

RESEARCH ARTICLE | *Vascular Biology and Microcirculation*

Advanced age results in a diminished endothelial glycocalyx

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¹Geriatric Research, Education, and Clinical Center, Veterans Affairs Salt Lake City, Salt Lake City, Utah; ²Department of Internal Medicine, University of Utah, Salt Lake City, Utah; ³Department of Nutrition and Integrative Physiology, University of Utah, Salt Lake City, Utah; ⁴Molecular Medicine, University of Utah, Salt Lake City, Utah; and ⁵Department of Biochemistry, University of Utah, Salt Lake City, Utah

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Machin DR, Bloom SI, Campbell RA, Phuong TTT, Gates PE, Lesniewski LA, Rondina MT, Donato AJ. Advanced age results in a diminished endothelial glycocalyx. *Am J Physiol Heart Circ Physiol* 315: H531–H539, 2018. First published May 11, 2018; doi:10.1152/ajpheart.00104.2018.—Age-related microvascular dysfunction is well characterized in rodents and humans, but little is known about the properties of the microvascular endothelial glycocalyx in advanced age. We examined the glycocalyx in microvessels of young and old male C57BL6 mice (young: 6.1 ± 0.1 mo vs. old: 24.6 ± 0.2 mo) using intravital microscopy and transmission electron microscopy and in human participants (young: 29 ± 1 yr vs. old: 60 ± 2 yr) using intravital microscopy. Glycocalyx thickness in mesenteric and skeletal muscle microvessels was 51–54% lower in old compared with young mice. We also observed 33% lower glycocalyx thickness in the sublingual microcirculation of humans in advanced age. The perfused boundary region, a marker of glycocalyx barrier function, was also obtained using an automated capture and analysis system. In advanced age, we observed a 10–22% greater perfused boundary region in mice and humans, indicating a more penetrable glycocalyx. Finally, using this automated analysis system, we examined perfused microvascular density and red blood cell (RBC) fraction. Perfused microvascular density is a marker of microvascular function that reflects the length of perfused microvessel segments in a given area; RBC fraction represents the heterogeneity in RBC presence between microvessel segments. Compared with young, the perfused microvascular density was 16–21% lower and RBC fraction was 5–14% lower in older mice and in older humans. These data provide novel evidence that, across mammalian species, a diminished glycocalyx is present in advanced age and is accompanied by markers of impaired microvascular perfusion. Age-related glycocalyx deterioration may be an important contributor to microvascular dysfunction in older adults and subsequent pathophysiology.

NEW & NOTEWORTHY Advanced age is characterized by microvascular dysfunction that contributes to age-related cardiovascular diseases, but little is known about endothelial glycocalyx properties in advanced age. This study reveals, for the first time, lower glycocalyx thickness and barrier function that is accompanied by impaired microvascular perfusion in both mice and humans in advanced age.

aging; electron microscopy; intravital microscopy; microcirculation; microvascular perfusion

INTRODUCTION

Age-related microvascular dysfunction is well characterized in rodents and humans, and the aged microcirculation exhibits impaired perfusion and lower capillary density (1, 12, 37). However, little is known about the structure and function of the microvascular endothelial glycocalyx in advanced age. The glycocalyx is a gel-like structure that lines the luminal surface of the vascular endothelium (33, 46), and it is composed of proteoglycans, glycoproteins, and glycolipids (7). Within the microvasculature, the glycocalyx has several important functions, including modulating flow resistance to maintain microcirculatory blood flow homogeneity (19, 32), stimulating nitric oxide release by mechanotransduction of fluid shear stress to the endothelium (33, 48), and protecting endothelial cells from oxidative stress (18), inflammation (25), and platelet (45) and leukocyte adhesions (26). A diminished glycocalyx is present in several age-related chronic diseases, such as cardiovascular disease (CVD) (23, 35), end-stage renal disease (47), and diabetes (2, 12, 29). However, it is not known if a diminished glycocalyx occurs in primary aging.

In the present study, we sought to determine if the glycocalyx is diminished in advanced age. We hypothesized that glycocalyx thickness and barrier function would be lower in advanced age and that the hypothesized age-related glycocalyx deterioration would be accompanied by markers of impaired microvascular perfusion. To test this hypothesis, we examined glycocalyx thickness using intravital microscopy and transmission electron microscopy in the microvasculature of multiple tissues in mice. To gain insight into glycocalyx function, we used intravital microscopy equipped with an automated capture and analysis system to measure markers of glycocalyx barrier function and microvascular perfusion. These intravital microscopy measurements were also measured in the sublingual microcirculation of humans.

METHODS

Animal Intravital Microscopy

Animals. Young (4–6 mo) male C57BL6 mice were obtained from Charles River Laboratories, and old (23–27 mo) male C57BL6 mice were obtained from the National Institute of Aging colony maintained by Charles River Laboratories. Animals used in this study were housed in an animal care facility at the Veterans Affairs Medical Center-Salt Lake City on a 12:12-h light-dark cycle and fed standard rodent chow (Teklad Diet no. 8604, Envigo) ad libitum. Animal experiments were performed under nonfasting conditions, conformed

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with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (8th ed.) (27), and were approved by the University of Utah and Veterans Affairs Medical Center-Salt Lake City Institutional Animal Care and Use Committees.

Intravital microscopy. The mesenteric and gastrocnemius microcirculations were observed using intravital microscopy. For the mesenteric preparation, mice were anesthetized with 2% isoflurane and placed in the supine position on a heated platform (37°C). An incision was made along the linea alba, and the intestines were mobilized, gently exteriorized, and placed into a warm isotonic 0.9% saline bath (37°C). Subsequently, the cecum was identified and positioned for intravital microscopy. For the gastrocnemius preparation, mice were anesthetized with 2% isoflurane and placed in the prone position on a heated platform (37°C). An incision was made along the posterior hindlimb, and the biceps femoris posterior was dissected to expose the gastrocnemius; an isotonic 0.9% saline solution was used to suffuse the muscle. Subsequently, the medial gastrocnemius was identified and positioned for intravital microscopy. Intravital microscopy was performed with a CapiScope handheld video capillary microscope (KK Research Technology, Honiton, UK) to view the mesenteric and gastrocnemius microcirculation. The intravital microscope uses a sidestream dark field camera that uses green light-emitting diodes and captures video recordings of the microcirculation at 20 frames/s. The green light is primarily absorbed by hemoglobin in red blood cells (RBCs) in the microcirculation, which allows RBCs to be viewed in contrast to the background.

Glycocalyx thickness. We derived an estimate of glycocalyx thickness by measuring the change in perfused diameter immediately before and after the passage of a spontaneous leukocyte in individual microvessels, as previously described and validated (19, 28). Leukocytes cannot freely penetrate the glycocalyx, and because of their size they compress the glycocalyx as they flow through microvessels, increasing the RBC flow width. The perfused diameter was determined with ImageJ by drawing a line segment perpendicular to the microvessel. To minimize experimental error, we determined glycocalyx thickness in microvessels smaller than 8 μm in diameter. Additionally, pre- and postleukocyte perfused diameter measurements were made at the same site, one to three frames before and one to five frames after leukocyte passage, respectively. Glycocalyx thickness was calculated using the following equation (28):

$$\text{Glycocalyx thickness} = \frac{(\text{postleukocyte perfused diameter} - \text{preleukocyte perfused diameter})}{2}$$

Automated measurement of microvascular function. Video of the microcirculation was recorded and analyzed using an automated capture and analysis system (GlycoCheck, MicroVascular Health Solutions, Alpine, UT). We have described these automated measurements of microvascular function in detail previously (20). Briefly, each trial lasts 2–3 min and consists of several 2-s video recordings that are 40 frames in length. In each recording, microvessels with a 5- to 25-μm lumen diameter are identified by differences in contrast between RBCs and the background and are subsequently divided into 10-μm-long microvessel segments. Video recordings are repeated until at least 3,000 microvessel segments have been acquired, concluding the trial. Thereafter, the automated analysis system determines functional outcomes in perfused microvessel segments, defined as any segment with sufficient contrast that contains RBCs in ≥50% of its length in the first frame of a 40-frame video recording session.

Perfused boundary region. Perfused boundary region (PBR) represents the depth of RBC penetration into the endothelial glycocalyx and is taken as a marker of glycocalyx barrier function, with a larger PBR indicating greater glycocalyx penetrability. The automated analysis algorithm identifies the median RBC flow width and maximal RBC flow width in each perfused microvessel segment. PBR was reported as an average across microvessel segments with a 5- to

25-μm lumen diameter and was calculated using the following equation (17):

$$\text{PBR} = \frac{(\text{maximal RBC flow width} - \text{median RBC flow width})}{2}$$

Perfused microvascular density. Perfused microvascular density represents the cumulative length of perfused microvessel segments in a given area of tissue. Perfused microvascular density was reported as a sum across microvessel segments with a 5- to 25-μm lumen diameter as well as at each individual microvessel segment diameter. Perfused microvascular density was calculated using the following equation (17):

$$\text{Perfused microvascular density} = \frac{(\text{number of perfused microvessel segments} \times 10)}{\text{tissue area recorded}}$$

RBC fraction. RBC fraction represents an estimate of the longitudinal tube hematocrit in perfused microvessel segments. To determine RBC fraction, the automated system places 10 marker lines spaced 0.5 μm apart on each side of the perfused microvessel segment marker line (21 marker lines total). RBC fraction was calculated as the percentage of marker lines crossed by RBCs in each perfused microvessel segment. This was repeated in the same microvessel segment in all 40 frames of a video recording and then averaged. RBC fraction was reported as an average across microvessel segments with a 5- to 25-μm lumen diameter.

Animal Electron Microscopy

Animals. Young and old male C57BL6 mice were used in this study, as described in *Animal Intravital Microscopy* above.

Transmission electron microscopy. Transmission electron microscopy was used to assess ex vivo glycocalyx thickness in the soleus muscle. The soleus muscle was selected as it is a longitudinal muscle with capillaries that are easily imaged with an electron microscope. Thoracotomy was performed on the animal under isoflurane anesthesia, and ~10 ml of primary fixative [2% glutaraldehyde, 2% sucrose, and 2% nitrate lanthanum hexahydrate in 0.1 M cacodylate buffer (pH 7.4)] was perfused through the left ventricle of the heart with a 21-gauge needle connected to a container filled with the primary fixative that was suspended above the animal at a height of 40 cm, which corresponds to a hydrostatic pressure of ~30 mmHg. After needle placement in the left ventricle, the inferior vena cava was cut to allow for outflow of blood and perfused fixative. Immediately after perfusion, tissues were dissected, placed in the primary fixative, and shielded from light on a rocker for 24 h at 4°C. Tissues were brought to the University of Utah Electron Microscopy Core Laboratory for further processing (6), where the samples were later imaged using a JEOL-1400 plus (JEOL) transmission electron microscope equipped with a charge-coupled device Gatan camera.

Glycocalyx thickness. After measuring the lumen perimeter and glycocalyx area, we calculated glycocalyx thickness in individual soleus microvessels using the following equations:

$$\text{Lumen perimeter} = \text{lumen diameter} \times \pi$$

$$\text{Lumen area} = \text{lumen radius}^2 \times \pi$$

$$\text{Glycocalyx-free diameter} = 2 \times \sqrt{\frac{(\text{lumen area} - \text{glycocalyx area})}{\pi}}$$

Quantitative PCR

mRNA expression of hyaluronan synthase isoforms 1, 2, and 3 (*Has1*, *Has2*, and *Has3*), hyaluronidase isoforms 1 and 2 (*Hyal1* and *Hyal2*), and hyaluronan and proteoglycan link protein 1 (*Hapln1*) were measured in aortas of young and old male C57BL6 mice by quantitative PCR. Briefly, RNA isolated from aortic tissue was used to

synthesize cDNA via a QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA). Quantitative PCR was performed using RT² SYBR Green quantitative PCR Master Mix (Qiagen). The fold change in mRNA expression was calculated as the fold difference in expression of target mRNA to 18S rRNA using the following equation: $2^{-(\text{target } C_t - 18S C_t)}$, where C_t is threshold cycle, and normalized to young values. Primer sequences were as follows: *18S*, forward 5'-TAGAGGGACAAGTGGCGTTC-3' and reverse 5'-CGCTGAGC-CAGTCAGTGT-3'; *Has1*, forward 5'-AGAACAAGACGGAGAA-GAGAGA-3' and reverse 5'-CTCTGAGGGCTTTGGCATGT-3'; *Has2*, forward 5'-TCAGCGAAGTTATGGGCAGG-3' and reverse 5'-TCTGTCTCACCAGGTCCCTT-3'; *Has3*, 5'-TAACACCTTC-TTTGACCAGCC-3' and reverse 5'-AAACGGGCTACCCAGAA-AAA-3'; *Hyal1*, forward 5'-TCTGTTACTGCCTCAATAAAA-CCC-3' and reverse 5'-AAAGACTGCCCGGCTAGGT-3'; *Hyal2*, forward 5'-CGCTGGTTCGAGTTCCTG-3' and reverse 5'-CAT-TCTTGTGTGGGCACGTT-3'; and *Hapln1*, forward 5'-CCCCGT-CTACTTGTGGAAG-3' and reverse 5'-TCCTGAGCCAAATGCT-GTAGG-3'.

Human Intravital Microscopy

Participants. A total of 60 adults (30 young and 30 old participants) were recruited from the general population. Participants were recruited based on no evidence of previous vascular disease or chronic medical conditions and were not taking any medications that would impact vascular function (i.e., participants on vasodilatory medications were excluded). All procedures were approved by the Institutional Review Board of the University of Utah. The nature, benefits, and risks of the study were explained to the participants, and their written informed consent was obtained before participation. All participants were nonsmokers and were asked to refrain from alcohol and/or caffeine consumption within 12 h of testing as well as food consumption within 1 h of testing.

Participant characteristics. For all participants, body mass index was calculated from body mass and height. Brachial arterial blood pressure measurements were made with a semiautomated blood pressure device (Omron Healthcare, Lake Forest, IL) in the upright seated position, as previously described (21).

Intravital microscopy. The sublingual microcirculation was observed using a CapiScope handheld video capillary microscope (KK Research Technology). We have previously described this technique

in humans (20). Determination of glycolyx thickness, as well as automated measurements of PBR, perfused microvascular density, and RBC fraction, was analyzed using the procedures described in *Animal Intravital Microscopy* above.

Statistical Analysis

Statistics were performed using SPSS software (IBM, Chicago, IL). Unpaired *t*-tests were used to evaluate differences between young and old groups. Because systolic blood pressure was significantly higher in old compared with young human participants, we used analysis of covariance with systolic blood pressure or sex as covariates to determine if they had any effect on the dependent variables. Two-way repeated-measures ANOVA was also used to evaluate differences in perfused microvascular density across microvessel segment lumen diameters between young and old groups, and a least-significant-difference unpaired *t*-test identified values that were significantly different. The Pearson correlation coefficient was used to assess correlations between PBR and glycolyx thickness in animal and human models. Statistical significance was set at $P < 0.05$ for all analyses. Data are presented as means \pm SE.

RESULTS

Animal Intravital Microscopy

Animals. Young and old male C57BL6 mice used for intravital microscopy studies were 6.1 ± 0.3 mo ($n = 20$) and 24.6 ± 0.2 mo ($n = 20$) for mesenteric microcirculation experiments and 5.9 ± 0.3 mo ($n = 10$) and 25.2 ± 0.1 mo ($n = 10$) for gastrocnemius microcirculation experiments. Compared with young mice, old mice had significantly higher body mass in both mesenteric (young: 28.6 ± 0.7 g vs. old: 30.9 ± 0.6 g, $P < 0.05$) and gastrocnemius (young: 29.1 ± 0.7 g vs. old: 30.9 ± 0.6 g, $P < 0.05$) microcirculation experiments.

Glycolyx properties. Glycolyx thickness was 51–54% lower in the mesenteric and gastrocnemius microcirculations of old compared with young mice ($P < 0.05$; Fig. 1, A and B). Additionally, we observed no difference in average postleukocyte perfused diameter between the age groups in both mes-

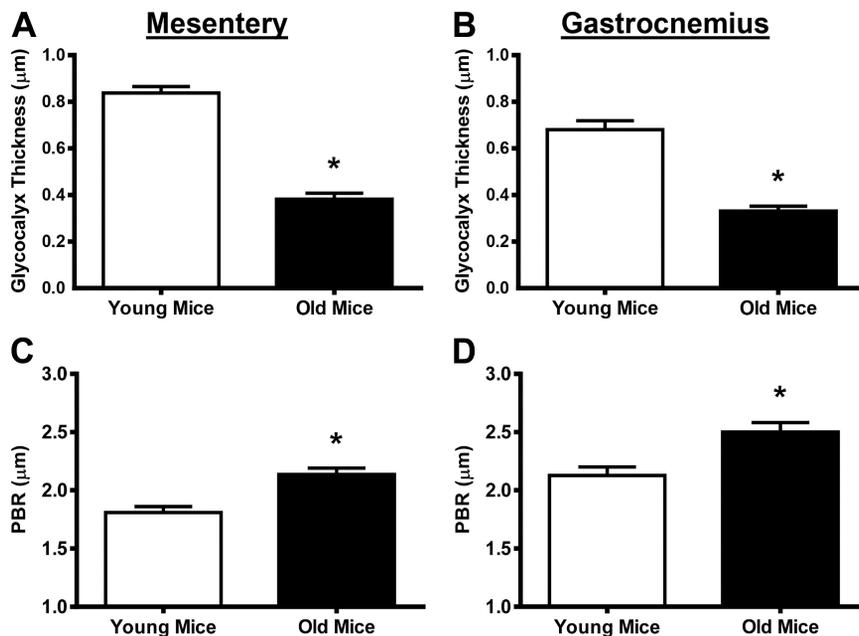


Fig. 1. Glycolyx thickness in mesenteric (A) and gastrocnemius (B) microvessel segments (4–8 μm and 4–6 μm , respectively) and perfused boundary region (PBR) in mesenteric (C) and gastrocnemius (D) microvessel segments (5–25 μm) of young and old C57BL6 mice ($n = 10$ –20 per group). * $P < 0.05$, significant difference vs. young mice. All data are means \pm SE.

enteric (young: $5.5 \pm 0.2 \mu\text{m}$ vs. old: $5.4 \pm 0.2 \mu\text{m}$, $P > 0.05$) and gastrocnemius (young: $5.1 \pm 0.2 \mu\text{m}$ vs. old: $4.9 \pm 0.2 \mu\text{m}$, $P > 0.05$) microcirculations. Mean PBR was 15–22% higher in old compared with young mice in both mesenteric and gastrocnemius microcirculations ($P < 0.05$; Fig. 1, C and D). There was a significant inverse relationship between PBR and glycocalyx thickness in the mesenteric and gastrocnemius microcirculations of mice ($R = -0.39$ and -0.47 , respectively, $P < 0.05$).

Markers of microvascular perfusion. Perfused microvascular density was 16–21% lower in old compared with young mice in both mesenteric (young: $3,446 \pm 143 \mu\text{m}/\text{mm}^2$ vs. old: $2,735 \pm 170 \mu\text{m}/\text{mm}^2$, $P < 0.05$) and gastrocnemius (young: $3,902 \pm 335 \mu\text{m}/\text{mm}^2$ vs. old: $3,277 \pm 125 \mu\text{m}/\text{mm}^2$, $P < 0.05$) microcirculations. Assessment of perfused microvascular density in microvessel segments of different lumen diameters revealed that differences between young and old mice tended to be more prevalent in the smallest size microvessel segments (i.e., 5–9 μm in diameter) for both mesenteric and gastrocnemius microcirculations ($P < 0.05$; Fig. 2, A and B). Compared with young mice, the RBC fraction was 7–14% lower in old mice in both mesenteric and gastrocnemius microcirculations ($P < 0.05$; Fig. 2, C and D).

Animal Electron Microscopy

Animals. Young and old male C57BL6 mice used for electron microscopy experiments were 5.4 ± 0.6 mo ($n = 6$) and 27.2 ± 1.2 mo ($n = 6$). There were no differences in body mass between young and old mice for electron microscopy experiments (young: 30.1 ± 0.8 g vs. old: 30.2 ± 1.1 g, $P > 0.05$).

Glycocalyx thickness. Transmission electron microscopy of the soleus muscle microvessels revealed that glycocalyx thickness was 53% lower in old compared with young mice ($P < 0.05$; Fig. 3A). On average, glycocalyx thickness was determined in 6 microvessels/animal. We observed no difference in

average microvessel lumen diameters used for the determination of glycocalyx thickness between the age groups (young: $4.9 \pm 0.5 \mu\text{m}$ vs. old: $4.9 \pm 0.5 \mu\text{m}$, $P > 0.05$).

Quantitative PCR

Animals. Young and old male C57BL6 mice used for gene expression experiments were 5.4 ± 0.1 mo ($n = 10$) and 24.3 ± 0.2 mo ($n = 10$). Compared with young mice, old mice used for quantitative PCR had a significantly higher body mass (young: 29.0 ± 0.4 g vs. old: 30.5 ± 0.7 g, $P < 0.05$).

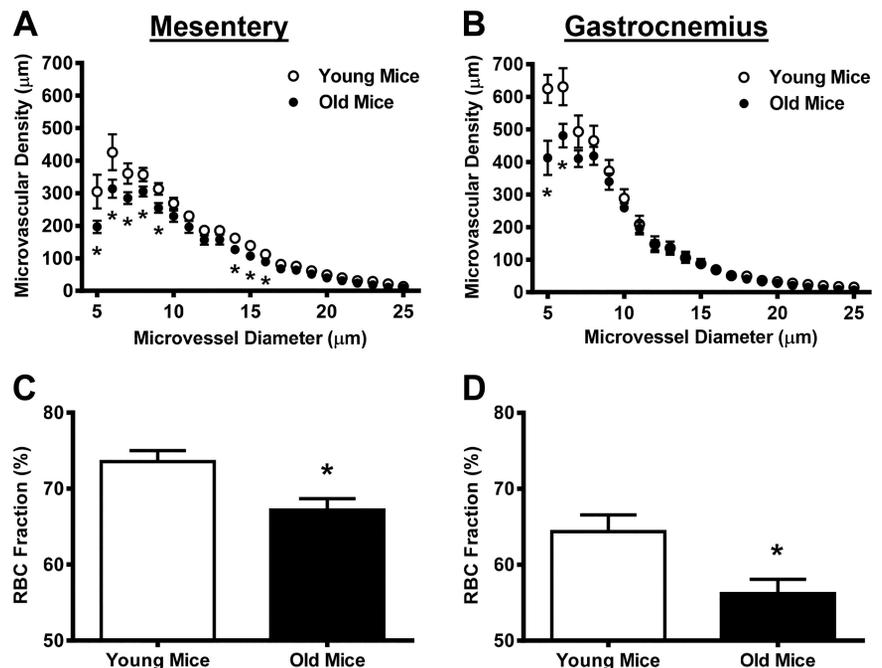
Gene expression. Aortic gene expression of *Has2* was lower in old compared with young mice ($P < 0.05$; Fig. 3C), whereas *Has1* and *Has3* gene expression was similar in old and young mice ($P > 0.05$). *Hyal1* and *Hyal2* gene expression was lower in old compared with young mice ($P < 0.05$). Finally, there was a trend for greater *Hapln1* gene expression in old compared with young mice ($P = 0.06$).

Human Intravital Microscopy Experiments

Participants. Participant characteristics are shown in Table 1. Older participants were similar in body stature; however, they had significantly higher systolic blood pressure than young participants, although still within the normotensive range ($P < 0.05$; Table 1). Thus, we used analysis of covariance with systolic blood pressure as a covariate, but the results from these models did not differ from those reported below.

Glycocalyx properties. Glycocalyx thickness was 33% lower in old compared with young participants ($P < 0.05$; Fig. 4A). We observed no difference in average postleukocyte perfused diameter between the age groups in the sublingual (young: $7.2 \pm 0.1 \mu\text{m}$ vs. old: $7.1 \pm 0.1 \mu\text{m}$, $P > 0.05$) microcirculation. Mean PBR was significantly higher in old compared with young participants ($P < 0.05$; Fig. 4B). There was a significant inverse relationship between PBR and glycocalyx thickness in the sublingual microcirculation of humans ($R = -0.27$, $P < 0.05$).

Fig. 2. Perfused microvascular density in mesenteric (A) and gastrocnemius (B) microvessel segments (5–25 μm) and red blood cell (RBC) fraction in mesenteric (C) and gastrocnemius (D) microvessel segments (5–25 μm) of young and old C57BL6 mice ($n = 10$ –20 per group). * $P < 0.05$, significant difference vs. young mice. All data are means \pm SE.



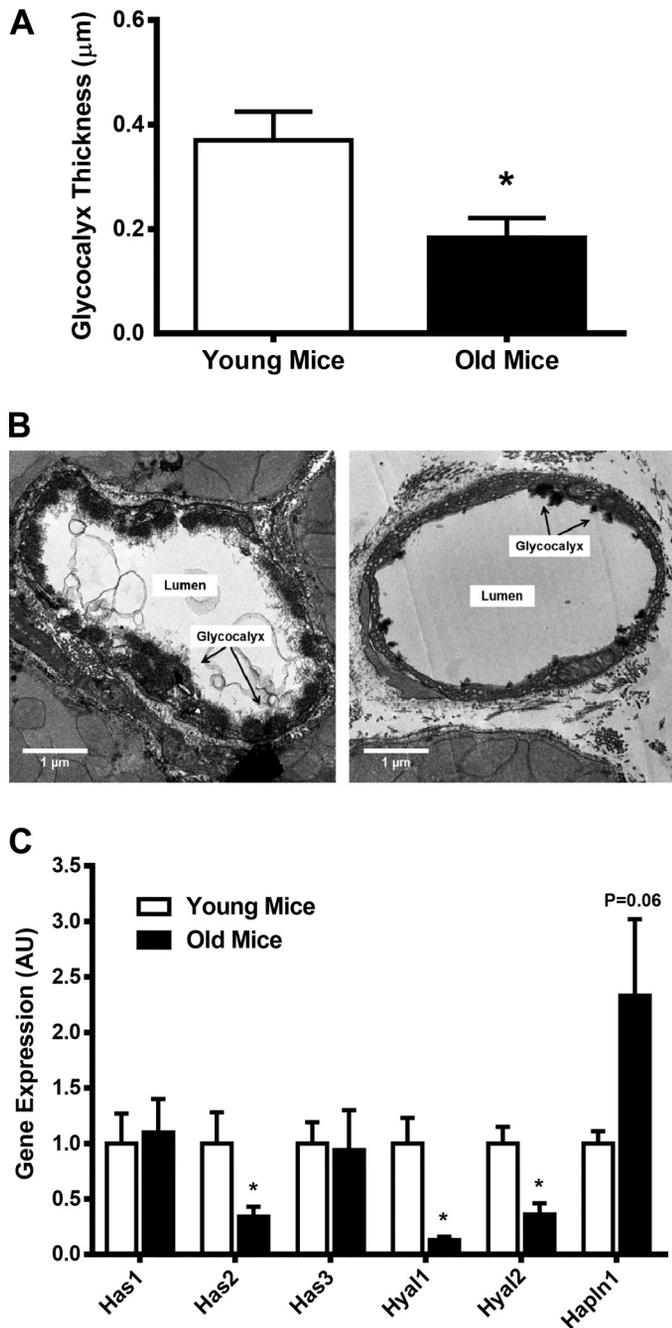


Fig. 3. *A*: glycocalyx thickness in soleus capillaries (4–6 μm) of young and old C57BL/6 mice ($n = 6$ mice/group). *B*: representative image of the glycocalyx in a soleus microvessel of a young (*left*) and old (*right*) mouse. *C*: aortic gene expression was expressed relative to *18S* gene expression to account for differences in cDNA concentration and is shown normalized to young mice ($n = 8$ –10 mice/group). * $P < 0.05$, significant difference vs. young mice. All data are means \pm SE.

Markers of microvascular perfusion. Perfused microvascular density was 18% lower in old compared with young participants (young: $2,890 \pm 175 \mu\text{m}/\text{mm}^2$ vs. old: $2,468 \pm 141 \mu\text{m}/\text{mm}^2$, $P < 0.05$). Measurement of perfused microvascular density in microvessels of different lumen diameter indicated that differences between young and old participants appeared to only be present in microvessel segments that were 6–11 μm in diameter ($P < 0.05$; Fig. 5A). Compared with

young participants, RBC fraction was 5% lower in old participants ($P < 0.05$; Fig. 5B).

DISCUSSION

To our knowledge, this is the first study to demonstrate age-related differences in glycocalyx thickness in multiple tissues of mice and human participants. We observed lower glycocalyx thickness in advanced age using intravital microscopy and transmission electron microscopy in the mesenteric and skeletal muscle microcirculation of mice as well as in the sublingual microcirculation of humans using intravital microscopy. We also observed greater glycocalyx penetrability and lower markers of microvascular perfusion in both older mice and humans using an automated capture and analysis system. Finally, we found that the aorta mice had age-related alterations in the expression of genes that affect production, degradation, and handling of hyaluronan, a major component of the glycocalyx. Taken together, these findings indicate that aging is associated with significant glycocalyx deterioration that is accompanied by markers of impaired microvascular perfusion that may be influenced by age-related alterations in hyaluronan synthesis.

Glycocalyx Structure and Function Are Altered in Advanced Age

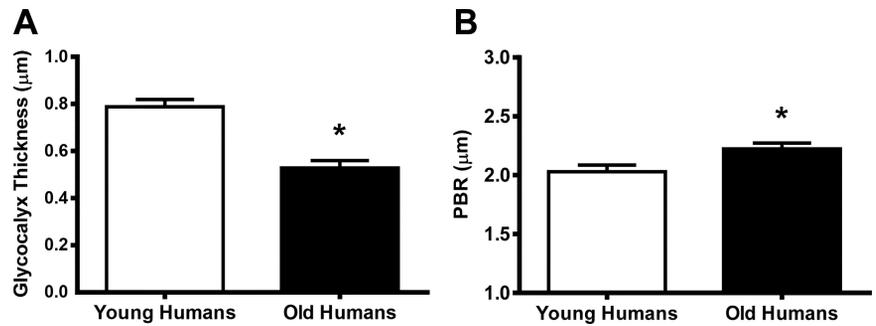
In advanced age, glycocalyx thickness and glycocalyx barrier function are markedly altered, suggesting age-related deterioration of the glycocalyx. We observed that these glycocalyx alterations occur in the microcirculation of multiple tissues using intravital and electron microscopy. Although a deteriorated glycocalyx has been identified in a multitude of age-related chronic diseases, such as CVD (23, 35), end-stage renal disease (47), and diabetes (2, 12, 29), the present study is the first study to demonstrate a diminished glycocalyx in advanced age in humans and mice. Collectively, this study provides novel evidence to support the idea that glycocalyx deterioration occurs in advanced age and in the absence of age-related diseases. Importantly, our findings are consistent with the notion that glycocalyx deterioration may be an important component of and/or predecessor to age-related diseases that involve microvascular dysfunction (2, 12, 23, 29, 35, 47).

Table 1. *Human participant characteristics*

	Young	Old
Women/men, <i>n</i>	15/15	15/15
Age, yr	29 \pm 1	60 \pm 2*
Height, cm	169 \pm 2	171 \pm 2
Weight, kg	67.9 \pm 2.5	73.2 \pm 2.2
Body mass index, kg/m ²	23.6 \pm 0.6	25.2 \pm 0.8
Systolic blood pressure, mmHg	112 \pm 2	121 \pm 3*
Diastolic blood pressure, mmHg	75 \pm 1	75 \pm 2
Heart rate, beats/min	87 \pm 1	91 \pm 2
Medication usage		
Thiazide diuretic, <i>n</i> (%)	0 (0)	1 (3)
Angiotensin-converting enzyme inhibitor, <i>n</i> (%)	0 (0)	0 (0)
β -Blocker, <i>n</i> (%)	0 (0)	1 (3)
Ca ²⁺ channel blocker, <i>n</i> (%)	0 (0)	0 (0)
Statin, <i>n</i> (%)	1 (3)	2 (7)
Levothyroxine, <i>n</i> (%)	1 (3)	2 (7)

Values are means \pm SE; *n* = number of participants. * $P < 0.05$ vs. the young group.

Fig. 4. Glycocalyx thickness (A) in sublingual microvessel segments (5–8 μm) and perfused boundary region (PBR; B) in sublingual microvessel segments (5–25 μm) of younger and older human participants ($n = 30$ participants/group). * $P < 0.05$, significant difference vs. younger participants. All data are means \pm SE.



Thus, further study is warranted to determine if a diminished glycocalyx is a risk factor for age-related chronic diseases, as well as to determine if the glycocalyx is a potential therapeutic target to prevent these age-related chronic diseases.

In addition to diminished glycocalyx thickness, PBR, a marker of glycocalyx barrier function, was higher in older mice and in older humans, indicating a greater ability of RBCs to penetrate the glycocalyx. PBR is derived using an automated capture and analysis system that examines RBC positioning in thousands of microvessel segments over the course of several recordings. Thus, it is a fairly robust and unbiased marker of the glycocalyx, in which higher scores are thought to indicate greater glycocalyx penetrability. We have previously shown that glycocalyx thickness and PBR have a moderate, inverse relationship to each other (20), whereas others have observed higher PBR in patients with sepsis (8). Furthermore, glycocalyx thickness is reduced in experimental sepsis in mice (5) as well as in a rat model of chronic kidney disease (38). Taken together, these findings suggest that lower glycocalyx thickness and greater penetration of RBCs into the glycocalyx are characteristics of aged microvessels. The precise physiological significance of this is uncertain, but current hypotheses include a diminished protective endothelial barrier function as well as impaired mechanotransduction of fluid shear stress and the associated downstream signaling cascades. Studies that pharmacologically manipulate the glycocalyx are needed to elucidate the role of the glycocalyx in vascular physiology and pathology.

Impact of the Glycocalyx on Markers of Microvascular Perfusion

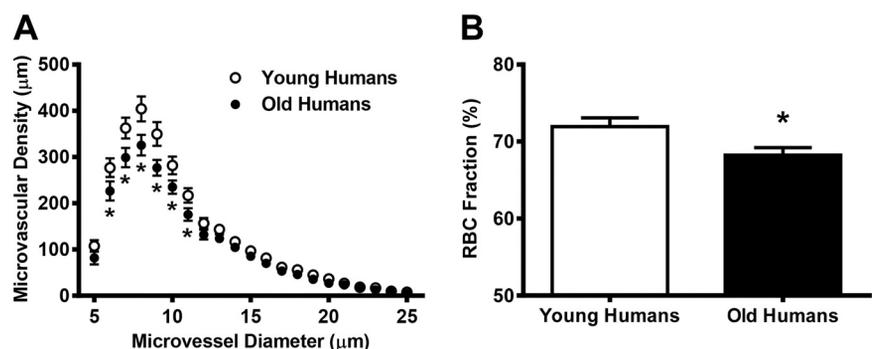
In addition to evaluating the glycocalyx, we examined two markers of microvascular perfusion: perfused microvascular density and RBC fraction. Although impaired microvascular perfusion in advanced age has been previously identified (1,

37), our use of an automated capture and analysis system provides an alternative approach to objectively measure age-related microvascular perfusion in a large number of microvessel segments while simultaneously measuring the glycocalyx. We observed lower perfused microvascular density and RBC fraction in older mice and humans compared with their younger counterparts. The difference in perfused microvascular density between young and old groups was primarily due to a higher density of the smallest perfused microvessel segments in both young mice and humans.

Because the intravital microscope used in this study identifies hemoglobin in RBCs, the microvessel segment diameter is representative of the RBC flow width, rather than its anatomic width. Thus, higher glycocalyx thickness in young groups could be responsible for a greater density of the smallest perfused microvessel segments, as a thicker and/or less penetrable glycocalyx would narrow the width of flowing RBCs, giving the appearance of increased perfusion (reading as a leftward shift in perfused microvascular density). Consistent with this, Groen et al. (12) used a similar imaging technique and automated capture and analysis system to evaluate glycocalyx properties in advanced age. They observed a trend toward higher glycocalyx penetrability in advanced age and also reported a similar leftward shift in perfused microvascular density in younger compared with older humans (12). Thus, it is possible that a higher density of the smallest perfused microvessel segments could be a direct consequence of greater glycocalyx thickness, establishing a link between the two.

RBC fraction, although similar to longitudinal tube hematocrit, does not account for individual RBCs. Rather, RBC fraction is calculated by determining the percentage of marker lines crossed by RBCs in each perfused microvessel segment. Thus, lower RBC fraction in advanced age could represent greater spacing between RBCs but may also be indicative of RBC deformability. A thicker and less penetrable glycocalyx

Fig. 5. Perfused microvascular density (A) and red blood cell (RBC) fraction (B) in the sublingual microvessel segments (5–25 μm) of younger and older human participants ($n = 30$ participants/group). * $P < 0.05$, significant difference vs. younger participants. All data are means \pm SE.



results in greater RBC deformability (39), which could promote elongation of RBCs as they flow through microvessels. While the physiological importance of RBC fraction in microvascular perfusion is unclear, a lower RBC fraction in advanced age suggests greater heterogeneity in RBC presence between microvessel segments, which may be a consequence of a deteriorated glycocalyx (24, 39).

Implications of Age-Related Glycocalyx Deterioration

The glycocalyx has been primarily studied for its role in the microvasculature (13, 43), but it is also functional in the central cardiovascular system. The glycocalyx plays a role in endothelium-dependent dilation by participating in the mechanotransduction of fluid shear stress to endothelial cells that stimulates the production of nitric oxide by endothelial nitric oxide synthase (33, 48). Additionally, the glycocalyx may play a role in blood pressure regulation and arterial stiffness, as pharmacologic glycocalyx deterioration with hyaluronidase induces arteriolar vasoconstriction (44). Finally, given its location at the interface between the vascular endothelium and flowing blood, the glycocalyx acts as a barrier, protecting endothelial cells from circulating oxidants (18) and inflammatory cytokines (25), as well as platelet (45) and leukocyte adhesions (26). Thus, in addition to its impact on the microcirculation, the glycocalyx may play a role in age-related arterial dysfunction. Glycocalyx deterioration is recognized in CVD (23, 35) and other age-related chronic diseases (1, 11, 24, 45), and our results contribute new evidence of a diminished glycocalyx in advanced age that might act as an important antecedent for these diseases. Further work is needed to fully explore the ramifications of a deteriorated glycocalyx on the aging vasculature.

Altered Expression of Genes That Regulate Hyaluronan

Hyaluronan is one of the major glycosaminoglycans that comprise the endothelial glycocalyx (3, 10, 13, 41, 43) and contributes to its barrier function (13). Thus, age-related alterations in the expression of genes that regulate the production (*Has2*), degradation (*Hyal1* and *Hyal2*), and handling (*Hapln1*) of hyaluronan may impact glycocalyx properties in advanced age. Arterial hyaluronan quantity has been shown to decrease across the lifespan in human cadaver arteries that range in size from the aorta to the radial artery (22). In addition to lower hyaluronan content, there appears to be a shift toward a lower hyaluronan molecular weight profile in advanced age (14, 16). Among the three hyaluronan synthase isoforms, *Has2* produces the highest molecular weight hyaluronan, whereas *Has1* and *Has3* produce small- to medium-sized hyaluronan (15). Interestingly, we observed a lower aortic *Has2* expression in old mice but no difference in *Has1* or *Has3*. Lower *Has2* expression in advanced age has been reported in other tissues (31, 40, 42). Alternatively, in a recent study, Reed et al. (34) found elevated *Has2* expression and hyaluronan content in the cerebral microvessels of old mice. Thus, further study is warranted regarding age-related changes in arterial *Has2* expression and how it would affect glycocalyx synthesis.

We also observed a lower gene expression of the hyaluronidase isoforms, *Hyal1* and *Hyal2*, in advanced age. This may be the result of an age-related reduction in arterial hyaluronan content (22) via lower *Has2* gene expression, as hyaluronidase

expression appears to be associated with hyaluronan content. In support of this, in patients with diabetes, a diminished glycocalyx is accompanied by elevated plasma hyaluronan and hyaluronidase activity (2, 4, 12, 29). Presently, it is unclear how age-related reductions in *Has2*, *Hyal1*, and *Hyal2* would impact the glycocalyx, as a reduction in hyaluronan synthesis would be offset by a reduction in hyaluronan degradation. However, a host of pathophysiological changes that occur in advanced age, such as elevations in blood glucose, cholesterol, and oxidative stress (9), have also been shown to degrade the glycocalyx (30, 36, 45). Therefore, it is plausible that these age-related changes in blood chemistry, as well as other factors, deteriorate the glycocalyx, which is then unable to be resynthesized because of a *Has2* deficiency-mediated lack of high molecular weight hyaluronan.

Experimental Considerations

This study is not without limitations. Although the magnitude of difference in variables between young and old mice was similar between mesenteric and gastrocnemius microcirculations, there appeared to be tissue-specific differences between measurement sites. Currently, it is unclear why tissue-specific differences would be present between measurement sites. However, future investigations that include these, as well as other, tissue beds may provide a greater physiological insight into the role of the glycocalyx, which may also elucidate novel risk factors for age-related vascular disease states. We measured glycocalyx thickness in microvessels of $<8 \mu\text{m}$ in diameter, as there was a concern that in microvessels larger than $8 \mu\text{m}$ there was more likely to be partial or no compression of the glycocalyx during spontaneous leukocyte passage. Although there may have been some partial compression of the glycocalyx in microvessels of $<8 \mu\text{m}$ in diameter, this would likely have a similar effect on glycocalyx thickness measurements in both young and old groups. In the present study, as well as in a previous study (20), we observed a moderate but significant inverse relationship between PBR and glycocalyx thickness, indicating that it is unlikely that an underestimation of glycocalyx thickness influenced the results of the present study. However, the physiological relationship between PBR and glycocalyx thickness remains unclear, as pharmacologic glycocalyx deterioration studies with subsequent glycocalyx thickness and PBR assessment have not yet been performed. One would assume that the absence of a glycocalyx would result in a PBR score that appears “healthy” as PBR is calculated using median and maximal RBC flow widths, both of which, in theory, would be similar when the glycocalyx is absent.

Conclusions

Glycocalyx thickness and barrier function were lower in advanced age in the microvasculature of mice and humans. This was evident in multiple tissue beds, and lower glycocalyx thickness was observed using both intravital microscopy and transmission electron microscopy. This age-related glycocalyx deterioration was accompanied by markers of impaired microvascular perfusion. Collectively, these data support the idea that deterioration of the glycocalyx is a characteristic of vascular aging, and this may be an antecedent of age-associated vascular pathophysiology. Whether glycocalyx deterioration

precedes microvascular dysfunction, or vice versa, warrants further study.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

D.R.M., R.A.C., P.E.G., L.A.L., M.T.R., and A.J.D. conceived and designed research; D.R.M., T.T.T.P., and P.E.G. performed experiments; D.R.M., S.I.B., and T.T.T.P. analyzed data; D.R.M., S.I.B., R.A.C., T.T.T.P., P.E.G., L.A.L., M.T.R., and A.J.D. interpreted results of experiments; D.R.M. and T.T.T.P. prepared figures; D.R.M., S.I.B., R.A.C., P.E.G., L.A.L., M.T.R., and A.J.D. drafted manuscript; D.R.M., S.I.B., R.A.C., T.T.T.P., P.E.G., L.A.L., M.T.R., and A.J.D. edited and revised manuscript; D.R.M., S.I.B., R.A.C., T.T.T.P., P.E.G., L.A.L., M.T.R., and A.J.D. approved final version of manuscript.

REFERENCES

- Behnke BJ, Delp MD, Dougherty PJ, Musch TI, Poole DC. Effects of aging on microvascular oxygen pressures in rat skeletal muscle. *Respir Physiol Neurobiol* 146: 259–268, 2005. doi:10.1016/j.resp.2004.12.009.
- Broekhuizen LN, Lemkes BA, Mooij HL, Meuwese MC, Verberne H, Holleman F, Schlingemann RO, Nieuwdorp M, Stroes ES, Vink H. Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus. *Diabetologia* 53: 2646–2655, 2010. doi:10.1007/s00125-010-1910-x.
- Cabrera P, Vázquez BY, Tsai AG, Intaglietta M. Microvascular and capillary perfusion following glycocalyx degradation. *J Appl Physiol* 102: 2251–2259, 2007. doi:10.1152/jappphysiol.01155.2006.
- Ceriello A, Giugliano D, Dello Russo P, Passariello N, Saccomanno F, Sgambato S. Glycosaminoglycans in human diabetes. *Diabete Metab* 9: 32–34, 1983.
- Chelazzi C, Villa G, Mancinelli P, De Gaudio AR, Adembris C. Glycocalyx and sepsis-induced alterations in vascular permeability. *Crit Care* 19: 26, 2015. doi:10.1186/s13054-015-0741-z.
- Chevalier L, Selim J, Genty D, Baste JM, Piton N, Boukhalfa I, Hamzaoui M, Pareige P, Richard V. Electron microscopy approach for the visualization of the epithelial and endothelial glycocalyx. *Morphologie* 101: 55–63, 2017. doi:10.1016/j.morpho.2017.04.001.
- Curry FE, Adamson RH. Endothelial glycocalyx: permeability barrier and mechanosensor. *Ann Biomed Eng* 40: 828–839, 2012. doi:10.1007/s10439-011-0429-8.
- Donati A, Damiani E, Domizi R, Romano R, Adrario E, Pelaja P, Ince C, Singer M. Alteration of the sublingual microvascular glycocalyx in critically ill patients. *Microvasc Res* 90: 86–89, 2013. doi:10.1016/j.mvr.2013.08.007.
- Donato AJ, Eskurza I, Silver AE, Levy AS, Pierce GL, Gates PE, Seals DR. Direct evidence of endothelial oxidative stress with aging in humans: relation to impaired endothelium-dependent dilation and upregulation of nuclear factor-kappaB. *Circ Res* 100: 1659–1666, 2007. doi:10.1161/01.RES.0000269183.13937.e8.
- Eskenz BJ, Mooij HL, Cleutjens JP, Roos JM, Cobelens JE, Vink H, Vanteffelen JW. Rapid insulin-mediated increase in microvascular glycocalyx accessibility in skeletal muscle may contribute to insulin-mediated glucose disposal in rats. *PLoS One* 8: e55399, 2013. doi:10.1371/journal.pone.0055399.
- Eskurza I, Monahan K, Robinson J, Seals D. Effect of acute and chronic ascorbic acid augmentation on flow-mediated dilation with physically active and sedentary aging. *J Physiol* 556: 215–224, 2004. doi:10.1113/jphysiol.2003.057042.
- Groen BB, Hamer HM, Snijders T, van Kranenburg J, Frijns D, Vink H, van Loon LJ. Skeletal muscle capillary density and microvascular function are compromised with aging and type 2 diabetes. *J Appl Physiol* 116: 998–1005, 2014. doi:10.1152/jappphysiol.00919.2013.
- Henry CB, Duling BR. Permeation of the luminal capillary glycocalyx is determined by hyaluronan. *Am J Physiol Heart Circ Physiol* 277: H508–H514, 1999.
- Holmes MW, Bayliss MT, Muir H. Hyaluronic acid in human articular cartilage. Age-related changes in content and size. *Biochem J* 250: 435–441, 1988. doi:10.1042/bj2500435.
- Itano N, Sawai T, Yoshida M, Lenas P, Yamada Y, Imagawa M, Shinomura T, Hamaguchi M, Yoshida Y, Ohnuki Y, Miyauchi S, Spicer AP, McDonald JA, Kimata K. Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem* 274: 25085–25092, 1999. doi:10.1074/jbc.274.35.25085.
- Kamada H, Masuda K, D'Souza AL, Lenz ME, Pietryla D, Otten L, Thonar EJ. Age-related differences in the accumulation and size of hyaluronan in alginate culture. *Arch Biochem Biophys* 408: 192–199, 2002. doi:10.1016/S0003-9861(02)00543-X.
- Lee DH, Dane MJ, van den Berg BM, Boels MG, van Teeffelen JW, de Mutsert R, den Heijer M, Rosendaal FR, van der Vlag J, van Zonneveld AJ, Vink H, Rabelink TJ; NEO study group. Deeper penetration of erythrocytes into the endothelial glycocalyx is associated with impaired microvascular perfusion. *PLoS One* 9: e96477, 2014. doi:10.1371/journal.pone.0096477.
- Li Q, Bolli R, Qiu Y, Tang X-L, Murphree SS, French BA. Gene therapy with extracellular superoxide dismutase attenuates myocardial stunning in conscious rabbits. *Circulation* 98: 1438–1448, 1998. doi:10.1161/01.CIR.98.14.1438.
- Liuhanen S, Sallisalmi M, Pettilä V, Oksala N, Tenhunen J. Indirect measurement of the vascular endothelial glycocalyx layer thickness in human submucosal capillaries with a plug-in for ImageJ. *Comput Methods Programs Biomed* 110: 38–47, 2013. doi:10.1016/j.cmpb.2012.10.019.
- Machin DR, Gates PE, Vink H, Frech TM, Donato AJ. Automated measurement of microvascular function reveals dysfunction in systemic sclerosis: a cross-sectional study. *J Rheumatol* 44: 1603–1611, 2017. doi:10.3899/jrheum.170120.
- Machin DR, Park W, Alkatan M, Mouton M, Tanaka H. Hypotensive effects of solitary addition of conventional nonfat dairy products to the routine diet: a randomized controlled trial. *Am J Clin Nutr* 100: 80–87, 2014. doi:10.3945/ajcn.114.085761.
- Manley G. Changes in vascular mucopolysaccharides with age and blood pressure. *Br J Exp Pathol* 46: 125–134, 1965.
- Martens RJ, Vink H, van Oostenbrugge RJ, Staals J. Sublingual microvascular glycocalyx dimensions in lacunar stroke patients. *Cerebrovasc Dis* 35: 451–454, 2013. doi:10.1159/000348854.
- McClatchey PM, Schafer M, Hunter KS, Reusch JE. The endothelial glycocalyx promotes homogenous blood flow distribution within the microvasculature. *Am J Physiol Heart Circ Physiol* 311: H168–H176, 2016. doi:10.1152/ajpheart.00132.2016.
- Mulivor AW, Lipowsky HH. Inflammation- and ischemia-induced shedding of venular glycocalyx. *Am J Physiol Heart Circ Physiol* 286: H1672–H1680, 2004. doi:10.1152/ajpheart.00832.2003.
- Mulivor AW, Lipowsky HH. Role of glycocalyx in leukocyte-endothelial cell adhesion. *Am J Physiol Heart Circ Physiol* 283: H1282–H1291, 2002. doi:10.1152/ajpheart.00117.2002.
- National Research Council; Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals*. Washington, DC: National Academies, 2011.
- Nieuwdorp M, Meuwese MC, Mooij HL, Ince C, Broekhuizen LN, Kastelein JJ, Stroes ES, Vink H. Measuring endothelial glycocalyx dimensions in humans: a potential novel tool to monitor vascular vulnerability. *J Appl Physiol* 104: 845–852, 2008. doi:10.1152/jappphysiol.00440.2007.
- Nieuwdorp M, Mooij HL, Kroon J, Atasever B, Spaan JA, Ince C, Holleman F, Diamant M, Heine RJ, Hoekstra JB, Kastelein JJ, Stroes ES, Vink H. Endothelial glycocalyx damage coincides with microalbuminuria in type 1 diabetes. *Diabetes* 55: 1127–1132, 2006. doi:10.2337/diabetes.55.04.06.db05-1619.
- Nieuwdorp M, van Haften TW, Gouverneur MC, Mooij HL, van Lieshout MH, Levi M, Meijers JC, Holleman F, Hoekstra JB, Vink H, Kastelein JJ, Stroes ES. Loss of endothelial glycocalyx during acute hyperglycemia coincides with endothelial dysfunction and coagulation

- activation in vivo. *Diabetes* 55: 480–486, 2006. doi:10.2337/diabetes.55.02.06.db05-1103.
31. Ohno T, Hirano S, Rousseau B. Age-associated changes in the expression and deposition of vocal fold collagen and hyaluronan. *Ann Otol Rhinol Laryngol* 118: 735–741, 2009. doi:10.1177/000348940911801009.
 32. Pries AR, Secomb TW. Microvascular blood viscosity in vivo and the endothelial surface layer. *Am J Physiol Heart Circ Physiol* 289: H2657–H2664, 2005. doi:10.1152/ajpheart.00297.2005.
 33. Pries AR, Secomb TW, Gaehtgens P. The endothelial surface layer. *Pflugers Arch* 440: 653–666, 2000. doi:10.1007/s004240000307.
 34. Reed MJ, Vernon RB, Damodarasamy M, Chan CK, Wight TN, Bentov I, Banks WA. Microvasculature of the mouse cerebral cortex exhibits increased accumulation and synthesis of hyaluronan with aging. *J Gerontol A Biol Sci Med Sci* 72: 740–746, 2017. doi:10.1093/gerona/glw213.
 35. Rehm M, Bruegger D, Christ F, Conzen P, Thiel M, Jacob M, Chappell D, Stoeckelhuber M, Welsch U, Reichart B, Peter K, Becker BF. Shedding of the endothelial glycocalyx in patients undergoing major vascular surgery with global and regional ischemia. *Circulation* 116: 1896–1906, 2007. doi:10.1161/CIRCULATIONAHA.106.684852.
 36. Rubio-Gayosso I, Platts SH, Duling BR. Reactive oxygen species mediate modification of glycocalyx during ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol* 290: H2247–H2256, 2006. doi:10.1152/ajpheart.00796.2005.
 37. Russell JA, Kindig CA, Behnke BJ, Poole DC, Musch TI. Effects of aging on capillary geometry and hemodynamics in rat spinotrapezius muscle. *Am J Physiol Heart Circ Physiol* 285: H251–H258, 2003. doi:10.1152/ajpheart.01086.2002.
 38. Salmon AH, Ferguson JK, Burford JL, Gevorgyan H, Nakano D, Harper SJ, Bates DO, Peti-Peterdi J. Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction. *J Am Soc Nephrol* 23: 1339–1350, 2012. doi:10.1681/ASN.2012010017.
 39. Secomb TW, Hsu R, Pries AR. Blood flow and red blood cell deformation in nonuniform capillaries: effects of the endothelial surface layer. *Microcirculation* 9: 189–196, 2002. doi:10.1038/sj.mn.7800132.
 40. Simpson RM, Meran S, Thomas D, Stephens P, Bowen T, Steadman R, Phillips A. Age-related changes in pericellular hyaluronan organization leads to impaired dermal fibroblast to myofibroblast differentiation. *Am J Pathol* 175: 1915–1928, 2009. doi:10.2353/ajpath.2009.090045.
 41. Tarbell JM, Pahakis MY. Mechanotransduction and the glycocalyx. *J Intern Med* 259: 339–350, 2006. doi:10.1111/j.1365-2796.2006.01620.x.
 42. Terazawa S, Nakajima H, Tobita K, Imokawa G. The decreased secretion of hyaluronan by older human fibroblasts under physiological conditions is mainly associated with the down-regulated expression of hyaluronan synthases but not with the expression levels of hyaluronidases. *Cytotechnology* 67: 609–620, 2015. doi:10.1007/s10616-014-9707-2.
 43. van den Berg BM, Vink H, Spaan JA. The endothelial glycocalyx protects against myocardial edema. *Circ Res* 92: 592–594, 2003. doi:10.1161/01.RES.0000065917.53950.75.
 44. VanTeeffelen JW, Brands J, Jansen C, Spaan JA, Vink H. Heparin impairs glycocalyx barrier properties and attenuates shear dependent vasodilation in mice. *Hypertension* 50: 261–267, 2007. doi:10.1161/HYPERTENSIONAHA.107.089250.
 45. Vink H, Constantinescu AA, Spaan JA. Oxidized lipoproteins degrade the endothelial surface layer : implications for platelet-endothelial cell adhesion. *Circulation* 101: 1500–1502, 2000. doi:10.1161/01.CIR.101.13.1500.
 46. Vink H, Duling BR. Identification of distinct luminal domains for macromolecules, erythrocytes, and leukocytes within mammalian capillaries. *Circ Res* 79: 581–589, 1996. doi:10.1161/01.RES.79.3.581.
 47. Vlahu CA, Lemkes BA, Struijk DG, Koopman MG, Krediet RT, Vink H. Damage of the endothelial glycocalyx in dialysis patients. *J Am Soc Nephrol* 23: 1900–1908, 2012. doi:10.1681/ASN.2011121181.
 48. Yao Y, Rabodzey A, Dewey CF Jr. Glycocalyx modulates the motility and proliferative response of vascular endothelium to fluid shear stress. *Am J Physiol Heart Circ Physiol* 293: H1023–H1030, 2007. doi:10.1152/ajpheart.00162.2007.