Metab Brain Dis (2016) 31:983–992
DOI 10.1007/s11011-016-9845-7

REVIEW ARTICLE

Hemoglobins as new players in multiple sclerosis: metabolic and immune aspects

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Received: 24 February 2016 / Accepted: 20 May 2016 / Published online: 28 May 2016

Abstract Basic science investigations and clinical observations in recent years indicate that hemoglobins (Hbs) may have important roles in the pathogenesis of multiple sclerosis (MS). These findings can be summarized as follows: 1- Erythrocyte fragility is higher in MS patients, the released free Hb damages blood-brain barrier, myelin basic protein and also triggers iron overload and inflammation. 2- Free Hb may further activate the inflammatory responses through Toll-like receptor 4 (TLR4), present on microglia and other innate immunocytes. 3- Hbs are expressed in neural cells including dopaminergic neurons. Also, several studies have demonstrated that Hbs are expressed in astrocytes and oligodendroglia. 4- Hb overexpression in neural cells upregulate mitochondrial complex I–V subunits. The comparison of the mitochondrial proteome between healthy and patients with MS revealed only four differentially expressed proteins including Hb β-chain. 5- Microarray analysis of 8300 genes in monocytes of twins with and without MS showed a difference in 25 genes that include genes encoding α- and β-globins as well. 6- β- and α-globin gene clusters reside at chromosomal regions 11p15.5 and 16p13.3, respectively. Whole genome screen (WGS) in Sardinian MS families using 327 markers revealed linkage in 3 regions including 11p15.5 loci. Further, 11p15.5 and 16p13.3 were part of the 17 regions identified in the WGS study of 136 sibling-pairs in Nordic countries analyzing 399 microsatellite markers. In the light of these findings, we propose that free Hb released from dying erythrocytes is detrimental. On the contrary, intracellular Hbs in neural cells are protective in MS. The genomic linkage findings can be explained by common haematologically-silent Hb variants that may lower the protective function of intracellular Hbs, and therefore, enhance the risk for MS. In the absence of such variants, aberrations in the translational and post-translational mechanisms controlling synthesis of neural Hbs may also enhance the vulnerability to MS. Alternatively, such genetic variants may perturb the metabolism of anti-inflammatory hemorphins produced via cleavage of Hbs.

Keywords Hemoglobin · Multiple sclerosis · Immunology · Neural injury

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Introduction

Multiple sclerosis (MS) is a chronic neuroinflammatory disease and the most common cause of neurological disability in young adults (Witte et al. 2014). First symptoms manifest in early adulthood, and a high percent of these patients need help with walking within 15 years (Witte et al. 2014). As the disease progresses, additional symptoms arise, such as cognitive deterioration (Witte et al. 2014). Current medicines are effective in reducing the MS attacks, but they are not completely sufficient to hinder the disease progress and the loss of neural tissue in long-term (Witte et al. 2014). Therefore, identifying novel pathways underlying MS pathogenesis is of essential

importance. In this review, we will discuss evidence on the likely contributions of hemoglobins (Hbs) in MS pathogenesis, which include free Hb (fr-Hb), Hb-cleavage products (hemorphins and hemocidins), and the recently discovered Hbs expressed in neural tissues. We propose that Hbs involve both in the "outside-in" and the "inside-out" pathogenesis models of MS. In the "outside-in" model, antibodies and immune cells against myelin are deemed responsible for demyelination, damage to neuropil, and axonal degeneration (Bamm and Harauz 2014). However, there are also clues suggesting that MS is triggered by cellular degeneration (Bamm and Harauz 2014). Given that, an "inside-out" model of MS was proposed. According to this model, autoimmunity in MS is secondary to the primary events, which cause the release of immunogenic cell debris and myelin degradation products (Bamm and Harauz 2014). In fact, both processes may contribute to the MS pathogenesis, such that abnormalities in homeostatic regulation of Hbs may be involved in both sides of the pathogenesis. We suggest that fr-Hb is detrimental in MS pathogenesis via causing damage to the myelin and blood brain barrier, while intracellular Hbs expressed in neural cells are protective.

Erythrocyte fragility, anemia, and chronic pathogenesis of MS. inherent and drug-induced causes of anemia

Almost half a century ago, it was shown that red blood cell (RBC) osmotic fragility in patients with MS was significantly higher than controls ($p < 0.05$) in a sample of 73 patients with MS and 38 healthy controls (Caspary et al. 1967). Further analysis, in which MS patients were categorized into active –that is, exacerbation within the last 4 months– and quiescent showed that active cases had more fragile cells ($p < 0.01$) (Caspary et al. 1967). However, until recently, the influence of the RBC fragility, anemia, and the released fr-Hb on MS have not been thoroughly investigated. In 2009, an impaired membrane fluidity of RBCs in MS was reported (Hon et al. 2009). Hon et al. further investigated differential full blood counts in 31 patients with MS and 30 age- and gender-matched healthy controls (Hon et al. 2012). The RBC count showed an inverse correlation both with the disease duration and the Expanded Disability Status Scale ($R = -0.41$; $p = 0.02$) (Hon et al. 2012). Small sample size precluded a definite conclusion that RBC lysis contributes to MS pathology. However, there exist some other clues supporting this possibility. Fragile RBCs and a sustaining hemolysis would cause a prominent release of fr-HB. As early as in 1968, some groups showed that RBC size is increased in MS (Prineas 1968). A significant increase in RBC-size was found to be linked to changes in disease activity (Prineas 1968). Enhanced RBC size is one of the features of increased RBC-turnover due to hemolysis (Prineas 1968). The authors proposed that lysolecithin, a powerful haemolytic agent existing in relatively small amounts in normal brain but increasing under hypoxic condition, might have led to this phenomenon (Prineas 1968). If exists, such a mechanism may contribute to the RBC fragility and fr-Hb release in MS. However, it should be noted that macrocytosis is also a feature of folate and cobalamin deficiency. A case-series of 10 patients with concurrent B12 deficiency and MS lead to intriguing revelations (Reynolds et al. 1991). Ages of the patients were younger than the general population with B12 deficiency (Reynolds et al. 1991). One case was previously treated with B12. Eight patients had macrocytosis and of two cases with normocytic anemia, one patient also became macrocytic following the treatment of comorbid iron deficiency (Reynolds et al. 1991). Surprisingly, only two patients were anemic but the marrow was megaloblastic in five of the six patients examined (Reynolds et al. 1991). One of the cases who had been on Vitamin B12 intermittently for 12 years to treat pernicious anemia had also strikingly low levels of Vitamin B12 at the time of presentation with neurological symptoms and macrocytic anemia (Reynolds et al. 1991). All patients excluding the case with pernicious anemia had no gastrointestinal disease and none were vegan (Reynolds et al. 1991). In the absence of a clear explanation for the deficiency in the majority of cases, a possibility of a Vitamin B12 transport and/or binding in RBC precursors was considered (Reynolds et al. 1991). In routine clinical practice, macrocytic cases without anemia may be neglected, and research on larger patient series examining Vitamin B12 levels in RBC-progenitors may provide new clues. The same group studied Vitamin B12 metabolism in 29 consecutively enrolled cases of MS, 17 neurological controls, and 31 normal subjects (Reynolds et al. 1992). Patients with MS had significantly lower serum B12 levels and higher unsaturated R-binder (transcobalamin) (Reynolds et al. 1992). Lower Vitamin B12 levels were also associated with earlier age of onset in MS (Sandyk and Awerbuch 1993). Serum Vitamin B12 and folate were evaluated in 35 MS patients during an acute attack and 30 healthy individuals (Kocer et al. 2009). No statistically significant differences between groups were observed for vitamin B12, folate, and homocysteine levels. On the other hand, vitamin B12 levels were low in seven patients with MS (20 %) compared to one healthy subject (3.3 %). Likewise, serum folate levels were low in 5 patients with MS (14.3 %) but only in 1 healthy subject (3.3 %), which suggest that a subtle chronic hemolysis may contribute to the pathogenesis of MS (Kocer et al. 2009). The National Health Insurance Research Dataset in Taiwan investigating the frequency of comorbid disorders in 898 patients with MS and 4490 randomly matched individuals without MS showed that patients with MS were more likely to have deficiency anemias than general population (OR =4.9, 95 % CI = 2.8–8.7) (Kang et al. 2010). This study provided strong

evidence that macrocytosis due to deficiencies of cobalamin and/or folate may contribute to anemia in MS.

In 2015, 187 patients with MS (51 males, mean age 44.5) and 200 controls (56 males, mean age 45.5) were analysed (Koudriavtseva et al. 2015). There was a significant difference in the prevalence of anemia between patients with MS and controls ($p = 0.009$). Moreover, the difference in risk for MS in anemic females and males was striking –risk was doubled in females while it was more than 7 times in males. Although, the latter finding did not reach statistical significance ($p = 0.07$) due to the small number of males (Koudriavtseva et al. 2015). The gender difference is important in both anemia and MS prevalence. If the rate of Hb synthesis is regulated by the same transcriptional machinery in marrow reticulocytes and neural cells; women, who have lower Hb levels than men generally, may benefit less from the protective effects of intracellular Hbs. This phenomenon may be one of the contributing factors to the preponderance of MS in women. Further, anabolic androgens stimulate synthesis of Hbs and it would be tempting to study whether stimulation of Hb in response to androgens is reduced in male patients with MS.

In vitamin deficiency-caused macrocytosis, three different pathogenetic mechanisms may co-occur. First, reduced deformability of macrocytic RBCs passing through microcapillaries may cause chronic RBC lysis and release of fr-Hb. Second, elevation of homocysteine due to Vitamin B12 and/or folate deficiency might enhance RBC-lysis via triggering lipid peroxidation (Ventura et al. 2004). These two phenomena may contribute to the "outside-in pathogenesis" of MS via myelin damage caused by fr-Hb. Third, if these vitamins activate Hb synthesis in neural cells at similar rates to marrow reticulocytes, their deficiency may cause depletion of intracellular Hbs in neural cells and enhance susceptibility to cell death. Such a process would contribute to an "inside-out pathogenesis" of MS.

MS treatment may also induce anemia. In a meta-analysis of six randomised, controlled clinical trials of subcutaneous IFNβ-1a in MS, five of which were placebo-controlled, haematological abnormalities were analysed (Rieckmann et al. 2004). Grade-1 anemia (Hb lower than 100 g/L as determined by National Cancer Institute Common Toxicity Criteria) was observed in 15 %, 20 %, and 27 % of patients receiving placebo, 22 μg IFNβ-1a three times weekly, and 44 μg IFNβ-1a three times weekly, respectively (Rieckmann et al. 2004). The reason of this slightly enhanced risk of anemia is unknown but some case reports which defined anemia following IFNβ-1a treatment in MS mentioned that these cases suffered from Coomb's positive autoimmune hemolytic anemia (Alanoglu et al. 2007, Saeedi et al. 2011). Similarly, anemia following fingolimod treatment in an MS patient was identified as an autoimmune hemolytic anemia (Lysandropoulos and Benghiat 2013).

Free hemoglobin involvement in the "outside-in pathogenesis" of multiple sclerosis

Using an exchange transfusion model, the actions of fr-Hb on the endothelial tight junction proteins (zonula occludens 1 (ZO-1), claudin-5), astrocyte activation, IgG extravasation, heme oxygenase (HO), iron deposition and oxidative endproducts were identified (Butt et al. 2011). ZO-1 expression reduced after fr-Hb transfusion. The distribution of Claudin-5, but not the total expression, was shifted from small- to medium-sized vessels (Butt et al. 2011). Transfusion of fr-Hb enhanced astrocytic activity and IgG extravasation (Butt et al. 2011). Prominent increase of HO-1 immunoreactivity in $CD163^+$ perivascular cells and infiltrating monocytes were observed (Butt et al. 2011). These changes were accompanied with significant increases of oxidative end-products in BBB-endothelia. Free-Hb neurotoxicity is a major pathophysiological component of brain hemorrhage (Butt et al. 2011). The lysis of extravasated RBCs exposes the central nervous system (CNS) to high quantities of fr-Hb and its breakdown products, thereby triggering oxidative and inflammatory cascades (Butt et al. 2011). Fr-Hb (and/or its chains) exert oxidative activity as a classic peroxidase (similar to horse radish peroxidase). More importantly, fr-Hb can be oxidized to methemoglobin (ferric, Fe^{3+}), ferryl heme intermediate (Fe^{4+}), hemichromes, and free heme or iron, which cause oxidative injury to lipids, nucleic acids and proteins (Butt et al. 2011). Heme dissociates more readily from Hb as ferric (Fe^{3+}) heme relative to ferrous (Fe^{2+}) heme and the endothelium is exposed to higher amounts of free (Fe^{3+}) heme (Butt et al. 2011). Uncomplexed heme is instable and either decomposes or be enzymatically oxidized to biliverdin, which is rapidly metabolized to bilirubin, ferrous iron (Fe^{2+}) and carbon monoxide (CO) (Bamm and Harauz 2014).

BBB dysfunction due to the "loosening" of tight junctions (TJs) leads to the entry of leukocytes into the neuronal parenchyma. For example, reduced ZO-1 expression correlates with higher monocyte infiltration (Butt et al. 2011). CD163, the monocyte/macrophage receptor for Hb-haptoglobin complexes, is not only expressed in peripheral blood monocytes but also in perivascular/meningeal macrophages and pericytes in significant amounts (Butt et al. 2011). Hb- and heme-triggered inflammatory events also increase endothelial adhesion molecules including vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), which facilitate leukocyte adhesion and transmigration (Butt et al. 2011). As inflammatory leukocytes also release oxidizing intermediates, this will further enhance BBB damage and trigger a vicious cycle. An intact BBB plays a key role in preventing the entry of neurotoxic fr-Hb into the CNS (Butt et al. 2011). However, a continuing hemolysis may saturate the buffering mechanisms of fr-Hb (such as plasma haptoglobin), which may facilitate its own entry to the CNS via

damaging BBB if it exists at a constantly high concentration in blood circulation. Neurologic complications are indeed frequently observed in conditions such as chronic or intermittent intravascular hemolysis, genetic and drug-induced hemolytic anemias and microbial infections (Butt et al. 2011).

In the human body, the most abundant iron source is heme, and iron released from heme plays a key role in multiple pathologies, such as reperfusion injury, trauma, and hemolysis (Bamm et al. 2015). Iron normally exists in the CNS in high concentrations, since it is essential for normal development of oligodendroglia and myelin. However, extremely high levels of iron accumulate in the walls of dilated veins in MS plaques, macrophages, microglia and adjacent demyelinated plaques both in grey and white matter (Bamm and Harauz 2014). The extent of iron accumulation in grey matter predicts disability progression, the intensity of lesion accumulation and cell loss (Bamm and Harauz 2014). In advanced phases of the disease, none-heme iron released from dying oligodendrocytes further increases free iron with high oxidative potential (Bamm and Harauz 2014). Hb also directly leads to oxidative damage to oligodendrocytes, MBP and myelin lipids, which involves formation of globin radicals and heme transfer (Bamm et al. 2015). Myelin, with its high content of polyunsaturated lipids, is especially vulnerable to Hb-induced oxidative stress (Bamm et al. 2015). Oxidized lipoproteins trigger further inflammation, oxidizing events and neuronal degeneration (Bamm et al. 2015). Oxidized cholesterol leads the exposure of MBP to the cytoplasm and induces its proteolytic cleavage (Bamm et al. 2015). The MBP is an important autoantigen in MS. Overexposure to immunodominant epitopes of MBP may aberrantly activate immunity (Bamm et al. 2015). Furthermore, both fr-Hb and heme exacerbate inflammatory responses and activate the innate immunity through Toll-like receptor 4 (TLR4) that is present on microglia and other innate immunocytes (Bamm and Harauz 2014).

Another detrimental impact of fr-Hb in MS may also occur when it exists in forms with supraphysiologically high oxygen affinity. Clues for this theory can be based on observations regarding peripheral neuropathy. Peripheral neuropathy is a major complication of diabetes and endoneurial hypoxia may be among the causes (Farber et al. 1991). An aberrant rise in Hb's oxygen affinity, which involves in diabetic microangiopathy, may cause tissue hypoxia (Farber et al. 1991). In a streptozotocin(STZ)-induced rat diabetes model, high and normal oxygen affinity of Hb were produced by backcrossing animals with higher and lower levels of 2,3-diphosphoglycerate (2,3-DPG) (Farber et al. 1991). In normal physiology, 2,3-DPG lowers Hb's oxygen affinity to facilitate its transfer to tissues (Graham et al. 2014). Diabetes was induced in high and low DPG animals and half of the animals in each group were given insulin while the remaining animals left untreated (Farber et al. 1991). Untreated animals, regardless of 2,3-DPG levels, demonstrated no neuropathy, while all insulin-treated animals showed degeneration that were inversely correlated with 2,3-DPG levels (Farber et al. 1991).

Insulin lowers the partial oxygen pressure required to half saturate Hb, decreases the arteriovenous oxygen difference and lowers tissue oxygen delivery, which explain the nerve injury only observed in insulin-treated animals (Farber et al. 1991). This diabetic neuropathy study demonstrated that lower 2,3-DPG levels and subsequent higher oxygen affinity of Hb may contribute to the neuronal tissue injury. High affinity Hbs may result from mutations in globin-encoding genes which increase Hb binding to oxygen. Hb's reduced ability to unload it to tissues may cause compensatory elevations in circulating Hb and 2,3-DPG (Graham et al. 2014). Notably, patients with Parkinson's Disease (PD) have higher Hb and 2,3-DPG levels (Graham et al. 2014). Thus, it was hypothesized that some clinically silent Hb variants with enhanced oxygen affinity may cause PD (Graham et al. 2014). Interestingly, it was shown that adrenocorticotropic hormone (ACTH) treatment of MS (an outdated treatment strategy) enhanced 2,3-DPG levels in RBCs (Chmielewski et al. 1987). Similar to the assumption relevant to the PD, some silent Hb variants with enhanced oxygen affinity may also be responsible for MS pathogenesis. The reduction of the aberrantly elevated Hb's oxygen-affinity in MS may have therefore contributed to therapeuticity of ACTH.

Intracellular hemoglobins in neural tissues. Protection against "inside-out pathogenesis" of MS

In 2009, Hb α-and β-globin chains were unexpectedly discovered in A9 dopaminergic neurons (Biagioli et al. 2009). Interestingly, Hb exists in the majority of A9 cells, whereas only in 5 % of A10 neurons (Biagioli et al. 2009). Brain Hb has been considered as an oxygen-storage molecule in hypoxia, which is especially essential for neurons with higher energy demand (Biagioli et al. 2009). Despite some inconsistent results regarding to a lack of Hb expression in glia, Biagioli et al. showed that Hbs were expressed in cortical and hippocampal astrocytes and in oligodendroglia residing almost in all brain regions including striatum, corpus callosum, and medulla oblongata (Biagioli et al. 2009). Gene expression analysis of dopaminergic neurons transfected with α- and β-chains revealed changes in genes that are involved in oxygen homeostasis and oxidative phosphorylation, and thereby suggesting a link between Hbs and mitochondrial activity (Biagioli et al. 2009). In neurons overexpressing Hb chains, 46 % of the induced-genes encode subunits of mitochondrial complex I–V (Biagioli et al. 2009). Both α- and β-chain are present in the neuronal soma, while mainly β-chains exist in dendrites (Richter et al. 2009). Co-localization in the cell soma may indicate an assembly of α and β-chains into a Hb tetramer as in RBCs. Using Western blots, the strongest signal was

found for monomers, but bands with a lower signal of dimers and tetramers were also demonstrated, enhancing the possibility of Hb-chain assembly in neurons (Richter et al. 2009). Nonetheless, an assembly is not essentially required for Hb function in neurons since the oxygen affinity of the α- or β-Hb monomers is much higher in comparison to that in the α2β2 tetramer. Hb monomers also bind to CO and nitric oxide (NO) with a very high affinity (Richter et al. 2009).

NO is the strongest ligand of the ferrous iron of Hb, with 500,000 times higher affinity than oxygen (Richter et al. 2009). The roles of Hbs in mitochondrial respiration and redox system have further been supported by the finding that neuronal Hb expression is strongly depleted in rats treated with the toxin, rotenone (Richter et al. 2009). Rotenone inhibits the mitochondrial complex I and inhibition of this complex under physiological NO-levels inactivates the transcription factor HIF1α, which subsequently lowers Hb mRNA (Richter et al. 2009). Another mechanism underlying Hb depletion may be the reduction of free heme, which is a strong activator of globin chain transcription (Richter et al. 2009). Heme is synthesized in the mitochondria via ferrochelatase, which inserts the ferrous iron into a tetrapyrrole (Richter et al. 2009). Inhibition of mitochondrial function might, therefore, lead to decreased heme synthesis, which in turn might lead to the reduced transcription of globin chains (Richter et al. 2009).

Congruent with the proposed expression of Hbs in all oligodendroglia, proteomic comparison of brains of healthy individuals and patients with MS revealed striking findings about Hbs, mitochondria, and MS. The mitochondrial fractions in postmortem MS and control cortex gray matter were measured using SELDI-TOF Mass Spectrometry (Broadwater et al. 2011). Principal component analysis and hierarchical clustering revealed that four proteins differentiate MS from controls, and notably, one of these was Hb β-chain (Broadwater et al. 2011). Inflammatory microglia express inducible NO-synthase (iNOS) and release NO in MS lesions which reacts with superoxide, a byproduct of mitochondrial respiration. This reaction produces a highly reactive and hazardous peroxynitrite ($ONOO^-$) radical. Hence, Hbs may protect neural tissues also from nitrosative stress, since Hb scavenges peroxynitrite in a variety of oxygen-bound states (Broadwater et al. 2011).

In human postmortem brain samples, Hb β-chain expressing neurons were more frequent in the internal layers of the cortex (layers IV–VI) compared to the external layers (layers I–III), while their overall percentage and distribution were not found to be different between the MS and control (Brown et al. 2016). The majority of Hb β-chain expressing neurons exhibited pyramidal morphology with large size, apical dendrites and long axons (Brown et al. 2016). Hb β-chain exists mostly in the cytoplasm but to a lesser extent in the nuclei (but not in nucleoli) of these cells (Brown et al. 2016). Interestingly, while the level of Hb β-chain was higher in mitochondria of MS tissue samples by about 30 %, it was reduced in cell nuclei by 38 % (Brown et al. 2016). With co-immunoprecipitation (co-IP) and mass spectrometry (MS), proteins specifically interacting with the Hb β-chain in neurons were identified (Brown et al. 2016). Five of the fourteen Hb β-chain interacting proteins were mitochondrial, including ATP synthase subunits α and β (ATP5A1 and ATP5B), mitochondrial malate dehydrogenase (MDH2), ADP/ATP translocase 4 (SLC25A31), and a mitochondrial phosphate carrier (SLC25A3) (Brown et al. 2016). Overall, these findings suggest an important role of Hb-chains in mitochondrial respiration of neurons and their involvement in MS.

Co-IP and MS analyses revealed other unexpected interacting partners of Hb β-chain including histone H3 (HIST2H3A) and lysine-specific histone demethylase 8 (KDM8) (Brown et al. 2016). Co-IP and MS analyses were repeated on proteins isolated from cultured rat primary neurons, because the protein lysates isolated from the whole brain samples may also contain proteins originated from RBCs, endothelia and macrophages. Same binding partners of Hb β-chain were determined; and furthermore, 2-oxoglutarate dehydrogenase was identified as another interacting protein. This is of interest since histone demethylases require 2-oxoglutarate as a cofactor (Brown et al. 2016).

These data suggest that Hbs may transmit signals between the neuronal mitochondria and nuclei. To test whether Hb β-chain could modify histone methylation, human SH-SY5Y neuroblastoma cells lacking endogenous Hb β-chain were transfected either with an empty vector or a vector driving Hb β-chain. Thereafter, histone H3 methylation was measured (Brown et al. 2016). It was found that this methylated histone was increased by about twofold in Hb β-chain expressing cells, followed by a reduction to physiological histone H3 levels (Brown et al. 2016). Of note, H3K4me3 is a histone mark of actively transcribed chromatin regions and controls expression of oxidative phosphorylation genes (Brown et al. 2016). These findings also explain higher Hb β-chain expression in projection neurons in deeper cortical layers to maintain adequate ATP supply by upregulating H3K4me3 and subsequently the genes driving oxidative phosphorylation (Brown et al. 2016).

In a recent study investigating the effect of systemic growth hormone (GH) injections on the gene expression in rat brains, the most robust increase was observed in the neuronal Hb β-chain (Walser et al. 2014). This finding is noteworthy considering its parallelity with an interesting study, which measured the serum and cerebrospinal fluid (CSF) concentrations of IGF-I and GH in 46 patients with MS and 49 patients with no evidence of demyelinating disease (Poljakovic et al. 2006). The only positive finding was a deficiency of GH in the CSF of MS patients (Poljakovic et al. 2006). It would be tempting to investigate whether a GH-Hb signaling pathway exists in neural tissues, and if so, whether this involves in MS pathogenesis.

Unlike the other regions of the eye's posterior pole, the optic nerve head (ONH) contains a high amount of just one pigment, Hb, and its colour essentially depends on it (Bambo et al. 2013). The Laguna ONhE (optic nerve head hemoglobin) software uses algorithms to delimitate the ONH border and retinal vessels (Bambo et al. 2013). The results obtained from the vessels were employed as reference values to calculate the Hb-content specifically in the nerve tissue (Bambo et al. 2013). MS patients showed significant reductions in the optic nerve Hb in almost all sectors, particularly temporal sectors (Bambo et al. 2013). Future studies are required to understand whether altered Hb metabolism plays a role in the optic nerve injury during the course of MS.

Clinically silent Hb mutations/variants may associate with "inside-out pathogenesis" of multiple sclerosis. Convergence of mitochondrial pathways with innate immunity

In this section, evidence is discussed which suggest that silent Hb mutations/variants which cause mild anemia or no laboratory abnormalities may associate with the inside-out pathogenesis of MS. There exist about 1720 different Hb variants in various globin chains (Giardine et al. 2014), yet only a minor percentage of these (5.2 % of the world population, about 37 million) cause clinical signs (Modell and Darlison 2015). This figure reaches to 70 million worldwide when carriers of hemoglobinopathies are taken into account (Cousens et al. 2010). Thalassemias are caused by mutations modifying Hb-chain synthesis rather than Hb-function –yet a minor percentage of mutations could affect both. β-thalassemia is the most common single-gene inherited condition across the world. Almost 70,000 infants are born with β-thalassemia major worldwide each year (Cousens et al. 2010). Many cases of β-thalassemia trait are overlooked or misdiagnosed as iron deficiency because both conditions are the most frequent causes of microcytic anemia (Ntaios et al. 2007, Usman et al. 2011). Since there exist data indicating MS linkage to 11p15.5 and 16p13.3 chromosomal regions, where α and β-globin gene cluster reside, it would be very tempting to investigate whether MS patients carry specific Hb variants which do not exert haematological abnormalities.

Some silent Hb variants/mutants may cause abnormally low or high binding and utilization of oxygen in neural tissues. MS is considered solely as a neuroinflammatory disease, yet an examination of MS lesions revealed infarct like lesions, which were attributed to vasoactive compounds released from activated macrophages and microglia (Lassmann 2003). In progressive stages of the disease, histotoxic hypoxia may cause diffuse neurodegeneration and release of inflammatory mediators triggering mitochondrial damage (Aboul-Enein and Lassmann 2005). In recent years, studies of perfusion MRI showed that Type-3 focal demyelinating lesions were white matter hypoperfusion regions and linked to MS-associated cognitive decline (De Keyser et al. 2008). Hypoxic mechanisms also involve in necrosis of demyelinated axons (Trapp and Stys 2009). Cellular necrosis causes a release of antigenic peptides, which do not normally exist in blood circulation and subsequently activates inflammation and innate immunity (Brines and Cerami 2012). Toll-like receptors have recently been proposed as key players in MS (O'Brien et al. 2008).

Silent Hb variants may perturb the optimal mitochondrial respiration in neuronal cells with high energy demand, which subsequently triggers cell death and cause release of antigenic peptides. An interesting link between the mitochondrial damage and activation of innate immunity in MS may also be proposed based on the therapeutic efficacy of dimethylfumarate in MS. Dimethyl fumarate attenuates inflammatory innate immunity in MS (Giunti et al. 2014) and protects mitochondria against several noxious stimuli. For instance, dimethylfumarate prevents the electron chain inhibition and cytochrome-C release following excess exposure to tetrahydropiopterin. Further, dimethylfumarate hinders the decrease of mitochondrial transmembrane potential and overproduction of O_2^- and H_2O_2 following 7-ketocholesterol exposure in oligodendroglia. From an evolutionary viewpoint, endosymbiosis theory assumes that mitochondria represent formerly free-living bacteria that were received as endosymbionts into eukaryotes (Dyall et al. 2004). There exists several lines of evidence supporting this theory including that mitochondrial outer-membrane proteins exert similarities to bacterial antigens (Baum 1995).

An assumption would be that mitochondrial protection and alleviation of innate immune responses by dimethylfumarate may associate with each other. Hindering energy failure and maintaining mitochondrial integrity may attenuate the release of mitochondrial degradation products from necrotic and late apoptotic cell debris. This would reduce activation of innate immune responses against debris molecules that exert antigenic similarities to bacteria. As partly mentioned above, Hbs also exert a danger-associated molecular pattern (DAMP), which binds to pathogen-associated molecular pattern (PAMP)-proteins and is a prominent trigger of innate immunity via Toll-Like receptors (Lee and Ding 2013). Therefore, Hb variants, reducing optimal mitochondrial metabolism and causing cell death and axonal injury, may work in a synergy with cell debris to activate the inflammation cascade.

It is also possible that variations or mutations in Hb encoding genes per se may not be frequent in MS patients. Defective signaling mechanisms controlling the translational and post-translational kinetics of Hbs may also perturb intracellular Hb homeostasis in neural cell lineages. As an alternative or a complementary theory, transcription factors that involve in the induction of Hb in neuronal cell types may be not optimal due to mutations or variations. Hb variants may also

derange the formation of anti-inflammatory endogenous hemorphins that are produced via cleavage of Hbs. Such variants may also explain the association between MS and chromosomal regions, where α- and β-globin gene cluster reside. The cleavage of Hbs leads to the formation of hemorphins, biologically-active peptides with anti-inflammatory properties and affinity towards opioid receptors (Yamamoto et al. 2002; Sanderson et al. 1998). Lowered hemorphins correlate with diabetes-associated cognitive decline (Song et al. 2012). Moreover, the differential cleavage of Hbs leads to the formation of hemocidins that act as immune defence molecules against gram positive and negative bacteria, fungi, and yeasts (Sheshadri and Abraham 2012; Liepke et al. 2003). Hemocidins composed of 17 to 36-amino acid peptides are formed via cleavage of the C-terminal of β- or γ-globin chains (Liepke et al. 2003). In clams, Hbs are the first elements in immune defence against bacteria (Ullal et al. 2011), while in sea bass, the most significantly and rapidly increased protein against microbial stress is the hemoglobin-β-like protein (Hb-LP) (Terova et al. 2011). These contemporary findings show that Hbs exert important immune roles emerging from their phylogenetic functions. In adults, HbA2 and fetal Hb (HbF) are expressed as minor Hbs. Although HbA2 levels are normal in cancer, myelofibrosis, and infections (Alperin et al. 1977), they are increased in certain types of psoriasis (Reddi et al. 1976) and rheumatismal fever (Zlatkov et al. 1990). Currently, it is not clear whether these increases are caused by immune activation or merely compensatory responses to inflammation. Interestingly, a recent systematic review of the comorbid autoimmune disorders in MS revealed that psoriasis is the most prevalent comorbid pathology (Marrie et al. 2015). In accordance, our recent findings show that HbA2 has a protective role in MS (manuscript submitted for publication).

The γ-globin chain of the minor HbF may augment the release of pro-inflammatory cytokines (Zlatkov et al. 1990) and modify dendritic cells towards more immunogenic or tolerant responses in a dose-dependent manner (Khatri et al. 2009). In a recent study (Brown 2014), Hb β-globin chain is found to reside predominantly on the surface of $CD68^{+}$ microglia in brain samples of patients with MS but not in the cytoplasm. It is more likely that this Hb was originated from the lysed fragile RBCs, which triggered inflammation rather than neuroprotection. Overall, a better understanding of immunological roles of Hbs and their cleavage products, hemorphins and hemocidins, may pave the way to the discovery of novel mechanisms underlying MS immunopathology.

Genetic studies in MS revealed associations with loci where β- and α-globin gene clusters reside

β- and α-globin gene clusters reside at chromosomal regions of 11p15.5 and 16p13.3, respectively. The prevalence of MS in Sardinia (140 per 100,000) is much higher than in neighbouring Mediterranean countries, implying that the isolated population concentrated genetic elements increasing susceptibility to MS (Coraddu et al. 2001). The distinct HLA association of MS in Sardinia supports this argument. A Whole Genome Screen (WGS) for linkage in 49 Sardinian multiplex families applying nonparametric linkage analysis of 327 markers demonstrated suggestive linkage in 3 regions, and notably, one of these was 11p15.5 (Coraddu et al. 2001). A WGS for linkage in 136 sibling-pairs with MS from 4 Nordic countries, with an exceptionally high prevalence of MS, was performed by typing 399 microsatellite markers (Akesson et al. 2002). Seventeen regions were found where the lod score exceeded the nominal 5 % significance threshold, and strikingly two of these were 11p15.5 and 16p13.3.

These studies provide futher argument to investigate whether some Hb variants/mutations are linked to MS pathogenesis. In accordance, a gene expression study analyzing 8300 genes in blood monocytes in a sample of eight monozygotic twin pairs discordant for MS showed that 25 genes were at least two-fold upregulated in twins with MS in comparison to their healthy siblings (Särkijärvi et al. 2006). Of the six most constantly overexpressed genes, two were genes encoding Hb α- and β-globin chains (Särkijärvi et al. 2006). The impact of mutations associated with minor thalassemia forms on Hb transcription may be different in RBC-progenitors than in other cells including monocytes and neurons. The involvement of differing transcription factors and preferred binding sites may be responsible for this diversity. Abnormal Hb synthesis in certain cell lines or suboptimal respiratory functions in silent hemoglobinopathies may augment compensatory Hb synthesis in different cell lineages.

May we benefit from the knowledge on Hb-involvement in MS-pathology for future therapeutic strategies?

Antioxidant agents that increase RBC stability (Marcel and George 1983) and molecules, e.g. pentoxyfylline that enhance RBC deformability and provide better blood rheology (Jilani and Iqbal 2011) – perhaps in tandem – may lower lysis of RBCs, the release of fr-Hb, and alleviate chronic toxicity. Better monitoring and treatment of vitamin deficiencies that trigger RBC lysis may also help to reduce fr-Hb. Valproic acid, a commonly used drug for the treatment of bipolar disorder and epilepsy, with likely neuroprotective effects, enhances intracellular levels of minor hemoglobin HbF (Lagace and Eisch 2005, Monti et al. 2009). Remarkably, valproic acid was recently shown to attenuate disease symptoms and increase endogenous myelin repair by recruiting oligodendrocyte progenitors in an experimental MS model (Pazhoohan et al. 2014). Furthermore, valproic acid in

conjunction with Oct4 transcription factor was also shown to accelerate myelin repair in demyelinated optic chiasm in mice (Dehghan et al. 2016). Further investigation is required to understand whether the induction of Hbs is involved in these therapeutic effects of valproic acid.

Lastly, erythropoietin, which neuroprotective effects are thoroughly investigated in recent years, may involve in both the "inside-out" and "outside-in" actions of Hbs in MS pathogenesis. First, erythropoietin is a significant inhibitor of eryptosis (programmed death of RBCs) (Lang and Lang 2015), which would attenuate the release of toxic fr-Hb. Besides stimulating erythropoiesis, erythropoietin also stimulates Hb synthesis (Papayannopoulou and Finch 1972) and could trigger the synthesis of HbF in manifest thalassemia and thalassemia trait during pregnancy (Breymann et al. 1999). Strikingly, EH-201 – a chemical inducer of erythropoietin synthesis – was recently shown to enhance Hb-synthesis in mice hippocampus, restore mitochondrial functioning and to improve memory following neurotoxic insults (Horng et al. 2015). EH-201 or erythropoietin may be tested whether they could improve neuronal and oligodendroglial Hb synthesis in the context of MS. Recent studies showed that erythropoietin alleviates experimental MS severity not only via reducing inflammation but also via inhibiting axonal damage, increasing oligodendroglial cells and inducing myelin synthesis (Cervellini et al. 2013a, b).

A randomized double-blind pilot study investigating the therapeutic role of erythropoietin with methylprednisolone (MPred) in 20 patients with severe motor relapsing-remitting MS (RR-MS) in relapse [10 patients receiving i.v. MPred (1 g/24 h) and i.v. EPO (20,000 U/24 h) for 5 days and 10 patients receiving only MPred (1 g/24 h)] showed an improvement in erythropoietin + MPred group. These improvements included increases in maximal distance walking and reductions in ambulatory index and Expanded Disability Status Scales at the 2nd month that endured after 3 months (Najmi Varzaneh et al. 2014). Furthermore, MRI showed a significant reduction in the number of T2WI lesions with the combination treatment (Najmi Varzaneh et al. 2014). Using transgenic mouse models, which over- or under-express Hbs in different neuronal cells, neuroprotective actions of erythropoietin, in relation to Hbs, might be tested.

Conclusions

Despite many advances achieved in the management of MS, it is still one of the major causes of neurological disability. Therefore, there is an urgent need to investigate novel detrimental/protective pathways underlying the pathogenesis of MS. It is plausible to argue that intracellular Hbs are protective in MS, regarding their roles in oligodendroglial expression, mitochondrial localization, induction of mitochondrial metabolic genes to maintain cellular energy demands and scavenging free radicals. Considering that fr-Hb damages myelin and changes its antigenicity, extracellular Hb released from fragile RBCs may be a detrimental factor in the "outside-in pathogenesis" of MS. In the "inside-out pathway" of MS pathogenesis, silent Hb mutations and/or improper transcriptionalregulation of Hbs may increase susceptibility to MS. This may occur due to the distruption of Hbs' protective roles in mitochondria that leads to enhancing cell death and the subsequent release of intracellular peptides with high antigenicity. A better understanding of the role of these pathways in MS may pave the way to innovative treatment strategies.

Compliance with ethical standards

Conflicts of interest The authors report no conflict of interest.

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