DEVELOPMENT OF Atherosclerotic Plaque Ultrasound Phantoms For the Investigation of Vulnerable Plaque Features

by

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Abstract

As the burden of atherosclerotic cardiovascular disease (ACVD) continues to rise, increasing efforts are directed to innovate screening tools for the detection of vulnerable plaque and the vulnerable patient. Vulnerable plaque lesions are thought to be specific lesions responsible for the majority of cardiovascular events, and are generally characterized by a large lipid core and a thin fibrous cap. Recent advances in ultrasound offer novel techniques to characterize various aspects of plaque vulnerability.

This study investigated two approaches for evaluating plaque vulnerability. Specifically, a density-based plaque phantom was developed for the evaluation of plaque echogenicity, a feature of plaque vulnerability that can be characterized by Grayscale Median (GSM) analysis. It was found that plaque density has a significant positive linear correlation with plaque echogenicity. Another phantom model of simulated heterogeneous plaque lesions was developed for the evaluation of plaque composition, a feature of plaque vulnerability that can be characterized by colourized Pixel Distribution Analysis (PDA), a novel tissue characterization technique. Two sets of GSM ranges associated with specific tissue types were established. Percentages of gray pixels identified in the correct tissue GSM range from colourized PDA were compared between the two sets of GSM ranges we established, and a previously reported set of tissue GSM ranges. Our tissue GSM ranges of: echolucent plastisol 0-4, muscle 68-86 (Neck muscle and leg muscle) and 104-108 (abdomen muscle), fat 87-100 (visceral fat) and bone 145-175, detected a greater percentage of pixels within the correct tissue type in comparison to the two other sets of ranges.

It is anticipated that the phantoms developed will serve as an ex vivo platform for the optimization of vulnerable plaque imaging technologies, allowing for timely and cost-effective development of ultrasound probes, software, contrast agents, and drug delivery systems for the treatment of atherosclerosis. The research presented in this thesis has focused on developing ultrasound analysis techniques to better stratify patients by their risk of ACVD. Atherosclerotic plaque lesions, specifically
vulnerable plaque lesions, may not be apparent by conventional diagnostic modalities. This research can improve diagnosis and treatment of cardiovascular diseases.
Co-Authorship

The research present in this thesis has been performed and written by Olivia Yau with the following co-authorships and technical assistance (see below). Dr. Amer M. Johri, Dr. Marie-France Hetu, Dr. Michael A. Adams, helped: conceive and design the research, and in developing the discussion of all experiments. Dr. Marie-France Hetu provided addition assistance with statistical analysis.

Chapter 2: Co-authored by Michael A. Adams, Marie-France Hetu, Amer M. Johri

Dr. Michael A Adams provided access to his research facility and equipment.


Chapter 3: Co-authored by Marie-France Hetu, Julia E. Herr, Michael A. Adams, Amer M. Johri

Dr. Michael A Adams provided access to his research facility, equipment, and tissue samples.

Julia Herr provided assistance with CoPDA protocol.

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<th>Description</th>
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<tbody>
<tr>
<td>ACVD</td>
<td>Atherosclerotic cardiovascular disease</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
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<tr>
<td>OCT</td>
<td>Optical coherence tomography</td>
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<td>ESS</td>
<td>Endothelial shear stress</td>
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<td>PVA-C</td>
<td>Poly(vinyl alcohol) cryogel</td>
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<tr>
<td>CIMT</td>
<td>Carotid intima-media thickness</td>
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<tr>
<td>B-mode</td>
<td>Brightness mode</td>
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<td>CAD</td>
<td>Coronary artery disease</td>
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<td>GSM</td>
<td>Gray scale median</td>
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<td>PDA</td>
<td>Pixel distribution analysis</td>
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<tr>
<td>ROI</td>
<td>Region of interest</td>
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<tr>
<td>r</td>
<td>Reflection coefficient</td>
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<tr>
<td>Z</td>
<td>Acoustic impedance</td>
</tr>
<tr>
<td>p</td>
<td>Density</td>
</tr>
<tr>
<td>c</td>
<td>Speed of sound wave</td>
</tr>
<tr>
<td>v</td>
<td>Ultrasound wave velocity</td>
</tr>
<tr>
<td>f</td>
<td>Ultrasound wave frequency</td>
</tr>
<tr>
<td>λ</td>
<td>Wavelength</td>
</tr>
<tr>
<td>IB</td>
<td>Integrate backscatter</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>PVA</td>
<td>Polyvinyl chloride</td>
</tr>
<tr>
<td>ICAROS</td>
<td>Imaging in carotid angioplasty and risk of stroke</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TBE</td>
<td>Tris-Borate-EDTA</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>P</td>
<td>Mass of specimen</td>
</tr>
<tr>
<td>S</td>
<td>Fluid density</td>
</tr>
<tr>
<td>F</td>
<td>Combined mass of specimen, support, and thread</td>
</tr>
<tr>
<td>K</td>
<td>Combined mass of specimen, support, and thread after immersing specimen into fluid of known density</td>
</tr>
<tr>
<td>TGC</td>
<td>Time gain compensation</td>
</tr>
<tr>
<td>SWE</td>
<td>Shear wave elastography</td>
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<tr>
<td>ARFI</td>
<td>Acoustic radiation force impulse imaging</td>
</tr>
<tr>
<td>CV</td>
<td>Cardiovascular</td>
</tr>
<tr>
<td>IVUS</td>
<td>Intravascular ultrasound</td>
</tr>
<tr>
<td>USVH</td>
<td>Ultrasound virtual histology</td>
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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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<td>CEUS</td>
<td>Contrast enhanced ultrasound</td>
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Chapter 1

Introduction

1.1 Atherosclerosis and the vulnerable plaque

Atherosclerosis is a silent, progressive disease characterized by hardening of arterial walls and the presence of plaque build-ups. It results from the combined processes of damaged arterial endothelium wall, lipid accumulation, oxidative damage and inflammatory responses. This atherosclerotic process is the main cause of atherosclerotic cardiovascular disease (ACVD), which comprises of heart attacks, strokes, and peripheral vascular disease, and is one of the leading causes of death in Canadians(1). Though plaque development is part of the atherosclerotic process, not all atherosclerotic plaque lesions behave in a similar manner. It is thought that the majority of cardiovascular events are caused by vulnerable plaque lesions, characterized as soft, rupture-prone with thin fibrous caps and large lipid cores(2) (Figure 1.1). Earlier identification of vulnerable plaque lesions can allow for treatment of existing cardiac risk factors in patients, and prevent future cardiovascular events. Non-ultrasound based imaging modalities such as magnetic resonance imaging (MRI), nuclear imaging, multi-detector computed tomography, optical coherence tomography (OCT), and positron emission tomography have been extensively reviewed and investigated(3–6). These imaging modalities tend to be expensive, stationary, and expose patients to radiation. In contrast, ultrasound may serve as an inexpensive, portable, and safe tool to address the challenge of detecting plaque vulnerability through a number of emerging techniques. Currently, many of these ultrasound techniques lack sufficient validation. Thus, the development of ultrasound phantom models that simulate different features of plaque vulnerability can provide a controlled, safe, and stable platform for the testing and advancement of these techniques.
Figure 1.2. Features of the vulnerable plaque.

A vulnerable plaque is characterized by a large lipid core, a thin fibrous cap, an aggregation of inflammatory cells, irregular surface features, and neovascularization. Reprinted from Journal of the American Society of Echocardiography, 30, Amer M. Johri, Julia E. Herr, Terry Y. Li, Olivia Yau, and Vijay Nambi(10), Novel Ultrasound Methods to Investigate Carotid Artery Plaque Vulnerability, 139–148., Copyright (2017), with permission from Elsevier (Appendix A).
1.2 Pathogenesis of the vulnerable plaque

Plaque development is part of the atherosclerotic process, which begins with a damaged endothelium of a blood vessel. This usually occurs at predilection anatomical sites located near branch points, such as the carotid bulb, coronary vessels, and the femoral arteries, where low or oscillatory endothelial shear stress (ESS) and turbulent blood flow are prevalent. Low ESS modulates gene expression of the endothelial wall through mechano-reception processes that induce an atherogenic phenotype, resulting in the formation of an early atherosclerotic plaque(7). Persistence of ESS induces compensatory expansive remodeling of the vessels, which is important in the preservation of lumen dimensions during plaque progression. However, this expansive remodeling process can become excessive, promoting a continual influx of lipids into the vessel wall(8). Cellular waste products and substances such as lipids and low-density lipoprotein (LDL) particles can infiltrate and accumulate inside the damaged areas of the arterial wall (Figure 1.2A). Oxidation of the LDL initiates an inflammatory response where endothelial cells express leukocyte adhesion molecules, thus facilitating the recruitment of monocytes that migrate into the intima. The monocytes then differentiate into macrophages that engulf the LDL particles and transform into foam cells. The foam cells eventually die, leaving behind the accumulated lipid as a soft, unstable, necrotic core(9). A thin fibrous cap is often present as part of a vulnerable plaque, and is formed by smooth muscle cells that have migrated from the vessel media, to the subendothelial space, where they proliferate and produce cellular matrix proteins. Simultaneously, endothelial cells, macrophages, and smooth muscle cells underneath the fibrous cap can undergo apoptosis or necrosis, further contributing to the formation of a necrotic core(10).
Figure 1.2. Pathogenesis of the vulnerable plaque.

A) Lipids and low-density lipoprotein (LDL) particles infiltrate and accumulate inside damaged arterial wall, where epithelial cells express leukocyte adhesion molecules. B) Monocytes differentiate into macrophages, which take up oxidized LDL particles and transform into foam cells. C) Smooth muscle cells migrate from the vessel media, to the intimal space, producing matrix proteins, forming a thin fibrous cap. D) Neovascularization originating from the adventitial vasa vasorum may be present in advanced plaque lesions. Reprinted from Journal of the American Society of Echocardiography, 30, Amer M. Johri, Julia E. Herr, Terry Y. Li, Olivia Yau, and Vijay Nambi, Novel Ultrasound Methods to Investigate Carotid Artery Plaque Vulnerability(10), 139–148., Copyright (2017), with permission from Elsevier (Appendix A).
1.3 Medical imaging modalities and phantoms

Current non-invasive medical imaging modalities clinically used to diagnose atherosclerosis and identify vulnerable plaque lesions include MRI, and OCT. These modalities tend to be expensive, non-portable, or expose patients to radiation. Ultrasound can serve as an economic, portable, and safe tool in the diagnosis of atherosclerosis with various emerging ultrasound techniques. However, the freehand manual component of ultrasound is a major limitation to the development, testing, and eventually clinical application of these techniques. Therefore, the development of a robust phantom model is a key step in the testing, development, and teaching of these novel ultrasound techniques. Despite the interest from various imaging societies for improved standardization of advanced ultrasound analytical techniques, there are limited studies reporting the creation of phantom models, and even fewer reporting on plaque simulation. This knowledge gap becomes more important as carotid plaque characterization continues to emerge as an important risk stratification tool, and more vendors develop off-line analytical software. It is important to realize that the freehand manual aspect of sonography is a major limitation to the testing, development, and eventually the clinical application of new techniques and analytical software. In particular, the application of angling, steering, as well as variability in various technical parameters, can result in interobservation inconsistency. Medical imaging phantoms can provide a stable, safe, and controlled environment for the advancement and teaching of leading-edge ultrasound analytical techniques. However, most carotid vessel phantoms previously developed are short-lived and semi-permanent. Studies have been conducted where items including corn kernels, olives, modeling compound (Play-Doh), animal meat and coagulated milk, were tested in the attempt of creating carotid artery plaque lesions(11,12). A limited number of commercial phantoms are available on the market with a high cost, but none of which simulates specific features of plaque vulnerability.

Historically, agar and gelatin were the material of choice commonly used to create phantom models mimicking human soft tissues(13–16). However, agar or gelatin base phantoms are extremely degradable, and are therefore unsustainable for long term usage - an alternative approach is required. Poly
(vinyl alcohol) cryogel (PVA-C) has been identified as a good candidate as it possesses realistic mechanical properties comparable to the human biological tissues (17,18). It is known to possess long-term structural stability, and can be used in thermal and radiation dosimetry applications (13).

1.4 Atherosclerotic plaque in the carotid artery

It has been shown that carotid plaque has a stronger association with cardiovascular events than the more conventional ultrasound assessment of carotid intima-media thickness (CIMT)(19,20). CIMT has been widely used as a surrogate marker of atherosclerotic disease, and is measured by B-mode ultrasound of the common carotid artery. Its accuracy as a marker of atherosclerosis and predictor of cardiovascular events has been questioned, as age and hypertension are the main factors resulting in the medial hypertrophy or intimal thickening of the carotid vessel, thus suggesting that CIMT may not be indicative of atherosclerosis (21). Numerous comparative studies have been conducted in investigating carotid plaque and CIMT’s association with coronary artery disease. It was shown that the presence of carotid plaque is a superior predictor of coronary artery disease (CAD)(20,22). Furthermore, while conducting ultrasound within the coronary arteries requires intravascular ultrasound, an invasive and expensive procedure, vascular ultrasound can be easily conducted to evaluate carotid plaque lesions due to the superficial location and the larger diameter of the carotid vessel. Carotid plaque lesions tend to occur predominantly at anatomical sites with turbulent blood flow such as the carotid bulb, and the proximal internal carotid artery. Since atherosclerosis and plaque instability are not local vascular phenomenon, but rather exist simultaneously at multiple sites throughout the vascular bed, it has been suggested that the investigation of carotid plaque lesions can act as a surrogate for coronary plaque vulnerability (23). Assessment of carotid plaque lesions can also provide insight into the disease progression of atherosclerosis, and ultimately the identification of a vulnerable patient.
1.5 Diagnosis of the vulnerable plaque

The diagnosis of vulnerable plaque lesions remains a difficult task due to the numerous features associated with plaque vulnerability. Not all vulnerable plaque lesions possess the following vulnerability features, but the presence of one or more of these features may indicate plaque vulnerability. The features of plaque vulnerability include high lipid content, low echogenicity, an irregular shape, heterogeneity in plaque composition, presence of micro-calcifications, and neovascularization. However, such plaque lesions often appear as non-stenotic, and may be overlooked despite being at high risk for rupture. There are currently no standardized, validated guidelines that can be utilized universally to identify vulnerable plaque by ultrasound(24). In addition to the lack of standardized guidelines, there are also currently no targeted therapies for vulnerable plaque beyond treatment with statins, as stenting and endarterectomy procedures are only recommended for the treatment of symptomatic patients with a high degree of stenosis(25). Most studies examining vulnerable plaque lesions have focused on various morphological aspects, including shape, size, volume, or height(26,27). Guidelines of vulnerability characterization based on plaque morphology may prove to be less effective as both vulnerable and stable plaque lesions can be morphologically similar. Despite their morphological similarities, vulnerable and stable plaque lesions differ greatly in their composition (density), activity and physiology. This thesis focuses on investigating two main features of plaque vulnerability: 1) Plaque density/echogenicity, which can be characterized using grayscale median (GSM) values, and 2) Plaque composition, using a novel approach of tissue characterization based on the different echogenicity of specific tissue types known as colourized pixel distribution analysis (PDA).

1.6 Plaque echodensity and ultrasound physics

Sound is a transverse, mechanical wave that travels through a medium. In diagnostic ultrasonography, a transducer sends out ultrasound waves produced by electrically stimulated vibrations
of piezoelectric crystals. These crystals in the transducer display the Piezoelectric Effect by generating an electric charge in response to an applied mechanical stress resulted from the application of voltage. The produced ultrasound waves encounter molecules in the region of interest (ROI), where a fraction of the original waves known as echoes are reflected and received by the transducer. The echoes are converted into electrical impulses, which are displayed on an oscilloscope, producing a black and white ultrasound image of the ROI. The amount of echoes reflected is dependent on the reflection coefficient (r) of the medium. The reflection coefficient can be calculated from the following equation:

$$ r = \frac{(Z_1 - Z_2)}{(Z_1 + Z_2)} $$

From the equation, one observes that the reflection coefficient is dependent on the acoustic impedance (Z) of the medium. Z represents the amount of resistance an ultrasound beam encounters in the medium, where the effect of Z is especially of relevance at the interface of different mediums. The ability of an ultrasound wave to transfer from one tissue (Z₁) to another (Z₂) depends on the Z difference between the two mediums (Z₁-Z₂). The larger the Z difference between the two mediums, the greater the amount of ultrasound waves will be reflected. Z itself depends on: the density of the tissue (ρ, in kg/m³) and the speed of the sound wave (c, in m/s). These two variables are related by:

$$ z = \rho c $$

From basic physics principles, it is known that all sound waves travel at the same speed in the same medium regardless of wave frequency, as any differences in frequency is compensated by a corresponding change in wavelength (ν = fλ), where ν represents with speed of sound wave, f represents wave frequency, and λ represents wavelength. The constant speed of an ultrasound wave in the same medium leads us to investigate the density variable. According to the acoustic impedance equation, the denser the structure, the larger its acoustic impedance will be. A larger Z₁ will result in a larger reflection coefficient, suggesting that a larger fraction of the original ultrasound waves sent out by the transducer will be reflected and received.

In ultrasonography, it was first noted by Reilly et al. that echo patterns of carotid plaque lesions
could be related to the lesions’ tissue composition(28). It was then qualitatively defined by the same group that plaque echogenicity is the degree of acoustic brightness seen on B-mode ultrasound images. An association of dimmer, echolucent plaque lesions, with neurological symptoms was subsequently reported. Meanwhile, brighter, echogenic plaque lesions were reported to be asymptomatic(29). Overall, denser, fibrous or calcified structures will appear brighter, and are characterized as echogenic, while less dense, fatty or hemorrhagic structures appear dimmer, and are characterized as echolucent. In vascular ultrasound, the computerized analysis of carotid plaque GSM has been proposed to quantitatively evaluate plaque echogenicity(30–32), and have since been shown to associate with cardiovascular events such as cerebral infarction(33–35)

1.7 Tissue characterization and plaque composition analysis

Up until the 1980s, the evaluation and characterization of atherosclerotic plaque components were considered “virtually impossible” without surgical removal of the plaque lesion or examination at autopsy(36). Since the 1980s, there has been a progression of three main approaches to characterizing tissue components in atherosclerotic plaque lesions: 1) The visual “eyeballing” approach, 2) the videodensitometry approach, and 3) the integrated backscatter (IB) approach(28,37–39). The visual “eyeballing” approach is the oldest and simplest approach. However, this approach is subjected to obvious limitations in terms of its operator-dependent subjectivity, and the degree of sensitivity in detecting smaller regional differences. IB analysis is the most contemporary approach. It directly measures raw radiofrequency signals upstream of video processing, and relies on the scattering of acoustic waves in all spatial directions reflected from the ROI. The quantity and the intensity of scattered radiofrequency signals vary among different media. The unprocessed radiofrequency signals are displayed in decibels, serving as quantitative ultrasonic indices that are undistorted by post-processing. Thus, IB analysis is able to maintain a linear relationship between the received signal and the subsequently displayed IB image. The usage of IB in plaque composition analysis takes advantage of the
small overlap between IB value ranges of different tissue types. Specifically, a proprietary software name iPlaque was developed, and visualizes IB values spatially distributed within a single plaque, resulting in a map-like image that describes plaque composition(40,41). However, this approach is not without limitations. The procedure converting raw radiofrequency data into usable quantitative values displayed in decibels is complex, and is subjected to artifacts related to image settings and the exact location of the plaque lesion(42). These reasons account for the rare clinical use of IB in current practices.

This thesis investigates the videodensitometry approach, which is a quantitative method using mean gray level to describe spatial distribution of plaque texture. The underlying hypothesis in tissue characterization, and plaque composition analysis via videodensitometry is that normal tissue, diseased tissue, and the different tissue components within an atherosclerotic plaque have fundamentally different biochemical structures, internal architectural arrangements, and physiological states. These differences can affect physical properties such as density, and can therefore be detected by ultrasound using a variety of acoustic parameters including acoustic backscatter, attenuation, angular variability, and acoustic internal homogeneity of spatial gray-level distribution(36). The differences in tissue echogenicity will provide insight into the spatial gray-level distribution of acoustic internal homogeneity.

1.8 Project overview

There is a high global demand to develop imaging expertise for earlier identification and diagnosis of atherosclerosis and vulnerable plaque, to quantify the atherosclerotic disease progression, and to guide prevention. As researchers continue to develop approaches to target atherosclerosis and its complications through advanced imaging modalities, there is a lack of reliable and non-invasive platform to test these strategies. This study aimed to develop two variations of ultrasound phantom models simulating vulnerable plaque lesions. The aims are:

1. To develop a density-based plaque model to evaluate plaque echogenicity characterization using Grayscale Median (GSM) analysis. It was hypothesized that plaque density will have a positive
linear correlation with plaque echogenicity.

2. To develop a heterogeneous plaque model to investigate a tissue composition analysis algorithm previously reported as colourized Pixel Distribution Analysis (PDA). It was hypothesized that colourized PDA will effectively identify the correct tissue types within the simulated heterogeneous plaque lesions.

Each aim will address three objectives. The three objectives for the first aim are as follow:

1. Develop a density-based atherosclerotic plaque ultrasound phantom model.
2. Sample three types of plaque mimic mixtures.
3. Delineate the relationship between density, an intrinsic physical property, with echogenicity.

The three objectives for the second aim are as follow:

1. Develop a heterogeneous atherosclerotic plaque ultrasound phantom model.
2. Establish new tissue GSM ranges to be used for the PDA analysis method.
3. Compare PDA results between new GSM ranges with a previously reported set of ranges.

It is anticipated that the phantoms developed will serve as an ex vivo platform for the optimization of vulnerable plaque imaging technologies, allowing for timely and cost-effective development of ultrasound probes, software, contrast agents, and drug delivery systems for the treatment of atherosclerosis. The research presented in this thesis has focused on developing ultrasound analysis techniques to better stratify patients by their risk of ACVD. Atherosclerotic plaque lesions, specifically vulnerable plaque lesions, may not be apparent by conventional diagnostic modalities. This research can improve diagnosis and treatment of cardiovascular diseases.
Chapter 2

Quantitative Validation of Gray Scale Median Analysis Using a Density Based Arterial Plaque Ultrasound Reference Phantom Model

2.1 Abstract

Background: Arterial plaque is characterized as vulnerable if having a large lipid core, a thin fibrous cap, making it prone to rupture. Developing non-invasive tools to target plaque vulnerability is important for earlier identification and treatment of cardiac risk patients. Ultrasound and off-line analytic software techniques, such as gray scale median (GSM) analysis, may help identify vulnerability. This study aims to delineate the relationship between plaque density and echogenicity quantified by GSM, thus providing insight into physical properties of plaque as a potential marker of vulnerability.

Methods: A soft tissue phantom surrounding a lumen, denoted as the simulated carotid artery, along with simulated plaque lesions with varying densities created from various polymers, was developed. Two-dimensional (2D) B-mode ultrasound images were acquired with a Vivid E9 ultrasound machine, and plaque echogenicity was characterized using GSM analysis. Linear regression analysis was conducted and Pearson correlation between plaque echogenicity and density was determined.

Results: A significant positive correlation was found between GSM and density, correlations varied slightly for each material: agarose mixtures ($r^2=0.595$, $p<0.001$), plastisol and salt mixtures ($r^2=0.603$, $p<0.001$), and polyvinyl chloride (PVC) and softener mixtures ($r^2=0.502$, $p<0.001$). The regression slope differed between different plaque mixtures and was specific to the particular polymer used.

Conclusions: Plaque density had a positive linear correlation with plaque echogenicity. This correlation was independent of the regression slope. The correlation between plaque echogenicity and density could introduce a potential new diagnostic parameter and provide a systematic, practical approach for assessing plaque vulnerability of patients. A standardized density metrics system could lay a foundation for the development of quantitative techniques for ultrasonic plaque characterization.

Keywords: Plaque vulnerability, plaque density, echogenicity, gray scale median, phantom.
Abbreviations

GSM – GrayScale Median

PVC – Polyvinyl chloride

ACVD - atherosclerotic cardiovascular disease

2.2 Introduction

Current prevention strategies of atherosclerotic cardiovascular disease (ACVD) focus on the identification and management of patient risk factors. However, stratifying the high-risk population by quantifying the occurrence and progression of subclinical (asymptomatic) atherosclerotic burden can allow for earlier identification and treatment. Studies such as the Imaging in Carotid Angioplasty and Risk of Stroke (ICAROS) registry examined the relationship between plaque echogenicity and the clinical risk of stroke(33), demonstrating that plaque echolucency increased risk of stroke in carotid stenting(35). Despite great interest in the development of non-invasive modalities for vulnerable plaque detection, there is currently no clinical standard for vulnerable plaque identification. The computerized analysis of carotid plaque grayscale median (GSM) has been proposed to quantitatively evaluate plaque echogenicity(30–32). GSM measurement represents the frequency distribution of gray values of the pixels within a structure. Analyses of the gray values are usually conducted by offline software. Previous studies have shown that stable plaque lesions, rich in calcium and fibrous tissue, tend to be more echogenic with higher GSM values, while plaque containing a large lipid or blood core is more echolucent with lower GSM values(37,42,43). Echolucent carotid plaque with a low GSM value is characterized as vulnerable and is associated with cardiovascular events(24,33).

Since GSM analysis of carotid plaque lesions currently cannot be conducted online in real time while images are being acquired, it is rarely applied to everyday clinical practice. Several studies have correlated GSM with either visual evaluation of carotid plaque lesions(44,45) or various cardiovascular risk factors(35,46). Our study aimed to delineate the relationship between density, an intrinsic property of
plaque, and plaque echogenicity using GSM analysis. This is the first study targeting density to validate GSM analysis using a phantom model of atherosclerotic plaque. The development of a standard curve based on plaque density and echogenicity can be used as a standard metrics system. Such a tool can help translate the use of GSM analysis into everyday clinical practice.

2.3 Materials and Methods

We first created an ultrasound phantom consisting of simulated carotid artery plaque with a range of known densities using different materials, we then validated the GSM method using this reference phantom.

2.3.1 Creation of a Phantom Model – Soft Tissue

Poly(vinyl alcohol) cryogel (PVA-C) was selected as the tissue-mimicking material. PVA-C soft tissue was created as described by Surry et al(18) where a 10% by weight aqueous PVA (Sigma-Aldrich, Oakville, ON, Canada) solution was used to form the simulated soft tissue. PVA solution was heated to 121°C while stirred on a magnetic stir plate until the solution turned clear. The clear gel solution was left on the magnetic stir plate and cooled to room temperature (RT). It was then poured into a mold containing a 1.0 cm diameter rubber tube. The gel solution was left to rest in a sealed container for 12-24 hours in order to allow for air bubbles to rise to the surface. This aqueous PVA solution was then gelled into a cross-linked hydrogel by the formation of crystallites during a freeze-thaw cycle between -20°C and RT. The freezing process required 2 hours, after which the phantom was maintained at this temperature for an additional 10 hours, for a total freeze stage time of 12 hours. At the end of the 12 hours, the phantom was thawed at RT over 8-9 hours. Removal of the rubber tube left behind a cast denoted as the carotid artery lumen (Figure 2.1). Previous studies have reported PVA cryogel’s physical, mechanical and acoustic properties as comparable to those of the arterial wall(17,18).
Figure 2.1. Phantom model of the carotid artery.

Model was created with polyvinyl alcohol cryogel (PVA-C) to mimic soft tissue. The vessel lumen was created by pouring the PVA-C around a rubber tube, and removing it after gelling of PVA-C.
2.3.2 Creation of Phantom Model – Simulated Plaque

Various materials were chosen to create simulated plaque for the phantom model. Materials that can be manipulated easily and cross-linked into hydrogels like agar were selected. Other materials selected, such as polyvinyl chloride (PVC), are polymers that can closely simulate components of the human biological system. Simulated plaque lesions with average densities ranging from 0.92g/cm$^3$ to 1.26g/cm$^3$ were created. The densities of the simulated plaque lesions were manipulated by varying the concentration of the various components in the plaque mixture and dissolving various salts into the solution to further increase the density. Ten plaque replicates of each density were created.

2.3.3 Agarose plaque specimens

A standard agarose gel protocol used for gel electrophoresis was used to create agarose plaque specimens with reagent grade agarose (Sigma-Aldrich, Oakville, ON, Canada), 1X Tris/Borate/EDTA (TBE) buffer. The density of agarose plaque specimens was manipulated by varying the concentration of agarose in the agarose mixture. Agarose mixtures were poured into a 1.0 mL mold and left to set. Simulated agarose plaque specimens containing 2%, 4%, 6%, and 8% agarose with various salts were created, with details listed in Table 2.1.
Table 2.1 Average plaque densities and corresponding GSM.

<table>
<thead>
<tr>
<th>Plaque Labels</th>
<th>Description</th>
<th>N</th>
<th>Density (g/cm³) Mean</th>
<th>Density (g/cm³) Std. Dev.</th>
<th>Avg GSM Mean</th>
<th>Avg GSM Std. Dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Agarose</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2% AG</td>
<td>2% Agarose</td>
<td>4</td>
<td>1.027</td>
<td>0.007</td>
<td>33.92</td>
<td>11.42</td>
</tr>
<tr>
<td>4% AG</td>
<td>4% Agarose</td>
<td>4</td>
<td>1.033</td>
<td>0.008</td>
<td>39.75</td>
<td>13.69</td>
</tr>
<tr>
<td>6% AG</td>
<td>6% Agarose</td>
<td>4</td>
<td>1.039</td>
<td>0.020</td>
<td>61.33</td>
<td>21.91</td>
</tr>
<tr>
<td>8% AG</td>
<td>8% Agarose</td>
<td>4</td>
<td>1.066</td>
<td>0.014</td>
<td>98.50</td>
<td>35.07</td>
</tr>
<tr>
<td>AG2C</td>
<td>6% Agarose + 1% Hydroxyapatite</td>
<td>7</td>
<td>1.077</td>
<td>0.005</td>
<td>103.05</td>
<td>18.22</td>
</tr>
<tr>
<td>AG3A</td>
<td>6% Agarose + 3% Hydroxyapatite</td>
<td>7</td>
<td>1.080</td>
<td>0.003</td>
<td>118.29</td>
<td>19.73</td>
</tr>
<tr>
<td>AG8C</td>
<td>10% Agarose + 2.5% Sodium monophosphate</td>
<td>6</td>
<td>1.085</td>
<td>0.010</td>
<td>121.11</td>
<td>27.68</td>
</tr>
<tr>
<td>AG5A</td>
<td>8% Agarose + 2.5% Hydroxyapatite</td>
<td>7</td>
<td>1.086</td>
<td>0.006</td>
<td>127.52</td>
<td>27.68</td>
</tr>
<tr>
<td>AG1A</td>
<td>8% Agarose + 3.5% Hydroxyapatite</td>
<td>7</td>
<td>1.089</td>
<td>0.021</td>
<td>129.19</td>
<td>26.77</td>
</tr>
<tr>
<td>AG4A</td>
<td>6% Agarose + 4% Hydroxyapatite</td>
<td>7</td>
<td>1.137</td>
<td>0.066</td>
<td>140.81</td>
<td>23.14</td>
</tr>
<tr>
<td></td>
<td><strong>Plastisol + salt mixture</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A mix</td>
<td>25% porous silicon + pre-mix plastisol</td>
<td>7</td>
<td>0.918</td>
<td>0.005</td>
<td>75.05</td>
<td>30.46</td>
</tr>
<tr>
<td>B mix</td>
<td>Pre-mix plastisol</td>
<td>7</td>
<td>0.995</td>
<td>0.004</td>
<td>84.67</td>
<td>19.36</td>
</tr>
<tr>
<td>C mix</td>
<td>Pre-mix plastisol + 25% NaCl</td>
<td>7</td>
<td>1.077</td>
<td>0.004</td>
<td>103.05</td>
<td>22.62</td>
</tr>
<tr>
<td>D mix</td>
<td>Pre-mix plastisol + 50% NaCl</td>
<td>7</td>
<td>1.173</td>
<td>0.026</td>
<td>117.57</td>
<td>19.50</td>
</tr>
<tr>
<td>E mix</td>
<td>Pre-mix plastisol + 12.5% hardener + 50% NaCl</td>
<td>7</td>
<td>1.251</td>
<td>0.027</td>
<td>136.48</td>
<td>16.10</td>
</tr>
<tr>
<td></td>
<td><strong>PVC + Softener</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 to 1</td>
<td>PVC + 50% softener</td>
<td>7</td>
<td>0.960</td>
<td>0.008</td>
<td>32.05</td>
<td>12.73</td>
</tr>
<tr>
<td>3 to 1</td>
<td>PVC + 33% softener</td>
<td>7</td>
<td>0.965</td>
<td>0.001</td>
<td>38.57</td>
<td>20.65</td>
</tr>
<tr>
<td>4 to 1</td>
<td>PVC + 25% softener</td>
<td>7</td>
<td>0.969</td>
<td>0.004</td>
<td>41.90</td>
<td>20.16</td>
</tr>
<tr>
<td>5 to 1</td>
<td>PVC + 20% softener</td>
<td>7</td>
<td>0.974</td>
<td>0.004</td>
<td>47.10</td>
<td>19.35</td>
</tr>
<tr>
<td>6 to 1</td>
<td>PVC + 15% softener</td>
<td>7</td>
<td>0.979</td>
<td>0.007</td>
<td>55.86</td>
<td>27.11</td>
</tr>
</tbody>
</table>
2.3.4 Plastisol and salt plaque specimens

Plastisol plaque specimens were created from custom mixed plastisol liquid plastic (Chromalux “Lunker sol”, F.H. & Sons Manufacturing Ltd, Etobicoke, ON, Canada). Concentration of plastisol, hardener containing epoxy resins (Spike-It Lureworks™, Brooklet, GA, USA), reagent grade sodium chloride (Sigma-Aldrich, Oakville, ON, Canada), and silicon dioxide (Sigma-Aldrich, Oakville, ON, Canada) were manipulated to create plaque specimens of various densities. Plastisol plaque specimens with density of 0.9144g/cm³ were first created with the custom mixed plastisol mixture. Sodium chloride (NaCl) was then added to increase the density of plastisol specimens. To further increase the density of the plaque specimens, standard plastisol hardener (Spike-It Lureworks™, Brooklet, GA, USA) was added. Silicon dioxide was added to further increase the density of the plaque specimens following the addition of NaCl and plastisol hardener. Plastisol mixtures were heated to 310-350°F depending on the specific mixture. Heated plastisol mixtures were then poured into a 1.0 ml mold and left to set at RT. Simulated plastisol plaque specimens with 5 different densities were created with details listed in Table 2.1.

2.3.5 PVC and softener plaque specimens

PVC plaque specimens were created from a formula of polyvinyl chloride particles suspended in a liquid plasticizer (M-F Manufacturing, Fort Worth, TX, USA). Density of PVC plaque specimens was manipulated by varying the ratio between PVC and polycarboxylic acid esters acting as softener (M-F Manufacturing, Fort Worth, TX, USA). PVC mixtures were heated to 325°F and poured into a 1.0 mL mold. PVA mixtures were then left to set in cool water (5°C) to ensure a faster process of cooling and preclude air bubbles from forming within the plaque specimens. Simulated PVC plaque specimens containing 6:1, 5:1, 4:1, 3:1, and 2:1 ratio of PVC to softener were created with details listed in Table 2.1.

2.3.6 Density measurements
Densities of simulated plaque specimens were calculated by the buoyancy density measuring method, which has been previously used to measure the density of excised human atherosclerotic plaque(47). The simulated plaque sample of interest was tied to a lightweight silk thread and submerged into a fluid with known density (water, 1g/cm$^3$; ethanol, 0.789g/cm$^3$). The weight of the plaque was measured before and after the application of buoyancy force. The density was calculated with the following equation:

$$\rho_{sp} = \frac{P(S)}{(F - K)}$$  \hspace{1cm} (1)

Where $\rho_{sp}$ is the density of the plaque specimen of interest, $P$ is the mass of the specimen, $S$ is the fluid density, $F$ is the combined mass of specimen, support and thread, and $K$ is the combined mass of specimen, support and thread after immersing specimen into the fluid of known density. Densities of various types of rat tissues were also measured to serve as a standard for comparison.

2.3.7 Image acquisition and analysis

Simulated plaque specimens were assembled into the phantom vessel. The phantom model was then immersed in water, with the water filling the lumen of the vessel. We did not create blood mimic for the phantom model as water has a similar echogenicity as blood. Although blood is a colloid, ultrasound waves do not possess enough sensitivity to detect the plasma component of blood. B-mode ultrasound images were then acquired with a GE Vivid E9 ultrasound machine and a 9L-D linear probe (GE Healthcare, Mississauga, ON, Canada). Images of each plaque specimen were taken at three different time gain compensations (TGC) at below medium, medium, and above medium TGCs. In this manner, the variation in echogenicity of the images was minimized. Though normalization of gray scale was conducted during offline image analysis, variation in echogenicity was still frequently observed between the same plaque specimens with the same density. Using the average GSM value of the three different
TGCs, along with gray scale normalization, we accounted for variation in echogenicity resulted from the freehand manual aspect of image acquisition in ultrasound. The distance between the linear probe and the simulated plaque of interest was fixed at 1.5 cm. Following image acquisition, ultrasound images were exported from EchoPAC (v. 113, GE Healthcare, Mississauga, ON, Canada) as high quality TIF files. Photoshop (v. CC 2015.2.2, Adobe Systems Incorporated) was then used to analyze ultrasound images and calculate GSM. Plaque echogenicity was characterized using GSM measurements (grayscale range: 0-225; black = 0, white = 225). A standard curve correlating to plaque echogenicity and density was developed. Images of simulated plaque specimens were normalized with linear scaling by the software, where the blood/lumen was assigned a GSM scale of 0-5 and the adventitia of the vessel was assigned a GSM scale of 185-195(31). A hardened layer of PVA-C outlining the simulated carotid vessel was chosen as the surrogate for adventitia in grayscale normalization. After normalization, the simulated plaque was outlined and its GSM calculated from the histogram (Figure 2. 2A). The same process was repeated for the various types of rat tissues that were previously measured for density to serve as a standard for comparison.
Figure 2.2. GSM analysis and optimization of simulated plaque lesions.

A) Grayscale histogram of outlined plaque. A median value of grayscale is shown within red box. B) Echolucent plastisol plaque outlined with an echogenic material allowing for accurate outlining of simulated plaque lesion during GSM analysis.
2.3.8 GSM optimization

GSM optimization was conducted on all echolucent plaque specimens that did not show a clear outline in their respective images, and thus were unable to be outlined accurately during GSM analysis. Optimization was conducted by coating echolucent plaque specimens with a thin layer of echogenic material with a synthetic sable brush. The echogenic material is a mixture of propylene glycol, titanium dioxide, carbon black, nickel, cadmium sulfide, and cadmium selenide. The coated plaque specimens were then left to dry for 24 hours to ensure proper hardening of the echogenic material. The resulting image showed a plaque with a clear echogenic border encasing the echolucent plaque that could be accurately outlined as demonstrated by Figure 2.2B. After GSM optimization, GSM analysis was conducted on echolucent plaque according to the protocol described.

2.3.9 Statistical Analysis

The GSM value of each plaque specimen was calculated as the average GSM value of the three normalized images taken for each specimen at below medium, medium, and slightly above medium TGCs. Data from each simulated plaque replicate of all plaque types were used for the linear regression analysis with JMP (v. 12.0.1., SAS Institute Inc., North Carolina, USA). Linear regression analysis was also conducted separately with data from each individual plaque mixture in order to determine whether the density of a specific mixture was better able to predict echogenicity. Pearson’s correlation coefficient was determined between the average mass density of the simulated plaque specimens and their GSM values.
2.4 Results

2.4.1 Visual evaluation of simulated plaque specimens

To begin, differences in echogenicity between simulated plaque lesions were assessed qualitatively from initial ultrasound images. Figure 2.3 illustrates 5 simulated plaque lesions (A mix, B mix, C mix, D mix, E mix) created from the plastisol and salt mixture with increasing density from A mix – E mix. It was observed that there was a correlating increase in brightness (echogenicity) with the increase in plaque density.

2.4.2 Linear Regression Analysis

The average mass density of the simulated plaque lesions had a significant positive coefficient of determination with plaque GSM ($r^2=0.554$, $p<0.0001$, Figure 2.4A). Upon further investigation of the data presented in Figure 2.4A, each data point was color coded according to the specific plaque mixture to demonstrate individual trends (Figure 2.4B). Although the linear regression of all simulated plaque lesions had a significant positive $r^2$ value, individual linear regression analysis of each specific material provided even stronger linear fits, as shown by Figure 2.5. Of the 3 different plaque mixtures, the plastisol & salt simulated plaque ($r^2=0.726$) compared to the other two plaque mixtures (Figure 2.5).
Figure 2.3. Qualitative assessment of simulated plaque lesions. Ultrasound image of five simulated plastisol & salt plaque lesions with increasing density from top to bottom, placed into a simulated vessel, surrounded top and bottom by PVA simulated soft tissue.
Figure 2.4. Linear regression analysis.

A) Linear fit of all simulated plaque types combined. P<0.0001. B) Linear fit of all simulated plaque types combined – colour-coded according to specific plaque mimic mixtures. P<0.0001.
Figure 2.5. Linear fit of each plaque mixture.

Agarose simulated plaque lesions, plastisol & salt simulated plaque lesions, PVC & softener simulated plaque lesions display a coefficient of determination ($r^2$) of 0.595, 0.603, 0.502, respectively, compared to the linear regression of rat tissues with a $r^2$ of 0.726. $P<0.0001$ all. The red circles denote a data point from simulated plaque lesions of different plaque mimic mixtures with a density of 1g/cm$^3$, but with variable GSM values, showing regression slopes that are dependent on the specific plaque mimic mixture.
Corresponding to the coefficient of determination, the Pearson’s linear correlation coefficient \( r \) of all plaque mixtures also displayed significant positive linear correlation with \( r \) values of 0.772, 0.777, 0.709 for agarose simulated plaque lesions, plastisol & salt simulated plaque lesions, and PVC & softener simulated plaque lesions, respectively, as shown in Table 2.2. Simulated plaque lesions created from the plastisol & salt plaque mixture displayed a correlation coefficient of \( r=0.777 \) closest to real tissues.

**Table 2.2. Correlation between density and GSM for each plaque type.**

<table>
<thead>
<tr>
<th>Plaque Type</th>
<th>Pearson's r</th>
<th>CI Lower 95%</th>
<th>CI Upper 95%</th>
<th>Coefficient of determination ( r^2 )</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose</td>
<td>0.772</td>
<td>0.640</td>
<td>0.859</td>
<td>0.595</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plastisol+salt</td>
<td>0.777</td>
<td>0.598</td>
<td>0.882</td>
<td>0.603</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PVC+softner</td>
<td>0.709</td>
<td>0.492</td>
<td>0.843</td>
<td>0.502</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Rat tissue</td>
<td>0.852</td>
<td>0.678</td>
<td>0.936</td>
<td>0.726</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

**2.5 Discussion**

Despite the call from various imaging societies for improved standardization of advanced ultrasound analytical techniques, there are limited studies reporting the creation of phantom models, and certainly even fewer on plaque simulation. This gap becomes more important as carotid plaque characterization continues to emerge as an important risk stratification tool, and more vendors develop off-line analytical software. An *ex vivo* phantom study is advantageous in that it allows for the evaluation of the relationship between density and plaque echogenicity without the pressure of time constraints, patient variability present in clinical settings, as well as provides well-controlled and reproducible conditions.

We first created a reference standard ultrasound phantom consisting of simulated carotid artery plaque lesions with a range of known densities. We then validated the GSM method against these known
densities. We demonstrated that GSM is a highly reproducible index of echogenicity with a significant linear correlation between plaque density and GSM. Plaque density was found to have a significant positive linear correlation with GSM in all three plaque mixtures. Interestingly, simulated plaque lesions created from the plastisol & salt mixture has a relationship between average mass density and echogenicity most similar to that of real tissues, where the coefficient of determination ($r^2$) and the Pearson’s correlation coefficient ($r$) are the closest to that of real tissues. This suggests that while density of all three plaque mixtures predicted the simulated plaque structures’ echogenicity, the plastisol & salt mixture exhibited mechanical properties most similar to real biological tissues, thus providing insight into any future plaque mimic studies. In accordance to the coefficient of determination, the Pearson’s correlation coefficients of all plaque samples combined and of each individual plaque mixture also demonstrated a significant positive association between the average mass density and the echogenicity of the simulated plaque lesions ($p<0.0001$).

In addressing the difference in coefficient of determination and the slope of correlation between the three different plaque mixtures, we suggests the possible influence of other material constants impacting the echogenicity of our simulated plaque structures. We had synthesized our hypothesis based on the concept of acoustic impedance ($Z$), which directly affects the reflection coefficient ($r$) of the medium as shown by the following equation(48).

$$r = \frac{(Z_1-Z_2)}{(Z_1+Z_2)}$$  \hspace{1cm} (2)

The reflection coefficient of the medium represents the proportion of ultrasound waves that bounce back, and therefore directly affect the echogenicity of the structure of interest. The acoustic impedance equation states that the acoustic impedance of a medium is dependent on the density of the tissue ($p$, in kg/m$^3$) and the speed of the sound wave ($c$, in m/s). It is fundamental physics knowledge that sound waves travel at the same speed in the same medium regardless of wave frequency. The constant
value of the speed of an ultrasound wave in the same medium directs our attention to investigate the density variable as we have done in this phantom model. According to the acoustic impedance equation, the denser the structure, the larger its acoustic impedance will be. A larger $Z_1$ will result in a larger reflection coefficient, suggesting that a larger fraction of the original ultrasound waves sent out will be reflected and received. This is indicative of a positive linear relationship between the density and the echogenicity of the medium (higher GSM value).

The differences in the coefficient of determination and the slope of regression between the three plaque mixtures suggest that the change in the expected value of echogenicity corresponds differently to 1-unit increase in the average mass density of the simulated plaque, depending on the specific plaque mixture. This indicates that there are variables other than speed of sound wave ($c$, in m/s) and average mass density ($p$, in kg/m$^3$) that can have an effect on the acoustic impedance of the medium. These variables are likely dependent on the medium composition of each individual plaque mixture. A potential variable, differing between the materials we used, could be “stiffness”.

Stiffness and density are fundamentally two different properties of materials. Stiffness is a material constant measured by Young’s modulus, where it represents the elastic modulus per mass density of a material in resistance to an applied force. Though it is often associated with density, where the “stiffness” of a material tends to increase with density, the relationship between these two variables is not necessarily a simple linear relationship. In ultrasound, wave velocity is the same within the same medium. Therefore, the physical properties of the specific medium including density and stiffness can have an effect on wave velocity. Sound waves travel faster in a stiffer medium but slower in a denser medium. Since we are unsure as to whether the stiffness of our simulated plaque specimens is directly proportional to its respective density, it can have unpredictable effects on the acoustic impedance that is dependent on the medium composition of the specific plaque mixture. The degree to which the stiffness of the simulated plaque lesions may increase in response to a unit increase in density is unknown in our plaque mimic mixtures. This can affect the portion of waves that bounces back and are received by the
transducer, potentially resulting in a less reliable quantification of echogenicity. The unpredictable effects of the stiffness variable in our different materials on acoustic impedance may account for the discrepancy observed in the estimated change in the dependent variable (echogenicity) for a give unit change of the independent variable (average mass density) between the three different plaque mixtures.

Shear wave elastography (SWE) was previously used to quantify the Young’s Modulus, or the stiffness, of carotid plaque lesions (49). It was demonstrated that the Young’s Modulus value of unstable plaque lesions was significantly lower than that of stable plaque lesions. However, no protocols or recommendations exist for SWE in vascular application as it is considered to be a relatively novel technique. In comparison, GSM analysis proves to be a much more accessible and user-friendly method. SWE studies also tend to focus on differentiating between diseased and normal tissue on the basis of tissue stiffness rather than echogenicity (49, 50). This study suggests that tissue stiffness may be related to plaque echogenicity, and the focus should perhaps be directed to investigate the relationship between these two variables.

Previous studies have shown that GSM measurements of carotid plaque lesions can be a useful reference index for cardiovascular disease events and cerebrovascular events (30, 45) in high-risk atherosclerotic diabetics (46). Other studies have attempted to correlate GSM with atherosclerotic plaque activity (51, 52). This is the first study using GSM analysis to target an intrinsic physical property of plaque lesions – density, with the idea that density can be used as an indicator that is reflective of plaque vulnerability. We understand that unlike our simulated plaque lesions, real human atherosclerotic plaque lesions are often heterogeneous, composing of various tissue types including lipid tissues, fibrous tissues, calcified tissues, and fibromuscular tissues (53, 54). Traditionally, the composition of heterogeneous atherosclerotic plaque lesions have been investigated with experimental methods like optical coherence tomography (55, 56), intravascular catheter based thermography (54, 57), or computed tomography (58, 59). Until recently, a study determined GSM ranges for different tissue types, and utilized these GSM ranges to develop the pixel distribution analysis (29, 60). This study identified various components of
atherosclerotic plaque lesions including intraplaque hemorrhage, lipid, fibromuscular tissue and calcium. The pixel distribution analysis method is in concordance with our concept that the echogenicity of a medium, characterized by GSM values, is reflective of the intrinsic properties of a medium. This study has demonstrated that changes in echogenicity correspond to changes in density of our simulated plaque lesions. Knowing that different tissue types in the body have different densities, future studies can be conducted to validate the pixel distribution analysis with heterogeneous simulated plaque lesions composing of different components where densities correspond to different tissue types.

2.6 Limitations

The simulated plaque specimens developed and investigated in this present study were homogenous, in contrast to real human atherosclerotic plaque lesions, which are often heterogeneous. Though the main focus of the present study was density - a fundamental intrinsic physical property, it may limit the study’s clinical applicability. This limitation can be overcome by developing heterogeneous plaque, and conducting plaque composition analysis based on GSM values(51). Additionally, real plaque specimens have rougher surfaces than simulated plaque, especially those developed from materials like plastisol. The irregular surfaces on atherosclerotic plaque can result in diffuse reflection, where ultrasound beams are scattered to produce weaker images(61). Most ultrasound machines currently available on the market are calibrated to account for this scattering effect. However, in our simulated plaque specimens, the smooth surfaces can lead to strong specular reflections, resulting in hyper-echoic images. This can result in a skewed measurement of the echogenicity of our simulated plaque. This limitation can be overcome by etching the smooth surfaces of our simulated plaque using plasma, laser, chemical or electrochemical techniques(62) to optimize simulated plaque texture. In the future, the echogenicity measured from our simulated plaque can be cross-referenced to the GSM measured from real atherosclerotic plaque of various plaque types, including calcified, fibrotic, or fatty to provide insight into the density of these plaque types.
Finally, the unpredictable effect that stiffness has on the acoustic impedance of our simulated plaque specimens can potentially result in a less reliable quantification of echogenicity. This limitation can be overcome by investigating simulated plaque specimens based solely on plaque stiffness. This can be accomplished by using various ultrasound elastography techniques, including acoustic radiation force impulse imaging (ARFI), shear wave elasticity imaging (SWEI), supersonic shear imaging (SSI) or transient elastography (63). A future phantom model could allow for controlling the density variable while assessing the effect of the stiffness variable on the GSM value.

2.7 Conclusion

In the present study, a ultrasound phantom model of simulated atherosclerotic plaque lesions was created. With this phantom model, it was shown that GSM had a positive, linear correlation with the density of the simulated plaque. The slope of this correlation was impacted by other acoustic factors of the material beyond density and could include stiffness. The relationship delineated in this study between plaque density and echogenicity using GSM analysis proved that ultrasound is able to detect a difference in the physical properties of our simulated plaque lesions. This finding corresponds to the underlying hypothesis of tissue characterization that a different biochemical structure, internal architectural arrangement, or physiological state of normal tissue or pathological tissue, can affect the physical properties of the tissue. Tissue characterization is listed by the American Society of Echocardiography roadmap to 2020 as one of the most promising fields in cardiovascular ultrasound imaging. Our finding can assist in the development of guidelines for vulnerable plaque diagnosis and tissue characterization. The concept that the echogenicity of a structure, characterized by GSM values, is reflective of an intrinsic property of a medium like density – can be used as an indicator that is reflective of plaque vulnerability. Since echolucent plaque lesions with a lower GSM value, is indicative of vulnerability, a potential approach for assessing patient risk based on plaque vulnerability would be to assess plaque density.
2.8 Acknowledgments

We would like to thank Ms. Julia Herr for her expertise in GSM analysis and the GSM analysis protocol used in this study, and Mr. Mike Deslauriers of Lip Locked Baits for providing his advice and expertise on working with plastisol.

2.9 Funding Sources

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Chapter 3

Development of an Ultrasound Heterogeneous Plaque Phantom in the evaluation of Colourized Pixel Distribution Analysis

3.1 Abstract

**Background:** Colourized pixel distribution analysis (PDA) is a novel method of assigning gray scale median (GSM) ranges to pattern the composition of a heterogeneous plaque. This study aimed to develop a phantom model of heterogeneous plaque, and to establish a new set of tissue GSM ranges for colourized PDA to be tested in a controlled, in-vitro phantom environment.

**Methods:** Simulated heterogeneous plaque lesions were created by encasing different rat tissue types in polyvinyl chloride. Two sets of tissue GSM ranges were established from two-dimensional (2D) ultrasound images of rat tissues in two ways: 1) All muscle subtypes were combined to establish a GSM range in addition to fat tissue and bone tissue 2) GSM ranges of each muscle subtypes were individually calculated in addition to fat tissue and bone tissue. Using the two sets of GSM ranges, colourized PDA was conducted on 2D ultrasound images of five simulated heterogeneous plaque types. Percent of pixels identified in the correct tissue range were compared between the two sets of ranges.

**Results:** The two sets of tissue GSM ranges were established (95% confidence interval lower, upper): 1) echolucent plastisol 0-4, muscle 84-95, fat 99-113 (visceral fat), and bone 145-175; 2) echolucent plastisol 0-4, muscle 68-86 (Neck muscle and leg muscle) and 104-108 (abdominal muscle), fat 91-100 (visceral fat) and bone 145-175. Of the two sets of ranges established, the second method detected a greater percentage of pixels within the correct tissue type.

**Conclusion:** A heterogeneous plaque phantom for ultrasound has been developed. We established a new set of tissue GSM ranges representing potential tissue types that may be identified within a heterogeneous plaque. We tested the colourized PDA algorithm in a controlled, in-vitro environment using these ranges, and detected the highest percent of pixels in the correct tissue range in four out of five types of simulated heterogeneous plaque lesions. The phantom developed can serve as a platform to advance plaque characterization techniques.
3.2 Introduction

Plaque vulnerability is a highly investigated field, with recent studies investigating vulnerability features associated with cardiovascular (CV) events. Vulnerability features including plaque morphology such as plaque size, shape, volume\(^{(24,64)}\), or plaque pathophysiology such as inflammation, proteolysis, apoptosis and angiogenesis\(^{(65,66)}\), were previously addressed. Vascular ultrasound is a non-invasive, portable, and cost-effective imaging modality to investigate plaque vulnerability, with plaque echogenicity and tissue composition characterization as two common techniques used to investigate vulnerability features.

Plaque echogenicity, a plaque vulnerability feature that can be characterized by gray scale median (GSM) values, has been demonstrated to be a useful index for predicting CV events\(^{(30–32,35,46)}\). However, using GSM to assess plaque vulnerability is subject to several limitations. GSM measures the median brightness of an entire plaque lesion and does not account for regional variability associated with the heterogeneous nature of most atherosclerotic plaque\(^{(10)}\). Tissue characterization analysis was proposed to be an effective approach to characterize the regional variability of plaque density and composition.

Ultrasound methods of tissue characterization in plaque composition investigation has mostly been conducted using intravascular ultrasound (IVUS) of coronary artery lesions\(^{(67–69)}\). While these studies demonstrated the accuracy of this technique, IVUS has limited clinical usage due to its invasiveness and cost. Colourized pixel distribution analysis (PDA), also known as colour mapping, or ultrasound virtual histology (USVH), is a non-invasive novel technique of tissue characterization. In this
method, a surface ultrasound of a plaque lesion can be used to assign GSM ranges associated with specific tissue types to pattern the composition of a heterogeneous plaque lesion. Previous studies have shown correlation between plaque composition determined by histology and colourized PDA(29,60,65). However, there are limitations to histological correlation studies. Current histological analyses are conducted using only a thin micrometric cross-section that may not be representative of the whole plaque. While conventional 2D ultrasound images are also taken as a “slice” of the plaque lesion, it is difficult to match the histological slice with the 2D ultrasound image, weakening the correlation. A controlled, objective, and reproducible way of validating the computerized assessment of components of plaque heterogeneity is needed.

This study aims to develop a heterogeneous atherosclerotic plaque lesion phantom, where the composition and location of the tissues within the simulated heterogeneous plaque are carefully designed. We then aim to establish a detailed set of tissue GSM ranges. These ranges represent potential tissue types that can be identified within atherosclerotic plaque lesions, addressing the previously suggested concern that a colour scale with GSM ranges representing more specific tissue types is needed for the improvement of the colourized PDA method(65). Finally, we aim to test the colourized PDA algorithm with our established tissue GSM ranges in a well-controlled, ex-vivo environment using the heterogeneous plaque phantom created.

3.3 Materials and Methods

3.3.1 Creation of a phantom model – soft tissue

To simulate the carotid vessel, a vascular phantom was constructed. Poly(vinyl Alcohol) Cryogel (PVA-C)(18) was selected to create the soft tissue surrounding the simulated vessel lumen. A 10% by weight aqueous PVA (Sigma-Aldrich, Oakville, ON, Canada) solution was first weighed, and then heated to 121°C on a magnetic stir plate to fully dissolve aggregates of PVA powder. The clear PVA solution
was left to cool to room temperature (RT). The solution was again weighed, where any loss in mass was replaced with de-ionized water to ensure the consistency of the 10% PVA solution. The PVA solution was poured into a mold containing a 1.0 cm diameter tube and left to set in a sealed container for 12-24 hours, allowing for air bubbles to rise to the surface. This ensures the formation of a homogeneous soft tissue phantom, avoiding potential interference of ultrasound signals from echogenic air bubbles. The PVA solution was gelled via a freeze-thaw cycle between -20°C and RT, where crystallites were formed, cross-linking the solution into a homogeneous hydrogel. The freezing process occurred over the course of 2 hours, after which the phantom was maintained at freezing temperature for an additional 10 hours, totaling to 12 hours of freeze stage time. After the freeze stage, the phantom was thawed at RT over the course of 8-9 hours. The carotid artery lumen was created by careful removal of the rubber tube. The cryogel was stored while wrapped in cling film, with the vessel lumen exposed to air. A dry membrane was formed where the cryogel was exposed to air, resulting in a simulated vessel wall.

3.3.2 Creation of a phantom model – heterogeneous plaque lesions

Heterogeneous phantom plaque lesions were created by immersing rat tissues such as *acromiotrapezius muscle* (neck), *biceps femoris* (leg), *rectus abdominus muscle* (abdomen), epididymal fat (visceral fat), and the femur bone tissues into a custom mixed polyvinyl chloride, also known as plastisol liquid plastic (Chromalux “Lunker sol”, F.H. & Sons Manufacturing Ltd, Etobicoke, ON, Canada). The echolucent plastisol serve to simulate the echolucent blood/hemorrhagic component of heterogeneous plaque lesions. Gelation process of the plastisol solution was activated with heated (150-180°C). The solution was heated and stirred until viscous, indicating a system change from a suspension of polyvinyl chloride particles in a liquid medium to a fused state(70). A thin layer of polymerized plastisol solution was dispensed into a metal mold containing 40 spherical cavities with a diameter of 0.80 cm. One rat tissue type was then placed within each cavity. The metal mold was screwed together, and fused state plastisol solution was injected into the mold. The mold was left to chill at 4°C for 1 hour.
After the chilling process, the metal mold was unscrewed, and simulated heterogeneous plaque lesions were retrieved. Each simulated heterogeneous plaque was composed of a specific type of rat tissue suspended in echolucent plastisol. A total of five types of simulated heterogeneous plaque lesions were created as follow: 1) *acromiotorrpezius* (neck) muscle heterogeneous plaque, 2) *biceps femoris* (leg) muscle heterogeneous plaque, 3) *rectus abdominus* (abdomen) muscle heterogeneous plaque, 4) epididymal (visceral) fat tissue heterogeneous plaque, and 5) femur bone heterogeneous plaque.

### 3.3.3 Establishing grayscale ranges for control tissues

All rat muscle, lipid, and bone tissue types were individually placed in the carotid vessel phantom. The phantom model was immersed in water and imaged with a GE Vivid E9 ultrasound machine and a 9L-D linear transducer (GE Healthcare, Mississauga, ON, Canada). Tissue subtypes were used for the tissue category of muscle, as described by Table 3.1. Ten images were obtained for each type of tissue, and exported from EchoPAC (v. 113, GE Healthcare, Mississauga, ON, Canada) as high quality TIF files. Photoshop (v. CC 2015.2.2, Adobe Systems Incorporated) was used to analyze ultrasound images and calculate associated GSM ranges. Image normalization was conducted, and tissue samples were outlined. The median value of the pixel intensity generated by the outlined tissue sample was determined. Two sets of GSM ranges were established with a 95% upper and lower confidence interval by different methods: 1) GSM of all muscle subtypes were combined to calculate a GSM range for the tissue category of muscle. GSM ranges of fat, and bone were established with epididymal fat tissue samples and femur bone tissue samples, respectively. Each tissue category (muscles, fats, bones), in addition to the echolucent plastisol used to encase the tissue samples, was assigned a specific colour. 2) GSM ranges of all muscle tissue subtypes (leg, neck, abdominal), in addition to the fat tissues (epididymal) and bone (femur) tissues were individually calculated. All tissues in the same tissue category (muscle, fat, bone) were assigned the same colour.
Table 3.1. Tissue GSM ranges.

<table>
<thead>
<tr>
<th>Tissue Category</th>
<th>Tissue Subtype</th>
<th>First set of tissue GSM ranges</th>
<th>Second set of tissue GSM ranges</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plastisol</td>
<td>Plastisol</td>
<td>0-4</td>
<td>0-4</td>
</tr>
<tr>
<td>Muscle</td>
<td>Neck (Acromiotrapezius)</td>
<td>84-95</td>
<td>68-87</td>
</tr>
<tr>
<td>Muscle</td>
<td>Leg (Biceps femoris)</td>
<td>84-95</td>
<td>76-90</td>
</tr>
<tr>
<td>Muscle</td>
<td>Abdomen (rectus abdominus)</td>
<td>84-95</td>
<td>104-108</td>
</tr>
<tr>
<td>Fat</td>
<td>Visceral (epididymal fat)</td>
<td>99-113</td>
<td>87-100</td>
</tr>
<tr>
<td>Bone</td>
<td>Long bone (femur)</td>
<td>145-175</td>
<td>145-175</td>
</tr>
</tbody>
</table>

3.3.4 Ultrasound scan of heterogeneous plaque phantoms

Simulated heterogeneous plaque lesions were placed in the carotid vessel phantom. The phantom model was immersed in water and imaged. B-mode images were taken at medium time gain compensation across the full depth of the image. The distance between the linear transducer and the simulated plaque of interest was fixed at 1.5 cm. Blood mimic for the phantom model was not necessary as the microscopic plasma components are beyond the resolution limitation of the ultrasound. Each image exemplified 1) a simulated heterogeneous plaque composed of a specific rat tissue type suspended in echolucent plastisol, 2) a tissue sample corresponding to the suspended tissue shown in the heterogeneous plaque as a control, and 3) a sphere of plain plastisol as a control (Figure 3.1).
Figure 3.1. B-mode ultrasound image of phantom model.

From left to right 1) Simulated heterogeneous plaque with abdominal muscle, 2) Abdominal muscle tissue sample control, and 3) Plastisol control.
3.3.5 Validation of phantom standards of normalization

Images of simulated plaque specimens were normalized with linear scaling by Photoshop. This results in a shift in all pixels in the image according to new assigned standards from two reference areas in longitudinal views of the vessel: the echo-free luminal area, and an echogenic area in the simulated far vessel wall. The blood/lumen GSM value was adjusted to a value between 0-5 and the adventitia of the vessel was assigned a GSM scale of 185-195 (31). GSM values of the stimulated vessel wall were validated with human adventitia prior to image normalization. GSM values of the phantom adventitia were calculated by selecting a section of the simulated adventitia from 20 random images of the phantom model. Twenty ultrasound images of carotid vessels from human subjects were obtained, and GSM values of these vessels were likewise calculated. An independent two-sample t-test was conducted to determine any significant difference in GSM between the far vessel wall of the phantom model and the human subjects.

3.3.6 Image analysis – colourized pixel distribution analysis of simulated heterogeneous plaque with control tissue grayscale ranges

In the two sets of tissue GSM ranges established, the echolucent plastisol used was assigned red, fat tissues were assigned yellow, muscle tissues were assigned green, and bone tissues representing calcifications were assigned blue, as shown in Figure 3.2. Any regions of GSM overlap between the tissue ranges were assigned black. Colourized PDA was applied using the two established tissue GSM ranges to ultrasound images of heterogeneous plaque phantoms and controls (Figure 3.2).
Figure 3.2. Colourized PDA on epididymal fat heterogeneous plaque, epididymal fat tissue control, and plastisol control.

The three structures observed in the simulated vessel starting from the left is an epididymal fat heterogeneous simulated plaque, an epididymal fat tissue control, and a plastisol sphere control. A) Colourized PDA using first set of tissue GSM ranges. B) Colourized PDA using second set of tissue GSM ranges.
3.3.7 Statistical Comparison of tissue GSM ranges

Percent of pixels detected in the correct tissue range in the tissue sample control was compared with the tissue sample encased within the simulated heterogeneous plaque using two-sample independent t-tests. A matched-pair t-test was conducted between the two sets of tissue GSM ranges to determine any significant difference between the two methods. The set of tissue GSM ranges with higher percent of pixels detected within the correct tissue ranges was selected to be compared with a set of previously reported tissue GSM ranges(60). Percent of pixels detected in each tissue GSM range in the simulated heterogeneous plaque lesions were determined. The tissue range with the highest percent of pixels detected was identified in the five types of simulated heterogeneous plaque. Data within each tissue GSM range, including medians with interquartile ranges were compared and presented in a boxplot. All P values were two sided, and values of <0.05 were considered to indicate statistical significance. All data were analyzed with JMP version 12.0 (SAS Institute Inc., North Carolina, USA).

3.4 Results

3.4.1 Validation of Phantom Standards of normalization

An independent two-sample t-test was conducted to determine any significant difference between GSM of the phantom far vessel wall and that of human carotid vessels. The mean GSM of the phantom far vessel wall was found to be 148, while the mean GSM of human carotid vessel far vessel walls was found to be 145. No significant difference was observed between the GSM values of the far vessel wall of the phantom model and the human carotid vessels (p=0.3358; p>0.05).
3.4.2 Establishing grayscale ranges for control tissues

Two sets of GSM ranges representing muscle, fat, bone, and the echolucent plastisol encasing the tissue types were determined. Within the second set of GSM ranges where ranges of all muscle subtypes (leg, neck, abdominal), fat tissues (epididymal) and bone (femur) tissues were individually calculated, the GSM ranges of neck muscles and leg muscles overlap (Figure 3.3). These two ranges were combined to establish a broader GSM range. At the same time, an overlap between the tissue categories of muscle and fat was observed (Figure 3.3). The first set of GSM ranges includes: echolucent plastisol 0-4, muscle 84-95, fat 99-113 (visceral fat), and bone 145-175. The second set of GSM ranges includes: echolucent plastisol 0-4, muscle 68-86 (Neck muscle and leg muscle) and 104-108 (abdomen muscle), fat 91-100 (visceral fat) and bone 145-175. The overlap GSM range between the tissue categories of muscle and fat was 87-90.
Figure 3.3. GSM ranges by tissue type.

GSM ranges by tissue type, colour coded according to the specific colour assigned to the tissue type in the colourized PDA method.
3.4.3 Qualitative evaluation of simulated heterogeneous plaque lesions

Five types of simulated heterogeneous plaque lesions were assessed qualitatively from colourized images. Figure 3.2 illustrates 1) an epididymal fat simulated heterogeneous plaque, 2) an epididymal fat tissue control, and 3) a plastisol control. Both methods of colour assignment identified areas of yellow representing visceral fat using colourized PDA. However, colours of other tissue types were also observed within the known epididymal fat tissue. Specifically in Figure 3.2A, the colour green representing muscle tissues (3.7% in simulated heterogeneous plaque; 5.2% in tissue control), blue representing bone tissue or calcification (16.0% in simulated heterogeneous plaque; 30.0% in tissue control) were observed with the first set of GSM ranges, with only 11.1% of the whole heterogeneous plaque, and 18.0% of the tissue control identified as fat tissue. In Figure 3.2B, the colour green representing muscle tissues (9% in simulated heterogeneous plaque; 23.5% in tissue control), blue representing bone tissue or calcification (13.0% in simulated heterogeneous plaque; 22.2% in tissue control), and black representing overlap between tissue GSM ranges (5.4% in simulated heterogeneous plaque; 8.5% in tissue control) were observed using the second set of GSM ranges, with only 6.6% of the whole heterogeneous plaque, and 17.3% of the tissue control identified as fat tissue.

3.4.4 Statistical comparison of colourized PDA

Percent of pixels detected in the correct tissue range in the tissue control, and in the encased tissue sample within the simulated heterogeneous plaque are presented as mean ± SD in Table 3.2. Simulated heterogeneous plaque with epididymal fat tissue, and leg muscle tissue presented p values < 0.05 in both sets of tissue GSM ranges used, indicating a significant difference in percent of pixels detected in the correct tissue range between the simulated heterogeneous plaque and the tissue control. Simulated heterogeneous plaque lesions with neck muscles, abdominal muscles, and bone samples presented p values >0.05 in both sets of tissue GSM ranges used, indicating no significant difference in percent of pixels detected in the correct tissue range between the simulated heterogeneous plaque and the
tissue control. A matched-pair t-test presented p values <0.0001 in all five types of simulated heterogeneous plaque, indicating a significant difference in percent pixels detected in the correct tissue GSM range between the two established sets of ranges. The second set of ranges provided greater percentages of pixels detected in the correct tissue range.

With greater percentages of pixels detected in the correct tissue range, the second set of tissue GSM ranges were selected as more comprehensive. The percent of pixels detected in all GSM ranges in the simulated heterogeneous plaque lesions were presented as mean ± SD in Table 3.3 and was compared to a previously reported set of ranges(60). In both sets of ranges, tissue GSM ranges with the highest percent of pixels detected were identified and bolded in Table 3.3, for all five types of simulated heterogeneous plaque. The percent of pixels detected in the plastisol GSM range were not included as the range with the highest percent of pixels detected, as these percentages represent the plastisol encasing the tissue sample in the simulated heterogeneous plaque lesions. Using the second set of tissue GSM ranges, the specific tissue GSM range with the highest percent of pixels detected corresponds to the specific tissue type encased within the echolucent plastisol in four out of the five simulated heterogeneous plaque types developed, namely the heterogeneous plaque with leg muscle tissue, heterogeneous plaque with neck muscle tissue, heterogeneous plaque with abdominal tissue, and heterogeneous plaque with bone tissue. On the contrary, using the previously established set of tissue GSM ranges, none of the heterogeneous plaque types developed has the highest percent of pixels detected within a tissue GSM range that corresponds to the specific tissue encased within the echolucent plastisol.
Table 3.2. Percent of pixels detected in the correct tissue range in tissue sample controls, and in tissue encased within plastisol in both sets of tissue GSM ranges.

<table>
<thead>
<tr>
<th>Simulated heterogeneous plaque type</th>
<th>First set of tissue GSM ranges</th>
<th>Tissue sample control (% ± SD)</th>
<th>Tissue encased in plastisol (% ± SD)</th>
<th>p-value</th>
<th>Second set of tissue GSM ranges</th>
<th>Tissue sample control (% ± SD)</th>
<th>Tissue encased in plastisol (% ± SD)</th>
<th>p-value</th>
<th>Matched-pair t-test (p-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque with Epididymal fat tissue</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plaque with leg muscle tissue</td>
<td>12.7 ± 2.13</td>
<td>8.90 ± 2.10</td>
<td>0.01</td>
<td></td>
<td>16.0 ± 3.20</td>
<td>11.7 ± 1.34</td>
<td>0.04</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plaque with neck muscle tissue</td>
<td>2.13</td>
<td>2.10</td>
<td>3.20</td>
<td></td>
<td>1.34</td>
<td>1.34</td>
<td>0.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plaque with abdominal muscle tissue</td>
<td>9.99 ± 2.12</td>
<td>5.46 ± 1.35</td>
<td>0.02</td>
<td></td>
<td>23.0 ± 3.06</td>
<td>12.7 ± 2.96</td>
<td>0.003</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plaque with bone tissue</td>
<td>10.3 ± 1.34</td>
<td>8.47 ± 1.05</td>
<td>0.08</td>
<td></td>
<td>18.8 ± 4.41</td>
<td>18.0 ± 2.47</td>
<td>0.75</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Plaque with bone tissue</td>
<td>39.7 ± 9.52</td>
<td>39.7 ± 9.16</td>
<td>1.00</td>
<td></td>
<td>39.7 ± 39.7 ± 9.16</td>
<td>39.7 ± 9.16</td>
<td>0.96</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table 3.3. Percent of pixels detected in each tissue GSM range in heterogeneous plaque using the second set of tissue GSM ranges and a previously reported set of tissue GSM ranges.

<table>
<thead>
<tr>
<th>Simulated heterogeneous plaque type</th>
<th>Second set of tissue GSM ranges</th>
<th>Previously reported tissue GSM ranges established by Lal et al.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plastisol (% ± SD)</td>
<td>Muscle (% ± SD)</td>
</tr>
<tr>
<td></td>
<td>Fat (% ± SD)</td>
<td>Bone (% ± SD)</td>
</tr>
<tr>
<td></td>
<td>Overlap (% ± SD)</td>
<td>Blood (% ± SD)</td>
</tr>
<tr>
<td></td>
<td>Lipid (% ± SD)</td>
<td>Muscle (% ± SD)</td>
</tr>
<tr>
<td></td>
<td>Fibrous (% ± SD)</td>
<td>Bone (% ± SD)</td>
</tr>
<tr>
<td>1) Plaque with Epididymal fat tissue</td>
<td>48.0 ± 8.30 ±</td>
<td>4.75 ± 4.02 ± 2.78 ±</td>
</tr>
<tr>
<td></td>
<td>7.94 ± 4.41 ±</td>
<td>2.51 ± 1.81 ± 1.21 ±</td>
</tr>
<tr>
<td></td>
<td>14.1 ± 12.7 ± 1.69 ±</td>
<td>45.9 ± 4.06 ± 2.96 ±</td>
</tr>
<tr>
<td>2) Plaque with leg muscle tissue</td>
<td>29.6 ± 7.14 ±</td>
<td>4.62 ± 6.20 ± 3.07 ±</td>
</tr>
<tr>
<td></td>
<td>15.7 ± 1.72 ±</td>
<td>1.07 ± 2.00 ± 0.94 ±</td>
</tr>
<tr>
<td></td>
<td>16.7 ± 7.21 ±</td>
<td>4.06 ± 2.54 ± 2.54 ±</td>
</tr>
<tr>
<td>3) Plaque with neck muscle tissue</td>
<td>45.9 ± 6.58 ±</td>
<td>4.06 ± 2.96 ± 2.54 ±</td>
</tr>
<tr>
<td></td>
<td>6.97 ± 0.87 ±</td>
<td>0.67 ± 1.39 ± 0.37 ±</td>
</tr>
<tr>
<td></td>
<td>9.19 ± 1.70 ±</td>
<td>6.97 ± 3.89 ± 2.16 ±</td>
</tr>
<tr>
<td>4) Plaque with abdominal muscle tissue</td>
<td>50.4 ± 8.49 ±</td>
<td>5.31 ± 4.42 ± 3.42 ±</td>
</tr>
<tr>
<td></td>
<td>2.93 ± 3.41 ±</td>
<td>2.00 ± 1.39 ± 0.59 ±</td>
</tr>
<tr>
<td></td>
<td>13.1 ± 3.30 ±</td>
<td>2.93 ± 3.03 ± 4.55 ±</td>
</tr>
<tr>
<td>5) Plaque with bone tissue</td>
<td>42.8 ± 8.54 ±</td>
<td>5.95 ± 3.70 ± 17.4 ±</td>
</tr>
<tr>
<td></td>
<td>8.37 ± 2.53 ±</td>
<td>2.07 ± 1.37 ± 8.37 ±</td>
</tr>
<tr>
<td></td>
<td>20.3 ± 5.07 ±</td>
<td>4.63 ± 3.16 ± 3.91 ±</td>
</tr>
</tbody>
</table>
3.5 Discussion

Studies have suggested that plaque lesions containing heterogeneity in composition, microcalcifications, neovascularization, surface irregularities, or large regions of echolucency are more vulnerable to cardiovascular events and complications as opposed to echogenic, homogeneous plaque lesions(31,36,42,71). Various techniques and tools have been developed to investigate specific features of plaque vulnerability, including contrast-enhanced ultrasound for the detection of neovascularization and surface irregularities, three-dimensional (3D) ultrasound to characterize surface features such as endothelial denudation or fissuring, integrated backscatter and radiofrequency analysis to characterize plaque texture, and GSM analysis for the investigation of plaque echogenicity. However, there are limited practical techniques validated for plaque tissue characterization to investigate plaque composition. To date, most tissue characterization studies have been conducted using three primary approaches: 1) The visual “eyeballing” approach, 2) the videodensitometry approach, and 3) the integrated backscatter (IB) approach(28,37–39). The visual “eyeballing” approach was the first approach reported in an attempt to differentiate components of plaque lesions(28,37–39). However, this approach is subjected to obvious limitations in terms of its operator-dependent subjectivity, and the degree of sensitivity in detecting smaller regional differences. IB analysis is the most contemporary approach. It utilizes raw radiofrequency signal from ultrasound waves reflected from the medium, known as backscatter analysis(72–74). This approach is considered to be the most accurate, as data are sampled upstream of the video display processor, providing comprehensive data that is undistorted by post-processing(10). Ultrasound tools such as IVUS can utilize radiofrequency backscattered signals to provide detailed quantitative information on plaque composition(75). However, this approach is not without limitations. The procedure converting the raw radiofrequency data into usable quantitative values displayed in decibels is complex, and is subjected to artifacts related to image settings, and the exact location of the plaque lesion(10,42). These reasons account for the rare clinical use of integrated backscatter in current practices.
Videodensitometry, is an approach developed in the mid 1990s(36). It is an objective method that utilizes GSM values to characterize spatial distribution of pixels, providing insight into plaque composition using easily accessible equipment and software. This study investigates a digital image-analysis algorithm of videodensitometry known as colourized PDA. The colourized PDA algorithm was used to map the composition of atherosclerotic plaque lesions from pre-endarterectomy patients. The virtual histological images produced from this algorithm was then correlated with histological analysis. Pixel intensities of specific tissue types are assigned specific colours in order to map the tissue composition of plaque in the colourized PDA method(29). However, a decade has passed since the development of this algorithm, with limited studies done to further validate and advance this technique. This is the first study to validate the colourized PDA algorithm using a highly controlled phantom model, where the algorithm is directly tested with simulated heterogeneous plaque lesion with known composition and location of different tissue types pre-determined. Furthermore, the tissue GSM ranges previously reported were established using equipment now obsolete. The field has called for a detailed set of ranges associated with more specific tissue types to facilitate the practical use of the PDA method(65). Our study meets this need by establishing an updated range of GSM values using various tissue types.

In our second set of GSM ranges, different tissue types such as muscle and fat overlap with one another. This is different from the previously reported ranges, where GSM ranges of different tissue types are discrete, with grayscale voids between them. The overlap observed in our GSM ranges suggests that muscles and fats do not differ in their biochemical structure significantly enough to result in a difference in echogenicity that can be detected by ultrasound. Though myocytes and adipose cells are significantly different from one another, their cellular differences may be beyond the sensitivity limitations of ultrasound. Specifically, these differences may not result in a big difference in their density. Density is ultimately what affects the acoustic impedance of the ultrasound wave in the tissue medium, directly affecting the refection coefficient, and subsequently the echogenicity of the tissue structure(48). Additionally, various speckle noise reduction technology recently integrated into ultrasonography has
allowed a greater dynamic range in gray scale values to be detected (76–79). This is reflected on the ultrasound imaging of various disease types such as kidney disease (78), Poly Cystic Ovarian Syndrome (PCOS) (79), and liver disease (77), where noises previously observed in the ultrasound images of these disease types have been reduced or are no longer present due to the increase in gray scale dynamic range. The increase in gray scale values detected may result in the overlap observed in our GSM ranges. It is also possible that the overlaps observed between tissue ranges are due to the type of tissues used. In this study, rat tissues, rather than human tissues were used to establish the tissue GSM ranges. Physical properties of rat tissues may be different from that of human tissues.

The GSM range of bone established in this study (145-175) is lower than that previously reported (211-255) (60). This could be explained by the attenuation of ultrasound signals from the bone samples used to establish the bone GSM range. This effect is similar to the clinical echo finding of acoustic shadowing caused by focal calcification in atherosclerotic plaque lesions, where the area underneath the calcification appears to be dark and unclear. It can be seen in Figure 3.4 that the bone tissue appeared to be echogenic in the upper area of the tissue, while the immediate areas underneath appeared to be echolucent due to acoustic shadowing, where most sound waves encountering the structure of interest are reflected and unable to penetrate any further. To partially account for acoustic shadowing, we selected a small square region of the bone tissue sample closer to the transducer in our GSM calculations instead of outlining the entire tissue sample (Figure 3.5). This avoids the inclusion of regions affected by acoustic shadowing in establishing the bone GSM range.
Figure 3.4. Acoustic shadowing in bone tissue samples in simulated carotid vessel.

Echogenic upper region of bone tissue can be observed within the circled region of bone tissues inserted into the simulated carotid vessel. Darker regions underneath the circled region are observed due to acoustic shadowing.
Figure 3.5. Region selected in bone tissue samples in establishing bone tissue range.

Sampling grey pixels closer to the transducer, as outlined by the dotted red box, can partially account for the effects of acoustic shadowing in establishing bone GSM ranges, as the echolucent lower regions of the bone tissue affected by acoustic shadowing was not included in the calculation of the GSM value.
In addition to lower bone GSM ranges, we also observed a higher fat GSM range than previously reported (60). The GSM range of the epididymal fat used was found to be 91-100. This is higher than that of neck muscle (68-87) and leg muscle (76-90), and the lipid GSM range previously reported (8-26). This is counter-intuitive, given the widely accepted fact that plaque lesions with a large lipid core are echolucent. However, this observation may again be a result of rat tissues being used rather than human tissues. The observation may also be due to the specific type of fat tissue used. Epididymal fat is a visceral fat, which is different from a non-visceral fat, such as subcutaneous fat. Visceral fat such as the epididymal fat we used in this study, is more cellular, vascular, innervated and contains a larger number of inflammatory and immune cells (80). Visceral fat also possesses lesser pre-adipocyte differentiating capacity and a greater percentage of large adipocytes (80). These cellular, molecular, and physiological differences can result in a higher tissue structure density. A denser structure will reflect greater amounts of ultrasound waves, producing an echogenic image with higher GSM values. This raises the question of whether visceral fat or non-visceral fat should be used to characterize the lipid content of atherosclerotic plaque lesions in the colourized PDA algorithm.

Ideally, colourized PDA should identify similar percentages of pixels detected within the correct tissue range in the tissue controls, and in the encased tissue samples. This was observed in our neck muscle heterogeneous plaque, abdominal muscle heterogeneous plaque, and bone tissue heterogeneous plaque lesions. However, the percent of pixels detected within the correct tissue range in the epididymal fat heterogeneous plaque, and the leg muscle heterogeneous plaque were significantly lower than their respective tissue sample controls in both of our established tissue GSM ranges. This observation may be due to the introduction of noise from the plastisol encasing the epididymal fat tissue and the leg muscle tissue. In developing the simulated heterogeneous plaque lesions, tissue samples were exposed to an increase in temperature during the injection of plastisol. The heat from the plastisol solution may have an effect on the tissue structure of the low-density epididymal fats, resulting in an unreliable percent of pixels detected within the correct tissue range in encased tissue samples. This theory can be applied to the
leg muscle heterogeneous plaque. The *bicep femoris*, or leg muscle, is a muscle that is utilized frequently. In comparison to the neck muscles and abdominal muscles, the leg muscle is likely to have enlarged muscle cells. This hypertrophic difference in muscular tissue structure may account for the difference in how the muscles respond to the heat from the plastisol in the tissue encasing process.

Of the two sets of tissue GSM ranges established, the second set of ranges detected a significantly greater percent of pixels in the correct tissue range than the first set of tissue GSM ranges. We selected the second set of GSM ranges to compare with a previously reported set of tissue ranges (60). In our tissue GSM ranges, the tissue range with the highest percent of pixels detected corresponds to the specific type of tissue used in the simulated heterogeneous plaque four out of the five simulated heterogeneous plaque types. These include the leg muscle heterogeneous plaque, neck muscle heterogeneous plaque, abdominal muscle heterogeneous plaque, and bone tissue heterogeneous plaque. In the epididymal fat heterogeneous plaque, the muscle tissue range was identified as the tissue range with the highest percent of pixels detected. This can again be due to the potential change in epididymal fat tissue structure in response to the heat from the plastisol solution. In the previously reported set of tissue GSM ranges, none of the five simulated heterogeneous plaque types had the highest percent of pixels detected in a tissue range that correspond to the specific tissue type used in the simulated heterogeneous plaque. Though our established ranges is more successful in detecting the correct tissue components, it is important to note that the previously reported ranges were established using human tissues, while our ranges were established using rat tissues. Different types of muscle and fat tissues were also used to establish the tissue GSM ranges. The previously reported ranges examined subcutaneous fat from the abdomen (non-visceral fat), biceps muscle, and the tibia and skull in establishing their ranges. An additional category of fibrous tissue was also included by examining human iliotibial tracts. We were unable to obtain fibrous tissues from our rat samples due to the small size of the animal. Since we developed our simulated plaque model using rat tissues, the colourized PDA results of our plaque model are more consistent with our established rat tissue ranges.
Of note, in establishing our own tissue GSM ranges, we question the reproducibility of the colourized PDA method. We observed a high level of heterogeneity even within the same tissue sample. As shown in Figure 3.2, where colour mapping was conducted on the simulated heterogeneous plaque, the tissue control, and the plastisol control, a variety of colours were observed within the same visceral fat tissue sample. Though the colourized PDA method identified the correct tissue type in our phantom model, we question the practicality in analyzing real human atherosclerotic plaque lesions, where plaque lesions are composed of an amalgamation of various pseudo-tissue types including inflammatory cells, vascular tissue, mixed connective tissue, and amorphous ground substance(60), rather than defined tissue types as displayed by our simulated plaque model. We further investigated this issue and carried out ultrasound on anatomical areas of volunteers, including the gastrocnemius muscle and subcutaneous fat (abdomen). We conducted colourized PDA on B-mode images of these tissues using the second set of ranges we established, as well as ranges previously reported by Lal et al(29,60). As seen in Figure 3.6, a high level of heterogeneity exists within the same tissue type, regardless of which set of tissue GSM ranges was used. Though the coloured PDA method generally identified the correct tissue type, it also displayed colours that represent other tissue categories. More work is required in establishing appropriate tissue GSM ranges for the colourized PDA algorithm. As observed in our established ranges, different muscle tissue subtypes (neck, leg, abdomen) have different GSM ranges. Knowing that tissue subtypes belonging to the same tissue category can have different GSM ranges, we aim to expand on the
Figure 3.6. Heterogeneity in colourized PDA images of tissues.

Heterogeneity observed within the same tissue colourized PDA produced images of *in vivo* 2D ultrasound images of human volunteers: A) Gastrocnemius muscle using the first set of tissue GSM ranges; B) Gastrocnemius muscle using a previously reported set of tissue GSM ranges; C) Subcutaneous fat using the first set of tissue GSM range; D) Subcutaneous fat using a previously reported set of tissue GSM ranges.
current study by scanning additional anatomical areas. In future studies, we can include fibrous tissues such as the iliotibial tract in human subjects to establish a detailed set of human tissue GSM ranges accounting for more tissue types. We would then like to use this set of tissue GSM ranges to validate the colourized PDA technique with real human atherosclerotic plaque lesions from endarterectomy procedures.

In the present study, an ultrasound phantom model of simulated heterogeneous plaque lesions was created. With this phantom model, we established a new, updated set of tissue GSM ranges for colourized PDA. In establishing this set of new tissue GSM ranges, we found that the second set of established tissue GSM ranges of: echolucent plastisol 0-4, muscle 68-86 (Neck muscle and leg muscle) and 104-108 (abdomen muscle), fat 91-100 (visceral fat) and bone 145-175, was more accurate in characterizing tissue components of the simulated heterogeneous plaque lesions as opposed to our first set of established GSM ranges. To our knowledge, this is the first study to investigate plaque vulnerability via analysis of tissue heterogeneity using an ultrasound phantom model. Though more work is required to refine this method before this technique can be applied to clinical practices, this study has demonstrated the potential and applicability of colourized PDA, and warrants further efforts into the development and standardization of this technique. The future development of techniques to detect features of plaque vulnerability, including tissue characterization, will allow for targeted treatment of patients at highest risk of cardiovascular events.

The phantom model developed in this study is valuable for advancing plaque analysis techniques. The developed phantom can serve as controlled and safe platform for vulnerable plaque mimics that can now be available for testing of novel ultrasound tools such as ultrasound guided contrast agents that also act as drug delivery systems(81). Currently, no similar platforms are available for such ultrasound testing.

3.6 Conclusion

While various ultrasound tissue characterization techniques are still currently in its experimental stage, it is listed by the American Society of Echocardiography roadmap to 2020 as one of the most
promising fields of application in cardiovascular imaging. Tissue characterization can help identify vulnerable plaque lesions, and also provide insight in understanding the pathophysiology of atherosclerotic disease and plaque development. For colourized PDA, additional work is required to refine GSM ranges that represent correct tissue types in identifying tissue components of atherosclerotic plaque lesions. Once these ranges have been standardized, we can apply this technique in future randomized trials to evaluate its use in identifying vulnerable plaque, and therefore in identifying vulnerable patients. Adding colourized PDA analysis to carotid duplex doppler examination, or strain imaging can potentially assist in identifying patients who require more aggressive treatment to prevent future cardiovascular events.

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Chapter 4

Summary and Future Directions

4.1 Summary

Though ultrasonic tissue characterization of atherosclerotic plaque is not yet ready for clinical use, it is widely recognized as one of the most promising fields of application in cardiovascular ultrasound imaging. Both the GSM and PDA methods have the potential to identify vulnerable, unstable plaque lesions prone to embolization and rupture, regardless of the degree of stenosis present in the vessels. This is particularly useful for the development of drugs to treat atherosclerosis, as vulnerable plaque lesions are also the type of atherosclerotic lesions that receive the greatest benefit from pharmacological treatments to prevent cardiovascular events. It is important to note that despite of its potential, diagnostic ultrasonography has inherent limitations. In particular, not all ruptured plaque lesions have corresponding histologic features of vulnerability, and not all vulnerable plaque lesions by histology criteria will rupture in their natural history. Regardless, the imaging of plaque vulnerability remains an important approach to bridging the current gap in understanding clinical manifestations of atherosclerotic disease. New quantitative methods will be developed to include variables such as attenuation, strain, and temperature. These image methods may offer opportunities for the early detection and treatment of the disease. Once the methodology and analysis have been standardized, future prospective randomized trials can be conducted to evaluate whether quantitative tissue characterization-based information on plaque vulnerability can be used to customize treatment in patients with clinically symptomatic as well as high-risk asymptomatic atherosclerosis.

In this thesis, two variations of ultrasound phantom models simulating specific features of plaque vulnerability have been developed. The current research has the potential to improve advanced technologies in the identification and diagnosis of vulnerable atherosclerotic plaque lesions. However, techniques such as GSM analysis and PDA analysis are still considered to be relatively novel and have
limitations to overcome before they can be translated to clinical practice. As the technology and tools for
diagnostic ultrasonography image acquisition is becoming more automated, the assessment and analysis
of these images requires standardization, and is subject to variability. From a clinical perspective, GSM
analysis and PDA analysis lack the long-term epidemiologic data that would inform our understanding of
how well the vulnerability features of plaque echogenicity, and plaque tissue composition can predict
cardiovascular events 5, 10, or 15 years later. Although some of these methods have been validated using
histology, rigorous evaluation of standardized techniques is still necessary. The development of robust,
long-lasting phantom models is a key step in the development, testing, and standardization of such
techniques.

4.2 Future Directions

A few experiments can be done to further expand on the research outlined in this thesis. First, GSM data from the phantom models developed can be compared with in vivo GSM data from pre-endarterectomy patients. The relationship between density and echogenicity can then be investigated by measuring the density of the atherosclerotic plaque lesions harvested from endarterectomy patients. Histological analysis can be conducted to correlate plaque density with cell types in order to provide insight into the specific cellular structural differences that contribute to intrinsic physical properties, such as tissue density. The Young’s modulus of plaque lesions can be investigated using various ultrasound elastography techniques. A detailed set of tissue GSM ranges should also be established using more specific subtypes of human tissues, where PDA can then be conducted on real human atherosclerotic plaque lesions using this detailed set of tissue GSM ranges. Finally, the simulated heterogeneous plaque lesions developed in this thesis contained only one specific type of tissue encased within echolucent plastisol.
Simulated heterogeneous plaque lesions with two or more tissue types encased should be the next step in expanding this phantom work.
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