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Histopathological Validation of Grayscale Carotid Plaque Characteristics Related to Plaque Vulnerability

Carol C. Mitchell, PhD[a], **James H. Stein, MD, FAHA**[a], **Thomas D. Cook, PhD**[b], **Shahriar Salamat, MD, PhD**[c], **Xiao Wang, PhD**[d], **Tomy Varghese, PhD**[d], **Daren C. Jackson, PhD**[e,*], **Carolina Sandoval Garcia, MD**[f], **Stephanie M. Wilbrand, PhD**[f], and **Robert J. Dempsey, MD**[f]

[a]Department of Medicine, Cardiovascular Medicine Division, University of Wisconsin School of Medicine and Public Health, USA, Madison WI, 53792

[b]Department of Biostatistics and Medical Informatics, University of Wisconsin School of Medicine and Public Health, USA, Madison WI, 53792

[c]Department of Pathology and Laboratory Medicine, University of Wisconsin School of Medicine and Public Health, USA, Madison WI, 53792

[d]Department of Medical Physics, University of Wisconsin School of Medicine and Public Health, USA, Madison, WI 53705

[e]Department of Neurology, University of Wisconsin School of Medicine and Public Health, USA, Madison, WI 53705

[f]Department of Neurological Surgery, University of Wisconsin School of Medicine and Public Health, USA, Madison, WI 53792

Abstract

Inflammation and angiogenesis play major roles in carotid plaque vulnerability. The purpose of this study was to determine if grayscale features of carotid plaques are associated with histological markers for inflammation. Thirty-eight individuals completed a dedicated research carotid ultrasound exam prior to carotid endarterectomy. Grayscale analysis was performed on plaque

Corresponding Author: Carol Mitchell, University of Wisconsin, Department of Medicine/Division of Cardiovascular Medicine, University of Wisconsin School of Medicine and Public Health, K6/322 Clinical Science Center, 600 Highland Ave MC 3248, Madison, Wisconsin 53792, Phone: 608-262-0680, Fax: 608-263-0405, ccm@medicine.wisc.edu.

*now at Wisconsin Surgical Outcomes Research Program, Department of Surgery, University of Wisconsin School of Medicine and Public Health, USA, Masison, WI 53792

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Conflicts of Interest

C.C. Mitchell: Other; Davies Publishing Inc., authorship for two echocardiography textbooks, currently under review, may have future royalties. Elsevier, Wolters Kluwer, author textbook chapters, may have future royalties.
J.H. Stein: Other; Wisconsin Alumni Research Foundation-patent related to carotid wall thickness and vascular age.
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images to measure plaque echogenicity (grayscale median [GSM] pixel brightness), plaque area, presence of discrete white areas [DWAs], and the percent of black area near the lumen on any one component of the plaque. Plaques with higher ultrasound GSM had greater percent calcification (p=0.013) on histopathology. Presence of an ultrasound DWA was associated with more plaque hemosiderin (p=0.0005) and inflammation (p=0.019) on histopathology examination. The percent of plaque black area in any one component was associated with a higher score for macroscopic ulceration (p=0.028). Ultrasound plaque characteristics (GSM, DWAs and Black areas) represent histopathological markers associated with plaque vulnerability.

Keywords

carotid plaque; vulnerable plaque; ultrasound grayscale imaging

Introduction

Treatment options for patients with carotid artery atherosclerosis are largely influenced by the presence of clinical symptoms and medical imaging estimates of the degree of stenosis and plaque surface characteristics (Liapis et al. 2009; Dempsey et al. 2010; Brott et al. 2011; Ricotta et al. 2011; Salem et al. 2014). However, these criteria do not account for cumulative arterial damage that can lead to clinically unrecognized (“silent”) strokes due to the presence of vulnerable plaques and microemboli (Dempsey et al. 2010). It is estimated that for every clinical stroke diagnosis there are approximately five silent strokes which go unnoticed and are associated with cognitive decline, especially in executive function skills (Seshadri 2006; Dempsey et al. 2010; Rocque et al. 2012; Wang et al. 2013). Therefore, better ways to evaluate plaque vulnerability are essential to optimize patient treatment and management not only for major stroke prevention but also to delay cognitive impairment due to arterial injury.

Vulnerable plaques are associated with thin fibrous caps, large lipid cores, intraplaque hemorrhage, inflammation (Stary et al. 1995; Fleiner et al. 2004; Salem et al. 2013; Marnane et al. 2014; Salem et al. 2014), and in some reports calcification (Shaalan et al. 2004). Ultrasound methods used to assess carotid plaque for features of vulnerability include; integrated backscatter (IBC) (Bridal et al. 2000; Kawasaki et al. 2001; Nagano et al. 2008), midband, slope and intercept values of straight-line fit (MBF) to the apparent backscatter transfer function, (Waters et al. 2003) carotid strain imaging, (Maurice et al. 2005; Shi et al. 2008; Shi et al. 2009; Wang et al. 2013; Wang et al. 2016a; Wang et al. 2016b), acoustic radiation impulse force imaging (ARFI) based approaches (Czernuszewicz et al. 2015), shearwave elastography (Garrard et al. 2015) and grayscale analyses of plaque features (El-Barghouty et al. 1996; Tegos et al. 2000; Grogan et al. 2005; Salem et al. 2014). IBC, MBF, zero frequency intercept, and carotid strain imaging utilize the raw radiofrequency echo signal to determine associations between scattering properties of the plaque tissues and plaque composition. In grayscale analyses, B-mode ultrasound images are digitized and processed with specialized software to calculate a grayscale median (GSM) value and to demonstrate pixel brightness distribution based on the grayscale value of groups of pixels (Lal et al. 2002; Nicolaides et al. 2010). Images are normalized with blood as the reference for black, and the vessel wall (adventitia) as the reference point for white. Comparing pixel

brightness values to histology specimens allows for determination of what type of tissue is most likely composing a plaque. Lal et al. (2002) imaged volunteers and patients undergoing carotid endarterectomy (CEA). Volunteers were scanned to determine pixel brightness values of bone (utilizing the tibial head and cranium), fat (utilizing the sub-cutaneous fat of the abdomen), muscle (biceps muscle), and fibrous tissue (iliotibial tract). A blood vessel was imaged in the same frame as all of these different tissue types so that images could be normalized with blood representing a grayscale value of 0 and adventitia representing a grayscale value of 190. Patients undergoing CEA had their plaques analyzed and the pixel brightness distribution was compared to values attained in normal tissue of volunteers and histopathology specimens. Findings demonstrated good agreement of ultrasound grayscale values predicting the type of tissue present in plaques (i.e. calcium, blood, lipid, fibrous tissue), with a significant higher amount of blood and lipid associated with symptomatic plaques compared to asymptomatic plaques ($p=0.0048$ for blood, and $p=0.026$ for lipid) (Lal et al. 2002). Evaluation of plaque surface characteristics have also been utilized to characterize features of plaques associated with vulnerability (Kanber et al. 2013a). Kanber et al 2013a utilized a plaque surface irregularity index to objectively quantify plaque surface irregularities. The authors found that plaques with a higher surface irregularity index were associated with ipsilateral cerebrovascular symptoms (Kanber et al. 2013a).

Grayscale findings that have been associated with plaque vulnerability are 1) low grayscale median (GSM) values (Nicolaides et al. 2010; Ruiz-Ares et al. 2011; Ibrahimi et al. 2014; Ruiz-Ares et al. 2014), 2) juxtaluminal black area (JBA) size (Griffin et al. 2010; Kakkos et al. 2013), 3) discrete white area (DWA) presence (Nicolaides et al. 2010), and 4) large plaque area (Nicolaides et al. 2010; Salem et al. 2014). Studies using these parameters have demonstrated correlations between low echogenicity and worrisome histopathological plaque features, few have described how grayscale features (Salem et al. 2014) are associated with inflammation (Griffin et al. 2010; Nicolaides et al. 2010; Kakkos et al. 2013; Salem et al. 2014; Kanber et al. 2015), except when ultrasound contrast agents are used (Shah et al. 2007; Hjelmgren et al. 2014). The purpose of this study was to determine if grayscale features of carotid plaques are associated with histological markers for inflammation.

Methods

Participants

Patients were recruited to subjects in the “Structural Stability of Carotid Plaque and Symptomatology” (NIH funded study: R01 NS064034) study from 2010–2015. This study was approved by the University of Wisconsin Health Sciences Institutional Review Boards and all subjects provided informed consent. All subjects met criteria for surgical carotid endarterectomy (>60% carotid arterial stenosis [percent stenosis determined by computed tomography angiography, magnetic resonance angiography, or ultrasound imaging]; symptomatic patients had symptoms of motor and/or language deficits on examination and asymptomatic patients had no deficits but may have had silent strokes seen on imaging) (North American Symptomatic Carotid Endarterectomy Trial (NASCET) Steering Committee; North American Symptomatic Carotid Endarterectomy Trial Collaborators

(NASCET), Walker et al. 1995). Subjects were recruited from the neurosurgery clinics and inpatient units. Potential participants were excluded if they had prior carotid artery surgery, prior carotid artery endovascular procedures, prior cervical radiation, or were otherwise deemed unsuitable for carotid endarterectomy. Thirty-eight patients completed the ultrasound research clinical imaging session and had pathology results available for comparison at the time of this analysis.

Ultrasound Imaging

Ultrasound images were acquired on average (standard deviation) 10.6 (13.8) days prior to carotid endarterectomy. The clinical imaging portion of the protocol was performed with an Acuson S2000 ultrasound system (Siemens Medical Solutions USA, Inc., Malvern, PA) and 9L4 transducer. The ultrasound clinical imaging protocol consisted of transverse and longitudinal imaging of the common carotid, carotid bulb, internal carotid and external carotid arteries. Pulsed wave Doppler velocities were recorded in the common carotid artery (proximal, mid and distal segments), carotid bulb, internal carotid artery (proximal, mid and distal segments) and external carotid artery. Color Doppler images were acquired in the transverse and longitudinal planes in the common carotid, carotid bulb, internal and external carotid arteries. All images were saved digitally in DICOM format and analyzed with Access Point Software (Freeland Systems, LLC, Alpharetta, GA) to select the frames most representative of the plaque, which were then saved as a bitmap for plaque grayscale analysis. All scans were performed by two certified ultrasonographers.

Carotid Plaque Grayscale Analysis

Ultrasound images were analyzed using the “Plaque Texture Analysis Software” package (LifeQ Medical, Nicosia, Cyprus). Images were normalized with the blackest area of the blood representing a grayscale value of 0 and the brightest white area of the adventitia representing a grayscale value of 190 (Nicolaides et al. 2010). After normalization, images were standardized to a pixel density of 20 pixels per mm (Nicolaides et al. 2010). The following grayscale features were analyzed for each plaque image: GSM, plaque area, presence of a discrete white area (DWA), presence of a black area adjacent to the lumen either with or without an echogenic border, and the percent black area of any one component of the plaque. The GSM value was calculated using the median grayscale value of the traced plaque. The presence of a DWA was noted by the identification of a white area in the plaque without acoustic shadowing and a grayscale value greater than 126 (Nicolaides et al. 2010) (color coded red, figure 1). Juxtaluminal black areas (JBAs) without an echogenic border and black areas with a border (BABs) were identified as areas with a grayscale value of less than 25 near the lumen and color coded as black with the plaque analysis software. In subjects with more than one black area, the largest black area was measured. The black area was traced to obtain an area measure (figure 2) and from this measurement the percent black area of any one component was calculated. The entire plaque was manually traced and the plaque area calculated (reported in mm^2). In images with acoustic shadowing, the plaque was traced to include the echogenic portion of the plaque and exclude the portion containing acoustic shadowing. All image selection and analyses were performed by a single, trained investigator.

Histopathology

Paraffin-embedded plaque sections were stained using standard hematoxylin and eosin methods. Sections were assessed by a single pathologist blinded to subject characteristics for the percent calcification, cholesterol content, hemorrhage, hemosiderin content, and inflammatory cell infiltration. Histologic classification of plaques was made using the updated classification of atherosclerotic plaques recommended by the American Heart Association (Tureyen et al. 2006). Plaque specimens also were scored on an ordinal scale (0–3) for the presence of hemosiderin and inflammation (figure 3). A score of 0 represented no hemosiderin or inflammation, 1 was minimal, 2 was moderate and 3 represented extensive presence of hemosiderin and/or inflammation.

Surgical Ulceration Scoring

All carotid endarterectomies and ulceration scores were provided by a board-certified physician. At the time of carotid endarterectomy, the surgeon scored the plaque on a subjective, ordinal scale of 1 to 4. A score of 1 represented minimal ulceration and a score of 4 represented extensive ulceration.

Statistical Analysis

Carotid plaque characteristics (GSM, presence of DWA, presence and size of JBA or BAB, and plaque area) were analyzed and compared to histopathology. Statistical calculations were performed using R Core Team (R Development Core Team 2015). Kendall's Rank Correlation Tau was utilized to assess the percent calcification reported in the plaque histopathology and its relationship to the GSM. The Wilcoxon Rank Sum Test was utilized to assess the relationships between diffuse histopathological inflammation and hemosiderin and the presence of DWAs, the surgeon's ulceration score, and the percent black areas of a component of a plaque.

Results

Subjects

There were 38 subjects that completed the ultrasound research protocol that had histopathology results available for analysis. Subjects were mean (standard deviation) 69 (9.5) years old (range 43–87 years); 16 (42%) were female; 24 (63%) were symptomatic. Characteristics for all patients are presented in Table 1. For each subject a single plaque image, that was the most representative of the grayscale features, was selected for analysis, except for one patient in which two images were selected. Most representative was defined as the longitudinal image plane in which the plaque appeared the largest, based on maximum lumen encroachment, maximum length extended and best border definition. Color Doppler loops were also used to ensure that the image selected also defined the plaque borders the best. In one patient two images were required to demonstrate the entire length of the plaque. One image demonstrated plaque extending from the distal CCA to the bulb and the other image demonstrated the plaque extending into the ICA. In this patient, the plaque extending into the ICA demonstrated the DWAs. Grayscale features of GSM, presence of DWA and/or black area and plaque area were selected based on the correlates of these features with

vulnerable plaque characteristics and clinical symptomology reported in other studies (El-Barghouty et al. 1996; Tegos et al. 2000; Grogan et al. 2005; Shah et al. 2007; Griffin et al. 2010; Nicolaides et al. 2010; Ruiz-Ares et al. 2011; Kakkos et al. 2013; Salem et al. 2014).

Grayscale Median Pixel Brightness and Plaque Calcification

The mean (standard deviation) GSM pixel brightness was 70.5 (22.6), (range 28–116). The percent plaque calcification (by histopathology examination) mean percentage was 17.6% (17%) (range 0 – 60%). Compared to histopathology examination, plaques with a higher GSM value were associated with a higher percent of calcium in the plaque specimen ($z=2.33$, p- = 0.013). There were no statistically significant relationships between GSM and percent cholesterol or diffuse hemorrhage on histopathology.

Discrete White Areas, Plaque Hemosiderin, and Plaque Inflammation

The presence of a DWA was associated with a higher hemosiderin grade ($p=0.0005$) and inflammation on histopathology examination ($p= 0.019$) (see figure 4). Seven participants (18.4%) were noted to have a DWA on ultrasound grayscale analysis.

Percent Black Areas and Surgical Ulceration

Plaques that had a black area (JBA or BAB, n=17, 44.7%) were associated with a higher surgical ulceration score ($p=0.028$), (see figure 5). The percent black area in any one component in the plaque ranged from 0.00–23.28%.

Plaque Area

Plaque area was not significantly associated with any histopathology finding.

Reproducibility

Reproducibility was evaluated by re-measuring 20 plaque images (9 of which were intentionally selected as complex plaques with large black areas). The mean at the second reading was a value of 0.30 higher than the mean at the first reading, this was not significant ($p=0.73$). The within-subject standard deviation was 2.656, and the intra-class correlation coefficient was 0.986 (0.965, 0.99). Plaque area was assessed in the same 20 plaque images as GSM. The mean plaque area at the second reading was of $2.23mm^2$ smaller than the mean at the first reading, this was significantly different ($p=0.007$), however this difference represents a small percent difference of the total plaque area (mean percent difference = 2.74%). Also, the within-subject standard deviation was 2.773 and the intra-class correlation coefficient was 0.995 (0.987, 1.00) for plaque area. For black areas, the mean plaque area at the second reading was $0.37mm^2$ higher than the mean at the first reading, this was not significantly different ($p=0.24$), the within-subject standard deviation was 0.781 and the intra-class correlation coefficient was 0.975 (0.921, 0.99). There was 100 percent agreement between both readings with identification of a discrete white area and black area near the lumen.

Discussion

We demonstrated that ultrasound plaque characteristics are associated with histopathological markers of plaque vulnerability such as inflammation, ulceration, hemorrhage, and focal calcification. Previous reports (Griffin et al. 2010; Nicolaides et al. 2010; Kakkos et al. 2013; Ibrahimi et al. 2014; Ruiz-Ares et al. 2014; Salem et al. 2014; Sztajzel et al. 2005) have demonstrated the following relationships between ultrasound grayscale characteristics, symptoms and histopathologic examination; 1) plaque area and JBA size are associated with unstable plaques (Salem et al. 2014), 2) lower GSM values at the surface of the plaque are associated with unstable plaques (Grogan et al. 2005; Sztajzel et al. 2005), 3) lower GSM values are associated with larger lipid cores (Salem et al. 2012) and 4) higher GSM values are associated with a higher percent calcium and fibrous content in plaques (El-Barghouty et al. 1996; Grogan et al. 2005; Sztajzel et al. 2005). Similar to others (Grogan et al. 2005; Sztajzel et al. 2005), we demonstrated that a larger percent black area (JBA or BAB) in any one component of the plaque, near the surface of the plaque, was associated with a higher ulceration score and that higher GSM values were associated with a higher percent calcium in the plaque. We also demonstrated previously unrecognized associations between DWAs on grayscale imaging with plaque inflammation. Our findings demonstrate the potential for adding grayscale analysis to carotid ultrasound imaging protocols to provide additional information that could guide patient treatment by providing additive value for assessing clinical or silent stroke risk and progressive cognitive decline.

Atherosclerotic plaque vulnerability is related to its chemical composition and mechanical properties of the arterial wall (Fleiner et al. 2004; Dempsey et al. 2010; Wang et al. 2013). Carotid strain elastography, shear wave elastography and ARFI are ultrasound methods that can assess mechanical properties of the arterial wall. High strain values are associated with softer plaques (those with characteristics of vulnerable plaque; large lipid core, intraplaque hemorrhage, thin fibrous cap) (Shi et al. 2008; Shi et al. 2009), increased white matter hyperintensities on magnetic resonance imaging (Berman et al 2015), and poor performance on cognitive function testing (Wang et al. 2013; Wang et al. 2014). Shear wave elastography has been shown to provide information regarding plaque stiffness, with lower Young's Modulus (YM) associated with vulnerable plaques (Garrard et al. 2015). The purpose of this study was to determine if grayscale features of carotid plaques are associated with histological markers for inflammation. Confirming that ultrasound grayscale plaque features are associated with histopathology findings is important in that it provides information about the plaque composition. Thus, adding grayscale analysis of plaque to carotid duplex Doppler examinations and/or strain imaging/ARFI could assist in identifying individuals who may benefit from more aggressive management, to halt or prevent future risk of stroke and silent stroke leading to cognitive decline, due to the presence of vulnerable plaque. Combining these ultrasound techniques may provide information both on composition (to include inflammation) and mechanical properties of the arterial wall.

To our knowledge, this is one of the first studies to demonstrate an association between the ultrasound presence of DWAs and histopathological hemosiderin presence (a marker of old hemorrhage and inflammation based on the presence of lymphocytes). Previously DWAs have been utilized in risk stratification models (Nicolaides et al. 2010) and thought to

represent areas of increased inflammation and angiogenesis. Ultrasound imaging studies with contrast have demonstrated associations between contrast uptake and increased plaque inflammation and angiogenesis (Shah et al. 2007; Hjelmgren et al. 2014). Indeed, inflammation can independently predict the risk of early stroke recurrence (Marnane et al. 2014). A larger percent black area in the plaque was associated with a higher surgical ulceration score, which also is a marker for plaque vulnerability. Presence of a black area along the lumen and the percentage of the black area in that component were associated with a higher ulceration score.

Limitations

All participants had advanced atherosclerosis. All participants were patients scheduled to undergo carotid endarterectomy, and most were symptomatic. This potential selection bias was required to obtain tissue for histopathological analysis, but could limit generalizability of the findings to individuals without advanced atherosclerosis. Individuals with earlier degrees of arterial injury may have different associations between ultrasound and histological markers of plaque vulnerability. All grayscale analyses were performed with 2D ultrasound, utilizing single slice, still images to depict a pulsatile three-dimensional plaque structure, thus leaving room for error in representing the entire plaque throughout the cardiac cycle (Kanber et al. 2013b). 3D ultrasound grayscale plaque characterization might offer additional information about the plaque and may be able to provide additional accuracy in evaluating plaque features that are more representative of the histopathology findings (vanEngelen A 2014; AlMuhanna et al. 2015).

An additional limitation was that we were not able to perform a point to point analysis between the ultrasound image and the plaque specimen. Our results could be improved with utilization of 3D ultrasound imaging and 3D plaque histopathology examination to enable us to do a point to point comparison between plaque tissue specimen and ultrasound images.

Conclusions

Ultrasound plaque characteristics such as GSM, DWAs, and percent black area represent histopathological markers of plaque vulnerability such as inflammation, ulceration, focal calcification, and hemorrhage, validating their potential value as clinical and research surrogates for plaque vulnerability.

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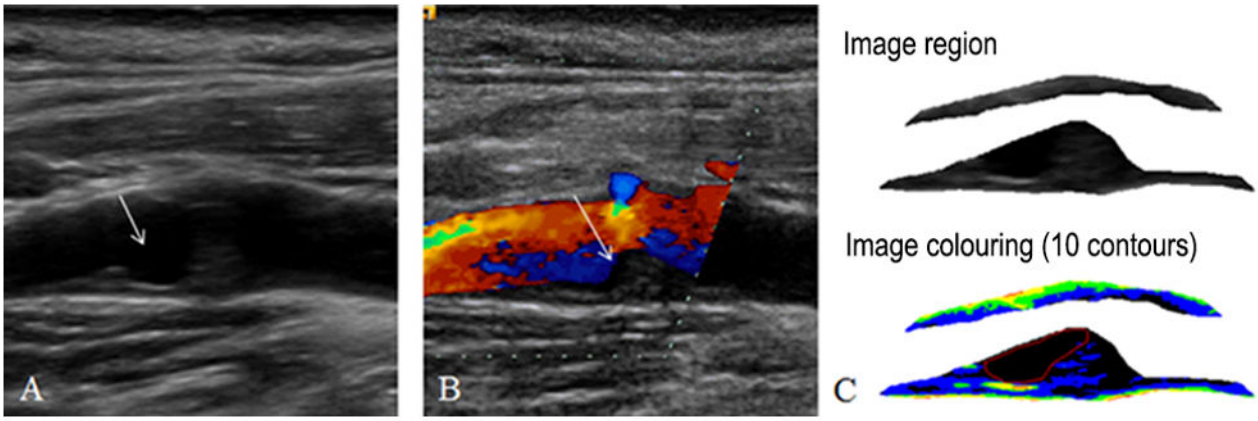

Figure 1.

demonstrates a juxtaluminal black area. Panel A is the B-mode grayscale image. Note the absence of an echogenic border on this juxtaluminal black area on the plaque (arrow). Panel B demonstrates the corresponding color Doppler image taken from a DICOM cine clip. The white arrow is pointing to the black area in this image. Panel C demonstrates the cropped grayscale plaque and its corresponding color coded image. The colors represent the grayscale values. For this software the color black corresponds to a grayscale value of <25. The juxtaluminal black area is outlined in red.

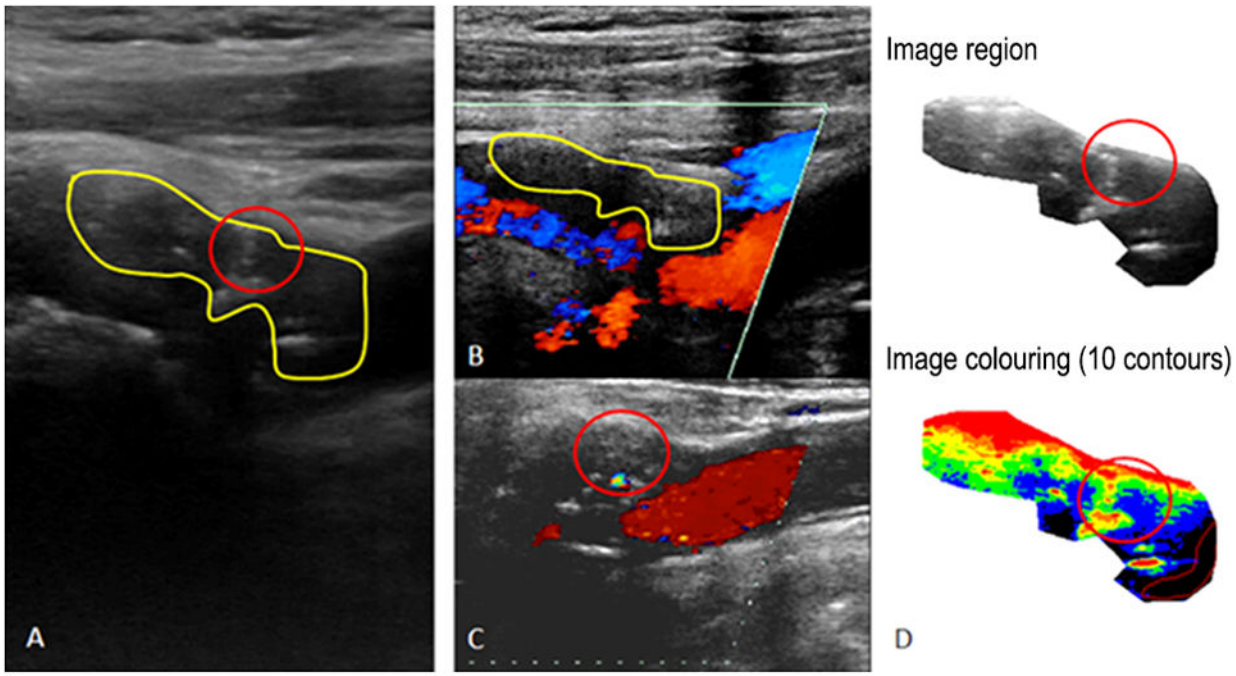

Figure 2.

demonstrates the outline of a plaque (yellow tracing) in panel A. The red circle highlights DWAs identified in this plaque image. Panels B and C demonstrate corresponding color Doppler images outlining the plaque borders and the presence of the DWAs (red circle). Panel D is the traced, cropped, plaque image and its color coded plaque image. Note the DWA bright red areas in the color contoured plaque with the red circle demonstrating the presence of the DWAs. This software identifies grayscale values >126 as red. This plaque also has a black area with a border (BAB) (which could be appreciated on real-time imaging and cine clips), outlined in red.

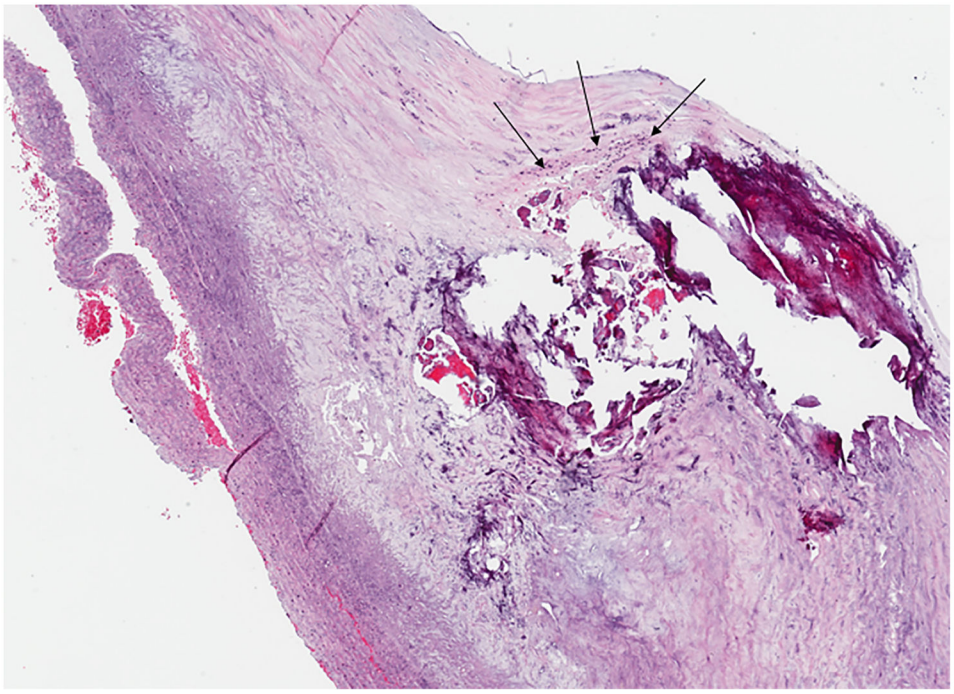

Figure 3.
Histopathology specimen (higher magnification 20xoriginal) from the same patient as in Figure 2 revealing an aggregate of lymphocytes and macrophages (arrow).

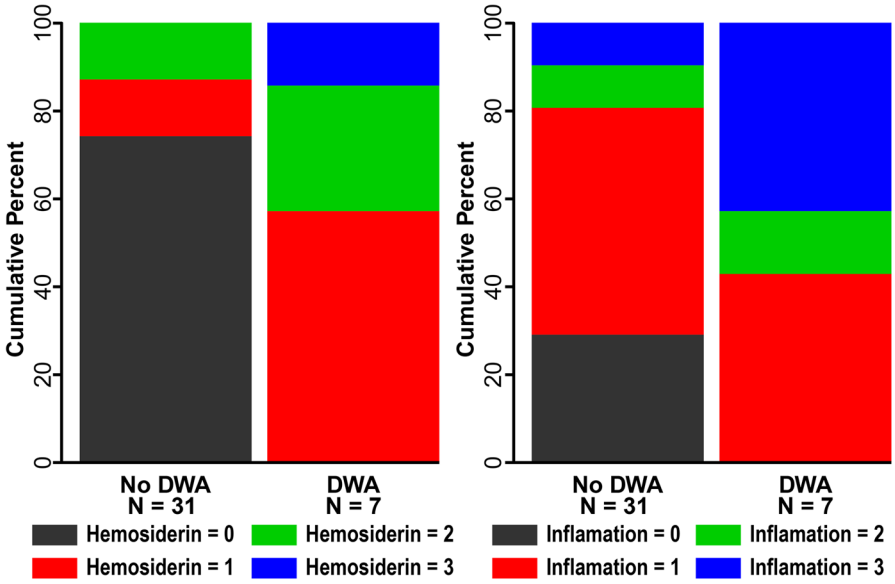

Figure 4.

Panel A, plaques with DWAs present have more hemosiderin on histopathology. Panel B, plaques with DWAs have more inflammation. Reprinted with permission from the American Society of Echocardiography, Abstracts, J Am Soc Echocardiogr 2016;29(6): B72 (Mitchell et al. 2016).

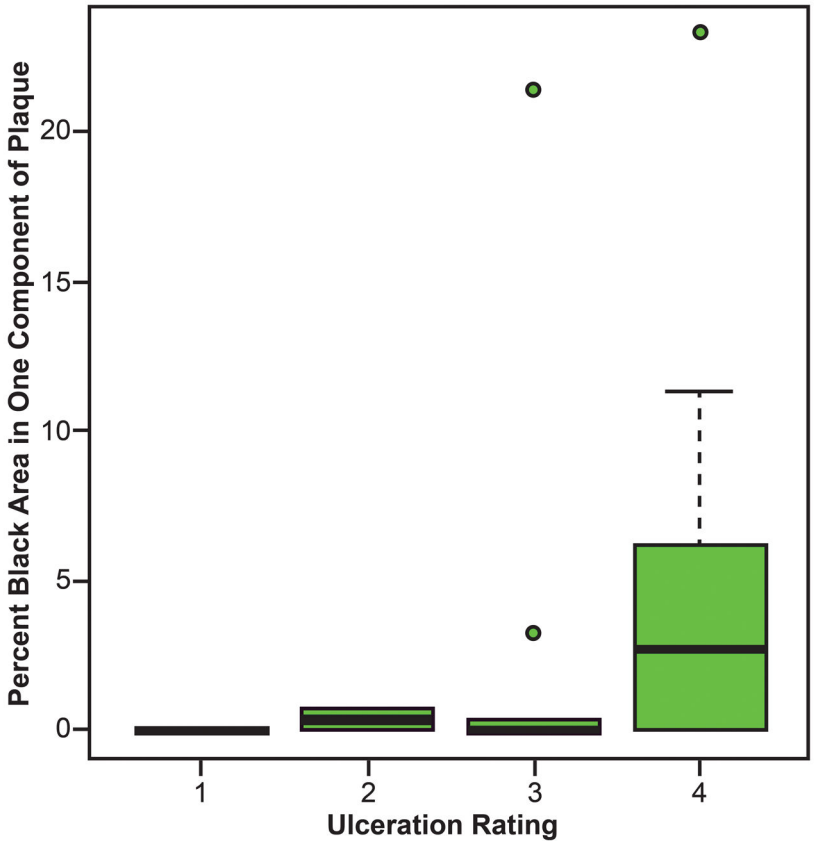

Figure 5.
Plaques with a larger percent black area in any one component are associated with a higher surgeon ulceration score.

Table 1

Subject Demographics, Histopathology and Ultrasound Findings

Subject Characteristic	**Subjects n=, (%) Mean [Standard Deviation]**
Total n	38
Female n (%)	16 (42%)
Age mean [SD]	69 [9.5]
Plaque area mean [SD]	56.2 [33.7]
DWA present (n,% of classification)	7 (18.4)
Black area close to the lumen present (n,%)	17 (44.7%)
Black area mm2 mean [SD]	3.04 [3.6]
% Black area of one component mean [SD]	7.4 [6.3]
GSM mean value [SD]	70.5 [22.6]
Histopathology % calcium, mean [SD]	17.6 [17]
Histopathology hemosiderin score , n, %	Score 3, n = 1 (2.6%)
	Score 2, n = 6, (15.8%)
	Score 1, n= 8, (21.1%)
	Score 0, n=23, (60.5%)
Histopathology inflammation score , n, %	Score 3, n = 6, (15.8%)
	Score 2, n =4, (10.5%)
	Score 1, n = 19, (50.0%)
	Score 0, n = 9, (23.7%)
Diffuse hemorrhage	5.1 [10.8]
Diffuse percent cholesterol	59.1 [21.8]
Ulceration score, n, (%)	Score 4, n =22, (57.9%)
	Score 3, n = 8, (21.1%)
	Score 2, n = 4, (10.5%)
	Score 1, n =3, (7.9%)
	1 no ulceration NA (2.6%)