Dissecting abdominal aortic aneurysm in Ang II-infused mice: the importance of imaging

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Abstract

Introduction. Since the initial publication in 2000, Angiotensin II-infused mice have become the model of choice to study abdominal aortic aneurysm in a pre-clinical setting. We recently used phase contrast X-ray based computed tomography to challenge the existing paradigm and demonstrated that these animals develop an apparent luminal dilatation and an intramural hematoma, both related to mural ruptures in the tunica media in the vicinity of suprarenal side branches, providing evidence that the main lesion is rather a mere dissection without previous dilation.

Aims. The aim of this narrative review was to provide an extensive overview of small animal applicable techniques that have provided relevant insight into the pathogenesis and morphology of dissecting AAA in mice, and to relate findings from these techniques to each other and to our recent PCXTM-based results. Combining insights from recent and consolidated publications we aimed to enhance our understanding of dissecting AAA morphology and anatomy.

Results and conclusions. We analyzed in vivo and ex vivo images of aortas obtained from macroscopic anatomy, histology, high-frequency ultrasound, contrast-enhanced micro-CT, micro-MRI and PCXTM. We demonstrate how in almost all publications the aorta has been subdivided into a part in which an intact lumen lies adjacent to a remodeled wall/hematoma, and a part in which elastic lamellae are ruptured and the lumen appears to be dilated. We show how the novel paradigm fits within the existing one, and how 3D images can explain and connect previously published 2D structures that had never been correctly interpreted. We conclude that PCXTM-based findings are in line with previous results, and all evidence points towards the fact that

dissecting AAAs in Angiotensin II-infused mice are actually caused by ruptures of the tunica media in the immediate vicinity of small side branches.

Keywords: abdominal aortic aneurysms, small animal imaging, dissecting aneurysm, high-frequency ultrasound, PCXTM, micro-CT, MRI, angiotensin II, mouse model

Introduction

Abdominal aortic aneurysm (AAA) is defined as a local dilation of aortic diameter that exceeds 150% of the initial size (1). Since the first paper published by Daugherty et al in 2000 (2), the Angiotensin (Ang) II-infused mouse model for AAA formation has been the golden standard for pre-clinical aneurysm research, and was used in (roughly) 200 papers studying abdominal aortic aneurysm in a preclinical setting. The model has been reported to replicate many features of human aneurysm formation, such as (amongst others) elastin degradation, luminal dilatation, and thrombus formation. Nevertheless, it has long been unknown why the murine AAA occurs suprarenally rather than infrarenally, why there is a high variability in aneurysm shape, and why the animals develop intramural rather than intraluminal thrombus. Notwithstanding the fact that most – if not all – early publications (approximately up to 2010) focused on the many similarities between human and murine AAAs, we have recently published results that challenge the existing paradigm of Ang II-induced AAAs in mice. Using a novel imaging technique called PCXTM (phase contrast X-ray computed tomography) we demonstrated that these animals in fact develop dissecting AAAs, and present an intramural hematoma due to ruptures of the aortic tunica media in the vicinity of small supraceliac side branches. Moreover, we demonstrated that the apparent primary luminal dilatation in these dissecting AAAs is actually due to the mural dissection of the tunica media in the segment proximal to the celiac artery.

At first sight, these observations contradicted a large number of previous studies, all of which have reported “true” luminal dilatation and none of which reported branch-related ruptures leading to intramural hematoma. Understanding and interpretation are however limited by how much can be directly observed on a given sample, hence also to the intrinsic properties of the imaging technique that is used. Despite the fact that preclinical imaging technologies are usually

technically much more advanced than the clinical devices, they could often not achieve sufficient temporal and spatial resolution to compensate for the increased demands on size (up to 10x smaller) and heart rate (up to 10x faster) in these small animals. Improvements in small animal imaging – be it at the macro-, micro-anatomical, physiological or molecular level – have resulted in a progressively enhanced insight into the pathophysiology underlying dissecting AAA formation. In the current review article, we aim to provide an extensive overview of the 2D and 3D techniques that have been used to visualize different aspects of dissecting AAA formation in Angiotensin II-infused mice throughout the last 15 years. We discuss results obtained from ex vivo macroscopic observations, ex vivo histopathology and immunohistochemistry, in vivo high-frequency ultrasound, in vivo contrast-enhanced micro-CT and in vivo micro-MRI. We give an overview of how dissecting AAA in these mice has been detected, characterized and interpreted, discuss advantages and disadvantages of each technique and merge observations coming from researchers with different backgrounds, professional and technical expertise.

We conclude with our recent advancements in the field using ex vivo PCXTM and PCXTM-guided histology (2D). We relate our findings to previous observations in the field, and demonstrate how the existing paradigm fits within the newly proposed one.

Methods

This narrative review is based on bibliographic data available on MEDLINE and PubMed up to December 2014. Used search terms were: “Angiotensin II, imaging, ultrasound, micro-CT, MRI and PCXTM” in combination with “abdominal aortic aneurysm, pathophysiology and mouse model”. We describe all imaging techniques that provide insight into Ang II-induced aneurysms, irrespective of the mice genotype (e.g. ApoE^{-/-}, LdL^{-/-}, or wild type). Aneurysms that were induced by combined Angiotensin II-infusion and intraperitoneal injection of anti-TGF-β or BNP were also included in the analysis. A total of 194 manuscripts was analyzed.

Results

Ex vivo macroscopic and microscopic anatomy

In their seminal paper from 2000, Daugherty *et al.* were the first to report that a bulbous aortic abdominal shape occurred in 20% and 33% of ApoE^{-/-} mice infused with 500 and 1000 ng/min/kg of Ang II, respectively (2). Already in 2001, Daugherty *et al.* classified Ang II-induced aneurysm morphology into 4 grades: Grade I was defined as a dilated lumen in the supra-renal region of the aorta with no thrombus, grade II as a remodeled tissue in the suprarenal region that frequently contains a thrombus, grade III as a pronounced bulbous form of type II that contains a thrombus, and grade IV as a form in which there are multiple aneurysms containing a thrombus, some overlapping, in the suprarenal area of the aorta (3) (Fig. 1). Fourteen years later, 134 of the 194 analyzed manuscripts have published pictures showing the gross morphology of the abdominal aortic aneurysm. As described above, aneurysm incidence is often based on macroscopic evaluation: either via a qualitative pathological evaluation of the morphology (4-18), or via a

quantitative analysis of the external diameter that is obtained from caliper measurements on either the aneurysmal tissue itself or a digitized picture of it (19-25).

Ex vivo detailed anatomy – histology

Most of the observations that have contributed to our understanding of the pathology of murine dissecting AAA formation have been made by histological or immunohistochemical evaluation of the specimens. Since 2001, 112 studies published pictures of stained sections in dissecting AAAs, and 21 of these studies were designed specifically to describe dissecting AAA formation in Angiotensin II-infused mice. In the current review, we have chosen to focus on those images in which histology added insight into the existing paradigm of anatomy and pathophysiology in Ang II-induced dissecting AAAs.

General dissecting AAA anatomy

In their first publication, Daugherty *et al.* mentioned the existence of two regions of distinct characteristics within murine dissecting AAAs (2). The proximal dissecting AAA region had an intact cross-sectional lumen area and intact elastin layers in the media, but was associated with pronounced remodeling in the adventitial layer. The distal dissecting AAA region was characterized by a complete medial rupture that resulted in marked dilation of the lumen (2) (Fig. 2a). In 2003, Saraff *et al.* made a major contribution to the field as they observed that the early phase of Ang II infusion was characterized by a dissection and consequent vascular hematoma that occurred 4-10 days after the onset of infusion (26). They confirmed that a rupture occurred in the tunica media of the distal part of the dissecting AAA, and further reported that the bleeding was constrained by adventitial tissue in most mice, although in some animals transmural arterial rupture led to hemoabdomen and sudden death. Nevertheless, the authors concluded that after 28

days of Ang II infusion, aneurysmal tissue had many features of the human disease, including luminal dilation, extracellular matrix fragmentation, leukocyte accumulation, and thrombus (26). In 2011, Rateri *et al.* serially sectioned the full length of several dissecting AAAs, and stained every 10th slice (de facto staining at 100 µm intervals) with Movat's pentachrome. They observed luminal dilatation and mural rupture of elastin fibers in the central part of the lesion, which was the only part they termed 'AAA' (27). Both proximally and distally of the dilated segment, a region with unchanged aortic lumen was observed adjacent to thickened adventitial regions that contained fibrous material and clotted blood, but was not considered part of the dissecting AAA (27) (Fig. 2b). The authors concluded that continuous infusion of Ang II into ApoE^{-/-} mice led to gradual luminal expansion of the suprarenal aorta and that studies could be designed to test effects of interventions on aneurysm progression using this experimental model.

Meanwhile, however, some authors questioned the term 'aneurysm' for a cardiovascular phenotype that does not show any consistent primary luminal dilatation. In 2007 Jiang *et al.* reported that Ang II infusion led to aneurysm-like expansive lesions that could not be classified as true aneurysms, as they were essentially encapsulated extramural thrombi (pseudoaneurysms)(28). They also reported a rupture of the tunica media in the distal part of the lesion, and postulated that the distal rupture in the tunica media may be associated with the proximal thrombus, confirming the original hypothesis by Saraff *et al.* (26). In 2012 Schriebl *et al.* performed serial histological sectioning of Ang II-induced dissecting AAAs (29). Similar to earlier reports, they observed that the central part of the dissecting AAA was characterized by completely ruptured elastic fibers and smooth muscle, while the distal and proximal ends of the dissecting AAA showed an intact true lumen adjacent to an intramural thrombus. Contrasting earlier reports, however, they also referred the existence of a third region, located just distally and

proximally to the site of the dilated lumen (Fig. 2c). This region was characterized by a separate channel that ran parallel to the intact lumen, and later on joined the latter at the central, dilated part of the dissecting AAA (29). Interestingly, a similar parallel channel was visible on published images by Rateri *et al.* (27), but not classified as such (Fig. 2b). A similar structure can also be observed (often without interpretation) on published histology pictures by other authors (11, 30-34) (Fig. 4b, 5). To discriminate murine AAAs from human AAAs (which demonstrate a true, luminal dilatation), Schriefl *et al.* used the term ‘dissecting AAA’ to describe the pathophysiology of this particular mouse model, which is also the preferred term in the current manuscript.

Ruptured tunica media leading to luminal dilatation

As the ruptured tunica media corresponds best to the luminal dilatation observed in human AAA, most reports in literature include histology images of this region. Disruption of the media has been associated with fragmentation of the elastic lamellae (2, 26, 33, 35-40), apoptosis of the smooth muscle cells (26, 29, 41-44) and infiltration of a variety of inflammatory cells (11, 19, 20, 26, 40, 44-53). The ruptured media occurs in the suprarenal part of the aorta (15, 17, 19, 26, 32, 37, 53-56), and is consistently found within the region of maximum dilatation (17, 19, 29, 37, 53, 57).

In 2012, Gavish *et al.* were the first to report that transmural disruptions of the tunica media occur throughout the entire aorta in Ang II-infused mice, with a distinct predilection for branch orifices (36). They further demonstrated that at such sites of transmural disruption the extent of macrophage infiltration was increased and an attempted repair by newly deposited collagen could be observed (36). In follow-up research from 2014, Gavish *et al.* performed serial histology near

the opening of celiac, mesenteric and both renal arteries(37). Transmedial disruptions occurred in 126 of 325 sections that included at least part of a branch orifice but, surprisingly, in none of the 479 sections without branch points. These disruptions ranged from small focal lesions to larger defects up to 1000 μm . There was no significant difference in branch-related transmedial disruptions between Ang II-infused mice that developed suprarenal dissecting AAAs and those that did not (37).

Remodeled aortic wall adjacent to intact lumen

A dilated wall adjacent to an intact tunica media has been reported by many authors (2, 18, 26, 27, 32, 33, 37, 58-63). In most publications, the dilated part was interpreted as an intramural thrombus (26-29, 32, 40, 46, 63, 64) or a remodeled adventitia (2, 31, 61, 65). Jiang *et al.* observed many infiltrating leukocytes within the thrombus after 4 weeks, indicating its organization (28). Cao *et al.* used Movat's pentachrome staining to demonstrate remodeling events of collagen within the dilated aortic wall (61). Schriebl *et al.* observed deposition of glycosaminoglycans (GAGs) and fibrillar collagen in regions devoid of fibrin, which implies a "remodeling" process, typical of hematoma resorption and organization. The authors further speculated that the youngest thrombus existed closest to the center of the lesion where the merged lumen could have continued to provide flowing blood and hence fibrinogen and platelets, whereas the oldest region of the same thrombus was abluminal hence far from the flowing blood (29).

While gross anatomy and detailed histopathology evaluations have provided substantial insight into the pathophysiology of dissecting AAA, a number of questions (such as the reason for the suprarenal location and large variation in aneurysm morphology) remained unaddressed.

Histopathology allows a morphologic evaluation, on specific 2D slices, and typically post mortem. Serial sectioning has provided some additional information (by allowing partial 3D reconstruction), but it was only with the development of a dedicated high-frequency ultrasound device (VisualSonics, 2005) that in vivo integrated functional and anatomical imaging of Ang II-induced dissecting AAA became feasible.

In vivo physiology and anatomy – high-frequency ultrasound

Since 2005, we found 56 manuscripts in which high-frequency ultrasound was used to visualize Ang II-induced dissecting AAA in vivo. Dedicated devices include the Vevo 660 (27, 55, 56, 66, 67), the Vevo 770 (36, 59, 61, 68-72) the Vevo2100 (30, 73-79) (all manufactured by VisualSonics) or the Mylab TM70 (80-83)(Esaote). Some used ultrasound to describe dissecting AAA formation *an sich* (25, 27, 30, 55, 56, 59, 61, 68, 70, 71, 76, 79, 84), while many other studies applied it to quantify lumen diameter in treatment studies (66, 67, 69, 73-75, 78, 80-82) . As was the case for histology, the current review does not claim to give a complete overview of all available images, but focuses on those published images that – in our opinion – shed new light on anatomy and pathophysiology of dissecting AAA.

In vivo dissecting AAA anatomy – BMode imaging

The first ultrasound recordings in AngII-infused mice were made using 10 MHz ultrasound probes suitable for humans (85), but the first recordings with dedicated high-frequency probes of 40 MHz was presented by Martin-McNulty *et al.* in 2005 (55) (Fig 3a). They reported that ultrasound images showed a bulge-like expansion in the suprarenal region of the abdominal aorta, with a shape that was strikingly similar to the gross picture obtained *post mortem*. Barisione *et al.* soon took advantage of this novel possibility for in vivo imaging to demonstrate that Ang II

infusion leads to a concentric luminal dilatation in the suprarenal part of the aorta (56). The dilatation increased rapidly the initial 7 days of infusion, and continued at a more modest rate for the remaining 21 days of observation. Interestingly, the authors also observed that luminal diameters increased several days before death in the Ang II-infused mice that succumbed to aortic rupture, and therefore concluded that transmural rupture was precipitated by a mechanism that occurred subsequent to the transmedial breaks that lead to luminal dilatation (56). Nevertheless, Cao *et al.* later reported that 28% of dissecting AAAs present at necropsy went undetected with ultrasound (61). In the same manuscript, Cao *et al.* conducted Power Doppler acquisitions to obtain 3D geometry parameters. In the resulting (rudimentary) 3D volume, two distinct zones (one with an intact lumen and dilated outer wall, and another with a dilated lumen) could be observed (Fig 3b) (61). Cao *et al.* also used time-averaged EKV (ECG-based kilohertz visualization) to show the *in vivo* existence of an aortic dissection “flap” at locations where the elastic media had broken down (Fig 3b) (61). The existence of this flap has been confirmed in long axis BMode images published by Gavish *et al.* (35) and Spin *et al.* (72) (Fig. 3c). The latter classified these aneurysms into a different group, termed ‘contained rupture’, as they observed that the medial dissection led to two differently contrasted regions on BMode imaging: one black region indicating free flowing blood, and one grey speckled region in which no blood seemed to be flowing. The authors performed histology in the same region to show that the speckled zone, which they termed ‘contained rupture’, corresponded to the dilated wall that was found adjacent to an intact aortic lumen in earlier histology studies. The existence of two differently contrasted regions within the dissecting AAA can also be observed on short axis BMode images from other groups (61, 70, 71, 86) (Fig 3b) and was even visible on the very first images published by Martin-McNulty *et al.* (55) (Fig 3a).

In vivo dissecting AAA physiology – Blood flow direction

Ultrasound is not limited to imaging anatomy, it also allows (amongst other applications) to quantify and visualize diameter distention (MMode imaging) and aortic blood flow patterns (Pulsed Doppler and Color Doppler imaging). While VisualSonics' most recent high-frequency ultrasound device (Vevo2100) allows for color-coded Doppler imaging, the earliest version (Vevo 660) did not offer Doppler imaging, while the Vevo 770 was limited to Power Doppler. Cao *et al.* used short axis Power Doppler imaging to visualize the blood flow within the proximal part of the dissecting AAA, in which histology had showed an intact aortic lumen adjacent to a remodeled aortic wall (61). They found that in this region, blood flow was restricted to the intact lumen, and no blood flow was entering into the adjacent region, which corresponded to the grey, speckled region that was observed in BMode imaging (as discussed before). These observations were later confirmed with both short axis (70) (Fig 3d) and long axis (73) (Fig. 3e) Color Doppler imaging. In order to visualize the flow patterns within the dilated part of the dissecting AAA, Ford *et al.* used Power Doppler 3D acquisitions to reconstruct 3D mouse-specific geometries (86). Numerical CFD computations based on these models showed a small vortex at the proximal edge of the dissecting flap. Later research by the same group, making use of Color Doppler to visualize the direction of main flow patterns in vivo, demonstrated that 2 counter-directional flow patterns take place in the dilated part of the murine dissecting AAA: in the true lumen (colored in red) there is an antegrade blood flow toward the distal (caudal) abdominal aorta, while in the second lumen (colored in blue) there is a retrograde blood flow towards the proximal (cranial) thoracic aorta. This double channel formation was observed in Ang II-infused ApoE^{-/-} mice (75) (Fig 3e) as well as in Ang II-infused C57Bl6 mice that received anti-TGF- β antibodies (77). The

latter manuscript also showed that blood velocity measured with pulsed Doppler was much higher in the forward flowing than in the backward flowing channel.

In vivo dissecting AAA physiology – Aortic wall strain and stiffness

Several authors used M-mode ultrasound to report that both the aortic distention and the strain of the outer wall decrease as dissecting aneurysms develop (33, 35, 87). Goergen *et al.* further observed that the anterior wall displacement was 2.5 to 4.0 times greater than the posterior wall displacement, suggesting that increased strain (corresponding to increased elastin fatigue) may explain why dissecting AAAs initially develop anteriorly (88). Favreau *et al.* used VisualSonics' speckle tracking algorithm to demonstrate that strains already decreased after 3 days of Ang II infusion, and continued to do so throughout dissecting AAA development (79). Finally, Luo *et al.* introduced a new technique (PWI or Pulse wave imaging) to quantify and visualize the propagation of the pulse wave along the aortic wall (84). While the pulse wave propagated uniformly in normal and sham mice, in Ang II-infused mice they observed that at the level of the dissecting AAA (i) the pulse wave moved with much lower velocity, indicating a local inhomogeneity in the aortic wall properties, and (ii) the wall displacement was significantly reduced, indicating local stiffening of the aneurysmal wall. Using the same PWI technique, Nandlall *et al.* subsequently subdivided dissecting AAAs into two categories (68). Some mice developed dissecting AAAs with luminal dilatation, and in these mice the wave speeds and wave-induced wall displacements obtained with PWI were much lower in the dilated region compared to the normal vessel. In some other mice they observed so-called 'fissures' or 'medial ruptures' at the sites of their dissecting AAA, which resulted in high displacements directed out of the lumen, while a clearly discernible wave pattern was lacking.

While ultrasound is by far the most used and versatile *in vivo* imaging modality in Ang II-infused mice, it has some limitations. In addition to intrinsic spatial resolution limitations, noise caused by reverberations, reflections and air bubbles within the acoustic gel can impair imaging. Moreover, measurements are strongly affected by manual positioning of the imaging plane, which makes the technique operator-dependent and thus prone to subjective interpretation. These disadvantages can be overcome by the use of dedicated automated 3D imaging techniques such as micro-CT or MRI.

In vivo anatomy - contrast-enhanced micro-CT

While micro-CT is restricted to visualizing anatomy, it does offer the possibility to do so in 3D, with an isotropic pixel size up to 50 micron. Despite the fact that the first commercial small animal micro-CT has been on the market since 1999 (89), the first manuscript using it to visualize Ang II-induced dissecting AAAs was published only in 2011 (76). The reason is that dedicated small animal contrast agents are required to contrast the blood-filled lumen from its surroundings, and human iodine-based contrast agents were cleared too fast by mice (90). Since 2011, we found only 4 manuscripts in which contrast-enhanced micro-CT was used to visualize Ang II-induced dissecting AAA *in vivo* (30, 51, 59, 76). In 3 of these, micro-CT was combined with PET (Positron Emission Tomography), a technique that uses FDG (fluorodeoxyglucose) molecules to visualize and quantify metabolism *in vivo* (30, 51, 59). Nahrendorf *et al.* imaged Ang II-induced dissecting AAAs with micro-CT and PET using macrophage-targeted nanoparticles labeled with fluorine-18 (^{18}F) (51). Micro-CT confirmed the existence of a dilated luminal area, while PET revealed an increased macrophage activity in the dissecting AAA region. Kitagawa *et al.* combined contrast-enhanced micro-CT with ^{18}F -labeled FPPRGD₂ particles to show increased vascular inflammation and neo-angiogenesis at the site of dissecting AAA (59). However, both

groups only published 2D transversal slices of the CT images, and Kitagawa *et al.* still used high-frequency ultrasound to confirm and quantify dissecting AAA presence. In 2011, we presented 3D segmentations of dissecting AAAs at several time points, based on sequential contrast-enhanced micro-CT scans (Fig. 4a). We used these data to compare CFD (Computational Fluid Dynamics) simulations at baseline with the presence of dilated regions after 28 days of Ang II infusion (76). Locally disturbed wall shear stress patterns were found near abdominal branches prior to Ang II infusion, but could not be related to the exact dissecting AAA location at end stage. In some animals no luminal dilatation could be observed with micro-CT while a dissecting AAA was clearly present post mortem (Fig. 4a). In a second follow-up study based on 3D micro-CT (30), histology confirmed that micro-CT does not allow to detect dissecting AAA presence in all mice (Fig. 4b). Moreover, in mice with a dilated lumen the latter was found to have a saccular, circumferentially asymmetric shape, extending parallel to the true lumen both cranially and caudally of the dilated region (Fig. 4b).

While micro-CT allows for a 3D visualization of the dissecting AAA, the obtained results are restricted to the blood-filled aortic lumen. Contrast agents are needed to differentiate the blood from surrounding soft tissues, and the dilation within the aortic wall cannot be captured. Magnetic resonance imaging (MRI) offers a valuable alternative for these limitations.

In vivo physiology and anatomy – micro-MRI

As for ultrasound and CT, clinical MRI applications needed significant technical enhancements before they could be used in small animal imaging. Most notably, the magnetic field necessary to achieve sufficient resolution (usually 1.5T or 3T in clinical practice) is much higher. Since 2009, we found 10 manuscripts in which small animal MRI has been used to visualize Ang II-induced

dissecting AAAs in mice (24, 31, 50, 60, 62, 91-95). Of these, one study used a 3T magnet (94), four used 4.7T (24, 91-93), one used 7T (62), three used 9.4T (31, 50, 60) and one used an 11.7T (95) magnet .

The first use of MRI to visualize Ang II-induced dissecting AAAs in vivo was published by Turner *et al.* (60). They reported that in the proximal part of the dissecting AAA, the lumen was stenotic and a dark region was presented adjacent to the lumen, indicating vessel remodeling without any medial wall rupture. Moreover, differently contrasted structures could be observed within the dilated wall, which was confirmed with ex vivo histology at similar locations (Fig. 5a). In a distal section of the same dissecting AAA a rupture of the aorta medial wall could be visualized, allowing blood to flow into an eccentric false lumen parallel to the original lumen (Fig. 5a). The existence of an eccentric false channel was confirmed by other publications showing either 2D axial or MIP (maximum intensity projection) MRI (31, 50, 62, 91-93). In a follow-up manuscript, Turner *et al.* combined MRI with an ultrasmall superparamagnetic iron oxide (USPIO) contrast agent that allowed them to visualize macrophage distribution in vivo (50). The areas of reduced signal were primarily along the aneurysm shoulder and outer perianeurysmal areas. This corresponded to regions of macrophage infiltration observed on histology, and was later confirmed in vivo by Yao *et al.* (62). Amirbekian *et al.* did not measure aneurysm bearing animals, but used phase contrast MRI to demonstrate that unlike in humans, there is no reversal of flow in the infra-renal aorta of wild type or ApoE^{-/-} mice (96). Goergen *et al.* used MRI to show that aortic motion and curvature are increased at the suprarenal region (93), and later demonstrated that suprarenal aortic motion is correlated to the location of dissecting AAAs in Ang II-infused mice (92). Klink *et al.* followed anti-TGF-beta injected mice with consecutive in vivo MRI, and demonstrated how the eccentric false channel “grew” quickly in

size from day 3 to days 6 and 8 (31) (Fig 5b). The authors also showed the first MRI-based 3D reconstruction of a murine dissecting AAA (Fig 5c), and imaged Ang II-induced dissecting AAAs with MRI after injection of CNA-35 micelles (31). They reported the existence of a collagen-rich and a collagen-poor region within the remodeled aortic wall, and an increased uptake of CNA-35 micelles was found in stable dissecting AAA lesions compared to dissecting AAAs that were likely to rapidly progress or rupture. Zampetaki *et al.* combined MRI with an elastin-specific contrast agent to obtain detailed 3D reconstructions of the dissecting AAA wall (94). Two different dissecting AAA shapes were shown: one fusiform, dilated wall, and another where the dilatation was much more eccentric (Fig 5d). Finally, in 2014 Fan et al published detailed 3D-reconstructions of murine dissecting AAAs using 11.7T MRI (95). Since their images were obtained ex vivo (and thus did not suffer from motion artifacts) they achieved much better 3D image quality. In the proximal region of the dissecting AAA an intramural hematoma was observed parallel to an intact lumen, while a false channel was observed in the distal region. A dissection of the outer wall tracked along the entire length of the descending aorta (Fig 5e). Interestingly, the point of rupture was located in the suprarenal segment of aorta, halfway between the diaphragm and the renal arteries, and thus much more cranially than what had previously been assumed.

While MRI thus increased the insight into the murine dissecting AAA morphology, applying 3D techniques and including the remodeled wall, it still has some disadvantages. Soft tissue contrast is good, but not optimal. Moreover the resolution is hampered by the large axial inter-slice distance. Recently we have introduced PCXTM (Phase Contrast X-ray Tomographic Microscopy), a novel ex vivo imaging technique that helped us overcome all of these limitations.

Ex vivo anatomy – PCXTM and PCXTM-guided histology

PCXTM is a relatively new technique that was first introduced in 2009. It uses X-ray synchrotron radiation to obtain a high, isotropic resolution on ex vivo samples (97), and combines it with advanced grating interferometry to obtain differential phase contrast (98). The combination of both features results in 6.5 μm pixel size images with sufficient soft tissue contrast to differentiate between most constituents of the intramural hematoma and aortic wall (Fig. 6). Moreover, since the inter-slice distance in the ex vivo images (6.5 μm) is similar to the slice thickness of paraffin sections (4 μm), PCXTM-guided histology can be performed to stain exactly at the region of interest.

We recently used PCXTM and PCXTM-guided histology to image murine dissecting AAAs that were obtained ex vivo from 15 twelve-weeks-old, male, Ang II-infused, anti-TGF- β injected C57BL/6 mice. We observed that an intramural hematoma forms in the proximal part of the dissecting AAA due to ruptures of the tunica media in the vicinity of suprarenal side branches, while apparent luminal dilatation is the result of a large tear in the vicinity of the celiac artery.

In the proximal part of the dissecting AAAs, PCXTM and PCXTM-guided histology demonstrated an intramural hematoma adjacent to an intact aortic lumen and tunica media (Fig. 6a, 6g, 6h). In vivo ultrasound and micro-CT showed that free flowing blood was restricted to the aortic lumen in the proximal part of the lesion (Fig. 6g). In contrast to previous reports, however, PCXTM also revealed the source of the intramural hematoma that caused the adventitia to dissect from the aortic wall. In 13 out of 15 scanned samples, one or several medial ruptures were detected near the ostium of suprarenal side branches such as intercostal arteries or the left superior suprarenal artery (Fig. 6a, 6f, 6g, 6h). PCXTM-guided histology demonstrated that at these locations, the elastic fibers and smooth muscle cells were fragmented, which allowed erythrocytes to enter the intramural breach (Fig 6g). ExiTron, the micro-CT contrast agent that

had been injected in vivo prior to sacrifice, was also detected near branch sites where it had leaked forming the intramural hematoma (Fig. 6a). In 7 out of 15 dissecting aneurysms, multiple independent hematomas occurred at different branches. In these cases the dissected wall extended into the thoracic aorta and resulted in a polymorphic shape (Fig. 6d, 6h), a finding explaining the existence of so-called 'grade IV aneurysms' that had first been described by Daugherty *et al.* in 2001 (Fig. 1b).

In the distal part of the dissecting AAAs, a large tear in the tunica media was observed in the vicinity of the celiac artery in all of 15 scanned dissecting AAAs. PCXTM-guided histology at this region showed a local disruption of elastin fibers (Fig. 6f). In some cases, the tear in the tunica media led to the formation of a locally dilated lumen (Fig. 6b, 6c, 6f, 6g). In some cases the false channel protruded cranially into the intramural hematoma, thus forming a parallel channel (Fig. 6b, 6c, 6f, 6g). Histology performed at these locations showed a concentric channel parallel to the original lumen (Fig. 6f, top). In some cross-sections, part of the ruptured media was observed to protrude into the false channel (Fig. 6f, middle). In vivo Color Doppler imaging with high-frequency ultrasound demonstrated cranial, retrograde flow at the level of the false channel, while no flow was recorded at zones that were later detected as intramural hematoma (Fig. 6g). Depending on the number of ruptured suprarenal side branches, the locally dilated lumen co-existed with a small or a large intramural hematoma, thus explaining the existence of so-called 'grade II' and 'grade III' aneurysms (Fig. 6b, 6c, 6f, 6g) that had first been described by Daugherty *et al.* in 2001 (Fig. 1b). In some cases no additional side branch ruptures occurred. In these cases the tear near the celiac artery was covered by fibrin and a remodeled adventitia and only led to a small bulge (Fig. 6e), a finding explaining the existence of so-called grade I aneurysms' (Fig. 1b).

Discussion

In this review we visualized how technical improvements in both in vivo and ex vivo imaging have influenced the interpretation and understanding of dissecting AAA morphology throughout the last 15 years. We started our overview with a categorization of dissecting AAA morphology into 4 different grades (Fig. 1) which, in the wording of Daugherty *et al.*, ‘arbitrarily defined aneurysms based largely on their visual characteristics’(3). We ended the overview with PCXTM images of the same Grade I-Grade IV aneurysms (Fig. 6), providing a comprehensive insight into why such large variability in their morphology exists.

For reasons of clarity and conciseness, our review did not provide an exhaustive overview of all available small animal imaging techniques, nor did we provide an exhaustive overview of all imaging techniques that have been used to visualize Ang II-induced dissecting AAAs. Instead, we focused on those publications that increased insight in either the anatomy (aortic lumen and wall) or physiology (blood velocities and wall stiffness) of dissecting aneurysms in Ang II-infused mice. More precisely, we reviewed images and insights obtained from gross anatomy, histology, ultrasound, micro-CT, micro-MRI and PCXTM imaging. “Functional” histology (e.g. macrophage infiltration, molecular pathways) and in vivo molecular imaging techniques (e.g. PET, NIRF, molecular MRI) were only discussed when the results were judged relevant to improve insight into dissecting AAA anatomy or physiology.

An important observation is that while the PCXTM-based observations change the existing paradigm, they are perfectly in line with many – if not all – previously published findings, both in the proximal and in the distal part of the dissecting AAA.

Proximal dissecting AAA – intramural hematoma formation

In the proximal part of the aorta, the PCXTM observation of an intact lumen adjacent to an intramural hematoma contained by a distended and previous dissecting adventitia (Fig. 6f, 6g, 6h) merely confirmed previous results. Using histology, an intact aortic lumen had been identified adjacent to a dilated structure (defined as either remodeled wall or intramural hematoma) in numerous papers (2, 18, 26, 27, 32, 33, 37, 58-63) (Fig. 2a, 2b, 2c). This corresponded to a black zone (lumen) that was reported adjacent to a grey speckled grey zone (hematoma) on ultrasound BMode images (Fig. 3a, 3b, 3c). PCXTM images demonstrating a dissected adventitia in the proximal aorta were also in line with the absence of flow in the dilated part of the proximal dissecting AAA that was previously demonstrated with Color Doppler ultrasound (Fig. 3d) , micro-CT (Fig. 4b, 4c) and MRI (Fig. 5a), and was confirmed by our in vivo measurements (Fig. 6g). The co-existence of several branch-related ruptures, leading to different sources of intramural hematoma, was compatible with axial MRI slices and histology that reported the presence of differentially contrasted regions within the remodeled wall (Fig. 5a). In addition to the enhanced insight into previously published images, PCXTM managed to reveal the source of this intramural hematoma to be local ruptures in the tunica media, in the vicinity of small branches.

Distal dissecting AAA – apparent luminal dilatation

In the distal part of the AAA, PCXTM revealed that a mural tear of the tunica media led to the formation of a so-called pseudo-aneurysm in the vicinity of the celiac artery. The apparent dilatation of the aortic lumen was directly related to the tear, and blood flow in this region was only contained by the remodeled adventitia. Again, this was perfectly in line with previously published histological stainings that showed a local disruption of elastin fibers in the distal part of the lesion (Fig. 2a, 2b, 2c). It was also compatible with observations that the region of maximal

dilatation occurs superior to the right renal artery (15, 17, 19, 26, 32, 37, 53-56), and coincides with the region where elastin fibers were fragmented (17, 19, 29, 37, 53, 57). As 3D allows to visualize axial connections that 2D leaves open for interpretation, PCXTM was the first technique demonstrating the existence of a parallel channel that was connected to the blood flowing out of the tear in the tunica media. These data confirmed the existence of a false channel parallel to the intact lumen that was first described using sequential histology by Schriebl *et al.* (29), and was visible on published images of many other authors (11, 30-34). PCXTM also confirmed earlier in vivo reports of an eccentric false channel by micro-CT (Fig. 4b, 4c) and MRI (Fig. 5a, 5b). The latter was also confirmed by our own in vivo micro-CT images (99). Moreover, our sequential in vivo 3D ultrasound imaging demonstrated how in some mice the false channel slowly protruded cranially over time, into the intramural hematoma that formed the proximal part of the dissecting AAA (99). This was compatible with an increase in 'luminal diameter' over time that was previously measured with BMode ultrasound (56). Furthermore our in vivo Color Doppler measurements demonstrated that retrograde, cranially directed flow was present in the dilated part of the dissecting AAA (Fig. 6g), which confirmed earlier findings by Takahashi *et al.* (75)(Fig 3e) and Klink *et al.* (31). Finally, the 'dissecting flap' that had been observed in vivo by several authors (35, 61, 72) (Fig. 3b) was reproduced by PCXTM-guided histology as part of the ruptured media that protruded into the luminal space (Fig. 6f).

Hypotheses on dissecting AAA pathobiology and pathogenesis

Importantly, our PCXTM data confirm the important role for micro-ruptures in elastin fibers near abdominal side branches, that was first reported by Gavish *et al.* (37). In addition to their analysis, which was focused on the 4 largest abdominal side branches (celiac, mesenteric and both renal arteries) we identified an important role for small, supraceliac and thoracic side

branches. We hypothesize that the pivotal role of small branches might be related to (i) locally increased mechanical stresses, (ii) local changes in structural material properties induced by a change in collagen fiber directions, (iii) the local change in the number of elastic lamellae from main aorta to side branches, or (iv) locally disturbed wall shear stresses that may lead to endothelial dysfunction. Deeper investigation into the local mechanobiology near side branches is needed, and will be the subject of future research.

Another important observation is in the role of intramural hematoma remodeling. PCXTM revealed the presence of newly synthesized collagen in the outer regions of the oldest dissecting AAAs (Figure 6), which confirmed earlier *in vivo* observations (31). Klink *et al.* imaged collagen within the intramural hematoma using CNA-35 micelles with *in vivo* MRI, and postulated that collagen-rich Grade II lesions were better protected from transmural rupture than collagen-poor Grade III and Grade IV lesions (31). In retrospect, we hypothesize that Klink *et al.* observed these collagen rich lesions near the source of the intramural hematoma in the proximal part of the dissecting AAA of Grade II lesions, while they observed collagen-poor regions at the outer layer of the dilated lumen in grade III lesions. Together, these data confirm the histology-based hypothesis for the protective role of fibrin and collagen deposition in hematoma remodeling that was initially put forward by Schriebl *et al.* (29). A better understanding of the remodeling processes that take place during the different phases of dissecting aneurysm development is needed, and will be further investigated in the future..

Throughout the manuscript, and also in previous work, we used the term ‘dissecting aneurysm’ to describe Ang II-induced lesions in mice. This term, first introduced by Xanthouleas *et al.* (63), has been used by several authors that wanted to express the difference in morphology between humans and mice (29, 100). Most authors describe the lesions as ‘murine AAAs’, but we believe

it is important to make a distinction between the true, luminal expansion that is typical for human aneurysm formation, and the apparent luminal expansion that is the result of the dissection of the tunica media in Ang II-infused mice. Confronted with similar observations, some authors termed the Ang II-induced lesions as ‘pseudo-aneurysm’ (28) or ‘aortic dissection’ (8, 18, 95, 101, 102). However, human pseudo-aneurysms are no longer contained by aortic wall, while human type A or type B dissections have a re-entry point for the false channel to rejoin the true lumen. In murine dissecting AAAs on the other hand, the false channel is constrained by the tunica adventitia, while a local vortex with retrograde flow is observed within the false channel (Fig. 3e), similar to aneurysm flow in human AAA. Precisely because of the lack of an exact clinical counterpart, we believe that the term ‘dissecting AAA’ is suited: it captures the most important characteristics and avoids confusion with other clinical pathologies.

Small animal imaging of dissecting AAA: state of the art and future developments

Providing an unprecedented combination of high resolution and soft tissue contrast, PCXTM yielded valuable information into many previously published images of murine dissecting AAA. Future research will apply these techniques to obtain a better understanding of (i) the mechanobiology leading to mural ruptures in the vicinity of aortic branches, and (ii) the biological processes taking place during hematoma remodeling. We strongly believe that, even if ‘dissecting AAAs’ do not have an exact counterpart in human pathophysiology, a better understanding of this mouse model could lead to important translational insights for both aortic aneurysm and aortic dissection. Since it provides access to the earliest stages of the disease, there is a unique opportunity to elucidate the role of side branches in the onset of cardiovascular disease. In this respect an important question that remains to be addressed is why some animals do not develop any branch-related medial ruptures, while other animals have medial ruptures that

are limited to a tear near the celiac artery (leading to ‘Grade I aneurysms’), and others seem much more susceptible to additional ruptures in the vicinity of suprarenal branches such as intercostals and superior suprarenal arteries (leading to ‘Grade II, Grade III and Grade IV aneurysms’).

Like most imaging modalities, however, the advantages of PCXTM come at a cost. In vivo PCXTM imaging on live mice is not feasible, while high-throughput ex vivo scanning is hampered by (i) the strong competition for and hence difficult access to dedicated synchrotron beam time, (ii) the relatively long scanning time per sample (1.5 hours per axial length of 4 mm), and (iii) the sample size limitation of 10x10x4mm (although stacked scans are possible). In vivo imaging techniques will therefore remain an indispensable source of information for cardiovascular small animal research in general, and dissecting aneurysm research in particular. High-frequency ultrasound is needed to assess functional parameters such as local circumferential strain, artery stiffness and blood velocity in vivo. Micro-CT and MRI will be needed to obtain the in vivo, pressurized geometry of the blood lumen, which is necessary as an input for computational models that might elucidate the role of mechanical stresses in branch-related medial ruptures. Additionally the development of in vivo contrast agents for PET, NIRF or molecular MRI might shed further light on ongoing biological remodeling processes within the intramural hematoma.

Conclusions

In this narrative review we demonstrated how PCXTM and PCXTM-guided histology relates to (and provide a posteriori insight into) previously published images of Ang II-induced dissecting AAA in mice. Within the novel paradigm of branch-related ruptures leading to apparent luminal dilatation and intramural hematoma formation, we explained several open issues regarding the morphology and anatomy of dissecting AAA in these mice. More research will further exploit these techniques to unveil the role of aortic side branches in the onset of cardiovascular disease.

Conflict of Interest

None declared.

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Figures

Daugherty *et al.*, J Br Pharmacol, 2001

Figure 1: dissecting AAA anatomy. Aneurysms are divided into Grade I (dilated lumen in the supra-renal region of the aorta with no thrombus), grade II (a remodeled tissue in the suprarenal region that frequently contains thrombus), grade III (a pronounced bulbous form of type II that contains thrombus), and grade IV (multiple aneurysms containing thrombus, some overlapping). Image reproduced with permission from (3).

Daugherty *et al.*, J Clin Invest, 2000

Rateri *et al.*, Am J Pathol, 2011

Schriebl *et al.*, Thromb Res, 2012

Figure 2: dissecting AAA histology. **a.** Stainings published in the original paper by Daugherty *et al.* Intramural thrombus is seen adjacent to intact aortic lumen (top left, top right, middle left, middle right) and a tear in tunica media occurs at the level of luminal dilatation (bottom left, bottom right). Sections are stained or immuno-stained with the following methods: Verhoeff (top left); Gomori (top right); Oil Red O (middle left, bottom left); presence of macrophages using antisera from Accurate Chemical and Scientific Corp. (dilution 1:10,000) and counterstained with hematoxylin (middle right, bottom right). Image reproduced with permission from (2). **b.** Serial sectioning of the full length of a dissecting AAA, every tenth slide was stained with Movat's pentachrome. The three top sections are reported to start 'proximally to the AAA', showing an intact lumen adjacent to an intramural thrombus (indicated by a letter T). The bottom left and middle sections are reported 'inside the AAA', showing a dilated lumen and elastin breaks indicated by arrows. The bottom right section is reported to be 'distal to the AAA', and shows a clearly delineated false channel parallel to the original lumen. Image reproduced with permission from (27). **c.** Left: photograph of a representative suprarenal dissecting aneurysm with photographs of cross-sections obtained from regions indicated by the dashed lines. Right: representative Verhoeff Van Gieson (VVG)-stained cross-sections that reveal the three distinct formations that were observed in each of the five dissecting aneurysms studied by the authors. Top right: merged lumen (ML), with arrows indicating locations where the media ruptured.

Middle right: intact lumen and cavity separated by remodeled tissue. Middle bottom: intact lumen (L) adjacent to intramural thrombus (T). The authors hypothesized (but could not demonstrate due to the lack of 3D imaging) that the cavity C belonged to a false lumen that merged with the true lumen L at the region where elastin breaks occurred in the tunica media. Image reproduced with permission from (29).

Figure 3: high-frequency ultrasound. **a.** The first published BMode image of an Ang II-induced murine dissecting AAA (Vevo 660), in both transverse (left) and longitudinal (right) views. A dilated lumen can be observed in the suprarenal aorta (middle left). Note that a remodeled wall is also visible adjacent to the lumen. Image reproduced with permission from (55). **b.** BMode Ultrasound images obtained with Vevo 770. Left: the white arrow indicates an aortic dissection “flap” where the elastic media has broken down; the red dotted arrows show altered flow; the blue arrow indicates a region with re-circulating slow flow where the authors hypothesized that thrombosis may occur. Middle: A representative diameter measurement in the suprarenal aorta. Note that the grey, speckled region is included into the measurement. Right: 3D representation of the dissecting AAA obtained with 3D ultrasound. Remark the difference in dilated inner lumen between the three represented specimen. Image reproduced with permission from (61). **c.** BMode Ultrasound images obtained with Vevo 770. Longitudinal (left) and transversal (middle) ultrasound image of the suprarenal aorta of a dissecting aneurysm with so-called ‘contained rupture’. The intramural hematoma is indicated with white arrows, and by * on the co-localized hematoxylin-eosin staining (right). Image reproduced with permission from (72). **d.** Transversal Power Doppler ultrasound of the suprarenal aorta in an Ang II-induced dissecting aneurysm (Vevo 770). Remark that blood flow is restricted to the aortic lumen. Image reproduced with

permission from (70). **e.** Longitudinal Color Doppler ultrasound of the suprarenal aorta in an Ang II-infused dissecting aneurysm (Vevo 2100). Remark the retrograde flow (termed 'turbulent flow' by the authors), indicated by white arrows. Image reproduced with permission from (75).

Figure 4: contrast-enhanced micro-CT. **a.** 3D representation of the aortic lumen obtained with contrast-enhanced micro-CT (left) compared to the post mortem gross anatomy (right) for 4 different Ang II-induced dissecting AAAs. Note the absence of luminal dilatation in top right and bottom right geometries. Image reproduced with permission from (76). **b.** Top: Longitudinal follow-up at 3 different time points (7, 14 and 28 days after pump implantation) of the aortic lumen scanned in vivo with contrast-enhanced micro-CT. Bottom: Zoomed micro-CT of the dilated lumen at day 28 (left) and co-located Orcein stainings (right). Note the presence of intramural thrombus adjacent to intact lumen (middle), and the presence of a false channel parallel to the intact lumen in the distal part of the dissecting AAA (bottom). Image reproduced with permission from (30).

Klink *et al.*, JACC, 2011

Figure 5: micro-MRI. Top left: T1-weighted (T1W) black-blood imaging of dissecting AAA progression over the course of 8 days. Medial rupture and false channel formation are visible as soon as 3 days after the start of Ang II-infusion in the upper-right corner, and the false channel increases in size at days 6 (in the lower-left corner) and day 8 (in the lower-right corner). Bottom left: combined Masson elastin staining showing medial rupture and degradation of the elastic lamina as well as the extreme remodeling of the adventitia and formation of a false channel parallel to the original lumen. Right: RD magnetic resonance (MR) time-of-flight angiography of a healthy aorta (left panel) and dissecting AAA (right panel). Moderate supraceliac aortic dilation is visible (arrow). Image reproduced with permission from (31).

Figure 6: PCXTM. **a.** Transversal PCXTM images show ExiTron aggregates (in white) near the ostium of an intact intercostal artery (top left), near the ruptured ostium of the superior suprarenal artery (top right) and at the edges of the tear (bottom right). The longitudinal PCXTM image (bottom left) shows the dissecting 'grade I aneurysm' depicted in panel e. **b.** longitudinal PCXTM image of the dissecting aneurysm depicted in panel f. Note the formation of a false channel that connects to the true lumen at the region of the ruptured tunica media. Also note the ruptured suprarenal artery that led to intramural thrombus. **c.** longitudinal PCXTM image of the dissecting aneurysm depicted in panel g. Note the formation of a false channel that connects to the true lumen at the region of the ruptured tunica media. Also note the ruptured suprarenal artery that led to a small false channel and intramural thrombus. **d.** longitudinal PCXTM image of the dissecting 'grade IV aneurysm' depicted in panel h. Note the rupture of an intercostal artery (leaving the outer wall) led to intramural blood accumulation with flow lines clearly visible

concentric around the branch. **e.** 3D representation based on PCXTM images of the dissecting 'grade I aneurysm' shown in the lower-left corner of panel a. Top left: tear in the tunica media, on the left side of the celiac artery (tunica media in non-transparent white, blood filled lumen in red, adventitia is hidden). Bottom left: 3D with tunica media in (transparent) white, intramural hematoma in orange, blood filled lumen in (transparent) red. Top right: PCXTM-guided combined Sirius Red (SR) and Miller stain shows destruction of the elastic lamellae and remodeled fibrotic adventitia. Middle right: Martius, Scarlet and Blue (MSB) stain shows the presence of fibrin (in red) within the hemostatic plug, while smooth muscle α -actin stain shows loss of smooth muscle cells, hence of medial architecture along the tear. Bottom right: gross anatomy showing only a small dilation of the wall. **f.** 3D representation based on PCXTM images of the dissecting aneurysm shown in panel b. Left: 3D with tunica media in (transparent) white, intramural hematoma in orange, blood filled lumen in (transparent) red. Top right: PCXTM-guided CD-31 stain shows endothelialization of the false channel parallel to an unchanged aortic lumen. Middle right: combined SR and Miller stain shows apparent dilatation as the hemorrhage resulting from the tear in the tunica media is enclosed within the distended adventitia. Bottom right: gross anatomy shows an abdominal dilatation. **g.** 3D representation based on PCXTM images of the dissecting aneurysm shown in panel c. Left: 3D with tunica media in (transparent) white, intramural hematoma in orange, blood filled lumen in (transparent) red. Top right: Color Doppler ultrasound shows apparent luminal dilation and retrograde (cranially directed) flow are present at the level of the tear in the tunica media (right panel), but not at the intramural hematoma (left panel). Middle right: PCXTM-guided H&E stain shows the ostium of the ruptured superior suprarenal artery. Bottom right: gross anatomy shows an abdominal dilatation. **h.** 3D representation based on PCXTM images of the dissecting 'grade IV aneurysm' shown in panel d. Left: 3D with tunica media in (transparent) white, intramural hematoma in orange, blood

filled lumen in (transparent) red. Top right: PCXTM-guided combined SR and Miller stains shows an intact tunica media adjacent to an intramural hematoma that has dissected the tunica adventitia from the medial layer. Bottom right: gross anatomy shows a polymorphic dilatation extending into the thoracic aorta.

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