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Cerebral malaria
Mysteries at the blood-brain barrier

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Cerebral malaria is the most severe pathology caused by the malaria parasite, *Plasmodium falciparum*. The pathogenic mechanisms leading to cerebral malaria are still poorly defined as studies have been hampered by limited accessibility to human tissues. Nevertheless, histopathology of post-mortem human tissues and mouse models of cerebral malaria have indicated involvement of the blood-brain barrier in cerebral malaria. In contrast to viruses and bacteria, malaria parasites do not infiltrate and infect the brain parenchyma. Instead, rupture of the blood-brain barrier occurs and may lead to hemorrhages resulting in neurological alterations. Here, we review the most recent findings from human studies and mouse models on the interactions of malaria parasites and the blood-brain barrier, shedding light on the pathogenesis of cerebral malaria, which may provide directions for possible interventions.

Malaria remains one of the most prevalent infectious diseases in the world. The World Health Organization (WHO) reports that 50% of the world's population living in 109 countries are still at risk of malaria.[1] More than 300 million clinical cases of malaria and one million deaths occur annually. Children under the age of 5 and pregnant women are most vulnerable to the disease. Hence, malaria continues to be a major global health problem, posing an enormous burden on mankind socially and economically.[2]

The Malaria Parasite

Plasmodium, the infectious agent responsible for malaria, is transmitted by *Anopheles* spp mosquitoes. There are five species of *Plasmodium* spp infecting humans, with *P. falciparum* and *P. vivax* being the two most widespread.[1] The infection begins when an infected female Anopheles mosquito injects 5 to 50 sporozoites into the skin, which migrate to the liver.[3] Inside parenchymal hepatocyte, each sporozoite transforms to an exoerythrocytic parasite that multiplies giving rise to thousands of liver merozoites during the asymptomatic pre-erythrocytic phase. After maturation, liver merozoites are released into the blood stream where they infect red blood cells (RBC) and initiate the erythrocytic stage of the infection. The invading merozoite forms a vacuole, develops into a uninucleated ring form, then matures and divides into a multinucleated schizont. When the schizont ruptures, 4 to 16 merozoites are released into the bloodstream and infect new RBCs. During the blood stage, a subpopulation of merozoites will develop into gametocytes that will be taken up during a blood meal by mosquitoes, in which the sexual stage of the life cycle is completed.

The blood phase of the infection is responsible for the pathology of this disease. Symptoms of malaria usually develop 10–15 d after being bitten and include high fever, muscle aches and chills. In the majority of cases, infections are cleared by the use of appropriate treatments but in some patients, severe pathologies can develop and lead to death. Severe malaria includes a wide array of pathologies, ranging from metabolic alterations, renal failure, liver and lung dysfunctions, and anemia to cerebral malaria.[4]

Human Cerebral Malaria

Human cerebral malaria (HCM) is the most severe complication of *P. falciparum* infection and has attracted the attention of both clinicians and scientists since the discovery of the malaria parasite.[4-6] HCM can occur in less than two weeks after a mosquito bite and may develop after 2 to 7 d of fever.[4] The commonly accepted clinical definition of HCM is the neurological syndrome with patients in unrousable coma.[4,7] Seizures, retinopathy and brainstem alterations due to elevated intracranial pressure and brain swelling are also clinical features frequently observed during HCM.[8,9] To meet the HCM definition, the *P. falciparum* infection has to be confirmed and other causes of encephalitis (of viral or bacterial origins) to excluded. However, in field settings with limited resources, only easily diagnosed or obvious infectious diseases with brain involvement are investigated.[10-12] Many viral, bacterial and parasitic infections can alter Plasmodium infections or pathologies and the reverse is also true.[12]

Neurological symptoms are also frequently associated with severe metabolic acidosis, anemia and hypoglycemia.[13,14] Thus exclusion of all these factors is important for the definition of "true HCM" and for making comparisons between different studies. It is well known that differences between pediatric and adult HCM exist.[15] Geographical differences in clinical patterns and prevalence of the syndrome are recognized and are likely due

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to differences in parasite and host genetics, immune status of the host, or epidemiological conditions.[4,15] In many patients with HCM, death occurs rapidly before treatment can be administered[16] and patients with HCM usually have a poor prognosis.[17] Patients who survive HCM may develop long-term neurological sequelae[17,18] and cognition and behavioral deficits.[19]

There is no consensus on the pathogenesis of HCM. Indeed, this topic has been one of the most dogmatic and divisive in malaria research. This is due to the fact that limited studies can be performed in humans, while the common mouse model of cerebral malaria does not reproduce all aspects of HCM. The first attempt to uncover the pathogenesis of this syndrome has relied heavily on histopathology of brain tissues from patients who died of HCM. Since 1900, a series of studies with a limited number of patients have reported brain capillary occlusions, swelling of the endothelium, and sequestration mainly of infected red blood cells (IRBC).[20–25] In the past 20 years, studies with larger numbers of samples from patients with a more rigorous HCM definition have supported the original findings in most cases.[25–28] All these findings have led to the prevailing dogma that cerebral sequestration of IRBC is the etiologic mechanism leading to HCM.

The mechanisms by which sequestration leads to neurological complications and death are not yet clearly defined. It has been postulated that IRBC sequestration causes cerebral occlusion of brain capillaries, reduction of microvascular flow, decrease of nutrient supply to the brain, and damage to the vessel wall, leading to hemorrhages and neuronal alterations.[29] However, the most rigorous histology study performed so far on clinically confirmed HCM, showed no evidence of cerebral sequestration of *P. falciparum* in a proportion of the post-mortem brain tissue collected from Malawian children.[28] Still, it cannot be excluded that IRBC were sequestered before the patients were treated, and antimalarial treatment cleared the sequestered parasites but could not halt the cascade of events leading to HCM.

Leukocytes and platelets were found in the brain tissue of some of these Malawian children.[27,30] Leukocytes have also been observed in the brains of adult patients from India[31] but not from Thailand.[26,32,33] These different observations suggest that cerebral sequestration of IRBC may not always be uniquely responsible for HCM and that different or alternative pathological pathways leading to HCM may exist. They also demonstrate the limitations of making inferences based only on post-mortem histological studies, which cannot give any insight into the kinetics of the pathogenic processes occurring locally in the brain in order to identify the players involved. Other factors likely to be involved in HCM pathogenesis are proinflammatory and anti-inflammatory cytokines. However, their roles in HCM are still not clearly established. In some studies, HCM was associated with proinflammatory cytokines with HCM patients having higher levels of circulating TNF-α and/or IFN-γ,[34,35] while in other studies, this was not observed.[36] One possible explanation for this discrepancy is the fact that in most reports, cytokine measurements were only performed at one time point at the time of diagnosis just before treatment. It is likely that some patients having high cytokine levels but not diagnosed as having HCM would have developed the syndrome if not treated. Another confounding factor is differences in HCM definition, in particular, the level of rigor in excluding co-infection (common in field studies) with pathogens that also modify the peripheral and local cytokine profiles. Nevertheless, the association of polymorphisms of pro-inflammatory cytokines or their receptors[37] lends support to a role for these mediators.

Mouse Cerebral Malaria

A large body of research on cerebral malaria (CM) has been performed using mouse models of CM even though there have been strong controversies over the relevance of these models over the years.[38-43] Although it is evident that mouse models do not replicate all aspects of the human disease, they have provided basic knowledge that can be applied to humans. Much of the debate on the value of mouse models is due to misconception and incomplete understanding concerning these models by clinicians or scientists working in HCM. However, on the other hand, a lack of rigorous characterization of these models and over-interpretation of data by researchers working on experimental cerebral malaria (ECM) further fueled the controversy. However, careful and balanced analyses of the data coupled with thorough understanding of parasite and mouse biology have allowed the validation of relevant hypotheses and the generation of new concepts applicable to HCM.

Of the four species of rodent malaria parasites, only a few *P. berghei* strains are able to induce ECM in mice with evident neurological involvement. The ANKA strain (PbA) has been the well-studied since its genome has been sequenced and analyzed extensively.[44] In addition, this is the strain of choice for genetic studies since transfection methods were first established for this strain.[45] ECM in PbA-infected susceptible mouse is characterized by the following neurological symptoms: paralysis, ataxia, deviation of the head and convulsion and/or coma.

In most susceptible mouse strains, 60 to 100 percent of the mice die of ECM during days 6 to 14 post-infection. The remaining mice die later at the end of week 2 or during the third week of infection due to hyperparasitemia and anemia.[46] ECM outcome can vary depending on the PbA parasite clone used,[47] the parasite form used for inoculation (sporozoite vs. IRBC),[48,49] the dose of IRBC inoculated[47] and the mode of propagation of the parasites, i.e., passage between different mouse strains.[47] PbA parasites, like their human Plasmodium counterparts, display phenotypic variations[50,51] and this influence the development of ECM.[47]

Histopathological analyses of the brains of mice that developed ECM showed an accumulation of leukocytes and, to a lesser extent, of IRBC and normal RBC.[52-54] Unlike in viral or bacterial infections, leukocytes do not infiltrate the parenchyma and are confined intravascularly. Normal RBC and IRBC are also located intravascularly unless hemorrhages have occurred.

Although accumulation of IRBC in the brains of PbA-infected mice is not as striking as in the brains of *P. falciparum*-infected patients who died of HCM, its occurrence has conclusively been demonstrated by quantitative PCR[53,54] and dynamic bioluminescence imaging of transgenic PbA parasites expressing luciferase.[55–57]

PbA IRBC sequestration in mice is essential for ECM to occur since drug treatment just before neurological signs are expected prevents ECM.[57–59] Sequestration is higher in susceptible C57BL/6J at the time of ECM signs than in resistant BALB/cJ mice (Claser and Renia, unpublished results) and is regulated by $CD8^+$ T cells and IFN-γ.[55,56] Very recent data have also shown that mature PbA IRBC cytoadhere to endothelial cells (Gruner, Ong and Renia, unpublished results), and mediate parasite sequestration. However, it has to be noted that only a fraction of PbA IRBC cytoadhere, explaining why mature blood forms of the parasite can be seen in the circulation unlike their *P. falciparum* counterparts.

Sequestered leukocytes are composed of monocytes and neutrophils (together representing 50 to 80% of the sequestered cells), $CD4^+$ and $CD8^+$ T cells, NK cells, platelets and few dendritic cells.[50,60–63] Depletion of monocytes, neutrophils and platelets by antibody treatment a day or two before disease onset does not prevent the occurrence of ECM, suggesting that they have no effector functions during ECM.[46,60,63] However, they might have a role early in the infection through the production of cytokines[63–66] or through their interaction with brain endothelial cells (see below). The role of NK cells is still unclear and debated. In one study, depletion of NK cells using anti-NK1.1 antibodies had no effect,[67] while in another study anti-asialo-GM1 antibodies prevented ECM by abrogating the migration of $CD8^+$ T cells to the brain.[68] More work is needed using mice with specific deletion of NK cells[69] to resolve this discrepancy since antibodies used for depletion can also deplete activated antigen-specific $CD8^+$ T cells.[70]

Depletion studies with specific antibodies and the use of mice deficient of immune cell subsets have shown that $CD8^+$ T cells are the principal effector cells.[48,60,67,71] Only 50,000 to 100,000 sequestered $CD8^+$ T cells are found in a whole mouse brain at the time of ECM.[60] In addition, it is not known how many of these sequestered $CD8^+$ T cells are parasite-specific, since PbA infection also induces non-specific activation of unrelated $CD8^+$ T cells.[72] Depending on the parasite/mouse combination, $CD4^+$ T cells can sometimes also behave as effector cells.[46,63] However, in most combinations tested so far, $CD4^+$ T cells play a role in the induction of ECM[60,67,73,76] possibly by providing help for $CD8^+$ T cells to fully mature.[74] Leukocytes are sequestered intravascularly and are in direct contact with endothelial cells. Thus, it has been postulated that $CD8^+$ T cells might kill activated endothelial cells which have ingested and cross-presented cytoadherent parasites or parasite-derived material, thus disrupting the blood-brain barrier (BBB) and leading to neuronal dysfunction and death.[74,75]

The Blood-Brain Barrier and Malaria

The blood-brain barrier at homeostasis. The brain contains a network of blood vessels which are necessary for providing nutrients, and oxygen, and for removing carbon dioxide and waste (i.e., urea, creatinine, etc.). This network of capillaries together with the glia form a protective barrier called the blood-brain barrier (BBB). This barrier prevents large molecules and pathogens in the blood from entering the brain tissues and from altering the brain's functions.[76,77] The brain is very sensitive to blood chemistry variations and its homeostasis is tightly regulated.[78] The BBB is regarded as a part of the neurovascular unit, a concept that stresses a cross-talk between the different brain components for optimal functions of the brain. Maintenance of homeostasis is principally due to the brain endothelial cells, which are on the luminal side of the blood-brain barrier and correspond to the actual barrier site. Brain endothelial cells differ from those found in other tissues in many ways. They are attached by tight junctions of high electrical resistance preventing intercellular passage of molecules, and do not contain small openings called slit pores that allow the diffusion of molecules. Thus to reach the brain parenchyma, essential nutrients need to be actively transported by carrier systems to pass through the capillary wall. Brain endothelial cells also have important functions in mediating and regulating the immune response in the nervous system.[79] The inner part of the BBB is composed of pericytes, glial cells and astrocytes that essentially shield the capillaries from the neurons. The pericytes by themselves do not have a barrier function but contribute to the barrier function and phenotype of the endothelial cells. Astrocytes and glial cells contribute to homeostasis for neurological functioning by contributing to and regulating brain endothelial cell phenotype. In particular, endothelial cells are in contact with foot processes of astrocytes. These cellular structures provide an additional barrier protecting neurons from toxic products in the blood. Astrocytes can also form a barrier called the glia limitance at sites where the endothelial barrier is absent, such as the postrema.

The blood-brain barrier in HCM. A role for the BBB in HCM was postulated 40 years ago[80] and since then, many studies have been performed to uncover the extent of BBB alterations and their relationship to HCM pathogenic processes.[81-83] Post-mortem observations on the accumulation of cytoadherent late-stage IRBC and of normal RBC in brain capillaries of infected humans have led to the prominent hypothesis that sequestration leads to capillary obstruction, reduced perfusion of the brain parenchyma and reduced delivery of necessary nutrients to neurons.[29] In addition, plugging of microvessels may also alter the function of BBB by creating local hypertension which increases pressure on tight junctions. If hypertension persists, tight junctions might break and this leads to the rupture of the BBB, causing hemorrhages. In addition, adhesion of IRBC to endothelial cells generates intracellular signaling in these cells, leading to activation and damage of the BBB. Although this hypothesis is attractive, it marginalizes the effects of systemic and local production of cytokines and parasite toxins such as malaria pigment, as well as the possible involvement of platelets and leukocytes that can also be found in the brain.[27,30,31]

In an effort to determine if the BBB was altered in *P. falciparum* patients, measurements of molecules such albumin or immunoglobulins in the cerebrospinal fluid (CSF) and molecules excluded from the brain during homeostasis have been performed. In one study, radioactive-labeled albumin levels were not increased in the CSF after intravenous injection in Thai adult patients during and after coma.[84] In another study, albumin levels in the CSF of Vietnamese adult patients with HCM were

not different from control subjects.[85] In Malawian children, the levels of albumin in the CSF were not different between children who died vs. those who survived, although they differ from UK adult controls.[89] When IgG was investigated, higher levels were detected in the CSF of a significant proportion in Thai patients with HCM,[87] but not in patients from Vietnam.[85] Discrepancies between these studies are probably due to measurements of samples obtained at a single time point. All together, these data suggest that although some BBB alterations occur during infection, major modifications leading to increased concentrations of plasma proteins in CSF as seen in bacterial or viral infections do not occur during HCM. However, focal ruptures of the BBB take place in the brain during HCM,[27] and may result from endothelial cell activation leading to the alteration of endothelial tight junctions, increased intercellular permeability, detachment from the basal matrix and/or death of endothelial cells. There is ample evidence that endothelial cells are activated during *P. falciparum* infection and during HCM as they show characteristic morphological modifications,[26] upregulate numerous surface antigens such as adhesion molecules[32,88] and produce a large variety of mediators.[89,90]

Activated endothelial cells in capillaries containing IRBC have also been shown to display a reduction but not a complete disappearance of cell-to-cell junction as shown by junction protein staining.[86,91] In addition, perivascular macrophages in the vicinity of modified endothelial cells were also positive for macrophage activation markers[92] suggesting the passage of blood proteins into the perivascular space. This phenomenon seems to be restricted locally since it does not result in detectable leakage into the CNS. Nevertheless, the accumulation of localized increased permeability may lead to the activation of the microglia and/or astrocytes, which in turn may produce various mediators affecting vascular and neuronal cells. However, just an increase in permeability because of loosened tight junctions does not explain the hemorrhages observed in HCM histology studies. Hemorrhages result from focal rupture of the BBB and allow a large influx of plasma and plasma proteins to the interstitial brain tissue at these sites. This may explain the brain swelling, associated with coma, death or neurological sequela, which is frequently observed in patients with HCM.[93,94] However, it has to be noted that brain swelling has been observed in the absence of hemorrhages[8] and that coma is not caused by cerebral edema in Vietnamese patients who died of HCM.[95,96]

Numerous studies have been conducted to identify the mechanism involved in endothelial cell modifications. Proinflammatory plasma cytokines such as TNF-γ, Lymphotoxin, IFN-γ and IL-1β, which are increased during HCM[34,35] can activate endothelial cells and modify vascular endothelium permeability.[96–99] There has been a lot of speculation regarding the role of nitric oxide (NO) in HCM.[100–104] The detection of inducible nitric oxide synthase in post-mortem brain tissues from African children[105] and Thai adults[106] has suggested a pathogenic role for NO. However, although NO might participate in neuronal dysfunctions, it may have a protective role at the BBB level. It has been shown in vitro that NO can decrease permeability of the tight junctions induced by TNF-α or IFN-γ treatment.[107]

Parasite cytoadherence can also directly activate endothelial cells as shown in vitro using brain endothelial cell lines.[108–114] This also leads to the production of chemokines and pro-inflammatory cytokines (IL-6, IL-8 and TNF-α), creating an activation loop.[110] Local productions of inflammatory cytokines and chemokines by endothelial cells and also microglial cells have been detected intravascularly in the brain of patients with HCM.[110,114]

P. falciparum IRBC express at their surface a variable parasite-derived antigen, *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), encoded by the *var* multigene gene family.[115-117] PfEMP-1 proteins have been shown to interact with a variety of surface receptors such as ICAM-1, thrombospondin, VCAM-1, PECAM-1, αvβ3 integrin, g1CR, endoglin, P-selectin, CD36 and fractalkine.[118-121] Expression of some of these molecules is increased after cytokine stimulation.[79] When engaged, adhesion molecules like ICAM-1 can induce intracellular signaling which leads to the modification of cytoskeletal rearrangements.[122] However, co-engagement of CD31/PECAM can counteract ICAM-1 induced signaling.[123] During *P. falciparum* infection, clones expressing different PfEMP-1 variants with different adhesion receptor specificities can co-exist. A recent and elegant study has shown that upon interaction of IRBC with endothelial cells, materials can be transferred from the IRBC to the endothelial cell plasma membrane, which then endocytosed these materials. This is associated with opening of the intercellular tight junctions.[75] A more drastic effect of cytoadherence is induction of endothelial cell apoptosis.[124] This phenomenon has been described in vitro, is parasite strain-specific,[125] depends on host genetic factors,[126] and is mediated by a unique set of parasite proteins[127] Different mechanisms leading to apoptosis have been described, involving the induction of oxidative stress and caspase-3.[102,128]

Platelet adhesion to brain endothelial cells can also lead to modifications of the BBB. They can facilitate IRBC adhesion by forming bridges between endothelial cells and IRBC, and they have been shown in vitro to potentiate endothelial cell apoptosis through TGF-β.[129] Expression of TGF-β has been detected in the brains of patients who died of HCM.[130] Leukocytes attracted by chemokines released at the site of IRBC adhesion can further induce modifications in endothelial cells by engaging ICAM-1 molecules and secreting proinflammatory cytokines and mediators locally.

An additional effect of sequestration of IRBC, platelets and leukocytes is a local reduction of blood flow.[131,132] This creates a dysfunctional environment where toxins produced by metabolically-active parasites are concentrated and supply of oxygen and nutrients such as glucose and amino acids is decreased. A recent in vitro study has demonstrated that metabolic acidosis can increase endothelial intercellular permeability and disorganization of tight junctions.[113]

The blood-brain barrier and ECM. Pioneer studies from Maegraith and collaborators first demonstrated that movement of both proteins and water did occur across the BBB during *P. berghei* infections using disulfine dye.[133] This phenomenon was further confirmed in later studies using Evans Blue, radio-labeled albumin, radio-labeled antibody or horseradish peroxidase, and

was associated with brain edema in mice with ECM.[134-136] Using retinal wholemounts, which allow the study of functional properties of microvasculature in a three-dimensional context, focal sites of BBB breakdown were observed in ECM-susceptible mice early in the infection.[137,138] At later times of ECM, more extensive rupture of the BBB is seen and is associated with modifications of endothelial cell morphology such as swelling and signs of cell death, explaining the increase of protein and water transfer into the brain parenchyma.[134] This transfer led to an activation of astrocytes and microglia as shown in the retinal whole mount system.[139] In more recent studies, it was shown that during ECM, brain edema resulting from leakage and accumulation of fluid into the parenchymal extracellular space was observed[140,141] and was associated with enlarged perivascular spaces.[142]

Modifications of the BBB during ECM can be caused by various mechanisms. The early alterations of BBB may be caused by the activation of endothelial cells due to proinflammatory cytokines[96,143] produced in the first week of PbA infection.[64] TNF-α, lymphotoxin or IFN-γ affect endothelial cells by decreasing tight junction proteins while inducing an increase in the expression of adhesion molecules[144] that mediate binding of leukocytes. Monocytes have been shown to adhere to endothelial cells in the retina as early as 2 d before disease onset, inducing morphology modifications of endothelial cells and provoking reduction of local blood flow.[137] Activated platelets also adhere to the brain microvasculature following TNF-α stimulation.[145,146] They can also fuse with endothelial cells to increase leukocyte adhesion.[145] Of interest, an active role for NO has been discarded since ECM is characterized by low NO bioavailability like in HCM. And moreover, treatments with exogenous NO-donors prevent BBB rupture and protect against ECM.[147,148]

Although these early and focal modifications are important, they are not responsible for late ECM deaths. $CD8^+$ T cells which migrate at the time of neurological signs are responsible for the ensuing lethality. It is not yet known if and how $CD8^+$ T cells induce the rupture of the BBB, but it has been proposed that $CD8^+$ T cells kill endothelial cells after they phagocytosed and processed parasite-derived antigens.[74] This hypothesis is supported by the fact that mice deficient for perforin or granzyme B, two CD8 cytotoxic effector molecules, are resistant to ECM.[58,71,149] However, a recent study in a mouse model of acute hemorrhagic leukoencephalomyelitis showed that $CD8^+$ T cells altered BBB tight junction proteins through a perforin-dependent mechanism without inducing endothelial apoptosis.[150] It remains to be determined if such a mechanism exists in ECM.

Relevance of the ECM findings to HCM. The mouse model of malaria does not reproduce all the features of *P. falciparum* infection predominantly due to differences in parasite biology. However, similarities need to be recognized since they help to generate hypotheses that can be tested in humans. In summary, several pathologic changes occur mainly intravascularly in both ECM and HCM. The BBB is altered during infection and changes in permeability and/or rupture appear to be restricted locally, with BBB rupture leading to hemorrhages. The exact causes of BBB alterations are still unknown and may involve cytoadherent parasites, cytokines, platelets and/or leukocytes. The role of $CD8^+$ T cells, which have been clearly demonstrated in the mouse model, is still strongly debated in HCM. The main refutation for a role of these cells is based on their rarity in post-mortem histology samples that represent a limited snapshot of the brain in both space and time. However, taking into account the low frequency of brain-sequestered mouse $CD8^+$ T cells in ECM, human studies performed thus far may not have been sufficiently sensitive. Quantitative and carefully controlled immunohistological studies are needed, or alternatively, new molecular approaches such as quantification of CD8 mRNA in the brain[63] should be performed.

Conclusion

The BBB is an important protective barrier for the human host during malaria infections. Although the intra-erythrocytic parasites do not penetrate the brain parenchyma outside of local ruptures, there is a constant and dynamic interplay between IRBC, parasite-derived materials, host leukocytes and the BBB that can lead to neurological alterations and death. However, till now, it has not been firmly established if alterations of the BBB during HCM are key pathogenic factors. As such, there is a need to describe and understand the interactions of IRBC with the brain endothelium and their downstream effects on the microglia, astrocytes, and neurons and how they influence the morbidity and mortality during HCM. Comprehension of these pathways may pave the way for the development of new adjuvant therapies.

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