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REVIEW ARTICLE

Pathology-supported genetic testing as a method for disability prevention in multiple sclerosis (MS). Part II. Insights from two MS cases

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Abstract

In Part I of this Review we evaluated the scientific evidence for a Metabolic Model of multiple sclerosis (MS). Part II outlines the implementation of an adaptive pathology-supported genetic testing (PSGT) algorithm aimed at preventing/reversing disability in two illustrative MS cases, starting with a questionnaire-based risk assessment, including family history and lifestyle factors. Measurement of iron, vitamin B12, vitamin D, cholesterol and homocysteine levels identified biochemical deficits in both cases. Case 1, after following the PSGT program for 15 years, had an expanded disability status scale (EDSS) of 2.0 (no neurological sequelae) together with preserved brain volume on magnetic resonance imaging (MRI). A novel form of iron deficiency was identified in Case 1, as biochemical testing at each hospital submission due to MS symptoms showed low serum iron, ferritin and transferrin saturation, while hematological status and erythrocyte sedimentation rate measurement of systemic inflammation remained normal. Case 2 was unable to walk unaided until her EDSS improved from 6.5 to 4.0 over 12 months after implementation of the PSGT program, with amelioration of her suboptimal biochemical markers and changes to her diet and lifestyle, allowing her to regain independence. Genotype-phenotype correlation using a pathway panel of functional single nucleotide variants (SNVs) to facilitate clinical interpretation of whole exome sequencing (WES), elucidated the underlying metabolic pathways related to the biochemical deficits. A cure for MS will remain an elusive goal if separated from nutritional support required for production and maintenance of myelin, which can only be achieved by a lifelong investment in wellness.

Keywords Multiple sclerosis · Pathology-supported genetic testing (PSGT) · Disability prevention · Nutritional reserve · Biochemical markers · Whole exome sequencing (WES)

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Introduction

Multiple sclerosis (MS) is a demyelinating disorder of the central nervous system (CNS) that causes disruption of signal transmission from the brain to peripheral nerves that may lead to disability. The diagnosis of MS is based on clinical criteria, as well as white matter dysfunction disseminated in time and space, the radiological McDonald diagnostic criteria for MS (Thompson et al. 2018). The gold standard for measuring MS-related disability is the Expanded Disability Status Scale (EDSS) that quantifies disease severity (0 to 10), with higher values denoting worsening disability (Kurtzke 1983). The EDSS is also a useful tool to monitor changes in the level of disability over time. It is widely used in clinical trials and in the assessment of people with MS-related disability.

Many questions concerning MS-related disability are still unanswered. In Part 1 of this Review we proposed a Metabolic Model, developed using a pathology-supported genetic testing (PSGT) method, to investigate differences in disability progression in people with MS (pwMS). MS is widely regarded as a progressive neurodegenerative disorder; however, evidence collected over 20 years led us to conclude that personalized lifestyle factors are important determinants of disability outcomes in MS. The PSGT Model hypothesizes that by maintaining adequate nutritional reserves, through provision of biochemical building blocks essential for myelin maintenance and regeneration, disability may be prevented or even reversed. PSGT identifies risk factors for demyelination and the data obtained is utilized in personalized treatment programs. Such a systematically collected database may provide valuable information that can be validated in large patient cohorts.

In Part II of this Review presented here, our proposed Model for prevention and reversal of disability in MS was demonstrated in two test Cases. PSGT incorporating whole exome sequencing (WES) of two pediatric MS patients, previously indicated that clinical laboratory science, including genetic and biochemistry tests, elucidated metabolic pathways related to myelination, including iron metabolism (van Toorn et al. 2010; van Rensburg et al. 2019). In the present study, we describe 2 adult females with relapsing-remitting MS (RRMS) who were similarly investigated and treated. The differing radiological-neurological profile of the two Cases highlights the potential benefit PSGT may offer in reducing MS-related disability.

Methods

Ethics

The study was approved by the Human Research Ethics Committee (HREC) of the University of Stellenbosch (N07/09/203). Both participants gave written informed consent for study participation and for the publication of the case reports.

Diet questionnaire

The Gknowmix health questionnaire (Davis et al. 2014) was completed for both participants at baseline and follow-up. This questionnaire is a screening tool that determines the individual's typical intake of saturated/trans fats, fruit and vegetables, fibre and folate in common and easily available foods in 7 days over a period of 3 months. Alcohol intake is also scored with the questionnaire.

Biochemical analysis and personalized nutritional intervention

Full blood count, iron parameters, ferritin, C-reactive protein (CRP), lipid profile, serum folate, vitamin B12 as well as vitamin D levels were determined as previously described (Davis et al. 2014; van Rensburg et al. 2016). The selection of the biochemical tests was determined by the PSGT protocol (Table 1). The need for supplementation was guided by Decision (Optimal) Levels (Tables 1 and 2) and personalized according to individual requirements. Preferred blood levels, also known as Optimal Health Reference Ranges (Travica et al. 2015), Decision Levels, or Values for Optimal Myelination (*VFOM*), were deemed clinically more relevant for MS than laboratory reference ranges which are less useful for clinical decision-making (Travica et al. 2015).

The proposed optimal level of vitamin B12 should be >500 pmol/l (Chandy 2019; Mitsuyama and Kogoh 1988) and vitamin D 40 ng/ml (100 nmol/l) (Munger et al. 2006; Bischoff-Ferrari et al. 2006; Holick 2007). The nutrients to be taken daily to promote iron absorption and myelin maintenance are listed in Table 2 (adapted from van Rensburg et al. 2006). The two cases were followed up to check that their values remained within the *VFOM* range (Table 3).

MRI

Magnetic resonance imaging (MRI) was performed on a Siemens Skyra 3-Tesla scanner at the Cape Universities Body Imaging Centre at Cape Town University, South Africa. MRI 3D was used to measure gyral volume. In addition, midsagittal MRI was used to measure the thickness of the corpus callosum (Figueira et al. 2007), which has been shown to be a surrogate marker of cerebral white matter volume (Andronikou et al. 2015) (Fig. 1).

Genetic studies

Genetic studies consisted of a pathway panel of functional single nucleotide variants (SNVs) to facilitate clinical

Table 1 Biochemical tests for determining personalized intervention in people diagnosed with MS.

	Laboratory reference values	*Decision values (VFOM)*	References
1. **Iron parameters.**			
Hemoglobin (g/dL)	7.4–10; 12.0–15.0	*12.5–15.0*	Weiss et al. 2019
Serum iron (μmol/L)	10.0–30.0	*12.5–30.0*	Van Rensburg et al. 2012
Transferrin (Tf) (g/L)	2.0–3.60	*2.0–3.60*	Van Toorn et al. 2010
Tf saturation %	15–30	*22–35*	Herbert et al. 2018
Ferritin (μg/L)	10–120	*40–200*	Van Rensburg et al. 2006
2. **Methylation metabolism**			
Vitamin B12 (pmol/L); (pg/ml)	156–698; 200–700	*500–1000*	Chandy 2019; Travica et al. 2015
Serum folate (nmol/L)	10.4–42.4	*> 45*	Serapinas et al. 2017; Iso et al. 2004
Homocysteine (μmol/L)	2.1–15.7	*5–7*	Oliveira et al. 2018; Teunissen et al. 2008
3. **Inflammation**			
C-reactive protein (CRP) (mg/L)	0.0–5.0	*0.0–5.0*	
Vitamin D (nmol/L); (ng/mL)	25–75; 10–30	*99.1; 40–100*	Holick 2007; Munger et al. 2006
4. **Lipid metabolism**			
Total cholesterol (mmol/L); (mg/dL)	< 5; < 200	*< 5; < 200*	Zhornitsky et al. 2016

Units are given as (SI Units); (C Units)

VFOM Values for optimal myelination

interpretation of whole exome sequencing (WES) (Davis et al. 2014; van Rensburg et al. 2016). WES was performed and variants were annotated by wANNOVAR (http://wannovar.wglab.org/) and prioritised based on their role in iron and methylation metabolism, by using an in-house pipeline of clinically relevant genes selected from the approximately 20,000 human genes. Mean exome-wide depth of coverage was at least 136x and uniformity was >93%. The genotype and coverage average of all variants reported were verified in the integrative genomics viewer (IGV) 2.8.13 tool. Real-time polymerase chain reaction (PCR)-based analysis, using haplotype tagging by rs9271366, was employed to investigate the presence of the *HLA-DRB1*1501* allele associated with increased risk for MS (Field et al. 2010).

Outcome measures

The EDSS was used to quantify the degree of MS-related disability and to monitor changes in the level of disability over time. It is widely used in clinical trials in the assessment of pwMS. The EDSS scale ranges from 0 to 10 in 0.5 unit increments that represent higher levels of disability. Scoring is based on measures of impairment in eight functional systems (FS): pyramidal, cerebellar, brainstem, sensory, bowel and bladder function, visual function, cerebral functions and other systems involved. Each FS is scored on a scale of 0 (no disability) to 5 or 6 (more severe disability).

Case descriptions

Case 1

This 61-year-old female was diagnosed in 2003 with RRMS at the age of 46 when she presented with paresthesia and loss of sensation/weakness of the left leg. Her family history includes cardiovascular disease, Parkinson's disease and schizophrenia and a distant relative with MS. Risk factors of MS included childhood anemia, cigarette smoking till the age of 40 years and heavy menstrual bleeding which led to a hysterectomy.

Initial treatment of the RRMS consisted of high dose corticosteroids as well as Interferon beta-1b. The latter was discontinued after 3 months due to severe adverse effects. She also voluntarily enrolled in a pilot study which investigated the role of iron deficiency in MS. At the time her iron parameters were low (Table 3) and she started taking iron supplements, together with nutrients to promote iron absorption and myelin maintenance (van Rensburg et al. 2006). Later, vitamin D 500 IU per day was added to the regimen. Over the next 14 years (2004–2018) she experienced clinical relapses (fatigue, paresthesias and muscle weakness) and during this time serial MRI scans (18 in total) with gadolinium demonstrated an increase in the number of white matter lesions. Of note was that radiological gadolinium enhancement was only evident on two occasions (2007 and 2012). Preservation of age-appropriate brain volume was reported in 2005, 2007, 2011, 2016, 2017, and in 2018 (Fig. 1). Her EDSS score in 2018 was 2.0 which implies no neurological

Table 2 List of nutrients taken daily to prevent disability progression (The Rapha Regimen) (van Rensburg et al. 2006)

	Nutrient	Recommended amounts
• Iron	Iron as supplements, injections or IV infusions under appropriate medical protocols [a]	Amount required determined by blood tests. If iron values are normal or high no iron is taken.
• Important Vitamins	Vitamin B12. Can be taken as injections or sublingual tablets if values are low.	Amount required determined by blood tests. Maintenance amount: 50 μg /day [b]
	Vitamin D3. During allergies/infections higher amounts may be required[c]	Amount required determined by blood tests. Maintenance amount: 500 IU /day [d]
• Lipids	**Salmon Oil**	1000 mg
	EPA	180 mg
	DHA	120 mg
	Evening Primrose Oil	500 mg
	GLA	45 mg
	LA	350 mg
	Lecithin (Choline)	300 mg
• Amino acids		30 ml/day as a protein shake
• Multivitamin-multiminerals at Recommended Dietary Allowance amounts.		
• Antioxidant supplements are helpful if dietary intakes of fruits and vegetables are suboptimal.		

[a] DeLoughery 2019; [b] Del Bo' et al. 2019; [c] Bartosik-Psujek and Psujek 2019; [d] Munger et al. 2004

sequelae (Table 3). She has lived an independent life without neurological disability (despite frequent exacerbations) and has remained on the PSGT program. Her iron levels remained in the normal range as long as she took iron; however, whenever she discontinued iron supplementation her serum iron dropped and she experienced exacerbations during those times. Table 4 summarises her iron status from 2015 to 2018. During this time period she had 5 episodes of recurring MS

Table 3 Clinical characteristics of the two Cases at baseline and follow-up, including EDSS, Biochemistry and Diet Scores

	CASE 1		CASE 2			
Dates of study entry and follow-up	2003	2018	2018	2019	*Decision levels VFOM*	Reference Range
EDSS	0	2	**6.5**	4.0	*0–4*	0–10
Hemoglobin (g/dL)	12.7	12.7	13.4	12.7	*12.5–15.0*	12.0–15.0
Serum iron (μmol/L)	**11.4**	15.7	14.6	**11.6**	*12.5–30.0*	10.0–30.0
Transferrin (Tf)	3.25	3.14	2.73	2.12	*2.0–3.60*	2.0–3.60
Tf saturation (%)	**15.6**	**21**	**21**	**22**	*25–35*	15–30
Ferritin (μg/L)	70.8	127	82	91	*40–200*	10–120
Vitamin B12 (pmol/L)	ND	810	**236**	661	*500–1000*	156–698
Serum folate (nmol/L)	ND	45.4	**20.7**	45.4	*> 45*	10.4–42.4
Homocysteine (μmol/L)	ND	**8.8**	**13.8**	5.9	*5.0–7.0*	2.1–15.7
CRP (mg/L)	ND	**10.7**	4	1	*0.0–5.0*	0.0–5.0
Cholesterol (mmol/L)	ND	**5.67**	**5.81**	**5.39**	*< 5*	< 5
Vitamin D (ng/mL)	ND	**23.6**	**37.3**	**38.32**	*40–50*	10–30
Saturated/Trans Fat score	ND	15	**38**	8	*< 16*	NA
Folate score	ND	15	**2**	**10**	*> 14*	NA
Fruit/veg/fibre score	ND	**9**	**5**	**7**	*> 19*	NA
5 Fruits/veg (d/w)	ND	**4**	**0**	**4**	*7*	NA

VFOM Values for optimal myelination. Values in bold denote results that are lower or higher than the decision levels

ND Not Determined, *NA* Not Applicable, *EDSS* Expanded Disability Status Scale (Kurtzke 1983)

symptoms, which led to hospitalisation with MRI investigations together with gadolinium contrast to gauge whether she was having relapses. However, no contrast enhancement was recorded over this time period, while brain volume was reported to be normal for age in 2016 and 2017, and confirmed in 2018 (Fig. 1).

Her iron biochemistry is of interest, since it represents a novel type of iron deficiency. With each hospital admission for MS symptoms, her serum iron, ferritin and transferrin (Tf) saturation values were low, while her hematological status and erythrocyte sedimentation rate (ESR, a measure of systemic inflammation) remained normal (Table 4).

Case 2

A 52 year old female was diagnosed at the age of 37 years (2004) with RRMS, following symptoms that included paresthesia, heat intolerance, fatigue, muscle spasms in the back and weakness of the left hand (poor grip which led to dropping of objects). There was a family history of diabetes, hypertension and cancer. Risk factors of MS included 23 years of cigarette smoking.

Initial treatment of the RRMS consisted of high dose corticosteroids as well as Interferon beta-1b. The latter was discontinued due to financial constraints. Four years after the MS diagnosis her EDDS was noted as 3.0. Five years thereafter she experienced a relapse (intermittent urge incontinence, ataxia and right lower limb spasticity) which necessitated a plasma exchange and intravenous methylprednisolone. At the time the EDDS was noted as 3.0–3.5. Blood investigations revealed a low serum iron concentration (10.2 μmol/l) with vitamin B12 levels noted as "normal" according to the laboratory reference range. Supplements administered at the time included vitamin D, calcium and thiamine (without the addition of iron or vitamin B12). Four years later her EDSS had worsened to 6.5 (+3) and she was not able to walk unaided or drive a car.

A further relapse experienced 7 months later required hospitalization and immune modulation with high dose intravenous methylprednisolone. Blood investigations at the time revealed an insufficient Tf saturation (21%) and low vitamin B12 (236 pmol/L) together with high cholesterol (5.81 mmol/l) and homocysteine (13.8 μmol/l) levels (Table 3). The Gknowmix questionnaire revealed that her

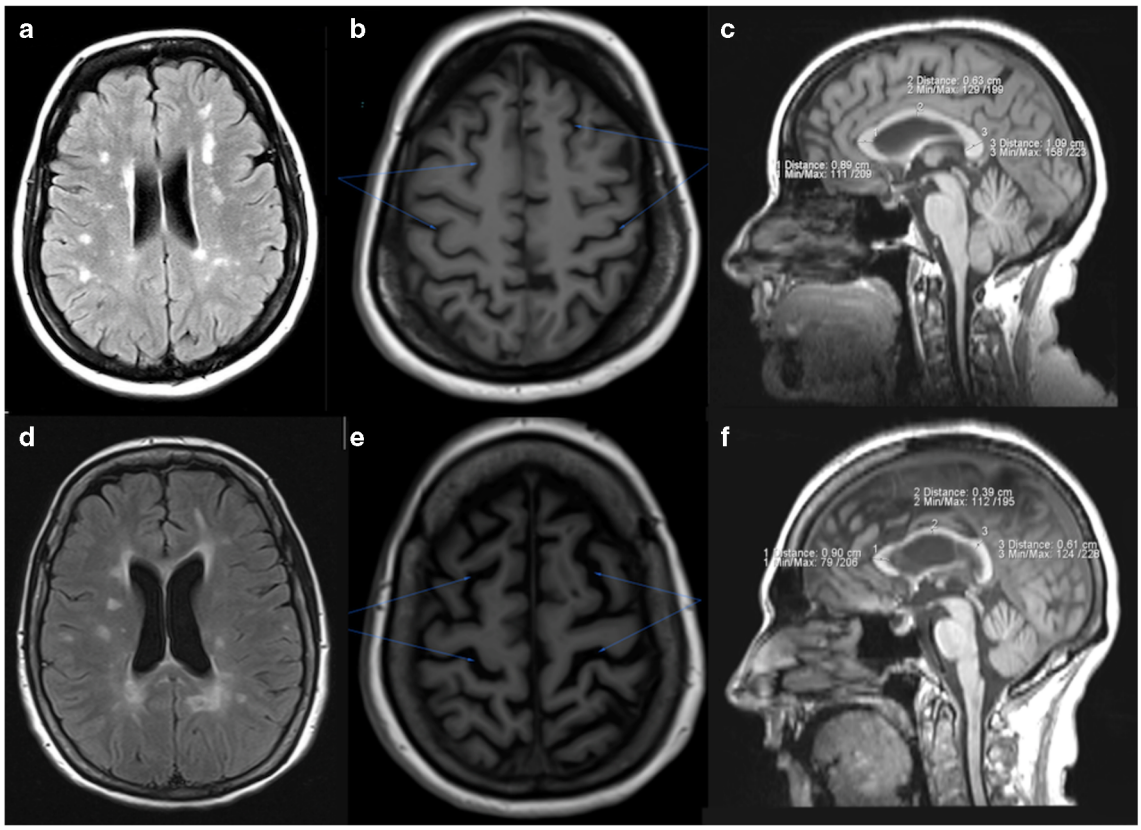

Fig. 1 MRI results of Case 1 (a, b and c) and Case 2 (d, e and f).a and d: MRI Axial T2 FLAIR at the level of the lateral ventricles showing multiple hyperintense periventricular white matter lesions which led to a diagnosis of MS in both Cases. b and e: MRI 3D T1 axial measurements of brain volumes: Case 1 (b) exhibits preserved sulcal proportions and gyral volume 15 years after MS diagnosis. Case 2 (e): arrows point to severe sulcal dilatation and reduction of gyral volume 13 years after diagnosis of MS. c and f: Corpus callosum thickness. Case 2 (f) shows diffuse thinning of the corpus callosum compared to Case 1 (c)

Table 4 Serum iron deficiency recorded during MS flare-ups in Case 1

Hematology and Iron parameters	Reference Range	*VFOM*	May 2015	Feb 2016	Oct 2016	Aug 2017	Apr 2018	Aug 2018
Red Cell Count	3.7–5.3 (×10^12/L)	*3.7–5.3 (×10^12/l)*	4.29	4.26	4.63	4.28	4.32	4.30
Hemoglobin	11.5–15.5 (g/dL)	*12.5–15.0* (g/dL)	12.7	13.1	13.8	12.8	12.8	12.8
Hematocrit	0.35–0.45 (L/L)	*0.35–0.45 (L/L)*	0.39	0.39	0.42	0.39	0.39	0.38
MCV	81–96 (fL)	*81–96 (fL)*	91	92	90	91	90	89
MCH	28–35 (pg)	*28–35 (pg)*	30	31	30	30	30	30
MCHC	32–37 (g/dL)	*32–37 (g/dL)*	33	33	33	33	33	33
RDW	10–15 (%)	*10–15 (%)*	13.6	12.9	13.8	13.4	13.8	13.9
Serum Iron	10.7–32.2 (μmol/L)	*12.5–30.0* (μmol/L)	**10.6**	**9.6**	**7.3**	**7.4**	**11.8**	**8.0**
Transferrin	2.00–3.60 (g/L)	*2.0–3.60* (g/L)	3.6	3.50	3.16	3.26	2.76	3.44
Tf saturation	16–50 (%)	*25–35* (%)	**12**	**11**	**9**	**9**	**17**	**9**
Ferritin	20–300 (μg/L)	*40–200* (μg/L)	**39**	**39**	**39**	**26**	55	**39**
ESR	2–39 (mm/h)	*2–39 (mm/h)*	ND	ND	16	17	23	24
CKD-EPI eGFR	>90 (ml/min/1.73 m2)	*>90 (ml/min/1.73 m2)*	84	77	87	82	95	87

VFOM Values for optimal myelination. Low values are in bold. *MCV* Mean cell volume, *MCH* Mean cell hematocrit, *MCHC* Mean corpuscular hemoglobin concentration, *RDW* Red cell distribution width, *ESR* Erythrocyte sedimentation rate. CKD-EPI eGFR (Chronic Kidney Disease Epidemiology Collaboration glomerular filtration rate) – values are normal when adjusted for age

dietary pattern was sub-optimal, showing high saturated/trans fat and low folate intake. She rarely ate fruits and vegetables, with no days per week of intake of at least five different fruits and/or vegetables (Table 3). Her BMI was 32 kg/m^2, indicating obesity.

Following counseling, she consented to following the PSGT lifestyle program which included dietary modification and nutrient supplementation (Rapha Regimen outlined in Table 2). In addition, she received monthly vitamin B12 injections. She gradually transitioned to a predominantly plant-based diet, low in saturated/trans fat. These interventions resulted in a decrease in her BMI to 27 kg/m^2. Her EDSS was re-tested in February 2019, and she now had a score of 4.0. This enabled her to walk unaided and to drive again. A repeat EDSS assessment in November 2019 confirmed the score of 4.0. The restored driving abilities markedly improved her functional ability and quality of life (Dickerson et al. 2011), confirming that the improvement in the EDSS was not merely an artefact of measurement (Ontaneda and Cohen 2012). Notably, her bladder function also improved markedly. When her EDSS stood at 6.5 she had experienced frequent bouts of nocturnal micturition, which ceased when her EDSS improved to 4.0 allowing her to sleep undisturbed. She was also able to perform outdoor activities such as shopping without fear of urgency incontinence.

Results

The clinical data of the two cases at baseline and follow-up are summarized in Table 3.

Case 1 hematology, iron status and ESR during 2015–2018 is shown in Table 4. Non-hematological iron deficiency was recorded during hospital admissions for recurring MS symptoms.

MRI findings

Figure 1 shows MRI Axial T2 FLAIR with multiple hyperintense periventricular white matter lesions of Case 1 (a) and 2 (d) respectively, confirming diagnosis of MS. MRI 3D T1 axial and T1 mid sagittal corpus callosum thickness measurements were employed to evaluate brain volumes. Preserved sulcal proportions, gyral volume (b) and corpus callosum thickness (c) was noted in Case 1, 15 years after MS diagnosis. In contrast, case 2 exhibited severe sulcal dilatation, reduction of gyral volume (e) and diffuse thinning of the corpus callosum (f) supportive of severe reduction in brain volume, 13 years after MS diagnosis. At the time of the brain volume measurements, Case 1 had an EDSS of 2.0, while the EDSS of Case 2 was 6.5. Images c and f show sagittal MRI's of the corpus callosum thickness in Case 1 and Case 2 respectively. Measurements were done using the best mid-sagittal T1W image of the genu, midbody and splenium. Case 1: genu 0.89 cm, midbody 0.63 cm, and splenium 1.09 cm. Case 2 genu 0.90, midbody 0.39 cm and splenium 0.61 cm, indicating a more severe reduction in corpus callosum thickness in Case 2 compared to Case 1. The annotation indicating min/max on the image refers to the signal intensity.

Genetic studies

Case 1 is heterozygous for *HLA-DRB1*1501* while Case 2 is a wildtype for this allele determined by tag SNV rs9271366. WES revealed several genetic variants in metabolic pathways involved in iron metabolism and the folate-vitamin B12 methylation pathway. Five of the variants found in the two adult cases were similar to those found in the two children with MS (van Rensburg et al. 2019), namely *CUBN* rs41289305 and rs7905349, *TMPRSS6* rs855791 and rs2543519, and *SLC25A37* rs3736032 (Table 5).

Discussion

In Part II of this Review two representative cases were tested to evaluate the PSGT Metabolic Model outlined in Part I, which included biochemical tests, EDSS assessments, brain volume differences on MRI, as well as genetic studies. The results confirmed that the goals of "stopping/reversing disability", outlined in Part I, could be achieved by implementing the PSGT method (Table 6). Case 1 remained stable at an EDSS of 2.0 for more than 15 years, thus qualifying as "benign MS" (Banwell et al. 2013), while Case 2 improved from an EDSS of 6.5 to 4.0 in 12 months which was sustained when assessed again 9 months later. According to MRI (Fig. 1) and clinical history, correct diagnosis could be confirmed for both pwMS, i.e. misdiagnosis could be excluded. Case 2 could have experienced "natural innate improvements" (Tremlett et al. 2012) due to lifestyle changes including smoking cessation, higher physical activity, personalized supplementation and weight loss by following a plant-based, low saturated/trans fat diet. The optimal diet for MS may be a Mediterranean-style diet, as suggested by a recent randomized clinical trial (RCT) that found a significant improvement in the EDSS in those pwMS following the diet which encourages intake of fresh fruits, vegetables, whole grains and food containing poly- and monounsaturated fats such as fish instead of saturated/trans fats from meat, cream and processed foods (Katz Sand et al. 2019). However, we have consistently found that dietary intervention alone is not sufficient to address disability in MS, since metabolic deficiencies of iron and vitamin B12 would

Table 5 Genetic variants in iron- and methylation metabolic pathways found in Case1 and Case 2 using whole exome sequencing (WES)

Gene	rs number	DNA change	Protein change	Case 1	Case 2	Effect
IRON METABOLISM						
CUBN	rs1801222	c.758 T>C	p.F253S	HET	.	Missense
	rs78201384	c.910G>A	p.E304K	HET	.	Missense
	rs1801224	c.1165C>A	p.P389T	HET	.	Missense
	rs1801231	c.4675C>T	p.P1559S	HET	.	Missense
	rs62619939	c.6459G>C	p.L2153F	HET	HET	Missense
	rs41289305	c.5803A>G	p.S1935G	.	HET	Missense
	rs7905349	c.2188C>T	p.H730Y	.	HET	Missense
	rs1801239	c.8950A>G	p.I2984V	.	HET	Missense
	rs1801240	c.9005A>G	p.E3002G	.	HET	Missense
IRON DEFICIENCY						
TMPRSS6	rs855791	c.2207C>T	p.V736A	HET	HET	Missense
	rs2235324	c.757A>G	p.K253E	HET	HET	Missense
	rs200332661	c.2101C>T	p.H701Y	HET	.	
	rs2543519	c.1262 T>C	p.I421T	HET	.	
	rs60484081	c.1869-31CCCCA[5]		HET	HET	Indel in Splice Acceptor
IRON IMPORT INTO MITOCHONDRIA						
SLC25A37	rs2942194	c.259A>G	p.I87V	HOM	.	Missense
	rs3736032	c.287G>A	p.R96Q	.	HET	Missense
METHYLATION METABOLISM						
MTHFR	rs1801131	c.1298A>C	p.E429A	.	HET	Missense
MTR	rs1805087	c.2756A>G	p.D919G	HET	.	Missense
MTRR	rs1801394	c.66A>G	p.I22M	.	HET	Missense
	rs162036	c.1049A>G	p.K350R	HET	HET	Missense
	rs10380	c.1783C>T	p.H595Y	HET	HET	Missense

HET Heterozygous, *HOM* Homozygous (risk-associated allele)

Table 6 The Pathology Supported Genetic Testing (PSGT) Protocol

All pwMS entering the PSGT study were previously diagnosed by their respective neurologists according to clinical criteria and MRI scans (Thompson et al. 2018). At enrollment, the participants are provided with all the information about the study approved by the Human Research Ethics Committee (HREC) of the University of Stellenbosch (N07/09/203) so that they can make an informed decision about whether they want to follow the protocol. They can select to proceed with steps 1–6 based on the Informed Consent approved for the PSGT Program:

1. To provide data about their medical and family history, their lifestyle, including physical activity, smoking/alcohol intake and diet. They sign informed consent for their data to be included in the Gknowmix database. They will then receive feedback about how to improve their diet and lifestyle free of charge.
2. To have the blood tests listed in Table 1, to find out whether there are deficits in any of the key biochemical pathways. The results are then combined with the lifestyle data, as shown in Table 3, and information is provided on how to ameliorate the deficits (Table 2).
3. To give consent for genetic tests. The tests are listed in the Demyelinating Diseases Genescreen at www.gknowmix.com and include SNVs involved in the metabolic pathways of iron regulation, methylation (vitamin B12/folate/homocysteine), lipids/lipoproteins, hemostasis/thrombophilia, as well as chronic inflammation related to obesity, hypertension, insulin resistance, detoxification, oxidative stress, dopamine/estrogen and drug metabolism. The results of the genetic tests provide explanations for the biochemical deficits revealed by the blood tests, as well as inform further testing using next generation genetic tests, such as whole exome sequencing (WES).
4. To have an assessment on entering the study by a clinician using the Expanded Disability Status Scale (EDSS) (Kurtzke 1983) to provide a baseline value for disability. This value can then be compared during follow-up assessments to evaluate study outcomes and address problems encountered. The EDSS provides an objective measure of improvement or regression to inform future inputs.
5. To evaluate reasons for relapse through follow-up assessments as required. These include blood tests to monitor biochemical changes (improvements/regressions if any), administering the health questionnaire to check for changes in the diet, lifestyle, and personal health as well as monitoring supplementation intake, i.e. adequate amounts at the correct time. Relapses could also occur as a result of infections/inflammation or severely stressful life events. The data are recorded for future reference. It is important to note that although the biochemical tests are not absolutely pathognomonic since the results may vary, they may be used to direct treatment.
6. To return research results to the referring clinician using relevant data entered in the research database which were extracted semi-automatically into adaptable reports using the Gknowmix research translation tool (Kotze et al. 2013). Patient reports are authorized for disclosure of actionable results by registered medical scientists as part of a multidisciplinary team responsible for the dissemination of genetic results.

Cases 1 and 2 are illustrative examples of the practical implementation of the PSGT Protocol.

Timeline for Case 1:

2003: Diagnosis with MS at age 46 and inclusion in the research study. At that time, low iron parameters were identified (Table 3). She started taking iron supplements as part of the Rapha Regimen (van Rensburg et al. 2006). Her EDSS was 0.

2004–2018: Clinical relapses were experienced every time she discontinued iron; however, she recovered fully when resuming iron supplementation and her blood iron parameters increased (Table 3). Her brain volume remained normal for age.

2018: Her iron levels were within the Decision Levels range, and her EDSS was 2.0. The increase of 2 EDSS points over 15 years is negligible (benign MS; normal neurological test over 15 years).

Timeline for Case 2:

2004: Diagnosis with MS at age 37.

2018: Entry into the research study. Her EDSS was 6.5, indicating that she could not walk without support and she could not drive. Her iron levels were within the Decision Levels range, but vitamin B12 and serum folate levels were low, with elevated homocysteine (Table 3). The diet questionnaire recorded high saturated/trans fat and low folate intake, and 0 days of eating >5 portions of fruits and vegetables. Her BMI was 32 kg/m^2. Following discussion of the results with Case 2, she started having monthly vitamin B12 injections, changed to a plant-based low saturated fat diet and the Rapha Regimen without iron (Table 2) since her iron values were within the Decision Levels range at that time. At the follow-up assessment, her vitamin B12, serum folate and homocysteine levels were within the Decision Levels range, although no significant change in cholesterol, vitamin D, and Tf saturation levels and a decrease in serum iron levels were recorded. Iron and higher vitamin D supplementation were therefore initiated.

February 2019: Follow-up EDSS was 4.0. This enabled her to walk unaided and to drive again, while her bladder function improved allowing her to sleep undisturbed.

November 2019: The EDSS of 4.0 was sustained.

Case 1 is an example of someone who has to be particularly aware of maintaining her iron levels. Case 2 showed a marked improvement in EDSS when her vitamin B12 and folate levels were restored and homocysteine decreased. After iron supplementation was additionally initiated, the EDSS of 4.0 was maintained. These perceptions are confirmed by the genetic variants detected in these two Cases, especially iron-related variants in Case 1, which may impede iron absorption and import into mitochondria, and heterozygosity of *MTRR* rs1801394 in Case 2, which is associated with decreased capacity to reduce oxidized cobalamin (Olteanu et al. 2002).

arise if meat is eliminated from the diet. On the other hand, modified Paleolithic and modified Ketogenic diet-interventions have shown variable outcomes of MS symptoms but not significant disability improvements (Bisht et al. 2017; Irish et al. 2017; Lee et al. 2020), suggesting that saturated fat intake from meat or coconut oil may have adverse effects in pwMS who have high cholesterol levels. Therefore it seems reasonable to combine an individualized supplementation program following biochemical tests with a low saturated fat diet. The need for supplementation in the present study was thus guided by Optimal/Decision Levels (Tables 1 and 2) and personalized according to individual requirements. Follow-up should ideally be at 6-month intervals, and during relapses. Relapses/flare-ups may be triggered by (a) a change in blood level markers such as iron (Table 4) or vitamin B12 (Chandy 2019); or (b) an allergy/infection necessitating a higher level of vitamin D which prevents excessive inflammation (Bartosik-Psujek and Psujek 2019); or (c) intake of saturated

fat (Swank 1991), which is associated with elevated cholesterol (Zhornitsky et al. 2016), atherosclerosis, hypoperfusion and hypoxia (Roher et al. 2011; Martinez Sosa and Smith 2017).

As seen from these two Case Reports, pwMS may differ regarding their biochemical deficits, rendering the discovery of a universal biomarker for MS unlikely. Vitamin D levels were suboptimal in both cases. Both Case 1 and Case 2 had low iron parameters at diagnosis and further variable iron levels. Case 2 also had a low vitamin B12 level, as well as a very low folate intake in her diet as revealed by the Diet Score (Table 3), which resulted in a homocysteine level of 13.8 μmol/l. Either iron or vitamin B12/folate deficiencies can inhibit oligodendrocyte maintenance, since iron metabolism and the folate-vitamin B12-methylation pathway converge in the mitochondria to sustain energy production in all brain cells, including oligodendrocytes and neurons, outlined in Part I of this Review (van Rensburg et al. 2021).

According to Travica et al. (2015) blood concentrations should reflect levels required for optimal health rather than minimal levels to avoid disease. Vitamin B12 values within "normal" levels may not be sufficient to provide methylation capacity for remyelinating the axons. This may be due to the limitations of the serum vitamin B12 test that measures the total amount of cobalamin in the blood; however, only about 10–30% thereof is "active B12" (B12 bound to transcobalamin), the only form that is internalyzed by cells via receptor-mediated uptake (Harrington 2017). The rest is bound to haptocorrin, which is biologically inert. The serum vitamin B12 assay does not discriminate between these two forms, so that the test may overestimate the amount of vitamin B12 available for metabolism (Harrington 2017; Chandy 2019). In addition, microwave cooking may cause a loss of 30–40% of vitamin B12 activity (Watanabe et al. 1998). Furthermore, oxidation of the cobalt in vitamin B12 inactivates the vitamin (Bratman and Harkness 2000; Selzer et al. 2003). The function of methionine synthase reductase (MTRR) is to constantly reduce the oxidized cobalt; however, some genetic variants of MTRR such as A66G are associated with impaired reduction capacity (Olteanu et al. 2002). Smoking in addition significantly reduces the amount of active vitamin B12 relative to total serum B12 (Shekoohi et al. 2017). Thus values that fall within the reference range may be interpreted by clinicians as being "normal" when in fact they are biologically inert and therefore insufficient for myelination or for remyelination during a relapse. Therefore, while the vitamin B12 standard reference range is 200–700 pg/ml, the proposed optimal level of vitamin B12 should be at least 500 pg/ml (Chandy 2019; Mitsuyama and Kogoh 1988; Hvas and Nexo 2006). Optimal vitamin B12 levels increase cognitive function and decrease brain atrophy, confusion, weakness and depression (Travica et al. 2015). In a randomised clinical trial with B vitamins in patients with mild cognitive impairment, treatment resulted in a significant reduction in the rate of brain atrophy (Smith et al. 2010) and with a reduction, by as much as seven fold, of atrophy in grey matter regions (Douaud et al. 2013). This finding is also important for MS, since disability worsening in MS is significantly associated with the atrophy rate of the deep grey matter (Eshaghi et al. 2018). Vitamin B deficiency may be treated with injections or sublingual tablets (Chandy 2019). Iron parameters need to be carefully evaluated in pwMS to ensure personalized intervention. If iron status is low, iron may be taken as supplements, preferably designed to prevent gastrointestinal discomfort. Intravenous iron infusions are safe when administered according to medical protocols (DeLoughery 2019). Dietary factors such as ascorbic acid or calcium (e.g. in milk) may enhance or inhibit iron absorption respectively (Abbaspour et al. 2014). Not only do iron levels vary as a result of circadian rhythms (van Rensburg et al. 2012), but they are also influenced by genetic variations and dietary intake (van Rensburg et al. 2019). The iron status tests requested by clinicians often only include hemoglobin and ferritin. However, as is shown in Table 4, the iron parameter that is most useful in demonstrating the true condition of iron insufficiency in MS is the Tf saturation, which should be at least 20% (Phatlhane et al. 2016), and preferably 25%, since it determines the amount of iron available to the brain for myelin synthesis, as demonstrated by an inverse association ($p = 0.05$) of Tf saturation with the EDSS (Herbert et al. 2018). The non-anemic iron deficit in MS represents a novel type of iron deficiency in that hemoglobin synthesis is prioritised. Even when serum iron parameters are low, hemoglobin levels tend to remain within the normal range (but will eventually decrease after prolonged iron deficiency). This raises the question as to whether the CNS hypoxia primarily associated with MS (Martinez Sosa and Smith 2017) could alter the balance of iron metabolism, such that iron availability is directed towards erythrocyte production in reticulocytes to prioritize oxygen delivery to the brain, to the detriment of iron delivery to oligodendrocytes when iron levels are insufficient. The classic physiological response to hypoxia is increased red cell production orchestrated by hypoxia-inducible factors (HIFs) (Haase 2013) and differs from "anemia of inflammation" (Ganz 2019). This imbalance of iron in MS, underpinned by brain hypoxia, may possibly explain why iron deficiency does not always lead to MS. The biochemical results of Case 1 are similar to those described by van Toorn et al. (2010) of two children

diagnosed with MS who had non-anemic iron deficiency whenever iron supplementation was discontinued.

Vitamin D also plays an important role in MS. Munger et al. (2006) discovered in the Nurses' Health Study, that vitamin D blood levels were inversely associated with the risk of MS diagnosis; levels higher than 99.1 nmol/l (40 ng/ml) significantly decreased MS risk (OR 0.38, $P = 0.006$), and that the relative risk (RR) comparing women with intake of vitamin D supplements of more than 400 IU/day with women with no supplemental intake was 0.59 (p for trend = 0.006). Vitamin D intake from food was not associated with MS risk (Munger et al. 2004).

Brain volume differences in the two cases reflected the EDSS values (Fig. 1). While brain volume is preserved for age in Case 1, atrophy is evident in Case 2. This is an illustration of how the use of the PSGT method allows for preserving brain volume in MS. Case 1 followed the Rapha Regimen outlined in Table 2 since 2003 when she participated in the original MS pilot study (van Rensburg et al. 2006), demonstrating that even flare-ups and relapses do not necessarily lead to increased disability (Confavreux et al. 2000). Case 1 avoided disability progression because her clinicians prescribed iron for her every time iron deficiency was detected during the flare-ups she experienced. The optimization of iron together with the other nutrients of the Rapha Regimen enabled remyelination of the damaged axons by the oligodendrocyte precursor cells (OPCs), the designated stem cells resident in the brain (Barnett and Prineas 2004; van Rensburg 2014). Case 2 joined the Program when her EDSS was 6.5. After changing her diet and lifestyle, and following the PSGT program for 12 months, her EDSS improved to 4.0, allowing her to regain independence through improved walking as well as the ability to resume driving. Since the atrophy rate of the deep grey matter is significantly associated with the EDSS (Eshaghi et al. 2018), future MRIs would have to be performed to ascertain whether the improvement in the EDSS in Case 2 was due to resolution of white matter lesions, improvement in brain volume, or whether it was due to cognitive reserve (Fenu et al. 2018).

The genetic studies revealed that Case 1 is heterozygous for *HLA-DRB1*1501* while Case 2 is a wildtype for this allele as determined by tag SNV rs9271366. It has been shown that this immune-related allele increases the risk of MS diagnosis about three times, but does not predict disability (Field et al. 2010; Van der Walt et al. 2011). A study by Yates et al. (2015) found that *HLA-DRB1*15* status has a significant association with inflammation in autopsy samples in the MS motor cortex. Inflammation has a suppressive effect on iron absorption through hepcidin function in pwMS, outlined in Part I of this Review (van Rensburg et al. 2021), which may suggest a connection between *HLA-DRB1*1501* and iron in the Metabolic Model. This study also highlights the positive role that comprehensive genetic tests such as WES can play to elucidate the underlying reasons for biochemical deficits in pwMS. The WES analysis indicated that the two adult Cases had variations in the same genes, but not necessarily the same SNVs. Similar to the results of the two children with MS (van Rensburg et al. 2019) most variations related to iron metabolism were found in the *CUBN* gene, followed by *TMPRSS6* and *SLC25A37*. *CUBN* codes for cubilin, a 46 kDa receptor that is involved in vitamin B12 absorption, as well as the re-uptake of iron, vitamin B12 and vitamin D in the kidney. Exley et al. (2006) found significantly increased iron excretion in pwMS, which needs to be further investigated due to the suggested link between *CUBN* dysfunction and iron loss (van Rensburg et al. 2019). *CUBN* variants may also impact vitamin D signaling in MS (Pytel et al. 2019). The polymorphisms in *TMPRSS6* detected are associated with reduced absorption of iron from the gut, while *SLC25A37* variants impede iron transport into mitochondria, which could have caused mitochondrial iron deficiency in these two pwMS. The genetic variation in several genes in the iron metabolism pathway in Case 1, could have contributed to the flare-ups she experienced when she became iron deficient.

Genetic variations were also detected in metabolic pathways involving vitamin B12 metabolism, methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*) and methionine synthase reductase (*MTRR*). These genes are related to impaired synthesis of phosphatidylcholine which is required for myelin synthesis, necessitating a higher intake of choline (lecithin) (Ganz et al. 2016). Variants in *MTRR* (especially rs1801394; 66A > G) have been associated with severely defective vitamin B12 function due to impaired reduction of oxidized cobalt (Olteanu et al. 2002), suggesting that these variants could have played a role in the susceptibility of Case 2 to vitamin B12 deficiency and that the improvement in her EDSS could be attributed primarily to the increase in vitamin B12, serum folate and decreased homocysteine and her improved dietary Folate Score.

Limitations and strengths of the study

Study limitations include the small sample size (2 case reports), lack of ability to generalize, no possibility to establish cause-effect relationship and the retrospective design. In addition, the follow-up period in case 2 is short. Although the functional significance of all the variants detected in Case 1 and 2 was not previously demonstrated by in vivo studies, the clinical relevance of the metabolic pathways underpinned by genetic variation is undisputed.

The strength of the study is that multiple data were available in both these cases, facilitating integration and evaluation using the PSGT approach which enabled us to derive these conclusions. To our knowledge, there are few studies that have utilized biochemical data to correlate with EDSS outcomes and MRI.

Conclusions

The PSGT Metabolic Model demonstrates that a diagnosis of MS is not a catastrophe, but rather a wake-up call for a paradigm shift towards taking personal responsibility for maintenance of brain cells. Case 1 illustrates that even flare-ups and relapses can be viewed positively as an opportunity for restoration of the myelin network with improved functioning by providing adequate nutrition for remyelination by the OPCs. A lack of nutritional reserve could result in cell death, demyelination and inhibition of signal transmission. Case 2 confirms that restoration of function is possible when the necessary building blocks for remyelination are provided. The PSGT Model therefore predicts that a cure for MS is an unlikely goal if separated from a lifelong investment in wellness, since oligodendrocytes and OPCs will always require nutrients, such as iron and methylation capacity to produce and maintain myelin. Disability in MS may occur due to neglected maintenance of the power stations (mitochondria) in the oligodendrocytes, the factories that synthesize myelin. Since mitochondria are the providers of energy to all brain cells including neurons, restoration of energy production may also lead to improved neural function and less MS-related fatigue. Future studies are warranted to investigate the non-anemic iron deficiency observed in Case 1 and be expanded to explore the benefit of the PSGT Model utilizing more cases.

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Code availability Not Applicable.

Authors' contributions SJVR, RVT, RTE and CJH wrote the manuscript. AEZ, PEH, FCVP, AAK and MJK revised the manuscript. MJK and SJVR designed the PSGT Method. CJH designed the specifications for MRI. CJH and MJ interpreted the MRI data. CJ did clinical and EDSS assessments. LW designed the Diet Questionnaire and reviewed the manuscript. MCK collected the biochemical and lifestyle data. KEM performed genetic testing and evaluated the results. AVP and CJVH evaluated the genetic data and reviewed the manuscript. All authors approved the final manuscript. The two last authors contributed equally in supervising the study.

Data availability Not Applicable.

Declarations

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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.

Ethics approval The study was approved by the Human Research Ethics Committee (HREC) of the University of Stellenbosch (N07/09/203).

Consent to participate Both participants gave written informed consent for study participation.

Consent for publication Both participants gave written informed consent for the publication of the case reports.

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