Published in final edited form as:

Biorheology. 2019 ; 56(2-3): 131–149. doi:10.3233/BIR-180205.

Endothelial barrier reinforcement relies on flow-regulated glycocalyx, a potential therapeutic target

Ian C. Harding^a, Ronodeep Mitra^b, Solomon A. Mensah^a, Alina Nersesyan^a, Nandita N. Bal^b, Eno E. Ebong^{a,b,c,*}

^aDepartment of Bioengineering, Northeastern University, Boston, MA, USA

^bDepartment of Chemical Engineering, Northeastern University, Boston, MA, USA

^cDepartment of Neuroscience, Albert Einstein College of Medicine, New York, NY, USA

Abstract

BACKGROUND: The onset of many disease processes depends on the function of the endothelial cell (EC) glycocalyx (GCX) which acts as a flow-dependent barrier to cellular infiltration and molecular transport across the blood vessel wall.

OBJECTIVE: This review aims to examine these processes with the potential end goal of implementing GCX repair to restore EC barrier function and slow the progression of disease.

METHODS: Cell and mouse studies were employed to examine the state of EC GCX in healthy versus disruptive flow conditions. Correlates of observations of the GCX with a number of EC functions were sought with an emphasis on studies of trans-endothelial barrier integrity against vessel wall infiltration of cells and molecules from the circulation. To demonstrate the importance of GCX as a regulator of trans-endothelial infiltration, assays were performed using ECs with an intact GCX and compared to assays of ECs with an experimentally degraded GCX. Studies were also conducted of ECs in which a degraded GCX was repaired.

RESULTS: In healthy flow conditions, the EC GCX was found to be thick and substantially covered the endothelial surface. GCX expression dropped significantly in complex flow conditions and coincided with a disease-like cellular and molecular accumulation in the endothelium or within the blood vessel wall. Therapeutic repair of the GCX abolished this accumulation.

CONCLUSIONS: Regenerating the degraded GCX reverses EC barrier dysfunction and may attenuate the progression of vascular disease.

Keywords

Endothelial glycocalyx; fluid shear stress; endothelial dysfunction; atherosclerosis; vascular inflammation; cancer metastasis

*Corresponding author: Eno E. Ebong, Ph.D., Department of Chemical Engineering, Northeastern University, 360 Huntington Avenue, 313 Snell Engineering Building, Boston, MA, 02115, USA. Tel.: +1 617-373-8744; Fax: +1 617-373-2209; e.ebong@northeastern.edu.

Disclosure statement

A provisional patent application entitled "GlycoFix (Structurally and Functionally Repaired Endothelial Glycocalyx)" (No. 62/534,660) has been filed. The authors have no other conflicts of interest to declare.

1. Background and motivation of the presented research

At Jagiellonian University, Kraków, Poland on July 2–6, 2018, the European Society for Clinical Hemorheology and Microcirculation, the International Society for Clinical Hemorheology, and the International Society of Biorheology held a joint meeting of the 3 societies. This joint meeting brought together scientists and clinicians from diverse areas of bio- and hemo-rheology research, to share their latest discoveries and engage in interactive discussions. This review paper is a summary of work that was shared by the Ebong research group in a session entitled “The Glycocalyx – Its Role in Disease.”

The Ebong laboratory studies mechanisms of mechanical regulation of endothelial cells (ECs), which normally line and protect blood vessels in order to maintain vascular health. These ECs are coated with molecules that together form a complex sugar structure, called glycocalyx (GCX). The GCX is a fibrous mesh that has been mainly attributed to the direct detection of blood flow-derived forces and transduction of these forces into EC responses to trigger events that are protective for the blood vessels in healthy conditions. Disruptions in blood flow contribute to vascular-associated disease conditions, including atherosclerosis and potentially cancer metastasis, which coincide with dysfunctional ECs [1]. The Ebong laboratory is focused on revealing that these disease conditions also coincide with and are in large part caused by degraded EC GCX.

Atherosclerosis is initiated by transition of ECs from an anti-inflammatory to a pro-inflammatory state [2,3]. Pro-inflammatory ECs exhibit impairment in their vasoregulatory functions [4,5], increased expression of cell adhesion receptors [6,7], and increased production of reactive oxygen species [8,9]. A major next step in the initiation and formation of atherosclerosis, after EC-dependent lipid trapping, is EC recruitment of circulating inflammatory cells that become retained in the blood vessel wall [10,11]. EC-dependent monocyte recruitment to affected vascular areas occurs in an attempt to reduce inflammation and, subsequently, reverse the disease process. However, in atherosclerosis, a chronic inflammatory state occurs whereby there is excessive blood vessel wall infiltration by inflammatory monocytes that become macrophages and promote lipid-filled, necrotic plaque formation. Previous studies have suggested that GCX degradation in vascular regions prone to atherosclerosis may lead to increased monocyte infiltration at the onset of plaque development [12–17].

Cancer disease mechanisms, particularly the formation of secondary tumors via metastasis, strongly resemble atherosclerosis disease mechanisms. Specifically, both diseases exhibit changes in expression of cell adhesion receptors, the influx of cells, and chronic inflammation, among other similarities [18]. This review draws attention to the metastatic stage of cancer, which is responsible for approximately 90% of cancer-related deaths [19–21]. During metastasis, primary tumor cells move from the parent tumor to neighboring or distant tissues to form secondary tumors [22]. This occurs when cells from the primary tumor migrate to and intravasate nearby blood vessels, travel through these vessels, and eventually extravasate the vessels at distant tissues where secondary tumors can form [23]. Preceding secondary tumor formation, the cancer cells must initially home to and cross the adhesive endothelium, in order to colonize and grow a secondary tumor. This trans-

endothelial infiltration to form secondary tumors could occur due to dysfunction of the endothelium's GCX coating [24,25].

The goal is to understand GCX-mediated EC mechanisms of these two diseases conditions, atherosclerosis and cancer metastasis, so that GCX-targeted treatments can be developed in a manner that heals ECs and effectively slows disease progression.

2. Introduction to the endothelial glycocalyx: Structure and function

The endothelial GCX is a mesh-like proteoglycan and glycoprotein layer on the luminal membrane of ECs [26,27] (Fig. 1). Structurally, the proteoglycans are the major component of the GCX. These proteoglycans consist of glycosaminoglycan (GAG) chains attached to the cell membrane via core proteins or receptors. GAGs of the EC GCX include heparan sulfate (HS), hyaluronic acid (HA), and chondroitin sulfate (CS) (Fig. 1). Of these, HS is the most abundant, constituting 50–90% of all GAGs [26]. HA is also a large constituent [28,29]. The core protein groups that many of these GAGs are attached to include syndecans, glypicans, perlecans, and versicans, among others [26,27]. However, syndecans and glypicans are the most prominent on ECs (Fig. 1). Furthermore, while syndecans and glypicans are attached to the endothelial membrane, perlecans and versicans are secreted and therefore likely do not provide structure to the surface GCX [30]. Additionally, various receptors act in lieu of core proteins when it comes to tethering HA to the membrane [26,27] (Fig. 1). Cluster of differentiation 44 (CD44), for example, is an important HA bound receptor [26,27] (Fig. 1). The other major structural components of the GCX include sialic acid (SA) moieties, glycoproteins, and soluble molecules [26,27] (Fig. 1). Glycoproteins of the GCX include the cell adhesion molecule groups selectins, integrins, and immunoglobulins [31,32]. Serum albumin is an example of a common soluble molecule. Serum albumin is adsorbed within the GCX mesh pores (which have been estimated at 7 nm or less in size [33]) and is believed to stabilize the GCX structure, sometimes even in the presence of GCX-degrading stimuli [34].

The GCX is an inherently difficult structure to study because it is extremely susceptible to degradation by a variety of stimuli including dehydration [35], chemical reagents [35], inflammatory stimuli [36,37], and ischemia/reperfusion [16,38]. As such, its dimensions are largely debated. Initially, the GCX was estimated to be on the order of nanometers [39–41]. More recent reports have indicated that the GCX layer can extend to several microns in thickness [35]. In actuality, considering that morphological and functional differences exist between ECs of different vascular origin (with respect to species, vascular bed, etc.) [35,41], the same must be true when comparing the thicknesses of GCX from different origin.

GCX attachment to the cell membrane, its composition, organization, and its dimensions, as described above, play a role in determining its function. HS, HA, and SA have been the focus of many reports for their specific roles in dictating EC GCX function [28,29,42–44]. Glypican and syndecan core proteins have also been reported to play significant roles in several GCX functions, consistent with their direct links to and, sometimes, across the EC membrane, [26,27,45]. The role of CS is understudied. These components of the endothelial

GCX work together, when intact, to promote endothelial and vascular health and, consequently, may prevent dysfunction and disease.

Under healthy conditions in which the GCX is abundantly expressed, the GCX functions by sensing and transducing blood flow-derived shear stresses into EC biochemical activities. This is known as EC mechanotransduction [46] and has been of great research interest. GCX-mediated mechanotransduction influences key endothelium functions. The GCX has been shown to regulate EC signaling pathways related to angiogenesis [47,48], vasoregulation [45,49], and regulation of inflammation [14,50]. One of the most well studied GCX-mediated mechanotransduction events is GCX-mediated flow-regulation of endothelial production of nitric oxide, which serves as a potent vasodilator while also promoting an anti-inflammatory endothelial state [28,45,49,51].

In addition to GCX's function as an endothelial mechanotransducer, it also plays a vital function in the protection of ECs from interactions with molecules and cells that reside in the blood circulation. To adhere to and permeate the endothelium, circulating cells, such as leukocytes and neutrophils, require adhesion molecules including E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and a variety of other glycoprotein cell adhesion molecules that are contained within the GCX [15–17,52–54]. In cell culture and animal studies, decreased expression of the proteoglycan components of the GCX has been shown to facilitate increased leukocyte adhesion to the endothelium [15,16,52,55], a phenomenon that is thought to extend to other circulating cell types and molecules. An adhesive and permeable endothelial state is the underlying cause of a wide array of medical conditions. These conditions include atherosclerotic plaque formation [56], coagulation and thrombosis [57,58], and extravasation of metastatic cancer cells [54,59,60].

In this review, recent findings regarding GCX response to flow-derived shear stress, as indicated by flow-dependent differences in GCX structure on the EC surface, are discussed. Specifically, it will be established that disturbed flow (DF) patterns, compared to uniform flow (UF) patterns, can induce GCX degradation. Subsequently, it will be demonstrated that GCX, when it is disabled due to flow-induced degradation, adversely impacts EC function. EC mechanotransduction function is of great interest, but was the subject of a recent review paper [61] and numerous other review articles. Therefore, herein the focus will be on the implications of flow-induced GCX degradation on exposure of the endothelium and vascular wall to adhesion and accumulation of circulating cells and molecules. Data will be presented to show that GCX degradation coincides with blood vessel wall infiltration by inflammatory cells in regions of atherosclerosis development. It will also be shown that GCX degradation promotes circulating cancer cell adhesion to the endothelium, which is a first step in secondary tumor formation. Lastly, the possibility of developing new drugs that target the GCX to limit trans-endothelial permeability and potentially treat disease will be discussed.

3. Review of *in vitro* and *in vivo* observations of flow-induced GCX remodeling

Previous studies showed thinning of the GCX overlying atherosclerotic plaques when compared to the GCX overlying non-plaque filled blood vessel walls [14]. Therefore, one

might assume that GCX thinning occurs as a consequence of the atherosclerotic process. However, in the present work it was considered that GCX thinning could occur prior to the onset of atherosclerosis. Interestingly, plaques consistently occur in predictable vascular regions, such as branches, constrictions, and curvatures [62–64]. Rheological studies of atheroprone vascular regions described the presence of unique blood flow patterns when compared to regions of infrequent atherosclerosis development [62,63]. Specifically, the 3-dimensional pattern of blood flow within atherosclerosis-prone areas is complex, characterized by low time-averaged shear stress magnitudes, high shear stress gradients, and occasional flow reversal, altogether referred to as DF [62,63]. In contrast, blood flow within atherosclerosis-resistant areas of the vasculature exhibits high time-averaged shear stress magnitudes, low shear stress gradients, and unidirectional flow, altogether referred to as UF [62,63]. A number of *in vitro* and *in vivo* studies performed by the research groups of Rubanyi [65], Gimbrone and Garcia-Cardena [66,67], Jo [68–72] and others have shown that DF patterns, in comparison to UF patterns, significantly induce atherosclerosis-associated endothelial inflammation. From these studies, clear indicators of DF-induced EC inflammation include, but are not limited to, impaired vasoregulatory functions, elevation of cytokines and chemokines and their receptors, induced expression of cell adhesion receptors, stimulation of morphogenesis and proliferation, increased expression and activation of inflammatory transcription factors, and production of reactive oxygen species via the reactive oxygen species synthesizing subunit p47^{phox} [4–9,65–72]. A GCX that is degraded by DF could be the open door to these pro-inflammatory events and, subsequently, to atherosclerotic plaque formation.

In the present work, it was hypothesized that GCX expression could be regulated by disturbed, atherosclerosis-prone flow patterns, resulting in a decrease in GCX expression. To test this hypothesis, both *in vitro* and *in vivo* methods were utilized to study GCX structure in relation to DF versus UF conditions [73,74]. This hypothesis was investigated using a custom, parallel-plate, *in vitro* flow chamber in which GCX-expressing ECs were exposed to flow patterns characteristic of the carotid artery bifurcation, one of the most common areas of atherosclerosis development [62,63,75]. In another experiment, this hypothesis was tested *in vivo* by performing surgical reconstruction of mouse carotid arteries to induce an acute disturbance in blood flow. This acute DF animal model, developed by the groups of Berk [76] and Jo [71], is well established. In a third experiment, the effects of chronic *in vivo* DF on EC GCX were assessed in mouse aortas along the inner curvature of the aortic arch and descending aorta, which are exposed to low shear stress magnitudes and DF that can predispose the vessel to atherosclerosis [77,78]. In the *in vitro* and *in vivo* experiments, EC GCX expression was also examined in UF conditions for reference. EC GCX expression was also examined in static (no-flow) control conditions for the *in vitro* experiments.

The cell culture experimental results confirmed expectations. Based on a measure of relative GCX fluorescence intensity, an increase in EC GCX coverage and thickness was observed after exposure to less than a day of UF when compared to static controls [73]. Specifically, GCX was labeled with wheat germ agglutinin (WGA) lectin, which broadly labels the GCX because it targets more than one GCX constituent, including SA and N-acetylglucosamine, a component of HS and HA. WGA-labeling of the GCX revealed that short term (less than a day) UF stimulated ECs express increased GCX coverage and thickness by 16% and 14%,

respectively, when compared to static controls (Fig. 2A, 2C, 2G, 2H) [73]. The effects of UF on the GCX were further investigated by labeling the HS component exclusively, as it has previously been implicated in GCX mechanotransduction and vascular health. These experiments revealed a 48% and 10.8% UF-induced increase in HS coverage and thickness, respectively (Fig. 2D, 2F, 2I, 2J) [73]. Collectively, these WGA and HS results confirmed that UF patterns can increase GCX expression from baseline levels in static conditions [73]. Further, the results suggest individual GCX components may be differentially regulated by UF [73]. These results are in agreement with previous one-, three-, and seven- day UF studies conducted by Gouverneur et al. [79] and Koo et al. [80], which similarly found elevation of GCX expression in a GCX component-specific manner in UF conditions compared to static conditions [79,80]. More interesting results were obtained in studies of GCX coverage and thickness in response to DF, which is relevant to atherosclerosis. Compared to static controls, it was found that exposure of ECs to DF led to a 44% decrease in overall WGA-labeled GCX coverage and 14% decrease in WGA-labeled GCX thickness (Fig. 2B, 2G, 2H) [73]. Similarly, DF induced an 18% and 10.9% decrease in HS coverage and thickness, respectively (Fig. 2E, 2I, 2J) [73]. While these results are similar to observations reported by Koo et al. [80], who found three and seven days of DF to induce decreased expression of various GCX components [80], the present study is the first study to examine the effects of DF on GCX expression using a geometrically relevant flow model with juxtaposed DF and UF profiles.

The cell culture results were validated by two different types of mouse studies. The first study involved a study of two cohorts of C57BL/6-background apolipoprotein E knockout (ApoE KO) mice on a chow diet, in which the left carotid arteries of the first cohort were normal and the second were partially ligated to induce an acute disturbance in blood flow [74]. Typically, flow within the common carotid arteries is uniform. However, ligating 3 of the 4 caudal branches of the left carotid artery induces DF patterns characterized by low time-averaged shear stresses within the left common carotid artery [71,74]. Mitra et al. fluorescently labeled the GCX and identified a significant change in GCX expression on ligated left carotid arteries vs. non-ligated left carotid arteries (Fig. 3A–3C) [74]. Specifically, the chow-fed ApoE KO mice with ligated left carotid arteries, when compared to chow-fed ApoE KO mice with non-ligated left carotid arteries, exhibited approximately 50% less WGA-labelled GCX within one week after left carotid artery ligation (Fig. 3C) [74]. This work provides strong evidence that disturbed flow alone is sufficient to induce GCX degradation even in the absence of high fat diet conditions, which is a well-known disease risk factor and typically incorporated into mouse studies of vascular disease. In another experiment in which the GCX was fluorescently labeled with HA binding protein, performed by Tarbell and Cancel, the superimposition of high fat in the diet led to complete removal of GCX from the wall of the ligated left carotid artery [81].

The second mouse study involved *en face* imaging of various endothelium regions of the aorta in C57BL/6 mice [73]. Due to the geometry of the aorta, ECs along the aortic vessel wall are chronically exposed to UF patterns adjacent to DF patterns [77,78]. For example, the high curvature of the aortic arch leads to DF patterns within the inner curvature of the distal aortic arch and proximal descending aorta. Furthermore, this region is also susceptible to the formation of atherosclerotic plaques [82,83]. In contrast, the straight geometry of the

abdominal aorta, which is resistant to atherosclerosis development, contains chronic UF patterns [84]. Fluorescent WGA labeling of the GCX within both the distal aortic arch and abdominal aorta corroborated previous *in vitro* findings: GCX expression was weaker within the distal aortic arch (Fig. 4A), which is exposed to chronic DF, than it was in the abdominal aorta (Fig. 4B), which is exposed to chronic UF [73]. While it is possible that variability in GCX expression along the aorta (and non-ligated vs. ligated carotid artery) is due to differences in pressure or hoop stresses, there has been no prior demonstration of a causal relationship between GCX expression and blood pressure or hoop stress. Considering the numerous studies that have presented evidence that shear stress regulates GCX expression [73,79,85], it is clear that the observed *in vivo* differences in GCX expression can be attributed in large part to changes in shear stresses (both acute and chronic).

The collective *in vitro* and *in vivo* results summarized here [73,74] show that the endothelial GCX is degraded through exposure to atherosclerosis-prone blood flow. GCX degradation, therefore, precedes atherosclerosis and is not a consequence of atherosclerosis. The pronounced differences in GCX structure in UF conditions compared to DF conditions highlight the implications of pro- versus anti-atherosclerotic flow patterns on GCX remodeling, which should translate to implications for EC and vascular function. GCX degradation was previously implicated in inhibited flow-induced nitric oxide production [45,61,86], impaired inter-endothelial communication [61,86], uncontrolled reactive oxygen species expression [51], and up-regulated adhesion molecule expression [87], which are all associated with pro-atherosclerotic endothelial dysfunction. Further research will hopefully elucidate the relationship between GCX structure, its function, and endothelial phenotype.

4. Implications of GCX degradation on circulating cell adhesion to the endothelium

GCX degradation may be directly responsible for circulating cell adhesion to the endothelium. Degraded GCX expression could lead to a reduced physical adhesion barrier between ECs and circulating cells, as presented by Mitchell and King [88]. To examine this notion, the aforementioned experimental mouse model with ligated left carotid arteries was revisited. Left carotid arteries that were GCX-deficient due to DF conditions produced by artery ligation were compared to left carotid arteries that were GCX-rich due to the typical UF conditions present in the non-ligated artery (Fig. 3A–3C) [71,74]. Most related studies have been conducted using leukocytes, such as neutrophils and macrophages [15,16,52,55]. Consistent with this, immunohistochemical analysis of cluster of differentiation 68 (CD68), a macrophage marker, was performed. It was found that decreased GCX expression in ligated left carotid arteries coincides with increased uptake of macrophages (Fig. 3D–3F) derived from monocytes of the circulation [74]. Specifically, macrophage uptake increased 20-fold within GCX-deficient, DF-conditioned, ligated left carotid arteries compared to GCX-rich, UF-conditioned, non-ligated left carotid arteries [74]. Previous studies, although limited, are in agreement with these findings. For instance, Schmidt and colleagues found increased inflammatory neutrophil adhesion in areas of pulmonary sepsis-associated GCX degradation [17]. Similarly, in ischemia/reperfusion patients, Chappell and coworkers

observed that inflammatory leukocyte adhesion correlated to shedding of HS and syndecan along with GCX thinning [16].

Additional experimentation was required to fully investigate the notion that GCX degradation is a direct cause for circulating cell adhesion to the endothelium and, further, to determine the specific GCX components responsible. Schmidt's group laid the foundation for demonstrating the causal relationship by blocking GCX degradation and subsequent cell adhesion through inhibition of HS-specific heparinase III (Hep III) enzyme [17]. Contrarily, Ebong's group explored the causal relationship by inducing EC GCX degradation. This was achieved through treatment of ECs with SA-specific *Clostridium perfringens* neuraminidase (Neur) enzyme [29,89], which degrades a broad variety of SA residues of the GCX. Subsequently, the GCX-deficient ECs were co-cultured with metastatic cancer cells as a model circulating cell (Mensah et al., manuscript in preparation). As previously mentioned, metastatic cancer cells are derived by cancer cell migration from primary tumor sites into the blood circulation. Preceding secondary tumor formation, the circulating cancer cells must adhere to the endothelium in order to begin their migration into the secondary tumor site. This process involves a range of endothelial and cancer receptors and ligands including selectins, cadherins, integrins, CD44, and immunoglobulin superfamily receptors [54,90]. Few studies have investigated the role of the EC GCX in circulating cancer cell adhesion to the endothelium [59,60].

Recent data revealed that after 4-hour exposure to GCX-degrading DF conditions, ECs interacting with circulating cancer cells for 1 hour resulted in an approximate 60% increase in cancer cell adhesion (Fig. 5B, 5J, 5M–5O) when compared to ECs pre-exposed to 4 hours of GCX-rich UF conditions (Fig. 5C, 5K, 5M–5O) obtained by Ebong's group (Mensah et al., manuscript in preparation). To confirm GCX degradation as a direct cause of increased adhesion, the Neur enzyme was added to the EC environment in UF conditions. After Neur enzyme treatment, the Neur-containing UF perfusate was exchanged with media containing circulating cancer cells. It was found that Neur-induced degradation of the SA component of the EC GCX in UF conditions does indeed substantially increase circulating cancer cell attachment to the endothelium (Fig. 5D, 5L, 5O) (Mensah et al., manuscript in preparation). This confirms prior speculation that SA, in comparison to other GCX components, such as HS and HA, is an important mediator of cancer cell adhesion and potentially cancer cell biology and signaling [91,92]. Further, taking Ebong's experiment together with Schmidt's [17] shows that intact GCX plays an important role in blocking adhesion of any circulating cell to the endothelium and degradation of the HS and SA components of the GCX promotes adhesion to the endothelium.

It is plausible that the increase in GCX penetration by circulating cells can be attributed to an increased void ratio created by the eliminated HS and SA content. This increased void ratio will enable passive and non-receptor mediated interactions with ECs. It is also likely that there is GCX collapse [93], due to reduced electrostatic repulsive force that occurs with loss of negatively charged GCX. This GCX collapse will expose to circulating cells ECs adhesion ligands, such as e-selectin, for active and receptor mediated interactions. In addition to reducing the physical adhesion barrier between ECs and circulating cells [88], the degradation of GCX components likely impairs mechanotransduction, resulting in up-

regulated expression of adhesion molecules [87], which further facilitates increased circulating cell adhesion to the endothelium and increased retention in the blood vessel wall.

5. Development of GCX-based therapeutics to prevent vascular-related diseases

This review has focused on EC GCX structure and function as a barrier to trans-endothelial infiltration, which breaks down in atherosclerosis and cancer. EC GCX degradation has also been implicated in the development of other vascular-related diseases and conditions such as diabetes [94], sepsis [17], and chronic kidney disease [95]. It is therefore of interest to investigate whether EC GCX restoration can improve vascular function.

Ebong's research group is contributing to this endeavor by developing an approach to repair the EC GCX layer. Demonstration of the efficacy of using an EC GCX regeneration approach to block trans-endothelial circulating cell infiltration of the blood vessel wall is currently in progress. To date, proof of concept experiments have been performed in cell culture settings, demonstrating the feasibility of repairing degraded EC GCX and subsequently restoring nanoscale trans-endothelial barrier function [96] and inter-endothelial communication [86].

In an initial experiment, ECs with artificially regenerated GCX were exposed to nanoparticles [96], laying the foundation for experiments in which ECs would be exposed to circulating inflammatory and/or cancer cells in the future. Given that the GCX mesh pores are estimated at less than or equal to 7 nm in size [33], infiltration of the nanoparticles, which are much smaller than circulating inflammatory or cancer cells, can reveal GCX degradation as it begins to occur. Exclusion of the same nanoparticles, in contrast, can reveal complete restoration of the trans-endothelial barrier. A proof of concept experiment utilized 10-nm-sized, red fluorescent, polymer-coated gold nanoparticles [96]. To observe passive infiltration regulated exclusively by the GCX and not by non-GCX cell surface structures and adhesion molecules, the nanoparticles were not conjugated to any ligand, which would typically target endothelial surface adhesion molecules and encourage active nanoparticle internalization by the ECs [97]. First the effect of intact GCX on nanoparticle infiltration into the endothelium was investigated, and minimal infiltration was observed (Fig. 6) [96]. GCX degradation, achieved by treating ECs with Hep III, led to a more than 6-fold increase in nanoparticle infiltration into ECs (Fig. 6) [96]. This trend is similar to observations on the effect of GCX degradation on macrophage and cancer cell attachment [17,25,33,98–103]. To determine whether GCX restoration can reduce endothelium permeability to nanoparticles, endogenous HS was used to restore GCX expression on Hep III-treated ECs (Fig. 6) [96]. After restoration, nanoparticle infiltration of ECs was observed to rebound from infiltration levels of degraded samples and return to levels of nanoparticle infiltration in untreated controls (Fig. 6) [96].

Prior studies have also successfully prevented GCX degradation or restored GCX expression to maintain endothelial function. In these studies, GCX-regenerating therapeutics included TNF- α inhibitors [58], angiopoietin-1 [104], sulodexide [94], sevoflurane [16], hydrocortisone [52], and heparin [105]. One prior study conducted by Fu's group found that

protection of the endothelial GCX through sphingosine- 1-phosphate (S1P) treatment or matrix metalloproteinase (MMP) inhibition resulted in regenerated GCX expression and reduced cancer cell adhesion [60]. This led Ebong and colleagues to incorporate S1P into the GCX regeneration strategy. The treatment of ECs with combined endogenous HS and S1P has been demonstrated, to date, to be effective in regenerating GCX and in restoring a complex EC function: cell-to-cell communication [86]. Preclinical animal studies are currently being performed to determine the potential of *in vivo* GCX regeneration to restore endothelial function. It is anticipated that the animal studies will show that GCX regeneration is able to restore the trans-endothelial infiltration barrier and a variety of other endothelial functions, ultimately promoting vascular health.

6. Conclusion

The endothelial GCX is a complex, protein-polysaccharide layer that assists in various endothelial functions including regulation of the barrier at the interface between the blood circulation and the tissue of the blood vessel wall. This review summarizes recent studies performed by Ebong's group, which confirmed that EC GCX is thick and substantially covers the endothelial surface in healthy flow conditions. Ebong and colleagues have also shown that atherosclerosis relevant flow patterns can significantly impair GCX expression [73]. This decrease in GCX expression is correlated with an increase in macrophage accumulation within the blood vessel wall, a key step in the development of atherosclerosis plaques [74]. Additionally, GCX loss is linked to other vascular-related processes including recruitment of metastatic cancer cells to the endothelium (Mensah et al., manuscript in preparation). Studies of the effect of loss of function of certain GCX components via treatment with Neur and Hep III demonstrated that disease-like molecular and cellular accumulation in the endothelium or within the blood vessel wall can be attributed to loss of SA and HS [86,96]. Lastly, therapeutic strategies targeting the GCX have demonstrated an ability to restore vascular endothelial function in the presence of detrimental stimuli [86,96]. Future studies on GCX structure and function will further develop and substantiate the benefits of GCX-targeted therapeutics.

Acknowledgements:

Sources of funding

This work was funded by (i) the National Institutes of Health (K01 HL125499 awarded to E. Ebong), (ii) the American Heart Association (18PRE33960461 awarded to I. Harding), (iii) the National Science Foundation (DGE-1451070 awarded to S. Mensah), and (iv) Northeastern University (startup funds and Tier 1 Provost Grant awarded to E. Ebong; Engineering Dean's Fellowship awarded to I. Harding). The funders had no role in data or information collection and analysis, decision to publish, or preparation of the manuscript.

Other acknowledgements

The Northeastern University Institutional Animal Care and Use Committee (NU-IACUC) approved the animal studies reported here, under protocol number 17-0824R. Other support was provided by Dr. Ming Cheng (one of the co-authors of the provisional patent application) and the Northeastern University Departments of Chemical Engineering and Bioengineering.

Abbreviations

4T1	Stage IV Metastatic Breast Cancer Cells
CD44	Cluster of Differentiation 44
CD68	Cluster of Differentiation 68 (a macrophage marker)
DF	Disturbed Flow
ECs	Endothelial Cells
GCX	Glycocalyx
GAGs	Glycosaminoglycan
Hep III	Heparinase III (heparan sulfate degradation enzyme)
HS	Heparan Sulfate
MMP	Matrix Metalloproteinase
Neur	Neuraminidase (sialic acid degradation enzyme)
S1P	Sphingosine-1-Phosphate
SA	Sialic Acid
UF	Uniform Flow
WGA	Wheat Germ Agglutinin (labels sialic acid and N-acetylglucosamine)

References

- [1]. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation*. 2004;109(23 Suppl 1):III27–32. [PubMed: 15198963]
- [2]. Bonetti PO, Lerman LO, Lerman A. Endothelial dysfunction - A marker of atherosclerotic risk. *Arteriosclerosis Thrombosis and Vascular Biology*. 2003;23(2):168–75.
- [3]. Shimokawa H Primary endothelial dysfunction: Atherosclerosis. *Journal of Molecular and Cellular Cardiology*. 1999;31(1):23–37. [PubMed: 10072713]
- [4]. Kawashima S, Yokoyama M. Dysfunction of endothelial nitric oxide synthase and atherosclerosis. *Arteriosclerosis Thrombosis and Vascular Biology*. 2004;24(6):998–1005.
- [5]. Oemar BS, Tschudi MR, Godoy N, Brovkovich V, Malinski T, Luscher TF. Reduced endothelial nitric oxide synthase expression and production in human atherosclerosis. *Circulation*. 1998;97(25):2494–8. [PubMed: 9657467]
- [6]. O'Brien KD, Allen MD, McDonald TO, Chait A, Harlan JM, Fishbein D et al. Vascular cell-adhesion molecule-1 is expressed in human coronary atherosclerotic plaques - implications for the mode of progression of advanced coronary atherosclerosis. *Journal of Clinical Investigation*. 1993;92(2):945–51. [PubMed: 7688768]
- [7]. Cybulsky MI, Gimbrone MA. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science*. 1991;251(4995):788–91. [PubMed: 1990440]
- [8]. Dimmeler S, Zeiher AM. Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. *Regul Pept*. 2000;90(1–3):19–25. [PubMed: 10828488]

- [9]. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res*. 2000;87(10):840–4. [PubMed: 11073878]
- [10]. Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, Nasri H. Atherosclerosis: process, indicators, risk factors and new hopes. *International Journal of Preventive Medicine*. 2014;5(8): 927–46. [PubMed: 25489440]
- [11]. Libby P, Ridker PM, Hansson GK. Leducq Transatlantic Network on A. Inflammation in atherosclerosis: from pathophysiology to practice. *J Am Coll Cardiol*. 2009;54(23):2129–38. [PubMed: 19942084]
- [12]. Grundmann S, Schirmer SH, Hekking LHP, Post JA, Ionita MG, de Groot D et al. Endothelial glycocalyx dimensions are reduced in growing collateral arteries and modulate leucocyte adhesion in arteriogenesis. *Journal of Cellular and Molecular Medicine*. 2009;13(9b):3463–74. [PubMed: 19438808]
- [13]. Pahwa R, Nallasamy P, Jialal I. Toll-like receptors 2 and 4 mediate hyperglycemia induced macrovascular aortic endothelial cell inflammation and perturbation of the endothelial glycocalyx. *Journal of Diabetes and its Complications*. 2016;30(4):563–72. [PubMed: 26908090]
- [14]. Cancel LM, Ebong EE, Mensah S, Hirschberg C, Tarbell JM. Endothelial glycocalyx, apoptosis and inflammation in an atherosclerotic mouse model. *Atherosclerosis*. 2016;252:136–46. [PubMed: 27529818]
- [15]. Constantinescu AA, Vink H, Spaan JA. Endothelial cell glycocalyx modulates immobilization of leukocytes at the endothelial surface. *Arterioscler Thromb Vasc Biol*. 2003;23(9):1541–7. [PubMed: 12855481]
- [16]. Chappell D, Heindl B, Jacob M, Annecke T, Chen C, Rehm M et al. Sevoflurane reduces leukocyte and platelet adhesion after ischemia-reperfusion by protecting the endothelial glycocalyx. *Anesthesiology*. 2011;115(3):483–91. [PubMed: 21785339]
- [17]. Schmidt EP, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L et al. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med*. 2012;18(8):1217–23. [PubMed: 22820644]
- [18]. Tapia-Vieyra JV, Delgado-Coello B, Mas-Oliva J. Atherosclerosis and cancer; a resemblance with far-reaching implications. *Archives of Medical Research*. 2017;48(1):12–26. [PubMed: 28577865]
- [19]. Tian Q, Wang Y, Guo H, Xie G, Li J, Zhang M et al. Recent perspectives of management of breast cancer metastasis - an update. *J Buon*. 2017;22(2):295–300. [PubMed: 28534347]
- [20]. Sharma R, Sharma R, Khaket TP, Dutta C, Chakraborty B, Mukherjee TK. Breast cancer metastasis: Putative therapeutic role of vascular cell adhesion molecule-1. *Cell Oncol (Dordr)*. 2017;40(3):199–208. [PubMed: 28534212]
- [21]. Seyfried TN, Huysentruyt LC. On the origin of cancer metastasis. *Crit Rev Oncog*. 2013;18(1–2): 43–73. [PubMed: 23237552]
- [22]. Li J, King MR. Adhesion receptors as therapeutic targets for circulating tumor cells. *Front Oncol*. 2012;2:79. [PubMed: 22837985]
- [23]. Steeg PS. Targeting metastasis. *Nat Rev Cancer*. 2016;16(4):201–18. [PubMed: 27009393]
- [24]. Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer*. 2013;13(12):858–70. [PubMed: 24263189]
- [25]. Cai B, Fan J, Zeng M, Zhang L, Fu BM. Adhesion of malignant mammary tumor cells MDA-MB-231 to microvessel wall increases microvascular permeability via degradation of endothelial surface glycocalyx. *J Appl Physiol (1985)*. 2012;113(7):1141–53. [PubMed: 22858626]
- [26]. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. *Annu Rev Biomed Eng*. 2007;9:121–67. [PubMed: 17373886]
- [27]. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch*. 2007;454(3):345–59. [PubMed: 17256154]
- [28]. Mochizuki S, Vink H, Hiramatsu O, Kajita T, Shigeto F, Spaan JA et al. Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release. *Am J Physiol Heart Circ Physiol*. 2003;285(2):H722–6. [PubMed: 12730059]

- [29]. Pahakis MY, Kosky JR, Dull RO, Tarbell JM. The role of endothelial glycocalyx components in mechanotransduction of fluid shear stress. *Biochemical and Biophysical Research Communications*. 2007;355(1):228–33. [PubMed: 17291452]
- [30]. Wight TN, Merrilees MJ. Proteoglycans in atherosclerosis and restenosis: key roles for versican. *Circ Res*. 2004;94(9):1158–67. [PubMed: 15142969]
- [31]. Tedder TF, Steeber DA, Chen A, Engel P. The selectins - vascular adhesion molecules. *Faseb Journal*. 1995;9(10):866–73. [PubMed: 7542213]
- [32]. Albelda SM, Buck CA. Integrins and other cell-adhesion molecules. *Faseb Journal*. 1990;4(11):2868–80. [PubMed: 2199285]
- [33]. Anderson AO, Shaw S. T cell adhesion to endothelium: the FRC conduit system and other anatomic and molecular features which facilitate the adhesion cascade in lymph node. *Semin Immunol*. 1993;5(4):271–82. [PubMed: 8219105]
- [34]. Jacob M, Rehm M, Loetsch M, Paul JO, Bruegger D, Welsch U et al. The endothelial glycocalyx prefers albumin for evoking shear stress-induced, nitric oxide-mediated coronary dilatation. *J Vasc Res*. 2007;44(6):435–43. [PubMed: 17622736]
- [35]. Ebong EE, Macaluso FP, Spray DC, Tarbell JM. Imaging the endothelial glycocalyx in vitro by rapid freezing/freeze substitution transmission electron microscopy. *Arteriosclerosis Thrombosis and Vascular Biology*. 2011;31(8):1908–15.
- [36]. Singh A, Ramnath RD, Foster RR, Wylie EC, Friden V, Dasgupta I et al. Reactive oxygen species modulate the barrier function of the human glomerular endothelial glycocalyx. *Plos One*. 2013;8(2).
- [37]. Chappell D, Hofmann-Kiefer K, Jacob M, Rehm M, Briegel J, Welsch U et al. TNF-alpha induced shedding of the endothelial glycocalyx is prevented by hydrocortisone and antithrombin. *Basic Res Cardiol*. 2009;104(1):78–89. [PubMed: 18836678]
- [38]. Rubio-Gayosso I, Platts SH, Duling BR. Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol*. 2006;290(6):H2247–56. [PubMed: 16399871]
- [39]. Luft JH. Fine structures of capillary and endocapillary layer as revealed by ruthenium red. *Fed Proc*. 1966;25(6):1773–83. [PubMed: 5927412]
- [40]. Potter DR, Damiano ER. The hydrodynamically relevant endothelial cell glycocalyx observed in vivo is absent in vitro. *Circulation Research*. 2008;102(7):770–6. [PubMed: 18258858]
- [41]. Tse D, Stan RV. Morphological heterogeneity of endothelium. *Semin Thromb Hemost*. 2010;36(3):236–45. [PubMed: 20490976]
- [42]. Schauer R. Sialic acids as regulators of molecular and cellular interactions. *Current Opinion in Structural Biology*. 2009;19(5):507–14. [PubMed: 19699080]
- [43]. Nightingale TD, Frayne MEF, Clasper S, Banerji S, Jackson DG. A mechanism of sialylation functionally silences the hyaluronan receptor LYVE-1 in lymphatic endothelium. *Journal of Biological Chemistry*. 2009;284(6):3935–45. [PubMed: 19033446]
- [44]. Kerfoot SM, Mcrae K, Lam F, McAvoy EF, Clark S, Brain M et al. A novel mechanism of erythrocyte capture from circulation in humans. *Experimental Hematology*. 2008;36(2):111–8. [PubMed: 18037227]
- [45]. Ebong EE, Lopez-Quintero SV, Rizzo V, Spray DC, Tarbell JM. Shear-induced endothelial NOS activation and remodeling via heparan sulfate, glypican-1, and syndecan-1. *Integr Biol (Camb)*. 2014;6(3):338–47. [PubMed: 24480876]
- [46]. Davies PF. Flow-mediated endothelial mechanotransduction. *Physiol Rev*. 1995;75(3):519–60. [PubMed: 7624393]
- [47]. Shintani Y, Takashima S, Asano Y, Kato H, Liao Y, Yamazaki S et al. Glycosaminoglycan modification of neuropilin-1 modulates VEGFR2 signaling. *EMBO J*. 2006;25(13):3045–55. [PubMed: 16763549]
- [48]. Qiao DH, Meyer K, Mundhenke C, Drew SA, Friedl A. Heparan sulfate proteoglycans as regulators of fibroblast growth factor-2 signaling in brain endothelial cells. *Journal of Biological Chemistry*. 2003;278(18):16045–53. [PubMed: 12591930]
- [49]. Ebong EE, Spray DC, Tarbell JM. Glycocalyx core proteins selectively mediate endothelial NOS activation and cell alignment in response to shear stress. *Faseb Journal*. 2013;27.

- [50]. Proudfoot AEI, Handel TM, Johnson Z, Lau EK, Liwang P, Clark-Lewis I et al. Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;100(4):1885–90. [PubMed: 12571364]
- [51]. Kumagai R, Lu X, Kassab GS. Role of glycocalyx in flow-induced production of nitric oxide and reactive oxygen species. *Free Radic Biol Med*. 2009;47(5):600–7. [PubMed: 19500664]
- [52]. Chappell D, Dorfler N, Jacob M, Rehm M, Welsch U, Conzen P et al. Glycocalyx protection reduces leukocyte adhesion after ischemia/reperfusion. *Shock*. 2010;34(2):133–9. [PubMed: 20634656]
- [53]. Haraldsen G, Kvale D, Lien B, Farstad IN, Brandtzaeg P. Cytokine-regulated expression of E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) in human intestinal microvascular endothelial cells. *Journal of Immunology*. 1996;156(7):2558–65.
- [54]. Reymond N, d'Agua BB, Ridley AJ. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer*. 2013;13(12):858–70. [PubMed: 24263189]
- [55]. Lipowsky HH. The endothelial glycocalyx as a barrier to leukocyte adhesion and its mediation by extracellular proteases. *Ann Biomed Eng*. 2012;40(4):840–8. [PubMed: 21984514]
- [56]. Nieuwdorp M, Meuwese MC, Vink H, Hoekstra JB, Kastelein JJ, Stroes ES. The endothelial glycocalyx: a potential barrier between health and vascular disease. *Curr Opin Lipidol*. 2005;16(5):507–11. [PubMed: 16148534]
- [57]. Nieuwdorp M, van Haeften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M et al. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation activation in vivo. *Diabetes*. 2006;55(2):480–6. [PubMed: 16443784]
- [58]. Nieuwdorp M, Meuwese MC, Mooij HL, van Lieshout MH, Hayden A, Levi M et al. Tumor necrosis factor- α inhibition protects against endotoxin-induced endothelial glycocalyx perturbation. *Atherosclerosis*. 2009;202(1):296–303. [PubMed: 18550063]
- [59]. Rai S, Nejadhamzeeigilani Z, Gutowski NJ, Whatmore JL. Loss of the endothelial glycocalyx is associated with increased E-selectin mediated adhesion of lung tumour cells to the brain microvascular endothelium. *Journal of Experimental & Clinical Cancer Research*. 2015;34.
- [60]. Zhang L, Zeng M, Fu BM. Sphingosine-1-phosphate reduces adhesion of malignant mammary tumor cells MDA-MB- 231 to microvessel walls by protecting endothelial surface glycocalyx. *Cell Mol Biol (Noisy-le-grand)*. 2017;63(4):16–22. [PubMed: 28478798]
- [61]. Mitra R, O'Neil GL, Harding IC, Cheng MJ, Mensah SA, Ebong EE. Glycocalyx in atherosclerosis-relevant endothelium function and as a therapeutic target. *Curr Atheroscler Rep*. 2017;19(12):63. [PubMed: 29127504]
- [62]. Zarins CK, Giddens DP, Bharadvaj BK, Sottiurai VS, Mabon RF, Glagov S. Carotid bifurcation atherosclerosis. Quantitative correlation of plaque localization with flow velocity profiles and wall shear stress. *Circ Res*. 1983;53(4):502–14. [PubMed: 6627609]
- [63]. Ku DN, Giddens DP, Zarins CK, Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation. Positive correlation between plaque location and low oscillating shear stress. *Arteriosclerosis*. 1985;5(3):293–302. [PubMed: 3994585]
- [64]. Bond AR, Jackson CL. The fat-fed apolipoprotein E knockout mouse brachiocephalic artery in the study of atherosclerotic plaque rupture. *Journal of Biomedicine and Biotechnology*. 2011.
- [65]. Brooks AR, Lelkes PI, Rubanyi GM. Gene expression profiling of human aortic endothelial cells exposed to disturbed flow and steady laminar flow. *Physiol Genomics*. 2002;9(1):27–41 [PubMed: 11948288]
- [66]. Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN et al. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *J Clin Invest*. 2006;116(1):49–58. [PubMed: 16341264]
- [67]. Dai G, Kaazempur-Mofrad MR, Natarajan S, Zhang Y, Vaughn S, Blackman BR et al. Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proc Natl Acad Sci USA*. 2004;101(41):14871–6. [PubMed: 15466704]

- [68]. Go YM, Son DJ, Park H, Orr M, Hao L, Takabe W et al. Disturbed flow enhances inflammatory signaling and atherogenesis by increasing thioredoxin-1 level in endothelial cell nuclei. *PLoS One*. 2014;9(9):e108346. [PubMed: 25265386]
- [69]. Chang K, Weiss D, Suo J, Vega JD, Giddens D, Taylor WR et al. Bone morphogenic protein antagonists are coexpressed with bone morphogenic protein 4 in endothelial cells exposed to unstable flow in vitro in mouse aortas and in human coronary arteries: role of bone morphogenic protein antagonists in inflammation and atherosclerosis. *Circulation*. 2007;116(11):1258–66. [PubMed: 17785623]
- [70]. Jo H, Song H, Mowbray A. Role of NADPH oxidases in disturbed flow- and BMP4- induced inflammation and atherosclerosis. *Antioxid Redox Signal*. 2006;8(9–10):1609–19. [PubMed: 16987015]
- [71]. Nam D, Ni CW, Rezvan A, Suo J, Budzyn K, Llanos A et al. Partial carotid ligation is a model of acutely induced disturbed flow, leading to rapid endothelial dysfunction and atherosclerosis. *American Journal of Physiology-Heart and Circulatory Physiology*. 2009;297(4):H1535–H43. [PubMed: 19684185]
- [72]. Ni CW, Qiu H, Rezvan A, Kwon K, Nam D, Son DJ et al. Discovery of novel mechanosensitive genes in vivo using mouse carotid artery endothelium exposed to disturbed flow. *Blood*. 2010;116(15):e66–73. [PubMed: 20551377]
- [73]. Harding IC, Mitra R, Mensah SA, Herman IM, Ebong EE. Pro-atherosclerotic disturbed flow disrupts caveolin-1 expression, localization, and function via glycocalyx degradation. *Journal of Translational Medicine*. 2018;16.
- [74]. Mitra R, Qiao J, Madhavan S, O'Neil GL, Ritchie B, Kulkarni P et al. The comparative effects of high fat diet or disturbed blood flow on glycocalyx integrity and vascular inflammation. *Translational Medicine Communications*. 2018;3(1):10. [PubMed: 30957020]
- [75]. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA*. 1999;282(21):2035–42. [PubMed: 10591386]
- [76]. Korshunov VA, Berk BC. Flow-induced vascular remodeling in the mouse: a model for carotid intima-media thickening. *Arterioscler Thromb Vasc Biol*. 2003;23(12):2185–91. [PubMed: 14576075]
- [77]. Shahcheraghi N, Dwyer HA, Cheer AY, Barakat AI, Rutaganira T. Unsteady and three-dimensional simulation of blood flow in the human aortic arch. *J Biomech Eng*. 2002;124(4):378–87. [PubMed: 12188204]
- [78]. Rodkiewicz CM. Localization of early atherosclerotic lesions in the aortic arch in the light of fluid flow. *J Biomech*. 1975;8(2):149–56. [PubMed: 1150681]
- [79]. Gouverneur M, Spaan JA, Pannekoek H, Fontijn RD, Vink H. Fluid shear stress stimulates incorporation of hyaluronan into endothelial cell glycocalyx. *Am J Physiol Heart Circ Physiol*. 2006;290(1):H458–2. [PubMed: 16126814]
- [80]. Koo A, Dewey CF, Garcia-Cardena G. Hemodynamic shear stress characteristic of atherosclerosis-resistant regions promotes glycocalyx formation in cultured endothelial cells. *American Journal of Physiology-Cell Physiology*. 2013;304(2):C137–C46. [PubMed: 23114962]
- [81]. Tarbell JM, Cancel LM. The glycocalyx and its significance in human medicine. *J Intern Med*. 2016;280(1):97–113. [PubMed: 26749537]
- [82]. Amarenco P, Heinzlef O, Lucas C, Touboul PJ, Gerard JL, Adrai V et al. Atherosclerotic disease of the aortic arch as a risk factor for recurrent ischemic stroke. *New England Journal of Medicine*. 1996;334(19):1216–21. [PubMed: 8606716]
- [83]. Amarenco P, Cohen A. Atherosclerotic disease of the aortic-arch and the risk of ischemic stroke - a case-control study (preliminary-results of the french study of aortic plaques in stroke). *Stroke*. 1994;25(1):259-.
- [84]. Taylor CA, Hughes TJR, Zarins CK. Finite element modeling of three-dimensional pulsatile flow in the abdominal aorta: Relevance to atherosclerosis. *Annals of Biomedical Engineering*. 1998;26(6):975–87. [PubMed: 9846936]
- [85]. Zeng Y, Tarbell JM. The adaptive remodeling of endothelial glycocalyx in response to fluid shear stress. *PLoS One*. 2014;9(1):e86249. [PubMed: 24465988]

- [86]. Mensah SA, Cheng MJ, Homayoni H, Plouffe BD, Coury AJ, Ebong EE. Regeneration of glycocalyx by heparan sulfate and sphingosine 1-phosphate restores inter-endothelial communication. *PLoS One*. 2017;12(10):e0186116. [PubMed: 29023478]
- [87]. McDonald KK, Cooper S, Danielzak L, Leask RL. Glycocalyx degradation induces a proinflammatory phenotype and increased leukocyte adhesion in cultured endothelial cells under flow. *PLoS One*. 2016;11(12):e0167576. [PubMed: 27907146]
- [88]. Mitchell MJ, King MR. Physical biology in cancer. 3. The role of cell glycocalyx in vascular transport of circulating tumor cells. *Am J Physiol Cell Physiol*. 2014;306(2):C89–97. [PubMed: 24133067]
- [89]. Tarbell JM, Ebong EE. The endothelial glycocalyx: a mechano-sensor and -transducer. *Sci Signal*. 2008;1(40):pt8. [PubMed: 18840877]
- [90]. Strell C, Entschladen F. Extravasation of leukocytes in comparison to tumor cells. *Cell Commun Signal*. 2008;6:10. [PubMed: 19055814]
- [91]. Dennis J, Waller C, Timpl R, Schirmmacher V. Surface sialic-acid reduces attachment of metastatic tumor-cells to collagen type-Iv and fibronectin. *Nature*. 1982;300(5889):274–6. [PubMed: 7144883]
- [92]. Kannagi R, Izawa M, Koike T, Miyazaki K, Kimura N. Carbohydrate-mediated cell adhesion in cancer metastasis and angiogenesis. *Cancer Science*. 2004;95(5):377–84. [PubMed: 15132763]
- [93]. Betteridge K, Arkill K, Neal C, Harper S, Foster B, Satchell S et al. Sialic acids regulate microvessel permeability, revealed by novel in vivo studies of endothelial glycocalyx structure and function. *J Physiol*. 2017;19.
- [94]. Broekhuizen LN, Lemkes BA, Mooij HL, Meuwese MC, Verberne H, Holleman F et al. Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus. *Diabetologia*. 2010;53(12):2646–55. [PubMed: 20865240]
- [95]. Padberg JS, Wiesinger A, di Marco GS, Reuter S, Grabner A, Kentrup D et al. Damage of the endothelial glycocalyx in chronic kidney disease. *Atherosclerosis*. 2014;234(2):335–43. [PubMed: 24727235]
- [96]. Cheng MJ, Kumar R, Sridhar S, Webster TJ, Ebong EE. Endothelial glycocalyx conditions influence nanoparticle uptake for passive targeting. *International Journal of Nanomedicine*. 2016;11:3305–15. [PubMed: 27499624]
- [97]. Singh R, Lillard JW Jr. Nanoparticle-based targeted drug delivery. *Exp Mol Pathol*. 2009;86(3):215–23. [PubMed: 19186176]
- [98]. Celie JW, Beelen RH, van den Born J. Heparan sulfate proteoglycans in extravasation: assisting leukocyte guidance. *Front Biosci (Landmark Ed)*. 2009;14:4932–49. [PubMed: 19482596]
- [99]. Fan J, Fu BM. Quantification of malignant breast cancer cell MDA-MB-231 transmigration across brain and lung microvascular endothelium. *Annals of Biomedical Engineering*. 2016;44(7):2189–201. [PubMed: 26603751]
- [100]. Gasic G, Gasic T. Removal of sialic acid from the cell coat in tumor cells and vascular endothelium, and its effects on metastasis. *Proc Natl Acad Sci USA*. 1962;48:1172–7. [PubMed: 13897149]
- [101]. Malek-Zietek KE, Targosz-Korecka M, Szymonski M. The impact of hyperglycemia on adhesion between endothelial and cancer cells revealed by single-cell force spectroscopy. *J Mol Recognit*. 2017;30(9).
- [102]. Mulivor AW, Lipowsky HH. Role of glycocalyx in leukocyte-endothelial cell adhesion. *Am J Physiol Heart Circ Physiol*. 2002;283(4):H1282–91. [PubMed: 12234777]
- [103]. Voyvodic PL, Min D, Liu R, Williams E, Chitalia V, Dunn AK et al. Loss of syndecan-1 induces a pro-inflammatory phenotype in endothelial cells with a dysregulated response to atheroprotective flow. *J Biol Chem*. 2014;289(14):9547–59. [PubMed: 24554698]
- [104]. Salmon AH, Neal CR, Sage LM, Glass CA, Harper SJ, Bates DO. Angiopoietin-1 alters microvascular permeability coefficients in vivo via modification of endothelial glycocalyx. *Cardiovasc Res*. 2009;83(1):24–33. [PubMed: 19297368]
- [105]. Lipowsky HH, Lescanic A. Inhibition of inflammation induced shedding of the endothelial glycocalyx with low molecular weight heparin. *Microvascular Research*. 2017;112:72–8. [PubMed: 28347755]

Fig. 1. Schematic of the EC GCX structure.

The GCX is mainly composed of glycosaminoglycans, such as heparan sulfate (HS), hyaluronic acid (HA), and chondroitin sulfate (CS), attached to core proteins or receptors, such as glypicans, syndecans, and CD44. The GCX also abundantly contains sialic acid (SA) residues and soluble proteins, such as serum albumin. Glycoproteins, including selectins, integrins, and immunoglobulins, lie at the base of the GCX. These components contribute to various functions of the GCX, which is mainly implicated in endothelial mechanotransduction and trans-endothelial barrier regulation. Through these functions, the GCX assists in regulating vascular activities and health.

Fig. 2. It has been found that, with exposure of cultured ECs to 6-hour flow conditions, GCX is differentially expressed in uniform versus disturbed flow (UF vs. DF) patterns [73]. A–C: WGA serves as a general GCX label because it targets more than one GCX constituent including SA and N-acetylglucosamine, a component of HS and HA. As determined by laser scanning confocal microscopy of fluorescent green WGA staining, GCX coverage and thickness on confluent monolayers of rat fat pad ECs is shown to be increased in regions of UF but decreased in DF (both compared to static conditions) [73]. D–F: Using an anti-HS antibody to look more specifically at the most abundant GCX component, HS, demonstrates similar trends in GCX coverage and thickness in response to flow [73]. G–J: Quantification of WGA- and anti-HS-labeled GCX coverage and thickness. Values were obtained using previously published methods [73]. Results were normalized to static controls. Statistically significant differences between static-, UF-, and DF-conditioned

GCX are indicated as $*P < 0.05$ and $***P < 0.001$, which were determined after performing one-way ANOVAs followed by post-hoc Tukey's multiple comparison test (G-J). (This figure is adapted from [73], which is allowed by the publisher without formal written permission.)

Fig. 3. It has been found *in vivo* that GCX degradation in response to acute DF correlates to vascular wall infiltration by circulating cells [74]. Acute DF is induced *in vivo*, by using a partial carotid ligation mouse model. A–C: As shown by laser scanning confocal micrographs of fluorescent red WGA labeling, GCX is found to be expressed continuously and more significantly (ANOVA, *** $P < 0.001$) in non-ligated left carotid arteries, which experience UF, than in ligated left carotid arteries, which experience DF [74]. D–F: Here, inflammatory cells (fluorescent yellow) are examined because they represent an important circulating cell population. Uptake of macrophages, a key inflammatory cell type, was examined through immunohistochemistry of macrophage-specific CD68. Macrophages of interest were those residing in the vessel wall and not those that lie outside of the dotted line that marks the outer vessel wall. Macrophage uptake was found to correlate to GCX expression. Significantly less macrophage uptake (ANOVA, *** $P < 0.001$) was found in non-ligated, UF regions than in ligated, DF regions [74]. (This figure is adapted from [74], which is allowed by the publisher without formal written permission.)

Fig. 4. It has been shown *in vivo* that chronically different flow conditions differentially regulate GCX expression

[73]. GCX expression is indicated by fluorescent green WGA labeling. A: GCX fluorescence intensity is weaker in the distal aortic arch of C57BL/6 mice where flow patterns are chronically disturbed. B: GCX fluorescence is more intense in the abdominal aorta where flow patterns are chronically uniform [73]. (This figure is adapted from [73], which is allowed by the publisher without formal written permission.)

Fig. 5. In a 4-hour flow study, DF-induced remodeling of the GCX, with respect to the SA component in particular, is found to enable adhesion of circulating cells to the endothelium (Mensah et al., manuscript in preparation). Fluorescent green WGA is again used as a general GCX label, to collectively visualize SA and N-acetylglucosamine, a component of HS and HA. A–C, M–N: Consistent with what is shown in Fig. 2, WGA labeled GCX is differentially regulated by DF versus UF in human umbilical vein ECs after 4 hours of flow. D, M–N: In UF conditions, treatment with Neur, which specifically degrades SA residues of the GCX, significantly reduces GCX expression as viewed by lesser WGA fluorescence. E–H: EC layers (in phase contrast images) maintain integrity in all conditions. E–L, O: Here, stage IV metastatic breast cancer cells (4T1; fluorescent red) are examined as a representative circulating cell population via static attachment over a 1-hour incubation period. Metastatic cancer cell adhesion differs under various GCX conditions. Specifically, a significant increase in adhesion was observed in GCX- deficient conditions of DF or GCX- deficient conditions of UF combined with Neur treatment. GCX expression and cancer cell attachment to ECs were quantified in accordance with previously published methods [96].

For statistical analysis of the data, all results were normalized to control conditions and reduced, ANOVA was performed between groups, and significance is indicated as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

Fig. 6. GCX regeneration may recover trans-endothelial barrier against molecular and cellular infiltration.

A: Here, HS expression and thickness on rat fat pad ECs, when compared to control conditions, is shown to be reduced by treatment of the cells with Hep III, which degrades HS from the GCX [96]. After Hep III treatment, HS is successfully regenerated through treatment of ECs with endogenous HS [96]. A, B: HS expression is correlated with infiltration of nanoparticles (in fluorescent red). Specifically, nanoparticle infiltration is significantly increased after Hep III-induced HS degradation and abolished with GCX regeneration due to endogenous HS treatment [96]. Infiltration values were obtained using previously published methods [96] and results were normalized to control conditions. To determine statistical significance of differences between infiltration on various GCX conditions, ANOVAs were performed. Significance is indicated as * $P < 0.05$. (This figure is adapted from [96], with formal written permission from the publisher.)