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Review

# Regeneration and Assessment of the Endothelial Glycocalyx To Address Cardiovascular Disease

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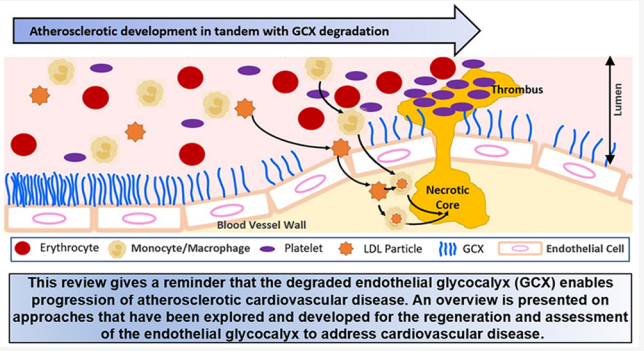
**ABSTRACT:** Endothelial cell (EC) glycocalyx (GCX) loss permits blood circulating particle infiltration of the vessel walls and leads to vascular dysfunction, atherosclerosis, and serious downstream cardiovascular events, motivating EC GCX regeneration to treat cardiovascular disease. This review discusses the benefits and drawbacks of current options for EC GCX regeneration and assessment. Existing pharmaceutical therapies are being explored for their applicability to EC GCX regeneration, while nutraceuticals are under development as primary EC GCX regeneration approaches, and novel therapies continue to emerge. Promotion of increased efficacy of these therapies by using novel targeted drug delivery approaches is proposed. In addition, development of intravital (and intravascular, if possible) detection tools for assessment of GCX health and GCX regeneration efficacy is recommended. The work presented in this review encourages continued development of GCX regeneration and detection approaches, which could lead to breakthrough solutions for addressing cardiovascular disease.






## 1. INTRODUCTION

The endothelial cell (EC) glycocalyx (GCX) is a sugar-rich layer that has a hair-like resemblance and lines the endothelium of the inner wall of the vasculature (Figure 1A). The EC GCX is negatively charged and heavily hydrated, consisting of a variety of glycosaminoglycan (GAG) chains, such as heparan sulfate (HS), hyaluronic acid (HA), and chondroitin sulfate (CS) (Figure 1B).[1−3] It also consists of a sialoglycoprotein named sialic acid (SA) (Figure 1B).[1,4,5] These sugar chains are bound to core proteins, such as syndecans, glypicans, and CD44, which are anchored to the EC membrane (Figure 1B).[6−8] Blood-borne proteins, such as albumin, are also embedded within the GCX (Figure 1B). They are tightly bound to the GCX because of their amphoteric nature, and they contribute to the extended thickness of the GCX, thus supporting GCX-mediated maintenance of vascular integrity.[9,10]

The EC GCX is an important contributor to the physical and biochemical health of the endothelium and the vasculature,[6,11−13] while mediating mechanotransduction and vascular signaling.[11,12] For example, when exposed to physiological (unidirectional and uniform in magnitude) levels of shear stress derived from the mechanical force of blood flow, the GCX is in its best condition (Figure 2E) and aids in the production of nitric oxide (NO) that regulates vascular tone (Figure 2F).[13,14] When the GCX is shed (Figure 2A), because of alterations in the chemical microenvironment or disturbances in blood flow that lead to pathological levels and patterns of shear stress, deregulated NO production can occur, causing a loss of vascular tone (Figure 2B).[6,14] As another example, the dynamics of the flow-regulated GCX determine the structure of the connexin proteins that comprised interendothelial gap junctions and control the flow of communication between neighboring ECs.[15] Specifically, GCX degradation alters interendothelial communication.[15] The most well-known example of the importance of the intact GCX comes from its action as a physical barrier to numerous substances in the blood, including low-density lipoprotein (LDL) particles, inflammatory cells such as monocytes that evolve into macrophages, and platelets (see Figures 2G, 2H, and 3).

Loss of the GCX renders the endothelium as adhesive and permeable, and this permits LDL, inflammatory cells, and platelets to penetrate or adhere to the inner blood vessel wall, leading to the formation of atherosclerotic plaque and thrombosis (see Figures 2C, 2D, and 3).[1,6] Plaque accumulation causes the blood vessel wall to become rigid and stiff.[6,16] This plaque eventually becomes unstable and ruptures into the

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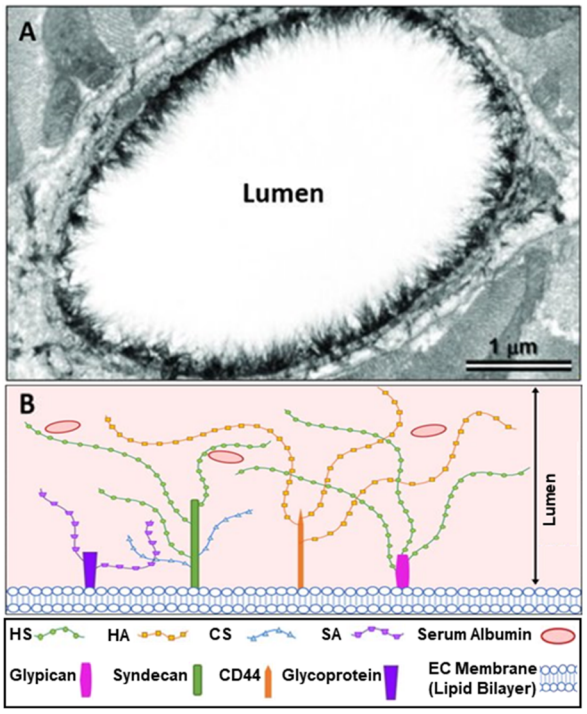


**Figure 1.** Introducing the EC GCX. (A) Transmission electron microscopy (TEM) photomicrograph showing that the EC GCX, which looks like a hair-like structure, provides a barrier between the EC membrane and the lumen of blood vessels. [Reproduced with permission from ref 140. Copyright 2003, Wolters Kluwer Health, Inc.] (B) Depiction of the composed structure of core proteins glypican, syndecan, and CD44, along with glycoproteins, which are embedded into the EC membrane (lipid bilayer). These structures have binding sites for associated SA and GAGs that include HS, HA, and CS. Albumin is also absorbed in this structure.

vascular lumen, where it can form a thrombus on the surface of the vessel wall (Figure 3) or plaque debris can travel through the bloodstream to block a vessel at a distant location. This causes serious downstream cardiovascular events, including myocardial infarction and stroke,[16] which are among the leading causes of death worldwide, affecting over 121 million adults in the United States alone.[17,18] Therefore, the EC GCX has emerged as a critical target in understanding, and potentially treating, cardiovascular diseases.[1,6]

Given the consequences of a degraded EC GCX, researchers have made numerous attempts (the most prominent are shown in Figure 4) to regenerate its structure in cell culture studies (*in vitro*) and in animal and human studies (*in vivo*).[19−25] This review will provide an overview of new GCX regeneration treatments that are under exploration through *in vitro* and *in vivo* research.[4,19,26−32] Efforts to regenerate the EC GCX have not yet resulted in a gold-standard therapeutic approach to GCX regeneration. The best options that exist are two nutritional supplements, but full characterization and study of these supplements is incomplete.[24,33] Therefore, further exploration and development of these and other potentially effective EC GCX therapies is necessary. At a minimum, there is a need to study existing therapies that are not currently indicated for GCX regeneration to determine if there is anything that may be applicable.[22,25,30,34−41] Another recommended avenue for the development of GCX regeneration therapies is the implementation of novel drug delivery approaches to specifically target therapies to the endothelium and only at sites of EC GCX degradation.[42−46]

While striving to develop GCX regeneration approaches, researchers are also in search of ways to clinically assess the EC GCX thickness, volume, and molecular composition for diagnostic purposes in order to evaluate therapeutic efficacy. Toward this aim, researchers have employed numerous methods that vary widely based on the study environment.[47−71] This review will summarize animal and human studies that employ GCX imaging techniques such as orthogonal phase spectroscopy (OPS) and sidestream dark-field (SDF) imaging.[51,52,55−59,64,72−75] These techniques have merit but are limited in their ability to precisely quantify the presence of the GCX as possible via microscopic methods that are commonly used for *in vitro* and *ex vivo* detection of the EC GCX.[47−50,53−59,64,76,77] Herein, we call for the development of clinically relevant detection methods that provide the precision offered by *in vitro* methods.

## 2. ENDOTHELIAL GLYCOCALYX REGENERATION

**2.1. Pharmaceutical Therapies: Is Reapplication to EC GCX Regeneration Possible?** There are many available pharmaceutical therapies that were not originally indicated for GCX regeneration but have great potential to be repurposed for GCX regeneration (see Table 1). These therapies, if successful in GCX regeneration, would leverage one of five strategies: replacement of GCX components and/or stabilizers, lipid level reduction, glucose concentration reduction, immunosuppression, or anticoagulation.

*2.1.1. Atherosclerotic Plaque Stabilizers.* Sulodexide (Figure 4, Table 1), used in patients with peripheral vascular disease, such as diabetic nephropathy, is the most obvious pharmaceutical option that is suitable for use in EC GCX regeneration. Sulodexide is composed of heparin (80%) and dermatan sulfate (20%), which are closely related to and could replace components of the EC GCX.[34,79,80] This formulation has been reported by Song et al. to effectively regenerate GCX in a disease model of cultured immortalized mouse brain microvascular ECs (bEND.3).[34] To model septic conditions, the cultured cells were subjected to lipopolysaccharide (LPS) endotoxin exposure.[34] The septic condition greatly diminished the GCX, as evidenced by a reduction in binding of fluorescein isothiocyanate (FITC) conjugated wheat germ agglutinin (WGA) lectin.[34] When Sulodexide was administered, the fluorescence intensity of the FITC WGA lectin increased, confirming that EC GCX was restored.[34] In a related experiment, bEND.3 were exposed to HA-specific hyaluronidase and HS-specific heparinase I/III enzymes, which like LPS endotoxin, reduced the thickness of the GCX, as observed with atomic force microscopy (AFM).[34] When enzymatic treatment was followed by Sulodexide treatment, AFM observations revealed that the thickness of the GCX was fully restored.[34] In addition to these *in vitro* studies, *in vivo* experiments were performed in animals and human subjects to demonstrate the GCX regeneration efficacy of Sulodexide. Song et al. induced sepsis in male C57BL/6 mice via injection of the LPS endotoxin.[34] GCX degradation occurred as a result of sepsis, as confirmed by visualizing the extent of dextran penetration of the vascular wall and by staining the aorta with FITC WGA lectin.[34] GCX was restored by Sulodexide treatment.[34]

In clinical studies performed on human subjects, Broekhuizen et al. orally administered Sulodexide to males with type II diabetes mellitus, and to healthy individuals, for two months.[79]


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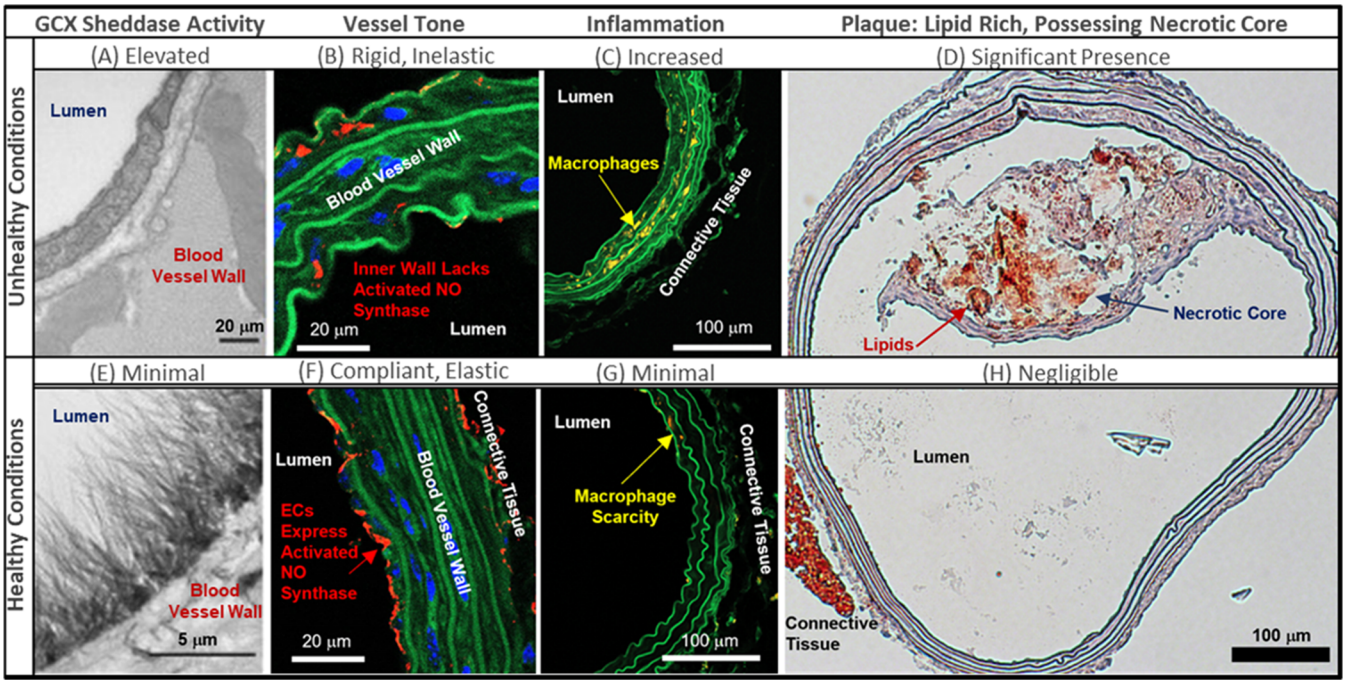


**Figure 2.** As the EC GCX responds to the microenvironment, it aids in regulation of blood vessel tone, mediates inflammation, and can control the formation of atherosclerotic plaques. (A) TEM micrograph that reveals the details of remaining rat endocapillary GCX when hyaluronidase levels are high in the bloodstream. (B) The vascular wall can become rigid and inelastic, as a result of reduced nitric oxide (NO) production due to EC inactivation of its synthase (red color shows synthase or nonspecific staining), which occurs when GCX is degraded.[141,142] The picture shown comes from mouse tissue, green shows autofluorescent elastin, and blue shows cell nuclei. (C) The vascular wall can become inflamed with infiltrated macrophages (yellow stain) when the EC GCX is in its degraded condition.[143] The picture shown comes from mouse tissue and green represents autofluorescent elastin. (D) Degradation of the GCX allows substances from the blood, such as lipids and inflammatory cells, to adhere to and eventually pass through the endothelium into the subendothelial space.[144] Over time, these substances interact to form plaque and eventually die, leaving necrotic cores.[6] (E) Unlike that observed in panel (A), here, the rat endocapillary GCX is normal. (F) Comparison with panel (B) shows that when the GCX is healthy and intact, there is EC activation of endothelial NO synthase (red color shows synthase or nonspecific staining) which will translate to ample NO production and a compliant and elastic vascular wall.[142] (G) Comparison with panel (C) shows that macrophages are scarce in the vessel wall, since the EC GCX blocks their entry. (H) Comparison with panel (D) shows that when the GCX is whole, it physically blocks substances from infiltrating the blood vessel wall and forming plaques.[144] [Panels (A) and (E) were reproduced with permission from ref 145. Copyright 2020, Elsevier. Panels (D) and (H) are original but come from a previous experiment and resemble images published in a report on that experiment; therefore, permission has been obtained from ref 144. Copyright 2016, Elsevier.]

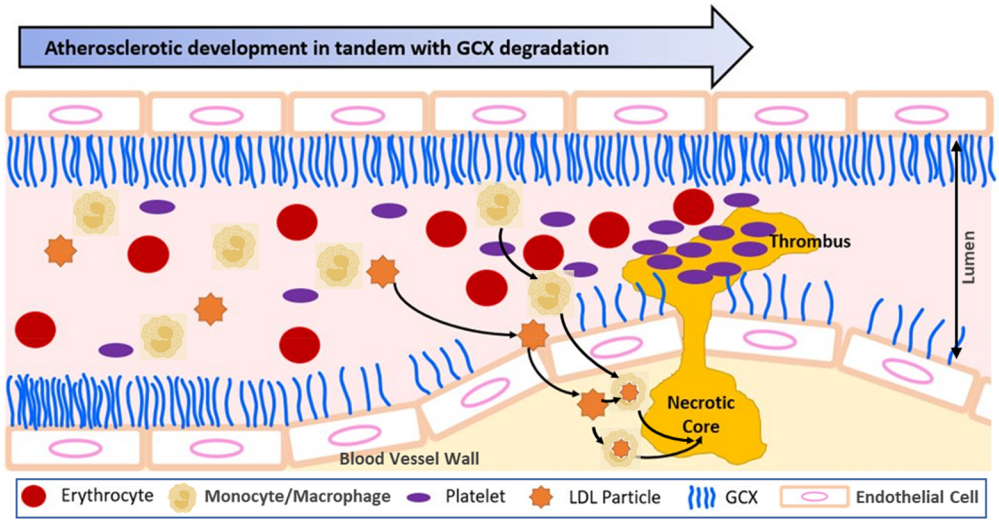


**Figure 3.** The most appreciated role of the EC GCX is that of physical barrier and protector of the blood vessel wall. A degraded EC GCX allows LDL particles to pass through the EC layer and enter the subendothelial space. Upon entry, the LDL particles aggregate together. This is followed by the passage of monocytes through the EC layer and into the subendothelial space, so that the monocytes can become macrophages and try to remove the LDL. The LDL-filled macrophages aggregate, begin to die, and then leave behind a necrotic core. The necrotic core grows over time to eventually rupture through the endothelium and into the lumen of the blood vessel, forming a thrombus. Platelets from the blood accumulate around this thrombus, reinforcing it.



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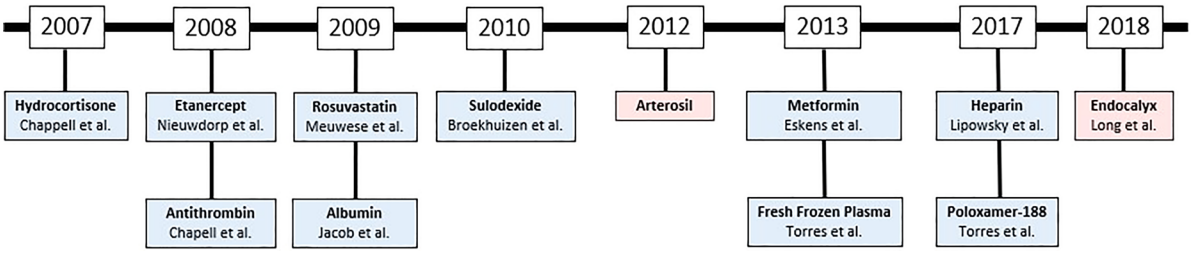


**Figure 4.** Chronological depiction of prominent EC GCX regenerative therapy developments. All reapplied therapies (blue) are ordered according to the year in which their beneficial effects on the EC GCX were published. Nutraceuticals (pink) Endocalyx and Arterosil are ordered according to the year of its patent approval and to the year it was first available for use, respectively. Of note, there is no published source confirming the earliest availability of Arterosil.

The endothelial vasculature in the sublingual and retinal vessels of the human subjects was imaged intravitally to visualize the perfused boundary region (PBR). As shown in Figure 5,[86] the PBR is the extent to which red blood cells can penetrate the EC GCX. In other words, the PBR is half of the difference between the diameter of the erythrocyte-perfused vascular lumen and the diameter from the erythrocyte column.[24,69,74] A Sulodexide-induced increase in GCX regeneration would result in a decrease of the PBR. This decrease would be indicated by the resistance to red blood cell perturbations, thereby demonstrating a marker of improved GCX thickness. In the Broekhuizen et al. study, before Sulodexide treatment, the diabetic patients were found to have increased retinal and sublingual PBR, indicating decreased GCX thickness, compared to healthy patients.[79] The decrease in GCX thickness coincided with elevation in markers responsible for HA catabolism as well as an increased transcapillary escape rate of albumin, which demonstrates an increase in the microvascular permeability.[79] After two months of Sulodexide treatment, GCX thickness significantly improved in diabetic patients, approaching the level of GCX thickness in healthy patients.[79] Similarly, a reduction in HA catabolism markers and a decreased transcapillary escape rate of albumin was observed, thereby showing improvement in GCX function. Taken together, the cell culture, animal, and human subject studies of Sulodexide show significant beneficial effects of Sulodexide on the EC GCX.

However, there are limitations. First, the heparin portion of Sulodexide can induce shedding of the HS component of the GCX, because of competitive binding to GCX-associated proteins, increasing permeability and impairing vasodilation during reactive hyperemia.[41,87] Second, the dermatan sulfate portion of Sulodexide is not naturally present in the EC GCX and can be considered a foreign substance. Third, there is currently no evidence that Sulodexide can restore the most complicated and vital GCX-mediated EC functions, such as gap junctional EC-to-EC communication,[6] which translates to limited information on the Sulodexide's efficacy for restoring healthy whole vessel function.

Another atherosclerotic plaque stabilizer, Rosuvastatin (Figure 4, Table 1), which is representative of statins that are common pharmaceutical drugs for treating hyperlipidemia, has also been explored to assess statin potential for preserving the EC GCX under diseased conditions. Meuwese et al. studied the effect of Rosuvastatin on GCX volume, thickness, and permeability in patients with familial hypercholesterolemia, compared to patients with normal cholesterol levels. The volume of EC GCX in these patients was examined by subtracting the difference between the intravascular distribution volume of GCX-permeable Dextran 40 and the intravascular distribution volume of GCX impermeable erythrocytes. The thickness of the EC GCX in capillaries was probed using orthogonal polarization spectroscopy (OPS) imaging (Figure 6[35]) to record and then calculate half of the difference between the diameter of the erythrocyte-perfused vascular lumen in the presence of leukocytes and the diameter of the erythrocyte-perfused vascular lumen in the absence of leukocytes. GCX permeability was determined based on clearance of Dextran 40 from the vasculature. Rosuvastatin significantly impacted EC GCX volume. Specifically, under untreated conditions, hypercholesterolemic patients expressed low volumes of EC GCX, compared to normocholesterolemic patients, while, under Rosuvastatin-treated conditions, EC GCX volumes in hypercholesterolemic patients increased, although to a limited extent that did not reach normocholesterolemic levels.[35] Rosuvastatin treatment appeared to also increase capillary GCX thickness in hypercholesterolemic patients; however, there was no statistically significant difference when comparing OPS data on GCX thickness for untreated hypercholesterolemia, Rosuvastatin-treated hypercholesterolemia, and normocholesterolemic conditions.[35] Unexpectedly, Rosuvastatin did not decrease hypercholesterolemic GCX permeability, as indicated by the sustained high rate of Dextran 40 clearance.[35] These results suggest that statins may have limitations as EC GCX regeneration agents.

*2.1.2. Anti-Diabetic Drug Treatments.* Metformin (Figure 4, Table 1), named for its active ingredient metformin hydrochloride, is historically an antidiabetes drug that has recently been proposed to apply to anticancer. It acts by lowering glucose levels, and has shown the ability to provide a protective effect against vascular complications.[21,22,82] Few studies have explored the effects of Metformin on the EC GCX. In a recent study performed by Targosz-Gorecka et al., the GCX regeneration efficacy of Metformin was examined in immortalized and hyperglycemic (diabetic) human umbilical vein ECs (HUVECs).[22] Metformin was shown to counter the effects of hyperglycemia (diabetes) and enhance HUVEC GCX density and length as detected via AFM along with immunoconfocal microscopy (of WGA lectin and *Maackia amurensis* lectin II (MAL II) stained cells).[22] Here, length can be taken to be the thickness, although thickness refers to the configuration of the GCX that is not pulled on by AFM.[22] The effects of Metformin on GCX structure were accompanied by effects on the GCX function. For example, the Metformin-induced recovery of the GCX was shown to reduce the hyperglycemia-induced EC surface expression of adhesion molecules E-selectin and intercellular adhesion molecule-1 (ICAM-1). Also, it attenuated the hyperglycemia-induced adhesion of human lung carcinoma cells to HUVECs.[22] In addition, GCX-dependent HUVEC stiffness and actin polymerization were reduced after Metformin


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**Table 1. Treatments That Are Either Specifically Intended for GCX Regeneration or Intended for Other Uses But Show Promise for GCX Regeneration**[a]

| Existing Therapy | Overview | FDA Approved? | GCX-related Research and Development Setting | Source(s) |
|---|---|---|---|---|
| | **Nutraceutical (Intended for GCX Regeneration)** | | | |
| Endocalyx | • Active ingredients: glucosamine (for HS and HA synthesis), exogenous HA, fucoidan (HS-like), superoxide dismutase and catalase (antioxidants)<br>• Indications: maintains GCX (thickness) and slows aging | No | • Human subjects (limited)<br>• Mice (B6D2F1, cross between C57BL/6 and DBA/2) | 24,103 |
| Arterosil | • Active ingredient: rhamnan sulfate (RS) (mimics GCX GAGs; has HS-like structure)<br>• Indications: repairs and restores GCX structure (thickness); improves arterial elasticity; reduces production of atherosclerotic plaques filled with lipid and necrotic cores | No (patent is pending) | • Human subjects (limited) | 33, 102 |
| | **Pharmaceutical (Repurposed for GCX Regeneration)** | | | |
| Sulodexide | • Active ingredients: dermatan sulfate and HS<br>• Indications: repairs and restores GCX (thickness, coverage, volume); downregulates endothelial inflammatory response and attenuates atherosclerotic factors, such as the expression of adhesion molecules VCAM and ICAM; inhibits thrombosis; treats peripheral vascular diseases such as diabetic nephropathy | No (approved outside the USA) | • Human subjects (limited)<br>• C57BL/6 mice<br>• Immortalized mouse brain microvascular ECs (bEND.3) | 34, 79,81 |
| Rosuvastatin | • Active ingredient: rosuvastatin calcium<br>• Indications: lowers cholesterol and triglyceride levels in patients suffering from hyperlipidemia, a cardiovascular disease risk factor; repairs and restores GCX (thickness; volume; barrier functionality) | Yes | • Human subjects | 35 |
| Metformin | • Active ingredient: metformin hydrochloride<br>• Indications: reduces polymerization of actin and thereby decreases EC stiffness; facilitates GCX barrier (thickness and volume) repair and reconstruction; yields antidiabetes effects via lowering glucose levels and protecting against vascular complications; produces anticancer effects via attenuation of adhesion between cancer cells and ECs | Yes | • DB/DB mice<br>• Human lung carcinoma cells | 21, 22 |
| Hydrocortisone | • Active ingredient: hydrocortisone steroid<br>• Indications: reduces inflammation and related oxidative damage, thereby alleviating shedding of GCX components (i.e., HS and Syndecan-1); reinforces GCX barrier | Yes | • Guinea pig hearts | 36, 37 |
| Antithrombin | • Active ingredient: recombinant human antithrombin<br>• Indications: decelerates blood clotting response; increases protease inhibitor function and decreases inflammatory responses, both of which alleviate shedding of GCX components (i.e., HS and Syndecan-1); reinforces GCX barrier | Yes | • Guinea pig hearts<br>• CD1 mice | 36, 38, 98 |
| Etanercept | • Active ingredient: etanercept, a TNF-$\alpha$ inhibitor<br>• Indications: ameliorates the effects of autoimmune diseases by inhibiting inflammatory response; delays oxidative damage seen during inflammation; alleviates GCX component HA shedding | Yes | • Human subjects | 39 |
| Poloxamer-188 | • Active ingredients: copolymer of polyoxyethylene and polyoxypropylene<br>• Indications: reduces inflammation; increases tissue oxygenation; as a consequence of inflammation reduction delays oxidative damage, alleviates GCX component Syndecan-1 shedding, repairs and restores GCX thickness, and reinforces GCX barrier | Yes | • Sprague–Dawley rats | 30 |
| Fresh frozen plasma (FFP) | • Active ingredients: clotting factors, fibrinogen, plasma proteins (i.e., albumin), electrolytes, anticoagulants (i.e., protein C, protein S, antithrombin, tissue factor pathway inhibitor)<br>• Indications: treats hemorrhagic shock by promoting coagulation; decreases GCX Syndecan-1 shedding; repairs and restores GCX thickness | Yes | • Sprague–Dawley rats | 25, 92 |



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**Table 1. continued**

| Existing Therapy | Overview | FDA Approved? | GCX-related Research and Development Setting | Source(s) |
|---|---|---|---|---|
| **Pharmaceutical (Repurposed for GCX Regeneration)** | | | | |
| Albumin | • Active ingredient: albumin (varying percent) along with other inactive ingredients<br>• Indications: restores plasma colloid pressure and thus the volume of circulating blood; promotes water retention; scavenges reactive oxygen species for antioxidant purposes; modulates cytokine production and antigen-presenting cell function for anti-inflammatory purposes; alleviates shedding of GCX HS and Syndecan-1, and reinforces GCX barrier | Yes | • Guinea pig hearts | 9, 40 |
| Heparin | • Active ingredient: heparin<br>• Indications: activates antithrombin III to inhibit coagulation factors and prevent blood clotting; attenuates adhesion between circulating inflammatory cells (i.e., leukocytes) and ECs; shown to alleviate shedding of GCX HS and Syndecan-1 and suppress inflammation-induced structural changes in the GCX in an antithrombin manner; in contrast, also shown to impair GCX barrier functionality and induce GCX HS shedding | Yes | • Male Wistar rats | 41 |

[a]These treatments all require further research and development into their effects on GCX structure and on the various functions of the GCX in facilitating vascular health.

treatment. In another study, conducted by Eskens et al., male diabetic (db/db) mice were supplied Metformin in their water.[21] As a result, there was an increase in whole-body GCX volume in the Metformin-treated animals, compared to the untreated animals, and the Metformin-treated diabetic mice exhibited GCX volume that was similar to that of non-diabetic control (C57BL/6).[21] More studies are warranted to build upon this data and further characterize the potential of Metformin as a therapy for EC GCX regeneration, particularly in human subject studies. However, since the purpose here is to regenerate EC GCX as an avenue to treat cardiovascular disease, it is important to note that the use of Metformin may pose a severe risk for patients with acute or unstable cardiovascular conditions, especially pertaining to heart disease and failure.[88,89]

*2.1.3. Anti-Inflammatory Therapies.* Anti-inflammatory pharmaceutical therapies have also been considered for EC GCX regeneration. In the inflammation cascade, inflammatory stimuli activate intracellular signals that increase the production of inflammatory mediators such as reactive oxygen species or cytokines (interleukins, tumor necrosis factors, gamma-interferons).[90] These pro-inflammatory molecules then activate metalloproteinases, which are known to cleave proteoglycans, thereby degrading the GCX.[72,90] Thus, therapeutics that can interrupt this cascade at any stage may minimize or stop GCX degradation.[90] Examples of such therapeutics include hydrocortisone steroid, Etanercept, and Poloxamer-188[36,37,39] (see Figure 4 and Table 1).

Regarding hydrocortisone (Figure 4, Table 1), Chappell et al. found that it alleviates GCX damage due to tumor necrosis factor-alpha in ischemic or inflamed guinea pig hearts.[36,37] Following hydrocortisone treatment of guinea pig hearts during reperfusion after ischemia, enzyme-linked immunosorbent assay (ELISA) was used to probe plasma levels of syndecan-1, HS, and HA. These markers of GCX degradation were reduced, compared to untreated post-ischemic conditions.[37] The ELISA assay also showed that, for guinea pig hearts in inflammatory conditions induced by tumor necrosis factor-alpha, hydrocortisone treatment completely suppressed shedding of syndecan-1 and HS.[36] Using TEM, the integrity of the EC GCX was confirmed to be most intact with hydrocortisone treatment post-ischemia or under inflammatory conditions, compared to effects of no treatment.[36,37] Furthermore, hydrocortisone resulted in a 4- to 5-fold reduction in coronary artery permeability to ∼250 kD hydroxyethyl starch, indicating the reinforcement of GCX barrier functionality, given the large size of hydroxyethyl starch molecules.[36,37] Meanwhile, Etanercept (Figure 4, Table 1), which is a tumor necrosis factor-alpha inhibitor, has also been shown to reduce EC GCX shedding. In a study by Niewdorp et al., healthy male volunteers received a low-dose endotoxin (*Esecherichia coli* lipopolysaccharide) to induce EC GCX degradation.[39] One cohort of volunteers then received a treatment of Etanercept, while another cohort received no treatment following the endotoxin. Two effects of Etanercept treatment were noted. First, the endotoxin-induced elevation in plasma levels of HA and hyaluronidase was abolished by Etanercept.[39] Second, the endotoxin-induced reduction in microvascular GCX thickness was significantly limited by Etanercept, although GCX thickness did not reach pre-endotoxin levels.[39]

In addition to hydrocortisone and Etanercept, the anti-inflammatory Poloxamer-188 (Figure 4, Table 1) has also been suggested to have beneficial effects on the EC GCX.[30] It has been approved for use to increase tissue oxygenation and reduce



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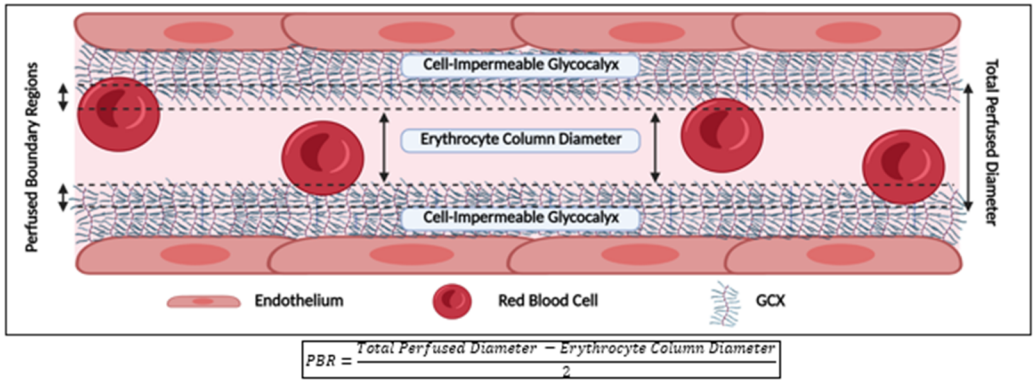


**Figure 5.** GCX function, as a result of regeneration treatment, can be assessed by determining the perfused boundary region (PBR).[86] A robust GCX will be less perturbable, restricting the lumen diameter through which erythrocytes are able to travel. A degraded and/or dysfunctional GCX will be more perturbable, allowing the erythrocytes more lateral movement. [This figure was created with BioRender.com.]

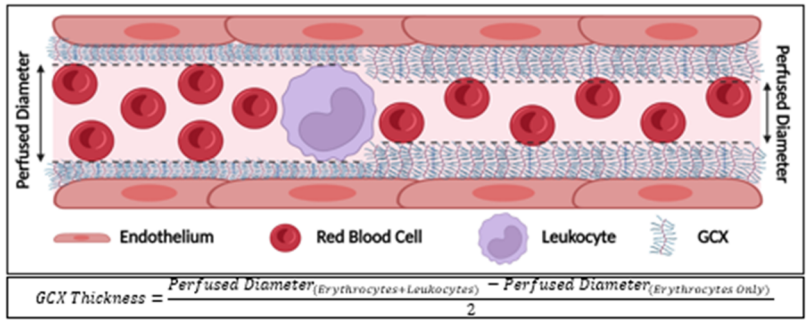


**Figure 6.** GCX thickness as a result of regeneration treatment can be deduced via orthogonal polarization spectroscopy (OPS) imaging.[86] From OPS recordings, an initial measurement of erythrocyte column diameter is taken within the vessel. Leukocyte concentration is low and the EC GCX is not compressed. After infusion with leukocytes, the column diameter is measured a second time. The leukocytes cause an apparent expansion of the erythrocyte column and compress the EC GCX. Thus, the difference between the initial and final diameters of this erythrocyte column, divided in half, provides an estimate of the relative thickness of the GCX layer. Resolution of OPS-derived GCX estimates is limited. [This figure was created with BioRender.com.]

painful episodes seen in sickle cell disease.[91] Torres et al. has studied the effects of Poloxamer-188 in Sprague−Dawley male rats recovering from hemorrhagic shock. The GCX is degraded, thinned, and exhibits limited barrier functionality post-hemorrhage. Torres et al. found that post-hemorraghic Poloxamer-188 treatment restored GCX thickness back to ∼85% of pre-hemorrhagic baseline conditions. It also significantly lowered plasma syndecan-1 close to the level measured under pre-hemorrhagic baseline conditions. Finally, it decreased GCX-associated vascular permeability to the pre-hemorrhagic baseline level.[30] These hydrocortisone, Etanercept, and Poloxamer-188 results, taken together, are encouraging and motivate further research into anti-inflammatory therapeutics as GCX regeneration agents. However, it is important to consider that their usefulness could be limited especially if high doses are required for effective regeneration of the EC GCX. Therapeutic approaches that aggressively suppress the immune system can make patients more prone to GCX-endangering infection than usual.

*2.1.4. Protein Supplementation-Based Therapies.* There are two particularly relevant FDA approved blood products for pharmaceutical use that have been applied for GCX regenerating purposes: fresh frozen plasma (FFP) and albumin (Figure 4, Table 1). FFP, which is typically used to maintain blood supply and promote coagulation in patients with various vascular conditions,[25] contains all of the plasma proteins that the GCX must absorb to be robust. Torres et al. performed a hemorrhage study in Sprague−Dawley male rats, in which GCX thickness and plasma levels of syndecan-1 were assessed,[25] similar to the study described above. Compared to the microcirculatory data before inducing hemorrhagic shock, the introduction of FFP aided in blood volume expansion, restoration of total protein level, and most importantly, reconstitution of the GCX structure. FFP treatment resulted in post-hemorrhage increased GCX thickness and a level of plasma syndecan-1 that equaled pre-hemorrhage values.[25] These results are promising. One component of FFP is fibrinogen, typically present at a concentration of 400−900 mg/unit. Studies suggest that the effects of FFP may be partly attributable to the effects of fibrinogen. In a study by Wu et al., human lung microvascular endothelial cells were treated with FFP as well as fibrinogen.[92] Equivalent significant increases in syndecan-1 protein expression were detected in each group.[92] In addition, fibrinogen-depleted FFP did not produce this increase in syndecan-1 expression.[92] To explore the potential mechanism behind this finding, the study performed immunostainings which demonstrated that fibrinogen and syndecan-1 colocalize on the cell membrane.[92] This therapeutic potential of fibrinogen in FFP is particularly interesting and worth further exploration.[92,93]

A major component of FFP is albumin, which alone can be applied as a standard of treatment for addressing low blood volume and promoting water retention.[94] For the GCX, there is a major physical benefit of albumin treatment. Recall that the amphoteric nature of albumin allows it to be tightly bound to


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and embedded in the GCX (Figure 1B), thus physically reinforcing the structure and the barrier function of the GCX. Similarly, it also physically restricts the flow of protein components and fluid across the endothelium.[9] There is also a biochemical benefit of albumin treatment in the perspective of the GCX. As evidence of this, Jacob et al. observed that cleavage of covalently linked GCX elements was reduced when guinea pig hearts were perfused with albumin.[40] It is presumed that this reduction in cleavage is due to the fact that albumin transports sphingosine-1-phosphate (S1P), a bioactive lipid metabolite that has been shown to stabilize the GCX, prevent its shedding, and ensure endothelial monolayer barrier integrity.[19,31,32] Confirming this presumption, Adamson et al. observed decreases in albumin content to be accompanied by decreased S1P content in Sprague−Dawley male rats.[9,84,95,96] For human subjects in the clinic, albumin treatment with this intention of GCX regeneration has not been explored. However, given the positive findings of the guinea pig and rat studies mentioned here (and there is other evidence), it would be worthwhile to determine the impact of albumin on the human GCX and investigate whether there are also positive effects toward GCX regeneration.

*2.1.5. Anticoagulants.* Another class of therapies that has been shown to affect the EC GCX, with varied results, is anticoagulants. One anticoagulant, antithrombin (Figure 4, Table 1),[83,97] was shown by Chappell et al. to protect the GCX.[38] In a study that was already described above, Chappell et al. modeled both ischemia and inflammation in guinea pig hearts.[36,38] In lieu of hydrocortisone, as previously mentioned, this time Chappell et al. administered antithrombin and compared its impact on the GCX to that of other treatments or no treatment.[36,38] Regardless of whether the disease conditions were characterized as ischemia and inflammation, the impact of antithrombin on the GCX was the same. Antithrombin treatment suppressed ischemia- or inflammation-induced shedding of syndecan-1 and HS components of the GCX, as detected via ELISA.[36,38] Antithrombin treatment also blocked ischemia- or inflammation-induced vessel leakage and permeability to hydroxyethyl starch.[36,38] TEM studies confirmed that antithrombin restored GCX structural integrity that was destabilized by ischemia or inflammation.[36,38] In addition, antithrombin has also shown barrier-protective qualities, given its role in preventing leukocyte adhesion. A study by El Saadani et al. showed that intravenous antithrombin treatment reduced penumbral leukocyte-EC interactions in the blood brain barrier after traumatic brain injury.[98] In the study, adult male CD1 mice underwent either severe traumatic brain injury via controlled cortical impact or sham craniotomy, and they were treated with either intravenous antithrombin-III, subcutaneous enoxaparin (a low-molecular-weight heparin, as a second anticoagulant), or intravenous normal saline.[98] As examined by pial intravital microscopy videos at 48 h post-injury, leukocyte adhesion and transit were reduced after treatment with antithrombin or enoxaparin, which suggests that these treatments may be of use in the restoration of GCX barrier functionality.[98] However, these GCX regenerative effects of antithrombin are not consistent.

Heparin (Figure 4, Table 1), which was previously mentioned in the context of Sulodexide and in the discussion of the El Saadani study, has been shown to have ambiguous effects on the GCX. These effects may vary by the type of heparin used. Low-molecular-weight heparin can inhibit GCX shedding, as observed by Lipowsky et al. in male Wistar rats that received increasing doses of low-molecular-weight heparin. Subsequently, these rats exhibited reduced glycan shedding (GCX degradation).[41] Therefore, low-molecular-weight heparin can impair GCX barrier functionality. The same study by Lipowsky et al. demonstrated a decrease in leukocyte adhesion to the walls of blood vessels in rats treated with low-molecular-weight heparin.[41] However, Vanteeffelen et al. observed that heparin competes with the HS component of the GCX, thus releasing the proteins bound to HS, degrading the GCX structure, and impairing its function as a barrier to transendothelial permeability.[85] In humans, Karlsson et al. observed that intravenous injection of low-molecular-weight heparin resulted in a 3-fold increase in the enzymatic activity responsible for the release of embedded protein from the EC GCX.[99] Similarly, Myrup et al. found increased protein detachment from the GCX into the plasma following low-molecular-weight heparin injection in control and diabetes patients.[100] Thus, despite heparin's prevention of EC GCX shedding,[41] it is also important to consider the negative effects that it has on barrier functionality.[41,100] For this reason, research is needed to further characterize these contradictory observations in order to use heparin and other anticoagulants for EC GCX regenerative therapy.

**2.1.6. Summary on the State of Reapplication of Pharmaceutical Therapies to EC GCX Regeneration.** Although significant developments have been made in EC GCX regeneration, further studies are needed to establish the robustness of reapplied pharmaceutical therapies in achieving positive EC GCX modifications and to look into their drawbacks. Given that these therapies are already FDA-approved, it may be worthwhile to expedite the research to provide GCX-targeted therapy within a faster timeline. In this expedited research, focus should be placed primarily on *in vivo* and clinical studies.

**2.2. Nutraceutical Therapies Specifically Designed for GCX Regeneration.** Currently, there are only two therapies that have been described that are specifically intended for GCX regeneration: Arterosil and Endocalyx (Figure 4, Table 1).[24,33,78] Both of these options are considered to be nutraceuticals or dietary supplements, rather than pharmaceuticals or medicinal drugs.

Arterosil (Figure 4, Table 1) was the first commercially available nutraceutical or dietary supplement developed for the protection and restoration of the EC GCX.[33] The patent-pending Arterosil contains rhamnan sulfate (RS), a heparin, and HS mimicking polysaccharide extracted from the algae *Monostroma nitidum*. The efficacy of Arterosil was first demonstrated in a cell culture study conducted by Cancel et al.[101] It was found that human coronary artery EC (HCAEC) monolayers treated with the Arterosil ingredient RS, specifically RS1 and RS2, exhibited a significant increase in HS coverage, compared to untreated monolayers.[101] Treatment of HCAECs with RS also reinforced the barrier formed by the HCAEC monolayers, resulting in a decrease in monolayer permeability to both water and LDL.[101,102] In support of studying the efficacy of Arterosil in human subjects, Daniels et al. reported, in the patent, that RS has oral bioactivity, unlike heparin, and has been prepared for oral administration.[102] The oral composition of Arterosil was applied in a set of pilot studies on human patients and found to localize to both arterial and venous endothelium, depending on the administered dose, distinguishing RS from other GCX-regenerating compounds.[102] In addition, Arterosil was shown to repair the glucose-damaged endothelial GCX in


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**Table 2. Future Directions for GCX Regeneration Therapy**[a]

| Other therapeutic options | Overview | FDA approved? | GCX-related research and development setting | Source(s) |
|---|---|---|---|---|
| Exogenous heparan sulfate (HS) | • Glycosaminoglycan (GAG), native to the EC GCX<br>• Repairs GCX structure (coverage and thickness); promotes GCX barrier function | No | • Rat fat pad ECs (RFPECs) | 19,108 |
| Exogenous hyaluronic acid (HA) and exogenous chondroitin sulfate (CS) | • GAGs, native to the EC GCX; must be administered in combination for peak efficacy<br>• Repairs GCX structure (volume and coverage) | No | • Syrian golden hamsters<br>• Human umbilical vein endothelial cells (HUVECs) | 26,27 |
| HS and S1P | • Combines a GAG native to the EC GCX with a bioactive lipid metabolite that is essential for EC membrane and vascular wall repair; must be administered in combination for peak efficacy<br>• Repairs GCX structure (coverage)<br>• Maintains active gap junctional interendothelial communication | No | • RFPECs | 19 |
| Beta-Hydroxybutyrate and Melatonin (BHB/M) | • Combines an energy source (BHB) with an antioxidant (M); the antioxidant is particular important for inhibition of GCX shedding<br>• Repairs GCX structure (thickness); alleviates shedding of the Syndecan-1 component of the GCX | Yes | • Sprague–Dawley rats | 30 |
| Adenosine, Lidocaine, and Magnesium (ALM) | • Subcomponents of ALM are important regulators of metabolism, immunomodulation, inflammation, and coagulation<br>• Repairs GCX structure (thickness); alleviates shedding of the Syndecan-1 component of the GCX; promotes GCX barrier function | Yes (subcomponents are individually approved) | • Sprague–Dawley rats | 30 |

[a]There are promising new approaches under development to promote proper EC GCX regeneration.



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three manners. It limited leukocyte-endothelium adhesion that would be caused by the enzymatic removal of the GCX, enhanced endothelial-dependent arterial elasticity by an average of 89.6%, and significantly reduced carotid atherosclerotic plaque severity, as indicated by the presence of fewer lipid-rich necrotic cores in the plaque.[33] However, the scope of the human subject study is limited and the extent to which the reported human subjects data have been peer-reviewed is unclear. Further investigation is necessary, especially given that the active ingredient in Arterosil, RS, is not a component found naturally in the body and may bring about an overactive immune response for some individuals, restricting the benefits of Arterosil treatment. Nevertheless, the demonstration of Arterosil efficacy to date is encouraging and suggests that it is a viable drug candidate for GCX repair in the clinic.[102]

Arterosil was recently joined by Endocalyx (Figure 4, Table 1), another nutraceutical or dietary supplement option developed for the protection and restoration of the EC GCX. Endocalyx is patented by Long et al. and consists of polysaccharides including the HA component of the EC GCX.[24] Endocalyx also contains amino sugars such as glucosamine for HS and HA synthesis, antioxidants, and HS-like fucoidan.[24] This makes Endocalyx a multistrategy therapeutic. The fact that the Endocalyx formulation primarily accounts for only two of the major GCX components, HA and HS, can be perceived as a limitation. It is possible that GCX regeneration can be maximized by incorporating more of the relevant GCX components. However, as Endocalyx is a multistrategy therapeutic, the developers of Endocalyx take an approach that is more aggressive than approaches that have been utilized for the development of other GCX regeneration therapies. Hence, expectations for successful outcomes with Endocalyx treatment are high. Indeed, an animal study by Machin et al. observed positive results after treating aged mice (30 months old) with Endocalyx for 10 weeks.[103] There was a decrease in PBR in Endocalyx-treated aged mice, compared to untreated aged mice.[103] These reported data suggest that Endocalyx regenerates and reinforces the protective GCX coat.[103] The study also showed that in the Endocalyx-treated mice the GCX barrier function was restored.[103] Furthermore, NO bioavailability was increased in the carotid artery of treated mice, in comparison to control mice, suggestive of improved arterial elasticity.[103] For reference, Endocalyx had no effect on the GCX of young and healthy mice, with similar PBR observed for untreated versus treated mice. It is presumed that (i) this is because the GCX in the young and healthy mice was not degraded prior to treatment and (ii) the feasibility of regeneration beyond healthy levels is limited. Endocalyx has also been shown to successfully regenerate GCX in clinical studies, as shown in the Long et al. patent that reports on a decrease in the extent of the PBR in patients who underwent Endocalyx treatment for 3 months.[24] Since this was a pilot study that only included 13 individuals and the published data does not appear to be peer-reviewed,[24] further investigation is necessary. As such, clinical trials are currently in progress[104] and anticipated to assist in fully characterizing the efficacy of Endocalyx.

Given the insufficient levels of clinical data, more robust peer-reviewed research investigating the effects and optimizing the designs of both Arterosil and Endocalyx are necessary in order to establish and fulfill their maximum potential as GCX regeneration agents. First, it is necessary to fill any gaps in knowledge about the individual components of Arterosil and Endocalyx—i.e., exogenous glucosamine, exogenous HA, superoxide dismutase, superoxide catalase, and RS—with respect to their bioactivity and independent contributions to GCX regeneration. Second, Arterosil and Endocalyx should be characterized in preclinical and clinical settings, where it will be possible to obtain information about their effects on systemic GCX volume, GCX shedding, and GCX-dependent blood vessel functionality. Third, there is much more to be learned about their timelines for GCX regeneration and potential dose regimens, the factors that interact and interfere with their efficacy, and potential side effects. Fourth, it would be ideal if Arterosil and Endocalyx could be developed and approved for pharmaceutical use. As nutraceuticals, Arterosil and Endocalyx are not currently FDA-approved because the U.S. government only acknowledges the effects of pharmaceuticals.[105] Residing outside of the FDA-approved pharmaceutical milieu, Arterosil and Endocalyx have limited capability of reaching and impacting individuals affected by cardiovascular diseases. Further development of Arterosil and Endocalyx is necessary in order to achieve widespread and effective clinical adoption of GCX regeneration therapy to address cardiovascular disease.

**2.3. Other Possibilities for EC GCX Regeneration.** The most ideal GCX regeneration therapies would trigger ECs to self-produce and overexpress their deficient GCX components. Such therapeutic approaches may be available in the future as an extension of current approaches to engineer Chinese hamster ovary cells to synthesize EC GCX-relevant GAGs.[106] Until then, supplementation of the EC microenvironment with exogenous components of the GCX, to replace what was shed, continues. Clearly this is not a new approach to GCX regeneration, considering that Sulodexide, Arterosil, and Endocalyx, for example, utilize this method of therapy. However, since the Sulodexide, Arterosil, and Endocalyx are not yet considered as standard-of-care, researchers continue to dream of and develop new GCX subcomponent supplementation therapies, many of which show promise *in vitro* and *in vivo* but have yet to advance to clinical stages.

Motivated by the prominence of HS in the EC GCX (HS is 50−90% of the GCX[107]), HS supplementation continues to be a primary focus of GCX regeneration therapy. As shown by Mensah et al. and Cheng et al., following enzymatic degradation of HS by heparinase III, cultured rat fat pad endothelial cells (RFPECs) were exposed to exogenous HS (see Table 2) and responded by re-expressing abundant HS in their GCX coat.[19,108] The repaired GCX was shown to regain its usual barrier function but could not regain its control over complicated and vital GCX-mediated EC functions, such as gap junctional EC-to-EC communication.[19] Perhaps exogenous delivery of another GCX component would be more effective. Although they are less prominent in the EC GCX, other major components, such as SA, HA, and CS, warrant exploration in exogenous form as potential GCX regenerating agents. Exogenous SA, for example, could be very useful, because of its important role in EC GCX structure and microvessel permeability to water and albumin.[4] In an *in vivo* study by Betteridge et al., blood vessels of Sprague−Dawley rats were perfused with neuraminidase, which is an enzyme that specifically degrades SA.[4] Glycocalyx depth and coverage were significantly decreased following 20 min exposure to the enzyme. Thus, it is possible that exogenous SA therapy may contribute to the maintenance or regeneration of GCX structure and its protective barrier function.[4] However, despite its evidenced contribution to the EC GCX, exogenous SA


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treatment has never been attempted for the purpose of GCX regeneration. To our knowledge, the same is true for HA and CS.

The truth is that the power of exogenous delivery of single components of the GCX is dwarfed by therapies that incorporate multiple GCX components and other GCX stabilizing agents simultaneously, such as Endocalyx. The approach of combining multiple compounds into one EC GCX regeneration agent promises to be optimally effective, because of the synergy between the individual compounds, which is essential to EC GCX structure and function. Acknowledging this notion, GCX research pioneers Henry and Duling combined HA with CS (see Table 2).[26] They studied an animal model of male Syrian golden hamsters, enzymatically degraded the EC GCX using hyaluronidase, followed up with an infusion of a mixture of HA and CS, and observed reconstitution of the GCX.[26] Infusions of either HA or CS separately had no effect.[26] Years later, in an *in vitro* study on HUVECs, Potter and Damiano noted an undetectable GCX on cultured ECs.[27] However, when supplemented with combined HA and CS to the cell culture environment at hyperphysiological concentrations, the GCX was detectable.[27] More recently, in a study by Mensah et al., another combination treatment, which consists of HS and S1P (Table 2), was shown to be effective.[19] Via fluorescence confocal microscopy, it was observed that following heparinase III-induced GCX degradation, delivery of HS and S1P successfully regenerated the GCX within a short time frame.[19] Not only did the HS and S1P rebuild the structure of the GCX, but it had the added and unique benefit of restoring complicated and vital GCX-mediated EC functions, such as gap junctional EC-to-EC communication.[19]

There has also been significant effort invested into the development of GCX regeneration therapies that combine only GCX stabilizing agents (excluding any native components of the GCX). For example, Tunac et al. has a patented approach that combines the compounds FTX-214 (melatonin 6,$\beta$-D-xyloside), FTX-218 (lipoate-choline), and FTX-219 (lipoate-cysteine-glutamic tripeptide) for restoration and maintenance of the GCX.[29] FTX-214 has shown protective effects by preventing the buildup of reactive oxygen species, which are known to damage the GCX by boosting antioxidant enzymes.[29] FTX-218 has shown anti-inflammatory properties by neutralizing cytokines and promoting the synthesis of the GCX.FTX-219 has shown GCX-repairing abilities by restoring and synthesizing various components found within the GCX.[29] As another example, Torres et al. utilized an approach that combines beta-hydroxybutyrate with melatonin (BHB/M) (Table 2).[30] BHB is a ketone body synthesized in the liver from fatty acids and acts as an energy carrier when the supply of glucose is low.[30,109] Melatonin is an antioxidant hormone associated with the sleep-wake cycle.[30] Individually, both are available as nutritional supplements.[30] Torres et al. studied the effects of this combination on GCX restoration in Sprague−Dawley male rats. During recovery from hemorrhagic shock, BHB/M treatment restored GCX thickness close to baseline conditions, as measured by the detection of fluorescently labeled dextran solutions. Furthermore, BHB/M treatment resulted in a significant lowering of plasma syndecan-1 levels to those measured in pre-hemorrhagic conditions.[30] Interestingly, BHB/M and the previously mentioned FTX-214 (melatonin 6,$\beta$-D-xyloside) share a commonality in their inclusion of melatonin or its derivatives. Therefore, further research investigating the effects of melatonin and its derivatives on the GCX may be worthwhile.[29,30] For one last example of an approach that combines GCX stabilizing compounds, Torres et al. also studied the combination of adenosine, lidocaine, and magnesium (ALM) (Table 2), which has historically been explored for its relevance to cardioplegia.[110,111] As the subcomponents of ALM are important regulators of metabolism, immunomodulation, inflammation, and coagulation, it should come as no surprise that ALM significantly restored GCX thickness, lowered plasma syndecan-1, and decreased GCX permeability to a level similar to baseline conditions.[30]

Since combination therapies have shown promising GCX regeneration results, because of the synergistic effects of the therapeutic components, as presented above,[19,26,27] continued research efforts are critical to advance the development of combination cocktails that are in progress, or explore new ones.

**2.4. Proposed Targeted Therapy Delivery Approaches for Future Optimization of EC GCX Regeneration.** For future optimization of EC GCX regeneration, researchers must consider implementing strategies to specifically deliver GCX regeneration therapies to the right place. First, strategies are needed for delivery of GCX regeneration therapies specifically to the endothelium, which is a component of the complex systemic vasculature that contains multiple cell types, each of which possesses a GCX, and a plethora of extracellular components, which may resemble the GCX.[43,112−114] Second, strategies are needed to deliver GCX regeneration therapies to only the endothelium of the disease-prone regions of the vasculature where GCX is most eroded, such as the branches of the coronary arteries and the curves of the aortic arch.[6,112] Executing these strategies could increase the efficacy and reduce side effects of the GCX regeneration therapies.[42−46]

Avenues to explore for targeted GCX regeneration therapy delivery include (i) targeting the receptors on the EC surface, (ii) targeting the loss of net negative charge that occurs for a degraded GCX, or (iii) targeting the conformational change and increase in porosity of the GCX. Of these avenues, the most recommended approach to delivering GCX regeneration therapies in a targeted manner is to take advantage of the receptors on the EC surface. Intact EC GCX is a barrier to ligand−receptor bond formation while a degraded GCX exposes EC surface receptors to facilitate ligand−receptor bonds.[112] Therefore, to target endothelium at disease-prone vascular locations where GCX damage is most common, it is possible to package GCX regeneration therapies with ligands that target the various exposed EC surface receptors. The most commonly considered ligands consist of antibodies, specifically immunoglobulin G (IgG)-type antibodies, but if antibodies are too large for the application, then peptides offer alternative options.[115] Antibodies or peptides are available for targeting EC surface adhesion molecules such as E-selectin, intercellular adhesion molecule (ICAM), and vascular cell adhesion molecules (VCAM).[116−119] However, an uncommon approach, simultaneous targeting of E-selectin or ICAM or VCAM with the highly EC-specific Tie2 (receptor for angiopoietin),[120,121] is highly recommended. E-selectin, ICAM, and VCAM have occasionally been found on non-ECs, but there is no other EC surface receptor reported to be as specific for EC-targeting as Tie2.[122−124] Therefore, optimized delivery of GCX regeneration therapy is anticipated with an approach that simultaneously targets Tie2 along with another EC receptor. Once successful targeted therapy delivery is achieved for any GCX regeneration therapy that contains a GCX stabilizing agent (i.e., anti-inflammatory or antioxidant), the EC surface adhesion receptors will provide the added benefit of facilitating endocytosis of the delivered therapeutics into ECs.[112]



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**Table 3. GCX Regenerative Therapies**[a]

| GCX detection method | Overview | Source(s) |
|---|---|---|
| Orthogonal polarization spectroscopy (OPS) imaging | • Estimates perfused boundary region (PBR), an indicator of GCX health<br>• Limited to the superficial vasculature and cannot visualize deeper blood vessels | 68,126,146 |
| Sidestream darkfield (SDF) imaging | • Estimates capillary GCX thickness<br>• Better resolution than OPS, but SDF vessels viewed are still those that are superficial and not very deep | 65,66,127,128,129 |
| Liquid chromatography−mass spectroscopy (LC-MS) | • Quantifies GCX composition and molecular structure; comprehensive<br>• Cannot measure GCX content *in situ*, but can only measure degraded GCX content | 55,131,132,133 |
| Colorimetric assays | • Quantifies GCX composition; inexpensive; rapid<br>• Cannot measure GCX content *in situ*, but can only measure degraded GCX content | 131,134,135,136 |
| Tracer dilution | • Estimates systemic GCX volume<br>• Limited by the reliance on several assumptions to be accurate | 68,137,138 |
| Microparticle image velocimetry ($\mu$-PIV) | • Quantifies velocity of microsphere flow through vasculature and applies fitting function to estimate GCX thickness<br>• Limited to the superficial vasculature and cannot visualize deeper blood vessels | 28,71,147 |

[a]To determine the clinical efficacy of the GCX regenerative therapies, researchers must be able to precisely detect the EC GCX and assess changes in its condition. This can be accomplished clinically via the listed GCX detection methods.

## 3. ENDOTHELIAL GLYCOCALYX ASSESSMENT

As the GCX regenerative approaches discussed above are adopted as the standard of patient care, it will be important for clinicians to be able to precisely detect the EC GCX and assess its changes in vascular health and disease conditions. Currently, there are several detection methods used to visualize, detect, and/or quantify the GCX *in vitro* and *ex vivo*, including traditional molecular biology techniques, such as Western blotting and PCR, as well as microscopy techniques, such as confocal and electron microscopy. However, since these methods are invasive end-point detection techniques, they cannot be used clinically in their current states and, therefore, cannot be applied to assess the GCX health of patients. Available clinical GCX detection methods are very limited compared to more widely used *in vitro* and *ex vivo* methods, especially in their quantifiability and precision. This section highlights the GCX detection methods that are relevant to the clinical realm (see Table 3). In addition, further developments are suggested for increasing the resolution of GCX detection in the clinic.

**3.1. Intravital Microscopy.** Intravital microscopy is the gold standard for visualization of capillary microcirculation throughout the body.[125] One form of intravital microscopy, OPS imaging (Table 3), which was already introduced earlier in this review, offers the capability of visualizing vessels within the body by using linearly polarized light to illuminate the vessel tissue of focus.[126] With OPS, light is cast on tissue with a polarizer orthogonal to the plane of illuminating light, and the photons that do not become polarized create the tissue image.[126] Using OPS imaging, Nieuwdorp et al. devised a technique that approximated the GCX thickness of the individual blood vessels of the sublingual microcirculation.[68] This method, described earlier and presented in Figure 6, is known to underestimate GCX thickness, given that GCX compression does not entirely eliminate its dimension from the erythrocyte region diameter. However, this method offers the ability to show relative differences in GCX thickness, enabling detection of positive or negative changes in GCX health and assessment of GCX regeneration efficacy.

Building upon the approach of OPS is another form of intravital microscopy, SDF imaging (Table 3),[125] which was also introduced earlier (Figure 5). SDF is considered to be an advanced and favored approach because it is able to image microvasculature even deeper within the body than OPS technology.[65] SDF is also considered to offer higher capillary contrast as compared to OPS imaging (Figure 7).[68] The two

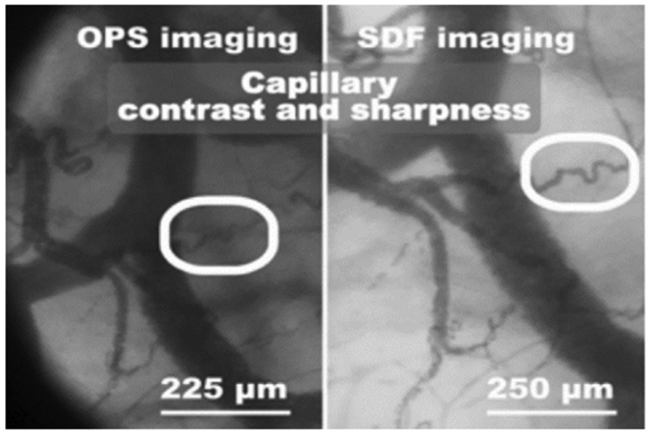


**Figure 7.** Microvessels can be imaged using OPS and SDF imaging. Compared to OPS, SDF offers more contrast and SDF can image and measure deeper microvessels.[125,146] The OPS or SDF images can be used to estimate the microvascular GCX thickness. Reproduced with permission from reference.[65] **Copyright 2008 Springer Nature.**

methods were compared in a study by Bezemer et al., and it was concluded that SDF imaging provides higher quality images with improved capillary contrast and sharpness for better eventual analysis.[65] The SDF probe consists of diodes which emit light of a wavelength which is absorbed by hemoglobin in erythrocytes.[127] Thus, the lateral movement of these erythrocytes can be visualized within blood vessels. SDF technology has since been adapted to a software called GlycoCheck, which analyzes the images captured by SDF to determine a PBR, as described earlier and presented in Figure 5.[69] In a study by Rovas et al. on


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patients in the emergency room and intensive care unit, noninvasive measurements were taken of the sublingual microcirculatory GCX by SDF imaging. GlycoCheck software was used to analyze the PBR, and it was concluded that this method could become a clinical standard in daily patient assessment protocols and clinical trials.[69] Furthermore, in a study by Lee et al., the PBR was assessed in blood vessels between an erythrocyte column width of 5 and 25 $\mu$m via SDF imaging in conjunction with the GlycoCheck software.[67] Therefore, the use of SDF imaging and GlycoCheck has become standard for GCX detection in clinical arenas. Unfortunately, neither SDF or its predecessor OPS can estimate the EC GCX in deep vessels beyond the sublingual regions, although the deeper vessels are of significant clinical relevance.[65,66] These deeper regions of the vasculature, especially when marked by disturbed blood flow due to branching and other geometric adaptations, are among the most susceptible to atherosclerotic development and vascular disease.[128]

Thus, to expand the capabilities and benefits offered by SDF, it is advisible to explore ways to expand this technology for visualization of the vasculature deeper within the body, specifically regions susceptible to GCX degradation and plaque formation such as the branches of the carotid and coronary arteries, as well as the aortic arch. For example, Berhouma et al. used SDF imaging intraoperatively in order to visualize the intracranial meningiomas during surgery.[129] This has been one of the first successful attempts to use SDF imaging beyond the sublingual vasculature, though this study did not specifically look at the GCX.[129] An alternative expansion of SDF to the deeper vasculature may lie in a probe technology delivered through a catheter intravascularly. The first such attempt, made by Bec et al., combined ultrasound with multispectral fluorescence lifetime imaging (FLIM) in order to gather morphological and biochemical information, as well as characterize atherosclerotic plaque pathophysiology on the swine coronary vessels *in vivo*.[130] Unfortunately, the method does not currently allow for detection of the GCX, but this could change if it were possible to integrate the technology of SDF and GlycoCheck with this catheter approach.[129,130]

**3.2. Analytical Techniques.** Beyond SDF and OPS, there are alternate clinical detection methods that gather information about the health of the GCX without using qualitative intravital imaging technology. One such method, liquid chromatography−mass spectroscopy (LC-MS) (Table 3), is an analytical detection method that can determine information about a sample's molecular weight and structure.[55,131] Often used to analyze numerous biological molecules, it can detect the quantity and structure of various glycans found within the GCX.[55,131,132] The LC component is used to separate the different components of the blood plasma or urine.[131] These components are then analyzed via MS, where they are fragmented to determine GAG composition and quantity. For example, LC-MS has been used by Sun et al. to analyze the urine of patients suffering from acute respiratory distress syndrome (ARDS).[132,133] The authors demonstrated that the elevated level of urinary GAG fragmentation predicted the development of acute kidney injury. Additionally, this elevation of GAGs in the urine could be tied to glomerular endothelial GCX disruption or the release of GAGs (which are filtered by the damaged glomerulus) from endothelium in other organs.[132,133] Similarly, Yeo et al. used LC-MS to detect elevated urinary GAG compounds in the patients suffering from cerebral malaria.[131] Thus, LC-MS offers researchers with a quantitative and structure-focused avenue to measure GCX degradation.

Further within the analytical detection field, colorimetric assays (Table 3) offer a simpler quantification method to detect systemic GAG content using a sample's absorbance or reflectance of light at specific wavelengths.[131,134] One such assay uses dimethyl methylene blue (DMMB) to detect highly sulfated GAGs, including HS and CS, within the urine or blood. Yeo et al. used this assay to observe GCX breakdown products in urine for patients exhibiting kidney failure.[134] Additionally, ELISA colorimetric assay can also be a resource to detect systemic GAG shedding within urine or blood plasma samples.[135] Rahbar et al. was able to observe increased shedding of three GAG proteins (CS, HS, and HA) and one core protein (syndecan-1) in severely injured trauma patients upon admission to the emergency department in comparison to healthy consented volunteers.[135] Thus, colorimetric assays, like DMMB or ELISA, can help offer clinicians a fast and cost-efficient method of GCX assessment. Note, however, that colorimetric assays only offer a specific quantitative measurement rather than the comprehensive structural analysis provided through LC-MS.

Additionally, specificity of antibody based detection methods like ELISA for detecting GAGs is dependent on the type of antibody (monoclonal vs polyclonal) that is utilized in the test. For instance, Tomatsu et al.[136] observed that the monoclonal antibody used for sandwich ELISA is specific for Galactose l$\beta$1(Gal$\beta$1), where both galactose and N-acetyl-glucosamine have to be sulfated. Hence, the analytical sandwich ELISA method did not provide quantification of total keratan sulfate (KS), which is another GAG.[136] Tomatsu et al.[136] noticed total KS measured through LC-MS was 10−100 times higher than measured through sandwich ELISA method, which showed that the ELISA method could underestimate total and relative levels of KS due to poor quantitation of different degrees of KS polymerization. Finally, LC-MS method is also more sensitive than the ELISA method. Tomatsu et al.[136] observed that the ELISA method could not detect below 2.5 ng/mL of KS; however, LC-MS method could detect 0.2 ng/mL of KS. Even though ELISA is another feasible and reproducible method, improvements in the variety of measurable GAGs, multi-GAG ELISA detection, and its sensitivity are needed.

**3.3. Speculative Methods.** An alternative method that should be mentioned, though it should be considered as purely speculative as it may have limited efficacy for GCX detection in the clinic, is tracer dilution (Table 3). This method that was first described by Nieuwdorp et al. was previously discussed in this review. To recap with some elaboration, the tracer dilution method relies on a series of blood draws and estimates systemic GCX volume by comparing the intravascular distribution volume of a GCX-permeable tracer, dextran 40.[68,70,137,138] The first blood draw offers an approximation of the circulating plasma volume in the blood. Subjects are then infused with the GCX permeable tracer dextran 40. Dextran 40 is assumed to fill the entire lumen of the micro vessel including the space occupied by the GCX (due to its GCX permeability); the distribution of dextran 40 is assumed to correspond to the cumulative volume of the plasma and the GCX. After infusion, a second blood draw is performed. The difference between the two blood draw volume measurements can then be used to approximate GCX volume.[68] In a review paper, Michel et al. offers further analysis of this technique as well as the work done


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by Nieuwdorp et al., suggesting experimental protocol changes to improve the accuracy of estimation.[68,70,137,138]

Another speculative method of interest is fluorescent microparticle image velocimetry ($\mu$-PIV), which works in a manner similar to OPS or SDF. Potter et al. implemented $\mu$-PIV to measure fluorescent microparticle velocities and then to compile the measurements to estimate the velocity profile of the particles.[28] To create an accurate velocity profile, there was simultaneous viewing of the vessel or channel wall with the flowing microparticles. This was achieved by using a beam splitter to separate the infrared transillumination (to view the microparticles) from the epifluorescent red light pathway (to view the vessel or channel wall).[28] The views were superimposed, and researchers were then able to measure the radial position of each microsphere relative to the vessel wall (Figure 8). The finalized velocity profile was then used to perform a

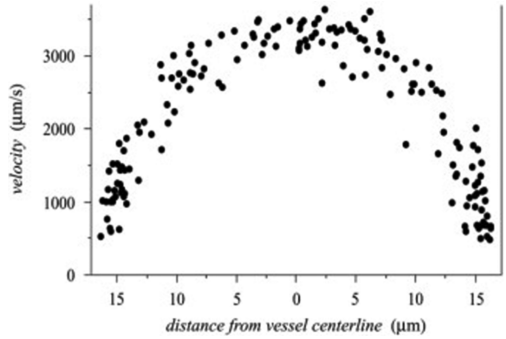


**Figure 8.** EC GCX thickness can be estimated by hydrodynamically analyzing the shown velocity profile and taking the region of highest hydraulic resistivity to motion as the EC GCX. The shown velocity profile comes from $\mu$-PIV and indicates the translational speed of each microsphere, as a function of its measured radial position relative to the vessel centerline. The velocity profile decreases with increasing radial position. The closer a particle is to the impermeable GCX, the slower its velocity. [Reproduced with permission from ref 147. Copyright 2004, Cambridge University Press..]

hydrodynamic analysis and the region of highest hydraulic resistivity to particle motion was taken as the EC GCX.[28,71] This method was applied to successfully track the recovery of the hydrodynamically relevant mouse cremaster-muscle venule GCX after enzymatic or cytokine-mediated degradation, for 1, 3, 5, and 7 days following the degradative input.[28] Unfortunately, similar to OPS and SDF, $\mu$-PIV usage is limited to the superficial vasculature and cannot visualize deeper blood vessels.

Dreaming big about the future of GCX detection in the clinic, the ideal method would be a magnetic resonance imaging (MRI) and/or magnetic resonance angiography (MRA) protocol. MRI and MRA offer excellent visualization of blood vessels that are most affected by atherosclerosis and responsible for cardiovascular disease. However, methods for labeling and rendering the GCX or its subcomponents detectable with MRI/MRA do not exist. Given that albumin is a component of the intact GCX, the closest option might be to use an intravascular contrast agent that binds to albumin, Gadafosveset.[139] Clinical trials show that Gadofosveset is safe and well tolerated in patients with vascular disease,[139] making it a viable contrast agent to enable GCX detection (indirectly) using MRI/MRA. Given the role that the GCX plays in vascular health, it would be valuable for clinicians to be able to precisely visualize and quantify the GCX structure.

## 4. CONCLUSION

In closing, significant progress has been made to develop GCX regenerative therapies. The most promising therapies combine multiple compounds to replace missing GCX components and overexpress GCX stabilizing factors (i.e. immunosuppressants or antioxidants). Optimizing the success of these therapies will require their targeted delivery to the endothelium in vascular regions that are specifically prone to or affected by cardiovascular disease, *in lieu* of systemic delivery. Clinical assessment of the success of these therapies will require improvement of existing GCX detection methods to facilitate visualization and quantification of the GCX structure in deep blood vessels and enable *in situ* measurement of GCX composition and molecular structure. Successfully overcoming the described challenges will result in tremendous advances toward the treatment of debilitating and widespread cardiovascular diseases.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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### Notes

The authors declare no competing financial interest.

## Biographies

**Selina Banerjee** received her bachelor's degree in Chemical Engineering from Northeastern University in 2021. She completed her undergraduate honors research project on endothelial glycocalyx characterization and regeneration under the supervision of Professor Eno Ebong. Selina is currently a Ph.D. student at Northeastern University in the Department of Chemical Engineering and is working on vascular tissue engineering and regeneration.

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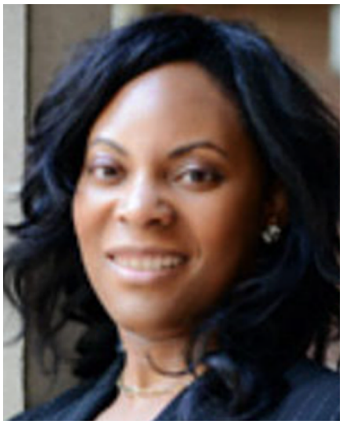
**Theodora Stanley** is a student at Northeastern University, completing her bachelor's degree in health science. Her research attempts to synthesize a drug compound for endothelial cell glycocalyx regeneration.

**Ronodeep Mitra** is a fourth-year PhD student in the Department of Chemical Engineering at Northeastern University. He received his bachelor's degree in chemical engineering from Georgia Institute of Technology (2016) where he conducted research in Ajit Yoganathan's Cardiovascular Fluid Mechanics Lab, providing clinicians with an engineering basis of optimal placement of a transcatheter aortic valve replacement within a patient via fluid dynamic parameters. Additionally, he earned a master's degree in bioengineering at Northeastern University (2018), where he completed a research thesis in Eno Ebong's Mechanobiology Lab determining the overall impact of two risk factors of atherosclerosis, high fat diet and blood flow patterns, on endothelial glycocalyx health. In Fall 2018, Ronodeep started his doctoral studies in chemical engineering at Northeastern University under the direction of Eno Ebong. His dissertation research focuses on improving cardiovascular disease outcomes via early disease intervention, which can be achieved by understanding, targeting, and rebuilding the endothelial glycocalyx.



**Dr. Eno Essien Ebong** earned her bachelor's degree (1999) in Mechanical Engineering from the Massachusetts Institute of Technology in Boston, MA and her master's (2001) and doctoral (2006) degrees in the area of biomechanics from the Department of Biomedical Engineering at Rensselaer Polytechnic Institute in Troy, NY. She was a National Institutes of Health Cardiovascular Research Fellow (2007 to 2012) at the Albert Einstein College of Medicine in New York, NY. Currently, Dr. Ebong is an Associate Professor of Chemical Engineering, Bioengineering, and Biology at Northeastern University. She recently received the prestigious NSF CAREER Award, for her "EMBRACE STEM (Endothelial MechanoBiology Research And multiCultural Education in STEM)" project. She was previously awarded the NIH Mentored Research Career Development Award (K01), for studying "Atheroprotective vs Atherogenic Glycocalyx Mechanotransduction Mechanisms".



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## ■ ABBREVIATIONS

AFM = atomic force microscopy
bEND.3 = immortalized mouse brain microvascular endothelial cells
CS = chondroitin sulfate
EC = endothelial cell
ELISA = enzyme-linked immunosorbent assay
FFP = fresh frozen plasma
FITC = fluorescein isothiocyanate
GAG = glycosaminoglycan
GCX = glycocalyx
HA = hyaluronic acid
HS = heparan sulfate
HUVEC = human umbilical vein endothelial cell
LDL = low-density lipoprotein
LPS = lipopolysaccharide
$\mu$-PIV = microparticle image velocimetry
NO = nitric oxide
OPS = orthogonal polarization spectroscopy (imaging)
PBR = perfused boundary region
RFPEC = rat fat pad endothelial cell
RS = rhamnan sulfate
SA = sialic acid
SDF = sidestream darkfield (imaging)
S1P = sphingosine-1-phosphate
WGA = wheat germ agglutinin

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