Supplement 1 (S1)

Extended methods for PBR Measurements

Imaging of microcirculation: To detect the dynamic lateral RBC movement into the glycocalyx of the microcirculation (expressed as PBR), sidestream dark field (SDF) intravital microscopy (MicroVision Medical Inc., Wallingford, PA) was performed to visualize the sublingual microvasculature. The SDF camera uses green light emitting diodes (540nm) to detect the haemoglobin of passing RBC. The images were captured using a 5x objective with a 0.2 NA (numerical aperture), providing a 325- fold magnification in 720 x 576 pixels at 23 frames per second. The image acquisition is automatically mediated through the Glycocheck software (Glycocheck BV, Maastricht, the Netherlands). With this method it is possible to detect increased penetration of RBCs into the glycocalyx (increased PBR) when the glycocalyx is perturbed or degraded.

Calculating the PBR: The software automatically identifies all available measurable micro-vessels, in focus and without movement of the imaging unit and defines vascular segments every 10μm along the length of these vessels (Figure S1A,B). Subsequently, a sequence of 40 frames is recorded in time, containing on average 300 vascular segments. Then the observer moves the SDF imaging unit to a different location for another recording session of 40 frames, until a total of minimal 3,000 vascular segments are recorded. After these measurements, 21 line markers are placed at an interval of 0.5μm around all vascular segments (figure S1C,D).

From these line markers in the recorded movies, the PBR is calculated. First, the width of the red blood cell column is measured by determining both inflection points of the intensity plot profiles of all line markers (Figure S1E). This results in 840 red blood cell column widths per vascular segment (21 line markers * 40 frames). Second, for every vascular segment, the measurements of all these RBC column widths are combined into a graph as shown in figure S1F. The number of observed RBC positions (as a percentage of the total) is plotted against the measured column width. From this graph the median column width is derived, being the 50^{th} percentile of the curve. By linear regression analysis, the slope of the line between the 25th and the 75^{th} percentile is measured. The point where this line intersects with the x-axis is a reliable marker (based on all 840 measurements) of the most outward location of the RBC, the perfused diameter (PD).

The PBR is defined as the distance between RBC column width and PD. Because the PBR is present on both sides of the RBC column, it is calculated using the equation: [PD – median RBC column width] / 2. The calculated PBR values, classified according to their corresponding RBC column width

between 5 – 25 μm, are averaged to provide a single PBR value for each participant. Finally, the PBR is schematically shown in figure S1G.

Quality checks: To ensure a reliable and reproducible measurement quality checks are performed during the whole analysis procedure. First, the software only selects the vascular segments of which at least 11 of the 21 line markers have a positive signal for the presence of an RBC (>50% of the vascular segment is filled with RBCs) to use for the subsequent analyses from the first frame of the different recordings. By selecting only the vascular segments that are filled for more than 50%, the influence of haematocrit on the PBR measurements is minimized. The second quality check is performed during the measurement of all RBC column widths (figure S1E). In the second quality check all measured segments are tested on minimal RBC width-, position of the column (centred or not) and the signal to noise ratio. In the third quality check the curve fit for calculating the median RBC column width and PD is tested (R^2 of 25^{th} to 75^{th} percentile > 0.75) (figure S1F). Measurement points that do not fulfil the criteria of these quality checks are not used for the calculation of the PBR.

Calculating perfused microvascular length: The acquisition software automatically places measurement sites at every 10 μm along the detected microvessels to be able to measure the PBR in these regions. After the first quality check, the program calculates the total amount of measurement sites present after all quality checks. Because all segments are 10 micron long, we know that there are x measurement sites * 10 micron of perfused microvessel length in the recordings. This can be adjusted per square mm of recorded sublingual area. In this way we have measured the perfused microvascular length of microvessels in micron per square mm of tongue bed.

Calculating RBC filling percentage: After the second quality check, the program determines if an RBC is present on every line marker that has been placed on all the detected microvessels that passed the first 2 quality checks in the recorded frames. The amount of line markers where an RBC is detected is shown as a percentage of the total of placed line markers and shown as the RBC filling percentage.

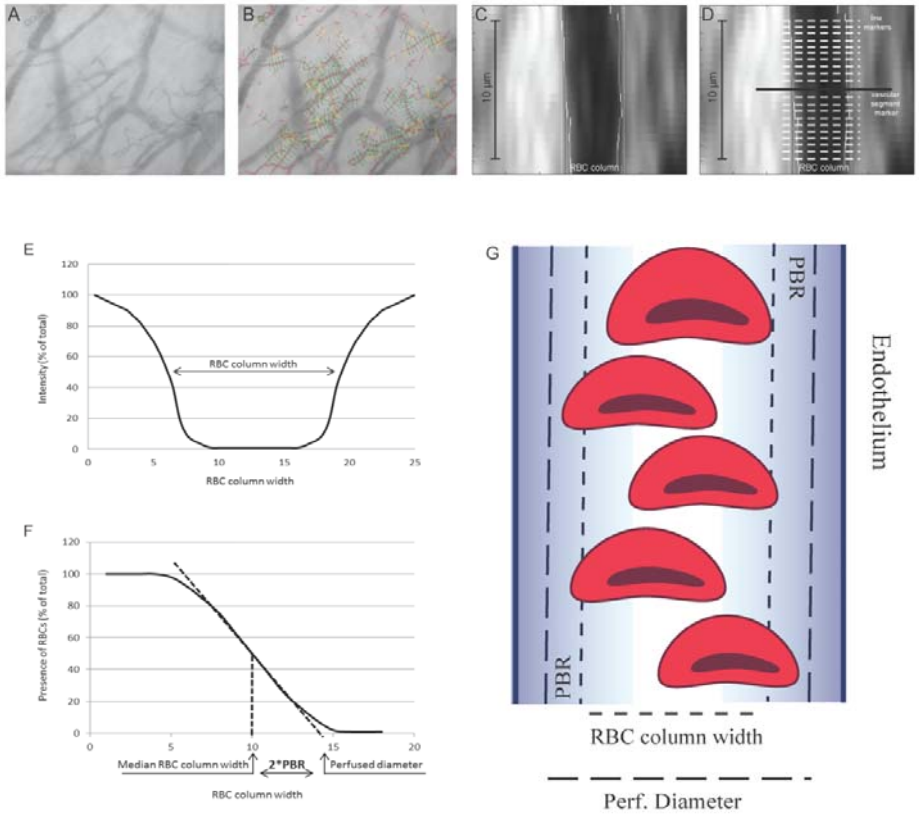

Figure S1: Image acquisition and logarithm to calculate PBR in sublingual microvasculature. A: Example of one frame made using the SDF camera. The camera detects the haemoglobin of passing RBCs by green light emitting diodes (540nm) and the software automatically identifies all available measurable micro-vessels in focus. B: Example of the same frame of the acquired image after vascular segments have been defined every 10µm along the length of these vessels, until at least 3000 vascular segments are recorded. C: Zoomed in example of one vascular segment of the RBC column. D. Schematic example with the vascular segment marker and the 21 line markers on and around the vascular segment marker. E. Example of a plot profile of the RBC column width measured at one line marker. F: Graph of all RBC column widths measured at one vascular segment. From this plot the median RBC column width can be calculated. The perfused diameter can be determined by using linear regression on the area of the curve from the 25th to the 75th percentile. Next the PBR can be calculated from these two parameters. G: Schematic image of cross section of a blood vessel with the RBC column width, perfused diameter and PBR explained.

Supplement 2 (S2)

Validation study in mice

Methods

To validate the measurements, a direct test to determine whether loss of glycocalyx dimension is reflected by outward radial displacement of circulating RBCs has been performed using intravital microscopy. For this experiment, B6.Cg-Tg(TIE2GFP)287Sato/1J mice were used (Jackson Laboratories, Bar Harbor, ME). In these mice endothelial cells (EC) can be imaged by the specific expression of GFP in ECs. These GFP-EC mice were prepared for intravital microscopic observation of the cremasteric microcirculation as described before.[1] The preparation was transferred to the stage of an intravital microscope (Leitz, Wetzlar, Germany), coupled to a cooled CCD video camera (C9100; Hamamatsu, Hamamatsu City, Japan). Microvessels were alternately observed using bright-field microscopy with a 435 nm band pass interference filter (blue light) in the light path for depiction of the RBC column and epi-illumination for examination of the GFP signal using the appropriate filters for fluorescein. A salt water immersion objective lens (x50, n.a. 1.0) was used. From these two images from the same microvessel segment (figure S2), the anatomic vessel width was determined using the endothelial position by the GFP intensity peak while the perfused diameter was determined by the width of the RBC profile at half height intensity. The RBC-EC gap, or the space between endothelial cell and RBC column, is calculated from the difference between vessel diameter and corresponding perfused diameter, divided by two (as the gap is present on both sides of the RBC column). To determine the effect of glycocalyx degradation on the outward displacement of the RBC column, paired measurements in a total 16 vessels in 7 mice have been performed before and 30 minutes after hyaluronidase treatment (35 U, jugular vein infusion).

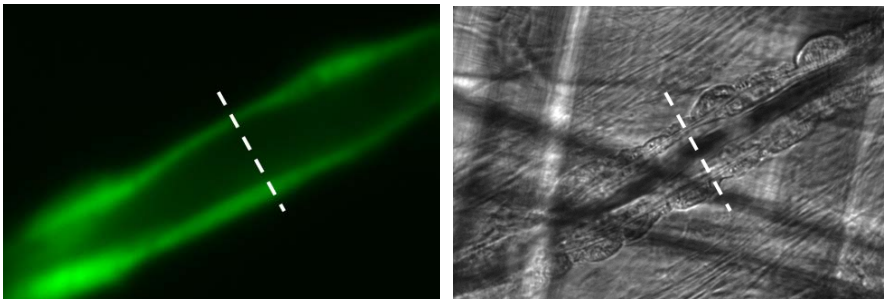

Figure S2: Example of the epifluorescence- (GFP, green) and trans-illumination (RBC, black) images made for measurement of both the vessel width using the endothelial cell GFP signal and the perfused diameter using the RBC column width.

Reference

1. Constantinescu A, Spaan JA, Arkenbout EK, Vink H, Vanteeffelen JW: Degradation of the endothelial glycocalyx is associated with chylomicron leakage in mouse cremaster muscle microcirculation. *Thromb Haemost,* 105: 790-801, 2011

Results

RBC-EC gaps ranged from 0.3 – 2.6 microns (vessel diameters 5 – 35 microns). Hyaluronidase treatment reduced the RBC-EC gap from 1.30 to 0.52 microns (average vessel diameter 16.0 microns) (figure S3). To exclude that changes in the RBC-EC gap originate from changes in the vessel diameter, the vessel diameter was measured before and after hyaluronidase treatment (figure S4). Because no changes could be observed in these paired measurements (p=0.91) the influence of vessel diameter can be excluded, thereby confirming that the observed changes originate from the changes in RBC column size as a result of the degradation of the ESL.

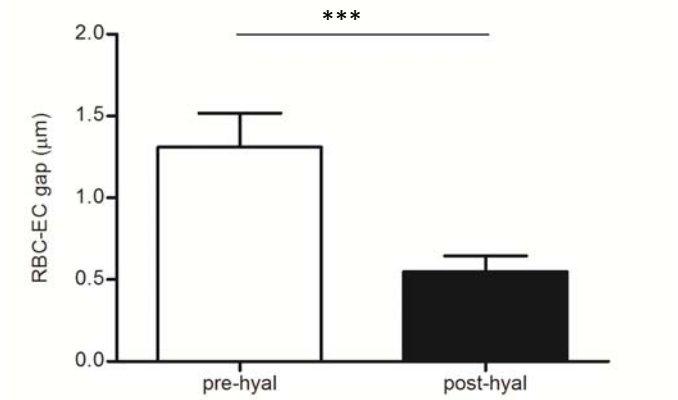

*Figure S3: Paired measurements of changes in EC-RBC gap before and after hyaluronidase treatment. (*** P<0,001)*

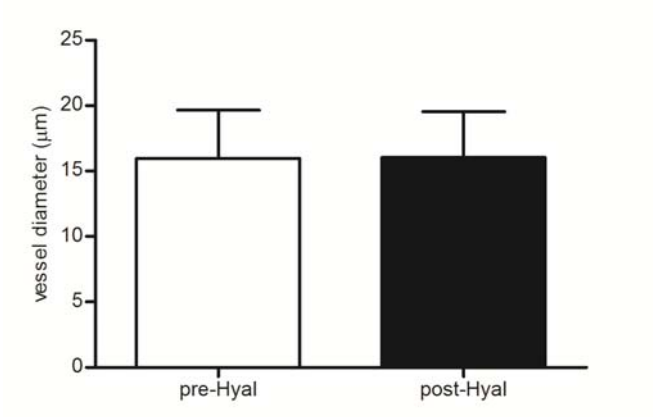

Figure S4: Paired measurements of changes in vessel diameter before and after hyaluronidase treatment show no differences.