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

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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, desferroxamine 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; MGA1, 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

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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) demyelinaton 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 (SAMe) 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, SAMe 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 SAMe 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 ProtonTM 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	*TMPRSS6*	rs855791, 2207C > T, V736A	HET	WT	*TMPRSS6* 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]
		rs2543519, 1223 + 66 T > C	HET	HOM	
Iron transport	*TF*	rs1130459, −2 G > A	HET	HOM	*TF* variants have previously been associated with decreased iron concentrations [56,57], including rs1880669 [58]
		rs1880669, 1298–23 T > C	HET	HOM	
Iron loss	*CUBN*	rs74431427, 9986C > T, S3329 L	WT	HET	*CUBN* encodes cubulin. 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].
		rs557482398, 8773 A > G, T2925A	HET	WT	
		rs41289305, 5803 A > G, S1935G	HET	HET	
		rs2271462, 5518 G > A, G1840S	WT	HET	
		rs116114483, 5098 G > A, D1700N	WT	HET	
		rs7905349, 2188C > T, H730Y	HET	HET	
Iron import into mitochondria	*SLC25A37*	rs3736032, 287 G > A, R96Q	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	*CD163*	rs61729512, 2702C > T, T901 M	HET	WT	*CD163* encodes a receptor on macrophages that endocytose iron from ruptured erythrocytes. [64] [65]
		rs567350690, 1939 A > G, R647G	WT	HOM	
Coenzyme Q10 synthesis	*COQ3*	rs7764793, 486 + 35A > C	HET	WT	*COQ3* encodes an *O*-methylase that forms part of a complex that synthesises CoQ in mitochondria [44]. rs11548336 encodes a missense variant, K134E
		rs11548336, A > G K134E	HET	HOM	
		rs9376137, −1G > T	WT	HET	

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 detection 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 desferroxamine 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 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 $CoQH_2$, 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 Horssen (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 (sCD163) 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 exretion 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.

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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.

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