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An investigation into diffusion tensor imaging-derived metrics in arterial tissue as biomarkers for disease progression, plaque rupture and graft recellularisation

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A thesis submitted to the University of Dublin in partial fulfilment of the requirements for the degree of

Doctor in Philosophy

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External examiner: Prof. David Pierce
Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work.

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Brooke Tornifoglio

September 14th, 2022
Summary
Non-invasive imaging offers great potential for several clinical applications, both in disease and treatment monitoring. Specifically, within vascular tissue better clinical indicators for vulnerable atherosclerotic plaque, as well as tracking of vascular graft integration, could benefit from insights gained using non-invasive imaging. The aim of this thesis was to establish a non-invasive characterisation technique, diffusion tensor imaging (DTI), within vascular tissue for a multitude of applications.

To achieve this, the sensitivity of DTI-derived metrics to specific microstructural changes in arterial tissue models was first investigated. Of note, a strong sensitivity to cell and elastin content was discovered – establishing that the measurable anisotropic diffusion within arterial tissue is predominantly driven by these components. This sensitivity was then explored within human atherosclerotic tissue for anatomical, microstructural, morphological, and mechanical characterisations. Within human cadaveric carotid arteries, a strong correlation between DTI-derived metrics and elastin content was found – a significant finding for any pathology driven by changes in elastin content. DTI-derived metrics were also explored in early atherosclerosis – and found to identify the thickened intima. Tractography yielded significant insight into the mechanical integrity of mechanically tested fresh human atherosclerotic plaques, whereby it was able to differentiate between more stable microstructures and those which would be more vulnerable to failure at lower stresses and strains. The sensitivity to cell content was revisited and demonstrated clearly in cultured vascular grafts which were characterised non-invasively and non-destructively. Tractography and DTI-derived fractional anisotropy were able to distinguish between acellular and recellularised vascular grafts and tracked that recellularisation over two weeks.

This imaging technique was also tested in human cadaveric aortic morphologies and distinct differences between healthy, aneurysmal, and chronic dissected aortae were found – highlighting its potential in more than one vascular application. Lastly, DTI was investigated in explanted abdominal tissue to identify the presence or absence of a fibrous capsule around implanted devices. It proved capable of identifying fibrous capsules and the underlying microstructure within them accurately and non-destructively. Together, the investigations presented in this thesis not only establish DTI within vascular tissue but also highlight its potential in disease diagnosis, tissue engineering, and even in the foreign body response around device integration.
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List of publications, conference proceedings and prizes

**First-author journal publications**


**Publications not included in this thesis**


Hanly, A.*, Johnston, R. D.*, Lemass, C., Jose, A., Tornifoglio, B., Lally, C., Phosphotungstic acid (PTA) preferentially binds to collagen-rich regions of porcine carotid arteries and human atherosclerotic plaques using 3D micro-computed tomography (CE-μCT). (2022) [Submitted].

Conference proceedings


diffusion tensor imaging to examine the foreign body response to explanted medical devices’, in 2nd Joint Meeting of the MSI and SMS. Galway, Ireland.


**Awards**

Finalist for the Engineers Ireland Biomedical Research Medal at the 27th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland.

Recipient of Women in STEM Conference Support Award at the 11th European Solid Mechanics Conference (ESMC 2022) at the National University of Ireland, Galway.

Nominated for The Trinity Teaching Award for Postgraduate Students 2021.

2nd place for Best non-neuro oral presentation at MRFest21 at the British and Irish Chapter of ISMRMB 2021.

1st place for Best Diffusion Talk at the Postgraduate meeting of the British and Irish Chapter of ISMRMB 2020.

1st place for Best Cardiovascular Research Oral Presentation (mature researcher category), 26th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland (2020).

Recipient of Student Support Grant at the 36th Annual Scientific Meeting of ESMRMB 2019, Rotterdam, Netherlands.

1st place for Best Cardiovascular Research Oral Presentation (early researcher category), 25th Annual Conference of the Section of Bioengineering of the Royal Academy of Medicine in Ireland (2019).
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<tbody>
<tr>
<td>AAA</td>
<td>Abdominal aortic aneurysm</td>
</tr>
<tr>
<td>AAD</td>
<td>Acute aortic dissection</td>
</tr>
<tr>
<td>ADC</td>
<td>Apparent diffusion coefficient</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>CEA</td>
<td>Carotid endarterectomy</td>
</tr>
<tr>
<td>COD</td>
<td>Cause of death</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>ddPCaA</td>
<td>Decellularised, de-intima porcine carotid artery</td>
</tr>
<tr>
<td>DIC</td>
<td>Digital image correlation</td>
</tr>
<tr>
<td>DWI</td>
<td>Diffusion weighted imaging</td>
</tr>
<tr>
<td>DTI</td>
<td>Diffusion tensor imaging</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EPI</td>
<td>Echo planar imaging</td>
</tr>
<tr>
<td>FA</td>
<td>Fractional anisotropy</td>
</tr>
<tr>
<td>FBR</td>
<td>Foreign body response</td>
</tr>
<tr>
<td>FD ddPCaA</td>
<td>Freeze dried decellularised, de-intima porcine carotid artery</td>
</tr>
<tr>
<td>FEFA</td>
<td>First-eigenvector fractional anisotropy</td>
</tr>
<tr>
<td>FOV</td>
<td>Field of view</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared microscopy</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>GE</td>
<td>Gradient echo</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin &amp; eosin</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischaemic heart disease</td>
</tr>
<tr>
<td>IPH</td>
<td>Intraplaque haemorrhage</td>
</tr>
<tr>
<td>IVOCT</td>
<td>Intravascular optical coherence tomography</td>
</tr>
<tr>
<td>IVUS</td>
<td>Intravascular ultrasound</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LRNC</td>
<td>Lipid-rich necrotic core</td>
</tr>
<tr>
<td>MD</td>
<td>Mean diffusivity</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCaA</td>
<td>Porcine carotid artery</td>
</tr>
<tr>
<td>PLM</td>
<td>Polarised light microscopy</td>
</tr>
<tr>
<td>PGSE</td>
<td>Pulsed gradient spin echo</td>
</tr>
<tr>
<td>pSMCs</td>
<td>Porcine smooth muscle cells</td>
</tr>
<tr>
<td>PSR</td>
<td>Picrosirius red</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency pulse</td>
</tr>
<tr>
<td>SE</td>
<td>Spin echo</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cells</td>
</tr>
<tr>
<td>SNR</td>
<td>Signal-to-noise ratio</td>
</tr>
<tr>
<td>TAA</td>
<td>Thoracic aortic aneurysm</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TEVG</td>
<td>Tissue engineered vascular grafts</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>UT</td>
<td>Ultimate tensile</td>
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Chapter 1 Introduction

1.1 Research motivation

While cardiovascular disease (CVD) incorporates death and disability from 11 cardiovascular causes, ischaemic heart disease (IHD) and stroke are the main contributors\textsuperscript{3,4}. IHD has seen a profound reduction in mortality in recent years\textsuperscript{3}; however, the incidence of stroke is expected to increase by 27\% in the next 30 years\textsuperscript{5}. The World Health Organisation defines stroke as the rapid development of clinical signs of cerebral function disturbance lasting $>24$ hours or leading to death\textsuperscript{6}. Specifically, carotid atherosclerosis accounts for approximately 10-20\% of ischemic strokes\textsuperscript{7,8} and symptomatic patients with a large burden of atherosclerosis have the highest odds of recurrence in the first 30 days\textsuperscript{9,10}.

Atherosclerosis is the accumulation of lipids and fibrous elements in large arteries\textsuperscript{11} and contributes to the thickening of the arterial wall with plaque progression. Current diagnostic criteria, established in the 1980s and 1990s\textsuperscript{12,13}, depend entirely on the percent stenosis. That is, if the luminal area is blocked by more than 50-70\%, patients are referred for surgical intervention\textsuperscript{14,15}. While carotid endarterectomy (CEA) (surgical plaque removal) is the standard of care for symptomatic, low risk patients, carotid artery stenting and medical therapy alone can be recommended for high-risk or asymptomatic patients\textsuperscript{10,16,17}. For severely stenosed symptomatic patients, the timing of surgical intervention is in question; however, for asymptomatic patients it is the benefit of surgical intervention that is in question\textsuperscript{18}.

Through the decades, percent stenosis as the main intervention criterion has not changed and still remains the only accepted clinical indicator used to determine the likelihood of an atherosclerotic plaque to rupture. Recently however, there has been a growing body of evidence that plaque microstructure and composition could provide a better indication of the likelihood of a plaque to rupture\textsuperscript{19–23}. The healthy arterial wall is composed predominantly of collagen, elastin, and smooth muscle cells (SMCs) – together they are responsible for the mechanical integrity of the wall. Alterations in these components, as well as the development of atherosclerotic morphologies, will ultimately have mechanical implications\textsuperscript{24}. 

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While more advanced stages of atherosclerosis can be characterised by the presence of intraplaque haemorrhage (IPH) and lipid-rich necrotic cores (LRNC), early intimal thickening is characterised by decreased elastin and SMC content. While the more advanced morphologies have been associated with increased stroke risk—so too have early-stage, low-grade stenosed plaques. Imaging biomarkers sensitive to these microstructures have the potential to shift the current landscape of diagnostic criterion to better capitulate plaque vulnerability.

More importantly, not only do non-invasive biomarkers have potential in disease characterisation but they also offer significant potential in monitoring the efficacy of tissue engineered vascular grafts (TEVG). Non-invasive, non-destructive characterisation of TEVG recellularisation would impact vascular tissue engineering research from in vitro studies to preclinical animal models and finally to clinical translation. To date, there are no such techniques which allow for this. The sensitivity of non-invasive metrics for arterial tissue characterisation needs to be systematically assessed and interpreted in controlled ex vivo scenarios prior to motivating progression to clinical translation.

1.2 Objectives

The fundamental goal of this thesis is to establish the use of a non-invasive magnetic resonance imaging (MRI) technique, diffusion tensor imaging (DTI), in vascular tissue. Ex vivo imaging of healthy and diseased arterial tissue, as well as recellularised TEVG, will identify novel microstructural, morphological, and ultimately mechanical insights in arterial tissue. The coupling of ex vivo imaging and mechanical testing will identify the role this technique can play in offering improved diagnostic criteria. Ultimately, these studies will highlight the potential this technique has in both early- and late-stage vascular diseases, as well as in the field of tissue engineering. A graphical summary of the key research objectives in this thesis is given in Figure 1.1.

1. Identify how key microstructural components in healthy arterial tissue influence common DTI-derived metrics.
2. Investigate DTI-derived metrics in whole cadaveric human carotid arteries to establish clinically relevant microstructural and morphological sensitivities.
3. Explore the mechanical characterisation of fresh, human atherosclerotic plaques with insights on microstructure provided by DTI.
4. Based on previous experimental data, use DTI to non-invasively and non-destructively track recellularisation of TEVG.

Figure 1.1. Graphical summary of key research objectives.
1.3 Thesis structure

This thesis consists of eight chapters centred around four main studies and two collaborative studies. From these studies, six separate first-author journal papers will emanate (one published, three in review, two in preparation). A brief description of each chapter can be found below:

Chapter 1: Outlines the foundations of the motivation for the work completed as part of this thesis, as well as identifies the key objectives to be addressed in Chapter 3 - Chapter 6.

Chapter 2: Begins with a review of cardiovascular anatomy and physiology and the prevalence of atherosclerosis and introduces the concept of a ‘vulnerable’ plaque. Next, an in-depth review of mechanical and non-invasive imaging characterisation studies in atherosclerotic tissue is carried out, followed by a general overview of MRI where DTI is introduced and explained. The literature review concludes with addressing the lack of non-invasive characterisation techniques within vascular tissue engineering.

Chapter 3: Explores the feasibility of DTI to non-invasively characterise specific microstructural changes in arterial tissue.

Chapter 4: Assesses the sensitivity of DTI-derived metrics within cadaveric human arterial tissue – both healthy and diseased – by investigating anatomical, microstructural, and morphological changes.

Chapter 5: Further investigates DTI-derived metrics within fresh human atherosclerotic plaque tissue by ascertaining the microstructural arrangement non-invasively using DTI and linking it to the mechanical integrity of the tissue.

Chapter 6: Examines the feasibility of using DTI to non-invasively and non-destructively identify recellularisation within two different types of TEVG.

Chapter 7 and Chapter 8: Conclude with a final discussion to unify the novel findings from Chapters 3-6, address limitations of the work alongside future perspectives, then highlight the contribution of this work to the field of non-invasive imaging, cardiovascular biomechanics, and tissue engineering.
Chapter 2 Literature review

2.1 Cardiovascular system

Together the heart and blood vessels form the cardiovascular system which is responsible for continuous blood supply throughout the body. Blood circulation is divided into two parts – the systemic and pulmonary circuits – and together they deliver nutrients and oxygen through the body. In systemic circulation, oxygen-rich blood is pumped from the left ventricle into the aorta and further travels to large and small arteries and the capillary networks. This cycle deposits oxygen and nutrients while picking up carbon dioxide and waste products. The now oxygen-poor blood is carried through veins and heads to the right atrium and into the right ventricle. The pulmonary circuit begins at this point, with the right ventricle pumping blood into the pulmonary artery which branches into small arteries and capillaries around the pulmonary vessels. Carbon dioxide is released, and fresh oxygen enters the bloodstream once again, Figure 2.1.

Figure 2.1. The pulmonary circuit transports blood between the heart and lungs to oxygenate the oxygen-poor blood, in blue. The systemic circuit delivers the oxygen-rich blood, in red, throughout the blood and then returns it to the pulmonary circuit to be re-oxygenated.
2.1.1 Carotid arteries

Located on both sides of the neck, Figure 2.2, the carotid arteries are large sized arteries which supply blood to the neck, face, and brain. The common carotid artery ascends from the brachiocephalic artery on the right and the aortic arch on the left side of the neck. Around the thyroid cartilage, the common carotid bifurcates into the internal and external carotid arteries. This bifurcation is where the carotid body and sinus are located. The carotid body, a chemoreceptor, is sensitive to alterations in oxygen, carbon dioxide, and hydrogen ion concentrations while aiding in respiratory control. The carotid sinus, a baroreceptor, is located at the origin of the internal carotid artery and detects and corrects changes in blood pressure. The external carotid supplies blood throughout the face and neck while the internal carotid passes through the temporal canal and supplies the cerebral arteries.

![Figure 2.2. The internal and external carotid arteries branch from the bifurcation of the common carotid, seen in red.

2.1.2 Arterial structure and function

Existing in a dynamic environment, the structure of arterial tissue is critical to the ability to function properly. Arteries undergo between 35 – 40 million load cycles per year and the capability of the tissue to repeatability deform dictates successful blood circulation. Arterial tissue is divided into two main subdivisions: elastic and muscular arteries. Larger diameter arteries with prominent elastic laminae and SMCs, referred to as elastic arteries, are typically located proximal to the heart. Arteries whose diameters decrease as they approach
the periphery have a less elastic media and are deemed muscular arteries\textsuperscript{33}. Regardless, the vessel wall is composed of SMCs, elastin and collagen fibres as well as ground matrix which includes glycosaminoglycans (GAGs)\textsuperscript{34}. While both types of arteries have these components, the amount and ratio of them varies. The aorta is an example of elastic artery, as too are the carotids proximal to the heart but they gradually transition into a more muscular structure distal to the heart.

Arterial tissue is predominantly composed of three main layers. Starting from the lumen, and moving radially outward there is the intima, media, and adventitia, Figure 2.3. The intima is composed of an endothelial layer of a single layer of cells and a thin basal lamina. These endothelial cells are flat, aligned parallel to blood flow and proliferate concentrically, leading to the collagen rich extracellular matrix (ECM)\textsuperscript{24}. The intima protects the other layers of arterial tissue from plasma lipids and lipoproteins in the circulating blood. Vascular tone is controlled by the intimal layer, but the overall mechanical contribution is minimal\textsuperscript{35}.

![Figure 2.3. Elastic (top) and muscular (bottom) arteries have key differences between the arrangement of collagen and elastin in the media\textsuperscript{33}.](image)
Chapter 2

The medial layer is the middle layer of the vascular wall, composed of collagen, elastin, and SMCs. In large arteries, between 40 – 60 elastic laminae are concentrically arranged and equally spaced, approximately 29% by volume. Interlamellar elastic fibres connect the laminae, 0.1-0.2 μm thick\textsuperscript{36}, giving way to a highly structured framework and accounting for two thirds of the total elastin volume\textsuperscript{37}. SMCs are directly connected to this framework of interlamellar elastin fibres, generally arranged obliquely between the concentric elastic laminae. Additionally, two types of collagens – type I and III – are found within the media. These collagen bundles range between 1-20 μm in diameter\textsuperscript{36} and are made up of 25 ± 14 fibres per bundle. Flat collagen sheets located near SMCs and tightly wound collagenous bundles are located between the adjacent elastic laminae, but are not directly connected to either elastin or SMCs\textsuperscript{37}. These collagen fibres, 47% by volume, surround the spindle shaped SMCs. SMCs have been reported to take up a volume of 24% with a cell density of 3.7 ± 0.6 x 10\textsuperscript{5} cells/mm\textsuperscript{3} in rabbit aorta\textsuperscript{37}. Vascular SMCs are approximately 89 ± 3% cytoplasm and 11 ± 3% nucleus, with the nucleus averaging 19 ± 3 μm long\textsuperscript{37} and overall cell size reaching lengths up to 200 μm with a 5 μm diameter\textsuperscript{38}. Meanwhile, muscular arteries have a predominant internal and external elastic lamina and few medial elastic laminae. The close relationship between elastin, SMCs, and collagen yield a continuous helically arranged matrix\textsuperscript{33,37}. This arrangement is capable of withstanding forces both circumferentially and axially and contributes to the mechanical behaviour of arterial tissue.

The outermost layer, the adventitia, is a loose network of elastin, type I collagen fibres, ground matrix, fibroblasts and fibrocytes\textsuperscript{24,33}. The varying thicknesses across blood vessels are dependent on the location and function of the vessel. In elastic arteries there is no distinct external elastic lamina; however, as vessels transition toward a more muscular phenotype the external elastic lamina reappears. In more muscular arteries, the adventitia has a more defined structure of thick collagenous bundles outside the external elastic lamina. While SMCs are not present in the adventitia, there are fibroblasts, fibrocytes and occasionally Schwann cells and macrophages.

Arterial tissue microstructure can vary in the composition of the vessel wall depending on location in the body. This variability is a key factor in the mechanical response of blood vessels and yields insight into the relationship between structure and function.

### 2.1.3 Biomechanical properties

The physical equilibrium maintained within blood vessels is a result of the balance between the blood pressure within the lumen, vessel wall expansion, and the tension resisting
expansion\textsuperscript{39}. The prominence of elastic fibres in the media and adventitia contribute elastic and highly extensible properties at lower loads, while the collagen fibres have only a slight distensibility and very high elastic modulus which are evident by fibre recruitment at higher loadings\textsuperscript{24,39}. Collagen, most abundantly type I, is the main load-bearing component in arterial tissue with a tensile strength much greater than those of elastin or SMCs. GAGs have been shown to contribute to the viscoelastic properties of arterial tissue\textsuperscript{34}. The swelling stress of proteoglycans in arterial tissue puts the vessel wall in tension and contributes to a stiffer tissue response in layers with higher GAG content\textsuperscript{40}. It is generally assumed arterial tissue volume is preserved under loading, i.e., incompressible, resulting in only isochoric deformations\textsuperscript{41}. This assumes that the water in arterial tissue is bound and not lost under loading. However, work by Nolan and McGarry have shown that this is not necessarily the case\textsuperscript{41}. Bound water acts as a plasticizer in biological tissue and a decrease in bound water can lead to an increase in stiffness\textsuperscript{42}.

The elliptical shape of SMCs and elongated inner nucleus surrounded by a more flexible cytoplasm suggests that the cytoplasm transfers tensile forces to the nucleus\textsuperscript{37}. Pulsatile cyclic forces applied to cell surfaces can induce gene transcription and lead to a contractile phenotype for the SMCs\textsuperscript{43}. This phenotype decreases the production of matrix metalloproteases (MMPs), matrix degrading enzymes, and maintains the ECM. Conversely, zero stress on the SMCs leads to cell apoptosis and a synthetic phenotype\textsuperscript{44}. The synthetic phenotype increases ECM turnover by an increased production in MMPs and increased matrix synthesis. The link between mechanical stimuli on SMCs and mural function is critical for functioning arterial tissue. Numerous studies have investigated the role arterial components play in the mechanical integrity of blood vessels; a summary is presented in Table 2.1.
Table 2.1. Summary of current literature investigating arterial mechanics and the influence of specific components.

<table>
<thead>
<tr>
<th>Results</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tension-radius response of native (control), elastin digested, and collagen digested human iliac arteries. Reproduced from Holzapfel, 2008.</td>
<td>Elastin and collagen digestion on human iliac arteries. Native arteries showed the typical J-shaped curve, while a strain-dependent transfer of load was evident by the stiffness of elastin at low loads and the stiffness of collagen at higher loads.</td>
<td>Roach and Burton, 1957.</td>
</tr>
<tr>
<td>Representative circumferential stress (10^1 MPa) vs mid-wall diameter (mm) at 1.8 extension. Controls (left), elastase (middle, solid circles) and collagenase (right, solid circles) treated carotids.</td>
<td>Elastin and collagen treatments on dog carotids. Pressurisation of the carotids showed elastin bears both circumferential and axial loads while collagen only bears a portion of the circumferential load.</td>
<td>Dobrin and Canfield, 1984.</td>
</tr>
<tr>
<td>Pressure-diameter curves for Triton®X-100 treated porcine carotids.</td>
<td>SMC, elastin, and collagen treatments on porcine carotids. Decreased SMC content led to increased outer diameter of the arterial wall up to 120 mmHg, followed by stiffening.</td>
<td>Kochová, et al., 2012.</td>
</tr>
</tbody>
</table>
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Elastase treated porcine aorta displayed a clear decrease in stiffness in the medial layer.

**Elastin, collagen, and GAG treatments on porcine aortas.**
Elastin removal reduced stiffness in the media, while collagen removal reduced stiffness in the adventitia and media. GAG removal reduced stiffness in both layers.

Beenakker, *et al.*, 2012\(^{40}\)

**Static stretch during elastin degradation on porcine aortas.**
Elastin degraded faster in the presence of stretch and the stress-strain behaviour transitions from J-shaped to S-shaped curves during equibiaxial testing. Elastin removal resulted in collagen fibre disorganisation.

Chow, *et al.*, 2013\(^{48}\)

**Elastin and collagen removal on porcine aortas.**
Elastase treatment yielded a stiffer response in equibiaxial testing, while collagenase treated tissues showed decreased nonlinearity compared to control tissue. These results suggest elastin distributes tensile stresses while collagen reinforces the wall circumferentially.

Gundiah, *et al.*, 2013\(^{39}\)

**Elastin and collagen removal on human abdominal aortas.**
Results confirmed elastin is responsible for initial stiffness in elastic arteries.

Schriefl, *et al.*, 2015\(^{50}\)
The studies summarised above not only highlight the typical-J shaped stress-strain response of arterial tissue, but how that mechanical response alters significantly with alterations in the quantity and quality of different compositional components. The initial ‘elastic’ region of the curve relies on the presence of elastin and the recruitment and uncirmping of collagen fibres ultimately leads to the stiffening ‘collagen’ region of the curve\textsuperscript{24}. The interaction of ECM molecules in arterial tissue determines the mechanical response and ultimately the functionality of the tissue. The reorientation of collagen fibres under load\textsuperscript{24,51} demonstrates how these components can adapt to best withstand the experienced mechanics. Cellular orientation affects the organisation of newly laid down ECM and ultimately dictates the structure of these anisotropic tissues\textsuperscript{52}. Understanding the role each component plays and how they interact and adapt from different biomechanical cues is important for appreciating the relation between structure and function in both healthy and diseased arterial tissue.

2.2 Cardiovascular disease

In 2017 it was reported that CVD accounts for 45% of all deaths in Europe, amounting to roughly 3.8 million people\textsuperscript{53}. CVD is the leading cause of death (COD) globally, with the most prevalent forms being IHD and stroke\textsuperscript{3}. Stroke accounted for 12% of all deaths in females and 8% in males, Figure 2.4, second only to IHD\textsuperscript{3}. IHD has seen a profound reduction in mortality in recent years\textsuperscript{3}; however, the incidence of stroke is expected to increase by 27% in the next 30 years\textsuperscript{5}. In 2013, CVD was responsible for 32% of deaths in Ireland – the leading COD\textsuperscript{53}. In the United States, it is expected that by 2035 the total costs

\textit{GAG removal in porcine aortas.}

GAG removal did not alter tissue stiffness significantly but did yield an earlier non-linear response in biaxial tensile testing. Elastin and collagen fibres were recruited at lower strain levels upon GAG removal.\textsuperscript{Mattson, et al., 2017}
from CVD will reach $1.1 trillion, while direct healthcare costs are projected to hit $748.7 billion\textsuperscript{54}.

![Image of bar chart showing national causes of death in females and males in European Society of Cardiology member countries.]

**Figure 2.4. National causes of death in females and males in European Society of Cardiology member countries.**

### 2.2.1 Atherosclerosis

Atherosclerosis is widely acknowledged as one of the most significant contributors to the burden of CVD\textsuperscript{55}. Some of the risk factors for atherosclerosis include obesity, smoking, diabetes, and hypertension. Aside from these genetic risk factors, vessel geometry and fluid dynamics precede altered biomechanics and can play a role in the progression of atherosclerosis\textsuperscript{56}. Typically atherosclerosis occurs at the carotid artery bifurcation\textsuperscript{56}. While it is rarely fatal on its own, the atherosclerotic plaque that silently forms can erode or rupture giving way to acute catastrophic events such as thrombosis and stroke\textsuperscript{57}.

While numerous risk factors are documented, elevated cholesterol levels in blood plasma alone are believed to be sufficient to initiate atherosclerosis\textsuperscript{57}. The relationship is not entirely understood, but low-density lipoproteins (LDLs) could increase in number, size or change the composition or the permeability of the arterial wall could change\textsuperscript{57}. Flow related mechanics could be a possible trigger for altered wall permeability. Hemodynamic stimuli which produce flow and circumferential stresses on the arterial wall induce remodelling of the endothelium which maintains homeostasis\textsuperscript{58}. It has been reported that atherosclerotic plaques are more likely to form in regions exposed to low shear stress as well as high oscillatory flow, with the carotid bifurcation being a prime example of this type of environment\textsuperscript{59}. One study showed that after just one month of altered shear stress, the vessel wall remodeled to adapt and maintain a singular level of shear stress\textsuperscript{60}. Arterial
remodelling requires the turnover of the ECM, led by the upregulation of MMPs. Hemodynamic forces like low flow have been shown to up-regulate MMP-2 more than arterial wall injury, which could be one pathway that allows for the remodelling of the arterial wall.

The binding of LDL molecules and their subsequent oxidation is an important step in the progression of the immunoinflammatory, fibroproliferative disease that is atherosclerosis. Atherosclerotic lesions start to develop when LDLs adhere to the endothelium and travel through the leaky or defective endothelium towards the medial layer. Entrapped LDLs allow for the infiltration of monocytes and their subsequent conversion to macrophages and foam cells. The oxidation of LDLs increases the expression of adhesion molecules, such as intercellular adhesion molecule-1, vascular cell adhesion molecule-1 and endothelial leukocyte adhesion molecule, allowing for the attachment of monocytes in the vessel wall.

Endothelial cells, macrophages and even T-cells are all contributors in early lesion development. A fibroproliferative response mediated by SMCs is triggered by the immunoinflammatory response in disease progression. SMCs are responsible for healing and repair in arterial injury; however, only in their recruitment, proliferation and synthetic activities is this beneficial. Impaired function, senescence, or apoptosis are believed to be detrimental. The majority of SMCs originate from medial SMCs which de-differentiate into synthetic phenotypes which modulate the ECM components. Vascular SMCs can undergo phenotypic modulation in atherosclerosis. The synthetic vascular SMCs secreting ECM components can increase LDL retention initially but stabilise more advanced plaques. The microenvironment can also lead to modulation to a more osteochondrogenic-like phenotype which excrete mineral deposits or even transition to foam cell like phenotypes. The continued progression of atherosclerotic plaques and their variable structure is of great interest as, despite the wealth of research being invested into understanding their development, there remains significant questions on the mechanisms behind their advancement and ultimately their stability.

2.2.1.1 Atherosclerotic plaque
Atherosclerosis presents itself as the accumulation of lipid proteins in the vessel wall. This accumulation is complex, and the structure of the atherosclerotic plaque has many forms as it adapts over time. Characterising the different lesion types is no small task and due to unstandardised histological methods, the American Heart Association (AHA) published a
three-part report defining types of atherosclerotic lesions alongside the histological classifications \(^{26,69,70}\). Table 2.2 outlines the key characteristics of these classifications as well as their onset and clinical correlation.

**Table 2.2. Different classifications of atherosclerotic lesions\(^{25,26}\).**

<table>
<thead>
<tr>
<th>Type and key features</th>
<th>Growth mechanism</th>
<th>Earliest onset</th>
<th>Clinical correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I (initial lesion): small, isolated macrophages with lipid deposits</td>
<td></td>
<td>From first decade</td>
<td>Clinically silent</td>
</tr>
<tr>
<td>Type II (fatty streak): stratified macrophage foam cells, SMCs with intracellular lipid droplets (cholesterol esters)</td>
<td>Lipid accumulation</td>
<td>From third decade</td>
<td></td>
</tr>
<tr>
<td>Type III (intermediate): pools of extracellular lipid deposits separating layers of SMCs, intimal thickening</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type IV (atheroma): presence of well-defined lipid core, disorganised intima, displacement of SMCs, some calcifications</td>
<td>Increased SMC presence and high collagen content in fibrous cap</td>
<td>From fourth decade</td>
<td>Clinically silent or apparent</td>
</tr>
<tr>
<td>Type V (fibroatheroma): formation of fibrous cap over lipid core, potentially numerous cores and caps, possible calcifications, decreased SMCs in media</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type VI (complicated): surface defects, hematoma, haemorrhage, thrombus</td>
<td>Thrombosis, hematoma</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Small lipid deposits in the intima of the vessel wall are defined as type I and type II lesions, sometimes called fatty streaks, Figure 2.5\(^{26}\). In the early-stage lesions, an increased cell density is typically seen alongside lipid deposits in macrophages without affecting the media or adventitia\(^{26}\). Type II lesions typically have more stratified macrophage foam cells but also show intracellular lipid droplets in SMCs. T lymphocytes and mast cells have also been identified as type II markers, while most of the intracellular lipid droplets are cholesterol esters\(^{26}\). Type II lesions are also most commonly the type of lesion that is formed in diseased models in animals. While different imaging modalities yield varying resolutions – making in vivo measurements variable – the wall thickness of a healthy carotid artery can range
between 0.5 to 1.53 mm\textsuperscript{71,72} and this increases with lesion type, as demonstrated in Figure 2.5.

Type III lesions, Figure 2.5, are so named purely to bridge Type II plaques to atheromas, the first type of advanced lesion. Extracellular lipid droplets are characteristic of Type III lesions and are located below the macrophages and foam cells, taking the place of intracellular proteoglycans and fibres as well as separating SMCs\textsuperscript{26}. The well-known lipid core of advanced lesions is formed from these lipid pools in Type III plaques.

Cellular morphological changes have been documented in these early lesions, most of which are likely due to the accumulation of macrophage foam cells and lipid in the ECM. Specifically, the anisotropy of the cells in the direction of blood flow is lost, the cells round, stress fibre content increases and multinucleated cells become more prominent\textsuperscript{73}.

The transition from early stage lesions to advanced lesions is defined as when the accumulation of lipids, cells and matrix components coincides with microstructural disorganisation, repair and deformativ remodelling\textsuperscript{25}. More specifically, type IV lesions are characterised by a dense, well-defined region of extracellular lipid, more commonly known as the lipid core, Figure 2.6\textsuperscript{25}. These lesions show severe disorganisation in the intima due to the lipid core formation and presence of extracellular lipid molecules displacing SMCs.

\begin{center}
\textbf{Figure 2.5. Sequence of lesion progression from early stage lesions (I-III) to more advanced lesions (IV-VI)}\textsuperscript{25}.
\end{center}
The displaced cells are typically elongated with uniquely thick basement membranes, and in some cases, calcifications may be present. When the surface of the lipid core, which at this point has not thickened and is still largely intima, increases into a more fibrous tissue, known as the fibrous cap, the lesion becomes type V\textsuperscript{25}. The difference between type IV lesion disorganisation and fibrous caps is not always obvious which is why they are both referred to as fibrous plaques. Type IV lesions can have significant clinical side-effects; however, they do not typically result in narrowing of the lumen or endothelial thickening and are therefore difficult to diagnose.

Type V lesions are formed when the fibrous connective tissue has formed a fibrous cap over the lipid core and can be subdivided further. The fibrous cap is defined, and well-known, as the layer of connective tissue completely covering the lipid-necrotic core\textsuperscript{74}. The newly formed fibrous tissue that forms around the lipid core is entirely different from the healthy intima with a high presence of SMCs and collagen. Type Va, or fibroatheromas, differ from type Vb lesions which have calcified regions, while type Vc lesions may lack a lipid core\textsuperscript{25}. These advanced lesions result in luminal narrowing as well as may present with fissures, hematomas, or thrombus. Type Va fibroatheromas can be multi-layered with several lipid cores separated by thick fibrous tissue, which ultimately can induce asymmetrical luminal narrowing and affect the hemodynamic and tensile forces on the plaque\textsuperscript{59}. Disrupted hematomas or thrombi could also lead to multilayer fibroatheromas. Large calcium concentrations, in type Vb lesions, are typically found by fibrous tissue and can be the result of mineral deposits replacing dead cell remnants or extracellular lipid\textsuperscript{25}. Fibrotic lesions, type Vc, can form after the organisation of thrombi or resorption of the lipid core. Overall, type V lesions have disorganised and decreased SMC content in the media with increased lymphocytes, macrophages, and macrophage foam cells.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.6.png}
\caption{Histological representations of atherosclerotic lesions: (a) type II, (b) type III, (c) type IV and (d) type VI. A: adventitia, M: media, Tmb: thrombus. Adapted from\textsuperscript{25}.}
\end{figure}
Type VI lesions are subdivided based on the lesion surface disruptions like fissures or ulcerations (type V\text{ia}), hematomas or haemorrhages (type V\text{ib}), and thrombi (type V\text{ic})\textsuperscript{25}.

2.2.1.2 Vulnerable plaque

A vulnerable plaque is an atherosclerotic lesion that has a high-risk of rupture or likelihood to cause thrombotic events. There are many overlapping suggestions in the literature on how to define the stability of plaques; however, new metrics and considerations surrounding biomechanics have emerged and highlight suggesting a change in focus\textsuperscript{75}. Table 2.3 outlines the different definitions existing in the literature on defining vulnerable plaques.
Table 2.3. Summary of the different indicators for vulnerable plaque throughout the literature.

<table>
<thead>
<tr>
<th>Indicators of a vulnerable plaque</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Fibroatheroma</td>
<td>Schroeder and Falk, 1995&lt;sup&gt;76&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Intra-plaque haemorrhage</td>
<td>Finn, &lt;i&gt;et al.&lt;/i&gt;, 2010&lt;sup&gt;77&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Inflammation</td>
<td></td>
</tr>
<tr>
<td>• Neovascularisation</td>
<td></td>
</tr>
<tr>
<td><strong>Major:</strong></td>
<td></td>
</tr>
<tr>
<td>• Thin cap with large lipid-necrotic core</td>
<td>Naghavi, &lt;i&gt;et al.&lt;/i&gt;, 2003&lt;sup&gt;78&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Surface fissures</td>
<td>Saam, &lt;i&gt;et al.&lt;/i&gt;, 2007&lt;sup&gt;79&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Endothelial denudation with superficial platelet aggregation</td>
<td>Naim, &lt;i&gt;et al.&lt;/i&gt;, 2014&lt;sup&gt;80&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Active inflammation</td>
<td></td>
</tr>
<tr>
<td>• Stenosis &gt;90%</td>
<td>Stefanadis, &lt;i&gt;et al.&lt;/i&gt;, 2017&lt;sup&gt;75&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Minor:</strong></td>
<td></td>
</tr>
<tr>
<td>• Superficial calcifications</td>
<td></td>
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<tr>
<td>• Yellow plaque visible in angiography</td>
<td></td>
</tr>
<tr>
<td>• IPH</td>
<td></td>
</tr>
<tr>
<td>• Endothelial disfunction</td>
<td></td>
</tr>
<tr>
<td>• Positive remodelling</td>
<td></td>
</tr>
<tr>
<td><strong>Biological and mechanical markers:</strong></td>
<td></td>
</tr>
<tr>
<td>• Systemic factors:</td>
<td>Nighoghossian, Derex, and Douek, 2005&lt;sup&gt;81&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Leukocyte and monocyte count</td>
<td></td>
</tr>
<tr>
<td>• Fibrinogen levels</td>
<td></td>
</tr>
<tr>
<td>• C-reactive protein levels</td>
<td></td>
</tr>
<tr>
<td>• Local factors:</td>
<td></td>
</tr>
<tr>
<td>• ICAM-1 and cytokine presence</td>
<td></td>
</tr>
<tr>
<td>• MMPs</td>
<td></td>
</tr>
<tr>
<td>• Growth factor-β1</td>
<td></td>
</tr>
<tr>
<td>• Neovasculature</td>
<td></td>
</tr>
<tr>
<td>• Shear stress</td>
<td></td>
</tr>
<tr>
<td>• Thin cap fibroatheroma</td>
<td>Fishbein, 2010&lt;sup&gt;82&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Inflammation</td>
<td></td>
</tr>
<tr>
<td>• Fibroatheroma</td>
<td>van Lammeren, &lt;i&gt;et al.&lt;/i&gt;, 2011&lt;sup&gt;83&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Low SMC content</td>
<td></td>
</tr>
<tr>
<td>• Thin cap fibroatheroma</td>
<td>Adamson, Dweck, and Newby, 2015&lt;sup&gt;84&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Plaque burden</td>
<td>Arbab-Zadeh and Fuster, 2015&lt;sup&gt;85&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Metabolic activity</td>
<td></td>
</tr>
<tr>
<td>• IPH</td>
<td>Huang, &lt;i&gt;et al.&lt;/i&gt;, 2017&lt;sup&gt;86&lt;/sup&gt;</td>
</tr>
<tr>
<td>• LRNC area &gt;70%</td>
<td></td>
</tr>
<tr>
<td>• Thin cap fibroatheroma</td>
<td>Ferencik, &lt;i&gt;et al.&lt;/i&gt;, 2018&lt;sup&gt;87&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Plaque burden &gt;70%</td>
<td></td>
</tr>
<tr>
<td>• Local stress concentrations</td>
<td>Barret, &lt;i&gt;et al.&lt;/i&gt;, 2019&lt;sup&gt;88&lt;/sup&gt;</td>
</tr>
<tr>
<td>• Tissue strength properties</td>
<td></td>
</tr>
</tbody>
</table>
In general, there is a common agreement that thin cap fibroatheromas and the distinguishing features of this type of lesion, the thin fibrous cap and large lipid core, characterise a vulnerable plaque. Alongside plaque composition, the biomechanics surrounding plaque components have gained heightened interest\textsuperscript{89}. Specifically, the biomechanical relevance around calcifications\textsuperscript{88} and at the shoulders of the fibrous cap\textsuperscript{75,90,91} have been shown to influence the rupture risk of atherosclerotic lesions. The differentiation between stable and rupture-prone plaques remains a challenging area with great interest as it ultimately could decrease the morbidity and mortality of CVD.

2.2.1.3 Mechanical properties

Initial studies investigating the composition of atherosclerosis date back to the 1960s\textsuperscript{92}, while in the last 30 years mechanical characterisation of plaque tissue has come to the forefront with an aim to better define rupture risk. A few initial studies of aortic tissue highlighted the comprised mechanical integrity of calcified regions\textsuperscript{93,94}; calcified regions consistently fail at lower strains. As increasing studies have been published investigating carotid, coronary, femoral and iliac atherosclerotic plaques – the highly variable nature of these tissues has become more and more apparent\textsuperscript{94–98}. Due to the complex and dynamic microstructure within these tissues, this variability is to be expected. Table 2.4 outlines the relevant literature surrounding atherosclerotic plaque mechanics.

<table>
<thead>
<tr>
<th>Results</th>
<th>Significance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress-strain for calcified and uncalcified circumferentially cut thoracic aorta strips.</td>
<td>Tensile tests on calcified and uncalcified thoracic and abdominal aortic plaques. Calcified specimens rupture at lower stretch both circumferentially and axially and failed at lower stresses in most cases.</td>
<td>Sherebrin, Bernans, and Roach, 1987\textsuperscript{93}</td>
</tr>
</tbody>
</table>
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Tensile testing of intimal layer of coronary plaques. Results showed considerable variability in both directions. Born and Richardson, 1990<sup>95</sup>

**Stress-stretch for axial and circumferential intimal strips from coronary plaques**<sup>99</sup>.

Tensile testing of aortic plaque caps of intact and ulcerated plaques. Plaque caps of ruptured plaques showed significantly increased extensibility and macrophage density and decreased maximum stress when compared to unruptured plaques. Lendon, et al., 1991<sup>100</sup>

Macrophage density, maximum extension, and maximum stress of plaque caps from ulcerated and non-ulcerated plaques.

Tensile testing of fibrous caps from aortic plaques. The tangential modulus of atherosclerotic plaque is not affected by the degree of cellularity or calcification. All strips demonstrated a significant increase in tangential modulus with increasing applied circumferential stress. Loree, et al., 1994<sup>94</sup>

Tangential modulus vs stress for cellular, hypocellular, and calcified caps.

Tensile testing of intimal and medial tissue from iliac plaques. Results showed anisotropic and nonlinear tissue properties, and high variability between subjects. The adventitia showed the highest mechanical strength, while the non-diseased media the lowest. Holzapfel, Sommer, and Regitnig, 2004<sup>98</sup>

**Stress-stretch response of fibrous cap, fibrotic intima, and diseased media of circumferentially cut strips**<sup>97</sup>.
Tensile tests on carotid plaques.
Used ultrasound to group specimens by calcified, echolucent, or mixed. Found calcified plaques had the stiffest response, while echolucent are least stiff. 

Maher, et al., 2009

Tensile testing of carotid plaque tissue.
Intact plaque strips showed similar failure stress to the media in circumferential direction, but twice that of media in the axial direction. The adventitia, media, and intact specimens showed similar extensibility in both directions.

Teng, et al., 2009

Failure strain (left) and stress (right) grouped by post-operative mechanical stiffnesses.

Tensile testing of carotid plaque tissue.
Post-operative grouping of samples into hard, mixed, or soft tissue did not yield mechanical insight beyond the high variability.

Lawlor, et al., 2011

Pure shear tests on carotid plaque tissue.
Pre-operative classifications by ultrasound yielded no insight but post-operative FTIR showed a significant relationship between failure stress and stretch and the level of calcification.

Mulvihill, et al., 2013
The initial stress in the un-cracked segment correlated to both total collagen content (left) and total macrophage content (right).

Single edge notched tensile tests of carotid plaque fibrous caps. Higher initial stress in the un-cracked segment was significantly correlated to higher collagen content and lower macrophage content. (Davis, et al., 2016)

Tensile testing of femoral plaque tissue. Highly calcified samples failed at a significantly lower stress, while moderately calcified samples show increased stiffness and lightly calcified samples fail at relatively high stretch. (Cunnane, et al., 2015)

Stress-stretch of samples characterised by FTIR.

Tensile testing of adventitia, media, and intima of type II and III lesions in carotid arteries. The adventitia was significantly stiffer than the media in both circumferential and axial directions. The stiffness of whole specimens was nearly the same in both directions. (Hoffman, et al., 2017)

Stress-strain for axial (left) and circumferential (right) strips from adventitia (A), media (M), and intima (I).

Tensile testing of calcified, lipid, and fibrotic aortic plaques. Compared to normal aorta, lipidic plaques were weakest and calcified were stiffest. Fibrotic plaques were also stiffer than normal aorta. (Kobielerz, et al., 2020)

Stress-strain curves for normal aorta (NA) and calcified (APC), lipid (APL), and fibrotic (APF) plaques.
Lendon, et al. showed early on that aortic plaque caps are weaker at locations with high densities of macrophages, a conflicting finding to more recent work by Davis, et al. in carotid plaques caps. Specifically, Davis, et al. showed that a higher initial stress in the plaque cap is correlated with lower macrophage content and increased collagen content. Johnston, et al. showed that collagen orientation, not content, ultimately dictates the mechanical integrity of carotid plaque caps. While some of these studies used specific microstructural characterisation techniques, such as small angle light scattering, Fourier transform infrared (FTIR) and scanning electron microscopy (SEM), very few have utilised non-invasive, clinically relevant imaging.

2.3 Current diagnostics and treatment

There is a growing body of evidence suggesting that the percent stenosis, a diagnostic criterion for atherosclerotic plaque vulnerability established in the 1980s and 1990s, is not an adequate indicator. The percent stenosis refers to the amount of luminal blockage by an atherosclerotic plaque. While studies have investigated geometric features of plaques and their usefulness in determining vulnerability, there is a recent acknowledgement that plaque composition and microstructure could better capitate rupture risk. More specifically, the percent stenosis falls short both in low grade stenosed plaques
and asymptomatic patients\textsuperscript{114}. A recent study identified that only 62.9\% of stroke patients had carotid stenosis, and of that, 54.5\% had only had mild stenosis (30 – 49\%)\textsuperscript{115}.

The distinction between symptomatic and asymptomatic carotid atherosclerosis plays an important role in treatment planning. Typically, there are three routes for the treatment for atherosclerosis: (1) lifestyle changes, (2) pharmacological agents and (3) surgical intervention\textsuperscript{56}. Patients are considered symptomatic if they present with a transient ischaemic attack or stroke in the last six months. For these patients, there is evidence to intervene as soon as possible after symptom onset – via either CEA or carotid artery stenting\textsuperscript{17}. The European Society for Vascular Surgery advised that both options have a role in the treatment of carotid stenosis, but evidence is slightly higher for CEA. Patients with low-grade carotid artery stenosis, less than 50\%, are generally prescribed lipid lowering or antihypertensive agents\textsuperscript{56}. Past 50-60\% stenosis, CEA surgery is recommended\textsuperscript{56}. CEA involves the invasive procedure of opening a patient’s carotid artery and removing the atherosclerotic tissue. Dissimilar to symptomatic patients, the optimal treatment for asymptomatic patients with carotid stenosis is highly controversial\textsuperscript{10}. A large majority of patients will present asymptptomatically which further highlights the need to accurately determine what the best treatment plan is for an individual. It has been shown that only about 5\% of asymptomatic patients who undergo this high-risk surgery benefit over medical treatment alone\textsuperscript{116}. Table 2.5 outlines a recently published algorithm for the management of patients with asymptomatic carotid stenosis.
Table 2.5. Algorithm for treatment of carotid stenosis in asymptomatic patients\textsuperscript{19}.

<table>
<thead>
<tr>
<th>Patient type</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any patient with (&gt;50%) carotid stenosis</td>
<td>Initiate best medical therapy and lifestyle changes (weight-loss, smoking cessation, physical activity)</td>
</tr>
<tr>
<td>Frail patient with several comorbidities and estimated life expectancy (&lt;5) years</td>
<td>Best medical therapy alone and lifestyle measures</td>
</tr>
</tbody>
</table>
| Patient with life expectancy \(>5\) years and few comorbidities | Best medical therapy and consider revascularisation based on\textsuperscript{19,117}:  
  - Presence of silent infarction on brain CT?  
  - Impaired cerebrovascular reserve?  
  - Plaque echolucency on Duplex ultrasound?  
  - Spontaneous embolization on transcranial Doppler?  
  - Contralateral cerebrovascular event?  
  - Asymptomatic carotid stenosis progression despite medical therapy  
  - High-risk plaque features?  
  - Individual patient preferences  
If yes to \(>1\), consider revascularisation |

Treatment ranges from lifestyle changes and medical therapy to surgical interventions\textsuperscript{56}. The Society for Vascular Surgery recently published guidelines recommending CEA surgery with best medical therapy for ‘low surgical risk patients’\textsuperscript{118}. Similarly, the European Stroke Organisation recommended surgical intervention for asymptomatic patients with a percent stenosis \(>60\%\)\textsuperscript{119}. Population level screening would allow for the potential to identify asymptomatic patients early on, but is not currently recommended\textsuperscript{120}. However, this begs the question – if we had a better indicator for the likelihood of a plaque to rupture, would screening be beneficial? As with all diseases, accurate diagnosis is essential to determine adequate treatment plans and prognoses. In recent years, imaging techniques have advanced showing the potential for better clinical indicators and improved clinical diagnosis; however, imaging the vulnerable plaque remains elusive.

2.3.1 Invasive imaging

While intravascular imaging was previously only used for post-operative visualisation, it also can be used prior to interventions to measure vessel and lumen size\textsuperscript{121}. Specifically, intravascular ultrasound (IVUS) and intravascular optical coherence tomography (IVOCT) are in practical use. IVUS uses a transluminal catheter to gain insight into the thickness and acoustic density of the vessel wall and has since been used to look at the composition of atherosclerotic lesions\textsuperscript{81,122,123}. Additionally, “virtual histology” IVUS uses the backscatter
from ultrasound radiofrequencies to look at the spectral analysis of different tissue types\textsuperscript{124,125}.

Optical coherence tomography, similar to IVUS but with the addition of a high-bandwidth infrared light source rather than an ultrasound crystal, has been used to produce high resolution images. Within coronary arteries, IVOCT has shown sufficient resolution to image cap thickness as well as thin cap fibroatheromas\textsuperscript{127}. IVOCT also shows features of inflammation and microchannels\textsuperscript{128}, but the evidence level of these findings is low. Due to issues with attenuation from blood and limited penetration depth, it has been used more frequently and successfully in research applications\textsuperscript{129}. Ultimately, non-invasive imaging techniques would be more advantageous\textsuperscript{130}.

### 2.3.2 Non-invasive imaging

Non-invasive imaging techniques have advanced dramatically in recent years to better facilitate diagnostic capabilities for both symptomatic and asymptomatic atherosclerosis patients. Angiography is the longest standing available method for \textit{in vivo} visualisation of the coronary arteries. After the injection of an iodine-based contrast agent, x-ray fluoroscopic examination of the heart allows for the visualisation of vessel space occupied by blood\textsuperscript{131}. Angiography is limited in that it only yields information on luminal stenosis, not vessel wall thickness, and it has been established that percent stenosis is not indicative of plaque vulnerability\textsuperscript{132,133}.
2.3.2.1 Ultrasound

Ultrasound, the first method to offer in vivo visualisation of vessels and plaques\textsuperscript{134}, offers a fast, inexpensive technique for monitoring plaque progression through vascular scanning. Ultrasound is typically the first method used to assess carotid artery stenosis in patients\textsuperscript{123}. With longitudinal positioning along the arteries, intimal thickening or plaque growth can be monitored\textsuperscript{126}. Ultrasound imaging quality has improved considerably with increasing bandwidth of transducers and the development of more sensitive transducer materials. Strain imaging is possible with ultrasound by scanning both the longitudinal and transverse planes. It has been shown that hard lesions demonstrate low strains and soft lesions show high strains\textsuperscript{135}. Strain measurements from ultrasound have also been used in combination with inverse finite element analysis to investigate the mechanical characteristics of plaques\textsuperscript{136}. A study by Huang, et al. showed that with strain measurements obtained from ultrasound a more stable plaque could be characterised by uniform velocity and axial strain\textsuperscript{86}. Meanwhile, a classically defined ‘vulnerable’ plaque demonstrated both a heterogenous velocity and strain map, Figure 2.8.

![Figure 2.8](image-url)

Figure 2.8. (a) Velocity and (b) axial strain estimations from (a-c) stable and (d-f) vulnerable plaques. The tissue velocity appeared relatively uniform, and the strain remains small and homogenous in the stable plaque. There is a large variation in velocity estimation for the vulnerable and regions of high strain\textsuperscript{86}.

2.3.2.2 Computed tomography

Computed tomography (CT), specifically multidetector CT, allows for evaluation of plaque composition. While multidetector CT has short acquisition time, 50 – 220 ms, it deposits a
high radiation dose to the patient\textsuperscript{131}. Calcified plaque regions appear highly attenuated and bright while LRNC or fibrous regions are hypoattenuated dark regions. Multidetector CT is not effective when distinguishing different lipid cores or haemorrhages; however, it can accurately detect ulcerations and thin fibrous caps\textsuperscript{137}. CT angiography has been shown to identify high-risk plaques by low attenuation, napkin-ring signs and positive remodelling\textsuperscript{138,139}.

\subsection*{2.3.2.3 Magnetic resonance imaging}

MRI offers unparalleled soft tissue contrast, and, despite its high operational costs, it is widely used clinically as it poses no safety threats. MRI has been explored both \textit{in vivo} and \textit{ex vivo} for cardiovascular findings as well as an investigative tool for atherosclerosis. Table 2.6 summarises \textit{in vivo} MRI studies focused on atherosclerotic plaques from the literature.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Results} & \textbf{Significance} & \textbf{Reference} \\
\hline
MR of plaque with fibrous cap rupture. & \textit{In vivo 3D MOTSA imaging of carotid plaques at 1.5 T.} & Hatsukami, \textit{et al.}, 2000\textsuperscript{140} \\
3D MOTSA sequence can distinguish intact, thick fibrous caps from intact thin and disrupted caps in carotid plaques. & \textit{In vivo TOF, T1-, T2-, PD weighted imaging of carotid arteries at 1.5 T.} & Cai, \textit{et al.}, 2002\textsuperscript{141} \\
Example of type I-II lesion, asterisk is lumen, \textit{JV} is jugular vein. Arrows indicate carotid wall. & \textit{Investigated type I-VI lesions with multicontrast MRI. Lesion classification by MR and histology showed good agreement.} & \\
\hline
\end{tabular}
\caption{Summary of relevant studies utilising MRI \textit{in vivo} for atherosclerotic plaque investigations and research. Abbreviations are: MOTSA (multiple overlapping thin slab angiography), TOF (time of flight), PD (proton density), DW (diffusion weighted), ADC (apparent diffusion coefficient), DP-TSE (diffusion-prepared turbo spin echo), EPI (echo-planar imaging), DPsti-TSE (diffusion-prepared stimulated-echo turbo spin echo), QSM (quantitative susceptibility mapping), MEDIC (multi-echo data image combination), DW-SOS (diffusion weighted stack-of-stars).}
\end{table}
Mean percent change from baseline in carotid volumes at 16 and 24 months.

In vivo TOF, $T_1$-, $T_2$-, PD weighted imaging, longitudinally of patients after CEA at 1.5 T. Showed that MRI can be used to non-invasively track changes in carotid atherosclerosis – with notable changes at 16 and 24 months. Adams, et al., 2004\textsuperscript{142}

In vivo and ex vivo TOF, $T_1$-, $T_2$-, PD-weighted imaging on carotid plaques at 3.0 T. 70% of patients with vulnerable plaques had mild to moderate stenosis (<70%), while type VI lesions saw the highest degree of stenosis. In stroke patients, the number of patients with thin or ruptured fibrous caps was double that of intact, thick caps. Gao, et al., 2009\textsuperscript{143}

ADC values for LRNC (red) versus the fibrous cap (white).

In vivo DWI of lipid rich cores in carotid plaques at 1.5 T. In moderate to severe stenosed patients, DWI was used to differentiate the LRNC from fibrous cap. Also suggested a correlation between the ADC and histological lipid core gradings. Young, et al., 2010\textsuperscript{144}

In vivo and ex vivo DWI for ADCs for lipid necrotic core and haemorrhage at 3.0 T. Demonstrated ability to compare in vivo ADC to ex vivo measurements. Kim, et al., 2011\textsuperscript{145}
Type VI lesions were most common in symptomatic patients, while asymptomatic was more variable.

*In vivo* black-blood TOF, T1-, T2-, PD-weighted images of carotid plaques at 3.0 T. Symptomatic patients showed mostly type VI lesions with ruptured fibrous caps, thrombus, and IPH. Asymptomatic patient lesion type varied considerably. 

Grimm, *et al.*, 2013

Carotid plaque composition with time after stroke; FC – fibrous cap, GE – gadolinium enhancement, IP – intraplaque; LC – lipid-rich necrotic core.

*In vivo* TOF, T1-, and PD weighted imaging of symptomatic and asymptomatic patients at 3.0 T. Presence of IPH and ruptured fibrous cap was significantly higher in symptomatic patients than asymptomatic.

Millon, *et al.*, 2013

Improved image quality and resolution of the carotids at two b-values.

*In vivo* black-blood DP-TSE imaging of healthy volunteers and patients with carotid atherosclerosis at 3.0 T. Diffusion-prepared turbo spin echo sequence demonstrated improved resolution and image quality of the vessel wall.

Xie, *et al.*, 2014

Ex vivo (top row) and corresponding in vivo (bottom row) plaques.

*In vivo* (3.0 T) and ex vivo (7.0 T) T2-, T1-, and DW imaging correlative study on atherosclerotic plaques. The difference in plaque area between ex vivo and histology was found to be 16.1 ± 16% while *in vivo* to ex vivo resulted in a 29.6 ± 5% area difference.

In vivo DTI using read-out segmented EPI on healthy carotid arteries at 3.0 T. Demonstrated feasibility of a 2D DTI scan for in vivo carotid imaging. Found a negative correlation between fractional anisotropy and age in the carotid vessels of volunteers. Opriessnig, *et al.*, 2016\(^{148}\)

In vivo quantitative T2 imaging of carotid plaques at 3.0 T. Strong correlation between lipid area in MR and histology, with lipid area being significantly higher in symptomatic patients despite similar degrees of stenosis. Chai, *et al.*, 2017\(^{149}\)

In vivo cross-sections comparing DWI sequences at two b-values and the resultant ADC maps. Zhang, *et al.*, 2017\(^{150}\)
As shown in Table 2.6, there have been a considerable number of studies using MRI to characterise plaque components for the past 20+ years. Multicontrast studies have shown...
the capability to identify plaque components\textsuperscript{20,21,141–143,147,154}, but there is a clear shift in the last five years where studies have started to focus on the promise of individual contrasts\textsuperscript{113,149–153}. However, to be able to interpret what is seen \textit{in vivo}, basic research needs to be conducted to identify specific changes and make sense of the contrast observed. \textit{Ex vivo} investigations allow for high-resolution, in-depth studies into MR contrast in arterial tissue, which ultimately can be used to infer clinical observations. For this reason, high-field MRI machines, including 4.7, 7.0, 9.4, 11.7, 16.4, 17.6, and 21.1 T\textsuperscript{155–157}, are used. Table 2.7 summarises MRI studies which also look specifically at arterial tissue and atherosclerotic plaques, but in \textit{ex vivo} scenarios.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
\textbf{Results} & \textbf{Significance} & \textbf{Reference} \\
\hline
Diffusion maps with gradient direction oriented parallel (left) and perpendicular (right) to luminal surface. & \textit{Ex vivo pulse field gradient nuclear magnetic resonance for atherosclerosis and thrombosis.} The ADC was calculated for different regions of iliac and femoral lesions. The lipid core was found to have isotropic diffusion. & Toussaint, et al., 1997\textsuperscript{158} \\
\hline
Cross-sections of carotid plaque with H&E staining, MR contracts, and segmented ROIs. & \textit{Ex vivo} \textit{T}1, \textit{T}2, DW, PD, and partial \textit{T}2 weightings for atherosclerotic plaques at 9.4 T. Proton density highlighted calcification, \textit{T}2-weighting identified the lipid core, DWI showed thrombus, and parametric \textit{T}2 fibro-cellular tissue. & Shinnar, et al., 1999\textsuperscript{159} \\
\hline
\end{tabular}
\end{table}
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- **MR microimage and corresponding histological slice.**
  - Ex vivo $T1$ and $T2$ weighted imaging of carotid plaques at 9.4 $T$.
  - Area differences between histology and MRI ranged from 4.5–14.2% depending on component. Calcified areas had a short $T1$ and $T2$, while lipid had a long $T1$ and longer $T2$.
  - Morrisett, et al., 2003\textsuperscript{160}

- **Normalised signal intensity for different components for each contrast (in order listed to right).**
  - Ex vivo $PD$, partial $T2$, $T2$, $FIESTA$, $T1$ SPGR, $MT$, $DW$ imaging of carotid plaques at 1.5 $T$.
  - Used multiple MR contrasts and a minimum distance classifier to train segmentation tool which could identify calcifications and necrotic cores most accurately.
  - Clarke, et al., 2003\textsuperscript{161}

- **Rabbit aorta with white thrombus (top row) and platelet aggregates (bottom row).**
  - Ex vivo DWI on plaque ruptured thrombus at 11.7 $T$.
  - Investigated white versus red thrombus in plaques. DWI allowed for the cell, platelet/fibrin-rich thrombus to be distinguished from the vessel wall.
  - Viereck, et al., 2005\textsuperscript{162}

- **T1, T2, and DW images of lipid rich core.**
  - Ex vivo DWI of lipid rich core at 11.7 $T$.
  - Lipid diffusion in the plaque is consistently slow, highlighting the capabilities of DWI to identify lipid content in plaques.
  - Qiao, et al., 2007\textsuperscript{163}

- **Multicontrast MR slices (top), T2 map, cluster map and histology (bottom).**
  - Ex vivo PD, $T2$, $T1$ weighted imaging of coronary plaques for automatic segmentation at 4.7 $T$.
  - Use of a fuzzy C-means based clustering algorithm showed promise using multicontrast MRI to detect plaque components.
  - Sun, et al., 2008\textsuperscript{164}
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*Ex vivo DTI of aortic tissue at 7.0 T.*
Investigated the fibrous structure of aortic tissue using the eigenvectors from the diffusion tensor and found helically arranged fibres (15 ± 2.5° and 175 ± 2.5°).

Flamini, et al., 2010

*Ex vivo comparison between DTI and SHG in carotids at 6.3 T.*
Both methods illustrated circumferential orientation of fibres in the outer adventitia and media of arteries, confirming that DTI can identify the dominant fibre direction.

Ghazanfari, et al., 2012

*Ex vivo DTI on fresh and frozen aortic tissue at 7.0 T.*
Demonstrated that DTI was sensitive to the changes in fibre structure between fresh and crudely frozen tissue where the fibres are disrupted.

Flamini, et al., 2013

*Ex vivo phase contrast MRI of aortic tissue at 7.0 T.*
Used phase contrast MRI to measure circumferential strain in tissue and inform computation models. Showed feasibility of adapting image originated parameters to derive in situ mechanical properties.

Flamini, et al., 2015

*Ex vivo T1, T2, T2*, and PD weighted imaging of intracranial plaques at 7.0 T.*
T1 maps showed the most differences between individual components – with significant differences between lipid, fibrous tissue, fibrous cap, calcification, and vessel wall.

Harteveld, et al., 2016
Multicontrast MR images and T1, T2, T2* maps alongside representative histology

Ex vivo T1, T2, T2*, PD weighted and STIR imaging of intracranial plaques at 3.0 T. T2 and T2* values of the lipid core are significantly different from those of the fibrous cap, but comparable to fibrous tissue and healthy wall. Jiang, et al., 2016

Colour-coded tractography of carotid plaque. Circumferential alignment is blue-red, while longitudinal (left to right) is green.

Ex vivo DTI on carotid atherosclerotic plaques at 9.4 T. Used DTI to investigate fibre distribution in atherosclerotic plaques; in the examined plaques 52% of the fibre distribution was circumferential, 34% longitudinal, and 14% radial. Akyildiz, et al., 2017

Projected helical angles on tractography and ellipsoidal representation of the main fibre orientation.

Ex vivo 2D SE DTI-EPI on carotid arteries at 7.0 T. Found significant variations in FA and MD across the medial layer in intact arterial rings, but not for rings cut open. Demonstrated near circumferential alignment in intact samples by tractography and the helical angles. Shahid, et al., 2017

QSM and MPRAGE images of carotid plaques and histology located in the yellow outline.

Ex vivo QSM of carotid plaques at 3.0 T. Found different susceptibilities between calcification, necrotic core without IPH or iron deposits, iron deposits without IPH, and IPH without iron deposits. IPH had the highest susceptibility while calcification had the lowest. Azuma, et al., 2020
Ex vivo studies have allowed for improved understanding of pathological features of atherosclerotic plaques and provide a link between histology and diagnostic MRI\cite{Ex vivo studies}. Figure 2.9 and Figure 2.10 attempt to consolidate literature values for the plaque components characterised by DWI, yielding apparent diffusion coefficients (ADC). While the ADC cannot clearly distinguish all plaque components, it most successfully differentiates LRNC from the vessel wall as the LRNC has significantly more restricted diffusion\cite{LRNC DWI, LRNC ADC, LRNC ADC1, LRNC ADC2}. Figure 2.11 consolidates literature values for T1 and T2. Despite the AHA classifications for

Multicontrast MR images with corresponding histology and T2* vs T1 plot.

Ex vivo T1 and T2* mapping at 11.7 T.
Saw good agreement between high field MRI and histology and used quadratic discriminant analysis to classify components but found a high degree of misclassifications when plaques had IPH or inflammation. Truong, et al., 2021\cite{Truong et al.}

Comparison of DTI tensor shapes for different tissue models.

Ex vivo DTI on carotid arteries at 7.0 T.
Demonstrated that non-gaussian schemes can characterise microstructural changes in arterial tissue. The stretched exponential model being more sensitive to changes in SMC, while the bi-exponential model was more sensitive to changes in collagen. Shahid, et al., 2021\cite{Shahid et al.}

Susceptibility values for various porcine tissue models.

Ex vivo QSM of carotid arteries at 7.0 T.
QSM results from numerous degraded porcine arterial tissue models shows a specific sensitivity to collagen. Also saw no significant difference between healthy fixed porcine tissue and fixed human carotids. Stone, et al., 2021\cite{Stone et al.}
Chapter 2

atherosclerotic lesions, there is a wide range of terminology used when classifying regions of plaques which affects the precision of comparing results from literature.

Figure 2.9. Compiled ADC values from in vivo studies in the literature\textsuperscript{113,144,145,148,150,153}. Mean values and standard deviations presented. LRNC – lipid-rich necrotic core, FC – fibrous cap, VW – vessel wall, T – thrombus, H – haemorrhage. B-values differ between studies.

![Diagram of ADC values from in vivo studies](image)

Figure 2.10. Compiled ADC values from ex vivo studies in the literature\textsuperscript{145,158,159,162,163,172}. Mean values and standard deviations presented. The grey box represents a threshold. LRNC – lipid-rich necrotic core, FC – fibrous cap, VW – vessel wall, T – thrombus, H – haemorrhage. B-values differ between studies.

![Diagram of ADC values from ex vivo studies](image)
rupture prone plaques. From MRI it would have the potential to offer a more definitive delineation of stable versus composition which links to a more biomechanical perspective of atherosclerosis. It has been suggested that the definition classifications of vulnerable plaques, MRI offers an abundance of valuable information with respect to the mechanics of atherosclerosis. However, as discussed previously in the literature, the reanalysis of published data, reported by the red boxes. Black circles are plotted on the left y-axis for T1 values, red on the right y-axis for T2 values. LC – lipid core, FC – fibrous cap, VW – vessel wall, T – thrombus, C – calcification.

MRI offers an abundance of valuable information with respect to investigating atherosclerosis and the cardiovascular system. However, as discussed previously in the classifications of vulnerable plaques, MRI – at current – does not inform on the mechanics of atherosclerosis. It has been suggested that the definition of vulnerable plaques needs to shift to a more biomechanical perspective. If further information on plaque composition which links to mechanical integrity, such as fibre orientation, could be acquired from MRI it would have the potential to offer a more definitive delineation of stable versus rupture prone plaques.
2.4 Magnetic resonance imaging

As previously mentioned, MRI offers unparalleled soft tissue contrast which is invaluable when it comes to both diagnostics for and research relevant to CVD. To get the most of the information obtained, it is critical to understand the mechanisms behind this state-of-the-art imaging technique.

MRI works due to the abundance of hydrogen nuclei, which have a magnetic moment, in the body. These hydrogen protons will attempt to align parallel, in a low energy state, or anti-parallel, a higher energy state, in a magnetic field, $B_0$ (Figure 2.12). Torque prevents these protons from aligning exactly so they precess in the direction of the field. This precession is proportional to the strength of the magnetic field and can be seen in the Larmor equation, Equation 2-1, where $\omega_0$ is the precession frequency [Hz], $\gamma$ is the gyromagnetic ratio, 42.57 MHz/T, and $B_0$ is the strength of the magnetic field [T].

$$\omega_0 = \gamma B_0$$

Equation 2-1

These protons spin out of phase in equilibrium and form a net magnetization vector, $M_0$, which aligns with $B_0$. This vector, $M_0$, is manipulated in pulse sequences to get measurable information.

![Figure 2.12. Upon insertion into a magnetic field, protons align (a) parallel or anti-parallel to $B_0$ and (b) precess (c) out of phase in the direction of the main field, forming a net magnetisation vector.](image)

The manipulation of $M_0$, tipping it into the transverse plane, creates a signal which can be recorded. Radiofrequency pulses (RF) applied at flip angles, 90° in spin-echo (SE) and <90° in gradient-echo (GE) sequences, bring all the proton spins into phase in the transverse plane with a fixed magnetic field, $B_1$.

The creation of echoes, either SE or GE, generate the specific measurable signal by the receiver. GE sequences work by the application of a negative gradient after the RF pulse in
order to rapidly dephase the protons, followed by a positive gradient to reverse the magnetic field gradient, Figure 2.13\textsuperscript{179}.

![Gradient echo sequence](image)

Figure 2.13. (a) Gradient echo sequence. (b) Spins dephase after a negative gradient (c) and begin to rephase after the positive gradient (d) which generates an echo (e). If the gradient remains on the dephasing occurs again (f)\textsuperscript{179}.

Dephasing spins begin to rephase forming a gradient echo. The positive gradient only rephases protons which were dephasing from the negative gradient, not any from field inhomogeneities or spin-spin relaxations. The echo height, $S_{GE}$ in Equation 2-2, depends on $T_2^*$ relaxation, where $S_0$ is the initial height of the free induction decay.

$$S_{GE} = S_0 \exp \left( -\frac{TE}{T_2^*} \right)$$

*Equation 2-2*

$T_2^*$ relaxation is a composite of $T_2$ relaxation that accounts for field inhomogeneities, tissue susceptibilities, and diffusion of protons\textsuperscript{179}, where $\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_{2\text{inhomogeneities}}}$.

In SE sequences, the protons dephase naturally after a 90° RF pulse for a certain time, half the echo time (TE), and then another RF pulse is applied at a flip angle of 180°. This RF pulse does not alter the precession frequencies, only the phase angles. This means that dephasing, which was anticlockwise, is now clockwise and vice versa, Figure 2.14.
Figure 2.14. (a) Spin echo sequence. The in-phase spins (b) dephase naturally after 90° RF pulse (c) until a 180° RF pulse (d) reverses the phase angles (e), generating an echo (f) before final dephasing again (g).

After the full TE, the spins come back into phase and forms the spin echo. This sequence allows for the decay curve to solely depend on $T_2$ and diffusion. Equation 2-3 shows this relationship, where $\Delta B$ is the magnetic field inhomogeneities and $D$ is the ADC. However, TE is typically short enough compared to $T_2$ that the second term is dropped, yielding Equation 2-4, for simplification. Table 2.8 and Table 2.9 outline the contrast weightings possible with each sequence.

\[
S_{SE} = S_0 \exp \left[ -\frac{TE}{T_2} - \frac{2}{3} \gamma^2 \Delta B^2 D \left( \frac{TE}{2} \right)^3 \right]
\]

Equation 2-3

\[
S_{SE} = S_0 \exp \left( -\frac{TE}{T_2} \right)
\]

Equation 2-4

Table 2.8. GE sequences: contrast weightings from TE/TR (repetition time) combinations\textsuperscript{179}.

<table>
<thead>
<tr>
<th>Flip angle, $\alpha$</th>
<th>TE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small ($\alpha &lt; 40^\circ$)</td>
<td>Proton density, $T_2$</td>
</tr>
<tr>
<td>Long ($\alpha &gt; 50^\circ$)</td>
<td>$T_1$, Not useful</td>
</tr>
</tbody>
</table>

Table 2.9. SE sequences: contrast weightings from TE/TR combinations\textsuperscript{179}.

<table>
<thead>
<tr>
<th>TR</th>
<th>TE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Short (&lt; 40 ms)</td>
<td>$T_1$, Not useful</td>
</tr>
<tr>
<td>Long (&gt; 1500 ms)</td>
<td>Proton density, $T_2$</td>
</tr>
</tbody>
</table>
Different adaptations of these sequences have led to the development of advanced imaging sequences that probe intrinsic and physical properties of tissues.

Field strength, acquisition time, spatial resolution, and signal-to-noise (SNR) are significant considerations within all MR imaging and are all connected. Clinical scan times are typically under 10 minutes and are performed routinely with 1.5 and 3.0 T scanners\textsuperscript{180,181}. \textit{In vivo} resolution varies depending on the MR sequence but can range between 0.5 to 2 mm with promising studies showing potential for higher resolution imaging\textsuperscript{182}. One benefit of \textit{ex vivo} MR imaging is the potential for increased field strength, SNR and higher resolution facilitating in-depth investigations into tissue. Spatial resolution is determined by the imaging field of view (FOV), image size and slice thickness. The resulting voxel sizes are the main determinants of SNR. As the voxel size increases, so too does the SNR linearly. Smaller voxels (higher resolution) aid in mitigating partial voluming effects, where one voxel’s signal is attenuated by multiple potentially different tissues.

2.4.1 Diffusion weighted imaging

Based off work from Hahn\textsuperscript{183} and Carr and Purcell\textsuperscript{184}, Stejskal and Tanner developed the pulsed gradient spin echo (PGSE) which is the basis of the current state of the art diffusion weighted imaging (DWI) sequences\textsuperscript{185}. The PGSE was derived in order to control the time over which diffusion is being measured, feasible by the addition of two gradients on either side of the 180° RF pulse, Figure 2.15. By manipulating the parameters of these gradients – $\delta$, the duration of the diffusion gradient, and $\Delta$, the time between gradients – the two diffusion gradients induce and control a diffusion weighting – termed the $b$-factor\textsuperscript{179,185,186}. 


Figure 2.15. PGSE sequence for diffusion weighting. $\delta$ denotes the diffusion gradient duration and $\Delta$ is the spacing between gradients. $G$ is the magnitude of the diffusion-weighting gradient.

The signal strength of DWI is based on Equation 2-5, where $S_b$ is the signal at one specific b-value and $D$ is the ADC.

$$S_b = S_0 \exp(-bD)$$

Equation 2-5

The b-value, s/mm$^2$, is calculated by $G$ (gradient strength), $\delta$, and $\Delta$ in Equation 2-6.

$$b = \gamma^2 G^2 \delta^2 \left( \Delta - \frac{\delta}{3} \right)$$

Equation 2-6

DWI works because of Brownian motion, i.e. random molecular motion, during the applied diffusion gradients. In the presence of a strong diffusion gradient, protons will diffuse and result in a loss of signal. Using Einstein’s relation for Brownian motion and Fick’s second law of diffusion, it is possible to estimate the displacement of water molecule in a certain diffusive time, Equation 2-7. $D$ is the ADC (mm$^2$/s), $t$ is the time for diffusion, and $\lambda_x$ is the displacement in the x-direction.

$$\lambda_x = \sqrt{2Dt}$$

Equation 2-7

Water diffusion at body temperature is typically estimated to be $10^{-3}$ mm$^2$/s, such that in a diffusion time of 50 ms free water can diffuse approximately 10 $\mu$m – roughly the size of...
tissue components, like cells. Diffusion can be restricted where physical barriers inhibit this random motion (Figure 2.16), such as cell walls or fibres. DWI is one-dimensional and allows for the calculation of the ADC in a volume based on an unweighted diffusion image and multiple diffusion weighted images.

Anisotropy refers to differing values in different directions. We know water has isotropic diffusion properties, that is the molecules diffuse freely and equally in all directions. One could expect due to the underlying architecture of a tissue, the diffusion might be anisotropic.

### 2.4.2 Diffusion tensor imaging

One step up from DWI is DTI. DTI uses the same principles as DWI but applies the diffusion gradients spatially in at least six non-coplanar, non-collinear directions so to model the diffusion tensor. This means that within one volume (one pixel) there is a tensor of information on the diffusion occurring in each direction – any anisotropic properties of a tissue become apparent. The diffusion is no longer defined by a singular scalar coefficient, but by a symmetric tensor, $\mathbf{D}$, which describes the molecular motion along each axis, Equation 2-8.

\[
\mathbf{D} = \begin{bmatrix}
D_{xx} & D_{xy} & D_{xz} \\
D_{xy} & D_{yy} & D_{yz} \\
D_{xz} & D_{yz} & D_{zz}
\end{bmatrix}
\]

Equation 2-8

The signal attenuation can then be calculated by
\[
\ln \frac{S}{S_0} = \left( \sum_{i=x,y,z} \sum_{j=x,y,z} b_{ij} D_{ij} \right)
\]

Equation 2-9

where \( b_{ij} \) are the elements of the b-matrix, replacing the b-factor from Equation 2-5. In a virtual reference frame that coincides with the principal directions of diffusivity, the tensor is reduced to only the diagonal terms\(^{186}\).

Most commonly the diffusion tensor is modelled as the so-called diffusion ellipsoid, Figure 2.17\(^{189}\). The diffusion ellipsoid represents the tensor in the x-, y-, and z-directions and is described by the eigenvectors and eigenvalues. The eigenvectors represent the vectors which describe the orientation of the diffusion tensor, while the eigenvalues are scalars which describe the size and shape of the tensor. More plainly, the eigenvalues tell you how the diffusion is weighted per direction while the eigenvectors describe the principle, radial, and transverse diffusion directions\(^{190}\).

![Diagram of diffusion tensor and eigenvectors](image)

From this tensor several useful metrics can be derived. The eigenvalues are used to calculate a number of rotationally invariant metrics. The trace, calculated in Equation 2-10\(^{189}\), is the sum of the three eigenvalues. The trace is indicative of the overall diffusion in a given volume.

\[
\text{Tr}(D) = \lambda_1 + \lambda_2 + \lambda_3
\]

Equation 2-10

A scaled version of the trace, called mean diffusivity (MD)\(^{189}\), represents the average displacement of protons, Equation 2-11. It represents the overall mean squared displacement of molecules within one volume, with no regard to directionality.
One of the most widely used metrics from DTI, fractional anisotropy (FA), is calculated in Equation 2-12. The FA is indicative of the degree of anisotropy in a volume, how much of the diffusion tensor’s magnitude is anisotropic. On a scale of 0-1, it quantifies the ratio between the anisotropic diffusion and the overall diffusion. Purely isotropic media would have a FA of 0, and with more alignment this would increase until fully anisotropic in one direction yields an FA of 1.

\[
FA = \frac{\sqrt[3]{(\lambda_1 - \lambda)^2 + (\lambda_2 - \lambda)^2 + (\lambda_3 - \lambda)^2}}{\sqrt{\lambda_1^2 + \lambda_2^2 + \lambda_3^2}}
\]

Equation 2-12

There are a number of other orientation independent metrics which can be derived and calculated from the diffusion tensor; however, the MD and FA typically are sufficient to characterise the diffusion within a tissue.

Not only can the tensor yield rotationally independent measures of anisotropy, but it also details the predominant direction of water diffusion within a voxel. Tractography operates under the assumption each voxel is characterised by a single predominant fibre orientation and as such, a global fibre trajectory in a volume can be modelled. The local orientations can be referred to as 3D vector fields, while their global trajectories are known as streamlines. A streamline is defined as any curve that along its trajectory is tangent to the vector field and can be represented as a curve in 3D space, \( r(s) \), parameterised by the arc length \( s \). For the streamline to align with the vector field, the tangent arc length must equal at the vector at the corresponding position:

\[
r(s) = \int_{s_0}^{s} v[r(s)]ds,
\]

Equation 2-13

where \( r(s) \) denotes the 3D position along the streamline, \( r(s_0) = r_0 \) is the starting point (also referred to as the seed point) and \( v \) is the 3D vector field. Deterministic tractography assumes one unique fibre direction in each voxel and therefore provides one pathway from each seed point. Continuity between points is considered with respect to the
angle between them and results can change drastically when parameters such as that angle, an FA threshold, tract length, and step size are changed.

2.5 Vascular tissue engineering

On the other side of diagnostics and tools for catching disease at an early stage, lies the need for better medical therapies and treatment options for patients. Research interest in small-diameter (<6 mm) vascular grafts has steadily been gaining intense traction over the past 50 years.\textsuperscript{194–196} Vascular tissue engineering is believed to be a viable strategy for the treatment of vascular disease in small vessels, for reconstruction or bypass of occlusions and aneurysms or even haemodialysis.\textsuperscript{194,197,198} In the absence of the favourable autologous grafts\textsuperscript{199–201}, which are limited in supply, and in cases where synthetic materials such as Dacron\textsuperscript{®} and polytetrafluoroethylene are not suitable\textsuperscript{202,203}, TEVG could be the answer. The overarching aim of tissue engineering is to create a 3D scaffold which when implanted integrates into native tissue and ultimately becomes indistinguishable in form and function. For small-diameter TEVG this means having similar viscoelastic properties to native arterial tissue, remaining nonthrombogenic, and maintaining adequate patency during its’ lifetime.\textsuperscript{195,201,204}

Table 2.10 illustrates the variety in compliance required for vessels and the current synthetic replacements.

<table>
<thead>
<tr>
<th>Vessel Type</th>
<th>Compliance (%/100 mmHg)</th>
<th>Suture retention strength (grams-Force)</th>
<th>Burst Pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td>8.0 – 17.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carotid</td>
<td>5.0 – 14.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Femoral</td>
<td>6.0 – 14.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popliteal</td>
<td>4.7 – 8.5</td>
<td>2200 – 4225</td>
<td></td>
</tr>
<tr>
<td>Internal thoracic</td>
<td>6.5 – 12.0</td>
<td>88 – 200</td>
<td></td>
</tr>
<tr>
<td>PTFE</td>
<td>0.2 – 0.9</td>
<td></td>
<td>2580 – 8270</td>
</tr>
<tr>
<td>Dacron</td>
<td>0.76 – 1.9</td>
<td>250 – 1200</td>
<td></td>
</tr>
</tbody>
</table>

Both natural\textsuperscript{206–209} and synthetic\textsuperscript{208,210–213} polymers as well as decellularised exogenous grafts\textsuperscript{214–222} have been extensively explored as viable options for small-diameter TEVG. Despite the advances in 3D printing\textsuperscript{208} and electrospinning\textsuperscript{210,213}, the native architecture and ECM of decellularised conduits remains desirable.\textsuperscript{223} Maintaining native biomechanical
properties in arterial grafts means preserving collagen and elastin’s architecture\textsuperscript{39}; however, this dense network poses challenges with cell infiltration past a luminal monolayer both \textit{in vitro}\textsuperscript{219,224,225} and \textit{in vivo}\textsuperscript{217}. Even when cells were injected directly into this dense extracellular network, cell proliferation through the media was limited\textsuperscript{215}. A handful of studies suggest the need for biomechanical preconditioning \textit{in vitro} in order to aid in cell infiltration\textsuperscript{210,212,221}; however, infiltration is still limited and seemingly elusive until reaching \textit{in vivo} studies\textsuperscript{214,216,218,222}. With the clear advancement of numerous strategies in vascular tissue engineering, there is a newfound need for versatile, non-invasive, and non-destructive imaging techniques to characterise the TEVG efficacy from \textit{in vitro} to \textit{in vivo}.

\subsection*{2.5.1 Non-destructive imaging}

While each imaging modality offers its own benefits, MRI is one of the few which offers potential for \textit{in vitro}, preclinical, and clinical applications\textsuperscript{226}. MRI offers unparalleled soft-tissue contrast, imaging penetration, safety, and most importantly – can provide anatomical, functional, and cellular information\textsuperscript{226–228}. In the last 15 years, a number of studies have been published investigating cell labelling contrasts such as super paramagnetic iron oxide particles\textsuperscript{229–234} or the molecule manganese porphyrin\textsuperscript{235,236}. These contrasts have been used with conventional T1-, T2-, and T2*-weighted imaging sequences and all show great cell viability as well as the ability to colocalise changes in measured signal with cell migration. Kerans et al. even showed the ability to genetically modify mesenchymal stem cells to produce intracytoplasmic magnetic nanoparticles, a promising methodology which could facilitate MR-based cell tracking\textsuperscript{237}. A benchtop-MRI has been developed which provided detailed insight into diffusion within 3D matrices and inhomogeneities in scaffolds\textsuperscript{233}. Multimodal MRI has also been used in cartilage and bone tissue engineering\textsuperscript{228,238–241}. Kotecha et al. found that T1 relaxation time and the ADC decreased in alginate beads seeded with human chondrocytes over a four week culture, while T2 relaxation times decreased up to week 3 before increasing 8\% the initial value\textsuperscript{228}. Li et al. demonstrated that quantitative magnetisation transfer imaging metrics, over T1-, T2-, and DW imaging metrics, correlated with increased GAGs in gelatin sponges seeded with mesenchymal stem cells. Cheng et al. similarly used T1-, T2-, and DW imaging to successfully differentiate between acellular matrices with and without hyaluronic acid\textsuperscript{242}. Studies have also used spatial variation in MRI contrasts to investigate how well constructs integrate into host tissue\textsuperscript{239,240,243} for cartilage and bone regeneration. DTI, to the author’s knowledge, has only been applied to tissue engineering studies twice\textsuperscript{211,244}. Ghazanfari et al. used DTI, validated
by confocal laser scanning microscopy, to monitor the evolution and reorientation of collagen fibres on polyglycolic acid meshes of different aspect ratios seeded with human vascular derived cells for up to six weeks\textsuperscript{211}, Figure 2.18. Alternatively, Gimenez et al. used in and ex vivo DTI to detect glioma cell migration in mice brains\textsuperscript{244}.

![Figure 2.18. Diffusion ellipsoid visualisation at different time points for cultured polyglycolic meshes seeded with human vascular derived cells\textsuperscript{211}.](image)

Non-invasive and non-destructive characterisation within tissue engineering is not only an advantageous goal, but also an attainable one. The ability to track recellularisation – both in quantity of cell repopulation and in quality of recellularised microstructure – would allow for non-destructive, non-terminal time points which can dictate experimental planning. The potential benefits, while significant in the transition from in vitro to preclinical studies, are also relevant in the clinical translation. It would be useful and convenient to use one consistent, reliable characterisation technique during translation, but could also positively address the 3Rs (Refinement, Reduction and Replacement) for animal studies\textsuperscript{245}. MRI offers a safe and non-ionizing imaging method which has the potential to impact preclinical experimental design by reducing the sample numbers needed by eliminating early terminal time points.

2.6 Summary

Atherosclerosis is defined as the build-up of lipid deposits and fibrous tissue in the arterial wall, progressing towards a more advanced lesion which is termed the ‘vulnerable’ plaque. Randomised trials in the 1980s and 1990s identified that the degree to which this plaque blocks the lumen – the percent stenosis – could be an adequate clinical measure to determine if a patient needs surgical intervention. However, not only is this measure only useful at the most advanced stages of disease, it also does not indicate the likelihood of a
plaque to rupture. In the past 40 years extensive research has focused on better identifying a vulnerable plaque.

As evidenced in both Table 2.1 and Table 2.4, the mechanical integrity of healthy and diseased arterial tissue has been widely investigated. Healthy arterial tissue is composed of elastin, collagen, and SMCs – which are together responsible for the mechanical integrity of the vessel wall. Specifically, the J-shaped curve seen for healthy arterial tissue is formed due to the initial extension of elastin in the wall, the gradual recruitment of collagen fibres within a transition region and ultimately leading to the stiff collagenous final part of the curve. In atherosclerosis, where the microstructure is constantly changing, so too is the mechanical environment. The complex, dynamic microstructure gives way to a complex, variable mechanical response.

There have also been numerous studies trying to address the characterisation of vulnerable plaques from an imaging standpoint. While there is a surplus of in vivo studies, see Table 2.6, there is also a need for ex vivo studies to understand and interpret the changes in imaging contrast observed, see Table 2.7. DTI is an MRI technique that shows unique promise as it measures the diffusion of protons within a tissue which is ultimately indicative of the microstructure – something we know to be critically important in arterial tissue. Despite the extensive research, no studies to date have tried to directly link the mechanical integrity of the arterial tissue to quantitative, non-invasive imaging metrics.

A similar issue arises when considering vascular tissue engineering. Natural and synthetic polymers as well as exogenous grafts have been explored as small-diameter options for TEVG. However, again we are met with this unmet need to be able to characterise their cellularisation and mechanical integrity, both non-invasively and non-destructively. MRI offers this potential in in vitro settings, as well in the translation from preclinical to clinical applications. There remains a clear need for a non-invasive imaging technique which can characterise vascular tissue, both in disease diagnosis and treatment monitoring.
Chapter 3 Diffusion tensor imaging and arterial tissue: establishing the influence of arterial tissue microstructure on fractional anisotropy, mean diffusivity and tractography

3.1 Introduction
To date, a handful of studies have explored the application of DTI to arterial tissue\textsuperscript{165,166} and demonstrated its sensitivity to changes in tissue integrity\textsuperscript{167,172,246}. These studies have laid the groundwork and demonstrated both the feasibility and promise of DTI to effectively investigate underlying tissue microstructure in arterial vessels. However, the specificity of FA, MD and tractography to individual microstructural components, such as SMCs, collagen, and elastin, remains unclear, therefore impeding the full interpretation and application of such metrics to clinical presentation. In a multifaceted microstructure like that of arterial tissue, understanding the impact of the different tissue constituents is critical to inferring any clinically relevant insights from DTI measurements.

The aim of this study is to investigate the potential of DTI to provide specific insight into microstructural changes in arterial tissue by exploring the influence of key components on FA, MD, and tractography. This is achieved using \textit{ex vivo} porcine carotid artery (PCaA) models, developed to selectively remove individual elements of arterial microstructure – SMCs, elastin, and collagen. Comparing FA, MD and tractography across these models allows for microstructural insight using DTI metrics. These metrics have the potential to yield novel characterisation of both arterial health and disease progression.

3.2 Materials and methods
All methods were carried out in accordance with relevant guidelines but as the animal tissue used in these experiments was obtained from a licensed slaughterhouse, additional approval for use by a licensing committee was not required.
3.2.1 Specimen preparation

PCaA of 6-month-old healthy Large White pigs, all from the same abattoir, were excised and within three hours of sacrifice all arteries were cleaned of connective tissue and cryopreserved together at a controlled rate of -1°C/min to -80°C in tissue freezing media. Porcine arterial tissue was chosen as a surrogate tissue due to its wide use in literature and same three-layer structure to human carotids. Tissue freezing medium constituted of Gibco RPMI 1640 Medium (21875034, BioSciences) supplemented with 0.1 M sucrose (S0389, Sigma) and 1.8 M DMSO (PIER20688, VWR International). DMSO is a cryoprotectant which has been shown to prevent the formation of ice crystals and therefore maintains tissue microstructure during freezing\textsuperscript{225,247}. Upon thawing at 37°C, vessels were rinsed in phosphate buffered saline (PBS) to remove any excess tissue freezing medium. All vessels were cryopreserved prior to treatment and imaged directly after treatment to ensure consistent preparation. Five tissue models were used in this study (n=6 for each): native, fixed native, collagen degraded, elastin degraded and decellularised PCaA. Native, decellularised, collagen degraded and elastin degraded PCaA were imaged fresh (unfixed) while the fixed native was imaged after formalin fixation. Table 3.1 and Figure 3.1 outline these models and their respective treatments. Upon thawing and after tissue treatments all samples underwent five PBS rinses in order to ensure any excess reagents were removed prior to being placed in fresh PBS for imaging.

Table 3.1. Five different PCaA tissue models and the respective treatments.

<table>
<thead>
<tr>
<th>PCaA tissue model</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>N/A</td>
</tr>
<tr>
<td>Fixed native</td>
<td>4% formalin (HT501128, Sigma) fixation for 7 days at 4°C</td>
</tr>
<tr>
<td>Collagen degraded</td>
<td>1000 U/ml purified collagenase (CLSPA, Worthington Biochemical Corporation) in MgCl$_2$ + CaCl$_2$ supplemented PBS (D8662, Sigma) at 37°C for 28 hours on a rotator</td>
</tr>
<tr>
<td>Elastin degraded</td>
<td>10 U/ml purified elastase (ESFF, Worthington Biochemical Corporation) with 0.35 mg/ml trypsin inhibitor (10109886001, Sigma) in Dulbecco’s Modified Eagle Medium, high glucose, GlutaMAX (61965026, BioSciences) at 37°C for 3.5 hours</td>
</tr>
<tr>
<td>Decellularised</td>
<td>0.1 M sodium hydroxide (S8045, Sigma) perfused through native vessels via a peristaltic pump at 2 Hz for 15 hours, followed by 0.1 M sodium chloride (S3014, Sigma) for 32 hours – all with a pressure of 100 mmHg during perfusion; then treated with 10 μl/ml DNAase (LS006343, Worthington Biochemical Corporation) and 2 μl/ml primicin (Ant-pm-2, InvivoGen) at 37°C for 19 hours\textsuperscript{225}</td>
</tr>
</tbody>
</table>
Figure 3.1. Schematic of the arterial tissue models used in this study. Four models were made from fresh tissue and had the selective removal of components – native, collagen degraded, elastin degraded and decellularised PCaA. Fixed native PCaA was also investigated.

3.2.2 MR data acquisition

A small bore (35 cm) horizontal 7T Bruker BioSpec 70/30 USR system (Bruker, Ettlingen Germany) equipped with a receive only 8-channel surface array coil, birdcage design transmit coil, shielded gradients (maximum strength 770 mT/m) and Paravision 6 software was used for all imaging. While not routinely used in clinical practice, 7T scanners have been approved for human clinical use. Its use in this study facilitated high resolution investigations into arterial tissue to establish the potential of DTI in this tissue. All vessels were positioned using a custom-made 3D printed holder placed in a 50-ml falcon tube and immersed in fresh PBS prior to imaging at room temperature. A conventional 3D SE DTI sequence with monopolar gradients was used and the parameters can be seen in Table 3.2.

Table 3.2. Parameters for the 3D DTI sequence.

<table>
<thead>
<tr>
<th>3D DTI sequence</th>
<th>17.682/1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE/TR [ms]</td>
<td></td>
</tr>
<tr>
<td>Image size</td>
<td>96 x 96 x 60</td>
</tr>
<tr>
<td>FOV [mm³]</td>
<td>30 x 30 x 18.75</td>
</tr>
<tr>
<td>Resolution [mm³]</td>
<td>0.3125 x 0.3125 x 0.3125</td>
</tr>
<tr>
<td>b-values [s/mm²]</td>
<td>0, 800</td>
</tr>
<tr>
<td>b-directions</td>
<td>10</td>
</tr>
<tr>
<td>δ, gradient duration [ms]</td>
<td>3.8</td>
</tr>
<tr>
<td>Δ, gradient separation [ms]</td>
<td>8.802</td>
</tr>
<tr>
<td>Acquisition time</td>
<td>17 hours 36 minutes</td>
</tr>
</tbody>
</table>
A 3D DTI sequence was used to take advantage of the high, isotropic resolution. It facilitates short TE which allows for the capturing of the fast-decaying signal from collagenous tissues. Standard gradient duration and separation values were used. The b-value was chosen based on previous studies optimising the b-value for arterial tissue ex vivo\textsuperscript{165,172}. Similarly, 10 b-directions were used as they have previously shown very comparable results to higher angular resolution studies as well as additional directions pose limitations regarding increasing scan time\textsuperscript{166,172}.

3.2.3 Image reconstruction and processing
All raw data was denoised\textsuperscript{248} and corrected for Gibbs ringing\textsuperscript{249} in MRtrix3\textsuperscript{250} (www.mrtrix3.org) prior to the mono-exponential tensor model\textsuperscript{185,186} fitting in ExploreDTI\textsuperscript{251}. The mono-exponential equation expands to incorporate the diffusion tensor and b-matrix – which characterises the diffusion sensitivity from the effects of the diffusion gradients, imaging gradients and cross-terms\textsuperscript{186}. From the tensor, the MD and FA were calculated in ExploreDTI. The MD represents the total diffusion within a voxel, while FA is indicative of the degree of anisotropic diffusion occurring within a voxel on a scale of 0-1\textsuperscript{190,252}.

3.2.4 Regional analysis and tractography
Regions of interest (ROIs) were manually drawn within the media of each vessel using an image created from the mean of the b = 800 s/mm\textsuperscript{2} images. Mean values of FA and MD were calculated from multiple slices within these regions for each vessel. Tractography was similarly performed for all vessels (n=6 per tissue model group) within ExploreDTI and all tissue model tractographies were modelled with the following parameters: seed point resolution: 0.3125 mm x 0.3125 mm x 0.3125 mm, FA threshold: 0.1, FA tracking threshold: 0.1 – 1, tract length: 0.5 – 50 mm, angular threshold: 30° and step size of 0.3125 mm. Representative vessels are presented for each group alongside first eigenvector-fractional anisotropy (FEFA) maps and quantitative tractography metrics were calculated for all vessels.

3.2.5 Histology
Standard histological processing was carried out for all vessels. Tissue model samples were fixed in 4% formalin for seven days at 4°C prior to stepwise dehydration in ethanol to xylene. Tissue fixation with formalin covalently binds proteins while maintaining the tissue structure\textsuperscript{253}. The main consideration is the potential for tissue shrinkage\textsuperscript{254}, whereby the vessel wall thickness may decrease. Once dehydrated, all samples were embedded in
paraffin wax and sectioned at 8 μm thick slices prior to staining. All stains, their purpose and required imaging are listed in Table 3.3. All imaging was done using an Olympus BX41 microscope with Ocular V2.0 software. Polarised light microscopy (PLM) maximises the birefringence of collagen via a polarised filter to polarise the light passing through the specimen. Two images 90° to each other were taken to adequately capture the fibre directionality. Representative histological images are presented.

Table 3.3. Histological stains used in this study, their visualisation and how they are imaged.

<table>
<thead>
<tr>
<th>Stain</th>
<th>Visualisation</th>
<th>Imaging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcian blue</td>
<td>Sulphated mucans, such as GAGs, stain blue</td>
<td>Brightfield</td>
</tr>
<tr>
<td>Haematoxylin and eosin</td>
<td>Haematoxylin stains acidic structures, like cell nuclei, purple-blue and eosin stains basic features, like cytoplasmic filaments, membranes and fibres, pink</td>
<td>Brightfield</td>
</tr>
<tr>
<td>Picrosirius red (PSR)</td>
<td>Collagen stains red, while PLM selectively highlights collagen birefringence allowing for visualisation of the orientation</td>
<td>Brightfield + PLM^{255}</td>
</tr>
<tr>
<td>Verhoeff’s</td>
<td>Elastin stains black</td>
<td>Brightfield</td>
</tr>
</tbody>
</table>

3.2.6 Statistical analysis

Statistical analysis was performed with GraphPad Prism (Version 8). One-way ANOVAs with Tukey’s post hoc test for multiple comparisons were used to analyse the variance between groups and determine significance. All numerical and graphical significance is shown as mean ± standard deviation, n=6 within each tissue model group and α=0.05 for all tests.

3.3 Results

3.3.1 Tissue model validation

Five tissue models were used in this study to investigate the sensitivity of DTI to the microstructural components of arterial tissue. The fixed native PCaA model is not presented in the histological figures as all PCaA models are fixed prior to histological processing, making it redundant compared to histology of native PCaA. Figure 3.2 shows H&E, Verhoeff’s and Alcian blue staining for the tissue models. In order to truly understand the influence of each of the tissue components, the selective removal of individual microstructural components was necessary and is confirmed here. H&E verifies cellular
content remained in all model tissues, with the exception of decellularised (Figure 3.2(d), top row) – where the complete removal of cells is confirmed. Similarly, the Verhoeff’s elastin stain validates that elastin was removed in the elastin degraded model only (Figure 3.2(c), middle row). Alcian blue (Figure 3.2, bottom row) shows a variety of GAG concentrations throughout the models. While this hasn’t been investigated in arterial tissue, GAGs have been shown to leach out of tissue when immersed in PBS in order to establish homeostasis\textsuperscript{256}. 

Figure 3.2. Histological representations of tissue models. Representative cell, elastin, and GAG in (a) native, (b) collagen degraded, (c) elastin degraded and (d) decellularised PCaA. Top to bottom: cell content visible by purple-blue nuclei in H&E, elastin shown in Verhoeff’s elastin stain in black and GAGs stained blue by Alcian blue. All imaged using brightfield microscopy. Scale bar 250 μm.

Figure 3.3 similarly validates the tissue models, but with respect to collagen content by PSR staining. The top row shows brightfield imaging of the models where collagen is visualised in red. PLM, in the second row, has a specificity for the birefringence of collagen fibres and therefore gives a representation of collagen fibre orientations. Together these confirm that the collagen degraded model (Figure 3.3(b)) removed all collagen content. These also confirm that while collagen content was not affected in the other models, neither was the collagen orientation.
Figure 3.3. Histological representation of collagen content and orientation. Collagen in (a) native, (b) collagen degraded, (c) elastin degraded and (d) decellularised PCaA. Brightfield microscopy (top row) shows all tissue stained red, where the PLM on the bottom row has a specificity for the birefringence of collagen. Scale bar 250 μm.

3.3.2 Fractional anisotropy and mean diffusivity

The mean FA measured in the media of each vessel is grouped by tissue model and presented in Figure 3.4 alongside parametric maps of FA in a representative slice for each model. Visually, the FA maps show stark differences between select tissue models. Native, fixed native and collagen degraded PCaA (Figure 3.4(a, b, and c)) appear similar. In contrast, both the elastin degraded and decellularised PCaA (Figure 3.4(e)) show lower FA ranges. The elastin degraded PCaA (Figure 3.4(d)) shows the expansion of the vessels and the apparent loss of tightly bound structure seen in native PCaA – this increase in extracellular space can be seen histologically in Figure 3.2(c). These observations were confirmed by the mean FA measured in the media of each vessel. Native and fixed PCaA demonstrated significantly higher FA than both the elastin degraded and decellularised PCaA. Additionally, the collagen degraded PCaA maintained a significantly higher FA than elastin degraded and decellularised. No significant differences were seen between native, fixed or collagen degraded PCaA.
Chapter 3

Figure 3.4. Parametric maps of FA in a representative slice for each of the tissue models. As measured in vessel media, both (a) native (N) and (b) fixed native (F) PCaA showed significantly higher FA than both the (d) elastin degraded (E) and (e) decellularised (D) tissue models. (c) Collagen degraded PCaA also showed a significantly higher FA than both elastin degraded and decellularised PCaA. FA maps scaled to show 0 to 0.5 (**p=0.0018 (C vs. E), **p=0.0016 (C vs. D), *** p<0.0001).

Parametric maps of MD and regional values of MD extracted from the vessel media for each tissue model are presented in Figure 3.5. Native and fixed PCaA showed significantly lower MD values than the elastin degraded model, which can be seen visually in the MD maps (Figure 3.5(a, b)). Decellularised PCaA (Figure 3.5(e)) had a significantly higher overall diffusion than both native and fixed tissue. Fixed PCaA had a lower mean MD than native PCaA, however no significant difference was found. The collagen degraded PCaA showed a significantly lower MD than the elastin degraded model, which is evidenced in the MD maps as well (Figure 3.5(c, d)). As the samples were imaged at room temperature, the MD for PBS was found to be 0.00185 ± 0.00001 mm²/s.

Figure 3.5. Parametric maps of MD in a representative slice for each of the tissue models. As measured in vessel media, both (a) native (N) and (b) fixed native (F) PCaA showed a significantly lower MD than both the (d) elastin degraded (E) and (e) decellularised (D) PCaA. The elastin degraded PCaA had a significantly higher MD than the (c) collagen degraded PCaA. (**p=0.0032, ***p=0.0001 (C vs. E), ****p=0.0003 (F vs. D), ***p<0.0001).
3.3.3 Tractography

Tractography was performed to visualise the diffusion pathways within the tissue models. Keeping tractography parameters constant, Figure 3.6 demonstrates the varying results obtained across tissue models alongside the FEFA maps. Fresh and fixed native and collagen degraded PCaA (Figure 3.6(a, b, c)) illustrate coherent and helical arrangements of tracts which align with the known helical arterial tissue structure. Using the same parameters, the elastin degraded and decellularised PCaA models (Figure 3.6(d, e)) show fewer tracts and lack continuity. Tract volume, the number of tracts and mean tract length were quantified and both fresh and fixed native showed the highest volume and number of tracts as well as the longest tracts (Figure 3.6(f, g, h)).

![Figure 3.6. Tractography from tissue models. Tracts from (a) native, (b) fixed native, (c) collagen degraded, (d) elastin degraded and (e) decellularised PCaA alongside the FEFA maps. Fresh and fixed native PCaA show the most continuous tractography which was verified by (f) tract volume (**p=0.0051 (N vs. C), **p=0.0029 (F vs. C), **p=0.0046 (E vs. D), ****p<0.0001) (g) the number of tracts (**p=0.0025 (N vs. C), **p=0.0021 (F vs. C), **p=0.0054 (E vs. D), ****p<0.0001) and (h) and the mean tract length (**p=0.0002, ****p<0.0001).
Comparing native and collagen degraded PCaA models, Figure 3.7 shows tractography for a representative vessel of each model alongside H&E and PLM histology. A cross-sectional view of native PCaA shows the circumferentially aligned cell and collagen fibre content (Figure 3.7(b, c)). The histologically verified orientation of both cells and collagen coincide well with the arrangement of the tracts of native PCaA (Figure 3.7(a)). The collagen degraded PCaA resulted in similar tract orientation (Figure 3.7(d)), despite the lack of any collagen (Figure 3.7(f)), while the circumferentially aligned cell content is still visible (Figure 3.7(e)).

Figure 3.7. Tractography and histology representations for native and collagen degraded vessels. Tractography of representative (a) native and (d) collagen degraded PCaA. Both models were obtained with the following parameters: seed point resolution: 0.3125 mm x 0.3125 mm x 0.3125 mm, FA threshold: 0.2, FA tracking threshold: 0.2 – 1, tract length: 0.5 – 5.0 mm, angular threshold: 30° and step size of 0.3125 mm. Representative cross-sectional histology shows (b, e) cellular arrangement by H&E for native and collagen degraded PCaA, respectively. (c) PLM shows the similar orientation of collagen in native PCaA,constituting thereof in (f) collagen degraded PCaA. Scale bar is 250 μm.

3.4 Discussion

In the present study we investigated the sensitivity of DTI derived FA, MD and tractography to changes in arterial tissue microstructure. By selectively removing SMCs, elastin and collagen we explored how each component plays a part in the typically anisotropic diffusion profile of arterial tissue (Figure 3.4). Differences between native and decellularised arterial tissue demonstrate that the main contributor to this anisotropic diffusion in arterial tissue is the presence of cell content. While the elastin degraded model suggests a similar
contribution from elastin – the removal of elastin resulted in a significant increase in extracellular space and decrease in SMC density. The degree of extracellular space increase in the elastin degraded model was far greater than any other tissue model, making it difficult to elucidate the exact impact elastin has on the anisotropic diffusion of arterial tissue. While previous studies highlight the role of collagen fibres in diffusion derived metrics\textsuperscript{172,211,257}, here, we evidence the co-dependency of collagen and cell content and characterise their influence on FA, MD and tractography. With the removal of collagen there is no significant change in FA or MD compared to native arterial tissue. However, the loss of cellular content results in predominantly more isotropic diffusion, seen by increased MD and decreased FA, despite the presence of collagen fibres as confirmed by histology. This becomes even more evident in the tractography results – where the decellularised vessels yield few helically arranged tracts (Figure 3.6(e-h)). Fresh and fixed native vessels resulted in the most uniform, continuously helical tracts (Figure 3.6(a, f, g, h)). Given the resolution of the DTI sequence, looking only in the media of the vessels, allowed for the characterisation of the highly aligned network via both FA and tractography. DTI within arterial tissue, specifically within the media, has previously been shown to accurately capture the fibre orientations when compared to second harmonic generation imaging\textsuperscript{166}. This result highlights the significance of cellular content and corroborates findings in a previous ex vivo DTI study on cell migration in brain tumours\textsuperscript{244}, where the authors saw a decrease in FA and increase in MD when cells migrated out of a region of interest. While this finding highlights the novel significance of cellular content on anisotropic diffusion, something not seen previously, when considering the overall composition of arterial tissue, it can be explained. While collagen contributes to 47% of the arterial wall by volume, it is followed by elastin at 29% and SMCs at 27%\textsuperscript{37}. Compared to cartilage tissue, where collagen orientation is typically elucidated by DTI metrics\textsuperscript{258}, arterial tissue has a significantly higher cell density. Therefore, the influence on diffusion within the tissue from cell content would be expected to be more significant.

Vascular SMCs on average are 200 μm long and 5 μm in diameter\textsuperscript{38}, while a single collagen fibril diameter is 80 ± 11 nm and a fibre bundle is approximately 5.1 ± 6.1 μm\textsuperscript{259}. SMCs are responsible for the turnover of the ECM, including collagen, and therefore their orientation aligns with that of collagen. Together these components form the helical matrix of arterial tissue which has been well documented\textsuperscript{165,166}. Using the 3D root-mean square equation\textsuperscript{260} ($r = \sqrt{6D\Delta}$), the gradient interval time used in this study ($\Delta= 8.802$ ms) and the measured
MD in PBS, a diffusing water molecule could travel approximately 9.8 μm, suggesting sensitivity to diffusion at the scale of both SMCs and collagen.

In the absence of obstructing boundaries, protons diffuse freely in all directions. The presence of boundaries, such as SMCs or collagen fibres, cause either restricted or hindered diffusion. Intracellular diffusion is typically regarded as restricted diffusion as the molecules are trapped within the cell membrane and have limited space to diffuse within\textsuperscript{261}. Hindered diffusion arises when the diffusion of water molecules is impeded by boundaries, such as collagen fibres, SMCs, or elastin fibres, which reduce their net displacement. Generally, extracellular diffusion in biological tissue is characterised as hindered diffusion\textsuperscript{179,261}. The cell membrane is composed of a phospholipid bilayer which is selectively permeable and, due to the polar nature of water molecules, limits the exchange rate of molecules across the membrane from intra- to extracellular space and vice versa\textsuperscript{262}. In contrast, aquaporins are channel proteins that act as transmembrane water channels, and their gating (open or closed) is a result of biochemical signalling. It has been shown in both rat and human vascular SMCs that aquaporin-1 plays an important role in vascular function – specifically in development and injury\textsuperscript{263}. Within the scale of diffusion presented here, we see the combined effects of both intra- and extracellular diffusion\textsuperscript{264} and changes in their profile as components are removed. It should be noted that the exact effect the enzymatic treatments have on the integrity of the cell membrane – for example on aquaporin gating – is unknown and requires further research. There has also been significant research investigating the choice and use of higher order DTI models to specifically characterise different diffusion compartments, which may provide further insight into the microstructural changes seen in the arterial models presented in this study\textsuperscript{265–267}.

The removal of SMCs from arterial tissue resulted in a drastic decrease in FA, as can be seen in the decellularised tissue model (Figure 3.4). The high FA measured in the collagen degraded vessels suggests that SMCs are the main contributors to the overall anisotropic diffusion. Both the intra- and extracellular hindered diffusion associated with their presence have a greater impact on anisotropic diffusion than hinderance from interactions with collagen fibres alone. Removal of elastin from the artery resulted in the most isotropic diffusion of all models, seen by low FA and the highest MD. While the quantity of cells did not change in the elastin degraded model, as previously mentioned, the removal of elastin resulted in drastically increased extracellular space and decreased cell density – this can be seen histologically by H&E (Figure 3.2(a, c)). SMCs attach to the concentric elastin
lamellae and are embedded between collagen fibres\textsuperscript{37}. The removal of elastin disrupted this relationship and with the drastically increased extracellular space and lower cell density, proton mobility increased and, therefore, increased the MD\textsuperscript{268}. This structural change can be seen plainly in the FA (Figure 3.4(d)) and MD (Figure 3.5(d)) maps and is confirmed histologically (Figure 3.2(c)). The MD of the elastin degraded model is higher than that of the decellularised model suggesting that not only does the less densely packed cell content affect the DTI metrics compared to native, but the decrease in hindered diffusion from the removed elastin molecules also plays a significant role in this isotropic response.

It is worth noting that all the tissue models displayed a decrease in GAG content (Figure 3.2, bottom row). GAGs are anionic unbranched heteropolysaccharides which constitute both structural and functional ECM components of connective tissues. These chains carry a high concentration of negatively charged groups and attract positive ions. GAGs together form distinct proteoglycan families, providing the vasculature with viscoelasticity and turgor. Not only do these molecules contribute to vascular permeability, lipid metabolism, haemostasis and thrombosis, but they also interact with vascular cells to modulate cell adhesion, migration and proliferation\textsuperscript{269}. While the overall amount of proteoglycans in the arterial wall is low, it increases in early phases of atherosclerotic lesion formation\textsuperscript{26}. A study by Bartholomew and Anderson\textsuperscript{270} demonstrated that proteoglycans coat collagen type III in the media, which in turn coats the elastin fibres. This suggests it is not possible to avoid the disruption and depletion of GAGs in arterial tissue when removing collagen or elastin. In cartilage, Xia, et al.\textsuperscript{268} illustrated that the MD has no direct correlation to GAG content but instead they proposed that the space left from degraded and removed macromolecules allows for increased diffusivity – which has since been demonstrated\textsuperscript{271}. To the author’s knowledge, no studies exist examining the influence that GAGs have on diffusion in arterial tissue; however, while proteoglycans show no preferred orientation and therefore shouldn’t influence FA, as previously suggested, their removal could increase MD as a result of the open extracellular space left behind upon their removal. Additionally, in more physiological diseased morphologies where their concentration is increased, the consequent increase in negatively charged groups could influence a number of factors, such as osmotic pressure and vascular permeability, which could impact the diffusive properties in the tissue.

The main constituents of native PCaA are SMCs, elastin, and collagen. When fixing native arterial tissue, these constituents were unaffected and there was no effect on the FA, MD or tractography. While multiple studies have reported increased MD in cardiac tissue when
fixed\textsuperscript{246,272,273}, the length of fixation, concentration of fixative and time after fixation in cardiac tissue have resulted in considerable variation in measured FA and MD\textsuperscript{274,275}. One study on fixed tissue observed an initial decrease in the MD followed by an increase after 15 days\textsuperscript{274}, whilst it has also been shown that increased exposure to fixatives can cause cell membrane degradation by the depletion of lipid membranes through carbon double bond reactions\textsuperscript{276}. The effect of fixation on sample dimensions has been well documented, with tissue shrinkage a known side effect\textsuperscript{254,277}. Our results showed no significant difference between fresh and fixed tissue with respect to the FA, MD and tractography. The length of our fixation protocol, seven days, is likely short enough to avoid any membrane degradation and therefore had no significant effect on diffusivity. This time frame has also been shown to minimise the effect of shrinkage from fixation, however different concentrations of formalin should be investigated in the future\textsuperscript{277}.

Previous studies in arterial tissue have looked at the structure of native arterial tissue as well as how storage and preparation for imaging affect the diffusion profiles. While the fibre angles have not been quantified in the present study, the helically aligned tractography of native and collagen degraded tissue corroborate previous studies highlighting the helical arrangement of SMCs and collagen\textsuperscript{165,166}. The results from the tractography analysis provide visualisation of the significance of cell content on the diffusion profile. It is demonstrated in this study that, within arterial tissue, tractography is sensitive to cellular orientation, more so than just collagen fibre arrangement (Figure 3.6(a, c, e)). Histological analysis demonstrates that SMCs and collagen follow the same circumferential arrangement in native arterial tissue (Figure 3.7(a)) and tractography yields good visualisation of that structure. However, for the collagen degraded vessels (Figure 3.7(d)) the tractography is a representation of cellular alignment alone.

All samples in this study were PCaA; however, the study did not control for the differences between proximal or distal sections of these carotids. Carotids are elastic arteries; however, more distal sections can be more muscular, thus containing less elastin in the medial layer\textsuperscript{33}. While this can explain visual differences between arteries in Figure 3.4 and Figure 3.5 (c and e), the treatments performed were designed and confirmed to selectively remove all of a specific microstructural component from a given vessel.

While a 2D slice selective scan would certainly be faster, crosstalk between slices can occur and may require slice gaps which are not ideal for investigating tractography. Additionally, while EPI acquisitions offer faster scan times, they can also introduce further distortions and
artefacts. With these variables in mind, the 3D spin echo DTI sequence was used in this study as it provided high resolution images with minimal distortion or artefacts and good SNR. 7 T has been approved for human use\textsuperscript{155} and while not yet routinely used clinically, its use in this study allowed for this high resolution, high SNR investigation. Additionally, the lengthy duration of the scan posed multiple limitations. It would be expected to have some tissue degradation during the scan time; however, all samples were treated the same and imaged for the same scan time and there was no evidence of degradation histologically. Additionally, a limited number of diffusion directions and unweighted images were acquired due to the long scan duration. While the same number of diffusion directions and unweighted diffusion images has been used previously in arterial tissue and shown microstructurally accurate results\textsuperscript{166,211}, it should be noted that the ratio of unweighted to weighted diffusion scans is not optimal in this study\textsuperscript{278}. Ranges of FA and MD from a previous study on arterial tissue using multiple b-values and up to 128 b-directions agree well with the measurements made in this study\textsuperscript{172}. While healthy arterial tissue microstructure is quite homogeneous and crossing fibres would not be common, the low number of diffusion directions can bias DTI derived metrics and should be investigated further to prevent potential bias in diseased arterial tissue where the homogeneity of microstructure is disrupted\textsuperscript{279}.

Microstructural changes in arterial tissue can have significant implications for the mechanical functionality of the tissue\textsuperscript{45}, as well as often being a precursor to disease progression\textsuperscript{29}. On this basis and considering the characterisation of arterial microstructure presented in this study, DTI has the potential to provide unique biomarkers for the integrity of arterial tissue. Morphologically, the first signs of an atherosclerotic plaque are the thickening of the intima, followed by the well-known formation of a lipid core and fibrous cap\textsuperscript{25,26}. Microstructurally, these different regions have altered quantities and arrangements of SMCs, collagen, and elastin. The thickened intima typically shows a decrease in SMC content\textsuperscript{26}, the lipid core highlights the displacement of SMCs by foam cells\textsuperscript{25}, and the fibrous cap, which covers the lipid core\textsuperscript{74}, has been shown to have quite variable SMC content depending on location\textsuperscript{57}. The demonstrated sensitivity of DTI to SMC content in arterial tissue in this study suggests that it may be an ideal metric to identify such early indicators of disease driven microstructural changes. Additionally, other CVDs – such as aneurysms – have shown significant fragmentation of the elastic lamellae which can cause catastrophic failure of the arterial wall\textsuperscript{280}. Changes in the key microstructural components of arterial vessels can lead to significant mechanical failings\textsuperscript{45} and identifying these changes using
imaging biomarkers, offers potential insight into the mechanical integrity of the arterial wall in atherosclerotic and/or aneurysmal tissue.

The high resolution, lack of physiological motion and extended scan time in this study allowed for a detailed look at the vessel microstructure, which would be necessary for investigating regions of atherosclerotic plaques. Despite the idealised ex vivo experimental set up, the work presented here highlights the promise for DTI metrics to yield valuable insight into arterial microstructure which could ultimately provide novel insight into diseased tissue morphologies. This study establishes the influence of key microstructural components on diffusion metrics in arterial tissue and highlights the potential of DTI for identifying disease driven changes in arterial microstructure.
Chapter 4 An *ex vivo* study using diffusion tensor imaging to identify biomarkers of atherosclerotic disease in human cadaveric carotid arteries

4.1 Introduction

With the sensitivity to specific, key microstructural components in arterial tissue established in Chapter 3, this study aims to leverage this sensitivity to help identify cardiovascular disease progression by imaging human healthy and diseased arterial tissue.

Many studies have looked to *ex vivo* MRI to obtain high-resolution images of atherosclerotic microstructure. Multi-contrast MRI protocols, such as proton-density weighted-, T1-, T2-, and DW imaging have been implemented with a view to characterising plaque components. While some studies show the promise of using all these sequences in combination to characterise morphological regions[147,160,161], other studies point to the sensitivity of individual MR contrasts to specific atherosclerotic morphologies[147,159,160,162,169,170]. Karmonik, et al. showed the promise of using multi-contrast MRI in conjunction with unsupervised segmentation to identify major plaque components such as lipid, calcifications, fibrotic tissue, thrombus, and normal vessel wall[283]. These morphologies are dictated by changes in microstructural components and their quantity and quality vary significantly throughout plaque progression[284–286]. DTI alone has shown a strong sensitivity to cell and elastin content in arterial tissue models in Chapter 3, and has recently been used to identify altered microstructural organisation in tissue models[172,175] and plaque tissue[171]. A strong negative correlation between FA and age has also been seen *in vivo* in the common carotid[148].

This chapter focuses on investigating aged human carotid arteries. DTI-derived metrics, namely FA and MD, are used to gauge the feasibility of this imaging technique to identify disease driven microstructural changes. Multiple analyses are carried out in order to both investigate bulk changes in these non-invasive metrics as well as microstructure and morphology specific changes. Together the different approaches aim to establish whether microstructurally sensitive DTI metrics are suitable biomarkers of atherosclerosis.
progression, and if so, what microstructural changes are driving measurable changes in the metrics.

4.2 Materials and methods

An overview of the methods used in this study are shown in Figure 4.1.

Figure 4.1. Brief overview of methods. A) Excised cadaveric carotid arteries; scale bars are 5 mm. B) Example of carotid prior to ex vivo imaging, held in place by a 3D printed holder. C) Sectioning of a carotid for histological processing, three sections were taken for each subject; scale bar is 10 mm. D) Five histological stains were used for all carotids; left to right: H&E, Verhoeff’s elastin, PSR and PLM, Alcian blue, and Alizarin red. Scale bar is 2 mm. E) MRI data processing flow. F) Carotids were investigated as a whole, by anatomical region and by healthy (H) and diseased (D) regions non-specifically with histology visually used to confirm. G) Histology slices were registered to common carotids, k-means clustering with different inputs was investigated as well as specific morphological features. Scale bar is 0.5 mm.
4.2.1 Human carotid arteries

Carotid arteries (n=8) were excised from six embalmed cadavers. Carotids were excised to include the common carotid, bifurcation and both internal and external carotid arteries, see Figure 4.1(A). All human cadaveric tissue was provided by The Royal College of Surgeons in Ireland and was used with approval from the Department of Anatomy, Royal College of Surgeons in Ireland institutional review board. Out of the eight arteries from the six cadavers, four left carotids were excised from four different cadavers and the remaining four from two cadavers (both left and right carotids). The subjects (three females and three males) ranged from 70 to 103 years in age (mean 78.25 ± 11.2 years). Table 4.1 details the subjects used in this study. All eight carotids were considered independent entities in this study as the focus was on identifying changes in arterial microstructure by DTI, not subject specific trends. The COD for all subjects was unrelated to CVD. The arteries were cleaned of connective tissue after excision and stored in PBS until imaging.

<table>
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<tr>
<th>Sex</th>
<th>Age</th>
<th>COD</th>
<th>Side</th>
<th>AHA Classification</th>
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<tr>
<td>1</td>
<td>F</td>
<td>103 Aspiration pneumonia and Alzheimer’s</td>
<td>L</td>
<td>V</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>70 Chronic obstructive airway disease</td>
<td>L</td>
<td>IV-V</td>
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<tr>
<td>3</td>
<td>M</td>
<td>80 Pneumonia</td>
<td>R</td>
<td>VI</td>
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<tr>
<td>4</td>
<td>M</td>
<td>75 Metastatic prostate cancer</td>
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<td>IV-V</td>
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<tr>
<td>5</td>
<td>F</td>
<td>80 Cerebellar syndrome</td>
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<td>7</td>
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<td>8</td>
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Table 4.1. Details of the cadaver carotids used in this study. AHA classifications made based off morphological criteria60. AHA = American Heart Association, COD = cause of death.

4.2.2 MR imaging

The common carotid of the arteries was secured to a 3D printed holder which was secured in a horizontal 50 ml falcon tube with fresh PBS for image acquisition, see Figure 4.1(B). Due to the fixation of the carotids, their stiffness prevented any drooping or movement during scanning. Carotids were imaged using a similar sequence to that in Table 3.2 outlined in Chapter 3, but with slight changes. The FOV was increased, which in turn increased acquisition time (image size: 96 x 96 x 96, FOV: 30 x 30 x 30 mm, isotropic resolution: 0.3125 x 0.3125 x 0.3125 mm and acquisition time: 28 hours and 9 minutes). This resolution allowed for approximately 3-5 voxels through the wall thickness in the common, internal and
bifurcation and 1-3 voxels in the thinner walled external artery, with diseased regions allowing for the most voxels through due to the wall thickening. FA and MD were calculated from the diffusion tensor. Tractography was also performed to visualise the tissue microstructure using the following parameters: seed point resolution and step size: 0.3125 mm, seed FA threshold: 0.075, tracking FA range: 0.075-1, fibre length: 1-50 mm, and angular threshold: 90°.

4.2.3 Histological analysis

4.2.3.1 Histological processing
For histological processing, 2-3 mm sections from the common carotid, bifurcation and internal and external carotids were sectioned from whole arteries for all subjects, see Figure 4.1(C). Samples were processed for histological staining as previously described in Chapter 3. All sections were stained with the same stains mentioned in Table 3.3, but with the addition of Alizarin red for calcium content, Figure 4.1(D) and all brightfield imaging was done on an Aperio CS2 microscope with ImageScope software V12.3 (Leica Biosystems Imaging, Inc., Vista, California).

4.2.3.2 Quantification of histology
Quantification of histology measures were measured using QuPath287 (version 0.2.3). Digital whole histology slides were imported into QuPath software where the following procedure was followed for Verhoeff’s elastin, Alcian blue and Alizarin red: 1) the stain vector was set288 for each histological stain, 2) the whole tissue region was delineated by thresholding the tissue slice from the background and 3) the stained area fraction was then identified using further thresholding. Thresholds were kept consistent for each stain between all subjects. After this, both the images of the tissue region and the stained area fraction were exported and brought into MATLAB where the percent content was calculated. PSR and the respective PLM images were analysed similarly using an in-house built script described previously108. For cell density analysis, specific ROIs were identified and exported from MATLAB, and subsequently manually drawn on the H&E image in QuPath where cell detection analysis was performed to yield 2D cell density measures. Quantitative measures were performed in ROIs either determined by k-means clustering, section 4.2.4.3, or by morphology, section 4.2.4.4.
4.2.4 Data processing

4.2.4.1 Regions of interest

Whole carotid arteries (n=8) were analysed with visual confirmation of tissue integrity from histology, see Figure 4.1(F). Regions with low signal-to-noise ratios and high residuals from the tensor model fitting were excluded from any of the following analyses to avoid any biasing the DTI metrics\textsuperscript{289}. Specifically, pixels with signal below the 50\% percentile in the non-DW image were excluded as well as any pixels with a mean residual above the 99\% percentile. Lastly, the mean MD of background PBS was used to remove PBS and yield the final tissue mask. Together these criteria were optimised to removed calcifications and air bubbles from all data (Figure A. 1). They also limited partial voluming effects by removing the background media, PBS, and excluding voxels with noise around calcifications. Changes in these values could lead to further minimising partial voluming effects or conversely, not minimising them enough. Mean FA and MD values were compared across the eight vessels as well as within manually segmented anatomical regions. One mean value per vessel was compared when looking at entire subjects. When looking anatomically, the ROIs were identified as the common carotid (n=7), bifurcation (n=8), internal carotid (n=8), and external carotid (n=8). Similarly, one mean value per anatomical region per subject was compared. The bifurcation was defined as the area where the common carotid transitioned from circular to oblique up to the apex of the bifurcation where the internal and external carotids split into individual branches. These anatomical regions where further separated into healthy and diseased ROIs throughout the entire carotid. These delineations were manually drawn, with diseased regions classified as vessel wall regions that showed any deviation at all from the standard aged vessel wall as confirmed by histology (Figure A. 2). Figure A. 3 and Table A. 1 contain more detail on the relative size of the healthy and diseased regions within each vessel. Metrics in diseased regions were then normalised by their respective anatomical healthy region within the same subject to account for anatomical differences. For all regions, one mean value and standard deviation of FA and MD are reported.

4.2.4.2 Image registration

For quantitative histological measures, common carotid (n=4 subjects) histological slices (one slice per stain) were registered to one MR slice. Registration was only performed on common carotid sections of four different subjects due to deformations in histological slices in more diseased regions of the carotids. Anatomical landmarks (the apex of the bifurcation,
the base of the common carotid at the 3D printed holder and notable anatomical features) allowed for manual matching of histology slices (0.495 x 0.495 μm) to a specific slice in high-resolution MR images. The tissue area of the identified MRI slice was then manually masked\(^{290}\). The corresponding brightfield histology image was then manually aligned to the identified high-resolution MRI slice by rotating and scaling providing an initial alignment for further calculated transformations. The manual alignment of histology to the high-resolution MRI was visually confirmed with reference to the anatomical landmarks prior to using Elastix\(^{291,292}\) (version 5.0.1) to perform nonrigid registration based on BSpline deformation. Details of the registration can be found in Appendix A.

4.2.4.3 k-means clustering
A k-means clustering algorithm was used as an unsupervised segmentation tool on the common carotids which had histology registered to the MRI data (n=4 subjects, n=1 MR slice per subject, n=5 different histological stained slices). The registered histology images mentioned above were imported into MATLAB alongside the parametric FA and MD maps for the specific slice. The elbow method was used to determine the appropriate number of clusters\(^{293}\), with k=5 proving to be sufficient for this data (Figure A. 4). Three different clustering inputs were investigated – FA, MD, and the combination of both FA and MD (Figure A. 5). Once the cluster regions were determined, mean FA and MD values were extracted for each region, along with the percent content of each of the five microstructural components for correlation analyses. It is worth noting that the background medium, in our case – PBS, was classified as a cluster so four regions are presented per sample. Quantitative histology, outlined in section 4.2.3.2, was performed on the registered histology slices in the determined clusters in order to correlate microstructural components to DTI-derived metrics.

4.2.4.4 Morphological regions of interest
The thickened intima (n=3) and the media (n=4) were identified and manually drawn on relevant DTI-registered H&E histology images (n=4 slices), Figure 4.1(G). These morphological regions were then used to investigate DTI metrics and microstructural content between the registered histology and MR data in these two morphologies present in the common carotid.

4.2.5 Statistical methods
Statistical analysis was performed using GraphPad Prism (Version 8). Sample size was determined by the availability of human tissue. All data was tested for normality using
D’Agostino-Pearson normality tests and equality of group variances with Brown-Forsythe ANOVAs. If the data did not pass normality (alpha=0.05) or had too small a sample size (n<8), nonparametric tests were used. For mean FA and MD in the different anatomical locations, the nonparametric Kruskal-Wallis test was used. Comparing healthy and diseased ROIs across the eight vessels, unpaired t-tests were used as the data passed normality and equal variance tests. A 2way ANOVA was used for investigating differences between healthy and diseased ROIs with respect to anatomical location. Pearson’s correlations were used to determine the relationship between microstructural components and DTI metrics. R values below 0.3 were considered a weak correlation, up to 0.7 a moderate correlation and above 0.7 a strong correlation. Lastly, nonparametric Mann-Whitney tests were used when investigating morphological ROIs in the common carotids due to limited sample numbers.

4.3 Results

4.3.1 DTI metrics of cadaver carotids

Figure 4.2 shows (A) 3D representations of the n=8 carotids used in this study alongside the mean (B) FA and (C) MD in each carotid for the whole sample (1 mean value per vessel). The mean FA between all vessels was 0.105 ± 0.05 and the mean MD was 0.00117 ± 0.0003 mm²/s (both marked by dotted lines). Anatomical delineations of the common carotid, bifurcation, internal carotid, and external carotid can be seen in 3D renders in Figure 4.2(D). The mean FA and MD for all subjects (1 mean value per vessel, n=8 for the bifurcation, internal and external, n=7 for the common) are presented in Figure 4.2(E, F). The common (0.115 ± 0.05) had the highest anisotropy and was significantly higher than that in the external carotid (0.0914 ± 0.04). The mean FA at the bifurcation was 0.104 ± 0.05 and 0.106 ± 0.05 in the internal carotid. For MD no significant differences were seen between anatomical regions (common: 0.00115 ± 0.00006 mm²/s, bifurcation: 0.00115 ± 0.00005 mm²/s, internal: 0.00117 ± 0.00003 mm²/s, and external: 0.00125 ± 0.00006 mm²/s).
Figure 4.2. DTI metrics of n=8 cadaveric carotids. (A) 3D representations of the 8 individual carotid arteries. (B) FA and (C) MD across the entire carotid for each subject. Each point represents the mean across the entire vessel (n=1 per vessel) with error bars showing the standard deviation across each individual vessel. (D) 3D representations of each carotid with the anatomical ROIs colour coded: common carotid in purple, bifurcation in red, internal carotid in orange, and external carotid in green. (E) FA and (F) MD of separate anatomical ROIs (C – common, B – bifurcation, I – internal carotid, E – external carotid). Each point represents the mean value within the specified anatomical region for individual vessels, n=8 for B, I and E and n=7 for C. Significance tested by Kruskal-Wallis test, *p=0.0310.

All subjects included in this study showed varying signs of atherosclerosis despite the fact that CVD was not implicated as a COD for any subject, see Figure A. 3. Histology was used to confirm healthy and diseased delineated regions for examination; however, it is important to remember this ‘healthy’ tissue is aged. Figure 4.3 shows representative 3D renders of a vessel with both anatomical and healthy and diseased regions shown. Figure 4.3(A) shows examples of healthy vessel wall histology from the dashed line location in the 3D render. Figure 4.3(B) shows histological cross-sections at the dashed line in the 3D render – where various diseased morphologies are present.
Figure 4.3. Representative healthy and diseased ROIs. (A) One representative slice of common carotid with a blue mask conveying the healthy ROI. The dashed line in the 3D render shows the location of the slice. Representative H&E, Verhoeff's and PSR and PLM of healthy common carotid. Scale bars are 200 μm. (B) One representative slice of common carotid with a blue mask over the healthy ROI and the white mask over the diseased ROI. Similarly, representative histology of the various disease morphologies is shown. The asterisk marks where a calcification was. Scale bars are 1 mm. In the 3D renders, anatomical regions which are coloured (blue – common, red – bifurcation, yellow – internal, and green – external) are healthy ROIs, whereas greyed out regions are diseased. MR images are the non-DW image.

Diseased regions, confirmed by histology as seen in Figure 4.3, were compared against healthy tissue. Figure 4.4(A, D) presents the mean healthy and diseased FA and MD values for each subject (n=8). The mean FA measured within healthy regions was 0.0996 ± 0.02 while the mean FA diseased regions was slightly higher at 0.109 ± 0.01. The MD measured in healthy regions, 0.00123 ± 0.00009 mm²/s, was significantly higher than that measured in diseased regions, 0.00112 ± 0.00008 mm²/s. When investigating healthy and diseased ROIs based on anatomical location, there was a significance difference in (B) FA based on anatomical location and a significant difference between healthy and diseased regions for (E) MD regardless of location. Diseased regions were normalised by their respective healthy anatomical region per subject and normalised FA and MD values are presented in Figure 4.4(C, F). Deviation from a value of 1 signifies how much the diseased tissue differs from healthy tissue in that specific location. For example, when looking at subject 7 in Figure 4.4(C) all anatomical regions show a value greater than 1 – indicating that the FA was higher.
in diseased regions than healthy. The opposite is seen in subject 4. The normalised MD values presented in Figure 4.4(F) are all below 1, indicating that all diseased tissue, regardless of subject or anatomical location, had a lower MD value than the healthy tissue. Some subjects have less than four anatomical regions presented in Figure 4.4(C, F) – indicating that the missing anatomical regions were either entirely healthy or diseased. Details can be seen in Table A.1. Figure 4.5 shows examples of more advanced diseased ROIs located in the bifurcation and internal carotid arteries. Fibrous tissue as well as large lipid cores are visible by H&E staining.

Figure 4.4. DTI metrics in healthy and diseased ROIs. (A) FA and (D) MD in healthy (solid bars) and diseased (checkered bars) ROIs of each vessel, n=8, regardless of anatomical location. Significance tested by unpaired t-tests, *p=0.0406. (B) FA and (E) MD in healthy and diseased ROIs within specific anatomical locations. Significance was determined by a 2-way ANOVA, *p<0.05 between anatomical locations for FA and ***p<0.001 between healthy and diseased regions for MD (n=7 healthy ROIs in the common, bifurcation and internal and n=8 in the external carotid; n=7 diseased ROIs in the common and internal carotid, n=8 in the bifurcation, and n=6 in the external carotid). (B) Normalized FA and (C) MD (diseased by healthy) for n=8 subjects. Missing bars mean the anatomical region was either completely healthy or diseased. Insert in (D) shows visual representation of the different anatomical ROIs in one vessel.
Figure 4.5. Examples of advanced diseased in the bifurcation and internal carotids for non-registered histology. Asterisk colouring on histology matches arrow colouring on non-diffusion weighted MR slices from same region. Yellow = lipid core, blue = fibrous tissue, purple = calcification location, red = red blood cells, green = possible rupture site or luminal calcified disruption (visible on MR image), and orange = cholesterol crystals. Scale bars are 1 mm for (A-E). High resolution regions are presented in (F-I): (F) lipid core, (G) fibrous tissue, (H) red blood cells, (I) cholesterol crystals, scale bars = 200 μm.
4.3.2 Quantitative histology and DTI metrics

4.3.2.1 DTI metrics and microstructural components

DTI metrics and quantitative histology were investigated within the common carotids of four subjects. Figure 4.6 presents the strong and moderate correlations for FA in the first column (Figure 4.6(A, C, E)) and MD (Figure 4.6(B, D, F)) in the second column against different microstructural components. Different rows correspond to the different clustering inputs: FA input is presented on the top row (Figure 4.6 (A, B)), MD input in the middle (Figure 4.6(C, D)) and both FA and MD on the third row (Figure 4.6(E, F)). Detailed summaries of the Pearson’s correlations are presented in Table 4.2 and Table 4.3. Additionally, correlations between microstructural components can be found in Figure A. 7 and Figure A. 8 and details of their Pearson’s correlations in Table A. 2 – Table A. 5.

![Diagram](image)

Figure 4.6. Strong and moderate correlations of microstructural components and DTI metrics based on clustering input, namely (A, B) FA, (C, D) MD and (E, F) FA and MD together. Weak correlations can be seen in Figure A. 6. Cell densities are plotted on right y-axis, all other components are plotted on left y-axis.
### Table 4.2. Summary of Pearson’s correlations between FA and microstructural components.

<table>
<thead>
<tr>
<th>Input</th>
<th>r</th>
<th>95% confidence interval</th>
<th>R²</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>0.7197</td>
<td>0.3482 to 0.8958</td>
<td>0.5179</td>
<td>0.0017**</td>
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<tr>
<td>MD</td>
<td>0.7970</td>
<td>0.4980 to 0.9266</td>
<td>0.6351</td>
<td>0.0002***</td>
</tr>
<tr>
<td>FA+MD</td>
<td>0.7410</td>
<td>0.3876 to 0.9045</td>
<td>0.5490</td>
<td>0.0010**</td>
</tr>
<tr>
<td>Collagen</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>0.3380</td>
<td>-0.1894 to 0.7141</td>
<td>0.1142</td>
<td>0.2004</td>
</tr>
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</tr>
<tr>
<td>FA+MD</td>
<td>0.4339</td>
<td>-0.07875 to 0.7650</td>
<td>0.1883</td>
<td>0.0931</td>
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<tr>
<td>GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
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<td>0.3081</td>
<td>0.0256*</td>
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</tr>
<tr>
<td>Calcium</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
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<td>0.02649</td>
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<tr>
<td>MD</td>
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</tr>
<tr>
<td>FA+MD</td>
<td>-0.08352</td>
<td>-0.5562 to 0.4300</td>
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</tr>
<tr>
<td>Cell density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>-0.4836</td>
<td>-0.7899 to 0.01593</td>
<td>0.2339</td>
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<tr>
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<td>0.0839</td>
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<tr>
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<td>-0.2060</td>
<td>-0.6367 to 0.3227</td>
<td>0.04243</td>
<td>0.4441</td>
</tr>
</tbody>
</table>

### Table 4.3. Summary of Pearson’s correlations between MD and microstructural components.

<table>
<thead>
<tr>
<th>Input</th>
<th>r</th>
<th>95% confidence interval</th>
<th>R²</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Elastin</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>-0.7996</td>
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<td>&lt;0.0001****</td>
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<td>0.6448</td>
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</tr>
<tr>
<td>Collagen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
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<td>0.1360</td>
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</tr>
<tr>
<td>GAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FA</td>
<td>0.2783</td>
<td>-0.2522 to 0.6802</td>
<td>0.07744</td>
<td>0.2956</td>
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<td>0.03141</td>
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<td>Calcium</td>
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<td></td>
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</tr>
<tr>
<td>FA</td>
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<tr>
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<td>0.6418</td>
</tr>
<tr>
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<td>-0.5281 to 0.4618</td>
<td>0.001928</td>
<td>0.8717</td>
</tr>
<tr>
<td>Cell density</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>FA</td>
<td>0.3035</td>
<td>-0.2262 to 0.6947</td>
<td>0.09214</td>
<td>0.2531</td>
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<tr>
<td>MD</td>
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<td>0.2326</td>
<td>0.0585</td>
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<tr>
<td>FA+MD</td>
<td>0.4475</td>
<td>-0.06189 to 0.7720</td>
<td>0.2003</td>
<td>0.0822</td>
</tr>
</tbody>
</table>
4.3.2.2 DTI metrics and atherosclerotic morphologies

DTI metrics between specific atherosclerotic morphologies were investigated using registered histology. In four subjects, thickened intima was present in common carotids (n=3) and compared to the media (n=4). Figure 4.7(A) presents the media and thickened intima in a H&E cross section and the mean (B) FA and (C) MD for both regions as well as the (D) cell density, (E) elastin, (F) collagen, (G) GAG, and (H) calcium content. FA, cell density and elastin content were all higher in the vessel media than in the thickened intima and the MD was lower in the vessel media. Figure 4.7(I) shows the differences in total microstructural content between the two regions for elastin (black), collagen (red), GAG (blue), and calcium (beige). Cell density was excluded from total content in Figure 4.7(I) as it is a density unlike the other components which are represented by a stained area fraction. The total stained area fraction of all components for thickened intima was 94.59% and 98.26% stained for the media.
Figure 4.7. Morphological assessment of DTI metrics. (A) Registered histology informed the presence of the thickened intima (TI) and media (M) within the common carotids. Scale bar 300 um. (B) FA and (C) MD in the media and thickened intima. (D-H) Microstructural differences between the media and thickened intima. (I) Collagen (red), elastin (black), GAGs (blue) and calcium (beige) parts of a whole for both thickened intima and media. No significant differences were found using Mann-Whitney tests; n=4 media and n=3 for thickened intima.

4.3.3 Tractography of cadaveric carotids

Tractography revealed varying degrees of microstructural alignment within each subject, shown in Figure 4.8. All subjects showed some degree of circumferential alignment;
however, some showed significant axial alignment and disorganisation, Figure 4.8(G, H). Regions of advanced disease, such as calcifications, were colocalised with incoherent and non-continuous tracts in some subjects.

Figure 4.8. Tractography of cadaveric carotid arteries. Top row of (A-H) shows an axial view of the carotids, looking from the base of the common into the lumen towards the bifurcation and branches. Green-red tracts represent in-plane alignment (circumferential) while blue tracts are indicative of axial alignment.

4.4 Discussion

4.4.1 DTI metrics of cadaver carotids

DTI has been used to investigate arterial tissue in both ex vivo and in vivo studies. Studies specifically investigating DTI metrics in human atherosclerotic tissue are limited. When investigating the potential of in vivo DTI, Opriessnig et al. found a strong negative correlation between FA and patient age, with a mean age of the 12 subjects being 38 years old. All subjects in the current study were above 70 years old and low anisotropy was apparent across all carotids. The FA is significantly lower in these aged samples than
in previous studies investigating healthy porcine arterial tissue\textsuperscript{172,175,295}. No subjects in this study died of CVD; however, there were varying signs of atherosclerosis present for all subjects. While it is not possible to know for sure these subjects were asymptomatic, the signs of advanced disease present in most subjects combined with non-cardiovascular related CODs suggests it was in fact silent progression of atherosclerosis. Based on percent stenosis alone, it is likely some of these subjects would have been recommended for an intervention; however, we know atherosclerosis was not the COD for any of the subjects. Vascular aging is characterized by the loss of arterial elasticity and reduced arterial compliance\textsuperscript{296}, which may be an early cardiovascular risk indicator\textsuperscript{297}. The imbalance of microstructural components in the vessel wall, governed by elastin fragmentation, immune cell filtration, cytokine accumulation and collagen deposition, coupled with chronic inflammation, dysregulation of cellular homeostatic systems and senescence inhibits arterial relaxation, changing the mechanical environment and ultimately allowing for atherosclerotic lesion progression\textsuperscript{298}. Therefore, it is possible that the significant drop in FA present in all subjects, regardless of anatomical location or healthy or diseased ranking, could be an early indicator for vessel wall degradation.

When looking at the samples without considering the health of the tissue, a significant difference was found between the FA of the common carotid and external carotid. It is important to note that these diseased regions were nonspecific and included many different morphologies, as evidenced in Figure 4.3 and Figure A. 3. While some atherosclerotic morphologies might show disorganization in the changing vessel wall, highly aligned fibre patterns either encircling, attached to and/or pushed around calcifications have been seen\textsuperscript{299}. As seen in the normalized FA values (Figure 4.4(C)), there is no clear trend suggesting either specific microstructural changes or disease morphologies are present across all subjects. While outside the scope of the current study, it is possible further identifying and classifying of these diseased regions could highlight clearer trends between DTI metrics and specific morphologies. Additionally, the MD was significantly lower in diseased compared to healthy regions. While Chapter 3 suggested that increased cell content contributes to a decreased MD, lipid has also been shown to reduce diffusivity and is lower when compared to fibrous plaque tissue and vessel wall in vivo\textsuperscript{145,146}. The MD of healthy arterial tissue found in this study agrees well with previously reported values of normal carotid wall\textsuperscript{146} and the diseased MD falls between reported values for lipid core and fibrous plaque\textsuperscript{146}. Histological deformation prevented the registration of histology past the
common carotid in this study; however, lipid rich cores can be seen in H&E-stained slices from diseased regions (Figure 4.5).

### 4.4.2 DTI metrics and microstructural components

Following image registration and the use of an unsupervised segmentation algorithm, a strong correlation was found between both FA and MD and elastin. Regardless of clustering input, there was a strong correlation with elastin. Elastin fragmentation is not only evident in vascular aging but also present in the earliest signs of atherosclerosis. Its complete depletion in Chapter 3 showed a drastic impact on DTI metrics; however, it was not fully understood the impact elastin had within physiological tissue conditions. From initial endothelial dysfunction to formation of atherosclerotic lesions, the ECM is drastically modified – of particular interest here are the elastic fibers. Elastin chains rarely turnover due to their stability and longevity, so any damage, such as proteolytic degradation from lipid and calcium deposits, tends to be irreversible. The quality and quantity of elastin is a good indicator of vessel wall health, and therefore the strong correlation it has with DTI metrics is very promising. Furthermore, any pathologies associated with the focal loss of elastin, impacted elastin synthesis, or altered turnover – such as Williams Syndrome, cutis laxa or anuerysms – may be promising, advantageous avenues for the application of DTI beyond carotid atherosclerosis. Chapter 3 highlighted the sensitivity of these metrics to cell content when elastin and collagen are unaffected. The findings in this chapter show that within the dynamic microstructural changes in atherosclerotic progression, it is elastin content which actually is the predominant contributor in the DTI-metric’s sensitivity.

Outside of the strong correlation with elastin content, moderate correlations were found between FA and collagen, GAG content, and cell density when FA was used as a clustering input. As the elastin and collagen content decrease and GAG and cell content increase, the FA decreases (Figure 4.6(A)). While it is clear elastin content showed the most dominant correlation with DTI metrics when using k-means clustering segmentation, it did not identify known morphologies with decreased elastin content – such as the thickened intima or lipid core. However, the combination of multiple microstructural components influenced measurable anisotropy. Collagen content and cell density both showed moderate correlations with MD regardless of clustering input and were correlated with each other for all inputs. As the MD increased, collagen content decreased, and cell density increased. Additionally, while calcium showed no correlation with DTI metrics it did maintain a moderate negative correlation with collagen content. It has been shown that collagen type II content
is significantly higher around calcifications\textsuperscript{303}; however, the calcium content in this study signified calcium deposits, potentially preceding microcalcifications, rather than calcifications themselves. While unsupervised segmentation of DTI metrics for common carotids does not yield morphological regions, it is instead highly correlated to elastin specifically.

4.4.3 DTI metrics and atherosclerotic morphologies

After looking for fundamental microstructurally driven changes in FA and MD, distinct atherosclerotic morphologies were investigated. In the aged common carotids, the only atherosclerotic morphology present within the vessel wall was the thickened intima – classified as an intermediate lesion, type III, in plaque development\textsuperscript{26}. While DTI metrics were not found to be sensitive to individual microstructural changes outside of elastin (Figure 4.6) they appear to be sensitive to bulk microstructural changes in atherosclerotic morphologies, as evidenced in Figure 4.7. The decreased FA and increased MD in the thickened intima corroborates the strong correlation observed between elastin and these metrics and knowledge on the importance of cell content established in Chapter 3\textsuperscript{295}. The decreased cell and elastin content characterize the thickened intima and are distinguishable from the aged vessel media. These findings agree well with the previously seen sensitivity to cell content, Chapter 3, and the newfound understanding of elastin’s contribution to DTI-metrics. Cell content in this study was not investigated with respect to specific cell types, SMCs or macrophages, but the cell density in the thickened intima is comparable to that seen in type II lesions\textsuperscript{284}. There was an increase in GAG content that suggests that it’s possible these morphologies are an intermediate step between GAG rich fatty streaks\textsuperscript{304}, type II, and type III lesions. While previous studies have used DWI to identify advanced lipid necrotic cores\textsuperscript{144–146}, to the authors’ knowledge, this is the first to use DTI to identify early-stage atherosclerosis in human carotid arteries.

4.4.4 Study limitations

While the results of this study are promising, there are limitations to be considered. Most notably, the number of diffusion directions is at the lower limit and outside the optimal ratio of unweighted to weighted diffusion scans\textsuperscript{278}. Given the unexpected diseased nature of the subjects used in this study, it is possible the limited number of directions did not allow for an adequate representation of the microstructural organization in areas of advanced disease. However, tractography results were able to show overall representations of organisation, or lack thereof, for individual subjects. While the fixed nature of the carotids was ideal for the
long scan times that come with 3D sequences and facilitated an in-depth look at the microstructure, it is worth commenting on the embalming process and possible variations in the embalming of different subjects. The time from embalming to excising could not be controlled for; however, after excision all carotids were stored in PBS. Additionally, the extreme scan times in this study are not clinically feasible; however, other studies have identified different DTI acquisition techniques which speed up acquisition\textsuperscript{148,172,175}. While there is still work to be done on the clinical translation of this work, this study highlights the potential of this imaging technique for clinical use. Future work focuses on bringing this imaging method to more clinically relevant fresh tissue. It is also worth mentioning that dimensions of different plaque components may change during embalming, freezing, and thawing. While the samples were never frozen, it is important to note that the phase properties of some cholesterol esters may alter between liquid and crystalline phases near body temperature\textsuperscript{305}. Specifically, for the liquid phase the melting point is 44°C; however, typically in plaque tissue the presence of multiple different lipids lowers this melting temperature\textsuperscript{306}. All samples were stored at 4°C and allowed to equilibrate to room temperature prior to scanning at room temperature (approx. 25°C). All quantitative microstructural measures were taken from 2D histological slices and compared to 3D MRI volumes, but this was kept consistent across all samples. Additionally, between the registration of MR images and histology, there are distortions that occur in histological processing which could affect how accurately and precisely the histological slice can be registered to the MRI slice\textsuperscript{307}. Lastly, this study did not look at inflammation histologically; however, inflammation has been shown to be indicative of increased stress in coronary plaques\textsuperscript{308} and should be investigated in carotid plaques the future.
Chapter 5 Microstructural and mechanical insight of atherosclerotic plaques – an ex vivo DTI study to better assess plaque vulnerability

5.1 Introduction

Chapter 4 looked at implementing the sensitivity of DTI metrics to arterial tissue microstructure, seen in Chapter 3, to cadaveric human healthy and diseased arterial tissue. The following study furthers this by microstructurally and mechanically characterising fresh human atherosclerotic plaques.

While some studies on atherosclerotic plaque mechanics have used specific microstructural characterisation techniques, such as small angle light scattering\textsuperscript{108}, FTIR and SEM\textsuperscript{102,104}, very few have utilised non-invasive, clinically relevant imaging\textsuperscript{96–98}. Conversely, a number of ex vivo MRI studies have characterised plaque components\textsuperscript{159–161,169,173,174,283,309}. These studies employ different combinations of T1-, T2-, proton-density, and DW imaging to gain insight into the plaque composition. The presence of necrotic cores\textsuperscript{161,309}, lipid cores\textsuperscript{158,159,169,174,283,309}, calcifications\textsuperscript{159–161,169,173,283,309}, fibrous tissue\textsuperscript{159–161,169,173,174,283} and caps\textsuperscript{158,169,309}, inflammation\textsuperscript{174,309}, hemorrhage\textsuperscript{174}, red blood cells\textsuperscript{173}, hemosiderin\textsuperscript{173,309}, neovascularization\textsuperscript{309}, thrombus\textsuperscript{283}, and solid-state and liquid-lipid\textsuperscript{160} have been investigated. Despite all these compositions being explored, there still lacks a direct connection between composition and mechanical integrity. As there is no current clinical imaging technique which correlates composition to mechanics, ex vivo mechanical characterisation of post-operative excised specimens must be investigated with clinically relevant imaging.

The aim of this study is to bridge the gap between clinically relevant imaging technique and mechanical integrity in carotid atherosclerotic plaques. To achieve this, fresh carotid plaques from CEA surgeries were explored ex vivo by DTI to characterise the microstructure, mechanically tested to failure, and histologically processed. Altogether, the work presented here sought to investigate and establish if there is potential for non-invasive
imaging metrics to inform the vulnerability of a plaque and ultimately improve upon the use of percent stenosis as a clinical indicator.

5.2 Materials and methods

5.2.1 Sample acquisition
Carotid atherosclerotic plaques (n=7) were obtained from symptomatic CEA patients at St. James’s Hospital Dublin. All patients had a percent stenosis greater than 50%. Ethical approval was obtained from St. James’s Hospital ethical committee in compliance with the declaration of Helsinki. Carotid plaques were rinsed in PBS to remove residual blood and cryopreserved as detailed in Chapter 3 and as previously reported\textsuperscript{108}. Samples remained at -80°C until imaging and were cryopreserved between experimental steps.

5.2.2 Ex vivo imaging
On the day of ex vivo imaging, specimens were thawed at 37°C and rinsed in PBS. Fresh plaques were secured to a custom-made 3D printed holder in a 15 ml falcon tube with fresh PBS for imaging, see Figure 5.1. All plaques were imaged individually and at ambient room temperature (approximately 25°C). All imaging was performed at 7 T as outlined in Table 3.2, but with a reduced FOV to increase resolution and shorten acquisition time (image size: 64 x 64 x 64, FOV: 16 x 16 x 16 mm, isotropic resolution: 250 x 250 x 250 μm, and acquisition time: 12 hours and 30 minutes). After imaging, plaques were cryopreserved again at -80°C until mechanical testing.
5.2.3 Mechanical testing

5.2.3.1 Circumferential strips

Samples were thawed at 37°C and rinsed in PBS. Circumferential strips (n=32) were sectioned from the plaques, each with a width of 2 mm, as seen in Figure 5.1. Due to the heterogeneity of the plaque tissue, dog-bone shapes, which avoid stress concentrations at the edges by promoting failure in the centre of the gauge length, were not possible. To this end, rectangular strips were cut and any samples which failed near the grips were excluded from further analyses. Images of each strip were taken so to measure dimensions in ImageJ. Three measurements were taken, and mean width and thickness were used for the calculation of the cross-sectional area. Of 32 tested strips, 15 strips were excluded due to either failure near the grips or inability to be referenced back to MR data. Of the 17 strips presented in this study, 14 strips were taken from the plaque within the common and three strips from plaques in the internal carotid branch. In order to use digital image correlation (DIC), strips were sprayed with a tissue marking dye (Epredia™, Fisher Scientific) using an airbrush (Kkmoon Airbrush) compressor (ABEST Single Cylinder Piston Compressor).
5.2.3.2 Uniaxial tensile tests and DIC

Strips were uniaxially extended to failure using a uniaxial test machine (Zwick Z005, Zwick GmbH & Co. Ulm, Germany). Due to inconsistent dimensions from longitudinal incisions on the plaque (acquired during surgical excision) multi-axial inflation and biaxial testing were not feasible. Uniaxial extension tests on circumferentially cut strips allowed for insight into the mechanical behaviour in the dominant load bearing orientation. All tests were performed in a PBS bath at 37°C. The testing protocol included a preload to 0.01 N, after which the force was zeroed and followed by five preconditioning cycles to 5% strain, then extension to failure. All steps were done at a strain rate of 20 mm/min. DIC was used during all tests in order to track local strain deformations. A two-camera set-up was used (Dantec Dynamics GmbH, Denmark), and camera calibration was performed prior to testing. Images were acquired from both cameras at 5 Hz. After failure all samples were fixed in 10% formalin for histological processing.

5.2.4 Histological analysis

After fixation, strips were processed for histology as described in Chapter 3. Strips were either embedded to get 1) axial cross-sections – such that the lumen was oriented perpendicular to the face of the wax block or 2) with the luminal side of the strip flush with the face of the wax block in order to achieve cross-sections radially through the wall thickness. The integrity of the strip after mechanical testing dictated which of these options was most feasible.

5.2.5 Registration

For the co-registration a number of reference points were used, namely (i) the bifurcation, (ii) the base of the plaque in the common carotid, (iii) notable calcifications, and (iv) the 3D printed holder. These reference points allowed the MR data to be segmented into individual volumes for each mechanically tested strip. Figure 5.1 shows an example of the MR data overlaid on the plaque, with the red lines denoting the imaging FOV. By knowing the width of the samples measured in ImageJ (around 2 mm) and the slice thickness of the DTI images (0.250 mm), each strip corresponded to approximately eight MR slices.
5.2.6 Data processing

5.2.6.1 DTI data analysis

All raw data was denoised and corrected as outlined in Chapter 3 and the FA, MD, and helical angles (HA) were calculated from the diffusion tensor. The first eigenvector was used to calculate the HA:

\[ HA = \arctan \frac{\epsilon_{1z}}{\epsilon_{1x}} \]

where \( \epsilon_{1z} \) and \( \epsilon_{1x} \) are the z- and x-components of the first eigenvector. The calculated HA represents the angle between the predominant direction of diffusion, the first eigenvector, and the plane normal to \( B_0 \), the main magnetic field. Due to the presence of calcifications, unusable MR data was removed using the same methods outlined in Chapter 4. After registration, as described in section 5.2.5, 17 DTI volumes represented the 17 mechanically tested specimens. Due to significant heterogeneity within tissue strips, regions which were held in the grips during testing were manually removed through visual inspection of images of the sample taken prior to testing and the high-resolution DIC images. Figure B. 1 shows the visual removal of MR data throughout these steps. At this point the final tissue regions for each mechanically tested specimen were obtained and average values of FA, MD, and HA were extracted for each specimen (one mean value and standard deviation).

5.2.6.2 Tractography

Deterministic tractography was performed in ExploreDTI both on whole plaques as well as individual strips. For whole plaques the following parameters were used: seed point resolution: 0.25 x 0.25 x 0.25 mm, seed FA threshold: 0.075, FA tracking threshold range: 0.075-1, MD tracking threshold range: 0-infinity, linear, planar, and spherical geometry tracking threshold range: 0-1, fibre length: 2-50 mm, angular threshold: 90°, linear interpolation, and no random perturbation of seed points. For individual specimens the same parameters were refined slightly: FA threshold (0.05), FA tracking range (0.05-1), and the fibre length (1-50 mm). This refinement allowed more fibres (at a lower FA and shorter length) to be tracked. This wasn’t necessary for the larger whole plaques but gave more detail for individual strips. Specimens were grouped into four groups based on tractography: (1) predominantly circumferentially aligned with sparse axial tracts on the luminal side, (2) predominantly circumferential with the presence of a plaque cap shoulder, (3)
circumferentially aligned medial layer with a mixed region on the luminal side, and (4) overall mixed alignment.

5.2.6.3 Mechanical data

The cross-sectional area of each specimen was used to calculate engineering stress and the force-deformation curves after preconditioning were used to establish the stress-strain behaviour of the samples. Failure was defined as the point at the first evidence of failure\textsuperscript{101}. Specifically, when the force decreased by 5%, the ultimate tensile (UT) stress and UT strain values were extracted. Stiffness was also calculated for each sample by taking 30 data points before the final 20% of the curve and calculating the slope of the final linear region in the stress-strain curves\textsuperscript{311}. DIC analysis was performed on Istra4D (x64 V4.4.6.534). Evaluations were done with the following parameters: facet size: 69, 3D residuum: 10, grid spacing: 15 pixels and a low outlier tolerance. The reference frame in all analysis was the frame at the end of preconditioning cycles prior to the start of extension to failure. Engineering strain was investigated from DIC as both (1) the average strain across the gauge length on the tissue surface, called DIC strain, and (2) mean strain locally at the point of failure, called DIC local failure strain (Figure B. 2).

5.2.7 Statistics

Statistical analyses were performed using GraphPad Prism (Version 8). All data was tested for normality using D'Agostino-Pearson normality tests and equality of group variances using Brown-Forsythe ANOVAs. If normality was not passed, nonparametric tests were used. Pearson’s correlations were used to determine the relationship between mechanical properties and DTI metrics, with $r$ values $< 0.3$ considered weak, $0.3 < r < 0.7$ considered moderate, and $r > 0.7$ a strong correlation.

5.3 Results

Tractography of whole plaques can be seen in Figure 5.2. Red-green tracts indicate in-plane circumferential alignment, while the blue tracts represent out-of-plane axial alignment. While all specimens show some degree of axial tract alignment, there are specimens which exhibit an overall more disorganised alignment, such as the first three plaques in Figure 5.2.
Looking across the mechanical properties and DTI-derived metrics of the 17 strips, a high degree of variability was observed, see Figure 5.3. The mean UT stress and strain across the samples was $0.293 \pm 0.191$ MPa and $38.3 \pm 18.6\%$ strain, respectively. The mean stiffness across all samples was $1.26 \pm 0.6$ MPa. The mean DTI-derived MD, FA and HA were $0.00109 \pm 0.0001$ mm$^2$/s, $0.115 \pm 0.014$, and $46.69 \pm 6.14^\circ$, respectively.

Figure 5.3. Mechanical properties and DTI-derived metrics of carotid atherosclerotic plaque strips. (A) UT stress, (B) UT strain and (C) final stiffness of carotid atherosclerotic plaque strips and the corresponding DTI-derived (D) MD, (E) FA, and (F) HA. Dashed line in (F) is at $45^\circ$. 

Figure 5.2. Whole plaque tractography of the seven specimens imaged and tested in this study. Red-green tracts indicate in-plane circumferential alignment whereas blue tracts represent axial out-of-plane diffusion.
The UT strain calculated from the grip-to-grip separation significantly overestimated the DIC strain on the tissue surface, see Figure 5.4(A). However, the strains seen at the local failure locations on DIC were in turn significantly higher than the mean strain across the tissue surface. When looking at how these strains correlate to DTI-derived HA, the DIC strain demonstrated the strongest correlation (Figure 5.4(C)), followed by grip-to-grip strain (Figure 5.4(B)), and then the DIC local failure strain (Figure 5.4(D)).

![Figure 5.4](image-url)

Figure 5.4. Strain measures and their correlations to DTI-derived HA. (A) Engineering strain from grip-to-grip (Grip), the gauge length from DIC (DIC-G), and local failure on DIC (DIC-L). Significance determined by repeated measures one-way ANOVA with Tukey’s post hoc multiple comparisons; ****p<0.0001. Correlations between DTI-derived HA and (B) grip-to-grip strain, (C) DIC gauge strain, and (D) DIC local failure strain.

Tractography of individual circumferentially cut strips highlighted the presence of four different microstructural alignments, see Figure 5.5. While most samples showed some circumferential alignment (Figure 5.5(A-C)), n=4 strips showed predominantly circumferential alignment with sparse axial tracts on the luminal side (Figure 5.5(A)) and n=4 strips were circumferentially aligned with the presence of a plaque cap shoulder (Figure
5.5(B)). Figure 5.5(C) shows n=5 strips which had circumferentially aligned tracts on the more medial side and a mixed alignment visible on the luminal edge. Finally, Figure 5.5(D) shows n=4 strips with no clear alignment.

![Figure 5.5](image)

*Figure 5.5. Tractography groupings of atherosclerotic plaque strips. (A) Predominantly circumferentially aligned tracts with sparse axial tracts on the luminal edge (concave side). (B) Predominantly circumferentially aligned tracts with the presence of a plaque cap shoulder – visible at the junction between fibres on the luminal side. (C) Circumferentially aligned medial tracts with large regions of mixed microstructural alignment on the luminal side and (D) overall mixed samples with no clear alignment. Red-green tracts indicate circumferential alignment, while blue is out-of-plane, axially aligned tracts.*

Using the tractography based grouping shown in Figure 5.5, significant mechanical insight was discovered, see Figure 5.6. The FA in mixed Group 4 strips (0.102 ± 0.014) was significantly lower than that in Group 3 (0.135 ± 0.008) and 2 (0.128 ± 0.009) strips, see Figure 5.6(B). The MD in Group 3 strips (0.0009 ± 0.00006 mm²/s) was significantly lower than that in Group 2 strips (0.0011 ± 0.00004 mm²/s). The HA was only significantly different between Group 2 and 4 strips, at 39.34 ± 5.32° and 53.26 ± 4.98°, respectively (Figure 5.6(C)).

The stress-strain curves presented in Figure 5.6(D) illustrate the different mechanical behaviours between these strip specimens. Predominantly circumferentially aligned strips in Group 1 have a significantly higher UT stress (0.559 ± 0.147 MPa), than those in Group 2 (0.264 ± 0.089 MPa), Group 3 (0.124 ± 0.02 MPa), and Group 4 (0.254 ± 0.161 MPa), see Figure 5.6(E). The UT strain (Figure 5.6(F)) in Group 3 strips (24.53 ± 8.43%) was significantly lower than that of Group 2 (51.82 ± 14.6%) and Group 1 (55.45 ± 9.11%) strips. Group 4 strips also had a significantly lower UT strain (24.93 ± 16.23%) than Groups 2 and 1. Figure 5.6(G) highlights the significantly stiffer response of Group 1 strips (2.06 ± 0.316 MPa) compared to both Group 2 (0.838 ± 0.260 MPa) and Group 3 (0.815 ± 0.214 MPa).
Figure 5.6. Mechanical properties of atherosclerotic plaque strips when informed by DTI-derived tractography. (A) Representative tractography of strip samples in each group. (B) FA and MD values within these groups; FA: significance determined by an ordinary one-way ANOVA with Tukey's post hoc multiple comparisons, **p=0.0023, *p=0.0237. MD: significance determined by Brown-Forsythe and Welch ANOVA with Dunnett's T3 post hoc multiple comparisons, **p=0.0089. (C) Mean HA for each group – the dotted line is at 45°; significance determined by ordinary one-way ANOVA with Tukey's post hoc multiple comparisons, *p=0.0227. (D) Stress-strain curves for n=17 strips, colour coded by their respective groupings. (E) UT stress; significance determined by ordinary one-way ANOVA with Tukey's post hoc multiple comparisons, Group 1 and 2 *p=0.0138, Group 1 and 3 ***p=0.0005, and Group 1 and 4 *p=0.0112. (F) UT strain; significance determined by an ordinary one-way ANOVA with Tukey's post hoc multiple comparisons, Group 3 and 2 *p=0.0225, Group 3 and 1 *p=0.0145, Group 4 and 2 *p=0.0255, and Group 4 and 1 *p=0.0191. (G) Final stiffnesses; significance determined by Brown-Forsythe and Welch ANOVA with Dunnett's T3 post hoc multiple comparisons, Group 1 and 2 **p=0.0051 and Group 1 and 3 **p=0.0053.
DIC not only allowed for strain contours to be displayed on the tissue surface but allowed for retrospective insight into how the strips failed. Figure 5.7 presents strain maps on representative strips for each group. High strain (shown in red) can be seen at the point of failure in Group 1 followed by abrupt failure (Figure 5.7(1)). Specimens in Group 2 consistently failed at the junction between differing microstructures present at the plaque cap before delaminating behind the lipid core (Figure 5.7(2)). Interestingly, the specimens in Group 3 similarly showed initial intimal tearing; however, delaminated through the thickness of the mixed region (Figure 5.7(3)). Specimens in Group 4 failed quite variably. In the example strip shown the location of failure did not show the highest local strain, showing that higher strains are not always co-located with failure.

![Figure 5.7. DIC strain contours and failure insights based on tractography groupings. For each grouping, (i) representative tractography is shown at the top, alongside DIC images at the (ii) reference frame (after preconditioning), (iii) right before failure, and (iv) right after failure. White arrows point to location of failure both on tractography and on strain maps.](image)

Representative histology of the strips shown in Figure 5.7 are shown in Figure 5.8-Figure 5.10. Figure 5.8 shows axial cross-sections for both strips in both Group 1 and 2. For both groups, failure occurred at the junction of differing microstructures, as pointed out by the green arrows. In the Group 1 sample, intimal thickening can be seen in H&E and decreased elastin is also visible in the Verhoeff’s stained cross-section. PSR and PLM highlight the circumferential arrangement of this sample, as seen in tractography. Similarly, circumferential arrangement can be seen on the more medial side (left side of image) in the Group 2 sample. However, there is a distinct delineation of differing microstructure, where failure occurred, at the location of the lipid core.
Figure 5.9 similarly shows representative histology for the Group 3 specimen shown in Figure 5.7. Unlike the cross-sections in Figure 5.8, these are radial slices through the thickness of the plaque wall due to difficulties orienting and embedding these mechanically tested strips. This specimen failed at the bottom edge of the strip, located at the bottom of the images, at what appears to be a plaque cap shoulder. The green box points to the centre of the strip (located in the centre of the gauge length) which is acellular, low in elastin, and has disorganised collagen (seen in PLM). The blue box highlights a plaque cap shoulder which could be similar to the shoulder at which the sample failed. Increased cell density is seen in this region, with nuclear alignment tending to be more longitudinally oriented. Distinct longitudinally aligned collagen fibres can be seen in PLM alongside regions of disorganisation.

Lastly, Figure 5.10 highlights radial cross-sections of the Group 4 specimen seen in Figure 5.7. Failure delaminated down the length of the specimen, seen in Figure 5.7(4), and H&E presents the variable cell densities circumferentially. PLM shows collagen alignment tending towards circumferential but overall, quite disjointed.

![Figure 5.8. Representative histology of strips in Group 1 and 2. H&E, Verhoeff's elastin, PSR and PLM are presented for each sample. Green arrows point to location of failure. Histology oriented to show axial cross-sections, moving from luminal edge towards media right to left and circumferential orientation top to bottom. All scale bars are 300 μm.](image)
Chapter 5

5.4 Discussion

This study used DTI-derived metrics to investigate the mechanical integrity of carotid atherosclerotic plaques. One previous study has looked at tractography-derived fibre orientation in carotid plaques and, while variable, found the predominant direction to be circumferential. When assessing the overall tractography of plaques in this study, varying degrees of alignment can be qualitatively seen (Figure 5.2). Variations in the microstructure can be seen both circumferentially, as well as longitudinally through the length of the plaque.
These qualitative insights highlight the degree of disorganization and microstructural variation not only between plaques, but within individual plaques.

The well-documented variable mechanical response of carotid plaques\textsuperscript{96,97,99,102,103} was also seen in this study. Comparatively, the UT stresses and strains of circumferentially cut strips in this study align well with those determined previously\textsuperscript{96,97,102}. These studies pre-operatively used ultrasound to classify plaques as calcified, echolucent, or mixed and found no significance between groups. Only when using post-operative FTIR, was Mulvihill et al. able to differentiate plaque compositions and their mechanical properties in pure shear tests\textsuperscript{102}. Similarly, it was only when using tractography of individually tested atherosclerotic strips that different microstructures became apparent in this study (Figure 5.5). Ultimately, these microstructures yielded significant mechanical insight into the plaque tissue. The earliest sign of progressive atherosclerosis is the thickening of the intima, classified as an AHA Type III lesion\textsuperscript{26,312}. Group 1 strips in this study, defined as having predominantly circumferential tracts with sparse axial diffusion on the luminal edge, show signs of intimal thickening histologically (Figure 5.5(A) and Figure 5.8). These samples failed at significantly higher stresses and strains than the other microstructures in this study.

Following the thickening of the intima AHA Type IV plaques or fibroatheromas can develop\textsuperscript{25}. Thin cap fibroatheromas exhibit low SMC density in the plaque cap which occludes a lipid or necrotic core\textsuperscript{313}; however these atheromas often fail to narrow the vascular lumen despite thickening the arterial wall\textsuperscript{314}. Both Groups 2 and 3 in this study exhibited the circumferential alignment known in the medial layers of the arterial wall, see Figure 5.5(B and C), but also showed signs of more advanced plaque development. Group 2 strips were similar in microstructure to Group 1 but had the distinct presence of a plaque cap shoulder. Tractography was capable of visualising the plaque cap and showed circumferential alignment, see arrow in Figure 5.7(2), which was corroborated by the PLM histological images seen in Figure 5.8. Circumferential alignment is clear on both the luminal and medial edges, and surrounds an elastin-poor, disorganised (with respect to cell and collagen content) region. There also appears to be evidence of cholesterol crystals, which are believed to arise from cellular apoptosis\textsuperscript{312}. Mechanically, these strips appeared to initially strain similar to Group 1 strips. When looking to the DIC images it appears the circumferentially aligned regions are bearing the load, until ultimately failure occurs at a significantly lower stress at the junction between the circumferential medial regions and the plaque cap shoulder (Figure 5.7(2)). Group 3 strips showed varying degrees of alignment in
the plaque cap, but all show a thick, mixed region between the cap and the medial layers of the plaque. While both Groups 2 and 3 failed on the luminal edge of the plaque, Group 3 failed through the mixed region whereas Group 2 strips delaminated behind the lipid core – seen both in the DIC images and histologically (Figure 5.7(2 and 3) and Figure 5.8). Mechanically, Group 3 strips failed at significantly lower stresses and strains than both Groups 1 and 2 – identifying them as the most vulnerable microstructure of those seen in this study (Figure 5.6(E and F)). When looking histologically, it becomes clear that Group 3 strips also failed at the plaque cap shoulder – where there is a distinct difference in cell density and collagen orientation (Figure 5.9). The mixed microstructures in Group 4, unsurprisingly, show highly variable mechanical properties and a highly disordered microstructure via tractography (Figure 5.5(D)) and histology (Figure 5.10). Together, these results suggest that under the same physiological conditions, plaque tissue with a microstructure resembling that of Group 3 strips would be the most vulnerable to rupture. Conversely, a microstructural alignment mimicking that in Group 1 would be the least vulnerable and more stable – suggesting intervention may not be needed.

Another interesting finding from this work highlighted differences in the strain across the tissue. The strain calculated from the grip-to-grip separation overestimated the strain across the tissue surface measured using DIC. However, the local failure strains on the tissue surface are significantly higher than these mean strains on the tissue surface. Previous work has shown that strain, rather than stress, might be a better indicator for plaque cap vulnerability\(^\text{103,108}\). The work presented here shows that the strain at local regions of plaque rupture might be significantly higher than the overall strain of the tissue.

In this study fresh, human atherosclerotic plaques from CEA were imaged \textit{ex vivo} with a DTI sequence, mechanically tested, and investigated histologically. For the first time, this work identified a non-invasive imaging technique which yields microstructural insight into atherosclerotic plaques which ultimately was used to identify microstructures more at risk of rupture. These novel findings have the potential to drive continued research in non-invasive imaging techniques linked with mechanical characterisation to better capitulate plaque vulnerability.
Chapter 6 Using DTI-derived metrics to non-invasively track recellularisation in vascular tissue engineering

6.1 Introduction

Having explored the potential of DTI to identify diseased vascular tissues in Chapter 4 and Chapter 5, this study refocused on the initial DTI sensitivity to cell content uncovered in Chapter 3. Here, that sensitivity to cell content is exploited to ascertain the role of DTI in the field of tissue engineering.

The aim of this study was to evaluate, non-invasively, recellularisation in two TEVGs, specifically decellularised and freeze-dried decellularised PCaA, over a two-week culture period with DTI. The results presented in this work are explained and validated by quantitative histological analysis. This work highlights the promise of quantitative DTI-derived metrics in the field of vascular tissue engineering.

6.2 Materials and methods

A brief overview of the methods in this study are presented in Figure 6.1.

6.2.1 TEVG preparation

Fresh PCaA was collected as in Chapter 3 and decellularised using the same protocol as that outlined in Table 3.1. Decellularised vessels were cut into 10 mm long segments and inverted in order to remove the intimal layer. This exposed the highly aligned microstructure of the medial layer of the arterial wall. A scalpel was carefully dragged along the intima layer, with no pressure applied, and fine tipped forceps were used to peel the intimal layer off. Any vessels which had uneven intimal layer removal were excluded.

Vessels up to this point constitute one group used in this study – decellularised, de-intima porcine carotid arteries (ddPCaA), seen in Figure 6.1(a). Other vessels were lyophilized using a similar protocol to one previously reported which preserves the mechanical properties of the scaffold. Briefly, vessels were allowed to air-dry then snap-frozen in liquid nitrogen and transferred immediately into a precooled freeze drier (FreeZone, Labconco Corporation, Kansas City, MO) at -40°C. The samples were kept at -40°C for two
hours and then ice crystal sublimation was induced by decreasing the pressure to 0.266 mbar and increasing the temperature to 0°C at 1°C/min. After 18 hours, a secondary drying phase to 20°C at 1°C/min concluded the lyophilization process. These vessels constitute the second graft group in this study – freeze-dried, decellularised, de-intima porcine carotid arteries (FD ddPCaA), seen in Figure 6.1(a). All vessels were sterilised in 100% ethanol for one hour, rinsed in sterile PBS and then transferred into sterile culture medium and incubated statically at 37°C for 24 hours prior to seeding.

Figure 6.1. General overview of the presented study. (a) Two TEVGs were used in this study: ddPCaA and FD ddPCaA. Scale bars 10 mm. (b) TEVGs were seeded with pSMC and cultured in individual eppendorf tubes on a roller for up to two weeks. (c) DTI was performed on both TEVG groups in order to calculate the FA and MD as well as perform tractography. (d) Qualitative and quantitative histological analysis was performed on each graft. Scale bars are 500 μm.
6.2.2 Cell seeding and dynamic culture

Porcine smooth muscle cells (pSMC) were isolated using a similar protocol used previously for rat SMCs\(^{317}\). PCaA were obtained from the abattoir, cleaned of connective tissue, and the adventitia was removed. Carotids were cut into segments no larger than 1 mm\(^3\) and placed into MgCl\(_2\) + CaCl\(_2\) supplemented PBS with 0.7 mg/ml collagenase type 1A from Clostridium histolyticum (Sigma) and 0.25 mg/ml elastase type III from porcine pancreas (Sigma). The tissue was digested under constant agitation at 37°C until totally digested, approximately 7 hours. The resulting cell suspension was spun down at 400 g and the digestion solution removed. The pSMC were cultured in high glucose DMEM with Glutamax (Biosciences) with 10% FBS (Gibco) and 0.2% primicin at 37°C, 5% CO\(_2\), 20% O\(_2\) and used at p6 for all experiments. For TEVG seeding, a 1 ml eppendorf was used for individual vessels, which were seeded at a concentration of 1.5·10\(^6\) cells/ml. Each eppendorf was fully closed and placed on a roller in an incubator at 37°C (Figure 6.1(b)). Due to the closed nature of the culture, media was changed daily in the A.M. and the vessels were vented in the P.M., approximately 10 hours later, for 5-10 minutes in sterile conditions. The dynamic culture lasted two weeks with terminal time points on day 3, 7, and 14. There were four vessels per time point for both ddPCaA and FD ddPCaA and one unseeded control for both groups at each time point. Vessels were fixed in 10% formalin for three hours at their respective time points then transferred to PBS until imaging.

6.2.3 DTI acquisition and analysis

All MR imaging was performed using the same sequence described in Chapter 4. Dental floss was tied onto a custom-made 3D printed holder which allowed for 12 vessels – three time points (n=3 per time point) and one control per time point – to be imaged together (Figure 6.1(c)). This set up was secured into a 50 ml falcon tube with fresh PBS for imaging. All ddPCaA vessels were imaged in one scan session and similarly all FD ddPCaA vessels were imaged together: two scan sessions in total.

All raw data was processed as outlined in Chapter 3 and FA and MD were calculated from the diffusion tensor. Due to variations in sample lengths and regions outside the imaging FOV, all vessels were analysed for the same number of slices (eight slices – 2.5 mm of length); these slices started at one end of the vessel and moved inwards longitudinally towards the centre of the vessel. FA and MD were averaged across the slices for each vessel, yielding one mean and standard deviation per vessel. Tractography was performed in ExploreDTI and was done on the same eight slices. The tracking parameters were as
follows: seed point resolution: 0.3125 x 0.3125 x 0.3125 mm, seed FA threshold: 0.1; FA tracking threshold range: 0.1-1, MD tracking threshold: 0-infinity, linear, planar, and spherical geometry tracking threshold range: 0-1, fibre length range: 0.5-50 mm, angle threshold: 45°, step size: 0.3125 mm, linear interpolation method, and no random perturbation of seed points. Representative slices are presented in parametric maps (FA and MD) and representative vessels shown for tractography.

6.2.4 Microstructural quantification

One vessel at each time point was qualitatively imaged with DAPI (Sigma) staining prior to MR imaging on a Leica SP8 scanning confocal microscope. TEVG were sectioned into 2 mm long segments and if possible, images were taken both at the end of the vessel (exposed edge) and the middle of the vessel. All images were taken at an image size of 1024 x 1024 and a scan speed of 400 Hz. These images were used to check for successful cell seeding and monitor cell infiltration during culture; these vessels were not imaged with MRI or used for quantitative histology. They are qualitative 3D representations of recellularisation for each time point.

Quantitative histological analysis was performed after MR imaging for all vessels. TEVG were brought to histology and elastin and collagen were quantified as described in Chapter 4. Elastin and collagen content were determined across three ROIs per vessel and averaged to yield one value per vessel. DAPI stained histology slides were imaged on an Olympus BX51 upright microscope (exposure time 200 ms). Cell counting was done manually using the cell counter ImageJ plug-in and tissue area measured using tissue detection in QuPath. Due to the low cell densities in this study, this plug-in was sufficient; however, methods similar to those established in Chapter 4 should be adapted in the future or with higher cell densities. Cell densities were calculated across nine ROIs per vessel and averaged to yield one density per vessel. Alcian blue staining was also performed to qualitatively look at the GAG content in the control grafts.

6.2.5 Statistical analysis

All statistical analysis was performed using GraphPad Prism V9.3.1. Normality was tested using D’Agostino-Pearson tests (alpha=0.5) and equal variances using Brown-Forsythe tests. FA, MD and tractography measures all passed both normality and equal variances so ordinary one-way ANOVAs with Tukey’s post hoc multiple comparisons were used. All DTI-derived data is presented as mean per vessel (averaged over eight slices) and standard deviation across the three vessels per time point. Similarly, quantitative histology measures
were tested for normality and equal variances; all data passed both tests and ordinary one-way ANOVAs with Tukey’s post hoc multiple comparisons test were used. To investigate the relationship between FA and MD with cell density and collagen-to-elastin ratio, Pearson’s correlations were tested, where an r value greater than 0.7 was considered a strong correlation. The ROUT method (Q=1) was used to check for outliers, from which none were found.

6.3 Results

6.3.1 DTI-derived metrics of recellularised porcine carotid arteries

In this study, two different TEVGs, ddPCaA and FD ddPCaA, were tested in an attempt to facilitate recellularisation and explored via DTI to non-invasively track this during a two-week culture.

Figure 6.2 presents DTI-derived FA and MD parametric maps and mean values for each time point within the two groups. Panel (a) shows the results for ddPCaA. Visually, both parametric maps, (a-i) FA and (b-ii) MD, show qualitative differences between unseeded controls (top left within each slice, marked with asterisks) and different time points in the two-week culture. These differences were quantified and are presented in (a-iii) and (a-iv). The mean FA of vessels cultured for 3 days was 0.19 ± 0.008, which was significantly lower than that of day 7 vessels, 0.23 ± 0.02, and day 14 vessels, 0.22 ± 0.004. Interestingly, the mean FA of the unseeded controls consistently decreased between time points. The day 3 unseeded control had an FA of 0.19, the day 7 – 0.12, and the day 14 vessel – 0.08 (visualised by the coloured lines in (a-iii)). The opposite trend was seen with MD for the different time points. Day 3 seeded vessels had the highest MD, 0.0011 ± 0.00009 mm²/s, followed by day 14 vessels, 0.0010 ± 0.00004 mm²/s, and day 7, 0.00098 ± 0.00003 mm²/s. Increasing MD was also observed with the unseeded controls from day 3 to 14, 0.00099, 0.0011, and 0.0014 mm²/s, respectively.

The same metrics for the FD ddPCaA are presented in Figure 6.2 panel (b). Parametric maps of FA and MD are shown in (b-i) and (b-ii). When quantified, the FA of day 7 vessels was significantly higher than day 3 vessels, 0.25 ± 0.02 and 0.14 ± 0.06, respectively. No significant difference was seen when compared to day 14 vessels, 0.22 ± 0.02. Unlike ddPCaA, the FA of the unseeded FD ddPCaA controls remained constant from day 3 to 14: 0.19, 0.21, 0.22, respectively. No significant differences were seen from day 3 to 14 with respect to MD of the pSMC seeded FD ddPCaA, 0.0011 ± 0.0002, 0.00089 ± 0.00007, and
0.00092 ± 0.00001 mm²/s, respectively. Again, the unseeded controls remained constant from day 3 to 14: 0.00089, 0.00093, and 0.00093 mm²/s, respectively.

Figure 6.2. DTI-derived metrics for (a) ddPCaA and (b) FD ddPCaA. (a, b-i) FA and (a, b-ii) MD parametric maps and mean (a, b-iii) FA and (a, b-iv) MD metrics for each time point. Windowing of parametric maps match the axes in graphs. Coloured asterisks (in parametric maps) and lines (in graphs) represent unseeded controls for each time point (n=1). Red signifies day 3 control, blue is day 7, and green day 14. Significance between TEVG (n=3 per time point) was determined with ordinary one-way ANOVAs with Tukey’s multiple comparisons. (a-iii) *p=0.0216 between d3 and d7 and *p=0.0248 between d3 and 14. (b-iii) *p=0.0308.

Tractography was performed for all TEVGs and results for unseeded controls as well as representative grafts for each time point are presented in Figure 6.3. In both panel (a) and (b) the top row presents the unseeded controls while the second row is pSMC seeded grafts. Qualitatively, for both groups there is a lack of continuity between tracks in the controls that is not as apparent in the recellularised vessels. Differences between time points was quantified in (a, b-ii-iv). The coloured lines in (ii-iv) are the unseeded controls which, save the day 3 control, are all well below the means for the pSMC seeded vessels at day 7 and 14 for ddPCaA (Figure 6.3((a)ii-iv)). Day 7 pSMC seeded ddPCaA had a significantly higher (a-ii) tract volume and (a-iii) number of tracts than day 3 vessels. Day 14 ddPCaA also had a significantly higher (a-iii) number of tracts than day 3 vessels. Similarly, day 7 FD ddPCaA had significantly higher (b-ii) tract volume and (b-iii) number of tracts than day 3 vessels. Day 14 FD ddPCaA vessels also had significantly higher (b-ii) tract volume and (b-iii) number of tracts than day 3 vessels.
Figure 6.3. Tractography for both (a) ddPCaA and (b) FD ddPCaA TEVGs. (a, b-i) Unseeded control vessels are shown in the top rows, with representative recellularised grafts in the second row for each group. (a, b-ii) Mean tract volume, (a, b-iii) number the tracts, and (a, b-iv) mean tract length for both groups. Significance was determined with ordinary one-way ANOVAs with Tukey’s post hoc multiple comparisons: (a-ii) *p=0.0160, (a-iii) *p=0.0204, **p=0.0039, (b-ii) *p=0.0144 between d3 and d7, *p=0.0163 between d3 and d14, (b-iii) **p=0.0100 between d3 and d7, **p=0.0099 between d3 and d14. Coloured lined are unseeded controls for each time point (n=1).
6.3.2 Qualitative histology

At day 3, 7, and 14 one TEVG from each group was DAPI stained and imaged for a qualitative look at cell infiltration. Figure 6.4 highlights these findings. For both groups, cell infiltration through the thickness was seen on day 3. Thorough infiltration through the wall thickness was seen in Figure 6.4(a) day 7 and 14 ddPCaA grafts at the exposed edges; however, when looking at a more central region of the graft, cells were only present on the luminal and adventitial sides. Similar results were seen for FD ddPCaA; however, the day 14 graft showed no infiltration through the wall thickness (Figure 6.4(b)). Additionally, the luminal cell layer was thicker than a monolayer for both day 7 and 14.

Alcian blue staining of unseeded controls showed a slight decrease in the already low GAG content of the control grafts, see Figure 6.5. The ddPCaA grafts appear to have increased intra-wall separation when comparing to the FD ddPCaA grafts. The FD ddPCaA grafts appear more densely compact within the vessel wall.
Figure 6.4. Qualitative scanning confocal microscopy of DAPI stained TEVG at each time point. (a) ddPCaA and (b) FD ddPCaA both showed significant cell infiltration up to a depth of 400 μm at the exposed ends of the grafts (top rows for day 7 and 14). However, when looking at edges from the centres of the grafts, this infiltration was less apparent, and a luminal layer of cells was present (bottom row for day 7 and day 14). Scale bars are 200 μm.
6.3.3 Quantitative histology

Representative histology is shown for ddPCaA in Figure 6.6 and for FD ddPCaA in Figure 6.7. While no significant difference was found, increasing cell density was observed for ddPCaA (Figure 6.6(a)). The collagen-to-elastin ratio remained constant during culture for ddPCaA, also with no significant differences. Due to visibly inconsistent cell seeding observed in the FD ddPCaA, the top row of Figure 6.7 has both unseeded controls in the top right subfigure and examples of localised high cell density regions in the bottom subfigure. While, similar to ddPCaA, no significant differences were found (Figure 6.7(a, b)), the cell density and collagen-to-elastin ratio both decreased at day 7 before increasing again at day 14 but this was not significant.

Figure 6.5. Alcian blue staining of unseeded vascular grafts. Scale bars are 200 μm.
Figure 6.6. Representative histology of ddPCaA. DAPI stained cross-sections (top row), PSR stained sections imaged with PLM (second row) and Verhoeff’s stained cross-sections (third row) at each time point. Subfigures are unseeded controls at each time point. All scale bars are 500 μm. (a) Cell densities (b) and collagen-to-elastin ratios for all ddPCaA TEVG. Coloured lines are unseeded controls. No significant differences were found using ordinary one-way ANOVAs with Tukey’s multiple comparisons.
Figure 6.7. Representative histology of FD ddPCaA. DAPI stained cross-sections (top row), PSR stained sections imaged with PLM (second row) and Verhoeff’s stained cross-sections (third row) at each time point. Subfigures are unseeded controls at each time point. DAPI subfigures are unseeded control (top) and examples of densely seeded localised regions in recellularised grafts (bottom). All scale bars are 500 μm. (a) Cell densities (b) and collagen-to-elastin ratios for all FD ddPCaA TEVG. Coloured lines are unseeded controls. No significant differences were found using ordinary one-way ANOVAs with Tukey’s multiple comparisons.
Figure 6.8 presents the correlations between DTI-derived FA and MD with quantitative histology measures. Panel (a) shows ddPCaA and FD ddPCaA is shown in panel (b). For ddPCaA grafts, cell density was strongly and significantly correlated to FA (Figure 6.8(a-i)). For the FD ddPCaA the collagen-to-elastin ratio was strongly and significantly correlated with both FA and MD (Figure 6.8(b-i, ii)).

![Figure 6.8. DTI-derived FA and MD correlations with quantitative histology.](image)

6.4 Discussion

To date, the biomedical field has very few examples of MRI for tissue engineering applications. When it comes to solely using the intrinsic contrast provided by the abundance of \(^1\)H protons, the majority of studies use multiple contrasts\(^{228,238,241,242}\) and it can be difficult to elucidate what changes in the tissues are resulting in the consequent changes in measurable signal. Chemical exchange saturation transfer has shown an ability to measure specific concentrations of different molecules\(^{281,319}\), however, its use in tissue engineering
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is limited\textsuperscript{320}. DTI has been used in a handful of focused studies in different tissues to understand how different microstructural components affect the measurable signal\textsuperscript{175,257,295}. A significant benefit of DTI is the ability to get directional information about the underlying microstructure – a feature which is highly relevant in both cardiac\textsuperscript{213,321–323} and cartilaginous\textsuperscript{324–326} tissues.

The results in this study showed, for the first time, that DTI-metrics are capable of tracking recellularisation in arterial tissue. Specifically, for ddPCaA the FA was significantly higher in day 7 and 14 grafts compared to day 3 grafts. As collagen and elastin content did not change between time points, the increasing cell density was the dominant microstructural change driving this FA increase. This relationship was confirmed by the strong correlation between FA and cell density. While the mono-exponential DTI model has shown that the main contributor to the measurable anisotropic diffusion in arterial tissue is cellular content, established in Chapter 3\textsuperscript{295}, non-gaussian schemes, such as the stretched-exponential model, have been used to further hone in on alterations in the tissue microstructure\textsuperscript{175}. Only one study has looked at the relationship with cell density related to tumour progression and DTI-derived perpendicular diffusivity. The authors identified both perpendicular diffusivity and FA as potential markers for glioma cell migration and invasion\textsuperscript{244}. Glioma cells migrate along white fibre bundles in the brain, and when these are disrupted due to erosion, destruction, impingement, and peritumoral edema in tumour progression, the disordered microstructure is identifiable by decreased FA\textsuperscript{327,328}.

Interestingly, for the FD ddPCaA, while the FA was significantly higher in day 7 grafts, there was a drop in cell density and collagen content with an increase in elastin compared to day three grafts. Previous work in Chapter 4 has shown a strong correlation between FA and elastin which likely explains this finding. With the layered structure and highly aligned collagen and elastin present within the arterial wall, the sublimation of ice crystals does not necessarily yield pore formation from lyophilisation\textsuperscript{316}, as typically seen in homogenous solutions, and no disruption to this microstructure was seen histologically. While lyophilisation is typically used to achieve high levels of porosity and anisotropy\textsuperscript{322}, the protocol used in this study instead focused on maintaining mechanical integrity of the graft rather than creating larger pores\textsuperscript{316}. Further studies should be done to investigate varying porosities in TEVGs and the influence on DTI-derived parameters.

It is interesting to note the decreasing FA and increasing MD in ddPCaA unseeded controls alongside qualitatively less continuous tractography. Over a two-week period in culture
medium, at 37°C, and on a dynamic roller, it would not be surprising to have tissue
degradation. However, no clear degradation was observed regarding collagen or elastin in
the unseeded controls. While GAG content was not quantitatively investigated, unseeded
controls qualitatively appeared to have less GAG from day 3 to day 14 which could explain
this result (Figure 6.5). Upon visual inspection of tractography of the unseeded control
vessels between the two groups it becomes clear that the overall quality decreases in
ddPCaA while no clear trend is seen in the FD ddPCaA controls. When looking between the
two different graft types, the FD ddPCaA grafts appear more densely packed within the wall,
seen in Figure 6.5, compared to the ddPCaA grafts. It is possible that the lyophilisation
compacts the wall, leading to a denser tissue which protects the graft from degradation as
seen in the DTI-metrics for the ddPCaA grafts (Figure 6.2 and Figure 6.3). Unquestionably,
however, the tractography of the recellularised grafts for both groups at all time points is
more representative of native arterial tissue than the decellularised control TEVGs. These
differences were further shown in the quantification of tractography based measures.
Tractography has previously been shown to be a useful visualisation tool when it comes to
crude and specific microstructural changes in healthy and diseased arterial tissue. While it is typically used in the brain, DTI tractography has demonstrated sensitivity to pathological processes in the spinal cord and results from this study demonstrate its potential use in the field of tissue engineering.

The samples in this study were fixed at each time point due to the lengthy scan time at
ambient temperature, in a non-sterile environment – all concerns for continuing a cell culture
experiment. Future work aims to develop an MRI compatible bioreactor which would allow
for TEVG to be taken out of culture, imaged, and returned to culture. Novel MRI-compatible
bioreactors already exist and with the wide-spread use of 3D printing and MRI-compatible
sensors, there are exciting opportunities to harness MRI as a longitudinal imaging
technique in tissue engineering. It has been shown how vascular stem cells align with
underlying collagen fibres regardless of the direction of strain imposed; however, when this
alignment is parallel to the direction of strain – it resulted in increased proliferation.
Ghazanfari et al. also found that higher aspect ratio constructs resulted in more highly
aligned collagen, and likely cells. While this study only used circumferential rotation during
culture, and it proved sufficient to facilitate cellular alignment with the TEVG microstructure,
future studies should investigate more physiological mechanical loading conditions. DTI is
notoriously tricky to implement in vivo in the carotid arteries due to physiological motion and
lengthy scan times required to obtain high angular resolution data, making ex vivo tissue engineering applications the ideal application for this imaging technique in the interim.

The results presented in this study clearly demonstrate the use of DTI-derived metrics in the field of tissue engineering. We showed that both FA and tractography measures were sensitive to recellularisation of TEVG, establishing the feasibility of DTI to be used in tissue engineering and highlights the exciting potential of this technique.
Chapter 7 Final discussion

With population ageing and improved survival rates, the number of people living with stroke is expected to increase 27% in the next 25 years within the European Union\(^5\). Stroke is second only to ischaemic heart disease within CVD, which accounts for more than half the deaths across Europe\(^3\). Improving screening methods, both in diagnosis and treatment efficacy, is key to reducing the burden of CVD. Non-invasive imaging offers the most benefits in both these areas. Currently, duplex ultrasound is the standard of care and first-line imaging technique used in the clinical workflow for atherosclerosis, whereas MRI and CT are reserved for additional confirmation of ultrasound assessment if necessary or for intracranial pathologies\(^333\). While ultrasound is faster, cheaper, and more readily available than MRI – a recent study by Eli et al. modelled the cost-effectiveness of MRI as a first-line imaging strategy for asymptomatic patients with carotid stenosis and IPH. Results showed a 16.8% relative stroke risk reduction when MRI was used as a first-line tool to identify asymptomatic, high-risk patients requiring annual surveillance when compared to duplex ultrasound alone\(^334\). A duplex ultrasound scan costs approximately $171.10 and a sensitivity analysis yielded a cost of $212.65 for a two-sequence MRI scan without contrast for the head and neck. While this would vary country-to-country, with this study based in Canada, it found that MRI remained the cost-effective first-line modality below a willingness-to-pay threshold of $50,000 – set by health care providers\(^334\). Currently, annual screening is not implemented for atherosclerotic disease progression, but research into the composition of plaques and how they correlate to vulnerability aims to identify if this could improve current clinical workflows. It is also widely accepted that MRI is the preferrable imaging modality for the assessment of atherosclerotic plaques due to its reliability in the detection of plaque components\(^335\).

In 2000, Hatsukami et al. was one of the first to use MRI to identify components of atherosclerotic plaques which may indicate a high likelihood for rupture\(^140\). Since then, a multitude of studies, both in vivo (Table 2.6) and ex vivo (Table 2.7), have tried to characterise the so-called vulnerable plaque. Interestingly, despite the promise in disease characterisation, only one study has used non-invasive imaging to characterise vascular
tissue engineering\textsuperscript{211}. The objective of this thesis was to investigate a singular non-invasive imaging technique, DTI, in vascular tissue to establish non-invasive biomarkers indicative of microstructural changes. To do this, a combination of experiments on animal and human, healthy, and diseased tissue were carried out in combination with microstructural, morphological, and mechanical characterisation.

Chapter 3 outlined the development of arterial tissue models to investigate the impact of specific microstructural components on DTI-derived metrics\textsuperscript{295}. By selectively removing SMCs, elastin and collagen we explored how each component plays a part in the typically anisotropic diffusion profile of arterial tissue\textsuperscript{172}. Differences between native and decellularised arterial tissue demonstrate that the main contributor to this anisotropic diffusion in arterial tissue is the presence of cell content. While the elastin degraded model suggests a similar contribution – the removal of elastin resulted in a significant increase in extracellular space and decrease in SMC density. The increased extracellular space in the elastin degraded model was far greater than any other tissue model, making it difficult to elucidate the exact impact of elastin removal. While previous studies highlight the role of collagen fibres in diffusion derived metrics\textsuperscript{172,211,257}, here, we establish the impact of cell, collagen, and elastin content and characterise their influence on FA, MD and tractography. This result highlights the significance of cellular content and corroborates findings in a previous \textit{ex vivo} DTI study on cell migration in brain tumours\textsuperscript{244}. The findings from this initial study form the foundation for the studies which follow, where the sensitivity to specific microstructural changes in arterial tissue are investigated in more clinically relevant applications.

Investigating the sensitivity of DTI to microstructural changes in human cadaveric tissue in Chapter 4 brought the sensitivity established in Chapter 3 to a more clinically relevant space. Following image registration and the use of an unsupervised segmentation algorithm, a strong correlation was found between both DTI-derived FA and MD and elastin. Regardless of clustering input, there was a strong correlation with elastin. Elastin fragmentation is not only evident in vascular aging but also present in the earliest signs of atherosclerosis. While the impact of elastin in its extreme degradation in Chapter 3 was not clear, this study demonstrates its significant impact. Elastin chains rarely turnover due to their stability and longevity, so any damage tends to be irreversible. The quality and quantity of elastin is a good indicator of vessel wall health, and therefore the strong correlation it has with DTI metrics is very promising. This finding has considerable applications for any pathology
associated with the loss of elastin or altered elastin synthesis and turnover and may be an advantageous avenue for the application of DTI beyond carotid atherosclerosis. After looking at fundamental microstructurally driven changes in FA and MD, the thickened intima, classified as an intermediate lesion, type III, in plaque development\textsuperscript{26}, was investigated in comparison to the aged media within the common carotids. The decreased FA and increased MD alongside the decreased cell density and elastin content in the thickened intima corroborates the strong correlation observed between elastin and these metrics and previous knowledge on the importance of cell content from Chapter 3. These changes are distinguishable from the vessel media, and while numbers are low, this shows great initial promise. While previous studies have used DWI to identify advanced lipid necrotic cores\textsuperscript{144–146}, this is the first time DTI has been both linked to elastin content and used to identify early-stage atherosclerosis in human carotid arteries.

To further extend the potential of these metrics within atherosclerotic tissue and investigate their potential to aid in clinical diagnostics, Chapter 5 linked non-invasive microstructural characterisation to the mechanical integrity of atherosclerotic plaques. The well-documented variable mechanical response of carotid plaques\textsuperscript{96,97,99,102,103} was similarly observed in this study. However, it was only when using DTI-derived tractography of individually tested atherosclerotic strips that different microstructures became apparent which ultimately yielded significant mechanical insight. The earliest sign of progressive atherosclerosis is the thickening of the intima which was identified non-invasively in Chapter 4 and was also present in this study. Group 1 strips showed predominantly circumferential tracts with sparse axial diffusion on the luminal edge and intimal thickening histologically. These samples failed at significantly higher stresses and strains than the other microstructures in this study – suggesting that of the observed microstructures, these would be the most stable. The presence of circumferentially aligned plaque caps was identified via tractography in Group 2 strips. While these strips strained comparably to those in Group 1, they failed at a lower stress and locally at the plaque cap shoulder where the plaque cap meets the circumferential medial regions. Plaque cap alignment was quite variable in Group 3 strips, but all strips had a thick, mixed region between the cap and the medial layers of the plaque. These strips failed at significantly lower stresses and strains than both Groups 1 and 2. This microstructure, of a disorganised plaque cap over a thick, mixed region, was the weakest and therefore the most vulnerable microstructure of those seen in this study. Through microstructural characterisation by DTI-derived tractography and mechanical
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characterisation of these plaques, these results show the ability to distinguish between more stable, Group 1, and more vulnerable, Group 3, microstructures.

Chapter 6 returned and refocused on the cell sensitivity established in Chapter 3 and explored this sensitivity within the field of tissue engineering. By investigating two different vascular grafts, Chapter 6 demonstrated that DTI-metrics can track recellularisation in TEVG. In the two separate grafts the FA was significantly higher in day 7 compared to day 3 grafts. While collagen and elastin remained constant through culture, the increasing cell density on the grafts was the main microstructural change. This increasing cell density had a strong, significant correlation with increasing FA for the ddPCaA grafts. Not only was it possible to track the increasing recellularisation on the grafts, but FA, MD and tractography all showed considerable differences between the recellularised grafts and the acellular control grafts. This sensitivity to non-invasively and non-destructively monitor the degree of recellularisation as well as differentiate between acellular and recellularised grafts has significant potential in tissue engineering, from in vitro to in vivo.

Understanding and interpreting DTI-derived metrics within arterial tissue allowed for their application in early disease, advanced disease, and tissue engineering applications. While it is no small task to bring this technique to the clinic, this work lays the foundation to motivate its continued use in research and its eventual translation to the clinic.

Limitations and future perspectives

While promising, the findings presented in this thesis are not without limitations. This section aims to address the limitations of each study and propose future work which looks to address them.

Chapter 3 highlighted the significance of cell content on the anisotropic diffusion in arterial tissue. However, one critical limitation of this study is the confounding effects of decreased GAG content in all tissue models. While GAG content did decrease across all models, this was an unintentional side effect caused by the native structure of the tissue. It is not feasible to remove individual components, namely cells, collagen, or elastin, without affecting the GAG content in the arterial wall. Additionally, it is not known how the different enzymatic treatments might impact the permeability of the vessel wall or cellular membranes. Rather than the top-down approach used in this study, a bottom-up approach could aid in elucidating how these constituents are intertwined. Specifically, highly aligned scaffolds from collagen-GAG suspensions have been previously fabricated. In a similar manner to
Chapter 3, such a scaffold could be investigated alongside a collagen only vessel, and cell-seeded scaffolds. A similar methodology could be adapted for elastin-dominant scaffolds. Additionally, looking towards graded degradations alongside varying levels of alignment could yield useful insight into different constituent contributions on DTI-derived metrics in different microstructures. Lastly, the incorporation of the stretched-exponential, bi-exponential, and diffusion kurtosis models to investigate the diffusion data would be advantageous as different models have shown sensitivities to differing components.

Chapter 3 highlighted that DTI-derived metrics are sensitive to specific changes in arterial microstructure, and this can be further explored in a multitude of scenarios.

In Chapter 4 this sensitivity was explored in human cadaveric carotid arteries. While the sensitivity to elastin content and early atherosclerotic pathologies demonstrated in this study are promising, the main limitation is the low n numbers. When increasing n numbers, future work should also look to include additional clinically relevant atherosclerotic features. Specifically, lipid, IPH, and neovascularisation should be investigated histologically as each have been shown to be key features in identifying so-called vulnerable plaques. More so, while advantageous, the methodologies between Chapter 4 and Chapter 5 should ideally be combined. The mechanical and microstructural characterisation of fresh human atherosclerotic plaque tissue would facilitate not only an overview of the overall microstructure of these tissues, but aid in identifying key constituents and how they influence plaque strength. At present, Chapter 4 identifies that elastin drives DTI-metrics in atherosclerotic tissue, whereas Chapter 5 identifies microstructural alignments which are more or less vulnerable. The combination of the two studies would be more valuable in linking what aspects of a microstructural organisation, or lack thereof, make it vulnerable.

Additionally, Chapter 5 utilised uniaxial extension of circumferentially cut strips from atherosclerotic plaques. A more physiologically relevant mechanical characterisation technique, such as bi-axial tensile testing or inflation testing, would yield more clinically relevant insights. Given the diseased nature of these specimens, the underlying microstructural arrangement does not necessarily mimic the well-known, load-bearing circumferential arrangement of arterial tissue. One can imagine a future study where an unloaded atherosclerotic plaque is imaged to obtain the unloaded microstructural organisation via DTI and is then consequently loaded to physiological pressures and re-imaged. The use of an MRI compatible bioreactor and optimisation of already faster EPI sequences could allow for this entire experimental set-up to occur within the MRI scanner.
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Not only would this yield novel insight into how the microstructural alignment of an atherosclerotic plaque is changing in physiological loading conditions, but also pave the way for DTI-derived metrics which can be implemented into patient specific finite element models.

The use of an MRI compatible bioreactor also has significant promise in tissue engineering. Chapter 6 revisited the significance to cell content on DTI-metrics on TEVGs. Due to the lengthy scan time of the sequence used in this work, the grafts were fixed once out of culture and imaged at terminal time points. An MRI compatible bioreactor would be advantageous for a number of reasons. Firstly, it would allow for longitudinal studies where TEVGs could be taken out of culture, imaged, and placed back into culture – all under sterile conditions while maintaining viability. Additionally, previous studies have looked at how varying strain levels and the underlying microstructural alignment alter both how the cells orient and proliferate. The development of an MRI compatible bioreactor which can come out and re-enter cell culture would allow for the longitudinal examination of how cells are reorienting with respect to both strain and underlying microstructure. Lastly, the combination of a physiological, MRI compatible bioreactor and the ex vivo DT imaging could be translated to a number of different tissue engineering applications – both in vascular tissue engineering via different TEVGs and cell types and outside vascular tissue engineering where alignment is equally as important, such as in cardiac or cartilage tissue engineering.

Consistent to all studies in this thesis, Chapters 3 – 6, is the main limitation of an idealised ex vivo DTI sequence. While this sequence was specifically chosen to facilitate 3D high-resolution, full coverage of the imaged specimens, it constitutes significant limitations. The use of a high-field preclinical scanner made this sequence possible and maintained adequate SNR. The 3D sequence used varied in resolution (a by-product of image size and FOV) from $312.5 \, \mu m^3$ isotropic to $250 \, \mu m^3$ isotropic and 28.15 to 12.5 hours, respectively. The combination of field strength, resolution, and scan time make this sequence far from clinically feasible. Other acquisition techniques, such as EPI, have been investigated at 7 T and, in combination with a reduced FOV, have made it possible to reduce acquisition time significantly (96 minutes total). While outside the scope of this work, the clinical feasibility of DTI at the carotid bifurcation needs to be critically assessed to put the significance of this work into context.
Clinical significance and translation

It would be naïve to not address the hurdle of clinical translation for a DTI sequence in the carotid arteries. Acquisition challenges stemming from cardiac and respiratory motion and scan duration make translation a non-trivial task. However, the work in this thesis provides fundamental insights into DTI-derived metrics within vascular tissues.

While realised in an idealised ex vivo scenario, the findings from this work hold significant potential to alter current clinical diagnostics in atherosclerosis and other pathologies. First and foremost, the benefits of non-invasive imaging to characterise a tissue’s underlying microstructure – with specific insight into the components – is invaluable for innumerable biomedical applications. The sensitivity of DTI metrics to key underlying microstructural components – namely, cells and elastin – was established in Chapters 3 and 4. The ability to capture changes in the underlying components of a tissue’s microstructure at early stages of disease could aid in better treatment planning and prevent significant disease progression. Specific to atherosclerosis, changes in cell and elastin content are implicated at early signs of disease progression. Additionally, the underlying microstructural alignment, as seen in Chapter 5, is indicative of the overall strength of a plaque. Together the implications of these findings suggest biomarkers sensitive to early signs of disease development, as well as biomarkers indicative of the stability of an atherosclerotic lesion.

From a more basic research standpoint, other tissues within the human body could benefit from similar explorations by DTI. With arterial tissue having been investigated in this work and articular cartilage having been explored in previously published studies[^257]–[^338], the overall methodology shown in this work could be applied to tissues and organs which display changes in microstructure in disease progression: for example, skin, skeletal muscle, liver, or the kidneys. The following discussion will detail the potential workflow for the translation of a DTI sequence, and understanding its usefulness and potential pitfalls, at the carotid bifurcation for atherosclerotic diagnostics.

Experimentally, there are a number of steps which can be outlined to aid in assessing the clinical feasibility of DTI in the carotid arteries. However, it is important to note that one study has already applied DTI in vivo at the carotid arteries – illustrating the potential of this technique in the clinic. Opriessnig et al., for the first time, demonstrated the feasibility of using in vivo DTI to measure diffusion anisotropy in cross-sections of the carotid arteries[^148]. By utilising a read-out segmented echo planar pulse sequence with a 2D acquisition on a 3 T whole body Siemens Prisma MR scanner, the authors were able to achieve a clinically...
acceptable scan time, 12 minutes, and sufficient signal-to-noise for DTI data. More specifically, the acquired in-plane resolution, 0.55 x 0.55 mm$^2$, was interpolated further to obtain an in-plane resolution of 0.2 x 0.2 mm$^2$ using zero-padding. While not included in the results, it would be worth directly comparing the real resolution with the interpolated resolution to further understand the impact of resolution and potential partial voluming effects. The authors also used peripheral pulse triggering to combat pulsatile vessel motion and cardiac triggering to acquire images in the diastolic phase. Rigid registration of the individual diffusion images was applied to the unweighted diffusion image to combat neck muscle relaxation during scanning. This was followed by B-spline registration, similar to the methodology used in Chapter 4, to correct for eddy currents, breathing, swallowing, and residual vessel motion. As a first pass at DTI in the carotid arteries, this study shows great potential in repeated measures of DTI metrics in the carotids of healthy male volunteers.

With this study in mind, there are still a number of translational studies which should supersede it to gauge the usefulness of DTI at the carotid bifurcation for atherosclerotic plaque characterisation. Performing additional high-resolution, high-field strength 3D DTI scans on cadaveric carotid arteries with high numbers of b-directions would better characterise the sequence requirements for diseased arterial tissue. The b-value, 800 s/mm$^2$, and number of directions, 10, chosen for this thesis had been previously established in arterial tissue$^{165,172}$. The cadaveric carotid specimens offer a unique combination of both healthy and diseased regions. It is possible the diseased regions may require a higher angular resolution than the 10 b-directions used to better capitulate the disorganisation seen in the microstructure. While previous work has looked at porcine carotid arteries with up to 128 directions$^{172}$, the results using only 10-directions are directly comparable; however, this has not been explored in advanced atherosclerotic tissue.

While there are a number of studies which have investigated arterial tissue ex vivo at high-field strengths (Table 2.7), these studies maintain the advantage of smaller FOVs and higher resolution while maintaining high SNR. Following on from the angular resolution study, imaging ex vivo human carotids both at 7 T and a more clinically relevant 1.5 or 3.0 T would be required. This would facilitate a direct comparison of the SNR loss expected at a lower field strength as well as any impact from partial voluming from inadequate resolution. A similar study has been done using DWI on in vivo and ex vivo excised atherosclerotic carotid plaques by Kim et al.$^{339}$. In that study, Kim et al. performed scanning on a Siemens Trio 3 T scanner. Using a 2D single shot interleaved multislice inner volume DW EPI sequence with
32 averages, the authors achieved an *in vivo* real resolution of $1.0 \times 1.0$ mm$^2$ which was zero-padded to a higher resolution in-plane resolution of $0.5 \times 0.5$ mm$^2$. This *in vivo* sequence took 3 minutes and 20 seconds to acquire. The subsequent *ex vivo* MR scan, performed on the same scanner, was performed using a 3D multishot-inner volume-DW-EPI technique achieving isotropic resolution of $0.5$ mm. The results from this study, show very comparable ADC values for the LRNC and haemorrhage between *in* and *ex vivo* measurements.

Furthermore, a number of *in vivo* studies have used DWI to investigate plaque components$^{20,113,144-147,153}$, already establishing the usefulness of measuring water diffusion within these tissues. Specifically, work by Xie et al., Zhang et al., Kim et al., and Alex et al. have all recently looked at different acquisition techniques to improve DWI$^{113,146,150,153}$. Xie et al. performed both 3D diffusion prepared turbo spin echo and 2D single shot DW EPI scanning on both healthy volunteers and patients with carotid atherosclerosis$^{146}$. While both scans were performed in under 6 minutes, the 3D sequence had a higher in-plane resolution of $0.6 \times 0.6$ mm$^2$ and also outperformed the 2D sequence in terms of image quality. Partial voluming effects were also minimised with the 3D diffusion prepared turbo spin echo sequence, allowing for ADC measurements in the vessel wall, LRNC and fibrous plaque. Zhang et al. similarly investigated both a diffusion prepared turbo spin echo and diffusion prepared stimulated-echo turbo spin echo sequence *in vivo* in the carotids$^{150}$. The authors demonstrated undistorted, 3D high resolution ($0.6 \times 0.6$ mm$^2$) diffusion images *in vivo* at the carotid arteries obtained in 12 minutes. Most recently, Kim et al. investigated a 3D high resolution DW stack of stars technique in symptomatic and asymptomatic carotid arteries with IPH$^{153}$. The authors similarly achieved an in-plane resolution of $0.6 \times 0.6$ mm$^2$ using this 3D sequence, in under 3.5 minutes. Together these studies highlight the ongoing clinical advancements in diffusion imaging at the carotid bifurcation. These studies also shed a light on the clinically feasible resolutions, approximately twice that of the work presented in this thesis, and the acquisition timeframe for DWI. Extending these acquisitions to incorporate directionality will inevitably increase scan time, but ultimately have the potential to better capitulate vascular tissue microstructure. Specifically, DTI identifies early stage disease as shown in Chapter 4, plaque vulnerability which links directly to mechanical integrity as shown in Chapter 5, and recellularisation of TEVGs as seen in Chapter 6. The fundamental work in this thesis outlines the value of DTI clinically in arterial tissue and motivates this translation from *ex vivo* to *in vivo*. 

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Chapter 7

While the transition of DTI to the clinic for use at the carotid bifurcation remains elusive at present, the application of DTI within the field of tissue engineering is readily available. The work presented in Chapter 6 illustrated that DTI-derived metrics can non-invasively track recellularisation in vascular grafts. This sensitivity has the potential to be exploited in a number of tissue engineering applications, specifically any scaffold-based construct which involves the temporal alignment of microstructural components, namely cells, collagen, or elastin. Often times in tissue engineering large sample sizes are required to adequately characterise constructs via destructive histological and biochemical analyses at different time points. Chapter 6 highlighted DTI as a non-invasive, non-destructive method to probe recellularisation over time. Furthermore, the translation from in vitro constructs, imaged in similar conditions to those in this thesis, to in vivo small animal models at 7 T is a feasible, as it is already done in practice for preclinical models. High-field MR scanners are most often used for small animal experiments and DTI has been explored in a multitude of applications in vivo in preclinical animal models\textsuperscript{244,340–345}. In this sense, DTI – either in in vitro scenarios, excised constructs from in vivo studies and even in in vivo preclinical small animal models – has the potential to non-invasively probe the microstructural changes within tissue engineered constructs.

Altogether, the work from this thesis provides fundamental understandings of DTI-metrics in arterial tissue, as well as the promise of such biomarkers for microstructural and mechanical integrity of atherosclerotic tissue. These insights offer the underpinnings for both the interpretation and ultimately the motivation for the clinical translation of DTI from preclinical to clinical use.
The fundamental goal of this thesis was to establish the use of a non-invasive imaging technique, DTI, in arterial tissue. *Ex vivo* imaging of healthy and diseased arterial tissue, as well as TEVG, identified novel microstructural, morphological, and ultimately mechanical insights in arterial tissue. The key contributions to the biomedical field emanating from the research presented in this thesis are summarised below:

- Optimised MR imaging set up, DTI protocols, and analysis pipeline for arterial tissue, capable of being adopted and/or adapted for *ex vivo* non-invasive characterisation of numerous tissue types.
- A strong sensitivity to cellular content was established in arterial tissue using DTI derived FA, MD, and tractography. The measurable anisotropic signal of arterial tissue is lost with the removal of cells.
- A strong correlation between elastin content and FA and MD was discovered in human atherosclerotic tissue – highlighting that in a complex, dynamic microstructure it is elastin which dominates the change in DTI signal. While promising in atherosclerosis, this finding has significant potential in other elastin-driven pathologies.
- Early signs of atherosclerotic disease – shown in the thickened intima – were distinguishable by DTI-derived metrics owing to the sensitivities to cellular and elastin content.
- Key mechanical insights into fresh atherosclerotic plaques were identified when grouped according to tractography. Tractography accurately captured the presence of plaque caps and microstructural disorganisation, ultimately identifying the most stable and at-risk microstructures.
- Increasing cell density in TEVG strongly correlated to increasing FA. FA, MD, and tractography are all capable of distinguishing between acellular and recellularised vascular grafts.
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References


References


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References


A Study 2

A.1 Image registration

A 3D multi-gradient echo sequence which has previously been used on arterial tissue\textsuperscript{176} was acquired and provided high resolution images of the carotids to aid in image registration. The parameters were: TEs = 5, 13.1, 21.2, and 29.3 ms with monopolar readout gradients, TR = 150 ms, flip angle = 30°, bandwidth = 34722 Hz, and averages = 2. An isotropic voxel resolution of 0.117 x 0.117 x 0.117 mm\textsuperscript{3} was achieved by matching the FOV to the DTI acquisition (30 mm x 30 mm x 30 mm) and using a 256 x 256 x 256 matrix size. Total scan time for this sequence was 5 hours 27 minutes. The BSpline registration was performed with an adaptive stochastic gradient descent optimizer and a maximum of 4000 iterations and maximum step length of 0.1 were used alongside a BSpline interpolation order of three. Finally, the corresponding slices from the FA and MD maps were registered to the high-resolution multi-echo gradient echo images.

Figure A. 1. Two representative non-diffusion weighted images from different specimens. The red mask overlays pixels that were used. From left to right: the original data, after low signal removal, after high residual removal, and the final used area after PBS was subtracted.
Figure A. 2. Histological representations of one cadaveric common carotid. (a) Different stains of common carotid. Left to right, top to bottom: H&E, Verhoeff’s elastin, PSR, PLM, Alcian blue, and Alizarin red. Scale bar 2 mm. (b) Healthy cross-sections of vessel wall. Scale bar 500 μm. (c) Histological confirmation of diseased region (highlighted in yellow dashed line). Scale bar 500 μm.
Figure A. 3. Non-DW images for each carotid artery in the common (C), bifurcation (B), and internal and external carotids (I+E). Various stages and morphologies of disease are present across the vessels. Blue overlays were considered diseased ROIs, while white overlays are healthy ROIs.

Figure A. 4. The elbow method was used to determine an adequate number of clusters (k) for the k-means clustering. Elbow plots are presented for (a) FA, (b) MD and (c) FA and MD as inputs into the clustering algorithm. k=5 was determined appropriate.

Figure A. 5. k-means clustering ROIs for k=5 for n=4 cadaveric common carotids. (a) FA, (b) MD and (c) FA and MD were used as the clustering inputs.
Figure A. 6. Correlation of microstructural components, determined by quantitative histology, and DTI metrics based on clustering input, namely (A, B) FA, (C, D) MD and (E, F) FA and MD. DTI metrics and GAGs, calcium and cell density displayed weak to no correlations for different clustering inputs.
Figure A. 7. Correlations of different microstructural components based on different cluster inputs. Collagen (first column) and elastin (second column) content correlations when (a, b) FA, (c, d) MD and (e, f) FA and MD are inputs for clustering. Pearson’s correlations were used to determine the strength of correlations and are presented in Supplementary tables.
Correlations of different microstructural components based on different cluster inputs. GAG (first column) and calcium (second column) content correlations when (a, b) FA, (c, d) MD and (e, f) FA and MD are inputs for clustering. Pearson's correlations were used to determine the strength of correlations and are presented in Supplementary tables.
Table A. 1. The percent disease in each vessel divided by anatomical location, presented alongside the pixel ratio between diseased and healthy ROIs in each anatomical location per vessel.

<table>
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<th>C</th>
<th>B</th>
<th>I</th>
<th>E</th>
<th>% Diseased</th>
<th>Pixel ratio (diseased:healthy)</th>
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Table A. 2. Summary of Pearson’s correlations between collagen content and other microstructural components.

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<th>R²</th>
<th>P value</th>
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<td></td>
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<td></td>
</tr>
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<td>-0.4777 to 0.5133</td>
<td>0.0005559</td>
<td>0.9309</td>
</tr>
<tr>
<td>MD</td>
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<td>0.08893</td>
<td>0.2619</td>
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<td>0.04002</td>
<td>0.4576</td>
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<td></td>
<td></td>
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<td></td>
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<td>FA</td>
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<td>0.06204</td>
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</tr>
<tr>
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<tr>
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<td></td>
<td></td>
<td></td>
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Table A. 3. Summary of Pearson’s correlations between elastin content and other microstructural components.

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Table A. 4. Summary of Pearson’s correlations between GAG content and other microstructural components.

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Table A. 5. Summary of Pearson’s correlations between calcium content and other microstructural components.

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B Study 3

Figure B. 1. Masking removal of MR data. Green overlay is usable MR data mask after (A) low signal removal, (B) high tensor residuals, (C) PBS removal, (D) manual stray pixel removal, and (E) removal of tissue within the grips during uniaxial extension.

Figure B. 2. Solid lined boxes surround the region of interest for local strain measurements at the location of failure, while dashed lined boxes surround the region of interest for strain measurements across the gauge length. Two representative (A) and (B) samples shown.
C Further applications of DTI

This appendix includes two studies where collaborators supplied ex vivo tissue which was investigated through DTI and analysed for microstructural insight. First, three human aortae are investigated – specifically one healthy, two aneurysmal, and one chronic dissected aorta. Next, DTI-derived insight is explored in explanted porcine abdominal tissue with implanted medical devices with an aim to characterise the presence or absence of a fibrous capsule at the tissue-device boundary.

C.1 An ex vivo MRI investigation into three distinct aortic morphologies: microstructural insight for better clinical indicators

This study was a collaborative effort where the human tissue was supplied from the Duffy Lab (NUIG) and Avril Lab (Saint-Étienne). All MR imaging, analysis, interpretations, and writing was performed by B. Tornifoglio. Histology images were supplied by Dr. Ruth Levey from NUIG.

C.1.1 Introduction

Thoracic aortic aneurysms (TAA), abdominal aortic aneurysms (AAA), and acute aortic syndromes such as acute aortic dissections (AAD) are all classified as aortic diseases. These diseases are relatively uncommon, with a reported death rate of 2.78 per 100,000\textsuperscript{346}. However, if misdiagnosed, or not diagnosed in a timely manner, these disorders have high morbidity and mortality rates\textsuperscript{347–349}. It is also believed that cases and mortalities are highly underreported due to undiagnosed fatal dissections and ruptures both in and outside of the hospital setting\textsuperscript{350,351}. Clinical presentations of these aortic diseases are diverse and confound efficient diagnoses. In the absence of routine screening coupled with the asymptomatic nature of AAA, diagnosis is often an incidental finding during imaging for other medical complaints\textsuperscript{352}. Meanwhile, AAD have been reported to be misdiagnosed on initial presentation in 39% of cases\textsuperscript{353} and are therefore associated with a longer time to correct diagnoses.
Hyperacute (<24 hours from symptom onset) and acute (1-14 days) classifications are typically diagnosed by anatomical imaging via ultrasound, CT or MRI\(^{354}\). Subacute (15-90 days) and chronic (<90 days) classifications similarly rely on anatomical imaging due to the lack of approved diagnostic and prognostic molecular markers\(^{355}\); however, to date there are still many challenges in identifying rupture-prone regions\(^{350,352,353}\). The only current anatomical criterion for AAA or AAD is the aortic diameter. Clinical surveillance with ultrasound or CT is carried out until the aortic diameter approaches 5-5.5 cm\(^{356}\), with specific ‘hinge points’ of 6 cm for the ascending and 7 cm for the descending aorta\(^{350}\). For type B dissections, changes in the intimal dissection flap have been observed in the transition from AAD to chronic AD\(^{357,358}\); however, these observations have not been incorporated into any guidelines on rupture-risk.

Extensive research has gone into characterising the influence of specific microstructural components on the mechanics of aortic tissue\(^{34,49,50,358–363}\). One common risk factor across all aortic diseases is arterial hypertension. This affects the microstructure of the arterial wall and can lead to accelerated ECM degradation, apoptosis and elastolysis with hyalinisation of collagen\(^{364}\). Elastin distributes tensile stresses while collagen reinforces the vessel wall, together forming the load bearing components in the arterial wall\(^{49,359,363,365}\). Ascending TAAs have been shown to have altered collagen orientation and significantly less elastin than healthy aortic tissue, which has direct mechanical implications on the tissue\(^{360–362}\). Duprey et al. found that the quality of collagen and elastin networks in the aortic wall can prevent rupture\(^{365}\). Specifically, the quality of elastin allows for extensibility of the tissue and delays collagen recruitment. The quality of collagen delays fibre damage and upholds the strength of the tissue. Panpho et al. found that the dissection flap was stiffer than the true and false lumen, had the lowest collagen concentration and the lowest collagen to elastin ratio\(^{358}\). Additionally, significant elastin loss was clear in the false lumen, while highly aligned, long elastin fibres remained in the dissection flap and true lumen. Given the clear connection between microstructural components and the surveillant imaging already in place to monitor chronic aortic diseases, herein lies an opportunity to better capitate clinical rupture-risks.

*In vivo* MRI to investigate the integrity of the aortic wall is limited to a handful of studies\(^{366–369}\). Richards et al. found that ultrasmall superparamagnetic particles colocalised in areas of cellular inflammation in 29 symptomatic patients with AAAs using T2* -weighting imaging\(^{366}\) and this was able to identify patients with rapid AAA expansion. Takahashi et
al. used 4D-flow MRI to investigate turbulent flow in the false lumen and found that patients with high-volume turbulent flow in the false lumen need close monitoring due to risk of complications\(^{369}\). Lastly, Botnar et al. used an elastin specific MR molecular imaging agent in mice and were able to obtain high spatial resolution of regions with ruptured elastic laminae\(^{368}\). A number of \textit{ex vivo} MRI studies have investigated arterial tissue with an aim to characterise the microstructural environment; specifically using DTI\(^{165,167,168,172,175,295}\), as demonstrated in Chapter 3 - Chapter 6. These studies not only identify the capability of DTI to non-invasively capitulate the microstructure of arterial tissue but highlight the sensitivity to specific components – such as collagen, elastin, and SMCs.

In this study we investigate three different aortic morphologies, healthy descending, ascending aneurysmal and descending type B chronic dissected, with an aim to use DTI-derived metrics to differentiate these morphologies. By addressing the feasibility of DTI to aid in microstructural insight into aortic tissue, this study aims to establish the foundation to motivate further studies and research in order to get this sequence to a clinical setting and ultimately improve clinical indicators for rupture-risk.

\textbf{C.1.2 Materials and methods}

\textit{C.1.2.1 Human aortae}

Three different aortic morphologies were investigated in this study. A healthy descending thoracic aortic segment from a 53-year-old male who died from a glioblastoma multiforme was obtained, see Figure C.1(a). A Type B chronically dissected descending thoracic aorta was obtained from an 83-year-old female who died from pneumonia, congestive cardiac failure, and atrial fibrillation, see Figure C.1(b). Both (a) and (b) being from cadaveric human donors, they were embalmed prior to collection. Additionally, two aneurysmal ascending aortic segments were harvested during surgery; however, the clinical details on these two donors are not available, see Figure C.1(c) and (d). The aneurysmal aortae were also fixed prior to collection.
C Further applications of DTI

Figure C. 1. Aortic tissue segments. (a) Healthy, (b) chronic dissection, and (c, d) aneurysmal aortic samples. All scale bars are 10 mm.

C.1.2.1 MR imaging

All samples were imaged individually in a small-bore (30 cm) horizontal 7 Tesla Bruker BioSpec 70/30 USR system (Bruker, Ettlinger, Germany) with a transmit-receive volume coil, shielded gradients (maximum strength 770 mT/m) and Paravision 6 software (Bruker, Ettlinger, Germany). Specimens were secured to a 3D printed holder which was secured in a horizontal tube with fresh PBS for imaging. Conventional proton-density, T2-, and T1-weighted scans were acquired. Proton-density weighted parameters were as follows: TE/TR: 7/830.632 ms, 4 averages, flip angle: 30°, 60 slices, image size: 256 x 256, FOV: 60 mm x 60 mm, and acquisition time: 9 minutes 31 seconds. T2-weighted parameters were as follows: TE/TR: 24/9137.56 ms, 4 averages, echo spacing: 8 ms, RARE factor: 8, 60 slices, image size: 256 x 256, FOV: 60 mm x 60 mm, and acquisition time: 14 minutes 37 seconds. T1-weighted images were obtained from a high-resolution 3D multi-gradient echo sequence with the following parameters: TE/TR: 4.886/150 ms, 4 echoes with echo spacing: 7.95 ms, 1 average, flip angle: 30°, image size: 256 x 256 x 256, FOV: 60 mm x 60 mm x 60 mm and acquisition time: 2 hours and 43 minutes.

A 2D spin echo DTI sequence was used for all four samples. The healthy aorta was imaged with the following parameters: TE/TR: 18.182/1500 ms, 5 averages, 5 slices, image size: 128 x 128, FOV: 64 mm x 64 mm, b-values: 0, 800 s/mm², 64 diffusion directions, gradient duration 3.8 ms, gradient separation: 8.802 ms and acquisition time: 17 hours and 20 minutes. The chronic dissection aorta had the same parameters save TR: 1182.335 ms, 55 slices and acquisition time of 13 hours and 39 minutes. Both aneurysmal samples were imaged with the following parameters: TE/TR: 18.182/4000 ms, 4 averages, 20 slices, image size: 140 x 140, FOV: 70 mm x 70 mm, fat suppression on, b-values: 0, 800 s/mm², 32 diffusion directions, gradient duration 3.8 ms, gradient separation: 8.802 ms and acquisition time: 20 hours and 32 minutes. The number of
Further applications of DTI

directions in the healthy aorta sample was down sampled to 32 directions in post processing in order to compare to the other specimens. This yielded no impact on the DTI-derived metrics.

C.1.2.3 Regions of interest
Data was denoised and corrected using the same methods established in Chapter 3. All aortae were compared against each other without differentiating any specific ROIs. However, ROIs within the chronic dissection sample were identified by an experienced physician and compared against each other and against healthy aorta. Four regions were identified – thrombus, media, adventitial dissected media, and luminal dissected media. The tissue surrounding the false lumen was defined as the dissected media and it was classified based on location – either on the luminal side or adventitial side of the arterial wall.

C.1.2.4 Histological processing
Standard histological analysis was performed on tissue sections from each aorta. The sections were stained with Masson’s Trichrome where collagen is stained blue, muscle tissue red, and cytoplasm is pink. This staining was performed by Dr. Ruth Levey, NUIG.

C.1.2.5 Statistical methods
Statistical analysis was performed using GraphPad Prism (Version 9.3.1). Data was tested for normality using D’Agostino-Pearson normality tests and due to the small sample numbers (n<8), nonparametric tests were used for analyses between different aortas. Data was also analysed using the averages per slice within the imaging data set in order to highlight variance within each sample; one-way ANOVAs with Kruskal-Wallis multiple comparison tests were used. For the healthy aorta n=5 slices, for the chronic dissection aorta n=31 slices, and for the aneurysmal aortas n=18 slices each. For analyses within different regions in the chronic dissected aorta, all data passed normality tests via a D’Agostino-Pearson test. Using Brown-Forsythe equal variance tests, FA passed while MD and HA did not. Brown-Forsythe and Welch ANOVAs with Dunnett’s multiple comparison test were used for MD and HA, while an ordinary one-way ANOVA with Tukey’s comparisons was used for FA when analysing the regions within the chronic dissected aorta. For media, adventitial and luminal dissected media n=31 slices and n=30 slices for thrombus. All data is presented as mean and standard deviation.
C Further applications of DTI

C.1.3 Results

C.1.3.1 Multicontrast MRI

Figure C.2 highlights the different aortic segments imaged in this study under different contrast weightings. Most notable is the significant size difference between the healthy aortic segment in the first column when compared to both the two aneurysmal segments and the chronic dissected aorta. Two different slices of the chronic dissected aorta are shown to highlight the heterogeneity throughout the sample.

Figure C.3 similarly shows the same representative slices of each sample but with no diffusion weighting (b=0 s/mm$^2$) and the mean diffusion weighting (b=800 s/mm$^2$, 32 directions).

Figure C.2. Multicontrast MRI images of different aortic segments. One representative slice is shown for the healthy and aneurysmal aortic segments and two representative slices shown for the chronic dissection sample.
C Further applications of DTI

![Figure C.3](image)

Figure C.3. B=0 s/mm² images (top row) and mean DW images (b=800 s/mm², 32 directions) of each sample. One representative slice shown for the healthy and aneurysmal samples and two representative slices shown for the chronic dissection sample.

C.1.3.2 DTI metrics across three different aortic morphologies

Figure C.4 presents parametric maps of FA and MD as well as the mean FA and MD measured across the entire aortic segment for each specimen. Healthy aortic tissue had the highest degree of anisotropic diffusion, with an FA of 0.244 ± 0.069, Figure C.4(b). This was higher than the FA in both aneurysmal aortas, 0.199 ± 0.074 and 0.195 ± 0.068, and the chronic dissected aorta, 0.134 ± 0.096. When looking at the average FA values by slice (Figure C.4(c)), rather than across the entire specimen (Figure C.4(b)), the same trend is seen. The FA in the healthy aorta and the aneurysmal aortas were significantly higher than that in the chronic dissected aorta. The healthy aorta showed the lowest MD at 0.000717 ± 0.0001 mm²/s, followed by the aneurysmal samples, 0.00104 ± 0.0002 and 0.000987 ± 0.0003 mm²/s, and finally the chronic dissection sample with the highest MD at 0.00109 ± 0.0005 mm²/s. Again, when looking at individual slice averages, the same trend was seen with varying degrees of significance. The chronic dissected aorta had a significantly higher MD than both aneurysmal aortas and the healthy aorta. Additionally, the healthy aorta and second aneurysmal aorta (A2) has significantly lower MD values than the first aneurysmal aorta (A1).
Figure C. 4. DTI metrics across three separate aortic morphologies. (a) Parametric maps of FA and the (b) mean values across each sample and (c) within each slice of the DTI dataset. (d) Parametric maps of MD and (e) the mean values across each sample and (f) within each slice. Scaling of the parametric maps matches the y-axis of the bar graphs. H represents healthy aorta, A1 and A2 are the aneurysmal aortas, and CD is chronic dissected aorta. N=1 for healthy and chronic dissection samples and n=2 for the aneurysmal in (b) and (e). In (c) and (f) n=5 slices for healthy, n=18 slices for A1 and A2, and n=31 slices for CD. Significance determined by one-way ANOVAs with Kruskal-Wallis multiple comparisons; *p<0.01, **p<0.01, ****p<0.0001.

Figure C.5 presents representative FEFA maps and the mean HA. The FEFA maps in Figure C.5(a) highlight the circumferential (red to green) arrangement clearly in the healthy aorta and both aneurysmal aortas. Out-of-plane axial (blue) diffusion and more disorganised microstructure can be seen in the chronic dissected aorta alongside underlying circumferential alignment. When looking at the average HA across entire specimens, the healthy aorta had an average angle of 26.32 ± 22.19°, the aneurysmal aortas slightly lower at 25.11 ± 22.34° and 20.87 ± 16.16° and the chronic dissection sample higher at 38.97 ± 27.32°. Interestingly, when looking at the averages by slice it became clear how much the HA of the chronic dissected aorta changes lengthwise through the slices. The HA of the chronic dissected aorta was significantly higher than that of both aneurysmal aortas.
Figure C.5. DTI derived (a) FEFA maps and (b) mean HA across entire aorta and (c) by slices. Colour coding in (a) represents the directionality of the first eigenvector. Green is left-right, red is top-bottom – together representing circumferential alignment – and blue is in and out of the page (axial alignment). (b) Mean HA for each specimen; H represents healthy aorta, A1 and A2 are aneurysmal aortas, and CD is chronic dissected aorta. N=1 for healthy and chronic dissection samples and n=2 for the aneurysmal. (c) Mean HA for each slice within the imaging dataset for each specimen. The dashed line in is at 45° - above which is considered more axial and below which is more circumferential. Significance was tested with a one-way ANOVA with Kruskal-Wallis multiple comparisons; ***p=0.0005 and ****p<0.0001.

C.1.3.3 DTI metrics within a chronic dissected aorta

The presence of multiple different morphological features within the chronic dissection aorta motivated further investigations into these distinct regions. Figure C.6 presents these insights. Figure C.6(a) shows the four ROIs within the chronic dissection aorta – thrombus (red), media (purple), luminal dissected media (green) and adventitial dissected media (yellow). The true and false lumen are also marked. Figure C.6(b-d) show the DTI-derived metrics as averages across the entire specimen. The dotted line on the graphs represents the mean metric from the healthy aorta. Figure C.6(e-g) similarly show the same metrics but with the mean metric per slice. The FA for each regional morphology was significantly different not only from each other, but also from the FA of healthy aortic tissue. When looking at the MD, the media was significantly higher than the adventitial and luminal dissected media and thrombus. The adventitial dissected media had a significantly lower MD than the luminal dissected media, which has a significantly higher MD than the thrombus area. All regions had a significantly higher MD than healthy aorta. Looking at the HA between regions revealed that the thrombus had a significantly higher HA than the luminal dissected media. Only the adventitial dissected media and thrombus were significantly different to healthy aorta. Masson’s trichrome staining of the three morphologies, shown in Figure C.7, varies considerably between the specimens. Healthy aortic tissue shows strong staining for collagen (Figure C.7(a)), which is not nearly as apparent in the aneurysmal tissue (Figure C.7(b)). Within the chronic dissection aorta,
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clear variations in the overall microstructural alignment and collagen quantity are apparent (Figure C.7(c)).

Figure C. 6. Investigation of distinct regions within a chronic dissected aorta. (a) Four ROIs were looked at – the media (M, in purple), adventitial dissected media (Adv-DM, yellow), luminal dissected media (L-DM, green) and thrombus (T, in red). (b-d) Mean DTI-derived metrics across the entire specimen. (e-g) Mean DTI-derived metrics per slice within the chronic dissected aorta. (e) Significance was determined by an ordinary one-way ANOVA with Tukey’s multiple comparisons. ****p<0.0001, ####p<0.0001. (f, g) Significance determined by Brown-Forsythe and Welch ANOVAs with Dunnett’s T3 multiple comparisons. **p=0.0048, ***p=0.0008, ****p<0.0001, ####p<0.0001. (g) *p=0.0465, #p=0.0126, ##p=0.0028. In all graphs the dotted line represents the mean metric for healthy aorta, and in (e-g) dashed lines show the standard deviation across the slices.
C.1.4 Discussion

C.1.4.1 DTI-derived metrics in aneurysmal tissue

The need for better clinical indicators of the stability, or lack thereof, of the aortic wall in aortic diseases is as prevalent as ever as the burden of aortic diseases is increasing. Currently, anatomical imaging, namely ultrasound, CT, or MRI, is standard and required in order to assess the only approved clinical indicator – the aortic diameter. While the two aortic diseases presented in this study tend to be diagnosed from incidental findings, the imaging technique presented here could be advantageous in surveillance screening of at risk cohorts, such as those with Marfan’s syndrome. Rupture or dilation of the aorta occurs from biomechanical weakening of the wall and therefore, mechanically sensitive clinical indicators are advantageous. The influence of microstructure on the mechanics of the aortic wall is well established, furthering the motivation for non-invasive microstructural insight.
into the wall. DTI has shown sensitivity to specific microstructural components in arterial tissue\textsuperscript{175,295} and the current study aimed to build on and harness this sensitivity.

The multi-contrast MR imaging performed in this study yielded detailed anatomical images of the different specimens. The clinical indicator of aortic diameter – while nicely displayed in these images – is not informative of the wall stability. The DTI-derived metrics allowed for a more quantitative characterisation of the aortic morphologies. The arterial wall is a highly organised, anisotropic structure composed of elastin, collagen, and SMCs\textsuperscript{37,49}. The calculated HA, indicative of the dominant angle of diffusion, of healthy aorta in this study, see Figure C.5, agrees very well with previously published results\textsuperscript{167,168}. Anisotropic diffusion has previously been measured in healthy porcine carotids in Chapter 3\textsuperscript{295} and aged cadaveric carotids in Chapter 4. The FA values in healthy aorta presented in this study (Figure C.4) fall below healthy porcine tissue but higher than the aged cadaveric carotids (mean age 78.25 ± 11.2 years). Vascular ageing affects both mechanical and structural properties of the arterial wall, specifically the loss of elastin leading to a reduction in wall compliance\textsuperscript{297,298}. Future studies would be needed to decouple the observed decrease in FA due to vascular aging and the decrease in FA due to disease related changes. Chapter 4 found FA to be highly correlated with elastin content – with decreasing elastin yielding decreasing measurable anisotropic diffusion. While not significantly lower, the FA in the aneurysmal samples was less than that in the healthy aorta. This suggests that both FA and MD might be useful metrics in surveillance imaging of aneurysmal tissue. Rather than relying solely on the aortic diameter, these metrics could offer insight, with decreasing FA and increasing MD, into progressive elastin fragmentation and depletion. The loss of elastin in the arterial wall increases the stiffness of the wall\textsuperscript{49} and releases intrinsic compressive stresses on the collagen fibres\textsuperscript{359}, allowing for them to become disordered\textsuperscript{48}. Elastin fragmentation, fibrosis, and medial necrosis are believed to be adaptations to stress and trauma in the arterial wall\textsuperscript{357,372}; however, how these microstructural changes develop and progress over time has not been studied. The potential for non-invasive metrics to characterise changes in the microstructure over time could offer novel insights which ultimately can be linked to mechanics.

C.1.4.2 DTI-derived metrics in a type B chronic dissected aorta

Uncomplicated type B aortic dissections are typically treated medically and in due time transition from acute to chronic dissections\textsuperscript{373}. Aortic diameter expansion is still relevant for patients with type B dissections and the chronic AD presented in this study is visibly dilated,
but arguably stable with the thrombosed false lumen\textsuperscript{374}. While the results from this study would not necessarily alter treatment of this specific morphology, the differences seen here suggest that DTI-derived metrics are sensitive to microstructural changes which occur in the false lumen, where rupture would occur in AAD. Morphological predictors have been suggested to predict aortic dilation\textsuperscript{375}. A thrombosed false lumen, when compared to a patent false lumen, has a beneficial effect, whereas a false lumen with both flow and thrombosis is associated with increased mortality\textsuperscript{374}. Dissection flap stiffness is accepted as a marker for chronicity; however, this has not been systematically observed\textsuperscript{376}. Microstructurally, this stiffness could be likely due to increased elastin fragmentation present and cystic medial necrosis, both of which occur with natural aging and chronic dissection progression\textsuperscript{357,372,377}. Loss of elastin coupled with elastin fragmentation has been shown to cause biomechanical weakening in the false lumen\textsuperscript{358}, while the dissection flap shows stiffer behaviour compared to the false and true lumen and the lowest collagen to elastin ratio\textsuperscript{358}. The luminal dissected media presented in this study, otherwise referred to as the dissection flap, demonstrated an increased FA similar to that of healthy aorta and the media (true lumen). This suggests that the microstructure in this region maintains key microstructural components that drive measurable anisotropic diffusion – elastin, cells, and collagen. With respect to collagen and overall alignment, this speculation is confirmed by Masson’s trichrome staining, see Figure C.7. In the adventitial dissected media, located behind the false lumen, a drop in FA and increase in MD was observed compared to healthy aorta. This highlights a compromised microstructure which lacks anisotropy and might be mechanically more susceptible to rupture.

With full acknowledgment of the translation hurdles, addressed in the final discussion, the results from this study highlight the previously established sensitivity of DTI in arterial tissue (Chapter 3-Chapter 6) in healthy and diseased aortic tissue morphologies and the microstructural insight which can be gained non-invasively.
C Further applications of DTI

C.2 Towards a whole sample imaging approach using diffusion tensor imaging to examine the foreign body response to medical devices

This study was a collaborative effort, and the resulting paper is joint co-first authored with Dr. Ruth Levey. Device fabrication, the animal study, SEM, MicroCT, and histological analysis were performed by collaborators while all MR imaging, analysis and interpretation was performed by B. Tornifoglio.

C.2.1 Introduction

No medical device is immune to its fate of being, to some extent, encapsulated as a result of the foreign body response (FBR). This response is immune-mediated, whereby a foreign body, or implanted material, stimulates a plethora of inflammatory events and wound-healing processes causing cellular and collagenous deposition. Formation of a dense fibrous capsule surrounding the implant is also common\textsuperscript{378–380}. This collagenous encapsulation can compromise the performance and endurance of the medical implant by impairing biosensing capabilities, causing pain, isolating cell-based implants from vascularisation and nourishment, hindering drug elution, and eventually causing implant/device failure\textsuperscript{378–380}.

Currently the standard practice for fibrous capsule examination lies in histological analysis. This approach for evaluating the microstructure of the fibrous capsule often relies on random sampling. This method is excellent for acquiring representative measurements, however, cannot account for the unique spatial distribution of features in 3D. Not to mention, this method can often require large numbers of animals for accurate analysis and additional qualitative imaging. All this becomes even more difficult when analysing human-sized devices used in large animal studies. A high-resolution, non-invasive, volumetric imaging technique for large tissue samples would be extremely beneficial in the characterisation of the fibrous capsule.

While existing non-invasive imaging techniques can probe for information regarding inflammation which would be relevant for monitoring the FBR, these methods lack any further microstructural information\textsuperscript{381–383} on the fibrous capsule. The application of DTI to examine the fibrous capsule has yet to be explored; however, it has been used to investigate myocardial\textsuperscript{384} and caesarean\textsuperscript{385} scar tissue, which are similar in composition to the fibrous capsule. Currently, a variety of anatomical sequences, such as T1- and T2-weighted scans, are used to identify the presence of a fibrous capsule around silicone breast implants\textsuperscript{386}.
however, DTI offers the potential to not only identify the presence of a fibrous capsule but yield valuable insight into its microstructural organisation.

Here we examine DTI as a potential technique for high-resolution imaging of the fibrous capsule surrounding two unique large-scale devices which have been implanted in a porcine model for 21 days. DTI enables 3D visualisation of the tissue microstructure and is validated using the standard means of fibrous capsule investigation: histological analysis and qualitative microCT and SEM imaging. DTI offers a promising non-invasive, non-destructive imaging modality that can yield novel insight into the formation of fibrous capsule and tissue-device integration.

C.2.2 Materials and methods

C.2.2.1 Device fabrication and animal study
In this study we examined two novel human-sized macroencapsulation devices that were implanted in the anterior abdominal wall of a diabetic porcine model for 21 days\textsuperscript{1,2}. Both devices were unique in their polymer composition, porosity, and topography (Figure C.8). The first device, the “Multiscale Porosity Device”, was composed of silicone, with both microporosity and macroporosity, in order to promote cellular attachment and tissue integration, respectively. The second device, the “Smooth Device”, was composed of a soft, flexible thermoplastic polymer (thermoplastic polyurethane). Its surface was smooth. Porcine studies were approved by the Italian Ministry of Health (Authorisation No. 976/2017-PR). Four female Landrace pigs, weighing 25-30 kg were enrolled in the study. Devices were implanted in the anterior abdominal wall as previously described\textsuperscript{1,2}; n=2 per group. Following euthanasia, the devices were removed en bloc with surrounding muscle tissue and fixed in 4% paraformaldehyde for 48 hrs.
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Figure C. 8. Volumetric renderings of devices after microCT imaging with associated high magnification images demonstrating both multiscale porosity surface\(^1\) and smooth surface\(^2\) respectively. Images courtesy of Dr. Ruth Levey, NUIG.

C.2.2.2 MR imaging and analysis

Fixed devices were stored at 4\(^\circ\)C in 70% ethanol until MR imaging. All samples were placed in a 3D printed holder, made from polylactic acid, and custom made to fit the MRI coil. For all imaging the holder was filled with fresh PBS at room temperature and devices were imaged individually. A 7T Bruker BioSpec 70/30 USR system (Bruker, Ettinger, Germany) equipped with Paravision 6 (Bruker, Ettinger, Germany) and a volume coil were used for all imaging sequences. T1- and T2- weighted scans were used to visualize the device and the surrounding tissue. T1-weighted parameters were as follows: TE/TR: 7/1452 ms, 20 averages, image size: 256 x 256 x 100, FOV: 70 x 70 x 50 mm, resolution: 0.273 x 0.273 x 0.5 mm and acquisition time: 1 hour and 23 minutes. T2-weighted parameters were as follows: TE/TR: 19.55/9137.6 ms, 20 averages, echo spacing: 6.518 ms, RARE factor: 8, image size: 256 x 256 x 30, FOV: 70 x 70 x 15 mm, resolution: 0.273 x 0.273 x 0.5 mm, and acquisition time: 1 hour and 13 minutes. A 2D spin echo DTI sequence was used for all four samples. One multiscale porosity device was imaged with the following parameters: TE/TR: 18.182/1011 ms, 5 averages, image size: 128 x 128 x 47, FOV: 64 x 64 x 23.5 mm, isotropic resolution: 0.5 x 0.5 x 0.5 mm, b-values: 0, 800 s/mm\(^2\), 32 diffusion directions, gradient duration: 3.8 ms, gradient separation: 8.802 ms and acquisition time 5 hours and 55
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minutes. The second multiscale porosity device and the two smooth devices were imaged with a refined 2D DTI sequence with the following parameters: TE/TR: 18.182/4000 ms, 4 averages, image size: 140 x 140 x 20, FOV: 70 x 70 x 10 mm, isotropic resolution 0.5 x 0.5 x 0.5 mm, acquisition time of 20 hours and 32 minutes and the same diffusion parameters. While the SNR of the refined 2D DTI sequence was higher than the initial sequence, the SNR ratio between B0:B800 was comparable between sequences.

All data was processed as outlined in Chapter 3 and the FA and MD calculated. ROIs (capsule and tissue) were defined based on the T1- and T2-weighted scans. The capsule was identified as the tissue adjacent to the device while muscle tissue not near the device was simply termed tissue. Mean values for FA and MD are reported. Normalised FA and MD values were calculated by dividing the metric of interest in the capsule by the value in the tissue of the same device. Tractography was done in ExploreDTI and parameters were kept consistent between all devices and are as follows: seed point resolution: 0.5 x 0.5 x 0.5 mm, FA threshold: 0.1, FA tracking threshold: 0.1 – 1, tract length: 2 – 20 mm, angular threshold: 30º and step size: 0.5 mm.

C.2.2.3 Tissue processing and histology
Core biopsy samples were taken systematically at five locations across each device using an 8 mm punch biopsy and placed in a 2% agarose to maintain structure. Four of these cores were for histological analysis while the 5th was processed for SEM imaging. Coronal sections for histological analysis were processed and embedded in paraffin wax blocks. Sections of 5 μm were cut and stained with Masson’s Trichrome with Gomori’s Aldehyde Fuchsin, PSR and αSMA and imaged for fibrous capsule analysis as detailed previously. Quantitative analysis of the collagen content for coherency and relative integrated colour densities was carried out on PSR stained sections using a previously reported technique.

C.2.2.4 Scanning electron microscopy (SEM)
Each core was bisected longitudinally to create a cross-section of the device and surrounding tissue. Samples were post-fixed overnight in glutaraldehyde and washed with PBS before being dehydrated and dried using an EMITECH K850 critical point dryer and mounted onto aluminium stubs using carbon adhesive tabs. An Emscope SC500 was used to lightly sputter coat the samples. Specimens were imaged using a Hitachi S2600N Scanning Electron Microscope using a secondary electron detector (Vacuum 15 kV, electron Beam 50, resolution 1280x960 PPI). SEM images were pseudo-coloured using MountainsMap® SEM Color 7.3.7984.
C.2.2.5 MicroCT
Whole tissue samples were stained with 2.5% phosphomolybdic acid solution in 70% ethanol for 7 days, then washed, and stored in fresh 70% ethanol. A microCT 100 scanner (Scanco) at 70 kVp and 85 μA with a 0.5-mm aluminum filter was used. MicroCT DICOM files were segmented using Mimics Research 18.0.0.525 software (Materialise) as described previously\textsuperscript{387}.

C.2.2.6 Statistical methods
Statistical analysis was performed with GraphPad Prism (Version 8). Kruskal-Wallis tests with Dunn’s multiple comparisons were performed on mean FA and MD values per sample (n=2 for both the multiscale porosity and smooth devices).

C.2.3 Results
C.2.3.1 Multicontrast MRI
Standard anatomical T1- and T2-weighted imaging was used to identify the implanted device. While the devices were identifiable, little distinguishable contrast was seen between the tissue surrounding the device and more distally located muscular tissue (Figure C.9). However, when looking at the mean DW images (b=800 s/mm\textsuperscript{2}, 32 directions) of both devices the tissue surrounding the smooth device showed distinct contrast compared to the tissue more distally located from the device.

Figure C.9. Multi-contrast MRI images of both multiscale porosity and smooth devices. T1- and T2-weighted and non-DW (b=0 s/mm\textsuperscript{2}) images showed little contrast within the samples, while the mean DW images showed distinct contrast.
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(b=800 s/mm², 32 directions) show contrast between the tissue around the smooth device (white arrow) and the more distal tissue (yellow arrow). Representative slices are shown, yellow dots mark the device location.

C.2.3.2 DTI metrics

Figure C.10 presents DTI metrics for the two devices. Parametric maps of FA (Figure C.10(a), top row) showed the stark difference in capsule delineation between devices; the regions of elevated FA surrounding the smooth devices highlight a highly anisotropic tissue around the device. Similarly, the MD maps (Figure C.10(a), bottom row) showed decreased diffusivity in the same region. Quantitatively, the mean FA in the tissue and capsule regions for the multiscale porosity device were 0.11 ± 0.0021 and 0.12 ± 0.033, respectively (Figure C.10(b)). For the smooth device the mean FA in the tissue was similar to the multiscale porous device at 0.12 ± 0.0086 but the capsule was much more anisotropic with an FA of 0.33 ± 0.11, see Figure C.10(b). The MD in the tissue regions for the multiscale porosity and smooth device, \(1.1 \pm 0.12 \times 10^{-3}\) mm\(^2\)/s and \(1.2 \pm 0.0019 \times 10^{-3}\) mm\(^2\)/s, were similar and also compared well with the MD in the capsule region for the multiscale porosity device, \(0.93 \pm 0.11 \times 10^{-3}\) mm\(^2\)/s, see Figure C.10(d). This was in stark contrast to the notably lower diffusivity in the fibrous capsule of the smooth device which had an MD of \(0.86 \pm 0.11 \times 10^{-3}\) mm\(^2\)/s. In order to ascertain differences between the tissue surrounding the device and tissue more distally located from the device, the capsule regions were normalised by these distal muscular tissue regions (Figure C.10(c, d)). The farther away from 1 the value was, the larger the difference between the two regions. From this, it became even more evident just how different the tissue around the smooth device was from the surrounding tissue. The elevated normalised FA in the smooth device highlighted a higher alignment (Figure C.10(c)) while the decreased normalised MD showed decreased diffusivity in the capsule compared to the normal tissue (Figure C.10(e)).
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Figure C. 10. DTI metrics for both devices. (a) FA (top) and MD (bottom) maps for both devices. Scales of images match the scales in (b, d), respectively. (b, d) FA and MD shown for the surrounding tissue and the tissue-device boundary (capsule). Capsule regions were normalised by the respective tissue regions per sample to give normalised (c) FA and (e) MD values. A value of 1 indicates no difference between the capsule and surrounding tissue. No significant difference was determined using Kruskal-Wallis tests with n=2 for each device. MSP – multiscale porosity device, SD – smooth device.

FEFA maps are shown in Figure C.11(a, c); where the colour coding indicates the principal direction of diffusion, and the intensity is weighted by the fractional anisotropy. The FEFA map for the multiscale porosity device (Figure C.11(a)) showed no coherent principal direction around the device, while the FEFA map of the smooth device illustrated a predominantly axial (blue) direction of diffusion. Tractography was performed to better visualise the diffusion pathways and microstructure between the capsule and surrounding tissue. Keeping the tracking parameters consistent, Figure C.11(b) and (d) show 3D representations of the microstructural arrangement. In the multiscale porosity device, no tracts were modelled around the device but instead only in the muscular tissue. Meanwhile, in the smooth device the capsule was clearly modelled at the tissue-device boundary. The blue tracts (Figure C.11(d)) highlight the longitudinal alignment in the capsule on the top and bottom of the device, while around the curvature of the lateral edges of the device a more circumferential alignment can be seen.
Figure C. 11. Vector maps and tractography for both devices. (a, c) First eigenvector-fractional anisotropy maps for both devices. (b, d) Tractography for the multiscale porosity device and smooth device, respectively. No clear delineation of a capsule is visible in the multiscale porosity device while the alignment of a capsule is clear in the smooth device. Holes are locations of biopsy punches for other analyses. Tractography was modelled with the following parameters: seed point resolution: 0.5 x 0.5 x 0.5 mm, FA threshold: 0.1, FA tracking threshold: 0.1 – 1, tract length: 2 – 20 mm, angular threshold: 30° and step size of 0.5 mm.

C.2.3.3 MicroCT and SEM imaging

MicroCT & SEM imaging was performed to gain insight into both the organisation of the fibrous capsules and also the relationship between the device topography and the surrounding tissue. The impact of the multiscale porosity devices’ surface features on the integration with the immediate surrounding tissue was strikingly apparent in the cross-sectional images from microCT (Figure C.12). Meanwhile, the smooth device showed decreased integration due to lack of surface features acting as a scaffold for the surrounding fibrous capsule tissue. The smooth device appears to be free of the surrounding tissue with a folded configuration (Figure C.12). These findings were further confirmed by SEM imaging. SEM allowed for a closer visualisation at the fibre organisation in the capsules. Fibres surrounding the multiscale porosity device appeared to have disorganised collagenous configuration as they envelope around the rope-coil structures at the device surface. In contrast, the fibrous capsule surrounding the smooth device appeared highly aligned and distinctively layered. The absence of any distinct surface features on the smooth device facilitated the formation of a fibrous capsule, which was highly aligned longitudinally down the length of the device.
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Figure C.12. MicroCT cross sectional images (left column) of multiscale porosity and smooth surfaced devices, demonstrating their relationship with the surrounding tissue. Scale bars = 10 mm. High magnification SEM images (right column) of rope-coil surface features (blue) integrated into the surrounding tissue and reduced integration surrounding the smooth device (green). Scale bar = 250 μm. Images courtesy of Dr. Ruth Levey, NUIG.

C.2.3.4 Histological analysis

Masson’s trichrome stain provided an overview of the capsule, its size, and its interaction with the implanted device. Specifically, this Masson’s Trichrome demonstrated the integration of the surrounding tissue with the multiscale porosity surface features, as imprints are seen within the tissue. Masson’s Trichrome also provided qualitative information on collagen structure and orientation. The collagenous capsule appears disorganised with no clear dominant direction of alignment around the multiscale porosity devices while the capsular fibres surrounding the smooth devices demonstrated a largely uniform alignment (Figure C.13(a)). Staining for αSMA allowed for insight into the quantity of myofibroblasts at the site of implantation as they are associated with the over-production of collagen in pro-inflammatory reactions, ultimately regulating tissue contraction and fibrosis⁵⁹⁰ and can be correlated to the capsule size³⁸⁷. Staining with CD31, an endothelial cell marker, enabled αSMA cells associated with blood vessels to be identified. Qualitatively this staining gives a nod towards collagen orientation and amounts of active collagen deposition (Figure C.13(b)). Quantification of the directional uniformity of the collagen fibres via PSR stained PLM demonstrated a higher coherency of the fibres in the capsule around the smooth device than the multiscale porosity device (Figure C.13(d)). Colour threshold segmentation was
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used to quantify both mature collagen type I fibres (red/orange) and thin, collagen type III-like fibres (green) present during the early phases of remodelling. No obvious differences between device groups were found (Figure C.13(e-f)), with both devices displaying more than 80% mature collagen fibres.

Figure C. 13. Histological analysis of fibrous capsule formation around multiscale porosity and smooth surfaced devices. (a) Masson’s trichrome images of core biopsy taken through device and adjacent surrounding tissue (Scale bar = 2 mm) with magnified representative image of the device space (*) and the fibrous capsule (Scale bar = 200 µm). (b) Immunofluorescent images for analysis of myofibroblast density within the fibrous capsule (Hoechst, blue; αSMA, green; CD31, red). Scale bar = 100 µm. (c) PLM images of the fibrous capsules. Scale bar = 100 µm. (d) Fibrous capsule coherency measurement using PLM. (e) Relative integrated density of red and orange collagen fibres (indicative of mature/permanent collagen deposition). (f) Relative integrated density of yellow and green fibres (indicative of immature/active collagen deposition). Images and analysis courtesy of Dr. Ruth Levey, NUIG.

C.2.4 Discussion

The present study illustrated, for the first time, the use of DTI for qualitative visualisation and quantitative characterisation of the microstructural organisation of the fibrous capsule formed at the tissue–device boundary with large animal samples. Characterisation of the microstructure and dominant components, such as collagen, within a fibrous capsule has the potential to yield significant insight into tissue integration and the FBR with respect to implanted devices. We explored this non-invasive and non-destructive imaging technique to analyse the fibrous capsules surrounding two unique human-sized macroencapsulation
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devices: the multiscale porosity device and smooth device after implantation in a porcine model for 21 days.

DTI investigated the presence of a fibrous capsule around these two devices with distinct topologies. T1- and T2-weighted images of the two devices yielded no discernible contrast between the presence of fibrous capsule and surrounding tissue. T1- and T2-weighted images have been used for identifying fibrous capsules and granulomas around breast implants; however, the envelope and capsule around the implants typically show low signal intensity. On qualitative inspection of the mean diffusion weighted images (b=800 s/mm², 32 directions), a large hyperintense region was apparent around the smooth device that is indicative of restricted diffusion. This contrast was absent surrounding the multiscale porosity device. This qualitative observation was quantified by regionally investigating the MD and FA – specifically in the capsules directly surrounding each device and in the more distally located tissue.

In the DTI-derived parametric maps, specifically the FA and MD, the regional differences within the tissues became even more apparent. A highly anisotropic capsule not only formed around the smooth device but showed no integration at the tissue-device boundary. In this highly aligned capsule, the diffusivity was decreased compared to the surrounding tissue. Tractography looked at the specific alignment of the microstructural organisation in the fibrous capsule. The fibres aligned longitudinally along the length of the device and showed some circumferential alignment at the lateral edge transition from top to bottom of the device. These non-invasive insights from DTI were further confirmed by traditional techniques. Standard histological tissue staining using Masson’s Trichrome and myofibroblast stains as well as imaging using SEM and microCT all qualitatively suggested that there appeared to be a difference in collagen organisation. MicroCT highlighted the same separation between device and fibrous capsule seen in DTI, while SEM and PLM confirmed the highly aligned fibre organisation within the fibrous capsule. Coherency measurements of the collagen fibres in PLM images demonstrated increased collagen anisotropy around the smooth device. These measurements agree strongly with the FA results whereby the fibrous capsule around the smooth devices showed a 2-fold increase in anisotropy when compared to more distal tissue.

Conversely, the multiscale porosity device showed no clear delineation of a fibrous capsule, but instead a highly integrated tissue at the tissue-device boundary. While the FA maps of the multiscale porosity device show some areas with increased anisotropy, they are
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sporadically on one face of the device and very localised. The normalised FA and MD values for the multiscale porosity device were around 1, indicating little difference between the tissue around the device and more distally located tissue. With the same tracking parameters used for the smooth device, tractography showed no coherent organisation around the multiscale porosity device, and this was further confirmed with low coherency measurements from PLM. MicroCT and SEM images of the multiscale porosity device also confirm this highly integrated fibrous capsule around the device.

Although microCT is a very useful large sample imaging modality for providing context into device tissue interactions, it lacks the quantitative microstructural insight gained by DTI allowing for in-depth tissue analysis in this circumstance. Additionally, while traditional histological processing is the gold standard in identifying the underlying microstructure in tissue, its destructive nature and limited size capabilities make it a less than ideal methodology for fully characterising large scale animal tissue samples. PLM using PSR stained sections enhances the birefringent properties of the collagen fibres enabling quantification of the directional uniformity (coherency) and colour segmentation of fibres; however the FA and tractography together yielded the same insight into the anisotropy of the tissues. Through the work presented in this study, DTI has non-invasively and non-destructively yielded volumetric information on the presence and microstructural organisation of fibrous capsules at the tissue-device boundary.

This study proved that DTI can not only detect the presence of the fibrous capsule surrounding two different devices but has the capabilities to accurately quantify microstructural differences in the capsules. The fact that the two devices were composed of different materials merely highlights that this method of imaging is versatile and has a broad range of applications. DTI is a non-destructive method for high-resolution imaging without the need for staining or special treatment of the tissue which could be used instead of, or in combination with, extensive histological staining, imaging, and analysis. The potential to non-invasively assess device integration during animal studies could lessen the need for increased numbers of animals as terminal time points could be decreased. It is particularly useful when analysing large tissue samples ex vivo, providing the viewer with faster insights into device integration, capsule organisation and unique spatial features that may otherwise be missed when using stereological sampling alone.

While this study focused on the presence and microstructural integration of fibrous capsules from two specific device topographies, the feasibility of using DTI for more general
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encapsulation of medical devices has been established. The potential to non-invasively ascertain fibre orientation within fibrous capsules has strong implications for understanding and predicting the efficacy of implanted devices. Understanding water diffusion around the device also has the potential to yield valuable insight for devices with drug delivery purposes.