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Bioengineered 3D microvessels for investigating *Plasmodium falciparum* pathogenesis

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Abstract

Plasmodium falciparum pathogenesis is complex and intimately connected to vascular physiology. This is exemplified by cerebral malaria (CM) a neurovascular complication that accounts for most of the malaria deaths worldwide. *P. falciparum* sequestration in the brain microvasculature is a hallmark of CM and is not replicated in animal models. Numerous aspects of disease are challenging to fully understand from clinical studies, such as parasite binding tropism or causal pathways in blood-brain barrier breakdown. Recent bioengineering approaches allow for the generation of 3D microvessels and organ-specific vasculature that provide precise control of vessel architecture and flow dynamics and hold great promise for malaria research. Here, we discuss recent and future applications of bioengineered microvessels in malaria pathogenesis research.

Keywords

Cerebral malaria; *Plasmodium falciparum*; PfEMP1; blood vessels; vascular engineering; 3D microvessels

Plasmodium falciparum interplay with blood vessels

Malaria parasites have a complex and intimate relationship with blood vessels through multiple stages of their life cycle (Figure 1). Sporozoites are introduced into the human body via mosquito bite into the dermis. Sporozoites migrate by gliding motility in the skin and traverse blood vessels to enter the blood circulation [1]. Once in the bloodstream, sporozoites travel to the liver, arrest, and cross the liver sinusoidal barrier to invade hepatocyte cells [2]. After undergoing asexual replication, hepatocyte-derived vesicles containing merozoites, known as merosomes, bud through endothelial cells [3] and are released into the blood circulation [4]. Merosomes become trapped in small blood vessels,

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such as lung capillaries [5], and are thought to release merozoites, to initiate the blood stage of infection.

Malaria disease symptoms appear after repeated rounds of parasite replication in red blood cells. Among the human malaria species, *P. falciparum* is unique for having highly cytoadhesive infected red blood cells (iRBC) that bind to the endothelial lining of blood vessels and cause microvascular obstruction. **Sequestration** (see **Glossary**) is thought to have evolved for iRBCs to avoid spleen-dependent clearance mechanisms and is associated with organ-specific complications when parasites reach high burdens. During the blood stage, sexually committed gametocyte-iRBCs develop within the bone marrow sinuses before returning to the blood circulation for uptake within a new mosquito blood meal [6]. These multiple interactions with the vasculature reveal that *P. falciparum* has evolved a set of complex and sophisticated strategies in synergy with blood vessels and distinct microvascular niches. Although cytoadhesion is central to *P. falciparum* disease, animals are non-natural hosts for *P. falciparum* and may not be fully optimal to study all endothelial binding interactions or organ niches. Novel vascular bioengineering approaches could provide new opportunities to study *P. falciparum* and human vessel interactions in a more physiological way.

Vasculature: a heterogenous system

The blood circulatory system provides nutrients and oxygen to all tissues and takes away waste and metabolites for disposal. Blood vessels are organized in a hierarchical branching network, ranging in size from large caliber arteries (from 100 μm to ≤ 1 cm internal diameter) to arterioles (15 – 100 μm) to a dense capillary plexus (4 – 12 μm) where transport interchange takes place. Deoxygenated blood gets drained into venules (10 – 100 μm) and veins (from 100 μm to ≤ 1 cm in diameter). These variations in blood vessel sizes correlate to variations in **vascular wall** composition, blood flow, and pressure, leading to heterogeneous biophysical forces on the vessel walls. In addition, the microvasculature in different organs has diverse functions to meet the unique demands of the tissue. Endothelial cells line the innermost layer of vessels, and possess heterogeneous phenotypes and functions to regulate the transport between the blood and the surrounding tissue. In dermis, heart, and brain, endothelium forms a continuous layer with low permeability, whereas endothelium in kidneys is **fenestrated** to allow for filtration and reabsorption of small solutes (see Box 1). Endothelial cells in liver, bone marrow, and spleen are **sinusoidal** and present large gaps in endothelial-endothelial junctions that facilitate macromolecular and cell diffusion. These heterogeneous organ-specific vessels provide unique interactions with *P. falciparum* and facilitate different stages of the infection as the parasites enter at the skin, move to the liver, and exploit multiple organ niches throughout the vascular system. To better understand *P. falciparum* pathogenesis or how it progresses through the human life cycle, future research needs to take vascular heterogeneity into account.

Small blood vessels and *P. falciparum* cytoadhesion

Cytoadherent asexual iRBCs predominantly sequester within small blood vessels with internal diameters smaller than 50 μm. *P. falciparum* exports proteins into the erythrocyte

cytoplasm, many of which are involved in erythrocyte cytoskeletal modifications and inducing knob-like cytoadhesive platforms on the red blood cell surface [7–9]. The main cytoadhesion ligand is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by a family of approximately 60 *var* genes per parasite genotype. PfEMP1 proteins are expressed in a mutually exclusive fashion and endow different binding properties to iRBCs [10]. The protein family is classified into three main groups (A, B, and C) that encode distinct binding properties for CD36 [11, 12], endothelial protein C receptor (EPCR) [13, 14], and intercellular adhesion molecule 1 (ICAM-1) [15]. CD36 and EPCR are mutually exclusive binding traits [16], and have ancient origins because they are also encoded in the related *var*-like gene family in the chimpanzee malaria parasite, *P. reichenowi* [17]. EPCR-binding PfEMP1 variants are enriched in severe malaria infections [18, 19] and linked to brain swelling in cerebral malaria, [16, 20], even though only representing a minor subset of the PfEMP1 repertoire (~11–15%).

Besides controlling blood-tissue exchanges, endothelial cells have a major function in vascular hemostasis by maintaining an anti-thrombotic and anti-inflammatory surface [21]. Endothelial cells respond to inflammatory cytokines by upregulating pro-coagulant and pro-inflammatory pathways [21] (Box 1). The barrier properties of blood vessels are regulated by both barrier disruptive and barrier restorative signaling pathways [22]. Chronic activation or hyperinflammation can result in endothelial dysfunction, an alteration in endothelial state from a resting or “calm” phenotype to a highly activated phenotype where it is unable to perform its normal functions [21]. iRBCs in high parasite biomass are pro-coagulant and interfere with anti-coagulant pathways. Sequestered iRBCs release products that activate endothelial cells and induce barrier disruption in endothelial cell monolayers [23, 24]. They also interfere with EPCR function, which normally counteracts coagulation and induces anti-inflammatory and barrier restorative pathways in endothelial cells [14, 25]. Moreover, *in vitro* assays provide evidence that products released by schizont-stage *P. falciparum*-iRBCs interact with thrombin to prolong barrier disruption in endothelial cell monolayers [26, 27]. A better understanding of how endothelial cells integrate inflammatory signals may guide new approaches to treat endothelial dysfunction and vascular leak in severe malaria.

The blood-brain barrier and cerebral malaria pathology

The **blood-brain barrier** (BBB) presents the lowest permeability coefficient in the human vasculature and is thus essential to preserve brain function. Brain microvascular endothelial cells present a strong barrier phenotype, which is accomplished by elevated expression of **tight junction** proteins, low levels of transcytosis, and controlled traffic of molecules through specific efflux pumps and solute transporters. The unique properties of the BBB are acquired by its structure and interactions with brain parenchyma cells [28]. Brain pericytes are thought to contribute to the mechanical stability of the capillary wall [29]. In addition, astrocytic end-feet contact and support the vascular bed and secrete factors that enhance the expression of endothelial tight junctional proteins, such as claudin-5 and occludin, and specific transporters, such as glucose transporter-1 (GLUT-1) (Figure 2) [30]. The key role of the BBB in maintaining brain function, makes cerebral microvasculature a highly pathogenic site of sequestration.

Our knowledge of cerebral malaria has been acquired through examination of autopsy samples and more recently through neuroimaging studies. Magnetic resonance imaging (MRI) has indicated that severe brain swelling is associated with fatality in pediatric cerebral malaria [31]. Although the pathophysiological changes leading to brain swelling in cerebral malaria are incompletely understood, venous congestion and **vascular engorgement** are common findings, and MRI has found strong evidence for **vasogenic edema** in children [32, 33]. Brain swelling also occurs in adult CM, although at milder levels [34, 35]. Fatality in adults has been recently associated to severe hypoxia, which might be triggered by *P. falciparum* microvascular obstruction [35]. In both children and adults, the neurovascular pathology on MRI is compatible with Posterior Reversible Encephalopathy Syndrome (PRES), a neurological disorder that is characterized by vasogenic edema and endothelial dysfunction including breakdown of the BBB. PRES is reversible and patient survivors recover from brain swelling in 48–72h [32, 33].

Histological examination of pediatric brain autopsies has confirmed evidence of BBB breakdown. Fatal pediatric CM cases are divided into two groups: dense sequestration only (CM1) and dense sequestration accompanied by extra-erythrocytic pigment, **ring hemorrhages**, and evidence of systemic activation of coagulation (CM2) [36, 37]. Both groups have fibrinogen leakage to brain parenchyma, indicating BBB breakdown (Figure 2) [36, 38]. In addition, high levels of extracellular histones and plasma cell-free DNA are associated with brain swelling on MRI [39, 40]. Accumulation of iRBCs is common in both white and grey matter of the brain. Sequestration is highest in capillary beds or marginalized in post-capillary venules [38, 41]. The same pattern is evident in retinal imaging with higher accumulation in smaller vessels and on the venular side of circulation (small venules > post-capillary venules > large venules > pre-capillary arterioles > small arterioles > large arterioles) [42]. This pattern may arise in part because cells experience deacceleration and lower **shear stress** as they exit the capillaries into the larger post-capillary venule spaces. Collectively, these findings suggest interactions between sequestration, coagulation, and BBB dysregulation in cerebral malaria, albeit the extent of brain coagulopathy varies in fatal cases.

Both CM1 and CM2 cases show perivascular brain activation and pathology including myelin damage in cortex, subcortex, white matter and brain stem, and reactive astrocytes in all brain regions except the cortex [36]. Ring hemorrhages are concentrated in the white matter and watershed areas where blood supply is decreased. It has been hypothesized that biophysical and biological components may account for these brain differences [43, 44]. White matter vasculature presents longer and less ramified small blood vessels and is more prone to upstream occlusive damage. By comparison, grey matter small vessels are organized in a dense and sponge-like network with multiple anastomosis and collateral pathways, which might prevent local increase in **flow resistance** after occlusion and hemorrhages [38]. In addition, it has been hypothesized that differences in perivascular cell composition might account for different responses between white and grey matter that could confer diverse barrier properties or influence the localized response to sequestered iRBCs (reviewed in [44]).

Host response and cerebral malaria pathogenesis

A pro-coagulant and hyperinflammatory host response might additionally contribute to vascular dysfunction in CM patients. For example, fibrin deposits are common in pediatric brain autopsies upstream of ring hemorrhages (Figure 2). Moreover, low circulating platelet levels has been linked to fatal brain swelling in pediatric CM [20]. Whereas thrombocytopenia might have multiple systemic causes, platelet accumulation in the brain vasculature might have pathogenic consequences [45, 46]. Platelets have both immunogenic and pro-coagulant functions, which might lead to a dual protective or damaging effect on blood vessels. Activated platelets secrete pro-inflammatory cytokines, including tumor necrosis factor alpha (TNFα) [47], transforming growth factor TGF-β1, and IL-1β [48], that enhance the local inflammatory milieu. They also release, microparticles that potentially inhibit parasite growth [49]. Platelets can bridge binding of iRBC without tropism to the brain endothelium [50], and may promote microvascular obstruction by platelet-mediated clumping of iRBC [51] or iRBC binding to platelet-**von Willebrand Factor** (vWF) multimers secreted upon endothelial activation or injury [52] (Figure 2).

Recent studies have revealed increased transcriptional signatures of neutrophil activation and the presence of neutrophil granular proteins in plasma of severe malaria patients [53, 54]. In addition, a recent study has shown that neutrophil extracellular traps (NETs) are found at sites of iRBC sequestration [55] (Figure 2). Like platelets, neutrophils and NETs might have a dual protective-harmful role (reviewed [56]). An additional cell type that has recently been associated to CM are CD8+ T cells. Autopsy studies have shown their enrichment in the choroid plexus [57] or the lumen of veins colocalizing with granzyme B staining [58]. ***In vitro* vascular engineered models** may provide a means to dissect and disentangle the individual and collective contribution of *P. falciparum* iRBC, platelets, neutrophils and CD8+ T cells in CM pathogenesis.

Organ-specific *P. falciparum*-iRBC cytoadhesion

Children who die from **cerebral malaria** have massive sequestration of iRBC in the brain, but also in the vasculature of the gastrointestinal tract and/or subcutaneous adipose tissue of the skin. Other sites of sequestration include the heart, lung, spleen, and to a lesser extent the kidney [59]. Despite evidence of a broad sequestration [59], parasite tropism for most organ/vascular sites remains terra incognito.

Although the gut is considered a relatively non-pathogenic sequestration site, malaria patients frequently have gastrointestinal symptoms, and the gastrointestinal vasculature (from the stomach to the large colon) is an intense site of sequestration in fatal pediatric cerebral malaria cases (Figure 1) [41, 59]. Gut sequestration has also been described in an adult CM autopsy series [38]. The gastrointestinal tract represents an enormous surface area and has voluminous fenestrated capillary beds for iRBC sequestration, and therefore makes a major contribution to the high parasite burdens in fatal cases [59]. In agreement with autopsy findings, the microcirculatory blood flow of the rectal mucosa is markedly disturbed in adult severe malaria patients [60]. The extent of mucosal microvascular obstruction and blocked capillaries by *in vivo* imaging is proportional to disease severity and correlates with

base deficit in plasma and the concentration of lactate [60]. Moreover, acidic microbial products contribute to metabolic acidosis in malaria, indicating that intestinal barrier function may be compromised [61]. Metabolic acidosis is associated with high mortality and significantly increases the risk of cerebral malaria mortality [62, 63]. Collectively, these findings implicate gut sequestration in high parasite burdens and raise the possibility of a gut-brain axis in CM. It remains to be determined if the same or different parasite binding variants sequester in gut and brain.

Conversely, the kidney is not a major sequestration site, but acute kidney injury and renal failure is frequent in children [64] an adult severe malaria [63], respectively. The kidney contains two highly specialized microvasculature, the glomerular capillaries and the tubular capillaries, with distinct cell structures and specialized functions. The glomerular endothelial cells are fenestrated and covered by a thick glycocalyx that facilitates the sieving properties of glomerular filtration. The peritubular capillary endothelial cells have a thin fenestrated diaphragm, which facilitates reabsorption and secretion of products between blood and adjacent tubular epithelial cells. The red average blood cell velocity is higher in the glomerular capillaries (~16.7 mm/s) compared to the peritubular capillaries (4.7 mm/s) [65]. Quantitative ultrastructure studies show that parasite sequestration is higher in the peritubular capillaries than glomerular capillaries, and that malaria-associated renal failure was associated with renal tubular injury rather than glomerulonephritis [66]. The parasite binding variants of the kidney remain unknown.

In pregnant women, *P. falciparum*-iRBCs sequester within the maternal intervillous spaces and bind to the syncytiotrophoblast lining causing placental malaria (Figure 1). The placenta architecture is highly complex and remodels during pregnancy. Blood flow is much slower in the placenta than other organs which may facilitate iRBC sequestration [67]. Whereas the mean flow velocity in a third trimester placenta is of 0.4 mm/s, other microvascular beds present a flow velocity gradient between 1 and 16 mm/s. Likewise, iRBCs are exposed to a much lower wall shear stress (0.5–2.3 dyn/cm^2) in the placenta than in the systemic microvasculature (1–6 dyn/cm^2) [68]. Placental binding parasites adhere to a unique low sulfated chondroitin sulfate A in the placenta [69, 70]. This causes placental malaria-derived complications, such as premature birth or miscarriage. The heterogeneity and complexity of organ microvasculature highlights the need for better *in vitro* models that recapitulate functional organ-specific microvessel beds and their varied flow dynamics to study parasite-vascular tropism in CM and severe malaria (Box 2).

Modeling *P. falciparum* cytoadhesion *in vitro*

The factors that determine iRBC-organ sequestration patterns are incompletely understood. The simplest assay and workhorse for investigating parasite binding is the static cytoadhesion assay [12, 15]. In this assay, iRBCs settle on cells or spotted proteins and then are gently washed. This technique has the significant advantage in that it is easy to implement, even in a low-tech field research setting, and therefore has seen abundant usage for characterizing receptor and endothelial-specific interactions [71–75]. For instance, this approach has shown that the same parasite variants can bind to primary human brain, lung, and heart microvascular endothelial cells [76]. However, a major disadvantage is that this

assay mostly measures the strength of iRBC attachment during washing steps and provides limited insights into iRBC capture from flow and other flow-based considerations that may be important for vascular tropism. To account for this limitation, flow-based microfluidic systems have been applied [77, 78]. While much more difficult to implement in a field setting, a recent study using commercial linear **flow chambers** found that increased number of iRBC from cerebral malaria patients could bind to primary brain endothelial cell monolayer under flow than uncomplicated malaria cases [79], suggesting expansion of brain-tropic parasites in cerebral malaria patients. While significant mechanistic insights have been gained from static and flow-based binding assays, 2D monolayer formats lack important microvascular parameters, such as lumen dimensions, branching architecture, and vessel curvature that influence microfluidic parameters and endothelial transcription and behavior. New *in vitro* vascular engineering models pave the way for more physiological studies for malaria pathogenesis research.

New avenues in vascular *in vitro* engineering

Recent advances in tissue engineering have allowed for the development of various modeling strategies to study vascular diseases and brings their potential to push the frontier of malaria research. Different vascular models have been generated with varying complexities. While vascular networks can be generated via **self-assembly** either as cells in a gel [80, 81] or **vascularized organoids** [82] from stem cells, the lack of control on vascular structure and perfusion has limited their usage in studies that require refined flow control. Recent advances have been focused on the creation of 3D **perfusable microvascular models** through a variety of manufacturing techniques. These methods offer different levels of cellular, structural and physiological complexity to investigate the interaction of molecular and cellular processes in different organ environments.

PDMS-based microfluidic channels and networks

Twenty years ago, **soft lithography** technology was developed to transfer the silicon-based microfabrication technology into silicone-based materials, such as polydimethylsiloxane (PDMS). This technology has been exploited to fabricate microchannels and transparent networks that can be studied under precise microfluidic flow when connected to a flow pump. PDMS microchannel networks extended the horizon of malaria research by providing tools to study rheology of *P. falciparum*-iRBC through geometries that mimicked capillary constrictions or splenic endothelial slits in PDMS models (reviewed in [83]). However, limitations exist in the use of PDMS and similar materials, due to the high chemical absorbability and its rigidness, which is far from recapitulating the biomechanical properties of the vessel wall or the lubricating properties of endothelial cells. Additionally, this platform imposes difficulties in studying the effect of the perivascular cell's extracellular matrix on endothelial cell phenotype and heterogeneity.

Hydrogel-based microvascular networks

To better mimic the microvascular environment, soft lithography techniques were later extended into **hydrogels** platforms to build microfluidic networks in cell-compatible biomimetic extracellular matrices, such as collagen and fibrin. The resultant channels can be

seeded with organ-specific endothelial cells in channels with different branching architecture, and perfused with defined flow conditions [84] (Figure 3). Conventional soft lithography techniques can generate robust vessels at diameters of 50 to 500 μm in collagen but they tend to collapse at smaller sizes [85]. An advantage of the hydrogel format is that perivascular cells, like pericytes, can be seeded into the biomatrix and directly interact with endothelial cells [84]. Other advantages of hydrogel-based vascular engineering platforms are described in Figure 3 and Box 2.

The microvessel format has been used in several recent studies for investigating vessel wall interactions that are relevant for malaria research. For example, a 13x13 grid network design based on human umbilical vein endothelial cells was used to create a large range of physiological and pathological flow velocities that have been used to study flow driven interactions of vWF and platelets [85]. This analysis showed that the ability of vWF to assemble into thicker fibers and more complex meshworks was influenced by vessel geometry and microfluidic properties (Box 1) The same grid-based 3D microvessel format was adapted to study *P. falciparum*-iRBC interactions with primary brain endothelial cells [86]. This study found that iRBC binding to 3D brain microvessels is strongly influenced by physiological differences in flow shear that exists within the brain microcirculation, and that parasite adhesion strength is highly sensitive to changes in EPCR and ICAM-1 expression levels on the endothelial cell surface caused by TNFα activation. Some PfEMP1-clonal parasite lines presented increased sequestration, while no difference was found in others, depending on their combinatorial binding properties [86]. These findings suggest a highly tuned strategy for parasite sequestration under different conditions experienced during human infection.

Endothelial-only 3D microvessel models have also been used to understand changes in endothelial permeability during malaria infections. For instance, the endothelial-cell only 3D microvessel 13x13 model has been used to study barrier function in response to human CM sera [87]. In a different endothelial cell-only model built from a crosslinked agarose–gelatin interpenetrating polymer-network (IPN) hydrogel, iRBCs caused occlusion of 20 μm microvessels followed by an immediate increase of permeability. Two days after occlusion, the microvessels recovered baseline permeability levels and endothelial cells were found to have engulfed the malaria pigment, hemozoin [88]. To better mimic brain pathogenic events in CM patients, future models might incorporate hypoxia.

Alternatives approaches to build perfusable tubes and networks in hydrogels, include **subtractive molding** [89] and **bioprinting** [90–92]. Success was demonstrated via subtractive molding to remove a needle from a hydrogel after gelation and form a hollow lumen for endothelial cell seeding and culture. Bioprinting has been demonstrated in either the direct printing of highly viscous biomaterials or sacrificial materials [90, 91] that can be removed after the surrounding hydrogel or scaffolds are cross-linked, followed by endothelial cell seeding and culture. This highly automated process has shown success in generating thick, complex 3D channels for endothelial cell seeding and culture to form defined networks. These approaches allow the integration of perivascular and extravascular cells, and controlled perfusion but are restricted to relatively large diameter vessels (100 μm

or larger), which are larger than the capillary and post-capillary venules where parasite sequestration usually occurs [36, 42].

More recently, multiphoton ablation technology has been used to create capillary networks (down to 5 μm) between two wider parallel microvessels [93]. Multiphoton ablation allows precise control of vessel diameter and exploits angiogenesis for endothelial cell in-growth to connect the two wider microvessels. We recently generated an arteriole-capillary-venule unit by combining soft-lithography based approach and multiphoton ablation, and used it to study the biomechanics of iRBC sequestration and microvascular occlusion. Whereas normal red blood cells readily traversed the capillary-sized constriction (5–10 μm) with negligible vessel wall interactions, iRBC displayed increased tumbling motions and accumulated. Sequestration was influenced by changes in red blood cell deformability, parasite adhesion properties, and changes in velocity experienced by cells as they move through different-sized vessels. Similar to human infections, iRBC predominantly sequester within the capillary and postcapillary-sized vessels of the arteriole-capillary-venule units [93]. Future models could include the presence of other host blood cells, including platelets, neutrophils or rosettes.

Blood brain barrier models

These new bioengineered microvessel models have shown promise in revealing new vascular biology phenomena and have provided molecular insights into molecular, microfluidic, and geometric factors that promote microvascular obstruction in malaria. Future CM models need to better recapitulate the unique cellular environment and barrier properties of the BBB. Many efforts have been focused to the development of 3D-BBB models with different degrees of success. Two promising models that combine PDMS and hydrogel based microfluidic fabrication methods have achieved physiological low permeability rates similar to human BBB [94, 95]. Both models incorporate endothelial cells differentiated from **induced pluripotent stem cells** and primary astrocytes and pericytes that increase the expression and strength of tight junctions and BBB specific transporters. These models have outstanding potential for studying barrier function. However, they present some limitations as either they do not reproduce the dimensions and branching architecture of the brain microvasculature [94], or do not offer a reproducible flow control which makes difficult the generation of biological replicates [95]. As flow has an important role to tune endothelial cell transcriptional networks [96] or BBB properties [97], future CM *in vitro* vascular models need to achieve low BBB permeability rates and consistently reproduce brain branching networks and flow properties.

Concluding remarks

P. falciparum sequestration is broadly distributed in diverse microvasculature beds in fatal CM cases [59]. Vascular dysfunction contributes to the complex array of multi-system organ complications that malaria patients can suffer. Along the circulatory system, the endothelial lining of blood vessels presents differences in receptor expression, and organ-specific microvessels present distinctive branching architectures and flow properties that may influence parasite sequestration burdens. Likewise, the unique functions of each

microvascular bed, given by distinct perivascular cell composition and endothelial cell phenotype, might influence how blood vessels react to parasite, inflammatory stimuli and immune cells. To better understand organ-specific disease mechanisms in malaria and evaluate therapeutic interventions, *in vitro* models need to mimic disease mechanisms found in patients and recapitulate functional and structural heterogeneity of organ-microvessel beds (see Outstanding questions).

Altogether, engineered i*n vitro* 3D microvessels offer a versatile opportunity to consistently reproduce and understand the heterogenous pathology of a complex disease such as malaria. Hydrogel-based methods provide an extracellular matrix that supports the growth of multiple cell types. Some of these methodologies allow control over network branching, microfluidic properties, and internal lumen dimensions with regulated perfusion of different parasite and blood components that, independently or collectively, might cause CM pathogenesis. In the last decade multiple organ-specific vascular models have been developed by using endothelial from primary origin or differentiated from iPSC, including kidney [98, 99], brain [86] or BBB models [94, 95]. These models hold great promise to gain a better understanding of the organ-specific disease mechanisms in CM patients. Likewise, although less explored, future models of the skin, liver, or bone marrow microvasculature might reveal new findings in the biology of pre-erythrocytic and transmission malaria stages.

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Glossary:

Bioprinting
use of 3D printing technology with materials that sustain viable living cells. The main limitation for vascular engineering is that current technologies do not allow the fabrication of small diameter vessels

Blood-brain barrier
Highly impermeable blood vessels that separate the blood from brain tissue. It is composed by endothelial cells, and surrounding pericytes, astrocytes and extracellular matrix

Cerebral malaria
Severe malaria complication characterized by patient coma and neurovascular pathology. Sequestration of *P. falciparum*-iRBC is a hallmark of cerebral malaria

Fenestrated (endothelium)
Fenestrations are round or oval 60–80 nm diameter transcellular pores in endothelial cells. Flow through the fenestrae is controlled by a diaphragm and allows for small protein and molecule diffusion

Flow chambers

Microfluidic devices that can be perfused through a flow pump. Endothelial cells grow in a 2D monolayer on plastic and glass. They can be commercial or self-fabricated and generally have a channel width of 1mm.

Flow resistance
The force opposing the flow of blood through a vessel which is dependent on vessel length and branching structure, vessel diameter, and viscosity of the blood

Hydrogel
biomaterial that sustains cellular growth in 3D. Examples of hydrogels are collagen, fibrin or Matrigel

***In vitro* vascular engineered models**
In vitro models that mimic the three-dimensional structure of blood vessels and that are perfusable with media and blood components

Induced Pluripotent Stem Cells (iPSC)
Pluripotent stem cells that can be generated from any somatic cell, for example white blood cells or dermal fibroblasts. They can propagate indefinitely and differentiate in any cell of the human body

Perfusable microvascular models
Engineered 3D microvessels that can be perfused with media and flow components in a controlled way

Placental malaria
Malaria complication associated with the adhesion of infected erythrocytes within the intervillous space of the placenta

Ring hemorrhages
Brain microvascular hemorrhages with a round shape that are usually found following a capillary blocked by fibrin depositions

Self-assembly/ Self-assembled models
Models where cells are seeded in hydrogels and self-organize organized replicas of tissue and organs. Organoids are examples of self-assembled models

Sequestration
Accumulation of *P. falciparum*-iRBC in the microvasculature caused by iRBC binding/ cytoadhesion to endothelial cells

Shear stress
Force that causes deformation of materials and cells by slippage along a plane, for example the endothelium. It can vary depending on fluid viscosity, flow rate, vessel diameter or length and branching network

Sinusoid

Small blood vessels of 30–40 um diameter with wide gaps in endothelial junctions, that take the place of capillaries and venules in the liver, bone marrow and spleen

Soft lithography
Fabrication technique to build microfluidic networks or devices that uses silicon-based materials and photomasks

Subtractive molding
Manufacturing technique used to create channels by the gelation of a material around a removable object, such as a needle, which is then removed from the material leaving an open lumen

Tight junction
Multiprotein complex at endothelial cell junctions that controls the passage of ions, fluids and small molecules through paracellular transport (in between cells)

Vascular engorgement
Local congestion and enlargement of blood vessels. In CM it is likely to be caused by sequestered *P. falciparum*-iRBC

Vascular permeability
Capacity of blood vessels to allow exchange of small molecules, or even cells, between blood and the surrounding tissue

Vascular wall
Blood vessel structure composed of endothelial cells, perivascular cells (pericytes or smooth muscle cells) and extracellular matrix

Vascularized organoids
Organoids are simplified version of organs grown *in vitro* with realistic cell types and tissue organization. Although different organoid types containing endothelial cells have been generated, they are generally not perfusable *in vitro,* which poses limitations for malaria research

Vasogenic (brain) edema
Brain swelling cause by blood-brain barrier breakdown

Von Willebrand Factor
glycoprotein released after endothelial activation. It forms long strings and has an important role in coagulation because it binds to platelets and coagulation factors

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Highlights

- Research into cytoadhesive complications of *Plasmodium falciparum* infection has been hampered by the lack of suitable animal models and limitations of endothelial cell monolayer models.
- Bioengineered microvessels offer precise control over vascular cell types, branching architecture, lumen diameter, and flow dynamics. They provide new opportunities for investigating *Plasmodium*-vessel interactions.
- 3D microvessels models have been used to explore how flow dynamics, vessel diameter, and endothelial activation influence sequestration. They can also model the endothelial cells response to host and parasite inflammatory products.
- Future bioengineered models could include tissue-specific vasculature to investigate organ-specific injury in severe malaria, evaluate novel therapeutic interactions, or improve our understanding of other key parasite-blood vessel interactions in the malaria life cycle.

Box 1.

Modeling parasite-vessel interaction in response to inflammation and hemodynamic factors.

The endothelium is a dynamic organ. It shifts from an anti-inflammatory and anti-coagulant phenotype in health to a pro-adhesive, procoagulant, and complement-activating phenotype in response to infection and injury. Most malaria infections are asymptomatic with low parasite burdens and limited vascular activation [100]. Only a subset of infections has high parasite burdens with extensive microvascular obstruction, widespread endothelial activation, and endothelial dysfunction. Although PfEMP1s encode diverse binding phenotypes, it is unknown whether the parasite cytoadhesion strategy adjusts to the differing microvascular environments in asymptomatic and symptomatic hosts [100]. Hemodynamic forces are a major driver of endothelial cell phenotypes. Shear stress lowers vascular permeability and the mechano-activated transcriptional programs, such as Kruppel-like factor 2 (KLF2), confer a barrier-strengthening, anti-inflammatory and anti-thrombotic phenotype on endothelial cells [96]. Flow forces have been binned into laminar flow (where vessel geometry is smooth and uniform) and disturbed flow (where vessels bifurcate, high curvature, or from microvascular obstruction). Vessel geometry and microfluidic forces play an important role in thrombosis. For instance, vWF fibers preferentially form near vessel bifurcations and vWF assembly is influenced by 3D vessel architecture, fluid shear stress and flow acceleration [85]. Exposure of 3D human microvessel models to physiological flow forces will enable study of how microvascular endothelial cells respond to inflammatory, pro-coagulant, and hemodynamic forces, as well as to explore the progression to endothelial dysfunction in malaria disease.

Box 2:

Engineered 3D microvessels: a versatile system and applications in malaria pathogenesis research.

Controlled vessel size and geometry: The fabrication of 3D microvessel networks is not limited to previously published 13 x 13 grids or parallel channels connected by capillary-size vessels (Figure 3). The combination of soft lithography, injection molding and laser photoablation allows the design of custom-made geometries. This provides control of vessel length and diameter, number of branches, branching angle, and vessel curvature. The 3D microvessel network will determine microfluidic properties which 1) regulate endothelial cell phenotype, including gene expression and transcriptional profile or 2) how blood cells interact with the vessel wall. These systems can be used to study parasite sequestration in engineered blood vessels that precisely mimic the shape and curve of natural organ-specific microvessels, as well as to explore how vessel geometry might determine parasite-mediated vascular damage.

Fabrication of organ-specific cell types and vasculature: These fabrication technologies support the growth and development of new vascular-specific microvasculature models, including human kidney fenestrated peritubular microvascular endothelial cells [98] or even models that combine two microfluidic networks, an endothelial and an epithelial, that recapitulate the vascular-tubular renal interface in the kidney [99]. Additionally, patient specific cells can be used, either primary cells or iPSC- differentiated cells. This offers controlled experimental conditions to study parasite-vessel tropism and investigate disease mechanisms leading to specific endothelial damage in different regions of the brain or in other organs.

Controlled perfusion of blood components: Attaching the 3D microvessel network into a flow pump allows for precise control of biological and biomechanical parameters in a step-wise fashion. For example, different blood cells or molecules (e.g. cytokines, clotting factors, patient blood samples) can be perfused independently, sequentially or in combination at controlled microfluidic conditions to dissect how human or *P. falciparum* factors contribute to severe disease, individually or in synergy.

Outstanding questions:

Malaria pathogenesis

- Do factors released by *P. falciparum*-iRBCs sequestration cause BBB breakdown?
- How do endothelial cells integrate inflammatory stimuli from malaria parasites, systemic factors and infiltrating host cells?
- How do host cells, including platelets, neutrophils, and CD8+ T cells contribute to brain endothelial activation and BBB breakdown?
- Is there cross-talk between endothelial cells, perivascular cells, and parenchymal cells in CM pathophysiology?
- Can we design adjunctive therapies that prevent vascular disfunction in severe and cerebral malaria?

Vascular engineering for malaria research:

- Can we use *in vitro* vascular engineering to develop BBB or neurovascular unit models that recapitulate brain microvascular structure and flow-induced properties to understand microvascular obstruction in the brain?
- Can these models (or simplified versions) be used to understand CM malaria pathogenesis in the BBB and brain parenchyma or to evaluate new therapeutic interventions?
- Can vascular tissue-specific models, including placenta, kidney and gut, be developed to understand other organ-complications in severe malaria?
- Can vascular engineered models be used to investigate other stages of the malaria life-cycle including sporozoite interactions with dermal or liver sinusoidal barrier or gametocyte-bone marrow interactions?

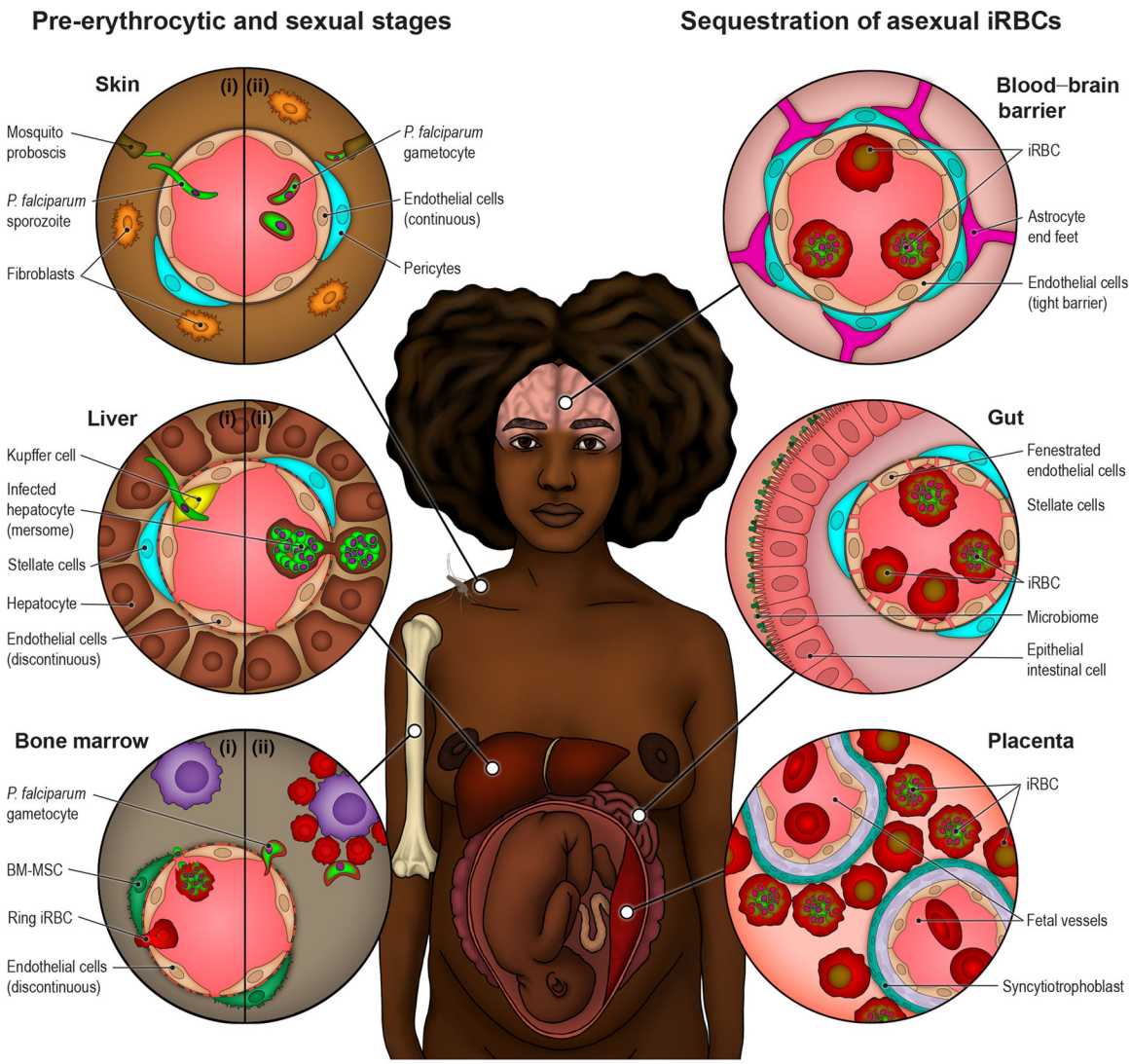

Figure 1. *P. falciparum* interactions with the vasculature along its life cycle.

Top left circle (Skin): *P. falciparum* parasites interact with skin blood vessels when initiating infection (I) and during transmission back to mosquitoes (II). The molecular mechanism of sporozoite crossing across blood vessels or whether mature gametocytes accumulate in skin capillaries remains unknown. Middle left circle (Liver): sporozoites arrest on endothelial cells in the liver and cross the liver sinuosoidal barrier probably through Kupffer cells to infect hepatocytes (I). There, they will asexually multiply into thousands of merozoites that will be released back to bloodstream through budding of membranous structures known as merosomes, containing merozoite parasite forms (II). Left bottom circle (Bone Marrow): *P. falciparum* sexual development occurs in the bone marrow. Immature ring stage iRBC or merozoites, asexual or sexually committed, have been proposed as candidates to cross the discontinuous bone marrow vasculature (I). Early stage gametocye-iRBCs mature on erythroblastic islands until they re-enter the blood circulation as less-rigid stage V gametocyte-iRBCs (II). Asexual *P. falciparum*-iRBC sequester in the microvasculature to avoid splenic clearance. There is widespread sequestration in fatal cases. Top right circle

(Blood-Brain Barrier): *P. falciparum* accumulation in the brain is highly pathogenic due to inherent properties of the blood-brain barrier to protect neuronal function. Middle right circle (Gut): One of the main parasite sequestration sites is the gut fenestrated microvasculature, which may contribute to high parasite biomass. Right bottom circle (Placenta): During pregnancy, *P. falciparum*-iRBCs accumulate in the maternal intervillous space and attached to the syncytiotrophoblast lining in the placenta.

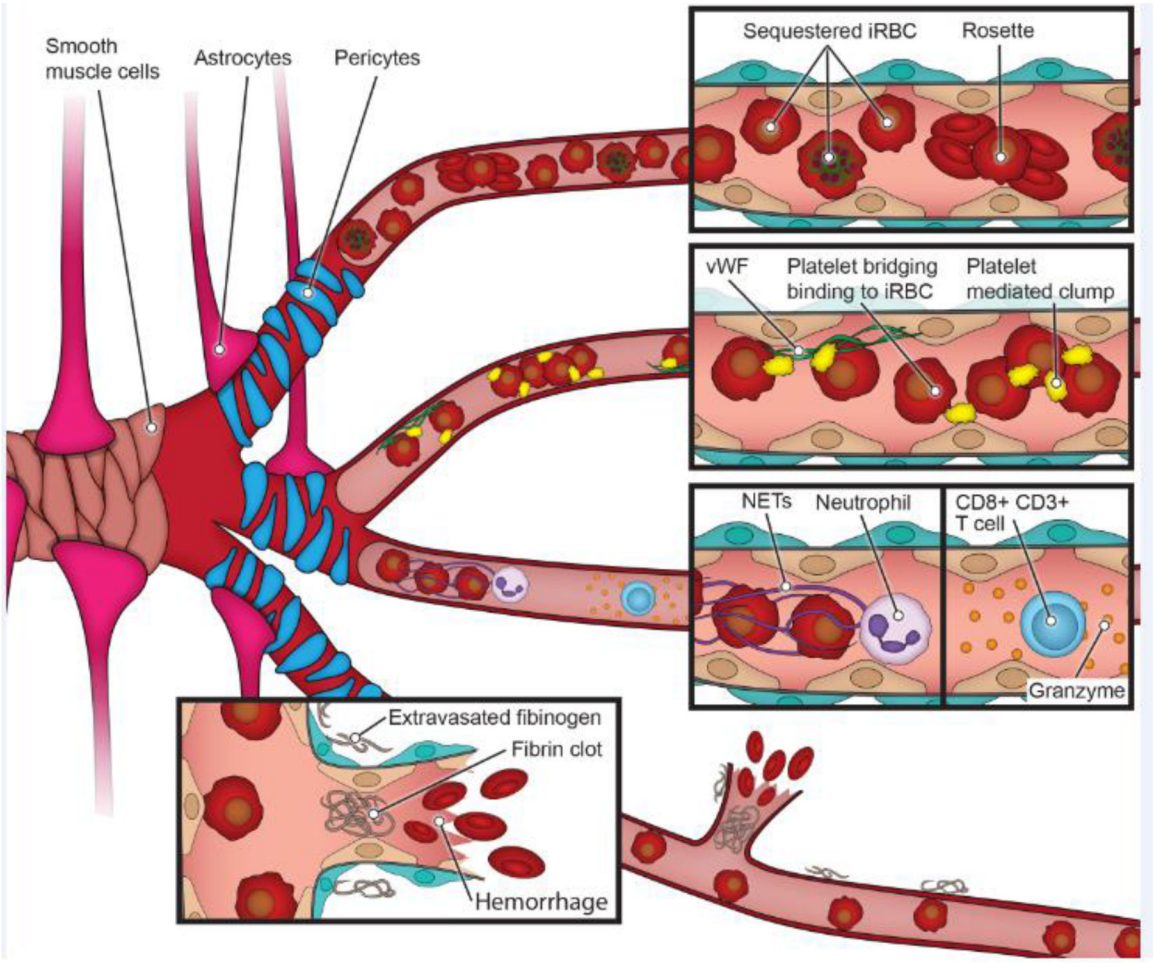

Figure 2. Cerebral malaria pathogenesis.

The unique properties of the BBB are achieved through interactions between endothelial cells, perivascular cells (smooth muscle cells in larger vessels and pericytes in the microvasculature) and astrocytes. *P. falciparum* sequestration in the brain microvasculature is a hallmark of CM and contributes to microvascular obstruction, probably along with rosettes, aggregates of iRBC to uninfected red blood cells. In autopsy studies, BBB disruption is exemplified by ring hemorrhages or fibrinogen deposition in the extravascular space. Platelets might also contribute to CM pathogenesis by bridging iRBC to vWF or endothelium or causing platelet-mediated iRBC clumps. Other cell types implicated in disease are neutrophils and CD8+ T cells. These pathogenic findings are sometimes localized in distinct regions of the brain, such as the white and grey matter [44].

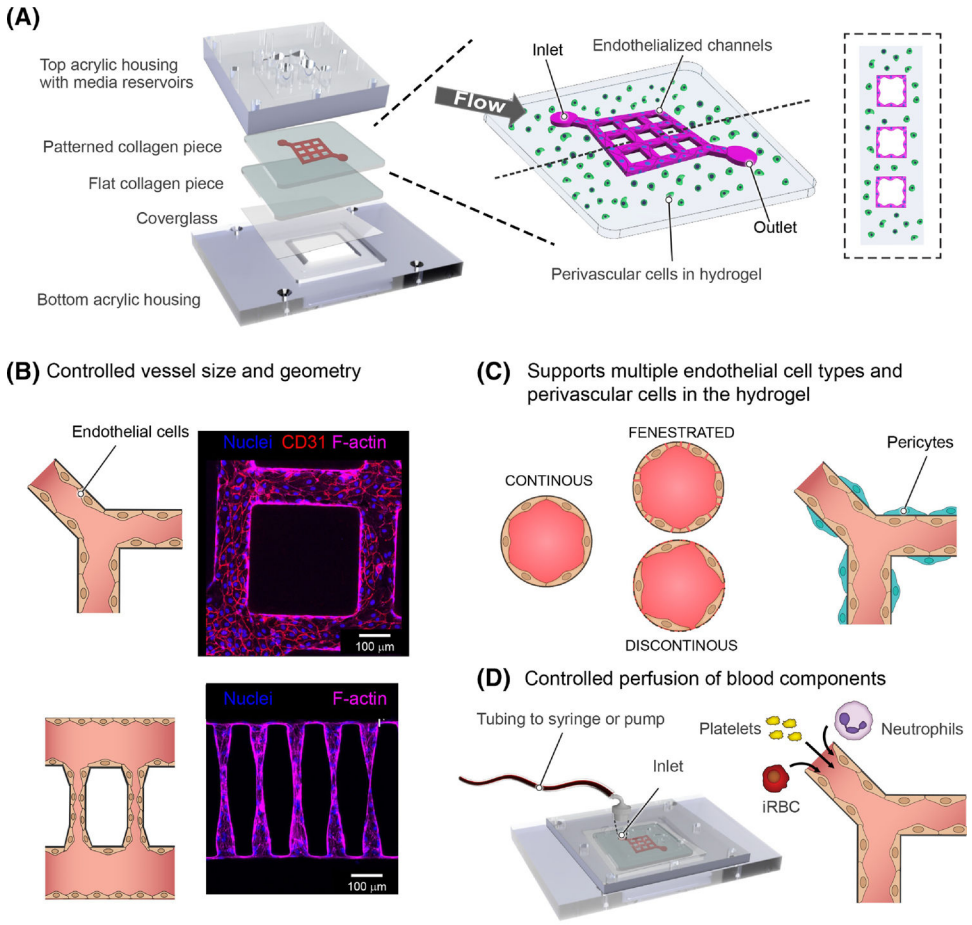

Figure 3. Bioengineered *in vitro* vascular models.

a. Rendering of device components (left), and schematic representation of 3D microvessels with a 3x3 grid microfluidic network (right). Bioengineered 3D microvessels are a versatile model. b. By combining fabrication methods like soft-lithography and photoablation control can be achieved over vessel size, diameter and network geometry. This is exemplified by immunofluorescences showing a portion of a 13X13 grid composed of a network of 100 μm diameter vessels (top), and vessels mimicking arteriole-capillary-venule transition with a diameter of 5–15 μm in the narrowest region (bottom). c. Engineered 3D microvessels are compatible with multiple endothelial cell types including continuous, fenestrated and sinusoidal endothelium, and support the growth of perivascular and parenchyma cells within the hydrogel. d. The use of a predetermined network provides refined control over microfluidic properties when connected to a flow pump. Perfusion of different blood components through the device can be independent, sequential or combined to understand the independent or synergistic contribution of parasite and host components in CM.