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Siobhan V. Glavey[1,7*], Alexandra Naba[2,¶,*], Salomon Manier[1*], Karl Clauser[3], Sabrin Tahri[1], Jihye Park[1], Michaela R. Reagan[1], Michele Moschetta[1], Yuji Mishima[1], Manuela Gambella[4], Alberto Rocci[5], Antonio Sacco[1,6], Michael E O'Dwyer[7], John M. Asara[8], Antonio Palumbo[4], Aldo M. Roccaro[1,6,#] Richard O. Hynes[2,9,#], Irene M. Ghobrial[1#]

[1]Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [2]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA; [3]Protemics Platform, Broad Institute, Cambridge, MA, USA; [4]University of Torino, Azienda Ospedaliero-Universitaria Città della Salute e della Scienza di Torino, Torino, Italy; [5]Manchester Royal Infirmary and Central Manchester University Hospital NHS Foundation, University of Manchester, Manchester, United Kingdom; [6] SST Spedali Civili, Dept. Medical Oncology, CREA Laboratory, Brescia, Italy; [7] Department of Hematology, National University of Ireland, Galway, Ireland; [8]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA; [9]Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA, USA.

[¶] AN Present address: Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL 60612, USA

[*] S.G, A.N and S.M. contributed equally to this study.

[#] AMR, ROH, IMG: co-last Authors

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Corresponding authors:

Irene M. Ghobrial, MD

Dana-Farber Cancer Institute, Boston, MA, USA

Tel: +1 617.632.6777

E-mail: Irene_ghobrial@dfci.harvard.edu

Richard O Hynes, PhD

Koch Institute for Integrative Cancer Research at MIT, Cambridge, MA, USA

Tel: +1 617.253.6422

E-mail: rohynes@mit.edu

ABSTRACT

The extracellular matrix (ECM) is a major component of the tumor microenvironment, contributing to the regulation of cell survival, proliferation, differentiation and metastasis. In multiple myeloma (MM), interactions between MM cells and the bone marrow (BM) microenvironment, including the BM ECM, are critical to the pathogenesis of the disease and the development of drug resistance. Nevertheless, composition of the ECM in MM and its role in supporting MM pathogenesis has not been reported. We have applied a novel proteomic-based strategy and defined the BM ECM composition in patients with monoclonal gammopathy of undetermined significance (MGUS), newly diagnosed and relapsed MM compared to healthy donor-derived BM ECM. In this study, we show that the tumor ECM is remodeled at the mRNA and protein level in MGUS and MM to allow development of a permissive microenvironment. We further demonstrate that two ECM-affiliated proteins, ANXA2 and LGALS1, are more abundant in MM and high expression is associated with a decreased overall survival. This study points to the importance of ECM remodeling in MM and provides a novel proteomic pipeline for interrogating the role of the ECM in cancers with BM tropism.

Keywords: Multiple Myeloma, Extracellular Matrix, Proteomics

INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy and accounts for approximately 10% of all hematological cancers (1). MM provides a model for the study of cancer cell metastasis as MM cells are known to traffic to distant bone marrow (BM) niches via hijacking the normal processes of cellular metastasis (2). Progression of the disease is mediated by intrinsic factors of the clonal cells along with factors that mediate a permissive tumor microenvironment (3). The MM tumor niche comprises the components of the BM microenvironment - cellular (stromal cells, osteoblasts, osteoclasts, endothelial cells and immune cells) and non-cellular (ECM) (4, 5). Despite advances in therapies in recent years, MM remains an incurable disease with a median survival of approximately 5-7 years for newly diagnosed patients (6). Identification of the molecular mechanisms leading to MM has the potential to lead to the development of novel prognostic tests and therapies for MM patients (7)(8).

The extracellular matrix (ECM) is a complex meshwork of proteins that serves as a scaffold for cells. In addition, it actively participates in cell functions such as proliferation, migration and survival via cell-to-matrix interactions (9, 10). The ECM is also rich in growth factors and cytokines thus supporting cell biology (9); therefore it follows that alterations in the ECM may be expected to occur in disease states such as inflammation and cancer. This is indeed the case in several tumor types where ECM alterations have been associated with changes in metastatic potential of tumor cells *in vivo* and in clinical outcomes (11, 12). The role of the ECM as a metabolic regulator of cell function also has implications for malignant processes (13) and molecular signals of metastasis have implicated ECM components and their receptors in tumor progression (14). In the past, attempts to systematically characterize the ECM were challenged by the vast diversity and number of ECM proteins and also by their insolubility and cross-linked nature. A new perspective on analysis of the ECM, on a relatively high throughput scale, came with the definition and *in-vivo* characterization of the "matrisome" (8). The term "matrisome", initially used by Martin and collaborators in 1984 was updated and refined by Naba et al in 2012. This study revealed tissue specific signatures of ECM proteins and importantly provided a method to determine the origin (tumor cell or stroma) of individual matrix proteins.

This, more in-depth knowledge of the ECM composition, was facilitated by whole genome sequencing. This was made possible due to the fact that ECM proteins are encoded by one or a few exons in the genome and whole genome sequencing has therefore provided a platform from which the ECM can be more clearly defined. Bio-informatic analysis of the proteome,

using a list of approximately 50 domains to identify a list of candidate ECM proteins, has facilitated the definition of ECM proteins into a more complete list, which has been named the “core matrisome’ – in mammals this comprises approximately 300 proteins. These proteins include large insoluble proteins such as collagens, proteoglycans and glycoproteins (15). In addition to these insoluble proteins, there are large numbers of ECM-modifying enzymes, ECM-binding growth factors, and other ECM-associated proteins, which for the most part are soluble in nature. These proteins have been included in a defined category of “matrisome-associated” proteins due to the integral role that they play in ECM function (15) . These different categories of ECM and ECM-associated proteins cooperate to assemble and remodel the matrix and bind to cells through ECM receptors. Together with receptors for ECM-bound growth factors, they serve to control survival, proliferation, differentiation, shape, polarity, and motility of cells (15).

This method of ECM profiling has yielded important insights into the composition of several solid tumor matrices along with potential novel therapeutic and prognostic targets (11, 16) however, this method has not been applied to hematological malignancies, where the process of cell migration from the primary niche is largely dependent on the tumor microenvironment (17-19). We characterize the unique extracellular matrices of patients with the pre-myeloma condition monoclonal gammopathy of undetermined significance (MGUS), newly diagnosed and relapsed MM patients and compare them to that of healthy human bone marrow. Our results demonstrate ECM remodeling with disease progression and identify ANXA2 and LGALS1 as ECM proteins that have prognostic relevance for MM patient overall survival.

MATERIALS AND METHODS

Human bone marrow aspirates. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. These studies were approved by the Dana-Farber Cancer Institute Institutional Review Board and the University of Torino, Italy. Whole bone marrow (BM) was obtained from healthy human donors (n=10), MGUS patients (n=3), newly diagnosed MM patients who had not yet received treatment (n=7) and patients with relapsed MM (n=6). Fresh BM samples (20ml/patient) were filtered and underwent red cell lysis, followed by centrifugation at 2500rpm for 10 minutes (see Supplementary Methods for full protocol). The isolated pellet was then processed through the sequential extraction methods outlined below.

Murine models and staining for collagen.

Tumors were established in mice using MM1s-GFP-Luc+ cells (5 × 106/ mouse), which were injected into the tail vein of SCID-Bg mice. Mice were sacrificed at 21 days post injection of tumor cells, femurs harvested and staining for collagens performed using Masson's trichrome stain as a surrogate marker for ECM proteins.

ECM protein enrichment and SDS gradient gel separation. ECM protein enrichment from of whole BM samples was achieved by sequentially depleting intracellular proteins as previously described (8, 20). In brief, whole BM samples were homogenized and subjected to incubations in different buffers to remove (1) cytosolic proteins, (2) nuclear proteins, (3) membrane proteins and (4) cytoskeletal proteins, leaving a final insoluble fraction enriched for ECM proteins.

Immunoblotting. For validation of the extraction process, the different fractions were separated on SDS-polyacrylamide gradient gels, transferred to nitrocellulose membranes and probed with relevant subcellular compartmental antibodies to confirm elimination of intracellular proteins and enrichment of ECM proteins. The antibodies used for immunoblotting included anti-GAPDH, anti-Histone (Cell Signaling Technology, Danvers, MA), anti-transferrin (Invitrogen, Camarillo, CA), anti-vimentin (Cell Signaling Technology, Danvers, MA), anti-fibronectin (Abcam, Cambridge, MA), anti-actin (Santa Cruz Biotechnology, Dallas, TX) or anti- α -tubulin (Cell Signaling Technology, Danvers, MA).

Protein digestion in peptides. The ECM-enriched protein fractions obtained after decellularization were solubilized and reduced in a solution of 8M urea in 100mM

ammonium bicarbonate containing 10mM dithiotreitol at 37°C for 30 minutes. The solubilized ECM proteins were then separated on 4-20% SDS-polyacrylamide gradient gels. Gels were stained with Gel Code Blue (Thermo Scientific) and washed in dH2O. Gels were then cut into 2 or 3 pieces using a clean scalpel and gel pieces were transferred into clean microcentrifuge tubes. Gel bands were wash twice with 200mL of 50% acetonitrile for 15 min. Protein gel samples were reduced with 10mM DTT, alkylated with 55mM iodoacetamide and digested overnight with 100ng of sequencing grade TPCK modified trypsin (Promega) at pH=8.3. Samples were acidified with 0.5% trifluoroacetic acid and desalted using C18 zip tips (Millipore) and concentrated to 10uL.

Liquid chromatography and Tandem Mass Spectrometry. 2 to 4uL of peptides were analyzed by microcapillary liquid chromatography (C18) tandem mass spectrometry (LC-MS/MS) using an EASY-nLCII nanoflow HPLC (Thermo Fisher Scientific) coupled to a hybrid Orbitrap Elite high-resolution mass spectrometer (Thermo Fisher Scientific) in Top 6 data-dependent acquisition positive ion mode at a flow rate of 300 nL/min. Please see supplemental methods for further information.

Protein and peptide identification. The LC-MS/MS datasets were analyzed with the Spectrum Mill software package, v 5.0 pre-release (Agilent Technologies, Santa Clara, CA). MS/MS spectra were searched against UniProt databases containing reference proteome sequences (including isoforms and excluding fragments) downloaded from the UniProt web site on October 17, 2014. Redundant sequences were removed, and a set of common laboratory contaminant proteins (150 sequences) was appended. The human database comprised 59,079 entries. Search criteria, described in the Supplementary Methods, yielded target-decoy-based false-discovery rate estimates for each patient's dataset of <0.8% at the peptide-spectrum match level and <1.3 % at the distinct peptide level. Across all datasets together the peptide-level FDR was 1.9%. Peptide-spectrum matches from all datasets together were assembled into proteins, and each protein was annotated as ECM-derived or not, as previously described (8, 21). The original mass spectra may be downloaded from MassIVE (http://massive.ucsd.edu) using the identifier: MSV000080451. The data is directly accessible via ftp://massive.ucsd.edu/MSV000080451.

Analysis of gene expression profiling and survival analysis. Publicly available gene expression profiles (GEP) were analyzed to evaluate the expression level of ANXA2 and LGALS1 in MM patient samples and cell lines (GSE6477)(22). We used the Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle/home)(23) to determine the expression level of ANXA2 and LGALS1 across over 1,000 cell lines, expressed by Robust Multi-array Average (RMA) values. We further studied GEP to assess the differential expression of ANXA2 and LGALS1 in MM vs. normal plasma cells – GSE6477. Gene levels were expressed by normalized expression values and 2-tailed t test were calculated to compare the 2 groups. Finally, we analyzed a large GEP - GSE2658 (24) – that enrolled 350 patients at diagnosis of MM. A Kaplan-Meier analysis was performed to compare overall survival (OS) of patients with low vs. high expression level of ANXA2 or LGALS1 based on the median expression of the cohort.

Gene set enrichment analysis (GSEA). We performed gene set enrichment analysis (GSEA)(25) to determine whether the identified ECM gene sets followed the same pattern at the mRNA level in MM patients compared to healthy donors. GSEA was performed following the developer's protocol (http://www.broad.mit.edu/gsea/) using the GSEA6477 dataset (22).

RESULTS

ECM protein enrichment from normal and diseased whole bone marrow samples.

We have examined murine and human BM, using Masson's trichrome to stain fibrillar collagens, as an assessment of ECM content (Fig. 1 and 2). We identified ECM within healthy murine BM niches and observed that the normal architecture of the ECM became disorganized in MM murine bone marrow but was still clearly present (Fig. 1A and B). Similarly, ECM was also identifiable within the BM of MM patients (Fig. 2 A and B), where morphology most resembled that seen in the MM murine BM. In order to isolate and characterize this ECM, we developed a protocol based on the previous decellularization method devised by Naba et al (8) specifically tailored to enrich for insoluble ECM proteins from the liquid marrow (Fig. 3A). Antibody markers, including anti-GAPDH, -histone, -transferrin receptor, and –vimentin were used for the cytosolic, nuclear, membrane and cytoskeletal compartments in order to confirm depletion of intracellular components at each step (Fig. 3B, left panel). In addition, we used an anti-fibronectin antibody to monitor the behavior of one exemplary ECM protein during the decellularization of bone marrow samples. Although we observed a portion of fibronectin to be partially depleted at each step, it was present in the final fraction (Fig. 3B, left panel). The protein fraction that remains insoluble after decellularization was then separated using gel electrophoresis, gels were stained using Coomassie blue stain (Fig. 3B, right panel). Gel bands were further cut from the gels and proteins were subjected to in-gel protein digestion to generate peptides to be separated by liquid chromatography and analyzed by mass spectrometry.

Proteomic characterization of healthy human donor, MGUS and MM ECM.

We aimed to define the proteomic signature of BM ECMs obtained from patients with MGUS and MM, as compared to healthy donors-derived BM ECMs. Complete data acquired on independent samples from healthy donors, MGUS patients and MM patients is shown in Supplementary Table S3. Peptide abundance, spectral count, number of unique peptides and number of proteins identified within the whole bone marrow were measured for both healthy donors and patients with MGUS or MM, either at first diagnosis or at relapse (Supplementary Fig. S1). In a representative healthy donor-derived sample, 24% of total spectral count signals were core matrisome or matrisome associated with 43% of the total precursor-ion intensity corresponding to proteins defined as ECM (Fig. S1A). A similar pattern was demonstrated for MGUS, newly diagnosed and relapsed MM BM ECMs (Fig. S1B-D. and

Supplementary Table S3B-E). In previous studies conducted on solid tumors (melanoma or mammary tumor xenografts, and human metastatic colorectal carcinomas), we have reported that the majority, typically (>75%) of the total precursor-ion intensities, correspond to matrisome proteins (8, 11, 16). This may indicate that the bone marrow ECM is comprised of a larger fraction of soluble ECM components, which by their nature are more readily extractable during the decellularization process than the ECM of normal tissues and solid tumors, where the ECM may play a greater structural role, and therefore be higher in in-soluble components. In addition, it is worth noting that the inter-patient variability was greater than what we previously observed in solid tumor [11]. Starting with 20 ml of bone marrow aspirate for each donor or patient, we observed that the proportion of the total precursor-ion intensities corresponding to matrisome proteins ranged from 10% to nearly 50%, (see Supplementary Fig. S2 and Supplementary Table S3A).

Characterization of the matrisome from healthy donor bone marrow.

We defined the matrisome of healthy-donor-derived BM as the ensemble of proteins detected in at least three independent biological replicates (i.e. patients) and by at least three peptides in one of the replicates. According to this definition the BM ECM from 10 independent healthy donors was composed of 62 proteins (Supplementary Table S1A). We previously proposed to classify proteins as part of the core matrisome, which comprises ECM glycoproteins, collagens, and proteoglycans, or being matrisome-associated proteins (ECM-affiliated proteins, ECM regulators, and ECM-associated secreted factors) (8, 21). The human bone marrow matrisome comprises 30 core matrisome proteins and 32 matrisome-associated proteins (Supplementary Table S1A, Supplementary Table S3B). The interrogation of the MatrisomeDB database (21) further revealed the identification of 11 proteins that have not been previously detected in any of the human tissues included in the database (Supplementary Table S1A). As human BM has not been previously subjected to this type of method, these proteins may be specific to this tissue.

Characterization of the matrisome from MGUS and MM patients.

We next sought to define the proteomic signature for BM ECMs derived from MGUS patients, using the same criteria outlined above, and found 11 proteins, two of which were core matrisome proteins, namely Bone Marrow Proteoglycan 2 (PRG2) and Bone Marrow proteoglycan 3 (PRG3) (Supplementary Table S1B). Nine of the proteins identified in the MGUS patient bone marrows were matrisome associated, including the ECM-affiliated

protein ficolin 1 (FCN1), the ECM-remodeling enzymes CTSG, Serpins, and the secreted factors HRNR, S100A8 and S100A9 (Supplementary Table S1B).

Analysis of the ECM signature of MM bone marrow identified 32 proteins (Supplementary Table S1C). Ten of these proteins were core matrisome proteins, with 22 being matrisome associated. Interestingly two matrisome associated proteins were identified, Annexin A2 (ANXA2) and Galectin-1 (LGALS1), which were not identified in healthy donor or MGUS bone marrow. The ECM of patients with relapsed MM comprised of 25 proteins, 10 of which are core matrisome proteins and 15 of which are matrisome associated (Supplementary Table S1D).

The analysis of the ECM identified signatures of MGUS and progressive MM.

By comparing the matrisomes of healthy donor bone marrow, MGUS and progressive stages of MM, we identified 11 proteins expressed by all four groups (Fig. 4A-B). Examples of these proteins are cathepsin-G (CTSG) and neutrophil elastase (ELANE). We further remarked that the normal-bone-marrow matrisome contained more proteins than diseased bone marrow samples. Because the proteomic pipeline employed analyzed only proteins that remained insoluble after decellularization, one hypothesis is that the ECM proteins in MGUS or MM bone marrow samples are more soluble. In agreement with this hypothesis, we observed the loss of several collagens and other fibrillar ECM glycoproteins such as fibronectin in MGUS and MM samples (Supplementary Table S2, Supplementary Table S3B-E). We also observed that some collagens and matrix metallo-proteinases (MMPs) found in healthy donor bone marrow and not detected at the MGUS stage are detected later in the disease progression pathway. This includes, among others, COL1A1, COL1A2, COL3A1, COL5A1, MMP8 and MMP9 (Supplementary Table S2). When comparing newly diagnosed MM to healthy donor BM and that of MGUS, Interestingly, two proteins, Annexin A2 and Galectin-1 are seen to emerge that are not present in earlier stages of the disease (Fig. 4B, Supplementary Table S2).

MM cells present with an enrichment for ECM-related genes.

We further validated the findings at the proteomic level by performing an analysis of the corresponding mRNA gene expression in MM plasma cells.

Using the signature of ECM proteins found to be present in the normal ECM, and absent in the MM tumor ECM, we performed GSEA to assess for enrichment of this gene signature in healthy donors vs. MM patients. Enrichment of the corresponding ECM gene signature was

demonstrated in healthy donors compared to MM patients (Fig. 5A-B; FDR<0.25). It has been shown that the majority of the tumor ECM is derived from the tumor cells in the main part, with stromal contribution at different stages of metastasis (16). Our findings of an altered ECM signature suggest that plasma cells may co-ordinate ECM remodeling in this disease. The interaction between the altered ECM and other bone marrow micro-environmental elements is likely to further augment the bone marrow milieu, creating a permissive environment for MM progression.

Annexin A2 and Galectin-1 and their prognostic relevance in MM.

ANXA2 and LGALS1, two ECM-affiliated proteins, were identified in newly diagnosed MM BM-ECMs, and not in healthy donor or MGUS BM-ECMs. We therefore analyzed the expression of ANXA2 and LGALS1 in MM cell lines, using the Cancer Cell Line Encyclopedia (CCLE); and found that both proteins were expressed in all MM cell lines (Fig. 6A and 7A). These observations were further corroborated by demonstrating the higher expression of both ANXA2 and LGALS1 in BM MM- as compared to healthy donor-derived plasma cells (GSE2658 and GSE6477; Fig. 6B and 7B) (22) (26).

Specifically, while ANXA2 levels were consistently higher in any given MGUS, smoldering-MM, MM newly diagnosed or MM relapsed patient as compared to healthy donors, LGALS1 was significantly higher in MGUS and newly diagnosed MM patients as compared to healthy individuals (Fig. 7B).

In order to further understand if these targets may be leading to a permissive tumor microenvironment in MM, we analyzed the Mulligan dataset (27) for co-expression of these genes in MM using the publically available Oncomine resource (www.oncomine.org); we found that ANXA2 is one of the most highly co-expressed genes with LGALS1 in 264 MM patients (Fig. 8A; correlation coefficient = 0.522).

We next interrogated the influence of both ANXA2 and LGALS1 in modulating survival in MM patients. By examining GSE2658 (28), and found that patients expressing higher levels of ANXA2 had a significantly reduced overall survival (OS) compared with those with lower expression levels (Fig. 8B; Log-rank p=4.9e-05). Similarly, high levels of LGALS1 were also associated with an inferior OS (Fig. 8C Log-rank P = 0.05)

DISCUSSION

In this study, we report the profiling of the ECM composition of normal bone marrow using mass spectrometry. We have compared this profile to the composition of the ECM of patients with MGUS, newly diagnosed, and relapsed multiple myeloma.

Our results demonstrate that significant remodeling of the bone marrow ECM is evident even at the MGUS stage, with loss of collagens and fibronectin (COL4A1-A3, COL4A6, COL5A2-3, COL6A2-3 COL10A1, COL11A2, COL16A1, COL27A1 and FN1). The absence of these proteins was consistent throughout the spectrum of MM from newly diagnosed to relapsed disease suggesting that the loss of these ECM components may contribute to a permissive tumor microenvironment supportive of the development and progression of MM. Indeed it has been shown that fibroblasts from patients with MGUS and MM demonstrate altered ECM profiles in comparison to those of healthy donors, supporting the hypothesis of early ECM remodeling (29).

Similarly, some collagens and matrix metallo-proteinases found in healthy donor bone marrow are not detected at the MGUS stage but are seen to re-emerge in the disease progression pathway indicating that they may be more supportive in aggressive phenotypes. This includes, among others, COL1A1, COL1A2, COL3A1, COL5A1, MMP8 and MMP9.

Interestingly, two proteins, Annexin A2 (ANXA2) and Galectin-1 (LGALS1) were detected in newly diagnosed MM but not in healthy donors or MGUS patients. These proteins were confirmed as being expressed in MM cell lines at the mRNA level in CCLE datasets. We hypothesized that these proteins may be required for myelomagenesis and further examined their presence in MM patient bone marrow.

Our analysis of GEP profiles reveals that these proteins are highly expressed in MM bone marrow plasma cells in comparison to healthy-donor-derived plasma cells. This may indicate that these proteins are produced by the tumor cells within the bone marrow niche and are specific to the malignant phenotype. If this is the case, then plasma cells themselves may contribute to bone marrow ECM remodeling as has been shown in other tumors (16). This may occur as a result of interactions between the remodeled ECM and other micro-environmental elements as altered ECMs in cancer deregulate the behavior of stromal cells, facilitate angiogenesis and promote a tumorigenic micro-environment (30). Altered ECM composition and topography indirectly affects cancer cells by influencing the effect of stromal cells, immune cells and fibroblasts which alters the tumor niche, this is also likely to be the case in multiple myeloma given our findings, however further work is needed to confirm this (30-32). Indeed, the galectins, of which LGALS1 is a member, are a family of beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix

interactions and recently Galectin-1 suppression has been shown to inhibit MM induced angiogenesis and tumor growth *in-vivo* (33). Annexin A2 is a calcium dependent phospholipid binding protein, which plays a role in cell growth and heightens osteoclast formation and bone resorption. Osteoclasts are known to contribute to an immune suppressive microenvironment in MM and are widely known to be associated with MM pathogenesis (34). Also, ANXA2 has previously been shown to promote MM cell growth and reduce apoptosis in MM cell lines (35). Identification of these targets, known to play a role in MM biology, helps to provide a wider validation of the remodeling of the ECM signature emerging using this novel strategy.

To further understand the implications of bone marrow remodeling in MM patients, we examined if there might be a common expression pattern for these two proteins within the MM bone marrow niche. Using data from Oncomine, we analyzed 264 MM patients and found that ANXA2 is one of the most highly co-expressed genes with LGALS1 in these patients. This leads us to hypothesize that the bone marrow remodeling seen in MM is likely to be an organized process that occurs as a result of multiple ECM protein switches being turned on and off within the bone marrow niche. Although both of these markers have previously been identified as being important in MM, we have further validated them as being part of an overall ECM remodeling process within the bone marrow.

The prognostic relevance of our findings was determined using GEP datasets, which provided survival outcomes for MM patients. This demonstrated that patients expressing higher levels of ANXA2 had a significantly reduced overall survival compared with those with lower expression levels. Similarly, high levels of LGALS1 were also associated with a shorter overall survival. This finding demonstrates that the bone marrow ECM plays an important role in MM and suggests that ECM remodeling is an active process within the bone marrow niche that has prognostic implications for MM patients. We can postulate that with the development of i) technologies to characterize the proteome and the matrisome with greater depth (including orthogonal peptide separation) and ii) methods to conduct quantitative analyses, we will be able to identify many more proteins, some of which may have prognostic values for MM patients. This previously under-evaluated area provides a potential resource for the identification of novel targets in this disease which may lead the way to preserving the normal ECM architecture and a less permissive niche.

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AUTHOR CONTRIBUTION

SG, AN, SM, JS, ROH and IG **designed research.**

SG, AN, SM, MRR, MM, YM, AS, and JMA **performed research**.

AR, MG and AP **contributed clinical samples to this study**.

SG, AN, SM, ST, JP, JMA, AMR and KRC **analyzed data.**

SG, AN, SM, MOD, ROH and IG **wrote the paper.**

CONFLICT-OF-INTEREST DISCLOSURES

The authors have no conflicts of interest to declare

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FIGURE LEGENDS

Figure 1. Staining of fibrillar collagens using Masson's trichrome (blue) of mouse bone marrow samples.

A. Healthy murine bone marrow.

B. Multiple myeloma murine bone marrow.

Figure 2. Staining of fibrillar collagens using Masson's trichrome (blue) of human bone marrow samples.

A. Healthy human bone marrow.

B. Multiple myeloma human bone marrow.

Figure 3. Proteomic pipeline to characterize the ECM compoistion of normal and diseased bone marrow samples.

A. Experimental pipeline.

B. Western blot to control the efficiency of the decellularization of bone marrow samples. Western blot shows the depletion of intracellular components (GAPDH, histones, Transferrin receptor and vimentin) during the four-step decellularization process.

Coomassie-stained gel shows the pattern of migration of proteins from the ECM-enriched samples.

Figure 4. Comparison of the matrisome of normal and diseased bone marrow samples.

A. Bar chart represents the number of proteins constituting the matrisome of normal, MGUS, newly diagnosed MM, and relapsed MM bone marrow.

B. Venn diagram illustrated the overlap between the matrisome proteins detected in normal, MGUS, newly diagnosed MM, and relapsed MM bone marrow (Supplementary Table S2).

Figure 5. Gene set enrichment analysis of matrisome gene signature in MM patients.

A. Gene set enrichment analysis (GSEA): demonstrating that (A) ECM proteins seen to be depleted in the bone marrow matrix with disease progression were also significantly down regulated at the gene level in normal donors (left) compared to MM patients (right).

B. Heatmap demonstrating enrichment profile of relevant ECM genes in healthy donors vs. MM patients.

Figure 6: Gene expression of ANXA2 in MM cell lines.

A. Analysis of CCLE MM cell lines (red bars) demonstrating the expression ANXA2 in MM cell lines at the mRNA level.

B. ANXA2 is significantly overexpressed at the mRNA level in MGUS, smoldering MM (SMM), newly diagnosed MM (MM, ndx) and relapsed MM (MM relapse) in comparison to normal donors (ND). Dataset reference: Cancer Res. 2007 Apr 1;67(7):2982-9. (GSE6477)

Figure 7: Gene expression of LGALS1 in MM cell lines.

A. LGALS1 is also significantly overexpressed at the mRNA level in newly diagnosed MM patients compared to normal donors in CCLE dataset.

B. LGALS1 is significantly overexpressed at the mRNA level in MGUS, smoldering MM (SMM), newly diagnosed MM (MM, ndx) and relapsed MM (MM relapse) in comparison to normal donors (ND). Dataset reference: Cancer Res. 2007 Apr 1;67(7):2982-9. (GSE6477)

Figure 8. Expression of ECM proteins in MM patients and impact on overall survival.

A. Co-expression of LGALS1 and ANXA2 in MM patients – data sourced from Oncomine (www.oncomine.org) Mulligan MM gene expression Blood. 2007 Apr 15;109(8):3177-88. Epub 2006 Dec 21.

B. Kaplan-Meier survival proportions analysis of patient outcome data from the GSE2658 MM GEP dataset demonstrating high levels of ANXA2 expression associated with inferior overall survival. Log-rank P = 4.9e-05

C. Kaplan-Meier survival proportions analysis of patient outcome data from the GSE2658 MM GEP dataset demonstrating high levels of LGALS1 expression associated with inferior overall survival. Log-rank P = 0.051.

Supplementary Table S1. Bone marrow matrisome. Matrisome of the bone marrow from healthy donors (A), MGUS patients (B), newly diagnosed multiple myeloma patients (C), and

relapsed multiple myeloma patients (D). * indicates ECM or ECM-associated proteins not detected before in any human tissue matrisome.

Supplementary Table S2. Comparison of the matrisome of normal, MGUS, newly diagnosed MM, and relapsed MM bone marrow

Supplementary Table S3. Proteomic data

A. Complete proteomic dataset. Proteins are sorted by matrisome categories and then by Entrez Gene Symbol.

B-E. The total precursor-ion intensity, number of spectra and unique peptides were calculated for each protein and in each sample. Proteins are sorted by number of observations (column D), then matrisome categories (column B) and gene symbols (column C). Proteins detected in at least 3 patients and with at least 3 peptides in one of the samples compose the matrisome of each of the four bone marrow sample types (bold).

B. All ECM and ECM-associated proteins detected in normal bone marrow samples.

C. All ECM and ECM-associated proteins detected in bone marrow samples from MGUS samples.

D. All ECM and ECM-associated proteins detected in bone marrow samples from newly diagnosed multiple myeloma patients.

E. All ECM and ECM-associated proteins detected in bone marrow samples from relapsed multiple myeloma patients.

F. Name of raw mass spectrometry files.

Supplementary Figure 1: Proteomic metrics.

Pie charts represent relative amount of core matrisome (blue), matrisome-associated (orange) and other (grey) proteins in terms of peptide abundance, and numbers of spectra, unique peptides and proteins.

A. Metrics for representative normal bone marrow sample.

B. Metrics for representative MGUS sample.

C. Metrics for representative newly diagnosed multiple myeloma sample.

D. Metrics for representative relapsed multiple myeloma sample.

Supplementary Figure 2: ECM peptide abundance

Bar charts represent the peptide abundance for ECM vs non-ECM proteins (**A**) and for core matrisome and matrisome-associated proteins (**B**) in each sample analyzed.

Proteomic characterization of human multiple myeloma bone marrow extracellular matrix

Siobhan V. Glavey[1,7*], Alexandra Naba[2,¶,*], Salomon Manier[1*], Karl Clauser[3], Sabrin Tahri[1], Jihye Park[1], Michaela R. Reagan[1], Michele Moschetta[1], Yuji Mishima[1], Manuela Gambella[4], Alberto Rocci[5], Antonio Sacco[1,6], Michael E O'Dwyer[7], John M. Asara[8], Antonio Palumbo[4], Aldo M. Roccaro[1,6,#] Richard O. Hynes[2,9,#], Irene M. Ghobrial[1#]

[1]Dana Farber Cancer Institute, Harvard Medical School, Boston, MA, USA; [2]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA; [3]Protemics Platform, Broad Institute, Cambridge, MA, USA; [4]University of Torino, Azienda Ospedaliero-Universitaria Città della Salute e della Scienza di Torino, Torino, Italy; [5]Manchester Royal Infirmary and Central Manchester University Hospital NHS Foundation, University of Manchester, Manchester, United Kingdom; [6] SST Spedali Civili, Dept. Medical Oncology, CREA Laboratory, Brescia, Italy; [7] Department of Hematology, National University of Ireland, Galway, Ireland; [8]Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA; [9]Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA, USA.

[¶] AN Present address: Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL 60612, USA

[*] S.G, A.N and S.M. contributed equally to this study.

[#] AMR, ROH, IMG: co-last Authors

Running title: Matrisome of human multiple myeloma

Corresponding authors:

Irene M. Ghobrial, MD

Dana-Farber Cancer Institute, Boston, MA, USA

Tel: +1 617.632.6777

E-mail: Irene_ghobrial@dfci.harvard.edu

Richard O Hynes, PhD

Koch Institute for Integrative Cancer Research at MIT, Cambridge, MA, USA

Tel: +1 617.253.6422

E-mail: rohynes@mit.edu

ABSTRACT

The extracellular matrix (ECM) is a major component of the tumor microenvironment, contributing to the regulation of cell survival, proliferation, differentiation and metastasis. In multiple myeloma (MM), interactions between MM cells and the bone marrow (BM) microenvironment, including the BM ECM, are critical to the pathogenesis of the disease and the development of drug resistance. Nevertheless, composition of the ECM in MM and its role in supporting MM pathogenesis has not been reported. We have applied a novel proteomic-based strategy and defined the BM ECM composition in patients with monoclonal gammopathy of undetermined significance (MGUS), newly diagnosed and relapsed MM compared to healthy donor-derived BM ECM. In this study, we show that the tumor ECM is remodeled at the mRNA and protein level in MGUS and MM to allow development of a permissive microenvironment. We further demonstrate that two ECM-affiliated proteins, ANXA2 and LGALS1, are more abundant in MM and high expression is associated with a decreased overall survival. This study points to the importance of ECM remodeling in MM and provides a novel proteomic pipeline for interrogating the role of the ECM in cancers with BM tropism.

Keywords: Multiple Myeloma, Extracellular Matrix, Proteomics

INTRODUCTION

Multiple myeloma (MM) is a plasma cell malignancy and accounts for approximately 10% of all hematological cancers (1). MM provides a model for the study of cancer cell metastasis as MM cells are known to traffic to distant bone marrow (BM) niches via hijacking the normal processes of cellular metastasis (2). Progression of the disease is mediated by intrinsic factors of the clonal cells along with factors that mediate a permissive tumor microenvironment (3). The MM tumor niche comprises the components of the BM microenvironment - cellular (stromal cells, osteoblasts, osteoclasts, endothelial cells and immune cells) and non-cellular (ECM) (4, 5). Despite advances in therapies in recent years, MM remains an incurable disease with a median survival of approximately 5-7 years for newly diagnosed patients (6). Identification of the molecular mechanisms leading to MM has the potential to lead to the development of novel prognostic tests and therapies for MM patients (7), (8).

The extracellular matrix (ECM) is a complex meshwork of proteins that serves as a scaffold for cells. In addition, it actively participates in cell functions such as proliferation, migration and survival via cell-to-matrix interactions (9, 10). The ECM is also rich in growth factors and cytokines thus supporting cell biology (9); therefore it follows that alterations in the ECM may be expected to occur in disease states such as inflammation and cancer. This is indeed the case in several tumor types where ECM alterations have been associated with changes in metastatic potential of tumor cells *in vivo* and in clinical outcomes (11, 12). The role of the ECM as a metabolic regulator of cell function also has implications for malignant processes (13) and molecular signals of metastasis have implicated ECM components and their receptors in tumor progression (14). In the past, attempts to systematically characterize the ECM were challenged by the vast diversity and number of ECM proteins and also by their biochemical nature. A new perspective on analysis of the ECM, on a relatively high throughput scale, came with the definition and *in-vivo* characterization of the “matrisome” (8). The term “matrisome”, initially used by Martin and collaborators in 1984 was updated and refined by Naba et al in 2012. This study revealed tissue specific signatures of ECM proteins and importantly provided a method to determine the origin (tumor cell or stroma) of individual matrix proteins. The “matrisome” itself is defined as the components constituting the ECM “core matrisome” and all associated components “matrisome associated”. This method has yielded important insights into the composition of several solid tumor matrices

along with potential novel therapeutic and prognostic targets (11, 15). ECM alterations that influence cellular metastasis have implications not only for solid tumors but also for hematological malignancies, where the process of cell migration from the primary niche is largely dependent on the tumor microenvironment (16-18).

In this study we apply a mass-spectrometry-based proteomic pipeline to study the tumor ECM from the bone marrow of MM patients. We characterize the unique extracellular matrices of patients with the pre-myeloma condition monoclonal gammopathy of undetermined significance (MGUS), newly diagnosed and relapsed MM patients and compare these ECM signatures to that of healthy human donor bone marrow. Our results demonstrate ECM remodeling with disease progression, as early at the MGUS stage. We further identify ANXA2 and LGALS1, two ECM-associated proteins as markers of the MM ECM and show the prognostic value of their expression for multiple-myeloma-patient overall survival.

MATERIALS AND METHODS

Human bone marrow aspirates. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki. These studies were approved by the Dana-Farber Cancer Institute Institutional Review Board and the University of Torino, Italy. Whole bone marrow (BM) was obtained from healthy human donors (n=10), MGUS patients (n=3), newly diagnosed MM patients who had not yet received treatment (n=7) and patients with relapsed MM (n=6). Fresh BM samples (20ml/patient) were filtered and underwent red cell lysis, followed by centrifugation at 2500rpm for 10 minutes (see Supplementary Methods for full protocol). The isolated pellet was then processed through the sequential extraction methods outlined below.

ECM protein enrichment and SDS gradient gel separation. ECM protein enrichment from of whole BM samples was achieved by sequentially depleting intracellular proteins as previously described (8, 19). In brief, whole BM samples were homogenized and subjected to incubations in different buffers to remove (1) cytosolic proteins, (2) nuclear proteins, (3) membrane proteins and (4) cytoskeletal proteins, leaving a final insoluble fraction enriched for ECM proteins.

Immunoblotting. For validation of the extraction process, the different fractions were separated on SDS-polyacrylamide gradient gels, transferred to nitrocellulose membranes and probed with relevant subcellular compartmental antibodies to confirm elimination of intracellular proteins and enrichment of ECM proteins. The antibodies used for immunoblotting included anti-GAPDH, anti-Histone (Cell Signaling Technology, Danvers, MA), anti-transferrin (Invitrogen, Camarillo, CA), anti-vimentin (Cell Signaling Technology, Danvers, MA), anti-fibronectin (Abcam, Cambridge, MA), anti-actin (Santa Cruz Biotechnology, Dallas, TX) or anti- α -tubulin (Cell Signaling Technology, Danvers, MA).

Protein digestion in peptides. The ECM-enriched protein fractions obtained after decellularization were solubilized and reduced in a solution of 8M urea in 100mM ammonium bicarbonate containing 10mM dithiotreitol at 37°C for 30 minutes. The solubilized ECM proteins were then separated on 4-20% SDS-polyacrylamide gradient gels. Gels were stained with Gel Code Blue (Thermo Scientific) and washed in dH2O. Gels were

then cut into 2 or 3 pieces using a clean scalpel and gel pieces were transferred into clean microcentrifuge tubes. Gel bands were wash twice with 200mL of 50% acetonitrile for 15 min. Protein gel samples were reduced with 10mM DTT, alkylated with 55mM iodoacetamide and digested overnight with 100ng of sequencing grade TPCK modified trypsin (Promega) at pH=8.3. Samples were acidified with 0.5% trifluoroacetic acid and desalted using C18 zip tips (Millipore) and concentrated to 10uL.

Liquid chromatography and Tandem Mass Spectrometry. 2 to 4uL of peptides were analyzed by microcapillary liquid chromatography (C18) tandem mass spectrometry (LC-MS/MS) using an EASY-nLCII nanoflow HPLC (Thermo Fisher Scientific) coupled to a hybrid Orbitrap Elite high-resolution mass spectrometer (Thermo Fisher Scientific) in Top 6 data-dependent acquisition positive ion mode at a flow rate of 300 nL/min.

Protein and peptide identification. The LC-MS/MS datasets were analyzed with the Spectrum Mill software package, v 5.0 pre-release (Agilent Technologies, Santa Clara, CA). MS/MS spectra were searched against UniProt databases containing reference proteome sequences (including isoforms and excluding fragments) downloaded from the UniProt web site on October 17, 2014. Redundant sequences were removed, and a set of common laboratory contaminant proteins (150 sequences) was appended. The human database comprised 59,079 entries. Search criteria, described in the Supplementary Methods, yielded target-decoy-based false-discovery rate estimates for each patient's dataset of <0.8% at the peptide-spectrum match level and <1.3 % at the distinct peptide level. Across all datasets together the peptide-level FDR was 1.9%. Peptide-spectrum matches from all datasets together were assembled into proteins, and each protein was annotated as ECM-derived or not, as previously described (8, 20).
The raw mass spectrometric data have been deposited to the ProteomeXchange Consortium.

Analysis of gene expression profiling and survival analysis. Publicly available gene expression profiles (GEP) were analyzed to evaluate the expression level of ANXA2 and LGALS1 in MM patient samples and cell lines (GSE6477)(21). We used the Cancer Cell Line Encyclopedia (CCLE) database (http://www.broadinstitute.org/ccle/home)(22) to determine the expression level of ANXA2 and LGALS1 across over 1,000 cell lines, expressed by Robust Multi-array Average (RMA) values. We further studied GEP to assess the differential expression of ANXA2 and LGALS1 in MM vs. normal plasma cells –

GSE6477. Gene levels were expressed by normalized expression values and 2-tailed t test were calculated to compare the 2 groups. Finally, we analyzed a large GEP - GSE2658 (23) – that enrolled 350 patients at diagnosis of MM. A Kaplan-Meier analysis was performed to compare overall survival (OS) of patients with low vs. high expression level of ANXA2 or LGALS1 based on the median expression of the cohort.

Gene set enrichment analysis (GSEA). We performed gene set enrichment analysis (GSEA)(24) to determine whether the identified ECM gene sets followed the same pattern at the mRNA level in MM patients compared to healthy donors. GSEA was performed following the developer's protocol (http://www.broad.mit.edu/gsea/) using the GSEA6477 dataset (21).

RESULTS

ECM protein enrichment from normal and diseased whole bone marrow samples.

We have examined murine and human BM, using Masson's trichrome to stain fibrillar collagens, as an assessment of ECM content (Fig. 1 and 2). We identified ECM within healthy murine BM niches and observed that the normal architecture of the ECM became disorganized in MM murine bone marrow but was still clearly present (Fig. 1A and B). Similarly, ECM was also identifiable within the BM of MM patients (Fig. 2 A and B), where morphology most resembled that seen in the MM murine BM. In order to isolate and characterize this ECM, we developed a protocol based on the previous decellularization method devised by Naba et al (8) specifically tailored to enrich for insoluble ECM proteins from the liquid marrow (Fig. 3A). Antibody markers, including anti-GAPDH, -histone, -transferrin receptor, and –vimentin were used for the cytosolic, nuclear, membrane and cytoskeletal compartments in order to confirm depletion of intracellular components at each step (Fig. 3B, left panel). In addition, we used an anti-fibronectin antibody to monitor the behavior of one exemplary ECM protein during the decellularization of bone marrow samples. Although we observed a portion of fibronectin to be partially depleted at each step, it was present in the final fraction (Fig. 3B, left panel). The protein fraction that remains insoluble after decellularization was then separated using gel electrophoresis, gels were stained using Coomassie blue stain (Fig. 3B, right panel). Gel bands were further cut from the gels and proteins were subjected to in-gel protein digestion to generate peptides to be separated by liquid chromatography and analyzed by mass spectrometry.

Proteomic characterization of healthy human donor, MGUS and MM ECM.

We aimed to define the proteomic signature of BM ECMs obtained from patients with MGUS and MM, as compared to healthy donors-derived BM ECMs. Complete data acquired on independent samples from healthy donors, MGUS patients and MM patients is shown in Supplementary Table S3. Peptide abundance, spectral count, number of unique peptides and number of proteins identified within the whole bone marrow were measured for both healthy donors and patients with MGUS or MM, either at first diagnosis or at relapse (Supplementary Fig. S1). In a representative healthy donor-derived sample, 24% of total spectral count signals were core matrisome or matrisome associated with 43% of the total precursor-ion intensity corresponding to proteins defined as ECM (Fig. S1A). A similar pattern was demonstrated for MGUS, newly diagnosed and relapsed MM BM ECMs (Fig. S1B-D. and

Supplementary Table S3B-E). In previous studies conducted on solid tumors (melanoma or mammary tumor xenografts, and human metastatic colorectal carcinomas), we have reported that the majority, typically (>75%) of the total precursor-ion intensities, correspond to matrisome proteins (8, 11, 15). This may reveal that the bone marrow ECM is more soluble and more readily extractable during the decellularization process than the ECM of normal tissues and solid tumors. In addition, it is worth noting that the inter-patient variability was greater than what we previously observed in solid tumor [11]. Starting with 20 ml of bone marrow aspirate for each donor or patient, we observed that the proportion of the total precursor-ion intensities corresponding to matrisome proteins ranged from 10% to nearly 50%, (see Supplementary Fig. S2 and Supplementary Table S3A).

Characterization of the matrisome from healthy donor bone marrow.

We defined the matrisome of healthy-donor-derived BM as the ensemble of proteins detected in at least three independent biological replicates (i.e. patients) and by at least three peptides in one of the replicates. According to this definition the BM ECM from 10 independent healthy donors was composed of 62 proteins (Supplementary Table S1A). We previously proposed to classify proteins as part of the core matrisome, which comprises ECM glycoproteins, collagens, and proteoglycans, or being matrisome-associated proteins (ECM-affiliated proteins, ECM regulators, and ECM-associated secreted factors) (8, 20). The human bone marrow matrisome comprises 30 core matrisome proteins and 32 matrisome-associated proteins (Supplementary Table S1A, Supplementary Table S3B). The interrogation of the MatrisomeDB database (20) further revealed the identification of 11 proteins that have not been previously detected in any of the human tissues included in the database (Supplementary Table S1A). As human BM has not been previously subjected to this type of method, these proteins may be specific to this tissue.

Characterization of the matrisome from MGUS and MM patients.

We next sought to define the proteomic signature for BM ECMs derived from MGUS patients, using the same criteria outlined above, and found 11 proteins, two of which were core matrisome proteins, namely Bone Marrow Proteoglycan 2 (PRG2) and Bone Marrow proteoglycan 3 (PRG3) (Supplementary Table S1B). Nine of the proteins identified in the MGUS patient bone marrows were matrisome associated, including the ECM-affiliated protein ficolin 1 (FCN1), the ECM-remodeling enzymes CTSG, Serpins, and the secreted factors HRNR, S100A8 and S100A9 (Supplementary Table S1B).

Analysis of the ECM signature of MM bone marrow identified 32 proteins (Supplementary Table S1C). Ten of these proteins were core matrisome proteins, with 22 being matrisome associated. Interestingly two proteins were identified as matrisome associated, Annexin A2 (ANXA2) and Galectin-1 (LGALS1), which were not identified at a significant level in healthy donor or MGUS bone marrow. The ECM of patients with relapsed MM comprised of 25 proteins, 10 of which are core matrisome proteins and 15 of which are matrisome associated (Supplementary Table S1D).

The analysis of the ECM identified signatures of MGUS and progressive MM.

By comparing the matrisomes of healthy donor bone marrow, MGUS and progressive stages of MM, we identified 11 proteins expressed by all four groups (Fig. 4A-B). Examples of these proteins are cathepsin-G (CTSG) and neutrophil elastase (ELANE). We further remarked that the normal-bone-marrow matrisome contained more proteins than diseased bone marrow samples. Because the proteomic pipeline employed analyzed only proteins that remained insoluble after decellularization, one hypothesis is that the ECM proteins in MGUS or MM bone marrow samples are more soluble. In agreement with this hypothesis, we observed the loss of several collagens and other fibrillar ECM glycoproteins such as fibronectin in MGUS and MM samples (Supplementary Table S2, Supplementary Table S3B-E). We also observed that some collagens and matrix metallo-proteinases (MMPs) found in healthy donor bone marrow and not detected at the MGUS stage are detected later in the disease progression pathway. This includes, among others, COL1A1, COL1A2, COL3A1, COL5A1, MMP8 and MMP9 (Supplementary Table S2). When comparing newly diagnosed MM to healthy donor BM and that of MGUS, Interestingly, two proteins, Annexin A2 and Galectin-1 are seen to emerge that are not present in earlier stages of the disease (Fig. 4B, Supplementary Table S2).

MM cells present with an enrichment for ECM-related genes.

We further validated the findings at the proteomic level by performing an analysis of the corresponding mRNA gene expression in MM plasma cells.

Using the signature of ECM proteins found to be present in the normal ECM, and absent in the MM tumor ECM, we performed GSEA to assess for enrichment of this gene signature in healthy donors vs. MM patients. Enrichment of the corresponding ECM gene signature was demonstrated in healthy donors compared to MM patients (Fig. 5A-B; FDR<0.25). It has been shown that the majority of the tumor ECM is derived from the tumor cells in the main

part, with stromal contribution at different stages of metastasis (15). Therefore, our findings suggest the ability of tumor cells to contribute to micro-environmental remodeling.

Annexin A2 and Galectin-1 and their prognostic relevance in MM.

ANXA2 and LGALS1, two ECM-affiliated proteins, were identified in newly diagnosed MM BM-ECMs, and not in healthy donor or MGUS BM-ECMs. We therefore analyzed the expression of ANXA2 and LGALS1 in MM cell lines, using the Cancer Cell Line Encyclopedia (CCLE); and found that both proteins were expressed in all MM cell lines (Fig. 6A and 7A). These observations were further corroborated by demonstrating the higher expression of both ANXA2 and LGALS1 in BM MM- as compared to healthy donor-derived plasma cells (GSE2658 and GSE6477; Fig. 6B and 7B) (21) (25).

Specifically, while ANXA2 levels were consistently higher in any given MGUS, smoldering-MM, MM newly diagnosed or MM relapsed patient as compared to healthy donors, LGALS1 was significantly higher in MGUS and newly diagnosed MM patients as compared to healthy individuals (Fig. 7B).

In order to further understand if these targets may be leading to a permissive tumor microenvironment in MM, we analyzed the Mulligan dataset (26) for co-expression of these genes in MM using the publically available Oncomine resource (www.oncomine.org); we found that ANXA2 is one of the most highly co-expressed genes with LGALS1 in 264 MM patients (Fig. 8A; correlation coefficient = 0.522).

We next interrogated the influence of both ANXA2 and LGALS1 in modulating survival in MM patients. By examining GSE2658 (27), and found that patients expressing higher levels of ANXA2 had a significantly reduced overall survival (OS) compared with those with lower expression levels (Fig. 8B; Log-rank p=4.9e-05). Similarly, high levels of LGALS1 were also associated with an inferior OS (Fig. 8C Log-rank P = 0.05)

DISCUSSION

In this study, we report the profiling of the ECM composition of normal bone marrow using mass spectrometry. We have compared this profile to the composition of the ECM of patients with MGUS, newly diagnosed, and relapsed multiple myeloma.

Our results demonstrate that significant remodeling of the bone marrow ECM is evident even at the MGUS stage, with loss of collagens and fibronectin (COL4A1-A3, COL4A6, COL5A2-3, COL6A2-3 COL10A1, COL11A2, COL16A1, COL27A1 and FN1). The absence of these proteins was consistent throughout the spectrum of MM from newly diagnosed to relapsed disease suggesting that the loss of these ECM components may contribute to a permissive tumor microenvironment supportive of the development and progression of MM. Indeed it has been shown that fibroblasts from patients with MGUS and MM demonstrate altered ECM profiles in comparison to those of healthy donors, supporting the hypothesis of early ECM remodeling (28).

Similarly, some collagens and matrix metallo-proteinases found in healthy donor bone marrow are not detected at the MGUS stage but are seen to re-emerge in the disease progression pathway indicating that they may be more supportive in aggressive phenotypes. This includes, among others, COL1A1, COL1A2, COL3A1, COL5A1, MMP8 and MMP9.

Interestingly, two proteins, Annexin A2 (ANXA2) and Galectin-1 (LGALS1) were detected in newly diagnosed MM but not in healthy donors or MGUS patients. These proteins were confirmed to be expressed in MM cell lines at the mRNA level in CCLE datasets. We hypothesized that these proteins may be required for myelomagenesis and further examined their presence in MM patient bone marrow.

Our analysis of GEP profiles reveals that these proteins are highly expressed in MM bone marrow plasma cells in comparison to healthy-donor-derived plasma cells. This may indicate that these proteins are produced by the tumor cells within the bone marrow niche and are specific to the malignant phenotype. If this is the case, then plasma cells themselves may contribute to bone marrow ECM remodeling as has been shown in other tumors (15).

Indeed, the galectins, of which LGALS1 is a member, are a family of beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions and recently Galectin-1 suppression has been shown to inhibit MM induced angiogenesis and tumor growth *in-vivo* (29). Annexin A2 is a calcium dependent phospholipid binding protein, which plays a role in cell growth and heightens osteoclast formation and bone resorption. Osteoclasts are known to contribute to an immune suppressive microenvironment in MM and

are widely known to be associated with MM pathogenesis (30). Also, ANXA2 has previously been shown to promote MM cell growth and reduce apoptosis in MM cell lines (31). Identification of these targets, known to play a role in MM biology, helps to provide a wider validation of the remodeling of the ECM signature emerging using this novel strategy.

To further understand the implications of bone marrow remodeling in MM patients, we examined if there might be a common expression pattern for these two proteins within the MM bone marrow niche. Using data from Oncomine, we analyzed 264 MM patients and found that ANXA2 is one of the most highly co-expressed genes with LGALS1 in these patients. This leads us to hypothesize that the bone marrow remodeling seen in MM is likely to be an organized process that occurs as a result of multiple ECM protein switches being turned on and off within the bone marrow niche. Although both of these markers have previously been identified as being important in MM, we have further validated them as being part of an overall ECM remodeling process within the bone marrow.

The prognostic relevance of our findings was determined using GEP datasets, which provided survival outcomes for MM patients. This demonstrated that patients expressing higher levels of ANXA2 had a significantly reduced overall survival compared with those with lower expression levels. Similarly, high levels of LGALS1 were also associated with a shorter overall survival. This finding demonstrates that the bone marrow ECM plays an important role in MM and suggests that ECM remodeling is an active process within the bone marrow niche that has prognostic implications for MM patients. We can postulate that with the development of i) technologies to characterize the proteome and the matrisome with greater depth (including orthogonal peptide separation) and ii) methods to conduct quantitative analyses, we will be able to identify many more proteins, some of which may have prognostic values for MM patients. This previously under-evaluated area provides a potential resource for the identification of novel targets in this disease which may lead the way to preserving the normal ECM architecture and a less permissive niche.

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AUTHOR CONTRIBUTION

SG, AN, SM, JS, ROH and IG **designed research.**

SG, AN, SM, MRR, MM, YM, AS, and JMA **performed research**.

AR, MG and AP **contributed clinical samples to this study**.

SG, AN, SM, ST, JP, JMA, AMR and KRC **analyzed data.**

SG, AN, SM, MOD, ROH and IG **wrote the paper.**

CONFLICT-OF-INTEREST DISCLOSURES

The authors have no conflicts of interest to declare

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FIGURE LEGENDS

Figure 1. Staining of fibrillar collagens using Masson's trichrome (blue) of mouse bone marrow samples.

A. Healthy murine bone marrow.

B. Multiple myeloma murine bone marrow.

Figure 2. Staining of fibrillar collagens using Masson's trichrome (blue) of human bone marrow samples.

A. Healthy human bone marrow.

B. Multiple myeloma human bone marrow.

Figure 3. Proteomic pipeline to characterize the ECM compoistion of normal and diseased bone marrow samples.

A. Experimental pipeline.

B. Western blot to control the efficiency of the decellularization of bone marrow samples. Western blot shows the depletion of intracellular components (GAPDH, histones, Transferrin receptor and vimentin) during the four-step decellularization process.

Coomassie-stained gel shows the pattern of migration of proteins from the ECM-enriched samples.

Figure 4. Comparison of the matrisome of normal and diseased bone marrow samples.

A. Bar chart represents the number of proteins constituting the matrisome of normal, MGUS, newly diagnosed MM, and relapsed MM bone marrow.

B. Venn diagram illustrated the overlap between the matrisome proteins detected in normal, MGUS, newly diagnosed MM, and relapsed MM bone marrow (Supplementary Table S2).

Figure 5. Gene set enrichment analysis of matrisome gene signature in MM patients.

A. Gene set enrichment analysis (GSEA): demonstrating that (A) ECM proteins seen to be depleted in the bone marrow matrix with disease progression were also significantly down regulated at the gene level in normal donors (left) compared to MM patients (right).

B. Heatmap demonstrating enrichment profile of relevant ECM genes in healthy donors vs. MM patients.

Figure 6: Gene expression of ANXA2 in MM cell lines.

A. Analysis of CCLE MM cell lines (red bars) demonstrating the expression ANXA2 in MM cell lines at the mRNA level.

B. ANXA2 is significantly overexpressed at the mRNA level in MGUS, smoldering MM (SMM), newly diagnosed MM (MM, ndx) and relapsed MM (MM relapse) in comparison to normal donors (ND). Dataset reference: Cancer Res. 2007 Apr 1;67(7):2982-9. (GSE6477)

Figure 7: Gene expression of LGALS1 in MM cell lines.

A. LGALS1 is also significantly overexpressed at the mRNA level in newly diagnosed MM patients compared to normal donors in CCLE dataset.

B. LGALS1 is significantly overexpressed at the mRNA level in MGUS, smoldering MM (SMM), newly diagnosed MM (MM, ndx) and relapsed MM (MM relapse) in comparison to normal donors (ND). Dataset reference: Cancer Res. 2007 Apr 1;67(7):2982-9. (GSE6477)

Figure 8. Expression of ECM proteins in MM patients and impact on overall survival.

A. Co-expression of LGALS1 and ANXA2 in MM patients – data sourced from Oncomine (www.oncomine.org) Mulligan MM gene expression Blood. 2007 Apr 15;109(8):3177-88. Epub 2006 Dec 21.

B. Kaplan-Meier survival proportions analysis of patient outcome data from the GSE2658 MM GEP dataset demonstrating high levels of ANXA2 expression associated with inferior overall survival. Log-rank P = 4.9e-05

C. Kaplan-Meier survival proportions analysis of patient outcome data from the GSE2658 MM GEP dataset demonstrating high levels of LGALS1 expression associated with inferior overall survival. Log-rank P = 0.051.

Supplementary Table S1. Bone marrow matrisome. Matrisome of the bone marrow from healthy donors (A), MGUS patients (B), newly diagnosed multiple myeloma patients (C), and relapsed multiple myeloma patients (D). * indicates ECM or ECM-associated proteins not detected before in any human tissue matrisome.

Supplementary Table S2. Comparison of the matrisome of normal, MGUS, newly diagnosed MM, and relapsed MM bone marrow

Supplementary Table S3. Proteomic data

A. Complete proteomic dataset. Proteins are sorted by matrisome categories and then by Entrez Gene Symbol.

B-E. The total precursor-ion intensity, number of spectra and unique peptides were calculated for each protein and in each sample. Proteins are sorted by number of observations (column D), then matrisome categories (column B) and gene symbols (column C). Proteins detected in at least 3 patients and with at least 3 peptides in one of the samples compose the matrisome of each of the four bone marrow sample types (bold).

B. All ECM and ECM-associated proteins detected in normal bone marrow samples.

C. All ECM and ECM-associated proteins detected in bone marrow samples from MGUS samples.

D. All ECM and ECM-associated proteins detected in bone marrow samples from newly diagnosed multiple myeloma patients.

E. All ECM and ECM-associated proteins detected in bone marrow samples from relapsed multiple myeloma patients.

F. Name of raw mass spectrometry files.

Supplementary Figure 1: Proteomic metrics.

Pie charts represent relative amount of core matrisome (blue), matrisome-associated (orange) and other (grey) proteins in terms of peptide abundance, and numbers of spectra, unique peptides and proteins.

A. Metrics for representative normal bone marrow sample.

B. Metrics for representative MGUS sample.

C. Metrics for representative newly diagnosed multiple myeloma sample.

D. Metrics for representative relapsed multiple myeloma sample.

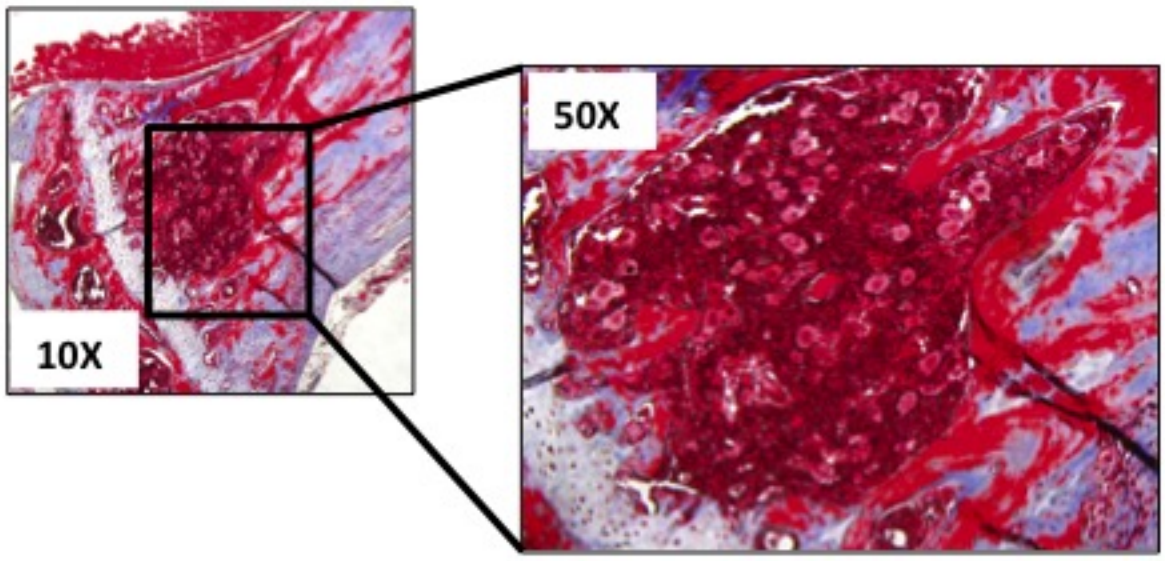
Supplementary Figure 2: ECM peptide abundance

Bar charts represent the peptide abundance for ECM vs non-ECM proteins (**A**) and for core matrisome and matrisome-associated proteins (**B**) in each sample analyzed.

Figure 1

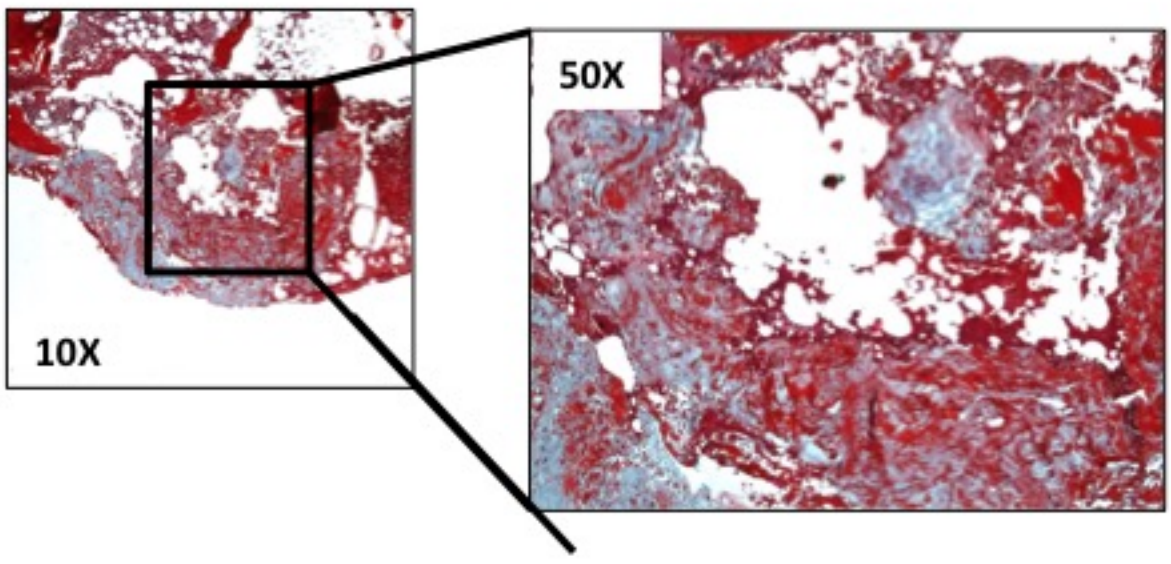
A.

Healthy murine bone marrow



B.

Multiple myeloma murine bone marrow

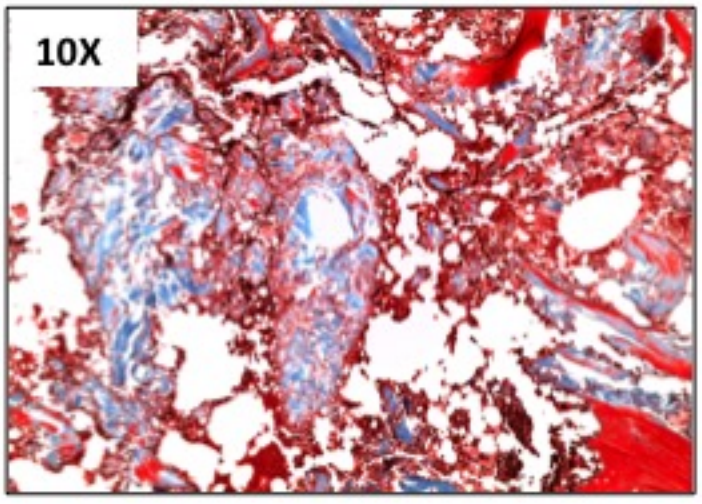


A.

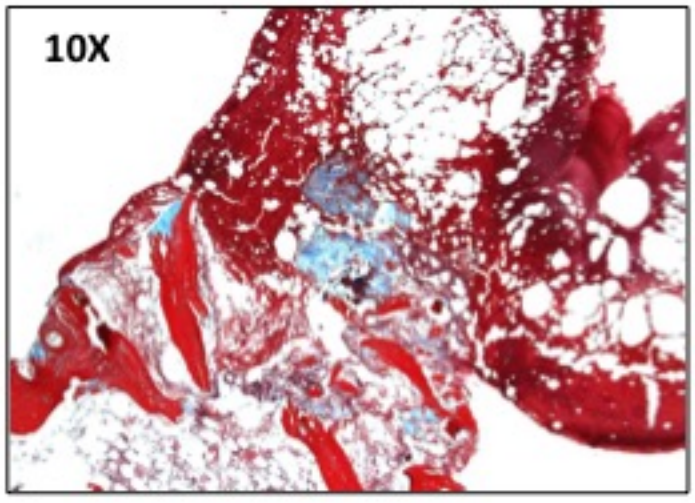
B.

Figure 2

Healthy human bone marrow



Multiple myeloma human bone marrow



A.

Figure 3

Sequential depletion of intracellular proteins/ECM-protein enrichment from whole fresh BM from normal or diseased human samples

↓

Protein separation by gel electrophoresis

↓

In-gel protein digestion

↓

Peptide fractionation by Liquid Chromatography

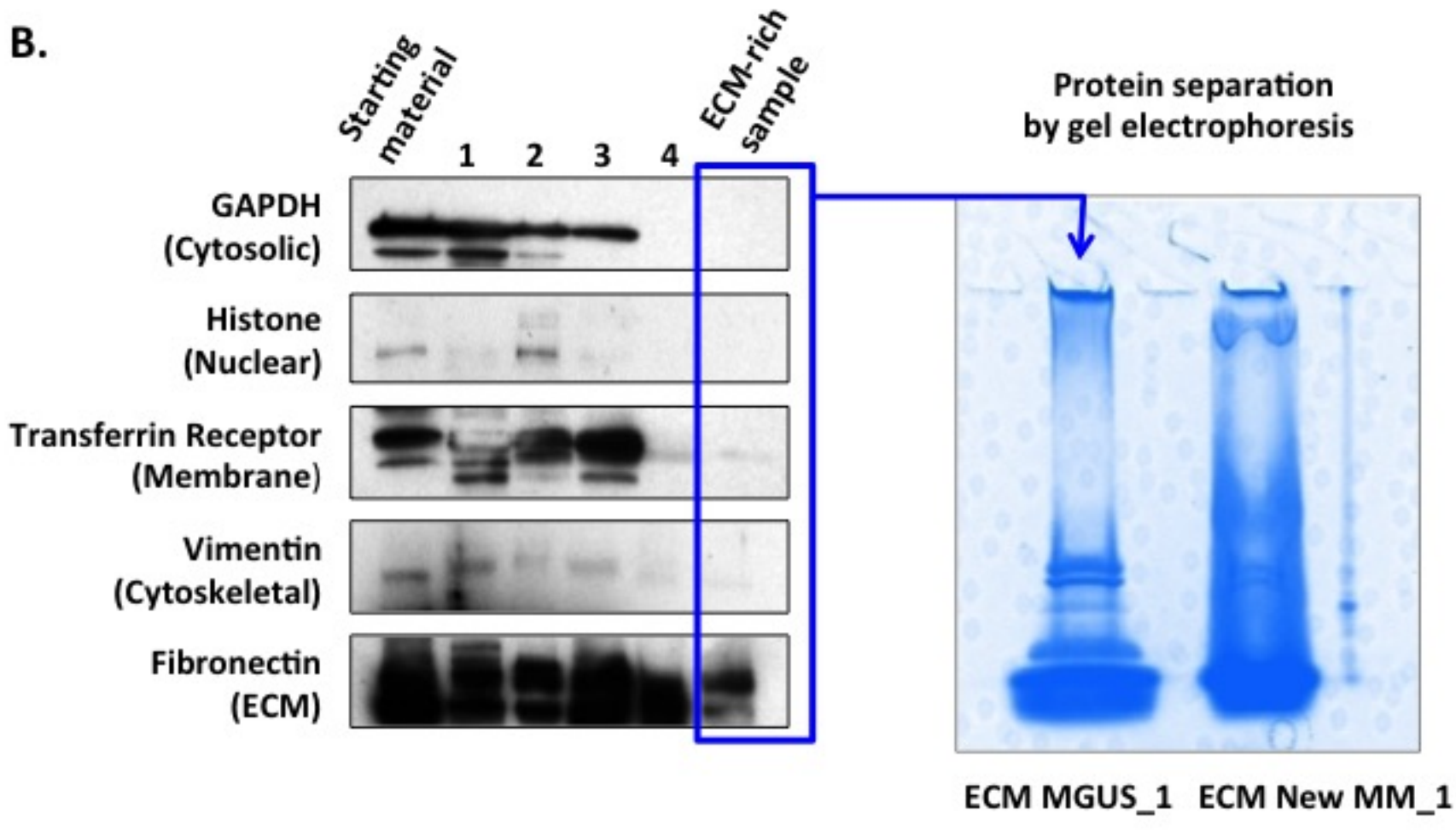
↓

Analysis by Tandem Mass Spectrometry (LTQ Orbitrap)

↓

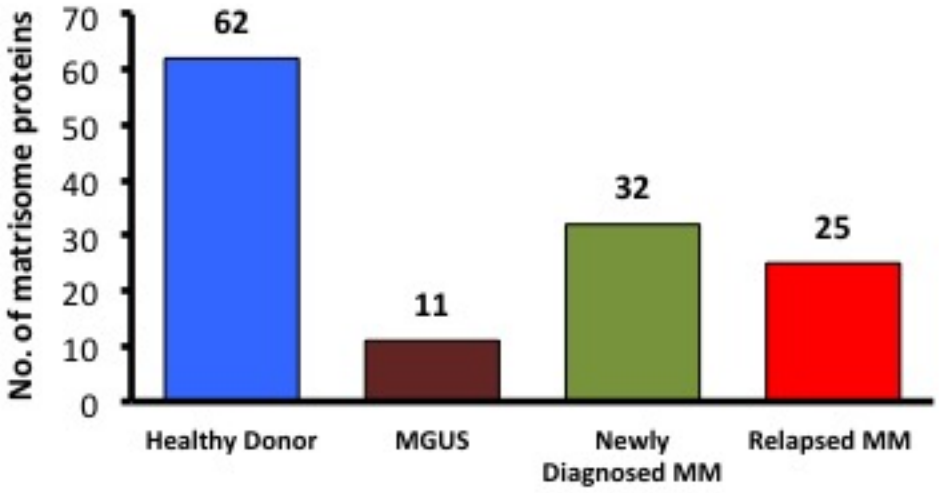
Protein Identification and Matrisome annotation

B.



A.

Figure 4


No. of matrisome proteins
70
60
50
40
30
20
10
0
62
11
32
25
Healthy Donor
MGUS
Newly Diagnosed MM
Relapsed MM

B.

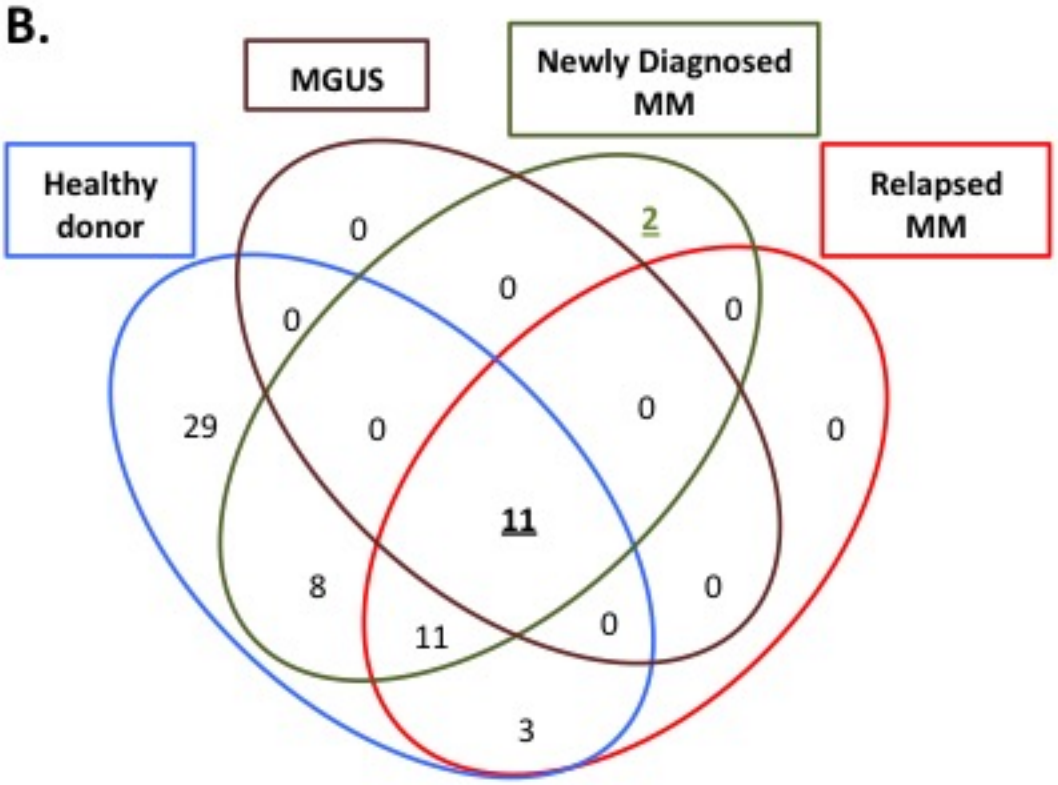
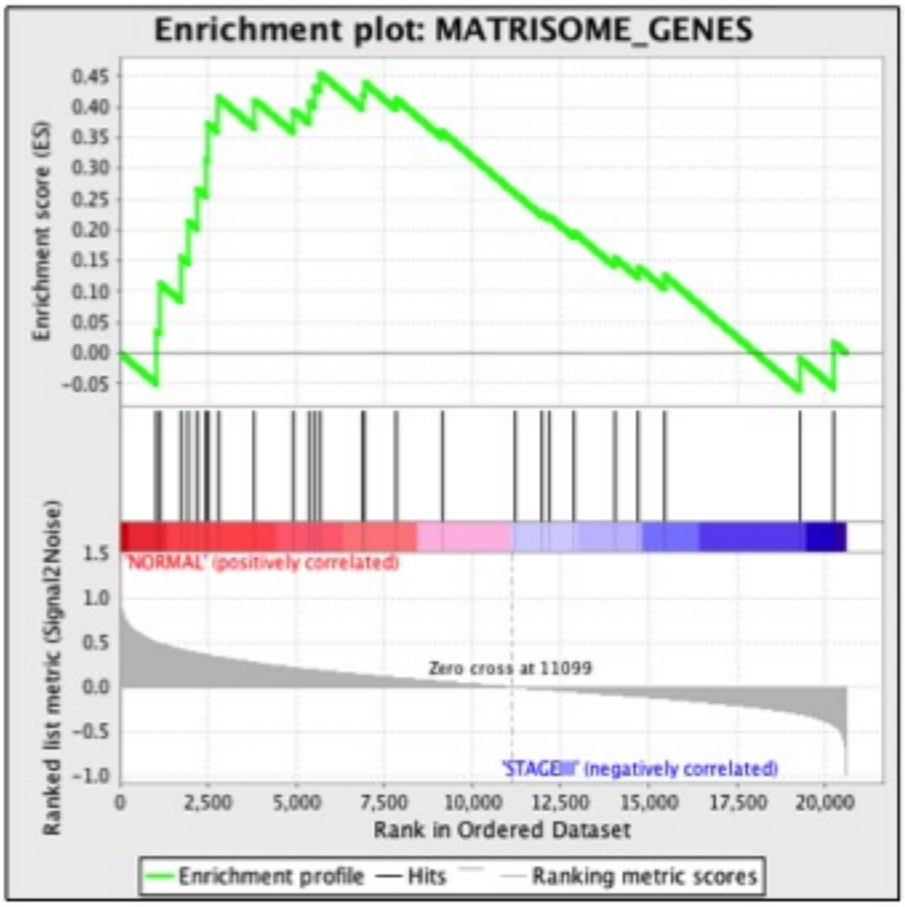

MGUS
Newly Diagnosed MM
Healthy donor
Relapsed MM
0
2
0
0
0
29
0
0
0
11
8
11
0
0
3

Figure 5

A.

FDR q-value 0.20041323



B.

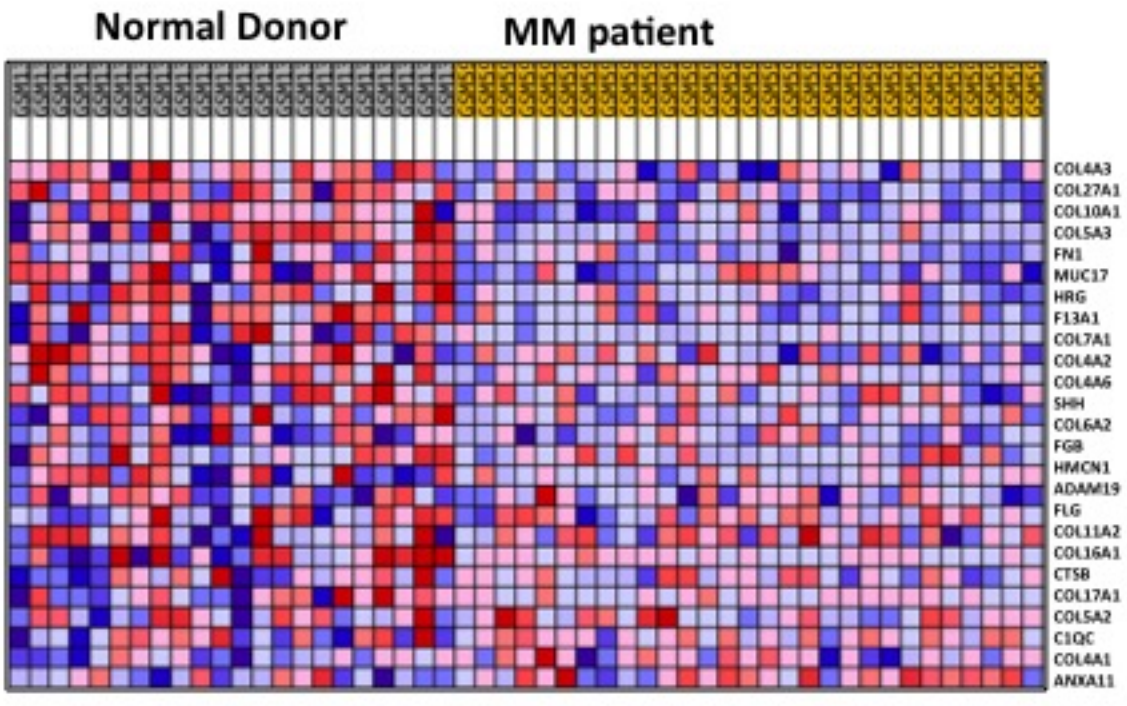
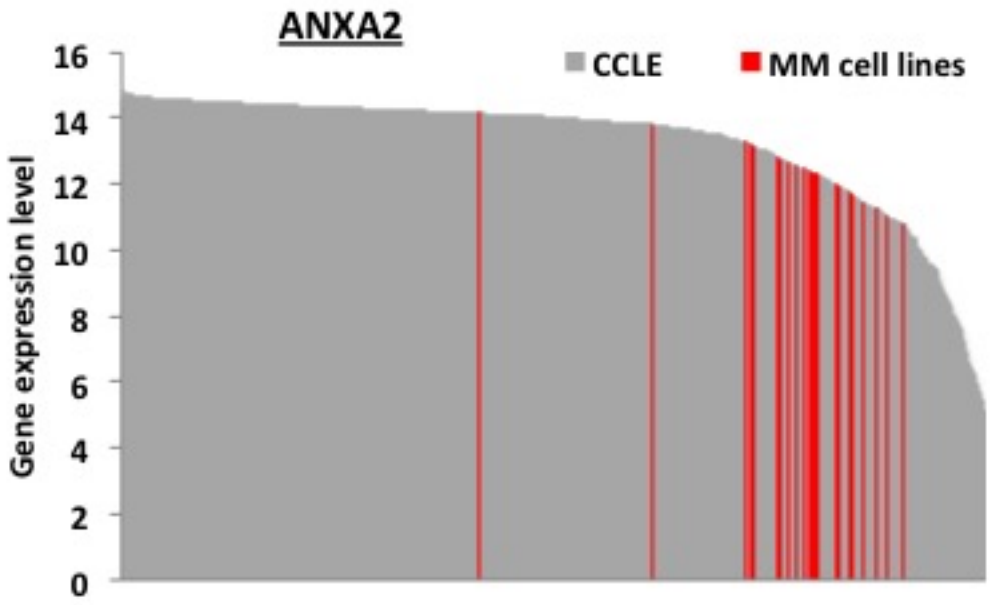
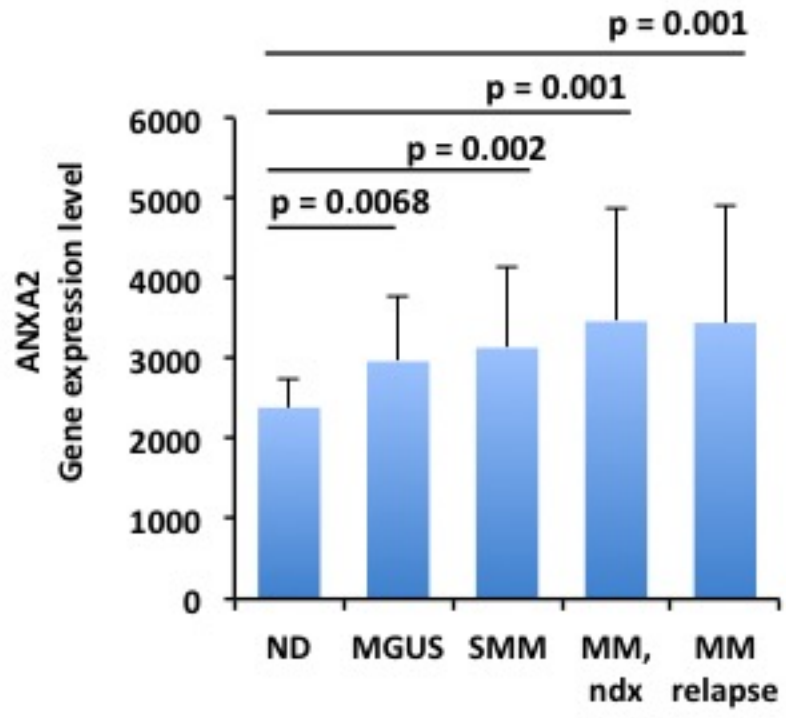


Figure 6

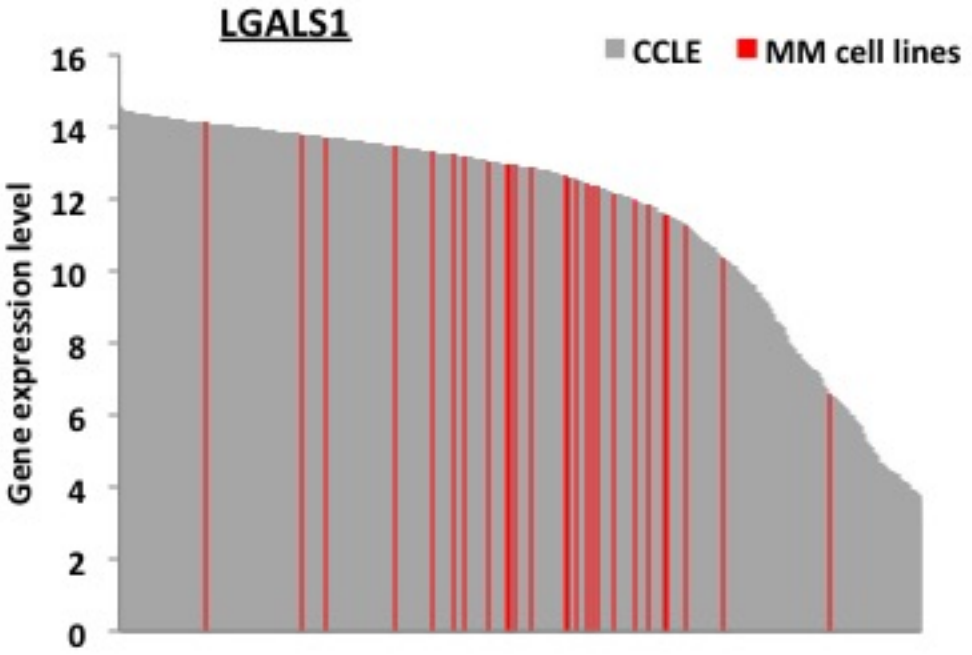
A.


ANXA2
CCLE
MM cell lines
Gene expression level
16
14
12
10
8
6
4
2
0

B.


p = 0.001
p = 0.001
p = 0.002
p = 0.0068
ANXA2
Gene expression level
6000
5000
4000
3000
2000
1000
0
ND
MGUS
SMM
MM, ndx
MM relapse

A.


LGALS1
CCLE
MM cell lines
Gene expression level
16
14
12
10
8
6
4
2
0

B.

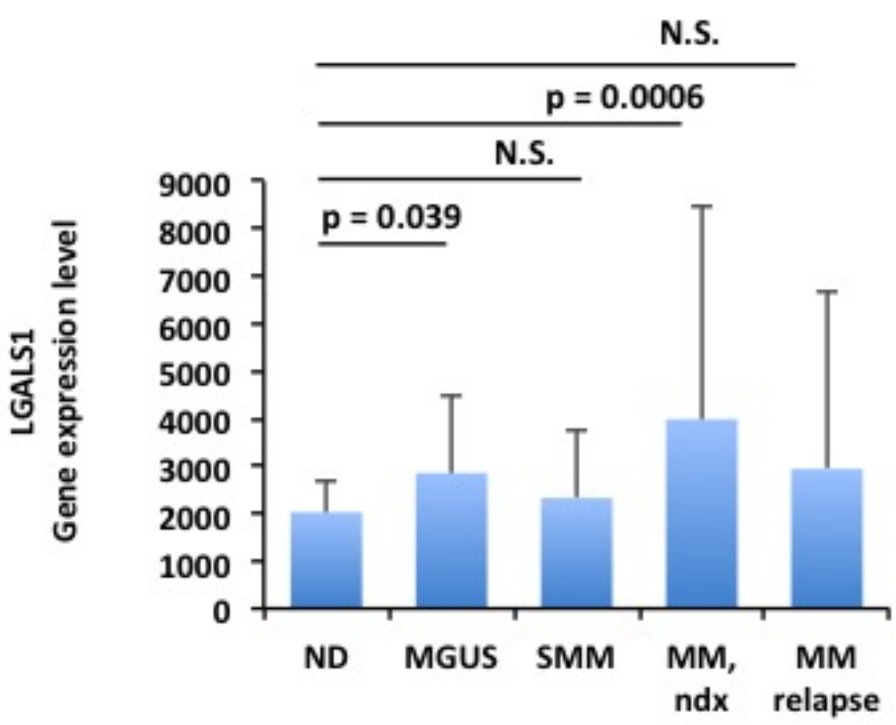
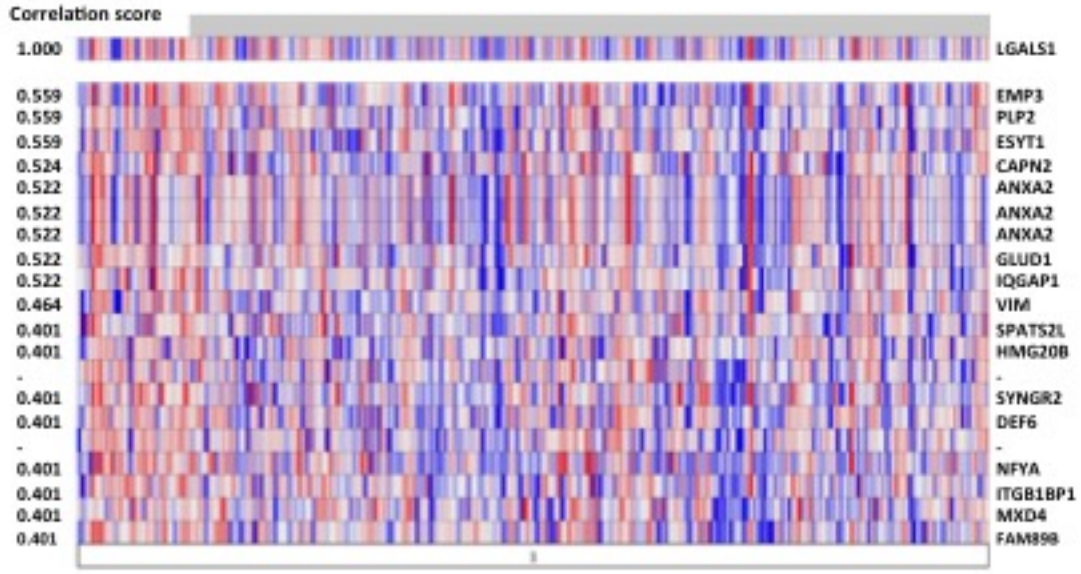
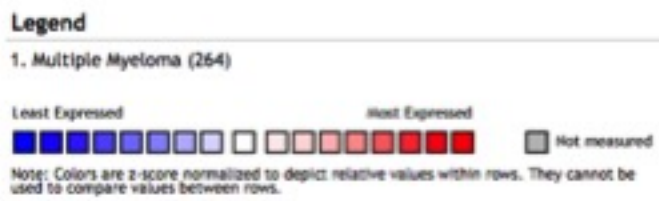

N.S.
p = 0.0006
N.S.
p = 0.039
LGALS1
Gene expression level
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ND
MGUS
SMM
MM, ndx
MM relapse

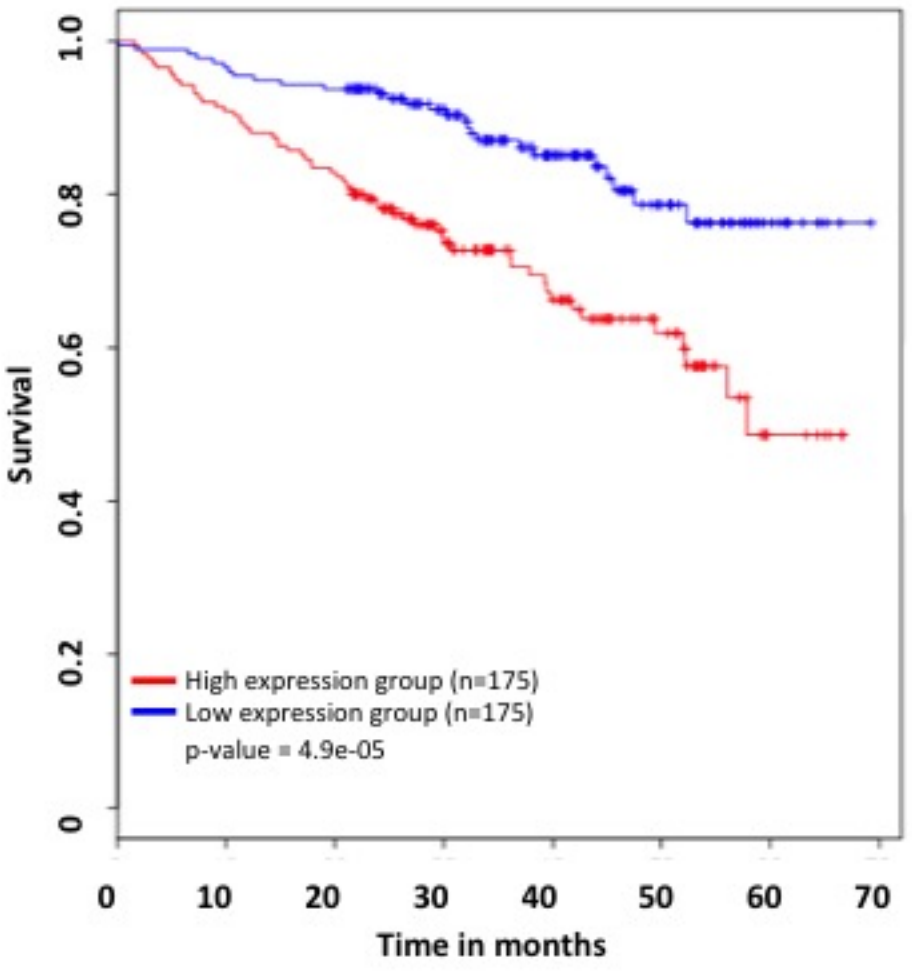
Figure 8

A.

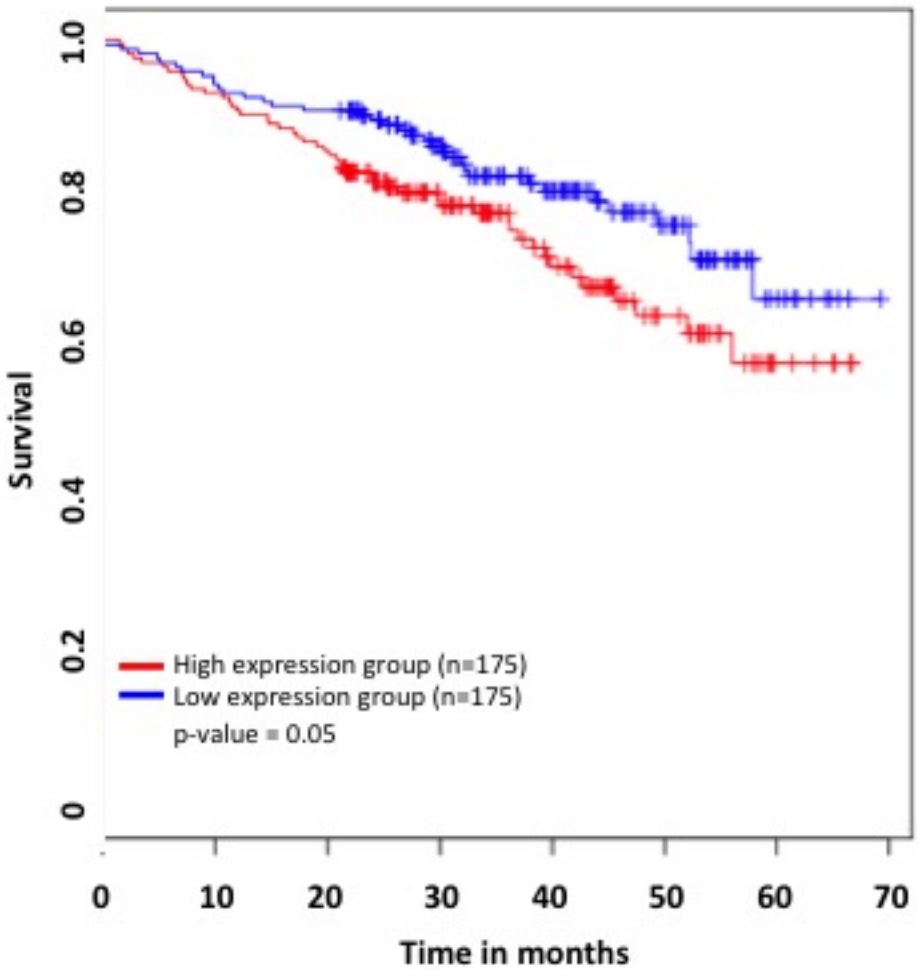

Correlation score
1.000
LGALS1
0.559
EMP3
0.559
PLP2
0.559
ESYT1
0.524
CAPN2
0.522
ANXA2
0.522
ANXA2
0.522
ANXA2
0.522
GLUD1
0.522
IQGAP1
0.464
VIM
0.401
SPATS2L
0.401
HMG20B
0.401
SYNGR2
0.401
DEF6
0.401
NFYA
0.401
ITGB1BP1
0.401
MXD4
0.401
FAM89B


Legend
1. Multiple Myeloma (264)
Least Expressed
Most Expressed
Not measured
Note: Colors are z-score normalized to depict relative values within rows. They cannot be used to compare values between rows.

B.


Overall survival MM patients – high vs. low ANXA2
Survival
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
Time in months
High expression group (n=175)
Low expression group (n=175)
p-value = 4.9e-05

C.


Overall survival MM patients – high vs. low LGALS1
Survival
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
Time in months
High expression group (n=175)
Low expression group (n=175)
p-value = 0.05