Tampere University

EETU NIINIMÄKI

Heterogenic Ascending Aortic Wall Tissue

A Translational Approach

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Medicine and Health Technology
of Tampere University,
for public discussion in the Lecture room F114
of the Arvo Building, Arvo Ylpön katu 34, Tampere,
on 03 March 2023, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finland

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The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

Cover design: Roihu Inc.

ISBN 978-952-03-2754-5 (print)
ISBN 978-952-03-2755-2 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-2755-2

Carbon dioxide emissions from printing Tampere University dissertations have been compensated.

PunaMusta Oy – Yliopistopaino
Joensuu 2023

For Susanna,

"My most brilliant achievement was my ability to be able to persuade my wife to marry me."

-Winston Churchill

ACKNOWLEDGEMENTS

This study was carried out during the years 2010-2014 at Tampere University Hospital, Tays Heart Hospital, and the University of Tampere.

I would like to thank Professor Timo Paavonen for making the thesis possible as well as for providing the facilities and environment to perform research. I would also like to thank Professor Paavonen for nurturing my excitement to perform this research.

I express my deepest gratitude to Ari Mennander (M.D., Ph. D.) for tireless and continued guidance in creating this thesis and teaching me the principles and joys of medical research.

I would like to thank supervision committee members Ivana Kholova (M.D, Ph. D.) and Teemu Tolonen (M.D, Ph. D.) for providing guidance with my research.

The official reviewers of the thesis manuscript, Vesa Anttila (MD, PhD) from the Department of Cardiothoracic Surgery, Turku University Hospital and Mikko Rönty (MD, PhD) from the Department of Pathology, Helsinki University, are warmly thanked for their constructive criticism and valuable comments for this thesis.

I respectfully express my thanks and appreciation to the following fellow authors in the field: Timo Valo (M.D), Professor Matti Tarkka, Henri Kajander (M.D.), Thanos Sioris (M.D, Ph. D.), Ville Pynnönen (M.D.), Petteri Muola (M.D.), Professor Seppo Parkkila, Hannu Haapasalo (M.D., Ph.D.), Silvia Pastorekova (M.D, Ph.D.), and Jaromir Pastorek (M.D., Ph.D.)

Without support from Ms. Eini Eskola, I would not have been able to conduct this research. I thank you for your kind and understanding support in the laboratory.

I would also like to thank my teachers Pekka Tiainen (M.D., Ph.D) and Merja Kokki (M.D., Ph. D) for support and encouragement in the clinical work.

I express my gratitude to Paul W. Harrison for the grammar check.

Finally, I would like to thank my children L, K, T and T for demonstrating the importance of future generations.

This thesis was financially supported by Etelä-Karjala Tuberculosis Foundation and The Finnish Heart Association.

ABSTRACT

Background: Successful surgery of the ascending aorta requires knowledge on tissue demeanor. Ascending aortic dilatation, tissue degeneration and inflammation may predispose to aortic events such as aortic dissection. We investigated the presence of complement C4d, Carbonic anhydrase 9 (CAIX), Immunoglobulin G4 (IgG4) and endothelial CD31 of the ascending aortic wall, and sought the interrelation of ascending aortic dilatation and ascending aortic wall histology.

Material and methods: After institutional review board approval and patient consent, ascending aortic tissue samples were procured from patients undergoing surgery for ascending aorta at Tampere University Hospital, Tays Heart Hospital. The samples were stained with Hematoxylin and Eosin (H&E), Verhoeff-van Gieson (VVG), underwent immunohistochemistry, and were processed for systematic semi-quantification.

Results: Intimal inflammation, thickness, cellularity, and a number of plasma cells were decreased in C4d-positive as compared with C4d-negative aortas (I). IgG4-positivity revealed concealed aortitis (II). Adventitial inflammation consisting of macrophages, B-cells and cellular proliferation, together with intimal macrophages and media elastin degradation, was increased in CA IX-positive as compared with CAIX negative aortas (III). CD31 positivity of the ascending aortic wall reflected neovascularization associated with aortic dissection (IV).

Conclusions: The lack of aortic wall C4d, increased IgG4- and CD31-positivity suggest increased risk of dissection, while CAIX-positivity suggests aortic wall stability. The aortic tissue immunohistochemistry reveals the heterogenic nature of the ascending aorta.

TIIVISTELMÄ

Tausta: Onnistunut nousevan aortan kirurgia vaatii kudostuntemusta. Nousevan aortan dilataatio, seinämän degeneraatio ja inflammaatio voivat altistaa aortan seinämän repeämiselle ja dissekaatiolle. Tutkimme komplementin osatekijän C4d:tä, karboanhydraasi IX:ä (CAIX), immunoglobuliini 4:ää (IgG4) sekä endoteelisolun CD31:ä aortan seinämässä tavoitteena arvioida nousevan aortan laajentuman ja seinämän kudoksen yhteyttä.

Potilaat ja menetelmät: Tutkimusnäytteet kerättiin sairaalan eettisen toimikunnan ja potilaan luvalla niiltä potilailta, joille tehtiin nousevaan aorttaan kohdistuva leikkaus Tampereen Yliopistollisessa Sydänsairaalassa. Näytteet värjättiin hematoksyliini-eosiini:lla, Verhoeff van Gieson -metodilla. Lisäksi näytteistä tehtiin immunohistokemiallinen analyysi. Näytteet arvioitiin semikvantitatiivisesti.

Tulokset: Intiman inflammaatio, sen paksuus, solumäärä sekä plasmasolujen määrä koko aortan alueella oli vähentynyt ns. C4d-positiivisissa aortan seinämissä (I). IgG4-positiivisuus aortan seinämässä vahvisti aortiitin (II). Makrofagien, B-solujen ja solujakautumisen määrän avulla arvioitu inflammaatio adventitiassa oli lisääntynyt CAIX-positiivisissa aortoissa verrattuna CAIX-negatiivisiin. Lisäksi intiman makrofagien määrä sekä median elastiinimäärä oli vähentynyt CAIX-positiivisissa aortoissa (III). Lisääntynyt CD31 positiivisuus aortan seinämän uloimmassa kolmanneksessa esiintyi aortan dissekaatiossa (IV).

Loppupäätelmät: C4d:n puuttuminen, lisääntyneet IgG4- ja CD31-positiivisuudet voivat viitata lisääntyneeseen nousevan aortan dissekaation riskiin toisin kuin CAIX-positiivisuus. Aorttakudoksen immunohistokemia paljastaa nousevan aorttakudoksen monimuotoisuuden.

Kirjoittajan osuus osajulkaisuissa

I Niinimäki E, Paavonen T, Valo T, Tarkka M, Mennander A. Lack of C4d deposition may reveal susceptibility for ascending aortic dissection. Scandinavian. Scand Cardiovasc J 2012;46:177-182.

Väittelijä osallistui tutkimuksen suunnitteluun ryhmän osana. Potilasaineiston keräämiseen väittelijä ei osallistunut. Laboratoriomenetelmien käytännön toteutus ja mukauttaminen on väittelijän itsensä toteuttama. Olennaiset patologiset analyysit koko aineistosta on toteuttanut väittelijä. Tilastollisten analyysien tulkintaan ja arviointiin osallistuminen sekä julkaistun tekstin kirjoittamiseen osallistuminen olivat väittelijän osuutta tutkimuksen toteuttamisessa ja raportoinnissa. Väittelijä raportoi tulokset kansainvälisessä sydänkirurgian kongressissa.

II Niinimäki E, Kajander H, Paavonen T, Sioris T, Mennander A. Aiming at One-Stage Corrective Surgery for Extended Thoracic Aortic Dilatation. Int J Angiol 2014;23:101–106.

Potilasaineisto on kerätty Sydänsairaalan leikatuista potilaista. Väittelijä osallistui tutkimusasetelman suunnitteluun ryhmän osana. Immunhistokemialliset värjäykset ja tähän liittyvät analyysit ovat väittelijän toteuttamia. Tutkimus toteutettiin tapausselostussarjana eikä tilastollista analyysia tehty. Väittelijä osallistui raportin kirjottamiseen sekä raportoi tulokset pohjoismaisessa tutkijakongressissa.

III Niinimäki E, Muola P, Parkkila S, Kholová I, Haapasalo H, Pastorekova S, Pastorek J, Paavonen T, Mennander A. Carbonic anhydrase IX deposits are associated with increased ascending aortic dilatation. Scand Cardiovasc J. 2016;50:162-6.

Väittelijä osallistui tutkimuksen suunnitteluun ryhmän jäsenenä. Histopatologinen arviointi on väittelijän toteuttama. Tulosten tulkinta on tehty osana ryhmää. Tilastolliset laskelmat ovat tutkimusryhmän jäsenen toteuttamia. Väittelijä on osallistunut raportin kirjoittamiseen sekä julkaisemiseen.

IV Niinimäki E, Pynnönen V, Kholová I, Paavonen T, Mennander A. Neovascularization with chronic inflammation characterizes ascending aortic dissection. Anatol J Cardiol. 2018;20: 289–295.

Väittelijä osallistui tutkimuksen suunnitteluun ryhmän jäsenenä. Immunohistokemialliset värjäykset ja näytteiden analyysi on väittelijän toteuttama, samalla väittelijä perehdytti syventävän työn tekijää ks. menetelmiin. Tilastollinen analyysi on toteutettu ryhmän toimesta. Tulosten tulkinta, artikkelin kirjoitus on tehty väittelijän ja ryhmän yhteistyönä.

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ABBREVIATIONS

AAA	Abdominal aortic aneurysm
ACTA2	Actin alpha 2
AUC	Area under the curve
C4d	Complement component C4d
CA	Carbonic anhydrase
CAIX	Carbonic anhydrase IX
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CD20	Cluster of differentiation 20
CD31	Cluster of differentiation 31
CD34	Cluster of differentiation 34
CD68	Cluster of differentiation 68
CD138	Cluster of differentiation 138
D2-40	Podoplanin, lymphatic endothelial marker
FGF	Fibroblast growth factor -family
H&E	Hematoxylin & Eosin stain
HIF-1	Hypoxia-inducible factor 1

IgG4	Immunoglobulin G4
IRAD	The International Registry of Acute Aortic Dissections
Ki-67	Ki-67 protein, marker for cell proliferation
MMP	Matrix metalloproteinase
MYH11	Myosin heavy chain 11
NF- ϰB	Nuclear factor κ-light-chain-enhancer of activated B cells
PECAM-1	platelet-endothelial cell adhesion molecule-1
PDGF	platelet derived growth factor
PROX1	Prospero homeobox protein 1
ROC	Receiving operating characteristic
TAA	Thoracic aortic aneurysm
TAD	Thoracic aortic dissection
TEVAR	Thoracic endovascular aortic repair
TNF-α	Tumor necrosis factor alpha
VEGF	vascular endothelial growth factor -family
VVG	Verhoeff-Van Gieson stain
vWf	von Willebrand factor

ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals [I-IV].

I Niinimäki E, Paavonen T, Valo T, Tarkka M, Mennander A. Lack of C4d deposition may reveal susceptibility for ascending aortic dissection. Scandinavian. Scand Cardiovasc J 2012;46:177-182.

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III Niinimäki E, Muola P, Parkkila S, Kholová I, Haapasalo H, Pastorekova S, Pastorek J, Paavonen T, Mennander A. Carbonic anhydrase IX deposits are associated with increased ascending aortic dilatation. Scand Cardiovasc J. 2016;50:162-6.

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1 INTRODUCTION

Aortopathy leading to ascending aortic events such as dissection is a major clinical challenge. Ascending aortic dilatation and tissue degeneration may predispose to aortic wall rupture, and aortic dissection often occurs acutely without warning.

Histological and immunohistochemical tissue analysis of the ascending aorta suggest that the aorta is not merely a passive blood vessel. The interplay of the various aortic wall layers – the intima, media and adventitia – all participate in the remodeling of the tissue during aortic dilatation and dissection. Understanding aortopathy, such as the development of dissection, could help the clinician to intervene on time.

Inflammation, cellular and molecular interplay between endothelial cells, tissue matrix and connective tissue are key players during aortic wall remodeling. Complement C4d, Immunoglobulin G4 (IgG4), Carbonic anhydrase 9 (CAIX) and endothelial CD31 are some variables reflecting tissue remodeling during inflammation and angiogenesis.

We hypothesized that systematic histological tissue analysis aids in revealing the heterogenic characteristics of the ascending aortic tissue.

2 REVIEW OF THE LITERATURE

2.1 Definitions- the ascending aorta

The human thoracic aorta encompasses the aortic root, ascending aorta, aortic arch and descending aorta. Embryologically, the thoracic aorta originates mainly from the neural crest (Cheung et al., 2012). The healthy ascending aortic diameter does not exceed 40 mm (Devereux et al., 2012). Besides delivering blood, the thoracic aorta regulates blood pressure via baroreceptors and the vagus nerve. The aortic wall is rich in elastin, forms an elastic reservoir for blood during systole, and releases blood during diastole (Westerhof et al., 2009). The elasticity and smooth muscle cells of the ascending aorta maintain blood pressure, while withstanding aortic wall shear stress (Eoh et al., 2017; Wanjare et al., 2015). Rough anatomy of thoracic aorta is presented in Figure 1.

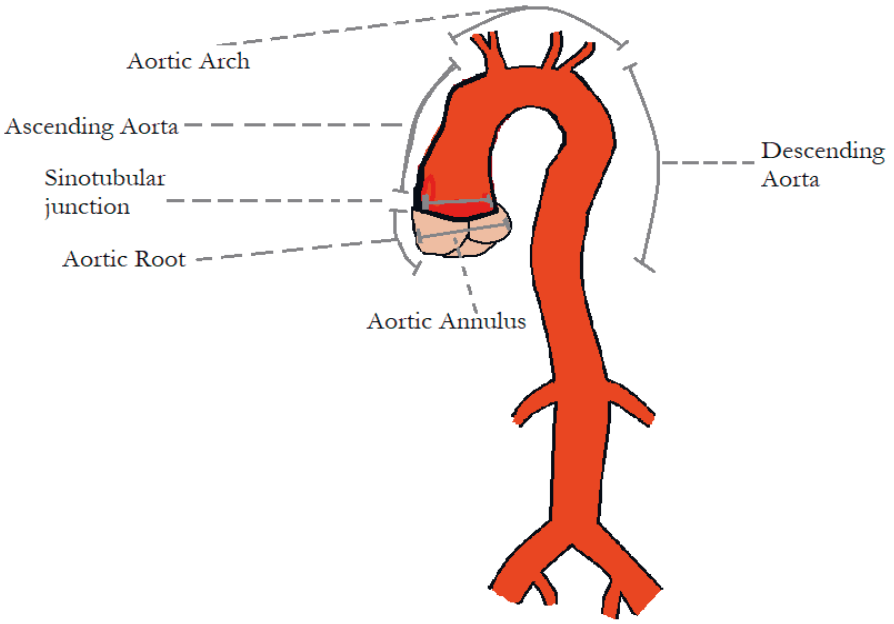

Figure 1. Anatomy of thoracic aorta

2.1.1 Dilatation, aneurysm and pseudoaneurysm

Aortic dilatation encompasses symmetrical increase of aortic cross-sectional diameter. Aortic aneurysm includes varied forms of aortic dilatation with all three layers of the aortic wall. Aortic pseudoaneurysm involves protrusion of the aortic wall due to weakening in the aortic wall. These terms are often intermixed in the literature.

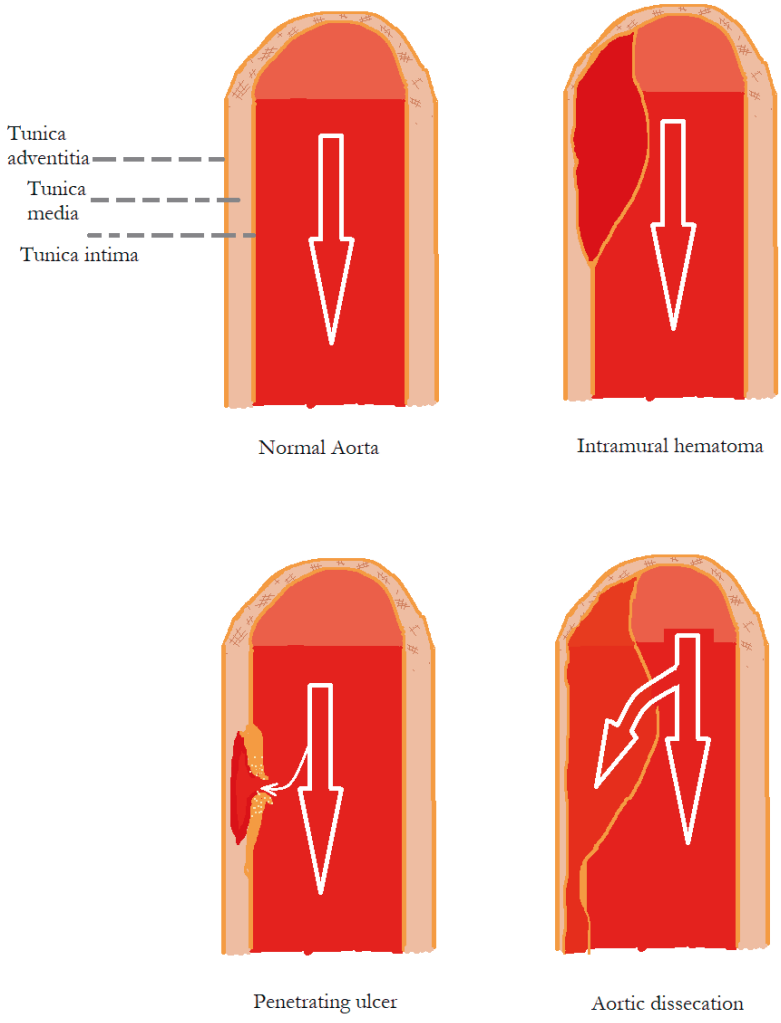

Figure 2. Layers of the aortic wall, intramural hematoma, dissection, and penetrating ulcer

2.1.2 Intramural hematoma and penetrating ulcer

Intramural hematoma includes an inner disruption of the aortic wall with a penetrating ulcer or without intimal tear, presumably resulting either from rupture of the vasa vasorum of the aortic wall or a penetrating intimal lesion lacking a longitudinal tear (Harris et al., 1994; Robbins et al., 1993; T. Yamada et al., 1988).

2.1.3 Aortic dissection and rupture

Aortic dissection includes longitudinal aortic wall rupture that may be contained in the aortic wall. Free aortic wall rupture signifies disruption of the aortic wall with blood flow out of the outer aortic wall layer. The aortic dissection includes an intimal tear. The intimal tear allows blood flow between layers of the aorta and a false lumen in the aortic wall may ensue. Depending on the timing of the diagnosis, aortic dissection is categorized as acute dissection, if it has been diagnosed and has occurred in less than two weeks, as subacute dissection with a time interval of 14-90 days, and as chronic dissection as diagnosed after 90 days (Erbel et al., 2014).

2.1.4 Aortopathy and aortic events

Aortopathy is a general term describing the pathological development of aortic tissue that may lead to aortic dilatation, aneurysm, pseudoaneurysm, or to aortic events such as intramural hematoma, penetrating ulcer, dissection, and rupture.

2.2 Epidemiology

Incidence for dissection in thoracic aorta is 4-6/100 000/year. Incidence in the elderly has been reported as high as 30/100 000/year (Howard et al., 2013; Olsson et al., 2007) .The Finnish National Institute for Health and Welfare reports 300 to 400 operations yearly for the thoracic aorta in Finland (THL, 2020). In a Swedish population-based study, the incidence of thoracic aortic events was noted as rising (Hagan et al., 2000; Olsson et al., 2007). The rise in incidence is most likely due to increasing life-expectancy and awareness of thoracic aortic events. Anatomic location of dissection plays a major role in prognosis and the decision of treatment

strategy. Ascending aorta is affected in ~60% of cases, descending aorta in ~30% of patients, and aortic arch is involved in less than ten percent of the cases (Lempel et al., 2014; Patel et al., 2014).

2.3 Risk factors of aortopathy

2.3.1 Acquired risk factors

Hypertension is the most common comorbidity associated with aortopathies of thoracic aorta. A total of 60–80% of patients with acute dissection have a history of high blood pressure. Smoking is established as a risk factor for aortic events (Landenhed et al., 2015). Mean age of patients with thoracic aortic dissection is 60 years and affects more males than females with a 60/40-ratio. The risk for aortopathy increases with age (Hagan et al., 2000; Homme et al., 2006; Howard et al., 2013; Landenhed et al., 2015). The role of atherosclerosis in thoracic aortopathies is not clear (Yang et al., 2019).

Atypical, acquired risk factors include drugs, pregnancy, trauma, and iatrogenic causes. Cocaine and amphetamine abuse have been associated with acute thoracic aortic dissections (Barth et al., 1986; Daniel et al., 2007; Palmiere et al., 2004; Rashid et al., 1996; Westover & Nakonezny, 2010). The third semester and postpartum period of pregnancy is associated with increased risk of dissection of thoracic aorta (Immer et al., 2003; Jovic et al., 2014; Kamel et al., 2016; Srettabunjong, 2013; Zeebregts et al., 1997). Oral fluoroquinolones have been associated with fibroblast-mediated extracellular matrix dysregulation and matrix metalloproteinase activation; conversely, there may be an association with prolonged use of oral fluoroquinolones and risk of aortic dissection(Daneman et al., 2015; Guzzardi et al., 2019; Lee et al., 2015; Nautiyal, 2017).

2.3.2 Congenital risk factors

Aortopathy is observed in up to 25% of patients with bicuspid aortic valve (Michelena et al., 2011). The incidence of bicuspid aortic valve is 0.5–1.5% of the whole population (Michelena et al., 2011; Sillesen et al., 2021). A bicuspid aortic valve is present in up to 6%, and TAA in 9% of first-degree relatives (Galian-Gay et al.,

2019). An eccentric tubular blood flow in patients with a bicuspid aortic valve may be associated with the presence of aortopathy (Manchester et al., 2021). Matrix metalloproteinases, smooth muscle cell apoptosis, and elastic fragmentation of the media layer may all act during aortopathy (Longo et al., 2002), but increased aortic wall degeneration per se in patients with a bicuspid vs tricuspid aortic wall remains controversial (Heng et al., 2015; Mennander et al., 2022). Aortic valve insufficiency per se may increase the risk for aortic dissection (Farag et al., 2018). Turner syndrome is associated with a bicuspid aortic valve, ischemic heart disease, hypertension, stroke, coarctation of the aorta, and thoracic vessel abnormalities, and may increase the risk for aortic dissection(Carlson & Silberbach, 2007; Gravholt et al., 1998, 2006; Ho et al., 2004).

2.3.3 Inherited connective tissue diseases

Inherited connective tissue diseases are a significant predisposing factor for aortopathies (Homme et al., 2006; Pape et al., 2007; Roman et al., 1993). Marfan's syndrome, a mutation affecting the FBN1-gene which codes fibrillin-1, has an incidence estimate of 6.5/100 000 (Groth et al., 2015). At least 5% of all patients with thoracic aortic dissection have genetically confirmed Marfan's syndrome (Homme et al., 2006; Pape et al., 2007). Other inherited connective tissue diseases are Ehlers-Danlos syndrome and Loeys-Dietz syndrome (especially Type IV). Ehlers-Danlos and Loeys-Dietz syndromes both have multiple subtypes. Each mutation corresponding to different type. Mutations affect connective tissue by affecting gene coding integral protein in connective tissue or by affecting the homeostasis by affecting growth factors in connective tissue. (Loeys et al., 2006). Cystic medial degeneration characterizes the thoracic aorta in patients with inherited connective tissue diseases (Homme et al., 2006).

2.3.4 Genetics and dissection of thoracic aorta

The integrity of the aortic wall is not only associated with fibrillin; current estimates associate 20% of patients having thoracic aortic dissection with a first-degree relative having a thoracic aortopathy (Albornoz et al., 2006). These aortic diseases are described as non-syndromic familial aortopathy. Notable genes in this category include ACTA2 and MYH11. Both genes code muscle proteins, ACTA2 encodes the α-actin, and MYH11 encodes β-myosin heavy chain. Both mutations have been

associated with thoracic aortic dissection, smooth muscle cell hyperplasia, and vasa vasorum occlusion (Guo et al., 2009; Isselbacher et al., 2016; Zhu et al., 2006).

2.4 Ascending aortic dilatation and dissection

There is an association between aortic dilatation and the development of aortic dissection; the ageing aortic wall becomes thinner, dilates, and may increase the risk for aortic dissection upon high blood pressure (Coady et al., 1997; Erbel et al., 2014; Johansson et al., 1995; Masuda et al., 1992). On the other hand, aortic dilatation is not a prerequisite for aortic dissection (Pape et al., 2007), and remodeling of the aortic wall after dissection may lead to aortic dilatation (Durham et al., 2015; Fattori et al., 2013).

2.4.1 Ascending aortic dilatation is a clinical challenge

Aortic dilatation or aneurysm is a risk factor for potentially fatal dissection or aortic rupture. (Kim et al., 2016). Whereas it would be tempting to intervene on every dilated aorta, morbidity and mortality associated with surgery also remains high (DeBakey et al., 1982; Hagan et al., 2000). Symptomatic aortopathy may always be considered for intervention (Erbel et al., 2014). The growth speed of the aortic diameter determines decision-making for surgery or follow-up (Yiu & Cheng, 2016). The International Registry of Aortic Dissection (IRAD) shows that 60% of patients with acute type A aortic dissection have an aortic diameter less than 55mm (Pape et al., 2007). Aortic wall stiffness and loss of elasticity may impact the clinical outcome (Angouras et al., 2000; Prakash et al., 2012; H. Yamada et al., 2015). Dilatation and the diameter of aneurysm is not the only identified predictor for an aortic event in patients with TAA. While the diameter of the aorta is important, chronic lung disease, the presence of aortic thrombosis, decreased aortic wall elasticity, and increased intraluminal pressure are all associated with the increased risk of aortic events (Kim et al., 2015).

The association of increased intraluminal pressure with increased risk of aortic events may theoretically be explained by Laplace's law (Z. Wang et al., 2021).

$$\sigma \propto \frac{PR}{2t}$$

Figure 3. Law of Laplace. σ = wall tension, P = blood pressure, R = vessel radius, t = thickness of vessel wall

2.4.2 Classification of aortic dissection

Several classification systems for aortic events have emerged from academic and clinical needs. DeBakey and Stanford classifications are most popular. In DeBakey type I and II dissection, the intimal tear is located in the ascending aorta and the extent of dissection continues to the descending aorta in type I; while in type II, the extent does not reach distally to the innominate artery (De Bakey et al. 1961). According to the Stanford classification, type A aortic dissection includes the ascending aorta, and type B dissection encompasses the aorta distally from the left subclavian artery (Daily et al., 1970). The Svensson classification focuses on etiology (Svensson et al., 1999). Already in 1986, Roux and Guilmet proposed a classification based on the aortic segment, the primary entry site, the distal extent of antegrade false channel propagation, and the proximal extent of retrograde false channel propagation (Roux & Guilmet, 1986).

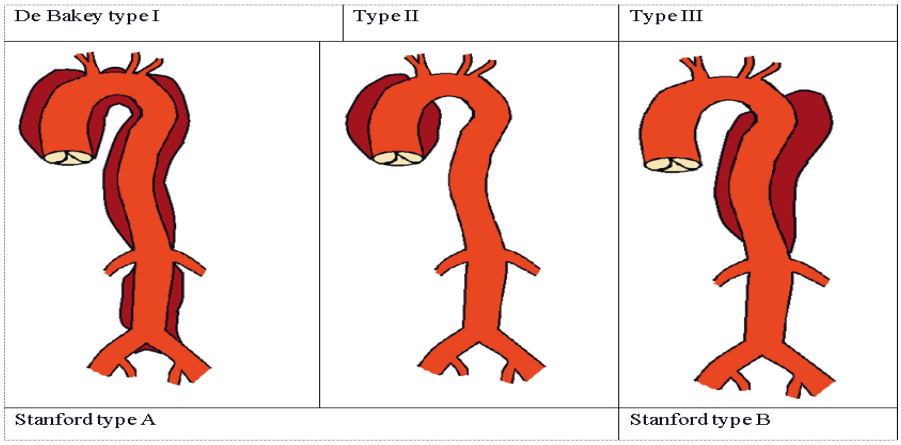

Figure 4. DeBakey and Stanford classification for aortic dissection

2.5 Diagnosis

High clinical vigilance of major symptoms such as hypotension, severe pain, cardiogenic collapse, and end organ symptoms due to hypoperfusion may suggest aortic events and dilatation. Imaging techniques, such as computed tomography angiography, ultrasound imaging, magnetic resonance imaging, and chest X-ray confirm the diagnosis.

2.5.1 Chest X-ray

Chest X-ray is easily available, but an unspecific means to diagnose aortopathy accurately (Von Kodolitsch et al., 2004).

2.5.2 Ultrasound imaging

Transthoracic and transesophageal ultrasound methodology afford viewing of the ascending aorta for prompt diagnosis of aortic events (Kabirdas et al., 2010; Labovitz et al., 2010).

2.5.3 Computed tomography angiography

Computed tomography angiography is widely available and relatively fast with high sensitivity and specificity of nearly 100% for the diagnosis of aortic events and aortopathy (Vardhanabhuti et al., 2016).

2.5.4 Magnetic resonance imaging

Magnetic resonance imaging remains an option for accurate imaging, especially if use of iodinated contrast dyes is contraindicated in some patients (Andreucci et al., 2017). However, magnetic resonance imaging is often unavailable during on-calls (Krishnam et al., 2010).

2.6 Treatment

Surgical resection and reconstruction continue to be the cornerstone of the treatment of aortic dissection and dilatation. Conservative treatment only without acute surgery is considered in Stanford type B -dissection (Erbel et al., 2014; Merola et al., 2013; Nienaber et al., 2009) without retrograde dissection and circulatory malperfusion. Current guidelines suggest performing surgery when the risk of rupture exceeds the risks of operation. This encompasses all symptomatic TAAs and TADs and TAAs with diameter over 55mm. In addition, the growth rate, location of aneurysm, family history of dissection should be weighed. Patients with known connective tissue disease should have it corrected with smaller diameters, the cut-off point dependent on the site of the aneurysm. The efficacy of the current guidelines has been demonstrated in clinical settings since their implementation (Saeyeldin et al., 2019). In acute and symptomatic dissections, prompt decision making is a prerequisite. Furthermore, slowly increasing dilatation of thoracic aorta requires close follow-up for good outcome (Erbel et al., 2014).

2.6.1 Surgical techniques

The extension of disease and possible valve pathologies define the chosen surgical technique. Techniques include replacement of the ascending aorta only; or with surgery of the aortic valve, the so-called Bentall procedure, David and Yacoub operations. The aortic arch may be replaced with or without reimplantation of the major aortic vessels, and either a traditional elephant trunk or a frozen elephant trunk-type of prosthesis may be used. Bentall, David and Yacoub procedures are applicable when an ascending aorta is affected. When an aortic arch or descending aorta are affected, separate techniques are required.

Replacement of the ascending aorta with a straight prosthesis may also include surgery for the aortic valve, if necessary, without replacing the aortic root. Most of the aortic procedures include replacement of the ascending aorta only (Gudbjartsson et al., 2019).

2.6.1.1 Bentall procedure (Conduit replacement of ascending aorta)

During the so-called Bentall procedure, the aortic root, aortic valve and ascending aorta are replaced with prosthesis. Coronary arteries are implanted to the graft via punched holes. The aortic valve prosthesis may include a bioprosthesis or a mechanical prosthesis. (Bentall & De Bono, 1968).

2.6.1.2 David procedure (valve-sparing aortic root replacement)

If the extent of aortopathy does not affect the aortic valve, a valve-sparing aortic root replacement may be considered. The aorta is resected just above, and distal to the aortic valve. Coronary buttons are resected, and a Dacron aortic prosthesis of aorta is sewn to the aortic annulus encompassing the sinuses and valves. Coronary buttons are sewn to the prosthesis. Aortic valve repair is performed when needed. (David & Feindel, 1992; David, 2014; David et al., 2015; Demers & Miller, 2004; Nezafati et al., 2015).

2.6.1.3 Yacoub procedure

The Yacoub procedure is another technique to spare the aortic valve during replacement of the ascending aorta together with the aortic root; the Yacoub operation also spares part of the aortic sinuses (Sarsam & Yacoub, 1993).

2.6.1.4 Surgery on the aortic arch

Aortic arch surgery includes meticulous planning and straightforward execution. Systemic hypothermia, selective cerebral perfusion, and circulatory arrest are often all needed to secure safe surgery. The brachiocephalic artery, left carotid artery and left subclavian artery stem straight from the aortic arch. These arteries often need to be implanted in the arch prosthesis during the surgery. A hanging trunk of prosthesis is inserted into the patient's aorta. Part of the prosthesis that is inserted into the patient's aorta is not sutured; hence the name "elephant trunk" (De la Cruz et al., 2012; Griepp et al., 1975).

2.6.1.5 Frozen elephant trunk

Endovascular treatment techniques may be considered for the treatment of the thoracic aorta originating distally to the left subclavian artery (Eggebrecht et al., 2006; Makaroun et al., 2005). Surgery may encompass a one-stage or two-stage operation (Gombert et al., 2019). In the case of circulatory malperfusion, a fenestration procedure may also be considered. A frozen elephant trunk prosthesis has recently been introduced as an option for some aortopathy encompassing the descending aorta. Contrary to the traditional elephant trunk, the frozen elephant trunk encompasses a meshed aorta prosthesis which is dilated into the aorta and which adheres to the aortic wall (Borst, Walterbusch, and Schaps 1983; Crawford et al. 1990; Orihashi et al. 2001; Suto et al. 1996). In the two-stage operation, usually the descending aorta is operated on later, using a separate endovascular stent or prosthesis (Borst et al., 1983; Carrel & Althaus, 1997; Inoue et al., 1997).

2.7 Mortality and outcome after surgery

Without immediate surgery, mortality is as high as 50% at the first two days after onset of symptoms in patients with acute type A aortic dissection (Mehta et al., 2002). Uncomplicated Type B has a mortality of 10% in the first 30 days without surgical intervention (Hagan et al., 2000). If the thoracic aorta ruptures, mortality varies between 27-100% (Goodney et al., 2011; Higgins et al., 2014). Both perioperative and long-term mortality and morbidity vary, depending on the site of the disease and surgery (Higgins et al., 2014).

2.8 Histology of thoracic aorta

The aortic wall consists of three distinct layers. The internal and external elastic laminae are rarely prominent in the thoracic aorta (Halushka et al., 2016).

2.8.1 Tunica Intima

The tunica intima is next to the bloodstream and includes mainly endothelial cells. The intimal endothelial layer is in contact with blood circulation and circulating blood cells (Dela Paz & D'Amore, 2009). Intima thickening of the thoracic aorta is

often due to atherosclerosis (Homme et al., 2006). An intimal entry tear is observed during dissection.

2.8.2 Tunica media

The tunica media consists of vascular smooth muscle cells, extracellular matrix, and small vessels. Vascular smooth muscle cells and two elastin fibers form a basic functional unit, the lamellar unit (Wolinsky & Glagov, 1967). The elastin fibers are further connected to microfibrils, collagen and proteoglycans, and together form the environment for lamellar units, the extracellular matrix. The lamellar unit produces the recoil capabilities of aorta (El-Hamamsy & Yacoub, 2009; Humphrey et al., 2014). Other components of extracellular matrix include fibulins and growth factors. Collagens (Types I and III) maintain aortic wall stability. Microfibrils form a mesh for the extracellular matrix. Fibulins are part of the lamellar unit with vascular smooth muscle cells and elastin (El-Hamamsy & Yacoub, 2009; Halper & Kjaer, 2014). Elastin is synthesized and regenerated by vascular smooth muscle cells (Eoh et al., 2017; Wanjare et al., 2015). Some matrix metalloproteinases and their endogenous tissue inhibitors (TIMPs) are secreted by vascular smooth muscle cells, neutrophils, macrophages (Johnson & Galis, 2004; Kurihara et al., 2012; Welgus et al., 1979). Substrates in the tunica media for MMPs include collagen, elastin, glycoprotein in the extracellular matrix and proteoglycans (Shen et al., 2015). Increased mechanical stress and increased matrix metalloproteinase expression may explain the association of hypertension with aortopathy (Ishii & Asuwa, 2000; Kurihara et al., 2012; Proietta et al., 2014; Seo et al., 2013; Xiong et al., 2012).

2.8.3 Tunica adventitia

The adventitia consists mainly of loose connective tissue and anchors the aorta to adjacent organs. Two thirds of vasa vasorum reside in the adventitia (Heistad et al., 1981). The adventitia includes the autonomic nervous system responsible for vasodilatation (Damon, 2005; DiBona & Jones, 2003), and includes progenitor cells, fibroblasts and growth factors milieu (Alberding et al., 2004; Greve et al., 2006; Howson et al., 2005; Weaver et al., 2006). T- and B-cells, macrophages, neutrophils and immunoglobulin activity have been described in the adventitia (Parums and Mitchinson 1981; Ramshaw and Parums 1990; Forney Prescott, Karboski McBride, and Court 1989).

2.8.4 Atherosclerosis

Atherosclerosis of the ascending aorta has traditionally been of minor concern when with TAA and TAD. An aneurysm in the abdominal aorta is often encountered with atherosclerosis, but the thoracic aorta is traditionally considered to be more resistant to atherosclerosis (Gu et al., 2011; Itani et al., 2004; Klima et al., 1983). Stroke may be associated with atherosclerosis in the thoracic aorta (Amarengo et al., 1992).

2.8.5 Cystic medial degeneration

Cystic medial degeneration describes the loss of vascular smooth cells, elastin degradation and accumulation of proteoglycans (Erdheim, 1930). Synonyms are Erdheim's necrosis, cystic medial necrosis, cystic necrosis, cystic degeneration, mucoid degeneration, and mucoid extracellular matrix accumulation. Cystic medial degeneration is a common finding in surgically resected thoracic aortas; found in 50–70% of all surgically resected aortas (Grewal et al., 2016; Homme et al., 2006; Ihling et al., 1999) including mucoid extracellular matrix accumulation, elastic fiber fragmentation and/or loss, elastic fiber thinning, elastic fiber disorganization, smooth muscle cell nuclei loss, laminar medial collapse, smooth muscle cell disorganization and medial fibrosis (Halushka et al., 2016).

2.8.6 Vasa vasorum

Vasa vasorum interna originate from the aortic lumen or media, whereas vasa vasorum externa originate from the adventitia reaching the media (Heistad et al., 1981; Kwon et al., 1998; Sano et al., 2014). The outside-in theory suggests that initial cellular damage occurs in the adventitia and the media. The inflammatory adventitia together with the outer media layer cause degeneration of the aortic wall described as cystic medial degeneration (Maiellaro & Taylor, 2007; Marcus et al., 1985; Osada et al., 2013). The inside-out theory suggests that the intimal damage via inflammation or mechanical stress causes aortopathy (Okamoto et al., 2001). Hypertension has been shown to promote vasa vasorum angiogenesis through hypoxia-related pathway (Kuwahara et al., 2002).

2.8.7 Angio- and lymphangiogenesis of vasa vasorum

The formation of new vessels from an existing vascular bed (angiogenesis) or from circulating progenitor cells (vasculogenesis) is a tightly controlled process in physiological conditions. Inflammation is related to angio- and vasculogenesis as pro-inflammatory factors such as vascular endothelial growth factor family (VEGF) and platelet-derived (PDGF) and fibroblast growth factor family (FGF), and tumor necrosis factor alpha (TNF-α) promotes neovessels growth (Dvorak, 2002; Enholm et al., 1997; Forsythe et al., 1996; Giraudo et al., 1998; Murakami & Simons, 2008; Sano et al., 2014; Scaldaferri et al., 2009; Taruya et al., 2015). The balance between the proangiogenic factors and antiangiogenic factors thereby determines the state of neovessels-based formation. Among these, proangiogenic factors include, e.g., Prospero homeobox protein 1 (PROX1) and vascular endothelial growth factor 3 (VEGF-3), both of which are induced by the nuclear factor kappa beta pathway and are considered major mediators in angiogenesis induced by inflammation. Antiangiogenic or angiostatic factors include multitude of mediators; among others, MMPs have been identified. The angiogenic balance does not seem to differentiate between lymphangiogenesis and angiogenesis, but both processes occur simultaneously when the balance shifts to angiogenesis (Flister et al., 2010; Kessler et al., 2014). Angiogenesis in vasa vasorum has been noted during vessel wall thickening, inflammatory processes and atherosclerosis (Mollmark et al., 2011; Moulton et al., 1999).

2.9 Aortic inflammation

Etiologies of non-infective aortitis include all several types of autoimmune vasculitis, autoimmune diseases with aortic manifestations, isolated aortitis, and idiopathic vasculitis (H. Wang et al., 2012). Giant cell activity, cystic medial degeneration and atherosclerosis may be present (Liang et al., 2009; Miller et al., 2006). IgG4-related systemic disease (Koo et al., 2014), giant cell arteritis and Takayasu (Svensson et al., 2015) are identified as rare causes of TAA. Infective aortitis of the thoracic aorta is very rare (Iimori et al., 2010; Lopes et al., 2009; Ranganath et al., 2013). Inflammation may be associated with risk of dissection and dilatation (Evans et al., 1995; Guard et al., 1995; Ishikawa et al., 2018; Kassar et al., 2017; Nuenninghoff et al., 2003). Inflammation increases oxygen consumption and tissue hypoxia (Eltzschig & Carmeliet, 2011). During thoracic aortic dissection, macrophages, neutrophils, T-

cells and mast cells are present (He et al., 2008; Wu et al., 2014). Activated macrophages are known to produce matrix metalloproteinases, cause smooth muscle cell apoptosis, and excrete growth factors and reactive oxygen species (Newby, 2006). Immunoglobulins may be increased during ascending aortic dilatation (Capella et al., 1996). Complement activation has been previously associated with the formation of intracranial aneurysms, including an interplay between chronic inflammation and locally impaired complement regulation (Capella et al., 1996; Glick et al., 2006; Niculescu et al., 2004; Tulamo et al., 2010).

2.10 Selected markers for analysis

Aortic wall degeneration, inflammation and hypoxia characterize aortopathy. Hypoxia may be associated with carbonic anhydrases, induction of complement activation, and inflammation. While the role of complements during aortopathy may be investigated by C4d, the presence of IgG4 may confirm tissue inflammation. Ongoing inflammation may induce aortic wall angiogenesis with endothelial CD31-positivity.

2.10.1 Complement component C4d (C4d)

C4d is a component in complement cascade. Its thioester bond binds it covalently to surrounding tissues. The covalent thioester bond produces long half-life and produces a complement 'footprint' ability which can be evaluated with, for example, immunohistochemistry (Zwirner et al., 1989). C4d was introduced as a marker for acute kidney and heart transplant rejection. C4d-deposition has been associated in aneurysms in smaller vessels. The presence of C4d in aortic samples has not been previously described (Regele et al., 2002; Rodriguez et al., 2005; Tulamo et al., 2010). C4d has no known biological function and is considered a remnant for complement activation. It should be noted that an alternative complement activation pathway does not produce C4d, as it does not cleave C4 (Murata & Baldwin, 2009).

2.10.2 Immunoglobulin G4 (IgG4)

Whereas Immunoglobulin G produced in plasma cells accounts for up to 75% of antibodies in human serum, the IgG4-subgroup is the most infrequent of the

immunoglobulin antibodies, accounting typically for less than 5% of serum Immunoglobulins (Bonilla, 2008). IgG4 seems to be more profoundly expressed in humans undergoing prolonged exposure to antigens and the effect of increased IgG4 has been demonstrated previously in chronic hay fever and novice beekeepers. (Devey, Wilson, and Wheeler 1976; Aalberse, van der Gaag, and van Leeuwen 1983). IgG4-related disease is associated with inflammatory sclerosing lesions storiform in histological appearance and is rich in IgG4-positive plasma cells, often with high IgG4 serum concentrations. Lesions have been found in organs within almost every system, such as the biliary system, salivary glands, orbital tissue, kidneys, lungs, lymph nodes, meninges, aorta, breast, prostate, thyroid, and skin (Dahlgren et al., 2010; Hamano et al., 2001; Kamisawa et al., 2010; Kitagawa et al., 2005; Saeki et al., 2006; Stone et al., 2009). (Stone et al., 2012). IgG4 sclerosing disease or IgG4-related systemic disease typically affect older men, and improvement during immunosuppressive therapy has been observed (Khosroshahi et al., 2010). IgG4-related aortitis is a significant subtype of aortitis and high serum levels of IgG4 may be measured (Kajander et al., 2013; Peng et al., 2020; Pérez-García et al., 2019).

2.10.3 Carbonic anhydrase IX (CAIX)

Carbonic anhydrase II and XII and their expression have been described in atherosclerotic lesions of arterial samples (Oksala et al., 2010). Carbonic anhydrases form a group of isoenzymes that catalyze the reaction from carbon dioxide and water to bicarbonate and water, and vice versa. Fifteen carbonic anhydrase isoenzymes have been found (Aggarwal et al., 2012; Hilvo et al., 2005). Among them, CAIX is expressed in multitude of cell lines which suggests a possibility for a role also in the aortic wall (Loncaster, 2002). The CAIX gene expression is regulated by the hypoxia induced factor 1 (HIF-1)- pathway. As HIF-1 is stabilized during hypoxia, CAIX may be a considered a surrogate of hypoxia (Chu et al., 2016; Logsdon et al., 2016).

2.10.4 Cluster of differentiation 31 (CD31)

CD31 is also known as platelet-endothelial cell adhesion molecule-1 (PECAM-1) (Newman et al., 1990). CD31 is a glycoprotein and is used to identify endothelial cells and thus vessels in immunohistochemistry (Müller et al., 2002; Pusztaszeri et al., 2006). Specifically, CD31 is found in endothelial cells and their junctions, additionally it can be identified in platelets and some inflammatory cells (Kimmig et

al., 2002; McKenney et al., 2001; Newman et al., 1990). CD31 is in clinical use for identifying vascular tumors, e.g., angiosarcomas (Sulliva et al., 2015). The use for identifying blood vessels through CD31 in immunohistochemistry has been well defined, and the extent of CD31-positivity correlates with angiogenesis (DeLisser et al., 1997). CD31 has been previously associated with angiogenesis (DeLisser et al., 1997).

3 AIM OF THE STUDY

The aim of this study was to compare systematic ascending aortic wall histology with some parameters of tissue inflammation and degeneration in patients undergoing ascending aortic surgery.

More specifically, we investigated:

1. Whether C4d aortic wall deposition is present during degenerative aortopathy?

2. Whether histological evaluation of the aortic wall and IgG4 investigation reveals concealed aortitis?

3. Whether CAIX aortic wall deposition is associated with aortopathy?

4. Whether CD31 aortic wall deposition and chronic inflammation are associated with aortopathy?

4 MATERIALS AND METHODS

4.1 Patients and surgery

After institutional review board (ETL 99204) approval and patient consent were secured, tissue samples were procured from patients undergoing surgery for thoracic aorta in Tays Heart Hospital. The decision for surgery and the surgical technique were at the discretion of the operating surgeon; computed tomography was performed on all patients preoperatively. The results of transthoracic cardiac ultrasounds were gathered from patient data when applicable. Altogether, there were 160 patients. The surgery period and total number of patients are shown on Table 1.

Table 1. Period of surgery and total number of patients in original publications

Study	Period of surgery	Total number of patients	Notable criteria
I (C4d)	2006–2009	91	-
II (IgG4)	2010–2012	5	only extended aortic dilatation
III (CA IX)	2008–2009	30	-
IV (CD31)	2009–2014	35	-

4.2 Tissue samples

Two to five aortic samples were obtained during surgery from representative resected parts of the ascending aorta. The samples procured during surgery were embedded in paraffin and cut to 4-5µm thick segments for further preparation and analysis.

4.3 Histology and immunohistochemistry

After preparation, the samples were stained with Hematoxylin and Eosin (H&E) and Verhoeff-van Gieson (VVG). Immunohistochemistry was performed using Ventana Lifesciences Benchmark XT© Staining module (Ventana Lifesciences, Tucson, Arizona, United States). Ventana Lifesciences Antibody Dilution Buffer© was utilized for the dilution media. Lab Vision Autostainer 480 (Lab Vision Corporation, Fremont, CA) was used for CAIX-immunostaining. The immunostainings were performed using Bright Vision Histostaining reagents (ImmunoLogic, Duiven, Netherlands). All utilized antigens are presented in Table 2. Primary parameters were C4d-positivity (I), CAIX -positivity in different layers (II), IgG4-positivity in plasma cells (III), and CD31-positivity and depth in the aortic wall (IV). For more detailed histological inspection, a set of secondary parameters was used. The secondary parameters evaluated for each aortic sample in all the studies were T-cells (CD3), B-cells (CD20), macrophages (CD68), plasma cells (CD138), proliferation (Ki-67), inflammation, medial degeneration, medial elastase, intimal thickness, and intimal cellularity. Von Willebrand factor, podoplanin, D2-40 and CD31 reflected capillary vessels, and actin showed the presence of smooth muscle cells.

Table 2. Names, sources and dilutions for antibodies used

Antibody	Dilution	Manufacturer	Source	Study
anti-C4d	1:50	Biomedica Gruppe	Rabbit, polyclonal antibody	I
anti-IgG4	1:100	Ventana	Sheep, polyclonal antibody	II
anti-CAIX	1:1000	Non-commercial product	Mouse, monoclonal	III
anti-CD31	1:30	DakoCytomation	Mouse, monoclonal antibody	I-IV
anti-vWf	1:2500	DakoCytomation	Rabbit, polyclonal antibody	I
anti-podoplanin	1:50	Angiobio co.	Mouse, monoclonal antibody	I
anti-D2-40	1:50	DakoCytomation	Mouse, monoclonal antibody	I
anti-CD68	1:70	DakoCytomation	Mouse, monoclonal antibody	I-IV
anti-CD3	1:50	Novocastra	Mouse, monoclonal antibody	I-IV
anti-CD20	1:1000	DakoCytomation	Mouse, monoclonal antibody	I-IV
anti-CD138	1:150	Serotec	Mouse, monoclonal antibody	I-IV
MIB-1	1:200	DakoCytomation	Mouse, monoclonal antibody	I-IV
Muscle actin	1:70	DakoCytomation	Mouse, monoclonal antibody	I-IV

4.4 Systematic quantification

The samples were semi-quantified and photographed using an Olympus DP25 (Olympus Optical Co., London, UK) microscope camera for T-cells, B-cells, macrophages, plasma cells, inflammation and cell proliferation, in accordance with the point score units from zero to three in all three anatomical layers (adventitia, media and intima) separately: 0 indicated no changes or evaluated cells; 1, mild changes or occasional target cells; 2, moderate changes or a moderate number of target cells; and 3, severe changes or great quantities of target cells. Medial degeneration, including elastase of the tunica media, were graded from 0–3; 0, no degeneration or not present; 1, patchy; 2, moderate; and 3, severe. The tunica intima, intimal thickness and cellularity were estimated using the same scale. For intimal cellularity, 0 encompassed a single endothelial layer with normal intima; 1, intimal cellularity and thickness, less than 25% as compared with the media; 2, intimal cellularity and thickness more than 25% but less than 50% as compared with the media; 3, intensive intima cellularity and thickness more than 50% as compared with the medial layer.

C4d-positivity was confirmed when intramural arterial vessels were present versus not present (I). The aortic wall was considered IgG4-positive if IgG4 were positively stained together with aortic wall plasma cells (II). CAIX-positivity included CAIX-staining within the aortic wall (III). The hotspots including CD31-positive blood vessels within the aortic wall were encountered, and the quantification was performed using Olympus Cellsens software (Olympus Soft Imaging System, version 1.6; IV).

4.5 Statistical analysis

Continuous variables are presented as mean and standard error of the mean, and categorial variables using percentages. The Mann-Whitney test was used for continuous variables and the chi-square test for categorical analysis (I, III and IV). The receiving operating characteristic curve was used to delineate the predictive value of the investigated marker (I, III and IV). The commercial statistical software SPSS (Chicago, IL) versions 19.0, 21.0 and 22.0 were used in studies I, III and IV, respectively. Statistical significance was defined as $P< 0.05$ (I, III and IV).

For comparison of histological and immunohistological analysis, the patients are grouped in accordance with the presence of aortic wall C4d-positivity and CAIX-positivity (I and III, respectively. The patients in Study IV are grouped in accordance with the presence of acute aortic dissection (IV).

5 RESULTS

5.1 Patient characteristics

The patient characteristics are shown on Table 3. The mean age was 64 ± 5 years, the patients were dominantly male, and hypertension was the most common comorbidity. Vasculitis was noted in one patient in studies I, III and IV. Previous CABG was confirmed in six, three and two patients in studies I, III and IV, respectively. The mean mid-ascending aortic diameter was 57 ±5 mm. Notable patient demographics are shown in Table 3.

Table 3. Patient demographics				
	I (C4d) n=91	II (IgG4) n=5	III (CAIX) n=30	IV (CD31) n=35
Age (years)	62 ± 13	64 ± 3	64 ± 3	64 ± 3
Male, n	61	3	20	24
Marfan, n	7	0	1	3
Hypertension, n	28	5	9	13
Diabetes, n	4	0	1	1
Hypercholesterolemia, n	11	0	4	3
Atherosclerosis, n	n/a	1	n/a	n/a
CAD, infarction, n	16	1	10	7
Obese, n	3	0	1	n/a
Tobacco, n	n/a	1	n/a	n/a
Vasculitis, n	1	0	1	1
Arthritis, arthrosis n	10	1	1	3
Asthma, n	0	0	3	2
History of malignancy, n	0	1	n/a	n/a
Myasthenia, n	1	0	n/a	n/a
Myositis, n	3	0	n/a	n/a
Dry eye syndrome, n	1	0	n/a	n/a
Diverticulitis, n	4	0	2	n/a
Gastritis, n	1	0	n/a	n/a
Gingivitis, n	2	0	1	n/a
Abdominal aneurysm, n	3	0	1	n/a
Hypothyroidism, n	n/a	0	1	n/a
Previous CABG, n	6	0	3	2
Previous aortic valve replacement, n	3	0	1	1
Previous aortic coarctation, n	3	0	2	0
Previous AAA surgery, n	1	0	0	1
Mid-ascending aorta diameter, mm	57 ± 9	54 ± 7	59 ± 2	59 ± 2
2-cusp aortic valve, n	26	1	8	9
Aortic valve insufficiency Moderate to severe, n	44	n/a	18	15
Aortic valve stenosis Moderate to severe, n	28	n/a	9	12

n/a = data is not available, CABG= coronary artery bypass grafting, AAA= abdominal aorta aneurysm

5.2 Surgical procedures

Graft replacement of the aortic root and ascending aorta was the slightly more common procedure in all studies in this thesis as compared with graft replacement of the ascending aorta only. The David operation was performed in four patients in study I, and one patient in study III. A mechanical valve was the most common choice for valve replacement in all studies. The surgical procedures and prosthesis utilized in the studies (studies I-IV) are summarized in Table 4.

Table 4. Surgery

Operation	I (C4d)	II (IgG4)	III (CAIX)	IV (CD31)
Total	91	5	30	35
Graft replacement of root and ascending aorta	52	0	16	20
Mechanical conduit	33	0	11	12
Biological conduit	15	0	4	8
David Operation	4	0	1	0
Graft replacement of ascending aorta	39	5	14	15
Mechanical valve with prosthesis	11	0	1	2
Biological valve with prosthesis	8	0	5	3
Prosthesis	20	5*	8	10

*Frozen elephant trunk prosthesis

5.3 Histology and immunohistochemistry

The main results of histology and immunohistochemistry are displayed in Table 5. Intimal inflammation, thickness, cellularity, and plasma cells were decreased in C4d-positive vs C4d-negative aortas. Concomitantly, the presence of vasa vasorum blood and lymphatic vessels was increased in C4d-positive aortas, as shown by vWf, D2-40 and Podoplanin. C4d-positive vessels were prominently found in adventitia when present. The intima did not show any C4d-positive vessels. (I). IgG4-positivity revealed previously concealed aortitis (II). Adventitial inflammation was increased in CA IX-positive aortas. The inflammation consisted of macrophages, B-cells and

cellular proliferation. Elastin degradation in tunica media was increased when CA IX -positivity was found. The intima showed increased macrophages in CA IX positive aortas vs CA IX negative aortas. No difference between the groups was found in intimal thickness (III). The outer third layer of the media at the vicinity of the adventitia showed an increased number of CD31-positive cells during aortic dissection as compared without aortic dissection (5.7 ± 1.3 vs 2.4 ± 0.7, $p < 0.016$), and corresponded to the site of the tear. Generally, histological assessment showed more inflammation in patients with aortic dissection as compared to patients without. Statistical significance was observed in adventitial and medial inflammation (2.2 ± 0.3 vs 1.3 ± 0.3, $p < 0.03$ and 1.5 ± 0.2 vs 0.3 ± 0.1, $p = 0$, respectively). An increased number of macrophages and T-cells of the intima were found during aortic dissection as compared to without (1.8 ± 0.2 vs 1.1 ± 0.2 $p<0.027$ and 1.3 ± 0.2 vs 0.6 ± 0.1, $p< 0.008$, respectively). Distinctive histologic features of ascending aortic dissection are shown in Table 5.

Table 5. Patient demographics										
Mean grade of		C4d+ (I)	C4d- (I)	p-value (I)	CA9+ (III)	CA9- (III)	p-value	AD+ (IV)	AD- (IV)	p-value (IV)
Adventitia	B cells	0.8 ± 0.8	1.0 ±1.1	Ns	1.5 ±0.2	0.7 ±0.2	<0.04	1.0 ±	1.0 ±	Ns
	Macrophages	1.7 ± 0.8	1.8 ±	Ns	1.9 ± 0.1	1.4 ±	<0.03	2.1 ±	1.5 ±	Ns
	Inflammation	1.8 ± 0.5	2.0 ±	Ns	2.2 ± 0.1	1.5 ±	<0.01	2.2 ±	1.3 ±	<0.003
	Proliferation	1.0 ± 0.8	1.2 ±	Ns	1.7 ± 0.1	0.7 ±	<0.02	1.5 ±	1.3 ±	Ns
	vWf density	25.0 ±	14.3 ±	<0.0001	----------	-------	--------	------	------	-------
	D2-40 density	5.1 ± 4.0	2.5 ±	<0.003	----------	-------	--------	------	------	-------
	Podo density	5.4 ± 5.2	2.5 ±	<0.004	-------	------	-------	-----	-----	-------
Media	Inflammation	1.0 ± 0.8	1.3 ±	Ns	1.3 ± 0.2	0.7 ±0.2	Ns	1.5 ±	0.3 ±	<0.0001
	Proliferation	0.9 ± 0.8	1.2 ±	Ns	1.3 ± 0.2	0.7 ±	Ns	1.5 ±	0.4 ±	<0.005
	Elastin**	1.4 ± 1.0	1.6 ±	Ns	1.7 ± 0.2	0.7 ±	<0.03	1.7 ±	1.6 ±	Ns
	CD31+ outer* third	----------	----------	----------	----------	-------	--------	5.8 ±	2.4 ±	<0.02
Intima	T cells	1.2 ± 0.9	1.4 ±	Ns	1.4 ± 0.1	1.0 ±	Ns	1.3 ±	0.6 ±	<0.01
	Macrophages	1.5 ± 0.9	1.8 ±	Ns	2.0 ± 0.1	1.2 ±	<0.01	1.8 ±	1.2 ±	<0.05
	Plasma cells	0.3 ± 0.6	0.6 ±	<0.05	0.5 ± 0.1	0.4 ±	Ns	0.6 ±	0.5 ±	Ns
	Inflammation**	1.3 ± 0.9	1.8 ±	<0.05	1.7 ± 0.1	1.3 ±	Ns	1.7 ±	0.7 ±	<0.05
	Thickness**	1.7 ± 0.9	2.1 ±	< 0.05	2.2 ± 0.2	1.7 ±	Ns	1.9 ±	2.1 ±	Ns
	Cellularity	1.5 ± 0.7	1.9 ±	<0.05	1.8 ± 0.1	1.4 ±	Ns	1.8 ±	1.3 ±	Ns

C4d+: Complement 4d-positive, C4d-: Complement 4d-negative, CAIX+: Carbonic anhydrase IX-positive, CAIX-: Carbonic anhydrase-negative, AD+: Aortic dissection present, AD-: Aortic dissection not identified, vWf: von Willebrand factor. *outer third indicating the nearest segment to adventitia. **Inflammation, thickness and elastin were estimated from 0–3 as described in 4.4. Others are expressed as arbitrary point scoring units 0–3.

5.4 ROC-analysis

C4d and CA IX were significantly associated with increased ascending aortic dilatation (AUC 0.792; S.E. 0.053; P<0.001; 95% C.I. 0.688-0.895, I and AUC 0.766; S.E. 0.0090; P=0.020; 95% C.I. 0.590-0.941, III, respectively). C4d was not associated with histologically identified ascending aortitis (AUC 0.523; S.E. 0.069; P=0.752; 95% C.I. 0.388-0.658, I). Local CD31 was associated with aortic dissection (AUC 0.750; S.E. 0.092; P=0.022; 95% C.I. 0.570-0.930, IV).

5.5 Early outcome

Early mortality comprises immediate perioperative mortality. Four patients died in study I, 3 in the C4d-negative group, and one patient in the C4d-positive group. In study III, which assessed CAIX-positivity in the aortic wall, two patients expired during the perioperative period. Both patients showed CAIX-positivity. In the study group in which CD31 was assessed, four patients died. Two with confirmed aortic dissection and two without dissection. Early mortality of the patients is presented in Table 6.

Table 6. Early outcome

Study I			
C4d-positive		53	1.9 % (1)
C4d-negative		38	7.9 % (3)
Total		91	4.4 % (4)
Study II			
IgG4-positive		1	0
IgG4-negative		4	0
Total		5	0
Study III			
CAIX-positive		20	10.0 % (2)
CAIX-negative		10	0
Total		30	6.6% (2)
Study IV			
Confirmed aortic dissection		14	14.3% (2)
No evidence of aortic dissection		18	11.1 % (2)
Total		32	12.5 % (4)

6 DISCUSSION

This translational study addresses some pertinent histopathologic features of the ascending aortic wall changes. Dissection of the thoracic aorta is an acute life-threatening condition necessitating prompt decision for surgery. Dilatation of the aorta is traditionally considered a risk factor for rupture and dissection, though aortic events may also occur without dilatation (Pape et al., 2007). The arbitrary borderline diameter of up to 5 cm of the ascending aorta may not solely be an absolute indication for surgery. After chronic dissection, the aorta may further dilate, and surgery is carefully considered. On the other hand, aortic surgery, least to say emergent or salvage surgery, encompasses major surgical and postoperative risks.

6.1 Rationale for tissue sampling

Research on aortic wall tissue changes and molecular indices associated with aortic dilatation and dissection may aid the clinician to identify and predict aortic events in the future. The methods used in this study aim to increase understanding of the process leading to aortic dilatation and dissection. The common denominator in patients with chronic dissection and ongoing aortic dilatation was aortic wall inflammation, including occasional plasma cells, macrophages, and T and B cells (Study II).

Though the rationale for the clinical adoption of a frozen-elephant trunk technique for the treatment of extensive aortic dilatation is still debated world-wide, tissue analysis aids in understanding the ongoing histopathological features of diseases leading to aortic dilatation. Systematic evaluation of aortic wall inflammation also revealed concealed aortitis (Study II). The presence of IgG4 positivity confirmed that a patient without chronic dissection had IgG4-positive aortitis; this led to close postoperative surveillance and conservative long-term medical treatment and suggests that serum IgG4-level measurements should be evaluated for possible underlying disease. Clinically, it is well-advised to plan a patient-tailored

postoperative treatment based on histopathology after extensive aortic surgery (Study II).

6.2 Chronic inflammation, hypoxia, and ascending aortic wall dilatation

An ongoing chronic inflammation is often associated with ascending aortic wall dilatation (I, III, IV). CAIX was found during aortic wall remodeling and adventitial inflammation that were present in the dilated aortic wall (III). The presence of macrophages and local scattered aortic wall inflammation with CAIX positivity suggest chronic aortic wall degeneration during aortic dilatation (III). CAIX is present during hypoxic conditions (Shin et al., 2008, 2011), and may thus reflect ongoing aortic remodeling and dilatation.

In study I, C4d-positivity of the aortic wall indicated stabile non-dissecting ascending aortic dilatation and was not associated with aortitis. We also noted that angiogenesis and lymphangiogenesis was increased in C4d-positive aortas. Increased microvasculature has been previously associated with decreased aortic wall strength and risk for TAD, but our study suggests a more complex role of complement in TAD as a possible stabilizing mechanism (I). The protective ability of complement activation may involve solubilization and degradation of cell debris and apoptotic cells that are proinflammatory by nature (Zhang et al., 2003). Complements have been associated with the development of atherosclerosis, the intracranial saccular aneurysms, and the myocardium of ischemic and cardiomyopathy (Ge et al., 2018; Tulamo et al., 2010).

6.3 Acute dissection and aortic dilatation

Tissue frailty, as observed during chronic hypertension, aortic dilatation and trauma predisposes to acute aortic wall tear during aortic dissection. Neovascularization of the aortic wall during chronic inflammation may trigger the essentially frail borderline of the media and adventitia to sudden rupture and longitudinal aortic wall tear during aortic dissection (IV). Vasa vasorum and disturbances of their blood flow may lead to aortic events (Kajander et al., 2013). The predisposing factor being aortic wall changes prior to neovascularization indicates that systematic, metabolic, and

inflammatory factors, together with connective and genetic tissue disorders, add to the development of aortic remodeling that initiates chronic aortic wall inflammation. In turn, chronic aortic wall inflammation, an increased number of proliferative cells, and macrophages at local vulnerable sites of hypoxia initiate angiogenesis and neovascularization, further increasing tissue wall frailty and susceptibility to dissection (IV). Interestingly, in our study (I) we found that remnants of complement activation were associated with stabile but degenerative aortas and showed increased microvasculature as compared to the C4d-negative aortas. The presence of increased microvasculature during some degenerative aortopathy may characterize the stiff atherosclerotic aorta that is not prone to dissection. Vascular stiffness during periadventitial activation of inflammation may produce C4d deposition that binds to collagen and elastin fibers through covalent thioester bonds, leading to increased aorta wall stiffness (Shields et al., 2011).

6.4 Study limitations

Limitations of this study include the limitations of histology with nature of aortopathies. Histology was only available after surgery. Currently no possibility for safe aortic biopsy is available. For the same retrospective nature, during AD it is possible that the histological findings represent the hyperacute response to dissection, not the process behind rupture. The limited number of patients and samples from a single surgical center determine the limitations of the retrospective analyses. The site of sample procurement was decided at the discretion of the surgeon but included the resected part of the ascending aorta (I, II, III) and the aortic arch (IV). The macroscopically most vulnerable resected aortic wall was procured for analyses during surgery. Inflammation during subacute and chronic ascending aortic dissection are distinctively different from the acute phase of dissection. The nature of aortitis may have also been affected by temporal changes during various inflammatory phases. Though specifically medical anti-inflammatory treatment was not evaluated in the study, aortitis was only diagnosed after the tissue analyses (IV). Systematic long-term follow-up of the patients was not investigated.

6.5 Clinical implications

The etiology of ascending aortic dilatation and dissection is complex and heterogeneous. Ascending aortic dilatation and related degeneration form a dynamic process that may lead to aortic dissection and rupture. We speculate that immunology impacts aortic dilatation and dissection. Our observations suggest a critical role of complement C4d, vasa vasorum angiogenesis, hypoxia and IgG4 in aortic events (I-IV). Surgery for the ascending aorta and aortic wall tissue analysis combines clinical and translational research that adheres to follow-up of the patients, ensures quality control after surgery, and enables the investigation of novel molecular pathways affecting the development of aortic wall dilatation and dissection. The early aortic disease outcome may be predicted using immunohistochemical analysis that aids systematic patient follow-up planning after surgery for the ascending aorta.

Tissue sample immunohistochemistry enhances awareness of the heterogenic nature of the ascending aortic disease. The aim of this study was to compare systematic ascending aortic wall histology with some parameters of tissue inflammation and degeneration. The presence of complement C4d, IgG4, CAIX, and CD31 in the ascending aortic wall reflect tissue remodeling associated with dissection and aortic wall degeneration. The lack of C4d, increased IgG4 and CD31 suggest for increased risk of dissection, while CAIX may indicate aortic wall stability. These initial one-center analyses warrant further molecular investigation of the aortic tissue pathology to clarify the risk for aortic events such as dissection.

7 CONCLUSIONS

Taken together, we showed that:

1. C4d aortic wall deposition is present during some aortopathy.
2. Histological evaluation of the aortic wall may reveal concealed aortitis.
3. CAIX aortic wall deposition is associated with ascending aortic dilatation.
4. CD31 aortic wall deposition and chronic inflammation add to the understanding of the aortic remodeling process.

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9 ORIGINAL COMMUNICATIONS

PUBLICATION I

Lack of C4d deposition may reveal susceptibility for ascending aortic dissection

Niinimäki E, Paavonen T, Valo T, Tarkka M, Mennander A.

Scand Cardiovasc J 2012;46:177-182.
doi: 10.3109/14017431.2012.663502..

Lack of C4d deposition may reveal susceptibility for ascending aortic dissection

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Abstract

Objectives. Complement activation as evidenced by C4d deposition indicates immunological tissue reactivity. We sought to study the vascular reactivity of the aortic wall by characterizing C4d deposits.

Design. Aortic wall histology and immunohistochemistry for C4d, leukocytes, T- and B-lymphocytes, plasma cells, macrophages, endothelial cells, smooth muscle cells, cell proliferation, elastase and Van-Gieson-staining were performed to 91 consecutive patients that underwent surgery for ascending aorta, and the samples were grouped according to presence of C4d deposits.

Results. Fifty-three out of 91 patients had C4d deposits mainly within the adventitia (C4d+), whereas 38 patients lacked C4d deposits (C4d-) including decreased staining of intra-aortic vessels ($p < 0.005$). Intimal thickness and cellularity, together with inflammation consisting of plasma cells were increased in C4d- as compared with C4d+ ($p < 0.05$). Receiver operating characteristic curve (ROC) analysis showed that C4d was associated with stabile non-dissecting ascending aorta (AUC 0.792; S.E. 0.053; $p = 0.000$; 95% C.I. 0.688-0.895), but not with presence of aortitis per se (AUC 0.523; SE 0.069; $p = 0.752$; 95 % CI 0.388-0.658).

Conclusions. Lack of C4d may indicate active remodeling of the aortic wall leading to AD. Immunologic complement factors may be amenable to diagnosis of instability after aortic surgery.

Keywords. C4d, ascending aortic dissection, inflammation

Introduction

Prompt surgery is required for ascending aortic dilatation (AA) prone to rupture to prevent life-threatening aortic dissection (AD) (1). While an arbitrary borderline of 5-cm enlargement of the diameter of the ascending aorta has been shown to increase the risk for AD, there is increasing awareness that aortic remodeling and subsequent AD may occur at an earlier stage of AA occasionally with aorta diameters less than 4.5 cm (1). Risk for AD may be associated with different molecular mechanisms of the aortic wall as compared with AA alone (2,3). AD is considered to initiate from a sudden tear in the aortic wall endothelium due to decreased resiliency (4). The origin may occur in the vasa vasorum of the aortic wall, which leads to intramural hematoma that induces subsequent AD (5).

Increasing evidence suggest that complement factors are involved during inflammatory remodeling of the arterial wall (6). Significant amounts of complement components are found in atherosclerotic lesions compared to healthy arteries (6,7). Increased amounts of complement end products are associated with development of intracranial saccular aneurysms (8). Pathogenesis of aortic valve stenosis (7) and experimental aortic dilatation (9) have been associated with complement activity. These findings may implement that complements play a crucial role in the extension of chronic lesion formation devoid of arterial wall rupture.

We hypothesized that the complement cascade is present during AA and AD indicating aortic wall remodeling. In this study, we define aortic wall remodeling as histologically confirmed aortic wall changes during AA and AD.

Materials and methods

Study protocol and surgery

After IRB approval, ascending aortic wall resection of 102 consecutive patients undergoing surgery for ascending aorta was obtained and processed for histology. AA was preoperatively confirmed and evaluated with computer tomography (CT). According to our institutional policy, AA included aortic diameter more than 5.5 cm wide or aortic growth more than 1 cm in a year. This definition was adjusted to presence of Marfan syndrome, gender, patient size, and symptoms including AD according to The Yale Center criteria (10). Surgery was performed between December 2006 and August 2009 in the Heart Center of Tampere University Hospital, and all cases of AA including AD processed for histology were enrolled. The samples of 11 patients were excluded due to technical failure during preparation of the samples.

Decision on the extension of resection and surgical technique was at the discretion of the operating surgeon. When AA including the sinotubular junction (STJ) was estimated as the reason for aortic regurgitation, STJ was tailored for a suitable graft in a supracoronary fashion. Whenever dilatation included the aorta root, a radical resection of the dilated ascending aorta together with the root and the aortic valve was performed in all but four patients, in whom a David-type valve-sparing operation was achieved. The graft size was estimated by the principal surgeon. Resection of the aortic arch was carried out depending on extension of distal aortic dilatation. Since the surgical procedure was performed upon surgical decision, the sample was procured from the middle of the resected diseased area of the ascending aorta at the vicinity of STJ.

Histology and immunochemistry

Two to five blocks of resected ascending aorta were embedded in paraffin and cut to 4-µm-thick segments and stained with hematoxylin and eosin (H&E), Verhoeff-van Gieson (VVG), elastase-van Gieson (EVG) and periodic acid-Schiff (PAS). A representative 1-cm-long piece of ascending aortic wall corresponding to all different staining was evaluated systematically for all resected samples procured during surgery. The heights of different layers (adventitia, media, and intima) were calculated for each sample. Inflammatory cells, intensity of inflammation, medial degeneration, intima cellularity, and thickness were estimated as previously described and expressed as point score units (PSU) (2).

Immunohistochemistry was performed using Ventana Lifesciences Benchmark XT© Staining module. Vasa vasorum were evaluated using Polyclonal Rabbit antibody for Von Willebrand factor (dilution 1:2500) (DakoCymation). Lymphatic vessels were evaluated using Podoplanin (dilution 1:50) (Angiobio Co.) and monoclonal mouse antibody D2 - 40 (dilution 1:50) (DakoCymation) (11, 12). C4d (dilution 1:50) (Biomedica Gruppe) was stained for slides, accordingly. Ventana Lifesciences Antibody Dilution Buffer© was utilized for dilution media.

Statistical Analysis

C4d + staining was predominantly found in the adventitia, at the border of the media including endothelial cell lining of small vessels (Figure 1). In order to seek for clinical relevance associated with histology, the patients were divided into two groups according to presence of C4d staining of small vessels of the aortic wall. Patients with C4d + staining of the ascending aortic wall were referred as C4d +, and those without C4d staining as C4d-Φ Quantitative variables are listed as mean and standard error of the mean. Categorical variables are stated as count and percentage. Statistical analysis was performed with SPSS version 19.0. Mann–Whitney-test was used for continuous variables and chi-square-test for categorical analysis. The predictive value of C4d to

identify AD among patients with AA only was assessed by receiver operating characteristic curve (ROC) analysis. P-values less than 0.05 were considered statistically relevant.

Figure 1. Representative photograph (× 40) of aortic adventitial wall immunohistochemistry of C4d during ascending aortic dilatation (A) and during ascending aortic dissection (B). Note positive C4d staining (arrows) in A suggesting stabile aortic wall during aortic dilatation.

Results

Patient characteristics (Table 1)

There were twice as many male than female in the study. The study population consisted of 61 male and 30 female. Mean age for the patients was 62 ± 13 years. Hypertension was diagnosed in only 28 patients, and no difference in its prevalence between the groups was found. There were seven patients (8%) with Marfan syndrome, of whom the majority had C4d positivity. The one patient with a nonspecific vasculitis in our study group was also C4d +. The frequency of preoperative inflammatory state, such as myositis and arthritis, showed no difference between the groups. The mean diameter of the ascending aorta at the sinotubular junction was 57 ± 9 mm for all patients. Interestingly, moderate to severe aortic valve stenosis (AVS) was found to be more frequent in C4d + group with 21 patients (40%) in contrast to 7 patients (19%) with C4d negativity, whereas aortic valve insufficiency (AVI) was equally present among the groups. The majority of the patients had tricuspid aortic valve. Ten patients with C4d positivity and 4 patients with C4d negativity underwent a previous cardiothoracic operation.

Operative technique (Table 2)

Graft replacement for the ascending aorta was performed either with root replacement (in 52 patients), or without encompassing the root (in 39 patients). The extension of root dilatation together with dilatation of the ascending aorta from STJ was remarkably equally distributed among the patients in both groups being 57% and 58% in C4d + and C4d −, respectively. A valve-sparing operation David operation was offered for four patients. Concomitant coronary artery bypass grafting was required to 12 patients.

Perioperative findings, histology and immunohistochemistry (Table 3 and 4)

Confirmed by histology, the aorta had acute or chronic dissection in 17 out of 38 patients (45%) with C4d − staining of the aorta. In contrast, only one patient had acute and three patients had chronic dissection in the group with C4d positivity (11%). Though dissection was undeniably diagnosed histologically, in six patients (five with C4d positivity and one with C4d negativity), the adventitia was difficult to evaluate for every inflammatory cells in every sample block due to blood stains and artifact. Altogether, 13 patients with C4d positivity and 6 patients with C4d negativity had histological findings of aortitis. Four patients died immediately, including 3 patients with AD and C4d negativity. In contrast, only one C4d + patient with a history of previous coronary artery bypass operation died due to perioperative heart failure.

In C4d − aortas, vasa vasorum small vessels of the adventitia, as detected by vWF density, were decreased in number as compared with C4d + aortas ($p < 0.0001$). Also, small lymphatic vessels were less numerous in C4d − group as observed with D2-40 and podoplanin stainings and compared with C4d + aortas ($p < 0.003$ and $p < 0.004$, respectively). The intima did not show any positivity of C4d, and C4d + vessels were observed in the media only whenever they were present in the adventitia. Intimal thickness and cellularity were increased in C4d − aortas (2.1±0.9 PSU and

1.9±0.7 PSU, respectively) as compared with C4d + aortas (1.7±0.9 PSU and 1.5±0.7 PSU, respectively), mostly owing to presence of inflammatory plasma cells in the intima ($p < 0.05$).

ROC curve analysis (Figure 2)

The predictive value of C4d + staining to identify AD from operated AA only was assessed by ROC analysis. C4d staining was significantly associated with nondissecting AA (AUC 0.792; SE 0.053; $p = 0.000$; 95% CI 0.688–0.895), but not with histologically identified ascending aortitis per se (AUC 0.523; SE 0.069; $p = 0.752$; 95% CI 0.388–0.658).

Discussion

In this study, C4d positivity was frequently present in patients who underwent surgery for ascending aorta. Patients with AA without AD showed clear C4d deposition indicating aortic wall remodeling associated with complement activation. Instead, C4d deposition was predominantly lacking in patients with AD.

The majority of the patients in our study were male, in whom hypertension was well-controlled. The extension of aortic dilatation, for example, to the root was not predictable by aortic wall C4d positivity. Histopathology revealed significant aortitis in 19 patients, of whom 7 had Marfan syndrome. Neither aortitis nor previous thoracic operation was statistically associated with either C4d positivity or negativity. C4d complement deposition was present during remodeling phase of the aortic wall without major aortic inflammation or intimal reactivity. According to ROC curve, C4d deposition predicted AA, but not aortitis. Activation of complement reaction has been proposed as an initiator of lesion formation, as modified lipoproteins initiate complement cascade in in vitro models (13). While the ascending aorta is relatively rarely affected with atherosclerosis as compared with the descending aorta, presumably due to embryological differences of the sites of the aorta (14), clinical observations approve for a protective nonactive aortic wall against AD during the degenerative AA (6,9).

Frequently, C4d depositions were devoid in patients with AD, while an important decreased number of adventitial lymphatic and vasa vasorum vessels together with inflammatory plasma cell infiltration in the intima were observed. The main difference in pathogenesis between AA and AD may be associated with vasa vasorum. Disturbances in media and adventitia vasa vasorum induce remodeling of the whole arterial wall (5). Rupture of aortic wall vasa vasorum vessels cause intramural hematoma, which eventually affects the integrity of aortic wall and leads to AD (5). It is

tempting to suggest that lack of C4d deposition is associated with changes of complement activation of the aorta during AD.

This study also revealed aortic disease undetected clinically before surgery. As stated above, diagnosis for AD was always confirmed by histology. Meticulous and systematic histopathological analysis was performed for all samples (

), and included widely acknowledged immunohistochemical methods to evaluate inflammatory cells. We acknowledge the challenge for the resection to include impeccably all three layers of aortic wall during AD; indeed, though AD was undeniably diagnosed histologically, the adventitia was difficult to evaluate for every inflammatory cells in every sample block due to blood stains and arterfact in six patients. We did not, however, exclude these patients, since statistical analysis was not confounded with or without these patients. Interestingly, immediate death occurred in four patients, including three with C4d negativity. All these three had AD, while only one C4d + patient with a history of previous coronary artery bypass operation died but only due to perioperative heart failure. Obviously, it is too early to associate early mortality with histology of the aortic wall, least to mention with C4d negativity, and subsequent follow-up of our patients is warranted.

To our best of knowledge, the association of complement factors has not been studied earlier during AA and AD. Complement factors are ubiquitous molecules, which are produced mainly in the liver, though some local production is observed during target tissue inflammation (15). Complement cascade has been proved to play an important role in cardiovascular diseases (13,16). Intracranial saccular aneurysms show increased complement activation (8). The protective ability of complement activation may involve solubilization and degradation of cell debris and apoptotic cells that are proinflammatory by nature (17). A study using rats devoid of complements showed that

experimental arterial lesions consisted of increased number of apoptotic cells as compared with control rats owing intact classical complement pathways (18). C4d positivity may therefore indicate vascular wall stiffness through a mechanism initiated within the adventitia (6). Vascular wall stiffness is tentatively defined as increased aortic wall degeneration related to atherosclerosis (6). Instead of a traditional “inside-out” theory of intima endothelial cell activation and subsequent aortitis, a potential “outside-in” mechanism of vascular stiffness during periadventitial activation of inflammatory adipose tissue may produce C4d deposition that bind to collagen and elastin fibers through covalent thiolester bonds leading to increased aorta wall stiffness (6). End products of complements, such as C5b-9, are prominent during atherosclerosis and aortic valve stenosis (19). Interestingly, 21 out of 53 patients (40%) with C4d aortic wall deposition and thus AA had also moderate to severe aortic valve stenosis, in contrast to 7 out of 38 patients (19%) without C4d aortic wall deposition. It remains to be investigated whether presence of important aortic valve stenosis and C4d aortic wall deposition are both associated with AA but not with AD.

A protective role of complements has previously been associated with the classical complement pathway (14). As C4d is the end product for classical and lectin pathways, the activation mechanics for C4d cannot be further differentiated (16,20). The complement activation of the alternative pathway may also be involved (20). To date, there has not been found any physiological role or receptor for C4d, but it is considered a remnant of C4b. On the other hand, complement activation may be associated with different complements than C4d. It is unfortunately beyond the scope of this study to further differentiate different complement pathways associated with AA and AD. Again, our study group is relatively small and postoperative follow-up includes only c.a. 1 year. However, as presence of complement C4d deposition seems beneficial for the integrity of aortic wall during AA, further investigation on complement activation associated with aortic remodeling is much appreciated based on this systematic pilot analysis.

Taken together, immunologic complement factors may be amenable to follow-up after surgery for AA. Since C4d is the end product of complement reaction and has a relatively long half-life due to its ability to form covalent bindings among other molecules, we speculate whether evaluation of presence of C4d deposition via immunohistochemistry enables one to identify aortic wall prone to AD. It remains to be investigated whether intervening with complement cascade may add an armament against AD..

A protective role of complements has previously been associated with the classical complement pathway (14). As C4d is the end product for classical and lectin pathways, the activation mechanics for C4d cannot be further differentiated (15, 19). The complement activation of the alternative pathway may also be involved (19). To date there has not been found any physiological role or receptor for C4d, but it is considered a remnant of C4b. On the other hand, complement activation may be associated with different complements than C4d. It is unfortunately beyond the scope of this study to further differentiate different complement pathways associated with AA and AD. Again, our study group is relatively small and postoperative follow-up includes only c.a. 1 year. However, as presence of complement C4d deposition seems beneficial for the integrity of aortic wall during AA, further investigation on complement activation associated with aortic remodeling is much appreciated based on this systematic pilot analysis.

Taken together, immunologic complement factors may be amenable to diagnosis of instability after surgery for AA. Lack of C4d deposition may suggest active aortic wall remodeling prone to AD. It remains to be investigated whether intervening with complement cascade may add an armament against AD.

Table 1

Patient demographics

	All patients	C4d+	C4d-
Number of patients	91 (100%)	53	38
Age (years)	62 ± 13	61 ± 13	64 ± 13
Male, n (%)	61 (67%)	37	24
Hypertension, n (%)	28 (31%)	15	13
Diabetes, n (%)	4 (5%)	2	2
Hypercholesterolemia, n (%)	11 (12%)	7	4
Marfan, n (%)	7 (8%)	5	2
Obese, n (%)	3 (4%)	2	1
Myasthenia, n (%)	1 (1%)	1	0
Myositis, n (%)	3 (4%)	1	2
Arthrosis, arthritis, n (%)	10 (11%)	4	6
Dry eye sydrome, n (%)	1 (1%)	1	0
Diverticulitis, n (%)	4 (5%)	3	1
Vasculitis, n (%)	1 (1%)	1	0
Gastritis, n (%)	1 (1%)	1	0
Gingivitis, n (%)	2 (2%)	1	1
Abdominal aneurysm, n (%)	3 (4%)	2	1
Myocardial coronary artery disease, infarction, n (%)	16 (18%)	9	7
Previous cardiothoracic operation			
Coronary artery bypass surgery, n (%)	6 (7%)	4	2
Aortic valve replacement, n (%)	3 (4%)	2	1
Correction of aortic coarctation, n (%)	3 (4%)	3	0
St post AAA operata	2 (2%)	1	1
Mid-ascending aorta diameter, mm	57 ± 9	55 ± 8	60 ± 17
3-cusp aortic valve, n (%)	65 (72%)	33	32
Aortic valve insufficiency			
Moderate to severe, n (%)	44 (49%)	28	16
Aortic valve stenosis			
Moderate to severe, n (%)	28 (31%)	21	7

Table 2

Operative details according to surgical evaluation of extension of diseased aorta

	All patients	C4d+	C4d-
	91 (100%)	53	38
Graft replacement of root and ascending aorta			
Mechanical conduit	33 (37%)	19	14
Biological conduit	15 (17%)	7*	8*
David operation	4 (5%)	4	0
Graft replacement of ascending aorta			
Mechanical valve + prosthesis	11 (12%)	9	2
Biological valve + prosthesis	8 (9%)	6	2
Prosthesis	20 (22%)	8*	12*
Additional procedures			
Thymectomy	1 (1%)	1	0
Ablation	2 (2%)	2	0
Mitral valve plasty	1 (1%)	0	1
Coronary artery bypass surgery	12 (13%)	7	5

* includes 1 patient with prosthesis extending up to the aortic arch

Table 3. Histology and quantitative immunohistochemistry

Mean grade of staining		All patients	C4d+	C4d-	p- value
Adventitia	T cells	1.6 ± 0.8	1.5 ± 0.7	1.7 ± 0.8	Ns
	B cells	0.9 ± 0.9	0.8 ± 0.8	1.0 ± 1.1	Ns
	Macrophages	1.8 ± 0.8	1.7 ± 0.8	1.8 ± 0.7	Ns
	Plasma cells	1.1 ± 0.9	1.1 ± 0.8	1.2 ± 1.1	Ns
	Inflammation	1.9 ± 0.7	1.8 ± 0.5	2.0 ± 0.8	Ns
	Thickness	5.5 ± 4.8	5.0 ± 2.5	6.2 ± 6.9	Ns
	Proliferation	1.1± 0.8	1.0 ± 0.8	1.2 ± 1.0	Ns
	vWF density	20.3 ± 12.3	25.0 ± 11.7	14.3 ± 10.9	< 0.0001
	D240 density	4.1 ± 3.8	5.1 ± 4.0	2.5 ± 2.8	< 0.003
	Podo density	4.2 ± 4.5	5.4 ± 5.2	2.5 ± 2.6	< 0.004
Media	T cells	0.6 ± 0.9	0.5 ± 0.8	0.7 ± 1.0	Ns
	B cells	0.2 ± 0.5	0.1 ± 0.4	0.2 ± 0.6	Ns
	Macrophages	1.3 ± 0.9	1.1 ± 0.8	1.5 ± 1.0	Ns
	Plasma cells	0.2 ± 0.7	0.3 ± 0.7	0.2 ± 0.6	Ns
	Inflammation	1.2 ± 0.9	1.0 ± 0.8	1.3 ± 1.0	Ns
	Proliferation	1.1 ± 0.9	0.9 ± 0.8	1.2 ± 1.0	Ns
	Degeneration	1.7 ± 1.0	1.6 ± 1.0	1.8 ± 1.0	Ns
	Elastase	1.5 ± 1.0	1.4 ± 1.0	1.6 ± 1.1	Ns
	vWF density	0.6 ± 1.1	0.5 ± 0.8	0.7 ± 1.3	Ns
	D240 density	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.3	Ns
	Podo density	0.1 ± 0.2	0	0.1 ± 0.3	Ns
Intima	T cells	1.3 ± 0.9	1.2 ± 0.9	1.4 ± 0.9	Ns
	B cells	0.2 ± 0.5	0.2 ± 0.5	0.2 ± 0.4	Ns
	Macrophages	1.6 ± 0.9	1.5 ± 0.9	1.8 ± 0.9	Ns
	Plasma cells	0.4 ± 0.7	0.3 ± 0.6	0.6 ± 0.9	< 0.05
	Inflammation	1.5 ± 0.9	1.3 ± 0.9	1.8 ± 0.9	< 0.05
	Proliferation	0.9 ± 0.8	0.8 ± 0.7	1.0 ± 0.8	Ns
	Thickness	1.9 ± 0.9	1.7 ± 0.9	2.1 ± 0.9	< 0.05
	Cellularity	1.7 ± 0.7	1.5 ± 0.7	1.9 ± 0.7	< 0.05
	vWF density	0.2 ± 1.2	0.1 ± 0.6	0.3 ± 1.6	Ns
	D240 density	0.1 ± 0.6	0.1 ± 0.5	0.1 ± 0.7	Ns
	Podo density	0.1 ± 0.4	0.1 ± 0.4	0.1 ± 0.4	Ns

Table 4

Postoperative outcome

	All patients	C4d+	C4d-
	91 (100%)	53	38
Acute dissection	15 (17%)	1	14
Subacute dissection	6 (7%)	3	3
Aortitis	19 (21%)	13*	6
Pulmonary embolism	1 (1%)	1	0
Mediastinitis	1 (1%)	1	0
Mortality	4 (5%)	1	3

* includes 1 patient with giant cell aortitis and 1 patient with temporal arteritis

Legends

Figure 1. Representative photograph (x 40) of aortic adventitial wall immunohistochemistry of C4d during ascending aortic dilatation (A) and during ascending aortic dissection (B). Note positive C4d staining (arrows) in A suggesting stabile aortic wall during aortic dilatation.

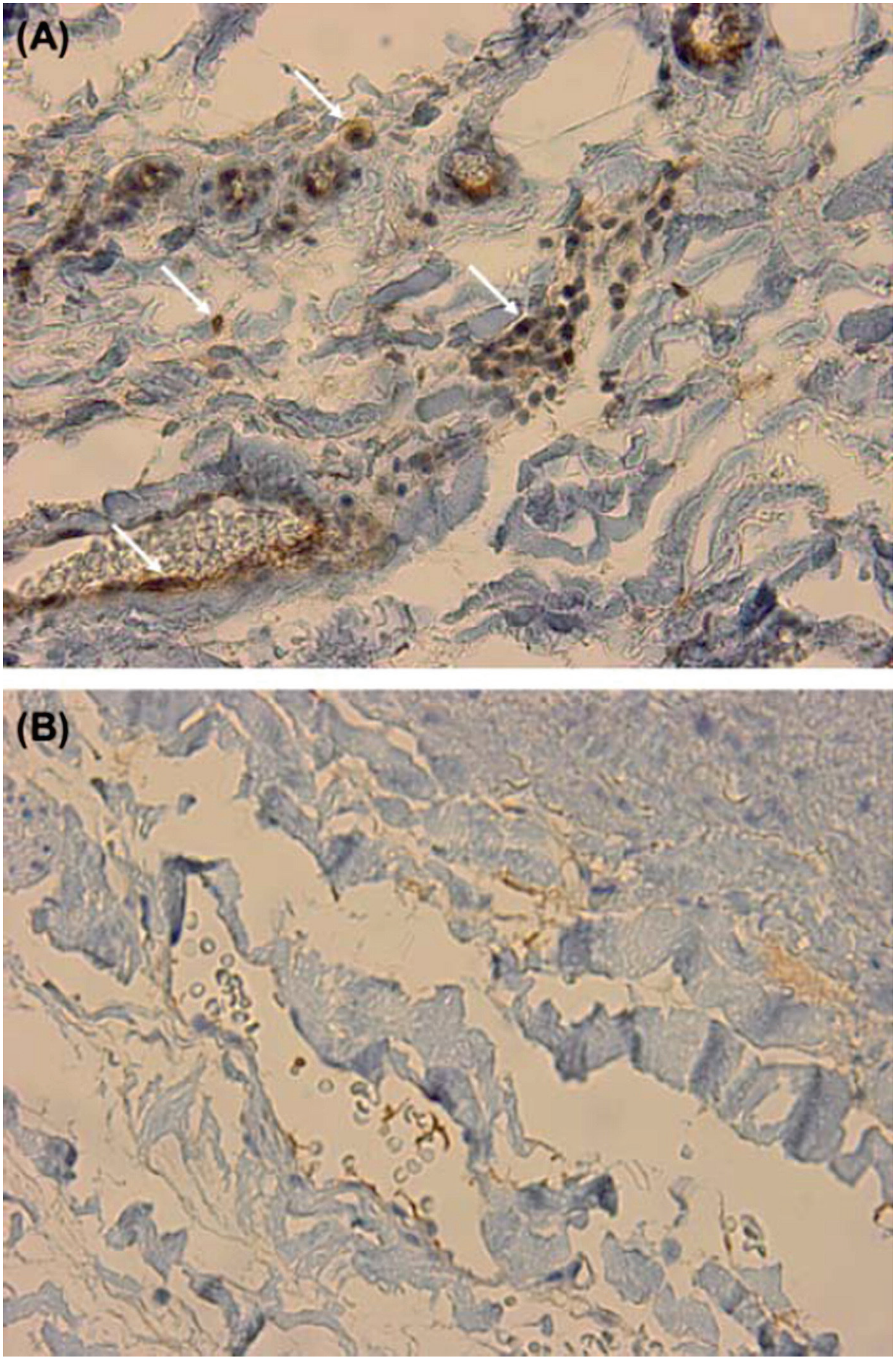
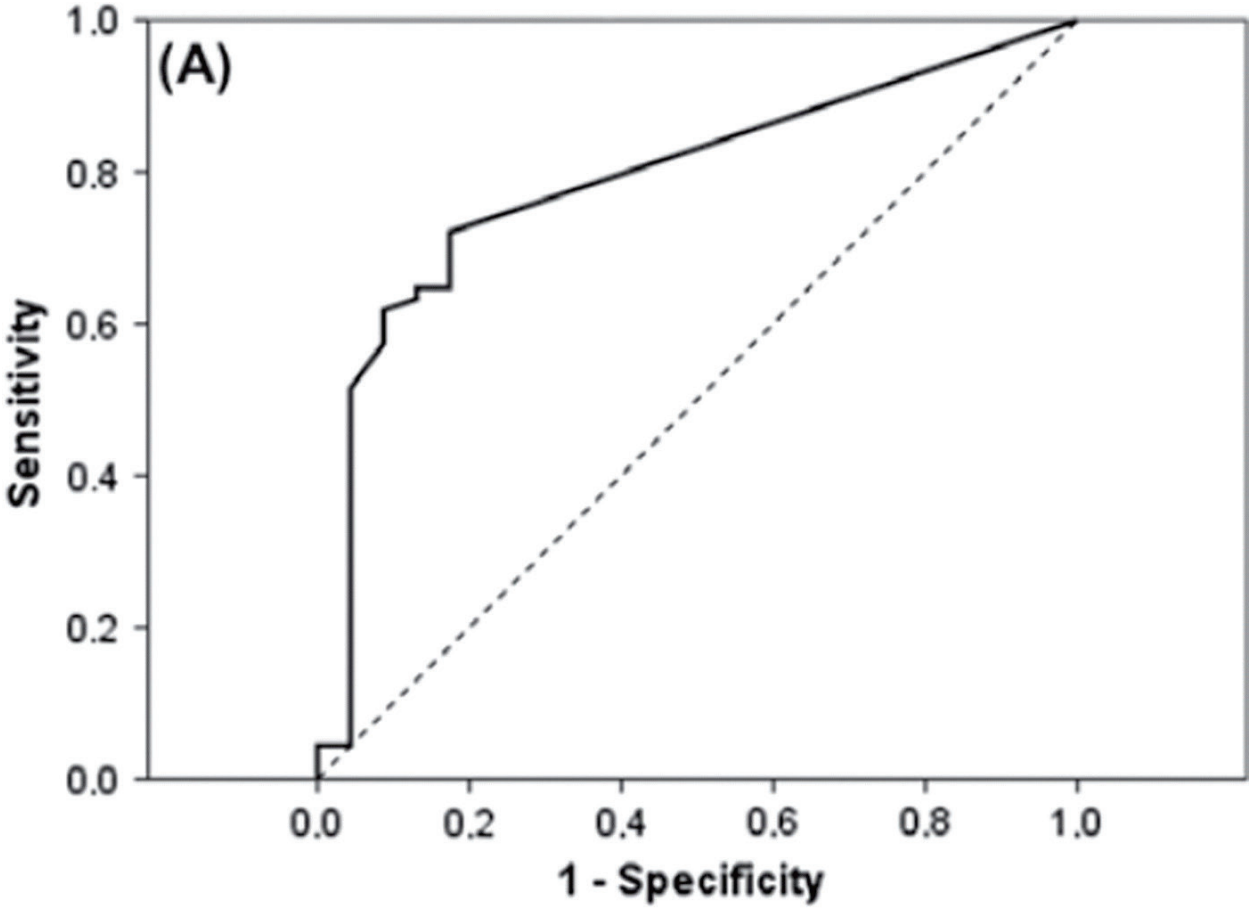
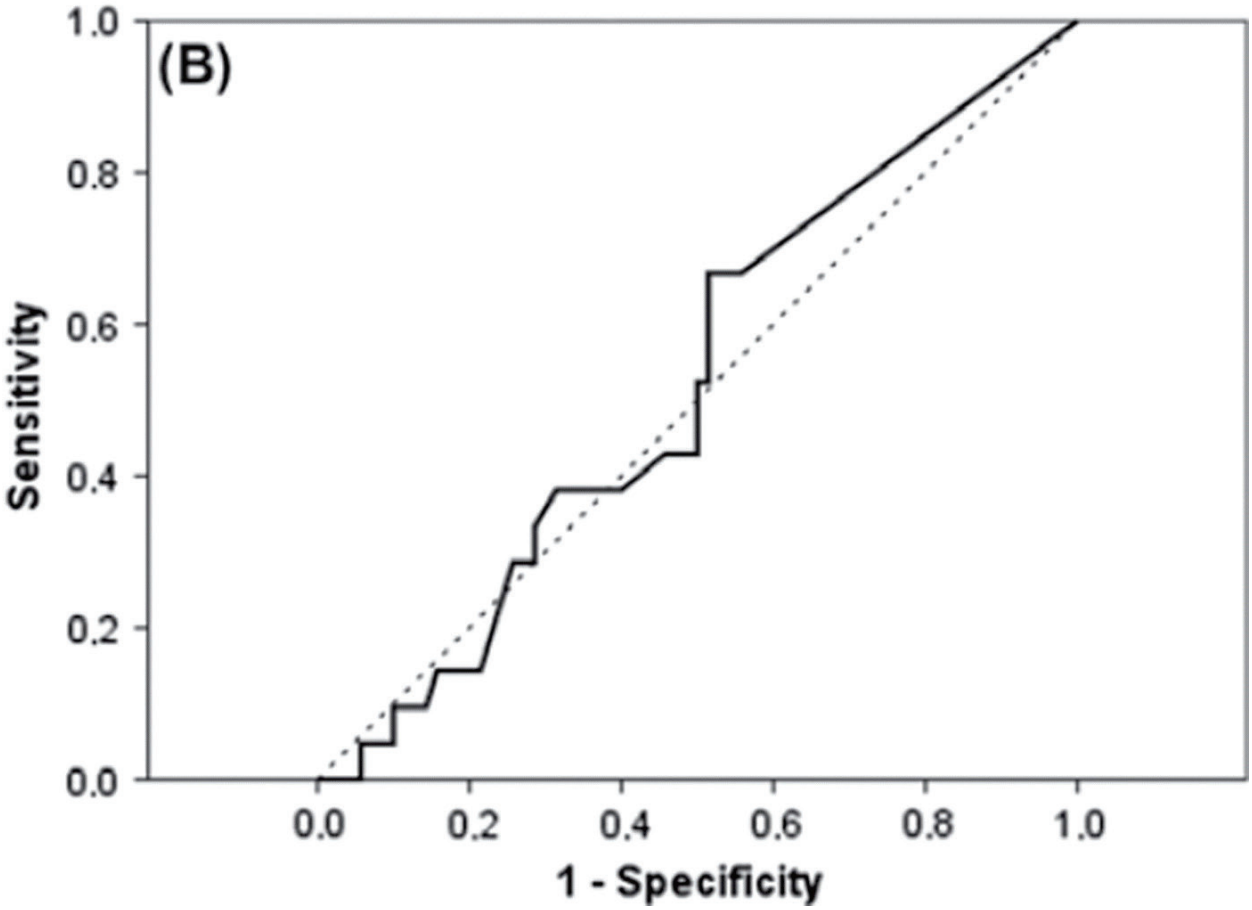

(A)
(B)

Figure 2. Receiver operating characteristic curve (ROC) analysis of C4d associated with stabile non-dissecting ascending aortic dilatation (A) and histologically defined aortitis (B). C4d is significantly associated with the prevalence of non-dissecting ascending aortic dilatation (AUC 0.792; S.E. 0.053; p = 0.000; 95% C.I. 0.688-0.895, A), but not with aortitis (AUC 0.523; SE 0.069; p = 0.752; 95 % CI 0.388-0.658, B).


(A)
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0


(B)
Sensitivity
1 - Specificity
0.0
0.2
0.4
0.6
0.8
1.0

Acknowledgements

This study was supported by research funding from The Competitive Research Foundation of Tampere University Hospital (Grant 9L058), Tuberculosis Foundation, The Finnish Heart Association and The Finnish Cultural Foundation. Dr A Mennander is the recipient of the Ingegeerd and Viking O Bjork award for Scandinavian Cardiovascular Research.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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PUBLICATION II

Aiming at One-Stage Corrective Surgery for Extended Thoracic Aortic Dilatation.

Niinimäki E, Kajander H, Paavonen T, Sioris T, Mennander A

Int J Angiol 2014;23:101–106
doi: 10.1055/s-0034-1370887

Aiming at one-stage corrective surgery for extended thoracic aortic dilatation

Running title: Extended thoracic aortic dilatation

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No conflict of interest

Abstract

Background and Aims: Definitive treatment of extended thoracic aortic dilatation is a major surgical challenge. Histopathology of resected thoracic aortic wall may reveal undiagnosed aortitis affecting outcome.

Material and Methods: Five patients underwent one-stage corrective surgery using the hybrid open arch repair by the frozen elephant trunk together with endovascular aortic grafting. A representative sample of the resected aortic arch was procured for histology. T- and B-lymphocytes, plasma cells, macrophages and Immunoglobulin G4 (IgG4) positivity were evaluated by immunohistochemistry.

Results: The mean preoperative maximum aortic diameter was 54 mm (range 41-79 mm). The mean follow-up was 18 months (range 1-24 months). Complete thrombosis of the false lumen at the level of the frozen elephant trunk was achieved in patients with dissection, and successful exclusion of the atherosclerotic dilatation in one; this 75-year-old male was diagnosed with IgG4-positive aortitis and experienced unexpected blindness after surgery without evidence of emboli or long-term neurological impairment upon repeated brain CT.

Conclusion: The hybrid open arch repair by the frozen elephant trunk and simultaneous endovascular repair is a feasible choice for one-stage surgery through sternotomy aiming at definitive treatment of extended thoracic aortic pathology. Systematic evaluation of inflammation may reveal concealed aortitis affecting postoperative outcome.

Keywords: Extended thoracic aortic dilatation; IgG4; frozen elephant trunk; one-stage surgery

Introduction

The incidence of extended thoracic aortic dilatation is relatively rare.1 One-stage surgery for definitive treatment of extended thoracic aortic dilatation may be achieved using the hybrid open arch repair by the frozen elephant trunk prosthesis (1, 2, 3, 4, 5). We have recently adapted the technique by applying endovascular grafting via the frozen elephant trunk component for distal aortic remodeling. The aim is to perform both ascending thoracic aortic and arch surgery through sternotomy while achieving descending thoracic and distal aorta remodeling with the frozen elephant trunk and additional endovascular repair.

Patients with extensive thoracic aorta disease such as dilatation and dissection may suffer from a systematic disease not only causing high blood pressure but affecting extension of aortic disease and recovery after surgery (6, 7). Aortic inflammation may predispose to future extension of dilatation or dissection of the aorta (8, 9, 10, 11). We sought to investigate whether thorough histological evaluation of the resected aorta would reveal concealed aortitis in patients with extended thoracic aortic dilatation.

Material and Methods

Study protocol and surgery

From October 2010 to August 2012, we encountered five patients with extended aortic dilatation. After the institutional review board approval, ascending aortic wall resection was obtained during surgery and processed for histology. Aortic dilatation was preoperatively confirmed and evaluated with computed tomography (CT). According to our institutional policy, aortic dilatation included an

aortic diameter more than 5.5 cm wide or aortic growth more than 1 cm in a year. This definition was adjusted to the presence of gender, patient size, and symptoms including aortic dissection, according to the Yale Center criteria. (12).

The decision on the extension of resection and surgical technique was at the discretion of the operating surgeon. Whenever dilatation included the aorta root, a radical resection of the dilated ascending aorta together with the root and the aortic valve was performed. The graft size was estimated by the principal surgeon. Resection of the aortic arch was performed using the Evita Open Plus (Hechingen, Germany) hybrid graft. The histological sample was procured from the middle of the resected diseased area of the aortic arch at the vicinity of the left subclavian artery.

Before surgery, a pig-tail catheter was inserted from the left femoral artery up to the aortic arch at the height of the left subclavian artery to identify the true or narrow atherosclerotic lumen of the aorta. The right femoral and right axillary arteries were cannulated for arterial access, and after sternotomy, the double-lumen venous cannula was inserted through the right atrial appendix. After initiation of cardiopulmonary bypass, cardioplegia was administered using the antegrade or retrograde routes, via the ascending aorta, coronary ostia, or sinus venous. During 20°C hypothermia, the dilated aortic arch was resected and the proximal part of the descending aorta was fashioned for the open anastomose of the Evita Open Plus hybrid graft. Circulatory arrest was established, the aortic arch was transacted, and bilateral selective antegrade cerebral perfusion was instituted using direct endoluminal cannulation of the arch vessels. Thereafter, the arch was resected, and the frozen elephant trunk of this device was launched into the diseased true lumen of the descending aorta according to the manufacturer's instructions using the pig-tail catheter to identify the true lumen. Immediately thereafter, a metallic endovascular mesh stent (Evita XL endograft [Hechingen, Germany]) was inserted via the frozen elephant trunk to further dilate the

true lumen against the false lumen. The metallic mesh structure of the stent endograft made it possible to prevent antegrade obstruction of allowed unobstructed flow into arterial branches of the distal aorta. The proximal part of the hybrid Evita Open Plus prosthesis was fashioned according to anatomical variances of the ascending aorta, the truncus, left carotid, and subclavian arteries.

Histology and immunohistochemistry

Up to five blocks of resected aorta tissue were embedded in paraffin and cut to 4-μm-thick segments. Histology was evaluated from the following: hematoxylin and eosin, Verhoeff–van Gieson or elastase-van Gieson, and periodic acid-Schiff. A representative 1-cm-long piece of aortic wall corresponding to all different staining was evaluated systematically for all resected samples procured during surgery. Immunohistochemistry was performed using Benchmark XT Staining module (Ventana Lifesciences, Tucson, Arizona, United States). The antibodies and dilutions, respectively, were immunoglobulin G4 (IgG4) (1:100, binding site), CD68 (1:200, Dako, Glostrup, Denmark), CD3 (1:50, Novocastra, Nusloch, Germany), CD31 (1:100, Dako), CD20 (1:1000, Dako), and CD138 (1:150, ABD Serotec, Kidlington, United Kingdom). Antibody Dilution Buffer (Ventana Lifesciences) was used for dilution media.

The samples were blindly evaluated for each primary antibody, categorized on a scale of 0 to 3 and expressed as point score unit (PSU) by four authors, and arbitrarily five fields (× 40) were reviewed for each 1-cm-long aortic sample. Inflammatory and endothelial cells, medial degeneration, and intima thickness were estimated as previously described and expressed as PSU (13Histological analysis included the evaluation of cystic medial degeneration (CMD) and intimal thickness. CMD was estimated on a scale from 0 to 3 (0, normal media; 1, mild degeneration; 2, moderate degeneration; and 3, severe degeneration). Intimal thickness were estimated according to an

arbitrary scale from 0 to 3, where 0 indicated normal intima with a single endothelial cell layer; 1, intima thickness less than 25% as compared with the media; 2, thickness more than 25% but less than 50% as compared with the media; and 3, intensive intima thickness more than 50% as compared with the media. If patchy lesions of the aortic wall were identified, we chose the field area including the respective media, intima, and adventitia layers as mapped according to the thickest intima layer.

Results

Tables 1 and and22 show preoperative and operative details, respectively. Mean cardiopulmonary bypass time was 326 minutes (range, 246–415 minutes), mean selective antegrade cerebral perfusion time was 79 minutes (range, 60–96 minutes), and mean cardioplegic arrest time was 238 minutes (range, 187–289 minutes). Four of the patients were operated due to aneurysmatic progression of dissection either antegrade or retrograde type A dissection. Two of these patients had previous surgery for acute dissection of the ascending aorta with a straight Dacron prosthesis from the sinotubular junction reaching to the proximal arch. All but one patient had a systemic disease such as adrenal adenoma with rheumatoid arthritis, hypophyseal tumor associated with hypothyreosis, and hypoaldosteronism. However, all patients suffered from malignant hypertension. The mean preoperative maximum aortic diameter was 54 mm (range, 41–79 mm).

The first patient was a 59-year-old man with onset of chronic B-type aortic dissection 6 years before the detection of new retrograde dissection at CT. Altogether, aortic arch dissection extended till both iliac arteries including a narrow true lumen with takeoff for coeliacus, superior and inferior mesenteric, and right renal arteries. Complete exclusion of the dilatation was performed using the hybrid open arch prosthesis, a distal stent endograft through the frozen elephant trunk, and resection

of the ascending aorta replaced by a straight Dacron prosthesis. During a 24-month follow-up, the patient has recovered uneventfully.

The second patient was a 63-year-old man referred to hospital with acute type B dissection. The patient suffered from seronegative rheumatoid arthritis, and soon developed delirium, bradycardia, pneumonia, and pulmonary embolism while nonoperative treatment was initially decided to apply. The dissection progressed in a retrograde fashion and instant surgery was executed. The dissection encompassed the whole of the aorta though sparing the root, and the hybrid open arch prosthesis, the distal stent endograft, and a proximal prosthesis were successfully used. However, 3 months after surgery, CT revealed a less than 1-cm gap between the frozen elephant trunk and the distal metallic stent endograft. A left adrenal adenoma was detected. During the 24-month follow-up, no endoleak has though been detected.

The following two patients, 65 and 56 years old, were both previously operated on due to acute ascending aortic dissection. The aortic valve insufficiency was dealt with an aortic valve replacement using a bioprosthesis, the aortic root was partly resected and replaced with a prosthesis, and the hybrid open arch prosthesis together with a distal stent endograft were applied in the 65 years old. This patient was soon diagnosed of hypothyroidism, transient epilepsy, and a hypophyseal tumor. The 56-year-old patient was postoperatively found to suffer from aldosteronism.

The fifth patient had a history of urinary bladder and rectum carcinoma without metastasis. Due to severe arteriosclerosis and extension of dilatation from the ascending aorta including the arch and the distal descending aorta with coronary artery stenosis, the patient was operated on using the hybrid open arch prosthesis, including two invaginated sequentially interposed aortic endografts to

exclude the distal dilatation and a prosthesis to replace the supracoronary ascending aorta excluding the aortic root. Simultaneous coronary artery bypass operation was performed. Postoperatively, total blindness occurred without any acute CT changes in the brain. Histological evaluation revealed global atherosclerosis with dilatation of the descending thoracic aorta including severe IgG4-positive aortitis (PSU 2; range, 0–2; Fig. 1).

Table 3 summarizes the histological findings and postoperative outcome. The mean follow-up was 18 months (range 1-24 months). All patients recovered from surgery, and none of the patients developed endoleaks or endotension postoperatively.

Discussion

On the basis of this pilot study, we were able to safely adapt and modify the hybrid open arch repair with the Evita prosthesis. After deployment of the hybrid graft, a bare-metal nitinol stent was inserted antegrade via the frozen elephant trunk for distal aortic remodeling of the true lumen in four patients with complicated type B dissection, of which two patients had been previously operated for acute type A dissection. Instead, one patient had a thoracic endograft for complete exclusion of an atherosclerotic aortic dilatation. Despite complete success with the one-stage surgical strategy employed, one patient out of five revealed severe IgG4-positive aortitis.

Extended thoracic aorta disease including dissection and dilatation requires meticulous surgery. Our patients with extended thoracic aorta disease were dealt using the hybrid open arch repair with the Evita prosthesis. The technique has recently been well established among clinics of expertise in Europe (1, 2, 3, 4, 5, 6). In our patients, an additional stent of the distal aorta was inserted to induce complete remodeling or exclusion of the entire diseased aorta whenever the frozen elephant trunk

Evita prosthesis itself was not sufficient in length. This strategy is in alignment with a recently presented technique including combined proximal stent grafting plus distal bare metal stenting for management of aortic dissection (7). Using the Evita prosthesis while inserting the distal stent endograft via the frozen elephant trunk, we aimed for definitive one-staged surgery. From a technical point, we discovered that only custom-made insertion knobs of the Evita device are appropriate to use, since otherwise the frozen elephant trunk part of the prosthesis may be difficult to apply distally to the descending aorta. Second, instead of using a hard wire during identification of the true lumen of the diseased aorta, we preoperatively used a pig-tail catheter that helped us to keep impeccable sterility.

We encountered four patients with dissection and one with atherosclerosis, all of which experienced extensive thoracic aorta disease together with hypertension, a well-known risk factor for aortic dilatation and dissection (14). All but one patient with dissection had a systematic disease such as rheumatoid arthritis with adrenal adenoma, hypoaldosteronism, and hypophyseal tumor upon closer examination. The common denominator for these patients upon thorough histopathological examination was aortic wall inflammation including occasional plasma cells, macrophages, and T and B cells. In addition, the patient with atherosclerosis had IgG4-positive aortitis and experienced unexpected postoperative blindness.

The degree of ascending aortic wall inflammation may determine the extension of aortic wall dilatation (15). The diagnosis of inflammation is fundamental as aortitis and ascending aortic dissection are associated with increased mortality (14, 15, 16). Recent attempts to elucidate the association of inflammation with aortic dilatation and dissection have generated theories on activation of aortic wall inflammation together with aortic wall remodeling (8, 17). IgG4 positivity has been linked earlier to various immunological conditions, and has recently also been introduced

to the literature of isolated thoracic aortitis (18, 19, 20). Interestingly, the association of extensive aortic dilatation with IgG4 positive aortitis has been proposed earlier (19, 20). It is tempting to speculate that the presence of IgG4 positivity of the aortic wall is associated with a developing aortitis predisposing to progression of aortic dilatation (19, 21). Currently, we have no proven explanation for the postoperative blindness, since repeated brain CT did not confirm evidence of emboli or long-term neurological impairment in our patient. Optic neuropathy and bilateral retinal infarction have been reported to occur in less than 0.1% of patients undergoing cardiopulmonary bypass (22). Clearly, as previously shown by Laco et al, microscopic examination of the aorta resected for dilatation is mandatory, as there are often no evident clinical signs of inflammation (18). We are to evaluate for IgG4 and total IgG antibody levels in the patient with atherosclerosis to seek for possible follow-up treatment aiming at eradicating IgG4 (23,24).

In conclusion, the hybrid open arch repair by the frozen elephant trunk and simultaneous endovascular repair seems a feasible choice for one-stage surgery through sternotomy aiming at definitive treatment of extended thoracic aortic pathology. However, despite the successful outcome of the one-stage surgical strategy, we strongly advocate for the importance of systematic histopathological evaluation of the aorta to further facilitate a tailored follow-up protocol and treatment of the individual patient. It remains to be proven whether the histological findings are associated with long-term outcome after major aortic surgery in these patients.

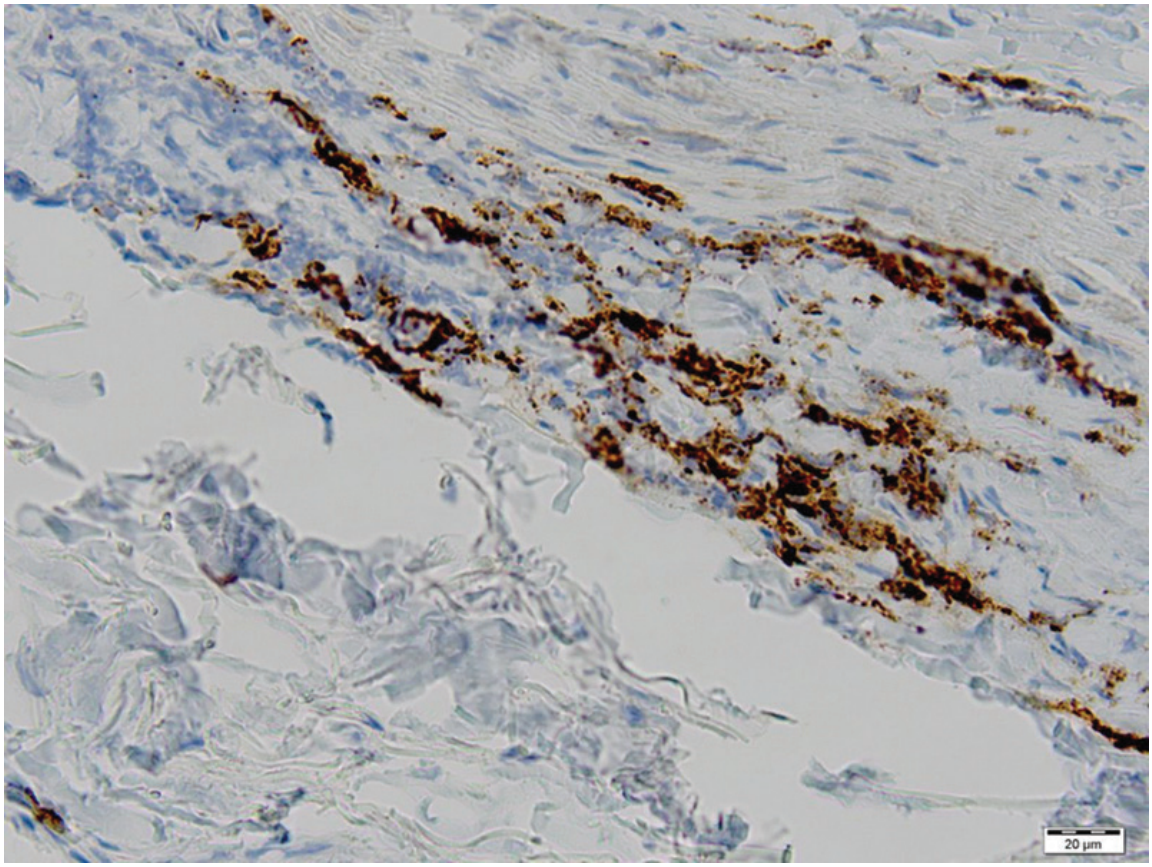
Acknowledgements

We gratefully thank The Competitive Research Foundation of Tampere University Hospital, Tuberculosis Foundation, The Finnish Heart Association and The Finnish Cultural Foundation. Dr A

Mennander is the recipient of the Ingegeerd and Viking O Bjork award for Scandinavian Cardiovascular Research.

Legend

Figure 1. Representative photograph of aortic wall during extended thoracic aortic dilatation (X40) showing IgG4 positive inflammation (dark staining colour) of patient #5.



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PUBLICATION III

Carbonic anhydrase IX deposits are associated with increased ascending aortic dilatation

Niinimäki E, Muola P, Parkkila S, Kholová I, Haapasalo H, Pastorekova S, Pastorek J, Paavonen T, Mennander A.

Scand Cardiovasc J. 2016;50:162-6.
doi: 10.3109/14017431.2016.1158416.

Carbonic anhydrase IX deposits are associated with increased ascending aortic dilatation

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Abstract

Objectives. Carbonic anhydrase IX (CA IX) expression is induced by local hypoxia. We studied whether CA IX deposits associate with ascending aortic dilatation.

Design. Aortic wall histology, CA IX expression, presence of leukocytes, plasma cells, macrophages, endothelial cells, smooth muscle cells, cell proliferation, elastin and collagen were studied in histological specimens collected from 30 patients who underwent surgery for ascending aorta. The samples were grouped according to presence of CA IX deposits.

Results. Twenty out of 30 patients had CA IX-positive deposits within the adventitia, whereas 10 specimens remained negative. Adventitial inflammation was increased in CA IX-positive samples as compared with CA IX-negative ones ($p < 0.01$). The mean diameter of the ascending aorta at the sinotubular junction increased significantly in patients with CA IX-positive staining as compared with CA IX-negative cases (63 ± 3 vs 53 ± 2 mm, $p < 0.02$). Receiver operating characteristic curve analysis confirmed the association of CA IX positivity with increased ascending aortic dilatation (AUC 0.766; S.E. 0.090; $p = 0.020$; 95% C.I. 0.590-0.941).

Conclusions. Positive CA IX staining in certain aortic specimens suggests that increased CA activity may contribute to ascending aortic dilatation.

Keywords. CA IX, ascending aortic dilatation, inflammation

Introduction

The ultimate aim of surgery for ascending aortic dilatation(AA) is to prevent aortic rupture or dissection. While an arbitrary borderline diameter of up to 5 cm of the ascending aorta has been shown to increase the risk for aortic rupture, there is increasing awareness that aortic remodeling and subsequent aortic rupture may occur at an earlier stage of AA occasionally with aortic diameters less than 4.5 cm.[1] The scarcity of atherosclerosis of the ascending aorta suggests that the risk for aortic dissection may be associated with different molecular mechanisms of the aortic wall as compared with AA alone.[2,3] Aortic rupture is considered to initiate from a sudden tear in the aortic wall due to decreased resiliency.[4]The loss of regulation of aortic wall dilatation may include factors defining aortic stiffness and elasticity associated with local hypoxia, implicating aortic wall remodeling and inflammation.[5] Increased AA may not solely lead to rupture or dissection of the aortic wall. While dissection is characterized as sudden onset of aortic wall rupture, aortic wall dilatation perse is predominantly characterized by chronic inflammation and associated medial degeneration.

Carbonic anhydrases (CAs) are involved during inflammatory and hypoxic remodeling of the arterial wall.[5] Several CA isozymes are present in vascular smooth muscle cells during vasodilatation.[6] Adventitial inflammation may initiate aortic dilatation in concert with intimal and medial microtrauma.[7]Arterial stiffness is characterized by the onset of calcium-phosphate mineral deposits within elastic lamellae of the medial arterial wall layer.[8] The membrane-bound isoform CA IV seems to induce the more acidic extracellular milieu of the artery thus allowing dissolution of excess mineral deposits,[8] and hence intervene with aortic wall remodeling. In this study, we define aortic wall remodeling as histologically confirmed aortic wall changes

during AA. We hypothesized that CA IX is present during aortic wall remodeling associated with local aortic wall inflammation.

Materials and methods

Study protocol and surgery

After institutional review board approval, ascending aortic wall resection of 30 randomly selected patients undergoing surgery for ascending aorta was performed and tissue samples were processed for histology. The patients were selected to represent various degrees of aortic wall degeneration as evaluated by elastin staining. AA was preoperatively confirmed and evaluated with computer tomography (CT). According to our institutional policy, AA included aortic diameter more than 5 cm wide or aortic diameter growth more than 1 cm in a year. This definition was adjusted to the presence of Marfa syndrome, gender, patient size and symptoms including aortic dissection according to the Yale Center criteria.[9]Surgery was performed between 2008 and 2009 in the Heart Center of Tampere University Hospital, and included 23patients with AA and seven with aortic dissection.

Decision on the extension of resection and surgical technique was at the discretion of the operating surgeon. When AA including the sinotubular junction (STJ) was estimated as the reason for aortic regurgitation, STJ was tailored for a suitable graft in a supracoronary fashion. Whenever dilatation included the aorta root, a radical resection of the dilated ascending aorta together with the root and the aortic valve was performed in all but one patient, in whom a David-type valve-sparing operation was achieved. The graft size was estimated by the principal surgeon. Resection of the aortic arch was carried out depending on the extension of distal aortic dilatation. Since the surgical

procedure was performed upon surgical decision, the sample was procured from the middle of the resected diseased area of the ascending aorta at the vicinity of STJ and processed for histology.

Histology and immunochemistry

Two to five blocks of resected ascending aorta were embedded in paraffin and cut to 5mm thick sections and stained with Hematoxylin and Eosin (H&E), Verhoeff-van Gieson (VVG),Elastin-van Gieson (EVG) and Periodic Acid-Schiff (PAS). A representative 1 cm long piece of ascending aortic wall was evaluated systematically for all resected samples procured during surgery. The heights of different layers (adventitia, media and intima) were calculated for each sample. Inflammatory cells, intensity of inflammation, medial degeneration, intima cellularity and thickness were estimated as previously described and expressed as point score units(PSU).[2]

Immunohistochemistry was performed using the monoclonal antibody M75 that recognizes part of the N-terminal proteoglycan domain of human CA IX, as previously described.[10] Five-mm thick sections were processed for immunoperoxidase staining, which was performed using an automated Lab Vision Autostainer 480 (LabVisionCorporation, Fremont, CA). Automated immunostaining was performed using the Bright Vision Histostaining reagents(ImmunoLogic, Duiven, Netherlands) and included the following steps: (1) rinsing in wash buffer; (2) treatment in 3%H2O2in ddH2O for five minutes and rinsing with wash buffer;(3) blocking with cow colostrum diluted 1:2 in Tris-buffered saline (TBS) containing 0.05% Tween-20 for 10 min and rinsing in wash buffer; (4) incubation with the primary M75antibody diluted 1:1000 for 30 min; (5) rinsing in wash buffer three times for five minutes; (6) post-blocking for 20 min with post-antibody blocking reagent (ImmunoLogic) and rinsing in wash buffer three times for five minutes; (7) incubation in poly-HRP-conjugated anti-rabbit/mouse IgG for 30 min and rinsing in wash buffer three times for five minutes; (8)incubation in DAB(3,30-diaminobenzidinetetrahydrochloride) solution (one drop

of DAB solution A and one drop of DAB solution B in 1 ml of ddH2O) for five minutes; (9) CuSO4 treatment for five minutes to enhance the signal and (10) rinsing with ddH2O. All procedures were performed at room temperature.

Statistical Analysis

In order to seek for clinical relevance associated with histology, the patients were divided into two groups according to the presence of CA IX staining of small vessels of the aortic wall. Patients with CA IX-positive staining of the ascending aortic wall were referred as CA IX+, and those without CA IX staining as CA IX-. Quantitative variables are listed as mean and standard error of the mean. Categorical variables are stated as count and percentage. Statistical analysis was performed with SPSS version 21.0. Mann-Whitney –test was used for continuous variables and chi-square –test for categorical analysis. The diagnostic association of aortic diameter to identify patients with CA IX+ was assessed by Receiver operating characteristic curve (ROC) analysis. P-values less than 0.05 were considered statistically significant.

Results

Patient characteristics (Table 1)

Mean age of the patients was 643 years. The study population consisted of 20 male and 10 female patients. Hypertension was diagnosed in only nine patients and no difference in its prevalence between the groups was found. Only one patient had diabetes, while four had hypercholesterolemia. One patient had Marfan syndrome. One patient with anon-specific vasculitis was also CA IX-positive. The frequency of preoperative inflammatory state, such as asthma and arthritis, showed no difference between the groups. The mean diameter of the ascending aorta at the STJ was592 mm for all patients and was significantly increased inpatients with CA IX positivity as compared

with CA IX negativity (633vs532 mm, p50.02). Moderate to severe aortic valve stenosis (AVS) was found in four patients with CAIX-positive staining and in five with CA IX negativity. Aortic valve insufficiency (AVI) was present in 11 patients with CAIX positivity and in seven with CA IX negativity. The majority of the patients had tricuspid aortic valve. Seven patients had undergone a previous cardiothoracic operation: four patients with CA IX positivity and three with CA IX negativity.

Operative technique (Table 2)

Graft replacement for the ascending aorta was performed either with root replacement (in 16 patients), or without encompassing the root (in 14 patients). The extension of root dilatation together with dilatation of the ascending aorta from STJ was remarkably equally distributed among the patients in both groups being 50% and 60% in CA IX-positive and CA IX-negative, respectively. A valve-sparing David operation was offered for one patient with CA IX positivity. Concomitant coronary artery bypass grafting was required in five patients with CA IX positivity. One patient with CA IX negativity had an ablation procedure to treat atrial fibrillation.

Perioperative findings, histology and immunohistochemistry (Table 3 and 4)

Confirmed by histology, the aorta had acute or chronic dissection in 5 out of 20 patients (25%) with CA IX-positive staining. Two patients had chronic dissection in the group with CA IX negativity (20%). Altogether two patients with CA IX positivity and one patient with CA IX negativity had histological findings of aortitis. Two patients with CA IX positivity died immediately.

CA IX positive staining was predominantly found in macrophages of the adventitia, at the vicinity of the media (Figure 1). Medial degeneration was present in CA IX-positive aortas as evaluated by elastin staining and compared with CA IX-negative aortas (1.7 ± 0.2 PSU and 0.7 ± 0.2 PSU,

respectively, $p < 0.03$). Intimal macrophages were more numerous in CA IX-positive aortas as compared with CA IX-negative ones, though no significant changes in intimal thickness were observed among the patients. Adventitial inflammation was increased in CA IX-positive aortas together with macrophages and B cells as compared with CA IX-negative aortas, respectively (1.9 ± 0.1 PSU and 1.4 ± 0.1 PSU, $p < 0.03$) and (1.5 ± 0.2 PSU and 0.7 ± 0.2 PSU, $p < 0.04$). Interestingly, IgG4 positivity was present only in CA IX-positive aortas ($p < 0.01$).

ROC curve analysis (Figure 2)

The diagnostic association of CA IX-positive staining with increased diameter of aortic dilatation was assessed by ROC analysis. CA IX was significantly associated with increased ascending aortic dilatation (AUC 0.766; S.E. 0.090; $p = 0.020$; 95% C.I. 0.590-0.941).

Discussion

Remarkably, up to 20 out of 30 aorta specimens showed CA IX-positive staining in this study of randomly selected patients operated for AA. CA IX suggests the presence of local aortic wall hypoxia, which was associated with adventitial inflammation and aortic wall degeneration.

This study shows for the first time that ascending aortic wall degeneration determined by increased medial elastic stain is associated with CA IX positivity and increased aortic diameter. CA IX has been previously shown in advanced atherosclerotic lesions of femoral arteries combined with osteoclast activity and macrophages (11), but the degenerative ascending aortic wall has been considered devoid of intimal thickness, thus decreasing enthusiasm in research linked with traditional atherosclerosis.

Fifteen CA-related isoforms have been identified in human. Among various CA isozymes, CA IX is involved in unique regulatory pathways. It is induced in hypoxic areas through hypoxia-inducible factor (HIF)1α-mediated pathway which plays a key role in the regulation of hypoxia responses in many different tumor categories. Therefore, severe hypoxia in tumors is associated with CA IX deposits (12, 13). CA II is another CA isozyme which is induced in blood vessels during pathological processes. During the development of various cancers, it is an endothelium-associated antigen which might be targeted by dendritic cell therapy. CA II may play an important role in tumor angiogenesis, and thus CA inhibitors may decrease tumor growth (14). On the other hand, CAs are not present in healthy intimal cells in aorta of mice (15). These findings implement that different CAs may play a crucial role in various pathological processes, such as the development of chronic degenerative lesions of the ascending aorta.

Angouras et al showed earlier that low blood flow of the vasa vasorum by ligating the costal arteries resulted in decreasing medial integrity (16). The mechanism involved may include the HIF-1α pathway, leading to aortic wall remodeling and up-regulation of vascular endothelial growth factor-dependent angiogenesis (17). As CA IX is present during hypoxic conditions associated with HIF-1α, matrix metalloproteinases (18, 19) and vascular endothelial growth factor (20, 21, 22), it may be deduced that aortic dilatation ensues via remodeling of the aortic wall.

It would be essential for the clinician to differentiate between the pathogenesis of aortic dilatation with and without the risk for subsequent dissection. Increased aortic diameter with inflammation is not a prerequisite for dissection. IgG4 positivity, increased B cells and the adventitial proliferative response suggests for increased adventitial inflammation in patients with CA IX positivity, but dissection was statistically equally represented in both patient groups in this study. Therefore, aortic

wall degeneration itself does not indicate increased risk for dissection, and the concomitant nature of inflammation seems to have an impact as well. The presence of macrophages at the sites of inflammation with CA IX positivity confirms chronic degeneration during dilatation of the ascending aorta. On the other hand, increased aortic root dilatation with CA IX-positivity occurred statistically as frequently as compared with patients with CA IX negativity. Extending aortic surgery to the aortic root in fear of increased risk for dissection may not be justified based on dilatation and CA IX alone; it is tempting to speculate that CA IX and associated aortic remodeling may again emphasize the autonomy of local dilatation without determining enhanced risk for dissection.

Limitations of the study include the small number of patients. However, CA IX is easily identified and importantly associates the remodeling of the ascending aorta with evolving inflammation, thus emphasizing the need to further identify molecular pathways responsible for aortic dilatation.

In conclusion, positive CA IX is a common feature during ascending aortic dilatation. Intervening with CA IX may add an armament against aortic dilatation and extension of surgery.

Table 1

Patient demographics

	All patients	CA IX+	CAIX-
Number of patients	30 (100%)	20	10
Age (years)	64 ± 3	62 ± 3	69 ± 3
Male, n (%)	20 (67%)	16	4
Hypertension, n (%)	9 (30%)	6	3
Diabetes, n (%)	1 (3%)	1	0
Hypercholesterolemia, n (%)	4 (13%)	3	1
Marfan, n (%)	1 (3%)	1	0
Obese, n (%)	1 (3%)	0	1
Neurologic deficiency, n (%)	3 (10%)	2	1
Hypothyreosis, n (%)	1 (3%)	1	0
Arthritis, n (%)	4 (13%)	1	0
Asthma, n (%)	2 (7%)	2	1
Diverticulitis, n (%)	2 (7%)	1	1
Vasculitis, n (%)	1 (3%)	1	0
Gingivitis, n (%)	1 (3%)	1	0
Abdominal aortic aneurysm, n (%)	1 (3%)	0	1
Coronary artery disease, myocardial infarction, n (%)	10 (33%)	6	4
Previous cardiothoracic operation			
Coronary artery bypass surgery, n (%)	3 (10%)	1	2
Aortic valve replacement, n (%)	1 (3%)	1	0
Plication of aortic dilatation or coarctation, n (%)	2 (7%)	2	0
Resection of abdominal aortic aneurysm, n	1 (3%)	0	1
Mid-ascending aorta diameter, mm	59 ± 2	63 ± 3*	53 ± 2
2-cusp aortic valve, n (%)	8 (27%)	4	4
Aortic valve insufficiency			
Moderate to severe, n (%)	18 (60%)	11	7
Aortic valve stenosis			
Moderate to severe, n (%)	28 (93%)	4	5

* $p < 0.02$

Table 2

Operative details according to surgical evaluation of extension of diseased aorta

	All patients	CA IX+	CA IX-
	30 (100%)	20	10
Graft replacement of root and ascending aorta			
Mechanical conduit	11 (37%)	9	2*
Biological conduit	4 (13%)	1	3
David operation	1 (3%)	0	1
Graft replacement of ascending aorta			
Mechanical valve + prosthesis	1 (3%)	1	0
Biological valve + prosthesis	5 (17%)	2	3
Prosthesis	8 (27%)	7	1
Additional procedures			
Ablation	1 (%)	0	1
Coronary artery bypass surgery	5 (%)	5	0

* includes 1 patient with prosthesis extending up to the aortic arch

Table 3. Histology and quantitative immunohistochemistry

Mean grade of staining		All patients	CA IX+	CA IX-	p- value
Adventitia	T cells	1.7 ± 0.1	1.8 ± 0.1	1.6 ± 0.1	Ns
	B cells	1.2 ± 0.1	1.5 ± 0.2	0.7 ± 0.2	< 0.04
	Macrophages	1.7 ± 0.1	1.9 ± 0.1	1.4 ± 0.1	< 0.03
	Plasma cells	1.6 ± 0.1	1.7 ± 0.2	1.4 ± 0.2	Ns
	Inflammation	1.9 ± 0.1	2.2 ± 0.1	1.5 ± 0.1	< 0.01
	Thickness	6.5 ± 1.1	7.9 ± 1.6	3.9 ± 0.7	< 0.03
	Proliferation	1.3 ± 0.1	1.7 ± 0.1	0.7 ± 0.1	< 0.02
Media	T cells	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.2	Ns
	B cells	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.2	Ns
	Macrophages	1.6 ± 0.1	1.6 ± 0.2	1.5 ± 0.1	Ns
	Plasma cells	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	Ns
	Inflammation	1.1 ± 0.1	1.3 ± 0.2	0.7 ± 0.2	Ns
	Proliferation	1.1 ± 0.1	1.3 ± 0.2	0.7 ± 0.2	Ns
	Myxoid material	1.9 ± 0.2	2.1 ± 0.3	1.6 ± 0.4	Ns
	Elastin	1.3 ± 0.2	1.7 ± 0.2	0.7 ± 0.2	< 0.03
Intima	T cells	1.3 ± 0.1	1.4 ± 0.1	1.0 ± 0.2	Ns
	B cells	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	Ns
	Macrophages	1.7 ± 0.1	2.0 ± 0.1	1.2 ± 0.2	< 0.01
	Plasma cells	0.4 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	Ns
	Inflammation	1.6 ± 0.1	1.7 ± 0.1	1.3 ± 0.3	Ns
	Proliferation	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	Ns
	Thickness	2.0 ± 0.1	2.2 ± 0.2	1.7 ± 0.2	Ns
	Cellularity	1.7 ± 0.1	1.8 ± 0.1	1.4 ± 0.1	Ns
IgG4-positivity		0.31 ± 0.1	0.47 ± 0.1	0	< 0.01

Table 4

Immediate postoperative outcome

	All patients	CA IX+	CA IX-
	30 (100%)	20	10
Acute dissection	3 (10%)	3	0
Chronic dissection	4 (13%)	2	2
Aortitis	2 (7%)	2*	1
Mortality	4 (13%)	2	0

* includes 1 patient with giant cell aortitis

Legends to figures

Figure 1. Representative photograph (x 40) of aortic adventitial wall immunohistochemistry of CA IX during ascending aortic dilatation. Note positive CA IX staining (black arrows in the media)

Figure 2. Receiver operating characteristic curve (ROC) analysis showing the association of CA IX with increased ascending aortic dilatation (AUC 0.766; S.E. 0.090; p = 0.020; 95% C.I. 0.590-0.941).

Acknowledgements

This study was supported by research funding from Tampere University Hospital (Grant 9L058), Tuberculosis Foundation, Sigrid Juselius Foundation, Finnish Heart Association and Finnish Cultural Foundation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Funding Information

This study was supported by research funding from Tampere UniversityHospital (Grant 9L058), Tuberculosis Foundation, Sigrid JuseliusFoundation, Finnish Heart Association and Finnish Cultural Foundation.

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PUBLICATION IV

Neovascularization with chronic inflammation characterizes ascending aortic dissection

Niinimäki E, Pynnönen V, Kholová I, Paavonen T, Mennander A.

Anatol J Cardiol. 2018;20:289–295.
doi: 10.14744/AnatolJCardiol.2018.42223

Ascending aortic neovascularization reveals site at risk for dissection

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Abstract

Objective: Neovascularization of the aortic wall may be associated with aortic dissection (AD). Aortic wall endothelial CD31 deposition together with chronic inflammation indicates angiogenesis that may lead to tissue disruption. We studied the presence of neovascularization of the ascending aortic wall by characterizing CD31 positive endothelial cells.

Methods: Aortic wall routine histology and immunohistochemistry for CD31, T- and B-lymphocytes, plasma cells, macrophages, endothelial cells, smooth muscle cells and cell proliferation were performed on 35 selected patients who underwent surgery for the ascending aorta, and the samples were grouped according to the presence of AD.

Results: Three Marfans were excluded. A total of 14 out of 32 patients had AD. A total of 18 patients were operated on due to dilatation only. Chronic inflammation of the adventitia ($p = 0.003$), media ($p = 0.001$) and intima ($p = 0.005$) was increased in AD. Neovascularization was predominant in the outer third medial layer in AD ($p = 0.037$) corresponding to the site of aortic wall disruption. Receiver operating characteristic curve analysis showed that neovascularization was associated with AD (AUC 0.750; SE 0.092; $p = 0.022$; 95 % CI 0.570-0.930).

Conclusion: Endothelial immunohistochemistry confirms neovascularization of the outer third medial layer during AD. Aortic wall remodeling including neovascularization characterizes AD. Chronic inflammation and neovascularization of the dilated ascending aorta suggest susceptibility for AD.

Keywords: neovascularization, ascending aortic dissection, chronic inflammation, CD31

Introduction

The main goal for surgery of the dilated ascending aorta is to prevent aortic dissection (AD) and rupture (1, 2). AD consists of an aortic wall tear in a tangential fashion and represents the ultimate rupture due to aortic wall weakness. Pathophysiological AD and aortic rupture are interrelated and are manifested by the anatomical site of the aortic tear (2). Despite the sudden occurrence of the aortic tear, the ascending aorta may have undergone a chronic remodeling phase of tissue weakening, including aortic wall hypoxia, hypertension, and chronic inflammation. Although a borderline of a 5.5 cm diameter of the ascending aorta is regarded as the threshold in enhancing the risk for AD (2), there is increasing evidence that aortas with an even smaller diameter may lead to AD (3). The decision for the extension of resection of the aorta during surgery is challenging, as one would aim at preventing AD after surgery.

The perioperative evaluation of the resected aortic wall during surgery for ascending aorta may reveal susceptibility for AD necessitating further extension of surgery. Most AD occurs in the outer third of the media close to the adventitia (4). This site is characterized by vasa vasorum that participates in the nutrition of the aortic wall (4). The significance of endothelial activation of vasa vasorum in aortic pathogenesis is under discussion (5). Arterial neovascularization may be regulated by chronic inflammation, suggesting that hypoxia alone is not leading to tissue remodeling (6). Recent experimental studies suggest that the regulation of angiogenesis is dependent on endothelial activation (7).

We studied the vascular reactivity of the aortic wall by characterizing the angiogenic histology of the ascending aorta as expressed by CD31. We hypothesized that chronic inflammatory remodeling of the ascending aorta is associated with dilatation of the aortic wall, and neovascularization of the ascending aortic media may determine the fate of the dilated aortic wall. Using extensive

immunohistochemical analysis and detection of CD31-positive endothelial cells of the medial layer, we evaluated whether neovascularization is associated with AD.

Methods

Study protocol and surgery

After an institutional review board approval, the need for informed consent was waived. The ascending aortic wall resection of 35 consecutive patients undergoing surgery for ascending aorta was obtained and processed for histology. An ascending aortic aneurysm was preoperatively confirmed and evaluated with computed tomography (CT). According to our institutional policy, aortic aneurysm included an aortic diameter wider than 5.5 cm or aortic growth greater than 1 cm in a year. This definition was adjusted to the presence of Marfan syndrome, gender, patient size, and symptoms, including AD according to the Yale Center criteria (2). Surgery was performed between December 2009 and August 2014, and cases of ascending aortas including AD processed for histology were enrolled. Three patients with Marfan syndrome were excluded. There were 14 patients with acute AD including onset of symptoms that lasted less than 7 days.

The decision on the extension of resection and surgical technique was at the discretion of the operating surgeon. When an aortic aneurysm including the sinotubular junction (STJ) was estimated as the reason for aortic regurgitation, STJ was tailored for a suitable graft in a supracoronary fashion. Whenever dilatation included the aorta root, a radical resection of the dilated ascending aorta together with the root and the aortic valve was performed. The graft size was estimated by the principal surgeon. The entry tears were located in the middle portion of the ascending aorta according to pre-operative CT and intraoperative assessment. Since the surgical procedure was

performed upon surgical decision, the sample was procured from the middle of the resected diseased area of the ascending aorta at the vicinity of STJ.

Histology and immunohistochemistry

Two to five blocks of resected ascending aorta were embedded in paraffin, cut to 4-mm-thick segments, and stained with hematoxylin and eosin, Verhoeff-van Gieson, Elastase-van Gieson, and Periodic Acid-Schiff. A representative 1-cm-long piece of ascending aortic wall corresponding to all different staining was evaluated systematically for all resected samples procured during surgery.

Aortic wall histology and immunohistochemistry were performed using Ventana Lifesciences Benchmark XT Staining module for leukocytes, T- and B-lymphocytes, plasma cells, macrophages, smooth muscle cells, cell proliferation, elastase, and van Gieson staining. The samples were further investigated for presence and locality of neovascularity within the aortic wall; capillaries with endothelial cells were evaluated using a polyclonal rabbit antibody for CD31 (dilution 1:2500) (DakoCymation). Ventana Lifesciences Antibody Dilution Buffer was utilized for dilution media. The heights of different layers (adventitia, media, and intima) were calculated for each sample. Inflammatory cells, the intensity of inflammation, cell proliferation, medial degeneration, intima cellularity, and thickness were estimated as previously described and expressed as point score units (PSU) in the three aortic wall layers accordingly (8). Briefly, inflammation was graded as none, mild, moderate, or severe (0, 1, 2, or 3). Medial degeneration was graded as patchy, moderate, or severe again on a scale of 0–3. Intima cellularity and thickness were estimated according to an arbitrary scale from 0–3, where 0 indicated normal intima with a single endothelial cell layer; 1, intima cellularity and thickness less than 25% as compared with the media; 2, intima cellularity and thickness more than 25% but less than 50% as compared with the media; 3, intensive intima cellularity and thickness more than 50% as compared with the media.

Quantification of medial neovascularization

Platelet endothelial cell adhesion molecule 1 (PECAM-1), also known as CD31 (cluster of differentiation 31), is expressed in high amounts in endothelial cell junctions (9). As CD31 may be expressed by leukocytes and platelets (10), angiogenesis was defined by accounting CD31-positivity, including only capillary-like morphology with a continuous uninterrupted endothelial monolayer within the media layer. For local quantification of CD31-positivity, we categorized the media into three equal parts; the inner media consisting of the innermost media adjacent to the intima, the outer media adjacent to the adventitia, and the middle media between these two media layers, respectively. The total number of positively stained CD31 new vessels was counted per square millimeter in four arbitrarily selected areas, which showed the most increased density of capillary-like morphology (hot spots).

Follow-up protocol

Documentation of mortality and morbidity was available for all the patients. For the included study patients, follow-up consisted of physical examination and echocardiography at 3 months after surgery, and on-demand thereafter including CT.

Statistical analysis

CD31-positive staining was predominantly found in the media, at the border of the adventitia including formation of small vessels (Fig. 1). To seek clinical relevance associated with immunohistochemistry, the patients were divided into two groups in accordance with the histologically confirmed presence of AD. Although histopathology confirms AD, indices of inflammation, hemorrhage, or fibrosis do not facilitate the temporal diagnosis of AD (11). The patients were categorized in keeping with the presence of dissection (AD+) and dilatation only

(AD-). All study patients were followed for a period of 3 months. Quantitative variables are listed as the mean and standard error of the mean. Categorical variables are stated as count and percentage. Statistical analysis was performed with the SPSS version 22.0. The Mann-Whitney U test was used for continuous variables, and the chi-squared test for categorical analysis. The association of CD31 with AD was assessed by the receiver operating characteristic curve (ROC) analysis. P-values less than 0.05 were considered statistically relevant..

Results

Demographics

Eighteen patients had ascending AD, while 14 out of 32 patients were operated for acute AD (Table 1). The mean age was 64±2 years. Hypertension and coronary artery disease were equally distributed among both groups. One patient without AD had unspecified vasculitis of the aortic wall. Four patients with AD and eight without AD had aortic valve insufficiency. Eleven out of 18 patients without AD had aortic valve stenosis, including five patients with combined aortic valve disease, in contrast to only one aortic valve stenosis in a patient with AD. The mean aortic diameter was 58±2 mm for all patients.

Operative technique

In patients with AD, surgery included either a Bentall-type operation with an aortic valve prosthesis, or replacement of the ascending aorta only (Table 2). In eight patients without AD, the aortic root was not operated on. A mechanical or biologic valve was replaced together with a prosthesis encompassing the ascending aorta distally from the sinotubular junction in five patients without

AD. In three patients without AD and without aortic valve disease, only the ascending aorta was replaced.

Perioperative findings, histology and immunohistochemistry

Histology revealed three cases of aortitis, of which two had AD+ (Tables (Tables33 and and4).4). The intensity of chronic adventitial, medial, and intimal inflammation was increased in AD+ as compared with AD− (2.2±0.3 vs. 1.3±0.2, p=0.03, 1.4±0.3 vs. 0.3±0.1, p<0.001, and 1.6±0.3 vs. 0.7±0.2, p=0.005, respectively). The media showed increased cell proliferation in AD+ as compared with AD− (1.5±0.3 vs. 0.4±0.2, p=0.002). An increased number of macrophages and T-cells of the intima were found in AD+ as compared to AD− (1.9±0.2 vs. 1.2±0.2 p=0.032 and 1.4±0.2 vs. 0.6±0.2, p=0.006, respectively). The outer third layer of the media at the vicinity of the adventitia expressed an increased number of cytoplasmic CD31-positivity in AD+ as compared with AD− (5.1±1.1 vs. 2.4±0.7, p=0.037), and corresponded to the site of AD tear (Fig. 1).

ROC analysis and outcome

A ROC analysis showed that the local endothelial activity was associated with AD (AUC 0.750; SE 0.092; p=0.022; 95% CI 0.570–0.930, Fig. 2). Two patients with AD died shortly after surgery: a 55-year old preoperatively unconscious patient and an 88-year old that experienced AD rupture before the onset of hypothermia. Two patients without AD died within 1 week of surgery, due to cerebral infarction caused by hypotension and cerebral emboli at surgery.

Discussion

Based on this study, local neovascularization of the outer medial layer indicates active remodeling of the aortic wall associated with AD; histological characteristics of the ascending aortic wall may

be investigated to reveal endothelial activation of newly formed capillaries of the media layer according to the CD31 positivity. Together with neovascularization, an increased number of proliferative cells and macrophages is strongly suggestive of chronic inflammation in patients with AD.

Dilatation of the ascending aorta alone does not undeniably lead to AD. Hypertension and a family history for dilatation of the aorta may increase the risk for dilatation per se, but AD seems to occur in some instances quasi-unexpectedly, while the aortic diameter has not reached the threshold value of 5.5 cm2. According to a large referral center, AD was missed up to 38% of cases on initial evaluation and first established in 28% of patients only at postmortem examination (12). While traditional CT and echocardiography may not provide an accurate estimation of the risk for AD, molecular imaging has emerged as a plausible and promising option (13). Risk stratification of AD may benefit from understanding the heterogeneous pathogenesis of the inflammatory process and angiogenesis during aortic remodeling. Imaging modalities, such as positron emission tomography, single photon emission CT, and magnetic resonance imaging, with the aid of tracing chronic inflammation and neovascularization, are intensively studied for clinical translation (13).

The clinician craves for an applicable means to diagnose accurately an aorta prone for AD. This study emphasizes the importance of investigating both chronic inflammation and neovascularization that together may form a trigger for AD. This message importantly adds to the clinical transition of imaging modalities that are based on tracing chronic inflammation and neovascularization (13). An aortic site characterized by microvessel formation and inflammation during progression of ascending aortic dilatation (14) enhances awareness for the need of early surgical intervention to prevent AD.

The onset of AD includes vertical rupture of the aortic wall often at the junction of the media and adventitia (15). This aortic wall site suggests that the hypoxic environment of the media layer may attract chronic inflammatory components and eventually lead to angiogenesis and tissue tear. Endothelial cells form the inner lining of newly formed capillaries and render the media–adventitia border susceptible to the onset of AD. Inflammation alone without angiogenesis may not suffice to initiate AD. There are sparse data on the impact of CD31 immunohistochemistry on AD. Dysfunction of the microcirculation in the outer media layer of the aorta may suggest ischemia and malnutrition of the aortic wall, thus increasing the risk for AD (4). The impact of ischemia along with intimal hyperplasia and hypertension is not demonstrated in our study among the patients. It is important to distinguish CD31-stained endothelial cells from macrophages, since proinflammatory macrophages have experimentally been shown to influence the aortic wall during AD (16).

Neovascularization is a key factor to the pathophysiology of various arterial diseases, such as atherosclerosis (17), vasculitis (6), intracranial artery aneurysm (18), and abdominal aortic aneurysm (5). Neovascularization barely seems to be a consequence of hypoxia alone, but it involves mechanisms introduced by immunology and subsequent chronic inflammation (5, 6, 19). According to a previous experimental model (20), decreased circulation of vasa vasorum, which often occurs in arterial hypertension (17), may increase the stiffness of the outer media of the aorta, but an immunological activation of the aortic wall seems prerequisite to initiate an active tear, which leads to AD (17, 19, 21, 22). Chronic inflammation of all aortic layers was present in our study, together with neovascularization and AD. Comparably, acute plaque rupture of the atherosclerotic coronary artery (23) or the formation of microhemorrhages within intracranial artery aneurysms (18) may represent disease entities initiated by the activation of neovessels (19). It is

tempting to suggest that CD31 immunohistochemistry may reveal a weak aortic wall site prone to AD.

Study limitations

This is a study investigating the association of increased aortic neovascularization in a fairly low number of patients with or without AD. Unfortunately, the rate of expansion of the non-repaired segments of the aorta was not evaluated to correlate with immunohistochemistry, partly due to the small number of patients. As a paradigm of comparable tissue preparation in this study, we did not systematically investigate aortas obtained from autopsied cases. Only less than a third of the patients had bicuspid aortic valve disease, and it is beyond our scope to discuss the plausible interaction of specific heterogenic aortic valve diseases in the development of AD. Increased chronic aortic wall inflammation was associated with AD together with neovascularization, but specific immunological parameters such as complement activation remain to be elucidated.

Conclusion

Heterogeneity of the progression of aortic dilatation to AD is expected (14). Interacting with chronic inflammation and associated neovascularization may impact against the development of AD. Taken together, we suggest that CD31 immunohistochemistry adds to the understanding of the remodeling characteristics of the ascending aorta, although further studies are clearly recommended.

Acknowledgements

This study was supported by research funding from The Competitive Research Foundation of Tampere University Hospital (Grant 9L058), Tuberculosis Foundation, The Finnish Heart Association and The Finnish Cultural Foundation.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Tables

Table 1

Patient demographics

	All patients	AD+	AD-
Number of patients	35 (100%)	17	18
Age (years)	64 ± 3	62 ± 5	59 ± 3
Male, n (%)	24 (67%)	10	14
Hypertension, n (%)	13 (37%)	6	7
Diabetes, n (%)	1 (3%)	1	0
Hypercholesterolemia, n (%)	3 (13%)	0	3
Marfan, n (%)	3 (9%)	3	0
Vasculitis, n (%)	1 (3%)	0	1
Arthritis, n (%)	3 (9%)	3	0
Asthma, n (%)	2 (6%)	1	1
Myocardial coronary artery disease, infarction, n (%)	7 (20%)	3	4
Previous cardiothoracic operation			
Coronary artery bypass surgery, n (%)	2 (6%)	2	0
Correction of aortic coarctation, n (%)	1 (3%)	0	1
Correction of abdominal aorta aneurysm	1 (3%)	1	0
Mid-ascending aorta diameter, mm	59 ± 2	60 ± 3	57 ± 3
2-cusp aortic valve, n (%)	9 (27%)	2	7
Aortic valve insufficiency			
Moderate to severe, n (%)	15 (60%)	7	8
Aortic valve stenosis			
Moderate to severe, n (%)	12 (93%)	1	11*

* includes five patients with combined aortic valve disease, $p = 0.001$

Table 2

Operative details according to surgical evaluation of extension of diseased aorta

	All patients	AD+	AD-
	35 (100%)	17	18
Graft replacement of root and ascending aorta			
Mechanical conduit	12 (34%)	6	6
Biological conduit	9 (26%)	4	4
Graft replacement of ascending aorta			
Mechanical valve + prosthesis	2 (6%)	0	2
Biological valve + prosthesis	3 (9%)	0	3*
Prosthesis	10 (29%)	7	3
Additional procedure			
Coronary artery bypass surgery	4 (12%)	2	2

* includes aortoplasty

Table 3. Histology and quantitative immunohistochemistry

Mean grade of staining		All patients	AD+	AD-	*p*- value
Adventitia	T cells	1.4 ± 0.2	1.7 ± 0.3	1.1 ± 0.2	Ns
	B cells	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.2	Ns
	Macrophages	1.8 ± 0.2	2.1 ± 0.83	1.5 ± 0.2	Ns
	Plasma cells	0.6 ± 0.2	0.8 ± 0.3	0.6 ± 0.2	Ns
	Inflammation	1.7 ± 0.2	2.2 ± 0.3	1.3 ± 0.2	0.003
	Proliferation	1.5± 0.2	1.5 ± 0.2	1.3 ± 0.4	Ns
Media	T cells	0.6 ± 0.2	0.8 ± 0.3	0.5 ± 0.2	Ns
	B cells	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.6	Ns
	Macrophages	1.3 ± 0.2	1.5 ± 0.3	1.0 ± 0.3	Ns
	Plasma cells	0.3 ± 0.2	0.3 ± 0.2	0.5 ± 0.4	Ns
	Inflammation	0.9 ± 0.2	1.5 ± 0.3	0.3 ± 0.1	< 0.0001
	Proliferation	1.0 ± 0.2	1.5 ± 0.3	0.4 ± 0.2	< 0.005
	Degeneration	1.6 ± 0.2	1.8 ± 0.3	1.4 ± 0.3	Ns
	Elastase	1.7 ± 0.2	1.7 ± 0.2	1.6 ± 0.3	Ns
Intima	T cells	1.0 ± 0.2	1.3 ± 0.2	0.6 ± 0.2	< 0.01
	B cells	0.2 ± 0.1	0.3 ± 0.2	0	Ns
	Macrophages	1.5 ± 0.2	1.8 ± 0.2	1.2 ± 0.2	< 0.05
	Plasma cells	0.6 ± 0.2	0.6 ± 0.2	0.5 ± 0.4	Ns
	Inflammation	1,1 ± 0.2	1.7 ± 0.3	0.7 ± 0.2	< 0.05
	Proliferation	0.9 ± 0.2	1.1 ± 0.2	0.5 ± 0.4	Ns
	Thickness	2.0 ± 0.3	1.9 ± 0.3	2.1 ± 0.4	Ns
	Cellularity	1.6 ± 0.2	1.8 ± 0.2	1.3 ± 0.2	Ns

Table 4. Quantitative immunohistochemistry for CD31 according to location of staining

Mean grade of staining		All patients	AD+	AD-	*p*- value
Media	Outer layer	4.0 ± 0.8	5.8 ± 1.3	2.4 ± 0.7	0.016
	Middle layer	2.3 ± 0.7	3.3 ± 1.4	1.4 ± 0.4	0.145
	Inner layer	1.4 ± 0.4	1.7 ± 0.8	1.1 ± 0.4	0.780

LEGENDS

Figure 1. Representative immunohistochemistry (x 20) for CD31 of the ascending aorta. Note CD31 positivity (white arrow) in outer third medial layer of the ascending aorta suggesting susceptibility for aortic dissection in A. Onset of dissection (white brackets) in B at site of CD31 positivity (white arrow). Inlets (x40) at bottom left corner show site of interests in detail.

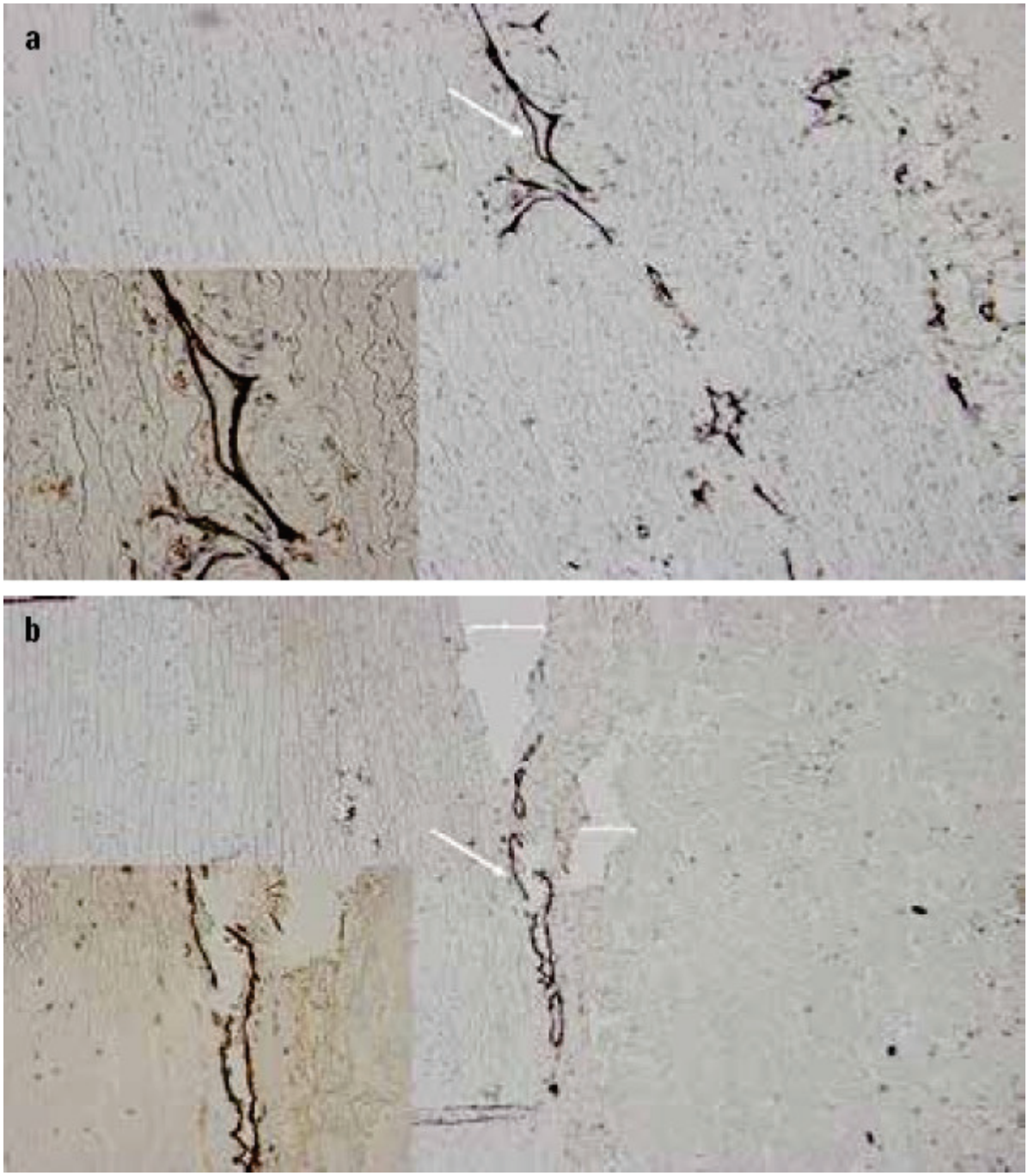
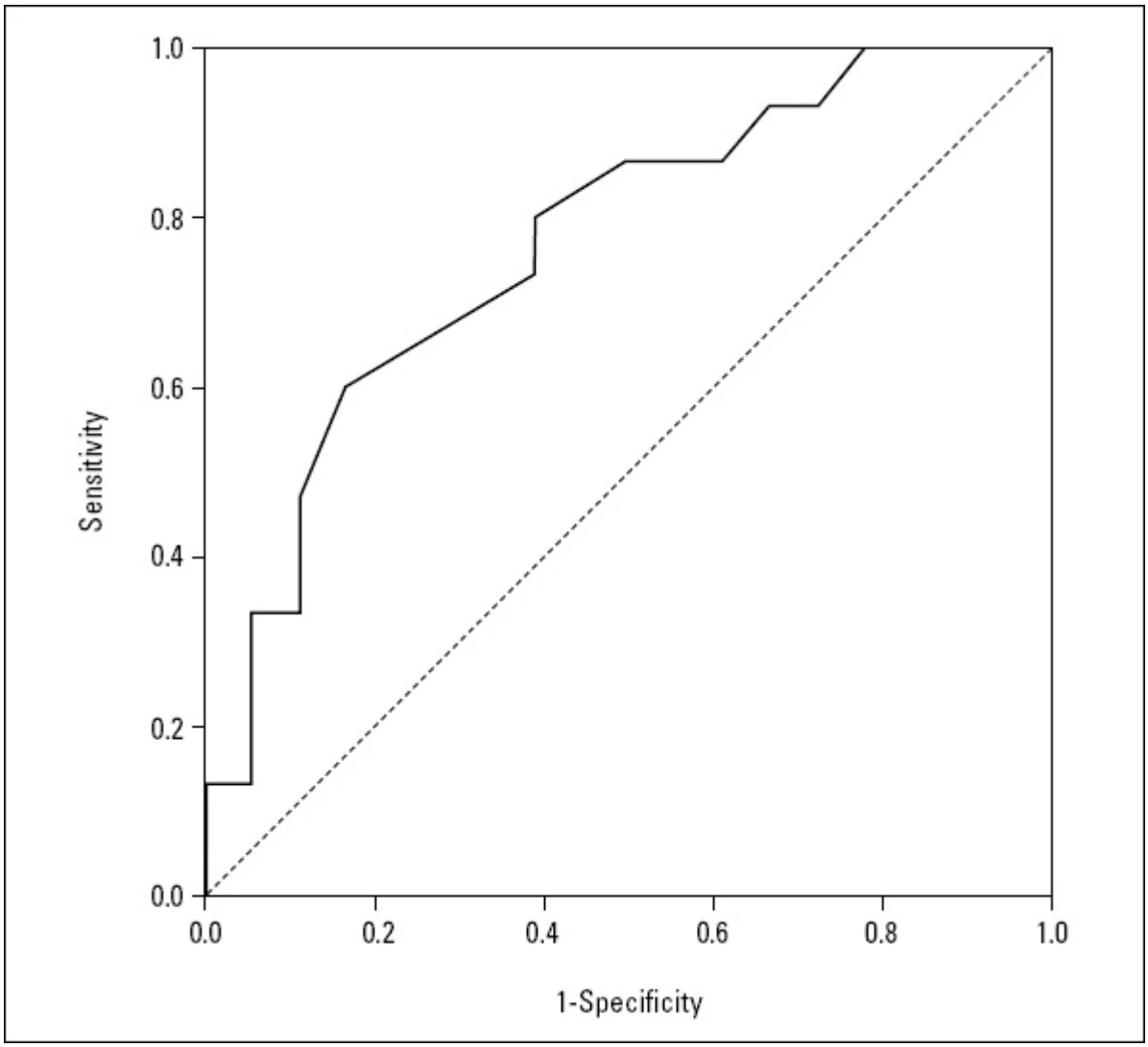


Figure 2. Receiver operating characteristic curve (ROC) analysis shows that local neovascularization of the aortic wall is associated with AD (AUC 0.750; SE 0.092; $p = 0.022$; 95 % CI 0.570-0.930).



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