



Identification of an iron-responsive subtype in two children diagnosed with relapsing-remitting multiple sclerosis using whole exome sequencing



Susan J. van Rensburg^{a,*}, Armand V. Peeters^b, Ronald van Toorn^c, Johan Schoeman^c, Kelebogile E. Moremi^a, Carel J. van Heerden^d, Maritha J. Kotze^e

^a Division of Chemical Pathology, Department of Pathology, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

^b Division of Anatomical Pathology, Department of Pathology, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

^c Paediatric Medicine and Child Health, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

^d Central Analytical Facility (CAF), DNA Sequencing Unit, Stellenbosch University, Stellenbosch, South Africa

^e Division of Chemical Pathology, Department of Pathology, Faculty of Medicine and Health Sciences, Stellenbosch University, National Health Laboratory Service (NHLS), Cape Town, South Africa

ARTICLE INFO

Keywords:

Pediatric onset multiple sclerosis
Genetic variants
Whole exome sequencing
Iron deficiency
Oxidative stress
Mitochondria

ABSTRACT

Background: Multiple sclerosis is a disorder related to demyelination of axons. Iron is an essential cofactor in myelin synthesis. Previously, we described two children (males of mixed ancestry) with relapsing-remitting multiple sclerosis (RRMS) where long-term remission was achieved by regular iron supplementation. A genetic defect in iron metabolism was postulated, suggesting that more advanced genetic studies could shed new light on disease pathophysiology related to iron.

Methods: Whole exome sequencing (WES) was performed to identify causal pathways. Blood tests were performed over a 10 year period to monitor the long-term effect of a supplementation regimen. Clinical wellbeing was assessed quarterly by a pediatric neurologist and regular feedback was obtained from the schoolteachers.

Results: WES revealed gene variants involved in iron absorption and transport, in the transmembrane protease, serine 6 (*TMPRSS6*) and transferrin (*TF*) genes; multiple genetic variants in *CUBN*, which encodes cubilin (a receptor involved in the absorption of vitamin B12 as well as the reabsorption of transferrin-bound iron and vitamin D in the kidneys); *SLC25A37* (involved in iron transport into mitochondria) and *CD163* (a scavenger receptor involved in hemorrhage resolution). Variants were also found in *COQ3*, involved with synthesis of Coenzyme Q10 in mitochondria. Neither of the children had the HLA-DRB1*1501 allele associated with increased genetic risk for MS, suggesting that the genetic contribution of iron-related genetic variants may be instrumental in childhood MS. In both children the RRMS has remained stable without activity over the last 10 years since initiation of nutritional supplementation and maintenance of normal iron levels, confirming the role of iron deficiency in disease pathogenesis in these patients.

Conclusion: Our findings highlight the potential value of WES to identify heritable risk factors that could affect the reabsorption of transferrin-bound iron in the kidneys causing sustained iron loss, together with inhibition of vitamin B12 absorption and vitamin D reabsorption (*CUBN*) and iron transport into mitochondria (*SLC25A37*) as the sole site of heme synthesis. This supports a model for RRMS in children with an apparent iron-deficient biochemical subtype of MS, with oligodendrocyte cell death and impaired myelination possibly caused by deficits of energy- and antioxidant capacity in mitochondria.

Abbreviations: CNS, central nervous system; CoQ, Coenzyme Q; DIS, dissemination in space; DIT, dissemination in time; DFO, desferrioxamine mesylate; DMT, disease modifying therapy; EDSS, Expanded Disability Status Scale; ETC, electron transport chain; GWAS, genome-wide association study; HERV-W, human endogenous retrovirus W; HDL, high density lipoprotein; HLA, human leukocyte antigen; HREC, human research ethics committee; IPMSSG, International Pediatric Multiple Sclerosis Study Group; IRE, iron-response element; MGAI1, juvenile hereditary megaloblastic anemia 1; MRI, magnetic resonance imaging; MS, Multiple sclerosis; MSRV, MS-associated retrovirus; MST1R, macrophage stimulating-1 receptor; PSGT, pathology supported genetic testing; ROS, reactive oxygen species; RRMS, relapsing-remitting MS; SAME, S-adenosyl methionine; SDHB, iron-protein subunit of Complex II; TF, transferrin; TMPRSS6, transmembrane protease, serine 6; WES, whole exome sequencing

* Corresponding author.

E-mail addresses: sjvr@sun.ac.za (S.J. van Rensburg), avpeeters@gmail.com (A.V. Peeters), vtoorn@sun.ac.za (R. van Toorn), jfs@sun.ac.za (J. Schoeman), lebomoremi@sun.ac.za (K.E. Moremi), cjvh@sun.ac.za (C.J. van Heerden), maritha@sun.ac.za (M.J. Kotze).

<https://doi.org/10.1016/j.ymgmr.2019.100465>

Received 7 January 2019; Received in revised form 16 March 2019; Accepted 16 March 2019

Available online 23 March 2019

2214-4269/ © 2019 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Multiple sclerosis (MS) is the most common cause of disability in young adults due to inflammatory demyelination of nerve axons. In approximately 3–5% of cases the diagnosis is made before the age of 18, referred to as pediatric MS. Consensus definitions for the diagnosis of pediatric MS and related disorders were proposed in 2007 by the International Pediatric Multiple Sclerosis Study Group (IPMSSG), and revised in 2012 to include components of the 2010 revision of the McDonald criteria [1]. Pediatric MS is defined by two or more demyelinating events separated in time and space. The criteria would be satisfied by new magnetic resonance imaging (MRI) lesions that appear three months or longer after the first clinical event together with clinical symptoms consistent with central nervous system (CNS) demyelination and after exclusion of differential diagnoses [2].

In MS, wide differences in age at diagnosis and disability may be indicative of subgroups of patients subject to different genetic and environmental influences [3,4]. Therefore, elucidating a common etiology to enable optimal treatment for disability prevention has been elusive [5]. An effective disease modifying therapy (DMT) for progressive MS has not been identified, and the results of clinical trials in pediatric MS to date have been generally disappointing and not without severe side-effects [6]. The notion of “this is the best treatment available – there are no other options” should never be accepted blindly. A translational model, pathology supported genetic testing (PSGT) [7], provides the necessary tools to evaluate clues presented by “black swan” cases that could shed new light on the etiology of MS. “Black swans” are unexpected, improbable events that challenge existing scientific theories, but in retrospect contribute to the elucidation of complex problems (the sighting of one black swan nullifies the theory that all swans are white) [8]. A case in point is the association of low iron status described in two children with relapsing-remitting MS (RRMS) [2]. Iron supplementation normalized the iron parameters and improved MS symptoms, but as soon as the supplementation was stopped, serum iron levels fell dramatically [2].

Low blood iron levels are generally attributed to lower iron absorption, since the current textbook view of iron retention by the body dismisses the notion that iron is lost through the kidneys, stating that less than 0.1% of body iron is lost daily, mostly as a result of desquamation and blood loss [9]. This view is so entrenched that validated tests for urinary iron are not available in pathology laboratories. The iron deficiency experienced by the two children was therefore hypothesized to involve genetic defects in iron absorption or iron metabolism, such as iron retention in macrophages as a result of chronic inflammation [10].

Inflammation is regarded to be the primary disease mechanism impacting both demyelination and neurodegeneration in MS [7,10]. MS is characterized by the presence of inflammatory demyelinating lesions and brain volume loss of approximately 0.7% per year compared to a loss of 0.1% to 0.3% in normal aging [11]. The association of MS with inflammation is corroborated by the fact that most genetic variations related to MS risk are found within immune system-related genes. The genetic marker with the largest effect size lies within the human leukocyte antigen (HLA) complex: the risk for a diagnosis of MS increases about three times in the presence of HLA-DRB1*1501. However, several studies have found that this allele does not predict disease severity or brain atrophy in MS [12–15]. Other non-HLA susceptibility genes with small individual effects implicated in MS risk by genome-wide association studies (GWAS) have also not shown associations with disease severity [16,17]. The finding that the HLA DRB1*1501 allele is not detected in all patients with MS while some people never develop MS despite inheritance of this allele, supports the suggestion of distinct subtypes involving other genes and/or environmental triggers. Suppression of inflammation by anti-inflammatory treatment has not been able to stop or reverse brain atrophy or disability progression [11,18], suggesting that multiple disease mechanisms are involved.

Currently, the most widely accepted theory of MS etiology (and the reason for DMTs) is that MS is an autoimmune disease, involving the migration of immune cells from the blood into the CNS where they attack normal myelin, after being activated by a myelin-related antigen [19]. However, the absence of a circulating diagnostic antigen/antibody argues against this hypothesis [20,21]. A thorough histopathology study by Prineas and Parratt (2018) using indirect immunofluorescence, *found no evidence for a diagnostic circulating anti-myelin or anti-oligodendrocyte autoantibody* [22]. This critical finding may hopefully redefine the concept of autoimmunity in the etiology of MS. These authors concluded that a more appropriate theory should include the phagocytosis of oligodendrocytes (the cells that produce myelin) and dysfunctional myelin by activated microglia and monocyte-derived macrophages, *secondary* to the death of the oligodendrocytes.

It is therefore imperative to elucidate the cause(s) of oligodendrocyte death leading to demyelination, so that hypotheses may be formulated for the mitigation of such insults. Six risk factors for demyelination and disability in MS have been identified, involving deficiencies or metabolic dysfunction of biochemical pathways and mechanisms: (1) Oxidative damage to lipids, DNA and mitochondria in oligodendrocytes and neurons [23]; (2) Vitamin D deficiency [24–27]; (3) S-adenosyl methionine (S-AdoMet) deficiency caused by deficits in folate-vitamin B12 metabolism [28–30]; (4) Copper deficiency (cuprizone mouse model of MS) [31]; (5) Hypoxia [32–34] and, notably, (6) Iron dysregulation [7].

Both iron overload and iron deficiency have been investigated in MS [35,36], reviewed in [7]. The two children evaluated in the present study who had severe iron deficiency, developed tumefactive demyelination (very large areas of demyelination) [2]. A recent multi-centre dietary study found that children with MS had a significantly lower intake of iron from their diet than children without MS, but with no differences in the other nutrients investigated [37]. Herbert et al. (2018) found that iron deficiency affected myelin parameters on MRI: Low blood iron status in females with MS was associated with lower fractional anisotropy (a measure of white matter microstructure) and worse disability, compared to females with higher iron parameters [38]. Notably, vitamin D deficiency, associated with both MS risk [24] and disability progression [25], is also associated with decreased iron levels and anemia in children with inflammatory bowel disease due to increased hepcidin levels [39]. Vitamin D reduces the circulating concentration of hepcidin, a protein that inhibits iron absorption by the enterocytes in the gut [40].

The six risk factors mentioned above, especially iron deficiency, may all lead to oligodendrocyte cell death and demyelination due to their effects on energy production in mitochondria. It has previously been shown that oligodendrocytes have a particularly large energy requirement for myelin synthesis [41]. It is of interest that iron, copper, S-AdoMet and oxygen are directly involved in the electron transport chain (ETC): Iron is a cofactor of Complexes I–IV [42], copper is involved in electron transport in Complex IV [43], while two enzymes (COQ3 and COQ5) use S-AdoMet as cofactor to methylate coenzyme Q (CoQ) [44]. The ultimate electron acceptor in the ETC is oxygen. Defective oxygen delivery to mitochondria (hypoxia) can be caused by vascular pathology leading to hypoperfusion [32–34], or by environmental toxins such as cigarette smoke that contains molecules such as cyanide, carbon monoxide and nitric oxide [45]. These toxins block the reduction site between iron and copper in Complex IV that should be occupied by oxygen [43]. It is evident that deficiencies (or defective metabolism) of any of the structural or active components of the ETC could cause inhibition of energy production in oligodendrocytes that could lead to their demise. Deficiencies could result from low dietary intake [37,46], and/or genetic variations that inhibit absorption or transport of these components in the blood or into mitochondria, or cause their enhanced excretion from the body.

The objectives of the present study were first to employ whole exome sequencing (WES) to investigate possible genetic causes for the

iron deficiency experienced by the two pediatric RRMS cases described by Van Toorn et al. [2]. Variations in known iron deficiency-related genes listed by Constantine et al. (2008) were included in the investigation [47]. Second, we describe the clinical wellbeing of the children, including scholastic progress and participation in extramural activities after a period of 10 years, during which time they followed a restorative program that included metabolic components to counteract the nutritional deficiencies and oxidative stress listed above [48].

2. Patients and methods

2.1. Subjects

The subjects were two pediatric RRMS cases previously described [2], who demonstrated severe recurrent iron deficiency when iron supplementation was discontinued. Variations in iron-related genes in the children were investigated. The clinical wellbeing of the children was monitored, including scholastic progress and participation in extramural activities over a period of 10 years.

2.2. Ethical clearance

Ethics Approval for the study was granted by the Human Research Ethics Committee (HREC) of Stellenbosch University under reference number N07/09/203. The parents provided written informed consent that their children may participate in the study and for publication of the results. All procedures performed were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments.

2.3. Clinical investigations

Serial MRI with associated clinical findings of the two children were described in 2010 [2]. The children (both males of mixed ancestry who were referred to as Case 1 and Case 2) were followed up every 4–6 months at the Tygerberg Children's Hospital, Cape Town, South Africa. Iron parameters performed at the National Health Laboratory Service, Tygerberg Hospital, Cape Town, were recorded every 4–6 months as described previously [2]. Both children described in the present study were exposed to second-hand smoke from their parents at the time of their diagnosis with MS.

2.4. Personalized nutritional supplementation

Both children presented with sustained iron deficiency and were prescribed iron supplementation (4 mg/kg/day). Since their iron levels dropped when the supplementation was stopped, they have continued to take iron supplements. In addition, considering the six risk-related metabolic pathways for disability referred to in the Introduction, the children followed a restorative supplementation program that contained essential nutrients considered beneficial for myelin production and maintenance [2,48].

2.5. Genetic studies

Whole exome sequencing (WES) was performed at the Central Analytical Facility (Stellenbosch University, Stellenbosch, South Africa) using the Ion Proton™ sequencing system (Thermo Fisher Scientific, Waltham, MA, USA) according to the method previously described by Van der Merwe et al. (2017) [49]. The Ion AmpliSeq™ Exome RDY kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to amplify the whole exome and subsequent library construction. Samples were sequenced with the One Touch workflow on an Ion P1™ Chip v3 using Hi Q™ Sequencing chemistry. Variant calling was performed on the TorrentServer against an ethnically concordant major allele reference genome based on Genome Reference Consortium Human Build 37 patch

release 13 (GRCh37.p13), NCBI *Homo sapiens* Annotation Release 105 [50]. Variants were annotated in IonReporter 5.6 and prioritised based on their role in iron deficiency [47,51], by using an in-house pipeline of clinically relevant genes selected from the approximately 20,000 human genes. All variants reported were verified by Sanger sequencing. Taqman analysis, using haplotype tagging by rs9271366, was employed to investigate the presence of the HLA-DRB1*1501 allele associated with increased risk for MS [52].

3. Results

3.1. Genetic investigations including whole exome sequencing

Using the method outlined above, variant calling using a high stringency setting, identified a total of 24,916 variants for Case 1, and 26,550 variants for Case 2. When the variants were annotated in IonReporter and prioritised with our pipeline based on their role in iron deficiency, several genetic variants were identified in metabolic pathways that may be associated with the MS disease process as outlined in the Introduction. Variants in iron metabolism were identified in both children that may have resulted in their iron deficiency, including pathways of absorption, transfer and excretion of iron (Table 1). The discovery of *CUBN* variants could relate to deficiencies or dysfunction of vitamin B12 and vitamin D as well. Genetic variants were also identified in the metabolic pathways of iron transport into mitochondria (SLC25A37) and of CoQ synthesis.

3.2. Clinical findings

Iron supplementation was initiated in both RRMS children following a third relapse. Case 1 first became symptomatic at 3 years 8 months of age and subsequently experienced relapses at 4 years 4 months and 5 years 1 months of age. The age of onset in case 2 was 5 years with relapses occurring at 5 years 8 months and 11 years 8 months. In both cases, serial MRI confirmed dissemination in space (DIS) and dissemination in time (DIT) [2].

Regular 4–6 monthly review by a pediatric neurologist revealed no further symptoms or signs suggestive of neurological relapse for 11 years (case 1) and 9 years (case 2). Repeat MRI was only performed when clinically indicated. Case 2 had repeat MRI studies at 12 years 3 months and 14 years 9 months of age due to epileptic seizures. The seizures were related to healed scar tissue from previous demyelination events and no active lesions were identified. Case 1 never warranted further neuroimaging studies.

3.3. Scholastic performance

Both children required remedial scholastic support due to the previous demyelinating episodes. Hearing, vision and motor ability appeared unaffected. Case 1 is currently coping in mainstream schooling with an interest in subjects such as computer applications for technology, business studies and art. He partakes in soccer, and excels in athletics, competing at a Provincial level against main-stream athletes in his age group.

Formal cognitive evaluation in Case 2 revealed mild intellectual disability with epilepsy. He attended a school for children with special needs until 18 years of age where he underwent training in beading and embroidery and took part in athletics. At present he lives with his mother and does care work.

4. Discussion

The present study was undertaken to ascertain whether genetic information from WES could shed light on the clinical profile of two children diagnosed with MS, including large areas of demyelination and a sustained decrease of serum iron parameters when supplementation

Table 1
Genetic variants found in Case 1 and Case 2.

Metabolic pathway	Gene	Gene variations	Result		Metabolic associations
			Case 1	Case 2	
Iron deficiency	<i>TMPRSS6</i>	rs855791, 2207C > T, V736A rs2543519, 1223 + 66 T > C	HET HET	WT HOM	<i>TMPRSS6</i> encodes a type II transmembrane serine proteinase that suppresses hepcidin expression. rs855791 is located close to both the catalytic and the specificity site of the serine protease and may therefore be a causal variant for iron deficiency [53]. Both rs855791 and rs2543519 are associated with iron deficiency [53–55] <i>TF</i> variants have previously been associated with decreased iron concentrations [56,57], including rs1880669 [58]
Iron transport	<i>TF</i>	rs1130459, -2 G > A rs1880669, 1298-23 T > C	HET HET	HOM HOM	
Iron loss	<i>CUBN</i>	rs74431427, 9986C > T, S3329 L rs557482398, 8773 A > G, T2925A rs41289305, 5803 A > G, S1935G rs2271462, 5518 G > A, G1840S rs116114483, 5098 G > A, D1700N rs7905349, 2188C > T, H730Y rs3736032, 287 G > A, R96Q	HET HET HET HET HET HET HET	WT WT WT HET HET HET HET	<i>CUBN</i> encodes cubilin. A cubilin-megalin complex is involved in the reuptake of iron-bound transferrin in the kidney [59]. Cubilin also facilitates the reabsorption of vitamin D in the kidney [60] and vitamin B12 absorption in the ileum. Compromised function of cubilin results in vitamin B12 malabsorption and enhanced excretion of transferrin-bound iron and vitamin D in the urine [61].
Iron import into mitochondria	<i>SLC25A37</i>		HET	HET	Mitoferrin-1 functions as an essential iron importer for the synthesis of mitochondrial heme and iron-sulfur clusters for the electron transport chain [62,63]
Hemorrhage resolution	<i>CD163</i>	rs61729512, 2702C > T, T901 M rs567350690, 1939 A > G, R647G	HET WT	WT HOM	<i>CD163</i> encodes a receptor on macrophages that endocytose iron from ruptured erythrocytes. [64] [65]
Coenzyme Q10 synthesis	<i>COQ3</i>	rs7764793, 486 + 35A > C rs11548336, A > G K134E rs9376137, -1G > T	HET HET WT	WT HOM HET	<i>COQ3</i> encodes an O-methylase that forms part of a complex that synthesises CoQ in mitochondria [44]. rs11548336 encodes a missense variant, K134E

HOM = homozygous (risk-associated allele), HET = heterozygous, WT = wild type (reference allele).

was discontinued [2]. The presence of genetic variants may have contributed to the diagnosis of RRMS in the two children, which was made according to clinical criteria presented by the consensus definitions of the IPMSSG [1,2]. The identification of the variants may provide insight into the impact of deficits in the biochemical pathways of the six risk factors for demyelination and disability mentioned in the Introduction. Addressing the deficits in these metabolic pathways led to clinical improvement as described above. Genetic variations in key pathways that could have impaired iron absorption in the children are shown in Table 1.

4.1. Iron absorption: *TMPRSS6*

TMPRSS6 has previously been shown to be associated with iron deficiency [53–55]. Case 1 is heterozygous for rs855791 and rs2543519, while Case 2 is homozygous for rs2543519 (Table 1). We have also demonstrated that homozygosity for the TT allele of rs855791 was associated with significantly lower blood iron parameters compared to wild-type CC in 107 South African MS patients [66]. Delbini et al. (2010) [53] found that rs2543519 was significantly associated with iron-refractory anemia; however in our study, Child 1 who is heterozygous for rs2543519, and Child 2 who is homozygous for this variant, both responded to sustained oral iron supplementation [2].

4.2. Iron transport: *TF*

Two genetic variations in the children were also detected in the *TF* gene, which encodes the main iron transport protein in the blood, transferrin. Case 1 is heterozygous for rs1130459 and rs1880669, while Case 2 is homozygous for both variants (Table 1). Many *TF* variants have previously been associated with decreased iron concentrations [56,57]. Constantine et al. [47] found an association of rs1880669 with iron parameters [58].

4.3. Iron loss: *CUBN*

Notably, WES revealed that both children have several genetic variants in *CUBN*, the gene that codes for cubilin, a 460 kDa receptor that binds to several ligands including the intrinsic factor (IF)-vitamin B12 complex, apolipoprotein A1, high density lipoprotein (HDL) and albumin [61]. Kozyraki et al. [59] discovered that a cubilin-megalin complex is involved in the reuptake of iron-bound transferrin in the kidney [59]. Furthermore, cubilin facilitates the reabsorption of vitamin D in the kidney where it is activated [60]. Cubilin lacks a transmembrane domain, and functions in concert with megalin and amnionless, which mediate endocytosis of cubilin-bound ligands [61]. Enhanced excretion of the ligands can be demonstrated in the urine when the function of any one of these three receptor proteins is compromised [61]. High-penetrance *CUBN* mutations such as 3916C → T and IVS-intraCUB6 C → G cause juvenile hereditary megaloblastic anemia 1 (MGA1, OMIM 261100; Imerslund-Gräsbeck syndrome), a rare autosomal recessive disorder characterized by an inability to absorb vitamin B12 in the terminal portion of the ileum and neurological symptoms [67]. Several functional polymorphisms in *CUBN* have unknown effects on megaloblastic anemia [67]. Neither of the two children in our study had symptoms of megaloblastic anemia; therefore the *CUBN* variants detected in the children are not clinically relevant for megaloblastic anemia. Case 1 is heterozygous for 3 variants, while Case 2 is heterozygous for 4 variants (Table 1).

4.4. Iron import into mitochondria: *SLC25A37*

Both children were heterozygous for *SLC25A37* rs3736032. This gene encodes mitoferrin-1, which functions as an essential iron importer for the synthesis of mitochondrial heme and iron-sulfur clusters for the electron transport chain [62,63]. Homozygosity for the

rs3736032 AA genotype is extremely rare with a frequency of less than 0.1 in all natural population groups [68], compatible with a severe impact on mortality. Considering that the two children already have an iron deficiency, impaired import of iron into their mitochondria may have an important impact on energy production and myelination.

4.4.1. Iron deficiency in Mitochondria: impact on energy production and oxidation – significance for MS

The significance of the genetic variations detected for the two children with MS, related to iron loss through the kidneys (*CUBN*) and decreased iron import into mitochondria (*SLC25A37*) becomes clear in light of the discovery by Yoon et al. [42]: In cultivated cells, iron chelation by desferrioxamine mesylate (DFO) caused down-regulation of the iron-protein subunit (SDHB) of Complex II of the ETC, resulting in a collapse of mitochondrial membrane potential, a significant decrease of intracellular ATP (53.8%) and irreversible growth arrest of the cells. The reduced expression of SDHB was due to an IRE in the 5' untranslated region of the mRNA, causing sensitivity to iron depletion. In addition, two AUUUA sequences on the 3'-UTR mRNA-destabilizing elements of the mRNA caused an even faster decrease of the mRNA level [42]. A similar downregulation of dehydrogenase activity as a result of iron depletion was found by Ackrell et al. (1985) in rat skeletal muscle mitochondria: both Complexes I and II showed 70% less activity [69]. The authors concluded that iron-deficient complexes were either not assembled or were lost after assembly. A study by Dutta et al. [70] demonstrated a reduction in the activities of respiratory chain complexes I (61%) and III (40%) in mitochondrial preparations from the motor cortex in postmortem tissue from MS patients, leading to a mismatch between energy demand and reduced synthesis of ATP. Since Complexes I and II synthesise CoQH₂, a powerful antioxidant in mitochondria [71], iron deficiency could paradoxically increase the oxidation level in mitochondria, contrary to the general perception that high iron, not low iron, causes oxidation. Lassmann and van Horsen (2016) describe the deleterious effects of mitochondrial oxidation in MS, including decreased ATP levels, oligodendrocyte apoptosis, demyelination, neurodegeneration and progressive disability [23]. Notably, high daily intake of fruits and vegetables in South African MS patients was significantly associated with improved disability scores [72] as measured by the Expanded Disability Status Scale (EDSS) [73]. This may possibly be related to their antioxidant content that would ameliorate the oxidative process.

4.5. Hemorrhage resolution: *CD163*

The oxidative process may furthermore have been enhanced in the two children by missense genetic variations in *CD163* (Table 1) which encodes a 130-kDa member of the scavenger receptor cysteine-rich (SRCR) superfamily [64,65]. *CD163* (also known as macrophage stimulating-1 receptor, *MST1R*) is a scavenger receptor exclusively expressed in monocytes, macrophages and microglia in response to hemoglobin released from ruptured erythrocytes. During hemorrhage resolution, free hemoglobin is bound by haptoglobin, and hemoglobin/haptoglobin complexes are endocytosed by the *CD163* macrophage receptors, enabling iron clearance and storage in ferritin, thereby protecting tissues from oxidative damage [64,65]. Evidence has been found of damage to cerebral veins and perivascular iron deposition due to hemorrhage, to be more common in MS than control brain samples [74,75]. Haptoglobin is expressed by oligodendrocytes following intracerebral hemorrhage [76]. Although it is not known whether genetic variations in *CD163* impair the function of *CD163/MST1R*, *MST1R* expression is significantly downregulated in brain material from MS patients compared with controls [77]. Impaired hemorrhage resolution by *CD163* may enhance inflammation and decrease termination of the immune response, leading to an overactivation of the immune system [78]. Soluble *CD163* (s*CD163*) measured in serum has been examined as a biomarker in MS [79], while their study also confirmed the

presence of human endogenous retrovirus (HERV-W) envelope epitopes on non-classical monocytes from patients with MS. It has previously been suggested that viral infections such as Epstein-Barr and subsequent activation of HERV-W, also known as the MS-associated retroviral element (MSRV), may be involved in the inflammatory demise of oligodendrocytes [80].

4.6. Biosynthesis of coenzyme Q: *COQ3*

Genetic variations were also found in *COQ3* (Table 1), which encodes an O-methylase that forms part of a complex that synthesises CoQ in mitochondria [44]. The importance of genetic variations in *COQ3* for MS is that CoQ is a powerful antioxidant in mitochondria [71], but it can only function as such if it has two methoxy groups, which are added by *COQ3* [44]. The two methoxy groups of CoQ are involved in the firm hydrogen bonding that takes place in the binding pockets of complexes I, II and III during electron transfer [81]. Methyl groups added to CoQ by the enzymes *COQ3* and *COQ5* are derived from SAME, which is transported into mitochondria [82]. While genetic variations in *COQ3* have not been related to primary CoQ10 deficiency in humans [83], homozygous mutations in *C. elegans coq3* that lack methyltransferase activity displayed delayed development and lethality at the embryonic stage in the next generation [84].

4.7. Significance for MS of the genetic variations detected in this study

Neither of the children had rs9271366 which tags the HLA DRB1*1501 allele associated with increased risk for MS, raising the possibility that the genetic contributions of the other gene variants identified in the context of the 6 disease risk pathways mentioned in the Introduction were instrumental in MS pathogenesis due to cumulative risk. These variants, especially *CUBN*, need to be investigated in a larger cohort of MS patients, together with biochemical determinations and measurements of renal excretion of iron, vitamin B12 and vitamin D. The possible roles of the other cubilin ligands such as HDL and Apolipoprotein A in MS should also be investigated.

The genetic variations found in the two children, although not identical, were present in the same disease pathways, and strongly suggest that WES could be implemented to identify impediments in the metabolic pathways involved in energy production in oligodendrocytes, causing reduced synthesis of myelin by oligodendrocytes, leading to their apoptosis and subsequent inflammation due to the scavenging functions of microglia [85]. Mitochondrial dysfunction may be particularly deleterious in demyelinated axons that have an increased energy demand [23]. Although the results of translational studies take long to become established in clinical practice, the finding of iron-deficient diets in children with MS compared to controls [37] could enhance the visibility of iron deficiency in MS. This finding provides support for the first description of iron deficiency in a subgroup of MS patients [86], paving the way for the current investigation. Considering the uncertain benefits of current anti-inflammatory treatment for children with MS, sustained nutritional intervention that supports mitochondrial function, myelin production and remyelination of axons seems reasonable [2,48]. This approach was successful in limiting disability progression in the two children with MS. During the follow-up period, the children did not relapse again and showed no MS-related neurological deficits, suggesting that deficiencies of these nutrients were involved in the pathogenesis.

4.8. Limitations of the study

The primary limitation of the present study is that urinary iron was not measured in the children, since the possibility of iron loss through the kidneys was not considered until the discovery of the *CUBN* variants in the children. Although the functional significance of all the variants in Table 1 were not demonstrated by *in vitro* studies, clinical relevance

for iron metabolism has been reported for *TMPRSS6* rs855791 [53–55] and *TF* rs1880669 [56–58] (Table 1).

4.9. Strength of the study

The strength of the study lies in the continued measurements of serum iron parameters in the children [2] that revealed a dramatic decrease in iron parameters following termination of iron supplementation. The detection of the *CUBN* variants is a novel finding that opens a new avenue for future research. It may therefore be advisable to include iron measurements in the recently proposed Pediatric MS Tool-Kit, that includes three risk factors: environmental tobacco smoke exposure, sun exposure and vitamin D intake [87].

5. Conclusion

WES provided evidence of genetic variations that could explain the sustained iron deficiency in the two cases of pediatric MS. The discovery of *CUBN* variants could relate to deficiencies of vitamin B12 and vitamin D as well. MS symptoms in both children were alleviated following supplementation with these nutrients together with antioxidants. Although some damage was sustained, supplementation resulted in an absence of relapses and restoration of function over 10 years, suggesting the following course of events in their MS etiology:

1. A combination of genetic and environmental factors causing a lack of substrates essential for ATP synthesis (e.g. iron) led to oxidative stress in mitochondria, apoptosis of oligodendrocytes and subsequent demyelination. Scavenging phagocytes caused further oxidative damage to lipids, proteins and DNA in oligodendrocytes and the demyelinated neurons. Exposure of the children to tobacco smoke from their parents could have exacerbated reactive oxygen species (ROS) generation.
2. Genetic variations in inflammatory pathways, such as ineffective scavenging of hemoglobin deposits at the blood-brain barrier, could have led to subsequent over-activation of the immune system and delayed termination of the inflammatory process.
3. Nutritional supplementation provided alleviation of oxidative stress and maintenance of adequate remyelination.

Further studies should investigate whether this model would be applicable to other children diagnosed with MS.

Financial disclosure

Funding: This study was funded by the National Health Laboratory Service Award Number 94139 and the South African BioDesign Initiative of the Department of Science and Technology and the Technology Innovation Agency, South Africa (Grant number 401/01). KE Moremi is supported by Winetech, South Africa (Grant Number N07/09/203), and AVP by the Strategic Health Innovation Partnerships Unit of the South African Medical Research Council (MRC), with funds received from the Department of Science and Technology, South Africa (Grant number S003665). Publication fees for Open Access are supported by the University of Stellenbosch, South Africa. The funding bodies had no role in the design of the study, collection, analysis, and interpretation of data or in writing of the manuscript.

Conflict of interest

SJVR and MJK are listed as inventors on patent number 2010/00058 filed by Stellenbosch University. MJK is also a director and shareholder of Gknowmix (Pty) Ltd., a spin out company of the South African Medical Research Council.

The remaining authors declared no conflict of interest. No writing assistance was utilised in the preparation of this manuscript.

This manuscript has not been published. It will not be submitted elsewhere while under consideration and, should it be published in Molecular Genetics and Metabolism Reports, it will not be published elsewhere – either in similar form or verbatim – without permission of the editors. Its publication is approved by all the authors.

Acknowledgments

The authors thank Dr. Lidia van Vuuren (registered Psychologist) for facilitating a meeting to verify scholastic outcomes. We thank the two children and their parents for permission to publish the data.

References

- [1] M. Tardieu, B. Banwell, J.S. Wolinsky, D. Pohl, L.B. Krupp, Consensus definitions for pediatric MS and other demyelinating disorders in childhood, *Neurology* 87 (Suppl. 2) (2016) S8–S11, <https://doi.org/10.1212/WNL.0000000000002877>.
- [2] R. van Toorn, J.F. Schoeman, R. Solomons, M.A. Rensburg, S.J. van Rensburg, Iron status in children with recurrent episodes of tumefactive cerebral demyelination, *J. Child Neurol.* 25 (2010) 1401–1407, <https://doi.org/10.1177/0883073810366179>.
- [3] H.B. Rasmussen, J. Clausen, Genetic risk factors in multiple sclerosis and approaches to their identification, *J. Neuro-Oncol.* 6 (Suppl. 2) (2000) 23–27.
- [4] C. Lucchinetti, W. Brück, J. Parisi, B. Scheithauer, M. Rodriguez, H. Lassmann, Heterogeneity of multiple sclerosis lesions: implications for the pathogenesis of demyelination, *Ann. Neurol.* 47 (2000) 707–717.
- [5] S.E. Hughes, O.M. Gray, Symptomatic therapy in multiple sclerosis: big pharma should do more - NO, *Mult. Scler.* 21 (2015) 978–979, <https://doi.org/10.1177/1352458515587930>.
- [6] T. Chitnis, D.L. Arnold, B. Banwell, W. Brück, A. Ghezzi, G. Giovannoni, B. Greenberg, L. Krupp, K. Rostásy, M. Tardieu, E. Waubant, J.S. Wolinsky, A. Bar-Or, T. Stites, Y. Chen, N. Putzki, M. Merschhemke, Gärtner J; PARADIG MS Study Group. Trial of Fingolimod versus Interferon Beta-1a in Pediatric Multiple Sclerosis, *N. Engl. J. Med.* 379 (2018) 1017–1027, <https://doi.org/10.1056/NEJMoa1800149>.
- [7] S.J. van Rensburg, M.J. Kotze, R. van Toorn, The conundrum of iron in multiple sclerosis - time for an individualised approach, *Metab. Brain Dis.* 27 (2012) 239–253, <https://doi.org/10.1007/s11011-012-9290-1>.
- [8] K. Popper, *Logik der Forschung*, Verlag von Julius Springer, Vienna, 1935.
- [9] T. Higgins, J.H. Eckfeldt, J.C. Barton, Doumas B.T. Hemoglobin, Iron and bilirubin, in: C.A. Burtis, E.R. Ashwood, D.E. Bruns (Eds.), *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*, Fifth edition, Elsevier Saunders, St Louis, 2012, p. 1007.
- [10] M.J. Kotze, J.N. de Villiers, R.N. Rooney, J.J. Grobelaar, E.P. Mansvelt, C.S. Bouwens, J. Carr, I. Stander, L. du Plessis, Analysis of the *NRAMP1* gene implicated in iron transport: association with multiple sclerosis and age effects, *Blood Cells Mol. Dis.* 27 (2001) 44–53.
- [11] T. Vollmer, J. Signorovitch, L. Huynh, P. Galebach, C. Kelley, A. DiBernardo, R. Sasane, The natural history of brain volume loss among patients with multiple sclerosis: a systematic literature review and meta-analysis, *J. Neurol. Sci.* 357 (2015) 8–18, <https://doi.org/10.1016/j.jns.2015.07.014>.
- [12] M.F. George, F.B. Briggs, X. Shao, M.A. Gianfrancesco, I. Kockum, H.F. Harbo, E.G. Celius, S.D. Bos, A. Hedström, L. Shen, A. Bernstein, L. Alfredsson, J. Hillert, T. Olsson, N.A. Patsopoulos, P.L. De Jager, A.B. Oturai, H.B. Söndergaard, F. Sellebjerg, P.S. Sorensen, R. Gomez, S.J. Caillier, B.A. Cree, J.R. Oksenberg, S.L. Hauser, S. D'Alfonso, M.A. Leone, F. Martinielli Boneschi, M. Sorosina, I. van der Meij, B.V. Taylor, Y. Zhou, C. Schaefer, L.F. Barcellos, Multiple sclerosis risk loci and disease severity in 7,125 individuals from 10 studies, *Neurol. Genet.* 2 (2016) e87, <https://doi.org/10.1212/NXG.0000000000000087> (eCollection 2016 Aug).
- [13] A. Van der Walt, J. Stankovich, M. Bahlo, B.V. Taylor, I.A. Van der Mei, S.J. Foote, J.P. Rubio, T.J. Kilpatrick, H. Butzkueven, Heterogeneity at the HLA-DRB1 allelic variation locus does not influence multiple sclerosis disease severity, brain atrophy or cognition, *Mult. Scler.* 17 (2011) 344–352, <https://doi.org/10.1177/1352458510389101>.
- [14] L.F. Barcellos, S. Sawcer, P.P. Ramsay, S.E. Baranzini, G. Thomson, F. Briggs, B.C. Cree, A.B. Begovich, P. Villoslada, X. Montalban, A. Uccelli, G. Savettieri, R.R. Lincoln, C. DeLoa, J.L. Haines, M.A. Pericak-Vance, A. Compston, S.L. Hauser, J.R. Oksenberg, Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis, *Hum. Mol. Genet.* 15 (18) (2006) 2813–2824.
- [15] International Multiple Sclerosis Genetics Consortium, Genome-wide association study of severity in multiple sclerosis, *Genes Immun.* 12 (2011) 615–625, <https://doi.org/10.1038/gene.2011.34>.
- [16] C.J. Jensen, J. Stankovich, A. Van der Walt, M. Bahlo, B.V. Taylor, I.A. van der Mei, S.J. Foote, T.J. Kilpatrick, L.J. Johnson, E. Wilkins, J. Field, P. Danoy, M.A. Brown, Australian and New Zealand Multiple Sclerosis Genetics Consortium (ANZgene), Rubio JP, Butzkueven H. Multiple sclerosis susceptibility-associated SNPs do not influence disease severity measures in a cohort of Australian MS patients, *PLoS One* 5 (2010) e10003, <https://doi.org/10.1371/journal.pone.0010003>.
- [17] W. Lundström, E. Greiner, F. Lundmark, H. Westerlind, C. Smestad, A.R. Lorentzen, I. Kockum, J. Link, B. Brynedal, E.G. Celius, H.F. Harbo, T. Masterman, J. Hillert, No influence on disease progression of non-HLA susceptibility genes in MS, *J.*

- Neuroimmunol. 237 (2011) 98–100, <https://doi.org/10.1016/j.jneuroim.2011.05.003>.
- [18] M. Rovaris, M. Filippi, Interventions for the prevention of brain atrophy in multiple sclerosis: current status, *CNS Drugs* 17 (2003) 563–575.
- [19] R. Hohlfeld, Gimme five: future challenges in multiple sclerosis. ECTRIMS Lecture 2009, *Mult. Scler.* 16 (2010) 3–14, <https://doi.org/10.1177/1352458509357355>.
- [20] M. Rodriguez, Have we finally identified an autoimmune demyelinating disease? *Ann. Neurol.* 66 (2009) 572–573, <https://doi.org/10.1002/ana.21838>.
- [21] J. Nakahara, M. Maeda, S. Aiso, N. Suzuki, Current concepts in multiple sclerosis: autoimmunity versus oligodendroglialopathy, *Clin. Rev. Allergy Immunol.* 42 (2012) 26–34, <https://doi.org/10.1007/s12016-011-8287-6>.
- [22] J.W. Prineas, J.D.E. Parratt, Multiple sclerosis: Serum anti-CNS autoantibodies, *Mult. Scler.* 24 (2018) 610–622, <https://doi.org/10.1177/1352458517706037>.
- [23] H. Lassmann, J. van Horsen, Oxidative stress and its impact on neurons and glia in multiple sclerosis lesions, *Biochim. Biophys. Acta* 1862 (2016) 506–510, <https://doi.org/10.1016/j.bbadis.2015.09.018>.
- [24] K.L. Munger, L.I. Levin, B.W. Hollis, N.S. Howard, A. Ascherio, Serum 25-hydroxyvitamin D levels and risk of multiple sclerosis, *JAMA.* 296 (2006) 2832–2838.
- [25] M.B. Sintzel, M. Rametta, A.T. Reder, Vitamin D and Multiple Sclerosis: a comprehensive review, *Neurol. Ther.* 7 (2018) 59–85, <https://doi.org/10.1007/s40120-017-0086-4>.
- [26] A.G. de la Fuente, O. Errea, P. van Wijngaarden, G.A. Gonzalez, C. Kerninon, A.A. Jarjour, H.J. Lewis, C.A. Jones, B. Nait-Oumesmar, C. Zhao, J.K. Huang, C. French-Constant, R.J. Franklin, Vitamin D receptor-retinoid X receptor heterodimer signaling regulates oligodendrocyte progenitor cell differentiation, *J. Cell Biol.* 211 (2015) 975–985, <https://doi.org/10.1083/jcb.201505119>.
- [27] R. McGregor, G. Li, H. Penny, G. Lombardi, B. Afzali, D.J. Goldsmith, Vitamin D in renal transplantation - from biological mechanisms to clinical benefits, *Am. J. Transplant.* 14 (2014) 1259–1270, <https://doi.org/10.1111/ajt.12738>.
- [28] R.R. Selzer, D.S. Rosenblatt, R. Laxova, K. Hogan, Adverse effect of nitrous oxide in a child with 5,10-methylenetetrahydrofolate reductase deficiency, *N. Engl. J. Med.* 349 (2003) 45–50.
- [29] R. Surtees, J. Leonard, S. Austin, Association of demyelination with deficiency of cerebrospinal-fluid S-adenosylmethionine in inborn errors of methyl-transfer pathway, *Lancet* 338 (1991) 1550–1554.
- [30] A. Miller, M. Korem, R. Almog, Y. Galboiz, Vitamin B12, demyelination, remyelination and repair in multiple sclerosis, *J. Neurol. Sci.* 233 (2005) 93–97.
- [31] N. Hibbits, R. Pannu, T.J. Wu, R.C. Armstrong, Cuprizone demyelination of the corpus callosum in mice correlates with altered social interaction and impaired bilateral sensorimotor coordination, *ASN Neurol.* 1 (2009), <https://doi.org/10.1042/AN20090032> (pii: e00013).
- [32] S. Martinez Sosa, K.J. Smith, Understanding a role for hypoxia in lesion formation and location in the deep and periventricular white matter in small vessel disease and multiple sclerosis, *Clin. Sci. (Lond)* 131 (2017) 2503–2524, <https://doi.org/10.1042/CS20170981>.
- [33] M.C. Nelson, F. Isaacs, M.S. Hassan, M. Kidd, F.J. Cronje, S.J. Van Rensburg, Prevalence of abnormal bloodflow patterns and effects of biochemistry and lifestyle factors on the major neck vessels in patients with Multiple Sclerosis in the Western Cape, *South Africa, Med. Technol. SA* 28 (2014) 28–33.
- [34] J.I. Spencer, J.S. Bell, G.C. DeLuca, Vascular pathology in multiple sclerosis: reframing pathogenesis around the blood-brain barrier, *J. Neurol. Neurosurg. Psychiatry* 89 (1) (2018) 42–52, <https://doi.org/10.1136/jnnp-2017-316011>.
- [35] D. Gemmati, G. Zerri, E. Orioli, F.E. De Gaetano, F. Salvi, I. Bartolomei, S. D'Alfonso, C. Dall'osso, M.A. Leone, A.V. Singh, R. Asselta, P. Zamboni, Polymorphisms in the genes coding for iron binding and transporting proteins are associated with disability, severity, and early progression in multiple sclerosis, *BMC Med. Genet.* 13 (2012) 70, <https://doi.org/10.1186/1471-2350-13-70>.
- [36] M.J. Kotze, J.N. de Villiers, L. Warnich, S. Schmidt, J. Carr, E. Mansvelt, E. Fourie, S.J. van Rensburg, Lack of clinical manifestation of hereditary haemochromatosis in South African patients with multiple sclerosis, *Metab. Brain Dis.* 21 (2006) 109–120.
- [37] J. Pakpoor, B. Seminatore, J.S. Graves, T. Schreiner, A.T. Waldman, T.E. Lotze, A. Belman, B.M. Greenberg, B. Weinstock-Guttman, G. Aen, J.M. Tillema, J.C. McDonald, J. Hart, J.M. Ness, Y. Harris, J. Rubin, M. Candee, L. Krupp, M. Gorman, L. Benson, M. Rodriguez, T. Chitnis, S. Mar, I. Kahn, J. Rose, S.L. Carmichael, S. Roalstad, M. Waltz, T.C. Casper, E. Waubant, US Network of Pediatric Multiple Sclerosis Centers. Dietary factors and pediatric multiple sclerosis: a case-control study, *Mult. Scler.* 24 (2018) 1067–1076, <https://doi.org/10.1177/1352458517713343>.
- [38] E. Herbert, P. Engel-Hills, C. Hattling, J.P. Fouché, M. Kidd, C. Lochner, M.J. Kotze, S.J. van Rensburg, Fractional anisotropy of white matter, disability and blood iron parameters in multiple sclerosis, *Metab. Brain Dis.* 33 (2018) 545–557, <https://doi.org/10.1007/s11011-017-0171-5>.
- [39] S. Syed, E.S. Michalski, V. Tangpricha, S. Chesdachai, A. Kumar, J. Prince, T.R. Ziegler, P.S. Suchdev, S. Kugathasan, Vitamin D status is associated with hepcidin and hemoglobin concentrations in children with inflammatory bowel disease, *Inflamm. Bowel Dis.* 23 (2017) 1650–1658, <https://doi.org/10.1097/MIB.0000000000001178>.
- [40] E.M. Smith, J.A. Alvarez, M.D. Kearns, L. Hao, J.H. Sloan, R.J. Konrad, T.R. Ziegler, S.M. Zughair, V. Tangpricha, High-dose vitamin D3 reduces circulating hepcidin concentrations: a pilot, randomized, double-blind, placebo-controlled trial in healthy adults, *Clin. Nutr.* 36 (4) (2017) 980–985, <https://doi.org/10.1016/j.clnu.2016.06.015>.
- [41] J.R. Connor, S.L. Menzies, Relationship of iron to oligodendrocytes and myelination, *Glia.* 17 (2) (1996 Jun) 83–93.
- [42] Y.S. Yoon, H.O. Byun, H. Cho, B.K. Kim, G. Yoon, Complex II defect via down-regulation of iron-sulfur subunit induces mitochondrial dysfunction and cell cycle delay in iron chelation-induced senescence-associated growth arrest, *J. Biol. Chem.* 278 (51) (2003 Dec) 51577–51586.
- [43] S. Yoshikawa, K. Muramoto, K. Shinzawa-Itoh, M. Mochizuki, Structural studies on bovine heart cytochrome c oxidase, *Biochim. Biophys. Acta* 1817 (2012) 579–589, <https://doi.org/10.1016/j.bbabi.2011.12.012>.
- [44] J.A. Stefely, D.J. Pagliarini, Biochemistry of mitochondrial coenzyme Q biosynthesis, *Trends Biochem. Sci.* 42 (10) (2017 Oct) 824–843, <https://doi.org/10.1016/j.tibs.2017.06.008>.
- [45] D. Moir, W.S. Rickert, G. Levasseur, Y. Larose, R. Maertens, P. White, S. Desjardins, A comparison of mainstream and sidestream marijuana and tobacco cigarette smoke produced under two machine smoking conditions, *Chem. Res. Toxicol.* 21 (2) (2008 Feb) 494–502.
- [46] A. Armon-Omer, C. Waldman, N. Simaan, H. Neuman, S. Tamir, R. Shahien, New insights on the nutrition status and antioxidant capacity in Multiple Sclerosis patients, *Nutrients* 11 (2) (2019 Feb 18), <https://doi.org/10.3390/nu11020427> pii: E427.
- [47] C.C. Constantine, L.C. Gurrin, C.E. McLaren, M. Bahlo, G.J. Anderson, C.D. Vulpe, S.M. Forrester, K.J. Allen, D.M. Gertig, HealthIron Investigators, SNP selection for genes of iron metabolism in a study of genetic modifiers of hemochromatosis, *BMC Med. Genet.* 9 (2008) 18, <https://doi.org/10.1186/1471-2350-9-18>.
- [48] van Rensburg SJ, Kotze MJ, Hon D, Haug P, Kuyler J, Hendricks M, Botha J, Potocnik FC, Matsha T, Erasmus RT. Iron and the folate-vitamin B12-methylation pathway in Multiple Sclerosis. *Metab. Brain Dis.* 2006;21:121–37.
- [49] N. van der Merwe, A.V. Peeters, F.M. Pienaar, J. Bezuidenhout, S.J. van Rensburg, M.J. Kotze, Exome sequencing in a family with luminal-type breast cancer underpinned by variation in the methylation pathway, *Int. J. Mol. Sci.* 18 (2) (2017), <https://doi.org/10.3390/ijms18020467> pii: E467.
- [50] F.E. Dewey, R. Chen, S.P. Cordero, K.E. Ormond, C. Caleshu, K.J. Karczewski, M. Whirl-Carrillo, M.T. Wheeler, J.T. Dudley, J.K. Byrnes, O.E. Cornejo, J.W. Knowles, M. Woon, K. Sangkuhl, L. Gong, C.F. Thorn, J.M. Hebert, E. Capriotti, S.P. David, A. Pavlovic, A. West, J.V. Thakuria, M.P. Ball, A.W. Zaranek, H.L. Rehm, G.M. Church, J.S. West, C.D. Bustamante, M. Snyder, R.B. Altman, T.E. Klein, A.J. Butte, E.A. Ashley, Phased whole-genome genetic risk in a family quartet using a major allele reference sequence, *PLoS Genet.* 7 (9) (2011) e1002280, <https://doi.org/10.1371/journal.pgen.1002280> (Epub 2011 Sep 15).
- [51] S.J. van Rensburg, R. van Toorn, K.E. Moremi, A.V. Peeters, A. Oguniyi, M.J. Kotze, Multiple sclerosis-like diagnosis as a complication of previously treated malaria in an iron and vitamin D deficient Nigerian patient, *Metab. Brain Dis.* 31 (1) (2016 Feb) 197–204, <https://doi.org/10.1007/s11011-015-9788-4>.
- [52] J. Field, S.R. Browning, L.J. Johnson, P. Danoy, M.D. Varney, B.D. Tait, K.S. Gandhi, J.C. Charlesworth, R.N. Heard, Australia and New Zealand Multiple Sclerosis Genetics Consortium, G.J. Stewart, T.J. Kilpatrick, S.J. Foote, M. Bahlo, H. Butzkueven, J. Wiley, D.R. Booth, B.V. Taylor, M.A. Brown, J.P. Rubio, J. Stankovich, A polymorphism in the HLA-DPB1 gene is associated with susceptibility to multiple sclerosis, *PLoS One* 5 (10) (2010) e13454, <https://doi.org/10.1371/journal.pone.0013454>.
- [53] P. Delbini, V. Vaja, G. Graziadei, L. Duca, I. Nava, C. Refaldi, M.D. Cappellini, Genetic variability of TMPRSS6 and its association with iron deficiency anaemia, *Br. J. Haematol.* 151 (2010) 281–284, <https://doi.org/10.1111/j.1365-2141.2010.08349.x>.
- [54] J.C. Chambers, W. Zhang, Y. Li, J. Sehmi, M.N. Wass, D. Zabaneh, C. Hoggart, H. Bayle, M.I. McCarthy, L. Peltonen, N.B. Freimer, S.K. Srivastava, P.H. Maxwell, M.J. Sternberg, A. Ruokonen, G. Abecasis, M.R. Jarvelin, J. Scott, P. Elliott, J.S. Koener, Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels, *Nat. Genet.* 41 (2009) 1170–1172, <https://doi.org/10.1038/ng.462>.
- [55] B. Benyamini, M.A. Ferreira, G. Willemsen, S. Gordon, R.P. Middelberg, B.P. McEvoy, J.J. Hottenga, A.K. Henders, M.J. Campbell, L. Wallace, I.H. Frazer, A.C. Heath, E.J. de Geus, D.R. Nyholt, P.M. Visscher, B.W. Penninx, D.I. Boomsma, N.G. Martin, G.W. Montgomery, J.B. Whitfield, Common variants in TMPRSS6 are associated with iron status and erythrocyte volume, *Nat. Genet.* 41 (2009) 1173–1175, <https://doi.org/10.1038/ng.456>.
- [56] P.L. Lee, C. Halloran, R. Trevino, V. Felitti, E. Beutler, Human transferrin G277S mutation: a risk factor for iron deficiency anaemia, *Br. J. Haematol.* 115 (2001) 329–333.
- [57] C.E. McLaren, S. McLachlan, C.P. Garner, C.D. Vulpe, V.R. Gordeuk, J.H. Eckfeldt, P.C. Adams, R.T. Acton, J.A. Murray, C. Leidencker-Foster, B.M. Snively, L.F. Barcellos, J.D. Cook, G.D. McLaren, Associations between single nucleotide polymorphisms in iron-related genes and iron status in multiethnic populations, *PLoS One* 7 (2012) e38339, <https://doi.org/10.1371/journal.pone.0038339>.
- [58] C.C. Constantine, G.J. Anderson, C.D. Vulpe, C.E. McLaren, M. Bahlo, H.L. Yeap, D.M. Gertig, N.J. Osborne, N.A. Bertalli, K.B. Beckman, V. Chen, P. Matak, A.T. McKie, M.B. Delatycki, J.K. Olynyk, D.R. English, M.C. Southery, G.G. Giles, J.L. Hopper, K.J. Allen, L.C. Gurrin, A novel association between a SNP in CYBRD1 and serum ferritin levels in a cohort study of HFE hereditary haemochromatosis, *Br. J. Haematol.* 147 (2009) 140–149, <https://doi.org/10.1111/j.1365-2141.2009.07843.x>.
- [59] R. Kozyraki, J. Fyfe, P.J. Verroust, C. Jacobsen, A. Dautry-Varsat, J. Gburek, T.E. Willnow, E.I. Christensen, S.K. Moestrup, Megalin-dependent cubilin-mediated endocytosis is a major pathway for the apical uptake of transferrin in polarized epithelia, *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001) 12491–12496.
- [60] A. Nykjaer, J.C. Fyfe, R. Kozyraki, J.R. Leheste, C. Jacobsen, M.S. Nielsen, P.J. Verroust, M. Aminoff, A. de la Chapelle, S.K. Moestrup, R. Ray, J. Gliemann, T.E. Willnow, E.I. Christensen, Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3), *Proc. Natl. Acad. Sci. U. S. A.* 98 (2001)

- 13895–13900.
- [61] S. Strope, R. Rivi, T. Metzger, K. Manova, E. Lacy, Mouse amnionless, which is required for primitive streak assembly, mediates cell-surface localization and endocytic function of cubilin on visceral endoderm and kidney proximal tubules, *Development* 131 (2004) 4787–4795.
- [62] E.T. Christenson, A.S. Gallegos, A. Banerjee, In vitro reconstitution, functional dissection, and mutational analysis of metal ion transport by mitoferrin-1, *J. Biol. Chem.* 293 (2018) 3819–3828, <https://doi.org/10.1074/jbc.M117.817478>.
- [63] D.J. Lane, A.M. Merlot, M.L. Huang, D.H. Bae, P.J. Jansson, S. Sahni, D.S. Kalinowski, D.R. Richardson, Cellular iron uptake, trafficking and metabolism: key molecules and mechanisms and their roles in disease, *Biochim. Biophys. Acta* 1853 (2015) 1130–1144, <https://doi.org/10.1016/j.bbamer.2015.01.021>.
- [64] T. Garton, R.F. Keep, Y. Hua, G. Xi, CD163, a hemoglobin/haptoglobin scavenger receptor, after intracerebral hemorrhage: functions in microglia/macrophages versus neurons, *Transl. Stroke Res.* 8 (2017) 612–616, <https://doi.org/10.1007/s12975-017-0535-5>.
- [65] M. Madsen, J.H. Graversen, S.K. Moestrup, Haptoglobin and CD163: captor and receptor gating hemoglobin to macrophage lysosomes, *Redox Rep.* 6 (2001) 386–388.
- [66] K. Moremi, S.J. van Rensburg, L.R. Fisher, W. Davis, F.J. Cronje, M. Jalali Sefid Dashti, J. Gamielidien, D. Geiger, M. Rensburg, van Toorn, M.J. de Klerk, G.M. Hon, T. Matsha, S. Hassan, R.T. Erasmus, M. Kidd, M.J. Kotze, Association of an iron-related genetic variant TMPRSS6 c.2207C > T (rs855791), with functional iron deficiency and its effect on multiple sclerosis risk in the South African population, *S. Afr. J. Psychiatry* 19 (2013) 120–121.
- [67] M. Aminoff, J.E. Carter, R.B. Chadwick, C. Johnson, R. Gräsbeck, M.A. Abdelaal, H. Broch, L.B. Jenner, P.J. Verroust, S.K. Moestrup, A. de la Chapelle, R. Krahe, Mutations in CUBN, encoding the intrinsic factor-vitamin B12 receptor, cubilin, cause hereditary megaloblastic anaemia 1, *Nat. Genet.* 21 (1999) 309–313.
- [68] NCBI dbSNP SLC25A37, Accessed 14/03/2019 https://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?searchType=ad hoc_search&type=rs&rs=rs3736032.
- [69] B.A. Ackrell, J.J. Maguire, P.R. Dallman, E.B. Kearney, Effect of iron deficiency on succinate- and NADH-ubiquinone oxidoreductases in skeletal muscle mitochondria, *J. Biol. Chem.* 259 (1984) 10053–10059.
- [70] R. Dutta, J. McDonough, X. Yin, J. Peterson, A. Chang, T. Torres, T. Guduz, W.B. Macklin, D.A. Lewis, R.J. Fox, R. Rudick, K. Mirnics, B.D. Trapp, Mitochondrial dysfunction as a cause of axonal degeneration in multiple sclerosis patients, *Ann. Neurol.* 59 (2006) 478–489.
- [71] A.P. Wojtovich, C.O. Smith, C.M. Haynes, K.W. Nehrke, P.S. Brookes, Physiological consequences of complex II inhibition for aging, disease, and the mKATP channel, *Biochim. Biophys. Acta* 1827 (5) (2013) 598–611, <https://doi.org/10.1016/j.bbabo.2012.12.007>.
- [72] W. Davis, S.J. van Rensburg, F.J. Cronje, L. Whati, L.R. Fisher, L. van der Merwe, D. Geiger, M.S. Hassan, T. Matsha, R.T. Erasmus, M.J. Kotze, The fat mass and obesity-associated FTO rs9393609 polymorphism is associated with elevated homocysteine levels in patients with multiple sclerosis screened for vascular risk factors, *Metab. Brain Dis.* 29 (2014) 409–419, <https://doi.org/10.1007/s11011-014-9486-7>.
- [73] J.F. Kurtzke, Rating neurologic impairment in multiple sclerosis: an expanded disability status scale (EDSS), *Neurology*. 33 (1983) 1444–1452.
- [74] C.W. Adams, Perivascular iron deposition and other vascular damage in multiple sclerosis, *J. Neurol. Neurosurg. Psychiatry* 51 (1988) 260–265.
- [75] S. Sheykhsari, K. Kozielski, J. Bill, M. Sitti, D. Gemmati, P. Zamboni, A.V. Singh, Redox metals homeostasis in multiple sclerosis and amyotrophic lateral sclerosis: a review, *Cell Death Dis.* 9 (3) (2018 Mar 1) 348, <https://doi.org/10.1038/s41419-018-0379-2>.
- [76] X. Zhao, S. Song, G. Sun, R. Strong, J. Zhang, J.C. Grotta, J. Aronowski, Neuroprotective role of haptoglobin after intracerebral hemorrhage, *J. Neurosci.* 29 (2009) 15819–15827, <https://doi.org/10.1523/JNEUROSCI.3776-09.2009>.
- [77] A. Márquez, M.C. Cénit, C. Núñez, J.L. Mendoza, C. Taxonera, M. Díaz-Rubio, M. Bartolomé, R. Arroyo, M. Fernández-Arquero, E.G. de la Concha, E. Urcelay, Effect of BSN-MST1 locus on inflammatory bowel disease and multiple sclerosis susceptibility, *Genes Immun.* 10 (2009) 631–635, <https://doi.org/10.1038/gene.2009.56>.
- [78] F. Gilli, N.D. Navone, S. Perga, F. Marnetto, M. Caldano, M. Capobianco, A. Pulizzi, S. Malucchi, A. Bertolotto, Loss of braking signals during inflammation: a factor affecting the development and disease course of multiple sclerosis, *Arch. Neurol.* 68 (2011) 879–888, <https://doi.org/10.1001/archneurol.2011.32>.
- [79] M.C. Gjelstrup, M. Stilund, T. Petersen, H.J. Møller, E.L. Petersen, T. Christensen, Subsets of activated monocytes and markers of inflammation in incipient and progressed multiple sclerosis, *Immunol. Cell Biol.* 96 (2018) 160–174, <https://doi.org/10.1111/imcb.1025>.
- [80] A. Dolei, The aliens inside us: HERV-W endogenous retroviruses and multiple sclerosis, *Mult. Scler.* 24 (2018) 42–47, <https://doi.org/10.1177/1352458517737370>.
- [81] V. Yankovskaya, R. Horsefield, S. Törnroth, C. Luna-Chavez, H. Miyoshi, C. Léger, B. Byrne, G. Cecchini, S. Iwata, Architecture of succinate dehydrogenase and reactive oxygen species generation, *Science* 299 (5607) (2003) 700–704.
- [82] G.G. van Dooren, L.M. Stimmler, G.I. McFadden, Metabolic maps and functions of the Plasmodium mitochondrion, *FEMS Microbiol. Rev.* 30 (4) (2006 Jul) 596–630.
- [83] A.M. Awad, M.C. Bradley, L. Fernández-Del-Río, A. Nag, H.S. Tsui, C.F. Clarke, Coenzyme Q10 deficiencies: pathways in yeast and humans, *Essays Biochem.* 62 (3) (2018 Jul 20) 361–376, <https://doi.org/10.1042/EBC20170106> (Print 2018 Jul 20).
- [84] A.K. Hibi, Y. Gao, S. Hekimi, Ubiquinone is necessary for *Caenorhabditis elegans* development at mitochondrial and non-mitochondrial sites, *J. Biol. Chem.* 277 (2002) 2202–2206.
- [85] M.H. Barnett, A.P. Henderson, J.W. Prineas, The macrophage in MS: just a scavenger after all? Pathology and pathogenesis of the acute MS lesion, *Mult. Scler.* 12 (2006) 121–132.
- [86] R.N. Rooney, M.J. Kotze, J.N. de Villiers, R. Hillermann, J.A. Cohen, Multiple sclerosis, porphyria-like symptoms, and a history of iron deficiency anemia in a family of Scottish descent, *Am. J. Med. Genet.* 86 (2) (1999) 194–196.
- [87] S. Magalhaes, B. Banwell, A. Bar-Or, I. Fortier, H.E. Hanwell, M. Lim, G.E. Matt, R.F. Neuteboom, D.L. O'Riordan, P.K. Schneider, M. Pugliatti, B. Shatenstein, C.M. Tansey, E. Wassmer, C. Wolfson, A framework for measurement and harmonization of pediatric multiple sclerosis etiologic research studies: The Pediatric MS Tool-Kit, *Mult. Scler.* (2018 Jun 1) 1352458518783345, <https://doi.org/10.1177/1352458518783345> [Epub ahead of print].