THE EXTRACELLULAR ROLE OF GRANZYME B IN ABDOMINAL AORTIC ANEURYSM

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
(Pathology and Laboratory Medicine)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

December 2013

ABSTRACT

Background: Abdominal aortic aneurysm (AAA) is an age-related disease characterized by progressive degradation of elastic lamellae, defective collagen architecture and medial smooth muscle cell loss. We previously demonstrated that knocking out the serine protease granzyme B (GZMB) reduces incidence and severity of AAA in mice; however, while GZMB is known for its role in apoptosis, it also accumulates extracellularly during inflammation and can cleave extracellular matrix (ECM) components such as decorin and fibrillin-1. We hypothesized that GZMB contributes to AAA development through the degradation of vascular ECM and that the inhibition of extracellular GZMB would reduce the incidence and severity of AAA progression.

Methods: Human aneurysmal samples were obtained and apolipoprotein E(apoE)-knockout (KO), GZMB/apoE-double knockout (GDKO) and perforin/apoE-DKO (PDKO) mice were implanted with osmotic minipumps releasing angiotensin II for 28 days to induce AAA formation. Additional apoE-KO mice were injected with the GZMB inhibitor, serpin A3N (SA3N, 4-120 µg/kg) or anti-GZMB neutralizing antibody (1 mg/kg) prior to pump implantation. Tissues were assessed for aneurysm pathology, inflammation and ECM composition. Collagen content was analysed by second harmonic generation and transmission electron microscopy.

Results: Human aneurysmal tissues showed elevated levels of GZMB immunopositivity compared to controls. A significant reduction in AAA incidence and severity was observed in GDKO mice compared to apoE-KO, whereas perforin deficiency was not protective against AAA. A dose-dependent reduction in the frequency of aortic rupture was observed in mice that received SA3N or anti-GZMB antibody treatment. Pre-incubation with SA3N prevented decorin cleavage by GZMB *in vitro.* Reduced GZMB and a corresponding reduction in loss of adventitial decorin were observed in SA3N and anti-GZMB-treated mice while collagen density was increased. Adventitial collagen from SA3N-treated mice exhibited significantly higher fibre density and reduced fibril size irregularity.

Conclusions: GZMB promotes destruction of the elastic lamellae via degradation of fibrillin-1 and destabilization of elastic microfibrils while GZMB-mediated degradation of decorin contributes to loss of adventitial collagen organization and density. The extracellular inhibition of GZMB prevented decorin loss and enabled a beneficial remodelling of adventitial collagen in response to medial injury, leading to higher vessel tensile strength and increased resistance to aortic rupture.

PREFACE

Chapter 1 contains text from a review entitled "*Inflammaging* and proteases in abdominal aortic aneurysm" by Hendel A, Ang LS, Granville DJ (*Curr Vasc Pharmacol. 2012 Jun 22*). Figure 1 was taken from "Granzyme B in injury, inflammation and repair" by Hiebert PR and Granville DJ (*Trends Mol Med. 2012 Dec;18(12):732-41*) and was drawn by Paul Hiebert.

Chapter 4 contains text and images from "Perforin-independent extracellular granzyme B activity contributes to abdominal aortic aneurysm" by Chamberlain CM, Ang LS, Boivin WA, Cooper DM, Williams SJ, Zhao H, Hendel A, Folkesson M, Swedenborg J, Allard MF, McManus BM, Granville DJ (*Am J Pathol. 2010 Feb;176(2):1038-49*). Immunohistochemistry was performed by Hongyan Zhao.

Chapter 5 contains text and images from "Perforin-independent extracellular granzyme B activity contributes to abdominal aortic aneurysm" by Chamberlain CM, Ang LS, Boivin WA, Cooper DM, Williams SJ, Zhao H, Hendel A, Folkesson M, Swedenborg J, Allard MF, McManus BM, Granville DJ (*Am J Pathol. 2010 Feb;176(2):1038-49*). Movat's Pentachrome staining was performed by Amrit Samra. Immunohistochemistry was performed with assistance from Tyler Varnals and Hongyan Zhao. Immunoprecipitation of mouse serum proteins was performed by Hongyan Zhao. Fibrillin-1 transcription levels were measured by Dawn Cooper. Confocal images of immune cells were taken by Alon Hendel.

Chapter 6 contains text and images from "Serpina3n attenuates granzyme B-mediated decorin cleavage and rupture in a murine model of aortic aneurysm" by Ang LS, Boivin WA, Williams SJ, Zhao H, Abraham T, Carmine-Simmen K, McManus BM, Bleackley RC, Granville DJ (*Cell Death Dis. 2011 Sep 8;2:e209*). Serpin A3N was kindly provided by Dr. R. Chris Bleackley. Movat's Pentachrome staining was performed by Amrit Samra. Assessment of serpin inhibition was performed by Sarah Williams. Decorin cleavage assays were performed by Wendy Boivin. Second harmonic generation studies were performed

in collaboration with Dr. Thomas Abraham, and electron microscopy was performed in collaboration with Dr. David Walker and Fanny Chu.

All animal work was performed in accordance with the guidelines for animal experimentation approved by the Animal Experimentation Committee of the University of British Columbia. All research studies were conducted with the approval of the University of British Columbia Animal Care Committee and research was carried out under the certificates A10-0119, A06-0158 and A10-0084.

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α_1AT	Alpha-1-antitrypsin
AAA	Abdominal aortic aneurysm
ACT	Alpha-1-antichymotrypsin
ADAMTS-13	A disintegrin and metalloproteinase with thrombospondin motifs-13
AIF	Apoptosis-inducing factor
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of variance
Anti-GZMB	Anti-GZMB antibody
AP	ABC-alkaline phosphatase
AP-1	Activator protein-1
APC	Antigen presenting cell
apoE	Apolipoprotein E
AT1	Angiotensin II receptor, type 1
AT2	Angiotensin II receptor, type 2
ATF	Activating transcription factor
AU	Arbitrary units
CBF	Core binding factor
CG	Cathepsin G
CMA-1	Mast cell chymase-1
COPD	Chronic obstructive pulmonary disease
CREB	Cyclic AMP-responsive element-binding protein
crmA	Cytokine response modifier-A
CTL	Cytotoxic T lymphocyte
CTLA-1	Cytotoxic T lymphocyte-associated serine esterase-1
DKO	Double knockout
DPPI	Dipeptidyl peptidase I

ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FGF-2	Fibroblast growth factor-2
GAG	Glycosaminoglycan
GDKO	Granzyme B/apolipoprotein E-double knockout mice
GZMA	Granzyme A
GZMB	Granzyme B
GZMH	Granzyme H
GZMM	Granzyme M
H&E	Hematoxylin and eosin
HCASMC	Human coronary artery smooth muscle cell
HDL	High-density lipoprotein
HIV-1	Human immunodeficiency virus-1
HMG CoA	3-Hydroxy-3-methylglutaryl coenzyme A
IFN-γ	Interferon-gamma
IHC	Immunohistochemistry
IL-13	Interleukin-13
IL-1α	Interleukin-1-alpha
IL-1β	Interleukin-1-beta
IL-2	Interleukin-2
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
IS	Immunological synapse
KO	Knockout
LDL	Low-density lipoprotein
5-LO	5-lipoxygenase

LIX	Lipopolysaccharide-induced CXC
LLC	Large latent complex
LOX	Lysyl oxidase
LTC4	Leukotriene C4
MCL-1	Myeloid cell leukemia sequence-1
MCP-1	Monocyte attractant protein-1
MHC-II	Major histocompatibility complex class II
MIP-2	Macrophage inflammatory protein-2
mMCP-4	Mouse mast cell protease- 4
mMCP-6	Mouse mast cell protease- 6
MMP-1	Matrix metalloproteinase-1
MMP-12	Matrix metalloproteinase-12
MMP-13	Matrix metalloproteinase-13
MMP-2	Matrix metalloproteinase-2
MMP-9	Matrix metalloproteinase-9
MPR	Mannose-6-phosphate receptor
MT1-MMP	Membrane-type 1 matrix metalloproteinase
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
NK	Natural killer
NO	Nitric oxide
NR	Non-ruptured
NSAID	Non-steroidal anti-inflammatory drug
PA	Phosphatidic acid
PAI-1	Plasminogen activator-1
PAR-2	Protease activated receptor-2
PDGF	Platelet-derived growth factor
PDKO	Perforin/apolipoprotein E-double knockout mice
PEBP2	Polyomavirus enhancer binding protein 2

PGE2	Prostaglandin E_2
PGG	Pentagalloyl glucose
PI-9	Protease inhibitor-9
PLD	Phospholipase D
RCL	Reactive centre loop
RGD	Arg-Gly-Asp
ROS	Reactive oxygen species
SA3N	Serpin A3N
SAAAVE Act	Screening for Abdominal Aortic Aneurysm Very Efficiently Act
Serpin	Serine protease inhibitor
SHG	Second harmonic generation
SMBM	Smooth muscle cell basal medium
SMC	Smooth muscle cell
SPI-6	Serine protease inhibitor-6
TAA	Thoracic aortic aneurysm
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor-beta
TIMP-1	Tissue inhibitor of metalloproteinase-1
TNF-α	Tumour necrosis factor-alpha
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
uPA	Urokinase-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGFR-2	Vascular endothelial growth factor receptor-2
VSMC	Vascular smooth muscle cell
vWF	von Willebrand factor
WT	Wild type
α_1AT	Alpha-1-antitrypsin

ACKNOWLEDGEMENTS

I would like to thank my family for their support and encouragement, and especially my parents, Richard and Sarah Ang, who brought me up to value knowledge and the pursuit of truth. I want to acknowledge my friends at the James Hogg Research Centre who have shared this seminal experience in graduate school with me, and helped to make it the most memorable time of my life thus far: Seti Boroomand, Hayley Hiebert, Paul Hiebert, Stephanie Warner, Wendy Boivin, Anna Meredith, Alon Hendel and Cleo Leung.

I want to thank my supervisor, Dr. David Granville, who has been a supportive mentor since the very beginning, and who never gave up on me. I will be ever grateful to Hongyan Zhao, for her patience and willingness to help with every experiment. This project would not have been nearly as successful without her assistance and instruction. I would also like to thank all the members of the Granville laboratory, both past and present, for their support and solidarity. It has been an honour to work alongside you. I would like to thank the members of my advisory committee for their invaluable time and willingness to share their expertise, and for the constructive support and advice given to me over the years: Dr. David Walker, Dr. Ismail Laher, Dr. Bruce McManus and Dr. Michael Allard.

I would like to acknowledge the members of the Genetically Engineered Models facility at the James Hogg Research Centre, with especial thanks to Lubos Bohunek and Tatjana Bozin, who selflessly gave of their time and effort and made all the animal work in this dissertation possible. I want to thank Fanny Chu for going the extra mile to help me obtain the electon microscopy images in this dissertation. The beautiful results speak for themselves.

Last, but not least, I would like to thank my husband, Sorin Alexander, for his unconditional love, support and endless patience with me. You were my inspiration all along.

CHAPTER 1: INTRODUCTION

1.1 AORTA: STRUCTURE AND FUNCTION

The aorta is the largest artery in the body and extends from the aortic valve in the heart to the iliac bifurcation. It is the main vessel through which oxygenated blood is distributed to all parts of the body and is thus crucial to the circulatory system.

1.1.1 EMBRYOLOGY

The development of the aorta is an integral part of the development of the conotruncus, or the cardiac outflow tract. Malformations in the conotruncus account for a large percentage of clinically relevant congenital heart defects such as persistent truncus arteriosus, transposition of the great arteries, double outlet of the right ventricle, and tetralogy of Fallot.[1] The cardiac outflow tract begins as a single tube that extends from the distal end of the primitive right ventricle to the end of the pericardial cavity. It is composed of three layers: an outer layer of myocytes, a middle layer of cardiac jelly and a single layer of endothelial cells lining the lumen.[2]

The outflow tract develops a characteristic bend with proximal and distal portions,[3] with the distal portion connecting to the bilateral dorsal aortae through the first pair of pharyngeal arch arteries at the aortic sac.[4] In humans, the pharyngeal arches develop during the fourth week of gestation as a series of mesodermal outpouchings on either side of the pharynx. The second, third, fourth and sixth pairs of pharyngeal arches appear in sequence and arise from the ventral side of the aortic sac, from where they develop to meet the dorsal aortae.[4]

The first and second pairs of arches degenerate early, with a remnant of the first arch developing into the maxillary artery. The third arches form the origin for the left and right common carotid arteries, while the left fourth arch is retained as a part of the mature left aortic arch and the

right fourth arch forms the most proximal part of the right subclavian artery. The aortic sac is partitioned by the aortopulmonary septum resulting in the separation of the intrapericardial aorta and the pulmonary trunk, as well as the origins of the fourth and sixth arches. The proximal part of the sixth arches form the right and left pulmonary arteries. While the distal section of the right arch degenerates, the distal part of the left arch forms the ductus arteriosus.[4] In the developing foetus, this duct remains open and allows for blood to bypass the immature pulmonary system, but should close within a few days of birth and develop into the ligamentum arteriosum, a fibrous band connecting the left pulmonary artery to the inferior surface of the aortic arch. The seventh intersegmental arteries enlarge and migrate towards the head along the dorsal aortae. The right dorsal aorta and first through sixth intersegmental arteries degenerate, leaving the seventh right intersegmental artery to become the right subclavian artery. The seventh left intersegmental artery arrives at the fourth arch, in between the ductus arteriosus and the left common carotid artery (third arch) and becomes the left subclavian artery. This leaves the mature aorta to derive from the persistence of the intrapericardial aorta, the left fourth arch and the left dorsal aorta.[4]

1.1.2 Anatomy

The aorta can be divided into four main sections: the aortic root and ascending aorta, the aortic arch, the thoracic descending aorta and the abdominal aorta.

At the aortic root, the aortic valve sits at the origin of the aorta and guards the left ventricular outflow tract. The three semilunar leaflets normally bridge the anatomic ventriculo-arterial junction. Immediately above the aortic valve are the three aortic sinuses, from which the left posterior aortic sinus gives rise to the left coronary artery and the anterior aortic sinus gives rise to the right coronary artery.[4] The valve leaflets are typically nearly equal in size, and are thin and pliable in young, healthy individuals but often become thick and stiff with age.[5] Like the sinuses, the ascending aorta is enclosed

within the pericardium and in adults, it measures approximately 3 cm across in diameter and 5 cm in length.[4]

The aortic arch begins at the level of the superior attachment to the fibrous pericardium and is entirely external to the pericardial sac. The arch curves cephalad and then posterior and to the left to cross in front of the trachea before descending into the thorax to become the thoracic aorta. The three major arteries that arise from the arch and supply the head, neck and arms are the brachiocephalic, left common carotid and left subclavian arteries.[4] The thoracic aorta continues from the arch and begins descent approximately level with the fourth thoracic vertebra, beginning on the left and shifting to the midline at the twelfth vertebra. There, it passes through the aortic hiatus in the diaphragm and becomes the abdominal aorta. The thoracic aorta gives rise to the visceral, intercostal, subcostal and superior phrenic arteries.[4] The abdominal aorta begins at the aortic hiatus and ends at the iliac bifurcation. It gives rise to major arteries such as the iliac, renal and mesenteric arteries, and narrows to approximately two-thirds the width of the thoracic aorta.[4]

Throughout its length, the aorta is composed of three layers: a thin tunica intima made up of a single layer of endothelial cells sitting on a basement membrane, a thick tunica media comprised of smooth muscle cells (SMC) embedded in a dense matrix of fibrillar structural proteins such as elastin, and lastly, the tunica adventitia, which contains fibroblasts and collagen fibres.[4] It is the media that provides the aorta with its viscoelastic properties via concentric bands of elastin filaments that associate with collagen fibres and SMC into lamellar units or lamellae.[6] In mammals, the first 28 to 30 lamellar units from luminal surface are an avascular zone that does not have a dedicated blood supply, and instead must rely on diffusion of oxygen and nutrients across the intima from the lumen.[7] If additional lamellar units are present, they must be supplied by a network of vasa vasorum which penetrates the media from the adventitia. As such, the abdominal aorta with 28 to 32 lamellar units, is typically

completely avascular and lacking a vasa vasorum, whereas the thoracic aorta contains 55 to 60 lamellar units and contains both vascular and avascular zones. [7] Aortic medial thickness increases from birth to adulthood; however, this is achieved by synthesizing lamellar units in the thoracic aorta,[8] whereas expansion occurs by widening existing lamellar units in the abdominal aorta.[9] While both mechanisms maintain the same ratio of aortic diameter to medial thickness, the difference in assembly may account for the reduced elastin content in the abdominal aorta compared to the thoracic segment.

1.1.3 Aortic Function

The arterial system can be seen as passive structure that accepts blood in waves from the left ventricle and distributes it to the organs and tissues of the body. As such, it can be said to have two major roles:

Conduit: to distribute blood to the organs and tissues

Cushion: to moderate flow pulsations arriving via left ventricular discontinuous ejection

Both positions are essential in a functional arterial system and when healthy, the human arterial system is relatively efficient conduit and cushion. Mean blood pressure loss is less than 1 mmHg between the aorta and the radial artery, and further decreases by approximately 2 mmHg for arteries that are less than 0.5 mm in diameter,[10] while pulsatile energy loss in the circulation has been shown to be only 10% of steady energy loss.[11]

One of the earliest models of the arterial system was described in 1733 by Stephen Hales in his treatise on blood pressure, entitled *Haemastaticks*.[12] Interestingly, he was also the first person to measure arterial blood pressure in an animal.[10] Hales compared the arterial system to a contemporary fire engine of his day, where an air-filled dome, or Windkessel, would function as a cushion, with an attached hose as a conduit.[13]In this system, the Windkessel converted pulsatile flow into constant flow,

and Hales likened arterial distensibility to the Windkessel, the distributing arteries to the hose, and peripheral resistance to the hose nozzle. This simple system is essentially correct but now viewed as oversimplified because cushion and conduit functions cannot be separated into specific arteries and are performed by the entire system.

A more modern model was proposed by McDonald, Taylor and Womersley in the 1950s,[14, 15] where the arterial system was described as a simple distensible tube which connects the heart to peripheral resistance. The analogy used was that of an electrical transmission line with high resistance at the end (peripheral resistance) that creates a mean voltage (pressure). This model can account for both differences in pressure at different points at the same time, and for amplification and reflection of the pressure wave; however, peripheral resistance is restricted to a single reflection point and it is assumed that the distance between the heart and the peripheral resistance is always constant, which is not true for different organs and tissue systems.[10]

With regards to the aorta, it has been suggested that its main function is to act as the chief cushion in the arterial tree, providing up to 70% of the entire cushioning capacity of the system. Compared to the peripheral arteries, the aorta is passive and does not constrict or dilate.[10] Distensibility of the aorta is largely dependent on its elastic components, and the ratio of elastin to collagen in the vessel wall. In the thoracic aorta, this ratio is 6:4 compared to 3:7 in the rest of the arterial tree.[16] As the elastic modulus of collagen is greater than 300 times that of elastin,[17, 18] the proximal thoracic aorta is typically much more distensible than the distal abdominal aorta. When measured, abdominal aortic stiffness was documented at 569 ± 138 N/cm^2, compared to 261 ± 26 N/cm^2 in thoracic aorta,[19, 20] and this can readily account for the increase in pulse wave velocity from the proximal aorta to the distal abdominal aorta.[21-23]

Additionally, the elastic properties, or the relationship between arterial pressure and diameter in the aorta is not linear and is attributed to different loading on different components in the aorta.[18, 24] At low levels of pressure, wall stress is typically supported by the more distensible elastin fibres, whereas at high pressure, support of wall stress is taken on by the stiffer collagen fibres, and these properties help maintain structural integrity of the aortic wall.[10]

1.2 The Pathophysiology of Abdominal Aortic Aneurysm

Aneurysm is derived from the Greek word ανεύρυσμα, meaning widening,[25] and is clinically defined as a permanent focal dilation of an artery over 50% from its original diameter and is characterized by chronic inflammation, destructive remodeling of the ECM and progressive weakening of the vessel wall.[26] Approximately 80% of all aortic aneurysms occur in the abdominal region.[27] Abdominal aortic aneurysm (AAA) is an increasingly common and frequently fatal clinical condition, responsible for over 15 000 deaths per year in the United States.[27] The incidence of AAA measuring 2.9-4.9 cm in diameter ranges from 1.3% in men 45-54 years of age to 12.5% in men 75-84 years of age. In women, prevalence ranges from 0% to 5.2% in the same age groups.[28] Rupture occurs in approximately 20% of AAA exceeding 5 cm in diameter but is expected in over 50% of AAA greater than 7 cm.[28] Currently, the only effective intervention is open surgical or endovascular repair, however, operative mortality for elective open surgical repair is 5.6%.[29] Endovascular repair is associated with lower operative mortality, but carries an increased risk of rupture after four years post-surgery and a greater probability of reintervention.[30] Total mortality for rupture has been reported to be as high as 90%;[28] many such patients do not reach emergency treatment in time and those that do have high surgical mortality.[31] Due to the risk of rupture in combination with the asymptomatic nature of AAA, in 2006 the US Congress passed the Screening for Abdominal Aortic Aneurysm Very Efficiently (SAAAVE) Act for men over the age of 65 as they enrol in Medicare, men between the ages of 65 and 75 who have smoked over 100 cigarettes in their lifetime, and both men and women with a family history of AAA. In

September 2008, the Canadian Society for Vascular Surgery recommended a similar screening program. As such, as patient and provider awareness and education increases, it is anticipated that early stage, pharmaceutical options geared towards slowing or preventing aneurysm progression and rupture will be in great demand.[27]

1.2.1 GENETICS

In 1977, a report by Clifton[32] about a family of three brothers who all suffered ruptured AAA sparked off an period of intensive research into the genetic basis for the disease. It is now understood that having a first-degree relative with AAA increases lifetime risk approximately 10 fold, with estimates ranging from 18-22%[33-35] but no specific gene or mutation has been definitively identified as the cause of familial AAA. Various inheritance patterns have been noted, including male expression bias,[36] autosomal recessive,[37] and autosomal dominant with low penetrance,[38] but given the state of knowledge regarding AAA today, it is far more likely that AAA is a complex, multifactoral disorder with multiple genetic and environmental risk factors[39] and possibly multiple pathological mechanisms.

Suggested targets include genes involved in extracellular matrix (ECM) production and degradation, inflammation, cell adhesion, atherosclerosis, SMC contraction, intracellular protein turnover and cell signalling that have been observed to be either up- or downregulated in AAA.[40] Additionally, specific polymorphisms of heme oxygenase 1, angiotensin converting enzyme, apolipoprotein E (apoE), plasminogen activator-1 (PAI-1), tissue inhibitor of metalloproteinase-1 (TIMP-1) and many others have been associated with AAA development and pathogenesis.[40] Of note, a genome wide linkage analysis mapped a locus for familial AAA to a specific location on chromosome 19q13.3,[41] further supporting the hypothesis that development of AAA may have a significant genetic aspect.

More recently, several groups have made a concerted effort to further identify circulating biomarkers for AAA.[42-44] While not directly looking for genetic risk factors, the identification of novel biomarkers may shed further light on the actual culprits involved in AAA pathogenesis, and may aid with the development of genetic screening assays to identify patients at greatest risk for developing AAA.

1.2.2 Classification and Etiology

AAA may be classified according to various categorization schemes. These include classification by etiology, location or shape.

Typical etiological categories include:[45]

- Congenital or developmental e.g. Ehlers-Danlos, Marfan's syndrome
- Mechanical or hemodynamic e.g. Arteriovenus fistula-associated, postenotic
- Traumatic e.g. blunt trauma
- Inflammatory, non-infectious e.g. Kawasaki's disease, polyarteritis, Takayasu's disease
- Infectious e.g. Bacterial, fungal, spirochete infection
- Degenerative, non specific e.g. Arteriosclerotic
- Anastomotic or postarteriostomy

Typical location categories include:[25]

- Infrarenal (entirely below the renal arteries)
- Juxtarenal (infrarenal aorta adjacent to or including the lower margin of renal artery origins)
- Pararenal (including the juxtarenal aorta and entire renal artery origins)
- Suprarenal (involves the aorta above the renal arteries)

Typical shape categories include: [25]

- Fusiform
- Saccular
- Pseudoaneurysm, external diameter dilation without internal dilation

Etiologically, the vast majority of AAA is degenerative, [25] and has traditionally been described as a complication of advanced atherosclerosis; however, it is more recently being viewed as a separate entity with similar risk factors. That said, the most significant risk factors for AAA are male gender, cigarette smoking, age, family history, atherosclerotic disease, low high-density lipoprotein (HDL) and high low-

density lipoprotein (LDL).[28, 46, 47] Hypertension is only weakly associated with AAA[25] while diabetes may even be protective against development of AAA.[48]

1.2.2 PATHOPHYSIOLOGY

The hallmark of degenerative AAA is a persistent proteolytic imbalance that results in excessive matrix destruction and progressive loss of vessel wall structural integrity.[49] AAA are histologically characterized by fragmentation of elastic lamellae and loss of elastin. The medial layer grows thinner with progressive elastin and SMC loss and is eventually remodelled with collagen. Atherosclerosis is often present, but the thin media maybe used to differentiate AAA from atherosclerotic occlusive disease where intimal-medial thickening is prominent.[25] A concentric laminated thrombus frequently lines the intimal surface and may preserve normal blood flow but has also been suggested to exacerbate proteolytic degradation of the vascular wall beneath it.[50] Another differentiating factor between AAA and atherosclerosis is the location of the inflammatory infiltrate. In AAA, chronic transmural inflammation is a principal characteristic, with mononuclear cells, lymphocytes, blood plasma cells and mast cells infiltrating the media and adventitia, while in atherosclerosis, infiltrating inflammatory cells are largely restricted to the intima. [25]

Hemodynamic factors within the abdominal aorta are critical in the development of AAA.[51] Not only does the reflection of pulse waves at the iliac bifurcation increase wall stress on the infrarenal aorta,[52] but the abdominal aorta is already much less compliant than the thoracic aorta with far fewer elastic lamellar units and a greatly reduced elastin:collagen ratio to begin with.[6] Following initial aneurysm formation, further dilation of the aorta is promoted by the increase in tangential stress.[25] As aneurysmal disease involves elastin fragmentation and SMC loss, an aortic region that experiences higher wall stress, has fewer lamellar units, decreased elastin content and poorer nutrient delivery to the endogenous cell population is at greater risk of developing AAA.

Chronic transmural inflammation is one of the hallmarks of degenerative AAA and while the exact mechanisms involved are unclear, the inflammatory milieu is very likely responsible for the destruction of vascular wall components, directly leading to loss of structural integrity. Many inflammatory molecules have been implicated, such as the chemokines monocyte attractant protein-1 (MCP-1) and interleukin-8 (IL-8).[53] It has not yet been determined whether these are the cause of, or the result of inflammation in the aneurysmal aorta. With the destruction of the elastic lamellae, it has been hypothesised that the production of ECM peptide fragments may present chemoattractant activity for macrophages and other inflammatory cells. [25] It is also well documented that the inflammatory milieu surrounding degenerative AAAs produce significant amounts of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interferon-γ (IFN-γ).[54]

While the lack of a vasa vasorum in the abdominal aorta initially results in poor nutrient delivery to medial SMC, chronic inflammation often results in medial neovascularisation of the aneurysmal wall. This further contributes to AAA development by recruiting more inflammatory cells and providing a direct microvascular route to the tissue for the newly summoned infiltrate.[51] Medial neoangiogenesis has been shown to be spatially associated with areas of greatest elastin degradation and inflammatory cell infiltrate.[25]

SMCs play a fundamental, protective role in maintaining aortic architecture and regulating physiological remodelling processes, however, aneurysmal media can lose up to 74% of SMC numbers.[55] Previous studies have determined that this loss is mostly due to SMC apoptosis. Indeed, the expression of various mediators of programmed cell death such as p53 and p21 have been shown to be upregulated in AAA tissues.[56] Infiltrating T cells are also sources of cytotoxic mediators such as perforin and Fas, further promoting SMC loss.[57] It has been suggested that ischemia may further damage SMC due to the lack of a vasa vasorum in the abdominal aorta combined with the presence of intramural thrombus and

intimal plaque development. As advanced age is a factor in AAA development, SMC senescence may also play a role in SMC loss. Similarly, medial SMC isolated from AAA display a distinct morphological appearance when cultured *in vitro*, and furthermore have a diminished proliferative ability compared to SMC from controls.[58]

The effect of the intraluminal thrombus on wall stress is the subject of much controversy. While some believe it may transmit pulsatile stress to the underlying wall, there is also evidence that the presence of thrombus may diminish the tensile stress present because it normalizes blood flow.[59, 60] Presence of thrombus results in hypoxia and diminish oxygen and nutrient diffusion to the media,[61] and has been associated with rapid dilation[62] and increased inflammation, revascularization and wall weakening.[63] It has been proposed that the intraluminal thrombus may act as a trap for proteases such as matrix metalloproteinase-9 (MMP-9) and result in increased proteolytic degradation.[50, 64] The presence of plasminogen and its activator, urokinase-type plasminogen activator (uPA) has been observed and may result in further activation of MMPs which are known elastolytic and collagenolytic enzymes.[50]

1.3 The Extracellular Matrix

The extracellular matrix (ECM) is the intricate network of diverse macromolecules that surround the cells in all tissues. It was commonly believed that the matrix served in a purely structural capacity but it is now understood that the ECM takes a very active role in regulating the cells that are embedded within it and is capable of influencing cell development, survival, migration, proliferation, phenotype and function. [65]

The ECM is by and large composed of two main groups of macromolecules: 1. fibrous proteins such as elastin, collagen, fibronectin and laminin, and 2. glycosaminoglycans (GAGs).

GAGs are long, unbranched polysaccharide chains with a repeating disaccharide unit. Based on dissacharide structure and sulfation, there are four types of GAGs: 1. hyaluronan, 2. chondroitin/dermatan sulfate, 3. heparan sulfate, 4. keratan sulfate. [65]

Because of the stiffness and hydrophilicity of the polysaccharide chains, GAGs tend to occupy a very large volume relative to their mass. Their highly negatively charge attracts cations such as Na^+, that cause the diffusion of water into the ECM providing the turgor that enables the ECM to withstand compressive forces. [65] GAGs are typically found covalently bound to protein cores except for hyaluronan, forming a class of proteins known as the proteoglycans.

Proteoglycans are believed to function as a reservoir for growth factors and cytokines in the ECM and may mediate the growth factor gradients that are critical for guidance in many developmental processes.[66] Extracellular proteases, such as the MMPs, may then regulate growth factor bioavailability by degrading ECM components and releasing sequestered cytokines and activating latent growth factors. [67, 68] However, some growth factors require heparin sulfate as a cofactor in binding their receptors[69] and numerous ECM proteins can bind growth factors directly without the involvement of GAGs, [66] while many ECM receptors such as the integrins function as signal transduction receptors, [66] further supporting the active role of the ECM in mediating cell processes eg. vascular endothelial growth factor (VEGF) binds to fibronectin type III domains in fibronectin and tenascin-C, which promotes cell proliferation.[70, 71]

1.3.1 Elastin

Elastic fibres in the ECM are found in almost all organ systems and are of critical importance and great abundance in tissues that must withstand periodic stress.[72] These fibres are responsible for the pliancy and distensibility in connective tissues and arterial walls, and influence both structure and hemodynamic function in these tissues.[73] They consist of an internal core of cross-linked elastin

surrounded by a sheath of 10-12nm microfibrils that are largely comprised of two large glycoproteins called fibrillin-1 and -2.[72] Although fibrillin-1 and -2 share partially overlapping expression patterns, fibrillin-2 tends to be strongly expressed in developing tissues, with fibrillin-1 being the most abundant isoform throughout life.[74] Fibrillin-1 in particular is believed to serve as a scaffold for the deposition of tropoelastin during elastic fibrogenesis,[75] may mediate cell binding via $\alpha_v\beta_3$- and $\alpha_5\beta_1$-integrins[76, 77] and has been shown to associate with focal adhesions.[78] Fibrillin-1 regulates the bioavailability of transforming growth factor-β (TGF-β) and mutations in fibrillin-1 result in Marfan syndrome, a connective tissue disorder which is associated with the development of arterial aneurysms, elevated TGF-β signalling and other severe complications in the skeletal, ocular, pulmonary and central nervous systems.[79, 80]

1.3.2 Collagen

Collagens are one of the most important structural proteins in vertebrates and typically the most common, constituting approximately 25% of total protein mass in mammals.[65] Within vascular ECM, fibrillar collagens (type I and III) are responsible for the provision of tensile strength and also contribute to the extensile properties of the vessel wall.[81] Collagen proteins are characterized by the presence of a triple-helical domain consisting of polypeptide chains with a repeating Gly-X-Y sequence and the ability to form supramolecule aggregates.[82] Collagens are comprised of three polypeptide α chains that each coil into a left-handed helix and associate together to form a right-handed super helix.[83]

After transcription of procollagen genes, the α chains are synthesized by endoplasmic ribosomes and the pre-pro-collagen peptide undergoes N-terminal processing to remove signal peptides after translocation across the membrane of the rough endoplasmic reticulum.[84] After the hydroxylation and glycosylation of lysines and prolines, 3 pro-peptides associate and twist together to form the procollagen triple helix with the assistance of molecular chaperones such as HSP47.[85] The procollagen

triple helix is then transported to the Golgi complex, where phosphorylation of serine and sulfation of tyrosine occurs, after which it is secreted outside of the cell. Collagen peptidases cleave off the propeptide to produce tropocollagen which undergo polymerization by lysyl oxidase to form collagen fibrils. Collagen fibrils then group together to form larger collagen fibre bundles. [82, 84]

Decorin is a small leucine-rich proteoglycan expressed by fibroblasts, myocytes and SMC [86, 87] and is believed to regulate numerous functions in the ECM, including the regulation of collagen fibrillogenesis,[88] collagen degradation,[89] cell growth[90-92] and cell signalling.[93, 94] Decorin is composed of a 40kDa core protein called decoron, which is attached to a single chondroitin/dermatan sulfate GAG chain.[95] Decoron functions as an anchor between decorin molecules and collagen fibrils, providing elasticity and improving the tensile properties of collagen.[96, 97] During collagen fibrillogenesis, decorin is believed to regulate fibril formation, fusion and organization.[88] In addition to these structural roles, decorin binds and sequesters all three isoforms of TGF-β. When bound to collagen, this interaction with TGF-β occurs directly to decoron and not via the GAG chain. The degradation of decorin during inflammation and remodelling increases the bioavailability of TGF-β[68, 98, 99] and has implications for the mediation of inflammation, and the inhibition of cell proliferation.

1.4 The Role of Immune-Derived Proteases in AAA

AAA is characterized by a persistent proteolytic imbalance that results in the destruction of the vascular wall.[49] In section 1.4 and 1.5, the role of key immune-derived proteases involved in the pathogenesis of AAA will be described. They are summarized in Table 1.

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TABLE 1: IMMUNE PROTEASES IN AAA

Enzyme	Source within the aneurysmal aorta	Pathological contribution	Extracellular Endogenous inhibitor
Elastase	• Macrophages • Neutrophils • SMC	• Cleavage of elastin and destruction of elastic lamellae • Generates proinflammatory elastin fragments (elastokines)	• α-1-antitrypsin
Chymase	• Mast cells	• Activating MMPs and cathepsins – resulting in elastic lamellar fragmentation • Promote immune infiltration • SMC apoptosis • Neovascularization • Convert Ang I to Ang II	• α_1-antichymotrypsin • α_2-macroglobulin • α_1-protease inhibitor
Tryptase	• Mast cells	• Activating MMPs and cathepsins – resulting in elastic lamellae fragmentation • Promote immune infiltration • SMC apoptosis	Unknown
Cathepsins	• Macrophages • Neutrophils • SMC • Mast cells	• Cleavage of elastin and destruction of elastic lamellae • Generates proinflammatory elastin fragments (elastokines) • Promote immune infiltration • Promoting angiogenesis • Activating MMPs	• Cystatin C
MMP-9	• Macrophages • Neutrophils	• Cleavage of collagens (I, IV, V, VII, X, XI, XIV), elastin, fibronectin and plasminogen • Promote rupture • Activate VEGF, angiogenesis • Activate, liberate TGF-β • Macrophage migration	• TIMP-1 • TIMP-2 • TIMP-3 • TIMP-4

TABLE 1: IMMUNE PROTEASES IN AAA (cont'd)

Enzyme	Source within the aneurysmal aorta	Pathological contribution	Extracellular Endogenous inhibitor
MMP-2	• SMC • Fibroblasts • Macrophages	• Cleavage of collagens (I, IV, V, VII, X, XI, XIV) elastin, fibronectin, laminin, MMP-1, MMP-9 and MMP-13 • Activate, liberate TGF-β • Promotes invasive migration of VSMCs	• TIMP-1 • TIMP-2 • TIMP-3 • TIMP-4
MMP-1	• Fibroblasts • SMC • Endothelial cells • Monocytes	• Cleavage of collagen (I, II, III, VII, VIII, X), aggrecan, gelatine, MMP-2 and MMP-9	• TIMP-1 • TIMP-2 • TIMP-3 • TIMP-4
MMP-12	• Macrophage • Foam cell	• Cleavage of collagen IV, gelatine, elastin, fibronectin, casein, fibrinogen, plasminogen, MMP-2 • Highest affinity for elastin and elastolytic cleavage	• TIMP-1
MMP-13	• SMC	• Cleavage of Collagens (I, II, III, IV, IX, X, XIV) gelatin, MMP-9	• TIMP-1 • TIMP-2 • TIMP-3
MT1-MMP	• SMC • Macrophage	• Activate pro-MMP2 • Promote macrophage-dependent elastolysis	• TIMP-1
Granzyme B	• CTL • NK Cell • T_{reg} • Basophils • Type II pneumocytes • Macrophage • Mast cell • Keratinocytes • Chondrocytes	• Cleavage of fibrillin-1, decorin • Disruption of elastic lamellae • Dysregulation of adventitial remodelling • Potentiates pro-inflammatory activity of IL-1α	• Serpin A3N (only found in mice)

(Adapted from Hendel *et al*. *Curr Vasc Pharmacol*, (2012).[100]

1.4.1 Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a large family of endopeptidases that are required for physiological homeostasis and remodelling processes in the ECM but have also been implicated in pathological disease conditions involving tissue degradation and degeneration, such as rheumatoid and osteo-arthritis,[101, 102] osteoporosis,[103] and metastasis.[104] Given that dysregulation of ECM turnover is a hallmark of AAA pathogenesis, many studies have focused on the role of MMPs in AAA development and progression.

The link between MMPs with aortic degeneration was first noted in 1991, when Senior *et al*. demonstrated that both MMP-2 and MMP-9 were capable of cleaving elastin.[105] Since then, the investigation of MMPs in aneurysmal disease has largely focused on these two proteases and their corresponding endogenous inhibitors. Of the two, MMP-9 is arguably the most prominent in the pathogenesis of AAA.[106]

MMP-9 expression and activity is upregulated in human AAA,[107-109] and is significantly increased at the site of aneurysmal rupture.[110, 111] While absent in normal tissues,[112] it is the predominant source of gelatinolytic activity in the luminal and parietal thrombus.[64] Furthermore, plasma levels of MMP-9 are elevated in AAA patients[113] and Type A and B aortic dissection within an hour of onset of symptoms compared to controls.[114]

Given the nature of MMP involvement in aneurysm pathology, numerous studies have attempted to demonstrate an association between MMP levels, their proteolytic activity in the aorta and the size and frequency of rupture. Freestone *et al*. identified MMP-2 as the principal source of gelatinase activity in small aneurysms (4.0-5.5 cm), whereas zymography data indicated a size-associated increase in MMP-9 activity in larger aneurysms (5.5-10 cm).[115] A subsequent study by McMillan *et al*. reported a four-fold elevation in MMP-9 mRNA transcript levels as measured by

competitive PCR in medium-sized AAA (5.0-6.9 cm) compared to small AAA (3.0-4.9 cm), large AAA(>7 cm) and normal aortas.[116] In comparison, Petersen *et al*. measured MMP-2 and MMP-9 levels by ELISA in medium-sized ruptured AAA (5-7 cm) and large asymptomatic unruptured AAA (>7 cm).[111] They determined that MMP-9 levels were significantly higher in medium ruptured AAA compared to large asymptomatic AAA, whereas MMP-2 levels were higher in asymptomatic AAA and demonstrated a positive correlation with diameter of asymptomatic AAA. Interestingly, they did not find a correlation between MMP-9 levels and aortic diameter, suggesting that increased MMP-9 levels may influence aortic rupture independently of dilation.

MMP-9 colocalizes to infiltrating macrophages in AAA in the media and media-adventitia junction.[107, 117] The preferential localization to discrete areas adjacent to the adventitial vasa vasorum suggests that MMP-9 is involved in matrix remodelling processes associated with neovascularisation.[116] While macrophages are the primary source of MMP-9 in the aortic wall, polymorphonuclear leukocytes are responsible for the majority of MMP-9 release in the mural thrombus.[50] MMP-9 synthesis by vascular smooth muscle cells (VSMCs) is also elevated when compared to normal and atherosclerotic controls.[118]

Several animal models have been employed to investigate the role of MMP-9 in AAA. MMP-9 deficiency has proven protective against aortic dilation and progressive destruction of elastin induced by elastase infusion[119] and calcium chloride application.[120] Similar to human AAA, histological analysis confirmed that macrophages are the primary source for MMP-9 in the animal models. In the latter study, it was found that the reinfusion of competent macrophages from wild-type mice into knockout (KO) animals was sufficient to reconstitute AAA in MMP-9-KO but not MMP-2-KO mice, suggesting that these two MMPs may work in concert in the pathogenesis of AAA, with MMP-2 enhancing the degenerative abilities of MMP-9.

Much attention has been given to the gelatinolytic and elastinolytic activity of the MMPs; however, there is growing interest in other functions that MMPs may serve in AAA pathogenesis. VEGF bioavailability is regulated extracellularly by numerous MMPs through intramolecular processing. Lee *et al*. have demonstrated that MMP-3, MMP-7, MMP- 9 and MMP-19 are able to cleave VEGF in an efficient manner, releasing a soluble, biologically active 16 kDa fragment that phosphorylates VEGF receptor 2 (VEGFR2) and induces angiogenesis[121] VEGF has also been shown to be upregulated in human AAA [122, 123] and exacerbates the development of angiotensin-II-induced AAA in mice.[124] Similarly, MMP-2 and MMP-9 are capable of liberating and activating TGF-β from the inactive Large Latent Complex (LLC).[125] The exact role of TGF-β in the pathogenesis of AAA is unclear given that it has both angiogenic and anti-angiogenic properties,[126] and has been shown to promote both the synthesis and degradation of the ECM.[127] However, dysregulation of TGF-β signalling is recognised as a characteristic of Marfan syndrome,[128] where patients frequently suffer from vascular defects including dissection and aneurysm.

Compared to MMP-2 and MMP-9, little is known about the involvement of other MMPs in aneurysm pathogenesis. MMP-12 is selectively expressed in macrophages and foam cells in AAA and atherosclerotic lesions[129] and it has been suggested that MMP-12 is responsible for up to 95% of macrophage-mediated elastin degradation in AAA.[129] Like all other MMPs, MMP-12 is secreted in inactive pro-enzyme form and requires extracellular activation. Interestingly, mice deficient in both apoE and uPA exhibit reduced pro-MMP-12 activation and are protected against medial destruction and aneurysm formation.[130] IL-13 induction of MMP-2, MMP-9, MMP-13 and MMP-14 is decreased in the absence of MMP-12,[131] suggesting that IL-13-induced inflammatory infiltration is, at least in part, mediated by an MMP-12-dependent pathway. Taken together, there is sufficient evidence to support a role for MMP-12 in AAA pathogenesis; however, results from animal studies have been conflicting. Pyo *et al*. reported that MMP-12-KO mice were not protected against elastase-induced aneurysmal degeneration and dilation, with similar incidence rates and elastin loss compared to wild-type

controls;[119] whereas Longo *et al*. showed that MMP-12-KO mice exhibit a reduction in aortic dilation, loss of elastic lamellae and macrophage infiltration following calcium chloride treatment.[129] It is probable that these differences are model-specific and it is still unclear which model is a more accurate representation of human AAA.

Membrane-type-1 MMP (MT1-MMP) is 66 kDa[132] integral plasma membrane protein that functions at the cell surface and is the dominant activator of MMP-2. Nollendorfs *et al*. were able to localize MT1-MMP to both VSMCs and macrophages and show elevated levels of MT1-MMP mRNA and protein in human AAA.[133] In a rat model of elastase-infusion, early MT1-MMP expression was associated with increased activation of MMP-2[134] whereas MT1-MMP protein levels were shown to increase progressively in a murine calcium chloride-induced thoracic aneurysm model for 16 weeks post-induction.[135] While known substrates for MT1-MMP include collagen (I, II, III), gelatin, casein, elastin, fibronectin, laminin, MMP-2 and MMP-13,[106] MT1-MMP is of interest in the context of AAA pathogenesis primarily because of its ability to activate pro-MMP-2. However, a recent study by Xiong *et al*. has demonstrated that MT1-MMP can regulate macrophage-dependent elastolytic activity in AAA development independently of MMP-2 and MMP-9.[136]

Additional roles for MMP-1 and MMP-13 have also been implicated in AAA pathogenesis. Elevated levels of MMP-1 protein and mRNA have been observed in aneurysmal adventitia [137, 138] and elevated plasma levels of MMP-1 are associated with AAA rupture [139]. Lizarbe *et al*. found that inhibition of inducible NO synthase (iNOS) protects against elastase-induced AAA by inhibiting production of MMP-13[140] and that MMP-13-KO mice exhibit increased extracellular MMP inducer (CD147) and reduced aortic dilation following elastase infusion,[140] suggesting that NO may contribute to AAA pathogenesis by modulating the activity of MMP-13.

Due to the significant roles of MMPs in AAA pathogenesis it is essential to explore the role of their counterpart inhibitors during disease progression. Tissue inhibitors of matrix metalloproteinases (TIMPs) are the endogenous inhibitors of the MMPs and represent a fundamental level of MMP regulation. TIMPs comprise 4 known glycoproteins: TIMP-1, TIMP-2, TIMP-3 and TIMP-4, of which TIMP-1 has received the most scrutiny.

TIMP-1 inhibits MMP-1, MMP-2, MMP-3, MMP-9 and MT1-MMP [106] and its expression is localized to VSMCs[116, 141] but can be induced *in vitro* by numerous other cell types such as endothelial cells (EC) and fibroblasts in response to external stimuli.[142] By blocking MT1-MMP and MMP-3, TIMP-1 effectively downregulates the activation of MMP-2 and MMP-9 respectively.[143] Plasma levels of TIMP-1 are significantly elevated in AAA patients compared to atherosclerotic or healthy controls[144] but curiously, TIMP-1 mRNA levels are 250 times higher than MMP-1 and 11 times higher than MMP-9 levels but protein ratios of MMP-1:TIMP-1 and MMP-9:TIMP-1 are significantly higher in aneurysmal aortas.[138]

Some of the strongest experimental data supporting a protective role for TIMP-1 in aneurysm development comes from studies in mice where the effect of TIMP-1 deficiency and overexpression was assessed. Silence *et al*. were able to show that TIMP-1/apoE-double KO (DKO) mice on cholesterol rich diets had increased incidence of aneurysm compared to TIMP-1 $^{+/+}$ mice.[145] TIMP-1 deficiency resulted in enhanced MMP activity and macrophage infiltration.[145] These observations were confirmed by Lemaitre *et al*. who additionally reported increased medial degradation and incidence of pseudoaneurysm in TIMP-1/apoE-DKO mice.[146] TIMP-1 deficient mice also show increased susceptibility to aneurysm induction by elastase infusion, presenting with higher incidence and greater severity.[147] Finally, overexpression of TIMP-1 was able to prevent aortic degeneration, dilation and rupture in a rat xenograft model of AAA.[143]

Cultured VSMCs from both AAA and healthy patients constitutively express both MMP-2 and TIMP-2, its endogenous inhibitor [106] but increased expression of TIMP-2 and MMP-2 in AAA colocalizes to both VSMCs and macrophages.[106, 141] Because the activation of MMP-2 requires the formation of the trimolecular complex of MT1-MMP, TIMP-2 and pro-MMP-2, TIMP-2 can theoretically both promote and inhibit matrix degradation in AAA. Xiong et al. probed this question by inducing aneurysm in TIMP-$2^{-/-}$ mice by application of calcium chloride; however, they concluded that the targeted deletion of TIMP-2 attenuates aneurysmal development, in spite of its supposed role as an MMP inhibitor [148] implying that the relationships between the MMPs and TIMPs may be more complex than initially assumed, and warrants further investigation.

Of all the MMPs involved in AAA pathogenesis, MMP-2 and MMP-9 are the most well characterized and evidence from both experimental and observational studies may imply that inhibition of these proteases will be a beneficial treatment approach for AAA. In that respect, doxycycline has been examined as potential drug that can inhibit MMPs activity. Doxycycline is a synthetic tetracycline derivative that functions both as an antibiotic and a broad-spectrum MMP inhibitor. It binds to the MMP active site and calcium ion site and induces a conformational change that results in the loss of enzymatic activity.[149, 150] Doxycycline also suppresses activation of pro-MMP-2 and inhibits transcription of MMP-9 in macrophages.[151, 152] *In vitro* studies show that doxycycline reduces MMP-9 activity and prevents elastin degradation[153] while data from animal studies show that doxycycline treatment can inhibit MMP activity *in vivo*, preventing elastin degradation and aortic dilation in both an elastase-infusion rat model[154] and dose-dependently in a murine calcium chloride model.[155] A preliminary study in AAA patients (100 mg doxycycline twice daily for 3-6 months) achieved similar plasma levels to murine models where aortic dilation was reduced by 33-66%.[155] Curci *et al*. reported that patients who were given doxycycline for 3 weeks prior to conventional aneurysm repair presented with significantly lower MMP-9 expression and activity in the aortic wall compared to controls[152] while Lindeman *et al*. showed

that a 2-week regimen of doxycycline treatment prior to repair profoundly reduced aortic wall neutrophil and cytotoxic T cell infiltration.[156] A small study by Baxter *et al*. showed significant reduction in MMP-9 plasma levels after 6 months of doxycycline treatment[157] but in a follow-up study on aneurysmal growth rate, the difference between treated and control groups did not achieve statistical significance.[158]

While direct inhibition of MMP activity might appear to be an attractive target for therapeutic intervention in AAA, it would be unwise to ignore the results from the use of broad spectrum MMP inhibitors in anti-cancer drug development programs where immensely promising preclinical results were subsequently followed by complete failure in clinical trials.[159] Firstly, many MMPs play important physiological roles in maintaining cellular homeostasis in normal tissues and in regulation of extracellular signalling networks;[160-162] thus, the use of broad spectrum inhibitors to block the pathological aspects of MMPs may have deleterious effects on overall patient outcome that may be masked in small studies when these "anti-target" functions of MMPs are affected.[161] Significant proportions of patients in these clinical trials developed severe musculoskeletal side effects,[163-165] necessitating the lowering of drug dosage below minimum inhibitory levels, confounding the results and indicating a very low therapeutic index.[166] These clinical trials were also performed with patients with advanced progression of disease while preclinical data indicated that MMP inhibition would be preferentially effective in earlier stages of disease.[161, 167] Similarly in AAA, differential effects of MMPs have been observed at different stages of aneurysm growth,[106] suggesting that targeted inhibition of individual MMPs could prove more beneficial than broad spectrum inhibition with less severe side effects.

Besides blocking MMP activity directly, another option that has been considered is to target the inflammatory response which activates and induces MMP expression. Non-steroidal anti-inflammatory

drugs (NSAIDs), such as indomethacin, reduce IL-1β and IL-6 release in aortic explants and have been shown to reduce MMP-9 activity and attenuate elastase-induced aneurysm in rats.[168] Indomethacin also downregulates synthesis of prostaglandin E_2 (PGE_2), which in turn regulates MMP-9 expression, reducing the inflammatory response and aortic dilation.[169]

There has been much interest in the use of statins, or 3-Hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors as part of AAA therapeutic strategy because not only do they reduce MMP-9 secretion by macrophages,[170] but also because of its beneficial effects on endothelial function, prevention of lipoprotein cholesterol oxidation, inhibition of macrophage and VSMC migration stabilization of atherosclerotic plaque and anti-inflammatory effects.[171] Initial studies were performed with cerivastatin, which has since been withdrawn from the market. However, at maximum clinical doses, cerivastatin downregulated the production of MMP-9 in explanted AAA organ cultures.[172] Patients given a 3-week pre-operative regimen of simvastatin also demonstrated reduced MMP-9 activity in the aortic wall.[173] Observational studies following the growth of untreated AAA in patients have reported that statin use attenuates or even halts expansion completely[174, 175] independently of other cardiovascular risk factors.

1.4.2 Elastase

Destruction of the elastic lamellae is one of the major hallmarks of aneurysmal disease, and this can lead to weakening of the aortic wall and subsequent dilation.[176] Quantification of protein content indicates up to 90% loss of elastin in human AAA specimens compared to healthy controls.[177, 178] The degradation of elastin by elastase may contribute to AAA development not only through reduction in structural stability but also through the generation of elastin-derived peptides that may serve as chemoattractant for inflammatory cells, further exacerbating inflammation in the aneurysmal aorta.[179]

As such, these observations formed the basis for the development of the widely used rodent elastase infusion model of AAA.[180]

Although there are known inconsistencies in outcome with this model, primarily due to variation in the preparation of elastase,[181] as well as the relatively complicated and invasive experimental procedure required, many recent studies have relied heavily on this model to investigate AAA pathogenesis. Although the degradation of elastin may serve as a primary event in AAA pathogenesis, [182] the use of elastase infusion may overshadow other initiating factors leading to this disorder and hinder the study of endogenous elastases involved in the progression of disease.

Evidence for the role of elastase in AAA development originated from earlier studies examining both local and systemic levels of elastase in AAA patients. [183-185] Sources for elastase include neutrophils, macrophages (MMP-12, discussed previously) as well as pancreatic and smooth muscle cells [186] Neutrophil elastase is highly upregulated throughout the adventitia [187] and within the intraluminal thrombi of aneurysmal aorta,[188] providing two sources for this enzyme from opposite sides of the aortic wall. Studies performed with neutrophil-depleted mice have shown a significant reduction in aneurysm formation,[189] however the role of neutrophil elastase per se was not directly evaluated in the this study due to the use of the elastase infusion model. Interestingly, blood samples of AAA patients show increased neutrophil elastase levels in both pre-operative[183] as well as post-operative patients with an increase in neutrophil elastase secretion in the latter group when compared to patients with occlusive aortic disease.[190] These findings suggest that neutrophil elastase release may serve as a primary event in aneurysm development that is independent of the presence of the aneurysm itself as levels of this enzyme remain high six months after aortic repair surgery. [190] Most importantly, this may also highlight the need for continued anti-elastase therapy after aortic repair as elevated elastase levels will likely continue to exert its harmful effects, hastening the need for reintervention in the future. Interestingly, it

has also been found that the elastase activity in aortic tissue of aneurysm patients without concomitant occlusive disease was significantly higher than in aneurysm patients that were diagnosed with occlusive disease, or occlusive disease alone,[183] suggesting that elastase-dependent mechanisms may play a more significant role in non-atherosclerotic AAA, and may have ramifications for the development of treatment strategies in these patients.

The observation of elevated elastase levels in AAA patients led researchers to examine the relationship between this protease and its endogenous inhibitor, alpha-1-antitrypsin (α_1AT). α_1AT deficiency is known to play a major role in the development of lung emphysema. Reduced inhibition of neutrophil elastase leads to increased proteolytic activity resulting in degradation of the alveoli wall.[191] Thus, it was postulated that an imbalance between elastase and α_1AT may also play a role in AAA pathogenesis. This imbalance was indirectly demonstrated by comparing the inhibition of exogenous elastase by plasma derived from either healthy controls or AAA patients. Plasma from healthy controls demonstrated increased elastase inhibition compared to plasma from AAA patients, indicating a reduction of anti-protease activity in AAA plasma; however, a direct measurement of α_1AT in these samples was not performed.[182] On the other hand, there is no increase in the frequency of α_1AT gene deficiency in AAA patients compared to the general population, suggesting that changes in α_1AT activity in AAA patients are independent of gene expression.[192] A recent study demonstrated that α_1AT protein levels are reduced in human ascending aortic aneurysm and aortic dissection compared to control aortas, without significant changes in α_1AT mRNA levels between aneurysm tissues and control.[193] While augmentation therapy using exogenous purified α_1AT administration is approved by the US Food and Drug Administration (FDA) for α_1AT deficient patients with evidence of respiratory disease,[194] this treatment approach remains controversial with a major concern being the excessive cost for treatment. Interestingly, peri-adventitial treatment with an elastin-stabilizing agent, pentagalloyl glucose (PGG), protected rat aorta from developing AAA after peri-aortic application of calcium chloride ($CaCl_2$)

solution.[195] Furthermore, administration of PGG on established aneurysm, 28 days post-$CaCl_2$ application, resulted in attenuation of AAA progression and reduction in aortic diameter was observed. It was suggested that PGG binds to the elastic lamellae and prevent elastin degradation when faced with the mounting immune response that develops in the aorta of these rats.[195] While in its current state, PGG treatment must be administered peri-adventitially and thus rendered less relevant as a pharmacological agent, it may still be useful as an adjunct treatment during aortic surgical repair to minimize post-surgery aneurysm recurrence.[195]

1.4.3 CATHEPSINS

Cysteine cathepsins are a family of lysosomal proteases that are potent elastolytic and collagenolytic enzymes.[196] Due to their involvement in numerous chronic inflammatory disease such as atherosclerosis,[197] arthritis [198] and emphysema,[199] their expression and release by macrophages, SMC,[200] neutrophils and mast cells, and their potent elastolytic abilities,[201] cathepsins are strong candidates for key roles in AAA onset and/or progression.[202]

Of the 11 known cysteine cathepsins, cathepsin K has the most potent elastolytic ability,[196]while cathepsin K, L and S have demonstrated collagenolytic enzyme activity at physiologically relevant temperatures, a function that was previously only attributed to the MMPs.[203] Several studies have demonstrated a significant increase in both protein expression and activity of cathepsins B,H, S, K, and L in human AAA samples compared to control aortas.[49, 200, 202, 204, 205] Increased activity of cathepsins B, H, L, and S appear to be atheroma-independent as the activity of these proteases have been shown to be higher in AAA compared to atherosclerotic aortas.[205] Furthermore, elevated activity of cathepsin S was observed in both growing aneurysm and ruptured human AAA samples, indicating a potential causative role for this particular cathepsin in disease progression.[49]

The original evidence for cathepsin involvement in aneurysm pathogenesis came from experiments showing dysregulated cathepsin activity during disease progression. A number of studies have shown a significant reduction in cystatin C, the endogenous extracellular inhibitor of cathepsins, in human AAA specimens compared to controls and is associated with aneurysm progression and increased expansion rate.[49, 202, 205-208] Cystatin C deficiency was observed both at the tissue level and in the serum, indicating a potential imbalance between cathepsins and their inhibitors resulting in a net increase in cathepsin activity during AAA development.[202, 207, 208] Pro-inflammatory cytokines,[209] reduced TGF-β signalling[207] and degradation of cystatin C by neutrophil-derived proteases such as neutrophil elastase and MMP-8,[49] are all mechanisms that may alter the balance between cystatin C and cathepsins in aneurysmal aortas. Furthermore, it has been shown that cystatin C/apoE-DKO mice exhibit increased cathepsin activity compared to wild type (WT) control mice[210, 211] and experience greater aortic dilation, increased elastic lamina degradation and elevated immune infiltration. As chymase and tryptase can regulate the activity and expression of cathepsins, reduced cathepsin activity has also been attributed to chymase and tryptase regulation as deficiencies of the latter proteases are protective against AAA development in mice.[212, 213]

Cathepsin C (also referred as dipeptidyl peptidase I (DPPI), a protease responsible for the activation of zymogen granule enzymes, may also contribute to AAA pathogenesis through increased activation and release of granule enzymes and promoting neutrophil recruitment to the affected aorta.[214] Cathepsin C expression is associated with AAA development while deficiency of this protease results in reduced aneurysm formation, greater preservation of elastic lamellae and reduced inflammation.[214]

Various cathepsin-KO mice models have been developed and it has been demonstrated that cathepsin L deficiency leads to reduced AAA development, reduced elastin fragmentation and reduced

inflammation in elastase infused aortas and in periaortic $CaCl_2$ treated mice,[215] while cathepsin S deficiency reduced aortic lesion expression and activity of matrix metalloproteinase MMP-2, MMP-9, and cathepsin K as well as significantly reducing AAA lesion media SMC apoptosis and inflammatory cell accumulation and proliferation.[216] Cathepsin S was also shown to promote angiogenesis, monocyte and T-cell transmigration, and T-cell proliferation *in vitro*.[216]

Contradictory observations have been published regarding the effect of cathepsin K deficiency in animal models of AAA. Bai *et al*, reported that cathepsin K deficiency did not result in any difference in aneurysm development or changes to the elastic lamellae in Angiotensin II (Ang II)-infused apoE-KO mice,[217] while Sun *et al.* reported that cathepsin K deficiency did result in reduced aneurysm formation, reduced elastin fragmentation, reduced SMC apoptosis and lower inflammatory infiltrate using the elastase infusion model of AAA.[218] These contradicting results may be attributed to the difference in experimental models used to induce AAA in these mice. In elastase-infused mice, inflammation is stimulated locally, while in the Ang II model a more systemic induction occurs, which may promote peripheral inflammatory cell recruitment to the aorta.[218] Although not directly assessed, it was hypothesized in both manuscripts that changes in inflammatory status due to Ang II infusion might support further activation of immune cells at the site of aneurysm development leading to overexpression and activation of other proteases and cathepsins, such as cathepsin S and cathepsin C, leading to increased proteolytic activity exerted by these enzymes, potentially obscuring the effect of cathepsin K in AAA development.[217, 218]

Although the physiological inducers of cathepsin activation and release in human AAA are yet to be determined, the observations from both human AAA tissue and animal models of AAA demonstrate the importance of maintaining the balance between cysteine cathepsins and their inhibitors, and that

dysregulation of cathepsin activity may contribute to loss of arterial integrity, vascular remodelling and aortic dilation in AAA.

1.4.4 Chymase and Tryptase

Chymases and tryptases are serine proteases that are largely restricted to mast cells where they constitute the majority of endopeptidases stored within the secretory granules.[219] A potential role for chymases and tryptases in the development of AAA was suggested when an increase in mast cells was observed in the media and adventitia of AAA lesions.[220, 221] The number of mast cells in the outer media and adventitia of human AAA specimens was significantly higher than the numbers typically observed in both early and advanced atherosclerotic lesions, as well as healthy aorta. Mast cell count increased up to 4-fold in aneurysmal non-ruptured aorta compared with non-dilated aortas in autopsy cases, indicating the specific localization of mast cells to the aneurysmal tissue.[221] Furthermore, mast cell numbers in human AAA correlated with the degree of aortic dilation observed.[221] While the exact mechanism involved in mast cell recruitment is yet unclear, elevated mRNA and protein levels of leukotriene C4 (LTC4) and its three key enzymes (5-lipoxygenase (5-LO), 5-LO-activating protein, LTC4 synthase) were localized to regions of increased inflammatory cell infiltrate, suggesting that LTC4 participates in mast cell recruitment in aneurysmal development.

Direct involvement of mast cells in AAA has been shown in various experimental animal models. In both the periaortic $CaCl_2$ model and the elastase-infusion model, the lack of functional mast cells in $Kit^{W\text{-}sh/W\text{-}sh}$ mice protected the animals from AAA development.[212, 222] Mast cell deficiency reduced medial elastin degradation, SMC loss, adventitial angiogenesis and lesion activity of various cysteine cathepsins, MMP-2 and MMP-9. Reduced AAA formation, levels of inflammatory cytokines and protease activity was restored in $Kit^{W\text{-}sh/W\text{-}sh}$ mice that received exogenous WT mast cells.[222] Mice treated with mast cell activator, compound 48/80, exhibited increased AAA dilation, upregulated protease expression and

greater elastin loss compared to controls, while mice treated with mast cell stabilizer, Cromolyn, saw a suppression of similar phenotypes.[222] Similar results were observed in rats, where an increase in mast cells was observed at 3,7 and 14 days after periaortic $CaCl_2$ application.[221] The absence of mast cells in Ws/Ws rats or treatment with mast cell stabilizer, tranilast, preserved elastic lamellae, reduced MMP-2 and -9 activity and prevented AAA development.[221] These findings support a regulatory role for mast cells in AAA development and preventing mast cell degranulation may prove to be an attract therapeutic target, and justify further study of mast cell recruitment and activation mechanisms in the aneurysmal aorta.

The mast cell-specific proteases include chymase, tryptase and carboxypeptidase A and together they constitute 25% of total cellular protein, of which tryptase accounts for nearly 20%.[223] Mast cell accumulation in AAA lesions are typically confirmed by the use of chymase or tryptase antibody-mediated immunohistological analysis.[224] In a prospective clinical follow-up study, it was determined that plasma levels of chymase in Danish men correlated with annual AAA growth rate but was not associated with initial AAA size.[225] In a separate study, plasma tryptase levels were significantly elevated in AAA patients compared to controls, although many in the AAA group were smokers and had other cardiovascular complications; however, tryptase levels also correlated with annual growth rates and regression analysis confirmed that higher tryptase levels indicated a higher likelihood of requiring later surgical repair even after adjustment for more than 10 potential AAA risk factors.[213]

The substrate specificities of both tryptase and chymase makes them pertinent to AAA pathogenesis. These include several ECM proteins including procollagen, VE-cadherin, fibronectin and vitronectin (reviewed in [224, 226]). Cleavage of fibronectin and vitronectin may lead to the disruption of the SMC focal adhesion complex and subsequent apoptosis.[224] Chymase also inhibits SMC collagen synthesis[227] while tryptase induces the endothelial cell production of chemokines IL-8 and MCP-1[228] and

both have been demonstrated to activate endothelial protease activated receptor-2 (PAR-2)[224, 229] which may affect regulation of vascular tone and permeability and contribute to pro-inflammatory response as observed in atherosclerosis.[230] While the ability of these enzymes to directly degrade the elastic lamellae is unclear, the potential role of chymase and tryptase in aortic wall deterioration may also rely on their ability to regulate MMP and cathepsin activity.[231, 232] Both proteases activate MMP-1 and MMP-3, which subsequently activate MMP-9.[233, 234] Furthermore, chymase converts angiotensin I (Ang I) to Ang II .[235] Ang II may play a significant role in AAA development as it promotes inflammation within the aorta, while Ang II infusion is a widely used mouse model of AAA and is discussed in section 1.6.[236] In this model, oral administration of chymase inhibitor, NK3201 significantly reduced AAA development, monocyte recruitment, and MMP-9 expression and activity.[237] Similar results were obtained using this inhibitor in elastase-perfused dogs[238] and hamsters.[239]

In experiments using elastase-infused mice deficient in mouse mast cell protease- 4 (mMCP-4), the most relevant human chymase ortholog, it was shown that these mice demonstrate significant attenuation of aneurysm formation, reduced immune infiltration, higher medial SMC content and less neovascularisation within the adventitia.[212] Reduced expression and activity of cysteine cathepsins, chymase-mediated SMC apoptosis and chymase-mediated angiogenesis in these mice were also noted when compared to WT controls.[212]

Mice deficient in mouse mast cell protease-6 (mMCP-6) or murine tryptase, did not develop aortic dilation at 7, 14 or 56 days post elastase-perfusion. Lesions macrophage and T cell infiltration and major histocompatibility complex class II (MHC-II) molecules were also greatly reduced,[213] while endothelial cells from *Mcpt6*$^{-/-}$ mice had significant reduction in the production of TNF-α, IL-6 and of chemokine KC (IL-8 homolog), macrophage inflammatory protein-2 (MIP-2) and lipopolysaccharide-induced CXC (LIX); all of which may contribute to reduced leukocyte infiltration in the vascular tissues.[213]

Deficiency in mMCP6 has also resulted in reduced SMC apoptosis, reduced cysteinyl cathepsin activity and decreased immune infiltration. However, as opposed to mMCP4, mMCP6 mice exhibited no effect on neovascularization [213].

A discussion of the potential roles for chymase and tryptase in AAA would not be complete without an investigation of their endogenous inhibitors within the context of this disease; however, knowledge in this area is lacking due to scarcity of known specific endogenous inhibitors for these enzymes. Earlier studies have identified α_1-antichymotrypsin (ACT), α_2-macroglobulin and α1-protease inhibitor as probable inhibitors of chymase, however their inhibitory effect *in vivo* still requires clarification as chymase in its physiological role is conjugated with heparin proteoglycan and is resistant to inhibition by these inhibitors.[240] Endogenous inhibitors for tetrameric tryptase have yet to be identified.[226] For this reason, most work in this area has been devoted to the development of small molecule inhibitors against these enzymes. Although most of these synthetic inhibitors were developed to inhibit human chymase and tryptase, to date, they have only been tested in animal models of disease, thus the efficacy of these inhibitors in humans remains to be determined.[226] This issue may become more complicated when interpreting data from studies in mice, considering that there are multiple gene orthologs of chymases (mMCP-1, -2, -4, -5, and -9) that are present in mice but absent in humans.[226] Further, chymase and tryptase are involved in a complex regulatory network that controls of the activity and expression of other proteases (MMPs, cathepsins) both in mast cells and in other cell types (SMC, monocytes), regulates leukocyte migration and the modulation of the cytokine milieu during inflammation. Moreover, chymase and tryptase may also have anti-inflammatory roles as they can degrade key inflammatory cytokines and this is highly suggestive of a role for them in dampening the immune response.[219] Extreme caution will be required in pursuing chymase and tryptase inhibition as a therapeutic strategy to treat AAA since interruption of their physiological activity may have immense impact on several pathways which are currently either unknown or ill defined. Nevertheless,

the relative clinical success of mast cell stabilizers in asthma, as well as the promising results from studies using specific chymase inhibitors in several animal models of AAA,[237, 238, 241] do indicate significant potential for this treatment approach.

1.5 GRANZYME B

In 1978, Hatcher *et al*. isolated a cytotoxic protease from lymphocytes that was capable of inducing apoptosis in target cells.[242] Shortly after, the granzymes, or granule enzymes, were identified as a family of conserved serine proteases found in the granules of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells.[243-248] There are 5 known granzymes expressed in humans: A, B, H, K, and M; and 11 expressed in mice: A, B, C, D, E, F, G, K, L, M, and N.[249] Of these, granzymes A and B (GZMA and GZMB) are the most abundant and subsequently, also the most well-characterized. GZMB, in particular, has been studied extensively for its central role in mediating programmed cell death.

GZMB is a 32 kDa chymotrypsin-like serine protease and is characterized by a His-Asp-Ser catalytic triad in its active site.[250] It is variably known as cytotoxic T lymphocyte-associated serine esterase-1 (CTLA-1) or granzyme 2 and possesses the unusual ability to cleave substrates at aspartic acid residues. This specificity is reliant on the presence of an arginine residue at position 226 within the binding pocket.[251]

The GZMB cluster is located on chromosome 14 in both mice and humans, but where the cluster in humans comprises GZMB, granzyme H (GZMH), cathepsin G (CG), and mast cell chymase-1 (CMA-1), the cluster in mice consists of GZMB, granzymes C, D, E, F, G and N, CG and CMA-1. Within CTLs, GZMB transcription is induced by T cell receptor activation and co-stimulation with cytokines.[252] The upstream promoter region for GZMB contains binding sites for two general transcription factors: activating

transcription factor/cyclic AMP-responsive element-binding protein (ATF/CREB) and activator protein-1 (AP-1); and two lymphoid-specific transcription factors: Ikaros and core-binding factor (CBF/PEBP2).[253-256] Transcriptional activation requires all 4 factors to act in concert and a single mutation to any of the transcription factor binding sites will abrogate all expression of GZMB.[253, 255]

1.5.1 GZMB-INDUCED CELL DEATH

The granule-mediated cell death pathway is the primary means used for the clearance of pathogen-infected cells and the removal of tumours (reviewed in Russell and Ley[257]) and the primary effectors of this pathway are the granzymes. Most lymphocytes express GZMB constitutively and further upregulate transcription when activated. GZMB expression in CTLs and NK cells is regulated by many of the same factors that stimulate immune cell activation, such as the composition of the cytokine milieu, receptor engagement and presence of $CD4^+$ T cell populations.[252] GZMB is synthesized as a pro-peptide and is tagged with a mannose-6-phosphate receptor (MPR) to target it to lytic granules.[258] Upon entering the granule, GZMB is activated by cleavage of a 2 amino acid propeptide domain by cathepsin C and then stored in complex with the chondroitin sulfate proteoglycan, serglycin.[259-262] The acidic pH inside the lytic granules and storage with serglycin serve to minimize proteolytic activity until engagement.[252]

When the cytotoxic cell engages with its target, a rapid polarization of lytic granules occurs towards the immunological synapse (IS).[263] The movement of lytic granules and other soluble factors across the IS is directional and enabled by the microtubule-organizing centre.[264, 265] Upon delivery to the target site, the lytic granule fuses with the target cell plasma membrane and its contents are endocytosed into the target cytoplasm. Because GZMB must access intracellular substrates to initiate the apoptotic cascade, a key step in GZMB function is necessarily its entry into the targets cell

cytoplasm. By convention, it was believed that this was mediated by perforin; however the exact mechanism by which this occurs is still under investigation. Perforin is a calcium-dependent cytolytic protein that multimerizes in the target cell plasma membrane, forming a pore 5-20 nm across.[266] While traditional models suggest that perforin grants GZMB entry into the target cell cytoplasm through the formation of these pores, it has also been shown that GZMB can be endocytosed via binding to the mannose-6-phosphate receptor in the absence of perforin.[267] Although its exact role in facilitating GZMB entry into the target cell is still unclear, perforin is definitely required for GZMB-mediated apoptosis as perforin deficiency is associated with impaired lymphocyte-mediated cytotoxicity, while perforin-deficient mice are highly susceptible to viral infection[268, 269] and cancer.[270, 271]

Upon entry into the target cell cytoplasm, (see Figure 1) GZMB exhibits broad substrate specificity and has been shown to cleave and activate caspase-3, -7 and -8 *in vivo*.[272-274] Activation of executioner caspase-3 is integral to the apoptotic cascade as it processes the inhibitor of caspase-activated deoxyribonuclease, DNA damage sensor, nuclear lamins and others (reviewed in Hengartner[275]) GZMB also cleaves Bid to gtBid, which then translocates to the mitochondria where it interacts with Bax and Bak to disrupt mitochondrial membrane integrity,[276-278] leading to increased membrane permeability and the release of cytochrome c and apoptosis-inducing factor (AIF)[279, 280] which stimulates the formation of the apoptosome. GZMB has also been shown to cleave anti-apoptotic protein myeloid cell leukemia sequence-1 (MCL-1).[281] This leads to the release of pro-apoptotic Bcl-2 family member Bim which can also induce mitochondrial outer membrane permeabilization and cytochrome c release.

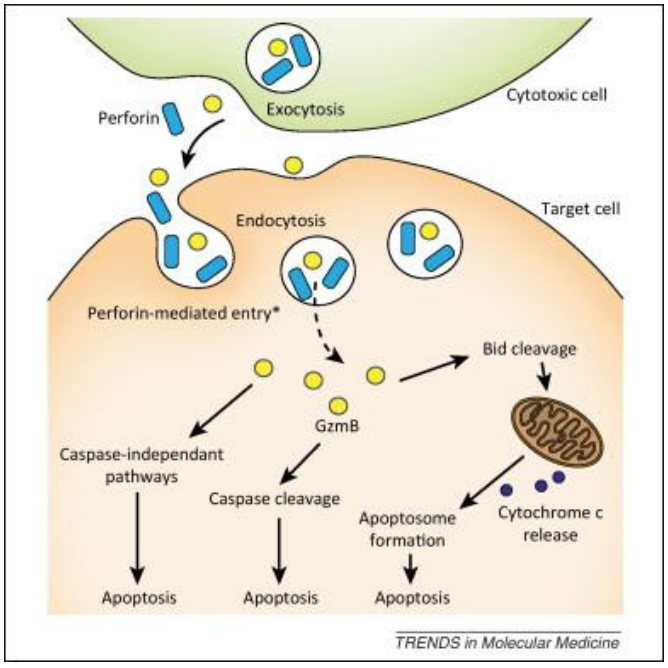


FIGURE 1: GZMB-MEDIATED APOPTOSIS

GZMB and perforin are released into the immunological synapse from the granules of the cytotoxic cell towards the target. They are taken into the target cell via endocytosis and GZMB gains access to the target cell cytoplasm via a perforin-mediated mechanism.GZMB cleaves various intracellular substrates initiating caspase-independent and caspase-dependent apoptosis. Reproduced with permission from Hiebert and Granville, *Trends Mol Med*, (2012).[282]

1.5.2 Extracellular GZMB Activity

Although the granzymes were originally discovered as both intracellular and extracellular proteases, the vast majority of research has focused on their intracellular functions; however, there has been a recent resurgence in interest regarding the extracellular abilities of GZMB in particular. While it was once believed that GZMB was expressed exclusively by CTLs and NK cells, it has been observed that under pro-inflammatory conditions, GZMB can be expressed by numerous immune ($CD4^+$ cells, mast cells, macrophages, neutrophils, basophils, dendritic cells, T regulatory cells) and non-immune cell types (SMC, chondrocytes, keratinocytes, pneumocytes, Sertoli cells, spermatocytes, granulosa cells and syncytial trophoblasts).[283-293]

Although certain cell types such as keratinocytes, chondrocytes and neutrophils can express both GZMB and perforin together, other types such as mast cells express GZMB in the absence of perforin, which would be indicative of GZMB having a purely extracellular function in these cells.[284, 293-295] Upon examining the composition of the GZMB gene cluster on chromosome 14, it becomes apparent

that GZMB is clustered together with numerous mast cell proteases apart from perforin and GZMA and as a result, GZMB can be expressed by myeloid cells independently of perforin.[288, 290, 296]

25 years ago, Kramer and Simon suggested that a proportion of granule proteases may leak from the IS during CTL degranulation.[297] It was now known that up to a third of GZMA and GZMB maybe be constitutively secreted by T cells,[298] with NK cells primarily releasing active proteases via the granule pathway and CTLs secreting inactive zymogen in a granule-independent manner, suggesting the possibility for the existence of extracellular GZMB activator(s).[299]

GZMB is present at low levels in the plasma of healthy individuals with the median reported levels ranging from 20-40pg/ml.[300, 301] Serum levels of GZMB are known to be elevated in numerous inflammatory diseases such as Epstein-Barr virus infection, arthritis, human immunodeficiency virus-1 (HIV-1) infection amongst others.[300-302] GZMB levels have also been reported to be elevated in the synovial fluid of rheumatoid arthritis patients,[303] cerebrospinal fluid of multiple sclerosis[304] and Rasmussen encephalitis patients,[305] and bronchoalveolar lavage in chronic obstructive pulmonary disease (COPD)[306] and lung inflammation.[307]

Furthermore, although the endogenous inhibitor of GZMB, protease inhibitor-9 (PI-9), is present in the plasma, GZMB retains up to 70% of its activity, suggesting that PI-9 is unable to inhibit GZMB effectively in the circulation,[308, 309] and in the absence of other extracellular inhibitors, it is possible that GZMB may be largely unregulated in comparison to other ECM proteases like the MMPs, which are tightly regulated by their inhibitors.

1.5.3 Extracellular GZMB Substrates

The earliest extracellular substrates for GZMB were identified in the 1990's, and it is now accepted that GZMB cleaves a variety of cell surface receptors, cytokines and has numerous targets in the ECM and the blood clotting cascade.

GZMB has been shown to cleave cell surface receptors such the nicotinic acetylcholine receptor,[310] the neuronal glutamate receptor,[311] Notch-1,[312] and fibroblast growth factor receptor-1.[312] Cleavage of the latter 2 receptors may affect cell signalling pathways involved in tumour survival and antiviral activity, while cleavage of the former two uncover cryptic epitopes that generate autoantigenic fragments. This may trigger an autoimmune response, further exacerbating or possibly inducing a state of chronic inflammation.

GZMB cleaves a number of targets involved in blood clotting, amongst them are plasmin,[313] plasminogen,[313] von Willebrand factor (vWF)[314] and matrix fibrinogen.[313, 314] Plasminogen is the precursor of plasmin, which mediates the dissolution of fibrin clots,[315] amongst other proteolytic functions. While plasmin is considered proangiogenic, cleavage of both plasminogen and plasmin by GZMB results in the production of a 38 kDa fragment called angiostatin, which is antiangiogenic[313] and has been shown to induce endothelial cell apoptosis.[316] GZMB cleaves vWF in the A1-3 domains with comparable efficiency to ADAMTS-13[314] As this region is necessary for interaction with platelets, GZMB cleavage can result in impaired platelet aggregation.[314]

Interleukin-1α (IL-1α) is a pro-inflammatory cytokine that is known to be elevated in AAA.[317, 318] It signals through the type I IL-1 receptor[319] and IL-1 signalling activates prostaglandin synthesis,[320] induces nitric oxide synthase in smooth muscle,[321] and promotes the release of other pro-inflammatory mediators, including IL-6,[322] a prominent cytokine observed consistently in aneurysmal tissue.[323] A recent study by Afonina *et al*. identified IL-1α as a substrate for GZMB, where cleavage by GZMB

potentiates the biological activity of this cytokine both *in vitro and in vivo.*[324] GZMB -processed IL-1α also exhibited greater potency as an immuno-adjuvant when injected into mice with ovalbumin.[324]

Numerous targets for GZMB cleavage have been identified in the ECM, namely aggrecan,[325] fibronectin,[326, 327] vitronectin, [76] decorin,[98] fibrillin-1,[328] biglycan,[98] betaglycan,[98] laminin α5[76] and others. The Arg–Gly–Asp (RGD) domain is the recognition sequence for integrin binding[329] in many ECM proteins and GZMB is known to or purported to cleave it in vitronectin and fibronectin,[326] indicating that GZMB-mediated proteolysis of these glycoproteins may alter cell–matrix interactions and affect cell migration and adhesion.[326]

Many ECM components serve in a structural capacity. For instance, decorin[88] and biglycan[330] regulate collagen fibrillogenesis while fibrillin-1 associates with elastin to form elastic microfibrils[331] in the vasculature. Degradation of these components may destabilize the vessel, weakening it and rendering it unable to withstand pulsatile blood flow. However, GZMB cleavage of ECM components does not only result in mechanical damage and direct loss of structural integrity in the target tissues. Of the known substrates, fibronectin, laminin and vitronectin have major roles in cell adhesion[326] and cleavage of these proteins can result in cell detachment and death by anoikis.[327] Choy *et al*. have shown that up to 30% of CTL-induced cell death was GZMB-dependent but perforin-independent after describing anoikis in cultured SMC following fibronectin cleavage.[327] Buzza *et al*. took this further by culturing endothelial cells on pure fibronectin, laminin and vitronectin matrices and also observed cell death by anoikis following incubation with GZMB in the absence of perforin.[326]

It has been suggested that ECM fragments generated by GZMB cleavage may possess chemotactic properties, recruiting inflammatory cells to the site of injury and further exacerbating the inflammatory state.[332] Fibronectin fragments have been shown to have chemotactic properties, attracting neutrophils and monocytes,[332-334] and additionally, have the ability to induce MMP expression

in chondrocytes.[335] ECM fragments may further serve as signalling molecules to neighbouring cells,[312] propagating the inflammatory phenotype in the vicinity of injury. The ECM also acts a reservoir for growth factors and cytokines, and the interaction between various ECM components, attendant proteases and these molecules can influence their storage, concentration, activation status, synthesis and degradation.[67, 68] For example, the TGF-β family can be sequestered in both active and latent forms by various proteoglycans in the ECM,[336] and degradation by GZMB could induce unregulated release of these molecules, elevating circulating levels as well as influencing and unbalancing various signalling pathways in the surrounding tissue.

A selection of AAA-relevant extracellular GZMB substrates are summarized in Table 2.

TABLE 2: EXTRACELLULAR GZMB SUBSTRATES AND POTENTIAL CONSEQUENCES IN AAA

Substrate	Role in vasculature and circulation	Potential consequences of cleavage	Status in AAA	References
Decorin	Regulates collagen fibril organization, sequesters growth factors, regulates cell proliferation and endothelial cell-matrix interactions during angiogenesis.	Impaired collagen fibrillogenesis, loss of vessel tensile strength and distensibility. Release of TGF-β.	Differential localization and altered ratio of decorin to other proteoglycans has been observed.	88, 98, 337-340
Biglycan	Regulates collagen assembly and cell proliferation, sequesters cytokines and growth factors.	Impaired collagen fibrillogenesis, loss of vessel tensile strength and distensibility. Release of TGF-β.	60% reduction in biglycan mRNA expression was observed in human AAA. Biglycan deficiency resulted in spontaneous aortic dissection and rupture in mice.	98, 330, 340-342
Fibrillin-1	Associates with elastin as a major scaffolding component of elastic microfibrils which provide load bearing support for the entire vessel.	Destruction of the elastic lamellar unit, loss of vessel elasticity and structural integrity.	Mutations in FBN-1 lead to inability of vessel wall to sustain hemodynamic stress due to mechanical collapse of the elastic lamellae resulting in dissecting aortic aneurysms in Marfan's syndrome.	331, 343, 344
IL-1α	Activates prostaglandin synthesis, induces iNOS in SMC, initiation of ischemia induced inflammation.	Cleavage augments proinflammatory biological activity, increases potency as immune-adjuvant.	IL-1α is elevated in AAA and promotes release of proinflammatory mediators eg. IL-6.	320, 322, 345, 346
Fibronectin	Cell adhesion, growth, differentiation and migration. Mediates SMC phenotype.	Impaired integrin binding. Impaired tissue repair, blood clotting, and cell migration and adhesion, SMC apoptosis and anoikis.	Loss of fibronectin in aneurysmal basement membrane observed, but is densely expressed in ruptured aneurysms and atherosclerotic lesions	326, 327, 347, 348
von Willebrand factor	Formation of platelet aggregates and clotting at sites of vascular endothelial cell injury.	Cleavage site within the domain responsible for platelet interaction, delaying thrombosis.	Peri-operative thrombocytopenia is associated with poor outcome in ruptured AAA.	349
Plasminogen	Precursor of plasmin, which is proangiogenic and has both proteolytic and fibrinolytic effects on the ECM, enabling cell migration.	Production of antiangiogenic angiostatin, defective clot clearance, impaired cell migration and angiogenesis, induces endothelial cell apoptosis.	PAI-1 reduces AAA incidence and rupture. tPA activation associated with greater expansion of AAA.	313, 316, 350, 351
Vitronectin	Cell adhesion, hemostasis, SMC migration and chemotaxis via Ras, ERK1/2, and p38. Binds PAI-1 and regulates plasminogen-mediated proteolysis of ECM.	Impaired SMC migration and proliferation. Disruption of growth factor binding.	Identified as a biomarker for AAA and correlated for AAA expansion rate.	326, 352-355

1.5.4 GZMB INHIBITORS

Because of the role GZMB plays in mediating cytotoxicity, cells expressing GZMB need a means of controlling extraneous GZMB in order to forestall their own autolysis. Currently, the only known endogenous inhibitor of GZMB in humans is the intracellular PI-9, which is a member of the large family of serine protease inhibitors (serpins) and is also known as serpin B9.[356]

Serpins inhibit serine protease activity by acting as a pseudo-substrate for their target protease. They contain a unique reactive centre loop (RCL) near the carboxy terminus that undergoes an irreversible conformational change when the serpin complexes with its protease, which cleaves at the RCL between two amino acid residues termed P_1 and P_1'. Serpin-protease specificity is typically dictated by the identity of P_1.[357]

Although GZMB, like the caspases, preferentially cleave substrates after aspartic acid, it is also able to cleave after various other acidic residues. So while GZMB is able to cleave after the P_1 glutamic acid in PI-9, caspases typically do not,[358] and this would appear to confer very high specificity of interaction between PI-9 and GZMB.[359] As expected, PI-9 is expressed by various immune cell types (T-cells, B-cells) and antigen presenting cells (APCs) [356, 359, 360] to protect themselves against misdirected GZMB, but it has also been observed that high levels of PI-9 expression may be found in VSMCs (VSMCs),[361] hepatocytes[362, 363] and endothelial cells,[364] as well as various cells types in immune-privileged tissues such as the eyes, testes, ovaries and placenta.[360]

In mice, the murine ortholog to PI-9 is known as serine protease inhibitor-6 (SPI-6) and is similarly capable of regulating intracellular murine GZMB activity.[365] Interestingly, human PI-9 is not capable of inhibiting murine GZMB, and neither is murine GZMB very effective at inducing apoptosis in human cells,[365] which would be indicative of a significant difference in functional specificities between the human and murine granzymes.

While no extracellular inhibitors of GZMB have been identified in humans, Sipione *et al*.[366] discovered an inhibitor of both murine and human GZMB, called serpin A3N (SA3N), that is expressed and secreted by mouse Sertoli cells in the seminiferous tubules, where it is believed to act in concert with SPI-6 to modulate activity of GZMB and protect islet grafts from allo-, auto-, and xenoimmune mechanisms of destruction. SA3N is now known to be expressed in the brain, testes, spleen, lung and thymus and has a high degree of homology with human ACT or serpin A3.[367] While in humans there is a single gene coding for ACT, repeated duplications have resulted in a cluster of 13 closely related genes at the same locus in mice.[367] Interestingly, while ACT shares homology with SA3N, it does not appear to have an inhibitory effect on GZMB.[368]

GZMB activity may also be indirectly affected by other granzymes. Granzyme M (GZMM) has been shown to inactivate PI-9,[369] and could therefore deregulate GZMB activity, while GZMH may potentiate GZMB antiviral capabilities by degrading adenoviral protein Ad5-100K, which is required for viral replication and also inhibits GZMB activity.[370] Several viral serpins have been found to inhibit GZMB, of which cytokine response modifier-A (crmA)[371-373] and Serp-2 are the most well-known. CrmA contains an aspartic acid at P_1[356] and is known to inhibit various intracellular cysteine proteases involved in apoptosis and to prevent the maturation of IL-1β. Myxoma virus serp2 has been shown to be a weak inhibitor of GZMB, but unlike crmA, is unable to prevent apoptosis in cowpox virus-infected cells.[374]

1.5.5 GZMB IN ATHEROSCLEROSIS

With the discovery that GZMB can be expressed and secreted by numerous immune and non-immune cell types, and the identification of novel extracellular substrates, the role of GZMB in disease is now being re-evaluated. During conditions of chronic inflammation, the infiltration of immune cells can result in the elevation of GZMB levels both at the site of injury and in the circulation.

Atherosclerosis is responsible for the majority of cerebrovascular disease, coronary artery disease, myocardial infarction and stroke[375] [376] and is currently the leading cause of death worldwide.[28] It can be described as a chronic inflammatory disease of the arterial wall that results in lipid-lesion formation and luminal narrowing of the vessel, typically at locations where laminar flow is disrupted. T lymphocytes and macrophages are the key immune mediators in atherosclerotic lesions and also the most abundant,[377-379] although smaller populations of neutrophils, mast cells, dendritic cells and NK cells are also involved. [380-382]

Atherosclerosis begins with endothelial dysfunction, which is characterized by reduced vasodilation, release of proinflammatory cytokines, enhanced pro-thrombotic functions and increased cell adhesion molecule expression.[383] It may be caused by hemodynamic stress, chronic hyperlipidemia or exposure to oxidative stress resulting from smoking, hypertension or diabetes mellitus.[384] The release of cytokines recruits immune cells to the region of endothelial dysfunction, where oxidative stress has lowered nitric oxide (NO) bioavailability and upregulated the expression of cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) which binds monocytes and T lymphocytes,[384] and induced MCP-1 expression which recruits mononuclear phagocytes.[385] As immune cells migrate into the intima, macrophages engulf oxidised lipoproteins and mature into lipid-laden foam cells,[283, 377, 386] which then secrete growth factors stimulating VSMC migration, proliferation and the synthesis of collagen and proteoglycans, forming a fibrous matrix that develops into a plaque with a fibrous cap. [384]

The level of GZMB expression has been shown to correlate with disease severity and has been observed in both the lipid rich and cellular areas as well as in the shoulder regions of atherosclerotic plaques.[283] Within the intima and media, GZMB has been found to colocalize to SMC, macrophages and T cells, but has also been observed extracellularly.[283] Additionally, elevated levels of circulating GZMB correspond to carotid plaque instability and incidence of cerebrovascular events,[387] while Tsuru *et al*.[388]

found that GZMB levels in peripheral blood mononuclear cells were elevated in patients with unstable angina pectoris compared to patients with stable angina pectoris, making it highly indicative that GZMB affects plaque stability.

GZMB colocalization to TUNEL-positive foam cells suggests that GZMB may be responsible for cell death in the lipid-rich necrotic core;[283] however, GZMB can cleave numerous ECM proteins found in the atherosclerotic plaque such as fibronectin, vitronectin and laminin and thereby contribute to ECM fragmentation [326, 327] and induce perforin-independent SMC apoptosis via anoikis.[327]

The effect of elevated GZMB activity in atherosclerosis may vary with the stage of lesion development, as in the early stages of atherosclerosis, GZMB-induced SMC apoptosis may act to reduce intimal hyperplasia,[389] while in more advanced lesions with fibrous caps, increased apoptosis of SMC and ECM degradation may undermine cap integrity and contribute to plaque rupture.

1.6 Murine Models of AAA

While the critical hallmarks of aneurysmal disease have been well characterized, there is still a general paucity of information on the actual sequence of events that result in the initiation, progression and rupture of the aneurysm. Given the nature of the disease, its location, and typical asymptomatic presentation until advanced dilation or rupture, acquisition of tissue in the formative stages of disease is not a viable option, even after the implementation of early screening programs. Researchers are thus restricted to observational studies of samples obtained during surgical repair for severe AAA over 5 cm,[180] where results may not necessarily reflect the processes involved in the formative stages of disease.

Various *in vitro* models have been developed to study the pathogenesis of AAA and to test novel treatment and diagnostic techniques. Thompson *et al.*[390, 391] published a model of aortic organ culture that was subjected to degradation by pancreatic elastase to determine the effect of leukocyte infiltration and shear stress on MMP production within the wall. Other *in vitro* methods have been described for the measurement of endoleakage[392, 393] and direct intra-aneurysm pressure.[394] These models have their uses, but in general, can only be maintained in culture for a limited time, and any *in vitro* system, no matter how controlled, will never fully be able to recapitulate the true state of the *in vivo* environment and will still require further validation prior to clinical use.

Animal models of disease ideally mimic the cellular and biochemical characteristics of the human form of the disease; however, in the case of AAA, there are numerous challenges to gauging the fidelity with which animal models replicate the human condition.[180] Even so, given the limitations of using human specimens and *in vitro* systems, various animal models of AAA have grown increasingly important in providing an experimental framework to test hypotheses relating to aneurysm initiation, progression and potential therapeutic strategies. While models in larger animals have been described, rodents and in particular, mice, have been the predominant model organism used to develop experimental models of AAA for various reasons. They are the most well-characterized laboratory research animal, with relatively short life cycle, documented genetic backgrounds and are easily manipulated for the regulation of specific genes.

Murine models of AAA can be classified into two general categories: Genetically engineered models, and chemically-induced models.

1.6.1 Genetically Engineered Mouse Models of AAA

The blotchy mouse has an X chromosome mutation that leads to abnormal copper absorption in the intestinal tract.[395] Copper is a required cofactor for lysyl oxidase (LOX) and thus, these mice exhibit

defective collagen and elastin crosslinking and which result in abnormalities in connective tissue architecture. [396]This predisposes them to develop aneurysms throught the aorta, but primarily in the thoracic region.[395] Based on these findings, it was suggested that altered copper metabolism may be a contributing factor to aneurysm pathogenesis in humans but no evidence of reduced copper levels has been observed in human AAA.[397, 398] While this model recapitulates some traits of the human disease, such as male gender bias for rupture,[399] other abnormalities such as emphysema and neurological dysfunction ultimately limit the usefulness of this model.[400] Similarly, mice with a genetically engineered deficiency in Lox are not viable models of AAA as they typically die in the perinatal period from rupture of thoracic aortic aneurysms (TAA).[401]

Various MMPs have been implicated in the pathogenesis of AAA, and mice deficient in MMP-3[402] and TIMP-1[145, 146] have been shown to develop medial dissections and small aneurysms in both the thoracic and the abdominal aorta. The lack of specificity may indicate a generalized destruction of aortic ECM and may obscure the true mechanism behind AAA development.[180]

ApoE and LDL receptor knockout (LDLR-KO) mice fed on high fat diets have both been shown to develop suprarenal AAA.[403] AAA development was reduced in apoE-KO mice that were also deficient for urokinase and this was determined to be due to an inability to activate MMP-12 via a plasmin-dependent pathway.[130] Elastin degradation and vessel dilation in LDLR-KO mice were reduced by treatment with a broad-spectrum MMP inhibitor, CGS 27023A, without affecting concurrent atherosclerotic lesions.[404]

The Tsukuba hypertensive mouse is produced by crossing transgenic mice that express human renin and angiotensinogen.[405] These mice developed chronic hypertension and died of ruptured AAA after 10 days of being fed drinking water containing 1% sodium chloride. Rupture was not associated

with hypertension, but warrants further study of the effects of high sodium diets on the renin-angiotensin system and association with AAA in humans.[180]

1.6.2 Chemically-Induced Mouse Models of AAA

Chemically-induced models of AAA have proven to be useful in elucidating the molecular mechanisms involved in aneurysm formation and for testing of potential pharmacotherapeutics. The three models described here are the most commonly employed animal models of AAA.

The elastase-induced model was first described by Anidjar *et al*. in rats[406] and is still a frequently used model of AAA today. It was developed based on the observation of disrupted elastin in AAA and knowledge of the critical role that elastin plays in providing vessel distensibility and maintaining structural integrity.[180] The model was adapted for use in mice,[119] whereby the abdominal aorta is isolated by a distal suture and a catheter is surgically inserted into the aorta at the iliac bifurcation. Porcine pancreatic elastase is infused into the aorta and incubated for 5 minutes before normal flow is restored. Elastase infusion via this method induces immediate dilation, presumed to be caused by the mechanical effects of the procedure, but by day 14, significantly greater dilation is observed in elastase-infused mice compared to heat-inactivated controls, accompanied by extensive destruction of elastic lamellae and adventitial nflammation mediated predominantly by macrophages. Aneurysm in this model is defined as a 100% increase in diameter to account for the initial mechanical dilation.[180] It should be noted that the source and method of preparation of the elastase are critical to the outcome in this method, and it has been suggested that the resulting inflammatory response is attributable to a contaminant in certain preparations of elastase, and not to the elastase itself.[181, 407] Decreased expression of both endothelial and neural nitric oxide synthase, but significantly increased iNOS has been observed in elastase-induced AAA. Interestingly, although iNOS has been implicated in inflammatory vascular diseases, male iNOS-deficient mice did not receive protection from development

of elastase-induced AAA, while female mice experienced greater incidence and developed larger AAA in the absence of iNOS. This effect was reversed by oophrectomy, suggesting an interaction between iNOS and gonadal hormones.[408]

The peri-aortic application of $CaCl_2$ to induce aneurysm formation was first described in rabbits[409] and has been adapted for induction of AAA in mice.[410] Using a cotton wool tip, 0.25M $CaCl_2$ is applied directly to the abdominal aorta for 15 minutes and aortic diameter was found to increase by 64% after 2 weeks, and 110% after 3 weeks.[410] Similar to human AAA, structural disruption of the medial elastic lamellae and activation of the inflammatory response in the media and on the luminal surface was observed, indicating that this might be a clinically relevant model of disease. The $CaCl_2$ model was also used to define the role of MMPs in AAA development. While MMP-2-KO and MMP-9-KO mice do not develop aneurysms,[120] aortic dilation is ablated in MMP-12-KO mice compared to WT controls after $CaCl_2$ application.

The systemic infusion of Ang II has been shown to induce a localized inflammatory response resulting in suprarenal aneurysm formation in hyperlipidemic LDLR-KO or apoE-KO mice.[411, 412] Ang II is typically administered at 500-1000ng/kg per minute via subcutaneously implanted osmotic pumps for 28 days.[180] This model also exhibits male gender bias, with males more susceptible than females. Androgen interaction may play a role as orchidectomy reduces incidence and severity in male mice to levels similar to that observed in females.[413]

Macrophage infiltration into the suprarenal aorta and the subsequent disruption of elastin fibres have been suggested as the precipitating event and may occur within a few days of pump implantation.[414] After 3-10 days, medial dissection occurs with the formation of a prominent vascular hematoma.[414] This thrombus is distinct from the laminated thrombus observed in human AAA as it results in only external diameter dilation, but may also exacerbate proteolytic activity in the dissected

aortic wall.[180] ECM deposition and leukocyte infiltration follow thrombus formation, and if rupture does not occur, the dilated aorta slowly regains circumferential elastin fibres and the lumen is re-endothelialized. Extensive neovascularization occurs throughout the remodelled tissue. If Ang II infusion is continued past 28 days, large atherosclerotic lesions characterized by lipid-laden foam cells, develop throughout the intima of the abdominal aorta.[414]

Co-infusion of angiotensin II receptor, type 1 (AT1) antagonist, Losartan, with Ang II is able to completely inhibit aneurysm formation in this model, whereas co-infusion with angiotensin II receptor, type 2 (AT2) receptor antagonist, PD123319 was shown to increase severity and incidence of AAA,[415] indicating that Ang II initiates aneurysm formation via signaling through the AT1 receptor. AT1 signaling in endothelial cells upregulates expression of E-selectin and VCAM-1, promoting leukocyte adhesion.[416, 417] In VSMC, Ang II induces the production of phosphatidic acid (PA) via phospholipase D (PLD)-mediated phosphatidylcholine hydrolysis. PA then activates nicotinamide adenine dinucleotide phosphate (NADPH) oxidase wich results in the production of reactive oxygen species (ROS).[418, 419] Elevated levels of ROS cause VSMC hypertrophy and hyperplasia, lipid peroxidation and the production of MMP-2 and -9.[418-420] This initiates a localized inflammatory response and recruits monocytes and lymphocytes to the vessel wall. AT1 receptor activation in macrophages leads to increased 12/15 lipoxygenase and ROS production, LDL oxidation, cholesterol synthesis and decreased cholesterol efflux, further promoting the inflammatory state. Therefore, aneurysm formation in this model is inflammatory, and has also been shown to be independent of blood pressure elevation, as treatment with hydralazine, which lowers systemic blood pressure, had no effect on AAA incidence.[421]

In conclusion, while *in vivo* animal models have been crucial to our current understanding of the pathogenesis of AAA and continue to be valuable tools for the development of new diagnostic tools, pharmacotherapy and repair techniques, they must be used with the understanding that each model

has its limitations and the purpose of the study is an important factor to consider when making an appropriate choice of model. These include but are not limited to interspecies variation in anatomy, genetics, physiology and pharmacodynamics.[422] However, the consistency of outcomes across various models in comparison to human AAA is strongly indicative that aneurysm formation involves a common mechanism of ECM destruction and loss of structural integrity.

CHAPTER 2: RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

2.1 RATIONALE

The exact etiology of AAA pathogenesis is yet unclear but it is currently believed that a key event in the development of aneurysms involves the proteolytic cleavage of ECM components such as collagen and elastin that results in a mechanical weakening of the vessel wall.[26] Analysis of human AAA tissue shows marked degradation of medial elastin fibres, thinning of the media, adventitial hypertrophy and an accumulation of T and B lymphocytes.[26] Atherosclerosis and the presence of thrombi are also frequently noted. Various proteases such as matrix metalloproteinases (MMP-2, 7, 9, 12),[106] cathepsins[202] and elastases[183] have all been put forward as possible contributors towards the degradation of vessel wall ECM.

It has also been suggested that medial SMC apoptosis plays a significant role in the pathogenesis of AAA. Substantial p53 mRNA expression has been observed in AAA tissue as well as a considerable decrease in SMC density.[423] Loss of SMCs would result in instability of the medial architecture and affect matrix remodelling processes, resulting in medial degradation that is a key characteristic of aneurysmal disease.

GZMB is a 32 kDa serine protease that plays an important role in immune cell-mediated apoptosis. In its traditional role as a mediator of cellular apoptosis, GZMB is released from the granules of an effector cell together with perforin, a pore-forming enzyme that is required for the entry of GZMB into the cytoplasm of the target cell where it can trigger the apoptotic cascade, resulting in cell death.[252] In addition to this intracellular pathway, it has been shown that GZMB is also released into the extracellular space during chronic inflammatory states and viral infections.[368] Outside of the cell, GZMB has been found to exhibit extracellular proteolytic abilities and is capable of cleaving numerous ECM

proteins such as fibronectin, vitronectin, laminin, aggrecan[326, 327] and the elastin scaffolding protein fibrillin-1[344] which would result in a deterioration of vessel wall integrity.

As further support for a pathogenic extracellular role, it has been observed that GZMB levels are elevated in advanced human atherosclerotic and allograft vasculopathy lesions compared to healthy arteries.[283] Additionally, high plasma GZMB levels correspond to carotid plaque instability and increased cerebrovascular events in humans,[387] while increased GZMB production in peripheral blood mononuclear cells was observed in patients with unstable versus stable angina.[388]

Given that GZMB possesses both intracellular and extracellular capabilities, it is possible that GZMB contributes to the development of AAA and dissection in our AngII-induced murine model via one of, or both, the perforin-mediated apoptotic pathway and its extracellular degradative ability. An experimental overview and chapter outline is described in Figure 2.

2.2 Hypothesis

I hypothesize that GZMB contributes to the pathogenesis of AAA through the proteolyses of extracellular matrix proteins leading to vessel wall instability and rupture.

2.3 Specific Aims

Aim 1: To assess the presence and role of GZMB in murine and human abdominal aortic aneurysm.

Aim 2: To determine if GZMB contributes to aneurysm formation through an intracellular, perforin-dependent and/or extracellular perforin-independent mechanism.

Aim 3: To determine if the administration of extracellular GZMB inhibitors can reduce the incidence and severity of disease in a mouse model of aortic aneurysm.

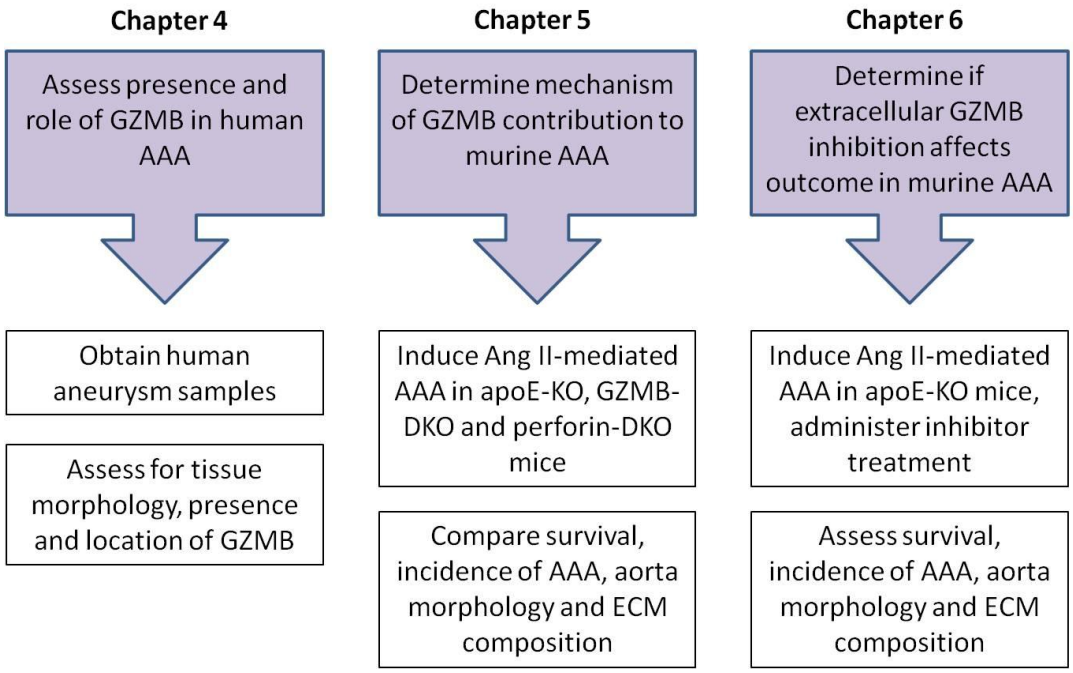


FIGURE 2: EXPERIMENTAL OVERVIEW AND CHAPTER OUTLINE

CHAPTER 3: METHODOLOGY

3.1 *IN VITRO* ASSAYS

3.1.1 FIBRILLIN-1 EXPRESSION ASSAY

Human coronary artery smooth muscle cells (HCASMC) (Cambrex, Walkersville, MD) were grown in Smooth muscle cell basal medium (SMBM) containing supplements and 5% FBS, according to the supplier's instructions (LONZA, Walkersville, MD), and were used after reaching 80% confluence. Prior to treatment, cells were starved for 24 hours in serum-starved media (supplemented SMBM (LONZA) + 0.2% FBS). Following starvation, cells were treated with 10 nM or 25 nM of GZMB (Alexis Biochemicals, Farmingdale, NY) for 24 hours. Controls were treated with PBS. At 24 hours, cells were lysed with TRIzol (Invitrogen, Carlsbad, CA), RNA was extracted according to manufacturer's instructions, and total RNA quantified by absorbance at 260 nm. To eliminate genomic DNA contamination, total RNA (1.5 μg) was DNAse I-treated (Invitrogen) according to manufacturer's instructions. cDNA was prepared from 1 μg of total RNA using 50 μM of oligo $(dT)_{20}$ primer and Superscript III reverse transcriptase (Invitrogen), according to manufacturer's instructions. All PCR reactions were carried out in 25 μl volumes using Platinum Taq DNA polymerase (Invitrogen) and previously published primers for human fibrillin-1 (Forward, 5'-GTGAGATCAACATCAATGGAGC-3'; Reverse, 5'-TTACACACTCCTGGGAACACTTC-3') and the universal GAPDH primers (Forward, 5'-CATGTTCGTCATGGGTGTGA-3'; Reverse, 5'-GACTGTGGTCATGAGTCCTT-3').[424] Equal aliquots of the PCR products were electrophoresed on a 2% agarose gel, stained with SafeView and photographed.

3.1.2 MURINE GZMB ENZYMATIC ACTIVITY ASSAY

Increasing concentrations of SA3N (0.625 nM – 80 nM) or anti-GZMB neutralizing antibody (0.113 - 14.15μg/ml, R&D Systems, Minneapolis, MN) were pre-incubated with active recombinant mGZMB (20nM, Sigma Aldrich, St Louis, MO) for 25 min in reaction buffer (50 mM HEPES, 10% sucrose, 0.1%

CHAPS, 5 mM DTT). Cleavage of the colorimetric GZMB substrate Ac-IEPD-pNA (1 mM, Calbiochem, EMD Chemicals, Gibbstown, NJ) in duplicate reactions was monitored by measuring absorbance at 405 nm using a plate reader (Magellan Tecan SaFire 2, Tecan Group Ltd., Männedorf, Switzerland) in kinetic mode. The initial rate of each reaction was calculated, and percent mGZMB activity for each concentration was determined relative to mGZMB activity in the absence of inhibitor. Inhibition was determined in 3 separate experiments.

3.1.3 Decorin Cleavage Assay

Western blot: Purified human GZMB (100 nM; Axxora, San Diego CA) was pre-incubated with or without recombinant SA3N (240 nM) in 50 mM Tris buffer, pH 7.4, for 25 min at room temperature (RT) prior to incubation with recombinant human decorin (0.16µg; Abnova, Walnut, CA) for 24 hours at RT. After incubation, proteins were denatured, separated on a 10% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and blocked with 10% skim milk. The membrane was probed using a mouse anti-human decorin antibody (1:200, R&D Systems) and IRDye® 800 conjugated affinity purified anti-mouse IgG (1:3000, Rockland Inc., Gilbertsville, PA). Bands were imaged using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).

Ponceau stain: Purified human GZMB (80 nM; Axxora) was pre-incubated with or without recombinant SA3N (240 nM) in 50 mM Tris buffer, pH 7.4, for 25 min at RT prior to incubation with recombinant human decorin (0.3 µg; Abnova) for 24 hours at RT. After incubation, proteins were denatured, separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Ponceau stain was used to identify cleavage fragments.

3.1.4 STATISTICS

Statistical analysis was compiled in Graphpad Prism 5 (Graphpad Software, La Jolla, CA). SA3N IC_{50} concentrations were determined using nonlinear regression analysis. For all tests, significant difference was set at $p < 0.05$.

3.2 *IN VIVO* ASSAYS

3.2.1 HUMAN AAA AND TAA

Formalin-fixed, paraffin-embedded human AAA samples were obtained in accordance with the ethical protocols at the Karolinska Institute, Sweden. Formalin-fixed, paraffin-embedded human TAA samples were obtained in accordance with the ethical protocols at the Biobank Cardiovascular Registry, St. Paul's Hospital, Vancouver, BC.

3.2.2 MICE

All procedures were done in accordance with the guidelines for animal experimentation approved by the Animal Experimentation Committee of the University of British Columbia. Male apoE-KO mice (C57Bl/6 background), GZMB-KO mice (C57Bl/6 background) and perforin-KO mice (C57Bl/6 background) were obtained from Jackson Laboratories, Bar Harbor, ME (Stock Numbers 002052, 002248, 002407). The GZMB/apoE-DKO (GDKO) mice and perforin/apoE-DKO (PDKO) mice were generated by crossing the apoE-KO and GZMB-KO or apoE-KO and perforin-KO mouse strains, respectively. Genotyping of the mice was performed using primers and polymerase chain reaction (PCR) protocols designed from Jackson Laboratories (GZMB primers: 5'-CTG CTA CTG CTG ACC TTG TCT-3', 5'-TGA GGA CAG CAA TTC CAT CTA-3' and 5'-TTC CTC GTG CTT TAC GGT ATC-3'; apoE primers:5'- GCC TAG CCG AGG GAG AGC CG -3', 5'- TGT GAC TTG GGA GCT CTG CAG C -3' and 5'- GCC GCC CCG ACT GCA TCT -3' and perforin primers: 5'- GCT ATC AGG ACA TAG CGT TGG -3', 5'- GGA GGC TCT GAG ACA GGC TA -3' and 5'- TAC CAC CAA ATG GGC CAA G -3') (Sigma Genosys, Oakville, ON). All mice were

housed at The Genetic Engineered Models (GEM) facility (UBC James Hogg Research Centre, St. Paul's Hospital, Vancouver, BC).

3.2.3 Murine Model of Angiotensin II-induced AAA

Mice aged 11-13 weeks received either 28 days of Ang II (Sigma Aldrich, St. Louis, MO) infusion at 1000 ng/min/kg or saline infusion from a subcutaneous 1004 model ALZET® mini osmotic pump (DURECT Corporation**,** Cupertino, CA) as previously described.[411] Briefly, an osmotic pump was filled with Ang II or saline solution, primed at 37ºC for 24 hours in saline, and surgically implanted subcutaneously posterior to the scapula of the mouse. During the implantation procedure, mice were anesthetized with gaseous anesthetic at a flow rate of 1.5 litres/min of oxygen with 2.5% isoflurane delivered via a Baines system using a calibrated tabletop anesthetic machine, administered from a rodent nose cone. Depth of anesthesia was monitored by toe pinch response and respiration. Eyes were protected using ocular lubricant. Post-surgical pain control consisted of a subcutaneous injection of buprenorphine. Animals were monitored daily for the duration of the experiment.

3.2.4 Serpin A3N Treatment

ApoE-KO mice were given a tail vein injection of sterile injectable saline (sham treatment) (n = 12) or recombinant SA3N diluted in sterile injectable saline at one of four doses: 120µg/kg per mouse (n = 11), 40 µg/kg per mouse (n = 18), 20 µg/kg per mouse (n = 10) or 4 µg/kg per mouse (n = 11) prior to Ang II pump implantation on day 0. An additional group of apoE-KO mice (n = 28) were given tail vein injections of recombinant SA3N diluted in sterile injectable saline at 40 µg/kg per mouse on day 0 prior to pump implantation and on day 4, 7, 14 and 21 post- pump implantation. SA3N was kindly provided by Dr. R. Chris Bleackley, University of Alberta.

3.2.5 Anti-GZMB Antibody Treatment

ApoE-KO mice were given a tail vein injection of control IgG or anti-GZMB neutralizing antibody diluted in sterile injectable saline at 1 mg/kg (n = 5 per group) on day 0 prior to pump implantation and on day 4, 7, 14 and 21 post-pump implantation.

3.2.6 Tissue Collection and Gross Pathological Characterization

At 28 days post-osmotic pump implantation, tissues from all surviving mice were collected. Blood was collected by cardiac puncture following CO_2 euthanasia in ethylenediaminetetraacetic acid (EDTA) (Sarstedt Monovette, Sarstedt, Nümbrecht, Germany) and RBC removed by centrifugation. The ventral cavity was opened, the right atrium was cut, and a catheter was inserted into the left ventricle. Sterile saline, and then 4% formalin (Fisher Scientific, Fairlawn, NJ), were perfused at a constant pressure of 100 mmHg using a pressurized tubing system until no blood was observed exiting the incision in the right atria. The heart, aorta to the iliac bifurcation, and kidneys were dissected from the mouse and photographed. At this point, a gross description of the aorta was made and grouped under the following categories: No visible pathology; Small localized saccular AAA below diaphragm with or without visible hematoma (28 day survival); Large dissecting AAA beginning in the suprarenal aorta and extending beyond the diaphragm, into the mid-thoracic aorta (28 day survival); Large ruptured AAA with death by exsanguination into abdominal cavity (<28 day survival). Observations were subsequently confirmed by a clinical and experimental pathologist blinded to treatment type. Necropsy was performed for all mice that died prior to 28 days to determine cause of death. Tissues were stored in fresh 10% formalin overnight before being embedded and serial sectioned into 5 or 10 µm sections.

3.2.7 HISTOLOGY

Formalin-fixed, paraffin-embedded murine abdominal aortic sections were sections were de-paraffinized and rehydrated in xylene and ethanol, then stained for hematoxylin and eosin (H&E), Movat's pentachrome, and picrosirius red as previously described. [283]

3.2.8 IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded murine abdominal aortic sections were sections were de-paraffinized and rehydrated in xylene and ethanol, then antigen retrieval was performed by boiling slides in citrate buffer (pH 6.0) to expose antigens masked in the fixation process as per recommended protocol from antibody supplier. Immunohistochemistry (IHC) was performed using goat anti-human fibrillin-1 Abs (N-19 and C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), rabbit anti-mouse GZMB (Abcam, Cambridge, MA), goat anti-mouse decorin (R&D Systems, Minneapolis, MN) as previously described.[425] Negative control staining was performed in the same manner for all tested antibodies, with absence of primary antibody. For the GrB/Mast cell double stains, slides were prepared by removing paraffin, hydrating, and treated with boiling citrate buffer. Blocking was done by adding 10% goat serum to all sections and incubating for 30 min. The primary antibody used was a rabbit anti-mouse GrB antibody (1:100 in 10% goat serum). After overnight incubation, a goat anti-rabbit secondary antibody was applied and incubated for 1 hour. An ABC-alkaline phosphatase (AP) reagent was then prepared and applied. After 30 minutes incubation, Vector Red substrate was prepared and applied to the sections to produce the red GrB stain. The sections were counterstained with alcian blue (in 0.7N HCL) for 20 minutes.

3.2.9 IMMUNOPRECIPITATION OF SERUM FIBRILLIN-1 FRAGMENTS

Fibrillin-1 fragments in mouse blood serum were isolated by immunoprecipitation then analyzed by western blotting. Briefly, proteins were denatured, separated on a 10% SDS-polyacrylamide gel,

transferred to a nitrocellulose membrane and blocked with 10% skim milk. The membrane was then probed using goat anti-human fibrillin-1 antibodies (N-19 and C-19, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and bands were imaged using the Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE).

3.2.10 Confocal Microscopy

Formalin-fixed, paraffin-embedded human TAA sections were de-paraffinized and rehydrated in xylene and ethanol. Antigen retrieval was performed by boiling slides in citrate buffer (pH 6.0) to expose antigens masked in the fixation process as per recommended protocol from antibody supplier. To demonstrate colocalization of GZMB in macrophages, background staining was blocked by incubation of sections in 10% goat serum. Sections were incubated in a 1:100 dilution of rabbit anti-GZMB (Abcam) overnight, followed by incubation in biotinylated goat anti-rabbit secondary antibody (Vector laboratories) and ABC reagent (Vector laboratories). GZMB staining was visualized with TSA$^{(TM)}$ Plus Fluorescence Systems cyanine 3 tyramide (PerkinElmer Life Sciences, Inc., Boston, MA). To assess for macrophages, slides were incubated with avidin and biotin to prevent interaction of labelling reagents and blocked with 10% horse serum. Sections were incubated in a 1:100 mouse anti-MAC387 (AbD Serotec, UK) overnight followed by incubation in biotinylated horse anti-mouse secondary antibody (Vector laboratories) and ABC reagent (Vector laboratories). MAC387 staining was visualized with TSA$^{(TM)}$ Plus Fluorescence Systems fluorescein tyramide (PerkinElmer Life Sciences, Inc.). Similarly, for co-localization of GZMB in lymphocytes, slides were first stained for GZMB with mouse anti-GZMB (Dr. Joseph Trapani) then incubated with avidin and biotin to prevent interaction of labelling reagents and blocked with 10% goat serum. Sections were incubated with 1:100 mouse anti-CD3 (Abcam) overnight followed by incubation in biotinylated goat anti-rabbit secondary antibody (Vector laboratories) and ABC reagent (Vector laboratories). CD3 staining was visualized with TSA$^{(TM)}$ Plus Fluorescence Systems fluorescein tyramide (PerkinElmer Life Sciences, Inc.). Confocal images of fluorescently labelled tissue

sections were acquired with a Leica AOBS SP2 laser scanning inverted confocal microscope (Leica, Heidelberg, Germany). Excitation beams were produced by Ar (488 nm for fluorescein) and HeNe (543 nm for cyanine 3) lasers (Leica AOBS SP2 module) respectively. Images from these dual stained samples were acquired sequentially to eliminate cross-talk between the emission signals and acquired images were overlaid using Volocity software (Improvisions, UK).

3.2.11 Second Harmonic Generation Microscopy

Collagen structures in formalin-fixed tissue specimens were visualized using second harmonic generation (SHG) microscopy as previously described.[426, 427] Ultra-short laser pulse was focused on the specimen through a Leica 63X/1.2 NA Plan-Apochromat water immersion objective. SHG signal in the forward direction originating from the histological specimens was captured using a non-descanned detector in the transmission geometry. In this non-descanned PMT detector (R6357, Hamamatsu, Shizuoka, Japan), a 440/20 nm band pass filter (MP 440/20, Chroma Technology, USA) was used to collect spectrally clean SHG signal. The gain and offset of the PMTs were adjusted for optimized detection using the color gradient to avoid pixel intensity saturation and background and these settings kept constant for all measurements. The 3D image restoration from the collected z-section images was performed using Volocity software (Improvisions, UK). For collagen signal density calculations, a noise removal filter with kernel size of 3X3 was used to define the boundary between foreground and background, and the lower threshold in the histogram was set to mean voxel intensity value. The total SHG signal intensity values thus generated were normalized by the total collagen volume (μm^3) and expressed in arbitrary units (AU).

3.2.12 Transmission Election Microscopy

All transmission electron microscopy (TEM) procedures were performed at the James Hogg Research Centre/Institute for Heart + Lung Health (JHRC/HLI). For sample processing, each mouse was perfused

with Krebs' buffer through the left ventricle until solution ran clear. Tissues were harvested and fixed overnight in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. After rinsing 3 times in 0.1M sodium cacodylate buffer, samples were post-fixed in a 1:1 mixture of 2% osmium tetroxide and 0.2M sodium cacodylate buffer then rinsed in distilled water and dehydrated in a graded series of acetone washes, then incubated with 1:1 solution of Epon and acetone for 2 hours. Samples were incubated overnight in a 2:1 solution of Epon and acetone then changed to 100% Epon for up to 8 hours, before embedded in pure Epon and polymerized at 60-65^{o}C for 24 hours. Sections with a thickness of 60 nm were cut using a Leica EM UC6 microtome (Leica) and collected on nickel grids. Samples were analyzed on a Tecnai 12 Transmission Electron Microscope (FEI, Hillsboro, OR) TEM at the JHRC/HLI. Aortas were divided into healthy and dilated segments. 20 images of collagen fibrils in cross section from each segment were taken and analyzed for morphology and diameter.

3.2.13 Immuno-Electron Microscopy

All immunogold electron microscopy procedures were performed at the James Hogg Research Centre/Institute for Heart + Lung Health (JHRC/HLI). For sample processing, each mouse was perfused with Krebs' buffer through the left ventricle until solution ran clear. Aortic rings of approximately 4 to 6 mm (length) x 2 to 3 mm (diameter) were excised and immediately fixed in 4% paraformaldehyde (Cat. 15710, Ted Pella Inc., Redding, CA) + 0.05% glutaraldehyde (Cat. 18427, Ted Pella Inc.) in PBS at pH 7 for 2 hours at 4°C. Then rings were further cross sectioned into several pieces, each having a length of 2 mm. Care was taken to keep tissue including thrombus intact. Samples were further fixed overnight at 4°C. The next day, samples were processed using a Pelco Biowave Microwave. Briefly, samples were dehydrated in a graded series of ethanol (50%, 70%, 90% and twice at 100%) for 40 seconds with temperature restriction to 37°C. Infiltration was carried out with 50% LR White resin (Cat. 14381, Electron Microscopy Sciences) in ethanol, then 100% LR White resin, both steps for 15 min with

temperature restriction of 45°C. Finally these steps were carried out without the microwave: Infiltration with a second change of 100% LR White resin at room temperature overnight and then polymerization at 50°C the next day. Sections with a thickness of 60 nm were cut using a Leica EM UC6 microtome (Leica) and collected on nickel grids.

For immunogold labeling of decorin, primary affinity-purified polyclonal goat anti-mouse decorin IgG antibody (Cat.AF1060, R&D Systems) was diluted at 1:20. Secondary antibody, F(ab') 2 Fragment of ultra-small rabbit-anti-goat IgG, H&L (Cat. 25220, Electron Microscopy Sciences, Hatfield, PA) was diluted at 1:50. The following procedure was carried out using the Pelco Biowave Microwave with temperature restriction to 37°C. First, free aldehydes in sections were blocked using 50 mM glycine (BP381-500, Fisher Scientific, Waltham, MA) in PBS at pH 7.4 for 5 min. Sections were then blocked in 5% rabbit serum blocking solution containing 0.18% Cold Water Fish Skin Gelatin (Cat. 15717, Ted Pella, Inc.) for 3 x 5 min with 2 min rest, washed 3 x 5 min in acetylated-BSA containing (BSA-c, Cat. 25557, Electron Microscopy Sciences) PBS buffer (0.15% BSA-c, pH 7.4) and incubated in primary antibody made in the same BSA-c buffer for 6 x 5 min with 2 min rest. Control sections were incubated in normal rabbit serum diluted at 1:20 or PBS buffer alone. Subsequently, sections were washed in BSA-c buffer for 3 x 5 min, and incubated in secondary antibody for 5 x 5 min with 2 min rest. The following was carried out at room temperature without microwave: BSA-c buffer for 6 x 5 min, PBS for 3 x 5 min, 2% glutaraldehyde in PBS for 5 min, distilled water for 5 x 2 min, and silver enhancement for gold labeling with Silver R-Gent SE-EM (Cat**.** 5000.033, Aurion, Wageningen, The Netherlands) for 40 min. Finally, sections were then washed 5 x 2 min in distilled water, stained in 2% uranyl acetate and then lead citrate, washed, air dried and finally analyzed on a Tecnai 12 Transmission Electron Microscope (FEI) TEM at the JHRC/HLI.

3.2.14 Statistics and Data Analysis

Statistical analysis was compiled in Graphpad Prism 5 (Graphpad Software). Survival curves were assessed using Log rank test for trend and Log rank/Mantel-Cox analysis. AAA incidence was assessed by Chi Square test for trend across all groups. SHG collagen signal density was assessed by one-way analysis of variance (ANOVA) and unpaired Student's *t*-test. Collagen fibril diameter was measured with Image ProPlus (Media Cybernetics, Inc., Rockville, MD) and standard deviation, standard error, range, mean and median were assessed with Graphpad Prism 5 (Graphpad Software). For all tests, significant difference was set at $p < 0.05$.

CHAPTER 4: GZMB IN HUMAN AAA AND TAA

4.1 INTRODUCTION

Elevated GZMB levels have been reported in advanced human atherosclerotic and allograft vasculopathy lesions but not in healthy coronary arteries.[283] In the latter study, GZMB was present in lymphocytes, macrophage foam cells, medial and intimal SMC and it was found that extracellular staining increased with disease severity.[283] High GZMB levels in the plasma correspond to increased carotid artery plaque instability and increased cerebrovascular events in humans.[428] Furthermore, elevated GZMB production is observed in peripheral blood mononuclear cells isolated from patients with unstable angina pectoris compared to cells from patients with stable angina pectoris.[388] As aneurysmal disease shares many risk factors with these conditions, GZMB is therefore a logical target for investigation as an effector agent in the pathogenesis of AAA. In the current chapter, human AAA and TAA samples were assessed for the presence of GZMB in the vessel wall and localization within various immune cell types.

4.2 GZMB IS ELEVATED IN HUMAN AAA

AAA tissue and healthy, non-atherosclerotic aorta tissue were stained for the presence of GZMB. No GZMB immunopositivity was observed in control healthy aorta (Figure 3A) while GZMB was abundantly present in AAA tissue (Figure 3B-F). GZMB positivity was noted in phagocytic cells trapped in the intima and fibrin platelet-red cell thrombus, and also in thrombic material attached to the deep atheroma. GZMB was also noted in cells in proximity to the medial neovasculature, in adventitial collagen layers, in remote adventitia containing nerves, and particularly in large collections of lymphocytes in the adventitia. Moderate staining was observed extracellularly and in the occasional SMC. GZMB positivity was also found in thrombic material with red cells, suggestive of GZMB in the

plasma. Intense granular cytoplasmic GZMB was also identified in neuroganglion cells of the remote adventitia.

4.3 GZMB Colocalizes to Immune Cells in Human TAA

Human TAA tissue was assessed for the presence of GZMB in various immune cell types (Figure 4 and Figure 5). GZMB was found to co-localize to mast cells in the adventitia when visualized by immunohistochemistry. Macrophages in the intima, adventitia and intraluminal thrombus, and CD3+ lymphocytes in the intima, media, adventitia and intraluminal thrombus were found to contain GZMB when visualized by confocal microscopy.

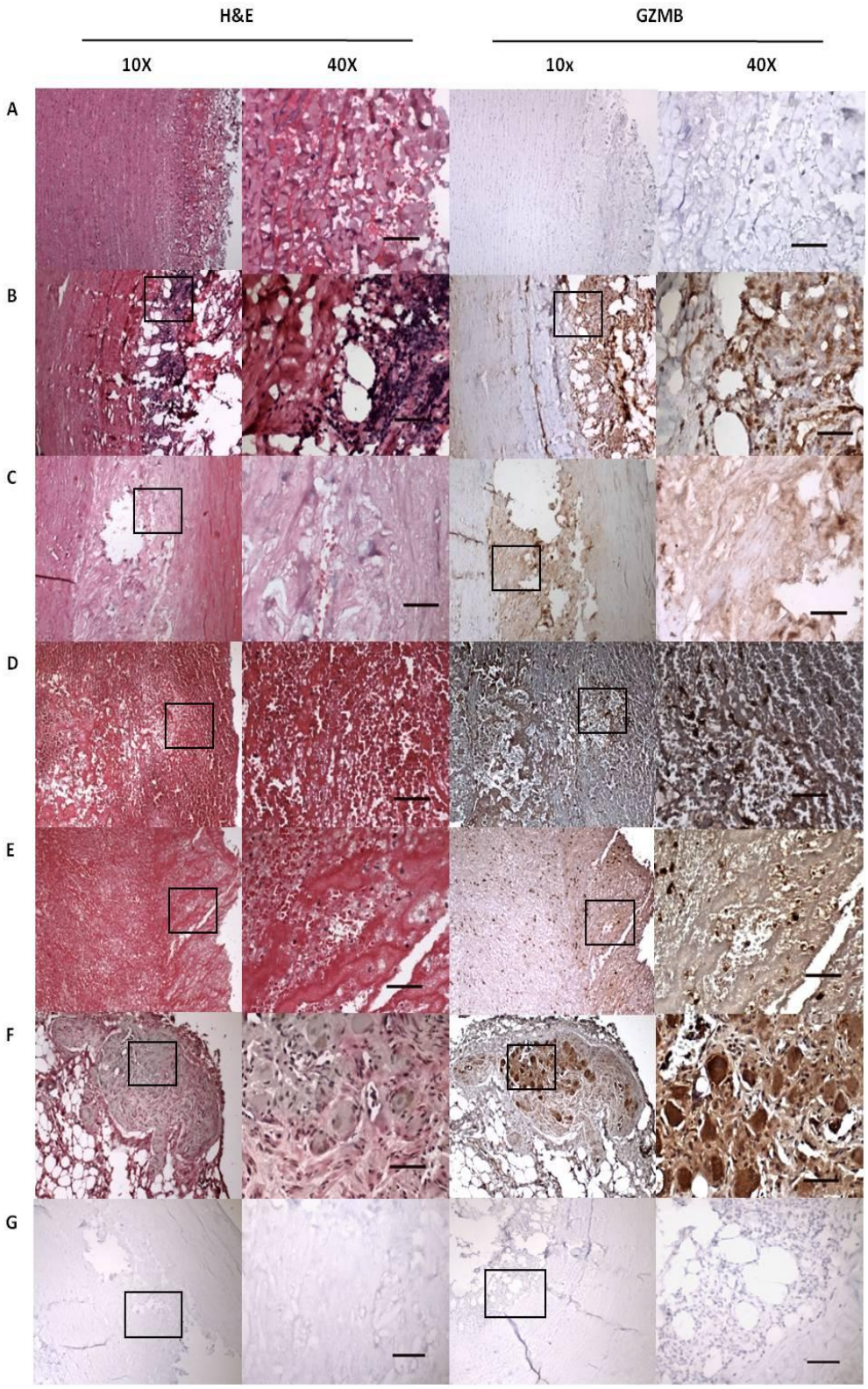

H&E
GZMB
10X
40X
10x
40X
A
B
C
D
E
F
G

FIGURE 3: GZMB IN NON-ATHEROSCLEROTIC HUMAN AORTA VERSUS HUMAN AAA.

No GZMB immunopositivity was observed in control healthy aorta (A) while GZMB was abundantly present in AAA tissue (B-F). GZMB positivity was observed in the media and adventitia but particularly in the adventitial lymphocytes and extracellular connective tissue layers of AAA tissue (B). Staining in the medial thrombus (C) was largely extracellular, however, within the intraluminal thrombus (D-E), GZMB positivity was noted in cellular elements trapped and scattered throughout the fibrin platelet red cell thrombus. GZMB was also noted in remote adventitia containing nerves, and in large collections of lymphocytes in the adventitia. Intense granular cytoplasmic GZMB was identified in nerve ganglion cells of the remote adventitia (F). Negative control, no GZMB primary antibody, shows lack of non-specific staining (G). Scale bar at 40X = 50 µm.

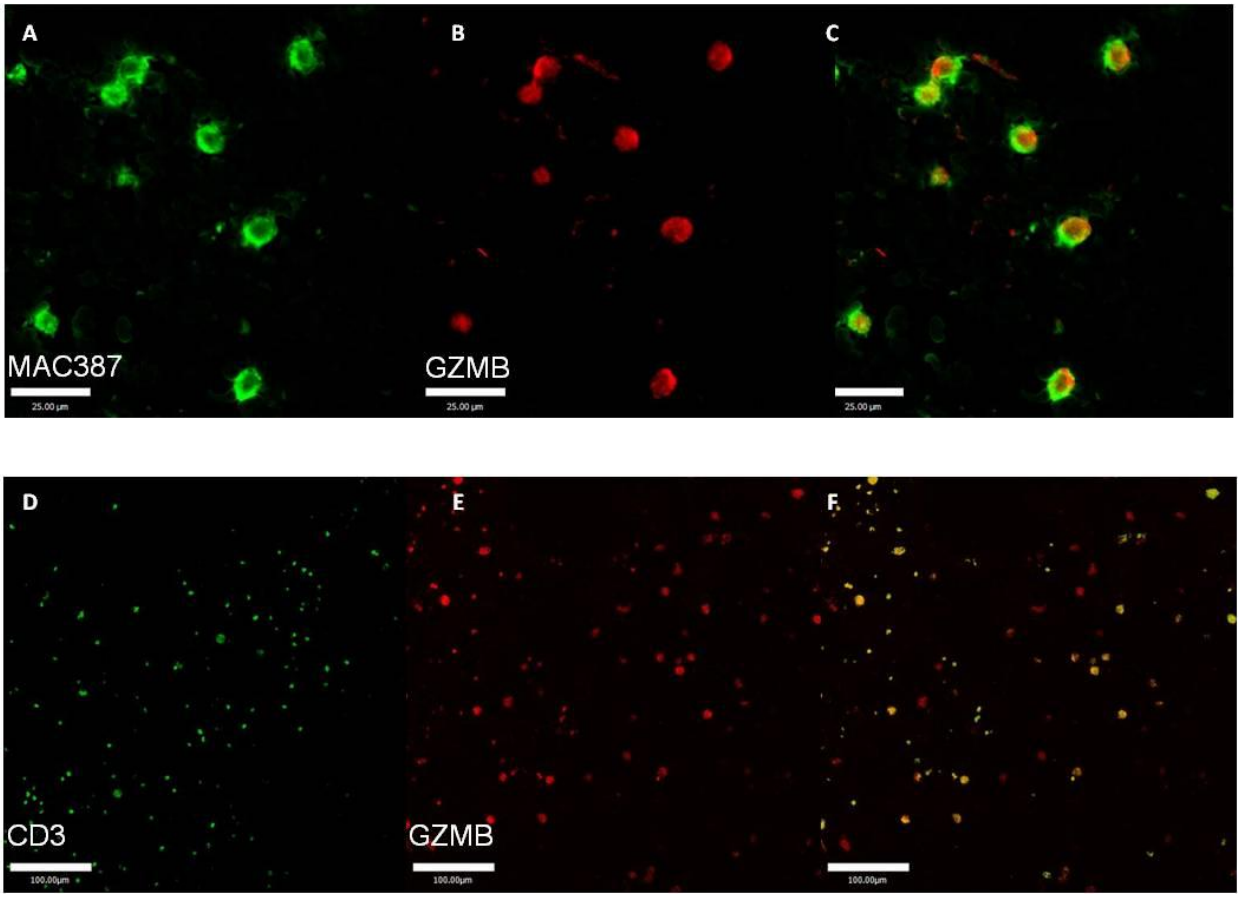


FIGURE 4: GZMB COLOCALIZATION WITH MACROPHAGES AND CD3$^+$ CELLS IN TAA

GZMB co-localization is observed in macrophages of the intraluminal thrombus when visualized by confocal microscopy. GZMB positivity is seen in red (B), macrophage marker MAC387 in green (A) and combined image in (C). Scale bar = 25 µm. GZMB also co-localizes to lymphocytes in the intraluminal thrombus. GZMB positivity is seen in red (E), CD3 in green (D) and combined image in (F). Scale bar = 100 µm.

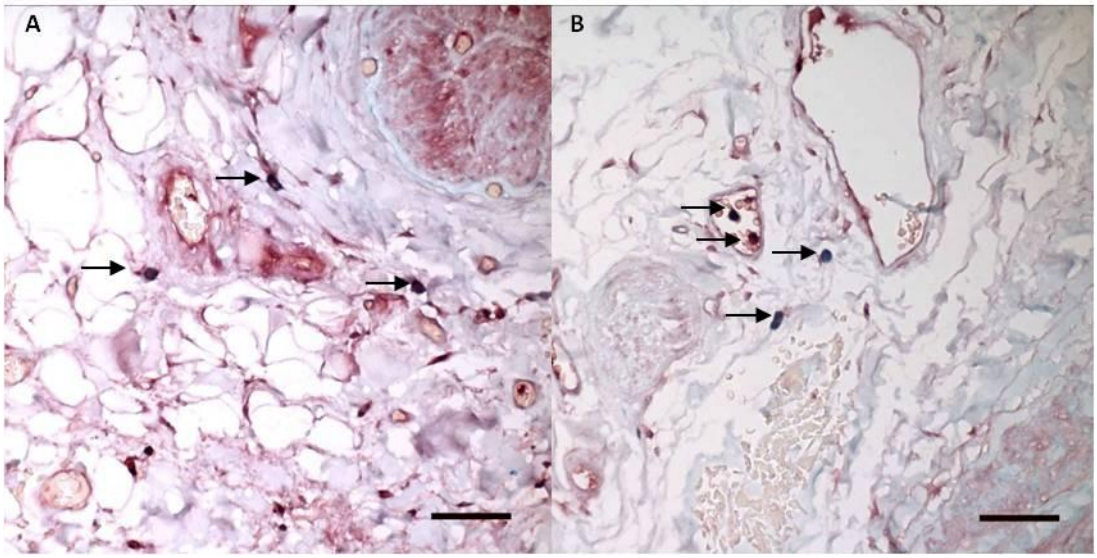


FIGURE 5: GZMB COLOCALIZATION WITH MAST CELLS IN TAA

GZMB expression was observed in mast cells (arrows in A, B) of the adventitia in sections of human TAA stained for GZMB (red) by IHC and counterstained with Alcian blue. Scale bar: 40X = 50 µm.

4.5 DISCUSSION

Although GZMB expression was initially thought to be restricted to CTL and NK cells, it is now clear that, under conditions of cellular stress, aging and disease, GZMB expression can be induced in, and secreted by other types of immune cells (macrophages, mast cells, neutrophils) and non-immune cells (keratinocytes, chondrocytes, VSMC).[283, 286, 429-432] As such, many cell types could potentially act as a source of GZMB in age-related degenerative diseases such as AAA. We observed substantial GZMB immunopositivity in human AAA whereas GZMB levels are minimal in corresponding healthy control aortas. Elevated GZMB was observed in lymphocytes trapped in the intraluminal thrombus of AAA. In addition, GZMB co-localized to macrophages (intima, adventitia and thrombus) and lymphocytes (intima, media, adventitia and thrombus) and mast cells (adventitia) in human TAA (Figure 4 and Figure 5), readily demonstrating a source of GZMB *in situ*. A role for GZMB in AAA rupture would be consistent with previous studies demonstrating that the aneurysm wall covered with thrombus exhibits increased inflammation, mast cell activation[221] and association with neovessels in the media and adventitia[220], macrophage infiltration,[414, 433-435] SMC apoptosis and ECM degradation, thus subjecting this region to greater risk of rupture.[436] Based on this immunohistochemical assessment of human aneurysmal disease, and given that GZMB is well characterized for its role in perforin-mediated apoptosis and is now known to degrade various extracellular targets in the ECM,[326, 327] we hypothesize that GZMB could contribute to AAA pathogenesis through medial and adventitial weakening in addition to the destabilizing effects initiated at the intraluminal thrombus and this warrants experimental testing in a suitable animal model for further elucidation.

CHAPTER 5: PERFORIN DEFICIENCY IS NOT PROTECTIVE AGAINST ANGIOTENSIN II-INDUCED MURINE AAA

5.1 INTRODUCTION

The proteolytic mechanisms that are associated with ECM degradation in AAA are areas of active investigation. Several types of proteases, including matrix metalloproteinases (MMP)-2, -7, -9, -12, cathepsins, plasminogen activators, and elastases, have been proposed to contribute to the degradation of fibrillar ECM proteins.[437] In the aorta, microfibrils associate with elastin in the tunica media to form the concentric lamellae that separate individual SMC layers and confer elasticity to the aortic wall. Microfibrils also act to stabilize the vessel wall by connecting lamellar rings to one another, to SMC, and to the sub-endothelial basement membrane.[343] Fibrillin-1 is the major scaffolding component of microfibrils and thus plays a key role in maintaining vessel wall stability. It has previously been shown to be a cleavage target for the serine protease GZMB[344] and in the current chapter, we shall examine possible roles for GZMB in aneurysmal pathogenesis via a well-established mouse model of Ang II-induced AAA.[438] In this model, macrophage accumulation in the media of the suprarenal aorta and dissection precede the formation of aneurysm and atherosclerosis in Ang II-infused apoE-KO mice. While thrombi are usually constrained by adventitial tissue, rupture of the abdominal aorta and subsequent death due to abdominal bleeding is often observed.[414] We hypothesize that GZMB is elevated in AAA and contributes to vessel wall instability and aneurysm formation. To test this, GDKO and PDKO mice were generated and infused with Ang II to induce AAA formation.

5.2 GZMB DEFICIENCY BUT NOT PERFORIN DEFICIENCY IMPROVES OUTCOMES IN AAA

5.2.1 SURVIVAL AND INCIDENCE OF AAA

Ang II-treated GDKO mice but not PDKO mice had a significant increase in 28-day survival (92.86%; n = 14, 56.25%, n = 16) when compared to apoE-KO mice (53.33%; n = 15). Survival curves

(Figure 6) were analysed by log-rank test and a statistically significant difference between survival curves was found (P = 0.002). Upon necropsy, all mice that died prematurely (<28 days) were found to have large aortic dissections and extensive blood clots in the abdominal cavity suggesting that the cause of death was rupture and exsanguination. When surviving mice from Ang II-treated groups were assessed, a further 40.0% of apoE-KO mice and 43.75% of PDKO mice were found to have AAA compared with only 28.57% of GDKO mice, reducing total incidence of aneurysm-related pathology from 86.67% in apoE-KO mice and 87.50% in PDKO mice to 35.71% in GDKO mice (Figure 7). Rupture was observed in only 1/14 GDKO mice and aneurysms that were observed in this group were all small and localized, suggesting that GZMB contributes to the onset and progression of aneurysm. No dissection, AAA or mortality was observed in any of the saline-infused groups. Representative images of aorta gross morphology and cross sections are shown in Figure 7.

5.2.2 Medial Integrity

As previously observed,[414] medial disruption characterised by the loss of elastin is a predominant pathological feature of AAA; both in human aneurysms and in Ang II-induced murine AAA. While incidence of AAA and dissection was much reduced in GZMB-deficient mice, severe elastin breakage was frequently observed in conjunction with aneurysm development in both apoE-KO and PDKO mice. Severe medial disruption, as seen in apoE-KO and PDKO mice, was not observed in GDKO mice (Figure 8).

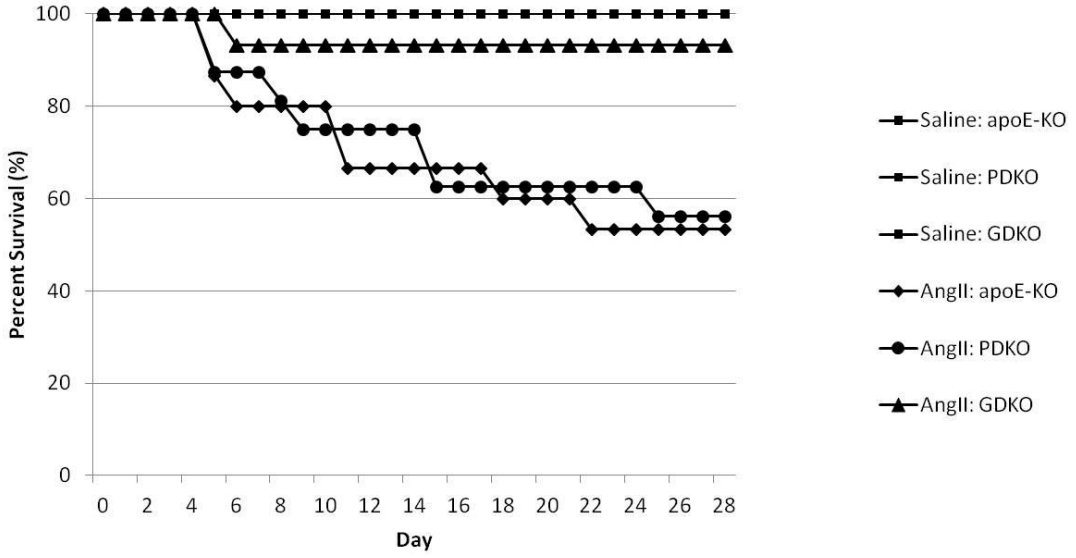


FIGURE 6: KAPLAN-MEIER SURVIVAL CURVE FOR APOE-KO, PDKO AND GDKO MICE ON ANG II

Mice were implanted with mini-osmotic pumps containing saline or Ang II as described to induce aneurysm formation. Lines represent percentage of mice alive on each day post-implantation. No death was observed in saline control groups (n = 8 for apoE-KO, n= 11 for GDKO, n = 5 for PDKO). In contrast, 92.86% of GDKO (n = 14), 53.33% of apoE-KO (n = 15) and 56.25% of PDKO (n = 16) infused with Ang II survived to 28 days. Survival curves were significantly different when analysed by log-rank test ($p = 0.002$).

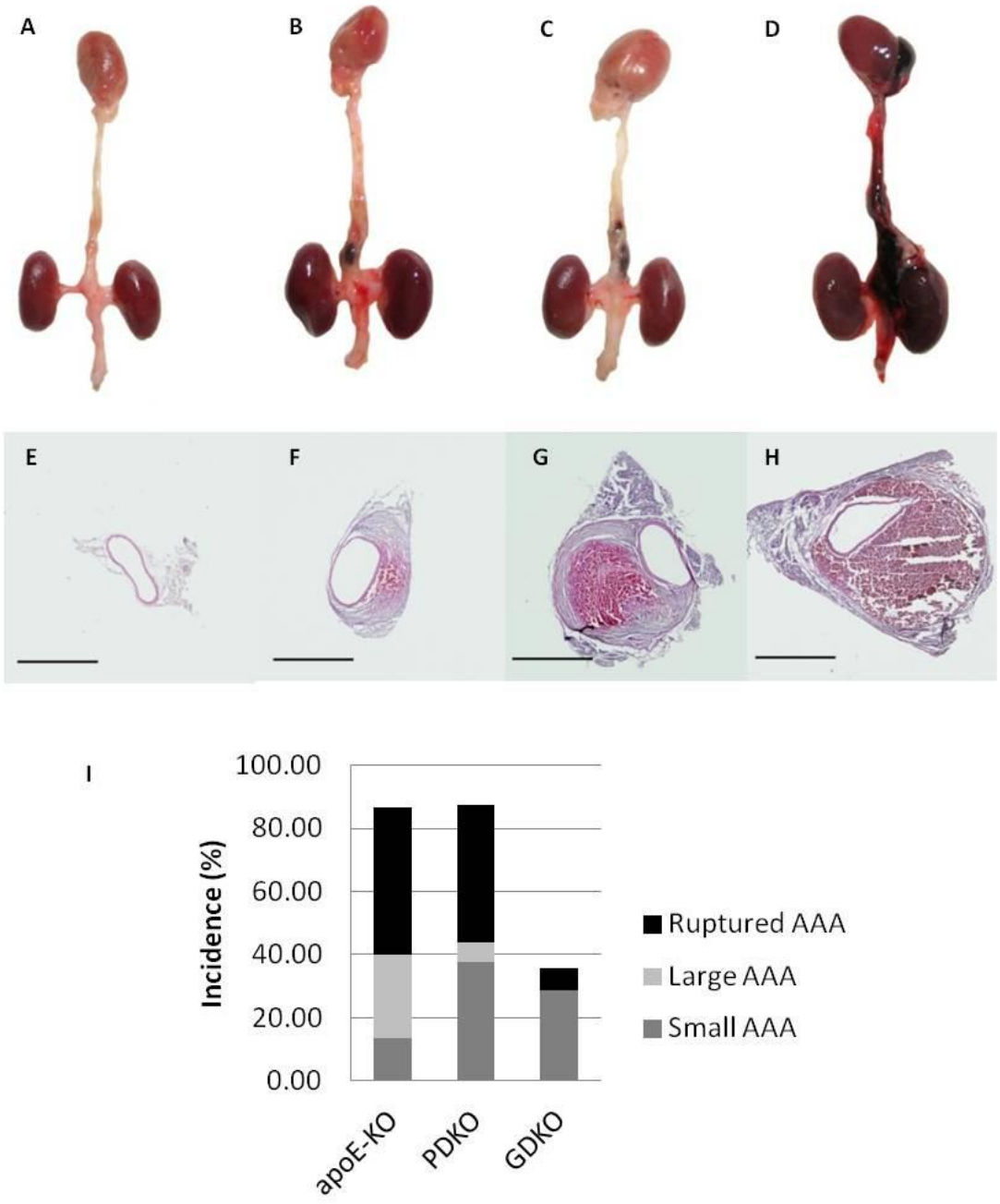


FIGURE 7: REPRESENTATIVE GROSS PATHOLOGY, MORPHOLOGY AND SUMMARY OF OUTCOMES

Upon euthanasia or necropsy, hearts and aortas were harvested and graded for severity of phenotype. Aortas were cross sectioned and stained with H&E to assess morphology. (A, E) Heart and aorta with no visible pathology (all saline groups, apoE 13.33%, GDKO 64.29%, PDKO 12.50%). (B, F) Small localized AAA with or without visible hematoma (apoE 13.3%, GDKO 28.57%, PDKO 37.50%). (C, G) Large dissecting AAA with visible hematoma in false lumen surrounding the abdominal aorta and extending above the diaphragm (apoE 26.67%, GDKO 0%, PDKO 6.25%). (D, H) Fatal ruptured dissecting AAA with abdominal bleeding (apoE 46.7%, GDKO 7.14%, PDKO 43.75%) was also observed. Outcomes are summarised in (I). Scale bar: 4X = 1mm.

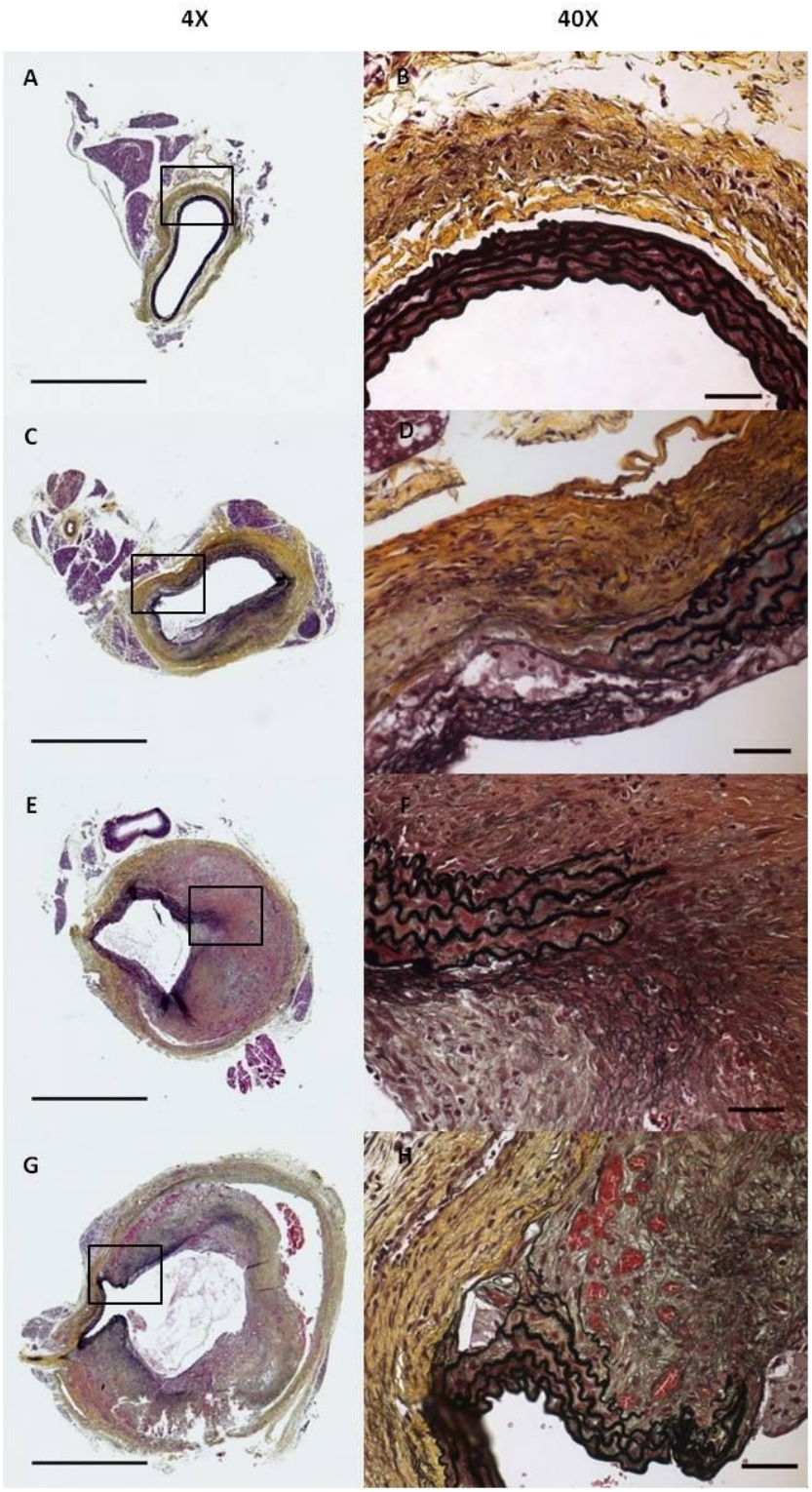


FIGURE 8: GZMB DEFICIENCY REDUCES MEDIAL DISRUPTION

Formalin-fixed tissues collected from mice that survived to 28 days were assessed using Movat's Pentachrome as described. (A, B) Representative Aorta from GDKO mouse with no medial disruption, minimal adventitial thickening. (C, D) Aorta from apoE-KO with elastin breakage, pronounced adventitial thickening, but no visible hematoma. (E, F) Aorta from PDKO mouse with small AAA, elastin breakage, remodelled medial hematoma. (G, H) Aorta from PDKO mouse with dissection, elastin breakage and pronounced dilation. Scale bars: 4X = 1mm, 40X = 50 µm

5.3 Perforin Deficiency Does Not Reduce GZMB Levels in Ang II-induced AAA

When assessed by IHC staining, GZMB was elevated in the aortas of apoE-KO and PDKO mice that developed AAA following Ang II infusion (Figure 10). No GZMB immunopositivity was observed in healthy, age-matched controls that received saline infusion. GZMB was particularly abundant in the thrombus, both in trapped lymphocytes and in the extracellular space, as well as in the adventitia of large, dissecting AAA. Furthermore, GZMB was shown to colocalize to mast cells in the adventitia of apoE-KO mice that developed AAA (Figure 9).

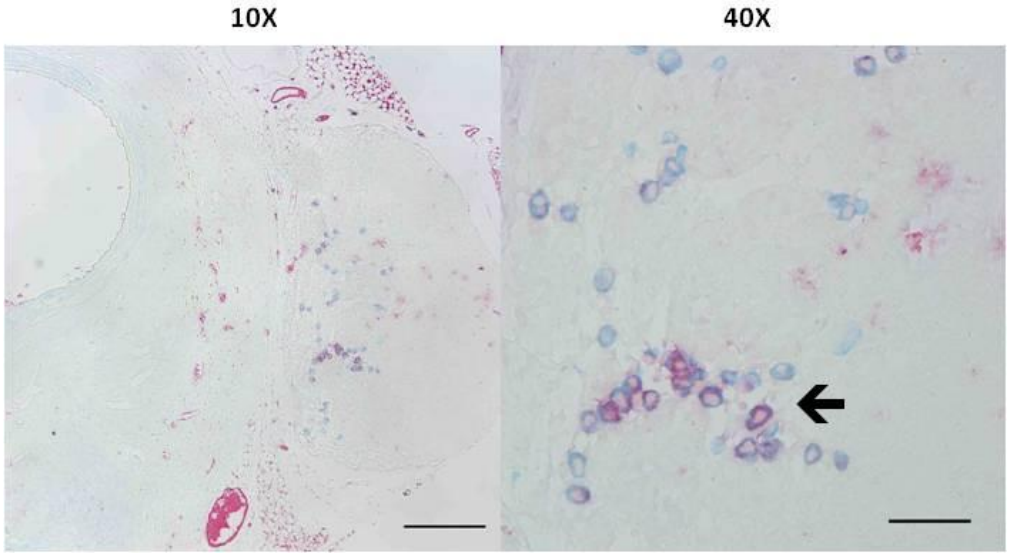


FIGURE 9: GZMB COLOCALIZES TO MAST CELLS

Aortas from apoE-KO mice that developed AAA were double-stained for GZMB and mast cells, GZMB was visualized by Vector Red and mast cells were counterstained with Alcian Blue. Mast cells are indicated by arrows. Scale bar 10X = 200μm, 40X = 50μm.

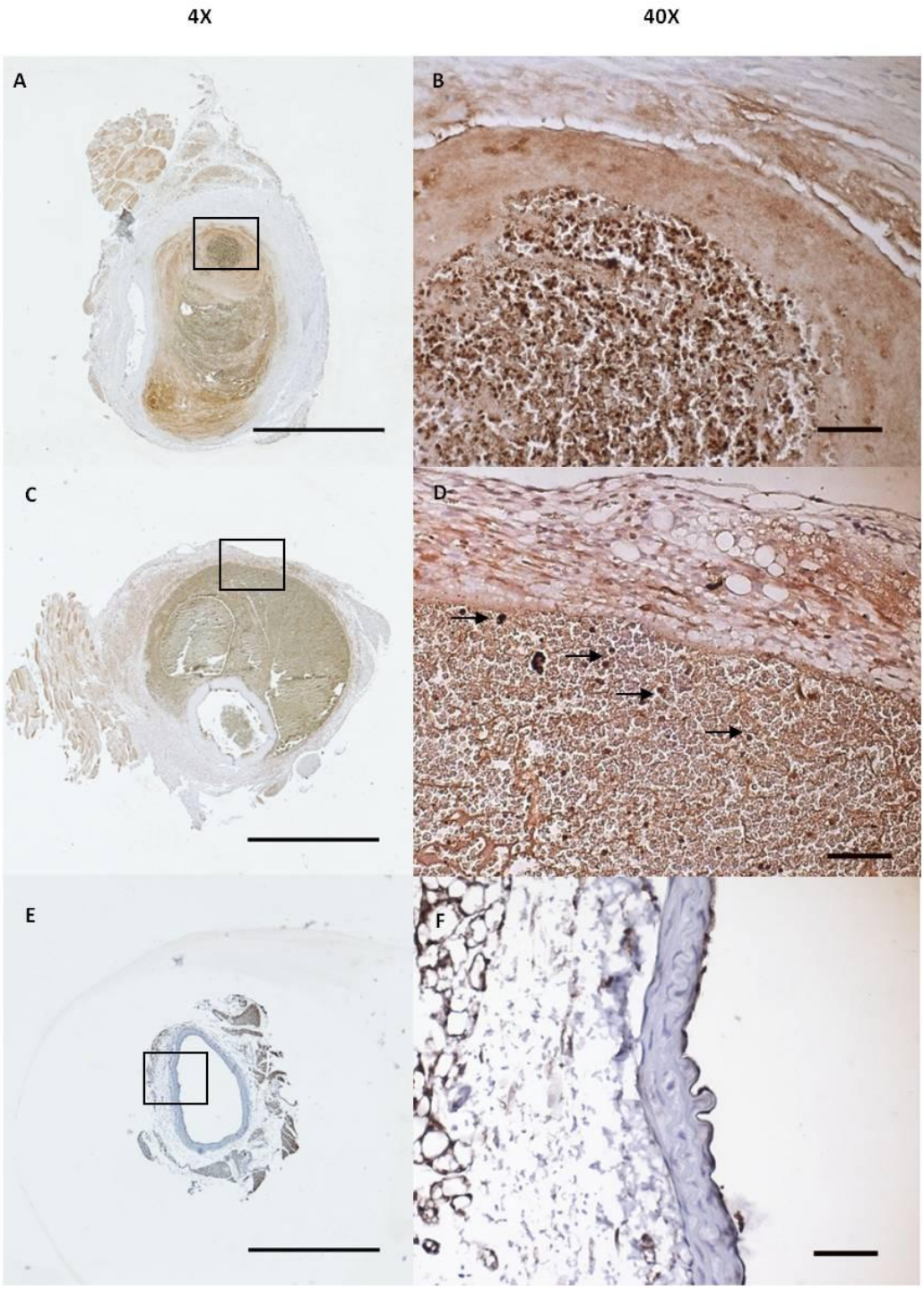


FIGURE 10: GZMB IMMUNOSTAINING IN APOE-KO AND PDKO

GZMB-immunopositivity is observed in the medial thrombus of an apoE-KO mouse that suffered a small, saccular AAA (A, B) and the adventitia of a PDKO mouse that suffered a large dissecting AAA (C, D). Trapped lymphocytes in the thrombus are indicated by arrows. GZMB staining is not observed in apoE-KO mice that received saline infusion (E, F). Scale bars: 4X = 1mm, 40X = 50 µm.

5.4 Perforin Deficiency Does Not Prevent Medial Fibrillin-1 Loss

The levels of fibrillin-1 immunopositivity in the tunica media of apoE-KO and PDKO mice that received Ang II infusion was frequently greatly reduced, especially in the tunica media of dilated, aneurysmal aorta in the regions adjacent to the thrombus compared to GDKO mice. (Figure 11)

5.5 GZMB Deficiency Reduces Fibrillin-1 Fragmentation in Mouse Serum

ApoE-KO and GDKO mice blood serum levels of fibrillin-1 were assessed by immunoprecipitation and western blotting. All samples tested have a band at approximately 50kD representing immunoglobulin heavy chain; however, it was observed that samples from apoE-KO mice with aneurysms following infusion with Ang II have a distinct second band at approximately 45 kD that is not seen in samples from GDKO mice that received similar treatment or in apoE-KO mice that received the saline control, suggesting that significant fibrillin-1 fragmentation is prevented in GZMB-deficient mice compared to apoE-KO controls following Ang II infusion (Figure 12).

5.6 GZMB Treatment Does Not Alter Fibrillin-1 Expression *In Vivo*

Fibrillin-1 transcription levels in HCASMC following treatment with GZMB were assessed by RT-PCR (Figure 12C). It was found that there was no difference in fibrillin-1 expression in HCASMC treated with GZMB compared to controls, suggesting that GZMB does not have any direct effect on fibrillin-1 transcription levels.

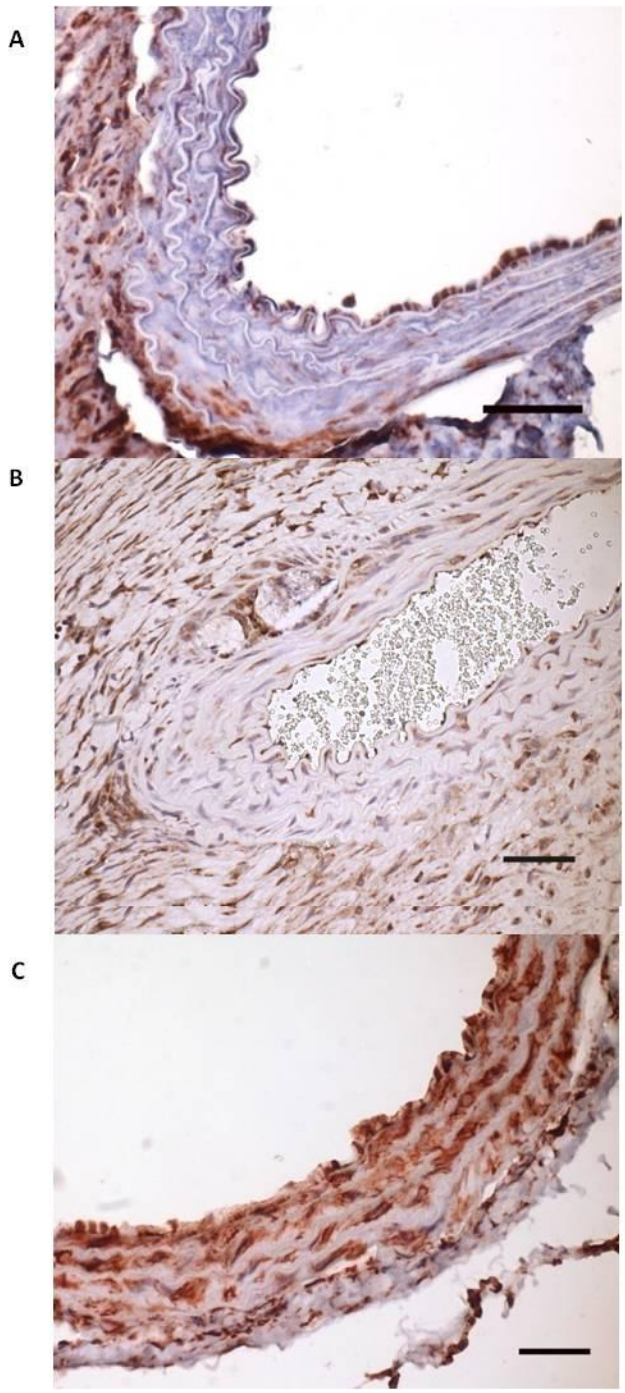


FIGURE 11: FIBRILLIN-1 STAINING IN APOE-KO AND PDKO IS REDUCED COMPARED TO GDKO

Decreased fibrillin-1 staining, as indicated by red colour, was observed in apoE-KO and PDKO mice (A and B respectively) compared to GDKO mice (C) that received Ang II. Scale bar = 50µm.

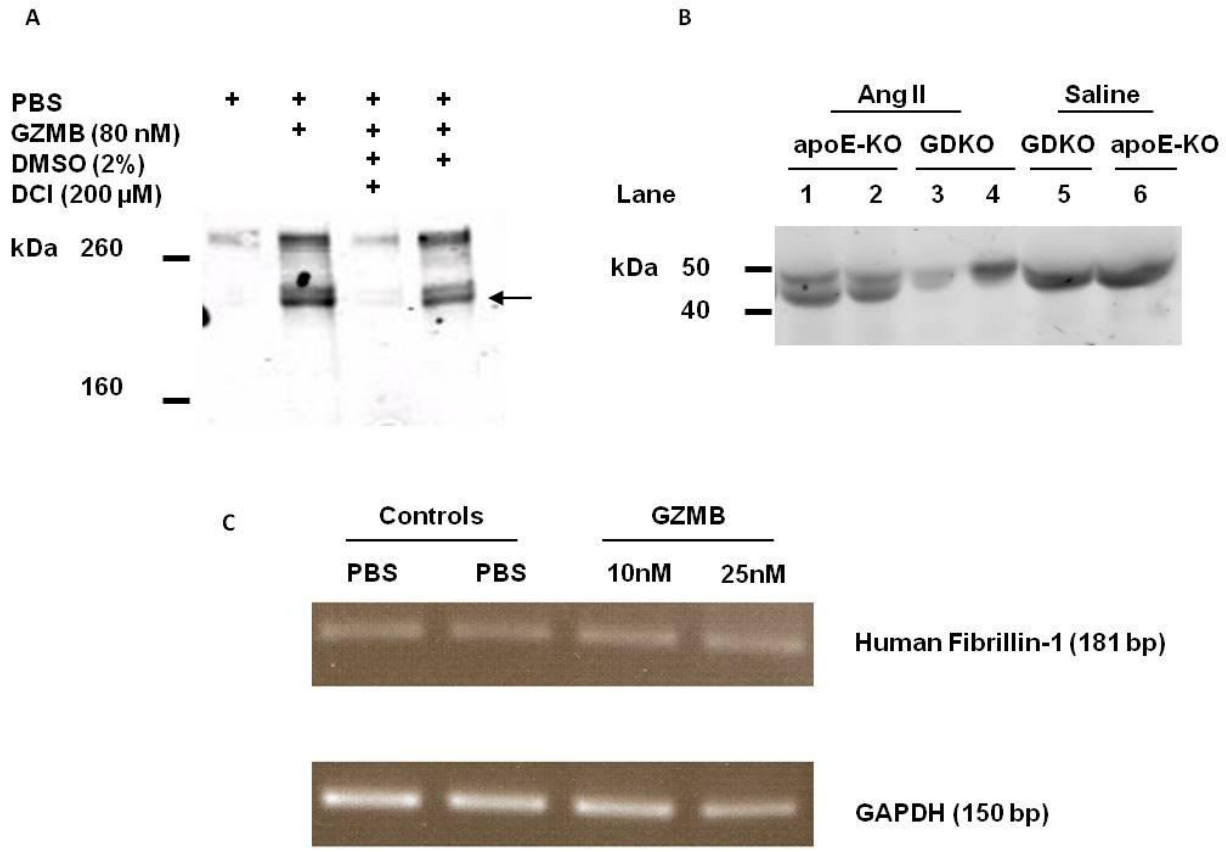


FIGURE 12: GZMB CLEAVES FIBRILLIN-1 *IN VITRO* AND *IN VIVO*

(A) GZMB cleaves fibrillin-1 from HCASMC-generated ECM *in vitro*. HCASMC were cultured to confluency, lysed, and ECM was biotinylated as described. Human GZMB, isolated from IL-2 stimulated lymphocytes was then added to biotinylated ECM and supernatants were collected to assess ECM fragments that were released from the plate. GZMB-mediated fibrillin-1 cleavage was attenuated by the GZMB inhibitor DCI but not by the inhibitor solvent control DMSO. Fibrillin-1 fragments from the supernatant are indicated by an arrow. Gel is representative of 6 independent experiments. **(B) Fibrillin-1 fragmentation is prevented in GDKO mouse serum compared to apoE-KO following Ang II infusion.** Fibrillin-1 in mouse serum was isolated by immunoprecipitation and analyzed by western blotting. ApoE-KO mice with aneurysm show a distinct band at approximately 45 kD that is not observed in GDKO on Ang II and apoE-KO on saline. **(C) *In vitro* fibrillin-1 expression is not directly affected by GZMB treatment.** HCASMC were treated with GZMB and assessed for fibrillin-1 transcription by RT-PCR. No difference was observed in cells that received treatment compared to controls.

5.7 DISCUSSION

While GZMB was absent in healthy, non-atherosclerotic aorta, intense immunopositivity was observed in AAA tissue from both humans (Figure 3) and mice (Figure 10). This complements previous studies where GZMB expression has been shown to increase in both the lesion and plasma as atherosclerotic disease severity increases[283, 428] however, the mechanism by which GZMB exerts its pathogenic effects are unclear. As it is difficult to demonstrate a causative relationship between GZMB and AAA in humans, we have utilized the well-established murine model of Ang II-induced AAA. In this model, macrophage accumulation in the media, medial disruption and dissection precede AAA and atherosclerosis.[414] GZMB deficiency resulted in a significant decrease in the total incidence of AAA from 86.67% in apoE-KO and 87.5% in PDKO mice to 35.71% in GDKO. Incidence of rupture was also reduced from 46.67% in apoE-KO and 43.75% in PDKO to 7.14% in GDKO resulting in a much improved survival rate for GZMB-deficient mice (Figure 6 and Figure 7). It is important to note that the GZMB-KO mouse[439] is a cluster knockdown in which some of the lesser abundant 'orphan' granzymes, unique to mice located close to GZMB on chromosome 14 (C, F, D, and G) have been reported to exhibit reduced expression.[440] However, it is unlikely that this would affect the outcome of this study as these granzymes are not present in humans and GZMB is highly expressed in the area of injury in both mice and humans while these other granzymes are not detectable in the vasculature (unpublished observations) and their physiological role in general has not been determined.

The results from the PDKO group are of particular note because perforin is essential for GZMB-mediated internalization and induction of apoptosis in target cells.[441] As GZMB is only one of multiple granzymes released towards target cells, many studies previously utilized perforin-KO mice to evaluate the role of the granule pathway in disease with the assumption that perforin is necessary for granzyme internalization and apoptosis. In this scenario, if perforin deficiency did not affect outcome, it was often indirectly concluded that granzymes were not involved and/or did not contribute to disease outcome.

One shortcoming of this approach, as demonstrated in the present study, was that it ignored the possibility that granzymes could exhibit perforin-independent, extracellular activity that might contribute to disease.

As perforin deficiency in this model does not provide any protective effect, it is unlikely that the classical GZMB/perforin-mediated apoptosis pathway is involved in AAA and this would suggest that the pathological effects exerted by GZMB are largely extracellular. In support of this concept, GZMB is capable of cleaving numerous extracellular proteins (reviewed in Boivin *et al*.[442]) This does not rule out a role for GZMB in cell death as extracellular GZMB can induce perforin-independent detachment-mediated apoptosis, or anoikis, of VSMC *in vitro* through the cleavage of ECM.[327] Although we did not observe a significant difference in SMC apoptosis at 28 days post-implant (data not shown), it is often difficult to capture apoptotic cells *in vivo* as apoptotic cell debris is rapidly removed by neighboring cells and it is possible that measurable levels of apoptosis may be observed at an earlier time-point. Nonetheless, the protective effect of GZMB deficiency on AAA is clearly evident.

The ability for GZMB to cleave ECM components such as fibrillin-1, decorin, fibronectin, vitronectin, laminin and aggrecan[98, 325-327] has been documented previously. Of note, fibrillin-1 is the major scaffolding component of microfibrils and plays a key role in maintaining vessel wall stability. In the aorta, fibrillin-1 associates with elastin to form the concentric elastic lamellae of the tunica media that confer elasticity to the vessel. In addition, microfibrils not associated with elastin act to stabilize the vessel wall by connecting lamellar rings to one another, to SMC, and to the sub-endothelial basement membrane.[331, 343] Together with collagen, fibrillin-1 microfibrils in the adventitia provide load-bearing support for the entire vessel.[443]

Increased GZMB staining and correspondingly reduced fibrillin-1 staining were observed in the aneurysmal aortas of apoE-KO and PDKO mice when compared to GDKO mice, suggesting that GZMB

degradation could contribute to the loss of fibrillin-1 (Figure 11). Furthermore, western blot analysis of mouse blood serum showed the presence of an extra fibrillin-1 fragment in apoE-KO samples that was not observed in GDKO mice following infusion of Ang II (Figure 12B) suggesting that GZMB deficiency prevents the degradation of fibrillin-1. In addition to this, *in vitro* GZMB treatment of HCASMC did not have any direct effect on fibrillin-1 transcription levels (Figure 12C) supporting the assertion that any decrease in fibrillin-1 observed in the aortas of apoE-KO mice was most likely attributed to proteolytic cleavage by GZMB. It should be noted that the fibrillin-1 fragment size detected in mouse serum differs from the fragments observed in human SMC-generated ECM supernatant. There could be a number of reasons for this discrepancy. Firstly, it is possible that human GZMB cleaves human fibrillin-1 at a different site on the protein compared to its murine counterpart. Indeed, previous studies have shown that mouse and human granzymes can differ in substrate specificities.[444] Secondly, different antibodies were required to detect mouse versus human fibrillin-1, as such, while we were unable to detect the larger fragment previously seen in the *in vitro* study, it is possible that fibrillin-1 might have multiple cleavage sites or that the murine antibody does not detect this fragment. These results may be of relevance to the fibrillin-1 deficiencies associated with Marfan syndrome, but whether structural changes in fibrillin-1 due to mutations associated with Marfan syndrome predispose it to GZMB cleavage is unknown.

Fibrillin-1 null mice die perinatally from ruptured aortic aneurysm and impaired lung function.[445] They have abnormally smooth elastic lamellae and exhibit a loss in VSMC attachments normally mediated by fibrillin-1.[446] Hence, the degradation of fibrillin-1 by GZMB could contribute to medial disruption and subsequent fragmentation of elastic lamellae that is commonly observed in aneurysms as shown in Figure 3, but also affect VSMC attachment and phenotype, ultimately resulting in a decrease in VSMC, loss of structural integrity and a predisposition towards dilation, dissection and the subsequent formation of aneurysms or rupture.[447]

One of the major risk factors for aneurysm formation is advanced age. During aging, the elastin to collagen ratio is reduced thereby leading to arterial stiffness and reduced compliance during contraction.[448-450] Chronic inflammation during atherosclerosis is associated with vessel wall remodelling and a loss of integrity. GZMB levels increase in the intima, media and adventitia with the severity of atherosclerotic disease,[283] a condition that is associated with increased risk for developing AAA. Elevated plasma levels of GZMB are found in patients with unstable versus stable carotid plaques and are associated with an increased occurrence of cerebral vascular events suggesting that GZMB contributes to plaque instability.[428] Furthermore, a recent study has suggested a link between GZMB and unstable angina pectoris in which mononuclear cells from unstable angina pectoris patients exhibited greater GZMB production compared to cells from stable angina pectoris or healthy controls.[388] As GZMB has been previously found to retain its activity in plasma,[451] it is not unreasonable to propose a mechanism whereby chronic inflammation with macrophage infiltration and mast cell activation could lead to increased extracellular GZMB levels in and around the vessel wall. GZMB then cleaves ECM components such as fibrillin-1, contributing to the loss of elastic lamellae, medial degeneration, vessel wall instability and subsequent aneurysm formation, after which further assault by GZMB on the adventitial layer that maintains the structural integrity of the vessel could lead to rupture of the aorta.

As such, the greatest concern upon diagnosis of an aneurysm is the significantly increased risk of fatal aortic rupture. GZMB-deficiency appears not only to reduce incidence of AAA formation but also considerably reduces the incidence of aortic rupture in our model. While premature fatal rupture occurred in over half of apoE-KO and PDKO mice, only 1 in 14 GDKO mice was found to have died from rupture and exsanguination.

In this model, the preliminary dissection that disrupts the media and results in a visible hematoma is usually constrained initially by the tunica adventitia, allowing for remodeling of the

thrombus and reforming of the endothelium.[414] Combining the observation of greatly reduced rupture in GDKO mice with the extensive GZMB immunopositivity observed in the adventitia, it is possible that increased GZMB activity contributes to adventitial degeneration and weakening thereby facilitating expansion and susceptibility to rupture. This could account for the fact that AAA observed in apoE-KO and PDKO were more likely to have expanded with loss of adventitia and progressed to premature rupture of the vessel wall. When the apoE-KO and PDKO mice that died prematurely were examined, large blood clots were always found in the abdominal cavity and thrombus material often spanned the entire length of the aorta from the kidneys to the heart. It is exciting to speculate that GZMB inhibition could limit aneurysm expansion by maintaining adventitial structural integrity; and shall be explored in the subsequent chapter.

CHAPTER 6: INHIBITION OF GZMB REDUCES RATE OF RUPTURE IN ANGIOTENSIN-II-INDUCED AAA

6.1 INTRODUCTION

In Chapter 5, it was shown, using GDKO and PDKO mice, that the serine protease GZMB contributes to AAA through an extracellular, perforin-independent mechanism and is abundantly expressed in the thrombus and adventitia of aneurysmal aortas,[328] making GZMB an attractive therapeutic target for the treatment of AAA.

GZMB has previously been shown to cleave the ECM proteoglycan decorin.[98] Decorin is a small chondroitin/dermatan sulphate proteoglycan belonging to the family of small leucine-rich proteoglycans. In healthy aorta, decorin localizes primarily to the adventitia, where it interacts extensively with other ECM proteins and plays an important role in ECM assembly. Decorin plays numerous roles in collagen organization, formation of tight collagen bundles and is thus considered a mediator of adventitial tensile strength. In the subsequent chapter, we explore the hypothesis that the inhibition of GZMB would reduce the incidence and severity of aneurysm development in the Ang II-induced murine model of AAA and examine the effect of GZMB inhibition on AAA rupture with two different inhibitors: Serpin A3N (SA3N) and a neutralizing anti-GZMB antibody (Anti-GZMB).

6.2 SA3N TREATMENT

6.2.1 SA3N INHIBITS MURINE GZMB ACTIVITY *IN VITRO*

Pre-incubation with SA3N dose-dependently inhibited cleavage of the GZMB tetrapeptide substrate IEPD-pNA by murine GZMB (Figure 13, IC_{50} = 11.83 nM, range 7.925 - 17.66 nM). These results are consistent with previous results demonstrating that SA3N inhibits both recombinant human GZMB and mouse cytotoxic T lymphocyte degranulate GZMB. [366]

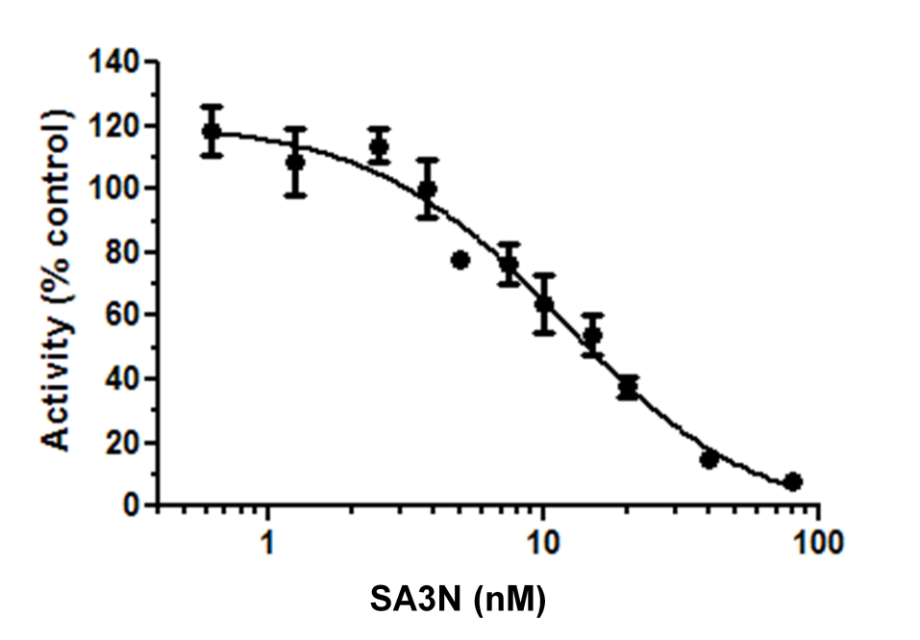


FIGURE 13: SA3N INHIBITS MURINE GZMB ENZYMATIC ACTIVITY.

Mouse GZMB (20 nM) mediated cleavage of the colorimetric substrate Ac-IEPD-pNA (1 mM) was inhibited by increasing concentrations of SA3N. Percent activity was determined as the change in initial rate relative to the initial rate in the absence of SA3N. IC_{50} = 11.83 nM (range 7.925 - 17.66 nM). Data represent the mean $\pm$ SEM of 3 experiments.

6.2.2 SA3N Dose-Dependently Improves Outcomes in AAA

Necropsy was performed on all mice that died prematurely before the 28-day time point and in all cases confirmed the presence of large ruptured aortic dissections and extensive blood clots in the abdominal cavity suggesting that death was caused by exsanguination following AAA rupture. The survival rate of mice that received a saline sham treatment was 50.0% while SA3N treatment improved survival of mice in a dose-dependent manner (Figure 14A, SA3N 120 µg/kg, n = 9/11, 81.8%; SA3N 40 µg/kg, n = 15/18, 83.3%; SA3N 20 µg/kg, n = 7/10, 70.0%; SA3N 4 µg/kg, n = 6/11, 54.5%; saline sham, n = 6/12, 50.0%). Log rank test for trend shows a significant increase in survival with dose of SA3N received ($p = 0.0207$). Mice that received 40 µg/kg SA3N demonstrated a significant increase in survival when compared to mice that received sham treatment (Log rank/Mantel-Cox test, $p = 0.0370$). Incidence of aneurysm rupture was reduced from 50.0% in saline controls to 18.2% in mice that received 120 µg/kg SA3N; 16.7% in those that received 40 µg/kg (16.7%); 30.0% in those that received 20 µg/kg and 45.5% in the group that received 4 µg/kg (Figure 14B).

6.2.3 GZMB Immunopositivity Corresponds to Regions of Medial Disruption and Loss of Fibrillin-1 and Decorin

Serial sections of abdominal aorta from a sham-treated mouse following aortic rupture were stained for Movat's Pentachrome (Figure 15A-C), GZMB (Figure 15D-F), and decorin (Figure 15G-I). Medial GZMB staining (Figure 15F) corresponded to the region of vessel dilation where profound loss of elastic lamellae was observed (Figure 15C). The same region exhibits a marked reduction in decorin (Figure 15I) proximal to the site of aneurysm. Minimal GZMB staining was noted on the non-dilated, healthy side of the vessel wall (Figure 15E) which displayed intact elastic lamellae (Figure 15B) and where robust decorin (Figure 15H) was observed.

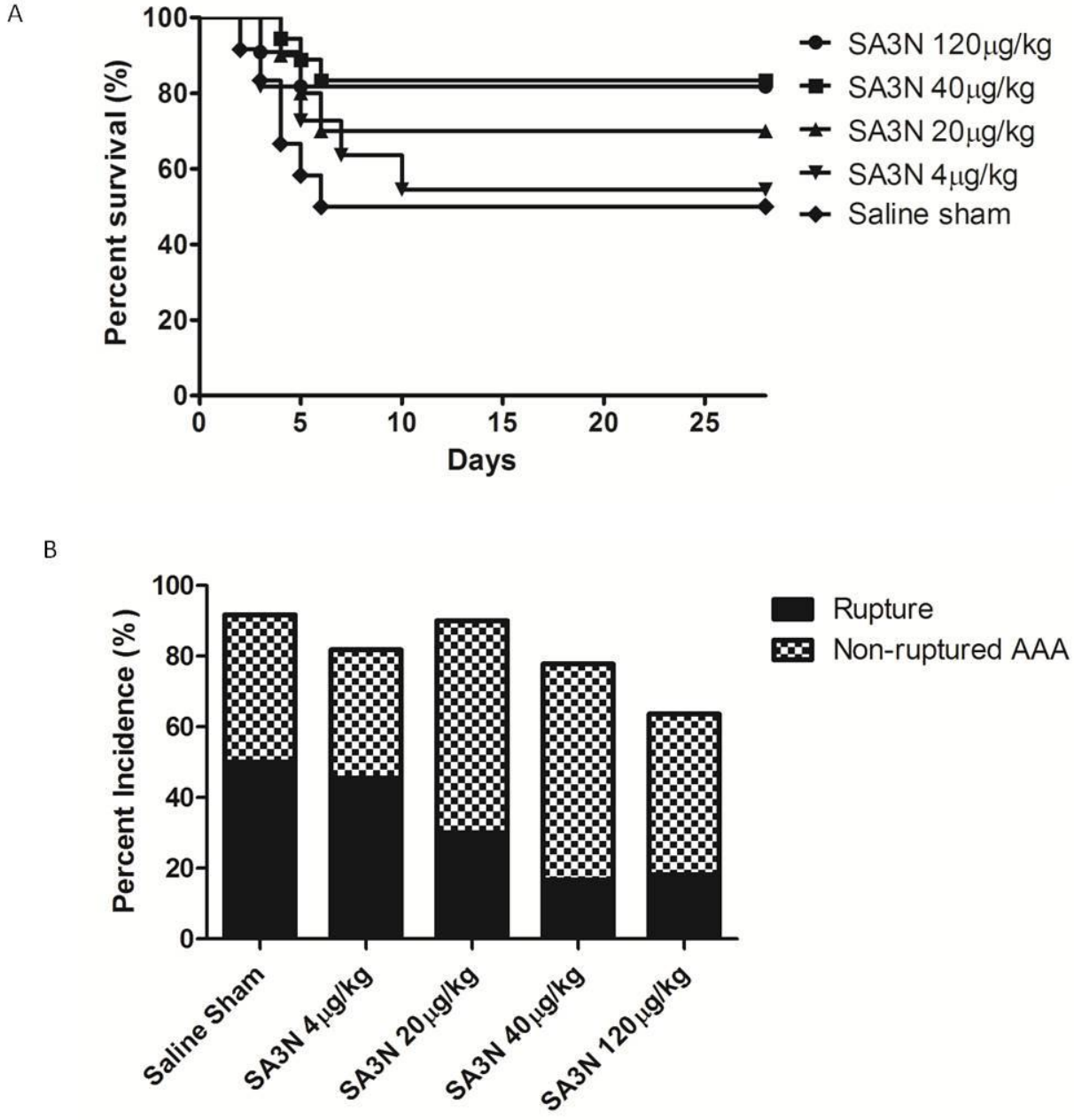


FIGURE 14: GZMB INHIBITION INCREASES 28-DAY SURVIVAL AND REDUCES RATE OF ANEURYSM RUPTURE.

(A) Survival. SA3N 120 μg/kg (81.8%, n = 11); SA3N 40 μg/kg (83.3%, n=18); SA3N 20 μg/kg (70.0%, n = 10); SA3N 4 μg/kg (54.5%, n = 11); Saline sham (50.0%, n = 12). Log rank test for trend shows a significant increase in survival with dose of SA3N received (p = 0.0207). (B) Total aneurysm incidence and rupture. SA3N 120 μg/kg (Incidence 63.3%; Rupture 18.2%); SA3N 40 μg/kg (77.8%; 16.7%); SA3N 20 μg/kg (90.0%; 30.0%); SA3N 4 μg/kg (81.8%; 45.5%); Saline sham (91.7%; 50.0%).

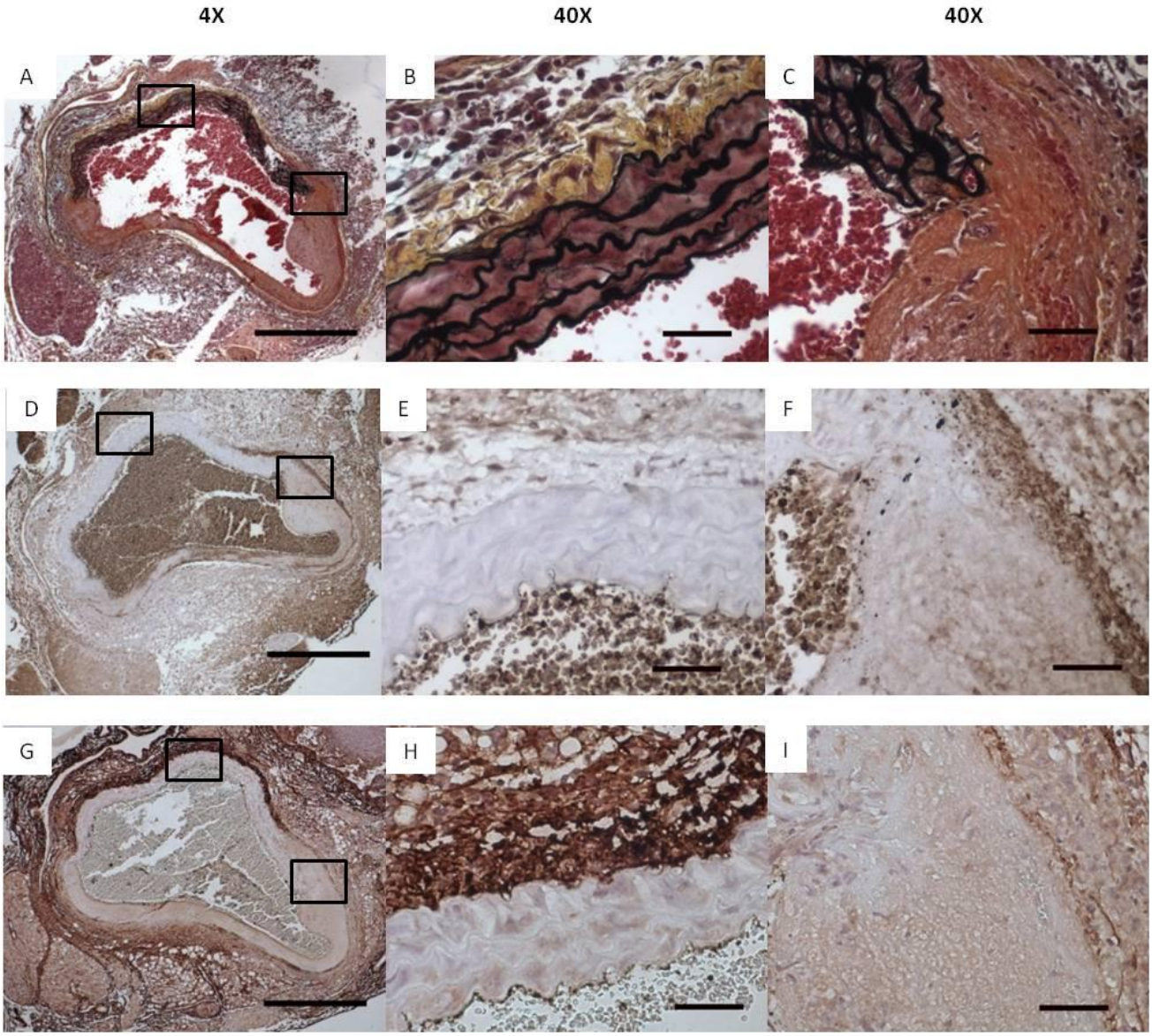


FIGURE 15: GZMB IS ABUNDANT IN VESSELS EXHIBITING MEDIAL DISRUPTION.

Serial sections of abdominal aorta were taken from a sham-treated mouse following aortic rupture and stained for Movat's Pentachrome (4X: A, 40X: B, C), GZMB (4X: D, 40X: E, F), and decorin (4X: G, 40X: H, I). GZMB staining by immunohistochemistry (D, F) corresponds to regions of medial disruption and elastin fragmentation (A, C) and loss of decorin in the adventitia (I). The non-dilated side of the aorta has reduced GZMB staining in the media and adventitia (D, E) and corresponds to intact elastic lamellae (A, B) and decorin (G,H). Scale bars: 4X = 500µm; 40X = 50 µm.

6.2.4 SA3N Reduces GZMB Immunopositivity in Mouse Aorta

GZMB was detected in the aortas of apoE-KO sham-treated mice (Figure 15 and Figure 16) that developed AAA and was particularly elevated in aneurysms that progressed to rupture. Conversely, immunodetection of GZMB was reduced in animals receiving SA3N treatment particularly in the adventitia even when small aneurysms were present (Figure 16). GZMB was found to colocalize to mast cells in murine aneurysmal tissue (Figure 9). These mast cells were often associated with areas of high density GZMB staining in the adventitia and thrombi. No clear dose-dependent trend was observed regarding the concentration of mast cells and co-localization with GZMB (data not shown). Non-ruptured aneurysms with similar pathology from both SA3N- and sham-treated groups were assessed for immune cell infiltration (CD3-positive T lymphocytes and activated macrophages); however no significant difference in the levels of T lymphocytes or macrophages was observed (data not shown).

6.2.5 SA3N Inhibits GZMB-mediated Cleavage of Decorin *In Vitro*

Active, purified human GZMB was incubated *in vitro* with recombinant human decorin protein. Decorin loss was observed by western blot (Figure 17A) and cleavage products were detected by Ponceau staining (Figure 17B). GZMB incubation resulted in production of numerous cleavage fragments ranging in size from approximately 15-55 kDa. GZMB-mediated decorin cleavage was abolished in the presence of SA3N.

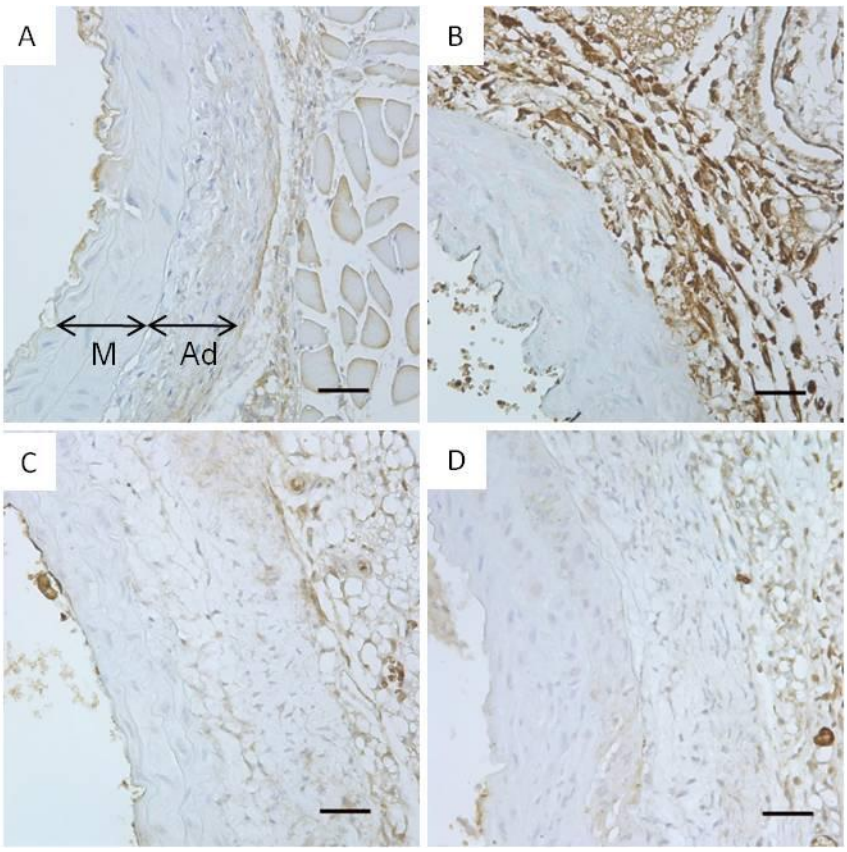


FIGURE 16: GZMB IMMUNOPOSITIVITY IS REDUCED IN SA3N-TREATED MICE.

GZMB staining is minimal in healthy aorta (A) in both the media (represented by “M”) and the adventitia (represented by “Ad”). GZMB expression is increased particularly in the adventitia of sham-treated mice that experienced rupture (B). Reduced GZMB was observed in SA3N-treated mice with no AAA (C) and small, remodelled AAA (D). Scale bars: 4X = 500 µm; 40X = 50 µm.

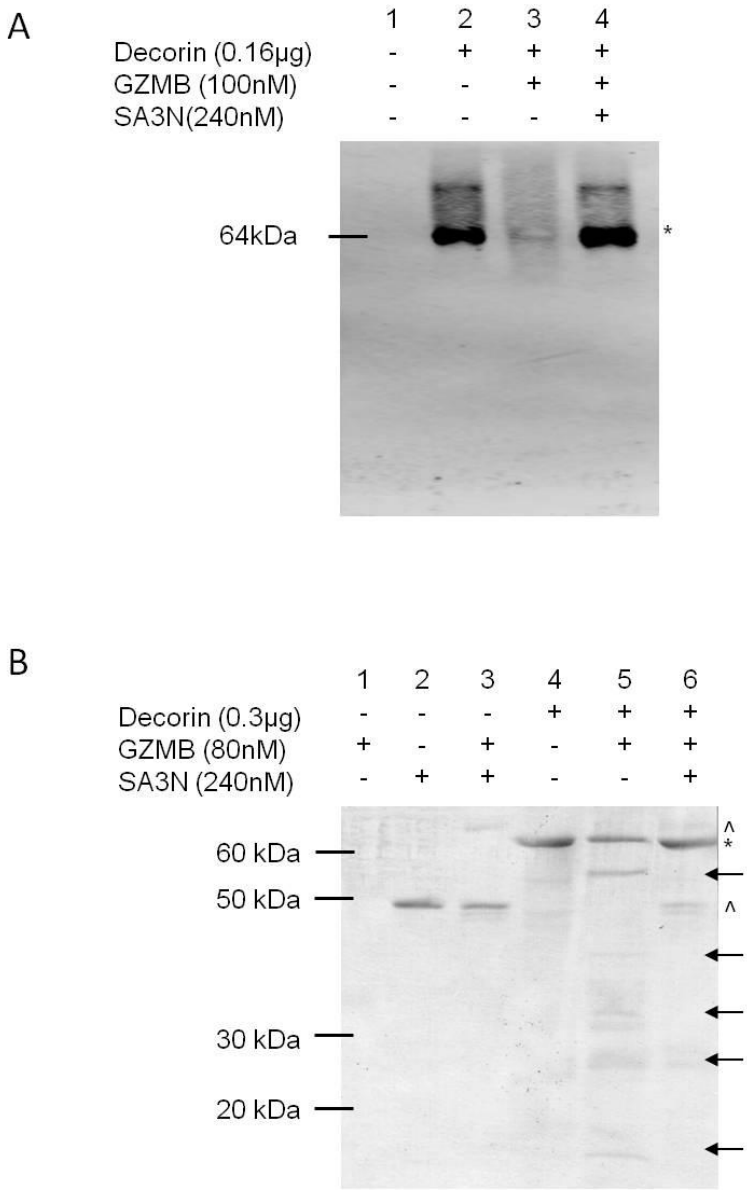


FIGURE 17: GZMB CLEAVAGE OF DECORIN IS PREVENTED BY PRE-INCUBATION WITH SA3N IN VITRO.

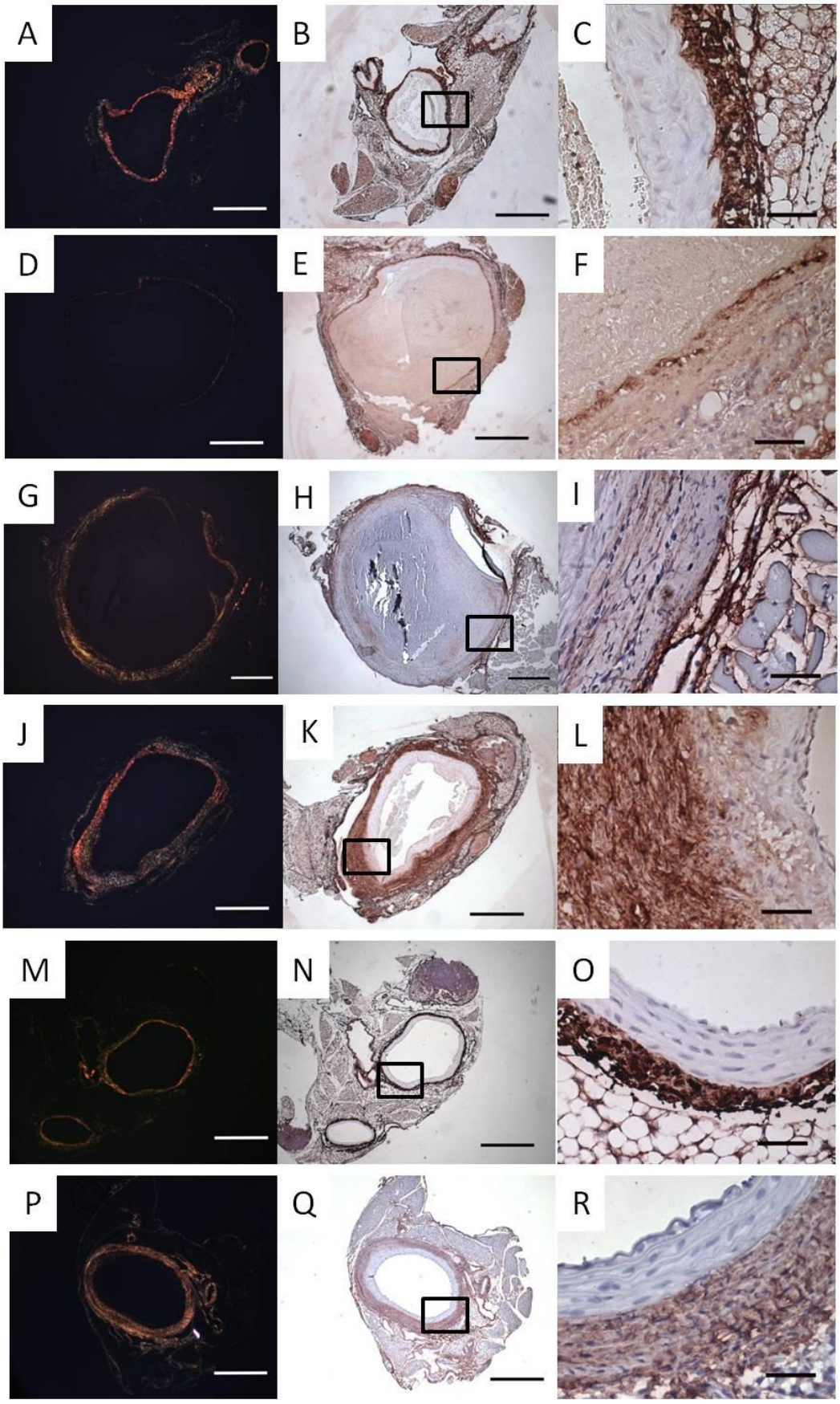
Purified GZMB was pre-incubated with or without SA3N for 25 minutes at RT prior to incubation with recombinant human decorin for 24 hr at RT. Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and visualized by western blotting for decorin (A) and Ponceau stain (B). In Figure 5A, full length decorin is indicated by an asterisk (*) and is visible at approximately 65 kDa in lane 1. Decorin is severely reduced following incubation with GZMB in lane 2. In lane 3, Decorin loss is not observed following pre-incubation of GZMB with SA3N. In Figure 5B, SA3N and SA3N/GZMB complex at approximately 47 KDa and 70 KDa, respectively (indicated by ^) are visible in lanes 2, 3 and 6. Full length decorin is indicated by the asterisk (*) at approximately 65 KDa in lanes 4, 5 and 6. Decorin cleavage fragments in lane 5 are marked by arrows on the right side of the membrane.

6.2.6 SA3N Promotes Adventitial Thickening and Prevents Loss of Decorin

Decorin was markedly reduced in the adventitial regions of aortas from saline sham-treated mice that developed aneurysm (Figure 18I) and exhibited rupture (Figure 18F) compared to aortas of healthy animals (Figure 18C). The adventitia surrounding the thrombus was thin and collagen content was also greatly reduced (Figure 18D, G). With picrosirius red staining under polarized light, adventitial collagen fibres from sham-treated ruptured and non-ruptured aneurysmal aorta (Figure 18D, G) appear green and yellow whereas adventitial collagen fibres from healthy aorta (Figure 18A) have a higher proportion of orange and red fibres, indicating higher fibre density. In comparison, the majority of aneurysms observed in the GDKO and SA3N-treated groups that received the two highest doses (120 and 40 μg/kg) were small aneurysms with minimal thrombus that did not progress to rupture and displayed thickened adventitia, increased decorin staining (Figure 18L, R) and collagen fibres of higher density (Figure 18J, P) compared to ruptured, sham-treated aortas.

6.2.7 SA3N Reduces Loss of Collagen Density in AAA

Collagen fibre imaging by SHG confirmed a reduction in collagen density loss in SA3N-treated mice compared to the saline sham-treated groups (Figure 19). Analysis by one-way ANOVA demonstrated a significant difference across all groups ($p = 0.0226$). SHG signal density representing fibre density was reduced in ruptured and non-ruptured (NR) sham-treated groups compared to normal and SA3N-treated mice, however only the difference between normal and sham-treated groups achieved statistical significance (sham NR, $p = 0.0268$; sham ruptured, $p = 0.0218$). Notable differences in collagen architecture and overall appearance were also observed in sham-treated aneurysmal aortas compared to healthy and SA3N-treated groups, with profound loss of collagen organization and thick bundle formation.


A
B
C
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R

FIGURE 18: SA3N AND GZMB DEFICIENCY PROMOTES ADVENTITIAL THICKENING AND INCREASED DECORIN CONTENT IN AAA.

Sections stained with picrosirius red are shown in the first column (A, D, G, J, M , P). Tissues immunostained for decorin are shown in the second column (B, E, H, K, N, Q) and enlarged for emphasis at 40X in the third column (C, F, I, L O, R). When stained with picrosirius red, thick collagen fibres evidenced by strong birefringence (red) under polarized light (A) are visible in the adventitia of healthy normal aorta, as well as robust decorin staining (B, C). Non-ruptured aneurysms from sham-treated mice exhibit bulbous thrombus, reduced decorin staining (H, I) and thinner collagen fibres of lower density (G) as evidenced by predominance of yellow colour when stained with picrosirius red and seen under polarized light. Ruptured aneurysms from sham-treated mice demonstrate even greater loss of collagen content (D) and a similarly reduced amount of decorin in the adventitia (E, F) compared to healthy aortas. In comparison, aortas from mice that received SA3N (120 μg/kg) prior to Ang II pump implantation demonstrate a significant increase in collagen and a thickened adventitial layer, with a greater proportion of red and orange fibres under polarized light (J). Decorin content in the adventitia is also greatly increased (K, L). Morphology and levels of collagen and decorin in normal, non-diseased aorta from GDKO mice resemble healthy controls. (M, N, O) while GDKO that develop small, localized aneurysm display thickened adventitia (P) and increased decorin content (Q, R) similar to SA3N-treated mice. Scale bars: 4X = 500 μm; 40X = 50 μm.

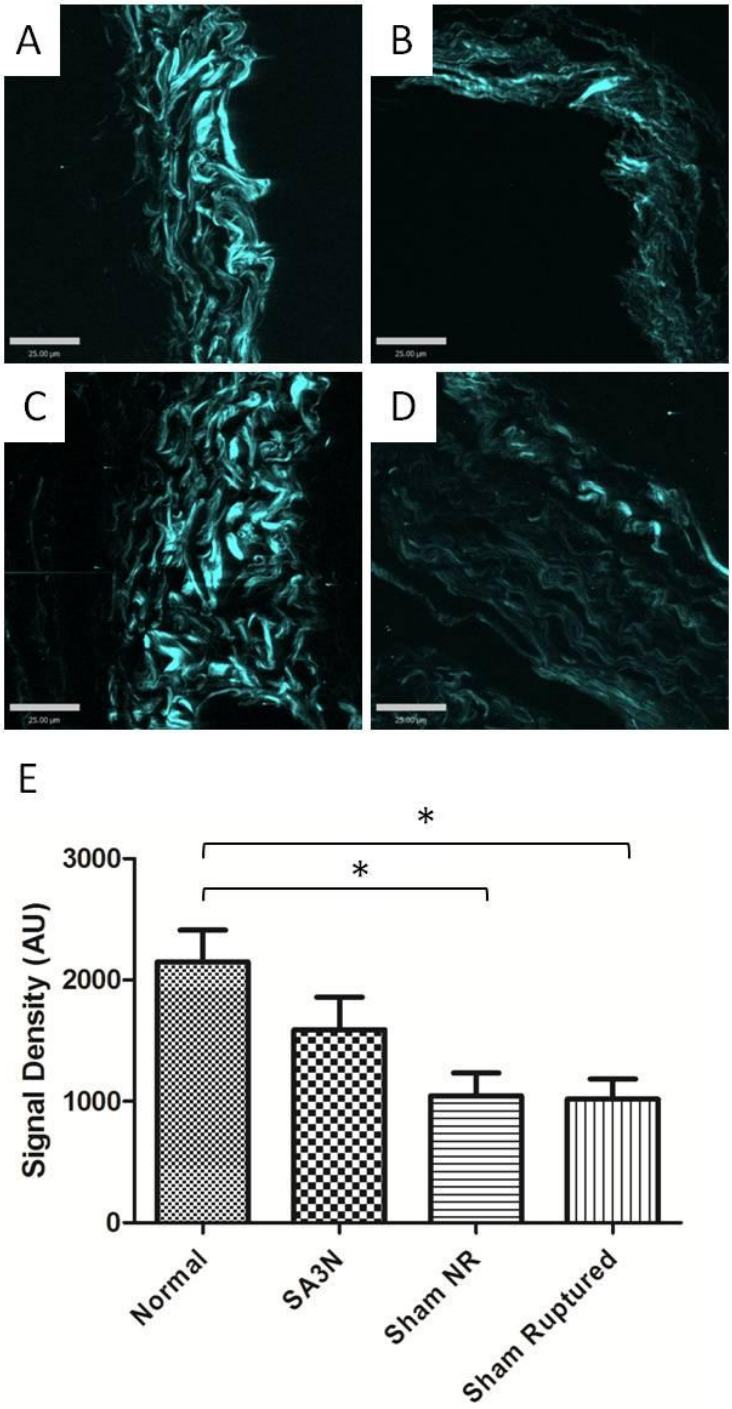


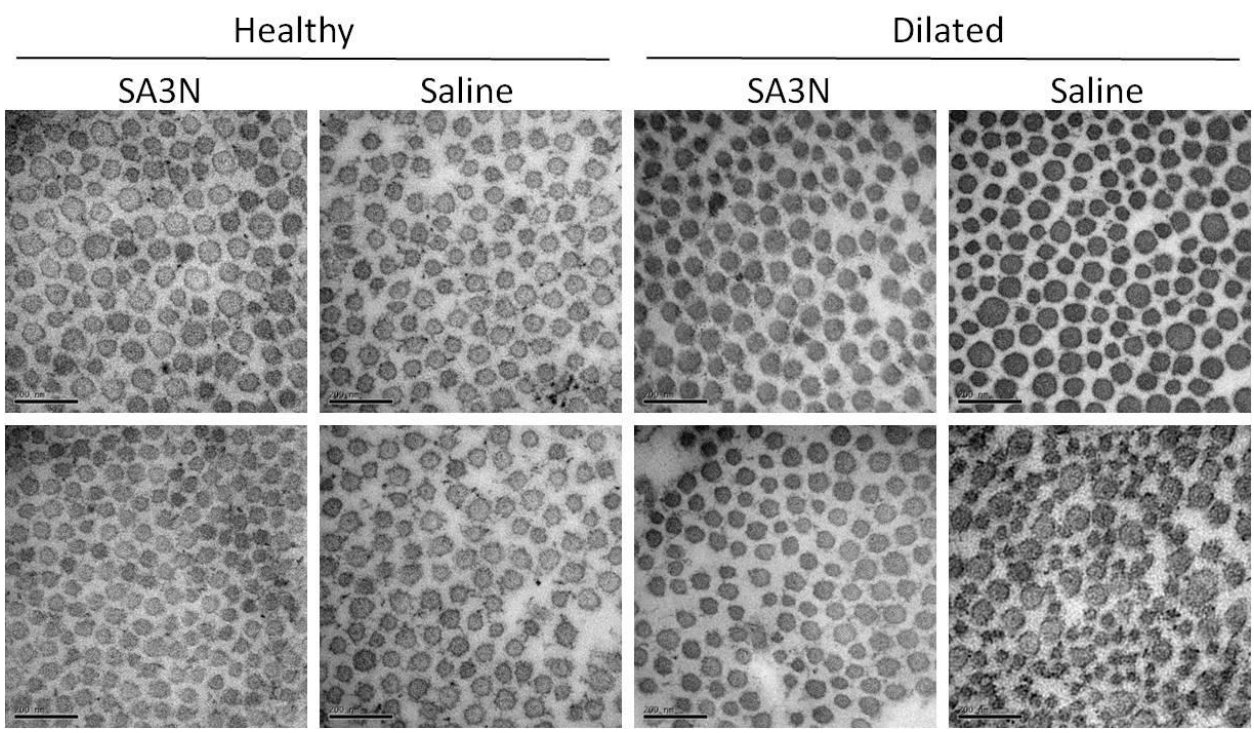
FIGURE 19: SA3N TREATMENT REDUCES THE LOSS OF COLLAGEN FIBRE DENSITY IN ANG II-TREATED APOE-KO MICE.

Adventitial collagen from healthy mouse (A), sham-treated mouse with ruptured aorta (B), SA3N-treated mouse (C), and sham-treated mouse, non-ruptured (NR) aorta (D) were assessed by SHG. (Scale bar: 25 µm). SHG signal densities are summarized in (E) (n = 3 per group, * $p < 0.05$). Error bars indicate SEM.

6.2.8 SA3N Reduces Collagen Fibril Diameter Irregularity and Adventitial Decorin Loss at the Ultrastructural Level

Adventitial collagen fibrils from both the healthy and dilated segments of aneurysmal aortas were visualized by TEM and analyzed for appearance and diameter. (Figure 20A) Collagen fibril diameter from the healthy segments of both SA3N and saline sham-treated were comparable (SA3N range = 24.80-67.98 nm, mean = 42.03 nm, median = 41.10, n = 4867; Saline sham range: 24.24-68.82 nm, mean: 42.05 nm, median = 40.93nm, n = 5099), however, collagen fibril diameter from the dilated segment of sham-treated aortas showed much greater range in size and appearance when compared with dilated segments of SA3N-treated aortas (SA3N range = 26.20-68.87 nm, mean = 47.87 nm, median = 47.82,n = 4179; Saline sham range: 8.32-106.1 nm, mean: 44.73 nm, median = 42.05, n = 5568) (Table 3) suggesting that SA3N treatment was able to reduce fibril diameter irregularity in aneurysmal aortas. In a preliminary pilot study, decorin at the ultrastructural level was visualized by immunogold labeling and imaged by TEM. Decorin immunopositivity was robust in the healthy segments of both SA3N and sham-treated aortas. Decorin loss in the dilated segments was profoundly reduced in the SA3N-treated aorta in comparison to the sham-treated aorta.

A



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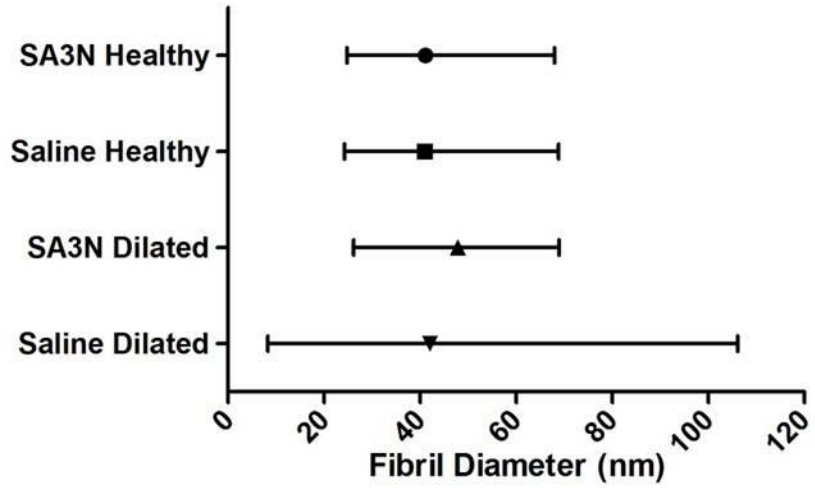


FIGURE 20: SA3N REDUCES COLLAGEN FIBRIL DIAMETER IRREGULARITY.

Adventitial collagen fibrils from the healthy and dilated segments of aneurysmal aortas from both SA3N- and saline-treated mice were visualized by TEM (A) and analyzed for appearance and diameter (B). 20 images per segment were obtained of collagen fibrils in cross section. Fibrils from the dilated segments of saline-treated mice exhibited much greater range in fibril size within the same field of view compared to fibrils from SA3N-treated mice.

TABLE 3: ADVENTITIAL COLLAGEN FIBRIL DIAMETERS

	SA3N Healthy	Saline Healthy	SA3N Dilated	Saline Dilated
Fibril Count	4867	5099	4179	5568
Minimum (nm)	24.8	24.24	26.2	8.32
25% Percentile (nm)	37.44	37.28	42.44	37.21
Median (nm)	41.1	40.93	47.82	42.05
75% Percentile (nm)	45.76	46.08	52.95	49.24
Maximum (nm)	67.98	68.82	68.87	106.1
Mean (nm)	42.03	42.05	47.87	44.73
Std. Deviation (nm)	6.407	6.408	7.145	11.62
Std. Error (nm)	0.09183	0.08974	0.1105	0.1557
Lower 95% CI of mean (nm)	41.85	41.88	47.65	44.43
Upper 95% CI of mean (nm)	42.21	42.23	48.08	45.04
Coefficient of variation	15.24%	15.24%	14.93%	25.97%

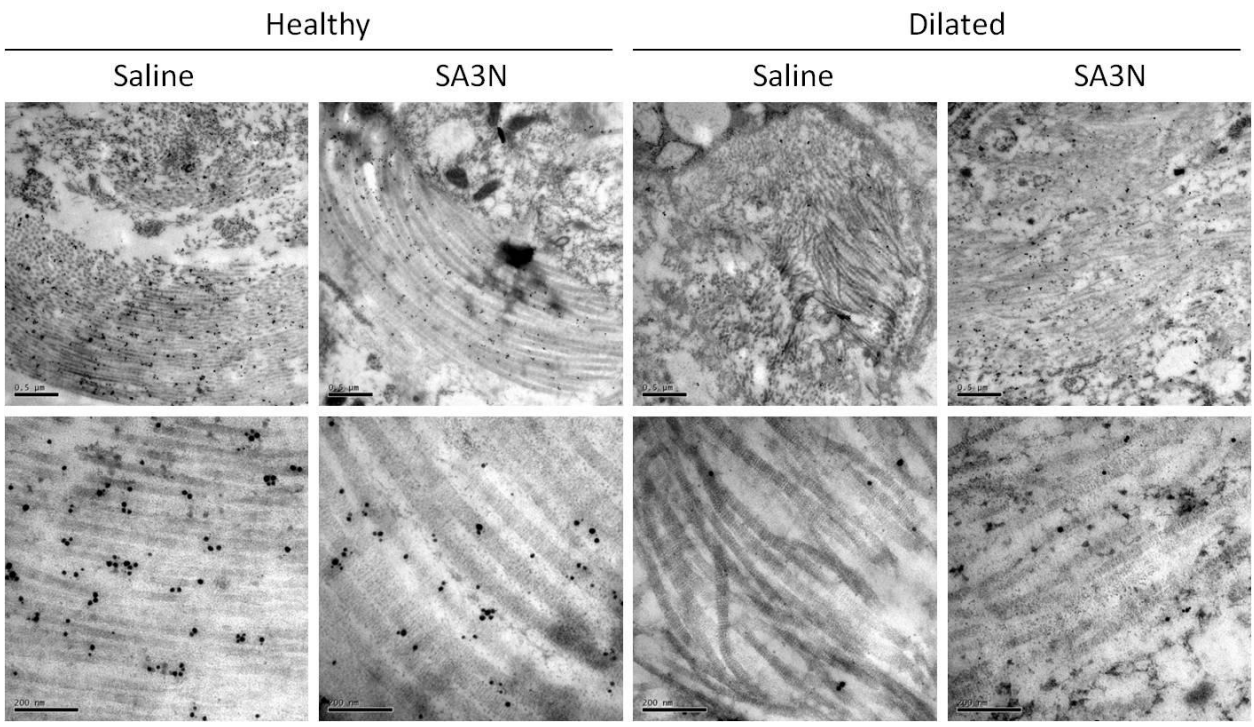


FIGURE 21: SA3N REDUCES DECORIN LOSS AT THE ULTRASTRUCTURAL LEVEL

Decorin at the ultrastructural level was visualized by immunogold staining. Decorin staining indicated by black spots is robust in the healthy segments of both saline sham- and SA3N-treated aortas, but is very much reduced in dilated segments of saline sham aortas. Decorin loss in the dilated segment is rescued by SA3N treatment.

6.3 Anti-GZMB Antibody Treatment

6.3.1 Anti-GZMB Antibody Treatment Improves Outcomes in AAA

Mice were given a tail vein injection of anti-GZMB (n = 9) or IgG control (n = 8) at 1 mg/kg on day 0, 4, 7, 14 and 21. Necropsy was performed on all mice that died prematurely before the 28-day time point and in all cases confirmed the presence of large ruptured aortic dissections and extensive blood clots in the abdominal cavity suggesting that death was caused by exsanguination following AAA rupture. Survival at 28 days was increased from 50.0% in the IgG control group to 77.8% in mice that received anti-GZMB, however, analysis of survival curves by Log-rank (Mantel Cox) test showed no

significant difference (p = 0.247). The incidence of aneurysmal pathology was reduced from 100% to 77.8% in mice that received anti-GZMB compared to IgG control. Incidence of aneurysm rupture and premature death was reduced from 50.0% in the IgG control group controls to 22.2% in mice that received anti-GZMB. (Figure 22).

6.3.2 Anti-GZMB Antibody Treatment Reduces GZMB Immunopositivity and Prevents Decorin Loss

Abdominal aorta from both IgG and anti-GZMB-treated mice were obtained and visualized with Movat's Pentachrome, and stained for GZMB and decorin content. Healthy aorta from the anti-GZMB-treated group is shown in Figure 23, non-ruptured aneurysmal aortas from IgG and anti-GZMB groups in Figure 25 and Figure 24 respectively, and ruptured aorta from the IgG-treated group in Figure 26. GZMB immunopositivity is minimal in healthy aorta and the healthy, non-dilated segments of aneurysmal aorta in both IgG and anti-GZMB-treated aortas. Similarly, decorin staining is robust in these areas. However, GZMB immunopositivity is greatly elevated in the dilated adventitia surrounding the thrombus in IgG control aortas compared to mice that received anti-GZMB treatment, and correspondingly, decorin content is much reduced in these areas. As previously observed in SA3N-treated mice, non-ruptured AAA that received anti-GZMB treatment developed a greatly thickened adventitia and did not display large hematoma or progress towards severe dissection extending past the diaphragm.

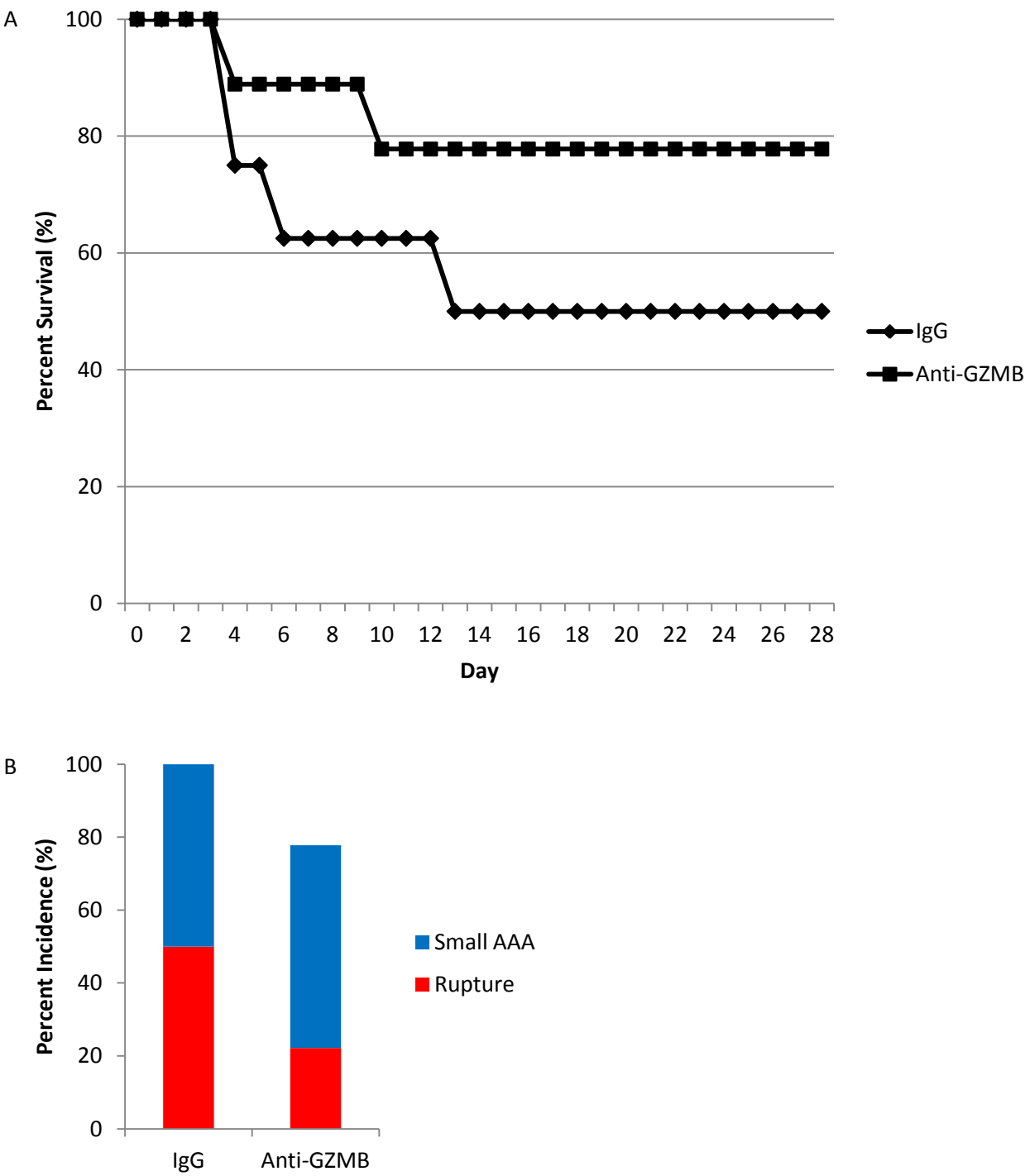


FIGURE 22: ANTI-GZMB ANTIBODY TREATMENT IMPROVES OUTCOME IN ANG II-INDUCED AAA

(A) Anti-GZMB antibody treatment increased survival from 50.0% to 77.8% and (B) reduced incidence of AAA from 100% to 77.8% and reduced rate of rupture from 50.0% to 22.2% compared to mice that received control IgG injections.

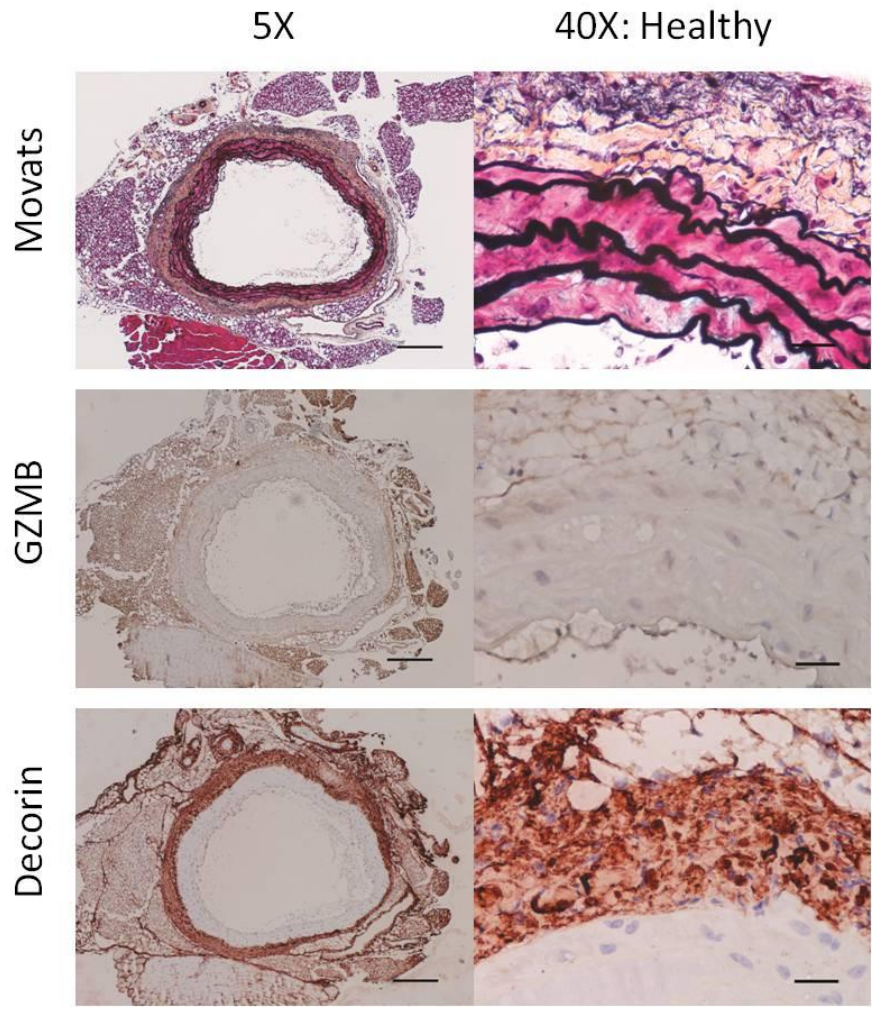


FIGURE 23: REPRESENTATIVE MOVAT'S PENTACHROME, GZMB AND DECORIN STAINING IN HEALTHY AORTA

Minimal GZMB staining and robust decorin staining is observed in the adventitia. Scale bars: 5X = 200 µm, 40X = 25 µm.

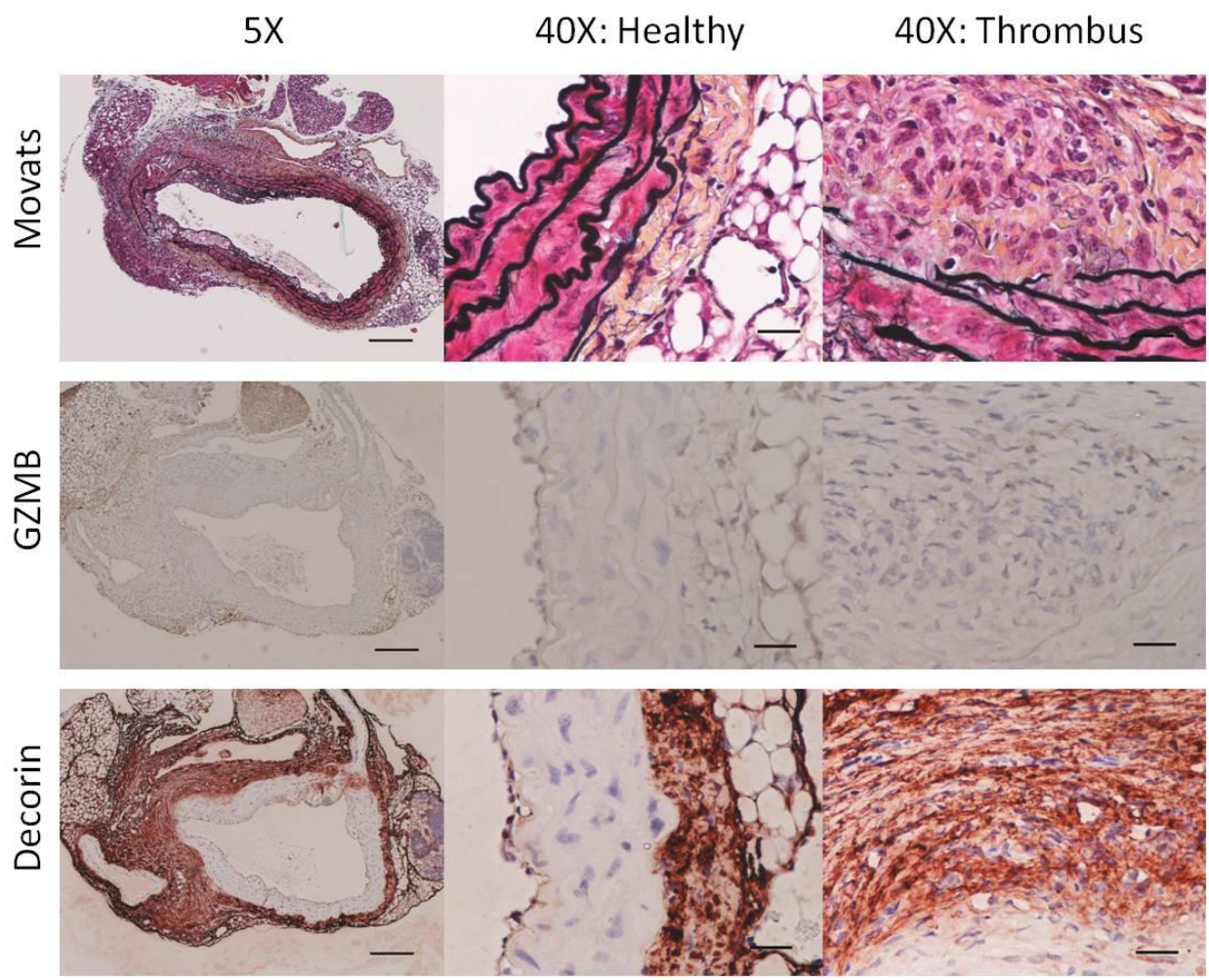


FIGURE 24: REPRESENTATIVE MOVAT'S PENTACHROME, GZMB AND DECORIN STAINING IN ANTI-GZMB TREATED NON-RUPTURED AORTA

Aortas from mice that received anti-GZMB antibody treatment and developed small non-ruptured AAA exhibited thickened adventitia surrounding regions of elastin degradation. GZMB staining is minimal in both the healthy and dilated segment, while decorin staining is robust. Scale bars: 5X = 200 µm, 40X = 25 µm.

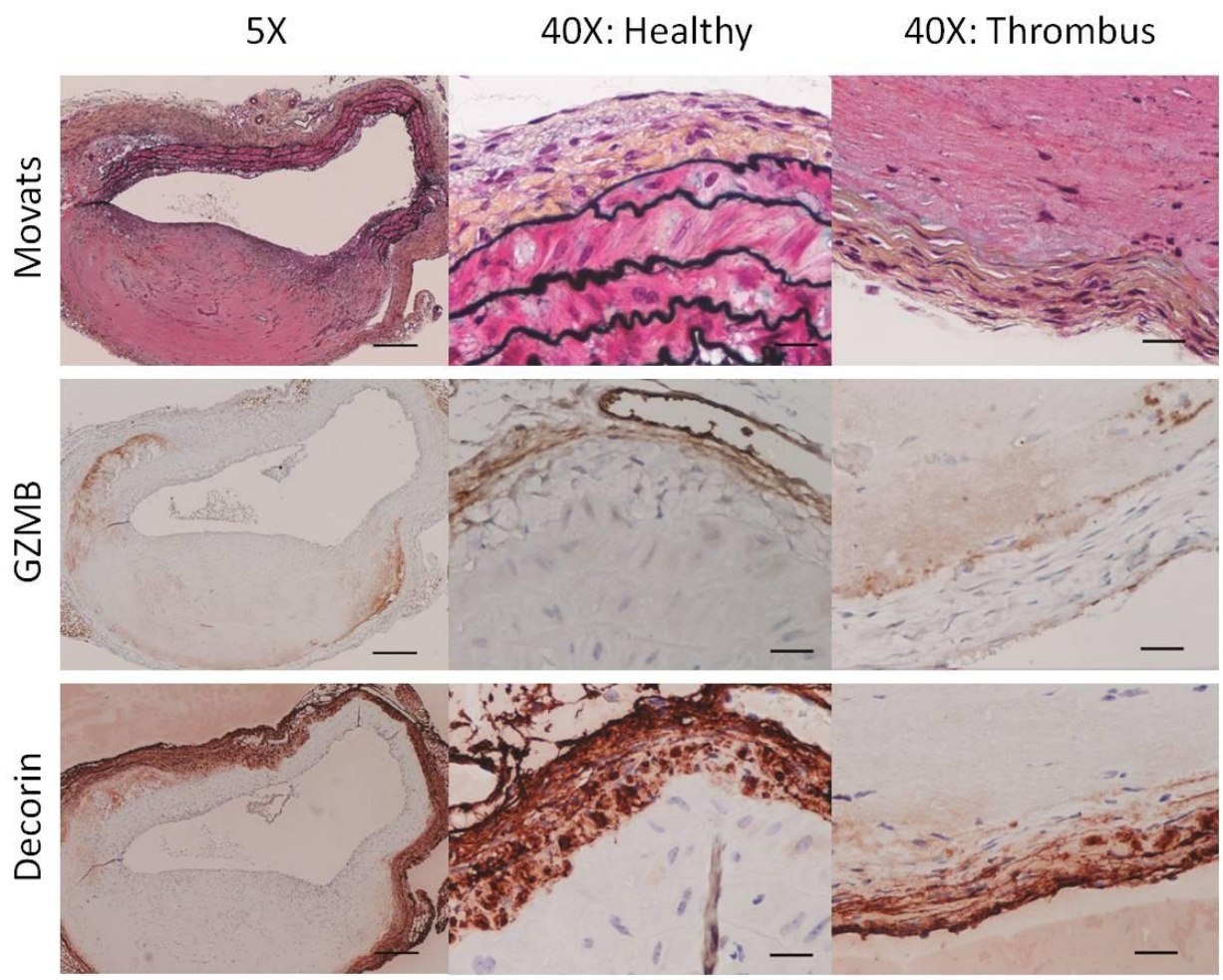


FIGURE 25: REPRESENTATIVE MOVAT'S PENTACHROME, GZMB AND DECORIN STAINING IN IGG-TREATED NON-RUPTURED AORTA

Aortas from mice that received IgG control antibody treatment and developed small non-ruptured AAA exhibited large hematoma and extensive elastin degradation. GZMB staining is pronounced in the medial thrombus while decorin staining is reduced in the thin adventitia surrounding the thrombus. Scale bars: 5X = 200 µm, 40X = 25 µm.

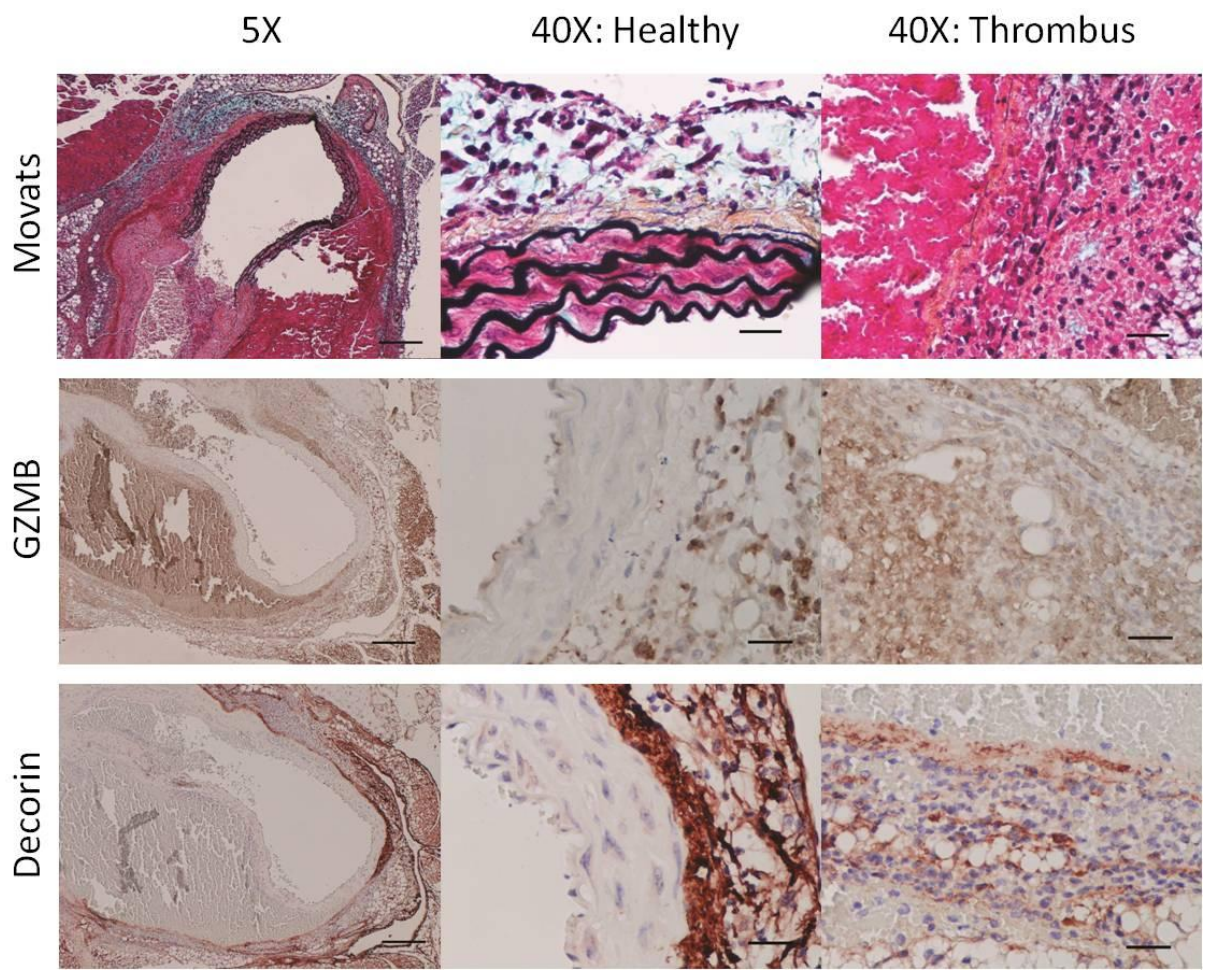


FIGURE 26: REPRESENTATIVE MOVAT'S PENTACHROME, GZMB AND DECORIN STAINING IN IGG-TREATED RUPTURED AORTA

Aortas from mice that received IgG control antibody treatment and developed ruptured AAA exhibited large hematoma and extensive elastin degradation. GZMB staining is pronounced both in the medial thrombus and adventitia while decorin staining is severely depleted in the thin adventitia surrounding the thrombus. Scale bars: 5X = 200 µm, 40X = 25 µm.

6.3.3 Anti-GZMB Antibody Treatment Reduces Loss of Collagen Density in AAA

Collagen fibre morphology and density were measured by picrosirius red (Figure 27) and SHG (Figure 28). With picrosirius red staining under polarized light, adventitial collagen fibres from IgG-treated ruptured and non-ruptured aneurysmal aorta appear green and yellow whereas adventitial collagen fibres from healthy aorta have a higher proportion of orange and red fibres, indicating higher fibre density. SHG signal density representing fibre density was significantly reduced in ruptured and non-ruptured IgG-treated mice compared to anti-GZMB-treated mice ($n = 4$, $p = 0.000927$). Notable differences in collagen architecture and overall appearance were also observed in IgG-treated aneurysmal aortas compared to anti-GZMB-treated mice, with profound loss of collagen organization and thick bundle formation, similar to that observed in SA3N vs. saline-treated mice.

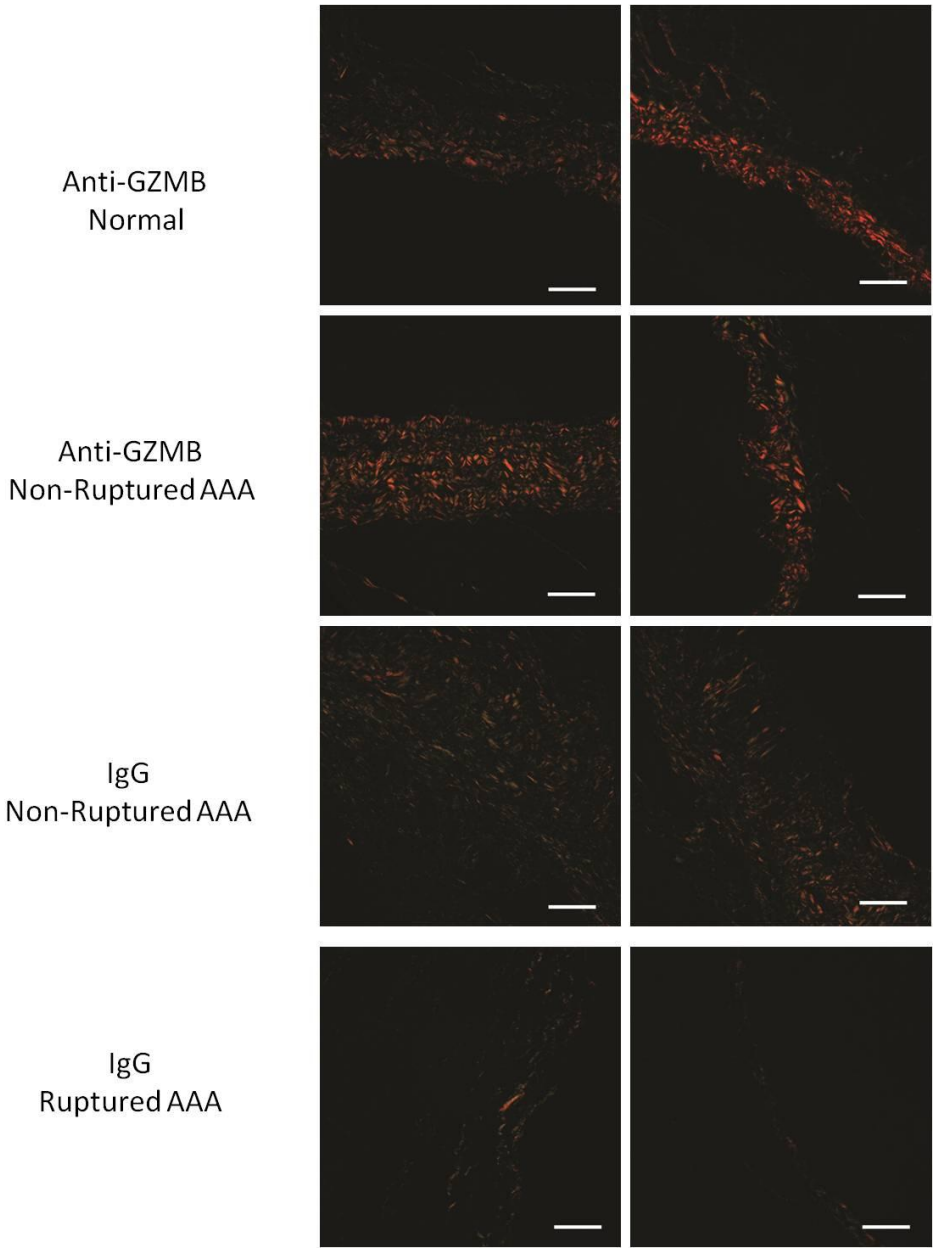
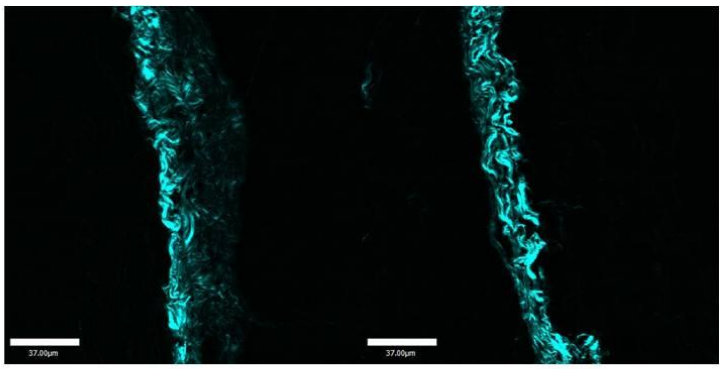


FIGURE 27: PICROSIRIUS RED: ANTI-GZMB ANTIBODY TREATMENT REDUCES LOSS OF COLLAGEN DENSITY IN AAA

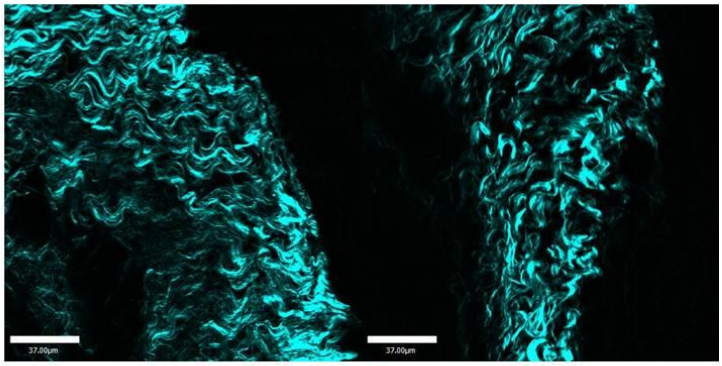
When stained with picrosirius red, thick collagen fibres evidenced by strong birefringence (red) under polarized light are visible in the adventitia of normal aorta. Non-ruptured and ruptured AAA from IgG-treated mice display thinner collagen fibres of lower density as evidenced by predominance of yellow colour when stained with picrosirius red and seen under polarized light. In comparison, non-ruptured AAA from mice that received anti-GZMB antibody display collagen that more greatly resembles healthy controls with a greater proportion of red and orange fibres under polarized light. Scale bars: 40X = 50 µm.

A

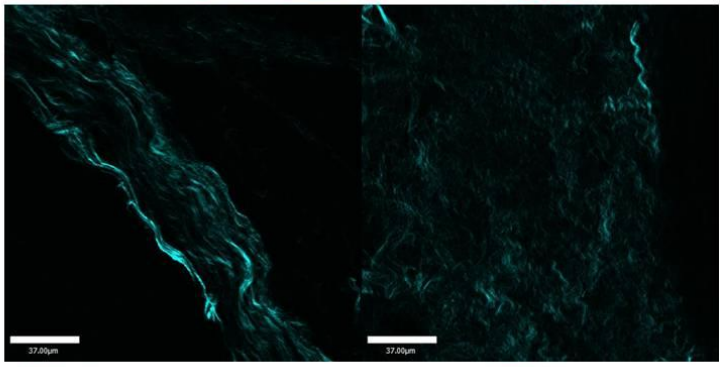
Anti-GZMB
Normal



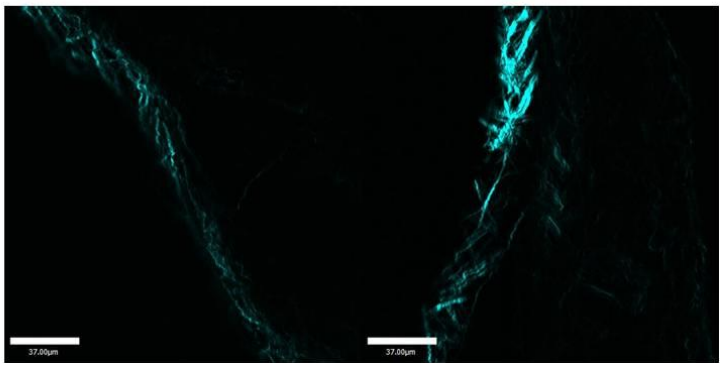
Anti-GZMB
Non-Ruptured AAA



IgG
Non-Ruptured AAA



IgG
Ruptured AAA



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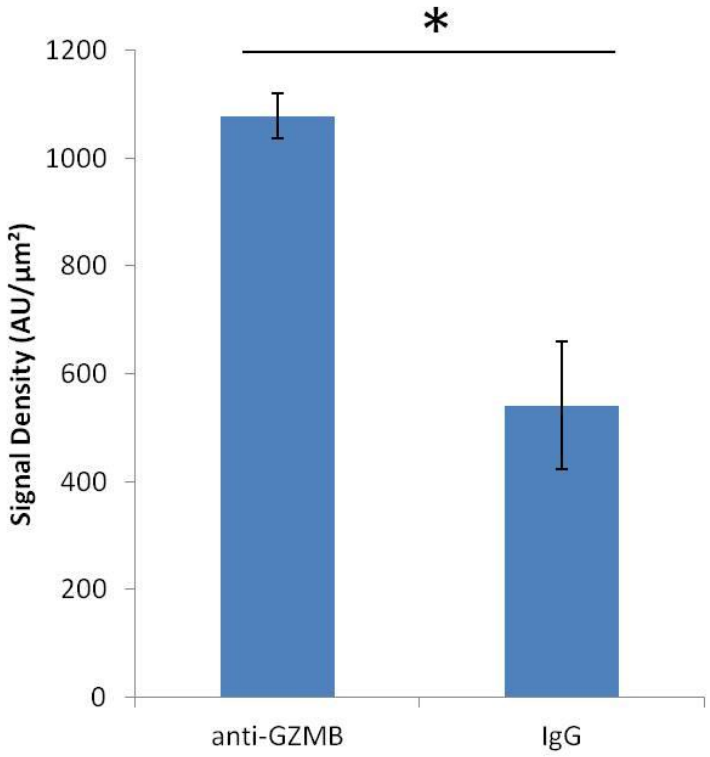


FIGURE 28: SHG: ANTI-GZMB ANTIBODY TREATMENT REDUCES LOSS OF COLLAGEN DENSITY IN AAA

Adventitial collagen from normal aorta, anti-GZMB-treated non-ruptured AAA, IgG-treated non-ruptured aorta and IgG-treated ruptured aorta were assessed by SHG (A). (Scale bar: 37 µm). SHG signal densities are summarized in (B) where anti-GZMB-treated mice exhibited significantly higher signal density compared to IgG-treated mice (n = 4 per group, * $p < 0.05$). Error bars indicate SD.

6.4 Decorin Loss and Collagen Density in Human TAA and Thoracic Dissection

6.4.1 GZMB Immunopositivity Corresponds to Decorin Loss in TAA

Tissues obtained from TAA and thoracic aortic dissection were assessed for GZMB immunopositivity relative to non-atherosclerotic, healthy thoracic aorta (Figure 29). Increased GZMB levels were observed both intracellularly and extracellularly in the thrombic material and infiltrating cells in the vessel wall of the false lumen in acute aortic dissections and corresponded with reduced staining for decorin. GZMB levels appeared to increase with severity of disease in TAA samples tested, with GZMB immunopositivity observed in infiltrating immune cells in the adventitia and adventitial-medial junction in mild TAA and significantly stronger staining both intracellularly and extracellularly in severe TAA. Decorin staining was reduced in intensity in both mild and severe TAA when compared to healthy aorta.

6.4.2 Disorganized Collagen Architecture in Human TAA and Dissection

Collagen fibres from healthy, non-atherosclerotic thoracic aorta (n = 4), TAA (n = 6) and thoracic dissection (n = 5) were visualized by Second Harmonic Generation (SHG) and assessed for signal density (Figure 30). Both TAA and dissection samples displayed profound differences in collagen architecture, with a marked realignment of the fibre bundles and a loss of the characteristic interweaving of adventitial aortic collagen as observed in healthy control samples. Signal density was lower in both TAA and thoracic dissections, and this reached significance in aortic dissections compared to healthy controls ($p = 4.46E\text{-}5$).

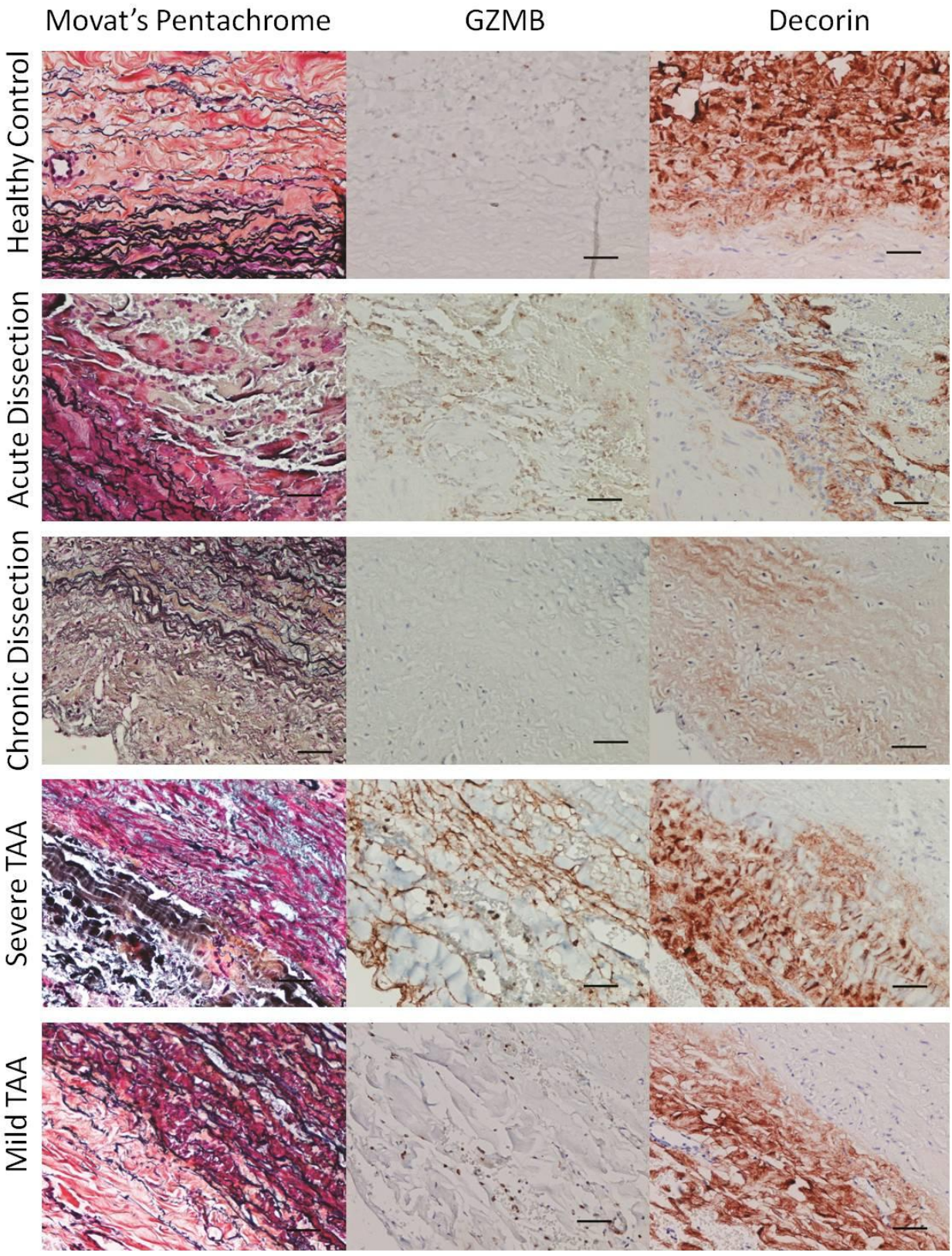
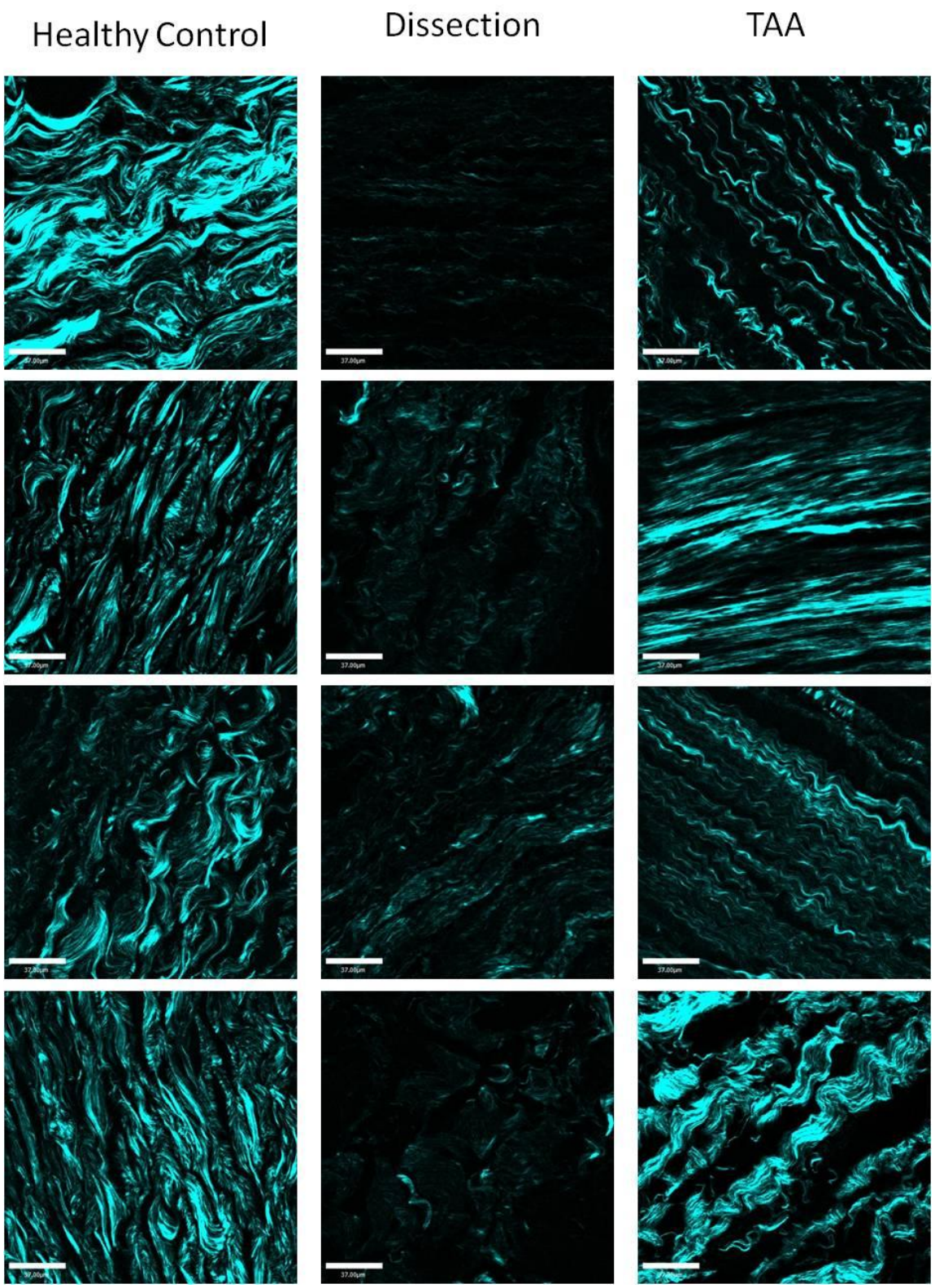

Movat's Pentachrome
GZMB
Decorin
Healthy Control
Acute Dissection
Chronic Dissection
Severe TAA
Mild TAA

FIGURE 29: GZMB IMMUNOPOSITIVITY CORRESPONDS TO DECORIN LOSS IN TAA

Increased GZMB levels were observed both intracellularly and extracellularly in the thrombic material and infiltrating cells in acute aortic dissections and corresponded with reduced staining for decorin. GZMB levels appeared to increase with severity of disease in TAA samples. Scale bar: 20X = 50 µm

A



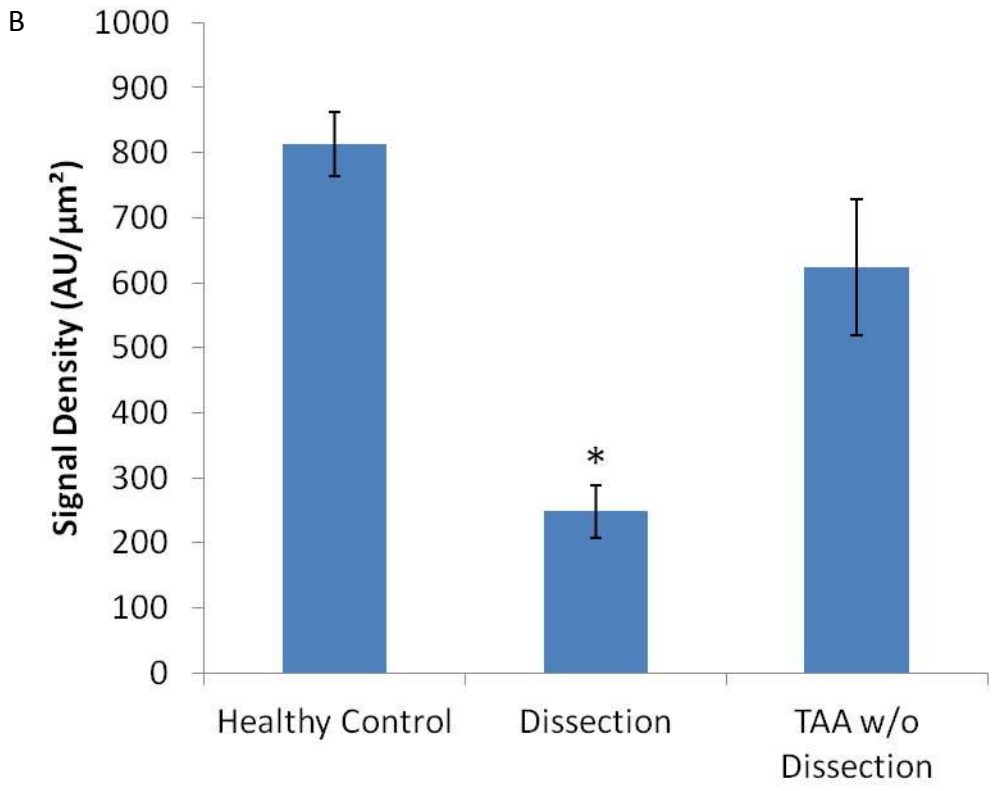


FIGURE 30: COLLAGEN DENSITY IN HUMAN TAA AND DISSECTION

Adventitial collagen from healthy control aortas, thoracic dissection, and TAA were assessed by SHG (A). (Scale bar: 37 µm). SHG signal densities are summarized in (B). Only the signal density from the dissections achieved statistical significance when compared to controls. (healthy control n = 4, dissection n = 5, TAA n = 6; * $p < 0.05$).

6.5 DISCUSSION

While once thought to function exclusively as a pro-apoptotic, immune-secreted serine protease, GZMB can exert other perforin- and/or apoptosis-independent roles in pathogenesis.[452] Indeed, it is now well-established that GZMB accumulates extracellularly in bodily fluids such as plasma, synovial fluid, cerebrospinal fluid, and bronchoalveolar lavage during conditions of aging and chronic inflammation (Reviewed in [453]). Furthermore, in addition to cytotoxic lymphocytes, other cell types, both immune and non-immune, are capable of expressing and secreting this protease alone, or in combination with perforin, into the extracellular milieu (reviewed in [453]). GZMB also retains its activity in plasma [308] and is capable of cleaving ECM *in vitro* (reviewed in [453]). As no extracellular inhibitors of GZMB have been identified in humans, in addition to being a marker of inflammation, extracellular GZMB activity may contribute to vascular pathogenesis. Indeed, in human atherosclerosis, elevated levels of GZMB are associated with increased disease severity and unstable plaque formation.[387] In further support of an extracellular role for GZMB in vascular disease, we demonstrate in Chapter 5 that GZMB contributes to Ang II-induced aortic aneurysm in a perforin-independent manner. While GDKO mice were afforded significant protection against both progression and rupture compared to apoE-KO controls, no protection was observed in PDKO mice. As this study suggested that extracellular GZMB was responsible for AAA in part through the cleavage of ECM, it was logical to determine if extracellular inhibitors of GZMB could elicit a similar effect.

Both SA3N and anti-GZMB significantly improved survival by reducing adventitial decorin degradation and preventing aneurysm rupture. These treatments also resulted in a decrease in overall incidence of aneurysm development that did not reach significance; however, the small unruptured aneurysms in the anti-GZMB and SA3N-treated groups were morphologically different to those observed in corresponding control animals. Aneurysms in the control groups consistently exhibited medial hematomas consistent to those previously observed by us and others [328, 411, 414] with a disordered,

fibrous adventitia displaying mostly yellow-green fibres when stained with picrosirius red, indicating less tightly packed collagen fibres of smaller diameter. In contrast, aneurysms in the GDKO, anti-GZMB and SA3N-treated groups, especially at the higher doses, demonstrated a remarkable thickening of the adventitial layer with red and orange collagen fibres when stained with picrosirius red. This suggests that the collagen fibres in the adventitia of these mice exhibited increased thickness, density and alignment[454] compared to their saline and IgG-treated counterparts. Indeed, when examined by SHG, collagen from saline and IgG-treated mice demonstrated a significant reduction in collagen fibre density and exhibited unidirectional realignment of fibres, whereas collagen morphology from SA3N and anti-GZMB-treated aortas more closely resembled the interwoven network as seen in healthy controls. Similar observations were made of the collagen architecture in the dilated portions of human TAA and dissected aortas, suggesting that this is not a feature that is unique to the murine model, and encouragingly, may also be remedied by the administration of GZMB inhibitors as a possible therapeutic agent in the human forms of disease.

The Law of Laplace states that for any given internal fluid pressure, vascular wall tension is proportional to the radius of the vessel, and inversely proportional to vessel wall thickness.[455] This implies that larger vessels require stronger, thicker walls than smaller vessels as a vessel of larger radius must be able to withstand greater tension. Similarly, dilated aneurysmal arteries experience increased wall tension and it follows that increasing wall thickness (as observed in the adventitia of GDKO, anti-GZMB and SA3N-treated animals that developed aneurysms), without overly compromising distensibility, will effectively reduce the tension experienced by the vessel.

The adventitia is responsible for maintaining the circumferential structural integrity of the vessel[456] and its tensile strength is largely reliant on the organization and morphology of its major constituent, type I collagen.[457] While medial elastin and fibrillin-1 loss is a critical factor in aneurysm

formation, aneurysmal growth and rupture is dependent on defective collagen homeostasis.[51] Consistent differences in the optical properties of aneurysmal collagen have been noted when compared to normal vessels, suggesting a difference in fibril organization.[458] To corroborate this, we have observed an increase in fibril diameter irregularity (Figure 20) in aneurysmal adventitia that is greatly reduced when treated with SA3N. Collagen content is both disorganized and reduced in human aneurysms and dissections of the ascending aorta, while in human AAA, turnover of collagen III is increased as evidenced by increased levels of aminoterminal propeptide of type III collagen and associated with impaired collagen fibrillogenesis.[459, 460] In a study on collagen cross-linking, Carmo *et al.* observed an overall decrease in collagen content in human AAA specimens and concluded that new collagen synthesis in aneurysmal tissues was defective and lacking proper cross-linking,[461] rendering it more susceptible to proteolytic attack.

Our findings in both murine and human aneurysmal disease related to the loss of collagen organization and packing are consistent with the recent findings of Lindeman *et al* [462] in which a loss of collagen microarchitecture was observed in the adventitia of AAA and Marfan syndrome patients. In the latter, it was proposed that the loose, ribbon-like appearance of collagen that is observed in a normal adventitia, but not aneurysmal adventitia, acts as a coherent network to maintain mechanical strength and allows for vessel dilation and that this is lost in growing aneurysms.[462] Given that we had also observed a loss of adventitial collagen organization in the mouse AAA model, we were interested in how GZMB might affect this process.

In saline sham and IgG treated mice that experienced rupture, strong intracellular and extracellular GZMB staining was apparent in the adventitia (Figure 16, Figure 25, Figure 26). In contrast, minimal staining was evident in the SA3N and anti-GZMB-treated groups. While these results suggest that SA3N administration could lower GZMB levels or that the non-functional GZMB-SA3N complex may

have been cleared from the vessel, it is also very possible that the non-competitive binding and/or conformational change of GZMB within the GZMB-SA3N complex results in reduced recognition and binding of the detection antibody to GZMB during IHC staining. It is, however, expected that these effects if present, should be largely alleviated in the anti-GZMB groups as the neutralizing antibody is polyclonal.

In analysis of non-ruptured AAA, normal levels of adventitial decorin staining were observed on the non-dilated side of the vessel where the elastic lamellae are still intact. Conversely, on the dilated side where aortic rupture occurred and GZMB staining was most prominent, decorin staining was nearly absent. It has been previously shown that GZMB degrades decorin *in vitro*[425] and here we demonstrate that pre-incubation with SA3N is capable of preventing cleavage by GZMB (Figure 17). Decorin staining was markedly elevated in GDKO, further implying that GZMB is responsible for decorin degradation. Decorin immunopositivity was strong in SA3N and anti-GZMB-treated mice (Figure 18 and Figure 24), which also displayed reduced levels of GZMB immunopositivity in the thickened adventitia compared to controls. GZMB staining was elevated in both saline and IgG controls (Figure 16, Figure 25, Figure 26), particularly when rupture was observed while decorin staining was nearly depleted in the adventitia surrounding the medial thrombus (Figure 18, Figure 25, Figure 26) and at zones of dilation. As further evidence, GZMB immunopositivity also corresponds to loss of decorin in human thoracic dissection and severity of disease in human TAA.

Because collagen is the main structural element of the adventitia, providing the mechanical and tensile strength, as well as forming the scaffold for the ECM, the organization and orientation of the collagen fibrils is critical and largely determined by interactions with proteoglycans such as decorin through interfibrillar bridges.[463] Decoron, the core protein of the decorin molecule, functions as a strong anchor for the elastic glycan bridge between each decorin molecule and collagen fibril, conveying

elasticity and allowing for reversible deformation during movement of the vessel wall.[97] Decorin is a key mediator of collagen fibrillogenesis [88, 337] and its incorporation into collagen fibrils improves tensile properties.[96] Decorin-deficient mice have fragile skin with markedly reduced tensile strength.[88, 464] Dermal collagen from these mice exhibit highly irregular fibril diameter and abnormal fibrillar organization,[88] suggesting that decorin plays a crucial role in fibril formation, fusion and maintaining stability. Furthermore, Iwasaki *et al* [338] have found that *in vitro* collagen gels cultured in the absence of, or with low concentrations of decorin produced highly porous fibre networks with poor elasticity, whereas gels cultured with higher concentrations of decorin produced fibre networks of significantly higher density and elastic properties. Reese *et al*.[465] have further demonstrated that the addition of decorin during the fibrillogenesis of Type I collagen increases the linear elastic modulus and tensile strength of collagen gels *in vitro*. This corroborates our own observation of higher adventitial collagen fibre density in SA3N and anti-GZMB-treated animals that also present with correspondingly higher decorin immunopositivity compared to controls.

Along with other proteoglycans, decorin can sequester and regulate the activity of growth factors such as TGF-β,[466] fibroblast growth factor-2 (FGF-2),[467] TNF-α,[468] and platelet-derived growth factor (PDGF)[469] making decorin a critical regulator of numerous receptor-specific signalling cascades and giving decorin the ability to influence repair and remodelling processes in damaged tissues. Under specific conditions, decorin binds to the forming collagen fibrils, functioning as a spacer and slowing down collagen fibril association and fusion. This allows sufficient time for optimal interactions and cross-linking to occur, and thus forces the formation of well-organized fibrils of uniform diameter optimized for local conditions.[88] In further support of this, we have observed that fibril-associated decorin is greatly reduced in the dilated regions of aneurysmal aorta which are more susceptible to rupture.

We have demonstrated that both human and murine aneurysmal disease exhibit a similar loss of collagen architecture and that GZMB is elevated in both conditions. We report that GZMB inhibition by SA3N was able to reduce decorin cleavage, subsequent collagen disorganization and aneurysmal rupture in murine AAA. These results were confirmed by the obtainment of comparable results with a more specific, neutralizing anti-GZMB antibody. Similar observations of adventitial thickening and reduced loss of decorin were also observed in GZMB-deficient mice following AAA induction, suggesting that the cleavage of decorin is most likely due to GZMB. As further support, SA3N treatment prevented GZMB cleavage of decorin *in vitro*. Based on these findings, we propose that reducing GZMB in the inflammatory milieu surrounding aneurysmal induction promotes beneficial adventitial collagen remodelling by preventing the degradation of decorin, thereby facilitating decorin-mediated fibrillogenesis and reinforcement of the adventitia, reducing wall tension and thus satisfying the Law of Laplace. The vessel consequently retains sufficient tensile strength and flexibility to maintain structural integrity, constrain dilation and prevent rupture.

Although we did not detect a change in $CD3^+$ lymphocytes or macrophages following SA3N treatment compared to non-ruptured sham-treated controls at 28 days post-induction, immune cell infiltration is difficult to accurately assess and quantify in ruptured aortas in this model and will require additional study at earlier time points. In Chapter 5, it was determined that GZMB deficiency, but not perforin deficiency protects against AAA development in this model.[328] While this rules out a role for GZMB/perforin-mediated apoptosis, there is indeed the possibility that GZMB could be contributing to aneurysm development through GZMB-mediated anoikis through ECM cleavage. Indeed, we and others have shown that GZMB cleavage of fibronectin can result in detachment-induced death of fibroblasts, [429] endothelial cells[326] and smooth muscle cells;[327] the loss of which may significantly contribute to aneurysm pathogenesis.

Although SA3N is a potent inhibitor of GZMB, SA3N is also known to bind and inhibit human leukocyte elastase (neutrophil elastase), [367] another protease implicated in AAA pathogenesis. Leukocyte elastase, however, does not cleave decorin.[340] As elastase-digested aortas are utilized to assess decorin and biglycan expression in AAA,[340] it is highly unlikely that inhibition of leukocyte elastase was responsible for prevention of decorin degradation in this model. This is further supported by the observation that decorin cleavage is also prevented in GZMB-deficient mice and similar results were achieved with a neutralizing anti-GZMB antibody. While the generation of small molecule GZMB inhibitors is in progress, at the present time, no other extracellular inhibitors of mouse GZMB are available.

In conclusion, because we have shown that small aneurysms in GDKO and GZMB-deficient mice develop similar morphology and show reduced propensity to decorin cleavage, adventitial collagen disorganization and rupture, we believe a strong argument can be made for pursuing further investigations into GZMB as a therapeutic target for reducing the progression and rupture of human aneurysms and dissections.

CHAPTER 7: CONCLUSION

AAA is a complex, multifactorial disease that is characterized by elastin degradation and impaired collagen turnover in the aortic wall, and is likely associated with the effects of multiple genes in combination with various lifestyle and environmental factors. In the present study, GZMB levels were found to be elevated in human AAA and that GZMB colocalizes to macrophages, lymphocytes and mast cells in aneurysmal lesions. Furthermore, we confirm that GZMB has the ability to cleave numerous ECM proteins such as fibrillin-1 and decorin.

Knocking out the serine protease GZMB reduced the incidence of aneurysmal disease in an Ang II-induced murine model of AAA, while perforin deficiency was not protective, indicating that GZMB contributes to the pathogenesis of AAA via its extracellular abilities. In the same model, GZMB deficiency prevented the loss of medial fibrillin-1 while a reduced level of fibrillin-1 fragments was observed in mouse serum. Because fibrillin-1 serves as a scaffold for elastin microfibrils, this would suggest that GZMB may play a role in the destruction of the elastic lamellae, a defining characteristic of aneurysmal disease.

Subsequently, we were able to show that inhibition of GZMB by both murine serpin A3N and a more specific neutralizing anti-GZMB antibody was able to reduce incidence of medial hematoma and decrease the rate of aneurysmal rupture in the same Ang II-induced model of AAA. In apoE-KO mice that received saline sham or IgG control treatment, GZMB expression corresponded to regions of decorin loss, and as decorin is a critical mediator of collagen fibrillogenesis, GZMB-mediated decorin degradation may be responsible for the irregularity of collagen fibril diameter and for the loss of collagen architecture and density in the adventitia seen in these mice. Correspondingly, GZMB deficiency prevented decorin loss, reduced collagen fibril irregularity, and preserved collagen architecture and

density. It is likely that this collagen remodelling constrained disease progression and accounted for the reduction in rupture rates seen in mice that received inhibitor treatment.

In this model, Ang II signals through the AT1 receptor and initiates a localized inflammatory response in the suprarenal aorta that is largely macrophage and T-cell-driven. It is still unclear whether the elevation in GZMB immunopositivity is a result or part of the cause of this inflammation; nonetheless, we propose that GZMB's role in AAA pathogenesis is two-fold: 1. GZMB-mediated cleavage of fibrillin-1 contributes to destruction of elastic lamellae, and 2. GZMB-mediated cleavage of decorin impairs the ability of the adventitia to respond to and compensate for medial injury and that collagen fibrillogenesis in the absence of decorin results in disorganized, fibrotic adventitial remodelling that is unable to support hemodynamic force or constrain further dilation of the aorta (Figure 31, part 1-4a).

In summary, the inhibition of GZMB in the aneurysmal wall as a potential treatment strategy could preserve fibrillin-1 and decorin content. Collagen fibrillogenesis following medial injury in the presence of sufficient decorin will promote healthier adventitial remodelling that retains more of its original tensile strength and thus, be better able to constrain dilation and prevent rupture of the aorta (Figure 31, part 1-4b).

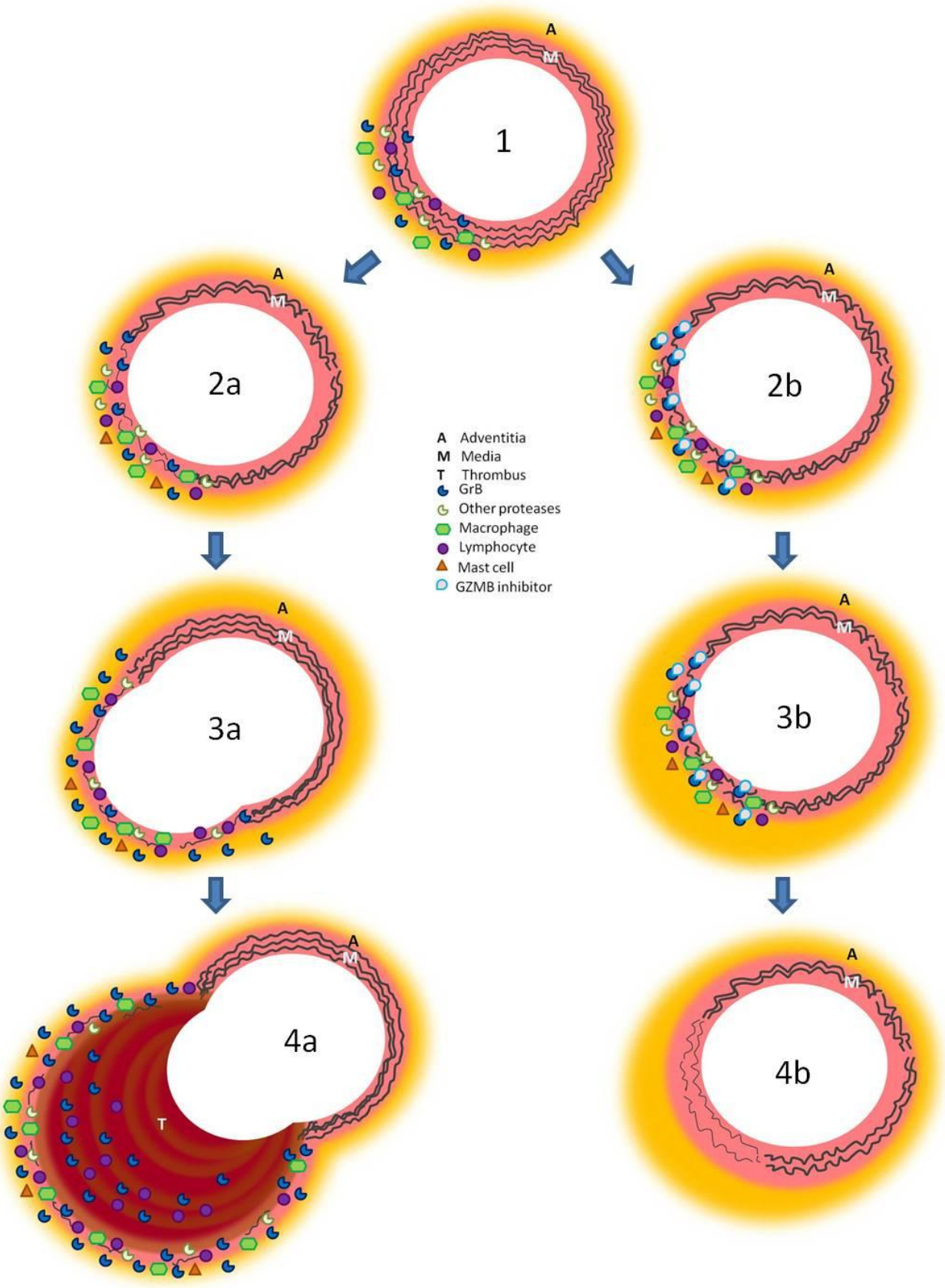

A
M
1
2a
2b
3a
3b
4a
4b
T
A Adventitia
M Media
T Thrombus
GrB
Other proteases
Macrophage
Lymphocyte
Mast cell
GZMB inhibitor

FIGURE 31: CURRENT PERSPECTIVE ON THE EXTRACELLULAR ROLE OF GZMB IN AAA

1) Inflammation and the presence of endogenous and infiltrating macrophages and leukocytes results in elevated protease activity in the aortic wall. **2a)** Protease (eg. MMPs, cathepsins, GZMB etc.) activity leads to destruction of elastic lamellae either via direct degradation of elastin (eg. MMPs) or degradation of microfibril components (eg. GZMB cleavage of fibrillin-1). **3a)** Inflammation advances, mast cells infiltrate the aneurysmal adventitia; GZMB levels remain elevated and continue degradation of vascular wall components (eg. decorin). Elastin loss confers larger percentage of tensile load on adventitial collagen and subsequent decrease in tensile strength causes dilation of aorta while release of TGF-β and other growth factors triggers collagen remodelling. **4a)** Localized dilation of the aorta and abnormal blood flow allow for the formation of laminated thrombus. Collagen remodelling in the absence of sufficient decorin results in disorganized adventitial collagen with poor density and abnormal architecture that has insufficient tensile strength to restrain further dilation. Eventual loss of structural integrity leads to aortic rupture. **2b)** Inhibition of GZMB in the inflammatory milieu lessens the degradation of the elastic microfibrils and subsequent loss of tensility. **3b)** GZMB inhibition prevents degradation of decorin and collagen remodelling in the presence of decorin promotes healthier, organized collagen architecture with regular fibril diameter and spacing that may provide sufficient tensile strength to constrain dilation. **4b)** Maintenance of adventitial integrity allows for re-endothelialization of the aorta and the re-formation of elastin fibres in the tunica media.

REFERENCES

1. Edmonds LD, James LM: Temporal trends in the birth prevalence of selected congenital malformations in the Birth Defects Monitoring Program/Commission on Professional and Hospital Activities, 1979-1989, Teratology 1993, 48:647-649

2. Manasek FJ: Embryonic development of the heart. I. A light and electron microscopic study of myocardial development in the early chick embryo, J Morphol 1968, 125:329-365

3. Goor DA, Dische R, Lillehei CW: The conotruncus. I. Its normal inversion and conus absorption, Circulation 1972, 46:375-384

4. Ho SY, Sheppard MN: The aorta:embryology, anatomy and pathology. Edited by Boudoulas H, Stefanadis C. New York, Informa Healthcare USA, Inc., 2009, p. pp. 6-21

5. Vollebergh FE, Becker AE: Minor congenital variations of cusp size in tricuspid aortic valves. Possible link with isolated aortic stenosis, Br Heart J 1977, 39:1006-1011

6. Ruddy JM, Jones JA, Spinale FG, Ikonomidis JS: Regional heterogeneity within the aorta: relevance to aneurysm disease, J Thorac Cardiovasc Surg 2008, 136:1123-1130

7. Wolinsky H, Glagov S: Comparison of abdominal and thoracic aortic medial structure in mammals. Deviation of man from the usual pattern, Circ Res 1969, 25:677-686

8. Wolinsky H: Comparison of medial growth of human thoracic and abdominal aortas, Circ Res 1970, 27:531-538

9. Halloran BG, Davis VA, McManus BM, Lynch TG, Baxter BT: Localization of aortic disease is associated with intrinsic differences in aortic structure, J Surg Res 1995, 59:17-22

10. Hashimoto J, O'Rourke MF: Basic concepts and regulation of aortic function. Edited by Boudoulas H, Stefanadis C. New York, Informa Healthcare USA, Inc., 2009, p. pp. 22-30

11. O'Rourke MF: Steady and pulsatile energy losses in the systemic circulation under normal conditions and in simulated arterial disease, Cardiovasc Res 1967, 1:313-326

12. Hales S: Haemastaticks. Edited by New York, Hafner Publishing, 1964

13. O'Rourke MF: Arterial function in health and disease. Edited by Edinburgh, UK, Churchill Livingstone, 1982

14. McDonald DA: The relation of pulsatile pressure to flow in arteries, J Physiol 1955, 127:533-552

15. McDonald DA, Taylor MG: The hydrodynamics of the arterial circulation, Prog Biophys Biophys Chem 1959, 9:107-173

16. Harkness ML, Harkness RD, McDonald DA: The collagen and elastin content of the arterial wall in the dog, Proc R Soc Lond B Biol Sci 1957, 146:541-551

17. Dobrin PB, Canfield TR: Elastase, collagenase, and the biaxial elastic properties of dog carotid artery, Am J Physiol 1984, 247:H124-131

18. Armentano RL, Levenson J, Barra JG, Fischer EI, Breitbart GJ, Pichel RH, Simon A: Assessment of elastin and collagen contribution to aortic elasticity in conscious dogs, Am J Physiol 1991, 260:H1870-1877

19. Vorp DA, Schiro BJ, Ehrlich MP, Juvonen TS, Ergin MA, Griffith BP: Effect of aneurysm on the tensile strength and biomechanical behavior of the ascending thoracic aorta, Ann Thorac Surg 2003, 75:1210-1214

20. He CM, Roach MR: The composition and mechanical properties of abdominal aortic aneurysms, J Vasc Surg 1994, 20:6-13

21. Nichols WW, McDonald DA: Wave-velocity in the proximal aorta, Med Biol Eng 1972, 10:327-335

22. Latham RD, Westerhof N, Sipkema P, Rubal BJ, Reuderink P, Murgo JP: Regional wave travel and reflections along the human aorta: a study with six simultaneous micromanometric pressures, Circulation 1985, 72:1257-1269

23. Latham RD, Rubal BJ, Westerhof N, Sipkema P, Walsh RA: Nonhuman primate model for regional wave travel and reflections along aortas, Am J Physiol 1987, 253:H299-306

24. Lanne T, Stale H, Bengtsson H, Gustafsson D, Bergqvist D, Sonesson B, Lecerof H, Dahl P: Noninvasive measurement of diameter changes in the distal abdominal aorta in man, Ultrasound Med Biol 1992, 18:451-457

25. Liapis CD, Kakisis JD: Abdominal aortic aneurysm: pathophysiology, clinical presentation, diagnostic evaluation and management. Edited by Boudoulas H, Stefanadis C. New York, Informa Healthcare USA, Inc., 2009, p. 214-227

26. Anidjar S, Dobrin PB, Eichorst M, Graham GP, Chejfec G: Correlation of inflammatory infiltrate with the enlargement of experimental aortic aneurysms, J Vasc Surg 1992, 16:139-147

27. Baxter BT, Terrin MC, Dalman RL: Medical management of small abdominal aortic aneurysms, Circulation 2008, 117:1883-1889

28. Lloyd-Jones D, Adams R, Carnethon M, De Simone G, Ferguson TB, Flegal K, Ford E, Furie K, Go A, Greenlund K, Haase N, Hailpern S, Ho M, Howard V, Kissela B, Kittner S, Lackland D, Lisabeth L, Marelli A, McDermott M, Meigs J, Mozaffarian D, Nichol G, O'Donnell C, Roger V, Rosamond W, Sacco R, Sorlie P, Stafford R, Steinberger J, Thom T, Wasserthiel-Smoller S, Wong N, Wylie-Rosett J, Hong Y: Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee, Circulation 2009, 119:480-486

29. Heller JA, Weinberg A, Arons R, Krishnasastry KV, Lyon RT, Deitch JS, Schulick AH, Bush HL, Jr., Kent KC: Two decades of abdominal aortic aneurysm repair: have we made any progress?, J Vasc Surg 2000, 32:1091-1100

30. Schermerhorn ML, O'Malley AJ, Jhaveri A, Cotterill P, Pomposelli F, Landon BE: Endovascular vs. open repair of abdominal aortic aneurysms in the Medicare population, N Engl J Med 2008, 358:464-474

31. Johansson G, Swedenborg J: Little impact of elective surgery on the incidence and mortality of ruptured aortic aneurysms, Eur J Vasc Surg 1994, 489-493

32. Clifton MA: Familial abdominal aortic aneurysms, Br J Surg 1977, 64:765-766

33. van Vlijmen-van Keulen CJ, Pals G, Rauwerda JA: Familial abdominal aortic aneurysm: a systematic review of a genetic background, Eur J Vasc Endovasc Surg 2002, 24:105-116

34. Ogata T, MacKean GL, Cole CW, Arthur C, Andreou P, Tromp G, Kuivaniemi H: The lifetime prevalence of abdominal aortic aneurysms among siblings of aneurysm patients is eightfold higher than among siblings of spouses: an analysis of 187 aneurysm families in Nova Scotia, Canada, J Vasc Surg 2005, 42:891-897

35. Rossaak JI, Hill TM, Jones GT, Phillips LV, Harris EL, van Rij AM: Familial abdominal aortic aneurysms in the Otago region of New Zealand, Cardiovasc Surg 2001, 9:241-248

36. Tilson MD, Seashore MR: Fifty families with abdominal aortic aneurysms in two or more first-order relatives, Am J Surg 1984, 147:551-553

37. Majumder PP, St Jean PL, Ferrell RE, Webster MW, Steed DL: On the inheritance of abdominal aortic aneurysm, Am J Hum Genet 1991, 48:164-170

38. Verloes A, Sakalihasan N, Koulischer L, Limet R: Aneurysms of the abdominal aorta: familial and genetic aspects in three hundred thirteen pedigrees, J Vasc Surg 1995, 21:646-655

39. Kuivaniemi H, Shibamura H, Arthur C, Berguer R, Cole CW, Juvonen T, Kline RA, Limet R, Mackean G, Norrgard O, Pals G, Powell JT, Rainio P, Sakalihasan N, van Vlijmen-van Keulen C, Verloes A, Tromp G: Familial abdominal aortic aneurysms: collection of 233 multiplex families, J Vasc Surg 2003, 37:340-345

40. Ailawadi G, Eliason JL, Upchurch GR, Jr.: Current concepts in the pathogenesis of abdominal aortic aneurysm, J Vasc Surg 2003, 38:584-588

41. Van Vlijmen-Van Keulen CJ, Rauwerda JA, Pals G: Genome-wide linkage in three Dutch families maps a locus for abdominal aortic aneurysms to chromosome 19q13.3, Eur J Vasc Endovasc Surg 2005, 30:29-35

42. Golledge J, Tsao PS, Dalman RL, Norman PE: Circulating markers of abdominal aortic aneurysm presence and progression, Circulation 2008, 118:2382-2392

43. Hellenthal FA, Buurman WA, Wodzig WK, Schurink GW: Biomarkers of AAA progression. Part 1: extracellular matrix degeneration, Nat Rev Cardiol 2009, 6:464-474

44. Hellenthal FA, Buurman WA, Wodzig WK, Schurink GW: Biomarkers of abdominal aortic aneurysm progression. Part 2: inflammation, Nat Rev Cardiol 2009, 6:543-552

45. Johnston KW, Rutherford RB, Tilson MD, Shah DM, Hollier L, Stanley JC: Suggested standards for reporting on arterial aneurysms. Subcommittee on Reporting Standards for Arterial Aneurysms, Ad Hoc Committee on Reporting Standards, Society for Vascular Surgery and North American Chapter, International Society for Cardiovascular Surgery, J Vasc Surg 1991, 13:452-458

46. Annambhotla S, Bourgeois S, Wang X, Lin PH, Yao Q, Chen C: Recent advances in molecular mechanisms of abdominal aortic aneurysm formation, World J Surg 2008, 32:976-986

47. Alcorn HG, Wolfson SK, Jr., Sutton-Tyrrell K, Kuller LH, O'Leary D: Risk factors for abdominal aortic aneurysms in older adults enrolled in The Cardiovascular Health Study, Arterioscler Thromb Vasc Biol 1996, 16:963-970

48. Shantikumar S, Ajjan R, Porter KE, Scott DJ: Diabetes and the abdominal aortic aneurysm, Eur J Vasc Endovasc Surg 2010, 39:200-207

49. Abdul-Hussien H, Soekhoe RG, Weber E, von der Thusen JH, Kleemann R, Mulder A, van Bockel JH, Hanemaaijer R, Lindeman JH: Collagen degradation in the abdominal aneurysm: a conspiracy of matrix metalloproteinase and cysteine collagenases, Am J Pathol 2007, 170:809-817

50. Fontaine V, Jacob MP, Houard X, Rossignol P, Plissonnier D, Angles-Cano E, Michel JB: Involvement of the mural thrombus as a site of protease release and activation in human aortic aneurysms, Am J Pathol 2002, 161:1701-1710

51. Thompson RW, Geraghty PJ, Lee JK: Abdominal aortic aneurysms: basic mechanisms and clinical implications, Curr Probl Surg 2002, 39:110-230

52. Vorp DA, Raghavan ML, Webster MW: Mechanical wall stress in abdominal aortic aneurysm: influence of diameter and asymmetry, J Vasc Surg 1998, 27:632-639

53. Koch AE, Kunkel SL, Pearce WH, Shah MR, Parikh D, Evanoff HL, Haines GK, Burdick MD, Strieter RM: Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemoattractant protein-1 in human abdominal aortic aneurysms, Am J Pathol 1993, 142:1423-1431

54. Newman KM, Jean-Claude J, Li H, Ramey WG, Tilson MD: Cytokines that activate proteolysis are increased in abdominal aortic aneurysms, Circulation 1994, 90:II224-227

55. Lopez-Candales A, Holmes DR, Liao S, Scott MJ, Wickline SA, Thompson RW: Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms, Am J Pathol 1997, 150:993-1007

56. Thompson RW, Liao S, Curci JA: Vascular smooth muscle cell apoptosis in abdominal aortic aneurysms, Coron Artery Dis 1997, 8:623-631

57. Henderson EL, Geng YJ, Sukhova GK, Whittemore AD, Knox J, Libby P: Death of smooth muscle cells and expression of mediators of apoptosis by T lymphocytes in human abdominal aortic aneurysms, Circulation 1999, 99:96-104

58. Liao S, Curci JA, Kelley BJ, Sicard GA, Thompson RW: Accelerated replicative senescence of medial smooth muscle cells derived from abdominal aortic aneurysms compared to the adjacent inferior mesenteric artery, J Surg Res 2000, 92:85-95

59. Mower WR, Quinones WJ, Gambhir SS: Effect of intraluminal thrombus on abdominal aortic aneurysm wall stress, J Vasc Surg 1997, 26:602-608

60. Di Martino E, Mantero S, Inzoli F, Melissano G, Astore D, Chiesa R, Fumero R: Biomechanics of abdominal aortic aneurysm in the presence of endoluminal thrombus: experimental characterisation and structural static computational analysis, Eur J Vasc Endovasc Surg 1998, 15:290-299

61. Vorp DA, Wang DH, Webster MW, Federspiel WJ: Effect of intraluminal thrombus thickness and bulge diameter on the oxygen diffusion in abdominal aortic aneurysm, J Biomech Eng 1998, 120:579-583

62. Wolf YG, Thomas WS, Brennan FJ, Goff WG, Sise MJ, Bernstein EF: Computed tomography scanning findings associated with rapid expansion of abdominal aortic aneurysms, J Vasc Surg 1994, 20:529-535; discussion 535-528

63. Vorp DA, Lee PC, Wang DH, Makaroun MS, Nemoto EM, Ogawa S, Webster MW: Association of intraluminal thrombus in abdominal aortic aneurysm with local hypoxia and wall weakening, J Vasc Surg 2001, 34:291-299

64. Sakalihasan N, Delvenne P, Nusgens BV, Limet R, Lapiere CM: Activated forms of MMP2 and MMP9 in abdominal aortic aneurysms, J Vasc Surg 1996, 24:127-133

65. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P: The Extracellular Matrix of Animals. Edited by New York, Garland Science, 2002

66. Hynes RO: The extracellular matrix: not just pretty fibrils, Science 2009, 326:1216-1219

67. Macri L, Silverstein D, Clark RA: Growth factor binding to the pericellular matrix and its importance in tissue engineering, Adv Drug Deliv Rev 2007, 59:1366-1381

68. Imai K, Hiramatsu A, Fukushima D, Pierschbacher MD, Okada Y: Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release, Biochem J 1997, 322 (Pt 3):809-814

69. Mohammadi M, Olsen SK, Goetz R: A protein canyon in the FGF-FGF receptor dimer selects from an a la carte menu of heparan sulfate motifs, Curr Opin Struct Biol 2005, 15:506-516

70. Wijelath ES, Rahman S, Namekata M, Murray J, Nishimura T, Mostafavi-Pour Z, Patel Y, Suda Y, Humphries MJ, Sobel M: Heparin-II domain of fibronectin is a vascular endothelial growth factor-binding domain: enhancement of VEGF biological activity by a singular growth factor/matrix protein synergism, Circ Res 2006, 99:853-860

71. Ishitsuka T, Ikuta T, Ariga H, Matsumoto K: Serum tenascin-X strongly binds to vascular endothelial growth factor, Biol Pharm Bull 2009, 32:1004-1011

72. Ramirez F: Pathophysiology of the microfibril/elastic fiber system: introduction, Matrix Biol 2000, 19:455-456

73. Hein S, Schaper J: The extracellular matrix in normal and diseased myocardium, J Nucl Cardiol 2001, 8:188-196

74. Charbonneau NL, Dzamba BJ, Ono RN, Keene DR, Corson GM, Reinhardt DP, Sakai LY: Fibrillins can co-assemble in fibrils, but fibrillin fibril composition displays cell-specific differences, J Biol Chem 2003, 278:2740-2749

75. Sakai LY, Keene DR, Engvall E: Fibrillin, a new 350-kD glycoprotein, is a component of extracellular microfibrils, J Cell Biol 1986, 103:2499-2509

76. Bax DV, Bernard SE, Lomas A, Morgan A, Humphries J, Shuttleworth CA, Humphries MJ, Kielty CM: Cell adhesion to fibrillin-1 molecules and microfibrils is mediated by alpha 5 beta 1 and alpha v beta 3 integrins, J Biol Chem 2003, 278:34605-34616

77. Pfaff M, Reinhardt DP, Sakai LY, Timpl R: Cell adhesion and integrin binding to recombinant human fibrillin-1, FEBS Lett 1996, 384:247-250

78. Weber E, Rossi A, Solito R, Sacchi G, Agliano M, Gerli R: Focal adhesion molecules expression and fibrillin deposition by lymphatic and blood vessel endothelial cells in culture, Microvasc Res 2002, 64:47-55

79. Robinson PN, Booms P: The molecular pathogenesis of the Marfan syndrome, Cell Mol Life Sci 2001, 58:1698-1707

80. Chaudhry SS, Cain SA, Morgan A, Dallas SL, Shuttleworth CA, Kielty CM: Fibrillin-1 regulates the bioavailability of TGFbeta1, J Cell Biol 2007, 176:355-367

81. Dobrin PB, Baker WH, Gley WC: Elastolytic and collagenolytic studies of arteries. Implications for the mechanical properties of aneurysms, Arch Surg 1984, 119:405-409

82. Prockop DJ, Kivirikko KI: Collagens: molecular biology, diseases, and potentials for therapy, Annu Rev Biochem 1995, 64:403-434

83. Engel J, Prockop DJ: The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper, Annu Rev Biophys Biophys Chem 1991, 20:137-152

84. Kielty CM, Hopkinson I, Grant ME: Collage: the collage family: structure, assembly, and organization in the extracellular matrix. Edited by Royce PM, Steinmann B. New York, Wiley-Liss Inc., 1993, p. pp. 103-147

85. Takechi H, Hirayoshi K, Nakai A, Kudo H, Saga S, Nagata K: Molecular cloning of a mouse 47-kDa heat-shock protein (HSP47), a collagen-binding stress protein, and its expression during the differentiation of F9 teratocarcinoma cells, Eur J Biochem 1992, 206:323-329

86. Thieszen SL, Rosenquist TH: Expression of collagens and decorin during aortic arch artery development: implications for matrix pattern formation, Matrix Biol 1995, 14:573-582

87. Hocking AM, Shinomura T, McQuillan DJ: Leucine-rich repeat glycoproteins of the extracellular matrix, Matrix Biol 1998, 17:1-19

88. Reed CC, Iozzo RV: The role of decorin in collagen fibrillogenesis and skin homeostasis, Glycoconj J 2002, 19:249-255

89. Bhide VM, Laschinger CA, Arora PD, Lee W, Hakkinen L, Larjava H, Sodek J, McCulloch CA: Collagen phagocytosis by fibroblasts is regulated by decorin, J Biol Chem 2005, 280:23103-23113

90. Kinsella MG, Bressler SL, Wight TN: The regulated synthesis of versican, decorin, and biglycan: extracellular matrix proteoglycans that influence cellular phenotype, Crit Rev Eukaryot Gene Expr 2004, 14:203-234

91. Fischer JW, Kinsella MG, Levkau B, Clowes AW, Wight TN: Retroviral overexpression of decorin differentially affects the response of arterial smooth muscle cells to growth factors, Arterioscler Thromb Vasc Biol 2001, 21:777-784

92. Moscatello DK, Santra M, Mann DM, McQuillan DJ, Wong AJ, Iozzo RV: Decorin suppresses tumor cell growth by activating the epidermal growth factor receptor, J Clin Invest 1998, 101:406-412

93. Seidler DG, Goldoni S, Agnew C, Cardi C, Thakur ML, Owens RT, McQuillan DJ, Iozzo RV: Decorin protein core inhibits in vivo cancer growth and metabolism by hindering epidermal growth factor receptor function and triggering apoptosis via caspase-3 activation, J Biol Chem 2006, 281:26408-26418

94. Schonherr E, Sunderkotter C, Iozzo RV, Schaefer L: Decorin, a novel player in the insulin-like growth factor system, J Biol Chem 2005, 280:15767-15772

95. Iozzo RV: Matrix proteoglycans: from molecular design to cellular function, Annu Rev Biochem 1998, 67:609-652

96. Pins GD, Christiansen DL, Patel R, Silver FH: Self-assembly of collagen fibers. Influence of fibrillar alignment and decorin on mechanical properties, Biophys J 1997, 73:2164-2172

97. Orgel JP, Eid A, Antipova O, Bella J, Scott JE: Decorin core protein (decoron) shape complements collagen fibril surface structure and mediates its binding, PLoS One 2009, 4:e7028

98. Boivin WA, Shackleford M, Vanden Hoek A, Zhao H, Hackett TL, Knight DA, Granville DJ: Granzyme B cleaves decorin, biglycan and soluble betaglycan, releasing active transforming growth factor-beta1, PLoS One 2012, 7:e33163

99. Al Haj Zen A, Lafont A, Durand E, Brasselet C, Lemarchand P, Godeau G, Gogly B: Effect of adenovirus-mediated overexpression of decorin on metalloproteinases, tissue inhibitors of metalloproteinases and cytokines secretion by human gingival fibroblasts, Matrix Biol 2003, 22:251-258

100. Hendel A, Ang LS, Granville DJ: Inflammaging and Proteases in Abdominal Aortic Aneurysm, Curr Vasc Pharmacol 2012, epub.

101. Vincenti MP, Brinckerhoff CE: Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors, Arthritis Res 2002, 4:157-164

102. Naito K, Takahashi M, Kushida K, Suzuki M, Ohishi T, Miura M, Inoue T, Nagano A: Measurement of matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in patients with knee osteoarthritis: comparison with generalized osteoarthritis, Rheumatology (Oxford) 1999, 38:510-515

103. Bolton CE, Stone MD, Edwards PH, Duckers JM, Evans WD, Shale DJ: Circulating matrix metalloproteinase-9 and osteoporosis in patients with chronic obstructive pulmonary disease, Chron Respir Dis 2009, 6:81-87

104. Deryugina EI, Quigley JP: Matrix metalloproteinases and tumor metastasis, Cancer Metastasis Rev 2006, 25:9-34

105. Senior RM, Griffin GL, Fliszar CJ, Shapiro SD, Goldberg GI, Welgus HG: Human 92- and 72-kilodalton type IV collagenases are elastases, J Biol Chem 1991, 266:7870-7875

106. Kadoglou NP, Liapis CD: Matrix metalloproteinases: contribution to pathogenesis, diagnosis, surveillance and treatment of abdominal aortic aneurysms, Curr Med Res Opin 2004, 20:419-432

107. Thompson RW, Holmes DR, Mertens RA, Liao S, Botney MD, Mecham RP, Welgus HG, Parks WC: Production and localization of 92-kilodalton gelatinase in abdominal aortic aneurysms. An elastolytic metalloproteinase expressed by aneurysm-infiltrating macrophages, J Clin Invest 1995, 96:318-326

108. Tung WS, Lee JK, Thompson RW: Simultaneous analysis of 1176 gene products in normal human aorta and abdominal aortic aneurysms using a membrane-based complementary DNA expression array, J Vasc Surg 2001, 34:143-150

109. Annabi B, Shedid D, Ghosn P, Kenigsberg RL, Desrosiers RR, Bojanowski MW, Beaulieu E, Nassif E, Moumdjian R, Beliveau R: Differential regulation of matrix metalloproteinase activities in abdominal aortic aneurysms, J Vasc Surg 2002, 35:539-546

110. Wilson WR, Anderton M, Schwalbe EC, Jones JL, Furness PN, Bell PR, Thompson MM: Matrix metalloproteinase-8 and -9 are increased at the site of abdominal aortic aneurysm rupture, Circulation 2006, 113:438-445

111. Petersen E, Wagberg F, Angquist KA: Proteolysis of the abdominal aortic aneurysm wall and the association with rupture, Eur J Vasc Endovasc Surg 2002, 23:153-157

112. Ogata Y, Pratta MA, Nagase H, Arner EC: Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) is induced in rabbit articular chondrocytes by cotreatment with interleukin 1 beta and a protein kinase C activator, Exp Cell Res 1992, 201:245-249

113. McMillan WD, Pearce WH: Increased plasma levels of metalloproteinase-9 are associated with abdominal aortic aneurysms, J Vasc Surg 1999, 29:122-127; discussion 127-129

114. Sangiorgi G, Trimarchi S, Mauriello A, Righini P, Bossone E, Suzuki T, Rampoldi V, Eagle KA: Plasma levels of metalloproteinases-9 and -2 in the acute and subacute phases of type A and type B aortic dissection, J Cardiovasc Med (Hagerstown) 2006, 7:307-315

115. Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, Powell JT: Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm, Arterioscler Thromb Vasc Biol 1995, 15:1145-1151

116. McMillan WD, Patterson BK, Keen RR, Shively VP, Cipollone M, Pearce WH: In situ localization and quantification of mRNA for 92-kD type IV collagenase and its inhibitor in aneurysmal, occlusive, and normal aorta, Arterioscler Thromb Vasc Biol 1995, 15:1139-1144

117. Newman KM, Jean-Claude J, Li H, Scholes JV, Ogata Y, Nagase H, Tilson MD: Cellular localization of matrix metalloproteinases in the abdominal aortic aneurysm wall, J Vasc Surg 1994, 20:814-820

118. Patel MI, Melrose J, Ghosh P, Appleberg M: Increased synthesis of matrix metalloproteinases by aortic smooth muscle cells is implicated in the etiopathogenesis of abdominal aortic aneurysms, J Vasc Surg 1996, 24:82-92

119. Pyo R, Lee JK, Shipley JM, Curci JA, Mao D, Ziporin SJ, Ennis TL, Shapiro SD, Senior RM, Thompson RW: Targeted gene disruption of matrix metalloproteinase-9 (gelatinase B) suppresses development of experimental abdominal aortic aneurysms, J Clin Invest 2000, 105:1641-1649

120. Longo GM, Xiong W, Greiner TC, Zhao Y, Fiotti N, Baxter BT: Matrix metalloproteinases 2 and 9 work in concert to produce aortic aneurysms, J Clin Invest 2002, 110:625-632

121. Lee S, Jilani SM, Nikolova GV, Carpizo D, Iruela-Arispe ML: Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors, J Cell Biol 2005, 169:681-691

122. Nishibe T, Dardik A, Kondo Y, Kudo F, Muto A, Nishi M, Nishibe M, Shigematsu H: Expression and localization of vascular endothelial growth factor in normal abdominal aorta and abdominal aortic aneurysm, Int Angiol 29:260-265

123. Kaneko H, Anzai T, Takahashi T, Kohno T, Shimoda M, Sasaki A, Shimizu H, Nagai T, Maekawa Y, Yoshimura K, Aoki H, Yoshikawa T, Okada Y, Yozu R, Ogawa S, Fukuda K: Role of vascular endothelial growth factor-A in development of abdominal aortic aneurysm, Cardiovasc Res 91:358-367

124. Choke E, Cockerill GW, Dawson J, Howe F, Wilson WR, Loftus IM, Thompson MM: Vascular endothelial growth factor enhances angiotensin II-induced aneurysm formation in apolipoprotein E-deficient mice, J Vasc Surg 52:159-166 e151

125. Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis, Genes Dev 2000, 14:163-176

126. Goumans MJ, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, ten Dijke P: Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors, Embo J 2002, 21:1743-1753

127. Jones JA, Spinale FG, Ikonomidis JS: Transforming growth factor-beta signaling in thoracic aortic aneurysm development: a paradox in pathogenesis, J Vasc Res 2009, 46:119-137

128. Neptune ER, Frischmeyer PA, Arking DE, Myers L, Bunton TE, Gayraud B, Ramirez F, Sakai LY, Dietz HC: Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome, Nat Genet 2003, 33:407-411

129. Longo GM, Buda SJ, Fiotta N, Xiong W, Griener T, Shapiro S, Baxter BT: MMP-12 has a role in abdominal aortic aneurysms in mice, Surgery 2005, 137:457-462

130. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D: Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation, Nat Genet 1997, 17:439-444

131. Lanone S, Zheng T, Zhu Z, Liu W, Lee CG, Ma B, Chen Q, Homer RJ, Wang J, Rabach LA, Rabach ME, Shipley JM, Shapiro SD, Senior RM, Elias JA: Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling, J Clin Invest 2002, 110:463-474

132. Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, Seiki M: A matrix metalloproteinase expressed on the surface of invasive tumour cells, Nature 1994, 370:61-65

133. Nollendorfs A, Greiner TC, Nagase H, Baxter BT: The expression and localization of membrane type-1 matrix metalloproteinase in human abdominal aortic aneurysms, J Vasc Surg 2001, 34:316-322

134. Sinha I, Hannawa KK, Eliason JL, Ailawadi G, Deogracias MP, Bethi S, Ford JW, Roelofs KJ, Grigoryants V, Henke PK, Stanley JC, Upchurch GR, Jr.: Early MT-1 MMP expression following elastase exposure is associated with increased cleaved MMP-2 activity in experimental rodent aortic aneurysms, Surgery 2004, 136:176-182

135. Jones JA, Ruddy JM, Bouges S, Zavadzkas JA, Brinsa TA, Stroud RE, Mukherjee R, Spinale FG, Ikonomidis JS: Alterations in membrane type-1 matrix metalloproteinase abundance after the induction of thoracic aortic aneurysm in a murine model, Am J Physiol Heart Circ Physiol 299:H114-124

136. Xiong W, Knispel R, MacTaggart J, Greiner TC, Weiss SJ, Baxter BT: Membrane-type 1 matrix metalloproteinase regulates macrophage-dependent elastolytic activity and aneurysm formation in vivo, J Biol Chem 2009, 284:1765-1771

137. Irizarry E, Newman KM, Gandhi RH, Nackman GB, Halpern V, Wishner S, Scholes JV, Tilson MD: Demonstration of interstitial collagenase in abdominal aortic aneurysm disease, J Surg Res 1993, 54:571-574

138. Tamarina NA, McMillan WD, Shively VP, Pearce WH: Expression of matrix metalloproteinases and their inhibitors in aneurysms and normal aorta, Surgery 1997, 122:264-271; discussion 271-262

139. Wilson WR, Anderton M, Choke EC, Dawson J, Loftus IM, Thompson MM: Elevated plasma MMP1 and MMP9 are associated with abdominal aortic aneurysm rupture, Eur J Vasc Endovasc Surg 2008, 35:580-584

140. Lizarbe TR, Tarin C, Gomez M, Lavin B, Aracil E, Orte LM, Zaragoza C: Nitric oxide induces the progression of abdominal aortic aneurysms through the matrix metalloproteinase inducer EMMPRIN, Am J Pathol 2009, 175:1421-1430

141. McMillan WD, Patterson BK, Keen RR, Pearce WH: In situ localization and quantification of seventy-two-kilodalton type IV collagenase in aneurysmal, occlusive, and normal aorta, J Vasc Surg 1995, 22:295-305

142. Lambert E, Dasse E, Haye B, Petitfrere E: TIMPs as multifacial proteins, Crit Rev Oncol Hematol 2004, 49:187-198

143. Allaire E, Forough R, Clowes M, Starcher B, Clowes AW: Local overexpression of TIMP-1 prevents aortic aneurysm degeneration and rupture in a rat model, J Clin Invest 1998, 102:1413-1420

144. Nakamura M, Tachieda R, Niinuma H, Ohira A, Endoh S, Hiramori K, Makita S: Circulating biochemical marker levels of collagen metabolism are abnormal in patients with abdominal aortic aneurysm, Angiology 2000, 51:385-392

145. Silence J, Collen D, Lijnen HR: Reduced atherosclerotic plaque but enhanced aneurysm formation in mice with inactivation of the tissue inhibitor of metalloproteinase-1 (TIMP-1) gene, Circ Res 2002, 90:897-903

146. Lemaitre V, Soloway PD, D'Armiento J: Increased medial degradation with pseudo-aneurysm formation in apolipoprotein E-knockout mice deficient in tissue inhibitor of metalloproteinases-1, Circulation 2003, 107:333-338

147. Eskandari MK, Vijungco JD, Flores A, Borensztajn J, Shively V, Pearce WH: Enhanced abdominal aortic aneurysm in TIMP-1-deficient mice, J Surg Res 2005, 123:289-293

148. Xiong W, Knispel R, Mactaggart J, Baxter BT: Effects of tissue inhibitor of metalloproteinase 2 deficiency on aneurysm formation, J Vasc Surg 2006, 44:1061-1066

149. Kaito K, Urayama H, Watanabe G: Doxycycline treatment in a model of early abdominal aortic aneurysm, Surg Today 2003, 33:426-433

150. Sorsa T, Ding Y, Salo T, Lauhio A, Teronen O, Ingman T, Ohtani H, Andoh N, Takeha S, Konttinen YT: Effects of tetracyclines on neutrophil, gingival, and salivary collagenases. A functional and western-blot assessment with special reference to their cellular sources in periodontal diseases, Ann N Y Acad Sci 1994, 732:112-131

151. Curci JA, Petrinec D, Liao S, Golub LM, Thompson RW: Pharmacologic suppression of experimental abdominal aortic aneurysms: acomparison of doxycycline and four chemically modified tetracyclines, J Vasc Surg 1998, 28:1082-1093

152. Curci JA, Mao D, Bohner DG, Allen BT, Rubin BG, Reilly JM, Sicard GA, Thompson RW: Preoperative treatment with doxycycline reduces aortic wall expression and activation of matrix metalloproteinases in patients with abdominal aortic aneurysms, J Vasc Surg 2000, 31:325-342

153. Boyle JR, McDermott E, Crowther M, Wills AD, Bell PR, Thompson MM: Doxycycline inhibits elastin degradation and reduces metalloproteinase activity in a model of aneurysmal disease, J Vasc Surg 1998, 27:354-361

154. Petrinec D, Liao S, Holmes DR, Reilly JM, Parks WC, Thompson RW: Doxycycline inhibition of aneurysmal degeneration in an elastase-induced rat model of abdominal aortic aneurysm: preservation of aortic elastin associated with suppressed production of 92 kD gelatinase, J Vasc Surg 1996, 23:336-346

155. Prall AK, Longo GM, Mayhan WG, Waltke EA, Fleckten B, Thompson RW, Baxter BT: Doxycycline in patients with abdominal aortic aneurysms and in mice: comparison of serum levels and effect on aneurysm growth in mice, J Vasc Surg 2002, 35:923-929

156. Lindeman JH, Abdul-Hussien H, van Bockel JH, Wolterbeek R, Kleemann R: Clinical trial of doxycycline for matrix metalloproteinase-9 inhibition in patients with an abdominal aneurysm: doxycycline selectively depletes aortic wall neutrophils and cytotoxic T cells, Circulation 2009, 119:2209-2216

157. Baxter BT, Pearce WH, Waltke EA, Littooy FN, Hallett JW, Jr., Kent KC, Upchurch GR, Jr., Chaikof EL, Mills JL, Fleckten B, Longo GM, Lee JK, Thompson RW: Prolonged administration of doxycycline in patients with small asymptomatic abdominal aortic aneurysms: report of a prospective (Phase II) multicenter study, J Vasc Surg 2002, 36:1-12

158. Mosorin M, Juvonen J, Biancari F, Satta J, Surcel HM, Leinonen M, Saikku P, Juvonen T: Use of doxycycline to decrease the growth rate of abdominal aortic aneurysms: a randomized, double-blind, placebo-controlled pilot study, J Vasc Surg 2001, 34:606-610

159. Pavlaki M, Zucker S: Matrix metalloproteinase inhibitors (MMPIs): the beginning of phase I or the termination of phase III clinical trials, Cancer Metastasis Rev 2003, 22:177-203

160. Morrison CJ, Butler GS, Rodriguez D, Overall CM: Matrix metalloproteinase proteomics: substrates, targets, and therapy, Curr Opin Cell Biol 2009, 21:645-653

161. Overall CM, Kleifeld O: Tumour microenvironment - opinion: validating matrix metalloproteinases as drug targets and anti-targets for cancer therapy, Nat Rev Cancer 2006, 6:227-239

162. Overall CM: Dilating the degradome: matrix metalloproteinase 2 (MMP-2) cuts to the heart of the matter, Biochem J 2004, 383:e5-7

163. Bramhall SR, Hallissey MT, Whiting J, Scholefield J, Tierney G, Stuart RC, Hawkins RE, McCulloch P, Maughan T, Brown PD, Baillet M, Fielding JW: Marimastat as maintenance therapy for patients with advanced gastric cancer: a randomised trial, Br J Cancer 2002, 86:1864-1870

164. Bramhall SR, Schulz J, Nemunaitis J, Brown PD, Baillet M, Buckels JA: A double-blind placebo-controlled, randomised study comparing gemcitabine and marimastat with gemcitabine and

placebo as first line therapy in patients with advanced pancreatic cancer, Br J Cancer 2002, 87:161-167

165. King J, Zhao J, Clingan P, Morris D: Randomised double blind placebo control study of adjuvant treatment with the metalloproteinase inhibitor, Marimastat in patients with inoperable colorectal hepatic metastases: significant survival advantage in patients with musculoskeletal side-effects, Anticancer Res 2003, 23:639-645

166. Fingleton B: Matrix metalloproteinase inhibitors for cancer therapy:the current situation and future prospects, Expert Opin Ther Targets 2003, 7:385-397

167. Fingleton B: MMP Inhibitor Clinical Trials - The Past, Present and Future. Edited by Edwards D, Hoyer-Hansen G, Blasi F, Sloane BF. New York, Springer, 2008, p. pp. 759-785

168. Holmes DR, Petrinec D, Wester W, Thompson RW, Reilly JM: Indomethacin prevents elastase-induced abdominal aortic aneurysms in the rat, J Surg Res 1996, 63:305-309

169. Walton LJ, Franklin IJ, Bayston T, Brown LC, Greenhalgh RM, Taylor GW, Powell JT: Inhibition of prostaglandin E2 synthesis in abdominal aortic aneurysms: implications for smooth muscle cell viability, inflammatory processes, and the expansion of abdominal aortic aneurysms, Circulation 1999, 100:48-54

170. Bellosta S, Via D, Canavesi M, Pfister P, Fumagalli R, Paoletti R, Bernini F: HMG-CoA reductase inhibitors reduce MMP-9 secretion by macrophages, Arterioscler Thromb Vasc Biol 1998, 18:1671-1678

171. Tsiara S, Elisaf M, Mikhailidis DP: Early vascular benefits of statin therapy, Curr Med Res Opin 2003, 19:540-556

172. Nagashima H, Aoka Y, Sakomura Y, Sakuta A, Aomi S, Ishizuka N, Hagiwara N, Kawana M, Kasanuki H: A 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, cerivastatin, suppresses production of matrix metalloproteinase-9 in human abdominal aortic aneurysm wall, J Vasc Surg 2002, 36:158-163

173. Evans J, Powell JT, Schwalbe E, Loftus IM, Thompson MM: Simvastatin attenuates the activity of matrix metalloprotease-9 in aneurysmal aortic tissue, Eur J Vasc Endovasc Surg 2007, 34:302-303

174. Schouten O, van Laanen JH, Boersma E, Vidakovic R, Feringa HH, Dunkelgrun M, Bax JJ, Koning J, van Urk H, Poldermans D: Statins are associated with a reduced infrarenal abdominal aortic aneurysm growth, Eur J Vasc Endovasc Surg 2006, 32:21-26

175. Sukhija R, Aronow WS, Sandhu R, Kakar P, Babu S: Mortality and size of abdominal aortic aneurysm at long-term follow-up of patients not treated surgically and treated with and without statins, Am J Cardiol 2006, 97:279-280

176. Golledge J, Muller J, Daugherty A, Norman P: Abdominal aortic aneurysm: pathogenesis and implications for management, Arterioscler Thromb Vasc Biol 2006, 26:2605-2613

177. Rizzo RJ, McCarthy WJ, Dixit SN, Lilly MP, Shively VP, Flinn WR, Yao JS: Collagen types and matrix protein content in human abdominal aortic aneurysms, J Vasc Surg 1989, 10:365-373

178. Gargiulo M, Stella A, Spina M, Faggioli G, Cenacchi G, Degani A, Guiducci G, Tonelli M, Bertoni F, D'Addato M: Content and turnover of extracellular matrix protein in human "nonspecific" and inflammatory abdominal aortic aneurysms, Eur J Vasc Surg 1993, 7:546-553

179. Antonicelli F, Bellon G, Debelle L, Hornebeck W: Elastin-elastases and inflamm-aging, Curr Top Dev Biol 2007, 79:99-155

180. Daugherty A, Cassis LA: Mouse models of abdominal aortic aneurysms, Arterioscler Thromb Vasc Biol 2004, 24:429-434

181. Curci JA, Thompson RW: "Variable induction of experimental abdominal aortic aneurysms with different preparations of porcine pancreatic elastase", J Vasc Surg 1999, 29:385

182. Rao SK, Mathrubutham M, Sherman D, Cerveira J, Cohen JR: Reduced capacity to inhibit elastase in abdominal aortic aneurysm, J Surg Res 1999, 82:24-27

183. Busuttil RW, Rinderbriecht H, Flesher A, Carmack C: Elastase activity: the role of elastase in aortic aneurysm formation, J Surg Res 1982, 32:214-217

184. Cohen JR, Mandell C, Chang JB, Wise L: Elastin metabolism of the infrarenal aorta, J Vasc Surg 1988, 7:210-214

185. Ahmed KS, Frank F, Gordon M: Elastase and Collagenase Activities in the Wall of the Abdominal Aortic Aneurysm, Vasc Endovascular Surg 1994, 28:311-317

186. Cohen JR, Mandell C, Wise L: Characterization of human aortic elastase found in patients with abdominal aortic aneurysms, Surg Gynecol Obstet 1987, 165:301-304

187. Cohen JR, Keegan L, Sarfati I, Danna D, Ilardi C, Wise L: Neutrophil chemotaxis and neutrophil elastase in the aortic wall in patients with abdominal aortic aneurysms, J Invest Surg 1991, 4:423-430

188. Folkesson M, Silveira A, Eriksson P, Swedenborg J: Protease activity in the multi-layered intra-luminal thrombus of abdominal aortic aneurysms, Atherosclerosis 2011, 218:294-299

189. Eliason JL, Hannawa KK, Ailawadi G, Sinha I, Ford JW, Deogracias MP, Roelofs KJ, Woodrum DT, Ennis TL, Henke PK, Stanley JC, Thompson RW, Upchurch GR, Jr.: Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation, Circulation 2005, 112:232-240

190. Cohen JR, Faust G, Tenenbaum N, Sarfati I, Rogowsky P, Wise L: The calcium messenger system and the kinetics of elastase release from human neutrophils in patients with abdominal aortic aneurysms, Ann Vasc Surg 1990, 4:570-574

191. Stoller JK, Aboussouan LS: Alpha1-antitrypsin deficiency, Lancet 2005, 365:2225-2236

192. Elzouki AN, Ryden Ahlgren A, Lanne T, Sonesson B, Eriksson S: Is there a relationship between abdominal aortic aneurysms and alpha1-antitrypsin deficiency (PiZ)?, Eur J Vasc Endovasc Surg 1999, 17:149-154

193. Schachner T, Golderer G, Sarg B, Lindner HH, Bonaros N, Mikuz G, Laufer G, Werner ER: The amounts of alpha 1 antitrypsin protein are reduced in the vascular wall of the acutely dissected human ascending aorta, Eur J Cardiothorac Surg 2010, 37:684-690

194. Tonelli AR, Brantly ML: Augmentation therapy in alpha-1 antitrypsin deficiency: advances and controversies, Ther Adv Respir Dis 2010, 4:289-312

195. Isenburg JC, Simionescu DT, Starcher BC, Vyavahare NR: Elastin stabilization for treatment of abdominal aortic aneurysms, Circulation 2007, 115:1729-1737

196. Chapman HA, Riese RJ, Shi GP: Emerging roles for cysteine proteases in human biology, Annu Rev Physiol 1997, 59:63-88

197. Cheng XW, Huang Z, Kuzuya M, Okumura K, Murohara T: Cysteine protease cathepsins in atherosclerosis-based vascular disease and its complications, Hypertension 2011, 58:978-986

198. Yasuda Y, Kaleta J, Bromme D: The role of cathepsins in osteoporosis and arthritis: rationale for the design of new therapeutics, Adv Drug Deliv Rev 2005, 57:973-993

199. Golovatch P, Mercer BA, Lemaitre V, Wallace A, Foronjy RF, D'Armiento J: Role for cathepsin K in emphysema in smoke-exposed guinea pigs, Exp Lung Res 2009, 35:631-645

200. Liu J, Sukhova GK, Yang JT, Sun J, Ma L, Ren A, Xu WH, Fu H, Dolganov GM, Hu C, Libby P, Shi GP: Cathepsin L expression and regulation in human abdominal aortic aneurysm, atherosclerosis, and vascular cells, Atherosclerosis 2006, 184:302-311

201. Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B, Turk D: Cysteine cathepsins: From structure, function and regulation to new frontiers, Biochim Biophys Acta 2012, 1824:68-88

202. Sukhova GK, Shi GP: Do cathepsins play a role in abdominal aortic aneurysm pathogenesis?, Ann N Y Acad Sci 2006, 1085:161-169

203. Li Z, Yasuda Y, Li W, Bogyo M, Katz N, Gordon RE, Fields GB, Bromme D: Regulation of collagenase activities of human cathepsins by glycosaminoglycans, J Biol Chem 2004, 279:5470-5479

204. Abdul-Hussien H, Hanemaaijer R, Kleemann R, Verhaaren BF, van Bockel JH, Lindeman JH: The pathophysiology of abdominal aortic aneurysm growth: corresponding and discordant inflammatory and proteolytic processes in abdominal aortic and popliteal artery aneurysms, J Vasc Surg 2011, 51:1479-1487

205. Abisi S, Burnand KG, Waltham M, Humphries J, Taylor PR, Smith A: Cysteine protease activity in the wall of abdominal aortic aneurysms, J Vasc Surg 2007, 46:1260-1266

206. Gacko M, Chyczewski L, Chrostek L: Distribution, activity and concentration of cathepsin B and cystatin C in the wall of aortic aneurysm, Pol J Pathol 1999, 50:83-86

207. Shi GP, Sukhova GK, Grubb A, Ducharme A, Rhode LH, Lee RT, Ridker PM, Libby P, Chapman HA: Cystatin C deficiency in human atherosclerosis and aortic aneurysms, J Clin Invest 1999, 104:1191-1197

208. Lindholt JS, Erlandsen EJ, Henneberg EW: Cystatin C deficiency is associated with the progression of small abdominal aortic aneurysms, Br J Surg 2001, 88:1472-1475

209. Chapman HA, Jr., Reilly JJ, Jr., Yee R, Grubb A: Identification of cystatin C, a cysteine proteinase inhibitor, as a major secretory product of human alveolar macrophages in vitro, Am Rev Respir Dis 1990, 141:698-705

210. Sukhova GK, Wang B, Libby P, Pan JH, Zhang Y, Grubb A, Fang K, Chapman HA, Shi GP: Cystatin C deficiency increases elastic lamina degradation and aortic dilatation in apolipoprotein E-null mice, Circ Res 2005, 96:368-375

211. Schulte S, Sun J, Libby P, Macfarlane L, Sun C, Lopez-Ilasaca M, Shi GP, Sukhova GK: Cystatin C deficiency promotes inflammation in angiotensin II-induced abdominal aortic aneurisms in atherosclerotic mice, Am J Pathol 2010, 177:456-463

212. Sun J, Zhang J, Lindholt JS, Sukhova GK, Liu J, He A, Abrink M, Pejler G, Stevens RL, Thompson RW, Ennis TL, Gurish MF, Libby P, Shi GP: Critical role of mast cell chymase in mouse abdominal aortic aneurysm formation, Circulation 2009, 120:973-982

213. Zhang J, Sun J, Lindholt JS, Sukhova GK, Sinnamon M, Stevens RL, Adachi R, Libby P, Thompson RW, Shi GP: Mast cell tryptase deficiency attenuates mouse abdominal aortic aneurysm formation, Circ Res 2011, 108:1316-1327

214. Pagano MB, Bartoli MA, Ennis TL, Mao D, Simmons PM, Thompson RW, Pham CT: Critical role of dipeptidyl peptidase I in neutrophil recruitment during the development of experimental abdominal aortic aneurysms, Proc Natl Acad Sci U S A 2007, 104:2855-2860

215. Sun J, Sukhova GK, Zhang J, Chen H, Sjoberg S, Libby P, Xiang M, Wang J, Peters C, Reinheckel T, Shi GP: Cathepsin L activity is essential to elastase perfusion-induced abdominal aortic aneurysms in mice, Arterioscler Thromb Vasc Biol 2011, 31:2500-2508

216. Qin Y, Cao X, Guo J, Zhang Y, Pan L, Zhang H, Li H, Tang C, Du J, Shi GP: Deficiency of cathepsin S attenuates angiotensin II-induced abdominal aortic aneurysm formation in apolipoprotein E-deficient mice, Cardiovasc Res 2012, 96:401-410

217. Bai L, Beckers L, Wijnands E, Lutgens SP, Herias MV, Saftig P, Daemen MJ, Cleutjens K, Lutgens E, Biessen EA, Heeneman S: Cathepsin K gene disruption does not affect murine aneurysm formation, Atherosclerosis 2010, 209:96-103

218. Sun J, Sukhova GK, Zhang J, Chen H, Sjoberg S, Libby P, Xia M, Xiong N, Gelb BD, Shi GP: Cathepsin k deficiency reduces elastase perfusion-induced abdominal aortic aneurysms in mice, Arterioscler Thromb Vasc Biol 2012, 32:15-23

219. Caughey GH: Mast cell tryptases and chymases in inflammation and host defense, Immunol Rev 2007, 217:141-154

220. Mayranpaa MI, Trosien JA, Fontaine V, Folkesson M, Kazi M, Eriksson P, Swedenborg J, Hedin U: Mast cells associate with neovessels in the media and adventitia of abdominal aortic aneurysms, J Vasc Surg 2009, 50:388-395; discussion 395-386

221. Tsuruda T, Kato J, Hatakeyama K, Kojima K, Yano M, Yano Y, Nakamura K, Nakamura-Uchiyama F, Matsushima Y, Imamura T, Onitsuka T, Asada Y, Nawa Y, Eto T, Kitamura K: Adventitial mast cells contribute to pathogenesis in the progression of abdominal aortic aneurysm, Circ Res 2008, 102:1368-1377

222. Sun J, Sukhova GK, Yang M, Wolters PJ, MacFarlane LA, Libby P, Sun C, Zhang Y, Liu J, Ennis TL, Knispel R, Xiong W, Thompson RW, Baxter BT, Shi GP: Mast cells modulate the pathogenesis of elastase-induced abdominal aortic aneurysms in mice, J Clin Invest 2007, 117:3359-3368

223. Schwartz LB, Irani AM, Roller K, Castells MC, Schechter NM: Quantitation of histamine, tryptase, and chymase in dispersed human T and TC mast cells, J Immunol 1987, 138:2611-2615

224. Wang Y, Shi GP: Mast cell chymase and tryptase in abdominal aortic aneurysm formation, Trends Cardiovasc Med 2012, 22:150-155

225. Pan JH, Lindholt JS, Sukhova GK, Baugh JA, Henneberg EW, Bucala R, Donnelly SC, Libby P, Metz C, Shi GP: Macrophage migration inhibitory factor is associated with aneurysmal expansion, J Vasc Surg 2003, 37:628-635

226. Pejler G, Abrink M, Ringvall M, Wernersson S: Mast cell proteases, Adv Immunol 2007, 95:167-255

227. Wang Y, Shiota N, Leskinen MJ, Lindstedt KA, Kovanen PT: Mast cell chymase inhibits smooth muscle cell growth and collagen expression in vitro: transforming growth factor-beta1-dependent and -independent effects, Arterioscler Thromb Vasc Biol 2001, 21:1928-1933

228. Kinoshita M, Okada M, Hara M, Furukawa Y, Matsumori A: Mast cell tryptase in mast cell granules enhances MCP-1 and interleukin-8 production in human endothelial cells, Arterioscler Thromb Vasc Biol 2005, 25:1858-1863

229. Sharma R, Prasad V, McCarthy ET, Savin VJ, Dileepan KN, Stechschulte DJ, Lianos E, Wiegmann T, Sharma M: Chymase increases glomerular albumin permeability via protease-activated receptor-2, Mol Cell Biochem 2007, 297:161-169

230. Martorell L, Martinez-Gonzalez J, Rodriguez C, Gentile M, Calvayrac O, Badimon L: Thrombin and protease-activated receptors (PARs) in atherothrombosis, Thromb Haemost 2008, 99:305-315

231. Sun J, Sukhova GK, Wolters PJ, Yang M, Kitamoto S, Libby P, MacFarlane LA, Mallen-St Clair J, Shi GP: Mast cells promote atherosclerosis by releasing proinflammatory cytokines, Nat Med 2007, 13:719-724

232. Tchougounova E, Lundequist A, Fajardo I, Winberg JO, Abrink M, Pejler G: A key role for mast cell chymase in the activation of pro-matrix metalloprotease-9 and pro-matrix metalloprotease-2, J Biol Chem 2005, 280:9291-9296

233. Gruber BL, Marchese MJ, Suzuki K, Schwartz LB, Okada Y, Nagase H, Ramamurthy NS: Synovial procollagenase activation by human mast cell tryptase dependence upon matrix metalloproteinase 3 activation, J Clin Invest 1989, 84:1657-1662

234. Saarinen J, Kalkkinen N, Welgus HG, Kovanen PT: Activation of human interstitial procollagenase through direct cleavage of the Leu83-Thr84 bond by mast cell chymase, J Biol Chem 1994, 269:18134-18140

235. Caughey GH, Raymond WW, Wolters PJ: Angiotensin II generation by mast cell alpha- and beta-chymases, Biochim Biophys Acta 2000, 1480:245-257

236. Daugherty A, Cassis L: Angiotensin II and abdominal aortic aneurysms, Curr Hypertens Rep 2004, 6:442-446

237. Inoue N, Muramatsu M, Jin D, Takai S, Hayashi T, Katayama H, Kitaura Y, Tamai H, Miyazaki M: Effects of chymase inhibitor on angiotensin II-induced abdominal aortic aneurysm development in apolipoprotein E-deficient mice, Atherosclerosis 2009, 204:359-364

238. Furubayashi K, Takai S, Jin D, Muramatsu M, Ibaraki T, Nishimoto M, Fukumoto H, Katsumata T, Miyazaki M: The significance of chymase in the progression of abdominal aortic aneurysms in dogs, Hypertens Res 2007, 30:349-357

239. Tsunemi K, Takai S, Nishimoto M, Jin D, Sakaguchi M, Muramatsu M, Yuda A, Sasaki S, Miyazaki M: A specific chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[[3,4-dioxo-1-pheny l-7-(2-pyridyloxy)]-2-heptyl]acetamide (NK3201), suppresses development of abdominal aortic aneurysm in hamsters, J Pharmacol Exp Ther 2004, 309:879-883

240. Pejler G, Berg L: Regulation of rat mast cell protease 1 activity. Protease inhibition is prevented by heparin proteoglycan, Eur J Biochem 1995, 233:192-199

241. Tsunemi K, Takai S, Nishimoto M, Jin D, Sakaguchi M, Muramatsu M, Yuda A, Sasaki S, Miyazaki M: A specific chymase inhibitor, 2-(5-formylamino-6-oxo-2-phenyl-1,6-dihydropyrimidine-1-yl)-N-[[3,4-dioxo- 1-phenyl-7-(2-pyridyloxy)]-2-heptyl]acetamide (NK3201), suppresses development of abdominal aortic aneurysm in hamsters, J Pharmacol Exp Ther 2004, 309:879-883

242. Hatcher VB, Oberman MS, Lazarus GS, Grayzel AI: A cytotoxic proteinase isolated from human lymphocytes, J Immunol 1978, 120:665-670

243. Simon MM, Hoschutzky H, Fruth U, Simon HG, Kramer MD: Purification and characterization of a T cell specific serine proteinase (TSP-1) from cloned cytolytic T lymphocytes, Embo J 1986, 5:3267-3274

244. Bleackley RC, Duggan B, Ehrman N, Lobe CG: Isolation of two cDNA sequences which encode cytotoxic cell proteases, FEBS Lett 1988, 234:153-159

245. Pasternack MS, Eisen HN: A novel serine esterase expressed by cytotoxic T lymphocytes, Nature 1985, 314:743-745

246. Masson D, Nabholz M, Estrade C, Tschopp J: Granules of cytolytic T-lymphocytes contain two serine esterases, Embo J 1986, 5:1595-1600

247. Brunet JF, Dosseto M, Denizot F, Mattei MG, Clark WR, Haqqi TM, Ferrier P, Nabholz M, Schmitt-Verhulst AM, Luciani MF, Golstein P: The inducible cytotoxic T-lymphocyte-associated gene transcript CTLA-1 sequence and gene localization to mouse chromosome 14, Nature 1986, 322:268-271

248. Young JD, Leong LG, Liu CC, Damiano A, Wall DA, Cohn ZA: Isolation and characterization of a serine esterase from cytolytic T cell granules, Cell 1986, 47:183-194

249. Bots M, Medema JP: Granzymes at a glance, J Cell Sci 2006, 119:5011-5014

250. Kam CM, Hudig D, Powers JC: Granzymes (lymphocyte serine proteases): characterization with natural and synthetic substrates and inhibitors, Biochim Biophys Acta 2000, 1477:307-323

251. Odake S, Kam CM, Narasimhan L, Poe M, Blake JT, Krahenbuhl O, Tschopp J, Powers JC: Human and murine cytotoxic T lymphocyte serine proteases: subsite mapping with peptide thioester substrates and inhibition of enzyme activity and cytolysis by isocoumarins, Biochemistry 1991, 30:2217-2227

252. Chowdhury D, Lieberman J: Death by a thousand cuts: granzyme pathways of programmed cell death, Annu Rev Immunol 2008, 26:389-420

253. Babichuk CK, Duggan BL, Bleackley RC: In vivo regulation of murine granzyme B gene transcription in activated primary T cells, J Biol Chem 1996, 271:16485-16493

254. Fregeau CJ, Bleackley RC: Transcription of two cytotoxic cell protease genes is under the control of different regulatory elements, Nucleic Acids Res 1991, 19:5583-5590

255. Wargnier A, Lafaurie C, Legros-Maida S, Bourge JF, Sigaux F, Sasportes M, Paul P: Down-regulation of human granzyme B expression by glucocorticoids. Dexamethasone inhibits binding to the Ikaros and AP-1 regulatory elements of the granzyme B promoter, J Biol Chem 1998, 273:35326-35331

256. Haddad P, Wargnier A, Bourge JF, Sasportes M, Paul P: A promoter element of the human serine esterase granzyme B gene controls specific transcription in activated T cells, Eur J Immunol 1993, 23:625-629

257. Russell JH, Ley TJ: Lymphocyte-mediated cytotoxicity, Annu Rev Immunol 2002, 20:323-370

258. Griffiths GM, Isaaz S: Granzymes A and B are targeted to the lytic granules of lymphocytes by the mannose-6-phosphate receptor, J Cell Biol 1993, 120:885-896

259. Smyth MJ, McGuire MJ, Thia KY: Expression of recombinant human granzyme B. A processing and activation role for dipeptidyl peptidase I, J Immunol 1995, 154:6299-6305

260. Galvin JP, Spaeny-Dekking LH, Wang B, Seth P, Hack CE, Froelich CJ: Apoptosis induced by granzyme B-glycosaminoglycan complexes: implications for granule-mediated apoptosis in vivo, J Immunol 1999, 162:5345-5350

261. Grujic M, Braga T, Lukinius A, Eloranta ML, Knight SD, Pejler G, Abrink M: Serglycin-deficient cytotoxic T lymphocytes display defective secretory granule maturation and granzyme B storage, J Biol Chem 2005, 280:33411-33418

262. Metkar SS, Wang B, Aguilar-Santelises M, Raja SM, Uhlin-Hansen L, Podack E, Trapani JA, Froelich CJ: Cytotoxic cell granule-mediated apoptosis: perforin delivers granzyme B-serglycin complexes into target cells without plasma membrane pore formation, Immunity 2002, 16:417-428

263. Bromley SK, Burack WR, Johnson KG, Somersalo K, Sims TN, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML: The immunological synapse, Annu Rev Immunol 2001, 19:375-396

264. Geiger B, Rosen D, Berke G: Spatial relationships of microtubule-organizing centers and the contact area of cytotoxic T lymphocytes and target cells, J Cell Biol 1982, 95:137-143

265. Kupfer A, Dennert G, Singer SJ: Polarization of the Golgi apparatus and the microtubule-organizing center within cloned natural killer cells bound to their targets, Proc Natl Acad Sci U S A 1983, 80:7224-7228

266. Sauer H, Pratsch L, Tschopp J, Bhakdi S, Peters R: Functional size of complement and perforin pores compared by confocal laser scanning microscopy and fluorescence microphotolysis, Biochim Biophys Acta 1991, 1063:137-146

267. Veugelers K, Motyka B, Goping IS, Shostak I, Sawchuk T, Bleackley RC: Granule-mediated killing by granzyme B and perforin requires a mannose 6-phosphate receptor and is augmented by cell surface heparan sulfate, Mol Biol Cell 2006, 17:623-633

268. Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H: Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice, Nature 1994, 369:31-37

269. Walsh CM, Matloubian M, Liu CC, Ueda R, Kurahara CG, Christensen JL, Huang MT, Young JD, Ahmed R, Clark WR: Immune function in mice lacking the perforin gene, Proc Natl Acad Sci U S A 1994, 91:10854-10858

270. Smyth MJ, Street SE, Trapani JA: Cutting edge: granzymes A and B are not essential for perforin-mediated tumor rejection, J Immunol 2003, 171:515-518

271. Smyth MJ, Thia KY, Street SE, MacGregor D, Godfrey DI, Trapani JA: Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma, J Exp Med 2000, 192:755-760

272. Adrain C, Murphy BM, Martin SJ: Molecular ordering of the caspase activation cascade initiated by the cytotoxic T lymphocyte/natural killer (CTL/NK) protease granzyme B, J Biol Chem 2005, 280:4663-4673

273. Medema JP, Toes RE, Scaffidi C, Zheng TS, Flavell RA, Melief CJ, Peter ME, Offringa R, Krammer PH: Cleavage of FLICE (caspase-8) by granzyme B during cytotoxic T lymphocyte-induced apoptosis, Eur J Immunol 1997, 27:3492-3498

274. Talanian RV, Yang X, Turbov J, Seth P, Ghayur T, Casiano CA, Orth K, Froelich CJ: Granule-mediated killing: pathways for granzyme B-initiated apoptosis, J Exp Med 1997, 186:1323-1331

275. Hengartner MO: The biochemistry of apoptosis, Nature 2000, 407:770-776

276. Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR, Bleackley RC: Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax, J Exp Med 2000, 192:1391-1402

277. Pinkoski MJ, Waterhouse NJ, Heibein JA, Wolf BB, Kuwana T, Goldstein JC, Newmeyer DD, Bleackley RC, Green DR: Granzyme B-mediated apoptosis proceeds predominantly through a Bcl-2-inhibitable mitochondrial pathway, J Biol Chem 2001, 276:12060-12067

278. Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K, Browne KA, Trapani JA: Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation, J Exp Med 2000, 192:1403-1414

279. Jiang X, Wang X: Cytochrome c promotes caspase-9 activation by inducing nucleotide binding to Apaf-1, J Biol Chem 2000, 275:31199-31203

280. Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X: Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade, Cell 1997, 91:479-489

281. Han J, Goldstein LA, Gastman BR, Froelich CJ, Yin XM, Rabinowich H: Degradation of Mcl-1 by granzyme B: implications for Bim-mediated mitochondrial apoptotic events, J Biol Chem 2004, 279:22020-22029

282. Hiebert PR, Granville DJ: Granzyme B in injury, inflammation, and repair, Trends Mol Med 2012, 18:732-741

283. Choy JC, McDonald PC, Suarez AC, Hung VH, Wilson JE, McManus BM, Granville DJ: Granzyme B in atherosclerosis and transplant vascular disease: association with cell death and atherosclerotic disease severity, Mod Pathol 2003, 16:460-470

284. Hernandez-Pigeon H, Jean C, Charruyer A, Haure MJ, Titeux M, Tonasso L, Quillet-Mary A, Baudouin C, Charveron M, Laurent G: Human keratinocytes acquire cellular cytotoxicity under UV-B irradiation. Implication of granzyme B and perforin, J Biol Chem 2006, 281:13525-13532

285. Hirst CE, Buzza MS, Sutton VR, Trapani JA, Loveland KL, Bird PI: Perforin-independent expression of granzyme B and proteinase inhibitor 9 in human testis and placenta suggests a role for granzyme B-mediated proteolysis in reproduction, Mol Hum Reprod 2001, 7:1133-1142

286. Horiuchi K, Saito S, Sasaki R, Tomatsu T, Toyama Y: Expression of granzyme B in human articular chondrocytes, J Rheumatol 2003, 30:1799-1810

287. Namekawa T, Wagner UG, Goronzy JJ, Weyand CM: Functional subsets of CD4 T cells in rheumatoid synovitis, Arthritis Rheum 1998, 41:2108-2116

288. Rissoan MC, Duhen T, Bridon JM, Bendriss-Vermare N, Peronne C, de Saint Vis B, Briere F, Bates EE: Subtractive hybridization reveals the expression of immunoglobulin-like transcript 7, Eph-B1, granzyme B, and 3 novel transcripts in human plasmacytoid dendritic cells, Blood 2002, 100:3295-3303

289. Sasson R, Dantes A, Tajima K, Amsterdam A: Novel genes modulated by FSH in normal and immortalized FSH-responsive cells: new insights into the mechanism of FSH action, Faseb J 2003, 17:1256-1266

290. Tschopp CM, Spiegl N, Didichenko S, Lutmann W, Julius P, Virchow JC, Hack CE, Dahinden CA: Granzyme B, a novel mediator of allergic inflammation: its induction and release in blood basophils and human asthma, Blood 2006, 108:2290-2299

291. Vernooy JH, Moller GM, van Suylen RJ, van Spijk MP, Cloots RH, Hoet PH, Pennings HJ, Wouters EF: Increased granzyme A expression in type II pneumocytes of patients with severe chronic obstructive pulmonary disease, Am J Respir Crit Care Med 2007, 175:464-472

292. Grossman WJ, Verbsky JW, Tollefsen BL, Kemper C, Atkinson JP, Ley TJ: Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells, Blood 2004, 104:2840-2848

293. Wagner C, Iking-Konert C, Denefleh B, Stegmaier S, Hug F, Hansch GM: Granzyme B and perforin: constitutive expression in human polymorphonuclear neutrophils, Blood 2004, 103:1099-1104

294. Saito S, Murakoshi K, Kotake S, Kamatani N, Tomatsu T: Granzyme B induces apoptosis of chondrocytes with natural killer cell-like cytotoxicity in rheumatoid arthritis, J Rheumatol 2008, 35:1932-1943

295. Froelich CJ, Pardo J, Simon MM: Granule-associated serine proteases: granzymes might not just be killer proteases, Trends Immunol 2009, 30:117-123

296. Caughey GH, Schaumberg TH, Zerweck EH, Butterfield JH, Hanson RD, Silverman GA, Ley TJ: The human mast cell chymase gene (CMA1): mapping to the cathepsin G/granzyme gene cluster and lineage-restricted expression, Genomics 1993, 15:614-620

297. Kramer MD, Simon MM: Are proteinases functional molecules of T lymphocytes, Immunol Today 1987, 8:140-142

298. Isaaz S, Baetz K, Olsen K, Podack E, Griffiths GM: Serial killing by cytotoxic T lymphocytes: T cell receptor triggers degranulation, re-filling of the lytic granules and secretion of lytic proteins via a non-granule pathway, Eur J Immunol 1995, 25:1071-1079

299. Prakash MD, Bird CH, Bird PI: Active and zymogen forms of granzyme B are constitutively released from cytotoxic lymphocytes in the absence of target cell engagement, Immunol Cell Biol 2009, 87:249-254

300. Spaeny-Dekking EH, Hanna WL, Wolbink AM, Wever PC, Kummer JA, Swaak AJ, Middeldorp JM, Huisman HG, Froelich CJ, Hack CE: Extracellular granzymes A and B in humans: detection of native species during CTL responses in vitro and in vivo, J Immunol 1998, 160:3610-3616

301. Buzza MS, Bird PI: Extracellular granzymes: current perspectives, Biol Chem 2006, 387:827-837

302. Tak PP, Spaeny-Dekking L, Kraan MC, Breedveld FC, Froelich CJ, Hack CE: The levels of soluble granzyme A and B are elevated in plasma and synovial fluid of patients with rheumatoid arthritis (RA), Clin Exp Immunol 1999, 116:366-370

303. Tak PP, Kummer JA, Hack CE, Daha MR, Smeets TJ, Erkelens GW, Meinders AE, Kluin PM, Breedveld FC: Granzyme-positive cytotoxic cells are specifically increased in early rheumatoid synovial tissue, Arthritis Rheum 1994, 37:1735-1743

304. Malmestrom C, Lycke J, Haghighi S, Andersen O, Carlsson L, Wadenvik H, Olsson B: Relapses in multiple sclerosis are associated with increased CD8+ T-cell mediated cytotoxicity in CSF, J Neuroimmunol 2008, 196:159-165

305. Takahashi Y, Mine J, Kubota Y, Yamazaki E, Fujiwara T: A substantial number of Rasmussen syndrome patients have increased IgG, CD4+ T cells, TNFalpha, and Granzyme B in CSF, Epilepsia 2009, 50:1419-1431

306. Bratke K, Bottcher B, Leeder K, Schmidt S, Kupper M, Virchow JC, Jr., Luttmann W: Increase in granzyme B+ lymphocytes and soluble granzyme B in bronchoalveolar lavage of allergen challenged patients with atopic asthma, Clin Exp Immunol 2004, 136:542-548

307. Tremblay GM, Wolbink AM, Cormier Y, Hack CE: Granzyme activity in the inflamed lung is not controlled by endogenous serine proteinase inhibitors, J Immunol 2000, 165:3966-3969

308. Kurschus FC, Kleinschmidt M, Fellows E, Dornmair K, Rudolph R, Lilie H, Jenne DE: Killing of target cells by redirected granzyme B in the absence of perforin, FEBS Lett 2004, 562:87-92

309. Rowshani AT, Strik MC, Molenaar R, Yong SL, Wolbink AM, Bemelman FJ, Hack CE, Ten Berge IJ: The granzyme B inhibitor SERPINB9 (protease inhibitor 9) circulates in blood and increases on primary cytomegalovirus infection after renal transplantation, J Infect Dis 2005, 192:1908-1911

310. Casciola-Rosen L, Miagkov A, Nagaraju K, Askin F, Jacobson L, Rosen A, Drachman DB: Granzyme B: evidence for a role in the origin of myasthenia gravis, J Neuroimmunol 2008, 201-202:33-40

311. Gahring L, Carlson NG, Meyer EL, Rogers SW: Granzyme B proteolysis of a neuronal glutamate receptor generates an autoantigen and is modulated by glycosylation, J Immunol 2001, 166:1433-1438

312. Loeb CR, Harris JL, Craik CS: Granzyme B proteolyzes receptors important to proliferation and survival, tipping the balance toward apoptosis, J Biol Chem 2006, 281:28326-28335

313. Mulligan-Kehoe MJ, Drinane MC, Mollmark J, Casciola-Rosen L, Hummers LK, Hall A, Rosen A, Wigley FM, Simons M: Antiangiogenic plasma activity in patients with systemic sclerosis, Arthritis Rheum 2007, 56:3448-3458

314. Buzza MS, Dyson JM, Choi H, Gardiner EE, Andrews RK, Kaiserman D, Mitchell CA, Berndt MC, Dong JF, Bird PI: Antihemostatic activity of human granzyme B mediated by cleavage of von Willebrand factor, J Biol Chem 2008, 283:22498-22504

315. Castellino FJ, Ploplis VA: Structure and function of the plasminogen/plasmin system, Thromb Haemost 2005, 93:647-654

316. Hanford HA, Wong CA, Kassan H, Cundiff DL, Chandel N, Underwood S, Mitchell CA, Soff GA: Angiostatin(4.5)-mediated apoptosis of vascular endothelial cells, Cancer Res 2003, 63:4275-4280

317. Ramshaw AL, Roskell DE, Parums DV: Cytokine gene expression in aortic adventitial inflammation associated with advanced atherosclerosis (chronic periaortitis), J Clin Pathol 1994, 47:721-727

318. Marculescu R, Sodeck G, Domanovits H, Hobusch G, Exner M, Heinzl H, Huber K, Mannhalter C, Minar E, Wagner O, Schillinger M: Interleukin-1 gene cluster variants and abdominal aortic aneurysms, Thromb Haemost 2005, 94:646-650

319. Sims JE, March CJ, Cosman D, Widmer MB, MacDonald HR, McMahan CJ, Grubin CE, Wignall JM, Jackson JL, Call SM, et al.: cDNA expression cloning of the IL-1 receptor, a member of the immunoglobulin superfamily, Science 1988, 241:585-589

320. Mizel SB, Mizel D: Purification to apparent homogeneity of murine interleukin 1, J Immunol 1981, 126:834-837

321. French JF, Lambert LE, Dage RC: Nitric oxide synthase inhibitors inhibit interleukin-1 beta-induced depression of vascular smooth muscle, J Pharmacol Exp Ther 1991, 259:260-264

322. Helle M, Brakenhoff JP, De Groot ER, Aarden LA: Interleukin 6 is involved in interleukin 1-induced activities, Eur J Immunol 1988, 18:957-959

323. Jones KG, Brull DJ, Brown LC, Sian M, Greenhalgh RM, Humphries SE, Powell JT: Interleukin-6 (IL-6) and the prognosis of abdominal aortic aneurysms, Circulation 2001, 103:2260-2265

324. Afonina IS, Tynan GA, Logue SE, Cullen SP, Bots M, Luthi AU, Reeves EP, McElvaney NG, Medema JP, Lavelle EC, Martin SJ: Granzyme B-dependent proteolysis acts as a switch to enhance the proinflammatory activity of IL-1alpha, Mol Cell 2011, 44:265-278

325. Froelich CJ, Zhang X, Turbov J, Hudig D, Winkler U, Hanna WL: Human granzyme B degrades aggrecan proteoglycan in matrix synthesized by chondrocytes, J Immunol 1993, 151:7161-7171

326. Buzza MS, Zamurs L, Sun J, Bird CH, Smith AI, Trapani JA, Froelich CJ, Nice EC, Bird PI: Extracellular matrix remodeling by human granzyme B via cleavage of vitronectin, fibronectin, and laminin, J Biol Chem 2005, 280:23549-23558

327. Choy JC, Hung VH, Hunter AL, Cheung PK, Motyka B, Goping IS, Sawchuk T, Bleackley RC, Podor TJ, McManus BM, Granville DJ: Granzyme B induces smooth muscle cell apoptosis in the absence of perforin: involvement of extracellular matrix degradation, Arterioscler Thromb Vasc Biol 2004, 24:2245-2250

328. Chamberlain CM, Ang LS, Boivin WA, Cooper DM, Williams SJ, Zhao H, Hendel A, Folkesson M, Swedenborg J, Allard MF, McManus BM, Granville DJ: Perforin-independent extracellular granzyme B activity contributes to abdominal aortic aneurysm, Am J Pathol 2010, 176:1038-1049

329. Ruoslahti E: RGD and other recognition sequences for integrins, Annu Rev Cell Dev Biol 1996, 12:697-715

330. Schonherr E, Witsch-Prehm P, Harrach B, Robenek H, Rauterberg J, Kresse H: Interaction of biglycan with type I collagen, J Biol Chem 1995, 270:2776-2783

331. Ramirez F, Sakai LY, Dietz HC, Rifkin DB: Fibrillin microfibrils: multipurpose extracellular networks in organismal physiology, Physiol Genomics 2004, 19:151-154

332. Barilla ML, Carsons SE: Fibronectin fragments and their role in inflammatory arthritis, Semin Arthritis Rheum 2000, 29:252-265

333. Norris DA, Clark RA, Swigart LM, Huff JC, Weston WL, Howell SE: Fibronectin fragment(s) are chemotactic for human peripheral blood monocytes, J Immunol 1982, 129:1612-1618

334. Odekon LE, Frewin MB, Del Vecchio P, Saba TM, Gudewicz PW: Fibronectin fragments released from phorbol ester-stimulated pulmonary artery endothelial cell monolayers promote neutrophil chemotaxis, Immunology 1991, 74:114-120

335. Stanton H, Ung L, Fosang AJ: The 45 kDa collagen-binding fragment of fibronectin induces matrix metalloproteinase-13 synthesis by chondrocytes and aggrecan degradation by aggrecanases, Biochem J 2002, 364:181-190

336. Ferdous Z, Wei VM, Iozzo R, Hook M, Grande-Allen KJ: Decorin-transforming growth factor-interaction regulates matrix organization and mechanical characteristics of three-dimensional collagen matrices, J Biol Chem 2007, 282:35887-35898

337. Zhang G, Ezura Y, Chervoneva I, Robinson PS, Beason DP, Carine ET, Soslowsky LJ, Iozzo RV, Birk DE: Decorin regulates assembly of collagen fibrils and acquisition of biomechanical properties during tendon development, J Cell Biochem 2006, 98:1436-1449

338. Iwasaki S, Hosaka Y, Iwasaki T, Yamamoto K, Nagayasu A, Ueda H, Kokai Y, Takehana K: The modulation of collagen fibril assembly and its structure by decorin: an electron microscopic study, Arch Histol Cytol 2008, 71:37-44

339. Fiedler LR, Eble JA: Decorin regulates endothelial cell-matrix interactions during angiogenesis, Cell Adh Migr 2009, 3:3-6

340. Theocharis AD, Karamanos NK: Decreased biglycan expression and differential decorin localization in human abdominal aortic aneurysms, Atherosclerosis 2002, 165:221-230

341. Heegaard AM, Corsi A, Danielsen CC, Nielsen KL, Jorgensen HL, Riminucci M, Young MF, Bianco P: Biglycan deficiency causes spontaneous aortic dissection and rupture in mice, Circulation 2007, 115:2731-2738

342. Tamarina NA, Grassi MA, Johnson DA, Pearce WH: Proteoglycan gene expression is decreased in abdominal aortic aneurysms, J Surg Res 1998, 74:76-80

343. Reinhardt DP, Keene DR, Corson GM, Poschl E, Bachinger HP, Gambee JE, Sakai LY: Fibrillin-1: organization in microfibrils and structural properties, J Mol Biol 1996, 258:104-116

344. Chamberlain CM: Granzyme B in abdominal aortic aneurysm and aortic dissection. Edited by Vancouver, University of British Columbia, 2009, p.

345. Dinarello CA: The IL-1 family and inflammatory diseases, Clin Exp Rheumatol 2002, 20:S1-13

346. Dinarello CA: Interleukin-1 in the pathogenesis and treatment of inflammatory diseases, Blood 2011, 117:3720-3732

347. Kittelberger R, Davis PF, Stehbens WE: Distribution of type IV collagen, laminin, nidogen and fibronectin in the haemodynamically stressed vascular wall, Histol Histopathol 1990, 5:161-167

348. Kilic T, Sohrabifar M, Kurtkaya O, Yildirim O, Elmaci I, Gunel M, Pamir MN: Expression of structural proteins and angiogenic factors in normal arterial and unruptured and ruptured aneurysm walls, Neurosurgery 2005, 57:997-1007; discussion 1997-1007

349. Adam DJ, Haggart PC, Ludlam CA, Bradbury AW: von Willebrand factor and platelet count in ruptured abdominal aortic aneurysm repair, Eur J Vasc Endovasc Surg 2003, 26:412-417

350. Lindholt JS: Activators of plasminogen and the progression of small abdominal aortic aneurysms, Ann N Y Acad Sci 2006, 1085:139-150

351. Allaire E, Hasenstab D, Kenagy RD, Starcher B, Clowes MM, Clowes AW: Prevention of aneurysm development and rupture by local overexpression of plasminogen activator inhibitor-1, Circulation 1998, 98:249-255

352. Willis AI, Sadowitz B, Fuse S, Maier KG, Lee TS, Wang XJ, Tuszynski GP, Sumpio BE, Gahtan V: Thrombospondin 1, fibronectin, and vitronectin are differentially dependent upon RAS, ERK1/2, and p38 for induction of vascular smooth muscle cell chemotaxis, Vasc Endovascular Surg 2011, 45:55-62

353. Urbonavicius S, Lindholt JS, Delbosc S, Urbonaviciene G, Henneberg EW, Vorum H, Meilhac O, Honore B: Proteins associated with the size and expansion rate of the abdominal aortic aneurysm wall as identified by proteomic analysis, Interact Cardiovasc Thorac Surg 2010, 11:433-441

354. Garg N, Goyal N, Strawn TL, Wu J, Mann KM, Lawrence DA, Fay WP: Plasminogen activator inhibitor-1 and vitronectin expression level and stoichiometry regulate vascular smooth muscle cell migration through physiological collagen matrices, J Thromb Haemost 8:1847-1854

355. Miyamoto S, Teramoto H, Gutkind JS, Yamada KM: Integrins can collaborate with growth factors for phosphorylation of receptor tyrosine kinases and MAP kinase activation: roles of integrin aggregation and occupancy of receptors, J Cell Biol 1996, 135:1633-1642

356. Sun J, Bird CH, Sutton V, McDonald L, Coughlin PB, De Jong TA, Trapani JA, Bird PI: A cytosolic granzyme B inhibitor related to the viral apoptotic regulator cytokine response modifier A is present in cytotoxic lymphocytes, J Biol Chem 1996, 271:27802-27809

357. Potempa J, Korzus E, Travis J: The serpin superfamily of proteinase inhibitors: structure, function, and regulation, J Biol Chem 1994, 269:15957-15960

358. Talanian RV, Quinlan C, Trautz S, Hackett MC, Mankovich JA, Banach D, Ghayur T, Brady KD, Wong WW: Substrate specificities of caspase family proteases, J Biol Chem 1997, 272:9677-9682

359. Bird CH, Sutton VR, Sun J, Hirst CE, Novak A, Kumar S, Trapani JA, Bird PI: Selective regulation of apoptosis: the cytotoxic lymphocyte serpin proteinase inhibitor 9 protects against granzyme B-mediated apoptosis without perturbing the Fas cell death pathway, Mol Cell Biol 1998, 18:6387-6398

360. Bladergroen BA, Strik MC, Bovenschen N, van Berkum O, Scheffer GL, Meijer CJ, Hack CE, Kummer JA: The granzyme B inhibitor, protease inhibitor 9, is mainly expressed by dendritic cells and at immune-privileged sites, J Immunol 2001, 166:3218-3225

361. Young JL, Sukhova GK, Foster D, Kisiel W, Libby P, Schonbeck U: The serpin proteinase inhibitor 9 is an endogenous inhibitor of interleukin 1beta-converting enzyme (caspase-1) activity in human vascular smooth muscle cells, J Exp Med 2000, 191:1535-1544

362. Barrie MB, Stout HW, Abougergi MS, Miller BC, Thiele DL: Antiviral cytokines induce hepatic expression of the granzyme B inhibitors, proteinase inhibitor 9 and serine proteinase inhibitor 6, J Immunol 2004, 172:6453-6459

363. Kanamori H, Krieg S, Mao C, Di Pippo VA, Wang S, Zajchowski DA, Shapiro DJ: Proteinase inhibitor 9, an inhibitor of granzyme B-mediated apoptosis, is a primary estrogen-inducible gene in human liver cells, J Biol Chem 2000, 275:5867-5873

364. Buzza MS, Hirst CE, Bird CH, Hosking P, McKendrick J, Bird PI: The granzyme B inhibitor, PI-9, is present in endothelial and mesothelial cells, suggesting that it protects bystander cells during immune responses, Cell Immunol 2001, 210:21-29

365. Bots M, L VANB, Rademaker MT, Offringa R, Medema JP: Serpins prevent granzyme-induced death in a species-specific manner, Immunol Cell Biol 2006, 84:79-86

366. Sipione S, Simmen KC, Lord SJ, Motyka B, Ewen C, Shostak I, Rayat GR, Dufour JM, Korbutt GS, Rajotte RV, Bleackley RC: Identification of a novel human granzyme B inhibitor secreted by cultured sertoli cells, J Immunol 2006, 177:5051-5058

367. Horvath AJ, Irving JA, Rossjohn J, Law RH, Bottomley SP, Quinsey NS, Pike RN, Coughlin PB, Whisstock JC: The murine orthologue of human antichymotrypsin: a structural paradigm for clade A3 serpins, J Biol Chem 2005, 280:43168-43178

368. Boivin WA, Cooper DM, Hiebert PR, Granville DJ: Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma, Lab Invest 2009, 89:1195-1220

369. Mahrus S, Kisiel W, Craik CS: Granzyme M is a regulatory protease that inactivates proteinase inhibitor 9, an endogenous inhibitor of granzyme B, J Biol Chem 2004, 279:54275-54282

370. Andrade F, Fellows E, Jenne DE, Rosen A, Young CS: Granzyme H destroys the function of critical adenoviral proteins required for viral DNA replication and granzyme B inhibition, Embo J 2007, 26:2148-2157

371. Macen JL, Garner RS, Musy PY, Brooks MA, Turner PC, Moyer RW, McFadden G, Bleackley RC: Differential inhibition of the Fas- and granule-mediated cytolysis pathways by the orthopoxvirus cytokine response modifier A/SPI-2 and SPI-1 protein, Proc Natl Acad Sci U S A 1996, 93:9108-9113

372. Quan LT, Caputo A, Bleackley RC, Pickup DJ, Salvesen GS: Granzyme B is inhibited by the cowpox virus serpin cytokine response modifier A, J Biol Chem 1995, 270:10377-10379

373. Tewari M, Telford WG, Miller RA, Dixit VM: CrmA, a poxvirus-encoded serpin, inhibits cytotoxic T-lymphocyte-mediated apoptosis, J Biol Chem 1995, 270:22705-22708

374. Turner PC, Sancho MC, Thoennes SR, Caputo A, Bleackley RC, Moyer RW: Myxoma virus Serp2 is a weak inhibitor of granzyme B and interleukin-1beta-converting enzyme in vitro and unlike CrmA cannot block apoptosis in cowpox virus-infected cells, J Virol 1999, 73:6394-6404

375. Weber C, Noels H: Atherosclerosis: current pathogenesis and therapeutic options, Nat Med 17:1410-1422

376. Hansson GK, Robertson AK, Soderberg-Naucler C: Inflammation and atherosclerosis, Annu Rev Pathol 2006, 1:297-329

377. Kim WJ, Kim H, Suk K, Lee WH: Macrophages express granzyme B in the lesion areas of atherosclerosis and rheumatoid arthritis, Immunol Lett 2007, 111:57-65

378. Daugherty A, Rateri DL: Atherosclerosis: cell biology and lipoproteins, Curr Opin Lipidol 2008, 19:328-329

379. Daugherty A, Rateri DL, Lu H: As macrophages indulge, atherosclerotic lesions bulge, Circ Res 2008, 102:1445-1447

380. Bobryshev YV, Lord RS: S-100 positive cells in human arterial intima and in atherosclerotic lesions, Cardiovasc Res 1995, 29:689-696

381. Jonasson L, Holm J, Skalli O, Bondjers G, Hansson GK: Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque, Arteriosclerosis 1986, 6:131-138

382. Kaartinen M, Penttila A, Kovanen PT: Accumulation of activated mast cells in the shoulder region of human coronary atheroma, the predilection site of atheromatous rupture, Circulation 1994, 90:1669-1678

383. Endemann DH, Schiffrin EL: Endothelial dysfunction, J Am Soc Nephrol 2004, 15:1983-1992

384. Libby P: Inflammation in atherosclerosis, Nature 2002, 420:868-874

385. Zeiher AM, Fisslthaler B, Schray-Utz B, Busse R: Nitric oxide modulates the expression of monocyte chemoattractant protein 1 in cultured human endothelial cells, Circ Res 1995, 76:980-986

386. Baba T, Ishizu A, Iwasaki S, Suzuki A, Tomaru U, Ikeda H, Yoshiki T, Kasahara M: CD4+/CD8+ macrophages infiltrating at inflammatory sites: a population of monocytes/macrophages with a cytotoxic phenotype, Blood 2006, 107:2004-2012

387. Skjelland M, Michelsen AE, Krohg-Sorensen K, Tennoe B, Dahl A, Bakke S, Brosstad F, Damas JK, Russell D, Halvorsen B, Aukrust P: Plasma levels of granzyme B are increased in patients with lipid-rich carotid plaques as determined by echogenicity, Atherosclerosis 2007, 195:e142-146

388. Tsuru R, Kondo H, Hojo Y, Gama M, Mizuno O, Katsuki T, Shimada K, Kikuchi M, Yashiro T: Increased granzyme B production from peripheral blood mononuclear cells in patients with acute coronary syndrome, Heart 2008, 94:305-310

389. Chamberlain CM, Granville DJ: The role of Granzyme B in atheromatous diseases, Can J Physiol Pharmacol 2007, 85:89-95

390. Wills A, Thompson MM, Crowther M, Brindle NP, Nasim A, Sayers RD, Bell PR: Elastase-induced matrix degradation in arterial organ cultures: an in vitro model of aneurysmal disease, J Vasc Surg 1996, 24:667-679

391. Thompson MM, Wills A, McDermott E, Crowther M, Brindle N, Bell PR: An in vitro model of aneurysmal disease: effect of leukocyte infiltration and shear stress on MMP production within the arterial wall, Ann N Y Acad Sci 1996, 800:270-273

392. Schurink GW, Aarts NJ, Wilde J, van Baalen JM, Chuter TA, Schultze Kool LJ, van Bockel JH: Endoleakage after stent-graft treatment of abdominal aneurysm: implications on pressure and imaging--an in vitro study, J Vasc Surg 1998, 28:234-241

393. Springer F, Schlierf R, Pfeffer JG, Mahnken AH, Schnakenberg U, Schmitz-Rode T: Detecting endoleaks after endovascular AAA repair with a minimally invasive, implantable, telemetric pressure sensor: an in vitro study, Eur Radiol 2007, 17:2589-2597

394. Dias NV, Ivancev K, Malina M, Hinnen JW, Visser M, Lindblad B, Sonesson B: Direct intra-aneurysm sac pressure measurement using tip-pressure sensors: in vivo and in vitro evaluation, J Vasc Surg 2004, 40:711-716

395. Andrews EJ, White WJ, Bullock LP: Spontaneous aortic aneurysms in blotchy mice, Am J Pathol 1975, 78:199-210

396. Hunt DM: Primary defect in copper transport underlies mottled mutants in the mouse, Nature 1974, 249:852-854

397. Senapati A, Carlsson LK, Fletcher CD, Browse NL, Thompson RP: Is tissue copper deficiency associated with aortic aneurysms?, Br J Surg 1985, 72:352-353

398. Jaakkola P, Hippelainen M, Kantola M: Copper and zinc concentrations of abdominal aorta and liver in patients with infrarenal abdominal aortic aneurysm or aortoiliacal occlusive disease, Ann Chir Gynaecol 1994, 83:304-308

399. Brophy CM, Tilson JE, Braverman IM, Tilson MD: Age of onset, pattern of distribution, and histology of aneurysm development in a genetically predisposed mouse model, J Vasc Surg 1988, 8:45-48

400. Carrell TW, Smith A, Burnand KG: Experimental techniques and models in the study of the development and treatment of abdominal aortic aneurysm, Br J Surg 1999, 86:305-312

401. Maki JM, Rasanen J, Tikkanen H, Sormunen R, Makikallio K, Kivirikko KI, Soininen R: Inactivation of the lysyl oxidase gene Lox leads to aortic aneurysms, cardiovascular dysfunction, and perinatal death in mice, Circulation 2002, 106:2503-2509

402. Silence J, Lupu F, Collen D, Lijnen HR: Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation, Arterioscler Thromb Vasc Biol 2001, 21:1440-1445

403. Tangirala RK, Rubin EM, Palinski W: Quantitation of atherosclerosis in murine models: correlation between lesions in the aortic origin and in the entire aorta, and differences in the extent of lesions between sexes in LDL receptor-deficient and apolipoprotein E-deficient mice, J Lipid Res 1995, 36:2320-2328

404. Prescott MF, Sawyer WK, Von Linden-Reed J, Jeune M, Chou M, Caplan SL, Jeng AY: Effect of matrix metalloproteinase inhibition on progression of atherosclerosis and aneurysm in LDL receptor-deficient mice overexpressing MMP-3, MMP-12, and MMP-13 and on restenosis in rats after balloon injury, Ann N Y Acad Sci 1999, 878:179-190

405. Fukamizu A, Sugimura K, Takimoto E, Sugiyama F, Seo MS, Takahashi S, Hatae T, Kajiwara N, Yagami K, Murakami K: Chimeric renin-angiotensin system demonstrates sustained increase in blood pressure of transgenic mice carrying both human renin and human angiotensinogen genes, J Biol Chem 1993, 268:11617-11621

406. Anidjar S, Osborne-Pellegrin M, Coutard M, Michel JB: Arterial hypertension and aneurysmal dilatation, Kidney Int Suppl 1992, 37:S61-66

407. Carsten CG, 3rd, Calton WC, Johanning JM, Armstrong PJ, Franklin DP, Carey DJ, Elmore JR: Elastase is not sufficient to induce experimental abdominal aortic aneurysms, J Vasc Surg 2001, 33:1255-1262

408. Lee JK, Borhani M, Ennis TL, Upchurch GR, Jr., Thompson RW: Experimental abdominal aortic aneurysms in mice lacking expression of inducible nitric oxide synthase, Arterioscler Thromb Vasc Biol 2001, 21:1393-1401

409. Gertz SD, Kurgan A, Eisenberg D: Aneurysm of the rabbit common carotid artery induced by periarterial application of calcium chloride in vivo, J Clin Invest 1988, 81:649-656

410. Chiou AC, Chiu B, Pearce WH: Murine aortic aneurysm produced by periarterial application of calcium chloride, J Surg Res 2001, 99:371-376

411. Daugherty A, Manning MW, Cassis LA: Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice, J Clin Invest 2000, 105:1605-1612

412. Manning MW, Cassi LA, Huang J, Szilvassy SJ, Daugherty A: Abdominal aortic aneurysms: fresh insights from a novel animal model of the disease, Vasc Med 2002, 7:45-54

413. Henriques TA, Huang J, D'Souza SS, Daugherty A, Cassis LA: Orchidectomy, but not ovariectomy, regulates angiotensin II-induced vascular diseases in apolipoprotein E-deficient mice, Endocrinology 2004, 145:3866-3872

414. Saraff K, Babamusta F, Cassis LA, Daugherty A: Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice, Arterioscler Thromb Vasc Biol 2003, 23:1621-1626

415. Daugherty A, Manning MW, Cassis LA: Antagonism of AT2 receptors augments angiotensin II-induced abdominal aortic aneurysms and atherosclerosis, Br J Pharmacol 2001, 134:865-870

416. Pueyo ME, Gonzalez W, Nicoletti A, Savoie F, Arnal JF, Michel JB: Angiotensin II stimulates endothelial vascular cell adhesion molecule-1 via nuclear factor-kappaB activation induced by intracellular oxidative stress, Arterioscler Thromb Vasc Biol 2000, 20:645-651

417. Harrison DG: Endothelial function and oxidant stress, Clin Cardiol 1997, 20:II-11-17

418. Griendling KK, Minieri CA, Ollerenshaw JD, Alexander RW: Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells, Circ Res 1994, 74:1141-1148

419. Griendling KK, Ushio-Fukai M: Reactive oxygen species as mediators of angiotensin II signaling, Regul Pept 2000, 91:21-27

420. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease, Circ Res 2000, 86:494-501

421. Cassis LA, Gupte M, Thayer S, Zhang X, Charnigo R, Howatt DA, Rateri DL, Daugherty A: ANG II infusion promotes abdominal aortic aneurysms independent of increased blood pressure in hypercholesterolemic mice, Am J Physiol Heart Circ Physiol 2009, 296:H1660-1665

422. Tsui JC: Experimental models of abdominal aortic aneurysms, Open Cardiovasc Med J 4:221-230

423. Holmes DR, Lopez-Candales A, Liao S, Thompson RW: Smooth muscle cell apoptosis and p53 expression in human abdominal aortic aneurysms, Ann N Y Acad Sci 1996, 800:286-287

424. Samuel CS, Sakai LY, Amento EP: Relaxin regulates fibrillin 2, but not fibrillin 1, mRNA and protein expression by human dermal fibroblasts and murine fetal skin, Arch Biochem Biophys 2003, 411:47-55

425. Hiebert PR, Boivin WA, Abraham T, Pazooki S, Zhao H, Granville DJ: Granzyme B contributes to extracellular matrix remodeling and skin aging in apolipoprotein E knockout mice, Exp Gerontol 2011, 46:489-499

426. Abraham T, Carthy J, McManus B: Collagen matrix remodeling in 3-dimensional cellular space resolved using second harmonic generation and multiphoton excitation fluorescence, J Struct Biol 169:36-44

427. Abraham T, Hogg J: Extracellular matrix remodeling of lung alveolar walls in three dimensional space identified using second harmonic generation and multiphoton excitation fluorescence, J Struct Biol 171:189-196

428. Skjelland M, Michelsen AE, Krohg-Sorensen K, Tennoe B, Dahl A, Bakke S, Brosstad F, Damas JK, Russell D, Halvorsen B, Aukrust P: Plasma levels of granzyme B are increased in patients with lipid-rich carotid plaques as determined by echogenicity, Atherosclerosis 2007, 195 142-146

429. Pardo J, Wallich R, Ebnet K, Iden S, Zentgraf H, Martin P, Ekiciler A, Prins A, Mullbacher A, Huber M, Simon MM: Granzyme B is expressed in mouse mast cells in vivo and in vitro and causes delayed cell death independent of perforin, Cell Death Differ 2007, 14:1768-1779

430. Wagner C, Stegmaier S, Hansch GM: Expression of granzyme B in peripheral blood polymorphonuclear neutrophils (PMN), myeloid cell lines and in PMN derived from haemotopoietic stem cells in vitro, Mol Immunol 2008, 45:1761-1766

431. Berthou C, Michel L, Soulie A, Jean-Louis F, Flageul B, Dubertret L, Sigaux F, Zhang Y, Sasportes M: Acquisition of granzyme B and Fas ligand proteins by human keratinocytes contributes to epidermal cell defense, J Immunol 1997, 159:5293-5300

432. Hernandez-Pigeon H, Jean C, Charruyer A, Haure MJ, Baudouin C, Charveron M, Quillet-Mary A, Laurent G: UVA induces granzyme B in human keratinocytes through MIF: implication in extracellular matrix remodeling, J Biol Chem 2007, 282:8157-8164

433. Maeda I, Mizoiri N, Briones MP, Okamoto K: Induction of macrophage migration through lactose-insensitive receptor by elastin-derived nonapeptides and their analog, J Pept Sci 2007, 13:263-268

434. Shiraya S, Miyake T, Aoki M, Yoshikazu F, Ohgi S, Nishimura M, Ogihara T, Morishita R: Inhibition of development of experimental aortic abdominal aneurysm in rat model by atorvastatin through inhibition of macrophage migration, Atherosclerosis 2009, 202:34-40

435. Hance KA, Tataria M, Ziporin SJ, Lee JK, Thompson RW: Monocyte chemotactic activity in human abdominal aortic aneurysms: role of elastin degradation peptides and the 67-kD cell surface elastin receptor, J Vasc Surg 2002, 35:254-261

436. Kazi M, Thyberg J, Religa P, Roy J, Eriksson P, Hedin U, Swedenborg J: Influence of intraluminal thrombus on structural and cellular composition of abdominal aortic aneurysm wall, J Vasc Surg 2003, 38:1283-1292

437. Wassef M, Baxter BT, Chisholm RL, Dalman RL, Fillinger MF, Heinecke J, Humphrey JD, Kuivaniemi H, Parks WC, Pearce WH, Platsoucas CD, Sukhova GK, Thompson RW, Tilson MD, Zarins CK:

Pathogenesis of abdominal aortic aneurysms: a multidisciplinary research program supported by the National Heart, Lung, and Blood Institute, J Vasc Surg 2001, 34:730-738

438. Daugherty A, Manning MW, Cassis LA: Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E–deficient mice, J. Clin. Invest. 2000, 105:1605–1612

439. Heusel JW, Wesselschmidt RL, Shresta S, Russell JH, Ley TJ: Cytotoxic lymphocytes require granzyme B for the rapid induction of DNA fragmentation and apoptosis in allogeneic target cells, Cell 1994, 76:977-987

440. Pham CT, MacIvor DM, Hug BA, Heusel JW, Ley TJ: Long-range disruption of gene expression by a selectable marker cassette, Proc Natl Acad Sci U S A 1996, 93:13090-13095

441. Voskoboinik I, Smyth MJ, Trapani JA: Perforin-mediated target-cell death and immune homeostasis, Nature Rev. Immunol. 2006, 6:940-952

442. Boivin WA, Cooper DM, Hiebert PR, Granville DJ: Intracellular versus extracellular granzyme B in immunity and disease: challenging the dogma, Lab Invest 2009, Epub 2009 Sept 21:1-26

443. Tilson DM EJ, Brophy CM: Tensile strength and collagen in abdominal aortic aneurysm disease. Edited by Greenhalgh M MJ. Plymouth, UK, Latimer Trend 1990, p. pp. 97–104

444. Kaiserman D, Bird CH, Sun J, Matthews A, Ung K, Whisstock JC, Thompson PE, Trapani JA, Bird PI: The major human and mouse granzymes are structurally and functionally divergent, J Cell Biol 2006, 175:619-630

445. Carta L, Pereira L, Arteaga-Solis E, Lee-Arteaga SY, Lenart B, Starcher B, Merkel CA, Sukoyan M, Kerkis A, Hazeki N, Keene DR, Sakai LY, Ramirez F: Fibrillins 1 and 2 perform partially overlapping functions during aortic development, J Biol Chem 2006, 281:8016-8023

446. Pereira L, Lee SY, Gayraud B, Andrikopoulos K, Shapiro SD, Bunton T, Biery NJ, Dietz HC, Sakai LY, Ramirez F: Pathogenetic sequence for aneurysm revealed in mice underexpressing fibrillin-1, Proc Natl Acad Sci U S A 1999, 96:3819-3823

447. Bunton TE, Biery NJ, Myers L, Gayraud B, Ramirez F, Dietz HC: Phenotypic alteration of vascular smooth muscle cells precedes elastolysis in a mouse model of Marfan syndrome, Circ Res 2001, 88:37-43

448. Greenwald SE: Ageing of the conduit arteries, J Pathol 2007, 211:157-172

449. Diez J: Arterial stiffness and extracellular matrix, Adv Cardiol 2007, 44:76-95

450. Agrotis A: The genetic basis for altered blood vessel function in disease: large artery stiffening, Vasc Health Risk Manag 2005, 1:333-344

451. Kurschus FC, Kleinschmidt M, Fellows E, Dornmair K, Rudolph R, Lilieb H, Jenne DE: Killing of target cells by redirected granzyme B in the absence of perforin, FEBS Lett. 2004, 562:87-92

452. Granville DJ: Granzymes in disease: bench to bedside, Cell Death Differ 17:565-566

453. Hendel A, Hiebert PR, Boivin WA, Williams SJ, Granville DJ: Granzymes in age-related cardiovascular and pulmonary diseases, Cell Death Differ 2010, 17:596-606

454. Dayan D, Hiss Y, Hirshberg A, Bubis JJ, Wolman M: Are the polarization colors of picrosirius red-stained collagen determined only by the diameter of the fibers?, Histochemistry 1989, 93:27-29

455. Katz AM: Physiology of the Heart. Philadelphia, PA, Lippincott Williams & Wilkins, 2011, p297-312

456. Sonesson B, Lanne T, Vernersson E, Hansen F: Sex difference in the mechanical properties of the abdominal aorta in human beings, J Vasc Surg 1994, 20:959-969

457. Holzapfel GA: Collagen in arterial walls: Biomechanical aspects. Edited by Fratzl P. New York, Springer, 2008, p. pp. 285-324

458. Whittaker P, Schwab ME, Canham PB: The molecular organization of collagen in saccular aneurysms assessed by polarized light microscopy, Connect Tissue Res 1988, 17:43-54

459. Satta J, Juvonen T, Haukipuro K, Juvonen M, Kairaluoma MI: Increased turnover of collagen in abdominal aortic aneurysms, demonstrated by measuring the concentration of the aminoterminal propeptide of type III procollagen in peripheral and aortal blood samples, J Vasc Surg 1995, 22:155-160

460. Bode MK, Soini Y, Melkko J, Satta J, Risteli L, Risteli J: Increased amount of type III pN-collagen in human abdominal aortic aneurysms: evidence for impaired type III collagen fibrillogenesis, J Vasc Surg 2000, 32:1201-1207

461. Carmo M, Colombo L, Bruno A, Corsi FR, Roncoroni L, Cuttin MS, Radice F, Mussini E, Settembrini PG: Alteration of elastin, collagen and their cross-links in abdominal aortic aneurysms, Eur J Vasc Endovasc Surg 2002, 23:543-549

462. Lindeman JH, Ashcroft BA, Beenakker JW, van Es M, Koekkoek NB, Prins FA, Tielemans JF, Abdul-Hussien H, Bank RA, Oosterkamp TH: Distinct defects in collagen microarchitecture underlie vessel-wall failure in advanced abdominal aneurysms and aneurysms in Marfan syndrome, Proc Natl Acad Sci U S A 107:862-865

463. Scott JE: Supramolecular organization of extracellular matrix glycosaminoglycans, in vitro and in the tissues, Faseb J 1992, 6:2639-2645

464. Danielson KG, Baribault H, Holmes DF, Graham H, Kadler KE, Iozzo RV: Targeted disruption of decorin leads to abnormal collagen fibril morphology and skin fragility, J Cell Biol 1997, 136:729-743

465. Reese SP, Underwood CJ, Weiss JA: Effects of decorin proteoglycan on fibrillogenesis, ultrastructure, and mechanics of type I collagen gels, Matrix Biol 2013,

466. Hildebrand A, Romaris M, Rasmussen LM, Heinegard D, Twardzik DR, Border WA, Ruoslahti E: Interaction of the small interstitial proteoglycans biglycan, decorin and fibromodulin with transforming growth factor beta, Biochem J 1994, 302 (Pt 2):527-534

467. Penc SF, Pomahac B, Winkler T, Dorschner RA, Eriksson E, Herndon M, Gallo RL: Dermatan sulfate released after injury is a potent promoter of fibroblast growth factor-2 function, J Biol Chem 1998, 273:28116-28121

468. Tufvesson E, Westergren-Thorsson G: Tumour necrosis factor-alpha interacts with biglycan and decorin, FEBS Lett 2002, 530:124-128

469. Nili N, Cheema AN, Giordano FJ, Barolet AW, Babaei S, Hickey R, Eskandarian MR, Smeets M, Butany J, Pasterkamp G, Strauss BH: Decorin inhibition of PDGF-stimulated vascular smooth muscle cell function: potential mechanism for inhibition of intimal hyperplasia after balloon angioplasty, Am J Pathol 2003, 163:869-878