Review Article

Atherofibrosis - a unique and common process of the disease pathogenesis of atherosclerosis and fibrosis - lessons for biomarker development

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Abstract: The hallmark of a variety of fibrotic diseases such as liver fibrosis, lung fibrosis, skin fibrosis and atherosclerosis is extensive extracellular matrix remodeling (ECMr) of the disease affected tissue. Inflammation often leads to tissue disruption and destruction, upon which locally released battery of proteases such as matrix metalloproteinases and cysteine proteases degrade the surrounding matrix. The degradation products of ECM proteins, the so-called neoepitopes, are released into the systemic circulation. By recent development of Enzyme-Linked Immunosorbent Assays (ELISAs) detecting the pathological tissue turnover in atherosclerosis and liver fibrosis, we have introduced a novel class of biomarkers into the field of fibrotic diseases, which have been proved efficient in the early diagnosis. This work has resulted in identification of common mechanisms involving specific cell types, proteins and proteases as well as pathways shared among the fibrotic diseases. In this analysis we seek to answer following questions: a) Are there common disease mechanisms and cell types involved in both atherosclerosis and fibrosis? b) Can the lessons learned in developing fibrosis biomarkers be used for the development of atherosclerosis biomarkers? Our hypothesis is that by answering the above questions, we may be able to improve general understanding of the early-stage disease initiation and progression of fibrotic diseases, which in turn may aid in early diagnosis, prognosis and ultimately patient management.

Keywords: Atherosclerosis, fibrosis, extracellular matrix, collagens, proteoglycans, matrix metalloproteinases, neoepitopes, biomarkers

Introduction

ECMr is a key process of tissue homeostasis [1]. Specific proteolytic activities are a prerequisite for a range of cellular functions and interactions with the extracellular matrix [1]. These specific activities are precisely coordinated under physiological situations, in which a specified sequence of events results in controlled tissue turnover. In pathological situations, including inflammation, atherosclerosis, fibrosis and cancer, the normal repair-response relationship is perturbed [2], leading to excessive remodeling, and tissue turnover.

ECM degradation is an important component of atherogenesis playing a central role in arterial remodeling important to clinical outcomes associated with coronary heart disease. Changes in the composition of ECM are in part due to elevated levels of proteases produced by resident and migratory inflammatory cells, such as macrophages [3]. The extracellular and pericellular matrix of arteries is mainly composed of fibrous proteins such as collagens (type I and type III) and proteoglycans [4]. Endopeptidases such as matrix metalloproteinases (MMPs) play major roles in the degradation of extracellular macromolecules such as collagens and proteoglycans [5, 6].

Liver fibrosis pathology is related to increased deposition and abnormal distribution of ECM proteins, mainly collagens and proteoglycans. Liver fibrosis is a serious complication of chronic inflammatory liver disorders with a diversity of infectious, inflammatory and toxic causes [7]. Progression of fibrosis eventually leads to
liver cirrhosis, which poses a major global health problem. During fibrogenesis, the quantity and composition of ECM proteins in the liver changes, resulting in excessive accumulation of fibrous tissue and an overall increase of ECM density [8]. The ECM of the cirrhotic livers contains approximately six times as much matrix mass as the normal liver [9], which is a result of increased levels of mainly type I, type III, and type IV collagen [10]. However, levels of matrix metalloproteinases (MMPs), such as MMP-9, also increase [11, 12].

The degradation of the ECM components by MMPs in both atherosclerosis and in liver fibrosis is accompanied by the release of breakdown products, called neoepitopes, into the circulation. Thus these neoepitopes may be useful indicators of arterial and hepatic enzymatic activity and the local matrix turnover in the atherosclerotic plaques and in diseased livers, respectively. Furthermore, these novel biomarkers may serve as predictors of future clinical outcomes and complications as well as markers of efficacy of drugs.

Neoepitope based biochemical markers found in urine and serum are receiving increased attention for their diagnostic and prognostic potential [13]. In particular for slowly progressing diseases, such as osteoporosis and osteoarthritis, bone resorption and cartilage degradation markers have been used extensively [14]. However at present, the use of neo-epitope based biochemical markers is restricted to those pathologies.

The aim of the current review is to present parallel insights of two fibrotic processes that involve pathological tissue turnover. We believe that we hereby may be able to identify common pathology denominators. This knowledge may be of great value in order to transfer valuable lessons from successful fibrosis biomarker development into the field of atherosclerosis.

**Atherosclerosis - vulnerable vs. stable lesions**

Atherosclerotic plaque formation in the arterial intima, results from complex cellular interactions mainly between resident cells of the vessel wall, such as smooth muscle cells (SMCs) and endothelial cells (ECs), and cells of the immune system, mainly leukocytes [15]. Disturbances in blood flow and increased lipid infiltration are major contributors to this process. When the atherosclerotic plaque has formed, it displays a very characteristic architecture composed of a fibrous cap covering a central core which mainly is composed of extracellular lipids and debris – the so called atheroma, which is soft, weak and highly thrombogenic. Unlimited phagocytosis of oxidized LDL by macrophages through scavenger receptors results in formation of foam cells, another hallmark of atherosclerosis [16]. Death of foam cells plays a central role in the formation of atheroma, together with extracellular binding of lipids to collagen fibers and proteoglycans [17, 18]. Histopathologic studies of atherosclerotic lesions in humans have revealed substantial variations in the thickness of fibrous caps, in the size of atheromas and the extent of calcifications and in relative amounts of inflammatory cells [19, 20]. Such studies contain valuable information and have contributed to understanding that only specific types of lesions appear to be associated with acute events of atherosclerotic disease.

**Key molecular mechanisms of the atherosclerotic plaque and fibrotic cap formation**

Extensive pathological analysis of fatally occluded arteries has led to realization that such fatal cardiovascular events are more likely due to artery occlusion by thrombi rather than due to simple obliteration of arterial lumen by atheroma [21-23]. During the evolution of atherosclerosis, the affected vessels undergo a continuous process of remodeling, during which they initially compensate for the lesion development in the intima by increasing overall diameter [15, 24, 25]. Later as the disease progresses, remodeling may continue toward destructive stages in which the tissue weakens and the plaque structure is compromised. Thrombi develop at the areas where the continuity of a plaque's fibrous cap is disrupted by fissures [23] or in places where the endothelial lining is eroded [26, 27]. The weakest areas of the plaque are plaque "shoulders", where the plaque meets the normal arterial wall [28]. Pathological determinants associated with plaque instability include high lipid content and increased number of macrophage-derived foam cells [23]. In addition, it is widely recognized that a dynamic process of weakening contributes to plaque rupture. Here the extensive arterial plaque remodeling by matrix
degrading enzymes leading to active destruction of the extracellular matrix is a process identified in many advanced lesions.

**Extracellular matrix remodeling in atherosclerosis**

Cardiovascular disease is predicted to be the leading cause of death by 2020. Despite a lot of progress in earlier diagnosis and better treatment regimens, CVD remains one of the leading causes of death both in men and women in the western world [29]. Atherosclerosis is a systemic disease of the vessel wall and is considered responsible for ischemic heart disease, ischemic strokes, and critical limb ischemia [30]. These events almost always start with stable clinical manifestations of atherosclerosis from progressive luminal narrowing by an atherosclerotic plaque development with inward remodeling (Figure 1).

The potential development of a thrombus over an underlying plaque is one of the major causes of acute coronary syndromes of unstable angina, myocardial infarction and ischemic sudden cardiac death [31, 32]. Thrombi forming near or on plaque may become incorporated into the plaque, where thrombin generation and the release of mediators by platelets may increase the rate of cell proliferation and thereby increase the production of extracellular matrix (ECM) proteins, further contributing to plaque growth [15, 33]. Many of the cell responses involved in plaque development have an inflammatory nature, further highlighting the pathophysiologic link between atherogenesis and thrombosis. The likelihood of thrombosis increases with disruption of atherosclerotic plaques, the process where the fibrous cap sealing the plaque and preventing plaque material to come in contact with the interior of the blood vessel and the circulating blood. Recent

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**Figure 1.** Cross section of an artery. Arterial structure is composed of 3 main layers, tunica adventitia which forms the outer layer of the structure, the tunica media which lie in the middle and the tunica intima which is in direct contact with blood circulation. During atherosclerosis progression, the arterial wall is gradually thickened and the lumen is narrowed. Complicated lesions are ultimately formed in an ongoing process which can last for decades ultimately blocking blood flow through the artery.
findings have indicated that inflammation influences the composition of the fibrous cap, increasing the risk of rupture [34, 35]. Various inflammatory cells, particularly monocytes infiltrate the atherosclerotic lesions and differentiate into macrophages and further into foam cells, thereby contributing to the development of the plaque [33]. Mast cells have also been identified in the atherosclerotic plaques, and may act in concert with macrophages promoting the inflammatory process [36]. Both macrophages/foam cells and mast cells contain a variety of enzymes such as serine proteases matrix-metalloproteinases, (MMPs), which are major participants in the degradation of ECM proteins of the atherosclerotic fibrous caps [37-39]. The biomechanical properties of blood vessels are largely dependent on ECM constituents and the absolute and relative quantities of these constituents. Thus the balance between tissue generation and degradation is a key modulator in the transition from physiology to pathology.

**ECM Proteins: collagens and proteoglycans**

Among many different collagen types described until now, type I and III collagen are the major fibrillar collagens detectable in vessels, representing 60% and 30% of vascular collagens, respectively [40, 41]. The remaining 10% include fibrillar type V collagen, fibril-associated collagens XII and XIV, microfibrillar collagen IV and type VIII collagen [40]. After secretion on the extracellular compartment and the removal of N- and C-terminal propeptides, collagen triple helices aggregate into fibrils [42]. During the formation of intermolecular cross-links, collagen fibers become increasingly insoluble, more refractory to the action of enzymes and show a progressive increase in tensile strength [40, 43]. The process of cross-linking is initiated by the lysyl oxidase (LOX) enzyme, through the oxidation of specific lysine or hydroxylysine residues in the N-terminal telopeptid regions. The resulting aldehydes undergo a series of reactions with adjacent reactive residues to give both inter- and intramolecular cross-links [44].

Proteoglycans, in particular versican, lumican, and biglycan have been identified in the ECM of the atherosclerotic arteries and implicated in facilitation of plaque rupture and thrombosis. However, at present, there is a complete lack of understanding of the protease generated fragments of these molecules, and the relation to atherosclerosis progression and outcome. Distribution and expression of proteoglycans change during atherogenesis due to the matrix remodeling.

The chondroitin sulfate proteoglycan (CSPG) versican is present in the arterial wall of normal blood vessels. Versican interacts with hyaluronan, a long chain molecular weight glycosaminoglycan (GAG) that is also present in the ECM of blood vessels and increases as versican in vascular disease [45]. Versican is also present in the advanced lesions and is prominent at the edges of the necrotic core. Furthermore, versican is also present in close proximity to deposited lipoproteins, suggesting a role in the retention of lipoproteins in the vessel wall [46].

Lumican, a keratan sulfate proteoglycan, has been involved in wound healing and tissue response injuries. In atherosclerotic arteries lumican is localized in the inner layer of the media, suggesting a local synthesis by intimal and medial smooth muscle cells of the atherosclerotic arteries [47]. Rodent models of atherosclerosis suggest an increase in lumican content by app. 2 fold in the intima of atherosclerotic arteries [48], suggesting high turnover. However, the role of lumican in human atherosclerotic tissues is not clearly elucidated.

Biglycan – a member of small leucine rich family, has been implicated in lipoprotein retention in human atherosclerosis, through the interactions of its glycosaminoglycan side chains [49]. Immunohistochemical staining for biglycan in arteries reveal prominent biglycan localization to the proteoglycan rich layer of the elastic laminae, but the distribution of biglycan seems to shift during atherosclerosis, since biglycan was mostly localized at the plaque shoulders and underneath the lesion.

**Proteases**

The MMP family posses fairly homologous amino acid domains and share several important features. All cell-secreted MMPs are produced as latent pro-enzymes or zymogens, thus a common feature of MMP activation is necessary in order to convert the enzymes to an active form. Furthermore, all MMPs are
dependent on the zinc ion for their proteolytic action [50]. The mechanism of activation, also known as the cysteine switch mechanism [51] can be initiated either through a conformational modifications of the proenzyme molecule or through the proteolytic removal of its pro-domain. The conformational modification leads to partially active enzyme that can bind and digest substrate and also, it can also initiate autocatalysis of the partially active enzyme. Through a series of consecutive series of autolytic steps, the MMPs are shortened going through a fully activated stage, and later with continuous processing loose the catalytic domain and thus the enzymatic activity. This molecular processing is characteristic of MMPs, whose enzymatic activity is absent under normal conditions because the stored zymogens are inactive and subsequent initiation of activation results in a limited life span for the enzyme. Moreover, the cells can also secrete natural MMP inhibitors, called tissue inhibitors of metalloproteinases (TIMPs) which can interfere with zymogen activation and with substrate degradation by activated MMPs [52].

MMPs have been classified into several classes. Each class shares high degree of sequence homology and substrate specificity. Some of the major classes of MMPs are collagenases, stromelysins, gelatinases, matrilysines and membrane-associated (MT) MMPs.

Morphological and cell culture studies have contributed to gaining knowledge on MMP expression in healthy and diseased tissues. In culture, endothelial cells (ECs) synthesize several MMPs and their natural inhibitors [53-55]. All cultured ECs seem to constitutively secrete MMP-2, which is thought to be necessary for the normal turnover of all cellular basement membranes. Expression of MMP-1 and MMP-3 by ECs is induced or enhanced depending on the vascular source of EC, by exposure to cytokines [56, 57]. Cytokines have also been shown to stimulated in vitro expression of MT1-MMP in human saphenous vein EC and in smooth muscle cells (SMCs) [58]. Vascular SMC constitute most of the cells in the blood vessel wall and are therefore likely the main contributors to protein synthesis in general. In vitro, SMC isolated from different vascular sources of different species have been shown to produce several types of MMPs [59-64]. In vitro, MMP-2 was found to be one of the major MMPs secreted by the SMCs, thus likely to be abundant in the vascular wall, and may be one of the main vascular MMPs under normal physiological conditions. Expression of pro-MMP9, pro-MMP1, and pro-MMP3 by human SMCs is induced in vitro by different growth factors and cytokines, which are expressed in atherosclerotic arteries in areas of atheroma infiltrated with inflammatory cells [65]. Furthermore, other mechanisms of activation of MMP-2 production by SMCs have been shown to be mediated by reactive oxygen species (ROS) [66]. In vitro ROS have been shown to be able to activate latent forms of gelatinases secreted by cultured human SMCs. Production of ROS known to be present in the atherosclerotic lesions is attributed to inflammatory cells [67].

Blood-borne cells are not a common component of the healthy vessel. However, the monocyte-derived macrophages have been identified in all atherosclerotic lesions and seem to be prevalent among the several types of inflammatory cells such as, T-lymphocytes and mast cells. Activated inflammatory cells have natural ability to penetrate tissues, largely due to the matrix degrading action of MMPs [68].

**Key molecular mechanisms of fibrogenesis- the example of liver**

Fibrosis results from chronic tissue injury and results in the formation of scar tissue formation. Even though fibrotic mechanisms may vary depending from the tissue affected, in all cases it the transition from an ECM balanced remodeling state to an unbalanced ECM remodeling (ECMR) state which is the main driver of shift to pathology [69]. During this process, ECM undergoes significant changes which alter the distribution, quantity and ultimately the quality of ECM [70-72]. The paradigm of ECM altered composition in the space of Disse where the predominant expression of collagen type III, IV, laminin and fibronectin under physiological conditions, is distorted during fibrosis initiation towards a state in which collagen type I is greatly upregulated is indicative of the ECM quantitative and qualitative changes that occur during fibrosis related ECMr (Figure 2) [73].

Since ECMr imbalances are in the centre of pathology creation, it is of great value to highlight some of the mechanisms, proteins, cells
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Myofibroblasts (MF) are the main matrix producing cells and due to this main function they are playing a key role in fibrosis processes in all tissues [69, 81, 82]. In liver, the MF precursor cells are the hepatic stellate cells (HSC) which are considered the main source of fibrotic processes, even though other mesenchymal cell types, also contribute to a lesser extend [83]. HSCs activation is a key event in liver fibrosis pathology as they are known to react to cellular injury and death as part of their constant interaction with their environment [84]. Activation is achieved by distinct stimuli, such as oxidative stress and apoptotic bodies, that can stimulate activation initiation and others that can ultimately result to fibrosis through contributing to HSC activation continuation, a process which include key events such as proliferation, fibrogenesis and ECM degradation that ultimately result in ECM accumulation and tissue fibrosis [85]. The multivariate role of HSCs in fibrotic processes and resulting inflammation is further highlighted by their role as antigen presenting cells (APCs) and containment of inflammasome components which can induce inflammatory responses as a reaction to cellular injury and death [84]. The involvement of the immune system in liver fibrosis processes is not limited to inflammatory events but is extended in ECMR

and proteases involved in different forms of fibrosis in order to highlight common mechanisms between atherofibrosis and other types of fibrotic events. For this reason, liver fibrosis mechanism will be used as an example that will facilitate the comparison with atherofibrotic events.

Liver extracellular matrix (ECM) consists of collagens, proteoglycans and glycoproteins, all of which play important, unique and interrelated functions in maintaining the physicochemical structure of liver tissue while they also possess signalling functions as both ligands and receptors [69, 70, 74]. Even though for years, the main focus was restricted on fibrillar collagens and the alterations in quantity and quality, the role of other proteins and proteoglycans such as fibronectin and laminin has become evident and brought additional attention to the important interplay of a wider variety of ECM constituents which collectively result in significantly increased, ECM derived protein in liver [75, 76]. An additional feature of this ECM centred interaction during liver fibrosis progression is the diverse post translational modification (PTM) and cross linking activities that concurrently have a direct impact on protein life cycle and pathology progression [77-80].

Figure 2. Quiescent HSC can be activated by a variety of chronic stress and/or signals such as alcohol, virus and auto-immune stimuli as well as by interaction from KC cytokines. When activated HSC can activate adjacent HSC and can produce MMPs and a number of different matrix constituents such as collagens and proteoglycans. These promote fibrogenesis and substantially alter tissue structure and ultimately function.
through the contribution of macrophages, lymphocytes and monocytes in ECM degradation and interaction with myofibroblasts. Additional implication of immune cells to ECMR stems from information on the activity of cytokines such as Th1 and Th2 have been found to have antifibrogenic and fibrogenic properties respectively, adding to our knowledge of participating cells in liver fibrosis related pathology [86].

Kupffer cells (KCs) are another cell type which is also an active participant in the transition from balanced to unbalanced ECMR which ultimately leads to liver fibrosis. KCs are involved in livers response to various stresses as they are the local liver macrophages which upon LPS mediated activation can release a range of cytokines which can contribute to stellate cell activation and stimulate ECM synthesis [87]. KC activation via toll-like receptor 4 (TLR-4) was recently described, suggesting that macrophages may be serving as a link between fibrotic and inflammatory processes [88]. KCs represent a vital source of chemoattractant molecules and they play a key role in HSC transformation to myofibroblasts, a feature which adds to their central part in activation of fibrotic processes and the transition from physiology to pathology [89]. KC cells have been described to be stimulated by apoptotic fragments, as in the case of HSC, and release fibrogenic mediators as a response while they have been found to also stimulate stellate cell apoptosis [90, 91].

An additional active participant in fibrotic progression is the sinusoidal endothelial cells (SEC), which are part of the endothelial wall and are interacting with HSCs during early liver fibrosis progression stages while also involved in sinusoidal capillarization through basement membrane proteins which is ultimately accompanying liver fibrosis development in central vein walls and is implicated to portal hypertension [92-95]. The morphological alterations

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**Figure 3.** Tissue fingerprint technology relies on the selection and generation of pathology relevant neoepitopes. Among different tissues ECM may have both tissue specific as well as common macromolecules and proteases. This may result in ECMr neoepitope formation which can be either tissue specific or overlap among tissues. In turn, certain neoepitopes may be useful as disease predictors for several pathologies. The proteins and enzymes presented in the figure are only indicative.
that SEC undergo after stress or injury it is believed to be an initial stimulus for fibrosis progression, since this is a process which precedes myofibroblast activation and ECM deposition [96].

Collagens, in terms of quantity, are the main ECM constituents. During fibrosis related pathology, increased collagen deposition is being counterbalanced by increased collagenolytic activity. This is mainly carried out by interstitial MMPs that degrade collagen fibers through TIMP regulation [97]. Proteases are active participants in both physiological and pathological related ECMr and their action directly affects the three dimensional structure of the matrix scaffold, the amount of fibrous and non-fibrous molecules as well as cell-matrix interactions. Alteration of the balance between TIMPs and MMPs contributes significantly to the progression of liver fibrosis [98]. More than 26 MMPs and 4 TIMPs have been identified so far and 11 of the MMPs have been found in the liver MMP levels are constantly changed during different stages of the progression of liver fibrosis [99].

Discussion

The first question that we seek to answer is whether there are common disease mechanisms and cell types involved in the pathologies of atherosclerosis and liver fibrosis. Both are ECMr related pathologies that involve and affect distinctly different tissues. However, both of these pathologies share some similar characteristics. Even though fibrosis in general can occur in a variety of tissues, the hallmark of the disease is the abnormal and unbalanced ECMr which is also the case in atherosclerosis. In order to highlight some of the common mechanisms that might be implicated in these two pathologies the example of liver fibrosis was used as a comparison to atherosclerosis. An overview of the mechanisms and key ECM participants of both pathologies was presented. From these it is evident that in both pathologies, a large number of ECM components are involved in the shift towards abnormal ECMr. Some of these active pathology participants seem to be common for both disease states since in both cases they form the ECM scaffold. An example of such an occurrence is collagen type I, III, IV and V as well as proteoglycans such as versican, biglycan, decorin and perlecan.

Concurrently, both pathologies seem to share a considerable amount of proteases such as MMP-1, -2, -3, -8, -9, -13 and -14. In both cases, the main aim of the macrophage derived increased protease levels is removal of the excessive ECM. The constant interaction of common ECM components across different tissues may create protein fingerprints that may overlap, while tissue specific interactions may create tissue specific fingerprints (Figure 3).

The second question we set to answer is whether lessons learned through the development of fibrosis markers can be used for atherosclerosis biomarker development. We have previously described and validated in pre-clinical and clinical studies a number of novel ECMr specific biomarkers for fibrotic diseases based on the tissue fingerprint technology [71, 72, 100-107]. This unique technology is based on precise measurements of specific protein fragments, neo-epitopes that are generated due to proteolytic action of proteases on tissue during pathology. The interaction of a specific ECM related protein with a particular protease could form a unique fragment that could be indicative of in vivo underlying ECMr.

The recognition that ECM constituent proteins found in both pathologies are interacting with a set of identical proteases leads to the probable suggestion that the valuable experience and positive results obtained so far in the field of fibrosis may also be applicable in the field of atherosclerosis. Early suggestions of neoepitope application in the field of atherosclerosis relied on the observation that macrophage mediated proteolysis of ECM constituents and their release into circulation is an important process in atherosclerogenesis [108]. The understanding of the above relationship lead to the suggestion that specific neo-epitope fragments may be of unique diagnostic and prognostic value for detailed atherosclerosis staging and biomarker development and maybe related with the cardiovascular continuum [15].

Early evidence of the possible opportunities for biomarker development indicates that the above approach may have some promising merits in the case of atherosclerosis. The examples of Mmcen and Collagen III markers are indicative of novel and promising ECM biomarkers that are related to atheromatic plaques and are utilising the concept of protein finger-
prints [104, 109]. The example of MMP-9 degraded Collagen III biomarker which is found to be upregulated in liver and skin fibrosis as well as in atherosclerosis is highly suggestive of the possible common mechanisms occurring in both diseases [72, 104, 105, 110]. Additionally, novel cardiovascular specific markers that are relying on the tissue fingerprint technology show some promising early results in clinical studies which should be investigated further [111].

The transition of ECMr from physiological to pathological processes is lengthy and complicated. In both fibrosis and atherosclerosis this transition involves a long period of transient state in which ECMr shift is asymptomatic and therefore almost impossible to diagnose. The above suggests that individuals that unknowingly are experiencing this transient underlying pathology of either fibrosis or atherosclerosis would await the first symptoms to appear before any diagnosis would be possible with the current diagnostic means. The vast majority of existing biomarkers utilised in clinics are unfortunately end stage disease markers, where few successful intervention options exist [15]. The requirement for new markers that can accurately describe the early and tissue specific shift to pathology is as important as ever. According to the world health organisation 17.3 million people have died from CVD related pathologies in 2008, and this number is expected to reach 23.6 million by the year 2030. Adding to the above numbers is individuals gradually developing fibrosis related pathology in any tissue (lung, liver etc.). The above numbers indicate that great numbers of individuals that are seemingly healthy today maybe already experiencing this transient ECMr shift to pathology which will eventually place them in high risk groups for either CVD or fibrotic diseases by 2030. It is therefore of great importance to characterise markers that can facilitate early diagnosis and can accurately monitor pathology related ECMr. Utilisation of protein fingerprint markers seems to have promising momentum towards this quest since they seem to be informative of protease specific protein cleavages that are related with pathology. Such fragments may become of great diagnostic and potentially prognostic value in the future. The example of Titin-12670 (TIM) marker is indicative of such promising potential, even though additional work is required to further validate these findings [112].

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