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Received: 21 November 2023 | Revised: 22 February 2024 | Accepted: 4 March 2024

DOI: 10.1111/jns.12620

RESEARCH REPORT

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A 21-bp deletion in the complement regulator *CD55* promotor region is associated with multifocal motor neuropathy and its disease course

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Abstract

Background and Aims: To further substantiate the role of antibody-mediated complement activation in multifocal motor neuropathy (MMN) immunopathology, we investigated the distribution of promotor polymorphisms of genes encoding the membrane-bound complement regulators CD46, CD55, and CD59 in patients with MMN and controls, and evaluated their association with disease course.

Methods: We used Sanger sequencing to genotype five common polymorphisms in the promotor regions of *CD46*, *CD55*, and *CD59* in 133 patients with MMN and 380 controls. We correlated each polymorphism to clinical parameters.

Results: The genotype frequencies of rs28371582, a 21-bp deletion in the *CD55* promotor region, were altered in patients with MMN as compared to controls (*p* .009; Del/Del genotype 16.8% vs. 7.7%, *p* .005, odds ratio: 2.43 [1.27–4.58]), and patients carrying this deletion had a more favorable disease course (mean difference 0.26 Medical Research Council [MRC] points/year; 95% confidence interval [CI]: 0.040–0.490, *p* .019). The presence of *CD59* rs141385724 was associated with less severe pre-diagnostic disease course (mean difference 0.940 MRC point/year; 95% CI: 0.083–1.80, *p* .032).

Interpretation: MMN susceptibility is associated with a 21-bp deletion in the *CD55* promotor region (rs2871582), which is associated with lower CD55 expression. Patients carrying this deletion may have a more favorable long-term disease outcome. Taken together, these results point out the relevance of the pre-C5 level of the complement cascade in the inflammatory processes underlying MMN.

KEYWORDS
CD55, complement system, DAF, genetics, multifocal motor neuropathy

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1 | INTRODUCTION

Multifocal motor neuropathy (MMN) is a rare, asymmetric, immune-mediated motor neuropathy that mostly affects the relatively young to middle-aged. Its natural history is characterized by progressive muscle weakness, particularly in distal muscle groups.[1–4] Although MMN responds to immunoglobulin treatment, approximately 20% of patients develop debilitating loss of hand and arm function.[1] Patient characteristics associated with the heterogeneity in MMN severity are largely unknown.[1,4,5]

The finding of nerve conduction block, thickening of peripheral nerves on ultrasound or MR imaging, and the presence of circulating IgM antibodies against the ganglioside GM1 are characteristic of MMN.[5–8] These antibodies activate the classical pathway of the complement cascade in solid phase immunoassays and after binding to induced pluripotent stem cell-derived motor neurons.[9–11] Higher anti-GM1 IgM antibody titers, increased complement deposition triggered by anti-GM1 IgM in vitro and higher innate activity of the classical pathway of complement are all associated with more severe muscle weakness.[8,9,11] These findings support the hypothesis that activation of complement is central in the inflammatory processes underlying MMN.

The contribution of variation in innate complement regulation in MMN has not been studied. Complement activation is tightly regulated at the fluid and tissue levels, among others by membrane-bound complement regulatory proteins (mCRPs), including CD46, CD55, and CD59.[12,13] These proteins play an important role in avoiding tissue damage through unchecked complement activation, as exemplified by the association of loss of CD55 with complement hyperactivation, angiopathic thrombosis, and protein-losing enteropathy, and by the loss of CD59 with paroxysmal nocturnal hemoglobinuria, and early-onset chronic axonal neuropathy, stroke and hemolysis.[14–16] Common polymorphisms in the promotor region of these mCRPs lead to variation between individuals in transcriptional activity and cellular mCRP expression. In general, higher mCRP expression will lead to higher thresholds for complement activity, and vice versa. The biological relevance of mCRP promotor polymorphisms is shown by their associations with inflammatory diseases.[17–21]

To further our understanding of the role of complement regulation in MMN immunopathology, we analyzed common polymorphisms in the promotor regions of *CD46*, *CD55*, and *CD59* in MMN patients and controls and determined their association with MMN susceptibility and its disease course.

2 | MATERIALS AND METHODS

2.1 | Study population

All patients with MMN were diagnosed and enrolled at the outpatient clinic of the University Medical Center Utrecht (UMCU), a tertiary neuromuscular referral center and national center for MMN. All patients fulfilled the most recent diagnostic criteria for definite, probable, or possible MMN.[22] These criteria rely on the combination of a typical clinical phenotype combined with conduction block found on nerve conduction studies or, in the absence of conduction block, on abnormal ancillary investigations and/or a response to treatment with immunoglobulins.

Dutch population-based controls were enrolled through the Prospective ALS study The Netherlands (PAN), a population-based case–control study performed in the UMCU.[23] Control subjects did not have a motor neuron disorder, and those enrolled between January 2012 and August 2018 were included in this study.

2.2 | Clinical data

We used the UMCU MMN database to extract clinical data for patients with MMN.[1–4] When necessary, we supplemented these data with the most recent data from the UMCU patient files.

Recorded baseline characteristics included sex, age at onset, diagnostic delay, treatment with IVIg, the presence of anti-GM1 IgM antibodies, and muscle strength testing on the first and last visit to the UMCU outpatient clinic.

We defined the onset of disease as the time of the first complaint of muscle weakness and diagnostic delay as the time between onset and diagnosis. We documented the presence or absence of anti-GM1 IgM antibodies as described previously.[24] At the first visit and at the last follow-up visit to the UMCU, we quantified muscle strength using the 6-point Medical Research Council (MRC) scale. The MRC scale ranges between 0 (no contraction) and 5 (normal muscle strength against resistance). MRC scores were documented for left and right shoulder abduction, elbow flexion and extension, wrist flexion and extension, finger flexion and extension, finger spreading, hip flexion, knee flexion and extension, and foot dorsal and plantar flexion. We calculated an MRC sum score (MRCss) by summation of the MRC scores of all tested muscle groups (range 0–130) for the first and last visit to our hospital.

2.3 | DNA samples

We used standard DNA isolation methods to extract genomic DNA from whole blood obtained during two national studies on MMN performed in 2007 and 2015.[1] We used control DNA samples that were obtained upon participation in the PAN study.

2.4 | Genotyping

We used two techniques to determine the presence of five *CD46*, *CD55*, and *CD59* promotor region variants, which were chosen because of both their common presence in healthy individuals (i.e., with a minor allele frequency [MAF] of at least 20%) and their previously described association with disease.

First, in patients with MMN only, we used the previously described Sanger sequencing methodology to genotype five common

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polymorphisms that have previously been found associated with disease or disease outcome.[17,18] Next, we designed a second primer set to specifically target the polymorphisms identified in patients with MMN to genotype the control cohort. These primers and PCR programs are summarized in Table S1. We determined optimal annealing temperatures using a temperature gradient PCR. For *CD46* rs2796267 and rs2796268, *CD55* rs28371583, and *CD59* rs141385724, we amplified genomic DNA by PCR, purified the PCR using Sephadex and subsequently performed Sanger sequencing.[25] We validated the sequencing result using eight MMN samples of which we had obtained the full promotor region sequence of *CD46*, *CD55*, and *CD59*. Results matched in all samples.

We genotyped *CD55* rs28371582 by gel electrophoresis of the PCR product. Since rs28371582 is a 21-base pair deletion, we designed primers predicted to provide products of 159 out of 180 base pairs, which could subsequently be resolved by gel electrophoresis to determine homozygous (Ins/Ins or Del/Del) and heterozygous (Ins/Del) genotypes. We included controls for each genotype (previously determined by Sanger sequencing) on each gel. We validated the genotyping results of 20 MMN samples by comparing results with the whole *CD55* promotor sequence. These results correlated 100%.

MMN samples that could not be genotyped by amplification of the whole promotor region of either *CD46*, *CD55*, or *CD59*, were retested once using the polymorphism-specific PCR approach.

TABLE 1 Baseline characteristics of patients with MMN and control subjects.

	MMN (N = 133)	Controls (N = 380)
Male sex (*n*, %)	101 (76)	234 (62)
Age[a]	43 (16)	67 (11)
Diagnostic delay in months[a]	42 (70)	
EFNS 2010 MMN diagnosis		
Definite MMN (*n*/*N*, %)	95/129 (74)	
Probable MMN (*n*/*N*, %)	24/129 (19)	
Possible MMN (*n*/*N*, %)	10/129 (8)	
Anti-GM1 IgM antibodies (*n*/*N*, %)	77/117 (66)	
IVIg treatment naïve at first visit (*n*, %)	103 (77)	
First visit MRC sum score[a]	122 (9)	
IVIg treatment after first visit (*n*/*N*, %)	96/103 (93)	
IVIg response	90/96 (94)	
Follow-up data (*n*/*N*, %)	102/103 (99)	
Follow-up duration in months[a]	101 (154)	
Last visit MRC sum score[a]	121.5 (11.5)	

Abbreviations: IQR, interquartile range; IVIg, intravenous immunoglobulins; MMN, multifocal motor neuropathy; MRC, Medical Research Council.
[a]Values displayed as median (IQR).

2.5 | Statistical analysis

We used R (version 3.5.1) to perform statistical analyses. We used a X^2 test to determine the association between the polymorphisms and MMN susceptibility. When a p value <.05 was found in the overall genotype comparison, post-hoc X^2 tests with Bonferroni p value adjustment were performed per genotype, and a p value <.017 was considered statistically significant. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated.

We used the continuous variables age at onset, first (i.e., baseline) visit, and last visit MRC sum score (FV MRCss and LV MRCss, respectively) as clinical parameters. We compared age at onset in all patients. The MRCss variables were compared in patients who were treatment-naïve at baseline only. In all comparisons, patients were grouped as either carriers (i.e., having a heterozygous or homozygous positive genotype) or non-carriers (i.e., having a homozygous negative genotype).

We compared age at onset using a Student's t test. Next, we determined the association between either of the polymorphisms and muscle strength. First, we used a linear regression analysis to determine the association between a polymorphism and the FV MRCss, including the FV MRCss as an outcome variable, disease duration at the first visit, and polymorphism status as independent variables. To avoid outlier-driven effects, patients with a disease duration longer than 20 years at their first visit were excluded from MRCss analyses. The mean MRCss deterioration over time and its mean difference between the polymorphism groups were obtained. Thus, modeling the pre-diagnostic disease course, the model's interaction term indicated whether the change in muscle strength over time was different for patients carrying any of the polymorphisms in question.Second, we determined the effect of a polymorphism on the MRCss change between the first and last visit to our hospital using a linear mixed effects model (R lme4 package), including the FV and LV MRCss as separate values. The fixed effect part contained polymorphism status, time since baseline in months and the interaction between polymorphism and time and included a random intercept per patient. In this analysis too, the interaction term indicated whether the change in muscle strength over time differed according to polymorphism status. Third, since it is known that anti-GM1 IgM antibody status is associated with MMN disease course, both MRCss analyses were performed in an anti-GM1 IgM antibody status independent and dependent way.[4,8,11] In the anti-GM1 IgM antibody status dependent way, antibody status was introduced as extra independent variable/fixed effect.In all three analyses, we obtained the mean MRCss deterioration over time for both groups, and calculated the mean difference between groups with its 95% CI and p value. Since we have selected the polymorphisms based on their association with the disease instead of studying all known polymorphisms in the *CD46*, *CD55*, and *CD59* promotor region, and since the clinical correlation analysis was exploratory, we did not perform a p value correction for these analyses. p Values <.05 were considered statistically significant.

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2.6 | Standard protocol approvals, registrations, and patient consents

The locally appointed ethics committee of the University Medical Center Utrecht approved this study (METC-NL.041.14528). All included patients gave written informed consent prior to inclusion in this study.

3 | RESULTS

3.1 | Study population

We included 133 patients with MMN and 380 control subjects. Baseline characteristics are shown in Table 1. Age at onset (MMN) or age at inclusion in the PAN study (controls) is shown. The first visit MRC sum score is depicted for patients with MMN who were treatment-naïve at their first visit ($n = 103$). The last visit MRC sum score is calculated only for patients with MMN who were treatment-naïve at baseline and for whom follow-up data were available.

3.2 | Genotyping

Genotyping was successful in at least 97.7% of patients with MMN and at least 99.5% of control subjects. Characteristics of the polymorphisms, MAFs, and genotyping results of patients and controls are shown in Table 2. Examples of Sanger sequencing and gel electrophoresis results are shown in Figure S1.

The largest difference in MAFs was found for *CD55* rs28371582 (controls vs. MMN 0.30 vs. 0.36, respectively), though this was not statistically significant ($X^2 = 2.50$, $p = .11$). To compare genotype frequencies, an overall X^2 test was performed, which showed a statistically significant different genotype distribution between patient and controls for *CD55* rs28371582 ($X^2 = 9.38$, $p = .009$). In a post-hoc analysis, we found that this effect was driven by patients with MMN

TABLE 2 Minor allele frequencies (MAFs) and genotype frequencies of rs2796267, rs2796268, rs28371582, rs28371583, and rs141385724 in patients with MMN ($n = 133$) and controls ($n = 380$).

Gene	Polymorphism	Alleles	MAF			Genotype frequencies (*n*, %)					
			Controls	MMN	*p*		Controls	MMN	*p*	OR (95% CI)	*p*
CD46	rs2796267	A versus G	0.42	0.39	.33	A/A	126 (33.2%)	45 (34.4%)	.24	-	-
						A/G	187 (49.2%)	71 (54.2%)		-	-
						G/G	67 (17.6%)	15 (11.5%)		-	-
						ND	0 (0%)	2 (1.5%)			
	rs2796268	A versus G	0.44	0.41	.40	A/A	122 (32.2%)	44 (33.8%)	.46	-	-
						A/G	179 (47.4%)	66 (50.8%)		-	-
						G/G	77 (20.4%)	20 (15.4%)		-	-
						ND	2 (0.5%)	3 (2.3%)			
CD55	rs28371582	Ins versus Del	0.30	0.36	.11	Ins/Ins	178 (47.0%)	59 (45.0%)	.009*	0.93 (0.61–1.41)	.70
						Ins/Del	172 (45.4%)	50 (38.2%)		0.74 (0.48–1.14)	.15
						Del/Del	29 (7.7%)	22 (16.8%)		2.43 (1.27–4.58)	.005*
						ND	1 (0.3%)	2 (1.5%)			
	rs28371583	A versus G	0.28	0.26	.67	A/A	203 (53.4%)	75 (56.8%)	.76	-	-
						A/G	143 (37.6%)	45 (34.1%)		-	-
						G/G	34 (8.9%)	12 (9.0%)		-	-
						ND	0 (0%)	1 (0.8%)			
CD59	rs141385724	- versus A	0.17	0.20	.25	−/−	261 (68.7%)	84 (64.1%)	.35	-	-
						−/A	110 (28.9%)	41 (31.2%)		-	-
						A/A	9 (2.4%)	6 (4.6%)		-	-
						ND	0 (0%)	2 (1.5%)			

Note: Chi-squared tests were performed and uncorrected *p* values are shown.
Abbreviations: CI, confidence interval; Del, rs28371582 21-base pair deletion (−); Ins, rs28371582 21-base pair insertion (TAGTTACTTCCCCTCCTTCCC); MAF, minor allele frequency (=n alleles/2N); MMN, multifocal motor neuropathy; ND, not determined; OR, odds ratio.
*Statistically significant after *p* value adjustment.

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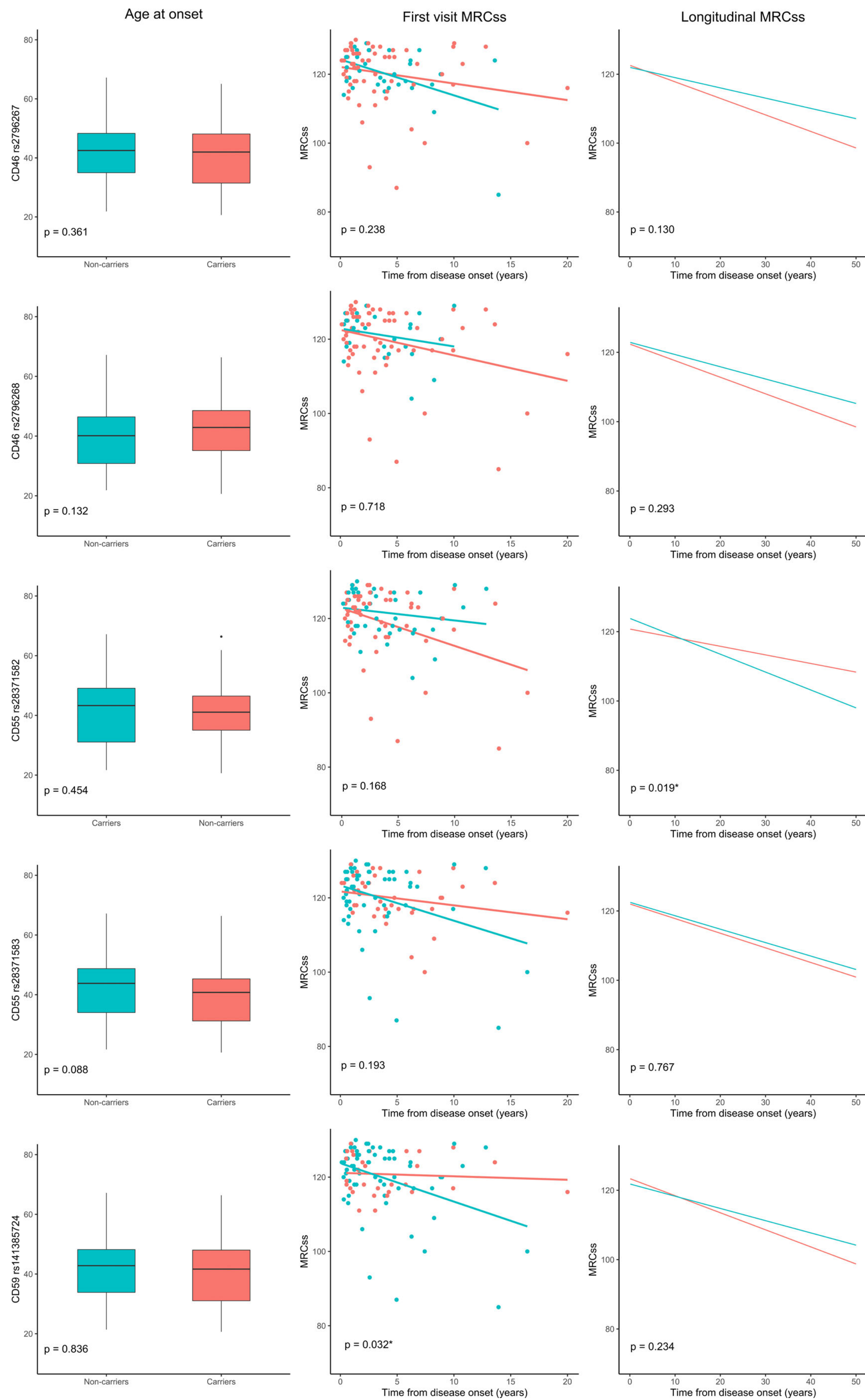

FIGURE 1 Legend on next page.

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more often carrying the Del/Del genotype ($X^2 = 8.05$, OR: 2.43 [1.27–4.58], $p = .005$).

3.3 | Clinical correlation

Next, we investigated whether specific genotypes of the complement regulatory proteins were associated with clinical features of MMN (Figure 1). We found no association between any polymorphism and age at onset. None of the polymorphisms in *CD46* and *CD55* were associated with MRC sum score deterioration until the first visit (FV MRCss), but patients carrying *CD59* rs141385724 showed a less severe MRCss decline until diagnosis (average deterioration rate 1.03 MRC points/year in non-carriers; average deterioration rate 0.093 MRC points/year in carriers; mean difference 0.93 MRC points/year; 95% CI: 0.08–1.80, *p* .019).

Among patients who were treatment-naïve at baseline, carriers of *CD55* rs28371582 showed a less severe decline in MRCss during follow-up than non-carriers (average deterioration rate 0.52 MRC points/year in non-carriers; average deterioration rate 0.25 MRC points/year in carriers; mean difference 0.27 MRC points/year; 95% CI: 0.04–0.49, *p* .019), suggesting a more favorable long-term disease course after initiation of IVIg treatment. All other polymorphisms were not associated with the MMN disease course. Neither the FV MRCss, nor the longitudinal MRCss data were different among patients with or without anti-GM1 IgM antibodies (*p* values >.05 for all analyses).

4 | DISCUSSION

In this study, we show that MMN susceptibility is associated with a polymorphism in the promotor region of the complement regulatory protein CD55, and its disease course with promotor polymorphisms of both CD55 and CD59. More specifically, we found that MMN was significantly associated with rs28371582, a 21-bp deletion in the promotor region of *CD55* ($p = .009$; Del/Del genotype 16.8 vs. 7.7%, OR: 2.43 [1.27–4.58], $p = .005$), while *CD59* rs141385724 was associated with less severe muscle weakness at baseline in treatment-naïve patients. Patients carrying *CD55* rs28371582 (55% of all patients) also showed a more favorable disease course after starting IVIg treatment.

Protection of tissues against the deleterious effects of inappropriate complement activation is mediated by membrane-bound complement regulators, including CD46 (MCP) and CD55 (DAF), which both regulate complement at a level upstream of the formation of the membrane-attack complex (MAC), and CD59 (MAC-IP), that regulates MAC formation (see Figure 2).[13,26–28] *CD55* encodes for decay-accelerating factor (DAF), a GPI-linked protein that regulates complement by enhancing the decay of the C3 convertases C4bC2a and C3bBb. *CD55* thus inhibits the formation and amplification of C3b, the central complement factor.[13,26,27]

The *CD55* promotor polymorphism rs28371582 has functional consequences. The *CD55* promotor region contains unique genetic features: it does not have a classical TATA box but rather contains frequent TC_n repeats that function as alternative transcription initiation sites, as typically seen in non-TATA-box promotor regions.[29–31] Interestingly, the *CD55* rs28371582 deletion contains three such possible TC_n elements. *CD55* rs28371582 is associated with a lower *CD55* transcriptional activity, suggesting lower cellular expression levels.[32,33] Reduced CD55 expression has been associated with multiple autoimmune diseases, including systemic lupus erythematosus, autoimmune hemocytopenia, and myasthenia gravis, the latter also being associated with a *CD55* promotor polymorphism.[20,21,34] Lower CD55 expression or reduced CD55 upregulation may promote the formation of C3 convertase and thus the deposition of C3 fragments on peripheral nerves.[35] This may explain the observed association of this CD55 promotor polymorphism with MMN susceptibility.

We found two associations of genetic variability of CRPs and MMN disease course. First, treatment-naïve patients carrying *CD59* rs141385724 showed a trend of less severe weakness at the first visit. *CD59* rs141385724 has previously been believed to have a tissue- and inflammation-specific functional effect and we hypothesize that it is associated with increased CD59 expression in motor neurons.[17,18] After initiation of IVIg therapy, patients carrying this polymorphism had a similar disease course as compared to non-carriers. Combined with the negative result of an open-label trial where IVIg-treated patients with MMN were treated with eculizumab, an anti-C5 monoclonal antibody that exerts a CD59-like effect on the terminal complement cascade, the results of our study may indicate that muscle strength deterioration in patients with MMN treated with IVIg is mediated through complement factors of the proximal (C2, C4, and C3), rather than the terminal pathway (C5–C9, i.e., MAC).[36] We hypothesize that CD59 expression on peripheral nerves is sufficiently

FIGURE 1 *CD46* rs2796267, *CD46* rs2796268, *CD55* rs28371582, *CD55* rs28371583, and *CD59* rs141385724, each assigned a separate row, correlated to clinical parameters in patients with MMN. The left column shows the age at onset in years. The middle column depicts the MRC sum score (MRCss) at the first visit in untreated patients, plotted against the disease duration at the first visit in years on the *x*-axis. The right column shows the course of the MRCss between patients' first and last visit in patients untreated at the first visit, plotted against MMN disease duration in years on the *x*-axis. Patients were grouped by either carrying or not carrying the polymorphism shown in each row (red: carriers; blue: non-carriers). Patients with MMN who were treatment-naïve at the time of their first visit and who carried *CD59* rs141385724 showed less severe muscle weakness at their first visit ($p = .032$). *CD55* rs28371582 was associated with less severe clinical deterioration during the follow-up period in patients with MMN who were treatment-naïve at baseline ($p = .019$). MMN, multifocal motor neuropathy; MRC, Medical Research Council.

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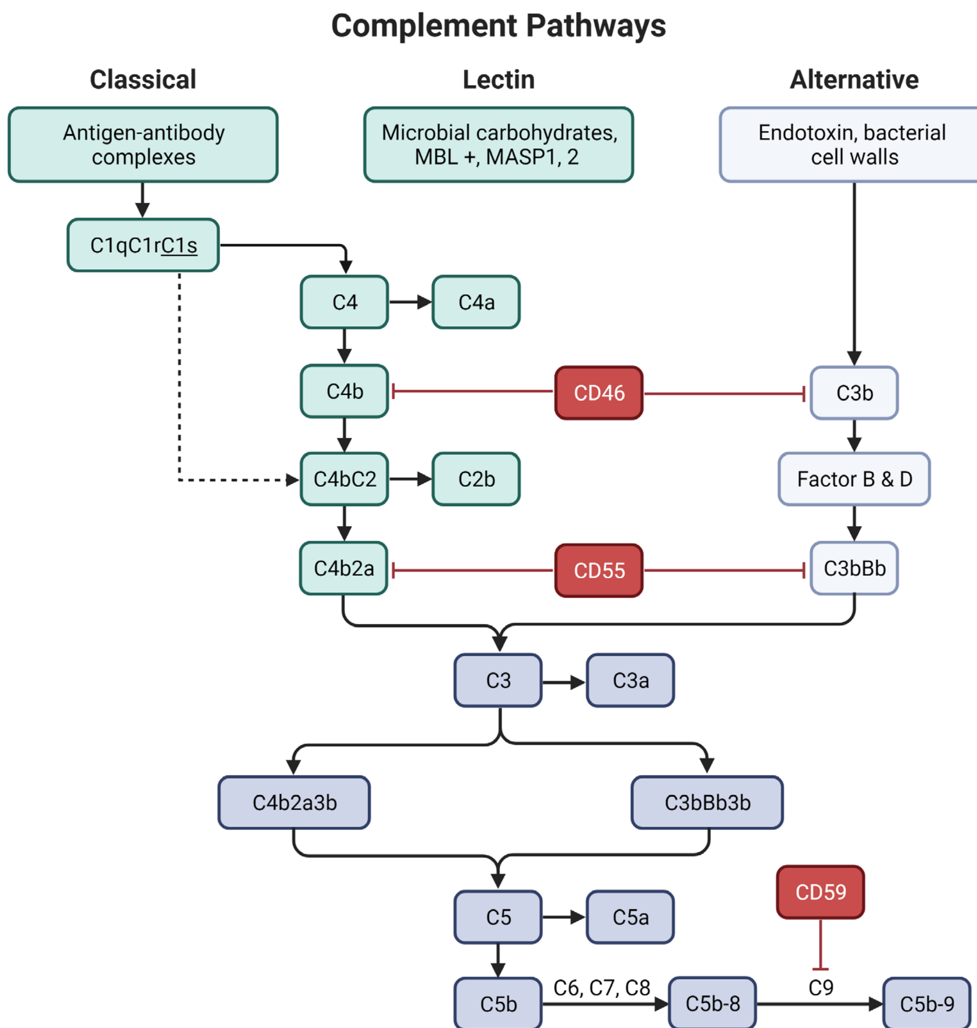

FIGURE 2 The complement pathways and cascades that follow upon activation. Anti-GM1 IgM antibodies activate the classical pathway, ultimately leading to formation of the membrane attack complex (MAC). The membrane-bound complement regulatory proteins CD46, CD55, and CD59 each inhibit the complement cascade at different levels. CD46 (membrane cofactor protein) acts as a cofactor for the cleavage-mediated inactivation of C3b and C4b. CD55 (decay accelerating factor) enhances the dissociation of the central C3 convertases C4bC2a and C3bBb, thereby inhibiting the formation of the central complement component C3b. Terminal complement activation is inhibited by CD59 (MAC inhibitory protein), which inhibits the polymerization of C9 and thus MAC formation.

high to—in combination with IVIg—hold MAC formation at bay after binding of anti-ganglioside antibodies to myelin or (para)nodes.The *CD55* promotor polymorphism rs28371582 not only associated with susceptibility, but also showed a second association with disease course. Treatment-naïve patients carrying *CD55* rs28371582 actually showed a trend to *less* severe muscle strength deterioration after IVIg therapy, despite the implication of lower CD55 expression and hence increased susceptibility to complement-mediated nerve damage. We believe that this association is best explained by the complement modulatory effects of IVIg. Monomeric IgG can compete directly with C1q, thereby inhibiting C1q-initiated complement activation.[37] IVIg, like CD55, also modulates complement at the C3 convertase level by scavenging activated C3 through binding to C3b. This inhibits the formation of the C3 convertases and attenuates further complement activation via the amplification loop.[38–41] If complement factors such as C3 and C4 play an important role in MMN pathogenesis as suggested by experimental models, carriership of *CD55* rs28371582 may provide a context in which IVIg may exert more pronounced beneficial effects.[10,35]

Our study is the first study on genetic susceptibility factors involved in innate complement regulation in MMN. As MMN is a rare disease, the size of our cohort is significant and includes a long follow-up time with a median duration of over 8 years. Additionally, our control cohort is large and population-based. Since the MRC sum scores were collected retrospectively, relatively small groups were compared in the analyses and any change in MRCss was reduced to a linear correlation; the results of these correlations should be interpreted with care. Future in vitro studies in motor neurons could aid in finding biological explanations for the associations found in this study.[10] Moreover, such studies could help in forming a stronger theoretical basis for treatment strategies targeting the pre-C5 level of the complement cascade in patients with MMN.

In conclusion, our study reports novel findings that point towards the relevance of complement regulation at the C3 level in multifocal motor neuropathy. We show that a 21-bp deletion in the promotor region of *CD55* is associated with MMN susceptibility and that patients carrying this polymorphism showed a trend to a better long-term clinical response upon IVIg treatment. With new therapies that target the early stages of the complement cascade emerging, future studies should aim to further our understanding of the importance of the proximal complement cascade in MMN immunopathology.

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ACKNOWLEDGMENTS

We thank Professor Jan Veldink MD, PhD, for his help in providing samples for this study's control cohort and Dr Sanne Piepers, Dr Elizabeth Cats, Dr Lotte Vlam, Dr Bas Jongbloed and Dr Ingrid Herraets for their help with systematically collecting patient data.

CONFLICT OF INTEREST STATEMENT

Jeroen W. Bos, Henny G. Otten, Keving Budding, Ruben P. A. van Eijk, Chantall Curial, Tineke Kardol-Hoefnagel, and H. Stephan Goedee report no disclosures relevant to the current manuscript. Ewout J. N. Groen serves on the scientific advisory board for SMA Europe. Leonard H. van den Berg received an educational grant from Takeda, serves on the editorial boards of Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration, and receives research support from the Netherlands ALS Foundation. W. Ludo van der Pol serves on the scientific advisory board for SMA Europe, provides ad hoc consultancy for Argenx, Biogen, Roche, and Novartis, is the local PI of the ARDA and ARDA+ trials for MMN, and receives research support from the Prinses Beatrix Spierfonds, Vriendenloterij, and Stichting Spieren voor Spieren.

DATA AVAILABILITY STATEMENT

The data that support the findings in this study will be available on reasonable request from the corresponding author.

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How to cite this article: Bos JW, Groen EJN, Otten HG, et al. A 21-bp deletion in the complement regulator *CD55* promotor region is associated with multifocal motor neuropathy and its disease course. *J Peripher Nerv Syst*. 2024;29(2):193-201. doi:10.1111/jns.12620

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