Endothelial Glycocalyx Biomarkers in Cardiovascular Disease

Abstract

Decades of cardiovascular disease (CVD) research have yielded numerous diagnostic targets – biomarkers – as surrogates for assessing CVD risk. While many of these biomarkers are now established clinical diagnostics, their utility in predicting CVD and cardiovascular outcomes is under scrutiny due to numerous factors, including the questionable clinical value of therapeutics designed to modulate specific pathways relating to these biomarkers. The limited success of these medicines emphasizes the need for continued study of this multifactorial disease. The purpose of this review is to highlight a newly emerging class of biomarkers: components of the endothelial glycocalyx (EGC), a fragile structure comprised of glycosaminoglycans, proteoglycans, and glycoproteins covering the apical surface of endothelial cells combined with certain proinflammatory markers. The EGC has a major role in maintaining healthy vascular function as a facilitator of mechanotransduction, modulation of cellular adhesion, resistance to oxidative stress, and induction of smooth muscle relaxation. When overwhelmed by a proatherogenic environment, EGC integrity is diminished and sheds components through a number of degradation pathways. This review will focus on the processes involved in EGC shedding and explore the utility of EGC degradation products and related enzymes as biomarkers for CVD.

Introduction

Given the prevalence of cardiovascular diseases (CVD) worldwide, there is a continuing need to identify diagnostic targets, so-called biomarkers, for assessing CVD risk. For decades, researchers, physicians, and publicly supported initiatives have devoted countless resources to examine CVD risk factors in seeking predictors to develop treatment plans and preventative strategies for patients. As a classic example, the observed relationship between elevated serum cholesterol and the development of atherosclerosis led to the persistent monitoring of low-density lipoprotein (LDL) and high-density lipoprotein (HDL), representing “bad” and “good” cholesterol, respectively. The presumed role of these lipoproteins spawned a billion-dollar industry with the advent of β-hydroxy β-methylglutaryl (HMG) CoA reductase inhibitors – statins – often touted for their effectiveness in attenuating the perceived role of cholesterol in contributing to the disease. However, evidence that this therapeutic approach is flawed has come to light in recent years, and the shortcomings of focusing on cholesterol as a biomarker for CVD, and as a target for its treatment, have been the topic of much research [1,2].

It has long been accepted that cholesterol is not the only biomarker of CVD. Among additional pathogenic pathways, inflammation is known to play a significant role in CVD development and progression. In one example, C-reactive protein (CRP), a non-specific inflammatory biomarker associated with lupus and rheumatoid arthritis, has a controversial relationship with coronary artery disease (CAD) and is believed by some to be a sensitive early-stage marker of atherosclerotic progression [3]. However, serum CRP levels have not been shown to predict impending thromboembolic events, and attempts to reduce CRP levels as a therapeutic intervention have not been successful. Additional examples of biomarkers with questionable

utility include cardiac troponin, creatinine kinase, myoglobin, and others [4]. These findings are a continuing reminder that CVD is a multi-factorial disease state that remains an unmet medical need. With decades of research and therapeutic development resulting in modest gains in treating this disease, identifying other biomarkers of CVD progression – and potential therapeutic targets – remains a priority.

Decades-old research describe the presence of a thin, non-cellular lining of blood vessels affixed to the luminal surface of vascular endothelium [5-7]. Subsequently, the term glycocalyx was introduced for this structure [8]. In the years since these early observations, it has been determined that the endothelial glycocalyx (EGC) is composed of fragile filaments identified as glycosaminoglycans (GAGs), the primary component of which is hyaluronan (HA), and proteoglycans, glycoproteins, and glycolipids [9]. Collectively, the structure plays a role in mechanotransduction, a process leading to downstream endothelial nitric oxide synthase (eNOS) activation that in turn induces smooth muscle relaxation, modulation of cellular adhesion (also involving NOS activation), resistance to oxidative stress, and control of vessel permeability, all core components of maintaining cardiovascular homeostasis [9-13].

Based in part on these academic findings and clinical observations, the EGC has become the focus of intense research owing to its perceived role as a facilitator for heathy endothelial function and as a target for numerous cardiovascular-related pathologies, topics covered by numerous recent reviews [14-17]. The EGC is now recognized as a key structure for maintaining vascular wall integrity and protecting the endothelial cell layer. This complex network forms a dynamic layer between circulating blood and the endothelium that continuously changes in thickness depending on shear force and blood pressure. Study of the EGC has yielded insights into constituents of the inner and outer layers of the EGC, which play an important role as a transducer of shear stress from blood flow to vascular intracellular signals [18]. Plasma levels of the EGC constituents, including GAGs, proteoglycans, and glycoproteins, have been implicated as key elements in assessing, preventing, and/or treating atherosclerosis.

In this review, the role of the glycocalyx as a modulator of cardiovascular health and as a target for CVD therapy will be explored, with an emphasis on glycocalyx-derived detritus and related inflammatory enzymes, and their potential utility as biomarkers for CVD.

Endothelial Function, Dysfunction, and the Glycocalyx: a Brief Overview

A healthy vascular endothelium provides a protective barrier to blood components, regulates vascular tone, modulates clotting mechanisms (including platelet adhesion), responds to substantial changes in blood flow-induced shear stress, possesses immune reactivity characteristics, regulates filtration of plasma components, and acts as a mediator of cell-cell communication[19]. In addition to vasoactive particles, including the eNOS -derived vasodilator nitric oxide (NO), and vasoconstrictors, such as endothelin and prostanoids that act on vascular smooth muscle and other targets [20], a robust EGC plays an important role in all of these processes.

Endothelial dysfunction refers to the impairment of the above-noted processes and is a consequence of prolonged exposure to CVD risk factors. These factors can include the presence

of excessive levels of inflammatory agents, reactive oxygen species (ROS) that include superoxide and peroxynitrite, and generalized risk factors associated with hypercholesterolemia, hypertension, and diabetes [21]. Homeostatic imbalances induced by these conditions can promote the formation of atherosclerotic lesions that initially develop in predictable locations, such as at bifurcations and curves in the arterial tree, with no clinical implications until the plaque destabilizes and ruptures. Unlike regions that experience uniform laminar shear stress, these areas are characterized by sharp spatial and temporal gradients leading to reduced shear stress due to reduced or turbulent blood flow [22,23]. These shear stress gradients ultimately result in an altered endothelial cell environment; rather than the protective coaxial alignment in the direction of flow beneath a thick glycocalyx, endothelial cells in susceptible regions tend to exhibit reduced integrity beneath the depleted glycocalyx [18]. This impaired barrier function promotes the retention of LDL within the intima and accelerates cell turnover that in turn attracts monocyte adhesion and entry with subsequent differentiation into macrophages [18]. These macrophages can then attract adhesion of cholesterol subtypes, forming "foam cells". The resulting atheroprone phenotype results in alterations in gene expression and mechanoactivated signaling pathways, among other CVD-related pathophysiologies [24]. Atheroprone regions of the vasculature such as bifurcation sites often exhibit compromised glycocalyx function that have been shown to lead to atherogenesis due to the dysfunction of the vasculoprotective characteristics of the endothelium. Here, we describe the major components of the endothelium with a focus on the EGC and how these components can become compromised and lead to the pathogenesis of CVD.

Key Structures and Function of the Healthy Vascular Lumen

The vascular endothelium is a cellular monolayer that is responsible for control of many processes necessary for healthy cardiovascular control and, as a consequence, is the target for disruption by proatherogenic pathologies. Figure 1 displays the major structures and enzymes involved in maintaining proper vascular function. The key structure is the endothelial cell monolayer that forms a protective and dynamic barrier between the smooth muscle cells and blood. On the luminal side of the endothelium, the EGC structure contains a network of GAGs, proteoglycans, glycoproteins, and glycolipids, which are clustered within invaginations called caveoli. The proteoglycan protein core with attached GAGs (mostly heparan sulfate; HS) possesses a net negative charge that is an important feature contributing to function. Also present is unbound HA present in sufficient quantity to create a viscous solution with water. The net negative charge on the glycocalyx repels red blood cells and helps attenuate the interaction of platelets and leucocytes with the endothelial wall [15].

Also featured on the luminal side are various receptors, including those that bind ligands such as endothelins [25], prostacyclins [26] vascular endothelial growth factor [27], other G-protein linked receptor ligands [28], and both calcium and potassium channels [29,30]. The smooth muscle layer, in addition to relaxing or constricting in response to messengers produced by the endothelium, also contains connective tissues, such as collagen and elastin. The key enzyme expressed in endothelial cells that is responsible for control of vascular tone is eNOS. When inactive, eNOS is bound to the protein caveolin, which is in turn anchored at the intracellular side of the caveoli invaginations [31]. eNOS has a bi-domain structure with a reductase domain that contain binding

sites for flavin-adenine dinucleotide, flavin mononucleotide, and nicotinamide adenine dinucleotide phosphate, and an oxygenase domain that binds heme, tetrahydrobiopterin, and the substrate L-arginine [32-34]. The reversible binding of calcium-calmodulin (Ca^{2+}-CAM) to eNOS is required for eNOS activation and is the control point for modulating NO generation.

Receptors and the EGC

The healthy EGC can respond to changes in its microenvironment and regulate functions such as vascular tone, circulating cell adhesion, coagulation, fibrinolysis, and vessel wall inflammation in response to hemodynamic changes at branches and curves in vessels. These functions are mediated by a host of receptors and adhesion molecules, some already mentioned, such as vascular cell adhesion proteins, platelet endothelial cell adhesion molecules, and intercellular adhesion molecules that play important roles in cell-cell interactions and diapedesis. An example of these receptors is CD44, a transmembrane glycoprotein involved in maintaining the barrier function of the EGC that binds hyaluronic acid moieties as its primary ligand [35]. CD44 is also known as the lymphocyte homing receptor. Other important receptors include the selectins and integrins the leucocytes use for adhesion and diapedesis. Leukocytes first tether to the glycocalyx using these receptors. They then "roll" towards the cell junctions where there is a higher concentration of ICAM to gain access in between cells. If the glycocalyx is compromised, this process becomes pathological and can lead to the development of plaques, particularly when LDLs are elevated while proinflammatory mediators are elevated. Another important function of these receptors is to act as a barrier to certain components in the blood while serving as a mesh to promote the accumulation of plasma proteins. For example, penetration studies have shown that despite the difference in molecular weight, vasculoprotective albumin and fibrinogen penetrate the outer layer of the glycocalyx at the same rate. These proteins are both anionic, and charge is unlikely to explain the similarity in penetration rate; however, to date, the explanation remains elusive [36].

Enzymes associated with the EGC

Part of the protective nature of the glycocalyx involves protecting the endothelial layer from shear forces. There are multiple ways this is accomplished. While components of the outer layer of the glycocalyx, such as hyaluronan and chondroitin sulfate, form a barrier and create a filtration system to protect the endothelium, a number of enzymes and core proteins are present within the inner layer to serve some of the more complex functions. Detailed studies have proposed that fluid shear stress and the passage of blood cells deform the core proteins in a way that physically transmit forces to actin filaments within the cytoskeleton of the endothelial cells [36]. Thus, one of the EGC's role in endothelial function is its involvement in mechanotransduction, by which increased shear stress activates the production of NO, which then dilates the vessels locally and reduces stress [37]. There are two components of shear stress that affect the endothelium: fluid shear in the direction of fluid flow and circumferential stretch due to changes in blood pressure. Deformation of the core proteins is the initial step in the mechanotransduction process. This deformation then activates transient receptor potential

channels. The close interaction between the EGC, caveoli, caveolin, and eNOS is in part responsible for the mechanotransduction property associated with EGC responses to changes in shear stress and flow. On sensing such changes, the EGC, which includes glycoproteins that are themselves receptors or are linked to receptors anchored to the plasma membrane [38], aids in facilitating the release of intracellular Ca^{2+}. [39] Stress-induced activation of EGC glycoprotein receptors facilitates the release of Ca^{2+} from intracellular stores.[30] The increase in intracellular Ca^{2+} availability activates calmodulin, forming a complex; the subsequent increase in Ca^{2+}-CAM facilitates eNOS dissociation from caveolin in a process that is further enhanced by heat shock protein-90 mediated processes [40]. These actions are key to forming a reversible Ca^{2+}-CAM-eNOS binding complex required for eNOS activity, thereby acting as a mechanotransducing switch that stimulates the endothelium's capacity to produce and release NO. Release of this freely diffusible neurotransmitter is subsequently crucial in facilitating such vasculoprotective processes as smooth muscle relaxation, inhibiting platelet aggregation, reducing inflammation, and attenuating adhesion molecule activity, among other benefits [41].

Additionally, there are a multitude of ligands and enzymes within the healthy glycocalyx that contribute to cell signaling and enzymatic modifications such as the regulation of fibroblast, anticoagulant and lipolytic activity. Another important contribution of the protective nature of the glycocalyx is that of antioxidant functions. The glycocalyx creates scaffolding for oxygen radical scavenging such as superoxide dismutase [42], which also helps maintain the bioavailability of NO, while reducing oxidative stress on the endothelium. An important group of enzymes that remain tethered within a healthy glycocalyx are antioxidants and antithrombotics. Extracellular superoxide dismutase (SOD3) is one such antioxidant critical to protecting the endothelium from ROS present in plasma and released upon adhesion of inflammatory mediators.

On the other hand, a group of proteolytic enzymes known as matrix metalloproteinases (MMPs) have been shown to mediate oxidative stress-related degradation of the syndecans, and inhibitors of this group of enzymes have recently been studied as potential therapies used to treat derangements of the endothelial glycocalyx. Oxidative stress has been shown to both increase the expression and activity of MMPs and subsequent loss of syndecans and SOD3 from the glycocalyx. Recent work has indicated that inhibition of MMPs decreases the loss of both the syndecans and SOD3 from the glycocalyx and reduces glycocalyx degradation [43]. Therefore, circulating levels of syndecan-1 correlates with cardiovascular disease progression; many cardiovascular diseases are linked to dysfunction of the endothelial glycocalyx, and inhibiting enzymes associated with high circulating syndecan-1 as well as glycocalyx degradation preserves glycocalyx function.

Endothelial Pathophysiology and the Role of the EGC

A healthy glycocalyx is very important to a properly functioning vascular endothelium. As such, the factors leading to glycocalyx degradation have come under intense scrutiny from both basic and clinical researchers. Endothelial dysfunction is the first step in the atherosclerotic process, and disruption of the glycocalyx is involved at its initial phase. The glycocalyx serves many roles from the mechanotransduction of shear stress to acting as a tether for enzymes and other

proteins. These roles put the endothelial glycocalyx front and center in the mediation of early inflammatory responses that lead to atherosclerosis. As important as the glycocalyx is to endothelial function, it is very dynamic and fragile despite its direct exposure to the shear stress created by non-laminar blood flow in non-linear sections of arteries and capillaries. Capillary leakage can result in regional edema; erosion of the outer layer of the glycocalyx can accelerate regional inflammation; loss of anticoagulant activities can result in platelet aggregation and hypercoagulation; and diminished NO responses can lead to the loss of vascular responsiveness. All of these disturbances can accumulate, making the region far more atheroprone [44].
The list of pathologies resulting in EGC injury is lengthy. Ischemia-reperfusion, hyperglycemia, hypervolemia, sepsis, hemorrhagic shock, bypass surgery, and other pathological and physiological insults lead to the disruption and degradation of the EGC [45]. The following discussion focuses on 3 broad CVD states and their observed impact on EGC structure and function.

Atherosclerosis

Dysfunction of the vasculo-protective EGC is a first step in the atherosclerotic process and may lead to disruption of the endothelium, a process initiated in part by enzymes upregulated under certain pathophysiological conditions. Examples of enzymes involved in the disruption of the EGC include metalloproteinases [35]. Endothelium disruption allows for changes in the subendothelial space and triggers inflammation. Inflammation leads to recruitment of monocytes across the endothelial monolayer into the intima where they proliferate and differentiate into macrophages. The macrophages ingest oxidized debris and lipoproteins, developing into foam cells and maturing into plaques. Inflammation-mediates changes to the glycocalyx and subsequent disruption of the endothelial layer of blood vessels, especially in areas associated with arterial bifurcations and curves. Invasion of the subendothelial intima by LDL is the initial hallmark of atherosclerotic progression, and evidence suggests that the overall positive charge of apoprotein B results in an attraction to negatively charged proteoglycans, thereby contributing to the accumulation of lipids within the intima where LDL deposition reaches high levels [46]. Once lipoproteins and associated lipids begin to accumulate within the intima, the lipids become susceptible to chemical modifications brought on by oxidation and enzymatic cleavage. As the glycocalyx is eroded, there is a concomitant loss of protective enzymes, including xanthine oxidoreductase (XOR), lipoprotein lipase (LPL), and tissue factor pathway inhibitor (TFPI). Loss of XOR in particular removes an important antioxidant from the region. The added loss of LPL removes the enzyme needed to perform lipolysis of the accumulating chylomicrons which then deprives the nearby parenchymal cells of free fatty acids. The erosion of TFPI then sets the stage for increased coagulation events by unregulated tissue factor [47]. Once the lipoproteins become chemically modified, the immune response greatly increases the pathogenic progression of plaque formation, which then further activates proinflammatory mediators and leads to a chronic inflammatory reaction. When the inflammatory reaction begins, macrophages move into the areas with oxidized LDLs and cholesterol. Macrophages begin to clean up the more dangerous forms of the lipids and acquire the characteristic foam cell morphology accompanied by arterial fatty streaks [47]. Once these foam cells become trapped within the endothelial intima, the

inflammatory response becomes exaggerated. The proliferation of foam cells causes the release of sufficient growth factors and cytokines; vascular smooth muscle cells migrate into the initima as well and begin to proliferate to form the fibrous cap [48]. It appears that the foam cells initially undergo apoptosis, and nearby macrophages scavenge the remnants. This process overwhelms the macrophages, and their death subsequently releases the lipids and proinflammatory and prothrombotic mediators. MMPs are activated, devouring the ECM scaffold and destabilizing the plaque [48].

Hypertension

Investigations into the association between hypertension and the EGC include animal models evaluating the effect of hypervolemia and hypertension on EGC thickness and retrospective human studies designed to assess the integrity of the EGC in hypertensive patients. In humans, hypertension is a multifactorial pathology. To begin to tease apart the wide range of contributing factors, a number of animal models have been developed. Electron microscopy studies in spontaneously hypertensive rats (SHR), stroke-prone spontaneously hypertensive (SHRSP), and Wistar Kyoto (WKY) rats were conducted by Ueno et al. [49,50] to evaluate EGC thickness and early changes in blood-brain barrier (BBB) vascular permeability. BBB disruption was observed in SHR and SHRSP rats but not the control WKY rats in parallel with damage to the EGC; however, this observation did not correlate with early changes in the BBB. Kumase et al. [51] also used electron microscopy to assess EGC integrity in both diabetic and hypertensive rat models. Measuring EGC thickness in retinal and choroidal capillaries, the authors observed a significant decrease in thickness in the retina of diabetic rats but not in choroidal capillaries compared with control animals; in hypertensive rats, both retinal and choroidal EGC were significantly decreased compared with controls. These observations were accompanied by increases in leucocyte and platelet adhesion at endothelial cell sites colocalized to EGC regions displaying marked degradation.

Another culprit for inducing a hypertensive state, chronic elevations in circulating NA+ concentrations, has been identified as a mediator of EGC shedding. Using in vivo and in vitro techniques, Schierke et al. [52] identified increased monocyte adhesion to the endothelial surface leading to decreased NO release paralleled by increases in pro-inflammatory cytokines, all signaling the degradation of the EGC. By contrast, acute conditions of Na+ excess had no effect on the EGC.

Among the few human studies, Bruegger et al. [53] assessed the effect of hypervolemia on EGC integrity in patients undergoing coronary artery bypass surgery. Focusing on hypervolemia-induced release of atrial natriuretic peptides (ANPs), the authors observed increases in ANP concentrations following on- and off-pump coronary artery bypass surgery and found that these changes coincided or preceded EGC shedding. Another study of relatively healthy older,

hypertensive patients by Triantafyllidi et al. found that circulating HDL levels had a positive correlation with healthy EGC [54].

Collectively, these data point to a complex relationship between hypertension, endothelial dysfunction, and EGC degradation that has yet to be fully understood.

Coronary Artery Disease

Endothelial dysfunction is at the core of atherosclerotic progression at every phase, and CAD is moderate to advanced atherosclerosis, which generally begins early in life. Initial exposure of the endothelial glycocalyx to turbulent flow alters the composition of the glycocalyx, leading to increased endothelial permeability to plasma constituents such as LDLs. When lipoproteins interact with the proteoglycans of the glycocalyx and intima, they become susceptible to chemical modifications, particularly oxidation. The fragile balance between NO and superoxide production by eNOS can be sufficiently perturbed to favor the production of peroxynitrite, which furthers the destruction of the glycocalyx and altered expression of adhesion molecules and metalloproteases [55]. As the glycocalyx deteriorates, the resulting increase in endothelial permeability provides the means for further barrier disruption; angiopoietin 2 then mediates further destruction of the glycocalyx, the shedding of syndecan-1 and a subsequent proinflammatory vascular response [15]. These indicators of glycocalyx shedding appears to allow for monocyte adhesion/infiltration, which then promotes further vascular inflammation. The infiltration and differentiation of monocytes under the influence of available proinflammatory signals are then accompanied by retention of increasingly available circulating lipids and the development of atherosclerotic plaques. These alterations appear to coincide with hyaluronan synthase inhibition, which can predispose the region to further leucocyte infiltration, enhanced inflammation and enhanced progression of the atherosclerotic plaque phenotype [24]. This progression subsequently upregulates what has come to be known as the sheddases, namely; “heparinase, hyaluronidase, matrix metalloproteinases activated in endothelial inflammation: thrombin, plasmin involved in thrombotic and fibrinolytic processes, elastase, and proteinase 3 released by endothelium-adherence leukocytes. One of the end results of this process can be CAD, which is the culmination of atherosclerosis in the arteries that bring the blood supply to the heart. If the build-up of plaque reaches the point of actual occlusion of a coronary artery, the result is a myocardial infarction. To date, physicians and researchers have found a way to reverse the atherosclerotic process with a very restrictive diet and exercise program, which has low patient compliance. Statins may have the same effect, but studies have been inconclusive in this regard, and the treatment requires very high doses, which also has a low patient are compliance. Because reversing CAD is so difficult, physicians attempt to use preventative strategies, such as non-invasive techniques to assess endothelial function including reactive hyperemia [55], and prescribing statins to maintain a low level of LDLs and VLDLs, while recommending exercise to encourage increased HDL. There is still substantial work to be done, but it appears that monitoring patients’ endothelial glycocalyx status could be indicative of their CAD status, even at early stages [56]. One difficulty with monitoring glycocalyx status is in the related imaging technology. Our work in analyzing the correlation of certain circulating components of the glycocalyx with major CVDs could provide physicians with a powerful and inexpensive tool.

EGC and Sheddases

Endothelial dysfunction is at the core of atherosclerotic progression at every phase. As mentioned above, atherosclerotic progression subsequently upregulates what has come to be known as the sheddases: heparinase, hyaluronidase, MMPs activated in endothelial inflammation, thrombin, plasmin involved in thrombotic and fibrinolytic processes, elastase, and proteinase 3 released by endothelium-adherence leukocytes. All of these enzymes and their products are indicative of different positions on the CVD spectrum. One of the end results of this process can be CAD. The extent to which key enzymes such as heparinase, hyaluronidase and chondroitinase erode the glycocalyx has been studied together and in combination. Individually, these enzymes can reduce the layer by about half; however, when used in combination, the glycocalyx is reduced to 10% of control depth [57]. This information is critical to understand the components of the glycocalyx that might be valuable for use as biomarkers. Imaging studies indicate that damaged glycocalyx does not mean merely diminished GAGs; those proteins are dynamic and are constantly being added and removed. Significant glycocalyx damage means diminished core proteins and their associated enzymes as well [56]. In addition, microvascular imaging studies appear to indicate that the technique would be far more useful in women than men. Thus, this review highlights the following particular glycocalyx components. Because the GAG components, as mentioned, are in constant flux, the background noise would be too high to clearly decern abnormal levels indicative of disease progression. Monitoring glycocalyx components that are the more superficial GAGs along with the core proteins and the associated enzymes, some transmembrane, would provide a more comprehensive picture of an individual's position on the CVD spectrum. Choosing glycocalyx components as biomarkers could actually provide a clear understanding of disease progression in individuals, which would make for a very valuable tool. Therefore, the following section outlines a summary of what could be considered promising biomarkers towards that end.

Promising Biomarkers of EGC Degradation

The past two decades has seen a rise in research on EGC degradation products in the context of CVD. Additional studies have explored relationships between and CVDs, mostly focusing on atherosclerotic disease. These studies attempt to correlate both qualitative and quantitative relationships between blood levels of these biomarkers with multiple CVD states. Table 1 presents seven such biomarkers and the CVDs for which a positive correlation has been described. Here, we discuss each of these molecular entities in detail.

Table 1. Promising Biomarkers of Endothelial Glycocalyx Dysfunction

Biomarker	Cardiovascular Disease State	Reference
Gamma fibrinogen	Myocardial Infarction, Stroke	Mannila.2007.JThrombHaemost.v5.p766; vandenHerik.2012.ThrombRes.v129.p807 Lovely. Thromb Haemost. 2002; 88:26; Uitte De Willige. Blood. 2005; 106:4176–4183; Uitte De Willige S. Blood. 2009; 114:3994

Growth differentiation factor-15	Heart Failure, Coronary Artery Disease, Atrial Fibrillation, Diabetes	Lind.2009.EurHeartJ.v30.p2346; Wallentin.2014.Circulation.130.1847; Rohatgi.2012.ClinChem.58.172.
Heparan sulfate[a]	Ischemia, Hemorrhagic Fever, Hypertension	Rehm.2007.Circulation.116.1896; Khedun.2002.ActaObstetGynecolScand.81.308; Tang.2017.SciRep.7.46191
Hyaluronan-1[a]	Stroke	Tang.2014.JNeuroinflammation.11.101; Kalay.2013.TohukoJExpMed.230.7
Plasminogen activator inhibitor-1[a]	Stroke	Kim.2005.JClinNeurol.1.142; Forood.214.AddictHealth.6.119; acute MI **Islam.**2014.IntJClinExpMed.7.1059
Pregnancy-associated plasma protein	Atheroma Plaque Instability	Wu.2016.Medicine (Baltimore).95.e2563; Lodh.2012.CardiovascJAfr.23.330; Zengin.2015.BiomarkMed.9.731; Cosin-Sales.2004.Circulation.109.1724; Mueller.2006.ClinChem.52.1096 Li.2017.CardiovascDiabetol.v16
Syndecan-1[a]	Heart Failure, Renal Failure, Ischemia/Reperfusion Injury, Acute Coronary Syndrome	Neves.2015.Circulation.79.1511; Miranda.2016.Atherosclerosis.247.184; Rehm.2007.Circulation.116.1896; Vlahu.2012.JASN.23.1900; Becker.2015.BrJClinPharmacol.80.389

[a] Shed components (detritus) from glycocalyx.

Gamma Fibrinogen

Fibrinogen is a plasma glycoprotein with an essential role in several biological functions, most notably wound healing, inflammation, angiogenesis, and hemostasis. While soluble in plasma, the conversion of fibrinogen to fibrin by the serine protease thrombin results in the formation of an insoluble clot, an essential component of wound healing. The fibrous material is subsequently degraded by a highly controlled fibrinolytic system [58].

Gamma (γ) fibrinogen is one of the 3 common fibrinogen chains found in humans. γ fibrinogen is dimeric, and several splice variants have been observed, resulting in a number of dimer variations. Among the splice variants, γ-prime (γ') fibrinogen is relatively common, comprising 8 to 15% of total fibrinogen [59], and has garnered significant research interest, in part due to its role in forming fibrin blood clots that are highly resistant to fibrinolysis.

Despite possessing antithrombotic properties [60,61], γ′ fibrinogen has been hypothesized to promote thrombosis [62,63], and elevated levels have been shown to be associated with CVD-related events, including myocardial infarction and stroke [64-68]. However, in at least one study, the correlation between γ′ fibrinogen and CHD, ischemic stroke, peripheral artery disease, heart failure, or CVD death has not been corroborated in older adults [69]. It has been suggested that the presence of elevated circulating γ′ fibrinogen in CVD may represent a non-specific marker of inflammation and be related to its antithrombotic role in combatting atherogenesis; however,

observed elevations are broadly associated with CVD deaths [70]. In fact, a recent review by Wang et al. found elevated fibrinogen levels to be indicative for myocardial infarction. Including g-fibrinogen in a hypothetical biomarker panel would be a valuable addition for detecting the patients impending risks of suffering a lethal myocardial infarction along with growth differentiation factor-15 (GDF-15).

Growth Differentiation Factor-15

GDF-15 is a cytokine known by many names, including macrophage-inhibitory cytokine-1, placental bone morphogenetic protein, placental transforming growth factor-beta, and nonsteroidal antiinflammatory drug-activated gene-1. GDF-15 is a cardioprotective enzyme upregulated by the presence of oxidative stress (reviewed by Adela et al. [71]), and its induction has been observed during cardiac ischemia-reperfusion injury, pressure overload, atherosclerosis, and heart failure [72-74]. Induction appears to involve NO-peroxynitrite-dependent signaling pathways [74]. Experiments in GDF-15-deficient mice show that these animals develop greater infarct sizes and exhibit more cardiomyocyte apoptosis than their wildtype litter mates following ischemia-reperfusion insult [74]. Further evidence supports GDF-15 as having a role in maintaining endothelial cell function under hyperglycemic conditions, which also induces its expression, leading to protection of endothelial cells from high glucose-induced apoptosis [75]. It has also been demonstrated that GDF-15 has a protective role against myocardial hypertrophy via a process involving GDF-15-mediated negative regulation of norepinephrine-induced myocardial hypertrophy by inhibiting epithelial growth factor receptor transactivation [76].

The association of GDF-15 with vascular pathologies renders it a prime candidate as a biomarker of CVD. Mazagova et al. have summarized supporting data for GDF-15 as an independent predictor of CVD, especially in the elderly [77]. Importantly, the association between GDF-15 levels and endothelial dysfunction is illustrated in a prospective study of over a thousand elderly participants [78]. In this study, participants were evaluated for CVD using ultrasound to assess carotid intima-media thickness, the presence of atherogenic plaques, and left ventricular geometry while venous occlusion plethysmography and ultrasound imaging were used to evaluate endothelial function in forearm resistance vessels and the brachial artery, respectively. GDF-15 and CRP were measured in serum. The authors found that elevated GDF-15 was related to several CV risk factors including male gender, current smoking status, elevated body mass index and waist circumference, diabetes, elevated fasting glucose and triglycerides, and low HDL. Following adjustment for CV risk factors, GDF-15 levels were found to be associated with reduced endothelium-dependent vasodilation, increased plaque burden, anomalies in left ventricular mass, hypertrophy, ejection fraction, and the clinical manifestation of CAD and heart failure.

In a large prospective study of patients with atrial fibrillation, Wallentin et al. [79] sought to evaluate the utility of GDF-15 as a biomarker when assessed both alone and in addition to

established clinical characteristics and other biomarkers in patients with atrial fibrillation. The investigators studied biomarkers from atrial fibrillation patients enrolled in a clinical trial that included biomarker data from 14,798 patients. Stroke, major bleeding episodes, and mortality were significantly higher in patients with elevated GDF-15 compared with lower GDF-15 levels, and GDF-15 was identified as an independent biomarker of clinical characteristics and risk scores. Adjustment of findings for other cardiac biomarkers reduced the prognostic value of GDF-15 for stroke, but its prognostic value for mortality and major bleeding remained.

In another large prospective study, data from participants in the Dallas Heart Study were analyzed for a potential relationship between GDF-15, atherosclerosis, and mortality. Plasma GDF-15 levels were measured in 3,219 participants with individuals grouped based on prespecified GDF-15 concentration categories. The authors found that elevated GDF-15 was associated with advanced age, being black, hypertension, diabetes, smoking, left ventricular (LV) mass/body surface area, and diminished renal function [80]. Overall, these preclinical and clinical studies support the utility of GDF-15, another enzyme implicated in endothelial response in vascular pathologies, as a strong biomarker candidate for a variety of CVD motifs.

Heparan Sulfate

Proteoglycans are found in a variety of forms. Heparan sulfate is a major constituent of the GAGs found as components of proteoglycans, comprising over 50% of GAGs in glycocalyx [81]. Those proteoglycans that are comprised of primarily chondroitin or dermatan sulfate residues are now considered to be atherogenic. However, proteoglycans that are comprised of primarily heparan sulfate residues have, until recently, been considered atheroprotective because they tend to inhibit both monocyte adhesion and the proliferation of vascular smooth muscle cells. Additionally, studies have indicated that heparan sulfate residues decrease both the retention of lipoproteins within the subendothelial matrix and endothelial permeability to LDLs [82]. More recently, studies in mice lacking the heparan sulfate proteoglycan perlecan show that these proteoglycans could also be atherogenic. In fact, circulating levels of heparan sulfate can be predictive of atherosclerotic progression due to the increase in expression and activity of heparanase as atheromas evolve from intermediate to advanced thin cap fibroatheromas [83]. Plasma heparan sulfate has also been used as evidence of endothelial glycocalyx degradation in studies of ischemia and reperfusion injury. Whether the ischemia/reperfusion injury is local or global, evidence indicates that glycocalyx endothelial is damaged; that evidence comes in the form of increased circulating heparan sulfate [84]. When plasma LDLs and VLDLs are chronically elevated, they start to impinge on the endothelial lining in areas of turbulent flow, which results in low and non-linear shear stress. When this stress is initiated, the lipids can become oxidized, and ROS will then activate enzymes such as heparinase. Activated heparinase begins to cleave heparan sulfate residues, which can be detected in the plasma. Any local pro-inflammatory state can result in the generation of cytokines, such as interleukin-beta and tumor necrosis factor

alpha, which then also activate metalloproteases. Therefore, what is found in plasma is heparan sulfates followed by syndecans as the atherogenic conditions prevail [44]. As mentioned, increased circulating levels of heparan sulfate is not just evidence that the glycocalyx is damaged; increased circulating levels also appears to provide a more atherogenic status to the endothelium by removing inhibition of leucocyte adhesion and vascular smooth muscle cell proliferation. This damage to the glycocalyx has shown sufficient pathological ramifications to warrant clinician concerns regarding current procedures used in treating a variety of serious conditions, such as the use of certain fluid resuscitation techniques for surgical procedures [44].

Hyaluronan Synthase-1

Hyaluronan synthases are integral membrane proteins that produce hyaluronan, an abundant polysaccharide component of the extracellular matrix essential for tissue homeostasis functions, such as acting as a lubricant in body fluids and promoting growth and healing. Certain splice variants of HAS1, though not lethal mutations, are associated with human diseases, such as multiple myeloma and bladder cancer [85]. As important as hyaluronan is to extracellular matrix function, it is understandably regulated by a number of growth factors and cytokines that act on hyaluronan synthase at the transcription level [85]. Hyaluronan is an abundant component of the endothelial glycocalyx; thus, finding it in circulation would not be very informative nor indicative of CVD progression. However, because hyaluronan synthase is an integral membrane protein, finding it in the circulation reflects advanced progression of atherosclerosis (preliminary unpublished data). Not until the endothelial glycocalyx is severely diminished in the location of a developing atherosclerotic plaque does endothelial dysfunction develop to a level of vascular permeability and impaired endothelial responsiveness that results in hyaluronan synthase shedding. This is relevant because the development of atherosclerosis is a precursor to most cardiovascular diseases, such as heart attack and stroke, and disruption of the endothelial glycocalyx coincides with the progression of atherosclerosis. Therefore, it would be useful to have biomarkers detectable in the plasma that give diagnosticians a reliable idea of where on the CVD spectrum a particular patient could be.

Plasminogen Activator Inhibitor-1

Circulating plasminogen activator inhibitor-1 (PAI-1) is a platelet-derived protein that binds tissue-type activator (tPA) irreversibly to prevent fibrin clot degradation. PAI-1 has been associated with type 2 diabetes, metabolic syndrome and CAD [86]. Along with the effect of PAI-1 on regulating fibrinolytic and thrombolytic activities, it has also been shown to play a role in the response to injury through the inhibition of cell migration and the degradation of the extracellular matrix [87]. However, in a number of animal models, the over-expression of PAI-1 contributed to the development of fibrosis. By extension, the studies performed by Corban et al. and the Framingham study appear to assign a role for PAI-1 contributing to hypertension due to its correlation with reduced vascular tone.

Elevated circulating levels of PAI-1 have recently been shown to reflect major adverse cardiovascular events in patients that have suffered death, myocardial infarction or a cerebrovascular accident [88]. The correlation was sufficient to explore the use of plasma PAI-1 as a biomarker for cardiovascular disease progression and the destabilization of atherosclerotic plaques. In another small study, Corban et al. found that PAI-1 positively correlated with early atherosclerosis and endothelial dysfunction. The authors studied PAI-1 in the coronary circulation and found that the positive correlation between increased PAI-1 and endothelial dysfunction was also associated with potential subclinical thrombosis and decreased fibrinolysis, which suggested the use of PAI-1 as a biomarker for early CAD. It is easy to see that the inappropriate formation of subclinical thrombus in the coronary circulation could lead to the upregulation of PAI-1 expression [87]. To date, the conclusion is that the use of PAI-1 as a biomarker or therapeutic target is supported; however, as is so often true in humans, larger cohorts are necessary for confirmation.

Pregnancy-associated Plasma Protein

Pregnancy-associated plasma protein-A (PAPP-A) is a zinc-binding MMP and glycoprotein belonging to the metzincins family of proteins that is expressed in fibroblasts, osteoblasts, ovarian granulosa cells, and vascular smooth muscle cells [89]. While initially identified in the plasma of women during pregnancy [90], increased PAPP-A expression is associated with conditions in which insulin-like growth factor activity is elevated. Evidence for this includes that observation of high PAPP-A expression in advanced, but not unstable, atherosclerotic plaques [91]. PAPP-A is therefore a regulatory protein in the insulin-like growth factor system and is important for cell proliferation and the regulation of fetal growth, and its proteolytic activity may have a role in the destabilization of atherosclerotic plaques [92].

Numerous recent studies provide data supporting the value of PAPP-A as a biomarker of CVD states. Zengin et al. found rising PAPP-A levels to be prognostic of cardiovascular mortality in CAD patients, an observation echoed by findings from Li et al. in diabetics [93]. Lohd et al. [94] found that both CRP and PAPP-A levels were predictive of atherosclerotic plaque instability in CAD patients, with the levels of these two biomarkers being highly correlative. In a study of 396 patients with chronic stable angina, the majority of whom had angiographically documented CAD, Cosin-Sales et al. (2004.Circulation.109.1724) evaluated the relationship of serum levels of PAPP-A and its endogenous inhibitor, the proform of eosinophil major basic protein (proMBP), with angiographic plaque complexity. After classifying the 531 identified coronary stenoses as smooth or complex, the authors assessed the predictive power of several parameters on the presence of unstable (i.e., complex) atheromatous plaques, including PAPP-A levels and the PAPP-A/proMBP ratio. They identified significantly higher PAPP-A/proMBP ratios and PAPP-A levels in patients with complex coronary stenoses compared with those without. Univariate analysis showed several parameters to correlate with the number of complex stenoses, including PAPP-A levels and PAPP-A/proMBP ratio, and a multiple regression analysis showed

that male gender, age, severe coronary artery disease, and PAPP-A/proMBP ratio independently predicted the number of complex stenoses.

Observations regarding the relationship between PAPP-A and plaque stability are not limited to CAD patients. In a study of 433 patients with atherosclerotic peripheral arterial disease (PAD) that included age- and sex-matched controls, Mueller et al. [95] investigated the relationship between serum PAPP-A concentrations and a diagnosis of atherosclerotic PAD. The authors found PAPP-A levels were significantly higher in the PAD population and added to the predictive value of other biomarkers, concluding that PAPP-A serum concentrations may be a biomarker for systemic atherosclerotic disease. Collectively these clinical studies suggest that PAPP-A is predictive of, and may have a pathophysiologic role in, formation of unstable atherosclerotic plaques that are a hallmark of EGC dysfunction.

Syndecan-1

A major component of the glycocalyx is the syndecans, primary heparan sulfate proteoglycans and abundant core proteins. As transmembrane proteins anchored to the cytoskeletal network within endothelial cells, syndecans provide structural integrity for the rest of the glycocalyx components. Syndecans have a role in tissue repair, metabolism, tumor formation, and immune response. They bind to and regulate heparin sulfate- and heparin-binding molecules [96,97] and can regulate ligand activity by affecting their stability, conformation, oligomerization, or compartmentalization [98]. Syndecans significantly contribute to the overall negative charge of the endothelial glycocalyx due to covalently bound glycosaminoglycans such as heparan and chondroitin sulfates [99]. While four syndecans have been identified, syndecan-1 has received significant interest among CVD researchers due to its links to multiple CVD states, including ischemia/reperfusion injury, ischemic heart failure, myocardial infarction, renal failure, and acute coronary syndrome [100,101].

Increased circulating levels of syndecans, and especially syndecan-1, have been associated with multiple cardiovascular diseases, such as atherosclerosis, heart failure, and hypertension. As with other biomarkers discussed here and elsewhere, elucidating the role of syndecan-1 in these diseases has been challenging, with some studies suggesting it facilitates cardiac fibrosis or atherogenesis, in part due to its significant correlation with angiotensin II activity, while other evidence shows a relationship between syndecan-1 and the regeneration and remodeling of myocardial tissue, in part due to a role in regulating inflammatory responses. Examples of syndecan-1's potential pathogenic roles in CVD include its high level of expression in early and developed aortic atheromas, where it is found in fatty streaks, fibrolipidic lesions, and intimal smooth muscle cells (Vo, N. Atherosclerosis, vol. 241, no. 1, p. e76, 2015), the independent association of syndecan-1 blood levels with EGC damage and mortality in patients with cardiogenic shock following infarction [102], and the observation that its presence exacerbates cardiac fibrosis in a mouse model of angiotensin II-induced cardiac fibrosis [103]. However, the

majority of studies point to syndecan-1 as being atheroprotective and that its elevated presence in blood is an indicator of inflammation and ultimately CVD as a consequence of it being shed during the disease process (REFS).

Conditions that lead to the production of atrial natriuretic peptide (ANP) have correlated with an increase in serum syndecan-1 levels [104]. The suggested mechanism by which these two observations could be related is by the receptor-mediated activation of metalloproteinases due to the parallel observations that syndecan-1 shedding can be mediated by both G-protein coupled and tyrosine kinase receptors and that shedding can be inhibited by the addition of tissue inhibitor of metalloproteinase type 3 [104]. Functionally, the ANP mediated shedding of syndecan-1 is associated with increased vessel permeability leading to tissue edema.

Clinical studies confirm an association between elevated syndecan-1 blood levels and several CVD states. Miranda et al. [105] found significantly higher levels of syndecan-1 in the patients with acute coronary syndrome compared with non-coronary chest pain patients and healthy controls; the authors found syndecan-1 levels higher than 148 ng/ml to be associated with an Acute Coronary Syndrome diagnosis (odds ratio = 14 [95% confidence interval: 1.8 to 102]). In a study of renal disease patients undergoing dialysis, Vlahu et al. [106] identified higher serum levels of HA and syndecan-1 in addition to increased hyaluronidase activity compared with healthy controls, and high levels of inflammation also correlated with greater EGC damage. In addition to their evaluation of heparan sulfate as noted above, Rehm et al. [84] evaluated the presence of serum syndecan-1 in patients undergoing cardiopulmonary bypass or aortic aneurysm intervention, finding basal levels, which at baseline where similar to healthy controls, to increase 42-fold in the first 15 minutes of reperfusion; this increase was especially pronounced in cardiopulmonary bypass (65-fold increase). In a study assessing acute kidney injury in heart failure patients admitted to an emergency department, Neves et al. [107] evaluated biomarkers of endothelial function including syndecan-1, CRP, byproducts of NO, and B-type natriuretic peptide. The authors found that syndecan-1 levels at admission were predictive of the development of acute kidney injury, especially severe injury, during the hospital stay even after adjusting for several confounding factors. Syndecan-1 levels were also associated with in-hospital and 6-month mortality rates.

Collectively, these studies provide compelling evidence for a role for syndecan-1 in CVDs, though none implicate syndecan-1 as a mediator of disease. Instead, these and other studies point to syndecan-1 as a casualty of inflammatory and other pathophysiologic processes in that it is one of the many “shed” components of the EGC that can predictably be found at elevated levels in those patients experiencing vascular disease.

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