THE EFFECT OF INTERSTITIAL PRESSURE ON TUMOUR STIFFNESS

by

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for the degree of Master of Science
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Abstract

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This work investigates the dependence of the stiffness of a solid tumour on its interstitial pressure. Supersonic shear imaging, a type of ultrasound elastography, was used for measurements of shear modulus. Experiments on in vitro gelatin phantoms demonstrated that distortion of a medium is necessary for stiffness changes. Experiments pressurizing ex vivo porcine kidneys showed that in unconstrained living tissue, shear modulus will increase with fluid pressure (median increase 0.7 kPa/mmHg). Preliminary experiments were performed on intramuscular VX2 tumours in rabbits. Both interstitial pressure and shear modulus showed large spatial variation. After lowering interstitial pressure with sacrifice, shear modulus was found to decrease (median drop 33%, p=0.04). These results suggest that fluid pressure contributes to tumour stiffness, though more work should be done to determine this relationship and the possible utility of its measurement in relation to cancer.
Acknowledgements

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<tr>
<td>ARFI</td>
<td>Acoustic radiation force impulse imaging</td>
</tr>
<tr>
<td>GPU</td>
<td>Graphics processing unit</td>
</tr>
<tr>
<td>IFP</td>
<td>Interstitial fluid pressure</td>
</tr>
<tr>
<td>MR</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>PRF</td>
<td>Pulse repetition frequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SSI</td>
<td>Supersonic shear imaging</td>
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<tr>
<td>SWEI</td>
<td>Shear wave elasticity imaging</td>
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<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<table>
<thead>
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<tr>
<td>$A$</td>
<td>Third order elastic modulus</td>
</tr>
<tr>
<td>$a$</td>
<td>Position of acoustic particle at equilibrium</td>
</tr>
<tr>
<td>$c$</td>
<td>Speed of sound</td>
</tr>
<tr>
<td>$c_s$</td>
<td>Shear wave speed</td>
</tr>
<tr>
<td>$E$</td>
<td>Young’s modulus</td>
</tr>
<tr>
<td>$\ell$</td>
<td>Half the effective aperture length</td>
</tr>
<tr>
<td>$P$</td>
<td>Fluid pressure</td>
</tr>
<tr>
<td>$\Delta P$</td>
<td>Change in pressure between steps</td>
</tr>
<tr>
<td>$P_{eff}$</td>
<td>Effective pressure</td>
</tr>
<tr>
<td>$P_i$</td>
<td>Initial pressure</td>
</tr>
<tr>
<td>$R$</td>
<td>Cross-correlation function</td>
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\begin{align*}
r & \quad \text{Position} \\
RF & \quad \text{Radio frequency signal} \\
s & \quad \text{Signal from coherent addition of echoes} \\
t & \quad \text{Time} \\
t_{\text{arr}} & \quad \text{Arrival time of shear wave} \\
t_d & \quad \text{Displacement of temporal window} \\
u & \quad \text{Particle displacement} \\
\vec{u}^D & \quad \text{Particle displacement due to wave travel} \\
\vec{u}^S & \quad \text{Particle displacement due to static stress} \\
x & \quad \text{Horizontal position} \\
x_1 & \quad \text{Horizontal position of transducer element} \\
x_{\text{ref}} & \quad \text{Horizontal position of reference point} \\
z & \quad \text{Depth} \\
\alpha & \quad \text{Signal of reference image} \\
\beta & \quad \text{Signal of comparison image} \\
\lambda & \quad \text{Wavelength of ultrasound wave} \\
\mu & \quad \text{Shear modulus} \\
\mu_0 & \quad \text{Shear modulus in the absence of pressure} \\
\mu_{\text{baseline}} & \quad \text{Shear modulus at equilibrium} \\
\Delta \mu & \quad \text{Change in shear modulus due to pressure} \\
\mu_{\text{max}} & \quad \text{Shear modulus at its greatest} \\
\rho & \quad \text{Density of medium} \\
\rho_0 & \quad \text{Equilibrium density} \\
\tau & \quad \text{Time constant of decay} \\
\tilde{\tau} & \quad \text{Time delay}
\end{align*}
Chapter 1

Introduction

1.1 Cancer, angiogenesis and interstitial fluid pressure

1.1.1 Cancer

Cancer is a disease characterized by unregulated cell growth. Malignant, cancerous tumours grow large and invade tissue, both nearby and in other parts of the body. These tumours grow more than normal and do not die at the rate they should [1],[2]. Additionally, they consume the resources of the body without contributing to bodily function. Ultimately, cancer is usually fatal, as the malignant tumours impede critical bodily functions to the extent that the patient can no longer survive.

Nearly half of Canadians, 41% of women and 46% of men, will develop cancer in their lifetime. A majority of these people will die of cancer, and it is responsible for 30% of the deaths in Canada each year, making it the single greatest cause of death in the country [3]. As might be expected, with such a large incidence, it is also among the costliest diseases for Canadian society; it costs $2.6 billion in direct healthcare costs each year, while lost productivity and premature death from cancer account for another $14.8 billion. This burden of $17.4 billion per year is very likely to increase as the Canadian population ages, putting further strain on society [3]. Cancer presents a substantial challenge, and improved diagnosis, characterization and treatment of cancer will aid in addressing this challenge.

There are many different methods that have been applied to the treatment of cancer, but none is without drawbacks. Each suffers from the dual concerns that it might not be effective enough at killing the cancer, while it might cause too much damage to healthy tissue. For this reason, the search for new treatments of greater efficacy is ongoing. Many effective treatments are based on exploiting the differences between cancer and healthy tissues, and using this to maximize damage to the former while minimizing damage to the latter.
1.1.2 Tumour vasculature and angiogenesis

Cells in the human body need oxygen, nutrients and waste removal to survive, and these functions are accomplished by the vasculature. As a result, almost all cells in the human body must be within 100 µm of a capillary [2]. In the case of solid tumours, unregulated cell growth creates new cells which have the same needs, and as a tumour grows, cells find themselves farther and farther from blood vessels, and are thus starved for oxygen and nutrients.

One of the hallmarks of cancer is the ability of the tumour to recruit blood vessels and to promote the development of blood vessels which better feed the tumour and allow it to grow [2]. Indeed a tumour is unable to grow to macroscopic size if it lacks the ability to signal for blood vessel creation. The process of blood vessel creation and proliferation is called angiogenesis.

There are numerous factors that promote angiogenesis and factors that inhibit angiogenesis. Prominent among the pro-angiogenic factors is the vascular endothelial growth factor (VEGF) pathway [4], though over two dozen pro-angiogenic factors are known [2]. In healthy tissues, there is a balance between the pro-angiogenic and anti-angiogenic factors, and the vasculature is stable [5]. In cancer, however, the process goes awry. There is excessive pro-angiogenic signalling causing an imbalance between the two. The resulting tumour vasculature is abnormal in nearly every aspect of its structure and function [6]. The network has a chaotic and ineffective morphology. The vessels are abnormally formed, have inadequate pericyte coverage, have sluggish blood flow, and tend to have holes in them, among other abnormalities. Vessels have inconsistent diameters partially due to their walls being compressed by tumour or stromal cells; some develop to be oversized while others are immature or lacking altogether. These structural problems cause the blood supply to be inconsistent both in time and space; this leaves the cells chronically oxygen starved and continually signalling for the creation of more of this disorganized vasculature, which compounds the problems with the vasculature's effectiveness [6].

It might initially seem like a good thing that the malignant cells are deprived of the benefits of functional vasculature. One problem is that hypoxic tumour cells are more likely to metastasize [7]. Further, due to the leaky vasculature, the interstitial pressure of the tumour tends to be higher than that of healthy tissue, meaning there is a reduced pressure gradient available for drug delivery, including chemotherapeutics. Hypoxic cells are additionally more resistant to radiotherapy due to the lack of oxygen [7]. This means that with chemotherapy or radiotherapy, the tumour cells that have access to the best functioning vasculature will be killed off, and replaced by cells that are more resilient and more likely to metastasize. On the whole, the deficient vasculature of the tumour microenvironment presents an obstacle for effective cancer treatment.
1.1.3 Anti-angiogenic therapies

Understanding the importance of angiogenesis in the formation of the tumour vasculature and the tumour microenvironment has led to a class of therapies that attack or change the tumour vasculature; these are called anti-angiogenic therapies.

Anti-angiogenic therapies have been demonstrated to be effective in numerous cases. Different anti-angiogenic agents have been approved by the FDA for clinical therapy. As monotherapies, they have been approved for treatment of gastrointestinal stromal tumours, hepatocellular carcinoma glioblastoma multiforme and metastatic renal cell carcinoma. In combination with chemotherapy, an anti-angiogenic agent has been approved for use as a first-line therapy for colorectal cancer, metastatic breast cancer, non-small-cell lung cancer and metastatic renal cell carcinoma [8]. The exact mechanism by which these drugs improve outcomes, however, is not entirely clear. Many theories have been put forward to resolve this question [4].

At first it was thought that by blocking the vascularization or destroying existing vessels in a tumour, it could be starved for oxygen and nutrients and would die, be unable to proliferate, or grow at a reduced rate, thus addressing the problem [5], [6], [9], [10]. As mentioned above, however, simply worsening the effectiveness of the vasculature in the tumour microenvironment could prove ineffective or even counterproductive at increasing patient survival [8]. It is also paradoxical at first glance that therapies which attack the vasculature should improve the effectiveness of chemotherapeutics, which depend on the vasculature for their delivery.

One hypothesis to account for this, put forward by Rakesh Jain, is the vascular normalization hypothesis [11]. Under this view, anti-angiogenic therapies in appropriate doses serve to improve or normalize the chaotic vasculature, allowing more effective vascular function.

By blocking VEGF signalling, the vasculature is passively ‘pruned.’ The idea is that the ill-formed, immature and ineffective vessels characteristic of the tumour microenvironment would be pruned away because they are most sensitive to the therapy. Those vessels remaining would contain a greater proportion of mature vessels, while the remaining immature vessels would be able to mature rapidly, and would be able to be more effective in their structure and function [10]. The improvements in the remaining vessels include increased pericyte coverage, less dilation, less tortuosity and less leakiness; see Figure 1.1. In short, the vasculature better resembles that of normal, healthy tissue, hence ‘normalization.’ These changes allow the vasculature to improve delivery of drugs and oxygen. This effect would explain why anti-angiogenic therapies can increase the effectiveness of chemotherapy and radiotherapy [6].

Anti-angiogenic therapies do not, however, always prove effective. Tumours will often not respond to an anti-angiogenic drug, or will show an initial response and improvement followed by a return to progression. Identification of resistance is crucial; in response, the dose can be increased so effectiveness is regained, or a different drug can be used to treat the disease [12].
1.1.4 Interstitial pressure

In addition to the irregularities of the tumour vasculature listed above, the interstitial fluid pressure (IFP) tends to be much higher in a solid tumour than in healthy tissues; indeed the interstitial pressure of most solid tumours is known to be elevated [13]. There are a great many factors that affect interstitial pressure, including solute concentration, the state of the interstitial matrix, and particularly the pressure of the blood in the microvasculature. Combined with the absence of lymphatic vessels in the tumour to drain excess fluid, the tumour has and maintains an elevated interstitial pressure. As mentioned before, the vasculature of a tumour is known to be leaky and have holes in it. This leakiness allows blood plasma at the vascular pressure to enter the interstitium, thus raising the pressure. Quantitatively, interstitial fluid pressure is generally in the neighbourhood of \( \sim 0 \) mmHg in healthy tissue, but is much higher in tumours, at \( \sim 15-20 \) mmHg for many types of tumour [14].

As a result of this high fluid pressure and poor drainage, the interstitial space is much larger in tumours than in healthy tissues [15]; about three to five times larger. While the relative volume of the interstitium is only around \( \sim 16\% \) in normal tissue, in a malignant tumour it is around \( \sim 45\% \) [14][16], see Figure 1.2. The interstitial fluid is also more mobile and free in tumours; while most interstitial fluid is freely moving in tumours, in healthy tissue the vast majority of the fluid is found in a gel phase.

After administration of anti-angiogenic therapy, the normalization hypothesis predicts that the remodelling of the vasculature will result in a reduction in interstitial pressure. With fewer leaky vessels, and those that remain leaking less, it seems reasonable that less plasma should leak out of the blood vessels, causing the interstitial pressure to drop, and increasing the pressure gradient between the vessels and the interstitium, aiding in drug delivery. Indeed, there is evidence, both pre-clinical [17],[18],[19] and clinical, that the IFP is reduced after targeted anti-angiogenic therapy.

Figure 1.1: The tumour vasculature is abnormal, both structurally and functionally. According to the vascular normalization hypothesis, an appropriate dose of anti-angiogenic drugs modifies the tumour vasculature to better resemble that of normal tissue. From Jain [5].
In terms of clinical evidence, three studies, two phase 1 and one phase 2, found a reduction in IFP in human rectal cancers with bevacizumab administration [20],[21],[22]. In each case, it was found that IFP was reduced from an average of \(~15\) mmHg before treatment to an average of \(~5\) mmHg after 12 days.

As mentioned above, however, anti-angiogenic therapy does not always prove effective. It has been suggested that interstitial pressure can be used as an early-response marker or surrogate marker of therapy effectiveness [19],[20],[21]. The primary interest in doing this over established methods, such as measurements of tumour volume, is the difference in timescale of the response. Changes in tumour volume occur on the order of several months [23]. Changes in interstitial fluid pressure, on the other hand, occur rapidly, on the order of days, making investigation of it as an early-response marker attractive. Further, interstitial fluid pressure has been shown to be the single best prognostic indicator in patients with cervical cancer [24].

One serious obstacle for adoption of interstitial fluid pressure as a marker of tumour status is that it is an invasive method. The standard method for measurement is called the wick-in-needle technique [25], and consists essentially in placing a fluid-filled needle tip at the region of desired measurement. Ideally, a non-invasive substitute for this method could be found.
1.1.5 Tumour stiffness

It is well-known that tumours are usually harder or stiffer than healthy tissue [26]. There are many factors that contribute to the mechanical properties of tissue in general and tumours specifically. Included in this are the mechanical properties of the cells themselves, and the mechanical properties of the interstitium, including the extracellular matrix. It is known that tumours induce a stromal reaction in normal tissue, increasing the amount of fibrotic tissue in the tumour, thereby increasing the tumour’s stiffness. Fibrosis is certainly a determinant of tumour stiffness, but it is perhaps not the only major one.

As mentioned earlier, interstitial pressure is known to be elevated in tumours. This increased fluid pressure might also contribute to the stiffness of a tumour, similar to the way that a highly inflated basketball is harder than one under less pressure. In the same vein, it could be that the reduction in interstitial fluid pressure after anti-angiogenic therapy will cause the tumour to be less pressurized, thereby making the tumour less stiff. Indeed, it is well-known that the stiffness of a tissue can change with the amount of mechanical stress to which it is subjected [26]. There is additional evidence that the stiffness of tissues increases with increasing internal fluid pressure [27],[28],[29]. A precedent for therapies changing elastographic properties also exists, with thermal ablation known to increase the stiffness of tissue [30],[31],[32]. The consideration of changes in stiffness in tumours from anti-angiogenic therapy is further prompted by anecdotal evidence from clinicians, who found that tumours seemed to soften to the touch after these therapies.

1.1.6 Research objective

The objective of this work is to shed light on how the stiffness of a tumour depends on interstitial fluid pressure. This is motivated by the drop in interstitial fluid pressure expected from anti-angiogenic therapy.

At the most basic level, knowledge of the contribution of interstitial pressure to stiffness would be helpful in the interpretation of elastographic images of tumours in general. At the same time, understanding the contribution of interstitial pressure changes to stiffness changes would give insight into monitoring tumours over time or following therapy. Though the focus of this work is on anti-angiogenic therapy, chemotherapy is also known to decrease interstitial pressure [19].

It was suggested in Section 1.1.4 that interstitial pressure could be used as a surrogate biomarker to assess the effectiveness of anti-angiogenic therapy on a tumour. This measurement is painful and invasive, however, and it samples fluid pressure at only a handful of points in the tumour, leaving such a measurement not ideal for monitoring tumour status. If, on the other hand, a clear relationship between stiffness and interstitial pressure could be found, monitoring of tumour status and prognosis could potentially be achieved noninvasively using ultrasound stiffness imaging.
The method we use to assess the stiffness of tumours is a type of ultrasound elastography called supersonic shear imaging, a novel method that uses radiation force to induce a shear wave, which is then tracked with ultrafast ultrasound imaging, to determine the stiffness of a tissue.

1.2 Ultrasound elastography - supersonic shear imaging

1.2.1 Historical background

Palpation is the practice of feeling the stiffness of a patient’s tissues with the practitioner’s hands. The chief idea is that whether the tissue is hard or soft will give diagnostic information about the presence or status of disease. Manual palpation dates back at least to 1500 BC, with the Egyptian Ebers Papyrus and Edwin Smith Papyrus both giving instructions on diagnosis with palpation. Hippocrates gave instructions on many forms of diagnosis using palpation, including palpation of the breasts, wounds, bowels, ulcers, uterus, skin and tumours. In the modern Western world, palpation became considered a respectable method of diagnosis in the 1930s [26]. Since then, the practice of palpation has become widespread, and it is considered an effective method of detecting tumours.

Manual palpation, however, suffers from several important limitations: it is limited to tissues accessible to the physician’s hand, it is distorted by any intervening tissue, and it is qualitative but not quantitative. Elastography, the measurement of tissue stiffness, seeks to address these challenges.

1.2.2 Supersonic shear imaging (SSI) - Introduction

There are many different ways of doing elastography. At the present time, ultrasound and magnetic resonance imaging (MRI) methods dominate the field [26]. Some methods are more popular than others, and a treatment of various elastographic methods appears in Section 1.2.6. We will begin, however, by discussing the elastographic method used in this work: supersonic shear imaging.

Supersonic shear imaging (SSI) is an ultrasound-based technique that uses acoustic radiation force to induce displacements in tissue in a region of interest; the perturbations from these induced displacements travel through the tissue, creating a shear wave. The shear wave travels through the tissue at a speed dependent on the local shear modulus, which is a measure of stiffness. The shear wave’s travel is imaged using an ultrafast ultrasound imaging technique called plane wave imaging. From the motion of the shear wave, the stiffness of the tissue is computed at each point in the region of interest (ROI). A map of the tissue’s stiffness is then displayed in real time.

Supersonic shear imaging is an appealing method for several reasons. As just mentioned, the stiffness is reported in real time, allowing the easier acquisition of a large number of measurements, rather than suffering from long processing delays. The resulting map (rather than a single value) also
allows the distinguishing of different stiffnesses at different regions, improving characterization of heterogeneous tissue. The induction of shear waves in the tissue itself rather than the surface of the body provides superior wave amplitudes and therefore signal to noise ratio in deeper tissues. Most importantly, it is a quantitative method, which allows meaningful comparisons of shear modulus across different tissue types, individuals and fluid pressures.

What follows is a more detailed explanation of how supersonic shear imaging works. The following three sections describe how plane wave imaging works, how the shear wave is generated, and how it is tracked and the shear modulus inferred.

1.2.3 Plane wave imaging

In conventional B-mode ultrasound, a two-dimensional image is built up one vertical line (A-line) of the image at a time. Each A-line is obtained by sending out a pulse of ultrasound and ‘listening’ for the echo; the depth of the echo’s origin is determined by the time it takes the sound to get back to the transducer, and a measurement of the intensity of the scattered sound at each depth is displayed on the screen. A B-mode image is built up of a number of such A-lines acquired in succession, typically in the neighbourhood of 128 or 256.

Let us now consider the frame rate of B-mode ultrasound. The pulse repetition frequency (PRF) is the measure of how often pulses are sent out. For a single A-line, the PRF is limited by the amount of time it takes ultrasound to reach the greatest depth in the image, and then come back. Mathematically:

\[
\text{Max PRF} = \frac{\text{Speed of sound}}{2 \times \text{Max depth}}
\]  

(1.1)

As a B-mode image is composed of many A-lines, the frame rate will decrease according to the number of A-lines per B-mode frame. Sometimes each A-line is acquired multiple times with foci at different depths to improve the lateral resolution. Consequently, if multiple foci are used per A-line, then the maximum frame rate is divided by the number of foci. This gives:

\[
\text{Max frame rate} = \frac{\text{Speed of sound}}{2 \times \text{Max depth} \times \text{Number of A-lines} \times \text{Foci per A-line}}
\]  

(1.2)

If the maximum depth in the image is 10 cm and 128 A-lines are acquired without multiple focusing, then this gives a maximum frame rate of 60 frames per second.

B-mode ultrasound functions well for a great many applications, and its frame rate is considered fast compared to many other modalities, including magnetic resonance imaging. Nonetheless, this frame rate is not sufficient for some applications, including shear wave tracking. For example, if you wish to track a shear wave where the highest frequency component is 500 Hz or 2000 Hz, then by the Nyquist sampling theorem, it must be sampled at a rate of 1000 Hz or 4000 Hz, which is much
Figure 1.3: Dynamic focusing on reception. The wavefronts of the echo from a shallow point (a) and a deep point (b) are shown. The time delay applied to the signal received at each element is adjusted to match the time it takes for the signal to arrive at that element from the scatterer of interest. From Cobbold [33].

higher than the capability of standard B-mode ultrasound for a full image.

Plane wave imaging is a type of synthetic aperture imaging done to acquire images at much higher frame rates, roughly two orders of magnitude higher than conventional B-mode ultrasound. Before explaining how plane wave imaging works, however, it will be instructive to consider how an A-line is acquired in B-mode ultrasound.

When acquiring a vertical line for a B-mode image, the goal is to only include the echoes from within the vertical line being considered, or to get as close to this as possible. To this end, the ultrasound sent out is focused on transmission. For effective focusing, the signal from each element of the transducer should arrive at the point of interest at the same time, creating a focus similar to that of a concave transducer. To achieve this, the elements from the array are activated with time delays corresponding to the distance between them and the desired focal point.

Focusing is also done on reception in a similar way, except the point of interest is dynamically changed during the listening. The echo from the point of interest will arrive at each element of the transducer after a time dictated by the distance between the transducer and the point. Knowing this, the signal from a given depth is the sum of the signals from each element at the specific time that the echo reaches each element. During the listening, the focal point and corresponding time delays are changed dynamically; see Figure 1.3. In B-mode ultrasound, this gives a crisper image.

In plane wave imaging, on the other hand, focusing on reception does not merely give a crisper image, it is what allows the formation of the image in the first place. In plane wave imaging, there is no focusing at all on transmission; the entire target volume is insonated by all array elements firing, and the focusing is done entirely on reception. This focusing is only partly similar to the focusing
on reception done for an A-line, in that it has the added complication that the scatter can come from a number of different lateral positions.

Consider the echo from a plane wave insonification. If a plane wave insonification is applied to a volume with a single scatterer, then the received signal will be from the backscatter off of it, and will arrive at each element after a time corresponding to the location of the scatterer; see figure 1.4. By coherently adding all of the echoes from each element at the time it would receive the echo from that location, the echogenicity at that point can be determined. With this in view, it can be seen that in any plane wave image, the echogenicity at each point in the image can be inferred by adding all of the echoes attributable to that spot by using appropriate time delays.

Formally, the time delay $\hat{\tau}$ at an element at horizontal position $x_1$ from a particle at horizontal position $x$ and depth $z$ is [34]:

$$\hat{\tau}(x_1, x, z) = \left( z + \sqrt{z^2 + (x - x_1)^2} \right) / c \quad (1.3)$$

where $c$ is the speed of sound in the medium. By appropriately applying these time delays to the different elements, the signal $s$ from $(x, z)$ is calculated according to:

$$s(x, z) = \int_{x-\ell}^{x+\ell} RF(x_1, \hat{\tau}(x_1, x, z))dx_1 \quad (1.4)$$

where $2\ell$ is the effective length of the aperture and $RF(x_1, \hat{\tau}(x_1, x, z))$ is the radio frequency signal at the element $x_1$ with the time delay applied.

It must be remembered the signal at each element at that point in time is only in a small part
due to the scatterer of interest, with contributions from many scatterers across the image. The assumption is made that the RF signals from the scatterer of interest will be coherent, while those from other scatterers will be incoherent, meaning that most (but not all) of the signal computed for a point in space will be from the scatterer at the point of interest. Equation 1.4 is computed for every point \((x, z)\) in the region of interest, giving a complete 2D map (image) of the location and echogenicity of the scatterers, just like a conventional B-mode image. It must be noted that computing Equation 1.4 for each point in the image, and doing so several thousand times per second, is extremely computationally demanding, and was only made possible by the advent of GPU (graphics processing unit) processing.

The result of this processing is that from a single insonification a full 2D image can be acquired, rather than only one A-line of an image. Consequently, the frame rate dramatically increases, and the maximum frame rate is in fact equivalent to the maximum PRF shown in Equation 1.1. For an image with a maximum depth of 10 cm, this gives a maximum frame rate of close to 8000 fps, which is a dramatic improvement. Plane wave imaging thereby affords frame rates that are high enough to track the travel of a shear wave through tissue.

1.2.4 Shear wave generation

In order to monitor the travel of a shear wave through a medium, the shear wave must first be induced in the medium. The same transducer used for imaging is used to focus a pulse of high intensity ultrasound at one point in the tissue. With the high intensity ultrasound comes a high radiation pressure, much larger than the very slight radiation pressure exerted by normal diagnostic ultrasound. This radiation pressure pushes the tissue in the axial direction, i.e. in the direction of the pulse. The resulting displacement is on the order of tens of microns [35]; by contrast the displacements from conventional ultrasound imaging are very small, less than one micron in size [36].

From this sudden perturbation in the tissue, a perturbation is induced in neighbouring tissue. The propagation of this perturbation through the tissue is a shear wave. The shear wave is polarized in the axial direction of the ultrasound beam, and it travels in all directions away from the source, though the amplitude is largest for waves travelling in directions perpendicular to the axial direction, while the amplitude of waves travelling in the axial direction is negligibly small. For this reason, among others, shear waves along the axis where radiation force is applied are not included in the computation of the shear modulus from that push [35]. The frequency content of the shear wave is determined by the length of the excitation generated from the radiation force, meaning the shear wave is at a much lower frequency than the compressional ultrasonic waves, typically in the range of a few hundred hertz.

The technique of supersonic shear imaging uses multiple pushes from acoustic radiation force
Figure 1.5: The generation of the plane shear waves that are tracked in supersonic shear imaging. From Bercoff et al. [35].

in multiple locations in order to create a wave of larger and more uniform amplitude to facilitate tracking and achieve an improved signal-to-noise ratio. Specifically, it induces a shear wave in at a point in the tissue as described above. Very shortly after, it induces another shear wave at a point deeper in the tissue, and it induces three to five such shear waves at successive depths; see Figure 1.5.

The term ‘supersonic’ arises from the fact that the source of the shear waves, i.e. the focus, moves faster through the medium than the shear waves themselves do; the situation is analogous to a sonic boom created behind an aircraft moving at supersonic speeds. This is possible because the compressional waves used to create the push that is used to track the shear waves are several orders of magnitude faster than the shear waves themselves. Note that the term ‘supersonic’ refers only to the shear waves, and the focus moves at only subsonic speeds compared to compressional waves. Inducing many such sources supersonically causes the shear waves from each to constructively interfere, creating a shear wavefront that is essentially a Mach cone propagating outwards from the axis on which the pushes are applied. Indeed, one section of this wavefront can be considered to be effectively a plane wave, simplifying analysis of its travel. Because the cone is symmetrical, in the plane of the ultrasound image we now have two plane shear waves travelling in opposite directions. It is these “plane” shear waves that will be tracked in order to compute the elastic properties of the tissue.

1.2.5 Shear wave tracking

Once the shear wave has been created, it must be tracked and computations must be performed in order to determine the elastic modulus of the medium. The first step in the tracking is using very high frame rate ultrasound images to monitor the medium during the shear wave’s travel. Once a
video has been created, the displacements of the tissue in the axial direction are computed using a cross-correlation method. This gives a video of the tissue’s displacement from its rest position at every point in time, i.e. a video of the shear wave’s travel. From this video, the time-of-flight of the centre of the shear wave between two points in space is computed. Comparing the time of flight with distance travelled gives the wave speed throughout the medium. This map of wave speeds is converted into a map of elastic moduli using a simple mathematical relationship, along with some processing and compounding. The following sections will explain each of these steps in more detail.

1.2.5.1 Calculation of displacements

The calculation of movement in supersonic shear imaging uses a technique called ultrasound speckle interferometry [37]. It is based on existing techniques for Doppler ultrasound, which also tracks movement. The specific algorithm for measuring the displacements of tissue is similar to that of colour Doppler, with two important distinctions. For one, it is able to estimate one displacement per image rather than having to obtain multiple images to estimate a velocity/displacement. Second, the movement detected is not limited by a maximum calculable amplitude due to the algorithm used [37].

Before the shear waves are induced, a reference plane-wave image of the medium is obtained. Next, the shear waves are induced, and then a series of images are acquired and compared to the reference image. The displacements are computed from the difference between these and the reference image. First, an axial line of the reference image is considered; this is analogous to an A-line, though it is not acquired in the same way. Next, it is cut into temporal windows of interest; these correspond to small clusters of scatterers at different depths [38], see Figure 1.6. In Bercoff’s thesis [37], the length of the temporal window was chosen to be around \(8\lambda\), where \(\lambda\) is the wavelength of the ultrasound waves. For the transducer used throughout this thesis, the centre frequency is 7.5 MHz, giving a wavelength of about 100 ns or 200 \(\mu\)m, and would give a window of 1 \(\mu\)s or 1.5 mm. These temporal windows may also be taken to overlap, and generally an overlap between subsequent windows of 50% was used [37].

Next, each temporal window from the initial signal is cross-correlated with the same axial line in the second image acquired; this is the first image following the creation of the shear wave, see Figure 1.7. The cross-correlation function \(R(t_d)\) is defined by:

\[
R(t_d) = \left[ \frac{\alpha \star \beta}{\sqrt{(\alpha \star \alpha)(\beta \star \beta)}} \right](t)
\]  \hspace{1cm} (1.5)

where \(t_d\) is the amount the temporal window is displaced, and \(\alpha \star \beta\) is the cross-correlation between the signals \(\alpha(t)\) of the reference image and \(\beta(t)\) of the one being compared. The value for which the cross-correlation function is maximum indicates the offset for which the two images best line up, and
therefore indicates the displacement of the region under consideration between the reference image and the subsequent image. In practice, however, finding the maximum precisely would require a computationally demanding number of cross-correlations. To save on computing power, the cross-correlation function is evaluated for a somewhat granular set of window displacements, a parabola is fit to several points near the maximum value, and the maximum is taken to be the maximum of this parabola. From the value of $t_d$ at this maximum, the axial displacement $u$ of the volume considered is calculated according to the simple expression

$$u = \frac{ct_d}{2}$$

(1.6)

which like Equation 1.1 simply reflects the time it takes sound to travel the distance in question [38]. By following this procedure for every temporal window on every axial line in every image (within the region of interest at least), a video of the local axial displacement of the medium at each time is obtained. It is from this that the shear wave’s travel is characterized.

### 1.2.5.2 Tracking of travel and calculation of elastic moduli

Once the maps of displacement in the axial direction have been obtained, it is possible to characterize the shear wave’s travel. The method used for this consists of determining the shear wave’s speed locally using a time-of-flight method [39].

The time-of-flight method used to determine the arrival times is based on cross-correlations, and is broadly similar to the method used to find the axial displacements, though this time the cross-correlations are computed along the lateral direction, which is the direction of the shear wave’s travel. Several assumptions are made about the behaviour of the shear wave in these computations. Though tissue is a viscoelastic medium, the viscous and elastic effects are treated separately. At the frequency of the shear wave, a few hundred hertz, the tissue is treated as a linear elastic medium and viscosity is mostly neglected. Viscosity is considered to act only to reduce the amplitude of the shear wave as it travels through the tissue [36]. With this in mind, we can expect the shear wave to
have the same approximate shape as it passes through two subsequent points; see Figure 1.8. The effects of dispersion are also neglected.

The axial displacement $u$ induced by the shear wave is a function of the position $x$ and the time $t$. First, a reference point $u(x_{ref}, t)$ near the point where the shear wave was generated is chosen. Then for each point in the region of interest, $u(x, t)$ is compared to the reference point. More specifically, it is cross-correlated with $u(x_{ref}, t)$, and the time for which the cross-correlation is maximum is taken to be the arrival time, $t_{arr}$ \[40\].

With the arrival time of the shear wave calculated for each point, local wave speeds can be determined everywhere. Because speed is merely distance covered per time, the speed of the shear wave $c_s$ can therefore be calculated from

$$\frac{dt_{arr}}{dx} = \frac{1}{c_s} \tag{1.7}$$

It should be noted that in Equation 1.7 $c_s$ is inversely proportional to $dt_{arr}/dx$, so the computed speeds are sensitive to noise in the computed arrival times, which can lead to large outliers and much variation in the results. Consequently, a more involved method based on this is used, reducing variation in the computed speeds due to noise while also reducing computation times; it is described in detail in \[40\].

If the shear wavefront is approximated as a plane, the shear modulus can then be found. In an isotropic linearly elastic medium, the speed $c_s$ of a travelling plane shear wave is simply
Figure 1.8: a) Maps of tissue displacements in a breast during supersonic shear imaging. b) The displacement as a function of time seen at points A, B and C. It is from these curves that the local shear wave speed is calculated. From Tanter et al. [39].

\[ c_s = \sqrt{\frac{\mu}{\rho}} \]  

(1.8)

where \( \mu \) is the shear modulus and \( \rho \) is the density of the medium, taken to be that of water.

As a final note, the shear modulus is often then converted into a Young’s modulus in order to match some other methods of elastography, methods for which the Young’s modulus is the quantity that is really being measured. For a nearly incompressible isotropic soft solid, the Young’s modulus \( E \) can be calculated from the shear modulus according to

\[ E \approx 3\mu \]  

(1.9)

The scanner used in experiments in this thesis displays this calculated Young’s modulus, though throughout this thesis we will instead quote the shear modulus, as that is closer to what is really being measured.

1.2.6 Competing elastographic techniques

Supersonic shear imaging is not the only method used to measure the mechanical properties of tissue. I will include an overview of other selected prominent elastography techniques, including their advantages and limitations. We will focus on quasistatic elastography, magnetic resonance elastography, acoustic radiation force impulse imaging and shear wave elasticity imaging, and we will include a brief survey of selected other techniques.
1.2.6.1 Quasistatic elastography

Quasistatic elastography is a pioneering elastography technique. In this technique, an external compression is applied to tissue, and the ultrasound images before and after the compression are compared. The areas of the image that are least deformed are the ones that are the stiffest, while the most deformed areas are the least stiff [41]. More specifically, the A-lines that constitute the image from before the compression are cross-correlated with the A-lines after compression; the cross-correlation gives the displacement at each depth of tissue, and displacement and depth together give the strain. Generally, what is given is a relative strain image, which is often of clinical utility [26].

From the relative strain image, however, making a quantitative stiffness map is often desired. To do this requires that assumptions be made about the nature of the soft tissue being imaged and about the boundary conditions, since the stress at the surface is known, but the stress at the deepest part of the image is not. Additionally, under compression, objects can move into or out of the plane, or in the azimuthal direction, causing problems with interpretation. Another limit of this technique is that it has difficulty with organs or tissues that are not close to the surface or easily compressed [42].

1.2.6.2 Magnetic resonance elastography

Magnetic resonance elastography (MRE) is a major player in elastography, and magnetic resonance imaging techniques are the only ones in serious competition with ultrasound elastography [26]. Though there are methods that function differently, most MR elastography is done by mechanically inducing shear waves in a patient, imaging those shear waves, and inferring the shear modulus. The shear waves are created by a mechanical actuator on the surface of the patient which vibrates at frequencies on the order of 50-1000 Hz [43].

A phase-contrast method is used to determine the displacements of the induced harmonic shear waves. More specifically, a motion-sensitizing gradient is used to measure the motion in each direction at a given frequency. The harmonically vibrating tissue accumulates a phase that is directly proportional to its displacement, giving a map of the displacement throughout the field of view [44]. By repeating this process three times, with the motion-sensitizing gradient in each of the three spatial directions, a three-dimensional map of the displacement field can be obtained. From the map of the displacement, the shear modulus is obtained either by estimating the wavelength and therefore the wave speed, or by solving the wave equation directly. An MR elastogram can be seen in Figure 1.9.

One strength of MR elastography is the resulting 3D elasticity map, which can cover an entire organ [43]. While the field of view in ultrasound elastography is constrained by where acoustic windows are available, the field of view in MR elastography can be freely oriented [45]. It also has the advantage of being less dependent on operator skill than most methods of ultrasound elastography.
Figure 1.9: Magnetic resonance elastography in a healthy liver (top) and cirrhotic liver (bottom). (a) and (d) show conventional MR magnitude images and no difference between the two. (b) and (e) show images of the waves during acquisition; the longer wavelength in (e) is reflective of stiffer tissue. (c) and (f) show the corresponding maps of the shear moduli; the cirrhotic liver is an order of magnitude stiffer. From Mariappan et al. [44].

MR elastography suffers from long acquisition times, in the neighbourhood of 15 minutes per direction. This makes it time-consuming, and also impractical for targets that undergo motion or are close to other tissues that undergo motion. Also, because the shear waves are induced on the surface, they can undergo very profound attenuation by the time they reach deeper tissues; this results in a greatly reduced signal to noise ratio, necessitating that lower frequencies, and thus lower resolutions, be used for deep tissues. The shear waves induced by the actuator are further limited in their propagation by bone, air and interfaces, as is the case with ultrasound’s limitations. MR imaging is also more expensive than ultrasound and less convenient for patients and physicians [26].

1.2.6.3 Acoustic radiation force impulse (ARFI) imaging

Acoustic radiation force impulse (ARFI) imaging is another ultrasound-based elastographic technique. It uses acoustic radiation force to push a small region of tissue, and monitors the response of the tissue to the push using ultrasound. The mechanical properties of the tissue will change how it responds to the push, and the restoring force and the displacements achieved vary with tissue stiffness. Like supersonic shear imaging, it uses a focused acoustic radiation force to create a displacement in tissue. Unlike supersonic shear imaging, however, ARFI looks at the displacements
along the axis of the pushing beam rather than the effect of waves in off-axis locations.

More specifically, one vertical line (A-line) of the medium is acquired for reference. Subsequently, a pushing pulse is applied along the same axis, inducing a displacement [26]. A series of tracking A-lines are then acquired from within the excited region; from these data, a number of different parameters can be used to characterize tissue response. These include the peak displacement of the tissue achieved, the time to achieve the peak displacement and the time for the tissue to return to equilibrium. Softer tissues will in general achieve a greater peak displacement, take longer to reach that displacement and take longer to reach equilibrium [36]. The peak displacement is the parameter most commonly displayed in images [43]. To achieve a full image, the sequence described above can be translated to multiple lateral locations throughout the image, building up a full elasticity map in much the same way as a B-mode ultrasound image is built up from individual A-lines [36].

Because ARFI applies the radiation force as a body force in the region of interest, it does not suffer greatly from concerns about forces induced on the surface effectively propagating to the region of interest. Indeed, wherever ultrasound can be focused well enough to generate the pushing pulse, ARFI can be used [42]. One of ARFI’s drawbacks, however, is that it is a qualitative, not quantitative imaging method, and displays only relative stiffness of tissue in the region of interest. Aside from the problem that this makes comparison with reference values impossible, it also means that the apparent stiffness of a lesion will depend on the health and stiffness of the surrounding background tissue. On the other hand, as the data do not require as much processing as quantitative imaging methods, these images can generally achieve higher spatial resolutions without suffering as greatly from SNR concerns as quantitative methods [46].

A further concern of ARFI imaging is tissue heating. Because ARF is a relatively weak effect, it necessitates high intensities and thus high heating [42]. For each application of the radiation force, the temperature increase can be as high as 0.2 °C, and a full image requires many such applications [36].

1.2.6.4 Shear wave elasticity imaging (SWEI)

Shear wave elasticity imaging (SWEI) [47] is a name given to ultrasound elastography methods that induce a shear wave with acoustic radiation force and track its arrival times at lateral positions, thereby inferring wave speed and tissue stiffness. It is substantially similar to supersonic shear imaging, which could be seen as a subset of SWEI, though the two are generally treated as separate. Notable differences from SSI include the use of only one shear wave source at a time rather than multiple, the tracking of only a few lateral locations (∼5), and that the result from a single acquisition is only one elasticity value or several in a horizontal line, rather than a real time two-dimensional map of elasticity values. Because much of its development was done by the same group developing ARFI, it is sometimes grouped with ARFI or considered a form of ARFI, even being referred to as
Chapter 1. Introduction

1.2.6.5 Other elastographic techniques

There are many other elastographic techniques in addition to these methods, many of which have substantial similarities to others. In sonoelastography, harmonic shear waves at slightly different frequencies are mechanically generated and the interference pattern at the beat frequency is measured using ultrasound [48]. In vibro-acoustography, two ultrasound beams of slightly different frequencies are focused on the same spot, causing the tissue to vibrate at the beat frequency; the acoustic response is reflective of the stiffness and detected by a hydrophone [49]. In transient elastography, ultrasound is used to track a transient (single cycle) mechanically induced shear wave [50]. Endogenous motion imaging is a family of methods which image displacements or waves caused by motion endogenous to the body, such as the heartbeat or pulsatility in blood vessels [43]. Ultimately, each method is characterized by how it disturbs the tissue, how it detects the disturbance, what is shown in the image, the imaging modality used, and perhaps a handful of other parameters to distinguish otherwise similar imaging methods. There is much overlap among different imaging methods in terms of which of these aspects are used.

1.3 Thesis outline

In a solid tumour, there is a proliferation of blood vessels that are leaky and permeable compared to vessels in healthy tissue. This allows high-pressure blood plasma to leak out into the interstitial space, which raises the interstitial fluid pressure. This increased fluid pressure might make the tumour stiffer.

Anti-angiogenic cancer therapies are known to reduce a tumour’s interstitial pressure. They are expensive and have toxic side-effects, however, so it is important to know as soon as possible whether or not they are working as desired. Changes in fluid pressure occur on the order of days, and they could be used as an early-response marker of therapy effectiveness. If interstitial pressure changes stiffness, then stiffness measurements might be able to determine changes in interstitial fluid pressure noninvasively. The first overall aim of this work is to determine if tumour stiffness can be used as a surrogate biomarker to monitor the effectiveness of anti-angiogenic therapies. The second overall aim is to determine the contribution of interstitial pressure to tumour stiffness, which will aid in interpreting elastographic measurements of tumours in general.

Considering the mechanical properties of cancer is nonetheless a complex problem, as cancer is a complex disease. Cancerous tissue is heterogeneous spatially, temporally, and across different instances of the disease. Following anti-angiogenic therapy, the interstitial pressure of the tissue will likely decrease, but a number of other changes to the tissue will also take place, including vascular
remodelling, hypoxia and apoptosis of some cells, each of which could contribute to changes in the tumour's elastic properties. To better understand how a change in interstitial pressure specifically could affect the elastic properties of tissue, in this work three specific aims are set out, each of which is to determine the behaviour of a simpler system with fewer variables in order to gain increased insight into the problem.

The first specific aim of this thesis is to determine how the shear modulus depends on hydrostatic pressure, stress and strain in a viscoelastic soft solid; these experiments are found in Chapter 2. The phantoms are subjected to increasing hydrostatic pressure, to uniaxial applied stresses and distortions, and to uniaxial applied stresses in the absence of distortions; the changes in shear modulus are observed. The second specific aim is to determine how shear modulus depends on internal fluid pressure in \textit{ex vivo} tissue. In Chapter 3, the hydrostatic pressure \textit{ex vivo} kidneys’ vasculature is increased, and the resulting changes in shear modulus are compared to the changes in pressure. The third specific aim is to determine how shear modulus depends on interstitial pressure in \textit{in vivo} tumours. Chapter 4 contains preliminary work investigating changes in shear modulus with interstitial pressure in a VX2 tumour implanted in a rabbit thigh. The shear modulus is compared to interstitial pressure locally, as well as being compared over time following a drop in interstitial pressure with sacrifice, and local comparisons before and after sacrifice are made. Chapter 5 contains a summary of the results of the thesis, and a discussion of future directions and the challenges and potential for investigating tumour pressure, response and status with ultrasound elastography.
Chapter 2

Stiffness changes in gelatin phantoms under pressure and compression

2.1 Introduction

Before investigating what change in stiffness will come with a change in interstitial pressure, it is important to first ask what change would be expected. Unfortunately, the question of how elasticity changes with interstitial pressure in a tumour in vivo is quite complex from a physical standpoint. An investigation into the basic physics of tissue and how it changes with pressure is complicated by the fact that physically characterizing the mechanical properties of “tissue” is difficult, as “tissue” is anisotropic, heterogeneous, and different between different individuals or even different sites within an individual. For a first approach, a simpler model is used.

Because tissue, including tumour tissue, is a viscoelastic medium, a model used to imitate tissue should also be viscoelastic. To this end, gelatin was chosen, as it is known to be viscoelastic and suitable for use with ultrasound, including elastographic experiments, provided an acoustic scatterer is added. Gelatin is primarily composed of water, and is therefore nearly incompressible, which are two further properties it shares with tissue. Of course, it has a number of important distinctions from tissue. It is homogeneous and isotropic, and its mechanical properties are relatively reproducible and easy to characterize. Though these are important qualities in a simple model, the distinctions will of course somewhat limit the applicability of the results of the gelatin experiments to tumours and other tissue.

The tumour microenvironment is known to contain anisotropic stresses; these stresses arise from a combination of cell proliferation at the centre of the tumour, causing it to grow and be packed more densely, and distortion and stretching of the periphery of the tumour to accommodate this growth and stress. Stylianopoulos et al. [51],[52] investigated the distribution of residual stresses in spherical
tumours with a combination of experiment and computer modelling. They also found that depletion of cancer cells following a therapy will cause the solid stresses to decrease \[51\]. Sarntinoranont et al. \[53\] identify IFP as a factor contributing to the solid stresses in spherical tumours. Gennisson et al. \[54\] showed anisotropic changes in stiffness under uniaxial compression of viscoelastic media. In terms of direct pressure dependence, Rotemberg et al. \[28\], \[29\] demonstrated that stiffness changes from pressurization occur in \textit{ex vivo} canine livers, but only if the livers are allowed to expand.

In this section, we consider how the stiffness of gelatin phantoms changes with hydrostatic pressure, how stiffness changes with stress, the anisotropy of this change, and whether it is stress or strain that is responsible for any changes in stiffness seen.

### 2.1.1 Theoretical considerations

As a water-based soft solid, gelatin is essentially incompressible, inheriting the bulk modulus of water. At 2 GPa, the bulk modulus is six orders of magnitude higher than the Young’s or shear moduli. The highest stresses or pressures in this experiment are on the order of 10 kPa (i.e. 75 mmHg), meaning the volume of the gel under bulk compression changes only a negligibly small amount.

Gennisson et al. \[54\] derive an equation for a transient shear wave travelling through an incompressible soft solid under uniaxial stress. The total displacement \(u\) of an acoustic particle is broken down into the displacement due to the static stress and the displacement due to the transient (dynamic) travel of the wave:

\[
\vec{u}^{TOT} = \vec{u}^D + \vec{u}^S
\]  

The wave equation is then written

\[
\rho_0 \frac{\partial^2 u_i^D}{\partial t^2} = \mu \left( \frac{\partial^2 u_i^D}{\partial a_k^2} + \frac{\partial^2 u_k^D}{\partial a_i \partial a_k} \right) + \frac{A}{4} \left( \frac{\partial^2 u_k^D}{\partial a_i \partial a_l} \frac{\partial u_i^S}{\partial a_l} + \frac{\partial u_k^S}{\partial a_i} \frac{\partial^2 u_i^D}{\partial a_l \partial a_k} + \frac{\partial u_k^D}{\partial a_i} \frac{\partial^2 u_i^S}{\partial a_l \partial a_k} + \frac{\partial^2 u_k^D}{\partial a_i \partial a_k} \frac{\partial u_i^S}{\partial a_l} + 2 \frac{\partial u_k^S}{\partial a_i} \frac{\partial^2 u_i^D}{\partial a_k \partial a_l} \right)
\]  

where \(\rho_0\) is the equilibrium density, \(t\) is time, \(\mu\) is the shear modulus of the \textit{unstressed} medium, \(A\) is a property of the material (specifically the third order elastic modulus), and \(a\) is the position of the acoustic particle at equilibrium. The quantity \(\frac{\partial^2 u_i^D}{\partial t^2}\) on the left side of the equation is the square of the speed of the shear wave. Given that in supersonic shear imaging, the shear modulus is calculated according to Equation 1.8, the quantity \(\rho_0 \frac{\partial^2 u_i^D}{\partial t^2}\) on the left side can be considered the effective shear modulus, and that practice is followed here, with the ‘effective’ descriptor dropped for brevity.

Consider the case of a medium under stress that does not undergo strain. In the absence of
strain, there will be no distortion, meaning that the static displacement $\mathbf{u}^S$ will be 0 throughout the medium, making all derivatives of it also 0, and reducing Equation 2.2 to

$$\rho_0 \frac{\partial^2 u_j^D}{\partial t^2} = \mu \left( \frac{\partial^2 u_j^D}{\partial u_k^D} + \frac{\partial^2 u_k^D}{\partial a_i \partial a_k} \right)$$  \hspace{1cm} (2.3)$$

In the case of a linearly polarized plane shear wave, coordinates can be chosen such that $i$ is the direction of polarization of the shear wave. This means $u_k^D$ is 0 unless $k = i$. Because in a shear wave polarization is perpendicular to wave travel, $\frac{\partial u_k^D}{\partial a_i}$ is 0. This implies that the rightmost term in Equation 2.3 is 0, and that Equation 2.2 reduces to

$$\rho_0 \frac{\partial^2 u_j^D}{\partial t^2} = \mu \frac{\partial^2 u_i^D}{\partial a_k^2}$$ \hspace{1cm} (2.4)$$

which is simply the wave equation for an unstressed medium. This implies travel of a shear wave in a soft solid under stress but without strain is the same as in an unstressed medium, and the shear modulus should show no dependence on stress at all.

2.2 Methods

2.2.1 Introduction

2.2.1.1 Pressure chamber experiment

Perhaps the most natural question with which to begin is the question of how a viscoelastic medium behaves when subjected to a hydrostatic pressure. To answer this question, an experiment was devised whereby a slab of gelatin was placed in an acoustically transparent chamber. The gelatin was surrounded by water, and the pressure of the water in the chamber could be changed by applying an external pressure. The shear modulus was measured with supersonic shear imaging and compared to the applied pressure.

2.2.1.2 Gelatin compression experiment

In addition to considering how gelatin behaves under isotropic hydrostatic pressure, we considered how a soft solid behaves when there is anisotropic stress on it. In order to test the effect of anisotropic stresses on stiffness, cylindrical gelatin phantoms were subjected to a uniform applied stress along a single axis. As this results in an anisotropy of the stresses in the medium, the shear modulus was measured in the three physically different resulting planes, similar to the experiment that was performed on different viscoelastic media by Gennisson et al. [54]. To differentiate the effect of the applied stress from the strain resulting from it, we compare a ‘constrained’ case where the phantom was encased in a mould and unable to expand laterally to an ‘unconstrained’ case where the phantom
was free to deform under stress. The constrained case is physically similar to the pressurization experiment, but is included here for the purposes of direct comparison with the unconstrained case.

2.2.1.3 Temperature control experiment

In preparation for early versions of the experiments described in Sections 2.2.1.1 and 2.2.1.2, it was remarked that the stiffness of the gelatin patties would change substantially over time following their transfer from the refrigerator to a warm water bath. This was responsible for undesired variability in the stiffness of the patties without changes in stress or pressure, and this variability needed to be understood and eliminated. It was hypothesized that this change was due to temperature change, or perhaps due to water absorption. An experiment was therefore devised to test the temperature dependence of the shear modulus of gelatin phantoms, while a second was performed to rule out water absorption as a cause.

2.2.2 Apparatus

2.2.2.1 The pressure chamber

The pressurization experiment used a small chamber designed to withstand high pressures, yet be transparent to ultrasound, see Figure 2.1. The chamber, described in Tremblay-Darveau et al. [55], was made on a square acrylic frame with two ports on one side of the frame. A Mylar window was attached to each side, held in place by square sections of metal and rubber that were bolted to the frame. The tight fit between the rubber, the Mylar acoustic window, and the frame left the chamber watertight, even at pressures of several hundred mmHg. The acoustic window was 3.8 cm × 3.8 cm in size, and the space between the two windows was approximately 1.4 cm.

The chamber features two ports to the outside. For this experiment, one was occluded, and the other connected to a tub of water whose height could be varied to change the applied pressure. The pressure was measured with a manometer (MediaGauge, SSI Technologies, Janesville WI) placed at the same height as the midline of the phantom.

2.2.2.2 Cylinder and piston

To achieve a uniform uniaxial compression in the compression experiments, a cylindrical mould was used. A piston was used to apply the stress to the phantom, serving as a platform for the weights that apply the stress. The piston was same size as the mould and therefore the resulting phantom, allowing the stresses to be uniformly distributed.

An acrylic cylinder of inner diameter 11.34 cm was machined, and a bottom glued on. A piston was machined to a diameter of 11.26 cm, leaving a space between the piston and cylinder of approximately 0.5 mm. Because the acrylic was thick, ultrasound images could not be taken through the
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2.1 Setup of the pressure chamber experiment

Figure 2.1: A diagram of the setup of the pressure chamber experiment. The gelatin phantom was behind an acoustically transparent window, and was imaged with the transducer pointing into the plane of the page.

piston. To allow for imaging of the phantom while the piston was in place, a hole of appropriate size for the transducer used was machined in the piston; see Figure 2.2. The bottom surface of the piston had a thick layer of inflexible Mylar attached to it, forming a window. The window was acoustically transparent, yet did not bend when the piston was pressed strongly against a gelatin sample, ensuring uniform distribution of any forces applied by the piston and window. Further, the window was found to be watertight.

2.2.3 Gelatin preparation

600 mL of deionized water was measured, and 6% gelatin w/v (gel strength 300, Sigma-Aldrich, St. Louis MO) and 0.5% silica w/v (99%, 0.5-10 µm, 80% between 1-5 µm, Sigma-Aldrich, St. Louis MO) were mixed in. A gelatin concentration of 6% was chosen as it was found to have a Young's modulus at room temperature close to 10 kPa, which is the approximate stiffness of healthy liver or kidney tissue [26]. The silica was included as an acoustic scatterer. The mix was heated on a hot plate to 70-80°C while stirring with a magnetic stirrer, or heated in the microwave to approximately 80°C, measured with a thermocouple. After this temperature was reached, the mixture was stirred as it was allowed to cool to about 30°C. It was then poured into the cylindrical mould, resulting in a patty 60 mm in height, and any bubbles were skimmed off the surface. It was covered with Saran wrap to prevent evaporation then put in a fridge at about 5°C overnight to set.

In the case of the compression experiments, care was taken to make sure the surface of the gelatin was parallel to the base of the container. This prevented the phantom from being uneven, which
Figure 2.2: Acrylic piston with 15-4 transducer. A layer of inflexible Mylar covers the hole cut for the piston, providing an acoustic window while making the surface uniform and flat.
would result in an uneven distribution of stresses and strains once under compression.

In the case of the temperature control experiment, the tip of a thermocouple (Type K, glass braid insulation, Omega Engineering, Laval QC) was positioned at the centre of the gel during casting, allowing measurement of the internal temperature of the gel using a multimeter (TrueRMS Supermeter, Omega Engineering, Laval QC).

One or two days later, the gelatin was removed from the fridge. The patty was carefully removed from the cylinder while still cold and stiff, and it was ensured that the cylinder was fully intact and suitable for experiments. After removal, the patty was then restored to the cylinder, and placed in a bath of room temperature water. The structure of the mould ensured that no water would be absorbed or released by the gel into the water bath. It was allowed to warm to room temperature over the course of 16-24 hours. Based on measurements in Section 2.3.3, it had been determined that the gelatin can be considered to be in temperature equilibrium with its water bath at the end of this period. Room temperature was generally about 22°C. In the case of the temperature control experiment, the patty was not removed from the cylinder, nor was it allowed any time to thermally equilibrate with the water before imaging began.

2.2.4 Imaging and analysis

2.2.4.1 Pressurization experiment

For the pressurization experiment, the gel was cut into chunks of an appropriate size to fit in the chamber, and the chamber was closed around the gel. Quantitatively, the gelatin block was cut slightly larger than 1 cm × 3 cm × 3 cm, and this size was chosen such that it fit in the chamber without pressing against any of the walls. This ensured that there were no unnecessary stresses in the gel that could confound the results, that the gel was free to expand or contract, and that the gel was in full contact with the surrounding water everywhere except the bottom face. The gel was slightly denser than water, but very close to neutrally buoyant, so any stresses in the gel due to it supporting its own weight were minimal.

The pressure chamber containing the gel was placed in the same large bath of room temperature water, and the chamber was also filled with water. This served to keep the temperature nearly constant, while also providing a medium that allowed efficient conduction of ultrasound between the transducer and the phantom. The ultrasound system (Aixplorer, SuperSonic Imagine, Aix-en-Provence, France) was set up to image through the acoustic window. The pressure chamber was bolted to the floor of the tank, and the ultrasound transducer used (15-4 linear array) was held in place with a mechanical arm. With both fixed in place, there was little relative motion between the two as the pressure was increased, ensuring an almost unchanging field of view between subsequent frames. To accommodate the range of angles required for imaging, some of which required the
transducer to be submerged in water, a transducer cover was used to protect the transducer.

One of the two ports of the chamber was occluded and the other pressurized with liquid water. The applied pressure ranged from about 0 to about 80 mmHg in intervals of approximately 10 mbar (7 mmHg), and stiffness maps were taken at each applied pressure. Six pieces of gelatin were each imaged from four different angles, each angle probing a different cross-section of the phantom. Shear modulus was not expected to change with angle as the phantom was isotropic.

Stiffness was evaluated on circular ROIs using the built-in software (Q-box). These were 6-8 mm in diameter, and chosen to be large, yet still far enough from the edges of the gel to avoid artifacts. Because none of the position of the gel, the field of view, the location of the stiffness map, or position or size of the region of interest in the stiffness map changed substantially between images, the location measured was identical or almost identical in every image for a given angle in each gelatin phantom.

The shear modulus was plotted against the applied pressure for each angle. To evaluate how the shear modulus changes with pressure, the slope of the data from each angle was calculated by a linear regression. The slopes were then compared, and the mean and median slope of all angles across all gels was calculated.

\subsection*{2.2.4.2 Compression experiment}

The gelatin phantom was imaged in one orientation in the constrained case and in three orientations in the unconstrained case, following Figure 2.3. In the constrained case, the transducer can only be positioned in the ‘top-down’ configuration because the thick acrylic walls of the cylinder are not acoustically transparent. In the unconstrained case, however, the transducer was positioned in planes of three different orientations relative to the gel. The orientations differed physically in the relative orientation of the applied stress, the polarization of the shear wave and the plane in which the shear wave’s travel was monitored. In each case the shear wave was polarized in the direction of travel of the ultrasound, while the shear wave’s travel was tracked in the plane of the image. On imaging, each measurement was taken from three different planes in each of the three configurations at each applied weight.

In the unconstrained case, the gelatin was immersed in water; this was again for reasons of buoyancy, temperature stability, and ultrasound conduction. It also had the advantage of providing a medium through which the ultrasound can travel, and a transducer cover was again used. The phantom or the cylinder containing it was lightly fixed in place to ensure there was no wandering of the field of view during image acquisition. The ultrasound transducer used was also held in place by a mechanical arm, ensuring minimal movement during acquisitions. In the case of the ‘top-down’ orientation, for both the unconstrained and constrained cases, the transducer was suspended over the window, and sat in a depth of water in the window recess in the piston without touching the piston
Figure 2.3: Different configurations under which the gelatin patty was imaged. In the constrained case, a), the transducer was positioned only in the ‘top-down’ configuration, whereby the image was taken through the window in the piston, and the plane of imaging was vertical. In the unconstrained case, the transducer was positioned in b) the ‘horizontal’ configuration, where the plane of the image was parallel to the ground, c) the ‘top-down’ configuration, and d) the ‘vertical’ configuration, where the plane of the image was again vertical, but the image was from the side rather than the top. In each case the shear wave was polarized in the direction of travel of the ultrasound, while the shear wave's travel was tracked in the plane of the image. Photographs of the horizontal and top-down configurations are shown in Figure 2.4.
itself. This ensured that the weight of the transducer did not contribute to the stress experienced by the phantom; see Figure 2.4. Unlike the pressurization experiment, however, due to repeatedly changing the position of the transducer between measurements, the field of view was not exactly the same under different applied stresses, even when the transducer was in the same orientation.

To apply the stress to the phantom, lead weights were set on top of the piston. These were ensured to be balanced such that the stress on the phantom was uniform. In the constrained case, the weight was increased by a several hundred grams at a time, corresponding to several hundred pascals, up to around 7 kg (7 kPa). In the unconstrained case, weight was increased by a few hundred pascals up to just under 2 kPa. Because it was noticed that there was substantial distortion in the patty in the unconstrained case under these amplitudes of stress, experiments on the unconstrained case were also performed with lower stresses; this was in increments of around 100 Pa up to a maximum of around 600 Pa. The constrained case was observed to cause less damage and distortion to the gel even under larger applied stresses, so it was performed before the unconstrained case in every gel.

The 15-4 transducer was again used. On each stiffness map, a circular region of interest (Q-box) was drawn on each image to measure the stiffness. These were 10-15 mm in diameter, and were centred in the stiffness map. The shear modulus was plotted against the applied stress for each angle. As mentioned above, for each configuration of the transducer relative to the gel, three different planes were chosen; all three of these measurements were plotted and used. To evaluate how the shear modulus changes with stress, the slope of the data for each orientation in each experiment
was calculated by a linear regression.

2.2.4.3 Temperature control experiment

In the temperature control experiment, imaging begins right after introduction of the cylinder to the water bath; the experimental setup was shown in Figure 2.5. No water was allowed to enter the cylinder except a 1 cm layer to prevent the gelatin from drying out over the course of the experiment and to aid in acoustic coupling. Imaging was performed in the ‘top-down’ configuration, though without the piston. An imaging plane was chosen such that it was very close to the thermocouple in the gel but did not include the thermocouple itself; this was both to improve the quality of the stiffness map and to avoid heating the thermocouple with the ultrasound waves. A Q-box of 1 cm diameter was drawn in a region of interest at the centre of the field of view. As with the pressurization experiments, neither the field of view nor the Q-box was moved at all over the course of the experiment to minimize variation due to regional variation in gel stiffness. The transducer’s acoustic output was suspended between measurements except for a few seconds before each measurement to avoid heating the medium or the thermocouple. The gelatin was monitored for a period of 7 hours, over which time the centre temperature changed from 7°C to 21°C.
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Figure 2.6: Normalized shear modulus as a function of applied hydrostatic pressure from all 24 fields of view across all 6 gelatin phantoms. The shear moduli have been normalized to the mean from that field of view. The standard error on the mean is shown, though barely visible at this scale.

2.3 Results

2.3.1 Pressurization experiment

The normalized shear modulus as a function of applied hydrostatic pressure from all 24 fields of view across all 6 gelatin phantoms are combined and shown in Figure 2.6. From the data in each field of view in each gel, the slopes were computed and compared. The individual slopes are plotted on Figure 2.7. The slopes were seen to be normally distributed. The mean ± standard error was determined to be \((-4.2 \pm 13.2) \times 10^{-5}\) kPa (shear)/mmHg. For comparison with later data, the median and interquartile range were determined to be \(-7.9 \times 10^{-5}\) and \(54.4 \times 10^{-5}\) kPa (shear)/mmHg, respectively. An unpaired t-test was performed on the slopes, to see if the average slope was statistically significantly different than 0. This returned \(p=0.75\).

An upper bound on the intrinsic variability of the measurements was investigated by computing the coefficient of variation of the shear modulus at all pressures in each field of view. The mean coefficient of variation was 0.7%.

2.3.2 Compression experiments

The data from a typical gelatin phantom are shown in Figure 2.8. From the linear regression on each case in each gel, the slopes were recorded. These were compared across the different cases, and the median and interquartile range of the slopes in each case were computed. The results are shown
Figure 2.7: Slopes of the change in shear modulus with applied hydrostatic pressure in 24 gelatin phantom cross-sections. The dashed line marks a slope of 0. Note that the vertical axis is in Pa/mmHg, not kPa/mmHg.

<table>
<thead>
<tr>
<th>Case</th>
<th>n</th>
<th>Slope median (interquartile range) [kPa (shear)/kPa (applied)]</th>
<th>Slope median (interquartile range) [kPa (shear)/mmHg equivalent]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horizontal</td>
<td>8</td>
<td>0.81 (0.15)</td>
<td>0.11 (0.02)</td>
</tr>
<tr>
<td>Vertical</td>
<td>8</td>
<td>-0.63 (0.18)</td>
<td>-0.08 (0.02)</td>
</tr>
<tr>
<td>Top-down</td>
<td>9</td>
<td>0.34 (0.15)</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>Constrained</td>
<td>4</td>
<td>-0.003 (0.006)</td>
<td>-0.0004 (0.0008)</td>
</tr>
<tr>
<td>Pressurized</td>
<td>24</td>
<td>-0.0006 (0.0041)</td>
<td>-0.00008 (0.00054)</td>
</tr>
</tbody>
</table>

Table 2.1: The median and interquartile range of the slopes from gels under uniaxial stress in 4 different cases. The first column shows the median slope and interquartile range in units of kPa of shear modulus per kPa of applied stress. The second column shows the slopes with the applied stress converted to the equivalent value in mmHg. The 'pressurized' case is that computed in Section 2.3.1 and is shown for reference. These results are shown graphically in Figure 2.9.

in Figure 2.9 and Table 2.1. The results from compressions at low stresses were not found to be substantially different to those at normal stresses, so they have been combined.

An unpaired t-test was performed on the slopes in the constrained case to see if the average slope is statistically significantly different than 0. This returned $p=0.82$. An upper bound on the intrinsic variability of the measurements was investigated by computing the coefficient of variation of the shear modulus in each gel, again in the constrained case. The mean coefficient of variation was 1.4%.
Figure 2.8: The relationship between shear modulus and applied uniaxial stress viewed in three different orientations in a gelatin phantom. Top - unconstrained only. Bottom - constrained also included; note the change in horizontal axis.
Figure 2.9: The slopes from gels under uniaxial stress in 4 different cases. The ‘pressurized’ case is that computed in Section 2.3.1 and is shown for reference. These results are shown numerically in Table 2.1. Note that the amplitude and confidence intervals of the constrained and pressurized cases are small enough that the full forms of the boxes and whiskers are not visible at this scale.

2.3.3 Temperature control experiment

Figure 2.10a shows the changes in the internal temperature of the gel and the changes in shear modulus in the gel with time. It was remarked that each of the two resembles an exponential return to some asymptotic equilibrium value. The shear modulus and the temperature of the gels were then compared directly; see Figure 2.10b. The shear modulus of the gel appears to decrease with temperature in an approximately linear fashion, though with a shoulder around 15°C.

Two separate slopes were calculated on the data - one for points below 15°C, one for points above 15°C. In the region below 15°C, the shear modulus was found to decrease by 0.32 kPa/°C, while in the region above 15 °C, shear modulus was found to decrease by 0.74 kPa/°C; these fits are shown in Figure 2.10b.

To determine if water absorption and thus change in volume was responsible for the stiffness change, the height of the patty was also measured before and after. The change in height, if there was any, was observed to be less than 1 mm in a patty 62 mm tall. This represents a maximum potential strain of less than 2%. A separate experiment on gelatin patties after several hours fully immersed in water with which they were in thermal equilibrium showed that each dimension changed by at most 1%, and that the total mass of the patty increased by less than 3%. No changes in stiffness were observed.
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Figure 2.10: (a) Change in centre temperature and shear modulus over time in a gelatin phantom during temperature equilibration. (b) Shear modulus plotted directly against temperature. Lines fit to the data above and below 15°C are shown.

2.4 Discussion

2.4.1 Pressurization experiment

The mean coefficient of variation of the stiffness in this experiment was 0.7%. This means that the intrinsic variability of the measurements in this experiment was small.

An unpaired t-test was performed on the set of all the slopes found to see if the average slope is statistically significantly different than 0. This returned $p=0.75$, implying the null hypothesis is reasonable in this case. Even if the null hypothesis is false, it should be emphasized just how small the average slope really is. As was perhaps visible in Figure 2.6, the changes in each case with pressure are very very slight. Note that although Figure 2.7 appears to show a large variation in the slopes, this is because the axes have been set to show the differences in the data acquired, and these differences are themselves quite small. The average slope, with a change in shear modulus with pressure of $-4.2 \times 10^{-5}$ kPa/mmHg, works out to a change of only 0.0004 kPa over a 10 mmHg change in pressure, the clinically relevant pressure range as described in Section 1.1.4. Even over 100 mmHg, ten times the clinically relevant range, this would give a change in shear modulus of only 0.004 kPa, or a change in “Young’s modulus” of just over 0.01 kPa. Given that the scanner used only displays the Young’s modulus in increments of 0.1 kPa, even over such a large pressure range the change in stiffness due to pressure would likely not be detectable even under ideal conditions.

In short, it is likely that there is no change in shear modulus with external hydrostatic pressure in gelatin phantoms, and if there is any change, it is negligibly small.

Gelatin essentially consists of a network of modified collagen fibres and of water. Given that the gelatin used here was 94% water by mass, it was hypothesized prior to the experiment that the
high-pressure water might infiltrate the gelatin and cause it to swell, or perhaps that under stress, the fibrous network of the gelatin might shrink, expelling some water. Neither of these occurred, however, and the gelatin phantoms remained the same size. The gelatin behaved as a soft solid, and its interactions with the water around it appear to be limited to mechanical ones. As described in Section 2.1.1, the pressures in this experiment should induce only a negligibly small strain. With this in view, the prediction made Section 2.1.1 that shear modulus will change under stress if there is no resulting strain explains the data here quite well, even though the circumstances here are slightly different.

Applying a hydrostatic pressure to a block of gelatin showed how a water-based soft solid's shear modulus changes with an external hydrostatic pressure. Though this gives some insight into how tissue might behave, it is also disanalogous in a few important respects. In tissue, the interstitial fluid pressure effectively comes from the inside, not the outside. To whatever extent there are soft solids in tissue to which the interstitial pressure can be considered external, for example cells or patches of tissue, these have a lengthscale closer to the order of microns than centimetres. This experiment also fails to capture whatever complex interplay there is between anisotropic pressures, media and distortions.

2.4.2 Compression experiments

The mean coefficient of variation of the stiffness in this experiment was only 1.4%. This means that the intrinsic variability of the measurements in this experiment was small.

2.4.2.1 Unconstrained cases

The horizontal case shows the largest increase in shear modulus with applied stress, while the top-down case shows a change about half that seen in the horizontal case. For the vertical case, the magnitude of the change is slightly smaller than that seen in the horizontal case, though it is opposite in sign.

The results of the unconstrained case can be compared with those found in the study by Gennisson et al. [54]. Though Gennisson et al.'s phantoms were agar-gelatin instead of gelatin (and silica), and had higher initial shear moduli than those in this experiment, there were many similarities in the results obtained. In most of his phantoms, slopes from the horizontal and vertical configurations were roughly equal in size to one-another, but opposite in sign, with the vertical case showing a decrease in shear modulus with stress and the horizontal case showing an increase. For the softest phantoms, the magnitudes of these were generally within a factor of two of those found here. The top-down case, however, was found to have a slope that was sometimes positive and sometimes negative, and almost always smaller than the other two. Considering the difference between the materials considered in this study and in that done by Gennisson et al., the results can be considered to be in
It is perhaps counterintuitive that in the vertical direction, the shear modulus decreases as the applied stress increases. While Gennisson et al. [54] give a mathematical explanation for this, they offer little in the way of qualitative explanation. One possibility is that the decrease is due to the fibres being relieved of tension or even buckling under the vertical strain, lowering the medium's resistance to shear strain in this direction. A similar phenomenon has been observed in coated bubbles, described by Marmottant et al. [56].

Let us now consider the magnitude of the effect seen. One way to consider the size of the effect is to convert the applied stress in kPa to the equivalent hydrostatic pressure that would exert such a stress if applied to one face of the gel. If we consider the clinically relevant pressure change of 10 mmHg, the difference in shear modulus for the horizontal case corresponds to a change in shear modulus of 1.2 kPa. This is a change of 30% compared to the initial stiffness, and a difference of shear modulus of 30% should be large enough to pick out. On the other hand, if the absolute differences turn out to be more relevant, then an absolute difference of only 1.2 kPa of shear modulus over 10 mmHg might be difficult to detect in a tumour with a shear stiffness of, say, 20 kPa.

Stylianopoulos et al. investigated the distribution of residual stresses in spherical tumours with a combination of experiment and computer modelling [51], [52]. These stresses arise from a combination of cell proliferation at the centre of the tumour, causing it to grow and be packed more densely, and the periphery of the tumour distorting and stretching to accommodate this growth and stress. They determined that the tumour tissue would be under a compressive radial stress on the order of 20 kPa throughout the tumour. The tissue would also be under a compressive circumferential stress in the interior of the tumour, again around 20 kPa, while the stretched tissue at the periphery would be under a tensile circumferential stress on the order of 50 kPa or perhaps more.

The situation on the tumour periphery, where the stresses are anisotropic, could be considered partially analogous to the gelatin compression experiment performed here. If the stresses are relaxed following therapy and/or a relief of interstitial pressure, as mentioned in Section 2.1, it could be that the solid stresses on the periphery would decrease. Given the magnitude of these stresses, even small changes in them could potentially create measurable changes in the shear modulus.

Complicating matters, however, is that not only the magnitude, but also the sign of the change in shear modulus with pressure was found to change with the relative orientation of the transducer and the medium. In imaging the tumour, different sections of the periphery would be in an orientation corresponding to each of the top-down, vertical and horizontal cases, making results more difficult to interpret. The behaviour of the centre of the tumour, which is under compressive stress in all directions, is not predicted by this uniaxial compression experiment. As a further complication, the results quoted above assume spherical tumours, and in tumours with more complicated geometry, the stresses, their changes, and their effects on the shear modulus could be considerably different.
and much more difficult to predict.

2.4.2.2 Constrained case

In terms of the constrained case, the change in shear modulus with applied stress was very, very small. As with the pressurized case in Section 2.3.1, an unpaired t-test was performed on the slopes, to see if the average slope is statistically significantly different than 0. Here it returned \( p=0.82 \), implying the null hypothesis is probable in this case as well. Quantitatively, the median slope in the constrained case, with a change in shear modulus with equivalent pressure of \( -4 \times 10^{-5} \text{kPa/mmHg} \), works out to a change of only 0.004 kPa over the clinically relevant 10 mmHg change in pressure, or a change of 0.04 kPa over 100 mmHg. In this case, as with the pressurized case, it is somewhat likely that there is no change in shear modulus with uniaxial compression of gelatin phantoms when they are not allowed to expand laterally, and if there is any change, it is negligibly small.

Comparing the constrained case and the pressurized case with the unconstrained case, as well as with the theoretical predictions in Section 2.1.1, it can be concluded that this viscoelastic medium must undergo strain in order for its shear modulus to change. This is also in excellent agreement with a study done by Rotemberg et al. [28] where \textit{ex vivo} canine livers were subjected to varying portal venous pressures with both the hepatic vein and hepatic artery closed off, and the stiffness was measured with SWEI. In the case where the livers were allowed to freely expand, the shear modulus substantially increased with applied pressure, but in the case where the livers were unable to expand, the shear modulus remained unchanged even under an applied pressure of over 40 mmHg. In a similar experiment by the same group, canine livers that did not deform under applied portal venous pressure were found to not experience a change in shear modulus, while those found to deform again experienced substantial changes [29].

An implication of this in the scope of the current project is that a change in interstitial pressure following anti-angiogenic therapy in a tumour might or might not result in a relative displacement of part or all of the tumour as solid and fluid stresses relax. If there is no change in shape at all in the tumour, there is no change in strain state, and it would be expected that no change in tumour shear modulus would be seen. On the other hand, if changes in IFP effect changes in strain state, even if small, it is quite possible that changes in tumour shear modulus would be seen. The nature and magnitude of these changes will depend on the specific mechanical properties of the tumour.

2.4.3 Temperature control experiment

At the temperatures at which the other gelatin experiments are performed, the shear modulus was found to decrease by 0.74 kPa/°C, a substantial amount. In particular, it should be noted that for each degree of temperature change, the shear modulus changes by around 20% of its unstressed value at room temperature - a change for each degree comparable in scale to the change seen at the
highest external stresses applied in the unconstrained case in Section 2.3.2. This underscores the importance of temperature equilibrium being maintained throughout the experiment.

2.5 Conclusion

Experiments on gelatin phantoms gave mixed results. The shear modulus of gelatin, a viscoelastic soft solid, was seen to change with a uniaxially applied stress. The changes seen, however, were modest. If in a tumour, the shear modulus changes with pressure by the same fraction of its initial value as was seen in gelatin, changes in stiffness might very well be seen. The actual magnitudes of the slopes, however, at \( \sim 0.8 \text{kPa (shear)/kPa (applied)} \) or \( \sim 0.1 \text{kPa (shear)/mmHg (equivalent)} \) or less was modest, and a change in shear modulus in a tumour might be difficult to detect if changes in stress or strain after therapy in the tumour are small. Further muddying any predictions of stiffness change following tumour response to therapy was the problem of orientation. The change in stiffness in gelatin phantoms had its magnitude and even its sign vary with relative orientation of the transducer and the stresses in the medium. Each of these conditions could be seen in the periphery of a tumour. Compounding this lack of clarity, the centre of the tumour is likely under stresses disanalogous to those considered here.

The shear modulus was seen to exhibit no change with pressure or stress in the absence of strain. This suggests that strain is necessary for changes in stiffness in soft solids. It can be hypothesized that tumours will see no change in shear modulus if no strains in the tumour change. Strains, however, are likely to change in the tumour following a therapy that changes pressures and kills some cells.

As a whole, the experiments on gelatin phantoms establish that shear modulus changes with stresses, and does so anisotropically, but will not change in the absence of strain.
Chapter 3

Stiffness changes of *ex vivo* kidneys under pressurization

3.1 Introduction

Experiments on gelatin phantoms represent a simple physical system that can be described and understood with relative simplicity. As the previous chapter showed, however, there are many limitations in the comparison of compression or pressurization of gelatin phantoms to the changes in interstitial pressure or other possible changes that might result from anti-angiogenic therapy in living tumour tissue. Although it was predicted that changes in tumour stiffness with interstitial pressure could potentially be visible, there are substantial limitations to this, and no clear conclusion was drawn about what changes should be expected in living tumour tissue following a drop in interstitial pressure.

As a result of this ambiguity, we determined that to understand how actual tissue responds to changes in interstitial pressure, it is appropriate to also investigate changes in stiffness with fluid pressure in actual tissue. Gennisson *et al.* [27] studied shear modulus changes in *in vivo* pig kidneys, with two discrete vascular pressurizations and a range of applied pressures in the ureter. As mentioned in Chapter 2, Rotemberg *et al.* [28],[29] studied stiffness changes in *ex vivo* canine livers as a function of pressurization of the portal vein. All found changes in tissue stiffness with various forms of internal fluid pressure.

In the present work, the shear modulus in *ex vivo* porcine kidneys was measured as a function of applied vascular pressure. The kidney is an attractive organ to consider due to the fact that it can be pressurized with relative simplicity. It has only one vessel entering it, the renal artery, and only two tubes exiting it, the renal vein and the ureter. (No kidneys used in this experiment had supernumerary renal arteries.) It also has a solid capsule, providing it with a relatively simple
geometry compared to other organs. This further affords it stability, in that a kidney should retain its shape when removed from the body and placed on a flat surface to a much greater degree than most other organs, limiting unexpected stresses and geometric deformations.

3.2 Methods

Experiments are performed on the kidneys of pigs used for other experiments to reduce the number of animals used.

Before its sacrifice, the pig was given Heparin to ensure that the vasculature was not compromised by blood clots. The kidney was removed as quickly as possible from the pig, and the renal artery was cannulated using a custom-made cannula, and the cannula was securely fastened to the renal artery using surgical sutures. The renal vein and ureter were neither cannulated nor closed off, as fluid flow through them was negligibly small. The kidney was placed in a bath of ice water to slow the death of the tissue, and the blood was flushed out of the kidney using chilled sterile 0.9% saline solution. Great care was taken to ensure that no air bubbles were introduced to the vasculature, as these could prevent effective perfusion. After approximately 1 L of saline has been flushed through the kidney, its colour changed reflecting the fact that the blood has been flushed out.

The kidneys were then placed in the container used for imaging, itself filled with ice-cold saline solution. The container was lined with rubber mats to minimize ultrasound reflections from the edge of the container. To prevent the kidney from moving during imaging (it was approximately neutrally buoyant), it was held in place using small weights.

The cannulated renal artery was now attached to a tub, also filled with ice-cold saline, whose height could be varied to adjust the hydrostatic pressure, similar to the pressure chamber experiment in Section 2.2.2.1. A manometer (MediaGauge, SSI Technologies, Janesville, WI) was attached to this line to measure the pressure. A schematic diagram of the experimental setup can be seen in Figure 3.1. The 15-4 transducer of the Aixplorer ultrasound scanner was fitted with a protective cover, and ultrasound imaging began, including elastography. In terms of orientation, the kidney lay flat on the bottom of the container. Images were taken along what would have been a sagittal plane were the kidney still in the organism; see Figure 3.1.

The first step was to raise the pressure in the kidney relatively quickly, and take sporadic images of this. This was done mainly to ensure that the connection was good and the kidney was pressurizing as expected. The pressure was raised to the maximum value, roughly 35 mmHg.

The next step was to abruptly drop the pressure from the maximum value to a low value, only a few millimeters of mercury. Though the change in pressure as measured by the manometer was nearly instant (on the order of seconds), the stiffness of the kidney took a great deal of time to return to its equilibrium value. The stiffness of the kidney was tracked as a function of time to monitor its
return to the equilibrium value over the course of 30 minutes or more. In order to quantitatively characterize the timescale over which the stiffness decreases, curves of the function

\[ \mu(t) = (\mu_{\text{max}} - \mu_{\text{baseline}}) \exp\left(-\frac{t}{\tau}\right) + \mu_{\text{baseline}} \]  

were fit to the data as the falloff resembles exponential decay. Here \( \mu(t) \) is the shear modulus as a function of time, \( \mu_{\text{max}} \) is the shear modulus at its greatest, \( \mu_{\text{baseline}} \) is the shear modulus at equilibrium (i.e. after a long time), making \( (\mu_{\text{max}} - \mu_{\text{baseline}}) \) the increase in shear modulus over the equilibrium value, and \( \tau \) is a time constant characterizing the decay.

Following this stage of the experiment, the pressure and stiffness of the kidney were taken to be returned to their equilibrium values. From this point, the primary measurement of the kidney’s stiffness as a function of the applied pressure began. Beginning with a baseline pressure of a few mmHg, the stiffness was measured at multiple locations in the kidney’s anatomy, predominantly different areas and orientations of the cortex. Following these measurements, the pressure in the kidney was increased in a step of approximately 2-3 mmHg. It was then allowed to rest for at least 5 minutes to account for the latency between change in applied pressure and change in stiffness demonstrated in Section 3.3.1. After the rest, the stiffness was measured again, the pressure raised again, and the kidney allowed another 5 minutes to rest. This continued until the pressure in the renal artery had reached around 35 mmHg. Analysis of this is detailed in Section 3.3.2.
Chapter 3. Stiffness changes of \textit{ex vivo} kidneys under pressurization

Figure 3.2: The shear modulus in different regions of the cortex of kidney 2 following an abrupt drop in applied pressure from 32 mmHg to 2 mmHg. (Letters do not correspond to the same regions as in Figure 3.4.)

Analysis of the images obtained during the experiment was performed after the experiment. As many circular regions of interest (Q-Box) as possible were drawn in the cortex using the Aixplorer’s software; an example can be seen in Figure 3.4. In kidney #1, this was 7 ROIs and in kidney #2 this was 4. In kidney #3, three parallel sagittal planes were used, each with 7 ROIs, for a total of 21 ROIs. The location of each region of interest remained the same across the range of pressurizations or time of depressurization. For each region of interest in each kidney, the shear modulus was plotted against the applied pressure in the renal artery; see Figure 3.4 for an example. Using linear regression, the slope of each line was calculated. The first point (lowest pressure) was excluded from the calculation of the slope; the rationale for this is explained in Sections 3.4.1 and 3.4.2.

In Section 1.1.4, it was mentioned that following anti-angiogenic therapy, human rectal cancers were found to have their IFP drop from an average of \(\sim15\) mmHg before treatment to an average of \(\sim5\) mmHg afterwards. The median shear moduli of each kidney at each of these pressures was calculated, as was the median difference and fractional difference between the local shear moduli at these pressures.

3.3 Results

3.3.1 Compliance measurements

Figure 3.2 shows the falloff of stiffness in kidney 2 following an abrupt drop in applied pressure from 32 mmHg to 2 mmHg. An initially abrupt drop in stiffness with respect to time decreased with
Chapter 3. Stiffness changes of *ex vivo* kidneys under pressurization

Figure 3.3: The first 10 minutes of the decrease in stiffness in kidney 2 shown in Figure 3.2. The calculated fits have been superimposed on the data.

<table>
<thead>
<tr>
<th>Median time constant $\tau$ (interquartile range) [minutes]</th>
<th>Kidney 1 (n=3)</th>
<th>Kidney 2 (n=5)</th>
<th>Kidney 3 (n=7)</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.8 (2.5)</td>
<td>3.4 (0.8)</td>
<td>5.0 (0.8)</td>
<td>4.6 (1.1)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Time constants $\tau$ of the decrease in shear modulus over time in each kidney. The numbers of regions of interest tracked varied between kidneys.

the stiffness and time. The equation does not perfectly match the observed phenomena, but is a reasonable approximation from which a timescale can be extracted. Fits were constrained to the first 10 minutes of data; this is reasonable as 90% of the decrease in stiffness has occurred by then. Figure 3.3 shows the fits in this period in kidney 2. Table 3.1 shows the mean time constant computed from each kidney and the associated standard deviation across the regions of interest considered.

The intrinsic variability of the measurements was investigated by computing the coefficient of variation of the stiffness in each region of interest in Figure 3.2. Because the change in shear modulus with time from 20 to 60 minutes was small but nonzero, this gives a reasonable upper bound on the variability. The mean coefficient of variation was found to be 2%.

### 3.3.2 Pressurization measurements

The slopes were very good fits to the data (see figure 3.4 for an example): the coefficient of determination $r^2$ was greater than 0.94 for all fits, and 90% of fits had $r^2 > 0.97$. Similarly, $p < 0.001$ for all fits, and 90% had $p < 10^{-6}$. The results were compared within kidneys, and an overall median was computed from the data from all three of the kidneys; these results appear in Table 3.2, while Figure 3.5 shows a box-and-whisker plot of the results from each kidney. Note that different numbers of
Chapter 3. Stiffness changes of *ex vivo* kidneys under pressurization

Figure 3.4: Left: Drawing of the regions of interest in which the Young’s modulus was computed in kidney 2 during pressurization. Multiple regions of interest are drawn based on the B-mode image (bottom) and are used to compute the elasticity map (top). The scale is in cm and the elasticity map is in kPa of Young’s modulus. Right: Shear modulus changes in kidney 2 with pressurization. Lines correspond to regions drawn at left.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Kidney 1</th>
<th>Kidney 2</th>
<th>Kidney 3</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median slope (interquartile range) [kPa (shear)/mmHg]</td>
<td>0.66 (0.14)</td>
<td>0.91 (0.30)</td>
<td>0.64 (0.17)</td>
<td>0.66 (0.17)</td>
</tr>
</tbody>
</table>

Table 3.2: Median values and interquartile ranges of the change in shear modulus with applied pressure (slopes) in each kidney and an overall value from all slopes.

Regions of interest were drawn in each kidney: kidney #1 had 7, kidney #2 had 4, and kidney #3 had 21.

Table 3.3 compares the median shear moduli of each kidney at 5 mmHg and 15 mmHg, as well as the median difference and fractional difference. Figure 3.6 shows the mean shear modulus in each kidney at each pressure, and a fit to those values for comparison.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Kidney 1</th>
<th>Kidney 2</th>
<th>Kidney 3</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear modulus at 5 mmHg [kPa]</td>
<td>3.7 (1.0)</td>
<td>12.1 (5.5)</td>
<td>5.6 (1.3)</td>
<td>5.3 (2.2)</td>
</tr>
<tr>
<td>Shear modulus at 15 mmHg [kPa]</td>
<td>10.0 (1.9)</td>
<td>21.1 (8.5)</td>
<td>12.0 (2.6)</td>
<td>11.8 (3.0)</td>
</tr>
<tr>
<td>Difference [kPa]</td>
<td>6.6 (1.4)</td>
<td>9.1 (3.0)</td>
<td>6.4 (1.7)</td>
<td>6.6 (1.7)</td>
</tr>
<tr>
<td>Fractional difference</td>
<td>2.7 (0.6)</td>
<td>1.7 (0.1)</td>
<td>2.2 (0.3)</td>
<td>2.2 (0.4)</td>
</tr>
</tbody>
</table>

Table 3.3: Comparisons of calculated shear moduli between 5 mmHg and 15 mmHg in each kidney.
Chapter 3. Stiffness changes of *ex vivo* kidneys under pressurization

Figure 3.5: Changes in shear modulus with applied pressure (slopes) in each kidney.

Figure 3.6: Mean shear modulus changes in the renal cortex of three different kidneys with pressurization. The standard deviation between the different regions of interest is shown.
3.4 Discussion

3.4.1 Compliance measurements

As Table 3.1 shows, the time constants were determined to be roughly 5 minutes. Let us now investigate the effect allowing 5 minute intervals between pressurizations. Assuming that the timescales for compliance on pressurization are similar to depressurization, we consider the shear modulus as a function of an ‘effective’ pressure experienced in the kidney, the pressure being a function of time.

In other words, we recast Equation 3.1 as:

\[ \mu(t) = \mu(P_{\text{eff}}(t)) \]  \hspace{1cm} (3.2)

\[ P_{\text{eff}}(t) = P_i + \Delta P(1 - \exp(-\frac{t}{\tau})) \]  \hspace{1cm} (3.3)

where \( P_{\text{eff}}(t) \) is the effective pressurization, \( P_i \) is the initial pressure and \( \Delta P \) is the change in applied pressure between the two steps. With \( \Delta P \) of 2-3 mmHg, and \( t = \tau = 5 \) minutes, the difference between the effective pressure and the pressure on the manometer for the first increase (second point on graphs like Figure 3.4) is \( \sim 1 \) mmHg from the previous step. For subsequent measurements, the inaccuracy due to the first step will be even less, and can be neglected.

The effect of this is a systematic disparity between the applied pressure and the effective pressure, such that the effective pressure is just over 1 mmHg lower than the applied pressure. This does not apply to measurements done at the first pressure in each kidney, which is part of the reason that the first point was neglected in each fit. The effect of this on the remainder of measurements, such as those shown in Figure 3.4 or Figure 3.6 would simply be to shift all points about 1 mmHg to the left, and the slopes would not be affected.

Based on this, allowing a 5 minute interval between measurements was deemed to be appropriate. Were it shorter, less equilibration of pressure/shear modulus would take place; were it longer, the experiment would take much longer, increasing the extent of the changes that the kidney undergoes after removal from the animal. The 5 minute interval between pressurizations described in Section 3.2 achieves a balance between these factors.

The mean coefficient of variation was found to be 2%, which implies the variability due to the measurement technique in this experiment was small.

3.4.2 Pressurization measurements

The median change in shear modulus with applied hydrostatic pressure was 0.66 kPa (shear)/mmHg; this is a strong dependence of shear modulus on pressure, with large changes in stiffness coming over modest changes in fluid pressure. In particular, this is a change over 5 times larger than the largest
changes in the gelatin phantoms.

Table 3.3 compares the median shear moduli of each kidney over the clinically relevant pressure range. The median difference in shear modulus is 6.6 kPa, which corresponds to a median change that more than doubles the shear modulus. If the tumour behaves like the kidney in response to hydrostatic pressure, such a drop in stiffness should be easily detectable. That being said, it is important that the same location be compared before and after the therapy, otherwise the difference in initial stiffness between the two could mask the overall changes in stiffness following a drop in fluid pressure.

**Comparison with prior work**

In Gennisson *et al.* [27], live pigs were opened such that their kidneys were exposed, allowing easier access for ultrasound imaging. The fluid pressure in the kidney was then modulated in three different ways: the renal artery was occluded, thereby dropping the vascular pressure; the renal vein was occluded, thereby raising the vascular pressure; and the ureter was catheterized and pressurized with serum at discrete pressures ranging from 5 to 40 mmHg.

In the case of occlusion of the renal artery or renal vein, both effect a change in vascular pressure, but the quantitative value of the pressure is not clear, and was not measured. For reference, the mean arterial blood pressure in a pig is around 100 mmHg [57],[58],[59], and this also roughly the pressure in the renal artery [60]. The quantitative pressure in the kidney with the renal artery occluded is likely between the $\sim 15$ mmHg typical of venules and the $0$ mmHg in the right atrium of the heart.

On occlusion of the renal vein, Gennisson *et al.* found the shear moduli to massively increase, the resulting median elasticity ranging from 4 to 6 times that of the unoccluded case. On occlusion of the renal artery, they found the shear moduli to drop, the resulting median elasticity ranging from 0.5 to 0.75 times that of the unoccluded case. Clearly, in both of these cases, there is a noticeable change in stiffness with vascular pressure.

For the pressurization of the ureter, the magnitudes of the shear moduli at varying pressures were in broad agreement with those found in this experiment. In [27], a distinction was made between the outer and inner cortex and between different orientations of the transducer relative to the kidney. Though a similar orientation or location dependence was noticeable in the results of the experiments in this thesis, in this thesis they were not separated for characterization. Table 3.4 shows the change in elastic modulus with applied pressure as found by Gennisson *et al.* Although that experiment varied urinary pressure rather than vascular pressure, the results are nonetheless in good agreement with those found in this study.

Rotemberg *et al.* [28],[29] measured stiffness as a function of portal venous pressure in *ex vivo* canine livers using SWEI. Rotemberg *et al.* quote their results as wave speeds, rather than shear
Table 3.4: Change in shear modulus with urinary pressure in the cortex of in vivo kidneys, from Gennisson et al. [27]. The slopes in kPa (Young’s modulus)/mmHg have been converted into kPa (shear modulus)/mmHg for ease of comparison.

<table>
<thead>
<tr>
<th>Slope [kPa (shear)/mmHg]</th>
<th>Axial outer cortex</th>
<th>Transverse outer cortex</th>
<th>Axial inner cortex</th>
<th>Transverse inner cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.43</td>
<td>0.75</td>
<td>0.52</td>
<td>0.95</td>
</tr>
</tbody>
</table>

moduli, so we will present both the former and an estimate of the latter following Equation 1.8. In
the first of their experiments, the shear wave speed was found to increase from \(~1.5\text{m/s}\) at 0 mmHg
to \(~4\text{ m/s}\) at 45 mmHg, corresponding to an increase from \(~2\text{ kPa}\) to \(~20\text{ kPa}\) of shear modulus.
In the second of their experiments, the shear wave speed was found to increase from 1.5-2 m/s
at 0-5 mmHg to 3.25-3.5 m/s at 20 mmHg in the majority of their specimens, corresponding to
an increase from 2-4 kPa to 11-12 kPa of shear modulus. Each of these corresponds loosely to a
change in shear modulus with pressure of \(~0.5\text{ kPa/mmHg}\). In the first experiment, Rotemberg et
al. estimated a linear fit for the change in shear wave speed with pressure as 0.08 m/s/mmHg.
Note that this would correspond to a quadratic fit to the shear modulus, as the shear modulus is
derived from the square of the shear wave speed in Equation 1.8. A loose approximation of this as a
change in shear modulus with pressure yields \(~0.5\text{ kPa/mmHg}\). These results are slightly smaller,
though similar in magnitude to the results from the in vivo kidneys in this thesis and in Gennisson
et al. Given that the comparison is between different organs from different animals pressurized in
different ways, the agreement between the results here and those in the two studies in canine livers
is reasonably good.

It should be noted that Rotemberg et al. found a minimal stiffness change in the livers over the
first \(~10\text{ mmHg}\) of applied pressure. This could correspond to a preloading effect, where in some soft
solids, the characteristic elastic response only begins after a certain initial stress (preload) is applied
[26],[45]. Any preloading effect in the present experiment was much smaller, only corresponding to
the first step of applied pressure in the kidneys. This concern was part of the reason that the first
point was neglected in each fit; the other reason is due to compliance as explained in Section 3.3.1.

3.5 Conclusion

Though gelatin experiments performed gave no clear prediction of what changes in stiffness with
fluid pressure might be seen in a tumour, the kidney experiments gave much clearer and more
encouraging results. Given that the structure of living tissue is much closer to the structure of
tumours than is a simple block of gelatin, these results should also be given much greater weight
in predicting the behaviour of tumours. Ex vivo tissue was subjected to hydrostatic pressure in the
vasculature, and it underwent notable changes in shear modulus with pressure. The change was
a substantial $\sim 0.7$ kPa (shear)/mmHg, and over the clinically relevant range in pressures, a large difference in stiffness was seen. Although pressurizing the vasculature of an ex vivo kidney is not a perfect analogue to reducing the interstitial pressure of a tumour with therapy, the systems are similar enough and the contribution of pressure change was large enough to be encouraging. In view of the kidney experiments, in addition to similar work done by other groups, it seems likely that stiffness will change with fluid pressure in a tumour as well.
Chapter 4

In Vivo VX2 Tumour Experiments

4.1 Introduction

In this chapter we present a preliminary investigation into the relationship between stiffness and interstitial fluid pressure in a rabbit VX2 tumour model. Compared to the experiment in ex vivo kidneys in Chapter 3, this experiment should give better insight into the dependence of stiffness on IFP in a human tumour for three reasons. First, and most obviously, it is a tumour, rather than a phantom or a different tissue type. Notably, the interstitial pressure should play a substantial role, in view of the large volume occupied by the interstitium shown in Figure 1.2. Second, the tissue is in a living organism and surrounded by other tissues. Given the importance of the tumour’s environment and surroundings to its characteristics, this is an improvement. Third, in this experiment, the interstitial pressure itself is actually measured for the first time, rather than using some other measure of pressurization as an approximation.

The relationship between stiffness and interstitial fluid pressure is investigated in three ways. First, the locally measured stiffness is compared to locally measured interstitial pressure; these measurements explore how local stiffness depends on local IFP. Second, IFP has been shown to drop in tumours following removal of blood pressure [61] and following sacrifice of the animal [62]. Using sacrifice and exsanguination as a method of modulating the IFP, the stiffness in many locations and the IFP in one location are recorded over time following sacrifice of the rabbit and compared. These measurements explore how stiffness in a given region changes with the overall IFP in the tumour over time, where the central IFP is taken as an (imperfect) indicator of IFP change throughout the tumour. Third and last, the stiffness is compared before and after sacrifice. This experiment too is an indicator of how the stiffness changes at each location following a drop in IFP.
4.1.1 A mathematical model of shear modulus and its dependence on pressure and location

In this section, we investigate the mathematical relationships that describe tissue stiffness and the factors on which it depends. By explicitly stating these equations and their underlying assumptions, we are able to better examine the strengths and weaknesses of each of the experimental techniques.

Though this thesis investigates the effect of interstitial fluid pressure on stiffness of biological tissue, fluid pressure is certainly not the only determinant of tissue stiffness. In an organ, the relative local concentration of different tissue and cell types, as well as the mechanical properties of each, will have an effect on the local stiffness. In general, it is expected that the stiffness will vary by location. It will possibly also depend on fluid pressure, but even the nature of this dependence will depend on the local environment. Mathematically, we can cast this as

\[
\mu(r, P(r)) = \mu_0(r) + \Delta \mu(r, P(r))
\]  

(4.1)

where \( \mu(r, P) \) is the local shear modulus of the tissue, \( r \) is the position under consideration, \( P(r) \) is the local fluid pressure, possibly interstitial fluid pressure, \( \mu_0(r) \) is the local shear modulus of the tumour in the absence of any pressure, and \( \Delta \mu(r, P) \) is the change in local shear modulus due to pressure, which is the quantity of interest in this thesis. Though shear modulus very likely depends on other parameters as well, including strain, in this equation those dependences are taken as implicit rather than shown explicitly. Note also that Equation 4.1 is general enough that it should apply to the pressurized gelatin experiments in Chapter 2 and the pressurization of kidneys in Chapter 3 as well.

Ideally, we would be able to vary the local interstitial fluid pressure in tissue in a known and controlled way. From this, \( \Delta \mu(r, P(r)) \) could be determined with relative ease. Indeed, aside from the fact that applied pressure rather than local pressure was known, the experiments on \textit{ex vivo} tissue in Chapter 3 did just that. Unfortunately, there is no clear method to change the local fluid pressure in a tumour in a controlled manner, especially in a manner that is similar enough to actual physiological pressure changes to be clinically relevant. As a result, other means must be investigated to determine \( \Delta \mu(r, P(r)) \).

Consider now the shear modulus of tissue in an animal when sacrificed. The death of an animal is known to change the vascular pressure and interstitial pressure. It may, however, have other effects as well. The mechanical properties will change in reaction to this, and do so as a function of time. Equation 4.1 must now be expanded to

\[
\mu(r, P(r, t), t) = \mu_0(r, t) + \Delta \mu(r, P(r, t), t)
\]  

(4.2)

where \( t \) is the time since death. If we assume that over a timescale of less than an hour, the tissue
does not change enough to see substantial changes in its mechanical properties, aside from those due to pressure, we can recast Equation 4.2 as:

\[ \mu(r, P(r,t)) = \mu_0(r) + \Delta \mu(r, P(r,t)) \]  

(4.3)

In principle, \( \Delta \mu(r, P) \) could be arbitrarily complex. That being said, in this case, earlier experiments can inform our prediction of its behaviour. In the pressurization of kidneys in Chapter 3, shear modulus was found to increase linearly with fluid pressure. This gives us reason to think that \( \mu(P(r)) \) could be linear or reasonably approximated as linear, at least in the range of 0-40 mmHg. If Equations 4.1 and 4.3 are linear, then \( \Delta \mu(r, P(r)) \) could be computed from fitting the data to them.

### 4.1.2 Determining the change in stiffness with pressure from experimental data

In the VX2 tumour experiments, we attempt to determine \( \Delta \mu(r, P(r)) \) in three different ways.

In Section 4.3.2, we compare the local shear modulus and IFP throughout several tumours. For measurement number \( i \), according to Equation 4.1,

\[ \mu(r_i, P(r_i)) = \mu_0(r_i) + \Delta \mu(r_i, P(r_i)) \]  

(4.4)

where \( \mu(r_i, P(r_i)) \) and \( P(r_i) \) are measured. We cannot, therefore, determine \( \mu_0(r_i) \) or \( \Delta \mu(r_i, P(r_i)) \) individually, only their combination. It is hoped that across many measurements, if \( \Delta \mu(r, P(r)) \) is sufficiently large and sufficiently similar across locations, then it will be visible even across varying \( \mu_0(r) \). Of course, if variations in \( \mu_0(r) \) are large, and/or \( \Delta \mu(r, P(r)) \) is dissimilar across locations, then inferring the properties of \( \Delta \mu(P(r)) \) will be quite difficult.

In Section 4.3.3, we compare local stiffness to a local pressure at a different location, and observe their evolution over time. For a stiffness at location \( i \), a pressure reading at location \( j \), and both taken at time \( t_n \), according to Equation 4.3,

\[ \mu(r_i, P(r_i, t_n)) = \mu_0(r_i) + \Delta \mu(r_i, P(r_i, t_n)) \]  

(4.5)

where \( \mu(r_i, P(r_i, t_n)) \) and \( P(r_j, t_n) \) (instead of \( P(r_i, t_n) \)) are measured. Here \( \mu_0(r_i) \) will not vary across the time measurements, according to the assumptions above, though it will vary across the different locations measured. In this case, however, we do not have \( P(r_i, t) \), only \( P(r_j, t) \). It is hoped that \( P(r_j, t) \) will be similar to \( P(r_i, t) \), or more likely that the changes in \( P(r_j) \) with time will be similar to the changes in \( P(r_i) \) with time. Comparing the changes of \( \mu(r_i, P(r_i, t)) \) and \( P(r_j, t) \) with time should give insight into how the two are related.
In Section 4.3.4, we compare local stiffness in many planes before and after sacrifice. Similar to the experiment just described, Equation 4.5 characterizes the measurements taken. The pressure, while still only corresponding to one location, is only known at two times: just before sacrifice and long after sacrifice. The different time points $P(r, t_n)$ are then considered merely different instances of pressure measurements $P_n(r)$, so the dependence on $t$ is dropped. Because this method permits many more imaging planes to be taken, it allows the comparison of many more spatial locations, improving the amount of data used to estimate $\Delta\mu(r, P(r))$ from Equation 4.5.

4.2 Methods

4.2.1 Animal and tumour model

A rabbit VX2 tumour model was chosen for this experiment. Mice and rats were judged to be too small for effective elastography in a system designed for humans, so a rabbit was chosen as the minimum appropriate size. The VX2 is a squamous cell carcinoma in domestic rabbits \[63\], and was chosen for its rapid growth rate and our lab’s prior experience with them.

The tumours used in this experiment were grown from chunks of tumour kept in a freezer at -80°C for about 15 years. A pathologist verified with histology that the tumours grown from this stock were indeed cancers, and not, for example, fibromas.

Six total rabbits (New Zealand white, 3-4.5 kg) were used in this experiment. Two were used only to grow the tumours and propagate the line, and their tumours were frozen and harvested for subsequent injection into other rabbits. The other four were used for the imaging portion of the experiment; the tumours in all four of these were grown from the same single tumour.

For each rabbit, chunks of frozen VX2 tumour were thawed and chopped into a slurry. 1 mL of this VX2 slurry was injected under ultrasound guidance into the rabbit’s right thigh with a 16 gauge needle. Beginning seven days later, the tumour’s growth was monitored with ultrasound every 3 days. A few days after the tumour was found to exceed 1 cm in longest dimension, the main experiment was performed.

For all ultrasound imaging, all fur on the lateral side of the thigh was removed, to ensure good coupling and good image quality. For monitoring, this was achieved with a wet shave, while on the day of the experiment, it was done with a wet shave or with a chemical depilatory.

The rabbit was sedated during injection, monitoring, and imaging. It was induced with ketamine/xylazine (50 mg/kg, 5 mg/kg) or dexmedetomidine. For the main experiment, it was maintained with isoflurane. The rabbit’s condition was monitored with a pulse oximeter. Sacrifice was achieved with euthanyl; this was followed by the severing of one or more major blood vessels.
4.2.2 Interstitial fluid pressure measurements and the wick-in-needle technique

Interstitial fluid pressure was measured using the wick-in-needle technique [25]. A 23-gauge or 25-gauge needle with a side port ground near the tip was used. A "wick" was placed in the distal portion of the needle. The needle was connected to a pressure transducer (Model P23XL, Harvard Instruments) and an electronic data acquisition and recording system (Model MP 100, World Precision Instruments, Sarasota, FL) through 470 mm of polyethylene tubing. The entire system was flushed with a heparin sulphate/saline solution (1:10).

To aid in penetration to the region of interest, a small incision was made in the dermis to allow the needle easier access to the tumour.

4.2.3 Imaging and fluid pressure measurements

On the day of the experiment, many different shear wave maps and interstitial fluid pressure measurements were performed. Because tumour stiffness and IFP both change with the amount of pressure applied to the tissue, (see, for example, Section 4.4.4.1) care was taken to press on the tumour as little as possible. As much acoustic gel as necessary to accomplish this was used.

4.2.3.1 Comparison of local stiffness pre- and post-mortem

The first measurements taken in the tumour were B-mode ultrasound sweeps over the tumour in frontal and transverse planes to give measurements that characterize all cross-sections of the tumor in these planes, and that give an idea of the tumour's three-dimensional layout. These aided in confirming later localization of acquired planes. Following this, stiffness images were taken in at least three frontal sections and at least three transverse sections of the tumour; see Figure 4.1.

The experiments described in Sections 4.2.3.2 and 4.2.3.3 were then performed, including the sacrifice of the animal. The last measurements taken in the animal were another set of stiffness images, taken in the same configuration of planes as above. These images were compared to images taken before sacrifice in the same plane. The pre-sacrifice images used for comparison were not necessarily the earlier images taken in the grid pattern; if images from Section 4.2.3.2 corresponded better to the final geometry they were instead used as the pre-sacrifice reference. For each post-mortem image, only the pre-mortem reference that best corresponded was used. If no pre-sacrifice image was found to have good correspondence, the post-sacrifice image was not used.

Multiple 3 mm diameter regions of interest are compared across the pairs of images, as well as one large region of interest that encompasses as much of the tumour as possible while not including other tissues; the large region of interest was on the order of 10 mm in diameter. As the IFP has been recorded before and after sacrifice, we have an idea of what the change in IFP was between
Figure 4.1: Diagram of the grid of planes taken to characterize the tumour. More than three planes were sometimes taken in each direction. One set was taken at the beginning of the imaging, and one set after sacrifice.

the two cases. These measurements were performed on rabbits 3, 5 and 6.

4.2.3.2 Local IFP and stiffness comparison

Following the initial characterization of the tumour described in Section 4.2.3.1, the interstitial fluid pressure was then measured at various locations in the tumour. The needle was inserted under image guidance to different locations of interest across the volume of the tumour. For each measurement, the needle was inserted, the tip was found with ultrasound, and the plane in which it sits was recorded, as was the IFP. The needle was then retracted slightly, and an ultrasound stiffness image was taken of the plane found earlier. A 3 mm diameter region of interest was centred on the previous location of the needle tip, and the stiffness was measured in this region. 8-15 such measurements were performed in the tumour. These measurements were performed on rabbits 3, 4, 5 and 6.

4.2.3.3 Imaging IFP and stiffness over time following sacrifice

Following the completion of the measurements in Section 4.2.3.2, the rabbit was readied for sacrifice. Just prior to sacrifice, the IFP needle was placed at a fixed location in the tumour, and the IFP began being recorded. A plane containing the tip of the needle was found, and the ultrasound transducer was held in place in this plane with a mechanical arm, ensuring a fixed field of view over the time period.

Both IFP and stiffness were measured for 20-40 minutes after sacrifice. The IFP was recorded every second, while stiffness images are taken approximately once every minute. The stiffness images
are reviewed after the experiment. Several 3 mm diameter regions of interest are chosen, as well as one large region of interest that encompasses as much of the tumour as possible. These regions of interest were drawn in every image, and their stiffness in each image recorded. IFP and stiffness were compared with time after sacrifice. These measurements were performed on rabbits 4, 5 and 6.

4.2.3.4 Evaluation of variability of measurements

To better understand the nature of variations seen in our results, measurements were made to determine the temporal and spatial variability of IFP and stiffness measurements made in tumour tissue and healthy tissue. To determine temporal variability in the absence of deliberate external changes, the experiment described in Section 4.2.3.3 was performed on a tumour for 10 minutes prior to sacrifice. This should also give an upper bound on how much variation arises in the measurements of a single location due to the measurement technique itself. To put the spatial variability seen in tumours in context, the same measurements as performed in Section 4.2.3.2 were performed in one rabbit in a relatively homogeneous tissue, namely contralateral healthy muscle. These measurements were taken within a few centimetres of one-another. This should give insight into whether spatial variation in measurements made in tumours reflects true physical heterogeneity or limitations of the measurement technique.

4.3 Preliminary results

4.3.1 Variations due to the measurement technique

Figure 4.2 shows images from monitoring 8 ROIs in one plane for 10 minutes before sacrifice, as described in Section 4.2.3.4. Figure 4.3 shows the results graphically. It can be seen that changes in shear modulus and interstitial fluid pressure over this time period are relatively small. The standard
deviation of the IFP was found to be 0.9 mmHg, while the mean standard deviation for the small ROIs was 1.2 kPa, and for the large ROIs 0.6 kPa, while the coefficient of variation was 7% for the small ROIs and 3% for large ROIs. (No coefficient of variation for IFP is calculated, as IFP is an interval variable, not a ratio variable.) In addition to showing variability over time without sacrifice, these values give an upper bound on the intrinsic variability of the measurement technique.

The IFP and shear modulus were found in local measurements of these in the contralateral muscle, as described in Section 4.2.3.2; sample images are shown in Figure 4.4, and the results are shown graphically in Figure 4.5. From this data, the standard deviation of the IFP was 2.2 mmHg, while the standard deviation of the shear modulus was 1.8 kPa and the coefficient of variation of the shear modulus was 28%.

4.3.2 Local IFP and stiffness comparison

Figure 4.6 shows sample images used in the measurement of local IFP and local shear modulus in rabbit 6. The results of the comparison of these in all rabbits are shown in Figure 4.7. For rabbit 4, two points with negative IFP have been removed, because there is little reason to think that trends in a positive pressure regime will be the same as in a negative pressure regime.

For the data from each rabbit, a simple linear regression was performed. All of the data from the rabbits were also combined, and a linear regression was performed on this combined data. Slopes ranged from 0.1 kPa (shear)/mmHg to 1.4 kPa (shear)/mmHg, with a median of 0.5 kPa (shear)/mmHg, but the fits to the data were poor; none of the fits had $p < 0.05$, and for all but one, $r^2$ was less than 0.1. For the aggregate fit, the slope was 0.26 kPa (shear)/mmHg, with
Figure 4.4: Sample images from the measurement of IFP and stiffness in muscle in the contralateral thigh of rabbit 6. The top pair of rows show the image wherein the needle’s location during IFP measurement was recorded; the bottom pair of rows show the region where stiffness was measured following the retraction of the needle. Elasticity is given as the Young’s modulus.

Figure 4.5: Variability of IFP and stiffness in muscle in the contralateral thigh of rabbit 6.
Figure 4.6: Sample images from the measurement of local IFP and stiffness in rabbit 6. The top pair of rows show the image where the needle’s location during IFP measurement was recorded; the bottom pair of rows show the region where stiffness was measured following the retraction of the needle. Elasticity is given as the Young’s modulus.
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Figure 4.7: Local measurements of IFP and stiffness in all 4 rabbits.

\[ p = 0.20, \text{ and } r^2 = 0.04. \]

The mean standard deviation of the local IFP in each of the rabbit tumours was 6.7 mmHg, while the mean standard deviation of the shear stiffness at the needle locations was 8.7 kPa and the mean coefficient of variation was 62%.

4.3.3 IFP and stiffness over time following sacrifice

Sample images from monitoring a tumour after sacrifice are shown in Figure 4.8, while sample quantitative results are shown in Figure 4.9. Figure 4.9 shows the evolution of IFP at one location  

Figure 4.8: The tumour in rabbit 5 over 40 minutes post-mortem. Images are 8, 20, 31 and 41 minutes after sacrifice. Elasticity is given as the Young’s modulus.
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Figure 4.9: Post-mortem measurements at 8 locations over time in rabbit 5.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>IFP before sacrifice [mmHg]</th>
<th>IFP after sacrifice [mmHg]</th>
<th>Time after sacrifice [min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>18</td>
<td>-2</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>23</td>
<td>2</td>
<td>40</td>
</tr>
<tr>
<td>6*</td>
<td>~23</td>
<td>~13</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 4.1: Comparisons of IFP before and after sacrifice, and the time after sacrifice when the ‘after’ measurement was taken. *In rabbit 6, the measurement was interrupted and the needle was placed elsewhere, so these do not represent the same location.

and shear modulus at 8 locations in rabbit 5 as a function of time. The same analysis was performed on data from rabbits 4 and 6. In rabbit 6, the needle was found to have clogged (i.e. no longer give IFP measurements reflective of its environment) at some point in the first 15 minutes following sacrifice, so the IFP measurements acquired could not be taken as accurate. As a result, the time series used for its analysis was from 17-26 minutes after sacrifice.

The IFP was found to decrease over time in all 4 rabbits; see Table 4.1. The amount of this decrease varied between rabbits; it was found to be large in rabbits 3 and 5, and more modest in rabbits 4 and 6.

For all of the small ROIs, lines were fit to find the change in shear modulus over time. No clear overall trends were seen; while some regions showed a decrease in stiffness with time, many saw no change, and some saw an increase. Across all 3 rabbits for which data was obtained, the median slope was 0.00 kPa (shear)/min with an interquartile range from -0.16 to +0.13 kPa (shear)/min, a large amount of variation. For the large regions of interest, the slopes are shown in Table 4.2. The decreases in the IFPs over time are of the same order of magnitude.
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<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Slope [kPa (shear)/min]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>-0.082</td>
</tr>
<tr>
<td>5</td>
<td>-0.025</td>
</tr>
<tr>
<td>6</td>
<td>-0.070</td>
</tr>
</tbody>
</table>

Table 4.2: Change of shear modulus over time in large ROIs.

4.3.4 Comparison of local stiffness pre- and post-mortem

Figures 4.10 and 4.11 show sample images of the stiffness before and after sacrifice in tumours in rabbits 5 and 6, while the data is shown graphically in Figure 4.12. Figure 4.13 shows a histogram of the percentage change in stiffness after sacrifice. It can be seen that in a majority of ROIs the stiffness after sacrifice, corresponding to a lower IFP, is lower than the stiffness before sacrifice. Quantitatively, for the small regions of interest, the median stiffness change was a drop of 15% of the initial value; in the larger regions this median drop was 33%. A Wilcoxon signed-rank test was performed on the data aggregated from all 3 rabbits, for both small ROIs and large ROIs. The change was found to be statistically significant in both cases, with p=0.04 in large ROIs, and p=0.004 in small ROIs.

4.4 Discussion

4.4.1 Local IFP and stiffness comparison

The mean standard deviation of the local IFP in each of the rabbit tumours was 6.7 mmHg, while the mean standard deviation of the shear stiffness at the needle locations was 8.7 kPa and the mean coefficient of variation was 62%. These are large compared to the values of 2.2 mmHg, 1.8 kPa and 28% that were found in contralateral muscle. Because the variation in these measurements is so much larger in the tumours, we can conclude that the variation in the data shown in Figure 4.7 is due to true physical heterogeneity of the tumours rather than due to limitations of the measurement technique.

It should be noted that negative IFP measurements in the range found in Figure 4.5 are unusual. The cause for this is unknown, though the measurement system was ensured to be calibrated such that atmospheric pressure was 0 mmHg. Comparable values of IFP were observed in measurements performed in the muscle of rabbit #4 as well. It is possible that these negative values are due to dehydration of the animals, due to hypertonicity of the interstitial fluid, or due to an artifact of negative pressures in the venous system.

It is quite possible that there are problems in co-registration of the ultrasound images with the measurements made with the IFP needle. The volume interrogated by the IFP needle is a few cubic millimetres. The exact lengthscale over which IFP varies in a VX2 tumour is not precisely
Figure 4.10: Sample images from pre- and post-mortem stiffness comparisons in rabbit 5. The top pair of rows show the image taken before sacrifice, while the bottom pair of rows show the image taken after. Elasticity is given as the Young’s modulus.
Figure 4.11: Sample images from pre- and post-mortem stiffness comparisons in rabbit 6. The top pair of rows show the image taken before sacrifice, while the bottom pair of rows show the image taken after. Elasticity is given as the Young’s modulus.

Figure 4.12: Shear modulus before and after sacrifice in (a) small ROIs and (b) large ROIs.
known, though it could be that from one end of the 3 mm ROI to the other, IFP changes by up to ~5 mmHg. This means that errors could arise due to mismatches of IFP measurement location and stiffness measurement location, or due to averaging of stiffness over a volume where the IFP could be varying.

Aside from the basic difficulties in co-registering planes, there was the issue that pushing a needle into tissue was found to move the tissue around and shift the layout of the tissue in the ultrasound image. A perfect registration of the two planes is therefore impossible, as the initial cross-section no longer exists. It was for this reason that stiffness was measured where IFP had been measured rather than vice-versa. Nonetheless, even in this case, some difficulties were found in acquiring the same plane as was found earlier.

As Figure 4.7 shows, if there is any change in stiffness with fluid pressure, the effect is heavily obscured by the large amount of variability in stiffness independent of the fluid pressure, by possible poor co-registration of the location of IFP and stiffness measurements, and by the small number of measurements made. Indeed, it was argued in Section 4.1.2 that under these circumstances, change in stiffness with fluid pressure would be difficult to see.

Let us now consider the possibility that there is an underlying trend. Though none of the fits could exclude the null hypothesis i.e. that there is no trend, the slopes from all rabbits as well as the aggregate were found to be positive. The slopes found were on the same order of magnitude as those found in kidneys, so it is roughly what would be expected based on these previous experiments.

In terms of statistical power, the smallest trend that could have been detected 90% of the time based on 38 measurements would be 0.66 kPa (shear)/mmHg, which is more than double the
slop e of 0.26 kPa (shear)/mmHg found. To create a trial that would detect a difference of this size, i.e. 0.26 kPa (shear)/mmHg with a power of 0.9, 200 more measurements would have to be made. At an optimistic 15 usable measurements per rabbit, this would mean that around 15 rabbits would have to be used. That being said, even this will not address concerns about co-registration problems; if the true change is in the neighbourhood of 0.7 kPa (shear)/mmHg, as was found in the kidney experiments, then co-registration errors and their resulting uncorrelated data could cause the magnitude of the slope and correlation to be underestimated.

Consequently, due to large variability in shear modulus independent of fluid pressure in the tissue and due to concerns about co-registration of fluid pressure measurements and stiffness measurements, it seems more reasonable that a different approach should be taken for investigation of how stiffness depends on interstitial fluid pressure.

4.4.2 IFP and stiffness over time following sacrifice

It is clear that there is heterogeneity in the change in stiffness of the tumour after sacrifice. With the variability in behaviour in small ROIs seen, it appears that conclusions drawn will depend heavily on which particular ROIs are chosen. For this reason, we suggest that the use of a single large ROI encompassing as much of the tumour as possible is more appropriate. Keeping a fixed field of view allows us to eliminate the obscuring of trends by the heterogeneity in local stiffness that afflicted the local IFP and stiffness measurements in Section 4.3.2. This allows us to see trends in local IFP and in both local and more global shear modulus over time.

Unfortunately, due to the need to maintain a constant field of view over time, only one large ROI can be tracked in this experiment, a severe limitation on the amount of data that can be gained in each animal. From this data, we may note that both IFP and shear modulus in large ROIs tended to decrease over time in the three rabbit tumours measured, and that the decrease in shear modulus over time in each was of the same order of magnitude across these rabbits. Still, there is insufficient data to draw more expansive conclusions on the change in stiffness with IFP.

4.4.3 Comparison of local stiffness pre- and post-mortem

Following sacrifice, a significant decrease in stiffness was found in both large ROIs (p=0.04) and small ROIs (p=0.004). Note, however, that only three rabbits were used, and each plane and each ROI does not represent a truly independent measurement. Larger ROIs are again likely better metric, as they include more data and are less sensitive to biasing from the specific locations chosen.

A calculation was performed to estimate the rough change in stiffness with IFP quantitatively. The change in the shear modulus for each of the large ROIs, shown in figure 4.12b, was compared with the IFP changes from Table 4.1 in IFP in each rabbit. The median change in stiffness with fluid pressure was 0.78 kPa (shear)/mmHg, with an interquartile range from -0.05 to 1.40 kPa (shear)/mmHg.
While there is a fair amount of variation here, the median value is remarkably close to the median value found in the kidneys, as well as the values found in [27],[28] and [29]. It is therefore plausible that changes in fluid pressure could be at least partially responsible for the changes in stiffness seen.

Anecdotally, it appears based on smaller ROIs in both pre- and post-mortem comparisons and the time series experiments, that for the stiffest points, with $\mu > 25$ kPa (shear), the shear modulus tends to decrease after sacrifice. For the softest points, with $\mu \sim 5$ kPa (shear), the shear modulus tends to remain unchanged. Values in between can exhibit increases, decreases or no change; see Figures 4.9 and 4.12a for examples. Nevertheless, it must be remembered that this observation is based on only a handful of points in a handful of tumours, and may not hold when more data is acquired.

4.4.4 Challenges and concerns

4.4.4.1 Methodological concerns

There were several issues with the measurement techniques used. One problem is that the stiffness of a tissue depends on how much stress is applied to it by the ultrasound transducer. This reflects the experiments in Section 2.3.2, among others. A pair of images was taken of the same plane of the tumour under two different conditions; see Figure 4.14. In Figure 4.14a, no force at all was applied to the tumour, and the transducer was held just above it. In Figure 4.14b, by contrast, the weight of the transducer and the author’s forearm were rested on the tumour, with his elbow resting on the table; no additional force was applied. The resulting shear modulus was dramatically different, increasing from 13.9 kPa to 19.6 kPa, a 40% increase. The concurrent fluid pressure change was from 11.4 to 18.5 mmHg. With such a large difference in stiffness and pressure arising from small differences in applied force, it is evidently important to minimize this source of error by applying no pressure to the tumour. While efforts were made to minimize the force applied to the tumour, any deviation from this could create substantial undesirable changes in both stiffness and fluid pressure.

At the same time, satisfying this requirement with large, superficial tumours is difficult. With a flat transducer and a curved, bulging tumour, copious quantities of gel were required to create an acoustically conductive layer in the large gap between the transducer and the tumour. With so much gel, there is the increased possibility of inaccuracies in ultrasound transmission or reception due to bubbles or gaps in the mound of gel.

As mentioned in Section 4.3.2, there are also difficulties co-registering the location of stiffness measurements with the location where the IFP was measured.

One other limitation is the potential for needle blockage. In the case of the local IFP and stiffness measurements this is only a minor nuisance, as another measurement can easily be made. In the case of tracking the IFP in the rabbit over time after sacrifice, a blockage renders the data unusable,
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4.4.4.2 Limitations to experiments performed and underlying assumptions

Section 4.1.2 described several assumptions behind the experiments in an effort to solve Equation 4.1. An attempt to extract $\Delta \mu(P)$ from Equation 4.4, will be vulnerable to large variability in $\mu_0(r)$. As described above, in Section 4.3.2, large variability was indeed found. Determining the contribution of IFP to shear modulus was not successful, and even if co-registration problems were not an issue, many more rabbits would need to be used to determine this relationship.

More must also be said about the assumption that over a timescale of less than an hour, the tissue’s mechanical properties change only due to changes in fluid pressure; rigor mortis, for example, takes well over an hour to begin in rabbits [64]. While this assumption is not wholly unreasonable, it has some limitations. For one, the temperature of the tissue changes over time as the deceased, exsanguinated rabbit is no longer able to regulate its body heat. Sapin-de Brosses et al. [65] found that while ex vivo (butcher shop) liver tissue showed no change in shear modulus with temperature in this range, muscle tissue saw its shear modulus decrease by $\sim 25\%$ when heated from $25^\circ C$ to $37^\circ C$. While the exact temperature change following sacrifice is not known, a similar trend in the tumours could be of the same magnitude as stiffness changes seen, and could be potentially masking a great deal of the change in shear modulus after sacrifice due to other factors, notably fluid pressure changes. Future experiments should attempt to keep the temperature of the leg constant.

It must be noted that in this experiment, even where correlations between shear modulus and

Figure 4.14: The same field of view in a tumour with (a) no force at all applied, and (b) force applied from resting the transducer and imager’s forearm on the tumour, with his elbow resting on the table.

and that information cannot be re-acquired. Indeed, this occurred in rabbit 6, as described above.
fluid pressure were found, causation was not definitively established. Especially in the imaging after sacrifice, there are potential confounding factors. Notably, vascular pressure is known to change after sacrifice, and indeed it was changes in vascular pressure that were expected to drive changes in interstitial pressure. It is possible that the changes in stiffness are due to vascular pressure, and that if interstitial pressure were to change in the absence of vascular pressure, that no change in stiffness would be seen. Hopefully, the fact that interstitial pressure represents such a large fraction of the volume of tumours, as shown in Figure 1.2, should mean that IFP changes are the dominant contributor to stiffness changes in this case.

4.4.5 Implications for future work

Several things can be learned from this experiment with a view to possible future work. While preliminary investigation has shown modest evidence that IFP affects shear modulus, it seems unlikely that an absolute IFP can accurately be determined from shear modulus; this effect is too weak compared to the variation in shear modulus to furnish an accurate prediction. That being said, it is still quite possible that changes in IFP are detectable with elastography.

Difficulties are also likely to arise in monitoring tumour response to therapy or evolution over time. Although in this experiment, some difficulty was encountered in matching planes before and after sacrifice, the tumour's geometry remained essentially the same. In a situation where the tumour grows (or shrinks) and its geometry changes, matching regions for an exact comparison of stiffness will be more difficult still, if not impossible. It could be possible to take multiple planes to characterize the tumour, average them, and compare two time points like this, hoping the error from non-matching planes is minimal. Any changes occurring, however, would not be easy to attribute to fluid pressure changes rather than a difference in stiffness due to biological changes. If such a measurement were performed, it might be better suited to an elastographic method that can measure stiffnesses over volumes. Magnetic resonance elastography is one possibility, though it would be ideal to use an extension of supersonic shear imaging to 3 dimensions.

4.5 Conclusion

Comparing local stiffness to local fluid pressure showed a great variation in both parameters across the tumour. Linear regressions were performed, but no statistically significant trend was seen. Measuring the tumour’s stiffness and IFP over time following sacrifice showed that the IFP drops in the tumour over the first \(\sim 30\) minutes. Much variability was seen in the behaviour of small regions of interest over time, and insufficient independent measurements were made to draw further conclusions here. Comparing multiple planes before and after sacrifice was much more effective. A set of planes from three tumours before sacrifice was found to be significantly stiffer (\(p=0.04\))
than those same planes after sacrifice. Though much variability was again seen, the median drop in stiffness was 33% in large ROIs. This corresponds to a change in stiffness with fluid pressure of roughly 0.8 kPa (shear)/mmHg, which is similar in size to the change seen in ex vivo kidneys in Chapter 3. More experiments would be needed to evaluate the robustness of this relationship.

Based on the preliminary experiments performed thus far, there is modest evidence suggesting that stiffness might change with interstitial pressure in tumours, and an estimate of the magnitude of the change has been made.
Chapter 5

Summary, future work and conclusion

5.1 Summary

In a solid tumour, there is a proliferation of blood vessels that are leaky and permeable compared to vessels in healthy tissue. This allows high-pressure blood plasma to leak out into the interstitial space, which raises the interstitial fluid pressure. This increased fluid pressure might make the tumour stiffer.

Anti-angiogenic cancer therapies are known to reduce a tumour’s interstitial pressure. They are expensive and have toxic side-effects, however, so it is important to know as soon as possible whether or not the they are working as desired. Changes in fluid pressure occur on the order of days, and they could be used as an early-response marker of therapy effectiveness. If interstitial pressure changes stiffness, then stiffness measurements might be able to determine changes in interstitial fluid pressure noninvasively. In addition to this, understanding the contribution of interstitial pressure to tumour stiffness will aid in interpreting elastographic measurements of tumours in general.

To better understand how a change in interstitial pressure specifically could affect the elastic properties of tissue, and to control for confounding factors, we performed several experiments to clarify the basic science of the relationship. Initial experiments were in a readily understood and simple system, and these built up to later experiments, trading increased complexity for increased biological accuracy.

Experiments on in vitro gelatin phantoms demonstrated that in soft solids, changes in shear modulus can occur only in the presence of a strain or deformation. Consequently, changes in tumour shear modulus should only be expected to occur if there is some deformation. Compression of gelatin phantoms showed that under unconstrained compression, direction-dependent changes in shear modulus are seen. Experiments pressurizing ex vivo kidneys showed that in living tissue, shear modulus will increase with fluid pressure, and the magnitude of the effect was estimated.
Notably, the change in stiffness with pressure over the clinically relevant range was found to be substantial. Preliminary experiments on intramuscular VX2 tumours in a rabbit investigated how stiffness depends on interstitial fluid pressure. Local measurements of shear modulus and interstitial pressure established that there is a great deal of spatial variability in the shear modulus independent of interstitial pressure in these tumours. In a comparison of the shear modulus and interstitial pressure before and after sacrifice, it was found that there is a significant change in shear modulus with a change in fluid pressure in these tumours. Though much variability was found, these results modestly suggest that fluid pressure contributes to stiffness in tumours. More work should be done to determine this relationship in greater detail.

5.2 Future work

5.2.1 Preclinical animal models

The most obvious direction for future work is to continue the experiments described in Chapter 4; with more data the nature of any relationships found will become more clear. It is thought, however, that this experiment is not the most advantageous of all options.

While the work in Chapter 4 suffered from an inability to modulate the fluid pressure, the work in Chapter 3 was partially limited in its applicability to tumours as it was a different tissue type. One option would be to fuse the strengths of each of these techniques by implanting a VX2 tumour in the kidney of a rabbit, or another animal model. By cannulating it \textit{in vivo}, the fluid pressure could be modulated as with the experiments on pig kidneys; this would give improved knowledge of the exact fluid pressure while at the same time removing many of the potentially confounding factors due to using sacrifice to achieve an IFP change. In other words, it would allow the solution of Equation 4.1 directly. Though the fluid pressure would change for different reasons than those in a naturally evolving or treated tumour, such an experiment would better establish a relationship between fluid pressure and stiffness in tumours.

Another potential direction for future work is the investigation of stiffness in tumours in smaller animal models. The VX2 tumours in the experiment above were implanted intramuscularly, and they developed into irregular shapes. They were further found to grow multiple lobes, making the spatial distribution of interstitial pressure less clear. In spherical tumours, however, the distribution of IFP as a function of position is well-characterized in the literature, showing a nearly constant IFP in the centre, dropping rapidly in the periphery [62],[66]. Use of such a model should improve knowledge of the spatial IFP distribution, which would improve on the corresponding limitations of the experiment above. Measurements in small animals would be facilitated with the new high-frequency probe our lab has recently received from SuperSonic Imagine, with a centre frequency of 15 MHz. The higher frequencies should allow stiffness maps with a finer resolution to be taken,
which would improve the suitability of the technique in small animal models.

5.2.2 Alternative therapy: dexamethasone

One issue raised in Section 4.1.1 is the difficulty of modulating IFP in a physiologically appropriate way. In either rabbits or rats, this could be done with a drug that is known to change IFP, and do so without changing the vascular pressure. Multiple agents are known to do so; a list of these can be found in [13]. Of note is dexamethasone, which has been shown to reduce IFP in human colon adenocarcinomas [67] and in rat gliomas [68]. Dexamethasone is a corticosteroid normally used to reduce inflammation. It was shown that vascular pressure is unaffected by the dexamethasone administration [67]. It is therefore believed to reduce interstitial pressure by reducing vascular permeability, a mechanism of action analogous to that of anti-angiogenic therapeutics. Nonetheless, it is also possible that dexamethasone reduces IFP by reducing the number of molecules in the interstitium, modifying the oncotic gradient [13]. This could very well change the mechanical properties of the tumour, which would be an undesirable effect.

In fact, approval has already been granted for the use of dexamethasone in the rabbit imaging protocol. Based on body surface area normalization [69], the dose in mice found in [67] was adjusted to a 3 mg/kg daily dose, and the imaging would be performed after 4 days of dexamethasone administration. While using dexamethasone would allow IFP changes due to vascular permeability, sacrifice was instead used to modulate the IFP due to two principle concerns. One is that the effect of dexamethasone on rabbits is unclear; side-effects could create undesired changes in stiffness or other biological parameters, or dexamethasone might not work at all in rabbits, at least at the first doses attempted. Second, comparing stiffness before dexamethasone administration to stiffness after four days of tumour evolution would be difficult. As described above, it would be quite difficult to meaningfully co-register locations or planes in the tumour when its geometry, biological properties and mechanical properties have changed due to growth in addition to changes due to drug administration. Indeed, as with the local comparisons of stiffness and IFP described in Section 4.2.3.2, this experiment would involve attempting to extract changes in shear modulus with pressure from Equation 4.4. Part of the initial impetus to use dexamethasone arose from concern that there would not be a sufficient spatial variation in IFP to compare it with stiffness over a meaningful range. As was shown in Section 4.3.2, there is a great deal of spatial variation in IFP, so such concerns were laid to rest, and experiments continued without dexamethasone.

5.2.3 Anti-angiogenic therapies in preclinical animal models

Naturally, the best drugs to use in an animal model to determine how the stiffness and IFP change after anti-angiogenic therapies would be anti-angiogenic therapies themselves. To investigate the
potential as an early-response marker, tumour response should be monitored using IFP, elastography and conventional volume-based measurements. The efficacy of each could then be properly compared.

5.2.4 Anti-angiogenic therapies in humans

Clearly, the only way to truly see if differences in interstitial fluid pressure in human tumours after anti-angiogenic therapy are detectable with ultrasound elastography is to investigate that itself. At the current time, whether or not this is valuable is too speculative to justify subjecting a large number of people to invasive wick-in-needle measurements of interstitial pressure. Should preclinical experiments performed provide solid evidence, then this situation could change.

Among the great strengths of ultrasound, including ultrasound shear wave elastography, are the facts that it is believed to not be harmful, that it is low-cost and that it is convenient. With this in view, measurements of tumour stiffness without measurements of interstitial pressure could well be performed on patients. A study on anti-angiogenic therapy in renal cell carcinoma is being performed at this hospital, and it would be possible to monitor how tumour stiffness changes as a response to the therapies.

It was suggested that ultrasound elastography could be used to measure IFP changes noninvasively, and thereby serve as an early-response marker of tumour response to therapy. It is possible that ultrasound elastography is capable of serving as an early response marker on its own without making predictions of specific interstitial pressure changes. Any weaknesses in determination of IFP could thereby be irrelevant. It should be noted, however, that such a possibility is quite speculative at this point.

If stronger evidence emerges of IFP as an early-response marker, then this would reinforce the possibility of using ultrasound elastography as an early-response marker. In practical terms, elastographic measurements could be done by piggy-backing on an experiment measuring IFP as an early response marker such as [24]; this would give evidence of how well elastography corresponds to clinical IFP measurements as well.

Though anti-angiogenic therapy has the strongest theoretical reasons for expecting elastographic measurements to be useful, the experiments proposed in this section could equally well be done to investigate therapies that are not anti-angiogenic, particularly chemotherapeutics, as these too are known to change the interstitial pressure.

5.3 Future potential

It appears that changes in interstitial pressure effect changes in stiffness. Though there are confounding factors and difficulties in measurement, it could very well be that this technique will be of clinical utility, and this question should be pursued further.
The experiment investigating local IFP and local shear modulus in Section 4.3.1 could very possibly have suffered from poor co-registration. Nonetheless, it is the author's opinion that even if this were not an issue, it is unlikely that ultrasound elastography could be used to reliably determine a specific interstitial fluid pressure in a clinical setting, be it in tumours or other tissue. The variation in stiffness due to other factors is too great for this; the strain state of the tissue in the tumour, for example, will depend heavily on the properties of the surrounding tissue. Determination of changes in interstitial pressure, on the other hand, remains much more plausible.

Further, it is very clear based on the experiments in Chapter 3, as well as [27], [28] and [29], that stiffness depends on fluid pressure in soft tissue. In solid tumours, for whatever difficulty was seen in Chapter 4 in accurately measuring the magnitude of this phenomenon, it is highly likely that this dependence is still present. Whenever elastography of any type is used to evaluate the state of tumours, it will be important to understand what the resulting stiffness means: how much of it is due to properties of the solid parts of the tissue, like fibrosis or cell density, and how much of it is due to fluid pressure? In a similar vein, Rotemberg et al. [28] identify the importance of distinguishing between the effects of fibrosis and the effects of elevated portal venous pressure in using an elastogram to determine the health of the liver. Regardless of whether elastography is capable of making standalone clinically useful predictions of changes in interstitial pressure after therapy, the results of this research should prove important in evaluating and understanding the state of tumours with elastographic techniques in general.

5.4 Conclusion

This work investigated the dependence of shear modulus of a tumour on its interstitial pressure. The primary motivation for this was the drop in interstitial pressure expected after anti-angiogenic therapy, supported by anecdotal clinical evidence.

Much progress was made towards the investigation of this phenomenon. The basic behaviour of a soft solid under hydrostatic pressure, stress and strain was determined. Experiments on ex vivo tissue showed consistent and substantial changes in stiffness with fluid pressure, and determined the size of the effect. In preliminary experiments in in vivo tumours, changes in stiffness were found to correlate with changes in fluid pressure, and the available evidence suggests that the change in stiffness with fluid pressure is of the same general size as those found in other tissue types. The heterogeneity of both interstitial pressure and stiffness was characterized. More work should be done to determine the relationship between tumour stiffness and interstitial pressure in greater detail.

The next step in determining this relationship would be to investigate tumour tissue under an internal fluid pressure that can be directly and manually controlled; this would give more insight into the biophysics of the pressure-stiffness relationship. Following this, work should be done with
relatively spherical tumours in small animal models to improve understanding of the in vivo relationship. To investigate the potential of elastography as an early-response marker for anti-angiogenics, it should be compared with interstitial pressure and volume measurements for tumour monitoring in a pre-clinical model. Clinical experiments could then establish the relationship between interstitial fluid pressure and stiffness in human cancers, and its diagnostic utility.
References


