Hyaluronidase-1-mediated glycocalyx impairment underlies endothelial abnormalities in polypoidal choroidal vasculopathy

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Abstract

Background: Polypoidal choroidal vasculopathy (PCV), a subtype of age-related macular degeneration (AMD), is a global leading cause of vision loss in older populations. Distinct from typical AMD, PCV is characterized by polyp-like dilatation of blood vessels and turbulent blood flow in the choroid of the eye. Gold standard anti-vascular endothelial growth factor (anti-VEGF) therapy often fails to regress polypoidal lesions in patients. Current animal models have also been hampered by their inability to recapitulate such vascular lesions. These underscore the need to identify VEGF-independent pathways in PCV pathogenesis.

Results: We cultivated blood outgrowth endothelial cells (BOECs) from PCV patients and normal controls to serve as our experimental disease models. When BOECs were exposed to heterogeneous flow, single-cell transcriptomic analysis revealed that PCV BOECs preferentially adopted migratory-angiogenic cell state, while normal BOECs undertook proinflammatory cell state. PCV BOECs also had a repressed protective response to flow stress by demonstrating lower mitochondrial functions. We uncovered that elevated hyaluronidase-1 in PCV BOECs led to increased degradation of hyaluronan, a major component of glycocalyx that interfaces between flow stress and vascular endothelium. Notably, knockdown of hyaluronidase-1 in PCV BOECs led to increased degradation of hyaluronan, a major component of glycocalyx that interfaces between flow stress and vascular endothelium. Notably, knockdown of hyaluronidase-1 in PCV BOECs improved mechanosensitivity, as demonstrated by a significant 1.5-fold upregulation of Krüppel-like factor 2 (KLF2) expression, a flow-responsive transcription factor. Activation of KLF2 might in turn modulate PCV BOEC migration. Barrier permeability due to glycocalyx impairment in PCV BOECs was also reversed by hyaluronidase-1 knockdown. Correspondingly, hyaluronidase-1 was detected in PCV patient vitreous humor and plasma samples.

Conclusions: Hyaluronidase-1 inhibition could be a potential therapeutic modality in preserving glycocalyx integrity and endothelial stability in ocular diseases with vascular origin.

Keywords: Endothelial dysfunction, Glycocalyx, Hyaluronidase-1, Polypoidal choroidal vasculopathy, Age-related macular degeneration
of vascular dilatation as hyperfluorescent nodules under indocyanine green angiography or orange-red subretinal nodules in routine ophthalmoscopic examinations [3–7]. Similar to typical neovascular age-related macular degeneration (AMD), PCV patients suffer from serous-guineous pigment epithelial detachment and submacular exudations resulting in a gradual loss of visual acuity [8, 9]. A background of choroidal vascular hyperpermeability is more frequently reported in PCV than in AMD [10]. Importantly, response of PCV to anti-vascular endothelial growth factor (anti-VEGF) therapy has been less consistent. In particular, while anti-VEGF controls the exudation, the underlying polypoidal lesion often fails to regress [8]. This current lack of effective therapeutic options for PCV that is refractory to anti-VEGF treatments reflects unresolved questions in the etiology of PCV.

Epidemiologic studies of PCV to date revealed a greater prevalence of PCV in populations of Asian (22–55% of neovascular AMD cases) and African descent [11] than Caucasian populations (8–13% of neovascular AMD cases) [12, 13]. Several cohort-based genome-wide association studies (GWAS) have revealed neovascular AMD to be a multifactorial disease with many single-nucleotide polymorphisms (SNPs) identified to be significantly associated with the risk of disease development [14, 15]. However, GWAS studies have reported similar associations of SNPs in both PCV and neovascular AMD subtypes, with only rs10490924 in the age-related maculopathy susceptibility 2/high-temperature requirement A serine peptidase 1 (ARMS2/HTRA1) region showing significantly stronger association with PCV than neovascular AMD [14, 16–18]. The current understanding suggests that pathogenesis of PCV is likely an interplay of polygenic, biological, and environmental factors [19].

The defining clinical feature of vascular dilatation in PCV suggests a unique perturbation to blood flow experienced by the choroidal endothelia. Indeed, optical coherence tomography angiography of PCV eyes, in combination with variable interscan time analysis revealed the varied nature of blood flow velocities within polyps with the center of polyps experiencing slower flow than the periphery, thus providing evidence for non-uniform flow within these vascular dilatations [20, 21]. The causes of these vascular malformations and the effects of non-uniform blood flow on the endothelium of these polyps are currently unknown.

Moreover, while several murine models for macular degeneration have been described, the mouse eye lacks a defined macula [22, 23] and laser-induced choroidal neovascularization models largely do not recapitulate polypoidal lesions in PCV [24]. In order to capture some of these genetic and environmental complexities in a human-relevant disease model, we leveraged on the use of patient-derived blood outgrowth endothelial cells (BOECs). BOECs can be derived in vitro from circulating endothelial colony-forming cells that originate from bone marrow or vessel resident stem cells [25–29]. With minimal manipulation, these cells give rise to mature endothelial cells in culture that are more likely to retain the genetic and epigenetic landscape of individuals [30]. Since it is well-known that endothelial cells are mechano-sensors that respond to shear stress by blood flow [31], we subject BOECs to variable and pulsatile flow conditions in order to recapitulate the dynamics within PCV polyps. Through single-cell analysis, we were able to discern transcriptional signatures of endothelial cells in response to heterogeneous flow and show that PCV and normal BOECs adopt distinct cell states under these conditions. Our findings demonstrate the powerful utility of patient-derived BOECs in modelling a complex vascular disease and illuminate molecular differences that can underlie the pathogenesis of PCV.

Results

Derivation and characterization of human blood outgrowth endothelial cells

We developed our BOEC models from peripheral blood mononuclear cell (PBMC) fractions isolated from PCV and normal donors according to established protocol [32]. Early colonies of BOECs emerged generally 7–14 days post-seeding of PBMCs (Fig. 1a). BOEC colonies were expanded for 1 week prior to passaging. On average for both normal and PCV groups, we obtained 1–3 colonies from every 10 million PBMCs (Fig. 1b). The proliferation capacity of BOECs were monitored from passages 3–8 when most of the BOEC lines demonstrated steady cell population doubling time (Fig. 1c). In our experiments, we excluded potentially senescent BOECs if there was substantiate increase in their cell doubling time. To confirm endothelial identity, our derived BOECs were highly enriched for endothelial cell markers such as CD31 (> 99%) and CD144 (> 94%), but they had negligible expressions for leukocyte markers CD45 and CD68, and progenitor cell marker CD133, suggesting purity of our BOEC cultures (Fig. 1d). Both PCV and normal BOECs were also positive for VWF and VE-cadherin (CDH5) expressions (Fig. 1e). We further performed functional characterization of BOECs. PCV and normal BOECs were able to form tubular networks and showed comparable attributes (i.e., number of junctions, number of loops, branching length) with the positive control, human umbilical vein endothelial cells (HUVECs) (Fig. 1f). In a three-dimensional fibrin gel bead sprouting assay, our BOECs also displayed sprouting with filopodia,
Fig. 1 Derivation and characterization of human blood outgrowth endothelial cells. 

a Workflow illustrating the generation of BOECs. Images show BOEC colonies emerging during days 7–14 post-seeding of PBMCs, followed by characteristic cobblestone-like endothelial cells after passaging of colonies (scale bar, 100 μm).

b Number of BOEC colonies per million PBMCs obtained from normal controls and PCV patients. Proliferation dynamics of BOEC lines measured by cell doubling duration over passages.

c Flow cytometry characterization of BOECs for endothelial, leukocyte, and progenitor cell markers (gray—isotype control; red/blue—cell lineage marker staining). Immunostaining of endothelial markers VWF and CDH5 in BOECs. HeLa cells and HUVECs are negative and positive controls respectively. Scale bars, 50 μm.

d Tube formation assay with representative images of tube formation ability of BOECs at 4 h. Bottom panel shows quantification of junctions, loops (tubes), and total branching length (total length of loops and branches) over time (quantified from n = 12 optical fields per timepoint from each cell line). Data are mean ± s.e.m. Scale bars, 200 μm.

e Fibrin gel bead sprouting assay of BOECs at 24 h. BOECs were immunostained for F-actin (red) and DAPI (cyan). Bottom panel shows measurements of relevant sprouting parameters (quantified from n = 10 beads from each individual, BOECs from 3 PCV and 3 normal individuals). Data are nested graphs showing mean ± s.e.m. of each individual donor. No significant difference was found between PCV and Normal (two-tailed t-tests with Welch's correction). Scale bars, 100 μm.
characteristic of endothelial protrusions in mediating guidance cues during angiogenesis (Fig. 1g).

Based on marker expressions and functional characterization, both PCV and normal BOECs demonstrated comparable attributes. Extrinsic factors such as complement dysregulation and oxidative stress play key roles in pathological endothelial behaviors [33]. We postulated that there could be other systemic mediators affecting endothelial health. Hence, we exposed PCV and normal BOECs to autologous plasma and found that some angiogenic attributes could be intensified by plasma stimulation (Additional file 1: Fig. S1). While there could be a multitude of paracrine influences which are known to cause endothelial dysfunction, the knowledge gaps in PCV endothelial cell autonomous effects remain understudied. We hereby focused on deciphering intrinsic endothelial mechanisms which could be further interrelated in our PCV BOEC disease model.

**Blood outgrowth endothelial cells adopt diverse cell states under heterogeneous flow**

Among our derived BOEC lines (13 PCV, 11 normal), we prioritized those that passed quality controls in terms of endothelial marker expressions and functional attributes (Fig. 1). In addition, we genotyped the BOEC lines for AMD/PCV genetic risk loci in ARMS2/HTRA1 (rs10490924 and rs11200638) and CFH (rs800292). Collectively, we selected 4 PCV and 6 normal lines for further experimentation (Additional file 1: Table S1). Three PCV donors were homozygous for the risk alleles in at least 1 locus. All the normal controls harbored protective alleles in at least 1 locus.

We introduced heterogeneous flow as a stress paradigm to PCV and normal BOECs. To recapitulate the variable flow conditions in PCV polyps, we utilized an orbital flow setup to generate a continuum of shear forces with high magnitude, uniaxial shear stresses at the edge of wells and low magnitude, multidirectional shear stresses in the center [34]. PCV and normal BOECs were exposed to 24 h of rotation (Fig. 2a). Peak fluid shear in our setup was estimated to be around 10 dyne/cm² using \( \tau_{\text{max}} = \alpha \sqrt{\rho \eta (2\pi f)^3} \), with \( \alpha \) being the orbital radius (1 cm), \( \rho \) as density of medium (assumed 0.9973 g/mL) [35], \( \eta \) as the medium viscosity (assumed 0.0101 poise) [35], and \( f \) as the frequency of rotation (210/60rps) [35, 36]. In a similar setup using a 6-well plate, optical Doppler velocimetry measured shear stress of 5 dyne/cm² at the center of the well and 11 dyne/cm² at the periphery [35]. This range of shear stress magnitudes is well within reported physiological range where shear stresses have been described at ± 4 dyne/cm² around curvatures, bifurcations, and branches, while straight arterial regions experience shear forces of approximately 10–20 dyne/cm², at times reaching 40 dyne/cm² [37]. The effects of this heterogeneous flow can be seen from the staining of vascular endothelial cadherin (CDH5) (Fig. 2b), where both PCV and normal BOECs demonstrated alignment to flow direction at well periphery but not in the center. Correspondingly, caveolin-1 (CAV1) which forms part of the endothelial mechanosensing machinery [38] showed re-distribution to BOEC cell edges as a response to higher shear stress at the well periphery (Fig. 2b).

To uncover the molecular underpinning of PCV endothelial abnormalities, we performed single-cell RNA sequencing (scRNA-seq) to resolve transcriptomic differences between PCV and normal BOECs in response to heterogeneous flow. Our single-cell analysis revealed 5 clusters of transcriptionally distinct cell states that we classified as (1) Proinflammatory, (2) Migratory-Angiogenic, (3) Transitory-Proliferative, (4) Proliferative, and (5) Quiescent cells (Fig. 2c). These cell states were determined firstly by analysis of cluster-enriched marker genes. For example, representative genes such as inflammatory markers CXCL8 and TGFβ2, as well as cell cycle/proliferation markers MKI67, PCLAF, and TOP2A were among the top 10 cluster-enriched marker genes where the highest average expression for each gene corresponded to their identified cell states (Fig. 2d). Furthermore, expression patterns of cell state-defining marker genes were conserved between PCV and normal datasets (Additional file 1: Fig. S2c).

Secondly, we confirmed cell state identities by the predominant processes found from gene enrichment analyses of top cluster-specific marker genes using Gene Ontology [39, 40] and Reactome [41] databases. Enriched
Fig. 2 (See legend on previous page.)
processes for the Proinflammatory cell state included myeloid leukocyte adhesion, neutrophil activation, and platelet degranulation, while those for Migratory-Angiogenic cell state included positive regulation of endothelial cell migration, angiogenesis, and hyaluronan (HA) uptake and degradation (Fig. 2e, Additional file 1: Fig. S2d and e). The Transitory-Proliferative and Prolifeative cell states shared several processes such as nuclear division and cell cycle (Additional file 1: Fig. S2d and e). Taken together, both PCV and normal BOECs adopted diverse transcriptomic cell states in response to heterogeneous flow.

PCV and normal endothelial cells demonstrate differential responses to heterogeneous flow

In discerning meaningful differences between PCV and normal BOECs, we found a majority of normal BOECs adopt Proinflammatory cell state (50.36%) under flow treatment. Notably, we noticed a distinct departure in PCV BOECs, with the majority of cells found in Migratory-Angiogenic cell state instead (41.69%) (Fig. 3a). These differences represented a primary shift between Proinflammatory and Migratory-Angiogenic cell states as the other cell states remain comparable between PCV and normal in terms of cell proportions. These changes in cell proportions were found largely conserved across each of the PCV and normal BOEC samples, ruling out bias arising from inter-individual variabilities (Additional file 1: Fig. S3a). As different durations of fluid shear stress may induce distinct sets of flow response genes in endothelial cells [42], we sought to profile those cell state-enriched genes in BOECs following short durations of heterogeneous flow (6 h and 1 day), as well as prolonged exposure to 7 days of flow. In accordance to normal BOECs preferentially adoption the Proinflammatory cell state at initial time points (6 h and 1 day), but these differences did not persist till 7 days of flow (Additional file 1: Fig. S3b). Interestingly, prolonged period of flow appeared to strengthen Migratory-Angiogenic cluster-enriched genes in PCV BOECs. We also examined the flow-responsive transcription factor, Krüppel-like factor 2 (KLF2) [43] and found that normal BOEC responded most pronouncedly with KLF2 upregulation at 1 day of flow (Additional file 1: Fig.S3b). While KLF2 expression decreased gradually over 7 days of prolonged flow exposure, normal BOEC still sustained a significantly higher level of KLF2 activation than PCV BOEC, suggesting that PCV BOEC might have lower sensitivity to flow stress.

Next, we performed differential expression analysis between PCV and normal datasets with a focus on combined Proinflammatory and Migratory-Angiogenic cell states. Gene set enrichment analyses of PCV downregulated genes revealed largely inflammation-related events such as neutrophil activation and neutrophil-mediated immunity (adj. \( p < 0.01 \)) (Fig. 3b), indicating that PCV BOECs expressed a weaker proinflammatory profile than their normal counterparts. Gene set enrichment analysis was expanded to include databases from KEGG [44] and MsigDB [45] to reveal enrichment of key pathways such as Cytokine Signaling in Immune system (R-HAS-1280215, Reactome), Inflammatory response (Hallmark, MsigDB), Fluid shear stress and atherosclerosis (hsa05418, KEGG), and Cellular responses to external stimuli (R-HAS-8953897). Module scores for the average expression of genes in these pathways demonstrated the degree of differential expression between PCV and normal BOECs for these gene sets (Fig. 3c). Upregulation of proinflammatory genes (e.g. CXCL8, ICAM) are well-described processes in endothelial cells subjected to disturbed, multidirectional flow [46]. Our findings suggested reduced sensitivity to flow by PCV BOECs in contrast to normal BOECs.

In addition, we found significant enrichment in oxidative stress response and oxidative phosphorylation related
Fig. 3 (See legend on previous page.)
processes with PCV BOECs expressing lower levels of genes involved in detoxification of reactive oxygen species and respiratory electron transport chain (Fig. 3d(i)). MitoSOX Red staining for superoxide and mitochondrial activity measurements validated these transcriptomic findings with PCV BOECs showing significantly higher levels of oxidative stress and lower mitochondrial functions than normal BOECs (Fig. 3d(ii) and (iii)) (p < 0.05). Antioxidant response genes such as superoxide dismutases (SOD1) are upregulated in endothelial cells as a protective response to oxidative stress in oscillatory flow [46]. Our MitoSOX staining and lower mitochondrial functions might explain a repressed protective response in PCV BOECs towards heterogeneous flow.

**PCV endothelial cells have increased migratory capacity and barrier permeability**

In addition to reduced flow response, PCV BOECs had a stronger migratory transcriptomic profile than normal BOECs. Our gene set enrichment analyses of the PCV upregulated genes showed that the major enriched processes revolved around cell migration and cell locomotion, in particular blood vessel endothelial cell migration (GO:0043534) (Fig. 4a). To functionally validate these transcriptomic differences, we went on to assess the migratory capacity of PCV and normal BOECs in a wound healing assay. PCV BOECs demonstrated significantly greater wound closure than normal BOECs with and without exposure to orbital flow, although the differences between PCV and normal were greater after 24 h of heterogeneous flow (23.17% ± 3.892, p < 0.0001) than static cultures (20.75% ± 5.865, p = 0.0046) (Fig. 4b). Intimately linked to the process of cell migration, extracellular matrix (ECM) modifying processes were also found to be significantly enriched in the PCV upregulated gene set (Fig. 4c), in particular proteases such as MMP1, MMP16, and ADAMTS18 (Additional file 1: Fig. S3c).

In linking reduced flow response to increased migratory capacity in PCV BOECs, we hypothesized a perturbed extracellular milieu that interfaced between flow and endothelial cells. Further to the enrichment of ECM-degrading processes in PCV upregulated genes, syndecan interactions (R-HAS-300170) was found to be significantly downregulated in PCV BOECs (Fig. 4c). Syndecans are part of the endothelial glyocalyx, which is a mechanosensing meshwork of glycosaminoglycans covering the luminal surface of endothelial cells, held covalently by proteoglycan core proteins [40]. Its apical positioning and extensive coverage of the cell surface enables sensing and transduction of hemodynamic forces to interacting partners on the plasma membrane [47, 48].

Here, we looked at the expression profiles of glyocalyx-related genes and found significantly lowered expression in PCV for heparan sulfate (HS) core proteins (GPC1, SDC2, SDC3, and SDC4) and HA core protein (CD44), while genes for HA degrading enzymes (HYAL1 and CEMIP2) were significantly higher in PCV (Fig. 4d). Also, β1 integrin (ITGB1), a reported mechanosensor of blood flow usually upregulated in the presence of flow [49], was found to be downregulated in PCV relative to normal BOECs.

**Increased HYAL1 levels in PCV endothelial cells impair glyocalyx**

The integrity and composition of endothelial glyocalyx can determine the efficiency and extent of mechanosensitivity and force transduction [47, 48]. Of the 6 known genes coding for hyaluronidases in humans [50], we found HYAL1 to be expressed at a significantly higher
Fig. 4 (See legend on previous page.)
level in PCV than normal BOECs across both Proinflammatory and Migratory-Angiogenic clusters (Fig. 4d and Additional file 1: Fig. S3c). Hence, we selected HYAL1 for further validation due to the strong differential expression in the transcriptomic data and its active functional role in modifying glycocalyx composition through its hyaluronan (HA)-degrading activities. Western blot analyses of BOEC lysates, subjected to heterogeneous flow, validated the increased expression of HYAL1 in PCV BOECs at the proteomic level (Fig. 5a). HYAL1 is a secreted protein that is endocytosed and activated at low pH in lysosomes [51, 52]. As such, we probed the proteolytic activity of secreted HYAL1 and found greater degradation of HA ($p = 0.0194$) using conditioned media from PCV BOECs subjected to heterogeneous flow (Fig. 5b). Higher levels of HYAL1 were also detected in these conditioned media of PCV BOECs as quantified by ELISA (Fig. 5c). Subsequently, HYAL1 was detectable in PCV patients’ plasma and eye vitreous humor extracts (Fig. 5d).

Using a biotinylated-HA binding protein (HABP), we were able to visualize the HA component of BOEC glycocalyx and observed an overall decrease in HA staining in PCV BOECs relative to normal BOECs after heterogeneous flow (Fig. 5e). Volumetric analyses of HA staining across z-stacks revealed a significantly lower HA volume in PCV BOECs exposed to heterogeneous flow at the center of well (Fig. 5e). While we observed the same trend in BOECs at the periphery of well, significant difference of HA content between PCV and normal BOECs was not achieved. As aforementioned, flow conditions in the center of well had relatively lower shear stress and higher multidimensionality than that found at the periphery of well. Collectively, these results validated the transcriptomic data of higher HYAL1 expression in PCV BOECs and suggest that PCV BOECs may experience a higher HA turnover and breakdown under pathological flow conditions.

Syndecans also form an integral part of endothelial glycocalyx. There were significant downregulation of syndecan genes with syndecan-2 (SDC2) most profoundly reduced in PCV BOECs (Fig. 4d). We observed lower SDC2 protein levels in PCV BOECs than normal BOECs although they were not statistically significant (Additional file 1: Fig. S4a). Since syndecans are one of the major heparan sulfate proteoglycans, reduction of syndecan core proteins may translate into reduced heparan sulfate content. Hence, heparan sulfate treatment has been effective in alleviating the effect due to syndecan deficiency in mice and human epithelial cells [53]. To understand if heparan sulfate supplementation might compensate for the reduction of syndecans in PCV BOECs, we measured KLF2 as an indicator of flow response in BOECs treated with heparan sulfate. Consistent with before, KLF2 was activated under flow condition, with normal BOECs showing significantly greater KLF2 level than PCV BOECs (Additional file 1: Fig. S4b). Even though heparan sulfate supplementation seemed to offset differences between normal and PCV BOECs, there was insignificant change of KLF2 levels in PCV BOECs between vehicle control and heparan sulfate supplementation. Since the effect of heparan sulfate supplementation was modest, restoring part of the damaged glycocalyx through syndecans or heparan sulfate proteoglycans might not substantially improve flow sensitivity in this PCV context. Therefore, we focused on HYAL1 as a modifiable target.

**Modulation of HYAL1 restores normal cell migration and barrier integrity in PCV endothelial cells**

Finally, we evaluated if the increased expression of HYAL1 in PCV BOECs can play a role in mediating the functional phenotypes of increased migratory capacity and barrier permeability. We used small-interfering RNA (siRNA) to silence gene expressions of HYAL1 that were confirmed at protein levels in the human BOECs (Fig. 6a). HYAL1 knockdown (siHYAL1) was able to reduce wound closure percentage in PCV samples significantly ($p = 0.046$) at 50 nM, while normal BOECs remained

![Fig. 5 HYAL1 enzymatic activities and perturbation of glycocalyx hyaluronan in PCV endothelial cells.](image)
Fig. 5 (See legend on previous page.)
unperturbed (Fig. 6b). The knockdown of HYAL1 was also able to restore PCV migratory capacity to the similar level as normal BOECs (Fig. 6b). We postulated that knockdown of HYAL1 might improve mechanosensing ability in PCV BOECs, in part through preserving HA in endothelial glycocalyx. We found that KLF2 expressions were indeed activated in BOECs exposed to heterogeneous flow compared to static condition (Fig. 6c). HYAL1 knockdown further upregulated KLF2 level significantly in PCV BOECs (7.03 ± 2.52, siHYAL1 versus 4.65 ± 1.45, non-targeting control) in response to flow (Fig. 6c). This might explain the aforementioned observation that PCV BOEC migratory capacity could be effectively modulated by HYAL1 knockdown, possibly through activation of KLF2, which in turn exerted anti-migratory effect on endothelial cells [54]. Furthermore, HYAL1 knockdown significantly reduced barrier permeability in PCV BOECs to a level similar to normal BOECs transfected with non-targeting siRNA (NT) (p = 0.0483) (Fig. 6d).

Hence, HYAL1 modulation could reverse abnormal PCV endothelial cell migration and barrier permeability.

Discussion

We have addressed a major knowledge gap in the endothelial underpinning of PCV as most ocular disease modelling studies have focused on the biology of retinal pigment epithelial cells. Previously, the difficulty of culturing human primary choroidal endothelial cells could underlie the dearth of studies on the role of endothelial cells. Here, derivation of BOECs represents a minimally invasive method of establishing disease-relevant endothelial cells for experimentations. We report single-cell analysis of the differential responses of human endothelial cells from healthy controls and PCV patients to heterogeneous flow. We found that PCV BOECs are abnormally migratory and have increased barrier permeability. This is due in part to their enhanced expression of HYAL1, whose knockdown restores endothelial stability in PCV. Our key finding explains an intrinsic mechanism of endothelial dysfunction, potentially contributing to leaky choroidal vessels and structurally abnormal vascular dilatation in PCV.

ECM-modifying factors form the central network in our PCV endothelial autocrine mechanism that may drive the hyperpermeability and vessel dilatations observed in PCV eyes. Our comparative single-cell analysis identified increased expressions of ECM modifiers with established roles in angiogenesis and vascular permeability, which corroborated earlier studies that implicated ECM degradation in PCV pathogenesis [55]. Intriguingly, the heterogeneous flow response in PCV BOECs and differential levels of glycocalyx-related genes led us to hypothesize potential perturbations in the flow-sensing extracellular components of PCV endothelial cells. Glycocalyx, a carbohydrate-rich layer on the luminal surface of vascular endothelium, creates a cell-free, permeable zone between the blood flow and endothelial cells, regulating permeability of the endothelium through size and steric hindrance [56, 57], signaling by plasma-borne endothrine factors, and reducing attachment of inflammatory immune cells [58, 59]. The glycocalyx plays an important role in the mechanotransduction of shear stresses. Enzymatic degradation or shear-induced shedding of any component of the glycocalyx can severely impact some of these functions [59, 60]. Infusion of canine femoral arteries with hyaluronidase or cultured endothelial cells with heparitinase both resulted in reduction of shear-induced nitric oxide (NO) production [61, 62]. The application of shear stress to human umbilical vein endothelial cells (HUVEC) also resulted in an increase in HA in the glycocalyx in a postulated positive feedback mechanism [63]. Hyaluronidase-1 is an endocytosed, acid-active enzyme that can break down HA chains of any size into tetrasaccharides [64, 65]. While plasma-borne HYAL1 has been found to have a short half-life of around 2–3 min [66, 67], we found very low levels of HYAL1 in our PCV vitreous humor samples in contrast to plasma, therefore indicating a likely autocrine role for the increased HYAL1 produced by PCV endothelial cells.

Elevated levels of HYAL1 and HA have also been reported in systemic diseases such as severe dengue [68] and diabetes [69] where glycocalyx degradation, vascular instability, and hyperpermeability effects have been associated. Of note, age-related reduction in HA

(See figure on next page.)

**Fig. 6** Modulating HYAL1 normalizes abnormal cell migration and barrier permeability of PCV endothelial cells. a siRNA knockdown of HYAL1 in BOECs. BOECs were transfected with siRNA for 4 days, including 24 h heterogeneous flow treatment. Cell lysates were harvested and analyzed for HYAL1 levels. Bar graphs show mean actin-normalized HYAL1 band intensities with error bars representing standard deviations (siHYAL1, short-interfering RNA of HYAL1; NT, non-targeting siRNA). b Wound healing assays were carried out to assess migratory capacity of BOECs at 21 h post-scratch after 24 h of static or rotated culture. siRNA knockdown was carried out 48 h prior to orbital rotation. Top panel shows representative image set from the flow condition with yellow outlines indicating cell-free wound regions at 21 h post-scratch, scale bar, 200μm. c Relative KLF2 gene expressions in BOECs in static condition and after 6 h of heterogeneous flow exposure, with treatment of either NT or siHYAL1. d In vitro vascular permeability imaging assay using biotinylated-gelatin-coated surfaces. Intercellular gaps were revealed by Neutavadin-FITC staining after 24 h of static or rotated culture. siRNA knockdown was carried out 48 h prior to orbital rotation. Shown are representative frames from different BOEC lines, scale bar, 500 μm. Area was normalized against average FITC area of normal BOEC lines. All bar graphs showing means with standard deviations. n indicates number of cell lines evaluated for each group while p value is from two-tailed t-test, *p < 0.05
Fig. 6 (See legend on previous page.)
in the Bruch's membrane have been observed in human eyes [70]. Consistent with reports where short-chain HA deposits can direct and drive endothelial cell migration [69, 71], our data shows that HYAL1 levels can mediate endothelial cell migration. Both increased barrier permeability and endothelial cell migration are processes linked to overall vascular instability. We are mindful that we do not have typical neovascular AMD as a comparison in the current study. Our patient cell-based studies may paint an incomplete picture for the development of aneurysmal dilatations in PCV. Nonetheless, we were able to uncover and demonstrate a previously unreported role for glycocalyx integrity in PCV pathogenesis and present HYAL1 as an autocrine mediator of endothelial dysfunctions in PCV endothelial cells (Fig. 7).

Conclusions
Understanding mechanisms of diseases affecting ocular integrity is an important area. Our patient endothelial model provides molecular and phenotypic insights into PCV pathophysiological processes which would inform further development of in vivo models, as well as pave the way for therapeutic advancement. The fundamental endothelial mechanism presented here could be far-reaching beyond PCV, and potentially a contributor to the pathogenesis of ocular diseases with a vascular origin, suggesting that many pathologies could be ameliorated by better knowledge of endothelial disease biology.

Methods
Patient selection and sample collection
We enrolled subjects from the retina clinic of the Singapore National Eye Center. Inclusion criteria were age 40–80 years (demographics detailed in Additional file 1: Tables S1 and S2). Written informed consent was obtained from each participant. This study was approved by the Local Ethics Committee of SingHealth Centralised Institutional Review Board (CIRB Refs: R1496 and 2018/2004) and Nanyang Technological University Singapore Institutional Review Board (IRB-2018-01-026 and IRB-2019-03-011-01).

For patients with PCV, the clinical diagnosis was confirmed on fundus examination and fluorescein and indocyanine green angiography. PCV was confirmed based on the presence of polypoidal dilatations on ICGA. Healthy volunteers were recruited for the control group. Controls were further selected based on absence of AMD or PCV from clinical examination. For sample collection, 10 mL of fresh blood was collected from each participant and processed in the laboratory within 6 h. Upon ficoll centrifugation of the blood specimen, a buffy coat layer containing peripheral blood mononuclear cells (PBMCs) was isolated from which DNA extraction was performed for genotyping with the OmniExpress chip. The rest of the PBMCs was used for cultivation in cell culture to derive blood outgrowth endothelial cells (BOECs).

Fig. 7 Increased expression of hyaluronidase-1 drives endothelial abnormalities in PCV. Phenotypes indicative of vascular instability were observed in PCV patient-derived endothelial cells under heterogeneous flow conditions. Increased HYAL1 expression in PCV cells led to impaired glycocalyx through the degradation of HA components, giving rise to an altered response to flow, increased cell migration and barrier permeability.
Derivation of BOECs and culture conditions

Blood samples were collected from donors as detailed in Additional file 1: Table S1. BOECs were generated as per described [32] with modifications. Briefly, peripheral blood samples (5–9 mL per donor) were diluted 1:1 with phosphate-buffered saline (PBS) and separated to obtain buffy coat by density gradient centrifugation over Ficoll® Paque (GE Healthcare). The buffy coat, which was enriched with peripheral blood mononuclear cells (PBMCs), was carefully collected, washed with PBS, resuspended in heparin-free, EGM-2 medium (Lonza) supplemented with 16% defined fetal bovine serum (FBS; Hyclone), and counted. Plasma was also collected and stored at –80 °C. Then, the PBMCs were seeded into collagen I-coated well(s) accordingly so that the cell density was ≥1.5 × 10^6 cells/cm^2_. Medium was changed every 2 to 3 days. Outgrowth colonies should appear between 7 and 14 days post-seeding. The cells were expanded to passage 3 before any applications were performed on them, including phenotyping and functional evaluation, in order to opt out unwanted leukocytes. After passage 3, BOECs were cultured on collagen I-coated tissue culture dishes in heparin-free, EGM-2 with 10% heat-inactivated FBS with media change every 2–3 days. BOECs from passages 4 to 8 were used in experiments.

Endothelial tube formation

Tube formation assay was performed according to the manufacturer’s instructions (Endothelial Cell Tube Formation Assay, Corning). BOECs were seeded onto Matrigel as 20,000 cells per well in serum-free EGM-2 medium, and H9 embryonic stem cell line (H9-ESC) and human umbilical vein endothelial cells (HUVECs) were seeded as a control. Cells were incubated for 24 h and gels containing sprouts were fixed with 4% paraformaldehyde (200 μL/well) overnight at 4 °C. Gels in 8-well chamber slides were washed by rinsing twice in 100 μL/well 1× PBS and gentle orbital shaking (agitation) in 1× PBS for 30 min. Permeabilization was performed with agitation in 0.5% Triton X-100 for 20 min and staining with agitating in TRITC-Phalloidin in 1× PBS with 1% BSA for 30 min and 500 ng/ml DAPI for 10 min. Final rinses were performed with agitation in PBS/T (0.1% v/v Tween-20 in 1× PBS) and subsequently in 1× PBS. Stained gels were stored in 1× PBS at 4 °C before confocal imaging.

Fibrin-embedded BOECs were imaged using an inverted laser scanning confocal microscope (LSM800, Carl Zeiss) using a Plan-Apochromat ×20/0.8 objective lens. Two-channel Z-stack images (AF568 and DAPI) of whole beads were captured using the ZEN software (blue edition, Carl Zeiss). Images of 1024 × 1024-pixel resolution were acquired from ×0.6 optical zoom at Z-intervals of 1.11 μm. Approximately 100–200 Z-slices were acquired for each bead. For each individual, 2–4 of the most well-formed individual filopodia were imaged. Sprouting parameters were quantified using Imaris 3.0 (Oxford Instruments). The number of sprouts per bead, “Filament Tracer” sprout length and number of filopodia branch points per sprout were measured.

Endothelial phenotyping by flow cytometry

Cell surface markers were quantified using flow cytometry to phenotypically confirm the endothelial identity of the derived BOECs. CD31 and CD144 were selected as endothelial markers; CD45 and CD68 as leukocyte exclusion markers, and CD133 as a progenitor marker. Briefly, the BOEC monolayers were trypsinized and washed with DPBS prior to staining with the marker antibodies in the dark for 15 min, room temperature. Fluorescence data were collected on a BD LSR Fortessa X-20 cell analyzer (Becton Dickinson) and analyzed using FlowJo software (Becton Dickinson).

Orbital flow setup

BOECs were trypsinized and counted with 0.4% Trypan Blue (Gibco, Thermo Fisher Scientific) staining on an automated cell counter (Countess II, Thermo Fisher Scientific) before diluting and seeding onto rat-tail collagen Type1 (Corning)-coated 12-well plates to give 120,000 viable cells per well. Seeded wells were incubated for 48 h in a 5% CO2, 37 °C, humidified incubator before overlying media was replaced with 800 μl of fresh heparin-free,
EGM-2 medium (10% heat-inactivated FBS) to give a liquid height of ~ 2 mm per well. Plates were then replaced onto an orbital shaker in a 5% CO₂, 37 °C, humidified incubator and rotated at 210 rpm for 24 h. Static controls were set up in the same manner under the same conditions, with 800 μl of fresh heparin-free, EGM-2 medium (10% heat-inactivated FBS) replaced after 48 h post-seeding and replaced into the incubator without rotation.

**Single-cell RNA sequencing and analysis**

BOECs from 2 PCV and 2 normal lines were seeded and subjected to orbital flow as described in Orbital Flow Setup. After 24 h of orbital flow, the BOECs were trypsinized, resuspended in heparin-free, EGM-2 medium (10% heat-inactivated FBS), and counted using Trypan blue and an automated cell counter (Countess II, Thermo Fisher Scientific) and resuspended appropriately for loading onto 10X Genomics Chromium Controller chip by facility personnel at Single-cell Omics Centre (SCOC), Genome Institute Singapore (GIS). Each BOEC cell line was prepared as a separate scRNA-seq library using Chromium Single Cell 3’ v3 Reagent Kit (10X Genomics) by SCOC GIS and the final ready-to-sequence libraries were handed over with quantification and quality assessment reports from Bioanalyzer Agilent 2100 using the High Sensitivity DNA chip (Agilent Genomics). Individual libraries were pooled equimolarly and sent for sequencing by NovogeneAIT Genomics (Singapore). Raw sequencing data was also processed by NovogeneAIT Genomics (Singapore) using CellRanger (10X Genomics) with reads mapped to the human genome assembly (GRCh38).

We performed secondary analysis on the resultant filtered matrix files using Seurat (v 3.2.0) [74]. Data was filtered for dead/poor-quality cells based on low number of genes detected (< 200) or potential doublets (> 9500) as recommended by Seurat’s tutorial (sattijalab.com) and inspection of nFeature spread for each sample (Additional file 1: Fig. S2a). Cells with high percentage of mitochondrial genes were also removed with the threshold of less than 20% informed by a previously reported percentage of mitochondrial gene content in endothelial cells [75]. In order to inspect for any cell cycle heterogeneity between samples, cell cycle states for each sample were determined by the CellCycleScoring function in Seurat (Additional file 1: Fig. S2b). These filtered datasets were then scaled and normalized using SCTransform individually before integrated based on 3000 integration features. Clusters were identified in the integrated dataset using the FindClusters function at resolution 0.2, after PCA analysis, RunUMAP and FindNeighbours at 1:30 dimensions. Marker genes were then identified for each cluster using FindAllMarkers with MAST [76] (R package) as the selected test of choice. Different expression analysis between PCV and normal datasets were performed using FindMarkers with MAST for each individual cluster. Gene enrichment analysis was carried out for both marker genes of clusters and differential expression genes between PCV and normal in each clusters using clusterProfiler (R package, v 3.17.0.) [77]. AddModuleScore function (Seurat) was used to present overall relative expression profiles between PCV and normal, for genes found in the indicated enriched processes.

**Mitochondrial function assay by Seahorse analyzer**

BOECs were reseeded onto collagen-I-coated 96-well Seahorse microplates at 20,000 cells per well and incubated in a humidified incubator at 37 °C, 5% CO₂ for 5 h. Thereafter, they were prepared and assayed for mitochondrial function assessment according to the manufacturer’s instructions (Seahorse XF Cell Mito Stress Test Kit, Agilent Technologies). The drugs oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/ antimycin A (Rot/AA) were used in the assay at concentrations of 1 μM, 1.5 μM, and 0.5 μM respectively. To obtain post-assay cell counts for normalization, assayed cells were rinsed once with 1× DPBS, fixed with 4% PFA, stained with DAPI (500 ng/ml) for 10 min and counted automatically using an imaging reader with the Gen5 software (Cytation 3, BioTek Instruments). Thereafter, OCR values were normalized to these counts in individual wells, processed, and analyzed in Wave 2.6.1 software according to the manufacturer’s instructions.
(Agilent Technologies). Wells containing uneven cell distribution or displaying outlier OCR were excluded from analysis.

**Wound healing assay**

BOEC cultures, prepared as described in Orbital Flow Setup section, were scratched across the horizontal diameter of each well with a P200 micropipette tip before imaging on an automated microscope (Celldiscoverer 7, ZEISS) using a × 5 objective over time. Multi-tile images were captured to encompass the entire scratch wound at time = 0 h, under live-cell imaging conditions (5% CO₂, 37 °C). Culture plates were then replaced into a 5% CO₂, 37 °C, humidified incubator and left to recover without rotation (for both rotated and static conditions) for 21 h. The same tiling positions determined for each well at 0 h were reused for the imaging at 21 h. Wound area was obtained using manual region-of-interest (R.O.I) annotation of cell-free areas across each tiled image set before area of R.O.I. was obtained using Measure in Fiji [72]. Wound closure percentage is shown by calculating the difference between cell-free areas for t = 0 h and t = 16 h and dividing it by t = 0 h % per well. All data points were collected over a total of 3 independent experiments.

**Permeability imaging assay**

BOEC cultures were prepared as described in Orbital Flow Setup section with the exception of biotinylated gelatin coating replacing rat-tail collagen coat. Biotinylated gelatin was prepared as described [79], and plates were coated with 10 mg/ml of biotinylated gelatin diluted in 0.1 M sodium bicarbonate solution (pH 8.3) at 4 °C overnight. Solutions were removed and wells washed with PBS before preparing cultures as describe in Orbital Flow Setup section. After 24 h of rotated/static incubation, culture plates were left to recover without rotation (for both rotated and static conditions) for 21 h in a 5% CO₂, 37 °C, humidified incubator. After which, overlying medium was removed, rinsed with PBS, before staining with 2 μg/ml FITC-Neutravidin (A2662, Thermo Fisher Scientific) for 3 min in a 5% CO₂, 37 °C, humidified incubator. Stained wells were washed thrice with PBS before they were fixed with 4% paraformaldehyde phosphate-buffered solution (Nacalai Tesque) for 10 min at room temperature. Fixed cell layers were washed with PBS once before automated imaging using a × 5 objective (Celldiscoverer7) to capture a tiled image of each entire well. FITC-stained areas per well were obtained using ZEN BLUE Image Analysis tool’s interactive Segmentation module. Relative FITC area was determined by normalizing total area per well against the average area obtained across all normal BOEC lines used in that experiment. All data points were collected over a total of 3 independent experiments.

**Hyaluronidase activity assay**

Hyaluronidase activity from rotated conditioned media was measured using an ELISA-like assay described in Lokeshwar et al. [80] with modifications. As shown in the graphical workflow (Fig. 5b, top panel), high molecular weight (1.5M-1.75M) HA (63357, Sigma-Aldrich) was coated onto black, clear-bottom 96-well plates at a concentration of 500 μg/ml overnight at 4 °C. HA solutions were removed and wells were washed with PBS twice. Each HA-coated well was incubated with 10 μl of conditioned media and 90 μl of HAase assay buffer (0.1 M sodium formate, 0.15 M NaCl, 0.2 mg/ml BSA, pH 4.2) at 37 °C for 24 h. Incubation solutions were removed and wells washed with PBS twice before incubated with 2 μg/ml biotinylated HABP (PBS, 1% BSA) for 30 min at room temperature. Staining solution was removed and wells washed twice with PBS. PBS was replaced at 100 μl per well before readout at 490 nm excitation and 525 nm emission (fixed gain of 150) on a Synergy H1 plate reader (Biotek). Conditioned media from at least 2 independent experiments per cell line were analyzed and shown.

**Glycocalyx HA staining**

Rotated BOEC cultures were prepared as indicated in Orbital Flow Setup with the exception of glass-bottom 12-well plates (Cellvis) replacing polypropylene 12-well plates used in other experiments. Overlying medium was removed and cell monolayers were briefly but gently washed once with cold sterile PBS once before fixation with cold methanol for 10 mins at – 20 °C. Fixed cell layers were washed gently with PBS once before addition of endogenous biotin blocking solutions (Endogenous Biotin Blocking kit, Thermo Fisher Scientific) according to the manufacturer’s instructions. Biotin and avidin-blocked cell layers were then washed with PBS and stained with 1 μg/ml of biotinylated-HABP (versican G1 domain, Affirmus Biosource) diluted in PBS at 4 °C overnight. Cell layers were then washed twice with PBS before counterstaining with Hoechst 33342 (2 drops per ml, Ready Flow, Thermo Fisher Scientific) and 20 μg/ml FITC-Neutravidin (A2662, Thermo Fisher Scientific) for 30 min. Staining solution was removed and cells washed with PBS before z-stack imaging using confocal microscopy. Start of z-stacks was determined using Hoechst 33342 signal staining for nuclei and last positions were determined by the last visible FITC signal per sample. Three frames per region per cell line were imaged using a × 40 objective. FITC-stained areas per frame were obtained and summed across all stacks using ZEN BLUE Image Analysis tool’s interactive Segmentation module.
Immunostaining

Cells were fixed with 4% paraformaldehyde phosphate (09154-85, Nacalai Tesque) at room temperature for 20 min, then washed with DPBS without Ca and Mg (SH3002803, Hyclone) and stored at 4 °C. Before staining, cells were permeabilized with 0.2% Triton X-100 (Sigma) and blocked with blocking buffer (4% FBS in DPBS) for 60 min. Cells were then incubated overnight at 4 °C with primary antibodies (Additional Table S3) diluted in blocking buffer. Next day, cells were washed three times with wash buffer (TBS + 0.05% Tween20 in water) before incubating with secondary antibodies diluted in blocking buffer for 1 h at room temperature. Cells were then washed three times with wash buffer followed by keeping in Hoechst dye (1:10,000 in PBS). Immunocytochemistry was analyzed using an Olympus IX71 inverted fluorescence microscope fitted with an Olympus digital camera.

Western blot

Cell lysates were prepared by lysing PBS-rinsed cell layers with 150 μl of 1× LDS buffer (NuPAGE, Thermo Fisher Scientific) at 4 °C for 10 min with gentle rocking. Lysates were harvested and heated at 95 °C for 10 min before being resolved in 10% SDS polyacrylamide gels (Bio-Rad). Resolved proteins were then transferred onto nitrocellulose membranes using TransBlot Turbo System (Bio-Rad) and membranes were then blocked with 5% bovine serum albumin (BSA, Hyclone), tris-buffered saline with Tween-20 (TBST) solution. Blots were probed with primary antibodies diluted according to the manufacturer’s recommended concentrations in 1% BSA/TBST overnight at 4 °C with constant agitation. Blots were then washed 4 times with TBST and incubated with secondary antibodies (1:5000, 1%BSA/TBST) for 1 h at room temperature. Washing steps were repeated as with primary antibodies (Additional Table S3) and development was achieved using Clarity Western ECL substrate (Bio-Rad) and membranes were then incubated in blocking buffer. Band intensities were analyzed using Image Lab (Bio-rad) software. Uncropped scans of Western blots can be found in Additional information.

siRNA knockdown

siRNA against human HYAL1 (ON-TARGETplus SMARTpool, Dharmacon) and a non-targeting control (ON-TARGETplus NT#4, Dharmacon) were prepared in 1× siRNA buffer (Dharmacon) according to the manufacturer’s instructions to give a stock concentration of 20 μM. Employing reverse transfection, siRNA and transfection reagent (Dharmafect-1, Dharmacon) were first complexed together in serum-free heparin-free, EGM-2 medium for 20 min at room temperature before adding into empty, collagen-coated plates. BOECs were then prepared as described in Orbital Flow Setup and seeded into each well to give a final siRNA concentration of 25 or 50 nM and 2.5 μl of Dharmafect-1 per well. Experiments proceed as described in Orbital Flow Setup, Wound Healing Assay or Permeability Imaging Assay.

ELISA

Plasma samples were isolated and stored at –80°C after density gradient centrifugation of peripheral blood samples as mentioned under Derivation of BOECs and cell culture. Cell-free BOEC supernatant were harvested and frozen at –80°C after centrifuging at 13,000g for 10 min after supernatant were collected following Orbital flow setup above. Vitreous humor samples were given by Singapore National Eye Center. HYAL-1 was quantified using Human Hyaluronidase 1/HYAL1 DuoSet ELISA and DuoSet ELISA Ancillary Reagent Kit 2 (DY7358 and DY008 respectively from R&D Systems), in accordance with the manufacturer’s protocol. All plates were read by spectrophotometry at 450 nm, followed by a subtraction at 540 nm for optical correction using Synergy H1 (BioTek). HYAL-1 concentrations were determined from standard curves generated from four-parameter logistic (4-PL) curve fit (“Quest Graph™ Four Parameter Logistic (4PL) Curve Calculator,” AAT Bioquest, Inc) and multiplied by the dilution factor. Mann-Whitney test was selected to identify significantly different HYAL-1 levels across plasma, vitreous humor, and BOEC supernatant samples in both PCV and normal samples.

Heparan sulfate supplementation

To treat BOECs with heparan sulfate, BOECs were seeded on 12-well plates, 120,000 cells per well, in heparin-free EGM-2 media (CC-3162, Lonza) containing 10% heat-inactivated FBS. One day later, BOECs were serum-starved in EGM-2 (heparin-free) with 2% heat-inactivated FBS. One day later, BOECs were subjected to orbital flow as described above. Static controls were set up in the same conditions but without orbital rotation. At specific time points, BOEC cell lysates were collected for downstream gene expression analyses.

Confocal and automated microscopy imaging

All confocal imaging were carried out at NTU-Optical Bio-Imaging Centre on an Inverted Confocal Airyscan Microscope (LSM800, ZEISS), and automated imaging was carried out on the CellDiscoverer7 (ZEISS).
**Statistics**

Data analysis (excluding all single-cell RNA sequencing analyses) was performed with GraphPad Prism version 9.0.2. Data were tested for normality using the D’Agostino & Pearson test or Shapiro-Wilk test. \( P \) values for data with a single factor were obtained using a two-tailed \( t \)-test (parametric) or two-tailed Mann-Whitney test (non-parametric) as indicated. \( P \) values for data with 2 factors were assessed using a two-way ANOVA with Tukey’s multiple comparisons test. A value of \( P < 0.05 \) was considered statistically significant. Other relevant statistical considerations have been elaborated in figure legends.

**Abbreviations**

AMD: Age-related macular degeneration; BOEC: Blood outgrowth endothelial cell; GWAS: Genome-wide association studies; PBMC: Peripheral blood mononuclear cell; PCV: Polypoidal choroidal vasculopathy; siRNA: Small-interfering RNA; VEGF: Anti-vascular endothelial growth factor.

**Supplementary Information**

The online version contains supplementary material available at [https://doi.org/10.1186/s12915-022-01244-z](https://doi.org/10.1186/s12915-022-01244-z).

**Additional file 1: Figure S1.**

Extrinsic mediators influence sprouting angiogenesis of BOECs. Figure S2. scRNA-seq data quality checks and cluster marker enrichments. Figure S3. Differential expression in PCV and normal BOECs after heterogeneous flow. Figure S4. Differential expression in PCV and normal BOECs after heterogeneous flow. Table S1. Demographics details of PCV patients and normal individuals. Table S2. HYAL1 validation in clinical samples. Table S3. Key resources used in this study.

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**Authors’ contributions**

Conceptualization: CC. Data curation: KXW, NJYY, CYN, FWJC, FQ. Formal analysis: KXW, NJYY, CC. Investigation: KXW, NJYY, CYN, FWJC, FQ. Methodology: KXW, NJYY, CYN, FWJC, FQ, XFT, YBX, GN, HMT. Resources: CC, CMGC, HWH. Validation: KXW, NJYY, CYN, CC. Funding acquisition: CC, CMGC, XS. Project administration: CC, CMGC. Supervision: CC, CMGC, XS, NRD, HWH. Writing—original draft: KXW, CC. Writing—review & editing: All authors. All authors read and approved the manuscript.

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**Availability of data and materials**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christine Cheung (cheung@ntu.edu.sg).

Most materials used in this study are commercially procured. There are restrictions to the availability of blood outgrowth endothelial cell lines derived from human patients and normal donors due to ethics considerations for use of these materials within the current scope of study. Requests can be made to the lead contact as we will explore use of materials subject to new ethics approval and research collaboration agreement (including material transfer). The authors declare that all data supporting the findings of this study are available within the paper and additional information that includes original data of Western blots. Specifically, single-cell sequencing (scRNA-seq) dataset that support the findings of this study are available in Gene Expression Omnibus repository, Accession number GSE179631 ([https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179631](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE179631)) [81].

**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Local Ethics Committee of SingHealth Centralised Institutional Review Board (CIRB Refs: R1/496 and 2018/2004) and Nanyang Technological University Singapore Institutional Review Board (IRB-2018-01-026 and IRB-2019-03-011-01). Informed consent was obtained from all individual participants included in the study.

**Consent for publication**

Informed consent was obtained from all individual participants. There is no personally identifiable information in this article.

**Competing interests**

The authors declare that they have no competing interests.

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**References**

1. Wong RL, Lai TY. Polypoidal choroidal vasculopathy: an update on therapeu tic approaches. J Ophthalmic Vis Res. 2013;8(4):359–71.
2. Yuzawa M, Mori R, Kawamura A. The origins of polypoidal choroidal vasculopathy. Br J Ophthalmol. 2005;89(5):602–7.
3. Yannuzzi LA, Sorenson J, Spaide RF, Lipson B. Idiopathic polypoidal cho roidal vasculopathy (IPCV). Retina. 1990;10(1):1–8.
4. Yannuzzi LA, Sorenson J, Spaide RF, Lipson B. Idiopathic polypoidal cho roidal vasculopathy (IPCV). Retina. 1999;19(7):1503–10.
5. Yannuzzi LA, Sorenson J, Spaide RF, Lipson B. Idiopathic polypoidal cho roidal vasculopathy (IPCV). Retina. 1999;19(7):1503–10.
6. Japanese Study Group of Polypoidal Choroidal Vasculopathy. Criteria for diagnosis of polypoidal choroidal vasculopathy. Nippon Ganka Gakkai Zasshi. 2005;109(7):417.
7. Lim T, Laude A, Tan C. Polypoidal choroidal vasculopathy: an angiographic discussion. Eye. 2010;24(3):483.
8. Kumar A, Kumawat D, Sundar MD, Gagrani M, Gupta B, Roop P, et al. Polypoidal choroidal vasculopathy: a comprehensive clinical update. Ther Adv Ophthalmol. 2019;11:2515841419831152.
9. Tsujikawa A, Sasahara M, Otani A, Gotoh N, Kameda T, Iwashima D, et al. Pigment epithelial detachment in polypoidal choroidal vasculopathy. Am J Ophthalmol. 2007;143(1):102–11.
10. Dansingani KK, Balaratnasingam C, Naysan J, Freund KB. En face imaging of pachychoroid spectrum disorders with swept-source optical coherence tomography. Retina. 2016;36(3):499–516.
11. Lorentzen TD, Subhi Y, Sorensen TL. Prevalence of polypoidal choroidal vasculopathy in white patients with exudative age-related macular degeneration: systematic review and meta-analysis. Retina. 2018;38(12):2363–71.
12. Coscas G, Yamashiro K, Coscas F, De Benedetto U, Tsujikawa A, Miyake M, et al. Comparison of exudative age-related macular degeneration subtypes in Japanese and French Patients: multicenter diagnosis with multimodal imaging. Am J Ophthalmol. 2014;158(2):309–318.e302.
13. Imamura Y, Engelbert M, Iida T, Freund KB, Yannuzzi LA. Polypoidal choroidal vasculopathy: a review. Surv Ophthalmol. 2010;55(6):501–15.
14. Chen H, Liu K, Chen LJ, Hou P, Chen W, Pang CP. Genetic associations in polypoidal choroidal vasculopathy: a systematic review and meta-analysis. Mol Vis. 2012;18:816–29.
15. Fritsche LG, Igl W, Bailey NJ, Grassmann F, Sengupta S, Bragg-Gresham JL, et al. A large genome-wide association study of age-related macular degeneration highlights contributions of rare and common variants. Nat Genet. 2016;48(2):134–43.
16. Liang XY, Lai TY, Liu DT, Fan AH, Chen LJ, Tam PO, et al. Differentiation of exudative age-related macular degeneration and polypoidal choroidal vasculopathy in the ARMS2/HTRA1 locus. Invest Ophthalmol Vis Sci. 2012;53(6):3175–82.
17. Yanagisawa S, Kondo N, Miki A, Matsumiya W, Kusuhara S, Tsukahara Y, et al. Difference between age-related macular degeneration and polypoidal choroidal vasculopathy in the hereditary contribution of the A695 variant of the age-related maculopathy susceptibility 2 gene (ARMS2). Mol Vis. 2011;17(383-85):3574–82.
18. Ng TK, Liang XY, Lai TY, Ma L, Tam PO, Wang JX, et al. HTRA1 promoter variant differentiates polypoidal choroidal vasculopathy from exudative age-related macular degeneration. Sci Rep. 2016;6:28639.
19. Fleckenstein M, Keenan TDL, Guymer RH, Chakravarthy U, Schmitz-Valckenberg S, Klaver CC, et al. Age-related macular degeneration. Nat Rev Dis Prim. 2021;7(1):31.
20. Rebhun CB, Moulit EM, Novaia EA, Moreira-Neto C, Ploner SB, Louzada RN, et al. Polypoidal choroidal vasculopathy on swept-source optical coherence tomography angiography with variable interscan time analysis. Transl Vis Sci Technol. 2017;6(6):4–4.
21. Wang M, Zhou Y, Gao SS, Liu W, Huang Y, Huang D, et al. Evaluating polypoidal choroidal vasculopathy with optical coherence tomography angiography. Invest Ophthalmol Vis Sci. 2016;57(9):OCT526–32.
22. Volland S, Esteve-Rudd J, Hoo J, Yee C, Williams DS. A comparison of some organizational characteristics of the mouse central retina and the human macula. PLoS One. 2015;10(4):e0125631.
23. Marmormstein AD, Marmormstein L. The challenge of modeling macular degeneration in mice. Trends Genet. 2007;23(5):225–31.
24. Liang XY, Lai TY, Liu DT, Fan AH, Chen LJ, Tam PO, et al. Polypoidal choroidal vasculopathy on swept-source optical coherence tomography angiography with variable interscan time analysis. Invest Ophthalmol Vis Sci. 2016;57(10):OCTT1341–50.
25. Tobe T, Ortega S, Luna JD, Ozaki H, Okamoto N, Derevjanik NL, et al. Targeted disruption of the FGF2 gene does not prevent choroidal neovascularization in mice. Trends Genet. 2007;23(5):225–31.
26. Dardik A, Chen L, Frattini J, Asada H, Aziz F, Kudo FA, et al. Differential effects of orbital and laminar shear stress on endothelial cells. J Vasc Surg. 2005;41(5):869–80.
27. Ley K, Lundgren E, Berger E, Afors KE. Shear-dependent inhibition of granulocyte adhesion to cultured endothelium by dextran sulfate. Blood. 1989;73(5):1324–30.
28. Schefflin CG, Nair DM, Cristalloantoja JA, Zweier JL, Aleviadrou BR. Fluid mechanical forces and endothelial mitochondria: a bioengineering perspective. Cell Mol Bioeng. 2014;7(4):483–96.
29. Sun RJ, Muller S, Stoltz JF, Wang X. Shear stress induces caveolin-1 translocation in cultured endothelial cells. Eur Biophys J. 2002;30(8):605–11.
30. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet. 2000;25(1):25–9.
31. Gene Ontology C. The Gene Ontology resource: enriching a GOld mine. Nucleic Acids Res. 2012;41(D1):D323–34.
32. Jassal B, Matthews L, Viteri G, Gong C, Lorente F, Fabregat A, et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2020;48(D1):D498–d503.
33. Dekker RJ, van Soest F, Fontijn RD, Salamanca S, de Groet PG, VanBavel E, et al. Prolonged fluid shear stress induces a distinct set of endothelial cell genes, most specifically lung Kruppel-like factor (KLK2). Blood. 2002;100(5):1689–98.
34. Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, et al. Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. J Clin Invest. 2006;116(1):49–58.
35. Kanehisa M, Goto S. KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. 2000;28(1):27–30.
36. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillett MA, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102(43):15545–50.
37. Ajami NE, Gupta S, Mauzy MR, Nguyen P, Li JY, Shyu JY, et al. Systems biology analysis of longitudinal functional response of endothelial cells to shear stress. Proc Natl Acad Sci U S A 2017;114(41):10990–S.
38. Tarbell JM, Pahakis MY. Mechanotransduction and the glyocalyx. J Intern Med. 2006;259(4):339–50.
39. Fu BM, Tarbell JM. Mechanosensing and transduction by endothelial surface glyocalyx: composition, structure, and function. Wiley Interdiscip Rev Syst Biol Med. 2013;5(3):381–90.
40. Xanthis I, Souilhol C, Serbanovic-Canic J, Rodee H, Kalli AC, Fragiadaki M, et al. beta1 integrin is a sensor of blood flow direction. J Cell Sci. 2019;132(11):jc022954.
41. Bourguignon Y, Flaimon B. Respective roles of hyaluronidases 1 and 2 in endogenous hyaluronan turnover. FASEB J. 2016;30(6):2108–14.
42. Puisant E, Gils F, Dogne S, Flaimon B, Jadot M, Boonen M. Subcellular trafficking and activity of Hyal-1 and its processed forms in murine macrophages. Traffic. 2014;15(5):500–15.
43. Gasingirwa MC, Thiron J, Mertiens-Strijthagen J, Watiaux-De Coninck S, Flaimon B, Watiaux R, et al. Endocytosis of hyaluronidase-1 by the liver. Biochim Biochim Acta Struct Mol Biol. 2010;1800(2):305–13.
44. Bode L, Salvestrini C, Park PW, Li JP, Esko JD, Yamaguchi Y, et al. Heparan sulfate and syndecan-1 are essential in maintaining mucus and human intestinal epithelial barrier function. J Clin Invest. 2008;118(1):229–38.
54. Atkins GB, Jain MK. Role of Kruppel-like transcription factors in endothelial biology. Circ Res. 2007;100(12):1686–95.

55. Yanagir Y, Foo VHY, Yoshida A. Asian age-related macular degeneration: from basic science research perspective. Eye. 2019;33(1):34–49.

56. Henry CB, Duling BR. Permeation of the luminal capillary glycocalyx is determined by hyaluronan. Am J Phys. 1999;277(2):H508–14.

57. Vink H, Duling BR. Capillary endothelial surface layer selectively reduces plasma solute distribution volume. Am J Physiol Heart Circ Physiol. 2000;278(1):H285–9.

58. Patel KD, Nollert MU, McEver RP. P-selectin must extend a sufficient length from the plasma membrane to mediate rolling of neutrophils. J Cell Biol. 1995;131(6 Pt 2):1893–902.

59. Reitsma S, Slaff DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. Pflugers Arch. 2007;454(3):345–59.

60. Tarbell JM, Weinbaum S, Kamm RD. Cellular fluid mechanics and mechano-transduction. Ann Biomed Eng. 2005;33(12):1719–23.

61. Florian JL, Kosky JR, Ainslie K, Pang Z, Dull RD, Tarbell JM. Heparan sulfate proteoglycan is a mechanosensor on endothelial cells. Circ Res. 2003;93(10):e136–42.

62. Mochizuki S, Vink H, Hirama MS, Kajita T, Shiogeto F, Spaan JA, et al. Role of hyaluronic acid glycosaminoglycans in shear-induced endothelium-derived nitric oxide release. Am J Physiol Heart Circ Physiol. 2003;285(2):H722–6.

63. Gouverneur M, Spaan JA, Pannekoek H, Fontijn RD, Vink H. Fluid shear stress stimulates incorporation of hyaluronan into endothelial cell glycocalyx. Am J Physiol Heart Circ Physiol. 2006;290(1):H458–2.

64. Atfly AM, Stern M, Guntenhoner M, Stern R. Purification and characterization of human serum hyaluronidase. Arch Biochem Biophys. 1993;305(2):434–41.

65. frost GI, Ciska AB, Wong T, Stern R. Purification, cloning, and characterizing heterogeneity in single-cell RNA sequencing data. Cell. 2019;177(7):1888–902.e1821.

66. McQuin C, Goodman A, Chernyshev V, Kamentsky L, Cimini BA, Karhohs KW, et al. CellProfiler 3.0: next-generation image processing for biology. PLoS Biol. 2018;16(7):e2005970.

67. Lukowiak SW, Patell J, Andersen SB, Sim SL, Wongs HY, Tay J, et al. Single-cell transcriptional profiling of aortic endothelium identifies a hierarchy from endovascular progenitors to differentiated cells. Cell Rep. 2019;27(9):2748–2758.e2743.

68. Finak G, McDavid A, Yajima M, Deng J, Gerschkov V, Shahak AK, et al. MAST: a flexible statistical framework for assessing transcriptional changes and characterizing heterogeneity in single-cell RNA sequencing data. Genome Biol. 2015;16:278.

69. Yu G, Wang LG, Han Y, He QY. clusterProfiler: an R package for comparing biological themes among gene clusters. OMICS. 2012;16(5):284–7.