

# The conundrum of iron in multiple sclerosis – time for an individualised approach

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**Abstract** Although the involvement of immune mechanisms in multiple sclerosis (MS) is undisputed, some argue that there is insufficient evidence to support the hypothesis that MS is an autoimmune disease, and that the difference between immune- and autoimmune disease mechanisms has yet to be clearly delineated. Uncertainties surrounding MS disease pathogenesis and the modest efficacy of currently used disease modifying treatments (DMTs) in the prevention of disability, warrant the need to explore other possibilities. It is evident from the literature that people diagnosed with MS differ widely in symptoms and clinical outcome - some patients have a benign disease course over many years without requiring any DMTs. Attempting to include all patients into a single entity is an oversimplification and may obscure important observations with therapeutic consequences. In this review we advocate an individualised approach named Pathology Supported Genetic Testing (PSGT), in which genetic tests are combined with biochemical measurements in order to identify subgroups of patients requiring different treatments. Iron dysregulation in MS is used as an example of how this approach may benefit

patients. The theory that iron deposition in the brain contributes to MS pathogenesis has caused uncertainty among patients as to whether they should avoid iron. However, the fact that a subgroup of people diagnosed with MS show clinical improvement when they are on iron supplementation emphasises the importance of individualised therapy, based on genetic and biochemical determinations.

**Keywords** Myelin · Autoimmunity · Biochemistry · Genetics · Personalised medicine

## Introduction

Whether iron supplementation is beneficial or harmful to people with multiple sclerosis (MS) has been debated for several years. The answer is of critical importance as it may guide physicians to optimal treatment of a very complex disorder (van Rensburg and van Toorn 2010). The intensity of the iron-MS debate escalated substantially when Dr Paolo Zamboni, a vascular surgeon, presented a hypothesis to the Royal Society of Medicine that iron deposition in the brain may contribute to the development of MS (Zamboni 2006). This theory states that periventricular iron deposits are formed in the brain, similar to those that form around veins in the leg in chronic deep vein thrombosis. Iron released from the deposits may cause oxidative damage resulting in the recruitment of white blood cells into the brain matrix (Singh and Zamboni 2009). The deposits are thought to result from an inhibition of blood outflow from the brain through the jugular veins due to venous obstructions. The hypothesis is called chronic cerebrospinal venous insufficiency (CCSVI), and it is suggested that balloon dilatation or stents may be used to improve cerebral venous drainage.

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The hypothesis and procedure were received with much scepticism by the majority of the medical community. The Cardiovascular and Interventional Radiological Society of Europe (CIRSE) issued a commentary (Reekers et al. 2011) in which they expressed the belief that the lack of clinical trial data did not offer a sound basis for such treatments. Filippi et al. (2011) stated that clinicians were generally satisfied with the current prevailing theory of MS, and since some studies have not confirmed the original results, “endo-vascular treatment of presumed vascular abnormalities in MS should be discouraged vigorously”.

The hypothesis however generated much optimism amongst people with MS, since correction of cerebral venous drainage is anecdotally related to rapid improvement in clinical outcome (reduced disability). Public urgency led the US National MS Society to fund a number of independent studies to investigate the relationship between CCSVI and MS. The above scenario emphasises the complexity of MS research, the diversity of treatment modalities available and the uncertainty all of this creates among MS sufferers. Evidence of this uncertainty is seen on the Internet where patients and MS support groups actively communicate with one another via Facebook, YouTube and blogs, raising optimism for “liberation treatment”, as the CCSVI treatment is called, contrary to the advice of clinicians (Filippi et al. 2011).

This raises the question: Is this not the time for the medical fraternity to acknowledge patient perception that conventional medicine does not benefit all patients? Recent studies, for example, found that fewer than half of patients benefit from interferon- $\beta$ , one of the standard treatments for MS (Byun et al. 2008; Wekerle and Hohlfeld 2010). In non-responders, interferon- $\beta$  may even cause worsening of disease outcome due to a Th17 immune response (Axtell et al. 2011). Treatment with disease modifying agents has been shown to reduce relapse rates, but has limited effect on disability progression. Such medications often have unpleasant side effects (Walther and Hohlfeld 1999), and patient resistance to taking the injections is not infrequently reported (Hancock et al. 2011). Unfortunately, these findings are not always reflected in randomised controlled trials, which may be attributed to selection bias, i.e. enrollment of an enriched study sample that differs from true population-based samples (Pittock and Rodriguez 2008; Caplan 2011). The controversies surrounding disease pathogenesis in MS and lack of efficacy of the currently used disease modifying agents to prevent disability, warrant the need to re-explore MS disease mechanisms, such as gaining an in-depth understanding of the basic biochemical processes that take place during demyelination. This would include the role played by genetic defects and biochemical deficiencies, and subsequent intervention to alleviate them. Investigating the role of iron in MS is especially important due to the

uncertainty of whether it is safe for MS patients to take iron supplements, or whether they should avoid ingesting iron.

### Is iron harmful or helpful in MS?

Proponents of the “iron is harmful” theory advocate reduced iron intake or removal of iron by chelation therapy as a means of reducing iron deposition in the brain (LeVine and Chakrabarty 2004). Their justification is based on the fact that poorly liganded iron generates free radicals via the Fenton reaction that could damage various molecules and tissues, including myelin (Kell 2009). Much of the research in this field is performed on an animal model of brain inflammation, experimental allergic (more recently: autoimmune) encephalomyelitis (EAE), which is widely believed to be a model for MS. EAE is generated by injecting an animal with an antigen such as myelin basic protein, together with an adjuvant such as *Mycobacterium tuberculosis* and pertussis toxin. In a study frequently cited to prove the benefit of iron deprivation, mice did not develop EAE when they were fed a low-iron diet (Grant et al. 2003). However, the authors did point out that the severity of EAE was not exacerbated in mice that were fed a high iron diet, and that the positive outcome of the iron deprivation was related to the impairment of CD4+ T cell development, rather than having a central effect. Furthermore, the authors emphasised that the potential harmful consequences of iron deficiency anaemia should preclude the translation of this research to humans, a warning that has not always been heeded.

Iron deprivation in MS would have been justified if substantiated by studies demonstrating improved disease outcome in people with MS who were iron restricted as in the case of the EAE animals. However, a clinical trial involving 9 chronic progressive patients with MS, who received up to 8 courses of the iron chelator desferrioxamine over 2 years, had a variable outcome (Lynch et al. 2000). One patient improved, 3 remained stable, and 5 worsened on the Expanded Disability Status Scale (EDSS) (Kurtzke 1983), the “gold-standard test” for neurological outcome. The authors attributed the negative outcome of the chelation therapy to an inadequate dosage schedule (Lynch et al. 2000). However, a more plausible explanation is that EAE is not a model for human MS (Sriram and Steiner 2005). It was designed to be an animal model for autoimmunity years ago, when it was first postulated that MS was an autoimmune disease (Behan and Chaudhuri 2010). Rodriguez (2009) has however pointed out that it is extremely difficult to classify MS as an autoimmune disease, since extensive research over more than 50 years has failed to confirm that MS fulfills the criteria for autoimmunity as proposed by Schwartz and Datta (1989; Table 1). A comparison with

**Table 1** Criteria for determining a disease as autoimmune

1. Demonstration of an immune response to a precise autoantigen in all patients with the disease
2. Reproduction of the lesion by administration of autoantibody or T cells into a normal animal
3. Induction of lesion by immunizing an animal with relevant purified autoantigen
4. Isolation or presence of autoantibody or autoreactive T cell from lesion (or serum)
5. Correlation of autoantibody or autoreactive T cell with disease activity
6. Presence of other autoimmune disorders or autoantigens associated with disease
7. Immune absorption with purified autoantigen abrogates pathogenic autoantibody or autoreactive T cell
8. Reduction of pathogenic autoantibody or T cell associated with clinical improvement

Table adapted from Rodriguez M. Have we finally identified an autoimmune demyelinating disease? *Ann Neurol.* 2009; 66 (5):572–573. Reproduced with permission of John Wiley & Sons, Inc

neuromyelitis optica shows that whilst the latter fulfills these criteria, MS does not (Rodriguez 2009).

### Mind the gap

The autoimmune hypothesis, based to a large extent on results obtained with EAE, states that potentially autoaggressive T cells exist in the blood of patients with MS, having been activated by an antigen that mimics myelin proteins. These T cells then traverse the blood–brain barrier (BBB) and migrate into the central nervous system (CNS) where they “orchestrate a complex series of interactions culminating in the destruction of both myelin and axons” (Hohlfeld 2010). A huge amount of literature, becoming ever more complex, exists in this field, although “there is still a wide gap between research in human MS and its animal models” (Hohlfeld 2010). The reason for this gap is that the 8 criteria for autoimmunity (Table 1) are all based on the premise that a precise autoantigen should be identified for the disease, similar to rheumatoid factor in rheumatoid arthritis. However, such an antigen has not yet been found in MS (Rodriguez 2009). While several self- and environmental antigens activate immune cells in patients with MS (Kimball et al. 2011), none of them meet the criteria listed in Table 1. In EAE the antigen is provided by the researcher, who decides which of the myelin proteins to inject. Therefore, the evidence for autoimmunity in MS remains elusive (Behan and Chaudhuri 2010; Steiner and Mosberg-Galili 2010; Trapp 2004; Miravalle and Corboy 2010; Corthals 2011). In addition, it has been shown that the oligoclonal B-cell response in the cerebrospinal fluid (CSF) of patients with MS is not targeted against the myelin proteins generally thought to direct the autoimmune attack: myelin basic protein, proteolipid protein, or myelin oligodendrocyte glycoprotein (Owens et al. 2009). Therefore, although the involvement of immune mechanisms in MS is confirmed (The International Multiple Sclerosis Genetics Consortium & the Wellcome Trust Case Control Consortium 2011), this does not necessarily provide proof for autoimmunity. Since there appears to be a general misunderstanding regarding the difference between immune- and autoimmune disease mechanisms, Table 1 may help to stimulate discussion in this regard.

The evidence for immune involvement in MS is that alleles of the major histocompatibility complex (MHC) exert the single strongest genetic effect in MS disease susceptibility (Ramagopalan and Ebers 2009; Ramagopalan et al. 2009). MHC molecules usually present antigen to CD4+ T cells. However, it was demonstrated in mice that Class II MHC molecules could exacerbate demyelination independently of T cells (see below) (Hiremath et al. 2008), indicating that there may be other mechanisms besides autoimmunity by which the immune system may exert negative effects. It may be possible that the immune response in MS is secondary to a primary disease mechanism residing within the CNS (Trapp 2004). For example, in people predisposed to MS, Epstein-Barr virus (EBV) (Levin et al. 2010) and other infections may activate the human endogenous retrovirus (HERV), thereby producing active virus particles which may in turn activate the immune system (Perron and Lang 2010).

Contemplating the possibility that MS may not be an autoimmune disease after all requires a huge mind shift, since autoimmunity in MS has become an entrenched dogma over the years. The assumption of MS as autoimmune has created a mindset of a typically progressive disabling condition which is incurable, since the origin of the disease emanates from a permanently dysfunctional immune system. Accordingly, the only hope is to delay disease progression by de-activating the immune system or to stop peripheral immune cells from entering the brain by using disease modifying treatments (DMTs). However, the autoimmune hypothesis cannot explain “benign” MS, defined as patients who have an EDSS score of 3 or less after 10 years, and have a >90% chance of remaining free from disability for >20 years without taking DMTs (Pittock et al. 2004; Mastorodemos et al. 2010). The number of patients having a favorable disease outcome may be significant, up to 30% (Pittock and Rodriguez 2008).

Besides benign MS, four subtypes of MS were standardised in 1996: relapsing-remitting- (RRMS), secondary progressive- (SPMS), primary progressive- (PPMS) and progressive relapsing MS (Lublin and Reingold 1996). However, despite attempts to classify disease subtypes, the diagnosis of MS remains confusing due to the heterogeneity of the disease (Steiner and Mosberg-Galili 2010).



patients may need supplementation, and others attenuation of iron intake depending on their genetic make-up, making the treatment of patients with MS particularly amenable to the concept of Pathology Supported Genetic Testing (PSGT), involving genetics as well as biochemical determinations (Kotze et al. 2010).

### The hypothesis of oligodendrocyte apoptosis

The role of iron in MS may be linked to an important alternative hypothesis for the aetiology of MS. Investigations of early MS brain lesions by Barnett and Prineas (2004) showed evidence of apoptosis (controlled cell death) of oligodendrocytes, leading to the extensive demyelination seen at diagnosis and during relapses. In early lesions the demyelination is initiated not by peripheral immune cells, but by microglia, the immune cells resident in the brain. During apoptosis, phosphatidylserine is externalised on cell membranes, which acts as an ‘eat me’ signal to phagocytes such as microglia (Barnett et al. 2006). The activated microglia clear the demised oligodendrocytes and the resulting dysfunctional myelin from the axons (Barnett and Prineas 2004). Subsequently, peripheral macrophages are recruited into the brain as scavengers, amplifying the inflammatory response (Barnett et al. 2006). Remarkable is the fact that oligodendrocyte precursors (adult stem cells) were observed in the immediate vicinity of the apoptotic process (Barnett and Prineas 2004). These cells, resident in the brain, mature into new oligodendrocytes and remyelinate the axons.

These findings have important clinical implications for disease outcome in MS since remyelination of the axons by oligodendrocyte precursor cells (OPCs) would induce remission and restoration of function, whilst lack of survival of these cells would lead to progressive myelin loss, neuronal injury and clinical disability.

Several questions arise:

- (1) What causes oligodendrocyte apoptosis?
- (2) Is oligodendrocyte demise preventable?
- (3) Can remission be achieved and sustained by preventing oligodendrocyte cell death and promoting OPC survival and maturation?

From a biochemical point of view, oligodendrocyte apoptosis may be caused by several factors (Fig. 1): (1) infective agents, (2) inflammatory mediators such as cytokines (Cammer 2002), (3) poisons or toxins, e.g. components of cigarette smoke (Healy et al. 2009), (4) deficiencies of essential nutrients (van Rensburg et al. 2006), (5) oxygen radicals (Kim and Kim 1991), depletion of antioxidants and oxidative stress leading to the release of ceramide (Jana and Pahan 2007), or (6) mitochondrial failure (Ly et al. 2003).

Iron may play a role in all of these insults as both iron overload and iron depletion can induce apoptosis, albeit by different mechanisms (Fassl et al. 2003). While iron excess may increase oxidative stress, iron depletion causes an energy deficit and loss of membrane potential in mitochondria (Koc et al. 2005). During infection/inflammation, iron is sequestered by the immune system to overcome the infection and may therefore decrease the availability of iron to oligodendrocytes. Iron deficiency may paradoxically also generate free radicals (van Rensburg et al. 2004). Catalase, one of the foremost antioxidant enzymes in oligodendrocytes contains 4 haem groups; its production and maintenance is therefore dependent on a constant supply of iron. If the catalase antioxidant system fails, oligodendroglial cell death may follow (Kim and Kim 1991). Furthermore, ingestion of medication or toxic substances causes the induction of cytochrome P450 which would suddenly have to be mass-produced to metabolise these chemicals. Iron deficiency could inhibit this process since each cytochrome P450 molecule contains a haem group.

The hypothesis of oligodendrocyte apoptosis unlocks the possibility that the MS disease process may be influenced by meeting the metabolic requirements of oligodendrocytes. While these cells are extremely complex, their needs for survival may be relatively simple to comprehend, since they have been studied extensively and a wealth of information already exists in the literature.

### Iron requirement by oligodendrocytes

The essential requirement for iron in oligodendrocytes has been systematically investigated in NIH-funded research by the group of Connor since the 1980’s. They showed that staining normal brain tissue with a dye that detects iron, identifies oligodendrocytes as the main iron-containing cells in the brain and that under normal circumstances these cells accumulate iron (Gerber and Connor 1989). Oligodendrocytes need iron for the extremely high energy requirements of producing and maintaining the complex myelin sheath which is many times larger than the cell body (Connor and Menzies 1996), indicating that iron deficiency could seriously compromise the viability of these cells. The list of iron-requiring enzymes in oligodendrocytes is extensive (Todorich et al. 2009). Myelin consists mainly of lipids and proteins synthesised by the oligodendrocytes themselves for their own requirements. Iron is essential for many of these biochemical reactions, such as cholesterol synthesis, which occurs at a higher rate in oligodendrocytes than any other cell type in the brain (Pleasure et al. 1977).

Iron is also a prerequisite for the maturation of OPCs into oligodendrocytes (Morath and Mayer-Pröschel 2001;2002), while Tf and ferritin also play crucial roles in this process

(Connor 1994; Badaracco et al. 2010). In culture, iron deficiency results in loss of viability of oligodendrocyte precursors (Todorich et al. 2011). As may be expected in such a high iron environment, OPCs are very sensitive to oxidation and to the depletion of antioxidants such as glutathione, even more so than mature oligodendrocytes (Back et al. 1998; French et al. 2009). The survival and maturation of OPCs may be especially vulnerable to cigarette smoke – even passive smoking (Sundström et al. 2008). This would imply that these cells would need antioxidant protection during patient relapses, when there is an increased concentration of inflammatory mediators and reactive oxygen species (ROS).

### Iron and microglia

Microglia have three functions with regard to oligodendrocytes: (1) they provide soluble survival factors to them, including iron, under normal conditions (Zhang et al. 2006), (2) they clear away dysfunctional oligodendrocytes and myelin by phagocytosis (Gray et al. 2008), and (3) they assist in remyelination (Matsushima and Morell 2001). During brain development, microglia release iron bound to Tf to OPCs for a limited time as a trophic factor, but as the OPCs mature into oligodendrocytes there is a down-regulation of transferrin receptors (TfR). The main iron transfer method for mature oligodendrocytes is through H-ferritin, which accumulates in microglia in the vicinity of oligodendrocytes when they start to produce myelin (Todorich et al. 2011). The H-ferritin iron uptake system is unique to oligodendrocytes, since neurons and other cells use the TfR-Tf system to acquire iron. This is consistent with the fact that oligodendrocytes need higher amounts of iron – ferritin can supply more iron to oligodendrocytes than Tf, which transports 2 iron atoms only (Hulet et al. 1999). A transport mechanism for H-ferritin has also been identified on brain microvasculature (Fisher et al. 2007).

### Iron deposits in the brain

Examining putative brain iron content in healthy subjects and in patients with MS using sophisticated imaging technology is a subject of ongoing research (Haacke et al. 2009;2010; Khalil et al. 2009; Ceccarelli et al. 2009); however, these novel techniques have not been considered for diagnostic purposes since they are not yet standardised (Poloni et al. 2011). The iron measured includes hemoglobin in blood vessels, ferritin in brain parenchyma and hemosiderin as iron deposits, as well as iron in MS lesions and amyloid plaques. Iron may vary with respect to age, brain

region, hypertension, as well as microbleeds (Rodrigue et al. 2011). Adams (1988) found that the iron deposition in MS reflected damage to vessel walls and “old” hemorrhages in the vicinity of plaques, and that the incidence of hemosiderin deposition in MS was 30% compared to 6% in non-MS cases; furthermore, he suggested that inflammation in vessel walls might be exacerbated by surges in intracranial venous pressure. Haacke et al. (2010) found that increased iron content in MS basal ganglia and thalamus formed a particular pattern of iron deposition in the medial venous drainage system at the confluence of the veins draining these structures. Zamboni (2006) postulated that iron mobilised from such deposits may enter the brain parenchyma and become the source of damage to neurons, thereby promoting disability progression. Worthington et al. (2010) investigated this hypothesis, reasoning that if iron from haemolysed erythrocytes were released into brain parenchyma, it should be reflected by increased ferritin in the CSF. Ferritin is one of the pivotal antioxidant defenses in the brain, providing protection against labile iron. Their results showed that CSF ferritin levels in patients with RRMS and PPMS were essentially normal, largely remaining within the range of less than 12 ng/ml. Unexpectedly, in SPMS a relative increase of the concentration of CSF ferritin from baseline to follow-up was related to the degree of improvement of lower limb function and improvement of the T1 lesion volume on MRI. The authors speculated that ferritin in these patients may reflect a physiologic reaction promoting remyelination (Worthington et al. 2010). Furthermore, there was no correlation of baseline CSF ferritin levels with the baseline EDSS, and no significant increase of CSF ferritin levels over a 3-year follow-up period, arguing against a gradual build-up of iron.

If deposition of iron in the brain in MS were an important predictor of morbidity, it should correlate with disability. However, a study by Ceccarelli et al. (2009) demonstrated that patients with benign MS and SPMS had similar amounts of iron deposition, suggesting that disability in MS may be related to factors other than iron deposition. Furthermore, if the procedure of relieving venous congestion as proposed by Zamboni (2006) is indeed followed by rapid relief of MS symptoms, it is unlikely to be related to immediate resolution of iron deposits. The fact that clinically benign MS may occur despite large lesion loads on MRI has been described as paradoxical (Strasser-Fuchs et al. 2008); the ‘clinical-radiologic paradox’ (Poloni et al. 2011).

Iron deposition has also been described in other brain disorders, such as Parkinson’s disease (PD) (Elolf et al. 2007; Michaeli et al. 2007). However, investigations of systemic iron concentrations revealed significantly lower blood iron parameters in PD compared to controls (Logroscino et al. 1997; Marder et al. 1998). These authors hypothesised that a shift of iron from the blood compartment to the brain compartment had taken place due to iron

dysregulation, leading to iron deposition due to excess iron in the brain. They furthermore hypothesised that iron removal by blood donation should limit the negative effects of excess iron and lower the risk of PD (Logroscino et al. 2006). To test this hypothesis, they studied 38,411 participants in the Health Professionals Follow-Up Study who provided details of blood donation. The results were unexpected: although the number of blood donations was inversely related to the ferritin levels, no association was found between the number of blood donations and risk of PD ( $P$  for trend=0.6); however, the risk of PD was *higher* among men who had reported recent multiple blood donations ( $P$  for trend=0.05). Since dopamine synthesis is dependent on iron availability, iron restriction may cause apoptotic demise of dopaminergic neurons in susceptible individuals (Levenson et al. 2004). Furthermore, Marder et al. (1998) observed that lower serum iron concentrations and elevated serum Tf receptors were associated with mortality in patients with PD, but not in controls. These unexpected findings may emphasise the essential requirement of iron for the viability of dopaminergic neurons and that the iron deposition in PD may represent cells that suffered damage paradoxically as a result of inadequate iron availability. This may confirm that additional biochemical factors need to be considered when iron restriction or removal by chelation is advocated (Levenson et al. 2004).

### The role of iron in the cellular antioxidant system

Contrary to the belief that iron is harmful and invariably causes oxidative damage, it may paradoxically represent the key component of the entire antioxidant protection system of the oligodendrocyte, since:

- (1) Iron is required for the production of ATP, which is essential for the synthesis of NADPH, the reducing power of the cell. NADPH constantly regenerates the reduced form of glutathione, an important component of oligodendrocyte antioxidant protection. NADPH is produced in oligodendrocytes by the pentose phosphate pathway (PPP) (Todorich et al. 2009). The phosphate group of glucose-6-phosphate, the substrate of the rate limiting enzyme of the PPP (glucose-6-phosphate dehydrogenase), comes from ATP (Voet and Voet, 2004). A genetic variation in hexose-6-phosphate dehydrogenase, which generates NADPH in the endoplasmic reticulum, was found to be associated with MS (Alcina et al. 2010).
- (2) More directly, iron forms part of the structure of catalase, an antioxidant enzyme that converts  $H_2O_2$  to water and oxygen (Kirkman and Gaetani 2007). NADPH is responsible for maintaining the active form of the 4 haem groups of catalase.

- (3) NADPH and iron are also involved in anabolic reactions. NADPH provides the reducing power for the synthesis of lipids such as cholesterol, which are produced by oligodendrocytes for their own membranes (Morell and Jurevics 1996; Todorich et al. 2009). The synthesis of one cholesterol molecule requires 19 reductions by NADPH (Voet and Voet 2004). To make one molecule of palmitic acid, a component of sphingomyelin, requires 12 NADPH reductions. Haem cofactors in cytochrome P450 enzymes catalyse the essential hydroxylation reactions in the synthesis of cholesterol. Furthermore, the hydroxylations that produce  $1,25(OH)_2 D_3$  (active vitamin D) from cholesterol are carried out by a cytochrome P450 enzyme called CYP27B1. A significant association of a genetic variant of CYP27B1 with MS has been found (Sundqvist et al. 2010; Ramagopalan et al. 2011). Oligodendrocytes express vitamin  $D_3$  receptors and respond to  $1,25(OH)_2 D_3$  (Baas et al. 2000). Some cells can produce antibacterial proteins to overcome infections by synthesizing  $1,25(OH)_2 D_3$  (Kamen and Tangpricha 2010), but they will only be able to do so in the presence of sufficient iron, because the relevant cytochrome P450 enzyme has to be synthesized as well, and each enzyme would need a haem group.

### Iron deficiency in MS

Iron deficiency may contribute to the finding that more females than males are diagnosed with MS (Warren et al. 2008). In females, deficiencies of iron availability to the brain may arise by (1) iron use elsewhere in the body, such as before and during menstruation when iron is re-routed to synthesise erythrocytes, and (2) iron loss during pregnancy and childbirth, which may amount to 20–25% of maternal iron (de Jong et al. 1990). Relapses are often experienced after childbirth, leading some clinicians to advise women with MS not to have children. In an ongoing study we found that relapses are prevented when women take iron supplements (together with other nutrients) during and after pregnancy (results to be published elsewhere). Furthermore, fatigue is a frequent symptom of MS. Some patients present at diagnosis with such low iron levels that intravenous iron may be required, and if given timeously, demyelination may be reversed and disease progression stopped (Kotze et al. 2010). In our study population two females and a male were diagnosed with MS after blood donation. The male reported frequent multiple blood donations. Although his hemoglobin was normal (12.1 g/dl), all his other iron measures were extremely low: his serum iron fell to 1.9  $\mu\text{mol/l}$  (range 10.0–30.0), his Tf saturation to 2% (20–50) and his ferritin to 7.6  $\mu\text{g/l}$  (22–322). He recovered rapidly following iron supplementation and has remained in remission, an indication

of the importance of taking a detailed history at diagnosis and doing biochemical measurements (Haug PD and van Rensburg SJ, unpublished case studies). Notably, Newman (2006) has suggested that blood donation may cause harm to menstruating females, including neurological sequelae, and the REDS-II Donor Iron Status Evaluation (RISE) study has issued recommendations for iron supplementation in blood donors (Cable et al. 2011).

The link between iron deficiency and obesity may also be of relevance in MS, since obesity may be a risk factor for MS in adolescent females (Munger et al. 2009). Iron deficiency in obese women has been ascribed to chronic, low grade inflammation (Cepeda-Lopez et al. 2011).

### Other requirements of oligodendrocytes

Iron, vitamin B12 and folate influence the absorption of one another. Myelination grinds to a halt if the folate-vitamin B12 pathway is blocked by mutations or by deficiencies of the substrates (Surtees et al. 1991; Ilniczky et al. 2002; Selzer et al. 2003), because folate-vitamin B12 metabolism is required for methylation of DNA, lipids (such as the production of phosphatidyl choline from phosphatidyl ethanolamine) and proteins, such as myelin basic protein (Baldwin and Carnegie 1971). The oligodendrocyte also requires amino acids for protein synthesis and for protection against retrovirus HERV's envelope protein ERV, which blocks amino acid uptake receptors (Perron and Lang 2010). Glycine and glutathione (glutamate-cysteine-glycine) are used in cellular detoxification reactions. Oligodendrocytes also need antioxidants and essential fatty acids for synthesising lipids (Nordvik et al. 2000). Sufficient vitamin D is protective against MS diagnosis (Ascherio et al. 2010) and is associated with improved clinical and MRI outcomes (Weinstock-Guttman et al. 2011), although the biochemical mechanism has not yet been elucidated. Since the life of oligodendrocytes depends on adequate blood flow for provision of nutrients and removal of toxic waste products, it is evident that all components of the cerebral vascular system need to function optimally.

### Genetics of iron metabolism in MS

The role of iron in MS will only be fully elucidated once more information becomes available about the effects of genetic variations regulating iron metabolism, including those leading to iron deficiency. The regulation of iron uptake and transport is extremely complex and involves many different biochemical factors, some of which are unexpectedly involved.

The genetics of iron metabolism in MS has to date largely focused on the analysis of the *SLC11A1* (*NRAMP1*) and

*HFE* genes involved in iron transport and absorption (Table 2). Insights gained from studies performed in a genetically homogeneous South African population paved the way to a better understanding of the close relationship between iron absorption and immune function in MS (Kotze et al. 2001; 2003; 2006). The statistically significant association observed between MS and a functional promoter polymorphism in the *SLC11A1* gene, supports the notion that iron trapping in macrophages upon infection may be detrimental in a subgroup of MS patients that are iron deficient (Kotze et al. 2003; van Rensburg et al. 2006). The *HFE* gene may be in linkage disequilibrium with an MS susceptibility allele in the HLA complex on chromosome 6 (Rubio et al. 2004; Kotze et al. 2006). Lack of clinical manifestation of hereditary hemochromatosis (HH) without any signs of organ damage in two South African sisters with MS found to be homozygous for the C282Y mutation in the *HFE* gene, further substantiates the role of iron dysregulation in the aetiology of MS (Kotze et al. 2006). Notably, iron deficiency anaemia was reported in one of these patients during her child-bearing years. Iron regulation by hepcidin may account for these contradictory findings.

Hepcidin is an iron-regulatory hormone which together with its receptor ferroportin, controls the dietary absorption, storage and tissue distribution of iron (Ganz 2011). Infection and inflammation (strongly implicated in MS) rapidly increases hepcidin synthesis and leads to decreased iron absorption and retention of iron in macrophages. Although genetic variation affecting the hepcidin-ferroportin axis is a major cause of iron overload, the presence of inflammation could lead to iron-restricted anaemia. Differential expression of hepcidin and ferroportin during infection and inflammation links iron metabolism to host defence and decreases iron availability to invading pathogens. This response also restricts the iron supply to erythropoietic precursors, thereby contributing to anaemia of chronic disease (the anaemia associated with infections and inflammatory disorders). The resultant hypoferraemia limits iron availability to microorganisms but may therefore also lead to cellular iron deficiency in MS during inflammatory episodes. Recently, it was discovered that the *TMPRSS6* gene encodes a protein that negatively regulates hepcidin expression (Cau et al. 2010). Some polymorphisms or sporadic mutations in this gene cause hepcidin overexpression and iron deficiency anaemia (Beutler et al. 2010).

An important development in the field of genetics in MS has been the use of a novel approach to investigate the effects of genetic variation on clinical outcome (Ramagopalan et al. 2008a). When comparisons are made of MS cases selected from opposite extremes of the disease outcome spectrum, i.e. the prognostic best 5% (benign MS) and worst 5% (malignant MS) as assessed by the EDSS, the



**Table 2** Summary of susceptibility and disease outcome studies performed in MS patients and controls in relation to genetic variation in the iron-related *SLC11A1* and *HFE* genes

Gene	Country	Study size	Association	References
<i>SLC11A1</i>	South Africa	104 MS patients, 522 controls including 32 parental alleles as family-based controls	Yes	Kotze et al. 2001
	Spain	195 MS patients, 125 controls	No	Comabella et al. 2004
	Sardinia	66 MS patients, 60 controls	Yes	Gazouli et al. 2008
	Turkey	100 MS patients, 100 controls	No	Ates et al. 2010
<i>HFE</i>	Tasmania	166 MS families, 489 MS patients, 104 control families	No clinical, despite 3-fold increase of C282Y in MS	Rubio et al. 2004
	Slovenia	314 MS patients, 400 controls	No, although earlier onset of MS symptoms noted in C282Y carriers	Ristic et al. 2005
	South Africa	118 MS patients, 102 controls	No, although two sisters with MS (without HH) were C282Y++	Kotze et al. 2006
	UK	112 benign and 51 malignant MS patients	No	Ramagopalan et al. 2008a, b
	Portugal	373 MS patients	No, although MS patients with mutation C282Y had a worse prognosis	Bettencourt et al. 2011

*HH* hereditary hemochromatosis

effective sample size increases about 40 times, and provides more than 99% power to detect a quantitative trait locus contributing to only 2% of the phenotypic variance (DeLuca et al. 2007). Using this method, no evidence was found that mutations in *HFE* have any effect on clinical outcome in MS (Ramagopalan et al. 2008a). The likelihood that a diagnosis of MS affects the clinical manifestation of HH could however not be excluded (Kotze et al. 2006). A further 45 different genes, reviewed by Ramagopalan et al. (2008b), both MHC and non-MHC-related, had no effect on clinical outcome: the genes selected were related to immune function, neurobiology, autoimmunity and myelin proteins. Interestingly, the -308 A allelic variant in the promoter region of *TNF- $\alpha$*  gene significantly decreased the risk of MS (Yang et al. 2010), while in another study this variant was shown to increase iron uptake (Krayenbuehl et al. 2006). These subtle effects of iron regulation in MS should be further investigated.

### The role of Human Leukocyte Antigen (HLA) in MS

Risk for MS diagnosis has previously been attributed to specific haplotypes of the MHC II immune system (HLA in humans) which may increase MS risk with an odds ratio of more than 30 (Ramagopalan and Ebers, 2009). Whilst MS susceptibility is linked to *HLA-DRB1\*1501*, protection against disease progression may be afforded by *HLA-DRB1\*01* (DeLuca et al. 2007). The classical role of MHC II (e.g. in EAE) is to activate T cells by antigen presentation. However, an *in vivo* cuprizone model in mice (Hiremath et al. 2008; Matsushima and Morell 2001) revealed that MHC II

may be involved in demyelination independently of T cells. Cuprizone is a copper chelator which exclusively affects oligodendrocytes causing mitochondrial injury, downregulation of mRNA and subsequent apoptosis. This occurs in the absence of T cells, since the BBB stays intact. It was assumed (Matsushima and Morell 2001) that the susceptibility of oligodendrocytes to cuprizone was due to their extreme dependence on energy production for maintenance of the vast myelin sheath, a situation that parallels iron deficiency as discussed above.

MHC II molecules are expressed on activated microglia. In the cuprizone model, microglia are activated not by antigen presentation but through cell signaling (Hiremath et al. 2008). Activated microglia produce TNF- $\alpha$ , IL-1  $\beta$  and NO which exacerbate the process of demyelination. Interestingly, microglia of mice in which MHC II was absent (*I-A $\beta$ <sup>-/-</sup>*) showed reduced activation and cytokine production, while other mice with truncated  $\beta$  chains (*I-A $\beta$ <sup>tr</sup>*) showed similar results, indicating that the cytoplasmic tail of the MHC II molecule was necessary for microglial activation, cell signaling and cytokine production. The mutant mice exhibited significantly reduced demyelination at week 3, however after 5 weeks of cuprizone treatment the amount of demyelination was similar between wild type and mutant mice. If mice were transferred to a cuprizone-free diet within 6 weeks, full remyelination occurred (Matsushima and Morell 2001), demonstrating that an energy deficiency in oligodendrocytes can initiate demyelination and microglial activation. These intriguing results tempt us to speculate that different genetic variants of HLA II may contribute to the underlying genetic predisposition to MS (Fig. 1) and affect MS outcome differentially due to the

effects of these alleles on microglial activation - some genetic variants may cause over-activation or an inability of microglia to terminate their activation when needed (“water damage caused by the fire brigade”). We furthermore hypothesise that if oligodendrocytes and OPCs were protected against apoptosis, microglial activation would be prevented, leading to prolonged remission in MS (Fig. 1). Such an approach would help to shift the focus in MS towards preventative care (Collins 2010). However, it also has implications for MS treatment. Microglial activation is attenuated by cortisone (Kaur et al. 1994), CD200 (Koning et al. 2009), antipsychotic drugs (Doorduyn et al. 2009), polyunsaturated fatty acids (Liuzzi et al. 2007) and vitamin D (Lefebvre d’Hellencourt et al. 2003). Microglia express vitamin D receptors, and are able to synthesize  $1,25(\text{OH})_2 \text{D}_3$  (Neveu et al. 1994); again, iron would be required for the relevant cytochrome P450 enzyme.

It would be of interest to establish whether HLA alleles affect the role of microglia in remyelination, whereby they deliver insulin-like growth factor-1 (IGF-1) (Matsushima and Morell 2001), TNF- $\alpha$  (Arnett et al. 2001) and iron (Zhang et al. 2006) to oligodendrocyte precursors.

### Randomised clinical trials for iron in MS?

Medical regulations dictate that treatment of MS patients should be guided by Evidence-based Medicine (EBM), i.e. by the highest available standard of evidence obtained from randomised clinical trials (RCTs). However, Caplan (2011) argues that the effective clinical neurologist should ask the question: “How well does the evidence from trials apply to the care of individual patients?” For most clinicians, the requirement for iron (or not) in MS should be relatively simple to investigate by doing RCTs. Unfortunately, this method may probably not provide the answer. Although iron chelation therapy has been promoted as a potential treatment, the trial by Lynch et al. (2000), in which one patient improved while others worsened, illustrates the point that iron requirements differ, and raises the question of whether it is useful to treat all MS patients according to the one-size-should-fit-all approach of RCTs, that determines similar intervention in all patients because a significant number of patients (but not all) responded during the trial. Steiner and Mosberg-Galili (2010) have questioned the reliability of even testing DMTs with RCTs in MS, since it is not known whether MS is a single disease or rather a group of various disease entities. When such an extremely heterogeneous population is recruited into RCTs, results are difficult to interpret. Steiner and Wirguin (2000) have argued that “the almost identical results of clinical trials using different agents, and their inability to go beyond the 33%

line, raise the possibility that the entire observed benefit is only a placebo effect, and that the significant deviation from the true placebo might be the outcome of partial unblinding of patients by the side-effects”. The failure of DMTs to stop disability progression contributes to this view (Filippini et al. 2008). RCTs of iron in an unselected population of MS patients cannot be informative, since iron deficiency does not occur in all patients with MS; if iron deficiency is identified in a particular patient, it would be unethical not to prescribe iron. The same is true for haemochromatosis: even though HFE screening is one of the most common genetic tests requested in general practice, no RCT has yet been done to determine treatment strategy, since excessive iron levels already necessitate clinical intervention. Furthermore, supplying the oligodendrocyte with only one essential element when it needs multiple different nutrients would be like inundating a car factory with rubber. Once enough wheels have been manufactured, more rubber will only clog the system. Future RCTs for MS should take cognizance of the existence of different MS subgroups that may respond differently to different intervention modalities.

### The advantages of individualised treatment

In the light of the heterogeneity of MS (Steiner and Mosberg-Galili 2010) and the extreme variability of disease outcome (DeLuca et al. 2007), it has been suggested that individualised treatment of MS patients would have more success (Wekerle and Hohlfeld 2010), but this should extend further than deciding which drug to prescribe. Rather than waiting for autoimmune biomarkers to be found (Hohlfeld 2010), biochemical biomarkers (such as iron, vitamin B12 and vitamin D) are already available and should be utilised to alleviate deficiencies and to ensure that blood levels remain within the optimal range for myelination. The symptoms of vitamin B12 deficiency may mimic those of MS (Ungley and Campbell 1951); misdiagnosis of MS with consequent disability is particularly tragic in light of the fact that vitamin B12 deficiency is an easily diagnosed and treatable disorder. Objective evidence for improvement is provided by doing the EDSS; differences can already be seen over 3–6 months and can be confirmed over longer periods (Nordvik et al. 2000; van Rensburg et al. 2006). Since MS patients differ *it should never be assumed that disease progression is inevitable*. Do all health professionals take note of the evidence for MS risk factors such as smoking and vitamin D deficiency? Since it is assumed that patients will inevitably experience disease progression, smoking is sometimes tolerated on the basis of “it is their only remaining pleasure”, instead of actively working with each patient to minimize their risk. Furthermore, pharmacogenetics may provide useful information, since

immunosuppressive drugs such as azathioprine and methotrexate will have adverse effects in patients with mutations in the methylation pathway. Increased relapse rates have been recorded for patients on some of these drugs (Confavreux et al. 2000).

In the clinical setting, the needs of clinicians and patients differ. Patients need encouragement instead of being informed at diagnosis that they need to prepare themselves for inevitable disability. Many patients lose hope at that point, some even dropping out of the workplace, while the truth is that rehabilitation is possible (Fink et al. 2010) and that up to a third of patients may have a benign disease course (Pittock et al. 2004). Does the fact that benign patients have minimal disability after more than 20 years indicate that these patients are doing something right, possibly protecting their oligodendrocytes against cell death? Research should be encouraged to investigate whether the favorable outcome of these patients is related to genetics, or to environmental factors, or both (Mastorodemos et al. 2010). A recent study reported that sequencing the genome of monozygotic twins discordant for MS provided no evidence that the DNA of the twins differed in DNA sequence, DNA methylation or gene expression (Baranzini et al. 2010). Among our own patients, investigation of a similar monozygotic pair of twins discordant for MS revealed that the unaffected twin does not smoke, while the twin with MS smokes and has low blood levels of essential biochemicals, due to the fact that she was under the false impression that nutrient supplementation would “activate her immune system”. Since it may be difficult to defend autoimmunity in MS at this time (Rodriguez 2009; Steiner and Mosberg-Galili 2010), it may need to be reconsidered whether it is reasonable to motivate patients to administer DMTs if they have fewer exacerbations when they abstain (Hancock et al. 2011).

## Conclusion

The conundrum of iron in MS may be solved when it is approached from an individualised point of view. The heterogeneity of the disease warrants the following question: What factors are present that may impact on demyelination and how should they be alleviated in individual patients? Adequate research already exists on essential requirements for oligodendrocyte survival and remyelination. The value of biochemical testing performed in conjunction with a medical, lifestyle and genetic assessment when appropriate (Pathology Supported Genetic Testing), should not be under-estimated. Further studies are essential to establish the validity of these concepts.

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