortem circulation can be installed [64]. With regard to a time- and cost-efficient diagnostic tool, the use of a pump might be promising for improving the technique from the practicable point of view. In contrast, given its time-consuming nature, postmortem circulation has mainly been proposed in the context of training clinicians and testing devices [64].

#### 4.5.2. Specimen processing

Methylene blue was removed during the dehydration procedure prior to paraffin embedding of the tissue. Thus, for histological analysis of the perfused and stained vessels, cryosectioned tissue could be more suitable. In its present form, the technique does not provide additional information for the sampling of histological specimens and in the histological analysis.

#### 4.5.3. Postmortem interval and fixation of the body donors

The aortic root and valve are complex structures essential for a functioning aortic valve [10]. One of their components is the aorto-ventricular junction [15]. As this component basically comprises myocardium of the left ventricular outflow tract, it might be affected by postmortem phenomena such as rigor mortis. During organ autopsy in this study, no rigor mortis was present. Therefore, the postmortem relaxation of the myocardium might lead to "relaxation" of the aortic root, potentially resulting in "widening" of the aortic root, although this would not be detectable. Thus, the retrograde dye perfusion might detect postmortem artifacts as aortic regurgitation, but vice versa if there is no regurgitation of dye into the left ventricular cavity this reflects proper aortic root geometry and valve coaptability despite postmortem changes.

Further, computed tomographic studies revealed that the ascending aorta appeared to shrink after death [65]. As the ascending aorta in particular exhibits viscoelastic properties [66], such shrinkage is the only logical consequence of circulatory breakdown due to subsequent loss of wall tension. As the presented perfusion aimed for ballooning and thus restoration of the wall tension, this postmortem artifact is likely to be ruled out by the method. Nevertheless, it has to be taken into consideration that the aortic tissue also degenerates after death [67]. Therefore, the elastic properties of the aortic wall appear to be hardly comparable ante- and postmortem.

To avoid the described possibility of misleading postmortem widening of the aortic root compared with the antemortem dimensions, we performed fixation of the body donors. Owing to the perfusion, the fixation solution reached the aforementioned structures of the proximal aorta. Formaldehyde induces several cross-links [57] and thus hardens the tissue with the induction of moderate shrinkage [68]. Theoretically, these effects could either negatively influence the movement of the structures relevant for aortic valve competence during the perfusion and/or somehow provide a counterbalance to the postmortem relaxation of the aortic root after loosening of the rigor mortis. However, previous studies showed, different fixation solutions are associated with different effects on the vasculature and the heart [69]. Hereby, it has been shown that formaldehyde appears to be crucial for maintaining the diameter and silhouette of the organs and vessels assessed [69]. The fixation applied to the cadavers used in this study comprised formaldehyde (see supplement S.4). Thus, it appears likely that the fixation did not severely alter the structures of the proximal aorta and the aortic valve. Overall, further studies are required to determine how postmortem phenomena and the application of fixation interfere with aortic root geometry and aortic valve competence.

### 4.6. Future perspectives

#### 4.6.1. Improvement of the technique

To address the limitations discussed, in the future a second cannula could be placed in the left ventricle (resembling the venting cannula of a heart-lung machine). This would allow approximation of the regurgitant dye volume. After systematic establishment in a postmortem setting, this could allow the quantification of aortic regurgitation in a manner analogous to echocardiography [9, 70].

#### 4.6.2. Diversification of the analysis

The present study focused on the aortic valve, while visualization of the vasa vasorum of the great arteries was also achieved. However, it is not only the cardiovascular structures that are relevant to cardiovascular disease; nervous structures, especially the autonomous nervous system, are linked with heart disease [71]. For example, it has been shown in a rabbit model that the numerical density of ganglia [72] and the arterial supply of the small vagal network [73] are crucial for cardiac survival [72] and heart rhythm maintenance [73] following subarachnoid hemorrhage [72, 73]. Thus, in further developing the method and its subsequent processing, the nerve structures around the heart, the coronaries and great arteries should also be analyzed. For example, the proximal aorta with the proximal coronaries could be obtained as samples without having to perform prior open preparation, preserving the superficial tissue containing nerve structures. Following on from previous research, the identified nerve structures could be differentiated regarding their origin (i.e., parasympathetic or sympathetic), as was already reported for the renal arteries [74]. From a translational point of view, such studies would help to obtain a better understanding of potential ablation targets for vascular denervation.

#### 4.6.3. Interdisciplinary and translational research

Targeting a disease via different disciplines leads to analyses on different levels (e.g., tissue and genetics) and provides deeper knowledge. For example, this was impressively demonstrated by Geisterfer-Lowrance and colleagues by their research on hypertrophic cardiomyopathy [75]. An interdisciplinary approach may help to increase the case number and boost research on topics such as the associations of aortic valve disease [76] or malformation [77], or end-stage heart failure with an implanted left ventricular assist device [78] and aortic wall changes. Such interdisciplinary and translational research has the potential to improve understanding of cardiovascular disease and thus sudden cardiac death. Finally, this would also allow increased safety in diagnosing sudden cardiac death and its underlying cause.

## 5. Conclusion

Understanding sudden cardiac death is an interdisciplinary challenge as well as part of routine casework in legal medicine and pathology. A variety of technologies, especially postmortem computed tomography, have been established to “enhance” autopsies. Nevertheless, methods to functionally analyze aortic valve competency are lacking so far in a postmortem setting. Retrograde dye perfusion of the proximal aorta can address this gap. Regarding the coronary arteries, the simple use of methylene blue in cadavers following fixation does not provide additional information. Methylene blue perfusion of the brachiocephalic trunk is a tool to identify large vasa vasorum of the truncus pulmonalis and the ascending aorta. We were able to demonstrate that, in humans, the vasa vasorum of the pulmonary trunk originate from the conal coronary artery branches. Visualization of the coronary vasa vasorum was not possible by retrograde dye perfusion. Dye extravasation approximating myocardial perfusion was mainly induced by xylene and did match with the coronary branches that were reached by the dye. Owing to postmortem artifacts and washing out of the staining during tissue processing, further studies are needed to determine the diagnostic power of optical clearing regarding the microcirculation of the heart and the great arteries.

## Declarations

### Author contribution statement

Jan Michael Federspiel: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Constantin Lux: Conceived and designed the experiments; Wrote the paper.

Katrin Burkhard, Mattias Kettner and Frank Ramsthaler: Analyzed and interpreted the data; Wrote the paper.

Marcel A Verhoff: Analyzed and interpreted the data.

Thomas Tschernig: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Data availability statement

Data included in article/supp. material/referenced in article.

### Declaration of interest's statement

The authors declare no competing interests.

### Additional information

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