Published in final edited form as:

J Mol Histol. 2020 October ; 51(5): 559–571. doi:10.1007/s10735-020-09905-5.

Lipid mass spectrometry imaging and proteomic analysis of severe aortic stenosis

Jihyeon Lim, PhD^{1,*}, Jennifer T. Aguilan, PhD^{2,3,*}, Rani S. Sellers, DVM, PhD⁴, Fnu Nagajyothi, PhD⁵, Louis M. Weiss, MD, MPH², Ruth Hogue Angeletti, PhD^{2,6,7}, Anna E. Bortnick, MD, PhD, MSc^{8,9}

¹Janssen Research and Development, Malvern, Pennsylvania

²Laboratory for Macromolecular Analysis & Proteomics, Montefiore Health System and Albert Einstein College of Medicine, Bronx, NY

³Department of Pathology, Montefiore Health System and Albert Einstein College of Medicine, Bronx, NY

⁴Pfizer, Inc. Groton, CT

⁵Department of Pathology, Rutgers New Jersey Medical School, Newark, NJ

⁶Department of Biochemistry, Montefiore Health System and Albert Einstein College of Medicine, Bronx, NY

⁷Department of Developmental and Molecular Biology, Montefiore Health System and Albert Einstein College of Medicine, Bronx, NY

⁸Department of Medicine, Division of Cardiology, Montefiore Health System and Albert Einstein College of Medicine, Bronx, NY

⁹Division of Geriatrics, Montefiore Health System and Albert Einstein College of Medicine, Bronx, NY

Abstract

Correspondence to: Anna E. Bortnick MD, PhD, MSc, Jack D. Weiler Hospital, 1825 Eastchester Road, Suite 2S-46, Bronx, NY 10461, Phone: 718-904-3388, Fax: 718-904-3133, abortnic@montefiore.org.

* Co-first authors

Authors' contributions

JL, JA, LMW, RHA, AEB: conception and design, collection and assembly of data, and data analysis and interpretation, manuscript writing, manuscript editing and final approval of manuscript; RS, FN: collection and assembly of data and final approval of manuscript.

Conflicts of interest/Competing interests

AEB served as site principal investigator for multi-center trials sponsored by Abbott and CSL-Behring in the past year, for which her institution received compensation and received an honorarium from ClearView Healthcare Partners, LLC.

Availability of data and material

Available upon request.

Code availability

Not applicable.

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Severe aortic stenosis (AS) is prevalent in adults > 65 years, a significant cause of morbidity and mortality, with no medical therapy. Lipid and proteomic alterations of human AS tissue were determined using mass spectrometry imaging (MSI) and liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to understand histopathology, potential biomarkers of disease, and progression from non-calcified to calcified phenotype. A reproducible MSI method was developed using healthy murine aortic valves (n=3) and subsequently applied to human AS (n=2). Relative lipid levels were spatially mapped and associated with different microdomains. Proteomics for non-calcified and calcified microdomains were performed to ascertain differences in expression. Increased pro-osteogenic and inflammatory lysophosphatidylcholine (LPC) 16:0 and 18:0 were co-localized with calcified microdomains. Proteomics analysis identified differential patterns in calcified microdomains with high LPC and low cholesterol as compared to non-calcified microdomains with low LPC and high cholesterol. Calcified microdomains had higher levels of: apolipoproteins (Apo) B-100 (p<0.001) and Apo A-IV (p<0.001), complement C3 and C4-B (p<0.001), C5 (p=0.007), C8 beta chain (p=0.013) and C9 (p=0.010), antithrombotic proteins alpha-2-macroglobulin (p<0.0001) and antithrombin III (p=0.002), and higher anti-calcific fetuin-A (p=0.02), while the osteoblast differentiating factor transgelin (p<0.0001), extracellular matrix proteins versican, prolargin, and lumican (p<0.001) and regulator protein complement factor H (p<0.001) were higher in non-calcified microdomains. A combined lipidomic and proteomic approach provided insight into factors potentially contributing to progression from non-calcified to calcific disease in severe AS. Additional studies of these candidates and protein networks could yield new targets for slowing progression of AS.

Keywords

cholesterol; calcific aortic valve stenosis; mass spectrometry imaging; proteomics

Introduction

Aortic valve stenosis (AS) is progressive and a leading cause of morbidity and mortality in older adults, for which there is no approved medical therapy (Lindman et al., 2016). Rheumatic, congenital, or calcific changes contribute to severe AS and obstruction of left ventricular outflow (Lindman et al., 2016). In older adults living in the West, calcification is the most common pathology of valves replaced for severe AS (Lindman et al., 2016). Severe flow limitation across the aortic valve leads to clinical deterioration and death, for which calcification, fibrosis and scarring are major pathogenic factors (Rajamannan et al., 2011). Computed tomography-derived calcium burden predicted aortic valve replacement and death independent of other predictors in individuals with severe AS (Pawade et al., 2018). Despite a causal association with low density lipoprotein (LDL), statins were not successful in slowing AVC progression (Smith et al., 2014, Zhao et al., 2016). Hence there is need to identify proteins associated with progression and to uncover new therapeutic targets. Pathways involved in aortic valve calcification (AVC) may be targets for intervention.

Mineralization of the aortic valve (AV) is an active process (Lindman et al., 2016). The AV is an avascular structure comprised of fibrosa, spongiosa, and ventricularis layers with endothelial and valve interstitial cell types (VICs) in extracellular matrix (ECM) (Lindman

et al., 2016). Cholesterol and lipid deposition is thought to trigger inflammation, reactivation of developmental programs, and osteoblast transformation of VICs, leading to calcium salt precipitation.

Proteomics on whole AS lysate vs. control, necropsy AV demonstrated changes in ECM (Martin-Rojas et al., 2015). A study using mass spectrometry imaging (MSI) demonstrated similar findings (Mourino-Alvarez et al., 2016). Proteomics and transcriptomics have been used to map changes by layer and cell type in severe AS (Schlotter et al., 2018). MSI combines the multichannel molecular weight measurement capability of mass spectrometers with a surface sampling process in a cell type specific manner (Murphy et al., 2009, Chaurand, 2012). It allows rapid probing and mapping of the molecular content of tissues in situ and selectively visualizes lipids which are often washed out during the conventional staining process (Zaima et al., 2011, Castro-Perez et al., 2014). Lipid-mapping has been performed for human congenital AS, which demonstrated changes in lipid localization with increasing disarray of ECM (Angel et al., 2016). However, it has not been reported for adult human AS, nor has it been used as a molecular tool to differentiate calcific vs. non-calcified microdomains for proteomic analysis. We hypothesized that the lipid and protein profile of calcific vs. non-calcified microdomains would demonstrate differences which could potentially elucidate factors associated with progression of AS.

Materials and methods

Murine Valve

Optimization of MSI was performed on frozen sections of mouse AV (n=3). C57BL/6 mice were maintained under SPF barrier conditions at the Albert Einstein College of Medicine Barrier. Mice were fed autoclaved Purina Rodent Chow 5053 ad libitum and autoclaved water. They were euthanized with CO₂ and exsanguinated. Hearts were flash-frozen by immersion in liquid nitrogen. Animals were handled according to the European Convention on animal care, Institute for Laboratory Animal Research Guide for Care and Use of Laboratory Animals and the Albert Einstein IACUC committee.

Human Valve

Resected AV tissue specimens were obtained from 2 patients undergoing elective replacement for non-rheumatic, severe AS at Montefiore Medical Center. Diagnosis was confirmed by clinical pathology. AVs were placed in 0.9% sodium chloride (Cat. No. S3014, Sigma-Aldrich, St. Louis, MO), removed and flash-frozen by slow immersion in liquid nitrogen. They were stored at -80°C. The study was approved by the Albert Einstein College of Medicine Institutional Review Board.

MSI sample preparations and mass spectral acquisition

Tissues were prepared at -20 °C as 8-µm sections using a Leica CM3050 cryostat (Leica Microsystems GmbH, Wetzlar, Germany, see Fig.1 for a description of the workflow). Cryosections were thaw-mounted onto indium tin oxide-coated conductive glass slides (Cat. No. CG-811N-S115, Delta Technologies, Loveland, CO). Murine hearts were sectioned continuously to the region of the AV. Slides were stained with diff-quick (Diff-stain Kit

7128, IMEB Inc, San Marcos, CA), the location of the valve microscopically confirmed, and $8 \times 8 \mu\text{m}$ sections were identically prepared. Slides were dried under vacuum for 20 minutes followed by slow immersion in 50 mM ammonium formate (Cat. No. A666-500, Fisher Scientific, Pittsburgh, PA) at 4°C for 30 seconds without agitation to desalt without losing lipid (Angel et al., 2012). After washing, excess liquid was blotted away and slides were dried under vacuum for 20 minutes. Slides were scanned (ScanMaker 9800XL, Microtek, Hsinchu, Taiwan) and coated with 2,5-dihydroxybenzoic acid (DHB, Cat. No. 149357-25G, Sigma-Aldrich, St. Louis, MO) in a sublimation apparatus (Chemglass Life Science, Vineland, NJ) (Thomas et al., 2012). Matrix-assisted laser desorption/ionization time of flight (MALDI-ToF) mass spectra were acquired from tissue on an UltrafleXtreme™ MALDI ToF/ToF mass spectrometer (MS) (Bruker, Billerica, MA). Data acquisition was performed in a positive reflectron mode at a constant laser power, optimized for detection of peaks in the m/z 300–1000 range. A total of 600 laser shots were accumulated to generate a mass spectrum from each pixel of $30 \mu\text{m} \times 30 \mu\text{m}$. XY coordinates of each pixel were mapped on the scanned optical images by matching fiducial marks on glass slides and images.

MSI data analysis

Data were rendered into spectra with XY coordinates. Peak intensities were normalized using total ion current to account for variation. Images (ion maps) were visualized with selected m/z of ions (± 0.1 Da) and normalized intensities (FlexImaging version 4.0, Bruker, Billerica, MA). Once spectra were acquired, slides were washed with ethanol, stained with hematoxylin and eosin (H&E, Cat. No. S216 and Cat. No. S2781, PolyScientific R&D Corp., Bay Shore, NY) and photomicrographed (Leica DM2000 microscope, Leica Microsystems GmbH, Wetzlar, Germany). Photomicrographs were co-registered with optical images, marked for regions of interest, overlaid with spectra and clustering analysis was performed (ClinproTools version 3.0, Bruker, Billerica, MA). Spectra were baseline subtracted using Convex Hull baseline and smoothed with Savitzky–Golay filter. Null spectra were excluded and the remainder recalibrated to match 20% of calibrant peaks. Peaks were picked from the total average spectrum with the threshold of $S/N = 4$ and the normalized peak intensities $>1\%$. Matrix arrays with peaks and intensities were subjected to unsupervised clustering scaled with unit variance and the distance computed with Ward's linkage. Dendrograms were used to plot the original location of mass spectra from each cluster (FlexImaging). Lipids were analyzed in LipidBlast (<http://fiehnlab.ucdavis.edu/projects/LipidBlast>) (Kind et al., 2013). MS/MS spectra of target lipids were acquired.

Proteomic analysis of severe AS by LC-ESI-MS/MS

Proteins from calcified and non-calcified microdomains were manually microdissected, extracted by heating at 98°C for 1.5 h and sonication for 1 h followed by reduction with 1,4-dithiothreitol (Cat. No. D0632, Sigma-Aldrich, St. Louis, MO) and alkylation with iodoacetamide (Cat. No. I6125, Sigma-Aldrich, St. Louis, MO), then digested with trypsin (Cat. No. T6567, Sigma-Aldrich, St. Louis, MA) overnight at 37°C . Peptide digests were desalted using a Pierce C-18 spin column (Cat. No. 89870, ThermoScientific, Waltham, MA). Peptides were separated and analyzed by LC-ESI-MS/MS using a nanoACQUITY

Waters UPLC (Waters Corporation, Milford, MA) connected to the LTQ-Orbitrap Velos mass spectrometer (ThermoScientific, Waltham, MA).

Mascot (version 2.5.1, Matrix Science Inc., Boston, MA) protein database search against Swissprot AC human protein database (version Swissprot_AC_2016_07) was performed using the following parameters: carbamidomethylation on cysteine for fixed modification; oxidation on methionine and deamidation on glutamine and/or asparagine for variable modifications. The peptide precursor mass tolerance was 25 ppm and 0.5 Da for the MS/MS tolerance. The Mascot search results were further processed using Scaffold Q+/Q+S (version 4.8.7, Proteome Software, Portland, OR). Protein identification results were based on a minimum of 1 peptide at 1% peptide and protein false discovery rates (FDR), then analyzed by Ingenuity Pathways Analysis (IPA version 44691306, Qiagen Bioinformatics, Redwood City, CA) for core and molecular networks analyses as described in the Supplemental Information. To generate the heat map, protein search results from Scaffold Q+/Q+S were converted to mzIdentML and processed by Scaffold Perspectives (version 2.1.0, Proteome Software, Portland, OR) using a minimum of 2 peptides filter, 5% Protein and 1% Peptide FDR and Fisher's Exact Test with confidence level based on controlled family-wise error rate using Hochberg's step-up and Holm's step-down analysis with Benjamini-Hochberg correction for significance level $\alpha < 0.01544$.

Results

Reproducible detection of lipids on serial sections of murine AV using MSI

In order to develop a reproducible method for detecting differences in calcified and non-calcified microdomains, we used sections from murine AV and then applied the method to analysis of human AV. A schema describing the overall workflow is shown in Fig. 1. Positive ion maps were constructed from murine AV (n=3). Supplemental Fig. 1 shows a) a representative ion map constructed from a peak at m/z 524.4 and b) H & E stained AV post MSI. In addition to visual assessment, section to section variability was evaluated (Karlsson et al., 2014). H&E stained images post MSI were co-registered on optical images. Mass spectra belonging to the co-registered AV were extracted and constructed into a matrix array. Peaks displayed linearity ($R^2 = 0.835-0.982$, Supplemental Fig. 1c). This protocol was then applied to characterize changes in lipid species associated with human tissue alterations.

Lysophosphatidylcholine (LPC) 16:0 and 18:0 co-localize with calcified microdomains in severe AS by MSI

MSI was performed to map which lipids were located within different microdomains of AS tissue. MSI data were acquired from 3 replicate slides and constructed with positive ion maps. Peaks at m/z 369.2, 496.3, 524.4, and 895.6 showed association with various microdomains (Fig. 2). Hexadecanoyl-*sn*-glycero-3-phosphocholine (LPC (16:0, 16 carbons and no double bonds, m/z 496.3)) and stearoyl-*sn*-glycero-3-phosphocholine (LPC (18:0, m/z 524.4)) (pink in Fig. 2a, b, c, h, i, j) were increased in calcified microdomains (white, Fig. 2e, g and deep red, Fig. 2d, f). A phosphocholine (PC) (at m/z 734.6) (green, Fig. 2b, j) and increased cholesterol (m/z 369.2, blue, Fig. 2c, h) differentiated non-calcified microdomains. The differences in tissue shapes between the optical and H&E images are

due to the loss or slight shifting of mineralized tissues post MSI, as there is tissue damage from laser ion generation. Both images confirmed the original location of the calcified microdomain.

Tandem MS spectra of the 4 peaks were acquired in situ by generating ions only within the several pixels of matching XY coordinates to confirm their identity. Fig. 3a–c displays MS/MS spectra which are assigned to LPC (16:0) (m/z 496.3, $[M+H]^+$), LPC (18:0) (m/z 524.4, $[M+H]^+$), and cholesterol (m/z 369.2, $[M+H-H_2O]^+$), respectively. Signature fragments at m/z 103.9 and 183.9 originated from the phosphate head group, verifying the presence of the LPCs (Fig. 3a, b). Spectra were compared with MS/MS peak patterns acquired in other studies and LipidBlast (Liebisch et al., 2002, Rossmann B, 2007, Kind et al., 2013), confirming the specific identities of each, except for the peak at m/z 895.6. This peak differentiated calcified from non-calcified microdomains (Supplemental Fig. 2a, b) and may be ascribed to triglyceride (TG) (Schiller et al., 2001).

Unsupervised hierarchical clustering of MS spectra differentiates non-calcified from calcified microdomains in severe AS

The MSI profile obtained for various microdomains allows them to be biochemically differentiated and they can be visually mapped by ascribing color to various compounds. A single tissue section was chosen to test if the unsupervised clustering of the spectra acquired could produce visual patterns comparable to histological annotation. To test this automatic interpretation process, 999/10852 acquired from each pixel were randomly selected to minimize the size of the distance matrix array, then hierarchically clustered in an unsupervised manner to generate a dendrogram for interactive analysis. Spectra, 927/999, were clustered in 3 major groups: 313 (cluster 1, green), 343 (cluster 2, pink), and 271 (cluster 3, blue, Fig. 4a). The plot shows the increase of cholesterol (m/z 369.2) in cluster 3; LPCs (m/z 496.3 and 524.4), a peak (m/z 895.6) in clusters 2; and a PC (m/z 734.6) in cluster 1. Spectra were plotted on the optical image (Fig. 4b). Two major clusters in blue and pink illustrate visual segmentations analogous to ion maps (Fig. 2h) with cholesterol and LPC distributions. Tissue outside the non-calcified microdomain is represented by the third major cluster of the MS spectra in green (Figs. 2j, 4b). These data confirm comparable visual and statistical differentiation of microdomains, supporting semi-automated interpretation of MSI.

In sum, there were patterns of major lipid species that differentiated microdomains. Calcified microdomains were associated with increased LPCs (16:0) and (18:0), and a peak at m/z 895.6 (putative TG (Schiller et al., 2001)). In contrast, non-calcified microdomains were associated with higher cholesterol levels and a PC at m/z 734.6.

Proteomic analysis of non-calcified and calcified microdomains in severe AS

Once MSI maps were created, we then studied which proteins were localized to different microdomains with the hypothesis that this may reveal networks involved in either pathophysiology of disease or counterregulatory mechanisms. Proteins (148) were identified and grouped into those (1) unique to the calcified microdomain; (2) unique to the non-calcified microdomain and; (3) common to both (Supplemental Tables S1–S3). For the

calcified microdomain, the top 3 networks (Fig. 5a, Supplemental Table 1 and Fig. S3) were organismal injury and abnormalities, inflammatory response and cardiovascular disease, system development and function pathways. Other interacting network proteins were found in pathways for migration, survival, proliferation and vasculogenesis of endothelial cells. For the non-calcified microdomain, the top 3 networks of interacting proteins involve pathways in free radical scavenging, connective tissue, developmental and hereditary disorders, cell death, survival and cellular movement (Fig. 5b, Supplemental Table 2 and Fig. S4). Proteins from the non-calcified region were related to protein binding, migration, response to stimuli, extracellular matrix organization and wound healing. For the proteins found in both microdomains, the top 3 networks involved pathways in hematological system development and function, cell-to-cell signaling, interaction and developmental disorder, humoral immune response, inflammatory response, developmental disorder and cell morphology and cardiovascular disease (Supplemental Table S3, Fig. S5).

Total spectrum counts from the calcified versus non-calcified microdomains were compared (Supplemental Table S4) and a heat map was constructed (Fig. 6). Calcified microdomains had statistically significant higher levels of: apolipoproteins (Apo) B-100 ($p<0.001$) and Apo A-IV ($p<0.001$), complement C3 and C4-B ($p<0.001$), C5 ($p=0.007$), C8 beta chain ($p=0.013$) and C9 ($p=0.010$), antithrombotic proteins alpha-2-macroglobulin ($p<0.0001$), antithrombin III ($p=0.002$) and plasminogen ($p=0.007$), and higher anti-calcific fetuin-A (alpha-2-HS-glycoprotein, $p=0.02$). In contrast, non-calcified microdomains had statistically significant higher levels of: tropomyosin and myosin ($p<0.0001$), actin-binding proteins ($p<0.001$), the osteoblast differentiating factor transgelin ($p<0.0001$), ECM proteins versican, prolargin, and lumican ($p<0.001$) and regulator protein complement factor H ($p<0.001$).

Discussion

We investigated the distribution of lipid species by mass spectroscopy imaging in calcified aortic valves from individuals undergoing surgical aortic valve replacement for severe aortic stenosis, a prevalent disease of older adults with no medical therapy. An advantage of MSI is that it does not require a priori information regarding histology, lipid or protein profiling and visual investigation of distinctive patterns on multiple ion distribution maps prevents over-normalization (Kind et al., 2013, Karlsson et al., 2014). We observed; 1) increased LPC 16:0 and 18:0, and decreased cholesterol in calcified microdomains of AS, as compared to non-calcified microdomains, which had the opposite profile, and 2) differential distribution of proteins associated with calcified and non-calcified microdomains pointing to potential pathways involved in the progression of disease. Regionality of lipid distribution that we observed in calcific AS is concordant with previous MSI studies of atherosclerotic lesions (Zaima et al., 2011) and in congenital AS, where varying patterns of lipid species were associated with ECM disarray (Angel et al., 2016). Additionally, many of the proteins, their localization, and protein networks we identified corroborate previous studies (Martin-Rojas et al., 2015, Mourino-Alvarez et al., 2016, Schlotter et al., 2018). Schlotter et al., used MSI to map proteins localized in different tissue layers of calcific AS separated by laser microdissection, while we separated calcified and non-calcified microdomains based on

their molecular lipid signatures. A potential advantage of our method includes identification of lipid species which may play a pro-calcific role through cellular signaling.

LPCs are generated from partial hydrolysis by LpPLA₂ which removes one of the fatty acid groups. LPCs (16:0) and (18:0) consist of a polar choline headgroup, a glycerol backbone and fatty acyl chain of 16 or 18 carbons. (Schmitz and Ruebsaamen, 2010) They are highly pro-inflammatory; signaling via multiple protein receptors to stimulate neutrophil and eosinophil activity, endothelial dysfunction, and cytokine release. Consistent with their pro-inflammatory properties, LPC (16:0) and (18:0) were significantly elevated in broncho-alveolar lavage fluid from individuals with more active asthma, as well as in atherosclerotic carotid and aorta. (Thukkani et al., 2003, Stegmann et al., 2011, Yoder et al., 2014) LpPLA₂ increases mineralization of smooth muscle cells and modulates inflammation (Vickers et al., 2010) and is known to be elevated in tissue and serum of individuals with AVC (Mahmut et al., 2014, Bortnick et al., 2016). LpPLA₂ associates with LDL and Lp(a), a complex of Apo (a), Apo B-100, cholesterol, PC, and TG. LDL and Lp(a) are causal for AVC from Mendelian randomization genetic studies (Thanassoulis et al., 2013, Smith et al., 2014). Thus, lipid deposition from Lp(a), LDL, increased LpPLA₂ activity and the pro-inflammatory effects of LPC (16:0) and (18:0), may work in concert to trigger or propagate AVC. Aggressive lowering Lp(a) with antisense oligonucleotides could be a useful strategy in the treatment or prevention of AVC (Viney et al., 2016, Youssef et al., 2020).

Using the distribution of lipids to define calcified vs. non-calcified domains, we determined proteins which could be important in AVC progression. Our findings strengthen the observation that high density lipoprotein (HDL)-associated proteins (Apo A1, Apo A2, Apo-AIV and Clusterin) are localized to calcified microdomains (Schlotter et al., 2018), which may indicate upregulation of cholesterol efflux as a mechanism for macrophages to rid themselves of cytotoxic cholesterol (Bortnick et al., 2000) or mark anti-inflammatory modulation by mature HDL's protein or lipid cargo (Furtado et al., 2018). Boosting cholesterol efflux could be a novel strategy for treatment of AS or AVC, using Apo A1 mimetics or infusions (Busseuil et al., 2008), however, genetic polymorphisms linked to higher plasma HDL cholesterol levels were not associated with decreased AVC in one study (Smith et al., 2014).

Accumulation of oxidized lipids, LPCs and cholesterol may be activating the complement cascade. Complement has been linked to bone turnover, as osteoclasts and osteoblasts produce and have receptors for it (Schoengraf et al., 2013). We identified proteins in the alternative and classical pathways, supporting previously reported associations in AVC and AS (Helske et al., 2008, Schlotter et al., 2018). C5b to C9 co-localize with atherosclerotic plaque and AV damage may similarly be occurring with activation of the membrane attack complex (Yasojima et al., 2001, Helske et al., 2008). Complement factor H was exclusively identified in non-calcified microdomains, but its counterregulatory activity may be overwhelmed, leading to progression of disease, as seen in atherosclerosis (Yasojima et al., 2001, Sarma and Ward, 2011). Anti-complement drugs such as eculizumab, anti-C5a antibody, C1 esterase inhibitors and related compounds, could be investigated for use against AVC, but immunosuppression is a risk.

Fetuin-A (alpha-2-HS-glycoprotein) was identified in calcified microdomains. Fetuin-A is a major binder of calcium-phosphate which prevents ectopic calcification and has been localized to AS tissue (Schafer et al., 2003, Schlotter et al., 2018). Interestingly, epidemiologic data associating serum levels of fetuin-A with AVC have been mixed. Lower serum levels of fetuin-A have been associated with prevalent AVC and faster AS progression in some, but not all studies, which may relate to the severity of disease in the population studied (Koos et al., 2009, Bortnick et al., 2016, Kubota et al., 2018). Studies of both serum and tissue biomarkers are thus complimentary, as serum levels of measured proteins may not always reflect tissue-level changes occurring in AVC.

Our study also identified antithrombotic/antiproliferative proteins α -2 macroglobulin, antithrombin III, and plasminogen in the calcified microdomain, similar to Schlotter et al., 2018. Antithrombin-III belongs to the family of serpins which inhibit thrombin and Factor Xa to decrease endothelial cell proliferation, but is also an anticoagulant (Satoh et al., 2015). Whether it functions as a counterregulatory mechanism in AVC is unknown. The finding of plasminogen in calcified microdomains points to degradation of ECM and anikis, a form of programmed cell death, as potential mechanisms not only in atherosclerosis, but also in AVC progression (Kochtebane et al., 2010, Carter, 2012, Romagnuolo et al., 2014). Plasminogen activator cleaves plasminogen to plasmin, which in turn, binds to cell surfaces and digests ECM glycoproteins. This leads to anikis when cells detach from ECM. Plasmin also cleaves pro-matrix metalloproteinase (MMP) 9 to active enzyme, increasing digestion of ECM (Natorska and Undas, 2015). Thus, anti-MMP drugs might be inhibitors of progression in AVC, by blocking ECM destruction.

In contrast to calcified microdomains, ECM proteins (versican, tenascin-X, decorin, prolargin, and lumican) and actin-binding proteins (tropomyosin, filamin-A, vinculin) involved in adhesion, development and remodeling, as well as transgelin, were more abundant in non-calcified microdomains. This is in line with the finding of increased plasminogen in the calcified area where degradation of ECM proteins may have already occurred.

Earlier in the course of disease, ECM proteins may accelerate progression of AVC by binding growth factors which induce osteoblastic trans-differentiation (Chen and Simmons, 2011, Elsafadi et al., 2016). ECM proteins also interact with periostin, a vitamin K dependent protein we found in both calcified and non-calcified microdomains, to promote tissue remodeling (Coutu et al., 2008). Low vitamin K reduces the activity of the calcification inhibitor, matrix Gla protein, which has been linked to worse AVC (Chiyoya et al., 2018). Warfarin has been implicated in worsening calcification through vitamin K antagonism as compared to direct oral anticoagulants (Rattazzi et al., 2018) and clinical data suggests that there is less AVC progression in individuals on direct oral anticoagulants as compared to warfarin (Di Lullo et al., 2019). Vitamin K repletion is being investigated as a treatment for AVC (Brandenburg et al., 2017).

This study has several limitations. First, the small n reduces statistical power to detect significant differences in total spectrum counts. Results should be validated with a larger sample size. Many of the proteins and networks we identified corroborate previous studies

(Martin-Rojas et al., 2015, Mourino-Alvarez et al., 2016, Schlotter et al., 2018). Others may not have been identified because of differences in statistical power or experimental approach, as we did not delipidate our samples and trypsin-digested proteins were extracted after manual microdissection. Second, calcium salt from AVC itself could potentially suppress the ionization of cholesterol, reducing its intensity in calcified microdomains (Lou et al., 2009). Regardless, the observed step-down in cholesterol content was useful in differentiating microdomains and aided proteomic analysis. Third, non-calcified domains contained both valvular and a minor amount of muscular tissue, which may contribute to the increased presence of myocyte-related proteins. Lastly, the peak at m/z 895.6 requires additional fragmentation in order to confirm if it is indeed TG.

In conclusion, our study used MSI, lipid spatial mapping and proteomics to analyze alterations in severe AS at the molecular level. Calcified microdomains had higher LPC (16:0) and (18:0) and lower cholesterol as compared to non-calcified microdomains, which had the opposite profile. Using the differential lipid profile to separate calcified and non-calcified microdomains, we were able to identify proteins potentially involved in pathologic mineralization and counterregulatory mechanisms. Based on this and others' findings, we hypothesize that tissue injury from repetitive shear stress leads to inflammation and oxidation, with thickening and fibrosis as an initial response, potentially accounting for increased proteins in networks related to free radical scavenging, cellular signaling and connective tissue seen in the non-calcified microdomains. Accumulation of LPCs produced by the action of LpPLA2 on LDL and Lp(a) may forward propagate inflammation and complement activation in areas that go on to form calcific nodules. Enzymatic degradation of ECM from plasmin and MMPs may weaken those areas, favoring growth of microcalcific deposits originating from extracellular vesicles shed by transdifferentiated cells into calcific nodules, eventually leading to worsened calcification and valvular dysfunction (Krohn et al., 2016). MSI analysis of valvular changes in an animal model of calcific AS could be useful to more precisely study the time course of lipid and protein alterations at different stages of disease. Candidate lipids, proteins, and protein networks identified from this study should be further investigated as biomarkers or targets for intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We acknowledge Edward Nieves' assistance with mass spectrometry, Amanda Beck, Jeffrey Harding and Barbara Canella's assistance in sectioning and photomicrography (all from the Montefiore Health System and Albert Einstein College of Medicine). We thank Genevieve T. Aguilan for composing Figure 1.

Funding

Funded by the Department of Medicine, Division of Cardiology at Montefiore Health System and Albert Einstein College of Medicine, with additional support from American Heart Association Mentored Clinical and Population Award 17MCPRP33630098, the Empire Clinical Research Investigator Program, and National Institutes of Health 1K23HL146982-01A1 (AEB), National Institutes of Health UL1TR001073, National Institutes of Health 1S10RR025128 and Shared Instrumentation Grant 1S10RR029398-01 (RHA).

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Fig. 1.

(a) *Lipid analysis by matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI)*: Flash frozen heart valve tissue sections (8-µm) were mounted onto indium tin oxide-coated (ITO) conductive glass slides and dried under vacuum. Tissue sections were washed by slow immersion in 50 mM ammonium formate and coated with 2,5-dihydroxybenzoic acid (DHB) in a sublimation apparatus. MALDI-time of flight (MALDI-ToF) mass spectra were acquired from tissue on an UltrafleXtreme™ MALDI ToF/ToF mass spectrometer with a spatial resolution of 30 µm x 30 µm. XY coordinates of each pixel were mapped. Images of lipid ion maps were visualized with selected mass/charge (m/z) of ions.

(b) *Proteomics analysis by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)*: Proteins from calcified and non-calcified areas were manually microdissected and extracted by heating at 98°C for 1.5 h and sonication for 1 h. Reduction and alkylation of the extracted proteins were performed followed by trypsin digestion of the proteins into peptides. The peptides were analyzed by nanoflow-LC-ESI-MS/MS using a nanoACQUITY Waters (Ultra Performance Liquid Chromatography) UPLC connected to the LTQ-Orbitrap Velos mass spectrometer. Protein identification was performed by submitting the MS/MS spectra for automated protein database search using the Mascot search engine. Protein heat maps were generated using Scaffold Q+/Q+S and Scaffold Perspective. Protein networks were identified by Qiagen Ingenuity Pathways Analysis

Fig. 2.

Patient 1: Distribution of LPCs and cholesterol in severe AS. (a) distribution of LPC (18:0) at m/z 524.4, $[M+H]^+$ (pink) and b) overlay of LPC (18:0) (pink) with a PC at m/z 734.6, $[M+H]^+$ (green); (c) overlay of cholesterol at m/z 369.2, $[M+H-H_2O]^+$ (blue) with LPC (16:0) at m/z 496.3 $[M+H]^+$ (pink); (d) H&E stained post MSI; and (e) Optical image of the section pre-MSI. **Patient 2:** Distribution of LPCs and cholesterol in severe AS. (f) H&E stained post MSI; (g) Optical image of the section pre-MSI; (h) overlay of cholesterol at m/z 369.2 (blue) with LPC (16:0) at m/z 496.3, $[M+H]^+$ (pink); (i) distribution of LPC (18:0) at m/z 524.4 (pink); (j) overlay of a PC at m/z 734.6, $[M+H]^+$ (green) with LPC (18:0) at m/z 524.4 (pink). Sections from Patient 1 and 2 were made on the same slide subject to identical preparation. Some of the calcified microdomain was lost during staining

Fig. 3.

To confirm the identity of lipids localized to the calcific microdomain, tandem MS spectra were acquired in situ and fragmentation patterns were compared to MS/MS peak patterns acquired in other studies and LipidBlast. Tandem mass spectra of severe AS displaying a) LPC (16:0) (m/z 496.3, $[M+H]^+$), b) LPC (18:0) (m/z 524.4, $[M+H]^+$), and c) cholesterol (m/z 369.2, $[M+H-H_2O]^+$) acquired in situ

Fig. 4.

The lipid species found on MS/MS were ascribed a color and visually mapped as part of the semi-automated process for MSI of AS. a) Overlaid mass spectra averaged using 3 major clusters from patient 2, showing increased LPC (16:0) and LPC (18:0) in the calcified microdomain (darker red in Fig. 2f). b) Distribution of the spectra from the 3 clusters plotted on the optical image (Fig. 2g)

Fig. 5.

Top protein networks identified from Ingenuity Pathway Core network analysis (IPA) of proteins (in yellow) in a) calcified and b) non-calcified microdomains. The solid lines represent direct connection while the dashed lines represent indirect interaction. a) Network (Score: 30). IPA identified 14 proteins from the calcified region in pathways involving organismal injury and inflammatory response. b) Network (Score: 52): IPA identified 22 proteins from the non-calcified region in pathways involving free radical scavenging and small molecule biochemistry

Fig. 6.

Protein heatmap of the most common proteins localized to calcified and non-calcified microdomains of AS. ($p < 0.05$, * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$). The probability of a protein being present is indicated green: 8 TSC (total spectrum counts), yellow: 4–7 TSC, orange: 2–3 TSC; red: 1 TSC, white: <1 TSC