Compartment-resolved Proteomic Analysis of Mouse Aorta during Atherosclerotic Plaque Formation Reveals Osteoclast-specific Protein Expression

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Atherosclerosis leads to vascular lesions that involve major rearrangements of the vascular proteome, especially of the extracellular matrix (ECM). Using single aortas from ApoE knock out mice, we quantified formation of plaques by single-run, high-resolution mass spectrometry (MS)-based proteomics. To probe localization on a proteome-wide scale we employed quantitative detergent solubility profiling. This compartment- and time-resolved resource of atherogenesis comprised 5117 proteins, 182 of which changed their expression status in response to vessel maturation and atherosclerotic plaque development. In the insoluble ECM proteome, 65 proteins significantly changed, including relevant collagens, matrix metalloproteinases and macrophage derived proteins. Among novel factors in atherosclerosis, we identified matrilin-2, the collagen IV crosslinking enzyme peroxidasin as well as the poorly characterized MAM-domain containing 2 (Mamdc2) protein as being up-regulated in the ECM during atherogenesis. Intriguingly, three subunits of the osteoclast specific V-ATPase complex were strongly increased in mature plaques with an enrichment in macrophages thus implying an active de-mineralization function.

Vascular diseases result in almost 18 million fatalities worldwide per year, making them the leading cause of death (1). The underlying pathophysiology is often atherosclerosis and the most common clinical manifestations are stroke and coronary artery disease (CAD) (2–5). Atherosclerosis is a chronic inflammatory condition characterized by an accumulation of lipids in the arterial wall, together with infiltration of monocytes and other immune cells, leading to the development of an atheromatous plaque. Over time, apoptotic and necrotic cells together with cholesterol crystals form a necrotic core. The surface of advanced plaques is covered by a fibrous cap of variable thickness, composed of a dense collagen-rich extracellular matrix (ECM) and sparsely distributed cells, in particular, vascular smooth muscle cells (VSMCs). In late stages, the expression of proteases can destabilize the cap, inducing plaque rupture and thrombosis, which may manifest as stroke or myocardial infarction (6–11). Thus, plaque stability is directly related to clinical events (12).

The arterial wall structure of vertebrates is subdivided into the tunica intima, media and adventitia, comprising endothel-
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...and VSMCs, and the differentiation of macrophages to foam cells elicit cytokine and chemokine secretion, leading to inflammation and to the development of an atherosclerotic plaque. The arterial ECM is critically involved in signaling processes relevant to atherosclerosis and undergoes substantial changes during atherogenesis (17–23). Several studies link ECM components to vascular inflammation and atherosclerotic plaque development (reviewed in (22)), yet our understanding of the underlying protein expression changes is mainly limited to the study of individual factors or small sets of proteins.

Recent application of “omics” methodologies (24), especially of high resolution mass spectrometry (MS), has allowed the categorization of the ECM constituents in “core matrisome” and “matrisome-associated” proteins (25–27). The core matrisome is a multifunctional matrix composed of collagens, proteoglycans and glycoproteins. It provides a biomechanical scaffold, with space-filling and lubrication functions, and plays a role in signal transduction, cell adhesion and many additional physiological processes (28, 29). The matrisome-associated proteins include secreted factors (such as cytokines and chemokines some of which are tightly bound to the ECM), ECM regulators (such as proteinases) and ECM-affiliated proteins (such as galectins) (25).

Although the proteome and secretome in atherosclerosis has been studied by proteomics in mouse models and human samples (30–32), an overall and in-depth picture of the progressive changes during atherogenesis of the protein component of the plaque in general and the ECM is currently lacking (33). MS based analysis of the ECM is challenging because of the insolubility of ECM components as well as contamination from more abundant intracellular proteins (33). Stepwise extraction protocols, in which a biological sample is fractionated according to the solubility of the contained proteins have been described, partially overcoming these limitations (26, 34–37).

To increase our understanding of protein changes in atherogenesis, we sought to study proteome changes with special focus on ECM proteins in an unbiased way. Using advances in MS-based proteomics (38), our laboratory has recently developed a very sensitive and in-depth approach termed quantitative detergent solubility profiling (QDSP) and applied it to unravel matrisome changes during lung injury in great depth (39). Here we applied the QDSP protocol to ApoE knock out mice (ApoE−/−), a classical mouse model for atherosclerosis. These mice develop intermediate and advanced plaques at 16 and 24 weeks, respectively, when fed a high-fat, “western-type” diet (40, 41). We generated a quantitative and in-depth resource of time- and compartment-resolved proteome changes of the aorta during atherogenesis. Analysis and replication in an independent cohort retrieved key known factors and uncovered novel proteins pointing to hitherto unknown activities in atherosclerotic plaques.
All four fractions were desalted using stage-tips containing a Poly-styrene-divinylbenzene copolymer modified with sulfonic acid groups (SDB-RPS) material (3M, St. Paul, MN) as previously described (42).

**LC-MS/MS Analysis**—Peptides were separated on a reverse phase column (50 cm length, 75 μm inner diameter) packed in-house with ReproSil-Pur C18-AQ 1.9 (Dr. Maisch GmbH, Ammerbuch, Germany). Reverse-phase chromatography was performed with an EASY-nLC 1000 ultrahigh pressure system, coupled to a Q-Exactive HF Mass Spectrometer (Thermo Scientific). Peptides were loaded with buffer A (0.1% (v/v) formic acid) and eluted with a nonlinear 120-min gradient of 5–60% buffer B (0.1% (v/v) formic acid, 80% (v/v) acetonitrile) at a flow rate of 250 nl/min. After each gradient, the column was washed with 95% buffer B and re-equilibrated with buffer A. Column temperature was kept at 50 °C by an in-house designed oven with a Peltier element and operational parameters were monitored in real time by the SprayQc software. MS data were acquired with a top15 shotgun proteomics method, where in each cycle a full scan is followed by up to 15 data-dependent MS/MS scans. Target value for the full scan MS spectra was 3 x 105 charges in the 300–1650 m/z range with a maximum injection time of 20 ms and a resolution of 60,000. Isolation of precursors was performed with the quadrupole at window of 1.4 m/z. Precursors were fragmented by higher-energy collisional dissociation (HCD) with normalized collision energy (NCE)/stepped NCE of 27. MS/MS scans were acquired at a fragmentation energy of 20 eV.

**Computational MS-data Analysis**—MS raw files were analyzed by the MaxQuant software (43) (version 1.5.3.29) and peak lists were searched against the mouse Uniprot FASTA database (UP00000589_10090.fasta and UP00000589_10090_additional.fasta, version June 2015), and a common contaminants database (247 entries) by the Andromeda search engine. Cysteine carbamidomethylation was set as fixed modification, methionine oxidation and N-terminal protein acetylation as variable modifications. False discovery rate was 1% for proteins and peptides (minimum length of 7 amino acids) and was determined by searching a reverse database. Peptide specificity was set to trypsin requiring C-terminal arginine or lysine with a maximum of two missed cleavages. Maximal allowed precursor mass deviation for peptide identification was 4.5 ppm after time-dependent mass calibration and maximal fragment mass deviation was 20 ppm. “Match between runs” was activated with a retention time alignment window of 20 min, and a match time window of 0.7 min to increase protein identification. For total proteome analyses, solubility fractions were defined in MaxQuant for a combined protein MaxLFQ output, with a minimum ratio setting of 2. For fraction specific analyses no fraction information was predefined and the minimum ratio setting was 1. Protein abundance estimates were calculated by dividing MaxLFQ values by the theoretical number of tryptic peptides per protein.

**Statistics**—All downstream bioinformatic analyses were performed with Perseus (44) (versions 1.5.3.0 and 1.5.5.5) and R (version 3.2.2). Protein groups only identified by site FDR, only from peptides identified also in the reverse database, or belonging to IgG subtypes were excluded from the analyses. For fraction specific analyses, MaxLFQ protein intensity values were filtered for at least two different peptides. For PCA, ANOVA and Student’s t test, missing values were imputed with a width of 0.2 and a downshift of 1.8 over the total matrix. ANOVA was performed in Perseus with s0 set to 0 and subsequent filtering for a minimum fold change of 1.5-fold. Gene annotation enrichment analyses were done with Fisher’s exact test including GOMF, GOCC, GOBP, and Corum annotations, curated gene lists of matrisome (25, 26), immune cell signatures (45, 46), and gene lists taken from Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany). Minimum FDR was set to 0.1 and annotation lists filtered for the presence for at least three proteins in the intersection. Student’s t test was performed with the SAM package in R (47). The logarithmized MaxLFQ intensities were filtered to have at least two valid values in one of the replicates of a single time point. Missing values were imputed as described before and t test statistics calculated using the samr function (s0 = 0.2, nperm = 1000). Delta tables were computed using the samr function with a minimal fold change of 2 and significant outliers defined by the smallest delta value allowing identification of the highest number of significant outliers with a median FDR < 0.1.

**RESULTS**

**Quantitative Detergent Solubility Profiling (QDSP) of Mouse Aorta**—To follow atherosclerotic plaque development by proteomics, we fed 8 week old atherosclerosis prone Apoe−/− mice for 8 and 16 weeks with a high-fat, western-type diet (Material and Methods) (Fig. 1A) resulting in consistent formation of atherosclerotic lesions (supplemental Fig. S1A). As controls, we used age- and sex-matched wild-type mice fed with chow diet, avoiding spontaneous plaque formation. For an independent evaluation of our data, we included a second Apoe−/− mouse cohort, which was kept in a different mouse facility. Per cohort, each biological condition consisted of three to five individual animals.

To gain insight into the cellular localization of proteins we applied the QDSP protocol (39). Briefly, we lysed individual aortas in buffer with low detergent strength, pelleted homogenized material by centrifugation and performed consecutive extraction steps with buffers containing increasing detergent concentrations. The resulting insoluble fraction was mechanically sheared and all fractions enzymatically digested by trypsin and LysC. Although the first QDSP fractions contained about 50–100 μg, peptide amounts in the more insoluble ones were about hundred-fold lower. For each aorta we quantified 3756–4728 proteins (median 4,265) using label-free val-
Fig. 1. **Quantitative detergent solubility profiling of mouse aorta.** A, Outline of the experimental setup. Two independent ApoE−/− mouse cohorts and one paired wild-type mouse cohort were analyzed at the age of 8, 16 and 24 weeks (n = 3 - 5 mice per time point). Aortas were prepared and proteins sequentially extracted with buffers of increasing detergent strength. Proteins from the four different fractions were digested with trypsin and analyzed by high-resolution mass spectrometry in a label free shotgun approach. B, Principal component analysis (PCA) reveals a clear separation of the four fractions in the first two components. C, Protein loadings of the PCA shown in (B). D, QDSP profiles for selected proteins of cohort #1. Data points are filtered for the presence of at least two valid values and are averages. Error bars represent S.E.
changes should only manifest in the ApoE −/− mice. We achieved high technical and biological reproducibility as reflected in Pearson correlation coefficients above 0.90 for all aorta proteomes (supplemental Fig. S1C).

Reproducibility was also high at the level of individual fractions as evident from principal component analysis (PCA) (Fig. 1B). The four fractions clearly separated in the PCA plot in the first two components, despite being collected from two different and independently processed cohorts. Cytosolic proteins such as the proteasome complex core members showed specific enrichment toward the first fraction (FR1), membrane proteins such as the NADH dehydrogenase complex were localized between FR1 and FR2 (Fig. 1C). The tropomyosin associated troponin complex specifically purified in FR2, and nucleosomal proteins distributed over FR2 and FR3. As expected, the ECM proteins such as collagens were preferentially enriched toward the insoluble fraction (FR4). The robustness of the protocol at the level of individual protein is exemplified by the highly reproducible profiles of Transforming Growth Factor Beta Induced (Tgfbi), Proteasome Subunit Beta 5 (Psmb5), and Histone H3.3 (H3f3a) across different time points, conditions and cohorts analyzed (Fig. 1D, supplemental Fig. S1D). This in-depth proteomic data set of mouse aorta during atherogenesis robustly reports on the compartmentalization of proteins within the tissue based on their detergent solubility profiles.

Atherogenesis and Vessel Maturation Lead to Distinct Proteome Changes in the Aorta—The changes in the aortas of our mouse cohorts are because of normal maturation and atherogenesis induced changes in these vessels as well as to plaque formation in the ApoE −/− mice. Note that these plaques constitute only a few percent of aorta mass, requiring accurate and in-depth differential quantification of the proteomes. From our data set, we first calculated protein changes of all and in-depth differential quantification of the proteomes.

Cluster 1 and 4 were specifically regulated during atherogenesis in ApoE −/− mice. Cluster 4 contained a large number of known factors related to atherosclerotic plaque development that were up-regulated at the 16 week and particularly the 24 week time points in the knock-out mice. This included the endothelial expressed vascular cell adhesion molecule-1 (Vcam-1) responsible for recruitment of immune cells (49), the macrophage derived Cathepsin B and D (Ctsb/Ctsd) (50), Galectin-3 (Mac2) (51), as well as apoptosis inhibitor of macrophage (Aim/Cd5l) (52), serving as positive controls for atherogenesis mediated recruitment and invasion of monocytes. The proteins with highest regulation in response to the genotype were also robustly enriched in individual t test comparisons of ApoE −/− and wild type aortas at the three different time points (supplemental Fig. S2A–S2C).

Bioinformatic “annotation enrichment analysis” in Cluster 4 revealed a high prevalence of proteins of the matrisome compartment (25, 26), immune cell gene signatures (45, 46) (p < 0.01), as well as of the gene lists “atherosclerosis” and “vascular lesion” from Ingenuity pathway analysis (IPA) (p < 10−11; Fig. 2C, supplemental Table S3). Further supporting our analysis, Cluster 4 was specifically enriched for Gene Ontology (GO) annotations related to known molecular processes in atherogenesis, including “chemotaxis” (p < 10−4), “integrin binding” (p < 10−5) and “secreted” protein (p < 10−35). Interestingly, proteins related to “morphology of bone” (p < 10−7) and “mineralization of bone” (p < 10−9) were also enriched, pointing to vascular calcification a crucial event in atherogenesis. Our proteomic data directly provides the identity of the proteins responsible for these annotation enrichments (supplemental Table S4).

In addition to a large set of proteins that are biologically linked to atherogenesis, we discovered several factors without previous connection to atherosclerosis. One of these is Coactosin-like protein (Cotl1), a chaperone for the Arachidonate 5-lipoxygenase (Alox5) (Fig. 2D). Alox5 catalyzes an initial step in the biosynthesis of leukotrienes, mediators of smooth muscle cell contraction that act as strong activators of inflammation when overexpressed. Polymorphisms in genes involved in the leukotriene pathway have been linked to atherosclerosis in humans and Alox5 itself is a key molecule for atherosclerosis susceptibility in mice (7, 53). As Alox5 stabilization by Cotl1 leads to increased activity because of an inactivation of protein turnover (54), the up-regulation of Cotl1 observed here may contribute to the activation of the leukotriene pathway during atherogenesis. Another up-regulated protein not previously linked to atherogenesis is Annexin A8 (Anxa8) (Fig. 2D). Loss of Anxa8 results in a reduction of the leukocyte adhesion factor P-selectin and its cofactor CD36 at the cell surface (55). Conversely, the up-regulation of Anxa8 during atherogenesis observed here may increase leukocyte recruitment to aortic endothelial cells.

We also identified proteins that were specifically downregulated in atherogenic ApoE −/− mice compared with nonathero-
Fig. 2. **Total proteome changes during atherogenesis.** A, Unsupervised hierarchical clustering of significantly (ANOVA, FDR < 0.05, fold change > 1.5 < -1.5) regulated proteins (z-scored MaxLFQ values) analyzing all fractions of the first cohort together. B, Correlation of protein fold changes (ApoE−/−, 24 weeks versus 8 weeks) of both cohorts reveals low variation. C, Gene annotation enrichment analysis of proteins from each cluster versus all quantified proteins. In addition to Gene Ontology (GO) annotations (no shading), annotations included extracted gene lists from Ingenuity pathway analysis (IPA) (yellow shading), immune cell gene signatures (Gautier et al., 2012; Jojic et al., 2013) (purple shading), as well as curated lists of matrisomal proteins (Naba et al., 2012a; Naba et al., 2012b) (blue shading) obtained from the literature. D, Cot1 QDPS profile of cohort #1. Data points are filtered for the presence of at least two valid values and are averages. Error bars represent S.E.
genic wild type mice at the age of 16 and 24 weeks (Fig. 2A). Podocan (Pdn), is an inhibitor for the migration and proliferation of VMSCs in mice and humans (56). As Pdn−/− mice show increased neointima formation after arterial injury, its down-regulation might be functionally associated with plaque development. Similarly, the GTPase Rrad (Ras associated with diabetes) suppresses the attachment and migration of VSMCs (57).

Atherosclerosis in General and the ApoE−/− Phenotype in Particular are Closely Linked to Lipid Metabolism and Transport—In our data set, 25 out of the 90 proteins that were differentially regulated between wild-type and the ApoE−/− mice on the high fat diet were related to lipid metabolism and transport (supplemental Fig. S2D). Most of those proteins were up-regulated specifically during atherogenesis - such as Apolipoprotein B (Apob) and Apolipoprotein A-IV (Apoa4). In contrast, Apolipoprotein A-I (Apoa1) was about 2-fold down-regulated and we attribute this to the ApoE knock out rather than to atherogenesis because it was the case at all time points.

Interestingly, palmitoyl protein thioesterase 1 (Ppt1) was specifically up-regulated in ApoE−/− mice after 16 weeks, corresponding to 8 weeks of high-fat diet (supplemental Fig. S2D). Ppt1 is a lysosomal hydrolase that removes long fatty acid sidechains such as palmitate from proteins. Mutations in the Ppt1 genes cause infantile neuronal ceroid lipofuscinosis (58), a severe neurodegenerative disease characterized by an accumulation of ceroid aggregates in lysosomes and leading to neuronal death. As ceroid formation has been also observed in atherosclerotic plaques (59), the up-regulation of Ppt1 during atherogenesis may represent a regulatory mechanism aimed at the removal of ceroids.

The Osteoclastic V-ATPase Complex is Expressed in Macrophages of Mature Atherosclerotic Plaques—Vascular calcification is a hallmark of atherogenesis, leading to arterial stiffening and mineralization of atherosclerotic plaques (60). Several proteins known to promote calcification were specifically expressed in the ApoE−/− mice during the latest time points of atherogenesis, including Osteoactivin (Gpnmb), Osteopontin (Spp1), Bone sialoprotein 2 (Isbp), matrix Gla pro-
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tein (Mgp) and proteoglycan 4 (Prg4) (Fig. 3A). Interestingly, two inhibitors of calcification, Osteoprotegerin (Tnfrsf11b) (61) and Cartilage oligomeric matrix protein (Comp) (62) were also increased, suggesting an interplay of positive and negative regulators of vascular calcification.

The mechanisms of vascular calcification resembles the calcification processes in bone (63). Bones are continuously remodeled by decalcifying osteoclast cells derived from the monocyte-macrophage lineage, which balance calcification by the osteoblasts. Aside from secreting enzymes that degrade organic matrix, osteoclasts release H+ and Cl- ions to dissolve inorganic matrix by means of a vacular-type H+-ATPase in the plasma membrane, containing three subunits specific to osteoclasts (Tcirg1, Atp6v0d2, and Atp6v1b2). Unexpectedly, we identified all three subunits to be specifically present and up-regulated in atherosclerotic aortas (Fig. 3B). Although we detected Tcirg1 and Atp6v0d2 exclusively in 24-week-old ApoE−/− mice, Atp6v1b2 was expressed in all conditions, with a specific increase in 24-week-old ApoE−/− mice. We next asked which cell type expressed this complex. Immunohistochemistry (IHC) against Tcirg1 showed a clear Tcirg1 staining of Mac2-marked macrophages in the plaque, exclusively in 24-week-old mice (Fig. 3C). The granular staining further suggested a localization in vesicles in addition to a patchy localization at the plasma membrane. The absence of Tcirg1 in the less developed plaques of 16-week-old ApoE−/− mice establishes a late manifestation of this osteoclastic function during plaque formation, as already suggested by our proteomic results.

Matrisome Remodeling During Atherosclerotic Plaque Formation—Structural changes in ECM have been implicated in the pathogenesis of atherosclerosis and other cardiovascular diseases (22). Accordingly, we identified matrisomal proteins to be enriched among significantly regulated proteins during atherogenesis (p < 10−16). To investigate the remodeling of the matrisome in more detail, we first determined matrisome associated proteins in our data set on the basis of their QDSP expression profiles (Fig. 4A, Methods). To this end, we filtered all quantified proteins for at least 2-fold higher expression in the insoluble fraction (FR4) compared with the average expression in FR2 and FR3. We also required at least 2-fold higher MS-signals compared with the average of FR1–3 in at least one biological condition, which resulted in a set of 532 proteins of a total of 3401 proteins initially identified in FR4 (supplemental Fig. S3, supplemental Table S5). As expected, there was high enrichment in ECM annotated proteins (p < 10−40) (supplemental Table S6), with 25% of proteins being previously designated as core matrisome or matrisome associated proteins (25, 26) (Fig. 4A). Collagens are the main structural proteins of the ECM and in terms of absolute abundance, Collagen types VI alpha 1–3 (Col6a1–3) were the most highly expressed ones in aorta, exceeding Collagen types I alpha 1 and 2 (Col1a1,2) 4-fold and Collagen XV type alpha 1 about eight times (Fig. 4B). Protein-lysine 6-oxidase (Lox) and Lysyl oxidase homolog 1 (Loxl1), with pivotal roles in the generation of covalently cross-linked collagen structures were the most abundant ECM associated enzymes and had levels comparable to these collagens.

To determine proteins within our ECM related group associated with matrisome remodeling, we performed ANOVA on their expression values in the insoluble fraction over the biological conditions. This resulted in 65 significantly regulated matrisome associated proteins in cohort 1 (FDR < 0.1, minimum fold change 1.5), whose regulation was closely reproduced in cohort 2 (r = 0.89) (Fig. 4C, 4D, supplemental Table S7). Among these proteins we observed strong atherogenesis dependent up-regulation of Collagen types II alpha1 (Col2a1) and XII alpha 1 (Col12a1). Collagen types X alpha 1 (Col10a1), and VIII alpha 1 (Col8a1) were exclusively detected in mature atherosclerotic aortas from ApoE−/− mice. This makes them a specific constituent that contributes to the structure of the plaque matrisome (supplemental Fig. S4).

Proteases actively shape the ECM and, matrix metalloproteinases (Mmps) target both core ECM and matrisome associated proteins (23). Mmp3, Mmp12, and Mmp19 were increased during atherogenesis (Fig. 4C; supplemental Fig. S4). Up-regulation of Mmp19 has not been reported before, whereas this is already known for Mmp3 and Mmp12 and likely derives from macrophage secretion (22). ECM-associated protease inhibitors were also significantly up-regulated in atherogenesis, including the tissue inhibitor of metalloproteinase 3 (Timp3), which was the most highly expressed one, as well as two subunits of the Inter-Alpha-Trypsin Inhibitor, Itih1 and Itih2. Although there is evidence for a protective role of Timp3 in atherosclerosis (64, 65), Itih1 and Itih2 have not yet been associated with plaque development. The fact that Itih1 was expressed in atherosclerotic aorta in very similar amounts to Timp3, may further support a functional role (Fig. 4B).

Apart from proteins connected to known features of plaque matrisomes, our data set contains factors whose involvement in this process was completely unknown. For instance, three matrisomal proteins—Peroxidasin (Pxdn), Matrilin 2 (Matn2), and MAM Domain Containing 2 (Mamdc2), were expressed at low levels in wild-type and young ApoE−/− mice, but were specifically up-regulated in ApoE−/− mice in the course of atherogenesis (Fig. 5A, 5B). Mamdc2 is an ECM-associated protein of unknown function containing four MAM domains, which can serve as adhesion domains in surface receptors (66). We validated its up-regulation by IHC and found that it was expressed in endothelium and tunica media in non-atherosclerotic aortas and in plaques, concordant with the increase during atherogenesis in the proteomics data (Fig. 5C).

DISCUSSION

Atherosclerosis is a multifactorial disease with a high incidence and mortality, and often clinically manifests as stroke
Fig. 4. Matrisome remodeling during atherosclerotic plaque formation. A, Definition of ECM associated proteins based on solubility profiles. B, Ranked protein abundance of ECM associated proteins defined in (A) in the insoluble fraction of 24 week old ApoE−/− mice. Pale blue: core matrisome proteins, orange: matrisome-associated proteins. C, Hierarchical clustering of protein expression values (MaxLFQ, z-scored) in the insoluble fraction of significantly (FDR < 0.1, s0 < 1) regulated ECM-associated proteins defined in (A). D, Correlation of fold expression changes (24 week versus 8 week old ApoE−/− mice) between both cohorts of significantly regulated proteins from the insoluble fraction of cohort #1 reveals low variation.
or myocardial infarction (1–5). A classical animal model for atherosclerosis is the ApoE/H11002 mouse (40, 41). Depletion of the ApoE protein leads to spontaneous hypercholesterolemia and atherogenesis manifested in mature atherosclerotic plaques. Feeding the mice with high fat diet (“Western diet”) accelerates atheroprogression. Molecular events related to vessel maturation happen in conjunction with those related to atherogenesis. To distinguish between them we followed a control cohort (wild-type mice on a chow diet) and contrasted this to an ApoE/H11002 cohort, fed high fat diet from week eight.

Because of the proteinaceous nature of plaques, it would be most promising to investigate how proteins change in atherogenesis, but so far this has only been done for individual or small sets of candidate proteins. Here, we employed the latest advances in proteomics technology to explore this question. Using aortas from single animals, we obtained quantitative profiles of thousands of proteins across solubility.

Fig. 5. Identification of novel atherosclerosis-associated ECM proteins. A, B, QDSP profiles for ECM-associated proteins with a novel role in atherogenesis in both cohort #1 (A) and cohort #2 (B). Data points are filtered for the presence of at least two valid values and are averages. Error bars represent S.E. C, IHC of Mamdc2 in young healthy (left panel) and mature atherosclerotic aortas (right panel) from ApoE/H11002 mice. Dashed lines delineate the inner vessel wall (EC: endothelial cell layer).
fractions. Although atherosclerotic plaques only constitute a few percent of aorta mass, their proteome was unambiguously captured by comparison to the wild-type mice. Investigation of three time points was crucial, as it provided a proteome base line in wild-type mice as well as early, purely genotype related changes, which we contrasted to both genotype and diet-related changes at the point of fully developed atherosclerosis. Overall, the large majority of atherogenic proteome changes was confined to the 24 week time point—16 weeks after high fat feeding. However, many interesting effects were clearly apparent at 16 weeks already, such as those related to lipid metabolism. Apart from providing a resource of proteome changes in this important disease to the community, our results suggest that many of the established mouse models of human diseases can now fruitfully be interrogated by MS-based proteomics.

The protein expression changes in individual aortas in response to atherogenesis were overwhelmingly upregulations, whereas only few proteins showed decrease in expression. This nonsymmetry can be explained by the de novo development of the atherosclerotic plaque, which reflects a buildup of ECM components by VSMCs together with an infiltration of immune cells. The increase in protein expression is therefore the sum of high abundant proteins from immune cells together with the secretion products of all cell types involved in plaque formation.

The quantitative detergent solubility profiling (QDSP) method allowed us to directly measure the compartment association of the aorta proteome. Although previous studies focused on soluble driver proteins in atherogenesis, little is known about changes in the ECM compartment. In addition to the increase in Col2a1, Col10a1, and Col12a1, which are osteoblastic and chondrocytic marker proteins and therefore likely related to plaque calcification (67, 68), we observed an atherogenesis specific up-regulation of Col8a1, a protein that has recently been implicated in fibrous cap formation in atherosclerosis (69). Collagens are organized as highly interlinked networks containing both inter- and intramolecular covalent linkages (29). Peroxidase Pxdn was significantly up-regulated in atherosclerotic aortas both at the total protein level and in the insoluble fraction, which is interesting because it forms collagen IV crosslinked networks in the basement membrane (70, 71). Thus, an increase in Pxdn might contribute to the arterial stiffening that is observed in atherosclerosis (72).

We identified family members of matrix metalloproteinases (Mmps) with both protective and proatherogenic functions to be up-regulated during atherogenesis. Although Mmp3 has been reported to reduce atherosclerotic plaque formation and rupture (73, 74), Mmp12 promotes plaque development and is a risk locus for large artery atherosclerotic stroke (75). Mmp19 was found to be protective against bleomycin induced lung injury in mice (76), therefore its up-regulation during atherogenesis raises the possibility that it has an atheroprotective effect. In addition, we found the Mmp inhibitor Timp3 to be enriched in the insoluble fraction, which has been reported to protect against atherosclerosis (65).

Among the ECM associated and up-regulated proteins that have not yet been related to atherogenesis are Matn2 and Mamdc2. Matn2 is a filament-forming protein widely distributed in ECM of various tissues, with largely uncharacterized function that has been linked to muscle and nerve regeneration as well as inflammation (77, 78). Mamdc2 is a proteoglycan with completely unknown function containing five MAM domains, which mediate interactions and stability and are commonly found in surface receptors (79). Our expression profiles in atherosclerosis make both proteins promising targets for future studies.

Vascular calcification is a crucial hallmark of atherogenic plaques that is causally related to clinical outcomes (60, 63) and here we identified upregulated proteins with known roles in this process. Vascular calcification is an analogous process to bone mineralization, involving osteoblast-like cells that are likely derived from vascular smooth muscle cells (VSMCs) (80). Up-regulated proteins included the bone matrix protein Tnfrsf11b, as well as the cartilage matrix proteins Prg4 and Comp, which serve as intermediates of endochondral bone formation. In addition, Col12a1 and Col10a1, which mark sites of bone and cartilage mineralization, respectively, were enriched in the atherosclerotic ECM. Collectively, these results support an active osteogenesis and chondrogenesis calcification model in atherosclerotic plaque development (63).

In bone, mineralization is a balanced process, which is negatively regulated by osteoclasts. This has led to long-standing speculation that similar osteoclast-like cells might be present in atherogenic plaques and counteract vascular calcification, yet clear evidence for the existence of those cells is missing (63). Here, we unambiguously identified two subunits of the osteoclast specific V-ATPase - Tcirg1 (v0a3) and v0d2-as exclusively expressed in atherosclerotic plaques of 24-week-old ApoE−/− mice. The v1b2 subunit of the same complex, which is also highly expressed in osteoclasts, was likewise increased in atherosclerotic mouse aortas. Notably, we observed Tcirg1 expression in macrophages, thus adding to recent evidence for a role of those cells in mineral clearance within atherosclerotic plaques (81, 82) and suggesting that macrophages adapt an osteoclast-like gene expression program and providing a possible functional mechanism. Osteoclastic V-ATPase is directly involved in bone resorption as it dissolves minerals through continuous expulsion of H+ and Cl− ions (83). The osteoclastic V-ATPase might therefore have an important regulatory function against plaque calcification. This finding may have clinical implications, as inhibitors of the osteoclastic V-ATPase have been proposed for the treatment of osteoporosis (83, 84). Because patients with osteoporosis also often develop atherosclerosis (85), inhibiting osteoclastic V-ATPase could unintentionally lead to a detrimen-
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tal increase in plaque calcification. Although our results derive from an established mouse model, it would be of translational interest to follow them up in the human system. In this case, healthy aorta controls would not be available, but this could be addressed by laser capture based techniques to isolate human plaques specifically.

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DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomcentral.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD006752 (https://www.ebi.ac.uk/pride/archive/projects/PXD006752).

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REFERENCES

1. WHO. Cardiovascular diseases (CVDs), 2017; Available from: http://www.who.int/mediacentre/factsheets/fs317/en/.
2. Kannel, W. B., Wolf, P. A., and Vetter, J. (1983) Manifestations of coronary disease predisposing to stroke. The Framingham study. JAMA 250, 2942–2946
3. Iso, H., Jacobs, D. R., Jr., Wentworth, D., Neaton, J. D., and Cohen, J. D. (1989) Serum cholesterol levels and six-year mortality from stroke in 350,977 men screened for the multiple risk factor intervention trial. N. Engl. J. Med. 320, 904–910
4. Sacco, R. L., Benjamin, E. J., Broderick, J. P., Dyken, M., Easton, J. D., Feinberg, W. M., Goldstein, L. B., Gorelick, P. B., Howard, G., Kittner, S. J., Manolio, T. A., Whisnant, J. P., and Wolf, P. A. (1997) American Heart Association Prevention Conference. IV. Prevention and Rehabilitation of Stroke. Risk factors. Stroke 28, 1507–1517
5. Dichgans, M., Malik, R., Konig, I. R., Rosand, J., Clarke, R., Gretsadottir, S., Thorleifsson, G., Mitchell, B. D., Assimes, T. L., Levi, C., O’Donnell, C. J., Fornage, M., Thorsteinsdottir, U., Psaty, B. M., Hengstenberg, C., Seshadri, S., Erdmann, J., Bis, J. C., Peters, A., Boncoraglio, G. B., Marz, W., Meschia, J. F., Kathiresan, S., Ikram, M. A., McPherson, R., Stefansson, K., Sudlow, C., Reilly, M. P., Thompson, J. R., Sharma, P., Hopewell, J. C., Chambers, J. C., Watkins, H., Rothwell, P. M., Roberts, R., Markus, H. S., Samani, N. J., Farrall, M., and Schunkert, H. (2014) Shared genetic susceptibility to ischemic stroke and coronary artery disease: a genome-wide analysis of common variants. Stroke 45, 24–36
6. Hansson, G. K. (2005) Inflammation, atherosclerosis, and coronary artery disease. N. Engl. J. Med. 352, 1685–1695
7. Hansson, G. K., and Hermansson, A. (2011) The immune system in atherosclerosis. Nat. Immunol. 12, 204–212
8. Weber, C., Zernecke, A., and Libby, P. (2008) The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. Nat. Rev. Immunol. 8, 802–815
9. Weber, C., and Noels, H. (2011) Atherosclerosis: current pathogenesis and therapeutic options. Nat. Med. 17, 1410–1422
10. Hansson, G. K., and Libby, P. (2006) The immune response in atherosclerosis: a double-edged sword. Nat. Rev. Immunol. 6, 508–519
11. Woollard, J. K., and Geissmann, F. (2010) Monocytes in atherosclerosis: subsets and functions. Nat. Rev. Cardiol. 7, 77–86
12. Libby, P. (2001) Current concepts of the pathogenesis of the acute coronary syndromes. Circulation 104, 365–372
13. Dobrin, P. B. (1978) Mechanical properties of arteries. Physiol. Rev. 58, 301–343
14. Slow, R. C., and Churchman, A. T. (2007) Advenitial growth factor signaling and vascular remodelling: potential of perivascular gene transfer from the outside-in. Cardiovasc. Res. 75, 659–668
15. Wagensel, J. E., and Mecham, R. P. (2009) Vascular extracellular matrix and arterial mechanics. Physiol. Rev. 89, 957–989
16. Libby, P., and Hansson, G. K. (2015) Inflammation and immunity in diseases of the arterial tree: players and layers. Circ. Res. 116, 307–311
17. Amento, E. P., Ehsani, N., Palmer, H., and Libby, P. (1991) Cytokines and growth factors positively and negatively regulate interstitial collagen gene expression in human vascular smooth muscle cells. Arteriosclerosis. Thromb. 11, 1223–1230
18. Hahn, C., and Schwartz, M. A. (2009) Mechanotransduction in vascular pathology and atherosclerosis. Nat. Rev. Mol. Cell Biol. 10, 53–62
19. Hynes, R. O. (2009) The extracellular matrix: not just pretty fibrils. Science 326, 1216–1219
20. Schwartz, M. A. (2010) Integrins and extracellular matrix in mechanotransduction. Cold Spring Harb. Perspect. Biol. 2:a005066
21. Hynes, R. O. (2012) The evolution of metazoan extracellular matrix. J. Cell Biol. 196, 671–679
22. Chistiakov, D. A., Sobchen, I. A., and Orekhov, A. N. (2013) Vascular extracellular matrix in atherosclerosis. Cardio. Res. 21, 270–288
23. Bonnans, C., Chau, J., and Werb, Z. (2014) Remodelling the extracellular matrix in development and disease. Nat. Rev. Mol. Cell Biol. 15, 786–801
24. Naba, A., Clauser, K. R., Ding, H., Whittaker, C. A., Carr, S. A., and Hynes, R. O. (2016) The extracellular matrix: Tools and insights for the “omics” era. Matrix Biol. 49, 10–24
25. Naba, A., Hoersch, S., and Hynes, R. O. (2012) Towards definition of an ECM parts list: an advance on GO categories. Matrix Biol. 31, 371–372
26. Naba, A., Clauser, K. R., Hoersch, S., Liu, H., Carr, S. A., and Hynes, R. O. (2012) The matrisome: in silico definition and in vivo characterization by proteomics of normal and tumor extracellular matrices. Mol. Cell. Proteomics 11, M111.014647
27. Orom, G. L., Xiong, X., Chaudart, E., Ricard-Blum, S., and Parkinson, J. (2012) Toward a systems level view of the ECM and related proteins: a framework for the systematic definition and analysis of biological systems. Proteins 80, 1522–1544
28. Frantz, C., Stewart, K. M., and Weaver, V. M. (2010) The extracellular matrix at a glance. J. Cell Sci. 123, 4195–4200
29. Hynes, R. O., and Naba, A. (2012) Overview of the matrisome–an inventory of extracellular matrix constituents and functions. Cold Spring Harb. Perspect. Biol. 4:a004903
30. Mayr, M., Chung, Y. L., Mayr, U., Yin, X., Ly, L., Troy, H., Fredericks, S., Hu, Y., Griffiths, J. R., and Xu, Q. (2005) Proteomic and metabolomic analyses of atherosclerotic vessels from apolipoprotein E-deficient mice reveal alterations in inflammation, oxidative stress, and energy metabolism. Arteriosclerosis. Thromb. Vasc. Biol. 25, 2135–2142
31. de la Cuesta, F., Barderas, M. G., Calvo, E., Zubiri, I., Maroto, A. S., Martin-Rojas, T., Gil-Dones, F., Posada-Ayala, M., Tejerina, T., Lopez, J. A., Vivancos, F., and Alvarez-Llamas, G. (2012) Secretome analysis of atherosclerotic and non-atherosclerotic arteries reveals dynamic extracellular remodeling during pathogenesis. J. Proteomics 75, 2960–2971
32. Bagnato, C., Thumar, J., Mayya, V., Huang, S. I., Zebroski, H., Claffey, K. P., Haendenschil, C., Eng, J. K., Lundgren, D. H., and Han, D. K. 332

Molecular & Cellular Proteomics 17.2
(2007) Proteomics analysis of human coronary atherosclerotic plaque: a feasibility study of direct tissue proteomics by liquid chromatography and tandem mass spectrometry. Mol. Cell. Proteomics 6, 1088–1102

33. Lynch, M., Barabollo-Barreiro, J., Jahangiri, M., and Mayr, M. (2016) Vascular proteomics in metabolic and cardiovascular diseases. J. Intern. Med. 280, 325–338

34. Lennon, R., Byron, A., Humphries, J. D., Randles, M. J., Carisye, A., Murphy, S., Knight, D., Brenchley, P. E., Zent, R., and Humphries, M. J. (2014) Global analysis reveals the complexity of the human glomerular extracellular matrix. J. Am. Soc. Nephrol. 25, 939–951

35. Randles, J. M., Woolf, A. S., Huang, J. L., Byron, A., Humphries, J. D., Price, K. L., Kolati-Joannou, M., Collinson, S., Denny, T., Knight, D., Mironov, A., Starbog, T., Korstanje, R., Humphries, M. J., Long, D. A., and Lennon, R. (2015) Genetic background is a key determinant of glomerular extracellular matrix cellular composition and organization. J. Am. Soc. Nephrol. 26, 3021–3034

36. Didangelos, A., Yin, X., Mands, K., Baumert, M., Jahangiri, M., and Mayr, M. (2010) Proteomics characterization of extracellular space components in the human aorta. Mol. Cell. Proteomics 9, 2048–2062

37. Langley, S. R., Willett, K., Didangelos, A., Matic, L. P., Skrobin, P., Baralobbe-Barreiro, J., Lengquist, M., Runnger, G., Kapustin, A., Kedenko, L., Mouton, C., Lux, S., Ross, N., Scancy, J., Fieder, B., Willett, P., Shahloob, J., Pastorkepam, G., Davies, A. H., Monaco, C., Hedin, U., Shanahan, C. M., Willett, J., Kiechl, S., and Mayr, M. (2015) Extracellular matrix proteomics identifies molecular signature of symptomatic carotid plaques. J. Clin. Invest. 127, 1546–1560

38. Aebersold, R., and Mann, M. (2016) Mass-spectrometric exploration of proteome structure and function. Nature 537, 347–355

39. Schiller, H. B., Fernandez, I. E., Burgstaller, G., Schaab, C., Scheltema, R. A., Schwarzmayr, T., Strom, T. M., Eickelberg, O., and Mann, M. (2015) Time- and compartment-resolved proteome profiling of the extra-cellular niche in lung injury and repair. Mol. Syst. Biol. 11, 819

40. Piedrahita, J. A., Zhang, S. H., Hagaman, J. R., Oliver, P. M., and Maeda, N. (1992) Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells. Proc. Natl. Acad. Sci. U.S.A. 89, 4471–4475

41. Plump, A. S., Smith, J. D., Hayek, T., Aalto-Setala, K., Walsh, A., Verstuyft, J. G., Rubin, E. M., and Breslow, J. L. (1992) Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by delayed normalization and maximal peptide ratio extraction. Proc. Natl. Acad. Sci. U.S.A. 89, 1604–1608

42. Chua, W. J., Hansen, T. H., Merad, M., and Randolph, G. J. (2003) Extracellular matrix remodeling in inflamed tissues: novel insights into inflammation and tissue repair. Circulation 107, 2513–2526

43. Chen, Y., Tian, Q., Liu, C. J., Zhang, T., Xu, Q., Arai, S., Shelton, J. M., Chen, M., Bradley, M. N., Castello, A., Bookout, O. L., Mak, P. A., Edwards, P. A., Mangelsdorf, D. J., Tortorente, P., and Yen, H. C. (2005) A role for the apoptosis inhibitory factor AIM/Spalix/Api6 in atherosclerosis development. Cell Metab. 1, 201–213

44. Nakashima, Y., et al. (1998) Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the Apoe-deficient mouse. Arterioscler. Thromb. Vasc. Biol. 18, 842–851

45. Zhao, C. F., and Herrington, D. M. (2016) The function of cathepsins B, D, and X in atherosclerosis. Am. J. Cardi. 6, 163–170

46. Jojic, V., Shay, T., Sylvia, K., Zuk, O., Sun, X., Kang, J., Regev, A., Koller, D., Immunological Genome Project. North, A. J., Knell, J., Goldbrath, A. Joic, V., Koller, D., Shay, T., Regev, A., Cohen, N., Brenner, P., Brenner, M., Kim, F., Rao, T. N., Wagers, A., Heng, T., Ericsson, J., Rothamel, K., Ortiz-Lopez, A., Mathis, D., Benoist, C., Benoist, M. A., Sun, J. C., Min-Oo, G., Kim, C. C., Lanier, L. L., Miller, J., Brown, B., Merad, M., Gautier, E. L., Jakubczik, C., Randolph, G. J., Monach, P., Blair, D. A., Dustin, M. L., Shinton, S. A., Hardy, R. Y., Laidlaw, D., Collins, J., Gazzit, R., Rossi, D. J., Malhotra, N., Sylvia, K., Kang, J., Kreslavs, J., T. Fletcher, A. Elpek, C., Bellemarte-Pelletier, A., Malhotra, D., and Turley, S. (2013) Identification of transcriptional regulators in the mouse immune system. Nat. Immunol. 14, 632–643

47. Tusher, V. G., Tibshirani, R., and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. U.S.A. 98, 5116–5121

48. Cox, J., Hein, M. Y., Luber, C. A., Paron, I., Nagaraj, N., and Mann, M. (2014) MaxLFQ allows accurate proteome-wide label-free quantification by delayed normalization and maximal peptide ratio extraction. Mol. Cell. Proteomics 2513–2526

49. Didangelos, A., Manoleuca, A., Thorleifsson, G., Gretarsdottir, S., Jonsdottir, H., Thorleifsson, U., Samani, N. J., Gudmundsson, G., Grant, S. F., Thorgerjonsdottir, G., Sveinbjornsdottir, S., Valdimarsson, E. M., Matthiasson, S. E., Johannsson, H., Gudmundsdottir, O., Guerin, M. E., Sanz, J., Thorhallsdottir, M., Andrewsdottir, M., Frigg, M. L., Topol, E. J., Kong, A., Guerin, V., Bakker, R., Balck, G. Ingelsson, R., and Stefansson, K. (2004) The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. Nat. Genet. 36, 233–239

50. Esser, J., Rakonjac, M., Hofmann, B., Fischer, L., Provost, P., Schneider, G., Steinhiber, D., Samuelsson, B., and Radmark, O. (2010) Coactosin-like protein functions as a stabilizing chaperone for 5-lipoxygenase: role of tryptophan 102. Biochem. J. 425, 265–274

51. Kawahara, T. H., Rosm, R. V., Goh, Y., Shahn, V., Skyrbain, B. V., Zarbock, A., Gerke, V., and Rescher, U. (2014) Annexin A1 controls leukocyte recruitment to activated endothelial cells via cell surface delivery of CD63. Nat. Commun. 5, 3738
Proteomics of Atherogenesis

(2014) Loss of TIMP3 exacerbates atherosclerosis in ApoE null mice. *Atherosclerosis* **235**, 438–443

66. Manabe, R., Tsutsui, K., Yamada, T., Kimura, M., Nakano, I., Shimojo, C., Sanzen, N., Furutani, Y., Fukuda, T., Oguri, Y., Shimamoto, K., Kiyozumi, D., Sato, Y., Sado, Y., Senoo, Y., Yamashina, S., Fukuda, S., Kawai, J., Sugiyama, N., Kimata, K., Hayashizaki, Y., and Sekiguchi, K. (2008) Transcriptome-based systematic identification of extracellular matrix proteins. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 12849–12854

67. Shao, J. S., Sierra, O. L., Cohen, R., Mecham, R. P., Kovacs, A., Wang, J., Distelhorst, K., Behrmann, A., Halstead, L. R., and Towler, D. A. (2011) Vascular calcification and aortic fibrosis: a bifunctional role for osteopontin in diabetic arteriosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **31**, 1821–1833

68. Izu, Y., Ezura, Y., Koch, M., Birk, D. E., and Noda, M. (2016) Collagens VI and XII form complexes mediating osteoblast interactions during osteogenesis. *Cell Tissue Res.* **364**, 623–635

69. Lopes, J., Addiguel, E., Gu, S., Liu, S. L., Hou, G., Heximer, S., Assoian, R. K., and Bendek, M. P. (2013) Type VIII collagen mediates vessel wall remodeling after arterial injury and fibrous cap formation in atherosclerosis. *Am. J. Pathol.* **182**, 2241–2253

70. Brandes, R. P. (2011) Vascular peroxidase 1/peroxidasin: a complex protein with a simple function? *Cardiovasc Res.* **91**, 1–2

71. Bhave, G., Cummings, C. F., Vanacore, R. M., Kumagai-Cresse, C., Ero-Silence, J., Lupu, F., Collen, D., and Lijnen, H. R. (2001) Persistence of stromelysin-1 (MMP-3) gene inactivation. *Arterioscler. Thromb.* **21**, 1821–1833

72. Kohn, J. C., Lampi, M. C., and Reinhart-King, C. A. (2015) Age-related vascular stiffening: causes and consequences. *Front. Genet.* **6**, 112

73. Silence, J., Lupu, F., Collen, D., and Lijnen, H. R. (2001) Persistence of atherosclerotic plaque but reduced aneurysm formation in mice with stromelysin-1 (MMP-3) gene inactivation. *Arterioscler. Thromb.* **21**, 1440–1445

74. Johnson, J. L., George, S. J., Newby, A. C., and Jackson, C. L. (2005) Divergent effects of matrix metalloproteinases 3, 7, 9, and 12 on atherosclerotic plaque stability in mouse brachiocephalic arteries. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 15575–15580

75. Traylor, M., Makela, K. M., Kilasics, L. L., Holliday, E. G., Devan, W. J., Nalls, M. A., Wiggins, K. L., Zhao, W., Cheng, Y. C., Achterberg, S., Malik, R., Sudlow, C., Bevan, S., Raitoharju, E., METASTROKE, International Stroke Genetics Consortium, Wellcome Trust Case Consortium 2 (WTCC2), Oksala, N., Thijs, V., Lemmens, R., Lindgren, A., Slowik, A., Maguire, J. M., Walters, M., Algra, A., Sharma, P., Attia, J. R., Boncorglio, G. B., Rothwell, P. M., de Bakker, P. I., Bis, J. C., Saleheen, D., Kittner, S. J., Mitchell, B. D., Rosand, J., Meschia, J. F., Levi, C., Dichgans, M., Lehtimaki, T., Lewis, C. M., and Markus, H. S. (2014) A novel MMP12 locus is associated with large artery atherosclerotic stroke using a genome-wide age-at-onset informed approach. *PLoS Genet.* **10**, e1004469

76. Yu, G., Kovkova-Naumovska, E., Jara, P., Parwani, A., Kass, D., Ruiz, V., Lopez-Otin, C., Rosas, I. O., Gibson, K. F., Cabrera, S., Ramirez, R., Yousem, S. A., Richards, T. J., Chensy, L. J., Selman, M., Kamiński, N., and Pardo, A. (2012) Matrix metalloproteinase-19 is a key regulator of lung fibrosis in mice and humans. *Am. J. Respir. Crit. Care Med.* **186**, 752–762

77. Jonas, A., Thiem, S., Kuhlmann, T., Wagener, R., Aszodi, A., Nowell, C., Hagemeier, K., Laverick, L., Perreau, V., Jokubaitis, V., Emery, B., Kilpatrick, T., Butzkueven, H., and Gresle, M. (2014) Aaxonally derived matrilin-2 induces proinflammatory responses that exacerbate autoimmune neuroinflammation. *J. Clin. Invest.* **124**, 5042–5056

78. Korpos, E., Deak, F., and Kiss, I. (2015) Matrilin-2, an extracellular adaptor protein, is needed for the regeneration of muscle, nerve and other tissues. *Neural Regen. Res.* **10**, 866–869

79. Beckmann, G., and Bork, P. (1993) An adhesive domain detected in functionally diverse receptors. *Trends Biochem. Sci.* **18**, 40–41

80. Alves, R. D., Eijken, M., van de Peppel, J., and van Leeuwen, J. P. (2014) Calcifying vascular smooth muscle cells and osteoblasts: independent cell types exhibiting extracellular matrix and bone mineralization-related mimics. *BMC Genomics* **15**, 965

81. Chinetti-Gbaguidi, G., Darouiche, R. M., Viñod, M., Louvet, L., Capón, C., Fanchon, M., Vanhoutte, J., Derudas, B., Belloy, L., Haillon, S., Zawadzki, C., Susen, S., Massy, Z. A., Eckhoute, J., and Staels, B. (2017) Human alternative macrophages populate calcified areas of atherosclerotic lesions and display impaired RANKL-induced osteoclastic bone resorption activity. *Circ. Res.* **121**, 19–30

82. Rogers, M. A., Aikawa, M., and Aikawa, E. (2017) Macrophage heterogeneity complicates reversal of calcification in cardiovascular tissues. *Circ. Res.* **121**, 5–7

83. Qin, A., Cheng, T. S., Pavlos, N. J., Lin, Z., Dai, K. R., and Zheng, M. H. (2017) Human alternative macrophages populate calcified areas of atherosclerotic lesions and display impaired RANKL-induced osteoclastic bone resorption activity. *Circ. Res.* **121**, 5–7

84. Yuan, F. L., Li, X., Lu, W. G., Li, C. W., Li, J. P., and Wang, Y. (2010) The vascular ATPase in bone cells: a potential therapeutic target in osteoporosis. *Mol. Biol. Rep.* **37**, 3561–3566

85. Hamerman, D. (2005) Osteoporosis and atherosclerosis: biological linkages and the emergence of dual-purpose therapies. *QJM* **98**, 467–484